WHERE NITRITE RESPIRATION MEETS ELECTROTROPHY: DIVERSITY STUDIES AND FUNCTIONAL CHARACTERIZATION OF AUTOTROPHIC BACTERIAL ISOLATES FROM BIOELECTROCHEMICAL SYSTEMS

Ariadna Vilar Sanz

Universitat de Girona

Doctoral thesis

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Dr. Lluís Bañeras Vives

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Universitat de Girona

El Dr. Lluís Bañeras Vives, professor titular del departament de Biologia de la Universitat de Girona.

DECLARA:

Que el treball titulat "*Where nitrite respiration meets electrotrophy: Diversity studies and functional characterization of autotrophic bacterial isolates from bioelectrochemical systems*", que presenta l'Ariadna Vilar Sanz per a l'obtenció del títol de doctor per la Universitat de Girona, ha estat realitzat sota la meva direcció i que compleix els requisits per poder optar a Menció Internacional.

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

Dr. Lluís Bañeras Vives

Girona, 2015

Dedicatòria

Tothom diu que la recta final d'escriure una tesi és dura, però crec que fins que no t'hi trobes no ets capaç d'adonar-te'n. Abans de començar a agrair a tots els que m'heu fet costat en aquest camí, permeteu-me que em posi una mica filosòfica. No sé si és pel fet d'haver escrit la tesi, o tot el que he viscut en aquest temps, el que fa que ara ho consideri com un llarg viatge, amb tots els seus alts i baixos. Potser el més apropiat és fer el símil amb la tempesta de sorra de la que parla Murakami, de la qual sobrevius però en surts transformat.

> "...Y cuando la tormenta de arena haya pasado, tú no comprenderás cómo has logrado cruzarla con vida. ¡No! Ni siquiera estarás seguro de que la tormenta haya cesado de verdad. Pero una cosa sí quedará clara. Y es que la persona que surja de la tormenta no será la misma persona que penetró en ella. Y ahí estriba el significado de la tormenta de arena."
>
> *Kafka en la orilla*
> Haruki Murakami

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Llistat de publicacions

Denitrifying Bacterial Communities Affect Current Production and Nitrous Oxide Accumulation in a Microbial Fuel Cell

Ariadna Vilar-Sanz, Sebastià Puig, Arantzazu García-Lledó, Rosalia Trias, Maria Dolors Balaguer, Jesús Colprim and Lluís Bañeras

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Denitrifiers isolated from biocathodes showed different electrotrophic capacities

Ariadna Vilar-Sanz, Narcís Pous, Sebastià Puig, Maria Dolors Balaguer, Jesús Colprim and Lluís Bañeras

Submitted

Non conventional abbreviations

- **Ag/AgCl:** Silver chloride electrode
- **Anammox:** Anaerobic Ammonium Oxidation
- **AOA:** Ammonium Oxidizing Archaea
- **AOB:** Ammonium Oxidizing Bacteria
- **ARB:** Anode-Respiring Bacteria
- **BES:** BioElectrochemical System
- **BLAST:** Basic Local Alignment Search Tool
- **C/N:** Carbon/Nitrogen ratio
- **Cd:** Current density
- **CE:** Coulombic Efficiency
- **cfu:** colony forming unit
- **COD:** Chemical Oxygen Demand
- **CV:** Cyclic Voltammetry
- **DGGE:** Denaturing Gradient Gel Electrophoresis
- **dMFC:** denitrifying Microbial Fuel Cell
- **DNRA:** Dissimilatory nitrate reduction to ammonium
- **$E^{0'}$:** Reduction potential
- **EET:** Extracellular Electron Transfer
- **H':** Shannon diversity index
- **HGT:** Horizontal Gene Transfer
- **HOB:** Hydrogen Oxidizing Bacteria
- **MFC:** Microbial Fuel Cell
- **NAC:** Net Anodic Compartment
- ***napA*/NapA:** Periplasmatic nitrate reductase gene and protein
- ***narG*/NarG:** Membrane-bound nitrate reductase gene and protein
- **NCC:** Net Cathodic Compartment
- **NirK or Cu-NIR:** Copper-containing nitrite reductase
- ***nirK*:** Copper-containing nitrite reductase gene
- **NirS or Cd_1-NIR:** Cytochrome Cd_1 nitrite reductase
- ***nirS*:** Cytochrome Cd_1 nitrite reductase gene
- **NOB:** Nitrite Oxidizing Bacteria
- **NOR:** Nitric oxide reductase
- **NOS:** Nitrous oxide reductase
- ***nosZ*:** Nitrous oxide reductase gene
- **OD:** Optical Density
- **OTU:** Operational Taxonomic Unit
- **CEM:** Cation Exchange Membrane
- **PWPCR:** PlateWash-polymerase chain reaction
- ***q*PCR:** quantitative PCR
- **R-NH_2:** Organic Nitrogen
- **S_{Chao1}:** Expected richness
- **SHE:** Standard Hydrogen Electrode
- **S_{obs}:** Observed richness
- **$\Delta i/\Delta E$:** First derivative of the voltammetric curve over the potential

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Resum

Les piles de combustible microbianes amb càtodes desnitrificants (dMFCs) són una bona alternativa als tractaments convencionals de desnitrificació heterotròfica quan les aigües residuals contenen una baixa concentració de carboni respecte el nitrogen. La capacitat de les dMFCs per reduir el nitrat o nitrit present a les aigües, recau completament en l'activitat dels bacteris desnitrificants del càtode, que utilitzen el poder reductor dels electrons que flueixen des de l'ànode fins al càtode per reduir el nitrat. Malgrat les comunitats presents en els càtodes han estat caracteritzades anteriorment, en cap estudi s'han utilitzat gens funcionals de la desnitrificació com a marcadors. L'ús de d'aquests gens com a marcadors específics, s'utilitza de manera freqüent per l'estudi de comunitats de bacteris desnitrificants, ja que aquesta capacitat està present en grups filogenètics molts diversos.

En la present tesi doctoral, s'ha analitzat la composició de la comunitat de bacteris desnitrificants en el càtode d'una dMFC durant diferents condicions d'operació, utilitzant diferents acceptors d'electrons: nitrat o nitrit; i en presència de donadors alternatius d'electrons: matèria orgànica. La presència de bacteris reductors de nitrat i nitrit, estava molt afectada per les diferents condicions d'operació. El nombre d'unitats taxonòmiques operacionals (OTUs), definides a nivell d'espècie, pels gens *narG, napA, nirS i nirK* va ser de 11, 10, 31 i 22, respectivament. En canvi, les comunitats formades pels bacteris reductors d'òxid nitrós (*nosZ*), es mantenien pràcticament invariables en totes les condicions assajades. La majoria de seqüències de *nosZ*, un 90%, estaven agrupades en un únic OTU que tenia una elevada similitud amb el gen *nosZ* d'*Oligotropha carboxidovorans* i d'*Hyphomicrobium nitrativorans*. El nombre de còpies dels diferents gens, va revelar que la comunitat del càtode, estava dominada per bacteris que contenien el gen *nirS*. No només la comunitat bacteriana estava afectada, l'eficiència elèctrica de la pila també es va veure afectada per les condicions aplicades. La producció de corrent va decrèixer de 15.0 $A \cdot m^{-3}$NCC, quan es va utilitzar el nitrat com a acceptor d'electrons, a 11.0 $A \cdot m^{-3}$NCC amb nitrit. A més a més, el canvi a nitrit com a acceptor, també va afavorir l'acumulació d'òxid nitrós (N_2O), fins a representar el 70% dels gasos acumulats. Les emissions d'òxid nitrós, estaven correlacionades positivament amb la ratio (q*nirS*+q*nirK*)/q*nosZ*. Les dades indicaven que diferents espècies bacterianes eren responsables de la reducció complerta del nitrat a nitrogen gas, i dels canvis en la producció eléctrica i l'acumulació de N_2O.

La comunitat formada per bacteris que contenen el gen *nosZ* no estava afectada per aquests canvis. L'estabilitat i l'elevada abundància relativa de bacteris que contenen aquest gen, fa plantejar-nos el rol que aquests bacteris poden tenir en els processos electrotròfics. La caracterització per DGGE de diferents comunitats procedents d'ànodes i càtodes de MFCs, utilitzant el gen *nosZ* com a marcador, va mostrar que les espècies dominants eren comunes en tots dos compartiments, i que per tant, biofilms procedents de qualsevol

d'ambdós compartiments es podien utilitzar com a inòculs per a realitzar enriquiments. Es van utilitzar diferents donadors d'electrons inorgànics: tiosulfat, sulfit i hidrogen, per incrementar les probabilitats d'enriquir desnitrificants autotròfics. Al final de l'aïllament, es van obtenir 119 cultius purs, dels quals 37 contenien el gen *nosZ*. El 56.8% dels aïllats es va classificar en l'OTU més abundant identificat en les MFCs segon la similitud amb el gen *nosZ*. Es van escollir 5 aïllats representatius, C2S229.1 (identificat com a *Hyphomicrobium denitrificans*), C2T108.3 (*Rhodopseudomonas palustris*), C1S131/132.1 (*Sinorhizobium fredii*), C1S131/132.2 (*Oligotropha carboxidovorans*) i C1S119.2 (*Rhodopseudomonas palustris*).

Tots els aïllats, excepte C2T108.3 i C1S119.2, que no tenien cap nitrat reductasa, posseïen els gens que codifiquen per una ruta desnitrificativa complerta. La capacitat de créixer heterotròficament o autotròficament, es va testar en totes les soques, així com per la soca tipus. Tots els bacteris eren capaços de créixer en totes les condicions, excepte la C1S119.2. La funcionalitat de les nitrat, nitrit i òxid nitrós reductases es va demostrar per tots els bacteris tot i que no per totes les condicions assajades. A més a més, es va observar que les soques C2T108.3, C2S229.1 i *Oligotropha carboxidovorans* OM5^{T} eren capcaces d'utiilitzar acetilè com a font de carboni addicional, i per tant, impedia l'acumulació d'òxid nitrós utilitzant el mètode de bloqueig per acetilè.

La caracterització electroquímica de totes aquestes soques va demostrar la seva capacitat electrotròfica, encara que en la majoria de casos, limitada a un únic pas del procés de desnitrificació. *Oligotropha carboxidovorans* OM5^{T}, C2S229.1, C2T108.3 i C1S131/132.2, van ser capaces de reduir el nitrit electrotròficament a un potencial de -520 mV. En canvi, les soques C1S131/132.2 i C1S119.2, no van mostrar cap canvi en la seva activitat electroquímica en resposta a l'addició d'òxids de nitrogen. El potencial de reducció, es va estimar a -450 mV i possiblement estava correlacionat amb la producció d'hidrogen. Aquesta reacció, té lloc independenment de la preséncia de nitrat, nitrit i òxid nirós. La identificació d'habilitats totalment diferents en bacteris relacionats filogenèticament, com C2T108.3 i C1S119.2, ambdues identificades com *Rhodopseudomonas palustris*, i C1S131/132.2 i *Oligotropha carboxidovorans* OM5^{T}, reforça l'hipòtesi inicial que en els càtodes, l'activitat cooperativa de diferents espècies bacterianes és necessària per reduir completament el nitrat a nitrogen gas. Aquestes observacions ens permeten hipotetitzar sobre el funcionament global de la MFC i, malgrat els nostres resultats estan limitats a cinc aïllats, hem observat que reaccions complementaries com la producció d'hidrogen i la reducció del nitrit, podrien succeeir simultàniament en el càtode de les MFCs. D'acord amb això, la producció d'hidrogen podria complementar, a certs potencial catòdics, el procés de desnitrificació que té lloc en els càtodes desnitrificants.

Resumen

Las pilas de combustible microbianas con cátodos desnitrificantes (dMFCs) son una buena alternativa a los tratamientos convencionales de desnitrificación heterotrófica, cuando las aguas residuales contienen una baja concentración de carbono respecto al nitrógeno. La capacidad de las dMFCs para reducir el nitrato o el nitrito presentes en el agua, recae completamente en la actividad de las bacterias desnitrificantes presentes en el cátodo. Éstas bacterias, utilizan los electrones que fluyen des del ánodo hasta el cátodo cómo fuente de poder reductor para reducir el nitrato. A pesar de que las comunidades presentes en los cátodos se han caracterizado previamente, en ningún estudio se han utilizado los genes funcionales de la desnitrificación como marcadores. El uso de estos genes como marcadores específicos, se utiliza habitualmente en el estudio de las comunidades de bacterias desnitrificantes, como consecuencia de la dispersión de esta capacidad entre bacterias pertenecientes a distintos grupos filogenéticos.

En la presente tesis doctoral, se ha caracterizado la composición de la comunidad de bacterias desnitrificantes del cátodo de una dMFC durante diferentes condiciones de operación, usando distintos aceptores de electrones: nitrato o nitrito, y en presencia de donadores alternativos de electrones, la materia orgánica. La presencia de bacterias reductoras de nitrato y nitrito está muy afectada por las diferentes condiciones de operación. El número de unidades taxonómicas operacionales (OTUs), definidas a nivel de especie para los genes *narG, napA, nirS y nirK* fue de 11, 10, 31 y 22, respectivamente. Por el contrario, las comunidades formadas por bacterias reductoras de óxido nitroso (*nosZ*), se mantenían prácticamente invariables en todas las condiciones ensayadas. La mayoría de secuencias de *nosZ*, un 90%, se agruparon en un único OTU que presentava una elevada similitud con el gen *nosZ* de *Oligotropha carboxidovorans* y de *Hyphomicrobium nitrativorans*. La cuantificación del número de copias de los genes reveló que la comunidad del cátodo estaba dominada por bacterias que contenían el gen *nirS*. No solamente la comunidad bacteriana estaba afectada, también la eficiencia eléctrica se vio alterada por las condiciones aplicadas. La producción eléctrica disminuía de 15.0 $A \cdot m^{-3}$NCC, usando nitrato como aceptor de electrones, a 11.0 $A \cdot m^{-3}$NCC con nitrito. Además, el cambio a nitrito como aceptor de electrones, también afectó a la acumulación de óxido nitroso (N_2O), que llegó a representar el 70% de los gases acumulados. Las emisiones de óxido nitroso estaban correlacionadas positivamente con la ratio (q*nirS*+q*nirK*)/q*nosZ*. El conjunto de datos indicaba que distintas especies bacterianas eran las responsables de la reducción completa del nitrato a nitrógeno gas, y que los cambios en la producción eléctrica y en la acumulación de N_2O eran consecuencia de las condiciones de operación.

La comunidad compuesta por bacterias que contienen el gen *nosZ* no estaba afectada por estos cambios. La estabilidad y la elevada abundancia relativa de las bacterias que

contienen este gen, conduce a plantearnos el papel que pueden desempeñar en los procesos electrotróficos. La caracterización por DGGE de distintas comunidades bacterianas procedentes de ánodos y cátodos de MFCs, y utilizando el gen *nosZ* como marcador, mostró que las especies dominantes eran comunes en ambos compartimentos. Se utilizaron diferentes donadores de electrones inorgánicos: tiosulfato, sulfito e hidrógeno, para incrementar la probabilidad de enriquecer desnitrificantes autotróficos. Se obtuvieron 119 cultivos puros al finalizar el proceso de enriquecimiento, de los cuales solamente 37 contenían el gen *nosZ*. El 56.8% de los aislados se agrupaban en el OTU más abundante identificado en las MFCs en base a la similitud con el gen *nosZ*. Se escogieron 5 aislados representativos, C2S229.1 (identificado como *Hyphomicrobium denitrificans*), C2T108.3 (*Rhodopseudomonas palustris*), C1S131/132.1 (*Sinorhizobium fredii*), C1S131/132.2 (*Oligotropha carboxidovorans*) y C1S119.2 (*Rhodopseudomonas palustris*).

Todos los aislados excepto C2T108.3 y C1S119.2, que no contenían ninguna nitrato reductasa, poseían todos los genes responsables de completar el proceso de desnitrificación. La habilidad de crecer heterotróficamente o autotróficamente se testo para todos los aislados. Todas las bacterias eran capaces de crecer en todas las condiciones, exceptuando la C1S119.2. La funcionalidad de las nitrato, nitrito y óxido nitroso reductasas se demostró para todas las bacterias, pero no en todas las condiciones ensayadas. Además se observó que C2T108.3, C2S229.1 y *Oligotropha carboxidovorans* OM5^{T} eran capaces de utilizar el acetileno cómo fuente de carbono adicional, impidiendo la acumulación de óxido nitroso cuando se uso el método de bloqueo con acetileno.

La caracterización electroquímica de las cepas demostró su capacidad electrotrófica, aunque en la mayoría de casos limitada a un único paso de la ruta de la desnitrificación. *Oligotropha carboxidovorans* OM5^{T}, C2S229.1, C2T108.3 i C1S131/132.2, eran capaces de reducir el nitrito electrotróficamente a un potencial de -520 mV. En cambio, las cepas C1S131/132.2 y C1S119.2, no mostraron ningún cambió en su actividad electroquímica en respuesta a la presencia de óxidos de nitrógeno. El potencial reductivo se estimó en -450 mV y posiblemente estaba correlacionado con la producción de hidrogeno. Está reacción se produce independientemente a la presencia de nitrato, nitrito o óxido nitroso. La identificación de habilidades totalmente diferentes en bacterias filogenéticamente relacionadas, cómo C2T108.3 y C1S119.2, ambas identificadas como *Rhodopseudomonas palustris*, y C1S131/132.2 y *Oligotropha carboxidovorans* OM5^{T}, sustenta la hipótesis de que en los cátodos, la acción coordinada de distintas especies bacterianas es necesaria para completar la reducción del nitrato a nitrógeno gas. Estas observaciones, permiten hipotetizar sobre el funcionamiento global de la pila, y a pesar de que nuestros resultados están limitados a cinco aislados, hemos mostrado que reacciones complementarias, cómo la producción de hidrogeno y la reducción de nitrato, pueden ocurrir simultáneamente en los cátodos de MFCs. De acuerdo con nuestra hipótesis, la producción de hidrogeno podría complementar, a ciertos potenciales catódicos, el proceso de desnitrificación.

Summary

Microbial Fuel Cells with denitrifying cathodes (dMFCs) can be used as an alternative to heterotrophic denitrification in wastewater treatment when low amount of organic matter is present compared to nitrogen content. The dMFCs ability to reduce nitrate or nitrite present in water depends completely on the activity of denitrifying bacteria developed on the cathode. These bacteria use the electron flow from the anode to the cathode to reduce nitrate electrotrophically. Cathodic bacterial communities have been previously characterized, but there has been no study that has used the functional genes of denitrification as molecular markers. The use of specific markers for these genes have been widely used to study denitrifying bacterial communities because this ability is widespread among the phylogenetic tree.

