CARBON AND NITROGEN TREATMENT IN INDUSTRIAL WASTEWATERS USING BIOELECTROCHEMICAL SYSTEMS

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Carbon and nitrogen treatment in industrial wastewaters using bioelectrochemical systems

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   Author's contribution: Experimental design and chemical and electrochemical performance. Data monitoring and reactor operation. Writing the paper.

List of abbreviations

ADP	Adenosine diphosphate
AEM	Anion exchange membrane
AMO	Ammonia monooxygenase enzyme
Anammox	Anaerobic ammonia-oxidizing bacteria
AOA	Ammonia-oxidizing archaea
AOB	Ammonia-oxidizing bacteria
AQDS	Anthraquinone-2-6, disulfonate
ATP	Adenosine triphosphate
ATU	Allylthiourea
BES	Bioelectrochemical system
BES	2-Bromoethanesulfonate (Chapter 5)
BOD	Biochemical oxygen demand
BSA	Bovine serum albumin
CA	Chronoamperometry
CAPEX	Capital expenditures
CARD-FISH	Catalysed reporter deposition-FISH
CCV	Closed-circuit voltage
CE	Coulombic efficiency
CEM	Cation exchange membrane
CFD	Computational fluid dynamic
CLSM	Confocal laser scanning microscopy
COD	Chemical oxygen demand
CRM	Confocal Raman microscopy
Cu-NIR	Copper nitrite reductase
CV	Cyclic voltammetry
DC	Double compartment
DCE	Dichloroethene
DET	Direct electron transfer
DGGE	Denaturing gradient gel electrophoresis
DN	Denitrification
DO	Dissolved oxygen
EAB	Electrochemically active bacteria
EET	Extracellular electron transfer
Ef	Final potential
Ei	Initial potential
ENR	External nitrifying reactor
EPS	Extracellular polymeric substance

ET	Electron transfer
EUB	Total bacteria
F	Faraday's constant
FA	Free ammonia
FC	Flow cytometry
FDR	False detection rate
FISH	Fluorescent in situ hybridization
FNA	Free nitrous acid
Fw	Flow rate
GG	Granular graphite
H'	Shannon diversity
HAO	Hydroxylamine oxidoreductase enzyme
HDH	Hydrazine dehydrogenase
HNO	Nitroxyl hydride enzyme
HRT	Hydraulic retention time
HZS	Hydrazine oxide synthase
I	Current
ICET	Intracellular electron transport
IET	Indirect electron transfer
MC	Microcosm
MEC	Microbial electrolysis cell
MFC	Microbial fuel cell
MPN-PCR	Most probable number PCR
MPP	Maximum power point
MPPT	Maximum power point tracking
Ms	Molecular mass
n	Number of electrons
N	Nitrification
N_2OR	Nitrous oxide reductase
NAC	Net anode compartment
NAR	Nitrate reductase
NCC	Net cathode compartment
□COD	Organic matter removal efficiency
niBES	Nitrifying BES
NIR	Nitrite reductase
NLR	Nitrogen loading rate
NOB	Nitrite-oxidizing bacteria
NOR	Nitric oxide reductase
NRC	Net reactor compartment

NRR	Nitrogen removal rate
NxOR	Nitroxyl oxidoreductase enzyme
NXR	Nitrite oxidoreductase enzyme
OCV	Open circuit voltage
DO	Dissolved oxygen
OLR	Organic loading rate
OM	Organic matter
ORR	Organic removal rate
OTU	Operational taxonomic units
P	Power (W in Chapter 4)
PCR	Polymerase chain reaction
PD	Phylogenetic diversity
QIIME	Quantitative insights into microbial ecology
qPCR	Quantitative PCR
RT-PCR	Real time PCR
S	Substrate
SC	Single compartment
SIP	Stable isotope probing
SM	Swine manure
SND	Simultaneous nitrification and denitrification
SS	Stainless steel
TAN	Total ammonium as nitrogen
TC	Triple compartment
TCD	Thermal conductivity detector
TCE	Trichloroethene
TKN	Total Kjeldahl nitrogen
TN	Total nitrogen
T-RFLP	Terminal restriction fragment length polymorphism
TSS	Total suspended solids
V	Cell voltage
VFA	Volatile fatty acids
VSS	Volatile suspended solids
WWTP	Wastewater treatment plant

List of figures

List of tables

Certificate of thesis direction

El Dr. Jesús Colprim Galceran, la Dra. Maria Dolors Balaguer Condom i el Dr. Sebastià Puig Broch del Laboratori d'Enginyeria Química i Ambiental (LEQUIA) de la Universitat de Girona,

DECLAREM:

Que aquest treball, titulat "Carbon and nitrogen treatment in industrial wastewaters using bioelectrochemical systems", que presenta Anna Vilajeliu Pons per a l'obtenció del títol de doctor/a, ha estat realitzat sota la nostra direcció i que compleix els requeriments per ser publicada com a compendi de publicacions i per poder optar a Menció Internacional.

I perquè així consti i tingui els efectes oportuns, signem aquest document.

Dr. Jesús Colprim Galceran

Dra. Maria Dolors Balaguer Condom

Dr. Sebastià Puig Broch

Girona,

A la Sònia, la iaia i en Narcís,

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Resum

La presència de carboni (matèria orgànica) i nitrogen (amoni) en aigües residuals municipals i industrials (ramaderia, agricultura i fàbriques) és un problema greu a tot el món. Aquests productes no desitjats es generen contínuament en la nostra societat. Aquests, contribueixen a la contaminació del medi ambient, reduint la qualitat de l'aigua i augmentant la freqüència dels fenòmens d'eutrofització. Avui en dia, els fangs actius i la digestió anaeròbica són les tecnologies més utilitzades en les plantes de tractament d'aigües residuals (EDAR) per eliminar aquests contaminants. No obstant això, ambdues tecnologies no són eficients pel tractament del nitrogen. En els fangs actius, la nitrificació comporta un fort cost econòmic degut a l'aireació, consumint prop d'1/3 del balanç energètic de l'EDAR. Mentre que en la digestió anaeròbia, el nitrogen no és tractat i requereix d'un post-tractament addicional. Per aquestes raons, cal explorar noves tecnologies capaces d'eliminar simultàniament el carboni i el nitrogen de les aigües residuals de manera més eficient i sostenible.

La utilització de sistemes bioelectroquímics (BES) permet l'eliminació de la matèria orgànica i el nitrogen de les aigües residuals amb generació de bioelectricitat. En els bioanodes d'un BES, els microorganismes exoelectrogènics poden oxidar matèria orgànica, alliberant electrons i protons cap al càtode. En els biocathodes, els microorganismes electrotròfics són capaços de desnitrificar. A més, aquesta tecnologia aporta versatilitat operativa per a la nitrificació. Per aquestes raons, aquesta tesi té com a objectiu avaluar l'eliminació simultània de carboni i nitrogen en BES per la seva futura aplicació en el tractament d'aigües residuals industrials com els purins.

En els primers capítols, aquesta tesi doctoral demostra la viabilitat dels BES en el tractament de matrius complexes com els purins. A continuació, es van avaluar diferents dissenys de BES per optimitzar el tractament de nutrients i el rendiment electroquímic mitjançant la modificació de: I) les configuracions de l'ànode i el càtode, ii) el control de la resistència externa, III) els donants/receptors d'electrons de l'ànode/càtode i IV) el punt d'ajust d'oxigen de la nitrificació.

Per tal de buscar una aplicabilitat real, els reactors BES a escala mL van ser escalats i operats a llarg termini. Es va observar que els retiments de tractament i la producció

d'electricitat assolits a escala mL (0,6 L) es podien reproduir a escala L (65 L). L'avaluació de diferents materials pels elèctrodes (grafit granular i acer inoxidable) a gran escala va permetre la identificació de les seves limitacions i la seva futura aplicabilitat. El grafit granular (àmpliament utilitzat en BES a petita escala) va disminuir la seva eficiència al llarg del temps, a causa del seu aixafament. No obstant això, l'acer inoxidable va destacar per ser l'elèctrode més adequat a llarg termini pel tractament d'aigües residuals complexes en BES a escala L.

De cara augmentar la sostenibilitat dels BES, es van investigar estratègies per augmentar les eficiències electroquímiques. La seva potència va ser millorada mitjançant l'aplicació d'un control de resistència extern (Maximum power point tracking, MPPT) a escala mL i diferents connexions elèctriques a escala L. D'altra banda, l'MPPT va incrementar l'abundància de bacteris i va promoure la selecció dels suposadament exoelectrogenics.

Finalment, els microbiomes dels BES van ser avaluats des del punt de vista microbiològic i electroquímic. Diferents tècniques microbiològiques, quantitatives (qPCR) i qualitatives (PCR-DGGE, 454-piroseqüenciació, T-RFLP, FISH i SEM) van ser utilitzades per quantificar i identificar les comunitats microbianes. Les dades microbiològiques i enginyerils van ser utilitzades per relacionar els microorganismes amb el seu suposat paper en l'eliminació de nutrients i producció d'electricitat (electrogènics, nitrificants i desnitrificants). Finalment, els microcosmos es van utilitzar amb èxit per la caracterització de la termodinàmica de transferència extracel·lular d'electrons i els passos electroactius per a l'oxidació anòxica d'amoni.

En conclusió, els resultats presentats en aquesta tesi doctoral conclouen que els sistemes bioelectroquímics tenen potencial de ser una tecnologia alternativa pel tractament simultani de carboni i nitrogen amb producció d'electricitat en aigües residuals industrials com els purins. En un futur proper, pot esdevenir una alternativa més econòmica i més sostenible que els processos actuals.

Summary

The presence of carbon (organic matter) and nitrogen (ammonium) in municipal and industrial (livestock farming, agriculture and factories) wastewaters is a serious worldwide concern. These undesired products are being continuously generated in our society. They contribute to the environmental pollution, reducing the water quality and increasing the eutrophication phenomena frequency. Nowadays, activated sludge and anaerobic digestion are the most used technologies in wastewater treatment plants (WWTPs) to remove these contaminants. However, both technologies are not efficient for the nitrogen treatment. In activated sludge, nitrification entails a strong economic cost for aeration. It consumes about 1/3 of the WWTP energy balance. In anaerobic digestion, nitrogen is not treated and it requires an additional post-treatment. For these reasons, it is necessary to explore alternative technologies able to simultaneously remove carbon and nitrogen from wastewater in a more efficient and sustainable way.

The utilization of a bioelectrochemical system (BES) allows the removal organic matter and nitrogen from wastewater with concomitant bioelectricity generation. In bioanodes of a BES, exoelectrogenic microorganisms can oxidize organic matter, releasing electrons and protons to the cathode. In biocathodes, electrotrophic microorganisms can perform denitrification. Moreover, this technology gives versatile operational options for nitrification. For these reasons, this thesis aims to asses a simultaneous carbon and nitrogen removal BES looking for its future implementation treating industrial wastewater as swine manure.

In the first chapters, this PhD thesis demonstrates the viability of BES to treat complex matrices as swine manure, removing carbon and nitrogen and generating electricity. Then, different BES designs were evaluated to optimize both nutrient removal and electrochemical performance by modifying: I) the anode/ cathode configurations; II) the external resistance control; III) the anode/cathode electron donors/acceptors and IV) the nitrification oxygen set-point.

In order to look for a real applicability, the mL-scale BES reactors were scaled-up and operated at long-term. It was observed that both treatment performances and electricity production achieved at mL-scale (0.6 L) could be reproduced at L-scale (65 L).

The assessment of different electrode materials (granular graphite and stainless steel) at L-scale allowed the identification of their limitations and its future applicability. Granular graphite (widely used at mL-scale BES) declined its efficiency overtime at L-scale due to electrode crushing. However, stainless steel highlighted as a better appropriate electrode for scaled-up BES long-term operation of for treating complex wastewaters.

On the way to increase BES sustainability, strategies for increasing the electrochemical efficiencies were investigated. The BES power output was improved by applying either an external resistance control (Maximum power point tracking, MPPT) at mL-scale or different electric circuit connections at L-scale. Moreover, the MPPT incremented the bacterial abundance and promoted the selection of putatively exoelectrogenic bacteria.

Finally, the BES microbiomes were evaluated from the microbiological and electrochemical perspective. Different microbiological techniques from the quantitative (qPCR) and qualitative (PCR-DGGE, 454-pyrosequencing, T-RFLP, FISH and SEM) point of view were used to quantify and identify the microbial communities. Microbiological and engineering data were used to relate the microorganisms with their putative role on nutrient removal and electricity production (electrogenic, nitrifying and denitrifying community). Last but not least, microcosms were successfully used for characterizing the extracellular electron transfer thermodynamics and electroactive steps for anoxic ammonium oxidation.

In conclusion, results presented in this PhD thesis supports that bioelectrochemical systems have the potential of being an alternative technology for simultaneous carbon and nitrogen removal with concomitant electricity production treating industrial wastewaters as swine manure. In the near future, it can become a cheaper and more sustainable alternative than current processes for wastewater treatment.

Resumen

La presencia de carbono (materia orgánica) y nitrógeno (amonio) en las aguas residuales municipales e industriales (ganadería, agricultura y fábricas) es una seria preocupación mundial. Estos productos no deseados se generan continuamente en nuestra sociedad. Contribuyen a la contaminación ambiental, reduciendo la calidad del agua y aumentando la frecuencia de los fenómenos de eutrofización. Hoy en día, los lodos activados y la digestión anaeróbica son las tecnologías más utilizadas en las plantas de tratamiento de aguas residuales (EDAR) para eliminar estos contaminantes. Sin embargo, ambas tecnologías no son eficientes para el tratamiento del nitrógeno. En los lodos activados, la nitrificación conlleva un fuerte coste económico por la aireación. Consume cerca de 1/3 del balance energético de la EDAR. En la digestión anaerobia, el nitrógeno no se trata y requiere un post-tratamiento adicional. Por estas razones, es necesario explorar tecnologías alternativas capaces de eliminar simultáneamente el carbono y el nitrógeno de las aguas residuales de una manera más eficiente y sostenible.

La utilización de sistemas bioelectroquímicos (BES) permite eliminar la materia orgánica y el nitrógeno de las aguas residuales con generación de bioelectricidad. En los bioánodos de un BES, los microorganismos exoelectrogénicos pueden oxidar la materia orgánica, liberando electrones y protones al cátodo. En los biocátodos, los microorganismos electrotróficos pueden realizar la desnitrificación. Además, ésta tecnología es versátil en cuanto a la nitrificación. Por estas razones, esta tesis pretende evaluar la eliminación simultánea de carbono y nitrógeno en BES para su futura implementación tratando aguas residuales industriales como el purín.

En los primeros capítulos, esta tesis doctoral demuestra la viabilidad del BES para tratar matrices complejas como el purín. A continuación, se evaluaron diferentes diseños de BES para optimizar la eliminación de nutrientes i el rendimiento electroquímico modificando: I) las configuraciones ánodo / cátodo; II) el control de resistencia externa; III) los dadores/aceptores de electrones ánodo/cátodo y IV) el punto de referencia de oxígeno de nitrificación.

Con el fin de buscar una aplicabilidad real, los reactores BES escala mL fueron escalados y operados a largo plazo. Se observó que los rendimientos de tratamiento y la producción de electricidad alcanzada a escala mL (0,6 l) podían reproducirse a escala L (65 l). La evaluación de diferentes materiales de electrodo (grafito granular y acero inoxidable) a escala L permitió identificar sus limitaciones y futura aplicabilidad. El grafito granular (ampliamente utilizado en el BES de escala de mL) disminuyó su eficiencia en el tiempo debido a su aplastamiento. Sin embargo, el acero inoxidable destacó como el electrodo más apropiado a largo plazo para el tratamiento de aguas residuales complejas en BES a escala L.

En el camino para aumentar la sostenibilidad de los BES, se investigaron estrategias para aumentar las eficiencias electroquímicas. Su potencia mejoró aplicando un control de resistencia externo (Maximum power point tracking, MPPT) en escala mL i diferentes conexiones eléctricas a escala L. Por otra parte, el MPPT incrementó la abundancia bacteriana y promovió la selección de bacterias supuestamente exoelectrogénicas.

Finalmente, los microbiomas de los BES fueron evaluados desde la perspectiva microbiológica y electroquímica. Se utilizaron diferentes técnicas microbiológicas, cuantitativas (qPCR) y cualitativas (PCR-DGGE, 454-pirosequenciación, T-RFLP, FISH y SEM) para cuantificar e identificar las comunidades microbianas. Los datos microbiológicos y de ingeniería se utilizaron para relacionar los microorganismos con su supuesto papel en la eliminación de nutrientes y producción de electricidad (electrogénicos, nitrificantes y desnitrificantes). Por último, los microcosmos se utilizaron con éxito para caracterizar la termodinámica de transferencia extracelular de electrones y los pasos electroactivos para la oxidación anóxica del amonio.

En conclusión, los resultados presentados en esta tesis doctoral apoyan que los sistemas bioelectroquímicos tienen el potencial para ser una tecnología alternativa en la eliminación simultánea de carbono y nitrógeno con producción de electricidad tratando aguas residuales industriales como el purín. En un futuro próximo, puede convertirse en una alternativa más económica y sostenible que los procesos actuales.

Table of contents

Chapter 1

General Introduction

Adapted from Vilajeliu-Pons, A., Puig, S., Carmona-Martínez, A., Bernet, N., Coma, M., Aulenta, F., Colprim, J. and Balaguer, M. D. 2016. Electroactive Biofilms in Water and Air Pollution Treatment, in: Romaní, A.M., Guasch, H. and Balaguer, M. D. Aquatic Biofilms: Ecology, water quality and wastewater treatment. Caister academic press, United Kingdom, pg.183-204. ISBN: 978-1-910190-17-3

1.1. Background

The presence of carbon (organic matter) and nitrogen compounds in municipal and industrial (livestock farming, agriculture and factories) wastewater is a worldwide concern. The continuous and unsustainable animal and vegetal production resulted in the accumulation of undesired products. Both carbon and nitrogen sources contribute to environmental pollution, reduce water quality and increase eutrophication phenomena frequency (Camargo and Alonso, 2006; Constable *et al.*, 2003).

One of the main nitrogen contaminants is ammonium. This compound negatively affects surface water bodies quality, modifying the environment and fauna associated. The increase of population in urban areas increases wastewater loads and flows. In this case, ammonium (NH_4^+) is the main nitrogen compound discharged from different human activities (Duce *et al.*, 2008). The most well-known product from pig farming with high nitrogen content is swine manure, which is characterized by a high organic matter and nitrogen content (mainly ammonium (NH_4^+)), high salinity and it contains heavy metals and pathogens (Reddy *et al.*, 1981). Traditionally, this waste was discharged to the soil as a fertilizer without control, but it was regularized (RD 324/2000 and 3483/2000) to avoid excess waste in agricultural land. Swine manure together with other fertilizers applied contains high ammonium concentration. This ammonium is often rapidly transformed into other nitrogenous forms as nitrates (NO_3^-) in an oxygen-dependent reaction (nitrification) (WHO, 1986). The product is commonly infiltrated to the aquifer, contaminating the human drinking water resource (Mencio *et al.*, 2011).

Carbon and nitrogen compounds are removed in municipal wastewater treatment plants (WWTPs) (Kowalchuk and Stephen, 2001). Before discharging the treated wastewater to the environment, a good effluent quality must be reached according to the European legislation (DMA 2000/60/DE). The conventional biological process for the treatment of nitrogen in WWTPs requires two phases (nitrification and denitrification). Nitrification, commonly consist in a two-steps process to oxidize ammonium to nitrate, even one-step nitrification has been

recently demonstrated by van Kessel *et al.* (2015). Initially, ammonium is oxidized to nitrite/nitrate under aerobic conditions (Eq 1.1). The ammonia-oxidizing bacteria (AOB) are the responsible of the ammonium oxidation to nitrite (Eq 1.1.1), while nitrite-oxidizing bacteria (NOB) are the responsible of the nitrite oxidation to nitrate (Eq 1.1.2). In denitrification, nitrites and nitrates generated from the ammonium oxidation are reduced to the harmless dinitrogen gas (N_2) with possible addition of organic matter (Eq 1.2). Great diversity of bacterial classes can perform the nitrogen reduction processes (more than 60 genera)(Clauwaert *et al.*, 2007a).

$$\mathbf{NH_4^+ + 2\ O_2 \rightarrow NO_3^- + H_2O + 2\ H^+} \qquad \mathbf{(Eq\ 1.1)}$$

$$NH_4^+ + 1½\ O_2 \rightarrow NO_2^- + H_2O + 2\ H^+ \qquad (Eq\ 1.1.1)$$

$$NH_4^+ + ½\ O_2 \rightarrow NH_2OH + H^+$$
$$NH_2OH + O_2 \rightarrow NO_2^- + H_2O + H^+$$

$$NO_2^- + ½\ O_2 \rightarrow NO_3^- \qquad (Eq\ 1.1.2)$$

$$\mathbf{0.82\ CH_3COOH + NO_3^- \rightarrow 0.07\ C_5H_7NO_2 + HCO_3^- + 0.3\ CO_2 + 0.9\ H_20 + 0.47\ N_2} \qquad \mathbf{(Eq\ 1.2)}$$

These processes entail a strong economic cost in aeration, consuming approximately one third of the energy balance of the WWTP (Rieger, 2004). For example, the WWTP of Girona (Spain) spends half a million Euros per year in aeration for ammonium oxidation (0.58±0.17 € kg^{-1}N oxidized) according to the Catalan Water Agency (ACA, 2009). Moreover, the potential limitation of organic matter in the wastewater could imply its addition to complete the nitrogen treatment, increasing the costs of the WWTP.

Anaerobic digestion, and more recently co-digestion, have been proposed as alternative to oxidize the organic matter from industrial wastes, obtaining biogas and a liquid-solid digestate as end-products (Zhang *et al.*, 2011; Holm-Nielsen *et al.*, 2009). However, the nitrogen is not removed from de liquid phase (Chen *et al.*, 2008a), requiring additional treatment of the effluent, such as ammonia stripping (Zhang *et al.*, 2012) or ammonium recovery in the form of struvite (Liu *et al.*, 2013) which, invariably, increase the energy and operational costs.

In this situation, it is necessary to explore alternative technologies that can treat carbon and nitrogen sources simultaneously, avoiding additional treatments. In this sense, the nitrogen and carbon treatment using bioelectrochemical system (BES) is a promising technology that would allow simultaneous carbon and nitrogen treatment, an autotrophic nitrogen removal, less-space requirement and low-cost treatment.

1.2. Bioelectrochemical system (BES)

Bioelectrochemical system (BES) was described for the first time about 100 years ago (Potter, 1911; Arends and Verstraete, 2012; Schröder, 2011). In a very general sense, BES represents a group of technologies capable to 1) produce energy, 2) treat gas and liquid pollutants or 3) recover and/or produce chemical compounds. The first description of microbial electroactivity was pointed by Michael C. Potter, who described electric activity from bacterial and yeast cultures in 1911 (Potter, 1911). After this discovery, it took 20 years until an additional development in the novel concept of BES. BES improvements were possible with Bruce E. Cohen who, in a similar way as Michael C. Potter, reported a difference in the "reduction potential" in a bacterial culture after a few days of incubation (Cohen, 1931). Interestingly, at that time, it was strongly believed that such phenomenon of power production was only possible due to the presence of exogenous molecules as electron mediators. During the following decades, the idea of microbial electricity generation did not significantly progress, receiving little attention until 1980-2000. Since then, BES has been rapidly developed to remove pollutants, produce high value products and energy, clarify the microbial mechanisms that perform the reactions and apply the technology to real systems.

Bioelectrochemical system consists of two electrodes placed in an anode and a cathode compartments where oxidation and reduction reactions take place, respectively (Figure 1.1). The oxidation reactions deliver protons to the media and electrons to the electrode. Protons diffuse to the cathode through an ion exchange membrane, while electrons are transferred by an external electric connection. In the cathode, electrons are consumed to carry out reduction reactions. In contrast to

the well-known electrochemical cells, at least one of the two reactions is catalysed by microorganisms. In consequence, the terms *bioanode* and *biocathode* have been adopted to describe those anodes or cathodes where the oxidation/reduction reactions are catalysed by electroactive microorganisms.

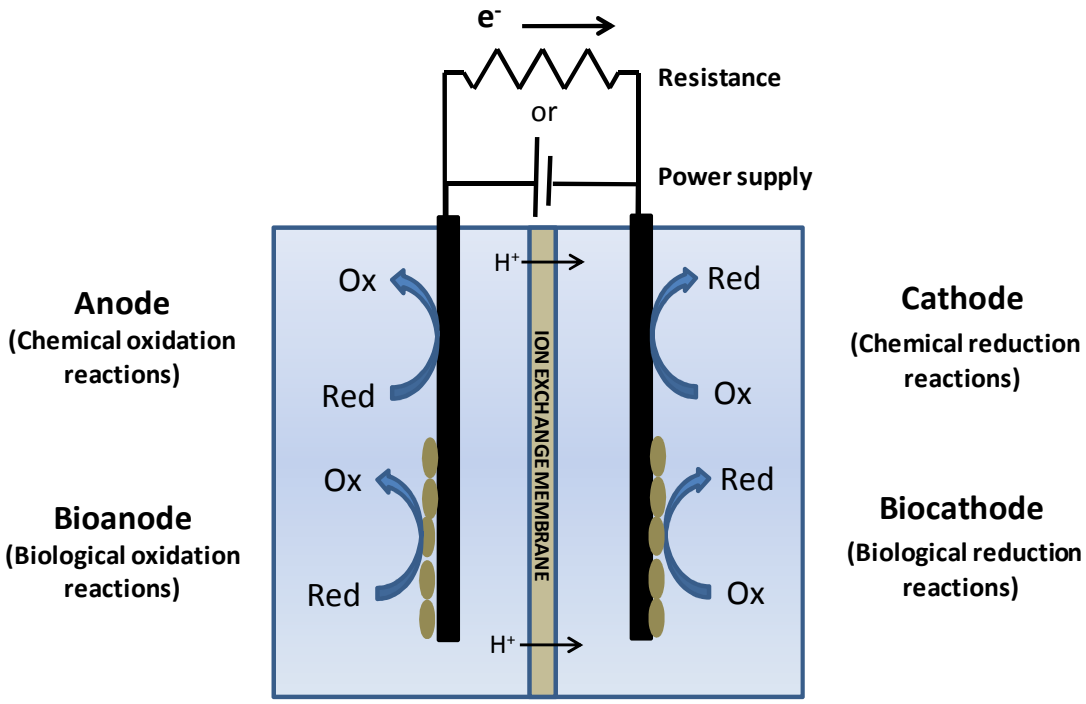


Figure 1.1 Basic scheme of a bioelectrochemical system (BES)

As an electrochemical process, the operation of each BES is determined by the potential at which each reaction occurs. If the overall cell potential, calculated by Eq 1.3, is positive, a thermodynamically spontaneous process is produced (Eq 1.4) and can generate electrical current.

$$E_{cell} = E_{cathode} - E_{anode} \qquad \text{(Eq 1.3)}$$

where E_{cell} is the cell voltage (V); $E_{cathode}$ is the cathode potential (V) and E_{anode} is the anode potential (V).

$$\Delta G = -n \cdot F \cdot E_{cell} \qquad \text{(Eq 1.4)}$$

where ΔG is the Gibbs free energy (J); n represents the number of electrons involved in the overall electrochemical process; F is Faraday's constant (96485 C·mol-e^{-1}) and E_{cell} is the cell voltage (V).

If the resulting Gibbs free energy is a negative value (ΔG<0), the process take place spontaneously, and energy will be produced in the form of electricity. These systems are known as Microbial Fuel Cells (MFC). Otherwise, if it is a positive value (ΔG>0), it means that the process is not taking place spontaneously, requiring energy to drive the process. These systems are known as Microbial Electrolysis Cells (MEC).

Microbial Fuel Cells (MFCs) were the first BES applied to remove pollutants with concomitant electricity production (Rabaey *et al.*, 2003). Thereafter, appeared Microbial Electrolysis Cells (MECs) which required applying a cell voltage to overcome the energy barrier to remove pollutants as uranium (Gregory and Lovley, 2005) and chlorinated compounds (Aulenta *et al.*, 2010) or to produce high value products (Rabaey and Rozendal, 2010).

In these systems, Coulombic Efficiency (CE) is the key parameter to determine the degree of microbial electroactivity in the system. The term CE correlates with the electrons flowing through the BES, with the process occurring in one of the compartments (i.e. anode or cathode). It is the ratio between the electrons actually transferred, and the electrons potentially transferred to the electrical circuit considering the reaction occurring at the bioelectrode (Eq 1.5).

$$CE = \frac{M_S\, I}{F\, n\, Q\, \Delta C} \cdot 100 \qquad \text{(Eq 1.5)}$$

Here, M_s is the molecular mass of the substrate (in g mol^{-1}), I is the current sourced from the MFC (in A) and F is the Faraday's constant (96485 C mol-e^{-1}). The term n identifies the number of electrons released for each mole of oxidized substrate, Q is the flow (L d^{-1}), while ΔC is the substrate concentration change (in mg L^{-1}) between influent and effluent streams.

1.3. Bioanode

1.3.1. Description

In bioanodes, the electroactive microorganisms are able to oxidize the substrate by using a solid electrode as a final electron acceptor (electrogenics). The use of bioanodes is usually seen as an alternative to biologic treatments that require oxygen supply. Thus, the suppression of aeration costs is pursued. As a general overview of multiple processes occurring in BES such as MFCs and MECs, bioanodes are usually described for breaking down organic material under anaerobic conditions from which mainly electrons and protons are released with certain amounts of biomass growth and CO_2 production (e.g. acetate oxidation in Eq 1.6).

$$\mathbf{CH_3COO^- + 2H_2O \rightarrow 2CO_2 + 7H^+ + 8\ e^-} \qquad \mathbf{E_0 = -0.280\ V\ vs.\ SHE} \qquad \mathbf{(Eq\ 1.6)}$$

This concept is applied on the bioremediation of contaminated sites since the early 90s (Lovley *et al.*, 1991; Nealson *et al.*, 1991). In BES, direct conversion of organic wastes into electricity is possible by bacteria (Figure 1.2). It is worth noticing that in order to improve bioenergy production, the research on BES should explore for instance the use of either robust mixed cultures or co-cultures able to break down complex substrates. Such approach should therefore continue since currently only a few attempts have been done to successfully address this aspect (Miceli *et al.*, 2014; Speers *et al.*, 2014).

The framework of microbial electrochemical technologies demonstrated that not only organic matter could be biologically oxidized using an electrode as electron acceptor, but also different kind of inorganic species can serve as electron donor for microbial bioanodes, as sulphide (Rabaey *et al.*, 2006), cis-DCE (Aulenta *et al.*, 2013) or ammonium (He *et al.*, 2009; Qu *et al.*, 2014) (Figure 1.2). The efforts have been focused on the treatment of waste compounds in order to remove them with the lowest operational cost. In the particular case of ammonium, the possibility of reducing the oxygen supply in the nitrogen treatment (approximately one third of the energy balance of the WWTP), has activated the interest of its application in MECs (Eq 1.7).

$$\mathbf{NH_4^+ + 3\ H_2O \rightarrow NO_3^- + 10\ H^+ + 8\ e^-} \qquad \mathbf{E_0 = 0{,}360\ V\ vs.\ SHE} \qquad \mathbf{(Eq\ 1.7)}$$

$$NH_4^+ + 2\ H_2O \rightarrow NO_2^- + 8\ H^+ + 6\ e^- \qquad E_0 = 0{,}440\ V\ vs.\ SHE$$
$$NO_2^- + H_2O \rightarrow NO_3^- + 2\ H^+ + 2\ e^- \qquad E_0 = 0{,}375\ V\ vs.\ SHE$$

Complete nitrogen removal to dinitrogen gas from ammonium was obtained but the metabolic route applied by the electrogenic bacteria has not been elucidated yet (Zhan *et al.*, 2014).

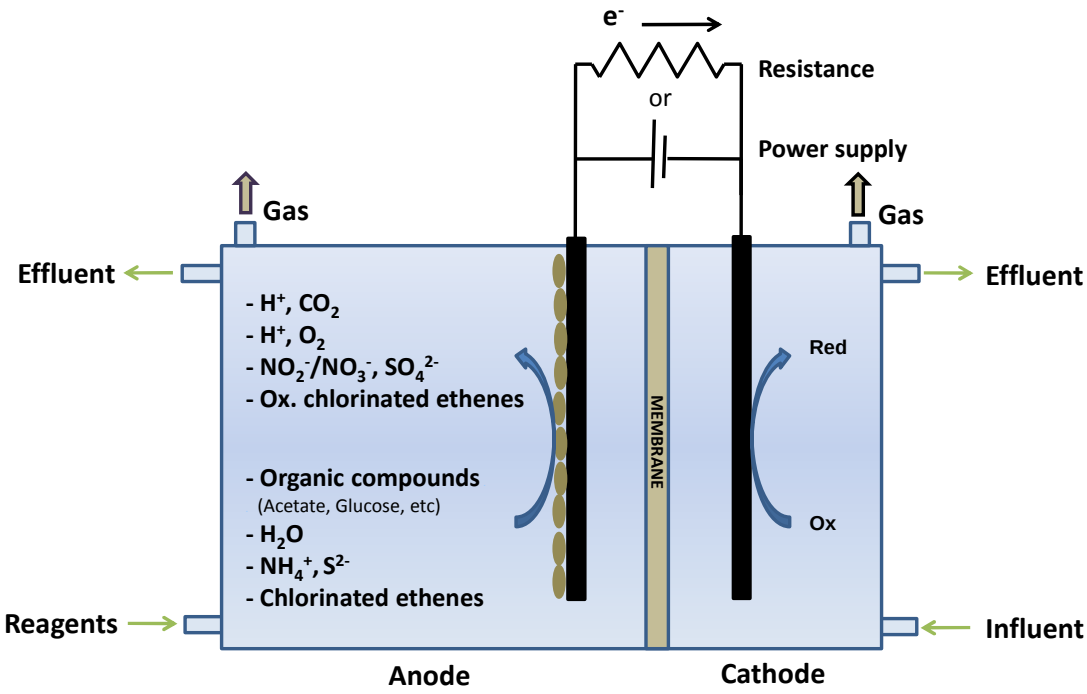


Figure 1.2 General overview of two configurations of a bioelectrochemical system (BES) performing anodic oxidation reactions.

In the anode compartment, the layer of biofilm contains microorganisms able to transfer electrons extracellularly (Lovley, 2008a). These bacteria, which are commonly called exoelectrogens (Logan and Regan, 2006), have an important role oxidizing and reducing metals in natural environments. In fact, the microbial electron transfer accomplished by some bacteria to insoluble metal electron acceptors is of special importance in several biogeochemical cycles.

1.3.2. Applicability of bioanodes for waste and nutrient treatment

First exoelectrogenic microorganisms applications were studied using synthetic media. The most used carbon source simulating wastewater was acetate, which is also used in genetic and molecular studies (Lovley *et al.*, 2011; Kiely *et al.*, 2011;

Logan *et al.*, 2006; Logan and Regan, 2006; Bond *et al.*, 2002). Table 1.1 shows a representative set of studies regarding exoelectrogenic organic wastes treated.

Table 1.1 Representative compilation of BES studies for the treatment of organic matter.

Substrate	Load (Kg COD m^{-3} d^{-1})	Surface/Volume (m^2 m^{-3})	Removal capacity (%)	CE (%)	Reference
Acetate	1.08	1290	94	91±2	Villano *et al.*, 2013
Acetate	1.10 2.10	817-2720	72 53	75±7 36±2	Rabaey *et al.*, 2005a
Glucose	2.40	7110	84	45±1	Freguia *et al.*, 2008
Glucose	1.10	817-2720	84	59±4	Rabaey *et al.*, 2005a
Glucose	0.50 5.00	125	85 41	89±4 10±2	Rabaey *et al.*, 2003
Municipal wastewater	0.90	22	76	89	Khalfbadam *et al.*, 2016
Municipal wastewater	10-20	NA	79	<5	He *et al.*, 2014
Municipal wastewater	1.50	NA	78-83	NA	Sevda and Sreekrishnan, 2014
Municipal wastewater	7.20	1023	80	NA	Puig *et al.*, 2011a
Municipal wastewater	9.77	1869	43	NA	Puig *et al.*, 2011c
Municipal wastewater	0.10	25	75	20	Liu and Logan, 2004
Industrial wastewater: food wastes	0.11	NA	80	27	Jia *et al.*, 2013
Industrial wastewater: Bakery Brewery Paper Dairy	0.05	44	86 85 78 82	2±1 2±1 26±6 2±1	Velasquez-Orta *et al.*, 2011
Industrial wastewater: Brewery	6.70	12	44	7	Wen *et al.*, 2010
Landfill leachate	2.20	1023	10	<2	Puig *et al.*, 2011b
Landfill leachate	3.20	40	13	NA	Greenman *et al.*, 2009
Swine manure	1.20 4.90	88	84 77	<1 <1	Zhuang *et al.*, 2012a
Swine manure	4.50	5	27	8	Min *et al.*, 2005

NA: Not available
CE: Coulombic efficiency

The bioanode CE treating organic matter is related to the percentage of the organic matter removed by exoelectrogenic bacteria. For example, in the study of Rabaey *et al.* (2005a), microorganisms were able to oxidize 72% of acetate with a CE of 75%. Several authors studied mechanisms involved in order to improve the efficiency of these systems. Villano *et al.* (2013) built a MEC able to oxidize 94% of acetate in the influent with a CE of 91%. In addition to acetate, a variety of other substrates have been used in the BES anode. For instance, glucose (Freguia *et al.*, 2008; Rabaey *et al.*, 2005a, 2003) or fructose (Liamleam and Annachhatre, 2007) have been tested. Interestingly, power outputs with both substances were lower than those of acetate due to a requirement of previous fermentation processes, which produces hydrogen and acetate, and the presence of methanogens that compete with the exoelectrogens.

Different sources of real wastewaters have also been tested (Table 1.1) as municipal wastewater (Khalfbadam *et al.*, 2016; He *et al.*, 2014; Sevda and Sreekrishnan, 2014; Puig *et al.*, 2011a and Liu and Logan, 2004) and industrial wastewaters from bakery, brewery, paper, dairy and food industries (Jia *et al.*, 2013; Velasquez-Orta *et al.*, 2011 and Wen *et al.*, 2010). Here, organic loading rate (OLR) has been generally higher based on degradable substrates than with the synthetic media. Despite the higher removal found when testing non-synthetic media, the relative exoelectrogenic activity (i.e. CE) was lower due to the complex organic matter (analysed in terms of chemical oxygen demand, COD) matrix in the influent, causing a more diversified range of side metabolic reactions that inevitably consume electrons.

Other substrates as landfill leachate (Puig *et al.*, 2011b and Greenman *et al.*, 2009), swine manure (Zhuang *et al.*, 2012a and Min *et al.*, 2005) and urine (Ieropoulos *et al.*, 2012) have been analysed with similar OLRs than wastewaters. The use of complex organic influents has resulted in lower CEs. However, such studies have proved the applicability of BES.

Nowadays, non-organic compounds are also starting to be studied as substrate for bioanode, such as sulphide, arsenite or ammonium. The application of BES appeared as an alternative to the current treatments in order to reduce the operational costs of treating this kind of compounds (Table 1.2).

Table 1.2 Representative compilation of BES studies for autotrophic anodic treatment of different inorganic substrates.

Substrate	Removal rate ($g\ S\ m^{-3}d^{-1}$)	Surface/ Volume ($m^2\ m^{-3}$)	Removal capacity (%)	CE (%)	DO (%)	Reference
Arsenite	0.016	4	100	NA	ND	Nguyen *et al.*, 2016
Arsenite	0.001	3	47	40±11	ND	Pous *et al.*, 2015a
Sulphide	0.007	817-2720	99	30	NA	Rabaey *et al.*, 2006
	0.021		99	15	NA	
Dechlorinated ethene	0.072	6	76	NA	NA	Aulenta *et al.*, 2013
Ammonium	0.040	NA	100	82	0.7-1.0	Zhan *et al.* 2012
	0.040		100	94	0.8-1.0	
Ammonium	0.028	12	41±3	80±2	ND	Zhan *et al.*, 2014
	0.056		56±6	44±2	ND	
	0.084		48±3	9±2	ND	
Ammonium	0.018	10	97	33±8	NA	Qu *et al.*, 2014
Ammonium	0.100	4	99	NA	NA[1]	Chen *et al.*, 2014

ND: Not detected; NA: Not available; [1] air supply
S: Substrates; CE: Coulombic efficiency; DO: Dissolved oxygen.

Sulphide production is an environmental hazard that involves high economic costs in anaerobic methane digester systems. The control of the sulphide oxidation process through the potential and/or current was demonstrated in BES (Rabaey *et al.*, 2006). The treatment of toxic metals present in water, especially in groundwater, like arsenite (As(III)) was also studied. Even its high toxicity established, remediation technologies are typically more effective removing arsenate (As(V)) than As(III). The BES technology was successfully applied in the bioremediation of As(III) (Nguyen *et al.*, 2016; Pous *et al.*, 2015a).

The elevated aeration costs of ammonium oxidation, makes BES as an alternative for nitrogen treatment with lower oxygen consumption than conventional treatments. Preliminary studies of ammonium removal were performed in aerated cathode compartment. Its treatment in BES reduced three orders of magnitude the oxygen consumption (Virdis *et al.*, 2008; Zhang and He, 2012), although it still

required oxygen supply. The development of a low-cost electrochemical system for nitrogen removal was necessary, for this reason the anoxic treatment of ammonium in the anode compartment was studied. The results suggested that anodic nitrification was viable (Qu *et al.*, 2014; Zhan *et al.*, 2014). Anodic nitrification, either coupled with anammox process (Zhu *et al.*, 2016) or with cathodic denitrification (Virdis *et al.*, 2008) could allow a complete ammonium removal without aeration. However, the knowledge available until now is scarce.

1.3.3. Scalability of bioanodes

Therefore, BES technology has been used to treat a great variety of pollutants including industrial wastewater in mL-scale reactors, obtaining relevant knowledge about fundamentals of BES (Zhuang *et al.*, 2012a; Puig *et al.*, 2011a; Velasquez-Orta *et al.*, 2011). Nevertheless, current research on BES should start moving from the small prototypes to scaled-up reactors in order to test its real applications and future implementations. However, there were some drawbacks in the practical feasibility of scaled-up BES, especially with respect to costs, system development and energy recovery (Li *et al.*, 2014). The firsts attempts of scaled BES started less than one decade ago, representing 22% of the total MFC publications in the Web of Science (Ferreira Mercuri *et al.*, 2016). The initial problem was an unappropriated design of scaled reactors in terms of total electrode surface area or electrode spacing. Liu *et al.*, (2008) demonstrated that power output can be maintained during reactor scale-up, increasing the anode surface area. The successive scaled-up were focused on different reactor configurations and media employed. A 2.5L square-BES treating acetate enriched medium was used to demonstrate that the system could be operational self-regulated. The results showed the viability of the technology removing over 70% of acetate at a removal rate of 0.2 kg COD m^{-3} d^{-1}, but achieving low power density (2.3 W m^{-3}) (Clauwaert *et al.*, 2009a). The group of He in Wisconsin (USA) evaluated 2L tubular-BES treating urban wastewater (Zhang *et al.* 2013a; Zhang *et al.* 2010). The organic matter removal efficiencies were maintained over 60% at a higher removal rate (0.4 kg COD m^{-3} d^{-1}) and current production (15 W m^{-3}) than in the previous study working in synthetic.

The energy recovered and the volumetric capacities for single scaled-up BES reactors were insufficient. Multiple stacked BES started to be tested in order to improve the systems. Different electrical configurations (i.e. series or parallel) were used in stacked BES to achieve higher voltage or current (Aelterman *et al.*, 2006a). The firsts attempts of stacked BES were performed at 1 L-scale MFCs, with a 12 pairs cassette-electrode BES, obtaining high organic matter removal rates (5.4 kg COD m^{-3} d^{-1}) and power production (129 W m^{-3}) (Shimoyama *et al.*, 2008). Increasing the volume of the reactor one order of magnitude (20 L), the power density was maintained respect to less-volume reactors (Dekker *et al.*, 2009). These values decreased when the stacked BES treated urban wastewater, obtaining low removal rates (0.2-1.0 kg COD m^{-3} d^{-1}) with low electricity production (0.4-0.9 W m^{-3}) in a 16 L stacked BES (Jiang *et al.*, 2011).

The success of stacking bench-scale BES encouraged the scaling up of the BES modules towards a large-scale system. A 90 L rectangular BES reactor vessel could achieve sufficient energy treating brewery wastewater to support its operation (Dong *et al.*, 2015). A modularized 200 L BES consisting of 96 tubular BES modules demonstrated the long-term performance (one year) treating municipal wastewater (Ge and He, 2016). The biggest BES pilot plant was constructed in Queensland (Australia) consisting of 12 tubular-BES modules with a total liquid volume of $1m^3$, based on Rabaey *et al.* (2005a). The pilot performance was unsuccessful due to low conductivity of brewery wastewater and high biomass proliferation on the cathode due to organic matter excess (Logan, 2010).

1.4. Biocathode

1.4.1. Description

Biocathodes are based on electroactive microorganisms that are able to obtain electrons from an electrode and perform reductive processes (electrotrophs). The investigation of biocathodes began with oxygen reduction for a sustainable anodic organic matter treatment because the potential made feasible the current production (Eq 1.8) (Chen *et al.*, 2008b; Clauwaert *et al.*, 2007b).

$O_2 + 4H^+ + 4e^- \rightarrow 2\ H_2O$ $E_0 = 0.816$ V vs. SHE (Eq 1.8)

Once its potential was demonstrated, two more fields of research bore (Figure 1.3): i) the bioremediation of pollutants, such as nitrate (Clauwaert *et al.*, 2007a; Gregory *et al.*, 2004; Virdis *et al.*, 2009; Pous *et al.*, 2015b), hexavalent uranium (Gregory and Lovley, 2005), sulphate (Coma *et al.*, 2013), chlorinated compounds (Aulenta *et al.*, 2008), perchlorate (Butler *et al.*, 2010), hexavalent chromium (Xafenias *et al.*, 2013) or nitrobenzene (Wang *et al.*, 2011); and ii) the production of commodity chemicals, such as hydrogen (Batlle-Vilanova *et al.*, 2014; Jeremiasse *et al.*, 2010; Rozendal *et al.*, 2008; Oh and Logan, 2005), methane (Villano *et al.*, 2011; Cheng *et al.*, 2009), acetate (Batlle-Vilanova *et al.*, 2015; Marshall *et al.*, 2012; Nevin *et al.*, 2010) or butyrate (Ganigué *et al.*, 2015).

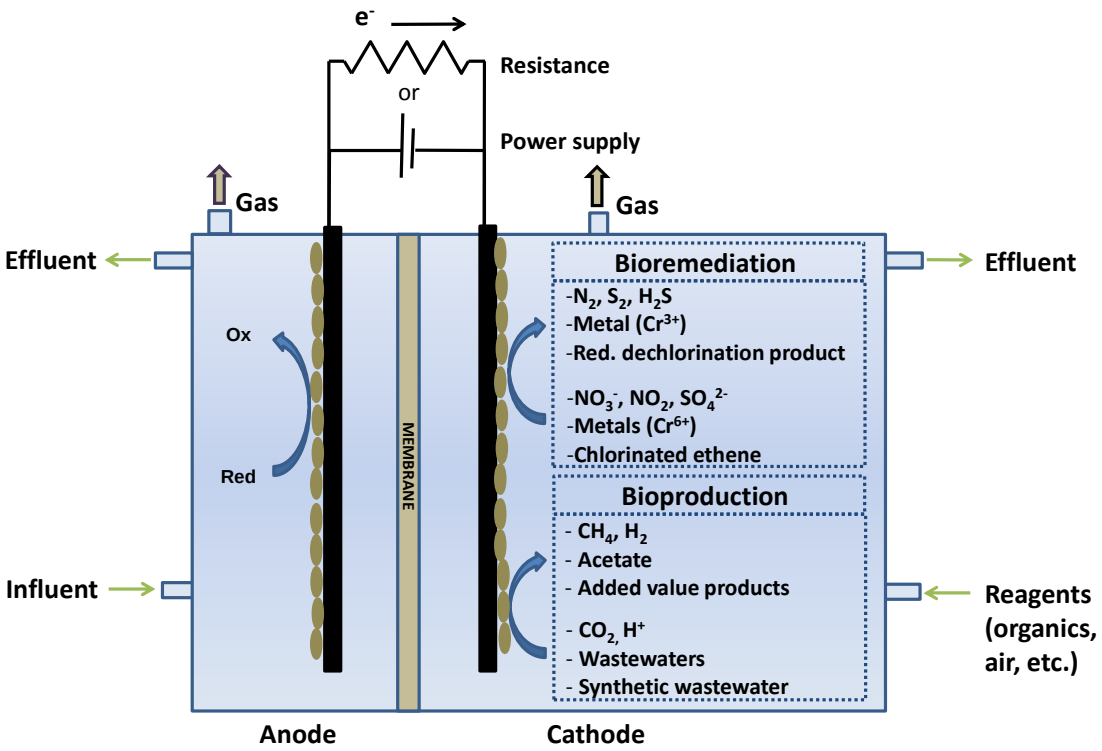


Figure 1.3 General overview of two configurations of a bioelectrochemical system (BES) performing cathodic reduction reactions.

In the specific case of nitrogen compounds, initially nitrogen was treated in the cathode chamber where nitrification and denitrification took place together (Virdis *et al.*, 2008; Zhang and He, 2012). Ammonium oxidation to nitrite and/or nitrate took place aerobically through microaeration in order to reduce costs. Finally, to complete the nitrogen treatment, the oxidized forms of nitrogen were reduced to

dinitrogen gas (N_2) through biologic denitrification in the cathode electrode. It involves four reduction steps (Eq 1.9) from nitrate (NO_3^-) to dinitrogen gas (N_2) through nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O). In this pathway, the accumulation of intermediate products due to their chemical stabilities produces undesirable consequences for human health and the environment. Nitrite impact on human health (WHO, 2011) and nitrous oxide is a greenhouse gas with 298 times more impact per unit weight than CO_2 (Forster *et al.*, 2007). For a sustainable process, nitrate should be converted to dinitrogen gas without the accumulation of any of these intermediates.

