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DE POSGRADO Y DOCTORADO

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(FOR EVALUATION OF THE ACT DOCTORAL THESIS)

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DOCTORANDO *(candidate PHD)*: **AGUIRRE SIERRA, MARÍA ARÁNZAZU**

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DEPARTAMENTO DE *(Department)*: **QUÍMICA ANALÍTICA, QUÍMICA FÍSICA E INGENIERÍA QUÍMICA**
TITULACIÓN DE DOCTOR EN *(Phd title)*: **DOCTOR/A POR LA UNIVERSIDAD DE ALCALÁ**

En el día de hoy 14/09/17, reunido el tribunal de evaluación, constituido por los miembros que suscriben el presente Acta, el aspirante defendió su Tesis Doctoral **con Mención Internacional** (*In today assessment met the court, consisting of the members who signed this Act, the candidate defended his doctoral thesis with mention as International Doctorate*), elaborada bajo la dirección de (*prepared under the direction of*) ABRAHAM ESTEVE NÚÑEZ // JUAN JOSÉ SALAS RODRÍGUEZ.

Sobre el siguiente tema *(Title of the doctoral thesis)*: *INTEGRATING MICROBIAL ELECTROCHEMICAL SYSTEMS IN CONSTRUCTED WETLANDS, A NEW PARADIGM FOR TREATING WASTEWATER IN SAMLL COMMUNITIES*

Finalizada la defensa y discusión de la tesis, el tribunal acordó otorgar la CALIFICACIÓN GLOBAL[1] de (**no apto, aprobado, notable y sobresaliente**) *(After the defense and defense of the thesis, the court agreed to grant the GLOBAL RATING (fail, pass, good and excellent)*: SOBRESALIENTE

Alcalá de Henares, a 14 de septiembre de 2017

Fdo. *(Signed)*: Jordi Más Fdo. *(Signed)*: Carlo Aris Fdo. *(Signed)*: Eloy Peña

FIRMA DEL ALUMNO (candidate's signature),

Fdo. *(Signed)*: Mª ARANZAZU AGUIRRE

Con fecha 4 de octubre de 2017 la Comisión Delegada de la Comisión de Estudios Oficiales de Posgrado, a la vista de los votos emitidos de manera anónima por el tribunal que ha juzgado la tesis, resuelve:

☒ Conceder la Mención de "Cum Laude"
☐ No conceder la Mención de "Cum Laude"

La Secretaria de la Comisión Delegada

[1] La calificación podrá ser "no apto" "aprobado" "notable" y "sobresaliente". El tribunal podrá otorgar la mención de "cum laude" si la calificación global es de sobresaliente y se emite en tal sentido el voto secreto positivo por unanimidad. (*The grade may be "**fail**" "**pass**" "**good**" or "**excellent**". The panel may confer the distinction of "cum laude" if the overall grade is "Excellent" and has been awarded unanimously as such after secret voting.).*

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En aplicación del art. 14.7 del RD. 99/2011 y el art. 14 del Reglamento de Elaboración, Autorización y Defensa de la Tesis Doctoral, la Comisión Delegada de la Comisión de Estudios Oficiales de Posgrado y Doctorado, en sesión pública de fecha 4 de octubre, procedió al escrutinio de los votos emitidos por los miembros del tribunal de la tesis defendida por *AGUIRRE SIERRA, MARÍA ARÁNZAZU*, el día 14 de septiembre de 2017, titulada *INTEGRATING MICROBIAL ELECTROCHEMICAL SYSTEMS IN CONSTRUCTED WETLANDS, A NEW PARADIGM FOR TREATING WASTEWATER IN SAMLL COMMUNITIES*, para determinar, si a la misma, se le concede la mención "cum laude", arrojando como resultado el voto favorable de todos los miembros del tribunal.

Por lo tanto, la Comisión de Estudios Oficiales de Posgrado **resuelve otorgar** a dicha tesis la

MENCIÓN "CUM LAUDE"

Alcalá de Henares, 10 de octubre de 2017
EL PRESIDENTE DE LA COMISIÓN DE ESTUDIOS
OFICIALES DE POSGRADO Y DOCTORADO

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Juan Ramón Velasco Pérez

Copia por e-mail a:
Doctorando: AGUIRRE SIERRA, MARÍA ARÁNZAZU
Secretario del Tribunal: ELOY GARCÍA CALVO.
Directores de Tesis: ABRAHAM ESTEVE NÚÑEZ // JUAN JOSÉ SALAS RODRÍGUEZ

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Comprobado que el expediente académico de D./Dª _____ reúne los requisitos exigidos para la presentación de la Tesis, de acuerdo a la normativa vigente, y habiendo presentado la misma en formato: ☐ soporte electrónico ☐ impreso en papel, para el depósito de la misma, en el Servicio de Estudios Oficiales de Posgrado, con el nº de páginas: _____ se procede, con fecha de hoy a registrar el depósito de la tesis.

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Facultad de Biología, Ciencias Ambientales y Química
Ctra. Madrid-Barcelona, Km. 33,600
28871 Alcalá de Henares (Madrid)
España

JESUS ALBERTO ESCARPA MIGUEL, Profesor Titular de Química Analítica y Director del Departamento de Química Analítica, Química Física e Ingeniería Química de la Universidad de Alcalá,

CERTIFICA:

Que el trabajo descrito en la presente memoria, titulado "**INTEGRATING MICROBIAL ELECTROCHEMICAL SYSTEMS IN CONSTRUCTED WETLANDS, A NEW PARADIGM FOR TREATING WASTEWATER IN SMALL COMMUNITIES**" ha sido realizado por Dña. María Aránzazu Aguirre Sierra bajo la dirección del Dr. Abraham Esteve Núñez, Profesor Titular del Área de Ingeniería Química del Departamento de Química Analítica, Química Física e Ingeniería Química de la Universidad de Alcalá, y del Dr. Juan José Salas Rodríguez, Director Técnico de la Fundación Centro de las Nuevas Tecnologías del Agua.

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 22 de abril de 2017.

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DEPARTAMENTO DE QUÍMICA ANALÍTICA, QUÍMICA FÍSICA E INGENIERÍA QUÍMICA

Facultad de Biología, Ciencias Ambientales y Química
Ctra. Madrid-Barcelona, Km. 33,600
28871 Alcalá de Henares (Madrid)
España

ABRAHAM ESTEVE NÚÑEZ, Profesor Titular de Ingeniería Química de la Universidad de Alcalá, y **JUAN JOSÉ SALAS RODRÍGUEZ**, Director Técnico de de la Fundación Centro de las Nuevas Tecnologías del Agua,

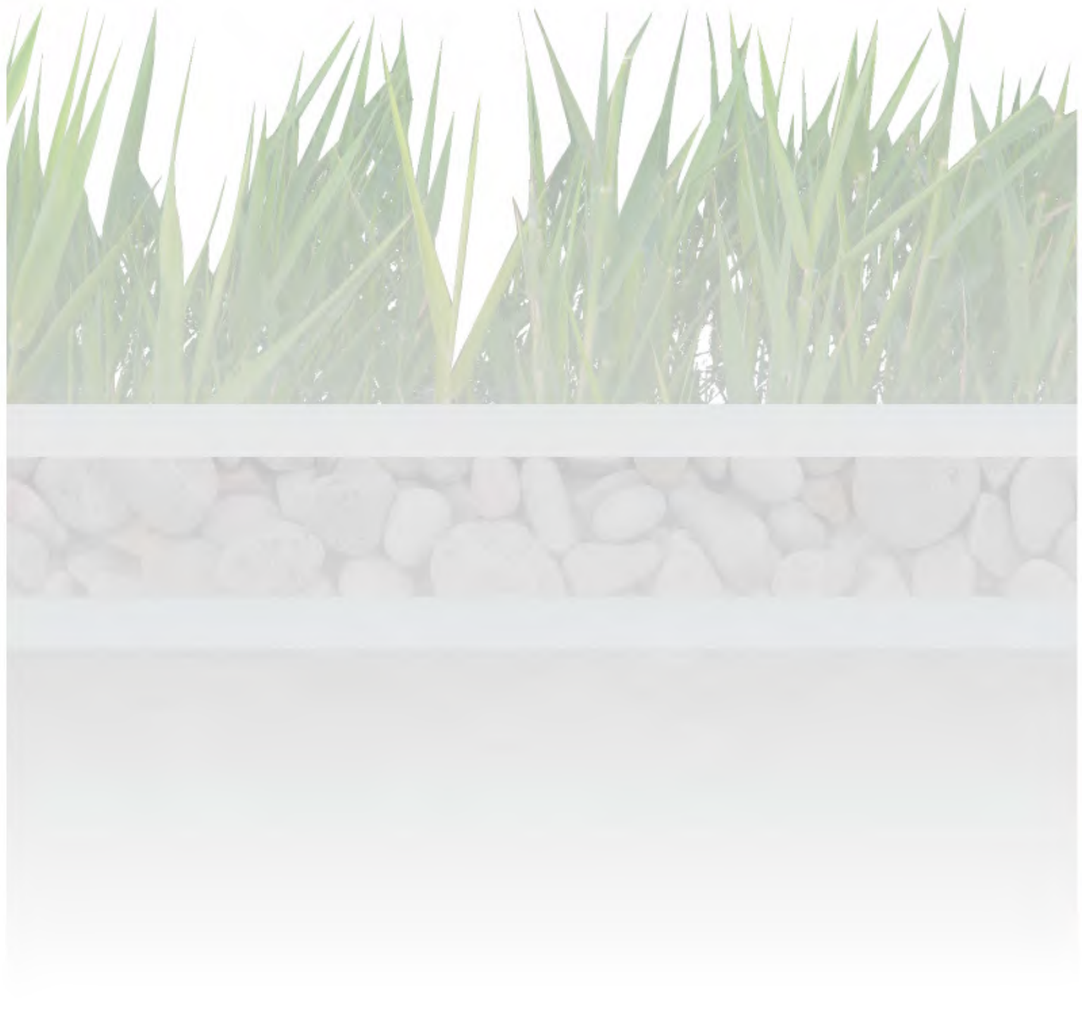
CERTIFICAN:

Que el trabajo descrito en la presente memoria, titulado "**INTEGRATING MICROBIAL ELECTROCHEMICAL SYSTEMS IN CONSTRUCTED WETLANDS, A NEW PARADIGM FOR TREATING WASTEWATER IN SMALL COMMUNITIES**", ha sido realizado bajo su dirección por **Dª. María Aránzazu Aguirre Sierra** en el Área de Ingeniería Química del Departamento de Química Analítica, Química Física e Ingeniería Química de la Universidad de Alcalá y en las instalaciones de la Fundación CENTA. Asimismo, autorizan su presentación para que sea defendido como Tesis Doctoral.

Y para que conste y surta los efectos oportunos, firman el presente en Alcalá de Henares a 22 de abril de 2017.

Abraham Esteve Núñez Juan José Salas Rodríguez

Microbial electrochemistry is an emerging discipline based on the interactions between microorganisms and electrically conductive materials. One of the applications of this technology consists in using the electrodes as unlimited electron acceptors or donors for wastewater treatment. In this thesis we have explored the integration of this emergent technology with constructed wetlands since they are a good alternative for wastewater treatment in small communities due to their low installation and operation cost. A new concept has been introduced both in the field of wastewater treatment and microbial electrochemical technologies: the concept of METland. The remarkable improvement in treatment efficiency makes METland a promising technology for wastewater treatment in small populations. Furthermore, the discovery that bioelectrochemical processes can take place in either anaerobic or aerobic environments has laid the groundwork for the design of future generation of METlands.



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MARÍA ARÁNZAZU AGUIRRE SIERRA

Integrating Microbial Electrochemical Systems in Constructed Wetlands, a New Paradigm for Treating Wastewater in Small Communities

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Integrating Microbial Electrochemical Systems in Constructed Wetlands,

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a new paradigm for treating wastewater in small communities

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Dirigida por:

Dr. Abraham Esteve Núñez
Dr. Juan José Salas Rodríguez

Departamento de Química Analítica, Química Física e Ingeniería Química

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A Mani, Lucas y Olmo

El agua es el símbolo por excelencia de la pureza, del amor y del misticismo, pero también de lo oculto en las simas, del mal escondido en los oscuros lagos subterráneos o de la desgracia representada en un albañal emponzoñado al que se arrumban nuestras esperanzas o desgracias, de la misma manera que arrojamos a las cloacas mil productos que envenenan la fuente de la vida (…)

Ríos de Letras. Antología de la imagen del Agua y de los Ríos en la Literatura
Pedro Brufao Curiel
Manuel García Castellón-Benarroch

"Nuestra recompensa se encuentra en el esfuerzo y no en el resultado. Un esfuerzo total es una victoria completa"

Mahatma Gandhi

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Table of contents

Summary 13

Resumen 17

CHAPTER 1: Introduction **21**

1.1 Urban wastewater treatment in small populations **23**

1.1.1 The need of wastewater treatment 23

1.1.2 The concept of small population 23

1.1.3 Legal framework of WWT 24

1.1.4 Special characteristics of small populations 25

1.1.5 Applicable technologies to WWT in small populations 28

1.1.6 Considerations to select the WWT technology in small populations 30

1.2 Treatment wetlands **32**

1.2.1 The concept of treatment wetland 32

1.2.2 Types of treatment wetlands 34

1.2.3 Surface flow constructed wetlands 36

1.2.4 Subsurface flow constructed wetlands 37

Horizontal subsurface flow CWs 38

Vertical flow CWs 38

Hybrid CWs 40

1.2.5 Treatment processes and performance in SSF CWs 40

Suspended solids removal 41

Redox potential behaviour 42

Removal or organic matter 42

Nitrogen removal 44

Phosphorous removal 46

1.3 Microbial electrochemical technologies (MET) **47**

1.3.1 Fundaments of microbial electrochemistry 47

1.3.2 Extracellular electron transfer (EET) 48

Direct extracellular electron transfer (DEET) 49

Mediated extracellular electron transfer (MEET) 53
1.3.3 The model electroactive bacteria: *Geobacter* 53
1.3.4 Types of METs by the operation mode 54
Microbial fuel cell (MFC) 55
Microbial electrolysis cell (MEC) 55
Microbial electrochemical snorkel (MES) 56
1.3.5 Wastewater treatment application of METs 57
1.3.6 Pollutants removal processes in METs 58
Bioelectrochemical organic matter oxidation 58
Bioelectrochemical nutrients removal 59
1.3.7 Scaling-up MET: the challenge 61

CHAPTER 2: Objectives and Research Framework 63

Objectives and Thesis Outline 65

Research Framework 67

CHAPTER 3: Microbial Electrochemical Systems Outperform Fixed-Bed Biofilters in Cleaning Up Urban Wastewater 69

3.1 Abstract 71

3.2 Introduction 72

3.3 Materials and methods 73
3.3.1 Design and construction of electroconductive biofilters 73
3.3.2 Physical, chemical and statistical analysis 75
3.3.3 Microbial communities 76
Sampling, DNA extraction and 16S rDNA sequencing 76
Bioinformatics analysis 76
Scanning electron microscopy analysis 77

3.4 Results And Discussion 77
3.4.1 Urban WWT by horizontal subsurface flow biofilters: electroconductive versus non electroconductive biofilters 78

Influence of the material on the wastewater pollutants removal 78
Electrochemical analysis using hybrid electroconductive setups 81
3.4.2 Microbial Communities 83
Presence of deltaproteobacteria as indicator of microbial electroactivity 84
Nitrogen cycle bacteria: nitrification and denitrification 87
3.5 Conclusions 87
3.6 Supplementary Information 88

CHAPTER 4: Scaling-Up METs: a Four Years Study of a Real METland 99

4.1 Abstract 101
4.2 Introduction 101
4.3 Materials and Methods 104
4.3.1 METland configuration 104
4.3.2 METland operation and water analysis 105
4.3.3 Determination of the loading rates 107
4.3.4 Study of the microbial community 108
Sampling, DNA extraction and 16S rDNA sequencing 108
Bioinformatics analysis 108
Chemical analysis of water 109
4.3.5 Study of oxygen release by wetland plants 110
Qualitative determination of oxygen release with methylene blue method 111
Root porosity determination by a pycnometer method 111
4.4 Results 113
4.4.1 Removal of pollutants from wastewater 115
Organic matter removal 115
Total suspended solids removal 115
Nutrients removal 118
4.4.2 Microbial community 122

First stage 16S rDNA sequencing 122
Second stage 16S rDNA sequencing 125
4.4.3 Oxygen released by plant roots 130
4.5 Discussion 133
4.5.1 Efficiency of the METland compared to conventional HSSF CWs 133
4.5.2 Microbial community 135
4.5.3 Oxygen released by wetland plants 142
4.5.4 Design parameters 143
4.6 Conclusions 149
4.7 Supplementary Information 149
CHAPTER 5: A New Concept In METlands: Assessment of Aerobic Electroactive Biofilters 165
5.1 Abstract 167
5.2 Introduction 168
5.3 Materials and methods 169
5.3.1 Experimental design 169
5.3.2 Biofilters operation 170
5.3.3 Physicochemical and statistical analysis 170
5.3.4 Microbial community study 171
Sampling, DNA extraction and 16S rDNA sequencing 172
Bioinformatics analysis 172
5.4 Results And Discussion 173
5.4.1 Organic matter removal in aerobic and anaerobic biofilters 173
5.4.2 Nutrients removal in aerobic and anaerobic biofilters 174
5.4.3 Analysis of microbial communities of aerobic and anaerobic systems 176
5.4.4 Analysis of microbial communities of conductive and inert materials 179
5.5 Conclusions 180
5.6 Supplementary Information 181

CHAPTER 6: Assessing The Design of a Vertical Aerobic MET Filter 189

6.1 Abstract 191

6.2 Introduction 191

6.3 Material And Methods 192

6.3.1 Experimental set up and operation 192

6.3.2 Physicochemical and statistical analysis 193

6.3.3 Scanning electron microscopy study 194

6.4 Results And Discussion 195

6.4.1 Organic matter removal 195

6.4.2 Nitrogen removal 196

6.4.3 Assessing the design of VF aerobic METlands 201

6.5 Conclusions 201

CHAPTER 7: General Discussion, Conclusions And Future Work 203

7.1 General Discussion 205

7.2 Conclusions 209

7.3 Recommendations And Future Work 210

References 213

Annex 231

List of Figures 233

List of Supplementary Figures 239

List of Tables 241

List of Supplementary Tables 243

Abbreviations 247

Summary

The wastewater treatment (WWT) in small populations constitutes nowadays one of the main challenges to achieve the good ecological status of surface waters established by the Water Framework Directive. The concept of good ecological status demands to ensure the physicochemical and hydromorphological conditions to allow the equilibrium of the biological communities of the surface waters. The special characteristics of small populations require sustainable and decentralized technologies with low energy costs. In this context microbial electrochemical technologies (METs) can provide energy efficient solutions.

METs constitute a set of technologies focused on the study of those processes produced by the interaction between microorganisms and materials conducting electricity, and which corresponds to the emerging field of electromicrobiology. The clear advantage of exploiting electroactive communities is that materials can boost microbial metabolism in anaerobic systems that are typically electron acceptor limited. WWT field has shown a vast potential for applying that innovative technologies from the very beginning. In spite of the strong potential the main challenge that microbial electrochemical technologies face is the scaling-up of the systems.

In this thesis we have explored the integration of this emergent technology with constructed wetlands since they are a good alternative for wastewater treatment in small communities due to their low installation and operation cost. However, they are not exempt from certain drawbacks such as the large area required per inhabitant.

The thesis is organized in seven chapters, four of them are devoted to develop the objectives and constitute the body of the research.

The dissertation begins by a first introductory chapter (**Chapter 1**) that offers a brief review of the special characteristics of the WWT in small populations, establishing its normative framework, together with the technologies commonly applied in this field with special attention to the constructed wetlands. This chapter also presents the fundamentals of microbial electrochemistry by briefly describing the mechanisms of electron transfer in electroactive microorganisms and the types of METs according to their mode of operation. Moreover, the application and bioelectrochemical processes related to pollutants removal in WWT are also included.

Chapter 2 sets out the objectives and framework for research. The research aimed to improve constructed wetlands (CWs) treatment by incorporating METs with the final goal of reducing their classical size, increasing the number of communities able to host the technology.

With this aim we have explored the conversion of the classical inert CW into an electroconductive bed in **Chapter 3**. This thesis presents for first time the integration of METs in biofilters used in horizontal flow CWs to form METlands, a new concept in WWT. We explored two different types of microbial electrochemical systems that are reviewed in the introduction: the microbial electrolysis cell (MEC), a three electrode configuration in which an electrode was poisoned to a certain potential; and the snorkel configuration, that constitutes a single electrode of conductive material that presents a redox potential gradient with depth. We also analysed the bacterial communities of the biofilms attached to the conductive and the inert materials under the different conditions to understand the role of electrically conductive material in selecting microbial populations. Our results revealed that the single electrode system represents a substantial advance since it can be operated a surface inlet load 4-fold higher than the standard systems, which suggest that surface area requirements of classical inert CWs could be significantly reduced.

In **Chapter 4** we show that METs can successfully improve the performance of CWs at pilot scale in terms of organic matter removal and we suggest that electroactive bacteria (EAB) are the main responsible of this effect. We present a pilot horizontal flow bioelectrochemical constructed wetland (METland), with a surface of 24 m^2 and a bed volume of 15 m^3, that was constructed in the facilities of the Foundation New Water Technologies (CENTA) in Carrión de los Céspedes (Sevilla, Spain) to treat real urban wastewater and we analyse the results of four years of operation, during which several electrochemical configurations were investigated. In the two 16S rDNA sequencing carried out an enrichment of electroactive bacteria in the biofilms of the electroconductive material was observed, whether the electrode was the anode or the cathode. We have also observed that the pollutants degradation state of wastewater affected taxa selection in the biofilms. The better degradation rates of the METland were confirmed when the BOD kinetic constant was calculated from the experimental data, being 2.4-fold the one established for CWs, especially when the anode was enlarged, revealing the important contribution of the electroconductive bed to the organic removal processes.

So far we have shown how METs have been implemented in horizontal anaerobic CWs. However, another type of CWs, those operating under vertical down-flow, intermittently lead to aerobic internal conditions and have a better efficiency in the elimination of pollutants, especially ammonium through aerobic metabolic processes. So, in **Chapter 5** we explore if bioelectrochemical processes can work in aerobic systems and if EAB are also enriched in the biofilms of the aerobic MET biofilters. Our results support the hypothesis that the conductive material enhances the development of electroactive bacteria even in aerobic systems and show that aerobic MET biofilters maximize the synergetic effects of both aerobic and anaerobic systems.

The **Chapter 6** is a short chapter that assess the removal of pollutants through the vertical column in a vertical down-flow MET biofilter compared to a conventional biofilter made of gravels, and try to evaluate how much could be reduced in depth. The differences we have found lead to establishing that the depth of conventional CWs could be reduced by a third at extremely high organic loading rates (until 400 g DBO_5 m^{-2} d^{-1}) when the inert bed is replaced by a conductive material, so that the volume would be reduced too, lowering the costs of implantation.

A general discussion, conclusions and future outlook are presented in **Chapter 7,** where final considerations of this thesis are expressed under a question-answer mode. The remarkable improvement in treatment efficiency makes METland a promising technology for wastewater treatment in small populations. Furthermore, a new concept has been introduced both in the field of WWT and the METs: the concept of METland. The discovery that bioelectrochemical processes can take place in either anaerobic or aerobic environments has laid the groundwork for the design of future generation of METlands.

Resumen

El tratamiento de aguas residuales en pequeñas poblaciones constituye actualmente uno de los principales retos para lograr el buen estado ecológico de las aguas superficiales establecido por la Directiva Marco del Agua. El concepto de buen estado ecológico exige garantizar las condiciones físico-químicas e hidromorfológicas que permitan el equilibrio de las comunidades biológicas de las aguas superficiales. Las características especiales de las pequeñas poblaciones hacen que estas requieran tecnologías sostenibles y descentralizadas, con bajos costes energéticos. En este contexto, las tecnologías electroquímicas microbianas (MET) pueden proporcionar soluciones energéticamente eficientes.

Las Tecnologías Electroquímicas Microbianas, constituyen un conjunto de tecnologías enfocadas al estudio de aquellos procesos producto de la interacción entre microorganismos y materiales conductores de la electricidad, y que corresponde al campo emergente de la electromicrobiología. La ventaja de emplear comunidades electroactivas es que los materiales electroconductores pueden acelerar el metabolismo microbiano en aquellos sistemas anaerobios típicamente limitados en aceptores de electrones. Uno de los campos en los que estas tecnologías han sido más investigadas es el tratamiento de aguas residuales. Pero el principal desafío al que se enfrentan las MET es la aplicación de estos sistemas a escala real.

En esta tesis se ha explorado la integración de esta tecnología emergente con humedales artificiales, ya que son una buena alternativa para el tratamiento de aguas residuales en pequeñas comunidades debido a su bajo coste de instalación y operación, no exenta de ciertos inconvenientes, principalmente la gran superficie requerida por habitante.

La tesis se organiza en siete capítulos, cuatro de los cuales desarrollan los objetivos y constituyen el cuerpo de la investigación.

La disertación comienza con un primer capítulo introductorio (**Capítulo 1**) que ofrece una breve revisión de las características especiales del tratamiento de aguas residuales en pequeñas poblaciones, establece su marco normativo, menciona las tecnologías comúnmente aplicadas en este campo y se centra en una de las tecnologías extensivas, los humedales artificiales.

De igual modo, en este capítulo se presentan los fundamentos de la electroquímica microbiana, describiéndose brevemente los mecanismos de

transferencia de electrones en microorganismos electroactivos y los tipos de METs según su modo de operación, así como sus aplicaciones y los procesos bioelectroquímicos relacionados con la eliminación de contaminantes en el tratamiento de aguas residuales.

El **Capítulo 2** establece los objetivos y el marco de trabajo. El objetivo general de la investigación se centró en mejorar el tratamiento de contaminantes en los humedales artificiales mediante la incorporación de METs, con el propósito final de reducir su tamaño clásico, permitiendo la instalación de la tecnología en cualquier pequeña población, con un coste energético nulo.

Con esta finalidad, en el **Capítulo 3** se ha evaluado la conversión del clásico lecho inerte de gravas de un humedal artificial en un lecho electroconductor. Esta tesis presenta, por primera vez, la integración de las METs en los biofiltros que se emplean en los humedales artificiales de flujo horizontal, para constituir un METland, un nuevo concepto en el tratamiento de aguas residuales. Se investigaron dos tipos diferentes de METs que se explican en la introducción: la célula de electrólisis microbiana (MEC), una configuración con tres electrodos en la que un electrodo se polariza a cierto potencial, y la configuración snorkel, que constituye un único electrodo de material conductor que presenta un gradiente de potencial redox con la profundidad.

También se analizaron las comunidades bacterianas de los biofilms, adheridos tanto a los materiales conductores como inertes, bajo las diferentes condiciones, para comprender el papel del material electroconductor en la selección de las poblaciones microbianas. Se encontró que la configuración más sencilla, la de un solo electrodo, representa un avance sustancial al hacerse funcionar con una carga orgánica superficial 4 veces superior a los sistemas estándar, lo que sugiere que los requerimientos de superficie de los humedales artificiales convencionales podrían ser reducidos significativamente.

En el **Capítulo 4** se muestra que las METs pueden mejorar con éxito el rendimiento de los humedales artificiales a escala piloto, en términos de eliminación de materia orgánica, y se sugiere que las bacterias electroactivas (EAB) son las principales responsables de este efecto.

Se trabajó con un humedal artificial bioelectroquímico de flujo horizontal (METland), con una superficie de 24 m^2 y un volumen de lecho de 15 m^3, construido en las instalaciones de la Fundación Nuevas Tecnologías del Agua (CENTA), en Carrión de los Céspedes (Sevilla, España), para el tratamiento de aguas residuales

urbanas reales y se analizaron los resultados de cuatro años de funcionamiento, durante los cuales se investigaron varias configuraciones electroquímicas.

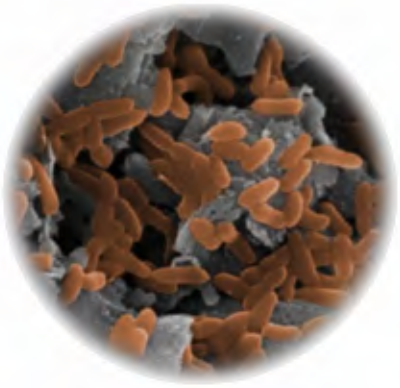
En los dos análisis de poblaciones llevados a cabo, se observó un enriquecimiento de bacterias electroactivas en los biofilms del material electroconductor, con independencia de la naturaleza electroquímica (anódica o catódica) del material. También se ha comprobado que el estado de degradación de contaminantes de las aguas residuales afectó a la selección de taxones en biofilms. Las mayores tasas de degradación del METland fueron confirmadas cuando se calculó la constante cinética de la DBO_5 a partir de los datos experimentales, resultando ser 2,4 veces superior a la establecida para los humedales artificiales de flujo horizontal, especialmente cuando se incrementaron las dimensiones del ánodo, revelando la importante contribución del lecho electroconductor a los procesos de eliminación de materia orgánica.

Tras las investigaciones llevadas a cabo sobre la implementación de las METs en humedales de flujo horizontal, en los que las condiciones de operación son, básicamente, anaerobias, se comenzó a trabajar con humedales de flujo vertical descendente, en los que imperan condiciones aerobias. El resultado fue un elevado grado de nitrificación de las formas amoniacales presentes en las aguas a tratar. Fruto de estas investigaciones, en el **Capítulo 5** se analiza si los procesos bioelectroquímicos pueden funcionar en sistemas aerobios y si los biofilms de los biofiltros MET aerobios también se enriquecen en EAB. Los resultados obtenidos apoyan la hipótesis de que el material conductor mejora el crecimiento de las EAB, incluso en sistemas aerobios, y muestran que los biofiltros MET aerobios maximizan los efectos sinérgicos de ambos sistemas aerobios y anaerobios.

En el **Capítulo 6,** de forma breve, se evalúa la eliminación de contaminantes a través de la columna vertical en un biofiltro vertical MET de flujo descendente, en comparación con un biofiltro convencional de grava, e intenta cuantificar cuánto se podría reducir la profundidad del lecho filtrante, sin afectar a los rendimientos. Las diferencias que se han encontrado conducen a establecer que la profundidad de los humedales artificiales de flujo vertical convencionales podría reducirse en un tercio, a tasas de carga orgánica extremadamente altas (hasta 400 g DBO_5 m^{-2} d^{-1}), cuando el lecho inerte es reemplazado por un material conductor, disminuyendo notablemente los costes de implantación.

Una discusión general, las conclusiones y perspectivas futuras se presentan en el **Capítulo 7**, donde las consideraciones finales de esta tesis se expresan a modo de pregunta-respuesta. Se discute el nuevo concepto de METland. La notable

mejora en la eficiencia del tratamiento hace de los METland una tecnología prometedora para el tratamiento de aguas residuales en pequeñas poblaciones. El descubrimiento de que los procesos bioelectroquímicos pueden tener lugar en ambientes tanto anaerobios como aerobios, ha sentado las bases para el diseño los futuros METlands



CHAPTER 1: Introduction

Introduction

1.1 Urban wastewater treatment in small populations

1.1.1 The need of wastewater treatment

Wastewater contains organic matter, pathogens (disease organisms), nutrients such as nitrogen and phosphorus, solids, chemicals from cleaners and disinfectants and even hazardous substances. Given all of the components of wastewater, it seems fairly obvious that we need to treat wastewater not only to recycle the water and nutrients but also to protect human and environmental health. Due to their volume, discharges of urban waste water are the second most serious cause of the pollution of waters by eutrophication.

Urban wastewater must be suitably treated whether it is going to be discharged into surface waters or reused. The main goals of wastewater treatment (WWT) are to protect the good ecological status of surface waters and to avoid risks for public health and living organisms that depend on them.

When speaking about water pollution and its effects on aquatic ecosystems, it is necessary to specify that their response is different depending on the kind of pollution, whether it is organic matter, inorganic nutrients, toxic substances or pathogens. With respect to pollution caused by untreated urban wastewater discharges, we refer in this thesis to organic matter and inorganic nutrients.

A current goal of our society is that of becoming sustainable in order to preserve the environment for future generations. WWT is a key element to reach this target, both because it mitigates the release of toxic compounds and because it alone consumes around 1.5% of electrical energy used yearly (Water Infrastructure Network, 2001), with its associated emission of greenhouse gases. The most efficient and widely implemented method to treat domestic and industrial wastewater is the activated sludge process, where oxygen is pumped in water tanks to allow microbial respiration and ultimately waste degradation. However, both aeration and disposal of residual sludge require high energetic inputs, and alternative technologies are sought.

1.1.2 The concept of small population

WWT is a universal process that is applicable to all kind of urban agglomerations, independently of its size. The size of a population can be

established according to the number of inhabitants and/or the organic load. In that sense, the common criteria applied to distinguish between small, medium and big populations is the number of population equivalents (p.e.). P.e., in WWT, is the number expressing the ratio of the sum of the pollution load produced during 24 hours by industrial facilities and services to the individual pollution load in household sewage produced by one person in the same time. In particular, one p.e. is the organic biodegradable load having a five-day biochemical oxygen demand (BOD_5) of 60 g of oxygen per day (Dir. 91/271/EEC of 21 May 1991).

There is not a global consensus on the number of inhabitants necessary to define a small population. Nevertheless, in the European Union, the term small population is often referred to those small agglomerations with less than 2,000 p.e., coinciding with the limit established by the Council Directive 91/271/EEC concerning urban WWT of 21 May 1991. Moreover, that limit was also used in the 7th International Water Association (IWA) Leading-Edge Conference on Water and Wastewater Technologies in 2010 (Ortega et al., 2010)

1.1.3 Legal framework of WWT

In Spain, as in the rest of member States of the European Union (EU), the basic regulation about WWT is the Council Directive 91/271/EEC concerning urban WWT that was adopted on 21 May 1991. Its objective is to protect the environment from the adverse effects of urban wastewater discharges and discharges from certain industrial sectors (see Annex III of the Directive). For that purpose, the Directive determines some minimal requirements for the collection and treatment of the wastewaters according to the size of the urban agglomeration and the characteristics of the receiving waters. Specifically the Directive requires:

- The collection and treatment of waste water in all agglomerations of >2,000 p.e.;
- Secondary treatment of all discharges from agglomerations of > 2,000 p.e., and more advanced treatment for agglomerations >10,000 population equivalents in designated sensitive areas and their catchments;
- In the case of agglomerations of less than 2,000 p.e., the urban wastewater entering collecting systems shall before discharge be subject to appropriate treatment.

Appropriate treatment means treatment by any process that after discharge allows the receiving waters to meet the relevant quality objectives and provisions of

the Community Directives (Art. 2.9 Dir. 91/271/EEC of 21 May 1991). Thus, there are not specific limits of discharge for small populations. Discharges from urban waste water treatment plants shall satisfy the relevant requirements of Annex I.b., that is, secondary treatment in normal areas (Table 1-1) and more advanced treatment in designated sensitive areas and their catchments (Table 1-2). Primary treatment as a whole complete treatment process is not recommended. Secondary treatment is recommended for all communities (García, 2001). This Directive has been transposed into Spanish law in the Real Decreto 11/1995 that established the norms applicable to urban WWT and the RD 509/1996 that develops it.

The European Water Framework Directive (WFD) (Directive 2000/60/CEE) establish that waters must achieve good ecological and chemical status, to protect human health, water supply, natural ecosystems and biodiversity. The concept of good ecological status demands to ensure the physicochemical and hydromorphological conditions to allow the equilibrium of the biological communities of the surface waters.

Thus, the treatment of wastewater in the small populations must be adequate to meet the goals established by the WFD, as well as the quality objectives of receiving waters established in other related European Directives (Bathing Water Directive, Drinking Water Directive, Shellfish Water Directive, Fish Directive) and other aspects as the reuse of purified wastewater, the future landscapes associated to climate change, etc. This requires that, prior to select the WWT, the water body must be perfectly characterised in terms of quality and the objectives to be reached. In summary, the selected technology must allow to comply with the defined objectives.

1.1.4 Special characteristics of small populations

In the large cities, it is easy to get enough efficiency with low cost per capita, but when treating wastewater in small communities, the solutions used in larger cities are not applicable (Fahd et al., 2007). Many small communities face significant barriers to building and maintaining effective WWT services, including:

- limited financial resources;
- geographically dispersed populations; and
- limited managerial capacity;
- extreme topography and climate; and
- geographic isolation.

Table 1-1: Requirements for discharges from urban waste water treatment plants of agglomerations of > 2,000 p.e. The values for concentration or for the percentage of reduction shall apply.

Parameters	Concentration	Minimum percentage of reduction (1)
Biochemical oxygen demand (BOD_5 at 20 °C) without nitrification (2)	25 mg/l O_2	70-90 40 under Article 4 (2)
Chemical oxygen demand (COD)	125 mg/l O_2	75
Total suspended solids	35 mg/l 35 under Article 4 (2) (more than 10,000 p.e.) 60 under Article 4 (2) (2,000-10,000 p.e.)	90 90 under Article 4 (2) (more than 10,000 p.e.) 70 under Article 4 (2) (2,000-10,000 p.e.)

(1) Reduction in relation to the load of the influent.
(2) The parameter can be replaced by another parameter: total organic carbon (TOC) or total oxygen demand (TOD) if a relationship can be established between BOD_5 and the substitute parameter.

Table 1-2: Requirements for discharges from urban WWT plants to identified sensitive areas which are subject to eutrophication. The values for concentration or for the percentage of reduction shall apply.

Parameters	Concentration	Minimum percentage of reduction (1)
Total phosphorous	2 mg/l P (10,000 – 100,000 p.e.) 1 mg/l P (more than 100,000 p.e.)	80
Total nitrogen (2)	15 mg/l N (10,000 – 100,000 p.e.) 10 mg/l N (more than 100,000 p.e.)	70-80

(1) Reduction in relation to the load of the influent.
(2) Total nitrogen means: the sum of total Kjeldahl-nitrogen (organic N + NH_3), nitrate (NO_3)-nitrogen and nitrite (NO_2)-nitrogen.

The higher the degree of dispersion of a population is the more expensive and complex are the collection and treatment of wastewater. If in a region there are numerous isolated and very small centres of population, the solution use to consist of collecting wastewaters in a unique point, with the aim of treating wastewater as whole and saving costs. This grouping is called an agglomeration. The degree of agglomeration is a determining factor to choose the WWT technology. In those

regions were the population is more dispersed, a variety of processes have been used, including extensive technologies and individualized treatments (Ortega et al., 2010).

Small populations cannot take advantage of the economy of the scale, and consequently the costs of construction, operation and maintenance (O&M) per capita are higher than in big towns. On top of that, many of these agglomerations discharge in designated sensitive areas and must meet very strict limits, which increase the costs. Small populations commonly have low technical and economical capacity to assure the costs of O&M.

Other inherent difficulty to conventional WWT plants is the management of sewage sludge produced in the biological treatments. The main problem is the difficulty to treat it in origin, due to the inefficiency of the small systems for dehydration, and so the transport to the treatment facilities. Except in exceptional cases, the usual trend is to establish integrated management systems of sewage sludge, with centralised facilities for dehydration or digestion, which cover a region and reduce the management costs.

The nature of the wastewater collection system, the population served and its variation, the site of construction, the properties and the surface area available, and the financial resources for O&M are the main particular local conditions that should be taken into account for the establishment of the appropriate WWT (García, 2001).

Wastewater generated in small populations differs from wastewater generated in medium and large agglomerations, mainly in two aspects: the volume of flow and the composition. The smaller the agglomeration is, the higher the oscillation, passing from almost null flow early in the morning to peak flows that overcome eight times the average flow rate, in individual homes (Salas et al., 2007).

Figure 1-1 shows the relationship between the ratio of maximum water flow and average water flow, and the number of inhabitants for agglomerations under 2000 inhabitants. It can be observed that the ratio of the water flows grows exponentially as the number of inhabitants decrease.

Also the lower supply of water registered in small urban agglomerations has an immediate effect in the composition of wastewater. The lower the water supply, the lower the dilution of pollutants, which means an increase in its concentration. Small populations are characterised by generating a small volume of wastewater, but much polluted (Table 1-3).

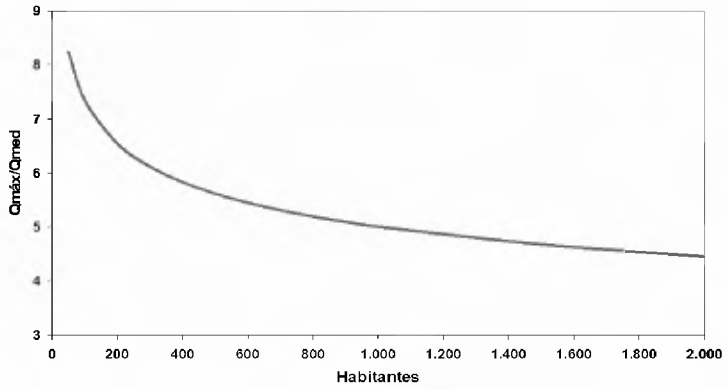


Figure 1-1: Relationship between water flow rate (Qmax/Qave) and number of inhabitants for populations under 2,000. From: (Salas et al., 2007).

Table 1-3: Composition of a model wastewater in small populations (Ortega et al., 2010)

Parameter	Concentration (mg L^{-1})
Suspended solids	250
BOD_5	300
COD	600
NH_4-N	30
TN	50
TP	10

1.1.5 Applicable technologies to WWT in small populations

WWT technologies can be classified in two large types: intensive and extensive ones. The difference between both types of systems consists in two fundamental aspects: the use of electromechanical devices that need energy and the area required to install the facilities. Intensive technologies are characterised by accelerate the WWT by means of external supply of energy, while extensive ones are based on natural purifying processes that occur in soils and water bodies and require larger areas. There is a third type of technologies in a half-way point between the two abovementioned ones.

The technologies that are mainly applied to treat wastewater in small populations are the following:

- *Primary treatments*
  - Septic tanks
  - Imhoff tanks
- *Extensive treatments*
  - Technologies that use soil as a filtering media: filtering trenches, filtering beds, vegetation filters.
  - Technologies that use filtration through a media: peat filters, intermittent sand filters. This last has been often implemented in other countries like USA, but not in Spain.
  - Technologies that simulate natural processes in wetlands: treatment wetlands (also called constructed wetlands). These systems have been widely used worldwide.
  - Technologies that imitate natural processes in rivers and lakes: stabilization ponds. These systems require large areas and the effluents quality is very variable, so they are in regression.
- *Intensive treatments*
  - Extended aeration activated sludge systems: is a method of sewage treatment using modified activated sludge procedures. It is preferred for relatively small waste loads, where lower operating efficiency is offset by mechanical simplicity. This process is typically used in prefabricated "package plants" intended to minimize design costs for waste disposal from small communities. Sludge must be periodically removed.
  - Sequencing batch reactors (SBR) and membrane bioreactors (MBR): these technologies are starting to be implemented in small populations.
- *Intermediate treatments*
  - Trickling filters: is a simple technology based on a filter bed of plastic pieces that have a long history.

- Rotating biological contactor (RBC): the use of adequate materials has allowed solving operational and mechanical problems of these systems.

All these technologies can be applied in centralised or decentralised systems, although certain technologies are more appropriate in either case.

The advantages of natural treatment methods lie mainly in the natural character of the sewage facility, the possibility of its inclusion in a favourable environment, the relatively simple technological implementation, lower operating costs, investment costs comparable with conventional WWT plants, low energy consumption, possibilities of being overload by ballast water, the possibility of short-term and long-term shutdown, the relatively rapid incorporation of the treatment process and achievement of the performance efficiency quality target in a short period of time after the start of operation, the removal of part of the nutrients, especially nitrogen and phosphorus, by biomass uptake and the treatment of organically low-loaded wastewater that cannot be treated by conventional methods (treatment plants based on activation processes) (Rozkosny et al., 2014).

1.1.6 Considerations to select the WWT technology in small populations

Conventional WWT requires high energy, O&M costs (Ghazy *et al.*, 2011). In addition, due to population growth and urban expansion, the volume of sewage sludge produced by WWT is constantly increasing. Thus, a different water-energy nexus is required to cope with the future global water demand.

Many small communities begin the process of addressing WWT needs by thinking that all they need to do is find the "recommended" treatment option and install it. However, each situation is unique and there are numerous treatment technologies available. Of course all treatment options have their advantages and disadvantages.

When deciding on the right treatment system, the community must have clear goals and specific criteria to use in making the decision. The system or systems chosen must provide the community with effective and manageable WWT at a reasonable cost. Depending on the overall population density, soil conditions and other factors, treatment systems follow one of two general concepts:

- *Decentralised*: individual and multiple-household (cluster) on-site systems using standard or “alternative” treatment technologies with subsurface discharge (may include pretreatment processes).

- *Centralised*: municipal style collection (gravity, pressure or vacuum) and centralized treatment with surface discharge (treatment involves primary, secondary, and perhaps tertiary processes).

A combination of these approaches is frequently the most viable solution. Sustainable sanitation systems require low cost, with low energy consumption and low mechanical technology, and the better choices of low cost treatment systems for rural areas are decentralized processes (Mahmood et al., 2013).

Management of a WWT plant includes monitoring, O&M. By providing a high level of management to all systems, communities can meet water quality and cost objectives using a variety of treatment systems. The benefits of good management include:

- Reduced overall costs
- Longer system life
- Improved system performance
- Increased reliability and overall satisfaction

Actually, most of WWT technologies are applicable to small populations, but some are more appropriate than others. The design and maintenance must be as effective as in big agglomerations, but other selection criteria should be employed.

The main criteria recommended for the selection of the technology for the WWT plants is simplicity of operation. Technical solutions that use a minimum of operator time and a minimum number of electromechanical facilities should be prioritised. The treatment process should be able to be operated by non-specialized staff. The energy consumption should be as low as possible, because energy cost represents one of the biggest costs in WWT plants. The treatment process should guarantee the effluent water quality, even during short periods of equipment failure and for a wide range of water flows and loading.

The technologies that best adapt to these criteria are the low cost or natural treatment systems. Natural treatment systems can obtain pollutants removal levels equivalent to conventional treatment systems. Furthermore, they involve lower O&M costs (Kadlec and Wallace, 2009; Lens et al., 2001; Ortega et al., 2010).

Primary treatments alone are discouraged because they are considered as only a partial treatment that does not guarantee public health; second, in water bodies with low water levels, the necessary appropriate treatment based on a contaminant loading balance could require effluents with more strict limits than those

stated in the Directive 91/271 for WWT plants treating waters from communities >2000 p-e. (García, 2001). This does not make sense because it is not logical to request a greater effort to the small WWT plants than that of the larger WWT plants. For this reason, it has been stated that the standards of the small WWTPs can only be as strict as the standards for larger plants.

1.2 Treatment wetlands

1.2.1 The concept of treatment wetlands

Wetlands have properties that make them unique among other ecosystems on Earth. Wetland plants are adapted to the abundant supply of water and to the lack of other essential chemical elements, such as oxygen. Because of this, wetlands are among the most biologically productive ecosystems on the planet and they are frequently inhabited by plants and are home to a multitude of animals including mammals, birds, reptiles, amphibians, and fish that are uncommon in other ecosystems. In addition, they can transform many of the common pollutants that occur in conventional wastewaters into harmless byproducts or essential nutrients that can be used for additional biological productivity (Kadlec and Wallace, 2009; Mitsch and Gosselink, 2007).

Treatment Wetlands for WWT have long attracted the attention because of their low installation and running costs, low production of sewage sludge (just in primary treatment), easy management, their overall technological simplicity (García et al., 2010, 2003b; Verhoeven and Meulemann, 1999) and good landscape integration (Knowles et al., 2011). Compared to conventional WWT methods, they tend to be simple, inexpensive, and environmentally friendly. They utilize a far smaller ecological footprint than other advanced WWT mechanisms. These systems are particularly viable options in places where the population density is low, because their main drawback is the large area required to set the wetland, which is not easily found in urban zones (Ferrer Medina et al., 2012; Ortega et al., 2010).

Many terms are used to denote man-made or artificial wetlands, such as reed beds (Arias et al., 2001; García et al., 2004), constructed wetlands (Nivala et al., 2013a; Vymazal, 2013, 2010), treatment wetlands (Faulwetter et al., 2009; Kadlec and Wallace, 2009), constructed treatment wetlands (Rozkosny et al., 2014) , etc. Beside "engineered" wetlands, the term of biofilter is found as well. A biofilter has many similarities with a constructed wetland, but it usually does not have plants. However, the term of constructed wetlands can also be used to describe restored

and recultivated land that was destroyed in the past through draining and converting into farmland, or mining.

For the author, the preferred term is "treatment wetland" that is used in one of the basic and most complete manuals to manage this kind of systems (Kadlec and Wallace, 2009), but most of literature used the term "constructed wetland" (CW), therefore is the term that is going to be used along this thesis.

Modern CWs are engineered systems that have been designed to emphasize specific characteristics of wetland ecosystems for improved treatment capacity under controlled conditions. They act as biofilters and remove solids and pollutants from the water, and may also serve as a habitat for native and migratory wildlife, although that is usually not their main purpose.

The artificial character is defined by some distinctive features:

- CWs are confined systems that are waterproofed to avoid water loses to the subsoil.
- The substrate material is different from the original land.
- Plants are selected on the basis of the most common aquatic plants in the region.

WWT take place when the wastewater flows through these artificial wetlands, where physicochemical and biological processes produce a treated effluent. These systems can be considered as a complex ecosystem in which the main actors are:

- The substrate: supports vegetation and allows the fixation of the microbial community, in the form of biofilm, which is the main contributor to the removal processes that take place in the CW.
- The vegetation (macrophytes) that contributes to the oxygenation of the substrate and the removal of nutrients, as well as the support of the biofilm in the root system.
- The water to treat that flows through the substrate and the vegetation.

The vegetation used in CWs is the same as colonise natural wetlands, emergent water plants as reeds, bulrush, cattails, etc., which grow in shallow waters, rooted to the ground.

CWs constitute a biological secondary stage of WWT plants, but they can also be used for tertiary treatment of effluents from other treatment plants. To prevent

clogging of the systems, a primary treatment is recommended to remove settle and floating solids (Ortega et al., 2010).

1.2.2 Types of treatment wetlands

CWs can be constructed in a variety of hydrologic modes (Fonder and Headley, 2010; Kadlec and Wallace, 2009). Hydrological traits are water position, flow direction and degree of saturation. Based on the predominant position of water in the system, two main groups of CW are identified:

- Those with surface flow (SF) above a benthic substrate,
- Those with subsurface flow (SSF) through a porous media.

The systems with surface flow are divided into three standards types, differentiated by vegetation type:

- Free water surface (FWS) CWs, have areas of open water and are similar in appearance to natural marshes (Figure 1-2 A).
- Free-floating macrophytes (FFM) CWs, containing free-floating vascular aquatic plants growing on the water surface (Figure 1-2 B).
- Floating emergent macrophytes (FEM) CWs, with emergent macrophytes growing on a buoyant structure (Figure 1-2 C).

SSF flow systems always contain sessile emergent macrophytes and are often divided into two standard types, based on flow direction:

- Horizontal subsurface flow (HSSF) CWs, which typically employ a gravel bed planted with wetland vegetation. The water, kept below the surface of the bed, flows horizontally from the inlet to the outlet (Figure 1-3).
- Vertical flow (VF) CWs distribute water across the surface of a bed made of sand or gravel, planted with wetland vegetation. The water is treated as it percolates through the media (Figure 1-4). Biosolids dewatering wetlands can be thought of as a type of VF wetland system.

Each of these major categories employs variants of the layout, media, plants, and flow patterns. For example, VF systems may be operated in continuous down-flow (DF), as is the case for anaerobic mine water wetlands, or they may be operated with intermittent dosing, that is the most common way.

Figure 1-2: Pictures of different surface flow constructed wetlands. A: Free Water Surface (FWS) at Knockholt Landfill, Region of Bulckley/Nechako, Houston, BC; B: Free Floating Macrophyte (FFM) C: Floating Emergent Macrophyte (FEM).

The term wetland seems a contradiction in the subsurface flow systems, given that the water is not visible, so it should be more appropriate the term planted biofilter.

Figure 1-3: An example of a subsurface flow constructed wetland to treat urban wastewater for 362 p.e. in Ondrejov, Czech Republic.

Figure 1-4: Water distribution in a vertical flow constructed wetland at the research centre CENTA, Carrión de los Céspedes, Sevilla, Spain. From: (Ortega et al., 2010).

1.2.3 Surface flow constructed wetlands

As the wastewater flows through the wetland, it is treated by the processes of sedimentation, filtration, oxidation, reduction, adsorption, and precipitation. The components in a typical FWS CW are shown in Figure 1-5.

These CWs are made up of channels with emergent vegetation and a scarce water depth (less than 40 cm). They are fed in continuous mode and the treatment process is favoured by the submerged parts of the plants that act as a substrate for the microbial growth. They usually take up several hectares and are commonly used as a refining treatment (Moshiri, 1993).

FFM and FEM CWs operate in the same way than FWS, with floating plants (Figure 1-6). Many wetland plants have the ability to grow without substrate, floating in the water (FFM) or made floating with supporting structures (FEM).

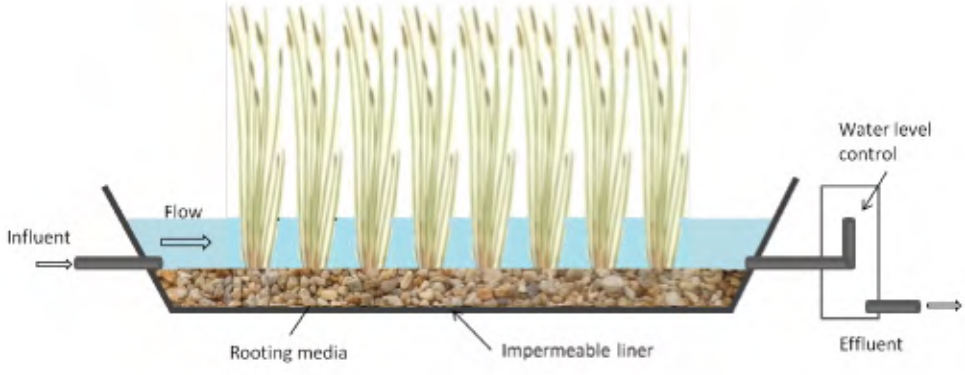


Figure 1-5: Basic elements of a free water surface constructed wetland

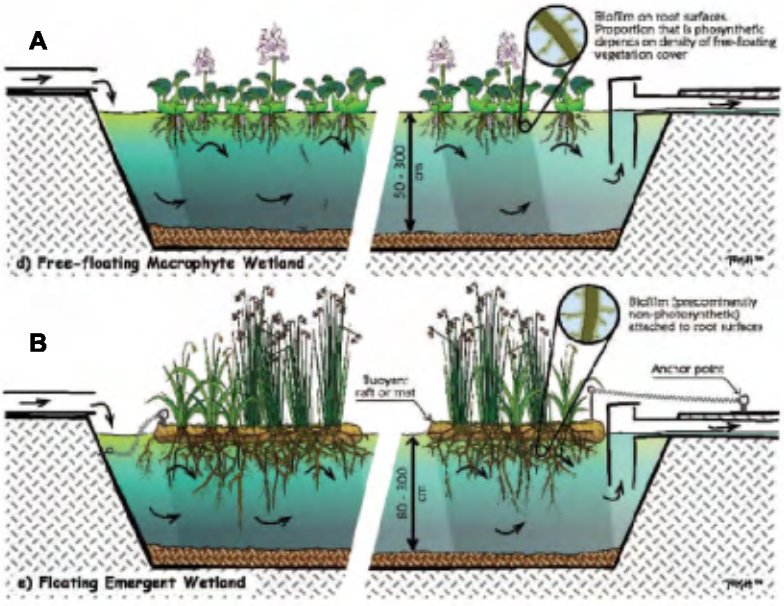


Figure 1-6: Schemes of floating macrophyte CW. A: free floating macrophyte (FFM); B: floating emergent macrophyte (FEM) with buoyant structure. From: (Headley and Tanner, 2012).

1.2.4 Subsurface flow constructed wetlands

In these systems water flows underground through a porous permeable media (gravel, grit) in contact with rhizomes and roots of the macrophytes (Vymazal and Kropfelova, 2008). The treatment processes occur in the surface of the porous media, where the microbial biofilm grows. Thus, some processes are similar to those in filtering treatments. They require less surface area than FWS CWs and in most cases they are used as a secondary treatment in small populations. However, there are many other applications to specialty wastewaters from industry (Calheiros et al., 2007; Nivala et al., 2007; Thut, 1993). So, they present certain advantages with respect to FWS CWs that also include thermal protection due to the underground flow and the vegetation coverage, which allows the use in cold climates, and avoid odours and mosquitoes. As drawbacks, the cost of construction is higher, due to the cost of the granular material (Collado, 2000; Kadlec and Wallace, 2009), and there is a risk of clogging, especially in HSSF systems (Caselles-Osorio et al., 2007; Knowles et al., 2011).

As mentioned above, two main types of SSF CWs exist: horizontal and vertical flow. The degree of flooding, temporary or permanent, confers different properties, affecting chiefly to oxygen transfer and so, the redox state.

Low cost O&M, low energy requirements, low production of sewage sludge (just in primary treatment) and good landscape integration are some of the most attractive advantages compared to conventional treatment systems (García et al., 2003b). However SSF CWs are constrained by limitations such as large land requirements (3-10 m^2 $p.e.^{-1}$ depending on design) (Tilley et al., 2008; Vymazal and Kropfelova, 2008) and clogging by the accumulation of solids (Rousseau *et al.*, 2004; Tanner and Sukias, 1995).

Horizontal subsurface flow (HSSF) CWs

These systems are often fed in continuous mode by gravity, although they can also work in intermittent way by pumping or siphons of controlled discharge. Water flows horizontally through the filtering media which has a depth between 40 and 60 cm, and where the vegetation is planted (Figure 1-7). At the outlet, a flexible pipe allows to control the water level, which is kept approximately 5 cm under the media level (Vymazal and Kropfelova, 2008).

HSSF CWs work under anaerobic conditions, producing effluents with scarce dissolved oxygen (DO) and so, a negative redox potential, depending on the organic load applied and the water depth (García et al., 2004). They operate with hydraulic retention time (HRT) of several days and are designed at a rate of 5 m^2 $p.e.^{-1}$ or more (Ortega et al., 2010) and a recommended organic loading rate (OLR) of 6 g DBO_5 m^{-2} d^{-1} (Kadlec and Wallace, 2009).

Hundreds of HSSF CWs are operating in Europe for urban WWT (Puigagut et al., 2007; Rousseau et al., 2004; Vymazal, 2009) and are currently increasing in number, especially in Mediterranean countries. Many are the researchers that have shown that one of the biggest problems in HHSF CWs is the clogging of the media that reduce the efficiency of the treatment and the lifetime of the systems (Caselles-Osorio et al., 2007; Pedescoll et al., 2013, 2011; Tanner and Sukias, 1995). But this problem can be solved by an adequate primary treatment and an appropriate OLR.

Vertical flow (VF) CWs

VF CWs (Figure 1-8) are commonly fed under discontinuous flow mode by pumping or, if the topography allows it, by siphons of controlled discharge. Water is distributed over the filtration surface (Figure 1-4) with perforated pipes that rely on the filtering bed. Water flows vertically trough the filtering media, which has a depth between 60 cm and 1 m. A drainage network in the bottom of the system, which collects the treated effluent, connect a combination of perforated pipes that stand

above the bed to increase the oxygenation of the filtering substrate by natural air circulation (Brix and Arias, 2005). In these systems, a small amount of oxygen is transferred by plants, comparing to the oxygen flow through the pipes. This air circulation makes the VF CWs aerobic environments that produce oxygenated and not smelly effluents even though OLR are higher (Cooper, 2003).

HRT in VF CWs takes several hours instead of days, as in the HSSF ones. They are usually designed at a rate of 3 m^2 $p.e.^{-1}$ (Ortega et al., 2010). OLR of 10 to 40 g DBO_5 m^{-2} d^{-1} are often used in VFCW, depending on factors such as external aeration, with most designs using loading rates of 20 g DBO_5 m^{-2} d^{-1} to achieve a concentration at the effluent under 30 mg BOD_5 L^{-1} (Kadlec and Wallace, 2009) and fulfil legal requirements for WWT (Dir. 91/271/EEC of 21 May 1991).

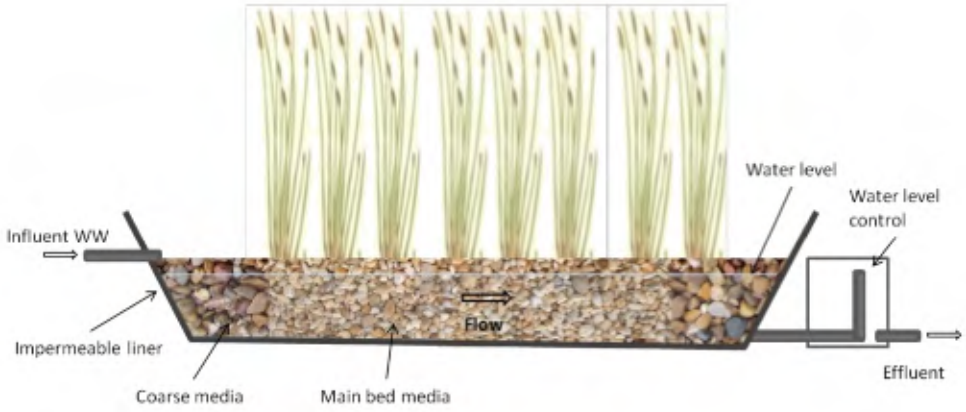


Figure 1-7: Schematic horizontal subsurface flow constructed wetland

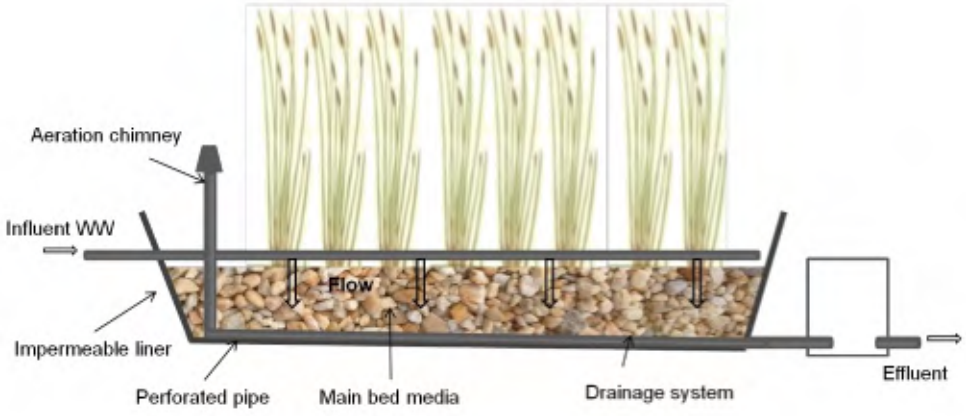


Figure 1-8: Schematic vertical flow constructed wetland

VF systems give better efficiencies with respect to removal of DBO_5 and nitrification of ammonia, due to the higher aerobic conditions compared to the HSSF systems, and also to a better hydraulic control (Brix and Arias, 2005; Cooper, 1999; Kadlec and Wallace, 2009). But phosphorous removal in these systems is low and depends of the bed material and the influent wastewater (Arias et al., 2001; Del Bubba et al., 2003). Because of this, last research in VF CWs is focused on compact VF systems and efficient phosphorous removal.

