**UNIVERSIDAD DE ALCALÁ**

Departamento de Química Analítica, Química Física e Ingeniería Química


# Bioelectrochemically-assisted remediation: a novel strategy for cleaning-up polluted soils

## TESIS DOCTORAL

Ainara Domínguez Garay

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Universidad de Alcalá

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**ABRAHAM ESTEVE NÚÑEZ, Profesor Titular de Ingeniería Química de la Universidad de Alcalá,**

**CERTIFICA**:

Que el trabajo descrito en la presente memoria, titulado "**BIOELECTROCHEMICALLY-ASSISTED REMEDIATION: A NOVEL STRATEGY FOR CLEANING-UP POLLUTED SOILS**", ha sido realizado bajo su dirección por Dña. Ainara Domínguez Garay 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 16 de mayo de 2016.

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Dicha tesis reúne los requisitos necesarios para su presentación y defensa.

Y para que conste y surta los efectos oportunos, firma el presente en Alcalá de Henares a 16 de mayo de 2016.

Jesús Alberto Escarpa Miguel

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

**TESIS DOCTORAL**

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

**Ainara Domínguez Garay**

Dirigida por:

**Abraham Esteve Núñez**

Departamento de Química Analítica, Química Física e Ingeniería Química

Universidad de Alcalá

Alcalá de Henares, 2016

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*“No nos atrevemos a muchas cosas porque son difíciles, pero son difíciles porque no nos atrevemos a hacerlas”.* Quizá fue esa la filosofía que apliqué al comienzo de esta tesis, cuando las dudas y la inseguridad de no saber si sería capaz o de si era éste el camino que quería emprender, me pusieron ante una decisión que hoy, no podría haber sido otra.

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*“No nos atrevemos a muchas cosas porque son difíciles,*
*pero son difíciles porque no nos atrevemos a hacerlas”*

**Luccio Anneo Séneca**

**A mis padres**

# Summary

Bioelectrochemically-assisted remediation is a novel tool for enhancing the degradation of soil pollutants. This application derives from the *Sediment Microbial Fuel Cell* (SMFC) concept, where an anode is buried in an anaerobic soil and connected through an electrical circuit to a cathode in the overlying water layer. The novelty of this technique lies in the unlimited character of soil-buried electrode as terminal electron acceptor (anode) allowing microorganisms to perform oxidative metabolism beyond the natural conditions found in soil. Unlike most of the standard Microbial Fuel Cells (MFCs) application developed in water matrix, implementing SMFC in the soil implies a very high resistance for proton transfer that limits the mass transfer, decreasing the efficiency in the degradation process. This is probably the reason why, since 2001, SMFC systems have been developed in ecological water bodies as marine sediments or freshwater flooded environments (rivers and waterlogged field crops), where saturated soil condition facilitates the ions transfer across soil matrix. However, this water dependence limits the application of microbial electrochemistry in soil because flooding is not a common situation for soil environments.

This dissertation begins with a first introductory chapter (***Chapter 1***) that provides a framework to introduce the topic and a literature review about the state of the art of this technology. All interactions and factors that affect the chemical and physical soil characteristics make the soil a complex and diverse habitat for living microorganisms. Nonetheless, the soil structure and conditions play an important role in the development of all processes in which they are present, e.g. the limitation in the degradation process of soil pollutants due to the lack of electron acceptors. In this way, conventional bioremediation techniques have

overcome this limitation by supplying to microorganisms with nutrients, co-metabolites, oxygen or other electron donors or acceptors (biostimulation) or by adding additional microorganisms able to degrade a specific pollutant (bioaugmentation). Nevertheless the requirement of constant supply makes these techniques an expensive alternative for accelerating any bioremediation process.

The aim of this thesis was to evaluate the application of microbial electrochemical systems in soil for either harvesting energy or bioremediating polluted soils. In this context, bioremediation assays were based on the use of he herbicide atrazine as model compound. All the objectives aimed in this thesis are outlined in ***Chapter 2***.

***Chapter 3*** shows how soil structure (particles distribution and the water content) can influence the performance of SMFC. Futhermore, a rice paddy soil with non suitable conditions for hosting SMFC (low organic matter, low content in salt etc.) was supplemented in fumed silica in order to stimulate the colloid formation. So thus, silica-supplemented SMFC showed a higher efficiency in power production derived from the influence of soil resistivity in the overall SMFC resistance. However, not only the resistance itself showed was responsible of the higher efficiency of these systems, but silica colloids also led to changes in soil structure that improve the nutrients utilization by soil microorganisms.

According to the main objective of this thesis, microbial electrochemistry application to remove pollutants from soil is presented in chapter 4, 5 and 6. ***Chapter 4*** suggests the use of term *Microbial Electroremediating Cell* (MERC) after investigating the impact of using conductive material in bioremediating an atrazine-polluted soil. Moreover, the study was complemented with different toxicological analyses for verifying a real clean-up of atrazine-polluted soil under flooded conditions.

The flexibility of these systems for operating under different electrodes configuration or redox conditions makes this technology a very versatile tool for operating with pollutants of different nature. ***Chapter 5***, indeed, demonstrate that atrazine mineralization to $CO_2$ was strongly enhanced by polarizing the anode at positive potentials. This chapter also reports an overall profile of the $^{14}$C-ATR metabolites, $^{14}$C mass balance, and ecotoxicological results of treated soils in response to the different treatments.

As a final scientific contribution, ***Chapter 6*** explores a new strategy for operating microbial electrochemical system in non flooded soil for expanding outdoor application of MERC in any real polluted environment. The proposed new design based on a ceramic barrier that result in a major proximity between anode and cathode, allows give-up the idea that the applicability of these systems is restricted to flooded soils.

A general discussion, conclusions and future outlook are presented in ***Chapter 7***, where final considerations of this thesis are presented under a question-answer mode. The remarkable impact of electrodes on soil bioremediation suggests a promising future for this emerging environmental technology. The work presented in this thesis supports the idea that this novel strategy for cleaning-up polluted soil might represent a potential alternative to conventional bioremediating techniques. This novel research field shows a wide spectrum of possibilities for its future application in real environments.

# Resumen

La biorrecuperación electroquímica supone una nueva herramienta en la degradación de compuestos contaminantes presentes en el suelo. Esta aplicación deriva del concepto *de Celda de Combustible Microbiana Sedimentaria* (SMFC, por sus siglas en inglés), en donde un electrodo (ánodo) ubicado en un suelo o sedimento bajo condiciones anaerobias, se conecta a otro electrodo (cátodo) situado, de forma estándar, en la columna de agua que cubre el suelo o el sedimento, dando lugar a un circuito eléctrico entre ambos electrodos. La novedad de esta técnica recae en el carácter ilimitado que tiene el electrodo del suelo (ánodo) como aceptor final de electrones, lo que permite a ciertos microorganismos llevar a cabo la oxidación metabólica independientemente de las condiciones naturales del suelo. Sin embargo, uno de los problemas más importantes que presenta utilizar esta tecnología en suelos es que, a diferencia de las aplicaciones en medios acuosos, el suelo presenta una alta resistencia al transporte de protones, lo que limita la transferencia de masa y la eficiencia en los procesos de degradación por parte de los microorganismos. Probablemente, tengamos aquí la razón por la cual, desde el año 2001, todas las SMFC han sido desarrolladas en ambientes anegados, como sedimentos marinos o ambientes de agua dulce como ríos o cultivos inundados. En estos ambientes, la condición de saturación de los suelos facilita la transferencia de iones a través de la matriz del suelo. Sin embargo, esta dependencia del agua limita enormemente la aplicación de estas tecnologías, ya que las condiciones de anegación no son un escenario muy común en los ambientes edáficos contaminados.

Esta memoria de tesis comienza con una sección introductoria (***Capítulo 1***) que proporciona un marco global para introducir el tema, así

como una revisión bibliográfica sobre el estado del arte de esta tecnología. Todas las interacciones y todos los factores que afectan a las características fisicoquímicas del suelo hacen de él un hábitat complejo y diverso para los microorganismos. Sin embargo, la estructura y las condiciones del suelo juegan un papel fundamental en el desarrollo de todos los procesos en los que se encuentran presentes los microorganismos; por ejemplo, la limitación en los procesos de degradación de los contaminantes del suelo por la falta de aceptores de electrones que completen el proceso metabólico. En este sentido, las técnicas de biodegradación convencionales son capaces de superar esta limitación proporcionando nutrientes, co-metabolitos, aceptores o donadores de electrones, a los microorganismos del suelo (bioestimulación); además de introducir microorganismos cuya capacidad de degradación de ese contaminante sea conocida (bioaumento). No obstante, la necesidad de proporcionar de forma artificial, estos compuestos o microorganismos de manera continuada al suelo, hace de estas técnicas alternativas costosas para acelerar el proceso de biodegradación.

El objetivo principal de este trabajo de tesis es evaluar a través de ensayos de laboratorio la aplicación de la electroquímica microbiana en sistemas edáficos para generar energía o bien biorrecuperar suelos contaminados. En este contexto se procedió a realizar ensayos de biorrecuperación utilizando el herbicida atrazina como compuesto contaminante modelo. Los objetivos perseguidos a lo largo de esta tesis doctoral se han recogido en el ***Capítulo 2***.

El ***Capítulo 3*** muestra cómo influye un cambio en la estructura del suelo en la respuesta bioelectroquímica. Para ello, el suelo procedente de un cultivo de arroz, no especialmente apto para aplicaciones electroquímicas (baja materia orgánica, bajo contenido en sal, etc.) se modificó a través de la formación de coloides resultantes de la adición de sílice pirolítico al suelo. Las SMFC que contenían sílice mostraron una mayor eficiencia en la producción de energía al disminuir la resistencia

del suelo. Sin embargo, no solo la resistencia *per se* afecta a la mejora, sino que también se da lugar a cambios en la estructura del suelo que facilitan la utilización de nutrientes por parte de los microorganismos.

El objetivo principal de esta tesis - la aplicación de la electroquímica microbiana a la degradación de contaminantes en el suelo - se ha desarrollado en los capítulos 4,5 y 6. En el ***Capítulo 4***, y tras investigar el efecto de los materiales conductores en la eliminación de atrazina, se propone el término *Microbial Electroremediating Cells* (MERC). La monitorización de los niveles de atrazina en el suelo se complementó con diferentes análisis toxicológicos del mismo, verificándose así la efectividad del tratamiento al término del mismo.

Estos sistemas presentan una alta flexibilidad para su operación, ya sea modificando la configuración o el escenario redox mediante variaciones en los electrodos, así como polarizando el ánodo. Esto convierte a esta tecnología en una herramienta versátil para operar con contaminantes orgánicos de distinta naturaleza. En este sentido el ***Capítulo 5***, demuestra que la mineralización de atrazina hasta $CO_2$ se vio favorecida, de forma notable, al polarizar el electrodo a potenciales positivos. Este capítulo también sugiere una posible ruta metabólica a partir del perfil de los metabolitos de $^{14}$C-atrazina. Asimismo incluye un balance de masa del carbono $^{14}$C, y una evaluación toxicológica de los suelos tratados.

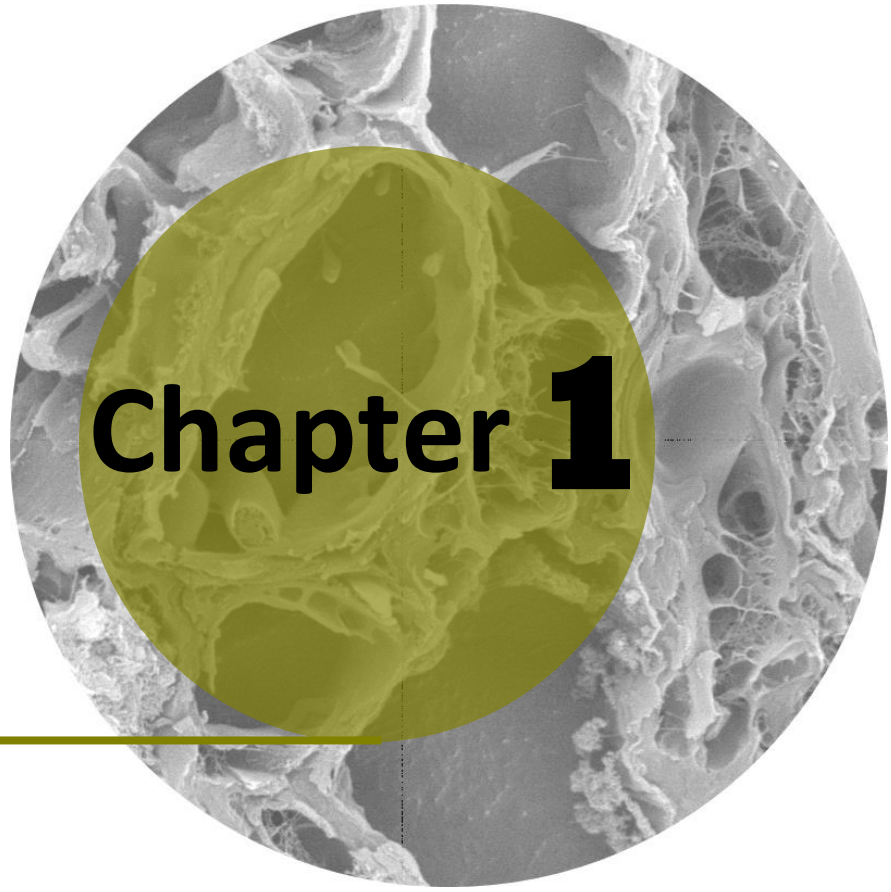
Como contribución científica final, el ***Capítulo 6*** explora una nueva estrategia para operar estos sistemas bajo suelos no inundados, expandiendo así las posibilidades de aplicación de las MERC en cualquier ambiente contaminado. El nuevo diseño propuesto, basado en el uso de una membrana cerámica que aproxima ánodo y cátodo, permite alejarse de la idea de que la aplicación de estos sistemas se restringe únicamente a ambientes con suelos anegados.

El ***Capítulo 7*** presenta una discusión y unas conclusiones generales de este trabajo de tesis, así como el análisis crítico de las perspectivas de futuro. Las consideraciones generales se presentan en un formato de pregunta-respuesta.

El impacto de utilizar material conductor en suelos contaminados sugiere un futuro prometedor para esta tecnología ambiental emergente. Además, este trabajo sostiene la idea de que esta nueva estrategia para la descontaminación de suelos podría representar una alternativa real respecto a las técnicas convencionales de biodegradación *in situ*. Así pues, creemos que este campo de investigación muestra un amplio espectro de posibilidades para su aplicación, en un futuro cercano, en ambientes reales.

# Contents

# Introduction



# Chapter 1

# General introduction

## 1.1. Soil and microorganisms

### 1.1.1. Soil as habitat

The soil (or pedosphere), the naturally occurring unconsolidated mineral and organic material at the earth's surface, provides an essential natural resource for living organisms (Voroney, 2007) as the result of the interaction between the lithosphere (rocks and minerals), biosphere (organic living), atmosphere and hydrosphere (nonliving components). Its complexity governs soil biodiversity and regulates the activity of the organisms responsible for ecosystem functioning and evolution (energy budget, water exchange, nutrients cycling and ecosystem productivity).

Soil habitat is defined as the totality of living organisms inhabiting soil, including plants, animals and microorganisms and their abiotic environment. As a habitat for microorganisms, soil is probably the most complex and diverse on the planet. This diversity arises, in part, through the wide variety of incoming substrates but the major factor is probably the spatially heterogeneous nature of soil (Powlson et al., 2001). The heterogeneity is mainly controlled by the soil structure that comprises mineral fragments covering a range of sizes that span several orders of magnitude. Mineral particles have a different chemical composition and surface properties that influence microbial survival and activity, soil solution composition and size and distribution of spaces between particles, that controls the transport and diffusion of solutes and gases, especially oxygen (Powlson et al., 2001). It provides a high range of habitats, and supports an enormous biomass, within the bacterial and archaeal cells are more than $10^9$ different species.

The pore space less than 10μm in diameter (micropores) can vary widely for a variety of reasons (soil mineralogy, bulk density, organic matter content and disturbance) and it plays an important role for water retention that restricts diffusion of exoenzymes and nutrients, and in consequence for providing an aqueous habitat for microorganisms (Figure 1.1). Although surface soils are typically about 50% pore space on a volume basis, only a quarter to a half of this pore space may be habitable by soil microorganisms due to restricted pore size.

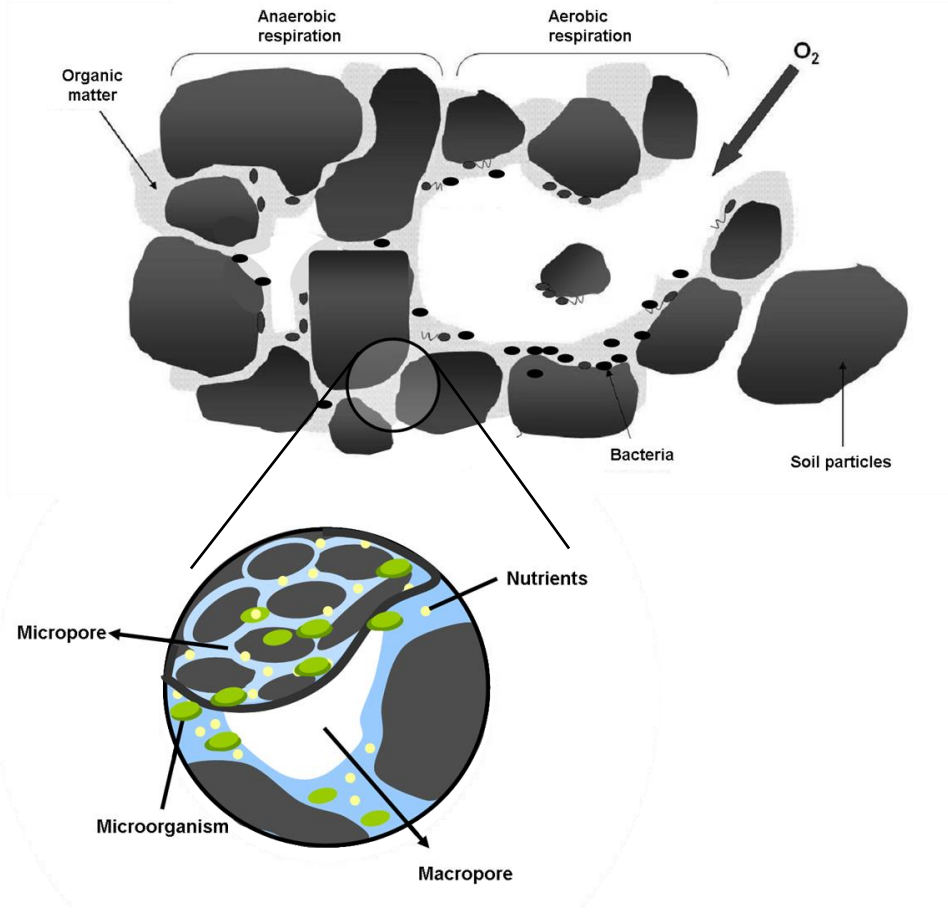



***Figure 1.1.*** General soil structure and microorganisms distribution along the soil particles.

### 1.1.2. Influence of soil parameters in living microorganisms' activity

The chemistry of soil is governed by many factors owing to soil solution (pH, soil redox, aeration) and the environmental conditions (moisture and temperature).

The soil solution provides an environment for soil microorganisms and its biogeochemistry is mainly determined by acid-base and redox reactions. The thermodynamic activities of protons and electrons in soil solution define the chemical environment that controls biotic activity:

**pH:**

Soil pH influences a number of factors affecting microbial activity, microbial biomass, microbial community structure and solubility and ionization of organic and inorganic soil solution constituent, which is turn affect soil enzyme activity, carbon (Andersson et al., 2000), nutrients availability (Aciego Pietri and Brookes, 2008) and the solubility of metals (Firestone et al., 1983).

The measurements of soil pH provide important data for predicting potential microbial reactions and enzymes activity in soil. Is important to mention that soil solution pH is very different comparing to the measured pH very next to soil particles. This is because concentrations of cations sorbed to the surfaces of negatively charged soil colloids are 10-100 times higher than those of the soil solution (Voroney, 2007).

**Soil redox:**

The metabolic activity of soil organisms produces electrons during the oxidation of the organic compounds. These electrons are transferred to an electron acceptor that could be the oxygen in aerobic soils. The $O_2$ contained in soil solution can be consumed within hours depending on the activity of soil microorganisms and it is replenished by oxygen diffusion.

When oxygen consumption rate by soil organisms is high or if $O_2$ diffusion into the soil is impeded, soil solution oxygen concentration continues to

decrease. When the available dissolved oxygen is exhausted, the solution changes from aerobic to anaerobic and microbial activity needs alternative electron acceptors. The activity of aerobic and facultative organisms decreases, and anaerobic organism increases, that promotes the reduction of $NO_3^-$, $Mn^{4+}$, $Fe^{3+}$, $SO_4^{2-}$ and $CO_2$.

Redox potential, *Eh*, is a measure of electron availability occurring as a result of the exchange of electrons between a redox couple during the process of reduction and oxidation of electron transfer between oxidized and reduced chemical species (Fiedler et al., 2007). It provides an indication of the soil aeration status and the measurements are often used to predict the most probable products of biological reactions.

Microbial aerobic activities reflect oxidizing conditions above an *Eh* of 300mV; facultative reducing microbes are active from 300 to -50 mV, or moderately reducing conditions. The preferred electron acceptors are first oxygen followed by $NO_3^-$, $Mn^{4+}$, and $Fe^{3+}$. Anaerobes microbes dominate at *Eh* levels below -50 mV. In these strongly reducing conditions, $SO_4^{2-}$ and $CO_2$ are the usual electron acceptors (Fiedler et al., 2007).

**Soil aeration:**
Molecular diffusion through the air-filled pores dominates the transport of gases in the soil and it maintains the gaseous exchange between the atmosphere and the soil, and diffusion through water films of varying thickness maintains the exchange of gases with soil organisms.

Environmental conditions, temperature and moisture mainly influence the physical, chemical and biological processes in soil:

**Temperature:**
The soil temperature influences in physical, chemical and biological processes in vastly different ways. e.g. molecular diffusion rate increases

with increasing temperature, but solubility of gases in soil solution does not and can even decrease.
The relationship between temperature and microorganisms activity is complicated as individual species differ in their optimal temperature response. Some microbial communities are able to adapt to temperature changes by altering their physiology and cellular mechanisms, membrane fluidity and permeability, and structural flexibility of enzymes and proteins (Wildung et al., 1975).

**Water content:**
Soil water content is essential for growth and multiplication of microorganisms and affects microbial degradation of organic contaminants by influencing aeration, contaminant diffusion and microorganism mobility. Studies done on aerobic microbial activity at different water-filled pore space showed an optimal water saturation of 60% for microbial activity (Linn and Doran, 1984). As the water content increased beyond 60%, the percentage of air-filled pores decreased and oxygen diffusion became limited, creating anaerobic conditions (Børresen and Rike, 2007).

Moisture content was expressed gravimetrically and without regard to the energy concept, in which soil moisture is expressed in term of the physical force with which it is held in soil rather than in terms of actual percentage content (Clark, 1967).

## 1.2. Microorganisms and pollutants

The continuous increase of industrial and agricultural development, environmental issues and considerations about sustainable development are becoming increasingly important. In consequence, the environmental pollution is stringent regulated, mostly concerning wastes, air, atmosphere and water. Nevertheless the soil has been subject to severe pollution as soon as agriculture began and mankind tried to control crops weeds. For

centuries the applied techniques were based on physical mechanisms such as tillage, however, from 1950 they began using all kinds of pesticides and fertilizers in order to increase and improve the crops production. Several years later the irrational use of these products and the increase of applied doses caused soil pollution problems and consequently aquifers and surface water pollution (Cerejeira et al., 2003).

Therefore a greater attention has been recently paid to soil pollution through the application of new laws, but the enforcement and the harmonization of these rules is still a crucial issue due to the immediate investments costs and to the high variability in the nature of polluted soils. Actually the polluted soil in Spain are regulated in 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. The environmental regulations are mainly focused by considering simultaneously the human health and economic, environmental and social factors, according to two basic principles in the environmental field: 1) the exposure levels reduction as low as reasonably achievable, 2) the prevention of conventional pollution through the best available techniques not entailing excessive costs.

### 1.2.1. Current perspective for polluted soil

The present treatment technology that involves physic-chemical and biological methods (natural attenuation) for cleaning up the soil is not efficient and/or effective to treat the pollutant to acceptable level and is in general very slow. So, according to the second principle, the major challenge is the treatment of polluted soil using environmentally friendly and efficient remediation technologies, considering the biotechnology as emerging science for environmental protection that involves the use of microorganisms for biological treatment of soil pollutants. The use and

manipulation of the detoxification abilities of living organisms is the main successful strategy to fight pollution (Lovley, 2003; Díaz Fernández, 2004). The most studied and the most frequently used for biodegradation strategies are bacteria. The bacteria are able to reduce, eliminate or transform the pollutants while their activity depends on the presence of many factors: specific microorganisms' existence, amount, combination, the availability of pollutants to the microbial population and the environmental condition (review section 1.1.2).

### 1.2.2. Bioremediation

Soil microorganisms have developed strategies for obtaining energy from virtually every compound and they play a crucial role in sustainable development of the biosphere and in biogeochemical cycles. Similarly, many microorganisms are able to use a wide array of organic pollutants as sole carbon and energy sources to gain energy for cell growth from the process. This ability of bacteria is related to the fact that most of these compounds are commonly present in the environment as a result of the recycling of plant derives material. These microorganisms are often very well adapted and they tend to utilize the soil nutrients and electron acceptors that are available. However, natural attenuation is generally slow due to the lack of suitable electron donors or acceptors that limits the microbial activity (Perelo, 2010) and the soil pollutants persist in waterlogged soil or sediment (Liu and Suflita, 1993). Furthermore, bioremediation strategies that are successful in one location might not work in another, and microbial processes that remediate pollutants in laboratory incubations might not function well in the field (Lovley, 2003).

Bioremediation is the use of living organisms, mainly microorganisms, to degrade the environmental pollutants into less toxic forms to human health and/or the environment. Bioremediation is a process that involves biotransformation and biodegradation. The first one is the alteration of the molecular or atomic structure of a compound by microorganisms and the second one is the breaking down of organic or bioaccumulation and

biotransformation of inorganic compounds into environmental friendly compounds (Head, 1998; Boopathy, 2000; Semple et al., 2001; Pandey, 2012). The biodegradation is often a result of the actions of multiple microorganisms that could be presented in the polluted soil or they could be imported to enhance the degradation (bioaugmentation). Ideally, bioremediation results in the complete mineralization of pollutants to $H_2O$ and $CO_2$.

Depending on the energy investment resulting from pollutants metabolism, the metabolic process could be classified as (Pandey, 2012): primary metabolism (the pollutant provides energy for cell maintenance and division), co-metabolism (the pollutant does not serve as a carbon and energy source and the cell obtains energy from another transformable compound).

The bioremediation process can be categorized in two groups: *ex situ*, that includes bioreactors, bio-filters, landfarming and composting methods; or *in situ*, that includes bioventing (oxygen in no saturated zone), bio-sparging (oxygen in saturated zone), bio-stimulation liquid delivery system and some composting methods. The *in situ* treatments require less equipment, so they have a lower cost and generate fewer alterations to the environment. For all this the *in situ* techniques tend to be more attractive (Boopathy, 2000).

### 1.2.3. Factors affecting microbial pollutants degradation

The factors that directly impact on bioremediation are energy sources (electron donors), nutrients, pH, temperature, inhibitory substrates or metabolites (Boopathy, 2000) and electron acceptors availability.

- The rate of pollutant degradation is generally not constant and dependent on the concentration of the pollutant (electron donors), the number of microorganisms able to metabolize the pollutant or the expression of specific enzymes by the cells. Bioremediation enhances the

rate of the natural microbial degradation due to the supply of additional microorganisms (bioaugmentation).

- The growth and activity of the microorganisms must be estimated by adequate maintenance and supply of nutrients: carbon, nitrogen and phosphorus, that are the most common molecules present in a cell (proteins, sugars and nucleic acids). Bioremediation enhances the rate of the natural microbial degradation by supplying of additional nutrients to soil (biostimulation) as humic acids (Lovley, 2000) or nitrates (Yu et al., 2014) that has been a common practice to remove organic pollutants, but this incurs extra cost and causes secondary pollution concerns (Pandey, 2012).

- Microbial activity is affected by a number of physicochemical environmental parameters like temperature, moisture and pH that affect directly on pollutants degradation by microorganisms. The temperature controls the rate of enzyme catalyzed reactions, and generally higher soil temperature results in higher microbial metabolic activity and higher rates of biodegradation. Moisture influences the solubility of materials that are available as well as osmotic pressure and pH, which affects the availability of nutrients.

Nevertheless, the presence of electron acceptors is indispensable to complete the respiration process.

### 1.2.4. Microbial respiration activity in bioremediation processes

The bioremediation process is based on the activities of aerobic or anaerobic heterotrophic microorganisms that is based on redox reactions, where a substrate is reduced and other one is oxidized. Although oxygen is the most common final electron acceptor for microbial respiration and aerobic processes provide the highest amount of energy to cells (higher oxidation states corresponds to higher energy yields)

microorganisms are able to use other final electron acceptors that provide less energetic incentive for microorganism degradation (Figure 1.2).

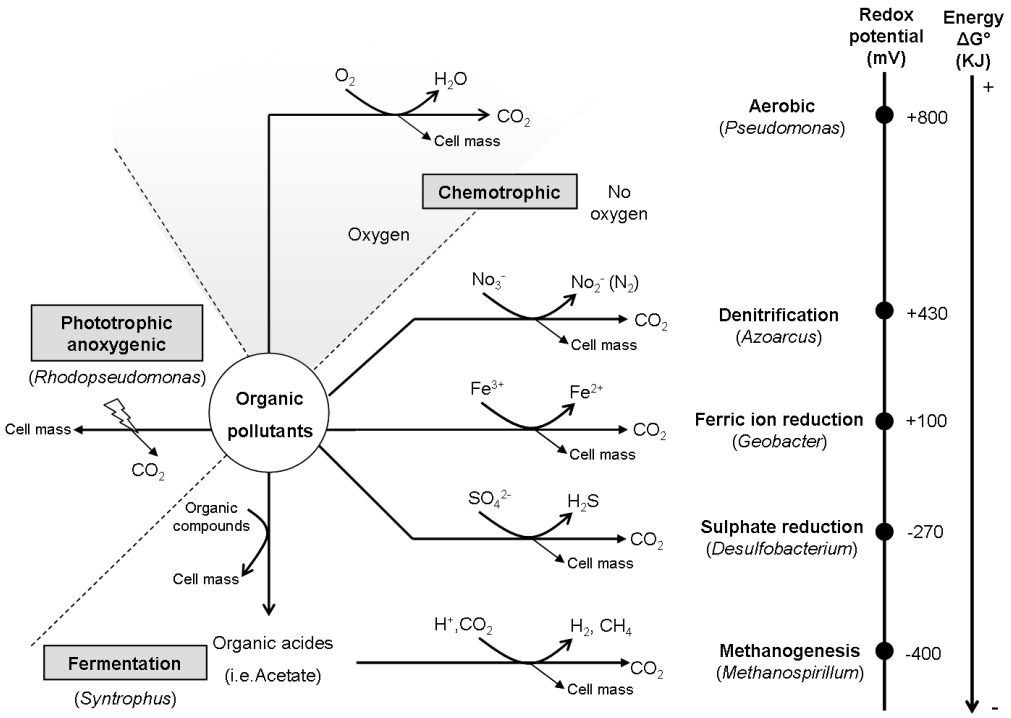



***Figure 1.2.*** Microbial utilization of pollutants by using different electron acceptors in respiration. Adapted from *Díaz Fernández, 2004.*

Surface soils have higher organic matter content and diversity of microbial populations, whereas those subsurface soils have lower levels of organic matter and bacteria become more dominant in the microbial community. This is attributed to the ability of bacteria to use alternative electron acceptors to oxygen (Boopathy, 2000). This is important because many polluted soil are often anoxic, e.g., submerged soils. In such environments, biodegradation is carried out by either strict anaerobes or facultative microorganisms using alternative electron acceptors (Gibson

and Harwood, 2002). The use of electron acceptors other than oxygen is based on: i) the availability and ii) the competition of different respiratory types of microorganisms for electron donors. These microorganisms use nitrate (denitrifying organisms), ferric iron (ferric-iron reducers), sulphate (sulphate reducers), $CO_2$ (methanogens) or other electron acceptors (chlorate, Mn, Cr, U, etc) for this anaerobic respiration (Widdel and Rabus, 2001; Singh and Ward, 2004).

**- Oxygen**

In aerobic respiration, oxygen no only is the electron acceptor but also participates in activation of the substrate via oxygenation reactions (Díaz Fernández, 2004). Although a wide phylogenetic diversity of microorganisms is capable of aerobic degradation of pollutants, *Pseudomonas* species and closely related organisms have been the most extensively studied owing to their ability to degrade so many different pollutants (Wackett, 2003). The addition of oxygen as electron acceptor (bioventing) in aerobic bioremediation process (Kabelitz et al., 2009; Frutos et al., 2010) has been a common practice to remove organic pollutants.

**- Nitrates**

Denitrification is a microbial respiratory process during which soluble nitrogen oxides are used as an alternative electron acceptor when oxygen is limiting. It results in considerable loss of nitrogen, which is the most limiting nutrient for crop production in agriculture (Philippot et al., 2007). This process has been thoroughly examined because of its importance in the global nitrogen cycle, the production of greenhouse gases and the removal of contaminants in the environment (Song and Ward, 2003). Denitrifiers use a wide variety of aromatic substrates under denitrifying conditions, such as benzoate and its hydroxylated derivatives, phenolic compounds, aromatic hydrocarbons, and halogenated benzoates, but not polyhalogenated aromatic (Song et al., 2001).

**- Sulphate**

It is a particularly important electron acceptor for the anaerobic degradation of pollutants in marine sediments owing to the high concentrations of sulphate in seawater. Its addition to groundwater can greatly accelerate pollutant degradation e.g. in aquifers (Anderson and Lovley, 2000). Sulphate-reducing microorganisms, such as *Desulfobacterium* species can oxidize hydrocarbons with sulphate as the electron acceptor.