Different BES designs were assessed in order to completely treat nitrogen such as the use of an external aerated reactor for ammonium oxidation together with an anoxic cathode for nitrate reduction (Virdis *et al.*, 2008). Moreover, multiple cathodes working under different conditions, aerated and anoxic, were also tested (Zhang and He, 2012) but the complete nitrogen removal did not exceeded the 50%. In order to reduce costs and increase the nitrogen treatment, the aeration was performed in the recirculation flow (Virdis *et al.*, 2010) or inside the cathode compartment (Virdis *et al.*, 2008), where simultaneous nitrification-denitrification (SND) took place. In both cases, the nitrogen removal was around 70-90% with oxygen supply similar to Zhang and He, (2012).

$NO_3^- + 5\ e^- + 6\ H^+ \rightarrow ½\ N_2 + 3\ H_2O$ **$E_0 = 0{,}740$ V vs. SHE** **(Eq 1.9)**

$NO_3^- + 2\ e^- + 2\ H^+ \rightarrow NO_2^- + H_2O$ $E_0 = 0{,}375$ V vs. SHE

$NO_2^- + e^- + 2\ H^+ \rightarrow NO + H_2O$ $E_0 = 0{,}420$ V vs. SHE

$NO + e^- + H^+ \rightarrow ½\ N_2O + ½\ H_2O$ $E_0 = 1{,}175$ V vs. SHE

$½\ N_2O + e^- + H^+ \rightarrow ½\ N_2 + ½\ H_2O$ $E_0 = 1{,}355$ V vs. SHE

In the cathode compartment, microorganisms are able to reduce compounds taking electrons from the electrode surface. These bacteria are called electrotroph (Lovley, 2011). The role of these microorganisms in such specific systems has been hardly studied. Therefore, it is important to gain further fundamental knowledge about these microorganisms for future removal of other pollutants from wastewaters and for the production of high value products (Patil *et al.*, 2012).

1.4.2. Applicability of biocathodes for waste and nutrient treatment

Electrotrophic microorganisms have been used in bioremediation and in the recovery/production of chemical compounds. A great variety of environmental pollutants has been treated by taking advantage of electrotrophic microorganisms in BES. For example, due to the intensive agriculture and livestock activities, the release of nitrate from water has become a serious problem, requiring large amounts of organic matter for its removal through conventional processes (denitrification). In this scenario, electrotrophs might provide a great benefit because they can reduce nitrates and/or nitrites from contaminated groundwater and wastewater to dinitrogen gas (Nguyen *et al.*, 2016; Pous *et al.*, 2015b; Pous *et al.*, 2013; Kondaveeti and Min, 2013; Vilar-Sanz *et al.*, 2013; Puig *et al.*, 2012, 2011b; Wrighton *et al.*, 2010; Virdis *et al.*, 2008). Electron transport increases in highly conductive media but is limited in media with low conductivities (Puig *et al.*, 2012). Despite this, the possibility of treating groundwater without affecting the drinking water quality by BES has been reported (Pous *et al.*, 2013). Biocathodes can also reduce other kinds of inorganic contaminants as sulphates (Coma *et al.*, 2013), chlorinated compounds (Aulenta *et al.*, 2010; Butler *et al.*, 2010) and different metals as uranium (Anderson *et al.*, 2003), chromium (VI) (Tandukar *et al.*, 2009), cadmium (II) (Colantonio and Kim, 2016), copper (II) (Heijne *et al.*, 2010) or selenite (Catal *et al.*, 2009). Table 1.3 provides a compilation of representative reducible pollutant studies using electrotroph treatments.

Table 1.3 Summary of literature studies about waste streams treated by electrotrophic bacteria for different substrates.

Substrate	Removal rate (Kg S m^{-3} d^{-1})	Surface/Volume (m^2 m^{-3})	Removal capacity (%)	CE (%)	Reference
Nitrate	3.10^{-3}	4	100	NA	Nguyen *et al.*, 2016
Nitrate	0.10	500	94	NA	Pous *et al.*, 2015b
Nitrate	0.06	500	64	60-80	Pous *et al.*, 2013
Nitrate	0.05	500	42	73±18	Puig *et al.*, 2012
Nitrate Nitrite	0.37 0.28	2483	15 77	85±11 41±17	Vilar-Sanz *et al.*, 2013
Nitrite	0.50	1869	35	48±11	Puig *et al.*, 2011c
Sulphates	0.21	1625	<1	NA	Coma *et al.*, 2013
Trichloroethene	$1{\cdot}10^{-6}$	2	69	70	Aulenta *et al.*, 2010
Perchlorate	0.02	NA	99	84	Butler *et al.*, 2010
Chromium (VI)	0.01	6	99	NA	Tandukar *et al.*, 2009
Uranium	$6{\cdot}10^{-6}$	NA	70	NA	Anderson *et al.*, 2003
Cadmium (II)	$2{\cdot}10^{-3}$	NA	95	2	Colantonio and Kim, 2016
Copper (II)	0.14	68	100	84	Ter Heijne *et al.*, 2010
Selenite	$4{\cdot}10^{-3}$ $7{\cdot}10^{-3}$	58	99 99	16 32	Catal *et al.*, 2009

NA: Not available
S: Substrate; CE: Coulombic efficiency (CE).

Electrotroph activity is not only linked to contaminants removal. Recent studies have suggested a promising future for these bacteria to produce high value products from wastes (Table 1.4). Different organic and inorganic products such as methane, hydrogen, acetate, ethanol, 1-3 propanediol, succinate and butanol have been obtained from the electrotroph activity (Logan and Rabaey, 2012; Ganigué *et al.*, 2015). Hydrogen is the main product of water electrolysis and occurs easily in electrochemical cells (Cheng *et al*., 2011). However, the presence of microorganisms in the cathode catalysed these reactions at higher redox potentials than not biocatalysed (Batlle-Vilanova *et al.*, 2014; Rozendal *et al.*, 2008).

Table 1.4 Summary of literature studies about different high value ending products production of electrotrophic bacteria from different initial products and cathode potentials.

Initial product (electron acceptor)	Final product	Potential (mV vs SHE)	Reference
H^+	Hydrogen	-1000	Batlle-Vilanova *et al.*, 2014
H^+	Hydrogen	-700	Rozendal *et al.*, 2008
Synthetic wastewater	CH_4, Acetate	< -1000	Xafenias and Mapelli, 2014
Wastewater CO_2, H_+	CH_4, H_2 Acetate	-590 to -900	Marshall *et al.*, 2013
CO_2, H_+	CH_4, H_2 Acetate, CH_4, H_2	-1047 to -1147 < -1147	Jiang *et al.*, 2013
CO_2, H^+	CH_4, H_2, Acetate	-590	Marshall *et al.*, 2012
CO_2	CH_4	<-700	Luo *et al.*, 2014
CO_2	CH_4	<-350	Fu *et al.*, 2015
CO_2	CH_4	-800	Batlle-Vilanova *et al.*, 2015
CO_2	Acetate	-400	Nevin *et al.*, 2011
CO_2	Acetate	-400	Nevin *et al.*, 2010
CO_2, H^+	H_2, Acetate, Formate	-600	LaBelle *et al.*, 2014
CO_2	Acetate, CH_4	-600	Batlle-Vilanova *et al.*, 2015a
CO_2, H^+	H_2, Acetate, Butyrate, Ethanol, Butanol	-800	Ganigué *et al.*, 2015
Acetic and butiric acid	Propanol, Butanol, Acetone	-645	Sharma *et al.,* 2013
Acetate and butyrate	Succinate, ethanol, H_2, glycerol, propionate, acetone, isopropanol, propanol, isobutyrate, isovalerate and heptanoate	-645	Sharma *et al.,* 2014

Methane has been investigated in BES due to its high energetic value. Recently, the interest on this type of methane production route has gained increased attention (Fu *et al.*, 2015; Batlle-Vilanova *et al.*, 2015b; Luo *et al.*, 2014). It has been demonstrated that methane production is feasible using synthetic and raw wastewaters and CO_2 carbon based substrates. Another interesting example of the production of added-value molecules has been recently described by Ganigué *et al.* (2015), with the production of multicarbon organic compounds such as butyrate. Butyrate was produced with a significant efficiency of 30% respect to the total products when the cathode potential was poised below -800 mV vs. SHE. Production of acetone and longer than 4-C alcohols were also observed opening up the potential for biofuel production (Sharma *et al.,* 2013; Sharma *et al.,* 2014).

1.4.3. Scalability of biocathodes

Microbial electrolysis cells (MECs) have been also evaluated at scaled-up level for hydrogen (Cusick *et al.*, 2011; Cotterill *et al.*, 2013; Heidrich *et al.*, 2013) and methane production (Cusick *et al.*, 2011). These works represented the first proof-of-concept demonstrations of the feasibility of large-scale BES for product generation, achieving 70% electrical energy recovery and producing almost 100% pure hydrogen gas per day (Cotterill *et al.*, 2013). The main challenges for the future development of the technology are enhancing the hydrogen-production rate and lowering the energy input. This includes increasing the performance of anodic biofilms in terms of current density, the development of efficient cathode electrode materials and novel architecture. Nevertheless, no tests of BES for nitrogen treatment at scaled-up level have been reported yet.

1.5. Biofilms in BES

1.5.1. Description

Microorganisms can establish a successful electron transfer by electrode surface as a biofilm. Biofilm structures consist of bacterial cells surrounded by self-produced extracellular polymeric substance (EPS). This biofilm can be a pure culture/single populations (Logan and Regan, 2006) or a mixed culture/mixed communities *i.e.* a complex microbial ecosystem (Zhan *et al.*, 2014). Microorganisms in the biofilm show certain heterogeneity due to interspersed distribution inside the EPS matrix (Davey and George, 2000). Mixed bacterial cultures are used to inoculate BES when the desired cultures (pure or enriched cultures) are not available. Simplifying the operational conditions used (such as single substrate, stable pH/temperature/ applied potential, etc.) a highly enriched biofilm is obtained (Harnisch *et al.*, 2011). Once dominating bacteria are found in BES studies, isolated or purchased pure cultures can be used to study and acquire more fundamental and specific knowledge about involved microorganisms and their metabolic pathways.

Mixed cultures have been used to treat complex organic matter usually contained in wastewaters due to their ability to adapt to changing conditions as the

ones normally observed in wastewater treatment plants. Consequently, a higher power output has been detected in BES studies based on mixed cultures, which seems to be more robust and resilient than pure cultures (Arends *et al.*, 2011). The maximum power output is not only related to the culture's source. In fact, there are many factors that can modify biofilm formation and its posterior behaviour, such as electrode surface, nutrient availability, pH (in pure cultures) and hydrodynamics, among others (Franks *et al.*, 2010).

The specific capacity of these organisms has been applied in the environmental biotechnology field in order to couple pollutants removal, mainly from water but also from air streams, to produce energy or valuable products. Bioelectrogenic biofilms are a bacterial consortium capable of performing electron transfer to the conductive material they are set on in BES. Microorganism-electrode interaction is not yet fully understood and still under research (Guo *et al.*, 2013). Different materials have been used as both electrode and carrier material as carbon, graphite, titanium and stainless steel (Wei *et al.*, 2011; Logan, 2008). Different ranges of conductive materials has been used (Logan *et al.*, 2007), they have been even modified in order to increase microorganism adhesion surface and the conductivity of the material (Guo *et al.*, 2013). For instance, granular graphite is one of the most common and cheap material used because of its natural conductivity properties and microorganism affinity (Vilar-Sanz *et al.*, 2013; Arends *et al.*, 2012; Puig *et al.*, 2011a; Gregory *et al.*, 2004; Logan, 2008; Rabaey *et al.*, 2006, 2005a).

Although the importance of the study of BES biofilms, it did not take off until 2005 (Rabaey and Verstraete, 2005). From that time on, it was considered that the knowledge about the role of microorganisms was important in order to maximize the energy production and nutrient removal capacity or the product production. Thereafter, biofilm studies increased significantly. Some microorganisms responsible of current production as *Geobacter sulfurreducens* (Reguera *et al.*, 2005) and *Shewanella oneidensis* (Gorby *et al.*, 2006) were identified, and they have become a model microorganisms in the field of BES. Since then, the mechanisms of extracellular electron transfer between bacteria and electrode materials have been

extensively studied by a broad diversity of research groups (Marsili and Zhang, 2009; Rosenbaum and Angenent, 2009; Lovley, 2011).

Nowadays, there is a special attention to the syntrophic interactions within the biofilm and the obtained end-product since such type of interactions seem to have a more pronounced relevance when working on the microbial electrosynthesis of added value products that not necessarily depend on direct uptake electron transfer (Arends *et al.*, 2013). In the context of BES, syntrophy is defined as the mutualistic interaction between microorganisms, where the main goal is maximizing resource utilization (Lovley *et al.*, 2011). Some microorganisms catabolize the production of metabolic substances which are further degraded by other organisms within the biofilm when treating pollutants. Single communities working individually are not capable to remove such substances. This concept is also related with communities present in extreme conditions (McInerney *et al.*, 2008). For example, the syntrophy was firstly described in communities that exchanged sulphur compounds in carbon and nitrogen cycles in natural environments (Morris *et al.*, 2013). In BES, syntrophic interactions have been described on exoelectrogenic and non-exoelectrogenic bacteria in anode biofilms (Parameswaran *et al.*, 2009).

1.5.2. Exoelectrogenic microorganisms

1.5.2.1. Description

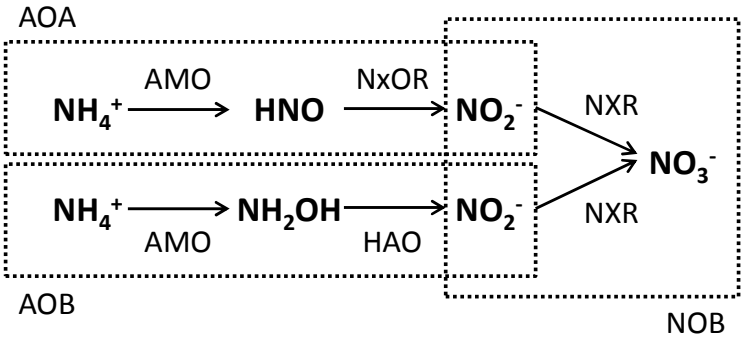
Lovley (2006) described a new form of microbial respiration in which microorganisms conserve energy for growing while transferring electrons extracellularly to the electrode. He called these bacteria **exoelectrogens**. Exoelectrogens are also known as anodophiles (Park and Zeikus, 2003), electrochemically active bacteria (EAB) (Chang *et al.*, 2006), anode-respiring bacteria (Torres *et al.*, 2007) or electrogenic microorganisms (Debabov, 2008). Currently, the study of exoelectrogens has been triggered through its application in BES. In this alternative treatment, exoelectrogen microorganisms transform acetate or complex organic matter from wastewater into electricity (Debabov, 2008; Lovley, 2008b).

The most extensively studied microorganism able to produce high current densities and thick biofilms in MFCs treating organic compounds is the Gram-negative *Geobacter sulfurreducens* (Bond and Lovley, 2003). Nowadays, 94 species are described as electroactive, but it has not been identified a singular ecological niche for them (Koch and Harnisch, 2016a). Among them, high current densities have also been described for *Rhodopseudomonas palustris* DX-1 (Xing *et al.*, 2008), *Thermincola ferriacetica* (Parameswaran *et al.*, 2013), *Geoalkalibacter ferrihydriticus* (Badalamenti *et al.*, 2013) and *Geoalkalibacter subterraneus* (Carmona-Martínez *et al.*, 2013). Such bacterial species have been well characterized but they are not the only ones with a corroborated electrical activity (Gorby *et al.*, 2006). Different Gram-positive (*Thermincola* sp.) and Gram-negative (*Shewanella oneidensis, Pseudomonas* sp.) bacteria have been identified in bioanodes. Therefore, their presence makes them very likely responsible of the extracellular electron transfer process at the electrode surface (Arends *et al.*, 2011).

The framework of microbial electrochemical technologies demonstrated that not only organic matter could be biologically oxidized using an electrode as electron acceptor, but also different kinds of inorganic species can serve as electron donors for microbial bioanodes (i.e. sulphide (Rabaey *et al.* 2006) or cis-DCE (Aulenta *et al.*, 2013)). For this reason, the oxidation of ammonium using the anode as a terminal electron acceptor started to be investigated.

Nitrification consists on a two aerated autotrophic steps catalysed by two categories of chemolithotrophic microorganisms (Eq 1.10): ammonia-oxidizing bacteria (AOB) and/or archaea (AOA) and nitrite-oxidizing bacteria (NOB). AOBs are responsible of the oxidation of ammonium (NH_4^+) to hydroxylamine (NH_2OH) and finally to nitrite (NO_2^-) by the membrane-associated ammonia monooxygenase (AMO) and the periplasmic hydroxylamine oxidoreductase (HAO) enzymes (Sayavedra-Soto *et al.*, 2011). AOAs convert NH_4^+ to NO_2^- as well, but the pathway followed is different. Ammonium is firstly oxidized to nitroxyl hydride (HNO) and secondly, to NO_2^- by archaea AMO and nitroxyl oxidoreductase (NxOR) enzymes (Stahl and de la Torre, 2012). In both cases (AOAs and AOBs), nitrite is further

oxidized to nitrate (NO_3^-) by nitrite-oxidizing bacteria (NOB) using the nitrite oxidoreductase (NXR) enzyme (Sorokin *et al.*, 2012).



(Eq 1.10)

Several authors demonstrated that ammonium could serve as a substrate for electricity generation (He *et al.* 2009; Qu *et al.* 2014). However, little is known about the mechanisms responsible of these processes and which extracellular electron pathways are involved. Chen *et al.* 2014 and Zhan *et al.* 2014 identified ammonium oxidation to nitrite as the initial step of a possible exoelectrogenic reaction. Further, they identified the genera of *Nitrosomonas*, *Comamonas* and *Paracocus* as possible important bacteria for the electron transfer mechanism of ammonium oxidation in the anode. However, there are two enzymes involved in the oxidation of ammonium to nitrite, and one intermediate product (hydroxylamine) that requires to be studied in deep in order to understand its electrogenic implication in the reaction.

1.5.2.2. History and identification of exoelectrogens

Potter's discovery (1911) of microbial catalysed electrode reduction revolutionized the world of microbiology. He observed and reported electricity production with *Escherichia coli* and yeast cultures. The knowledge on the respiration ability of the exoelectrogens was not studied until the end of the twentieth century, when Vargas *et al.* (1998) defined Fe (III) as the first external electron acceptor in MFC systems that allowed energy conservation to support their growth. Several techniques started being used to identify bacterial populations in the biofilms (Davey and George, 2000), as the polymerase chain reaction (PCR) and fluorescent in situ hybridization (FISH) techniques. These techniques have been used since then to identify the biofilms in BES systems.

Reimers *et al.* (2001) established microbes in seawater batteries, obtaining low power and voltage gradients. More specifically, one year later, Bond *et al.* (2002) reproduced the experiment of Reimers by sequencing the microbial community responsible of that electricity production. The 16S rRNA sequences obtained were analysed by most probable number-polymerase chain reaction (MPN-PCR) technique showing that the *Geobacteraceae* family of δ–proteobacteria was the most abundant. Finding a bacterial species within the *Geobacteraceae* family (i.e.: *Desulfuromonas acetoxidans*) has been of great importance for the BES field owing to the frequent appearance of these types of bacteria in electroactive biofilms (Yates *et al.*, 2012).

The interest on exoelectrogens has therefore rapidly increased with the identification of anode respiring bacteria in BES gradually achieving higher current densities. The complete nucleotide sequences of the genomes of *Shewanella oneidensis* (Heidelberg *et al.*, 2002) and *Geobacter sulfurreducens* (Methé *et al.*, 2003) have been described. The utilization of stable isotope probing (SIP) (Radajewski *et al.*, 2000), microarray technique (Nielsen *et al.*, 2003), polymerase chain reaction - denaturing gradient gel electrophoresis (PCR-DGGE) (Rabaey *et al.*, 2004), PhyloChip analysis (Wrighton *et al.*, 2008) and more recently, pyrosequencing (Kiely *et al.*, 2011) and flow cytometry (Koch *et al.*, 2013), between others, have all proven their usefulness in the physiological identification and characterisation of the microorganisms responsible for electricity generation (Table 1.5). For instance, microarray and genetic analysis have successfully been used for the study of electron transfer mechanisms in *Geobacter sulfurreducens* (Holmes *et al.*, 2006). However, cloning, sequencing, and assembly techniques are needed to improve the microbial knowledge about exoelectrogens.

Table 1.5 Compilation of methods used to quantify and to characterize microbial populations and communities.

Techniques	Description	Type of information extracted	Based on PCR	References
Structural				
SEM	Type of electron microscope that produces images of a sample by scanning it with a focused beam of electrons	Visualize intact biofilms and its structure Visualize the underlying (electrode) material	No	Cortizo and Cortizo, 2003
Microarray	Measures the expression of a large number of genes simultaneously. Probes target functional genes. Fluorescence is detected and quantified.	Community structure and relative quantification	No	Nielsen *et al.*, 2003 Wrighton *et al.*, 2008
CLSM	Optical imaging technique for increasing optical resolution and contrast of a micrograph	Monitor morphological changes in biofilms from the obtained images	No	Zhang and Fang, 2001
Qualitative				
DGGE	A method of DNA fragment separation based on its sequence and denaturizing properties. Coupled to sequencing can be used for identification. Fragments of rRNA or functional genes can be amplified	Community structure and function prediction	Yes	Rabaey *et al.*, 2004
Pyrosequencing	A method of DNA sequencing based on light emission once the commentary nucleotide is incorporated. The intensity of the light determines the incorporation. Fragments of rRNA or functional genes can be amplified	Community structure and functional prediction. More sensitive than DGGE	Yes	Kiely *et al.*, 2011
T-RFLP	A method based on the position of a restriction site closest to a labelled end of an amplified gene. The PCR mixture is digested with restriction enzymes.	Community structure	Yes	Liu *et al.*, 1997
SIP	Detection of the microorganisms able to metabolize a substrate labelled with stable isotopes and to incorporate it into their cells	Trace fluxes of nutrients by microorganisms	No	Radajewski *et al.*, 2000

Table 1.5 (Continued)

Techniques	Description	Type of information extracted	Based on PCR	References
Quantitative				
qPCR /RT-PCR	Monitor the amplification of a targeted DNA molecule during the PCR	Quantify the number of copies of a target gene	Yes	Albinana-Gimenez *et al.*, 2009
FISH / CARD-FISH	A cytogenetic technique that uses fluorescent rRNA oligonucleotide probes that hybridize complementary gene sequences in the ribosome	Localize the presence or absence of specific DNA sequences Cell distribution and relative quantification	No	Davey and George, 2000
FC	A laser technology employed in cell counting and sorting, biomarker detection and protein engineering, by suspending cells in a stream of fluid and detecting them by fluorescence	Analyse the physical and chemical characteristics of the cells, classifying them in groups/ functional groups	No	Koch *et al.*, 2013

PCR: Polymerase chain reaction; SEM: Scanning electron microscopy; CLSM: Confocal laser scanning microscopy; DGGE: Denaturing gradient gel electrophoresis; T-RFLP: Terminal restriction fragment length polymorphism; SIP: Stable isotope probing; Qpcr: Quantitative-PCR; RT-PCR: Real time-PCR; FISH: Fluorescence in situ hybridization; CARD-FISH: Catalysed reporter deposition-FISH; FC: Flow cytometry.

Once the first exoelectrogenic microorganisms were identified, scientists tried to unravel the mechanisms used to release electrons. The mechanisms used to transfer electrons can be direct (direct contact, conductive biofilm and conductive pili) or indirect (mediators). Cyclic voltammetry (CV) is a technique used to distinguish between the direct and indirect electron transfer processes in mixed or pure cultures (Harnisch and Freguia, 2012). Confocal Raman microscopy (CRM) has also been used to test variations in c-type cytochromes redox state (Virdis *et al.*, 2014).

Geobacter sulfurreducens has been widely studied as a model of exoelectrogenic bacterium frequently enriched in electroactive biofilms. Methé *et al.* (2003) interpreted the *G. sulfurreducens* genome, thus facilitating future studies about its electron transport mechanisms. The importance of conductive pili (also called nanowire) was realized when scientists began to understand their utility in electrons transport (Malvankar *et al.* 2011; Lovley *et al.* 2011; Reguera *et al.* 2005).

Nevin *et al.* (2008) compared pure (*G. sulfurreducens*) and mixed cultures in terms of power production in different designs of MFCs. Different proportions between anode and cathode compartments were tested, leading to the conclusion that the use of pure or mixed biofilm cultures was not the most important parameter for power production but reactor design. Other parameters have been evaluated like biofilm conductivity. Malvankar *et al.* (2012) demonstrated that mixed cultures biofilms had higher electrical conductivity than pure cultures of *Geobacter sulfurreducens.* High conductivity allows for rapid electron transport between electrodes. Recent studies have demonstrated syntrophic interactions between exoelectrogen and non-exoelectrogen microorganisms (Parameswaran *et al.*, 2009) and the synergy in communities to remove complex compounds (Kiely *et al.*, 2011). Kim *et al.* (2011) showed that the proportion of exoelectrogens to non-exoelectrogens was lower at higher organic loading rates.

Finally, the most recent studies have focused on the effects of different parameters over the microbial community. Aelterman *et al.*, (2008) observed that the community was capable of self-regulating extracellular electron transfer (EET) pathways at different anode potentials. Moreover, the workability of the system at the optimal anode potential regulated the bacteria activity and growth, obtaining enhanced current and power generation. In addition, the impact of different substrate loads and pH on the microbial community were studied by Puig *et al.* (2010a) and Behera and Ghangrekar (2009). The highest power production was achieved at low OLR, below 1 kg COD kg VSS^{-1} d^{-1} (volatile suspended solid; VSS) with a basic pH of 8-9.5.

1.5.3. Electrotrophic microorganisms

1.5.3.1. Description

An electrotroph has the microbial capacity to directly accept electrons from the electrode at the cathode compartment in the BES. These microorganisms have not been as extensively studied as exoelectrogenic microorganism (Rosenbaum *et al.*, 2011). Early studies focused mainly on the anode compartment and on the exoelectrogenic microorganisms. Currently, the interest in electrotrophs has

increased, as researchers aim to develop biocathodes for the removal of contaminants and for the production of different added value molecules (Marshall *et al.*, 2012; Rosenbaum *et al.*, 2011).

1.5.3.2. History and identification of electrotroph

The study of these microorganisms was not developed until recently. One of the main drawbacks of studying electrotrophic communities compared to the exoelectrogenic ones are the low microbial growth of the cathodic biofilms. On one hand, cathodic biofilms are autotrophic, which already implies a low microbial growth. On the other hand, cathodes have to face higher overpotentials (irreversible energy losses) become than anodes (Puig *et al.*, 2012). For these reasons, only few studies have focused on the characterization of electrotrophic microorganisms. The study of Wrighton *et al.* (2010) deeply analysed the denitrifying microbial communities of two different BES and examined their phylogenetic affiliation and community structures. The study performed by Vilar-Sanz *et al.* (2013) about denitrifying communities focused on the functional genes of denitrification pathways.

Once the microorganisms were identified, efforts became more focused on the identification of the electron transfer mechanisms utilized such electrotrophic microorganisms (Rosenbaum *et al.*, 2011; Lovley, 2011) and the relationship between members of the communities. Marshall *et al.* (2013) focused on the physical (scanning electron microscope) and molecular (RNA, DNA) analysis of microbiomes (synergic communities) for electrosynthesis purposes. On the other hand, Ross *et al.* (2011) studied *Shewanella oneidensis* strain MR-1 to learn about the complex oxidation/reduction reactions occurring at the electrodes. This genus uses the Mtr respiratory pathway to catalyse electron flow from cytoplasmic oxidative reactions to electrodes. It has the ability to drive microbial reductive metabolism. This result suggested the possibility of obtaining valuable fuels and chemicals from that pathway.

In contrast to exoelectrogenic microorganisms, few examples of biocathode electron transfer characterization are available in literature. *Mariprofundus ferrooxydans* was electrochemically characterized as oxygen reducing biocathode (Summers *et al.*, 2013). Moreover, two independent works characterized nitrate reducing biocathodes colonized by *betaproteobacteria*. Pous *et al.* (2014) characterized a biocathode predominantly covered by *Thiobacillus* sp., while Gregoire *et al.* (2014) characterized a biofilm dominated by *Rhodocyclales* and *Burkholderiales*, also within the *betaproteobacteria*. Through the use of CVs, both articles elucidated the thermodynamics for nitrate reduction and they observed Nernstian current–potential dependency for biocathode, which is a common shape observed in bacterial bioanodes.

1.6. Microbial electron transfer (ET) mechanisms

There are two types of electron transport (ET) mechanisms: 1) extracellular electron transport (EET), in which electrons are transferred outside cells by direct (DET) or indirect (IET) mechanisms, or intracellular electron transport (ICET), in which electrons are released inside the membranes of the microorganisms.

1.6.1. Microbial extracellular electron transfer (EET) mechanisms

Certain microorganisms that are considered strict anaerobes and that are able to reduce metallic oxides such as iron, are also considered to transfer electrons via extracellular electron transfer (EET) to an electrode material (exoelectrogen). However, such metal oxide reduction ability does not necessarily confer EET ability (Richter *et al.*, 2007). Due to the original focus on the improvement of MFC performance, EET mechanisms have long remained unknown, although they represent a critical step in understanding the power generation phenomenon in BES. Fortunately, advances in electrochemical techniques have clarified part of these EET mechanisms (Manohar *et al.*, 2008) through the use of polarization curves (Logan, 2008), cyclic voltammetry (Harnisch and Freguia, 2012), electrochemical impedance spectroscopy (Dominguez-Benetton *et al.*, 2012) or surface-enhanced resonance raman spectroscopy (Millo, 2012) among others (Franks and Nevin, 2010).

Recent biocathode studies have shown that electrochemically active bioanodes may be turned into biocathodes when the environmental and operation conditions change (Rozendal *et al.*, 2008), probably due to similar EET mechanisms as those occurring at bioanodes could take place at biocathodes. One of the main differences between anodic and cathodic EET mechanisms is that the redox active components could operate at higher redox potentials (Arends *et al.*, 2011). Nevertheless, bidirectional biofilms have already been described for anodic acetate oxidation and cathodic fumarate reduction (Strycharz *et al.*, 2011; Ross *et al.*, 2011; Dumas *et al.*, 2008), anodic acetate oxidation and cathodic hydrogen production (Jeremiasse *et al.*, 2012; Geelhoed and Stams, 2011), anodic acetate oxidation and cathodic oxygen reduction (Blanchet *et al.*, 2014; Cheng *et al.*, 2010) or anodic acetate oxidation and cathodic nitrate reduction (Pous *et al.*, 2016; Cheng *et al.*, 2012). Moreover, the electrochemical characterization of the biofilm developed by Pous *et al.* (2016) suggested that both acetate oxidation and nitrate reduction took place at a similar formal potential. Hence, this study suggested that electroactive bacteria within biofilms could use the same electron transfer conduit for catalysing anodic and cathodic reactions.

Due to the knowledge generated by these techniques, scientists have been able to distinguish between different EET mechanisms, such as direct or indirect extracellular electron transfer. Interestingly, in mixed microbial biofilms both mechanisms are believed to take place simultaneously in order to maximize the microbial benefits (Logan *et al.*, 2006).

1.6.1.1 Direct electron transfer (DET) mechanisms

Direct electron transfer (DET) is defined as the transport of electrons from the cofactor of a redox active enzyme (oxidoreductase) or a redox protein in the bacterial cell membrane to the electrode surface in the absence of redox mediators (Logan *et al.*, 2006). Thus, the main advantage of direct electron transport consists on the absence of diffusion limitations between microorganisms and electrode (Rabaey and Rozendal, 2010).

Initial investigations of microbial DET were based on pure cultures. The microorganisms selected for this type of studies were *Geobacter sulfurreducens* (Gregory *et al.*, 2004; Bond and Lovley, 2003) and *Shewanella oneidensis* (Gorby *et al.*, 2006) because they are well known for being dissimilatory metal reducing organisms in nature (Holmes *et al.*, 2004; Nealson *et al.*, 1991). Studies on EET have focused on Gram-negative bacteria because they have shown more efficient EET capacities in terms of their achieved currents (1A m^{-2}) and greater EET diversity in both direct and mediated mechanisms (Arends *et al.*, 2011). However, *Thermincola ferriacetica* has recently been reported to produce current values 8 A m^{-2}, indicating that Gram-positive bacteria can also efficiently conduct electrons via DET (Parameswaran *et al.*, 2013).

Microorganisms use three different direct electron transfer mechanisms, including the following: (1) direct contact between the membrane and the electrode, (2) conductive biofilms and (3) conductive pili called nanowire.

1) Direct cell membrane-electrode contact

Exoelectrogen microorganisms can attach to the electrode surface. *Geobacter sulfurreducens* is a clear example of this transfer mechanism. It has been demonstrated by spectroelectrochemical studies that electron transfer occur via c-type cytochromes displayed on the outer cell surface (Millo *et al.*, 2011; Busalmen *et al.*, 2008). Furthermore, *G. sulfurreducens* grows in layers, which allows for close contact between cells (Bond and Lovley, 2003) (Figure 1.4).

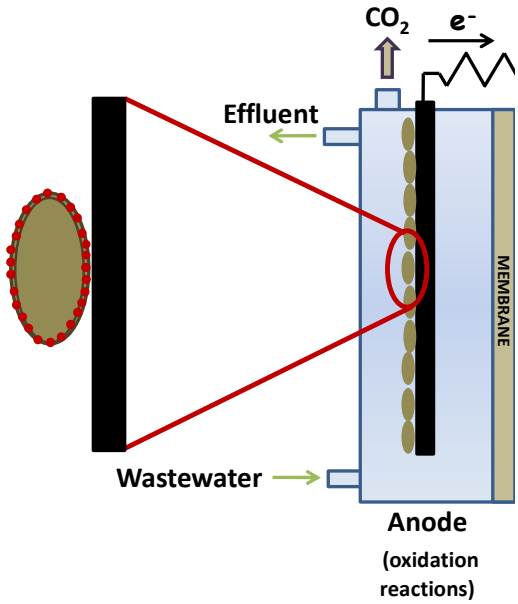


Figure 1.4 Scheme of direct electron transport mechanism. Small red circles represent c -type cytochromes.

On the other hand, *Shewanella oneidensis* is a well-known example of other anode respiring bacterium. Interestingly, it adheres to its substrate five times

stronger in anaerobic conditions than in aerobic conditions indicating that direct contact is a good fixation strategy for EET in adverse conditions. Therefore, based on such findings, EET studies between electrodes and microbes were initially accomplished under anaerobic conditions (Kim *et al.*, 1999).

2) Conductive biofilm

As mentioned above, exoelectrogen microorganisms within conductive biofilms attach to each other by EPS (Figure 1.5). This strategy results in the formation of thick and conductive biofilm layers that allow for high current productions in BES ($1A/m^2$). For instance, it has been recently demonstrated that in pure-culture biofilms of *Geobacter sulfurreducens*, the microorganisms are able to release cytochromes to the matrix (Lovley *et al.*, 2011), thereby increasing the conductivity of the biofilm structure.

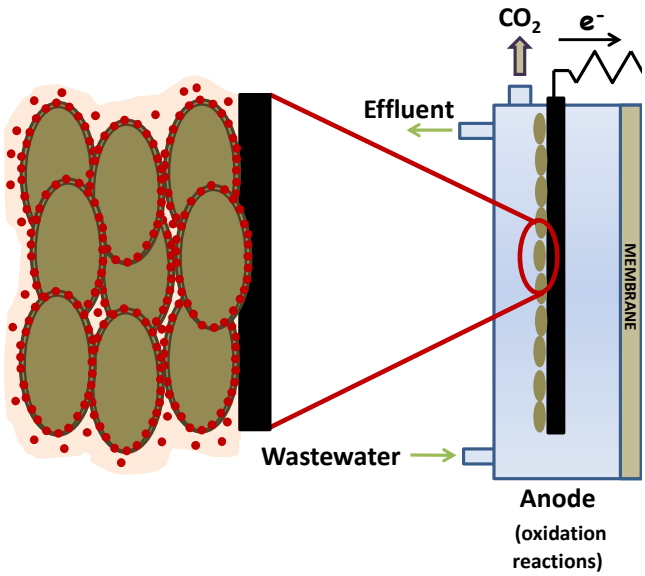


Figure 1.5 Scheme of direct electron transport mechanism of conductive biofilms. Orange matrix: EPS.

3) Conductive pili (Nanowires)

An additional mechanism of microbial DET in biofilms involves self-produced conductive hair-like filaments. These filaments are produced by some microorganisms attached to the cell wall and are known as nanowires (Malvankar and Lovley, 2014, 2012). Such nanowires are involved in long-range EET due to their high content of c-type cytochromes in their structure. Additionally, the nanowires are shaped as thin single strands that connect the microorganism to the solid electrode (Figure 1.6).

Nanowires have been proposed as a possible EET mechanism that is used by a variety of different microorganisms. For example, *Geobacteraceae* and *Shewanellaceae* species were studied as nanowires producers by Reguera *et al.* (2006) and Gorby *et al.* (2006), respectively. Later, the study of Malvankar and Lovley (2014) has revealed that the function of the nanowire in these two organisms is different. While the nanowires in *Geobacter sulfurreducens* have metal conductivity and transfer electrons, the electrons seem to "jump" between cytochromes located on the non-conductive pili in *Shewanella oneidensis*.

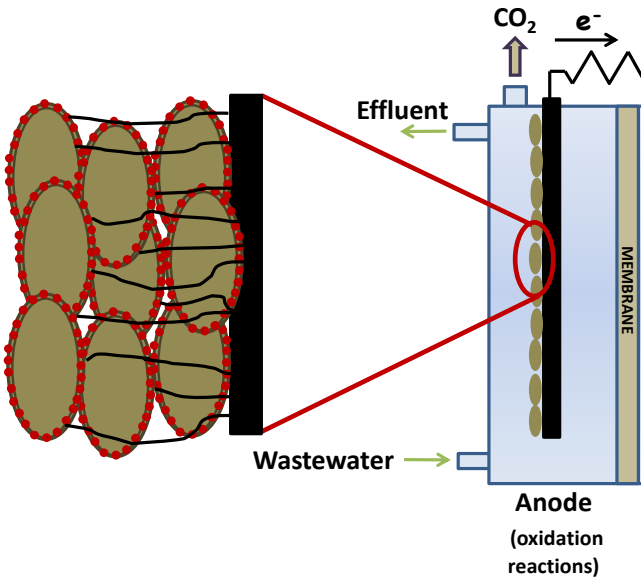


Figure 1.6 Scheme of direct electron transport mechanism by nanowires. Black lines: conductive pili.

1.6.1.2. Indirect electron transfer (IET) mechanisms

Indirect electron transfer (IET) is defined as the transport of electrons from the bacterial cell to the electrode surface or from the cell to another bacterial cell through redox mediators (Rabaey and Rozendal, 2010) (Figure 1.7).

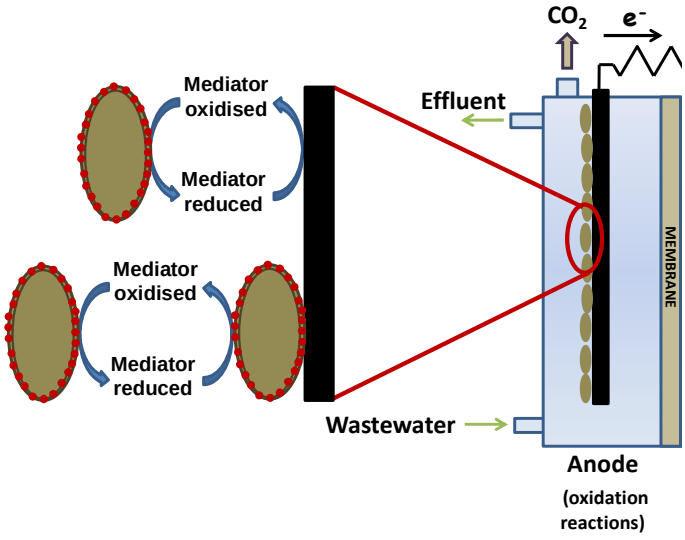


Figure 1.7. Scheme of indirect electron transport mechanism.

These mediators could be chemically added or self-produced by microorganisms as secondary metabolites (Arends *et al.*, 2011) (Table 1.6). For example, pyocyanin is produced by *Pseudomonas aeruginosa* (Rabaey *et al.*, 2004), while riboflavin is

secreted by *Shewanella* to mediate the electron transfer to the electrode (Marsili *et al.*, 2008). Moreover, free cell-derived enzymes can interact with electrode surfaces and catalyse the formation of intermediates that are rapidly consumed by microbial cells (Deutzmann *et al.*, 2015).

Table 1.6 Representative biogenic production of redox mediators.

Microorganism	Mediator molecule	Reference
Sphingomonas xenophaga	4-amino-1,2-naphthoquinone	Keck *et al.*, 2002
Pseudomonas aeruginosa	Phenazine-1-carboxylic acid	Price-Whelan *et al.*, 2006
Pseudomonas chlororaphis	Phenazine-1-carboxamide	van Rij *et al.*, 2004
Shewanella oneidensis	Flavin mononucleotide	von Canstein *et al.*, 2008
Shewanella algae	Melanin	Turick *et al.*, 2002
Bacillus pyocyaneus	Pyocyanine	Friedheim and Michaelis, 1931
Propionibacterium freundenreichii	2-Amino-3-carboxy-1,4-naphthoquinone	Hernandez and Newman, 2001
Shewanella algae	Cyanocobalamin	Workman *et al.*, 1997
Acetobacterium woodii	Hydroxycobalamin	Hashsham and Freedman, 1999
Pseudomonas stutzeri	Pyridine-2,6-bis	Lewis *et al.*, 2001
Methanosarcina thermophila	Porphorinogen-type molecules	Koons *et al.*, 2001
Shewanella oneidensis	1,4-Dihydroxy-2-naphthoate derivative	Ward *et al.*, 2004
Shewanella sp.	Riboflavins	Marsili *et al.*, 2008

[a] More detailed information can be found in the following references: (Hernandez and Newman, 2001; Li *et al.*, 2009; Marsili and Zhang, 2009; Schröder, 2007; Watanabe *et al.*, 2009).

A variety of chemicals have been also used to facilitate IET. These mediators include neutral red (Park *et al.*, 1999), anthraquinone-2-6, disulfonate (AQDS) (Holmes *et al.*, 2004), thionin, potassium ferricyanide (Bond *et al.*, 2002), methyl viologen, and others (Rabaey and Verstraete, 2005). The main problems of the chemical additions are related to the high cost of the mediators, their possible toxic effect and their consumption by wash-out.

Within the context of anodic biofilm, it is worth noticing that the study of DET mechanisms has gained more attention over IET mechanisms. This has resulted in the partial abandon of IET by the scientific community. However, recent preliminary studies on microbial electrosynthesis of acetate at the cathode and the absence of evident biofilms suggest that an electron uptake based on IET mechanism might be responsible for the observed conversions of CO_2 into acetate (Arends *et al.*, 2013)

and butyrate (Ganigué *et al.*, 2015). Therefore, it is expected that the interest on IET will soon increase again.

As in the case of anode IET mechanisms, cathode IET requires mediators to carry out the reduction of certain final electron acceptors (see above). Although little information is available at present, it can be assumed that the mediators responsible of cathode IET could be artificially added or biologically self-produced as has been described for the anode compartments. Another study demonstrated the use of methyl viologen as a mediator for the electrochemically assisted microbial dechlorination of trichloroethene (TCE) and cis -dichloroethene (cis -DCE) (Aulenta *et al.*, 2009). Other mediators have been applied to the cathodes as anthraquinone-2,6-disulfonate (Thrash *et al.*, 2007) and neutral red (Park and Zeikus, 1999) for the reduction of perchlorate and fumarate, respectively. Taking into account all available information, it is expected that the cathodic IET mechanism will be very similar to the anodic IET (Thrash and Coates, 2008).

1.6.2. Microbial intracellular electron transport (ICET) mechanisms

The internal microbial mechanism to transport and release electrons inside the membranes of microorganisms is currently being investigated. The study of these pathways is usually performed with pure cultures. One of the most studied species for ICET is *Geobacter sulfureducens*, which is studied due to its enrichment in multiple BES studies (Yates *et al.*, 2012) and due to its demonstrated ability to produce high current densities (Chen *et al.*, 2012). Despite these efforts, the mechanism of electron transfer remains unclear as unknown pathways exist in the process.

The mechanism that allows electron transfer from electron donor (reductant) to the electron acceptor (oxidant) occurs inside the lipid-membranes and involves the oxidoreductase enzyme (Hartshorne *et al.*, 2009). First, electrons are generated by the NADH dehydrogenase located in the inner mitochondrial membrane (Figure 1.8). This enzyme catalyses the conversion of NADH to NAD^+ and thereby releases

protons to the periplasm zone. NAD^+ is reduced through a route that recycles it back to the active form via the Krebs cycle.

Electrons are transferred to the cytochrome b-c by the coenzyme ubiquinone. Coenzyme ubiquinone, also called Q10, develops various functions related to its redox capacity, including electron transport between cytochromes. Once the electrons are in this cytochrome, more protons are released into the intermembrane space. Thereafter, electrons from the cytochrome b-c are transferred to the c-type cytochrome. The c-type cytochrome is mobile, connecting the inner membrane, the periplasm and the outer membrane. This cytochrome is the responsible for the electron transfer between both membranes (Lovley *et al.*, 1991). Then, electrons are transferred through different cytochromes until they reach the OmcS cytochrome, which is in contact with the surface of the cell (Lovley, 2008a).

During electron transfer, the gradient of protons between the cytoplasm and the periplasm is used to generate energy in form of adenosine triphosphate (ATP). The accumulation of protons is generated by proton pumping from the different cytochromes, decreasing the pH of the periplasm. Protons flow through the ATP synthase along the proton gradient leading to the synthesis of ATP from adenosine diphosphate (ADP) and inorganic phosphate. This ability has been intensively studied with the aim of increasing the power production of MFCs. Different genetic engineering modifications have been applied to *Geobacter sulfurreducens* (Lovley *et al.*, 2011): cell abilities to produce more cytochromes, which increase the amount of electron transfer within the membrane and therefore increase the nanowire expression levels. These effects in turn enhanced the contact cell-cell and/or cell-electrode contact, but not the current production. To improve the current production, Malvankar *et al.* (2011) increased the biofilm conductivity regulating the gene expression and varying the voltage, observing a positive correlation. These findings highlight the necessity of a better understanding of the processes occurring inside the bacteria.

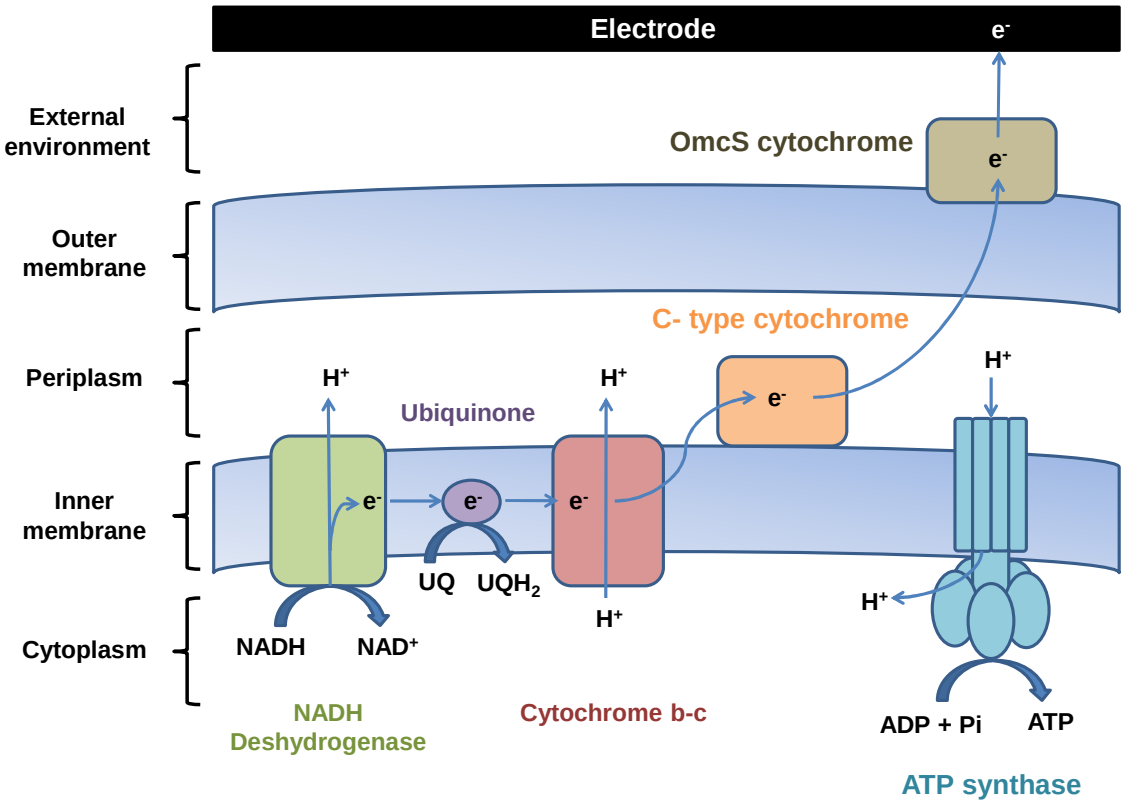


Figure 1.8 Electron transport chain to move electrons directly from the mitochondrial membrane to the external membrane until the electrode for *Geobacter sulfurreducens*.

It is believed that the ability of *Geobacter sulfurreducens* to create very thick biofilms (>50 µm) through the direct contact mechanism can be substituted by electron transport through conductive pili (Figure 1.6). Such an additional DET mechanism is actively being discussed by the scientific community. However, recent studies on microbial nanowires suggest that these pili-like structures, which are located along the membranes on the external environment of the cell, are covered by OmcS cytochromes (Leang *et al.*, 2010; Lovley, 2006). Interestingly, this nanowire DET pathway allows the microorganisms to transfer electrons between them without intermediates, enabling biofilm communication (Logan and Regan, 2006).