In the present PhD dissertation, denitrifying bacterial communities composition from dMFC cathode have been characterized under different operational conditions, using different electron acceptors: nitrate or nitrite, and the presence of alternative electron donors: organic matter. The presence of nitrate and nitrite reducing bacteria, are highly affected by the different operational conditions. The number of Operational Taxonomic Units (OTUs) defined at species level for *narG, napA, nirS* and *nirK* genes were of 11, 10, 31 and 22, respectively. On the contrary, the community of nitrous oxide reducing bacteria (*nosZ*), remained almost unvaried under all conditions tested. For most *nosZ* sequences, 90%, were grouped in a single OTU which had a high similarity with the *nosZ* gene of *Oligotropha carboxidovorans* and *Hyphomicrobium nitrativorans*. The amount of gene copy numbers revealed that the cathodic community was dominated by *nirS*-containing bacteria. Not only the bacterial community affected, but also the current efficiency was altered by the operational conditions. Current production decreased from 15 $A \cdot m^{-3}$NCC, using nitrate as an electron acceptor, to 11 $A \cdot m^{-3}$NCC with nitrite. Additionally, the change to nitrite as electron acceptor also affected the accumulation of nitrous oxide (N_2O), representing 70% of accumulated gases. Nitrous oxide emissions were correlated with the ratio (q*nirS*+q*nirK*)/q*nosZ*. Data indicated that different bacterial species were responsible for the complete nitrate reduction to nitrogen gas, and changes on current production and N_2O accumulation were a consequence of operational conditions.

The community composed of *nosZ*-containing bacteria was not affected by these changes. The stability and relative high abundance of bacteria containing this gene lead us to the question of the role of these bacteria in the electrotrophic processes. DGGE characterization of different bacterial communities from anodes and cathodes of MFCs, and using the gene *nosZ* as target, showed that the dominant bacterial species were common in both compartments. Different inorganic electron donors were used: thiosulphate, sulphide and hydrogen, to increase the chance to enrich autotrophic denitrifiers. At the end of the

isolation process we obtained 119 pure cultures, 37 of those containing the *nosZ* gene. The 56.8% of these isolates were classified in the most abundant OTU found in MFCs according to *nosZ* gene similarities. Five representative isolates were selected, C2S229.1 (identified as *Hyphomicrobium denitrificans*), C2T108.3 (*Rhodopseudomonas palustris*), C1S131/132.1 (*Sinorhizobium fredii*), C1S131/132.2 (*Oligotropha carboxidovorans*) and C1S119.2 (*Rhodopseudomonas palustris*).

All isolates, except C2T108.3 and C1S119.2, which lacked nitrate reductases, had a complete denitrification pathway. The ability to grow heterotrophically and autotrophically was tested for all strains. All bacteria were able to grow under all conditions, with the exception of C1S119.2. The functionality of nitrate, nitrite, and nitrous oxide reductases were demonstrated for all bacteria although not at all experimental conditions. Additionally, we observed that C2T108.3, C2S229.1 and *Oligotropha carboxidovorans* OM5^T were able to use acetylene as an additional carbon source, thus imparing nitrous oxide accumulation using the acetylene blockage method.

Electrochemical characterization of all these strains demonstrated their electrotrophic capacity, although in most cases limited to one step of the denitrification reaction. *Oligotropha carboxidovorans* OM5^T, C2S229.1, C2T108.3 and C1S131/132.2, were able to reduce nitrite electrotrophically at -520 mV (mid-point potential). Contrarily, the strains C1S131/132.2 and C1S119.2, did not show any change in the electrochemical activity in response to the addition nitrogen oxides. The reductive mid-point potential was estimated at -450 mV and possibly correlated to hydrogen production. This reaction occurred independently of the presence of nitrate, nitrite or nitrous oxide. The identification of totally different abilities in phylogenetically related bacteria, such as C2T108.3 and C1S119.2, both identified as *Rhodopseudomonas palustris*, and C1S131/132.2 and *Oligotropha carboxidovorans* OM5^T, reinforces the initial hypothesis that in cathodes the cooperative activity of different bacterial species is necessary to completely reduce nitrate to nitrogen gas. These observations allowed us to hypothesize about the global MFC performance and, although our results are limited to five isolates, we show that complementary reactions, such as hydrogen production and nitrite reduction, may occur simultaneously in a MFC cathode. According to our hypothesis hydrogen producing bacteria, at certain cathodic potentials, might fuel some denitrification steps.

1 Introduction

1.1 Denitrification, genes and activities

1.1.1 Overview of the Nitrogen cycle

Nitrogen is essential for life. Organisms have a central role in the transformation of nitrogen by complex metabolic pathways, which is known as the nitrogen cycle (Figure 1). Most of these reactions are mediated exclusively by *Bacteria* and *Archaea* (Knowles 1982, Zumft 1997).

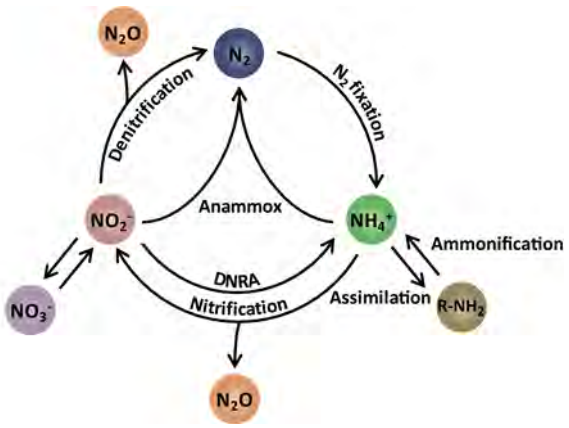


Figure 1. Nitrogen cycle. Schematic representation of metabolic pathways involved in the transformation of nitrogen connecting the most common nitrogen forms in nature.

Dinitrogen gas (N_2), the most abundant nitrogen form in nature, can be fixed by free living or symbiontic *Bacteria* and some *Archaea* into organic nitrogen ($R\text{-}NH_2$). Organisms that fix molecular nitrogen are diazotrophs, and many of them participate in other nitrogen transformations in the N-cycle. Diazotrophs have either molybdenum or vanadium dependent nitrogenases, which combine N_2 and hydrogen to produce highly reduced nitrogen forms (Dixon and Kahn 2004), which is fixed into the organic matter. The nitrogen contained in organic matter can be further mobilized to more soluble forms. Ammonification is the conversion process of organic nitrogen into ammonia (NH_4^+), which is highly soluble in water (Moir 2011). During nitrification, ammonia is oxidized to nitrate in two sequential steps conducted by phylogenetically unrelated groups of microorganisms, the ammonia-oxidizing bacteria and archaea (AOB or AOA), and nitrite oxidizing bacteria (NOB) (Bothe *et al.,* 2000, Kowalchuk and Stephen 2001, Treusch *et al.,* 2005). Nitrate can later be reduced back to N_2 by denitrification, a complex metabolic reaction catalyzed by many microorganisms, known as denitrifiers (Knowles 1982, Zumft 1997). These three metabolisms, N_2 fixation, nitrification and denitrification, constitute a true nutrient cycle that governs most nitrogen conversions that occur in nature. However, at least two other metabolic pathways have been described that may alter the cycle above under certain environmental conditions. Those pathways are the anaerobic oxydation of ammonia (anammox), and the nitrate reduction to ammonia (DNRA). Under anaerobic condicions, some members of *Planctomycetales*, can oxidize ammonium to N_2 using nitrite as the sole electron acceptor. The anammox reaction has been known for more than a decade and has been exploited in nitrogen removal (Kartal *et al.,* 2007). Dissimailatory nitrate reduction to ammonium (DNRA), in which nitrite is reduced to ammonium, is also a well known reaction and has recently centered the scientific interest since its activity may impact the efficiency of nitrogen removal facilities (Welsh *et al.,* 2014).

1.1.2 Denitrification pathway

Denitrification is a dissimilatory pathway based on a sequential reduction of nitrate coupled to electron transport phosphorilation. During denitrification, nitrogen oxides act as an alternative electron acceptors for energy production in the absence of oxygen (Zumft 1997). This alternative respiratory pathway is shared by many *Bacteria* and *Archaea* and is a facultative trait in most microorganisms (Knowles 1982, Zumft 1997).

All denitrifiers use a common pathway for their metabolism, consisting of four consecutive enzymatic reactions catalyzed by metalloproteins, that may differ from organism to organism (Park and Yoo 2009). Two enzyme groups participate in

dissimilatory nitrate reduction to nitrite, the membrane-bound nitrate reductase (NAR), and the free periplasmic nitrate reductases (NAP). Nitrite reduction is also catalyzed by two structurally different but metabolically equivalent enzymes, the periplasmic copper containing nitrite reductase (NirK or Cu-NIR) and the haem containing nitrite reductase (NirS or *cd*$_1$-NIR). Nitric oxide reductases (NOR) are generally membrane-bound and exist in a relatively large sequence heterogeneity among bacteria. Periplasmic nitrous oxide reductase (NOS) catalyses the final step in the denitrifiфacion reaction and is responsible for the N_2 formation (Figure 2) (Knowles 1982, Philippot 2002, Throbäck *et al.,* 2004).

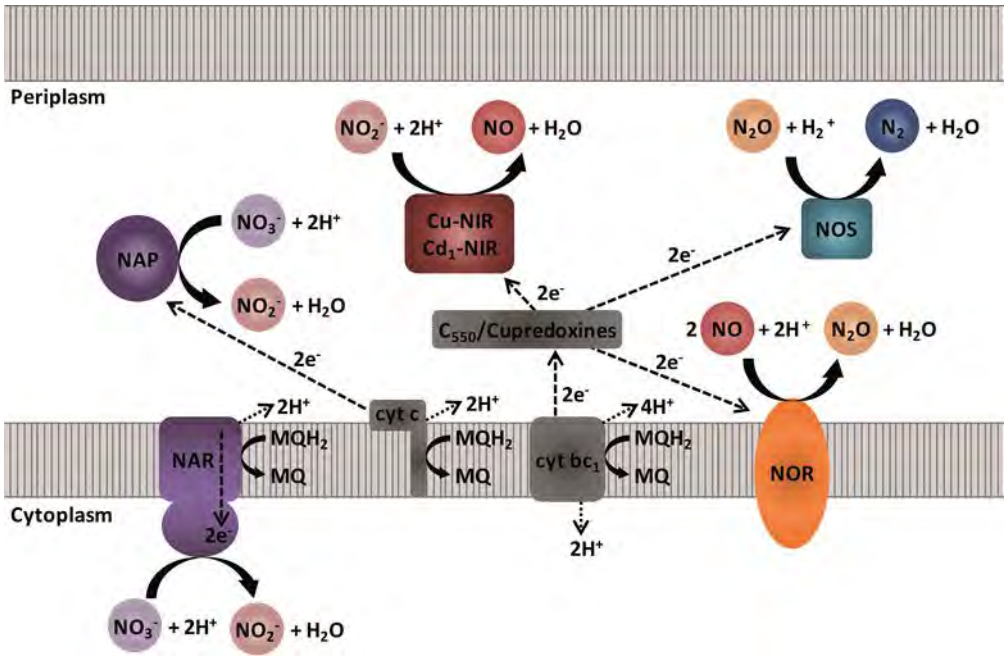


Figure 2. Denitrification pathway. Schematic representation of the most common metalloenzymes in the cell envelope. NAP, periplasmic nitrate reductase. NAR, membrane-bound nitrate reductase. Cu-NIR, periplasmic nitrite reductase copper containing. cd_1-NIR, periplasmic nitrite reductase haem containing. NOR, membrane-bound nitric-oxide reductase. NOS, periplasmic nitrous oxide reductase. Modified from (Cabello *et al.,* 2004).

Nitrate reduction is not an exclusive trait of denitrifying bacteria. NAR-type reductases are found in bacteria belonging to almost all bacterial divisions. Many non-denitrifying bacteria, such as *Escherichia coli* and other proteobacteria, can respire nitrate as an alternative in low oxygen conditions. NAR reductases are also present in *Archaea* (Afshar *et al.,* 2001). In contrast, NAP reductases are only present in Gram-negative bacteria (Philippot 2005a). The analysis of bacterial genomes reveals interesting differences in the

gene duplication events of both types of nitrate reductases. In the case of NarG, 1 to 3 copies of the gene may exist per genome (Philippot 2002), whereas a single copy of *napA* gene exists in most bacteria (Richardson *et al.,* 2001). Bacteria that catalyze exclusively the first denitrification step are named as nitrate respirers (or reducers) and are differentiated from true denitrifiers since they lack the ability to produce gaseous compounds. Nitrate-respiring microorganisms and true denitrifiers can harbour either NAP or NAR independently of their phylogeny. Moreover, the simultaneous presence of both types of reductases is also found in many bacterial genomes (Roussel-Delif *et al.,* 2005).

The *napA* gene sequences showed a good correlation with the 16S rRNA based taxonomy for the *Alpha*-, *Beta*-, *Gamma*-, and *Epsilonproteobacteria*. Nevertheless, some discrepancies were observed for strains such as *Pseudomonas aeruginosa*, *Bradyrhizobium japonicum* or *Magnetospirillum magnetotacticum* (Jones *et al.,* 2008, Philippot 2005a). In contrast, the phylogenies of *narG* were not consistent with that of 16S rRNA gene indicating that this gene is not highly reliable for taxonomic purposes (Gregory *et al.,* 2003, Philippot 2005a).

The first critical step in denitrification, and the key character that differentiates true denitrifiers, is the redution of nitrite to nitric oxide (NO) since this is the first step in the pathway that catalyzes the formation of a gaseous intermediate, thus contributing to N elimination. The two structurally different nitrite reductases found in denitrifiers differ in their active site. The *nirS* gene codes for a cytochrome *cd*1 nitrite reductase harboring a haem group in the active site, and differs significantly from the multi-copper oxidase metalloprotein encoded in *nirK* gene. It is generally assumed that *cd*1-NIR reductases are more common among dentitrifying bacteria, whereas copper containing reductases show a greater sequence variation (Zumft 1997). Relevant differences in function of the two nitrite reductases have been reported so far. This led to the formulation of an hypothesis in which the two types of nitrite reductases were functionally redundant, and thought to be mutually exclusive (Jones *et al.,* 2008). However, very recently, at least ten isolates have been found that possess both *nirK* and *nirS* genes in their genomes, although it has not yet been demonstrated that the two types of nitrite reductases are fully functional (Graf *et al.,* 2014).

A second degree of complexity among nitrite reductases reveal that two different classes of the *nirK* exist according to enzyme structure (Boulanger and Murphy 2003, Moir 2011). NirK-type I is predominantly found in the *Alpha*- and the *Gammaproteobacteria*, and in the halobacteria *Haloarcula marismortui*. Whereas, sequences NirK-type II copper-binding regions, occur mainly in *Firmicutes*, *Cytophaga-Flavobacterium*-

Bacteroidetes, and *Betaproteobacteria* (Jones *et al.,* 2008). NirK-type I has been largely used as a molecular proxy for bacterial denitrification in environmental studies and many sequences of uncultivated microorganisms are available in the databases. This has led scientists to believe that *nirK* containing bacteria were phylogenetically related (Throbäck *et al.,* 2004). Some *nirK* enzymes possess an additional N-terminal copper-containing cupredoxin domain with a T1Cu center that serve as a fused electron donor (Ellis *et al.,* 2007, Nojiri *et al.,* 2007). In others, NirK proteins are completed with a C-terminal monohaem cytochrome *c* domain contained in 160-190 extra amino acid residues which serves as an electron transfer site (Ellis *et al.,* 2007).

The periplasmatic cytochrome *cd*1 reductase (NirS) is a homodimeric, haem-containing protein with one *c* haem and one *d*1 group in each subunit. The group *c* haem accepts electrons from soluble electron carriers, similarly to *c*-type cytochromes, and then transfer the electrons to *d*1 haem where nitrite is reduced. The *d*1 haem is unique to the *cd*1-NIR and is synthesized by a specialized pathway only present in denitrifiers (Rinaldo *et al.,* 2011a, Rinaldo *et al.,* 2011b). The cytochrome *cd*1 nitrite reductase from *Paracoccus pantotrophus* and *Pseudomonas aeruginosa* have been thoroughly studied, and are considered as model examples at the structural and functional levels for this enzyme (Rinaldo *et al.,* 2011b).

Analyses of NIR genes provide interesting examples of bacterial evolution. The differences observed in *nirK* and *nirS* phylogenies, and also the species taxonomy between both structural groups of NirK, suggest the occurrence of convergent evolution, lineage sorting and Horizontal Gene Transfer (HGT) events (Jones *et al.,* 2008). In this sense, multi copper containing enzymes, such as Cu-NIR reductase, are thought to be the result of various duplication events or re-arrangements during evolution. This may have led to the wide range of functions found in these type of enzymes (Murphy *et al.,* 1997, Nakamura *et al.,* 2003). An interesting question arises when trying to decipher why two completely different alternatives (NirS and NirK) exist, and have been maintained through evolution for the same reaction. The most accepted hypothesis to explain this is the occurrence of alternative enzymatic functions that cannot be replaced for one or other enzyme. Isolates of *Rhizobium* sp. have shown the ability to reduce toxic selenite using NirK (Basaglia *et al.,* 2007). Another alternative function has been observed for NirS genes of *Roseobacter denitrificans*, with oxygen reductase activity and *Magnetospirillum* sp. which have ferrous nitrite oxidoreductase activity (Fukumori *et al.,* 1997).

Significant discrepancies on *nirS* and 16S rRNA phylogenies might be due to HGT and other gene rearrengement events. For example, two or more copies of the *nirS* gene have

been found in *Thiobacillus denitrificans*, *Dechloromonas aromaticum*, *Thaurea* species and *Magnetospirillum magneticum* (Jones *et al.,* 2008). However, the functionality of both gene copies is not unkikely since the two copies of *nirS* present in a *Thauera* sp. isolate were expressed under different conditions (Etchebehere and Tiedje 2005).

Nitric oxide (NO) can be reduced by two different nitric oxide reductases, the *c*Nor and *q*Nor, encoded in *cnorB* and *qnorB* genes, respectively. These two variants of the same gene have been proposed to be homologs, because *q*Nor may have resulted from a gene fusion of the *norC* and *norB* genes in which the *norC*-like region evolved (Braker and Tiedje 2003, Heylen *et al.,* 2007). NOR enzymes might rather play an additional role in detoxification of toxic nitrogen compounds. This has been shown in pathogen-host interactions where NO tolerance is affected by NO removal through denitrification (Zumft 2005). In addition to structural differences, the two enzymes occur in different bacterial species. Both NOR types can be found in *Betaproteobacteria,* whereas *Alphaproteobacteria* have *cnorB* genes exclusively (Heylen *et al.,* 2007). There is no evidence of the *norB* gene being transferred. However, the polyphyly in the *qnorB* clade together with the absence of this gene in the *Alphaproteobacteria* may indicate a different evolutionary history compared to *cnorB* (Jones *et al.,* 2008).