- **$CO_2$**

Methanogenic bacteria are specialized in the breakdown of a limited number of substrates. These include $H_2/CO_2$, formate, acetate and a few other $C_1$-compounds like methanol, ethanol, isopropanol, methylated amines, methylated sulphur compounds, and pyruvate (Stams, 1994). The methanogens species are very varied and each specie is able to use a different substrate i.e. *Methanospirillum hungatei* grows on $H_2/CO_2$ and formate (Shen et al., 2016). Due to this restricted metabolism of methanogens, organic compounds are degraded in methanogenic environments by associations of fermenting (reduction of the organic compounds to organic acids), acetogenic (acetate, $H_2$ and formate formation) and methanogenic bacteria (methane production). Finally, via fermentative, syntrophic and methanogenic microorganisms, biomass can in principle be completely converted to methane and $CO_2$.

**- Fe (III) oxides**

It is often the most abundant potential electron acceptor for the oxidation of organic and metals pollutants in subsurface environments (Lovley et al., 1989; Lovley, 1991).

*Geobacter* species, which are representative of the family of Fe (III) reducers that predominate in a wide diversity of sedimentary environments, require direct contact with Fe (III) oxides in order to reduce them. In contrast, *Shewanella* (Tang et al., 2007) and *Geothrix* (Nevin and Lovley, 2002a) species produce chelators that solubilise Fe (III) and

release electron-shuttling compounds that transfer electrons from the cell surface to the surface of Fe (III) oxides not in direct contact with the cells.

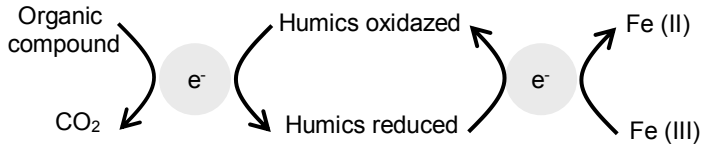



***Figure 1.3***. Role of humic substances as electron mediator in the reduction of Fe (III).

The rate of pollutant degradation coupled to Fe (III) reduction in aquifer sediments can be stimulated with the addition of compounds that make Fe (III) more accessible for microbial reduction such as: Fe (III) chelators (Nevin and Lovley, 2002b; Nevin and Lovley, 2002a), humic substances (Figure 1.3) and other extracellular quinones which shuttle electrons from the cell surface to the surface of Fe (III) oxides (McKnight et al., 2001; Bond and Lovley, 2002; Nevin and Lovley, 2002b; Stams et al., 2006).

For example in anthraquinone-2,6-disulfonate (AQDS), the quinone moieties in these compounds are reduced to the hydroquinone state that reacts abiotically with Fe (III) oxides, reducing it to Fe (II) and regenerating the quinone state of the molecule (Bond and Lovley, 2002), which can then undergo another cycle of reduction and oxidation.

*Geobacter* species have been also studied in the bioremediation of uranium-contaminated aquifer. U (VI) is the mobile valence state (soluble) of uranium while reduced uranium, U (IV), is insoluble as uraninite, preventing further downgradient spread of groundwater contamination (Anderson et al., 2003). A simple strategy for promoting U (VI) reduction is to add acetate as an electron donor to stimulate the activity of metal-reducing microorganisms that use the electrons from the oxidation of acetate to reduce U (VI) to U (IV).

### 1.3. Herbicide atrazine: a soil environmental problem

Atrazine (ATR) (Figure 1.4) is a member of chlorinated *s*-triazine group of herbicides, which is moderately mobile and highly persistent in the environment (Iriel et al., 2014).

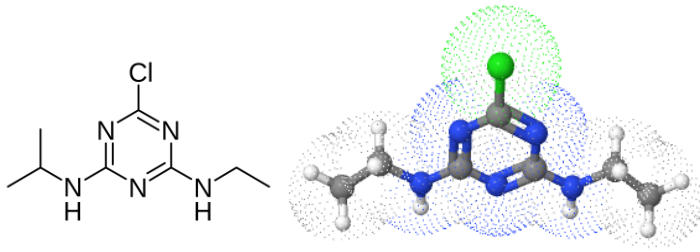



***Figure 1.4.*** Chemical structure of atrazine molecule

The Water Framework Directive (Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy) includes atrazine as one of 33 priority substances to monitor in European waters and although atrazine was banned in the European Union in 2003 (Bethsass and Colangelo, 2006) it is still widely used in other nations outside the European Union such as Brazil, China, India and Russia due to its low cost and high effectiveness for control of weeds in crops such as corn and sorghum. Over 3.4·104 *t* of ATR is applied each year in the USA (Sadler et al., 2014) and the expected amount of yearly atrazine usage in China in 2020 is 10820 *t* (Zhang et al. 2014).

#### 1.3.1. Environmental chemistry of atrazine

ATR is a white crystalline substance with an intermediate polarity, although it shows a low octanol/water partition coefficient (log $K_{ow}$ = 2.7) and a relatively high water solubility in water (33 mL·L$^{-1}$ at 27ºC). ATR is

usually applied in a water spray at concentrations of 2.2 to 4.5 kg·ha$^{-1}$ before weeds (Mudhoo and Garg, 2011).

***Table 1.1.*** Physical-chemical properties of ATR

| Property | Value |
|---|---|
| Molecular weight | 215.7 g·mol$^{-1}$ |
| Water solubility | 33 mg·L$^{-1}$ at 27ºC |
| Density | 1.23 g·mL$^{-1}$ at 22ºC |
| Vapour pressure | 2.89·10$^{-7}$ mm Hg at 25ºC |
| Octanol/water partition coefficient, logKow | 2.60 - 2.71 |
| Organic carbon/water partition coefficient, logKoc | 1.96 - 3.38 |
| Henry law constant | 2.96·10$^{-9}$ atm·m$^3$·mol$^{-1}$ at 25ºC |
| pKa | 1.68 |
| Melting point | 173 – 175 ºC |
| Boiling point | 200 ºC |

Some authors observed a high affinity of ATR to organic matter in soil systems (Binet et al., 2006; Correia et al., 2007) and its sorption to soils correlates positively with organic carbon content. Sorption of atrazine to organic matter lowers its bioavailability, which then increases its persistence despite its susceptibility to abiotic and biotic degradation (Mudhoo and Garg, 2011). Different physical and chemical properties of ATR are classified in Table 1.1.

Therefore often appears in ground and surface water, exposing a risk to human health. e.g. ATR concentrations in runoff waters from treated cornfields may exceed 740 µg·L$^{-1}$ (Graymore et al., 2001).

### 1.3.2. ATR as toxic compound

The toxicity of ATR is confirmed by the low allowable levels in water for human consumption (0.1 µg/L) and soil (0.2 mg/kg) established the European Union.

The herbicide was reported as an endrocrine-disrupting chemical, which could cause birth defects, reproductive tumors and weight loss. Thus, it is critical to eliminate residual ATR from environment. However, in spite of its recalcitrance recent studies have demonstrated that ATR biodegradation is feasible (Solomon et al., 2013).

Numerous studies have indicated that ATR inhibits growth and photosynthesis of freshwater algae and algal responses to ATR vary widely depending upon concentrations used, duration of exposure, and algal species tested (Tang et al., 1997; Weiner et al., 2004). Furthermore, ATR and other triazinic compounds have showed genotoxic and mutagenic actions in *Drosophila*, yeasts and plants, but not mutagenic actions in bacteria (de Campos Ventura et al., 2008). Recently, ATR and its chlorometabolites were reported to have the potential to influence the development and behaviour in the early life stages of zebrafish still remain unclear (Liu et al., 2016).

Several authors have already studied changes in the yield of chlorophyll fluorescence in the presence of herbicides. Conrad *et al* (1993) found that some triazines and derivatives of phenylurea produced variations in the yield of the *in vivo* fluorescence for PSII chlorophyll-a (Conrad et al., 1993; Iriel et al., 2014).

### 1.3.3. ATR degradation

In soils, ATR degradation is mainly a result of microbial activity. It could be biodegraded by either single functional bacterium or microbial consortium (Tortella et al., 2013). The biotic degradation of ATR can follow several metabolic pathways (Figure 1.5) that involve stepwise transformation mediated by individual strains or microbial consortia.

Among bacteria, there are reports on ATR degradation by individual strains such as *Pseudomonas* sp ADP (de Souza et al., 1998), *Agrabacterium radiobacter* J14a (Struthers et al., 1998), *Arthrobacter* sp.

.C3 (Wang et al., 2015), *Acinetobacter* spp. (Singh et al., 2004) or *Nicordioides* (Topp et al., 2000) that degrade this herbicide through co-metabolic processes that lead to the formation and accumulation of ATR metabolites and have been successfully isolated from agricultural soils, industrial wastewater, and other ATR polluted environment.

*Pseudomonas* sp. ADP (Figure 1.5) is the best-characterized bacterial strain capable to degrading the herbicide ATR through a catabolic pathway that contains six enzymatic steps (AtzABCDEF). This capacity has been shown to be widespread and plasmic borne in a number of bacterial isolates where the operon atzABC was present (de Souza et al., 1998; Sene et al., 2010). The cleavage of the cyanuric acid to carbon dioxide and ammonia (assimilated as a nitrogen source) are coded by operon atzDEF genes. The ATR metabolic pathway proceeds via three consecutive hydrolytic reactions that result in the formation of hydroxiatrazine (HA) when the chlorine is replaced with a hydroxyl group (hydrolytic dechlorination, catalysed by the enzyme atrazine chlorohydrolase, AtzA), N-isopropylammelide (hydrolytic deamidation catalysed by the enzyme ydroxyl-atrazine ethylaminohydrolase, AtzB) and cyanuric acid (hydrolytic deamidation catalysed by the enzyme N-isopropylammelide isopropylaminohydrolase, AtzC) (de Souza et al., 1998; Smith et al., 2005). Consecutively the atzDEF operon encodes cyanuric acid amidohydrolase (AtzD), biuret amidohydrolase (AtzE), and allophanate hydrolase (AtzF), involved in cleavage of the cyanuric acid to the complete mineralization to $CO_2$ and $NH_3$, which is assimilated as a nitrogen source.

Several atrazine-degrading bacteria in microbial consortia have been isolated and studied, revealing the presence of atzABC and atzDEF genes (Fang et al., 2015). E.g. *Nocordia* sp. is able to dechlorinate atrazine through the TzN enzyme (atrazine chlorohydrolase). Following dechlorination, the resulting hydroxyatrazine was afterwards degraded in N-isopropylammelide by *Rhyzobium* sp. which contained the gene atzB, and N-ethylammelide by *Nocordia* sp. which dealkylates hydroxyatrazine

to form N-ethylammelide (Figure 1.5) (Smith et al., 2005; Bouquard et al., 1997). However, none of these microorganisms showed to carry atzDEF genes. Other studies demonstrated that cyanuric acid may be transfer to biuret through *Arthrobacter* sp. by AtrzD (Fang et al., 2015) or directly to urea through *Rhizobium sp*. by TrzD. The presence of trzD (also present in other species as *Pseudomonas putida*) and ureasa (present in *Agrobacterium tumefaciencs*) suggests that mineralization of cyanuric acid proceeds through biuret rather than an allophanate intermediate (Cook et al., 1985).

In anaerobic systems, ATR is reported to be more recalcitrant to degradation; however *Pseudomonas* sp. ADP was shown to mineralize ATR under both aerobic and denitrifying conditions (Katz et al., 2000; Clausen et al., 2002). Some bacteria are able to use ATR as the sole carbon and nitrogen source under anoxic conditions, and it is the case of the strain *D. acidovorans* D24 that was been isolated from a sediment (Vargha et al., 2005). In these cases the atrazine biodegradation pathway seems to follow *N*-dealkylation and dechlorination processes (Kaufman and Blake, 1970; Fang et al., 2015) resulting in the formation of the most commonly observed compounds from ATR degradation: hydroxiatrazine (HA), desethylatrazine (DEA), deisopropylatrazine (DIA), deethylhydroxyatrazine (DEHA), and deisopropylhydroxyatrazine (DIHA). ATR-degrading strains were isolated from sediment of Danubio River, showing diverse ATR metabolism, leading to dealkylated (desethylatrazine, deisopropylatrazine, didealkylatrazine) and dechlorinated (deethylhydroxyatrazine, deisopropylhydroxyatrazine) metabolites that were transformed into ammeline and ammelide products (Figure 1.5). These metabolites are more easily degraded than ATR and HA, so subsequent mineralization could be performed by an adapted microbial consortium (Vargha et al., 2005). Thereby several bacterial isolates belonging to the genus *Rhodococcus* dealkylate and dechlorinate ATR but are unable to cleave the ring (Behki et al., 1993), but it can be completely degradable by the combined action of the *Pseudomonas* and *Rhodococcus* (Shao et al., 1995).

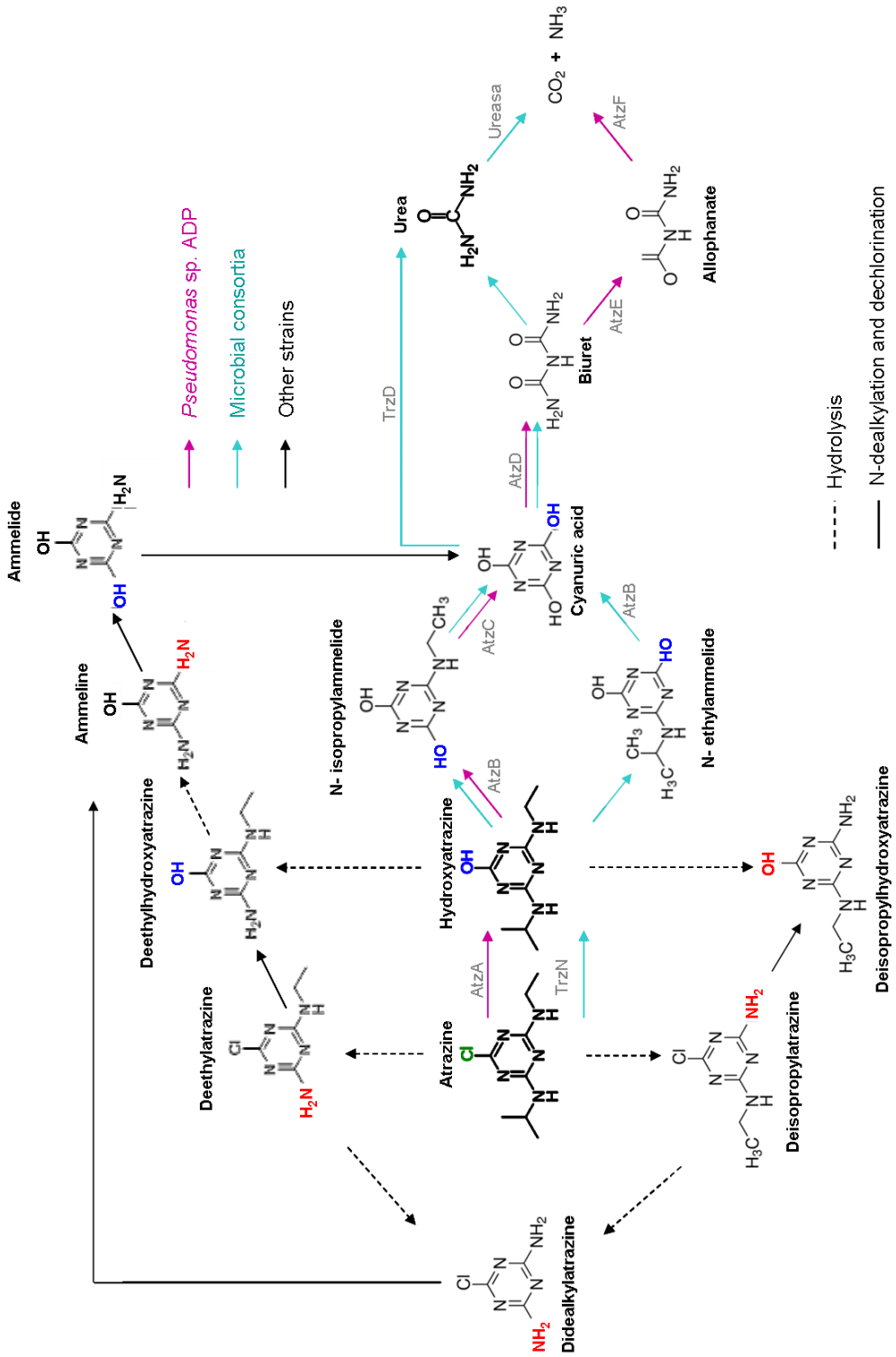



***Figure 1.5.*** Proposed metabolic pathways of ATR biodegradation

### 1.3.4. ATR transport in soil

- The movement of atrazine in soils is governed by two distinct and different processes: slow transport through the soil matrix and rapid movement through macropores (Mudhoo and Garg, 2011).

The first one is controlled by sorption kinetics and degradation reactions. Sorption term refers to the full range of processes whereby matter is partitioned between the gas, aqueous and solid phase (air-water interface and mineral or organic matter of soil).

The total organic carbon content plays a critical role in the sorption of organic pollutants and the sorption of hydrophobic organic compounds (HOC) to soil colloidal material is an important process when considering colloid-facilitated transport. However, depending on the organic carbon composition, the $K_{oc}$ (partition coefficient that indicates the sorption of atrazine to organic compounds in soil) it will be affect, e.g. humic acid characterized by higher values of $K_{oc}$ compared to fulvic acid (Mudhoo and Garg, 2011). However, the adsorption of ATR on humic substances changes considerably with the soil Ph (Wang et al., 2011) and both are inversely correlated due to ATR is easily protonated at Ph ≤ 4.
The adsorption of atrazine to humic substances is also decreased with the increase of soil salinity, whereby sodium and chlorine interact with these substances and change the organic matter composition and structure (González-Márquez and Hansen, 2009).

Pesticides and herbicides like ATR can interact with the surface sites through electrostatic interactions, ionexchange reactions or by surface complexation, and for these molecules sorption to mineral surfaces may be significant (Clausen et al., 2001). A large sorption of ATR to organic matter reduces its bioavailability for microorganisms (Binet et al., 2006). So thus, when the herbicide is irreversibly bound to a soil or when its

desorption is very slow, then its mobility become negligible and ATR mineralization is minimized.
Many substances with a large surface area, e.g. smectites, contain cations to provide electroneutrality. Eventually these cations are generally available to participate in cation exchange reaction unless they are large polyanions of aluminium or iron. The adsorption of ATR in these substances decreases by increasing temperature (Kovaios et al., 2006).

- The second one is controlled by hydraulic conductivity and the water retention capacity.

The hydraulic conductivity depends on soil texture, so that water infiltration is faster in sandly soil than in clayey soil, what influences the speed of ATR transport (Müller et al., 2012).

The soil water retention capacity and drying-rehydrating cycles can affect the migration of ATR in soil due the change in the organic matter structure (Hosse and Wilkinson, 2001).

## 1.4. Environmental microbial electrochemistry

### 1.4.1. New paradigm: a conductive material as electron acceptor

The ability of certain microorganisms to transfer electrons to insoluble iron resulted key for studying the redox reactions between environmental microorganisms and conductive materials. The first electronic activity by microorganisms has been described more than 100 years ago by Potter (1910) who showed that *E.coli* cultures could generate certain electric current in presence of platinum electrodes. In 1987, Derek Lovley (Lovley et al., 1987) discovered *Geobacter* family in sediment from Potomack River, that eventually became the model electroactive microorganisms. Nevertheless, many environmental microorganisms can establish a direct

electrochemical communication with an electrode (Lovley, 2006). All these discoveries resulted in the *Microbial Fuel Cell* (MFC) concept that can be distinguished from Fuel Cells (FC) or batteries by the fact that at least one of the reactions in MFC is catalysed by a biological component to convert chemical energy into electrical energy. These organic fuel sources cannot be used in currently conceived abiotic fuel cells because, unlike hydrogen or methanol, these fuels are not electrochemically active. However, microorganisms can catalyse the release of electrons from organic matter and transfer them to various electron carriers that are electrochemically active (Lovley, 2006).

The MFC (Figure 1.6) was the very first Microbial Electrochemical Technology (MET) (Schroder et al., 2015) to be explored and consists of two compartments, the anodic and cathodic chamber separated by a proton exchange membrane (PEM).

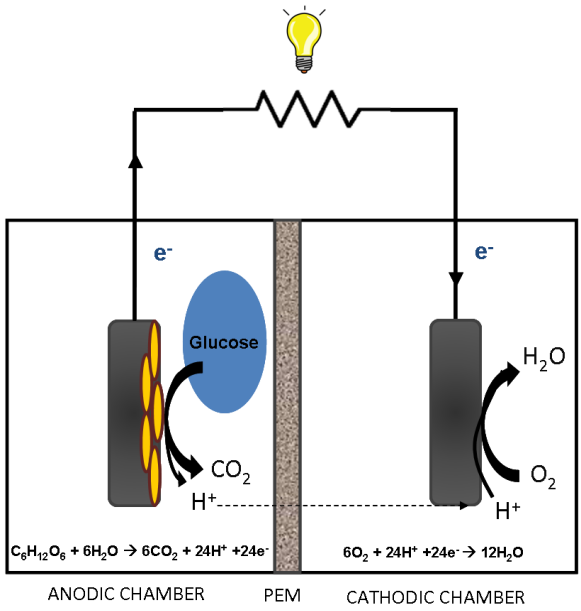



***Figure 1.6.*** Microbial Fuel Cell (MFC) scheme.

Each compartment hosts an electrode: the anode, which accepts electrons from microbial culture, and the cathode, which transfers electrons to an electron acceptor, typically oxygen for most perceived practical applications. The electron transfer from the anode to the cathode is conducted by an external electrical connection that typically includes a resistor, and the protons resulting from the degradation of the organic matter reach the cathode through the PEM membrane. The anodic compartment is typically maintained under anoxic conditions, whereas the cathodic chamber can be aerated since oxygen reacts with the protons and electrons from cathode to form water (Logan, 2009).

MFCs offer the possibility of extracting over 90% of the electrons from some organic compounds (e.g. acetate), but they are not commonly considered a part of the energy portfolio for the future due to the technology is not yet sufficiently developed to produce substantial quantities of power in a cost-effective manner.

### 1.4.2. Harvesting energy from the environment: the sediment microbial fuel cell

The concept of MFC requires a suitable supply of organic matter to proceed with the energy production, a situation that actually occurs in the environment where nutrients cycle operate. So the first step in the modern history of electrochemical microbiology was precisely the application of microbial fuel cells in ecological water bodies as 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) in order to harvest energy provided by natural bacteria populations that not require the addition of any exogenous microorganisms or electron shuttles. These devices were called Benthic or Sediment Microbial Fuel Cells (BMFCs or SMFCs) (Reimers et al., 2001). The anode is buried in the anoxic sediment and connected through an electrical circuit to a cathode in the overlying water layer (Figure 1.7). In this case, the interface between sediment and water column replaces the PEM from classical

MFC. The first practical device to be powered by SMFC technology was reported in 2008 by Tender *et al*. (2008). The power production was in the range of 50-100 mW/m$^2$ (Lowy et al., 2006; Dumas et al., 2008) due to the advantage of low ohmic internal resistance in these saline environments for the high concentration of salts.

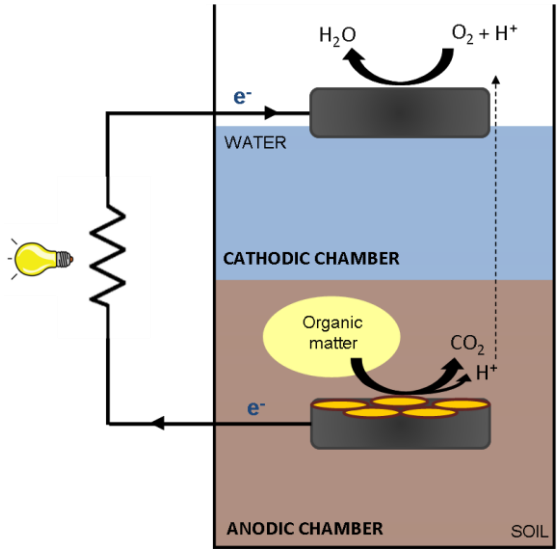



***Figure 1.7.*** Sediment microbial fuel cell (SMFC) scheme.

Freshwater environments can also produce electrical current, although lower output power values have been reported (ca.10−30 mW/m2), 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). Therefore, an expansion of the research focus is warranted in order to engineer SMFCs to perform as well in freshwater environments with low salt concentrations (De Schamphelaire et al., 2008), where simultaneously is created the opportunity for harvesting energy and the oxidation of sediment organic matter by two biologically mediated cathodic reactions respectively employing an oxygen reduction and a manganese cycle. Both reactions imply a low cost and a high electrode potential.

Other variations of SMFCs have been deployed in freshwater natural environments, such as rivers (Sacco et al., 2012; Wang et al., 2012a; Sajana et al., 2014). Sacco *et al*. (2012) used an indigenous microbial community contained in anaerobic sediments and two types of graphite materials that differ mainly in shape and size. The maximum power density observed was 20 and 9 $mW/m^2$ using rod and graphite disk electrodes respectively. The difference obtained between both electrodes may be due to a more efficient mass transport on the surface of the rod electrode. Wang *et al*. (2012) developed three dimensional floating biocathode (FBC) of graphite granules in order to avoid negative effect of dissolved oxygen (DO) depletion in aqueous environments. The maximum power density achieved was 1 $W/m^3$ and a 29% of the organic content was efficiency removed after 120 days operated. More recently Sajana *et al.* (2014) studied the effect of cellulose content in the aquaculture pond bottom sediment on performance of SMFC employed for in situ remediation of aquaculture water.

An additional environment to test SMFCs was the rice crop soil by means of using the root secretions as source of organic substrates (Kaku et al., 2008; Busalmen et al., 2010; Takanezawa et al., 2010; Chen et al., 2011; Chiranjeevi et al., 2012). Kaku *et al*. (2008) developed SMFCs at real scale using paddy fields with rice plants. They observed that resulting electricity (6 $mW/m^2$) was sunlight dependent and exhibited circadian oscillation, suggesting that the paddy field electricity generation system was an ecological solar cell in which the plant photosynthesis was coupled to the microbial conversion of organics to electricity. Chiranjeevi *et al.* (2012) evaluated the electrochemical activity to observe that current was inversely correlated with the varying anode distance from root in rhizosphere.

In addition, a number of substrates (e.g. vermiculite, graphite granules) and plant-growth medium have been used to support plant-MFCs (P-MFC) in soil-free applications (De Schamphelaire et al., 2008; Helder et al., 2010; Timmers et al., 2010; Arends et al., 2012; Helder et al., 2012).

The distance between the cathode and the anode in an SMFC often leads to changes in internal resistance, with a larger distance resulting in greater resistance. Additionally, the low conductivity of liquid medium difficult the ion transport from anode to the cathode and consequently the SMFC performance and the power output. Chen *et al*. (2011) reported fhe first cathode buried in a soil environment with an oxidizing redox potential. This was possible allocating the cathode in the vicinity of rice rhizodeposits where the oxygen secretion by the roots generates a microaerobic environment oxidizing (Figure 1.8).

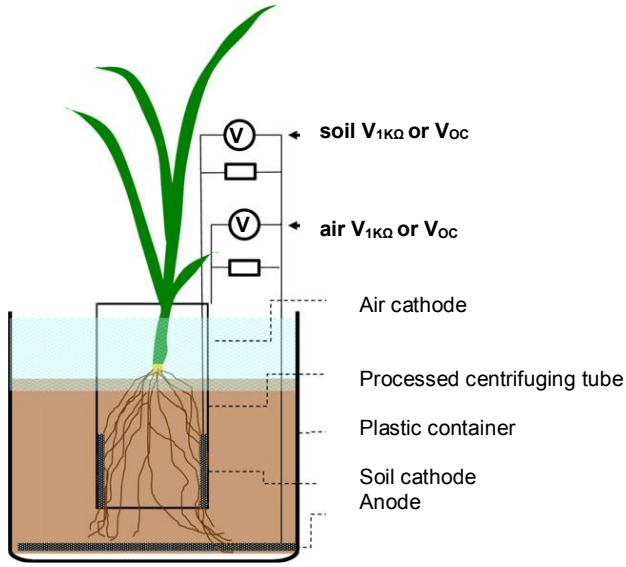



***Figure 1.8.*** Section scheme of the experimental setup of the plant-MFC with air cathode and soil cathode. The anode was a round graphite mat. Two different cathode configurations (air cathode and soil cathode) were set up. $V_{1K\Omega}$: the voltage between two electrodes with 1000 Ω resistor; $V_{OC}$: the voltage between two electrodes without resistances, open circuit. Extracted from *Chen et al. (2011).*

In spite of the low power generation by SMFC, it has been demonstrated that this system can be successfully use to power low-energy electronic devices in aquatic ecosystems (Donovan et al., 2008; Zhang et al., 2011; Thomas et al., 2013).

### 1.4.3. A novel tool for enhancing the biodegradation of soil pollutants

On top of that, one of the most promising SMFC application might be environmental bioremediation (Thrash and Coates, 2008). Zhang *et al.* (2010) proposed for first time a new concept for bioremediation of the terminal electron acceptor based on a conductive graphite electrode that could serve as an electron acceptor for the degradation of toluene and benzene in polluted slurries. Since then, several studies have reported the biodegradation enhancement of pollutants of different chemical nature (Table 1.2): PAHs (Huang et al., 2011; K. Chandrasekhar, 2012; Morris and Jin, 2012; Wang et al., 2012b; Yan et al., 2012; Lu et al., 2014; Rodrigo et al., 2014; Zhang et al., 2014; Li and Yu, 2015; Li et al., 2015; Sherafatmand and Ng, 2015; Daghio et al., 2016; Li et al., 2016; Venkidusamy et al., 2016), pesticides (Liang et al., 2014; Cao et al., 2015), chlorinated organics (Chun et al., 2013; Liu et al., 2013; Yu et al., 2016) and herbicides (Rodrigo Quejigo et al., 2016). Moreover, not just pollutant removal but effective clean-up was demonstrated by ecotoxicological analysis of a DBT-polluted soil after SMFC treatment (Rodrigo et al., 2014). Rodrigo *et al.* (2014) proposed the name of Microbial Electroremediating Cell (MERC), because this novel approach is based on the use of a SMFC variant to maximize bioremediation and not to harvest energy from soil. In this thesis MERC concept is used to refer the implementation of microbial electrochemical devices in soils for enhancing the biodegradation of soil pollutants.

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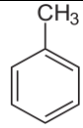
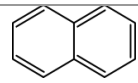
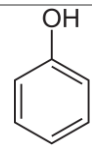
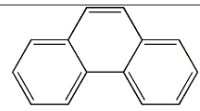
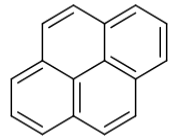
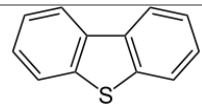
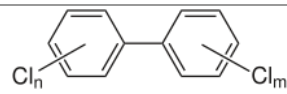
The MERC configuration is a key factor for enhancing pollutant biodegradation in sediments or soils. A significant effort has been devoted to the study of the anodic and cathodic material and the distance between

them in real environments for its application. For example Morris and Jin (2012) demonstrated that this technology can be applied to environments with either aerobic or anaerobic overlying water and an anaerobic matrix, such as shallow lagoon, ponds, and marshes, and groundwater. This conclusion was based on the use of a wicking air cathode that maintained dissolved oxygen concentrations 1-2 $mg \cdot L^{-1}$ higher than submerged cathodes. Zhang *et al*. (2014) studied the influence of anode arrangement (horizontal or vertical) in polluted soil for enhancing the efficiency degradation of TPHs. They concluded that SMFCs with anodes horizontally arranged were an effective design over vertically arranged systems to improve remediation efficiency of TPHs. 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.

However, performance of such systems can be limited by the inefficient mass transport in soil. Thereby, since 2012 a few studies have been developed to decrease the soil resistivity by modifying the soil moisture from 23% to 33% (Wang et al., 2012b), and soil structure with sand (Li et al., 2015) and glucose (Li et al., 2016) amendment. The higher moisture of soil decreased the internal resistance (from 42.6 to 7.4 Ω), and thus improved the current output and the removal rate of petroleum hydrocarbons. Sand particles enlarged pores of soil and provided more channels for ion and substrate transport that resulted in a higher remediation performance. Glucose was added as a co-substrate for simultaneous electricity generation and petroleum hydrocarbon degradation in air-cathode MFCs, the degradation of hydrocarbons as well as changes in bacterial community were investigated.

***Table 1.2.*** Studies reporting biodegradation of pollutants of different chemical nature: PAH (Polycyclic Aromatic Hydrocarbons), COC (Chlorinated Organics Compounds), P (Pesticides), H (Herbicides).

| Pollutant | Structural formula | Category | Reference |
|---|---|---|---|
| **Toluene** |  | PAH | Zhang et al., 2010<br>Daghio et al., 2016 |
| **Benzene** |  | PAH | Zhang et al., 2010 |
| **Naphthalene** |  | PAH | Zhang et al., 2010<br>Sherafatmand and Ng, 2015 |
| **Phenol** |  | PAH | Huang et al., 2011 |
| **Phenanthrene** |  | PAH | Yan et al., 2012<br>Sherafatmand and Ng, 2015 |
| **Pyrene** |  | PAH | Yan et al., 2012 |
| **Dibenzothiophene (DBT)** |  | PAH | Rodrigo et al., 2014 |
| **Total Petroleum Hydrocarbons (TPH)** | | PAH | Chandrasekhar and Mohan., 2012<br>Morris and Jin, 2012<br>Wang et al., 2012b<br>Zhang et al., 2014<br>Lu et al., 2014<br>Li et al., 2015<br>Li et al., 2016<br>Venkidusamy et al., 2016 |
| **Polychlorinated biphenyl (PCB)** |  | COC | Chun et al., 2013<br>Li and Yu, 2015<br>Yu et al., 2016 |

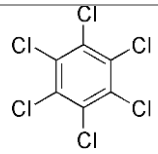
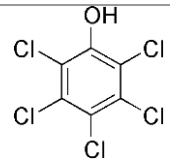
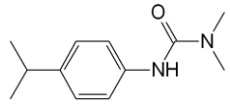
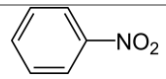
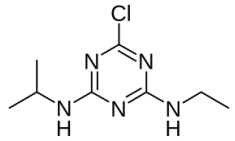
| | | | |
|---|---|---|---|
| **Hexachlorobenzene** |  | COC | Cao et al., 2015 |
| **Pentachlorophenol** |  | COC | Liu et al., 2013 |
| **Isoproturon** |  | P | Rodrigo et al., 2016 |
| **Nitrobenzene** |  | P | Liang et al., 2014 |
| **Atrazine** |  | H | Dominguez Garay A., et al (this thesis) |

***Table 1.2.*** (continued)

Other alternative to improve the efficiency of MERC in the degradation activity by microorganisms is to modify the electrode potential. The redox gradient in MERC is established spontaneously across the soil-water interphase as a result of spatially segmented reduction-oxidation reactions establishing an electron transport route between electrodes (Li and Yu, 2015). This electrode potential is typically negative and can result insufficient for leading to an effective transformation of recalcitrant compounds due to the high ohmic internal resistance of the system. To overcome this limitation, several studies have applied an external voltage between anode and cathode setting a most favourable redox scenario for soil microorganisms (Aulenta et al., 2007; Schrotta et al., 2011; Chun et al., 2013; Yu et al., 2016). Chun *et al*. (2013) studied the effect of applying low voltages (1.5 - 3V) on the microbial transformation of PCBs. The results indicate that both oxidative and reductive microbial transformation

of the spiked PCBs was stimulated (40-60%) but oxidation was dominant and most effective with higher voltage, where chlorobenzoates metabolites degradation was also enhanced. Yu *et al*. (2016) demonstrated that bioanode stimulation by applying a potential of 0.2 V substantially accelerated the dechlorination of PCB 61 (57%) as compared to the control experiments non-artificially polarized (2.3-fold lower). Analysis of the bacterial community showed significant community shifts in response to variations in treatment, resulted in substantially increased abundance of *Actinobacteria*, *Bacteroidetes*, and *Chloroflexi* either capable of PCB dechlorination. More recently, Rodrigo *et al.* (2016) even reported how certain electrode potential show a strong impact in the mineralization of $^{14}$C-isoproturon herbicide. The study concluded that using electrodes at a positive potential (+600 mV versus Ag/AgCl) enhanced the isoproturon mineralization by 20-fold respect the electrode-free control.