In the last years, research is focused on VF systems with recirculation to increase the total nitrogen removal efficiency (Arias et al., 2005), because the treated effluent is oxygen saturated and has a low availability of carbon, consequently the removal of total nitrogen is limited. The results have shown that recirculation improve the removal of total nitrogen.

Hybrid CWs

HSSF systems cannot nitrify because of their limited oxygen transfer capacity. On the other hand, VF systems provide good conditions for nitrification but no denitrification occurs in these systems. Therefore, there has been a growing interest in hybrid systems, where the advantages of both systems can be combined to complement each other in order to achieve higher treatment efficiency, especially for nitrogen. Most of them comprise VF and HF systems arranged in a staged manner (Vymazal, 2013, 2005).

When a VF system is followed by an HSSF system, nitrification is achieved in the former while denitrification can be achieved in the latter if a part of the influent is derived to provide organic matter. When a HSSF system is followed by a VF system (Figure 1-9) nitrification can be achieved in the second stage and denitrification in the first stage, while the ratio C/N in the denitrification stage is adequate. For that purpose, the recirculation of a fraction of the effluent of the VF system to the inflow of the HSSF system is carried out (Brix et al., 2003; Vymazal, 2005).

In the same way, the location of a FWS CWs in the queued of the hybrid system allows an improvement of the final quality of the effluent, increasing the removal of pathogens.

1.2.5 Treatment processes and performance in SSF CWs

Table 1-4 resumes average values of treatment performance that are achieved in SSF CWs. The final effluent characteristics are referred to a model wastewater (Table 1-3).

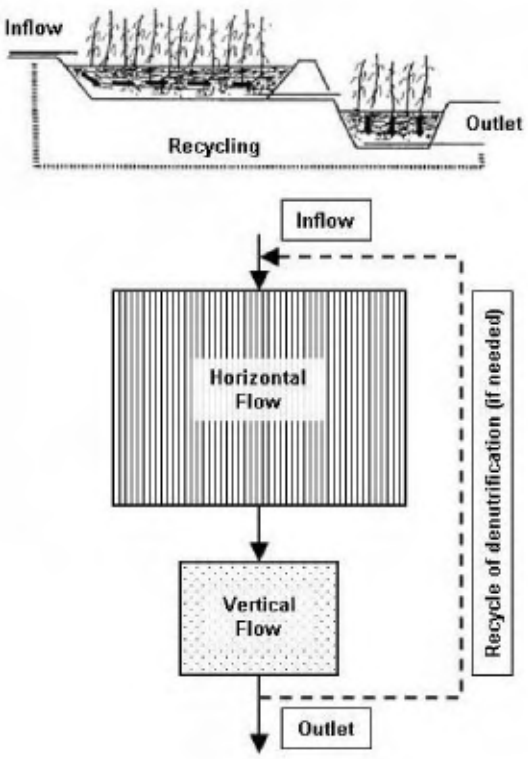


Figure 1-9: Scheme of a hybrid constructed wetland. From Vymazal, 2005.

Suspended solids removal

The processes that take part in the removal of suspended solids are:

- Sedimentation: is the tendency for particles in suspension to settle out of the fluid due to force of gravity.
- Flocculation: is a process wherein colloids come out of suspension in the form of floc or flake, either spontaneously or due to the addition of a clarifying agent, and can sediment.
- Filtration: is the process why suspended matter is retained in the substrate, the roots and the rhizomes of the plants.

Most of the SS removal in SSF CWs is due to filtration of the water through the substrate, mainly in the inlet zone (Kadlec and Wallace, 2009), because of the low speed of water flow, the adhesion forces between particles and the constriction of the granular media and the roots. SSF CWs are very efficient removing SS.

Table 1-4: Treatment performance values in subsurface slow constructed wetlands. From Ortega et al., 2010.

Parameter	Vertical CW		Horizontal CW	
	% Removal	Effluent (mg L^{-1})	% Removal	Effluent (mg L^{-1})
Suspended solids	90-95	13-25	90-95	13-25
BOD_5	90-95	15-25	85-90	15-30
COD	80-90	60-120	80-90	60-120
NH_4-N	60-70	9-12	20-25	22-24
TN	60-70	15-20	20-30	35-40
TP	20-30	7-8	20-30	7-8

Redox potential behaviour

The prevailing redox conditions in SSF CWs have a strong effect on removal mechanisms. SSF CWs are distinguished from other WWT processes by the simultaneous co-existence of areas with different redox status at the macro- and micro-scale, which allows different processes to occur at the same time (García et al., 2010). Besides the spatial changes, temporal variability of redox conditions also occurs like those closely related to the daily light cycle (Wießner et al., 2005). Macro-scale redox potential changes along the length of HSSF CWs are characterized by a general increase, so stronger reducing conditions are found near the inlet than at the outlet and it has been observed that redox potential decreased considerably with depth, because the processes that provide oxygen (surface aeration and plant release) occur mainly in the upper layers of the wetland media (García et al., 2003a). Redox variations at the micro-scale have been found at the surface of the roots (Münch et al., 2005) due to the oxygen release by plants.

Removal of organic matter

Organic matter (OM), which is in the form of suspended sedimentary matter and dissolved matter, pours gradually through the wetland and experiments biological degradation. Particulate matter is retained by filtration. Almost half of the BOD_5 is removed in the zone near the inlet of the wetland. The responsible processes of the removal of organic matter vary over the time and space and depend of factors as OLR, water depth and electron acceptors availability (García et al., 2004). Disintegration and hydrolysis are processes that occur either under aerobic, anoxic, or anaerobic conditions.

In the aerobic degradation processes, a fraction of the organic matter is oxidized by the bacteria, producing CO_2, water and energy. Simultaneously, other fraction is converted in cellular tissue (new bacteria), using the energy released in the oxidation. Finally, once the organic matter decreases, the new bacteria metabolise their own cell material, in a process known as "endogenous respiration". Oxygen supply for these processes is different depending on the type of CW. Wetland plants are able to release oxygen to the root systems, but it is widely accepted in the scientific community that the influence of the plants is only significant in systems that work with low OLR (US EPA, 2000).

In the anaerobic processes, degradation of organic matter occurs in a series of linked stages, in which compounds produced are the substrate for the next stage:

- Hydrolytic stage: complex organic compounds (carbohydrates, proteins, lipids) are transformed in more simple compounds like monosaccharides, amino acids, fat acids and glycerol.
- Acidogenic stage: simple organic compounds from the previous stage are transformed in organic volatile acids (mainly acetic, propionic and butyric acids) through fermentation by means of acidogenic and acetogenic microorganisms. The removal rates achieved in this process are very low.
- Methanogenic stage: the organic volatile acids released in the previous stage are transformed by methanogenic microorganisms in methane and CO_2. This is the process that removes most of the organic matter in anaerobic environments and is the limiting stage because methanogenic bacteria are more sensitive to environmental conditions (pH, temperature, toxics, etc.). There are also other processes that

Main degradation reactions of organic carbon that occurs in SSFCW are:

- Respiration, in aerobic zones:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \quad [1]$$

- Fermentation in anoxic or anaerobic zones:

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH \text{ (lactic acid)} \quad [2]$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH \text{ (ethanol)} + 2CO_2 \quad [3]$$

- Nitrate reduction (denitrification) in anoxic and anaerobic zones:

$$C_6H_{12}O_6 + 4NO_3^- \rightarrow 6CO_2 + 6H_2O + 2N_2 + 4e^- \quad [4]$$

- Iron reduction in anoxic and anaerobic zones:

$$CH_3COO^- \text{ (acetate)} + 8Fe^{3+} + 3H_2O \rightarrow 8Fe^{2+} + CO^2 + HCO_3^- + 2H_2O + 8H^+ \quad [5]$$

- Sulphate reduction in anaerobic zones:

$$2CH_3CHOHCOO^- + SO_4^{2-} + H^+ \rightarrow 2CH_3COO^- + 2CO_2 + 2H_2O + HS^- \quad [6]$$

$$CH_3COO^- + SO_4^{2-} + 2H^+ \rightarrow 2CO_2 + 2H_2O + HS^- \quad [7]$$

- Methanogenesis in anaerobic zones:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad [8]$$

$$CH_3COO^- + 4H_2 \rightarrow 2CH_4 + H_2O + OH^- \quad [9]$$

It is assumed that in CWs organic compounds are degraded simultaneously by means of aerobic and anaerobic processes, being difficult to quantify the proportion of each process.

Nitrogen removal

In urban wastewater, the main forms of nitrogen are organic nitrogen and ammonia nitrogen, and much less nitrites or nitrates.

Organic nitrogen is transformed in ammonia nitrogen by ammonification. Part of this ammonia nitrogen is assimilated by the microorganisms. Other part is assimilated by plants, but if they are not harvested most of the nutrients retained in plant tissues go back to the water through degradation processes.

Most of the ammonia nitrogen in CWs is removed by combined processes of nitrification and denitrification (Reddy and D'Angelo, 1994). Ammonia is adsorbed temporary over the granules surface and over organic electrically charged particles, by mechanisms of cationic exchange (Vymazal, 2007).

Nitrification is an autotrophic process that transforms the ammonia in nitrate in two stages. The first step of nitrification is carried out by the ammonia-oxidizing bacteria (AOB) that oxidize ammonia to nitrite (Eq 10). Due to the slow growth of these bacteria and their sensitivity to environmental conditions, nitrification has been often considered one of the most unreliable and unpredictable processes of WWT plants (Bellucci and Curtis, 2011). Bacteria from genus *Nitrosomonas* oxidize ammonia to nitrite, according to the reaction:

$$NH_4^+ + 3/2\ O_2 \rightarrow NO_2^- + 2H^+ + H_2O \quad [10]$$

Afterwards, bacteria from genus *Nitrobacter* oxidize nitrite to nitrate, according to the reaction:

$$NO_2^-- + 1/2\ O_2 \rightarrow NO_3^- \quad [11]$$

The total reaction is:

$$NH_4^+ + 2\ O_2 \rightarrow NO_3^- + 2\ H^+ + H_2O \quad [12]$$

being necessary 4.6 mg of oxygen to oxidize 1 mg of ammonia-N.

Nitrifying bacteria are very sensitive to inhibitor substances. The high concentration of ammonia or nitrite can inhibit them, but it is also important the effect of pH, which is optimal between 7.5 and 8.6. Also the temperature exerts a big influence on bacterial growth, and it is necessary a DO concentration over 1 mg L^{-1}. Thus, oxygen is a limiting factor of the process.

For the removal of total nitrogen, nitrification must be followed by denitrification processes that are performed by heterotrophic facultative bacteria in two stages and in anaerobic conditions. Firstly, the conversion of nitrate to nitrite, followed by the conversion of nitrite to gaseous forms of nitrogen (nitric oxide, nitrous oxide and dinitrogen) (eq. [13], [14] [15], [16]). The presence of oxygen suppresses the enzymatic system required to develop this process. Optimal pH is between 7 and 8, and the process is affected by the temperature. It is also required enough amount of organic carbon: to reduce 1 g of nitrate, 3 g of BOD_5 are necessary.

$$NO_3^- + 2e^- + H^+ \rightarrow NO_2^- + H_2O \quad [13]$$

$$NO_2^- + e^- + H^+ \rightarrow NO\ (g) + H_2O \quad [14]$$

$$NO\ (g) + e^- + H^+ \rightarrow ½\ N_2O\ (g) + ½\ H_2O \quad [15]$$

$$½\ N_2O\ (g) + e^- + H^+ \rightarrow ½\ N_2\ (g) + ½\ H_2O \quad [16]$$

Anammox (anaerobic ammonium oxidation) has been proposed as a more energy-efficient alternative to the conventional nitrification and denitrification processes (Op den Camp et al., 2007; van der Star et al., 2007). Anammox process consists of the combination of ammonia and nitrite directly into dinitrogen gas. The Anammox bacteria (planctomycete-like) oxidize NH_4^+ to N_2 using NO_2^- as electron acceptor under anaerobic condition (Strous et al., 1999) following the reaction:

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O\ (\Delta G_0 = -357\ kJ/mol) \quad [17]$$

The Gibbs free energy change associated with this reaction is even higher than for aerobic NH_4 oxidation and could support autotrophic growth. Compared to other nitrifiers, Anammox bacteria coexist with heterotrophic bacteria because heterotrophic consumption of oxygen creates a more anoxic environment beneficial to Anammox bacteria but in competition with nitrifiers (Faulwetter et al., 2009). Due to the environmental conditions favouring Anammox bacteria, it seems likely that they would exist in HSSF CWs, however direct evidence is limited (Dong and Sun, 2007; Li and Tao, 2017; Waki et al., 2015).

Phosphorous removal

Phosphorous in wastewater is in the form of organic phosphorous and inorganic orthophosphate. Between 10 and 20% is assimilated in microbial biomass. Three are the main processes to remove phosphorous in TWs (Reddy et al., 1999):

- Direct absorption by plants: plants adsorb much less phosphorous than nitrogen.
- Adsorption on the particles: in can be released under certain environmental conditions, especially by changes in redox potential.
- Physicochemical precipitation, by means of reactions of phosphorous with iron, aluminium and calcium of the wastewater, resulting in insoluble phosphates.

Phosphorous removal in CWs is approximately from 20 to 30%. This percentage can be improved with filtering specific substrates, i.e. containing iron. It has been observed that the retention of phosphorous decreases over time.

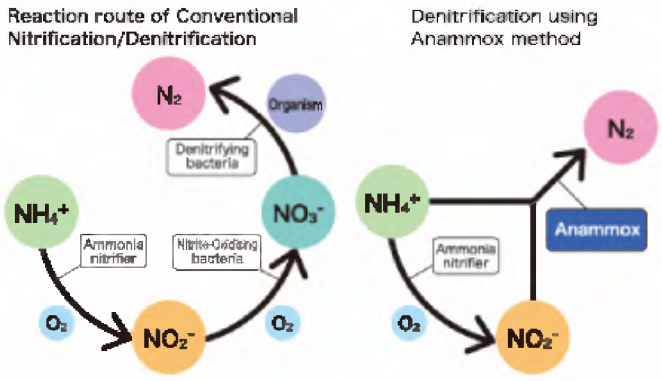


Figure 1-10: Schematic of nitrification /denitrification and Anammox processes.

1.3 Microbial electrochemical technologies (MET)

1.3.1 Fundaments of microbial electrochemistry

Organisms depend on the flow of electrons for key energy-generating cellular processes. Continuous electron flow is necessary for the formation of the electrochemical gradients that enable the synthesis of adenosine triphosphate (ATP), which is the life's energy currency. In eukaryotes, including animals, this power generation is the specialty of mitochondria. But the same process is also at play in domains of life that lack internal organelles, namely archaea and bacteria, from which mitochondria evolved.

In 1911 M.C. Potter described for the first time the ability of *Escherichia coli* to generate a voltage and deliver a current. For decades, microorganisms that turn food into a flow of electrons were a biological curiosity. But in the last fifteen years, Microbial Electrochemistry (ME), a subfield of the bioelectrochemistry, has emerged as a discipline that has gained the attention of many researchers and engineers (Schröder et al., 2015a). ME is the study and application of interactions between living microorganisms and electrodes and is based on the ability of certain microorganisms, called exoelectrogens or electroactive microorganisms, to exchange electrons with a terminal electron acceptor (TEA) or electron donor (ED) characterised for being a conductive and insoluble form (Tejedor-Sanz et al., 2017).

Many anaerobes can only transfer electrons to soluble compounds such as nitrate or sulphate that can diffuse across the cell membrane, but exoelectrogenic bacteria are distinguished from these anaerobes by their ability to directly transport electrons outside of the cell, through a mechanism currently known as extracellular electron transfer (EET), which permits them to function in a Microbial Fuel Cell (MFC) (Logan, 2008). This process were described for the first time in marine sediments, where electroactive microorganisms were able to transfer the electrons resulting from their metabolism to an electrode, generating an electric current (Bond et al., 2002; Reimers et al., 2001). These microorganisms were able to conserve energy to support their growth by oxidizing organic compounds in the marine sediments with an electrode serving as the sole TEA. And nowadays, the diversity of bacteria capable of exoelectrogenic activity is just beginning to be discovered.

The clear advantage of exploiting electroactive communities is that electrodes can boost microbial metabolism in anaerobic systems that are typically electron acceptor limited. Electroconductive material may represent an inexhaustible source of electron acceptors, hosting the additional advantage of providing a more easily

modulated redox potential compared to standard, low-reducing redox species that generally drive these systems (Kato Marcus *et al.*, 2007).

1.3.2 Extracellular electron transfer (EET)

Electrochemically active biofilms have great importance in the natural environment, namely in metal oxidation and reduction and the associated effects on mineral dissolution, the carbon cycle, and the sorption and complexation of phosphorus and heavy metals (Logan, 2008). Redox active minerals, such as those that contain iron and manganese, are abundant in soils and in aquatic and subsurface sediments (Shi et al., 2016). As the microbial cell envelope is neither physically permeable to minerals nor electrically conductive (Albers and Meyer, 2011; Shi et al., 2007), microorganisms have evolved strategies to exchange electrons with extracellular non soluble minerals when the ED or the TEA are limited in the environment.

EET is defined as the microbial metabolic process that enables electron transfer between microbial cells and extra-cellular solid materials and is a type of microbial respiration. Respiration converts redox potential differences between the oxidation and reduction of chemical compounds into a bio-available form of energy, generally ATP. In the respiration process, electrons derived from the oxidation of electron donors (as acetate, lactate, hydrogen, methane, ammonia, sulphides, etc.) are transferred to electron acceptors, that can be in the form of soluble oxidized compounds (as oxygen, carbon dioxide, nitrate or sulphate), insoluble forms (as humic acids) or solids (as minerals or electrodes) (Figure 1-11). By means of EET, microorganisms can transfer the electrons to the outer surface of the cell to reduce an extracellular solid terminal electron acceptor (Lovley, 2008). For example, dissimilatory iron-reducing bacteria (DMRB) (such as *Shewanella* and *Geobacter*) oxidize organics and transfer electrons to anodes, resulting in the current generation in microbial fuel cells (Bond and Lovley, 2003; Kim et al., 2002). On the other hand, *G. metallireducens* accepts electrons from graphite cathodes and use them for nitrate reduction (Gregory et al., 2004).

For years, the primary goal for many researches has been to understand the mechanisms of extracellular electron transfer and to determine how electrons are transferred to the TEA or from the ED and the factors controlling the rate and extent of this process. The curious aspect of extracellular electron transfer (EET) is that microbes are transferring electrons from the inside of the cell to the outside.

Our understanding of biological electron transport remains limited, especially when the distances travelled far exceed the length of a cell. Much is known about the mechanisms that enable electron transfer reactions between nearby molecules; but bacteria, the planet's oldest organisms, are able to transfer the charged particles to a variety of acceptors, including some at great distances. For instance, we now know that some anaerobic bacteria gain energy through electron transfer to inorganic minerals, and even to synthetic surfaces, hundreds of cell-body lengths away (Pfeffer et al., 2012). So how do they do it?

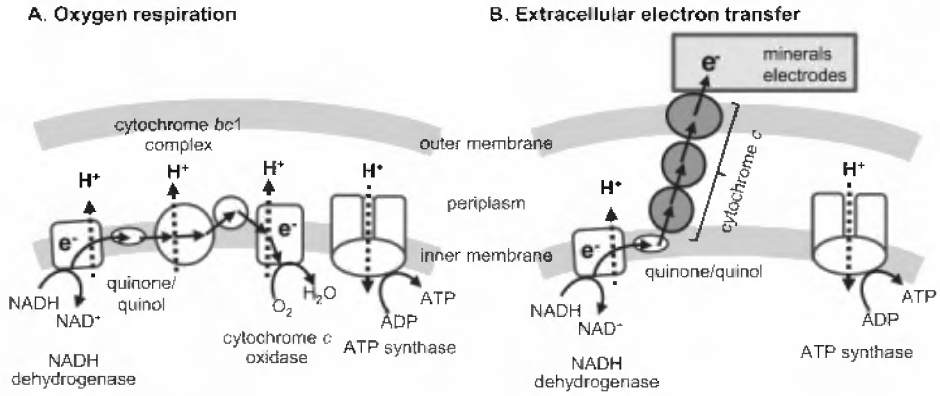


Figure 1-11: Schematic diagrams of respiration: (A) electron transfer to a soluble compound (as oxygen) and (B) microbial extracellular electron transfer. From (Kato et al., 2012).

Direct extracellular electron transfer (DEET)

One clue to how they do it has come from observations that metal-reducing bacteria localize electron transfer proteins called cytochromes to the outer cell membrane (Busalmen et al., 2008; Gorby et al., 2006; Lloyd et al., 2003; Myers and Myers, 1992). DEET requires a physical contact between the electrode and the microorganism that is usually attached to the electrode surface forming a biofilm. Cytochromes perform electron transfer reactions when the iron ions in their heme groups switch redox states, from reduced Fe^{2+} to oxidized Fe^{3+}, and vice versa. However, while most gram-negative bacteria confine these electron transfer reactions to the inner membrane of the cell, *Shewanella* and *Geobacter* locate some of their heme-containing cytochromes on the outer membrane, where the molecules can access external, solid electron acceptors, such as a mineral surface.

These solid minerals interact with electroactive bacteria in at least four different DEET ways (Figure 1-12): as electron sinks for heterotrophy-based

respiration (A), as energy sources for autotrophic growth (B), by enabling cell-to-cell transfer of electrons (C), and as electron storage materials (D) (Shi et al., 2016).

When the biofilm grows, only bacteria in the first monolayer at the electrode surface are able to transfer electrons by direct contact (Figure 1-12.A; Figure 1-13.D), which constitutes a limitation.

But in 2005 and 2006, Reguera and Gorby, reported another DEET pathway found in both *Geobacter* and *Shewanella* (Gorby et al., 2006; Reguera et al., 2005). Using atomic force and scanning tunnelling microscopy techniques, they found that some of the bacteria's extracellular protein nanofilaments, called pili, are electrically conductive. Specifically, they showed that electrons could travel a few nanometres across the width of the pili, now commonly referred to as bacterial nanowires (Malvankar and Lovley, 2012). In 2010, El-Naggar found that the nanowires are also conductive along their lengths, over micrometer-long distances, and suggested that the cytochromes are responsible for the nanowires' conductivity (Figure 1-13 C). Proteins typically have low electron mobility and are therefore regarded as insulators rather than conductors over micrometer-long distances, so how were these bacteria so efficient conducting electrons along these protein-based appendages? One proposed strategy is the multistep hopping that involves forming a conductive pathway to electrodes by incorporating redox components on outer cell membranes and along microbial nanowires within biofilms (Pirbadian and El-Naggar, 2012).

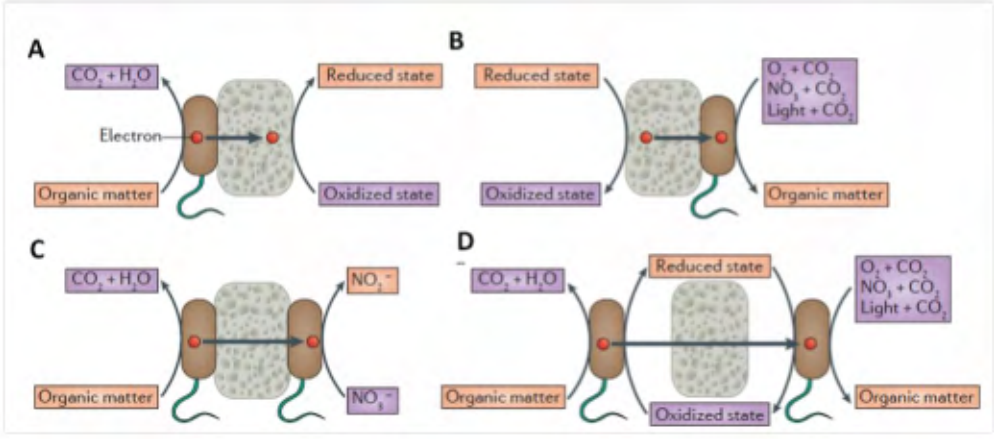


Figure 1-12: Electrical interplay between microorganisms and minerals. A: Microorganisms use minerals that contain metal ions as terminal electron acceptors for respiration; B: electron and/or energy sources for growth; C: electrical conductors that facilitate electron transfer between microbial cells of the same and different species and D: electron-storage materials, or batteries, to support microbial metabolism. From: Shi et al., 2016.

In 2012, a team of physicists and biologists reported the discovery of centimetre-long multicellular bacterial chains, consisting of thousands of cells lined end-to-end within the marine sediments (Pfeffer et al., 2012). Cells at one end of each "bacterial cable" (Figure 1-14 B) would oxidize hydrogen sulphide and supply electrons to the oxygen-consuming cells at the other end. Unlike nanowires (Figure 1-14 A), which serve to pass electrons between individual cells via external appendages, the cables serve to pass the electrons within a multicellular architecture. The bacteria, which belong to the family *Desulfobulbaceae*, share a common outer membrane, and the researchers found string-like structures running in the periplasmic space along the entire length of the cable just underneath this membrane, with a high capacity for storing and conducting charge.

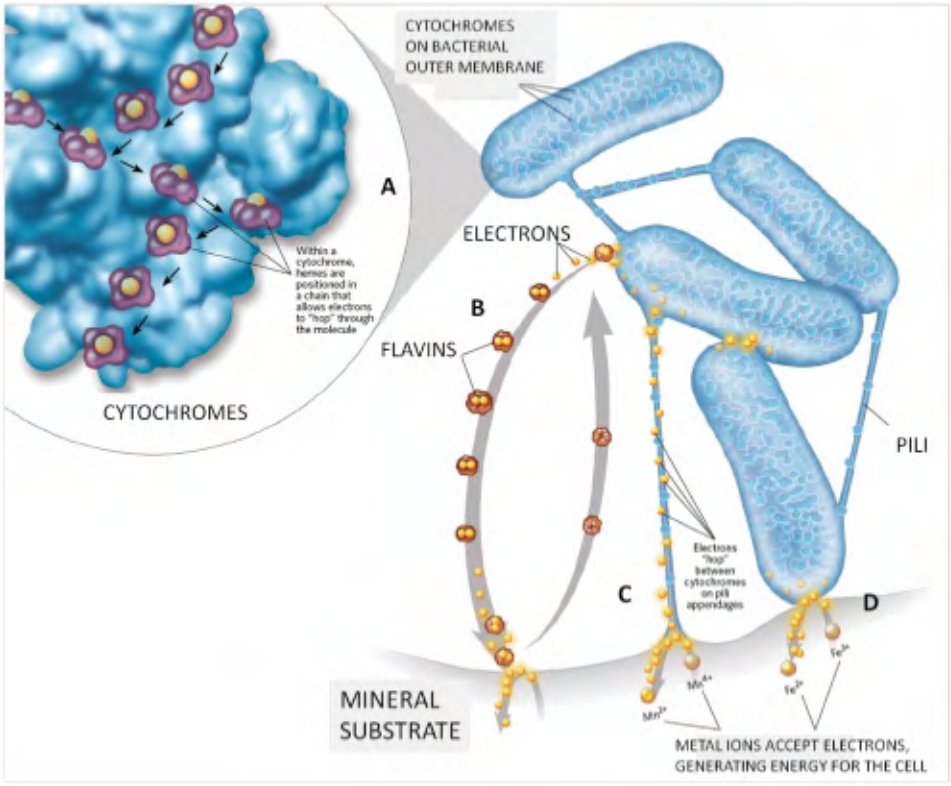


Figure 1-13: A: Within a cytochrome, hemes are positioned in a chain that allows electrons to hop through the molecule; **B**: Flavins carry electrons from the bacterium to the TEA and shuttle back to the bacterium to pick up more electrons; **C**: Electrons hop between cytochromes on pili appendages (DEET); **D**: DEET by direct attachment of cells to the surface. ©Tom Graves.

Another type of DEET is the direct interspecies electron transfer (DIET) in which one cell uses another cell as TEA. This mechanism was firstly described in *G. metallireducens* and *G. sulfurreducens* co-cultures, growing them in a medium with ethanol as electron donor (not utilized by the second species) and fumarate as electron acceptor (not utilized by the first species) (Summers et al., 2010). Recently, it has also been reported that some species of methanogens (*Methanosaeta* and *Methanosarcina barkeri*) are capable of performing DIET in co-cultures with *Geobacter* species (Morita et al., 2011; Rotaru et al., 2014a, 2014b), which can be important in methane production in anaerobic digesters. DIET can also be performed with a mineral as a mediator (Figure 1-12 C) between both microorganisms. Also conductive surfaces such as granular activated carbon can act as mediators between organisms (Kato et al., 2012; Liu et al., 2012; Rotaru et al., 2014a).

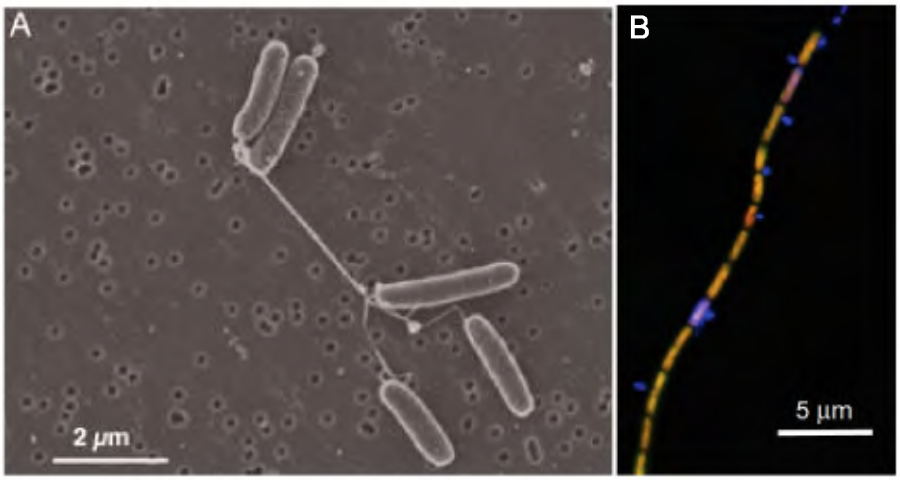


Figure 1-14: A: Cells of *Shewanella oneidensis* connected by microbial nanowires, composed of pili protein. From Gorby *et al*, 2006. B: Filamentous *Desulfobulbaceae* cells (yellow) identified by fluorescence in situ hybridization forming a micro-cable. From Pfeffer *et al*, 2012.

Cell-to-cell electron transfer could additionally serve a similar function to quorum sensing: allowing cells to communicate with each other. For example, in both *Shewanella* biofilms and the *Desulfobulbaceae* cable system, the flow of electrons occurs in one direction: toward the terminal electron acceptor. The direction will allow cells downstream in the redox gradient to be directly "informed" of the oxidation activity of their respiration partners upstream in the donor-rich regions. In this way, each cell along the electron transport pipeline can tune its local gene

expression in response to events occurring far away. Cells are communicating their metabolic state across the network.

Mediated extracellular electron transfer (MEET)

In addition to this direct cell interaction with the surface, it has also been proposed that *Shewanella* secretes small redox molecules called flavins to act as electron shuttles from the cell to more distant electron acceptors (Marsili et al., 2008). Flavins pick up electrons from the cell, drop them off at a solid electron acceptor, and come back to the cell for more—a cyclic process that is driven by diffusion (Figure 1-13.B).

Many electroactive bacteria exploit mediated extracellular electron transfer based on molecular redox compounds. In nature (in soil and in sediments) this involves humic compounds as electron carrier (Roden et al., 2010).

1.3.3 The model electroactive bacteria: *Geobacter*

Scientists discovered the first dissimilatory metal-reducing bacteria (DMRB), *Shewanella* and *Geobacter*, in the late 1980s. The first *Geobacter* species (initially designated strain GS-15) was isolated from the Potomac River, just downstream from Washington D.C. in 1987 (Lovley et al., 1987). This organism, known as *Geobacter metallireducens*, was the first organism found to oxidize organic compounds to carbon dioxide with iron oxides as the electron acceptor. In other words, *Geobacter metallireducens* gains its energy by using iron oxides in the same way that humans use oxygen. *Geobacter* species have been isolated from a diversity of soils and sediments and provide a model for microbial electrochemistry because of the ability to transfer electrons on to the surface of electrodes.

Today, up to 94 microbial species are assigned to be electroactive and presumably significantly more electroactive species exist in nature (Koch and Harnisch, 2016).

The DMRB metabolism, which couples biological electron transport to inorganic materials, gives us the opportunity to both study such redox reactions at conductive surfaces. In fact, if an electrode is poised at a favourable redox potential, it is possible to "trick" the DMRB into transferring their electrons to the electrode surface in the absence of any other electron acceptor. This not only provides an opportunity to study respiration, it gives researchers precise control of the energetic redox conditions, thereby allowing them to direct the growth of the microbes, and even to culture some bacteria that may be difficult to grow in standard media.

Geobacter species are in the family *Geobacteraceae*, which is within the domain Bacteria, phylum *Proteobacteria*, class *Deltaproteobacteria*, and order *Desulfuromonadales*.

1.3.4 Types of MET by the operation mode

Microbial Electrochemical Technologies (MET) can be derived as technologies or applications that utilize the electrochemical interaction of microbes and electrodes. In the great majority these interactions involve extracellular electron transfer (Schröder et al., 2015b). These MET are often denominated in literature as bioelectrochemical system (BES). In addition, to differentiate between MET and systems that are not MET, it has to be stated that in a MET at least one of the two electrodes (anode or cathode) has to be functionally connected with a respective microbial process (Schroder 2015) (Figure 1-15).

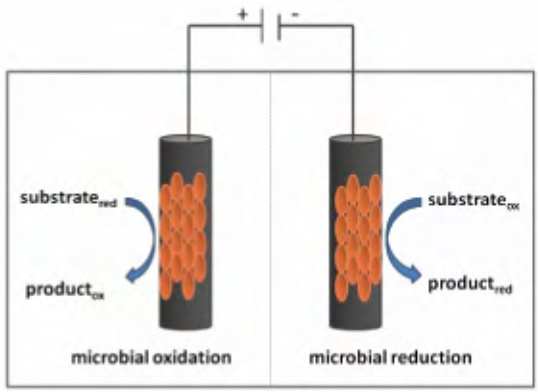


Figure 1-15: Microbial processes in a MET system

The redox potential of the anode depends on the chemistry and bioelectrochemistry around the electrode. Moreover, the electrochemical characteristics of those microbial-assisted devices can be simply controlled by altering their configuration. Thus, they can be operated in different configurations, such as:

a) Microbial Fuel Cell (MFC), able to harvest energy in presence of a resistor (Logan et al., 2006; Rabaey and Verstraete, 2005);

b) Short-circuit, with no resistors between electrodes (Erable et al., 2011; Wu et al., 2014);

c) Microbial Electrolysis Cell (MEC) by poising a certain potential through a potentiostat or a power source (Borjas et al., 2015; Kiely et al., 2011).

A group of derivative technologies, such as microbial desalination cells (MDCs), photomicrobial fuel cells (photo MFCs), microbial electroremediating cells (MERC) (Domínguez-Garay et al., 2016; Rodrigo et al., 2014; Rodrigo Quejigo et al., 2016) microbial electrosynthesis (MES) and microbial electrochemical fluidized bed reactors (ME-FBR) (Tejedor-Sanz et al., 2017) have been developed.

Microbial fuel cell (MFC)

MFCs are devices that use bacteria as the catalysts to oxidize organic and inorganic matter and generate current (Figure 1-16 A). In these devices, electrons produced by the microbial metabolism (the oxidation of a compound) are first transferred to a negative electrode (anode), and then flow to a second positive electrode (cathode), linked by a conductive material containing a resistor (Logan *et al.*, 2006), where a reduction reaction occurs. Anode compartment is usually kept anoxic while cathode compartment can be aerated to permit the reaction of oxygen with protons and electrons. Both anode and cathode compartments can be separated by an ion exchange membrane that allows protons to permeate from the anode to the cathode and impede the diffusion of oxygen to the anode. In this configuration the anode act as TEA, as any other natural acceptor like oxygen, nitrate or Fe (III), while cathode act as ED for a reduction reaction.

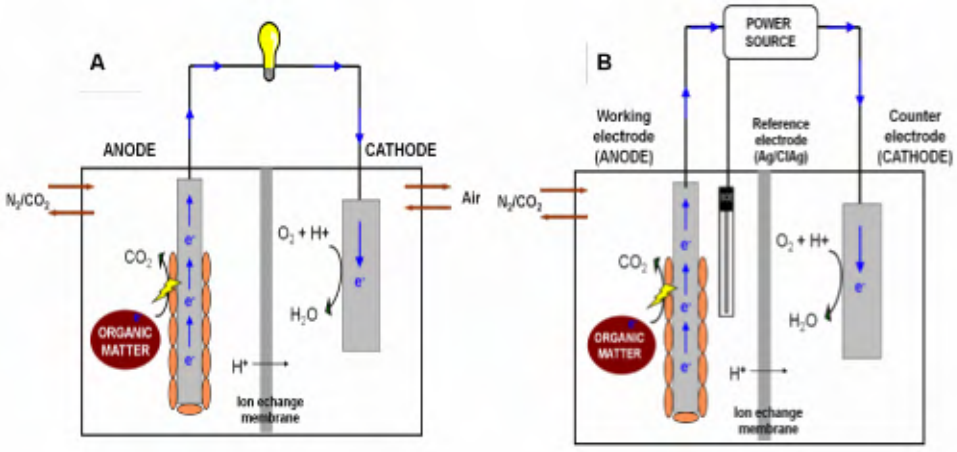


Figure 1-16: A: Schematic of a 2-chamber MFC with an anionic membrane separator. B: Schematic of a MEC configuration of 3 electrodes in which the anode is the working electrode. From Tejedor-Sanz, 2016.

Microbial electrolysis cell (MEC)

The MEC is based on modifying an MFC. Anodic reactions are almost the same in both configurations, but while in a MFC the reactions are thermodynamically

spontaneous, because oxygen (or other highly oxidant compound) is provided in the cathode compartment to be reduced and favour the oxidation of organic or inorganic matter in the anode, in a MEC the reactions are not spontaneous and must be induced by supplying external energy. Coupling organic matter oxidation at the anode with oxygen reduction at the cathode, are example of thermodynamically spontaneous processes. In contrast, organic matter oxidation coupled to water reduction is an example of not spontaneous processes.

From earlier studies, MECs were used to produce hydrogen from the chemical electrolysis of water in the cathode (D. F. Call et al., 2009; Cheng and Logan, 2007; Ditzig et al., 2007), where no oxygen was supplied, coupled to microbial oxidation of organic matter in the anode. That is the reason to the term MEC (Logan and Rabaey, 2012). But energy can be provided also to reduce other compounds than water in the cathode, such as nitrate (Clauwaert et al., 2007; Pous et al., 2013; Virdis et al., 2010), sulphate (Luo et al., 2014), CO_2, etc. Here the cathode should be kept anoxic to avoid the competition of oxygen with the chemical species we want to reduce.

The energy can be supplied by a power source or a potentiostat depending on the selected mode of operation: galvanostatic mode (current flow is fixed) or potentiostatic mode (the potential difference between two electrodes is fixed), respectively. In this latter case, if we want to maintain the potential of one of the electrodes under a selected value, i.e. for inducing a certain reaction, we need to work, close to the so-called working electrode (WE), with a reference electrode (RE) in a 3-electrode configuration (Figure 1-16). The other electrode is called counter or auxiliary electrode (AE) and its potential is dependent upon the current flow circulating through the system.

Microbial electrochemical snorkel (MES)

This configuration is actually a short-circuited MFC. This kind of system provides the highest currents, meaning that it ensures the highest rate for organic matter oxidation. In the microbial electrochemical snorkel, one of the sides of an electrode plays the role of an anode and the other side the role of a cathode (Erable et al., 2011). In theory, the anodic part should be exposed to anaerobic conditions and develop an electroactive biofilm over it, while a catalyst and/or an electroactive biofilm should form on the cathodic part exposed to the aerobic zone, as can be seen in Figure 1-17. The redox potential difference between both environments is the driving force of the electrons that circulate through the conductive material. The goal of this configuration is to maximize the pollutants removal, as is as been

reported before i.e. for nitrate removal (Yang et al., 2015) or petroleum hydrocarbons (Cruz Viggi et al., 2015), and not to harvest a current flow. Consequently, the system does not require complex electrochemical reactors with ionic exchange membranes or any other kind of separators. The design can be simplified to two connected electrodes, or even a single piece of conductive material.

1.3.5 Wastewater treatment applications of METs

Since the discovery of electroactive microorganisms, Microbial Fuel Cells (MFC) were proposed to play an important role in WWT for converting the waste into clean energy, by oxidizing organic and inorganic matter to generate electrical current (Liu *et al.*, 2004; Logan *et al.*, 2006). The researchers took the microbial fuel cell (MFC) from a concept to a technology. A variety of fuels have been utilised for this energy recovering: marine sediments (Reimers et al., 2006, 2001), rice paddy fields (Kaku et al., 2008), urban wastewater (Liu et al., 2004), food processing wastewaters (Aelterman et al., 2006; Wang et al., 2008), cattle industry wastewaters (Doherty et al., 2015b; Min et al., 2005) or even plant root exudates, as in plant-MFC devices (Helder et al., 2010; Strik et al., 2008).

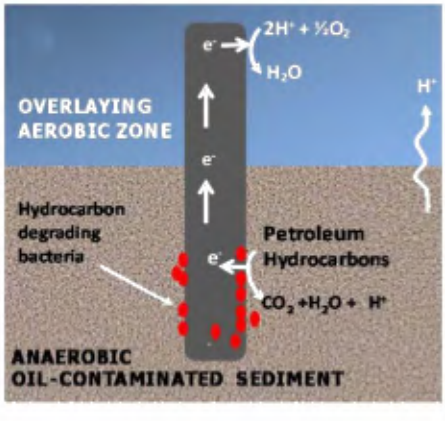


Figure 1-17: Schematic of a MES applied to bioremediation of a polluted soil with petroleum hydrocarbons. From: Cruz Viggi *et al.* (2015).

Oxidation of organic matter coupled to electron transfer to electrodes was a potential strategy for harvesting electricity from the environment and waste organic matter (Lovley, 2006). But energy recovering has demonstrated to be scarce, in spite of the efforts to increase energy efficiency and it seems to be more adequate to specific applications.

However, WWT remains an important field to scale up these technologies. Regarding the large amount of studies, this is probably the most relevant challenge that METs face. Many researchers are trying to find more efficient technologies that increase the energetic efficiency of the WWT, trying to accomplish the new objectives of the strategy Europe 2020 for smart, sustainable and inclusive growth.

A suitable scenario for testing this emergent technology is the CWs since they are a good alternative for WWT in small communities and are used worldwide (García *et al.*, 2010). HSSF CWs were initially presented as environments that could take advantage of depth-depending redox potential gradients (Corbella et al., 2014a; Villaseñor et al., 2013). Previous reports argued that redox conditions in CWs could be controlled by altering the organic loading rate, the hydraulic design and the mode of operation (Faulwetter *et al.*, 2009).

Following this strategy, different groups have integrated MFC elements to lab-scale CWs with the purpose of harvesting electricity (Fang *et al.*, 2015; Liu *et al.*, 2014; Zhao *et al.*, 2013). In spite of using wastewater as organic fuel, the power densities reported were as low as 1.84 – 44.63 mW m^{-2} (Doherty et al., 2015a), which is a range typical for sMFC operating in soil or sediments, but still far from 10 W m^{-2} values obtained using filter press bioelectrochemical reactors (Borjas *et al.*, 2015). This is mainly due to the fact that redox gradients are not broad enough in this kind of environments and *in situ* implementation of power-harvesting devices is indeed limited.

1.3.6 Pollutants removal processes in METs

Bioelectrochemical organic matter oxidation

Exoelectrogenic microorganisms can use organic and inorganic simple molecules in the anodic chamber of a MET to obtain energy in anaerobic conditions: acetate, ethanol, glucose, hydrogen gas, etc (Coppi, 2005; Mahadevan et al., 2006). If more complex substrates are present in the wastewater, then the electrogenic metabolism needs of a partner that breaks these compounds into more simple molecules (Ren et al., 2007).

When real wastewaters are treated, the electrochemical performance is importantly decreased compared to using synthetic water with easier biodegradable substrates. This is due to the low degradation rates of complex organic matter and to the appearance of competing processes as methanogenesis. The performance of the treatment depends highly on the wastewater composition. It has been observed

that at higher organic matter concentrations, increasing amounts of organic matter can be removed, but results in decreasing coulombic efficiencies. This is due to the stimulation of other microbial processes such as fermentation and methanogenesis (Freguia et al., 2007; Logan and Regan, 2006).

Bioelectrochemical nutrients removal

- *Nitrogen*

The disadvantage of the conventional nitrification/denitrification reaction is that it requires considerable amounts of energy, because the wastewater needs to be aerated to supply oxygen for the conversion of ammonium to nitrate, and a supply of electrons for denitrification in the form of COD is required (Rodriguez Arredondo et al., 2015)

Ammonium removal by MFC has focused on two different strategies: MFC ammonium nitrification under aerobic conditions in the cathode (Virdis et al., 2010) or, given that ammonia can be diffused from anode to cathode through the cation exchange membrane; it can be stripped with a suitable gas and subsequently absorbed (Desloover et al., 2012). Anodic oxidation of ammonium to nitrite has been reported by few authors (He et al., 2009; Zhan et al., 2014; Zhu et al., 2016) in two chamber MECs. Furthermore, it has been reported Anammox process to be enhanced by previous bioelectrochemical oxidation of ammonia to nitrate (Zhu et al., 2016).

In conventional WWT systems, the organic matter available in the wastewater is typically used as electron donor during denitrification. In contrast, METs can be a potential alternative for removing nitrate from wastewater with low organic matter content, even from groundwater (Pous et al., 2013; Tong and He, 2013). In METs, the cathode can serve as electron source for heterotrophic or autotrophic electroactive microorganisms to reduce the nitrate (Figure 1-18). The electrons provided by a biocathode can either come from acetate oxidation in a bioanode or from the abiotic electrochemical oxidation of other compounds such as water (Pous et al., 2014; Virdis et al., 2010). Given that in a MEC a flow of electrons can be supplied to the electrodes, denitrification could be controlled without adding organic matter and better results could be reached.

Different reactor designs have been tested for performing a complete nitrogen removal treatment. For instance, nitrification can be accomplished in a separate chamber, transforming the ammonium to nitrite or nitrate, while denitrification can be

carried out in the cathode, as previously described by Virdis et al. (2008). Other studies have investigated the simultaneous aerobic nitrification/bioelectrochemical denitrification in the same chamber (Sayess et al., 2013; Virdis et al., 2010).

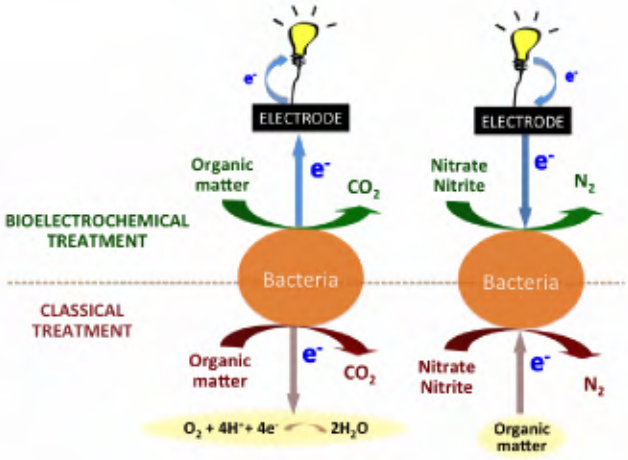


Figure 1-18: Bioelectrochemical nitrogen and organic matter removal process in wastewater. From Tejedor-Sanz, (2016).

- *Phosphorous*

Phosphorous is usually removed from the wastewater in the form of struvite. Struvite recovery from wastewater can be achieved by several approaches: chemical addition, carbon dioxide stripping, or electrolysis. Most struvite recovery studies have focused on increasing solution pH via chemical base addition (NaOH, $Mg(OH)_2$, $Ca(OH)_2$) and stripping carbon dioxide through aeration. These solutions are effective, but operational costs associated with continuous chemical addition or blower operation are high (Doyle and Parsons, 2002).

While much attention and further research has been focused on nitrogen removal and recovery through METs, only few studies investigated the removal or recovery of phosphorous from wastewaters in these systems. In an earlier study, digested sewage sludge was used to release the orthophosphate from iron phosphate. This was followed by the addition of magnesium and ammonium along with pH adjustments, which resulted in struvite formation (Fischer et al., 2011). Other studies combined the struvite formation with hydrogen production in an MEC unit to enhance the process benefits (Cusick et al., 2014; Cusick and Logan, 2012). This precipitation of phosphorous compounds is caused by the local pH increase at the vicinity of the cathode that results from the reduction reactions. Despite the scarce efforts being put thus far in phosphorous removal in METs, there is an increasing

interest due the new requirements in WWT and the valuable product that can be obtained.

1.3.7 Scaling up METs: the challenge

An intense race has taken place during the last years to scale-up METs. In spite of the effort to scale up the technology for the last 10 years, just pilot scale devices no larger than 1000L have been reported in scientific literature (Cusick et al., 2011; Ewing et al., 2014; Heidrich et al., 2014, 2013; Logan, 2010).

MFCs have been demonstrated at scales useful for powering remote devices in seawater applications (Tender et al. 2008; Figure 1-19 A). The first large-scale test of MFCs was conducted at Foster's brewery in Yatala, Queensland (Australia), by the Advanced Water Management Centre at the University of Queensland, conducted under the direction of Jurg Keller and Korneel Rabaey. The reactor consisted of 12 modules, each 3 m high, with a total volume of approximately 1 m^3 (Logan, 2010) (Figure 1-19 A). The reactor contained carbon fibre brush anodes inside tubular reactors, with flow up through the tubes and out over the outside of the reactor that was covered with graphite fibre brush cathodes. This design was similar to one tested in the laboratory with a ferricyanide catholyte (Rabaey et al., 2005).

The first demonstration of an MEC for hydrogen production was conducted at the Napa Wine Company, in Oakville, CA, USA, by Penn State researchers with engineering services by Brown and Caldwell (Walnut Creek, CA, USA; Figure 1-20). The reactor design is based on approach of immersing brush anodes and flat cathodes made of stainless steel into a tank (Call et al., 2009; Logan, 2008). The reactor contained 24 modules, each with six pairs of electrodes, and was approximately 1 m^3 in total volume.

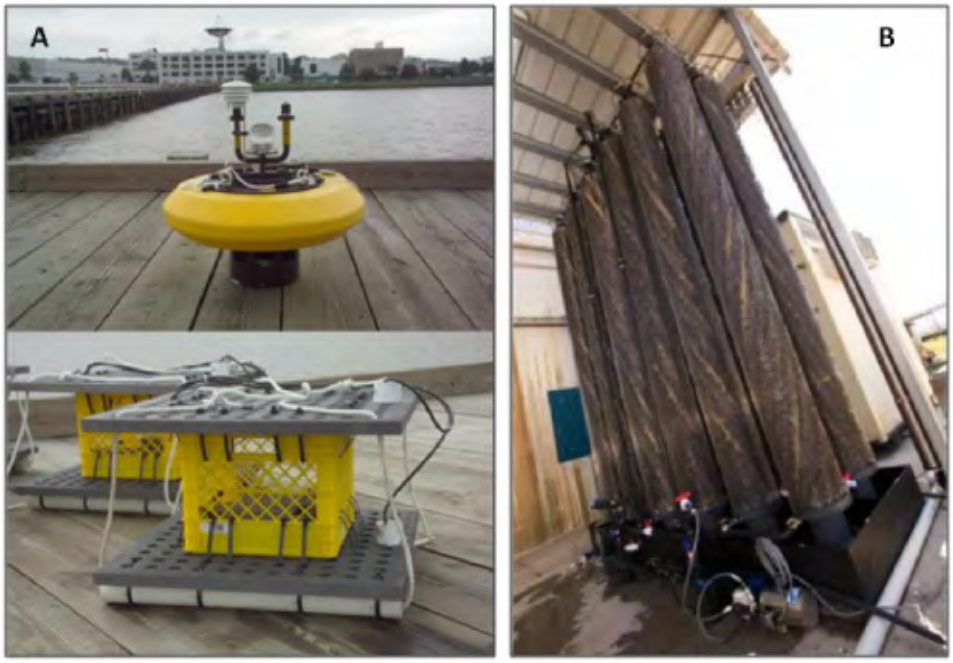


Figure 1-19: A: *Top*: meteorological data buoy used in demonstration on the pier of the Naval Research Laboratory in Washington, DC, being prior to deployment (mooring and RF transmitter antenna not yet configured). *Bottom*: one of the first generation BMFC subunits on pier prior to deployment. Seven subunits were electrically connected in parallel to provide sufficient power to operate buoy. From (Tender et al., 2008). **B**: Tubular microbial fuel cells tested for power production using wastewater produced at Foster's brewery in Yatala, Australia (www.microbialfuelcell.org). From (Logan, 2010)

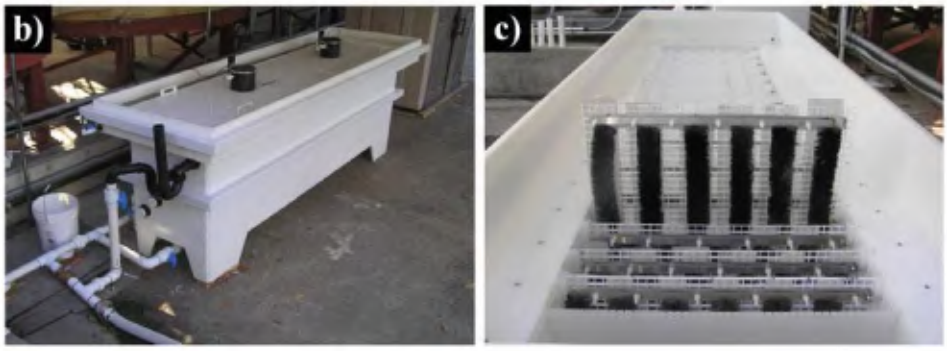
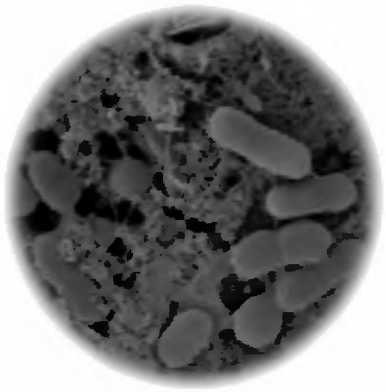


Figure 1-20: Pilot-scale microbial electrolysis cell being tested for hydrogen production using winery wastewater at the Napa Wine Company in California, USA. From (Cusick et al., 2011)



Chapter 2. Objectives and Research Framework

Objectives and Thesis Outline

The capacity of some bacteria to interchange electrons with electro-conductive material constitutes one the most fascinating discovery in the history of environmental biotechnology. In spite of the attractive concept the last 15 years of research are plenty of successful lab scale stories that have been no implemented so far at full scale. With the aim of accelerating the implementation of such microbial electrochemical technologies (METs) we have avoided the use of classical bioelectrochemical membrane-bed reactors (press-filter designs, two chamber electrochemical cells, etc); instead we have use the concept of constructed wetlands as a frame for testing and enhancing microbial electroactivity.

CWs are indeed a good alternative to classical intensive methods for wastewater treatment in small populations (until 2000 p.e.), mainly due to the low cost and simplicity of operation and maintenance of the technology. However, they show a large surface area requirement that is not always available.

Thus, this thesis aims to improve CWs performance by incorporating microbial electrochemical technologies (METs) with the final goal of reducing their classical size, allowing the installation of the technology in any population and, furthermore, make the most of any old facility that is in misuse, and all at once, achieve a null energy cost.

With this aim, three objectives have been pursued:

The first objective was to explore the conversion of the classical biofilter present in CW, either aerobic or anaerobic, into an electroconductive biofilter whose redox state could be tuned or controlled by electrochemical tools.

The second objective was to analyse the microbial communities responsible of using electroconductive material as novel supporting material.

The third objective was to design, construct and operate a full scale electroconductive wetland to treat real urban wastewater of 10 inhabitants.

To achieve these objectives the research has been developed both in the laboratory environment and in the field. Laboratory experiments have been carried out in the Chemical Engineering Department facilities at the University of Alcalá (UAH) (Alcalá de Henares, Madrid, Spain), and at the Foundation Centre for New Water Technologies (CENTA) (Carrión de los Céspedes, Seville, Spain). On top of that, the assays performed at full scale were developed at CENTA. Most of the

research (including activities at UAH) was assessed with real urban wastewater from the CENTA facilities.

Chapter 1 is an introduction that presents the general framework of this thesis. Chapter 3 to Chapter 6 correspond to material published or submitted to peer-review international journals prior to PhD defence. Chapter 7 discuss and synthesise the contribution of this thesis to wastewater treatment in small populations in the context of microbial electrochemical technologies.

Research Framework

This PhD thesis was developed within the context of three research projects, all focused on applying microbial electrochemical systems for treating wastewaters.

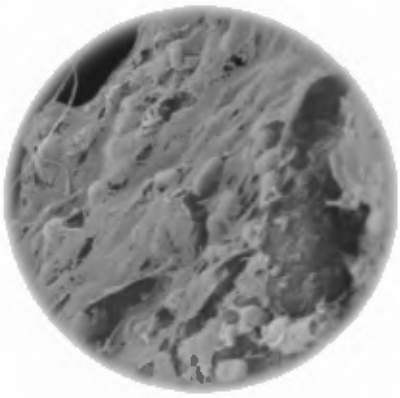
The project Aquaelectra (Bioelectrogenic treatments applied to wastewater treatment) was funded through the program INNPACTO from the Spanish Ministry of Science and Innovation, and was developed by a consortium of two research institutions - the Bioelectrogenesis group of IMDEA Water (Alcalá de Henares, Spain) and CENTA (Foundation Centre of New Water Technologies, Seville, Spain) - and three water engineering companies - DAM (Mediterranean Water Purification), JOCA and Euroestudios -. This initiative aimed to provide the application of new techniques to wastewater treatment, with three objectives: a) to develop a natural system of sewage treatment using MET-based wetlands, b) to establish a MET-based anaerobic system for treating wastewater and c) to build a MET-based nutrient (nitrogen) removal system.

The Smart Wetland Project pursued the aforementioned strategy conceived for the Aquaelectra Project while incorporating three new technological elements (self-monitoring, renewable energy and ITC) with the aim of improving treatment efficiency and management of these systems, without compromising one of their main principles, a null energy cost.

The most recent project, currently active, is a European Union's Horizon 2020 initiative called iMETland (www.imetland.eu), funded by the research and innovation program, currently ongoing, that aims to implement this technology in small communities at zero-energy cost and with remote control process.

The author stayed three months in the group of Dr Hans Brix and Dr Brian K. Sorrel in the Biosciences Department, Area of Aquatic Biology of the Aarhus University, Denmark, along 2014, as a visiting scientist. The purpose of the stay was to develop methodologies to measure the oxygen released by wetland plants through the rhizomes and roots.

For the development of the current research the author has received a PhD fellowship funded by the Formación de Profesorado Universitario (FPU) programme of the University of Alcalá.



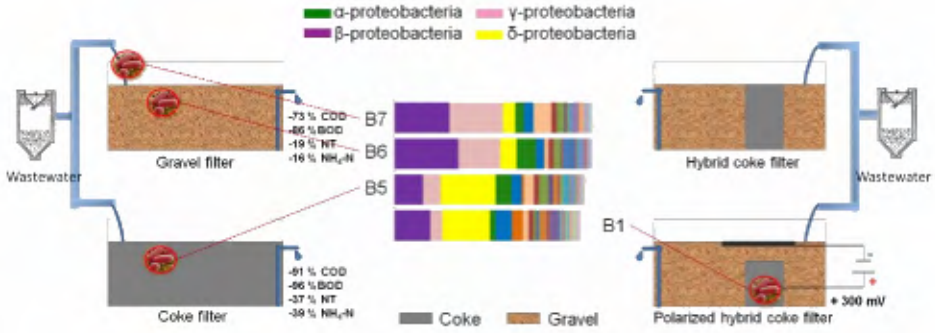
CHAPTER 3: Microbial Electrochemical Systems Outperform Fixed-Bed Biofilters In Cleaning Up Urban Wastewater

This section has been redrafted after:

Arantxa Aguirre-Sierra[a], Tristano Bacchetti-De Gregoris[b], Antonio Berná[b], Juan José Salas[c] and Abraham Esteve-Núñez[a,b]. 2016. *Microbial electrochemical systems outperform fixed-bed biofilters in cleaning up urban wastewater*. Environ. Sci.: Water Res. Technol. 2, 984–993

[a.] Department of Chemical Engineering, Universidad de Alcalá, Alcalá de Henares, Madrid, Spain.
[b.] IMDEA Agua, Parque Tecnológico de Alcalá, Alcalá de Henares, Madrid, Spain
[c.] Foundation Centre for New Water Technologies (CENTA), Carrión de los Céspedes, Sevilla, Spain

Microbial Electrochemical Systems Outperform Fixed-Bed Biofilters in Cleaning Up Urban Wastewater



3.1 Abstract

In this work we present for first time the concept of integrating Microbial Electrochemical Technologies (METs) with the natural wastewater treatment (WWT) biofilters used in constructed wetlands (CW) to form METlands. In order to validate this technology, four lab-scale horizontal subsurface flow (HSSF) biofilters, three hosting electroconductive material and one gravel biofilter (control), were operated for 525 days to define the best design and operational conditions to maximize removal of wastewater pollutants. Organic loading rates tested ranged from 2 to 24 g BOD_5 m^{-2} d^{-1} at hydraulic retention times (HRT) from 4 to as low as 0.5 days, respectively. The electroconductive biofilter showed the best COD and BOD_5 removal rates per volume of bed, achieving mean values of 213 g COD m^{-3} d^{-1} and 119 g BOD_5 m^{-3} d^{-1} at the lowest HRT (0.5 d). Ammonia and total nitrogen maximum removal efficiency at 3.4 days of HRT were 97 and 69 %, respectively, in the electroconductive biofilter. Bacterial communities were studied by 16S rDNA Illumina sequencing with the aim of understanding the role of the electrically conductive material in selecting microbial populations. *Deltaproteobacteria* (a known electroactive taxon) were enriched in presence of electrically conductive bed. *Geobacter* and *Geothrix* were the dominant genera in the deeper zone of the electrically conductive bed where oxidation of organic matter occurred. The results suggest that the enhancement in biodegradation rate will significantly reduce the area requirements of classical CW.