The last step involved in the denitrification process is the reduction of nitrous oxide (N_2O) to nitrogen gas (N_2). The core catalytic region of the N_2O reductase (NOS) contains two active sites. The C-terminal region may be lineage-specific and contains both catalytic regions, named CuZ and CuA. The CuZ site corresponds to the multinuclear copper catalytic site, whereas the CuA site is the cupredoxin active site in C-terminal. According to these two differences, two clades have been identified recently, clades I and II. The *nosZ* genes from each clade also contain a distinctive signal peptide that helps define the final location of the enzyme in the cell envelope through recognition of the transport mechanism. A twin-arginine translocation pathway (Tat) or the general secretory pathway (Sec) are used to transport NosZ clades I and II enzymes, respectively (Jones *et al.,* 2013).

The *nosZ* gene has been hypothesized as a likely candidate for HGT, since it has also being detected in bacterial plasmids together with genes encoding for transposases, recombinases and plasmid transfer proteins (Zumft 1997). However, the *nosZ* phylogenies show the best score when compared with 16S rRNA based taxonomies (Dandie *et al.,* 2007), and is the desired target for molecular studies of denitrification based on clade I. This clade is composed of *nosZ* sequences from members of the *Alpha*-, *Beta*-, and *Gammaproteobacteria*, togheter with *Haloarcula*, *Halorubrum* and *Halogeometricum*. Contrarily, bacteria classified in clade II of *nosZ* gene are scattered on

the phylogenetic tree, and members of the *Proteobacteria* (*Alpha*-, *Beta*-, *Gamma*- and *Epsilon*-), *Bacteroidetes* and *Archaea* have been described (Jones *et al.,* 2013).

1.1.3 Missing denitrification steps

The reason for the widespread distribution of the denitrifying genes among phylogenetically diverse microorganisms is a matter of debate. Either denitrification appeared only once in evolution and affected a common ancestor existing probably before the split between *Archaea* and *Bacteria* (Knowles 1982, Philippot 2002, Zumft 1997), or the spread of the denitrification genes in today's bacterial species is the result of intense and repeated gene transfer events (Clays-Josserand *et al.,* 1999, Philippot 2002). In both cases, several phenomena involving the selective loss or duplication of denitrification genes may have been occurred during evolution. The differential evolutionary events that might have occurred among denitrifying genes resulted in organisms that harbor a different combination of genes involved in the denitrification pathway that often result in incomplete or truncated pathways (Zumft and Kroneck 2007).

Some decades ago, it was proposed that genes coding for all 4 reductive steps were contained in the so called "denitrification genomic island", which could be transferred from one species to another by lateral gene transfer (Zumft 1997). This hypothesis has since been discarded by the description of new genotypes that show that incomplete denitrification pathways are common (Bergthorsson *et al.,* 2007, Jones *et al.,* 2008, Philippot 2002). Denitrification is observed as modular pathway in which an organism does not always possess the full set of enzymes to perform a complete denitrification. Theoretically, any combination of denitrification genes is possible and examples of almost all of these combinations can be found in bacteria (Figure 3). Despite this possibility, complete denitrification pathways, including *nir*, *nor* and *nos* genes are relatively common among sequenced bacterial genomes (Graf *et al.,* 2014).

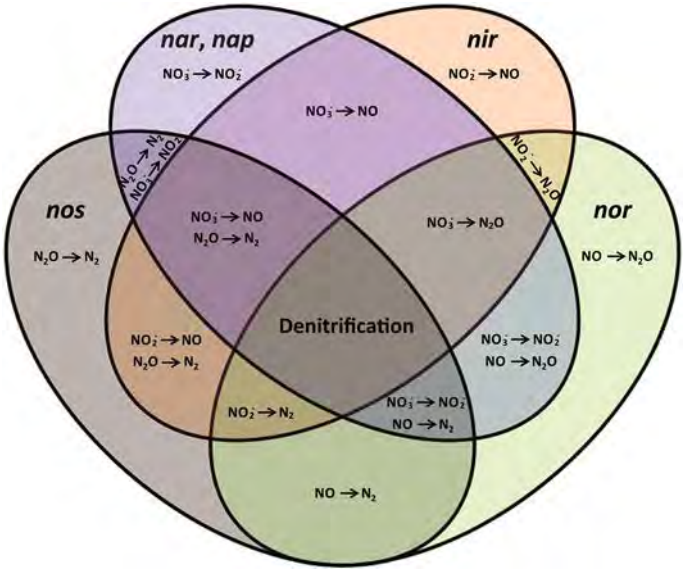


Figure 3. Genes included in modular denitrification pathways. Intersections show combinations of two or more metabolic steps. The genes implied in each pathway: nitrate reductases (*nar, nap*), nitrite reductases (*nir*), nitric oxide reductases (*nor*) and nitrous oxide reductases (*nos*), are indicated. Complete denitrifications is shown in the center of the figure.

Nitrate reduction is relatively common in bacteria as the first step in the denitrification pathway altough many nitrate reducers are not able to further metabolize nitrite down to gaseous compounds. This feature has been discussed earlier in this section. In general, denitrifying bacteria having NIR genes do also posses NOR activity, although some exceptions exist. Homologs of *qnorB* gene have been observed in non-denitrifying pathogenic species, and it is suspected to be involved in detoxification of nitric oxides (Philippot 2005b, Zumft 2005). The co-ocurrence of two or more genes catalyzing differen steps of the pathway might indicate a evolutionary link between them. Lack of NOR and NIR reductases, is found in *Bacteroidetes* and *Firmicutes* belonging to *nosZ* clade II. However, in some species, such as *Rhodothermus maritimus*, the lack of NOR gene is combined with the presence of NIR genes (Graf *et al.,* 2014).

The final step in the denitrification proces, NOS, can be either found or absent in a specific genome, independently of the presence of other genes of the pathway. According to the analyses of complete bacterial genomes, the *cd*1-NIR reductases are more common among dentitrifying bacteria, and higher co-ocurrence of NirS reductase with *nosZ* gene has been observed. Exceptions to this rule are *Cupriavidus eutropha* JMP134, and some members of the *Deinococcus-Thermus* and *Chlorofexi* phyla (Graf *et al.,* 2014, Jones *et al.,* 2008). *nirK*-containing bacteria may also have a truncated pathway. In this sense, the NirK-type II bacteria are mainly lacking the *nosZ* gene cluster and thus not participating actively in the complete reduction to nitrogen gas (Graf *et al.,* 2014, Jones *et al.,* 2008). The loss of *nosZ* enzyme can be associated to the little contribution of nitrous oxide reduction to the bioenergetic requirements of denitrifying bacteria (Jones *et al.,* 2008).

Many other bacteria, however, do exhibit the lack of NIR genes and participate exclusively in the reduction of nitrous oxide. Some interesting examples are *Anaeromyxobacter dehalogenans* or *Wolinella succinogenes* (Sanford *et al.,* 2002, Sanford *et al.,* 2012, Simon *et al.,* 2004), and some *Bacteroidetes*, *Deltaproteobacteria*, *Firmicutes* and *Euryarchaeota*, also exist (Graf *et al.,* 2014).

Although it seems that the co-ocurrence of different genes might be due to evolutionary events, non-random patterns of NIR/NOR/NOS genes occurrence have been observed and further research on physiological or evolutionary mechanisms would provide information to predict the bacterial capacities to develop N_2O mitigation strategies (Graf *et al.,* 2014).

The denitrification pathway is a widespread trait, and many different subsets of denitrifying enzymes can be found in bacterial species. It is difficult the use of 16S rRNA as a realistic molecular proxy to target denitrifiers. Functional genes need to be used instead, which could provide a good model for studying evolutionary relationship of denitrifying bacteria (Philippot 2002). Nevertheless, phylogenetic analysis of denitrifying gene sequences from bacterial isolates have showed incongruences when compared with 16S rRNA phylogenies (Dandie *et al.,* 2007, Delorme *et al.,* 2003, Gregory *et al.,* 2003, Heylen *et al.,* 2006a, Heylen *et al.,* 2007, Jones *et al.,* 2008). Although these incongruences can be critical for taxonomic purposes (Heylen *et al.,* 2007, Philippot 2002, Zumft 1997), we think the use of functional genes is a more realistic tool to study the composition of the denitrifying community.

1.1.4 Habitat preferences of Nir-containing denitrifiers

Despite the fact that there is a functional equivalence between NirK and NirS nitrite reductases, many experimental reports exist in which *nirS*:*nirK* ratios show significant differences suggesting a habitat selection due to environmental parameters (Bañeras *et al.,* 2012, García-Lledó *et al.,* 2011, Hallin *et al.,* 2006, Hallin *et al.,* 2009, Oakley *et al.,* 2007, Vilar-Sanz *et al.,* 2013)..

nirS-containing bacteria were found to be dominant in wastewater treatment plants (WWTPs) (Geets *et al.,* 2007, Wang *et al.,* 2014). Moreover, the diversity of *nirS* genotypes is always higher in cultured denitrifiers from municipal wastewater treatment plants compared to cultured *nirK* bacteria, suggesting that the concentration of organic matter is a major factor in the selection between both types (Hallin *et al.,* 2006). Not only the amount of organic matter but also the presence of certain organic compounds, such as methanol, may also affect the abundance of specific denitrifiers. Some specific

denitrifying bacteria, such as methylotrophs, mostly containing the *nirS* gene, are selected in the presence of C1 compounds and enriched in activated sludge (Hallin *et al.,* 2006). Many other parameters, such as salinity, pH, nitrate content and redox potential, also affect the enrichment of one of either type of NIR-containing bacteria. For example, communities from marine samples were dominated by *nirS*-type denitrifiers (Jones and Hallin 2010). However, soil samples showed differences on distribution of both NIR-types among environmental gradients. *nirS* denitrifiers are also dominant in the presence of clay, and higher nitrate concentrations, whereas *nirK* denitrifiers respond to more complex environmental parameters (Enwall *et al.,* 2010, Santoro *et al.,* 2006, Smith and Ogram 2008).

Community structure of *nirK* denitrifiers appear to be more stable in wasteweter treatment because they are less affected by external carbon sources (Hallin *et al.,* 2006). The dominance of *nirK* genes were found in special systems for treating wastewater, such as the OLAND (Oxygen limited autotrophic nitrifiying-denitrifying) and ABIL (Ammonium binding inoculum liquid) configurations (Geets *et al.,* 2007). Also, higher amounts of *nirK*-type denitrifiers were found in soil samples (Henry *et al.,* 2006), and in sediments from a free water surface constructed wetland treating nitrogen (García-Lledó *et al.,* 2011). *nirK*-type denitrifiers seem to be affected by a high nitrite amount and lower concentrations of organic matter and nitrate (Hallin *et al.,* 2006, Jones and Hallin 2010). The presence of copper is also a stronger ecological driver for the *nirK*-type denitrifiers, which fits with the fact of the *nirK* gene is a multicopper protein (Enwall *et al.,* 2010).

Differential distributions were also found in structured biofilm samples, being the *nirK* populations mainly located in the internal part of the biofilm, where the oxygen and nutrient concentrations are lower. The *nirS*-containing bacterial population, in contrast, was located in the outer region. Oxygen and nutrients (carbon and nitrogen) do play a role in shaping NirS and NirK-type bacteria distributions (Cole *et al.,* 2004). A similar effect was observed in groundwater, where nitrate, pH and DO, determined the proportion of *nirK* and *nirS* denitrifiers (Yan *et al.,* 2003).

Although it is not completely clear, previous results suggest a niche differentiation between bacteria harboring any of the two enzyme types and is a key character that may have helped in the maintenance of both types of bacteria through their evolution (Enwall *et al.,* 2010, Hallin *et al.,* 2009).

1.1.5 Nitrogen as a contaminant in water

Anthropogenic activity has increased contamination by nitrogen compounds. This not only increases the nitrogen concentration in water bodies but is also related with effects on a global scale, such as the increase on greenhouse gases emission and considerable effects on acid rain.

Nitrate (NO_3^-) is one of the most abundant contaminants in aquatic environments and affects both surface and ground waters. The major sources of nitrate are agricultural field runoff, leakage from septic tanks, sewage, and erosion of natural nitrate deposits (Nolan and Stoner 2000, Puckett *et al.,* 1999, van Egmond *et al.,* 2002). The increase of anthropogenic discharge coupled to the high stability and solubility of nitrate have led to accumulation at high concentrations in freshwater (Benedict *et al.,* 1997), limiting the use of natural water sources for human consumption (Park and Yoo 2009, Shrimali and Singh 2001, Till 1998).

Nitrate contamination not only impacts water quality, but has also been described as a hazard for human health implicated in methaemoglobinaemia or the "blue babies" syndrome (Knobeloch *et al.,* 2000). Additionally, in different parts of the body (e.g. oral cavity, stomach, bladder, or intestines) nitrate reducing bacteria can produce carcinogenic nitrosamines possibly related to non-Hodgkin's lymphoma and gastric cancers (Chang and Parsonnet 2010, Winneberger 1982). Environmental concerns are also associated to nitrite accumulation, because it can be toxic to aquatic organisms causing massive fish mortality and eventual losses of the aquatic plant beds or coral reefs, among other problems (Carpenter 1998, McIsaac 2003, Murphy 1991).

The increased concentration of N-pollutants such as NO_3^- and NO_2^- in water resources and the direct and indirect effects of these compounds on health and environment makes nitrogen removal a critical step in water treatment processes (Schnobrich *et al.,* 2007, Shrimali and Singh 2001). The removal of nitrogen compounds such as nitrate or nitrite from water can be accomplished by different techniques. Chemical and physical methods for nitrate removal are expensive because previous water treatments are required, or material replacement is needed (i.e. membranes in electro-dialysis, reverse osmosis and other concentration methods) (Monty C. Dozier 2008, Shrimali and Singh 2001). Other methods, such as chemical precipitation with rhodium, palladium and copper, produce ammonium as an end product limiting the overall nitrogen removal efficiency from water (Park and Yoo 2009, Shrimali and Singh 2001, Till 1998).

As an alternative, biologically driven processes are the desired choice for large scale applications. Biological denitrification is therefore used to finally remove nitrate from water in most European countries. Process control of biological denitrification is rather simple, selective and cost effective and is commonly used in wastewater treatment using a large series of adaptations and set-up configurations (Benedict *et al.,* 1997, Haugen *et al.,* 2002, Schnobrich *et al.,* 2007, Shrimali and Singh 2001, Sunger and Bose 2009). The most common biological methods for nitrogen removal in wastewater treatment plants (WWTPs) is based on the combination of two processes, autotrophic nitrification and heterotrophic denitrificatiton. The combination of both processes promotes the net loss of nitrogen from the system (Bernhard *et al.,* 2005). However, conventional denitrification respiratory processes become challenging to treat contaminated waters with high nitrogen and low carbon loads. Carbon dependence in heterotrophic denitrification produces additional costs involved in adding organic matter (mainly acetate) to water treatment (Puig *et al.,* 2008). To avoid this additional cost, autotrophic processes, such as partial nitritation-anammox, are used at full-scale installations (Lackner *et al.,* 2014). Another promising technology, denitrifying microbial fuel cells (dMFCs), is currently used to suppress the carbon dependence of denitrification (Clauwaert *et al.,* 2007, Puig *et al.,* 2011, Virdis *et al.,* 2008). MFC technology is based in the autotrophic denitrification process in which the addition of organic compounds is no longer required.

The application of the BES technology has been studied as a sustainable solution to treat wastewater, in a similar way to that proposed for anammox (anaerobic ammonium oxidation) treatment of nitrogen contaminated waters. BES and anammox driven technologies have been proposed as energetically efficient alternatives to conventional nitrification-denitrification processes to eliminate ammonium from wastewater (Rodriguez Arredondo *et al.*, 2015). Although anammox driven processes have been optimized and scaled-up in wastewater treatment (OLAND and PANAMMOX technologies, as improved examples), BES technologies are less developed so far. In addition to the application of BES technology to wastewater treatment, other alternatives have been successfully explored. Bioelectrochemical systems have the potential of being an alternative for treatment of nitrate-polluted groundwater in low organic matter content (Pous 2015). Despite these and other experimental efforts, the application of BES technology out of the laboratory conditions is challenging and scale-up tests have not been resulted in a significant outcome yet. Although a lot of limitations of this technology have been solved reducing costs of electrodes, and maximizing current

densities, process optimization is still needed. Field tests in pilot-scale reactors, checking operational parameters, performance of materials used over time, temperatures and system maintenance, are still required (Logan 2010). Additionally, a better understanding of electrotrophic denitrifiers implied in the process and the biochemical pathways used for these bacteria will be useful to scientific community to improve the applicability of bioelectrochemical systems devoted to nitrate elimination.

1.1.6 Autotrophic denitrifiers

Denitrification is a facultative respiratory pathway used by many bacteria when oxygen is limiting, in which sequential nitrate reduction is coupled to electron transport phosphorylation (Benedict *et al.,* 1997, Heylen *et al.,* 2006a, Jones *et al.,* 2008, Mahne and Tiedje 1995, Park and Yoo 2009). According to the electron donors used in the denitrification process, bacteria can be classified into heterotrophic and autotrophic denitrifiers, although many of them may be facultative. For autotrophic growth alternative electron donors, including hydrogen, sulphur compounds and metals, are used instead of organic molecules (Robertson and Kuenen 1990).

Heterotrophic denitrification has been largely used in wastewater treatment systems to remove nitrate. However, the diversity of autotrophic denitrifiers has been unattended to some extent, mainly because of their lower growth rates and the tricky requirements of strictly autotrophic bacteria for growing (Park and Yoo 2009, Till 1998, Zhang and Lampe 1999). In addition, many isolated denitrifiers have never been proven to grow autotrophically, although they may perfectly be facultative (Table 1) (Robertson and Kuenen 1990, Winterstein and Ludwig 1998).

Table 1. Examples of autotrophic denitrifying bacteria capable of chemolithoautotrophic growth. The trophic requirements as well the preferable electron donors are listed for each bacterial species.