### 1.4.4. The electrochemical analysis of microbial electrochemical systems

#### 1.4.4.1. Polarization and power curves

The performance of MFCs is characterized by polarization and power curves and these are obtained by different methods. A polarization curve is a linear sweep voltammetry (LSV) which is commonly used in MFC studies to obtain polarization data (Watson and Logan, 2011). However it has been reported that high scan rates (speed of potential change per unit of time, $mV \cdot s^{-1}$) can overestimate power production (Watson and Logan, 2011). An alternative method consists in varying the electrical resistance of the external circuit at fixed time intervals, ranging from 10 s to 24 h (Benziger et al., 2006).

**The *polarization curve*** (A in Figure 1.9) is helpful in explaining the chemistry and physics associated with fuel cell operation, and it is used to characterize power production, where:

*E, potential* (*volts*, V): is the driving force for the reaction.

$$\Delta E = E_C - E_A - I \cdot R_{internal} = (E_{eqC} + \eta_C) - (E_{eqA} + \eta_A) - I \cdot R_{internal}$$

$\eta_C$ and $\eta_A$ are the overpotentials of the cathode and the anode respectively, and the $I \cdot R_{INT}$ term includes all ohmic losses that are proportional to the generated current ($I$) and ohmic resistance of the system ($R_{INT}$).

*I, current* (*amperes*, A): Is the flow of electric charge or the rate of chemical reaction in the fuel cell. Generally it is measured or calculated from Ohm's law as $I = V/R_{ext}$.

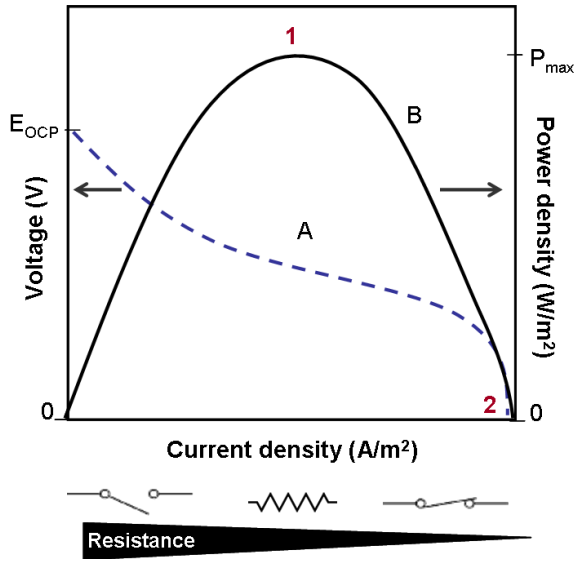



***Figure 1.9*** Representation of polarization (discontinuous line, A) and power (continuous line, B) curves. Point 1 corresponds to the maximum power generation by a MFC system. The point 2 corresponds to the maximum current intensity (short-circuit condition) to obtain the maximum degradation rate in MERC. The external resistance is decreasing from left to right of the curve.

The maximum difference of potential in polarization curve ($E_{OCP}$) corresponds to the difference between cathode and anode potentials in

open circuit (OC). In this point there is no current flow (I=0) because cathode and anode are not connected, therefore $R_{ext} = \infty$ and the ohmic losses ($I \cdot R_{INT}$) are 0.

The minimum voltage in polarization curve corresponds when cathode and anode are connected in short-circuit, therefore $R_{ext} = 0$.

***The power curve*** (B in Figure 1.9), describes the power as the function of the current and it is calculated from the polarization curve (Erable et al., 2011):

*P, Power* (*Watts*, W) is calculated from the measured voltage (V) and current (I) as:

$$P = V \cdot I$$

That considering the Ohm's law it can be calculated as:

$$P = V^2/R_{ext} = I^2 \cdot R_{ext}$$

The significant point in the power curve is the maximum power ($P_{max}$) of the system that corresponds to the top of the curve.

The representation of polarization and power curves is important for operating in the optimal point depending on the objective of microbial electrochemical devices.

For energy generation, the optimal functioning point for an MFC is the point of maximum power (1 in Figure 1.9). In contrast, it has been rarely reported that, with the objective of optimizing the degradation rate for operating as MERC, the optimal functioning point should be at short-circuit (2 in Figure 1.9).
At maximum voltage, both power and current production are null, a scenario that is possible when the external resistor is infinite. Decreasing

the resistor leads to a voltage decrease and then a current increase resulting in a bell-shaped curve. When the internal and external resistances are the same the system is operating at the maximum power point. A short-circuited MFC provides the highest currents, meaning that it ensures the highest rate for organic matter oxidation although is capacity for generating useful power is null. At this point the external resistor is very low comparing with the internal resistance of the MFC. If the power *versus* current bell-shaped curve were perfectly symmetrical, shifting from the maximum-power point to the maximum-current point then the rate of biodegradation would be doubled (Erable et al., 2011). However the symmetry of the power curve can be broken owing to a "power overshoot" that refers to the response of the device at high current densities where the cell voltage and current drop very quickly resulting in a doubling back of the power density curve (Watson and Logan, 2011). The overshoot resulted from a rapid increase in the anode potential as a resistance response to a decrease in current flow. This indicates an electron transfer limitation at the anode that is related to a slow response from the microbes to adjust to the new resistance.

In a conventional MFC the ohmic losses are due to the limitation in the electrons and protons transport, mainly as a consequence of the resistance from the electrodes, membrane, electrolytes or distance between anode and cathode. For the past 10 years, several studies have been focused on the study of anodic and cathodic materials to increase the efficiency of MFC. Nevertheless the internal resistance plays an important role in the performance of SMFCs that operate in soil. Unlike the MFC operating in liquid media or sediments with a high electrical conductivity, the soil is the barrier that ions must overcome in order to reach the SMFC cathode. Due to the lack of moisture or salts, the physical properties and high electric resistance of soil can restrain the transport of ions to the cathode and consequently limit SMFC performance. Some studies have shown the relationship between soil organic matter and nutrients (Dunaj et al., 2012), and the effect of

sediment pre-treatment on the performance of SMFCs (Song and Jiang, 2011). Other studies have focused on understanding what happens at the soil matrix, analyzing the influence of the soil internal resistance on SMFC efficiency, changing the anode-embedded in the sediment (An et al., 2015), and modifying the cathode set up by in order to capture the released oxygen from rice roots (Chen et al., 2011).

Despite all efforts to improve the efficiency of MERC, its application has been conducted under waterlogged conditions and little efforts have been devoted to the study of the physical chemistry of the soil affecting the performance of soil-MFC. Furthermore, the importance of water content in the OCV of SMFCs has been reported (Chiranjeevi et al., 2012) and all studies about SMFC applications were conducted under waterlogged conditions. This set-up requires a water layer on the surface or, at least, a certain moisture condition in soil to allow ions mobility. This is a limitation for the application of these systems in real contaminated soil as agricultural areas where flooding is not an option. *Chapter 5* of this thesis offered a practical solution in that sense. Some studies reported alternative SMFC configuration to decrease the high resistivity on low water content sediments by using a tubular air-cathode MFC (TAC-MFC) (Yuan et al., 2010) where anode and cathode are very closed (0.02 cm electrode spacing) and electrolytically connected with a catalytic and waterproof layer that replace the ionic exchange membrane present in standard MFC (Zhuang et al., 2009). However the poor permeability of sediments limited the proton transfer rate, resulting in an acidification of the anode chamber (Wang et al., 2012b). Another original configuration was based on a U-tube air cathode soil MFC system by inserting a hollow membrane electrode assembly (MEA). MFCs were filled with polluted soils under different water contents (Wang et al., 2012b).

#### 1.4.4.2. Cyclic voltammetry

Another tool for characterizing the soil redox reactions is by performing a cyclic voltammetry (CV) to identify e.g. electrochemical reduction and

oxidation of chemicals in the electrochemical cell. Usually, CV is the first experiment performed in an electrochemical study of a compound, a biological material, or an electrode surface (Kissinger and Heineman, 1983).

CV is a standard tool in electrochemistry and has regularly been exploited to study and to characterize the electron transfer interactions between microorganisms or microbial biofilms (Fricke et al., 2008; Harnisch and Freguia, 2012). It consists in a cyclic potential sweep that requires three-electrode set-up: a working electrode (WE), a reference electrode (RE), and a counter electrode (CE). Using this set-up, a current–potential polarization curve can be recorded using a potentiostat for controlling the voltage between the WE and the RE and for measuring the current flow between the WE and CE. A cyclic voltammetry scan starts at initial potential ($E_1$) that usually it coincides with the open circuit potential ($E_{OCP}$), proceeds to the final potential ($E_2$) and then proceeds back to other potential one ($E_3$) with a certain scan rate (Figure 1.10) thus the flowing current is recorded (Harnisch and Freguia, 2012).

By recording cyclic voltammograms at different stages of biofilm formation and substrate availability like substrate excess (turnover) and substrate limitation (non-turnover). The current production under this cyclic sweep can provide valuable information for understanding the microbial redox activity. For example, when the voltammograms are obtained from pure cultures like *G.sulfurreducens* at the maximum of the bioelectrocatalytic activity, a typical sigmoidal shape under turn-over conditions is obtained. So, when the mass transfer is not limited we can also observe a limiting current that doesn't change with the potential (Strycharz et al., 2011).

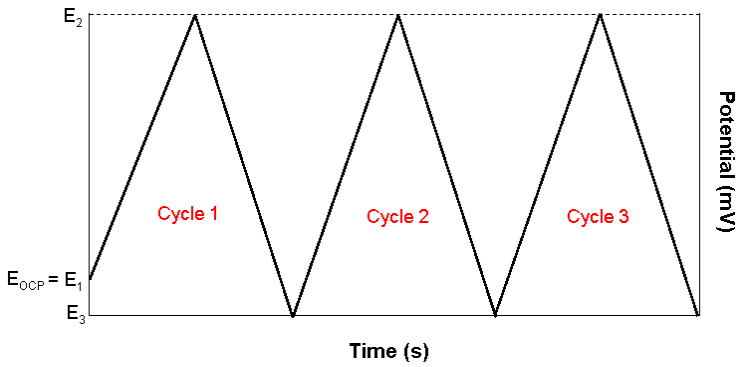



***Figure 1.10.*** Cyclic voltammetry waveform.

The height of the bioelectrocatalytic current strongly correlates with the peak currents measured under substrate depletion (Fricke et al., 2008). This indicates that the increase of the electrocatalytic biofilm activity is either caused by a growing cell density at the electrode surface, or by an increase in the number of membrane bound electron transfer proteins in the individual cells. Interestingly, not just anode reaction but also biological catalysis of the oxygen cathodic reduction can be verified by CV (Cournet et al., 2010).

The scan rate selected for operating de CV is a fundamental parameter to consider. In the case of soil-MFC the mass transfer is very limited due to the high resistivity that presents the soil. Thereby the CV must be operated at the minimum scan rate where the capacitive current (the current related to the change in the electrode surface charge, not related to an oxidation/reduction reaction) doesn't mask the faradaic response (the current generated from the oxidation, positive current or reduction, negative current , of chemical species) (Harnisch and Freguia, 2012).

#### 1.4.4.3. Chronoamperometry

Chronoamperometry (CA) is another electrochemical tool where the working electrode acquires a constant potential and the resulting current

from the oxidation or reduction of a compound on the surface of the working electrode is recorded as a function of time.

The applied potential depends on the experimental goal. When the objective is oxidizing the soil pollutants or other organic compounds, the polarization potential must be more positive than the OCP for the working electrode. In contrast, the polarization potential must be more negative when the goal is promoting the reduction at the working electrode (Babauta et al., 2012).

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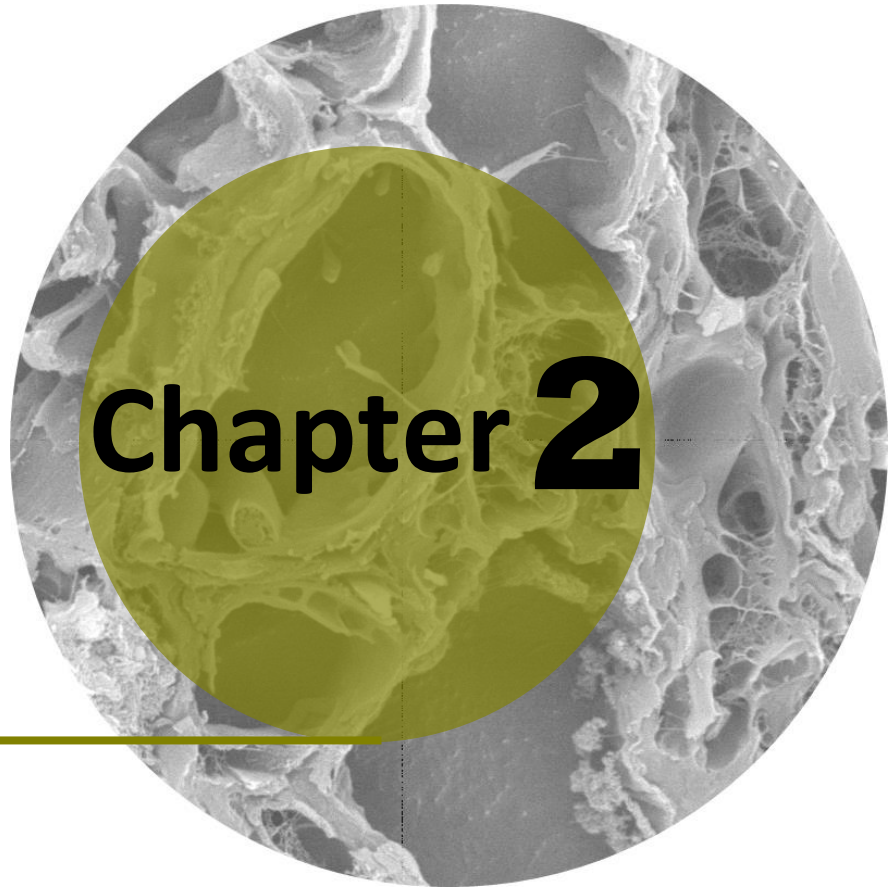
# Objectives and Thesis Outline


Chapter 2

# Objectives and Thesis Outline

The present thesis aims to evaluate the laboratory scale performance of microbial electrochemical systems as bioremediating tool in atrazine-polluted soils. The research effort mainly focuses on the strategies for enhancing the pollutant degradation rate by modifying the system configuration for its application in real polluted environments. Soil analysis of ATR and the corresponding metabolic products were combined with toxicological assessment in order to evaluate the efficiency of this promising technology.

The specific objectives and outlines were:

***1. To study the influence of soil structure in the efficiency of SMFC.***

This objective was developed through ***Chapter 3:*** *Silica colloid formation enhances performance of Sediment Microbial Fuel Cells in a low conductivity soil (Environmental Sciences and Technology 47 (4), 2117-2122, 2013).* After developing a complete physical-chemical analysis of selected soil, the physical properties were modified by stimulating colloid formation to enhance the proton mobility through preferential water channels formed between the soil particles. We aim to deeply explore the impact of adding silica colloid ($SiO_2$) on the soil moisture, internal resistance, nutrients mobility and power output. .

***2. To apply microbial electrochemical systems for stimulating the biodegradation of atrazine herbicide-polluted soil.***

This objective was developed in ***Chapter 4:*** *Cleaning-up atrazine - polluted soil by using Microbial Electroremediating Cells (MERC).* All electrode configurations were compared with natural attenuation conditions in order to evaluate the efficiency of MERC in the ATR removal. The developed toxicological analyses include ecotoxicity, genotoxicity and phytotoxicity test, using *Pseudokirchneriella subcapitata* algae*, Salmonella typhimurium* TA1535-pSK1002 bacteria and *Sorghum saccharatum* seeds respectively.

***3. To analyse the impact of the electrode potential on microbial electrochemical remediation of atrazine-polluted soil***.

This objective was developed in ***Chapter 5:*** *Bioelectroventing: an electrochemical-assisted bioremediation strategy for cleaning-up atrazine-polluted soils.* Our goal was to analyse the impact of the electrode configuration in the complete biodegradation of $^{14}$C-ATR to $^{14}$C-$CO_2$ using an agriculture soil matrix. An overall profile of the $^{14}$C-ATR metabolites was also performed. Ecotoxicological assays were developed in order to validate this technology as a useful tool for biodegrading pollutants in soil. Some of the activities described in this chapter were generated as part of collaboration with the Soil Ecology institute (Institut für Bodenökologie, IBOE) from Helmholtz Zentrum in Munich (Germany) under the supervision of Dr. Reiner Schroll.

***4. To design and construct a microbial electrochemical setup able to operate in non-flooded soils***

This objective was developed in ***Chapter 6:*** *Designing strategies for operating Microbial Electrochemical System in soil under non-flooded conditions*. We aimed to test set ups based on the use of a ceramic barrier to narrow the proximity between electrodes as well as keeping the soil moisture and the electrolytic contact. The efficiency of this new design

was evaluated by studying the ATR removal as well as through toxicological assays.

*Chapters 3* to *Chapter 6* of this thesis correspond to material published or submitted to peer-review international journals prior to PhD defense. ***Chapter 1*** is introductory and describes a general framework for this thesis, and ***Chapter 7***, summarizes and remarks the various contributions of this thesis in view of the scientific advances gained in the context of environmental microbial electrochemistry. The novel concept *bioelectroventing* was introduced and properly discussed as a term with a promising future in bioremediation.

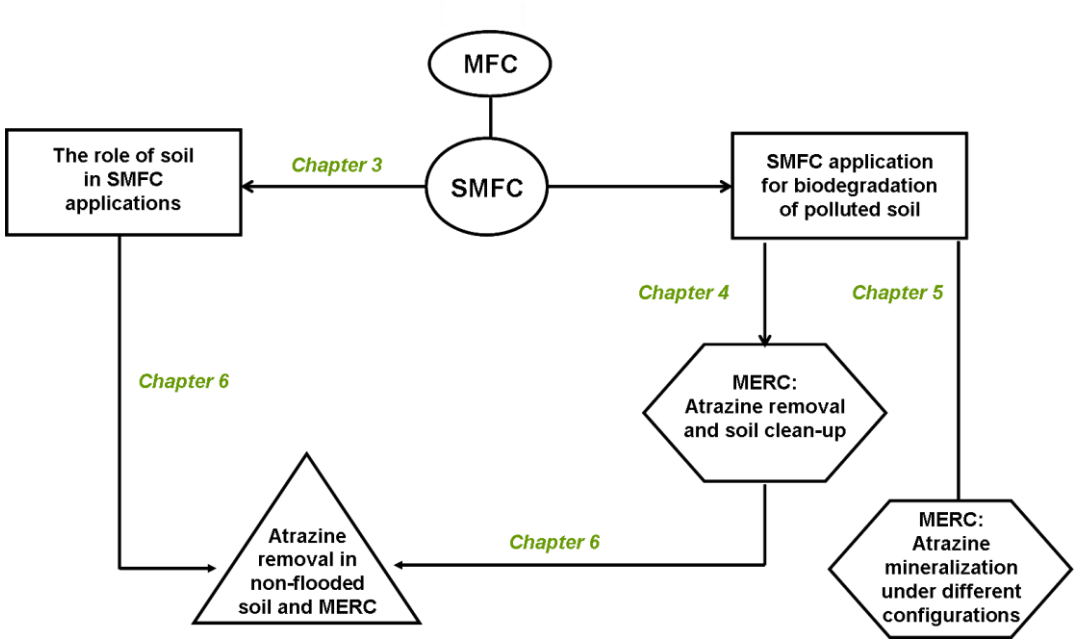



***Figure 2.1.*** General thesis outline diagram

# Silica colloid formation enhances performance of Sediment Microbial Fuel Cells in a low conductivity soil

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***Environ. Sci. Technol.*** 2013, *47* (4), 2117-2122.

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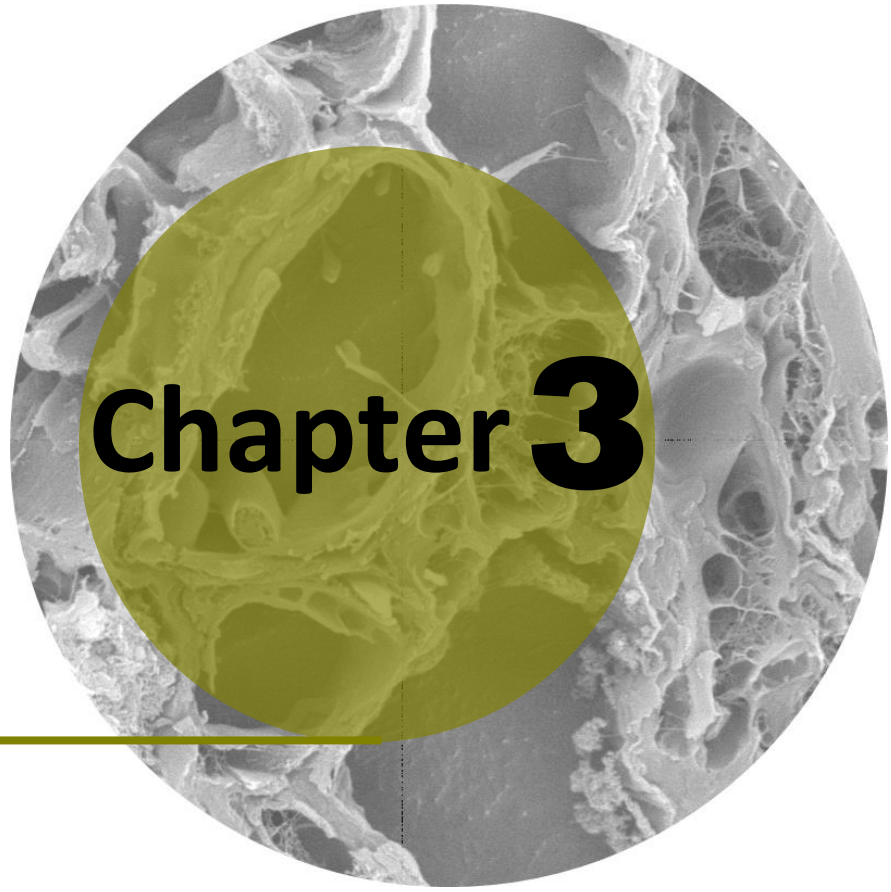
Chapter 3

# Silica colloid formation enhances performance of Sediment Microbial Fuel Cells in a low conductivity soil *

## 3.1. Abstract

The performance of sediment microbial fuel cells (SMFCs) is usually limited by the structure, moisture and salt content of the soil where they are allocated. Despite the influence of soil, so far most of efforts to improve SMFCs have been limited to the hardware design of the bioelectrochemical device. Our main objective was to enhance performance of SMFCs by stimulating the *in situ* formation of silica colloids in a low conductivity rice paddy soil. Our results have revealed that the presence of a silica colloid network, described by cryo-SEM analysis, reduced soil resistivity, enhanced ion mobility and consequently enhanced the power production by a factor of 10. Furthermore, our silica-supplemented soil showed better utilization of the electron donor, either acetate or natural rice root exudates, by electrogenic microbial populations. Sustainable manipulation of soil micromorphology using environmentally friendly reagents such as silica offers a novel approach for enhancing the performance of *in situ* microbial electrochemical applications in low conductivity soils, thus silica colloid geoengineering should be considered as part of future applications of SMFCs.

*The contents of this chapter have been published as:

Domínguez-Garay, A.; Berná, A.; Ortiz-Bernad, I.; Esteve-Núñez, A., Silica Colloid Formation Enhances Performance of Sediment Microbial Fuel Cells in a Low Conductivity Soil. *Environ. Sci. Technol.* 2013, *47* (4), 2117-2122.

## 3.2. Introduction

Microbial fuel cells (MFCs) are bioelectrochemical devices that can use bacterial metabolism to generate an electrical current from the biodegradation of a wide range of organic substrates (Franks and Nevin, 2010). Typically, these devices consist of an anaerobic anodic chamber where microorganisms oxidize organic substrates and transfer the electrons to a solid electrode, i.e., anode. Electrons are then conducted to an aerobic chamber through an external resistor connected to another solid electrode, i.e., cathode, where electrons are finally transferred to a final electron acceptor such as oxygen (Logan, 2009).

Benthic or sediment microbial fuel cells (SMFCs) are variants of MFC that are placed in ecological water bodies and be used to harvest energy provided by natural bacteria populations (Lowy et al., 2006). The anode is placed in the sediment and connected through an electrical circuit to a cathode in the overlying water layer, and energy is obtained by means of several reactions (De Schamphelaire et al., 2008): (1) the chemical oxidation of microbially produced reductants, such as humic acids, $Fe^{2+}$, and especially $S^{2-}$ at the anode (Ryckelynck et al., 2005) (2) the microbial oxidation of organics (Bond et al., 2002; Tender et al., 2002; Venkata Mohan et al., 2009) and (3) the microbial oxidation of $S^0$ to $SO_4^{2-}$ (Ryckelynck et al., 2005).

Most of the SMFCs described so far have been installed 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; Reimers et al., 2006). This environment has the advantage of low ohmic internal resistance due to its high concentration of salts, leading to power production in the range of 50-100 $mW/m^2$ (Dumas et al., 2008).

Freshwater environments can also produce electrical current, although lower output power values have been reported (ca. 10-30 $mW/m^2$), most likely to an increase in the internal resistance due to the lower ionic

strength and poor ionic conductivity of the electrolyte as a result of lower conductivity (Liu et al., 2005). Therefore, an expansion of the research focus is warranted in order to engineer SMFCs to perform as well in freshwater environments with low salt concentrations (De Schamphelaire et al., 2008). Other variations of SMFCs have been deployed in freshwater natural environments, such as rice crop soils, in which the plant provides a source of organic substrates through root secretions (Kaku et al., 2008; Busalmen et al., 2010; Takanezawa et al., 2010; Chen et al., 2011; Chiranjeevi et al., 2012). In addition, a number of substrates (e.g., vermiculite, graphite granules) and plant-growth medium have been used to support plant-MFCs in soil-free applications (De Schamphelaire et al., 2008; Helder et al., 2010; Timmers et al., 2010; Arends et al., 2012; Helder et al., 2012).

For the past ten years, several groups have used affordable materials, such as graphite, carbon felt, and stainless steel, to explore the effect of the anodic material on SMFC performance (Dumas et al., 2007; Venkata Mohan et al., 2008; Pant et al., 2011; Wei et al., 2011; Luckarift et al., 2012). Modifications in the cathode material have also recently been explored, improving the oxygen reduction and studying the effect of cathode material on the growth of oxygen-consuming heterotrophic microbes (Lowy et al., 2006; Orfei et al., 2006; Dumas et al., 2007; Rismani-Yazdi et al., 2008; An et al., 2011; Pant et al., 2011; Renslow et al., 2011). A novel and interesting concept is the use of a cathode buried in soil in order to capture the released oxygen from rice roots (Chen et al., 2011). In this configuration, anode and cathode become closer and the ohmic internal resistance decreases so power outputs up to ca. 5mW/m$^2$ can be achieved. Other studies have shown the relationship between soil organic matter, nutrients, water content, bacterial community structure and the effect of sediment pre-treatment on the performance of SMFCs (Song and Jiang, 2011; Chiranjeevi et al., 2012; Dunaj et al., 2012).

A number of constraints related to the efficiency of SMFCs fuelled by organic substrates (e.g., acetate (Dunaj et al., 2012), chitin and cellulose (Rezaei et al., 2007) have been reported. Power outputs as high as 105

mW/m$^2$ were reported by Lowy et al. (2006) after applying microbial oxidants (anthraquinone-1,6-disulfonic acid, $Ni^{2+}$, $Mn^{2+}$) into anode graphite disks as charge transfer mediators, but the duration was limited to a few days.

Despite the importance of the sediment matrix that hosts these processes; little effort has been devoted to the study of the physical chemistry of the sediment affecting the performance of SMFC.

The sediment is the barrier that ions must overcome in order to reach the SMFC cathode. Due to lack of moisture or salts, the physical properties and high electric resistance of the sediment can restrain the transport of ions to the cathode and consequently limit SMFC performance. Some authors have overcome this limitation by adding NaCl (Hong et al., 2009) to the sediment, however we do not believe this is a reasonable approach to apply in field assays.

Therefore, in this study, several soil parameters likely to influence SMFCs performance were examined with the aim of outperforming laboratory-scale SMFCs. For this purpose, soil physical properties were modified by stimulating colloid formation via the addition of silica colloid ($SiO_2$), a reagent regularly used in agriculture. The influence of such a colloid suitably enhanced the microbial electric power production in soils with low conductivity and low organic matter content, regardless of the presence of rice plants.

## 3.3. Materials and methods

### *3.3.1. Physical and chemical characterization of soil*

All laboratory-scale experiments were conducted using soil samples from an original soil profile located on an alluvial plain in Calasparra (Murcia, SE Spain) and classified as Haplic Fluvisol (Calcaric) (WRB, 2006) and Typic Xerofluvent (2010). This soil was formed on alluvial

sediments deposited by the Segura River and developed on rice fallow land. Soil samples were taken at 0-20 cm depth. They were stored in hermetically sealed plastic bags, air dried for three days at room temperature and sieved (2 mm screen size) for laboratory analysis.

Particle size distribution was determined by the pipette method after removal of organic matter with $H_2O_2$ and dispersion by shaking with sodium hexametaphosphate (Loveland,1991). A saturated extract of the soil was prepared following the guidelines of the US Salinity Laboratory Staff (1954), and its electrical conductivity was measured in a 10:1 distilled water:soil extract dilution with a Crison conductimeter at 25 ºC. Soil moisture content was determined by weight difference after drying the samples at 110 ºC. The pH was measured potentiometrically in a 2:5 soil:water suspension. The organic carbon content was determined using the method of Tyurin (1951). The $CaCO_3$ equivalent was determined according to Bascomb (1961).

### *3.3.2. Soil resistivity analysis*

The electrical resistance of a material is related to geometric parameters and to an inherent property of the material, resistivity, obeying the following relationship:

$$R = \rho \cdot (L/A) \qquad [1]$$

where, R is the electrical resistance (Ω), ρ is the resistivity (Ω·m), A is the cross-sectional area ($m^2$) and L is the length (m) of the material (Amato et al., 2008).

The Wenner-Schlumberger method was used in order to measure soil resistivity in both the presence and absence of silica dioxide colloids. This method employs four electrodes (Rhoades et al., 1977): two electrodes connected to a power source, (current electrodes, C1 and C2) and

another two used for the measurement of electric potential differences along the soil (reference electrodes, P1 and P2). In order to keep the operating conditions constant during the experiment, a system with a well-defined architecture was designed (Figure 3.1). It consisted of a glass hemicylinder (ø = 6 cm and L = 12 cm). Two stainless steel electrodes (AISI 304) were placed at both ends of the hemicylinder, whose contact area was 14.13 $cm^2$ (current electrodes surface area). Reference electrodes (Ag/AgCl, BASi) were situated at a distance of 1 cm away from each current electrode. The total distance between reference electrodes was 10 cm.

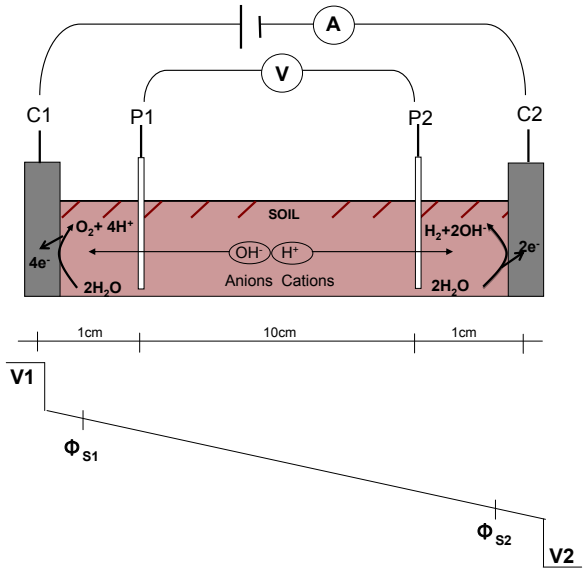


***Figure 3.1.*** Four electrodes system configuration for measuring the specific resistance of soil. **C1** and **C2** are current electrodes of stainless steel, **P1** and **P2** are reference electrodes of Ag/AgCl. The different of potential measured through the multimeter (V in the scheme) is calculated by the equation:

$$E = (V1 - \Phi_{S1}) - (V2 - \Phi_{S2}) + V_{internal} = V1 - V2 + I \cdot R_{interna}$$

The hemicilynder was filled with 150 g of soil and 75 mL of water. The same conditions were applied in the experiment with soil containing colloid, 1g of silica dioxide (*Aerosil 200,* Hydrophilic fumed silica).

Soil electrical resistance was measured by applying several electric potential differences (ranging from 2 to 9 V) between the current electrodes placed in the soil. A potentiostat *VoltaLab PGZ100* was used as power source. Electric potential differences between reference electrodes were measured with a multimeter Metrix.
Plots of potential difference and electric current between soil reference electrodes exhibit a linear behavior. This fact confirms that soil obeys Ohm's law, due to the ionic character of its electrical conductivity. Using Ohm's law, the value for soil electrical resistance can be obtained straightforward from the slope of these plots.

