BIOELECTROVENTING:

cleaning-up polluted sites using electrodes to stimulate microbial remediation activities

Ph.D. Thesis

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CERTIFICA:

Que el trabajo descrito en la presente memoria, titulado "**Bioelectroventing: cleaning up polluted sites using electrodes to stimulate microbial remediation activities**", ha sido realizado bajo su dirección por D. Jose Fernando Rodrigo Quejigo en el Área de Ingeniería Química del Departamento de Química Analítica, Química Física e Ingeniería Química de la Universidad de Alcalá. Asimismo, autorizo su presentación para que sea defendido como Tesis Doctoral.

Y para que conste y surta los efectos oportunos, firma el presente en Alcalá de Henares a 12 de Diciembre de 2016.

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Escuela de Posgrado de la Universidad de Alcalá
Programa de Doctorado en Hidrología y Gestión de los Recursos Hídricos

TESIS DOCTORAL

Bioelectroventing: cleaning-up polluted sites using electrodes to stimulate microbial remediation activities

Memoria presentada para optar al título de Doctor por la Universidad de Alcalá por:

Jose Fernando Rodrigo Quejigo

Dirigida por:

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Departamento de Química Analítica, Química Física e Ingeniería Química

Universidad de Alcalá

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At worst, one is in motion; and at best,
reaching no absolute, in which to rest,
one is always nearer by not keeping still.

Thom Gunn, from *Collected Poems*

Para Beatriz y Michael

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Summary

Microbial electrochemistry is an emerging biotechnological discipline based on the interactions between microorganisms and electrically conductive materials. All the assorted technologies based on this principle are referred as “Microbial Electrochemical Technologies” (METs) and their range of applications spans diverse fields. For instance, a system based on Microbial Electrochemistry conforms a novel strategy for enhancing the clean-up of polluted environments. The novelty of this application consists in the unlimited character of electrodes as terminal electron acceptor (anode) or donors (cathode), unchaining microbial metabolism activities beyond the natural conditions. The versatility of these systems allows the application of electrode-assisted bioremediation in matrices as different as wastewater, sediment, soil and manure. Nevertheless, overcoming microbial, technological and economical challenges is basic for the implementation of such a system in real-field applications.

In this thesis, we have conformed new redox scenarios supplied by electrodes for degrading 3 different pollutants in 3 different matrices, using the electrode as a terminal electron acceptor: **dibenzothiophene** (polycyclic aromatic hydrocarbon) in paddy soil, **isoproturon** (herbicide) in agricultural soil and **sulfamethazine** (antibiotic) in manure. The work has been organized in 5 chapters, 3 of them being experimental.

Chapter 1 establishes an introduction to microbial electrochemistry, its fundamentals and its applications. We centre the attention in the state of the art of Microbial Electrochemical Systems as bioremediation tools of polluted environments. Conventional bioremediation techniques have acted by supplying nutrients, co-metabolites, oxygen or other electron donors or acceptors (biostimulation) or by adding additional microorganisms able to degrade a specific pollutant (bioaugmentation). Electrode-assisted bioremediation means a novel strategy to stimulate microbial activity, avoiding the supply of costly chemical amendments that diffuse uncontrollably and might be consumed in unwanted metabolic reactions. Furthermore, electrodes

not only overcome the deficiency of suitable electron donors or acceptors in highly reductive environments, but also provide a battery of tailored redox scenarios that can be displayed and interchanged in reference to the different pollutant chemical nature, redox conditions and matrix. Additionally, the introduction presents the pollutants tested in this thesis, their ecological impact and their degradation current knowledge. One common feature of the compounds evaluated is the scarce research about their biodegradation under flooded and extreme reductive conditions. The common feature of the matrices is their high resistivity that restrains the transport of ions between electrodes, and makes them less favourable scenarios for electrode-assisted bioremediation. All the objectives aimed in this thesis are also outlined also in ***Chapter 1***.

In ***Chapter* 2** we establish the term Microbial Electroremediating Cell (MERCs) to refer to METs that in spite of harvesting energy, aim to maximize metabolic oxidation or reduction for enhancing the biodegradation of a pollutant. MERCs outperform the removal of dibenzothiophene (DBT) compared to the natural attenuation under flooded conditions. Moreover, the study was complemented with toxicological analyses for verifying a real clean-up of DBT-polluted soil. Green algae confirm a toxicity decrease of the treated soil during incubation in MERCs, in contrast to the unaltered values detected under natural conditions. Our results also reveal that the mere presence of conductive material enhanced the biodegradation of DBT, which raises this conductive material-assisted bioremediation as a very promising technique to treat polluted environments.

The flexibility of these systems for operating under different electrode configurations or redox conditions makes this technology a very versatile tool for cleaning up with pollutants of different nature. So the **first part of *Chapter 3*** demonstrates that isoproturon (IPU) total degradation to CO_2 can be enhanced by polarizing the anode at positive potentials. In this case the ^{14}C detection techniques provide a precise measurement of the stimulation capacity of electrodes to degrade IPU. We introduce the term **bioelectroventing** to designate the process to supply electrodes to stimulate

the oxidative metabolism of environmental microbial populations in allusion to the similarities with the traditional bioremediation technique *bioventing*, where oxygen is artificially applied as electron acceptor. This chapter also reports an overall profile of the ^{14}C-IPU metabolites and ^{14}C mass balance in response to the different treatments. In the **second part of Chapter 3**, we demonstrate how the use of electrodes at high anodic potential (+600 mV versus Ag/AgCl) in IPU-contaminated soil increases not only the IPU-removal, but also leads to effective clean-up demonstrated by ecotoxicological analysis of treated soils. Furthermore, electrode potential differences induced taxonomical shifts in the microbial community. So thus, microbial metabolism and community structure respond to the bioelectrochemically stimulation, linking functional differences (IPU-removal) with taxonomical microbial shifts. We also use the microbial community profile as a reporter of the electrode influence, suggesting that the electrode effect to change the communities profile is at least 0.5 cm.

As a final scientific contribution, ***Chapter 4*** explores the operation of MERCs in flooded manure to degrade sulfamethazine (SMZ). This compound is frequently used in veterinary medicine and enters the environment by using manure as soil fertilizer due to its incomplete absorption in the animal gut and its unmetabolized excretion. Our results have revealed how the use of electrodes at negative anodic potential (-400 mV versus Ag/AgCl) in SMZ-contaminated manure increases, not only the SMZ-removal, but also influence the compound fate. Similarly than in the study with DBT in polluted soils, our results also reveal that the mere presence of conductive material enhanced the mineralization to $^{14}CO_2$ of the radiolabeled antibiotic. We hypothesized that conducted material acts as assistance of a process called Direct Interspecies Electron Transfer (DIET), a metabolism that consists in interchanging electrons in the absence of electron shuttles or redox mediators, just through direct electrical connections between microbes.

Finally, in **Chapter 5** we introduce a general discussion, conclusions and future work based on our experimental results. The general discussion is presented under a question-answer mode. The remarkable impact of electrodes on bioremediation of polluted environments suggests a promising

future for this emerging environmental technology as a potential alternative to conventional bioremediating techniques, enforcing the establishment of electrodes as a conceivable cost-effective and environmentally friendly strategy for enhancing pollutants degradation under extreme reductive conditions.

Resumen

La electroquímica microbiana es una disciplina emergente basada en la interacción entre microorganismos y materiales conductores de la electricidad. Todas las tecnologías fundamentadas en este principio se denominan "Tecnologías Electroquímicas Microbianas" (METs, por sus siglas en inglés). Una de las múltiples aplicaciones de las METs consiste en la utilización de un electrodo como aceptor/donador de electrones, para estimular el metabolismo microbiano de compuestos químicos contaminantes. Además, la versatilidad de esta tecnología permite su uso en diferentes matrices y sustratos como aguas residuales, sedimentos, suelos y residuos ganaderos como los purines. Sin embargo, es fundamental resolver primero los desafíos de carácter microbiológico, tecnológico y económico, antes de lograr implementar estas técnicas bioelectroquímicas en aplicaciones a escala real.

En esta tesis se han utilizado electrodos como aceptor terminal de electrones (TEA, por sus siglas en inglés) con el objeto de estimular la degradación de contaminantes. En concreto se ha trabajado con 3 contaminantes y 3 matrices diferentes: **dibenzotiofeno** (hidrocarburo aromático policíclico) en un suelo de arrozal típicamente anegado, **isoproturón** (herbicida) en un suelo agrícola y **sulfametazina** (antibiótico) en purines ganaderos. El trabajo se ha organizado en 5 capítulos, 3 de ellos experimentales.

El **capítulo 1** presenta una introducción a la electroquímica microbiana, sus fundamentos y aplicaciones. Dentro de las aplicaciones, se concede especial atención a los Sistemas Electroquímicos Microbianos utilizados como herramientas de biorrecuperación de ambientes contaminados. Las técnicas de biorrecuperación convencionales han estimulado la biodegradación de contaminantes suministrando donadores/aceptores de electrones, como por ejemplo nutrientes, co-metabolitos u oxígeno (bio-estimulación). También han agregado microorganismos capaces de degradar un contaminante específico (bio-

aumento). La biorrecuperación asistida por electrodos evita el suministro de compuestos que difunden sin control y que pueden consumirse en reacciones metabólicas no deseadas. Además, los electrodos no sólo superan la deficiencia de donadores/aceptores de electrones en ambientes reductores, sino que pueden establecer nuevos escenarios redox en el ambiente contaminado. La adecuación de un escenario u otro va a depender de la naturaleza química del contaminante, el entorno contaminado y las condiciones redox del mismo. En la introducción se presentan, además, los contaminantes tratados en el desarrollo de esta tesis, su impacto ecológico y el estado del arte sobre sus mecanismos de biodegradación. Un rasgo común de los compuestos evaluados es la escasa información sobre su biodegradabilidad bajo condiciones reductoras. La característica común que presentan las matrices empleadas es su alta resistividad, que limita el transporte de iones entre electrodos y los convierte en escenarios no favorables para la biorrecuperación electrogénica. Por último, y para finalizar el capítulo 1, se proponen los objetivos principales de este trabajo de investigación.

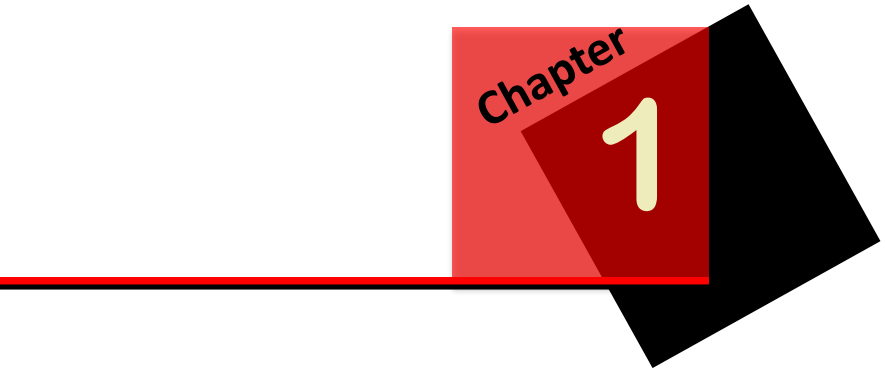
En el **capítulo 2** se presenta el término Celdas de Electrorrecuperación Microbiana (MERCs, por sus siglas en inglés). Las MERCs son Sistemas Electroquímicos Microbianos, que más allá de la producción de energía, tienen como objetivo principal maximizar la oxidación o reducción metabólica de un contaminante. Estos sistemas estimulan la eliminación de dibenzotiofeno (DBT) en referencia a la atenuación natural que experimenta este mismo compuesto bajo condiciones de anegación. Además, el estudio se complementa con análisis toxicológicos con el alga *Pseudokirchneriella subcapitata*, para ratificar la efectividad del tratamiento bioelectroquímico al término del mismo. Dichos análisis confirman una disminución de la toxicidad del suelo tratado con electrodos, en contraste con los valores toxicológicos inalterados que muestran los suelos no tratados de forma biolectroquímica. Nuestros resultados también revelan que la mera presencia de material conductor acelera la biodegradación de DBT. Así, el bajo coste y la sencillez de su aplicabilidad hacen de esta última intervención una estrategia prometedora para el tratamiento de ambientes contaminados.

La flexibilidad de las MERCs para operar bajo distintos diseños y configuraciones, estableciendo diferentes condiciones redox a través del uso de electrodos, convierten a esta tecnología en una herramienta muy versátil para degradar diferentes contaminantes. De este modo, la **primera parte del capítulo 3** muestra que la mineralización del isoproturón (IPU) a CO_2 aumenta al seleccionar potenciales positivos en el ánodo. El uso de ^{14}C-IPU permite una medición precisa de la capacidad de los electrodos para estimular la degradación completa de este compuesto. En este capítulo se introduce el término ***bioelectroventing***, que hace referencia a la utilización de electrodos para estimular, de modo específico, el metabolismo oxidativo de las poblaciones microbianas. El término alude a la similitud con la técnica *bioventing*, donde el oxígeno se aplica artificialmente como aceptor final de electrones. También se propone una posible ruta metabólica a partir del perfil general de los metabolitos procedentes del ^{14}C-IPU. Además se establece el balance de masas del ^{14}C en respuesta a los distintos tratamientos. En la **segunda parte del capítulo 3** se muestra como el uso de electrodos con alto potencial anódico (+600 mV frente a Ag / AgCl) no sólo aumenta la eliminación del IPU, sino que también reduce la toxicidad del suelo, como lo confirman los análisis ecotoxicológicos con *P. subcapitata*. Además, la presencia del electrodo y el potencial de funcionamiento de este, inducen cambios taxonómicos en la comunidad microbiana. Por lo tanto, el metabolismo y estructura de la comunidad microbiana responden claramente a la estimulación bioelectroquímica, vinculando las diferencias funcionales (eliminación de IPU) con variaciones taxonómicas. También se utilizan dichas diferencias como un indicador del área de influencia del electrodo. Así, los resultados sugieren que el efecto del electrodo sobre las comunidades microbianas es al menos de 0.5 cm, y por tanto también el área de influencia del mismo.

Como contribución científica final, el **Capítulo 4** explora el funcionamiento de las MERCs en purines contaminados con sulfametazina (SMZ), un antibiótico de uso veterinario. Este compuesto entra en el medio ambiente con la aplicación de estiércol como fertilizante orgánico del suelo agrícola. Dicho estiércol posee altas concentraciones de SMZ debido a su

absorción incompleta en el intestino animal y su posterior excreción no metabolizada. Los resultados experimentales establecen que el uso de electrodos trabajando a un potencial anódico negativo (-400 mV versus Ag / AgCl) aumentan no sólo la eliminación de SMZ sino que también influyen en el destino ambiental del compuesto. Del mismo modo que en el estudio con DBT en suelos contaminados, nuestros resultados también revelan que la mera presencia de material conductor aumenta la mineralización a $^{14}CO_2$ del antibiótico radio-marcado. Se plantea la hipótesis de que el material utilizado, concretamente fieltro de carbono, actúa como asistente de un proceso de transferencia directa de electrones entre especies (DIET, por sus siglas en inglés). Este proceso metabólico consiste en intercambiar electrones a través de conexiones eléctricas directas entre microorganismos.

Para finalizar, el **capítulo 5** presenta una discusión del trabajo de tesis bajo el formato de pregunta-respuesta. También se establecen unas conclusiones generales y una exposición de trabajos futuros basados en los resultados experimentales expuestos. El notable impacto que tienen los electrodos en la biorrecuperación de entornos contaminados sugiere un futuro prometedor para esta tecnología ambiental emergente, suponiendo una alternativa potencial a las técnicas convencionales de biorrecuperación *in situ*.


Chapter
1

Introduction, objectives and thesis outline

INTRODUCTION

1.1 Microbial Electrochemistry and Technology

1.1.1 Microbial electrochemistry: historical context

The idea of using the interaction between living microbial cells and electrodes in a fuel cell to produce electricity was first conceived in the early twentieth century when M.C. Potter, a professor of Botany at the University of Durham, came to the conclusion that electric energy could be liberated from the microbial degradation of organic compounds, by cultures of enteric bacterium *E. Coli* (Potter, 1911) (Fig.1.1). The idea of this new Microbial Fuel Cell (MFC) did not receive any major coverage probably given the limited knowledge about bacterial metabolism and the unappealing idea of investing in renewable energy sources during the fossil fuel booming. In 1931 the American chemist Barnet Cohen restarted Potter's work and created several half MFC that, when connected in series were capable of producing an output of 35 volts although the current output in this setup was only 2 milli-Amps (Cohen, 1931).

It was not until the 1960s that some American space programs in the framework of the NASA focused about the development of biological fuel cells as possible system for organic waste management and energy generation (Canfield *et al*., 1963). Nevertheless during these research efforts, the complexity of the underlying bioelectrochemical processes became evident. More than a decade later, Suzuki *et al*. (1976) presented the first MFC prototype similar to the current one and in the late seventies Di Salvo established the first computational model of biological fuel cell (Di Salvo *et al*., 1979).

In the 1980s, the interest around the microbial fuel cells grew significantly and the roll of microbial consortium as anode catalyst was investigated with pure cultures, electron mediators between bacteria and electrodes and complex carbon sources (Bennetto *et al*., 1985; Thurston *et al*., 1985; Delaney *et al*., 1984; Bennetto *et al*., 1983; Stirling *et al*., 1983;

Tanaka et al., 1983; Suzuki and Aizawa, 1980). During the 1990s proliferated experimentation with different reactor configurations and combinations of carbon sources, and current generation was increased considerably (Cooney *et al*., 1996; Bennetto, 1990). In those two decades (1980s and 1990s) is worth to underline the work of M.J Allen and H. P. Bennetto, which combined advancements in the understanding of the electron transport chain and technological advancements envisioning the MFC systems as a green energy supply, especially in developing countries (Allen and Bennetto, 1993).

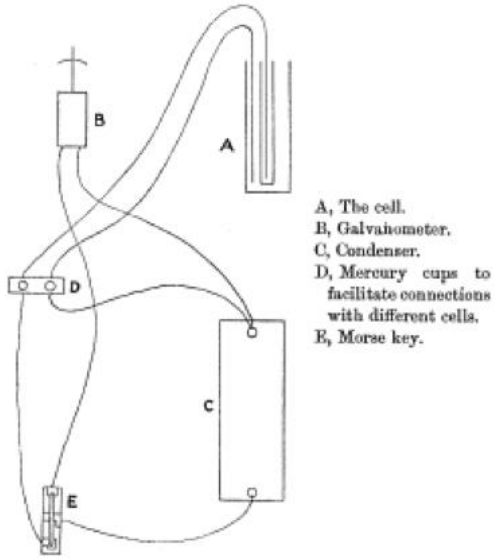


Figure 1.1: Original scheme of the MFC first prototype (from Potter, 1911). In the studies *E. Coli, B. Fluorescens, B. Violaceus* and *Sarcina Lutea* were used, but only *E.Coli* generated a cell electromotive force of 0.3 Volts recorded by the galvanometer.

However, it has taken nine decades from early Potter's research to establish that bacteria can transport electrons out of the cell without the aid of artificial electron shuttling compounds, in a process now called "Extracellular Electron Transfer" (EET) (Lovley and Phillips, 1988; Myers and Nealson, 1988). Moreover, it was discovered as recently as 1999 that cells can directly exchange electrons with electrodes acting as electron donors and acceptors

(Kim *et al*., 1999). Kim and his colleagues at the Korea Institute of Science and Technology showed that a bacterium known as *Shewanella oneidensis* did not require the use of a mediator molecule to transport electrons to the electrodes. The microorganisms with this capacity are denominated "electroactive microorganisms". The ability of these EET-capable bacteria to reduce electrodes has energized the discipline of microbial electrochemistry, which focuses in the study and application of interactions between living microbial cells and insoluble acceptors (i.e. physical electrodes and naturally occurring conductive materials such as metal oxides) (Schröder *et al*., 2015). After a century of moving slowly, microbial electrochemistry was rapidly morphing into a complex multidisciplinary area of its own, stems from different disciplines including environmental engineering and technology, biochemistry, electrochemistry, physics, mathematical modelling, and microbiology.

1.1.2 Microbial Electrochemical Systems: further than a MFC vision

From an engineering perspective, exploiting microbial reduction of minerals and in extension electrodes appears attractive to generate electrical power; such devices are commonly known as MFC (Tender *et al*., 2002). Although energy harvesting was the primary target, we have now explored additional scenarios and the range of applications of electroactive bacteria spans many diverse fields. All these assorted technologies are referred as "Microbial Electrochemical Technologies" (METs) or "Microbial Electrochemical Systems", where MFC is just one of several (Schröder *et al*., 2015).

1.1.2.1. Bacteria-electrode interaction

Bacteria grow by using the metabolic energy derived by catalysing chemical reactions and store energy in the form of adenosine triphosphate (ATP). In some bacteria, the electrons obtain with the substrates oxidation entry through NADH the respiratory chain, a series of enzymes that work to move protons across an internal membrane, creating a proton gradient (Fig.1.2). The protons flow back into the cell through the enzyme ATPase, creating ATP. The electrons are finally released to soluble terminal electron

acceptors, such as nitrate, sulphate, or oxygen or to insoluble terminal electron acceptor as minerals or electrodes (Lovley and Phillips, 1988; Myers and Nealson, 1988; Kim *et al*., 1999; Logan *et al*., 2006).

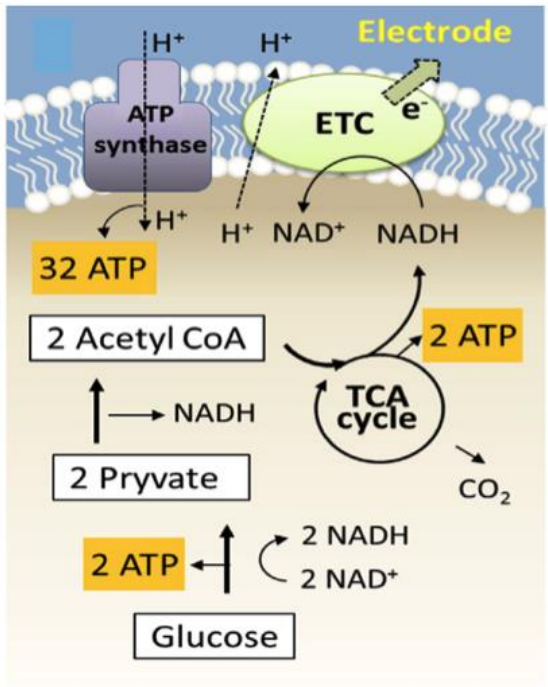


Figure 1.2: Microbial respiration and electron transfer to a solid substrate as an electrode. Special molecular mechanisms are required for extracellular electron transfer (ETT) because microorganisms cannot incorporate such insoluble materials into their cells and thus the electrons need to go through periplasm and over the outer membrane. The electrodes may alter the microbial dominant energy metabolic pathway shifting from substrate-level phosphorylation (i.e., anaerobic fermentation) to oxidative phosphorylation (i.e., electrode respiration), which leads to more ATP synthesis and hence increased activities for contaminant degradation (from Li and Yu, 2015).

Three proposed models of extracellular electron transfer (ETT) coexist since two microbial genus named *Shewanella* (Myers and Nealson, 1988) and *Geobacter* (Lovley and Phillips, 1988) were reported to utilize insoluble metal oxides as sole electron acceptors almost 30 years ago (Fig.1.3). The so-called ETT models here presented focus in the relation of electroactive microorganisms and electrodes, which has place in METs, giving insight into

the biological mechanisms underlying empirical observations of electrode microbial populations transforming organic molecules into CO_2 and generating an electron flow.

Numerous types of microorganisms can exchange electrons with an electrode, by donating or accepting them (Koch and Harnish, 2016). Nevertheless, the mechanisms for such outstanding interaction have been thoroughly studied primarily in two bacteria *Shewanella oneidensis* and *Geobacter sulfurreducens* (Carmona-Martínez *et al*., 2013; Marsili *et al*., 2008; Lovley, 2011a; Schrott *et al*., 2011; Estévez-Canales *et al*., 2015b). The interest in *Geobacter sulfurreducens* has exponentially grown, due to its easy culturing conditions, metabolism, its broad environmental relevance and because of its intrinsic capacity for establishing direct contact with electrodes. Hence it is not surprising that it has become the model organism for the study of Microbial Electrochemical Systems.

One of the features that share most of the reported electroactive bacteria is their natural capacity for reducing metals such as insoluble iron forms. However, the mechanisms for reducing metals and electrodes seem to differ. For instance, when some *Geobacte*r species are grown with Fe (III) oxides, they express flagella that use as a motility element for the search of the next source of Fe (III) (Childers *et al*., 2002). In contrast, when *Geobacter* is cultured in an electrochemical system with fixed electrodes, they permanently attach to the electrodes forming a biofilm (Bond and Lovley, 2003). Nevertheless, recent advances show for the first time that living in a biofilm is not a strict requirement for *Geobacter sulfurreducens* to exchange electrons with an electrode. The growth of planktonic electroactive *G.sulfurreducens* could be supported by a fluid-like anode as soluble electron acceptors do and with electron transfer rates similar to those reported for electroactive biofilms (Tejedor-Sanz *et al*., 2016b). Another aspect that questions the same mechanisms for reducing metals and electrodes is the fact that not all the metal-reducing microorganisms are able to respire electrodes. For instance, *Pelobacter carbinolicus*, which reduces Fe(III)

oxides, does not have the ability of transferring electrons to anodes (Richter *et al*., 2007).

Since electrodes have an insoluble nature that cannot penetrate the bacterial cells, a major requirement is that electrons must be transferred from the inside of the microbial cell membrane to its outside. In general EET mechanisms are commonly classified in mediated and direct EET, depending on the way microorganisms transport electrons into and out of the cell from or towards an electrode (Fig. 1.3).

Direct extracelular electron transfer (DEET) takes place through transmembrane redox active proteins or conductive pili with no diffusional redox species being involved in the electron transfer from the cell to the electrode (Reguera *et al*., 2005; Gorby *et al*., 2006; Lovley, 2006; Schröder, 2007). DEET does not involve any redox species or mediators. The bacteria should possess membrane bound electron transport proteins, which could help in the electron transfer from the outer membrane of the bacterial cell to the electrode. Another form of DEET is through the conductive pili (nanowire) formed on the bacterial cell surface connected to the cyctochrome which can transfer/conduct electron flow from internal layers of biofilm to the anode (Malvankar and Lovley, 2012; Malvankar *et al*., 2012a; Malvankar and Lovley, 2012b). These conductive pilus-like structures, identified so far in *Geobacter sulfurreducens* PCA (Reguera *et al*., 2005), *Shewanella oneidensis* MR-1 (Gorby *et al*., 2006), appear to be directly involved in extracellular electron transfer and introduces a whole new dimension allowing the direct reduction of a distant electron acceptor.

DEET also takes place when a cell uses another cell as terminal electron acceptor (TEA) via Direct Interspecies Electron Transfer (DIET), described as a mechanism based on an electrical connection among microbial species in order to develop syntrophic metabolisms through direct contact and in absence of electron shuttles or redox mediators (Summers *et al*., 2010; Lovley, 2011b). According to Liu *et al*. (2012) this metabolism may be accelerated in presence of a solid conductor material, demonstrating how

the anaerobic conversion of organic matter in a digester could be enhanced by promoting DIET between bacteria and methanogens, in presence of a conductive activated carbon, which permits better electrical connections among microorganisms. A more natural conductive material as magnetite has been also reported to promote DIET among *Geobacter sulfurreducens* and *Thiobacillus denitrificans* (Kato *et al*., 2012) so acetate oxidation can be coupled to nitrate respiration, both reactions that cannot be performed by the single bacterial strains. So DIET can be assisted and promoted with mineral as mediator, being the contact with these extracellular solids still performed via DEET.

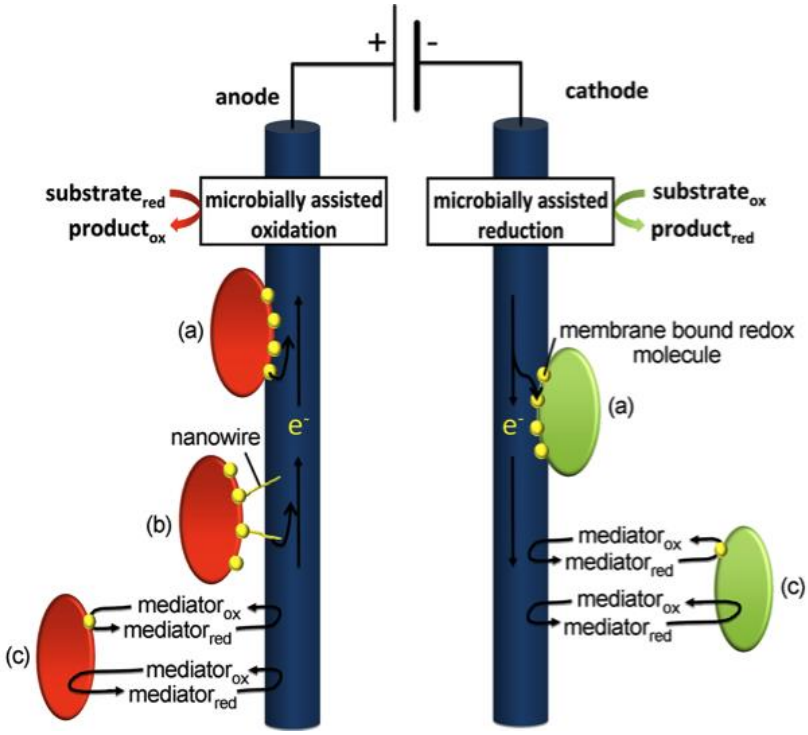


Figure 1.3: Principal extracellular electron transfer mechanisms in Microbial Electrochemical Systems. Microorganisms can perform anodic and cathodic interactions by means of extracellular electron transfer as follow: a) DEET through physical contact of the cell surface redox molecules and the electrode; b) DEET via microbial nanowires; and c) MEET through diffusive molecules that are reduced/oxidized at the cell surface or within the cells (from Koch and Harnisch, 2016).

On the other hand, Mediated Extracelular Electron Transfer (MEET) takes place through redox shuttles that mediate the electron flow from bacterial metabolism towards the electrode, acting as electron carriers. MEET occurs either by the addition of artificial mediators, by present in natural (as humic substances) or by secretion of soluble mediators such as primary and secondary metabolites from bacterial metabolism like flavins, Phenazines, phenoxazines or quinines compounds (Schröder, 2007).

EET is a bi-directional process, being already reported EET-capable microbes that utilize electrodes as an electron source (Rabaey and Rozendal, 2010; Rosenbaum *et al*., 2011; Ross *et al*., 2011; Rowe *et al*., 2015). Nevertheless, only little is known on the mechanisms of cathodic electron transfer and how these organisms obtain electrons from solid substrate electron donors.

1.1.2.2 Microbial Electrochemical Systems: applications

Although the bacterial capacity to couple the oxidation of organic matter to the reduction of electrodes was known since the origins of the 20th century (Potter, 1911), it was not considered a real technological alternative until the beginning 21st century. For a long time microbial electrochemistry awaked mainly the interest of fundamental researchers. This has considerably changed during the last decade and microbial electrochemistry gained interest from applied researchers and engineers. Indeed, it was in 2001 when the field went through a revolution whose origin was the key research from Reimers *et al*. (2001). They demonstrated that electric energy could be harvested from the natural voltage gradient generated between the anoxic zone and the overlaying oxygenic seawater in marine sediments. Since then, the increasing appearance of new applications and derive technologies, all named under the term Microbial Electrochemical Systems or Microbial Electrochemical Technologies (METs) (Schröder *et al*., 2015). In this section, it will be briefly describe those applications and its main achievements until now.

- ***Microbial Electrochemical Systems for power generation***

Normally named in the literature as MFC, have shown their potential for sustainable bioenergy production with a diverse number of fuels as urban wastewaters, food processing, and animal wastewaters (Logan and Regan, 2006; Angosto *et al*., 2015; Kelly and He, 2014; Rozendal *et al*., 2008).

MFCs are used as well as a bioenergy production tool in marine sediments (Reimers *et al*., 2001; Bond *et al*., 2002; Tender *et al*., 2002; Holmes *et al*., 2004; Ryckelynck *et al*., 2005; Lowy *et al*., 2006; Yuan *et al*., 2010) and freshwater sediments (Sacco *et al*., 2012; Wang *et al*., 2012a; Sajana *et al*., 2014), and paddy soils using the root secretions as source of organic substrates (Kaku *et al*., 2008; Busalmen *et al*., 2011; Takanezawa *et al*., 2010; Chen *et al*., 2012; Chiranjeevi *et al*., 2012). These METs are referred in literature as Benthic or Sediment Microbial Fuel Cells (BMFCs or SMFCs) (Reimers *et al*., 2001). Nevertheless lower output power values have been reported (ca.10−30 mW/m^2), most likely caused by an increase in the internal resistance due to the lower ionic strength and poor ionic conductivity of the electrolyte (Liu *et al*., 2005).

Due to the still limited energy production in MFCs, feeding small devices in remote locations is one of the current alternative scenarios explored. It has been already reported sediment MFCs as direct power source for an acoustic modem and seawater oxygen/temperature sensor system (Gong *et al*., 2011), for a wireless sensor (Donovan *et al*., 2008; Zhang *et al*., 2011) and for a wireless telecommunication system (Thomas *et al*., 2013).

Although energy harvesting is an important target, the simultaneous processes to current production e.g., wastewater treatments have become the most important environmental service dispose by MFCs. To date, Microbial Electrochemical Systems has been successfully used in lab-scale (Aelterman *et al*., 2006a; Capodaglio *et al*., 2013) and pre-pilot scale (Borjas *et al*., 2015) studies to treat domestic wastewater focussing on organic matter removal and energy production. The anaerobic nature of electrogenic bacteria works as an energy saving strategy in wastewater treatments,

avoiding the aeration supplies of the aerobic treatments and recovering part of the energy investment through green energy production (Logan and Rabaey, 2012). Beside this energy advantage, MFCs are capable of replacing the conversion of acetate to methane, a greenhouse-effect gas, for conversion of acetate into green electricity production.

Pursuing the same goal, the improvement of wastewater treatments and technology, Microbial Electrochemical Systems has been efficiently coupled to constructed wetlands (CWs) to generate a new hybrid concept so-called METland. CWs were an alternative wastewater treatment in small communities because of their low-cost operation and maintenance and low-energy requirements. However they were classically limited by a high area requirement. In contrast, the concept METland faces one of the disadvantages of this technology, the required surface area per inhabitant, and results in a powerful hybrid technology for enhancing the biodegradation rates in wastewater treatment (Aguirre-Sierra *et al*., 2016). Esteve-Nuñez *et al*., 2013 developed a full-scale METland in Carrión de los Céspedes, Spain, which treated cubic meters of real urban wastewater for over 2 years and attenuated the phenomena of clogging of the filter substrate. This research field will be further develop through the project iMETland (European Union's Horizon 2020 research and innovation program; www.imetland.eu), which aims to implement this technology in small communities at zero-energy cost and with remote control process based on monitoring the electricity generated by electroactive microorganisms.

- ***Microbial Electrochemical Systems for valuable products synthesis***

Microbial Electrochemical Systems dedicated to valuable products synthesis are normally referred in literature as Microbial Electrosynthesis System. In these systems, electroactive bacteria use the provided cathodic electrons to reduce carbon dioxide or convert other chemicals into different organic compounds. Thus, the cathodic electrons can be used for acetogenic bacteria to produce organic acids (Clauwaert *et al*., 2008) and for methanogens to produce hydrogen, which can be further converted to

methane in an external anaerobic digester (Clauwaert *et al*., 2008). From carbon dioxide can be synthesized Acetate (Jourdin *et al*., 2016; Patil *et al*., 2015) and butyrate (Ueki *et al*., 2014) and ethanol from acetate at the cathode (Steinbusch *et al*., 2010). Additionally, other inorganic chemicals as struvite have been produced in the cathode chamber (Cusick *et al*., 2014).

- ***Microbial Electrochemical Systems for water desalination***

They are normally referred in literature as *Microbial Desalination Cells* (MDCs). MDC became of great interest because it can be used as a stand-alone technology for simultaneously removing organics and salt with energy production (Saeed *et al*., 2015). The basic principle of these systems is to utilize the potential gradient generated across the anode and cathode to drive desalination *in-situ*. They show a variation regarding to the basic two chamber design in using a third chamber for desalination limited by an anion exchange membrane (AEM) and a cation exchange membrane (CEM) in between the anode and cathode chambers. As a consequence of organic substrate oxidation and by hence electron production by electroactive bacteria in the anode chamber, anions (e.g., Cl^-) from the salty water in the middle chamber migrate to the anode and the cations (e.g., Na^+) to the cathode for charge balance, thus the middle chamber solution is desalinated (Cao *et al*., 2009; Luo et al., 2012; Borjas Hernández, 2016, PhD dissertation).

- ***Microbial Electrochemical Systems as biosensors***

In the wastewater field, researchers have successfully developed miniaturized biosensors based on MFCs for measuring biological oxygen demand (BOD) (Peixoto *et al*., 2011), acetate (Li *et al*., 2011), pH (Uria *et al*., 2016) as well as toxic compounds (Dávila *et al*., 2011; Liu *et al*., 2014). A novel approach on that is the immobilization of cells inside silica gel and carbon felt as a new strategy for constructing ready-to-use artificial bioelectrodes of *Geobacter sulfurreducens* (Estévez-Canales M., 2016).

In addition to the use of biosensors for measuring a chemical property, it has also been developed the concept of biosensors as a tool for

assessing the microbial electroactivity by employing screen-printed electrodes, a novel low-cost platform at the microscale level (Estévez-Canales *et al*., 2015a). This was also used for characterizing the response of *Geobacter sulfurreducens* under diverse physiological states revealing different electron transfer responses.

In sediments and subsurface environment, sedimentary MFC has monitored microbial metabolism (Friedman *et al*., 2012; Wardman *et al*., 2014) and microbial activity during subsurface bioremediation (Williams *et al*., 2009).

- ***Microbial Electrochemical Systems for metal removal and recovery***

Microbial Electrochemical Systems have already demonstrated to remove and recover metals from different wastewaters through the oxidation and reduction reaction oriented processes in either the anode or cathode. For instance, it has been reported the uranium removal and recovery from contaminated groundwater with poised electrodes serving as electron donors for microorganisms (Gregory and Lovley, 2005) and also that Cu^{2+} is reduced to metallic copper on the cathode of a MFC coupled to the microbial oxidation of organic matter and the electricity generation (Heijne *et al*., 2010; Rodenas Motos *et al*., 2015).

The main drawback of the microbial mediated process is that high concentration metal solutions generally inhibit microbial activities and reduce system efficacy. The abiotic process in the cathode usually employs low catholyte pH in order to keep metal dissolved in acidic condition, which can be problematic from the environmental and the operational point of view.

- ***Microbial Electrochemical Systems for restoration of polluted environments***

Another emerging environmental application for Microbial Electrochemical Systems is using the electrodes to serve as electron acceptors or donors for removing contaminants from polluted environments. These systems have been traditionally referred as MFCs for pollutant degradation assisted by electrodes in liquid matrix, (e.g., wastewater) and sedimentary MFCs for pollutant degradation in soil and sediments. In 2014 these systems were firstly called Microbial Electroremediating Cells (MERCs) by Rodrigo *et al*. (2014) and described as devices to overcome electron acceptor/donor limitation and maximize metabolic oxidation/reduction of pollutants.

Microbial Electrochemical Systems have the potential of removing a wide range of organic compounds and has demonstrated efficient removal of nitrogen (Clauwaert *et al*., 2007a; Tejedor-Sanz *et al*., 2016a), sulphate (Coma *et al*., 2013), chlorinated hydrocarbons (Strycharz *et al.,* 2008; Aulenta *et al*., 2011), antibiotics (Harnisch *et al*., 2013; Guo et al., 2016), herbicides (Rodrigo *et al*., 2016; Domínguez-Garay *et al*., 2016) and petroleum hydrocarbon (Rodrigo *et al*., 2014; Zhang *et al*., 2015b). All bioremediation processes are influenced by the physical and chemical conditions of the contaminated matrix. MERCs have shown high versatility assisting bioremediation processes in wastewater (Tejedor-Sanz *et al*., 2016a), sediments (Yu *et al*., 2016), soil (Domínguez-Garay *et al*., 2016) and manure (Sotres *et al*., 2015). Soil/sediment remediation can be even more challenging than groundwater remediation because soil types and textures significantly affect the permeability of oxygen and water and therefore influence the mass transfer of ions and contaminants, leading to performance variations. On top of that, the electrodes are typically separated spatially according to the scale of natural redox gradients, which leads to a high internal resistance (Dominguez-Garay *et al*., 2013).

Thus, the next section has been entirely dedicated to describe the fundaments of MERCs and the state of the art of these systems applied to soil, sediment and manure bioremediation, the emphasis of this publication.

1.2. Microbial Electroremediating Cells (MERCs)

1.2.1 MERCs: Performing bioelectroventing

Traditional MFCs consist of an anode, a cathode and an optional separator, e.g., a cation membrane. Microorganisms in the anode chamber can oxidize substrates that act as electron donors. The anode is connected through an external resistance to a cathode where electrons are finally consumed by an electron acceptor as oxygen (Tender *et al*., 2002; Ventaka-Mohan *et al*., 2009; Dominguez *et al*., 2013). MFCs can be modified in interesting and valuable ways for sustainable environmental remediation. Microbial Electroremediating Cells (MERs) share some MFCs principles, but with different purpose. Although both are Microbial Electrochemical Systems, they differ in the operation mode. While MFCs aim to maximize the power generation (Watts), MERCs aim to reach maximum current production (Amperes) through maximizing metabolic oxidation/reduction of organic/inorganic soil compounds (Rodrigo *et al*., 2014).

In an experimental modus operandi this is achieved configuring the MERCs with external resistances close to the short circuit or setting potential electrodes that favour the current production. So, regarding to the power curve of a bioelectrochemical device (Fig.1.4), the MFCs work under an external resistance that allows performing at maximum power and MERCs work close to short circuit where the power is almost zero, offering an alternative to solve the constraints of electron acceptors and favouring the degradation of contaminants under extreme reductive conditions.

In MERCs, electroactive microorganisms oxidize an organic pollutant acting as an electron donor and use an anode as a sole and inexhaustible electron acceptor in a strategy so called *Bioelectroventing*, in allusion to the

similarities with the traditional bioremediation technique *bioventing* where oxygen is artificially applied as electron acceptor (Fig.1.5).

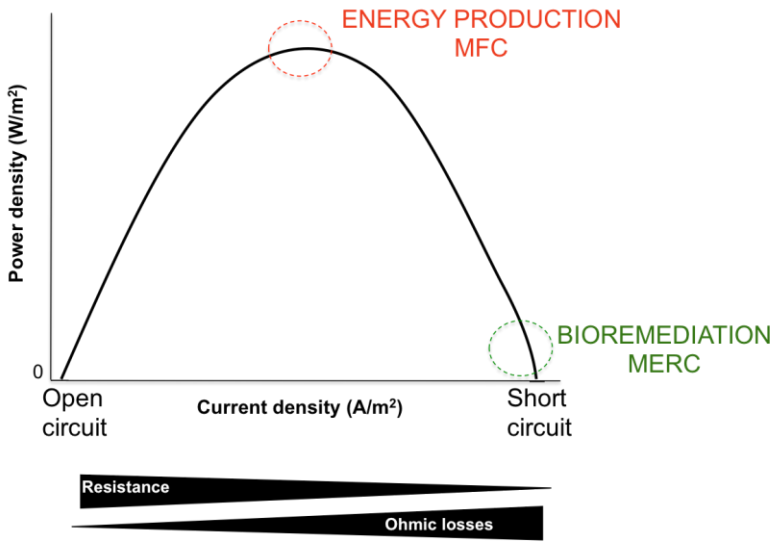


Figure 1.4: Power curve of a Microbial Electrochemical System. MERCs work at high current density and low power density to maximize metabolic oxidation of organic/inorganic soil compounds optimizing bioremediation processes.

Likewise, electroactive bacteria can also use electrodes as electron source for reducing pollutants (Rosenbaum *et al*., 2011). Feeding electrons to microorganisms living on electrode surfaces has significant potential to contribute to bioremediation of a diversity of contaminants, including radioactive and toxic metals (Gregory and Lovley, 2005; Tandukar *et al*., 2009), chlorinated compounds (Aulenta *et al*., 2009; Butler *et al*., 2010; Strycharz *et al*., 2010; Strycharz *et al*., 2008; Thrash *et al*., 2007), sulphate (Coma *et al*., 2013), and nitrate (Gregory *et al*., 2004; Park *et al*., 2005; Tejedor-Sanz *et al*., 2016a).