Genus	Species	obl/fac	Substrates	References
Thiobacillus	*denitrificans*	fac	S^{2-}, $S_2O_3^{2-}$, S^o, Fe^{2+}	(Robertson and Kuenen 1990, Straub *et al.,* 1996)
	versutus	fac	S^{2-}, $S_2O_3^{2-}$, S^o, org	(Oh *et al.,* 2001)
	thyasiris	fac	S^{2-}, $S_2O_3^{2-}$, S^o, org	(Oh *et al.,* 2001)
	delicates	fac	S^{2-}, $S_2O_3^{2-}$, S^o, org	(Lee *et al.,* 2013)
	pantotropha	fac	S^{2-}, $S_2O_3^{2-}$, S^o, org	(Lee *et al.,* 2013)
	thiophilus	obl	$S_2O_3^{2-}$,	(Kellermann and Griebler 2009)
Sulfurimonas	*denitrificans*	obl	S^{2-}, $S_2O_3^{2-}$, S^o	(Robertson and Kuenen 1990)
Thiomicrospira	CVO	fac	HS^-,S^0 , H_2	(Gevertz *et al.,* 2000)
Thiosphaera	*pantotropha*	fac	S^{2-}, $S_2O_3^{2-}$, H_2, org	(Robertson and Kuenen 1990)
Paracoccus	*denitrificans*	fac	H_2, Fe^{2+}, org	(Robertson and Kuenen 1990, Till 1998)
	pantotrophus	fac	H_2, $S_2O_3^{2-}$, HS^-, org	(Szekeres *et al.,* 2002)
	alcaliphilus	fac	S^{2-}, org	(Lee *et al.,* 2013)
Alcaligenes	*eutrophus*	fac	H_2, org	(Robertson and Kuenen 1990)
Pseudomonas	*saccharophilia*	fac	H_2, org	(Robertson and Kuenen 1990)
	pseudoflava	fac	H_2, org	(Robertson and Kuenen 1990)
	stutzeri	fac	H_2, Fe^{2+}, org	(Straub *et al.,* 1996, Szekeres *et al.,* 2002)
Rhodopseudomonas	*sphaeroides*	fac	H_2, org	(Park and Yoo 2009)
Paracoccus	*denitrificans*	fac	H_2, org	(Park and Yoo 2009)
Alcaligenes	*eutrophus*	fac	H_2, org	(Chang *et al.,* 1999)
	thiophilus	fac	H_2, org	(Vasiliadou *et al.,* 2006)
Ochrobactrum	*anthropi*	fac	H_2, org	(Szekeres *et al.,* 2002)
Agrobacterium	sp.	fac	S^{2-}, org	(Lee *et al.,* 2013)
Acinetobacter	sp.	fac	S^{2-}, org	(Lee *et al.,* 2013)
Sulfurimonas	*denitrificans*	fac	S^{2-}, S^o, $S_2O_3^{2-}$, org	(Burgin *et al.,* 2012)
	paralvinellae	fac	H_2, S^0 $S_2O_3^{2-}$, org	(Takai *et al.,* 2006)
Thermothrix	*thiopara*	fac	$S_2O_3^{2-}$, org	(Brannan and Caldwell 1980)
Thioalkalivibrio	*denitrificans*	fac	$S_2O_3^{2-}$, org	(Sorokin *et al.,* 2001)
	nitratireducens	fac	HS^-, $S_2O_3^{2-}$, org	(Sorokin *et al.,* 2003)
Thiohalomonas	*nitratireducens*	fac	$S_2O_3^{2-}$, org	(Sorokin *et al.,* 2007)
	denitrificans	fac	HS^-, $S_2O_3^{2-}$, org	(Sorokin *et al.,* 2007)
Acinetobacter	sp.	fac	H_2, org	(Vasiliadou *et al.,* 2006)
Acidovorax	*avenae*	fac	H_2, org	(Vasiliadou *et al.,* 2006)

obl: obligately autotrophic, **fac:** facultatively autotrophic, **org:** organic matter, **S^{2-}:** sulphide, **$S_2O_3^{2-}$:** thiosulphate, **S^o:** elemental sulphur, **Fe^{2+}:** iron (II), and **H_2:** hydrogen

According to the electron donor used autotrophic bacteria have been divided into hydrogen-based and sulphur-based denitrifiers (Zhang and Lampe 1999). Despite these capacities, other electron donors can be used alternatively, such as iron II (Fe^{2+}) or other metals (Straub *et al.,* 1996, Weber *et al.,* 2006).

Hydrogenotrophic denitrification is a biological process conducted by hydrogen oxidizing bacteria (HOB). Hydrogen is one of the most thermodynamically favorable electron donors for nitrate based respiration (Benedict *et al.,* 1997, Kurt *et al.,* 1987 , McCarty 1972, Park and Yoo 2009). On the other hand, sulphur-based denitrifiers gain energy from inorganic reduced sulphur compounds, such as sulphide (S_2^-), elemental sulphur (S^0), thiosulphate ($S_2O_3^{2-}$), tetrathionate ($S_4O_6^{2-}$), and sulphite (SO_3^{2-}), which serve as electron donors to reduce nitrate or nitrite (Batchelor and Lawrence 1978, Knowles 1982, Park and Yoo 2009, Sengupta and Ergas 2006). The weakest point of this metabolism, in addition to the sulphate and acid formation, is the low solubility of elemental sulphur, which can be replaced by thiosulphate (Liu and Koenig 2002, Moon *et al.,* 2008, Park *et al.,* 2002, Sahinkaya *et al.,* 2011). Thiosulphate is a good alternative because its high solubility and high energy yields (92.27 kJ/electron equivalent) (Koenig and Liu 2001, Park and Yoo 2009).

The use of inorganic electron donors has many advantages over heterotrophic denitrification in wastewater treatment plants. In this sense, an interesting source of electrons is electrical current, which has been exploited in Bioelectrochemical Systems (BES) to promote autotrophic denitrification (Rabaey *et al.,* 2007, Wrighton *et al.,* 2010).

1.2 Bioelectrochemical systems

1.2.1 Microbial Fuel Cell concept

Microbial Fuel Cells (MFCs) are electrochemical systems analogous to chemical batteries in which a difference in potential is created between the anode and the cathode. In MFCs current is generated by microbial metabolism and conducted through an open electric circuit (Logan *et al.,* 2006). Compared to chemically catalysed electrolysis, MFCs do not require expensive catalysts to promote oxidation of the electron donors at the anode because oxidation is naturally mediated by microorganisms. MFCs can be easily operated at a wide range of temperatures according to the physiological possibilities of microbes. Additional benefits of MFCs can be associated to the use of low-value fuels, such as wastewater or organic matter in soils or sediments which can be oxidized, further contributing to removal of contaminants.

Microbial Fuel Cells (Figure 4) usually contain two chambers, an anode and a cathode, physically separated by a cation exchange membrane (CEM). CEM restricts diffusion of oxygen and hydrogen from the cathode to the anode, and helps maintain anoxic conditions in the latter. CEM are also selectively permeable to protons and other positively charged molecules that are released during microbial oxidation of organic matter (Logan *et al.,* 2006, Lovley 2006).

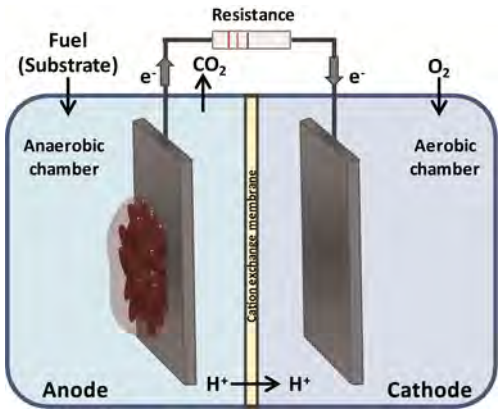


Figure 4. Microbial fuel cell with abiotic cathode. Microorganisms attached to anode oxidize the substrate generating electrons. The electrons flow across an electric circuit with a resistance to the cathode. These electrons can be used in the cathode to reduce an oxidized electron acceptor i.e. oxygen. Anode and cathode chambers are separated by a proton exchange membrane which allows diffusion of positive charged molecules (protons).

Anodic bacteria are either flowing-freely in the medium or adhered to the electrode surface forming a biofilm. Bacteria oxidize organic matter (or any other substrate available for bacterial oxydation) and produce CO_2, electrons and protons. Electrons are transferred to the anode and flow through an external circuit to the cathode. In the

cathode, the electrons are used by an electron acceptor, usually an oxidized molecule. Open air cathodes use atmospheric oxygen as the final electron acceptor (Bennetto 1990, He and Angenent 2006). Microbially catalysed electron production, and electron consumption in the cathode, are the two defining characteristics of a MFCs (Feng *et al.,* 2008, Liu and Logan 2004, Liu *et al.,* 2004, Liu *et al.,* 2005a, Logan *et al.,* 2006, Logan 2009, Lovley 2006, Oh and Logan 2006).

1.2.2 Evolution of Bioelectrochemical systems, from MFC to Microbial Electrolysis Cells (MEC)

MFCs and electrochemical systems (BES), benefit from natural bacterial interactions (He and Angenent 2006). The ability of bacteria to generate electricity has been known for many decades. Over a century ago in 1911, Michael C. Potter described the ability of a bacterial culture grown in sterile conditions to generate electricity. Two electrodes were submerged in a bacterial culture and a voltage between them could be generated, the first demonstration of the MFC concept (He and Angenent 2006, Potter 1910, Potter 1911).

One of the first designs of bioelectrochemical cells was based on a potensiostat-poised half-cell with artificial electron mediators (i.e. ferrycianide, neutral red, thionin, methyl viologen or benziquinone) in the anode to improve the current production (Cohen 1931). Although the concept of recycling organic waste into electric energy using microorganisms became popular during the 60s, it was not until the late 15 years when the demand of renewable energy sources focuse the interest of scientists into MFC research (Esteve-Nunez 2008, Logan 2004, Lovley 2006).

MFC designs were significantly improved and higher efficiencies, greater reaction rates, better electron transfer processes, and more stable bacterial communities were achieved (Allen and Bennetto 1993, Bennetto 1990). However, the MFC performance in its early stages of the new era was highly related to the presence of electron mediators which seemed to be responsible for the electron transfer mechanisms (Du *et al.,* 2007, Logan *et al.,* 2006, Rabaey *et al.,* 2005). The discovery of exoelectrogenic bacteria, able to transfer electrons directly to the anode via electrochemical active redox proteins, changed the MFCs design (Chaudhuri and Lovley 2003). MFCs became self-sustained, more efficient in electron-transfer and avoided the costs of catalysts, and prevented pollution risks of using artificial electron mediators (He *et al.,* 2009, Logan *et al.,* 2006, Lovley 2006, Rabaey *et al.,* 2004).

Initial MFCs designs were half biological, because the microorganisms were only present in the anode. However, abiotic cathodes were shown to be unsustainable on the long

term. Additionally, it was observed that the presence of bacteria in the cathode improved reductive reactions without the need of external mediators (Freguia *et al.,* 2007). The reductive capacity in the cathode is rather high, and microbe mediated reductive reactions may include a variety of compounds, such as fumarate, nitrate, uranium (VI), carbon dioxide or chloride compounds (Clauwaert *et al.,* 2007, He and Angenent 2006). The presence of bacteria in both chambers resulted in completely biological MFCs, in which the whole chamber volume could be replenished with a conductive material, increasing the electrode surface (Rosenbaum *et al.,* 2011).

Microbial metabolism is the limiting factor in current production, with the electric power generated from wastewater as yet insufficient to be exploited commercially as renewable energy sources (Logan 2009). However, it was reported that production of valuable chemicals (electrosynthesis), or the ellimination of contaminants (bioelectroremediation), could give additional environmental benefits of MFC (Foley *et al.,* 2010). Chemical products can also generate a larger monetary pay back if they are useful in wastewater treatment plants (Rosenbaum *et al.,* 2011).

The production of certain compounds, such as hydrogen, is thermodinamically challenging since increases of anode and cathode potentials are required (Rozendal *et al.,* 2006). In this sense, Microbial Electrolysis Cells (MEC) were developed with the purpose of stimulate the microbial metabolism applying a controlled current potential (Thrash and Coates 2008). MECs are based on the use of a potentiostat or a power supply, which overcomes cathodic reaction overpotentials by increasing the difference between the two electrodes (Cheng *et al.,* 2009, Rozendal *et al.,* 2006). These systems can be operated with two electrodes (Bioelectrochemical Systems, BES), or in combination with a reference electrode (microbial 3-electrode cell, M3C) (Aelterman *et al.,* 2008, Torres *et al.,* 2009).

Microbial electrosynthesis has allowed the production of organic compounds from carbon dioxide (CO_2), methane (CH_4), or hydrogen (H_2) production (He and Angenent 2006, Liu *et al.,* 2005b). BES have also been used for metal recovery from sludge or soils (He and Angenent 2006, Lovley 1991), and for the reduction of uranium, chlorinated compounds or nitrate from wastewater (Aulenta *et al.,* 2010, Clauwaert *et al.,* 2007, Gregory and Lovley 2005). The above examples reveal that BES could be an efficient and suitable technology for nitrogen removal from waters with a low organic matter content.

1.2.3 Denitrifying biocathodes

The nitrate reduction in biocathodes is a chemolithoautotrophic denitrification process in which nitrate is reduced to nitrogen gas without the presence of organic matter. This reaction is analogous to the chemolithoautotrophic processes found in the environment, however, instead of using inorganic electron donors, typically hydrogen or reduced sulphur compounds, the electrons are provided by an electrode (Clauwaert *et al.,* 2007, Virdis *et al.,* 2008, Wrighton *et al.,* 2010) (Figure 5).

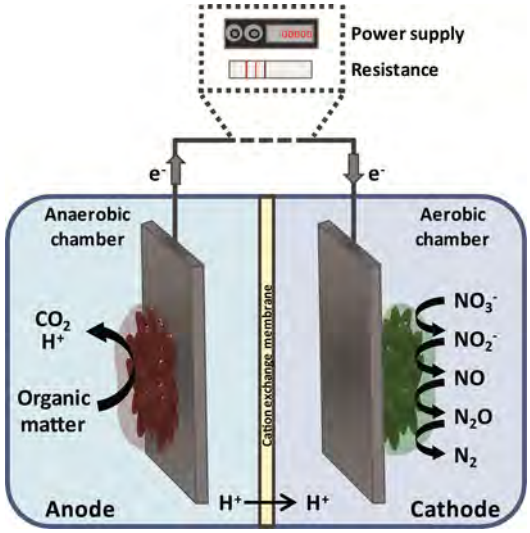


Figure 5. Denitrifying microbial fuel cell (dMFC). The anode compartment is filled with oxidizing bacteria which produce electrons from organic matter oxydation. Denitrifiers reduce nitrate to nitrogen gas in the cathode.

Biocathodes made use of biocathodic nitrate reduction, a reaction proposed about 50 years ago (Lewis 1966). However, it was not until 2004 when Holmes and co-workers found that microorganisms on the cathode of a sediment of a MFC participated in biological reactions coupled to the nitrogen cycle (Holmes *et al.,* 2004). Gregory *et al.* (Gregory *et al.,* 2004) demonstrated for the first time that electrodes could serve as the sole electron donor for nitrate reduction in a potensiostat-poised half-cell in the presence of *Geobacter metallireducens*. The complete nitrate reduction to nitrogen gas via electrode donor was demonstrated in another experiment performed in the absence of any organic substrate acting as an external electron donor (Park *et al.,* 2006). These studies and others, demonstrated that the use of denitrifying biocathodes raises the oportunity to combine biological wastewater treatments in which the energy generation is accomplished by the removal of complex organic matter (Clauwaert 2009, Liu *et al.,* 2004, Liu *et al.,* 2005a).

Although the denitrifying biocathodes are widely studied to optimize the operational conditions, limited information exists about the bacterial community structure and the electron-transfer mechanisms used by bacteria.

1.2.4 Electrochemical process driving BES

The performance of bioelectrochemical systems (BES) relies on the ability of bacteria to either transfer electrons to an electrode or accept them from the electrode surface (He and Angenent 2006). Exocellular transference of electrons in a cell-to-cell basis or from cells to an external acceptor is a common process (Lovley 2006). Cell respiration using solid metal oxides is one such a process catalysed by dissimilatory metal-reducing bacteria (DMRB). Several bacteria, such as *Clostridium, Geobacter, Aeromonas, Rhodoferax, Desulfobulbus* and *Shewanella*, have been classified as DMRB. As an example, *Shewanella oneidensis* MR-1 can reduce Mn(IV) and Fe(III) oxides, and produce electric current (Bretschger *et al.,* 2007). Additionally, the demonstration that the electron transfer mechanism (>90% efficiency) could be a respiratory process was performed using a non-fermentable substrate, such as acetate (Bond *et al.,* 2002, Bond and Lovley 2003).

Recent studies have suggested that the cell-to-cell exchange of energy may be much more sophisticated and effective than previously thought, this concept has been referred to as direct interspecies electron transfer (DIET). Recently, it has been found that two *Geobacter* species can form a conductive aggregate that exhibits faster ethanol utilization, demonstrating that microorganisms may exchange electrons through direct electrical connections rather than using electron carriers. This bacterial relationship has also been named syntrophy, referred to as critical interdependency or obligate mutualistic metabolism between two microorganisms (Dolfing 2014), leading to the development of the concept of DIET-based syntrophyc growth (Shrestha *et al.*, 2013, Summers *et al.*, 2010). An example of DIET-based syntrophyc growth occurs in electrically conductive methanogenic aggregates (Morita *et al.*, 2011). In the anodes, some examples of DIET processes have been described. Many exoelectrogenic bacteria have limited metabolic versatility and rely on other fermentative partners to produce usable molecules to be oxydized. Syntrophic interactions that exist in exoelectrogenic biofilms allow for the successful conversion of virtually any substrate into electrical current (Kiely *et al.*, 2011). Although the exoelectrogenic behaviour of *Geobacter* species is widely studied, electron exchange is a naturally occurring phenomenon and many other microorganisms may be able to make electrical contacts in anaerobic environments (Gorby *et al.*, 2006, Summers *et al.*, 2010). Because little is known about the kinetics behind syntrophic interactions

and the interplay between kinetics and thermodynamics in MFCs, the analysis of bacterial communities is a promising tools to tackle those issues (Dolfing 2014).

1.2.5 Bacteria feed the electric circuit

Models to explain electron transfer between bacteria vary according to the position of cells either in the biofilm or the bulk liquid. Direct contact of bacterial cells to the anode involve different mechanisms, such as bacterial pili, bacterial networks, and nanowires (Gorby *et al.,* 2006, Reguera *et al.,* 2005), multihaem outer cytochrome *c*-proteins (Shi *et al.,* 2007, Weber *et al.,* 2006), or extracellular polymeric substances (Torres *et al.,* 2007). Alternatively, contact-free mechanisms involving soluble redox-active mediators, that diffusively shuttle electrons (Gralnick and Newman 2007, Oh *et al.,* 2010, von Canstein *et al.,* 2008), are found in bulk or biofilm attached bacteria (Figure 6).