3.2 Introduction

The conventional WWT require high energy, operation and maintenance costs. In addition, due to population growth and urban expansion, the volume of sewage sludge produced by WWT is constantly increasing (Ghazy et al., 2011). So thus, a different water-energy nexus scenario is required to cope with the future global water demand.

From the outset of the discovery of electroactive bacteria (Bond et al., 2002), their use in Microbial Fuel Cells (MFC) were initially proposed to play an important role in WWT for converting the waste in clean energy, by means of those microorganism able to oxidize organic and inorganic matter and generate electrical current (Liu et al., 2004; Logan et al., 2006). In those devices, electrons produced by the microbial metabolism are first transferred to an electrode (anode), and then to a second electrode (cathode) both wired by a conductive material containing a resistor (Logan et al., 2006). In this configuration, the electrode act as terminal electron acceptor as any other natural acceptor like oxygen, nitrate or Fe(III). The clear advantage of exploiting electro-stimulated communities is that electrodes can boost microbial metabolism in anaerobic systems that are typically limited in electron acceptor. Electroconductive material may represent an inexhaustible source of electron acceptors, hosting the additional advantage of providing a more easily modulated redox potential compared to standard, low-reducing redox species that generally drive these systems (Kato Marcus et al., 2007). The redox potential of the anode depends on the chemistry and bioelectrochemistry around the electrode. Moreover, the electrochemical characteristics of those microbial-assisted devices can be simply controlled by altering their configuration. Thus, they can be operated in different configurations, such as i) short-circuit, no resistors between electrodes (Wu et al., 2014); ii) MFC, able to harvest energy in presence of a resistor (Rabaey and Verstraete, 2005); and iii)Microbial Electrolysis Cell (MEC) by poisoning a certain potential through a potentiostat or a power source (Borjas et al., 2015; Kiely et al., 2011).

A suitable scenario for testing METs is the CWs since they are a good alternative for WWT in small communities and are used worldwide (García et al., 2010). Low cost operation and maintenance, low energy requirements, low production of sewage sludge (just in primary treatment) and good landscape integration are some of the most attractive advantages of CWs compared to

conventional treatment systems (García et al., 2003b). However, CWs treatment is constrained by limitations such as large land requirements (3–10 m^2 per p.e. depending on the design) (Tilley et al., 2008; Vymazal and Kropfelova, 2008) and clogging by the accumulation of solids (Rousseau et al., 2004; Tanner and Sukias, 1995). Recommended surface organic inlet load for HSSF CW is reported as 6.0 g BOD_5 m^{-2} d^{-1} in order to achieve a value under 30 mg BOD_5 L^{-1} in the effluent and to avoid clogging (Kadlec and Wallace, 2009; US EPA, 2000). HSSF CWs were initially presented as environments that could take advantage of depth-depending redox potential gradients (Corbella et al., 2014b; Villaseñor et al., 2013). Previous reports argued that redox conditions in CWs could be controlled by altering the organic loading rate, the hydraulic design and the mode of operation (Faulwetter et al., 2009). Following this strategy, different groups have integrated MFC elements into lab-scale CWs with the purpose of harvesting electricity (Fang et al., 2015; Liu et al., 2014; Zhao et al., 2013). In spite of using wastewater as organic fuel, the power densities reported were as low as 1.84–44.63 mW m^{-2} (Doherty et al., 2015a) which is a range typical for sediment microbial fuel cells (sMFC) operating in soil or sediments, but still far from 10 W m^{-2} values obtained using filter press bioelectrochemical reactors (Borjas et al., 2015). This is mainly due to the fact that redox gradients are not broad enough in this kind of environment and in situ implementation of power-harvesting devices is indeed limited.

However, we still believe that CWs are a suitable environment for implementing microbial electrochemical systems. Our aim was not to harvest energy but to enhance the rate of pollutant removal by converting the classical inert biofilter into an electroconductive biofilter where its redox state could be tuned or controlled by electrochemical tools. Our results revealed how the integration of METs in wetlands resulted in a powerful hybrid technology, the so-called METland (Esteve-Núñez et al., 2013), that strongly outperforms the treatment of urban wastewater through the stimulation of different microbial populations.

3.3 Materials and Methods

3.3.1 Design and construction of electroconductive biofilters

In this study, four laboratory-scale Horizontal Subsurface Flow (HSSF) biofilters were constructed to determine the best design and operational conditions to maximize wastewater pollutant removal. A control unit used standard siliceous gravel (Ø 6–12 mm) as a biofiltering bed (Figure 3-1, A). An electroconductive bed

configuration (Figure 3-1, B) was constructed with a single material, acting as a whole electrode. This configuration did not allow the conversion of microbial metabolism into electrical current to be monitored, since the anode and cathode were not differentiated. In order to gain electrochemical information about the process, a three-electrode system was additionally constructed by using a hybrid unit made of inert gravel and a polarized coke bed (Figure 3-1, C). An additional hybrid unit operating under short-circuit (Figure 3-1, D) was constructed as control. In these hybrid biofilters, the conductive material was vertically inserted into the gravel. The short-circuit hybrid unit acted as a single electrode without differentiated anode and cathode.

The conductive material in the bed was coke granules (Ø 5–10 mm). The dimensions of the biofilters were 0.52 m long, 0.34 m wide and 0.30 m high, and the material layer was 0.20 m deep, with a total bed volume of 0.034 m^3 and a water volume of 0.011 m^3. Each biofilter had a drainage pipe, located on the flat bottom, for effluent discharge, and the water level was kept below the surface. The hybrid polarized biofilter hosted a coke anode of 0.006 m^3 as shown in Figure 3-1. A plate of graphite (3 cm × 3 cm × 0.5 cm, Sofacel) buried into the coke anode acted as an electron collector. The cathode was made of carbon cloth (0.34 m × 0.15 m, Resinas Castro, 420 g m^{-2}). The anode and cathode were connected by a copper wire to a potentiostat unit (Nanoelectra S.L., Spain). A third electrode (Ag/AgCl) buried in the anodic bed acted as reference to polarize the anode at 0.3 V (vs. Ag/AgCl). The anode potential and the current were periodically measured using a digital multimeter (Model 2700, Keithley Instruments, USA). The data were recorded every 10 s on a spreadsheet using ExceLINX (Keithley) via an interface card (GPIB Interface Boards, Keithley) linked to a personal computer. The performance of the polarized biofilter was evaluated in terms of coulombic efficiency (CE, %) comparing the total electrons harvested by the anode to the electrons possibly generated by the microbial oxidation of the substrate. For continuous flow through the system, we calculated CE based on the COD change and the flow rate, q (Logan, 2008), as follows:

$$CE = \frac{8I}{Fq\Delta COD}$$

where 8 is a constant used for COD, based on the conversion from g O_2 (MW = 32 g mol^{-1}) to mol e^- (4 mol e^- per mol O_2), I is the current obtained over time and F is the Faraday's constant.

The systems were operated in parallel and fed with real urban wastewater from the municipality of Carrión de los Céspedes (Sevilla, Spain) (2500 inhabitants) under discontinuous flow regime during 525 days (75 weeks). Wastewater was pretreated in an Imhoff tank in order to remove solids and prevent potential clogging of the systems. The feeding from the Imhoff tank was made by programmed pumping, by means of 12 daily periods, simulating the production of wastewater in small populations (Ortega *et al.*, 2010). Several organic loading rates were tested (2.0 ± 1.0, 4.2 ± 0.7, 9.2 ± 2.8, 13.8 ± 9.5 and 24.0 ± 12.7 g BOD_5 m^{-2} d^{-1} in average) at the following hydraulic retention times (HRT): 4.0, 3.4, 1.7, 0.8 and 0.5 days, respectively.

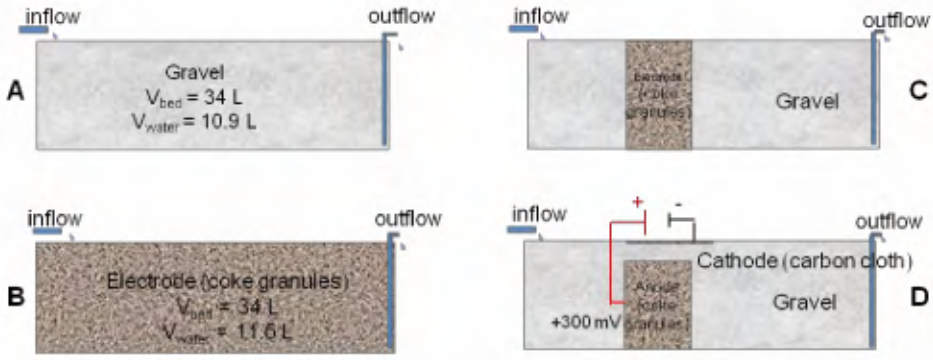


Figure 3-1: Simplified design of the four systems A) Gravel biofilter (control), B) Coke biofilter, C) Hybrid biofilter, D) Hybrid polarized biofilter.

3.3.2 Physical, chemical and statistical analyses

BOD_5, total suspended solids (TSS), total nitrogen (TN), ammonia (NH_4) and nitrate (NO_3) were analysed weekly; COD was analysed twice a week, following the standard methods (American Public Health Association, 2005). Temperature (T), pH, electrical conductivity (EC), dissolved oxygen (DO), and redox potential (ORP) were measured weekly using a handheld multiparameter instrument (YSI 556 MPS). Samples were taken at the inlet and the outlet of the systems and water flow was measured daily. Moreover, hybrid systems were also sampled through sampling tubes buried in the bed, before and after the electroconductive barrier (anode), in order to calculate the coulombic efficiency. Inlet wastewater analyses are shown in Table S 3-1. Removal rates were calculated as grams per cubic meter of bed material per day. Removal efficiencies were calculated as percentage.

Statistical procedures to evaluate the effect of HRT for every water quality parameter were conducted using the Statgraphics Centurion XVII statistical software package. T-test or Wilcoxon tests were used to determine the differences of every water quality parameter among the effluents, depending on the type of data (95% confidence).

3.3.3 Microbial communities

Sampling, DNA extraction and 16S rDNA sequencing.

Samples were taken from lab-scale biofilters and inlet wastewater to determine the composition of their microbial community at four different spots: anode in the hybrid polarized biofilter (B1), upper area of the coke biofilter (B5), upper area of the gravel biofilter (B6) and inlet wastewater (B7). Either granules of coke (B1, B5) or gravel pebbles (B6) were sampled with tweezers and loosely attached bacteria were removed by dipping them in 3 consecutive sterile saline solutions (50 ml, NaCl 7 g L^{-1}). Coke and gravel pebbles were then frozen for 1 week until DNA extraction was performed. Around 10 granules/pebbles were extracted per spot. DNA was extracted using PowerSoil spin columns (MO BIO Laboratories), suspended in 60 µL of sterile MilliQ water and quantified with PicoGreen (Invitrogen). A total of 3 ng of DNA were amplified with primers 515F-CS1 (ACACTGACGACATGGTTCTACAGTG CCAGCMGCCGCGGTAA) and 806R-CS2 (TACGGTAGCAGAGACTTGGTCTGGA CTACHVGGGTWTCTAAT). The polymerase used was Q5 Hot Start High-Fidelity (New England Biolabs), and the PCR conditions were as follows: initial denaturation at 98 °C for 30 s followed by 30 cycles of 98 °C × 10 s, 60 °C × 20 s and 72 °C × 20 s, and a final elongation step of 72 °C for 2 min. 1/100 dilutions of PCR products were then re-amplified (15 cycles) with Illumina's primers. Finally, products were run on a Bioanalyzer (Agilent), and the successful generation of equimolar pools was confirmed by qPCR. Sequencing was performed using MiSeq equipment in a 2 × 250 bp format and following Illumina's protocol.

The Illumina Miseq sequence reads have been deposited in the European Nucleotide Archive (ENA) database under accession Nr. PRJEB10685.

Bioinformatics analysis.

The total sequence reads were analysed with the QIIME 1.7 pipeline (Caporaso *et al.*, 2010) with few stitches along the way. Briefly, complementary reads were merged using fastq-join (Aronesty, 2011). Subsequently, our quality filtering strategy removed complemented sequences that had one of the following

characteristics: (i) deviated more than 10 bp from the expected length (292), (ii) contained primers with more tan 1 mismatch, or (iii) contained nucleotides with a Phred score of <20. Filtered seqs were organised in OTUs by de novo picking using Usearch (Edgar, 2010), and one representative sequence per OTU was chosen. Taxonomy was assigned using the GreenGenes database (DeSantis *et al.*, 2006) version 10_12 at the 97% identity rate. Furthermore, sequences were aligned and a tree was generated using FastTree 2.1.3 (Morgan N Price et al., 2010). Finally, in order to investigate alpha diversity with QIIME, OTUs containing less than 0.005% of the total sample reads were removed according to Bokulich (Bokulich *et al.*, 2013). The results have been represented as relative abundance of a specific sequence in every sample. Taking into account the possible effect of deviation introduced by the implemented protocol and that not all the bacterial species have the same number of copies of 16S rRNA gene in their genomes (Klappenbach, 2001), the values can be related to the percentage of cells of every species that were part of the sampled communities.

Scanning electron microscopy analysis

Samples of conductive material were taken to elucidate biofilm formation. Coke granules of the anode of the polarized hybrid coke filter and the single electrode of the coke filter were taken. Samples were fixed with 5% glutaraldehyde in 0.2 M Na-Cacodylate buffer, pH 7.4 and dehydrated with growing ethanol concentrations (25 %, 50 %, 50 %, 90 % and 100 %), acetone and anhydrous acetone. Then the samples were dried to the critical point, covered with gold and observed through the Scanning Electron Microscopy (SEM).

3.4 Results and discussion

HSSF CWs are biofilter setups that exploit the biofilm-based natural process by means of an inert material like gravel with the purpose of treating urban wastewater. Plants are typically integrated into CWs to oxygenate the root zone and to provide aerobic microorganisms a habitat within the anoxic environment (Brix, 1994). Our approach consists in substituting an inert material for an electroconductive material in order to stimulate electroactive microorganisms and consequently biodegradation rates. Due to the oxygen supply role of plants, we did not include vegetal species in our experimental set-up in order to achieve better control of the redox interaction between bacteria and the electroconductive bed.

3.4.1 Urban WWT by horizontal subsurface flow biofilters: electroconductive versus non electroconductive biofilters

Influence of the material on the wastewater pollutants removal

In order to quantify the influence of the material, we tested two independent HSSF biofilters fully constructed with electroconductive and inert material (Figure 3-1, A, B). The organic matter removal rates, in terms of COD and BOD_5, were similar under a low organic loading rate regardless of the material (Table S 3-2). However, significant differences among both systems appeared when the organic loading rate was increased. The coke biofilter performed removal efficiencies close to 100% despite increasing the organic loading rate, while the gravel biofilter efficiency decreased as the organic loading rate increased (Figure 3-2). Indeed, the coke biofilter showed the best COD and BOD_5 removal rates, achieving mean values of 213 g COD $m^{-3}d^{-1}$ and 119 g BOD_5 m^{-3} d^{-1} (Table S 3-2). Furthermore, the gravel biofilter showed a more variable performance.

Statistical tests revealed that there were significant differences ($p < 0.05$) in the effluent's concentration of COD and BOD_5 at every HRT (Table S 3-3) when the coke and gravel biofilters where compared. The coke biofilter biodegradation rates led to effluents with residual values up to 3-fold lower for COD and 4.5-fold lower for BOD_5 (Figure 3-3). COD and BOD_5 coke biofilter effluent values never exceeded the limits of discharge, which are 125 mg COD L^{-1} (or > 75 % removal) and 25 mg BOD_5 L^{-1} (or 70-90 % removal) (Dir. 91/271/EEC of 21 May 1991), in contrast with the gravel biofilter performance from 3.4 days of HRT onwards, which average effluent concentration exceeded 25 mg BOD_5 L^{-1} (Figure 3-3). Even at the lowest HRT the performance of the coke biofilter fulfilled the COD and BOD_5 discharge requirements in percentage (91 % and 96 %, respectively), compared to hardly 73% and 86 % for the gravel biofilter (Table S 3-2). Caselles-Osorio and García (2007) reported COD removal efficiencies of 71-85 % in intermittent fed HSSFCW experimental systems with a nominal HRT of 3.4 days, which is comparable to removal efficiencies of our control system at the same HRT (83 %). Coke biofilter achieved mean BOD_5 removal rates as high as 99 % at high HRT (3.4 days).

The BOD_5 surface inlet loads applied at 1.7, 0.8 and 0.5 days of HRT (Table S 3-2) were 1.5, 2.3 and 4-fold, respectively, the recommended load (6.0 g BOD_5 m^{-2} d^{-1}) and BOD_5 average values of the coke biofilter effluent were always under 10 mg L^{-1} (Figure 3-3). Even at very high inlet organic loads, the coke biofilter had a great capacity to remove organic matter, without any evidence of clogging during the long course (525 days) of the experiment. A remarkable conclusion is that just the coke

biofilter fulfilled the Directive for COD and BOD_5 at a HRT as low as 0.5 days. In contrast, for standard gravel biofilter a HRT as high as 3.5 days was required for fulfilling the limits. Moreover, there were not significant TSS differences in the effluents of the two biofilters, and both fulfilled the limit values of discharge (35 mg L^{-1}) (Table S 3-1).

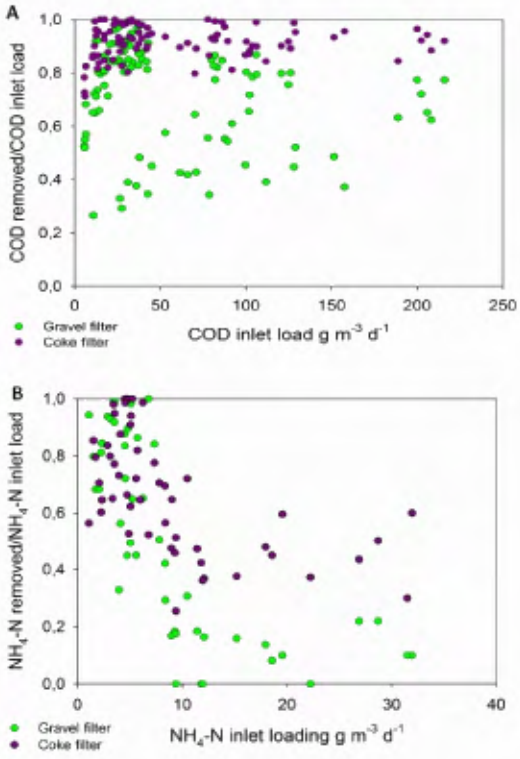


Figure 3-2: A) Relation between normalized COD removed and COD inlet loading of the coke and the gravel biofilters, B) Relation between normalized NH_4-N removed and NH_4-N inlet loading of the coke and the gravel biofilters.

Nitrogen removal was also analysed under both electroconductive and inert materials and a very similar result was found. Statistical analysis revealed significant differences ($p < 0.05$) among TN and NH_4-N effluent concentrations at every HRT. The coke biofilter exhibited the highest removal rates at every HRT (Table S 3-4). Interestingly, differences with gravel biofilter were more noticeable than those found for organic matter removal. In the coke biofilter, the maximum amount of nitrogen was removed at 0.5 days of HRT (TN: 11.9 g N $m^{-3}d^{-1}$; NH_4: 11.2 g N $m^{-3}d^{-1}$) although the removal efficiency (%) decreased with decreasing HRT. This trend has been reported in other studies (Kadlec and Wallace, 2009; Tanner *et al.*, 1998). The coke

biofilter showed maximum average removal efficiency values at 3.4 days, 97% of ammonia and 69% of total nitrogen, compared to 71% and 51%, respectively, in the gravel biofilter. The minimum values were reached at 0.5 days, 39 % of NH_4-N and 37 % of TN compared to 16 % and 19 %, respectively, in the gravel biofilter (Table S 3-4). Figure 3-2: B shows that the coke biofilter had a trend to maintain higher removal rates than gravel biofilter. The higher biodegradation rates generated effluents with residual TN and NH_4-N significantly lower (Figure S 3-1).

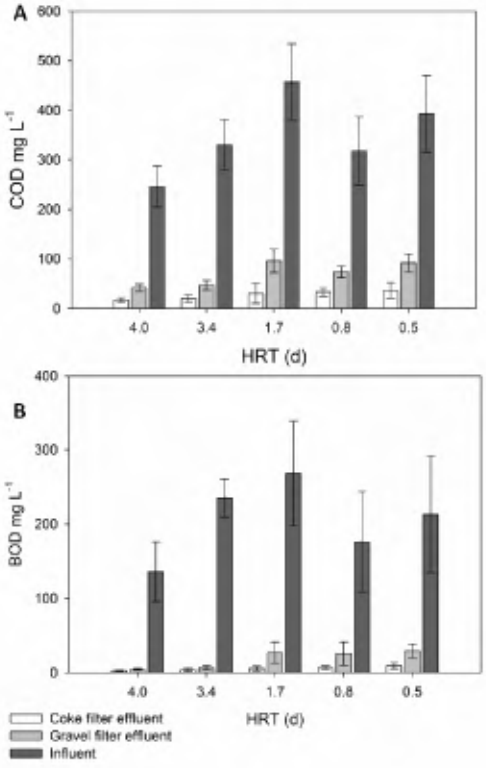


Figure 3-3: COD (A) and BOD_5 (B) influent and effluent average values of the coke and gravel filters. Error bars represent 95 % confidence interval.

The results demonstrated that the coke biofilter removed at least 2-fold the amount of TN and 2.5-fold the amount of NH_4 than removed by the gravel biofilter (HRT 0.5 days). Therefore, at HRT shorter than 4 days, nitrification was higher in the coke biofilter compared to the gravel biofilter. Moreover, at lower HRT, ammonia concentration in the effluent increased while nitrate was decreased (Figure S 3-1). The improvement of the conversion of ammonia to nitrate and nitrogen removal

suggests the enhancement of other metabolic pathways in the electroconductive bed.

Electrochemical analysis using hybrid electroconductive setups

In order to quantify the role of the electroconductive bed for accepting charge from microbial metabolism we constructed a hybrid polarized biofilter (Figure 3-1, D). In contrast with the sole-coke biofilter, this setup allows an accurate control of the electrical current by polarizing the system at 0.3 V (vs. Ag/AgCl).

The electrical current monitored throughout the assay revealed an expected profile, a stable value around 100 mA was measured (Figure 3-4). Interestingly, an increase in the organic loading rate did not result in a clear increase in electrical current, suggesting that the electroactive biofilm was not limited in electron donor. In contrast, the increase in the organic loading rates showed very good correlation with the organic removal rates only in the presence of electroconductive material so we concluded that some other biodegradation pathways, although not contributing to current production, are definitively being enhanced.

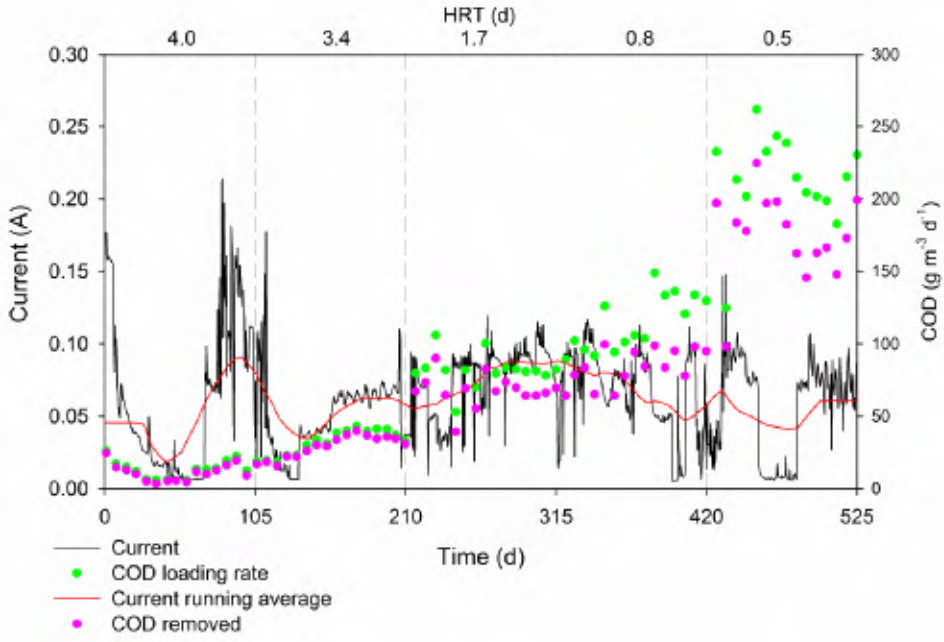


Figure 3-4: Profile of electrical current, COD loading rate (g $m^{-3}d^{-1}$) and COD removal rate (g $m^{-3}d^{-1}$) during long term operation of the hybrid biofilter polarized at 0.3 V (vs. Ag/AgCl).

As the electron donor is not a limiting factor, other degradation routes must have a major influence on the performance. In that sense, coulombic efficiency (CE) ranged from 37 % at low organic loading rate to 9 % at maximum organic loading rate, which indicates that low organic loading rates enhance the CE. The bacteria can biodegrade part of the COD through fermentation or the use of alternative electron acceptors (Rabaey *et al.*, 2005), such sulphate or nitrate. This is consistent with previous reports that showed how, under higher organic loading rates, electron flow is channelled towards methanogenesis or sulphate reduction so CE is reduced (Rabaey *et al.*, 2005). Methane emissions are common in HSSFCW because these systems present appropriate environmental conditions for methanogens and sulphate-reducing bacteria. These *Archaea* and *Eubacteria* require environments with similar redox potentials and use the same types of electron donors (i.e., hydrogen, methanol, and acetic acid) (García *et al.*, 2010). Methane emission rates are very variable and they are usually greater at the inlet than the outlet, given that methanogens activity is directly dependent of the organic load (Teiter and Mander, 2005). Further research about this topic should be carried out to evaluate the contribution of METlands to methane emissions.

Together with the hybrid polarized system, a non-polarized hybrid biofilter was also constructed (Figure 3-1, D) to evaluate the influence of the polarization versus the mere effect of the coke. Interestingly, despite polarizing the anode our assays did not reveal significant differences ($p>0.05$) in terms of COD and BOD_5 removal among the two hybrid configurations (Figure S 3-2). This fact strongly suggests that the electroconductivity of the material exert a positive influence on the microbial metabolism regardless of the existence of an electron flow among the different electrodes. Our hybrid biofilter is a single electrode configuration, a simplified design of a short-circuited system that cannot provide current but optimizes the pollutants removal. In that sense, our results are consistent with previous studies that reported how compact short-circuited system provided higher biodegradation performance than MFCs operating at maximum power (Erable *et al.*, 2011).

Redox potential was measured in both the electroconductive and the gravel biofilters. There was a noticeable redox potential gradient with depth and distance from the inlet in the systems which corresponded to COD and BOD_5. This gradient was greater in the electroconductive biofilter (Figure S 3-3) and suggests the presence of an electron flow from the deep bed to the more oxidized top layer of the coke bed.

In the hybrid systems the differences between materials were also remarkable. COD removal rates in the electroconductive bed (Table 3-1) were ca. 5-fold higher than in the gravel bed of the same hybrid device. Regarding nitrogen removal, both hybrid systems removed similar amounts of total nitrogen and ammonia at high and medium HRT (Table S 3-4).

Table 3-1: Urban wastewater treated by hybrid biofilter setups. COD overall averages ± SD, at HRT = 3.4 d. Removal efficiencies in the conductive bed (%) were referred to the COD before conductive bed.

COD levels (mg L^{-1})	Hybrid biofilter	Hybrid polarized biofilter
Influent	231 ± 58	231 ± 58
Before conductive bed	188 ± 55	182 ± 59
After conductive bed	89 ± 49	78 ± 31
Effluent	37 ± 20	35 ± 14
COD removal		
Removed in conductive bed (g m^{-3} d^{-1})	50.9 ± 24.8	55.5 ± 26.0
Removal efficiency in conductive bed (%)	52 ± 18	56 ± 14
Removed in gravel before conductive bed (g $m^{-3}d^{-1}$)	12.8 ± 7.8	15.8 ± 13.4
Removed in gravel after conductive bed (g $m^{-3}d^{-1}$)	10.4 ± 7.0	8.1 ± 4.8

3.4.2 Microbial communities

The analysis of four microbial communities revealed 696,288 raw reads that yielded a total of 689,911 high quality sequences with an average length of 292 bp (Table S 3-5). This volume of sequences is around one order of magnitude greater than previously reported studies of diversity in BESs (Miceli *et al.*, 2012), as result of improved sequencing technologies. Clustering these sequences generated 16,582 OTUs evenly distributed between the four samples. 2.33% of the sequence reads were not classified.

The classifiable sequences included members of 48 Phyla of which an average of 64 % were *Proteobacteria,* ranging between 52% (anode of the hybrid polarized biofilter) and 74% (gravel biofilter).

Rarefaction curves showed saturation, indicating that a reasonable number of sequence reads per sample were collected to reveal diversity at the sites (Figure S 3-4). Rarefaction curves indicate that predicted diversity was much less in the inlet wastewater than in the rest of the niches (around 70% of the number of identified

taxa). Diversity estimators such as observed OTUs, Chao1 and Shannon-Wienner, were significantly higher for coke granules samples when compared to the gravel samples (Table S 3-5). The Good's coverage estimator denoted that the sizes of the libraries were enough to cover almost 100% of the bacterial communities. Shannon diversity indexes (*H*), which includes the information of both richness (the number of species present) and evenness (how the abundance of each species is distributed) were obtained for our system. They were distinctly higher (between 6.27 and 7.38) than those in other studies on electrochemical CW treating urban wastewater (4.36-5.5 (Corbella *et al.*, 2015), 5.6 – 6.3 (Ahn *et al.*, 2014)) and similar to the results of Lu *et al.* (2015) (*H*: 7.33-7.47). These results, together with the high number of taxa found in the samples, indicated a very high diversity.

Weighted Fast UniFrac analysis and Correspondence analysis (CA) were used to identify the differences of the bacterial community structures based on their phylogenetic lineages. CA showed that the four communities separated distinctly from one another despite the same origin (Figure S 3-5). CA plot revealed that coke and hybrid polarized biofilters are closely related and that electroactive bacteria (*Deltaproteobacteria*) had the higher component weight in both systems. Another closely related taxa to these biofilters were the classes *Holophagae* (with the genus *Geothrix*, an electroactive bacteria of the phylum *Acidobacteria*), and *Brocadiae* (phylum *Planctomycetes*). The class *Brocadiae*, involved in annamox processes, only appeared in the anode of the polarized biofilter (Table S 3-6). *Alpha*, *Beta* and *Gammaproteobacteria* had the higher component weight in the inlet wastewater and the gravel biofilter.

Presence of Deltaproteobacteria as indicator of microbial electroactivity

Our analysis of microbial communities revealed the presence of similar taxonomic groups with the exception of some remarkable ones. An interesting finding was the high presence of *Deltaproteobacteria* (Figure 3-5:) when the electroconductive material was the substrate (27.2 % in the coke biofilter and 23.4% in the hybrid polarized biofilter) in comparison with the gravel biofilter (8.1 %). Bacteria belonging to this group have been reported associated to electroactive biofilm from the very beginning (Bicciato *et al.*, 2003) as they share the capacity for generating ATP from very low thermodynamic value reactions (Lovley, 2013; McInerney *et al.*, 2007). In the anaerobic treatment of wastewater, *Deltaproteobacteria* assures the removal of fatty acids of low energetic value as acetate which is typically the metabolic bottleneck of these systems (Zhao *et al.*, 2014). In addition, *Deltaproteobacteria* can compete with methanogenic

microorganisms and their preponderance may reduce methane emissions. However, we cannot confirm any outcompeting effect on methanogenic populations because, apparently, some of the taxa might have not been amplified with the primer sets 515F/806R utilised for the sequencing (Parada *et al.*, 2016). In fact, only 0.1 % of OTUs correspond to the Kingdom *Archaea*, which contains the main methanogenic groups. It must also be noted that community members with multiple 16S copies may be over-represented. Nevertheless, our main purpose was to estimate those genera associated with degradation processes and electroactive bacteria, groups that were adequately represented.

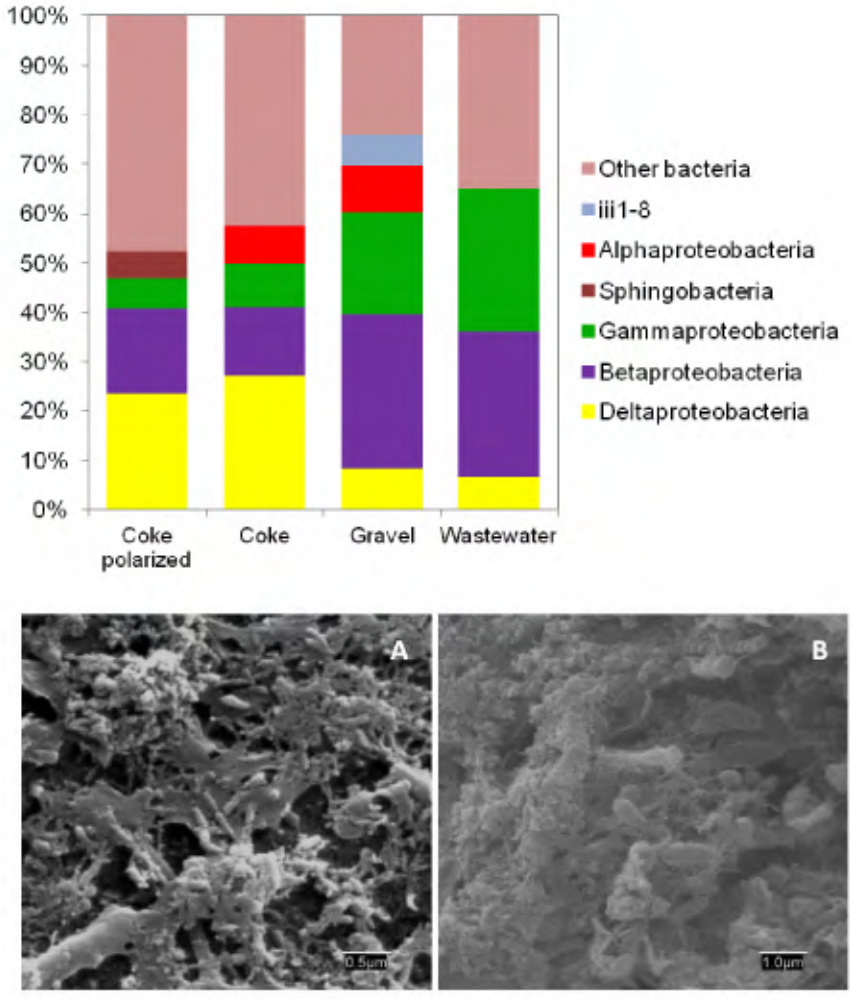


Figure 3-5: Relative abundances of OTUs at class level (larger than 5% in average). SEM images of coke granules of A) polarized hybrid coke filter. B) coke filter. A dense matrix covering the conductive material can be observed.

Some *Deltaproteobacteria*, like bacteria from the genus *Geobacter,* are able to transfer electrons to conductive materials (Bond *et al.*, 2002). Indeed, the largest presence of *Geobacter* (2.9%) was found in the coke biofilter (Table S 3-6). Surprisingly, although at lower levels, it was also found in the inlet wastewater (0.45 %) and in the gravel biofilter (0.3%). Some studies have previously reported the presence of *Geobacter* species in anaerobic digesters suggesting a role in performing direct interspecies electron transfer (DIET) (Morita *et al.*, 2011; Rotaru *et al.*, 2014b; Shrestha *et al.*, 2014; Zhao *et al.*, 2015) with a direct impact on methane production. Interestingly, inlet wastewater for our assays was generated in an Imhoff tank, which host environmental conditions similar to those found in an anaerobic digester. It seems reasonable to expect the presence of *Geobacter* associated with other biofilm species in our gravel biofilter.

In the *Deltaproteobacteria*, it is remarkable the dominance of some genera of the family *Desulfobulbaceae* (Table S 3-6) in both the anode of the hybrid polarized biofilter (20.8%) and also in the coke biofilter (16.8%), in contrast with low presence in the gravel biofilter (1.6%). Moreover, other studies also reported *Desulfobulbus* species colonizing anodes (Ahn *et al.*, 2014; De Schamphelaire *et al.*, 2010; Sun *et al.*, 2010; Wang *et al.*, 2014) and, for instance, *D. propionicus* was previously reported to use the electrode surface as an electron acceptor when pyruvate, lactate, propionate or hydrogen was provided as electron donor (Holmes *et al.*, 2004). The presence of *Desulfobulbus* is especially relevant due to its fascinating capacity for generating electrically conductive-microbial filaments (Larsen *et al.*, 2015; Pfeffer *et al.*, 2012). These microbial filaments transport electrons from the bottom of sediment, rich in hydrogen sulphide, up to the oxygen-rich sediment that is in contact with the water. Interestingly, this situation is similar to the one found in our METlands where a redox gradient is generated among bottom and upper layers of the electroconductive bed. So, our results have revealed that specific microbial consortia previously related to electrical current production were selected by our electroconductive biofilters from our inlet wastewater.

On top of that, other electroactive microorganism like *Geothrix*, an *Acidobacteria* (Bond and Lovley, 2005), were also found in all the systems (Table S 3-6), with a significant presence in the anode of the hybrid polarized biofilter (3.2 %) and in the coke biofilter (2.2 %). However, *Geothrix* was almost absent in the inlet wastewater and scarce in the gravel biofilter (0.2%).

Nitrogen cycle bacteria: nitrification and denitrification

Nitrogen removal is typically poorly achieved under anaerobic conditions, showing a bottleneck in the ammonium oxidizing process. Apparently this is not the case when electroconductive material is supporting the biofilm growth (Figure S 3-1) since this material outperforms gravel to remove nitrogen by 2-fold (Table S 3-4).

A deep analysis into the microbial communities' distribution may helps us to understand what different nitrogen metabolisms are active in our systems. The detection of ammonium oxidizers, like *Nitrosomonadaceae*, associated to the electroconductive material is remarkable if we consider that this family was absent in both the gravel and the inlet wastewater. Even more interesting was the presence of bacteria from the *Brocadiaceae* family (1.7%) in the anode of the polarized biofilter. This family of bacteria include several genera involved in the anaerobic oxidation of ammonia to dinitrogen via ANNAMOX (Kartal *et al.*, 2012).

Another nitrogen pathway that could be enhanced by the presence of the electroconductive material is based on DIET (Rotaru *et al.*, 2014a). Focusing on nitrogen removal, it has been reported that *Geobacter* bacteria can transfer electrons directly to *Thiobacillus* which in turn may reduce nitrate (Kato *et al.*, 2012). It is interesting that both microbial genera are colonizing our electroconductive biofilters although further research is required to find out if these redox syntrophic relationships are the ones after nitrogen removal in our systems.

3.5 Conclusions

Problems with WWT in small communities are different that in large cities owing to the scarcity of economical and technical resources. It is necessary to find solutions that generate minimum energy cost, simple maintenance and functional robustness. With this aim, the successful integration of microbial electrochemical technologies into well tested treatments, such as CWs, represents a substantial advance since the new system can be operated a surface inlet load 4-fold higher than the standard systems. Indeed, our lab scale METland design for treating urban wastewater was able to fulfil the Directive 91/271/EEC and produced water with BOD_5 levels as low as 6 mg L^{-1}. Our research suggests that surface area requirements of classical CWs can be significantly reduced.

3.6 Supplementary Information

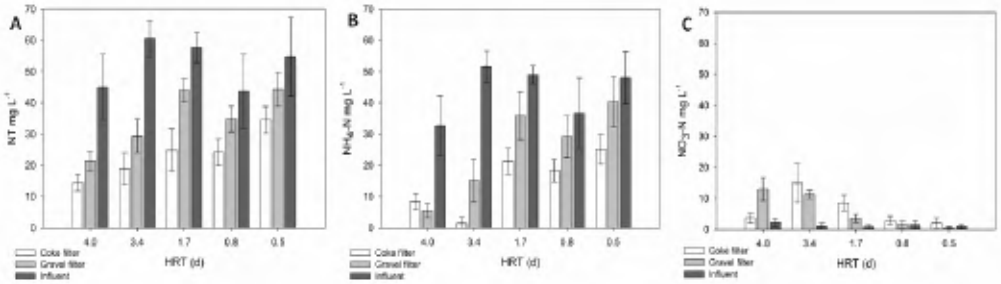


Figure S 3-1: Total nitrogen (A), ammonia (B) and nitrate (C) influent and effluent average values of the coke and the gravel biofilters. Error bars represent 95% confidence interval.

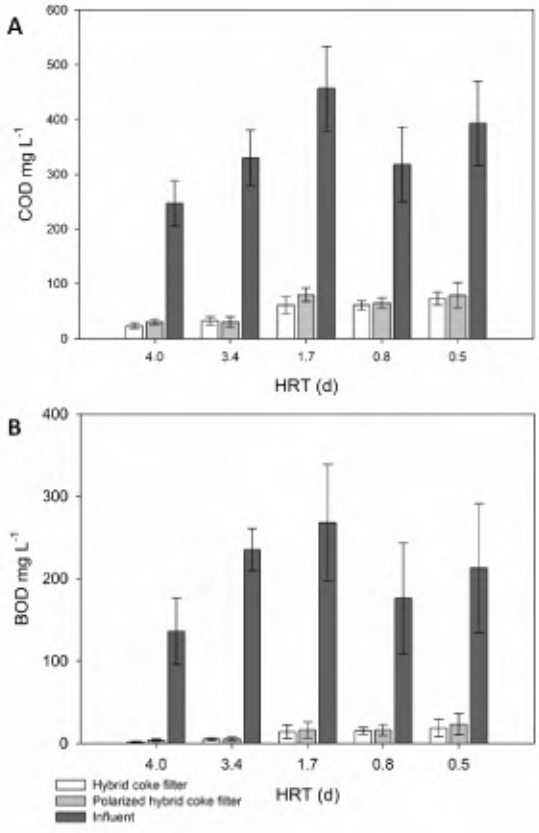


Figure S 3-2: Influent and effluent COD (A) and BOD_5 (B) average values of the hybrid biofilter and the hybrid polarized biofilter. Error bars represent 95% confidence interval.

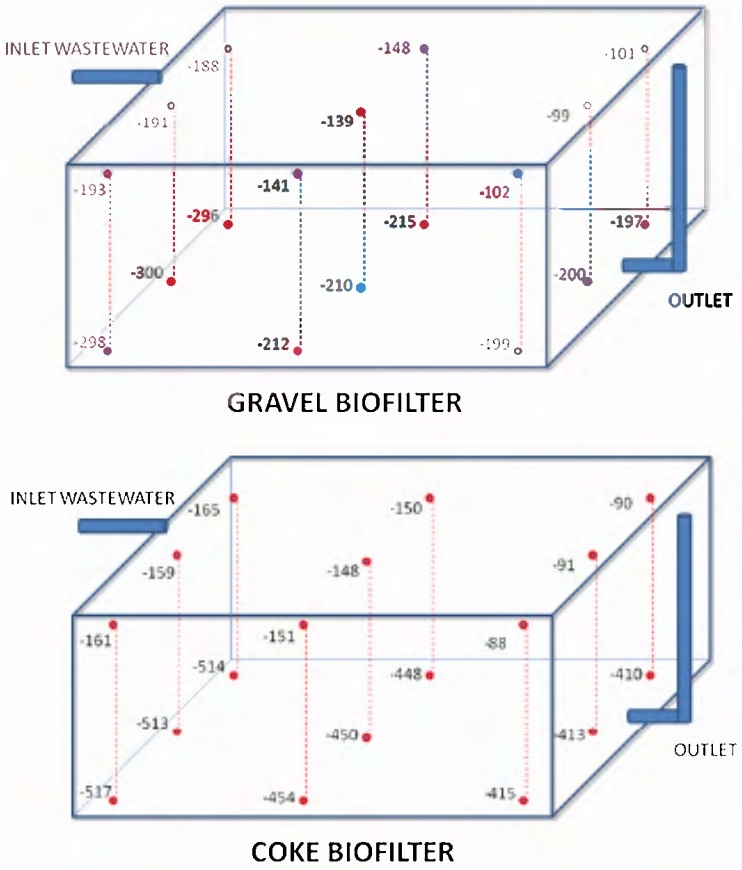


Figure S 3-3: Redox potential measurements in the coke and the gravel biofilters

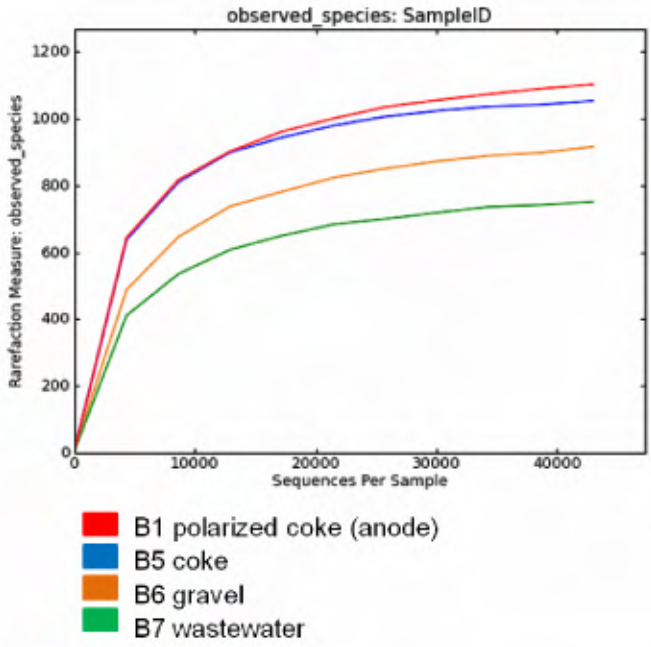


Figure S 3-4: Rarefaction curves calculated for each sample based on the OTU computations.

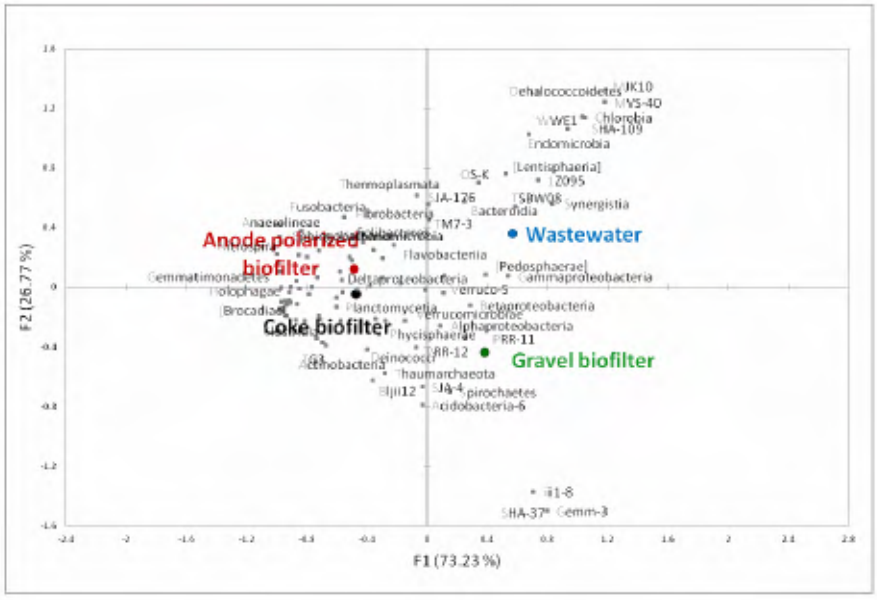


Figure S 3-5: Correspondence analysis biplot of classes' distribution from 16S rDNA analysis.

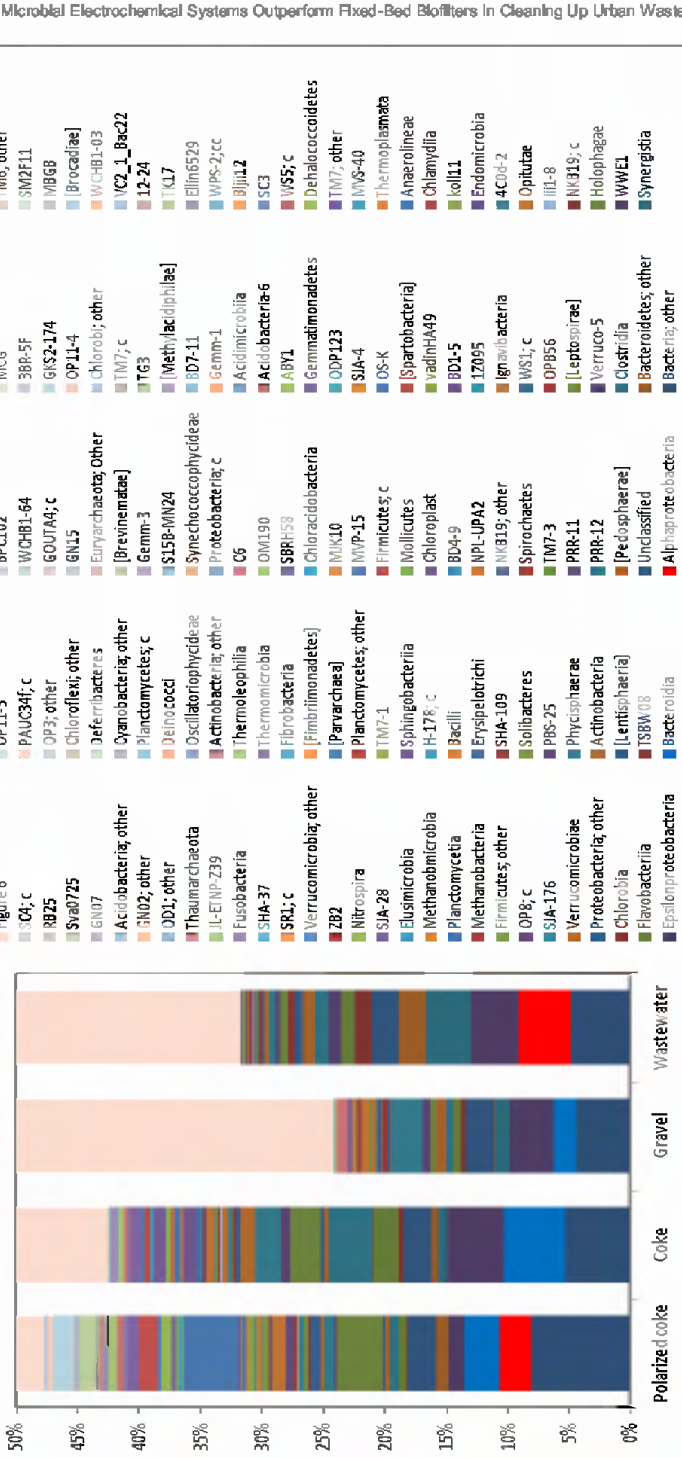


Figure S 3-6: Relative abundance of classes of the category 'other' at class level.

Table S 3-1: Influent pre-treated wastewater and effluents characteristics. Averages ± SD

HRT (d)	Parameter	4.0	3.4	1.7	0.8	0.5
Influent	COD (mg L^{-1})	246 ± 114	330 ± 107	457 ± 92	318 ± 143	393 ± 101
	BOD_5 (mg L^{-1})	136 ± 86	235 ± 36	268 ± 81	176 ± 127	213 ± 112
	TN (mg L^{-1})	45.0 ± 17.4	60.6 ± 7.5	57.7 ± 3.9	43.7 ± 16.5	54.8 ± 10.1
	NH_4-N (mg L^{-1})	32.7 ± 18.7	51.6 ± 6.5	49.0 ± 2.3	36.6 ± 15.9	47.0 ± 8.8
	NO_3-N (mg L^{-1})	2.3 ± 3.6	1.0 ± 1.6	0.8 ± 0.6	1.5 ± 2.0	0.9 ± 0.6
	TP (mg L^{-1})	6.1 ± 2.3	7.5 ± 1.1	7.3 ± 0.8	7.0 ± 1.3	8.6 ± 2.2
	TSS (mg L^{-1})	62 ± 36	102 ± 29	141 ± 32	207 ± 156	239 ± 168
	EC (µS cm^{-1})	1519 ± 209	1133 ± 66	1177 ± 31	1312 ± 93	1207 ± 130
	ORP (mV)	-220 ± 19	-180 ± 17	-178 ± 17	-78 ± 190	-101 ± 98
	pH	7.7 ± 0.2	7.5 ± 0.3	7.3 ± 0.3	7.5 ± 0.4	7.2 ± 0.2
	T (ºC)	19.5 ± 5.0	23.3 ± 2.8	20.1 ± 4.0	19.9 ± 2.3	21.8 ± 1.1
	DO (mg L^{-1})	0.6 ± 0.4	0.7 ± 0.3	0.6 ± 0.1	1.3 ± 0.6	1.3 ± 0.4
Coke Biofilter	TSS (mg L^{-1})	41 ± 21	2 ± 3	2 ± 2	15 ± 5	14 ± 9
	EC (µS cm^{-1})	1641 ± 283	1026 ± 56	1011 ± 64	1226 ± 107	1120 ± 69
	ORP (mV)	-44 ± 13	-72 ± 18	-111 ± 19	-98 ± 26	-61 ± 19
	pH	7.5 ± 0.3	7.7 ± 0.2	7.3 ± 0.1	7.5 ± 0.3	7.5 ± 0.1
	T (ºC)	18.5 ± 4.9	22.3 ± 2.8	19.8 ± 4.0	18.5 ± 2.2	20.9 ± 0.9
Gravel biofilter	TSS (mg L^{-1})	4 ± 3	2 ± 1	11 ± 7	13 ± 5	16 ± 6
	EC (µS cm^{-1})	1448 ± 235	971 ± 49	1102 ± 75	1288 ± 103	1179 ± 95
	ORP (mV)	-31 ± 11	-65 ± 15	-88 ± 13	-80 ± 20	-54 ± 18
	pH	8.0 ±0.2	7.7 ± 0.1	7.6 ± 0.1	7.7 ± 0.3	7.6 ± 0.2
	T (ºC)	18.5 ± 4.9	22.4 ±2.8	19.8 ± 4.0	18.5 ± 2.2	20.8 ± 0.8

Table S 3-2: Organic matter removal rates (COD and BOD_5) and percentage removal efficiencies (in brackets). Averages ± SD (COD: n=30; BOD_5: n=15)

HRT (d)		4.0	3.4	1.7	0.8	0.5
Surface Inlet load	COD	3.6 ± 1.7	6.0 ± 1.9	15.8 ± 3.2	25.1 ± 11.3	44.4 ± 11.4
	BOD_5	2.0 ± 1.0	4.2 ± 0.7	9.2 ± 2.8	13.8 ± 9.5	24.0 ± 12.7
Volume Inlet load	COD	19.0 ± 9.1	31.0 ± 10.1	82.0 ± 16.5	130.9 ± 59.0	231.2 ± 59.2
	BOD_5	10.4 ± 6.6	22.1 ± 3.4	48.1 ± 14.5	71.9 ± 49.4	125.0 ± 65.9
Coke biofilter	COD	17.6 ± 8.7 (91±8)	28.7 ± 10.1 (93±5)	76.6 ± 17.9 (93±6)	117.2 ± 58.8 (90±8)	213.5 ± 71.2 (90±8)
	BOD_5	10.1 ± 6.5 (97±3)	21.9 ± 3.4 (99±1)	47.0 ± 14.5 (98±2)	69.8 ± 52.6 (96±3)	119.4 ± 65.0 (95±2)
Gravel biofilter	COD	15.4 ± 8.7 (80±9)	25.8 ± 9.6 (83±7)	64.9 ± 19.3 (78±8)	95.6 ± 59.4 (73±10)	168.9 ± 67.5 (73±9)
	BOD_5	10.0 ± 6.6 (92±5)	21.0 ± 3.2 (95±2)	43.2 ± 14.4 (90±6)	62.0 ± 54.4 (78±10)	107.8 ± 65.0 (82±4)
Hybrid biofilter	COD	16.4 ± 8.7 (88±8)	27.9 ± 10.2 (89±8)	71.0 ± 18.5 (87±6)	107.6 ± 58.0 (80±8)	186.5 ± 57.4 (81±6)
	BOD_5	10.2 ± 6.1 (98±2)	21.7 ± 3.3 (98±1)	45.6 ± 14.9 (94±4)	66.5 ± 51.5 (88±7)	114.3 ± 66.1 (90±9)
Hybrid polarized biofilter	COD	16.7 ± 8.6 (86±8)	27.8 ± 10.5 (89±8)	67.7 ± 15.7 (83±4)	108.2 ± 55.8 (80±8)	182.8 ± 69.3 (79±9)
	BOD_5	10.1 ± 6.5 (97±3)	21.6 ± 3.2 (98±2)	45.2 ± 14.5 (94±4)	66.0 ± 51.2 (88±7)	111.5 ± 68.0 (88±8)

Surface Inlet loads are given in g $m^{-2}d^{-1}$. Rest of the values are given in g $m^{-3}d^{-1}$.

Table S 3-3: Statistical test and p-values of the coke and gravel biofilters effluents comparison (*significant differences). W = Wilcoxon test; t = T-test

HRT (d)		4.0	3.4	1.7	0.8	0.5
COD	Test	W=115.0	t=4.7358	t=4.9700	t=5.9989	t=6.0708
	P-value	3.39 E-7*	3.36 E-5*	2.056 E-4*	5.71 E-7*	2.141 E-5*
BOD_5	Test	t=3.3510	W=14.0	W=0.0	W=3.5	t=6.1501
	P-value	1.830 E-3*	6.683 E-3*	12.186 E-3*	1.182 E-3*	8.469 E-4*
NT	Test	t=3.7542	t=2.2767	t=3.2857	t=2.7879	t=3.5482
	P-value	9.782 E-4*	3.903 E-2*	1.109 E-2*	1.215 E-2*	7.529 E-2*
NH4-N	Test	t=-1.8113	W=1.0	t=3.3645	t=2.9074	W=0
	P-value	7.948 E-2	1.337 E-3*	1.201 E-2*	9.395 E-3*	1.219 E-2*
NO3-N	Test	W=20.0	W=40.0	t=-2.7704	T=-1.6426	W=20.0
	P-value	0.14367	0.43022	0.02428*	0.11782	0.14367

Table S 3-4: Overall averages ± SD (n=15) of total nitrogen and ammonia removal rates and average removal efficiencies (in brackets)

HRT (d)		4.0	3.4	1.7	0.8	0.5
Surface inlet load	TN	0.7 ± 0.3	1.1 ± 0.1	2.0 ± 0.1	3.5 ± 1.3	6.2 ± 1.1
	NH4-N	0.5 ± 0.3	0.9 ± 0.1	1.7 ± 0.1	2.9 ± 1.3	5.4 ± 0.8
Volume inlet load	TN	3.4 ± 1.3	5.7 ± 0.7	10.4 ± 0.7	18.0 ± 6.8	32.2 ± 6.0
	NH_4-N	2.5 ± 1.4	4.8 ± 0.6	8.8 ± 0.4	15.1 ± 6.5	28.2 ± 4.0
Coke biofilter	TN	2.3 ± 1.4 (68 ± 12)	4.0 ± 0.9 (69 ± 12)	4.1 ± 1.4 (40 ± 11)	8.0 ± 4.7 (45 ± 10)	11.9 ± 8.6 (37 ±12)
	NH_4-N	1.9 ± 1.5 (74 ± 21)	4.8 ± 0.7 (97 ± 5)	6.6 ± 1.4 (57 ±10)	7.6 ± 4.6 (49 ±10)	11.2 ± 6.8 (39 ±11)
Gravel biofilter	TN	1.8 ± 1.3 (52 ± 14)	3.0 ± 0.8 (51 ± 13)	2.5 ± 0.7 (24 ± 6)	3.7 ± 3.4 (20 ± 9)	6.6 ± 6.0 (19 ± 9)
	NH_4-N	2.1 ± 1.5 (83 ± 22)	3.5 ± 0.7 (71 ± 15)	2.3 ± 0.9 (27 ± 11)	3.0 ± 3.1 (20 ± 12)	4.6 ± 3.9 (16 ± 13)
Hybrid biofilter	TN	1.8 ± 1.3 (59 ± 14)	3.4 ± 0.9 (56 ± 13)	2.2 ± 0.8 (24 ± 14)	7.7 ± 5.6 (24 ±14)	8.4 ± 5.6 (23 ±12)
	NH_4-N	2.0 ± 1.5 (78 ± 25)	4.2 ± 0.8 (87 ± 14)	2.6 ± 0.6 (30 ± 8)	5.3 ± 4.4 (28 ± 13)	7.0 ± 4.1 (24 ± 12)
Hybrid polarized biofilter	TN	2.0 ± 1.4 (59 ± 14)	3.2 ± 0.7 (56 ± 13)	2.5 ± 1.6 (24 ± 14)	5.6 ± 5.6 (23 ± 13)	6.9 ± 5.3 (22 ±12)
	NH_4-N	2.2 ± 1.5 (81 ± 21)	4.2 ± 0.9 (87 ± 14)	2.3 ± 1.1 (27 ± 13)	4.9 ± 4.4 (25 ± 12)	5.7 ± 3.1 (20 ± 9)

Surface inlet loads are given in g m^{-2} d^{-1}. Rest of the values are given in g m^{-3} d^{-1}.

Table S 3-5: Alpha diversity metrics of the bacterial populations

Sample ID	Description	Total reads	Observed OTUs	Chao 1	Shannon	Good's Coverage
B1	Coke granules anode	167,678	1,219	1,263	7.13	0.99
B5	Coke granules single electrode	178,772	1,121	1,151	7.38	0.99
B6	Gravel	173,665	1,006	1,042	6.89	0.99
B7	Inlet wastewater	169,876	832	914	6.27	0.99

Table S 3-6: Main taxa of bacteria identified in the analysed communities (over 0.1), in percentage.