The mechanisms of electron transfer through the membrane in electrotrophic bacteria remain largely unknown. Scientists suspect that the process of moving electrons is similar to the electrogenic process. The reduction of these compounds consumes protons in the cytoplasm, generating a proton gradient across the inner membrane (Lovley, 2011). Therefore, proton gradient is necessary to generate

energy by ATP synthase, as it is the case for exoelectrogens. Ross *et al.* (2011) studied *Shewanella oneidensis* strain MR-1 and demonstrated that similar pathway can be used for oxidation and reduction reactions. In the case of *Shewanella*, similar electron transfer to *Geobacter sulfurreducens* was identified, differing on the cytochromes, known as Mtr, that catalyses electron flow from cytoplasmic oxidative reactions to electrodes (Bird *et al.*, 2011).

Chapter 2

Objectives

The main objective of this PhD thesis is to move forward in **simultaneous carbon and nitrogen removal in complex wastewaters using bioelectrochemical system (BES) and scaling-up it towards application**. The available knowledge on literature about pollutants treatment in synthetic wastewaters is remarkable (Chapter 1). However, once this thesis started, few studies regarding simultaneous carbon and nitrogen treatment in real streams had been reported. Moreover, no studies with swine manure were available. This PhD targets at the following specifics objectives (Figure 2.1):

- To determine the viability (robustness, reliability and resilience) of BES in the treatment of complex matrices as swine manure in mL-scale and L-scale in terms of carbon and nitrogen removal and electricity production (A).

- To elucidate the optimal operational conditions in mL-scale using different designs with and without external resistance control, cathode electron acceptors and oxygen set-point to obtain a more electroactive BES for the carbon and nitrogen treatment (B).

- To test at long-term scaled-up BES (C) and electrode materials (D) in real environments.

- To improve the electricity production in both BES sizes (mL and L-scale) by applying an external resistance control (mL-scale) and different electric circuit connection (L-scale) (E).

- To characterise the BES microbiomes treating swine manure, in both anode and cathode, relating the microorganisms identified with their putative role on nutrient removal and electricity production (electrogenic, nitrifier and denitrifier bacteria) (F).

- To stablish the first proof-of-concept of microbial anoxic ammonium oxidation in BES and to elucidate the extracellular electron transfer

thermodynamics for ammonium oxidation, identifying the electroactive steps (G).

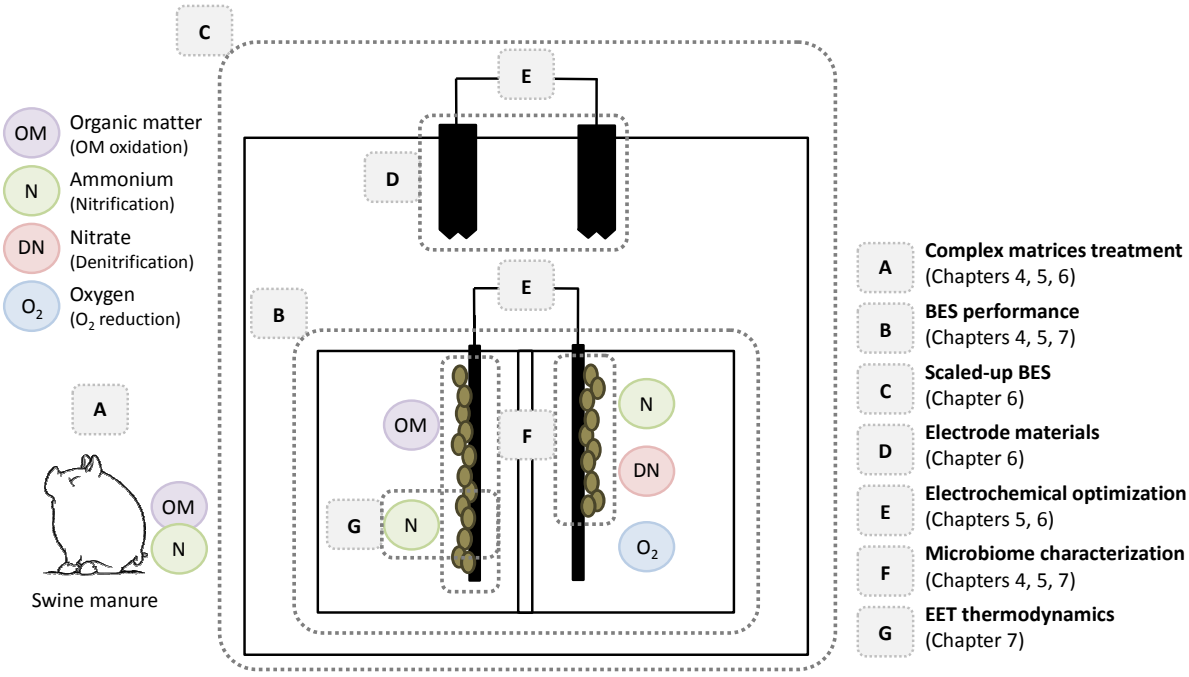


Figure 2.1. Scheme of the targets of this PhD thesis.

Chapter 3

Methodology

3.1. BES reactor set-up

Along the whole PhD thesis, different electrode materials were evaluated. Graphite, in form of granular graphite (diameter 1.5 – 5 mm, EnViro-Cell, Germany) and graphite rod (Sofacel, Spain in Chapters 4, 5; Mersen Iberica, Spain in Chapter 6; or CP-Graphite GmbH, Germany in Chapter 7), was the main conductive material support used in bioanodes and biocathodes in the different chapters (Chapters 4, 5, 6 and 7). Stainless steel in form of mesh (SS-316L, diameter 0.40 mm, path: 1 x 1 mm, Cisa, Spain) and filament (SS-316L, diameter 0.40 mm, Cisa, Spain) was also used in both compartments in Chapter 6 (Figure 3.1).

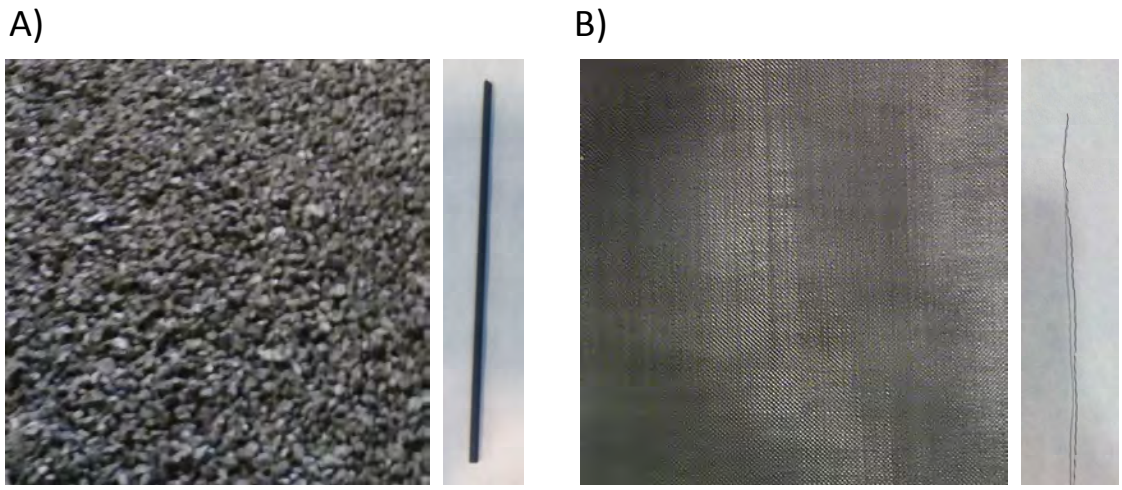


Figure 3.1 Materials used as electrodes where A) Graphite granules and rod, B) Stainless steel mesh and filament.

Three different BES designs were used during the experimental period: i) rectangular design (Figure 3.2); ii) scaled-up stack design (Figure 3.4) and iii) Microcosm design (Figure 3.6).

3.1.1. Rectangular design

The rectangular BES was used for Chapters 4, 5 and 7. It consisted of an anode and a cathode placed on opposite sides of a single methacrylate rectangular chamber (20 x 20 x 2.2 cm, Futura, Spain), separated by an ionic exchange. Anode and cathode compartments were filled with granular graphite (model 514, diameter 1.5–5 mm, EnViro-cell, Germany), which decreased the net anode and cathode compartment (NAC and NCC, respectively) volumes between 370 and 460 mL. Two thin graphite

rod electrodes (130 × 6 mm, Sofacel, Spain) were installed in both compartments. The electrodes (granules and rods) were previously washed for at least 1 h with 1 M HCl and then in 1 M NaOH to remove possible metal and organic contamination (Bond and Lovley, 2003). An internal recirculation loop (between 170 and 360 L d^{-1}) was placed in each compartment to maintain well-mixed conditions, and to minimize concentration gradients. In all cases, both anode and cathode were equipped with Ag/AgCl reference electrodes (+0.197 V vs standard hydrogen electrode (SHE), model RE-5B BASi, United Kingdom).

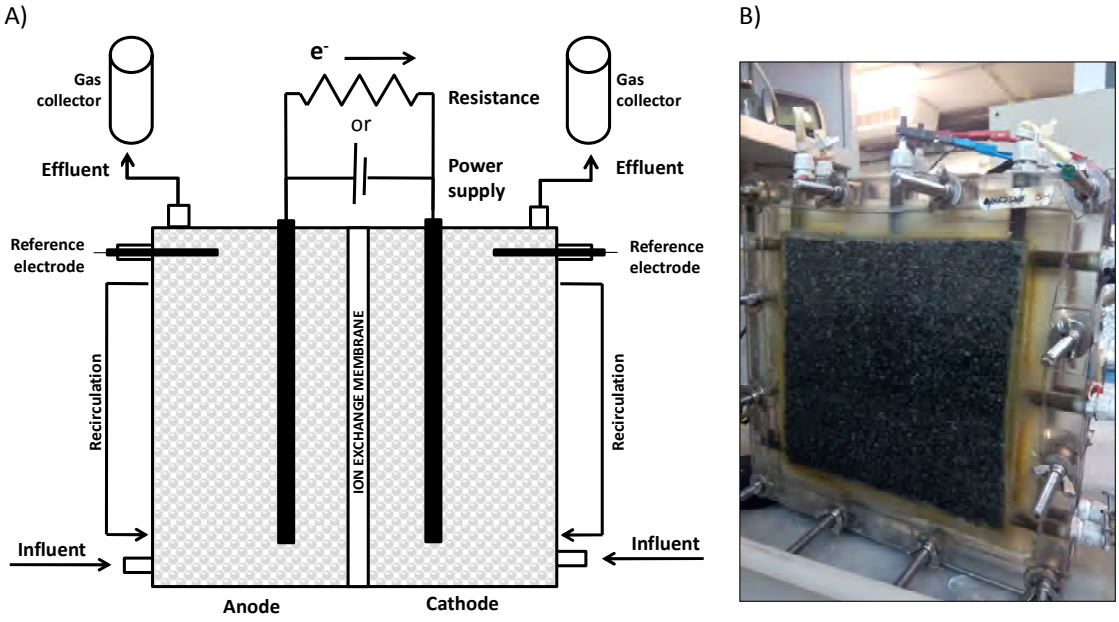


Figure 3.2 A) Schematic representation and B) picture of the rectangular reactor design.

All anode compartments followed the same configuration, but different cathode configurations and membranes were applied. Four different configurations were tested for the cathode compartments depending on oxygen supply position (Figure 3.3).

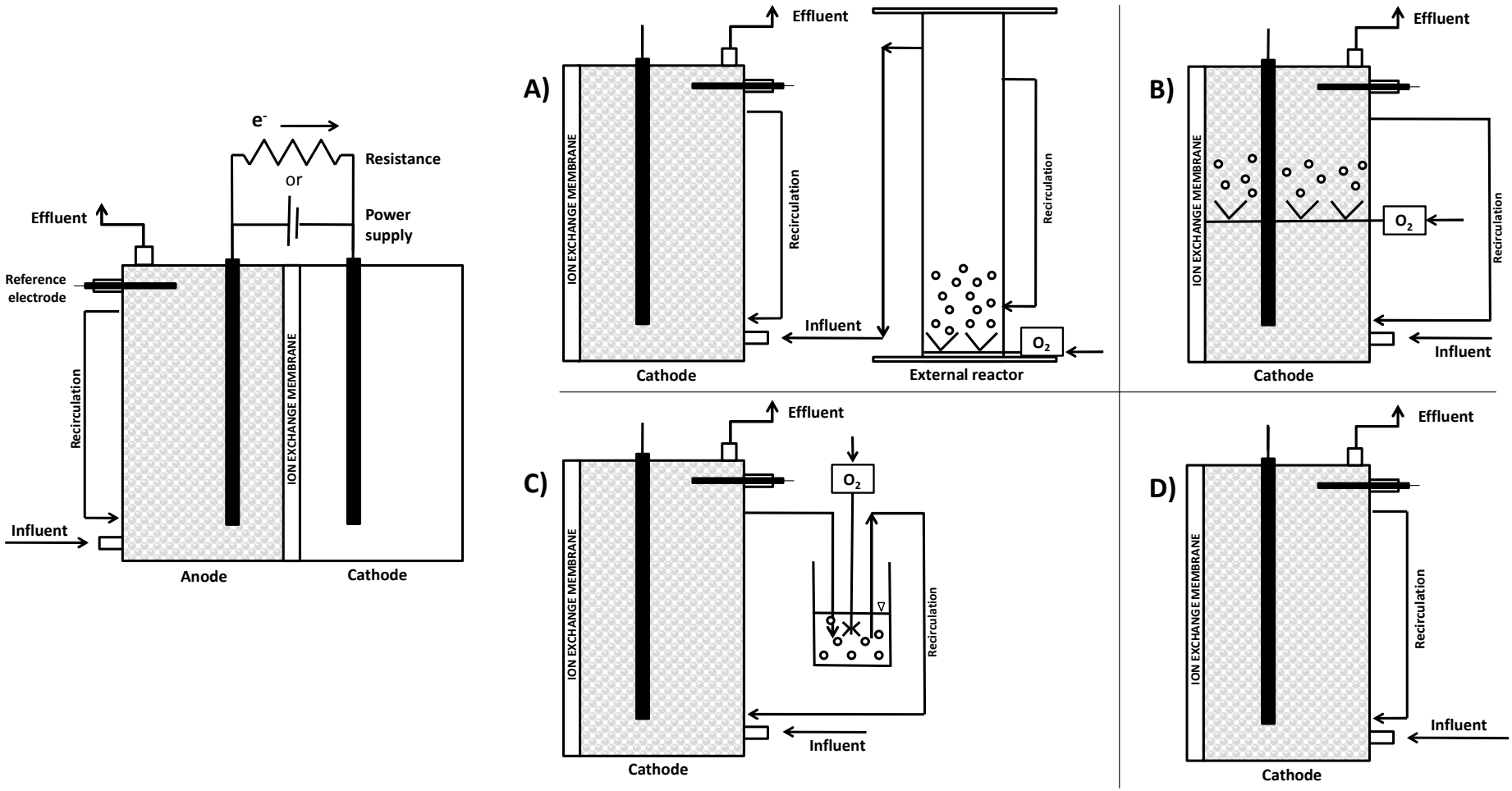


Figure 3.3 Cathode configurations where A) anoxic compartment with influence of an aerobic external reactor (Chapter 4); B) Half-compartment aerated (Chapter 4); C) Aerated recirculation (Chapter 5) and D) Anoxic cathode (Chapter 7).

Cathode **configuration A** was used in Chapter 4. It consisted of a cathode coupled to the external aerated reactor to promote two different reactions under clearly well differentiated conditions. In configuration A, denitrification and nitrification occurred in different compartments. The external reactor was filled with clay (diameter 0.8 cm) to promote the bacteria adhesion. Aeration was performed from the bottom and it was controlled at an oxygen set-point of 3.2±0.8 mg O_2 L^{-1}. The net reactor compartment (NRC) volume was 5 L. The **configuration B** was also used in Chapter 4. It allowed different internal conditions (i.e. aerobic and anoxic conditions) that will allow simultaneous processes. In configuration B, nitrification and denitrification occurred simultaneously in the same compartment. Aeration was performed at half of the height of the compartment (10 cm from the base) to separate an aerobic (upper) from an anoxic (bottom) zone in the cathode. The oxygen set-point was controlled at 1.3±0.3 mg O_2 L^{-1}. The **configuration C** was used in Chapter 5 to prevent overpressures working with an oxygen saturated solution. The **configuration D** was used in Chapter 7, where anoxic conditions were required. Specific conditions of each study can be found in the corresponding chapters.

An anion exchange membrane (AEM) was used to separate the anode and cathode compartments (AMI-7001, Membranes International Inc., USA) in Chapters 4 (configuration A), 5 (Configuration C) and 7 (Configuration D) to avoid ammonium diffusion to the cathode. However, a cation exchange membrane (CEM; CMI-7001, International Membranes Inc., USA) was used in one of the configurations of Chapter 4 (Configuration B) to promote ammonium diffusion to the cathode.

3.1.2. Stacked scaled-up design

The stacked BES was designed and used in Chapter 6 (Figure 3.4). The BES consisted of six anode and cathode compartments (90 x 40 x 1.5 cm each one) hydraulically connected to an external nitrifying reactor (150cm x 20cm diameter). The internal volume of the MFCs was 65 L while for the external tubular reactor was 50 L (system gross capacity of 115 L). The anode and cathode compartments were

placed on opposite sides of a polyvinyl chloride rectangular compartment, clamped transversely together by stainless steel bolts. The anode and cathode compartments were separated by an anion exchange membrane (AMI-7001, Membranes International Inc., USA) to avoid ammonium diffusion to the cathode.

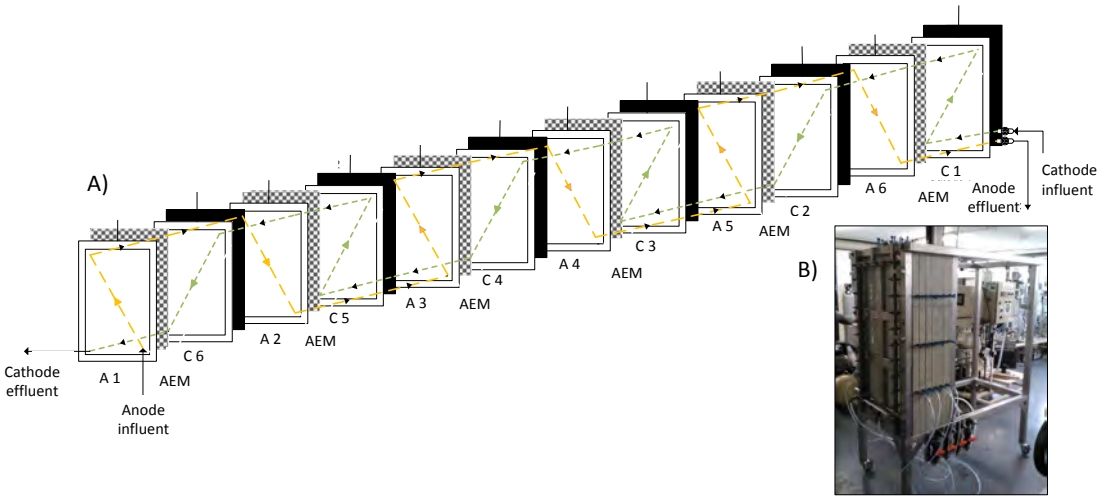


Figure 3.4 A) Schematic representation of the stacked scaled- up reactor set up, where anodes (A), cathodes (C) and anion exchange membrane (AEM) are represented, and B) picture of the stacked scaled- up reactor.

A couple of configurations were assessed depending on the electrode material (Figure 3.5). In one of the configurations, granular graphite (model 00514, diameter 1.5 - 5 mm, EnViro-cell, Germany) was used as electrode material (named as Scaled GG). Graphite rods (120 cm x 0.6 cm) were used as working and counter electrode collectors (Mersen Iberica, Spain). The filling material decreased the volumes of the compartments, amounting of 20 L NACs and NCCs, respectively. In the other configuration, a double layer of stainless steel mesh (90 x 40 x 0.1 cm every layer, model SS-316L, Cisa, Spain) was used as electrode material (Scaled SS). Stainless steel wire was used to connect the compartments. The filling material allowed a bigger net volume inside the compartments (37 L NACs and NCCs, respectively). All configurations had one Ag/AgCl reference electrode in each compartment (+0.197 V vs SHE, model RE-5B, BASi, United Kingdom).

In both configurations, an external tubular reactor of PVC (150 cm x 20cm diameter) with NRC volume of 20 L was built. The reactor was filled with clay as in

Chapter 4. Aeration from the bottom of the reactor was performed by an air compressor (B2800B/100 CM3 2 CIL, Ingersoll Rand, United Kingdom). Oxygen concentration was controlled and limited to values between 1-1.5 mg $O_2 \cdot L^{-1}$ by an oxygen probe (Model 50 60, Crison, Spain) to minimize oxygen input, avoid diffusions and reduce costs.

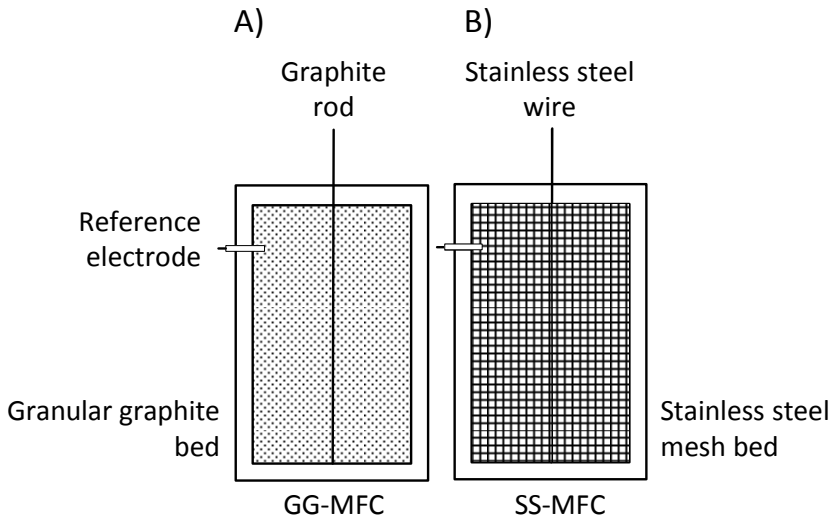


Figure 3.5 Compartment configurations in the stacked MFC filled with: A) granular graphite and a graphite rod, B) stainless steel mesh and a stainless steel wire.

3.1.3. Microcosm design

Microcosm design was used in Chapter 7 to further characterize the electrotroph activity of the nitrifying electroactive biofilms (Figure 3.6), based on Pous *et al.* (2014). Microcosms consisted in tailor-made single-chamber BES with a final working volume of 15 mL. Each microcosm contained two graphite rods (CP-Graphite GmbH, Germany) with a projected surface area of 6.68 cm^2, connected with stainless steel wire and one Ag/AgCl reference electrode (+0.197 V vs SHE, SE11 Sensortechnik Meinsberg, Germany).

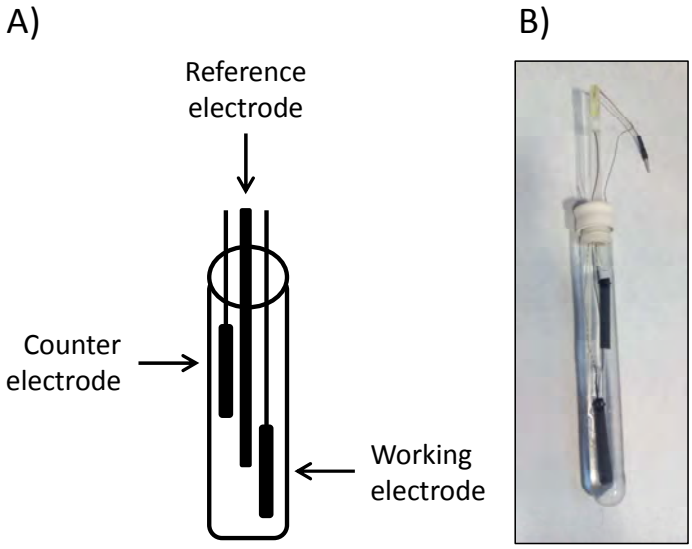


Figure 3.6 A) Schematic representation and B) picture of the microcosm used for electrochemical characterization.

3.2. Electrochemical configurations

A couple electrochemical configurations (MFC, MEC) were performed with the different reactor designs (rectangular, stacked scaled-up, microcosm). BES was operated as MFC in Chapters 4, 5 and 6, meanwhile in Chapter 7 BES was operated as MEC.

3.2.1. MFC electric configuration

The cell potential (V) of MFC configurations was monitored at 60 s intervals using an on-line multimeter (Alpha-P, Ditel, Spain) with a data acquisition system (Memograph® M RSG40, Endress + Hauser, Germany). Current (*I*) and power (*P*) were determined according to Ohm's laws (Eq 3.1). Power and current densities were calculated by dividing power and current by the net compartment volume (NAC or NCC) (Eq 3.2).

$$\text{Ohm's law: } I = \frac{V}{R} \quad ; \quad P = I \cdot V \qquad \text{(Eq 3.1)}$$

$$\text{Power density} = \frac{P}{NCC} \quad ; \quad \text{Current density} = \frac{I}{NAC \; or \; NCC} \qquad \text{(Eq 3.2)}$$

In Chapter 4, the electrodes were connected to an external resistance of 30 Ω to close the electric circuit, while in Chapter 5, the maximum power point tracking

(MPPT) control strategy was applied (Figure 3.7) instead of a fixed resistance. The implementation of the MPPT control system was supported by Prof. Giuseppe Venchi and the staff of the Power Electronics Laboratory of the University of Pavia. The MPPT control consisted of an array of parallel-connected potentiometers imposing resistance on the MFC, an amperometer and a voltmeter (Model 2000 6-1/2 Digit Multimeter, Keithley Instruments, USA) for current and voltage measurements. The applied external resistance could vary between 6 and 200 Ω via 2 Ω steps (ΔR). The implemented MPPT algorithm is classified as a perturbation-observation method. Basically, it was composed of a loop that periodically measured (every 2 min) the MFC output power (P). The power value measured at one iteration (step i) was compared with the value measured in the previous iteration (step i-1); the resistance *R* varied according to Eq 3.3.

$$R_{i+1} = R_i + \Delta R \, sign\left(\frac{P_i - P_{i-1}}{R_i - R_{i-1}}\right) \quad \text{(Eq 3.3)}$$

A) B)

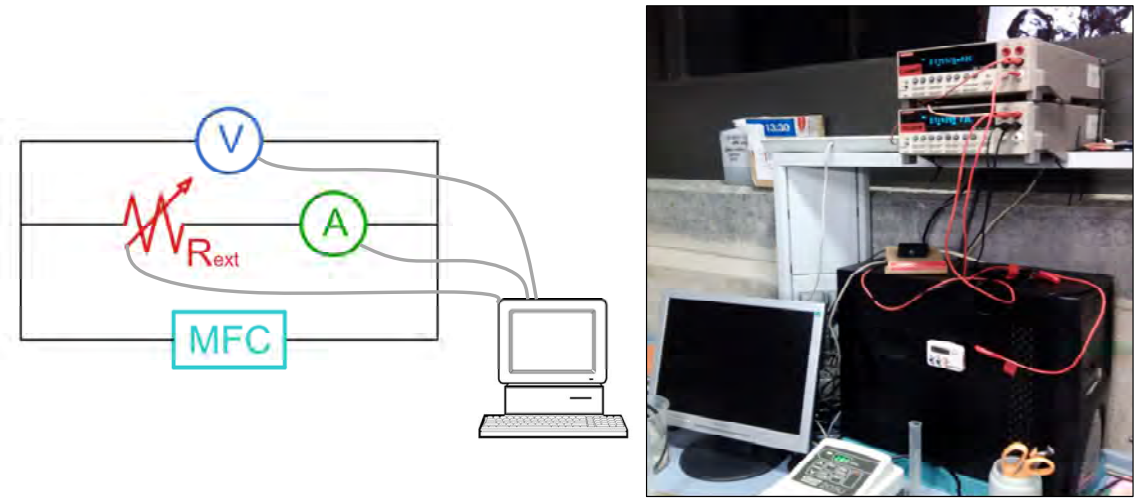


Figure 3.7 A) Schematic representation and B) picture of the Maximum power point tracking (MPPT) control system.

Different electrochemical connections were done in stacked MFC (Chapter 6). The electrical connections used for the 6-stacked MFC were (i) individual, (ii) in parallel, (iii) in series and (iv) mixed (3 MFCs in parallel and 3 in series), to step up

the current, voltage or both, respectively. The external resistances applied under individual connection was 1.5 Ohms, in parallel connection were 15 and 100 Ohms while in series connection was 2200 Ohms in order to be higher than the internal resistance. The mixed connection joined the MFCs depending on their internal resistances. MFC were connected in series by pairs (MFCs 1-6, 2-3 and 4-5) due to their similar internal resistance. One of the pairs was connected in parallel with two different sets of MFC in series. The resistance applied was 100 Ohms.

3.2.2. MEC electric configuration

In Chapter 7, a MEC configuration with a three-electrode arrangement was used (Figure 3.6). In this Chapter, biologic anoxic ammonium oxidation was evaluated by poising the anode potential at +0.8 V vs. SHE using a potentiostat (model SP50, Bio-Logic, France) (Figure 3.8).

Figure 3.8 Bio-logic potentiostat model SP50.

3.3. Analyses and calculations

3.3.1. Electrochemical analyses

Along the whole PhD thesis, different electrochemical analyses were performed using a potentiostat (model SP50, Bio-Logic, France).

In Chapters 4 and 6, polarization curves were recorded to analyse the MFC behaviour. Polarization curves were performed by imposing a linear potential decrease of 1 mV s^{-1} from open circuit voltage (OCV) to a cell voltage of 0 mV, followed by a linear voltage increase of 1 mV s^{-1} to the original OCV value.

In Chapter 7, cyclic voltammetries (CVs) were recorded to analyse the extracellular electron transfer thermodynamics in the MEC reactor. CVs consists on a voltage swept between two values (the initial (E_i) and final (E_f) vertex potential) at a fixed rate. Once the voltage match the E_f the scan is reversed and the voltage is swept back to the E_i. The parameters applied in this study for CV were as follows: scan rate: 1 mV s^{-1}; E_i = +0.00 V and E_f = +0.63 V. Four CV cycles were performed in each routine, but only data from the last cycle is shown. CV data was analysed with SOAS software19 to identify the oxidation and reduction peaks.

3.3.2. Electrochemical calculations

The bioelectrochemical performance was characterized in terms of coulombic efficiency (CE, %) for the oxidation (anode) and reduction (cathode) reactions. The anode and cathode CEs (Eq 3.4) were calculated as following:

$$CE = \frac{I\ measured}{I\ theoric} = \frac{I\ measured}{n\ F\ Q\ (C_{in} - C_{out})} \cdot 100 \qquad \text{(Eq 3.4)}$$

where *I* is the current (A), *n* is the number of electrons; *F* is Faraday's constant (96485 C mol^{-1}); *Q* is the flow rate (L s^{-1}) and C_{in} and C_{out} are the influent and effluent concentrations of substrate (mol S L^{-1}).

The energy consumption was only calculated for nitrogen removal in Chapter 7. The energy consumption (Wh $kg^{-1}N\text{-}NH_4^+$) was calculated as the electrical energy supplied by the power source per kilogram of nitrogen removed (Eq 3.5).

$$\text{Energy consumption} = \frac{\frac{I\ E_{cell}}{V}}{(C_{in} - C_{out})} \qquad \text{(Eq 3.5)}$$

where *I* is the current (A), *Ecell* is the cell voltage (V), *V* is the volume (L); C_{in} and C_{out} are the influent and effluent concentrations of ammonium (kg N L^{-1}).

3.3.3. Chemical analyses

Liquid phase standard wastewater measurements for organic matter, nitrogen and solid content were performed at regular intervals according to the American Public Health Association guidelines (APHA, 2005). Depending on the aim of the

experiment different analysis were performed. Standard wastewater measurements for organic matter were measured in form of total and soluble chemical oxygen demand (COD_t, COD_s) and 5-day biochemical oxygen demand (BOD), which represents the biodegradable organic matter. Respect to nitrogen content, in form of total Kjeldahl nitrogen (TKN-N), ammonium (NH_4^+–N), nitrite (NO_2^-–N) and nitrate (NO_3^-–N)). Finally, solid content was measured in form of total suspended solids (TSS) and volatile suspended solids (VSS).

The conductivity and the pH were measured with an EC-meter (EC- meter basic 30^+, Crison, Spain) and a pH-meter (pH-meter basic 20^+, Crison, Spain).

The gas phase collected was analysed to detect the presence of carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), dinitrogen gas (N_2), oxygen (O_2) and hydrogen (H_2) with an Agilent 7820A GC System equipped with Washed Molecular Sieve 5A and Porapak® Q columns and a Thermal Conductivity Detector (TCD). N_2O levels in the liquid-phase were analysed using a liquid microsensor (N_2O-100, UNISENSE, Denmark) in Chapter 4.

The continuous measurements of dissolved oxygen (DO) in the external reactor and cathodes were controlled using a dissolved oxygen probe (model 5060, Crison, Spain).

3.3.4. Chemical calculations

Carbon and nitrogen (Substrate, S) removal rates and efficiencies were evaluated based on COD and NH_4^+/NO_3^- influent and effluent concentrations, depending on the compartment analysed.

Substrate removal rates (kg S m^{-3} d^{-1}) were calculated as (Eq 3.6):

$$\text{S removal rate (kg S m}^{-3}\text{ d}^{-1}\text{)} = \frac{(C_{in} - C_{out})}{V \cdot 1000} \cdot Fw \qquad \text{(Eq 3.6)}$$

where, C_{in} and C_{out} are the influent and effluent concentrations of substrate (mg S L^{-1}). *Fw* is the flow rate (L d^{-1}) and *V* is the compartment volume (L).

Substrate removal efficiencies (%) were calculated as (Eq 3.7):

$$\text{S removal efficiency (\%)} = \frac{C_{in} - C_{out}}{C_{in}} \cdot 100 \quad \text{(Eq 3.7)}$$

While, substrate production rates and efficiencies were calculated as following:

Substrate production rates (kg N L^{-1} d^{-1}) were calculated as (Eq 3.8):

$$\text{S production rate (kg N L}^{-1}\text{ d}^{-1}\text{)} = \frac{(C_{out} - C_{in})}{V \cdot 1000} \cdot Fw \quad \text{(Eq 3.8)}$$

Substrate production efficiencies (%) were calculated as (Eq 3.9):

$$\text{S production efficiency (\%)} = \frac{C_{out} - C_{in}}{C_{in}} \cdot 100 \quad \text{(Eq 3.9)}$$

The gas measurement production efficiencies were calculated as the proportion between all gas compounds measured in the obtained gas flow (volume of gas in the column per unit of time).

The values of the free ammonia (FA) and the free nitrous acid (FNA) concentrations were calculated as a function of pH, temperature and total ammonium as nitrogen (TAN) for FA (Eq 3.10), or total nitrite (TNO_2^-) for FNA (Eq 3.11).

$$\text{FA (mg N L}^{-1}\text{)} = \frac{TAN}{1 + \left(\frac{10^{-pH}}{K_{e,NH_3}}\right)}; \quad \text{where; } K_{e,NH_3} = \frac{-6344}{e^{273+T}} \quad \text{(Eq 3.10)}$$

$$\text{FNA (mg N L}^{-1}\text{)} = \frac{TNO_2}{1 + \left(\frac{K_{e,HNO_2}}{10^{-pH}}\right)}; \quad \text{where; } K_{e,HNO_2} = \frac{-2300}{e^{273+T}} \quad \text{(Eq 3.11)}$$

3.4. Molecular analyses

The molecular analyses performed along this thesis were supported by the Molecular microbial ecology group (EcoAQUA) from the Institute of Aquatic Ecology (University of Girona, Spain) in Chapters 4 and 5, the Research Technical Services (University of Girona, Spain) in Chapter 4 and the Environmental microbiology department of Helmholtz-Zentrum für Umweltforschung (UFZ center, Germany) in Chapter 7.

Bacteria present in the BES compartments was analysed using different molecular or microscopic techniques from the qualitative and quantitative perspective to provide a complete description of the microbial community. Graphite and clay samples were taken from the different compartments for microbial analysis when steady state conditions were achieved. The bacterial morphology and structure was visualized by Scanning electron microscopy (SEM). The microbial composition was analysed by Polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE), PCR-pyrosequencing and Terminal-restriction fragment length polymorphism (T-RFLP) and the microbial quantification was analysed by quantitative PCR (qPCR) and by Fluorescence in situ hybridization (FISH).

3.4.1. Visualization of the microbial community

SEM analyses were performed with intact biofilms in Chapter 4. Graphite and clay samples were collected and immediately immersed in 2.5% (w/v) glutaraldehyde in a 0.1 M cacodylate buffer at pH 7.4. The samples were washed and dehydrated successively in an ethanol series. Fixed samples were dried with a critical-point drier and sputtered with a 40 nm gold layer. The coated samples were examined with a SEM (model DSM-960; Zeiss, Germany) at 20 kV, and the images were captured digitally.

3.4.2. Microbial community composition

Biofilm attached to the graphite surface was dislodged in an ultrasonic bath (P-Selecta) for 60 s in phosphate buffered saline (PBS). Liquid phase was collected after precipitation of graphite granules and cells were pelleted by centrifugation at 4000 rpm. Nucleic acids were extracted using the Fast DNA® SPIN Kit for soil (MP, Biomedicals).

In Chapter 4, the microorganisms attached to the graphite electrodes (anode and cathode) and clay adhesion surface (external reactor) were analysed by **PCR-DGGE** targeting the 16S rRNA gene. Bacterial 16S rRNA gene was amplified by PCR using universal primers 357F (5’ -CTCCTACGGGAGGCAGCAG- 3’) (Turner *et al.*, 1999) and 907R (5’ -CCGTCAATTCCTTTRAGTTT- 3’) (Lane, 1991a). A 41 base pair GC clamp was

added to the 5' end of the forward primer for DGGE analyses. PCR reactions were prepared in a total volume of 50 µL containing the following: 1× PCR buffer (Tris-HCL, KCl, 1.5 mM $MgCl_2$ and $(NH_4)_2SO_4$); 0.5 mM $MgCl_2$; 0.2 mM deoxynucleotides triphosphate; 1× Q Solution; 0.2 µM of each primer; 0.1 U of Taq polymerase. All chemicals and reagents were provided by Qiagen®. PCR amplification reactions were done in a Gene Amp® 2700 thermal cycler (Applied Biosystems) and consisted of an initial denaturation step at 94ºC for 4 min, followed by 10 cycles of 30 sec at 94ºC, 45 sec at 52ºC and 45 sec at 72ºC and another 15 cycles only changing the annealing temperature at 50ºC. PCR products were checked by electrophoresis in agarose gels.

Approximately, 100 ng of PCR amplified DNA was loaded on loaded on 6% (v/v) acrylamide–bis-acrylamide gels with a 35-65% urea–formamide denaturing gradient. DGGE analyses were performed using an INGENY phorU® system (Ingeny, The Netherlads). Known standards consisting of PCR amplified products of the microorganisms *Micrococcus luteus*, *Pseudomonas fluorescens*, *Sulfolobus acidocaldarius*¸ *Saccharomyces cerevisiae* and *Mucor* sp. were loaded at equidistant positions and used for comparison and standardization of DGGE bands in gels. Electrophoreses were run for 15 hours at 160 volts and 60ºC. Gels were stained with SYBR® Gold (Invitrogen, molecular Proves) for 45 minutes and visualized in a Herolab UVT-20M. Analysis of gel images and calculations was performed with the GelCompar II v.6.1 software. Intense and differential DGGE bands were excised using a sterile scalpel, purified using the QIAquick PCR purification Kit (Qiagen) and re-amplified by PCR using the above mentioned primers and conditions. Primer 357F was used without the GC clamp. 16S rRNA gene sequences were obtained in the reverse direction using the 907R primer from Macrogen (www.macrogen.com). Identification of the microbial species was done after a Basic Local Alignment Search Tool (BLAST) from the National Centre for Biotechnology Information (NCBI, USA) of the obtained representative sequences. Environmental sequences databases were excluded from BLAST searches except when needed (identification of uncultured microbial groups).

Pyrosequencing was applied in Chapter 5. DNA was extracted using Fast DNA® SPIN Kit for soil using conditions provided by the manufacturer. The DNA concentration was quantified using a Qubit 12.0 Fluorometer (Life Technologies Ltd., Paisley, UK). The bacterial 16S rRNA gene amplicon sequences were obtained using the bTEFAP method by 454GL FLX technology at the Research and Testing Laboratory (http://www.researchandtesting. com) (Dowd *et al.*, 2008) using primer set 341F-907R (Muyzer *et al.*, 1998). Primer sequences were modified to contain a 454 FLX Titanium Lib adapter and sample-specific barcode sequences (8bp) using standard protocols.

Raw sequence reads were trimmed to 200 bp and quality-filtered using the split-libraries.py implemented in QIIME (Quantitative Insights Into Microbial Ecology) (Caporaso *et al.*, 2010). High quality sequences were checked for chimeras using UCHIME and distributed into operational taxonomic units (OTUs) at a 97% level using either the UPARSE (Edgar, 2013). Representative sequences for each OTU cluster were identified and assigned taxonomy with reference to the Greengenes 16S rRNA gene database (release May 2013). OTU tables containing read counts and taxonomic assignments were generated using the script make_otu_table.py. OTUs containing less than 4 sequences in the whole dataset were removed to avoid potential effects of spurious diversity due to sequencing errors. When necessary, identification of relevant OTUs was done after screening in BLAST (NCBI) of the obtained representative sequences.

T-RFLP analysis was performed in Chapter 7. The graphite electrode rods were cut into pieces and stored at -20°C at the end of the experiments. DNA was extracted with the NucleoSpin Soil kit (Macherey-Nagel) following the manufacturer's instruction for genomic DNA purification from soil (lysis buffer 2 for DNA extraction of biofilms, lysis buffer 1 for DNA extraction of wastewater, sample lysis with FastPrep (Thermo Fisher Scientific) speed 4 for 20 sec). The final elution step was performed with 50 µL of elution buffer. The PCR was performed with the

primer set UniBac27f (FAM labeled) and Univ1492r targeting the 16S rRNA gene of bacteria (Lane, 1991b).

The PCR MasterMix contained 6.25 µL enzyme mix (MyTaq HS Red Mix, 2x, Bioline, Germany), 0.5 µL of each primer (MWG Biotech, Germany), 4.75 µL nuclease-free water, and 1 µL genomic DNA (about 20 ng). The PCR cycle parameters were as follows: 60 sec at 95ºC, 34 cycles of 15 sec at 95ºC, 15 sec at 54ºC, and 120 sec at 72ºC, followed by a 20 min extension at 72ºC. Afterwards, the PCR product was purified using Sure Clean (Bioline, Germany). For T-RFLP, the labeled PCR products were digested with the restriction endonucleases RsaI and HaeIII at 37ºC for 1 h, followed by product precipitation and T-RFLP analysis using an ABI PRISM Genetic Analyser 3130xl (Applied Biosystems, Germany) and MapMarker 1000 (BioVentures Inc., USA). For sequencing, unlabelled PCR products were cloned with Qiagen PCR Cloning Kit (Qiagen, Germany) following the manufacturer's instruction. Clones were selected for further PCR amplification using M13 primers (cycle parameters: 1 min at 958C and 25 cycles of 15 s at 958C, 15 s at 558C and 2 min at 728C followed by a 10 min extension step at 728C). Finally, 257 clones were investigated for their sequences and terminal restriction fragment (T-RF) length by using an ABI PRISM Genetic Analyser 3130xl (Applied Biosystems, Germany).

3.4.3. Quantification of the microbial community

Quantitative PCR (qPCR) analysis of bacterial 16S rRNA gene abundance was performed in Chapter 5. Quantification was done by targeting the 16S rRNA gene using the primers 341F (Muyzer *et al.*, 1998) and 534R (López-Gutiérrez *et al.*, 2004). Twenty-microliter reaction mixtures contained 1x SYBR green master mix, 1 µg μl^{-1} bovine serum albumin (BSA), and 10 ng of DNA. Primer concentrations were set to 1 µM. Reactions were performed in a 7500 Real Time PCR system (Applied Biosystems, USA) using the SYBR Green PCR Mastermix. qPCR amplification consisted of 120 sec at 50 ºC for carryover prevention, 900 sec at 95 ºC for enzyme activation, followed by 40 cycles of 15 sec at 95ºC for denaturation, 30 sec at 60ºC

for annealing and 35 sec at 72ºC for extension. For a final data acquisition step, the annealing temperature progressively decreased by 1 ºC down to 50 ºC. Standard curves were obtained using serial dilutions (10^2 to 10^7 copies) of linearized plasmids. The PCR efficiency ranged between 80 and 100%. Negative controls resulted in undetectable values in all cases. Inhibition tests were performed for each sample. Sample dilution was applied when necessary to avoid inhibition of PCR.

FISH analyses were performed in Chapters 4 and 7. Biofilms were dislodged as mentioned in the analysis of the microbial community composition. After extraction, biofilm samples for FISH determination were fixed with freshly prepared 4% paraformaldehyde in a PBS solution to reduce autofluorescence. Fixed samples were hybridized at 46ºC with 35% formamide. General and specific fluorescent probes were used to characterize the microbial community (Table 3.1). Image quantification of specific probes was performed with Matlab. The number of images evaluated for each compartment was about 15. Microbial population fractions were represented as percentages with respect to quantifications made with the EUB probe (total bacteria). Moreover, the organization of several genera and or microbial groups from inside the biofilms was also observed.

Table 3.1 FISH probes for sample hybridisation in the anode and cathode compartments and external reactor.

Name	Specificity	Probe	Indicator	% Form	Reference
EUBMIX	Bacteria domain	EUB338	Cy5	0-70	Amann *et al.*, 1990
		EUB338II		0-50	Daims *et al.*, 1999
		EUB338III		0-50	Daims *et al.*, 1999
AOB	Ammonia oxidising bacteria	NSO190	Fluos	40	Mobarry *et al.*, 1996
		NSO1225		35	
NOB	Nitrite oxidising bacteria	NIT3	Cy3	40	Wagner *et al.*, 1996
		CompNIT3	-	40	Wagner *et al.*, 1996
		Ntspa663	Cy3	35	Daims *et al.*, 1999
		CompNtspa663	-	35	Daims *et al.*, 1999
ANAMMOX	*Brocadia+Kuenenia*	Amx-0820	Cy3	40	Schmid *et al.*, 2000
	Brocadia	Amx-0223-a		40	
GEOBACTER	*Geobacter sulfurreducens*	SRB385	Cy3	30	Ren *et al.*, 2008
δ-PROTEOBACTERIA	-	GAM42a	Fluos	35	Manz *et al.*, 1992
α-PROTEOBACTERIA	-	ALF969	Fluos	35	Manz *et al.*, 1992
		ALF1B		20	
FIRMICUTES	-	LGC354A	Cy3	35	Meier *et al.*, 1999
		LGC354B		35	
		LGC354C		35	

EUB: Total bacteria; AOB: Ammonia-oxidizing bacteria; NOB: Nitrite-oxidizing bacteria; ANAMMOX: Anaerobic ammonia-oxidizing bacteria.

3.5. Measurements of bacterial diversity

All statistical analyses were performed using SPSS for Windows 19.0 (SPSS1, IBM). Significant differences for the chemical, electrochemical and microbial values between BES configurations and sampled positions were analysed using either parametric or non-parametric tests implemented in QIIME. The parametric tests used were ANOVA and Welch tests with the corresponding Games-Howell post hoc tests. Moreover, Pearson's correlation was calculated, identifying the linear dependence between two variables. In all cases rarefied OTU tables were used in order to standardize the number of sequences in each sample. Rarefaction was done after random subsampling of an equal number of sequences for each sample.

The microbial diversity indices were calculated by QIIME. Community alpha diversity indices: Shannon's (H') index (Eq 3.12), and maximum richness estimator (Chao1) (Eq 3.13). Differences on the complete microbial community structures, both at different sampling points and BES configurations, were inferred from beta-

diversity measures (non-parametric ANOSIM method and Jaccard index (J)) (Eq 3.14).

Shannon index: H' = $\sum - \mathbf{pi}\ \mathbf{ln}\ (\mathbf{pi})$ (Eq 3.12)

where *pi* is the relative abundance of each OTU group of organisms

Chao1 index: $\mathbf{Chao1} = \boldsymbol{S_{obs}} + \frac{\boldsymbol{F_1^2}}{\mathbf{2}\cdot \boldsymbol{F_2}}$ (Eq 3.13)

where *Sobs* is the number of OTUs in the sample, F_1 is the number of singletons (i.e., the number of OTUs with a single occurrence in the sample) and F_2 is the number of doubletons (the number of OTUs with two occurrences in the sample).

Jaccard index: J = $\frac{\boldsymbol{c}}{(\mathbf{a}+\mathbf{b}-\mathbf{c})}$ (Eq 3.14)

where, *a* is the number of OTUs present in sample a; *b* is the number of OTUs present in sample b; and *c* the OTUs present in both, samples a and b.

Chapter 4

Microbiome characterization of MFCs used for the treatment of swine manure

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Microbiome characterization of MFCs used for the treatment of swine manure

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HIGHLIGHTS

- Two MFC configurations treating swine manure were evaluated at long-term.
- Organic matter and nitrogen were removed from swine manure.
- The microbiomes treating complex wastewater were revealed for the first time.

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ABSTRACT

Conventional swine manure treatment is performed by anaerobic digestion, but nitrogen is not treated. Microbial Fuel Cells (MFCs) allow organic matter and nitrogen removal with concomitant electricity production. MFC microbiomes treating industrial wastewaters as swine manure have not been characterized. In this study, a multidisciplinary approach allowed microbiome relation with nutrient removal capacity and electricity production. Two different MFC configurations (C-1 and C-2) were used to treat swine manure. In C-1, the nitrification and denitrification processes took place in different compartments, while in C-2, simultaneous nitrification-denitrification occurred in the cathode. *Clostridium disporicum* and *Geobacter sulfurreducens* were identified in the anode compartments of both systems. *C. disporicum* was related to the degradation of complex organic matter compounds and *G. sulfurreducens* to electricity production. Different nitrifying bacteria populations were identified in both systems because of the different operational conditions. The highest microbial diversity was detected in cathode compartments of both configurations, including members of Bacteroidetes, Chloroflexiaceae and Proteobacteria. These communities allowed similar removal rates of organic matter (2.02–2.09 kg COD m^{-3} d^{-1}) and nitrogen (0.11–0.16 kg N m^{-3} d^{-1}) in both systems. However, they differed in the generation of electric energy (20 and 2 mW m^{-3} in C-1 and C-2, respectively).