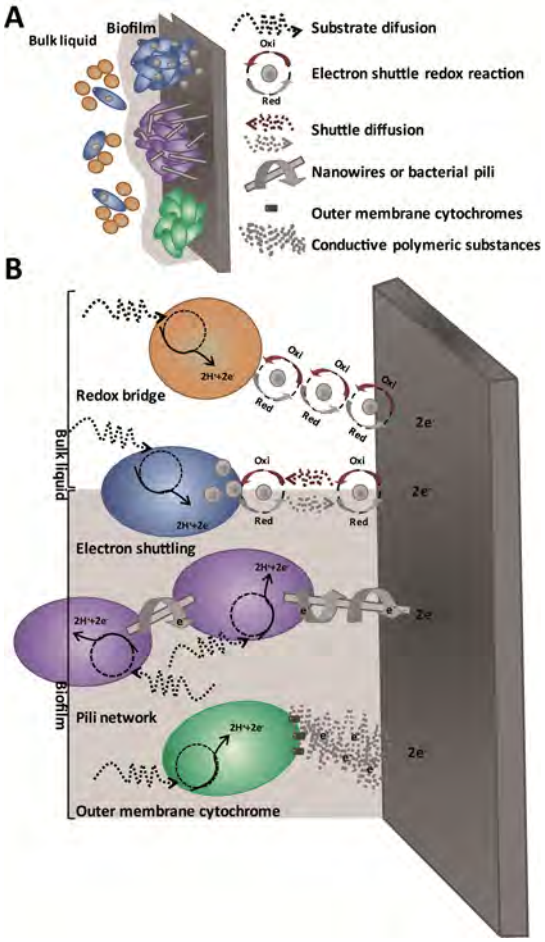


Figure 6. Proposed exoelectrotrophic mechanisms in anodic electron transfer. A) Schematic diagram of bulk and biofilm bacterial cells. **B)** Details of proposed mechanisms in electron transfer. Biochemical redox bridge occurring in bulk liquid, electron shuttling and diffusion across biofilm in bulk and biofilm cells, bacterial pili network and outer cytochrome or extracellular polymeric substances. There may be also other non-exoelectrogenic bacteria in the anode community which are not showed in the diagram. Modified from (Oh *et al.,* 2010).

Direct contact *c*-type cytochromes as an EET mechanism was described on the basis of the observation that cell growth was covering a significant part of the anode surface, suggesting that direct contact between bacteria and the electrode was required (Shi *et al.,* 2007, Weber *et al.,* 2006). Contact dependent electron transfer mechanisms occur in the absence of other terminal electron acceptor (Shi *et al.,* 2009). *c*-type cytochromes are located in the cell-envelope to interact with external substrates (Heidelberg *et al.,* 2004, Shi *et al.,* 2007). In the absence of strong complexity ligands, some DMRB have developed the ability to transfer electrons across the bacterial cell envelope to the surface of Fe(III)/Mn(IV) oxides external to the cell (Gralnick and Newman 2007, Lovley 2006, Shi *et al.,* 2009, Weber *et al.,* 2006). *Shewanella*, *Rhodoferax*, *Desulfuromonas acetoxidans* and some fermentative bacteria, such as *Clostridium butyricum* and *Aeromonas hydrophila* transfer electrons via a *c*-type cytochrome dependent process (Bond and Lovley 2003, Logan *et al.,* 2006, Logan 2009, Lovley 2006, Myers and Myers 1992, Oh and Logan 2006).

Nanowires are considered as electrically conductive pili. Recently, in *Shewanella oneidensis* outer membrane extrussions more than pili structures have been shown to be responsible for transferring electrical current between cells (Pirbadian *et al.,* 2014). Membrane extrusions also contribute to enhance cell cohesion within biofilms and thus allowing the development of thicker biofilms. In thicker biofilms, the proportion of cells in direct contact with the electrode is less, and for thus additional systems to favour electric conductance are needed to generate higher current levels. This strategy is used by many organisms but only have been studied in detail in *Geobacter sulfurreducens* and *Shewanella oneidensis* (Gorby *et al.,* 2006, Holmes *et al.,* 2006, Logan *et al.,* 2006, Logan 2009, Lovley 2006, Reguera *et al.,* 2005).

Studies have revealed the differences in electron transport mechanisms for both species (Malvankar and Lovley 2014, Shi *et al.,* 2009). In *Shewanella oneidensis* electron transport is based on a multistep hopping mechanism in which multihaeme cytochrome proteins are associated to outer membrane vesicles and act as electron carriers (Pirbadian *et al.,* 2014). In *Geobacter sulfurreducens*, distances between cytochromes are higher (100-200 nm) and electron hoping is no longer possible. In these conditions, the molecular structure of the specialyzed pili is likely to favour free electron displacement in a metal-like conductive manner (Giltner *et al.,* 2012, Vargas *et al.,* 2013).

Contact-independent mechanisms are based on the synthesis of specific mediators, named electron shuttles, these molecules transport electrons back and forth between cells and mineral surfaces and diffuse freely in the bulk liquid (Gralnick and Newman 2007). This mechanism has been described in *Shewanella oneidensis*, *Geothrix fermentas*,

or *Pseudomonas* spp.. Contact-independent mechanisms may also be relevant in biofilms, where most of the cells are not in direct contact with the mineral surface (Hernandez and Newman 2001). However, this electron transfer method is not likely to occur in open environments since electron shuttles are rapidly lost, representing a competitive disadvantage for producers (Logan *et al.,* 2006, Logan 2009, Lovley 2006, Rabaey *et al.,* 2004, Rabaey *et al.,* 2005).

1.2.6 Electron fed bacteria

The exoelectrogenic electron transfer mechanisms have been largely studied, but, the electron flow in the opposite direction, from electrodes to microorganisms, has not been studied in detail. In recent years, the number of studies focusing on biocathodes have increased, nevertheless, not much is known about the biochemical mechanisms underlying the electron uptake (Rosenbaum *et al.*, 2011).

In cathodes, the bacteria able to use electrons directly from the electrode are named electrotrophs (Lovley 2011). Growth with an electrode serving as an electron donor is theoretically possible when common electron acceptors are reduced on the inner side of the inner membrane or in the cytoplasm, this reaction must be specifically linked to a mechanism for generating a proton-motive force and energy conservation (Strycharz *et al.*, 2011). To date, all known electrotrophic microorganisms, except *Methanobacterium palustre*, are also electrogenic, suggesting that electron transfer mechanisms can be reversed (Finneran *et al.*, 2002, Lovley *et al.*, 1999). However, studies performed with *Geobacter sulfurreducens* demonstrated that the mechanisms used in current-consuming and current-producing biofilms are not equivalent (Strycharz *et al.,* 2010). Additionally, differences on the potential in anodes and cathodes suggest the involvement of distinct redox active components or substantial quasi-reversible catalytic behaviour of a single redox system (e.g. the same outer membrane cytochrome) (Rosenbaum *et al.*, 2011). Although this may not be the case for all microbial species and specifically be incorrect in complex biofilm structures in which various electron-transfer mechanisms coexist (Pous *et al.,* 2014)

Differences in electrotrophic and electrogenic mechanism in the same bacterial species were investigated at a molecular level using *Geobacter*. Interestingly, deletion of essential genes for current production (Cytochromes, OmcS and OmcZ, and other nanowire proteins) did not impact current-consumption biofilms and vice versa (Strycharz *et al.*, 2010). Electron consuming cells overexpressed the GSU3274 gene coding for a putative redox-active protein with homologies with cytochrome *c* family proteins. These results

indicated that different electron transfer mechanisms may exist in either direction. The current production is merely a pathway for electrons to flow down a potential gradient, in which once electrons are transferred across the inner membrane, the remaining steps do not require mechanisms for energy conservation. On the contrary, current consumption requires the generation of specifically linked mechanisms of proton driving force to maintain the reduced electron acceptors into the cell, being metabolic demands of the cell significantly different from those required in electrogenesis (Strycharz *et al.*, 2011).

According to these observations, Rosenbaum and co-workers (Rosenbaum *et al.*, 2011) summarized the different mechanisms proposed as posible bioelectrochemical electron-accepting reactions (Figure 7), in this sense different extracellular electron transfer mechanisms (EET) have been proposed. These mechanisms include direct electron transfer involving *c* type cytochromes, similar mechanism used by iron (II) and sulphur oxidation bacteria to uptake electrons from soil (Rosenbaum and Angenent 2010, Weber *et al.,* 2006), or during photosynthetic reaction to obtain ATP (Kappler *et al.,* 2005, Madigan *et al.,* 2004). EET mechanisms can also be mediated by perisplasmatic hydrogenases, because electrocatalytic activity of purified enzymes has been observed, and several studies have been reported in mediator-less hydrogen producing biocathodes (Batlle-Vilanova *et al.,* 2014, Rozendal *et al.,* 2008, Villano *et al.,* 2011). Another proposed mechanism is the direct electron transfer involving cytochrome-hydrogenase partnerships. Metal biocorrosion, associated to sulfate-reducing bacteria (SRB), use the outer membrane cytochromes (e.g. Hmc) as an "entrance point" and transfer electrons to hydrogenases, the redox partners (Van Ommen Kloeke *et al.*, 1995). Additionally, *Desulfovibrio vulgaris* genome revealed the presence of a pool of *c*-type cytochromes which interconnect multiple periplasmic enzymes, including hydrogenases, serving as a temporary capacitor for storage of low-potential electrons (Heidelberg *et al.*, 2004).

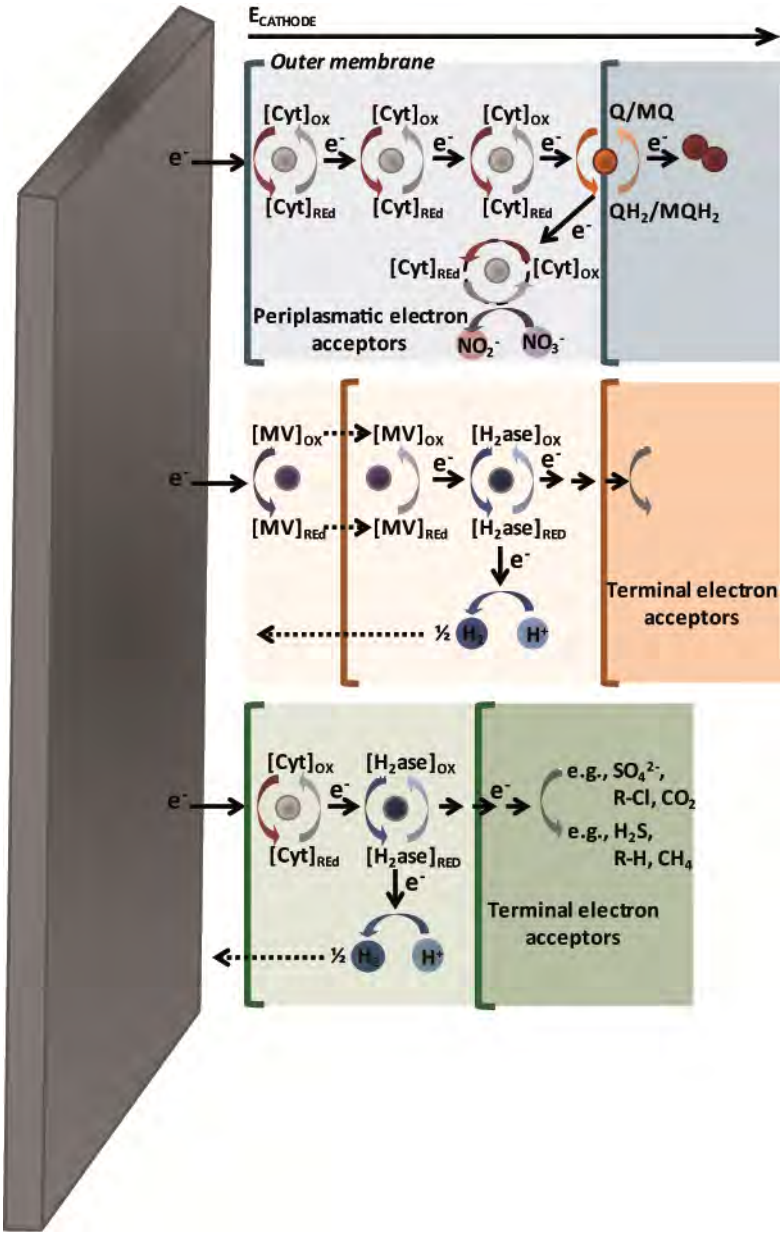


Figure 7. Proposed electrotrophic mechanisms in cathodic electron transfer. A) Direct electron transfer involving *c*-type cytochromes electron transfer chains. **B)** Mediated electron transfer to periplasmatic hidrogenase, different terminal electron acceptors can be used. **C)** Direct electron transfer involving cytochrome-hydrogenase partnerships.

In summary, the studies cited above show that the microbial energy gain of biocathodic reactions is strongly affected by the type and efficiency of the used external electron transfer (EET) mechanism (Rosenbaum *et al.*, 2011).

1.2.7 Electrochemical characterization of cells and biofilms

Cyclic voltammetry is an electrochemical method used to characterize electroactive microbial biofilms, such as those found in anodes and cathodes of MFCs (Harnisch and Freguia 2012). In addition to the application of these electrochemical methods, studies of the bacterial community composition are also essential to understand the interactions that exist between cells and can be helpful to elucidate mechanisms for electron uptake (Rosenbaum *et al.,* 2011).

Cyclic voltammetry has been used extensively to study charge transfer in electrochemical systems (Richter *et al.,* 2009). The principle of the technique is that the voltage is swept between a value range (V_1 and V_2) and the current produced is recorded and plotted as a function of voltage. The scan begins from the left hand site of a current/voltage plot, the forward sweep produces a response, because the reactant is oxidized, and can be visualized as a peak. When the scan reaches V_2, it is reversed and the scan voltage is swept back to V_1, which occurs in the opposite sense to the forward sweep. The voltage applied moves back gradually to the equilibrium position, where current flow is moving from the solution species to the electrode again, producing the electrolysis products back to a single electrochemical reactant (Andrienko 2008, Harnisch and Freguia 2012). At the first applied voltages almost no current flows, but at certain potential the current begins to increase up to a maximum value owing to an electrochemical oxidation reaction on the electrode surface, and afterwards it decreases because all the electrons are transferred to oxidase the protein, and the change of state produces a suddenly decreased current. This maximum current is called the peak current (i_P^a), where the maximum current occurs the respective peak potential appears (E_P^a), and could be observed on a voltammogram or a CV curve. An asociated reduction reaction can be identified (Harnisch and Freguia 2012). A typical CV recorded for a reversible single electrode transfer reaction is shown below (Figure 8).

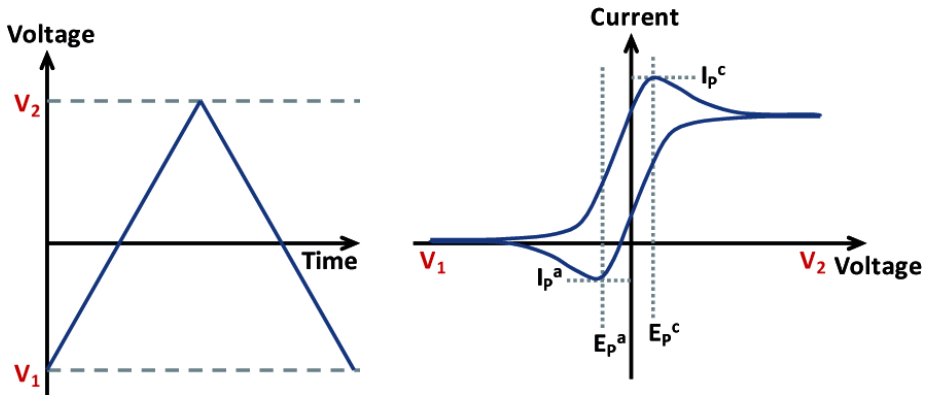


Figure 8. Cyclic voltammogram. Voltage variation as a function of time (left), and current as a function of voltage (right) in theoretical cyclic voltammetry experiments. Adapted from (Andrienko 2008).

Most common systems used to characterize electroactive microbial biofilms by CV are the three-electrode set-up. This set-up is composed of a working electrode (WE), a reference electrode (RE) and counter electrode (CE) (Figure 9). The electrochemical reaction of interest occurs in the WE, whereas a fixed potential is applied to the reference electrode, which is non-polarizable, and the potential of WE is measured. This measurement can occur because CE is accompanying the WE reaction with the respective reverse reaction. The current-potential polarization curve can be recorded using a potentiostat that controls the voltage application. The uncompensated resistance causes a drop in potential, in the solution and in the biofilm, during the current flow owing to Ohm's law. To avoid this problem, it is advisable to place the RE outside the path of migration of electrons between WE and CE, or if it is not possible, is better to place RE as close as possible to the WE. This avoids large potential drops, which cause incorrect potential measurements.

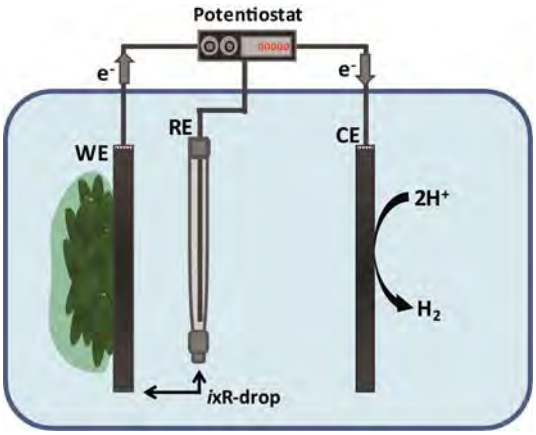


Figure 9. A three-electrode experiment housed in a single-chamber electrochemical cell. Working electrode (WE), reference electrode (RE) and counter electrode (CE) are connected to a potentiostat. Adapted from (Harnisch and Freguia 2012).