Taxon	Coke polarized	Coke	Gravel	Waste water
Bacteria;Other;Other;Other;Other;Other	8.04	5.35	4.35	4.91
Acidobacteria;Acidobacteria-6;iii1-15;;	0.43	0.21	0.94	0.00
Acidobacteria;Holophagae;Holophagales;Holophagaceae;	0.09	0.12	0.17	0.49
Acidobacteria;Holophagae;Holophagales;Holophagaceae;Geothrix	3.21	2.24	0.19	0.00
Acidobacteria;Holophagae;Holophagales;Other;Other	0.54	0.16	0.06	0.04
Acidobacteria;iii1-8;DS-18	0.02	0.00	0.35	0.00
Acidobacteria;iii1-8;SJA-36	0.10	0.17	5.67	0.09
Acidobacteria;iii1-8;Other;Other;Other	0.04	0.02	0.13	0.01
Acidobacteria;Solibacteres;Solibacterales;Solibacteraceae;CandidatusSolibacter	0.50	0.10	0.13	0.04
Actinobacteria;Acidimicrobiia;Acidimicrobiales;C111	0.10	0.02	0.01	0.00
Actinobacteria;Actinobacteria;Actinomycetales;Dietziaceae;Dietzia	0.00	0.23	0.00	0.00
Actinobacteria;Actinobacteria;Actinomycetales;Geodermatophilaceae	0.00	0.12	0.04	0.00
Actinobacteria;Actinobacteria;Actinomycetales;Microbacteriaceae;Other	0.00	0.22	0.02	0.06
Actinobacteria;Actinobacteria;Actinomycetales;Other;Other	0.03	0.46	0.01	0.20
Actinobacteria;Actinobacteria;Bifidobacteriales;Bifidobacteriaceae;Bifidobacterium	0.01	0.11	0.05	0.01
Bacteroidetes;Bacteroidia;Bacteroidales	2.47	3.33	1.20	6.73
Bacteroidetes;Bacteroidia;Bacteroidales;BA008	0.22	0.61	0.11	0.21
Bacteroidetes;Bacteroidia;Bacteroidales;Bacteroidaceae;Bacteroides	0.02	0.11	0.04	0.55
Bacteroidetes;Bacteroidia;Bacteroidales;GZB119	0.07	0.61	0.37	0.01
Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;Parabacteroides	0.01	0.20	0.06	1.30
Bacteroidetes;Flavobacteriia;Flavobacteriales;Cryomorphaceae	0.15	0.34	0.01	0.00
Bacteroidetes;Flavobacteriia;Flavobacteriales;Flavobacteriaceae;Flavobacterium	0.16	1.33	0.38	0.97
Bacteroidetes;Flavobacteriia;Flavobacteriales;Flavobacteriaceae;Other	0.02	0.31	0.21	0.13
Bacteroidetes;Flavobacteriia;Flavobacteriales;Other;Other	0.25	0.08	0.01	0.00
Bacteroidetes;Sphingobacteriia;Sphingobacteriales;;	4.79	0.24	0.01	0.00
Bacteroidetes;Sphingobacteriia;Sphingobacteriales;Chitinophagaceae;	0.15	0.15	0.01	0.00
Bacteroidetes;Sphingobacteriia;Sphingobacteriales;Cyclobacteriaceae;	0.02	0.42	0.00	0.00
Bacteroidetes;Sphingobacteriia;Sphingobacteriales;Flammeovirgaceae;A4	0.13	0.01	0.00	0.00
Bacteroidetes;Sphingobacteriia;Sphingobacteriales;Other;Other	0.23	0.09	0.12	0.00
Bacteroidetes;Other;Other;Other;Other	0.96	0.56	0.07	2.30
BRC1;PRR-11	0.03	0.18	0.28	0.11
Chlorobi;Chlorobia;Chlorobiales;Chlorobiaceae;	0.02	0.02	0.01	0.58
Chlorobi;Ignavibacteria;Ignavibacteriales;Ignavibacteriaceae	0.41	0.06	0.02	0.03
Chlorobi;OPB56	0.20	0.27	0.06	0.09
Chlorobi;SJA-28	0.34	0.00	0.00	0.00
Chloroflexi;Anaerolineae;Anaerolineales;Anaerolinaceae;	0.18	0.01	0.00	0.00
Chloroflexi;Anaerolineae;Anaerolineales;Anaerolinaceae;WCHB1-05	0.17	0.03	0.00	0.00
Chloroflexi;Anaerolineae;Caldilineales;Caldilineaceae	0.15	0.02	0.00	0.00
Chloroflexi;Anaerolineae;envOPS12	2.85	0.00	0.00	0.00
Chloroflexi;Anaerolineae;Other;Other;Other	0.78	0.00	0.00	0.00
Chloroflexi;TK17;mle1-48	1.26	0.00	0.00	0.00
Cyanobacteria;4C0d-2;MLE1-12	0.11	0.01	0.01	0.01
Cyanobacteria;4C0d-2;YS2	0.03	0.17	0.00	0.03
Cyanobacteria;Chloroplast;Stramenopiles	0.12	0.01	0.00	0.00
Cyanobacteria;Chloroplast;Streptophyta	0.12	0.00	0.00	0.01
Elusimicrobia;Elusimicrobia;Elusimicrobiales;Elusimicrobiaceae;Elusimicrobium	0.36	0.15	0.02	0.00
Firmicutes	0.02	0.59	0.01	0.03
Firmicutes;Clostridia;Clostridiales;Catabacteriaceae	0.07	0.61	0.01	0.15
Firmicutes;Clostridia;Clostridiales;Christensenellaceae	0.04	0.23	0.00	0.01
Firmicutes;Clostridia;Clostridiales;Clostridiaceae	0.05	0.29	0.04	0.09
Firmicutes;Clostridia;Clostridiales;Clostridiaceae;Clostridium	0.04	0.18	0.00	0.01
Firmicutes;Clostridia;Clostridiales;Clostridiaceae;Fusibacter	0.00	0.38	0.02	0.01
Firmicutes;Clostridia;Clostridiales;Peptococcaceae;	0.22	0.37	0.00	0.00
Firmicutes;Clostridia;Clostridiales;Peptostreptococcaceae;	0.02	0.14	0.10	0.08
Firmicutes;Clostridia;Clostridiales;Veillonellaceae;	0.00	0.31	0.26	0.36
Firmicutes;Clostridia;Clostridiales;Other;Other	0.05	0.15	0.01	0.11
Firmicutes;Clostridia;Coriobacteriales;Coriobacteriaceae;	0.10	0.12	0.01	0.00
Firmicutes;Clostridia;Other;Other;Other	0.03	0.11	0.02	0.02
Firmicutes;Erysipelotrichi;Erysipelotrichales;Erysipelotrichaceae;PSB-M-3	0.02	0.23	0.00	0.01
Fusobacteria;Fusobacteria	0.06	0.49	0.00	0.00

Taxon	Coke polarized	Coke	Gravel	Waste water
Gemmatimonadetes;Gemmatimonadetes	0.38	0.16	0.01	0.00
Gemmatimonadetes;Gemmatimonadetes;Gemmatimonadales;Gemmatimonadaceae;Gemmatimonas	0.00	0.51	0.00	0.00
Gemmatimonadetes;Gemmatimonadetes;KD8-87	0.45	0.04	0.00	0.00
GN02;BD1-5	0.02	1.02	0.00	0.02
Lentisphaerae;[Lentisphaeria];Victivallales;Victivallaceae;	0.01	0.08	0.03	0.62
Nitrospirae;Nitrospira;Nitrospirales;Thermodesulfovibrionaceae;GOUTA19	0.80	0.00	0.00	0.00
NKB19	0.10	0.08	0.30	0.18
NKB19;TSBW08	0.19	0.38	0.38	1.38
OD1;ZB2	1.68	0.01	0.00	0.00
OP11;OP11-4	0.36	0.00	0.00	0.00
OP3;koll11	0.44	0.00	0.00	0.00
OP3;PBS-25	0.56	0.03	0.00	0.06
Planctomycetes;[Brocadiae];Brocadiales;Brocadiaceae	1.71	0.00	0.00	0.00
Planctomycetes;C6;MVS-107	0.40	0.00	0.01	0.00
Planctomycetes;OM190;agg27	0.12	0.01	0.02	0.00
Planctomycetes;OM190;CL500-15	0.69	0.54	0.05	0.00
Planctomycete;Phycisphaerae;Phycisphaerales;Phycisphaeraceae;Phycisphaera	0.38	0.24	0.27	0.11
Planctomycetes;Planctomycetia;Pirellulales;Pirellulaceae	0.25	0.46	0.16	0.00
Proteobacteria;Alphaproteobacteria;BD7-3	0.50	0.87	0.52	0.02
Proteobacteria;Alphaproteobacteria;Caulobacterales;Caulobacteraceae;Brevundimonas	0.05	0.48	0.18	0.03
Proteobacteria;Alphaproteobacteria;Caulobacterales;Caulobacteraceae;Phenylobacterium	0.04	0.20	0.45	0.00
Proteobacteria;Alphaproteobacteria;Caulobacterales;Caulobacteraceae;Other	0.10	0.38	0.27	0.06
Proteobacteria;Alphaproteobacteria;Rhizobiales;Beijerinckiaceae;Other	0.00	0.01	0.00	0.17
Proteobacteria;Alphaproteobacteria;Rhizobiales;Bradyrhizobiaceae;Other	0.05	0.03	0.46	0.01
Proteobacteria;Alphaproteobacteria;Rhizobiales;Hyphomicrobiaceae;Devosia	0.02	0.19	0.03	0.00
Proteobacteria;Alphaproteobacteri;Rhizobiales;Hyphomicrobiaceae;Rhodoplanes	0.23	0.15	0.27	0.00
Proteobacteria;Alphaproteobacteria;Rhizobiales;Phyllobacteriaceae;Other	0.01	0.12	0.01	0.00
Proteobacteria;Alphaproteobacteria;Rhizobiales;Rhizobiaceae;	0.00	0.10	0.07	0.00
Proteobacteria;Alphaproteobacteria;Rhizobiales;Rhizobiaceae;Other	0.06	0.46	1.10	0.05
Proteobacteria;Alphaproteobacteria;Rhizobiales;Other;Other	0.16	0.96	0.72	0.03
Proteobacteria;Alphaproteobacteria;Rhodobacterales;Hyphomonadaceae;Hyphomonas	0.01	0.15	0.01	0.00
Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Rhodobacter	0.02	1.11	1.06	0.03
Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Other	0.13	0.90	2.16	0.03
Proteobacteria;Alphaproteobacteria;Rhodospirillales	0.17	0.03	0.79	0.00
Proteobacteria;Alphaproteobacteria;Rhodospirillales;Acetobacteraceae;Roseomonas	0.01	0.11	0.18	0.25
Proteobacteria;Alphaproteobacteria;Rhodospirillales;Rhodospirillaceae;	0.27	0.07	0.25	0.01
Proteobacteria;Alphaproteobacteria;Rhodospirillales;Rhodospirillaceae;Oleomonas	0.04	0.00	0.00	2.91
Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadaceae;Novosphingobium	0.03	0.18	0.14	0.04
Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadaceae;Sphingopyxis	0.02	0.22	0.09	0.00
Proteobacteria;Alphaproteobacteria;Other;Other;Other	0.22	0.17	0.14	0.18
Proteobacteria;Betaproteobacteria	0.17	0.00	0.00	0.00
Proteobacteria;Betaproteobacteria;Burkholderiales;Alcaligenaceae;Other	0.00	0.09	0.02	0.82
Proteobacteria;Betaproteobacteria;Burkholderiales;Burkholderiaceae;	0.31	0.31	0.77	0.00
Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Aquabacterium	0.02	0.13	0.21	0.02
Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Hydrogenophaga	0.43	0.65	0.35	5.47
Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Other	1.82	2.37	2.05	11.20
Proteobacteria;Betaproteobacteria;Burkholderiales;Oxalobacteraceae;Janthinobacterium	5.58	0.00	0.00	0.00
Proteobacteria;Betaproteobacteria;Burkholderiales;Oxalobacteraceae;Other	0.20	0.02	0.01	0.00
Proteobacteria;Betaproteobacteria;Ellin6067	0.48	0.01	0.16	0.00
Proteobacteria;Betaproteobacteria;Hydrogenophilales;Hydrogenophilaceae;	0.01	0.02	0.45	0.00
Proteobacteria;Betaproteobacteria;Hydrogenophilales;Hydrogenophilaceae;Thiobacillus	0.72	1.28	12.98	0.01

Taxon	Coke polarized	Coke	Gravel	Waste water
Proteobacteria;Betaproteobacter;Methylophilales;Methylophilaceae;Methylotenera	0.02	0.10	0.01	0.56
Proteobacteria;Betaproteobacteria;Methylophilales;Other;Other	0.01	1.03	0.01	0.00
Proteobacteria;Betaproteobacteria;Nitrosomonadales;Nitrosomonadaceae;	0.17	0.17	0.01	0.00
Proteobacteria;Betaproteobacteria;Procabacteriales;Procabacteriaceae;	0.00	0.02	0.00	0.19
Proteobacteria;Betaproteobacter;Rhodocyclales;Rhodocyclaceae;Dechloromonas	0.02	0.27	0.33	0.65
Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Denitratisoma	0.22	0.23	0.01	0.00
Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Dok59	1.63	0.94	2.05	0.00
Proteobacteria;Betaproteobacte;Rhodocyclales;Rhodocyclaceae;Methyloversatilis	0.11	0.24	0.51	0.07
Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Propionivibrio	0.01	0.10	0.14	0.24
Proteobacteria;Betaproteobacte;Rhodocyclales;Rhodocyclaceae;Sterolibacterium	0.39	0.00	0.00	0.00
Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Thauera	0.01	1.41	1.20	0.19
Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Zoogloea	0.00	0.02	0.10	0.17
Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Other	1.94	1.29	1.69	0.07
Proteobacteria;Betaproteobacteria;SC-I-84	0.12	0.01	0.04	0.00
Proteobacteria;Betaproteobacteria;Thiobacterales;;	0.42	0.22	2.58	0.00
Proteobacteria;Betaproteobacteria;Thiobacterales;Other;Other	0.03	0.43	1.02	0.03
Proteobacteria;Betaproteobacteria;Other;Other;Other	2.25	2.20	4.55	6.91
Proteobacteria;Deltaproteobacteria	0.05	0.18	0.00	0.06
Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bacteriovoracaceae;	0.07	0.20	0.02	0.00
Proteobacteria;Deltaproteobact;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio	0.20	0.04	0.02	0.00
Proteobacteria;Deltaproteobacteria;Desulfobacterales;Desulfobulbaceae;	20.72	17.83	2.23	0.01
Proteobacteria;Deltaproteobacteria;Desulfobacterales;Desulfobulbaceae;Desulfobulbus	0.01	0.09	0.24	0.12
Proteobacteria;Deltaproteobacteria;Desulfovibrionales;Desulfomicrobiaceae;Desulfomicrobium	0.03	0.40	0.75	0.41
Proteobacteria;Deltaproteobacteria;Desulfovibrionales;Desulfovibrionaceae;Desulfovibrio	0.01	0.16	0.11	0.51
Proteobacteria;Deltaproteobacteria;Desulfurellales	0.02	0.06	0.02	0.24
Proteobacteria;Deltaproteobact;Desulfuromonadales;Geobacteraceae;Geobacter	0.25	2.88	0.28	0.45
Proteobacteria;Deltaproteobacteria;Desulfuromonadales;Geobacteraceae;Other	0.14	2.72	1.36	3.39
Proteobacteria;Deltaproteobacteria;Desulfuromonadales;Pelobacteraceae;	0.06	0.96	1.30	0.00
Proteobacteria;Deltaproteobacteria;Desulfuromonadales;Other;Other	0.25	0.18	1.10	0.02
Proteobacteria;Deltaproteobacteria;Myxococcales	0.09	0.27	0.02	0.00
Proteobacteria;Deltaproteobacteria;Myxococcales;0319-6G20;	0.11	0.00	0.00	0.00
Proteobacteria;Deltaproteobacteria;Myxococcales;Myxococcaceae;Anaeromyxobacter	0.12	0.00	0.01	0.00
Proteobacteria;Deltaproteobacteria;Myxococcales;Other;Other	0.36	0.00	0.00	0.00
Proteobacteria;Deltaproteobacteria;Syntrophobacterales;Desulfobacteraceae;	0.01	0.45	0.20	0.08
Proteobacteria;Deltaproteobacte;Syntrophobacterales;Desulfobacteraceae;Desulfobacter	0.01	0.19	0.23	0.37
Proteobacteria;Deltaproteobacter;Syntrophobacterales;Desulfobacteraceae;Other	0.09	0.16	0.05	0.05
Proteobacteria;Deltaproteobacteria;Other;Other;Other	0.36	0.13	0.02	0.03
Proteobacteria;Epsilonproteobacte;Campylobacterales;Campylobacteraceae;Arcobacter	0.05	2.90	2.21	3.70
Proteobacteria;Epsilonproteobacter;Campylobacterales;Helicobacteraceae;Sulfurimonas	1.00	1.64	1.38	0.00
Proteobacteria;Gammaproteobacteria;Aeromonadales;Aeromonadaceae;	0.01	0.03	0.05	0.11
Proteobacteria;Gammaproteobacteria;Alteromonadales;[Chromatiaceae];Rheinheimera	0.57	0.01	0.00	0.21
Proteobacteria;Gammaproteobacteria;Alteromonadales;125ds10;	0.16	0.38	1.63	0.00
Proteobacteria;Gammaproteobacteria;Alteromonadales;Alteromonadaceae;Other	0.01	0.17	0.11	0.00
Proteobacteria;Gammaproteobacteria;Chromatiales;;	0.03	1.24	0.01	0.00
Proteobacteria;Gammaproteobacteria;Chromatiales;Halothiobacillaceae;Thiovirga	0.01	0.01	0.02	0.49
Proteobacteria;Gammaproteobacteri;Enterobacteriales;Enterobacteriaceae;Other	0.00	0.01	2.64	0.00
Proteobacteria;Gammaproteobacteria;FCPT525;FCPT525;	0.09	0.01	0.30	0.02
Proteobacteria;Gammaproteobacteria;HTCC2188;;	0.08	0.00	0.15	0.00
Proteobacteria;Gammaproteobacteria;HTCC2188;HTCC2188;HTCC	0.47	0.20	0.00	0.00
Proteobacteria;Gammaproteobacteria;Legionellales;Francisellaceae;Francisella	0.00	0.00	0.00	5.02
Proteobacteria;Gammaproteobacteria;Methylococcales;Methylococcaceae;Methylomonas	0.21	0.01	0.10	0.01
Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Acinetobacter	0.00	0.05	0.06	0.20
Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas	0.04	0.25	0.67	1.39

Taxon	Coke polarized	Coke	Gravel	Waste water
Proteobacteria;Gammaproteobact;Pseudomonadales;Pseudomonadaceae;Other	0.06	0.45	0.59	15.18
Proteobacteria;Gammaproteobacteria;Xanthomonadales;Sinobacteraceae;	0.29	0.04	0.04	0.27
Proteobacteria;Gammaproteobacteria;Xanthomonadales;Sinobacteraceae;Other	0.66	0.00	1.36	0.00
Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;	0.15	0.83	0.13	0.01
Proteobacteria;Gammaproteobacteri;Xanthomonadales;Xanthomonadaceae;Aquimonas	0.01	0.36	0.01	0.19
Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;Arenimonas	0.11	1.36	4.90	0.00
Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;Pseudoxanthomonas	0.05	0.04	0.11	0.01
Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;Stenotrophomonas	0.00	0.01	0.02	0.72
Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;Thermomonas	0.48	1.63	5.74	0.05
Proteobacteria;Gammaproteobacter;Xanthomonadales;Xanthomonadaceae;Other	0.11	0.39	0.55	0.16
Proteobacteria;Gammaproteobacteria;Other;Other;Other	2.09	0.64	0.98	2.11
Proteobacteria;Other;Other;Other;Other	0.90	0.34	0.26	0.29
Spirochaetes;[Leptospirae];[Leptospirales];Leptospiraceae;Leptospira	0.15	0.00	0.00	0.00
Spirochaetes;[Leptospirae];[Leptospirales];Sediment-4;	0.13	0.01	0.01	0.02
Spirochaetes;MVP-15;PL-11B10;;	0.04	0.24	0.02	0.00
Spirochaetes;Spirochaetes;Spirochaetales;Spirochaetaceae;Treponema	0.06	0.09	0.41	0.02
Spirochaetes;WWE1;[Cloacamonales];[Cloacamonaceae];W22	0.04	0.05	0.02	0.97
Synergistetes;Synergistia;Synergistales;Dethiosulfovibrionaceae;HA73	0.01	0.25	0.51	0.35
Synergistetes;Synergistia;Synergistales;Dethiosulfovibrionaceae;PD-UASB-13	0.00	0.06	0.09	0.16
Synergistetes;Synergistia;Synergistales;Synergistaceae;	0.00	0.02	0.01	0.67
Synergistetes;Synergistia;Synergistales;Synergistaceae;vadinCA02	0.03	0.25	0.45	2.20
Tenericutes;Mollicutes	0.11	0.04	0.00	0.00
Tenericutes;Mollicutes;Acholeplasmatales;Acholeplasmataceae;	0.01	0.37	0.00	0.00
Verrucomicrobia;[Methylacidiphilae]	0.11	0.00	0.00	0.00
Verrucomicrobia;[Pedosphaerae];[Pedosphaerales]	0.17	0.01	0.02	0.02
Verrucomicrobia;[Pedosphaerae];[Pedosphaerales];Ellin515;	0.05	0.15	0.43	0.04
Verrucomicrobia;[Pedosphaerae];[Pedosphaerales];R4-41B;	0.01	0.12	0.07	0.90
Verrucomicrobia;[Pedosphaerae];[Pedosphaerales];Other;Other	0.16	0.09	0.16	0.02
Verrucomicrobia;Opitutae;Opitutales;Opitutaceae;	0.44	0.43	0.44	0.01
Verrucomicrobia;Opitutae;Opitutales;Opitutaceae;Opitutus	0.68	0.23	0.16	0.01
Verrucomicrobia;Verruco-5;WCHB1-41;RFP12;	0.05	0.66	0.53	0.41
Verrucomicrobi;Verrucomicrobiae;Verrucomicrobiales;Verrucomicrobiaceae;Luteolibacter	0.00	0.05	0.13	0.09
Verrucomicr;Verrucomicrobiae;Verrucomicrobiales;Verrucomicrobiaceae;Prosthecobacter	0.08	0.35	0.18	0.01
WS3;PRR-12;GN03;;	0.19	1.98	2.11	0.47
WS3;PRR-12;GN03;KSB4;	0.56	0.01	0.31	0.00
WS3;PRR-12;GN03;Other;Other	0.14	0.00	0.00	0.00
WS5	0.04	0.11	0.00	0.00
Unclassified;Other;Other;Other;Other;Other	2.29	2.33	2.35	2.20

CHAPTER 4: Scaling-Up METs: a Four Years Study of a Real HSSF METland

This section has been redrafted after:

Arantxa Aguirre-Sierra[a], Antonio Berná[b] Tristano Bacchetti-De Gregoris[b], Alejandro Reija[b] Juan José Salas[c] and Abraham Esteve-Núñez[a,b]. Scaling-up METs: a four years study of a real horizontal subsurface flow METland

[a.] Department of Chemical Engineering, Universidad de Alcalá, Alcalá de Henares, Madrid, Spain.
[b.] IMDEA Agua, Parque Tecnológico de Alcalá, Alcalá de Henares, Madrid, Spain
[c.] Foundation Centre for New Water Technologies (CENTA), Carrión de los Céspedes, Sevilla, Spain

Scaling-Up METs: a Four Years Study of a Real HSSF METland

4.1 Abstract

In this chapter we show the largest scale application of a microbial electrochemical system described so far. It consisted in an electroconductive bed (2.6 m^3) integrated in a horizontal subsurface flow (HSSF) wetland constructed in the facilities of Foundation New Water Technologies (CENTA) in Carrión de los Céspedes (Sevilla, Spain) to treat urban wastewater. The resulting design was so-called METland, due to the merging of microbial electrochemical technologies (MET) and constructed wetlands (CWs) technology. During a 4 years period the system was tested under different electrochemical scenarios including different configurations for anodes, cathodes and planting of macrophytes. Microbial community analysis through massive sequencing was performed in different wetland environments to elucidate the impact of conductive and non-conductive materials on the microbial community profile.. The results show that and suggest that electroactive bacteria (EAB) are the main responsible for reaching COD removal rates as high as ca. 400 mg COD m^{-3} d^{-1}.

4.2 Introduction

Microbial Electrochemical Technologies (METs) exploit the ability of EAB to use conductive material as electron donor/acceptor (Rabaey, 2009). The classical textbook example of METs are microbial fuel cells, where wastewater treatment (WWT) can be coupled to electricity generation (Logan, 2005). Because the flow of electrons in a circuit follows Ohm's law, METs are generally designed to exploit natural differences in redox potential existing in separate areas or compartments of the system under investigation. With this approach, electrodes will provide a way in and out from the circuit for the electrons deriving from microbial catabolism. Alternatively, a specific electrochemical potential can be applied to one electrode using a potentiostat, which guarantees a better control of the system at the expense of energy consumption (Borjas et al., 2015; Kiely et al., 2011). Furthermore, this second strategy may be preferred because it has been demonstrated that the electrode potential represents a strong selective force for the microbial community that uses such electrode to respire (Larrosa-Guerrero et al., 2010; Read et al., 2010). Thus, polarized conductive material can be used to stimulate growth of

specific microbial groups, whose functional characteristics are only beginning to emerge and could be of great interest in the context of WWT.

A current goal of our society is that of becoming sustainable in order to preserve the environment for future generations. WWT is a key element to reach this target because it mitigates the release of toxic compounds and because it alone consumes around 1.5% of electrical energy used yearly (Water Infrastructure Network, 2001). The most efficient and widely implemented method to treat domestic and industrial wastewater is the activated sludge process, where oxygen is pumped in water tanks to stimulate microbial respiration and ultimately waste degradation. However, both aeration and disposal of residual sludge require high energetic inputs, and alternative technologies are sought.

Constructed wetlands (CWs) for WWT have long attracted the attention because of their low installation and running costs, low production of sewage sludge (just in primary treatment), easy management, and their overall technological simplicity (García et al., 2003b; Verhoeven and Meulemann, 1999). These systems are particularly viable options in places where the population density is low, because their main drawback is the large area required to set the wetland, which is not easily found in urban zones (Ferrer Medina et al., 2012; Ortega et al., 2010). Many types of CWs have been implemented, including vertical and horizontal CWs (Kadlec and Wallace, 2009), but they all exploit the activity of diverse microbial communities for pollutant transformation and removal (García et al., 2010). These communities show markedly different metabolic requirements and they are thought to spatially distribute according to the availability of nutrients and electron acceptors/donors (Ji et al., 2012; Samsó and García, 2013). Heterotrophic, nitrifying and sulphide oxidizing bacteria represent the main functional groups in the CWs zones where dissolved oxygen concentration is higher, while fermenting, sulphate reducing and methanogenic bacteria dominate anaerobic niches. The subsequent activity of these groups allows treatment processes to take place, and CW architecture and the inclusion of elements such as plants, air pipes or physical barriers are used to favour growth of specific populations. In this sense, vertical flow CWs (VF) favour aerobic metabolism, while horizontal subsurface flow CWs (HSSF) mainly develop anaerobic processes. However, low oxygen concentrations make these systems less efficient than the activated sludge process, and improved CW designs are required to increase the WWT rate and to make them practical in a larger number of scenarios. With this aim, the stimulation of bioelectrochemical processes in CWs seems to be an interesting way forward (Liu et al., 2013; Zhao et al., 2013).

Studies on the improvement of CWs by incorporating METs, so called METland (Aguirre-Sierra et al., 2016; Esteve-Núñez et al., 2013), originated from earlier investigation on plant microbial fuel cells, which aimed at transforming root exudates into electricity by the action of EAB (De Schamphelaire et al., 2008; Domínguez-Garay et al., 2013; Strik et al., 2008). In that context, planted system as constructed wetlands seemed the suitable scenario for hosting such new technology. From the very beginning the electroconductive material from METlands was conceived as an inexhaustible form of electron acceptor, that could be recruited to enhance microbial catabolism in reducing environments rather than to produce electricity as the main goal (Yadav et al., 2012; Zhao et al., 2013). Under this view, lab-scale METlands have shown to improve the sanitation rate of standard CWs for WWT by promoting the activity of EAB (Aguirre-Sierra et al., 2016), which are otherwise a minor group in natural wetlands (Peralta et al., 2013). These bacteria seem to have specialised in using low-energy fermentation products as electron donor and are therefore ideal to remove these residual pollutants from other WWT approaches (Esteve-Núñez et al., 2005; Miyatake et al., 2009). Furthermore, it is emerging that EAB form a network of interactions with other functional groups (Kato et al., 2012; Summers et al., 2010), which suggest that by stimulating these bacteria, a much broader effect on the functioning of the system can be achieved. However, a deeper understanding of mechanisms by which EAB are selected in METlands as well as their overall metabolic influence on the microbial ecosystem is required to fully exploit this technology.

In spite of the effort to scale up the technology for the last 15 years, just pilot scale devices no larger than 1000L have been reported in scientific literature (Cusick et al., 2011; Ewing et al., 2014; Heidrich et al., 2014, 2013). In this paper we present for the first time a full-scale, horizontal-flow METland for WWT that has been fed with real sewage from a small community in south of Spain for over four years. Analytical data were collected periodically to assess the treatment efficiency of the system for both carbon and nitrogen removal. Furthermore, 16S rDNA from different locations within the METland was massively sequenced. The structure of the communities was used to extrapolate the metabolic activities carried out at those different locations. These two lines of evidence clearly show that METs can improve the performance of CWs and suggest that EAB are the main actors of this effect.

4.3 Material and methods

4.3.1 METland configuration

One full scale METland was built in November 2011 in the facilities of Foundation Centre for New Water Technologies (CENTA), Carrión de los Céspedes, Seville, Spain (Picture S 4-1). The system consisted of several units: pretreatment (coarse screen bar and sand and oil separators), primary treatment (Imhoff tank for 20 p.e.[1]) (Picture S 4-1) and bioelectrochemical HSSF CW (METland), as a secondary treatment (Picture S 4-2). The dimensions of the CW were 6.5 m length; 3.7 m width and 0.6 m depth, with a surface area of 24 m^2 and a total volume of 14.5 m^3. An anode with a volume of 0.875 m^3 (3.7 m wide, 0.5 m long and 0.5 m height) made of coke granules (Ø 5-10 mm) was embedded into a standard siliceous gravel (Ø 6-12 mm) biofiltering bed (Figure 4-1.A), located at 1 metre of distance from the inlet and occupying a length of 0.5 m in the water flow direction. A plate of stainless steel (60 cm x 25 cm x 0.2 cm) buried into the bottom centre of the anode acted as electron collector. Reference electrodes (Ag/AgCl) were placed in both anode and cathode compartments to register potentials. Anode and cathode were connected by isolated copper wires, locating a power source between them. Anode potential was set to 0 V (vs. Ag/AgCl). The anode and cathode potentials and the current were periodically measured using data loggers (Tiny Tag Plus 2, Gemini, UK), recording data every 1 minute. Three different cathodes were tested (Picture S 4-3) and an enlargement of the anode was also performed. From now on, we are going to refer the results to two stages of operation:

1) In the first stage, with the small anode, a cathode of graphite cloth (3.0 m x 0.6 m, Resinas Castro, 420 g m^{-2}) was placed over the gravel bed 2 cm below the water level, which allowed cathode being flooded (Figure 4-1A).

After one year and a half of operation (May 2013), a new cathode made up of coke granules (3 m length x 3.7 m width x 0.1 m depth; volume 1.1 m^3) was established over the gravel surface and rhizomes of *Phragmites australis* were planted in (Figure 4-1B);

[1] p.e. (population equivalent) is the number expressing the ratio of the sum of the BOD load produced during 24 hours by industrial facilities and services to the individual BOD load in household sewage produced by one person at the same time. For practical calculations, it is assumed that one unit is equal to 60 g of BOD per 24 hours.

2) In a second stage, in February 2014, the gravel before the electrodes was substituted by coke granules, increasing the anode volume to 2.625 m^3 after more than two years of operation (Figure 4-1C). This was decided because the initial gravels were clogged and we wanted to test the performance of a bigger electrode. In June 2014, coke granules cathode was disconnected and a rolled-up carbon cloth cathode (2 m x 1 m, Resinas Castro, 420 g m^{-2}) was inserted into the anode inside a perforated PVC cylinder (Figure 4-1C).

4.3.2 METland operation and water analysis

The system was fed with real urban wastewater from the municipality of Carrión de los Céspedes (Sevilla, Spain) (2500 inhabitants) under discontinuous flow regime, with a daily hydraulic flow of 2 m^3 d^{-1}, following the pattern established by the European standard EN 12566-3, that regulates the EC mark for compact wastewater treatment systems with capacity for less than 50 p.e. Average physicochemical characteristics of the influent wastewater are shown in supplementary Table S 4-1. Wastewater was pretreated in an Imhoff tank in order to remove solids and prevent early clogging of the systems. The feeding from the Imhoff was made by programmed pumping, by means of 12 daily periods, simulating the production of wastewater in small populations (Ortega et al., 2010).

Several vertical perforated PVC tubes (Ø 20 cm) were inserted to the bottom in different points to take samples (Figure 4-2). Samples were taken in four points: first point was the Imhoff tank effluent (the inlet wastewater of the METland), point 2 before the anode (0.9 m from the inlet, after the initial gravel), point 5 after the anode and last point the effluent of the system. After the enlargement of the anode, point 2 was not used as sample point.

Chemical Oxygen Demand (COD), Biochemical Oxygen Demand (BOD_5), Total Suspended Solids (TSS), pH, Temperature (T), Electrical Conductivity (EC), Dissolved Oxygen (DO), Redox Potential (ORP), Total Nitrogen (TN), ammonium nitrogen (NH_4–N), nitrate nitrogen (NO_3–N), total phosphorous (TP) and phosphates (PO_4-P) were analysed weekly, following the standard methods (American Public Health Asociation, 2005). T, pH, EC, DO and ORP were measured with a handheld multiparameter (YSI 556 MPS). Averages and standard deviations were calculated for every parameter. Removal efficiencies were calculated as percentage with respect to the influent wastewater. Removal rates were referred to every zone volume and to the total wetland volume.

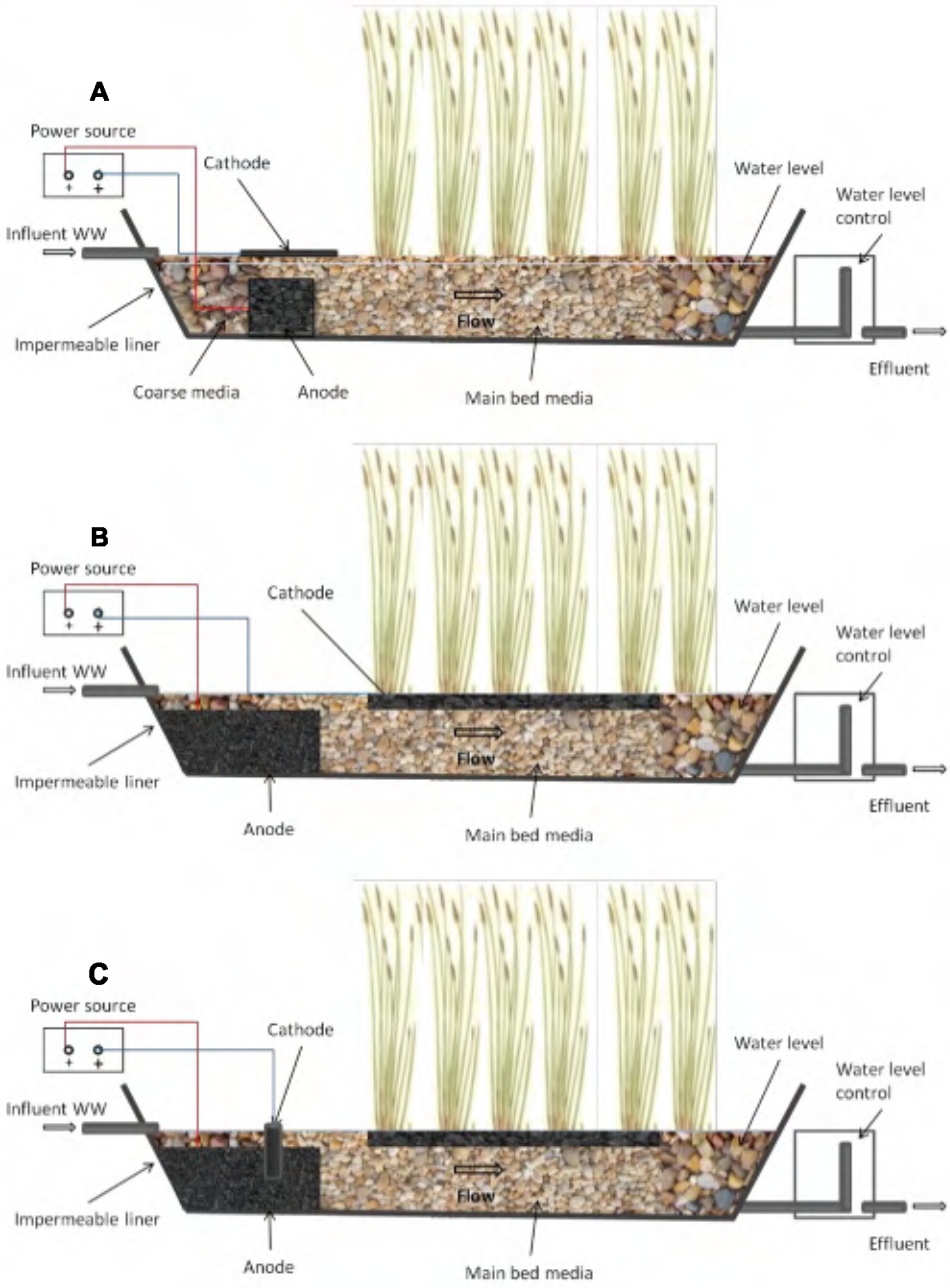


Figure 4-1: Schematic drawings of the tested configurations of cathode and anode of the bioelectrochemical constructed wetland (METland) through the period of study. A) Carbon cloth cathode over the surface; B) Coke granules cathode, with plants; C) Rolled-up carbon cloth cathode into the 2.6 m^3 anode.

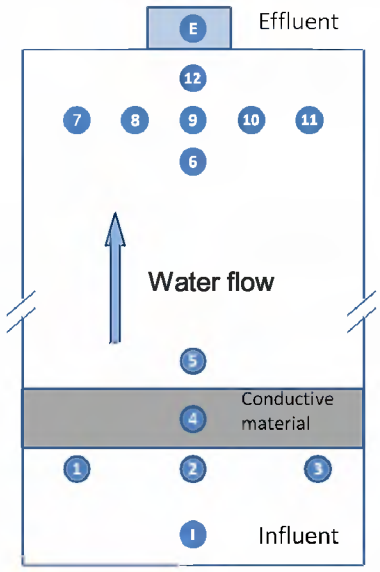


Figure 4-2: Schematic of water sample points in the METland

4.3.3 Determination of the loading rates

The organic loading rate (OLR) is defined as the amount of organic matter measured in BOD_5 that is applied by surface unit of the system.

$$OLR = {}^{g\ BOD_5}/_{m^2 \cdot d} \qquad [1]$$

The total suspended solids loading rate (SSLR) is the amount of TSS that is applied over the wetland by surface unit.

$$SSLR = {}^{g\ TSS}/_{m^2 \cdot d} \qquad [2]$$

The calculus of nitrogen and phosphorous loading rate is similar to the previous ones.

$$TNLR = {}^{g\ N}/_{m^2 \cdot d} \qquad [3]$$

$$TPLR = {}^{g\ P}/_{m^2 \cdot d} \qquad [4]$$

The hydraulic loading rate (HLR) is the daily flow rate that enters the system by surface unit.

$$HLR = {}^{m^3}/_{m^2 \cdot d} \qquad [5]$$

4.3.4 Study of the microbial community

Several studies were carried out to relate the structure of the microbial community with the treatment processes in the METland. During the first stage (small anode), in April 2013, a study of 16S rDNA sequencing was performed in the wastewater and four points of the METland to analyze the composition of the microbial community. In the second stage (large anode), two months after the anode enlargement (April 2014), another 16S rDNA exhaustive sequencing was carried out in samples of 24 points of the METland, and were related to physicochemical conditions of the water in those sampling points.

Sampling, DNA extraction and 16S rDNA sequencing

So, two campaigns of sampling and sequencing were developed. In the first campaign (April 2013), one sample of wastewater and of granular material (either gravels or coke granules) was taken from each of the five METland niches investigated in order to determine the composition of their microbial community (Figure 4-3) and corresponded to the stage with the small anode, after 15 months of operation. Cathode samples were taken from the carbon cloth fibres. In the second sampling and sequencing campaign (April 2014), samples of inlet wastewater and 22 different niches inside the METland were taken following the scheme of Figure 4-4. Anode samples were taken at 30 cm depth, gravel samples at 10 cm depth and cathode coke granules samples in the bottom of the material.

For the wastewater entering the system, 30 ml were filtered through a 0.2 µm nitrocellulose membrane and DNA extracted directly from the shredded filter. For the remaining samples, either granules of graphite coke or small gravels were taken with tweezers and dipped in three consecutives, sterile, 50 ml saline solutions (NaCl 7 g/l) in order to remove loosely attached bacteria. These rinsed biofilm were first frozen and then fully processed within a week. Around 10 granules/pebbles were extracted for each niche.

DNA was extracted and processed as explained elsewhere (Aguirre-Sierra et al., 2016). The primers used were 515F-CS1 (ACACTGACGACATGGTTCTACA *GTGCCAGCMGCCGCGGTAA*) and 806R-CS2 (TACGGTAGCAGAGACTT*GGTC TGGACTACHV GGGTWTCTAAT*).

Bioinformatics analysis

Sequence reads were analysed with the QIIME 1.7 pipeline (Caporaso et al., 2010) with few stitches along the way. Briefly, complementary reads were merged using fastq-join (Aronesty, 2011). Subsequently, our quality filtering strategy

removed complemented sequences that had one of the following characteristics: (i) deviated more than 10 bp from the expected length (292); (ii) contained primers with more than 1 mismatch or; (iii) contained nucleotides with Phred score <20. Filtered seqs were organised in OTUs by de novo picking using Usearch (Edgar, 2010) and one representative sequence per OTU was chosen. Taxonomy was assigned using the GreenGenes database (DeSantis et al., 2006) version 10_12 at the 97% identity rate. Furthermore, sequences were aligned and a tree generated using FastTree 2.1.3 (Morgan N. Price et al., 2010) Finally, in order to investigate alpha diversity and the network formed by communities members with QIIME, OTUs containing less than 0.005% of the total sample reads were removed according to Bokulich (Bokulich et al., 2013). The resulting network of the first sequencing was analysed and visualised using Cytoscape (Shannon et al., 2003).

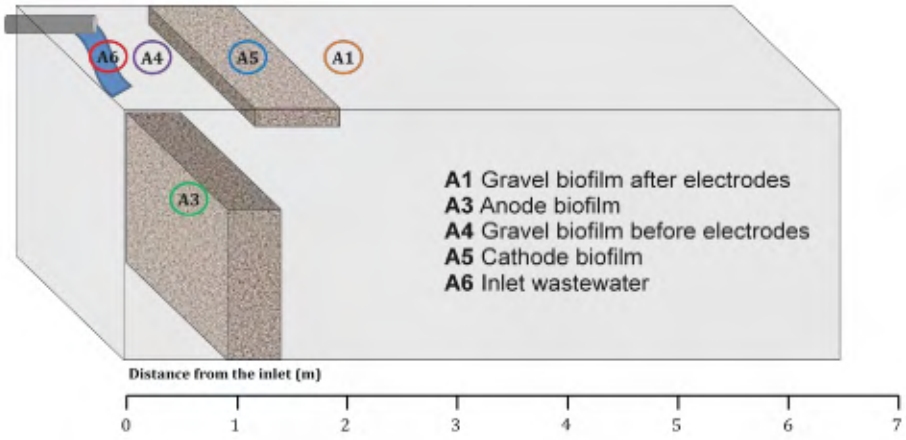


Figure 4-3: Schematic of the sample points for 16S rDNA sequencing analysis to study the bacterial community in the METland during the first stage. A1: Gravel biofilm after electrodes; A3: Anode biofilm; A4: gravel biofilm before electrodes; A5: Cathode biofilm; A6: Inlet wastewater.

Chemical Analysis of water

Samples of water were taken in the same sampling points that were used to sample granules material in order to relate in situ chemical composition with bacterial communities. Chemical Oxygen Demand (COD), ammonium, nitrate, and phosphate were analysed, following the standard methods (American Public Health Association, 2005). PH, Temperature (T), Electrical Conductivity (EC), Dissolved Oxygen (DO) and Redox Potential (ORP) were measured with a handheld multiparameter (YSI 556 MPS). Heat maps of COD, ammonium, nitrate and DO were elaborated to associate the physicochemical data with the bacterial community.

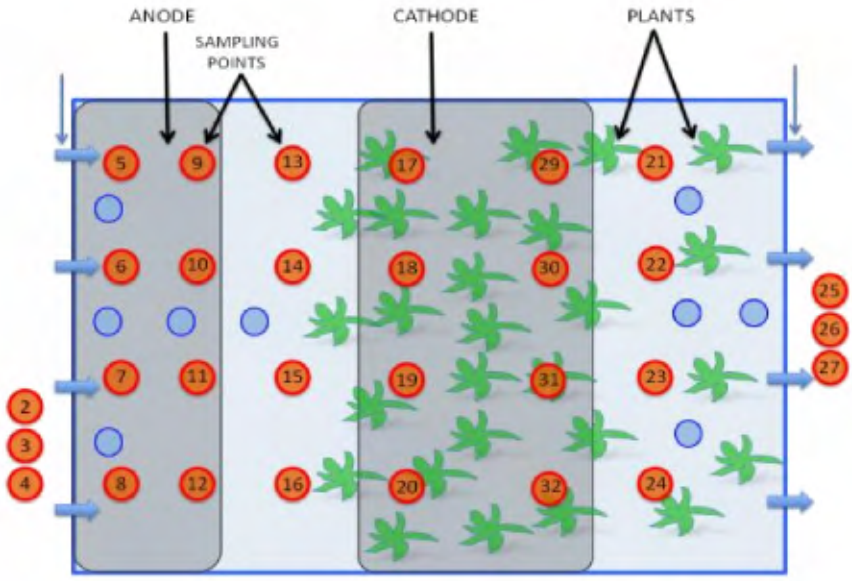


Figure 4-4: Schematic of the sample points for the 16S rDNA sequencing during the second stage. The dimensions are not proportional to the real ones.

4.3.5 Study of oxygen release by wetland plants

In flooded anoxic soils, wetland plant roots are known to release oxygen into their immediate environment (the rhizosphere), in a process called radial oxygen loss (ROL) (Jespersen et al., 1998; Sorrell, 1999; Sorrell and Armstrong, 1994). Oxygen creates an oxidized rhizosphere that protects the plant against toxic reduced compounds and allows it to survive. Differences in the location and amount of oxygen released reveal how well-adapted different plants are for surviving in flooded environments. The flux of O_2 from aerenchyma of root to the rhizosphere soil (ROL) is determined by many factors such as the physical resistance to O_2 diffusion (Armstrong, 1979). Aerenchyma is a spongy tissue that forms spaces or air channels in the leaves, stems and roots of some plants, which allows exchange of gases between the shoot and the root (Figure 4-5). The channels of air-filled cavities provide a low-resistance internal pathway for the exchange of gases such as oxygen and ethylene between the plant above the water and the submerged tissues. Aerenchyma is also widespread in aquatic and wetland plants which must grow in hypoxic soils.

Qualitative determination of oxygen release with methylene blue method

A study of oxygen released by plants, following the methylene blue gel method (Pi et al., 2009), was conducted to determine the best suitable species to plant in the cathode of METlands that was able to provide more oxygen to the electrode and improve the MET performance.

Hydroponically grown healthy plants of five species were used for the experiment, *Juncus effussus*, *Phragmites australis*, *Typha dominguensis*, *Cyperus laevigatus* and *Iris pseudoacorus* (Figure 4-6). A stock solution of methylene blue dye was prepared with a concentration of 0.6 g L^{-1}. One litre of agar gel was prepared for each plant and continuously sparged with nitrogen gas. When it began cooling 20 mL or stock methylene blue was added to the solution and was poured into a glass vessel (1 litre) and kept sparging with nitrogen gas. When it was cooled to below 40ºC 130 mg sodium dithionite was added to the solution to eliminate the remaining oxygen and it decoloured. When temperature arrived to 35ºC plant was mounted in the neck of the vessel, using a foam rubber sealed with coconut oil to avoid oxygen entrance to the head space, being careful that plant roots were fully immersed in the solution, and gas sparging was stopped. After a few minutes the solution started to get blue coloured due to the oxygen that the plants released throw their roots. Methylene blue is an indicator that is colourless in the absence of oxygen and turns blue when oxidized (Figure 4-7). Three replicates of each plant were used.

Figure 4-5: Cross-section of an aerenchymatous stalk of a typical aqueous plant

Root porosity determination by a pycnometer method

The air space fraction, or porosity, of plant roots is important to their internal aeration. Porosity determination by pycnometer is a very precise method (Jensen et al., 1969). It uses a working liquid with well-known density, such as water. The pycnometer is a glass flask with a close-fitting ground glass stopper with a capillary

hole through it that is used normally to determine density of liquids. This fine hole releases a spare liquid after closing a top-filled pycnometer and allows for obtaining a given volume of measured and/or working liquid with a high accuracy. Pycnometer can be used to determine the density of homogeneous solid object that does not dissolve in working liquid (water).

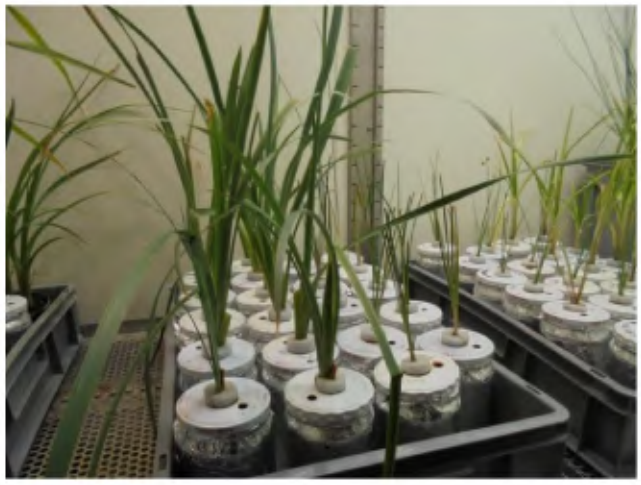


Figure 4-6: Hydroponically grown wetland plants in a growing chamber with humidity and temperature control.

Figure 4-7: Experimental set up of the methylene blue method and examples of plants mounted in two types of jar.

The sample of 10 pieces of adventitious roots, cut to 2 cm, was placed in a pycnometer bottle. The bottle was then filled with water (air bubbles trapped among the roots were freed), capped, and weighed on an analytical balance (Wr+w). Next, the water was poured from the bottle and the roots were removed and excess water

blotted from them. They were immediately weighed by themselves (Wr). This weight (Wr) was imprecise because of evaporation of water from the roots during weighing; but the error introduced was small. The roots were then placed in a mortar and crushed intensively. The resulting homogenate of the roots was returned to the pycnometer bottle, which was placed in a temperature bath to the original water temperature. The bottle was then topped up with water, and weighed again (Wh). The bottle was also weighed filled with water only (Ww). The temperature of the bottle contents was the same for all weighings. The porosity of the roots was then calculated with the formula (derived below):

$$\%\, porosity \;=\; 100(Wh - W_{r+w})/(Ww + Wr - W_{r+w}) \quad [6]$$

This procedure was repeated 5 times with every species to get accuracy and averages ± 1SD were calculated.

4.4 Results

In HSSF METlands WWT two main microbial processes are occurring. On one hand, anaerobic degradation processes that are produced in conventional CWs; On the other hand, the bioelectrochemical degradation processes that consist of the oxidation of organic and inorganic matter in the anode environment, and simultaneous reduction of oxygen or alternative compounds as nitrate in the cathode. As this METland system is an extensive WWT system, results will be normalized using the total volume of the wetland bed or the different compartments. Size is an important factor to be considered, as the reduction of the size of the wetland allows increasing the applicability of this technology.

Three areas of the METland have been established to analyse the removal efficiency. The first one is defined as the gravel bed between the inlet wastewater and the sample point 2 (Figure 4-2), that delimits the beginning of the electroconductive bed, where bioelectrochemical processes take place. The second is the area situated between sampling point 2 and 5 that encloses the bioelectrochemical region of the METland. The third region is the gravel bed comprised between sampling point 5 and the end of the METland. In a later testing period, the anode was enlarged and since then just, two regions can be recognized, the electroconductive bed and the inert bed (gravels) (Figure 4-1 C).

Throughout the course of the study different configurations of the METland have been tested. Thus, two big designed-based stages can be differentiated: first, with a small anodic bed (volume = 0.875 m^3), and a second stage in which the anode

was ca. 3-fold enlarged (2.625 m^3). Each of these stages can be subdivided into other shorter periods well described in Material and methods section and Table 4-1. So, in the first stage several periods can be defined: during the first twenty weeks the system was operated in MFC mode, with a resistor of 1KΩ. After 21 weeks of operation the system started to be poisoned to 0 V potential. After a long period of 25 weeks the poisoning was stopped and the system was left in open circuit (OC) mode during 5 weeks, after which the system was poisoned again. After 65 weeks symptoms of clogging were observed in the feeding area made of gravel, so the METland was drained to recover the permeability. At that time, the cathode was replaced by a planted-cathodic bed made of coke granules. The system continued operating until week 104; then gravels from feeding zone were removed and replaced by coke granules so the anode bed was substantially enlarged. Since then, the system was operated in open circuit during 10 weeks and then poisoned again. Results are shown from ca. 4 years of performance.

Table 4-1: Periods of operation and changes carried out in the METland throughout the time of study.

Stage	Periods	Weeks	Plants	Electrochemical	Cathode	Anode
1	1	11-21	No	MFC	Carbon cloth cathode over bed	0.875 m^3
	2	22-47	No	Polarized (0V)		
	3	48-51	No	Open circuit (OC)		
	4	52-71	No	Polarized (0V)		
	5	72-74	METland was drained			
	6	75-94	Yes	Polarized (0V)	Coke granules	0.875 m^3
	7	95-103	Yes	Open circuit (OC)		
		104-118	Substitution of initial gravels by conductive material			
2	8	119-138	Yes	Shortcircuit	Coke granules	2.625 m^3
	9	139-170	Yes	Polarized	Rolled cloth	

The system was poisoned with a power source that was consuming 36V and giving 4A. Measures were stable. The differences found in treatment performance were more related with the size of the anode than with changes in the electrochemical configuration.

4.4.1 Removal of pollutants from wastewater

Organic matter removal

In spite of using an organic loading rate (OLR) as high as 20 g BOD5 $m^{-3}d^{-1}$, what constitutes 3-fold the recommended OLR for HSSF wetlands (Kadlec and Wallace, 2009; Ortega et al., 2010), our METland fulfilled the WWT discharge limits (Figure 4-8) (Dir. 91/271/EEC of 21 May 1991). After 20 weeks of operation. COD average effluents ranged between 38 ± 29 mg L^{-1} and 76 ± 29 mg L^{-1} (Table S 4-3). BOD_5 average effluents were always well below the limit of 25 mg L^{-1}, achieving average concentrations as low as 9 mg L^{-1} during 25 continuous weeks (Table S 4-3).

The electroconductive bed showed an efficient performance regardless the size of the bed. Moreover, no differences were observed in COD or BOD removal under either polarized or short-circuit operation mode (Figure 4-8). The differences were only due to the lower organic load that led to lower removal rates. So thus, only two periods of treatment should be considered, one first was corresponding to the 0.9 m^3-anode and the second to the period of the 2.6 m^3-anode.

During the first period, the anodic bed performance was compared to the gravels bed at the feeding zone. The analysis revealed COD removal rates as high as 369 ± 196 g COD m^{-3} d^{-1} for the electronductive bed (Table S 4-4) what represent ca. 10-fold higher than the one shown by the previous gravel bed (Figure 4-9). Treated wastewater achieved an average COD concentration after the anodic bed of 115 ± 20 mg L^{-1} for over a year (Table S 4-3), which was under the legal limits of discharge. This is remarkable considering that the electroconductive bed is just 1/6 of the total wetland bed.

Figure 4-10 and Figure 4-11 show the relation between the inlet organic load and the load removed during both periods. During the first and second periods the METland removed 87% and 94% of BOD and 79% and 86% of COD, respectively.

Total suspended solids (TSS) removal

With respect to TSS, effluents fulfil discharge requirements (35 mg L^{-1}). Thus, in spite of the high OLR, just the conductive bed by itself was able to accomplish the limits by releasing an effluent with a concentration between 28 ± 14 and 35 ± 5 mg TSS L^{-1} (Table S 4-5). However, evidences of clogging were observed in the gravel bed located in the feeding zone (Figure 4-12). This is somehow expected since OLR in that feeding zone was 4-fold higher than recommended in literature for gravel beds in HSSF wetlands.

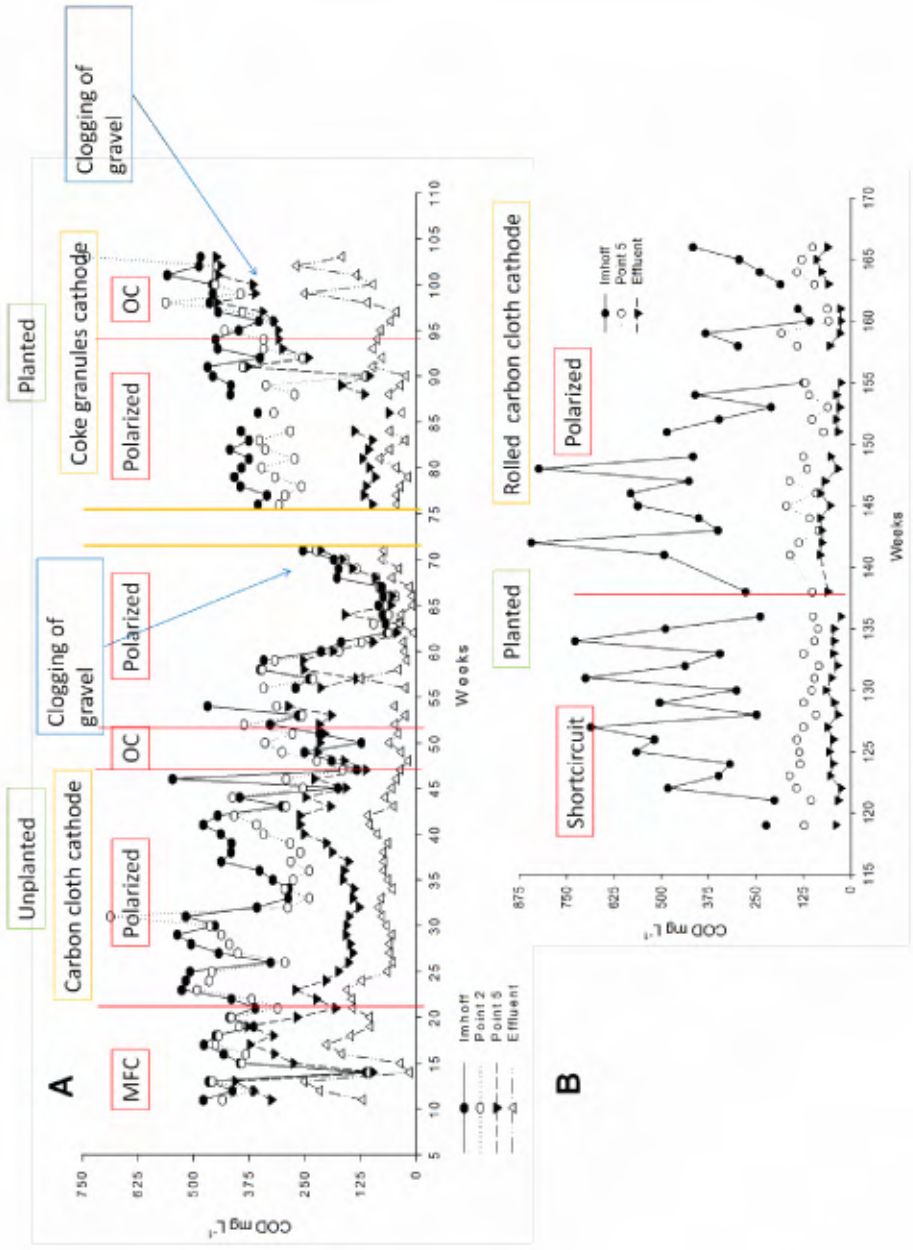


Figure 4-8: COD evolution throughout the period of study. The results have been scheduled in two main stages: a first period where the system was operating with a 0.9 m^3-anode (A) and a second period with a 2.6 m^3-anode (B).

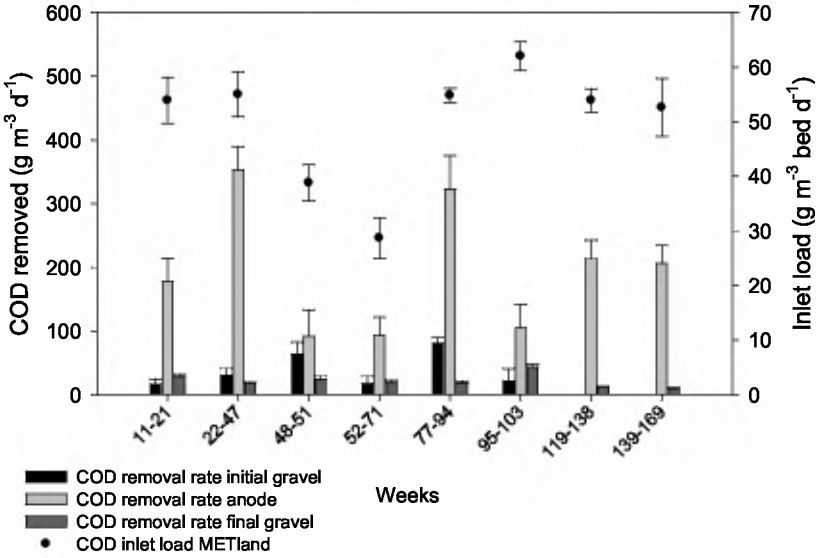


Figure 4-9: COD removal rates through the period of study in different locations of the METland. It is important to point out that data for removal rates are referred to the volume of certain beds, while COD loading rate is referred to the total volume of the METland bed.

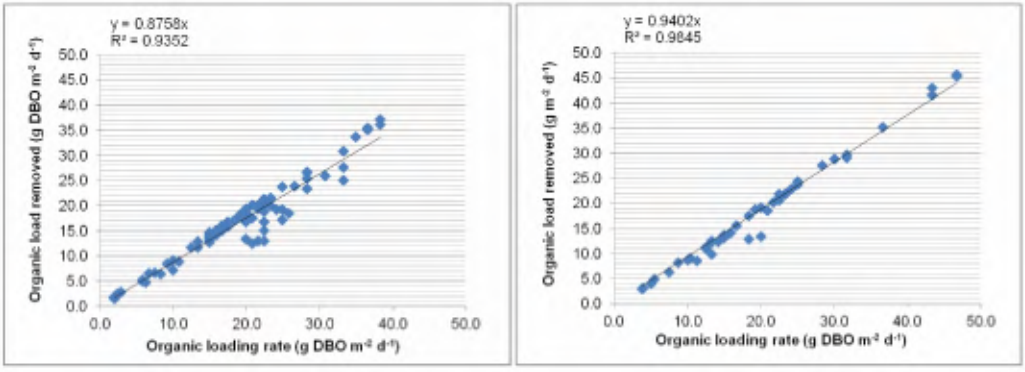


Figure 4-10: Relationship between BOD loading rate and BOD removal rate during the two stages (0.9 m^3-anode and 2.6 m^3-anode). The slope represents the percentage of BOD removed.