The linear shape of the plot confirms that soil behaves as an ohmic resistor. When all the parameters in equation [1] are known, resistivity is determined. The resistivity was determined using the expression formerly shown. Geometric parameters were fixed and defined the value of the cell constant.

The influence of soil moisture on soil resistivity was investigated in the absence of external irrigation. Water content was periodically determined by monitoring the weight loss due to evaporation over five days.

### *3.3.3. Mobility of chemicals in soil*

Ion mobility in soil was tested by following the transport of the ion in a water saturated soil column. To perform this test, an open tube (20mm in diameter and 95mm in height) was perforated at the bottom and partially filled with 24g of dry soil as a control. Water was added to the tubes until soil saturation was achieved, resulting in 1cm of overlying water. Then the tube was placed inside a Falcon conical tube (30mm in diameter and 100mm in height) containing 20mL of a solution of either potassium chloride 0.5M or sodium acetate 0.5M. The overlying water was periodically analyzed by ion chromatography (DIONEX AS9-HC 4mmx250mm, 9mM Sodium Bicarbonate at 1mL/min flow) to monitor

chloride and acetate concentration. In order to test the effect of silica colloid on ion mobility, an identical soil tube was prepared by mixing the soil with 0.16g of fumed silica (*Aerosil 200*, Hydrophilic fumed silica). Experiments were performed in at least triplicate, yielding differences lower than 10%.

### *3.3.4. Low Temperature-Scanning Electronic Microscopy (LT-SEM) analysis*

Low Temperature-Scanning Electronic Microscopy analysis (also known as cryo-SEM) was performed to explore the internal morphology of soil with the colloids. A paddy soil sample (3.2 g) was mixed with 0.02 g of fumed silica, while paddy soil that was not amended with silica was used as control. Both samples were placed in test-tubes and each was saturated with 1.6 mL of water. The water supernatant was then removed in order to determine the amount of water retained by the soil matrix. The pieces were mounted on a mechanical stub that clamps the frozen sediment in place in order to be transferred into the electron microscope (model DSM 960 with ZEISS model Oxford CT1500).

The sample was examined under vacuum at approximately -160 °C so that any surface water was removed by “heat etching”. The temperature of the microscope stage was raised to -90 °C in order to sublime water, avoiding the liquid phase. The etching process could be observed under the SEM at a low accelerating voltage (cf. 2 kV) until the desired amount of etching had taken place. The samples were then coated with gold while being maintained at low temperature (<-160 °C) and reexamined.

### *3.3.5. Sediment Microbial Fuel Cell Design*

The standard electrogenic microcosm configuration used in these studies consisted of a glass-made SMFC manifold with cylindrical shape (50 mm inner diameter and 250 mL volume). The standard electrogenic

microcosm was set up with 150 g of sieved dry paddy soil (≤ 2 mm). For silica-supplemented experiments, a defined amount of fumed silica in 100 mL 0.1M phosphate buffer solution was added to the soil. The buffer solution was required due to the acidic pH (3.7-4.7) of the fumed silica in solution (*Aerosil 200*, Hydrophilic fumed silica).

Plain graphite plates (*SOFACEL*, 3 x 0.8 cm; 1 mm thickness) without any surface treatment were used as electrodes for the anodes and buried at 5 cm in depth (geometric area: 4.8 $cm^2$). Graphite felt (*SOFACEL*, 13 x 4 cm and 5 mm thickness; 0.7 $m^2/g$ of surface area) was used as the electrode for the cathodes and allocated in the flooded water of the SMFC. Copper wires used for connections were sealed with a conductive epoxy resin (Circuit Works) and isolated with a non-conductive epoxy resin (Araldit Ceys ®). The electrodes were connected through a 1 KΩ resistor and the cell voltage was monitored with a multimeter (7700, *Keithley* instruments).

In order to investigate the influence of soil water content on the performance of the SMFC, a special two-chamber configuration was designed to allow for evaporation from the anodic open chamber. A plastic container (8 x 5 x 3 cm length, width, height) was converted into a two-chamber SMFC by using a Nafion separator. One of the chambers was used as an anodic open chamber by burying a graphite plate anode (*SOFACEL*, 3 x 0.8 cm; 1 mm thickness) in 100 g of soil, followed by the addition of 50 mL of 60 mM KCl solution. For silica-supplemented experiments, 0.6 g of fumed silica was mixed with 100 g of soil. The second chamber was also filled with 50 mL of 60 mM KCl and was used as a cathode compartment containing a graphite felt (*SOFACEL*, 6 x 3 cm and 5 mm thickness). The anode and cathode were connected through a 1KΩ resistor. The anodic microbial population was stimulated by adding 1mL of an anoxic solution containing 0.5 M sodium acetate. To test the effect of moisture in the SMFC performance, all of the overlying water in the anodic chamber was removed and the response of cell voltage to

water evaporation was periodically measured. All experiments were performed at least in three independent electrogenic microcosms.

### *3.3.6. Plant-SMFC*

Rice plants used in our assays belonged to *Oryza sativa* ssp. *Bomba,* the same variety that has been grown in our paddy soil since the fourteenth century. Rice seeds were provided by Cooperative "Virgen de la Esperanza" (Calasparra, Spain).

After 1 week humid incubation of the rice seeds at 25 °C and 4 weeks growth in soil, the plants were transplanted into the anode chamber of our two-chamber SMFC devices described above. Silica-supplemented and silica-free soils were used to construct these SMFCs. Plants were maintained under flooded conditions for 2 weeks, then irrigation was stopped and the current production ($mA/m^2$) was monitored over time.

The experiments were performed in at least triplicate to estimate the uncertainty of the observed trend.

### *3.3.7. Power curves analysis*

Using the two-chamber SMFC configuration, polarization curves were recorded with a potentiostat (model *μAutolab 300)* during a linear potential sweep at a scan rate of 0.1mV/s. The curves were obtained in the presence and absence of silica colloid, and with identical amounts of soil, water and silica colloid previously described in the two-chamber SMFC model. The power density (power=voltage*current) and current density were calculated based on the anode surface area.

## 3.4. Results and discussion

### *3.4.1. Soil analytical features*

The results of the physical and chemical soil analyses are summarized in *Table 3.1*. The physical and chemical analyses of the rice paddy soil used in this study showed a clay loam texture, which favored water storage, and chemical conditions that could stimulate microbial activity (i.e., high pH and calcium carbonate content).

***Table 3.1.*** Physical and chemical soil analysis.

| | | |
|---|---|---|
| Rice crop field Murcia-Spain | Depth (cm) | 0-20 |
| | Sand (%) | 30 |
| | Silt (%) | 18.9 |
| | Clay (%) | 32.1 |
| | pH | 8.0 |
| | EC ($dSm^{-1}$) | 0.24 |
| | $CaCO_3$ (%) | 52.0 |
| | OC (%) | 0.10 |
| | OM (%) | 0.18 |
| | Moisture (%) | 39.0 |

EC, electrical conductivity; OC, Organic Carbon; OM, organic matter

However, other parameters, such as very low organic matter content and ionic conductivity, could severely limit the performance of the MFC in an electrogenic microcosm.

Low ionic conductivity is a consequence of the low ionic strength that increases the internal resistance of the system (Liu et al., 2005).

More importantly, low organic matter content reduces the microbial electrogenic activity due to the reduced availability of the electron donor.

We therefore concluded that the paddy soil used in these studies was unfavourable for optimal performance of SMFCs in electrogenic microcosms under natural conditions, and thus some organic amendment is required.

### *3.4.2. Silica colloid formation reduces soil resistivity and prevents moisture lost*

Resistivity and moisture are inversely related soil parameters. In this sense, minimizing soil resistivity is key to achieving an optimal performance of SMFCs in electrogenic microcosms. Actually, we have evaluated from the maximum power point of the power curves that the contribution of soil ohmic resistance to the overall internal resistance of SMFCs accounts for a 20% of the value (Figure 3.2). Therefore, acting on this parameter will help to reduce the internal resistance value. Moisture control is another valid strategy to accomplish this aim. Indeed, water content was recently reported to be key for avoiding reductions in the OCV of SMFCs (Chiranjeevi et al., 2012). However, watering is not always feasible in remote sites and additional methods for increasing soil water content are required.

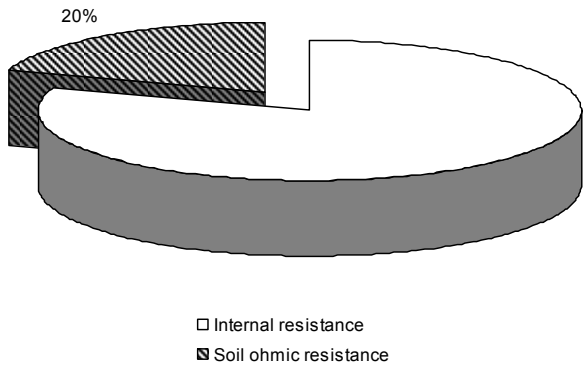


***Figure 3.2.*** Percentage of the ohmic resistance of soil in the overall internal resistance.

Fumed silica is a low cost nanostructure material with unique properties, including large surface area and exceptional adsorptive affinity for organic macromolecules and water (Cruz et al., 2011). Silica reacts with the soil water and is converted into a reversible gel ($H_4SiO_4$) that is able to accumulate charge. Due to the presence of negatively charged

silanol groups (SI-OH) at its surface, the silica colloid provides preferential adsorption pathways for a variety of molecules with low sorptive capacity (Cruz et al., 2011; Liu and Maciel, 1996) and the absolute storage capacity becomes increased (Gun'ko et al., 2011).

In order to evaluate the effect of silica colloid addition on the resistivity of the rice paddy soil, a series of assays were conducted in electrogenic microcosms. When soil was saturated with water, the resistivity values were not affected by the presence or absence of silica colloid. The differences became relevant after six days without irrigation, when resistivity increased from the original value of 10 ohm·m to 140 ohm·m in control soil and silica-supplemented soil just reached just 30 ohm·m (Figure 3.3).

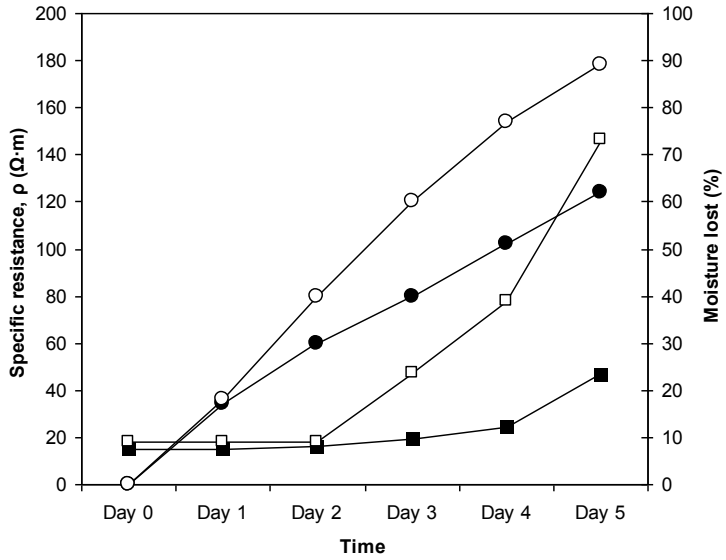


***Figure 3.3.*** Specific resistivity (square) and evolution of moisture loss (circles) evolution of a non-irrigated soil in the presence (closed symbol ● ■) and absence (open symbol ○□) of silica colloid during a five days period.

### *3.4.3. Low Temperature Scanning Electron Microscope (LT-SEM) analysis*

The advantages of LT-SEM in the study of hydrated sediments have been outlined by several authors (Perkins et al., 2006). The LTSEM consists of the SEM examination of samples whose microstructure has been fixed by plunge-freezing into a liquid with a high thermal conductivity, such as nitrogen slush (Nègre et al., 2004).

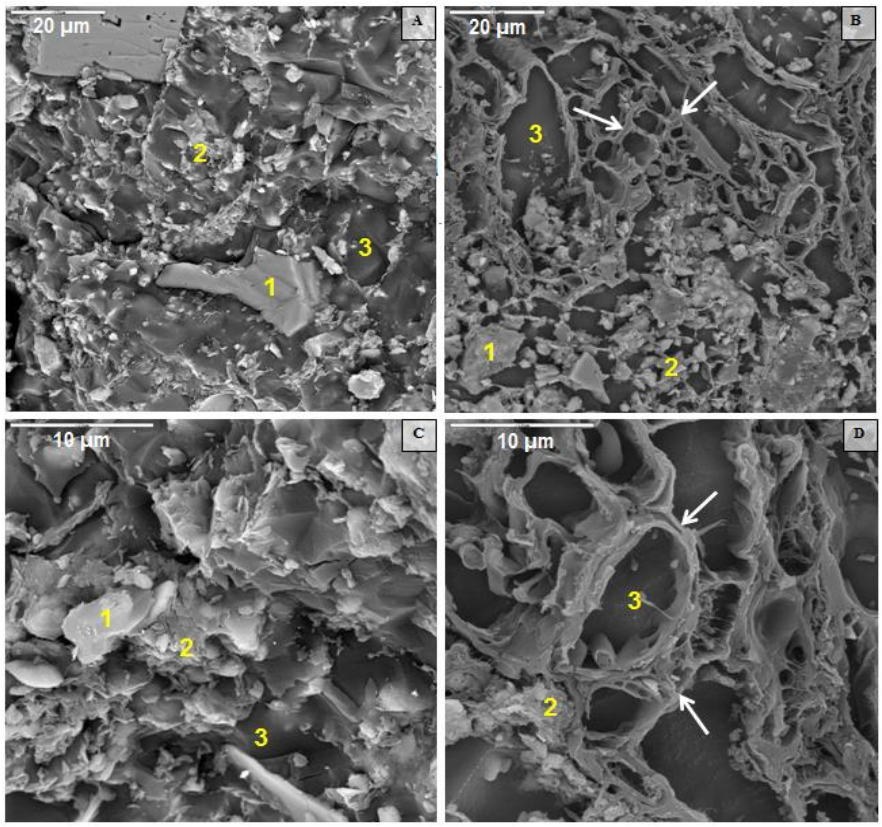


***Figure 3.4.*** Low-temperature scanning electron micrographs of control soil (**A and C**) and silica supplemented soil (**B and D**). ***(1, 2)*** Mineral soil particles, (***3***) Voids left by the water sublimation process, (***Arrows***) Network of silica colloid.

LTSEM micrographs showed a high content of carbonate particles (*number 1* in Figure 3.4), that confirm the high content of calcium

carbonate revealed by the soil chemical analysis. The images in the presence of fumed silica showed an interconnection of the soil matrix, consisting of sand and clay particles (*number 2* in Figure 3.4), and a colloidal network around the cavities left by the water sublimation process (*number 3* of B and D in Figure 3.4).

There was different soil morphology in the presence of the colloid (B and D in Figure 3.4), giving higher cohesion to the soil particles and greater retention of water. According to (Kalkan and Akbulut, 2004) fumed silica covered the surrounding silt and clay particles and filled the voids in the samples. The settled fumed silica particles reacted to form hydration products (flocculation products), and this generated water channels (arrow in Figure 3.4) that may facilitate the migration of ions from the anode to the cathode.

To provide evidence of this, we performed a mobility test in a column of our soil, which showed a higher mobility of chloride in the presence of the silica colloid compared with the control soil (Figure 3.5).

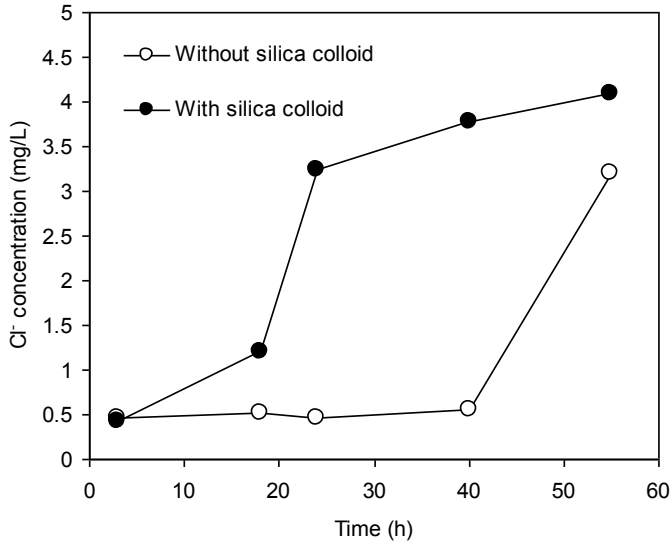


***Figure 3.5.*** Chloride mobility test. The plot shows the time required for chloride to diffuse along soil.

### *3.4.4. Sediment Microbial Fuel Cell performing enhanced by colloid-formation in soil*

Electrogenic microcosms were set up with the purpose of evaluating the effect of silica colloid on power production. The assays revealed that silica-supplemented electrogenic microcosms generated power (23mW/$m^2$) ca. 10-fold higher than the control electrogenic microcosm (2.4mW/$m^2$). The silica doses also appeared to be relevant because the study revealed an optimal concentration of 1.6 mg $SiO_2$/ g soil, whereas doses equal to or higher than 3 mg $SiO_2$/g soil decreased power generation. Doses over 6 mg $SiO_2$/g soil had a negative influence on power production, generating even lower power values than in the silica-free microcosm (Figure 3.6). This result is not unexpected because it has been recently reported that high doses of $SiO_2$ produces an acute and toxic effect on bacteria from activated sludge (Zheng et al., 2012).

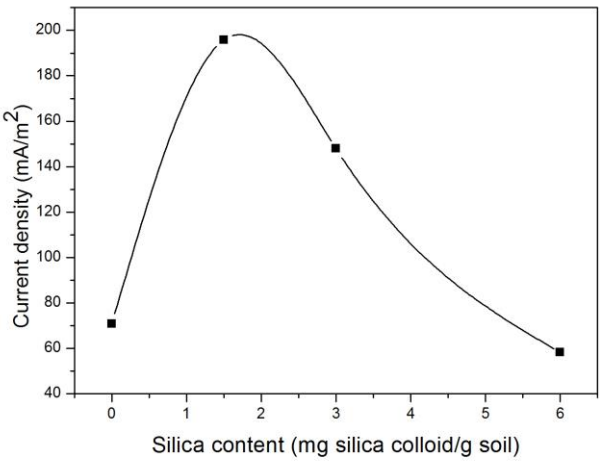


***Figure 3.6.*** Current density of SMFC after different doses of silica colloid in the standard SMFC model.

A special two-chamber SMFC configuration was set up to test the effect of the silica colloid under evaporation conditions. Due to the additional

nafion-associated resistivity, this specific setup achieved a lower power production compared with the standard SMFC described in this work. However, it was a useful tool to investigate the effect of halting irrigation for 30 hours, and revealed that the silica-supplemented electrogenic microcosms produced a higher (2-fold) electric current and power production than the control microcosm (Figure 3.7 and 3.8).

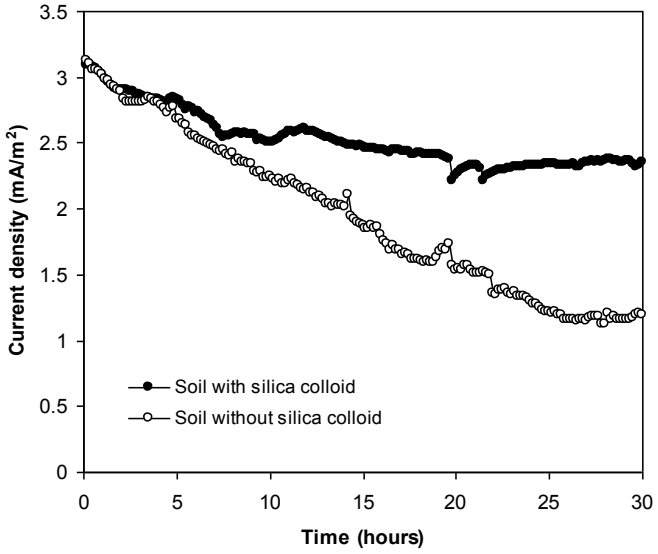


***Figure 3.7.*** Current production in presence and absence of silica colloid under evaporation conditions in the two-chamber SMFC model. The use of a Nafion membrane allowed for development of a system to test the effect of water evaporation. However, it increased the internal resistivity, leading to lower current density than observed for other SMFCs of this work.

This work demonstrated that silica colloid formation minimized both soil resistivity and water evaporation, thus enhancing SMFCs performance under non-optimal conditions. SMFCs have been classically shown to operate under flooded conditions, where the cathode is submerged under water. However a feasible alternative could be a cathode buried in a soil environment with an oxidizing redox potential. This situation has been

recently reported by allocating the cathode in the vicinity of rice rhizodeposits, where the oxygen secretion by the roots generates a microaerobic environment oxidizing (Chen et al., 2011).

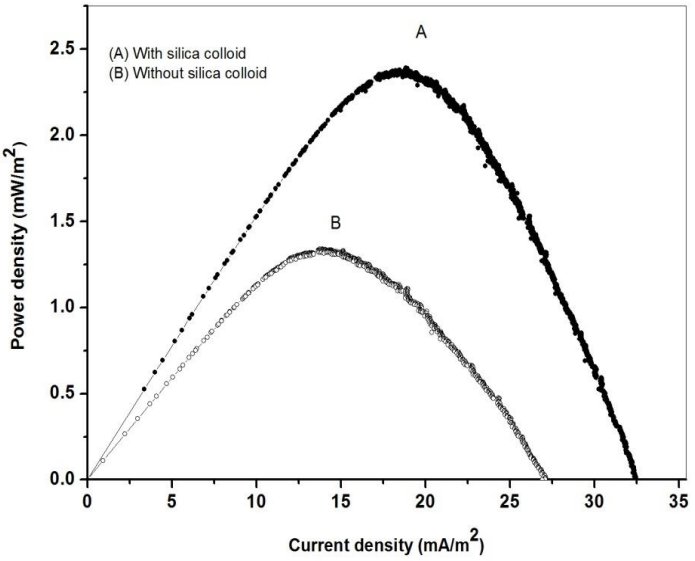


***Figure 3.8.*** Power curves of SMFCs in the two-chamber SMFC model under evaporation conditions, in presence (A) and absence (B) of silica colloid.

Another limitation for the suitable SMFC performance using our rice paddy soil was its low organic content. In order to provide an electron donor, acetate was artificially added to the electrogenic microcosm. Interestingly, only the silica-supplemented microcosm showed an immediate increase in current production, in contrast with the delayed and minor response in the control soil (Figure 3.9).

This immediate assimilation of acetate by electrogenic microorganisms in the presence of the silica colloid could be due to higher acetate mobility. To explore this concept, an acetate mobility test was performed in soil and

indicated that acetate migration in our paddy soil was increased 8-fold in presence of silica (Figure 3.10).

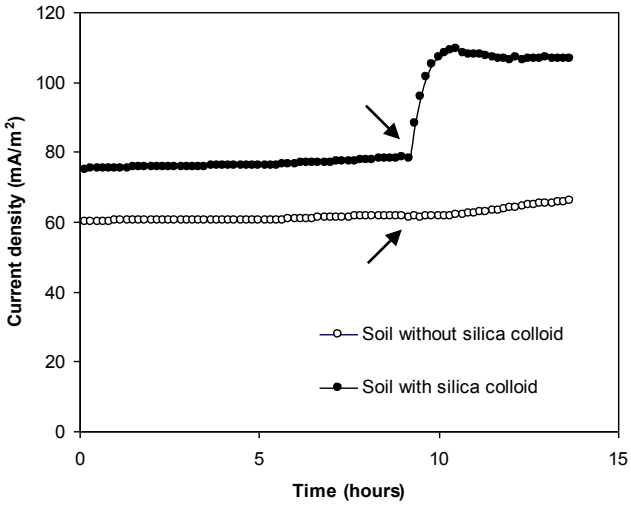


***Figure 3. 9.*** Current production in response to an acetate pulse (indicated by the arrows) in the absence (open symbol) and presence (closed symbol) of silica colloid using the standard SMFC configuration.

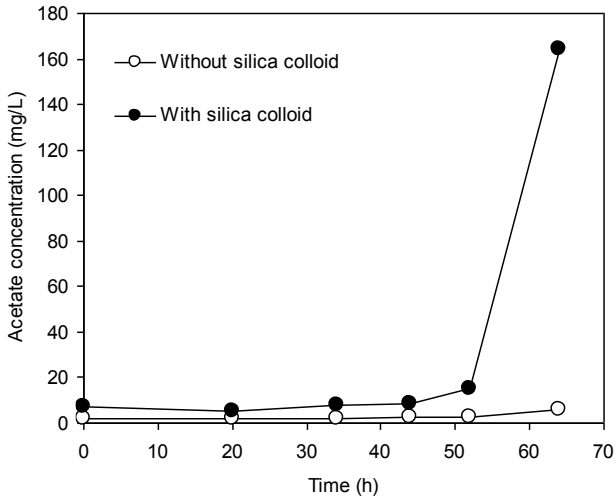


***Figure 3.10.*** Acetate mobility test. The plot shows the time required for acetate to diffuse along soil.

In addition, silicon is known to enhance the growth, development and yield of several plant species, including rice (Mitani and Ma, 2005). Moreover, it has been reported that the application of silica to rice plants increased resistance to blast fungus and increased silicon content in rice tissues (Hayasaka et al., 2008). Interestingly, another way to provide electron donors to SMFC in a natural manner is by supplying organic electron donors through the root secretions of plants such as rice (De Schamphelaire et al., 2008; Kaku et al., 2008; Busalmen et al., 2010; Takanezawa et al., 2010). Other authors have previously studied the plant-SMFC concept using rice and soil (Busalmen et al., 2010) but the effect of colloids in the plant-SMFC system has not yet been explored. We now have evidence that a rice-SMFC deployed under evaporation conditions produces a power density of 31 mW/m$^2$ in the presence of silica colloids, whilst only 8 mW/m$^2$ was generated by the Plant-SMFC in unamended soil (Figure 3.11).

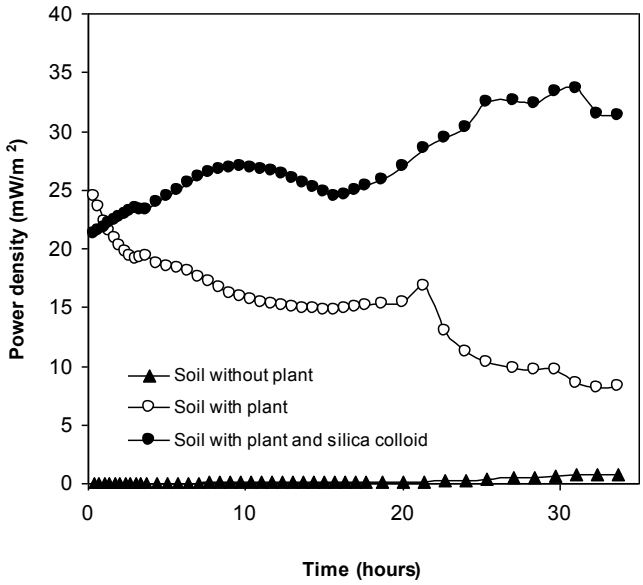


***Figure 3.11.*** Power density evolution of SMFC (circles) in the presence (closed symbol) and absence (open symbol) of silica colloid, in the two-chamber SMFC configuration model.

We conclude that the use of silica colloid can improve SMFC performance in soils with low conductivity and low content of organic matter. We believe that such a sustainable and safe reagent can extend the range of environments amenable to SMFC operation. Furthermore, the known benefit of silicon in agriculture also makes silica colloid an attractive tool to incorporate in the plant-SMFC concept.

### 3.5. Acknowledgments

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# Cleaning-up atrazine polluted soil by using Microbial Electroremediating Cells (MERC)

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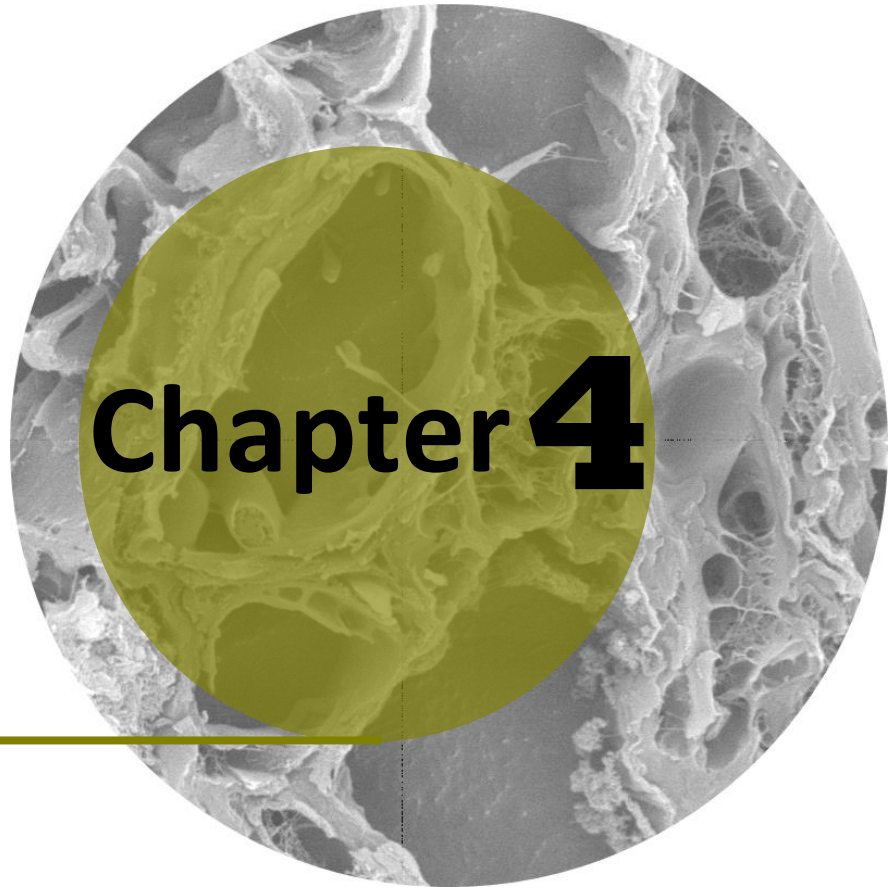

# Chapter 4

# Cleaning-up atrazine polluted soil by using Microbial Electroremediating Cells (MERC) *

## 4.1. Abstract

Biodegradation of pollutants in soil is greatly limited by the availability of terminal electron acceptors required for supporting microbial respiration. Such limitation can be overcome if soil-buried electrodes accept the electrons released in the microbial metabolism. We propose the term *bioelectroventing* for such a environmental treatment. The process would be performed in a device so-called *Microbial Electroremediating Cell* (MERC). Indeed, our studies demonstrate that the presence of electrodes as electron acceptors effectively stimulated by 5-fold the biodegradation rate of the herbicide atrazine (2-chloro-4-ethylamino-6-isopropyl amino-1,3,5-triazine) (ATR) in comparison with soil natural attenuation. Furthermore, a different set of toxicological test using *Pseudokirchneriella subcapitata* green algae, *Salmonella typhimorium* bacteria and *Sorghum saccharatum* plant seeds respectively, confirm that ATR polluted soil can be effectively cleaned-up in short time by the use of MERCs.

*The contents of this chapter have been submitted as:

Domínguez-Garay, A.; Boltes, K.; Esteve-Núñez, A., Cleaning-up atrazine polluted soil by using Microbial Electroremediating Cells.

## 4.2. Introduction

The organic pollutants can be degraded in nature by many microorganisms making biodegradation the main mechanism for their removal in the environment (Head, 1998; Semple et al., 2001). Sometimes the lack of suitable electron acceptors limits microbial respiration and a variety of organic pollutants persist in waterlogged soil or sediment (Liu and Suflita, 1993). The supply of additional terminal electron acceptors (TEA) like oxygen (bioventing) (Kabelitz et al., 2009; Frutos et al., 2010), humic acids (Lovley, 2000) or nitrates (Yu et al., 2014) to stimulate the microbial metabolism have been a common practice to remove organic pollutants, but this incurs extra cost and causes secondary pollution concerns (Pandey, 2012).

Such a TEA limitation can be overcome using electrically conductive material like the electrodes used in Microbial Electroremediating Cells (MERCs) (Rodrigo et al., 2014). MERCs are variants of Sediment Microbial Fuel Cells (SMFC). These bioelectrochemical devices use sediment- or a soil-buried electrode (anode) 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; Domínguez-Garay et al., 2013). In contrast with SMFC, MERCs aim to enhance *in situ* bioremediation through generation of current instead of harvesting energy as MFCs typically do (Rodrigo et al., 2014).

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), herbicides (Rodrigo Quejigo et al., 2016) and chlorinated organics (Chun et al., 2013) has been reported.

Moreover, not just pollutant removal but true clean-up of a DBT-polluted soil after MERCs treatment was demonstrated by ecotoxicological analysis (Rodrigo et al., 2014). More recently, Rodrigo *et al*. (2016) even reported how a fine tuning on the electrode potential can show a strong impact in the pollutant mineralization.

The absence of suitable TEA in environments like soil might be responsible of the limited *in situ* biodegradation of ATR (2-chloro-4-ethylamino-6-isopropyl amino-1,3,5-triazine), a member of chlorinated *s*-triazine group of herbicides, which is moderately mobile and highly persistent in the environment(Iriel et al., 2014). Although ATR was banned in the European Union in 2003 (Bethsass and Colangelo 2006), it is still widely used around the world due to its low cost and high effectiveness for control of weeds in crops such as corn and sorghum. Therefore often appears in ground and surface water, exposing a risk to human health. However, in spite of its recalcitrance recent studies have demonstrated that ATR biodegradation is feasible (Solomon et al., 2013). In soils, ATR degradation is mainly a result of microbial activity (Mudhoo and Garg, 2011) and a large variety of microorganisms such as *Pseudomonas* sp ADP (de Souza et al., 1998)*, Agrabacterium radiobacter* J14a (Struthers et al., 1998) or *Nicordioides* (Topp et al., 2000) degrade this herbicide through co-metabolic processes that lead to the formation and accumulation of ATR metabolites. ATR biodegradation pathway seem to follow *N*-dealkylation and dechlorination processes prior to ring cleavage (Fang et al., 2015). Either single species or microbial consortia are responsible for reactions that involves three hydrolases genes atzA, atzB, atzC that encode for dehalogenate and dealkylate ATR in a stepwise fashion (Smith et al., 2005).

Numerous studies have indicated that ATR inhibits growth and photosynthesis of freshwater algae and algal responses to ATR vary widely depending upon concentrations used, duration of exposure, and algal species tested (Tang et al., 1997; Weiner et al., 2004). Furthermore, ATR and other triazinic compounds have showed genotoxic and

mutagenic actions in *Drosophila*, yeasts and plants, but not mutagenic actions in bacteria (de Campos Ventura et al., 2008). Recently, have been reported that ATR and its chlorometabolites have the potential to influence the development and behaviour in the early life stages of zebrafish still remain unclear (Liu et al., 2016).