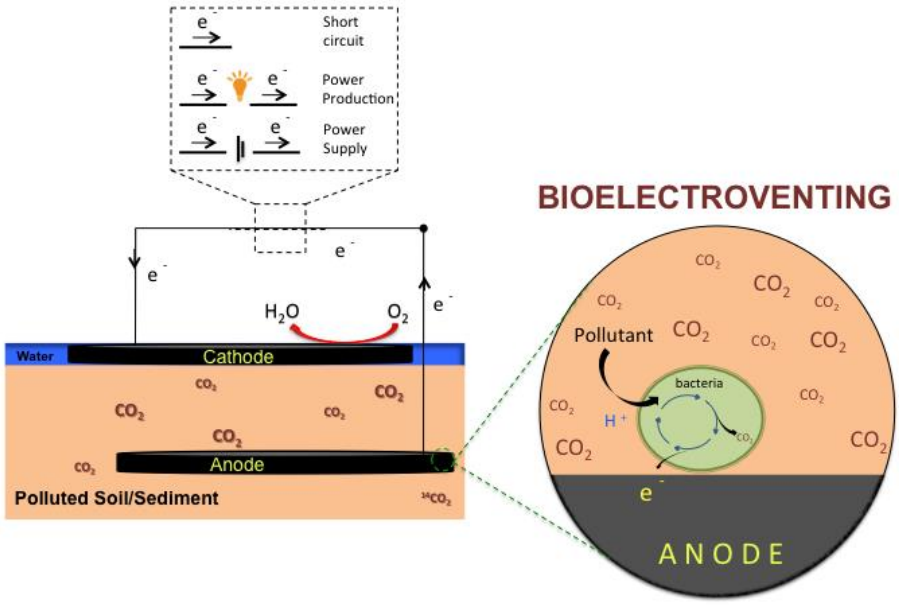


Figure 1.5: MERCs are a highly versatile technology and so a plethora of choices can be made regarding to the operation modus, tailoring the potential electrode for different bioremediation processes. The emphasis of this publication focuses in maximizing metabolic oxidation of organic compounds at the anode, used by electroactive microorganisms as a sole and inexhaustible electron acceptor in a strategy so called *Bioelectroventing*.

1.2.2 MERCs applied to soil/sediment: a new redox scenario

1.2.2.1 Fundamentals of MERCs for enhancing bioremediation in Soil/Sediment

Bioremediation based on the metabolic activities of microorganisms is gaining popularity due to its cost effectiveness and environmental benignity regarding to physicochemical methods, such as dredging, ozonation, and electrochemical degradation with high cost and energy consumption associated to an environmental aggressive nature. The introduction of xenobiotic chemicals into the environment drives microorganisms to evolve new catabolic pathways either to exploit the new carbon and energy sources. The diverse catabolic activities due to the presence of catabolic genes and

enzymes (De Carvalho *et al*., 2005; Khomenkov *et al*., 2008; Van der Geize and Dijkhuizen, 2004) and other adaptation-strategies such as the production of biosurfactants (Ron and Rosenberg, 2002), award the microorganisms with the capability to have a decisive roll in the bioremediation of contaminated sites.

One of the main difficulties in the bioremediation of subsurface environments contaminated with organic compounds or metals is the optimum delivery of an electron acceptor or donor to better promote the desired biodegradation. A number of experiences trying to supply additional electron acceptors like oxygen (bioventing) (García *et al*., 2010; Kabelitz *et al*., 2009) or humic acids (Lovley, 2000) to the environment have been developed as a bioremediation strategy. These approaches offer little control over the potential for unwanted metabolic reactions to occur and the accumulation of fermentation products as well as biomass. Furthermore incurs extra cost, causes secondary pollution concerns, sediment perturbation and accelerate contaminants release to overlying water (Pandey and Fulekar, 2012; Thrash and Coates, 2008). Alternative electron acceptors, such as Fe^{3+} and sulphate that support anaerobic respiration can also be exploited (Lovley, 1997; Anderson and lovley, 1997), but electrodes represent a very convenient alternative as electron acceptor. Employing MERCs provides a less aggressive, flexibly deployable and unceasing source of electron acceptor or donor, eliminating the need for costly chemical amendments and secondary pollution concerns. Furthermore electrodes have the unique capability of co-localizing the contaminants, electron acceptor/donor and microorganisms (degraders) on the same surface, causing minimal disturbance in soils/sediments structure and properties and allows a significant reduction in methane gas emission from aquatic environments (Ueno and Kitajima, 2012; Ueno and Kitajima, 2014).

So, electrode-assisted bioremediation (referred as well as “electrochemical biostimulation” or “bioelectrochemical remediation”) is a promising strategy to overcome the absence of suitable TEAs in anaerobic and strong reductive environments like flooded soils and sediments,

responsible of the limited biodegradation of organic pollutants (Megharaj *et al*., 2011), unlocking the *in-situ* microbial respiration rate. But electrodes not only overcome the TEA. Burying an electrode in flooded soil may remodel the redox scenario and as a consequence may influence the dominant energy metabolic pathways.

One of the key electrochemical parameters to trigger decisive biodegradation routes to enhance the biodegradation rate of polluted compounds is the electrode potential. This parameter, settled by the MERC operation mode, governs the microbial electron releasing capabilities, determining from a thermodynamic point of view the metabolic pathway used and the theoretical energy gain for the biocatalyst (Schröder, 2007). To date, most studies on electrode-based biostimulation used a soil-buried electrode with a typically negative potential, established spontaneously across the soil-water interphase as a result of spatially segmented reduction-oxidation reactions (Li and Yu, 2015), so it can be insufficient or inappropriate to drive the transformation for many recalcitrant organics (Zhao *et al*., 2006). Alternatively, an external voltage can be applied between anode and cathode for stimulating bacteria activity and consequently pollutant removal (Aulenta *et al*., 2007; Chun *et al*., 2013). However, those studies just set a constant voltage value between electrodes, but no control over the anode (the electron sink electrode) was performed.

A more advanced strategy for coping with the electron redox unbalance would be to set up electrodes at a concrete potential (Fig. 1.6). To maintain the potential of one of the electrodes under a selected value, we need to work with a 3-electrode configuration: the so-called working electrode (WE), the reference electrode (RE) and the counter or auxiliar electrode (AE). This is one of the most extended configurations since it allows controlling the anodic or cathodic reactions, which is crucial for the study of the microbial-electrode interaction and microorganisms may have more redox favourable TEA for performing the oxidative reactions. A higher anode potential may increase the energy per electron transferred which is available for growth and cell maintenance, resulting in a higher microbial density and current

generation (Aelterman *et al*., 2008; Finkelstein *et al*., 2006; Busalmen *et al*., 2008). Actually electrodes artificially polarized at potentials as high as +600 mV (versus Ag/AgCl) have been reported to show a high impact in the microbial degradation activity in herbicides-contaminated soils (Rodrigo *et al*., 2016).

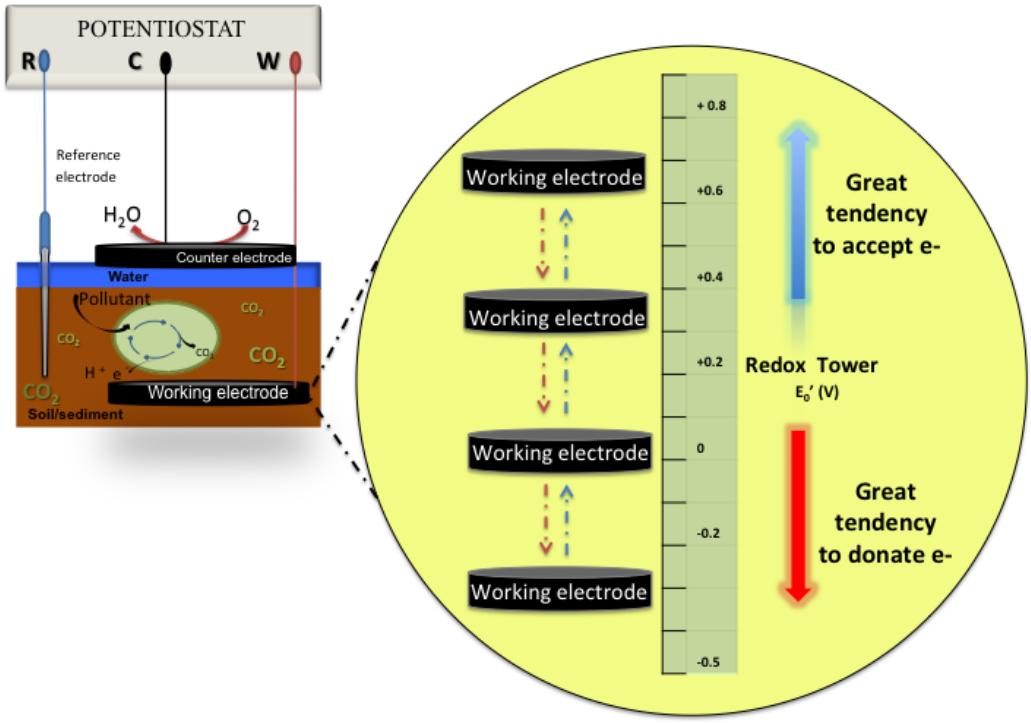


Figure 1.6: 3-electrode configuration MERCs allows the controllability of the bioremediation processes by tuning electrochemical parameters as the working electrode potential. Thus, it is possible to establish novel redox scenarios with untypical TEAs potential in anoxic and extreme reductive environment, stimulating new energy metabolic pathways in native microbial communities.

As is shown, the electrodes not only overcome the TEA limitation but also allow the controllability of the bioremediation processes by engineering the environmental redox conditions and selecting microbial activities that remediate contaminants of concern. In consequence, electrode-assisted

treatment offers a malleable terminal electron acceptor capable to adapt to different redox-dependent processes and perform *in-situ* bioremediation by avoiding chemicals consumption or soil manipulation with negative environmental consequences.

1.2.2.2 Current developments of MERCs in soil/sediment remediation

Recently, Microbial Electrochemical Systems employing electrodes as electron donors or acceptors have been proposed to stimulate biodegradation processes in soils and sediments, expanding the natural microbial metabolic activities of the native microorganisms and boosting the degradation of organic contaminants.

This section tries to review the bioelectrochemical remediation achievements in soils and sediments (marine and fresh water sediments) and Tables 1.1 summarize the literature published so far on this topic.

Degradation of organic compounds

Most persistent organic pollutants (POPs) in soils and sediments are difficult to break up due to usually insufficient availability of oxidation or reduction power, depending on their chemical nature (Huang *et al*., 2010). Zhang *et al.* (2010) reported for the first time a poised electrode (+300 mV vs. Ag/AgCl) that could serve as an electron acceptor for the degradation of toluene, benzene and naphthalene to CO_2 in polluted sediments, while Huang *et al*. (2011) found enhanced bioelectrochemical degradation of phenol, positively correlated with the removal of chemical oxygen demand (COD) in waterlogged soils.

As the primary energy source worldwide, petroleum hydrocarbon contamination is a widespread environmental problem. Several studies demonstrated that bioelectrochemical remediation stimulate and enhance hydrocarbon degradation in soil and sediments. As Table 1.1 shows, bioelectrochemical remediation studies has been systematically applied on hydrocarbon and its derivates including polycyclic aromatic hydrocarbons

(PAHs) (Zhang *et al*., 2010; Yan *et al*., 2012), diesel (Mao *et al*., 2016), phenol (Huang *et al*., 2011) and benzoate (Reija and Esteve-Núñez, 2011). All studies showed that the electrode approach could increase hydrocarbon degradation rate by multiple times without using any external energy which place this remediation process in a pre-commercial state.

Wang *et al*. (2012b) observed how the electrode led to duplicate the degradation rate of petroleum hydrocarbons in saline soil close to the anode (<1 cm). Very similar results were obtained by Lu *et al*. (2014a) that showed how total petroleum hydrocarbon (TPH) removal rate almost doubled in soils close to the anode (63.5− 78.7%) than that in the open circuit controls (systems with electrodes disconnected)(37.6− 43.4%). Morris and Jin (2012) reported as well an anode-assisted biodegradation study of petroleum hydrocarbons, this time in marine sediments, with nearly 12-fold faster degradation of sediment petroleum hydrocarbons compared to the natural attenuation control.

Enhanced oxidative degradation of PAHs by anode-assisted treatments has been reported in marine sediments (Zhang *et al*., 2010), fresh water sediments (Sherafatmand and Ng, 2015) and soil (Rodrigo *et al*., 2014). Yan *et al*. (2012) observed a significantly accelerated degradation of PAHs, concretely phenanthrene and pyrene in freshwater sediments in the presence of electrodes, showing an enhanced degradation compared with unamended soils and soils supplied with alternative TEA, i.e., Fe(III). Several studies have remarked that the presence of electroactive bacteria grown with easily degradable organics such as acetate or glucose can markedly stimulate the anaerobic degradation of contaminants as PAHs (Wang *et al*., 2012b). Indeed glucose amendment improved TPH biodegradation up to three times in anode-assisted bioremediation in saline soil (Li *et al*., 2016b) regarding to open circuit control, enhancing the anode remediation performance without glucose addition and increasing dehydrogenase and polyphenol oxidase activities, major microbial catalysts which are directly involved in decomposition of alkanes and aromatic hydrocarbons.

The produced electrical current from MERCs can as well offer an *in-*

situ real-time bioeremediation indicator with minimal environmental perturbation. Thus Reija and Esteve-Núñez (2011) registered the bioelectrogenic response of soil microbial populations, measured as current production, after adding benzoate to paddy soils, showing how the native microbial population previously exposed to benzoate showed a current production rate 5-fold higher than non- exposed populations after a pulse of benzoate. External remediation monitoring tools, e.g., electrical resistivity survey, might provide an accurate, real time, and easy remediation monitoring hydrocarbon tool. Mao *et al*. (2016) conducted studies for diesel removal in a tank experiment using resistivity geophysical survey, a non-invasive monitoring technique. Given the electrical insulating nature of diesel, the enhanced bioelectrochemical removal of diesel in sandy soils corresponded to an increase of conductivity, establishing a relationship between the diesel content and measured electrical conductivity variation.

Likewise it has been reported that cathodes can serve as a permanent and stable electron donor providing a low redox potential to drive reduction processes. Chun *et al*. (2013) investigated the eletrode-assisted degradation of weathered polychlorobiphenyl (PCBs) in river sediments. After ca. 90 incubation-days, natural attenuation reached a 15% PCB removal in contrast with over 60% PCB under electrochemical stimulation conditions (application of 1.5-V external voltage). The electrochemical system promoted synergies between reductive dechlorination of the 2,3,4,5-PCB at the cathode and a predominant further oxidation of the degradation intermediates to chlorobenzoates (CBA) and even ultimately to benzoate (BA), profiting the given sequential reductive and oxidative biodegradation that supplies the anode and the cathode. Not only biocathode stimulation is effective for enhancing the anaerobic reduction of PCBs. Yu *et al*. (2016) demonstrate the concept of bioanode stimulation towards PCB dechlorination in river sediments. Anodes with an applied potential of +0.2 V versus standard calomel electrode (SCE) (+0.441 V versus Standard hydrogen electrode SHE) substantially accelerated the dechlorination of PCB 61 (2.3 folds) compared to the open-circuit counterpart, transforming 58% of the total PCB 61 at the initial concentration, which overcome the 80% associated with acetate amendments

to the anode-embedded sediments. Bioanode stimulation would significantly improve the removal efficiency of the toxic refractory organic pesticide HCB in sandy soil, an organochloride used mainly as a fungicide (Cao *et al*., 2015). The addition of the anionic surfactant sodium dodecyl sulphate (SDS) into soil contributed to the improvement of HCB removal efficiency, reaching the 80% removal of the total initial HCB, 20% superior to systems with electrodes no connected (Cao *et al*., 2016). Xu *et al*. (2015) reported as well enhanced Total Organic Carbon (TOC) and PCBs degradation in sediments combining surfactants and bioelectrochemical remediation.

Several studies have shown that lab-scale Microbial Electrochemical Systems can be used to accelerate the biodegradation of contaminants of diverse chemical nature like cellulose and organic matter removal in freshwater sediments (Zhu *et al*., 2016) and high-polar organic chemicals such as alkanoates, sulfonic azo dyes, or phthalates in a contaminated environment (Xia *et al*., 2015). In this last study, Xia *et al*. (2015) found that nitrate additions enhance the bioremediation of contaminated sediment with low-polar chemicals (e.g., PAHs and polybromodiphenyl ethers). Viggi *et al*. (2015) stimulated the oxidative biodegradation of petroleum hydrocarbons in marine sediments creating an electrochemical connection between the anoxic sediments and the oxic-overlying water through a single conductive material peace, in a simple bioelectrochemical configuration denominated “snorkel”. The redox potential difference between both environments is the driving force of the electrons that circulate through the conductive material. After 200 days negligible degradation occurred under snorkel-free controls, while over 20% of the TPH was already removed by the snorkel-assisted assays. Yang *et al*. (2015) similarly inserted an iron rod into sediment with its lower part immersed in the sediment layer and its end attached with or without a carbon felt. By doing so, the lower portion of rod was served as the bioanode for MFC with substrate in sediment being decomposed to protons and electrons and the upper portion of iron rod as the biocathode conversion of nitrate into nitrogen.

Moreover, not just pollutant removal but effective clean-up was demonstrated by ecotoxicological analysis of a atrazine-polluted soil after

bioelectrochemical treatment (Dominguez Garay *et al*., 2016). More recently, Domínguez Garay, 2016 (PhD dissertation) concluded that using electrodes at a positive potential (+600 mV versus Ag/AgCl) enhanced the mineralization by 20-fold respect the electrode-free control, confirming that atrazine-polluted soil can be effectively cleaned-up in a short time by the use of MERCs.

Table 1.1: Microbial Electrochemical Systems achievements for remediation of soil/sediment contaminated environments.

Reference	Compound	Operation modus	Matrix	Biodegradation measurement	Treatment
Zhang *et al*., 2010	Aromatic hydrocarbon (benzene)	Working electrode at +0.3V	Marine sediments	Mineralization	Anodic
Zhang *et al*., 2010	Aromatic hydrocarbon (toluene)	Working electrode at +0.3V	Marine sediments	Mineralization	Anodic
Zhang *et al*., 2010	PAHs (naphthalene)	Working electrode at +0.3V	Marine sediments	Mineralization	Anodic
Huang *et al*., 2011	Aromatic organic compound (phenol)	External resistance 100 Ω	Paddy soil	Removal	Anodic
Reija and Esteve-Núñez, 2011	Organic Compound (benzoate)	External resistance 1000 Ω	Paddy soil	Current	Anodic
Wang *et al*., 2012b	Total Petroleum Hydrocarbon	External resistance 1000 Ω	Saline soil	Removal	Anodic

Morris and Jin, 2012	Total Petroleum Hydrocarbon	External resistance 1000 Ω	Marine sediments	Removal	Anodic
Yan *et al.*, 2012	PAHs (phenanthrene)	External resistance 100 Ω	Fresh water sediments	Removal	Anodic
Yan *et al.*, 2012	PAHs (pyrene)	External resistance 100 Ω	Fresh water sediments	Removal	Anodic
Zhang and Angelidaki, 2012	Nitrate	External resistance 470 Ω	Fresh water sediments	Removal	Cathodic
Chun *et al.*, 2013	Polychlorinated biphenyl	1.5 V electrode potential difference	Fresh water sediments	Removal	Cathodic
Reimers *et al.*, 2013	Refractory organic Matter	0.4 V electrode potential difference	Marine sediments	Removal	Cathodic
Rodrigo *et al.*, 2014 (at this thesis)	PAHs (dibenzo-thiophene)	External resistance 1000 Ω	Paddy soil	Removal	Anodic
Lu *et al.*, 2014a	Total Petroleum Hydrocarbon	External resistance 100 Ω	Soil	Removal	Anodic
Lu *et al.*, 2014b	Total Petroleum Hydrocarbon	External resistance 100 Ω	Soil	Removal	Anodic
Li *et al.*, 2014	Total Petroleum Hydrocarbon	External resistance 1000 Ω	Saline soil	Removal	Anodic

Zhang *et al.*, 2015b	Total Petroleum Hydrocarbon	External resistance 1000 Ω	Soil	Removal	Anodic
Sherafatmand and Yong NG, 2015	PAHs	External resistance 1500 Ω	Fresh water sediments	Removal	Anodic
Cao *et al.*, 2015	Hexachloro-benzene	External resistance 2000 /1000 /500 Ω	Soil	Removal	Anodic
Li *et al.*, 2015	Total Petroleum Hydrocarbon	External resistance 1000 Ω	Soil	Removal	Anodic
Song *et al.*, 2015	Biomass	External resistance 100 Ω	Fresh water sediments	Removal	Anodic
Zhou *et al.*, 2015	Biomass	Not specified	Fresh water sediments	Removal	Anodic
Xia *et al.*, 2015	High Polarity Chemical (alkanoates)	External resistance 1000 Ω	Fresh water sediments	Removal	Anodic
Xu *et al.*, 2015	Polychlorinated biphenyl	External resistance 1000 Ω	Fresh water sediments	Removal	Anodic
Yang *et al.*, 2015	Nitrates	Short circuit (snorkel)	Fresh water sediments	Removal	Short circuit (snorkel)
Zhu *et al.*, 2016	Cellulose	External resistance 100 Ω	Fresh water sediments	Removal	Anodic
Viggi *et al.*, 2016	Total Petroleum Hydrocarbon	Short circuit (snorkel)	Marine sediments	Removal	Short circuit (snorkel)

Rodrigo *et al.*, 2016 (at this thesis)	Isoproturon	Working electrode at +0.6V	Soil	Mineralization	Anodic
Yu *et al.*, 2016	Polychlorinated biphenyl	Working electrode at +0.25V	Fresh water sediments	Removal	Anodic
Mao *et al.*, 2016	Hydrocarbons (Diesel)	External resistance 100 /100 Ω	Soil	Removal	Anodic
Habibul *et al.*, 2016	Metals (Cd, Pb)	External resistance 1000 Ω	Soil	Removal	Anodic
Zhou *et al.*, 2016	Biomass	External resistance 1000 Ω	Fresh water sediments	Removal	Anodic
Domínguez -Garay *et al.*, 2016	Atrazine	External resistance 1000 Ω	Paddy soil	Removal	Anodic
Cao *et al.*, 2016	Hexachloro- benzene	External resistance 1000 Ω	Soil	Removal	Anodic
Ye *et al.*, 2016	Biomass	External resistance 100 Ω	Fresh water sediments	Removal	Anodic
Wang *et al.*, 2016	Nitrates	External resistance 1000/ 510/51/10/1 Ω	Fresh water sediments	Removal	Cathodic
Li *et al.*, 2016b	Total Petroleum Hydrocarbon	External resistance 1000 Ω	Saline soil	Removal	Anodic

Yang *et al.*, 2016	Phosphorus	External resistance 1000 Ω	Fresh water sediments	Removal	Anodic
Li *et al.*, 2016c	Total Petroleum Hydrocarbon	External resistance 100 Ω	Soil	Removal	Anodic
Domínguez-Garay,2016, PhD dissertation	Atrazine	working electrode at +0.6V	soil	Mineralization	Anodic

Attenuated eutrophication by MERCs

Application and subsequent run off of nitrogen and phosphorous rich fertilizers from agricultural lands can cause another form of pollution known as nutrient pollution, or eutrophication, in water bodies and sediments. The resulting algal blooms and subsequent dies off, settling and decay can result in water column anoxia and large-scale ecosystem disruption known as dead zones. A few studies actually investigated Microbial Electrochemical Systems to remediate eutrophication. Song *et al*. (2015), showed how anode can enhanced the macrophyte litter biodegradation in sediments of shallow lakes, opening the door of electrodes to be exploited as a new and promising tool to delay lake terrestrialization. So METs represent an alternative strategy for re-establishment of aquatic macrophytes in sediment-contaminated aquatic environments from eutrophic lakes, through an increased utilization of readily degradable but complex organic matters is sediments (Zhou *et al*., 2016; Ye *et al*., 2016). These organic matters are typically degraded anaerobically following the route of hydrolysis-fermentation methanogenesis and would lead to massive methane emission (Singh, 2001). Such processes could be readily interrupted by simply inserting a polarized electrode to raise the local redox potential (Ueno and Kitajima, 2014) altering oxidation-reduction potential and anaerobic metabolism pathways. Furthermore bioelectrochemical systems

has shown potential for eutrophication control of water bodies promoting the transfer of overlying water phosphorus P to the sediments, enhancing the microbial oxidation of Fe^{2+}, and increased the formation of stable phosphorus in sediment (Yang *et al*., 2016).

Zhang and Angelidaki, (2012) presented a new concept for *in-situ* nitrate and nitrite removal from eutrophic lakes by means of biocathode-based METs place in the overlying water, being the nitrogen removal in the system almost four times higher than under open-circuit operation. Wang *et al*. (2016) verified also the feasibility of *in-situ* denitrification via biocathode stimulation in eutrophic lake sediments showing the higher nitrate removal efficiencies can be achieved by using a low external resistance, optimizing the nitrate removal with external resistance as low as 1 ohm (60% of the initial nitrate concentration), while the nitrate concentration increased with external resistance of 1000 ohms.

Heavy metal detoxification by MERCs

Metal contaminated soil supposes a difficult environmental issue as most metals are not biodegradable and they can only be transformed to a different valence. The overall strategy of metal conversion in Microbial Electrochemical Systems is the use an electrode as the electron donor to reduce metal ions, and the metal products will either deposit on the cathode surface or precipitate at the bottom of the reactor for recovery. Additionally Habibul *et al*. (2016) studied the possibility to couple Microbial Electrochemical Systems and electrokinetic processes (direct electric potential application for removing the metal contaminants in soil *in-situ*) to move heavy metals (Cd and Pb) and other contaminates in the soil. The results indicated that the weak voltage electrical field generated at the anode could power the electrokinetic remediation. So thus, the electrode-electroactive bacteria interaction, further than direct pollutant biodegradation, might trigger a variety of reactions and pollutants transport processes in the contaminated soil due to electromigration, electroosmosis and electrolysis. The remediation and recovery of metals from soil and sediments using METs might be a new and promising approach. Given that many metals have high

market values, an ideal treatment strategy is not disposal but accumulating and recovering metals during the treatment process. However, when single chamber reactor is used, the high metal concentration may inhibit microbial activities and interfere with the biodegradation processes (Abourached *et al*., 2014; Catal *et al*., 2009).

1.2.2.3 Main factors influencing and limiting MERCs remediation performance

MERCs performance for soil and sediment remediation relies on engineering (system configuration, electrode selection) and environmental elements (microbial ecology and environmental conditions). In addition to the common thermodynamic and kinetic limitations of Microbial Electrochemical Systems, new challenges stand up from the distinctive features of soils and sediments, e.g., decreased pollutant mass transport and the subsequent limited bioavailability (Nielsen *et al*., 2007) and high internal system resistance (Rezaei *et al*., 2007). Over and above, the heterogeneous nature of soils and sediments strongly determined the bioremediation processes and the influence area of the electrode in contrast with standard Microbial Electrochemical Systems operating in bioreactors with homogenous solutions.

Engineering factors in MERCs for soil and sediment bioremediation

The maximal open circuit potentials (potential observed when no current is running through the MERCs electrical circuit) are below 0.8 V, the given natural redox potential difference between oxic water and anoxic soils or sediments (Lowy *et al*., 2006; Schulz, 2006; Stumm and Morgan, 1996). When the circuit is close, the system potential decreases, mainly because of the so-called overpotentials, which are potential losses due to the limitation in the electrons and protons transport because of internal system resistances. High overpotential takes place in MERCs especially with soils or sediments where the salt concentration and solution conductivity are low (Hong *et al*., 2009). This leads to lower redox potential of the anode and hence decreased anodic decontamination efficiency.

The distance between electrodes plays an important roll in MERCs performances. The more separated the electrodes are placed the higher is the system overpotentials, which determine negatively the electrochemical biostimulation of the electrodes on the pollutants. A tubular air-cathode design was proposed by Yuan *et al*. (2010) to overcome this overpotential constrain. In this prototype the cathode was covering the tube internal side, directly expose to the air and electrolytically connected to the anode with a catalytic and waterproof layer that replace the ionic exchange membrane present in standard MFC. This fact allows a satisfactory oxygen reduction reaction on the anode and overcomes the mandatory inter-space electrode to respect the separation of oxic and anoxic zones in flooded sediments and soils.

Another possible intervention to decrease the high overpotential in Microbial Electrochemical Systems is decreasing the high internal system resistance. Particularly this high internal resistance in soil/sediment is due to its low electrical conductivity that restrains the transport of ions between anode and cathode and consequently limit the performance. Some authors have overcome this limitation by changing the soil physical properties by stimulating colloid formation via the addition of silica (agricultural reagent) (Domínguez-Garay *et al*., 2013), decreasing the resistance and enhancing the proton mobility through preferential water channels formed between the soil particles and optimizing the nutrients utilization by soil microorganisms. Recently, Li *et al*. (2016c) enhanced bioelectrochemical remediation of petroleum hydrocarbons contaminated saline-alkali soil by 5 fold, decreasing the soil salinity by rinsing the soil, and decreasing the internal resistance by supplying carbon fibre that increased the soil conductive channels. So, after rinsed salt, the soil osmotic pressure decreased, which was beneficial to the microbial activity and the soil internal resistance significantly increased. This last effect was compensated by the increasing effect of carbon fiber over the high internal resistance and the charge output was enhanced by 110% while maximum current densities were increased from 81 to 304 $mA \cdot m^{-2}$. Other authors have decreased the soil resistivity (from 42.6 to 7.4 Ω) by increasing the soil moisture (from 23% to 33% water content), which improved the

current output and the removal rate of petroleum hydrocarbons (Wang *et al*., 2012b) (Fig.1.7).

Soil/sediment structure is a decisive factor in the bioelectrochemical bioremediation performance. Li *et al*. (2015) used soil with sand amendment bioelectrochemical assays, which led to change the soil structure, enlarged soil pores and provided more channels for ion and substrate transport. These modifications resulted in a higher remediation performance, increasing the TPH and **n**-alkanes removal 100% over open circuit (OC) assays. Zhang *et al*. (2015b) determined that anodes horizontally disposed improved remediation efficiency of TPHs, obtaining removals 50% superior to the vertical anode set up and 95% superior than with the disconnected anodes. The different electrodes position implied a change in the soil pH and conductivity due to the proton permeability in the waterlogged soil and the reduction of ion concentration.

A preliminary condition to obtain a high anode potential is the oxygen reduction at the cathode, which is not always optimal given the low oxygen concentrations and considerable oxygen consumption by heterotrophic aerobes. In the future introducing photosynthetic microorganisms, such as photosynthetic bacteria and algae, for *in-situ* oxygen generation (Cai *et al*., 2013; Xiao *et al*., 2012) should be an alternative to improve the bioremediation performances.

The constricted mass transfer in the sediments and soils also contributes significantly to the overpotential in Microbial Electrochemical Systems. Increasing the anode-soil/sediment contact might be a possible solution to decrease the mass transfer limitation. Increasing the total area of a unique electrode brings overpotential increases as a result of non-uniform potential distribution (Hsu *et al*., 2013). In this respect a few studies already established multi-anodes to overcome the mass transfer limitations without increasing the overpotential system. Li *et al*. (2014) demonstrated that the remediation could be extended to a larger range using three anodes systems with only one cathode, enhancing the total petroleum hydrocarbon, PAHs and n-alkanes. So multielectrodes might be one of the ways to balance the until

now limited influential electrode area, the barrier to demolish for an optimal full-scale field implementation. Another alternative might be column-type reactors with large-area anode directly exposed to the surrounding matrix, which face the mass transfer and the overpotential losses (Huang *et al*., 2011; Lu *et al*., 2014a; Yuan *et al*., 2010) (Fig.1.7). Lu *et al*. (2014b) using a column type reactor enhanced the TPH degradation until a distant of 90 cm regarding to the free-electrode control, which was more than ten times the electrochemical system radius. Nevertheless still further research should be conducted in this direction to face the internal resistivity and mass transfer constrains that brings the scaling up.

Until now all efforts pointed to improve the efficiency and configuration of Microbial Electrochemical Systems under waterlogged conditions because soil/sediment requires water saturation for a suitable proton transfer between electrodes. The greatest engineering challenge for the implementation of METs in *in-situ* environments is to overcome the need for the soil or sediment to be waterlogged. So it is the crucial importance the development of new system configuration that allows its application in non-flooded soils with the purpose of either harvesting energy of alternatively performing *bioelectroventing* to restore polluted environments. In that direction, the Bioe group Bioelectroegenesis from University of Alcalá developed a new configuration that overcomes this limitation by using a ceramic barrier. The low resistance of ceramic barrier, the optimal moisture of soil and the distance reduction between anode and cathode, resulted in a mass transfer loss reduction. This new free-flooded conditions configuration aims to implement the use of Microbial Electroremediating Cells in *in-situ* bioremediation processes (Domínguez-Garay, 2016, PhD dissertation).

Microbial factors in MERCs for soil and sediment bioremediation

Bioremediation depends strongly on microorganisms. However microorganisms involved in MERCs and their interactions with an inserted electrode are poorly understood. Burying an electrode in flooded soil or sediments may remodel the redox scenario and as a consequence may influence the dominant energy metabolic pathways. Predominance of anode

respiration over fermentation (Hunt *et al*., 2010; Pinchuk *et al*., 2011) and a suppression of several anaerobic species, such as archaea and sulphide producing populations at high redox potentials have been recently reported (Lu *et al*., 2014a; Ueno and Kitajima, 2014). Further research must be performed to unveil how electrodes at different potentials can affect the microbial communities and its bioremediation capabilities.

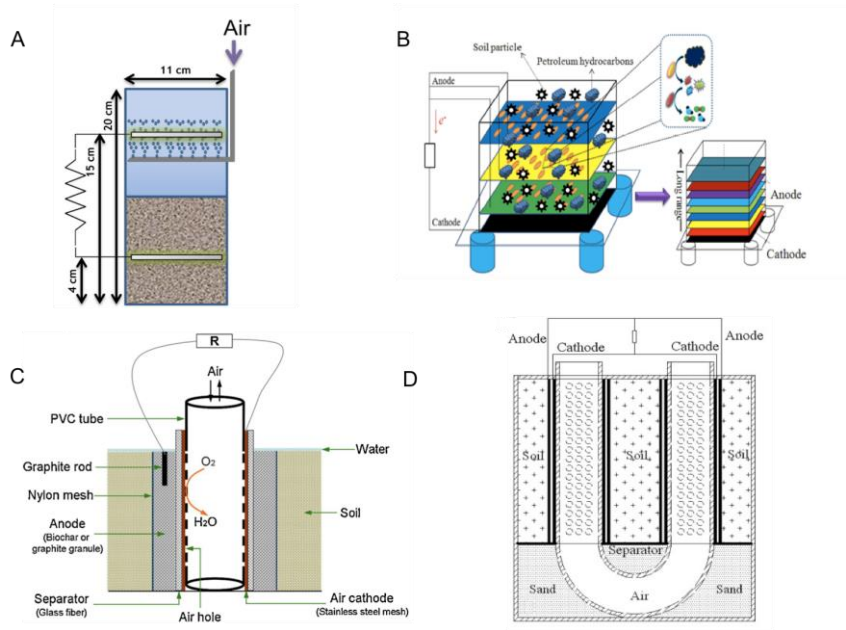


Figure 1.7: Schemes of different Microbial Electrochemical Systems configurations to stimulate the degradation of polluted soils/sediments. (A) Prototype with an air bubble cathode (from Sherafatmand and Yong NG, 2015), (B) multi-anode system (from Li *et al*., 2014, (C) column-type system (from lu *et al*., 2014a) (D) U-tube air-cathode soil system designed by inserting a hollow membrane electrode assembly into a rectangle box (from Wang *et al*., 2012b).

Many functional microbial groups are involved in different degradation and respiration processes, and electroactive bacteria are those that directly interact with the electrodes for extracellular electron transfer. So, the entire microbial community in METs comprises a complex net of the upstream fermenters that generate electron donors, breaking down complex organic

matters, and the downstream electron consumers that produce methane and/or electricity as end products, which role is mainly undertaken by electroactive bacteria (Li *et al*., 2014). Thus, most of the deposited organic matters cannot be directly utilized by electroactive bacteria (Lovley, 2006). The complex organic matters (OMs) is first broken down (through hydrolysis) to smaller size molecules such as long-chain fatty acids, fermentable sugars, aromatic compounds, amino acids, and then being further converted (through fermentation) to various volatile organic acids (VFAs) that are utilizable by electroactive bacteria (Fig.1.8). Acetate is typically the most important intermediary in carbon and electron flow in anoxic sediments (Lovley and Chapelle, 1995). The production and consumption of other organic substrates, as well as H_2, may also contribute to current production. Fermentation products can also be utilized by sulphate (SO_4^{2-}) reducing bacteria to produce sulfide (S^{2-}), that beside Fe(II), can diffuse through sediments and abiotically donate electrons to electrodes. Furthermore, sulfide can be abiotically oxidized at the anode surface to generate sulfur, which can serve as an electron donor for current production by microorganisms like *Desulfobulbus* (Holmes *et al*., 2004) and *Desulfuromonas* (Zhang *et al*., 2014) species. So, MERCs performance is not just a question of electroactive bacteria, able to transfer electrons from substrates to an electrode without aid of external mediators, but also relies in non-electrochemically-active microorganisms and its immense ensemble of syntrophic interactions.

Based on a literature survey 94 species are ascribed as electroactive (koch and Harnisch, 2016). There is not one specific ecological niche for electroactive microorganisms. Many species were found and isolated from soil, sediment and sludge but the natural habitat of the majority of electroactive microorganisms is described as “multiple” (77 species). For example, *Geobacter sulfurreducens* is usually found to dominate in environmentally stable sediments but at the same time it can also be enriched from wastewaters. *Delta*- and *Gammaproteobacteria* are generally the two major phylogenetic groups of electroative bacteria identified playing an important role in delivering electrons to the anode (Bond *et al*., 2002; Lovley, 2006; Nielsen *et al*., 2007). Information on the microbial diversity and their

detail functions associated with MERCs cathodes are still very limited. Given the oxic environment, both electroactive bacteria and some aerobic microbes such as *Pseudomonas* and *Novosphigobium*, also exit at the cathode (Clauwaert *et al*., 2007b; Erable *et al*., 2010).

Despite the microbial complexity, linking pollutant electrocatalysis in MERCs to the microbial communities involved may help to have a better insight and understanding of the implication of the anode-related microbial community in the pollutant degradation and therefore may help to optimize our envisioned application of MERCs (Daghio *et al*., 2016). *Geobacter metallireducens* was reported to oxidize toluene and benzoate while using the anode as electron acceptor (Zhang *et al*., 2010). Wang *et al*. (2012b) reported the enhanced degradation rate of petroleum hydrocarbons in soil close to the anode (<1 cm) with the concomitant hydrocarbon degradation bacteria (HDB) increased. In a hydrocarbon degradation study, *Comamonas testosteroni*, *Pseudomonas putida*, and *Ochrobactrum anthropi*, uncommon electrochemically active bacteria capable of hydrocarbon degradation, were selectively enriched on the anode, while hydrogen-oxidizing bacteria were dominant in soil samples (Lu *et al*., 2014a). During an enhanced bioelectrochemical remediation of petroleum hydrocarbons contaminated soil, Li *et al*. (2016b) reported an enrichment of species belonged to *Deltaproteobacteria* (*Proteobacteria*), *Flavobacteria* (*Bacteroidetes*) or *Clostridia* (*Firmicutes*). Moreover, oxigenase genes, widely used for the molecular monitoring of hydrocarbons biodegradation were quantified. Simultaneously comprehensive correlations were surveyed and the results confirmed that the bio-current stimulated the activities of naphthalene dioxygenase and xylene monooxygenase and thus the hydrocarbons degradation.

Marine sediment-hosted anodes poised at positive and oscillatory negative-positive electrode potential were enriched with *Deltaproteobacteria* parallel to the enhanced degradation of refractory organic matter. Interestingly *Deltaproteobacteria* were not enriched in the anode-adjacent sediments. In contrast phylotypes allied to the *Clostridia* dominated likely playing essential

roles in converting complex OM into simple fermentation products (Reimers *et al*., 2013). Pyrosequencing analysis showed a relatively high proportion of aromatic compounds-degrading bacteria and a growth methanogen inhibition in sediments adjacent to anodes employed to enhance litter biodegradation as a new and promising way to delay lake terrestrialization (Song *et al*., 2015). Zhou *et al*. (2016) reported an increased abundance of iron-reducing bacteria as *Geobacter*, *Desulfuromonas* and *Geothrix* using the bioanode stimulation as an alternative strategy for re-establishment of aquatic macrophytes in sediment from eutrophic Lakes. These distinct bacterial communities might regulate nutrients/trace metals availability to plants and then make some contribution to plant growth.

Bioanode stimulation for enhancing the anaerobic reduction of PCBs influenced significantly the microbial community (Yu *et al*., 2016). At genus level contained two types of microorganisms: electroactive bacteria represented by *Geobacter, Ignavibacterium, and Dysgonomonas*, and dechlorinating bacteria including *Hydrogenophaga, Alcanivorax, Sedimentibacter, Dehalogenimonas, Comamonas and Vibrio*. These results suggest that the presence of electroactive bacteria can promote the population of dechlorinating bacteria, which are responsible for PCB 61 transformation.

Thus, microbial metabolism and community structure distinctively respond to the bioelectrochemically stimulation. These distinct bacterial communities have an effect in the soil functional capabilities to degrade pollutants, relying not only on electroactive bacteria but also on synergies between the diverse local microbial communities in soil/sediment. So far, the molecule-level bioelectrochemical influences on remediation microbial activities are mostly unknown and a more comprehensive linking between the diverse microbial community and bioelectrochemical anode-influence is still to be established.

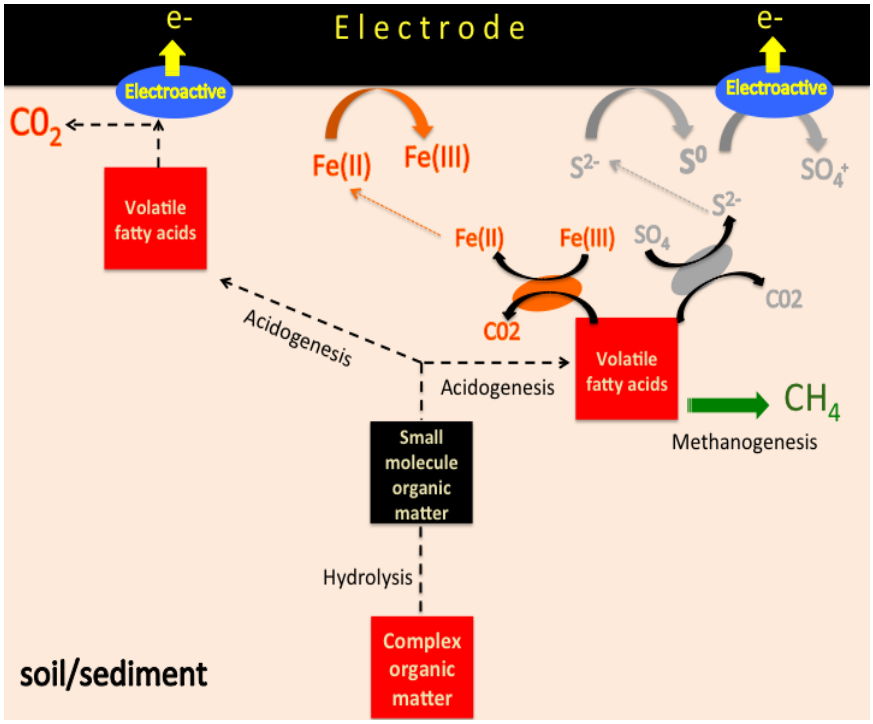


Figure 1.8: Metabolic processes involved in MERCs anodes. Fermentation products serve as electron donors for microbial current production at the anode surface. These fermentation products can be electron donors for methane production, sulfate reduction or Fe (III) reduction. Methane is not reactive with the anode, being an attenuated route in presence of electrochemical stimulation. The production of sulfide from sulfate could be suppressed at a raised potential. Fe (II) and sulfide can be abiotically oxidized at the anode. Elemental sulfur produced from the oxidation of sulfide can serve as an electron donor for additional microbially catalyzed current production. Figure adapted from Wardman *et al.*, 2014.