1. Introduction

Intensive pig farming during the last decades has significantly increased swine manure production. In 2013, four countries (Germany, Denmark, Spain and France) accounted for more than 54.4% (140 million of heads) of pig production in the European Union. This large number of pigs generates more than 250×10^6 m^3 of swine manure each year [1], which requires treatment before being spilled into the environment. Swine manure is characterized by a high content of organic matter and nitrogen (mainly ammonium), inorganic salts, heavy metals and pathogens, all of which contribute to environmental pollution [2].

Swine manure treatment typically consists of anaerobic digestion of the organic matter, obtaining biogas and a digestate as end-products [3]. Co-digestion has been used as an alternative system to increase biogas production and treat higher amounts of organic matter [4]. However, the nitrogen content is left virtually unchanged in both cases, requiring additional treatment of the effluent, such as ammonia stripping, and invariably increasing energy and operational costs. An ion exchange bioreactor has been recently explored as an alternative process, which produced high

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quality effluents but at high energetic costs and simultaneously generated concentrated nitrogen wastes [5].

Microbial fuel cells (MFCs) are a new sustainable technology used to remove organic matter and nitrogen from wastewater with concomitant electricity generation by microorganisms [6]. In MFCs, exoelectrogenic microorganisms oxidize organic matter in the anode and release electrons and protons. Electrons are transferred to the cathode through an external resistance, while protons diffuse to the cathode chamber through an ionic membrane. At the cathode, electrotrophic microorganisms reduce nitrogen compounds (nitrite or nitrate) to dinitrogen gas [7,8].

MFC technology was mainly applied to the removal of organic matter from urban wastewater, industrial wastewater and landfill leachate [9,10]. Swine manure treatment using MFCs have been poorly investigated. Min et al. [11] demonstrated the feasibility of using MFC technology to generate electricity and simultaneously remove organic matter and ammonia from swine manure. According to Kim et al. [12], unpleasant odors and volatile acids, such as acetate, butyrate and propionate, could be removed from swine manure using a single-chamber MFC. Thereafter, the studies were focused on optimizing chemical and electrical performances of single-chamber MFCs. Ammonium and volatile fatty acids (i.e., acetate) were removed satisfactorily from swine manure [13]. Kim et al. [14] observed that losses of ammonia from the anode chamber were accelerated with electricity generation. These losses occurred mainly due to pH increase at the cathode in a single-chamber MFC. Different MFC configurations and materials were designed and applied in order to remove pH gradients between both chambers [15]. Loop configuration MFC was the optimal to achieve a stable power density from swine manure at long term operation. Moreover, the study of hydraulic retention time (HRT) suggested an optimal HRT of 8 days for both the organic matter removal efficiency and the power density [16]. The latest studies were focused on the comparison between different animal wastes. Pig manure produced higher voltages and power densities than other animal wastes as cow, chicken and duck wastes due to mixed microbial communities [17,18].

Microbial characterization was analyzed in MFCs since the technology was applied. In 2008, Wrighton et al. [19] identified members of the Firmicutes phylum able to produce electricity in the anode of thermophilic MFCs. Two years later, Wrighton et al. [20] provided the first characterization of active bacterial communities in denitrifying cathodes. They concluded that the bacterial community structure corresponded to the cathode nitrate reduction performance. Despite these identifications, a microbiome analysis is required to understand the relationship between the biofilm and the final end product. In 2013, Kouzuma et al. [21] were the first to study anode associated bacterial communities, revealing several electrochemically active bacteria of the family Geobacteraceae that generated electricity in these systems through syntrophic interactions.

To date, the composition of the MFC microbiome in the treatment of swine manure has not been analyzed. In this study, microbiomes of the compartments of two different MFC configurations used for the treatment of swine manure were characterized phylogenetically. Composition of bacterial communities was related to removal capabilities and electricity production of MFCs.

2. Materials and methods

2.1. Swine manure

Swine manure was taken from the food and agricultural research institute (IRTA) of Monells (Spain). It was stored in a refrigerated tank (6 °C) to promote the settling of solids and minimize degradation over time. The supernatant was fed into the anode chambers of the MFCs. Table 1 presents the main characteristics of swine manure supernatant used during the experimental period. The supernatant was characterized by a high organic matter content (2200 ± 665 mg COD L^{-1}, 59% of biodegradability), high nitrogen content (650 ± 40 mg TKN-N L^{-1}, 83% as ammonium), high conductivity (8.6 ± 0.8 mS cm^{-1}) and a solid content of 425 ± 140 mg TSS L^{-1}. These characteristics of swine manure were suitable a priori for its treatment using MFCs.

Table 1
Swine manure characteristics. The results are presented as the means ± standard deviation ($n = 5$).

	Swine manure	Units
pH	8.5 ± 0.3	–
Conductivity	8.6 ± 0.8	mS cm^{-1}
Alkalinity	4745 ± 575	mg $CaCO_3$ L^{-1}
COD_{Total}	2200 ± 665	mg COD L^{-1}
$COD_{Soluble}$	1620 ± 360	mg COD L^{-1}
BOD_5	1302	mg BOD L^{-1}
TKN	650 ± 40	mg TKN–N L^{-1}
NH_4^+	540 ± 50	mg NH_4^+–N L^{-1}
NO_2^-	n.d	mg NO_2^-–N L^{-1}
NO_3^-	n.d	mg NO_3^-–N L^{-1}
N_2O	n.d	mg N_2O–N L^{-1}
TSS	425 ± 140	mg TSS L^{-1}

n.d: not detected.

2.2. Experimental set-up

Two MFC configurations were designed and operated to remove organic matter and nitrogen from swine manure with the concomitant production of bioelectricity. The MFCs consisted of an anode and a cathode placed on opposite sides of a single methacrylate rectangular chamber, separated by an ionic exchange membrane. Anode and cathode compartments were filled with granular graphite (model 514, diameter 1.5–5 mm, EnViro-cell, Germany), which decreased the net anode and cathode compartment volumes to 420 and 460 mL (NAC and NCC, respectively). Swine manure was continuously fed at a flow rate of 2.9 ± 0.1 L d^{-1}, with a hydraulic retention time (HRT) of 0.16 d^{-1}. An internal recirculation loop 34 times faster than the influent flow was placed in the anode and cathode chambers to maintain homogeneous conditions. Two thin graphite rod electrodes of 130×6 mm (Sofacel, Spain) were installed in both compartments. Both rods were connected to an external resistance of 30 Ω to close the electric circuit.

Moreover, an external reactor for nitrification purposes was filled with clay. The net reactor compartment (NRC) volume was 5 L. The reactor was operated at a flow rate of 2.9 ± 0.1 L d^{-1} (HRT of 1.72 d^{-1}). An internal recirculation loop was placed in the reactor and operated at a flow rate 124 times faster than the influent flow.

Fig. 1 shows a schematic diagram of the two configurations used in this study (C-1 and C-2). The C-1 configuration consisted of a MFC coupled to the external nitrifying reactor. Swine manure was fed into the anode for organic matter oxidation. The effluent of the anode was connected to the external aerobic nitrifying reactor for ammonium oxidation. The effluent from the external nitrifying reactor was fed to the cathode for nitrate reduction to dinitrogen gas. The anode and cathode chambers were separated by an anionic exchange membrane (AEM) (AMI-7001, International Membranes Inc., USA) to avoid ammonium diffusion to the cathode. The transport of nitrate through an AEM was assessed in H-type MFCs under open circuit conditions and in closed circuit conditions (current applied 11 mA). In both cases, nitrate diffusion through the membrane was not detected (data not shown). The dissolved oxygen (DO) concentration was controlled in the external reactor at

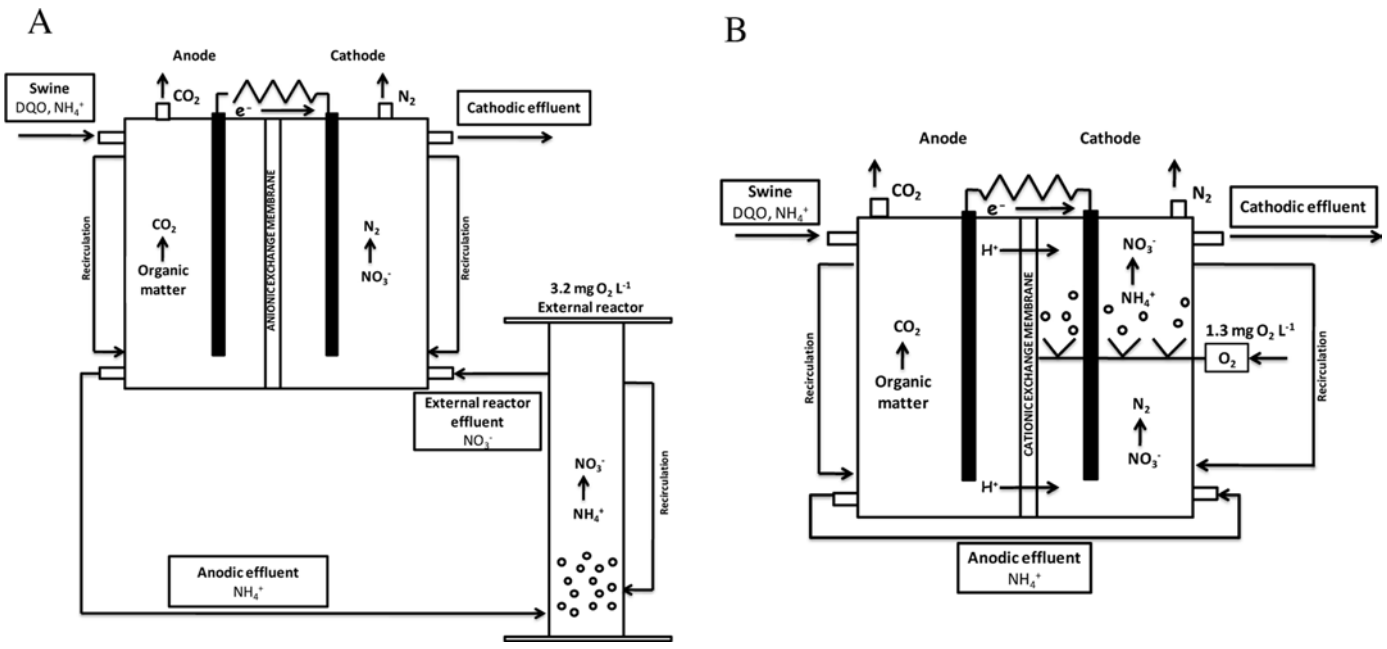

Fig. 1. Schematic diagrams of the two MFC configurations evaluated in this study (C-1 and C-2). (A) C-1 has an MFC with a denitrifying biocathode and external nitrifying reactor. (B) C-2 has an MFC with simultaneous nitrification-denitrification biocathode.

3.2 ± 0.8 mg O_2 L^{-1} using a dissolved oxygen probe (model 5060, Crison, Spain).

Simultaneous nitrification and denitrification (SND) processes occurred inside the cathode of the MFC in the second configuration (C-2). The anode was fed with swine manure as in C-1. The effluent was directly connected to the cathode for nitrogen treatment. The dissolved oxygen concentration was maintained at 1.3 ± 0.3 mg O_2 L^{-1} to ensure SND in the cathode. Aeration was performed at half of the height of the compartment (10 cm from the base) to separate an aerobic (upper) from an anoxic (bottom) zone in the cathode. Two reference electrodes were located at different heights (5 and 15 cm from the base). Anode and cathode compartments were separated by a cationic exchange membrane (CEM) (CMI-7001, International Membranes Inc., USA), which allowed ammonium diffusion through the membrane.

2.3. MFC operation

Anode compartments were inoculated with the anode effluent of a parent MFC treating 300 mg COD L^{-1} of acetate [22]. The external nitrifying reactor of C-1 was inoculated with activated sludge from a partial nitritation reactor treating high ammonium landfill leachate (6000 mg NH_4^+–N L^{-1}) [23]. Cathodes of both configurations were inoculated with effluent from a denitrifying MFC treating nitrate contaminated groundwater [24]. Additionally, in order to achieve nitrification and denitrification in the cathode of C-2, the cathode was also inoculated with C-1 external nitrifying reactor effluent.

2.4. Start-up

The start-up time was divided into two different periods, depending on the feeding of external reactor and cathodes. During 120 days, MFCs compartments were hydraulically disconnected. The first 70 days, first period, the anode compartment was fed with swine manure while the external reactor of C-1 and the cathode compartments of C-1 and C-2 were fed with synthetic media. The external reactor of C-1 and the cathode of C-2 configuration were fed with a synthetic medium amended with ammonium. An identical composition with nitrate instead of ammonium was fed to the cathode of C-1. During this period, nitrogen concentrations (ammonium or nitrate) were gradually increased in the nitrifying reactor and the cathodes from 100 to 500 mg N L^{-1} to reinforce the microbial community.

The second period started (from day 70 until day 120) once nitrification and denitrification efficiencies were above 90%. The percentage of swine manure in the external reactor and cathode chambers was gradually increased from 25% to 100%. At the end of the start-up phase (day 120), the operational conditions were stable and the compartments of the MFCs were hydraulically connected. The continuous operation of MFCs fed at 100% swine manure with the compartments hydraulically connected was evaluated during other 150 days.

2.5. Analyzes and calculations

Standard wastewater measurements for total and soluble organic matter (CODt, CODs and BOD_5), total suspended solids (TSS) and volatile suspended solids (VSS), and nitrogen (total Kjeldahl nitrogen (TKN-N), ammonium (NH_4^+–N), nitrite (NO_2^-–N) and nitrate (NO_3^-–N)) were performed at regular intervals according to the American Public Health Association guidelines [25]. Nitric oxide (NO) production was considered negligible in this study, according to Virdis et al. [7]. Nitrous oxide (N_2O) levels were analyzed using a liquid N_2O microsensor (N_2O-100, UNISENSE, Denmark). The total nitrogen removed was calculated by closing the nitrogen mass balance according to the methodology of Virdis et al. [7]. Concentrations of free ammonia (FA) and free nitrous acid (FNA) were calculated according to Anthonisen et al. [26]. The pH and conductivity (EC-Meter BASIC 20+ y EC-Meter BASIC 30+, Crison, Spain) were measured in the influent and effluent of the compartments.

Carbon and nitrogen removal rates (kg COD m^{-3} NAC d^{-1} and kg N m^{-3} NCC d^{-1}, respectively) were calculated as the difference between the influent and effluent loading rates. Anode and cathode Coulombic efficiencies (CE) were calculated as in Virdis et al. [6]. Nitrogen reduction at the cathode was determined according to Clauwaert et al. [27]. Nitrification rates were determined as the dif-

ference between the influent and effluent TKN rates in the external reactor of C-1 and the cathode of C-2.

Cell potential (V) in both MFC configurations was monitored at 60 s intervals using an on-line multimeter (Alpha-P, Ditel, Spain) with a data acquisition system (Memograph® M RSG40, Endress + Hauser, Germany). Current (I) and power (W) were determined according to Ohm's laws ($I = V/R$; $P = I \times V$). Power and current densities were calculated by dividing power and current by the net cathode volume. Anode and cathode potentials were monitored with Ag/AgCl reference electrodes (+0.197 V vs SHE, model RE-5B, BASi, United Kingdom). Polarization curves were performed using a potentiostat (model SP50, Bio-logic, France) and by imposing a linear potential decrease of 1 mV s^{-1} from open circuit voltage (OCV) to a cell voltage of 0 mV, followed by a linear voltage increase of 1 mV s^{-1} to the original OCV value.

2.6. Microbial community analyzes

Microscopic and molecular techniques were applied to provide a complete description of the microbial community. Graphite samples were taken from the different compartments for microbial analysis when steady state conditions were achieved. An integrated sample was collected from the anode and cathode compartments of both configurations (C-1 and C-2). Samples were extracted at three different distances from the inlet section (5, 40 and 90 cm) in the external reactor.

Scanning electron microscopy (SEM) analyzes were performed with intact biofilms. Graphite samples from the anodes and cathodes were collected and immediately immersed in 2.5% (w/v) glutaraldehyde in a 0.1 M cacodylate buffer at pH 7.4. The samples were washed and dehydrated successively in an ethanol series. Fixed samples were dried with a critical-point drier and sputtered with a 40 nm gold layer. The coated samples were examined with a SEM (model DSM-960; Zeiss, Germany) at 20 kV, and the images were captured digitally.

Biofilm attached to the graphite surface was dislodged in an ultrasonic bath (P-Selecta) in 1 cycle of 60 s followed by 120 s of centrifugation at 4000 rpm. Dislodged biofilms were analyzed by fluorescence in situ hybridization (FISH) and polymerase chain reaction – denaturing gradient gel electrophoresis (PCR– DGGE). After extraction, biofilm samples for FISH determination were fixed with freshly prepared 4% paraformaldehyde in a PBS solution to reduce autofluorescence. Fixed samples were hybridized at 46 °C with 35% formamide. General and specific fluorescent probes were used to characterize the microbial community (Table A1). Image quantification of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) probes was performed with Matlab. Approximately 15 images were evaluated for each compartment. As a result, the average fractions of microorganisms or families present in the corresponding samples were obtained. Microbial population fractions are represented as percentages with respect to quantifications made with the EUB probe. The sonication process can partly destroy the original biofilm architecture. Despite this, different flocs were preserved and fixed in order to distinguish the internal organization of the biofilms.

Nucleic acids were extracted for fingerprinting analysis (PCR–DGGE) using the Fast DNA® SPIN Kit for soil (MP, Biomedicals) according to the manufacturer's instructions. Bacterial 16S rRNA gene was amplified by PCR using universal primers 357F (5′ -CTCCTACGGGAGGCAGCAG- 3′) [28] and 907R (5′ -CCGTCAATTCCTTTRAGTTT- 3′) [29]. PCR products were analyzed by DGGE according to the method described by Prat et al. [30]. A 35–65% urea-formamide denaturing gradient was used. Jaccard similarity coefficients based on the presence/absence of defined DGGE bands were calculated pairwise and used to compare sample fingerprints. Analysis of gel images and calculations was performed with the GelComparII v.6.1 software. Intense and differential DGGE bands were excised, purified and re-amplified by PCR using the above mentioned primers and conditions. 16S rRNA sequences were obtained in the reverse direction using the 907R primer from Macrogen (www.macrogen.com). The sequences presented in this study have been submitted to the GenBank database with accession numbers KM359447–KM359476.

3. Results

3.1. Swine manure treatment

Once the start-up procedure finished, the two MFC configurations fed with 100% swine manure and hydraulically connected were studied during 150 days. The organic matter removal rate of both configurations was 2.02–2.09 kg COD m^{-3} d^{-1} (Summarized data in Table 2, evolution data in Fig. A1). Despite this high organic matter removal rate, both configurations presented low exoelectrogenic activity with an anodic CE of 24% and 5% in C-1 and C-2, respectively. Methane was not detected in the anode effluents, indicating that methanogenesis did not occur.

Table 2
Variation of parameters related to organic matter, nitrogen and electricity in both systems (C-1 and C-2).

Process	Analysed parameter	C-1	C-2	Units
Organic matter oxidation	Compartment	Anode	Anode	–
	Organic matter removal rate	2.09 ± 0.76	2.02 ± 0.57	kg COD m^{-3} d^{-1}
	Coulombic efficiency	24 ± 9	5 ± 4	%
	Anode potential	−235 ± 35	-420 ± 40	mV vs SHE
	HRT	0.16	0.16	*d*
Nitrification	Compartment	External reactor	Upper cathode	–
	Nitrification rate	0.26 ± 0.06	1.26 ± 0.29	kg N m^{-3} d^{-1}
	Nitrification efficiency	95 ± 3	49 ± 19	%
	Nitrite production	0.00 ± 0.00	0.31 ± 0.14	kg N m^{-3} d^{-1}
	Dissolved oxygen concentration	3.0	1.3	mg O_2 L^{-1}
	Cathode potential	n. a.	212 ± 51	mV vs SHE
	HRT	1.7	0.16	*d*
Denitrification	Compartment	Cathode	Lower cathode	–
	Nitrogen removal	0.16 ± 0.06	0.11 ± 0.05	kg N m^{-3} d^{-1}
	Nitrogen removal efficiency	7 ± 3	22 ± 10	%
	Nitrite production	0.00 ± 0.00	0.31 ± 0.14	kg N m^{-3} d^{-1}
	Coulombic efficiency	10 ± 4	n.d.	%
	Cathode potential	190 ± 105	40 ± 60	mV vs SHE
	HRT	0.16	0.16	*d*
Electricity production	Power density	20	2	mW m^{-3}

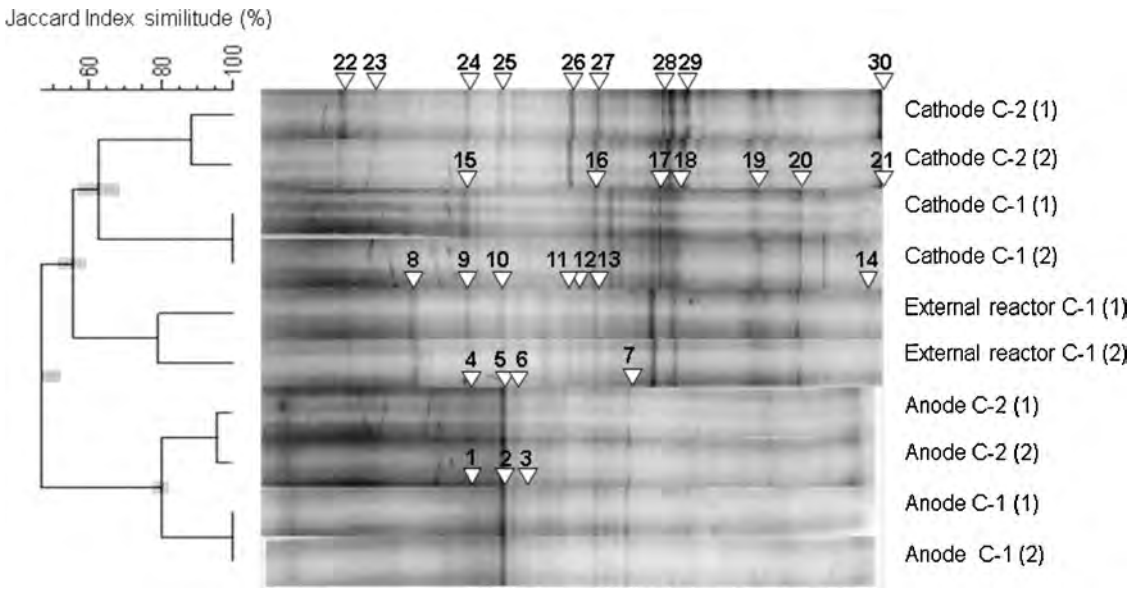

Fig. 2. Negative image composition of Sybr Gold® stained DGGE gels of partial 16S rRNA gene fragments. The dendrogram on the left is based on a Jaccard similarity matrix (presence/absence of bands). Samples were grouped using the UPGMA method. Each compartment (C-1 anode, C-1 external reactor, C-1 cathode, C-2 anode and C-2 cathode) was analysed in duplicate (sub index 1 or 2).

Nitrification activities were different in both configurations. The nitrification rate of C-1 was 0.26 ± 0.06 kg N m^{-3} d^{-1} (Table 2). Despite this low rate, the nitrification process was complete, with the total ammonium content of swine manure oxidized to nitrate. In C-2, where simultaneous nitrification and denitrification (SND) took place in the cathode, the nitrification rate was 1.26 ± 0.29 kg N m^{-3} d^{-1}. However, nitrite was accumulated at a rate of 0.31 ± 0.14 kg NO_2^-–N m^{-3} d^{-1}, revealing unbalanced nitrification and denitrification activities. The high pH value of swine manure (8.5 ± 0.3) corresponds to free ammonia concentrations of 17 ± 6 mg N–NH_3 L^{-1} in C-1 (external reactor) and 62 ± 24 mg N–NH_3 L^{-1} in C-2 (SND cathode). The tolerances of AOB and NOB were below the estimated FA concentrations [26].

The performances of both MFCs were similar in terms of nitrogen reduction capacity. The denitrification rates of C-1 and C-2 were 0.16 ± 0.06 kg N m^{-3} d^{-1} and 0.11 ± 0.05 kg N m^{-3} d^{-1}, respectively. Neither nitrite nor nitrous oxide was detected in the cathode of C-1. In the SND of the C-2 cathode, some nitrite accumulation was observed due to partial nitrification or partial denitrification.

3.2. Electrochemical performances as a function of the cathode design

The electrochemical performances of both anodes were completely different (Table 2). The anode potentials were -235 ± 35 and -420 ± 40 mV vs SHE for C-1 and C-2, respectively. These results are linked with the different CE achieved. A lower anode potential corresponded to a lower CE. In contrast, the cathodes of C-1 and C-2 followed a similar trend. Cathode potential in C-1 was $+190 \pm 105$ mV vs SHE, while the cathode potentials in C-2 were different in the aerobic (upper) and anoxic (bottom) zones. The zone below aeration had a cathode potential near $+40 \pm 60$ mV vs SHE mV vs SHE. In the upper zone, the cathode potential reached $+212 \pm 51$ mV vs SHE.

The differences in terms of anode and cathode potentials in both MFCs were linked with different energy production. The electricity production for the fully anoxic cathode (C-1) was 20 mW m^{-3}, ten times higher than the energy production calculated for the SND cathode (C-2; 2 mW m^{-3}).

3.3. Characterization of the MFC microbiomes

3.3.1. Anode biofilms

The anode biofilm presented a complex organization with a dominance of rod-shaped and filamentous cells tangled together, as shown by SEM analysis (Fig. A2). The aggregates were firmly attached to the electrode surface by the use of filamentous appendages. According to the results obtained by FISH, members of Firmicutes and alpha-, gamma- and delta-Proteobacteria (*Geobacter sulfurreducens*) were detected. Moreover, the microbial community of the anodes was analyzed by PCR–DGGE (Fig. 2). The obtained fingerprints were mainly composed of a dominant band and exhibited an apparent low diversity. The anode microbial communities of C-1 and C-2 were fairly similar (>80% similarity, Jaccard index). Sequences from DGGE bands 1–3 (C-1) and 4–6 (C-2) were found to be very similar (99%) to *Clostridium disporicum* (Firmicutes) (Table 3).

3.3.2. Nitrifying microorganisms

SEM images showed that the external reactor in C-1 and the cathode in C-2 presented a complex biofilm organization, as was observed in the anodes (Fig. A2). Using the FISH technique and Matlab post-analysis were used to detect and quantify the presence of AOB and NOB at three different heights from the inlet section in C-1, external reactor and C-2 cathode (Table A2). Relative abundances of AOB and NOB showed an opposite trend according to distance from the inlet in C-1. AOB decreased from 40% (5 cm above the influent zone) to 12% (90 cm), whereas the relative abundance of NOB increased from 2 to 10% at the same positions. Conversely, the cathode of C-2 with SND showed a similar percentage of AOB (38%), but fewer NOB (2%), than the influent zone of the external reactor of C-1. Moreover, FISH images allowed the observation of the distribution of selected bacterial groups. AOBs were distributed in the outer part of the biofilm where dissolved oxygen and ammonium were more available. Instead, NOBs were located in the inner part of the biofilm surrounded by AOB (Fig. 3).

DGGE profiles of cathode biofilms showed the presence of a more diverse microbial community compared to that found in the corresponding anodes. The nitrifying community of the external reactor (C-1) and cathode (C-2) showed significant differences,

Table 3
Most probable identification of microbial community members according to 16S rRNA sequences obtained from PCR–DGGE. Numeric codes for DGGE bands are identified in Fig. 2. GenBank accession numbers are provided in parentheses. Similarity values to the nearest identified species are given according to the best hit from blast analysis (reference RNA sequences database).

Phylum	Most probable identification	MFC-1						MFC -2			
		Anode		External reactor		Cathode		Anode		Cathode	
		Band number	(%) Sim.	Band number	(%) Sim.	Band number	% Sim.	Band number	% Sim.	Band number	% Sim.
Actinobacteriaceae	*Mycobacterium chelonae*					17 (KM359463)	99				
Bacteroidetes	*Fulvivirga kasyanovii*			8 (KM359454)	85						
	Ferruginibacter alkalilentus									22 (KM359468)	93
Chloroflexiaceae	*Sphaerobacter thermophilus*			14(KM359460)	90	21 (KM359467)	88			30 (KM359476)	90
	Longilinea arvoryzae					19 (KM359465)	83				
Deinococcaceae	*Truepera radiovictrix*					18 (KM359464)	88			28 (KM359474)	89
						20 (KM359466)	88			29 (KM359475)	88
Firmicutes	*Clostridium disporicum*	1 (KM359447)	99	9 (KM359455)	97	15 (KM359461)	96	4 (KM359450)	98	24 (KM359470)	98
		2 (KM359448)	99					5 (KM359451)	98		
		3 (KM359449)	98					6 (KM359452)	99		
Proteobacteria	*Cupriavidus* sp.							7 (KM359453)	99		
	Nitrobacter alkalicus			11 (KM359457)	95					25 (KM359471)	93
				13 (KM359459)	94					26 (KM359472)	99
										27 (KM359473)	98
	Uncultured *nitrosospira* sp.			10 (KM359456)	93						
	Nitrosomonas europaea									23 (KM359469)	99
	Ralstonia mannitolilytica					16 (KM359462)	93				
	Schlegelella thermodepolymerans			12 (KM359458)	91						

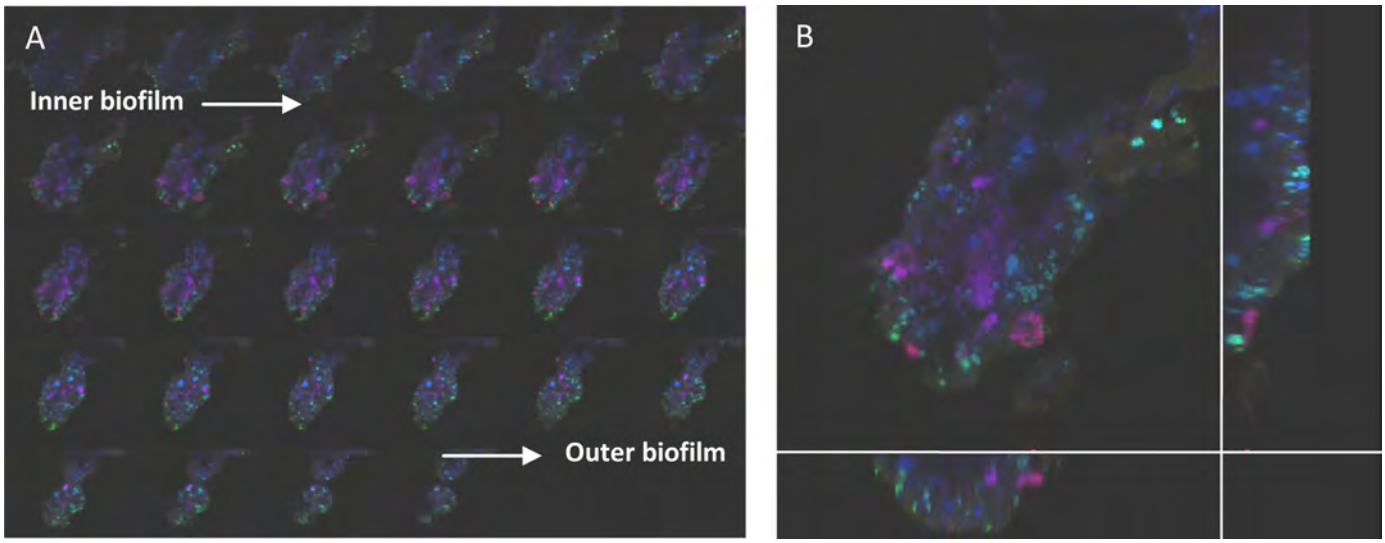

Fig. 3. Confocal laser micrographs for AOB in blue-green (NSO190 and NSO1225 probes), NOB in pink (NIT3, CompNIT3, Ntspa663 and CompNtspa663 probes) and EUB in dark blue (EUB338, 338-II and 338-III probes). (A) Sequences of images of the biofilm on the *Z*-axis (from the inner to the outer surface of the biofilm). (B) Compilation of images obtained from the external reactor of C-1 at day 118 of the study. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

both at the fingerprinting level (55% similarity, Jaccard index) and according to the identified bacteria (Table 3). The nitrifying community at the cathode of C-1 was composed of Betaproteobacterium *Nitrosospira* sp. (DGGE band 10) as the main AOB and Alphaproteobacterium *Nitrobacter alkalicus* (DGGE bands 11 and 13) as the main NOB bacteria. Conversely, AOB populations varied in the C-2 cathode (DGGE band 23 showing a high similarity to *Nitrosomonas europaea*), whereas the NOB populations remained unchanged (DGGE bands 25–27).

3.3.3. Denitrifying microorganisms

The denitrification process took place in the cathodes of both configurations. SEM images showed microorganisms with similar morphologies to those observed in the anodes and the external nitrifying reactor (Fig. A2). Members of the Firmicutes, and the alpha-, gamma-, and delta-Proteobacteria(*G. sulfurreducens*) were detected by FISH. Finally, 16S rRNA PCR–DGGE fingerprints showed that the microbial diversity on the cathodes was higher than in the other compartments (Fig. 2). Jaccard indices of the C-1 and C-2 cathode samples showed 65% similarity. Sequences from DGGE band 22 showed high similarity to a Bacteroidetes species, bands 15 and 24 belonged to Firmicutes, bands 16, 23 and 25–27 to the Proteobacteria and bands 19, 21 and 30 to the Chloroflexaceae, although the latter group could not be identified at the species level according to the sequences obtained (Table 3).

4. Discussion

4.1. The microbiome of exoelectrogenic communities

The anode compartments of the two configurations achieved similar organic matter removal rates (approximately 2 kg COD m^{-3} d^{-1}) by treating swine manure. Low CEs observed were in accordance with other studies using MFCs for swine manure treatment. For example, Min et al. [11] observed a CE of 8%. Organic matter could be used for other biological processes, such as methanogenesis, fermentation, or bacterial growth, simultaneously with the current generation. Methane was not detected throughout the experimental period, indicating that methanogenesis did not have a significant impact on organic matter depletion. In contrast, fermentation processes could be implicated according to the putatively fermentative bacteria identified in the anodes, such as *C. disporicum*.

Similar microbiomes were identified in both anodes (Firmicutes, alpha-, gamma- and delta-Proteobacteria phylums). A single population of *C. disporicum* clearly dominated the microbial community in both systems. *C. disporicum* is a fermentative bacterium able to grow on complex organic macromolecules [31]. Its presence was related to swine manure because it was not found in the compartments when they were feeding with synthetic media. Although no analysis of the manure used in this study was performed, an identification of the species present in similar batches of swine manure done by 16S rRNA amplicon pyrosequencing, revealed the conspicuous presence of *Clostridium* spp. at relatively high abundances (14–22%) (unpublished data).

Despite the similar removal capacity, CE showed significant differences (24% in C-1 compared to 5% in C-2). These results were in accordance to the higher electricity production measured in C-1, compared to C-2. *G. sulfurreducens* was present in both designs. This exoelectrogenic bacterium could be responsible of electricity production from organic matter oxidation [32]. However, the role of *C. disporicum* in the electrogenic process remained elusive, even though its abundance and persistence in the studied systems suggested an active role. Despite this, the identification of other exoelectrogenic bacteria or differences in the proportions between exoelectrogenic and non-exoelectrogenic bacteria could not be estimated, probably due to the high abundance of *C. disporicum* in the system that might mask the detection of bacterial representatives at lower relative concentrations.

However, the difference in electricity productions and Coulombic efficiencies were not only linked to the microbiome. Differences in cathode configuration and ionic exchange membrane selection could cause divergence in these parameters. In the aerated cathode of C-2, oxygen diffused through the membrane to the anode where could be used as an electron acceptor, decreasing the CE.

Different membranes were chosen in order to avoid or allow the ammonium diffusion through the membrane. In C-1, the anode and cathode compartments were separated by an AEM, while in C-2 they were separated by a CEM. The implementation of different membranes in the configurations was done considering the future applicability of the design, taking into account the knowledge achieved by Virdis et al. in 2008 [6]. Higher electrical production

with AEM was in agreement with the study of Kim et al. [33] that observed that the use of an AEM allowed larger power densities than a CEM. Moreover, power densities measured during wastewater treatment were in the range of values observed by other authors. Two different MFC reactors described by Virdis et al. [6] produced 13.6 and 22.5 mW m^{-3}, respectively (at a resistance of 50 Ω).

4.2. The microbiome of electrotrophic communities

The SND cathode (C-2) achieved a higher ammonium oxidation rate of 1.26 ± 0.29 kg N m^{-3} d^{-1} compared to the external reactor (C-1). The relative abundance of AOB was similar in both configurations (approximately 40%). The different AOB detected in both configurations could be related to the partial accumulation of nitrite in C-2. *Nitrosospira* sp. and *N. europaea* were identified in the external reactor of C-1 and cathode of C-2, respectively. Although it was not proven experimentally in this work, the observed divergence in bacterial AOB in the two systems could be a consequence of changes in the inert support (clay and graphite, respectively) and/or the operational dissolved oxygen concentration (3.2 ± 0.8 and 1.3 ± 0.3 mg O_2 L^{-1}, respectively). The same NOB species (*N. alkalicus*) was found in both compartments. In both cases, nitrifying communities (AOB and NOB) were inhibited by the presence of FA according to the inhibition ranks described by Anthonisen et al. [26]. *N. europaea* and *N. alkalicus* inhibition ranks were 10 and 0.1–1 mg $N–NH_3$ L^{-1}, respectively. FNA concentrations were lower than the inhibition rank due to the pH. Thus, nitrite oxidation activity was not inhibited.

Microbial organization of AOB and NOB in the nitrifying reactor was studied as a function of the flow direction and biofilm distribution. AOB and NOB distributed according to the concentration gradient. High ammonium concentration in the inlet zone positively impacted on AOB, instead for NOB was related with nitrite. Progressively, relative abundance of AOB tended to decrease while NOB and nitrite concentration increased toward the outlet. This biofilm distribution showed a microbial organization based on oxygen and resource (ammonium or nitrite) availability. NOB microorganisms were organized inside the biofilm, surrounded by AOB in order to optimize the nitrification process.

Similar nitrogen removal rates were exhibited in the cathodes of both configurations. C-1 and C-2 presented less similarity between the cathode microbial communities than those observed in the anodes, probably due to difference in the operational conditions. Denitrification occurred by electrotrophic microorganisms that triggered the biological reduction of nitrate to dinitrogen gas under anoxic conditions [27]. Nitrate reduction depends on the activity of chemolithoautotrophic nitrate reducers, which are phylogenetically diverse (over 60 genera). Most of these bacteria are able to use other compounds as electron donors (sulphide, hydrogen, or reduced iron compounds) for denitrification [34]. Some families with autotrophic denitrifiers belonging to the Bacteroidetes, the Chloroflexaceae and the Proteobacteria were found in both systems. Additionally, simultaneous processes in the cathode of C-2 allowed the establishment of species with the ability to perform different reactions. *N. europaea* was a clear example of an ammonia oxidizer that was also able to reduce nitrite to nitrous oxide [35].

5. Conclusions

The study confirmed that MFCs successfully treated swine manure. Applied operational conditions influenced the prevailing microbiome and organic matter and nitrogen removal capacities.

Anodes of both designs (C-1 and C-2) showed similar organic removal rates (2.02–2.09 kg COD m^{-3} d^{-1}). Microbiome was composed by *C. disporicum*, which was related to swine manure and *G. sulfurreducens* was involved in current production.

Different nitrification rates were linked to diverse AOB microbial composition. *N. europaea* was present in cathode of C-2, nitrifying faster (1.26 ± 0.29 kg N m^{-3} d^{-1}) than *Nitrosospira* sp. in the external reactor of C-1 (0.26 ± 0.06 kg N m^{-3} d^{-1}).

Similar denitrification rates in the cathode chambers were observed (0.11–0.16 kg N m^{-3} d^{-1}), which were linked to similar diversity of microorganisms observed in them (Bacteroidetes, Chloroflexiaceae and Proteobacteria).

C-1 system produced ten times more electricity than C-2 due to differences in cathode operational conditions and ionic exchange membrane selection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2015.02.014.

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Supporting information for manuscript

Appendix A

Microbiome characterisation of MFCs used for the treatment of swine manure

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Summary:

Fig. A1. Organic matter and nitrogen removal rates and electricity production during the experimental study (150 days).

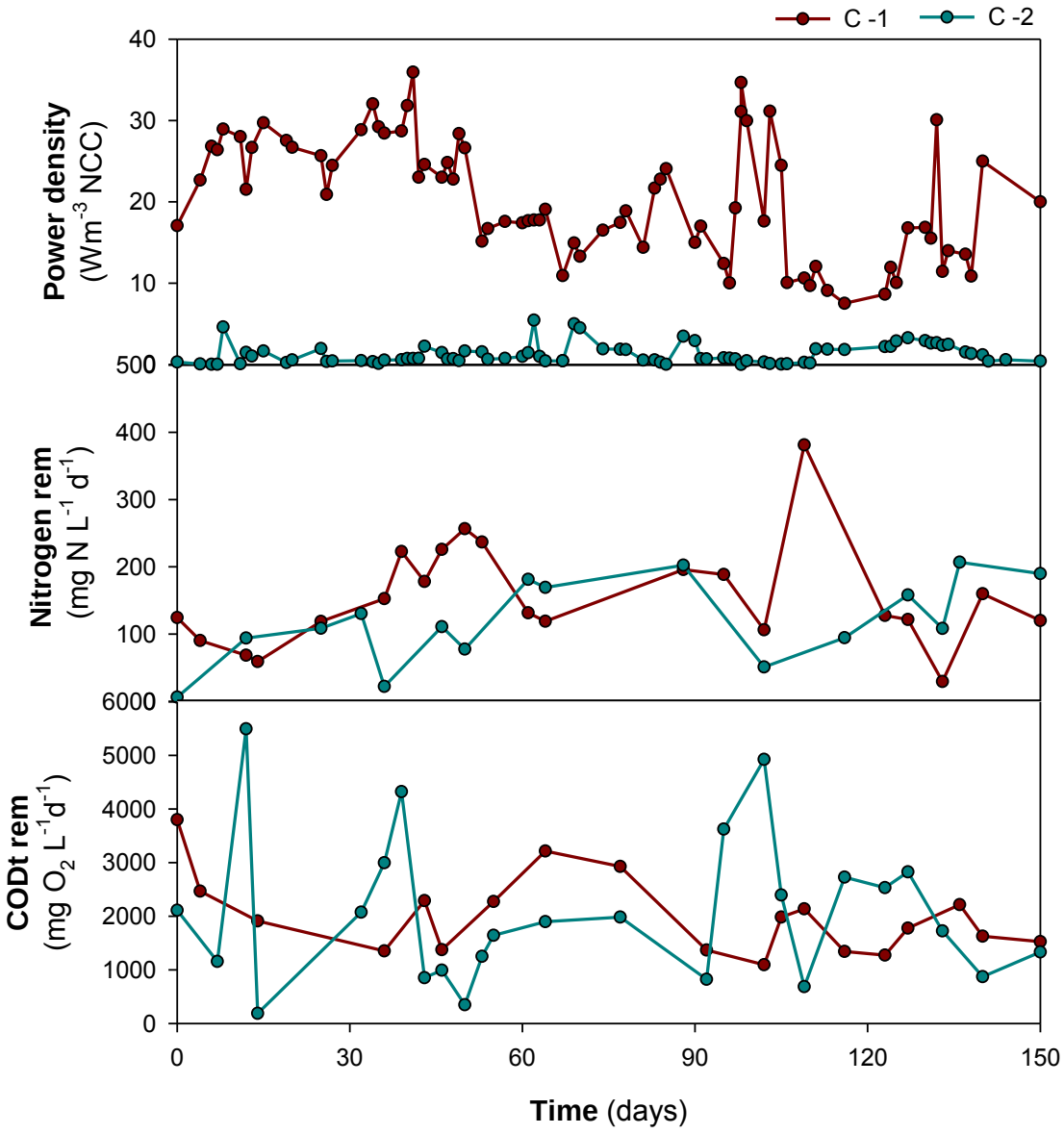

Fig. A.2 SEM analyses of the different compartments of C-1 and C-2.

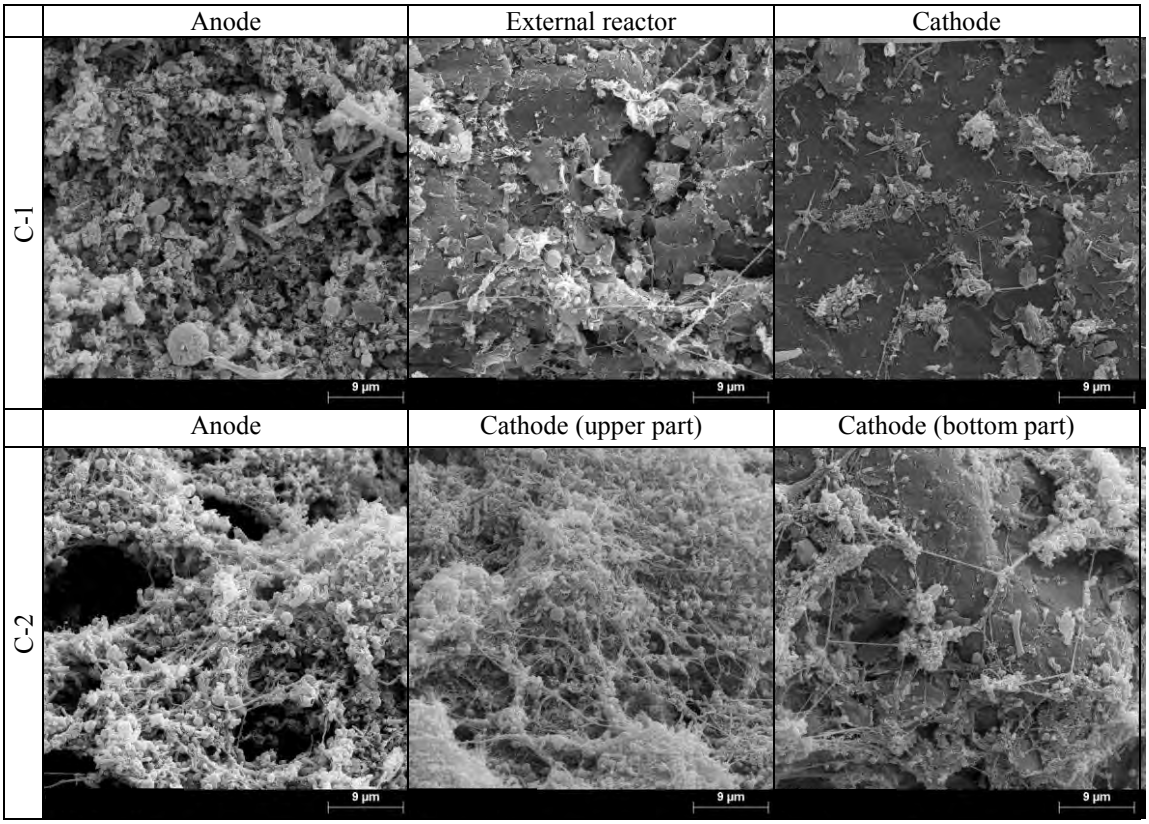

Table A.1 FISH probes for sample hybridisation in the anode and cathode compartments and external reactor of configurations of C-1 and C-2.

Probe	Organism	Sequence (5'->3')	Long	Indicator	% Form	Reference
EUB338	Eubacteria	GCT GCC TCC CGT AGG AGT	18	Cy5	0-70	[1]
EUB338II	*Planctomyces branch*	GCA GCC ACC CGT AGG TGT	18	Cy5	0-50	[2]
EUB338III	*Verrucomicrobia*	GCT GCC ACC CGT AGG TGT	18	Cy5	0-50	[2]
NSO190	Ammonia oxidising β-Proteobacteria	CGA TCC CCT GCT TTT CTC C	19	Fluos	40	[3]
NSO1225	Ammonia oxidising β-Proteobacteria	CGC CAT TGT ATT ACG TGT GA	20	Fluos	35	[3]
NIT3	*Nitrobacter*	CCT GTG CTC CAT GCT CCG	18	Cy3	40	[4]
CompNIT3	Competitors *Nitrobacter*	CCT GTG CTC CAG GCT CCG	18	-	40	[4]
Ntspa663	*Nitrospira*-like organism	GGA ATT CCG CGC TCC TCT	18	Cy3	35	[2]
CompNtspa663	Competitors *Nitrospira*-like organism	GGA ATT CCG CTC TCC TCT	18	-	35	[2]
SRB385	*Geobacter sulfurreducens*	CGG CGT YGC TGC GTC AGG	18	Cy3	30	[5]
GAM42a	Gamma Proteobacteria	GCC TTC CCA CAT CGT TT	17	Fluos	35	[6]
ALF969	Some α-Proteobacteria	TGG TAA GGT TCT GCG CGT	18	Fluos	35	[6]
ALF1B	α-Proteobacteria, some δ-Proteobacteria	CGT TCG YTC TGA GCC AG	17	Fluos	20	[6]
LGC354A	Firmicutes (Gram + Bacteria)	TGG AAG ATT CCC TAC TGC	18	Cy3	35	[7]
LGC354B	Firmicutes (Gram+ Bacteria)	CGG AAG ATT CCC TAC TGC	18	Cy3	35	[7]
LGC354C	Firmicutes (Gram + Bacteria)	CCG AAG ATT CCC TAC TGC	18	Cy3	35	[7]

Table A.2 AOB (NSO190 and NSO1225 probes) and NOB (NIT3, CompNIT3, Ntspa663 and CompNtspa663 probes) abundance percentages in respect to EUB probes (EUB338, 338-II and 338-III probes). FISH image quantification was applied to the samples of the external reactor (C-1) at three different altitudinal levels and at the upper zone of the cathode (C-2).

Compartment		AOB	NOB
External reactor C-1	Upper part	12±2	10±3
	Middle part	15±3	4±2
	Bottom part	40±3	2±1
Cathode C-2	Upper part	38±4	2±1

n.d: non-detected.