Several authors have already studied changes in the yield of chlorophyll fluorescence in the presence of herbicides. Conrad *et al.* (1993), found that some triazines and derivatives of phenylurea produced variations in the yield of the *in vivo* fluorescence for PSII chlorophyll-a (Conrad et al., 1993; Iriel et al., 2014).

So, the main aim of this work was to apply a MERC treatment for stimulating the biodegradation of ATR in soil. Moreover, a set of several toxicological analyses, including ecotoxicity, genotoxicity and phytotoxicity tests have revealed how the MERC-assisted treatment outperforms natural attenuation to accelerate the clean-up process of an ATR-polluted soil.

## 4.3. Material and methods

### *4.3.1. Chemicals*

ATR (CAS No. 1912-24-9, purity > 97%) was obtained from TCI. $Na_2HPO_4$ and $NaH_2PO_4$ were used to make the phosphate buffer solution, with purity > 98% and purchased from Scharlau. Deionized water was used to prepare all media and solutions and Methanol from Sigma Aldrich (purity > 99.9%) was used to soil extractions.

### *4.3.2. Soil sampling*

The laboratory-scale experiments were developed using a single soil collected from an uncontaminated soil on an alluvial plain in Calasparra (Murcia, SE Spain), deposited by the Segura River and developed on rice fallow land. A complete description of the physical and chemical

characteristics of soil is provided in *section 3.4.1. Soil analytical features,* in Chapter 3).

### *4.3.3. Construction and operation of the Microbial Electroremediating Cells* (MERCs)

MERC systems were constructed under a multichamber configuration in plastic containers. Each watertight compartment was 4 x 6.5 x 3.5cm (wide, length and deep) and it was filled with 60 g of sieved dry paddy soil (≤2 mm) (Figure 4.1 and 4.2).

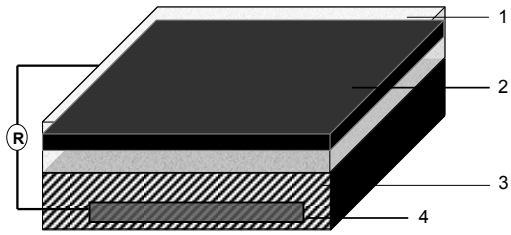


***Figure 4.1.*** Schematic diagram of the laboratory-scale MERC. The graphite plate anode **4** was buried in the sediment of the anodic chamber **3**, and connected with 1000 Ω resistor to graphite felt cathode **2** on the water surface of the cathodic chamber.

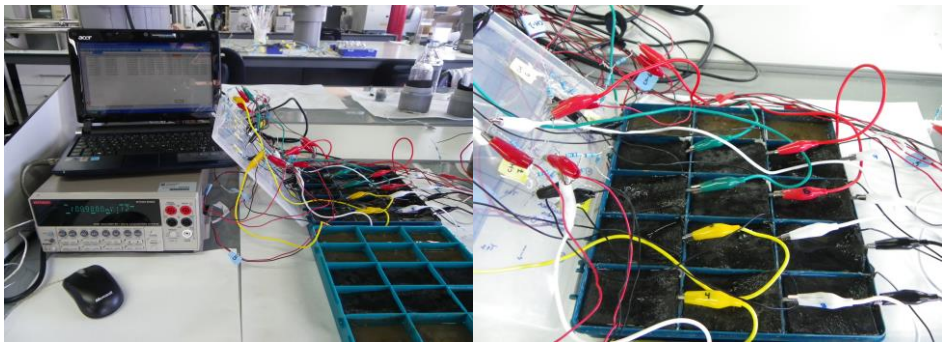

***Figure 4.2.*** Experimental multichamber configuration for bioremediating ATR-polluted soil.

Plain graphite plates (*Mersen,* 3 x 3 cm, 0.5 cm thickness) were used as electrode for the anodes that were buried in the middle of the soil layer. Copper wires used for connections were sealed with a conductive epoxy resin (Circuit Works) and isolated with a nonconductive epoxy resin (Araldit Ceys). Graphite felt (Mersen, 4 x 6.5 x 0.5cm; 0.7 $m^2/g$ of surface area) was used as cathode electrode and were allocated in the flooded deionized water of the MERC. The electrodes were connected through a 1KΩ resistor and the cell voltage was monitored with a multimeter (7700, Keithley instruments).

The MERC assays were compared with other experimental conditions to estimate the adsorption of the contaminant to the soil and to the electrode. So thus, electrode-free assays (natural attenuation) were compared with non-connected MERC assays (open circuit). Additionally, an electrode-free sterilized soil was compared with an electrode-free soil (natural attenuation). Sterilized MERCs treatments were performed as well to discard the existence of abiotic electrochemical reactions (Table 4.1).

***Table 4.1.*** Experimental conditions during the soil treatment.

| Assay | Electrodes | Electric connection | Biological Activity |
|---|---|---|---|
| Sterilized soil | ○ | ○ | ○ |
| Soil | ○ | ○ | ● |
| Soil + Electrode (OC) | ● | ○ | ● |
| Sterilized MERC | ● | ● | ○ |
| MERC | ● | ● | ● |

*(●) Presence; (○) Absence*

Each assay condition was simultaneously replicated three times at room temperature, and was stabilized for two weeks. After this stabilization

period and to estimate the presence or absence of biological activity a pulse of acetate 500 mM (1mL) was spiked to a soil-buried electrode from both sterilized and non sterilized-MERC. One week after acetate pulse, soil was artificially polluted (1.5 mg atrazine·$Kg^{-1}$ soil) by spiking a 3 mL anoxic solution of ATR (30 mg·$L^{-1}$) in phosphate buffer 20mM ($Na_2HPO_4/NaH_2PO_4$; pH 7). Soil samples were collected 24 hours, 1 and 2 weeks after the pollutant addition for further analysis.

### *4.3.4. Soil and chemical analyses*

The totality of the soil used in every assay was devoted to ATR extraction. So thus, 60 g of soil was first mixed with methanol solution (1mL·$g^{-1}$ of soil) and then shaken at room temperature during 24 hours on an orbital shaker. The soil solvent mixture was incubated in an ultrasonic bath for 60 minutes, in order to force the ATR extraction of soil. Finally, the mixture was centrifuged (Multifuge 3L-R Heraesus) at 5000*gs* for 10 minutes and the supernatant was completely evaporated. The extracted compounds were resuspended in 3 mL of phosphate buffer 20mM under stirring for one hour and then they were sonicated for ten seconds. All samples were filtered (Millex-GV, 0.22µm) and stored at 4 ° C. ATR concentration were determinated by HPLC-DAD analyses (Varian 9040) with C18 *Phenomenex Kromasil* column (*150x4.5mm, 5µm*) and UV detection at 220 nm. The mobile phase was 50:50 (v/v) acetonitrile-water mixture with a flow rate of 1 mL·$min^{-1}$. ATR concentration was calibrated in the range of 30- 0.625 mg/L.

### *4.3.5. Toxicity bioassays*

#### *4.3.5.1. Algal test*

Toxicity of extractable fraction was evaluated according to OECD Test Guide 201 (OECD 2008), using the green microalgae *Pseudokirchneriella subcapitata* (previously *Selenastrum capricornutum*) as it was reported

previously (Boltes et al., 2012; González-Pleiter et al., 2013). Microorganisms and culture media were purchased from MicroBioTest Inc., Belgium. Following the instructions of manufacturer, algal beads of *P. subcapitata* were reconstituted with the provided dissolving matrix and they were grown in 25 mL of the culture until reaching the logarithmic growing phase (three days). Then, the cells were used as inoculum for the toxicity test. Culture was performed at 22 ± 2 ºC under continuous light and 150 rpm of stirring. Cell growth was monitored by optical density (OD) of culture and was measured at 670 nm to control growth process (*Shimadzu UV-VIS 1800*). Test started using the prescribed amount of $5x10^4$ cells per mL and was performed into 96-well clear disposable microplates. Three replicates of each extracted soil sample, including negative control (atrazine-free soil) and blank (algae-free) were included. 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 than inoculums 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 were was refereed to the negative control assay.

#### *4.3.5.2. Genotoxicity test*

A commercial kit *UMU-Chromo Test* (EBPI-Canada), based on ISO 13829 protocol, was used to evaluate the genotoxicity effect of the soil extracts. Testing for mutagenicity was carried out as described previously (Maron and Ames, 1983). The genotoxicity of soil samples containing ATR was determined measuring the induction of umuC-gene in *Salmonella typhimurium* TA1535-pSK1002 after exposition a controlled exposition to soil extracts.

Procedure was as follows: cell culture of tester strain were grown at 37ºC overnight prior to the assay. The resulting bacterial suspension was used

to inoculate independent wells from a 96-well disposable microplate hosting the soil extracts dissolved in 10% DMSO. After inoculation the assay mixture was incubated at 37ºC for 2 h.

The expressed β-galactosidase activity in the tester strain was determined measuring absorbance of *ortho*-nitrophenyl-β-galactoside at 420 nm, after 30 min of incubation at 28ºC, using a microplate reader (RAYTO RT-2100 C).

The Induction Ratio (*IR*) that accounts for the genotoxin-dependent induction was calculated as:

$$I_R = \frac{1}{G} \cdot U$$

Where *G* was the growth factor of bacteria, which is related to the acute toxicity effects on protein synthesis. *Us* was the relative enzyme activity of cells in contact with toxicant compared to the enzyme activity of cells in blank assay (in absence of toxicants). Induction ratios greater than 1.5 indicate genotoxic activity in samples, so in order to consider a test sample not genotoxic, the induction ratio must be lower than 1.5.

#### *4.3.5.3. Plant toxicity test*

Plant toxicity assays were performed according to Seedling Emergence and Seedling Growth Test (OECD Test Guide 208) (OECD 2003). A modified Phytotoxkit test (Microbiotest, Belgium) was used to evaluate the inhibition of seed germination and root elongation in *Sorghum saccharatum* (monocotyl Sorgho). This specie is indeed included in the list of the OECD guideline for the testing of chemicals in terrestrial plants. Each test was performed in disposable petri plates with 60g of soil and 30mL of deionized water was added. Six seeds of the single specie were placed on a filter paper, separated each other by 1 cm distance. The petri

dishes were sealed with Parafilm to minimize water loss while allowing air diffusion.

Soil samples were collected 1, 7 and 14 days after the pollutant addition. The totality of the soil in every assay was immediately dried and refrigerated. Both polluted and non-polluted (control) soil samples after either MERC-treatment or natural attenuation were tested using the Phytotoxkit test. Each assay was simultaneously replicated three times at 25ºC and after 3-day of incubation both seed germination (*SI*) and root/stem elongation (*RI*) were measured for each plant.

Treatment and incubation time was calculated using the following formula:

$$SI/RI = \frac{A(A-B)}{A} \cdot 100$$

where *A* is the mean ratio between seed germination and root or stem elongation in the control soil , while *B* is the mean ratio between seed germination and root elongation in the polluted soil.

In order to monitor the activity of PSII system, plates were incubated for 3 days before they were placed in a light room at 25 °C. Then they were irrigated with distilled water for 7 days to obtain a leaf growth in order to measure the chlorophyll *a* fluorescence. The fluorescence parameters were calculated by a portable modulated fluorimeter, FMS-2 (Hansatech Instruments Ltd., UK) based on dark-adapted and light-adapted fluorescence measurements, obtaining the fluorescence induction curves, or Kautsky's curves.

The determined parameters are reported in (González-Naranjo et al., 2015). Quantum efficiency of PSII ($\Phi_{PSII}$), was calculated, that measures the proportion of light absorbed by chlorophyll associated with PSII.

## 4.4. Results and discussion

In order to validate the potential of MERC technology to restore a polluted soil we have selected an agriculture soil and the herbicide ATR as target for our clean-up strategy.

### *4.4.1. ATR-adsorption and desorption assays*

MERC-based treatment requires the use of electrically conductive material that may exhibit adsorption properties on the pollutant. So thus, both soil matrix and electrode material should be independently evaluated regarding adsorption.

The agriculture rice paddy soil used for our assays showed a high percentage of clay content (32%), which could facilitate adsorption effects of ATR on soil (Ahmad and Rahman, 2009). In contrast, the organic matter content was very low (0.10%) (Domínguez-Garay et al., 2013), which promotes a lower soil affinity for soil contaminant (Wang and Keller, 2009).

Our results with sterile soil assays confirmed that a 64% of the initial ATR added was reversible bound to the soil matrix through the applied extraction protocol. This value was used to normalize the rest of assays, excluding the physicochemical adsorption in the percentages of ATR removal.

Interestingly, the reported values for ATR showed an adsorption coefficient $K_f$ of 842.6 mL/kg , while the adsorption coefficient normalized by organic content ($\log K_{oc}$) was 1.832 (Zhong et al., 1999). The adsorption assay performed to evaluate the pollutant affinity for the soil matrix showed an adsorption of 16% of the total ATR after 90 hours of

incubation. Adsorption and desorption isotherms were determined using batch equilibration by Freundlich sorption isotherm. Desorption assay determined that only 0.47% of the adsorbed ATR can be recovered, suggesting a high hysteresis of the pollutant in this clayed soil (Ma and Selim, 1994).

Regarding the adsorption mechanism on the electrode materials (graphite plate and carbon felt), our results revealed that just 10% of the initial pollutant was adsorbed on them. All assays were corrected based on the values obtained for the sterile MERC as control, discarding a possible electroadsorption effect.

### *4.4.2 ATR removal can be enhanced by using MERCs*

We first evaluated the atrazine-removal capacity of our soil microbial population. The ATR removal after 7 days of incubation was just 4.5% higher than one found in sterilized soil (Figure 4.3) so we conclude that our paddy soil contain a microbial community with certain, although low, capacity to use ATR as substrate.

The challenge of our research is to stimulate such a population through bioelectrochemical methods. In order to evaluate the influence of an electrically conductive material buried in soil, an open circuit system was designed. In this setup electron transfer between electrodes could not flow, but electrodes still could supply microbes a redox active surface to interact with. Interestingly, the mere presence of the electrode in soil (Figure 4.3) stimulates the microbial activity leading to an increase (68.5%) of the ATR removal.

Similar results were reported for a DBT-polluted soil where 40% of the DBT was successfully removed through a process so-called “Graphite-assisted bioremediation (Rodrigo et al., 2014). A similar process had been previously described in methanogenic digesters where direct interspecies electron transfer (DIET) was elegantly demonstrated in

presence of electrically conductive material (Liu et al., 2010; Liu et al., 2012; Chen et al., 2014). Similar graphite-assisted DIET mechanism may also be playing a role for removing ATR in our polluted soils.

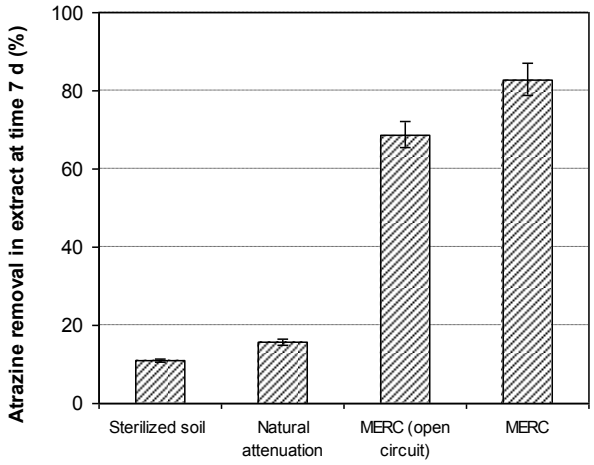


***Figure 4.3.*** Atrazine removal (excluding sorption to soil matrix) from polluted soils under different treatments. ATR removal by MERCs was represented in percentage after subtracting the control assay (Sterilized MERC). ATR removal under natural attenuation was represented in percentage after subtracting the control assay (sterilized soil). The error bars represent the standard deviation.

In spite of the efficient graphite redox support, microbes should be still more stimulated if electrons could flow among electrodes. Indeed, when electrodes were connected through a resistor (MERC systems) the ATR was efficiently removed at a rate 5-fold higher than the one obtained under natural attenuation conditions. During the first week that MERC systems were generating in soil a maximum current density of 66mA/$m^2$ while 83% of the ATR was efficiently removed. ATR removal is for sure key in the bioremediation process, but it cannot be necessarily lead to a fully restore environment due to the presence of toxic metabolites. So thus, we considered toxicological analysis of the treated soil as a “must” action in the bioremediation strategy.

### *4.4.3 Ecotoxicological analysis*

Numerous studies have indicated that ATR inhibits growth and photosynthesis of freshwater algae and algal responses to ATR vary widely depending upon concentrations used, duration of exposure, and algal species tested (Tang et al., 1997; Weiner et al., 2004).

In our hands, the growth rate $EC_{50}$ value of *P. subcapitata* was 70μg/L after 96h of algal incubation, which was determined using median-effect equation. This value was very similar to reported value in other studies with the same algal test, where EC50 after 96h was in the range 50-80 μg/L (Weiner et al., 2004; Yeh and Chen, 2006). Our ecotoxicity test showed that just one week of MERC-treatment was enough to reach toxicological level under non-toxic threshold (Figure 4.4).

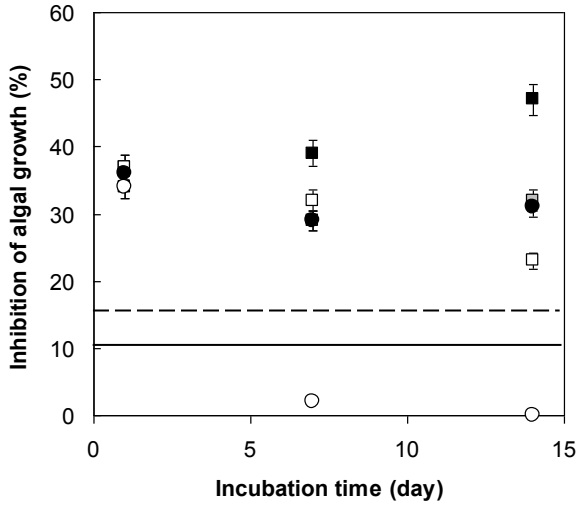


***Figure 4.4.*** Ecotoxicity of soil extractions from atrazine-polluted soil incubated under different treatments (sterile soil ■ , natural attenuation □ , MERC in open-circuit ⧈ , sterile MERC ● , MERC ○). The toxicity values were represented as inhibition of *P. Subcapitata* algal growth (%) at different periods of biodegradation treatments (1,7 and 14 days). Reference toxicity value was represented for non-atrazine-polluted soil (solid line) and non-toxic value was also provided (dashed line). The error bars represent the standard deviation.

None of the other treatments showed a similar trend. Moreover, after 14 days of MERC-treatment the ecotoxicity test indicated that the atrazine-polluted soil was fully detoxified in contrast with the rest of the treated soils that remain toxic after two weeks. The detoxification rate in MERC during the first week was 6-fold higher than under natural attenuation. No surprisingly, this value fits well with the 5-fold difference shown in ATR removal for both treatments in the same period of time.

Interestingly, our ecotoxicological analysis revealed that the electron flow facilitated in MERC treatment was required for a successful clean-up of the polluted soil.

### *4.4.4. Genotoxicological analysis*

The genotoxic effect of ATR was previously reported on aquatic species (de Campos Ventura et al., 2008; Cavas, 2011) by applying microsome mutagenicity test using different strains of *Salmonella typhimurium* (Plewa et al., 1984; Mersch-Sundermann et al., 1994; Ruiz and Marzin, 1997) or SOS chromotest using *E.coli* PQ37 (von der Hude et al., 1988; Mersch-Sundermann et al., 1994; Ruiz and Marzin, 1997).

Our genotoxicity analysis (Figure 4.5) based on umuC test revealed that a soil extract sample showed an induction ratio of 3 (note that values over 1.5 indicate genotoxicity) just after the pollutant spiking.

This value was greatly reduced in case of the MERC treatment, which after 7 days of incubation revealed complete genotoxicity elimination. The soil under open circuit conditions presented an induction ratio of 0.8 after 14 days of treatment, with a genotoxicity elimination of 77%.

Moreover, the sterilized controls (both for soil and MERC) showed no genotoxicity elimination in soil extracts after 14 days of treatment.

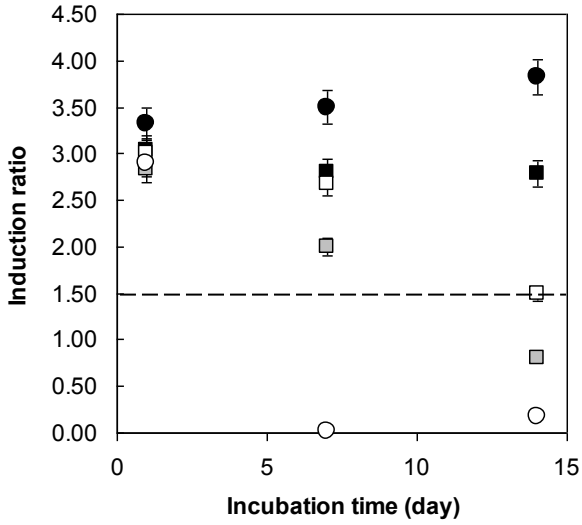


***Figure 4.5.*** Genotoxicity of soil extractions from atrazine-polluted soil incubated under different treatments (sterile soil ■ , natural attenuation □ , MERC in open-circuit ▫ , sterile MERC ● , MERC ○). The genotoxicity values were represented as induction ratio of β-galactosidase enzyme in *S. typhimurium* bacterium from the umu-assay during the different bioremediation treatment (1, 7 and 14 days). A Non-genotoxic value of 1.5 was provided (dashed line). The error bars represent the standard deviation.

### *4.4.5. Phytotoxicological analysis*

Root and stem elongation of *Sorghum saccharatum* species were presented (Figure 4.6) as measurement of the soil toxicity under different conditions and incubation periods. Soil samples collected 24h after ATR addition were evaluated and showed that plant root and stem elongation were inhibited, 45% and 28% respectively, in comparison with germinated seeds in contact with non-polluted soil. No significant difference was observed between natural attenuation and MERC soil systems in the root length after 14 days in incubation, where the inhibition on root length was between 20-47%. In contrast, the stem elongation revealed a noticeable difference between the different treatments. The soil under natural

attenuation conditions presented an inhibition of 55% in stem length, while the incubated soils under MERC settings showed and inhibition of just 20%.

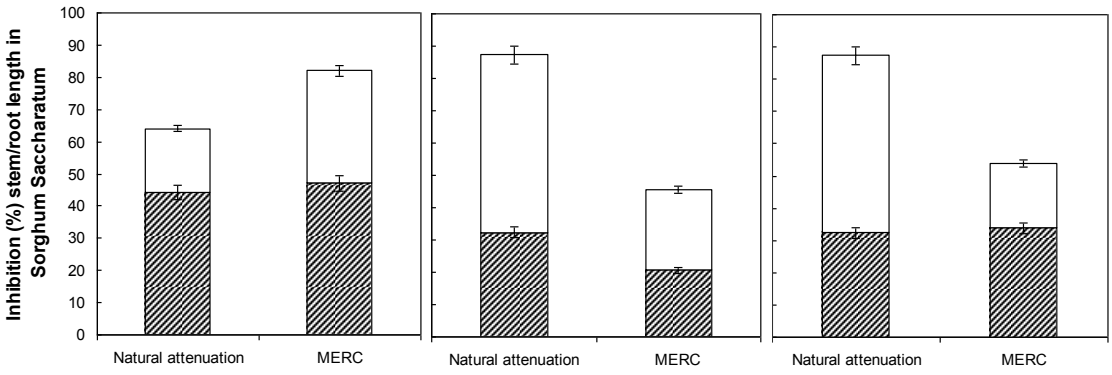


***Figure 4.6.*** Phytotoxicity of soil extractions from atrazine-polluted soil incubated under natural attenuation and MERC conditions. The phytotoxicity values were represented as inhibition % on stem (plain area) and root (striped area) length of *Sorghum saccharatum* in contact with soil under different periods of biodegradation treatments (1, 7 and 14 days). The error bars represent the standard deviation.

Using photosynthetic organisms to monitor pollutants in the environment is representative because they can also assess toxicity by identifying changes in their photophysical behaviour in the presence of a given toxicant. Thereby, it is known that ATR is an inhibitor of PSII activity (Rutherford and Krieger-Liszkay, 2001). Furthermore, (Conrad et al., 1993) reported how the yield of chlorophyll fluorescence in the presence of ATR, was successfully correlated with the ATR concentration.

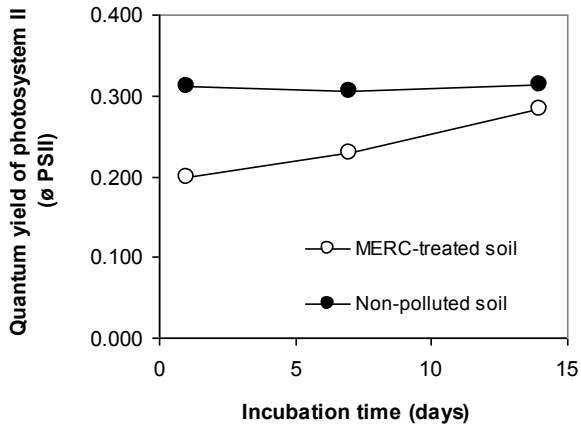


***Figure 4.7.*** Quantum yield of photosystem II (ΦPSII) for MERC-treated soil after two weeks in incubationn comparing with non-polluted soil.

However, most studies were developed with periphyton organisms (Laviale et al., 2011) and just a few studies have been developed with plant species (Ralph, 2000; Hussain and Reigosa, 2011; González-Naranjo et al., 2015) in presence of ATR herbicide (Iriel et al., 2014) to conclude that quantum yield of photosystem II (ΦPSII) was the most sensitive bioindicator for ATR.

Using *Sorghum saccharatum* species, the ΦPSII revealed very similar values for both non-polluted soil and MERC- soil after two weeks of treatment (Figure 4.7)

Our results demonstrate that MERCs are able to enhance the ATR microbial removal in a short incubation period, despite the unfavourable characteristics (low conductivity and content in organic matter) of our paddy soil. The ATR removal was well correlated with the detoxification results revealed by *P.subcapitata, S. typhimurium* and *S.saccharatum* tests, confirming the successful clean-up performance on polluted soils.

MERC system appears as a new tool for the *in situ* bioremediation of polluted environments, where the presence of unlimited terminal electron acceptor (anodes) would allow microorganisms to perform oxidative metabolism beyond the natural conditions found in soil. In consequence MERC offers a malleable terminal electron acceptor capable of adapting to *in situ* treatments and avoiding energy-associated cost of traditional *ex situ* methods (biopiling, landfarming).

## 4.5. Acknowledgments

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# Bioelectroventing: an electrochemical-assisted bioremediation strategy for cleaning-up atrazine polluted soils

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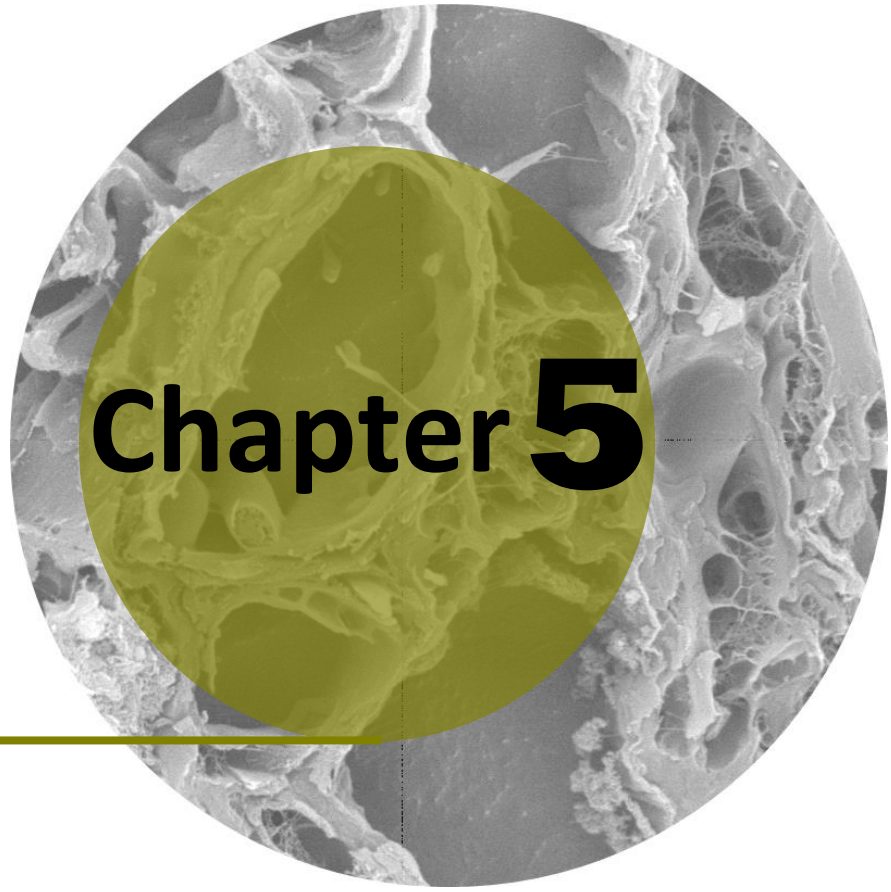
Chapter 5

# Bioelectroventing: an electrochemical-assisted bioremediation strategy for cleaning-up atrazine polluted soils *

## 5.1. Abstract

The absence of suitable terminal electron acceptors (TEA) in soil might limit the oxidative metabolism of environmental microbial populations. *Bioelectroventing* consists in a bioelectrochemical strategy that aims to overcome the electron acceptor limitation and maximize the metabolic oxidation with the purpose of enhancing the biodegradation of a pollutant in the environment. Microbial Electroremediating Cells (MERCs) are indeed the configurations to perform such a *bioelectroventing* treatment. The objective of this work was to use MERCs principles, under different configurations, for stimulating soil bacteria to achieve the complete biodegradation of the herbicide $^{14}$C-atrazine (ATR) to $^{14}CO_2$ in soils. Our study concludes that using electrodes at a positive potential (+600mV (vs. Ag/AgCl)) ATR-mineralization was enhanced by 20-fold respect the natural attenuation in electrode-free controls. Furthermore, an ecotoxicological analysis of soil after the bioelectroventing treatment revealed an effective clean-up task in less than 20 days. We also report an overall profile of the $^{14}$C-ATR metabolites and a $^{14}$C mass balance in response to the different treatments. The remarkable impact of electrodes on soil bioremediation suggests a promising future for this emerging environmental technology.

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## 5.2. Introduction

Biodegradation is a major process in the complete mineralization of aromatic compounds in the environment and is considered an in situ treatment that avoids excavation and emission control costs, therefore is considered as cheap and clean technology (Khan et al., 2004). However, only a certain proportion of these compounds can be degraded by the soil microbial community (Katayama et al., 2010), being the process highly dependent on the presence of indigenous degrading species as well as several abiotic factors (soil properties, temperature, plant presence, soil moisture) with considerable effects on the herbicide sorption and bioavailability (Ngigi et al., 2011). In flooded soils under strong reductive conditions the deficiency of suitable terminal electron acceptors (TEA) limits microbial respiration and in consequence a variety of organic pollutants persist in waterlogged soil or sediment (Liu and Suflita, 1993). Traditional bioremediation techniques have overcome this TEA constrain supplying additional electron acceptors like oxygen (bioventing) (Kabelitz et al., 2009; Frutos et al., 2010), humic acids (Lovley, 2000) or nitrates (Yu et al., 2014), to stimulate microbial metabolism but this actions result in reagents cost and causes secondary pollution concerns if nitrite is generated (Pandey, 2012).

MERCs devices are indeed bioelectrochemical variants of Sediment Microbial Fuel Cells (SMFCs), although they differ in the operation mode. While SMFCs aim to maximize the power generation (Watts), MERCs aim to reach maximum current production (Amperes) by maximizing metabolic oxidation of organic/inorganic soil compounds and consequently the degradation rate by microorganisms (Rodrigo et al., 2014).

So thus, we have now explored electrochemical-assisted scenarios where oxidative metabolism can be enhanced to bioremediate polluted environments. Since Zhang *et al*. (2010) reported for first time that a graphite electrode could serve as an electron acceptor for the degradation

of toluene and benzene in polluted slurries, several studies have reported microbial electrochemical system to enhance the biodegradation of pollutants of different chemical nature: petroleum hydrocarbons (Morris and Jin, 2012; Zhang et al., 2014; Daghio et al., 2016), PAHs (K. Chandrasekhar, 2012; Wang et al., 2012; Yan et al., 2012; Rodrigo et al., 2014; Li and Yu, 2015; Li et al., 2015; Sherafatmand and Ng; Li et al., 2016), phenol (Huang et al., 2011), nitrobenzene (Liang et al., 2014), pesticides (Cao et al., 2015), herbicides (Yeh and Chen, 2006; Rodrigo Quejigo et al., 2016) and chlorinated organics (Yeh and Chen, 2006; Chun et al., 2013; Liu et al., 2013; Yu et al., 2016). Moreover, not just pollutant removal but an efficient clean-up was demonstrated by ecotoxicological analysis of a DBT-polluted soil after MERC treatment (Rodrigo et al., 2014). Similar results were obtained after genotoxicological and phytotoxicological assays in atrazine- polluted soils when the microbial oxidation was stimulated with an electrode (*Chapter 4 of this thesis*).

The redox gradient in MERC is established spontaneously across the soil-water interphase as a result of spatially segmented reduction-oxidation reactions establishing an electron transport route between electrodes (Li and Yu, 2015). This electrode potential is typically negative and can result insufficient for leading to an effective transformation of recalcitrant compounds due to the high ohmic internal resistance that present this soil systems. This limitation has been analyzed in depth throughout the *Chapter 2* of this thesis. To overcome this limitation, several studies have applied an external voltage between anode and cathode setting a most favorable redox scenario for soil microorganisms (Aulenta et al., 2007; Chun et al., 2013). More recently Rodrigo *et al.* (2016), even reported that an electrode potential as high as 600mV (vs Ag/AgCl) can show a strong impact in the isoproturon herbicide mineralization.