1.2.3 MERCs applied to manure: soil preventive remediation treatments

Intensive farming during the last decades has significantly increased livestock and swine manure production (Vilajeliu-Pons et a., 2015; Zhang *et al.*, 2015a; Ma *et al.*, 2016). These farming residues must be treated before being spilled into the environment given its high content of organic matter and nitrogen (mainly ammonium), inorganic salts, heavy metals and pathogens. Pit storage, pile storage, composting and aerated lagoons are some of the

manure-handling practices to reduce manure environmental impact in surface waters, aquifers, soil quality and air deterioration. However, Anaerobic Digestion (AD) is the most extended emerging manure management technology providing waste stabilization, organic matter digestion, odour and pathogens reduction and a digestate as an end-product. Additionally this technique has the benefit to convert waste solids into methane gas (Holm-Nielsen *et al*., 2009; Mitchell *et al.,* 2013). So AD is well established as an energy recovering technology in terms of performance and is technically and economically feasible (Angenent *et al*., 2004).

Nevertheless, most AD processes require backup techniques to further remove the effluent chemical oxygen demand (COD) and/or nutrients (Deng *et al*., 2006, 2008). AD indeed does not modify total N content of digestates, requiring additional treatment of the effluent, such as chemical precipitation of ammonium and phosphate as struvite (Cerrillo *et al*., 2015), ammonia stripping and its subsequent absorption in an acid solution (Laureni *et al*., 2013) or thermal concentration of the digestate (Bonmatí *et al*., 2003). Besides this fact, AD frequently suffers instabilities given to organic overload or the presence of long chain fatty acids (Palatsi *et al*., 2009), ammonia (Yenigün and Demirel, 2013), sulphide, light metal ions (Na^+, K^+, Mg^{2+}, Ca^{2+} and Al^{3+}), heavy metals and organic compounds such as chlorophenols or halogenated aliphatic compounds (Chen *et al*., 2008), which derive in a reduced biogas production and/or biogas methane content, and accumulation of volatile fatty acids (VFA).

The Combination of AD and Microbial Electrochemical Systems has emerged as a new promising strategy for stabilizing AD reactors under high organic loading rates aiming to recover energy and nitrogen simultaneously and maintaining the AD effluent quality within the desired limits (Cerrillo *et al*., 2016b). Min *et al*. (2005) demonstrated the feasibility of using electrodes to generate electricity and simultaneously remove organic matter and ammonia from swine manure and Kim *et al.* (2008) removed volatile acids and unpleasant odours from swine manure using a single-chamber Microbial Electrochemical System. Since then, several studies have focused on

maximizing power generation from swine manure (Chen *et al*., 2014; Capodaglio *et al*., 2015: Ma *et al*., 2016), dairy manure (Scott *et al*., 2007; Kiely *et al*., 2011; Wang *et al*., 2014; Zhang *et al*., 2015a), cattle manure (Lee and Nirmalakhandan, 2011) and other animal waste (Kim *et al*., 2014; Angosto *et al*., 2015). Thus Microbial Electrochemical Systems can produce additional energy, profiting the high organic load AD effluents and parallel increasing the biogas production (Cerrillo *et al*., 2016a).

On the one hand, one advantage of Microbial Electrochemical Systems with a two-chamber configuration is that the oxidation and reduction products are produced in separated compartments, making possible to recover ‘‘clean’’ products out of wastes (Hamelers *et al*., 2010). So ammonium can be removed and recovered since it is transferred through the cation exchange membrane from the anode to the cathode compartment where it can be recovered (Kuntke *et al*., 2014; Zhang *et al*., 2013). The electrode potential has been revealed as an important factor to implement the nitrogen recovery. Thus, Sotres *et al*. (2015) reported increased ammonia migration from anode to cathode by an increase in voltage in pig slurries and Cerrillo *et al*. (2016a) used a polarized anode (0 mV vs. Standard hydrogen electrode, SHE), as well in pig slurries, to remove 46% of COD and recovering 40% of nitrogen, overcoming the inhibited AD by organic and nitrogen overload.

Despite the promising MERC results in remediating polluted environments, scarce research, besides nitrate elimination, has been conducted with manure in this direction. Antibiotic degradation is one of the most suggestive additional scenarios to apply MERCs in manure remediation. Antibiotics are massively used with veterinary purposes (Sarmah *et al*., 2006). Due to its incomplete absorption, a significant fraction of these antimicrobials might be excreted in an unchanged form or as bioactive metabolites through animal feces and urine (Chee-Sanford *et al*., 2009; Wei *et al*., 2011; Nelson *et al*., 2011). When these substances reach agricultural soils either directly by grazing animals or via the application of manure to land as fertilizer, the environmental problem becomes more complex to solve and the risk of

contaminating other environmental compartments like surface and underground water increases (Jørgensen and Halling-Sørensen, 2000; Winckler and Grafe, 2001). This is of special importance as non-lethal concentrations of antibiotics can already enrich resistant microorganisms (Andersson and Hughes, 2012; Gullberg *et al*., 2011). These resistances can then potentially be transferred to other bacteria in soils, even to human pathogens, via gene transfer (Kemper, 2008; Gaze *et al*., 2013).

Besides the advantage that means the accumulated knowledge about MERC configurations and operations applied to other matrix as soil and sediment, manure shows favourable features to apply MERC principles, as a high conductivity, which might decrease the overpotential in the electrochemical system, and high organic carbon content, which could promote high microbial activity. Given the limited capability of AD, pit storage, composting and other manure-handling practices for antibiotic degradation (Mohring *et al*., 2009; Mitchel *et al*., 2013) MERCs might suppose an alternative to eliminate them in manure under confined conditions before its application to agricultural fields, as a preventing soil bioremediation strategy, avoiding the contamination of further ecological niche and the disadvantages of *in-situ* bioremediation processes.

1.3 Degradation of three pollutants by MERCs: a proof of concept

Industrialization, animal husbandry and agricultural development bring associated environmental effects on water, air, and soil/sediment resources. In the face of this challenge, there is a need for better management practices and regulations. This thesis presents the biodegradation analysis of three different pollutants by means of MERCs. They represent different environmental pollution sources: **dibenzothiophene** is a fossil fuel component that causes punctual contamination through accidental spills and industrial processes. **Isoproturon** is an herbicide used in order to increase the crops production, but irremediably pollutes the soil and water resources. The last compound evaluated is **sulfamethazine**, an antibiotic used with veterinary purposes that given its incomplete absorption in animals reach the environment by manure soil application as the first stop to the surface and

subsurface water. One common feature of the compounds evaluated is the scarce research of their biodegradation under flooded and extreme reductive conditions, despite the many anoxic environments like groundwater aquifers, riparian zones, subsurface soil and seasonally flooded agricultural soils where they can be found.

The environmental regulations consider simultaneously the human health and economic, ecological and social factors, according to two basic principles, a pollutant exposure as reduced as possible and the application of the most cost-effective techniques to prevent contamination processes. Currently the polluted soils in Spain are regulated by the Law 22/2011, of July 28, on *waste and polluted soils* and in the RD 9/2005 of 14 January, which establishes *a list of potentially soil contaminating activities and criteria and standards for declaring that sites are contaminated*. These regulations are based on the Directive 2008/98/EC of the European Parliament and of the Council of 19 November 2008 on waste and repealing certain Directives.

1.3.1 Dibenzothiophene, a policyclic aromatic hydrocarbon

PAHs include a group of priority organic pollutants of significant concern due to their toxic, genotoxic, mutagenic and/or carcinogenic properties (Ghosal *et al*., 2016). In this family, dibenthotiophene ($C_{12}H_8S$) (DBT) was used as a model compound for more complex thiophenes in several biodegradation studies due to the high persistence in environment (Ansari *et al*., 2009; Singh *et al*., 2012; Deriase *et al*., 2012; Boltes *et al*., 2007). Structurally, DBT has two benzene rings fused to a thiophene ring at the centre. It has a molar mass of 184.26 g/mol (Fig.1.10).

DBT is a component of fossil fuels (coal and oil), which contains various heterocyclic organosulfur-containing compounds. Combustion of fossil fuels generates many waste products like sulfur dioxide, which have bad effects on environment, plants, humans and animals' health. Therefore, environmental regulations have been demanding a reduction in the sulfur contents, till concentrations lower than 15 ppm (Maass *et al*., 2015; Nuhu,

2013). Biological methods of desulphurization are emerging as an effective tool for removal of sulphur, using the microbes to metabolize sulphur for their own growth (in bacteria, sulphur contributes 0.5–1.0% to cell dry weight). Beside sulfur level requirements in industry, which has led DBT biodegradation research, ecological concerns arise because the ubiquitous presence of PAHs, particularly in industrial soils, sediments and oil-spill impacted marine environments (Wayland *et al*., 2008; Vila *et al*., 2015). For instance, Yin *et al*. (2015) reported DBT levels of 345 $mg.kg^{-1}$ in the oil spill occurred in 2014 (Galveston Bay) and 98 mg.kg-1 in the one occurred in 2010 (Deepwater Horizon accident), both in EEUU.

Owing to its high toxicity, inhalation of DBT causes lung disorders in human beings and causes inflammation if it comes in direct contact with the skin. Apart from its effects on human beings, it can also have harmful effects on aquatic life (Mishra *et al*., 2016).

Under aerobic conditions, bacterial oxidation of DBT has been shown to follow three distinct patterns and all of them include angular dioxygenase attack adjacent to the bridging atom, dioxygenase attack of the aromatic rings or monoxygenation attack at the methylene position at the sulfur atom (Bressler and Fedorak, 2000). As the caloric value is maintained because C-C bonds are not altered, the biocatalytic pathway known as “4S” results more attractive in the biodesulfuration processes for the fuel industry, removing the sulfur from DBT and its derivatives in a sulfur-specific manner without affecting the carbon skeleton via the 4S pathway (Gallager *et al*., 1993). Despite all these successful approaches, the achieved biodesulfurization rate of the currently used biocatalysts is still far away from what is required to develop a commercial BDS process (Mel *et al*. 2014).

Most of the research regarding biodegradation of DBT has been almost fully devoted to the aerobic metabolism. Kim *et al.,* (1995) reported in experiments with crude oil and distillates significant conversion of DBT under anaerobic conditions using a concentrated cell suspension of *Desulfovibrio desulfuricans* M6. However, those assays were performed in the presence of

an artificial electron donor, methyl viologen. *Desulfovibrio desulfuricans* M6, a sulphate reducing bacterium, was isolated from soil and selected for its high hydrogenase activity. Analysis of metabolites from DBT biodegradation found biphenyl, a sulfur free compound, as the major intermediate. The sulphate reduction pathway proceeded with DBT acting as the sole electron acceptor being the surfur selectively removed. Subsequently, Armstrong *et al*. (1997) studied sulphur removal from vacuum oil, de-asphalted oil, and bitumen, using sulphate-reducing bacterial cultures grown in the absence of methyl viologen, but none of these cultures desulphurized DBT extensively. Desulphurization of Kuwait crude oil was also carried out using *Desulfovibrio desulphuricans* M6, which resulted in 21% of sulphur reduction along with the release of H_2S (Kim *et al*., 1990). Armstrong *et al*. (1995) and McFarlan, (1999) utilized three different sulphate reducing bacteria including Desulphovibrio, Desulphotomaculum and Desulphomicrobium for the removal of sulphur from DBT. They found that all the three strains were capable of DBT biodegradation and produced H_2S and biphenyl.

Nevertheless, the anaerobic biodesulphurization process proceeds at a very slow pace, specifically when the reactants consist of organic compounds, as DBT, where only 10% of the energy generated as a result of substrate utilization is employed for microbial growth, while 50% under aerobic conditions (Mishra *et al*., 2016). Further research should be performed to implement anaerobic biodesulfuration because under the aerobic route is difficult to remove the final end product i.e. sulphate formed during the reaction, while H_2S formed in the anaerobic route can be easily treated in the desulphurization plants. Additionally, the cost of aeration is also minimized using the anaerobic process of biodesulphurization.

1.3.2 Isoproturon, an herbicide

Isoproturon (IPU, 3-(4-isopropylphenyl)-1,1-dimethylurea) ($C1_2H_{18}N_2O$) is a worldwide extensively used phenylurea herbicide for pre- and post-emergence control of annual grass and weeds in cereal crops (Hussain *et al*., 2015). It is constituted by a phenyl ring (C_6 H_4) with a C_1 - branched methyl urea and C_4 branched dimethyl (Fig.1.10). IPU acts inhibiting

the electron transfer to the photosystem II (PSII) and inducing oxidative stress. As a result, lipids, proteins and other cellular components are damaged, as evidenced by necrosis and chlorosis (Yin *et al*. 2008).

Isoproturon shows a Kd (distribution coefficient) value below 10mL g^{-1}, which suggests that is poorly retained in the soils (Chao *et al*., 2010). Hence, given its massive use, high mobility and water solubility (72 mg L^{-1} at 20 °C) IPU has been detected in both surface and ground waters exceeding the European threshold concentration of pesticides in drinking water (0.1 mg L^{-1}) (Fenner *et al*., 2013). Furthermore, IPU metabolites including 3-(4-isopropylphenyl)21-methylurea (MDIPU), N-(4-isopropylphenyl)urea (DDIPU) and 4- isopropylaniline (4-IA) have been commonly detected as contaminants in rivers, streams, lakes, marine water and groundwater around the world (Hussain *et al*., 2015). Toxicity of IPU metabolites such as MDIPU and DDIPU are getting increasing attention in risk assessment of IPU (EFSA, 2015) and indeed, aniline metabolite 4-IA was found to be 600 times more toxic than IPU (Tixier *et al*., 2002).

A new report from EFSA (2015) revealed that IPU is also an endocrine disrupting, in addition of being potentially carcinogenic, which led to its ban in EU since July 2016 (Commission Implementing Regulation (EU) No 2016/872, 2016). IPU has shown severe ecotoxicological effects as potential endocrine disruption (Orton *et al*., 2009) and mutagenic effects in mammalians (Behera and Bhunya, 1990). Numerous studies indicated that IPU adversely affected the algal growth and its metabolism in soil algal communities as diatom species (Pérés *et al.,* 1996), algal strains *Scenedesmus obliquus* and *Scenedesmus quadricauda* (Dosnon-Olette *et al*., 2010). Schmitt-Jansen and Altenburger (2005) conclude that IPU has toxic effects on periphyton communities even at sub-acute toxicity level. Furthermore, IPU has shown pronounced effects on non-target plants as wheat (Triticum aestivum) and beans (Yin *et al*., 2008; Liang *et al*., 2012) and bioacumulation in macrophytes as *Lemna minor*, which might suppose the food chain entry (Böttcher and Schroll, 2007). Regarding to animals, isoproturon showed sub-lethal effects on earthworms Tubifex, such as

reduced growth rate, decreased protein content and increased activity of antioxidant defence enzymes were also observed (Paris-Palacios *et al*., 2010; Mosleh *et al*., 2005; Mosleh, 2009). The use of IPU in agricultural soil also negatively affected the development of early larval stages of spawn and tadpoles of amphibians (Greulich *et al*., 2002), influencing as well the microbial activity and community composition, including bacteria and fungi (Widenfalk *et al*., 2008).

Degradation of IPU in soil environment primarily takes place through microbial processes (Ronhede *et al*., 2005). Since 2011 eleven IPU-mineralizing bacteria has been isolated and characterized from different regions of Europe and Asia, among them eight *Sphingomonas* (Li *et al*., 2016a). Given the similar intermediates detected during the IPU-mineralizing process, a common catabolic pathway has been proposed (Sørensen *et al*., 2003; Hussain *et al*., 2015). Two successive N-demethylations are followed by the cleavage of the urea side chain, generating 4-IA; 4-IA is transformed into 4-isopropylcatechol (4-IPC), which is the substrate for ring-cleavage (Fig. 1.9). Two alternative metabolic pathways were reported involving initial hydroxylation of the isopropyl side chain, resulting in either 1-OH-IPU, a dead-end metabolite just detected in bacterial cultures derived from soil, or 2-OH-IPU, described in agricultural soils (Lehr *et al*., 1996; Scheunert and Reuter, 2000). These metabolites were reported just under aerobic pathways. Just a few reports about the anaerobic degradation of this substance are available and they do not included information about either the microbial reactions or their metabolites accumulated under flooded conditions (Larsen *et al*., 2000; Larsen and Aamand, 2001; Janniche *et al*., 2010).

Although many microbial metabolites of IPU are known and IPU-mineralizing bacteria have been isolated, the molecular mechanism of IPU catabolism has just been elucidated in 2016 (Yan *et al*., 2016). In this study, genes encoding for the conserved IPU catabolic pathway were revealed, based on comparative analysis of the genomes of three IPU mineralizing *Sphingomonas* and subsequent experimental validation, enhancing the understanding of the microbial mineralization of IPU (Fig.1.9).

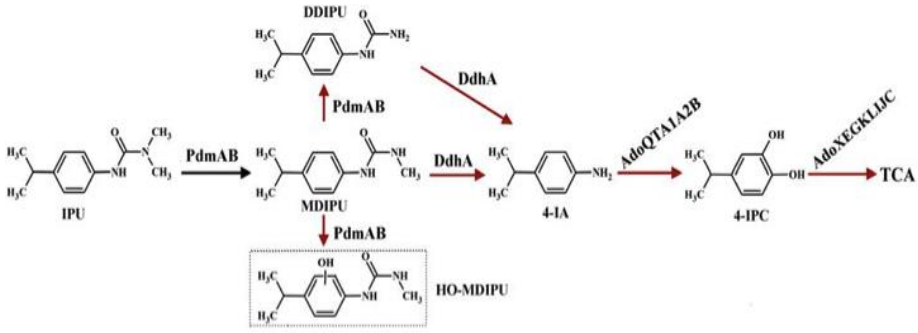


Figure 1.9: The IPU-mineralizing pathway in *Sphingomonas sp*.strain YBL2 and the enzymes involved. Black arrows referred to reaction characterized by Gu *et al*.2013 and red arrows reaction characterized by Yan *et al*. 2016. The dashed box indicates that the structure is predicted based on LC-MS/MS analysis. Figure from Yan *et al*., 2016.

1.3.3 Sulfamethazine, an antibiotic

Sulfamethazine ($C_{12}H_{14}0_2S$) (4-Amino-N-(4,6-dimethyl-2-pyrimidinyl) benzenesulfonamide; SMZ) is a veterinary antibiotic. It is used against infections of the respiratory tract, mainly in swine farming (Sarmah *et al*., 2006; Kim *et al*., 2011), impeding the production of folic acid in target microbes. It is constituted for a pyrimidine with methyl substituents at the 4- and 6-positions and a 4-aminobenzenesulfonamido group at the 2-position (fig.1.10).

Physical-chemical properties of SMZ include: molecular weight = 278.34, water solubility = 1.5 g L^{-1}, The octanol/water partition coefficient (Kow)= 0.89; pKa,1 = 2.65 ± 0.2, and pKa,2 = 7.4 ± 0.2. Sulfonamides have relatively high polarity and water solubility, which result in weak sorption affinity to soil particles and high mobility in soil (Thiele-Bruhn *et al*., 2004). Nevertheless this property is highly dependent on environmental factors, including but not limited to pH, redox condition, particle size distribution,

organic matter content, biological activity and temperature. SMZ is a diprotic acid and as a consequence, depending on the pH, SMZ can exist in cationic, neutral and anionic forms. The fraction of non-ionized and ionized forms of SMZ as a function of pH will affect the sorption of SMZ to soils and hence the fate and transport of this compound in the environment (Lertpaitoonpan *et al*., 2009). Therefore higher pH values increase the SMZ mobility. Contrarily higher organic carbon soil content impacts more dominantly than pH decreasing the mobility of SMZ (Lertpaitoonpan *et al*., 2009).

SMZ poor gastrointestinal absorption in animals can lead to the presence of SMZ in animal waste and its subsequent land application as a soil fertilizer may directly release it into the environment (Chee-Sanford *et al*., 2009; Wei *et al*., 2011; Nelson *et al*., 2011) being manure the most important vehicle for transporting veterinary antibiotics into the environment. SMZ has been already found in concentrations as high as 10 mg kg^{-1} on a manure dry matter basis (Aust *et al*., 2008). This fact acts as a trigger for the antibiotic contamination of water resources (Rajapaksha *et al*., 2014; Amarakoon *et al*., 2014; Hamscher *et al*., 2005; Barnes *et al*., 2008) and negative impacts to soil microbial communities (Nelson *et al*., 2011). Finally, the ubiquitous environmental presence of SMZ provokes its access to human food chain (Clark *et al*., 2005; Dolliver *et al*., 2007; Wang *et al*., 2006). Indeed, SMZ and its N_4-acetylated metabolite have been already found in human urine (Ji *et al*., 2010). In addition to chronic effects to human health, one of the greatest concerns with regards to antibiotics is the development and spread of antibiotic resistant bacteria (Kemper *et al*., 2008). Although antibiotic concentrations in the environment are below the ecotoxicologically effective mg L^{-1} levels, chronic environmental toxic effects and potentially synergistic effects cannot be excluded (Hernando *et al*., 2005).

Studies conducted by The National Center for Toxicological Research (NCTR) indicated that high doses of SMZ were associated with significant incidences of thyroid tumours in mice and rats and tumorigenic activity in rodents resulting in constant stimulation of the thyroid by thyroid-stimulating hormone (TSH) (Poirier *et al*., 1999). Humans, on the other hand, were found

to be insensitive to the SMZ-like inhibition of thyroid function. Several studies have reported SMZ as moderately toxic on the crustacea *Daphnia magna,* the marine bacteria *Vibrio fischeri* and the fish *Oryzias latipes* (De Liguoro *et al*., 2009; Kim *et al*., 2007).

Sulfamethazine is partly metabolized in liver (Vree *et al*., 1980) and excreted mainly beside metabolites through urine (Mitchell *et al*., 1986; Hardman *et al*., 2001). The major SMZ metabolites in swine are N_4-acetylsulfamethazine, desaminosulfamethazine, and N4-D-glucosyl sulfamethazine (Matusik *et al*., 1982; Nouws *et al*., 1985; Paulson *et al*., 1984; Adams, 2001, Grant *et al*., 2003). N_4-Acetylsulfamethazine was found presents the highest percentage in animal excretions and can be reconverted to the parent SMZ (Langhammer, 1989). N_1-methyl-SMZ was present in swine manure (Paulson *et al*., 1981) and other animal excreta (Garcia-Galán *et al*., 2011). Despite environmental SMZ removal has received considerable attention, mainly under aerobic conditions, information on the metabolites or metabolism mechanisms of SMZ is still limited. Lertpaitoonpan *et al*. (2015) reported presence of N_4-acetyl-SMZ and desamino SMZ in soils extracts which had been amended with manure (Lertpaitoonpan *et al*., 2015) and another 2 different SMZ degradation intermediates can be produced by fungal cultures (white-rot fungus *Trametes versicolor*) and purified laccase (desulfo-SMZ, N_4-formyl-SMZ). Thus the molecular mechanism of SMZ microbial catabolism is still far from being elucidated and further research is important to identify persistent transformation products, which may increase human or ecological risk when SMZ reach the environment.

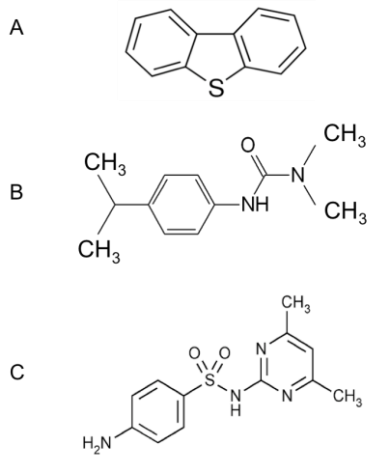


Figure 1.10: Chemical structure of DBT (A), IPU (B) and SMZ (C)

Objectives and Thesis Outline

The present thesis aims to evaluate the laboratory scale performance of Microbial Electroremediating Cells (MERCs) as versatile bioremediating tools in polluted environments. With this goal we have tested contaminants of different physicochemical nature, concretely a Polycyclic Aromatic Hydrocarbon (diobenzothiophene, DBT), an herbicide (isoproturon, IPU) and an antibiotic (sulfamethazine, SMZ). The research effort mainly focuses on the strategies for enhancing the pollutant degradation rate by remodelling the redox scenario of polluted soil and manure under extreme reductive conditions. The degradation assays were combined with the identification, quantification and fate of its degradation products and the subsequence toxicological assessment in order to evaluate the efficiency of this promising technology. Additionally we explore the role of both the electrode potential and the soil distance to the electrode, on selecting communities of heterotrophic bacteria participating in a bioelectroventing strategy for successfully cleaning-up an IPU-polluted soil. Thus, the following specific objectives were proposed:

1. To operate MERCs to stimulate the biodegradation and detoxification processes in polluted environments as DBT and IPU-polluted soil and SMZ-polluted manure.

This objective has been developed through chapter 2, chapter 3 (part I and II) and chapter 4. Different MERC configurations were compared with electrode-free attenuation in order to evaluate the efficiency of electrodes in pollutant degradation. In these chapters we present the concept of bioelectroventing as a strategy to supply inexhaustible electron acceptors as electrodes to maximise pollutant degradation. Ecotoxicological assays were developed in order to validate MERCs as a useful tool for biodegrading pollutants.

2. To evaluate the effect of the different redox scenarios established by the electrode potential on microbial electrochemical remediation of polluted environments.

This objective has been addressed through chapter 3 (part I and II) and chapter 4. Electrodes allow designing and remodelling unique redox scenarios in *a priori* close extreme reductive environments, supplying exclusive and scarce energy metabolic pathways to native microbial communities. Our goal was to analyse how the novel redox scenarios offer by the electrode and its different ways to be operated impact on the in pollutant biodegradation.

3. To examine the influence of plain conductive material on the microbial remediation metabolic activities.

This objective has been developed through chapter 2 and chapter 4. Our goal consisted in deepen about the stimulation that electroactive materials might have over syntrophic metabolisms as Direct Interspecies Electron Transfer (DIET) and how this stimulation can impact in the biodegradation of contaminants.

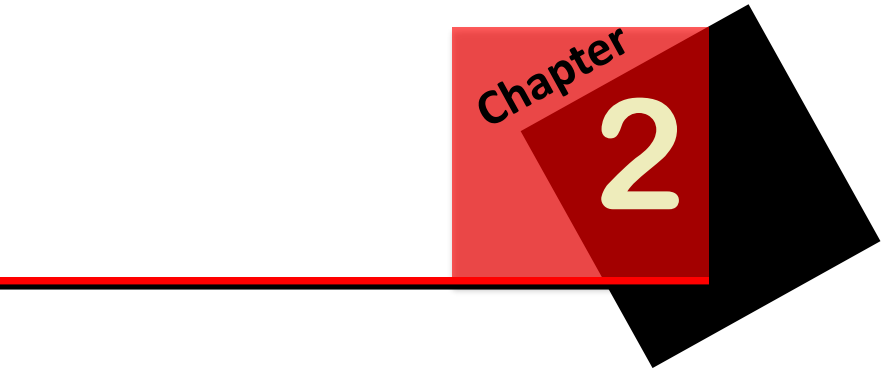
4. To assess the influence of electrochemical remediation in the pollutants environmental fate.

This objective has been addressed in chapter 3 (part I) and chapter 4. An overall profile and a mass balance of the ^{14}C-metabolites were performed to determine the environmental fate of the parent compound and its metabolites.

5. To explore the role of bioelectrochemical stimulation and soil distance to the electrode on selecting microbial communities.

This objective was developed in chapter 3 (part II). We aimed to analyse the distinct taxonomical shifts in the microbial community based on a high-throughput sequencing analysis. We also used the microbial community profile as reporter of the electrode influence area.

Chapters 2 **to** ***Chapter 4*** of this thesis pursue to achieve the objectives and correspond to material published or submitted to peer-review international journals prior to PhD defence. ***Chapter 1*** is introductory and describes a general framework for this thesis, and ***Chapter 5*** presents a general discussion, conclusions and future outlook. The researching activities described in chapter 3 and 4 were performed in collaboration with the institute of Soil Ecology and the institute of Microbe-Plant Interactions (Helmholtz Zentrum München) under the supervision of Dr. Reiner Schroll and Dr. Ulrike Dörfler.


Chapter
2

Microbial-electrochemical bioremediation and detoxification of dibenzothiophene-polluted soil

Chapter redrafted after:

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Microbial-electrochemical bioremediation and detoxification of dibenzothiophene-polluted soil

2.1 Abstract

Bioremediation is a relatively efficient and cost-effective technology for treating polluted soils. Nevertheless, the availability of suitable electron acceptors to sustain microbial respiration can reduce the microbial activity. This work aims to evaluate the impact of burying electrically conductive electron acceptors in soil for enhancing the removal of dibenzothiophene (DBT) by native electrogenic microbes. Although this novel approach is based on the use of a microbial electrochemical technology as Microbial Fuel Cells, our goal is not to harvest energy but to maximize bioremediation, so we concluded to name the device as Microbial Electroremediating Cell (MERC). Our results proved that stimulating the microbial electrogenic metabolism, DBT removal was enhanced by more than 3-fold compared to the natural attenuation. On top of that, ecotoxicological test using green algae confirms a decrease of 50% in the toxicity of the treated soil during incubation in MERC, in contrast to the unaltered values detected under natural conditions.

2.2 Introduction

Pollution of the environment by petroleum compounds is a matter of concern around the world. Significant impact on ecosystems and hazards to human health are caused by spills, leaks, and other releases of petroleum products in superficial water and soil (Boopathy, 2004; Onwurah *et al*., 2007). Biodegradation is the primary mechanism for removal of these pollutants in the environment. Nevertheless, the process is highly dependent on the existence of indigenous degrading species as well as several abiotic factors like temperature, nutrient availability and the nature of the compounds involved (Romantschuk *et al*., 2000; Haritash and Kaushik, 2009).

Polycyclic aromatic hydrocarbons (PAHs) are more recalcitrant to microbial attack than those aromatic compounds that have either single rings or branched hydrocarbons (Widdel and Rabus, 2001; Abha and Singh, 2012). Occurrence of PAHs in soil and sediments was extensively reported and condensed thiophenes are recognized as an important portion of PAHs containing sulphur in petroleum and products derivatives from fossil fuels (Banger *et al*., 2010; Leite *et al*., 2011; Ma and Zhou, 2011; Martins *et al*., 2012). In this family, dibenzothiophene (DBT) was used as a model compound in several biodegradation studies due to the high persistence in environment (Ansari *et al*., 2009; Singh *et al*., 2012; Deriase *et al*., 2012; Boltes *et al*., 2013).

Soil microorganisms can degrade a wide variety of compounds, including aromatic hydrocarbons under anaerobic conditions, but still, these compounds persist in sediments and soil in part due to the absence of a suitable electron acceptor to sustain microbial respiration (Widdel and Rabus, 2001). Therefore, the limitation in the availability of terminal electron acceptors (TEA) could reduce the success of soil bioremediation (Boopathy, 2004; Megharaj *et al*., 2011). A number of experiences trying to supply additional electron acceptors like oxygen (bioventing) (Kabelitz *et al*., 2009; García *et al*., 2010) or humic acids (Lovley, 2000) to the environment have been developed as a bioremediation strategy.

Such a TEA limitation could also be overcome using solid conductive electron acceptors like the electrodes used in sediment microbial fuel cells (sMFC). These bioelectrochemical devices use a sediment-buried electrode (anode) acting as electron sink for microbial oxidation of organic matter. The anode is connected through an external resistance to a cathode where electrons are finally consumed by an electron acceptor as oxygen (Tender *et al*., 2002; Venkata-Mohan *et al*., 2009; Domínguez-Garay *et al*., 2013). This system would offer an alternative to solve the constraints of electron acceptors, favoring the anaerobic degradation of contaminants. Although a diversity of electrode materials can serve as an electron acceptor for microbial respiration, graphite can provide a low-cost, low-maintenance and continuous

sink for electrons since it does not corrode or otherwise degrade during long-term deployments (Reimers *et al*., 2006; Tender *et al*., 2008), also it is easy to remove from the soil after the treatment, showing a low impact for the environment.

Our MERC-based methodology should not be confused with classical electroremediation process that involves passage through soil of direct current between appropriately distributed electrodes in order to force the migration of contaminants (e.g. PAHs, organochlorines) towards a confined zone (Pazos *et al*., 2010; Gomes *et al*., 2012).

Zhang *et al*. (2010) demonstrated for first time that graphite electrodes of a Microbial Electrochemical System could serve as an electron acceptor for the degradation of toluene and benzene in polluted slurries. Since then, bioelectrochemical-assisted sediment bioremediation has been reported for phenol (Huang *et al*., 2011), benzoate (Reija and Esteve-Núñez, 2011), petroleum hydrocarbons (Morris and Jin, 2012) and PAHs (Wang *et al*., 2012b).

The aim of this work was to use Microbial Electrochemical System principles for stimulating natural bacteria in order to enhance the biodegradation of DBT in soil. On top of that, we performed for the first time ecotoxicological analysis of a bioelectrochemical-assisted treated soil showing that it was successfully cleaned up. We propose to name it Microbial Electroremediating Cell (MERC) since the main purpose is not harvesting energy, but stimulating *in situ* bioremediation.

2.3 Experimental Procedure

Chemicals

The chemicals used were as follows: NaCl, $NaH_2PO_4 \cdot H_2O$, $K_2HPO_4 \cdot 3H_2O$, $MgCl_2 \cdot 6H_2O$ and sodium acetate, all with purity >98% and purchased from FLUKA. Acetonitrile (purity >99.93%) and dibenzothiophene (CAS 132-65-0, purity >99%) were obtained from Sigma Aldrich. Deionized water was used to prepare all media and solutions.

Soil

All laboratory-scale experiments were carried out using soil samples from an originally soil profile located on an alluvial plain in Calasparra (Murcia, SE Spain) and classified as HaplicFluvisol (Calcaric) and Typic Xerofluvent. It was formed on alluvial sediments deposited by the Segura River and developed on a rice fallow land. Soil samples were taken at 0-20 cm in depth. They were stored in hermetically sealed plastic bags, air dried for three d at room temperature and sieved (2 mm screen size) for laboratory analysis. A complete physical-chemical analysis of this soil was previously reported by Domínguez-Garay *et al*. (2013).

Microbial Electroremediating Cells (MERCs)

A MERC-multichamber station was disposed in plastic container. Each MERC unit was constructed by mixing 50 g of dry soil and 100 mL of deionized water. The chamber was 5 cm deep, 4 cm wide and 6 cm length. A 1 cm water body was maintained above the soil in order to ensure flooded conditions in the soil and to supply oxygen to the cathode. Water evaporation was compensated daily by the addition of deionized water. The cathode used in this experiment was graphite felt (SOFACEL), allocated in the water body. The dimension of the cathode was 5 cm x 3.5 cm x 5 mm (0.7 m^2 g^{-1} of surface area) and the anode was 3 cm x 3 cm x 0.5 cm (length x width x thickness). The material of the anode was graphite plate (SOFACEL) and it was buried 3 cm beneath the soil-water interface. Anode and cathode were connected by a copper wire using a 1 kΩ external resistor (R), while the contact area was sealed with a conductive epoxy material (Circuit Works) that was isolated with a non-conductive epoxy resin (Araldit Ceys®). The voltage difference between the two electrodes was periodically measured using a digital multimeter (Model 2700, Keithley Instruments, USA). Data were recorded every 10 min on a spreadsheet using ExceLINX_ (Keithley) via an interface card (GPIB Interface Boards, Keithley) linked to a personal computer. The recorded voltage was converted into current using Ohm's law (current = voltage/resistance). A scheme of MERC reaction principles and pictures of experimental set-up are shown in figure 2.1.

Degradation assays

Each assay condition (Table 2.1) was simultaneously replicated four times at 30±0.5°C. Soil was artificially polluted by spiking a 5 mL anoxic solution of DBT (5 mg L^{-1}) in phosphate buffer (pH 7). The ratio pollutant-soil was 0.5 mg DBT kg^{-1} soil.

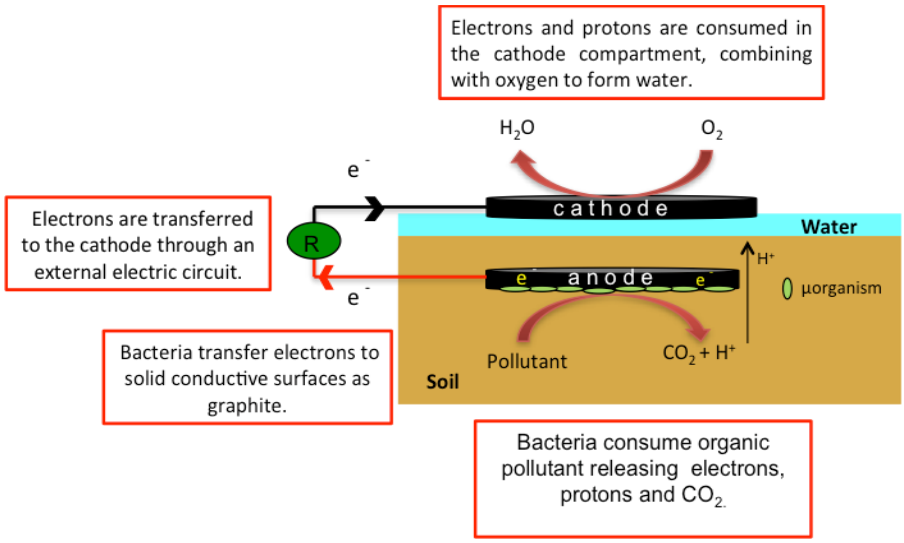


Figure 2.1: MERC scheme and main electrode reactions.

Samples were collected 3, 9, and 25 d after the pulse. In order to consider the abiotic physicochemical impact along the time, data from every series were normalized by subtracting the DBT-adsorption values from the sterile soil series at the corresponding sampling times.

In table 2.1, series 1 correspond to MERC operation in which electric connection was established between cathode and anode, while series 2 is identical but under sterile conditions. Physicochemical processes of contaminants are shown in a sterilized soil assay (series 3), which was taken as control assay. The natural attenuation processes using soil control was

shown in absence (series 4) or presence of a conductive material as carbon plate (series 5).

Analytical methods

DBT was analyzed after extraction of 50 g of soil with acetonitrile (5 mL g^{-1} of soil). Each soil-solvent mixture was sonicated during 1 h, dried under nitrogen and the residue was resuspended in 3 mL of the same solvent. Samples were filtered (0.2 µm filter, *Scharlau*) before HPLC-DAD analyses (Varian 9040) using a Kromasil C18 column (150 x 4.6mm, 5 µm, Phenomenex), with H_2O:ACN, 30:70 (v/v) as mobile phase (1.0 mL min^{-1}). Under these conditions, DBT was detected at 239 nm. The signal was calibrated in the range of 0.12-4.6 mg DBT L^{-1}. The same extraction procedure was carried out with electrodes to evaluate DBT adsorption on graphite.

Table 2.1: Experimental set-up of degradation studies.

Series	Electric connection	Graphite	Biological activity	Description
1	+	+	+	MERC
2	+	+	-	Sterilized MERC
3	-	-	-	Sterilized soil
4	-	-	+	Soil
5	-	+	+	Soil + graphite (Open circuit)

(+) presence; (-) absence

Control of cell viability in sterile soils

Biologic activity was tested by dish-plate inoculation. Eluates of soil were prepared using phosphate buffer (pH 8) in 1:10 soil-water ratio. 1:10 and 1:100 dilutions were carried out and incubated at 30 ºC in LB agar plates during 72 h. Colonies formation was detected only in plates seeding with eluates of non-sterilized soil.

Toxicity evaluation

Toxicity evaluation of soil extractions was carried out following the algal growth inhibition test (OECD TG201, 2008) with *Pseudokirchneriella subcapitata*. Algal beads of *P. subcapitata*, dissolving matrix and growing media were purchased from MicroBioTest Inc. (Belgium). Algal cells were de-immobilized according to the manufacturer's recommendations and cultured in 25 mL shaken flasks measuring the optical density (OD) at 670 nm to control growth process. 1.5 mL of extracted sample was evaporated until dryness under N_2 atmosphere and resuspended in algal culture medium. Exposition of algal cells to pollutants was performed in 96-well clear disposable microplates. A microplate reader (RAYTO RT-2100 C) was used to assess the growth of *P. subcapitata* measuring the OD at 640nm during 72 h. Plates were incubated in a culture chamber at 22 ± 2 ºC under continuous light. Three replicates of each extracted soil sample, negative control (DBT-free) and blank (algae-free) were assayed. Similarly, positive controls with DBT concentration from 0.25-5 mg L^{-1} were tested in order to evaluate toxicological parameters of this compound.

2.4 Results and discussion

Adsorption assays

The first series of experiments were developed to evaluate the physicochemical binding of the pollutant DBT to the matrix of soil. The reported values of the adsorption coefficient of DBT normalized by organic content (log K_{oc}) ranged from 5.2 to 20.5 in soil and sediments, while for the octanol-water partition coefficient (log K_{ow}) the value is 4.38 (Broholm *et al*.,

2000). Both parameters suggest that the pollutant tend to sorbs strongly onto the soil mass and this could have an impact on the bioavailability of DBT (Wilcke, 2000). Our results with sterile soil assays (series 3) confirmed that under the conditions of our extraction protocol an average value of 55%, of the initial DBT added was reversible bound to the soil matrix. The values of recovery DBT obtained in this series of assays were used to normalize the rest of series results so the physicochemical adsorption was excluded in the percentages of DBT removal. In spite of such an adsorption, our series of assays with natural soil showed how it did not prevent the biological activity for remediation, as is shown after 25 d of incubation in natural soil (Fig. 2.2a and Fig. 2.2b).

Stimulation of DBT-removal

Stimulating the microbial activity by the presence of electrical conductive material implies the addition of an extra component in the soil. Performing assays in sterile-MERC revealed how the sole presence of graphite (Fig. 2.2a and Fig. 2.2b) was responsible of a DBT sorption process leading to a 10% DBT removal along the course of the experiment. A similar result was previously reported by Zhang *et al*. (2010), although they reported a higher adsorption for benzene and toluene (70%) and also confirmed that toluene adsorbed on the graphite could be metabolized. Interestingly, when our DBT extraction protocol was applied to our buried graphite plate, no DBT was detected in the solvent in any of the assays (series 1, 2 and 5). This suggests that DBT could be metabolized directly on the anode surface or desorbed into the aqueous phase before being degraded.

In contrast with the sterile-MERC assays (series 2), when the MERC (series 1) was properly installed in natural soil, a stable current density of 20 mA m^{-2} was measured while 50% of the DBT was efficiently removed. In contrast just 11% of the initial DBT was removed under natural attenuation (series 4) during the same period of time. DBT-removal rate was diminished in the last phase of analysis (after 9 d of incubation) probably due to the fact that DBT and nutrient depletion were most rapid in MERC than in natural soil. In

addition, this trend should indicate a concentration dependence profile that should be explored in the future.

Previous studies also suggest that MERC technology may be used for enhancing biodegradation of petroleum contaminants in anoxic environments by providing an inexhaustible source of terminal electron acceptors to a polluted environment. Nevertheless, the experiments so far performed among a number of laboratories are difficult to compare due to differences in the device construction, characteristics of the sediment, microbial populations, and also the nature of the pollutants.

For instance, Zhang *et al*. (2010) demonstrated that graphite electrodes of a Microbial Electrochemical System can serve as an electron acceptor for the degradation of toluene and benzene, but the experiments were run with sediment slurry (1:4 (v/v) sediment: water sea. Huang *et al*. (2011) reported a phenol degradation rate 7-fold higher than the phenol removal obtained in soil without graphite, although phenol is more easily biodegradable xenobiotic (Van Hamme *et al*., 2003) compared with PAHs as DBT. Morris and Jin (2012) reported the application of METs to sandy beach sediments polluted with total petroleum hydrocarbons (TPH). They found that natural biodegradation of total n-alkanes was enhanced by 2- fold after 66 d of incubation, which is longer period time than we used in our series of assays.

In our system we enhanced natural attenuation by 3-fold despite the more recalcitrant nature of DBT, and the unfavourable low-conductivity value of our soil. For future assays, factors such as current density (lower external resistance), temperature, pH, bioaugmentation, etc. should be investigated to increase the degradation rates of PAHs.