Either turnover or non-turnover conditions can be applied experimentally when running a CV. Turnover conditions, also referred as bioelectrocatalytic conditions, are used to identify the active sites of bacterial biofilm. The reaction occurs in the presence of microbes. The biological reduction depends on the number of redox centres in microbial cells, on their activity, and also, on the concentration of the oxydized substrate. Microbial cells can continuously re-reduce the electron-transfer sites and thus can achieve an s-shaped oxidative CV. The analysis of inflection points of the obtained bioelectrocatalytic curve allows the identification of the formal potential of the catalytitic moiety responsible of the reaction performed. In contrast, the absence of substrates or electron acceptors avoids the mass transfer at the biofilm and non-turnover conditions apply. Under this conditions, no redox process is available to regenerate the reduced form of the compound (Fricke *et al.,* 2008, Harnisch and Freguia 2012).

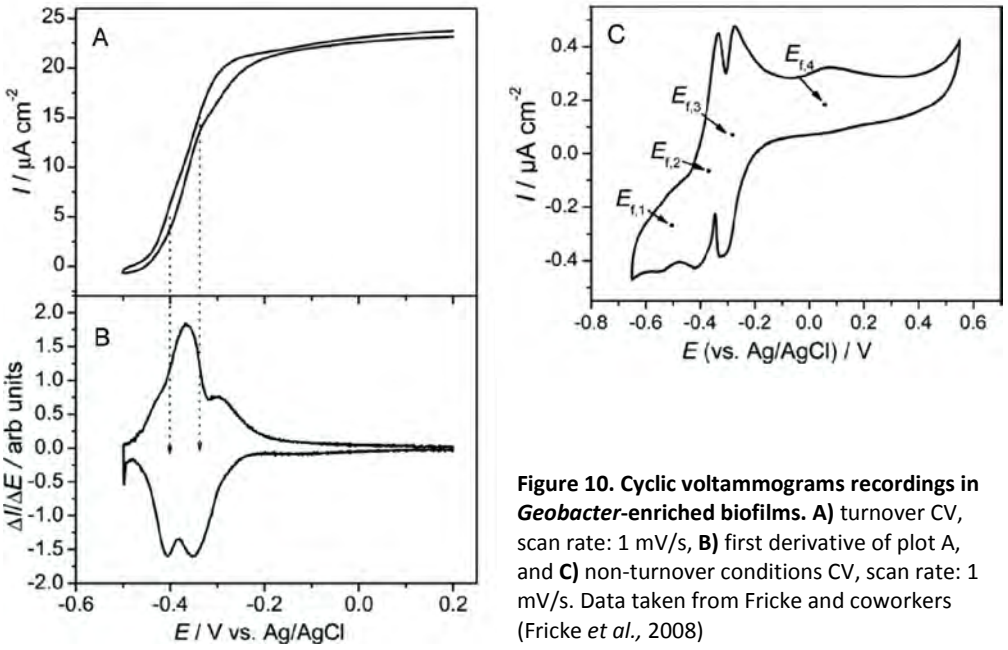


Figure 10. Cyclic voltammograms recordings in *Geobacter*-enriched biofilms. A) turnover CV, scan rate: 1 mV/s, **B)** first derivative of plot A, and **C)** non-turnover conditions CV, scan rate: 1 mV/s. Data taken from Fricke and coworkers (Fricke *et al.,* 2008)

1.2.8 Electrochemical characterization of biocathodes

Electrochemical characterization of denitrifying biofilms, could be an useful tool in order to elucidate the implied electron transfer mechanisms. According to the biological tower of electron donors an acceptors at pH 7, denitrification occurs at 0.323 V *vs* SHE (He *et al.,* 2009). However, the midpoint potential of the isolated denitrifying enzymes differs significantly from this value. Midpoint potentials for Nitrate reductase GHI, Cu-containing nitritrite reductase, and Cu nitrous oxide reductase, are estimated to be 0.007 V, 0.127 V and 0.183 V (*vs* SHE), respectively (Anderson *et al.,* 2001, Dell'Acqua *et al.,* 2010, Wijma *et al.,* 2006).

Extracellular electron transfer on biocathodes have been characterized in some studies, and the values indicate discrepancies in the working potentials of biofilms from those found for bacterial isolated enzymes. The potential for nitrate reduction (E_1= -0.103 V *vs* SHE) and nitrite reduction (E_2=-0.503 V *vs* SHE) found in actual biofilm samples differ significantly from the potentials observed for isolated enzymes (Pous *et al.,* 2014). Similar differences were observed in biocathodes operating at lower electrode potentials (E=–0.303 V *vs* SHE) (Gregory *et al.,* 2004, Virdis *et al.,* 2011, Virdis *et al.,* 2012).

1.2.9 Characterization of denitrifying bacterial communities

Denitrifying biocathodes have not been studied in detail and only some studies have characterized these communities using molecular techniques targeting the 16S rRNA genes. The microbial communities on denitrifying biocathodes are complex and may contain both denitrifiers and other species not involved in nitrogen transformations (Kelly and He 2014).

Nevertheless, in all performed studies on denitrifying biocathodes, *Proteobacteria* appear to be dominant despite changes in the composition of the community. Different techniques have been used to characterize bacterial biocathodes, including PCR-DGGE and clonning approaches (Chen *et al.,* 2010). In all of them, the inoculum community changed to a more specialized community dominated by denitrifying bacteria, mainly composed of *Proteobacteria* (He *et al*., 2009). *Betaproteobacteria* have been found as the most abundant group with abundances between 50 and 78% of the sequences identified in biocathodes (Chen *et al.,* 2008, Chen *et al.,* 2010, Gregoire *et al.,* 2014, He *et al.,* 2009). Other phylotypes occur at lower relative densities, *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Chlorobi*, indicating that cathode communities are complex (Chen *et al.,* 2010). Nevertheless, it is known that members of the phylum *Proteobacteria* are

associated with nitrogen cycling and denitrification, for thus it is assumed that they are the primer drivers of denitrification in the biocathode (Ginige *et al.,* 2005). Denitrifying ability has been defined in some Gram positive bacteria, indicating that the denitrification knowledge is still incomplete (Verbaendert *et al*., 2011). Unfortunately, primers used in this study are biased towards detection of *Proteobacteria* and Gram positive denitrifiers are not covered completely."

A more detailed analysis of active bacterial community of the denitrifying biocathodes was conducted through comparing the communities between two different enrichment approaches, an MFC with a loop connection (in which the anode effluent flowed into the cathode) and an MFC with separated anode and cathode streams (Wrighton *et al.,* 2010). Completely different communities were found to be associated to operational conditions. Greater bacterial richness and evenness were associated to higher performances in current generation in the loop reactor, which was dominated by *Firmicutes*. On the contrary, the non-loop reactor was dominated by *Proteobacteria*, with similar abundances between *Gamma*-, *Alpha*- and *Betaproteobacteria* (33%, 22% and 18%, respectively). Another study, in which microbial diversity was analysed by massive sequencing techniques, active bacterial communities were shown to be composed of *Alicycliphilus*, *Acidovorax*, *Simplicispira*, *Thermomonas* and *Aeromonas*, among the most abundant genera, all within the *Proteobacteria* (Van Doan *et al*., 2013).

All of these studies revealed that the denitrifying cathodes are mainly dominated by members of the *Proteobacteria* (most commonly *Beta*- class), although members of other phyla (*Firmicutes*) can also be found at reasonable densities. As shown in a previous section of this thesis the classical approach, targeting the 16S rRNA gene, is not suitable for studying denitrifiers due to the lack of clear monophyletic branches of denitrifying bacteria (Philippot 2002). A most valuable approach available to data is the use of functional genes although this is not exempt of criticism due to the lack of coverage of available PCR primers, and to multiple incongruences in phylogenies inferred with both methods (16S rRNA and functional genes) (Jones *et al.,* 2008). Additionally, the available sequences of denitrifiers mainly belong to *Proteobacteria*, producing a bias on available primers which do not allow the *in situ* monitoring of other phyla of potential denitrifiers, such as *Actinobacteria* or *Firmicutes* (Verbaendert *et al*., 2011). However, despite these pitfalls, the use of functional markers may be advisable to a deeply understanding of biocathodic denitrification process and to identify dominant members of the bacterial community in each denitrification step.

The use of bioelectrochemical systems and their application in a variety of processes, including production of chemicals (electrosynthesis) and removal of contaminants (bioelectroremediation), is gaining scientific interest. Nitrogen removal is a key example of such a methodology. Our main goals for this thesis was to study denitrifying bacterial communities in the cathode of a Microbial Fuel Cell (MFC) and unravel, using both culture dependent and independent methods, key players and processes in the nitrate reduction to nitrogen gas. The following specific objectives were defined:

1) To identify relevant players in cathodic denitrification testing the effect of different operational conditions and the electrochemical performance of a denitrifying MFC.

2) To isolate denitrifying bacteria belonging to representative populations found in biocathodes using strict autotrophic conditions.

3) To decipher the putative role of prevailing denitrifiers in biocathodes and to establish cooperative behaviours using the electric and physiological characterization of isolates.

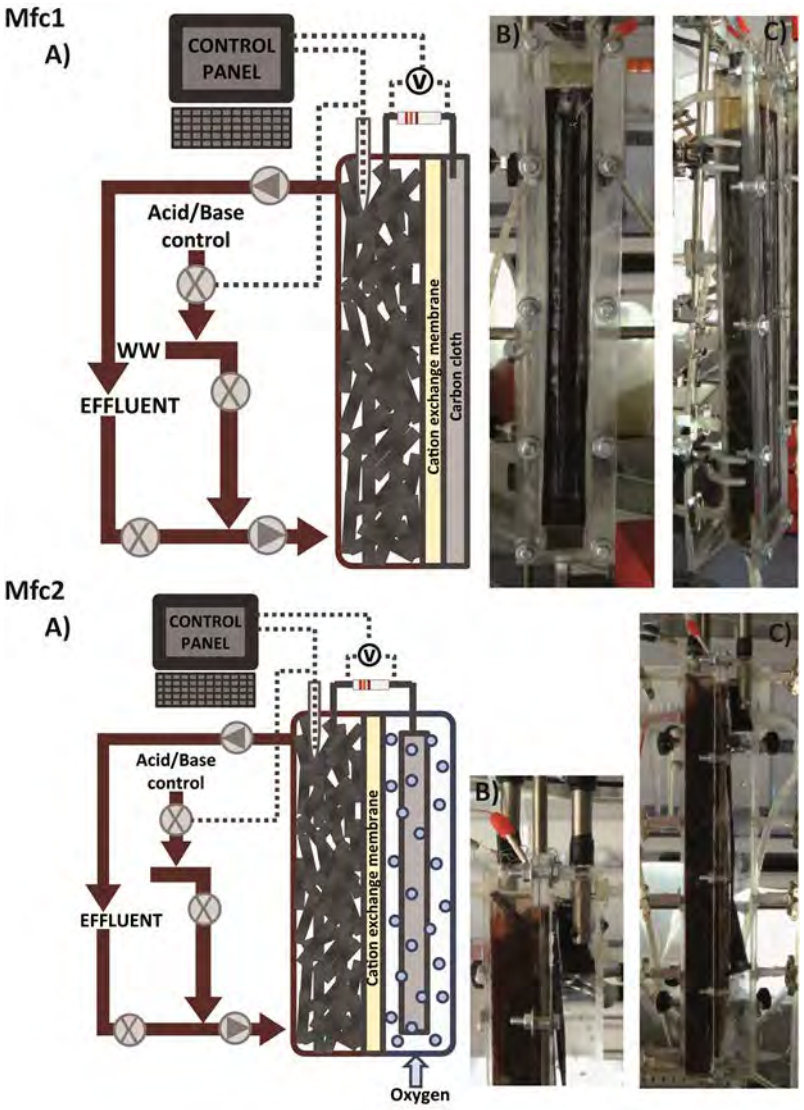
3.1 Microbial Fuel Cells set-up design and operational conditions

Four different Microbial Fuel Cells (Mfc1, Mfc2, Mfc3 and Mfc4), all operated at the Laboratory of Chemical and Environmental Engineering (LEQUIA) of the University of Girona, were studied in the present work. Different configurations were used for all of them. Mfc1 was an air-cathode MFC, and Mfc2 was mounted with an oxygen bubbled in cathode. Additionally, two denitrifying MFCs (dMFCs) were also studied: Mfc3 and Mfc4 which differed on the size and the type of connectiveaterial used.

3.1.1 MFC set-up configuration

All MFCs studied (except Mfc1) consisted of an anode and a cathode, placed on opposite sides of a single methacrylate rectangular chamber. In all cases, anodes and cathodes were separated by a cation exchange membrane (CEM, Nafion® 117, Dupont) treated according to Liu and Logan (Liu and Logan 2004). Membrane treatment has demonstrated to favour higher energy production. Anode and cathode, in all systems were connected to an external resistor (100 Ω) to close the electric circuit.

Two different types of MFCs were used according to the their chamber sizes. Mfc1, Mfc2 and Mfc4, consisted of rectangular chambers (32x26x400 mm) for anodes and cathodes, in case they were present (Figure 11). Anode chambers of Mfc1, Mfc2 and Mfc4 were filled with thin graphite rods (6 mm). Either carbon-cloth (0.35 $mg \cdot cm^{-2}$), or a Pt catalyst 30% wet-proofing (Clean Fuel Cell Energy LLC, USA), were used as cathode electrodes, respectively. Mfc4 cathode was filled with graphite rods. Mfc3 was a two-chambered denitrifying MFC (310 mL) Thin graphite rods were used as anode and cathode electrodes (28x35 mm, Sofacel, Spain) (Figure 12).



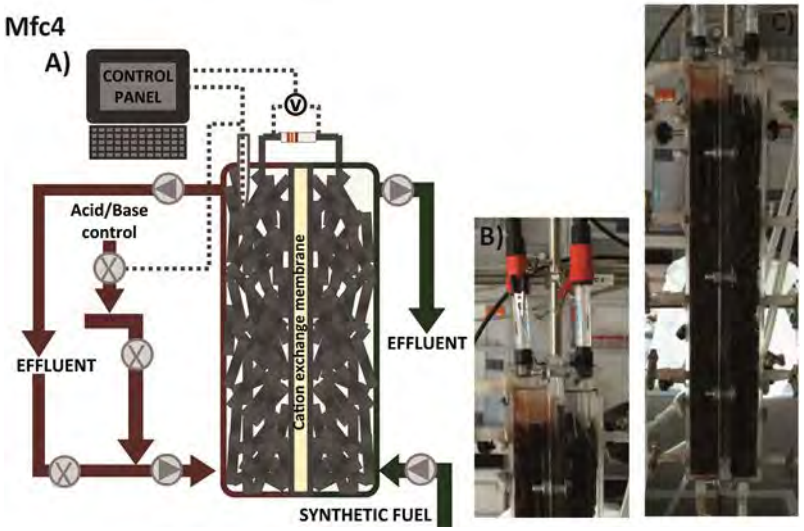


Figure 11. Mfc1, Mfc2 and Mfc4 chambers and connections. A) Schematic diagrams with influent and effluent flow directions indicated. **B)** and **C)** Pictures of MFCs. Mfc1 has an opened air-cathode, Mfc2 has a cathode chamber bubbled with oxygen and Mfc4 has a denitrifying cathode filled with graphite rods.

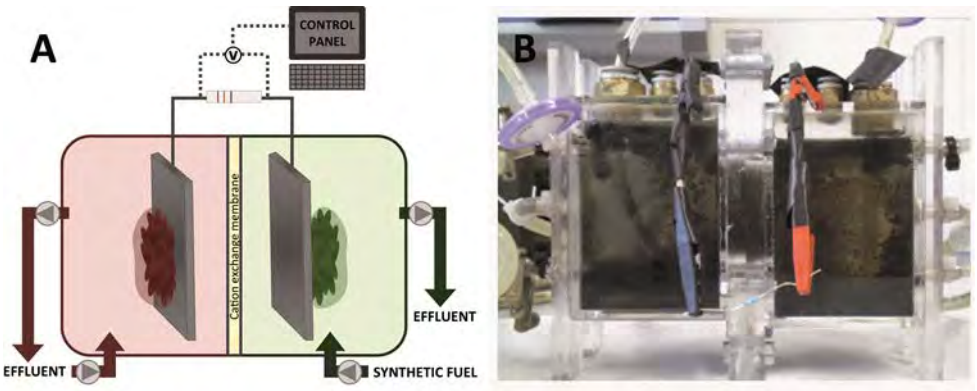


Figure 12. Denitrifying microbial fuel cell Mfc3 chambers and connections. A) Schematic diagram with influent and effluent flow directions indicated. **B)** Picture of Mfc3.

Medium was continuously fed into a recirculation loop to maintain well-mixed conditions and avoid concentration gradients within electrode chambers. All systems were thermostatically controlled at 23±2 °C. Prior to treatment, MFCs were inoculated with 50 mL of the effluent obtained from the anode of a parent MFC (Puig *et al.,* 2010). This parent MFC was previously used to treat synthetic wastewater primarily composed of sodium acetate and a buffer solution. The Mfc4 cathode was inoculated with 50 mL of Mfc3 cathode effluent.

In each chamber, graphite rods were used, with a subsequent net reduction of the working volume. Net anodic compartment (NAC) volumes were measured to be 242 mL for Mfc1, 395 mL for Mfc2, and 120 mL for Mfc4. Net cathodic compartment (NCC) volume were 145 mL for Mfc4.

3.1.2 Microbial Fuel Cell Operation

The operational conditions varied for each MFC according to their configuration and the objectives pursuit in the executed experiments. Different feeding regimes were used for the different MFCs configurations; Mfc1 anode was fed with waste water, whereas Mfc2, Mfc3 and Mfc4 anodes were fed with synthetic wastewater.

The Mfc1 anode was fed using wastewater, the amount of different compounds changed between the influent and effluent due to bacterial activity (Table 2). Mfc1 showed a significant organic matter removal capacity (COD decrease) while ammonium was partially oxydized (50%) to nitrate and nitrite.

Table 2. Characteristics of anode influent and effluent in Mfc1.

	Characteristics of wastewater	
Composition	**Influent** ($mg \cdot L^{-1}$)	**Effluent** ($mg \cdot L^{-1}$)
COD	556	123
TKN	104.73	93.3
NH_4^+	58.58	21.61
NO_2^-	0.4	2.3
NO_3^-	0.08	23.92

COD: chemical oxygen demand, **TKN**: total Kjehldahl nitrogen, **NH_4^+**: ammonium, **NO_2^-**: nitrite and **NO_3^-**: nitrate.

Anode compartments of Mfc2, Mfc3 and Mfc4, were fed with synthetic wastewater, which consisted of nitrogen-purged medium enriched with acetate (Table 3).

Table 3. Anode feed characteristics used as influent in Mfc2, Mfc3 and Mfc4. The composition of the microelements solution used is also indicated.