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Chapter 5

External resistances applied to MFC affect core microbiome and swine manure treatment efficiencies

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PLOS ONE

RESEARCH ARTICLE

External Resistances Applied to MFC Affect Core Microbiome and Swine Manure Treatment Efficiencies

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Abstract

Microbial fuel cells (MFCs) can be designed to combine water treatment with concomitant electricity production. Animal manure treatment has been poorly explored using MFCs, and its implementation at full-scale primarily relies on the bacterial distribution and activity within the treatment cell. This study reports the bacterial community changes at four positions within the anode of two almost identically operated MFCs fed swine manure. Changes in the microbiome structure are described according to the MFC fluid dynamics and the application of a maximum power point tracking system (MPPT) compared to a fixed resistance system (Ref-MFC). Both external resistance and cell hydrodynamics are thought to heavily influence MFC performance. The microbiome was characterised both quantitatively (qPCR) and qualitatively (454-pyrosequencing) by targeting bacterial 16S rRNA genes. The diversity of the microbial community in the MFC biofilm was reduced and differed from the influent swine manure. The adopted electric condition (MPPT vs fixed resistance) was more relevant than the fluid dynamics in shaping the MFC microbiome. MPPT control positively affected bacterial abundance and promoted the selection of putatively exoelectrogenic bacteria in the MFC core microbiome (***Sedimentibacter*** sp. and gammaproteobacteria). These differences in the microbiome may be responsible for the two-fold increase in power production achieved by the MPPT-MFC compared to the Ref-MFC.

Introduction

Continuous and unsustainable animal production causes an accumulation of undesirable products such as swine manure, which has a complex organic matter and nitrogen (primarily ammonium) content that contributes to environmental pollution. Annually, 1.4 billion tonnes of swine manure are generated in the European Union, where the main contributor country is France [1]. Many technologies have been proposed to treat these pollutants, of which the most commonly employed is anaerobic digestion. However, these processes entail high operation

Competing Interests: The authors have declared that no competing interests exist.

costs, large surface availability and low treatment efficiencies [2,3]. Microbial fuel cells (MFCs) are a newer biological technology for animal wastewater treatment with concomitant electricity production [4,5], and may be able to address limitations of anaerobic digestion.

Interest in MFCs has increased during the last decade, especially regarding their use in scaled-up applications [6–8] for *in situ* swine manure treatment. MFCs will become a recognisable alternative technology for green energy recovery from wastewater treatment in the near future. Scientific reports on MFCs include the evaluation of their nutrient removal capacities [9], power production [10], or microbial community characterization [11], which generally are analysed separately. A simultaneous, multi-disciplinary approach has rarely been performed, although this type of approach may be required to obtain an in-depth understanding and optimize the technology for use.

Microbial communities are essential for the bioelectrochemical processes in MFCs. In the anode chamber, exoelectrogenic microorganisms oxidise organic matter to release electrons to the anode electrode [12]. The exoelectrogenic respiration capacity has been thoroughly studied using model organisms growing on acetate, including *Geobacter sulfurreducens* PCA [13] and *Shewanella oneidensis* MR-1 [14]. The structural and biochemical properties of the different extracellular electron transfer (EET) mechanisms performed by these two strains have been studied [15,16]. However, MFC application to wastewater treatment increases bacterial community complexity, leading to interconnected relationships among the cells that make it difficult to study the exoelectrogenic capacities of each identified strain. In a few studies, identified microorganisms were specifically related to substrate degradation and electricity production in MFCs treating wastewater [17,18]. For example, Velvizhi and Mohan (2015) focused on the identification of EET sites and processes that were linked to the degradation of pharmaceutical wastewater [19].

Additional syntrophic relationships occur in biofilms and may be essential for the degradation of complex organic matrices [20], especially when combined with interspecies electron transfer events [21,22]. The development of structured microbial communities within MFC anodes showed significant advantages compared to pure exoelectrogenic communities in the treatment of complex organic matter matrices, such as urban wastewater [23], feedstock wastewater [24], landfill leachate [25] and more recently swine manure [26]. The reasons for the enhanced performance of complex-structured biofilms relied on the increased resilience of the cells, protection against toxic substances, and closer contact between cells, which might facilitate communication through biochemical signals and assist with nutrient distribution.

New methodologies, such as high-troughput amplicon sequencing [27], PhyloChip analysis [28], flow cytometry [29], and stable isotope probing (SIP) [30], have been applied to identify microorganisms performing specific metabolic processes within complex matrices. In this sense, cultivation independent methods have significantly added to the analysis of relevant taxa that have escaped cultivation so far. However, despite these technical advances, species identification and the determination of their active roles in the biofilm remain difficult. In most cases, indirect comparative methods must be used. Biofilms able to treat complex substrates are usually characterised in terms of the community (microbiome) instead of the individual species. The identification of microbial groups potentially responsible for electricity production in MFCs (i.e., exoelectrogenic groups) can be accomplished by comparing the microbial communities that develop under well-differentiated conditions.

Different control strategies can be applied to improve MFC functionality and enhance the activity of the microorganisms associated with the exoelectrogenic process. Strategies aimed at reaching and maintaining the maximum power point (MPP) of the MFC were developed. The MPP is reached when the MFC internal and external resistances coincide [31]. Its continuous tracking (called MPPT) has achieved several advantages as follows: enhanced exoelectrogenic

activity of microorganisms [32], higher electricity production and coulombic efficiency (CE) [33] and reduced side effects, such as methane production [34]. Nevertheless, the relationship between the structure of the microbial community and electricity production in MPPT-controlled MFCs remains unclear.

In addition to MPPT control, the reactor design is a determinant for fluid dynamics and therefore homogeneity and mass transfer kinetics within the cell [35][36]. Computational fluid dynamics methods can be used to study the fluid distribution but rarely have been applied to MFC research. Few studies are available to assist in reactor design and optimization. Kim et al. (2012) studied the effects of the influent flow rate and substrate concentration on MFC performance and concluded that high flow rates (7.5 mL min^{-1}) correlated with maximum power production (2.7 mW) [37]. Moreover, a recent study by Michie et al. (2014) showed a 40% increase in bacterial abundance when adopting a turbulent flow regime (shear rate of 237 s^{-1}). The microorganism distribution inside the MFCs was not investigated in any of these studies [38].

In this study, different external resistance control strategies were applied to two replicate MFCs fed swine manure. The effect on the development of the exoelectrogenic bacterial community was evaluated to optimise the internal MFC bioprocesses. A MPPT control strategy (based on dynamic resistance) was applied to one of the MFCs and compared with a Ref-MFC operated at fixed resistance. Microbial groups developing in the anode chamber were identified. The comparative analysis of the MFC core microbiomes allowed the identification of bacteria that were potentially responsible for the power generation. Moreover, the internal distribution of the bacterial abundance was analysed and related to the reactor fluid dynamics.

Materials and Methods

Experimental set-up and inoculation

Two replicate, dual-chamber MFCs were constructed and operated to remove organic matter from swine manure with bioelectricity production. Details of the MFC design and operation are described in the study of Molognoni *et al.* (2014). Briefly, each MFC consisted of a methacrylate, rectangular reactor with an anode and cathode placed on opposite sides of an Anionic Exchange Membrane (AMI-7001, International Membranes Inc., USA). Each anode and cathode chamber contained approximately 400 mL of liquid volume (S1 File) [39]. Swine manure collected at an experimental station of the Food and Agricultural Research Institute (IRTA, Girona, Spain) was continuously fed to the anode at a flow rate of 1.5 L d^{-1}. The continuous replacement of fresh swine manure (main characteristics in S1 Table) maintained the organic loading rate (OLR) at 10.5 ± 0.7 kg COD m^{-3} d^{-1} for the entire experimental period (43 days). The cathode was fed an oxygen-saturated inorganic solution (see Supplementary methods). The temperature was kept constant at 21 ± 1°C.

The two MFCs operated under the same hydraulic conditions and differed only in the electrical load application (Fig 1). The MPPT-MFC operated with an automatically controlled resistance, whereas the Ref-MFC operated at a fixed resistance of 30 Ω. The fixed resistance value was chosen to approximate the MFC internal resistance based on previous experience [26].

The anode and cathode chambers of both MFCs were inoculated as described by Molognoni *et al.* [39]. 2-Bromoethanesulfonate (BES) was added to prevent methanogen growth only during start-up. Four days after inoculation, the MFCs were continuously fed swine manure and the electric control was switched on for the MPPT-MFC.

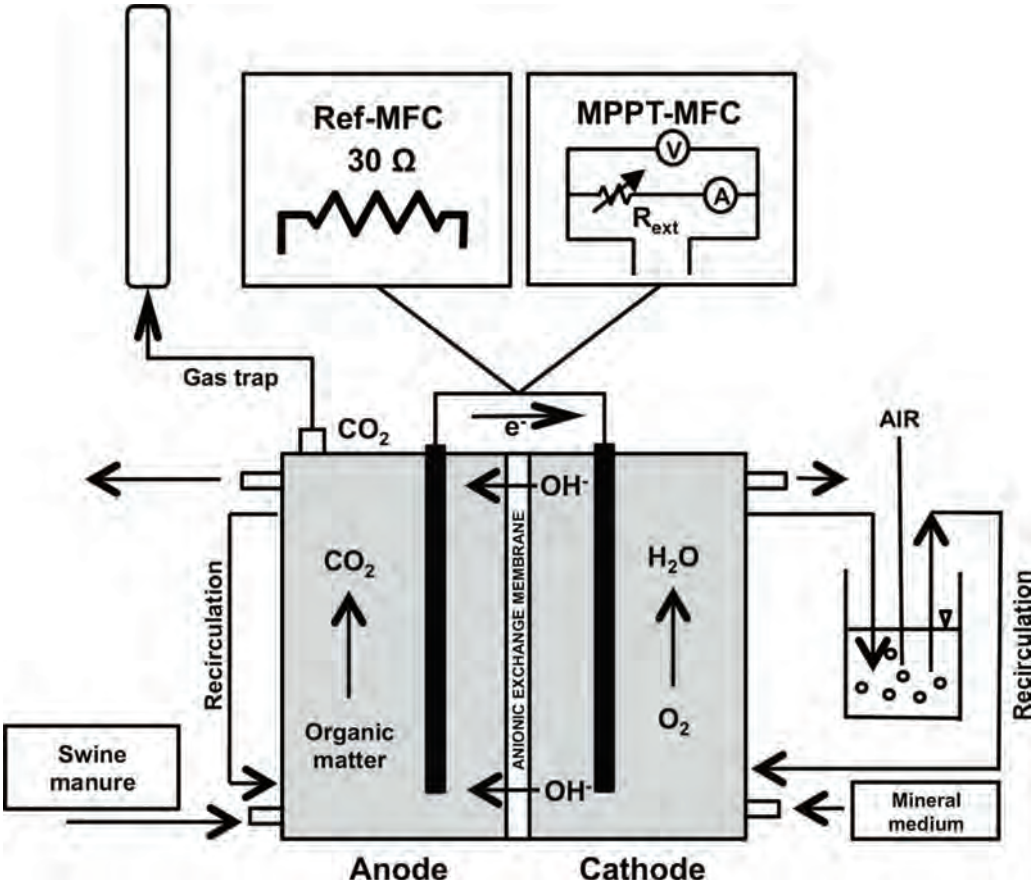

Fig 1. Schematic representation of replicate MFC configurations with the two evaluated electrical load conditions (Ref-MFC and MPPT-MFC).

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Maximum power point tracking control

The MPPT control consisted of an array of parallel-connected potentiometers imposing resistance on the MFC, an amperometer and a voltmeter (Model 2000 6-1/2 Digit Multimeter, Keithley Instruments, USA) for current and voltage measurements [39]. The applied external resistance could vary between 6 and 200 Ω via 2 Ω steps (ΔR). The implemented MPPT algorithm is classified as a perturbation-observation method. Basically, it was composed of a loop that periodically measured the MFC output power (P). The power value measured at one iteration (step i) was compared with the value measured in the previous iteration (step i-1); the resistance R varied according to Eq 1.

$$R_{i+1} = R_i + \Delta R \; sign\left(\frac{P_i - P_{i-1}}{R_i - R_{i-1}}\right) \quad (1)$$

Computational fluid dynamics

A fluid dynamic model of the MFC anode chamber was developed using the Ansys Fluent software (ANSYS® Academic Research, Release 12.1). The fluid dynamic equations and shear rate calculation were solved in Vilà-Rovira *et al.* [40]. Fig 2 shows the anode chamber

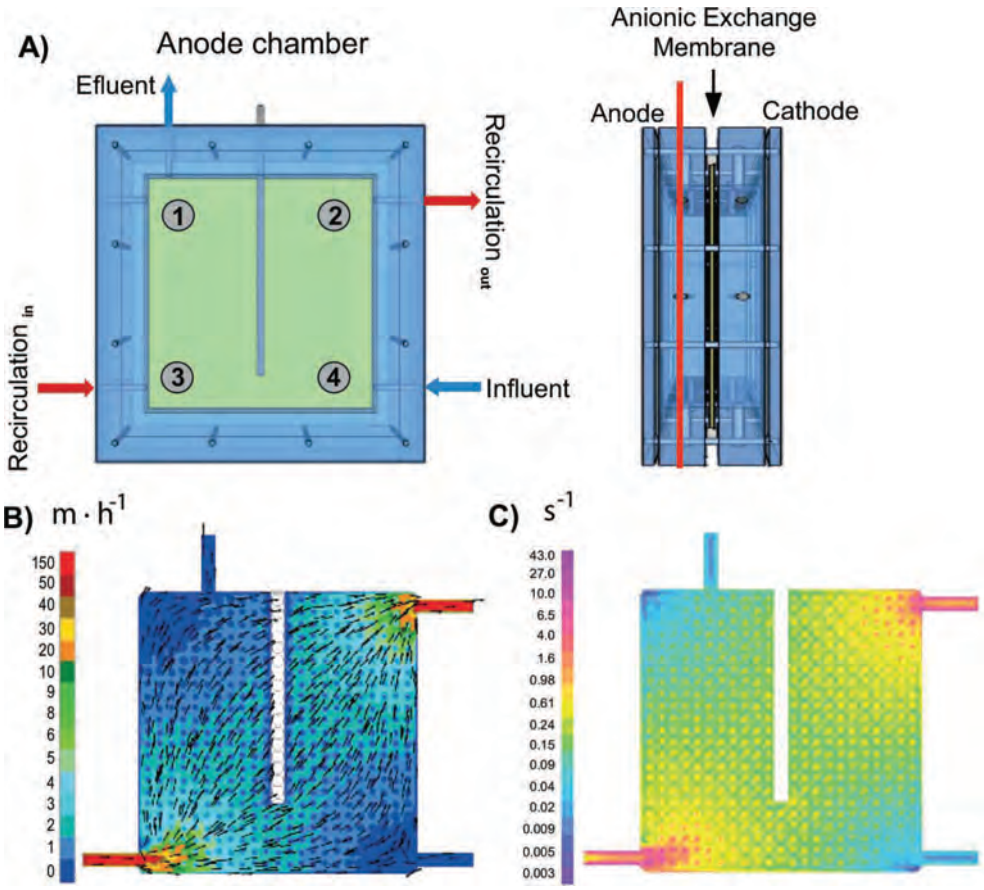

Fig 2. A) Schematic figure of the anode compartment where the 4 samples (1, 2, 3 and 4) for microbial analyses are represented. Hydrodynamic model results in terms of (B) fluid velocity and (C) shear rate profile distribution.

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configuration scheme together with the fluid velocity and shear rate internal distributions under the MFCs' operational conditions (influent flow rates of 1.5 $L \cdot d^{-1}$ and recirculation flow rates of 170 $L \cdot d^{-1}$).

Chemical and electrochemical analyses and calculations

Liquid phase standard wastewater measurements for organic matter, nitrogen and solid content were performed at regular intervals according to the American Public Health Association guidelines [41]. Samples were obtained from the (anode) influent and effluent sections of the MFCs. Detailed calculations of the chemical analysis performed are provided in the supplementary methods (S1 File). Gas samples were analysed to detect the presence of carbon dioxide and methane (CO_2 and CH_4) with an Agilent 7820A GC System equipped with the Washed Molecular Sieve 5A and Porapak® Q columns and a Thermal Conductivity Detector (TCD). Gas production rates were calculated by dividing the obtained gas volume per unit time. The current (mA) and power (mW) generations were derived from the cell voltage (mV) measurements according to Ohm's laws. The coulombic efficiency (CE) was calculated as described in Logan *et al.* [42].

DNA extraction and 16S rRNA gene amplicon sequencing

Samples were collected from the MFC feed (swine manure) and anode chambers once during steady-state operation (day 43). The MFCs were opened and 26 g of granular graphite was collected from four different positions (Fig 2). Biofilm was detached from the surface of graphite after incubation of samples in an ultrasonic bath (Selecta, Spain) for 60 s in Phosphate Buffered Saline. Liquid phase was collected after precipitation of graphite granules and cells pelleted by centrifugation at 4,000 rpm. Nucleic acids were extracted from the recovered cell pellets using the Fast DNA® SPIN Kit for soil (MP Biomedicals, USA) according to manufacturer's instructions. The DNA concentrations were verified using a Qubit® 2.0 Fluorometer (Life Technologies Ltd., Paisley, UK).

The bacterial 16S rRNA gene amplicon sequences were obtained using the bTEFAP method by 454GL FLX technology at the Research and Testing Laboratory (http://www.researchandtesting.com) [43] using primer set 341F-907R [44] modified to contain a 454 FLX Titanium Lib adapter. Sequence denoising, trimming and Operational Taxonomic Units (OTU) assignments (97%) were performed using QIIME (Quantitative Insights Into Microbial Ecology) pipelines [45]. Details of data processing and analysis, together with the calculation of alpha and beta-diversity indices of the bacterial communities at the different sampling positions, are described in the Supplementary Methods.

Core communities were defined for both MFCs using QIIME. OTUs consistently found in at least 3 of 4 samples were selected as members of the core community [46]. Beta-diversity indices were used to analyse differences between bacterial communities according to sampling points or MFCs. Clustering of samples was performed on the basis of the weighted UNIFRAC pairwise distance matrices and visualized as a dendogram [47]. Weighted UNIFRAC distances were calculated and used for the jackknife-resampling analysis. Dendograms of either sample distributions or OTU phylogenies generated in QIIME were visualized in the Interactive Tree of Life software [48].

Quantitative analysis of bacterial abundance

Bacterial 16S rRNA gene abundance was quantified by quantitative PCR (qPCR) as previously described [49]. Reactions were performed in a 7500 Real Time PCR system (Applied Biosystems, USA) using the SYBR Green PCR Master mix. Standard curves were obtained using serial dilutions (10^2 to 10^7 copies) of linearized plasmids. Inhibition tests were performed for each sample. Sample dilution was applied when necessary to avoid inhibition of PCR. Samples for the analysis of bacterial abundances were collected from the two MFC configurations at the four sampling points during steady state conditions. Sampling and quantification of bacterial abundance was performed after applying swine manure to the MFC at three OLRs (10.5 ± 0.7 kg COD m^{-3} d^{-1}, 5.3 ± 1.4 kg COD m^{-3} d^{-1}, 0.7 ± 0.1 kg COD m^{-3} d^{-1}). For all samples, qPCR analyses were performed twice.

Statistical analysis

All statistical analyses were performed using SPSS for Windows 19.0 (SPSS®, IBM). The ANOVA test was applied for the chemical and electrochemical values obtained from the MFC effluents. Bacterial abundances were related to fluid dynamics by Pearson's correlation test. The Welch and Games-Howell post hoc tests were used to identify differences in abundance between sample positions and MFCs. Significant differences among whole bacterial community compositions were analysed for the previous variables using the non-parametric ANOSIM method.

Sequence data submission

The sequences presented in this study have been submitted to the GenBank database with Bio-Project accession number PRJNA302844.

Results

Assessment of MFC performances

The two MFCs behaved similarly in terms of their organic matter removal rate (ORR) and no significant differences were found at an OLR of 10.5 ± 0.7 kg $_{COD}$ m^{-3} d^{-1} (ANOVA test, $p = 0.61$). In both systems, the COD removal efficiency ranged from 36 to 38% (Table 1, Fig 3). The methane (CH_4) flow rate in Ref-MFC was 10-fold higher than the carbon dioxide (CO_2) flow rate (48 mL CH_4 d^{-1} versus 5 mL CO_2 d^{-1}). The ratio between the CH_4 and CO_2 flow rates decreased to 2 in the MPPT-MFC primarily due to an increase in CO_2 production (17 mL d^{-1}). This increment of CO_2 emissions in the MPPT-MFC suggested a higher efficiency of the exoelectrogenic process. In terms of nitrogen, ammonium removal was negligible along the experimental period. Significant differences ($p < 0.05$) between the two MFCs were found for current and power generation and CE values (Table 1). Energy production from the MPPT-MFC (0.025 kWh m^{-3} at 17% CE) was almost double compared with the Ref-MFC (0.013 kWh m^{-3} at 6% CE).

Effect of fluid dynamics on bacterial abundance

The calculated fluid velocity was not uniform within the anode chambers despite the recirculation loop application (Fig 2). The position near the influent and effluent sections of the MFC (sampling positions 4 and 1) presented the lowest flow velocities (0.69 and 0.24 m h^{-1}, respectively), whereas the recirculation loop positions (positions 2 and 3) presented 10-fold higher values (6.88 and 7.82 m h^{-1}, respectively). The highest shear rate was observed at position 3 (16 s^{-1}).

The bacterial 16S rRNA gene abundance in the MPPT-MFC ($3.1 \cdot 10^6$ DNA copies $g_{graphite}^{-1}$) was higher compared to the Ref-MFC ($6.1 \cdot 10^5$ DNA copies $g_{graphite}^{-1}$), but the difference was

Table 1. MFC performances in terms of organic matter and solid removal, current and power generation, and gas production measured for both reactors (Ref-MFC and MPPT-MFC).

Parameter	Units	Ref—MFC	MPPT—MFC	p_value
OLR	$kg_{COD}m^{-3}d^{-1}$	9.9±2.5	11.2±2.8	0.15
ORR	$kg_{COD}m^{-3}d^{-1}$	4.0±2.5	4.4±2.7	0.61
η_{COD}	%	38±18	36±16	0.12
η_{TSS}	%	66±7	55±21	0.76
η_{VSS}	%	64±5	54±20	0.72
R_{ext}	Ω	30±0	8±3	0.02
$I_{density}$	A m^{-3}	14±2	33±10	0.01
$P_{density}$	W m^{-3}	2.5±0.8	5.0±1.0	0.01
CE_{CODs}	%	6±3	17±7	0.01
CH_4	mL d^{-1}	48±12	39±17	0.06
CO_2	mL d^{-1}	5±3	17±7	0.06
CH_4/CO_2	—	6.6±3.8	2.2±1.8	0.05

Values are expressed as the average ± standard deviation. The last column reports the p-value of the ANOVA test. ***S***ignificant differences between the microbial fuel cells (MFCs) were set at $\boldsymbol{p}$<0.05.

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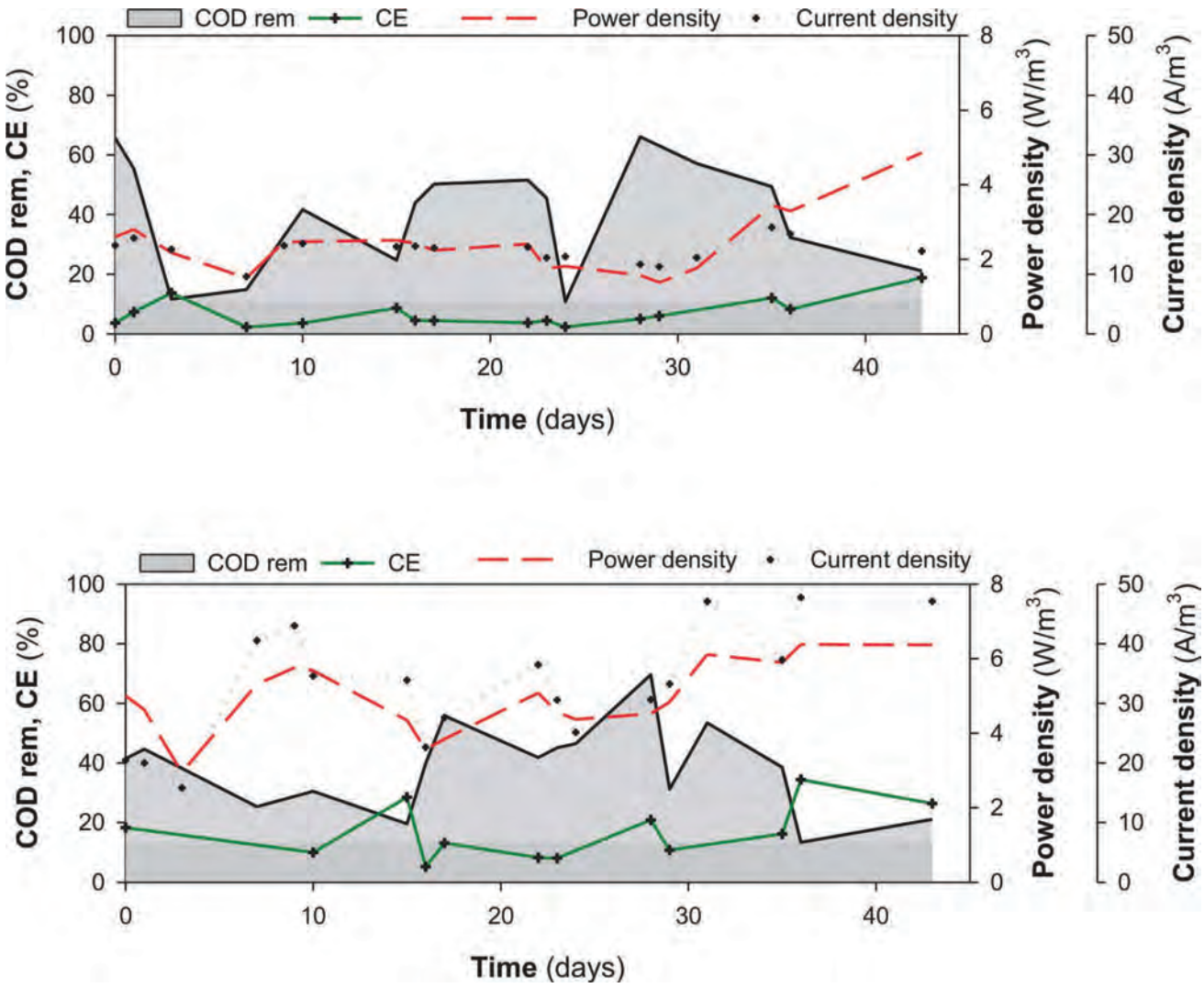

Fig 3. Time course of organic matter removal (COD rem) and electrical performance indicators (CE- coulombic efficiency, Current density and Power density) during the experimental period (43 days) in A) Ref-MFC and B) MPPT-MFC.

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not significant when all sampling points were considered together. Small differences in the abundance of 16S rRNA genes were detected for the four analysed positions in the two MFCs. Gene abundances were reduced by 20 to 50% at position 3 compared to the other sampled positions (Table 2). This situation remained constant in additional tests at a lower OLR (5.3 ± 1.4 kg COD m^{-3} d^{-1} for 5 weeks and 0.7 ± 0.1 kg COD m^{-3} d^{-1} for 2 weeks). These results demonstrated that the bacterial abundance distribution in the MFCs was influenced by the fluid velocity and shearing effect.

Bacterial community structure

A total of 22,680 sequences were obtained and used for diversity analyses. The number of sequences varied from a minimum of 554 sequences obtained in MPPT-MFC position 1, to a maximum of 4,868 sequences obtained in Ref-MFC position 4. On average, 2,268 sequences were obtained per sample. Valid sequences were clustered into 474 OTUs at a 97% similarity

Table 2. 16S rRNA gene abundances (Mean values ± SD, n = 2, technical replicates) at the four sampled positions within the Ref-MFC and MPPT-MFC operated at different organic loading rates (OLR).

Reactor	Position	Ribosomal RNA gene abundance per gram graphite (x10^5)			OLR effect
		High OLR	Intermediate OLR	Low OLR	
Ref-MFC	1	3.31±0.47 ab	2.05±0.80 a	42.60±3.36 a	***p***<0.05
	2	1.30±0.09 a	1.34±0.34 a	23.10±4.41 ab	NS
	3	0.16±0.07 b	0.07±0.06 a	0.12±0.08 ab	NS
	4	1.34±0.44 ab	0.07±0.05 a	5.10±1.02 b	NS
MPPT-MFC	1	13.64±1.00 a	0.87±0.13 a	79.70±5.14 a	***p***<0.05
	2	16.20±1.16 a	1.10±0.02 a	33.4±6.61 bc	***p***<0.05
	3	0.17±0.16 b	11.8±0.23 b	69.4±3.54 ab	***p***<0.01
	4	0.98±0.20 b	1.03±0.03 a	0.89±0.16 c	NS

Lower case letters next to quantification values show homogenous variance groups among the sampled positions according to pair-wise Games-Howell tests for every reactor and OLR condition. Differences of 16S rRNA gene abundances in every position according to changes of OLR for a single sampling point were analysed with a Welch test. High OLR- 10.5 ± 0.7 kg COD m^{-3} d^{-1}, Intermediate OLR- 5.3 ± 1.4 kg COD m^{-3} d^{-1}, Low OLR- 0.7 ± 0.1 kg COD m^{-3} d^{-1}.

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level, but only 221 OTUs contained at least four sequences and were considered for diversity calculations. Each sample was rarefied to 500 sequences for comparisons of alpha and beta diversity. Despite this reduction in sequence number, the species richness of all samples was considered to be enough covered in view of rarefaction curves (S1 Fig). Significant differences in species richness indicators (observed OTUs and Chao1 estimator) were found between swine manure and the two MFC configurations (Table 3). On the contrary, although Shannon's and phylogenetic diversity (PD) indices were higher in the swine manure (H' = 4.23±0.39,

Table 3. Observed OTUs and alpha diversity indices, Chao1, Shannon (H') and phylogenetic diversity (PD) diversity indices in swine manure (SM), Ref-MFC (Ref_1 to Ref_4) and MPPT-MFC (MPPT_1 to MPPT_4).

Samples	Observed OTUs	Chao1	Shannon (H')	PD
SM1	80.7	102.9	5.02	8.37
SM2	61.2	93.2	3.44	6.09
Mean ± SD	**70.95±9.75**	**98.03±4.85**	**4.23±0.39**	**7.22±1.13**
Ref_1	27.5	48.1	2.38	2.90
Ref_2	26.7	36.2	2.53	3.08
Ref_3	45.1	68.2	3.60	5.36
Ref_4	30.2	49.7	2.75	3.44
Mean ± SD	**32.37±7.46**	**50.57±11.43**	**2.81±0.47**	**3.69±0.98**
MPPT_1	40.9	41.1	4.50	4.75
MPPT_2	39.0	45.4	3.74	4.99
MPPT_3	34.6	40.6	3.74	3.94
MPPT_4	38.1	54.0	3.44	4.69
Mean ± SD	**38.15±2.28**	**45.27±5.39**	**3.85±0.39**	**4.59±0.39**
Pair-wise comparisons				
SM-Ref	***p*** = 0.035	***p*** = 0.002	NS (***p***>0.1)	NS (***p*** = 0.097)
SM-MPPT	***p*** = 0.019	***p*** = 0.030	NS (***p***>0.1)	NS (***p*** = 0.082)
Ref-MPPT	NS (***p***>0.1)	NS (***p***>0.1)	NS (***p*** = 0.079)	NS (***p***>0.1)

Indicated values for each sample are mean values of ten rarefied samples containing 500 sequences. Pair-wise differences of alpha diversity indices according to Feed (SM) or MFC Type were calculated using a t-test. Significance level was set to ***p***<0.05. NS- not significant.

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PD = 7.22±1.13, n = 2) compared to the MFCs samples (Ref-MFC H' = 2.81±0.47, PD = 3.69 ±0.98; MPPT-MFC H' = 3.85±0.39, PD = 4.59±0.39) no significant differences were found. No significant differences in alpha-diversity indicators of the microbial community structures were observed between the two MFC configurations.

Representative sequences for the 221 OTUs were aligned and taxonomy assigned down to the species level when possible. Taxonomy assignments were performed using the Greengenes reference database (v13.8). The swine manure microbial community was primarily composed of bacteria from the phylum *Proteobacteria* (41.8%, OTU 3, showing a high similarity to *Pseudomonas* spp. being the dominant genus) and *Firmicutes* (38.9% of sequences). The bacterial community in Ref-MFC was enriched with bacteria from the phylum *Firmicutes* (69.2% of sequences) and *Bacteroidetes* (29.8%) at all sampling positions, whereas *Proteobacteria* were present at very low relative abundance (less than 1%). In contrast, members of the latter group were consistently found in the MPPT-MFC samples and accounted for almost 7% of the sequences. However, those sequences annotated as *Pseudomonadaceae* found in MPPT-MFC (OTU 12) differed from those found in swine amnure (OTU 3). Members of the candidate division WWE1 (Waste Water of Evry 1) were consistently found at all positions in the MPPT-MFC and accounted for almost 4% of sequences, but rarely occurred in the Ref-MFC and swine manure. OTU 0 (*Turicibacteraceae*), OTU 1 (uncultured p-2534-18B5 gut group), and OTU 2 (*Porphyromonadaceae*) were found at the highest frequencies (35.7% of sequences) and appeared almost exclusively in the MFC samples. Conversely, OTU 3 (*Pseudomonadaceae*) and OTU 6 (*Carnobacteriaceae*) showed a higher relative abundance in the swine manure compared to the MFC biofilms. Only, twenty-four out of 221 OTUs were shared for the three sample types (Fig 4A). The number of shared OTUs increased to 74 when the MFC samples were considered independently.

A reconstructed phylogenetic tree was used to calculate differences in community compositions between sample types according to weighted Unifrac metrics of jacknifed subsamples (500 sequences each). Community structures at the OTU and genus levels were tested for homoscedasticity using betadisper ($F = 3.08$, $p = 0.115$ for OTU data; $F = 1.93$, $p = 0.224$ for genus level data). Distances to centroid of the four sampling points revealed significant differences in the bacterial community structure among MFC types (S2 Fig). The microbial community structure of the two MFC types was essentially different from the community structure found in the swine manure even at higher taxonomic levels (Fig 4B). Differences among sample groups (SM, Ref-MFC and MPPT-MFC) were tested using ANOSIM without any transformation of data. Results confirmed significantly different bacterial community structures between the feed (SM) and MFC biofilms ($R = 0.688$, $p = 0.01$). Differences between the two MFC types were lower ($R = 0.385$, $p > 0.031$). In order to test if variability of microbial communities within each MFC type (intra-group comparisons) was lower than variation among the two MFC types (inter-group comparisons), Bray-Curtis similarity indices were calculated pairwise and compared (S3 Fig). As expected, Ref-MFC yielded a homogeneous low variability group for all intra-group pair-wise comparisons. On the contrary, MPPT-MFC comparisons were highly variable and no significant differences in Bray-Curtis similarity indices were found when compared to inter-group comparisons. High variation in similarity indices in the MPPT- MFC type was due to differences observed in the sample close to the influent (M4).

The MFC core microbiomes

OTUs detected at relative abundances higher than 0.5% were considered to estimate the core community of SM, Ref-MFC and MPPT-MFC. Members of the core community of SM were considered if found in the two samples analysed. In the case of the two MFCs, core members

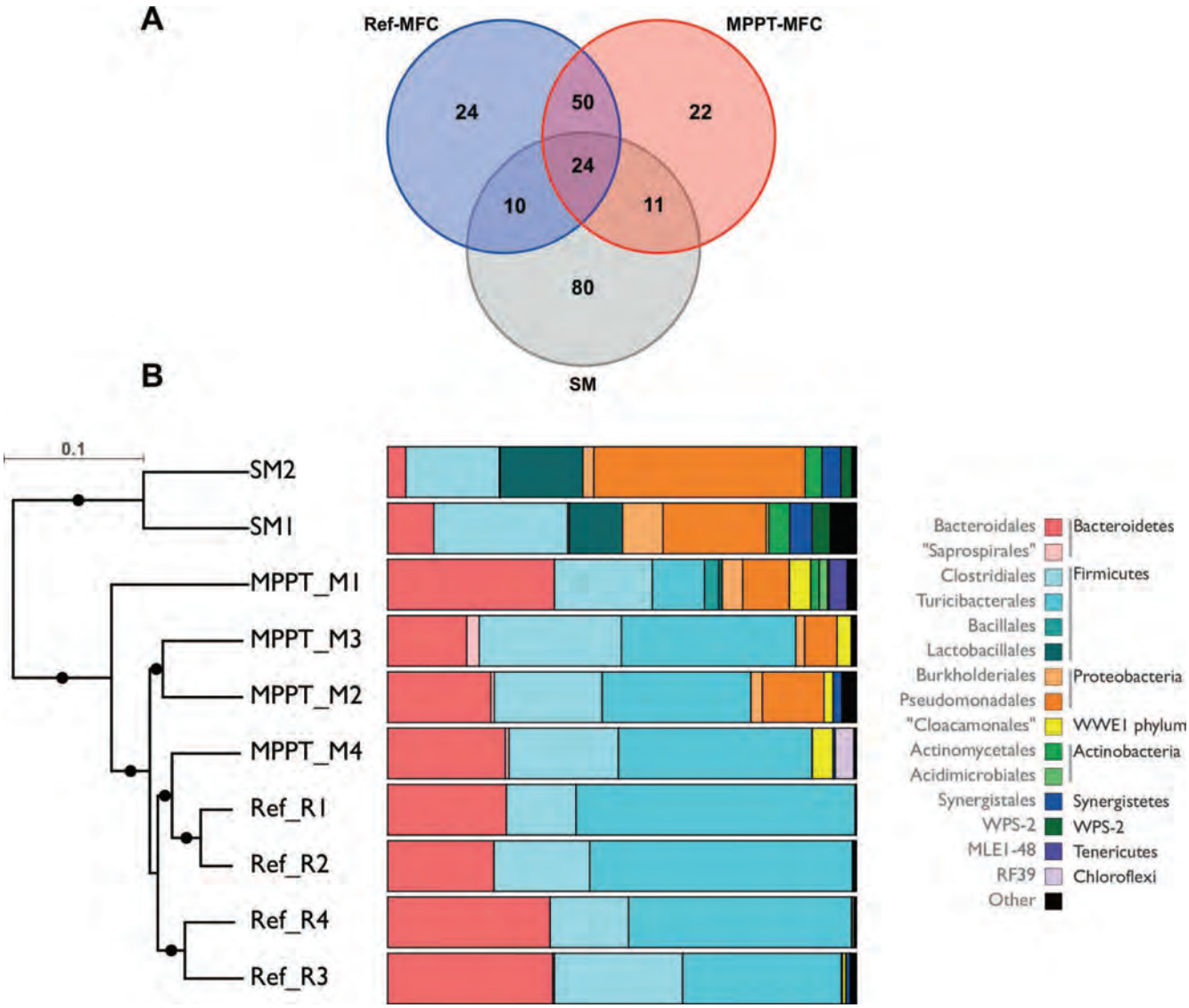

Fig 4. A) Venn diagram of the OTU distribution in swine manure and the two MFC configurations (MPPT and Ref). **B)** Clustering of samples based on weighted Unifrac measures (rarefied at 500 seqs per sample) calculated after phylogenetic reconstruction of detected OTUs. Black dots in the dendrogram show nodes at bootstrap supported levels above 80%. Bar charts show the relative abundance of main bacterial groups (Orders) found in each of the samples. Phyla representing less than 1% of the sequences in a sample have been grouped as Others.

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were identified as those OTUs found in a minimum of three sampling points within each MFC. The core community common for the SM and both MFCs was limited to three OTUs (OTU 0, 9 and 11), all belonging to the *Firmicutes*. Seven additional OTUs were found exclusively in swine manure. Ten extra OTUs were simultaneously defined as members of the core community of the two MFC configurations. Only two OTUs, the *Proteobacteria* OTU 12 (7.2% of sequences in MPPT-MFC) and 23 (2.0%), were found exclusively in MPPT, revealing some specificity between MFC configurations (Fig 5, S2 Table). No *Proteobacteria* were found and selected as members of the core community in the Ref-MFC.

Relative abundances of OTU 0 (*Turicibacter* sp.), OTU 4 (*Alkaliphilus* sp.) and OTUs 1, 2 and 7 (*Bacteroidetes*) were significantly higher in the two MFCs compared to the SM,

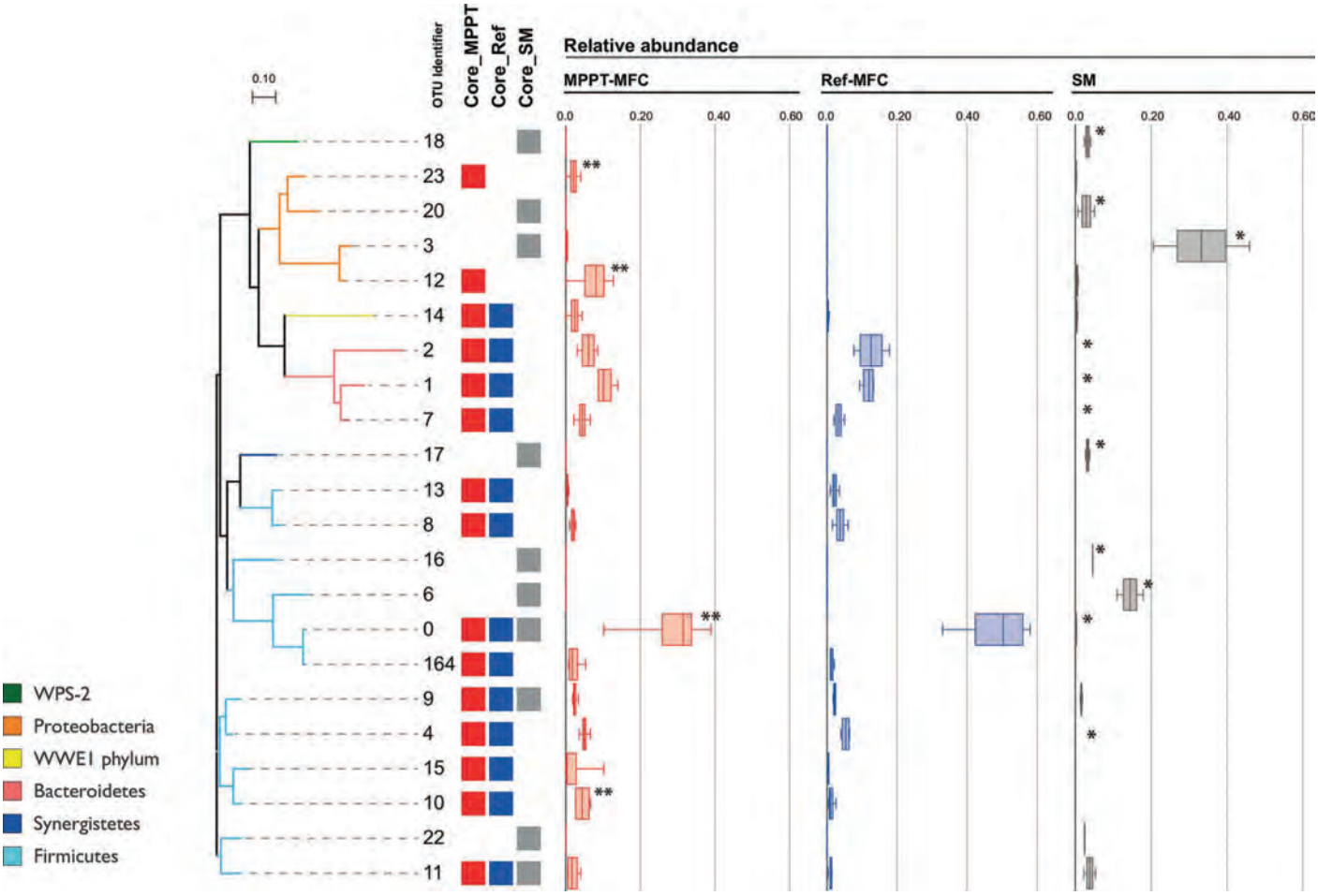

Fig 5. Phylogenetic relationship of OTUs (97% similarity level) belonging to core communities of SM, Ref-MFC, and MPPT-MFC biofilm samples. Members of core communities for each sample type are shown as coloured squares next to OTU identifier. Box plots show the relative abundance of OTUs in swine manure (grey), Ref-MFC (blue), and MPPT-MFC (red). * indicate OTUs showing significant differences on relative abundances between SM and MFCs. **indicate OTUs showing significant differences on relative abundances between Ref-MFC and MPPT-MFC.

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suggesting an implication of these bacteria on electrogenesis. The four OTUs accounted for approximately the 80% and 50% of Ref and MPPT-MFCs sequences, respectively. If the two MFC configurations were compared, OTU 10 (*Sedimentibacter* sp.), OTU 12 (*Pseudomonas* sp.) and OTU 23 (uncultured *Oxalobacteraceae*) were enriched in the MPPT-MFC (n = 4, t-test, $p<0.045$, False detection rate, FDR< 0.225, S2 Table), whereas OTU 0 (*Turicibacter* sp.) appeared at higher densities in the Ref-MFC compared to MPPT-MFC ($p = 0.049$, FDR = 0.237). Unfortunately, significant differences between the two MFCs could not be confirmed by FDR corrections of *p*-values, most likely caused by the low number of samples per MFC type (n = 4) and sequences per sample (500 seq.).

Discussion

Both anode chambers achieved similar ORRs (4.2 kg_{COD} $m^{-3}d^{-1}$), solid removal efficiencies (approximately 60% VSS) and gas production rates (in terms of CH_4 and CO_2). High OLRs such as those used here may have led to competition between the exoelectrogenic microorganisms and other bacteria for organic substrates, causing an accumulation of gaseous compounds.

This phenomenon was previously observed in MFCs by Oliveira *et al.* [50] and specifically for methanogenesis, which was shown to decrease the final CE of the system [51]. Min *et al.* (2005) obtained a 27% COD removal efficiency (1.3 Kg_{COD} $m^{-3}d^{-1}$) with a CE similar to that obtained by the Ref-MFC (8%) [5]. A later study improved the COD removal efficiency to 60–70% (3.5 Kg_{COD} $m^{-3}d^{-1}$) but the CE remained at values lower than 1.5%, indicating the prominent occurrence of side reactions [52]. The current study showed that the application of a variable resistance control in the MPPT-MFC improved organic matter treatment despite the use of a complex organic matrix for degradation, thereby achieving higher organic removal rates and electric performance, incrementing the CE by 40% and doubling the energy production compared to the MFC with fixed external resistance. These differences may be explained by an enhancement of electrons released when optimal resistances for electron transfer were consistently applied. Intermittent electric connection allowed higher current production, since both capacitive and faradaic currents are harvested. The same positive effect has been observed in a series of MFC configurations, using different solid matrices as electrodes, including granular graphite and marine sediment [53,54]. It has been stated that ammonia levels between 2–8 g $N\text{-}NH_3$ L^{-1}, would cause an inhibition of biofilm anaerobic digestion activity, resulting in less biogas production [55,56]. Similar effects could be hypothesized for electrogenic activity. However, the low influent concentrations used in this study (245±40 mg N-NH4+ L^{-1}) are unlikely to have an inhibitory effect on the bacterial community.

The key factor for the proper development of MFC technology relies on the formation of a stable electroactive biofilm. In this sense, the MPPT control has been proven to be more effective for the proliferation of exoelectrogenic bacteria [31] and reduces the start-up time for running a MFC at full capacity [33]. Similar results were obtained here, with the MPPT control exhibiting an increase in both the bacterial abundance at the anode (5-fold compared with Ref-MFC) and the current density (33 and 14 mA/m^3, respectively).

MFC fluid dynamics were shown to affect the mass transfer kinetics, biofilm structure and production of extracellular polymeric substances (EPS) [57]. In the studied anode chambers, fluid dynamics and shear rates influenced the internal biomass distribution, causing high biofilm detachment near the recirculation loop (position 3) compared to the other analysed positions. The fragility of the different biofilm layers on the electrode could explain the easy detachment that occurred at this position; this finding was in agreement with Shen *et al.*, [58] and Celmer *et al.*, [57], who reported that a 65% decrease in the biofilm thickness increased the flow rate from 1.3 to 24 mL min^{-1}.

The microbial community in swine manure is versatile and changeable depending on variables such as the pigs' diets or the sporadic use of antibiotics, which have an impact on the gut microbiota [59]. Uncontrolled differences in the microbial composition of the feed could not be avoided and were recorded in the two swine manure samples. Bacteria found in the influent had a limited influence on the community established within the MFCs. Only six OTUs from the MFC core community were identified in swine manure, and all of them belonged to *Clostridiales* (*Firmicutes*). According to 16S rRNA gene based identifications, the *Firmicutes* found were described as having a fermentative behaviour by Siegert *et al.*, [60] and Regueiro *et al.*, [61]. Previous studies identified *Firmicutes* (*Clostridia*), *Bacteroidetes* (*Bacteroidia*) and *Proteobacteria* (*Gammaproteobacteria*) as the main phyla in swine manure [62]. A recent study suggested a possible exoelectrogenic role for *Clostridium* bacteria because they were detected at a high amount within the exoelectrogenic biofilm of a MFC treating swine manure [26].

According to the OTU-based analysis, the MFC microbiome varied significantly compared to the influent swine manure. Although *Firmicutes* and *Bacteroidetes* also appeared as the dominant phyla in the MFC biofilms, the enrichment of specific OTUs suggested a number of bacteria putatively implicated in exoelectrogenesis. Among these, OTU 0 (*Turicibacter* sp.), OTU

1 (uncultured *Bacteroidetes*), and OTU 2 (*Parabacteroides* sp.) occurred at relative abundances higher than 10% in both of the MFC configurations. On the contrary, *Proteobacteria* were drastically reduced in the anode chambers and were found only in the MPPT-MFC samples. Microbial communities of MFC anodes are usually composed of different bacterial species from which electricity generation capabilities has not been described, but may be hypothesized from comparisons of microbiome structures in selective experimental conditions. The dominance of *Firmicutes* was detected in anode reactors treating swine manure in a previous study of our group [26]. The most abundant phylotype within the two MFCs was identified in base of the partial 16S rRNA sequence as *Turicibacter* sp. (OTU 0), which was barely represented in swine manure samples. *Turicibacter* spp. have been previously found in pig waste, using cultivation-independent molecular analyses [62]. Under strict anaerobic conditions, lactate is the main fermentation product from carbohydrates for *Turicibacter* spp. [63]. Unfortunately, no exoelectrogenic activity has been described for this species. Uncultured p-2534-18B5 gut group (OTU 1) and *Parabacteroides* sp. (OTU 2) were also found at high relative abundances in MFCs. OTU 1 can be related to intrinsic gut microbiota [64], whereas species with a 16S rRNA sequence similar to that of OTU 2 was involved in current generation [65].