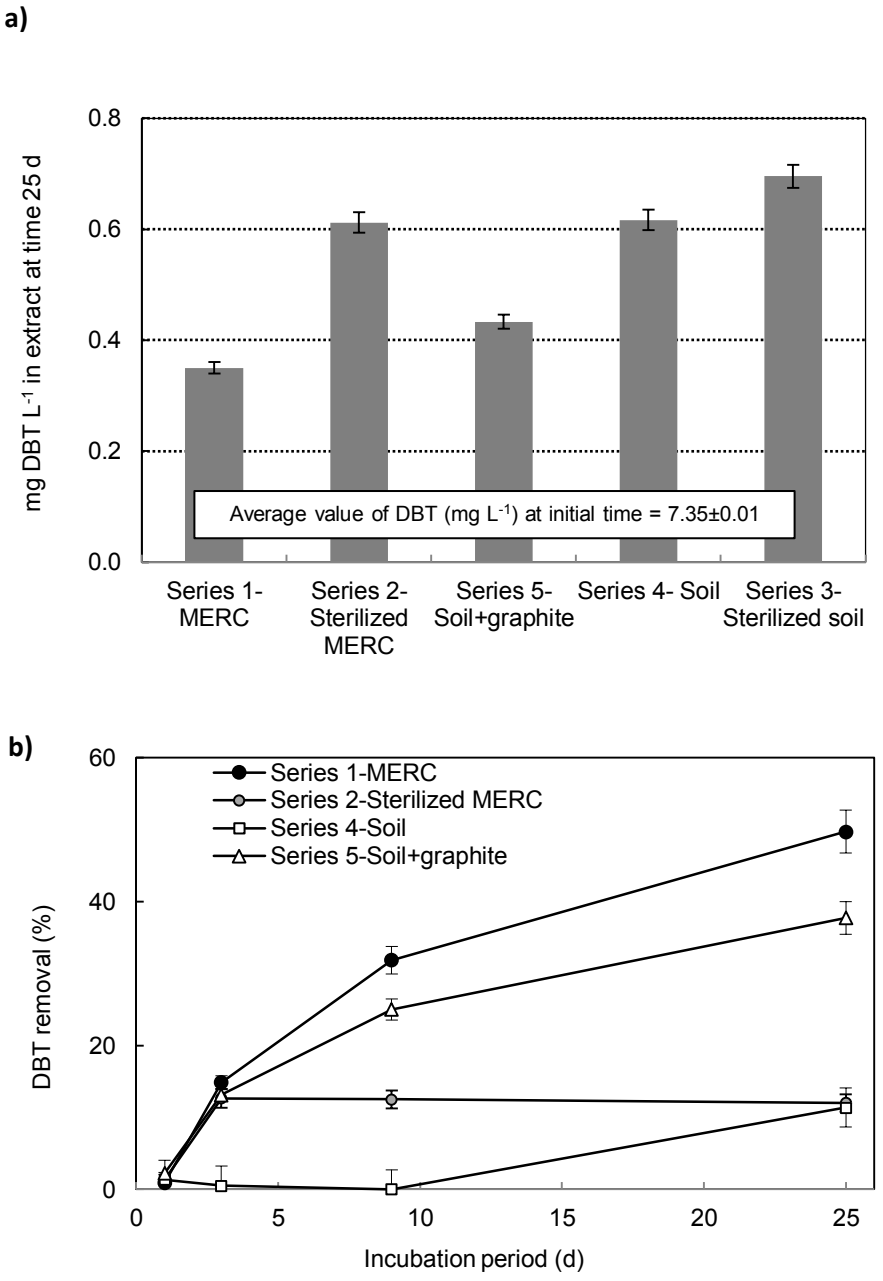


Figure 2.2: a) DBT recovery in extracts prepared with acetonitrile in all assays; b) DBT removal (excluding sorption to soil matrix) from polluted soil after different treatments. DBT removal was expressed in percentage related to the control assay (series 3). The error bars represent 5% of deviation of the mean value for triplicate assays.

Graphite-assisted bioremediation of DBT-polluted soil

In order to explore influence of using conductive material with soil bacteria we designed an experiment to prove if DBT bioremediation was also enhanced by the sole presence of a conductive material. For that purpose, we set up a series of assays using MERC operating in open circuit (series 5) so anode and cathode were not connected and electron cannot flow through the system. In that configuration the impact of the conductive graphite plate buried in the soil can be evaluated. Interestingly, the sole presence of graphite was denoted by a clear stimulation of microbial activity and 40% of the DBT was successfully removed at the final time (Fig. 2.2b).

Biodegradation of PAH as DBT requires a set of redox reactions that can be played by different strains, our results suggest that graphite would help these redox reactions to occur. In addition to this biodegradation enhancement, DBT removal was stimulated by an extra 11% when the graphite plate was connected to another electrode (cathode) as part of a MERC setup. A similar trend was shown by Wang *et al*. (2012b) when performing bioremediation of total n-alkanes (TNAs). Interestingly, a phenomenon called Direct Interspecies Electron Transfer (DIET) term was reported for first time by Lovley (2011b) to describe a mechanism based on a electrical connection between microbial species in order to develop syntrophic metabolisms through direct contact and in absence of electron shuttles or redox mediators (Summers *et al*., 2010). According to Liu *et al*. (2012) this metabolism may be accelerated in presence of a solid conductor material, demonstrating how the anaerobic conversion of organic matter in a digester could be enhanced by promoting DIET between bacteria and methanogens, in presence of a conductive activated carbon, which permits better electrical connections between microorganisms. A more natural conductive material as magnetite has been also reported to promote DIET among *Geobacter sulfurreducens* and *Thiobacillus denitrificans* (Kato *et al*., 2012) so acetate oxidation can be coupled to nitrate respiration, both reactions that cannot be performed by the single bacterial strains.

For a number of years it has been shown that biodegradation of petroleum compounds by native microbial population can be favoured especially by the addition of limiting nutrients (N and P) and organic residues (Haritash and Kaushik, 2009). Our results demonstrate the finding of novel tools for biostimulating based on insoluble amendments with low impact for the environment.

Ecotoxicological analysis

The disposal of soils contaminated by PAHs is typically carried out after reducing the levels of TPH, other organic compounds, metals, and toxic substances to specific concentrations marked by legislation as environmentally safe. In conclusion, a bioremediation task is not completed till the environment is cleaned up, and this is not necessarily linked to the absence of the original pollutant. Thus, remediation processes must be followed by ecotoxicity tests to ascertain that it has regained its natural integrity (Hamdi *et al*., 2007; Liu *et al*., 2010). Hankard *et al*. (2004) recognised ecotoxicology provide a better insight into ecological assessment of remediation and may support decisions for on-site amendments towards a successful site restoration.

In our work, ecotoxicity tests based on algal growth indicated diminished of 50% in the toxicological level during incubation in MERC in contrast to the unaltered values detected in the soil incubated under natural conditions. Figure 2.3 shows evolution of inhibition on algae growth due to the direct contact with soil extracts.

To the best of our knowledge, this is the first work in which MERC-assisted bioremediaton of PAHs was monitored in soil using ecotoxicological methods and the results demonstrate that the detoxification capacity of MERC outperforms the natural attenuation process, shortenings the time needed to clean-up a polluted terrestrial ecosystem.

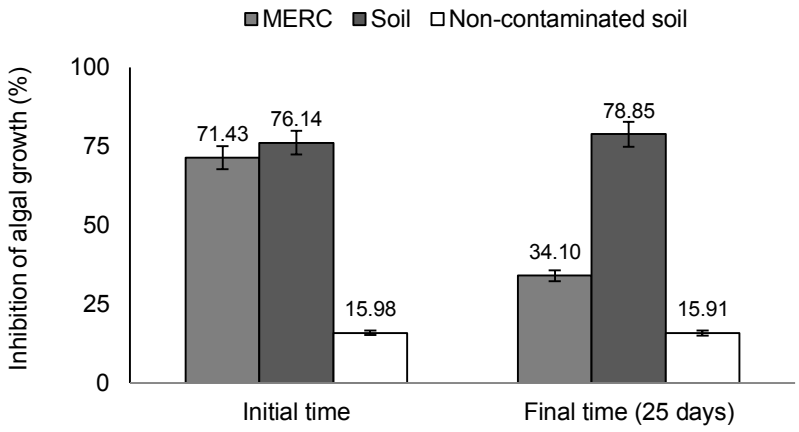


Figure 2.3: Toxicity levels in soil extractions of polluted soil with and without MERC-treatment. Reference toxicity values for non-polluted soils were also provided. The error bars represent standard deviation.

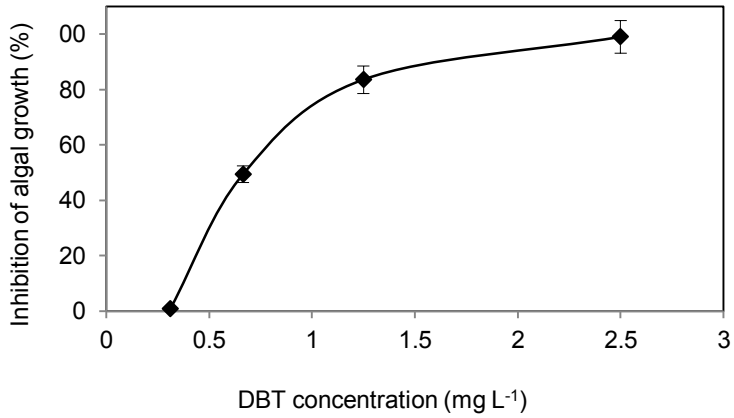
2.5 Conclusions

These results lead us to conclude that MERCs are able to enhance the DBT removal, reaching high efficiency in a shorter incubation time, even at the initial stage of the experiments. This technology may be used for *in situ* true decontamination of soils due to its proven detoxification capacity and present all the advantages of the others *in situ* bioremediation approaches, while avoiding bioaugmentation or artificial addition of nutrients with impact in the environment. Furthermore, MERC technology requires amendments of inert nature (graphite electrodes) with low cost and low impact for the environment because the electrode material can be removed and reused when the treatment is over. All this features make MERC an interesting and efficient bioremediating tool for stimulating native populations and overcoming the availability in electron acceptor that typically limits biodegradation of organic pollutants.

2.6 Supporting Information

Determination dose-effect parameters for dibenzothiophene on green algae

EC50 is defined as the concentration of DBT, which caused a 50% of reduction in the growing rate of *P. subcapitata* measured under high favourable conditions. Assays were carried out to get a reference point of maximum inhibition levels that could be expected because we did not found bibliographic report on toxicity parameters of DBT on green algae. Supporting figure 2.1 shows the response of algal growth to the presence of pollutant and Table 2.1 summarizes toxicity parameters of DBT for the algae used as biosensor in this work. Here, appear the calculated values for EC10, EC20 and EC50 with there 95% of confidence intervals.



Supporting figure 2.1: Dose–response for the growth inhibition of *Pseudokirchneriella subcapitata* after 72 h exposed to dibenzothiophene. The error bars represent the standard deviation.

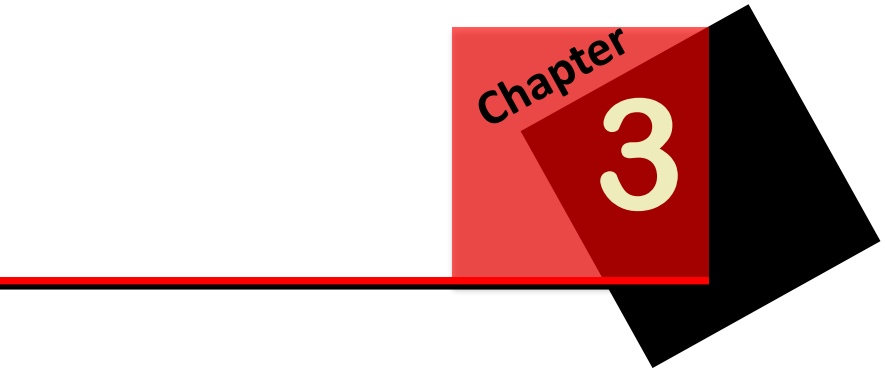
Eisentraeger *et al*. (2008) reported an EC50 = 0.2 mg L^{-1} for the invertebrate *Daphnia magna* and Hartnik *et al*. (2007) found an EC50 = 0.12 mg L^{-1} for the marine bacterium *Vibrio fischeri*, both similar to the result found for the photosynthetic microalgae *P. subcapitata*. In accordance to EU-directives 93/67/EC, 67/548/EEC, an EC50 value below to 1 mg L^{-1} indicates that this compound is highly toxic for aquatic organisms.

Supporting Table 2.1: Toxicity parameters for dibenzothiophene on *P. subcapitata.*

Parameter	mg L^{-1}	95% CI
EC10	0.339	(0.285-0.413)
EC20	0.435	(0.387-0.441)
EC50	0.666	(0.642-0.683)

Acknowledgment

This research was funded by Madrid Regional Government and University of Alcala Grant CCG10-UAH/AMB-5899 as well as by Spanish Ministry of Education through Grant RyC2008-03376.


Chapter
3

Supporting bioremediation and detoxification of isoproturon-polluted soil by using electrodes

PART I: Stimulating soil microorganisms for mineralizing the herbicide isoproturon by means of Microbial Electroremediating Cells

Chapter redrafted after:

Rodrigo Quejigo, J., Dörfler, U., Schroll, R., & Esteve-Núñez, A. (2016). Stimulating soil microorganisms for mineralizing the herbicide isoproturon by means of microbial electroremediating cells. **Microbial biotechnology**, 9(3), 369-380.

Stimulating soil microorganisms for mineralizing the herbicide isoproturon by means of Microbial Electroremediating Cells

3.1 Abstract

The absence of suitable terminal electron acceptors (TEA) in soil might limit the oxidative metabolism of environmental microbial populations. Microbial Electroremediating Cells (MERCs) consist in a variety of Microbial Electrochemical Systems that aim to overcome electron acceptor limitation and maximize metabolic oxidation with the purpose of enhancing the biodegradation of a pollutant in the environment. The objective of this work was to use MERCs principles for stimulating soil bacteria to achieve the complete biodegradation of the herbicide ^{14}C-isoproturon (IPU) to $^{14}CO_2$ in soils. Our study concludes that using electrodes at a positive potential (+600mV (vs. Ag/AgCl)) enhanced the mineralization by 20-fold respect the electrode-free control. We also report an overall profile of the ^{14}C-IPU metabolites and a ^{14}C mass balance in response to the different treatments. The remarkable impact of electrodes on the microbial activity of natural communities suggests a promising future for this emerging environmental technology that we propose to name bioelectroventing.

3.2 Introduction

Biodegradation based on metabolic activities of microorganisms is the primary mechanism for pollutant removal in the environment. Nevertheless, the process is highly dependent on the existence of indigenous degrading species as well as several abiotic factors (Vogt and Richnow, 2014). The absence of terminal electron acceptors (TEA) in soil might be responsible of the limited biodegradation of organic pollutants under anaerobic and strong reductive conditions (Megharaj *et al*., 2011) limiting the *in situ* microbial respiration. The supply of additional electron acceptors like oxygen (bioventing) (Kabelitz *et al*., 2009; García *et al*., 2010), humic acids (Lovley, 2000) or nitrates (Yu *et al*., 2013) to stimulate the microbial metabolism has

been a common practice to remove organic pollutants, but this incurs in extra cost and specially in the case of nitrates causes secondary pollution concerns (Pandey and Fulekar, 2012).

Such a TEA limitation could be overcome using solid conductive electron acceptors like the electrodes used in Microbial Electroremediating Cells (MERCs). MERCs are indeed variants of Microbial Fuel Cells (MFCs). These Microbial Electrochemical Systems use sediment-buried electrodes (anodes) acting as electron sink coupled to the microbial oxidation of organic matter. The anode is connected through an external resistance to a cathode where electrons are finally consumed by an electron acceptor as oxygen (Tender *et al*., 2002; Venkata-Mohan *et al*., 2009; Dominguez-Garay *et al*., 2013). Both MERCs and MFCs are Microbial Electrochemical Systems but they differ in the operation mode. While MFCs aim to maximize the power generation (Watts), MERCs aim to reach maximum current production (Amperes) through maximizing metabolic oxidation of organic/inorganic soil compounds (Rodrigo *et al*., 2014). In an experimental modus operandi this is achieved configuring the MERCs with external resistances close to the short circuit or setting potentials electrode that favor the current production. So, regarding to the power curve of a Microbial Electrochemical Systems, the MFCs work under an external resistance that allows performing at maximum power and MERCs work close to short circuit where the power is almost zero, offering an alternative to solve the constraints of electron acceptors and favoring the degradation of contaminants under soil-flooded conditions.

Electroactive microorganisms have been shown for almost a decade to interchange electrons with conductive materials (electrodes) buried in the soil and sediments (Reimers *et al*., 2001; Tender *et al*., 2008; Dominguez-Garay *et al*., 2013; Li and Yu, 2015). Although energy harvesting was the primary target, we have now explored additional scenarios where oxidative metabolism, eg. mineralization of ^{14}C-isoproturon (^{14}C-IPU), can be enhanced and subsequently used as new tools for stimulating environmental bacteria. Zhang *et al*. (2010) demonstrated for the first time that graphite electrodes could serve as an electron acceptor for the degradation of toluene and

benzene in polluted slurries. Since then, enhanced biodegradation of PAHs (Morris and Jin, 2012; Yan *et al*., 2012; Rodrigo *et al*., 2014; Sherafatmand and Ng., 2015), phenol (Huang *et al*., 2011), pesticides (Cao *et al*., 2015) and chlorinated organics (Chun *et al*., 2013) has been reported. It is important to point out that pollutant removal by bioelectrochemical-assisted tools as MERCs involves biodegradation and not just the migration of contaminants (e.g. organochlorines) as classical physicochemical soil electroremediation does (Gomes *et al*., 2012).

The redox gradient in MERCs is established spontaneously across the soil-water interphase as a result of spatially segmented reduction-oxidation reactions (Li and Yu, 2015) establishing an electron transport route between electrodes. However, the soil-buried electrode potential is typically negative as a response to the biochemical environment around it so it can be inappropriate to drive the transformation for many recalcitrant organics (Zhao *et al*., 2006). Alternatively, an external voltage can be applied between anode and cathode for stimulating bacteria activity and consequently pollutant removal (Aulenta *et al*., 2007; Chun *et al*., 2013). However, those studies just set a constant voltage value between electrodes, but no control over the anode (the electron sink electrode) was performed.

A more advanced strategy for coping with the electron redox unbalance would be to set up electrodes at a positive potential so microorganisms may have a more redox favorable TEA for performing the oxidative reactions. Actually, it has been reported an increase in the oxidative metabolism of *Geobacter sulfurreducens* by increasing electrode potentials to values as high as 600mV (vs Ag/AgCl) (Busalmen *et al*., 2008). Then the electrodes not only overcome the TEA limitation but also allow the controllability of the bioremediation processes that can be monitored and regulated by tuning electrochemical parameters.

The absence of suitable TEAs in anaerobic and strong reductive environments like flooded soils might be responsible of the limited biodegradation of a phenylurea herbicide as isoproturon (IPU) (Larsen and

Aamand, 2001). As a consequence, the presence of IPU in groundwater may exceed the approved critical value for drinking water (0.1 μg L^{-1}) set by the European Community Drinking Water Directive (Folberth *et al*., 2009).

The aim of our work was to use the mineralization of ^{14}C-IPU in soil as a proof of concept to demonstrate how setting electrodes at positive potentials can have a high impact in the microbial oxidation activity. We have proved that the presence of electrodes artificially polarized at potentials as high as 600mV (vs.Ag/AgCl) stimulate the biodegradation in flooded environments. A similar effect is achieved when oxygen (800mV) is artificially supplied in bioventing treatments. Thus, we propose the term *bioelectroventing* for referring to the process of enhancing bioremediation under soil-flooded conditions by using electrodes as microbial electron sink.

3.3 Experimental procedures

Chemicals

Uniformly ^{14}C ring-labeled isoproturon [3-(4-isopropylphenyl)-1,1-dimethylurea] (^{14}C-IPU) with a specific radioactivity of 9.96 kBq μg^{-1} and radiochemical purity of 98 % according to the producer was purchased from GE Healthcare (Little Chalfont, UK) and used as a representative pesticide for phenylurea herbicides. The ^{14}C-IPU was mixed with unlabeled IPU to provide a final concentration of 1.75 mg mL^{-1} and a specific radioactivity of 154 Bq μg^{-1}. The new mix was denominated "^{14}C-IPU standard mix". Non-labelled IPU, monodemethyl-isoproturon (MDIPU) [3-(4-isopropylphenyl)-1-methyl-urea], 2-OH-Mono-demethyl-Isoproturon (2-OH-MIPU) 3-(4-(2-hydroxyisopropylphenyl))1-methylurea, didemethyl-isoproturon (DD-IPU) [3-(4-isopropylphenyl)-urea] and 4-isopropyl-aniline (4-IPA) were purchased from Dr. Ehrenstorfer (Augsburg, Germany; purity 99.5%). Scintillation cocktails (Ultima Gold XR and Ultima Flo AF) were obtained from Packard (Dreieich, Germany). All other chemicals and solvents were of analytical grade and were purchased from Merck (Darmstadt, Germany).

Soil

The soil material was an Aric Anthrosol from an agricultural field (Hohenwart; latitude: 48.250, longitude: 11.567, elevation 472 m) in Germany without IPU history and with an organic matter content of 0.99 %. A complete physical-chemical analysis of this soil was previously reported by Grundmann *et al*. (2011). Soil samples were taken from 0-20 cm and were stored in plastic bags at -20 ºC according to guidelines of the Organization for Economic Cooperation and Development (OECD, 1995). The soil samples were unfrozen 2 weeks before the start of the experiment following the next incubation protocol: first they were kept at +4 ºC for 7 days, and then another 7 days at room temperature (20 ± 1 ºC). The first day at room temperature the soil was moistened to a water potential close but below -15 kPa and compacted to a density of 1.3 g cm^{-3} to equilibrate all microbial processes and ensure comparable conditions at the start of the experiments. Previous studies have shown that the microbial activity is at its optimum at these soil conditions (Schroll *et al*., 2006). The conductivity, 0.247 mS/cm, was measured just before the set up of the experiment with the commercially available ECa sensors UMP-1 (Umwelt-Geräte-Technik, Freising/Weihenstephan, Germany). This tool can be used for *in situ* EC measurements with minimal disruption because the sole requirement consists in burying the probe needs in the soil. The measurements were conducted under the same conditions (water content and temperature) than for the biodegradation assay.

Spiking of the soil samples

100 μL of ^{14}C-IPU standard mix was applied to an aliquot of 3 g dried-and-ground soil and homogeneously mixed. After evaporation of the organic solvent (methanol), the soil aliquot was mixed with 32 g (dry weight equivalent) of equilibrated soil with the goal to distribute the pollutant homogenously resulting in a concentration of 5 (± 0.1) μg g^{-1} soil (dry weight) and a radioactivity of 154 Bq μg^{-1}. The spiked soil sample was transferred to the opaque glass flask of the laboratory system described below, compacted

to a soil density of 1.3 g cm^{-3} and adjusted to flooded conditions (water holding capacity + 35 mL extra deionised water). As a result 2 cm water body was maintained above the soil in order to ensure flooded conditions. Water evaporation was compensated 3 times per week by the addition of deionized water.

Laboratory system

The mineralization experiment was conducted in a laboratory system built in approximation to the OECD guideline for testing of chemicals 304A (OECD, 1981). It consisted of opaque glass flasks (250 mL volume; neoLab, Heidelberg, Germany), which were closed with a rubber stopper (neoLab, Heidelberg, Germany); at the bottom of the stopper a plastic beaker of 25 mL volume (VWR International, Darmstadt, Germany) was attached. The plastic beaker was filled with 10 mL of 0.1 N NaOH (Merck, Darmstadt, Germany) to trap the $^{14}CO_2$ resulting from the mineralization of ^{14}C-IPU. The NaOH-solution was exchanged three times per week and from the collected NaOH solution an aliquot of 2 mL was taken, mixed with 3 mL Ultima FLO AF (PerkinElmer, Rodgau, Germany) and the radioactivity was measured in a liquid scintillation counter (Tricarb 1900 TR, Packard, Dreieich, Germany). A hollow needle (neoLab, Heidelberg, Germany) conducted through the rubber stopper allowed a constant supply of O_2. To prevent saturation of the NaOH-solution with atmospheric CO_2, a plastic reservoir (neoLab, Heidelberg, Germany) filled with soda lime (Merck, Darmstadt, Germany) was connected to the needle.

MERCs: Operating conditions.

MERCs were assembled in the laboratory system (Fig.3.1). The electrode used in this experiment was carbon felt (Sofacel, Barcelona, Spain), as it showed no IPU adsorption (Supporting Information) and very adequate mechanical properties to conform the system. Although a diversity of electrode materials can serve as an electron acceptor for microbial respiration, carbon felt as graphite can provide a low-cost, low-maintenance, continuous sink for electrons since it does not corrode or otherwise degrade

during long-term deployments (Reimers *et al*., 2006; Tender *et al*., 2008) so it is easy to remove from the soil after the treatment, showing a low impact for the environment. The electrodes were allocated at the bottom of the soil layer (anode) and above the water body (cathode). The geometrical area of the electrodes was 39 cm^2 (surface area: 0.7 $m^2\ g^{-1}$).

MERCs were continuously operated at a poised anode potential of + 600 mV versus Ag/AgCl reference electrode (RE-5B, BASi, United Kingdom) (+199 mV vs. SHE) by using a potentiostat (NEV2 nanoelectra). Henceforth these MERCs will be designated as pol-MERCs.

Abiotic electrochemical reactions on ^{14}C-IPU were evaluated by using sterile pol-MERCs. The soil was sterilized with γ-irradiation. The use of gamma γ-irradiation as a method for soil sterilization for laboratory experiments has been recommended over other sterilization techniques (Mcnamara *et al*., 2003) with advantages as highly effectiveness at sterilization while avoiding chemical contamination. Moreover, small doses of γ-irradiation are capable of sterilizing large soil samples without increasing soil temperature or pressure. Gammacell 220 cobalt-60 irradation unit was used to sterilize samples contained in vented polypropylene tubes for 96 hours at a rate of 8,33 $Gy.min^{-1}$ for a total γ-ray dosage of 60 KGy. Biologic activity was tested by dish-plate inoculation. Eluates of soil were prepared using phosphate buffer (pH 8) in 1:10 soil-water ratio. 1:10 and 1:100 dilutions were carried out and incubated at 30 ºC in LB agar plates during 72 h. Colonies formation was detected only in plates seeding with eluates of non sterilized soil.

Electrode-free controls were assembled in the laboratory system without the presence of the electrodes and under the same water content, temperature and ^{14}C-IPU concentrations than pol-MERCs.

Mineralization assays.

The mineralization of ^{14}C-IPU to $^{14}CO_2$ was studied in a closed aerated laboratory system, as described before. The soil samples were

incubated at 30 ± 0.1 ºC for 25 days in the dark. The first sampling of the NaOH trap was performed 24 hours after ^{14}C-IPU additions. Then sampling was performed three times per week.

The $^{14}CO_2$ production rate was expressed as μg d^{-1} g^{-1} of ^{14}C-IPU and the cumulative $^{14}CO_2$ as percentage of the applied ^{14}C-IPU. After 25 days of incubation, two of the four replicates of the pol-MERCs treatment were sacrificed for soil analysis. The other two replicates were shifted into MERCs conditions where anode and cathode were connected by a copper wire using a 56 Ω external resistor (R), where the redox potential of the anode in MERCs was set by the redox potential differences across sediment/water.^{14}C-IPU mineralization was monitored for another 12 weeks in absence of artificial anode poisoning.

^{14}C-IPU residue analysis

At the end of the incubation periods all soil samples were extracted, and the extracts were cleaned up and analyzed by HPLC. The ^{14}C non-extractable residues were quantified by combustion.

Soil aliquots and carbon felt were separately extracted with methanol in an accelerated solvent extractor (ASE 200, Dionex, Idstein, Germany) at 90 ºC, with a pressure of 10 MPa. One hundred microliter aliquots of each extract were mixed with 5 mL Ultima Gold XR and measured by liquid scintillation counting. The ASE extracts were concentrated on a rotary evaporator to remove the organic solvent. The concentrated extracts were adjusted to 250 mL with distilled water and subjected to solid phase extraction (SPE; Lichrolut ENV 200 mg, Varian, Darmstadt, Germany). After extraction, the SPE columns were dried under a gentle nitrogen stream and eluted with 10 mL methanol. The eluate was concentrated to a volume of one mL with a rotary evaporator and further concentrated under a gentle nitrogen-stream to volumes between 40 and 380 µL, depending on the total ^{14}C-radioactivity of the samples. The samples were immediately analyzed by HPLC. Twenty microliter of each of these samples were injected to a HPLC system,

consisting of a L-6200 Intelligent Pump (Merck-Hitachi, Darmstadt, Germany) a UV/VIS detector (240 nm, Merck, Darmstadt, Germany) and a radioactivity detector LB 506 C1 (Berthold, Wildbad, Germany.) The column used was a Lichrospher 100 RP-18, 5 µm, 4 x 250 mm (Merck, Darmstadt, Germany). The mobile phase consisted of A = acetonitrile (HPLC grade) and B = water (Lichrosolv water for chromatography, Merck, Darmstadt, Germany) at a flow rate of 1 mL min^{-1}. The gradient program was as follows: T0min 95% A, T15min 40% A, T20min 40% A, T25min 95% A, T30min 95% A. Parent compound and metabolites were identified by comparison of their retention times with reference substances.

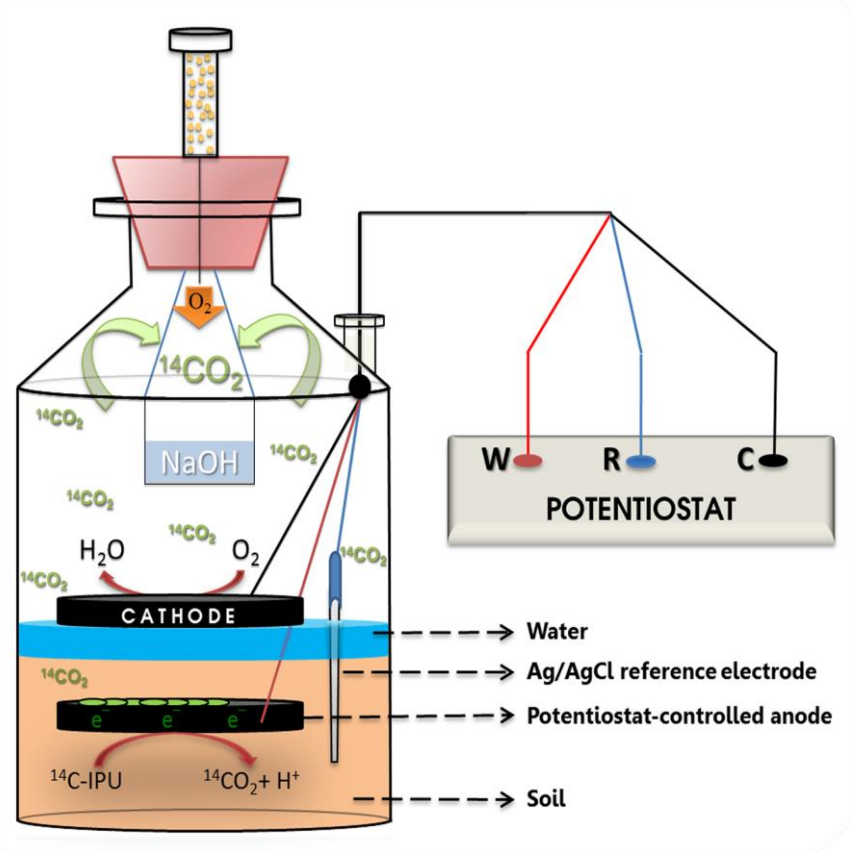


Figure 3.1: Scheme of a pol-MERC. The anode was polarized at 0.6 V vs Ag/AgCl (+197 mV versus normal hydrogen electrode (NHE)). $^{14}CO_2$ was trapped in a 0.1 N NaOH solution for allowing the measurement of ^{14}C-IPU.

After performing ASE, in order to achieve the quantification of ^{14}C-labelled non-extractable residues (NER), soil material was homogenized intensively before combusting. Three aliquots (each approximately 250 mg) of each soil sample were filled into combustion cups and mixed with 7–8 drops of saturated aqueous sugar solution to guarantee a complete oxidation of the ^{14}C. Carbon felt electrodes from each assay were cut in pieces and placed into combustion cups. Combustion was conducted with an automatic sample-oxidizer 306 (Packard, Dreieich, Germany). $^{14}CO_2$ was trapped in Carbo- Sorb E (Packard, Dreieich, Germany) and mixed with Permafluor E (Packard, Dreieich, Germany) prior to scintillation counting.

Electrochemical analysis

The anode potential in pol-MERCs was continuously poised at + 600 mV versus Ag/AgCl (+197 mV versus normal hydrogen electrode (NHE)) reference electrode (RE-5B, BASi, United Kingdom) using a potentiostat (NEV1-3 Nanoelectra, Madrid, Spain) and the resulting current was recorded every 60 seconds.

Cyclic voltammetry (CV) was recorded to qualitatively characterize the electrochemical activity of the soil by imposing at a scan rate of 1 mV/s from the open circuit voltage (OCV) potential by a potentiostat (NEV3 Nanoelectra, Madrid, Spain.)

3.4 Results and discussion

Enhanced isoproturon mineralization by using positive electrode potential

The first series of experiments started by investigating the influence of a positive potential electrode on the ^{14}C-IPU mineralization in soil. After the incubation period (25 days) the cumulative mineralization reached the value of 21.3% (average value of four experimental replicates), whereas ^{14}C-IPU mineralization in the electrode-free control was below 1% (Fig. 3.2A). Regarding the low proportion of radiochemical impurities of the ^{14}C-IPU

applied in this series (98% purity), most of the $^{14}CO_2$ derives from the radiochemical pollutant. Sterile pol-MERCs were assayed in order to prove that polarized electrodes by themselves were not performing abiotic electro-oxidation of ^{14}C-IPU. Actually, the sterilized system (1.49 ±0,05 % mineralization after 25 days of incubation) confirms the mineralization of ^{14}C-IPU by means of the native microbial population in our soil assays. Interestingly, the cumulative mineralization under both, electrode-free control and sterile pol-MERCs were lower than the impurities fraction in our ^{14}C-IPU (2%).

Thus, the enhancing effect of the anode in the microbial metabolic processes raised the mineralization over 20 fold in comparison with the electrode-free control under flooded conditions (1%). Moreover, the anodic enhancement outperformed the aerobic mineralization (8%) previously reported by Folberth *et al.,* 2009 using the same soil Aric Anthrosol. So thus, this fact underlines the doubtful implication of oxygen as a possible mechanism to enhance the mineralization of IPU under the presence of a polarized electrode, which is consistent with the strong reductive scenario-taking place under soil-flooded conditions.

Our pol-MERCs assays achieved a high cumulative mineralization together with a high mineralization rate (Fig. 3.2B), both common features present in biodegradation when the pollutant acts as a source of energy for the microbial degrading community (Grundmann *et al*., 2011). In consequence, our electrode-assisted treatment offers a malleable terminal electron acceptor capable to adapt to different redox-dependent processes and perform *in situ* bioremediation by avoiding chemicals consumption or soil manipulation with negative environmental consequences.

To the best of our knowledge, it is the first time that a significantly ^{14}C-IPU mineralization has been reported under soil-flooded conditions. Most of the research regarding biodegradation of IPU has been almost fully devoted to the aerobic metabolism, and just a few reports are available for degradation of this substance under strong reductive conditions, where significant

mineralization of IPU has not being reported elsewhere. Larsen *et al.* (2000) found no mineralization of IPU anaerobically in the presence of nitrate in microcosm experiments with a sandy aquifer sediment and in a subsequent study they detected no mineralization of IPU in different aquifer sediments under denitrifying, sulfate-reducing or methanogenic conditions following incubation for 312 days at 10ºC (Larsen and Aamand, 2001). The same results were obtained examining sub-surface limestone samples after 250 days (Janniche *et al*., 2010).

The neutral pH of our soil (6.8) and the flooding soil conditions of our assays allow CO_2 to dissolve in water and establish an equilibrium with carbonic acid leading to negative charged species as HCO_3^- and CO_3^{2-} that could be adsorbed on the surface of positive polarized electrodes. In order to evaluate this hypothesis, just after 25 days of assay, the electrode potential was shifted from positive potential (+600mV versus a Ag/AgCl) to negative one (-300mV versus a Ag/AgCl) in both pol-MERCs and sterile pol-MERCs (Fig.3.2B). The negative potential was kept for 12 hours to release and monitor the $^{14}CO_2$ electro-adsorbed in the anode. The mineralization rate increased in pol-MERCs from 0.01 to 0.05 $\mu g\ d^{-1}g^{-1}$ (dry soil) of ^{14}C-IPU mineralized but not increased was registered in sterile pol-MERCs.

The transitory assay of reversing the electrode potential was followed by a long-term assay for evaluating the response of the electroactive microbial community to a different electrode potential. So thus, two of the four pol-MERCs replicates were kept running for 12 additional weeks after converting the pol-MERCs into MERCs by substituting the potentiostat by a low external resistor (Fig. 3:2B). The new configuration led to a drop off in the redox anode potential from +600 mV (versus Ag/AgCl) to a negative anode potential (-200 mV (versus Ag/AgCl)) for the rest of the assay. The effect was evident from the shift in the slope for the cumulative ^{14}C-IPU mineralization, decreasing from 3- to 9-fold over the course of the second phase. This strongly supports the key role of setting a positive anode potential to achieve an optimal *in situ* biodegradation of IPU.

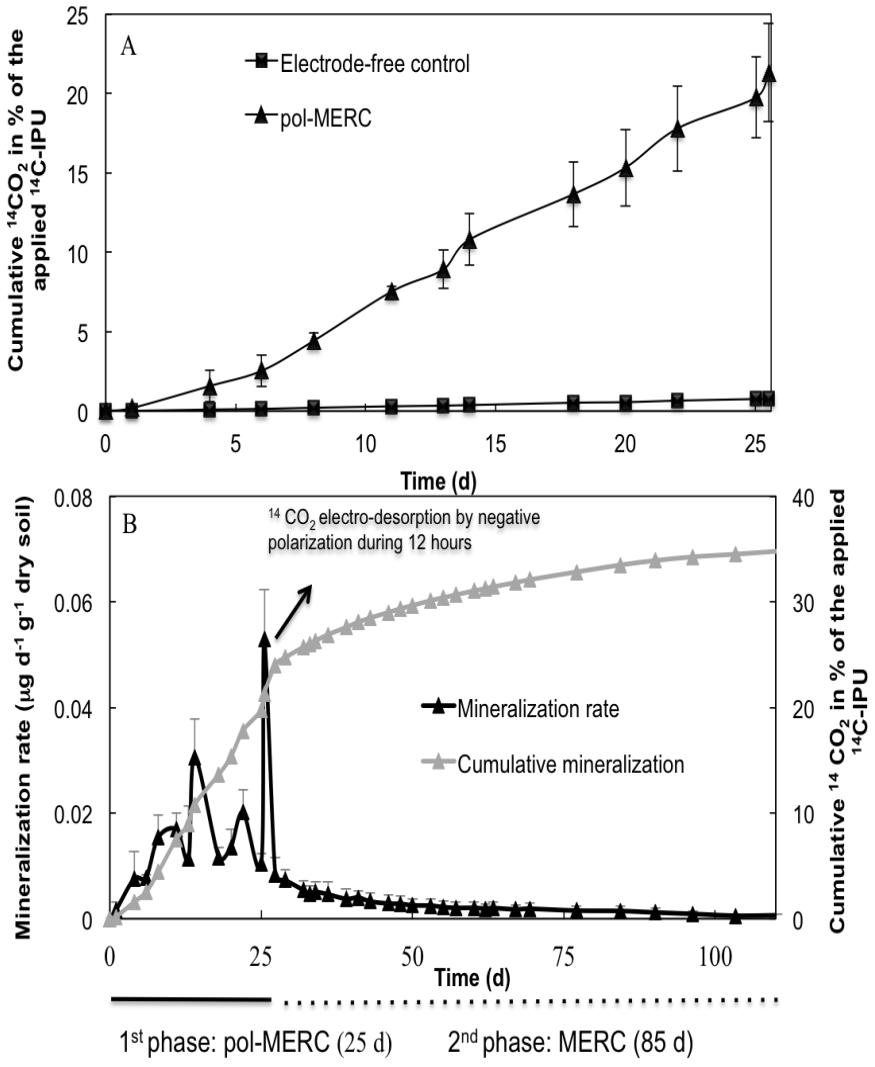


Figure 3.2: A) Cumulative mineralization of ^{14}C-IPU within 25 days in pol-MERCs (n=4, S.D.) compared with mineralization capability of the soil under the electrode-free conditions (n=3, S.D.); B) Cumulative mineralization and rate of ^{14}CO2 production from ^{14}C-IPU under long term MERCs treatment. During the first phase the polarized anode acted as TEA at 0.6 V vs Ag/AgCl (+197 mV versus normal hydrogen electrode (NHE)). This phase ended with the anode potential reversed to -300mV vs Ag/AgCl for 12 hours. During the second phase the anode potential was achieved by connecting the electrodes through a 56ohm resistor.

It is an accepted practice in traditional bioremediation techniques like bioventing (Kabelitz *et al*., 2009; García *et al*., 2010) to support microbial respiration by supplying a terminal electron acceptor with a positive redox potential like oxygen (+800mV). One disadvantage of these strategies is the necessity to supply continuously the oxidant, which spread out to the environment and negatively affect the native microbial community. In contrast pol-MERCs provide an endless, low priced and sustainable terminal electron sink showing minimal environmental disturbance (Logan *et al*., 2006). So thus, we propose the term *bioelectroventing* for referring to the process of enhancing bioremediation by using electrodes as microbial electron sink.

Electrochemical performance of pol-MERCs

In order to evaluate the electrochemical performance the current production was continuously registered in pol-MERCs (Fig. 3.3A). Soil under this treatment showed just one day of lag phase, reaching a maximum current density of 35 mA/m^2 before entering into steady-state (ca. 15mA/m^2).

To give insight into the nature of this response we performed cyclic voltammetries (CV) of the anodes from pol-MERCs. A different bioelectrochemical profile was observed after 25 days of incubation. In fact, Fig. 3.3B shows an inflexion peak centered at +0.300V (vs Ag/AgCl reference) in comparison with initial time anodes and with the CV of the sterile pol-MERCs. Thus, the intensity of the signals provides an indication of an enrichment of microbial communities with electron transfer capabilities.

In addition, CV analysis did not show any peak corresponding to the oxidation of water, even after performing the analysis under a wide voltage interval (from +1V to -1V). This issue confirmed that the electrode potential applied was not enough to drive oxygen production at the anode.

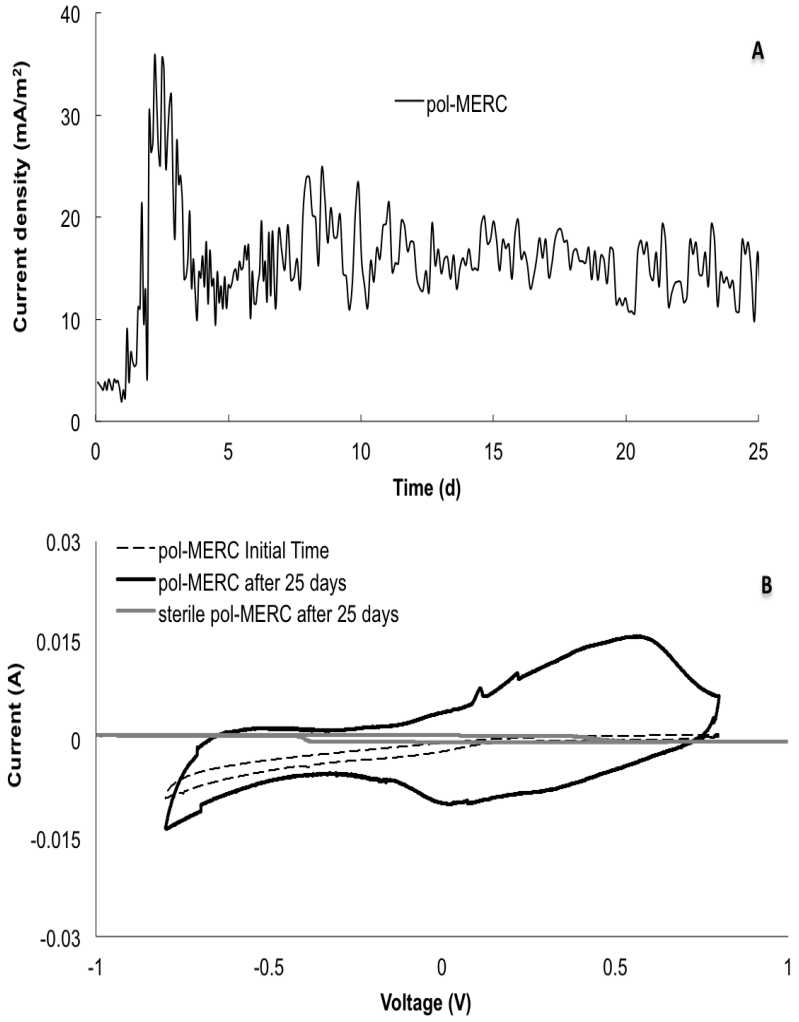


Figure 3.3: A) Chronoramperomety of pol-MERCs polarized at 0.6V (versus Ag/AgCl). Geometrical anode surface was used for calculating the current density B) Cyclic voltammetry tests (scan rate: 1 mV/s) carried out at initial experimental time and after 25 days under bioelectrochemical assisted soil (pol-MERCs and sterile pol-MERCs).