Medium composition		Microelements solution*	
Composition	($g \cdot L^{-1}$)	**Composition**	($mg \cdot L^{-1}$)
$NaCH_3COOH$	1.44	$FeSO_4 \cdot 7H_2O$	1000
$NaHCO_3$	0.488	$ZnCl_2$	70
NH_4Cl	0.03	$MnCl_2 \cdot 4H_2O$	100
$NaH_2PO_4 \cdot 2H_2O$	0.92	H_3BO_3	6
$CaCl_2 \cdot 2H_2O$	0.0056	$CoCl_2 \cdot 6H_2O$	190
$MgSO_4 \cdot 7H_2O$	0.035	$CuCl_2 \cdot 2H_2O$	2
KCl	0.0052	$NiCl_2 \cdot 6H_2O$	24
$NaNO_3$	0.044	$Na_2Mo_4 \cdot 2H_2O$	36
microelements solution*	$0.1\ mL \cdot L^{-1}$		

Neither nutrients nor electron acceptors were added in cathodes of Mfc1 (open air) or Mfc2. Only Mfc3 and Mfc4 cathodes were set as denitrifying cathodes and were fed with nitrate enriched synthetic wastewater (Table 4).

Table 4. Denitrifying cathodes feed characteristics. Nitrate amount was different in each dMFC and is indicated.

Medium composition		
Composition	**MFC type**	**($g \cdot L^{-1}$)**
$NaHCO_3$		0.488
$NaH_2PO_4 \cdot 2H_2O$		0.92
$CaCl_2 \cdot 2H_2O$		0.0056
$MgSO_4 \cdot 7H_2O$		0.036
KCl		0.0052
$NaNO_3$	Mfc3	0.148
$NaNO_3$	Mfc4	0.243
Microelements solution*		0.1 $mL \cdot L^{-1}$

*Composition of microelements solution is detailed in table 2.

MFCs were regularly monitored by experienced personnel at the LEQUIA group (Marc Serra and Dr. Sebastià Puig). Chemical and electrochemical performances were analysed regularly, and used to define steady-state conditions when no significant changes in the recorded variables were detected for a time longer than at least 3 times the HRT. Once steady-state was achieved functioning parameters were calculated (Table 5), and MFCs opened to take samples for microbial characterization.

Table 5. MFCs performance. Chemical and electrochemical parameters of the four types of microbial fuel cells at steady-state conditions.

	Anode			**Cathode**	
	Days of operation	**HRT** (h)	**COD removed** ($mg \cdot L^{-1}$)	**HRT** (h)	**Nitrate removed** ($mg\ NO_3^- \cdot L^{-1}$)
Mfc1	43	0.128	433	n.a.	n.a.
Mfc2	51	0.085	730	n.a.	n.a.
Mfc3	268	0.037	625	0.045	0.63
Mfc4	156	0.107	722	0.095	5.65

	Electrical parameters		
	Power density ($mW \cdot m^{-3}$)	**Current generation** (mA)	**Voltage** (mV)
Mfc1	7314	3.8	380
Mfc2	4357	3.43	343
Mfc3	106.5	2.25	225
Mfc4	2808	2.44	244

COD: chemical oxygen demand, **HRT**: hidraulic retention time, **n.a.** not applicable.

3.1.3 Shift experiments in denitrifying conditions

Mfc4 was operated for almost one year to treat acetate-enriched wastewater in the anode, and forced to eliminate nitrogen compounds in the cathode. During its life span two shift experiments were performed to determine changes in the abundance and composition of denitrifying bacteria. During the first period, the cathode was fed with nitrate as the electron acceptor (Table 4). At day 76 of operation (first shift experiment), nitrate was maintained in the feed composition but organic matter was added at the same time (64 ± 21 mg·L^{-1} COD). The increase on organic matter was caused because during this period the cathode was fed with the effluent of an air-cathode MFC used for treating urban wastewater. No analysis to determine the composition of organic compounds was done, but the measured COD concentration was similar to that of the non-biodegradable organic matter in urban wastewater, revealing its complex formulation (Puig *et al.,* 2010). At day 183 (second shift experiment), organic matter was removed and nitrate was replaced by nitrite as the electron acceptor in respiration. Table 6 shows the main influent characteristics of the anode and cathode during the three experimental periods.

Table 6. Influent characteristics of Mfc4. Chemical characteristics of the anode and cathode influents during the shift experiments in Mfc4. Values show means and SD (n=5) at steady-state conditions.

Period	Days of operation	Anode		Cathode			
		Flow ($L \cdot d^{-1}$)	COD (ppm)	Flow ($L \cdot d^{-1}$)	COD (ppm)	$N\text{-}NO_2^-$ (ppm)	$N\text{-}NO_3^-$ (ppm)
Period 1	0-76	2.4±0.3	745±231	2.0±0.3	n.d.	0.3±0.2	26.6±1.3
Period 2	77-182	1.5±0.6	895±175	1.4±0.5	64±21	1.0±0.4	29.6±5.3
Period 3	183-350	1.2±0.2	923±266	1.2±0.2	n.d.	20.3±1.7	2.9±4.7

COD: chemical oxygen demand; **n.d.**: not detected.

Period 1 will also be referred to as autotrophic conditions with nitrate, Period 2 as heterotrophic conditions, and Period 3 as autotrophic conditions with nitrite. During period 1, the cathode of the MFC was fed at 2.0±0.3 $L \cdot d^{-1}$, in period 2 at 1.4±0.5 $L \cdot d^{-1}$, and in period 3 at 1.2±0.2 $L \cdot d^{-1}$. The anodic feed consisted of an acetate enriched nitrogen-purged medium enriched with acetate (Table 3). The cathodic feed composition was detailed in table 4, with minor modifications on the electron acceptor. Schematic characteristics of each period, indicating the duration of the periods, the electron acceptor and the electron donor used for each period (Figure 13).

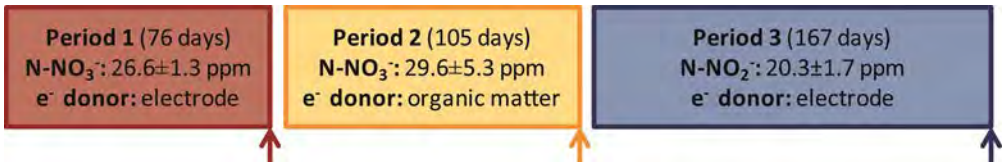


Figure 13. Shift experiments chronogram. Samples for microbiome characterization were taken at end of each period (arrows). Electron acceptors used are indicated as $N\text{-}NO_3^-$ (nitrate) and $N\text{-}NO_2^-$ (nitrite).

3.1.4 Analyses and calculations

Liquid-phase samples for organic matter (chemical oxygen demand, COD) and nitrogen (ammonium: $N\text{-}NH_4^+$; nitrite: $N\text{-}NO_2^-$ and nitrate: $N\text{-}NO_3^-$) were obtained regularly and analysed according to the Standard Methods for the Examination of Water and Wastewater (Eaton and Franson 2005). The levels of nitrous oxide ($N\text{-}N_2O$) production during the shift conditions experiments were estimated according to the electron balance at the cathode following the methodology of Virdis *et al.* (Virdis *et al.,* 2008). Experiments carried out using liquid- and gas-phase N_2O analysers demonstrated excellent fits between measured data and estimated data using the electron balance (Pous *et al.,* 2013, Virdis *et al.,* 2009). The nitric oxide (NO) production was considered to be negligible. To close the mass balance, the level of dinitrogen gas in the effluent was calculated from the current produced according to equation (1).

$$5 \cdot \Delta NO_3^- - 3 \cdot \Delta NO_2^- - 2 \cdot \Delta NO - 1 \cdot \Delta N_2O - \frac{3600 \cdot I}{F \cdot V} = 0 \qquad \text{Equation (1)}$$

Where ΔNO_3^- is the nitrate consumption rate, whereas ΔNO_2^-, ΔNO, and ΔN_2O are nitrite, nitric oxide, and nitrous oxide production rates, respectively. I indicates intensity, V voltage applied and F the Faraday's constant.

The cell potential (V) in the MFC circuit was monitored at one-minute intervals using an on-line multimetre (Alpha-P, Ditel) equipped with a data acquisition system (Memograph M RSG40, Endress + Hauser). The current (I) was calculated according to Ohm's law. The current density was calculated by dividing the current by the net cathodic volume ($A \cdot m^{-3}$ NCC). The Coulombic efficiencies for nitrate and nitrite reduction were calculated according to Virdis *et al.* (Virdis *et al.,* 2008).

3.1.5 Biofilm sampling strategy

Different types of samples for microbiological analyses were taken according to the MFC design. All samples were taken when the MFCs reached a steady state: Mfc1 at 43 days, Mfc2 at 51 days, and Mfc3 at 268 days of operation. Samples from Mfc4 were taken during different operational conditions at days 51 (autotrophic growth with nitrate), 106 (heterotrophic growth with nitrate) and 350 days (autotrophic growth with nitrite) of operation.

Samples for enrichment purposes (see chapter 4.2) were collected from Mfc1 anode and Mfc3 cathode, maintained at 4ºC, and processed within two hours after sampling to avoid cell decay as much as possible.

Biofilm samples from different positions were collected for molecular analysis. Samples from Mfc3 consisted of suspended cells and were collected directly from the electrode chamber. Volumes of 1 mL from detached bacterial biofilm present in the cathode were removed with a sterile syringe, distributed in microcentrifuge tubes and maintained at 4ºC, until processed (less than 24 hours from sampling).

Samples from Mfc1, Mfc2 and Mfc3, were obtained from the biofilms growing on the graphite rods either at the cathode or the anode, when it was applicable. In all cases, two graphite rods (6 × 38 mm) were collected aseptically from two positions of the electrode chamber. The two rods were replaced with two uncolonized rods to maintain the working volume of the cathode chamber. The graphite rods were immediately chilled on ice after collection and processed within less than 24 hours after sampling.

3.2 cultivation-dependent methods: enrichment and isolation procedures

Cultivation of microorganisms is fundamental to understand microbial physiology. It provides the opportunity to investigate the previously inaccessible resources that these microorganisms potentially harbor (Kaeberlein *et al.,* 2002). Additionally, pure cultures allow the examination of differences in cellular activity, establishing the relationship between function and structure, testing hypotheses formulated based on molecular analyses, and facilitating the interpretation of field data, objectives that tend to be rather unaccessible in natural populations of bacteria (Dahllof 2002, Ellis *et al.,* 2003, Nichols 2007).

Culture limitations have been described, because great biases between environmental diversity and the number of cultured species are found (Amann *et al.,* 1995, Staley and Konopka 1985). Conventional approaches result in the cultivation of a tiny subset of the wide diversity of microorganisms (Tyson and Banfield 2005).

Some variations in culturing techniques have been proposed to increase the number of bacteria isolated (Bruns *et al.,* 2002, Bruns *et al.,* 2003, Button *et al.,* 1993, Connon and Giovannoni 2002, Kaeberlein *et al.,* 2002). The selection of the appropriate media composition is essential because it have an inherent selective character for certain bacteria (Madigan *et al.,* 2004). The most used strategy is the use of media which emulate environmental conditions of sampling site (De Fede *et al.,* 2001, Kaeberlein *et al.,* 2002, Madigan *et al.,* 2004, Tyson and Banfield 2005). Also good results have been obtained using media with low nutrient concentration formulations (Button *et al.,* 1993, Connon and Giovannoni 2002, Zengler *et al.,* 2002), and incubations during long time periods (Kaeberlein *et al.,* 2002) are among the most popular approaches.

3.2.1 Enrichment of chemolithotrophic denitrifying bacteria

Samples from Mfc1 anode and Mfc3 cathode were used for the enrichment of chemolithoautotrophic denitrifying bacteria. The medium used for enrichment purposes was based on the composition of feeding solution of Mfc3 cathode (Table 4), and supplemented with a 1 $mL \cdot L^{-1}$ of vitamin solution (V7) (Pfennig 1992) and with 1 $mL \cdot L^{-1}$ of microelement solution (SL10) (Tschech and Pfennig 1984) (Table 7). This medium will be referred from here on as basal mineral medium (BMM). The same composition was used as solid media after the addition of 15 $g \cdot L^{-1}$ of rinsed high quality agar (Merck®,

Germany). The medium pH was adjusted to 6.8 ± 0.1 and sterilized by autoclaving prior to its distribution in 96-wells microplates (liquid medium) or Petri dishes (agar medium).

Table 7. Composition of vitamin and microelements solutions.

Vitamin solution (V7)		Microelement solution SL10	
Composition	($mg \cdot L^{-1}$)	**Composition**	($mg \cdot L^{-1}$)
Biotine	2	$FeSO_4 \cdot 7H_2O$	1000
p-aminobenzoate	10	$ZnCl_2$	70
Thiamine	10	$MnCl_2 \cdot 4H_2O$	100
Pantothenate	5	H_3BO_3	6
Pyridoxamine	50	$CoCl_2 \cdot 6H_2O$	190
Vitamine B12	20	$CuCl_2 \cdot 2H_2O$	2
Nicotinate	20	$NiCl_2 \cdot 6H_2O$	24
		$Na_2Mo_4 \cdot 2H_2O$	36
		Hcl (25%; 7.7M)	10 mL

Different electron donors were used to stimulate autotrophic denitrification. Thiosulphate ($Na_2S_2O_3$) was added to the medium formulation before autoclaving. The final concentration was 5 $g \cdot L^{-1}$. Hydrogen sulphur (H_2S) was continuously provided at approximately 100 µM from acidified (0.1M HCl) thioacetamide (C_2H_5NS) powder, following the procedure described by Butler *et al.* (Butler *et al.,* 1958). Finally, hydrogen (H_2) was provided by purging the jar atmosphere with a gas mixture composed by 5% of H_2, 5% of CO_2 and 90% of N_2, for 10 minutes. Media were named as BMM_H_2, BMM_H_2S and BMM_Na_2S2O_3, according to the electron donor used.

Cell and detached bacterial biofilm from Mfc1 anode and Mfc3 cathodes were serially diluted with an isotonic solution (Ringer solution for prokaryotes, Sharlau®, Barcelona, Spain) to 10^{-5} or 10^{-3}, respectively, and inoculated in triplicates on BMM_H_2, BMM_H_2S and BMM_$Na_2S_2O_3$ plates. Agar plates were incubated at 30ºC between 15 and 30 days depending on the sample origin. Incubations were done inside anaerobic jars (GENbox Har 7 liters, bioMérieux® S.A., France) previously purged with N_2 gas for 30 minutes. Anaerobic conditions were ensured using AnaeroGen bags (Oxoid, Hampshire, United Kingdom). Agar plates were visually inspected for growth of colonies in the surface.

Three to four representative colonies of different morphologies were selected from each agar plate and aseptically transferred to a 96 deep well microplate containing 1.2 mL of the corresponding liquid medium. Up to 991 different colonies were picked using pipette tips. The microplates were used in order to minimize the space needed for incubation. 96-wells microplates were incubated inside anaerobic jars using the same conditions and procedure above. Enrichment cultures were transferred to fresh media at the same incubation conditions (10% inoculum) every 30 to 36 days. All manipulations were

performed inside a CoyLab anaerobic chamber (Coy laboratory products, Inc., Grass Lake, Michigan, EUA).

Once selected, colonies were reinoculated individually in fresh media. The overall microbial diversity existing in agar plates was analyzed by plate wash-PCR (PW-PCR) (see below) (Ellis *et al.*, 2003, Stevenson *et al.*, 2004).

3.2.2 Isolation of chemolithoautotrophic denitrifying bacteria

Liquid enrichments in which a higher growth rate was detected (higher increase in optical density) were selected for isolation of autotrophic denitrifying bacteria. For every selected micro-well, 0.1 mL were collected and plated on agar plates. Composition of mineral media in agar plates was set according to the conditions used in the enrichment process, using nitrate as electron acceptor and different electron donors, i.e. H_2, H_2S and $Na_2S_2O_3$. After the incubation at 30ºC during 15 days, colonies with different morphologies were selected and re-inoculated on selective media. A single colony from every plate was selected each time to facilitate isolation. Once single colony morphology was observed on plates, single colonies were further re-inoculated for at least 4 times to ensure purity of isolated cultures. 16S rRNA gene sequences were obtained and used for identification of isolates (see below)."

Once isolates were obtained, BMM amended with organic matter (0.5 $g \cdot L^{-1}$ of ammonium acetate, 0.3 $g \cdot L^{-1}$ of propionic acid and 0.253 $mL \cdot L^{-1}$ of ethanol) was used to obtain reasonable culture densities in shorter times. The incubation time using heterotrophic conditions was reduced to 72 hours at 30ºC.

The isolates were maintained in an active state by re-inoculating them in agar plates every week. Additionally, deep-freezing of isolates in glycerol stocks was used for long term storage. Grown cells were suspended in two millilitres of tryptic soy broth (6 $g \cdot L^{-1}$), supplemented with nitrate 6 mM, in 21% of glycerol. Cell suspensions were incubated for two hours at 30ºC, and frozen at -80ºC. The culture viability after the freezing was assessed by plating on agar plates and incubating during 72 hours at 30ºC.

3.3 Molecular approach to bacterial diversity

Molecular methods are based on the analysis of the DNA extracted from environmental samples and provide information about the diversity and structure of microbial communities (Osborn and Smith 2005). Different molecular methods can be used to determine the presence or absence of functional genes in the environment (Osborn and Smith 2005). The successful application of molecular methods relies on the nucleic acid recovery efficiency from the environment as a sample that efficiently represents the microbial community (Hurt *et al.,* 2001, Osborn and Smith 2005).

The cell lysis can be achieved by different methods, enzymatic or chemical disintegration, and/or physical cell disruption (Johnson 1994, Roose-Amsaleg *et al.,* 2001, Sprott *et al.,* 1994). Chemical methods, such as a combination of detergents, which produce damage in gram negative bacteria, and lytic enzymes (i.e. lisozyme or proteinases), which digest gram positive bacteria, have been used as the most suitable option to ensure the effective disruption of most microbial cells (Johnson 1994, Roose-Amsaleg *et al.,* 2001, Sprott *et al.,* 1994). Currently, there are DNA purification kits that improve the efficiency, and reduce the time needed to obtain DNA extracts suitable for molecular analysis. However, a universal protocol for all sample types does no exist, and methods that are convenient for a sample type may be inconvenient for others (Chaudhuri *et al.,* 2006, Maciel *et al.,* 2009, Osborn and Smith 2005). In consequence, different extraction methods were used in this work.