Most known exoelectrogenic bacteria fall within the *Proteobacteria*, which have been detected as dominant members of the bacterial community in MFCs treating simple substrates, such as acetate and glucose [66][67][68], and wastes from industrial sources [69][19], revealing a substrate effect on dominant putative exoelectrogenic bacteria. *Proteobacteria* were in competitive disadvantage relative to *Firmicutes* under the experimental conditions applied in this study, as this was most likely related to the presence of highly recalcitrant components of the influent organic matter [70].

The effect of the MPPT control on the bacterial community structure was analysed by comparing the microbiome core communities and related to the increment of current density. The core community of MPPT-MFC contained three different OTUs that appeared at significantly higher relative abundances compared to Ref-MFC. Interestingly, the only OTU found at higher abundances in Ref-MFC was OTU 0, thus questioning its implication in exoelectrogenesis since significantly lower CE was found for Ref-MFC. More likely, *Turicibacter*-related species may be implicated in heterotrophic degradation of organic matter, probably through a fermentation process. However, additional molecular analyses, including shotgun metagenome and metatranscriptome sequencing, will be necessary to identify putative exoelectrogenic bacteria in MFCs based on functional capacity and activity and confirm the previous hypothesis.

Sedimentibacter spp. (OTU 10), *Pseudomonas* sp. (OTU 12) and an uncultured *Oxalobacteraceae* (OTU 23) were significantly enriched in the MPPT-MFC. Based on the analysis of 16S rRNA gene similarities, *Sedimentibacter* related species were identified in the core community of MFC systems with high power generation capabilities together with *Geobacter*, *Aminiphilus*, *Acetoanaerobium*, and *Spirochaeta* [71]. Although this was not proven experimentally with activity analyses, the enrichment of these bacteria at higher abundances is likely related to their exoelectrogenic role. Exoelectrogenic capacity has also been proven for some gammaproteobacteria, including *Pseudomonas* species [72].

The MFC microbial community diversity and abundance were studied in relation to the external resistance control and fluid dynamics. The bacterial community from the studied MFCs was more efficient at treating the complex organic matter than the community reported in previous studies. The application of a MPPT electric control in MPPT-MFC resulted in a 5-fold higher bacterial abundance compared to the Ref-MFC and doubled energy production and CE. The adopted electric condition (MPPT *vs* fixed resistance) was more relevant than the fluid dynamics in shaping the MFC microbiome. The MFC core community was primarily composed of the fermentative *Turicibacter* genus. The MPPT control was able to select specific

OTUs potentially harbouring higher exoelectrogenic capacities compared to the fixed resistance system. *Sedimentibacter* and gammaproteobacteria were among the most abundant phylotypes that being a member of the core community were enriched in the MPPT-MFC compared to Ref-MFC. Hence, it is likely that these organisms may be related to the extra electricity production in the MPPT-MFC. The optimization of the MFC systems together with the comprehension of the bacterial communities responsible for the internal processes will enable the implementation of MFC technology for *in situ* swine manure treatment. For this reason, future studies could focus on the physical relationship of the dominant taxa with the electrode (fluorescent in situ hybridization analyses) and the identification of active members of the MFC-associated community (shotgun metatranscriptome sequencing).

Supporting Information

S1 Fig. Rarefaction curves of observed OTUs (species) at 97% similarity level for swine manure (SM1 and SM2), Ref-MFC (R1, R2, R3 and R4) and MPPT-MFC (M1, M2, M3 and M4). Sequence subsampling at different number of sequences was performed ten times. Mean values are shown.
(DOC)

S2 Fig. Box plot graph showing the distances to centroid of microbial communities determined at a genus level (L6). n = 4 for Ref-MFC and MPPT-MFC, and n = 2 for Swine Manure (SM).
(DOC)

S3 Fig. Box plot showing dispersion of Bray-Curtis similarity indices of OTU distribution in Ref-MFC (n = 4) and MPPT-MFC (n = 4) reactor types. All pair-wise combinations of data-points have been organized as Inter-group or Intra-group comparisons and shown as individual points using different colours.
(DOC)

S1 File. Supplementary Methods.
(DOC)

S1 Table. Main characteristics of swine manure. The values are presented as average ± standard deviation (n = 7). n.d. not detected. * Acetic acid and Propionic Acid were the only VFA identified and detected above LOD.
(DOC)

S2 Table. Relative abundances of members of the core community in the two MFC types (Ref and MPPT). Core community members were limited to those OTU that were found in at least three of the four samples in the MFC. Differences were assayed using a t-test. FDR (False Detection Rate) correction of p_value. n.d. not detected. NS- not significant (p values >0.1).
(DOC)

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Supplemental Materials and methods

External resistances applied to MFC affect core microbiome and swine manure treatment efficiencies

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1.1. Composition of the cathode feed

The cathode was fed with an oxygen-saturated mineral medium with the following composition: 122 mg L^{-1} $NaHCO_3$, 7.6 mg L^{-1} NH_4Cl, 300 mg L^{-1} $NaH_2PO_4 \cdot 2H_2O$, 1.4 mg L^{-1} $CaCl_2$, 9 mgL^{-1} $MgSO_4 \cdot 7H_2O$, 1.3 mg L^{-1} KCl, 150 mg L^{-1} $KH_2PO_4 \cdot 2H_2O$ and 0.01 mL L^{-1} microelements solution.

1.2 MFCs configuration and operation

Details of MFCs design and operation are described in the study of Molognoni *et al.* (2014) and consisted (each one) of an anode and a cathode placed on the opposite sides of a single methacrylate rectangular chamber. The anode and cathode chambers were filled with granular graphite (model 00514, diameter 1.5-5 mm, EnViro-cell, Germany), which decreased the volumes to 370 ± 10 mL net anodic compartment (NAC) and 410 ±

10 mL net cathodic compartment (NCC) respectively. The electrodes were previously washed in 1 M HCl and 1 M NaOH to remove possible metal and organic contamination. Two thinner graphite rods electrodes (250 x 4 mm, Sofacel, Spain) were introduced in each chamber to allow an external electrical connection to the system. An Anion Exchange Membrane (AMI-7001, Membranes International Inc., USA) was placed between the anode and cathode frames.

The swine wastewater was continuously fed to the anode at a flow-rate of 1.5 L d^{-1}. Internal recirculation loops (170 L d^{-1}) in each compartment maintained well-mixed conditions and minimized concentration gradients. The temperature of the system was kept constant at 21 ± 1 ºC. The anode potential of each MFC was monitored with an Ag/AgCl reference electrode (+197 mV vs Standard Hydrogen Electrode, model RE-5B, BASI, United Kingdom).

1.3 Electrochemical analysis calculations

The OLR was calculated as the total organic matter concentration (COD) divided by the hydraulic retention time (HRT). The organic removal rate (ORR) was determined as the difference between influent and effluent OLRs. The organic matter removal efficiency (ηCOD) was calculated as the ratio between ORR and OLR.

The total suspended solid (TSS) and volatile suspended solid (VSS) removal efficiencies (ηTSS/ ηVSS) were determined as the ratio between solids removal and solids influent concentrations.

Energy production was calculated as the power (P) divided for the flow rate, where the power production is the product of the voltage by the intensity. Coulombic efficiency (CE) was calculated as in Logan *et al.* (2006).

1.4 Sequence trimming and processing with QIIME

Raw sequence reads were trimmed to 200 bp and quality-filtered using the split-libraries.py implemented in QIIME (Quantitative Insights Into Microbial Ecology) [3]. High quality sequences were checked for chimeras using UCHIME and distributed into operational taxonomic units (OTUs) at a 97% level using either the UPARSE [4], or the UCLUST algorithms. The latter was used to obtain a closed reference sequence file and predict the metagenome. Representative sequences for each OTU cluster were identified and assigned taxonomy with reference to the Greengenes 16S rRNA gene database (release May 2013). OTU tables containing read counts and taxonomic assignments were generated using the script make_otu_table.py. OTUs containing less than 4 sequences in the whole dataset were removed to avoid potential effects of spurious diversity due to sequencing errors. When necessary, identification of relevant OTUs was done after a BLAST search (NCIB) of the obtained representative sequences. Environmental sequences databases were excluded from BLAST searches except when needed (identification of uncultured microbial groups).

1.5 Structure of the communities

Community alpha diversity indices, Shannon's (H') and Phylogenetic diversity (PD) indices, and maximum richness estimator (S_{Chao}) were calculated after rarefying the

number of sequence reads in each sample to the lowest value obtained (sample with the lowest number of reads was 503 sequences; Figure S1). Community subsets were calculated at least ten times and mean values for diversity indices estimated. Differences among them were analysed using either parametric or non-parametric tests implemented in group_significance.py in QIIME. Differences were assayed for sample groups according to position of sampling point within the MFC, shear rate due to flow increases, and MFC type. Significant differences were set at $p<0.05$ level.

Differences on the complete microbial community structures, both at different sampling points and MFC configurations, were inferred from beta-diversity measures. Clustering of samples were performed on the basis of the weighted or un-weighted UNIFRAC pairwise distance matrices and visualized either as a dendogram or a Principal Coordinates plot. UNIFRAC distances were calculated and used for jackknife-resampling analyses with subsampled communities of 500 members.

Significant differences in OTU abundance between the two MFC configurations were analysed using either parametric or non-parametric t-tests implemented in QIIME, and based on rarefied OTU tables for each sample.

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Supporting information for manuscript

External resistances applied to MFC affect core microbiome and swine manure treatment efficiencies

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Summary:

Figure S1: Rarefaction curves of observed OTUs (species) at 97% similarity level for swine manure (SM1 and SM2), Ref-MFC (R1, R2, R3 and R4) and MPPT-MFC (M1, M2, M3 and M4). Sequence subsampling at different number of sequences was performed ten times. Mean values are shown.

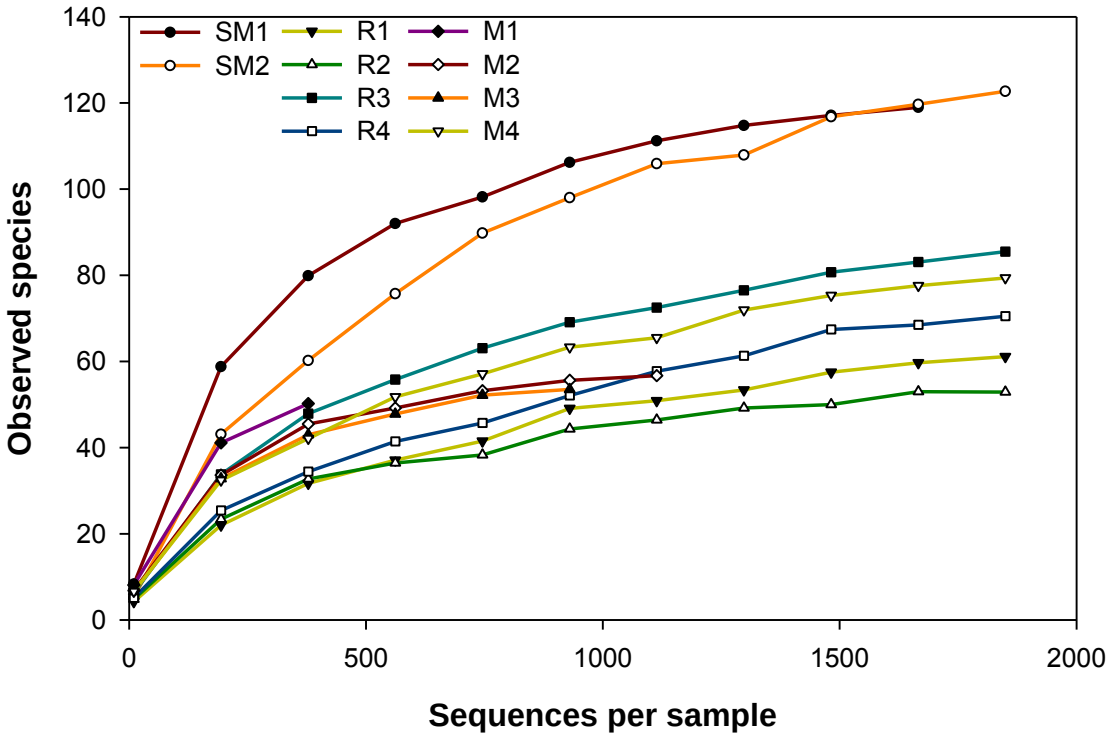
Figure S2. Box plot graph showing the distances to centroid of microbial communities determined at a genus level (L6). n=4 for Ref-MFC and MPPT-MFC, and n=2 for Swine Manure (SM).

Figure S3. Box plot showing dispersion of Bray-Curtis similarity indices of OTU distribution in Ref-MFC (n=4) and MPPT-MFC (n=4) reactor types. All pair-wise combinations of data-points have been organized as Inter-group or Intra-group comparisons and shown as individual points using different colours.

Table S1. Main characteristics of swine manure. The values are presented as average ± standard deviation (n=7). n.d. not detected. * Acetic acid and Propionic Acid were the only VFA identified and detected above LOD.

Table S2. Relative abundances of members of the core community in the two MFC types (Ref and MPPT). Core community members were limited to those OTU that were found in at least three of the four samples in the MFC. Differences were assayed using a t-test. FDR (False Detection Rate) correction of p_value. n.d. not detected.

Figure S1. Rarefaction curves of observed OTUs (species) at 97% similarity level for swine manure (SM1 and SM2), Ref-MFC (R1, R2, R3 and R4) and MPPT-MFC (M1, M2, M3 and M4). Sequence subsampling at different number of sequences was performed ten times. Mean values are shown.



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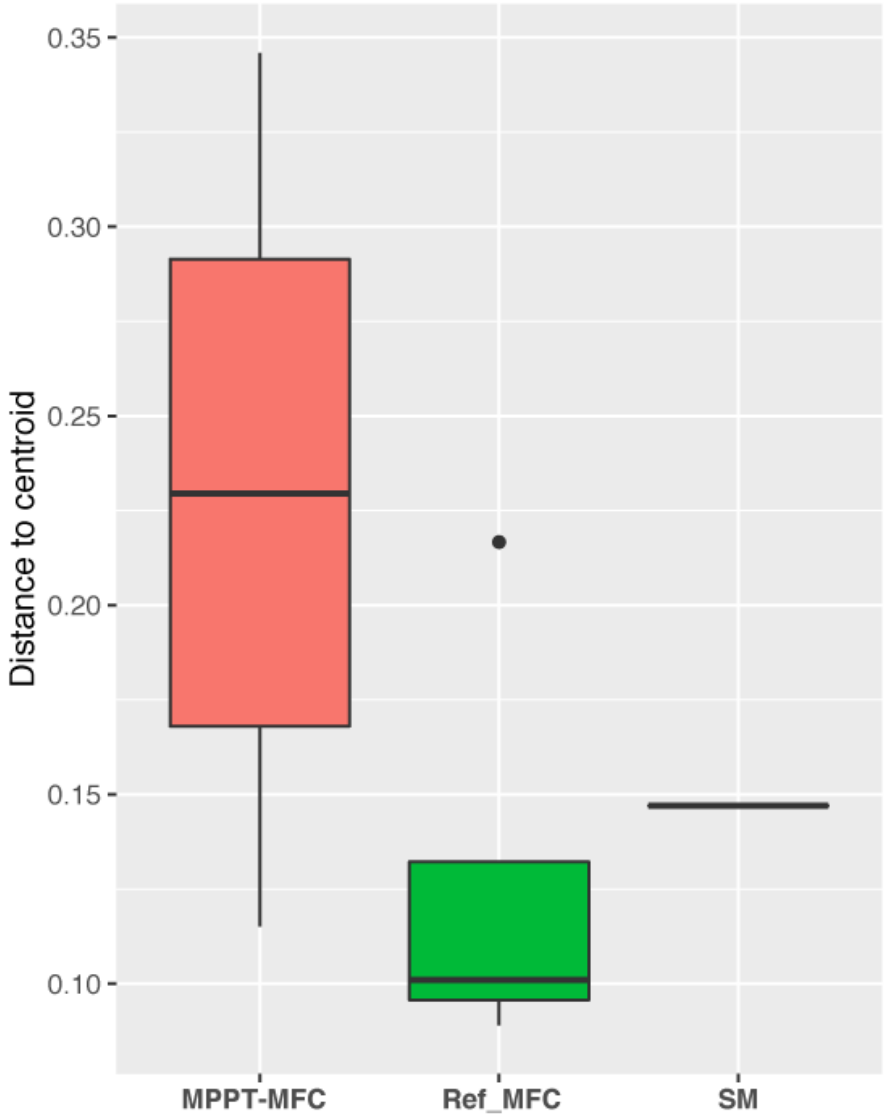

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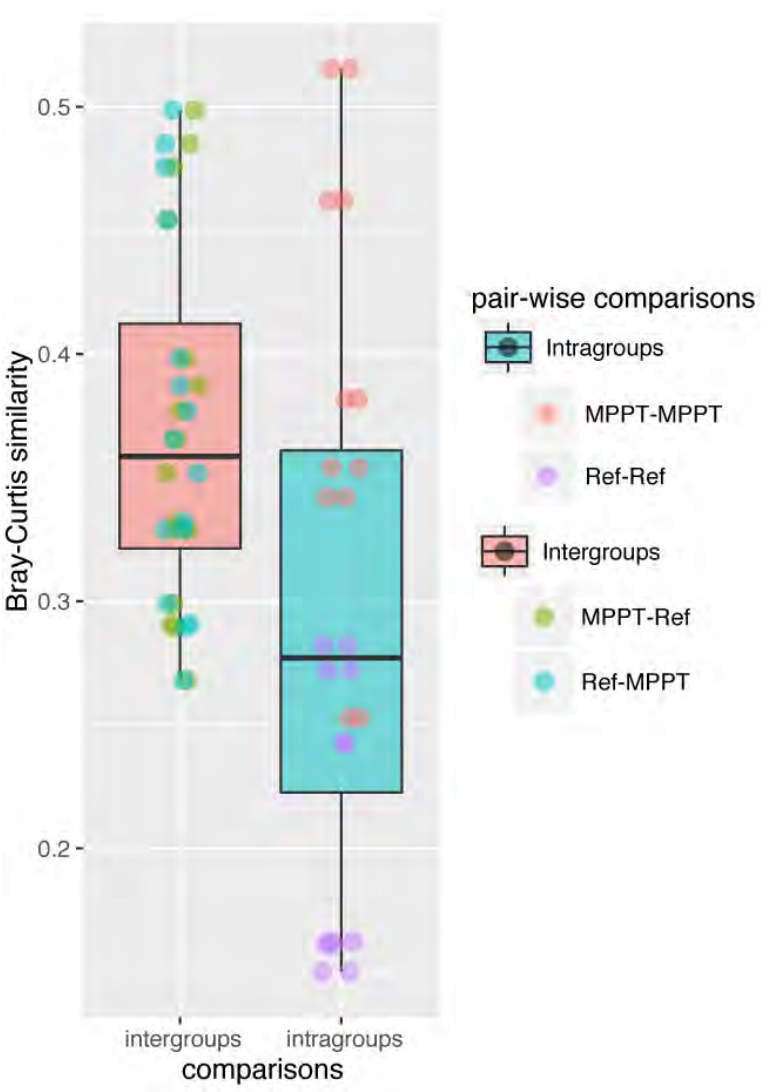

Table S1. Main characteristics of swine manure. The values are presented as average ± standard deviation (n=7). n.d. not detected. * Acetic acid and Propionic Acid were the only VFA identified and detected above LOD.

	Swine manure	**Units**
pH	7.9±0.6	-
Conductivity	2.7±0.3	mS cm^{-1}
COD_{Total}	2330±620	mg COD L^{-1}
$COD_{Soluble}$	1330±316	mg COD L^{-1}
$BOD_{5\ Total}$	895±220	mg BOD L^{-1}
$BOD_{5\ Soluble}$	560±140	mg BOD L^{-1}
VFA	13±7	mg C L^{-1}
TKN	280±50	mg N-TKN L^{-1}
NH_4^+	245±40	mg N-NH_4^+ L^{-1}
NO_2^-	n.d	mg N- NO_2^- L^{-1}
NO_3^-	n.d	mg N- NO_3^- L^{-1}
TSS	600±450	mg TSS L^{-1}
VSS	545±380	mg VSS L^{-1}

Table S2. Relative abundances of members of the core community in the two MFC types (Ref and MPPT). Core community members were limited to those OTU that were found in at least three of the four samples in the MFC. Differences were assayed using a t-test. FDR (False Detection Rate) correction of p_value. n.d. not detected.

		OTU ID	% Rel. Abund. *			SM vs MFC		Ref vs MPPT	
			SM	Ref-MFC	MPPT-MFC	*p* value	FDR corrected *p*	*p* value	FDR corrected *p*
Bacteroidetes	*Parabacteroides*	**2**	n.d.	12.6±4.6	5.9±2.4	0.027	0.075	0.054	0.238
	Uncultured *p-2534-18B5*	**7**	n.d.	3.3±1.3	4.4±1.8	0.014	0.047	>0.1	>0.3
	Uncultured *p-2534-18B5*	**1**	n.d.	11.6±1.9	10.7±2.5	0.000	0.002	>0.1	>0.3
Firmicutes	*Alkaliphilus*	**4**	n.d.	5.3±1.2	5.0±1.3	0.000	0.002	>0.1	>0.3
	Alakalibacculum bacchi	**16**	4.6±0.0	<0.1	<0.1	0.000	0.000	>0.1	>0.3
	Cryptoanaerobacter phenolicus	**18**	3.2±1.1	<0.1	n.d.	0.000	0.000	>0.1	>0.3
	Sedimentibacter	**10**	n.d.	1.2±0.9	4.5±2.2	>0.1	>0.3	0.042	0.224
	Syntrophomonas	**8**	n.d.	3.7±1.9	1.9±0.7	0.062	0.152	>0.1	>0.3
	Syntrophomonas	**13**	n.d.	2.1±1.1	0.5±0.2	>0.1	>0.3	0.061	0.227
	Tissierella	**15**	n.d.	0.4±0.2	2.7±2.0	>0.1	>0.3	>0.1	>0.3
	Thrichococcus pasteurii	**6**	14.4±4.9	<0.1	<0.1	0.000	0.000	>0.1	>0.3
	Turicibacter	**0**	0.2±0.0	47.9±11.3	28.0±12.4	0.011	0.039	0.049	0.237
	Turicibacter	**164**	<0.1	1.4±0.6	2.4±2.1	>0.1	>0.3	>0.1	>0.3
	Uncultured *Clostridiaceae*	**11**	3.8±2.2	1.0±0.5	1.9±1.7	0.092	0.208	>0.1	>0.3
	Uncultured *Clostridiales*	**9**	1.6±0.4	2.1±0.4	2.4±0.7	>0.1	>0.3	>0.1	>0.3
	Youngiibacter fragilis	**22**	2.4±0.0	n.d.	n.d.	>0.1	>0.3	>0.1	>0.3
Proteobacteria	Simplicispira metamorpha	**20**	2.9±3.1	n.d.	n.d.	0.012	0.024	>0.1	>0.3
	Pseudomonas caeni	**3**	33.3±18.0	<0.1	0.2±0.2	0.000	0.001	>0.1	>0.3
	Pseudomonas sp.	**12**	0.4±0.5	<0.1	7.2±5.4	>0.1	>0.3	0.042	0.224
	Uncultured *Oxalobacteraceae*	**23**	0.1±0.2	n.d.	2.0±1.6	>0.1	>0.3	0.045	0.224
WWE1	Uncultured *Cloacamonaceae*	**14**	0.3±0.4	0.3±0.2	2.3±1.8	>0.1	>0.3	0.091	0.241
Synergistetes	Cloacibacillum porcorum	**17**	3.2±0.8	n.d.	<0.1	0.000	0.000	>0.1	>0.3

Chapter 6

Long-term assessment of the six-stacked scaled-up MFCs treating swine manure with different electrode materials

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Abstract

Microbial fuel cells (MFCs) technology is a bio-approach to remove organic matter and nitrogen from wastewater with concomitant production of renewable electricity. Nowadays, it exists a clear interest in moving MFCs towards application. This study aimed to demonstrate MFCs technology feasibility treating swine manure. A couple of 6-stacked MFCs presenting a total volume of 115 L were designed and operated to treat swine manure at 50 $L \cdot d^{-1}$ for more than 6 months. Two different electrodes were tested, one for each stacked MFC: Granular graphite (GG-MFC) and stainless steel mesh (SS-MFC). Organic matter was oxidised in the anode compartments, ammonium was oxidized to nitrate in an external aerated reactor, and nitrate was reduced to dinitrogen gas in the biocathodes. GG and SS-MFCs reached similar organic matter and nitrogen removal rates (1.9±0.3 kg COD m^{-3} d^{-1}; 0.35±0.02 kg N m^{-3} d^{-1}) with power densities between 2-4 Wm^{-3}, being the central units the most electroactive. However, the GG-MFC performance declined overtime due to electrode crushing and the clogging of granular graphite which reduced its applicability in comparison with stainless steel. The application of stacked SS-MFC with mixed electric circuit is a feasible strategy to maintain or even improve treatment efficiencies and power densities when scaling-up MFCs.

Keywords: Organic matter and nitrogen treatment, electric circuit configuration, power production, granular graphite, stainless steel.

1. Introduction

Microbial fuel cells (MFCs) are a technology to treat wastewater while recovering bioenergy.[1] MFCs utilize exoelectrogenic microorganisms as catalysts to convert chemical energy of organic substrate into electricity in the anode compartment.[2,3] The protons and electrons generated migrate to the cathode where reduction reactions take place (either chemically or biologically catalysed).[4]

This technology has been widely tested to treat great variety of pollutants including industrial wastewater in mL-scale reactors, obtaining relevant knowledge about MFC fundamentals.[5,6,7] The ability of MFC is not limited to the treatment of organic matter. Several authors demonstrated simultaneous multiple pollutants treatment (i.e. carbon and nitrogen sources) using both, anode and cathode compartments.[8,9] MFCs became a sustainable wastewater treatment alternative technology with potential advantages over other technologies (i.e. anaerobic digestion), including multiple pollutants treatment, lower energy consumption, smaller environmental footprint and lower sludge generation.[10]

The small prototypes must be scale-up to generate enough electricity for practical applications and future implementations. However, there are some drawbacks in the practical feasibility of scaled-up MFCs, especially with respect to costs, system development and energy recovery.[11] The firsts attempts to scale-up MFCs started a few years ago and they have been developed until today, representing 22% of the total MFC publications in the Web of Science.[12] The initial problematic point was the "underdesigned" scaled-up reactors in terms of total electrode surface area or electrode spacing. Liu *et al.* demonstrated that power density could be maintained during reactor scale-up, increasing the anode surface area.[13] A new challenge on BES scaled-up was the reactor design, for this reason the successive studies were focused on different configurations and media employed. A 2.5L square-MFC treating acetate was used to demonstrate the viability of the technology with a 70% of acetate removed, but achieving low power density (2.3 W m^{-3} NAC) for the high ohmic cell resistance (1.4–1.7 mΩ m^{-3} NAC) of the MFC.[14]

Zhang et al. evaluated 2L tubular-MFCs treating acetate enriched wastewater and urban wastewater with bioelectricity production. Usually, the nitrogen in wastewater is in form of ammonium. It requires a previous oxidation step to nitrate before being removed by a MFC simultaneously with the organic matter. A couple of cathode electron acceptor (oxygen and nitrate) configurations were used to determine their effect on organic matter removal rates and power production.[15,16] The organic matter removal efficiencies were maintained over 60% treating urban wastewater and current production was almost 7 times higher (15 W m^{-3} NAC) than in the previous study. Once nitrogen (in form of nitrate) was incorporated to the medium, 76% of nitrogen was removed in the cathode but the energy production substantially decreased (8 W m^{-3} NAC) because of the coexistence of heterotrophic denitrification with the remaining anodic organic matter.

In spite of these promising attempts, the energy recovery and the volumetric capacities in scaled-up MFC reactors with single units were insufficient. Multiple stacked MFCs started to be tested in order to improve the systems. Different electrical configurations (i.e. series or parallel) were tested in stacked MFCs to achieve higher voltage or current.[17] However, series connection can suffer voltage reversal, contact voltage losses and erratic operation, while in parallel connection internal losses increase, which reduces the total power production.[18]

The firsts attempts of stacked MFCs were performed at 1 L-scale MFCs, including a 12 pairs cassette-electrode MFC, obtaining high organic matter removal rates (5.4 kg COD m^{-3} d^{-1}) and power production (129 W m^{-3} NAC).[19] It was reported a MFC consisting of 4-stacked MFC reactors with a total volume of 20 L, maintained the power density (140 W m^{-3} NAC) respect to mL-reactors.[20] Jiang *et al.* also operated a stacked MFC of 16L with urban wastewater obtaining low removal rates (0.2-1.0 kg COD m^{-3} d^{-1}) with low electricity production (0.4-0.9 W m^{-3} NAC) due to the precipitation of calcium and sodium carbonates in the cathode which increased the internal resistance.[21]

The success of stacking bench-scale MFCs encouraged the scaling-up of the MFC modules towards larger scale systems. In these cases, alternative parallel/series connections were applied in order to charge and discharge the capacitors successively. As a result, a 90 L rectangular MFC reactor vessel achieved sufficient energy treating brewery wastewater that supported its operation.[22] A 200 L modularized MFC system consisting of 96 tubular MFC modules was examined for long-term (one year) performance treating municipal wastewater. The MFC was able to achieve removal efficiencies over 65% for organic matter and nitrogen content but the electricity production was limited to 1W m^{-3} NAC.[23] The biggest MFC pilot plant was constructed in Queensland (Australia) consisting of 12 tubular-MFC modules with a total liquid volume of $1m^3$.[24] The pilot performance was unsuccessful due to low conductivity and high biomass proliferation due to organic matter excess.[25]

The applicability of the scaled-up stacked MFCs was tested working with municipal and brewery wastewater, but there are few examples focused on the treatment of complex matrices as swine manure at this scale. A 1.5L 5-stacked tubular air-cathode MFC was evaluated in terms of simultaneous real swine manure treatment and bioelectricity generation. Although the relatively fast removal rates (between 1.0-3.2 kg COD m^{-3} d^{-1}), the power density was reduced two orders of magnitude respect to simplest wastewater matrices, 4 W m^{-3} NAC.[26] The highest volumetric example consisted of a 3.7 L constructed wetland MFC with an aerated cathode. The achieved COD removal rate and power density achieved were lower to the predecessor study treating swine manure.[27] The low treatment and volumetric capacities of theses reactors, with flows below 4 L d^{-1}, threatens its real applicability.

This study aims to scale-up stacked MFCs for swine manure treatment. A couple of 6-stacked MFCs performances with different electrode, granular graphite (GG) and stainless steel (SS), were evaluated in terms of organic matter and nitrogen removal rates and efficiencies, energy production and material life expectancy. The

evolution of these compounds inside the 6 rectangular MFC units and their electrochemical behaviour were monitored in order to identify the activity differences between units. Finally, several electric connections as series, parallel and mixed (parallel-series) were tested in order to optimise the renewable electricity production.

2. Materials and methods

2.1. Stacked MFCs design

The stacked MFC was designed and operated to remove organic matter and nitrogen from swine manure with concomitant bioelectricity production. The MFCs consisted of six anodic and cathodic compartments (90 x 40 x 1.5 cm each one) hydraulically connected to an external nitrifying reactor (150 cm x 20 cm diameter). The total volume of the MFCs was 65 L while for the external tubular reactor was 50 L (system gross capacity of 115 L). The anode and cathode compartments were placed on opposite sides of a polyvinyl chloride rectangular compartment, clamped transversely together by stainless steel bolts (Fig. 1). Moreover, they were separated by an anion exchange membrane (AMI-7001, Membranes International Inc., USA) to avoid ammonium diffusion to the cathode.

Swine manure was stored in a sedimentation tank where the solid pre-settled. The stacked MFC was continuously fed at a flow rate of 50 L d^{-1}. Anodes and cathodes were connected with a counter current flux. The purpose of the system was to feed the swine manure through the anode set of compartments in order to oxidize the organic matter by exoelectrogenic bacteria. The influent was transferred from the 1^{st} anode to the 6^{th} anode compartment (orange flow, Fig. 1). Then, the anode effluent was used to feed the aerated external nitrifying reactor to oxidize ammonium into nitrate (nitrification) by nitrifying bacteria. Finally, the effluent of the nitrifying reactor was fed to the cathode set of compartments to reduce nitrates into dinitrogen gas (denitrification) by electrotroph bacteria. Cathodes followed the same hydraulic strategy than anode but in the opposite direction, starting the

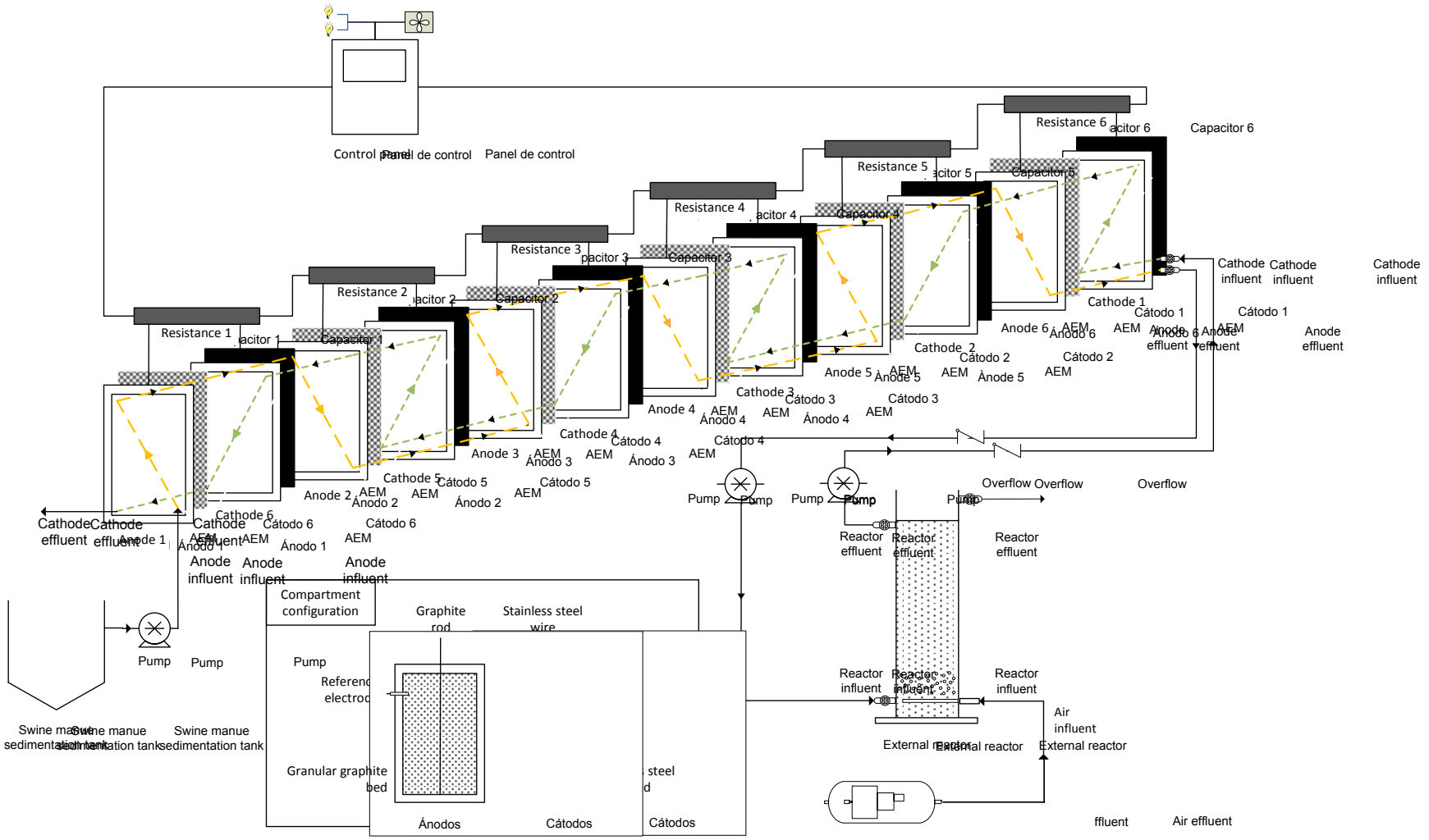

Fig. 1 Schematic diagram of the reactor set up with coloured hydraulic fluxes of the anodes (orange) and cathodes (green). The compartment configuration is also shown, where GG-MFC was filled with granular graphite and a graphite rod, while the SS-MFC was filled with stainless steel mesh and a stainless steel wire. [28]

treatment of nitrate from the 1st cathode and finishing at the 6th cathode (green flow in Fig. 1). The temperature was kept constant at 23±2 ºC.

A couple of configurations were assessed depending on the electrode material. In one of the configurations, granular graphite (model 00514, diameter 1.5e5 mm, EnViro-cell, Germany) was used as electrode material (GG-MFCs) and graphite rods (120 cm x 0.6 cm) as electrode collectors (Mersen Iberica, Spain). The filling material decreased the volumes of the compartments, reaching 20 L net anodic and cathodic compartments (NACs and NCCs, respectively). In the other configuration, a double layer of stainless steel mesh (90 x 40 x 0.1 cm every layer, model 316L, Cisa, Spain) was used as electrode material (SS-MFCs) and stainless steel wires as electrode collectors. The filling material reduced the volumes to 37 L of NACs and NCCs. All configurations had one Ag/AgCl reference electrode in each compartment (+0.197 V vs SHE, model RE-5B, BASi, United Kingdom). Anodes and cathodes were individually connected to an external resistance of 1.5 Ω to close the electric circuit.

In both configurations, an external tubular reactor of PVC with net reactor compartment (NRC) volume of 20 L was built to perform aerobic ammonium oxidation to nitrate (nitrification). The reactor was filled with clay (diameter 0.8 cm) to promote the bacteria adhesion.[9] Aeration from the bottom of the reactor was performed by an air compressor (B2800B/100 CM3 2 CIL, Ingersoll Rand, UK). Dissolved oxygen concentration was controlled and limited to values between 1-1.5 mg $O_2 \cdot L^{-1}$ by a dissolved oxygen probe (Model 50 60, Crison, Spain) to limit the oxygen influence into the anoxic cathodic compartment.

2.2 MFC operation

The anode compartments of MFC reactors were inoculated with the anode effluent of activated sludge from Girona wastewater treatment plant (WWTP) and a parent lab-scale MFC treating swine manure.[9] The nitrifying reactor was inoculated with biomass from a biologic treatment of the same WWTP and with activated sludge from a partial nitritation reactor treating high ammonium landfill leachate.[29] The

cathodes were inoculated with activated sludge and with the effluent of a parent lab-scale denitrifying bioelectrochemical system.[30] The start-up period of both configurations finished after a couple of weeks working at continuous mode (50 L d^{-1}). The continuous operation feeding with swine manure was evaluated during 6 months for each configuration (GG-MFC and SS-MFC).

2.3 Swine manure

Swine manure was taken from the food and agricultural research institute (IRTA) of Monells (Spain). It was stored in a refrigerated sedimentation tank (6 ºC) to promote the settling of solids and minimize degradation over time. The supernatant was fed into 1st anode chamber of the MFCs. Table 1 presents the main characteristics of swine manure supernatant used during the experimental period.

Table 1 Swine manure characteristics. The results are presented as the means ± standard deviation (n=5).

	Swine manure	Units
pH	8.5±0.2	-
Conductivity	8.3±0.4	mS cm^{-1}
Alkalinity	3330±900	mg $CaCO_3$ L^{-1}
COD $_{Total}$	2470±490	mg COD L^{-1}
COD $_{Soluble}$	2290±460	mg COD L^{-1}
BOD_5	1225±125	mg BOD L^{-1}
TKN	305±86	mg TKN-N L^{-1}
NH_4^+	245±50	mg NH_4^+-N L^{-1}
NO_2^-	n.d	mg NO_2^--N L^{-1}
NO_3^-	n.d	mg NO_3^--N L^{-1}
N_2O	n.d	mg N_2O-N L^{-1}
TSS	1150±100	mg TSS L^{-1}

n.d: not detected.

2.4 Electrochemical configuration

The electrochemical connection of stacked MFCs can be arranged in a number of different ways. The electrical connections used for the 6-stacked MFC were (i) individual (normal operation), (ii) in parallel, (iii) in series and (iv) mixed (3 MFCs in parallel and 3 in series), to step-up the current, voltage or both, respectively (Fig. 2).

The external resistances applied under individual MFC connection was 1.5 Ω, in parallel connection were 15 and 100 Ω while in series connection was 2200 Ω in order to be higher than the internal resistance found in polarization curves. The mixed connection joined the MFCs depending on their internal resistances. MFCs 1-6, 2-3 and 4-5 were arranged in series due to their similar internal resistance. Once the MFCs were connected in series, they were arranged in parallel. The resistance applied was 100 Ω.

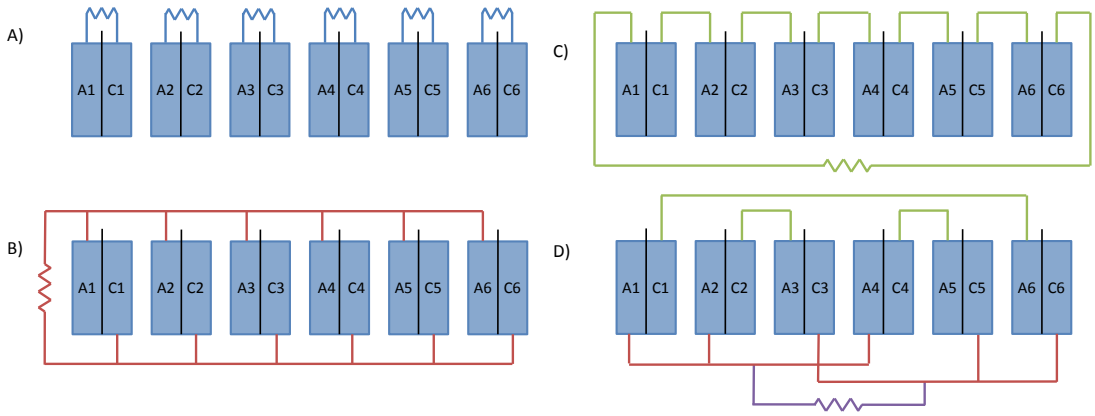

Fig. 2 Schematic representation of the electrical circuit connection in the 6-stacked MFCs. A) individual (1.5 Ω), B) in parallel (15 and 100 Ω), C) in series (2200 Ω) and D) mixed (parallel-series, 100 Ω).

2.5 Chemical and electrochemical analyses and calculations

Liquid-phase standard wastewater measurements for total and soluble organic matter (CODt and CODs), 5-days total and soluble biodegradable organic matter (BOD_5t and BOD_5s), total and volatile suspended solids (TSS and VSS), and nitrogen (total Kjeldahl nitrogen (TKN-N), ammonium (NH_4^+–N), nitrite (NO_2^-–N) and nitrate (NO_3^-–N)) were performed at regular intervals according to the American Public Health Association guidelines.[31] Samples were obtained from the influent (first anode and last cathode) and effluent (last anode and first cathode) sections of the BES reactors. For the analysis of every compartment, the samples were taken from a side opening of each one. The free ammonia (FA) and free nitrous acid (FNA) concentrations were calculated according to Anthonisen *et al.* [32]. Organic (ORR) and nitrogen (NRR) removal rates (kg COD m^{-3} NAC d^{-1} and kg N m^{-3} NCC d^{-1},

respectively) were calculated as the difference between the influent and effluent loading rates. Anode and cathode coulombic efficiencies (CE) were calculated as in Virdis *et al.* [33].

Gas samples were analysed for detecting the presence of carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O) and nitrogen (N_2) gases with an Agilent 7820A GC System equipped with Washed Molecular Sieve 5A and Porapak® Q columns and a Thermal Conductivity Detector (TCD). Nitric oxide (NO) production was considered negligible.[34] Gas production rates were calculated dividing the obtained gas volume per unit of time.

Anode and cathode potentials were monitored with Ag/AgCl reference electrodes (+0.197 V vs SHE, model RE-5B, BASi, United Kingdom). Current (I) and power (P) were determined according to Ohm's laws (I = V/R; P = I × V). Power and current densities were calculated by dividing power and current by the NAC. Polarization curves were performed using a potentiostat (model SP50, Bio-logic, France) and by imposing a linear potential decrease of 1 mV s^{-1} from open circuit voltage (OCV) to a cell voltage of 0 mV and vice versa. The electron balance in the MFC units was calculated as the ratio between the carbon and nitrogen removal concentrations (ratio of C/N) and then, compared to the stoichiometric/theoretical carbon and nitrogen ratio (2.86).

3. Results

3.1. Overall performance of stacked MFCs

3.1.1. Nutrient removal and electricity generation

The stacked MFC operated continuously feeding supernatant swine manure at a mean OLRs of 5.0±0.5 kg COD m^{-3} d^{-1} and NLRs of 0.6±0.2kg N m^{-3} d^{-1} in both configurations, GG-MFC and SS-MFC. Fig. 3 presents the mean values for organic matter and nitrogen compounds during steady state conditions for both

configurations. In terms of organic matter, 850±220 mg COD L^{-1} (36 ±7%) where removed in GG-MFC and 950±270 mg COD L^{-1} (40±15%) in SS-MFCs. Moreover, almost all biodegradable organic matter was consumed along the six units of the stacked MFC (90 and 95% of BOD in GG and SS-MFCs, respectively). Similar removal rate was obtained in GG-MFC (2.1±0.5 kg COD m^{-3} d^{-1}) and SS-MFC (1.6±0.7 kg COD m^{-3} d^{-1}). The anodic CEs of both configurations were similar, 17±4%, suggesting that side reactions as fermentation and/or methanogenesis and other biological

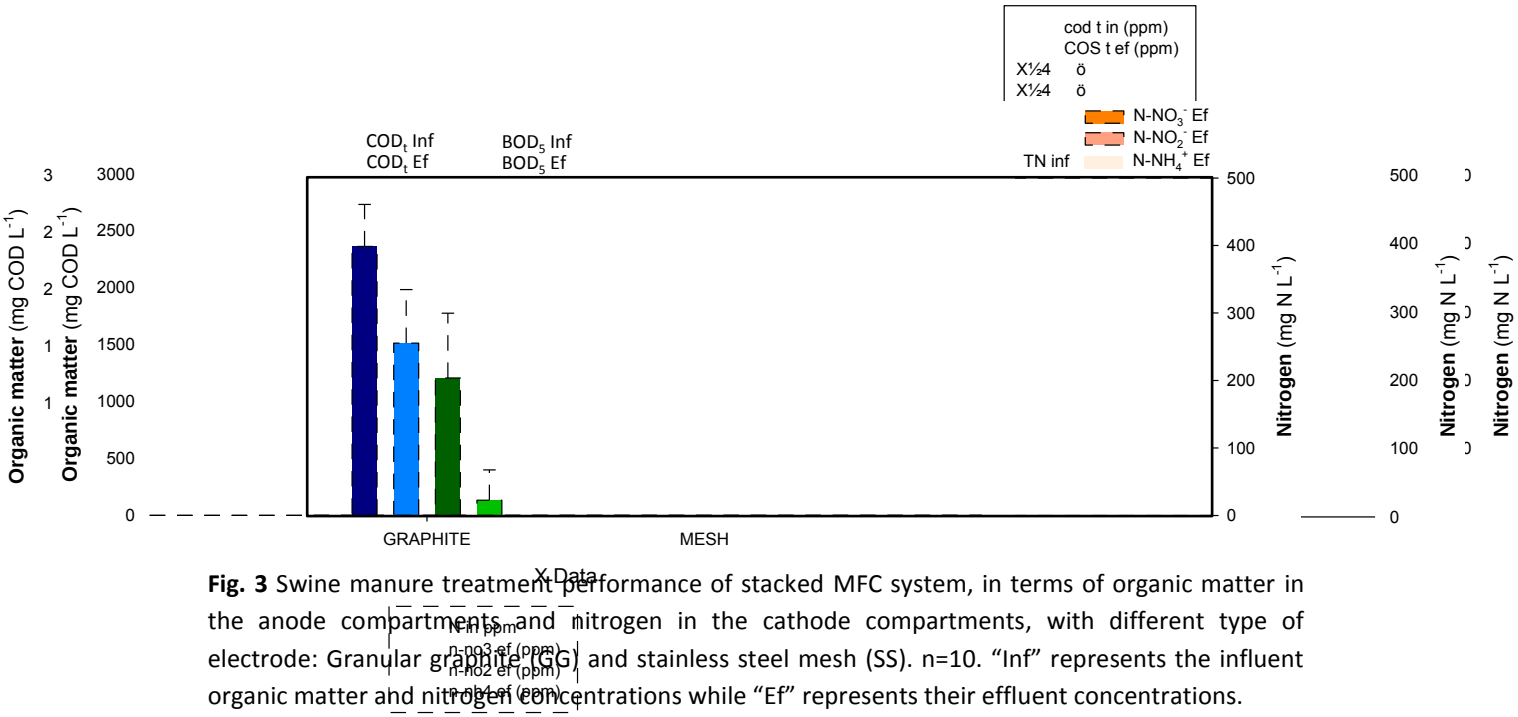

Fig. 3 Swine manure treatment performance of stacked MFC system, in terms of organic matter in the anode compartments and nitrogen in the cathode compartments, with different type of electrode: Granular graphite (GG) and stainless steel mesh (SS). n=10. "Inf" represents the influent organic matter and nitrogen concentrations while "Ef" represents their effluent concentrations.