Characterization of the ^{14}C-IPU mass balance

In addition to the cumulative $^{14}CO_2$ mineralization, the soil- and electrode-associated radioactivity should be measured in order to conform the

^{14}C mass balance established in our experimental system. Hence, methanol extractable residue (ER) and non-extractable residues (NER) of ^{14}C-IPU that remained in the soil and electrodes during the course of the assays were shown in Fig. 3.4A. Interestingly, these mass balances ranged between 91.2% and 98.4% of the initially supplied ^{14}C-IPU under pol-MERCs and electrode-free control respectively. This parameter was therefore an indicator of the effectiveness of our experimental design and underlines the presence of potential losses of $^{14}CO_2$, especially under conditions of high mineralization rates (pol-MERCs treatment). The extractable ^{14}C-residues in the soil samples varied considerably between the different experimental conditions after 25 days of incubation. The ER did reach 53.9% of the applied radioactivity for the electrode-free control but it did just 15% in soil under the pol-MERCs treatment. On the contrary, a similar distribution was observed for NER regardless the treatment: 43.6% for electrode-free control and 40.4% for pol-MERCs. Examining the ^{14}C mass balance of the long-term assay (110 days), we observed an increase in the NER in comparison with the standard pol-MERCs. The formation of NER are often explained by binding of a xenobiotic to the soil matrix, specially to the soil organic matter and has been reported that IPU-metabolites are mostly adsorbed onto organic matter in soils (Ertli *et al.*, 2004). Another possible pathway for the formation of NER is the ^{14}C-IPU-degradation by microorganisms that can use it as an energy source and for microbial growth; as a result, ^{14}C-residues are incorporated into biomolecules and e.g. subsequently bound to soil ("apparent NER") when microbes die (Grundmann *et al.*, 2011). Positive correlations between NER formation and IPU aerobic mineralization have been previously reported (Alletto *et al.*, 2006), which could explain the similar NER fraction under electrode-free control and pol-MERCs despite the broad ^{14}C-IPU cumulative mineralization.

Adsorption-desorption assays were conducted to exam the affinity of ^{14}C-IPU and different electro conductive materials. Carbon felt showed a convenient physic-mechanical properties and it was established as the electrode material for our assays due to the lack of IPU adsorption (Supporting Information). Nevertheless, to calculate the total recoveries of ^{14}C-radioactivity at the end of the experiments we extracted by accelerated

solvent extraction (ASE) and combusted the carbon felt electrodes, both the anode and the cathode separately (Fig. 3.4B). The total amount of ^{14}C radioactivity, extractable and non extractable, in the electrodes reached 13.7 % in pol-MERCs. In the long-term assay where pol-MERCs treatment was followed by MERC treatment, the percentage of total ^{14}C-residues in the electrodes was reduced to 7.8%. Interestingly, the amount of extractable residues (0.2%) was extremely low in those anodes that were used for such a long incubation period. A similar result was previously reported by Zhang *et al*. (2010) using polluted slurries, although they reported a higher adsorption for benzene and toluene in the electrodes (70%) and also confirmed that toluene adsorbed on the graphite could be metabolized.

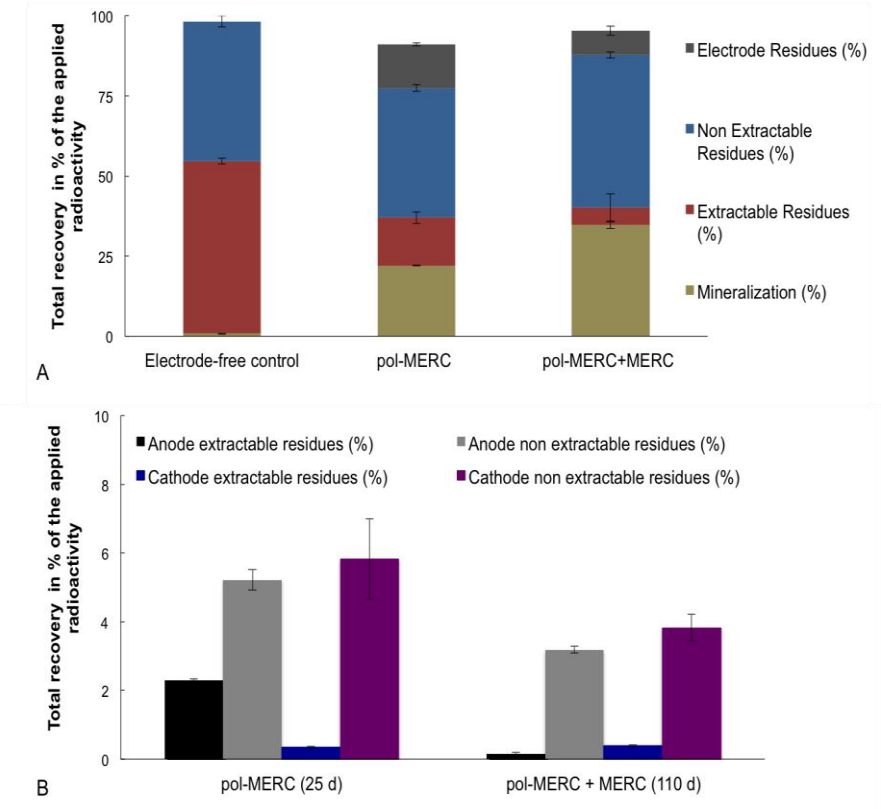


Figure 3.4: A) Distribution and mass balance of different soil treatments regarding initial radioactivity of ^{14}C-IPU B) Extractable and non-extractable ^{14}C residues in carbon felt electrodes used under different treatments. Bars represent standard deviation.

^{14}C-IPU biodegradation pathway

Since the discovery and marketing of phenylurea herbicides shortly after the Second World War, this group of chemicals has grown to be one of the most important classes of herbicides for agricultural use (Sørensen *et al*., 2003). The removal of IPU from the environment has received considerable attention, mainly under aerobic conditions and despite many anoxic environments like groundwater aquifers, subsurface soil and seasonally flooded agricultural soils, biodegradation of IPU under anaerobic and strong reductive conditions is largely unknown (Larsen and Aamand, 2001). The absence of suitable terminal electron acceptors (TEA) might be responsible of the limited biodegradation of IPU under strong reductive scenarios (Larsen and Aamand, 2001; Megharaj *et al*., 2011). Interestingly, this is the first time that IPU is reported to be mineralized under soil-flooded conditions so we can conclude that the use of polarized electrodes has accelerated the finding of new biodegradation pathways that were not found for decades.

Typical mineralization pathway of IPU by bacterial strains includes two successive N-demethylations, cleavage of both the urea side chain and the aromatic ring, and a final mineralization to CO_2 and production of biomass (Sørensen and Aarmand, 2001). Two alternative metabolic pathways were reported involving initial hydroxylation of the isopropyl side chain, resulting in either 1-OH-IPU, a dead-end metabolite just detected in bacterial cultures derived from soil, or 2-OH-IPU, described in agricultural soils (Lehr *et al*., 1996; Scheunert and Reuter, 2000). These metabolites were reported just under aerobic pathways. Just a few reports about the anaerobic degradation of this substance are available and they do not included information about either the microbial reactions or their metabolites accumulated under flooded conditions (Larsen *et al*., 2000; Larsen and Aamand 2001; Janniche *et al*., 2010).

Exhaustive analysis of our treated soil revealed six radioactive IPU metabolites that were compared with authentic analytical standards. In addition to the parent compound IPU, we have successfully identified three

metabolites and detected two unidentified metabolites among the degradation products (Table 3.1): monodemethyl-isoproturon (MDIPU), (3-(4-isopropylphenyl)-1-methyl-urea), didemethyl-isoproturon (DD-IPU) (3-(4-isopropylphenyl)-urea) and 2-OH-Mono-demethyl-Isoproturon (2-OH-MIPU) 3-(4-(2- hydroxyisopropylphenyl))1-methylurea. IPU as well as any other phenylurea is not subjected to chemical degradation within the pH range of 4–10. Consequently, chemical degradation of IPU in soils is of minor importance (Sørensen *et al*., 2003) making it a recalcitrant xenobiotic. Thus, the metabolites observed in this study therefore resulted from microbial attack of the parent compound.

The two most abundant metabolites were obtained after demethylation of the N,N-dimethylurea side chain to generate MDIPU and DDIPU (Fig.3.5, steps 1 and 2). MDIPU has been previously reported to be the most abundant metabolite after IPU biotransformation in agricultural soils, as well as in pure cultures assays by soil fungi and bacteria (Sørensen *et al*., 2003). Moreover, the abundance levels for MDIPU in pol-MERCs extracts were ca. five-fold higher than in environments under negative redox potential like the electrode-free control. On the contrary, the abundance of IPU in pol-MERCs extracts was ca. 5-fold lower, going down the concentration from the initial 5 ($\pm$ 0.1) $\mu g\ g^{-1}$ dry soil to 0.179 $\mu g\ g^{-1}$ dry soil, while under electrode-free control the decrease only reach the 1.9 ($\pm$ 0.006) $\mu g\ g^{-1}$ dry soil. These concentrations were calculated based in the ^{14}C chromatograms from the HPLC (coupled to a radioactivity detector) and the specific radioactivity of our standard applied in the soil (radioactivity per unit mass of the stated compound).

MDIPU has been reported to generate 4-IA before cleavage of the aromatic ring and final mineralization to CO_2 (Sørensen and Aarmand, 2001). So thus, low concentrations of 4-IA have been detected in IPU-treated agricultural soils (Lehr at al., 1996) and during the mineralization of IPU by *Sphingomonas* sp. strain SRS2 (Sørensen and Aarmand, 2001). On the contrary, none of our bioremediation treatments showed 4-IA among the radioactive metabolites from ^{14}C-IPU (Fig. 3.5). It has being reported the

irreversible bound of this metabolite to soil organic matter, increasing the NER and subsequently decreasing the availability of the compound to the degrading microbial communities (Scheunert and Reuter, 2000; Johannesen *et al.*, 2003). The hydroxyl derivative 2-OH-MIPU in our experiments has been detected at levels ca. five-fold higher in pol-MERCs extracts than under electrode-free control (Fig.3.5. step 4). This intermediate is part of an alternative metabolic path initiated with the hydroxylation of the isopropyl side chain. Regarding the unidentified metabolites, they were only observed in low levels and just slight changes were observed when the electrode was set at positive potential, suggesting that they are due to biological reactions from the soil.

Table 3.1: Profile composition of the methanol extractable residues (peaks areas appear as % of extract-^{14}C and metabolite concentrations as μg/g dry soil) for either, electrode-free control and polarized-MERCs.

Isoproturon and metabolites	RT (min)	**Electrode-free control**		**pol-MERC**	
		Area %	μg/g dry soil	Area %	μg/g dry soil
2-OH-MIPU[1]	11.90 ± 0.11	0.72 ± 0.16	0.024 ± 0.008	3.87	0.035
Unidentified	13.01 ± 0.05	2.75 ± 0.51	0.081 ± 0.014	6.53	0.058
Unidentified	13.94 ± 0.05	1.82 ± 0.04	0.040 ± 0.009	2.76	0.025
DD-IPU[2]	17.60 ± 0.06	1.39 ± 0.17	0.029 ± 0.005	6.52	0.058
MDIPU[3]	18.70 ± 0.02	16.82 ± 0.3	0.421 ± 0.006	60.77	0.543
IPU[4]	19.95 ± 0.02	76.5 ± 0.19	1.913 ± 0.006	19.98	0.179
4-IPA[5]	n.d	n.d	nd	n.d	n.d

n = 3 ± SD except pol-MERC which analysis was conducted with pooled samples replicates to exceed detection limit, therefore no standard deviation can be given. n.d: not detectable.

[1] 3-(4-(2- hydroxyisopropylphenyl)) 1-methylurea
[2] [3-(4-isopropylphenyl)-urea]
[3] [3-(4-isopropylphenyl)-1-methyl-urea]
[4] [3-(4-isopropylphenyl)-1,1-dimethylurea]
[5] 4-isopropyl-aniline

Although oxygen cannot be generated under our conditions, our assays revealed common metabolite patterns with aerobic conditions. Interestingly burying a positive potential electrode in flooded soil may remodel the redox scenario and as a consequence may influence the dominant energy

metabolic pathways leading to common metabolite patterns with aerobic conditions. This new redox context under the influence of the electric field of the polarized electrode may change the permeability of cell membrane, leading to the excessive absorbance of extracellular substances and further change the microbial metabolism (Rittmann and McCarty, 2001). In adittion, predominance of anode respiration over fermentation (Hunt *et al*., 2010; Pinchuk *et al*., 2011) and a suppression of several anaerobic species, such as archaea and sulphide producing populations at high redox potentials have been recently reported (Lu *et al*., 2014a; Ueno and Kitajima, 2014). Interestingly, bioenergetics and therefore the profile of metabolites during IPU biodegradation may change by shifting from substrate-level phosphorylation (i.e., anaerobic fermentation) to oxidative phosphorylation (i.e., electrode respiration).

Further research is currently being performed to unveil whether these processes or some others as electroosmotic flow inside the saturated soil could be affected by the polarized electrode presence. Moreover, it is still unclear how a polarized electrode affects the microbial community activity to enhance *in situ* bioremediation.

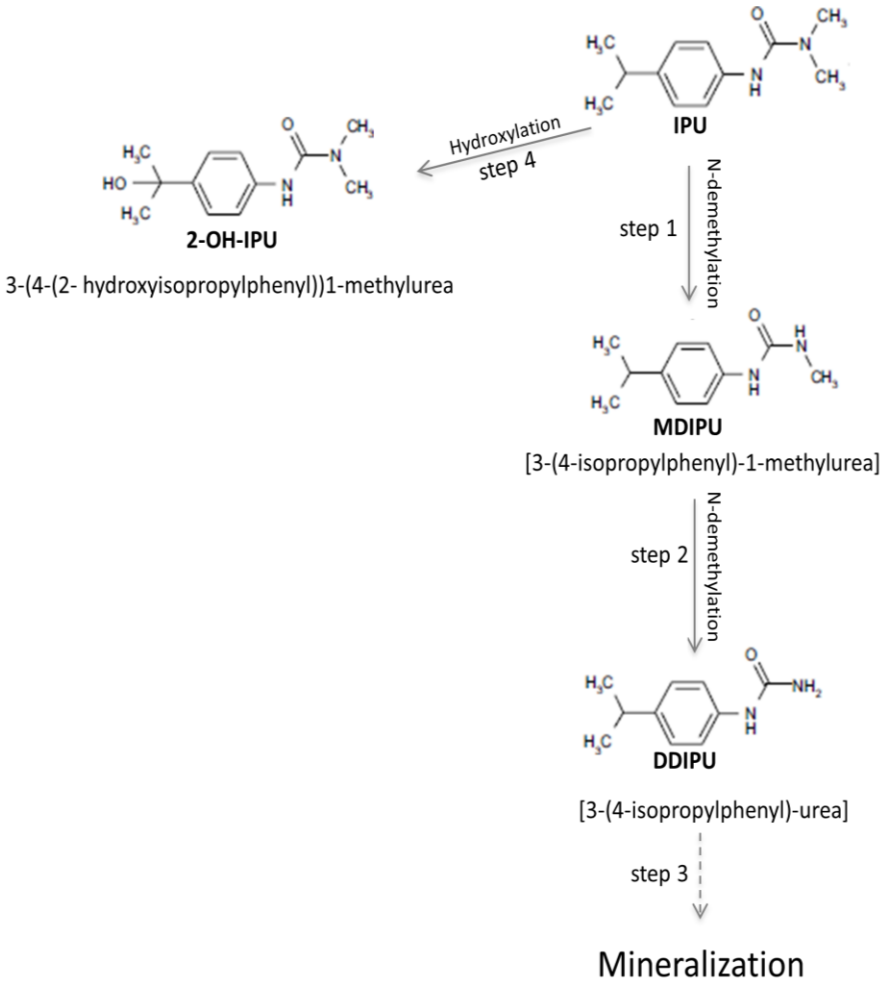


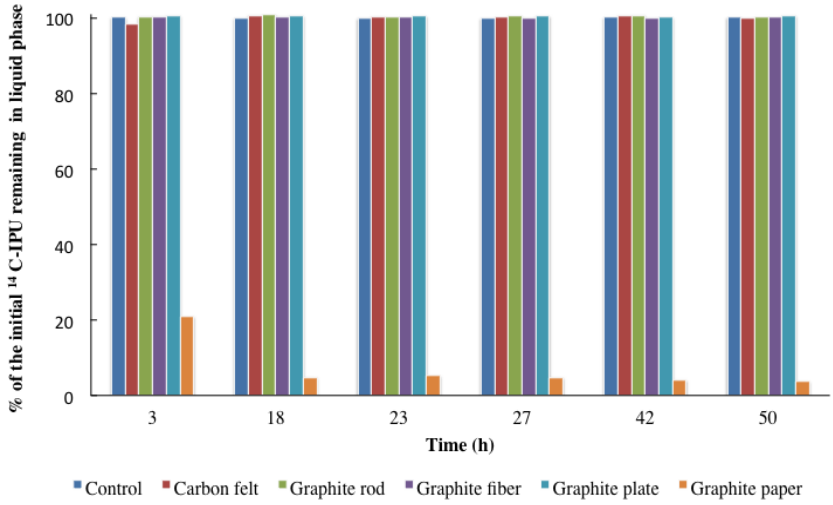
Figure 3.5: Proposed biodegradation pathways for IPU under polarized-MERCs conditions.

3.5 **Supporting information**

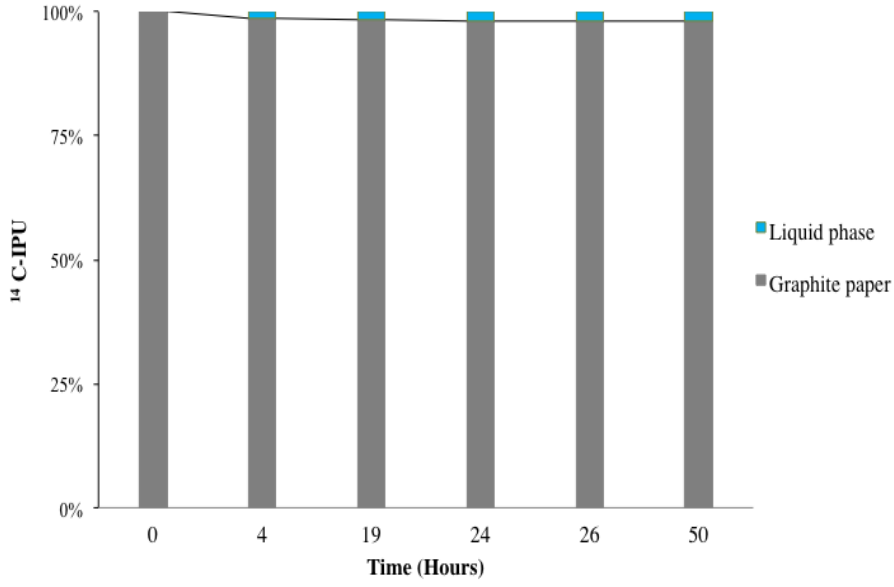
^{14}C-IPU adsorption-desorption assays in different electro-conductive materials

In this study, batch experiments were conducted in triplicates to investigate the adsorption-desorption kinetics of IPU in different electro-conductive materials (graphite paper, graphite plate, carbon felt, graphite rod and carbon fiber). 50 mL eppendorf tubes were filled with 35 mL deionized water and 100 μl ^{14}C-IPU standard was added to give a final concentration of 5 (± 0.1) μg mL and a specific radioactivity of 154 Bq μg-1. Samples of a total geometrical area of 39 cm^2 of each material (except graphite rod with 1,5 cm^2, the area that would be used to conform a Microbial Electroremediating Cell) were placed into the eppendorf tubes, which were shaken overhead continuously. The control was performed under the same conditions but without the presence of any material. At different time intervals aliquots of 0,1 mL were sampled to measure the radioactivity until the equilibrium for adsorption of ^{14}C-IPU was reached. For the determination of radioactivity, the 0,1 mL samples were mixed with 5 mL Ultima Gold XR and measured in a liquid scintillation counter (Tricarb 1900 TR, Packard, Dreieich, Germany). IPU adsorption was rapid at initial stages in graphite paper, reaching a stable phase just after 18 hours with 95% of the initial ^{14}C-IPU adsorbed in the material. For the rest of materials there was not adsorption processes, remaining the total amount of initial ^{14}C IPU in the liquid phase (Supporting figure 3.1). So thus, the desorption assay was only performed with paper graphite, which was transferred to plastic incubators with 35 mL fresh deionized water to investigate the reversibility of the process. During the desorption process the tubes were shaken overhead and proceeding as in the adsorption phase, aliquots of 0,1mL were taken to measure at different time intervals the radioactivity. Only 1.9 % of the total ^{14}C IPU adsorbed in the material was desorbed, showing an irreversible bond under the experimental conditions (Supporting figure 3.2). Regarding to the results and considering the convenience physic-mechanical properties, the high surface area, and the

inexpensive cost, the material used to conform the electrodes of the bioelectrochemical systems was carbon felt.



Supporting figure 3.1: ^{14}C-IPU adsorption in different electro-conductive materials



Supporting figure 3.2:^{14}C-IPU desorption in graphite paper.

PART II: Bioelectroventing: selecting microbial communities to detoxify an isoproturon-polluted soil by anode stimulation

Chapter redrafted after:

Rodrigo Quejigo, J., Dominguez-Garay, A., Dörfler, U., Schroll, R., & Esteve-Núñez, A. (2016). Bioelectroventing: selecting microbial communities to detoxify an isoproturon-polluted soil by anode stimulation. **Submitted**.

Bioelectroventing: selecting microbial comunities to detoxify an isoprturon-polluted soil by anode stimulation

3.7 Abstract

The biodegradation of pollutants in soil is limited by the availability of terminal electron acceptors required for supporting microbial respiration. Microbial Electroremediating Cells (MERCs) consist of a variety of bioelectrochemical devices that aim to overcome this electron acceptor limitation and maximize the biodegradation of pollutants in the environment. This electrode-based method to stimulate the oxidative metabolism of environmental microbial populations has recently been refereed as *bioelectroventing*. The current research uses MERCs principles, under different configurations, for stimulating native soil bacteria to achieve the complete removal of the herbicide isoproturon (IPU). Our studies conclude that the application of a high anodic potential (+600 mV versus Ag/AgCl) to contaminated soils increases, not only IPU-removal, but also leads to an effective clean-up as demonstrated by soil ecotoxicological analysis after the respective treatments. Furthermore, electrode potential differences induced taxonomical shifts in the microbial community as revealed by the high-throughput sequencing analysis. We also used microbial community diversity as reporter of electrode influence. Our results revealed that the electrode had an impact on the communities located as far as 0.5 cm away. The data provided here is evidence that polarized electrodes are a cost-effective and environmentally friendly strategy to select microbial communities for the successfully bioremediation of isoproturon-polluted soils.

3.8 Introduction

Environmental pollution by xenobiotic compounds is a matter of global concern (Folberth *et al*., 2009). Biodegradation based on microbial metabolic activities is the primary mechanism for pollutant removal in the environment due to the biodiversity and catabolic potential of the microbial communities. However, the bioremediation of a pollutant depends on the environmental conditions, the existence of indigenous degrading species, and the nature and chemical structure of the compound being degraded. In environments like soil, the absence of suitable terminal electron acceptors (TEA) to sustain microbial respiration might be responsible for the limited anaerobic biodegradation of pollutants (Larsen and Aamand, 2001; Megharaj *et al*., 2011).

Microbial Electrochemical Systems or Microbial Electrochemical Technologies (METs) have been shown as an alternative to the classical bioremediation strategy of supplying electron acceptors like oxygen (bioventing) (Kabelitz *et al*., 2009; García *et al*., 2010), humic acids (Lovley, 2000) or nitrates (Yu *et al*., 2013) to name a few. Rodrigo *et al*. (2014) referred to the microbial electrochemical devices that aim to maximize current production (Amperes) through maximizing metabolic degradation of organic/inorganic soil pollutants as Microbial Electroremediating Cells (MERCs). In MERCs, electroactive microorganisms oxidize the organic pollutant as an electron donor and use the anode as an inexhaustible electron acceptor. This strategy is called *Bioelectroventing*, in allusion to the similarities with the traditional bioremediation technique *bioventing* where oxygen is artificially applied as electron acceptor. Likewise, these bacteria can also use electrodes as an electron source for reducing pollutants (Rosenbaum *et al*., 2011).

The electrode´s potential governs the microbes' electron releasing capabilities, determining from a thermodynamic point of view, the metabolic pathway able to be used and the theoretical energy gain from the biocatalyst (Schröder 2007). To date, most studies on electrode-based biostimulation

used a soil-buried electrode with a negative potential as a response to the biochemical environment around it, which can be insufficient or inappropriate to drive the transformation of many recalcitrant organics (Zhao *et al*., 2006). A higher anode potential may increase the amount of energy, per electron transferred, available for growth and cell maintenance, increasing the reduction energy yield and resulting in higher microbial density and current generation (Aelterman *et al*., 2006b; Finkelstein *et al*. 2006; Busalmen *et al*., 2008). Actually electrodes artificially polarized at potentials as high as +600 mV (versus Ag/AgCl) had a high impact on the microbial degradation activity in herbicides-contaminated soils (Rodrigo *et al*., 2016). So, the electrodes not only overcame the TEA limitation but also allow for the controllability of the bioremediation processes by engineering the environmental redox conditions and selecting microbial activities that remediate contaminants of concern.

Nevertheless microorganisms involved in MERCs are poorly understood. The entire microbial community comprises a complex network of upstream fermenters that generate electron donors and downstream electron consumers that produce methane and/or electricity as end products. Thus, MERCs performance not only rely on electroactive bacteria's ability to transfer electrons from substrates to an electrode without the aid of external mediators, but on non-electrochemically active microorganisms and their immense ensemble of syntrophic interactions (Zhi *et al*., 2014). Despite this microbial complexity, linking pollutant electrocatalysis in MERCs to the microbial communities involved will lead to better insight and understanding of the implication of the anode-related microbial community in the pollutant degradation and therefore may help to optimize our envisioned application of MERCs (Daghio *et al*., 2016).

The absence of suitable TEAs in anaerobic and strongly reductive environments like flooded soils might be responsible for the limited biodegradation of isoproturon (IPU) (Larsen and Aamand, 2001). Isoproturon is one of the most extensively used herbicides in agriculture for pre- and post-emergence control of annual grasses and weeds in winter cereals. As a consequence, the presence of IPU in groundwater may exceed the approved

critical value for drinking water (0.1 μg L^{-1}) set by the European Community Drinking Water Directive (Folberth *et al*., 2009), leading to a significant impact on ecosystems and hazards to human health.

In the current work we explore the role of communities of heterotrophic bacteria participating in bioelectroventing for cleaning-up IPU-polluted soil and how they are affected by electrode potential and their distance from the electrode.

3.9 Experimental procedures

Chemicals

3-(4-isopropylphenyl)-1,1-dimethylurea (IPU), monodemethyl-isoproturon (MDIPU) [3-(4-isopropylphenyl)-1-methyl-urea], 2-OH-Mono-demethyl-Isoproturon (2-OH-MIPU) 3-(4-(2- hydroxyisopropylphenyl))1-methylurea, didemethyl-isoproturon (DD-IPU) [3-(4-isopropylphenyl)-urea] and 4-isopropyl-aniline (4-IPA) were purchased from Dr. Ehrenstorfer (Augsburg, Germany; purity 99.5%). All other chemicals and solvents were of analytical grade and were purchased from Merck (Darmstadt, Germany).

Soil

The soil material was an Aric Anthrosol from an agricultural field (Hohenwart; latitude: 48.250, longitude: 11.567, elevation 472 m) in Germany without IPU-history and with an organic matter content of 0.99 %. A complete physical-chemical analysis of this soil was previously reported by Grundmann *et al*. (2011). Soil samples were taken from 0-20 cm and were stored in plastic bags at -20 ºC according to guidelines of the Organization for Economic Cooperation and Development (OECD, 1995). The soil samples were unfrozen and equilibrated 2 weeks before the start of the experiment following the incubation protocol specified by Rodrigo *et al*. (2016).

Spiking of the soil samples

45 μL of 19 mM ^{14}C-IPU standard was applied to an aliquot of 3 g dried-and-ground soil and homogeneously mixed. After evaporation of the organic solvent (methanol), the soil aliquot was mixed with 32 g (dry weight equivalent) of equilibrated soil with the goal to distribute the pollutant homogenously, resulting in a concentration of 5 (± 0.1) μg g^{-1} soil (dry weight). The spiked soil sample was transferred to an opaque glass flask of the laboratory system described below, compacted to a soil density of 1.3 g cm^{-3} and flooded (water holding capacity + 35 mL extra deionised water). Water evaporation was compensated for 3 times per week by the addition of deionized water.

Laboratory system

The degradation experiment was conducted in a laboratory system built in approximation to the OECD guideline for testing of chemicals 304A (OECD, 1981). It consisted of opaque glass flasks (250 mL volume; neoLab, Heidelberg, Germany), which were closed with a rubber stopper (neoLab, Heidelberg, Germany). A hollow needle (neoLab, Heidelberg, Germany) conducted through the rubber stopper allowed a constant supply of O_2. Actually, it is common practice in Microbial Electrochemical Systems to expose the cathode electrode to atmospheric oxygen (He *et al*., 2007; Song *et al*., 2011) given that oxygen reduction reaction is the dominant cathodic process.

MERCs: Operating conditions

MERCs were assembled in the laboratory system (Supporting figure 3.3B and C). The electrode used in this experiment was carbon felt (Sofacel, Barcelona, Spain), as it showed no IPU-adsorption and very adequate mechanical properties to conform to the system (Rodrigo *et al*., 2016). The electrodes were located at the bottom of the soil layer (anode) and above the water body (cathode). The geometrical area of the electrodes was 39 cm^2 (surface area: 0.7 m^2 g^{-1}). MERCs were operated under 2 different conditions:

1) systems operated at a poised anode potential of + 600 mV versus Ag/AgCl reference electrode (RE-5B, BASi, United Kingdom) (+199 mV vs. SHE) by using a potentiostat (NEV2 nanoelectra), which are designated as **pol-MERCs** and 2) systems where the electrodes were connected by a copper wire using a 56Ω external resistor (R), with the redox potential of the anode set spontaneously by the redox potential differences across sediment/water, which are designated simply **MERCs**.

Electrode-free controls (Supporting figure 3.3A) were assembled in the laboratory system without electrodes and under the same water content, temperature and IPU-concentrations as the electrode-assisted treatments.

Abiotic reactions on IPU were evaluated by using sterile free-electrode control. The soil was sterilized with γ-irradiation in a Gammacell 220 cobalt-60 irradiation unit during 96 hours at a rate of 8.33 $Gy.min^{-1}$ for a total γ-ray dosage of 60 KGy. Biologic activity was tested by dish-plate inoculation. Elutes of soil were prepared using phosphate buffer (pH 8) in 1:10 soil-water ratio. 1:10 and 1:100 dilutions were carried out and incubated at 30 ºC in LB agar plates during 72 h. Colonies formation was detected only in plates seeding with elutes of non-sterilized soil.

Biodegradation assays

The IPU-removal was studied in a closed aerated laboratory system, as described above. The soil samples were incubated at 30 ± 0.1 ºC for 25 days in the dark. After 25 days of incubation, soil samples were extracted, and the extracts were cleaned up and analysed by HPLC. Soil aliquots were extracted with methanol in an accelerated solvent extractor (ASE 200, Dionex, Idstein, Germany) at 90 ºC, with a pressure of 10 MPa. The ASE extracts were concentrated by rotary evaporator to remove the organic solvent. The concentrated extracts were adjusted to 500 mL with distilled water and subjected to solid phase extraction (SPE; Lichrolut ENV 200 mg, Varian, Darmstadt, Germany). After extraction, the SPE columns were dried under a gentle nitrogen stream and eluted with 10 mL methanol. The elute was concentrated to one mL with a rotary evaporator and further concentrated

under a gentle nitrogen-stream to 400 µL. The samples were immediately analysed by HPLC. Twenty microliter of each of these samples were injected to a HPLC system, consisting of an L-6200 Intelligent Pump (Merck-Hitachi, Darmstadt, Germany) and an UV/VIS detector (240 nm, Merck, Darmstadt, Germany). The column used was a Lichrospher 100 RP-18, 5 µm, 4 x 250 mm (Merck, Darmstadt, Germany). The mobile phase consisted of A = acetonitrile (HPLC grade) and B = water (Lichrosolv water for chromatography, Merck, Darmstadt, Germany) at a flow rate of 1 mL min^{-1}. The gradient program was as follows: T0min 95% A, T15min 40% A, T20min 40% A, T25min 95% A, T30min 95% A. Parent compound and metabolites were identified by comparison of their retention times with reference substances.

DNA extraction, PCR amplification and sequencing using illumina MiSeq

Anode-adjacent soil was separately collected at the end of experiment by scraping the attached soil with a sterile razor blade and preserved at -80◦C until extraction of genomic DNA. Electrode-free control samples were as well taken from the same soil depth level than in the electrode-assisted treatments. In pol-MERCs extra samples at 0.5 cm above the anode were collected. Genomic DNA was extracted with a PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., USA), according to the manufacturer's instructions. DNA concentration was measured by Nanodrop 2000 instrument (NanoDrop Technologies, Wilmington, USA) and the V3-V4 hypervariable regions of the 16S rRNA gene were PCR amplified and used for illumina high-throughput sequencing by ERA7 Bioinformatics (Granada, Spain). The taxonomic assignment was based on the Best BLAST Hit obtained in the BLAST of each read against the NCBI **nt** database with over a threshold of similarity (e<1E-15).

Toxicity evaluation

Toxicity evaluation of soil extracts was carried out according to OECD Test Guide 201 (OECD 2008), using the green microalgae *Pseudokirchneriella subcapitata* as it was reported previously (Boltes *et al*.,

2012; González-Pleiter *et al*., 2013). Microorganisms and culture media were purchased from MicroBioTest Inc., Belgium. According to the instructions of manufacturer, algal beads were reconstituted and grown during 3 days at 22 ± 2 ºC under continuous light and 150 rpm of stirring until reached the logarithmic growing phase. Cell growth was monitored by the optical density (OD) of the culture at 670 nm to control growth process (Shimadzu UV-VIS 1800). Test started with the prescribed amount of $5x10^4$ cells per mL and was performed in 96-well clear disposable microplates. Three replicates of each soil extract, including negative control (non-IPU-polluted soil) and blank (algae-free) were assayed. Similarly, positive controls with IPU-concentration from 0.25 to 5 mg L^{-1} were tested in order to evaluate the EC50, a toxicological parameter that represent the effective concentration that cause 50% inhibition algal growth. The dilution factor for the polluted wells was 0.9268 (190 µL extracted soil sample + 10 µL growth media + 5 µL alga culture). Plates were incubated during three days under the same conditions of light and temperature as inoculum cultures were grown but in absence of stirring. The *in vivo* fluorescence emission of chlorophyll (excitation 450 nm; emission 672 nm) was measured daily using a FLUOROSKAN FL, Thermo Fisher. Then, the percentage of growth inhibition in test samples was produced by reference to the negative control assay.

3.10 Results and discussion

The bioremediation performance of IPU-polluted soil was previously reported under a positive electrode potential (Rodrigo *et al*., 2016). Nevertheless, we aim now to explore the role of the electrode potential on the clean-up (ecotoxicology) by operating the systems also at negative electrodes potentials.

Bioelectroventing IPU-polluted soil: the effect the electrode potential

This experimental series was developed in order to evaluate the microbial degradation of IPU under different scenarios: MERCs and pol-MERCs. The intrinsic capability of the soil for this function under flooded conditions (electrode-free control) was examined simultaneously. After the

incubation period (25 days) the highest IPU-removal was reached in pol-MERCs (+600 mV versus Ag/AgCl), where over 97% of the initial applied IPU was not found in the final soil extracts (Fig. 3.6A). In contrast, anodes hosted by MERC setups (-200 mV versus Ag/AgCl) removed just 66%. The electrode-free soil removed 59%. This increase is consistent with the higher cumulative mineralization under MERC conditions, which reached the 3.5% versus only 1% under electrode-free control (Supporting table 3.1).

Regarding IPU-metabolites, we have successfully identified monodemethyl-isoproturon (MDIPU). MDIPU can be generated after demethylation of the N,N-dimethylurea side-chain and it was reported as the most abundant metabolite after IPU-biotransformation in agricultural soils (Sørensen *et al*., 2003). At the end of the assay (Fig. 3.6A and B) 12.2% of the total applied IPU was transform to MDPU under electrode free control (0.63 µg g^{-1} dry soil extracted at the final time), 14.30% under MERC (0.74 µg g^{-1} dry soil extracted at the final time) and the lowest production, 8.50%, under pol-MERC (0.44 µg g^{-1} dry soil extracted at the final time). The extractable IPU-residues fraction in the soil extracts (IPU and MDIPU) was very variable between different experimental conditions, from the 50.1% (free-electrode control) and the 47.3% (MERC) to the scarce 12% under pol-MERC. This distribution is consistent with the results already reported by Rodrigo *et al*., (2016), where MDIPU was identified by ^{14}C detection techniques, besides another four ^{14}C-compounds that not exceeded the 4.5% of the total ^{14}C-IPU applied.

Sterile electrode-free control was assayed in order to determine the abiotic physicochemical impact on IPU-availability. After extracting the soil incubated under sterile conditions, ca 4.5µg g^{-1} dry soil of the initial 5µg g^{-1} was detected (ca. more than 91% of the total applied IPU) (Fig. 3.6A). This data suggested that IPU-availability is not a limiting factor for IPU-degradation and is consistent with the low IPU-sorption capacity (*Kd*) in soils, mainly controlled by the content of soil organic matter (Boivin *et al*., 2005; El Arfaoui *et al*., 2012). In spite of the oxidative potential, polarized electrodes by them-

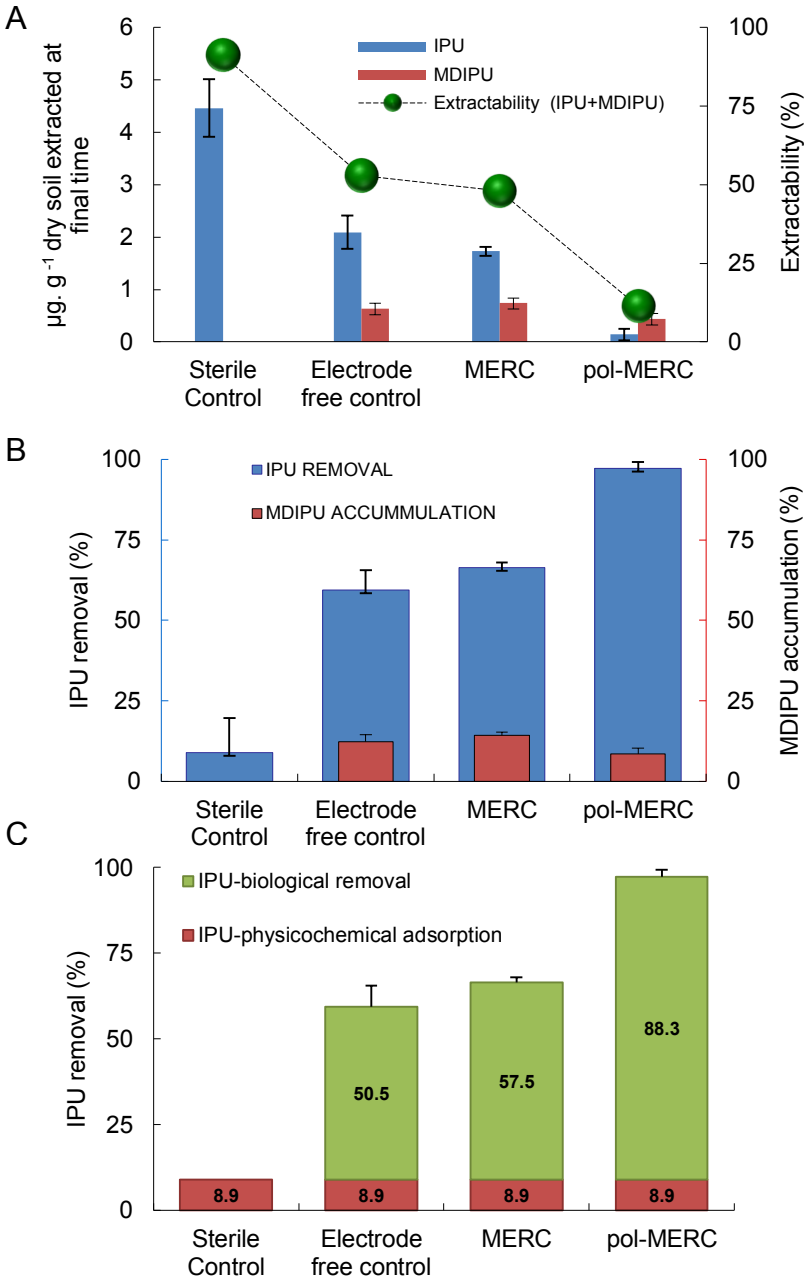


Figure 3.6: (A) IPU and MDIPU recovery in soil extracts after accelerated solvent extraction and the total extractability from IPU-polluted soil incubated under different treatments. (B) IPU-removal and MDIPU accumulation expressed in % of the initial IPU applied. (C) IPU biological removal expressed in percentage of the initial IPU applied after excluding the sorption to soil matrix in the sterile control. The error bars represent the standard deviation of the mean value for triplicate assays.

selves do not promote abiotic electro-oxidation of ^{14}C-IPU (Rodrigo *et al*., 2016). The sterilized system also reaffirms that native microbial population can remove IPU from soil and are a clear biological player in the IPU-removal in the electrode-free control as evidenced after normalizing IPU-removal data by subtracting the IPU-physicochemical adsorption (Fig. 3.6C). Thus, our hypothesis makes sense to stimulate such a native microbial community through alternative methods like bioelectroventing.

Interestingly, burying a positive potential electrode in a flooded soil may remodel the redox scenarios and as a consequence may affect the dominant metabolic processes. This may be the reason for an IPU-removal increase of over 38% in comparison with the electrode-free control. Despite the negative electrode potential, MERC as well increased the IPU-removal and the production of MDIPU when compared to the electrode-free control. However, IPU-removal was not as effective under MERC scenario, probably due to the negative anodic potential was insufficient or inappropriate to drive compound transformation (Zhao *et al*., 2006). In contrast with MERC, a strong effect on IPU removal was observed when the anode was poised at +600 mV versus Ag/AgCl (pol-MERC). Thus, the microbes might be more stimulated when the anode not only overcomes the TEA limitation but also offers a suitable redox scenario (positive potentials) to transform IPU. This situation is not very different from the classical supply of electron acceptors with high redox potentials (so, negative Gibss Free energy) to enhance bioremediation via bioventing (Kabelitz *et al*., 2009; García *et al*., 2010). Thus, pol-MERC, our electrode-assisted treatment, offers a malleable, endless, low priced and sustainable terminal electron sink (Logan *et al*., 2006) capable to adapt to different redox-dependent processes by tuning the electrochemical parameters, which clearly affect the pollutant removal activities. Indeed, bioelectroventing is a process of enhancing bioremediation by using electrodes as a microbial electron sink in order to achieve a minimal environmental disturbance. This contrasted with standard bioremediation techniques like bioventing (Kabelitz *et al*., 2009; García *et al*., 2010) that requires the continuous supply of an oxidant, which spreads through the environment and negatively affects the native microbial community.