Another pollutant that has been subject of study through bioelectrochemical strategies by MERCs is the atrazine (2-chloro-4-ethylamino-6-isopropyl amino-1,3,5-triazine) (*Chapter 4* of this thesis). ATR degradation is mainly a result of microbial activity, being a favourably degraded by soil microorganisms (Mudhoo and Garg, 2011) . A large variety of them such as *Pseudomonas* sp ADP (de Souza et al., 1998), *Agrabacterium radiobacter* J14a (Struthers et al., 1998) or *Nicordioides* (Topp et al., 2000) degrade ATR through co-metabolic processes that lead to the formation and accumulation of ATR metabolites. Although ATR was banned in the European Union in 2003 (Bethsass and Colangelo 2006), it is still widely used around the world due to its low cost and high effectiveness for control of weeds in crops such as corn and sorghum. Numerous studies have indicated that ATR inhibits growth and photosynthesis of freshwater algae and algal responses to ATR vary widely depending upon concentrations used, duration of exposure, and algal species tested (Tang et al., 1997; Weiner et al., 2004).

The main objective of our work was to stimulate soil native bacteria by *bioelectroventing* strategy in order to enhance the complete biodegradation of $^{14}$C-ATR to $^{14}C$-$CO_2$ using different electrode configuration, in combination with ecotoxicological analysis of treated soil.

## 5.3. Material and methods

### *5.3.1. Chemicals*

Uniformly $^{14}$C ring-labeled ATR (2-chloro-4-ethylamino-6-isopropyl amino-1,3,5-triazine) ($^{14}$C-ATR) with a specific radioactivity of 1.2 kBq $\mu g^{-1}$ and a radiochemical purity >98.5 % according to the producer was purchased from GE Healthcare (Little Chalfont, UK). $^{14}$C-labelled and non-labeled ATR (standard) were mixed and dissolved in methanol to reach a final concentration of 2.5 µg/µL and a specific radioactivity of 63 $Bq \cdot \mu g^{-1}$.

Non-labeled ATR and the metabolites standards 2-hydroxyatrazine (HA-ATR), deethylatrazine (DEA) and deisopropylatrazine (DIA) were obtained from Sigma Aldrich (Fluka). 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).

### *5.3.2. Soil sampling*

The soil material (aric anthrosol) was formed on an agricultural field (Hohenwart; latitude 48.250°, longitude: 11.567°, altitude 472 m) in Germany without ATR 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) and the conductivity, 0.247 mS/cm, was reported by Rodrigo *et al.* (2016). Soil samples were taken at 0-20 cm depth and were stored in hermetically sealed plastic bags at -20 ºC for laboratory analysis according to guidelines of the Organization for Economic Cooperation and Development (OECD) from 1995. The soil samples were unfrozen following the incubation protocol reported by Folberth *et al.* (2009).

### *5.3.3. Degradation experiments*

#### *5.3.3.1. $^{14}$C-ATR application*

0.1 mL of ATR standard with a total radioactivity of 15.75 kBq was applied dropwise with a Hamilton syinge to an aliquot of 3.5 g of oven-dry and pulverized soil sample and homogeneously mixed until a homogeneneous distribution of the herbicide was achieved. After evaporation of the organic solvent (methanol) the soil aliquot was mixed for another 2 minutes with fresh soil (46.5 g dry weight) resulting in a total sample amount of 50 g dry soil per experiment with a herbicide concentration of 5 (± 0.1) $\mu g \cdot g^{-1}$ soil (dry weight). The total spiked soil

sample was transferred to the opaque incubation 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 + 50 mL extra deionised water). Water evaporation was compensated weekly by the addition of deionized water.

### *5.3.3.2. Experimental set-up and operational conditions for mineralizing $^{14}$C-ATR*

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) that 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 process. The trapping solution was collected three times per week and the traps were filled with fresh NaOH solution. 2 mL aliquots from collected NaOH were mixed with 3 mL of scintillation cocktail 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 the exposition for the cathode electrode to the atmospheric oxygen without disturbing the anoxic environment surrounding the soil-buried anode. 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.

In order to evaluate the capability of soil microbial community to degrade $^{14}$C-ATR under flooded conditions, the following four different configurations (Figure 5.1) were set up:

a) MERC (open-circuit): anode and cathode were disconnected, so the redox potential of the anode was determined by the redox potential differences across sediment/water.

b) MERC (closed-circuit): A MERC configuration where anode and cathode were connected by a cooper wire using 5 Ω external resistor (R) This low resistor allows the system to operate at maximum current intensity. More technical details can be found in *section 4.3* of this thesis.

c) Snorkel (short-circuit): Carbon felt electrodes were placed vertically, so half of the electrode was buried in the soil and the other half was placed in the flooded water body. The electro-conductive material represents the complete redox potential spectrum across sediment/water/air. This configuration allows system to operate at maximum current intensity but, unlike configuration B, the redox potential is variable along the material.

d) pol-MERC: 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).*

All systems were compared with a system with the response of a soil microcosm under flooded conditions in absence of any conductive material (natural attenuation). Indeed, electrode-free soils were set up in the laboratory under the same water content, temperature and $^{14}$C-ATR concentration than the rest of the electrode-assisted assays.

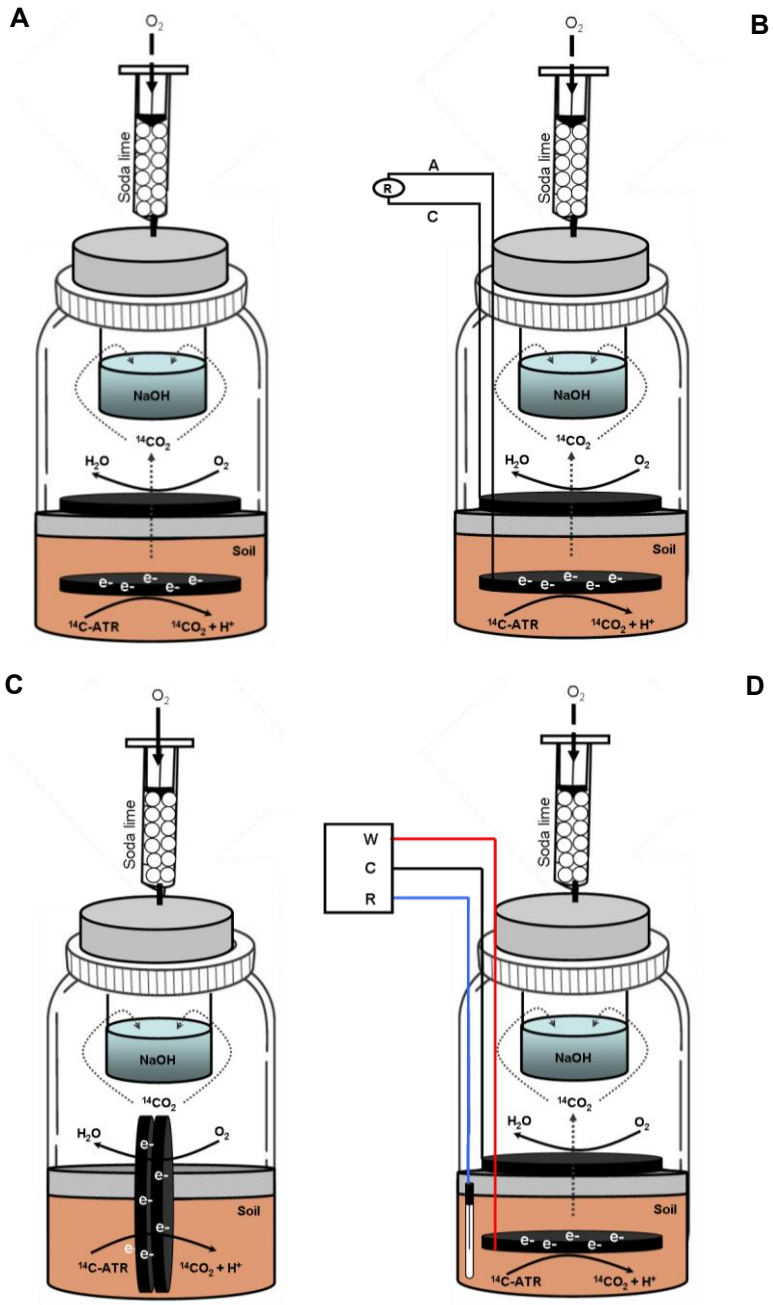


***Figure 5.1.*** Experimental design for monitoring the $^{14}$C-ATR mineralization under different configurations. A) MERC under open-circuit conditions, B) MERC under closed-circuit conditions, C) Snorkel configuration. D) pol-MERC, the 3 electrode system is controlled by a potentiostat for polarizing the anode (working electrode) at 0.6 V vs Ag/AgCl reference electrode.

Graphite felt was selected as electrical conductive material (*Mersen*, 4.5cm diameter, 0.5 cm thickness; 0.7 $m^2/g$ of surface area) for both anodes and cathodes. This material showed no ATR adsorption and very adequate mechanical properties to conform the system. Copper wires used for connections were sealed with a conductive epoxy resin (*Circuit Works*, USA) and isolated with a nonconductive epoxy resin (*Araldit Ceys*, Spain) to a graphite rod (*Mersen*, Spain) that was inserted in the graphite felt. The anode was buried at the bottom of the soil layer (anode), while the cathode was placed at the soil upper layer in direct contact with the water body.

#### *5.3.3.3. $^{14}$C-ATR adsorption assays in different electro-conductive materials*

Several adsorption assays were conducted to investigate the adsorption of $^{14}$C-ATR on different conductive materials: graphite plate, graphite paper, carbon felt and graphite rod. 50 mL eppendorf tubes were filled with 39 mL of deionized water and $^{14}$C-ATR standard was added to vide a final concentration of 2.5 µg/µL and a specific radioactivity of 63 $Bq \cdot \mu g^{-1}$. Three replicates were conducted for each electrode material and the eppendorf tubes were shaken overhead continuously. At different time intervals aliquots of 1 mL were sampled to measure the radioactivity until the equilibrium for adsorption of $^{14}$C-ATR was reached. Each sample was mixed with 4 mL Ultima Gold XR and measured in a liquid scintillation counter (*Tricarb* 1900 TR, Packard, Dreieich, Germany). A control was performed under the same conditions but in the absence of material.

#### *5.3.3.4. Mineralization assays*

The mineralization of $^{14}$C-ATR to $^{14}CO_2$ was studied in a closed aerated laboratory system (1 in Figure 5.2), as described above. The soil samples were incubated at 30 $\pm$ 0.1 ºC (4 in Figure 5.2) for 20 days (short-term assay). The first sampling of the NaOH trap was performed 24 hours after

$^{14}$C-ATR addition and then sampling was performed three times per week. The cumulative $^{14}CO_2$ was expressed as percentage of the applied $^{14}$C-ATR.

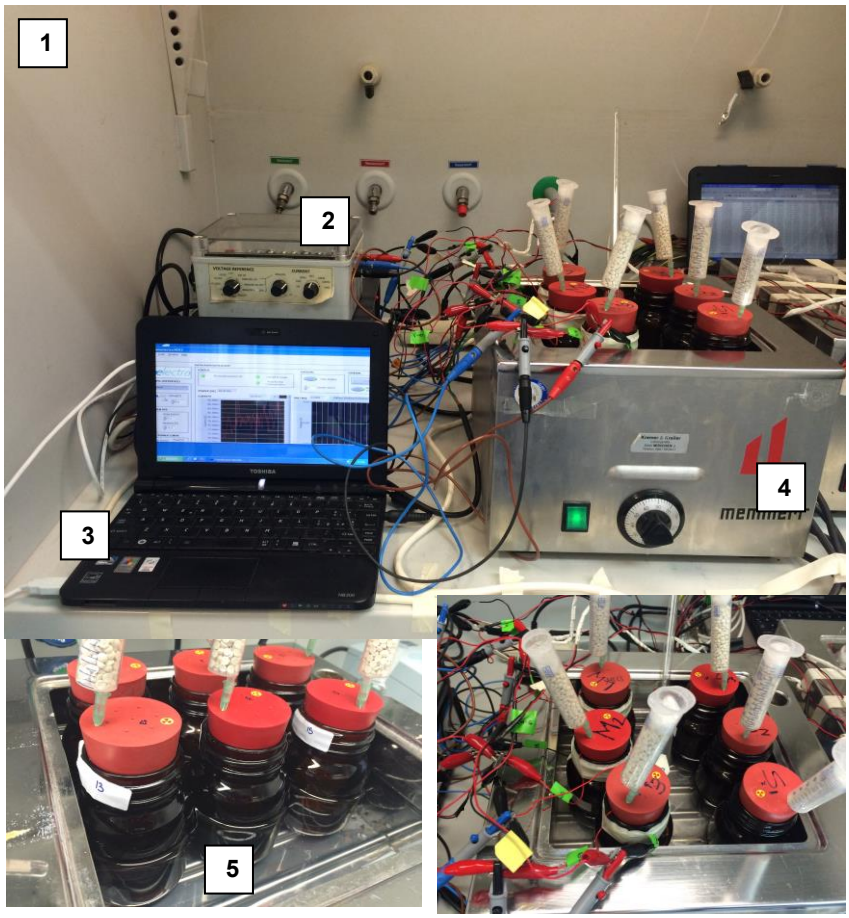


***Figure 5.2.*** Experimental set-up for bioremediating an atrazine polluted soil. 1) Fume hood; 2) Potentiostat; 3) Computer (NEV3 Software *Nanoelectra*); 4) Water bath at 30ºC; 5) Experimental microcosms.

After 20 days of incubation, three of the five open-circuit replicates were sacrificed for soil analysis. The other two replicates were shifted into pol-MERC conditions. So thus, the anode was poised at +600 mV versus Ag/AgCl reference electrode (RE-5B, *BASi*, United Kingdom) (+199 mV

vs. SHE) by using a potentiostat (NEV2 *Nanoelectra*). $^{14}$C-ATR mineralization was monitored comparing with control assays (natural attenuation) for another 80 days under the influence of the artificial anode poisoning (long-term assay).

Short-term parallel assays were performed under the same conditions (five different configurations) although using non-radioactive ATR. Each different configuration was set up with 6 experimental replicates; three of them were collected and extracted after 7 days and the rest after 20 days. The soil extracts sampled after 7 and 20 days were analyzed by HPLC to determine the metabolite pattern and to conduct ecotoxicological test.

#### *5.3.3.5. Analysis of radioactive soil extracts*

After 20 days of incubation soil aliquots and carbon felt electrodes were separately extracted with methanol in an accelerated solvent extractor (ASE 200, *Dionex*) at 90 ºC, with a pressure of 10 MPa. Aliquots of 0.1 mL of each extract were mixed with 5 mL Ultima Gold XR and measured by liquid scintillation counting. Subsequently, extracts were concentrated on a rotary evaporator to a volume of 2-3 mL and adjusted to 250 mL distilled water for cleaning up with Isolate Triazine columns (500 mg, *Separtis*, Grenzach-Wyhlen, 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 1 mL with a rotary evaporator and further concentrated under a gentle nitrogen-stream to volumes between 40 and 160 µL, depending on the total $^{14}$C-radioactivity of the samples. 20 µL of each soil extract were injected to a HPLC system equipped with a L-6200 Intelligent Pump (*Merck-Hitachi*, Darmstadt, Germany) a UV/VIS detector (220 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 0.003M $KH_2PO_4$, pH 3 (A) and acetonitrile (B) at a flow rate of 0.8 ml min-1. The gradient

program was: T 0min 20% A, T 10min 38% A, T 24min 75% A, T 29min 75% A, T 33min 20% A, T 40 min 20%A. Parent compound and metabolites were identified by comparison of their retention times with reference substances.

After ASE, soil material was homogenized intensively. Three aliquots (each 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.

#### *5.3.3.6. Electrochemical characterization*

The anode potential in MERC systems were continuously poised at + 600 mV versus Ag/AgCl (RE-5B, *BASi,* United Kingdom) using a potentiostat (NEV1-3 *Nanoelectra,* Madrid, Spain) (2 in Figure 5.2) and the resulting current was recorded every 60 seconds (3 in Figure 5.2). Both, anode and cathode potential in pol-MERC and the difference of potential between electrodes in MERCs were recorded continuously with a multimeter (7700, *Keithley* instruments).

Cyclic voltammetry (CV) was performed at the beginning of the experiment (24h) and at the end of the different times in incubation (7 and 20 days) to characterize the electrochemical activity of soil microorganisms by imposing at a scan rate of 1 mV/s from the open circuit potential by a potentiostat (NEV3 *Nanoelectra,* Madrid, Spain).

#### *5.3.3.7. Algal test*

Algal growth inhibition test was conducted for the extractable non labeled soil residues fraction according to OECD Test Guide 201 (OECD 2008), using the green microalgae *Pseudokirchneriella subcapitata* (previously *Selenastrum capricornutum*) as it was reported previously (Boltes et al., 2012; González-Pleiter et al., 2013). Microorganisms and culture media were purchased from MicroBioTest Inc., Belgium. Following the instructions of manufacturer, algal beads of *P. subcapitata* were reconstituted with the provided dissolving matrix and they were grown in 25 mL of the culture until reaching the logarithmic growing phase (three days). Then, the cells were used as inoculum for the toxicity test. Culture was performed at 22 ± 2 ºC under continuous light and 150 rpm of stirring. Cell growth was monitored by optical density (OD) of culture and was measured at 670 nm to control growth process (*Shimadzu UV-VIS 1800*). Test started using the prescribed amount of $5x10^4$ cells per mL and was performed into 96-well clear disposable microplates. Three replicates of each extracted soil sample, including negative control (atrazine-free soil) and blank (algae-free) were included. The dilution factor for the polluted wells was 0.9268 (190 µL extracted soil sample + 10 µL growth media + 5 µL alga culture containing $9 \cdot 10^4$ cells/mL). Plates were incubated during three days under the same conditions of light and temperature than inoculums 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 referred to the negative control assay.

## 5.4. Results and discussion

### *5.4.1. $^{14}$C-ATR adsorption in different electro-conductive materials.*

The higher adsorption was observed in graphite paper (A in Figure 5.3), that after 18 hours in incubation 86% of the applied $^{14}$C-ATR was retained in the material. The rest of materials showed lower adsorption behavior

than the graphite paper: 7.7% in graphite plate (B in Figure 3), 2.2% in carbon felt (C in Figure 5.3) and 1.2% in graphite rod (D in Figure 5.3).

Regarding to the results and considering the convenience physical-mechanical properties, the high surface area, and the inexpensive cost, the material used to the electrodes for mineralization assays was carbon felt and graphite rod.

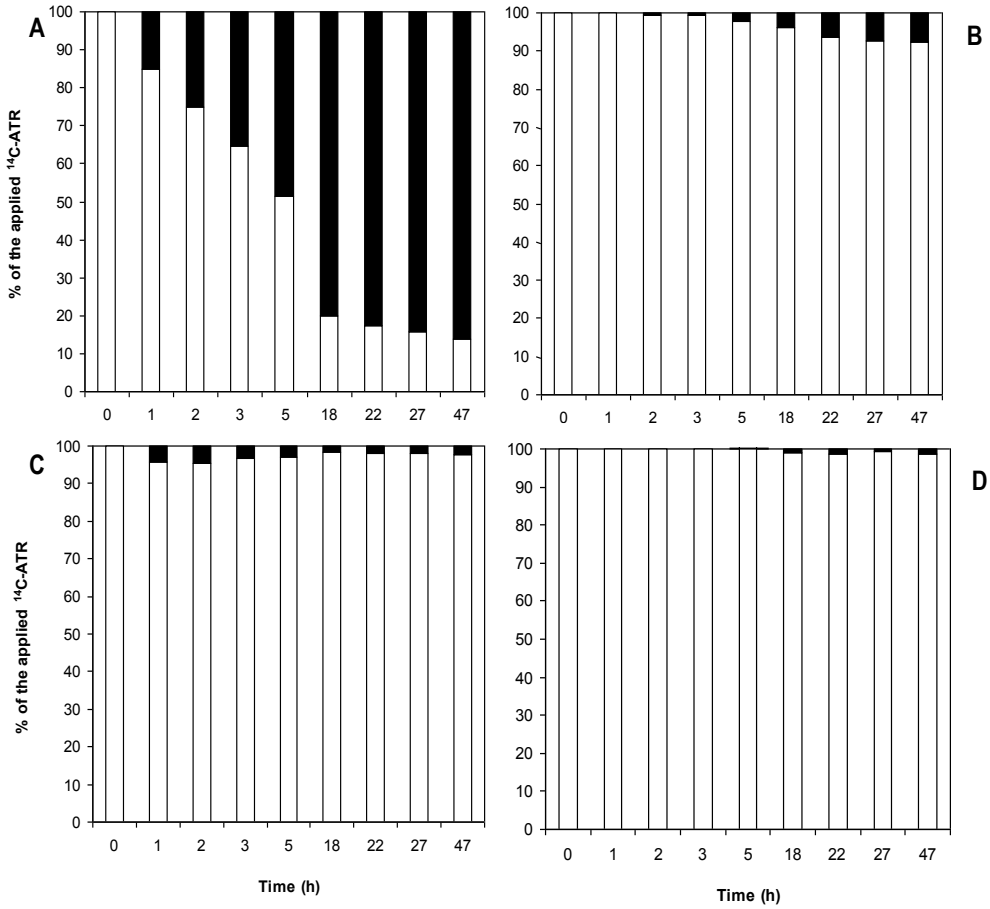


***Figure 5.3.*** $^{14}$C-ATR adsorption in different electrical conductive material: graphite paper (A), graphite plate (B), carbon felt (C) and graphite rod (D)

### *5.4.2. Bioelectrochemical interrogation of the microbial redox activity*

In order to evaluate the degradation activity of soil microorganisms exposed to different experimental conditions and for understanding the relationship between the cumulative mineralization and the increased biological activity, several cyclic voltammetries (CV) were developed along the experimental phase to study the electron transfer interactions between soil microorganisms and anodes at the different incubation stages (Figure 5.4). CV after 24h revealed redox peaks at approximately -400mV and 100 mV due to abiotic processes for the oxidation or reduction of soil compounds. These peaks were also detected at the different incubation times (7 and 20 days) in open-circuit MERC and closed-circuit MERC systems, showing similar current production under each of them. pol-MERC showed a peak at 0.2 V (vs Ag/AgCl) after 7 and 20 days, which appear as well in closed-circuit MERCs but with considerable lower intensity. This inflexion didn't appear in the open-circuit profile. The increased signal of the current peak (10-fold regarding to open-circuit MERC and closed-circuit MERC) revealed the anode enrichment of electroactive microbial communities, which may be due to an increase in the cell density on the electrode surface (Fricke et al., 2008) or to an increase in the microbial metabolism that accelerate the electron transfer.

The higher enrichment of electroactive microbial communities was confirmed by the measurement of anode potential in open-circuit MERC at the end of the experiment (20 days), that reached -480 mV potential in pol-MERC, comparing with open-circuit MERC and closed-circuit MERC systems where the potentials were –250 mV and – 300 mV respectively. So thus CVs reveal a different redox interaction and microbial activity occurring on the electrodes under different treatments what may explain the differences observed in terms of ATR biodegradation.

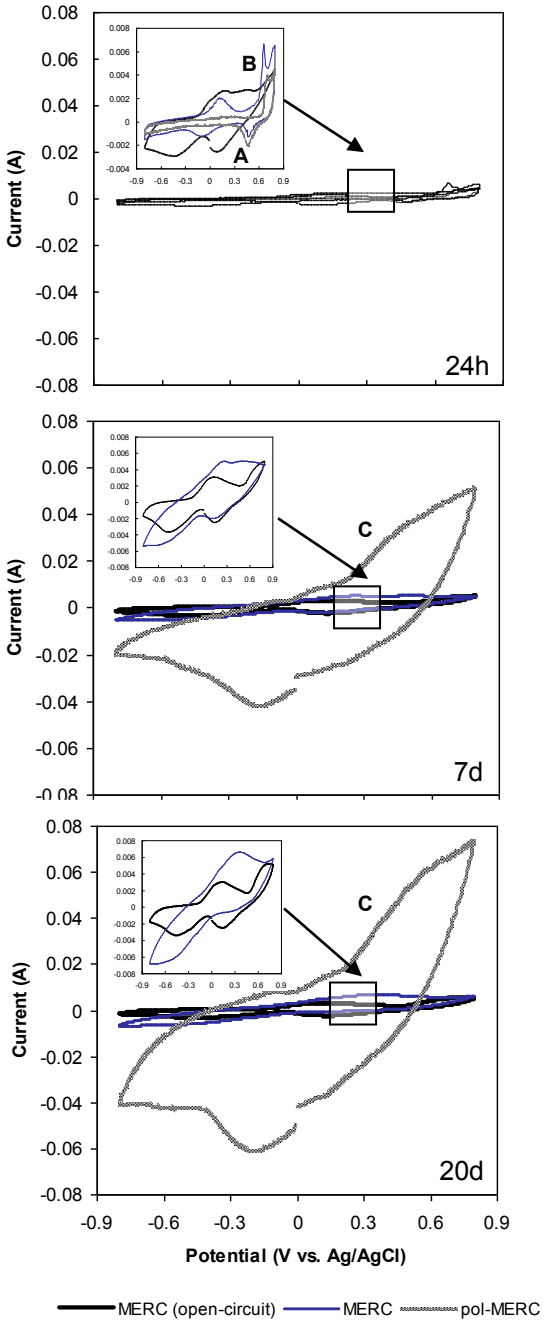


***Figure 5.4.*** Cyclic voltammetry analysis at different incubation periods (1, 7 and 20 days) for different configurations: A- MERC (open-circuit), B- MERC (closed-circuit) and C- pol-MERC recorded at 1mV/s from 0.8V and -0.8V and back to 0.8V (vs. Ag/AgCl).

### *5.4.3. $^{14}$C-ATR mineralization under short-term assay*

Independent short-term mineralization assays were performed to investigate the influence of the electrodes potential on the $^{14}$C-ATR degradation. After 20 days of incubation, the cumulative mineralization reached a value of 5% in pol-MERC whereas under the rest of treatments reached values below 1%, except the snorkel configuration that achieved a value of 3% (Figure 5.5).

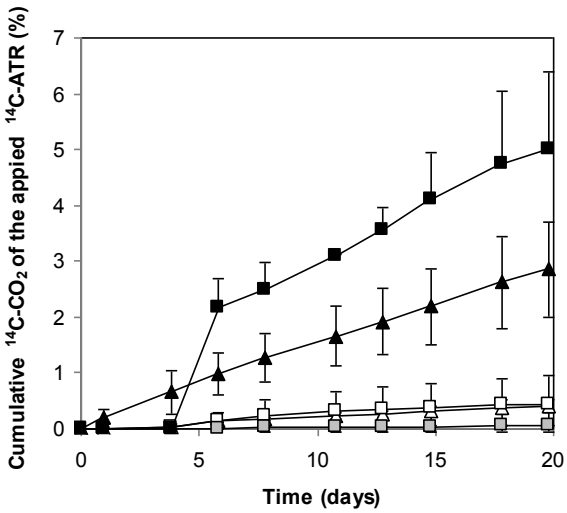


***Figure 5.5.*** Cumulative mineralization of $^{14}$C-ATR for short-term assay (20 days) at different configurations: △ Natural attenuation, □ MERC (open-circuit), ▫ MERC (closed-circuit), ▲ Snorkel and ■ pol-MERC. The error bars represent standard deviation.

So thus, setting an electrode at 600mV the mineralization was enhanced over 10-fold regarding to the electrode-free soil. The low cumulative mineralization in the electrode-free soil was an expected result given the absence of ATR application history in our soil in the past 20 years (Schroll et al., 2006; Getenga et al., 2009; Ngigi et al., 2011).

Although the highest mineralization occurred under the influence of a positive electrode potential (pol-MERC), the results shown by the snorkel

configuration suggest a favourable scenario for enhancing the microbial activity. Actually snorkel configuration, a system where the absence of ohmic internal resistance ($I \cdot R_{internal}$) promote the ion mobility (Domínguez-Garay et al., 2013), showed better response than MERC under either closed- or open-circuit (Figure 5.5).

### *5.4.4. $^{14}$C-ATR biodegradation pathway*

Although $^{14}$C-ATR was highly mineralized in our bioelectrochemical system, the analysis of the residual ATR metabolites will make us to suggest a biodegradation pathway. In that sense, ATR biodegradation has been reported to follow chemical hydrolysis through *N*-dealkylation and dechlorination processes prior to ring cleavage (Fang et al., 2015). Either single species or microbial consortia are responsible for reactions that involve three hydrolases genes atzA, atzB, atzC that encode for dehalogenate and dealkylate ATR in a stepwise fashion(Smith et al., 2005). Chemical hydrolysis results in the formation of hydroxyatrazine (HA-ATR) and *N*-dealkylation results in the formation of deethylatrazine (DEA-ATR) and/or deisopropylatrazine (DIA-ATR) (Loos and Niessner, 1999). Complete degradation of ATR has been observed through continued hydroxylation of the triazine ring and the formation of ammiline, ammelide, and cyanuric acid prior to ring cleavage, and finally to $CO_2$ and $NH_3$ (çWackett et al., 2002). It is known that ATR metabolites are relatively persistent in groundwater and soil/sediment (Enoch et al., 2007).

Analysis of soil samples subject to our treatments revealed the presence of ATR and three of its metabolites (Table 5.1): deisopropylatrazine (DIA-ATR), deethylatrazine (DEA-ATR) and hydroxyatrazine (HA-ATR). After 7 days of pol-MERC treatment, the ATR concentration was 2.6-fold lower (0.70 μg $g^{-1}$ dry soil) than under natural attenuation (electrode-free) (1.82 μg $g^{-1}$ dry soil). A similar situation occurred when pol-MERC was compared with the rest of configurations.

| Atrazine and metabolites | Structural formula | Incubation time (d) | Electrode free soil | MERC (open-circuit) | MERC (closed-circuit) | pol-MERC | Snorkel |
|---|---|---|---|---|---|---|---|
| ATR |  | 7 | 1.829 | 1.631 | 1.592 | 0.694 | 1.279 |
| | | 20 | 1.026 | 0.759 | 1.026 | 0.327 | 0.845 |
| HA-ATR |  | 7 | 0.103 | 0.093 | 0.092 | 0.159 | 0.081 |
| | | 20 | 0.147 | 0.134 | 0.123 | 0.227 | 0.131 |
| DEA-ATR |  | 7 | 0.017 | 0.022 | 0.024 | 0.026 | 0.015 |
| | | 20 | 0.019 | 0.020 | 0.039 | 0.045 | 0.029 |
| DIA-ATR |  | 7 | 0.006 | 0.011 | 0.014 | 0.015 | 0.005 |
| | | 20 | 0.008 | 0.017 | 0.019 | 0.195 | 0.016 |

***Table 5.1.*** Atrazine and its degradation products (µg/g dry soil) in methanol-soil extractions from the different treatments and incubation times (7 and 20 days).

The differences in soil ATR levels between pol-MERC and natural attenuation were kept to ca. 3-fold after 20 days of incubation.

Regarding ATR-metabolites, HA-ATR coming from hydrolysis reaction was most abundant regardless the treatment assayed. The highest concentration of HA-ATR was detected in pol-MERC treated soil, after 20 days of incubation. Interestingly, HA-ATR formation was associated with anaerobic or oxygen-limited conditions, like those found in flooded soil (Armstrong et al., 1967; Chung et al., 1996) reported a linear isotherm study where HA-ATR showed 6-fold higher adsorption to soil than ATR did. Regarding DIA-ATR metabolite, it was detected at high concentration in pol-MERC-treated soil, 10-fold higher than the rest electrodes-assisted systems (open-circuit MERC, closed-circuit MERC and snorkel) and 24-fold higher than the natural attenuation.

### *5.4.5. Ecotoxicity assays reveal an effective clean-up*

In order to evaluate the restoration degree of the soil, a setup of ecotoxicological tests were performed after the incubation period (Figure 5.6).

Interestingly, after treating the soil using pol-MERC for 20 days , soil extracts did not exhibit any inhibition of the algal growth in contrast with the inhibition (43%) shown under natural attenuation. Similar results were observed for MERC under either closed- or open-circuit, that presented an inhibition in algal growth ca. 35-40%,

It has been reported that ATR exhibited a higher toxicity on algal species than its chlorinated primary metabolites (Enoch et al., 2007; Ralston-Hooper et al., 2009). This fact is consistent with the lower toxicity shown in those treatments showing high level of ATR mineralization like pol-MERC and snorkel.

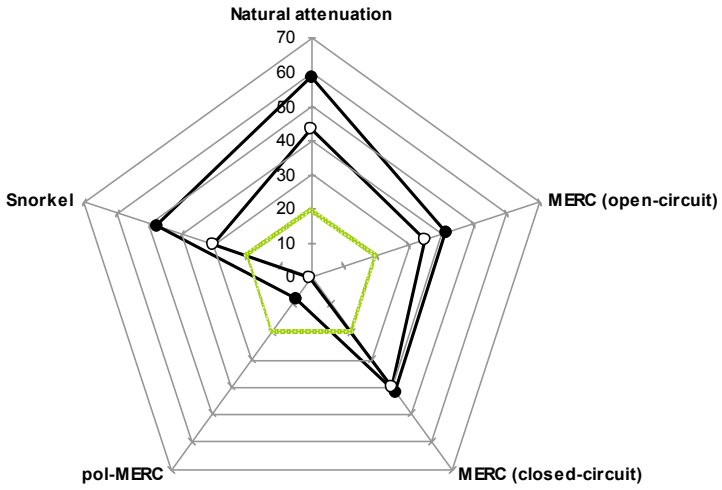


***Figure 5.6.*** Toxicity of soil treated under different configurations. The toxicity values were represented as inhibition of *P. subcapitata* algal growth (%) at different incubation times: 7 days (●) and 20 days (○). Reference non-toxic value was also provided (green line) to lower toxicity.

### *5.4.6. Characterization of the $^{14}$C-ATR mass balance*

In order to give insights into the factors governing ATR mineralization we proceed to perform a $^{14}$C- mass balance at the different experimental set-ups after 20 incubation days (Figure 5.7). So thus, we analysed the methanol extractable residues (ER) from soil and electrodes together with non-extractable residues (NER). The $^{14}$C-balances ranged between 82% and 88% of the applied $^{14}$C-ATR since volatilization of dealkylated amino metabolites may have led to $^{14}$C-losses (Loos and Niessner, 1999).

Soil ER levels showed a high variation regardless the treatments after 20 days in incubation (Figure 5.7). In pol-MERC systems, where the cumulative $^{14}$C-ATR mineralization was higher, the ER reached just 13%, while under natural attenuation in natural attenuation assays the ER was

slightly under 50%. Under closed-circuit MERC and snorkel configurations the ER reached intermediate values of approximately 37%.

Regarding to NER in soil, a similar distribution was observed for natural attenuation (38%), open-circuit MERC (34%) and snorkel (37.6%) configuration. Interestingly, lower values were obtained when electron flow flowed between electrodes, reaching 30% in closed-circuit MERCs and 27% in pol-MERC configuration. The decrease in soil-NER may be due to different metabolic pathways able to generate metabolites have a lower affinity for soil than for electrodes. In that sense, DEA, DIA, and HA-ATR, are strongly adsorbed to soil (Loos and Niessner, 1999), increasing the NER and decreasing the availability of the compound to continue with the mineralization pathway.