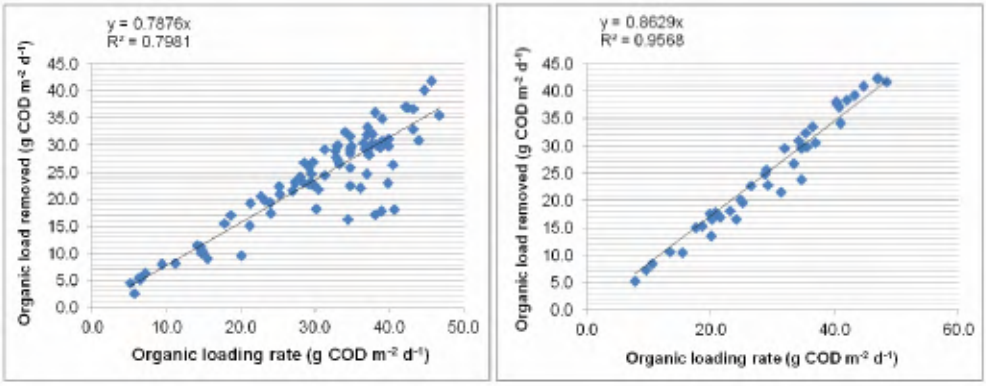


Figure 4-11: Relationship between COD loading rate and COD removal rate during the two stages (0.9 m^3-anode and 2.6 m^3-anode). The slope represents the percentage of COD removed.

Nutrients removal

A slight nitrification was observed in the electroconductive bed, although it was enhanced along the area of gravels after the conductive bed (Figure 4-13). Nitrogen removal was not stable, with values that ranged between 16 and 60% for total nitrogen and between 18 and 45% for ammonium (Table S 4-6). During the second stage it seemed that there was a slight increase in the percentage of total nitrogen removed by the anodic bed; no nitrogen was eliminated during the first weeks after the enlargement of the anode and then there was also an increase.

Regarding to phosphorous, in the first period 61% of the TP was removed although the removal performance was variable (Figure 4-14). Electroconductive material removed between 5 and 11 % during the first stage (0.9 m^3-anode) and after enlarging the electrode to 2.6 m^3-anode it removed 21% of the total phosphorous removed in the wetland. Total phosphorous removal in the METland achieved efficiencies as high as 64% but in the last period removal efficiency decreased to 30%, most of it removed in the anode (21%) (Table S 4-7). Mean percentage during the first and second period were around 29% and 33%, respectively (Figure 4-15).

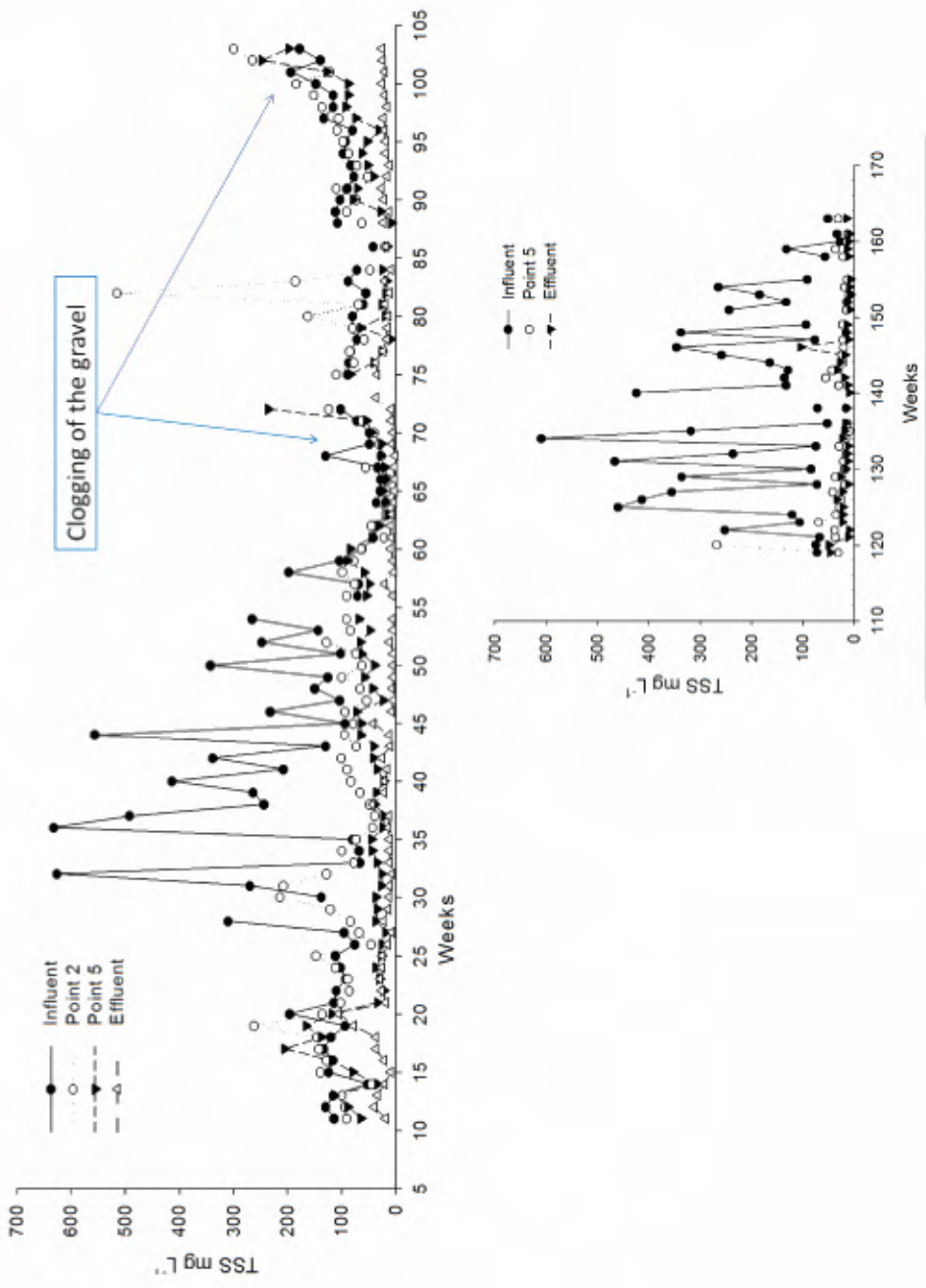


Figure 4-12: TSS evolution throughout the period of study. The results have been divided in two main stages: a first period where the system was operating with a 0.9 m^3-anode (A) and a second period with a 2.6 m^3-anode (B).

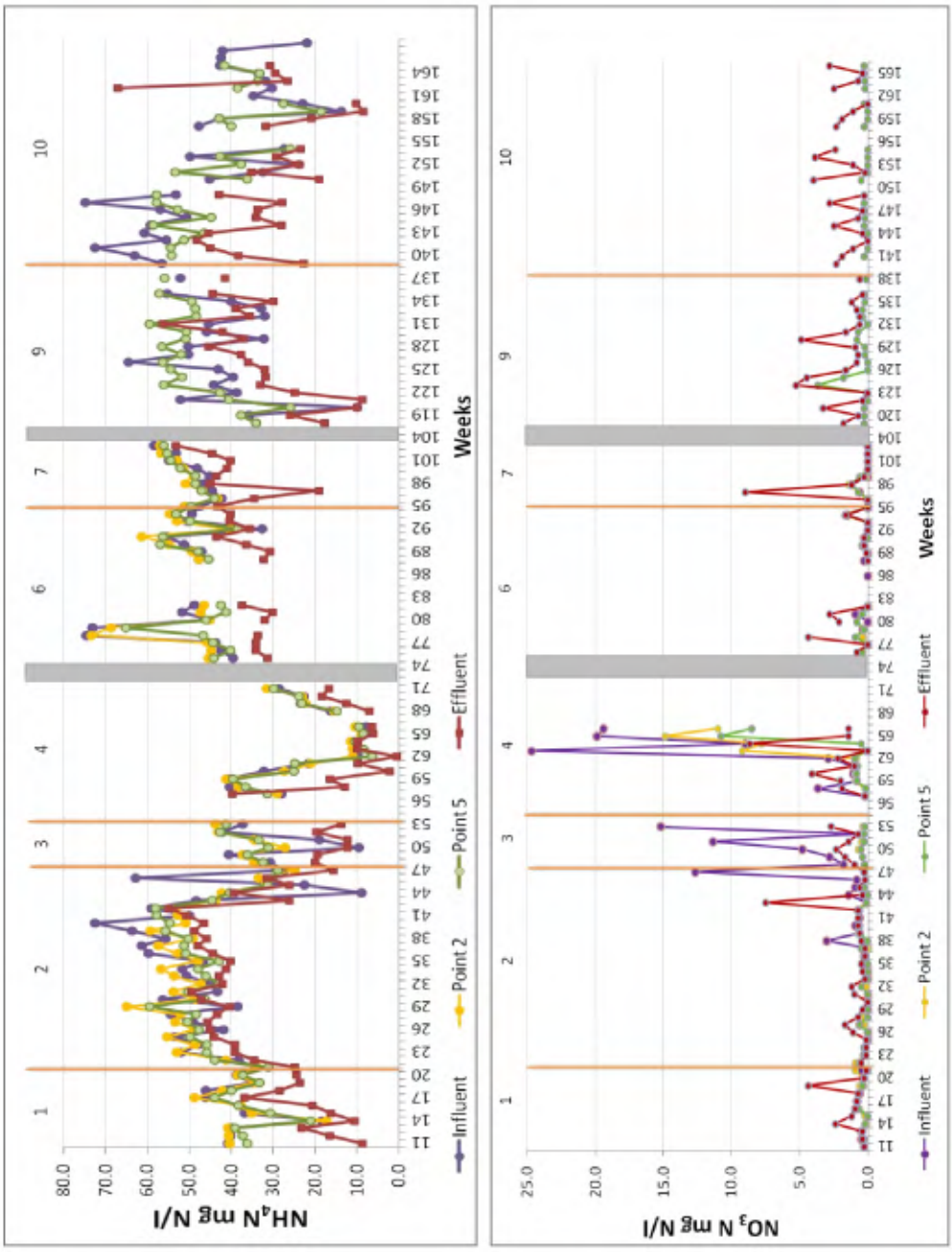


Figure 4-13: Nitrogen evolution throughout the period of study. Top graph: Ammonia nitrogen; Bottom graph: Nitrate nitrogen. The results have been divided in two main stages: a first period (weeks 11 to 103) where the system was operating with a 0.9 m^3-anode and a second period (weeks 119 to 166) with a 2.6 m^3-anode. Grey bars correspond to changes of configuration. Top numbers indicate the periods of operation.

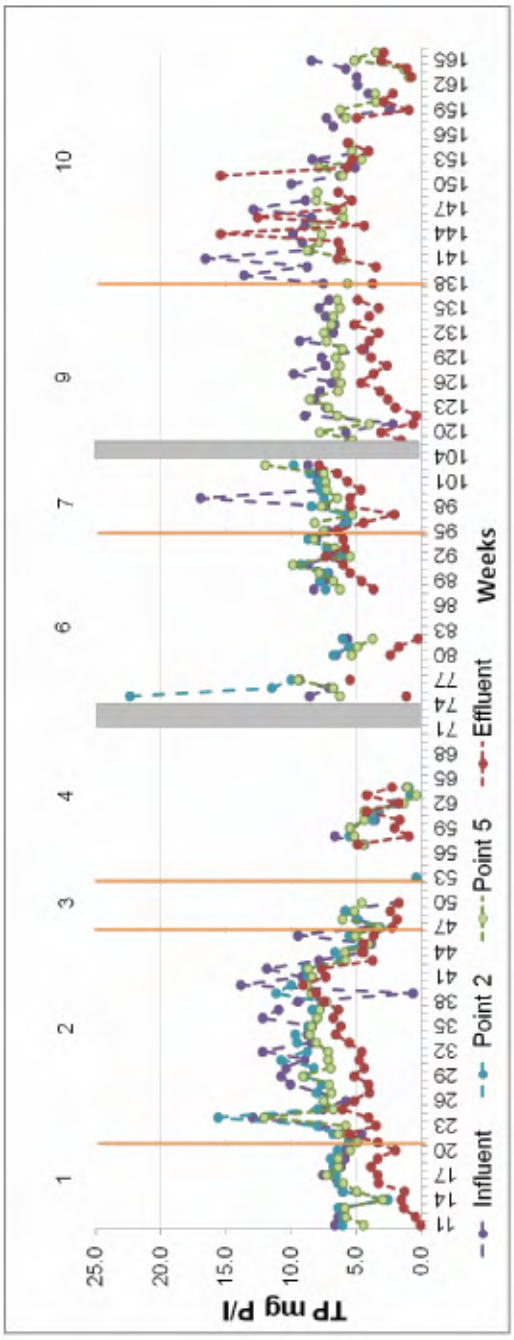


Figure 4-14: Total phosphorous evolution throughout the period of study. The results have been divided in two big stages: a first period (left graphs) where the system was operating with a 0.9 m^3-anode and a second period (right graphs) with a 2.6 m^3-anode.

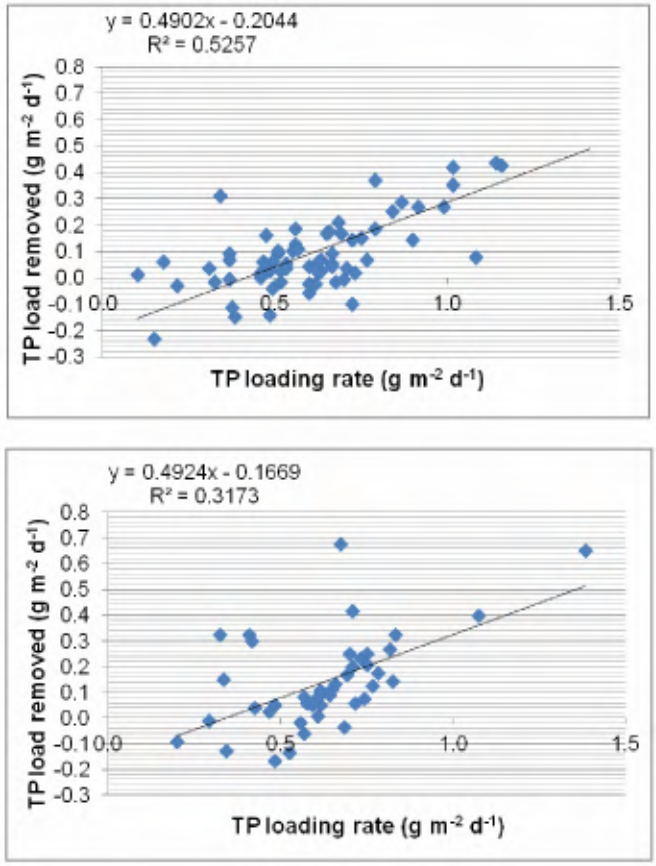


Figure 4-15: Relationship between TP loading rate and TP removal rate during the two stages (0.9 m^3-anode and 2.6 m^3-anode). The slope represents the percentage of COD removed. Line does not fit the data very closely.

4.4.2 Microbial community

First stage 16S rDNA sequencing

The microbial community was analysed in five independent locations generating 810,440 raw reads per sequencing direction, which yielded a total of 681,912 high quality, reverse-complemented sequences with an average length of 292 bp (171,663 for sample A1 (gravel after the electrodes); 132,886 for A3 (anode); 109,975 for A4 (gravel before electrode); 145,916 for A5 (cathode) and; 121,372 for A6 (inlet wastewater)). This volume of sequences was around one order of magnitude greater than previously reported studies of diversity in bioelectrochemical systems (Miceli et al., 2012), which reflects an improvement in sequencing technologies. Clustering these sequences generated 18,962 OTUs evenly distributed

in the 5 samples, with the exception of the inlet wastewater (A6) where the dominance of one OTU likely covered the detection of other less abundant species (Figure 4-16).

Bacterial community structures were characterised by the prevalence of *Proteobacteria* (over 60% of sequences) in all samples but in the anodic biofilm (Figure 4-17). However, while the alpha, beta and gamma subdivisions dominated samples A1, A4 and A5 (gravel samples and cathode), the wastewater community (A6) was enriched in epsilon (51.5%). Regarding the biofilm attached to the anode (A3), *Deltaproteobacteria* were the most abundant group (22%), followed by other recognized anaerobic groups such as *Clostridia* (8.2%), *Bacteroidia* (10.3%), *Synergistia* (12.9%) and *Archaea* (*Methanobacteria* 4.4% and *Methanomicrobia* 2.6%).

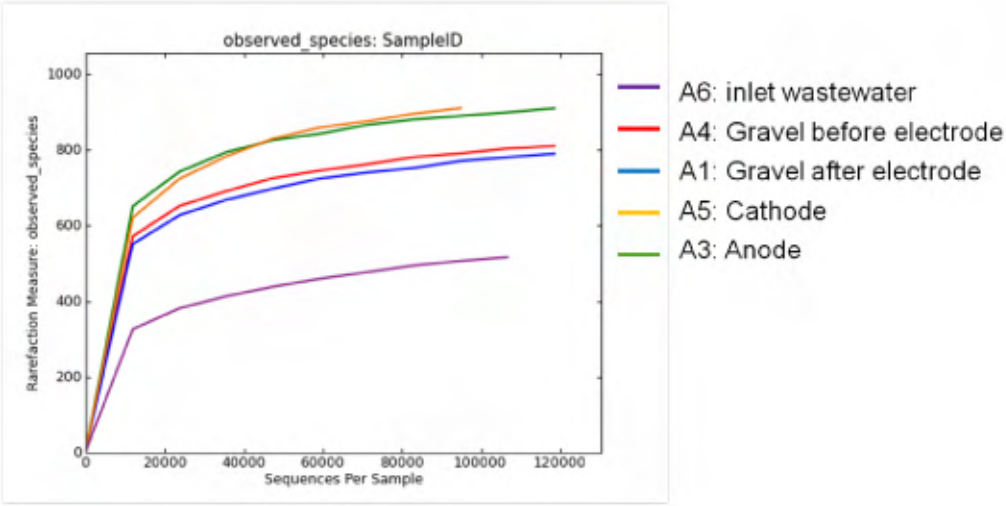


Figure 4-16: Rarefaction curves of the observed species in the five samples of the first sequencing.

The two most similar samples were A1 (gravel post electrodes) and A5 (cathode), which deviated from all others by higher proportions of *Actinobacteria* (4.1 ± 0.4% versus 0.9 ± 0.5%), *Acidomicrobia* (1.1 ± 0.3% versus 0.2 ± 0.2%), *Flavobacteria* (7.0 ± 0.5% versus 0.7 ± 1%), *Sphingobacteria* (3.3 ± 0.9% versus 0.3 ± 0.4 %), *Alphaproteobacteria* (19.5 ± 3.0% versus 2.2 ±1.9%), and by a smaller proportion of Clostridia (1.9 ± 0.7% versus 5.4 ± 2.4%). When comparing these two highly similar samples, in respect to sample A5 (cathode), sample A1 (gravel after the electrode) showed a greater prevalence of the classes *Synergistia* (3.6 versus

1.4%) and *Planctomycetia* (3.7 versus 0.9%), together with a reduced proportion of the phyla *Verrucomicrobia* (0.5 versus 2.5%) and *Cyanobacteria* (0.1 versus 1.5%). Despite these similarities at the class-level, the number of unique, dominant OTUs in either sample A1 or A5 is surprisingly high (Figure 4-18).

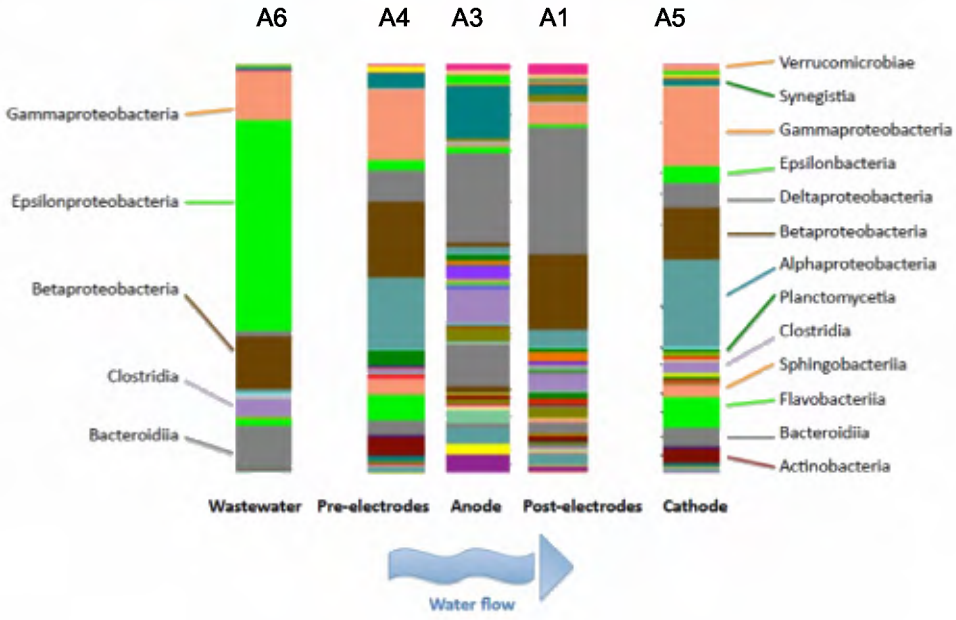


Figure 4-17: Higher taxa community composition in the samples of the first sequencing.

Moving down the taxonomic scale, results at the genus level (or best available taxonomic affiliation) are shown in Figure 4-19. Focusing on the anodic community, many dominant OTUs (> 1%) are shared with at least another niche, with those that reach concentration clearly higher in this sample that fall within the taxa *Methanobacterium*, *Methanosaeta*, *Holophagaceae*, *Desulfovibrio*, *Geobacteraceae*, *OP8*, *Syntrophus* and other poorly characterised *Synergistetes*. Few OTUs are shared uniquely between samples A3 and A5, while many more are common between A3 and A4, with *T780* and a tentatively assigned *Phycisphaerae* being the two most abundant.

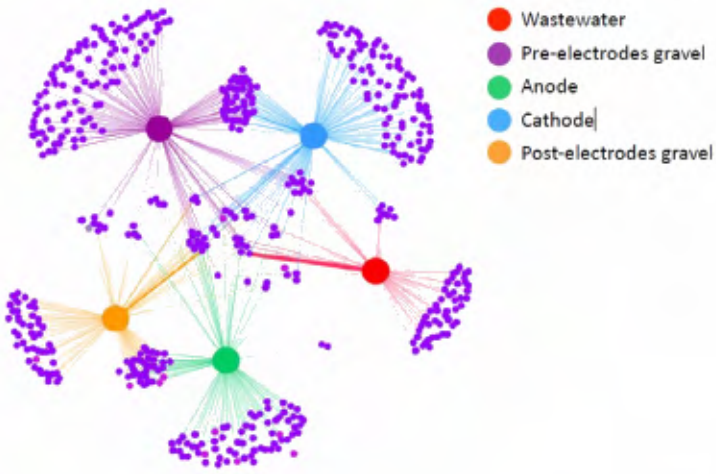


Figure 4-18: OTUs network of the five samples of the first stage. Purple nodes represent dominant bacterial OTUs (with proportion higher than 0.005%). Lilac nodes represent dominant Archaea OTUs (mostly methanogenic taxa).

Second stage 16S rDNA sequencing

The 24 microbial community samples analysed produced 1,953,395 raw reads per sequencing direction, which yielded a total of 1,591,605 high quality reverse-complemented sequences with an average length of 292 bp (Table 4-2). Clustering these sequences generated 27,450 OTUs evenly distributed in the 24 samples. Rarefaction curves showed saturation, indicating that a reasonable number of sequence reads per sample were collected to reveal diversity at the sites (Figure 4-20). Table 4-2 shows that observed species were less abundant in the inlet wastewater than in the rest of the niches.

Bacterial community structures were characterised by the prevalence of *Proteobacteria* in all samples, especially in wastewater samples (70%) (Figure S 4-3). As resulted in the previous sequencing, while the alpha, beta and gamma subdivisions dominated gravel and cathode samples, the wastewater community was enriched in epsilon (51.4% and 47.5%). Effluent treated wastewater showed a very similar composition than influent wastewater at class level, but the proportions of some taxa varied. So, *Epsylonproteobacteria* was lower (33.1-38.2%), *Bacteroidiia* increased from around 10% to around 20% and *Deltaproteobacteria* raised from 1.2-1.4% to 4.8-5.5%.

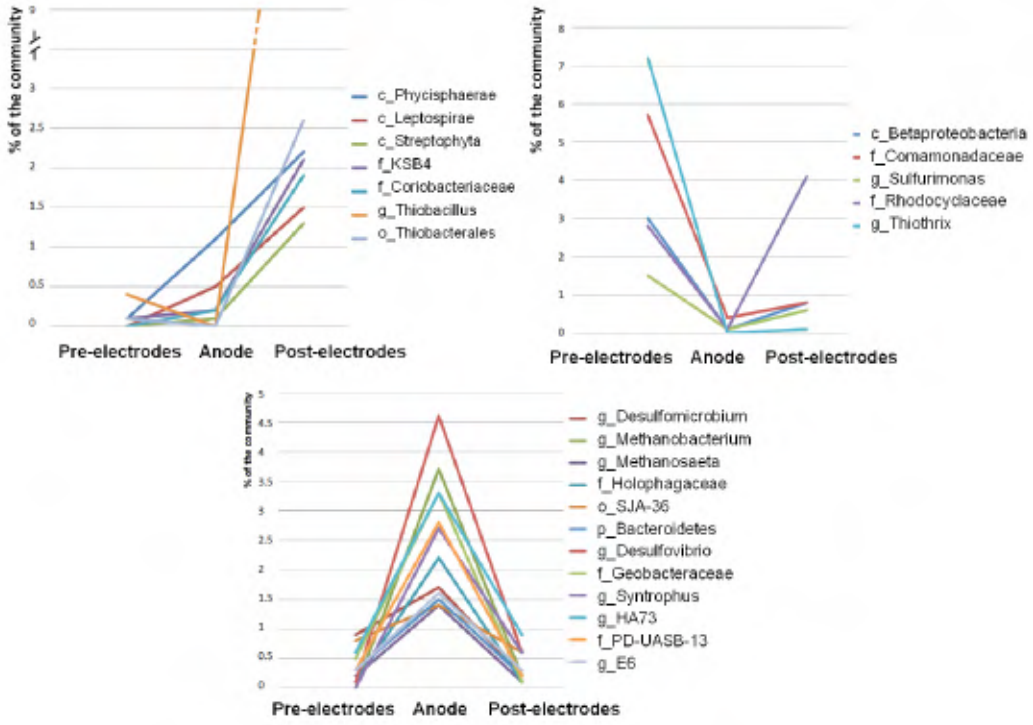


Figure 4-19: Dominant bacteria genera in the different environments of the METland in the sequencing of the first stage.

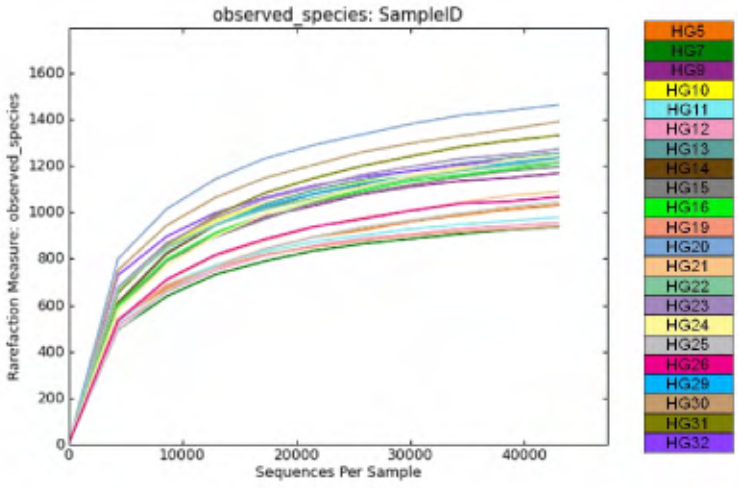


Figure 4-20: Rarefaction curves of the observed species in the 22 samples taking into the METland at the second stage.

Regarding the biofilm attached to the anode, *Deltaproteobacteria* were the most abundant class (between 18 and 38%), followed by other recognized anaerobic groups such as *Clostridia* (17.6-25.4%), *Bacteroidia* (7.8-14.7%), *Betaproteobacteria* (2.4-7.5%) and *Synergistia* (0.8-3.9%)(Figure 4-21). OTUs of the phylum *Planctomycetes* have been found in all the samples, especially abundant in cathode samples, ranging between 2.7 and 4.1%, but also in the anode (0.1-0.6%). Some genera of this phylum are related to Anammox process.

In the cathode, members of the classes *Betaproteobacteria* (11.0-22.8%), *Alphaproteobacteria* (18.0-27.5%), *Gammaproteobacteria* (8.5-14.8%), *Actinobacteria* (1.3-17.3%), *Deltaproteobacteria* (3.8-13.9%), *Sphingobacteriia* (1.5-9.4%), *Nitrospira* (1.0-2.8%) and *Anaerolineae* (1.2-2.8%) have been found to be the most abundant (Figure 4-21). Similar taxonomic groups were found in the final gravels, located after the cathode in the water flow direction (Figure 4-21).

Focussing on family level, anode biofilm is dominated by *Geobacteraceae* (2.8-15.3%) family, followed by *Pelobacteraceae* (0.9-9.5%), *Desulfobacteraceae* (3.7-8.6%), *Peptostreptococcaceae* (1.9-8.9%), *Desulfomicrobiaceae* (1.0-7.0%), and *Clostridiaceae* (4.4-6.8%) (Table S 4-9). *Pelobacteraceae* and *Geobacteraceae* are also the two dominant families in the intermediate gravel (Table S 4-10). Other bacteria that have been found in proportion bigger than 1% are *Nitrospiraceae*, *Nitrospirales* and *Nitrosomonadaceae*, all of them related to nitrogen processes. In the cathode, the most abundant family is *Hydrogenophilaceae*, followed by *Comamonadaceae*, *Rhodocyclaceae* and *Geobacteraceae*, which range between 0.3 and 6.9% (Table S 4-11). In the final area of gravels after the cathode, similar families but large variations in the proportion were found. An increase in the family *Xanthomonadaceae* (7.9-11-9%), *Nocardioidaceae* and *Nitrosomonadaceae* was observed, while decreasing *Hydrogenophilaceae*, *Geobacteraceae*, *Caulobacteraceae y Nitrospiraceae* (Table S 4-10).

To the genus level, the most abundant in the anodic biofilm is *Geobacter* (2.6-15.2%) and other *Deltaproteobacteria* such as *Desulfomicrobium*, *Desulfobacter* and *Desulfobulbus*, another reported electroactive bacteria (Ahn et al., 2014; De Schamphelaire et al., 2010) (Table S 4-12). Interestingly, *Geobacter* was one of the most abundant genera in the cathode (0.3-6.9%) together with *Thiobacillus*, a genus that interact with Geobacter through interspecies electron transfer (IET) (Kato et al., 2012) to reduce nitrite and nitrate. Figure 4-22 shows the distribution of *Geobacter* over the METland.

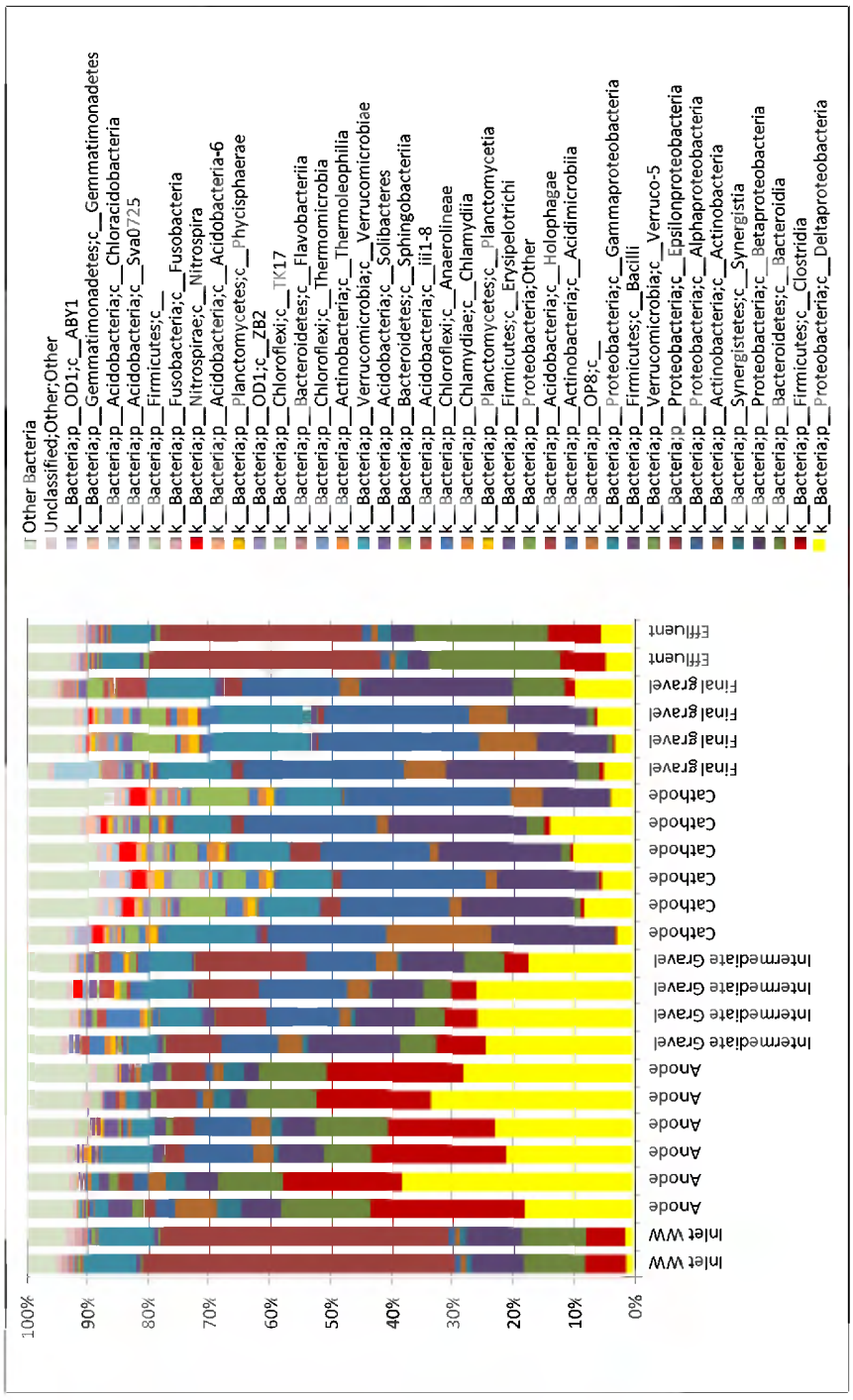


Figure 4-21: Taxa of OTUs at class level of the second sequencing, with more than 1% in some of the samples.

Table 4-2: Total and high quality reads and alpha diversity metrics of the bacterial populations in the samples of the second stage 16S rDNA sequencing.

Sample point	Description	Chao 1	Observed species	Total reads	High quality reads
HG3	Inlet WW	974	870	106487	89068
HG4	Inlet WW	1031	904	77545	63975
HG5	Anode	1177	1042	96263	76155
HG7	Anode	1009	937	94048	77708
HG9	Anode	1241	1167	72908	63525
HG10	Anode	1348	1217	88242	70607
HG11	Anode	1064	971	78992	69115
HG12	Anode	1023	950	92968	77415
HG13	Intermediate Gravel	1409	1252	95347	78994
HG14	Intermediate Gravel	1414	1232	98898	82504
HG15	Intermediate Gravel	1286	1191	91986	74307
HG16	Intermediate Gravel	1330	1199	92040	73480
HG19	Cathode	1015	1248	68180	54291
HG20	Cathode	1596	941	82498	59862
HG29	Cathode	1396	1459	55748	43844
HG30	Cathode	1558	1235	78340	58718
HG31	Cathode	1492	1393	61382	51389
HG32	Cathode	1343	1335	64852	44047
HG21	Final gravel	1247	1240	64337	56847
HG22	Final gravel	1355	1086	76863	59528
HG23	Final gravel	1409	1203	68332	55434
HG24	Final gravel	1426	1274	78775	68039
HG25	Effluent	1187	1038	94727	79958
HG26	Effluent	1202	1066	73637	62795

Other genera related to nitrogen metabolism appeared in the cathode, such as the nitrite-oxidant *Nitrospira* (1.0-2.4%) and *Candidatus Brocadia* (0.1-0.6%) involved in Anammox process (Table S 4-13). *Arcobacter* was the dominant genera in the inlet wastewater (41.4-45.6%), as was shown also in the first sequencing, and was reduced to a half in the effluent wastewater (Table S 4-14). On the contrary, genera as for example *Parabacteroides*, *Sulfurospirillum*, *Bacteroides*, *Sulfurimonas*, *Tolumonas* and *Geobacter* increased in percentage in the effluent with respect to the influent. *Geobacter* and *Thiobacillus* were the dominant genera in the intermediate area of gravels after the anode (Table S 4-15). In the final area of gravels after the

cathode, *Geobacter* appeared but in much less percentage (0.8-2.7%), as well as *Thiobacillus* in some samples, because in others was still high (Table S 4-15).

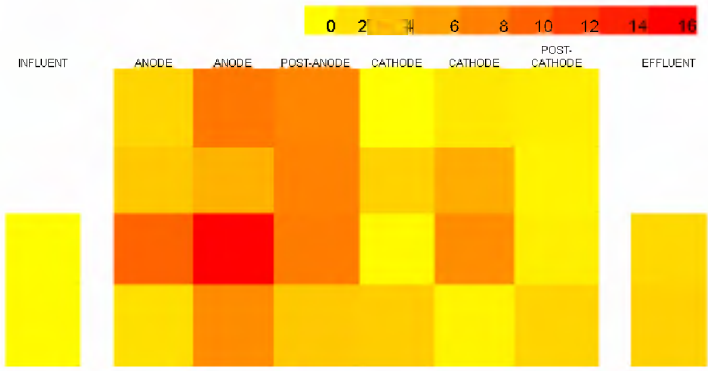
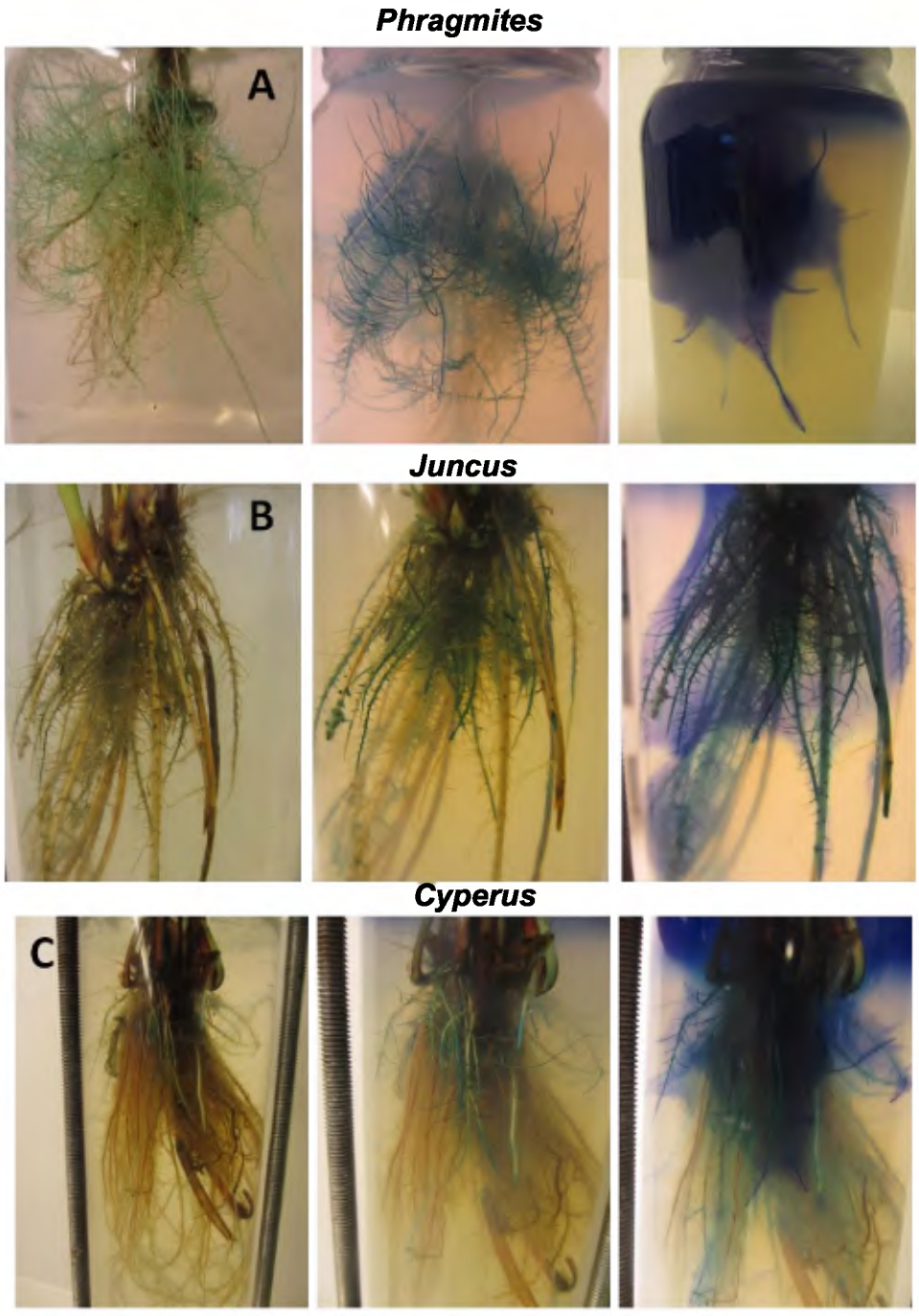


Figure 4-22: Heat map of *Geobacter* distribution in the METland in the second stage.

4.4.3 Oxygen released by plant roots

The methylene blue agar gel acts as a surrogate for a flooded soil, and allows the location of oxygen release from roots to be identified. There were visual differences in the oxygen released by the five species (Figure 4-23). All the species showed to release oxygen mainly through the tips of the new adventitious roots and the new secondary roots (Figure 4-24), but *Phragmites australis* and *Iris pseudacorus* have been seen to develop a large new root system and so, more new zones were able two transfer oxygen to the rhizosphere. *Typha latifolia* was the species that showed much less blue zones in their roots, while *Phragmites australis* showed the darkest blue zones and a distribution of the oxygen in all the numerous secondary roots and in most of the length of new adventitious roots. *Cyperus laevigatus* was seen to release oxygen mainly in the top part of the roots, where the new short adventitious roots grew, while *Iris pseudacorus* showed very long adventitious roots and most of the oxygen was released in the half bottom part of the root system. Finally, *Juncus effussus* released less oxygen than *Phragmites australis* but equally distributed throughout the root plant system.

With respect to porosity of the adventitious roots, *Juncus effussus* showed the highest porosity (39.5±4.1%), followed by *Iris pseudacorus* (33.9±1.6%) and *Phragmites australis* (30.2±2.2%) (Figure 4-25).


Phragmites
A
Juncus
B
Cyperus
C

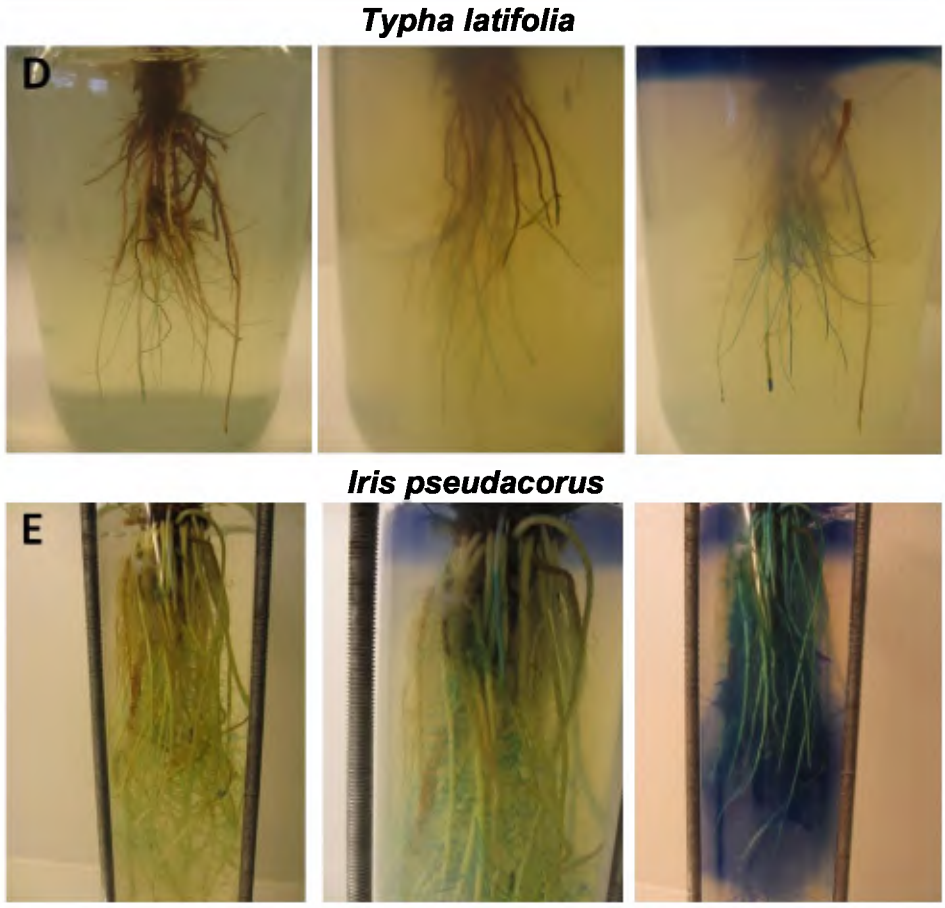


Figure 4-23: Pictures of ROL by plant roots, at 0 h, 2 h and 24 h since the introduction in the agar solution. A: *Phragmites australis*; B: *Juncus effussus*; C: *Cyperus laevigatus*; D: *Typha latifolia*; E: *Iris pseudacorus*

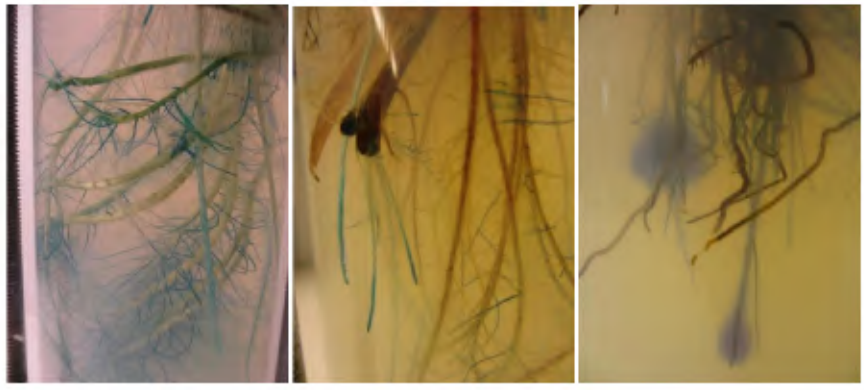


Figure 4-24: Pictures of ROL by plant roots showing that oxygen release was mainly produced throughout the tip of new adventitious roots and secondary roots.

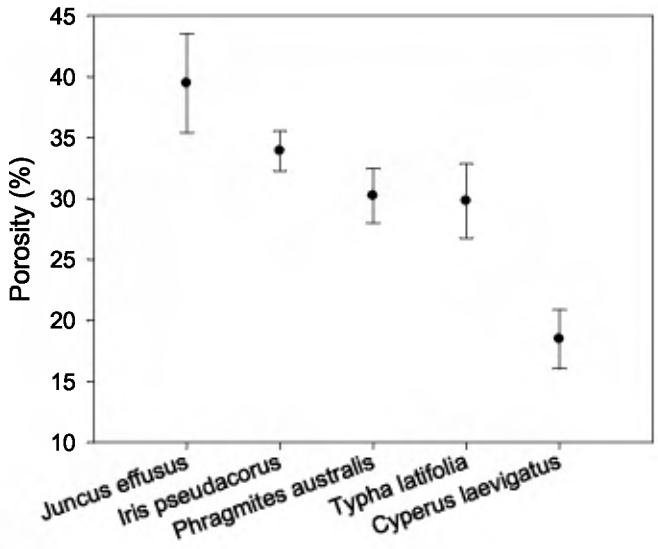


Figure 4-25: Porosity of the adventitious roots of the five wetland plants tested.

4.5 Discussion

4.5.1 Efficiency of the METland compared to conventional HSSF CW

During this four years period study, METland design was shifting according to the results and the troubleshooting detected under field operation. Finally, the METland has been operating with a 2.6 m^3 anode over a year and samples were weekly analyzed in order to generate representative data of the treatment performance. During this stage data have been grouped in two periods, corresponding to different cathodes (coke granules and rolled carbon fibre cathode) but also to different seasons. The first period took place between early April until the end of August (spring and summer seasons) and the second period corresponded to the weeks between early September and end of April (autumn and winter). Slight differences between the two periods were found with respect to COD and BOD_5 removal, but they were not significant. In the current section, we have compared the performance of METland configuration with data from real CWs reported in literature. In spite of the vast amount of literature data we have been cautious considering that a large variability exists between different CWs depending on issues such as pollutants concentration, HLR, depth, proportion length/width, plants species, climate, etc. (Kadlec and Wallace, 2009).

In a first approach, in spite of the high organic loading rates (20 g DBO_5 m^{-2} d^{-1}) METland configuration was showing higher BOD_5 removal, in comparison with

conventional CWs (Figure 4-26) reported in literature (Kadlec and Wallace, 2009). For BOD_5 inlet loads between 40 and 450 kg ha^{-1} d^{-1} the METland exhibited effluents between 5 and 28 mg L^{-1}.The analysis carried out by Kadlec and Wallace (2009) showed that for those inlet loads most of the effluent concentrations ranged between 30 and 400 mg L^{-1}. Regarding removal efficiency, in percentage, Vymazal and Kropfelova (2008) referred removal efficiencies of 81% and 63% for BOD_5 and COD, respectively, based on data from 746 and 556 HSSF wetlands with loading rates of 9.7 g BOD_5 m^{-2} d^{-1} and 23.7 g COD m^{-2} d^{-1}, respectively. The METland was operated at more than double BOD_5 inlet load and 1.5-fold the COD inlet load (Table S 4-4) and achieved higher removal efficiencies (BOD_5 = 92-88% and COD = 87-79%).

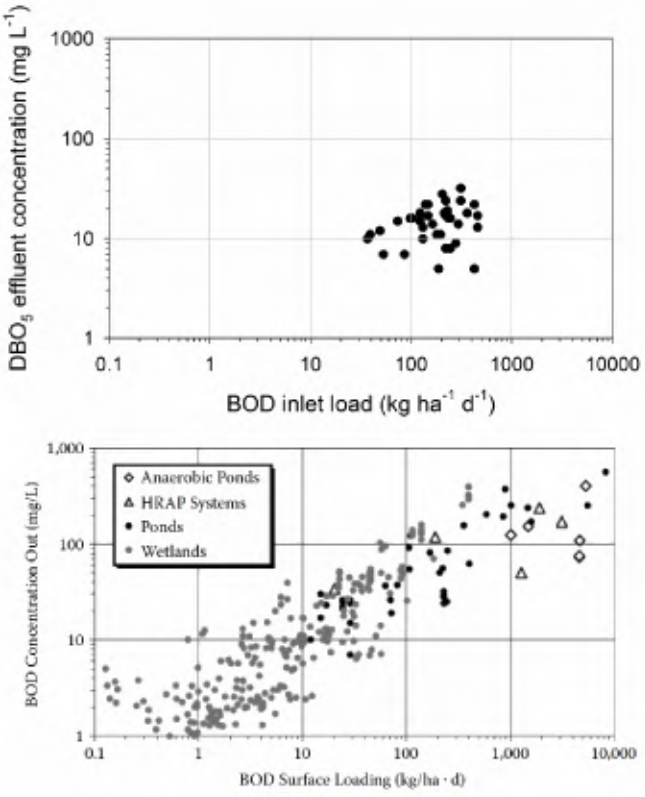


Figure 4-26: Response annual average effluent BOD_5 of WWT systems to increasing annual average BOD loadings. Top graph: Data of one year operation of our METland during the second stage (large anode); Bottom graph: Data of HSSF wetlands are represented by 265 years of data for 113 systems. From Kadlec & Wallace 2009

The longitudinal profile of BOD_5 and COD of the METland (Figure 4-27, top graph) contrast with reported data from actual longitudinal profile of BOD_5 and COD in CWs from Belgium and Czech Republic (Figure 4-27, bottom graph) (Vymazal and Kropfelova, 2008). All the graphs demonstrate that the highest removal of organics takes place within several meters of the inlet zone. Interestingly, our METland unit did remove around 65% of the COD and 70% of the BOD_5 in just 1.5 m of anodic bed (Table S 4-3), in contrast with the 3.5m reported in the case of standard CWs.

Regarding TSS, most of them were removed in the first 1.5 m of anodic bed. However, no differences have been observed when compared with conventional CWs.

Regarding total nitrogen, the nitrogen-removing mechanisms in HSSF CW are quite limited due to lack of oxygen caused by the continuous waterlogging of the bed. The lack of oxygen restricts oxidation of ammonia (nitrification) which is the major form of nitrogen in most wastewaters. Removal of TN and NH_4-N were in the range of those reported in the literature (Kadlec and Wallace, 2009; Vymazal and Kropfelova, 2008).

Phosphorus entering HSSF wetlands can be stored in either plant biomass, bed particles (generally gravels), or in accreted sediments. These storages have finite capacities, which place an upper limit on the amount of phosphorus that can be retained within HSSF. Low sorption capacity of the gravels used in conventional CW limits the phosphorous removal. The amount of phosphorus that can be adsorbed is a function of the inlet phosphorus concentration, and material properties of the bed media. Phosphorus sorption is strongly influenced by the account of calcium carbonates, aluminium oxides, and iron oxides present in the material. Coke material has shown more storage capacity than gravels. However, additional studies should be performed to study the properties of the material surface for trapping phosphorous.

4.5.2 Microbial community

While comparative taxonomic assignments based on 290 bp-long sequences suggests caution in interpreting the results, the detailed assessment of microbial community composition can provide important information on the metabolic processes occurring in any micro-environment. In this article, we present results regarding communities that acted at different (i.e. successive) points during the waterfront advance in the electrochemical wetland. Apart from wastewater samples, which were collected by filtering the wastewater feeding the wetland, all other DNA

templates were extracted from biofilms that contributed to the successful removal of contaminants.

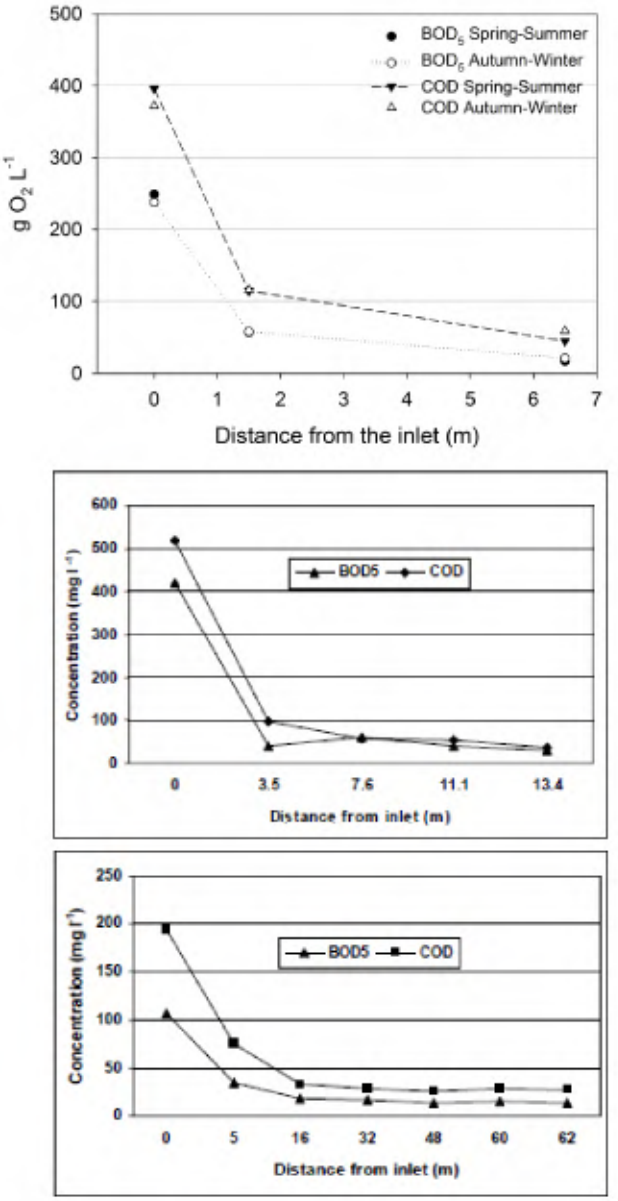


Figure 4-27: Longitudinal profiles of BOD_5 and COD. Top graph: at METland. Bottom graphs: data from Belgium and Czech Republic. From Vymazal and Kropfelova 2008.

METland was fed by wastewater primary-treated in a Imhoff tank. Interestingly, such wastewater was characterized by the dominance of *Arcobacter* sp. (49.8% of reads). This is not a genus of recognised faecal origin but it was included in the core taxa of 13 communities collected from sewer infrastructures (Shanks et al., 2013), suggesting that this bacteria is typically enriched either in the piping system or in the pre-treatment tank. Furthermore, Shanks and colleagues also identified *Tolumonas* sp., *Aeromonas* sp., *Pseudomonas* sp., *Acinetobacter* sp. and members of the *Comamonadaceae* as common sewage infrastructure inhabitants, which altogether made up 64.5% of our community. This enrichment of gamma- and beta-proteobacteria appear to be a characteristic of sewer systems (McLellan et al., 2010). On the other hand, looking at known faecal bacteria, *Bacteroides* sp. and other *Bacteroidia* constituted around 10% of reads from sample A6, while famous gut *Firmicutes* were under-represented in the wastewater analysed.

Of greater interest is the evolution of biofilm communities with the horizontal flow of wastewater through the METland. Interestingly, sample A4 was collected only 40 cm away from the feeding port and it already markedly differed from inlet wastewater sample. *Arcobacter* reduced its presence from almost 50% to 1.1% and the *Gammaproteobacteria* profile strongly shifted. While OTUs related to the *Tolumonas* sp., *Acidovorax* sp., *Thiovirga* sp., *Acinetobacter* sp., and a member of the *Aeromonadaceae* almost disappeared, *Pseudoxanthomonas* sp., a member of *Xanthomonadaceae* and *Thiothrix* sp. became dominant taxa. The latest in particular, which became the most abundant OTU, it is known for colonising flowing water containing a source of sulphide as well as for being a member of activated sludge (Williams and Unz, 1985). While the *Betaproteobacteria* remained similar between wastewater and gravel samples (A6 and A4), both in diversity and preponderance, the *Alphaproteobacteria* expanded their presence as soon as we entered the wetland. Around 11.2% of reads were tentatively classified among the orders *Rhodobacterales* and *Sphingomonadales*, known to metabolise a wide variety of carbon source as well as complex aromatic compounds (Haritash and Kaushik, 2009). Taken together, our results suggest that the *Proteobacteria*, together with the *Bacteroidetes*, were the largest groups within the microbial assemblage that performed the initial bulk degradation of pollutants in the tested METland.

A similar consideration can be made for sample A5, which was collected from the cathodic carbon cloth fibre located 110 cm from the inlet. The unusual high feeding rate made the wetland head transitory flooded, so it is not surprising that the proximity of samples A4 and A5 resulted in highly similar microbial profiles, particularly in respect to taxa proportions at the phylum and class level (Figure 4-17)

and to dominant bacteria diversity. It appeared that these two communities resembled that of a typical wetland with high organic load (Lv et al., 2014). However, the analysis of enriched, but not dominant, OTUs suggest greater differences between these samples, which is clearly evident from network configuration (Figure 4-18). Sample A5 was characterised by possessing unique (greater than 0.005%) OTUs tentatively assigned to the taxa *Erythromicrobium* sp., *Aquiflexum* sp., *Alishewanella* sp. *Acinetobacter* sp. and to *Cyclobacteriaceae* among others, but none of these have been reported to function as cathode-oxidizing bacteria. Nonetheless, it appears that the community on the cathode did not deviate much from its gravel-attached counterpart, at least in terms of most abundant taxa, which suggest that moving the cathode toward the tail area of the wetland may maximise its selective pressure on bacteria, as the nutrients get removed and other metabolic strategies need to appear.

Thus, it is reasonable to suggest that A4 and A5 communities acted as a first filter for the organic matter, which then shifted in a partially degraded state to reach following niches. Samples A3 (anode) and A1 (gravels after anode) confirmed this hypothesis as they were enriched in taxa capable of using reduced compounds as electron donors. Regarding the anode-attached community, dominating taxa were known anaerobic genera of the *Deltaproteobacteria*, the *Bacteroiidia*, the *Clostridia* and the *Euryarchaeota*. Some of the identified OTUs were fermenters, while close relatives of *Geobacter* sp., like *Syntrophus* sp. and *Desulfovibrio* sp. can couple the oxidation of organic acids using inorganic compounds as terminal electron acceptors. Members of the *Geobacteriaceae* are known to reduce insoluble materials like Fe-oxides but also to be electroactive (Lovley et al., 2011). Interestingly, *Geobacter* was abundant in the METland and therefore was facilitating the removal of fermentation products from the wastewater. Furthermore, electroactive bacteria have been reported to grow syntrophically with methanogens such as *Methanobacterium* sp. and *Methanosaeta* sp. using either hydrogen or conductive material as electron shuttle between the species involved (Call et al., 2009; Kato et al., 2012; Zhao et al., 2016). The anodic community was composed of many of these syntrophic taxa, suggesting that the presence of conductive material stimulated microbial activity in the reducing environment, favouring the WWT process. Furthermore, the importance of the class *Synergistia* in this assemblage deserves extra attention.