Nitrogen compounds remained invariable along the anode compartments. In the external aerated reactor more than 95% of ammonium was oxidised to nitrate. Nitrates were removed inside the cathodes, removing 130 mg N L^{-1} (44±10%) in GG-MFC and 170 mg N L^{-1} (56±15%) in SS-MFC. The denitrifying removal rates were similar between configurations, 0.37±0.1 Kg N m^{-3} d^{-1} in GG-MFC and 0.30±0.1 Kg N m^{-3} d^{-1} in SS-MFC. Nitrogen intermediates species presence in both configurations was almost negligible, less than 3% of nitrite was accumulated, while nitrous oxide was not detected. The low cathodic CE achieved (16±3% and 13±2% in GG and SS MFCs, respectively) indicated an alternative process to remove nitrate (e.g. heterotrophic denitrification). In terms of energy recovered, the GG-MFC achieved

slightly higher power densities (3.5±1.2 $W \cdot m^{-3}$ NAC) than SS-MFC (1.9±0.6 $W \cdot m^{-3}$ NAC).

3.1.2. Electrode material assessment for treating swine manure in MFCs

The present study compared two different electrode materials, granular graphite in GG-MFC and stainless steel in SS-MFC at long-term operation. Both materials were chosen for a scaled-up application for its high conductivity, good chemical stability and relatively low cost. Moreover, at mL-scale, both materials allowed a better water flow distribution through the reactors respect to other materials (graphite rod and graphite plate), favouring biomass attachment and consequently, removal efficiency and electricity production, as demonstrated by Vilà-Rovira *et al.* [35].

Granular graphite was used in GG-MFC for 6 months. The granules touch each other and have an intrinsic low porosity of 0.53. A potential clogging effect either from bacteria growth on the electrode or particles from wastewaters could negatively affect its structure. All these effects increased the overpotentials of the cells overtime with the concomitant decrease of energy production and removal rates. At the end of the experimental period, the granular graphite electrode was crushed. Therefore, another material (stainless steel) with similar characteristics to granular graphite in terms of conductivity and costs was used in the other configuration (SS-MFC). Stainless steel showed similar removal efficiencies in the anodes and cathodes, avoiding problematics of clogging or electrode compaction.

3.2 Unravelling dynamics in each MFC unit

The study of nutrient and electrical dynamics of each unit of the stacked MFC was performed with the GG-MFC configuration in steady state conditions. The influent swine manure crossed the anodes one by one and then, the cathodes in the same way but opposite direction, suggesting internal gradients of organic matter and nitrogen, respectively. Fig. 4 presents the evolutions of the concentrations of organic matter and nitrogen compounds along the stacked MFC units.

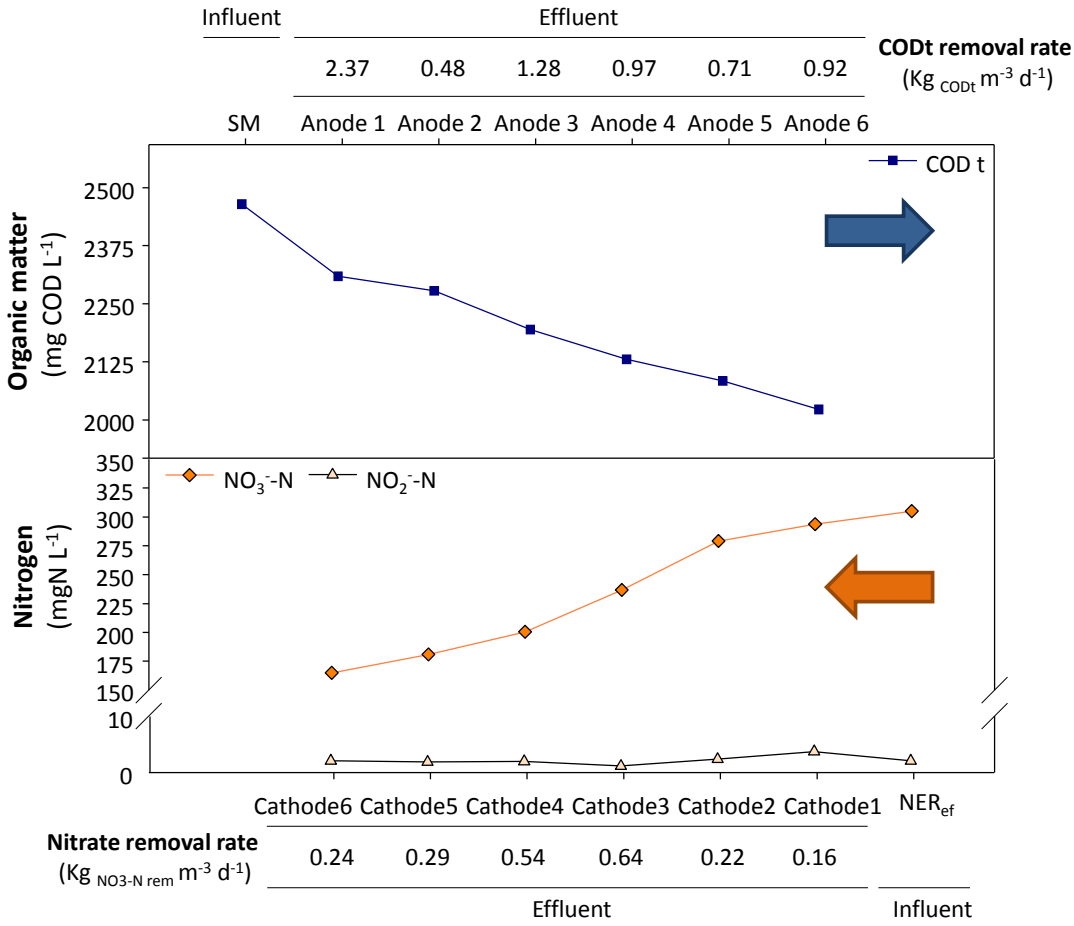

Fig. 4 Organic matter (blue) and nitrogen (orange) concentrations and rates evolution inside the anodes and cathodes units of the stacked GG-MFC, respectively. The samples were obtained from the effluent of each unit. The influent concentration was also analysed, where SM is swine manure and NERef is the effluent of the nitrifying external reactor. Arrows indicated the hydraulic flux direction.

The organic matter concentration inside of the anode compartment decreased gradually. The oxidation rate tendency showed high oxidation rates in the first compartments and lower at the last ones. The maximum organic matter removal rate (2.37 kg COD m^{-3} d^{-1}) was obtained in the first compartment. In this case, the solids from swine manure, not decanted in the refrigerated settler tank, were partially retained in the 1st anodic compartment. From then on, the central units (Anodes 3 and 4) showed higher removal rates (1.28 and 0.97 kg COD m^{-3} d^{-1}, respectively) than the contiguous units. In the last anode, the oxidation rate diminished slightly to 0.92 kg COD m^{-3} d^{-1}. Around 2 g L^{-1} COD of organic matter remained in the liquid due to the low anodic HRT applied (9.6 hours).

A different behaviour was observed for the nitrogen treatment. The central cathodic units (cathodes 3 and 4) were the units with the highest denitrifying rates (0.64 and 0.54 kg NO_3^--N m^{-3} d^{-1}, respectively). The lowest denitrifying rate was obtained at the 1st cathode, where the influence of aerated external reactor could negatively influence the process. Nevertheless, nitrate was not completely reduced in the cathodes (0.17 g L^{-1} NO_3^--N), which certainly indicates that some limitation existed for denitrifying bacteria in this system.

The theoretical electron balance between carbon (mg COD L^{-1}) and nitrogen (mg N L^{-1}) for complete removal is 2.86. The units 1 and 6 showed values far from the theoretical ratio (9.94 and 5.58, respectively), which is in line with high organic matter removal rates in the first unit due to the solid removal effect and, low denitrification removal rates in the last unit due to the influence of the external reactor. The central units had C/N values closer to 2.86 (1.63, 2.35, 1.53 and 3.23, for units 2, 3, 4 and 5, respectively) than the peripheral units, which indicated a better balanced electron flux between the anodes and cathodes. These results fitted with the electrochemical performance of each individual MFC (Fig. 5) where the fastest treatment rate corresponded to the unit 3 that showed the highest power density, 0.23 W m^{-3} NAC.

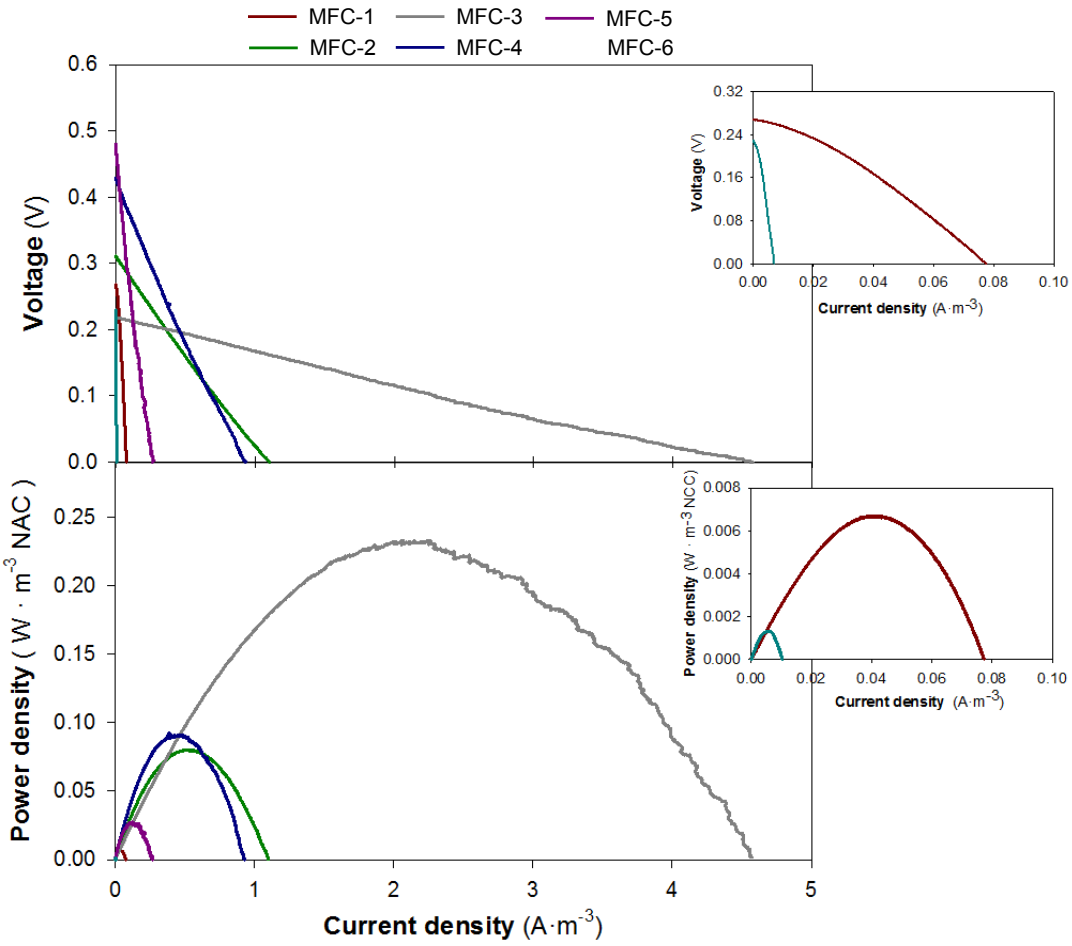

Fig. 5 Polarization and power density curves of the 6 units making up the stacked GG-MFC.

3.3. Electrochemical characterization of the stacked MFCs

Different electric circuit conditions were tested to enhance renewable electricity production. The electrical connections tested for the 6-stacked MFC were in parallel, in series and mixed (3 MFCs in parallel and 3 in series), to step-up the current, voltage or both, respectively. The parallel circuit connection under different resistances (15 and 100 Ω) showed the lowest internal resistance (< 4 Ω) and the highest current densities (2-3 A m^{-3} NAC in both cases) among the set of electric connections tested (Fig. 6). On the contrary, the series connection increased the internal resistance of the system and decreased the intensity and energy obtained. In addition, occasional problems with voltage reversal from two of the six MFCs (1st and 6th MFCs) indicated that this electrical configuration was not the ideal for this

system. The mixed circuit substantially reduced the internal resistance of the stacked MFC respect to series circuit, and maintained the current density (1.89 A m^{-3}) of parallel circuit. This combination resulted in the highest power density (0.33 W m^{-3}) achieved. Furthermore, there were no problems of voltage reversal as in series connection.

Electric circuit connection

	Parallel		Series	Mix
	15 Ω	100 Ω	2200 Ω	100 Ω
Current density (A m^{-3})	1.88	3.00	0.01	1.89
Cell voltage (V)	0.23	0.34	1.55	0.70
Internal resistance (Ω)	3.80	3.25	3419.84	11.61
Power density (W m^{-3})	0.12	0.27	0.03	0.33

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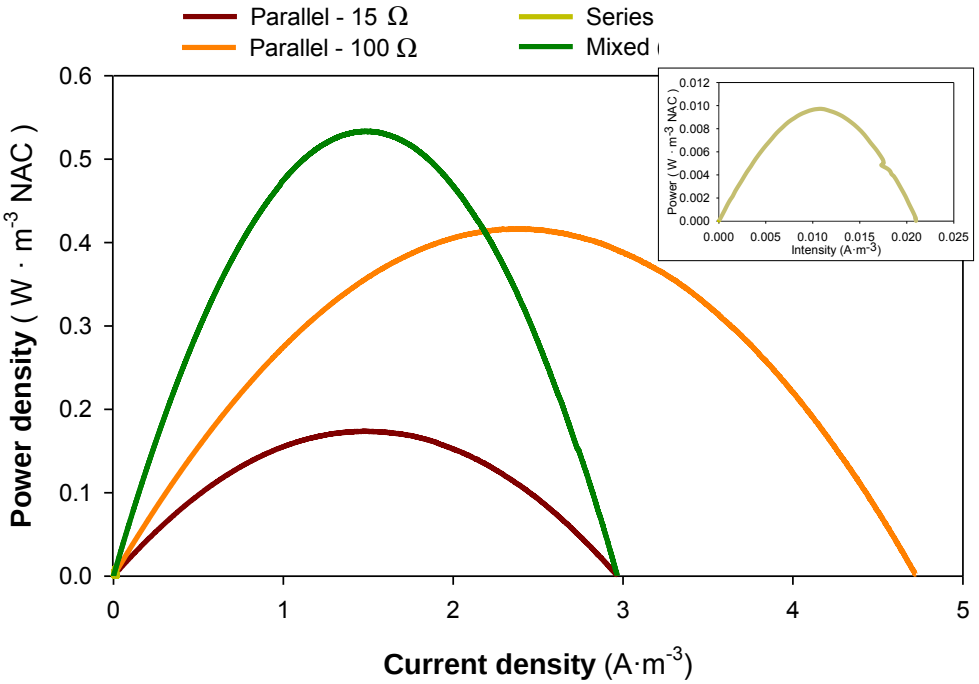

Fig. 6 Power density curves of the stacked MFCs connected at different electric circuit connections: parallel (15 Ω and 100 Ω), series (2200 Ω) or mixed (100 Ω).

4. Discussion

4.1. Assessment of electrode material for 6-stacked MFCs

The performance of the 6-stacked MFCs under different electrode materials were evaluated and compared with previous researches reports using synthetic, urban or industrial media as influent (Table 2). In this study, more than 90% of BOD (1.9±0.3

kg COD m^{-3} d^{-1} removal rate; CEs of 17±4%) and 50% of the total nitrogen content (0.35±0.02 kg N m^{-3} d^{-1} removal rate; CEs of 15±2%) were removed, achieving power densities between 2-4 Wm^{-3} in both configurations. The COD removal rates and CEs achieved were below the ranges reported using synthetic media, where the removal rates ranged between 2.5-5.5 Kg COD m^{-3} d^{-1} with the corresponding 48% and 28% of CE.[19] Real wastewater influents showed lower removal rates and CEs respect to synthetic media, achieving values between 0.1- 1.0 kg COD m^{-3} d^{-1} with CEs generally lower than 10%.[21,22,23] Several authors related it to the toxic effect of the intermediate products accumulation of the complex reactions taking place, which reduces the organic matter removal efficiency and to the high complex nature that promote side reactions as fermentation and/or methanogenesis and other biological processes, reducing the CEs achieved.[36,37]

No scaled-up BESs over 4 L for swine manure treatment have been reported in literature. A 1.5L 5-stacked tubular MFC working at a similar OLR was able to achieve higher organic rates than the ones presented in this study (3.23 kg COD m^{-3} d^{-1}; 66% of COD removed) but achieved CEs lower than 0.2%. These results indicated an almost non-electrogenic treatment of the organic matter. Once the OLR was reduced, the treatment efficiencies increased (83% COD removal) but the removal rate was reduced to 0.99 kg COD m^{-3} d^{-1} and the CEs did not improve. This effect was also demonstrated at mL-scale with other wastewaters, obtaining higher treatment efficiencies (over 80%) when the OLR decreased.[38,39] In these cases, the lower OLR corresponded with longer HRTs that allowed more exposition time of organic matter by bacteria, increasing their degradation.[40,41] The biggest BES tested was a constructed wetland MFC of 3.7 L. Increasing the volumetric capacity, the achieved COD removal rate was reduced to 0.19 kg COD m^{-3} d^{-1} and the CE was maintained at values lower than 1% (77% of COD removal efficiency) at a low OLR of 0.83 kg COD m^{-3} d^{-1}.[27] This result was in accordance with the tendency of all scale-up reactors, independently from the liquid source.[21,23] The 6-stacked MFC evaluated

Table 2 MFC configuration and operation, organic matter and nitrogen treatment performance of single and/or stacked scale-up MFCs. The power density results of the stacked MFCs were calculated taking into account an individual electric connection.

Influent wastewater	Configuration	Net Volume	Organic matter removal				Nitrogen removal				Power density	References
			OLR	ORR	ORE	Anode CE	NLR	NRR	NRE	Cathode CE		
		L	kg COD m^{-3} d^{-1}	kg COD m^{-3} d^{-1}	%	%	kg N m^{-3} d^{-1}	kg N m^{-3} d^{-1}	%	%	W m^{-3}	
Synthetic wastewater	12-chamber cassette MFC	1	2.90 5.80	2.70 5.39	95 93	48 28	No nitrogen treatment performed				117 129	[19]
Brewery wastewater	5-stacked dual chamber MFC	90	0.16 0.27	0.14 0.23	88 85	8 19	No nitrogen treatment performed				97 56	[22]
Urban wastewater	12-stacked dual chamber MFC	16	0.24 1.08	0.21 0.99	88 92	<1 <1	0.05 0.01	0.02 0.01	30 30	n.d. n.d.	<1 <1	[21]
	96-stacked dual chamber MFC	200	0.31	0.12	38	n.d.	0.05	0.03	68	n.d.	8	[23]
Swine manure	Single wetland MFC	3.7	0.83	0.19	77	<1	No nitrogen treatment performed				<1	[27]
	5-stacked single chamber MFC	1.5	1.20 4.90	0.99 3.23	83 66	<1 <1	0.11 0.52	0.09 0.41	87 80	n.d. n.d.	4 n.d.	[26]
	6-stacked dual chamber GG-MFC	60	5.00±0.50	2.10±0.50	36±7	17±3	0.75±0.30	0.37±0.10	44±10	16±3	4±1	This study
	6-stacked dual chamber SS-MFC	94	5.00±0.50	1.60±0.70	40±15	17±4	0.41±0.10	0.30±0.10	56±15	13±2	2±0	This study

in this study allowed the increase of the net volume reactor between 16 and 25 times respect to the biggest MFC designed to treat swine manure, achieving similar removal rates to 1.5 L MFC.[26,27] Moreover, the configuration used in this study was able to establish and maintain the electrogenic process at long-term.

BES was proposed as a new alternative for nitrogen treatment in swine manure wastes due to the lack of elimination of this compound in anaerobic digestion.[42] The development of a sustainable and robust system for nitrogen removal is still missing. No BES scaled-up attempt for nitrogen treatment has been reported until this study. Similar nitrifying efficiencies (over 90% in both cases) and denitrifying removal rates (0.37±0.1 Kg N m^{-3} d^{-1} in GG-MFC and 0.30±0.1 Kg N m^{-3} d^{-1} in SS-MFC) were achieved between the electrode materials, with cathodic CEs of 16±3% and 13±2% in GG and SS MFCs, respectively. In terms of nitrogen, the configuration showed in this study was already tested in mL-scale.[9,33,43] These studies were able to achieve similar efficiencies for ammonium oxidation (>90%) respect to the ones presented in this study, while lower nitrates reduction rates were achieved (0.40, 0.01 and 0.16 kg N m^{-3} d^{-1}, respectively with the corresponding CEs, 70-80%, 20% and 10%). The higher removal efficiencies corresponded to the higher HRTs (1-5 d) and lower nitrogen concentrations (40 mgN L^{-1}). The low CE achieved indicated an alternative process to remove nitrate. In the present study, the migration of nitrates to the anode through the membrane and heterotrophic nitrate removal could explain it. However, nitrogen species, as nitrate or nitrite, were not detected in the anode compartments. These results suggested that the bioelectrochemical nitrate reduction is the limiting step for nitrogen treatment from swine manure using BES.

In terms of energy recovered, the GG-MFC achieved a power density of 4±1 $W \cdot m^{-3}$ while in the SS-MFC was 2±1 $W \cdot m^{-3}$. The results obtained were in agreement with the other stacked MFC treating swine manure, which obtained a power density of 4 W m^{-3}

even if usually lower values were obtained treating swine manure (0.02 W m^{-3} in Zhao *et al.* [27]) and urban wastewater (0.35-0.90 W m^{-3} in Jiang *et al.* [21]).[26] The studied stacked MFC allowed the maintenance of the removal rates and the power output achieved for the mL-scale stacked MFCs treating complex wastewater matrices.

In all BES scaling-up to treat swine manure, a carbon electrode was used as electrode material bed, either for anode and cathode compartments.[26,27] A couple of electrode (GG-MFC and SS-MFC) were evaluated in the present study for organic matter and nitrogen treatment, obtaining similar removal rates and electricity generation. The results suggested that granular graphite electrode material was not appropriate at long-term operation for any compartment (anodes and cathodes), obtaining graphite blocks that did not allow the appropriate liquid distribution inside the compartment. Moreover, the presence of solids (1.2 ± 0.1 g TSS L^{-1}) caused packing of the anode compartments and it could also negatively influence the membrane functionality. Respect to the stainless steel electrode material, the high corrosion risk could compromises its applicability in scaled-MFCs, as demonstrated by Ledezma *et al.* [44], but it was not appreciated in the six anodes used in this study, besides its potentials (-0.15±0.06 V vs. SHE) were nearby its standard oxidation potential (-0.21 vs. SHE at pH 7).[45] The reductive nature of the cathodes protected the stainless steel against corrosion, being a good option for long-term treatment of wastewaters.

4.2. Performance of individual MFC units

The study of nutrient and electrical dynamics of each individual unit of the stacked MFC concluded that the central units were the most electro-active compartments, which had the highest organic matter and nitrogen removal rates (1.28 and 0.97 kg COD m^{-3} d^{-1}; 0.64 and 0.54 kg NO_3^--N m^{-3} d^{-1}) and power densities (0.23 and 0.09 W m^{-3} NAC). These results agreed with the electron balance performed between the anode and cathodes which were near the stoichiometric carbon/nitrogen ratio (central units),

indicating that, apparently, no electron limitation existed for carrying about the two bioelectrochemical processes (carbon oxidation and nitrate reduction). On the contrary, the high organic matter removal rate of the extreme anode compartments (peripheral units) could be explained by the high complexity of organic matter and the solid retention. The nitrogen treatment in the cathodes was limited by the dissolved oxygen in the liquid phase coming from the external reactor. In general, nitrate bioelectrochemical removal was limited, since nitrate was not completely reduced from the cathodes. The operational HRT applied as well as intrinsic overpotentials (reactor size, reactor configuration, etc.) could have played a role on this limitation. Zhuang *et al.* demonstrated that complete removal efficiencies could be achieved increasing the HRT but this negatively affect the power density generated.[26] Other authors that used a similar hydraulic fluid reactor observed different performances in terms of organic matter treatment and energy production between the stacked MFC units respect to the ones observed in this study.[5,26,46] Their studies support that the most electroactive compartment is the last one, where the substrate is more accessible. In our configuration, the solid retention and exhaustion of the BOD in the anodes and, the low HRT and influence of the external reactor in the cathodes made the central unit the most electroactive.

4.3. Electric configuration optimization

Several scaled-up reactors were electrically configured in parallel or in series circuits, achieving the highest current and power densities of their systems, respectively.[17,18,26,46] However, the mixed circuit substantially lowered the internal resistance of the stacked MFC, leading to a higher power density (0.33 W m^{-3}) at relatively higher current density (1.89 A m^{-3}). Furthermore, there were no problems of voltage reversal as in series connection. The high number of parallel connection applied in the mixed circuit greatly enhanced the power and current densities achieved, as

demonstrated by Papaharalabos *et al.* [47]. Consequently, the mixed circuit was considered the most appropriate connection due to its high applicability. The maximum power density achieved with this circuit will consequently reduce the number of units required for the other electric circuits to obtain the same power output while the high current densities will allow the better workability of the cathodic reaction, and thus a higher denitrification rates might be expected.

5. Conclusions

The results allowed a better understanding of the real potential of MFCs, highlighting the challenges of MFC scaling-up for swine manure treatment towards application. Low HRT applied (9.6 hours in anodes and cathodes) did not allow the complete degradation of the organic matter and nitrogen compounds, but the biodegradable fraction was almost completely consumed. High organic matter and nitrogen removal rates 2.1±0.5 and 1.6±0.7 kg COD m^{-3} d^{-1} and, 0.4±0.1 and 0.3±0.1 kg N m^{-3} d^{-1}, for GG and SS-MFCs, respectively were reached. In both cases, anodic and cathodic electrogenic activities were around 15%, achieving power densities of 4±1 and 2±0 W m^{-3} NAC for GG and SS-MFCs, respectively. The values were considered high due to the complexity of the organic matter in swine manure, which could have promoted side reactions. The clogging effect of the granular graphite bed used in GG-MFC reduced its applicability at long-term operation. The stainless steel used in SS-MFC should be considered as a promising material for scaled-up reactors due to its easy applicability, cheap price, high treatment efficiencies, no clogging effect neither corrosion problematic. The treatment capacity of the different units showed that the central units were the most electroactive of the stacked MFC. Moreover, the utilization of a mixed electric circuit between units increased the power and current density achieved. The application of this configuration could be a feasible strategy to maintain or even

improve treatment efficiencies and power densities when scaling-up MFCs towards application.

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Chapter 7

Microbial electricity driven anoxic ammonium removal

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Apart from the carbon cycle the globally most important nutrient cycle is the conversion of nitrogen species. Thereby the oxidation and reduction of nitrogen does not only play a key role in nature, but also in engineered systems. Most important is the high energy demand for removal of ammonium during conventional aerobic wastewater treatment, currently causing a consumption of 4.6 kWh per kg of nitrogen. Here we present the complete conversion of ammonium to dinitrogen gas (N_2) using bioelectrochemical systems without accumulation of intermediates like nitrate (NO_3^-), nitrite (NO_2^-) or nitrous oxide (N_2O). We demonstrate the feasibility of this electricity driven anoxic ammonium removal in continuously operated reactors with 35 g N m^{-3} d^{-1} at the litre-scale and elucidate the underlying bioelectrochemical transformations. The electricity driven process requires more than 35 times less energy as - in contrast to the conventional process – no aeration is needed. This article shows the technical feasibility and indicates the economic viability of the anoxic ammonium removal and discusses future engineering needs.

Wastewater treatment is a highly energy demanding process. The removal of organic matter and nitrogen (mainly ammonium; NH_4^+) as the main hazardous products in sewages is necessary for protecting the quality of the water bodies.[1] Activated sludge treatment is the main process for treating organic matter and nutrients (nitrogen and phosphorus).[2] Figure 1 schematically summarizes the most relevant steps for nitrogen removal. During conventional nitrification, ammonium is oxidized aerobically to nitrite (nitritation) and then to nitrate (nitratation) by two functional groups of microorganisms (ammonia-oxidizing bacteria, AOB and nitrite-oxidizing bacteria, NOB). Alternatively, complete nitrification (from ammonium to nitrate) is performed by single microorganisms.[3] Subsequently, nitrate is reduced to dinitrogen gas usually heterotrophically that is using organic carbon as electron source. In wastewater treatment plants (WWTP) about 4.6 kWh per kg of nitrogen are required for aeration.[4] This energy consumption sums up to about half a million Euros per year in an average European WWTP with influent flow rates of $5.5x10^4$ m^3 per day (0.58±0.17 € kg^{-1}N

oxidized) (See ESI, † 2.4, for calculation). This is up to one-third of the total operational cost of a WWTP, not yet considering a potential limitation of organic matter.[5]

An alternative approach for nitrogen removal is the use of anaerobic ammonium-oxidizing (anammox) bacteria.[6] These bacteria can oxidize ammonium using nitrite as electron acceptor to mainly dinitrogen gas and some nitrate (Fig. 1). The anammox process can deal with increasing nitrogen loads in a cost effective way in respect to the conventional treatment[7,8], but the growth of anammox bacteria is relatively slow (doubling time of 15-30 days, which could be optimized to 3 days when the adequate cultivation conditions are imposed, e.g. low solid retention time)[9]. Moreover, the limits of the operational conditions are narrow (temperature, pH) and a previous aerobic nitritation process to obtain nitrite is required.[10] The anammox technology is established with 109 full-scale installations operating in the world in 2014 (75% for side stream treatment of municipal wastewater)[11], but still the stable supply of appropriate nitrite levels is the most challenging factor.[12] Moreover, remaining intermediate products as nitrite (NO_2^-) and nitrate (NO_3^-) in the effluent entail additional treatments *a posteriori.* Avoiding the disadvantages of the so far established technologies, we suggest an alternative effective autotrophic and anoxic nitrogen removal strategy based on bioelectrochemical systems (BES).[13]

Chapter 8

Discussion

8.1. BES for carbon and nitrogen treatment from complex wastewater

The main objective of this PhD thesis was to develop a sustainable treatment for carbon (organic matter) and nitrogen from industrial wastewaters, such as swine manure. Bioelectrochemical systems (BES) were used as the technological framework to achieve this objective.

Before this PhD thesis started, some researchers had already reported simultaneous organic matter and nitrogen removal in BES (Virdis *et al.*, 2008; Zhang and He, 2012). In those studies, a synthetic influent was tested, containing acetate and ammonium. In all cases, ammonium removal was performed in a completely or partially aerated compartment in order to reduce oxygen supply costs (Zhang and He, 2012; Virdis *et al.*, 2008), but alternative anoxic treatment for ammonium was not studied. However, the treatment of industrial wastewater (e.g. swine manure) was limited to few studies (Kim *et al.*, 2008a; Min *et al.*, 2005a). In these cases, authors mainly focused on the organic matter treatment, paying little attention to nitrogen removal. The final objective of treating industrial wastewaters should be scaling-up BES towards its applicability. In literature, two main strategies have been used to scale-up BES for industrial wastewater treatment: i) Single BES (Zhao *et al.*, 2013) or ii) Stacked BES (Zhuang *et al.*, 2012a). These first scaled-up studies were a nice starting point, although they were still far away of the volumetric capacity and flow requirements for a real BES application. The hydraulic retention times (HRTs) were between 1-5 days, which threatens its real applicability because it would require large BES reactors, thus implying a higher CAPEX.

In this context, this PhD thesis uses a multidisciplinary approach (Chemistry, Biology and Engineering perspectives) for improving the BES understanding and the optimization for simultaneous carbon and nitrogen treatment in complex matrices. Following this objective, reactors presenting different configurations and scales were assembled, operated and analysed (Table 8.1).

Table 8.1 Reactor configurations set-up studied in this thesis.

	Chapter 4		Chapter 5		Chapter 6		Chapter 7
	C-1	**C-2**	**Ref**	**MPPT**	**Scaled GG**	**Scaled SS**	**niBES**
Reactor volume	mL-scale	mL-scale	mL-scale	mL-scale	L-scale	L-scale	mL-scale
Electron Donor	OM	OM	OM	OM	OM	OM	NH_4^+
Electron acceptor	NO_3^-	NO_3^-	O_2	O_2	NO_3^-	NO_3^-	H_2 / NO_3^-
Membrane	AEM	CEM	AEM	AEM	AEM	AEM	AEM
Electrochemical configuration	MFC	MFC	MFC	MFC	MFC	MFC	MEC 3-electrodes
External resistance	Fixed	Fixed	Fixed	Variable	Fixed	Fixed	-
Electrode material	GG	GG	GG	GG	GG	SS	GG

OM: Organic matter; AEM: Anion exchange membrane; CEM: Cation exchange membrane; MFC: Microbial fuel cell; MEC: Microbial electrolysis cell; GG: Granular graphite; SS: Stainless steel.

8.2. Carbon removal in a BES bioanode treating complex wastewater

8.2.1. Effect of the reactor configuration on organic carbon removal and energy production

Different reactor configurations were studied in order to determine the viability of BES for the treatment of complex matrices, such as swine manure. For these reasons, different cathode electron acceptors, membranes, external resistances controls, reactor sizes and electrode materials were tested from Chapters 4 to 6 (See Table 8.2 for set-up configuration).

Table 8.2 Reactor set-up, removal capabilities and electricity production of scale-up studies in this thesis.

	C-1 (Chapter 4) (Vilajeliu-Pons *et al.*, 2015)	**C-2 (Chapter 4)** (Vilajeliu-Pons *et al.*, 2015)	**Ref (Chapter 5)** (Vilajeliu-Pons *et al.*, 2016)	**MPPT (Chapter 5)** (Vilajeliu-Pons *et al.*, 2016)	**Scaled GG (Chapter 6)**	**Scaled SS (Chapter 6)**
Set-up						
Electrode	GG	GG	GG	GG	GG	SS
External resistance	Fixed, 30 Ω	Fixed, 30 Ω	Fixed, 30 Ω	Variable	Fixed, 1.5 Ω	Fixed, 1.5 Ω
Cathodic process	DN	SND	Oxygen reduction	Oxygen reduction	DN	DN
Membrane	AEM	CEM	AEM	AEM	AEM	AEM
Anode potential *(V vs SHE)*	−0.24 ± 0.04	-0.42 ± 0.04	-0.27 ± 0.02	-0.25 ± 0.06	-0.15 ± 0.06	-0.16 ± 0.06
Flow *($L\ d^{-1}$)*	2.9 ± 0.1	2.9 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	50.0 ± 1.0	50.0 ± 1.0
HRT *(d)*	0.16	0.16	0.26	0.26	0.40	0.74
Removal capabilities						
Organic matter removal rate *($Kg\ COD\ m^{-3}\ d^{-1}$)*	2.1 ± 0.7	2.0 ± 0.6	4.0 ± 2.5	4.4 ± 2.7	2.1 ± 0.5	1.6 ± 0.7
Organic matter removal efficiency *(%)*	15 ± 6	15 ± 3	38 ± 18	36 ± 16	36 ± 7	40 ± 15
Anodic CE *(%)*	24 ± 9	5 ± 4	6 ± 3	17 ± 7	16 ± 3	13 ± 2
Electricity production						
Current density *($A\ m^{-3}$ NAC)*	26 ± 3	6 ± 2	14 ± 2	33 ± 10	12 ± 5	7 ± 3
Power density *($W\ m^{-3}$ NAC)*	20 ± 2	2 ± 1	3 ± 1	5 ± 1	4 ± 1	2 ± 1

GG: Granular graphite; SS: Stainless steel; DN: Denitrification; SND: Simultaneous nitrification-denitrification; AEM: Anion exchange membrane; CEM: Cation exchange membrane.

The firsts reactors designed (C-1 and C-2, Chapter 4) were used to determine the viability of simultaneous organic matter oxidation in the anode and nitrogen removal (either in the external reactor and/or the cathode) with renewable electricity production. The success of the treatment relied not only on the bioanode performance (electron donor), but also on the biocathode (electron acceptor). Both configurations oxidised organic matter in the anode but they differed on the cathode configuration for nitrogen treatment. In C-1, nitrate was the sole cathode electron acceptor, while oxygen and nitrate were the cathode electron acceptors in C-2. The oxygen reductive power is higher (+0.8 V vs SHE) than the nitrate/nitrite reductive power (+0.4 V vs SHE) (Ferguson and Richardson, 2004). In consequence, C-2 was expected to present a higher cell voltage and, consequently, power output than C-1, but it was not.

Moreover, different membranes were chosen considering the future application (AEM in C-1 and CEM in C-2) and taking into account the knowledge achieved by Virdis *et al.*, (2008). On the one hand, AEM prevented the ammonium diffusion from the anode to the cathode and it could also imply larger power densities than using CEM (Kim *et al.*, 2007). For these reasons, AEM was chosen for the configuration with the external nitrifying reactor (C-1). On the other hand, CEM was proposed for the system with simultaneous cathodic nitrification-denitrification (SND). CEM would allow ammonium diffusion from the anode to the cathode, allowing a lower ammonium impact to anode biomass and higher substrate availability for ammonia-oxidizing bacteria present at the cathode. Table 8.2 summarizes the results obtained.

The anode compartments of C-1 and C-2 achieved similar organic matter removal rates (2.0±0.4 kg COD m^{-3} d^{-1}) and efficiencies (15±5%) by treating swine manure. The differences on the membranes applied to C-1 and C-2 did not influence the removal rates but the cathodic electron acceptor influenced on the electroactivity of the anode compartment microorganisms. The CE showed differences between configurations (24±9% in C-1 compared to 5±4% in C-2). These results were in accordance to the higher current and power densities measured in C-1 (26±3 A m^{-3}

NAC and 20±2 W m^{-3} NAC), compared to C-2 (6±2 A m^{-3} NAC and 2±1 W m^{-3} NAC). Besides C-2 presented the combination of O_2/NO_3^- as cathode electron acceptor, it had a lower electricity production than C-1 (NO_3^- as cathode electron acceptor). It could be hypothesized that C-2 presented lower power output because of the possible oxygen diffusion from the cathode to the anode through the membrane (diffusivity constant of 4.3 ·10^{-6} cm^2 s^{-1} (Kim *et al.*, 2007)).

Although both configurations (C-1 and C-2) treated organic matter at the same removal rate, C-1 achieved the highest power density. Hence, according to these results we can conclude that the configuration C-1, with an anoxic cathode and an external nitrifying reactor, was the most appropriate configuration for organic matter oxidation and electricity production.

In order to put into context the results obtained in C-1 and C-2, Table 8.3 summarizes the results obtained in the BES literature for organic matter treatment. In general, BES fed with complex matrices as swine manure wastewater showed lower organic matter removal efficiencies and electroactivities respect to synthetic media. Usually, CEs around 90% can be observed using synthetic medium, while it decreases to <10% when using complex wastewaters, such as municipal wastewater (He *et al.*, 2014), bakery, diary and brewery wastewater (Velasquez-Orta *et al.*, 2011; Wen *et al.*, 2010), landfill leachate (Puig *et al.*, 2011b) or swine manure (Kim *et al.*, 2008a; Min *et al.*, 2005) (Table 8.3). The values observed in C-2 (CE = 5%) are in accordance to this range, but CE in C-1 was notably higher (CE = 24%). The low CEs achieved with swine manure can be explained by its complexity, which may limits the substrate accessibility for the bacteria (Reddy *et al.*, 1981). Moreover, the accumulation of intermediate products can produce a toxic effect (e.g. volatile fatty acids) that deteriorates the organic matter removal efficiency (Behera and Ghangrekar, 2009). In the case of complex substrates, lower CEs indicate the coexistence of side reaction (i.e. methanogenesis, fermentation or bacterial growth). This phenomenon was previously observed in MFCs by Oliveira *et al.*, (2013) and specifically for methanogenesis, which was shown to decrease the final CE of the system (Sleutels *et al.*, 2011). However, methane was not detected in our

configurations (C-1 and C-2), indicating that methanogenesis did not have a significant impact on organic matter depletion.

Comparing the BES electroactive workability under different cathode electron acceptors like nitrate (NO_3^-) and oxygen (O_2), it can be observed that the use of O_2 at the cathode resulted in higher removal efficiencies and coulombic efficiencies (over 90% both) (Rabaey *et al.*, 2003) than using NO_3^- as electron acceptor (Zhang and He, 2012; Virdis *et al.*, 2008). The organic removal efficiencies were over 70% with coulombic efficiencies around 50% when NO_3^- was the electron acceptor. These results are in accordance to the higher oxygen reduction potential previously mentioned. However, the results found at C-1 and C-2 differed, since a higher anode CE was observed when using NO_3^- as cathode electron acceptor (C-1). The possible oxygen diffusion through the membrane in C-2 could explain its low power output.

In order to evaluate the bioanode behaviour treating swine manure without electron acceptor limitations, a MFC with the same architecture as C-1 and C-2 but with an oxygen reducing cathode was assembled (Ref, chapter 5) (Table 8.2). In this case, the oxygen was supplied until saturation. The results obtained in the Ref-MFC caused the doubling of the organic matter removal rate (around 4 kg COD m^{-3} d^{-1}) compared to C-1 and C-2 (around 2 kg COD m^{-3} d^{-1}) (Chapter 4). The CE observed in Ref (6%) was similar to C-2 (5%), which presented the SND cathode. However, C-2 (6 A m^{-3}) presented only half of the current density achieved by Ref (14 A m^{-3}). That difference could be explained by multiple processes co-occurring inside the compartment, where ammonium oxidation by nitrifiers caused a partial consumption of the oxygen, reducing the availability of this electron acceptor in the cathode. Our results confirmed the same behaviour than the observed by other authors working with complex matrices and oxygen electron acceptor (Table 8.3).

Table 8.3 Representative compilation of BES studies for the treatment of organic matter in the anode compartment (in continuous).

Electron donor	Electron acceptor	Configuration	External resistance (Ω)	Anodic HRT (days)	Anodic volume (L)	Electrode material	Organic matter removal rate (Kg COD m^{-3} d^{-1})	Removal capacity (%)	CE (%)	Power density (W m^{-3})	Reference
Acetate	Nitrate	DC-MFC	5 100	0.13	0.2	GG	1.72 1.29	94 70	52±0 14±0	16±0 13±0	Virdis *et al.*, 2008
Acetate	Nitrate	TC-MFC	10	4.00 (Batch)	0.1	Carbon brush	0.13	94	32±2	NA	Zhang and He, 2012
Glucose	Oxygen	DC-MFC	100	6.00 (Batch)	<0.1	Graphite plain	0.85	85	89±4	25	Rabaey *et al.*, 2003
Municipal wastewater	Oxygen	Stacked Scaled SC-MFC MFC	2	6.00	250	Carbon brush	0.03	79	<5	<1	He *et al.*, 2014
Bakery Dairy	Oxygen	SC-MFC	1000	2.17	0.5	Carbon cloth	0.04 0.04	86 82	2±1 2±1	<1 <1	Velasquez-Orta *et al.*, 2011
Brewery	Oxygen	SC-MFC	Variable	0.08	0.1	Carbon fiber	2.95	44	7	24	Wen *et al.*, 2010
Landfill leachate	Oxygen	SC-MFC	4610	0.34	0.3	GG	0.22	10	<2	<1	Puig *et al.*, 2011b
Swine manure	Oxygen	SC-MFC	1000	10.83	0.2	Carbon paper	0.64	84	NA	<1	Kim *et al.*, 2008a
Swine manure	Oxygen	SC-MFC	1000	1.83	<0.1	Carbon paper	1.27	27	8	2	Min *et al.*, 2005
Swine manure	Oxygen	DC-MFC	30	0.26	0.4	GG	4.00 ± 2.50	38 ± 18	6±3	3 ± 1	Ref (Chapter 5) (Vilajeliu-Pons et al., 2016)
Swine manure	Nitrate	DC-MFC	30	0.16	0.4	GG	2.10 ± 0.70	15 ± 6	24±9	20 ± 2	C-1 (Chapter 4) (Vilajeliu-Pons *et al.*, 2015)
Swine manure	Oxygen	Stacked SC-MFC	1000	0.20 0.05	1.5	Graphite felt	0.99 3.23	83 66	<1 <1	4 <1	Zhuang *et al.*, 2012a
Swine manure	Oxygen	Single wetland MFC	12000	1.06	3.7	Graphite plate	0.19	77	<1	<1	Zhao *et al.*, 2013
Swine manure	Nitrate	Stacked Scaled DC-MFC	2	0.40 0.74	20 37	GG SS	2.10 ± 0.50 1.60 ± 0.70	36 ± 7 40 ± 15	16±3 13±2	4 ± 1 2 ± 1	Scaled MFCs (Chapter 6)

NA: Not available

HRT: Hydraulic retention time; SC: Single compartment; DC: Double compartment; TC: Triple compartment; GG: Granular graphite; SS: Stainless steel

In order to improve the anode electroactivity (i.e. increase the CE) another configuration was evaluated and compared to the Ref. This configuration, called MPPT (Chapter 5), used a variable external resistance control instead of a fixed resistance (Ref; Chapter 5). The application of a variable resistance control in the MPPT did not improve the organic matter treatment rate and efficiency but incremented the CE by 40% (from 6 to 17%) and it doubled the energy production compared to Ref (from 14 to 33 A m^{-3}) (Table 8.2). These differences may be explained by an enhancement of electrons released when optimal resistances for electron transfer were consistently applied. Moreover, intermittent electric connection allowed higher current production, since both capacitive and faradaic currents could be harvested (Borsje *et al.*, 2016; Gardel *et al.*, 2012). However, MPPT configuration did not shown electrogenic activities higher than the initial configuration tested (C-1) (CE of 24%). For this reason, the scaling-up of the technology was performed following the C-1 configuration (Chapter 6).

Nowadays, scaling-up BES is one of the main bottlenecks of this technology. The feasibility of scaling-up MFCs has been investigated working with synthetic media (Dekker *et al.* 2009; Shimoyama *et al.* 2008), municipal wastewater (Ge and He, 2016) and brewery wastewaters (Dong *et al.* 2015). However, there are few examples focused on the treatment of complex matrices as swine manure at this scale (Zhao *et al.*, 2013; Zhuang *et al.* 2012a).

One of the main problematic points on scaling-up is to choose the appropriate reactor design. Firstly, scaled-up MFCs with single units were evaluated. These studies were performed treating acetate and the power densities obtained were below 10 W m^{-3} NAC (Zhang *et al.* 2013a; Zhang *et al.* 2010; Clauwaert *et al.* 2009a). In spite of these promising attempts, the energy recovered and the volumetric capacities were insufficient. For this reason, multiple stacked MFCs started to be tested. Different electrical configurations (i.e. series or parallel) were tested in stacked MFCs to achieve higher voltage or current (Aelterman *et al.* 2006a). However, series connection can suffer several issues such as voltage reversal,

contact voltage losses and erratic operation, while in parallel connection internal losses increase, which reduces the total power production (Oh and Logan 2007). By using stacked MFCs, power densities were increased by an order of magnitude (130-140 W m^{-3} NAC) if synthetic wastewater was treated (Dekker *et al.* 2009; Shimoyama *et al.* 2008) but reduced significantly if complex sources were treated. Although the relatively fast removal rates of 1.5L BES treating swine manure (between 1.0-3.2 kg COD m^{-3} d^{-1}), the power density was reduced two orders of magnitude respect to simplest wastewater matrices, 4 W m^{-3} NAC (Zhuang *et al.* 2012a). The highest volumetric example consisted of a 3.7 L constructed wetland MFC with an aerated cathode. The achieved COD removal rate and power density achieved (0.2 kg COD m^{-3} d^{-1} and 0.02W m^{-3} NAC, respectively) were lower to the predecessor study treating swine manure due to the negative effect of the reactor size (Zhao *et al.*, 2013).

The low treatment and volumetric capacities of theses reactors threatens its real applicability due to high CAPEX. It would imply that bigger reactors would be needed. The market demands high treatment capacities at low HRTs. For this reason, in this PhD thesis, the scaling-up of BES for treating swine manure was designed as an 6-stacked-MFC with a total anodic volume above 20L and HRT below 1 day (Chapter 6). The system was designed following the C-1 configuration. Two different electrode materials were tested: granular graphite (Scaled GG) and stainless steel (Scaled SS). In both cases, the system was operated at a high flow (50 L d^{-1}), which implied a low HRT for both configurations (0.40 and 0.74 d for Scaled GG and Scaled SS, respectively).

In terms of organic matter removal rates, the scaled GG presented a performance similar to C-1 (mL-scale) (2.1±0.5 and 2.1±0.7 kg COD m^{-3} d^{-1}, respectively) and it removed more than 90% of influent BOD. However, scaled GG presented a poorer electrochemical performance. Both CE (16±3%) and power density (4±1 W m^{-3} NAC) were lower than the values observed in C-1 (24±9% and 20±2 W m^{-3} NAC). Positively, the values observed at scaled GG were still higher than

the configuration discarded (C-2) (5±4% and 2±1 W m^{-3} NAC) and the values were similar or higher than the values observed in literature for scaled-up MFCs (Table 8.3). Hence, the results observed at the scaled GG can be considered as relatively positive in terms of organic matter oxidation rates and electroactivity. However, at long-terms, the scaled GG configuration showed issues on electrode stability because they crushed and compacted, changing the hydraulic distribution, which may difficult its applicability. The granules have an intrinsic low porosity of 0.53, which promote a potential clogging effect either from bacteria growth on the electrode or particles from wastewater (Rabaey *et al.* 2005b). Stainless steel from Scaled SS (Chapter 6) kept the structure and obtained similar organic removal rates than Scaled GG. Moreover, stainless steel has good mechanical properties, and no issues of electrode crushing were detected.

The lower values obtained in terms of power density by the scaled GG and SS indicated that the individual electric connection was not the optimal. Several electric connections as series, parallel and mixed (parallel-series) were tested in order to optimise the renewable electricity production. The mixed circuit maintained the current density of the parallel circuit and did not show problems of voltage reversal as in series circuit. This combination resulted in the highest power density output achieved between all electric circuits tested. Therefore, the application of stacked scaled SS MFC with mixed electric circuit was considered a feasible strategy to maintain or even improve treatment efficiencies and power densities while treating swine manure.

8.2.2. Anodic microbiome treating complex carbon waste

Microbial communities from the anodic compartments treating complex carbon wastes were analysed along this PhD thesis. Three different configurations with different cathode performance: denitrifying cathode (C-1, Chapter 4), simultaneous nitrifying-denitrifying cathode (C-2, Chapter 4), and oxygen reduction cathode (Ref and MPPT, Chapter 5), were characterised, trying to determine its putative role on electrogenesis.