Ecotoxicological analysis

Although IPU-removal is key to the bioremediation of polluted sites, it is not complete until the environment is no longer toxic, and this is not necessarily linked to the absence of the parent compound. The presence of metabolites might be more toxic than the parent compounds (Escher and Fenner, 2011). For instance 4-IA, an IPU-metabolite is 600-fold more toxic than IPU as tested by Microtox (Tixier *et al*., 2002). Nevertheless, this compound was not detected in our soil extracts. Thus, remediation strategies must be followed by ecotoxicity tests to ascertain if polluted sites have restored and regained its natural integrity (Hankard *et al*., 2004; Hamdi *et al*., 2007; Liu *et al*., 2010).

In accordance to EU-directives 93/67/EEC, 67/548/EEC, an EC50 value below to 1 mg L^{-1} indicates that this compound is highly toxic for aquatic organisms. In our hands, the growth rate EC50 value of *P. subcapitata* was 112 µg L^{-1} for IPU after 96h of algal incubation, which was determined using median-effect equation. In our study, ecotoxicity tests based on algal growth indicated that after treating the soil using pol-MERCs for 25 days, soil extracts exhibit just 3% inhibition of algal growth in contrast with the high inhibition (63%) shown under electrode-free control (Fig. 3.7). MERC presented an inhibition in algal growth of ca. 23%, as well a remarkably lower toxicity than the electrode-free control. Thus, 25 days of electrode-assisted treatments revealed that facilitated electron flow was required for a successful clean-up of the polluted soil, especially pol-MERC treatment where the IPU-polluted soil was fully detoxified. The detoxification in electrode-assisted treatments fits well with the higher IPU-removal for both treatments (MERC and pol-MERC) in the same period of time. Similar results have been already reported, where not just pollutant removal but true clean up after MERC treatments was demonstrated by ecotoxicological analysis in soil polluted with Polycyclic Aromatic Hydrocarbons (PAHs) (Rodrigo *et al*., 2014) and herbicides (Domínguez-Garay *et al*., 2016); these did not just remove the targeted pollutants but were a true clean-up as demonstrated by ecotoxicological analysis. Therefore, monitoring MERC-assisted bioremediaton of pollutants in

soils using ecotoxicological methods provides a better insight into the ecological assessment of remediation and the results demonstrate the detoxification capacity of pol-MERC outperforms that of the natural attenuation process, shortening the time needed to clean up a polluted terrestrial ecosystem.

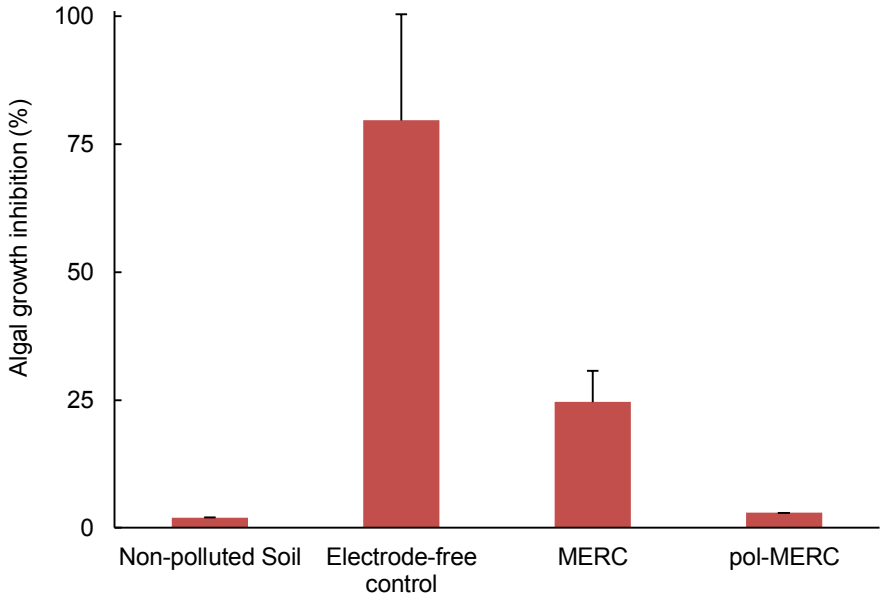


Figure 3.7: Toxicity levels in soil extractions from IPU-polluted soil incubated under different treatments. The toxicity values were represented as inhibition of *P.subcapitata* algal growth (%). Reference toxicity values for non-polluted soil (negative control) were also provided. The error bars represent the standard deviation (n=3, S.D).

Bacterial community variations in response to bioanode stimulation

At the end of the treatments, all soils were sampled and processed by illumina high-throughput sequencing of the V3-V4 region of the 16S-rRNA gene to detect how the bacterial communities reacted to bioanode stimulation, elucidating which bacteria populations are crucial for IPU-bioremediation and biocurrent generation.

Three replicates from each treatment, electrode-free control, MERC

and pol-MERC were selected for bacterial analysis. An average of 150.703 reads per sample were performed; 16S rRNA gene sequences were assigned into 7765 OTUs. Table Supporting Information 3.2 lists the values of merged and non-merged reads, assigned and non- assigned reads, the Shannon-Wiener diversity index and Simpson´s diversity index. The Shannon diversity index (H′) indicates the richness of information (i.e., the number of species present) and how the abundance of each species is distributed (the evenness of the species). It should be noted that both diversity indices are similar under each treatment and only a slight decrease can be observed in the Shannon-Wiener diversity index under anode-assisted treatments. This might suggest a selective enrichment of specific communities due to the anode.

The anode´s operational mode impacted the bacterial communities at the phylum level. Only 0.33-0.51 % of the total sequences among the test samples were not classified, and a total of 33 phyla were successfully identified from the libraries. The majority of the sequences belonged to 3 predominant phyla, *Proteobacteria*, *Firmicutes* and *Actinobacteria* (Fig. 3.8A). *Proteobacteria* accounts for the highest number of the total reads in each bacterial community, followed by *Firmicutes*, which showed higher differences between treatments. Other affected phyla included *Acidobacteria*, *Chloroflexi* and *Bacteroidetes*.

The comparison between the treatments revealed that the electrochemical effect of the anode resulted in a significant increased in abundance of *Firmicutes*. The presence of *Firmicutes* in the electrode-free control (18.5%) was significantly increased when electrodes were present, either under positive potential (pol-MERC, 27,5%) or under negative potential (MERC, 23.1%). The differences in the abundance of *Firmicutes* between the treatments were mainly in the classes *Clostridia* and *Bacili* (Fig. 3.8B). The presence of *Firmicutes* has been reported in Microbial Electrochemical Systems serving the role of converting complex carbon into simple molecules (Jung and Reagan, 2007; Lin and Lu, 2015). Moreover, previous studies have documented that some *Firmicutes* may also have electrochemical activity (Wrighton *et al*.,

2008). *Firmicutes* abundance increased during electrode assisted remediation of petroleum hydrocarbon contaminated soil (Lu *et al*., 2014a) and the degradation of macrophyte litter in sediments (Song *et al*., 2015).

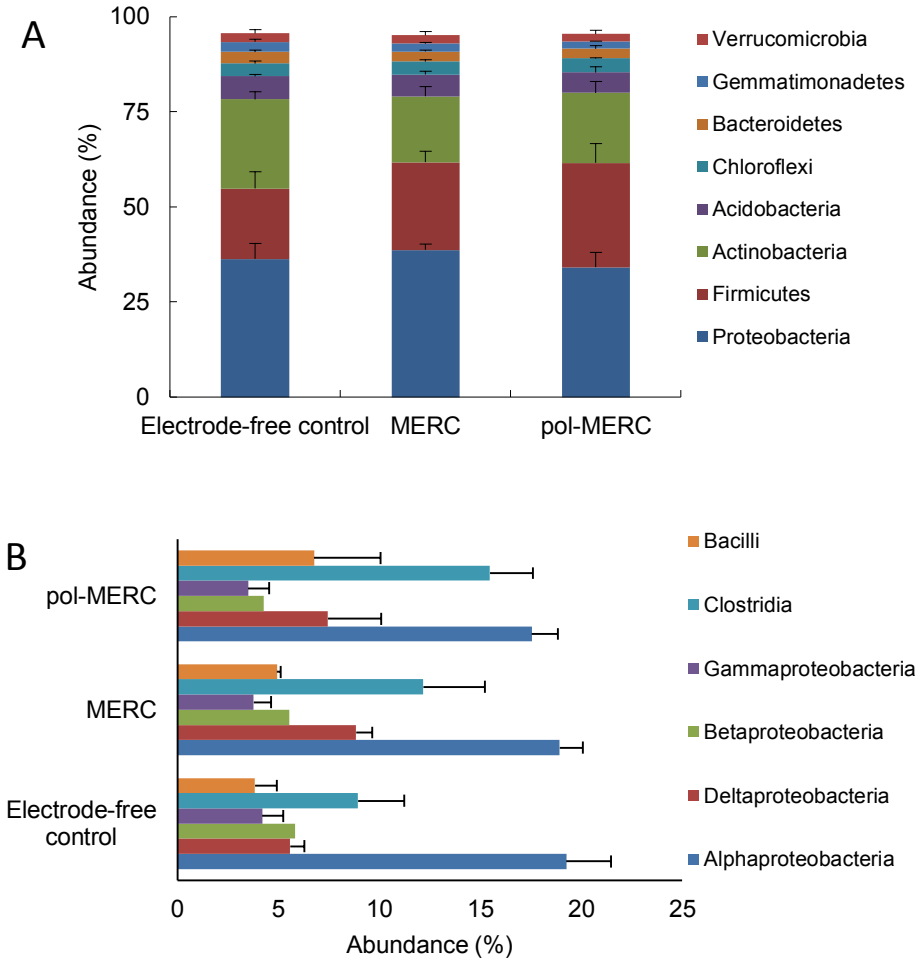


Figure 3.8: Taxonomic classification of 16S rRNA gene from bacterial communities of soil samples at phylum level (A) and the class level distribution of the most dominant phyla, *Proteobacteria* and *Firmicutes* (B). Bars represent standard deviation (n=3, S.D). Phylums with a relative abundance <2% and classes with a relative abundance <3% are not represented.

Although the *Proteobacteria* phylum showed similar abundances under all our treatments, an analysis at the class level revealed that while *Betaproteobacteria*, *Gammaproteobacteria* and *Alfaproteobacteria* reached similar abundances, *Deltaproteobacteria* increased under the influence of the anode, regardless of the electrode potential (8.8% for MERC and 7.4% for pol-MERC) (Fig. 3.8B) in comparison to the electrode-free control (5.6%). The enrichment of *Deltaproteobacteria* in these microbial communities agree with communities associated to sediment/soil embedded electrodes reported elsewhere (Bond *et al*., 2002; Reimers *et al*., 2006; Nielsen *et al*., 2007). Additionally, electrodes polarized at elevated and oscillatory redox potential (Reimers, *et al*., 2013), root exudates in plant-METs (Lin and Lu, 2015; Cabezas *et al*., 2015) and biomass amended sediments (Zhou *et al*., 2015) also led to the enrichment of *Deltaproteobacteria* on the sediment-hosted anodes. In contrast to *Deltaproteobacteria* and *Firmicutes*, a higher abundance of the phylum *Actinobacteria* was detected in those soils where electrodes were not present. *Actinobacteria* might be involved in the fermentation processes that generate electron donors from the degradation of recalcitrant components for downstream electron consumers, like those that produce methane and/or electricity as end products (Rui *et al*., 2009). Interestingly, Lu *et al.* (2014a) reported that although *Actinobacteria* are known to be a group of versatile petroleum hydrocarbon degraders, its presence decreased on electrodes buried in petroleum-polluted sediments. Inversely, the bioanode stimulation resulted in substantially increased abundance of *Actinobacteria* associated to the bioremediation of chlorinated organic compounds in river sediments (Yu *et al*., 2016).

Additionally, bacterial communities were also identified by class, order, family, genus and species level. Supporting figure 3.4 in Supporting Information (SI) displays a heat-map showing the relative abundance of different bacterial taxa above 2%. Genus-level characterization was performed to elucidate the key bacteria responsible for IPU-bioremediation allowing us to go deeper into the metabolic activities in the vicinity of the

electrode. *Symbiobacterium* was the preeminent genus in all soils although it was more abundant in MERC and pol-MERCs soil by a factor of 1.94 and 3.93 in comparison to the electrode-free control (Fig. 3.9). Currently, the genus *Symbiobacterium* is tentatively classified into family *XVIII incertae sedis* of the order *Clostridiales* within the class *clostridia* (Vos *et al*., 2011), but was recently proposed to the family *Symbiobacteriaceae* (Shiratori-Takano *et al*., 2014). *Symbiobacterium* was markedly found in flooding soils (Huang *et al*., 2015) and *Symbiobacterium thermophilum* that belongs to this genus has been reported as a unique bacterium living in symbiosis with *Bacillus sp.* and using formate as electron donor during anaerobic respiration (Ueda *et al*., 2004). Thus, it is important to note that the genus *Bacillus'* abundance increased in parallel to genus *Symbiobacterium*. *Bacillus* has the ability to degrade a wide range of organic compounds, including proteins and carbohydrates, being involved in fermentation processes (Rui *et al*., 2009; Wang *et al*., 2015). This genus has been commonly found at soil embedded electrodes (Chae *et al*., 2009; Sacco *et al*., 2012). Recent studies reported the presence of *Bacillus* species in an anodic biofilm during cis-dichloroethene oxidation (Aulenta *et al*., 2013) and members of the family *Bacillaceae* were relatively abundant in electrochemical reactors that stimulate anaerobic toluene degradation (Daghio *et al*., 2016). Furthermore, another study showed that *Bacillus subtilis* was able to use an electrode as the electron acceptor by excreting external mediators (Nimje *et al*., 2009).

Among all bacteria reported to play a role in Microbial Electrochemical Systems the genus *Geobacter* is one of the main actors. Hence, microbial communities associated to anodes are enriched in *Geobacter sp.* being abundant in sediment METs (Zhou *et al*., 2015), plant METs (De Schamphelaire *et al*., 2010; Timmers *et al*., 2012; Cabezas *et al*., 2015) and Paddy soil METs (Kouzuma *et al*., 2013; Wang *et al*., 2014). In the same way, *Geobacter* enrichment was reported during stimulation of pesticides degradation under electrode-assisted soil treatments (Cao *et al*., 2015). Our analysis also revealed the enrichment of *Geobacter* on the electrode-hosting soil compared with electrode-free control, suggesting the stimulation of *Geobacter*-related populations in current producing systems. This genus was

markedly observed under MERC (3.45%) and pol-MERC (2.32 %), both with higher abundances that electrode-free control (1.18%). So Anodes acting as TEA brought rapid acclimation of electrochemically active *Geobacter sp.* capable to transfer electrons to anodes. Although the abundance of these electroactive bacteria was higher in MERC than in pol-MERC setups, the electrochemical analysis revealed a higher electrochemical response in pol-MERC (Supporting figure 3.5). This suggests that other electro-active microbes but *Geobacter sp.* might be capable to improve the extracellular electron transfer processes under the effect of anodes poised at +600 mV versus Ag/AgCl.

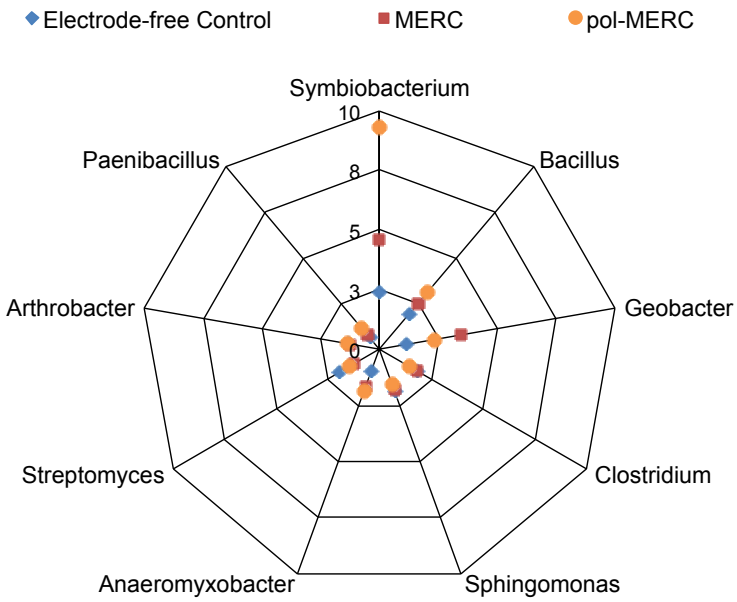


Figure 3.9: Taxonomic classification of bacterial sequences from communities of soil samples at genus level from the different treatments. Genus with a relative abundance <0.5 % are not represented.

Regarding other soil microbial communities associated to electrodes, the presence of the *Deltaproteobacteria Anaeromyxobacter sp.* has been previously detected on anodes from rice crops plant-METs (Cabezas *et al*., 2015), as well as on biocathodes able to perform dechlorination of aromatic compounds (Strycharz *et al*., 2010). Interestingly, our studies have revealed

how *Anaeromyxobacter* was increased by a factor of 1.7 in MERC and by 2 in pol-MERC relative to the electrode-free control. On top of that, *Clostridium*, *Sphyngomonas*, *Streptomyces* and *Arthrobacter* were detected at similar levels under all treatments with a relative abundance above 1%. Supporting Table 3.3 in SI displays a figure showing the relative abundance of the top 20 genera.

In contrast with standard Microbial Electrochemical Systems operating in bioreactors with homogenous solutions, the heterogeneous nature of soil strongly affected the influence area of the electrode. In that sense we decided to use the microbial community profile as a reporter of the electrode influence. Thus, we analysed communities from soil intimately associated to the anode and soil sampled at 0.5 cm above of the anode (so called pol-$MERC_{0.5}$) (Fig. 3.10A). The comparison between pol-MERC and pol-$MERCA_{0.5}$ samples revealed some bacterial abundance differences. The more explicit change consisted in the *Proteobacteria* decreased (34% in pol-MERC to 25% pol-$MERCA_{0.5}$) and the *Firmicutes* increased (27% pol-MERC to 40 % pol-$MERCA_{0.5}$) as a result of the distance between the microbial communities to the electrode. At genus level, *Geobacter* (1.5% in pol-$MERCA_{0.5}$ and 2.3% in pol-MERC) was less abundant in soil far from the anode, where we identified an identical abundance as in the electrode-free control. On the contrary, the abundance of genus *Symbiobacterium* increased more than sevenfold in soil samples pol-$MERC_{0.5}$ relative to the electrode-free control and twofold increase as compared to the soil in contact with the anode suggesting that the electrode influence to change the communities profile is higher than 0.5cm. Fig. 3.10B displays the microbial abundance at genus level at different sampling points.

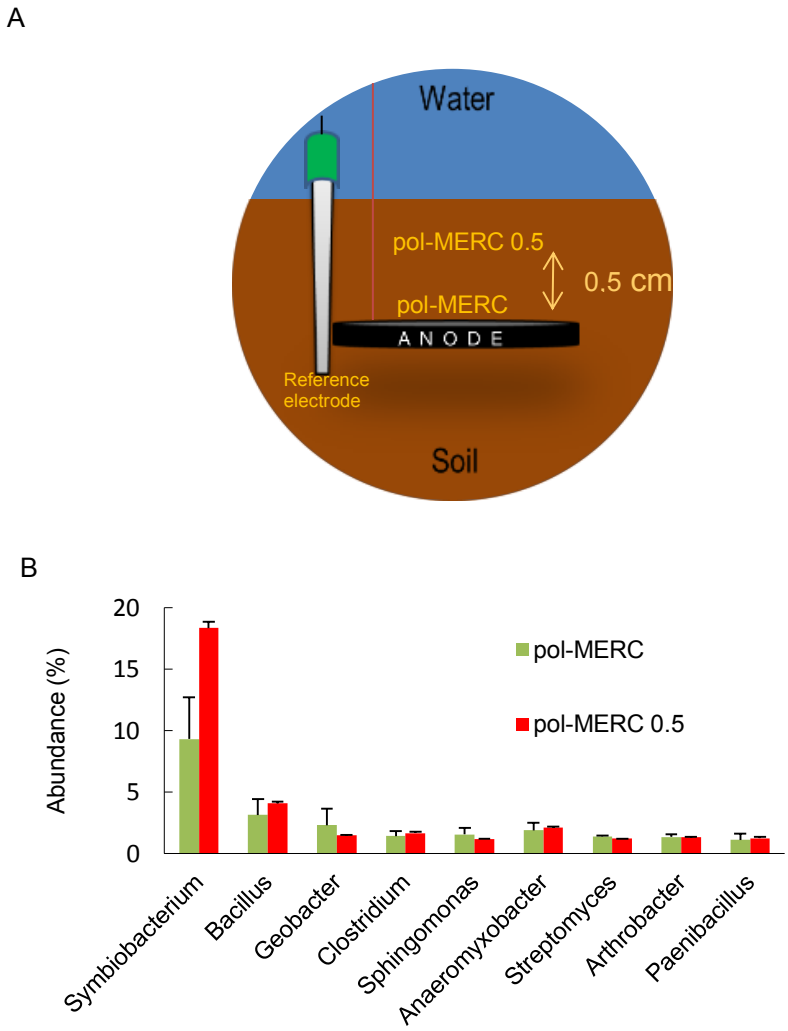
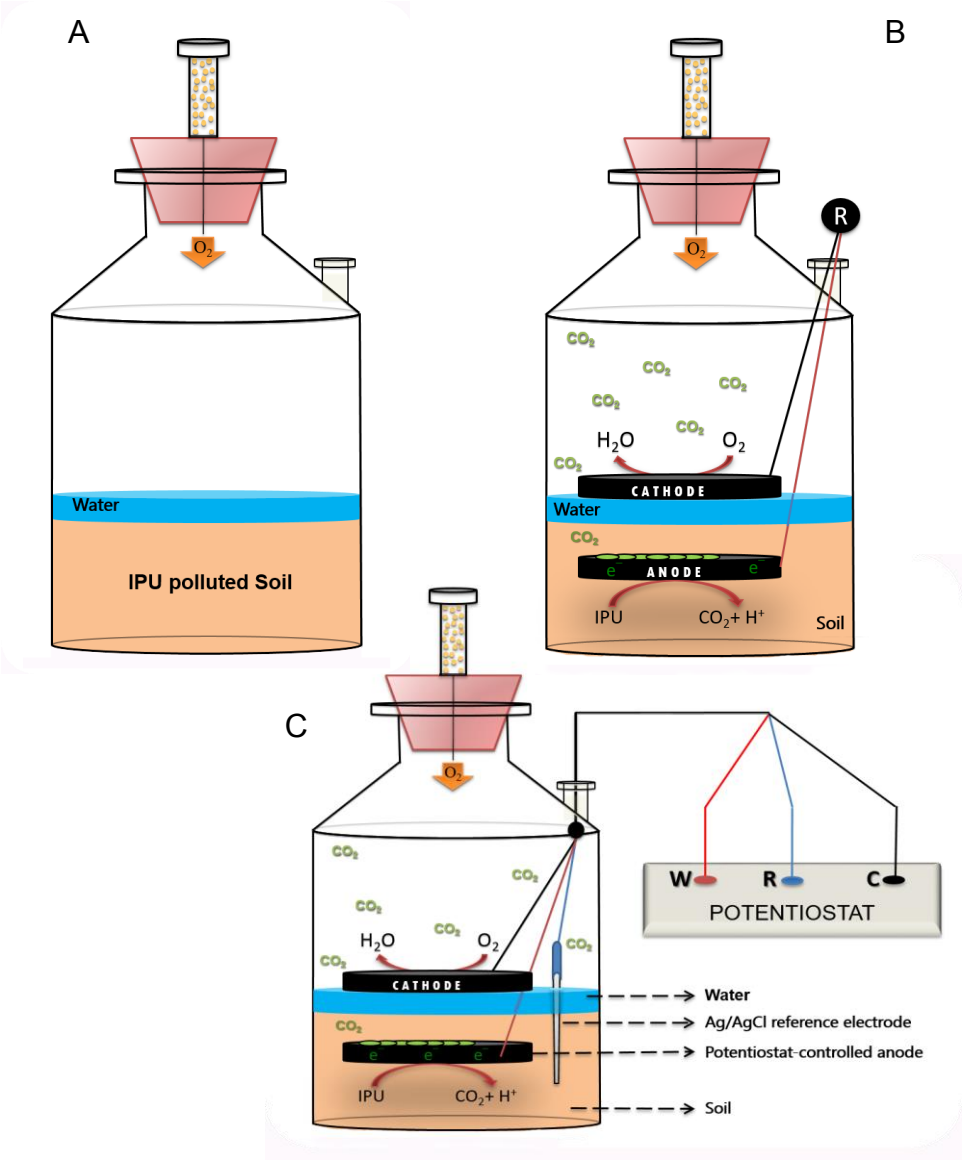


Figure 3.10: (A) Sampling points in pol-MERCs. Samples collected from the soil adjacent to the electrode were denominated pol-MERC and soil sampled 0.5 cm above the anode in the vertically section were denominated pol-MERC$_{0.5.}$ (B) Taxonomic classification of bacterial sequences from communities of soil samples collected at different sampling points in pol-MERC assays at genus level. Genus with a relative abundance <1% are not represented. Bars represent standard deviation (n=3, S.D).

3.11 Conclusions

In the present study, we demonstrate how the use of electrodes at high anodic potential (600 mV versus Ag/AgCl) in IPU-contaminated soil increases not only the IPU-removal, but also leads to effective clean-up demonstrated by ecotoxicological analysis of treated soils. These functional differences (IPU-removal and soil effective clean-up) were associated to taxonomical shifts in the microbial community revealed by the high-throughput sequencing analysis and reinforced the use of polarized electrodes as a conceivable cost-effective and environmentally friendly strategy for enhancing pollutant degradation under extremely reductive conditions.

3.12 Supporting Information

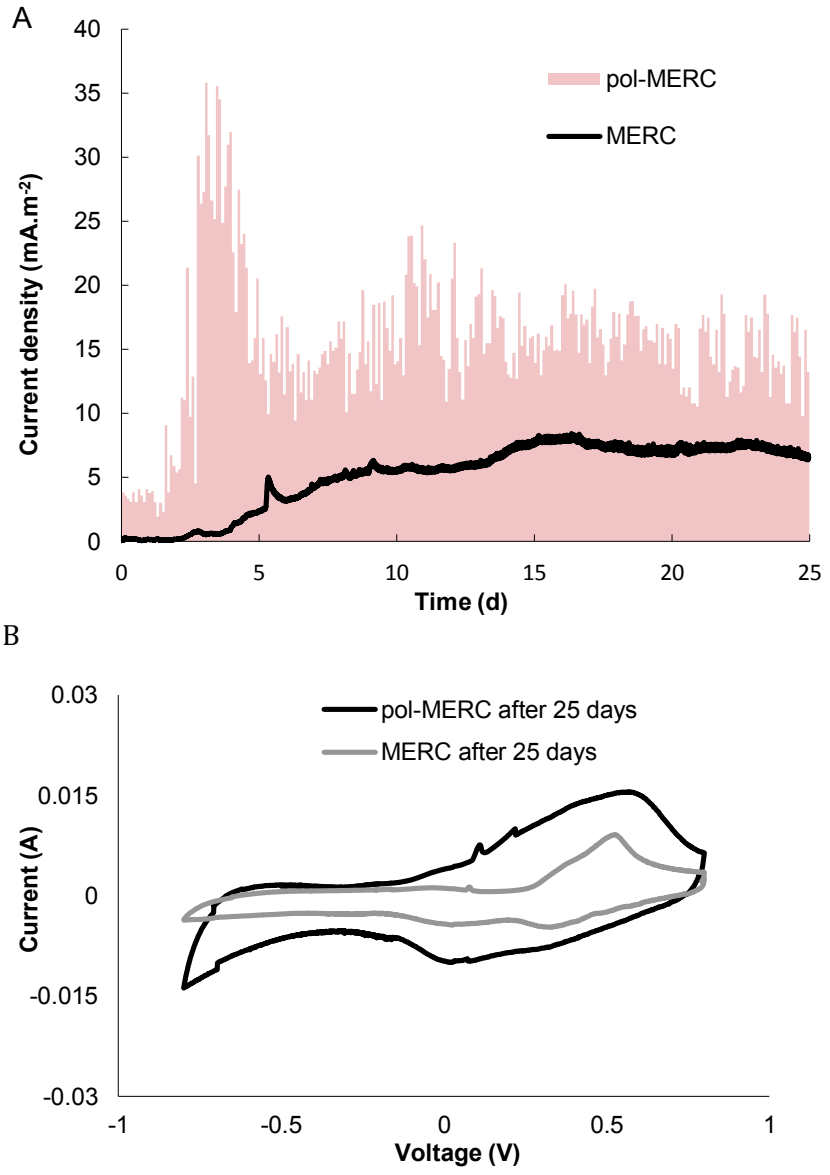


Supporting figure 3.3: Scheme of the different operation modus: (A) **electrode-free control**, assembled in the laboratory system without the presence of the electrodes, (B) **MERC**, where the electrodes were connected by a copper wire using a 56 Ω external resistor (R), and the redox potential of the anode was set spontaneously by the redox potential differences across sediment/water and (C) **pol-MERC**. The anodes in pol-MERCs were poised at 0.6 V vs Ag/AgCl (+197 mV versus normal hydrogen electrode (NHE)).

Supporting figure 3.4: Heatmap of tested samples under the different treatments for classified bacteria with relative abundances of top 40 taxa based on illumina sequencing. Taxa with a relative abundance <2% are not represented.

Electrochemical analysis

The anode potential in pol-MERCs was continuously poised at + 600 mV versus Ag/AgCl (+197 mV versus normal hydrogen electrode (NHE)) reference electrode (RE-5B, BASi, United Kingdom) using a potentiostat



Supporting figure 3.5: A) Chronoramperomety of pol-MERCs polarized at 0.6V (versus Ag/AgCl) and MERC connected to a 56 Ω resistor. Geometrical anode surface was used for calculating the current density B) Cyclic voltammetry tests (scan rate: 1 mV/s) carried out at initial experimental time and after 25 days under bioelectrochemical assisted soil (pol-MERCs and sterile MERCs). Pol-MERC amperometry and CV were already published by Rodrigo *et al*. (2016).

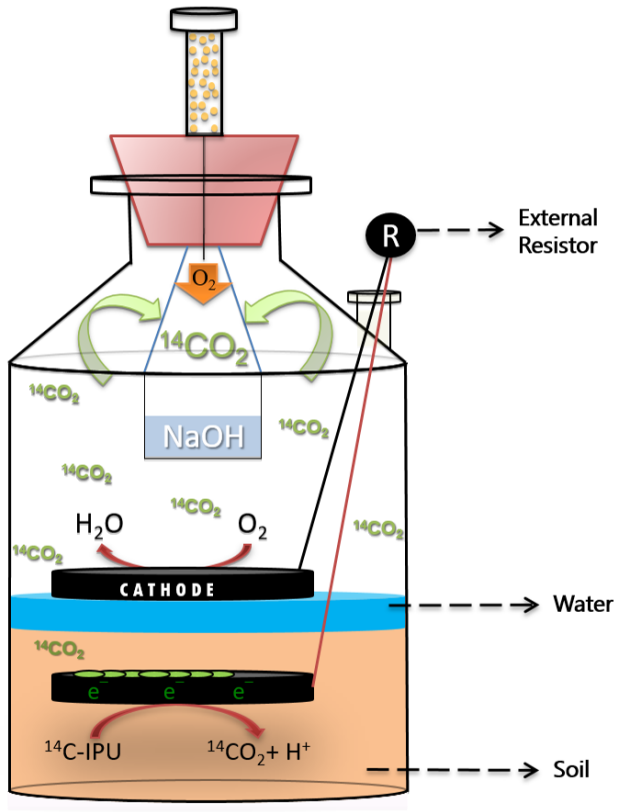
(NEV1-3 Nanoelectra, Madrid, Spain). Cyclic voltammetry (CV) was recorded to qualitatively characterize the electrochemical activity of the soil by imposing a scan rate of 1 mV/s from the open circuit voltage (OCV) potential by a potentiostat (NEV3 Nanoelectra, Madrid, Spain). In MERC the voltage difference between the two electrodes was periodically measured using a digital multimeter (Model 2700, Keithley Instruments, USA). Data were recorded on a spreadsheet using ExceLINX_ (Keithley) via an interface card (GPIB Interface Boards, Keithley) linked to a personal computer. The recorded voltage was converted into current using Ohm's law (current = voltage/resistance). The current density was calculated based on the anode surface area.

Mineralization assay

100 μL of ^{14}C-IPU standard mix was applied to an aliquot of 3 g dried-and-ground soil and homogeneously mixed. After evaporation of the organic solvent (methanol), the soil aliquot was mixed with 32 g (dry weight equivalent) of equilibrated soil with the goal to distribute the pollutant homogenously resulting in a concentration of 5 (± 0.1) μg g^{-1} soil (dry weight) and a radioactivity of 154 Bq μg^{-1}. The spiked soil sample was transferred to the opaque glass flask of the laboratory system described below, compacted to a soil density of 1.3 g cm^{-3} and adjusted to flooded conditions (water holding capacity + 35 mL extra deionised water). As a result 2 cm water body was maintained above the soil in order to ensure flooded conditions. Water evaporation was compensated 3 times per week by the addition of deionized water.

The mineralization experiment was conducted in a laboratory system built in approximation to the OECD guideline for testing of chemicals 304A (OECD, 1981) (Supporting figure 3.6). It consisted of opaque glass flasks (250 mL volume; neoLab, Heidelberg, Germany), which were closed with a rubber stopper (neoLab, Heidelberg, Germany); at the bottom of the stopper a plastic beaker of 25 mL volume (VWR International, Darmstadt, Germany) was attached. The plastic beaker was filled with 10 mL of 0.1 N NaOH (Merck,

Darmstadt, Germany) to trap the $^{14}CO_2$ resulting from the mineralization of ^{14}C-IPU. The NaOH-solution was exchanged three times per week and from the collected NaOH solution an aliquot of 2 mL was taken, mixed with 3 mL Ultima FLO AF (PerkinElmer, Rodgau, Germany) and the radioactivity was measured in a liquid scintillation counter (Tricarb 1900 TR, Packard, Dreieich, Germany). A hollow needle (neoLab, Heidelberg, Germany) conducted through the rubber stopper allowed a constant supply of O_2. To prevent saturation of the NaOH-solution with atmospheric CO_2, a plastic reservoir (neoLab, Heidelberg, Germany) filled with soda lime (Merck, Darmstadt, Germany) was connected to the needle.



Supporting figure 3.6: MERC Scheme. $^{14}CO_2$ was trapped in a 0.1 N NaOH solution for allowing the measurement of ^{14}C-IPU mineralization.

Supporting Table 3.1: Cumulative mineralization of ^{14}C-IPU within 25 days in MERCs (n=3, S.D.) compared with pol-MERCs (n=4, S.D.) and free-electrode control (n=3, S.D.). The mineralization of this 2 last treatment has been already reported in Rodrigo *et al.* (2016).

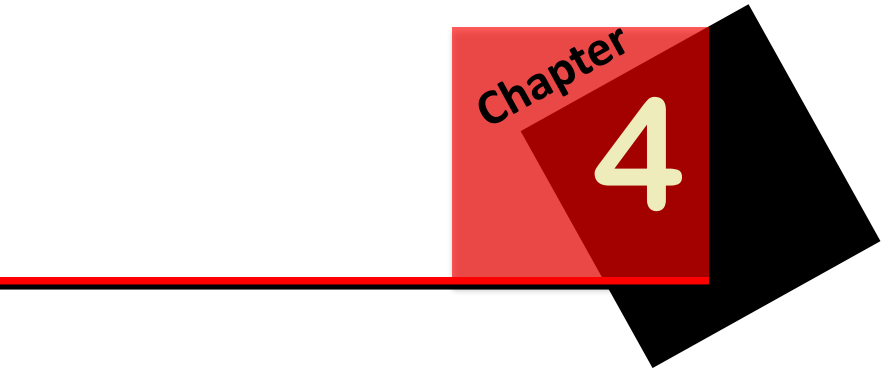
Treatment	Mineralization (%)
Electrode-free control	0.9 ± 0.1
MERC	**3.5 ± 0.2**
pol-MERCs	21.3 ± 1.1

Supporting Table 3.2: The number of merged and non-merged, assigned and non-assigned reads, Shannon´s and Simpson´s diversity index under the different treatments including pol-MERC$_{0.5}$ (soil sampled 0.5 cm above the anode in the vertically section).

	Merged	Non-Merged	Assigned	Non-Assigned	Shannon-Wiener Index	Simpson's Index
Electrode-free control	134723	7197	134361	362	5.0	0.97
MERC	144547	6851	144231	316	5.0	0.98
pol-MERC	144484	7282	144104	380	4.9	0.97
pol-MERC 0.5	150076	7654	149625	452	4.7	0.96

Supporting Table 3.3: Phylogenetic classification of the 16S rRNA gene sequences at genus level in soil samples under the different treatments.

		%	%	%
Taxa	**Genus**	Electrode-free Control	MERC	pol-MERC
Firmicutes	Symbiobacterium	2.38	4.60	9.33
	Bacillus	1.93	2.50	3.14
	Clostridium	1.80	1.83	1.44
	Paenibacillus	0.64	0.79	1.13
	Gracilibacter	0.62	0.96	0.68
	Sporomusa	0.43	0.43	0.34
Deltaproteobacteria	Geobacter	1.18	3.45	2.32
	Anaeromyxobacter	0.98	1.70	1.90
	Bdellovibrio	0.53	0.51	0.50
Alphaproteobacteria	Sphingomonas	1.88	1.81	1.57
	Microvirga	0.85	0.81	0.77
	Azospirillum	0.44	0.51	0.57
	Methylobacterium	0.63	0.53	0.33
Actinobacteria	Streptomyces	1.90	1.23	1.42
	Arthrobacter	1.31	1.26	1.37
	Solirubrobacter	1.05	0.59	0.60
	Rhodococcus	0.76	0.68	0.60
	Mycobacterium	0.79	0.61	0.67
	Nocardioides	0.71	0.40	0.47
Betaproteobacteria	Herbaspirillum	0.62	0.69	0.25


Chapter
4

Supporting microbial activities by using electrodes for mineralizing antibiotics in polluted manure

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Supporting microbial activities by using electrodes for mineralizing antibiotics in polluted manure

4.1 Abstract

Livestock manures are potential environmental sources of antibiotics. Sulfamethazine, frequently used in veterinary medicine, can enter the environment by using manure as soil fertilizer due to its incomplete absorption in the animal gut and its unmetabolized excretion. The objective of this study was to evaluate the mineralization of sulfamethazine in manure under different redox scenarios provided by Microbial Electroremediating Cells. Microbial Electroremediating Cells (MERCs) aim to overcome electron acceptor limitation and maximize metabolic oxidation to enhance the biodegradation of pollutants in the environment. This process to supply electrodes to stimulate the oxidative metabolism of environmental microbial populations is refereed as *bioelectroventing.* Our results proved that negative redox scenarios (-400mV vs. Ag/AgCl) enhanced sulfamethazine mineralization while positive polarized electrodes (+400mV (vs. Ag/AgCl) increased the microbial electrogenic metabolism. The different treatments have as well determined changes in ^{14}C-sulfamethazine metabolite pattern and ^{14}C mass balances. The impact of electrodes on environmental microorganisms and manure bioremediation suggests a promising future for this emerging technology to treat polluted livestock wastes having an important roll as a preventive soil bioremediation treatment.

4.2 Introduction

Antibiotics are extensively used not only in human and veterinary medicine but also as growth promoting agents in farming and aquaculture (Sarmah *et al*., 2006). Due to its incomplete absorption a significant fraction of these antimicrobials might be excreted in an unchanged form or bioactive metabolites through animal feces and urine (Chee-Sanford *et al*., 2009; Wei *et*

al., 2011; Nelson *et al*., 2011). Sulfamethazine (SMZ), a sulphonamide, is one of the most used antibiotics in the swine industry to treat infections and as growth promoter (Sarmah *et al*., 2006; Kim *et al*., 2011) and has been already found in concentrations as high as 10 mg kg^{-1} on a manure dry matter basis (Aust *et al*., 2008). Thus, when SMZ containing manure is used as a soil fertilizer on farmland, this high mobility drug easily leaches out and reaches different environmental zones (Haller *et al*., 2002; Hamscher *et al*., 2005). At present this sulfonamide has been already detected in soils (Rajapaksha *et al*., 2014), surface water (Rajapaksha *et al*., 2014; Amarakoon *et al*., 2014), wells (Batt *et al*., 2006) and groundwater (Hamscher *et al*., 2005; Barnes *et al*., 2008). Swine wastewaters present higher concentrations, ranging from 1 to 80 µg L^{-1} (Zhou *et al*., 2013; Ben *et al*., 2013), and eventually reaching 400 µg L^{-1} (Campagnolo *et al*., 2002). Although these concentrations are below the ecotoxicologically effective mg L^{-1} levels, chronic environmental toxic effects and potentially synergistic effects cannot be excluded (Hernando *et al*., 2005). The ubiquitous environmental presence of SMZ provokes its access to human food supplies such as milk, vegetables and pork products meaning a direct risk for human health (Clark *et al*., 2005; Dolliver *et al*., 2007; Wang *et al*., 2006). Indeed, SMZ and its N_4-acetylated metabolite have been already found in human urine (n = 541) at concentrations up to 448 pg mL^{-1} and 6000 pg mL^{-1} respectively (Ji *et al*., 2010).

To date studies has been almost totally devoted to SMZ removal in wastewater (García-Galán *et al*., 2011; Oliveira *et al*., 2016) and soil (Vithanage *et al*., 2015). All these remediation strategies could be avoided reducing the SMZ concentrations in manure before its application to agricultural fields, as a preventing bioremediation strategy. Storage pits and piles, composting and aerated lagoons are some of the manure-handling practices to reduce manure environmental impact, which requires treatment before disposal, but none of them have shown potential on further SMZ degradation. Anaerobic Digestion (AD) is the most extended emerging manure management technology providing waste stabilization, organic matter digestion, odour and pathogens reduction, a digestate as an end-product and a mixture of gases (biogas) (Holm-Nielsen *et al*., 2009; Mitchell *et al.,* 2013).

Nevertheless the few studies dedicated to asses the potential of manure AD in SMZ dissipation has not revealed promising results. Mohring *et al*., 2009 and Mitchell *et al*., 2013 reported not detectable SMZ removal during swine manure AD, while an anaerobic incubation of manure and maize silage using a semi-continuous fermenter led to a elimination of 47% and 48% respectively but without specifying the abiotic and biotic factors in the dissipation process (Spielmeyer *et al*., 2015).

Consequently, new methods for removing veterinary antibiotics in manure, especially SMZ, must be investigated. Microbial Electrochemical Systems open a new scenario of tools for enhancing biodegradation based on the fascinating interaction between living bacteria and electroconductive materials (Schröder *et al*., 2015). In particular, a variety of them so-called Microbial electroremediating Cells (MERC) has demonstrated efficient removal of nutrients (Tejedor-Sanz *et al*., 2016), chlorinated hydrocarbons (Aulenta *et al*., 2011), antibiotics (Harnisch *et al*., 2013; Guo et al., 2016), herbicides (Rodrigo *et al*., 2016, Domínguez-Garay *et al*., 2016) and petroleum hydrocarbons (Rodrigo *et al*., 2014; Zhang *et al*., 2015a) showing great versatility in wastewater, sludge, sediments or soil bioremediation. Furthermore, microbial electrochemistry in combination with anaerobic digestion has been already used to generate electricity and simultaneously remove organic matter aiming to recover energy and nitrogen simultaneously from livestock manures (Min *et al*., 2005; Zhang *et al*., 2015a; Ma *et al*., 2016; Cerrillo *et al*., 2016b) while eliminating unpleasant odours (Kim *et al*., 2008). In MERCs, electroactive microorganisms oxidize an organic pollutant acting as an electron donor and use an anode as an inexhaustible electron acceptor as part of a strategy so called *bioelectroventing*. This term alludes to the similarities with the traditional bioremediation technique *bioventing,* where oxygen is artificially applied as electron acceptor. Likewise, these bacteria can also use electrodes as electron source for reducing pollutants (Rosenbaum *et al*., 2011).