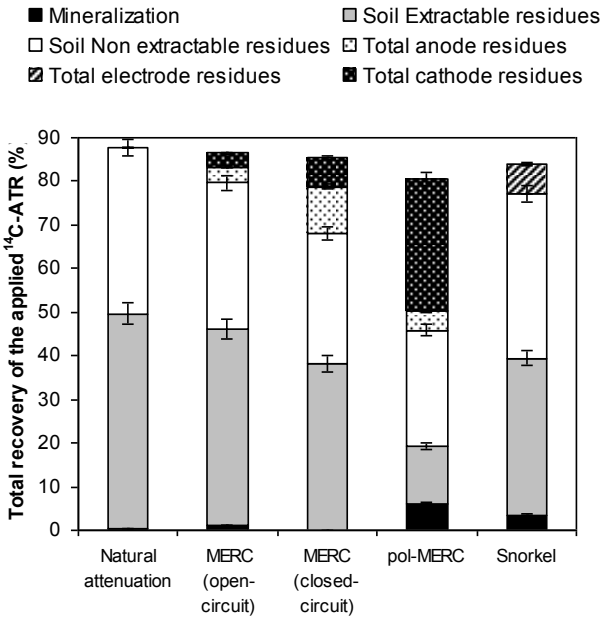


***Figure 5.7.*** Mass balance of $^{14}$C-ATR under the different treatments. The error bars represent the standard deviation for triplicate assays.

The percentage of extractable residues (ER) from the electrode was negligible (around 0.15%), so almost the total fraction of electrode residues corresponded to NER. Interestingly, the percentage of NER from electrodes was affected by the electron flow between electrodes. So thus, under open-circuit conditions 7% of the applied ATR was attached irreversibly to the electrodes (3.5% for both anode and cathode, respectively). These values are very similar to those obtained from $^{14}$C-ATR adsorption assays (2.2%) on carbon felt (C in Figure 5.3). Total electrode residues increased considerably under treatments where electrons are flowing between electrodes (pol-MERC) suggesting that ATR and its metabolites were attached to the electrodes as NER. While under closed-circuit MERCs the major NER fraction was attached at the anode (11%), under pol-MERC most of the NER were bound to the cathode (30%). Baskaran, S *et al.* (1998) (Baskaran and Bolan, 1998) determined that ATR molecule has a positive charge, that could cause a major adsorption to negatively charged electrode (Figure 5.7) like the anode in the case of closed-circuit MERC system (-250 mV) and the cathode in the case of pol-MERC system (-150 mV).

### *5.4.7. $^{14}$C-ATR long-term mineralization*

*Bioelectroventing* an ATR polluted soil was proved to be toxicologically clean it up after 20 days treatment using a pol-MERC. However, in order to evaluate the effect of *bioelectroventing* the polluted soil for longer periods we prolong the assay 80 additional days. Our strategy was to convert two experimental open-circuit MERC replicates just after finishing the short-term assay, into pol-MERC by shifting the electrode potential from negative (-250 mv vs Ag/AgCl) to positive (+600mV vs Ag/AgCl) (Figure 5.8).

At the end of the long term assay the cumulative mineralization under pol-MERC increased to reach 20% of the initial $^{14}$C-ATR whereas the natural attenuation reached just 1%. So thus, the *bioelectroventing*

performance was actually enhancing the mineralization capability of the soil native microbial community.

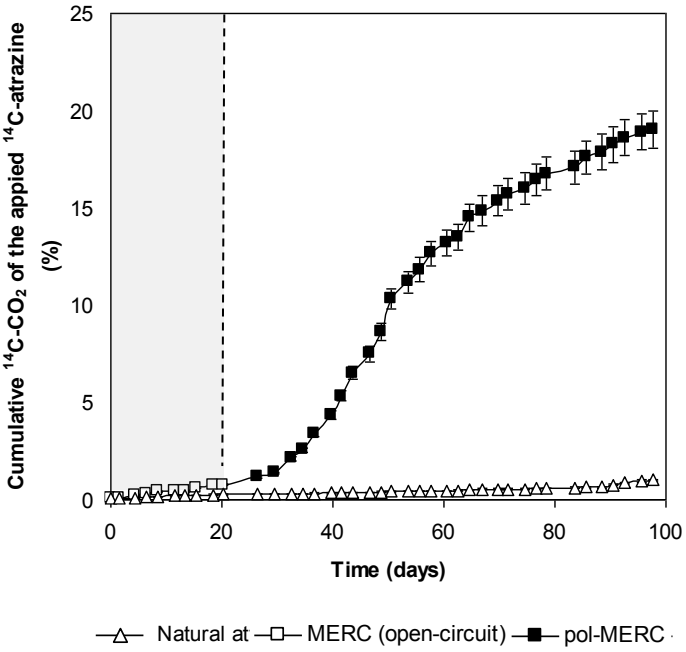


***Figure 5.8.*** Monitoring of the cumulative mineralization of $^{14}C$-ATR under different configurations for 100 days. A MERC (open-circuit) configuration (□) was converted to a pol-MERC (■) after 20 days of incubation (grey zone). Natural attenuation (soil without electrodes) (Δ) were showing a negligible ATR mineralization. The error bars represent standard deviation.

After both short- and long-term assays (Figure 5.8), a reversed potential was performed in pol-MERC. The flooding conditions of the systems could allow the water and $^{14}CO_2$ reaction establishing an equilibrium with carbonic acid leading negative charged species as $HCO_3^-$ and $CO_3^{2-}$. These species could be retained and adsorbed on the surface of positive polarized electrodes, so 12 hours before finishing the experiment the electrode potential was shifted from positive potential (+600mV vs Ag/AgCl) to negative one (-300mV vs Ag/AgCl) in pol-MERC systems.

The mineralization rate increased in pol-MERC from 0.2% to 0.7% of the initial $^{14}$C-ATR applied, indicating a low adsorption of $^{14}CO_2$ in the polarized anode.

Most of the research regarding biodegradation of ATR has been almost fully devoted to the aerobic metabolism. Furthermore, ATR mineralization in soil depends on the pollutant-exposed period. Actually, a significant ATR mineralization was reported by Getenga *et al*. (2009) by treating soil historically treated with the herbicide. In contrast, a low mineralization (3-5% of the initial ATR) was reached using soils non previously exposed to ATR (Schroll et al., 2006; Ngigi et al., 2011). Similarly, low mineralization of ATR has been reported under anaerobic and strong reductive conditions (Nair and Schnoor, 1992; DeLaune et al., 1997; Seybold et al., 2001). The low mineralization of ATR by native soil microbes has been attributed to the halogen on the pesticide ring, which impedes further microbial metabolism (çWackett et al., 2002). This is consistent with Crawford (1998) studies in anoxic sediment slurries where a non-significant mineralization was reported under denitrifying conditions (Crawford et al., 1998). Chung *et al.* (1996) reported 20% ATR transformation in anaerobic sediments after an incubation period as long as 30 weeks although mineralization was not reported. In spite of this oxygen-associated limitation, our pol-MERC was able to remove 93% of ATR in the first 20 days. In contrast a rapid mineralization under denitrifying conditions was reported by *Pseudomonas* sp. Strain ADP in aquifer sediments when it is amended with citrate and ATR was presented in high concentration (Zophel et al., 1991; Shapir et al., 1998).

Our study concludes that using electrodes at a positive potential (+600mV (vs. Ag/AgCl)) we can overcome electron acceptor limitation and maximize metabolic oxidation with the purpose of enhancing the biodegradation of a pollutant like ATR in the environment. Even more remarkable and according to our ecotoxicological assays, this so-called

*bioelectroventing* strategy was also successful for achieving an effective clean-up of the soil able to restore the pre-pollution conditions.

### 5.5. Acknowledgments

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# Designing strategies for operating Microbial Electrochemical System in soil under non-flooded conditions

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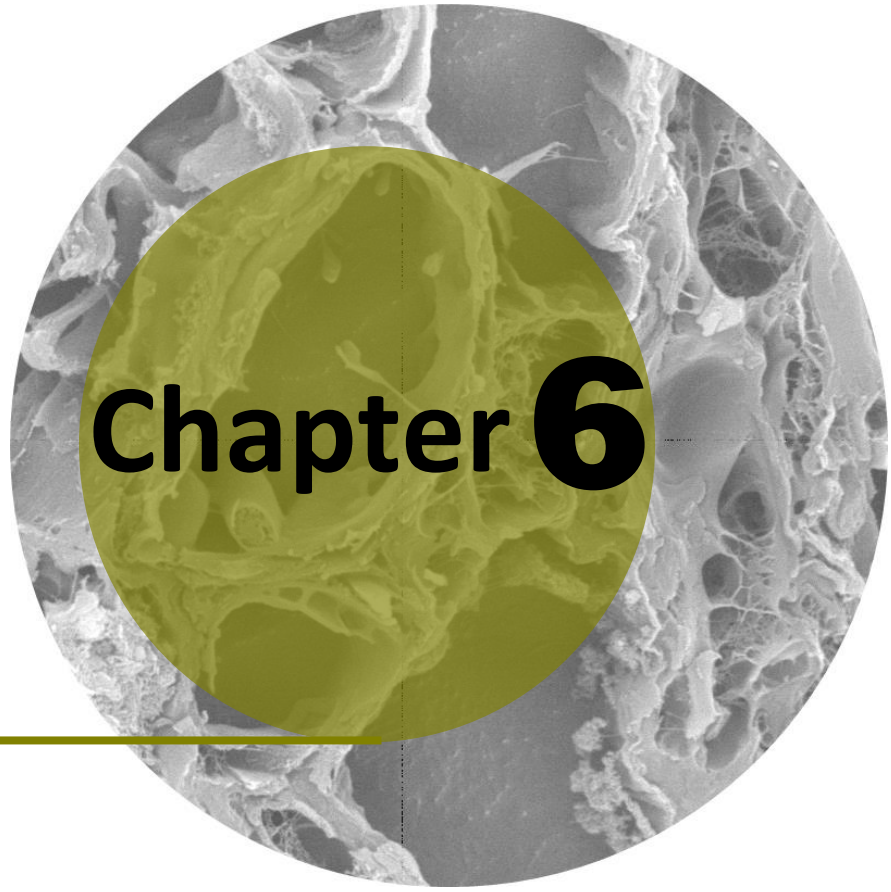
Chapter 6

# Designing strategies for operating Microbial Electrochemical System in soil under non-flooded conditions *

## 6.1. Abstract

Microbial electrochemical systems can be setup in a soil for either harvesting energy from microbial metabolism (Sediment Microbial Fuel Cell, SMFC) or for bioremediating polluted environments (Microbial Electroremediating Cell, MERC). However, the apparent need for locating this technology in flooded environments to keep the ionic contact between anode and cathode have limited the implementation of applications in standard soils.

This work describes a new configuration that overcomes this limitation by using a ceramic barrier so we can achieve a closed circuit system without flooding the soil. On top of harvesting energy we have outperformed natural bioremediation by restoring an atrazine-polluted soil as proof of concept. Moreover, a set of toxicological tests using green algae, *Salmonella typhimurium* and *Sorghum saccharatum* strongly confirmed a non-toxic scenario after the MERC treatment.

*The contents of this chapter have been submitted as:

Domínguez-Garay, A. and Esteve-Núñez, A. Designing strategies for operating Microbial Electroquemical System in soil under non-flooded conditions.

## 6.2. Introduction

We know that Sediment Microbial Fuel Cells (SMFCs) are bioelectrochemical devices, variants of microbial fuel cells (MFCs), which are placed in a variety of environments. A typical environment for a SMFC is a lake. In such a case the anode is located in the sediment and connected through a resistor to the cathode in the water column, where electrons are finally consumed by an electron acceptor such as oxygen (Tender et al., 2002; De Schamphelaire et al., 2008a; Venkata Mohan et al., 2009; Wang et al., 2012a; Domínguez-Garay et al., 2013). The low ohmic internal resistance of the saline sediments allowed power production in the range of 50-100 mW/m$^2$. In contrast, lower output values have been reported with SMFC installed in fresh water environments, where low conductivity increases the internal resistance of the system (Zhang et al., 2015). Additional studies have investigated SMFCs in rice paddies (De Schamphelaire et al., 2008b; Kaku et al., 2008; Takanezawa et al., 2010; Chen et al., 2011) or using other grown plant species under flooded conditions in soil-free applications (Helder et al., 2010; Timmers et al., 2010; Strik et al., 2011).

For the past 10 years, most of the studies about SMFC applications have been conducted in waterlogged soils or sediments. The greatest limitation for the implementation of SMFC in real-world environments is the need for the soil or sediment to be waterlogged. In that sense, the key role of organic matter and nutrients (Dunaj et al., 2012), the sediment pre-treatment (Song and Jiang, 2011), the water content (Chiranjeevi et al., 2012) or the influence of the soil internal resistance (Domínguez-Garay et al., 2013) have been extensively evaluated. Although most efforts to increase the efficiency of SMFC have been focused on the anodic and cathodic materials (Dumas et al., 2007; Venkata Mohan et al., 2008; Pant et al., 2011; Wei et al., 2011; Luckarift et al., 2012; Wang et al., 2012b; An et al., 2015), some studies have reported alternative SMFC configurations to decrease the resistance of soils. For instance, by using tubular air-

cathode designs (TAC-MFC) (Yuan et al., 2010) to narrow the distance between anode and cathode or by using a catalytic and waterproof layer to replace the ionic exchange membrane present in typical MFC (Zhuang et al., 2009). Unfortunately, the poor permeability of sediments limited the proton transfer rate, and the authors reported an acidification of the anode chamber (Wang et al., 2012b). Another reported approach was based on a soil-buried cathode to capture the oxygen released from rice roots (Chen et al., 2011).

In addition to the energy harvesting, soil-buried electrodes provide an alternative TEA to microorganisms for stimulating the anaerobic degradation and mineralization of organic contaminants (Li and Yu, 2015). In that context, Rodrigo *et al.* (2014) first proposed the term *Microbial Electroremediating Cell* (MERC) for a bioelectrochemical device designed to clean-up polluted environments. This novel approach of bioelectroventing (Rodrigo *et al*., 2016) showed a high efficiency in situations where lack of suitable electron acceptors limited microbial respiration leading to the persistence of pollutants in soils or sediments. Moreover, in *Chapter 4* a number of ecotoxicological and genotoxicological analysis have demonstrated how ATR polluted soils treated by MERCs can be efficiently cleaned-up of (Rodrigo et al., 2014; Domínguez-Garay et al., 2016).

The aim of this work was to construct a new design of microbial electrochemical device 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 sense, the system was tested to bioremediate an atrazine-polluted soil as a proof of concept.

## 6.3. Material and methods

### *6.3.1. Chemicals*

ATR (CAS No. 1912-24-9, purity > 97%) was obtained from TCI. $Na_2HPO_4$ and $NaH_2PO_4$ (Scharlau, purity > 98%) were used to make the phosphate buffer solution. Deionized water was used to prepare all media and solutions. Methanol (Sigma Aldrich, purity > 99.9%) was used for soil extractions.

### *6.3.2. Soil sampling*

The experiments used soil collected from an uncontaminated alluvial plain in Calasparra (Murcia, SE Spain), deposited by the Segura River. The land had been previously used for rice cultivation. A complete description of the physical and chemical characteristics of soil is provided in *section 3.4.1.*of this thesis.

### *6.3.3. Soil and chemical analyses*

ATR extractions were performed with the entirety of the soil from each of the treatments. 60 g of soil was mixed with a methanol solution ($1mL \cdot g^{-1}$ of soil) and was shaken for 24 hours on an orbital shaker at room temperature. The soil solvent mixture was incubated in an ultrasonic bath for 60 minutes, in order to complete the ATR extraction. Finally, the mixture was centrifuged (Multifuge 3L-R Heraesus) at 5000gs for 10 minutes and the supernatant was completely evaporated. The extracted compounds were resuspended in 3 mL of phosphate buffer 20mM, stirred for one hour, and finally they were sonicated for ten seconds. All samples were filtered (Millex-GV, 0.22µm) and stored at 4 ° C. The soil extractions were analyzed to quantify ATR. These values were references values to estimate the removal of ATR.

A series of solvent extractions, following the same protocol applied to the soil, were performed to estimate the adsorption of ATR by electrodes and

the ceramic material. ATR concentration was determinated by HPLC-DAD analyses (Varian 9040) with C18 *Phenomenex Kromasil* column (*150x4.5mm, 5µm*) and UV detection at 220 nm. The mobile phase was 50:50 (v/v) acetonitrile:water mixture with a flow rate of 1 $mL \cdot min^{-1}$. ATR concentration was calibrated in the range of 30- 0.625 mg/L.

### *6.3.4. Microbial Electrochemical Systems: construction and operation*

Our microbial electrochemical device (Figure 6.1) was designed and constructed to eliminate the need to flood the environment of interest. The cathodic chamber consisted in plastic reservoir (200ml) with a cathode of graphite felt (*Mersen*, 20 x 4 x 0.5; 0.7 $m^2/g$ of surface area) soaked in 190mL of KCl 30mM. The anode was a hollow carbon fiber cylinder (*ClipCarbono*, 9cm length, 0.5 mm thickness, 38mm diameter) embedded in soil. To connect the soil-buried anode and the out-of-soil cathodic chamber a novel cone-shaped ceramic barrier (*Aquasolo* ©) was used (Figure 6.1). The anode was embedded in 60g of sieved dry paddy soil (≤2 mm). The connection between the anode and the ceramic barrier was sealed tightly with silicon. The anode/cathode connection used copper wires attached to the electrodes with a conductive epoxy resin (Circuit Works) and isolated with a nonconductive epoxy resin (Araldit Ceys). The ceramic barrier allowed a water flow rate of 0.07 L/day. Furthermore, the resistance due to the ceramic barrier was determined by using the Wenner-Schlumberger method (described previously in *section 3.3.2.* of this thesis).

Three experimental controls were setup. First the electrode-free soil was used as a control for natural attenuation. Second, a non-connected MERC (open circuit) was set-up as control. Third, abiotic effects on ATR removal were evaluated by using sterilized soil with $HgCl_2$. The validation of this method for sterilizing the same soil has already been reported in (Domínguez-Garay and Esteve-Núñez, 2016). Each condition had the different incubation times (24h, 7day and 14 days) and was

simultaneously replicated four times at room temperature. The differing conditions were stabilized for three weeks. Soil was artificially polluted by spiking 3 mL of anoxic solution of ATR (30 $mg \cdot L^{-1}$) in phosphate buffer 20mM ($Na_2HPO_4/NaH_2PO_4$; pH 7) adding 1.5 mg atrazine$\cdot Kg^{-1}$ soil.

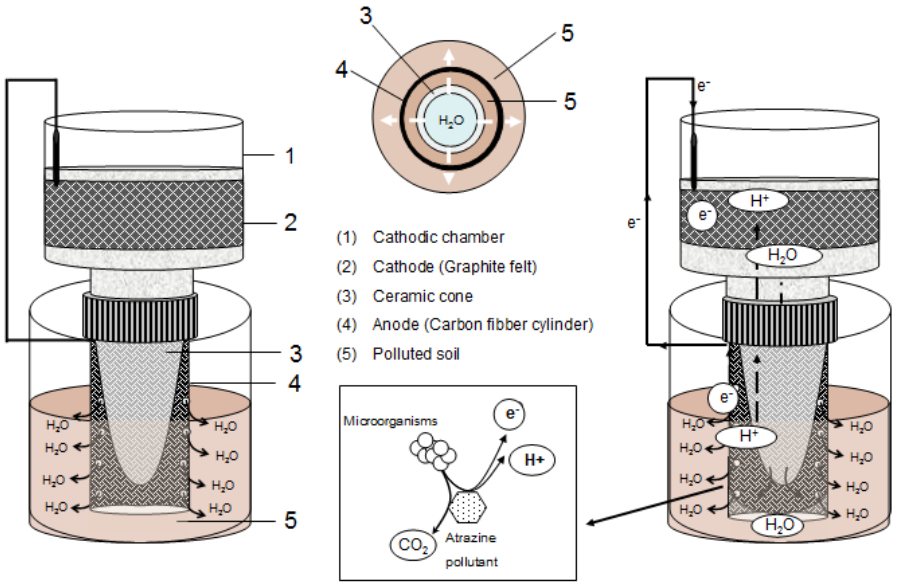


***Figure 6.1.*** Configuration of a MERC system including a ceramic barrier as interface between anode and cathode electrodes.

### *6.3.5. Power Curves Analysis*

The novel SMFs was electrochemically characterized by performing polarization curves recorded with a potentiostat (model μAutolab 300) using a linear potential sweep at a scan rate of 0.1 mV/s. The curves were obtained in the presence of acetate. The power density (power =voltage × current) and the current density were calculated based on the anode surface area. The internal resistance was estimated from the maximum of the power curve.

### *6.3.6. Toxicity bioassays*

#### *6.3.6.1. Algal test*

Toxicity of extractable fraction was evaluated according to OECD Test Guide 201 (OECD 2008), using the green microalgae *Pseudokirchneriella subcapitata* (previously *Selenastrum capricornutum*) as reported previously in *Chapter 4* and *Chapter 5.* The initial inoculum of the test was $5x10^4$ cells per mL and was performed into 96-well clear disposable microplates. Three replicates of each extracted soil sample, including negative control (atrazine-free soil) and blank (algae-free) were included. The polluted wells consisted of 190 µL extracted soil sample + 10 µL growth media + 5 µL alga culture. Plates were incubated during three days under the same conditions as the inoculum culture was 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 referenced to the negative control assay.

#### *6.3.6.2. Genotoxicity test*

A commercial kit *UMU-Chromo Test* (EBPI-Canada), based on ISO 13829 protocol, was used to evaluate the genotoxicity effect of the soil extracts. Testing for mutagenicity was carried out as described previously in *section 4.3.5.2* of this thesis.

The procedure was as follows: tester strain cultures were grown overnight at 37ºC. The resulting bacterial suspension was used to inoculate individual wells of a 96-well disposable microplate containing soil extracts dissolved in 10% dimethylsulfoxide (DMSO). After inoculation the assay mixture was incubated at 37ºC for 2 h. The β-galactosidase activity of the tester strain was determined measuring absorbance of *ortho*-nitrophenyl-β-galactoside at 420 nm, after 30 min of incubation at 28ºC, using a microplate reader (RAYTO RT-2100 C).

## 6.4. Results and discussion

In order to validate the electrochemical performance of our design we have selected an agriculture soil to perform assays under non-flooded conditions. Moreover, its bioremediation potential was tested with the herbicide ATR as proof of concept for cleaning up polluted soils.

### *6.4.1. Microbial Electrochemical Performance in soil under non-flooded conditions*

SMFC (Domínguez-Garay et al., 2013; Ewing et al., 2014) and MERC (Rodrigo et al., 2014; Li and Yu, 2015; Sherafatmand and Ng, 2015; Domínguez-Garay et al., 2016; Rodrigo Quejigo et al., 2016) have been classically operated in soils and sediments under flooded conditions to allow for protons from the anode to react with electrons in the cathode. Such a design is limited in its real-world applications because flooding is not a common situation for soil environments and it is not practical to flood all environments that need bioremediation. To overcome such an obstacle we have a novel design where an out-of-soil cathodic chamber in combination with a ceramic barrier allows the system to run in any soil (Figure 1). Interestingly, the new design did not show any major increase in electrical resistance in comparison with standard flooded SMFC. The ceramic barrier, acting an interface between soil-buried anode and cathode, showed a resistance of 50Ω which correspond to just 0.6% of the internal resistance of the microbial electrochemical system. The total internal resistance of this configuration (8KΩ) was very similar to the one reported for the same soil under flooded conditions using a standard SMFC configuration (Domínguez-Garay et al., 2013).

The analyses of the power curve (Figure 6.2) revealed a maximal power production of just 0.25mW/m$^2$ that was consistent with the low values for organic matter content (0.18%) and electrical conductivity (0.24dSm$^{-1}$) of our paddy soil. The electrochemical performance revealed a design

suitable for harvesting current in non-flooded soils. This extends the number of outdoor applications for the SMFCs.

In addition to harvesting electrical current from soil environments, microbial electrochemical technologies show also a promising application for *in situ* bioremediation when they operate as Microbial Electroremediating Cells (MERC) (Zhang et al., 2010; Rodrigo et al., 2014; Domínguez-Garay et al., 2016). In order to validate the bioremediation potential of our design will operate it as a MERC to restore an ATR polluted soil.

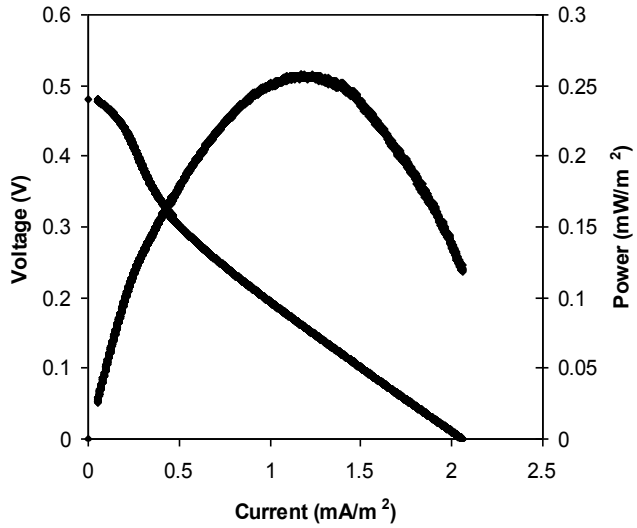


***Figure 6.2.*** Power curve of a MERC device under non-flooded configuration in presence of acetate.

### *6.4.2. Adsorption of ATR to soil and materials from MERC*

ATR (2-chloro-4-ethylamino-6-isopropyl amino-1,3,5-triazine) is a member of chlorinated *s*-triazine group of herbicides, which is moderately mobile and highly persistent in the environment (Iriel et al., 2014).

Understanding the interaction between ATR the soil matrix and the MERC materials is key for a proper validation of the process.

The rice paddy soil used for our MERC devices showed a high clay content (32%), which could facilitate adsorption effects of ATR on the soil (Ahmad and Rahman, 2009). The sterile soil extraction assay confirmed that 64% of the initial ATR was reversibly bound to the soil matrix. Regarding the adsorption process by the SMFC 10% of the initial pollutant was adsorbed in the case of the cathode material (carbon felt), in contrast with the lack of adsorption observed in the anode material (carbon fiber) or in the ceramic barrier.

### *6.4.3. Microbial Electrochemical Remediation of ATR-polluted soil under non-flooded conditions.*

Previous studies using this paddy soil already reported the presence of an atrazine-biodegrading microbial community that could be stimulated by electrochemical tools to enhance ATR removal (Domínguez-Garay et al., 2016). Such assays were performed by flooding the soil, a situation that rarely occurs in nature. The challenge of our research was to perform a similar strategy but avoiding soil flooding in order to reach more natural conditions.

In this new set of experiments, we first evaluated the atrazine-removal capacity under natural attenuation conditions. The ATR removal after 7 days of incubation was 20% of the initial ATR and 54% was removed after 2 weeks of incubation (Figure 6.3).

Previous studies reported that the mere presence of electrically conductive material in soil was able to enhance the bioremediation of pollutants such as dibenzothiophene (DBT) in soil through a process called *Graphite-assisted bioremediation* (Rodrigo et al., 2014). Similar phenomena have been reported for isoproturon (Rodrigo Quejigo et al., 2016) and ATR (Domínguez-Garay et al., 2016; Dominguez Garay et al., 2016b) by using graphite electrodes. In order to evaluate this effect, our

design with a carbon fiber-based anode was operated under open circuit conditions so the current could not flow from the anode to the cathode. Interestingly, the presence of electrically conductive carbon fiber for 1 week left the soil with lower ATR levels (ca. 55%) than natural attenuation achieved (Figure 6.3). The result was even more promising after 2 weeks of incubation since ATR-removal rate was enhanced by 4-fold by the sole presence of the electrically conductive material. A similar process had been previously described in sediment slurries for toluene and benzene degradation in methanogenic digesters (Zhang et al., 2010) where direct interspecies electron transfer (DIET) was demonstrated in the presence of electrically conductive material (Liu et al., 2010; Chen et al., 2014).

Interestingly, when anode and cathode were connected and operated under maximal current flow (short-circuit conditions), more than 98% of the initial ATR was removed after 2 weeks in contrast with 58% removal under natural attenuation.

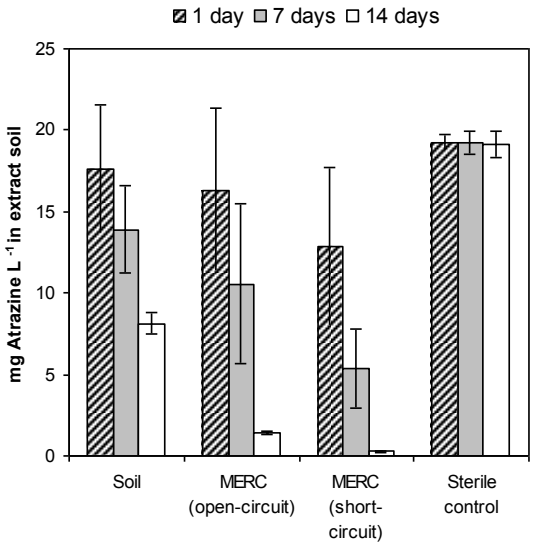


***Figure 6.3.*** Atrazine concentration in methanol-soil extracts. The error bars represent the deviation of the mean value for triplicate assays.

The most remarkable part was not just the efficient removal of waste but the fact that our design by-passed the classical concept of flooding the soil for installing microbial electrochemical systems. ATR removal is key in the bioremediation process, but it cannot be necessarily lead to a fully restore environment if additional toxic metabolites still persist. So thus, we included toxicological analysis of the treated soil as a “must” action in the bioremediation strategy.

### *6.4.4. Ecotoxicological analysis*

Numerous studies have indicated that ATR inhibits growth and photosynthesis of freshwater algae; actually algal responses to ATR vary widely depending upon concentrations, duration of exposure, and algal species tested (Tang et al., 1997; Weiner et al., 2004).

In our hands, the growth rate EC50 value of *P. subcapitata* was 70µg/L after 96h of algal incubation. This value was very similar to reported value in other studies with the same algal test, where EC50 after 96h was in the range 50-80 µg/L (Weiner et al., 2004; Yeh and Chen, 2006).

Our ecotoxicity test showed that 24 hours after ATR addition the soil extractions all showed an average of 65% inhibition of algal growth (Figure 6.4). This inhibition value was reduced to about 50% after 7 days of incubation under all conditions. However, after 14 days of incubation a significant difference between the different conditions was observed.

Natural attenuation, in absence of electrodes, remained in the range of 50% inhibition which did not fulfill the non-toxic level. In contrast, the detoxification capacity of soil microorganisms was increased with the presence of a conductive material, reaching values for inhibition of algal growth very closed to the non-toxic limit. Interestingly, 100% detoxification of soil was achieved when MERC was operated under short-circuit conditions and electrons from microbial metabolism flowed to the out of soil cathode chamber. The low concentrations of ATR observed under this

short-circuit configuration correlates well with the ecotoxicity results, suggesting a successful cleaned up process.

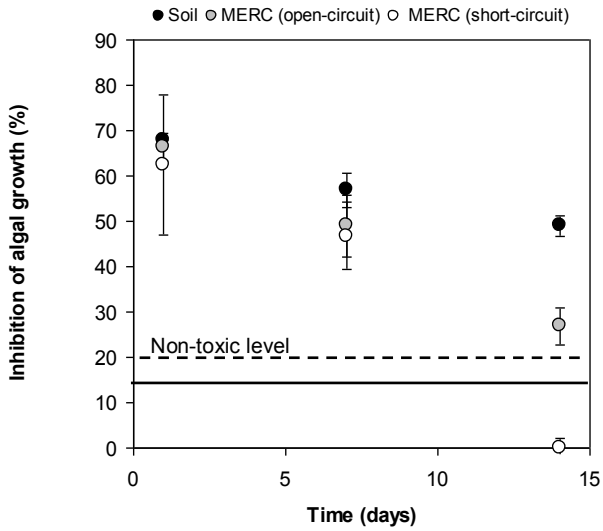


***Figure 6.4.*** Toxicity levels in soil extracts from non-treated soil, MERC (open circuit) and MERC (short-circuit) treatments. The toxicity values were represented as inhibition of *P.subcapitata* algal growth (%) at different periods of soil incubation (1, 7 and 14 days). Reference toxicity value was represented for non-atrazine-polluted soil (continuous line) and non-toxic value was also provided (discontinuous line). The error bars represent the deviation of the mean value for triplicate assays.

### *6.4.5. Genotoxicological analysis*

The genotoxic effect of ATR was previously reported on aquatic species (de Campos Ventura et al., 2008; Cavas, 2011) by applying microsome mutagenicity test using different strains of *Salmonella typhimurium* (Plewa et al., 1984; Mersch-Sundermann et al., 1994; Ruiz and Marzin, 1997) or SOS chromotest using *E.coli* PQ37 (von der Hude et al., 1988; Mersch-Sundermann et al., 1994; Ruiz and Marzin, 1997) . Our genotoxicity analysis based on umuC test (Figure 6.5) revealed that a soil extract

sample showed an initial induction ratio of 1.8 (note that values over 1.5 indicate genotoxicity) just after the pollutant spiking. The initial genotoxicity from soil extracts was not very high comparing with non-toxic induction ratio value, however an increase to values as high as 2.5 were detected in the case of natural attenuation configurations. In contrast, a decrease of genotoxicity was observed when electrode-mediated configurations were tested. This was especially remarkable by using MERC under short-circuit because 82% of the initial genotoxicity was erased and an induction ratio as low as 0.3 was reached after 2 weeks of incubation.