The microbial community in swine manure is versatile and changeable depending on many variables, e.g. diet of pigs, use of antibiotics, which have an impact on the gut microbiota (Sutton *et al.*, 1999). Few studies are available on microbiome identification treating swine manure using BES. Han *et al.*, (2011) identified *Firmicutes* (*Clostridia*), *Bacteroidetes* (*Bacteroidia*) and *Proteobacteria* (*Gammaproteobacteria*) as the main phyla. The complexity of the substrate makes harder the microbiome identification and putative role in comparison with the identification of model organisms like *Geobacter sulfurreducens* or *Shewanella oneidensis* in synthetic substrate (Kimura and Okabe, 2013; Jung and Regan, 2007; Gorby *et al.*, 2006). However, the bacteria found in the influent swine manure in this PhD thesis (all of them belonging to *Clostridiales* (*Firmicutes*)) had a limited influence on the community established within the MFCs, indicating an initial selection pressure of the reactors.

The anode biofilm organization of the configurations with nitrogen compounds in the cathode (C-1 and C-2, Chapter 4) showed a complex organization with a dominance of rod-shaped and filamentous cells. The aggregates were firmly attached to the electrode surface by the use of filamentous appendages. Similar microbiomes were identified in both anodes (*Firmicutes* and *alpha-*, *gamma-* and *delta-proteobacteria*) (Table 8.4). A single population of *Clostridium disporicum* (99%) clearly dominated the microbial community in both systems. *C. disporicum* is a fermentative bacterium able to grow on complex organic macromolecules (Miyahara *et al.*, 2013). *G. sulfurreducens* was present in both designs. This exoelectrogenic bacterium could be responsible of electricity production from organic matter oxidation (Lovley *et al.*, 2011). However, the role of *C. disporicum* in the electrogenic process remained elusive, even though its abundance and persistence in the studied systems suggested an active role.

Table 8.4 The most relevant microorganisms found during the specific studies of this PhD thesis.

Configuration	Detection technique	Phylum	Class	Order	Specie	Putative * electroactive	Reference
C-1	DGGE	Firmicutes	Clostridia	Clostridiales	*Clostridium disporicum*	No	Chapter 4 (Vilajeliu-Pons *et al.*, 2015)
	FISH	Proteobacteria	Deltaproteobacteria	Desulfuromonadales	*Geobacter sulfurreducens*	Yes	
C-2	DGGE	Firmicutes	Clostridia	Clostridiales	*Clostridium disporicum*	No	Chapter 4 (Vilajeliu-Pons *et al.*, 2015)
	FISH	Proteobacteria	Deltaproteobacteria	Desulfuromonadales	*Geobacter sulfurreducens*	Yes	
Ref	Pyrosequencing	Firmicutes	Erysipelotrichia	Erysipelotrichales	*Turicibacter* sp.	No	Chapter 5 (Vilajeliu-Pons *et al.*, 2016)
		Bacteroidetes	Bacteroidia	Bacteroidales	*Uncultured p-2534-18B5*	No	
		Bacteroidetes	Bacteroidia	Bacteroidales	*Parabacteroides* sp.	Yes	
MPPT	Pyrosequencing	Firmicutes	Erysipelotrichia	Erysipelotrichales	*Turicibacter* sp.	No	Chapter 5 (Vilajeliu-Pons *et al.*, 2016)
		Bacteroidetes	Bacteroidia	Bacteroidales	*Uncultured p-2534-18B5*	No	
		Bacteroidetes	Bacteroidia	Bacteroidales	*Parabacteroides* sp.	Yes	
		Firmicutes	Clostridia	Clostridiales	*Sedimentibacter* sp.	Yes	
		Proteobacteria	Gammaproteobacteria	Pseudomonadales	*Pseudomonas* sp.	Yes	
		Proteobacteria	Betaproteobacteria	Burkholderiales	*Uncultured Oxalobacteraceae*	No	

*Bibliographically based putative role

DGGE: Denaturing gradient gel electrophoresis; FISH: Fluorescent in situ hybridization.

The high abundance of *C. disporicum* in the system might mask the detection of bacterial representatives at lower relative concentrations, which made difficult the identification of other exoelectrogenic bacteria. However, the difference in power density generated (20±2% in C-1; 2±1% in C-2) and Coulombic efficiencies achieved (24±9% in C-1; 5±4% in C-2) were not only linked to the microbiome. The anoxic cathode conditions with an AEM improved the electricity production in C-1 respect to C-2.

The microbiome identified in the configurations with nitrogen compounds in the cathode (C-1 and C-2, Chapter 4) differed from the ones identified in the configurations with oxygen reductions cathodes (Ref and MPPT, Chapter 5). In this case, the community was clearly dominated by a single population of *Turicibacter* sp. (99%) (Ref and MPPT, Chapter 5). *Turicibacter* sp. has been previously found in pig waste, using cultivation- independent molecular analyses (Han *et al.*, 2011). More likely, *Turicibacter* related species may be implicated in heterotrophic degradation of organic matter, probably through a fermentation process. Under strict anaerobic conditions, lactate is the main fermentation product from carbohydrates for *Turicibacter* sp. (Bosshard *et al.*, 2002). However, its implication in exoelectrogenesis was dismissed since significantly lower CE was found for Ref in comparison with MPPT. *Bacteroidetes* were also identified at relative abundances higher than 10% in both of the MFC configurations (Uncultured *p-2534-18B5* and *Parabacteroides sp*.). Uncultured *p-2534-18B5* gut group can be related to intrinsic gut microbiota (Morton *et al.*, 2015), whereas species with a 16S rRNA sequence similar to that of *Parabacteroides* sp. has been previously reported as a potential current generator (Watanabe *et al.*, 2011).

The microbial communities of BES anodes were usually composed of different bacterial species from which electricity generation capabilities has not been described so far in the literature. However, a putative electrogenic role of some species can be hypothesized from comparisons of microbiome structures in selective experimental conditions. The MPPT microbiome of a BES reactor with a dynamic external resistance control was analysed and compared with the BES at

fixed resistance (Ref). The MPPT control has been proven to be more effective for the proliferation of exoelectrogenic bacteria (Premier *et al.*, 2011) and reduces the start-up time for running a MFC at full capacity (Molognoni *et al.*, 2014). Moreover, in this PhD thesis it is demonstrated a 5-fold increase on the bacterial abundance at the MPPT anode and doubled energy production and CE (compared with Ref).

The effect of the MPPT control on the bacterial community structure was mainly related to the enrichment of specific OTUs, specifically *Proteobacteria*, all of them missing in the Ref configuration. These enrichments suggested a number of bacteria putatively implicated in electrogenesis. *Sedimentibacter sp.*, *Pseudomonas sp*. and an uncultured *Oxalobacteraceae* were significantly enriched in the MPPT. Based on the analysis of 16S rRNA gene similarities, *Sedimentibacter* sp. related species were identified in the core community of MFC systems with high power generation capabilities together with *Geobacter*, *Aminiphilus*, *Acetoanaerobium*, and *Spirochaeta* (Lesnik *et al.*, 2014). The exoelectrogenic capacity has also been proven for some *gammaproteobacteria*, including *Pseudomonas* species (Rabaey *et al.*, 2004).

Proteobacteria were in competitive disadvantage relative to *Firmicutes* under the experimental conditions applied in this study, as this was most likely related to the presence of highly recalcitrant components of the influent organic matter (Velvizhi and Venkata Mohan, 2012). Most known exoelectrogenic bacteria fall within the *Proteobacteria*, which have been detected as dominant members of the bacterial community in MFCs treating simple substrates, such as acetate and glucose (Lee *et al.*, 2010; Aelterman *et al.*, 2006b; Logan *et al.*, 2006), and wastes from industrial sources (Velvizhi and Mohan, 2015; Patil *et al.*, 2009), revealing a substrate effect on dominant putative exoelectrogenic bacteria.

Taking all together, the microbial community found in all anode compartments (C-1, C-2, Ref and MPPT) has a high diversity and it is significantly different than the community found in the swine manure itself. The optimization of the MFC systems together with a better comprehension of the bacterial communities responsible for

the internal processes will help on enabling the implementation of MFC technology for *in situ* treatment of swine manure. Future studies should be focused on the physical relationship of the dominant taxa with the electrode (fluorescent *in situ* hybridization analyses) and the identification of active members of the MFC-associated community (shot gun metatranscriptome sequencing).

8.3. Complete nitrogen removal in BES for treating complex wastewaters

8.3.1. Strategies for removing nitrogen in BES

Different reactor configurations were studied in order to completely treat nitrogen compounds in complex matrices (as swine manure) using BES. The main objective was to achieve the highest electrotroph nitrogen removal rates and efficiency at a competitive cost. For this reason, different compartments (anode, cathode and external reactor) were evaluated for the independent or simultaneous nitrogen removal. In order to reduce costs of ammonium oxidation, the nitrification rates and efficiencies were studied at different aeration set-points (3.2, 1.3 mg O_2 L^{-1} and anoxic conditions (anode as electron acceptor)). Moreover, different reactor sizes and different electrode materials were evaluated (See Table 8.5 for set-up configuration).

In Chapter 4, the nitrogen removal in two independent compartments (nitrifying in the external reactor and denitrifying in the cathode) or in one compartment (simultaneously nitrifying and denitrifying in the cathode) at mL-scale were evaluated (C-1 and C-2 reactors, respectively). In both cases, the nitrification products (nitrite and/ or nitrate) were reduced in the cathode.

Table 8.5 Nitrogen oxidation and removal capabilities of the specific studies of this thesis.

	C-1 (Chapter 4) (Vilajeliu-Pons *et al.*, 2015)	**C-2 (Chapter 4)** (Vilajeliu-Pons *et al.*, 2015)	**Scaled GG (Chapter 6)**	**Scaled SS (Chapter 6)**	**niBES (Chapter 7)**
Set-up					
Strategy for nitrogen removal	Independent N-DN	SND	Independent N-DN	Independent N-DN	SND Anammox
Electrochemical configuration	MFC	MFC	MFC	MFC	MEC-three electrodes
Compartment	N: External reactor DN: Cathode	N: Upper cathode DN: Lower cathode	N: External reactor DN: Cathode	N: External reactor DN: Cathode	Anode
Potential *(V vs SHE)*	+0.19 ± 0.11	+0.21 ± 0.05	+0.22 ± 0.10	+0.20 ± 0.07	+0.80[1]
Flow *($L\ d^{-1}$)*	2.9 ± 0.1	2.9 ± 0.1	50.0	50.0	0.5 ± 0.0
HRT *(d)*	1.86	0.16	0.80	1.14	0.92
Removal capabilities					
Nitrification rate *($g\ N\ m^{-3}\ d^{-1}$)*	260 ± 60	1260 ± 290	190 ± 50	190 ± 50	35 ± 6
Nitrification efficiency *(%)*	95 ± 3	49 ± 19	95	95	32 ± 5
Nitrite production rate *($g\ N\ m^{-3}\ d^{-1}$)*	0 ± 0	310 ± 140	0 ± 0	0 ± 0	<0.01
Nitrogen removal rate *($g\ N\ m^{-3}\ d^{-1}$)*	160 ± 60	110 ± 50	370 ± 100	300 ± 100	NA
Nitrogen removal efficiency *(%)*	7 ± 3	22 ± 10	44 ± 10	56 ± 15	NA
Process CE *(%)*	10 ± 4	NA	16 ± 3	13 ± 2	28 ± 13[2]
Energetic requirements					
Aeration set-point *($mg\ O_2\ L^{-1}$)*	3.2 ± 0.8	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	Non aerated
Potential control	Not applied	Not applied	Not applied	Not applied	+0.8 V vs. SHE

NA: Not available; [1] Applied potential; [2] CE was calculated with electrons of hydroxylamine oxidation to nitrite

N: Nitrification; DN: Denitrification; SND: Simultaneous nitrification-denitrification; MFC: Microbial fuel cell; MEC: Microbial electrolysis cell;

C-1 configuration had the highest aeration set-point tested (3.2 ± 0.8 mg O_2 L^{-1}) (Table 8.5). Nevertheless, it was still below the ones tested in previous studies (above 5 mg O_2 L^{-1}) (Zhang *et al.*, 2013b; Virdis *et al.*, 2008). C-1 was able to completely oxidise ammonium without accumulation of intermediates (nitrite and nitrous oxide) at a high nitrifying rate (260 ± 60 g N m^{-3} d^{-1}). Positively, the denitrification process was also complete, achieving a high nitrate removal rate (160 ± 60 g N m^{-3} d^{-1}) but low cathode CE (10%). Other authors have worked with similar reactor configuration for nitrogen treatment in synthetic media (Zhang and He, 2012; Wrighton *et al.*, 2010; Virdis *et al.*, 2008). In all cases, nitrification was complete, but the ammonium oxidation rates were one or two orders of magnitude lower working with higher aeration set-points and HRTs than C-1 (Table 8.6). These results suggested a better availability of the substrate, enhanced aeration on the reactor, increase biomass activity or abundance in the system. In terms of nitrate removal, the reactors working in synthetic showed higher removal efficiencies and electrotroph activity, where the CEs were generally over 70%. However, the presence of available carbon in swine manure could have allowed heterotrophic denitrification, increasing the denitrifying rates but decreasing the CEs down to 10%.

In C-2, where simultaneous nitrification and denitrification occurred at the cathode, the nitrification rate was almost 5-fold higher than in C-1 even a lower aeration set-point was fixed to do not negatively affect the electrotroph denitrification (1.3 ± 0.3 mg O_2 L^{-1}) (Table 8.5). However, nitrite was accumulated at a rate of 310 ± 140 g NO_2^-–N m^{-3} d^{-1}, revealing unbalanced nitrification and denitrification activities probably caused by the low HRT (0.16 d) and the high FA levels in the reactor. The tolerances of nitrifying bacteria were below the estimated FA concentrations (Anthonisen *et al.*, 1976). Nevertheless, the performance of both MFCs (C-1 and C-2) was similar in terms of nitrogen reduction capability (160 and 110 g N m^{-3} d^{-1} for C-1 and C-2, respectively).

Table 8.6 Representative compilation of BES studies for the treatment of nitrogen compounds (in continuous).

					Nitrification				Denitrification			
Influent wastewater	**Configuration**	**HRT**	**Volume**	**Electrode material**	**Aeration set-point**	**Nitrification rate**	**Nitrification efficiency**	**Nitrite prod. rate**	**NRR**	**Removal capacity**	**Process CE**	**Reference**
		(days)	(L)		(mgO_2L^{-1})	(g N m^{-3} d^{-1})	(%)	(gN m^{-3} d^{-1})	(gN $m^{-3}d^{-1}$)	(%)	(%)	
Synthetic wastewater	MFC- ENR (5 and 100 Ω)	3.45	4.80	GG	Saturated	18 22	91 94	0.06 0.12	409 97	97 19	82±1 68±2	Virdis *et al.*, 2008
Synthetic wastewater	MFC- ENR (5 Ω)	3.45	4.80	GG	Saturated	3	100	0.00	76	100	87	Wrighton *et al.*, 2010
Synthetic wastewater	MFC- ENR (10 Ω)	4.00 (Batch)	0.24	Carbon brush	Saturated	14	91	0.00	9	44	21±2	Zhang and He, 2012
Swine manure	MFC-ENR (30 Ω)	1.86	5.60	GG	3.2±0.8	260±60	95±3	0.00 ± 0.00	160±60	7±3	10±4	C-1 (Chapter 4) (Vilajeliu-Pons *et al.*, 2015)
Municipal wastewater	Stacked Scaled MFC (3.3 Ω)	0.50	124	Carbon cloth	Saturated	70	68	3.60	70	68	NA	Ge and He, 2016
Swine manure	Stacked Scaled MFC (1.5 Ω)	0.80 1.14	40 57	GG SS	1.3±0.3 1.3±0.3	190±50 190±50	95 95	0.00±0.00 0.00±0.00	370±100 300±100	44±10 56±15	16±3 13±2	Scaled MFCs (Chapter 6)
Synthetic wastewater	MFC-SND (5 Ω)	0.57	0.40	GG	2.0±0.1 7.2±0.1	127 181	68 97	0.00 1.05	126 55	67 29	NA NA	Virdis *et al.*, 2010
Synthetic wastewater	MFC-SND (230 Ω)	5.41 (Batch)	1.20	Graphite plates	5.0±1.0	16	59	1.11	9	78	NA	Zhang *et al.*, 2013b
Synthetic wastewater	MFC-SND (1000 Ω)	4.17 (Batch)	0.25	Graphite felt	6.5±0.3	54	100	0.00	48	89	NA	Feng *et al.*, 2015
Swine manure	MFC-SND (30 Ω)	0.16	0.48	GG	1.3±0.3	1260±290	49 ± 19	310±140	110±50	22±10	NA	C-2 (Chapter 4) (Vilajeliu-Pons *et al.*, 2015)

Table 8.6 (Continued)

Influent wastewater	Configuration	HRT (days)	Volume (L)	Electrode material	Aeration set-point (mgO_2L^{-1})	Nitrification: Nitrification rate (g N m^{-3} d^{-1})	Nitrification: Nitrification efficiency (%)	Nitrification: Nitrite prod. rate (gN m^{-3} d^{-1})	Denitrification: NRR (gN $m^{-3}d^{-1}$)	Denitrification: Removal capacity (%)	Process CE (%)	Reference
Synthetic wastewater	MEC-AN (-0.70 V vs SHE)	4.00 (Batch)	0.20	Graphite plate	-	17	98	0.35	-	-	33±8[1]	Qu *et al.*, 2014
Synthetic wastewater	MEC-AN (+0.60 V vs SHE)	5.00 (Batch)	0.10	Carbon felt	-	11 40	41 48	0.00 0.00	-	-	80±2[2] 53±4	Zhan *et al.*, 2014
Synthetic wastewater	MEC-AN (+0.80 V vs SHE)	0.92	0.46	GG	-	35±6	32±5	1.00±0.60	-	-	28±13[3]	niBES (Chapter 7)
Synthetic wastewater	MFC (1.1 Ω)	12	0.11	GG	-	-	-	-	503	80	100	Clauwaert *et al.*, 2009b
Synthetic wastewater	MFC (25 Ω)	0.42	0.60	GG	-	-	-	-	51	42	73±18	Puig *et al.*, 2012
Synthetic wastewater	MEC (-0.10 V vs SHE)	0.29	0.20	GG	-	-	-	-	106	95	46	Virdis *et al.*, 2009
Groundwater	MFC (10 Ω)	0.50	0.01	Carbon paper	-	-	-	-	36	91	90	Zhang and Angelidaki, 2013
Groundwater	MEC (-0.12 V vs SHE)	0.21	0.60	GG	-	-	-	-	106	69	NA	Pous *et al.*, 2015b
Municipal wastewater	Scaled MFC (100 Ω)	0.44	5.10	Carbon cloth	-	-	-	-	20	30	NA	Jiang *et al.*, 2011

NA: Not available. [1] CE was calculated from NH_4^+ to NO_3^-; [2] CE was calculated from NH_4^+ to N_2; [3] CE was calculated from NH_4^+ to NH_2OH

HRT: Hydraulic retention time; NRR: Nitrogen removal rate; CE: Coulombic efficiency; MFC: Microbial fuel cell; MEC: Microbial electrolysis cell; AN: Anode; ENR: External nitrifying reactor; SND: Simultaneous nitrification and denitrification; GG: Granular graphite; SS: Stainless steel.

Comparing C-2 to similar configurations found in the literature, it highlights that the other authors operated the system at a higher aeration set-points (above 2 mg O_2 L^{-1}) and HRTs (above 0.57 d) (Feng *et al.*, 2015; Zhang *et al.*, 2013b; Virdis *et al.*, 2010). They observed no intermediates accumulation, but lower nitrification rates (below 181 g N m^{-3} d^{-1}) compared to C-2 (1260 g N m^{-3} d^{-1}). In general, the denitrification rates were below 50 g N m^{-3} d^{-1}, except in Virdis and co-workers work (2010), who achieved a denitrification rate of 126 g N m^{-3} d^{-1} treating synthetic wastewater. This value is similar to the one observed for C-2 (110 g N m^{-3} d^{-1}), but here swine manure instead of synthetic wastewater is treated.

Taking together all the results, it can be pointed out that the C-2 configuration (SND) did not perform efficiently for a simultaneous nitrification and denitrification and, as a result, nitrite was accumulated at the effluent. In consequence, the C-1 configuration (equipped with an external nitrifying reactor) was chosen as the most appropriate configuration for scaling-up from the nitrogen point of view.

When the BES system was scaled-up, the scaled GG (stacked-MFC with granular graphite as electrodes) and the scaled SS (stacked-MFC with stainless steel as electrodes) were working as C-1 configuration, but the aeration set-point was decreased to values similar to those used for C-2 (1.3 ± 0.3 mg O_2 L^{-1}). The reduction of aeration in scaled-up systems was implemented to reduce the operation costs of the process after observing that nitrification was feasible at this set-point. In the scaled-GG and SS, the nitrifying process was complete (95%) with nitrifying rates (190 ± 50 g N m^{-3} d^{-1}) slightly lower than the ones observed at mL-scale (C-1) (260 ± 60 g N m^{-3} d^{-1}). Moreover, intermediate products were not detected besides the oxygen set-point was lower.

The denitrifying rates in the scaled-GG improved, achieving almost double removal rates (370 g N m^{-3} d^{-1}) and CEs (16%) than C-1 (160 g N m^{-3} d^{-1} and 10%, respectively). The improvement of denitrifying performance when scaling-up could be related to the

decrease of the aeration set-point at the external reactor. The negative influence of oxygen in denitrifying cathodes was also stated by lower CE and removals to the receiving stream from the external reactors within the stacked MFC. The first cathode had lower efficiencies than the following. The denitrification results obtained in scaled GG were similar to the scaled SS (300 g N m^{-3} d^{-1}), indicating the low effect of the electrode material for denitrification.

Comparing the scaled-up reactors with literature, it has to be pointed that no specific cathodic nitrate reduction scaled-up systems are available. However, some studies have been published dealing with ammonium removal. An ammonium removal system working with municipal wastewater obtained ammonium removal rates (70 g N m^{-3} d^{-1}) lower than the values observed in this PhD thesis (370 and 300 g N m^{-3} d^{-1} in Scaled GG and SS, respectively). Moreover, the authors reported nitrite accumulation even though the medium was saturated with air (Ge and He, 2016).

In order to determine if denitrification in our system was competitive with other BES studies, different configurations treating nitrate (synthetic or groundwater) in the cathode have been selected for comparison (Table 8.6). The absence of organic matter in the reactors used for solely nitrate removal resulted in CEs higher (above 46%) than the scaled GG and SS (13-16%), independently of the electrochemical configuration (MFC or MEC). However, the configuration tested in this PhD thesis achieved higher removal rates (300-370 g N m^{-3} d^{-1}), indicating that either heterotrophic denitrification or the anode compartment electroactivity were limiting electrotroph denitrification.

The determination of a limiting behaviour of the anode compartment together with the increasing potential of MEC reactors for nitrogen treatment opened the possibility to evaluate the usage of a MEC configuration for the nitrogen removal. The MEC configuration implies that an external power input has to be supplied to the system. Therefore, for a complete nitrogen treatment it would imply a double source of operational costs: energy input for empowering organic matter oxidation and nitrate

reduction and aeration for the nitrification process. In order to make this process more affordable, it would be interesting to use the energy input to catalyse not only organic matter and denitrification, but also, to catalyse the ammonium oxidation anoxically. In consequence, in Chapter 7 of this PhD thesis, the anoxic ammonium oxidation was evaluated by poising the anode compartment at fixed potential of +0.8 V vs SHE (niBES). The anode potential was chosen to allow ammonium oxidation (>0.34 V vs. SHE (Madigan *et al.,* 2010)) but avoiding oxygen production (>+1.23 V *vs.* SHE (Herron *et al.*, 2015)). If ammonium could be oxidized bioelectrochemically, the aeration supply could be suppressed, reducing significantly the operational costs. The results obtained indicated that this process was feasible. Surprisingly, not only ammonium was oxidized at the anode compartment, but also complete ammonium conversion to dinitrogen gas was observed. Ammonium was removed to dinitrogen gas at a rate of 35 ± 6 g N m^{-3} d^{-1} with an anodic CE of 28±13% and with slight nitrite accumulation (1 g N m^{-3} d^{-1}). An abiotic reactor was used to ensure the absence of oxygen production through the potential applied. In this case, the use of granular graphite as electrode hindered the possible oxygen production at this potential (Lai *et al.*, 2017).

Some authors have worked on ammonium oxidation fixing an anode potential and achieving nitrification rates (11-40 g N m^{-3} d^{-1}) similar or lower than the values obtained in this thesis (35 g N m^{-3} d^{-1}). In all cases nitrite was almost negligible due to the operation in batch mode during 4 and 5 days, respectively (Qu *et al.*, 2014, Zhan *et al.*, 2014), while the results obtained in this thesis were obtained under continuous mode operation at an HRT of 0.92 d. The achievement of ammonium bioelectro-oxidation under continuous-flow mode increases its potential for future applications. Although the application of BES for carrying out ammonium bioelectro-oxidation is still in its infancy, it can imply a breakthrough on the treatment of organic matter and nitrogen from wastewater, making it a process completely aeration-independent.

8.3.2. Electrochemical performances for dealing with nitrogen in BES

8.3.2.1. Energetic overview

The complete sustainable nitrogen removal in BES at a competitive cost was one of the objectives of the PhD thesis. Different aeration set-points were tested, from 3.2±0.8 mgO_2L^{-1} in C-1 (Chapter 4), to 1.3±0.3 mgO_2L^{-1} in scaled SS (Chapter 6) and finally anoxic conditions in niBES (Chapter 7). Table 8.7 summarizes the results of the energetic consumption obtained in this thesis in comparison with other BES (Virdis *et al.*, 2008) and compares with the conventional treatments of complete or partial nitrification and denitrification in WWTPs (Rieger, 2004) and partial nitrification with Anammox (Wett, 2007; Van Dongen *et al.*, 2001; Third *et al.*, 2001).

Conventional nitrogen treatment in WWTPs, completely or partially, implies a high energy consumption (4.6 kWh $kg^{-1}_{N\text{-}NH4+}$) from the aeration point of view, without regard on the addition of organic sources (Rieger, 2004). Anammox process appeared 20 years ago with objective of reducing aeration costs of nitrification and avoids the high organic matter demand for denitrification. The technology optimization allowed to reach higher nitrogen removal rates (until 1.2 kg N m^{-3} d^{-1}) as well reduced energetic costs (1.16 ± 0.21 kWh $kg^{-1}_{N\text{-}NH4+}$) in comparison to the traditional treatments in WWTPs. However, nitrogen removal with anammox requires a previous oxidation of ammonium to nitrite by partial nitritation (Van Dongen *et al.*, 2001). Thus, the aeration costs are not completely avoided. Moreover, the anoxic-aerobic phases of partial nitritation process emits nitrous oxide (N_2O), which is a harmful greenhouse gas (Gabarró *et al.*, 2014; Brotto *et al.*, 2015).

Table 8.7 Comparison of energy consumption between conventional treatment technologies (aerobic and anaerobic) and BES technology (aerobic and anoxic), in terms of complete nitrogen removal.

Process	N removal rate ($g\ N\ m^{-3}\ d^{-1}$)	Energy consumption ($kWh\ kg^{-1}_{N\text{-}NH4+}$)	Ammonium removal products	Air supply	Addition of products	References
Conventional treatment I (Complete nitrification/denitrification)	21-58	4.6	NO_3^-, N_2	Yes	Yes, methanol	Rieger, 2004
Conventional treatment II (Partial nitrification/denitrification)	21-58	1.6	NO_2^-, N_2	Yes	Yes, methanol	Rieger, 2004
Partial nitritation - Anammox	1200 1200 563±48	NA NA 1.2 ± 0.2	NO_2^-, NO_3^-, N_2 NO_2^-, N_2 NO_2^-, N_2	Yes Yes Yes	NA NA NA	Van Dongen *et al.*, 2001 Third *et al.*, 2001 Wett, 2007
Conventional nitrification/ BES denitrification (Independent N-DN)	409	NA (4.6-1.2)	NO_2^-, NO_3^-, N_2O, N_2	Yes (Saturated)	No	Virdis *et al.*, 2008
Conventional nitrification/ BES denitrification (Independent N-DN)	160±60	NA (4.6-1.2)	NO_3^-, N_2	Yes ($3.2\pm0.8\ mgO_2L^{-1}$)	No	C-1 Chapter 4 (Vilajeliu-Pons *et al.*, 2015)
Conventional nitrification/ BES denitrification (Independent N-DN)	300±100	NA (4.6-1.2)	NO_3^-, N_2	Yes ($1.3\pm0.3\ mgO_2L^{-1}$)	No	Scaled SS Chapter 6
Conventional nitrification/ BES denitrification (SND)	127	NA (4.6-1.2)	NO_2^-, NO_3^-, N_2	Yes ($2.0\pm0.1\ mgO_2L^{-1}$)	No	Virdis *et al.*, 2010
Conventional nitrification/ BES denitrification (SND)	110±50	NA (4.6-1.2)	NO_2^-, NO_3^-, N_2	Yes ($1.3\pm0.3\ mgO_2L^{-1}$)	No	C-2 Chapter 4 (Vilajeliu-Pons *et al.*, 2015)
Electrogenic anoxic ammonium removal	35±6	0.13 ± 0.09*	N_2	No	No	niBES Chapter 7

NA: Not available

*The energy consumption was calculated as the energy required applying the anode potential.

N: Nitrification; DN: Denitrification; SND: Simultaneous nitrification-denitrification.

The firsts attempts of BES implementation were focused on the viability of the process, more than on the optimization of the aeration supply (Virdis *et al.*, 2010; Virdis *et al.*, 2008). Independent and simultaneous nitrogen removals were achieved at a faster removal rates than in WWTPs (130 - 400 g N m^{-3} d^{-1}). However, intermediate nitrogen species as nitrite were still present in the effluent. The use of BES for nitrogen treatment allowed electrotroph nitrogen removal, removing the addition of organic compounds from the equation. However, the energy consumption of these systems was calculated due to the constant air supply, but it indicated an energy consumption similar to the ones obtained in WWTPS and Anammox processes.

The BES configurations used in this PhD thesis aimed to reduce the costs of nitrogen removal. The reduction of the oxygen supply set-point (1.3 – 3.2 mg O_2 L^{-1}) allowed nitrogen removal but at removal rates slightly lower (100-300 g N m^{-3} d^{-1}) than BES with air saturation supply (Virdis *et al.*, 2010; Virdis *et al.*, 2008). The simultaneous treatment in one compartment (C-2, Chapter 4) resulted in the accumulation of intermediate products due to unbalanced nitrification and denitrification processes and lower nitrogen removal rates, due to the inhibitory effect of oxygen on denitrification process. On the contrary, the configurations with independent nitrogen treatment avoided the problems mentioned above (C-1, Chapter 4), even in scaled-up systems (Scaled SS, Chapter 6). However, air supply at a set-point of 1.3 mg O_2 L^{-1} was still required.

From the technical perspective, anoxic nitrogen removal was performed in anodic BES to remove air supply requirement (niBES, Chapter 7). NiBES presented the same nitrogen removal range than conventional treatments, but allowed the transformation of almost all ammonium (>96%) to dinitrogen gas. The absence of intermediate compounds, as nitrite, nitrate and nitrous oxide, as end-products gives a toxicological advantage in respect to the other systems. The operational costs of niBES were one order of magnitude lower (0.13 ± 0.09 kWh $kg^{-1}_{N\text{-}NH4^+}$) than conventional treatments (1.6 – 4.6 kWh $kg^{-1}_{N\text{-}NH4^+}$) (Table 8.7). Not only the niBES

would be a cheaper treatment, but also its application would simplify reactor operation in WWTPs through managing without air dispersers for nitrification and organic carbon addition (e.g. methanol) for denitrification. Therefore, the easy and fast workability of niBES makes it a promising alternative to current treatments.

8.3.2.2. Extracellular electron transfer thermodynamics in nitrifying anodes

In order to further investigate the electrochemical dependency of the bioelectrochemical ammonium oxidation, its extracellular electron transfer thermodynamics (i.e. identification of the redox potentials at which the electron transfer takes place) was investigated using microcosms.

The knowledge regarding the EET of heterotrophic bioanodes is abundant in literature. The best studied anodic EET is the extracellular respiration of dissimilatory metal-reducing bacteria of the genera Shewanella and Geobacter (Velvizhi and Venkata Mohan, 2014; Rosenbaum and Angenent, 2010; Weber *et al.*, 2006a). However, the EET mechanisms involved in ammonium oxidation is scarce (Zhan *et al.*, 2014), but this knowledge is a fundamental to understand and optimize the niBES operation.

In this PhD thesis, the use of microcosms, i.e. in tailor-made single-chamber BES based on Pous *et al.*, (2014), was necessary for studying the extracellular electron transfer in bioanodes of BES (Chapter 7). Samples from the working anode of niBES reactor were extracted to inoculate nitrifying microcosms. The smaller size of the microcosms allowed a more sensitive electrochemical analysis than in niBES reactors. For microcosm cultivation at electrodes, different media containing NH_4^+, NH_2OH, NO_2^- or no nitrogen containing buffer were tested under anaerobic and aerobic conditions including abiotic controls. The results revealed that microbial electroactivity was due to the oxidation of ammonium and hydroxylamine.

The potential extracellular electron transfer (EET) redox systems sites were identified by applying CVs. The methodology was adapted from the commonly used for bioanodes (Harnisch and Freguia, 2012). In this PhD thesis, CVs showed that the

oxidation of ammonium took place at a formal potential of +0.73±0.06 V vs. SHE, representing a thermodynamically feasible oxidation cascade to nitrite. Similar ammonium oxidation formal potential (+0.8 V vs. SHE) was obtained by Zhan *et al.*, (2014) indicating that some elements of the cell surface are close enough to the electrode to undergo the electron transfer. However, the oxidation of ammonium to nitrate is a sequential pathway that involves hydroxylamine (NH_2OH) and nitrite as stable intermediates. For this reason, in this PhD thesis, all the different nitrification steps were evaluated electrochemically. It is worth noticing that the maximum current density (4.02±0.46 μA cm^{-2}) was achieved when NH_2OH was used as electron donor, whereas NH_4^+ yielded 0.91±0.18 μA cm^{-2} and the abiotic control 0.06 μA cm^{-2}. These results suggest that hydroxylamine was the main substrate for the anoxic microbial electrochemical oxidation reaction in our system.

8.3.3. Microbiome associated to nitrogen dynamics in BES

Along this PhD thesis, the microbial communities related to nitrogen treatment were analysed in the different configurations used. Three different configurations for ammonium treatment (nitrifying microbiome): nitrifying external reactor (C-1, Chapter 4), nitrifying-denitrifying cathode (C-2, Chapter 4) and anoxic nitrogen removal anode (niBES, Chapter 7); and two configurations for nitrate removal (denitrifying microbiome): denitrifying cathode (C-1, Chapter 4) and nitrifying-denitrifying cathode (C-2, Chapter 4), were characterized, trying to determine its putative role on nitrification and denitrification. Table 8.8 summarizes the most relevant microorganisms identified in all configurations. Along the following sections the obtained results are discussed.

8.3.3.1. Nitrifying microbiome

Nitrification generally consists on a two-steps process to oxidise ammonium to nitrite in a process called nitritation (Sayavedra-Soto *et al.*, 2011) and then, to nitrate in a process called nitratation (Sorokin *et al.*, 2012) by two functional groups of microorganisms, ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), respectively. But complete nitrification (from ammonium to nitrate) can also

be performed by a single microorganism (van Kessel *et al.*, 2015; Daims *et al.*, 2015).

As presented in Table 8.8, different nitrifying microbiomes were identified depending on the operational characteristics applied as the inert support (clay in C-1 and granular graphite in C-2 and niBES, Chapters 4 and 7) and the operational oxygen set-point (3.2 ± 0.8 mg O_2 L^{-1} in C-1 (Chapter 4), 1.3 ± 0.3 mg O_2 L^{-1} in C-2 (Chapter 4) and anoxic conditions in niBES (Chapter 7)).

Similar relative abundances of AOB were observed in C-1 and C2 configurations, but different AOB species were identified depending on the nitrifying process. In C-1 nitritation was performed by *Nitrosospira* sp. while in C-2 was performed by *Nitrosomonas europaea*. The observed divergence in bacterial AOB resulted in a high nitrifying rate but partial accumulation of nitrite in C-2 configuration. In terms of nitratation, the same NOB species (*Nitrobacter alkalicus*) was found in both compartments, not showing differences between configurations. The AOB and NOB presence was already described in other reactors working with complex matrices treating nitrogen compounds in the anode (Hussain *et al.*, 2016;Chen *et al.*, 2014) and cathode (Sotres *et al.*, 2016) under aerobic conditions.

Additionally, the microbial distribution of AOB and NOB in C-1 external reactor was studied as a function of the flow direction and biofilm distribution (Chapter 4). AOB and NOB distributed according to the ammonium and nitrite concentration gradients. Progressively, relative abundance of AOB tended to decrease while NOB and nitrite concentration increased towards the outlet. In addition, NOB were situated inside the biofilm, surrounded by AOB in order to optimize the nitrification process. A stratified biofilm had also been observed previously when treating nitrogen compounds (Virdis *et al.*, 2011).

Table 8.8. The most relevant microorganisms found during the specific studies of this PhD thesis for the different nitrogen processes.

Configuration	Compartment	Detection technique	Phylum	Class	Order	Specie	Putative nitrogen role*	Reference
C-1	External reactor	DGGE	Proteobacteria	Betaproteobacteria	Nitrosomonadales	*Nitrosospira* sp.	N	Chapter 4 (Vilajeliu-Pons *et al.*, 2015)
			Proteobacteria	Alphaproteobacteria	Rhizobiales	*Nitrobacter alkalicus*	N	
C-1	Cathode	DGGE	Chloroflexi	Thermomicrobia	Sphaerobacterales	*Sphaerobacter thermophilus*	DN	Chapter 4 (Vilajeliu-Pons *et al.*, 2015)
			Proteobacteria	Betaproteobacteria	Burkholderiales	*Ralstonia mannitolilytica*	Unknown	
C-2	Cathode	DGGE	Proteobacteria	Betaproteobacteria	Nitrosomonadales	*Nitrosomonas europaea*	N	Chapter 4 (Vilajeliu-Pons *et al.*, 2015)
			Proteobacteria	Alphaproteobacteria	Rhizobiales	*Nitrobacter alkalicus*	N	
			Chloroflexi	Thermomicrobia	Sphaerobacterales	*Sphaerobacter thermophilus*	DN	
niBES	Anode	T-RFLP	Proteobacteria	Betaproteobacteria	Nitrosomonadales	*Nitrosomonas* sp.	N	Chapter 7
			Planctomycetes	Planctomycetia	Planctomycetales	*Brocardia* sp.	N	
			Planctomycetes	Planctomycetia	Planctomycetales	*Kuenenia* sp.	N	
			Proteobacteria	Alphaproteobacteria	Rhizobiales	*Uncultured Rhizobiales*	DN	

*Bibliographically based putative role

DGGE: Denaturing gradient gel electrophoresis; T-RFLP: Terminal restriction fragment length polymorphism; N: Nitrification; DN: Denitrification

The anoxic ammonium removal anode (niBES) clearly showed a clearly higher diverse community compared to C-1 and C-2 configurations. The microbiome was composed of members of different functional groups involved in the nitrogen cycle. Nitrifying bacteria (*Nitrosomonas*), Anammox (*Brocardia* and *Kuenenia*), denitrifying bacteria (*Bacteroidetes* and *Proteobacteria*), Feammox (*Actinobacteria*) and *Firmicutes* were identified, clearly demonstrating their coexistence inside the reactor. The entire diversity of bacteria was certainly not only involved in the ammonium oxidation. It suggested that the degradation of intermediate products to dinitrogen but also simultaneous cross reactions (e.g. anammox reaction) as well as transfer of metabolites (e.g. amino acids, organic carbon compounds) could be their functionality in the biofilm. This hypothesis was in accordance with the coulombic efficiency (35±13%). The extreme diversity of genera was in agreement with previous studies on bioelectrochemical ammonium oxidation, where *Nitrosomonas europaea* was the main specie identified in the community together with *Empedobacter* (Qu *et al.*, 2014), or it was part of the minority community dominated by the denitrifying *Thermomonas* (Zhan *et al.*, 2014).

8.3.3.2. Denitrifying microbiome

Denitrification process consisted on the reduction of nitrites and nitrates generated from the ammonium oxidation to the harmless dinitrogen gas (N_2). Traditionally the process requires organic matter (heterotrophically), but in BES the process is catalysed autotrophically (Clauwaert *et al.*, 2007a). Nitrate reduction depends on the activity of chemolithoautotrophic nitrate reducers, which are phylogenetically diverse (over 60 genera). Moreover, most of these bacteria are able to use other compounds as electron donors (sulphide, hydrogen, or reduced iron) for denitrification (Weber *et al.*, 2006b).

When comparing the denitrifying microbiome of C-1 and C-2 (Chapter 4), it was observed that the putative denitrifying microbiome were similar despite the differences in cathode operational conditions: independent denitrification in C-1 and simultaneous nitrification-denitrification in C-2 (Table 8.8). Some of the reasons

of these similarities could rely on the high diversity of genera identified that could perform the process and the presence of oxygen in both cathodes. Some families with autotrophic denitrifiers belonging to the *Bacteroidetes*, the *Chloroflexaceae* and the *Proteobacteria* were found in both systems. From them, only *Sphaerobacter thermophilus* had been described in literature as denitrifier (Jones *et al.*, 2013) while the putative role of the others remained unknown. Additionally, simultaneous processes in the cathode of C-2 allowed the establishment of species with the ability to perform different reactions. *Nitrosomonas europaea* was a clear example of an ammonia oxidizer that is also able to reduce nitrite to nitrous oxide (Schmidh *et al.*, 2004, Shrestha *et al.*, 2002).

8.4. Implications of this thesis

Swine manure is a hazardous wastewater rich on carbon (organic matter) and nitrogen (in the form of ammonium). This PhD thesis has demonstrated that the simultaneous treatment of both compounds with renewable bioelectricity production using BES is feasible (robust, reliable and resilient). Moreover, the scalability of the process, through the assembling of a stacked BES was also successful.

The multidisciplinary approach of this PhD thesis allowed a better comprehension of BES in terms of nutrient removal, electricity production and microbiome characterization. Different BES operational conditions were applied, identifying the optimal configuration for organic matter and nitrogen electroactive removal activity. In MFCs, the appropriate workability of anode and cathode is indispensable for not limiting the system. In order to obtain a more electroactive bioanode for treating complex wastewaters, an anaerobic cathode was demonstrated to present a better performance than the strategy of using an oxygen reduction cathode.

Nitrogen removal was evaluated under different aeration conditions (aerobic and anoxic conditions) for both nitrification and denitrification processes. In the configurations that required aeration, a physical separation of the compartments

performing the two reactions was the key to obtain high nitrification and denitrification rates with more electrotroph activity. This PhD thesis also demonstrated that nitrification can be performed in a configuration under strictly anoxic conditions. The treatment rates and efficiencies were still lower compared to aerobic conditions. However, this is the first approach that anodic nitrification has the potential to possess an energy consumption one order of magnitude lower than the classical aerobic nitrification.

The mL-scale reactors were moved towards application by using a stacked configuration. Two materials, the granular graphite and stainless steel, were evaluated as electrode. Positively, the good performances of mL-scale reactors were reproduced at the scaled-up BES in terms of organic matter and nitrogen removal efficiencies and electroactivity behaviour. Besides granular graphite has been widely used for BES applications, the scaled-up experiments detected that its efficiency declined over time due to electrode crushing. The use of stainless steel highlighted as a more appropriate electrode for long-term operation of scaled-up BES for treating complex wastewaters.

Highly diverse, but specialized communities were found in the different compartments. The microbiomes were characterised both quantitatively (qPCR) and qualitatively (PCR-DGGE, 454-pyrosequencing, T-RFLP, FISH and SEM) identifying the putative role of some members of the electrogenic, nitrifying and denitrifying community. Moreover, the microbial organization of nitrifying bacteria showed an AOB/NOB gradient according to the substrate and oxygen concentration gradients.

A strategy for monitoring reactor microbiomes of nitrifying BES from the electrochemical perspective has been successfully evaluated. The use of microcosms was shown as an effective way to characterise the extracellular electron transfer of bioanodes treating ammonium. In particular, hydroxylamine was identified as the main substrate for the anoxic microbial electrochemical oxidation reaction in our system.

The optimization of the electricity production was performed by an external

resistance control (MPPT) in mL-scale reactor. MPPT control positively affected bacterial abundance and promoted the selection of putatively exoelectrogenic bacteria in the MFC core microbiome (*Sedimentibacter* sp. and gammaproteobacteria). These differences in the microbiome may be responsible for the two-fold increase in power production achieved by this system. Once the reactor scaled up, the mixed (series-parallel) circuit connection presented the maximum power production and high current densities. In L-scale reactors, the optimization of electricity production was performed by evaluating different electric circuit connections (series, parallel and mixed). The results obtained suggested that a mixed electric circuit is the most appropriate operation.

The application of stacked BES equipped with an external nitrifying reactor, with stainless steel electrodes and a mixed electric circuit is proposed as a feasible strategy to maintain or even improve the carbon and nitrogen treatment efficiencies and power densities when scaling-up MFCs treating complex wastewaters as swine manure. However, the proof-of-concept of anoxic nitrification in a BES bioanode presented in this PhD thesis opens the window to a clear breakthrough on the current treatments of wastewaters containing ammonium. Although further studies are required to improve the nitrogen removal rates, the anodic nitrification has the potential to simplify the reactor operation in WWTPs in the future. It would suppress the requirements of air dispersers for aerobic nitrification, as well as the organic carbon addition (e.g. methanol) for denitrification.

Chapter 9

Conclusions

This PhD Thesis explores the simultaneous treatment of organic matter and nitrogen compounds in complex wastewaters. The results obtained using different BES configurations, reactor sizes and microbial techniques proved the robustness, reliability and resilience of the technology in order to move forward to application. The main conclusions could be summarized as follows:

- The electroactive abilities of bioanodes treating complex wastewaters was higher when using an anoxic denitrifying cathode (CE = 24%) instead of an aerobic cathode (either NO_3^-/O_2 or solely O_2 as cathode electron acceptor; CE = 5-6%). The best performance for nitrogen treatment (i.e. combination of nitrification rates, nitrogen removal rates and intermediates accumulation) was found when nitrification and denitrification were performed in separated compartments (nitrification rate of 260 g N m^{-3} d^{-1}, nitrogen removal rate of 160 g N m^{-3} d^{-1} without nitrite accumulation).

- The scaled-up systems were capable to maintain the carbon and nitrogen removal rates (1.2 kg COD m^{-3} d^{-1} and 300-370 g N m^{-3} d^{-1}, respectively) without intermediate species accumulation. Moreover, the electroactivity behaviour found in mL-scale was positively reproduced at L-scale (CE around 15 % in all cases). At short-term, no significant differences were seen when using granular graphite or stainless steel as electrode in the scaled-up system. However, at long-term it would be recommended the usage of stainless steel to avoid electrode crushing.

- The optimization of the electricity production was performed by an external resistance control (MPPT) in mL-scale reactor and by different electric circuit connection in scaled-up BES. MPPT control positively affected bacterial abundance and promoted the selection of putatively exoelectrogenic bacteria in the MFC core microbiome (*Sedimentibacter* sp. and gammaproteobacteria). These differences in the microbiome may be responsible for the two-fold increase in power production achieved in this system. Once the reactor scaled-up, the mixed (series-parallel) circuit

connection presented the maximum power production and high current densities.

- MFC microbiomes treating industrial wastewaters as swine manure were characterized and related with nutrient removal capacity and electricity production. The inherent bacteria from swine manure did not affect the core MFC microbiome. In the anodes, the species *Geobacter sulfurreducens* and *Sedimentibacter* sp. were related to putative electrogenic role and *Clostridium disporicum* and *Turicibacter* sp. to a fermentative behaviour. Different AOB nitrifying communities (*Nitrosospira* sp. and *Nitrosomonas europaea*) were identified from the different configurations studied. Moreover, the AOB and NOB biofilm distribution was related to concentration gradients. In the cathodes, high diverse communities were found.

- Anoxic nitrification using the anode as electron acceptor was demonstrated. The treatment rates and electrogenic efficiencies were lower than under aerobic conditions (35 g N m^{-3} d^{-1} and 28%, respectively) but this process have the potential to become an alternative technology to remove nitrogen. It possess a lower energy consumption (0.13 kWh $kg^{-1}_{N\text{-}NH4+}$) compared to the classical aerobic nitrification (between 1.2-4.6 kWh $kg^{-1}_{N\text{-}NH4+}$). Through the application of anodic nitrification, the aeration could be completely suppressed for nitrogen treatment.

- The extracellular electron transfer thermodynamics for ammonium oxidation was elucidated. A redox system located at +0.73 V vs SHE was related to ammonium and hydroxylamine oxidation to nitrite. Among both, hydroxylamine was identified as the main substrate for the anoxic microbial electrochemical oxidation reaction in our system, since higher current densities were observed when it was used as the sole electron donor (4.02±0.46 μA cm^{-2}).

The future directions for research in this area can have multiple perspectives.

In terms of applied research, further studies regarding the scalability of BES are required. The improvement of the initial scaled-up reactors performance in terms of electroactivity is essential for the successful future application in real systems. This PhD thesis was able to reproduce the electroactivity achieved at mL-scale into L-scale reactors. In order to improve the treatment of pollutants together with the power output, the study of electrode materials able to increase the biomass attachment surface and reduce its resistivity, as well as, the evolution to materials able to keep their structure and properties at long-term is crucial for the proper development of scaled-up reactors. The achievement of high and stable CEs will make BES stronger and more competitive than other processes, like anaerobic digestion or activated sludge. Moreover, more attention is required on reactor design. It influences on substrate distribution, where dead spaces must be avoided, and on the workability of the pilot plants, which can end up with higher removal capabilities and power outputs.

At mL- scale, further research is required from the fundamental and applied research points of view. There are some gaps to elucidate referring to the anoxic nitrification electron transfer mechanism and the microorganism responsible of that reaction. In this sense, the work presented in this PhD thesis contributed with the study of the mechanisms involved in the anoxic ammonium removal, characterizing the associated microbiome.

Chapter 10

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