In the current work we designed and performed a *bioelectroventing* strategy using MERC for stimulating swine manure native bacteria in order to

enhance the complete biodegradation of ^{14}C-SMZ to CO_2. Our study was also focus on an overall evaluation of the fate of ^{14}C-SMZ in a bioelectrochemical-assisted treated manure and in the identification and quantification of its degradation products.

4.3 Experimental procedures

Chemicals

Uniformly ^{14}C ring-labelled Sulfamethazine (4-Amino-N-(4,6-dimethyl-2-pyrimidinyl) benzenesulfonamide) (^{14}C-SMZ) with a radiochemical purity of 98% according to the producer was purchased from Campro Scientific (AH Veenendaal, Netherlands) and used as a representative antibiotic for sulfonamides. The ^{14}C-SMZ standard had a final concentration of 0.74 mg mL^{-1} and a specific radioactivity of 2.41 kBq. Non-labelled SMZ and N_4-acetylsulfamethazine were purchased from Santa Cruz biotechnology (Heidelberg, Germany). Scintillation cocktails (Ultima Gold XR and Ultima Flo AF) were obtained from Packard (Dreieich, Germany). All other chemicals and solvents, of analytical grade, were purchased from Merck (Darmstadt, Germany).

Manure

Swine manure was obtained from a local farmer and showed no presence of SMZ. Table 4.1 presents the main characteristics of swine manure used during the experimental period (results by wet basis). This matrix was characterized by high organic matter content (3.47±0.01 g L^{-1}, 54% biodegradable), a C/N ratio of 12.85±0.11 and high conductivity (4.2±0.3 mS cm^{-1}). The conductivity was measured just before the set up of the experiment with the commercially available ECa sensors UMP-1 (Umwelt-Geräte-Technik, Freising/Weihenstephan, Germany). This tool can be used for *in situ* conductivity measurements with minimal disruption because the sole requirement consists in burying the probe needs in the manure. The measurements were conducted under the same conditions (water content and temperature) than for the biodegradation assay. The pH was determined

using a pH meter after mixing 2 g of milled (<2mm) dry manure with 20 mL of 0.01 M $CaCl_2$ (McLean, 1982). The carbon and nitrogen content was analysed using Elemental Analyser (Euro EA, Eurovector, Milano, Italy).

Table 4.1: Swine manure characteristics. The results are presented as the means ±standard deviation (n=5). Results by wet basis.

Parameters	Swine manure
pH	7.2±0.1
Conductivity ($mS.cm^{-1}$)	4.2±0.3
Water content (%)	90.3%
COD total ($g\ L^{-1}$)	3.47±0.01
COD soluble ($g\ L^{-1}$)	1.87 ±0.2
TKN ($g\ L^{-1}$)	0.27±0.02
NH_4^+ ($g\ L^{-1}$)	0.171 ±0.012
C / N ratio	12.85 ±0.11
Copper (Cu)	1.39 mg/kg
Zinc (Zn)	5.21 mg/kg

Laboratory system

To assess the mineralization of ^{14}C-SMZ to $^{14}CO_2$ a special set up was designed in order to adapt MERC to a closed laboratory system (Fig. 4.1). The manure samples were disposed at incubation flasks at 30ºC in the dark and at the sampling times they were aerated for 1h with humidified free air ($1\ L\ h^{-1}$) and the evolved $^{14}CO2$ from mineralization of ^{14}C-SMZ were absorbed in special traps filled with 10 mL of 1 N NaOH (Merck, Darmstadt, Germany). First sampling of the liquids in the traps was three days after application and then three times per week. From the collected NaOH solution an aliquot of 2 mL was taken, mixed with 3 mL Ultima FLO AF (PerkinElmer,

Rodgau, Germany) and the radioactivity was measured in a liquid scintillation counter (Tricarb 1900 TR, Packard, Dreieich, Germany).

MERC operation

MERC were designed and operated under 3 different configurations to degrade ^{14}C-SMZ with the concomitant production of bioelectricity: systems operated at a poised anode potential of + 400 mV versus Ag/AgCl reference electrode (RE-5B, BASi, United Kingdom) (+197 mV vs. SHE) by using a potentiostat (NEV2 nanoelectra), where energy is supplied to promote non-spontaneous reactions. They were designated as **pol-MERC**. Systems where the electrodes were connected by a copper wire using a 5.6 Ω external resistor (R) and the redox potential of the anode set spontaneously by the redox potential differences across manure vertical section. They were designated simply **MERC**. At last systems operated in open circuit with the anode and cathode disconnected (designated as **MERC$_{open}$**). The electrodes were allocated, under each of the configurations, at the bottom of the manure layer (anode) and above the liquid body (cathode). The electrode used in this experiment was carbon felt (Sofacel, Barcelona, Spain), as it showed no SMZ adsorption (Supporting Information) and very adequate mechanical properties to conform the system. The geometrical area of the electrodes was 39 cm^2 (surface area: 0.7 m^2 g^{-1}). Although a diversity of electrode materials can serve as an electron acceptor for microbial respiration, carbon felt can provide a low-cost, low-maintenance, continuous sink for electrons since it does not corrode or otherwise degrade during long-term deployments (Reimers *et al*., 2006; Tender *et al*., 2008). Additionally, it is easy to remove from the manure after the treatment, showing a low impact for the environment. **Electrode-free controls** were assembled in the incubation flask without the presence of the electrodes and under the same water content, temperature and ^{14}C-SMZ concentrations than the other treatments.

Mineralization assays

All systems were equilibrated for 2 weeks before the compound application and monitored by measuring the bioelectrocatalytic oxidation

current. ^{14}C-SMZ standard was applied to 60 mL manure after theses 2 equilibration weeks to give the desired concentration of 1 (± 0.1) μg mL^{-1} of manure and a specific radioactivity of 2.41 kBq μg^{-1}.

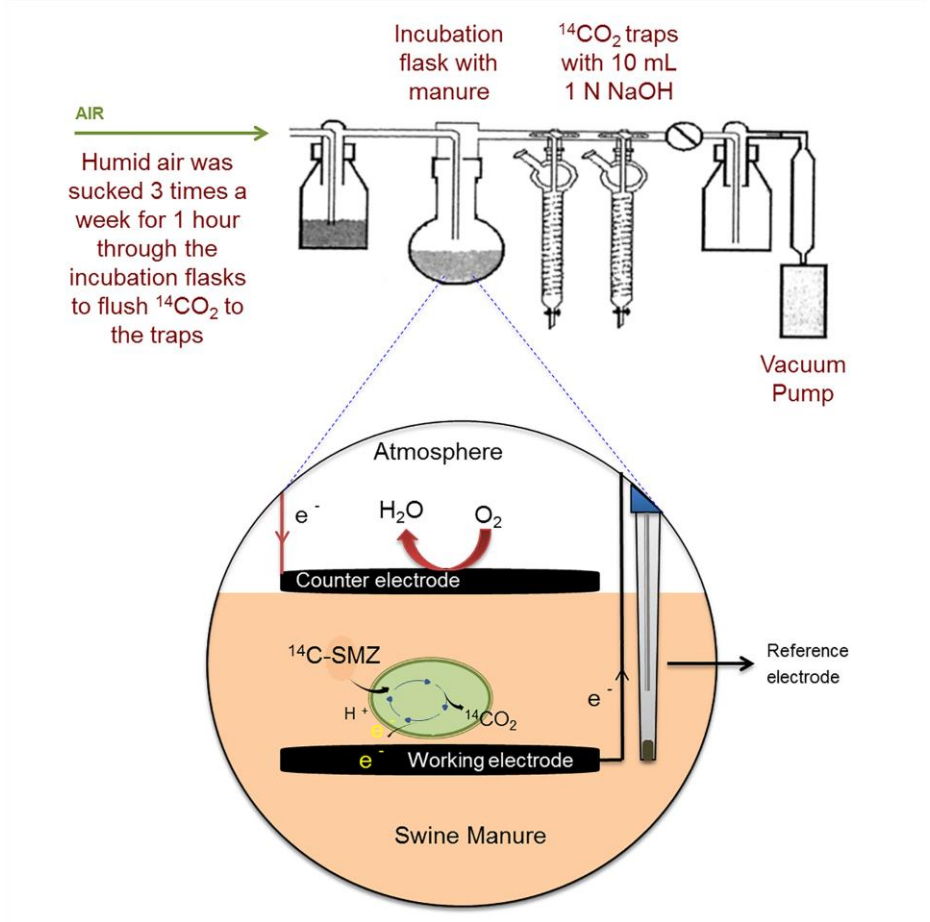


Figure 4.1: Laboratory system for monitoring the^{14}C-SMZ mineralization under different configurations. The zoom depicts a pol-MERC with a three-electrode system controlled for a potentiostat, polarizing the anode (working electrode) at +0.4 V vs Ag/AgCl reference electrode. 1N NaOH solution was harvested three times per week for measuring the $^{14}CO_2$ released from^{14}C-SMZ mineralization in manure. All systems were performed by triplicate.

This point of exposure is further referred to as initial time (t_0). The concentration was chosen to mimic concentration of SMZ found in swine manure (Aust *et al*., 2008). Water evaporation was compensated weekly by the addition of deionized water. The mineralization of ^{14}C-SMZ to $^{14}CO_2$ was studied in a closed aerated laboratory system, as described above. The $^{14}CO_2$ production rate and cumulative $^{14}CO_2$ were expressed as percentage of the applied ^{14}C-SMZ. After 32 days of incubation, pol-MERC treatment were shifted into MERC conditions where anode and cathode were connected by a copper wire using a 5,6 Ω external resistor (R), where the redox potential of the anode in MERC was set by the redox potential differences in the manure vertical section.^{14}C-SMZ mineralization was monitored until incubation day 62, in absence of artificial anode polarization.

^{14}C-SMZ-residue analysis

At the end of the incubation periods all samples were centrifuged and extracted. The aqueous supernatants from centrifugation were cleaned up and analysed by HPLC. The ^{14}C non-extractable residues were quantified by combustion.

All the samples were first centrifuged. The aqueous supernatant was retired and 100 µL of each one were mixed with 5 mL Ultima Gold XR and measured by liquid scintillation counting to measure the ^{14}C-SMZ residues fraction in water. After centrifugation solid manure and electrode material were extracted together as follow: They were vortexed for 1 min with 3 mL of citrate buffer (pH 5), subsequently vortexed for 2 min with 20 mL ethyl acetate and centrifuged for 8 min. 100 µL of each extract were mixed with 5 mL Ultima Gold XR and measured by liquid scintillation counting to measure the extractable ^{14}C-SMZ with organic solvents.

The aqueous supernatants (water residues) were adjusted to 250 mL with distilled water and subjected to solid phase extraction (SPE; Oasis HLB cartridge 6 mL, München, Germany) and equilibrated with 0.5 M citric acid buffer (pH 4.7). The cartridges were conditioned with 10 mL of HPLC grade water pH 2.5 (adjusted with acetic acid), followed by 10 mL methanol. The

loaded cartridge was washed with 10 mL distilled water. The retained sulfonamides were eluted with 10 mL acetonitrile in a second step. The elute was concentrated to a volume of one mL with a rotary evaporator and further concentrated under a gentle nitrogen-stream to volumes between 60 and 150 µL, depending on the total ^{14}C-radioactivity of the samples and subjected to HPLC analysis. Twenty microliter of each samples were injected to a HPLC system, consisting of a L-6200 Intelligent Pump (Merck-Hitachi, Darmstadt, Germany) a UV/VIS detector (240 nm, Merck, Darmstadt, Germany) and a radioactivity detector LB 506 C1 (Berthold, Wildbad, Germany). The column used was a Lichrospher 100 RP-18, 5 µm, 4 x 250 mm (Merck, Darmstadt, Germany). The mobile phase consisted of solvent A = 0.5% formic acid in water (Lichrosolv water for chromatography, Merck, Darmstadt, Germany) with 1mM ammonium acetate and solvent B = acetonitrile (HPLC grade) at a flow rate of 0.250 mL min^{-1}. The gradient program was as follows: T0min 90% A, T6min 85% A, T10min 85% A, T12min 74% A, T16min 35%, T30min 0%, T35min 0%, T40min 90%, T50min 90%. Parent compound and metabolites were identified by comparison of their retention times with reference substances.

After performing extraction, to achieve the quantification of ^{14}C-labelled non-extractable residues (NER), manure was homogenized intensively before combusting. Three aliquots (each approximately 100 mg) of each manure sample were filled into combustion cups and mixed with 7–8 drops of saturated aqueous sugar solution to guarantee a complete oxidation of the ^{14}C. Electrodes from each assay were cut in pieces and placed into combustion cups. Combustion was conducted with an automatic sample-oxidizer 306 (Packard, Dreieich, Germany). $^{14}CO_2$ was trapped in Carbo- Sorb E (Packard, Dreieich, Germany) and mixed with Permafluor E (Packard, Dreieich, Germany) prior to scintillation counting.

Electrochemical analysis

All electrochemical experiments were carried out under potentiostatic control, using a three-electrode arrangement consisting of the working electrode, an Ag/AgCl reference electrode (+197 mV versus normal hydrogen electrode (NHE)) (RE-5B, BASi, United Kingdom) and a counter electrode. The anode potential in pol-MERC was continuously poised at + 400 mV versus Ag/AgCl reference electrode using a potentiostat (NEV1-3 Nanoelectra, Madrid, Spain). Electrodes potential and current productions were continuously recorded with a multimeter (7700, Keithley instruments). Cyclic voltammetry (CV) was recorded before and after ^{14}C-SMZ pulses and after shifting pol-MERC into MERC to qualitatively characterize the electrochemical activity of the manure by imposing a scan rate of 1 mV/s from the open circuit voltage (OCV) potential by a potentiostat (NEV3 Nanoelectra, Madrid, Spain).

4.4 Results and discussion

Enhanced SMZ mineralization by using MERC

The experiment started by investigating the influence of a positive potential electrode on the ^{14}C-SMZ mineralization in manure. After 32 days the cumulative mineralization under the influence of polarized felt electrodes (pol-MERC) reached the value of 1.1%, whereas ^{14}C-SMZ mineralization in electrode-free control was over 17% (Fig. 4.2). Regarding the low proportion of radiochemical impurities of the ^{14}C-SMZ applied in this series (98% purity), most of the $^{14}CO_2$ derives from the radiochemical pollutant. Interestingly, the cumulative mineralization under open circuit conditions ($MERC_{open}$) was over 31% in the same period of time. So, the enhancing effect of the sole presence of the conductive carbon felt influence the microbial metabolic processes raising the mineralization in comparison with the electrode-free control, which nevertheless already shows a microbial native community capable to degrade SMZ.

The assays were running for another 4 weeks until day 62, where the maximum cumulative mineralization still took place under $MERC_{open}$ (37%), followed by electrode-free control (over 27 %). Displaying high anode potential increases the energy available to electroactive bacteria, giving them the possibility of outcompeting methanogens by means of their faster metabolism (Sleutels *et al*., 2012; Aelterman *et al*., 2008). Nevertheless, electrode may remodel the redox scenario and the influence of the electric field may change the permeability of cell membrane, leading to the excessive absorbance of extracellular substances and further change the microbial metabolism (Rittmann and McCarty, 2001). This factor might be key in the low mineralization capacity of the microbial community when the compound is an antimicrobial as SMZ. On the other hand, SMZ is a diprotic acid with pKa values of 2.6 and 7.4, respectively. Thus, depending on the pH, SMZ can exist in cationic, neutral and anionic forms. The percentages of anionic SMZ calculated by Lertpaitoonpan *et al*. (2009) at pH values of 6.0 and 8.0 were 3.8 and 79.9% respectively. Thus, at pH values above 7.4 (the manure pH is 7.2) the sulfonamide molecule exists primarily in anionic form. So negative charged SMZ might be intensively adsorbed on the surface of positive polarized electrodes conditioning their sorption behaviour and by hence their bioavailability. In order to evaluate whether the positive electrode potential might have adverse effects over the microbial degradation capacity, either by reducing the SMZ bioavailability, or damaging the microbial community, we followed the next strategy: 32 days old pol-MERC setups were converted into MERC by substituting the potentiostat by a low external resistor. The new configuration led to a drop off in the redox anode potential from +400 mV (versus Ag/AgCl) to a negative anode potential (-400 mV (versus Ag/AgCl)) for the rest of the assay. Despite a slope increased in the ^{14}C-SMZ mineralization rate, the mineralization just reached the 9,7% after 62 days, far from the mineralization figures under $MERC_{open}$ (Fig. 4.2). Additionally, we set up MERC assays and after 32 incubation days the cumulative mineralization reached the value of 43.5 % (average value of four experimental replicates), exceeding in more than 26% to the free-electrode control and more than 12 % to the $MERC_{open}$ after the same experimental time period. This result enforces

the hypothesis of the positive electrode potential as a damaging factor for the microbial community.

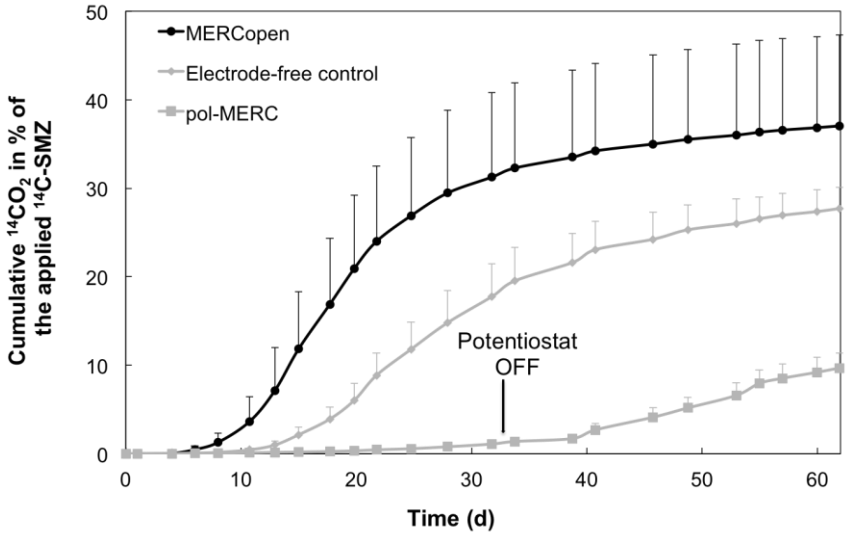


Figure 4.2: Cumulative mineralization of ^{14}C-SMZ at different configurations for 62 days. A pol-MERC configuration was converted to MERC (potentiostat off) after 32 days of incubation. During the first phase, the electrode acted as electron acceptor at +0.4 V versus Ag/AgCl [+197 mV versus normal hydrogen electrode (NHE)]. During the second phase, the anode potential was achieved by connecting the electrodes through a 5.6ohm resistor and monitored until day 62. MERCopen and electrode-free control were under the same operation conditions during the 62 days incubation time. Average value presented resulted from three experimental replicates. The error bars represent standard deviation.

The High cumulative and mineralization rates (Fig. 4.3) showed under MERC conditions are common and desired features when the pollutant acts as a source of energy for the microbial degrading community (Grundmann *et al.*, 2011). The analysis of $MERC_{open}$ assays revealed how ^{14}C-SMZ degradation was also enhanced by the sole presence of a conductive material, with the anode and cathode not connected and without electron flow through the system. Biodegradation of SMZ requires a set of redox reactions that can be played by different strains and our results suggest that conductive materials would help these redox reactions to occur. Interestingly, a

phenomenon called Direct Interspecies Electron Transfer (DIET) term was reported for first time by Lovley (2011b) to describe a mechanism based on an electrical connection between microbial species in order to develop syntrophic metabolisms through direct contact and in absence of electron shuttles or redox mediators (Summers *et al*., 2010).

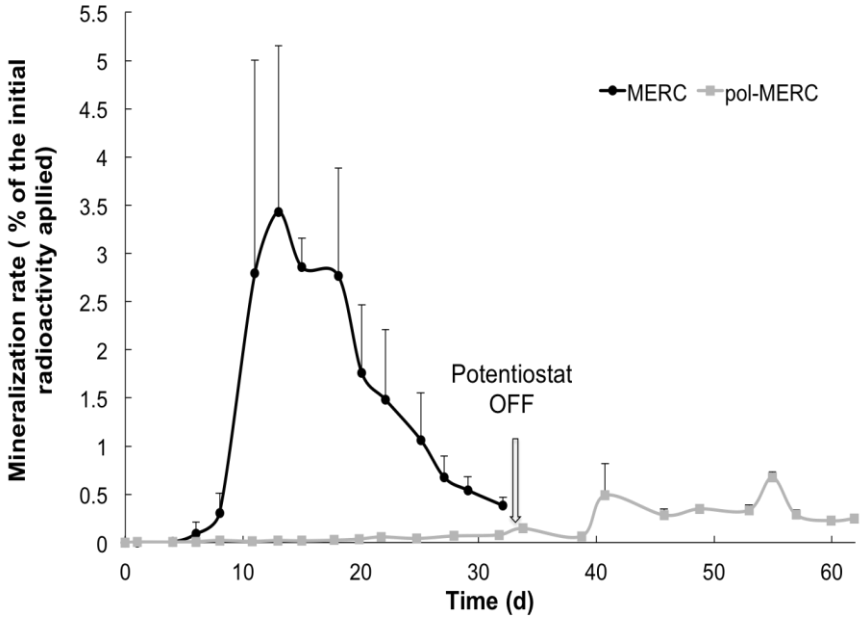


Figure 4.3: Mineralization rate of $^{14}CO_2$ production from ^{14}C-SMZ. In pol-MERC the working electrode was polarized at +0.4 V (versus Ag/AgCl) during 32 days, when was converted into MERC by substituting the potentiostat by a low external resistor (indicated by grey arrow) and monitored until day 62. Systems operated as MERC from the initial time showed higher mineralization rates and were operated for 32 days.

According to Liu *et al*. (2012) this metabolism may be accelerated in presence of a electrically conductor material, demonstrating how the anaerobic conversion of organic matter in a digester could be enhanced by promoting DIET between bacteria and methanogens, in presence of a conductive activated carbon, which permits better electrical connections

between microorganisms. A more natural conductive material as magnetite has been also reported to promote DIET among *Geobacter sulfurreducens* and *Thiobacillus denitrificans* (Kato *et al*., 2012) so acetate oxidation can be coupled to nitrate respiration, both reactions that cannot be performed by the single bacterial strains. In addition to this biodegradation enhancement, SMZ mineralization was, as explained above, stimulated by an extra 14% when the electrodes were connected as part of a MERC setup. Similar trends were shown performing bioremediation of total n-alkanes (TNAs) (Wang *et al*., 2012b) and polycyclic aromatic hydrocarbons (Rodrigo *et al*., 2014).

To the best of our knowledge, it is the first time that a significantly ^{14}C-SMZ mineralization has been reported in manure. Most of the research regarding biodegradation of SMZ has been almost fully devoted to the aerobic metabolism in water and soil and just a few reports are available for degradation of this substance under strong reductive conditions, where significant mineralization of SMZ has not being reported elsewhere (Mohring *et al*. 2009; Mitchell *et al*. 2013; Spielmeyer *et al*., 2015). Although addition of limiting nutrients (N and P) (Yu *et al*., 2013) and soluble electron acceptor (Kabelitz *et al*., 2009; García *et al*., 2010) have been classically reported for enhancing bioremediation, our results demonstrate the existence of novel tools for biostimulating based on insoluble amendments with low impact for the environment. In consequence, our electrode-assisted treatment performs *in situ* bioremediation by avoiding chemicals consumption or manure manipulation with negative environmental consequences providing an endless, low priced and sustainable terminal electron sink showing minimal environmental disturbance (Logan *et al*., 2006).

Microbial electrochemical activity

Electron transfer between manure microorganisms and electrodes were analysed by cyclic voltammetry (CV) in order to give insight to the relationship between cumulative mineralization and the bioelectrochemical activity (Fig. 4.4). A different bioelectrochemical profile was already observed during the equilibration period, providing inflexion peaks centred at different redox potential under the different treatments. These differences were

maintained constant along the incubation time. pol-MERCs showed a peak at -0.1 and +0.2 V (vs Ag/AgCl), before and after ^{14}C-SMZ applications but with considerable higher intensity under the presence of the antibiotic. In contrast, $MERC_{open}$ showed only a peak at -0.1 (vs Ag/AgCl) with a very low intensity, before and after the antibiotic pulse.

The current production was as well continuously registered in both pol-MERC and MERC (Fig. 4.5). Manure treated through pol-MERCs showed shorter lag phase in current production regarding with manure treated through MERCs. On top of that pol-MERCs showed a maximum current density of 15 $mA.m^{-2}$ while MERC hardly reached the 3.5 $mA.m^{-2}$, so positive potential accelerated the electroactive microbial activity. Under both treatments the current production increased after ^{14}C-SMZ pulses. This increase might be attributed to the fact that the electroactive community had not reached steady state within the equilibration time and/or ^{14}C-SMZ utilization as a carbon source for electroactive bacteria. Indeed it has been already reported increases in the electroactive microbial activity simultaneous to antibiotics removal in Microbial Electrochemical Systems (Harnisch *et al*., 2013; Guo *et al*., 2016). Further studies are needed to completely elucidate whether direct impacts (e.g., the graphite electrode served as a direct electron acceptor for SMZ-oxidation), indirect impacts (the electrode somehow stimulated the activity of oxidizing bacteria involved in biodegradation of SMZ for instance enforcing metabolic cooperation strategies) or both take place under the presence of electrodes.

After shifting from positive (+400mV versus a Ag/AgCl) to negative anode potential (-400mV versus a Ag/AgCl), acting the system as a MERC, the CVs inflexion peaks, mainly the one at +0.2 V showed considerable lower intensity (Fig. 4.4C) and the current production intensively decreased and become unstable. This instability might be related with the oxygen concentration dependency of the system at the cathode chamber when the system is not supplied with an external current (Fig. 4.5). Furthermore, when the system is operated as a MERC from the initial incubation moment, the only oxidation peak is centred at -0.1V (vs Ag/AgCl) but with an increased

signal regarded to the same peak under pol-MERC. Beside that fact, as specify above, the current production is much lower but the cumulative mineralization increased 40 fold regarding to the polarized system.

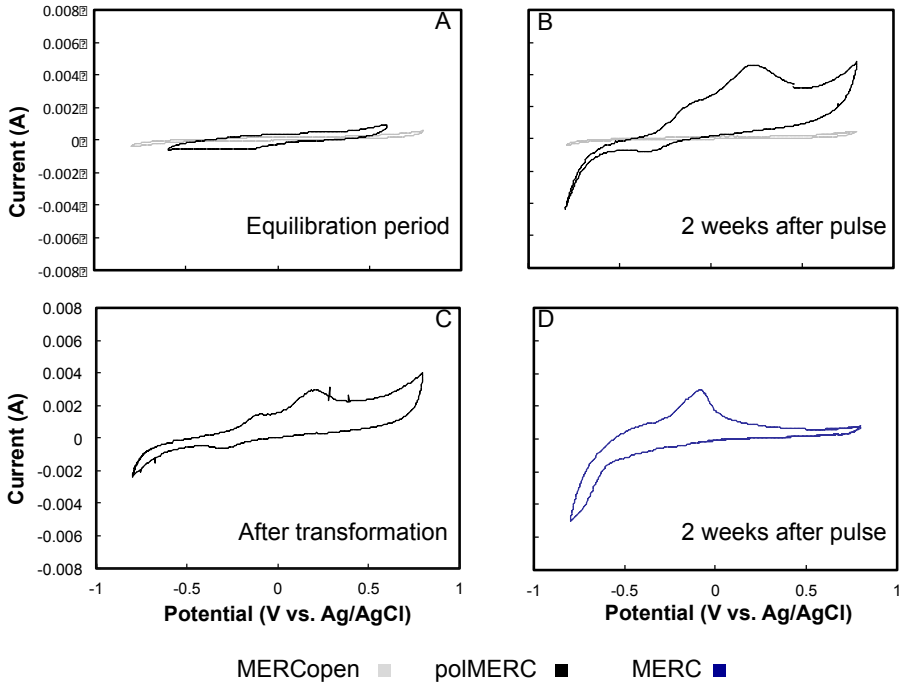


Figure 4.4: Cyclic voltammetry analysis at different incubation periods for different configurations (scan rate: 1 mV s^{-1}). **A.** CV carried out at equilibration period, 2 weeks after system set-up. **B.** CV carried out 2 weeks after ^{14}C-SMZ pulses. **C.** CV carried out after pol-MERC conversion into MERC by substituting the potentiostat by a low external resistor. **D.** CV carried out in systems operated as MERC from the initial time.

So, the data provided evidence of a higher electrochemical response of the manure microbial community assisted by polarized electrodes. This is an indication of an anode enrichment of electroactive microbial communities, due to an increase in the cell density on the electrode surface or to an increase in the microbial metabolism that accelerate the electron transfer. Nonetheless, the SMZ mineralization capability was not directly related with the electroactive bacteria performance, being under the influence of the

negative electrode potential where the antibiotic degradation reached higher results but lower current production. This fact leads to conclude that under MERC configurations, the electrodes stimulate more adequate biodegradation pathways to fully degrade SMZ.

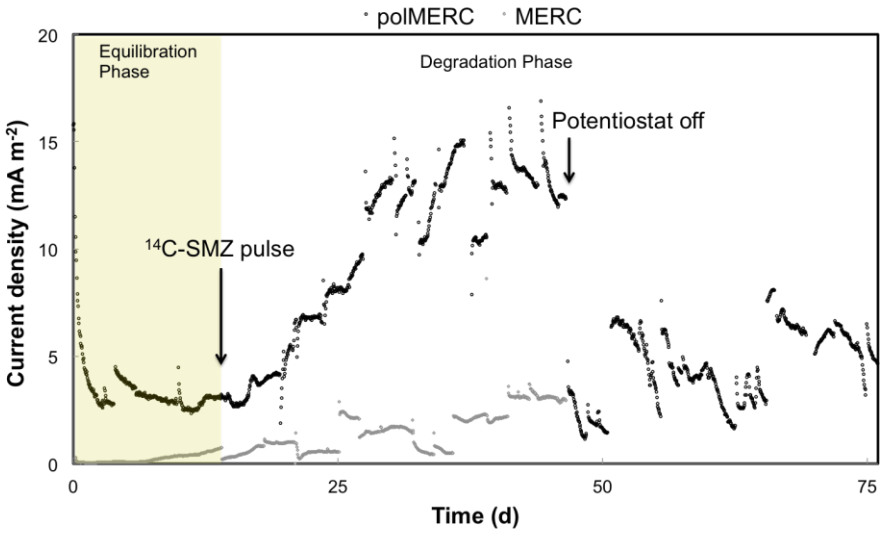


Figure 4.5: Chronoamperometry. Current production was continuously registered before and after ^{14}C-SMZ pulses. In pol-MERC (black color) the working electrode was polarized at +0.4 V (versus Ag/AgCl), and subsequently was converted into MERC by substituting the potentiostat by a low external resistor, which led to a drop in current production. Systems operated as MERC from the initial time (grey color) showed a lower current production than systems with polarized electrodes. Current data in this article are reported as current density (mA.m^{-2}).

Characterization of the ^{14}C-SMZ mass balances

A complete understanding of the bioremediation task impact requires a proper analysis about the pollutant destiny. Thus, in addition to the cumulative $^{14}CO_2$ mineralization, also the manure- and electrode-associated radioactivity should be measured in order to establish an accurate ^{14}C mass balance in our experimental system. Extractable residues (ER) and non-extractable residues (NER) of the ^{14}C-SMZ associated to manure and electrode fractions are shown in figure 4.6. Interestingly, these mass balances

ranged between 96% and 102% of the initially supplied ^{14}C-SMZ under MERC and pol-MERC respectively. This parameter was therefore an indicator of the effectiveness of our experimental design and underlines the low potential losses of $^{14}CO_2$.

The extractable ^{14}C-residues in the manure samples varied considerably between the different experimental conditions after 62 days of incubation. The ER, accounting the ^{14}C-residues available in the water fraction after centrifuging and the ^{14}C-residues after organic extract, did reach 19.2% of the applied radioactivity for the electrode-free control and 13% under $MERC_{open}$ treatment. Unexpectedly, the low mineralizing pol-MERC assay also showed low ER of ca. 15%, what could be a consequence of the higher residues bounded to the felt electrode (18% of the applied radioactivity). On the contrary, higher NER was observed under the influence of the polarized electrode (58.5%) followed by the electrode-free control (50.8%) and $MERC_{open}$ (45.2%).

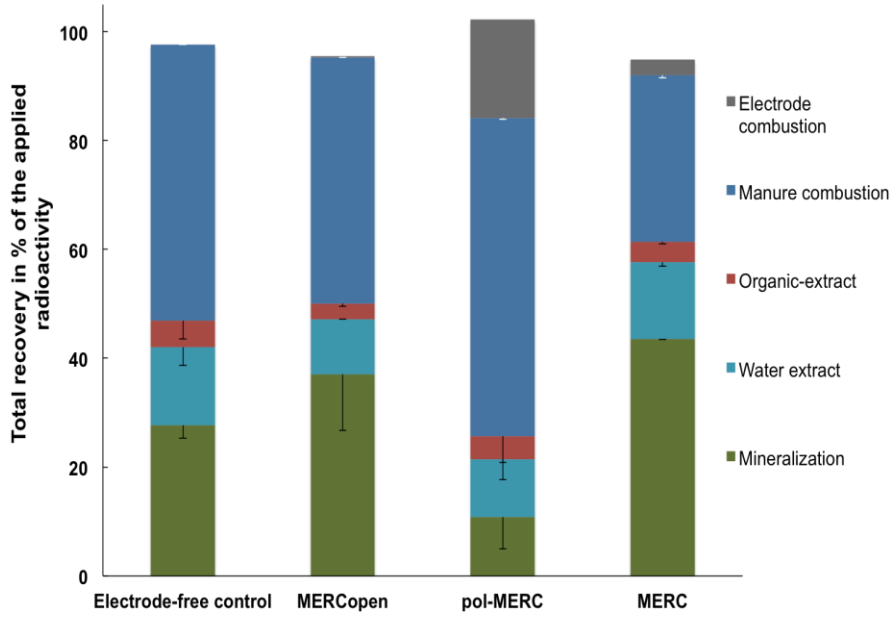


Figure 4.6: Mass balance of ^{14}C-SMZ under the different treatments. The error bars represent the standard deviation for triplicate assays.

Apparently, the matrix itself has an influence on the SMZ removal. Such an observable dissipation of the parent compound is often called "degradation" in studies reporting the fate of environmental pollutants. Nevertheless, the disappearance of the parent substance and its metabolites in soil does not necessarily correspond to formal degradation leading to mineralization; actually mineralization to carbon dioxide seems to play only a minor role in some of these studies (García-Galán *et al*., 2011). Examining the ^{14}C mass balance of the MERC assay, after only 32 days, we observed a decrease in the NER, only reaching 30%, while the ER reached over 17%. The radioactivity accumulated in the MERC electrodes did not get 3 %, in contrast with the higher value observed in pol-MERCs (18%). The last value contrasts with the previous adsorption-desorption assays conducted to confirm the lack of SMZ adsorption on carbon felt (Supporting Information). In the MERC assay, as has been specified, the percentage of total ^{14}C-residues attached the electrodes was much lower than in pol-MERCs, which suggests the electric field generated in polarized electrodes as reason for the high SMZ electro-adsorption.

^{14}C-SMZ biodegradation pathway

The removal of SMZ from the environment has received considerable attention, mainly under aerobic conditions and biodegradation of SMZ under anaerobic and strong reductive conditions is largely unknown. Currently, information on the metabolites or metabolism mechanisms of SMZ is still limited. N_4-acetyl-SMZ and desamino SMZ were found in anaerobic soil amended with swine manure (Lertpaitoonpan *et al*., 2015). The metabolite, N_4-acetyl SMZ has been reported to be a major metabolite of SMZ, frequently detected in water, soil, and manure (Grant *et al*. 2003). Beside N_4-acetyl-SMZ and des-amino-SMZ, the metabolite N_1-methyl-SMZ was the form of SMZ present in swine manure (Paulson *et al*. 1981) and other animal excreta (García-Galán *et al*. 2008). It is not clear whether these metabolites were formed solely by chemical reactions or by microbial degradation in the animal digestive systems. Another 2 different SMZ degradation intermediates can be produced by fungal cultures (white-rot fungus Trametes versicolor) and

purified laccase (desulfo-SMZ, N_4-formyl-SMZ). Nonetheless SMZ mineralization was not demonstrated under those fungal assays by using the isotopically labelled sulfamethazine-phenyl-$^{13}C_6$ (García-Galán *et al*., 2011).

Exhaustive analysis of aqueous supernatants (water residues) revealed seven radioactive SMZ metabolites. Analyses of the organic extracts were not performed given its low radioactivity content. SMZ and N_4-acetyl SMZ identification were made by comparing retention times to standards, prepared using analytical standards. The parent compound was abundant under each treatment (Table 4.2), even under MERC (22%), where the cumulative mineralization was over 43% of the total applied ^{14}C. This fact discards the lack of SMZ availability as constrain for further mineralization. It is under MERC configuration, where we identify N_4-acetyl SMZ (6%). None of the other treatments showed N_4-acetyl SMZ presence. The acetyl group of this metabolite is readily hydrolysed, being the ionized N_4-acetyl SMZ more polar than the parent SMZ (Grant *et al*. 2003). An unidentified metabolite (retention time 41.5 min) was as well exclusively detected under MERC treatments, with 0.179 μg mL^{-1} manure, which revealed the strong effect of the electrode potential on the metabolic activity of microorganisms, leading the degradation pathways to different metabolite patterns.

Table 4.2: Profile composition of the water residues, sulfamethazine and its degradation products, obtained from the different treatments (peaks areas appear as % of ^{14}C-water residues and metabolite concentrations appear as μg mL^{-1} wet matrix).

SMZ and metabolites	RT (min)	Electrode-free control		MERCopen		pol-MERC		MERC	
		Area %	mg mL^{-1}	Area %	mg mL^{-1}	Area %	mg mL^{-1}	Area %	mg mL^{-1}
Unidentified	9 ± 0.6	25.0	0.038	24.4	0.042	27.1	0.047	16.7	0.024
Unidentified	13.9 ± 0.8	17.7	0.027	40.4	0.070	23.1	0.040	30.5	0.044
Unidentified	20.5 ±2.2	12.9	0.019	13.7	0.024	12.7	0.022	7.3	0.011
N_4- acetylSMZ	29.7	n.d	n.d	n.d	n.d	n.d	n.d	6.2	0.009
SMZ	33.3 ±2.3	31.4	0.047	15.6	0.027	24.3	0.042	22.3	0.032
Unidentified	39.4 ± 0.4	13.03	0.020	5.9	0.010	12.8	0.022	10.3	0.015
Unidentified	41.5	n.d	n.d	n.d	n.d	n.d	n.d	6.65	0.010

n.d: non detectable.

Further research is currently being performed to identify the unknown metabolites of SMZ and the corresponding eco-toxicity of its metabolites become urgent due to their higher mobility when they are dispose in soils. Knowledge of degradation products is important to identify persistent transformation products, which may increase human or ecological risk when AD effluents are applied to the environment.

4.5 Conclusions

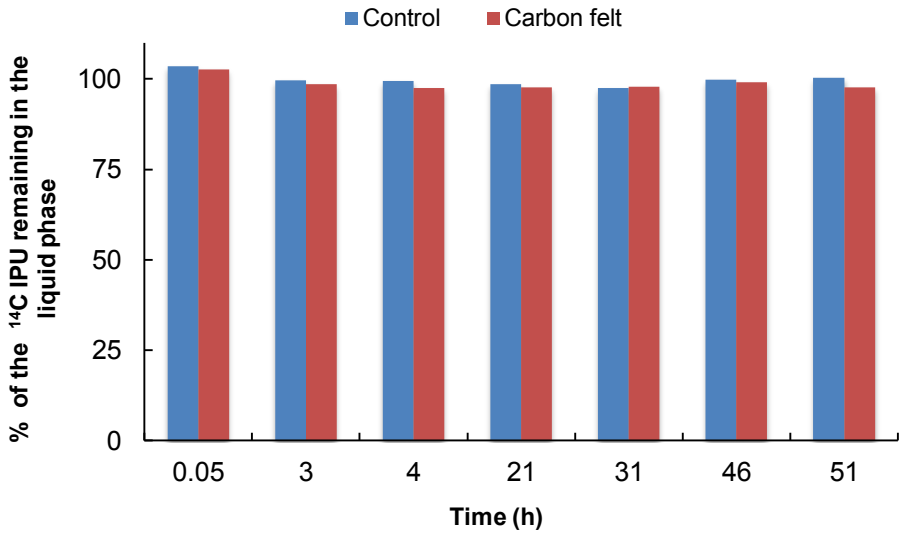
In the present study, we demonstrate how the use of electrodes at negative anodic potential (-400 mV versus Ag/AgCl) in SMZ-contaminated manure increases not only the SMZ-removal but also influence the ^{14}C mass balance, the ^{14}C-SMZ metabolite pattern and the pollutant fate. This technology may be used for *in situ* decontamination of manure due that presents all the advantages of the others *in situ* bioremediation approaches, while avoided bioaugmentation or artificial addition of nutrients with impact in the environment. Thus, the results presented here enforce the establishment of electrodes as a conceivable cost-effective and environmentally friendly strategy for enhancing pollutants anaerobic degradation.

4.6 Supporting Information

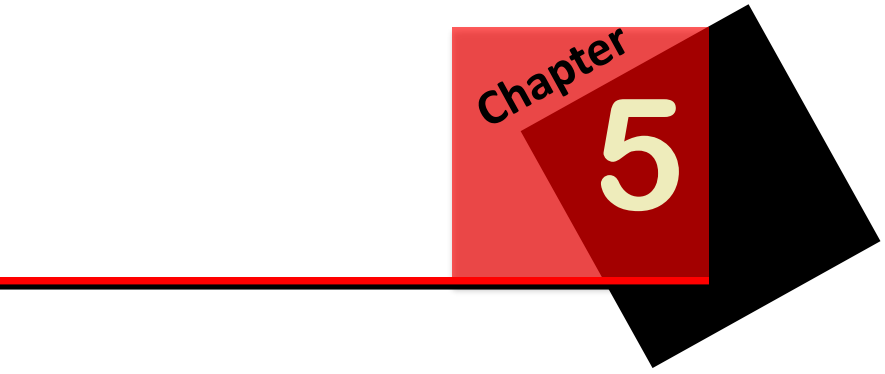
^{14}C-SMZ adsorption assays in carbon felt

In this study, batch experiments were conducted in triplicates to investigate the adsorption-desorption kinetics of SMZ in carbon felt, an electro-conductive material. 50 mL eppendorf tubes were filled with 40 mL deionized water and 40 μl ^{14}C-SMZ standard was added to give a final concentration of 1 (± 0.1) μg mL-1. Samples of a total geometrical area of 39 cm^2 of each material were placed into the eppendorf tubes, which were shaken overhead continuously. The control was performed under the same conditions but without the presence of any material. At different time intervals aliquots of 0.1 mL were sampled to measure the radioactivity until the equilibrium for adsorption of ^{14}C-SMZ was reached. For the determination of radioactivity, the 0.1 mL samples were mixed with 5 mL Ultima Gold XR and

measured in a liquid scintillation counter (Tricarb 1900 TR, Packard, Dreieich, Germany). There was not adsorption processes, remaining practically the total amount of initial ^{14}C-SMZ in the liquid phase (supporting figure 4.1). So thus desorption assay were not performed. Regarding with these results and considering the convenience physic-mechanical properties, the high surface area, and the inexpensive cost, the material used to conform the electrodes of the bioelectrochemical systems was carbon felt.



Supporting figure 4.1: ^{14}C-SMZ adsorption in carbon felt.


Chapter
5

General discussion, conclusions and future work

General Discussion, Conclusions And Future Work

The main objective of this thesis was to evaluate the performance and versatility of Microbial Electroremediating Cells at lab scale as bioremediation tools with polluted soil and manure. This work presents a novel alternative to conventional bioremediating techniques, focussing not only in pollutant removal but also in fully biodegradation (mineralization) and effective clean up demonstrated by ecotoxicological analysis. We expose the final considerations of this thesis as a question-answer mode.