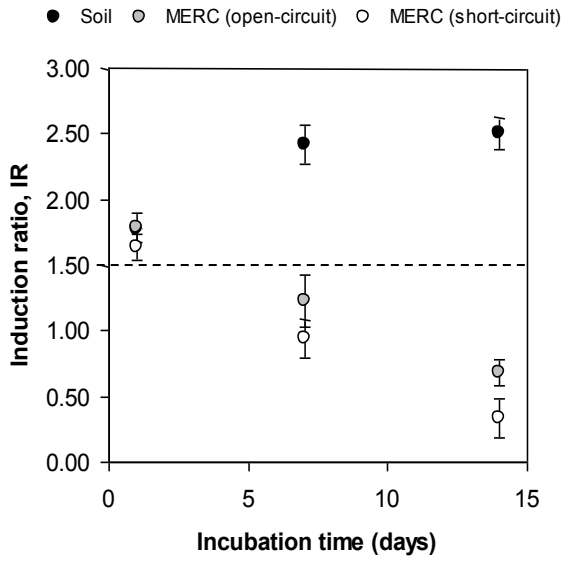


***Figure 6.5.*** Induction ratio as a measure of genotoxicity levels in soil extractions from polluted soil for non-treated soil. MERC (open circuit) and MERC (short-circuit) by using S9 enzyme fraction. The genotoxicity values were represented as induction ratio of β-galactosidase enzyme in *S.typhimurium* bacterium by umu-assay at different periods of soil incubation (1,7 and 14 days). Non-genotoxic value was provided (discontinuous line).The errors represent 5% of deviation of the mean value for triplicate assays.

So, using flooded soils to test SMFC makes implementing microbial electrochemical systems difficult to apply in the real-world. Our configuration overcomes this limitation with the use of a ceramic barrier that plays two important roles: a) a low-resistance barrier that allow for cathode avoiding the role of soil as the interface, and b) an irrigation role, keeping the soil moisture and the electrolytic contact through the permeable nature of the ceramic material. The result is SMFC/MERC able to both harvest energy without flooding the soil and to efficiently clean-up an ATR-polluted soil by stimulating natural electroactive populations.

## 6.5. Acknowledgments

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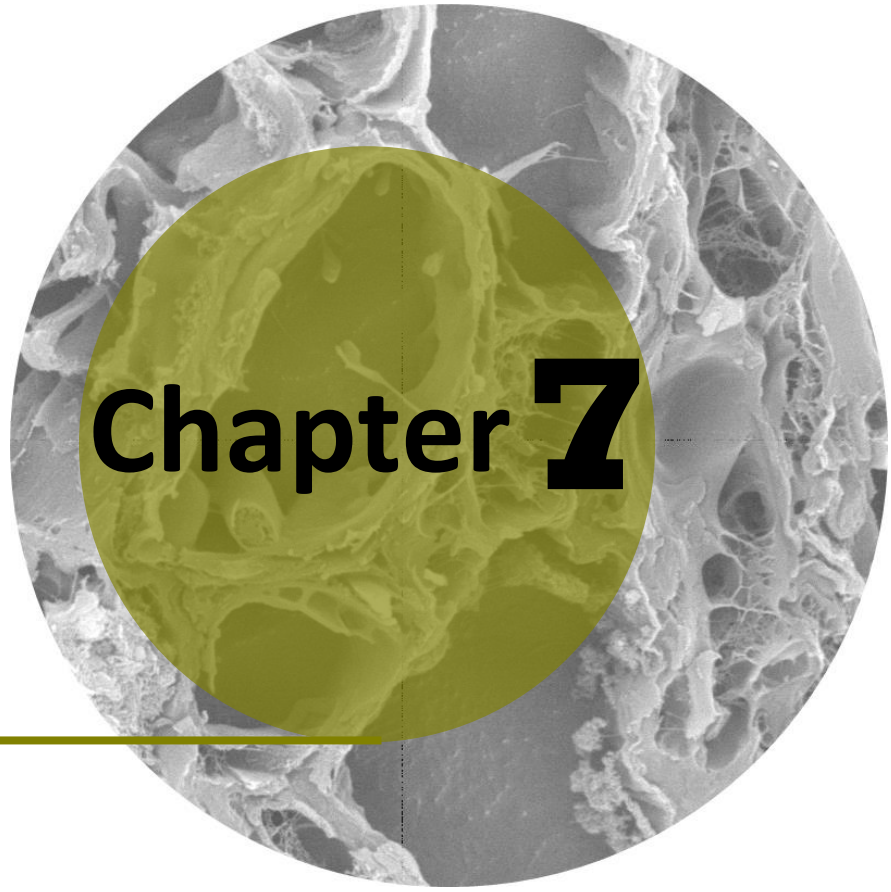
# Discussion, conclusions and future outlook


Chapter 7

# Discussion, conclusions and future outlook

The main objective of this thesis was to evaluate the performance of microbial electrochemical systems in soil at laboratory scale, with special emphasis in its role as bioremediating tool in atrazine-polluted soil. The work presented in this thesis supports the idea that this novel strategy for cleaning-up polluted soils might represent a potential alternative to conventional bioremediating techniques. Nevertheless, the soil nature supposes an important limitation for the efficiency of the technology although can be overcome using a new design. We have presented the final considerations of this thesis as question-answer mode:

## 7.1. Discussion and conclusions

● *How does soil structure affect SMFC efficiency? Is the soil resistance a limiting factor for operating SMFC technology?*

Most bioelectrochemical devices reviewed at the state of the art in this thesis have been developed in ecological water bodies, as 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). They all show the advantage of a low ohmic internal resistance due to the high concentration of salts. Freshwater flooded environments as rivers or waterlogged field crops (De Schamphelaire et al., 2008; Kaku et al., 2008; Busalmen et al., 2010; Takanezawa et al., 2010; Chen et al., 2011; Chiranjeevi et al., 2012; Sacco et al., 2012; Wang et al., 2012; Sajana et al., 2014) could be also possible scenarios for its application but they are less efficient due to the lower salinity (and higher internal resistance) of these environments.

A low publication record dealing with non-flooded conditions suggests the limitation of SMFC systems when water is not overlaying the soil. Nevertheless *Chapter 3* showed a new design that demonstrates how a water flooded environment is not necessarily the limiting factor in the development of SMFC. However, the water distribution between the soil particles still plays an important role in the SMFC efficiency.

An interesting approach for implementing SMFC technology in soil includes the use of plants (eg. rice) as fuel suppliers via root exudates (Kaku et al., 2008; Busalmen et al., 2010; Takanezawa et al., 2010; Chen et al., 2011; Chiranjeevi et al., 2012). Considering the relevance of rice crops in certain areas located in the southeast of Spain, we focused on exploring the use of rice crop soils using a paddy soil sampled in Calasparra (Murcia).

The unfavourable conditions (very low organic matter content and ionic conductivity) of the selected paddy soil for performing microbial electrochemistry, allowed us to investigate the importance of soil structure and water distribution and more importantly to develop strategies for overcoming such a limitation through the addition of silica colloid. The colloid formation, that gives higher cohesion to the soil particles and greater retention of water, results in a higher power production (ca.10-fold) when optimal silica dose is applied in SMFC microcosms. This is the result of multiple changes due to the colloid addition:

i) The formation of preferential water adsorption channels for minimizing soil resistivity, improving the ions mobility throughout the soil particles, that is key, to achieving an optimal performance of SMFC.

ii) A higher mobility of nutrients across the soil solution that allows a better assimilation by soil microorganisms.

iii) A high water and nutrients retention between soil particles enhances the development and yield of several plant species (Mitani and Ma,

2005). Interestingly, root secretions provide organic electron donors to soil microorganisms that transfer the resulting electrons to soil-buried electrode (Kaku et al., 2008; Busalmen et al., 2010; Takanezawa et al., 2010; Chen et al., 2011; Chiranjeevi et al., 2012). So thus, presence of silica enhanced the power ca. 4-fold in our rice-SMFC assays.

Therefore the soil resistivity reduction is key to achieving an optimal performance of SMFC in electrogenic microcosms. While the MFC studies are focused on the reduction of mass transfer loss due the electron transfer between bacteria and electrode (Torres et al., 2008) or the resistance and suitability of the electrode material (Oh and Logan, 2005), SMFC applications should also consider that soil ohmic resistance contribution is ca. 20% of the overall internal resistance. So, new strategies are required for enhancing the efficiency of this technology in soil applications.

● *What is the effect of using conductive material on the degradation of soil pollutants?*

*Chapter 4* shows the results obtained from operating SMFC applications for stimulating the biodegradation of ATR in soil.

The degradation assays conducted in the presence of the conductive material, exhibited over 60% of ATR removal after only 1 week of incubation.

Unexpectedly the mere presence of electrodes under open circuit configuration showed 50-70% removal of extractable ATR, after discarding adsorption processes on soil and electrodes material. We hypothesized that these results were related to a process called Direct Interspecies Electron Transfer (DIET) previously described by other authors (Aulenta et al., 2010; Liu et al., 2010; Liu et al., 2012; Aulenta et

al., 2013; Chen et al., 2014; Rodrigo et al., 2014). In sedimentary environments, microorganisms with Extracellular Electron Transfer (EET) capabilities may take advantage of the electric currents circulating through conductive minerals, which they connect spatially segregated bio-geochemical redox processes (Nielsen et al., 2010). Another possible explanation is the so-called natural geobatteries. Geobbateries are formed when graphite deposits in the subsurface are able to transfer electrons between anaerobic an oxic zones (Bigalke and Grabner, 1997). They may constitute a long-term electron acceptor for the surrounding microbes and share common elements with the anodes from a SMFC (Leung and Xuan, 2015). Considering that the mere presence of an electrically conductive material in soil increases the degradation efficiency ca. 4-fold versus natural attenuation (absence of electrodes) then this strategy is a promising bioremediation technique for accelerating the pollutants degradation processes in soil by boosting microbe-electrode interactions. However, this process shows a favourable scenario for MERC application because biodegradation can be expanded at long distances far away from the electrode.

The presence of electrodes in closed-circuit (connected with a resistor) increases the removal efficiency of soil pollutants in an 83% of extractable ATR, 5-fold higher than the one obtained under natural attenuation conditions. The electrodes connection with a resistor increases the anode potential (less negative) compared to open circuit-conditions, resulting in a higher current production that increases the electron transfer in the degradation activity by microorganisms.

Furthermore, it is important to consider that ATR removal is not enough for assure a fully clean-up process, due to the toxic effect of ATR derivatives. Thereby our toxicological analysis revealed that a successful bioremediation requires a proper electron flow facilitated by the MERC treatment (closed-circuit). Under this configuration the soil showed a complete detoxification while natural attenuation or MERC in open circuit

showed toxic values 6-fold higher that closed-circuit even 14 days of incubation.

All these results reveal that although a mere presence of electrodes facilitates the electron transfer between soil microorganisms due to conductivity of material surface; the redox processes occurring in soil are very different. This means that not only the oxidation pathway, able to release ATR metabolites, is different, but also the redox state of other compounds (nitrates, sulphates, etc).

● *Under which electrode configuration or redox scenario is more efficient the bioremediation process?*

The configuration of microbial electrochemical systems can be very flexible. *Chapter 5* shows comparative ATR mineralization experiments by modifying: i) electrodes architecture, ii) electrodes connection, iii) anode potential.

i) Electrodes architecture (horizontal or vertical) influences: a) the mass transfer of ions throughout the soil matrix and b) the redox potential spectrum across soil/water. In this way, two different electrode configurations were development: snorkel configuration that represents a single conductive material spans the anaerobic and aerobic zones, and conductive material buried horizontally in the soil with a suspended cathode in water. The first configuration (single electrode) shows a lower resistance to protons transfer and exhibits a broader redox scenario and, consequently, a greater number of microorganisms for degrading ATR. The results suggested that putting the anode and cathode in snorkel configuration forces the anode to the highest possible value of potential, which promotes the microbial oxidation reactions (Erable et al., 2011). The mineralization under this configuration was 6-fold higher comparing with the standard one where the electrodes were separated by the soil matrix.

ii) Electrodes connection (closed circuit) by a very low resistor: a) The resulting electric current flow increases the degradation rate of soil pollutant, due to the free electron transfer compared with open circuit configuration, where current cannot flow. b) The anode potential becomes more positive (or less negative), which involves a more favourable electron acceptors for soil microorganisms.

iii) Electrodes polarization: although the redox gradient in MERC is established spontaneously across the soil-water interphase, the electrode can be artificially polarized by using a potentiostat or, alternatively, a power source. Actually, previous authors have applied an external voltage between anode and cathode setting a more favourable redox scenario for soil microorganisms (Aulenta et al., 2007; Chun et al., 2013). Moreover, a recent report (Rodrigo et al., 2016) amend such methodology as *bioelectroventing*, showing how mineralization of the herbicide isoproturon was strongly enhanced by shifting the electrode potential from negative (-250 mv vs Ag/AgCl) to positive (+600mV vs Ag/AgCl). Following a similar strategy we concluded that using electrodes at a positive potential (+600mV (vs. Ag/AgCl)) we can overcome electron acceptor limitation and maximize ATR mineralization by ca.20-fold. Even more remarkable and according to our ecotoxicological assays, this so-called *bioelectroventing* strategy was also successful for achieving an effective clean-up of the soil able to restore the pre-pollution conditions.

● *What are the advantages of bioelectroventing through MERC systems over conventional bioremediating techniques?*

This electrochemical technique for degrading the soil pollutants is classified within *in situ* techniques. Therefore comparing with *ex situ* techniques as: land-farming or biopiles, *in situ* treatments require less equipment, so they have a lower cost and generate fewer alterations to the environment. For all this, *in situ* techniques are likely more attractive (Boopathy, 2000). Nevertheless these conventional bioremediation

technologies present limitations cause they are not applicable at some sites or pollutants (Watanabe, 2001).

For instance, biostimulation actives the native microbial metabolism by introducing nutrients, co-metabolites, oxygen or other electron donors or acceptors to accelerate the decontamination rate (Tyagi et al., 2010). However, nutrients, oxygen, electron acceptors or donors are exhaustible compounds in soil that requires a constant additional supply and its availability depends on the appropriate environmental conditions (pH, temperature, moisture, etc) (Ngigi et al., 2011). In this way, bioelectrochemically-assisted remediation overcomes this limitation due to the soil electrode behaves as never-ending TEA in anaerobic soil or sediments, allowing microorganisms to perform oxidative metabolism beyond the natural conditions found in soil. Moreover, the competitive role of an electrode may reduce the formation of hydrogen sulphide and even methane that cause secondary pollution concerns (Pandey, 2012).

Bioaugmentation, that increases the bioremediation rate with additional microorganisms, can be very expensive and depends on the pollutant nature. Moreover, it could cause other problems concerning the adaptation of the inoculated microorganisms, competition between introduced and indigenous biomass, the use of other organic substrates in preference to the pollutant, etc (Tyagi et al., 2010). In this thesis native microbial species of treated soil have been used for developing the biodegradation process. Although the soil has not been previously exposed to ATR, effective results have been obtained by stimulating electrochemically the soil bacteria, revealing a high versatility of soil microrganisms for adapting to these bioelectrochemical conditions.

Additionally, microbial electrochemical system can be readily monitored and regulated by tuning electrochemical parameters, endowing it a good controllability, i.e. the produced electrical current can serve as a real-time bioremediation indicator. Example of this real-time monitoring rate was

reported by Wardman (2015) by using acetate electro oxidation as prove of microbial activity. The power production by this bioelectrochemical devices could be exploited to supply low-power electrical devices in remote locations were the descontamination is given e.g. power wireless sensors for remote online monitoring.

- Can we *in situ* interrogate the microbial redox activity with electrochemical tools?

A tool for characterizing the soil redox reactions is by using cyclic voltammetry (CV) technique to identify e.g. electrochemical reduction and oxidation of several pollutants in soil. Usually, It is the first experiment performed in an electrochemical study of a compound, a biological material, or an electrode surface (Kissinger and Heineman, 1983).

*Chapter 5* shows several cyclic voltammetries developed for evaluating the degradation activity of soil microorganisms exposed to different experimental conditions and for understanding the relationship between the cumulative mineralization and the increased biological activity. In this CVs several peaks were detected at the different incubation times (7 and 20 days) and conditions. When pol-MERC configuration was operating after 20 days in incubation, an increased signal of the current peak (10-fold regarding to open-circuit MERC and closed-circuit MERC) revealed the anode enrichment of electroactive microbial communities, which may be due to an increase in the cell density on the electrode surface (Fricke et al., 2008) or to an increase in the microbial metabolism that accelerate the electron transfer.

This remarkable difference in pol-MERC voltammetry coincides with a higher mineralization respect to the other operating conditions. Therefore, this electrochemical technique seems to be a good strategy for interrogating the microbial redox activity. However, this technique should be combined with other electrochemical techniques and previous analyses in order to establish more reliable correlations.

● *Is it possible to scale up this bioelectrochemical technology for biodegradating real polluted environments?*

All bioremediation processes are influenced by the physical and chemical conditions of the polluted matrix (soil), especially for *in situ* bioremediation. Specifically for microbial electrochemical systems in soil, the polluted matrix requires water saturation for a suitable proton transfer from the anode to the cathode. This thesis has shown that it is possible to detoxify the polluted soil by using a new MERC architecture, that can implemented in non-flooded soil by constructing an out of soil cathodic chamber in combination with a ceramic barrier *(Chapter 6).* This is important because flooding is not a common situation for soil and it is definitively not practical to flood a polluted one.

By applying this new MERC model, several conclusions were obtained:

i) The total internal resistance of this configuration decreases just ca. 2-fold comparing with the MERC classical configuration under flooded conditions with the same soil. The low resistance of ceramic barrier, the optimal moisture of soil and the distance reduction between anode and cathode, result in a mass transfer loss reduction.

ii) When electrodes were connected in short-circuit 68% and 98% of extractable ATR was efficiently removed under this configuration after 7 and 14 days of incubation respectively.

iii) Toxicological analysis showed a favourable scenario after 14 days of incubation where ecotoxicity and genotoxicity of treated soils under this configuration were below non-toxic levels. Table 7.1 shows comparative results about ATR removal efficiency and detoxification levels between conventional MERC (under waterlogged conditions) and non-flooded MERC configuration with ceramic barrier. The ATR removal results are represented respect control soil under open-circuit configuration.

***Table 7.1.*** Comparative results about atrazine removal efficiency and detoxification between conventional MERC (in *chapter 4*) and non-flooded MERC configuration (in *chapter 6*).

| Design | Conventional MERC | | Non-flooded MERC | |
|---|---|---|---|---|
| Incubation time (d) | 7 | 14 | 7 | 14 |
| Atrazine removal | 15% | 15% | 45% | 75% |
| Ecotoxicity | Non toxic | Non toxic | Toxic | Non toxic |
| Genotoxicity | Non toxic | Non toxic | Non toxic | Non toxic |

## 7.2. Recommendations ad future outlook

Recommendations for further research are made based on the results exposed in this thesis. The general conclusion of this thesis is that microbial electrochemical systems have the potential to be applied as *in situ* bioremediating technology in saturated soils but not necessarily waterlogged. Nevertheless, some limitations should be overcome and optimization of operational configuration should be addressed prior to using BES technology for real world applications.

**i**) In order to obtain a better understanding of processes occurring in the anode, a number of additional assays should be performed.

**a)** Bacterial community study in response to anode stimulation by varying the electrode potential to a more positive one. In this thesis a possible change in microbial communities has been mentioned due to the increase in ATR removal and/or mineralization when the electrode configuration or electrode potential is modified. Nevertheless it is important to perform a genus-level characterization to identify the key bacteria or bacterial responsible for ATR (or other pollutants) degradation and/or mineralization. In this way, all the bacterial community

data could provide evidence that different operational modes (electrodes configuration or redox scenario) result in differences of soil bacterial community structure.

**b)** To consider the possibility of merging conventional bioremediation techniques with bioelectrochemically-assisted remediation. Thus, identifying soil microbial communities potentially capable of degrading a specific pollutant would be helpful to design enriched bioelectrodes for accelerating the biodegradation processes in a polluted soil.

**ii**) In order to scale-up this technology as a real bioremediating tool:

**a)** The *in situ* applications for soil bioremediation require flexible microbial electrochemical configurations to adapt to different depths, soil matrix types, and other physical-chemical parameters. The study should be completed by evaluating the electrodes radius of influence (ROI) in soil (diffusion layer) for its application in real polluted environments, which is a critical consideration for full-scale MET implementations. This is important to reduce operating costs and to maximize further treated surface by using different conductive material with different shape or surface area. Moreover the influence of ceramic barrier as interface between soil anode and aerated cathode should be studied in more depth. The design of all these elements is essential for the scale-up of this technology, considering the importance of anode and cathode proximity as well as optimal moisture conditions and proper cathode aeration. These electrodes and separator materials should be biocompatible, conductive, low-cost, and resistance to corrosion, due to the long operation time in the subsurface.

**b)** In this thesis a sole pollutant has been used for testing soil bioremediation process. Nonetheless, different pollutants generally co-exist in soil, which may require integrated remediation strategies. More studies should be performed to tackle mixtures of chemicals.

**c)** To develop strategies for harvesting the microbial-based electricity produced during remediation processes in case of being operated under closed circuit conditions.

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**Chapter 4** **Cleaning-up atrazine polluted soil by using Microbial Electroremediating Cells (MERC)**

**Chapter 5** **Bioelectroventing: an electrochemical-assisted bioremediation strategy for cleaning-up atrazine polluted soils**

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# Annex II

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# Annex III

## Abbreviations and Units

# Abbreviations and Units

**ABBREVIATIONS**

| Symbol | Meaning |
|---|---|
| **$^{14}$C-ATR** | Labeled atrazine |
| **$^{14}$C-$CO_2$** | Labeled carbon dioxide |
| **ASE** | Accelerated Solvent Extraction |
| **ATR** | Atrazine |
| **ATRA** | Atrazine chlorohydrolase enzyme |
| **ATRB** | Hydroxy-atrazine ethylaminohydrolase enzyme |
| **ATRC** | N-isopropylammelide isopropylaminohydrolase enzyme |
| **ATRD** | Cyanuric acid amidohydrolase enzyme |
| **ATRE** | Biuret amidohydrolase enzyme |
| **ATRF** | Allophanate hydrolase enzyme |
| **BMFC** | Benthic Microbial Fuel Cell |
| **CA** | Chronoamperometry |
| **CE** | Counter Electrode |
| **CV** | Cyclic Voltammetry |
| **DBT** | Dibenzothiophene |
| **DEA** | Desethylatrazine |
| **DEHA** | Deethylhydroxyatrazine |
| **DIA** | Deisopropylatrazine |
| **DIET** | Direct Interspecies Electron Transfer |
| **DIHA** | Deisopropylhydroxyatrazine |

| | |
|---|---|
| **DMSO** | Dimethyl Sulfoxide |
| **DO** | Dissolved Oxygen |
| **E** | Potential |
| **$E_1$** | Initial potential |
| **$E_2$** | Final potential |
| **$E_A$** | Anode potential |
| **$E_C$** | Cathode potential |
| **$EC_{50}$** | Half maximal effective concentration |
| **$E_{eq}$** | Equilibrium potential |
| **Eh** | Redox potential |
| **ER** | Extractable Residues |
| **FBC** | Floating biocathode |
| **FC** | Fuel Cell |
| **HA** | Hydroxyatrazine |
| **HOC** | Hydrophobic Organic Compound |
| **HPLC-DAD** | High Performance Liquid Chromatography with Diode Array Detection |
| **I** | Current |
| **$K_d$** | Distribution coefficient |
| **$K_f$** | Freundlich adsorption constant |
| **$K_{oc}$** | Soil organic carbon-water partitioning coefficient |
| **$K_{ow}$** | Octanol/water partitioning coefficient |
| **LSV** | Linear Sweep Voltammetry |
| **LT-SEM** | Low Temperature Scanning Electron Microscopy |
| **MEA** | Membrane Electrode Assembly |
| **MERC** | Microbial Electroremediating Cell |

| | |
|---|---|
| **MET** | Microbial Electrochemical Technology |
| **MFC** | Microbial Fuel Cell |
| **$\eta_A$** | Anode overpotential |
| **$\eta_C$** | Cathode overpotential |
| **NER** | Non Extractable Residues |
| **OC** | Open Circuit |
| **OCP** | Open Circuit Potential |
| **OCV** | Open Circuit Voltage |
| **OD** | Optical Density |
| **OECD** | Organization for Economic Cooperation and Development |
| **P** | Power |
| **PAH** | Polycyclic Aromatic Hydrocarbons |
| **PCB** | Polichlorobenzene |
| **PEM** | Proton Exchange Membrane |
| **P- MFC** | Plant Microbial Fuel Cell |
| **pol-MERC** | Polarized Microbial Electroremediating Cell |
| **PSII** | Photosystem II |
| **RE** | Reference Electrode |
| **$R_{EXT}$** | External resistance |
| **$R_{INT}$** | Internal resistance |
| **SHE** | Standard Hydrogen Electrode |
| **SMFC** | Sediment Microbial Fuel Cell |
| **SPE** | Solid Phase Extraction |
| **TAC-MFC** | Tubular Air Cathode Microbial Fuel Cell |
| **TEA** | Terminal Electron Acceptor |

| | |
|---|---|
| **TPH** | Total Petroleum Hydrocarbons |
| **TZN** | Atrazine chlorohydrolase enzyme |
| **V** | Voltage |
| **WE** | Working Electrode |
| **ΔE** | Difference of potential |
| **ΦPSII** | Quantum yield or photosystem II efficiency |

**UNITS**

| Symbol | Meaning |
|---|---|
| **A** | Ampere |
| **atm** | Atmosphere |
| **Bq** | Becquerel |
| **cells·mL$^{-1}$** | Cells per millilitre |
| **cm** | Centimeter |
| **cm$^2$** | Square centimeter |
| **dSm$^{-1}$** | DeciSiemens per meter |
| **g** | Gram |
| ***g*** | Relative centrifugal force |
| **g·mol$^{-1}$** | Grams per mole |
| **h** | Hour |
| **kBq** | Kilobecquerel |
| **Kg** | Kilogram |
| **KΩ** | KiloOhm |
| **L** | Liter |
| **M** | Molar |

| | |
|---|---|
| **m** | Meter |
| **$m^2$** | Square meter |
| **$m^3$** | Cubic meter |
| **mA** | MilliAmpere |
| **mg** | Milligram |
| **mL** | Milliliter |
| **mm** | Millimeter |
| **mM** | MilliMolar |
| **mm·Hg** | Millimeter of mercury |
| **mS** | MilliSiemen |
| **mV** | MilliVolt |
| **$mV \cdot s^{-1}$** | MilliVolt per second |
| **mW** | MilliWatt |
| **nm** | Nanometer |
| **ºC** | Degree Celsius |
| **V** | Volt |
| **W** | Watt |
| **µg** | Microgram |
| **µL** | Microliter |
| **µm** | Micrometer |
| **Ω** | Ohm |
| **Ω·m** | Ohm per meter |

# Annex IV

Curriculum Vitae

# Curriculum Vitae

## PERSONAL INFORMATION

Ainara Domínguez Garay

Born: May 7th 1986, Bilbao, Spain

Nationality: Spanish

Contact information: ainara.dominguez@gmail.com

## ACADEMIC BACKGROUND

| | |
|---|---|
| 2/2012 - | **PhD Student at the Bioelectrogenesis research group**<br><br>Department of Analytical Chemistry, Physical Chemistry and Chemical Engineering<br>University of Alcalá<br><br>Thesis: Bioelectrochemically-assisted remediation: a novel strategy for cleaning-up polluted soils |
| 10/2011 - 07/2012 | **Master degree in Hydrology and Water Resource Management**<br><br>MsC.Thesis: "Bioelectrogenic degradation of atrazine herbicide in soils"<br>Alcalá University and Rey Juan Carlos University |
| 3/2011 - 12/2011 | **Researcher in "Toxicological evaluation of herbicides polluted soils under bioremediation treatments"**<br><br>Funding: University of Alcalá and Community of Madrid<br>Promoter: Karina Boltes Espinola |
| 09/2009 - 12/2010 | **Researcher in practice**<br><br>Final degree project: "Bioelectricity generation from Sedimentary Microbial Fuel Cell (SMFC)"<br>Department of Analytical Chemistry, Physical Chemistry and Chemical Engineering<br>University of Alcalá<br>Supervisor: Prof. Abraham Esteve Núñez |

09/2005 – 09/2010 **Bachelor in Environmental Sciences**

University of Alcalá

## RESEARCH STAYS

10/2014 – 12/2014 Institut für Bodenökologie, Helmholtz Zentrum München. Munich (Germany)

06/2015 – 07/2015 Institut für Bodenökologie, Helmholtz Zentrum München. Munich (Germany)

## OTHER RELEVANT EXPERIENCE

### Dissemination projects

- Researchers´ night in Madrid (2010-2015)

  Funding: People Program (7th FP)

- Research Programme for Secondary Schools based on 3D strategies: Design-Develop-Disseminate (PICSI-3D) (from 2014-2015)

  Funding: FECYT, Fundación Española para la Ciencia Y la Tecnología.

- Semana de la ciencia (2009-2013)

  Funding: Community of Madrid

- Química en Acción (2012-2013)

  Funding: University of Alcalá

### Teaching experience

- Lectures in the course “Bioengineering and new sources of energies”
  Biology program
  Alcalá University

### Tutoring experience

- Master student: Hydrology and Water Resource Management
  Alcalá University

- Bachelor student: Environmental Sciences
  Alcalá University

## SCIENTIFIC CONTRIBUTIONS

### Publications

**Domínguez.Garay, A.**, Berná, A., Ortiz-Bernad, I. and Esteve-Núñez, A.
Colloid formation for outperforming sediment microbial fuel cells in a low conductivity soil. *Environ. Sci. Technol.*, 2013, *47* (4), pp 2117–2122.
DOI: 10.1021/es303436x

**Domínguez-Garay, A.**; Boltes, K.; Esteve-Núñez, A., Cleaning-up atrazine polluted soil by using Microbial Electroremediating Cells. *Submitted*

**Domínguez-Garay, A.**; Rodrigo Quejigo, J.; Dörfler, U.; Schroll, R.; Esteve-Núñez, A. Bioelectroventing: an electrochemical-assisted bioremediation strategy for cleaning-up atrazine polluted soils. *Submitted*

**Domínguez-Garay, A.** and Esteve-Núñez, A. Designing strategies for operating Microbial Electroquemical System in soil under non-flooded conditions. *Submitted*

### Participations in workshops & congress

**Dominguez-Garay, A**.; Boltes, K.; Esteve-Núñez, A. Strategies for cleaning up ATRAZINE-polluted soils using Microbial Electroremediating Cells (MERCs). SETAC Europe 25th Annual Meeting. Barcelona, May 3-7, 2015. Oral presentation.

Rodrigo-Quejigo, J.; **Dominguez-Garay, A**.; Boltes, K.; Esteve-Núñez, A. Microbial Electroremediating Cells (MERC): strategies to biominerilezing C herbicides in soils by stimulating microbial electrogenic communities. 2nd European meeting of the International Society for Microbial Electrochemistry and Technology. Alcalá de Henares, 3-5 September 2014. Poster.

**Dominguez-Garay, A.**; Rodrigo-Quejigo, J, Boltes, K.; Esteve-Núñez, A. Strategies for cleaning up Atrazine-polluted soils using Microbial Electroremediating Cells (MERCs). 2nd European meeting of the International Society for Microbial Electrochemistry and Technology. Alcalá de Henares, 3-5 September 2014. Oral presentation and poster.

**Dominguez-Garay, A**.; Berna, A.; Boltes, K.; Rodrigo-Quejigo, J. and Esteve-Núñez, A. Colloid formation for outperforming plant-sediment microbial fuel cells in soils with low conductivity. 2nd International PlantPower Symposium. Ghent, 22-23 November. Poster.

Boltes, K.; **Dominguez-Garay, A.**; Rodrigo-Quejigo, J.; Reija, A., Berna, A. and Esteve-Núñez, A. Microbial Remediating Cells (MRC): strategies for cleaning up polluted soils by stimulating the bioremediation capacity of microbial electrogenic communities. European International Society for Microbial Electrochemistry and Technology Meeting (EU-ISMET). Ghent, 27-28 September. Poster.

Esteve-Nuñez, A.; Boltes, K.; Reija, A.; Bragado, N.; **Dominguez-Garay, A.** ;Rodrigo-Quejigo, J.; Biel, M. and Berná, A. Detoxification of herbicides contaminated soil using Microbial Fuel Technology. 12Th Mediterranean congress of Chemicals engineering (12MCCE). Barcelona, 15-18 November, 2012. Poster.

Esteve-Nuñez, A.; Boltes, K.; Reija, A.; Bragado, N.; **Dominguez-Garay, A.**; Rodrigo-

Quejigo, J.; Miriam Biel and Berná, A.. Microbial conversion of pollutants into electric current: strategies for monitoring and stimulating the bioremediation capacity of soil microbial communities. 15th International Symposium on Biodeterioration and Biodegradation (IBBS-15). Vienna. 19-24 September, 2011. Oral presentation.

Esteve-Nuñez, A.; Boltes, K.; Reija, A.; Bragado N.; **Dominguez-Garay, A.**; Rodrigo-Quejigo, J.; Biel, M.; Ortiz-Bernad, I.; and Berná, A. Electrogenic microcosm: strategies for monitoring and stimulating the bioremediation capacity of soil microbial communities. 3rd International Microbial Fuel Cells conference. Leeuwarden, Holland. 6-8 June, 2011. Poster.

**Dominguez-Garay, A.**; Berná, A.; Esteve-Núñez, A. Influence of Soil Structure on Outperforming Sedimentary Microbial Fuel Cell in low conductivity soil. 3rd International Microbial Fuel Cells conference. Leeuwarden, Holland. 6-8 June, 2011. Oral presentation.

Reija, A.; Tejedor, S.; Berna, A.; Boltes, K.; Gutierrez, C.; Borjas, Z.; Estevez-Canales, M.; **Dominguez-Garay, A.**; Rodrigo-Quejigo, J.; Biel, M.; Montero, E.; Barroeta, B. and Esteve-Nuñez, A. Microbial electrogenesis: wastewater as an energy resource. 3rd International congress of Wastewater in small communities: Smallwat 11 .Sevilla, Spain. 2-5 May, 2011. Poster.

**Dominguez-Garay, A.**; Esteve-Nuñez, A. and Ortiz-Bernad, I. Harvesting electricity from natural environments: role of rooting in sedimentary microbial fuel cell fuelled by rice plants. 1st International PlantPower simposium. Ghent, Belgium, 10 February, 2010. Poster.

**Dominguez-Garay, A.**; Esteve-Nuñez, A. and Ortiz-Bernad, I. Harvesting electricity from natural environments: role of rooting in sedimentary microbial fuel cell fuelled by rice plants. IV Congreso Ibérico de Cienciads del Suelo (CICS). Granada, Spain. 21-24 September, 2010. Poster.

Esteve-Nuñez, A., Reija, A.; Boltes, K.; Bragado, N.; Tejedor, S.; **Dominguez-Garay, A.**; Gutierrez, C.; Mas, N.; Barroeta, B.; Ortiz-Bernad, I. Electrogenic microcosm: novel method for monitoring and stimulating the bioremediation capacity of soil microbial communities. SETAC Europe 20th Annual Meeting· Sevilla, Spain. April 2010. Poster.