Finally, sample A1 represents the biofilm colonising gravel at 400 cm from the inlet port. This community shared more OTUs with the anode-attached biofilm than with samples A4 or A5, pointing out that the degradation state of wastewater affected

taxa selection more than the inert material the communities grew on. The dominant OTU in this case (24.1% of reads) was tentatively assigned to *Pelobacter* sp., a member of *Deltaproteobacteria* closely related to *Geobacter* spp., known to use organic acids as electron donors while reducing iron and sulphur. However, it has been reported that *Pelobacter* spp. lack many cytochromes typical of other *Geobacteriaceae*, and that their metabolism is prevalently fermentative than respiratory (Lonergan et al., 1996). Another detected difference with sample A3 is that the post-electrode community saw a strong decrease in *Bacteroidiia* and *Synergistia*, equilibrated by an increase in *Betaproteobacteria*, and particularly of an OTU similar to *Thiobacillus* sp. This genus is known to be composed of obligate autotrophic organisms that need oxygen to grow. Interestingly, the sequencing-based suggestion that strict aerobes and anaerobes co-occurred pointed out a possible stratification within the community, where *Thiobacillus*-like species may occupy the external layer of the biofilm while *Pelobacter*-like species may reside the internal ones. If this was the case, it is evident that the evolution of an electron shuttling mechanism would provide advantageous for both members of the consortium. On the other side, many *Thiobacillus* spp. have genes for denitrification. Considering the wastewater characteristics at this stage, it is likely that denitrifiers were enriched, suggesting that a more efficient treatment at the beginning of the wetland facilitated other important processes to occur in following zones, altogether improving the quality of the effluent.

Community for the inlet wastewater presented the same profile regardless the set of sequencing. However, the community around the inlet port area changed with respect to the first sampling due to the material change (from gravel to electroconductive material) to enlarge the anode. Thus, anodic communities were enriched in taxa capable of using reduced compounds as electron donors. Majority OTUs were members of the *Geobacteraceae* and *Pelobacteraceae* (closely related to *Geobacter*), together with *Thiobacillus* that were also main taxa in the first analysis. In contrast, a lower percentage of *Archaea* in comparison with the first sequencing (*Methanobacteria* 0.02-0.2% and *Methanomicrobia* 0.2-0.7%) was observed.

Communities associated to the cathode were different from the first sequencing due to both the new nature of the electroconductive material (coke granules instead of carbon cloth) and its farthest location to the inlet port. The presence of *Geobacter* confirmed the positive effect that conductive material exerts over electroactive bacteria, even in areas where a noticeable concentration of oxygen could exist. This is not surprising because *Geobacter*, although wrongly

classified as anaerobe can actually use oxygen as electron acceptor at 0-1 mg L^{-1} (Lin et al., 2004). The *Hydrogenophilaceae* (the most abundant family in the cathode) are a family of Betaproteobacteria, with two genera, *Hydrogenophilus* and *Thiobacillus*. In this case all the members of the family correspond to the second genus. One interesting finding was the presence of species related to Anammox, as *Candidatus Brocadi*. Ammonia oxidation is also performed by Anammox organisms under anaerobic conditions. *Nitrospiraceae* is the only established family in the phylum *Nitrospirae* and comprises chemolithoautotrophic aerobic nitrite-oxidizing bacteria (*Nitrospira*), chemolithoautotrophic aerobic and acidophilic ferrous iron oxidizers (*Leptospirillum*), and anaerobic, thermophilic, chemoorganoheterotrophic or hydrogenotrophic sulphate reducers (*Thermodesulfovibrio*). More than 1% of a genus of this family (GOUTA 19) has been detected, as Nitrospira, a genus that is almost ubiquitously distributed in oxic habitats and represents the predominant known nitrite oxidizers in nature, which catalyze the second step of nitrification and thus are essential for biogeochemical nitrogen cycling. The genus *Nitrospira* contains the key nitrite oxidizers in biological wastewater treatment plants (Daims, 2014). The *Nitrosomonadaceae* (also found in the cathode samples) comprise a monophyletic phylogenetic group within the *Betaproteobacteria*, all of whose cultivated representatives are lithoautotrophic ammonia oxidizers. Ammonia oxidizers generally exert control over nitrification by oxidizing ammonia to nitrite, which is subsequently oxidized by bacterial nitrite oxidizers to nitrate (Prosser et al., 2014). Due to the slow growth of these bacteria and their sensitivity to environmental conditions, nitrification has been often considered one of the most unreliable and unpredictable processes of WWTPs.

Heat maps revealed that ammonium oxidizing activity was performed in the cathode because lower levels of ammonium were detected (Figure 4-28). Interestingly, chemolithoautotrophic ammonium oxidation requires oxygen which was indeed detected in the cathode (Figure 4-29) and provided by plants through their roots.

As a consequence of the nitrifying activity, an increase of nitrate levels was observed at the cathode area. However, nitrate concentrations became low again in the gravel area around the cathode where roots exudates could provide organic matter (Figure 4-30). Denitrification constitutes the microbial respiration of nitrate or nitrite to N_2 or N_2O, and it is carried out by a phylogenetically diverse group of bacteria, generally under anaerobic conditions. *Thiobacillus*, a known denitrifier genus was detected in the cathode.

As expected, microbial community associated to gravel bed after the anode showed the same community profile regardless the size of the anode, i.e. similar taxa appeared.

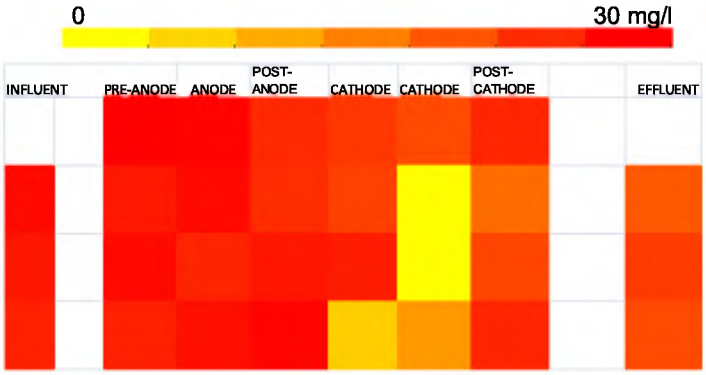


Figure 4-28: Ammonium heat-map of the METland at the second stage.

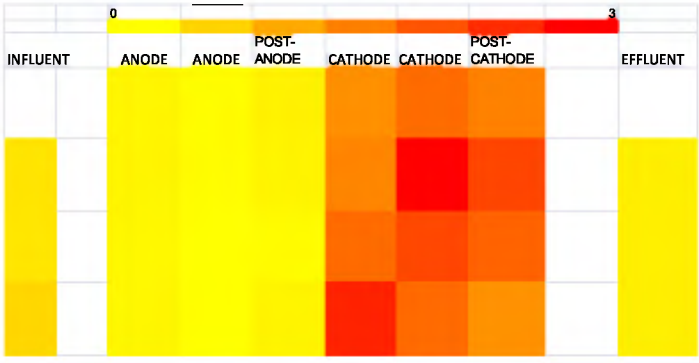


Figure 4-29: Dissolved oxygen heat-map of the METland at the second stage.

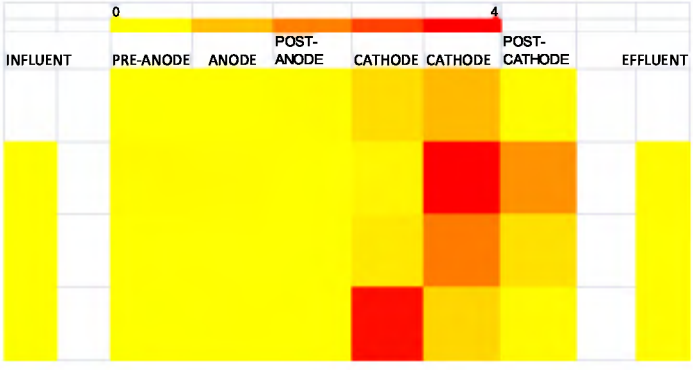


Figure 4-30: Nitrate distribution in the METland in the second stage.

4.5.3 Oxygen released by wetland plants

The root system of a plant is composed of the primary, lateral and adventitious roots. Lateral roots always develop from roots, whereas adventitious roots form from stem or leaf derived cells. Adventitious roots formation is part of the normal development of the plant and occurs naturally, like in most monocotyledonous for which they constitute the main root system or in many dicotyledonous species that propagate vegetatively. Adventitious rooting is an essential step for vegetative propagation (Bellini, 2001).

The roots of plant species native to wetlands usually display distinct morphological and anatomical characteristics that enable them to withstand the adverse conditions in water-saturated soils (Kawase, 1981). Common adaptations are large root diameters and high porosity of the cortical cell layers. In many wetland plants, aerenchyma is well developed even in drained conditions, and can be further enhanced in waterlogged conditions (Justin and Armstrong, 1987; Smirnoff and Crawford, 1983). Aerenchyma provides a low resistance internal pathway for the movement of O_2 (and other gases) between shoot and root extremities. Porosity (gas volume/tissue volume) in plant tissues can differ markedly between species. This results from the constitutive intercellular gas-filled spaces (Raven, 1996) and can be further enhanced by formation of aerenchyma (Armstrong, 1979).

Species possessing an internal convective through-flow ventilation system have higher internal oxygen concentrations in the rhizomes and roots than species relying exclusively on diffusive transfer of oxygen. Wetland plants with a convective through-flow mechanism therefore have the potential to release more oxygen from their roots compared to species without convective through-flow (Brix, 1994).

Brix et al., 1992 demonstrated that the resistance to airflow at the stem-rhizome junction was very high for some species, resulting in a low ability to convert internal pressurization into convective airflow through the rhizomes. Species with a high potential for internal pressurization and a low internal resistance to convective flow seem to have a competitive advantage over species that rely exclusively on diffusive gas transport, which allows them to grow in deeper waters. In this study, *P. australis* resulted to be the species with the highest efficiency for convective through-flow and the lowest resistance, whereas *Juncus* and *Cyperus* exhibited high resistance in the junctions between aerial culms and rhizomes.

Our results are consistent with the results of some studies that compare ROL in several plant species (Brix et al., 1992; Brix and Schierup, 1990). Other factors such as rooting depth, tolerance to high loads of water, plant productivity, etc., also

have to be taken into consideration when considering the suitability of plant species in constructed wetlands (Brix, 1994) *P. australis* is one of the most used species in CW due to their robustness and good adaptation to wastewater treatment (Gagnon et al., 2013; García et al., 2010) showing the highest ROL rates. Regarding the use of exotic species, every geographic zone should be studied to avoid planting invasive species considering the corresponding legislation.

In any case plant-mediated oxygen transfer has long been a subject of debate. Research to quantify rates of oxygen release by plants have led to the general consensus that plants do not release enough oxygen into their immediate root environment to remove all of the organic matter in standard urban waste- water in horizontal or vertical subsurface flow constructed wetlands (Brix, 1997; Nivala et al., 2013b). Oxygen is consumed very rapidly in the wetlands by several processes including aerobic respiration, nitrification and sulphide oxidation (Tyroller et al., 2010). Thus, it is difficult to predict if plants could release enough oxygen to improve the MET performance, thus more research would be necessary to assess this issue.

4.5.4 Design parameters

It is very easy to compare the inlet and outlet concentrations of pollutants of a wetland, and to calculate the percentage difference. Unfortunately, this information is of very limited use in design or in performance predictions, because it reflects none of the features of the ecosystem, which are the target of design (Kadlec and Wallace, 2009).

To design CW it is necessary to know:

- Average flow rate Q ($m^3\ d^{-1}$). For dimensioning the pretreatment and the primary treatment it would be also necessary to know the maximum and the minimum water flow.
- Concentration of BOD_5 and TN in wastewater in mg L^{-1}. This last if it is necessary.
- Concentration of BOD_5 and TN to be achieved in mg L^{-1}.
- Operation temperature (ºC): it is generally used the mean temperature of the coldest month.

The main variables to design are:

- Hydraulic retention time (HRT) in days
- Water depth inside the wetland (d) in metres
- Geometry: length (L) and width (W) in metres

- Surface organic loading rate (OLR) in g DBO_5 m^{-2} d^{-1}
- Surface hydraulic loading rate (HLR) in m^3 m^{-2} d^{-1} (m d^{-1})

Table 4-3 show the typical design parameters of the horizontal and vertical SSF CW. At present, the most commonly used models for the design of CW start from the basis of considering them as plug flow reactors, which follow first order kinetics for the elimination of the different pollutants.

Table 4-3: Design parameters of CW of subsurface flow (SSF)

Parameter	Value	
	Horizontal	Vertical
Surface organic loading rate (OLR) (g DBO_5 m^{-2} d^{-1})	6	20
Mean water depth inside the wetland	0,4-0,6	0,5-0,8

The Reed model (1995) use equations in which reaction constants by volume unit are considered, dependent of the temperature. The basic equation to calculate the removal of contaminants, DBO_5 as total nitrogen, ammonium and nitrate is:

$$\ln\left({}^{C_e}/_{C_i}\right) = K_t \cdot t \qquad [7]$$

where:

C_e: pollutant concentration in the effluent (mg L^{-1})

C_i : pollutant concentration in the influent (mg L^{-1})

K_t: reaction constant (d^{-1})

t: hydraulic retention time (d)

Taking into account that the hydraulic retention time is defined by the relationship between the volume filled with water inside the wetland and the feeding flow rate:

$$t = {}^{V_e}/_{Q_m} = S \cdot h \cdot {}^{\varphi_s}/_{Q_m} \qquad [8]$$

where:

V_e: effective volume (m^3)

Q_m: average feeding flow rate ($m^3\ d^{-1}$)

S: wetland surface (m^2)

h: water depth (m)

φ_s: porosity of the filtering substrate (given as a fraction of unity). In the case of SSF CW porosity varies in function of the size of the substrate. In our METland, porosity of the granular coke was 0.4 and the gravel 0.35

The dependence of the constant of the reaction on the temperature is given by the next expression:

$$K_t = K_R \theta_R^{(T_w - T_r)} \qquad [9]$$

where:

K_R = constant of the reaction at the reference temperature (d^{-1}).

T_w = temperature of water considered in the design (°C). It uses to be the average of the coldest month.

T_r = reference temperature at which θ_R has been calculated (°C). It uses to be 20 °C.

θ_R = temperature coefficient.

Table 4-4 shows the values of K_R and θ_R for every type of contaminant in SSF CW. Combining equations [7] and [8] the equation to calculate the needed surface in a CW is the following:

$$S = L \times W = \frac{Q_m \cdot t}{h \cdot \varphi_s} = \frac{Q_m \cdot \ln\left(C_i / C_e\right)}{K_t \cdot h \cdot \varphi_s} \qquad [10]$$

where:

L = length of the CW (m)

W = width of the CW (m)

A new kinetic constant of first order K_A can be defined from the last equation:

$$K_A = K_t \cdot h \cdot \varphi_s \qquad [11]$$

Table 4-4: Values of K_R and θ_R for every type of contaminant in SSF CW

	BOD_5	NH_4 (nitrification)	NO_3 (denitrification)
K_r (d^{-1})	1,104	0,01854 + 0,3922 $(h_r)^{2,6077}$	1
θ_R	1,06	1,048	1,15

And so, equation 10 would be like this:

$$S = \frac{Q_m}{K_A} ln\left[\frac{C_i}{C_e}\right] \qquad [12]$$

Knowing the flow rate, concentrations of the pollutant at the influent and the effluent and surface of the system, the K_A can be estimated. The K_A value is generally referred to BOD_5 and several authors have established different values. Schierup et al. (1990) established a value of 30.30 m yr^{-1}, Vymazal and Kropfelova (2008) 31.76 m yr^{-1}, Kadlec (2009) between 20 and 60 m yr^{-1} and García et al. (2004) 33.2 m yr^{-1}. It is a variable value that depends of the HLR, the OLR and the years of operation of the system (Brix, 1998). When BOD is removed, suspended matter will be removed too, due to the fact that CWs are more effective eliminating suspended matter than BOD (Garcia and Corzo, 2008). The value of K_A will logically vary depending on the pollutant and can be also calculated for TN.

This dimensioning allows a TN reduction of around 30%. For the efficient removal of ammonia and phosphorus it is necessary to choose the reaction constant considerably lower, about 0.025 m d^{-1} (Garcia and Corzo, 2008; Vymazal and Kropfelova, 2008) Those values are useful for concentrations in the influent under 250 mg BOD L^{-1}.

The parameter K_A has been calculated from the experimental data for every period for two pollutants during the two stages (small and large anode), BOD_5 and TN, and the results are shown in Table 4-5. HLR was 0.083 m d^{-1} and did not vary throughout the period of study, without taking into account the variations in the permeability of the wetland. HRT was 2.5 d.

BOD_5 K_A values are much higher than those reported in literature, except in period 1 (Table 4-5). During the rest of the first stage (0.9 m^3-anode) the K_{BOD5} values ranged between 53.35 and 89.38 m d^{-1}, with an average K_{BOD5} of 73.26±8.23 m yr^{-1}, being more than double. During the second stage (2.6 m^3-anode) the K_{BOD5} average value was 80.15±18.21 m yr^{-1} (0.22 m d^{-1}). This high BOD rate constant led

to a similar TN removal than conventional CWs (ca. 30%). This means that the efficiency of the METland for BOD_5 is much higher than a conventional CW, especially when the anode was enlarged, revealing the important contribution of the electroconductive bed to the organic removal processes.

TN K_A values were very variable during the first stage (0.9 m^3-anode) but reached a more stable value during the second stage (2.6 m^3-anode). This could be due to the bad performance of the gravel bed located by the inlet port during the first stage. During the second stage the average K_{TN} calculated from our experimental data was 0.029 m d^{-1} (10.66±0.51 m yr^{-1}) that is in the range of the rate constant recommended for the removal of ammonium, 0.025 m d^{-1} (9 m yr^{-1}).

Table 4-5: K_A values of BOD and TN calculated from experimental data for every period. OLR = organic loading rate; TNLR = total nitrogen loading rate. Average water temperature is shown in top table.

Stage	Period	Weeks	OLR (gm^2 d^{-1})	C_i (mg L^{-1})	C_e (mg L^{-1})	K_A (m d^{-1})	K_A (m yr^{-1})	water T (ºC)
0.9 m^3 anode	1	11-21	20	247	69	0.106	38.79	18.6±2.3
	2	22-47	24	300	29	0.195	71.07	24.5±3.0
	3	48-51	11	170	9	0.245	89.38	16.9±1.3
	4	52-71	7	90	9	0.192	70.04	15.6±2.2
	6	75-94	19	234	18	0.214	78.02	25.7±2.1
	7	95-103	21	260	45	0.146	53.35	17.1±3.1
2.6 m^3 anode	9	119-138	20	249	17	0.224	81.65	22.5±2.7
	10	139-166	20	238	21	0.202	73.84	17.4±4.9

Stage	Period	Weeks	TNLR (gm^2 d^{-1})	C_i (mg L^{-1})	C_e (mg L^{-1})	K_A (m d^{-1})	K_A (m yr^{-1})
0.9 m^3 anode	1	11-21	3.8	47	27.9	0.043	15.86
	2	22-47	4.8	58.4	43.4	0.025	9.03
	3	48-51	4	42.9	19.8	0.064	23.52
	4	52-71	3.3	40.7	16.1	0.077	28.21
	6	75-94	4.3	53.2	36.7	0.031	11.29
	7	95-103	4.6	56.1	47.1	0.015	5.32
2.6m^3 anode	9	119-138	4.3	52.9	37.7	0.028	10.30
	10	139-166	4.5	53.3	37.1	0.030	11.02

Average values (±SD) of both K_A are represented in figures 4-33 and 4-34. Our results revealed that using a 2.6 m^3-anode, both the OLR and the K_{BOD5} increased until reaching steady state values of ca. 100 m yr^{-1}. Regarding TN, the same pattern was found except for TNLR values lower than 3 g m^{-2} d^{-1}.

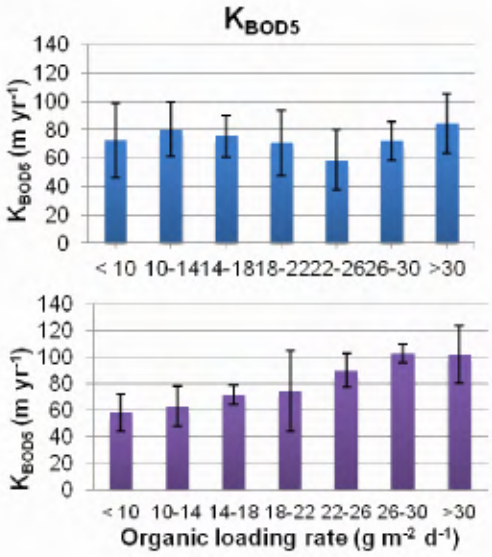


Figure 4-31: Average K_{BOD5} during the **first stage** (top graph) and the second stage (bottom graph) depending on the organic loading rate, calculated from experimental data.

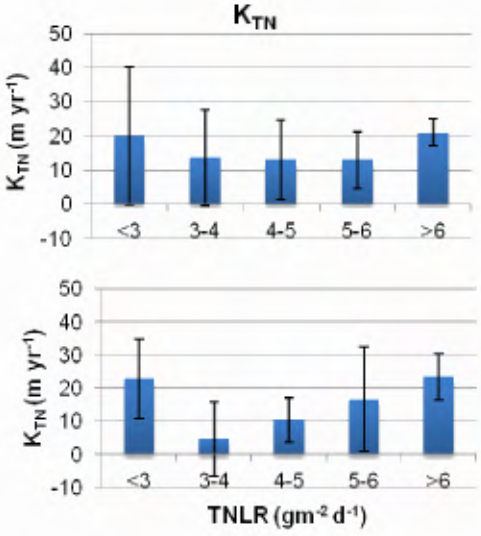


Figure 4-32: Average K_{TN} during the **first stage** (top graph) and the **second stage** (bottom graph) depending on the TN loading rate, calculated from experimental data.

4.6 Conclusions

METland constitutes a new concept in WWT. The results show that microbial electrochemical systems can successfully improve the performance of HSSF CWs in terms of pollutants removal due to the stimulation of electroactive bacteria. Interestingly, the METland design fulfilled the discharge requirements established in the legislation, in spite of being subjected to an organic loading rate more than 3-fold the recommended.

4.7 Supplementary information

Table S 4-1: Characteristics of influent wastewater after the Imhoff tank (average ± SD)

Parameter	Value
pH	7.2 ± 0.4
Conductivity (µS/cm)	1,335 ± 307
Temperature range (°C)	11 – 30
Dissolved oxygen (mg/l)	1.0 ± 0.6
Redox potential (ORP)	-175 ± 55
Total suspended solids (TSS) (mg/l)	166 ± 111
Volatile suspended solids (VSS) (mg/l)	117 ± 58
BOD_5 (mg/l)	238 ± 134
COD (mg/l)	393 ± 198
Total nitrogen (TN) (mg N/l)	51.7 ± 13.4
Ammonium nitrogen (NH_4N) (mg N/l)	41.4 ± 15.5
Nitrate nitrogen (NO_3N) (mg N/l)	2.0 ± 4.8
Total phosphorous (TP) (mg P/l)	7.1± 3.0
Phosphates (PO_4P) (mg P/l)	5.0 ± 2.0

Table S 4-2: Limits of discharge established in the Directive 91/271/CEE.

WWT	SS	BOD_5	COD	NH_4N	TP
Primary	> 50 %	> 20 %			
Secondary	< 35 mg L^{-1} > 90 %	< 25 mg L^{-1} > 70 %	< 125 mg L^{-1} > 75 %		
Secondary with nitrogen and phosphorous removal				< 15 mg L^{-1}	< 2 mg L^{-1}

Table S 4-3: COD and BOD_5 concentrations (in mg L^{-1}) of treated wastewater in sample points at different periods (average ± SD).

Concentration (mg/L)		Period 1 (11-21)	Period 2 (22-47)	Period 3 (48-51)	Period 4 (52-71)	Period 6 (75-94)	Period 7 (95-103)	Period 9 (119-138)	Period 10 (139-166)
Influent (Imhoff tank effluent)	COD	396±103	405±109	285±49	203±116	403±39	456±58	396±163	372±190
	BOD_5	247±61	300±111	170±18	90±61	234±30	260±65	249±121	238±144
Point 2 (before anode)	COD	379±101	353±110	218±25	185±114	302±63	465±122	--	--
	BOD_5	225±58	237±71	136±5	87±69	155±31	269±62	--	--
Point 5 (after anode)	COD	301±92	184±46	178±45	152±78	161±94	387±58	115±22	116±35
	BOD_5	162±42	101±43	111±21	66±44	82±55	209±65	57±16	58±24
Effluent	COD	136±72	76±29	38±18	38±29	55±27	134±80	45±11	59±28
	BOD_5	69±36	29±20	9±2	9±9	18±6	45±29	17±6	21±18

Table S 4-4: COD and BOD_5 removal rates in every region of the METland in grams per cubic meter of bed material and day (g m^{-3} d^{-1}), according to the period (average ± SD). Surface and volumetric loading rates are given in grams per square meter (g m^{-2} d^{-1}) or per cubic meter (g m^{-3} d^{-1}) of the total wetland and day, respectively.

Period		Surface loading rate gm^{-2} d^{-1}	Volumetric loading rate gm^{-3} d^{-1}	Initial gravel removal rate gm^{-3} d^{-1}	Anode removal rate gm^{-3} d^{-1}	Final gravel removal rate gm^{-3} d^{-1}	Total removal rate gm^{-3} d^{-1}
Period 1 (11-21)	COD	32±8	54±14	17±26	177±122	29±11	35±10
	BOD_5	21±5	34±8	21±6	143±24	16±2	24±7
Period 2 (22-47)	COD	33±9	55±15	57±13	369±196	19±6	45±14
	BOD_5	25±9	41±15	63±16	316±173	13±1	37±3
Period 3 (48-51)	COD	23±4	39±7	64±37	92±41	24±10	51±4
	BOD_5	14±2	19±1	0±14	146±33	17±2	17±1
Period 4 (52-71)	COD	17±9	29±16	18±12	93±30	19±3	23±4
	BOD_5	8±5	12±2	3±5	54±17	10±2	11±1
Period 6 (75-94)	COD	33±3	55±5	81±34	323±52	18±3	47±5
	BOD_5	19±2	32±4	75±23	168±28	11±2	29±4
Period 7 (95-103)	COD	37±5	62±8	22±57	105±37	44±13	44±10
	BOD_5	22±5	35±3	20±26	126±29	28±8	29±6
Period 9 (119-138)	COD	32±13	54±22	--	214±125	12±4	48±22
	BOD_5	21±10	34±4	--	147±24	7±3	32±4
Period 10 (139-166)	COD	32±16	53±26	--	206±138	10±6	44±25
	BOD_5	20±12	33±4	--	142±22	6±1	30±4

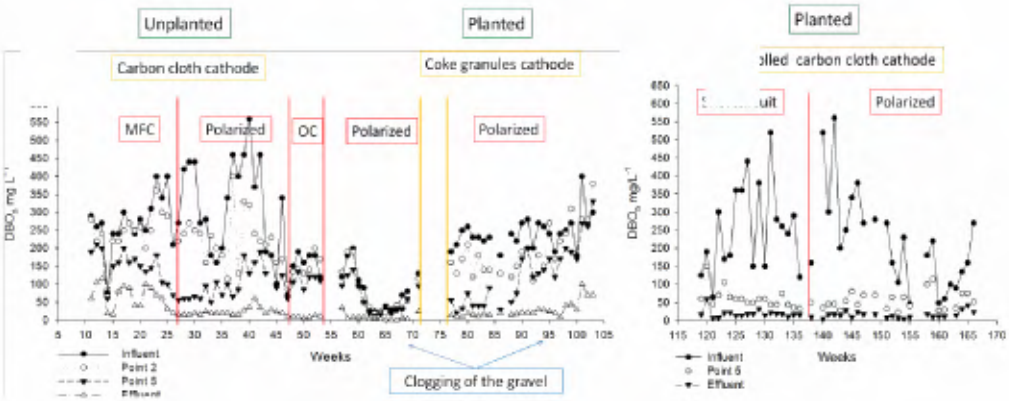


Figure S 4-1: BOD_5 evolution throughout the period of study. The results have been divided in two big stages: a first period where the system was operating with a 0.9 m^3-anode (A) and a second period with a 2.6 m^3-anode (B).

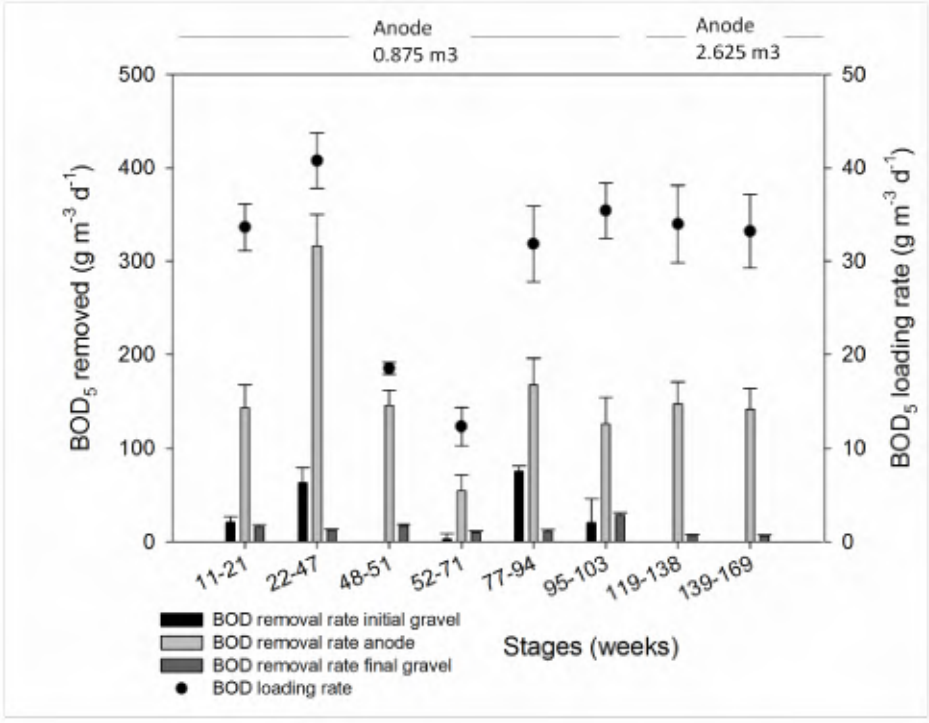


Figure S 4-2: BOD_5 average removal rates through the period of study in different regions of the METland, referred to the volume of each respective region. COD loading rate is referred to the total volume of the METland. Error bars represent 95% confidence interval.

Table S 4-5: Total suspended solids (TSS) concentrations in mg L^{-1} of treated wastewater in every sample point at different stages (average ± SD).

TSS (mg/L)	Period1 (11-21)	Period2 (22-47)	Period 3 (48-51)	Period 4 (52-71)	Period 6 (75-94)	Period 7 (95-103)	Period 9 (119-138)	Period 10 (139-166)
Influent (Imhoff tank)	119±34	185±98	180±55	89±75	82±19	133±37	202±152	151±105
Point 2 (after initial gravel)	126±54	93±43	76±17	56±34	76±32	164±73	--	--
Point 5 (after anode)	106±16	36±14	51±12	44±22	35±23	111±70	28±14	35±5
Effluent	38±9	18±10	6±1	7±4	18±5	22±3	22±3	24±4

Table S 4-6: Nitrogen (in the form of total nitrogen and ammonium nitrogen) removed in percentage referred to the influent, in every area of the METland at different periods (average ± SD).

% N removed		Period1 (11-21)	Period2 (22-47)	Period 3 (48-51)	Period 4 (52-71)	Period 6 (75-94)	Period 7 (95-103)	Period 9 (119-138)	Period 10 (139-166)
Initial Gravel	TN	0±1	0±6	13±4	13±4	0±2	0±2	--	--
	NH_4N	1±1	0±15	0±21	0±11	0±9	0±5	--	--
Anode	TN	5±3	8±2	8±2	9±3	15±5	1±4	0±5	10±4
	NH_4N	4±3	5±9	1±10	6±4	7±9	1±3	0±7	0±6
Final Gravel	TN	35±13	12±2	40±2	38±5	24±6	18±4	33±4	20±7
	NH_4N	38±16	14±3	64±17	40±8	25±10	21±6	25±8	22±7
Total	TN	40±16	21±5	49±11	60±6	37±6	16±5	29±6	29±7
	NH_4N	43±16	18±14	45±12	41±9	29±16	18±6	22±7	20±8

Table S 4-7: TP removed in percentage, referring to the influent, in every region of the METland at different periods (average ± SD).

% TP removed	Period1 (11-21)	Period2 (22-47)	Period 3 (48-51)	Period 4 (52-71)	Period 6 (77-94)	Period 7 (95-103)	Period 9 (119-138)	Period 10 (139-169)
Initial Gravel	0±9	4±24	10±12	20±25	0±7	4±8	--	--
Anode	8±10	11±10	6±6	5±8	10±13	10±9	12±4	21±6
Final Gravel	55±16	21±15	48±1	1±14	32±24	20±7	44±5	9±9
Total	61±23	35±24	64±7	25±15	41±28	34±7	56±5	30±8

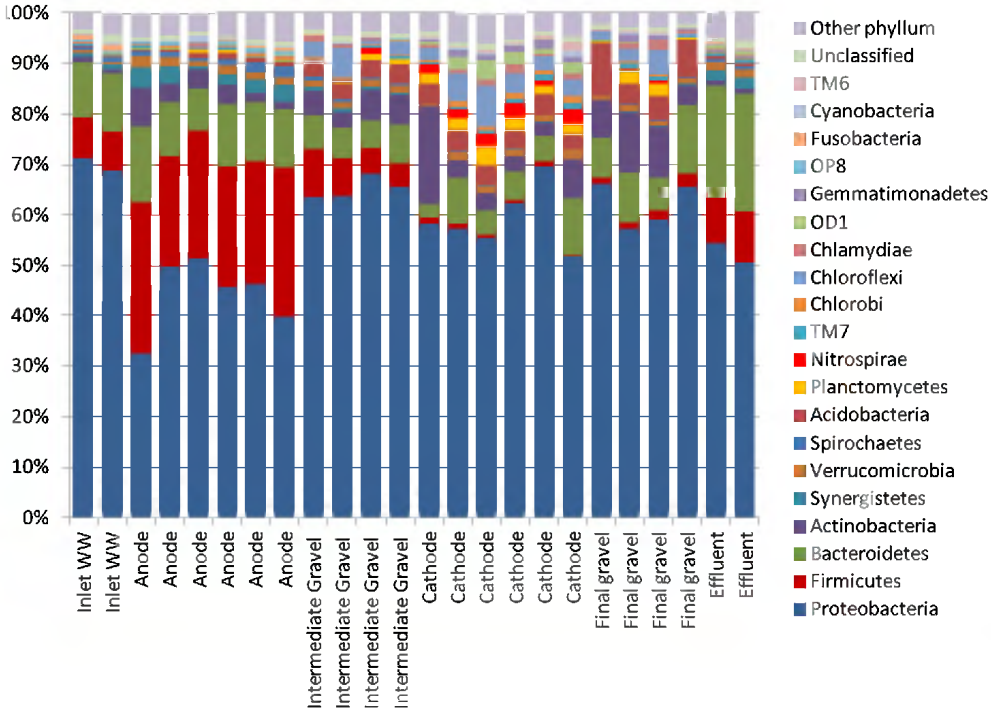


Figure S 4-3: Groups of OTUs at phylum level of the second stage 16S rDNA sequencing.

Table S 4-8: Dominant bacteria genera in the different environments of the METland in the first stage 16S rDNA sequencing (in %). Legend: * = p_ phylum; c_ class; o_ order; f_ family; g_ genus

Best taxonomic affiliation*	phylum/class	Waste Water	Pre-electrodes	Cathode	Anode	Post-electrodes
g_Arcobacter	*Epsilonproteobacteria*	49.8	1.1	4.1	1.1	0.2
g_Bacteroides	*Bacteroidia*	4.2	0.0	0.2	0.0	0.0
g_Parabacteroides	*Bacteroidia*	2.3	0.2	0.5	0.3	0.1
f_Aeromonadaceae	*Gammaproteobacteria*	1.2	0.0	1.3	0.0	0.0
g_Tolumonas	*Gammaproteobacteria*	1.0	0.0	0.0	0.0	0.0
g_Acidovorax	*Betaproteobacteria*	3.0	0.5	1.4	0.2	0.1
g_Thiovirga	*Gammaproteobacteria*	2.3	0.0	0.1	0.0	0.0
g_Acinetobacter	*Gammaproteobacteria*	1.6	0.0	0.7	0.0	0.0
f_Pseudomonadaceae	*Gammaproteobacteria*	3.8	0.7	0.7	0.1	0.1
f_Comamonadaceae	*Betaproteobacteria*	5.1	5.7	2.6	0.4	0.8
c_Betaproteobacteria	*Betaproteobacteria*	2.0	3.0	2.0	0.1	0.8
f_Flavobacteriaceae	*Flavobacteriia*	1.1	2.3	3.0	0.0	0.0
o_Bacteroidales	*Bacteroidia*	3.1	1.9	2.8	7.5	1.1
g_Sulfurimonas	*Epsilonproteobacteria*	1.2	1.5	0.0	0.1	0.6
f_Flammeovirgaceae	*Flavobacteriia*	0.0	1.1	0.0	0.0	0.5
c_Sphingobacteriia	*Sphingobacteriia*	0.0	1.3	0.1	0.0	0.0
g_Microbacterium	*Actinobacteria*	0.0	1.3	0.3	0.1	0.1
f_Pirellulaceae	*Planctomycetes*	0.0	3.6	0.7	1.5	0.5
f_Rhodocyclaceae	*Betaproteobacteria*	0.7	3.0	0.9	0.1	4.1
o_Rhizobiales	*Alphaproteobacteria*	0.0	1.2	0.7	0.1	0.2
o_Sphingomonadales	*Alphaproteobacteria*	0.0	1.4	0.5	0.0	0.0
o_Sphingomonadales	*Alphaproteobacteria*	0.0	1.0	0.6	0.0	0.0
g_Pseudoxanthomonas	*Gammaproteobacteria*	0.0	1.7	0.3	0.0	0.0
g_vadinCA02	*Synergistetes*	0.3	2.1	0.7	2.9	0.9
g_Sphingopyxis	*Alphaproteobacteria*	0.0	1.6	0.5	0.0	0.1
g_Hydrogenophaga	*Betaproteobacteria*	0.5	1.6	3.1	0.0	0.1
f_Rhodobacteraceae	*Alphaproteobacteria*	0.2	2.6	2.8	0.1	0.2
g_Rhodobacter	*Alphaproteobacteria*	0.1	3.3	6.5	0.1	0.2
g_Flavobacterium	*Flavobacteriia*	0.7	3.3	4.0	0.0	0.1
f_Pelobacteraceae	*Deltaproteobacteria*	0.0	1.5	2.4	1.3	24.1
g_Thiothrix	*Gammaproteobacteria*	0.0	7.2	9.1	0.0	0.1
f_Xanthomonadaceae	*Gammaproteobacteria*	0.1	4.0	2.5	0.0	0.1
g_Phycicoccus	*Actinobacteria*	0.0	0.7	1.4	0.0	0.0
f_Cyclobacteriaceae	*Flavobacteriia*	0.0	0.0	1.5	0.0	0.0
f_Sphingomonadaceae	*Alphaproteobacteria*	0.0	0.6	2.1	0.0	0.1
g_Novosphingobium	*Alphaproteobacteria*	0.0	0.7	2.2	0.0	0.0

g_Arenimonas	*Gammaproteobacteria*	0.0	0.1	2.0	0.0	0.0
g_Luteolibacter	*Verrucomicrobia*	0.0	0.1	1.0	0.0	0.0
g_Desulfomicrobium	*Deltaproteobacteria*	0.2	0.9	2.1	1.7	0.2
g_Methanobacterium	*Euryarchaeota*	0.0	0.0	0.1	3.7	0.2
g_Methanosaeta	*Euryarchaeota*	0.0	0.2	0.1	1.4	0.1
f_Holophagaceae	*Acidobacteria*	0.0	0.1	0.0	2.2	0.1
o_SJA-36	*Acidobacteria*	0.0	0.8	0.3	1.4	0.6
p_Bacteroidetes	*Bacteroidetes*	0.0	0.3	0.2	1.5	0.2
g_T780	*Chloroflexi*	0.0	0.0	0.0	2.6	1.7
f_Clostridiaceae	*Firmicutes*	0.1	0.1	0.4	1.3	0.4
f_Peptostreptococcaceae	*Firmicutes*	0.2	0.2	0.3	1.3	0.2
g_Desulfovibrio	*Deltaproteobacteria*	0.2	0.1	0.1	4.6	0.6
f_Geobacteraceae	*Deltaproteobacteria*	0.5	0.5	0.1	3.3	0.1
p_OP8	*OP8*	0.1	0.1	0.1	3.2	0.4
g_Syntrophus	*Deltaproteobacteria*	0.0	0.0	0.0	2.7	0.6
o_Synergistales	*Synergistetes*	0.0	0.1	0.0	1.0	0.1
g_HA73	*Synergistetes*	0.0	0.6	0.4	3.3	0.9
g_PD-UASB-13	*Synergistetes*	0.0	0.3	0.1	2.8	0.2
g_E6	*Synergistetes*	0.0	0.3	0.1	1.6	0.3
o_GN03	*WS3*	0.0	0.1	0.1	1.1	0.3
o_GW-28	*Deltaproteobacteria*	0.0	0.0	0.0	2.2	0.3
c_Phycisphaerae	*Planctomycetes*	0.0	0.1	0.0	1.1	2.2
o_Alteromonadales	*Gammaproteobacteria*	0.0	0.2	0.3	0.0	2.3
c_Leptospirae	*Spirochaetes*	0.1	0.0	0.0	0.5	1.5
c_Streptophyta	*Cyanobacteria*	0.0	0.0	0.0	0.1	1.3
f_KSB4	*WS3*	0.0	0.1	0.0	0.2	2.1
f_Coriobacteriaceae	*Firmicutes*	0.0	0.0	0.0	0.2	1.9
g_Thiobacillus	*Betaproteobacteria*	0.0	0.4	0.5	0.0	8.0
o_Thiobacterales	*Betaproteobacteria*	0.0	0.1	0.1	0.0	2.6

Table S 4-9: Dominant bacteria **family** (more than 1% in some sample) in the **anode** of the METland in the second sequencing (in %). Legend = c_ class; o_ order; f_ family.

Taxon	HG5	HG7	HG9	HG10	HG11	HG12
f_Geobacteraceae	*2.78*	*9.64*	*8.14*	*4.76*	*15.30*	*7.11*
f_Pelobacteraceae	*0.93*	*9.48*	*5.04*	*5.63*	*5.20*	*5.77*
f_Peptostreptococcaceae	*8.85*	*7.83*	*8.43*	*1.91*	*2.20*	*3.42*
f_Desulfobacteraceae	*3.67*	*6.71*	*3.73*	*6.61*	*7.10*	*8.60*
f_Desulfomicrobiaceae	*7.01*	*6.58*	*1.00*	*2.49*	*2.29*	*2.78*
o_Bacteroidales	*9.50*	*6.51*	*5.67*	*8.36*	*7.40*	*6.62*
f_Clostridiaceae	*5.23*	*5.13*	*6.79*	*4.37*	*4.54*	*5.73*
f_Porphyromonadaceae	*3.81*	*3.11*	*0.99*	*1.59*	*1.58*	*1.68*
f_Comamonadaceae	*3.73*	*1.94*	*3.97*	*2.89*	*1.72*	*1.35*
f_Campylobacteraceae	*1.65*	*1.88*	*2.32*	*3.17*	*6.26*	*5.39*
f_Desulfobulbaceae	*1.76*	*2.85*	*0.57*	*1.24*	*1.36*	*1.05*
f_Rhodocyclaceae	*1.62*	*2.11*	*0.43*	*0.68*	*0.62*	*0.58*
f_Synergistaceae	*2.67*	*1.87*	*0.43*	*1.04*	*1.15*	*1.05*
f_Eubacteriaceae	*3.52*	*1.65*	*1.15*	*1.75*	*1.86*	*1.78*
f_Desulfovibrionaceae	*1.23*	*1.55*	*0.53*	*1.08*	*1.65*	*1.74*
f_Bifidobacteriaceae	*2.90*	*1.41*	*0.42*	*1.10*	*0.99*	*0.76*
f_Ruminococcaceae	*1.86*	*1.21*	*1.39*	*2.14*	*1.71*	*1.40*
f_Dethiosulfovibrionaceae	*0.83*	*1.14*	*0.19*	*0.44*	*0.79*	*1.34*
f_Veillonellaceae	*1.06*	*1.10*	*0.50*	*0.99*	*1.45*	*1.66*
f_RFP12	*1.28*	*1.07*	*0.22*	*0.81*	*0.63*	*0.55*
f_Caulobacteraceae	*0.09*	*0.07*	*5.17*	*3.99*	*0.05*	*0.04*
p_Firmicutes	*0.00*	*0.01*	*0.36*	*1.65*	*1.62*	*2.95*
f_Carnobacteriaceae	*2.26*	*0.73*	*0.61*	*1.13*	*1.32*	*1.55*
o_Bacteroidales	0.46	0.37	0.58	1.10	1.35	1.93
f_Peptococcaceae	0.13	0.16	0.82	0.98	2.08	3.87
f_Erysipelotrichaceae	0.36	0.15	0.68	2.36	1.46	1.27
f_Xanthomonadaceae	0.23	0.12	3.17	0.96	0.11	0.10
f_Hyphomicrobiaceae	0.34	0.23	1.58	0.58	0.10	0.11
o_Desulfuromonadales	0.10	0.72	1.15	0.38	0.20	0.54
f_Catabacteriaceae	1.32	0.64	0.56	0.82	0.95	0.91
f_Helicobacteraceae	0.24	0.52	1.02	0.47	0.41	0.42
f_Lachnospiraceae	0.92	0.46	0.75	1.12	0.89	0.59

Table S 4-10: Dominant bacteria **family** (more than 1% in some sample) in the **intermediate area of gravel** after the anode (HG13, HG14, HG15, HG16) and in the **final area of gravel** after the cathode (HG21, HG22, HG23, HG24) of the METland in the second stage sequencing (in %). Legend = c_ class; o_ order; f_ family.

	Gravel after anode				Gravel after cathode			
Taxon	HG13	HG14	HG15	HG16	HG21	HG2	HG23	HG24
f_Pelobacteraceae	9.02	10.49	10.98	4.94	0.65	0.09	0.65	2.45
f_Geobacteraceae	7.41	7.63	7.95	3.40	1.09	0.84	1.07	2.77
f_Helicobacteraceae	2.72	6.09	9.41	16.97	0.32	0.02	0.03	0.45
f_Anaerolinaceae	2.47	5.44	0.28	1.58	0.77	0.15	0.16	0.12
f_Hydrogenophilaceae	9.88	5.13	4.52	6.40	2.73	0.14	0.61	10.27
f_Xanthomonadaceae	2.64	4.44	4.64	3.65	8.45	11.85	9.81	7.86
o_Bacteroidales	4.28	3.89	3.69	5.95	2.61	0.97	0.84	5.68
o_Rhodospirillales	1.65	2.26	3.37	0.95	1.92	1.74	2.38	1.54
f_Comamonadaceae	1.36	2.23	0.95	2.07	9.88	3.96	3.80	6.57
f_Clostridiaceae	3.12	2.13	1.60	1.48	0.47	0.10	0.14	1.06
f_Campylobacteraceae	6.46	2.13	1.05	1.03	1.23	0.25	0.91	2.65
f_Desulfobulbaceae	1.18	1.71	0.57	2.87	0.17	0.03	0.08	0.46
o_Desulfuromonadales	1.24	1.58	1.53	2.15	0.62	0.14	1.95	2.94
f_Desulfobacteraceae	2.32	1.51	2.47	1.43	0.32	0.02	0.13	0.18
f_Caulobacteraceae	0.83	1.42	0.71	1.16	4.40	3.52	2.36	1.50
f_Sphingomonadaceae	0.60	1.37	1.16	1.24	5.08	2.75	1.90	1.48
f_Peptostreptococcaceae	2.64	1.32	0.67	0.69	0.01	0.01	0.03	0.04
f_Rhodocyclaceae	2.38	1.21	0.53	0.89	6.07	2.80	2.18	5.31
f_Rhizobiaceae	0.99	1.14	0.71	1.28	3.38	0.71	0.37	0.95
f_Bradyrhizobiaceae	1.32	1.11	0.51	0.60	1.15	1.27	1.49	1.25
f_Syntrophobacteraceae	1.05	1.08	0.78	0.63	0.20	0.10	0.17	0.14
f_Solibacteraceae	0.60	1.07	1.01	1.21	1.25	1.64	1.42	1.08
f_Hyphomicrobiaceae	0.91	0.81	2.63	1.38	2.44	3.96	4.83	1.35
f_Nocardioidaceae	1.02	0.27	1.13	0.62	4.90	4.07	3.23	1.32
f_Rhodobacteraceae	0.64	0.62	1.18	0.87	2.24	3.33	4.38	2.94
f_Desulfomicrobiaceae	1.15	0.50	0.60	0.31	0.05	0.01	0.03	0.14
f_Bacillaceae	0.06	1.52	0.02	0.02	0.10	0.50	0.52	0.17
f_Sinobacteraceae	0.51	0.20	0.09	1.33	0.44	1.56	2.14	0.14
f_Nitrospiraceae	0.02	0.02	1.33	0.03	0.05	0.26	0.31	0.03
f_Nitrosomonadaceae	0.02	0.02	0.05	0.09	1.01	0.27	2.01	0.07
f_Chitinophagaceae	0.34	0.44	0.42	0.49	0.28	3.81	2.46	2.49
f_Flavobacteriaceae	0.01	0.14	0.10	0.16	2.72	1.39	1.03	2.25
f_Pirellulaceae	0.29	0.20	0.50	0.43	0.12	0.92	1.00	0.10
f_Acetobacteraceae	0.33	0.59	0.76	0.67	1.00	1.03	0.82	1.24

Table S 4-11: Dominant bacteria family (more than 1% in some sample) in the **cathode** of the METland in the second sequencing (in %). Legend = c_ class; o_ order; f_ family.

Taxon	HG19	HG20	HG29	HG30	HG31	HG32
f_Hydrogenophilaceae	*14.55*	*2.50*	*1.63*	*6.94*	*4.66*	*1.58*
f_Comamonadaceae	*1.47*	*4.65*	*5.50*	*3.13*	*8.17*	*4.57*
f_Hyphomicrobiaceae	*3.88*	*2.76*	*3.95*	*3.04*	*3.92*	*4.08*
f_Caulobacteraceae	*0.84*	*0.66*	*7.23*	*0.74*	*8.88*	*2.21*
f_Geobacteraceae	*0.26*	*3.15*	*1.50*	*5.11*	*6.93*	*0.76*
f_Rhodocyclaceae	*0.48*	*4.19*	*3.28*	*4.04*	*3.84*	*0.67*
o_Chromatiales	*6.44*	*2.22*	*1.18*	*2.49*	*1.33*	*1.74*
f_Xanthomonadaceae	*3.54*	*2.04*	*1.80*	*1.57*	*1.80*	*2.12*
f_Campylobacteraceae	*0.77*	*3.02*	*1.38*	*4.58*	*1.84*	*0.19*
f_Rhodospirillaceae	*3.20*	*2.01*	*1.09*	*2.54*	*0.53*	*1.89*
f_Sphingomonadaceae	*1.22*	*1.36*	*1.23*	*2.04*	*1.73*	*3.44*
f_Rhodobacteraceae	*1.30*	*2.21*	*1.42*	*1.51*	*0.77*	*2.66*
o_Rhodospirillales	*2.02*	*1.77*	*2.43*	*1.19*	*1.15*	*0.93*
f_Chitinophagaceae	*0.99*	*1.78*	*1.88*	*0.87*	*0.67*	*2.81*
f_Nitrospiraceae	*1.30*	*1.43*	*1.04*	*2.43*	*0.22*	*2.29*
o_mle1-48	*0.14*	*1.49*	*4.28*	*1.11*	*0.79*	*0.71*
f_Saprospiraceae	*0.39*	*3.22*	*0.56*	*1.47*	*0.18*	*2.35*
o_Ellin6067	*0.67*	*1.36*	*1.51*	*1.51*	*2.20*	*0.62*
f_Sinobacteraceae	*0.47*	*1.33*	*1.45*	*0.73*	*2.62*	*0.86*
o_Rhizobiales	*1.23*	*1.15*	*0.90*	*1.39*	*0.73*	*1.51*
o_Desulfuromonadales	*0.15*	*0.53*	*0.46*	*1.07*	*4.10*	*0.12*
f_Burkholderiaceae	*0.33*	*1.24*	*1.47*	*1.18*	*1.35*	*0.71*
o_SC-I-84	*0.53*	*0.83*	*1.42*	*0.80*	*1.03*	*0.74*
f_Nocardioidaceae	*0.24*	*0.38*	*1.18*	*0.43*	*0.96*	*2.07*
f_Acetobacteraceae	*0.38*	*1.10*	*1.01*	*0.84*	*1.12*	*0.78*
f_Solibacteraceae	*0.38*	*1.34*	*0.85*	*0.66*	*1.14*	*0.52*
o_Sphingomonadales	*0.24*	*0.34*	*0.23*	*0.88*	*0.20*	*2.56*
o_Sva0725	*2.26*	*0.57*	*0.58*	*0.25*	*0.55*	*0.24*
f_Bradyrhizobiaceae	*0.21*	*0.67*	*1.44*	*0.71*	*1.05*	*0.26*
f_Hyphomonadaceae	*0.87*	*1.15*	*0.17*	*0.67*	*0.11*	*1.27*
f_Methylococcaceae	*1.44*	*0.57*	*0.75*	*0.68*	*0.32*	*0.33*
o_Bacteroidales	*0.16*	*0.68*	*0.21*	*0.66*	*2.01*	*0.16*
c_Gemmatimonadetes	*0.13*	*0.74*	*0.76*	*0.53*	*1.11*	*0.47*
f_Beutenbergiaceae	*3.57*	*0.03*	*0.02*	*0.06*	*0.03*	*0.03*
o_Nitrospirales;f_Thermodesulfovibrionaceae	*0.22*	*0.30*	*1.37*	*0.23*	*0.82*	*0.31*
f_Nitrosomonadaceae	*0.26*	*0.67*	*0.18*	*0.41*	*0.03*	*0.52*

f_Micrococcaceae	*1.34*	*0.52*	*0.17*	*0.19*	*0.03*	*1.23*
f_Nocardiaceae	*11.20*	*0.06*	*0.04*	*0.08*	*0.04*	*0.04*
f_Verrucomicrobiaceae	*0.21*	*0.49*	*0.76*	*0.37*	*0.31*	*1.09*
f_Haliangiaceae	*0.37*	*0.91*	*1.10*	*0.35*	*0.17*	*0.27*
o_BD7-3	*0.74*	*0.27*	*0.21*	*0.48*	*0.21*	*1.01*
o_Chlamydiales	*0.18*	*0.41*	*0.20*	*1.09*	*0.08*	*0.40*
f_Alteromonadaceae	*0.11*	*0.62*	*0.01*	*0.15*	*0.01*	*1.36*
f_Holophagaceae	*0.02*	*0.13*	*0.18*	*0.43*	*1.19*	*0.07*
f_Sphingobacteriaceae	*0.00*	*0.14*	*0.17*	*0.03*	*0.03*	*1.29*
f_Erythrobacteraceae	*0.00*	*0.12*	*0.04*	*0.17*	*0.06*	*1.26*

Table S 4-12: Dominant **genus** (more than 0.5% in some sample) in the **anode** of the METland in the second stage 16S rDNA sequencing (in %).

Genus	HG5	HG7	HG9	HG10	HG11	HG12
Geobacter	2.56	9.37	8.10	4.67	15.20	6.97
Desulfomicrobium	6.99	6.56	1.00	2.49	2.29	2.76
Desulfobacter	1.56	3.91	1.81	2.65	1.98	2.30
Parabacteroides	3.71	3.04	0.90	1.48	1.55	1.64
Desulfobulbus	1.25	2.46	0.54	1.10	1.05	0.88
Fusibacter	1.18	1.86	1.53	1.11	0.92	0.92
Arcobacter	1.57	1.76	1.84	2.96	5.92	5.03
vadinCA02	2.19	1.54	0.37	0.89	0.95	0.91
Bifidobacterium	2.88	1.40	0.42	1.09	0.99	0.76
Acetobacterium	1.61	1.13	0.57	0.94	1.04	0.92
Desulfovibrio	0.86	1.12	0.47	0.98	1.59	1.66
Clostridium	1.95	1.11	3.68	1.03	1.49	2.48
Comamonas	1.51	0.79	0.36	0.67	0.62	0.43
Trichococcus	2.23	0.71	0.60	1.12	1.31	1.53
Acidovorax	0.67	0.40	1.15	0.93	0.36	0.23
Thiobacillus	0.02	0.00	2.42	0.74	0.01	0.01
PSB-M-3	0.05	0.03	0.61	2.18	1.29	1.17
Dehalobacter	0.01	0.00	0.70	0.77	1.77	3.58
Propionivibrio	0.57	0.73	0.16	0.20	0.19	0.16
Dechloromonas	0.40	0.64	0.05	0.16	0.11	0.08
HA73	0.42	0.60	0.05	0.17	0.52	0.96
Acinetobacter	0.39	0.26	0.15	0.85	0.63	0.51
Bacteroides	0.43	0.23	0.23	0.54	0.81	0.73
kBrevundimonas	0.01	0.01	1.91	0.22	0.03	0.02

Phenylobacterium	0.04	0.02	0.05	1.28	0.00	0.00
Rhodococcus	0.03	0.02	0.96	0.07	0.01	0.02
Halothiobacillus	0.00	0.00	1.96	0.02	0.00	0.00
Thiomonas	0.00	0.00	1.42	0.00	0.00	0.00
Parvibaculum	0.00	0.00	1.24	0.06	0.03	0.01
Streptococcus	0.63	0.28	0.23	0.21	0.25	0.21
Turicibacter	0.30	0.23	0.55	0.10	0.13	0.12
SJA-88	0.26	0.32	0.11	0.28	0.43	0.57

Table S 4-13: Dominant **genus** (more than 0.5% in some sample) in the **cathode** of the METland in the second stage 16S rDNA sequencing (in %).

Genus	HG19	HG20	HG29	HG30	HG31	HG32
Thiobacillus	14.45	2.43	1.60	6.85	4.63	1.57
Geobacter	0.26	3.12	1.49	5.08	6.89	0.75
Arcobacter	0.76	2.99	1.34	4.46	1.74	0.18
Nitrospira	1.30	1.43	1.04	2.43	0.22	2.29
Denitratisoma	0.00	0.52	0.95	1.53	1.47	0.03
Rhodoplanes	1.07	1.12	1.94	1.31	1.32	0.92
Candidatus Solibacter	0.38	1.34	0.85	0.66	1.14	0.52
Hydrogenophaga	0.54	1.18	1.19	0.61	1.37	1.34
Candidatus Brocadia	0.13	0.08	0.55	0.43	0.40	0.14
Anaeromyxobacter	0.00	0.04	0.05	0.76	0.32	0.11
Sulfuritalea	0.05	0.51	0.27	0.66	0.38	0.08
Haliscomenobacter	0.04	0.35	0.05	0.58	0.07	0.78
Hyphomicrobium	1.37	0.57	0.98	0.53	0.52	0.68
A4	0.47	0.65	0.15	0.52	0.12	0.64
Sphingobium	0.18	0.48	0.67	0.47	0.93	0.43
GOUTA19	0.22	0.30	1.36	0.21	0.79	0.27
Devosia	0.72	0.64	0.32	0.45	0.11	1.71
Rhodobacter	0.27	0.63	0.15	0.45	0.05	1.32
Rhodococcus	11.18	0.06	0.04	0.08	0.04	0.04
Arenimonas	1.34	0.10	0.07	0.07	0.03	0.15
Parvibaculum	0.00	0.02	0.16	0.04	1.68	0.04
Phaeospirillum	0.69	0.14	0.06	0.36	0.01	0.15
Methylocaldum	1.16	0.30	0.55	0.35	0.15	0.12
Aquicella	0.25	0.05	0.06	0.32	0.29	0.74
Thermomonas	0.13	0.36	0.61	0.25	0.55	0.32
Dechloromonas	0.05	1.26	0.15	0.30	0.16	0.18
Acidovorax	0.24	0.14	1.14	0.22	2.89	0.29

Flavobacterium	0.05	0.42	0.60	0.14	0.41	0.74
Geothrix	0.01	0.05	0.14	0.22	0.98	0.03
Sphingopyxis	0.30	0.24	0.07	0.19	0.07	0.55
Rhodanobacter	0.16	0.18	0.10	0.13	0.71	0.12
Mycoplana	0.04	0.08	0.09	0.09	0.03	0.81
Cellvibrio	0.11	0.56	0.01	0.09	0.01	1.26

Table S 4-14: Dominant **genus** (more than 0.5% in some sample) in the **influent and effluent wastewater** of the METland in the second stage 16S rDNA sequencing (in %). Legend = f_ family; g_genus.

Taxon	HG3	HG4	HG25	HG26
g_Arcobacter	45.59	41.38	28.00	23.49
g_Parabacteroides	3.60	3.61	10.03	9.91
g_Bacteroides	2.91	2.68	5.47	5.49
g_Comamonas	2.81	2.91	0.21	0.25
g_Acinetobacter	2.53	2.68	0.18	0.17
g__Geobacter	0.28	0.39	2.41	2.79
g_Sulfurimonas	2.36	2.41	3.53	3.54
g_Sulfuricurvum	1.95	2.09	0.11	0.10
f_Comamonadaceae	1.55	1.68	0.55	0.56
f_Aeromonadaceae	1.78	1.65	1.54	1.68
g_Acidovorax	1.26	1.30	0.34	0.44
g_Tolumonas	0.99	0.82	2.52	2.64
f_Peptostreptococcaceae	0.78	0.65	0.52	0.59
f_Flavobacteriaceae	0.74	0.72	0.46	0.58
g_Sulfurospirillum	0.72	0.78	5.66	5.32
f_Helicobacteraceae	0.66	0.65	0.72	0.57
f_Catabacteriaceae	0.58	0.50	1.60	1.82
f_Veillonellaceae	0.47	0.54	0.34	0.43
g__Trichococcus	0.47	0.48	0.38	0.50

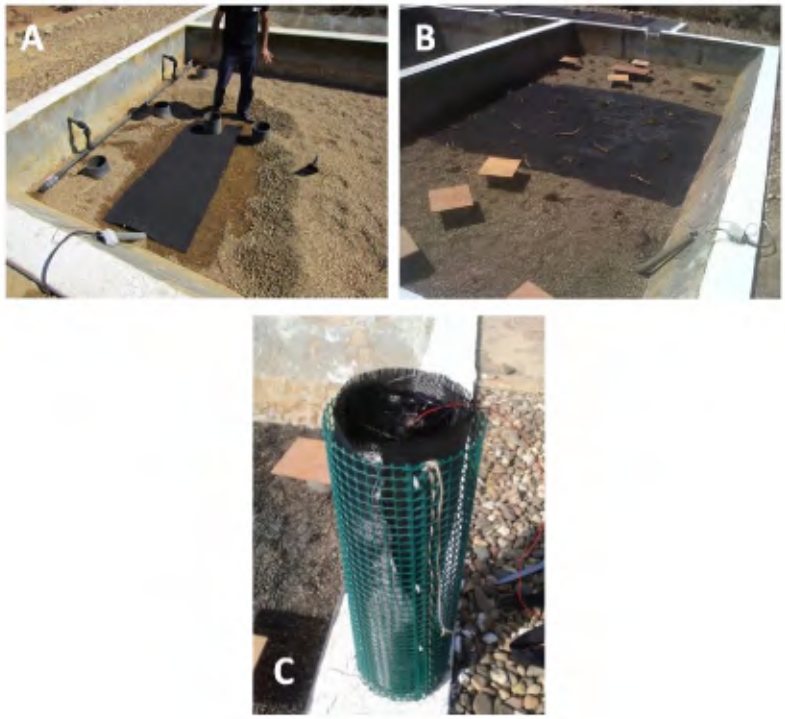
Table S 4-15: Dominant **genus** (more than 0.5% in some sample) in the **intermediate gravel** after the anode and **final gravel** after the cathode of the METland in the second stage 16S rDNA sequencing (in %). Legend = o_ order; f_ family; g_genus.