5.1. Discussion and conclusions

- **What are the advantages of using MERCs over conventional bioremediation techniques?**

Conventional bioremediation techniques have focussed on speeding up natural bioattenuation processes, optimising metabolic activities of microorganisms for removing or immobilizing environmental contaminants. To achieve this goal different actions have been taken in the last decades. For instance, biostimulation techniques have worked to overcome the deficiency of suitable electron donors or acceptors in highly reductive ambient as subsurface soils, flooded sediments and manures. This lack of suitable electron donors or acceptors limits the microbial activity and by hence the bioremediation processes. Thus, amendments of nutrients (Lovley, 2000), co-metabolites (Tyagi *et al*., 2010), nitrates (Yu *et al.,* 2013), oxygen (García *et al*., 2010; Kabelitz *et al*., 2009) or alternative electron donors or acceptors have been applied to the environment as a bioremediation strategy. Regardless to the success of the bioremediation task, these interventions incurs extra costs, causes secondary pollution concerns, matrix perturbation and accelerate contaminants release to overlying water (Pandey and Fulekar, 2012; Thrash and Coates, 2008). Furthermore, they stimulate unwanted metabolic reactions to occur and the accumulation of fermentation products

as well as biomass. Bioelectrochemically-assisted remediation overcomes this limitation offering the electrodes as a limitless and easily deployable and removable terminal electron donor or acceptor. The electrodes are supplied exclusively once during the bioremediation process avoiding continuous amendments and excluding unwanted diffusion processes of added compounds. Moreover allows a significant reduction in methane gas emission from aquatic environments (Ueno and Kitajima, 2012; Ueno and Kitajima, 2014). We have explored carbon and graphite electrodes acting as electron acceptors in soil (chapter 2 and 3) and manure (chapter 4). They bring the advantage of co-localizing the contaminants and microorganisms on the same surface to perform oxidative metabolism beyond the natural conditions.

Other bioremediation techniques have influenced on the pollutant degradation by employing specific microorganisms to degrade a concrete compound. Regardless to the high cost and legal restrictions for the *in-situ* application of non-native microorganisms, these strategies could present adaptation problems in the inoculated microorganisms given to the competition between introduced and indigenous populations *(*Tyagi *et al.,* 2010). On the contrary, the experiments presented in this thesis used the metabolic capabilities of native microbial species in paddy soil (chapter 2), agricultural soil (chapter 3) and swine manure (chapter 4) to degrade contaminants of different chemical nature. None of the matrices were previously exposed to these compounds revealing a high versatility of bacteria for adapting to these bioelectrochemical conditions and creating an astonishing expectation of control over the microbial catabolic processes.

Furthermore, Microbial Electrochemical Systems endow a high controlability over the bioremediation process by tuning electrochemical parameters as electrode potential. So electrodes not only overcome the TEA limitation but also allow the manipulation of biodegradation reactions, controlling the environmental redox conditions in pro to select microbial activities that remediate contaminants of concern. Additionally electrodes can serve as real time non-invasive bioremediation explorers by using parameters as current production as a proof of the microbial activity (Wardman, 2014).

- **How natural matrices affect MERCs application and performance?**

All bioremediation processes are influenced by the physical and chemical conditions of the contaminated matrix. Microbial Electroremediating Cells have revealed as a high versatility bioremediation techniques in wastewater (Tejedor-Sanz *et al*., 2016), sediments (Yu *et al*., 2016), soil (Domínguez-Garay *et al*., 2016) and manure (Sotres *et al*., 2015). High electrode overpotential due to the limitation in the electrons and protons transport takes place in MERCs operated in soils or sediments where solution conductivity is low (Hong *et al*., 2009). This leads to lower redox potential of the anode and hence decreased anodic decontamination efficiency (Dominguez-Garay *et al*., 2013).

Microbial Electrochemical System design (Huang *et al*., 2011; Lu *et al*., 2014a; Yuan *et al*., 2010), oxygen concentration at the cathode chamber (Cai *et al*., 2013; Xiao *et al*., 2012), mass transfer losses in electrode-bacteria interactions (Li *et al*., 2014) and the electrode resistance (Oh and Logan, 2006) has been studied with the goal to minimize the system internal resistance and to optimize the bioelectrochemical performance. The reduction of matrix resistivity is as well key to optimize the electrochemical performance in electrogenic microcosms (Wang *et al*., 2012b; Li *et al*., 2015; Li *et al*., 2016c). Marine sediments and manure show the advantage of a low ohmic internal resistance due to the high concentration of salts. The lower salinity and higher internal resistance in soils and fresh water sediments, that restrains the transport of ions between anode and cathode, make them less favourable scenarios for MERC application. Despite the bioelectrochemical limited performance in MERCs given these factors, this thesis and the state of the art reviewed within, shows electrode influences in the bioremediation task even in non-favourable matrices as soil and fresh water sediments (Fig. 5.1). Chapter 2 study a bioelectrochemically-assisted remediation performance in a paddy soil with unfavourable conditions (very low organic matter content (0.18%) and electrical conductivity (0.24 dSm^{-1}). Nevertheless, given that this soil has been used for decades under flooded conditions for growing

semiaquatic rice, presumably is a favourable matrix for microorganism well adapted to flooded and anaerobic environments, as the one where the anode is placed. Chapter 3 reports MERC application in agricultural soils with a higher organic matter content (0.99 %) but a similar electrical conductivity as the paddy soil (0.247 mS/cm). At last chapter 4 treats bioremediation processes by means of MERCs in manure, which regarding to MERCs overpotentials is a more favourable scenario given its high organic matter content (3.47±0.01) and a conductivity of 4.2±0.3. Nevertheless MERC electrochemical performance in both soils is higher, reaching current densities of 20 $mA.m^{-2}$ while MERC applied in manure hardly reached 3.5 $mA.m^{-2}$ when the electrodes are not polarized. The current density increases until 35 $mA.m^{-2}$ in agricultural soil and 15 $mA.m^{-2}$ in manure when electrodes were polarised at high positive potentials. This performance difference must be due to the different MERC design and the different matrices, which have different electroactive microbial populations.

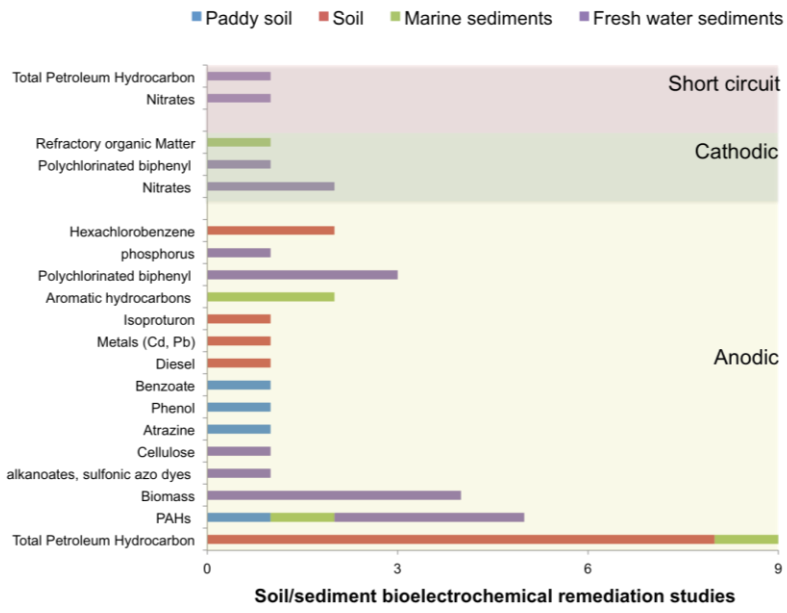


Figure 5.1: Soil & sediment bioremediation studies assisted by electrodes

- **Does *bioelectroventing* represent a suitable and versatile technology for remediating pollutants of different chemical nature?**

Bioelectrochemically-assisted remediation studies presented in this thesis show MERCs as a highly versatile bioremediation technique capable to enhance the degradation of three different pollutants (DBT, IPU and SMZ) in three different matrices (paddy soil, agricultural soil and swine manure), which represent different environmental pollution sources. This process to supply electrodes to stimulate the oxidative metabolism of environmental microbial populations is refereed as *bioelectroventing.* One of the key criteria to select these compounds has been the insufficient information about their degradation under reductive conditions despite the many anoxic environments like groundwater aquifers, riparian zones, subsurface soil and seasonally flooded agricultural soils where they can be found:

1. The removal of dibenzothiophene (DBT), an organosulfur compound, was increased 3-fold by MERCs after 25 days regarding the one obtained under intrinsic attenuation conditions despite the recalcitrant nature of DBT, and the unfavourable low-conductivity value of our soil (Chapter 2).

2. The highest removal of **isoproturon (IPU),** a phenylurea herbicide, was reached by means of MERCs, where over 97% of the initial applied IPU was not found in the final agricultural soil extracts when the electrode was polarised at high potentials (+600 mV versus Ag/AgCl). This figure increased in 38% the removal intrinsic capability of the soil under flooded conditions (electrode-free control) (Chapter 3 part II). Meanwhile, the total biodegradation of isoproturon (^{14}C-IPU mineralization) measured as the total accumulated $^{14}CO_2$ proceeding from the radiolabelled herbicide is still more conclusive (chapter 3 part I). The electrodes at a positive potential (+600mV (vs. Ag/AgCl)) enhanced the mineralization by 20fold respect the electrode-free control, enforcing the roll of the electrode in the total biodegradation

of IPU. In this case the ^{14}C techniques provide a precise measurement of the stimulation capacity of electrodes degrading IPU.

3. The mineralization of **sulfamethazine (SMZ)**, a veterinarian antibiotic, just confirmed the remarkable impact of electrodes on the microbial activity of natural communities, in this occasion in swine manure. MERCs with electrodes at negative potentials after 32 days increased more than 2-fold the mineralization obtained under intrinsic attenuation conditions.

Further studies are needed to completely elucidate whether direct impacts (e.g., the graphite electrode served as a direct electron acceptor for pollutant-oxidation), indirect impacts (the electrode somehow may stimulate the activity of oxidizing bacteria involved in biodegradation of pollutants for instance enforcing metabolic cooperation strategies) or both take place under the presence of electrodes. However, these results lead us to conclude that MERCs are able to act on pollution problems of different kind, enhancing the removal (DBT and IPU) and total degradation (IPU and SMZ) of pollutants of different chemical nature and reaching higher efficiency in a shorter incubation time. This time factor is decisive to minimise environmental pollution problems, for instance after a petroleum hydrocarbon spill or after the accumulation of stagnant polluted water mass. The remarkable impact of electrodes on the microbial activity of natural communities suggests a promising future for MERCs as an *in situ* environmental technology.

- **What are the implications of electrode potential in bioremediation processes?**

Electrodes acting as electron acceptors or donors remodel the redox scenario and by hence influence the dominant metabolic pathways of microorganisms. To date, most studies on electrode-based biostimulation used a buried electrode with a typically negative potential, established spontaneously across the matrix-water interphase as a result of spatially segmented reduction-oxidation reactions (Li and Yu, 2015). Electrodes

connection by a very low resistor lift up the anode potential that becomes more positive (or less negative), which mean a more favourable electron acceptors for soil microorganisms. So the redox scenario provided for such an electrode stimulated the DBT removal regarding to intrinsic attenuation conditions showing higher DBT-removal rate from the initial incubation time. The removal rate was 30-fold higher just after 9 days but diminished in the last phase of analysis. We hypothesise that this decreased removal rate may be due to the fact that DBT and nutrient depletion were most rapid under the influence of electrodes than in electrode-free control. Similarly, under this same configuration, electrodes stimulated ^{14}C-SMZ mineralization, this time multiplying per 2 the mineralization capabilities of the manure microbial community without electrodes.

Nevertheless, the negative potential provides by electrodes connected by resistors can be insufficient or inappropriate to drive the transformation of other many recalcitrant organics (Zhao *et al*., 2006). The implications of the electrode potential resides in its influence on microbial electron releasing capabilities, determining from a thermodynamic point of view the metabolic pathway used and the theoretical energy gain for the biocatalyst (Schröder, 2007). A higher anode potential may increase the energy per electron transferred available for growth and cell maintenance, increasing the yield energy and resulting in a higher microbial density and current generation (Aelterman *et al*., 2008; Finkelstein *et al*., 2006; Busalmen *et al*., 2008). So we have verify the influence of high anode potential in ^{14}C-IPU and ^{14}C-SMZ degradation assays. While the ^{14}C-IPU mineralization increased by more than 20-fold respect the electrode-free control, enforcing the roll of the electrode potential in the total IPU biodegradation (pol-MERC), ^{14}C-SMZ mineralization decreased under the influence of high electrode potential in comparison with the assays assisted by anodes at negative potential (MERC). Although oxygen cannot be electrochemically generated under our test conditions, our assays revealed ^{14}C-IPU metabolite patterns similar to those obtained under aerobic conditions. So burying a positive potential electrode in flooded soil may influence the dominant metabolic pathways leading to common metabolite patterns with aerobic conditions.

It is still unclear how a polarized electrode affects the microbial community and bioremediation activities. The electric field may change the permeability of cell membrane, leading to the excessive absorbance of extracellular substances and further change the microbial metabolism (Rittmann and McCarty, 2001). This increased compound uptake could be a negative feature in case of antimicrobial degradation, e.g., SMZ. Moreover electricity affects certain enzymes from electrogenic bacteria to promote organic removal reactions (Pitts *et al*., 2003). Beside those facts, predominance of anode respiration over fermentation (Hunt *et al.,* 2010; Pinchuk *et al.,* 2011) and a suppression of several anaerobic species, such as archaea and sulphide producing populations at high redox potentials have been recently reported (Lu *et al.,* 2014a; Ueno and Kitajima, 2014).

Thus, the electrodes not only overcome the TEA limitation but also offer the possibility through the potential electrode modification to accommodate to very different biodegradation scenarios. Consequently, they provide a battery of tailored redox setups that can be displayed and interchanged regarding to the different pollutants chemical nature, redox conditions and matrix, triggering decisive and specific biodegradation routes to enhance removal rate of pollutants.

- **Does the sole presence of conductive material have an effect in pollutant microbial bioremediation?**

The mere presence of conductive material enhanced the biodegradation of DBT in soil and SMZ in manure under open circuit configurations, so anode and cathode disconnected without electron flow through the system. In the case of DBT the graphite caused a clear stimulation of microbial activity and 40% of the DBT was successfully removed after 25 day, for just 10% in the graphite-free control. In the case of SMZ another conductive material (carbon felt) enhanced in 14% (after 32 days) and in 10% (after 62 days) the mineralization of the ^{14}C-SMZ in relation with the manure without carbon felt.

We hypothesized that these results were related to a process called Direct Interspecies Electron Transfer (DIET). This mechanism occurs when microbial species interchange electrons in absence of electron shuttles or redox mediators, just through direct electrical connections (Summers *et al*., 2010; Lovley, 2011b). This electron transfer may be accelerated in presence of an insoluble conductor material, which optimise electrical connections between microorganisms. Conductive activated carbon has been reported to promote this mineral mediated DIET between bacteria and methanogens enhancing the anaerobic conversion of organic matter in a digester (Liu *et al*., 2012). Similarly magnetite facilitated the electron transfer among *Geobacter sulfurreducens* and *Thiobacillus denitrificans* enhancing the coupling between acetate oxidation to nitrate respiration, both reactions that cannot be performed by one of those single bacterial strains (Kato *et al*., 2012). So taking into consideration that biodegradation of recalcitrant compound as DBT and antimicrobials as SMZ requires a set of redox reactions that can be played by different strains, our results suggest that conductive material would help these redox reactions to occur, facilitating the electron transfer between microorganisms due to conductivity of the material.

The phenomenon of conductive materials acting as electron conveyor belts is not new in nature. $MERC_{open}$ configuration might mimic the processes occurring in the so-called natural geobatteries, which consist of graphite deposits in the subsurface acting as electron acceptors for microorganisms. These geobatteries are capable to transport electrons among different redox zones (Bigalke and Grabner, 1997; Leung and Xuan, 2015). This ability to separate electron acceptors and donors in space for energy conservation (over centimetre distances) takes place as well with soluble electron acceptors like oxygen. Indeed, Pfeffer *et al*. (2012) reported electrode transportation via living micro-cables over long distances in the form of long filamentous bacteria of the *Desulfobulbaceae* family. The electrons generated by the microbial sulphide oxidation at the bottom layers of the sediment can be conducted through the wires to upper layers where soluble electron acceptors like oxygen are reduced.

The fact that the mere presence of an electrically conductive material increases the capability of native microbial communities to degrade different contaminants, rise this conductive material-assisted bioremediation as a very promising technique to treat polluted environments. Nevertheless DBT removal was stimulated by an extra 11% and SMZ by an extra 14% when the electrodes were connected as part of a MERC setup. A similar trend was shown by Wang *et al*. (2012b), when performing bioremediation of total n-alkanes (TNAs).

- **Can MERCs fully clean-up contaminated environments?**

Pollutant removal is not enough to assure a fully clean-up process in contaminated environments, so bioremediation processes must be followed by ecotoxicity tests to ascertain that it has restored its natural integrity (Hamdi *et al*., 2007; Liu *et al*., 2010).

MERCs not just have an effect in DBT and IPU removal but act as a true clean-up bioremediation strategy demonstrated by ecotoxicological analysis based on algal growth. In both cases, DBT and IPU, the detoxification under electrode-assisted treatments is consistent with the removal of the parent compound. It points to the electron flow between electrodes as a decisive factor to decrease the toxicity of both soil and manure extracts. Furthermore, detoxification capacity of electrodes polarised at high potentials (+600 mV versus Ag/AgCl) outperforms not just the natural IPU attenuation process, but also in lesser extent electrodes with negative potential, set spontaneously by the redox potential differences across soil/water.

Using ecotoxicological methods provides a better insight into ecological assessment of remediation and may support decisions for on-site amendments towards a successful site restoration (Hankard *et al*., 2004). The presence of toxic metabolites might be more toxic than the parent compounds (Escher and Fenner, 2011). For instance 4-IA, an IPU-metabolite is 600-fold more toxic than IPU as tested by Microtox (Tixier *et al*., 2002). Indeed IPU

metabolite pattern change with the treatment design, so an increased IPU removal not necessarily leads to a better detoxification performance.

- **Does bioelectrochemical stimulation influence the fate of pollutants?**

Pollutants dissipation or removal is often called “degradation” in studies reporting the fate of environmental pollutants. However, the disappearance of the parent substance and its metabolites does not necessarily correspond to formal degradation leading to mineralization. A complete understanding of the bioremediation task impact requires a proper analysis about the pollutant destiny. The use of the ^{14}C-isotopic analysis enabled the establishment of a ^{14}C mass balance where the different fates of the pollutant are totally specified. In this case not only mineralization ($^{14}CO_2$-formation resulting from the total degradation of ^{14}C SMZ) but also volatilization and extractable and non-extractable ^{14}C residues can be properly measured. All these variables conform an overall evaluation of the pollutant fate.

The extractable ^{14}C-residues (ER) in the soil and manure samples varied considerably between the different experimental conditions being always lower under the treatments that reached higher mineralization rates. So thus, we recovered less ER in MERCs polarised at high potentials (+600 mV versus Ag/AgCl) to treat ^{14}C-IPU contaminated soil and MERCs with negative anode potential to treat ^{14}C-SMZ polluted manure. In this last case the electrodes were connected by a copper wire using a 5.6Ω external resistor (R).

Generally non-extractable residues (NER) mean a lower environmental risk than ER because of their reduced mobility (Barraclough *et al*., 2005). Nevertheless, matrix properties changes or microbial activities can modify chemical bounds and pollutants may be eventually released (Liu *et al*., 2013; Barraclough *et al*., 2005). Positive correlations between NER formation and IPU aerobic mineralization have been previously reported (Alletto *et al.,* 2006). Furthermore, recent progress in the nature of NER using ^{13}C-isotope

label unravelled that NER formed during metabolic biodegradation (usually coupled with intensive mineralization) of organic pollutants in soil are mainly comprised of biomolecules originated from anabolism of xenobiotics (Nowak *et al*. (2010, 2013)); Poßberg *et al*., 2016). This type of environmental innocuous NER is defined as bioNER (Kästner *et al*., 2014) to distinguish from "real" NER, which are formed by xenobiotic parent compounds and/or metabolites (Roberts, 1984). This could explain the similar distribution observed for Non Extractable Residues (NER) despite the 20-fold higher ^{14}C-IPU mineralization regardless the presence or absence of electrode (40.4% for 43.6% respectively of the total ^{14}C-applied IPU). It is assumed that the microbial communities causing greater IPU mineralization resulted in formation of more bioNER and less "real" NER and therefore the environmental risks are reduced.

The time of contact between the pollutant and the matrix makes NER to increase regardless the assay configuration and operation modus. Thus, ^{14}C-IPU NER increased during long-term assay (110 day) regarding to shorter incubation assays (25 days). Similarly, NER are much higher in ^{14}C-SMZ mineralization assays after 62 days (pol-MERC (58.5%), electrode-free control (50.8%) and $MERC_{open}$ (45.2%) than after 32 days (30% in MERC). Another possible pathway for the formation of NER is by the binding of the xenobiotic to the soil matrix (eg. organic matter). Actually, it has been previously reported that IPU-metabolites are mostly adsorbed onto organic matter in soils (Ertli *et al*., 2004).

Carbon felt showed a convenient physic-mechanical properties and it was established as the electrode material due to the lack of ^{14}C-pollutants adsorption. This fact contrasts with the high ^{14}C-IPU and ^{14}C-SMZ residues adsorbed on the electrodes after the performance with anodes polarized at high potentials, which suggests the electric field generated by polarizing as the probable reason for such a high pollutant-adsorption rate. For instance, when we turned off polarization of anodes during ^{14}C-IPU degradation assays, by connecting the electrodes through a resistor then, the total ^{14}C-residues decrease from 13.7 % to 7.8% and the extractable residues from the anode

decreased as low as 0.2%. A similar result was previously reported by Zhang *et al*. (2010) using polluted slurries, although they reported a higher adsorption for benzene and toluene in the electrodes (70%) and also confirmed that toluene adsorbed on the graphite could be metabolized. Similarly, the radioactivity accumulated in the MERC with non artificially polarized electrodes did not reach the 3 % of the total ^{14}C-SMZ applied, in contrast with the higher value observed in pol-MERCs (18%) polarised at high potentials (+400 mV versus Ag/AgCl).

- **Can we interrogate *in situ* microbial activity by mean of MERCs?**

Monitoring *in situ* microbial activity in anoxic submerged environments can be an intensive and technically difficult labour. MERCs may provide a strategy for real-time monitoring of the natural activity of microorganisms meanwhile bioremediation processes take place without disturbance or necessity to sacrifice samples.

In order to evaluate the electrochemical performance the current production was continuously registered in MERCs. Maximum current densities were always higher in soil than in manure assays, which might be related not just to the matrix physic-chemical characteristics but also to matrices microbial population differences. The natural habitat of the majority of electroactive microorganisms is described as “multiple” but many species were found and isolated from soil and sediments (Koch and Harnisch, 2016). So the electroactive population in soil might be more abundant than in manure.

The current density was also influence for the configuration of the electrochemical system. Systems with electrodes polarized at high positive potential showed shorter lag phases and higher current productions than systems based on the configuration anode-resister-cathode, where anode potentials were negative and indeed more similar to reducing anaerobic environments. So positive potential accelerated the electroactive microbial activity.

Electroactive microbial activity is required for harvesting current by an electrode according to our sterile controls. Nevertheless, there is not evidence proving that the microorganisms degrading the pollutant are indeed electroactive. Actually, taking in account the IPU concentration in soil bioremediation assays (5 ($\pm$ 0.1) μg g^{-1} soil (dry weight)), this compound may contribute to the total current in a negligible range compared with the background current. The IPU applied (0.85 μMol per incubator) contains a potential charge of 0.65 Coulomb, ca 0.7% of the charge generated in the pol-MERC (131 Coulombs). So, the organic content of the soil (0.99 %) will be certainly key and responsible of such a background current. However it can not be questioned the fact that positive polarized electrodes stimulate the mineralization of the herbicide isoproturon and negative electrodes increased SMZ mineralization and DBT removal associated with a general microbial activity.

Cyclic voltammetry (CV) analysis was also performed for evaluating the activity of microorganisms exposed to different experimental conditions and to progress in understanding the relationship between the pollutant degradation and the increased biological activity. In these CVs several oxidation/reduction peaks were detected at different incubation times. The intensity of the signals increased with the time under each configuration, matrix and pollutant, providing an indication of an enrichment of microbial communities with electron transfer capabilities. This increase might be due to an increase in the cell density on the electrode surface or to an increase in the microbial metabolism that accelerate the electron transfer (Fricke *et al*., 2008). The pattern and intensity of the peaks varied between different configurations, with higher intensities under the influence of high electrode potential. These facts just support the circumstance that higher anode potential may increase the energy per electron transferred available for growth and cell maintenance (Aelterman *et al*., 2008; Finkelstein *et al*., 2006; Busalmen *et al*., 2008).

In bioremediation assays with IPU contaminated soil this remarkable difference in pol-MERC current production and voltammetry is consistent with a higher ^{14}C-IPU mineralization. Contrarily in the case of SMZ contaminated

manure the best mineralization rates took place under anode-resistor-cathode configuration where the current production and the signal intensity of oxidation/reduction peaks in CV were lower than under pol-MERCs. Therefore, cyclic voltammetry seems to be a good strategy to monitor microbial activity but should be combined with other electrochemical techniques and previous analyses in order to establish more direct correlations between pollutant degradation and electrochemical response.

- **Does the new redox scenario provided by MERCs influence the microbial community profile?**

The anode's operational mode not just unchains functional differences regarding to IPU-removal and soil effective clean-up but came associated to taxonomical shifts in the microbial community. High-throughput sequencing analysis underlines what kind of bacteria populations might be key for IPU-bioremediation and biocurrent generation.

Anodes poised at +600 mV versus Ag/AgCl stimulate the presence of *Firmicutes*, mainly the classes *Clostridia* and *Bacili.* Other studies have documented *Firmicutes* abundance increased during bioremediation processes associated to electrodes, e.g., petroleum hydrocarbon contaminated soil (Lu *et al*., 2014a) and degradation of macrophyte litter (Song *et al*., 2015). Similarly *Deltaproteobacteria* increased under the influence of the anode, regardless the electrode potential, in comparison to the electrode-free control. *Deltaproteobacteria* is an ubiquitous group in microbial communities associated to sediment/soil embedded electrodes (Bond *et al*., 2002; Reimers *et al*., 2006; Nielsen *et al*., 2007) and has been enriched in electrodes polarized at elevated and oscillatory redox potential (Reimers, *et al*., 2013), root exudates in plant-Microbial Electrochemical Systems (Lin and Lu, 2015; Cabezas *et al*., 2015) and biomass amended sediments (Zhou *et al*., 2015). Additionally, the presence of electrode inhibited the phylum *Actinobacteria*, involved in fermentation processes that generate electron donors for the downstream electron consumers, those that produce methane and/or electricity as end products (Rui *et al*., 2009). Interestingly, a

similar descent took place during bioremediation of petroleum-polluted sediments assisted for electrodes although *Actinobacteria* are known to be abundant as a group of versatile petroleum hydrocarbon degraders (Lu *et al.,* 2014a).

In genus level, *Symbiobacterium* (*Firmicutes*) presence was 4fold higher under the influence of high electrode potentials and its abundance increased parallel to genus *Bacillus sp.* Interestingly *Symbiobacterium thermophilum,* that belongs to genus *Symbiobacterium,* has been reported as a unique bacterium living in symbiosis with *Bacillus sp.* and using formate as electron donor during anaerobic respiration (Ueda *et al*., 2004). Recent studies reported the presence of *Bacillus* species in an anodic biofilm during cis-dichloroethene oxidation (Aulenta *et al*., 2013) and members of the family *Bacillaceae* were relatively abundant in electrochemical reactors that stimulate anaerobic toluene degradation (Daghio *et al*., 2016). Our analysis also revealed the enrichment of *Geobacter (Deltaproteobacteria)* on the electrode-hosting soil compared with electrode-free control, suggesting the stimulation of *Geobacter*-related populations in current producing systems. Among all bacteria reported to play a role in Microbial Electrochemical Systems, *genus Geobacter* is for sure one of the main actors. Hence, anodes acting as TEA brought rapid acclimation of electrochemically active *Geobacter sp.* capable to transfer electrons to anodes. The abundance of these electroactive bacteria was higher in MERC than in pol-MERC setups. Nevertheless the electrochemical analysis revealed a higher electrochemical response in pol-MERC in terms of electrical current and cyclic voltammetry performance. This result suggests that other electro-active microbes but *Geobacter sp.* might be capable to improve the extracellular electron transfer processes under the effect of anodes poised at +600 mV versus Ag/AgCl. Similar trends were follow for *Anaeromyxobacter sp.* also a *Deltaproteobacteria*, which has been previously detected on anodes from rice crops plant-METs (Cabezas *et al*., 2015), as well as on biocathodes able to perform dechlorination of aromatic compounds (Strycharz *et al*., 2010). Members of *Deltaproteobacteria*, as *Geobacter and Anaeromyxobacter sp.* are together with *Gammaproteobacteria* the major phylogenetic groups of

electroative bacteria identified playing an important role in delivering electrons to the anode (koch and Harnisch, 2016).

Despite the inherent microbial complexity, understanding the link between microbial communities and the electrocatalysis of pollutants in bioelectrochemically-assisted remediation may help to optimize our envisioned application of MERCs (Daghio *et al*., 2016). Thus far, the molecule-level bioelectrochemical influences on remediation microbial activities are mostly unknown and a more comprehensive linking between the diverse microbial community and bioelectrochemical anode-influence must be still established.

- **Does the microbial community change with the distance to the electrode?**

Microbial community profile was used as a reporter of the electrode influence during IPU-bioremediation assays. Soil sampled at 0.5 cm above of the anode where still favouring some microbial differences regarding to the communities from soil intimately associated to the anode and the soil from the electrode-free control. The more explicit change consisted in the *Proteobacteria* decreased and the *Firmicutes* increased as a result of the distance between the microbial communities to the electrode. At genus level, *Geobacter* was less abundant in soil far from the anode, where we identified an identical abundance as in the electrode-free control. On the contrary, the abundance of genus *Symbiobacterium* increased more than sevenfold in soil samples far from the anode regarding to the electrode-free control and twofold in reference to the soil in contact with the anode. This suggests that the electrode influence to change the communities profile is higher than 0.5 cm.

5.2. Final conclusions

The general conclusions that can be withdrawn from this thesis are the following:

- *Bioelectroventing,* defined as a process where soil is exposed to electrodes to stimulate the oxidative metabolism of environmental microbial populations, **enhance the DBT removal** assisted by anode-resistor-cathode systems (MERC), reaching high efficiency in a shorter incubation time, even at the initial stage of the experiments.

- Our results conclude that the application of high anodic potential (+600 mV versus Ag/AgCl) (pol-MERC) to contaminated soil **increases ^{14}C-IPU-mineralization by 20-fold**, enforcing the establishment of polarized electrodes as a conceivable cost-effective and environmentally friendly strategy for enhancing herbicides degradation under extreme reductive conditions.

- Functional differences (IPU-removal and mineralization) were associated to **taxonomical shifts in the microbial community** revealed by the high-throughput sequencing analysis. We also used the microbial community profile as reporter of the electrode influence suggesting that the electrode effect to change the communities profile is at least 0.5 cm.

- Our results proved that positive polarized electrodes (+400mV (vs. Ag/AgCl) increased the microbial electrogenic metabolism. However, negative redox scenarios (-400mV vs. Ag/AgCl) **enhanced sulfamethazine mineralization**.

- DBT-removal and ^{14}C-SMZ mineralization were also enhanced by the **sole presence of a conductive material**, a situation where anode and cathode are not connected, thus electron flow is not occurring

($MERC_{open}$). This fact opens the feasibility of efficient *in-situ* low equipment interventions as a bioremediation strategy.

- In the present study, we demonstrate how the use of MERC electrodes in IPU contaminated soil and SMZ-contaminated manure enhance, not just the biodegradation rates, but also influences the ^{14}C mass balance, the ^{14}C metabolite profile and **the fate of the pollutant,** decreasing the extractable residues, the most available and therefore hazardous pollutant fraction.

- MERCs performing *bioelectroventing* may be used for *in situ* effective decontamination of soils due to their **detoxification capacity.** They also present all the advantages of the others *in situ* bioremediation approaches, while avoiding bioaugmentation or artificial addition of nutrients with impact in the environment.

5.3 Recommendations and future work

Recommendations for future research are made based on the results presented in this thesis. The main outcome of this work is that Microbial Electroremediating Cells (MERCs) are a versatile, controllable and environmentally sustainable technology to clean up polluted environments with a high potential to be applied in real *in-situ* bioremediation scenarios. Nevertheless some limitations should be overcome and optimization of an operational configuration should be addressed prior to use MERCs for real open field applications. This challenge may need researching efforts in multiple disciplines including biotechnology, geochemistry, material science and electrochemistry.

- In order to achieve **energy-neutral design:**

Energy recovery is no longer the major goal in bioremediation processes assisted by Microbial Electrochemical Systems, and even a moderate input of external energy is allowed to favour the decontamination

reactions (Chun *et al*., 2013). Nevertheless a MERC energetically self-sustained operation for remediation is theoretically feasible using adequate equipment for harvesting the microbial-based electricity produced during remediation processes. Furthermore, the small energy input for MERCs may be readily provided *in situ* from solar and wind energy or another renewable energy sources.

- In order to obtain a **better understanding of the bioremediation processes** occurring in MERCs is necessary to emphasise on pollutant mixtures effects and microbial electrocatalysts and to implement the real-time monitoring of lab-scale microcosm systems.

    a) In the current work we have successfully presented bioelectrochemical remediation processes tested just with individual pollutants. Mixed interactions of contaminants may have an effect in their bioavailability and accumulation in the different matrices and electrode materials, modifying the risk and the site-specific bioremediation options. Given that most of the contaminated matrices as manure and soil contains not a single contaminant but a complex mixture of organic compounds or heavy metals, more studies to understand the effect of pollutant interactions should be performed underlying their influence in bioelectrochemical performance and their ecological impact in the microbial community.

    b) The clean up of contaminated environments remains a very challenging task. Challenges exist primarily because environmental conditions make each site unique. To achieve efficient, reliable and robust field remediation, the bioelectrochemical remediation strategy must be tailored to the specific site conditions. Implementing the real-time monitoring of lab-scale microcosm systems during bioelectrochemical remediation processes would refine the knowledge of how the different electrochemical parameters (such as electrode potential and current) influence the environmental conditions and microbial metabolic processes. This

knowledge could help to optimize of design site-specific system, which requires a deep understanding of the indigenous microbial population, the pollutant and the environmental conditions.

c) Bioremediation success assisted by Microbial Electrochemical Systems will depend on reactor design as well as operational aspects, but principally of the presence and activity of sufficient microbial electrocatalysts. So it is essential to identify and isolate and electroactive microorganisms involved in degrading pollutant and to understand the assorted metabolic routes in the presence of an electrode. This would help to have a better insight of the implication of the anode-related microbial community in the pollutant degradation and may help to conceive better bioremediation strategies.

- In order to scale up this technology as a real bioremediating tool *for real polluted environments* significant knowledge gaps and technological barriers remain to be addressed to transfer these processes to the field application.

a) Real pollutes sites bioremediation strategies require an intense control of the engineering aspects of Microbial Electroremediating Cells designs to adapt to different depths, matrix types, and other physical-chemical parameters. Additional problems as the passivation of electrode materials by electrochemical deposition, the corrosion of electronic connections and possible damages of the system set-up are factors that should be addressed to scale-up successfully bioelectrochemical systems under the dynamics of natural environments.

b) Another critical factor to take into account for full-scale systems is the radius of influence (ROI) of the electrode. The ROI can be influenced by physiochemical and biological properties of the matrix and the contaminants, but also by the system configuration and electrode materials. Thus, it is key the study of these variables to maximize the

distance from the anode where biodegradation is still increased regarding to natural attenuation.

c) Additionally to all the engineering and microbial aspects, the requirement of water content should be considered to improve the efficiency and applicability of the Microbial Electroremediating Cells in full-scale bioremediation processes. Currently bioremediation processes assisted by MERCs are confined to water saturation of matrices for a suitable proton transfer between electrodes. So thus this factor is currently the greatest engineering challenge for the implementation of MERCs in *in situ* environments. Recent progresses in this direction the group "Bioelectrogenesis" of University of Alcalá developed a new configuration by using MERC with ceramic barrier, which avoid the condition of waterlogged environments. The influence of this barrier as interface between matrix, anode and aerated cathode should be studied in more depth. This new free-flooded conditions configuration aims to implement Microbial Electrochemical System scale-up, concretely the use of Microbial Electroremediating Cells in *in situ* bioremediation processes (Domínguez-Garay, 2016).

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Annexes

Abbreviations

^{14}C-IPU	^{14}C-isoproturon
^{14}C-SMZ	^{14}C-Sulfamethazine
2-OH-MIPU	2-OH-Mono-demethyl-Isoproturon
4-IA	4- isopropylaniline
4-IPA	4-isopropyl-aniline
AD	Anaerobic Digestion
AE	Auxiliar electrode.
AEM	Anion exchange membrane
ASE	Accelerated solvent extraction
ATP	Adenosine triphosphate
BA	Benzoate
BMFCs	Benthic or Sediment Microbial Fuel Cells
CBA	Chlorobenzoates
CEM	Cation exchange membrane
COD	Chemical oxygen demand
CV	Cyclic voltammetry
CWs	Constructed wetlands
DBT	Dibenzothiophene
DD-IPU	Didemethyl-isoproturon
DEET	Direct extracelular electron transfer
DIET	Direct Interspecies Electron Transfer
EET	Extracellular Electron Transfer
ER	Extractable residue

H′	Shannon diversity index
IPU	Isoproturon
MDIPU	Monodemethyl-isoproturon
MEET	Mediated Extracelular Electron Transfer
MERC	Microbial Electroremediating Cell
METs	Microbial Electrochemical Technologies
MFC	Microbial Fuel Cell
NCTR	The National Center for Toxicological Research
NER	Non extractable residues
NHE	Normal hydrogen electrode
OC	Open circuit
OCV	Open circuit voltage
OD	Optical density
OECD	Organization for Economic Cooperation and Development
OMs	Organic matters
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorobiphenyl
POPs	Persistent organic pollutants
R	Resistor
RE	Reference electrode
SCE	Standard calomel electrode
SDS	Sodium dodecyl sulphate
SHE	Standard hydrogen electrode
SI	Supporting Information
SMFCs	Sediment Microbial Fuel Cells

SMZ	Sulfamethazine
SPE	Solid phase extraction
t_0	Initial time
TEA	Terminal electron acceptor
TOC	Total Organic Carbon
TPH	Total petroleum hydrocarbon
TSH	Thyroid-stimulating hormone
VFAs	Volatile organic acids
WE	Working electrode

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trapped in a 0.1 N NaOH solution for allowing the measurement of ^{14}C-IPU. ***P111.***

Figure 3.2: A) Cumulative mineralization of ^{14}C-IPU within 25 days in pol-MERCs (n=4, S.D.) compared with mineralization capability of the soil under the electrode-free conditions (n=3, S.D.); B) Cumulative mineralization and rate of ^{14}CO2 production from ^{14}C-IPU under long term MERCs treatment. During the first phase the polarized anode acted as TEA at 0.6 V vs Ag/AgCl (+197 mV versus normal hydrogen electrode (NHE)). This phase ended with the anode potential reversed to -300mV vs Ag/AgCl for 12 hours. During the second phase the anode potential was achieved by connecting the electrodes through a 56 ohm resistor. ***P115.***

Figure 3.3: A) Chronoramperomety of pol-MERCs polarized at 0.6V (versus Ag/AgCl). Geometrical anode surface was used for calculating the current density B) Cyclic voltammetry tests (scan rate: 1 mV/s) carried out at initial experimental time and after 25 days under bioelectrochemical assisted soil (pol-MERCs and sterile pol-MERCs). ***P117.***

Figure 3.4: A) Distribution and mass balance of different soil treatments regarding initial radioactivity of ^{14}C-IPU B) Extractable and non-extractable ^{14}C residues in carbon felt electrodes used under different treatments. Bars represent standard deviation. ***P119.***

Figure 3.5: Proposed biodegradation pathways for IPU under polarized-MERCs conditions. ***P124.***

Figure 3.6: (A) IPU and MDIPU recovery in soil extracts after accelerated solvent extraction and the total extractability from IPU-polluted soil incubated under different treatments. (B) IPU-removal and MDIPU accumulation expressed in % of the initial IPU applied. (C) IPU biological removal expressed in percentage of the initial IPU applied after excluding the sorption to soil matrix in the sterile control. The error bars represent the standard deviation of the mean value for triplicate assays. ***P138.***

Figure 3.7: Toxicity levels in soil extractions from IPU-polluted soil incubated under different treatments. The toxicity values were represented as inhibition of *P.subcapitata* algal growth (%). Reference toxicity values for non-polluted soil (negative control) were also provided. The error bars represent the standard deviation (n=3, S.D). ***P141.***

Figure 3.8: Taxonomic classification of 16S rRNA gene from bacterial

communities of soil samples at phylum level (A) and the class level distribution of the most dominant phylum, *Proteobacteria* and *Firmicutes* (B). Bars represent standard deviation (n=3, S.D). Phylums with a relative abundance <2% and classes with a relative abundance <3% are not represented. ***P143.***

Figure 3.9: Taxonomic classification of bacterial sequences from communities of soil samples at genus level from the different treatments. Genus with a relative abundance <0.5 % are not represented. ***P146.***

Figure 3.10: (A) sampling points in pol-MERCs. Samples collected from the soil adjacent to the electrode were denominated pol-MERC and soil sampled 0.5 cm above the anode in the vertically section were denominated pol-$MERC_{0.5.}$ (B) Taxonomic classification of bacterial sequences from communities of soil samples collected at different sampling points in pol-MERC assays at genus level. Genus with a relative abundance <1% are not represented. Bars represent standard deviation (n=3, S.D). ***P148.***

Figure 4.1: Laboratory system for monitoring the^{14}C-SMZ mineralization under different configurations. The zoom depicts a pol-MERC with a three-electrode system controlled for a potentiostat, polarizing the anode (working electrode) at +0.4 V vs Ag/AgCl reference electrode. 1N NaOH solution was harvested three times per week for measuring the $^{14}CO_2$ released from^{14}C-SMZ mineralization in manure. All systems were performed by triplicate. ***P167.***

Figure 4.2: Cumulative mineralization of ^{14}C-SMZ at different configurations for 62 days. A pol-MERC configuration was converted to MERC after 32 days of incubation. During the first phase, the electrode acted as electron acceptor at +0.4 V versus Ag/AgCl [+197 mV versus normal hydrogen electrode (NHE)]. During the second phase, the anode potential was achieved by connecting the electrodes through a 5.6 ohm resistor and monitored until day 62. MERCopen and electrode-free control were under the same operation conditions during the 62 days incubation time. Average value presented resulted from three experimental replicates. The error bars represent standard deviation. ***P172.***

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Supporting figure 3.6: MERC Scheme. $^{14}CO2$ was trapped in a 0.1 N NaOH solution for allowing the measurement of ^{14}C-IPU mineralization. ***P154.***

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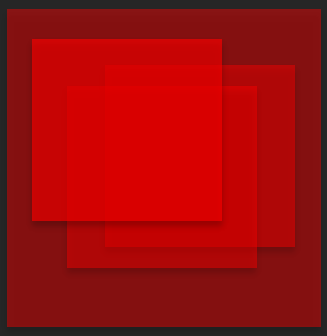

Microbial electrochemistry is an emerging discipline based on the interactions between microorganisms and electrically conductive materials. All the assorted technologies based on this principle are referred as “Microbial Electrochemical Technologies” (METs) and their range of applications spans diverse fields. One of these novel applications consists in using the electrodes as unlimited electron acceptors or donors for removing contaminants from polluted environments. METs with this purpose are named as Microbial Electroremediating Cells (MERCs) and in this thesis were operated to maximize metabolic oxidation and to enhance the biodegradation of pollutants. This process of supplying electrodes to stimulate the oxidative metabolism of environmental microbial populations is called *bioelectroventing*. The use of this strategy has disclosed as a versatile, controllable and environmentally sustainable technology to clean up polluted sites with a high potential to be applied in real *in-situ* bioremediation scenarios.

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