Taxon	HG13	HG14	HG15	HG16	HG21	HG2	HG23	HG24
f_Pelobacteraceae	8.99	10.46	10.93	4.90	0.65	0.09	0.65	2.44
g_Geobacter	7.37	7.59	7.89	3.34	1.08	0.82	1.06	2.70
g_Thiobacillus	9.85	5.10	4.50	6.36	2.70	0.14	0.60	10.22
g_T78	2.09	4.05	0.19	0.16	0.29	0.02	0.01	0.03
o_Bacteroidales	4.28	3.89	3.69	5.95	2.61	0.97	0.84	5.68
g_Sulfurimonas	1.14	3.32	1.60	15.48	0.16	0.01	0.01	0.27
o_Rhodospirillales	1.65	2.26	3.37	0.95	1.91	1.74	2.38	1.54
g_Sulfuricurvum	1.38	2.19	7.69	1.36	0.01	0.01	0.00	0.06
g_Arcobacter	6.05	1.77	1.00	0.91	0.98	0.25	0.90	2.40
g_Thermomonas	0.67	1.71	0.26	1.12	2.41	8.10	5.38	5.39
o_Desulfuromonadales	1.24	1.58	1.53	2.15	0.62	0.14	1.95	2.94
f_Desulfobulbaceae	0.68	1.43	0.15	2.50	0.13	0.02	0.05	0.26
f_Peptostreptococcaceae	2.60	1.31	0.65	0.68	0.01	0.01	0.02	0.04
f_Desulfobacteraceae	1.88	1.12	1.92	0.98	0.11	0.01	0.07	0.16
g_WCHB1-05	0.27	1.08	0.03	0.18	0.38	0.10	0.09	0.06
g_Candidatus Solibacter	0.60	1.07	1.01	1.21	1.25	1.64	1.42	1.08
g_Syntrophobacter	1.02	1.02	0.73	0.59	0.20	0.03	0.08	0.13
f_Bradyrhizobiaceae	1.19	0.95	0.41	0.52	1.06	1.20	1.42	1.10
g_Arenimonas	0.18	0.90	2.25	0.91	0.54	0.68	0.21	0.22
g_Acidovorax	0.37	0.90	0.31	0.32	1.93	0.36	0.27	0.53
f_Ellin515	0.47	0.73	0.68	0.52	0.11	0.04	0.05	0.26
g_Fusibacter	1.20	0.70	0.62	0.46	0.01	0.00	0.00	0.08
g_Brevundimonas	0.25	0.69	0.10	0.26	0.09	0.25	0.16	0.07
g_Clostridium	0.86	0.65	0.32	0.48	0.07	0.04	0.05	0.09
f_Comamonadaceae	0.62	0.65	0.35	1.40	6.48	2.85	2.86	5.50
f_Clostridiaceae	0.90	0.65	0.54	0.45	0.37	0.05	0.06	0.82
f_Xanthomonadaceae	0.20	0.59	1.70	0.91	0.03	0.38	0.30	0.05
g_Roseomonas	0.30	0.56	0.72	0.63	0.70	0.51	0.51	0.95
f_Rhodospirillaceae	0.50	0.55	1.17	0.82	0.26	0.49	0.33	0.36
f_Caulobacteraceae	0.48	0.52	0.20	0.37	2.90	1.64	1.18	1.04
g_Desulfomicrobium	1.14	0.50	0.59	0.31	0.05	0.01	0.03	0.13
g_Kaistia	0.51	0.45	0.32	0.35	0.07	0.04	0.00	0.14
g_Dechloromonas	1.16	0.44	0.16	0.13	1.30	0.62	0.33	0.85
o_Rhizobiales	0.36	0.43	0.68	0.56	1.74	2.14	1.27	0.94
g_Devosia	0.25	0.42	0.80	0.72	0.33	0.68	0.29	0.14

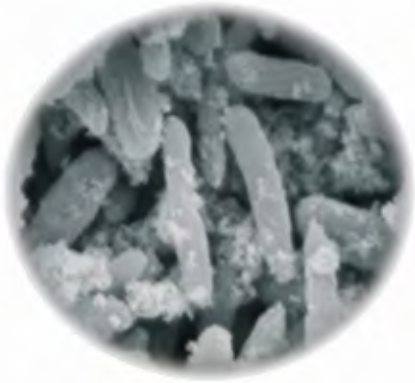
g_Parabacteroides	0.72	0.38	0.18	0.17	0.37	0.05	0.14	1.00
f_Xanthomonadaceae	1.19	0.37	0.29	0.33	4.68	1.14	2.07	0.60
g_Rhodobacter	0.26	0.28	0.38	0.51	0.87	0.59	0.59	0.76
f_Rhodobacteraceae	0.28	0.27	0.60	0.23	1.28	2.63	3.71	2.04
g_Phycicoccus	0.45	0.27	0.29	0.82	0.33	0.60	0.26	0.07
f_Sinobacteraceae	0.51	0.20	0.09	1.33	0.40	1.48	2.08	0.13
g_Rhodoplanes	0.31	0.18	1.10	0.46	1.21	1.57	1.59	0.47
f_Nocardioidaceae	0.64	0.25	1.03	0.52	4.83	3.88	3.13	1.26
g_Longilinea	0.01	0.13	0.01	0.54	0.02	0.00	0.02	0.00
f_Chitinophagaceae	0.18	0.30	0.18	0.22	0.23	3.12	1.50	0.20
g_Thauera	0.42	0.17	0.02	0.05	2.44	0.76	1.07	0.94
o_Sphingomonadales	0.27	0.35	0.32	0.37	0.50	1.93	0.52	0.76
g_Rhodanobacter	0.13	0.38	0.00	0.02	0.54	1.03	1.57	1.44
g_Flavobacterium	0.02	0.03	0.12	0.01	0.89	1.20	0.88	0.23
g_Mycoplana	0.02	0.11	0.13	0.16	0.52	0.91	0.19	0.24
g_Hyphomicrobium	0.16	0.05	0.40	0.07	0.20	0.66	1.45	0.16

Picture S 4-1: Photographs of the construction of the METland in CENTA, Carrión de los Céspedes, Sevilla. A: A peat filter pool in misuse was emptied and reused to install the METland; B) Imhoff tank; C) Construction of the conductive bed; D) General view of the METland just completed.

Picture S 4-2: Photograph of the METland with the vegetation



Picture S 4-3: Photographs of the three cathodes: A) Carbon cloth cathode; B) coke granules cathode with rhizomes; C) Tubular carbon cloth cathode



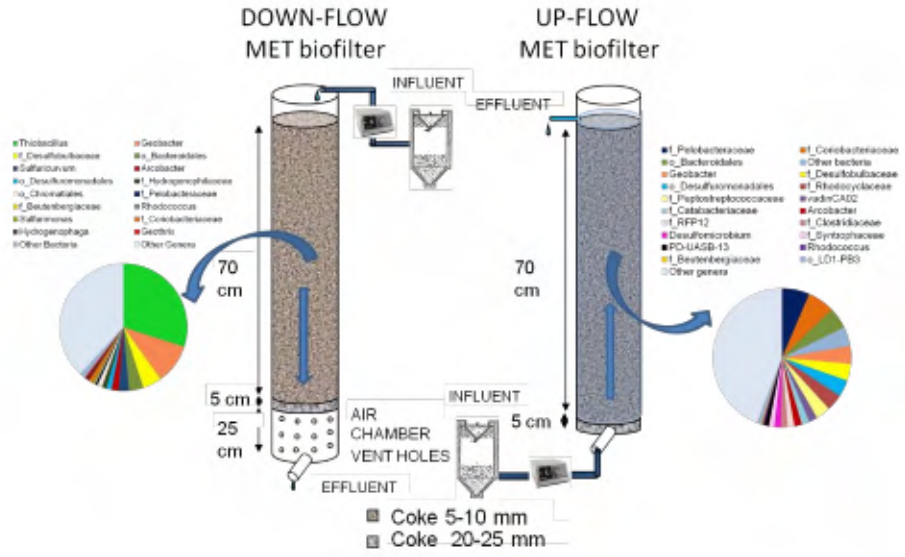
CHAPTER 5: A New Concept in METlands: Assessment of Aerobic Electroactive Biofilters

This section has been redrafted after:

Arantxa Aguirre-Sierra[a], Tristano Bacchetti-De Gregoris[b], Juan José Salas[c] and Abraham Esteve-Núñez[a,b].
A New Concept in METlands: Assessment of Aerobic Electroactive Biofilters. Submmited to Environmental ScienceWater Research & Technology

a. Department of Chemical Engineering, Universidad de Alcalá, Alcalá de Henares, Madrid, Spain.
b. IMDEA Agua, Parque Tecnológico de Alcalá, Alcalá de Henares, Madrid, Spain
c. Foundation Centre for New Water Technologies (CENTA), Carrión de los Céspedes, Sevilla, Spain

A New Concept in METlands: Assessment of Aerobic Electroactive Biofilters



5.1 Abstract

METland constitute a hybrid concept for treating wastewater where microbial electrochemical technologies (MET) are integrated into constructed wetlands in order to enhance the pollutant removal performance. Although electroactive bacteria are typically studied under anaerobic environments, it would be convenient explore alternative aerobic environments as biofilters operating in vertical constructed wetlands. Thus, two electroconductive biofilter fed with real urban wastewater were operated under down-flow (aerobic) and up-flow (anaerobic) conditions. The objective was to evaluate the impact of the operation mode in both the pollutants removal and the microbial community profile, with the aim of knowing if electroactive bacteria (EAB) were able to grow in aerobic constructed wetland (CW) systems and maximize the synergistic effects of METs and CWs for wastewater treatment (WWT). In spite of the aerobic nature of the downflow electroactive biofilter our results revealed the abundance of electroactive bacteria like *Geobacter* what open a new scenario for treating wastewater based on extracellular electron transfer. Moreover, the downflow electroconductive biofilter outperformed the anaerobic up flow one in terms of COD and nitrogen removal.

5.2 Introduction

Since the discovery of electroactive microorganisms, those able to directly interchange electrons with electrical conductive materials (Lovley, 2012) a number of innovative applications in the wastewater field has been extensively explored. For instance, the conversion of the chemical energy from wastewater into electrical energy by means of bioelectrochemical devices so-called microbial fuel cells (MFC), constitutes the most extensively reported concept at lab scale (Aelterman et al., 2006; Liu et al., 2004) and, occasionally, at pilot scale (Cusick et al., 2011; Ewing et al., 2014; Heidrich et al., 2014, 2013), However, in spite of the energy harvesting potential of this technology, alternative applications like nutrients removal (Clauwaert et al., 2007; Puig et al., 2011; Tejedor-Sanz et al., 2016) biosensors (Di Lorenzo et al., 2009; Estévez-Canales et al., 2017; Modin and Wilén, 2012) nutrients recovery (Kuntke et al., 2012; Zamora et al., 2017), sulphate reduction (Pozo et al., 2015) or metal recovery (Heijne et al., 2010; Rodenas Motos et al., 2015) to name a few are configuring an attractive platform so-called microbial electrochemical technologies.

In contrast with all that applications, it has been suggested that integrating MET into already existing systems like anaerobic digesters (Liu et al., 2012), oxic-anoxic systems from WWTPs (Tejedor-Sanz et al., 2016) may be an effective approach to accelerate the implementation of full scale bioelectrochemical treatments. In that context, the combination of Microbial Electrochemical Technologies (METs) and constructed wetlands (CWs) have been satisfactory implemented in few previous works leading to a hybrid technology so-called METland (Aguirre-Sierra et al., 2016; Esteve-Núñez et al., 2013). CWs are engineered systems made of a gravel or sand biofiltering bed with wetland plants that use natural functions of vegetation and organisms to remove pollutants from water. They have been widely used for decades in urban wastewater treatment (WWT) for small communities (García et al., 2010) and present the advantage of low energy requirements, low costs of operation and maintenance and good landscape integration in comparison to conventional WWT technologies (Knowles et al., 2011). Alternative studies have integrated MFC concepts into CW with the purpose of harvesting energy, although such a strategy of just burying electrodes into the gravel seemed to exhibit a minor impact in the wetland behaviour from the water purification perspective (Doherty et al., 2015b; Fang et al., 2015; Yadav et al., 2012). In contrast, the METland concept followed an alternative approach by replacing the classical biofiltering material (gravel, sand) by electroconductive material like coke (Aguirre-Sierra et al., 2016) so the electrons could flow through the material outperforming by 4-fold those standard CWs (García et al., 2010; Knowles et al.,

2011) that typically operate at a ratio of 5-10 m^{-2} p.e.$^{-1}$†. Interestingly, bacterial communities analysis revealed the enrichment of *Deltaproteobacteria* (a known electroactive taxon in presence of electrically conductive bed; moreover, *Geobacter*, the model electroactive bacteria most extensively studied, was the dominant genus in the deeper zone of the electrically conductive bed where oxidation of organic matter occurred (Aguirre-Sierra et al., 2016). The first METland design were based on Horizontal Subsurface Flow (HSSF) (Aguirre-Sierra et al., 2016), a saturated system in which anaerobic conditions prevailed. Although HSSF METland concept showed an effective response in terms of COD removal, the anaerobic nature of the process did not revealed as an optimal treatment for removing nitrogen. Actually, optimal nitrogen removal requires a different kind of CW called vertical sub-surface flow (VSSF). In this operation wastewater is dosed through the system intermittently so organic matter removal and nitrification by aerobic microorganisms are strongly favoured. Standard VSSF CWs show lower surface requirements (1-3 m^{-2} p.e^{-1}) and usually do not suffer from clogging, quite typical in HSSF CWs, when they are operated by intermittent downflow (Vymazal, 2010).

Interestingly, electroactive bacteria research is typically limited to anaerobic conditions in order to avoid the electron-accepting nature of oxygen and maximize the electron transfer to electroconductive material. So, in this scenario it may seem counterintuitive to study electroactive bacteria under a typical aerobic environment like a VHSF CW. However, the main goal of the current research was to assess the potential of constructing in a vertical down-flow electroconductive biofilter by adapting their configuration to explore the synergist effects of both electroconductive material and aerobic operation mode for enhancing organic matter and nutrients removal.

5.3 Materials and methods

5.3.1 Experimental design

Two semi-pilot tubular biofilters were constructed (Figure 5-1) using two operation modes: one of the biofilters were flooded- and operated up-flow (UF) while the other was non-flooded and operated down-flow (DF). Both UF and DF biofilters

† p.e. Population equivalent is the number expressing the ratio of the sum of the BOD load produced during 24 hours by industrial facilities and services to the individual BOD load in household sewage produced by one person in the same time. For practical calculations it is assumed that one unit equals to 60 g of BOD per 24 hours.

were constructed using electrically conductive material made of coke granules as biofiltering bed. Semi-pilot biofilters were made of PVC cylinders (diameter 20 cm and 90 cm height). From bottom to top, the first 5 cm of the biofilters were filled with coarse material (Ø 25 mm) followed by 70 cm of thin material (Ø 6-12 mm). DF biofilter also hosted a 1 cm layer of sand (Ø 0.27 mm) in the top to spread out the inlet wastewater. The bottom of the DF biofilter was perforated allowing the water to drain into an air chamber, perforated with 1 cm diameter holes, which provided a better circulation of air through the media. Biofilters had a total volume of 23.5 L including a water volume of 8 L and an inlet area of 0.031 m^2.

Plants are typically integrated in CW for oxygenating the root zone and for providing a habitat to aerobic microorganisms. The most common VFCW design approach is to size these systems as recirculating gravel filters and ignore the vegetation component from a treatment perspective (Kadlec and Wallace, 2009). Thus, we did not include plants in our experimental set up in order to achieve a better control of the redox interaction between bacteria and bed. BOD loading rates of 10 to 40 g m^{-2} d^{-1} are often used in VFCW depending on design, with most designs using loading rates of 25 g m^{-2} d^{-1} to achieve BOD_5 concentration at the effluent under 30 mg BOD L^{-1} (Kadlec and Wallace, 2009) and fulfil legal requirements for wastewater treatment (Dir. 91/271/EEC of 21 May 1991).

5.3.2 Biofilters operation

The semi-pilots were fed with urban wastewater from Carrión de los Céspedes WWTP (Seville, Spain, 2500 inhabitants) during 175 days (twenty five weeks). Wastewater passed a primary treatment in an Imhoff tank in order to remove solids and prevent early clogging of the systems. Wastewater characteristics are shown in Table S 5-1. The feeding from the Imhoff tank was made by means of programmed pumping: DF biofilter were fed from the top like intermittent sand filters, with 8 L d^{-1} by means of 16 pulses of 10 minutes (0.05 L min^{-1}), with a hydraulic loading rate (HLR) of 258 mm d^{-1} leading to alternating aerobic/anoxic conditions. In contrast, UF system received continuously 8 L d^{-1} at a flow rate of 5 mL min^{-1} from the bottom, giving a nominal hydraulic retention time (HRT) of 1 day, flooding all the porous of the media and keeping anaerobic conditions. Biofilters were operated during one month prior to sample analysis.

5.3.3 Physicochemical and statistical analysis

Samples of influent wastewater and effluents of the systems were weekly analyzed to monitor their performance in terms of biochemical oxygen demand

(BOD_5), total suspended solids (TSS), total phosphorous (TP), total nitrogen (TN), ammonia-nitrogen (NH_4-N) and nitrate-nitrogen (NO_3-N). Wastewater was sampled twice a week for chemical oxygen demand (COD) analysis. All the analysis were performed following the standard methods (American Public Health Asociation, 2005). Electrical conductivity (EC), pH, dissolved oxygen (DO), temperature (T) and redox potential (ORP) were measured with a handheld multiparameter (YSI 556 MPS). Inlet wastewater characteristics are shown in Table S 5-1 (Supplementary information).

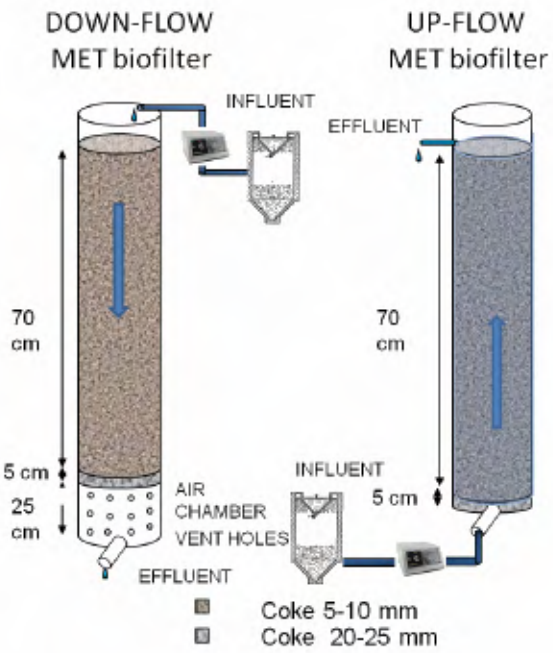


Figure 5-1: Schematic of the biofilters design and experimental set-up.

Statistical procedures for the evaluation of treatment performance for every water quality parameter were conducted using the Statgraphics Centurion XVII statistical software package. Kruskal-Wallis tests were developed to determine the differences of water quality parameters among the effluents of the biofilters.

5.3.4 Microbial community study

A third DF inert biofilter filled of gravel was constructed and operated with the same conditions as the electroconductive DF biofilter made or coke granules, during the same time prior to microbial sampling with the aim of comparing the microbial communities between a conductive and an inert biofilter.

Sampling, DNA extraction and 16S rDNA sequencing.

Samples were taken from each of the two biofilters (from 40 cm depth) by duplicate to determine the composition of their microbial communities. Granules of coke were sampled with tweezers and dipped in three consecutives, sterile, 50 ml saline solutions (NaCl 7 g/l) in order to remove loosely attached bacteria. These rinsed granules were first frozen and then fully processed within a week. Around 10 granules were extracted for each sample spot.

DNA was extracted with PowerSoil spin columns (MO BIO Laboratories) and amplified with primers 341F 5'-CCTACGGGNGGCWGCAG-3' and 785R 5'-GACTACHVGGGTATCTAATCC-3' (Klindworth et al., 2013), targeting the V3 and V4 region of the bacterial 16S rRNA gene. The polymerase used was 2x KAPA HiFi HotStart Ready Mix (KAPA Biosystems) and the PCR conditions were: initial denaturation at 95°C for 3' followed by 25 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, and a final elongation step of 72°C for 5 min. 1/50 dilution of PCR products were then re-amplified (15 cycles) with Illumina´s primers. Positive reactions were excised out of the gel in order to get rid of any possible primer-dimers and undesired products. Finally, products were run on a Bioanalyzer (Agilent) to estimate the concentration of each sample within the region of interest and the successful generation of equimolar pools was confirmed by qPCR. Sequencing was performed in a MiSeq equipment using the 2x250 bp format and following Illumina's protocol. The Illumina Miseq sequence reads have been deposited in the European Nucleotide Archive (ENA) databases under accession Nr. PRJEB15667.

Bioinformatics analysis

The total sequence reads were analysed with the QIIME 1.7 pipeline (Caporaso et al., 2010) with few stitches along the way. Briefly, complementary reads were merged using fastq-join (Aronesty, 2011). Subsequently, our quality filtering strategy removed complemented sequences that had one of the following characteristics: (i) deviated more than 10 bp from the expected length (292); (ii) contained primers with more than 1 mismatch; or (iii) contained nucleotides with Phred score <20. Filtered seqs were organised in OTUs by de novo picking using Usearch (Edgar, 2010) and one representative sequence per OTU was chosen. Taxonomy was assigned using the GreenGenes database (DeSantis et al., 2006) version 10_12 at the 97% identity rate. Furthermore, sequences were aligned and a tree generated using FastTree 2.1.3 (Morgan N Price et al., 2010). Finally, in order to investigate alpha diversity and the network formed by communities members with QIIME, OTUs containing less than 0.005% of the total sample reads were removed

according to Bokulich (Bokulich et al., 2013). The results have been represented as percentage of a specific sequence in every sample. Taking into account the possible effect of deviation introduced by the implemented protocol and that not all the bacterial species have the same number of copies of 16S gen in their genomes (Klappenbach, 2001), the values can be related to percentage of cells of every species that were part of the sampled communities. The alpha diversity indexes calculated were Chao1 richness index and Shannon index. Rarefaction curves and the coverage percentage by Good's method were used to assess whether the clone library reflected the actual bacterial diversity in the samples. Beta diversity was studied to show the degree of dissimilarity between any pair of bacteria communities. Weighted Fast UniFrac analysis and correspondence analysis (CA) were used to identify the differences in the bacterial community structures based on their phylogenetic lineages.

5.4 Results and Discussion

The effect of using electroconductive material instead of classical inert material for construction of vertical biofilters was evaluated at two different levels: biodegradation capacity and microbial ecology.

5.4.1 Organic matter removal in aerobic and anaerobic biofilters

In terms of COD removal significant statistical differences ($p<0.05$) were found among the two systems. The most efficient was the coke DF aerobic biofilter, with an average COD removal efficiency of 96% (Table 5-1), an average removal rate of 115 g COD m^{-3} d^{-1}, and achieving removal rates as high as 197 g COD m^{-3} d^{-1}. It is remarkable that our system was tested under a high COD inlet load, at least double than the standard load reported in literature for vertical CWs able to remove just 80-90% (Ávila et al., 2013; Ortega et al., 2010; Zhao et al., 2010).

UF anaerobic biofilter removed 109 g COD m^{-3} d^{-1} of the COD inlet load (120 g COD m^{-3} d^{-1}) and showed a COD removal efficiency of 90% (Table 5-1). These results are consistent with data reported by Aguirre-Sierra et al. (2016) for saturated anaerobic HSSF electrochemical biofilters (90% and 117 g COD m^{-3} d^{-1} of the inlet load (131 g COD m^{-3} d^{-1})) at similar HRT, and 17% higher than the gravel horizontal biofilter reported in the aforementioned study (96 of 131 g COD m^{-3} d^{-1} and 73%). BOD_5 removal efficiency did not show significant statistical differences ($p>0.05$) and was very high in both biofilters, achieving 98% (DF) and 97% (UF) (Table 5-1). This results leads to think that in the UF biofilter there were enough terminal electron

acceptors (TEA) to degrade the COD and no effect of introducing oxygen in the DF biofilter was observed at this retention time. On top of the pollutant removal, the key parameter in wastewater to fulfil the discharge legislation is the pollutant level in the treated wastewater. In this case the aerobic DF biofilter generated water where COD residual concentrations were 2-fold lower than those obtained with anaerobic UF biofilter and were also much lower than the limits of discharge even for BOD_5 (Table S 5-2). No significant statistical differences ($p>0.05$) appeared with regard to TSS (Table S 5-2).

Table 5-1: Removal rates (g m^{-3} d^{-1}) of the up-flow and down-flow coke biofilters (averages ±SD). Removal efficiencies (%) are shown in brackets.

	COD	BOD_5	TN	NH_4-N	TP
Surface inlet load (g m^{-2} d^{-1})	93 ± 11	51 ± 15	14.1 ± 1.6	11.8 ± 1.5	2.3 ± 0.3
Volume Inlet load (g m^{-3} d^{-1})	120 ± 14	65 ± 12	18.2 ± 1.2	15.2 ± 1.9	2.9 ± 0.4
Removal rates					
Up-flow biofilter	109 ± 15 (90 ± 2)	63 ± 12 (97 ± 1)	4.1 ± 2.1 (23 ± 8)	4.0 ± 2.2 (25 ± 9)	1.3 ± 0.4 (45 ± 9)
Down-flow biofilter	115 ± 14 (96 ± 2)	64 ± 12 (98 ± 2)	6.7 ± 2.5 (37 ± 9)	15.0 ± 1.4 (98 ± 1)	2.3 ± 0.6 (76 ± 11)

5.4.2 Nutrients removal in aerobic and anaerobic biofilters

In terms of nutrients removal there were statistical differences between both biofilters ($p<0.05$). Aerobic (DF) biofilter removed 98% of the ammonia, which was detected in the effluent at average values as low as 0.8 ± 0.4 mg L^{-1}, while NO_3-N concentration reached 32.9 ± 12.2 mg L^{-1} in average (Figure 5-2). This removal rate is between 30 and 40% higher than ammonia removal rates in the literature about VF CW, that range between 60 and 70% (Ávila et al., 2013; Kadlec and Wallace, 2009; Ortega et al., 2010). Zhao et al., (2010) reported TN removal efficiencies around 40% in vertical DF biofilters (CW without plants) operated with a TN loading rate of 5.5 g m^{-2} d^{-1}, which is 3-fold lower than the TN loading rate in our systems, which showed similar TN removal efficiencies (Table 5-1). Analysis of nitrifying rates revealed that coke DF biofilter accumulated just 72% of the ammonia into nitrate. This fact together with the TN removal (37%) in coke DF biofilter (Table 5-1) suggest

either assimilation of nitrogen into biomass or some denitrifiying activity in order to justify nitrogen removal from water, in spite of being an aerobic system.

With regard to ammonia, the anaerobic UF biofilter removed 25%, but only 9% was accumulated into nitrate. This low value of nitrification is consistent with the anaerobic environment of UF saturated biofilters where oxygen-based nitrification is strongly limited. UF biofilter was removing ca. 20%,of the TN in contrast to the aforementioned study about horizontal anaerobic electroactive biofilters that reported efficiencies of 45% (Aguirre-Sierra et al., 2016) One possible explanation to this difference could be that the redox potential difference between the bottom and the top layers of the HSSF biofilters were increased, due to the higher ratio inlet area over bed volume, and so bioelectrochemical nitrification were increased. But further research would be necessary to study this idea.

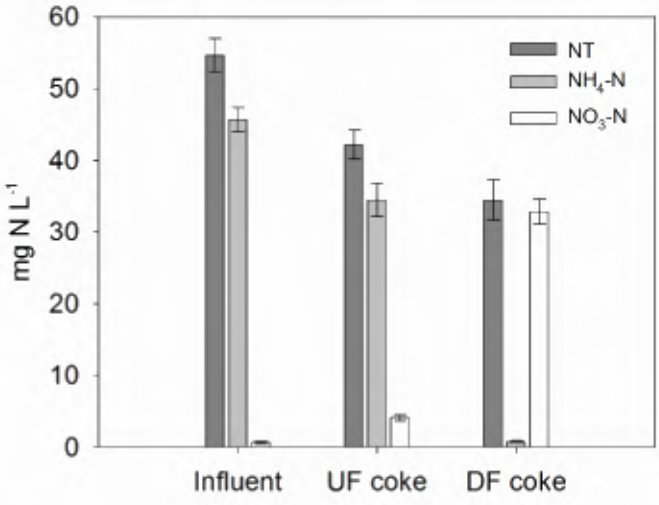


Figure 5-2: Influent and effluent average concentrations of TN, NH_4-N and NO_3-N of the anaerobic UF and aerobic DF electroactive biofilters. Error bars represent 95% confidence interval.

In terms of TP removal, the aerobic DF biofilter exhibited the best removal efficiency (Figure 5-3), achieving an average of 76% (Table 5-1), and punctual TP removal rates as high as 95%. Biofilters showed a 2-fold higher TP removal under aerobic DF in comparison with anaerobic UF operation. This result revealed that a positive impact of venting the electroconductive material may be associated to shift the redox state of the coke surface to more oxidative potentials.

As a consequence of a better WWT, other advantages of vertical aerobic DF versus anaerobic CWs are that the former have lower surface requirements (1-3 m^{-2}

p.e.$^{-1}$ depending on design) than the latter (5-10 m^{-2} p.e.^{1}) (Vymazal, 2010) and usually do not suffer from clogging, quite typical in HSSF CWs. (Rousseau et al., 2004; Tanner and Sukias, 1995).

5.4.3 Analysis of microbial communities of aerobic and anaerobic systems

The Illumina Miseq sequence reads revealed an important influence of operation mode. The analysis of the microbial communities (by duplicate) revealed 319,049 raw reads that yielded a total of 38,813 high quality sequences with an average length of 460 bp (Table S 5-3). 0.86% of the sequence reads were not classified. The classifiable sequences included members of 44 phyla of which the most abundant group were *Proteobacteria*, with an average of 55% (Figure S 5-1), and ranging between 38% and 73% in the UF and the DF biofilters, respectively. The *Proteobacteria* phylum includes a high level of bacterial metabolic diversity related to global carbon, nitrogen and sulphur cycling and has also been found as the most abundant group in other studies of the composition of bacterial communities in CWs (Adrados et al., 2014; Ansola et al., 2014; Arroyo et al., 2015).

Rarefaction curves showed saturation, indicating that an appropriate number of sequence reads per sample were collected to disclose diversity at the sites (Figure S 5-2). Diversity indexes, such as observed OTUs and Chao1 were significantly higher in the UF biofilter than in the down-flow one (Table S 5-3). The Good's coverage estimator denoted that the sizes of the libraries were enough to cover almost 100% of the bacterial communities. Shannon diversity indexes (H), which include the information of both richness (the number of species present) and evenness (how the abundance of each species is distributed), were distinctly higher (6.26 and 7.54) than those reported in other studies using electrochemical setups integrated in constructed wetlands treating urban wastewater (Corbella et al., 2015) and similar to the results of Aguirre-Sierra et al. (2016)(H: 6.27–7.47) and Lu et al., (2015) (H: 7.33–7.47). These results, revealed a very high diversity at those sites. Weighted Fast UniFrac analysis and correspondence analysis (CA) were used to identify the differences in the bacterial community structures based on their phylogenetic lineages. Constrained correspondence analysis showed that the adjusted explained variation was 79.3%. CA plot showed clear differences between communities of the aerobic DF and anaerobic UF biofilters (Figure S 5-3).

The analysis of the microbial communities revealed the presence of different taxonomic groups depending on the operation of the biofilter. The results showed a

high presence of *Deltaproteobacteria* (Figure 5-4), 27-28% in the UF coke biofilter and 20-24% in the DF coke biofilter. Bacteria belonging to this class have been reported to be associated with the electroactive biofilm from the very beginning (Bicciato et al., 2003) as they share the capacity for generating ATP from very low thermodynamic value reactions (Lovley, 2013; McInerney et al., 2007). Within the class *Deltaproteobacteria*, bacteria from the genus *Geobacter*, are able to transfer electrons to conductive materials (Bond et al., 2002). *Geobacter* species were found in the coke UF biofilter, ranging between 3.7 and 4.1% (Table S 5-5). These species have been found in anaerobic electroactive biofilters run under horizontal subsurface flow (Aguirre-Sierra et al., 2016). But *Geobacter* bacteria were also detected in an aerobic system like the DF biofilter, representing a 10% of the bacterial community (Table S 5-4). This result is consistent with the fact that some *Geobacter* species are able to respire oxygen when this terminal electron acceptor (TEA) is supplied at low concentrations (Lin et al., 2004). Contrary to the general idea that *Geobacter* species are strictly anaerobic (Koch and Harnisch, 2016), years of work with this genus in our laboratory have demonstrated that *Geobacter* is able to live in environments with oxygen concentrations in the range of 0 – 1 mg L^{-1} like the upper layer of CWs (Aguirre-Sierra et al., 2016). Another electroactive genus like *Geothrix*, was also found in the aerobic DF biofilter, although its abundance was lower than *Geobacter*'s (Table S 5-7).

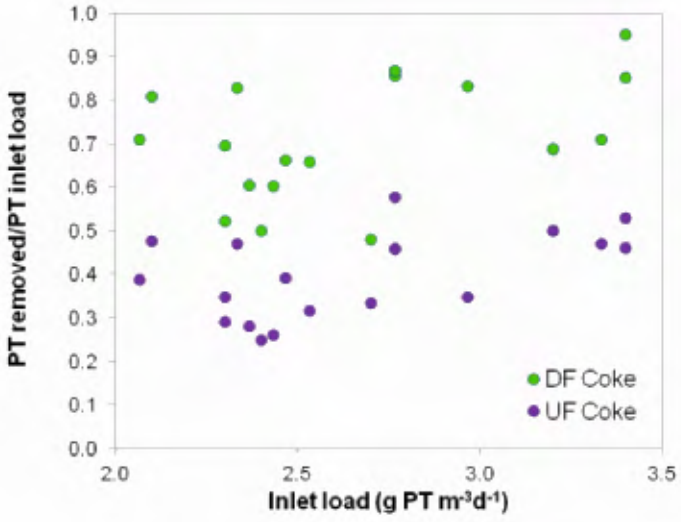


Figure 5-3: Relationship between normalized PT removed and PT inlet load of the anaerobic (UF) and aerobic (DF) electrochemical biofilters and their respective controls.

Total nitrogen removal is difficult to achieve in anaerobic systems, such as our UF biofilter, since nitrifying reaction becomes limited by the absence of available electron acceptors. In contrast, vertical DF aerated biofilters can behave very active in terms of nitrification, but scarce denitrification. Nitrification is a two step process, where ammonia in presence of oxygen, is first converted to nitrite by strictly chemolithotrophic *Nitrosomonas*, *Nitrosococcus* and *Nitrosospira* bacteria, and then to nitrate by facultative chemolithotrophic bacteria *Nitrospira*, and *Nitrobacter* (Reddy et al., 1984). A deep analysis into the bacterial community showed that *Nitrospira* y *Nitrosomonadaceae* family, both involved in aerobic oxidation of ammonia to nitrate were not abundant, in spite of the high nitrifying activity detected in the coke DF biofilter. On the other hand, the analysis showed a high relative abundance of *Thiobacillus* in the coke DF biofilter, ranging between 11 and 30%. In a study of the nitrogen transforming bacteria in VFCW, Pelissari et al. (2017) found that ammonia-oxidizing bacteria occurred only in the top layer (0 – 17 cm depth) of the wetland (8%), nitrite oxidizing bacteria (NOB) were found in top and intermediate layers (34 – 51 cm depth, 5%) and denitrifying bacteria were found in all layers, appearing *Thiobacillus denitrificans* in the mid layer (34 – 51 cm depth, 2%) and the bottom layer (> 51 cm, 10%) of a vertical DF wetland. In our systems the granules were sampled at 40 cm depth, which can explain the small proportion of ammonium oxidizing species in the samples of the coke DF biofilter, where the presence of *Thiobacillus* seemed to be enhanced. Novel nitrogen removal routes reported in wetland systems (Saeed and Sun, 2012), include partial nitrification-denitrification. This process includes conversion of ammonia to nitrite, followed by denitrification of nitrite to nitrogen gas. The advantage of partial nitrification and denitrification is that requires lower oxygen (approximately 25%), and lower organic matter (40%), in contrast with traditional nitrification and denitrification metabolism (Jianlong and Ning, 2004). The enhancement of nitrogen removal observed in the aerobic coke DF biofilter suggests the activation of a partial nitrification-denitrification reactions leading to N_2 formation. Although this may seem counterintuitive due to inhibitory effect of oxygen on denitrification, the biofilm layer in intimate contact with the coke can preserve an anoxic environment. This was confirmed by the detection of *Geobacter* and *Thiobacillus* genus associated to the coke material in such aerobic DF biofilter (Table S 5-4). In fact, *Geobacter* bacteria has been reported to transfer electrons directly to *Thiobacillus* which in turn may reduce nitrate or nitrite to dinitrogen (Kato et al., 2012). This interspecies electron transfer might be enhanced in presence of an electroconductive material like coke in a similar manner that reported elsewhere (Rotaru et al., 2014a).

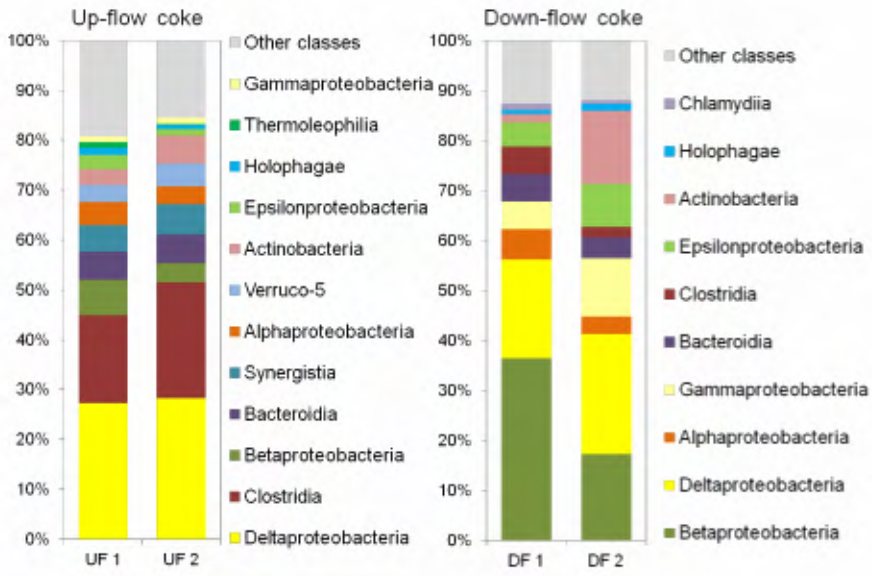


Figure 5-4: Relative abundances of OTUs at class level (more than 1%) comparing communities of the anaerobic (UF) and aerobic (DF) biofilters.

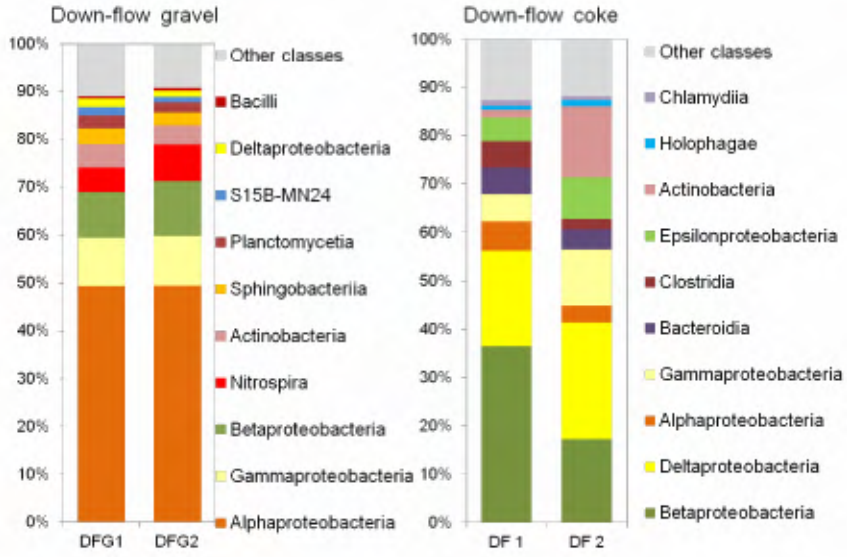


Figure 5-5: Relative abundances of OTUs at class level (larger than 1% in average) comparing communities of the aerobic DF coke and gravel biofilters.

5.4.4 Analysis of microbial communities of conductive and inert materials

When gravel and coke DF biofilters microbial communities were compared large differences appeared. While in the coke biofilter the dominant classes were *Beta* and

Deltaproteobacteria in the gravel biofilter a dominance of *Alphaproteobacteria* existed (Figure 5-5). It is also remarkable the appearance in the gravel DF biofilter of bacteria of the class *Nitrospira* that comprises chemolithoautotrophic aerobic nitrite-oxidizing bacteria. Moving down in the taxonomy the gravel DF biofilter showed a high presence of bacteria of the family *Nitrosomonadaceae* (3.29-3.26%), ammonium oxidizing bacteria and of the *Nitrospira*-like genus (4.97-7.54%) a nitrite oxidizer, both implied in the nitrifying activity, while in the coke DF biofilter samples they were very scarce (Table S 5-6). It is curious the high presence of *Phenylobacterium* (4.98-4.86%), a genus that comprises a single aerobic species called *P. immobile*, which is remarkable for its extremely limited nutritional spectrum. Strains of this bacterium have been isolated growing on artificial compounds like chloridazon (active ingredient of the herbicide Pyramin®), antipyrin and pyramidon (two analgesics)(Dworkin, M. et al., 2006). There were also a high proportion of the family *Sphingomonadaceae* and some genus as *Sphingopyxis* (2.96-4.61%) and *Sphingomonas* (1.13-1.40%) appeared. *Sphingomonadaceae* are a versatile group of aerobic or facultative anaerobic chemoorganotrophs occurring in various environments such as soil and water and are able of metabolize aromatic compounds (Dworkin, M. et al., 2006). The order *Rhizobiales* presents the highest proportion in the bacterial community of the gravel DF biofilter (23.10-20.64%). This order belongs to Alphaproteobacteria and is represented mainly by three families, *Bradyrhizobiaceae* (8.13-7.24%), *Phyllobacteriaceae* and *Rhizobiaceae*, many of the genera related to nitrogen fixing associated with plant roots. *Betaproteobacteria* and *Deltaproteobacteria* constitute a small proportion in the gravel biofilter but they are a large component of the coke biofilter, where *Geobacter* represents a 10% of the bacterial community. These results come to support once again the idea that the conductive material enhances the development of EAB, even in aerobic systems.

5.5 Conclusions

Aerobic electroactive biofilters improve the removal of organic matter when they are compared to anaerobic ones but moreover they increase nitrification. Aerobic electroactive biofilters enhance the presence of EAB as *Geobacter* even in a general aerobic environment and also promote the existence of bacteria that are reported to perform DIET with *Geobacter*. Bacterial communities are very different in aerobic and anaerobic electroconductive systems. When aerobic electroactive systems are compared with inert systems larger differences appear. Our results support the hypothesis that the conductive material enhances the development of EAB even in aerobic systems. Aerobic electroactive biofilters maximize the synergetic effects of both aerobic and anaerobic environments.

5.6 Supplementary information

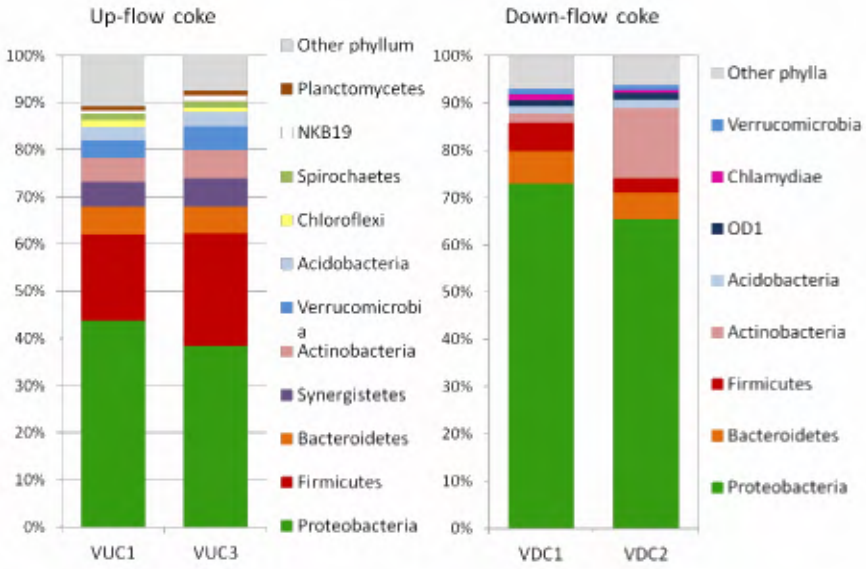


Figure S 5-1: Relative abundances of OTUs at the phylum level (larger than 1% in average

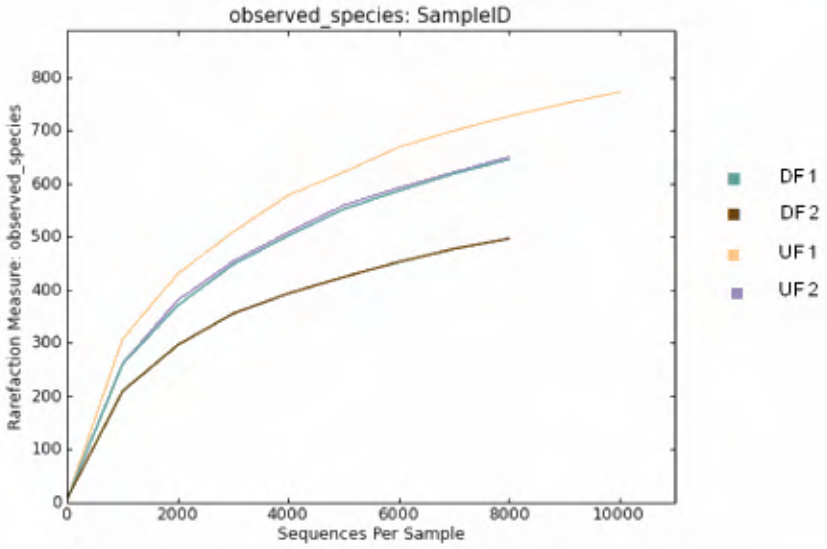


Figure S 5-2: Rarefaction curves calculated for each sample based on the OTU computations. DF = down-flow aerobic biofilter; UF = up-flow aerobic biofilter.

Table S 5-1: Influent wastewater characteristics

Parameter	Concentration
pH	7.4 ± 0.2
Conductivity (μS cm^{-1})	1,199 ± 102
Temperature (°C)	23.8 ± 2.3
DO (mg L^{-1})	5.4 ± 1.8
ORP (mV)	-166 ± 46
COD (mg L^{-1})	359 ± 42
BOD_5 (mg L^{-1})	196 ± 57
TSS (mg L^{-1})	293 ± 134
TN (mg L^{-1})	54.6 ± 6.1
NH_4-N (mg L^{-1})	45.7 ± 5.7
NO_3-N (mg L^{-1})	0.7 ± 0.7
TP (mg L^{-1})	8.8 ± 1.1

Table S 5-2: Influent and effluent residual concentrations of COD, BOD_5, TSS, TN, NH_4-N, NO_3-N and TP (average ± SD). Removal efficiencies (%) are shown in brackets. UF = up-flow; DF = down-flow.

	COD (mg L^{-1})	BOD_5 (mg L^{-1})	TSS (mg L^{-1})	TN (mg L^{-1})	NH_4-N (mg L^{-1})	NO_3-N (mg L^{-1})	TP (mg L^{-1})
Influent	359 ± 42	196 ± 57	293 ± 134	54.6 ± 6.1	45.7 ± 5.7	0.7 ± 0.7	8.8 ± 1.1
UF coke effluent	30 ± 6	6 ± 3	7 ± 5	42.2 ± 7.7	34.5 ± 7.9	4.1 ± 1.3	4.8 ± 0.7
DF coke effluent	15 ± 3	5 ± 3	11 ± 8	34.5 ± 6.6	0.8 ± 0.4	32.9 ± 9.2	2.0 ± 1.2

Table S 5-3: Alpha diversity metrics of the bacterial populations in the samples. UF = up-flow; DF = down-flow

Sample ID	Total sequence written	Median sequence length	Observed OTUs	Chao 1	Shannon	Good's coverage
DF 1	9720	465	688	901	7.47	0.98
DF 2	9217	464	517	686	6.26	0.98
UF 1	13645	462	835	986	7.54	0.98
UF 2	6231	449	694	903	7.07	0.97

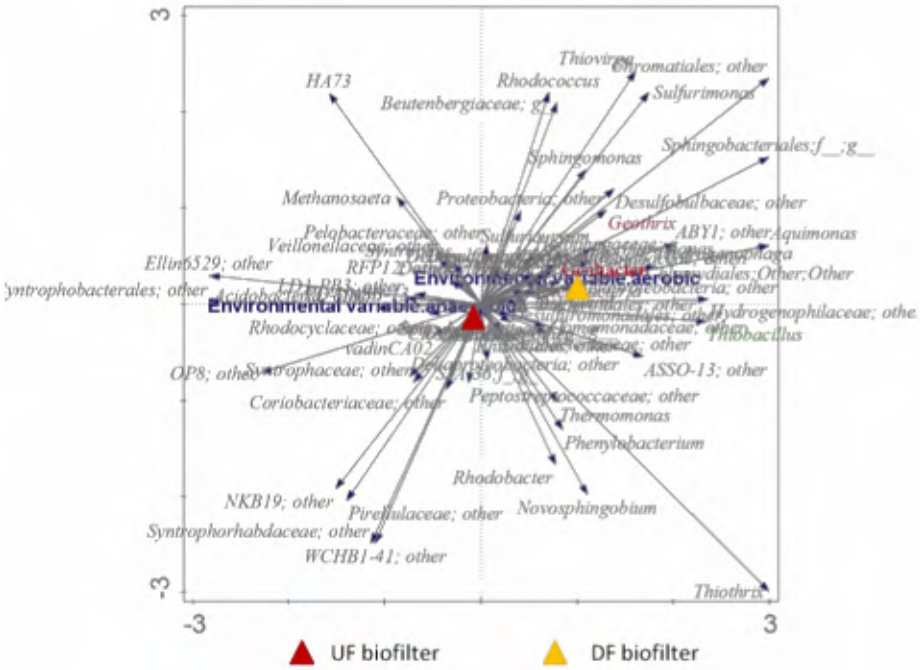


Figure S 5-3: Correspondence analysis plot of the bacterial communities in the two up-flow (anaerobic) and down-flow (aerobic) biofilters

Table S 5-4: OTUs at level genus of the aerobic DF biofilter (> 1% in some sample).

Taxon	DF 1	DF 2
Thiobacillus	30.21%	11.88%
Geobacter	10.22%	10.10%
f_Desulfobulbaceae	4.86%	9.04%
o_Bacteroidales	3.71%	2.98%
Sulfuricurvum	2.60%	4.00%
Arcobacter	1.86%	2.38%
f_Desulfuromonadales	1.29%	0.87%
f_Hydrogenophilaceae	1.42%	1.08%
o_Chromatiales	1.17%	7.93%
f_Pelobacteraceae	0.64%	1.49%
f_Comamonadaceae	0.50%	0.26%
Sulfurimonas	0.30%	2.04%
f_Beutenbergiaceae	0.29%	6.55%
Rhodococcus	0.25%	7.08%
f_Pseudomonaceae	0.24%	0.29%

Table S 5-5: OTUs at genus level of the anaerobic (UF) biofilter (> 1% in some sample). Legend: o = order; f = family; g =genus.

Taxon	UF1	UF 2
f_Pelobacteraceae	6.38%	9.76%
f_Coriobacteriaceae	6.25%	10.21%
o_Bacteroidales	4.95%	4.56%
Geobacter	**4.13%**	**3.70%**
f_Desulfobulbaceae	3.93%	1.44%
f_Desulfuromonadales	3.88%	3.62%
f_Rhodocyclaceae	3.84%	2.30%
f_Peptostreptococcaceae	3.37%	3.70%
vadinCA02	1.96%	2.42%
f_Catabacteriaceae	1.82%	2.39%
Arcobacter	1.73%	0.85%
f_RFP12	1.58%	1.85%
f_Clostridiaceae	1.55%	2.26%
Desulfomicrobium	1.48%	1.50%
f_Syntrophaceae	1.26%	1.36%
PD-UASB-13	1.11%	1.18%
Rhodococcus	0.49%	2.24%
f_Beutenbergiaceae	0.30%	2.00%

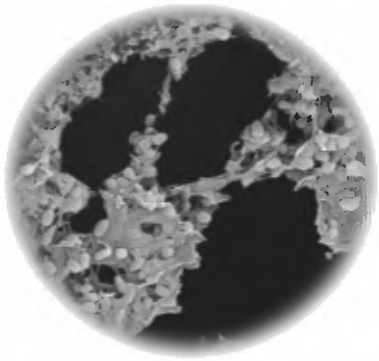
Table S 5-6: OTUs at genus level of the gravel down-flow (DF) biofilter (> 1% in some sample).

Taxon	DFG 1	DFG 2
f_Bradyrhizobiaceae	8.13%	7.34%
Phenylobacterium	4.98%	4.86%
Nitrospira	4.97%	7.54%
f_Sphingomonadaceae	4.10%	3.98%
o_Rhizobiales	4.06%	4.02%
Rhodoplanes	3.38%	3.50%
f_Nitrosomonadaceae	3.29%	3.26%
f_Xanthomonadaceae	3.27%	3.64%
Sphingopyxis	2.96%	4.61%
Thermomonas	2.57%	1.65%
f_Chitinophagaceae	2.49%	2.11%
f_Phyllobacteriaceae	2.25%	1.44%
f_Comamonadaceae	1.95%	2.76%
o_Sphingomonadales	1.66%	1.94%
f_Sinobacteraceae	1.60%	1.78%
o_Acidimicrobiales	1.48%	0.86%
f_Rhizobiaceae	1.47%	0.85%
Rhodobacter	1.40%	1.22%
f_Erythrobacteraceae	1.37%	1.63%
f_Rhodobacteraceae	1.34%	1.39%
f_Pirellulaceae	1.23%	0.85%
Sphingomonas	1.13%	1.40%
o_Actinomycetales	1.12%	0.71%
Other genera	38.15%	36.93%

Table S 5-7: Main genera of bacteria identified in the analysed coke biofilters communities (> 0.1%).

Bacteria genus	UF1	UF2	DF1	DF2
abr-29	0.3%	0.1%	0.0%	0.0%
Acetobacterium	0.1%	0.1%	0.1%	0.0%
Acidovorax	0.3%	0.1%	0.1%	0.1%
Acinetobacter	0.1%	0.1%	0.1%	0.1%
Agrobacterium	0.0%	0.0%	0.1%	0.0%
Aminiphiluss	0.5%	0.4%	0.0%	0.0%
Aminobacter	0.2%	0.0%	0.0%	0.0%
Anaerofustis	0.1%	0.0%	0.0%	0.0%
Aquimonas	0.0%	0.0%	0.6%	0.7%
Arcobacter	1.7%	0.9%	1.9%	2.4%
Arenimonas	0.0%	0.0%	0.4%	0.3%
Azoarcus	0.1%	0.0%	0.0%	0.0%
Azovibrio	0.1%	0.2%	0.0%	0.0%
Bacteroides	0.0%	0.1%	0.3%	0.1%
Bifidobacterium	0.0%	0.1%	0.1%	0.0%
Bosea	0.2%	0.2%	0.1%	0.0%
Brachybacterium	0.1%	0.0%	0.0%	0.0%
Brevibacterium	0.2%	0.0%	0.0%	0.0%
Brevundimonas	0.0%	0.1%	0.0%	0.1%
Candidatus Protochlamydia	0.0%	0.0%	0.2%	0.2%
Candidatus Solibacter	0.1%	0.1%	0.3%	0.1%
Chryseobacterium	0.0%	0.0%	0.0%	0.1%
Clostridium	0.8%	0.8%	0.4%	0.3%
Corynebacterium	0.1%	0.1%	0.1%	0.2%
Dechloromonas	0.2%	0.1%	0.5%	0.6%
Dehalobacterium	0.1%	0.3%	0.0%	0.0%
Denitratisoma	0.4%	0.2%	0.0%	0.0%
Desulfobacter	0.1%	0.1%	0.1%	0.1%
Desulfococcus	0.0%	0.2%	0.0%	0.0%
Desulfomicrobium	1.5%	1.5%	0.4%	0.5%
Desulfomonile	0.3%	0.4%	0.1%	0.0%
Desulfovibrio	1.0%	0.7%	0.1%	0.1%
Devosia	0.1%	0.1%	0.0%	0.0%
E6	0.1%	0.3%	0.0%	0.0%
Enterobacter	0.0%	0.1%	0.0%	0.1%
Escherichia	0.0%	0.0%	0.0%	0.2%
Fusibacter	0.1%	0.0%	0.0%	0.0%
Gemmata	0.0%	0.0%	0.1%	0.0%
Geobacter	**4.1%**	**3.7%**	**10.2%**	**10.1%**
Geothrix	**0.2%**	**0.0%**	**0.4%**	**0.7%**
Gordonia	0.1%	0.1%	0.1%	0.0%
HA73	0.7%	0.9%	0.0%	0.1%
Hydrogenophaga	0.1%	0.0%	0.9%	0.9%
Hylemonella	0.0%	0.0%	0.0%	0.1%
Hyphomicrobium	0.0%	0.0%	0.0%	0.1%
Kaistobacter	0.3%	0.4%	0.0%	0.0%

Lactobacillus	0.0%	0.0%	0.0%	0.4%
Leucobacter	0.2%	0.2%	0.0%	0.0%
Methylotenera	0.0%	0.0%	0.0%	0.1%
Methyloversatilis	0.0%	0.0%	0.1%	0.0%
Micrococcus	0.1%	0.0%	0.0%	0.2%
Mycobacterium	0.3%	0.2%	0.1%	0.0%
Nitrospira	0.1%	0.0%	0.0%	0.0%
Novosphingobium	0.1%	0.0%	0.5%	0.1%
Oleomonas	0.0%	0.0%	0.0%	0.3%
Opitutus	0.0%	0.0%	0.1%	0.2%
Parabacteroides	0.4%	0.7%	0.4%	0.5%
Paracoccus	0.1%	0.1%	0.0%	0.1%
PD-UASB-13	1.1%	1.2%	0.1%	0.1%
Phenylobacterium	0.1%	0.2%	0.6%	0.1%
Pseudomonas	0.0%	0.0%	0.1%	0.1%
Pseudoxanthomonas	0.0%	0.0%	0.1%	0.0%
Rhodobacter	0.2%	0.2%	0.7%	0.1%
Rhodococcus	0.5%	2.2%	0.3%	7.1%
Rhodoplanes	0.3%	0.1%	0.2%	0.0%
Roseococcus	0.0%	0.0%	0.1%	0.0%
Roseomonas	0.0%	0.0%	0.1%	0.1%
Ruminococcus	0.0%	0.1%	0.0%	0.0%
SJA-88	0.3%	0.3%	0.0%	0.0%
Soehngenia	0.1%	0.0%	0.0%	0.0%
Sphingomonas	0.1%	0.1%	0.2%	0.6%
Sphingopyxis	0.1%	0.0%	0.0%	0.1%
Staphylococcus	0.0%	0.0%	0.0%	0.1%
Streptococcus	0.0%	0.0%	0.0%	0.1%
Sulfuricurvum	0.6%	0.3%	2.6%	4.0%
Sulfurimonas	0.0%	0.0%	0.3%	2.0%
Sulfurospirillum	0.3%	0.1%	0.1%	0.0%
Suyntrophobacter	0.1%	0.2%	0.0%	0.0%
Syntrophus	0.5%	0.6%	0.1%	0.1%
T78	0.3%	0.5%	0.0%	0.1%
Thauera	0.1%	0.0%	0.1%	0.1%
Thermomonas	0.2%	0.2%	0.6%	0.1%
Thiobacillus	**0.0%**	**0.0%**	**30.2%**	**11.9%**
Thiothrix	0.0%	0.0%	0.6%	0.1%
Thiovirga	0.1%	0.0%	0.1%	0.5%
Treponema	0.4%	0.2%	0.4%	0.1%
Trichococcus	0.0%	0.2%	0.0%	0.0%
Turicibacter	0.1%	0.1%	0.0%	0.0%
vadinCA02	2.0%	2.4%	0.2%	0.1%
W22	0.1%	0.1%	0.1%	0.1%
WCHB1-05	0.0%	0.0%	0.1%	0.0%
Zooglea	0.1%	0.0%	0.3%	0.1%
Total genera	23.4%	23.1%	57.2%	47.5%



CHAPTER 6: Assessing the Design of a Vertical Aerobic MET filter

Assessing the Design of a Vertical Aerobic MET filter

6.1 Abstract

METland constitute a hybrid concept for treating wastewater where microbial electrochemical technologies (MET) are integrated into constructed wetlands (CWs) in order to enhance the pollutant removal performance. Previous research in this thesis (chapter 5) revealed that electroactive bacteria can unexpectedly grow attached to electroconductive bed in aerobic biofilters. Thus, we have now assessed a comparative study to explore the impact of the material (inert versus electroconductive material) in performing downflow aerobic biofilters for treating wastewater. Three hydraulic retention times (HRT) have been tested and samples were taken at different distances from the inlet (20 cm and 50 cm). The electroconductive biofilter fulfilled the COD legal limits of discharge even at values of low HRT (6 h) and a remarkable OLR 20-fold higher than recommended for vertical flow CWs. Regarding ammonium, the electroconductive material biofilter outperformed the classical inert biofilter producing effluents with , ammonium levels not higher than 15 mg NH_4N L^{-1}. According to the results, we suggest that the classical depth of Vertical Flow CWs might be shortened by at least one third (from 80 to 50 cm) while the HRT could be reduced to 6 hours, ensuring the fulfilments of legal WWT discharge.

6.2 Introduction

The combination of Microbial Electrochemical Technologies (METs) and constructed wetlands (CWs) has been satisfactory implemented in few previous works leading to a hybrid technology so-called METland (Aguirre-Sierra et al., 2016; Esteve-Núñez et al., 2013).

Since the first published study about CW-MFC (Yadav et al., 2012) nearly all the research work has been based in vertical up-flow CWs (Doherty et al., 2015b; Fang et al., 2015; Zhao et al., 2013) and they are mainly focused on harvesting electricity with low, if any, success. Vertical up-flow CWs are saturated systems in which anaerobic conditions prevail. Only one previous study has assessed VF aerobic METlands (Aguirre-Sierra et al. 2017, submitted) by comparing wastewater treatment (WWT) in anaerobic up-flow (UF) and aerobic down-flow (DF) biofilters.

This study showed that METs can work not only in an anaerobic environment but also in aerobic systems.

Given that one of the general objectives of this research was to reduce the classical size of CWs in order to maximize the feasibility of this WWT technology, our goal was to assess the impact of shifting the material in the design of vertical METland in comparison with a classical vertical CWs for removing pollutants (COD and ammonium).

This study analyses the performance of an electrochemical vertical down-flow biofilter (a CW without plants) to remove pollutants from wastewater, in particular the organic matter and the ammonia, by nitrification, comparing it with a classical non-conductive material biofilter.

6.3 Material and methods

6.3.1 Experimental set up and operation

A vertical biofilter, with conductive material (coke granules) as biofiltering bed, was built as a VF CW design but in absence of plants so we can focus on WWT process mainly due to microorganisms (Ortega et al., 2010). An identical biofilter made of inert gravel was constructed as control (Figure 6-1).

Two PVC pipes of 90 mm outer diameter were cut at 100 cm long and perforated in their lateral at 20 cm and 50 cm from the top end. Through every hole, a PVC tube of 16 mm diameter was inserted sloping as a collector to take water samples. Bottom caps were perforated to let the water drain and facilitate air convection; likewise, top caps were perforated as a showerhead to improve the homogeneous distribution of wastewater.

Systems were filled with granulated material (diameter 5-10 mm) up to 80 cm, one with graphite coke (electroconductive material), and the other one with gravel (non-electroconductive material) as a control, leaving a reservoir to avoid overflowing. Moreover, a column of rolled mesh, with a diameter of 3 cm and filled with the same material, was introduced into the PVC pipes in order to extract biological samples at different levels.

With the purpose of generating a real biological community, the biofilters were inoculated with real urban wastewater from the municipality of Carrión de los Céspedes (Sevilla, Spain) (2500 inhabitants), that was circulated during 3 days.

Then, they were inoculated with 50 ml of *Geobacter sulfurreducens* culture with 0.51 units of Optical Density at 600 nm.

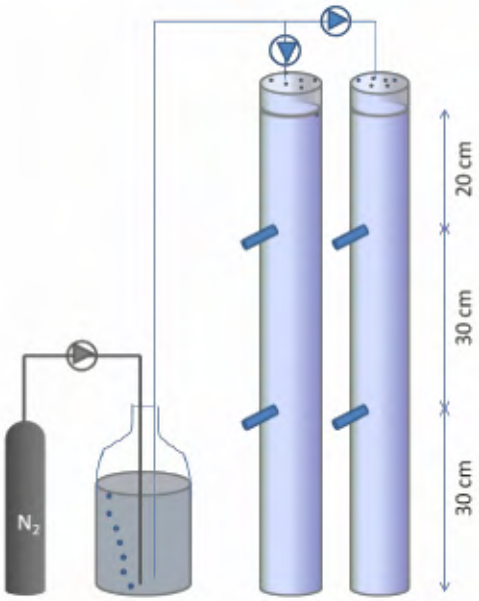


Figure 6-1: Design of the vertical down-flow biofilters set up

The biofilters had a total volume of 4.7 L and a water volume of 1.6 L. The systems were fed from the top using anoxic synthetic wastewater (SWW) sparged with nitrogen gas. A peristaltic pump was calibrated for a flow rate of 1.6, 3.2 and 4.8 L day^{-1} along the experiment (Aguirre-Sierra et al., 2016). Such flow rate implied hydraulic loading rates (HLR) of 280, 560 and 1120 mmd^{-1} that would correspond to 24, 12 and 6 hours of hydraulic retention time (HRT), respectively. Henceforth, although the water is not retained inside the biofilters (it just percolates through the media) we have used the term HRT for referring to the hydraulic operation.

6.3.2 Physicochemical and statistical analysis

SWW was prepared weekly, the pH was adjusted to 7 (Table 6-1) and it was autoclaved to avoid biological degradation of the substrates. Influent, effluent and intermediate sampling points, (20 cm and 50 cm from the inlet port) were daily sampled. The WWT performance of the biofilters was determined by analyzing the evolution of COD, ammonia nitrogen (NH_4-N), nitrate nitrogen (NO_3-N) and nitrite

nitrogen (NO_2-N). COD was analysed following standard methods (American Public Health Asociation, 2005). Nitrogen forms were analysed by ion chromatography (930 Compact IC Flex, Metrohm).

Removal efficiencies were calculated as percentage while removal rates were calculated in grams per cubic meter of biofiltering bed and day. Statistical procedures to evaluate the performance of the biofilters were conducted using the Statgraphics Centurion XVII statistical software package. ANOVA method was used to determine the differences of every water quality parameter among the sample points and pairs were compared by LSD multiple ranges comparisons (95% confidence).

Table 6-1: Composition of the synthetic wastewater

Compounds	Concentration (mg L^{-1})
Sodium acetate	600
Ferric citrate	23.1
Yeast extract	56
KH_2PO_4	44
$NaHCO_3$	310
$(NH_4)_2SO_4$	250
$MgCl_2 \cdot 2H_2O$	50
$CaCl_2 \cdot 2H_2O$	74
$ZnSO_4 \cdot 7H_2O$	0.4
NaCl	100

6.3.3 Scanning Electron Microscopy study

Granules of each material were harvested at the end of the experiment from the top layer (2 cm under the surface), 20 cm and 50 cm layers and from the final layer of material of both biofilters. Biofilm was fixed with 5% glutaraldehyde in 0.2 M Na-Cacodylate buffer, pH 7.4 and dehydrated with growing ethanol concentrations (25 %, 50 %, 50 %, 90 % and 100 %), acetone and anhydrous acetone. They were held into anhydrous acetate for 12 h and finally dehydrated through vacuum pump, getting their critic point of moisture. Then the samples were covered with gold and observed through Scanning Electron Microscopy (SEM) (Hitachi TM-1000).

6.4 Results and discussion

The electroconductive biofilter functioned as a whole electrode that accept the electrons produced in the oxidation processes and transfer these electrons for reduction processes. This short-circuited electrochemical scenario is so-called Microbial Electrochemical Snorkel (MES) and electrons flow inside a single material instead of having a two electrode system with anode and cathode.

6.4.1 Organic matter removal

During the first stage (HLR = 280 mm d^{-1}; HRT = 24 h) the biofilters supported an organic loading rate (OLR) of 175 g COD m^{-2} d^{-1} and an estimated theoretical BOD_5 loading rate of 105 g BOD_5 m^{-2} d^{-1} (60% COD) (Table 6-2). Recommended OLR for conventional VFCW is 20 g BOD_5 m^{-2} d^{-1}, with a maximum of 50 g BOD_5 m^{-2} d^{-1} in the case of some optimized systems with recirculation and aeration (Kadlec and Wallace, 2009) in order to fulfil discharge requirements (Dir. 91/271/EEC of 21 May 1991). Hence, our systems held up 5-fold the recommended OLR for conventional VF CWs. This OLR was duplicated and quadruplicated in the next two periods, corresponding to 12 and 6 hours of HRT, respectively (Table 6-2). Once the first stage was concluded it was decided not to sample at 20 cm in the next phases, given that the electroconductive biofilter showed analytical results close to the legal limits of discharge and lower retention times were going to be tested.

Table 6-2: Organic and ammonium loading rates corresponding to the different hydraulic loading rates tested. Theoretical BOD was estimated as the 60% of the COD concentration.

HRT	24 h	12 h	6 h
HLR mm d^{-1}	280	560	1120
COD loading rate g m^{-2} d^{-1}	175±16	372±22	695±12
Theoretical BOD_5 g m^{-2} d^{-1}	105±10	223±13	417±7
NH_4-N loading rate g m^{-2} d^{-1}	9.2±1.3	19.7±1.2	38.8±2.6

There were significant differences between both biofilters in every sample point ($p<0.05$), achieving the coke biofilter the best performance (Figure 6-2). Regarding to the highest HRT (24 h), the coke biofilter reached the legal limits of discharge at 20 cm from the inlet, and an average COD concentration very low (35 mg L^{-1}) at 50cm (Figure 6-2) with a removal efficiency of 94±5% at that point (Table

6-3). Even at the lowest HRT, this biofilter always fulfilled the limit values of discharge (125 mg L^{-1} or more than 75% removal) (Directive 91/271/EEC of 21 May 1991) in every sample point (Figure 6-2). On the other hand, at 24 h the gravel biofilter doubled the limit of discharge at 20 cm and showed an average COD concentration of 105 ± 30 mg L^{-1} at 50 cm, surpassing the limit values of discharge frequently, with a lower removal efficiency (83 ± 9%) in that point (Table 6-3), although the effluent was under the legal limit value. Concerning lower HRTs, the gravel biofilter never fulfilled the limits at 50 cm from the inlet (Figure 6-2).

The results are consistent with data obtained in previous studies about HSSF biofilters, in which a similar trend was observed when decreasing the HRT (Aguirre-Sierra et al., 2016). Though they were anaerobic environments, when the HRT was decreased, the differences between the electroconductive and the inert biofilters increased, being the electroconductive biofilter the only one that fulfilled the discharge requirements at 12 h and 6 h of HRT.

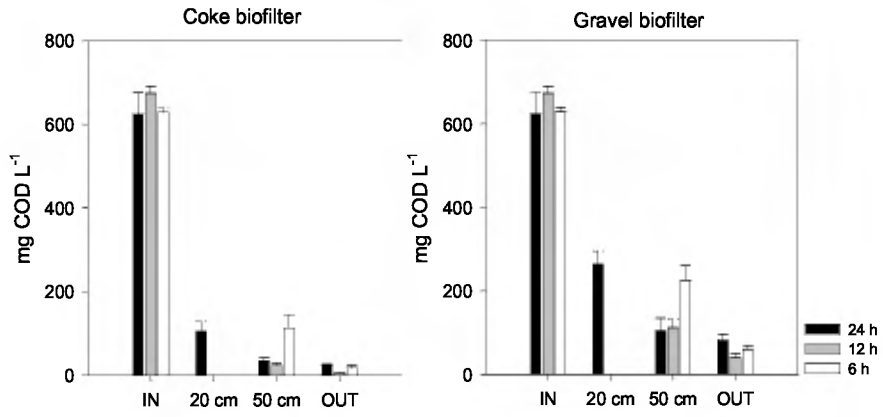


Figure 6-2: The graphs show COD concentration at different levels from the inlet, at every HRT in the electroconductive (left) and the gravel (right) biofilters.

6.4.2 Nitrogen removal

Regarding to ammonium nitrogen, differences are more remarkable than with COD, and the electroconductive biofilter outperformed the gravel biofilter. In the gravel biofilter, total removal efficiencies decreased from 97±1% to 57±7% when the HRT was reduced from 24 to 6 hours. In contrast, the electroconductive biofilter showed a more regular performance, with total removal efficiencies that shifted from

97±2% to 81±5% (Table 6-3). Significant statistical differences ($p<0.05$) were found in ammonium concentration in every sample point at every HRT, except between the effluents when the HRT was 24 h and 12 h (Figure 6-4). It can be also observed that the shorter the HRT the larger the differences of ammonium concentration between coke and gravel systems in every sample point.

Table 6-3: COD and ammonia removal efficiencies reached at every level for each HRT in both biofilters.

	Biofilter section	Coke biofilter			Gravel biofilter		
		24 h	12 h	6 h	24 h	12 h	6 h
NH_4N removal efficiency (%)	Inlet - 20 cm	81 ± 6	--	--	51 ± 7	--	--
	Inlet - 50 cm	95 ± 2	78 ± 4	70 ± 7	87 ± 2	54 ± 8	46 ± 4
	Inlet - Effluent	97 ± 2	90 ± 3	81 ± 5	97 ± 1	86 ± 4	57 ± 7
COD removal efficiency (%)	Inlet - 20 cm	83 ± 9	--	--	57 ± 8	--	--
	Inlet - 50 cm	94 ± 5	97 ± 4	82 ± 5	83 ± 6	87 ± 3	64 ± 5
	Inlet - Effluent	96 ± 3	99 ± 1	97 ± 2	88 ± 4	96 ± 4	90 ± 4

At a distance of 50 cm from the inlet the coke biofilter removed 9%, 44% and 52% more than the gravel biofilter at a HRT of 24 h, 12 h and 6 h, respectively (Table 6-3). At 24 h of HRT the electroconductive biofilter removed, in the first 50 cm, a 95% of the ammonium, resulting in an average concentration as low as 1.6±0.2 mg N L^{-1} at that sampling point. Then, at 12 and 6 h of HRT, the removal efficiency decreased until 70% at 50 cm, while gravel biofilter only reached 46%. The ammonium concentration at 50 cm in the electroconductive biofilter was always under 10 mg L^{-1} at any HRT, whereas the gravel biofilter exceeded this value in the effluent, with the exception of HRT of 24 h. As Figure 6-3 shows, punctual ammonium removal rates were not affected by the material nature at low loading rate; however at low HRT the material showed an impact and the electroconductive biofilter removed 38.9±3.0 g N m^{-3} d^{-1}, ca. 1.4-fold more ammonium than the gravel biofilter.

The removal efficiencies led to effluents more than 2-fold higher in the gravel biofilter at the shorter HRT. It should be pointed out that a large amount of ammonia can be converted to other forms of nitrogen, such as nitrite and nitrate, given that

these biofilters are aerobic systems where nitrification (and not denitrification) processes are enhanced. As shown in figure 6-4, the concentration of nitrate in every sample point was significantly higher ($p<0.05$) in the electroconductive biofilter than in the gravel one. Those data were well correlated with the amount of ammonium that was removed. As expected, nitrate concentration decreased at lower values of HRT, showing an impact in the nitrification performance.

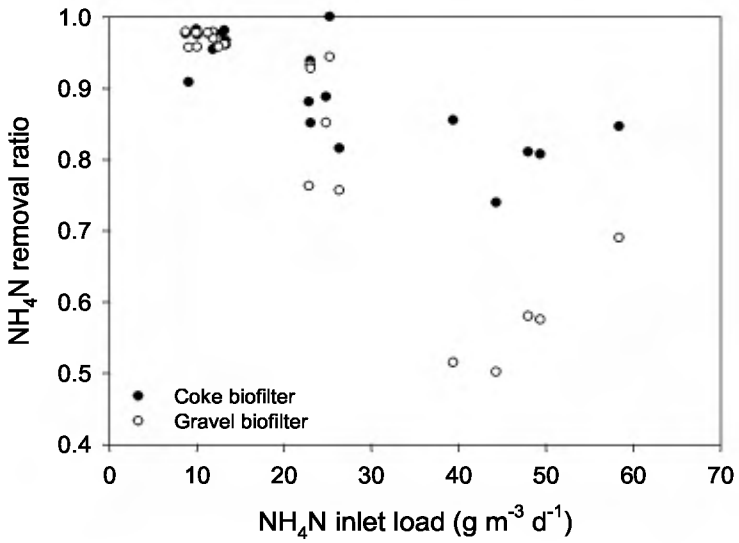


Figure 6-3: Ammonia removal rates in both biofilters at different loading rates that correlate with several HRT.

Our results suggest that some metabolic pathways like nitrification are indeed affected by the electroconductive bed. A similar pattern has been observed in a previous study that compared gravel and electroconductive horizontal subsurface flow (HSSF) anaerobic biofilters (Chapter 3). Interestingly, differences with the gravel biofilter were more noticeable under anaerobic conditions, where the electroconductive HSSF biofilter removed 39% of ammonium, while gravel system removed 16%.

In VF biofilters a predominant aerobic environment favours nitrification; however our community analysis results shown in Chapter 5 revealed that electroactive anaerobic bacteria such as *Geobacter* and *Geothrix* represented more than 10% of the bacterial community, which would imply that anaerobic or microaerobic environments can be found in the inner zone of the biofilms. This fact is supported by SEM images that illustrate a thick biofilm covering the surface of

electroconductive granules (Figure 6-5). Regarding nitrogen removal, it has been reported that *Geobacter* bacteria can transfer electrons directly to *Thiobacillus* which in turn may reduce nitrate and nitrite (Kato et al., 2012). This genus of bacteria was abundantly found in those previous aforementioned studies and there is nothing to suggest otherwise than it would not be present in our systems. Therefore a fluorescence in situ hybridization (FISH) study would be of interest to show if ammonium oxidizing bacteria (AOB) and denitrifying bacteria could be present at different locations inside the biofilm.

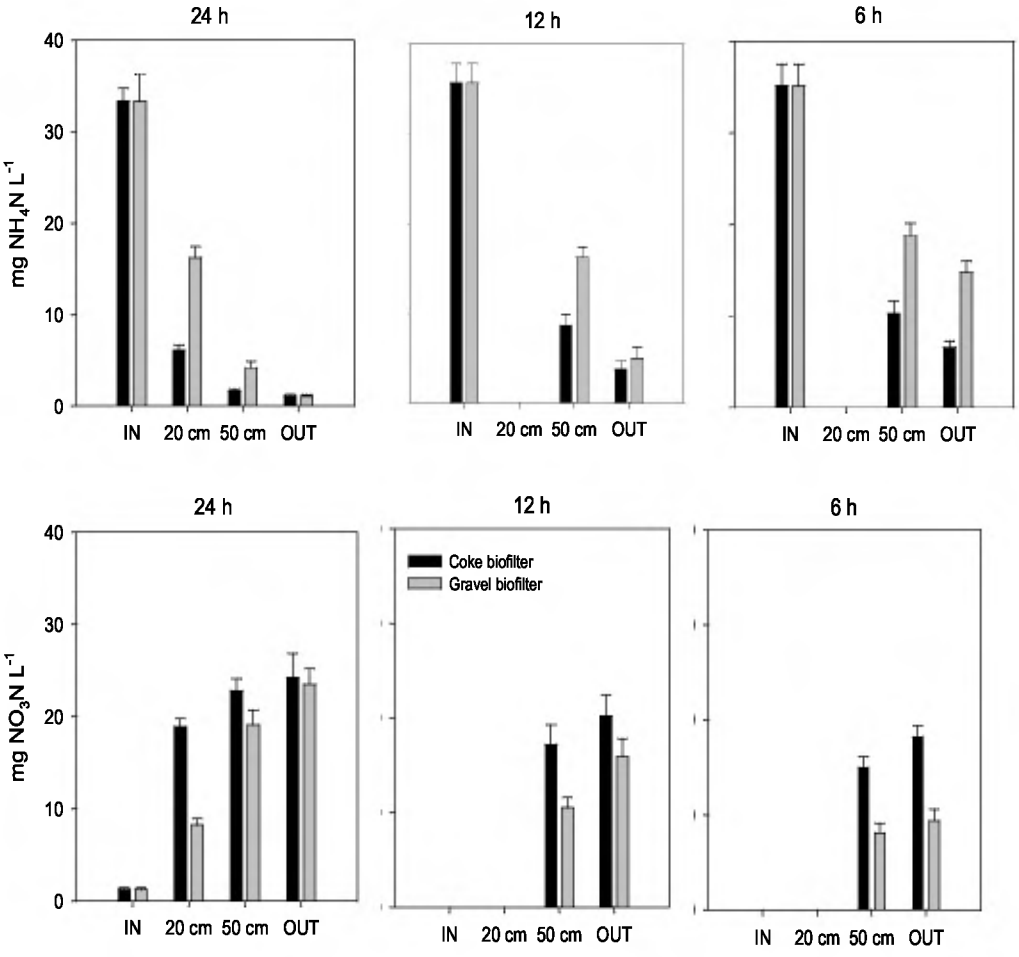


Figure 6-4 The graphs above illustrate ammonia-nitrogen and the graphs below nitrate-nitrogen concentrations in WW in every sample point at HRT of 24 h (left), 12 h (centre) and 6 h (right) comparing electroconductive (coke) and inert (gravel) biofilters.

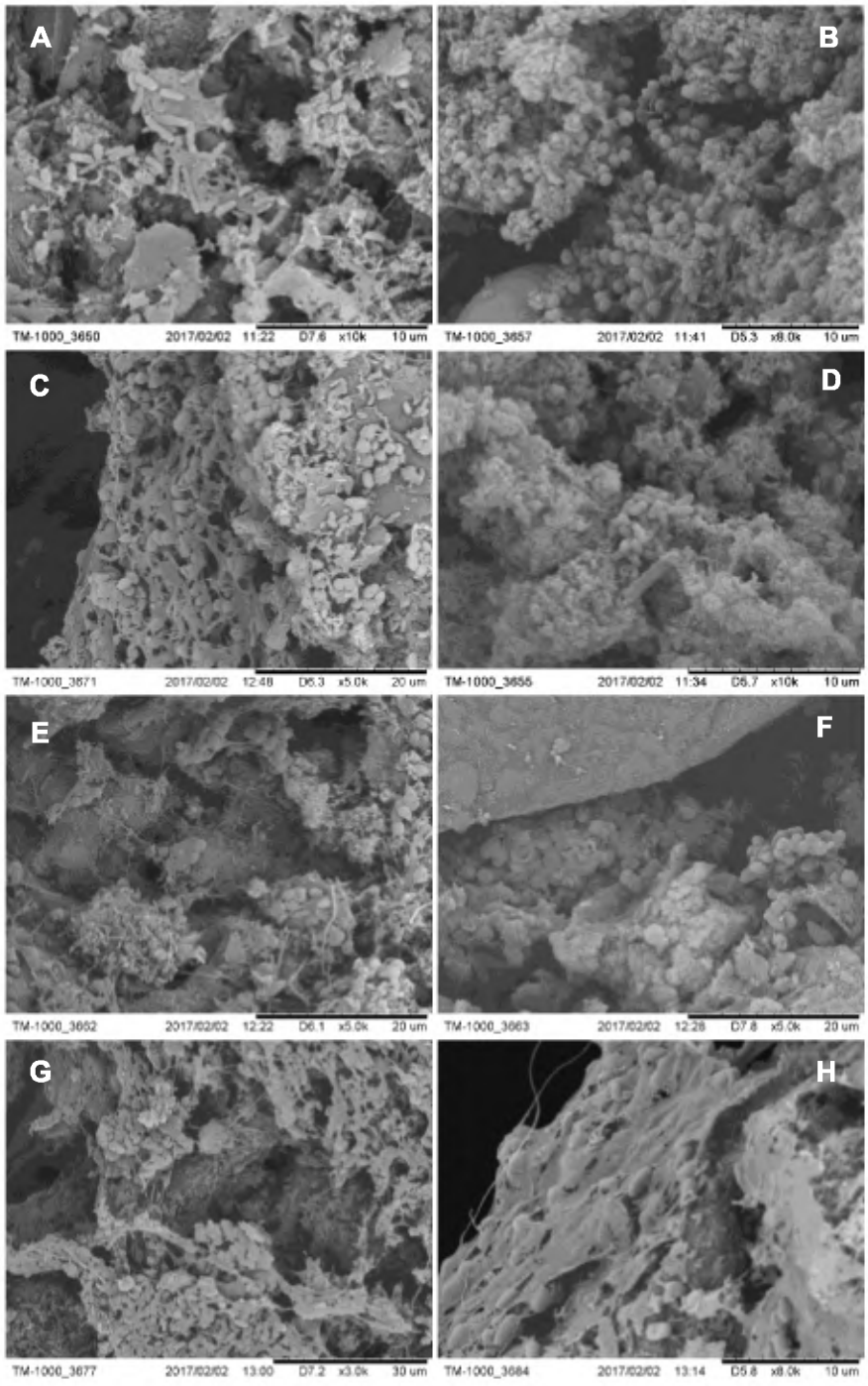


Figure 6-5: SEM images of the biofilm grown over the coke granules at different distances from the inlet. A: 2 cm; B, C: 20 cm; D, E, and F: 50 cm; G, H: 80 cm. A very thick biofilm can be observed.

6.4.3 Assessing the design of VF aerobic METlands

As previously shown in Chapter 1, VF CWs are usually designed at a rate of 3 m^2 p.e.$^{-1}$ (Brix and Arias, 2005; Kadlec and Wallace, 2009; Ortega et al., 2010). OLR of 10 to 40 g DBO_5 m^{-2} d^{-1} are often used in VFCW, depending on factors as external aeration (Fan et al., 2016; Uggetti et al., 2016), recirculation (Arias et al., 2005; Brix et al., 2003; Brix and Arias, 2005), with common designs using loading rates of 20 g DBO_5 m^{-2} d^{-1} to achieve a concentration at the effluent under 25 mg BOD_5 L^{-1} (Kadlec and Wallace, 2009; Ortega et al., 2010). The use of water or air pumps can result in systems with smaller area requirements (and better treatment performance), but it comes at the cost of increased electricity inputs (Nivala et al., 2013b). Common VF CWs have a filtering media depth between 75 cm and 1 m.

With an eye toward design of VF biofilters, we will take the recommended loading rate of 20 g DBO_5 m^{-2} d^{-1}, and taking into account that 1 population equivalent (p.e.) is 60 g DBO_5 m^{-2} d^{-1}, a conventional design would require 3 m^2 p.e.$^{-1}$ The VF electroconductive biofilter has shown to withstand a theoretical OLR of more than 400 g DBO_5 m^{-2} d^{-1} fulfilling COD requirements with 50 cm of material from the inlet and in a time equivalent to 6 hours. These figures would give a surface requirement of 0.15 m^2. Regarding solids, experts recommend the use of primary treatment systems as septic tanks or Imhoff tanks in order to remove over 50-60% of suspended solids, so it would be necessary to evaluate a long term performance with real wastewater to assess design parameters of the VF METlands.

The figures that we can manage are that 50 cm of depth is enough to fulfil COD legal requirements of discharge (125 mg COD L^{-1}) at a HRT of 6 hours. It is also adequate to generate effluents with an ammonium concentration under 10 mg N L^{-1}.

6.5 Conclusions

Aerobic VF MET biofilters provide a more efficient WWT than conventional VF gravel biofilters. It has been assessed that size can be reduced (one third of the volume of a conventional gravel biofilter) and still fulfil legal requirements of discharge established by the current legislation for organic matter.

Aerobic VF electroconductive biofilters achieve also higher nitrification rates; however, additional research is required to asses nitrate removal in combination with anaerobic biofilters such as HSSF ones, recirculating effluents or trying new MET applications as nitrate-reducing biocathodes artificially polarized at negative potentials.

Chapter 7. General Discussion, Conclusions and Future Work

General Discussion, Conclusions and Future Work

The work presented in this thesis supports the following hypothesis:

The performance of standard constructed wetlands (CWs) for treating urban wastewater can be enhanced by integrating microbial electrochemical systems. Thus, the term METland is presented here for first time as a hybrid concept that summarized the merging of two fields. Moreover, a general discussion is presented in the framework of a question-answer session, followed by a brief section of final conclusions, recommendations and future work.

7.1 General discussion

- Can METlands be a suitable technology for wastewater treatment? How do they work?

Our study reveals how the combination of Microbial Electrochemical Technologies (MET) and constructed wetlands (CW) can be satisfactory implemented for treating urban wastewater. The new concept of METland strongly outperforms classical treatments of urban wastewater through the stimulation of electroactive microbial populations, maximizing the synergetic effects of both technologies, as have been demonstrated in chapters 3, 4 and 5.

Although METs are classically related to energy producing concepts our aim was not to harvest energy but to enhance the rate of pollutants removal by converting the classical inert biofilter into an electroconductive biofilter that favoured the growth of electroactive bacteria. In this configuration, the electroconductive bed act as terminal electron acceptor as any other natural acceptor like oxygen, nitrate or Fe(III). The clear advantage of exploiting electro-stimulated communities is that electrodes can boost microbial metabolism in anaerobic systems that are typically limited in electron acceptors. Thus, electroconductive material may represent an inexhaustible source of electron acceptors, hosting the additional advantage of providing a more easily modulated redox potential compared to standard, low-reducing redox species that generally drive these systems (Kato Marcus et al., 2007). Electroconductive biofilters typically support biofilms enriched in *Geobacter* species together with other electroactive bacteria like *Geothrix*, *Desulfobulbus*, *Desulfovibrio*, etc. The increase of this bacterial genus triggers processes of interspecies electron transfer (IET), as reported in chapter 1 (Introduction section).

Interestingly, this IET was observed among different microbial species (Kouzuma et al., 2015; Liu et al., 2012; Morita et al., 2011; Rotaru et al., 2014a, 2014b; Summers et al., 2010) and it seems to contribute to the removal of pollutants. The fact that independent cells are capable of both extracellular electron donation and acceptance has remarkable implications for biofilms. In this biofilm scenario cells are in intimate contact and strong redox gradients may exist, which can represent appropriate environmental conditions for oxidation and reduction of chemical compounds boosted by IET.

- Why we do not construct METlands following the standard two-electrode configuration used in most MET?

Our first attempts for integrating microbial electrochemical systems in constructed wetlands were to follow the standard two electrodes (or even three by using a reference) configurations. Such systems allow to either have a control of the anode potential (microbial electrolysis cell configuration) or to harvest electrical power (microbial fuel cell configuration). Actually, chapter 3 and specially chapter 4 are a good prove of how the system can operate not just at the lab but also at field scale. However, our results revealed that integrating a two-electrode system operated as electrolytic mode in CWs did not improve WWT efficiency in comparison to the performance of a single electrode system (microbial electrochemical snorkel). On top of that, microbial electrolysis cell also entailed additional energy consumption due to the resistance exhibited by an electrochemical system with large electrodes at cubic meter scale. Our research revealed that the electroconductivity of the material by itself exert such a positive influence on the microbial metabolism that the electron flow among electrodes located at different environments (anode and cathode) is not a requirement to enhance biodegradation. In that sense, the single electrode configuration works as a simplified design of a short-circuited system that cannot monitor electrical current but stimulate electron transfer between bacteria. Our results with METlands are consistent with previous studies that reported how compact short-circuited system provided higher biodegradation performance than MFCs operating at maximum power (Erable et al., 2011). Similar examples have been also shown when short circuit systems are integrated in soil environments for enhancing bioremediation (Dominguez-Garay el al., 2007; Rodrigo et al., 2017). The driving force boosting microbial activity under single electrodes short-circuit systems could be supported by the redox gradient established between the bottom and the top environments of the systems in HSSF electroconductive bed. The redox

gradients with depth in that anaerobic single electrode biofilter may provide a chain of oxidation and reduction processes from the most electronegative to the most electropositive. Thus, the top layer of the METland could act as electron sink due to the presence of oxygen secreted from plants roots or diffused from atmosphere. Alternative other chemical species, such as nitrate, sulphate, CO_2, N_2 or even protons could be reduced at different levels depending on the redox state; and some species such as lactate, acetate or glucose will be oxidized at lower redox potentials.

- Can we still harvest electrical energy from CWs?

Since the first published study about CW-MFC (Yadav et al., 2012) a few researchers have made efforts to harvest energy integrating small electrodes in the biofiltering inert bed of anaerobic CWs. The goal was to maximize the redox gradient to increase the electrical current by burying an anode and locating a cathode in the surface or in the plant rhizosphere, with the aim of providing oxygen to the cathode and avoiding oxygen in the anode. In spite of the external aeration in the cathode these configurations result in large electrode separation that contributes to the ohmic resistance of the system, reducing the power generation of the MFC. Furthermore, such MFC integration did not pollutants removal in wastewater. Many problems have been found to reduce the resistance and increase the energy production and none of the studies seemed to offer a viable solution (Doherty et al., 2015a). Thus, electricity can be indeed harvested from CWs by means of electroactive bacteria but we doubt it can reach a value high enough to be of interest at industrial scale.

- Can electroactive bacteria be active in an aerobic environment? What advantages does an aerobic environment provide for treating wastewater in METlands?

HSSF and Vertical up-flow biofilters are saturated systems in which anaerobic conditions prevail. In a first overview they seem to be environments more appropriated for implementing METs because of the anaerobic conditions that favour the growth of anaerobic microorganisms. In fact, the model electroactive bacteria *Geobacter* was believed to be a strict anaerobe microorganism. However, *Geobacter* species have been reported to respire oxygen when this soluble terminal electron acceptor (TEA) is supplied at low concentrations (Lin et al., 2004). Contrary to the general idea that *Geobacter* species are strictly anaerobic (Koch and Harnisch, 2016), our experience have shown that *Geobacter* genus is able to live in

environments with oxygen concentrations in the range of 0 – 1 mg L^{-1} like those find in the upper layer of CWs. In that aerobic context, chapter 5 was fully devoted to explore if METs could work in an aerobic environment. Surprisingly, vertical down-flow non-flooded electroconductive biofilters constituted aerobic environments able of enhance the presence of electroactive bacteria and IEE processes. The presence of a redox gradient in biofilm mainly due to an oxygen gradient (Gieseke and de Beer, 2004; Li et al., 2015), in the case of thick biofilms could act as a protection for the inner anaerobic layers colonized by *Geobacter*.

Some studies have addressed that marine sediments become anoxic because oxygen is consumed by microbial processes at the surface. Without available oxygen the microorganisms living below the surface are supposed to depend on energetically less favourable anaerobic processes (Jørgensen, 2000). Actually, electrical currents harvested through marine sediment are generated by coupling oxygen consumption at the sediment surface with oxidation of hydrogen sulphide and organic carbon deep within the sediment (Nielsen et al., 2010). Mass balances indicated that more than 40% of total oxygen consumption in the sediment was driven by electrons conducted from the anoxic zone (Risgaard-Petersen et al., 2012). Microbial activity apparently drives the electrochemical half-reactions and the establishment of electron-conducting structures through the sediment (Nielsen et al., 2010; Pfeffer et al., 2012).

As a consequence, in these aerobic biofilters two types of processes can be achieved, the conventional aerobic processes in the outer layers of the biofilm plus the exoelectrogenic processes in the inner layer close to the electrode surface. Thus, oxidation of organic matter can be performed by a cooperative effect of aerobic bacteria and electroactive bacteria. Moreover, nitrification will be performed by aerobic bacteria and denitrification could be achieved by IET through electroactive bacteria and additional nitrate-reducing bacteria.

- What are the differences between the microbial communities colonizing electroconductive materials in comparison with those attached to classical inert bed?

Microbial community analysis by massive sequencing revealed vast differences between those attached to inert material (e.g. gravel) or electroconductive material (e.g. coke). Electroconductive material showed to enhance the growth of electroactive bacteria (EAB) such as *Geobacter*, but also others as *Geothrix* including members of the *Desulfobulbaceae* family.

Deltaproteobacteria, that are the class where many EAB are included, was the class group more represented in all the investigated systems when a conductive material was used as bed material. On the contrary, gravel-attached microbial communities were composed mainly by members of the classes *Alpha*, *Beta* and *Gammaproteobacteria*. At the order level, *Sphingomonadales* and *Rhizobiales* are some of the taxa more represented in the gravel microbial communities.

- Can we successfully scale-up the METland concept? What are the advantages of using METlands over conventional constructed wetlands?

As shown in Chapter 4, our scaled-up HSSF METland has been successfully implemented and operated during 4 years to treat 2 m^3 of real urban wastewater (the wastewater generated by ca. 15 inhabitants). The system was operated under organic loading rates ca. 4-fold higher than the recommended for standard horizontal subsurface constructed wetlands. The system fulfilled the water discharge legislation (Directive 91/271/EEC of WWT) with COD removal rates as high as ca. 400 g COD m^{-3} d^{-1} what allow to reduce the area requirement from ca. 5 m^2 $p.e^{-1}$ in standard HSSF CW to ca. 0.5 m^2 $p.e^{-1}$ in our new designs.

- Is it possible to reuse misused facilities to implement METlands?

Chapter 4 clearly shows that a HSSF METland can be easily implemented using standard CW facilities. Moreover, vertical flow METlands might use extended aeration activated sludge systems that have been abandoned during the last years in small populations due to the high costs of operation and maintenance. There are also many abandoned peat filters, for example in Andalusia (Ferrer Medina et al., 2012; Ortega et al., 2008) that are ideal pools for implementing METlands.

7.2 Conclusions

- METlands are a new efficient technology for treating urban wastewater by optimizing the synergetic effects of electroactive microorganism in a constructed wetland scenario.
- Electroconductive material stimulates the colonization of electroactive bacteria like those from the *Geobacter* genus.

- A single electrode system, like the one after the METland concept, can use the redox gradient as driving force stimulating microbial activity as any standard microbial electrochemical system.
- Microbial Electrochemical Systems for treating urban wastewater can be performed not just in anaerobic but also in aerobic environments.
- METland concepts can also be applied to enhance nitrification if they are operated under aerobic conditions.
- Surface area requirements of classical Constructed Wetlands (CW) can be significantly reduced with METlands design.

7.3 Recommendations and future work

The most important contribution of this research work has been to set the basis for the iMETland project, a project funded by the Horizon 2020 program of the EU which is currently being developed. The discovery that bioelectrochemical processes can take place in aerobic systems has laid the groundwork for the design of downstream vertical aerobic METlands, with the consequent energy saving that it entails when avoiding pumping.

Spain has a large number of small communities, as evidenced by the fact that of the 8,125 existing municipalities, according to the National Institute of Statistics, (INE 2015), 72% have a population of less than 2,000 inhabitants, with municipalities under 500 inhabitants rising to 3,800 (47%) (Ferrer Medina et al., 2012). Since 1 January 2006, urban agglomerations of less than 2,000 equivalent inhabitants, which discharge into inland waters or estuaries and which have a sanitation network, are obliged to subject their waste water to adequate treatment (Real Decreto Ley 11/1995 developed by RD 509/1996). The first National Sanitation and Treatment Plan (1995-2005) gave priority to medium and large agglomerations. Subsequently, the National Water Quality Plan (2007-2015) addressed the purification of small agglomerations, especially those located in protected areas. Also, the Autonomous Communities have begun to include the purification of small agglomerations in their sanitation plans.

In the WWT of small urban agglomerations in Spain, three distinct stages can be distinguished (Ferrer Medina et al., 2012): at the outset (prior to the 1980s), the purification technologies applied to small agglomerations were mere reproduction, on a smaller scale, than those applied in large cities, with the predominance of

installations based on the technology of prolonged aeration that were buried in smaller applications. Technical and economic deficiencies, in order to deal with the correct operation and maintenance of this type of installations, and lack of economic and technical resources caused that a large number of them were out of order, or did not meet the expected expectations. As an alternative, in the 1980s there was a "boom" of the so-called "low-cost technologies" or "soft technologies", which are now known as extensive technologies. In this context the technologies that reached a greater degree of implantation at national level were the stabilization ponds and peat filters. In most cases, these facilities also failed, due to poor designs or the applying of these technologies to population sizes much superior to the recommended. As a result many of these have been abandoned.

Nowadays, and after learning from the two previous stages, it is becoming aware that water purification in small agglomerations requires a more demanding approach, both from the technical level and from the management, to solve the problems that caused the previous failures, considering a whole range of possible technologies, depending on the specific characteristics of the agglomeration whose waste water is to be treated, and the requirements of discharge. It is necessary to find solutions that generate minimum energy cost, simple maintenance and functional robustness. Experts agree that the treatment of wastewater in small towns is, therefore, the great challenge of Spain in the management of urban water. In the current context, administrations must still devote many resources to the implementation and improvement of WWT in small populations.

It is therefore an appropriate moment to develop the implantation of this low-cost METland technology in small populations. But it is still necessary to improve aspects such as the elimination of nitrate and phosphorus that have not been addressed in the thesis, in order to allow the implementation in places where treated wastewater must to be discharged into sensitive areas and their catchments. Nitrate removal could be achieved by combining aerobic METlands followed by anaerobic ones to promote nitrification and denitrification. As this latter process needs organic matter, two options could be tested. One could be deriving a part of the inlet wastewater to the second stage and the second could be using a two electrodes configuration performing as a MEC that would allow reducing nitrate in the cathode.

In 1991, when the directive of WWT was approved, the authorities were concerned about organic matter and suspended solids and, in some cases, nitrogen and phosphorus, but in the 21st century there are new challenges to consider and add to the previous ones, such as organic microcontaminants called emerging

contaminants (biocides, cosmetics, hormones) and faecal microorganisms. Looking ahead into the future the laws will include also limitations to these contaminants. In view of this situation it will be necessary to test these systems in order to remove them from wastewater before their discharge to surface waters. And in the context of the climate change in Mediterranean climate areas, receiving waters are supposed to be reduced in quantity during the more and more extreme dry seasons, which will lead to more stringent regulations in the future.

Other interesting studies could be based on GEI emissions. It is known that anaerobic CW are systems where methanogenesis is produced and more research should be necessary to evaluate in which extent would the METlands reduce these emissions.

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ANNEX

List of Figures

Figure 1-1: Relationship between water flow rate (Qmax/Qave) and number of inhabitants for populations under 2,000. From: (Salas et al., 2007)..... 28

Figure 1-2: Pictures of different surface flow constructed wetlands. A: Free Water Surface (FWS) at Knockholt Landfill, Region of Bulckley/Nechako, Houston, BC; B: Free Floating Macrophyte (FFM) C: Floating Emergent Macrophyte (FEM). 35

Figure 1-3: An example of a subsurface flow constructed wetland to treat urban wastewater for 362 p.e. in Ondrejov, Czech Republic. 35

Figure 1-4: Water distribution in a vertical flow constructed wetland at the research centre CENTA, Carrión de los Céspedes, Sevilla, Spain. From: (Ortega et al., 2010). 36

Figure 1-5: Basic elements of a free water surface constructed wetland 36

Figure 1-6: Schemes of floating macrophyte CW. A: free floating macrophyte (FFM); B: floating emergent macrophyte (FEM) with buoyant structure. From: (Headley and Tanner, 2012). 37

Figure 1-7: Schematic horizontal subsurface flow constructed wetland 39

Figure 1-8: Schematic vertical flow constructed wetland 39

Figure 1-9: Scheme of a hybrid constructed wetland. From Vymazal, 2005. 41

Figure 1-10: Schematic of nitrification /denitrification and Anammox processes. ... 46

Figure 1-11: Schematic diagrams of respiration: (A) electron transfer to a soluble compound (as oxygen) and (B) microbial extracellular electron transfer. From (Kato et al., 2012). 49

Figure 1-12: Electrical interplay between microorganisms and minerals. A: Microorganisms use minerals that contain metal ions as terminal electron acceptors for respiration; B: electron and/or energy sources for growth; C: electrical conductors that facilitate electron transfer between microbial cells of the same and different species and D: electron-storage materials, or batteries, to support microbial metabolism. From: Shi et al., 2016. 50

Figure 1-13: A: Within a cytochrome, hemes are positioned in a chain that allows electrons to hop through the molecule; **B**: Flavins carry electrons from the bacterium to the TEA and shuttle back to the bacterium to pick up more

electrons; **C**: Electrons hop between cytochromes on pili appendages (DEET); **D**: DEET by direct attachment of cells to the surface. ©Tom Graves. 51

Figure 1-14: A: Cells of *Shewanella oneidensis* connected by microbial nanowires, composed of pili protein. From Gorby *et al*, 2006. B: Filamentous *Desulfobulbaceae* cells (yellow) identified by fluorescence in situ hybridization forming a micro-cable. From Pfeffer *et al*, 2012. 52

Figure 1-15: Microbial processes in a MET system 54

Figure 1-16: A: Schematic of a 2-chamber MFC with an anionic membrane separator. B: Schematic of a MEC configuration of 3 electrodes in which the anode is the working electrode. From Tejedor-Sanz, 2016 55

Figure 1-17: Schematic of a MES applied to bioremediation of a polluted soil with petroleum hydrocarbons. From: Cruz Viggi *et al.* (2015). 57

Figure 1-18: Bioelectrochemical nitrogen and organic matter removal process in wastewater. From Tejedor-Sanz, (2016). 60

Figure 1-19: A: *Top*: meteorological data buoy used in demonstration on the pier of the Naval Research Laboratory in Washington, DC, being prior to deployment (mooring and RF transmitter antenna not yet configured). *Bottom*: one of the first generation BMFC subunits on pier prior to deployment. Seven subunits were electrically connected in parallel to provide sufficient power to operate buoy. From (Tender et al., 2008). **B**: Tubular microbial fuel cells tested for power production using wastewater produced at Foster's brewery in Yatala, Australia (www.microbialfuelcell.org). From (Logan, 2010) 62

Figure 1-20: Pilot-scale microbial electrolysis cell being tested for hydrogen production using winery wastewater at the Napa Wine Company in California, USA. From (Cusick et al., 2011) 62

Figure 3-1: Simplified design of the four systems A) Gravel biofilter (control), B) Coke biofilter, C) Hybrid biofilter, D) Hybrid polarized biofilter 75

Figure 3-2: A) Relation between normalized COD removed and COD inlet loading of the coke and the gravel biofilters, B) Relation between normalized NH_4-N removed and NH_4-N inlet loading of the coke and the gravel biofilters. 79

Figure 3-3: COD (A) and BOD_5 (B) influent and effluent average values of the coke and gravel filters. Error bars represent 95 % confidence interval. 80

Figure 3-4: Profile of electrical current, COD loading rate (g $m^{-3}d^{-1}$) and COD removal rate (g $m^{-3}d^{-1}$) during long term operation of the hybrid biofilter polarized at 0.3 V (vs. Ag/AgCl). 81

Figure 3-5: Relative abundances of OTUs at class level (larger than 5% in average). SEM images of coke granules of A) polarized hybrid coke filter. B) coke filter. A dense matrix covering the conductive material can be observed. 85

Figure 4-1: Schematic drawings of the tested configurations of cathode and anode of the bioelectrochemical constructed wetland (METland) through the period of study. A) Carbon cloth cathode over the surface; B) Coke granules cathode, with plants; C) Rolled-up carbon cloth cathode into the 2.6 m^3 anode. 106

Figure 4-2: Schematic of water sample points in the METland 107

Figure 4-3: Schematic of the sample points for 16S rDNA sequencing analysis to study the bacterial community in the METland during the first stage. A1: Gravel biofilm after electrodes; A3: Anode biofilm; A4: gravel biofilm before electrodes; A5: Cathode biofilm; A6: Inlet wastewater. 109

Figure 4-4: Schematic of the sample points for the 16S rDNA sequencing during the second stage. The dimensions are not proportional to the real ones. 110

Figure 4-5: Cross-section of an aerenchymatous stalk of a typical aqueous plant 111

Figure 4-6: Hydroponically grown wetland plants in a growing chamber with humidity and temperature control. 112

Figure 4-7: Experimental set up of the methylene blue method and examples of plants mounted in two types of jar. 112

Figure 4-8: COD evolution throughout the period of study. The results have been scheduled in two main stages: a first period where the system was operating with a 0.9 m^3-anode (A) and a second period with a 2.6 m^3-anode (B). 116

Figure 4-9: COD removal rates through the period of study in different locations of the METland. It is important to point out that data for removal rates are referred to the volume of certain beds, while COD loading rate is referred to the total volume of the METland bed. 117

Figure 4-10: Relationship between BOD loading rate and BOD removal rate during the two stages (0.9 m^3-anode and 2.6 m^3-anode). The slope represents the percentage of BOD removed. 117

Figure 4-11: Relationship between COD loading rate and COD removal rate during the two stages (0.9 m^3-anode and 2.6 m^3-anode). The slope represents the percentage of COD removed. 118

Figure 4-12: TSS evolution throughout the period of study. The results have been divided in two main stages: a first period where the system was operating with a 0.9 m^3-anode (A) and a second period with a 2.6 m^3-anode (B). 119

Figure 4-13: Nitrogen evolution throughout the period of study. Top graph: Ammonia nitrogen; Bottom graph: Nitrate nitrogen. The results have been divided in two main stages: a first period (weeks 11 to 103) where the system was operating with a 0.9 m^3-anode and a second period (weeks 119 to 166) with a 2.6 m^3-anode. Grey bars correspond to changes of configuration. Top numbers indicate the periods of operation..... 120

Figure 4-14: Relationship between TP loading rate and TP removal rate during the two stages (0.9 m^3-anode and 2.6 m^3-anode). The slope represents the percentage of COD removed. Line does not fit the data very closely. 122

Figure 4-15: Total phosphorous evolution throughout the period of study. The results have been divided in two big stages: a first period (left graphs) where the system was operating with a 0.9 m^3-anode and a second period (right graphs) with a 2.6 m^3-anode..... 121

Figure 4-16: Rarefaction curves of the observed species in the five samples of the first sequencing. 123

Figure 4-17: Higher taxa community composition in the samples of the first sequencing. 124

Figure 4-18: OTUs network of the five samples of the first stage. Purple nodes represent dominant bacterial OTUs (with proportion higher than 0.005%). Lilac nodes represent dominant Archaea OTUs (mostly methanogenic taxa)..... 125

Figure 4-19: Dominant bacteria genera in the different environments of the METland in the sequencing of the first stage..... 126

Figure 4-20: Rarefaction curves of the observed species in the 22 samples taking into the METland at the second stage..... 126

Figure 4-21: Taxa of OTUs at class level of the second sequencing, with more than 1% in some of the samples. 128

Figure 4-22: Heat map of *Geobacter* distribution in the METland in the second stage..... 130

Figure 4-23: Pictures of ROL by plant roots, at 0 h, 2 h and 24 h since the introduction in the agar solution. A: *Phragmites australis*; B: *Juncus effussus*; C: *Cyperus laevigatus*; D: *Typha latifolia*; E: *Iris pseudacorus* 132

Figure 4-24: Pictures of ROL by plant roots showing that oxygen release was mainly produced throughout the tip of new adventitious roots and secondary roots.. 132

Figure 4-25: Porosity of the adventitious roots of the five wetland plants tested... 133

Figure 4-26: Response annual average effluent BOD_5 of WWT systems to increasing annual average BOD loadings. Top graph: Data of one year operation of our METland during the second stage (large anode); Bottom graph: Data of HSSF wetlands are represented by 265 years of data for 113 systems. From Kadlec & Wallace 2009..... 134

Figure 4-27: Longitudinal profiles of BOD_5 and COD. Top graph: at METland. Bottom graphs: data from Belgium and Czech Republic. From Vymazal and Kropfelova 2008. 136

Figure 4-28: Ammonium heat-map of the METland at the second stage..... 141

Figure 4-29: Dissolved oxygen heat-map of the METland at the second stage.... 141

Figure 4-30: Nitrate distribution in the METland in the second stage..... 141

Figure 4-33: Average K_{BOD5} during the **first stage** (top graph) and the second stage (bottom graph) depending on the organic loading rate, calculated from experimental data..... 148

Figure 4-34: Average K_{TN} during the **first stage** (top graph) and the **second stage** (bottom graph) depending on the TN loading rate, calculated from experimental data. 148

Figure 5-1: Schematic of the biofilters design and experimental set-up..... 171

Figure 5-2: Influent and effluent average concentrations of TN, NH_4-N and NO_3-N of the anaerobic UF and aerobic DF electroactive biofilters. Error bars represent 95% confidence interval. 175

Figure 5-3: Relationship between normalized PT removed and PT inlet load of the anaerobic (UF) and aerobic (DF) electrochemical biofilters and their respective controls..... 177

Figure 5-4: Relative abundances of OTUs at class level (more than 1%) comparing communities of the anaerobic (UF) and aerobic (DF) biofilters. 179

Figure 5-5: Relative abundances of OTUs at class level (larger than 1% in average) comparing communities of the aerobic DF coke and gravel biofilters. 179

Figure 6-1: Design of the vertical down-flow biofilters set up 193

Figure 6-2: The graphs show COD concentration at different levels from the inlet, at every HRT in the electroconductive (left) and the gravel (right) biofilters..... 196

Figure 6-3: Ammonia removal rates in both biofilters at different loading rates that correlate with several HRT. 198

Figure 6-4 The graphs above illustrate ammonia-nitrogen and the graphs below nitrate-nitrogen concentrations in WW in every sample point at HRT of 24 h (left), 12 h (centre) and 6 h (right) comparing electroconductive (coke) and inert (gravel) biofilters. 199

Figure 6-5: SEM images of the biofilm grown over the coke granules at different distances from the inlet. A: 2 cm; B, C: 20 cm; D, E, and F: 50 cm; G, H: 80 cm. A very thick biofilm can be observed. 200

Figure 7-1: The electron tower illustrating the electrode potentials (E_0') of various redox couples at environmentally relevant concentrations for microorganisms. From: Brock Biology of Microorganisms 11/e © 2006 Pearson Prentice Hall Inc. .....**¡Error! Marcador no definido.**

List of supplementary figures

Figure S 3-1: Total nitrogen (A), ammonia (B) and nitrate (C) influent and effluent average values of the coke and the gravel biofilters. Error bars represent 95% confidence interval. 88

Figure S 3-2: Influent and effluent COD (A) and BOD_5 (B) average values of the hybrid biofilter and the hybrid polarized biofilter. Error bars represent 95% confidence interval. 88

Figure S 3-3: Redox potential measurements in the coke and the gravel biofilters 89

Figure S 3-4: Rarefaction curves calculated for each sample based on the OTU computations. 90

Figure S 3-5: Correspondence analysis biplot of classes' distribution from 16S rDNA analysis. 90

Figure S 3-6: Relative abundance of classes of the category 'other' at class level. 91

Figure S 4-1: BOD_5 evolution throughout the period of study. The results have been divided in two big stages: a first period where the system was operating with a 0.9 m^3-anode (A) and a second period with a 2.6 m^3-anode (B). 151

Figure S 4-2: BOD_5 average removal rates through the period of study in different regions of the METland, referred to the volume of each respective region. COD loading rate is referred to the total volume of the METland. Error bars represent 95% confidence interval. 151

Figure S 4-3: Groups of OTUs at phylum level of the second stage 16S rDNA sequencing. 153

Figure S 5-1: Relative abundances of OTUs at the phylum level (larger than 1% in average 181

Figure S 5-2: Rarefaction curves calculated for each sample based on the OTU computations. DF = down-flow aerobic biofilter; UF = up-flow aerobic biofilter. 181

Figure S 5-3: Correspondence analysis plot of the bacterial communities in the two up-flow (anaerobic) and down-flow (aerobic) biofilters 183

List of Tables

Table 1-1: Requirements for discharges from urban waste water treatment plants of agglomerations of > 2,000 p.e. The values for concentration or for the percentage of reduction shall apply. 26

Table 1-2: Requirements for discharges from urban WWT plants to identified sensitive areas which are subject to eutrophication. The values for concentration or for the percentage of reduction shall apply. 26

Table 1-3: Composition of a model wastewater in small populations (Ortega et al., 2010) 28

Table 1-4: Treatment performance values in subsurface slow constructed wetlands. From Ortega et al., 2010. 42

Table 3-1: Urban wastewater treated by hybrid biofilter setups. COD overall averages ± SD, at HRT = 3.4 d. Removal efficiencies in the conductive bed (%) were referred to the COD before conductive bed. 83

Table 4-1: Periods of operation and changes carried out in the METland throughout the time of study. 114

Table 4-2: Total and high quality reads and alpha diversity metrics of the bacterial populations in the samples of the second stage 16S rDNA sequencing. 129

Table 4-3: Design parameters of CW of subsurface flow (SSF) 144

Table 4-4: Values of K_R and θ_R for every type of contaminant in SSF CW 146

Table 4-5: K_A values of BOD and TN calculated from experimental data for every period. OLR = organic loading rate; TNLR = total nitrogen loading rate. Average water temperature is shown in top table. 147

Table 5-1: Removal rates (g m^{-3} d^{-1}) of the up-flow and down-flow coke biofilters (averages ±SD). Removal efficiencies (%) are shown in brackets. 174

Table 6-1: Composition of the synthetic wastewater 194

Table 6-2: Organic and ammonium loading rates corresponding to the different hydraulic loading rates tested. Theoretical BOD was estimated as the 60% of the COD concentration. 195

Table 6-3: COD and ammonia removal efficiencies reached at every level for each HRT in both biofilters. 197

List of supplementary tables

Table S 3-1: Influent pre-treated wastewater and effluents characteristics. Averages ± SD..... 92

Table S 3-2: Organic matter removal rates (COD and BOD_5) and percentage removal efficiencies (in brackets). Averages ± SD (COD: n=30; BOD_5: n=15) . 93

Table S 3-3: Statistical test and p-values of the coke and gravel biofilters effluents comparison (*significant differences). W = Wilcoxon test; t = T-test 93

Table S 3-4: Overall averages ± SD (n=15) of total nitrogen and ammonia removal rates and average removal efficiencies (in brackets) 94

Table S 3-5: Alpha diversity metrics of the bacterial populations 94

Table S 3-6: Main taxa of bacteria identified in the analysed communities (over 0.1), in percentage..... 95

Table S 4-1: Characteristics of influent wastewater after the Imhoff tank (average ± SD) 149

Table S 4-2: Limits of discharge established in the Directive 91/271/CEE. 149

Table S 4-3: COD and BOD_5 concentrations (in mg L^{-1}) of treated wastewater in sample points at different periods (average ± SD). 150

Table S 4-4: COD and BOD_5 removal rates in every region of the METland in grams per cubic meter of bed material and day (g m^{-3} d^{-1}), according to the period (average ± SD). Surface and volumetric loading rates are given in grams per square meter (g m^{-2} d^{-1}) or per cubic meter (g m^{-3} d^{-1}) of the total wetland and day, respectively..... 150

Table S 4-5: Total suspended solids (TSS) concentrations in mg L^{-1} of treated wastewater in every sample point at different stages (average ± SD). 152

Table S 4-6: Nitrogen (in the form of total nitrogen and ammonium nitrogen) removed in percentage referred to the influent, in every area of the METland at different periods (average ± SD). 152

Table S 4-7: TP removed in percentage, referring to the influent, in every region of the METland at different periods (average ± SD). 152

Table S 4-8: Dominant bacteria genera in the different environments of the METland in the first stage 16S rDNA sequencing (in %). Legend: * = p_ phylum; c_ class; o_ order; f_ family; g_ genus 154

Table S 4-9: Dominant bacteria **family** (more than 1% in some sample) in the **anode** of the METland in the second sequencing (in %). Legend = c_ class; o_ order; f_ family. 156

Table S 4-10: Dominant bacteria **family** (more than 1% in some sample) in the **intermediate area of gravel** after the anode (HG13, HG14, HG15, HG16) and in the **final area of gravel** after the cathode (HG21, HG22, HG23, HG24) of the METland in the second stage sequencing (in %). Legend = c_ class; o_ order; f_ family. 157

Table S 4-11: Dominant bacteria family (more than 1% in some sample) in the **cathode** of the METland in the second sequencing (in %). Legend = c_ class; o_ order; f_ family. 158

Table S 4-12: Dominant **genus** (more than 0.5% in some sample) in the **anode** of the METland in the second stage 16S rDNA sequencing (in %). 159

Table S 4-13: Dominant **genus** (more than 0.5% in some sample) in the **cathode** of the METland in the second stage 16S rDNA sequencing (in %). 160

Table S 4-14: Dominant **genus** (more than 0.5% in some sample) in the **influent and effluent wastewater** of the METland in the second stage 16S rDNA sequencing (in %). Legend = f_ family; g_genus. 161

Table S 4-15: Dominant **genus** (more than 0.5% in some sample) in the **intermediate gravel** after the anode and **final gravel** after the cathode of the METland in the second stage 16S rDNA sequencing (in %). Legend = o_ order; f_ family; g_genus. 162

Table S 5-1: Influent wastewater characteristics 182

Table S 5-2: Influent and effluent residual concentrations of COD, BOD_5, TSS, TN, NH_4-N, NO_3-N and TP (average ± SD). Removal efficiencies (%) are shown in brackets. UF = up-flow; DF = down-flow. 182

Table S 5-3: Alpha diversity metrics of the bacterial populations in the samples. UF = up-flow; DF = down-flow 182

Table S 5-4: OTUs at level genus of the aerobic DF biofilter (> 1% in some sample). 183

Table S 5-5: OTUs at genus level of the anaerobic (UF) biofilter (> 1% in some sample). Legend: o = order; f = family; g =genus. 184

Table S 5-6: OTUs at genus level of the gravel down-flow (DF) biofilter (> 1% in some sample). 185

Table S 5-7: Main genera of bacteria identified in the analysed coke biofilters communities (> 0.1%). 186

Abbreviations

AE	Auxiliary or counter electrode
AOB	Ammonium oxidizing bacteria
BES	Bioelectrochemical system
BOD	Biological oxygen demand
CE	Coulombic efficiency
CEM	Cation exchange membrane
COD	Chemical oxygen demand
CW	Constructed wetland
DEET	Direct extracellular electron transfer
DF	Down-flow
DIET	Direct interspecies electron transfer
DMRB	Dissimilatory iron-reducing bacteria
DO	Dissolved oxygen
E_{anode}	Anode potential
$E_{cathode}$	Cathode potential
EAB	Electroactive bacteria
ED	Electron donor
EET	Extracellular electron transfer
FEM	Free emergent macrophytes
FFM	Free floating macrophytes
FISH	Fluorescence in situ hybridization
FWS	Free water surface
HSSF	Horizontal subsurface flow
HLR	Hydraulic loading rate
HRT	Hydraulic retention time
IET	Interspecies electron transfer
MBR	Membrane bioreactor
MDC	Microbial desalination cell
ME	Microbial electrochemistry
ME-FBR	Microbial electrochemical fluidized bed reactor
MEC	Microbial electrolysis cell
MEET	Mediated extracellular electron transfer
MERC	Microbial electroremediating cell
MES	Microbial electrochemical snorkel
MET	Microbial electrochemical technology

MFC	Microbial fuel cell
NOB	Nitrite oxidizing bacteria
OCP	Open circuit potential
OLR	Organic loading rate
OM	Organic matter
O&M	Operation and maintenance
p.e.	Population equivalent
RBC	Rotating biological contactor
RE	Reference electrode
ROL	Radial oxygen loss
SBR	Sequencing batch reactor
SF	Surface flow
SSF	Subsurface flow
SEM	Scanning electron microscopy
TEA	Terminal electron acceptor
TN	Total nitrogen
TNLR	Total nitrogen loading rate
TP	Total phosphorous
TSS	Total suspended solids
TW	Treatment wetland
UF	Up-flow
VF	Vertical flow
WE	Working electrode
WWT	Wastewater treatment
WFD	Water framework directive