3.3.1 DNA extraction from biofilm samples

Biofilms exist in a wide range of environments, in which microorganisms are associated in complex communities (Sutherland 2001). These communities excrete a sticky matrix, also called glycocalix, which protects them and allows the interaction of individuals with each other and with the surrounding environment (Davey and O'Toole G 2000, O'Toole *et al.*, 2000). The complexity of biofilm samples due to the great number of molecules present together with many recalcitrant substances that surround the cells may affect the DNA extraction process. The presence of enzyme inhibitors, such as humic substances, highly resistance cells to lysis, and acidic pH that may preclude the effectiveness of the DNA extraction, are conditions present in biofilm samples and have to be taken into consideration to choose for the appropriate protocol.

Graphite rods were washed three times in Ringer solution (Sharlau®, Barcelona, Spain) to eliminate loosely attached cells. Subsequently, rods were immersed in 4 mL of 0.1 M of

sodium pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$), and the biofilm dislodged using three consecutive sonication rounds for 20 seconds followed by 30 seconds on ice (Knief *et al.*, 2008). The suspended bacterial cells were pooled and centrifuged at 10,000 × g for 2 minutes.

Detached bacterial biofilms (anodes of Mfc1, Mfc2 and Mfc4 and cathodes of Mfc3 and Mfc4) were used for DNA extraction using the FastDNA® SPIN Kit for soil (MP, Biomedicals, Santa Ana, California, EUA) following the manufacturer's instructions. The obtained DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) and stored at -20ºC.

3.3.2 DNA extraction for Plate Wash PCR

A high-throughput screening method, Plate Wash PCR (PWPCR), was used to screen a large number of plates containing multiple colonies. PWPCR consists of washing the surface of a grown agar plate with a buffer in order to recover the maximum number of cells. The resulting cells suspension is used to extract DNA. PCR amplification is latter used to amplify the target microorganism (Ellis *et al.*, 2003, Stevenson *et al.*, 2004). Positive PCR results are used to select agar plates that may deserve more attention in order to try to isolate the desired microorganism.

In this work, PW-PCR was used as a fast screening method to detect plates harboring desired microorganisms for isolation. All biomass remaining in plates after transferring of selected colonies into 96-microwell plates was scraped from the agar surface with an inoculation loop and collected in microcentrifuge tubes containing 480 µl of 50 mM EDTA. Nucleic acids were extracted using the Wizard® Genomic DNA Isolation System Kit protocol G (Promega Corporation, Madison, WI, USA), following the manufacturer instructions. The obtained DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, EUA) and stored at -20ºC.

3.3.3 DNA extraction from liquid enrichments

A freeze-thaw method was used for DNA extraction from liquid cultures and enrichments (Tsai and Olson 1991). Cultures grown on 96 deep well plates were centrifuged (Eppendorf Centrifuge 5804R, Hamburg, Germany) for 30 minutes at 3,000 × g. At least 800 µL of the supernatant was discarded and the remaining cell pellet maintained at -20 ºC until the DNA extraction. Thawed pellets were homogenized and transferred to a capped 96 well PCR plates for nucleic acid extraction purposes. The plate

was sonicated for 10 seconds. The extraction was based on 3 cycles of 10 minutes at 85 ºC followed by 10 minutes at -80 ºC. Cross contamination of extracted DNA was checked with addition of negative controls in all plates used for extraction. In all cases no DNA was detected above detection limits and PCR amplificactions using bacterial universal primers always yielded negative results.

3.3.4 DNA extraction from bacterial isolates

Extractions of DNA from bacterial isolates were performed using Chelex® 100 Resin (Bio-Rad laboratories, Inc., Hercules, California, EUA). About twenty bacterial colonies grown on agar plates for every isolate were scraped and re-suspended into 100 µL of 6% of Chelex® 100 Resin. Suspensions were incubated for 20 minutes at 56ºC and for 5 minutes at 96ºC, followed by a thermal shock at -20ºC for 5 minutes. After extraction, samples were centrifuged for 5 minutes at 14,000 rpm and 40 µL of the supernatant were transferred into a clean sterile tube. The obtained DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) and stored at -20ºC.

3.3.5 Functional and 16S rRNA genes amplification by PCR

The use of molecular tools based on the use of DNA sequences analyses provide means to classify and compare genomic sequences (Donachie *et al.,* 2007). This allows the exploration of the structure, the function, and the dynamics of bacterial communities without a cultivation step (Dahllof 2002, Throback *et al.,* 2004).

Five functional genes of the denitrification pathway (*narG*, *napA*, *nirS*, *nirK* and *nosZ*), and the 16S rRNA gene were amplified by PCR. Functional genes amplifications and sequencing were used either to analyse the denitrifier community structure (chapter 4.1), or to determine the presence of selected functional genes in the genome of bacterial isolates (chapter 4.3). The 16S rRNA gene amplification and sequencing was used for taxonomic classification and phylogenetic inference of bacterial isolates (chapter 4.3). The primers used to amplify 16S rRNA and functional genes are listed in Table 8.

Table 8. Primers used for PCR amplificaions of 16S rRNA and functional genes.

Gene	Primers	Sequence (5' – 3')	Amplicon (bp)	References
16S rRNA	27F	AGAGTTTGATCMTGGCTCAG	1465	(Lane 1991)
	1492R	CGGTTACCTTGTTACGACTT		
	357F	CCTACGGGAGGCAGCAG	620	(Lane 1991)
	907R	AAACTTAAAGGAATTGACGG		
narG	1960F	TAYGTSGGSCARGARAA	650	(Philippot *et al.,* 2002)
	2650R	TTYTCRTACCABGTBGC		
napA	V67m	AAYATGGCVGARATGCACCC	514	(Henry *et al.,* 2008)
	V17m	GRTTRAARCCCATSGTCCA		
nirS	cd3aF	GTSAACGTSAAGGARACSGG	425	(Michotey *et al.,* 2000, Throback *et al.,* 2004)
	R3cd	GASTTCGGRTGSGTCTTG		
nirK	F1aCu	ATCATGGTSCTGCCGCG	472	(Hallin *et al.,* 2009)
	R3Cu	GCCTCGATCAGRTTGTGGTT		
nosZ	nosZ-F	CGYTGTTCMTCGACAGCCAG	453	(Kloos 2001, Throback *et al.,* 2004)
	nosZ1622R	CGSACCTTSTTGCCSTYGCG		

The PCR conditions used for each primer combination have been previously published and were applied with minor modifications (Table 9). In all cases, PCR reactions were performed in a total volume of 50 µL according to the conditions described in table 8. All chemicals and reagents used were from Qiagen (Qiagen, Germany). PCR amplifications were performed in a Gene Amp® 2700 thermal cycler (Applied Biosystems). PCR products were checked for its correct amplification and size using electrophoresis on a 1.5% agarose gel and visualised through ethidium bromide staining.

Table 9. PCR conditions for 16S rRNA and functional genes. The final concentration of the reaction reagents and temperature conditions for the amplification of the studied genes are detailed.

		16S rRNA			Functional genes				
		Complete	Partial		*narG*	*napA*	*nirS*	*nirK*	*nosZ*
		27F-1492R	357F-907R		1960F-2650R	V67m-V17m	cd3aF - R3cd	F1aCu - R3Cu	nosZ-F - nosZ1622R
Reaction reagents		**Final concentration in 50 µL of reaction**							
10X Buffer solution		1X	1X		1X	1X	1X	1X	1X
25 mM $MgCl_2$		0.5 mM	0.5 mM		1 mM	1 mM	1 mM	1 mM	1 mM
5X Q Solution		na	1X		na	na	1X	na	1X
BSA (10 ng·mL^{-1})		na	na		na	na	na	0.2 ng·mL^{-1}	0.8 ng mL^{-1}
Primers		0.4 µM	0.5 µM		0.5 µM	0.5 µM	0.25µM	1 µM	0.8 µM
10mM dNTPs		0.8 mM	0.2 mM		0.2 mM	0.2 mM	0.8 mM	0.8 mM	0.2 mM
Taq (5U·µL)		1U	1U		0.5U	0,625U	0.5U	0.5U	0.25U
Amplification conditions									
Start		95ºC 4 m	94ºC 4 m		95ºC 5 m	94ºC 3 m	94ºC 2 m	94ºC 3 m	94ºC 3 m
Denaturation	× num of cycles	95ºC 30 s	94ºC 30 s		95ºC 30 s	94ºC 30 s	94ºC 30 s	94ºC 30 s	94ºC 30 s
Annealing		52ºC 60 s ×30	52ºC 45 s	×10	60ºC* 30 s ×10	59ºC* 45 s	×8 57ºC 60 s	×35 60ºC 60 s	×35 60ºC 60 s ×35
Extension		72ºC 2 m	72ºC 45s		72ºC 45 s	72ºC 45 s	72ºC 60 s	72ºC 60 s	72ºC 60 s
Denaturation	× num of cycles		94ºC 30 s		94ºC 30 s	94ºC 30 s			
Annealing			50ºC 45 s	×15	55ºC 30 s ×30	55ºC 45 s	×30		
Extension			72ºC 45 s		72ºC 45 s	72ºC 45 s			
Final extension		72ºC 4 m	72ºC 25 m		72ºC 6 m	72ºC 10 m	72ºC 10 m	72ºC 10 m	72ºC 10 m
Storage		4ºC	4ºC		4ºC	4ºC	4ºC	4ºC	4ºC

na: not applicable, **BSA**: bovine serum albumin, ***** step down reaction, in which the temperature decrease 0.5ºC at each cycle.

3.3.6.Quantification of gene copies using *q*PCR

Gene abundances were determined using quantitative PCR (*q*PCR). The *q*PCR amplification was performed for the functional genes *narG*, *napA*, *nirS*, *nirK* and *nosZ*. Additionally, the bacterial 16S rRNA gene was also quantified and used as a proxy for total bacterial abundance. All reactions were performed in a 7500 Real Time PCR system (Applied Biosystems) using the SYBR® Green PCR Mastermix (Applied Biosystems, Foster City, CA, USA). The reactions were performed with a 20 µL final volume containing 1X SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA).), 1 µg/µL BSA, 10 ng of sample DNA, and 1µM of each primer. Primers for *q*PCR differed in some cases from those used for conventional PCR amplification (Table 10). In all cases, *q*PCR primers were obtained from Biomers.

The standard curves were generated using serial dilutions (from 10^2 to 10^9 copies/reaction) of plasmids containing known sequences of the targeted genes. For each gene, a clone containing the gene sequence without any mistmatch in its priming sequence was used to perform the standard curve (Figure 14 A). Additionally, to check for the specificity of the *q*PCR reaction, melting curves were analyzed to ensure all of them produce a single dissociation peak (Figure 14 B).

Table 10. Primers and conditions used for quantitative PCR

Gene and Primers	Sequence (5' – 3')	Amplicon (bp)	Thermal conditions	Reference
16S rRNA 341F 534R	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGGC A	194	▪ 1 cycle: 95°C for 15 min. ▪ 35 cycles: 95°C for 15 s, 60°C for 30 s, 72°C for 30 s, and 80°C for 30 s.	(Hallin *et al.,* 2009, López-Gutiérrez *et al.,* 2004)
narG narG-F narG-R	TCGCCSATYCCGGCSATGTC GAGTTGTACCAGTCRGCSGAYTCS G	173	▪ 1 cycle: 95°C, 15 min. ▪ 6 cycles: 95°C for 30 s, 63 to 58°C (-1°C by cycle) for 30 s, 72ºC for 30s. ▪ 40 cycles: 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, 80°C for 30 s.	(Bru *et al.,* 2007)
napA napA-V17 napA4r	TGGACVATGGGYTTYAAYC ACYTCRCGHGCVGTRCCRCA	152	▪ 1 cycle: 95°C, 15 min. ▪ 6 cycles: 95°C for 30 s, 61 to 56°C for 30 s (-1°C by cycle), 72ºC for 30s. ▪ 40 cycles: 95°C for 15 s, 56°C for 30 s, 72°C for 30 s, 80°C for 30 s.	(Bru *et al.,* 2007)
nirS Cd3aFm R3cdm	AACGYSAAGGARACSGG GASTTCGGRTGSGTCTTSAYGAA	425	▪ 1 cycle: 95°C, 15 min. ▪ 6 cycles: 95°C for 15 s, 65 to 60°C for 30 s (-1°C by cycle), 72°C for 1 min, 80°C for 15 s. ▪ 40 cycles: 95°C for 15 s, 60°C for 30 s, 72°C for 30 s, 80°C for 15 s.	(Hallin *et al.,* 2009, Throback *et al.,* 2004)
nirK nirK876 nirK1040	ATYGGCGGVCAYGGCGA GCCTCGATCAGRTTRTGGTT	164	▪ 1 cycle: 95°C, 15 min. ▪ 6 cycles: 95°C for 15 s, 63 to 58°C for 30 s (-1°C by cycle), 72°C for 30 s, 80°C for 15 s. ▪ 40 cycles: 95°C for 15 s, 60°C for 30 s, 72°C for 30 s, 80°C for 15 s.	(Hallin *et al.,* 2009, Henry *et al.,* 2006)
nosZ nosZ2F nosZ2R	CGCRACGGCAASAAGGTSMSSGT CAKRTGCAKSGCRTGGCAGAA	267	▪ 1 cycle: 95°C, 15 min. ▪ 6 cycles: 95°C for 15 s, 65 to 60°C for 30 s (-1°C by cycle), 72°C for 30 s, 80°C for 15 s. ▪ 40 cycles: 95°C for 15 s, 60°C for 30 s, 72°C for 30 s, 80°C for 15 s.	(Hallin *et al.,* 2009, Henry *et al.,* 2006)

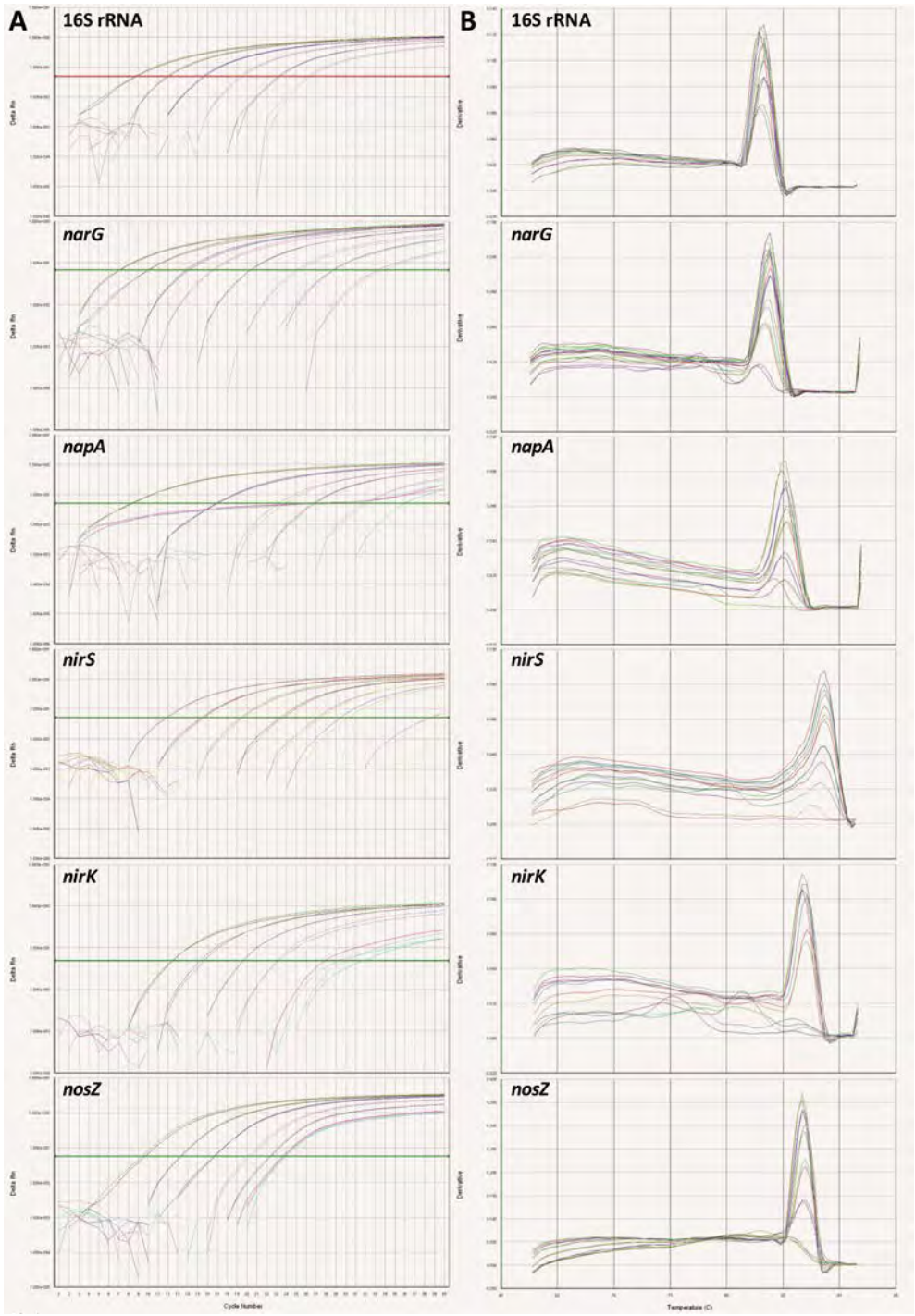


Figure 14. Amplification plots and dissociation peaks of *q*PCR standards. Serially diluted standards (10^7 to 10^3 copies) are plotted for each gene. Red or green horizontal lines in the left pannels indicate the fluorescence treshold at which Ct values are calculated.

The *q*PCR efficiencies for all analyzed genes are listed in table 11. The negative controls resulted in undetectable values in all *q*PCR reactions.

Table 11. Efficiency of *q*PCR standard curves. Values of efficiency in percentage and the lineal adjustment of the standard curves: R^2.

Primers	*q*PCR efficiency	R^2
16S rRNA	90.03%	0.999
narG	88.59%	0.994
napA	82.08%	0.995
nirS	86.61%	0.993
nirK	89.56%	0.998
nosZ	102.52%	0.991

Inhibition tests were performed before *q*PCR assays were done. Every sample was evaluated for inhibition independently. For inhibition tests a known number of copies of the plasmid DNA (pGEM-TEasy, Promega, Madison, WI), were added to the extracted DNA samples in a ratio 1:100. This ratio is considered to be sufficient to detect significant increase in cycle thresholds (Ct) in case of PCR inhibition (Hallin *et al.,* 2009). Independently of the sample added to the plasmid solution containing a known number of copies, Ct quantifications, using plasmid specific PCR primers (Table 12), differed for less than 0.08 ± 0.23 when compared with values obtained for the plasmid solution alone. This indicates that no inhibition occurred at the sample concentrations used in the three periods.

Table 12. Primer sequences used to amplify plasmid sequences.

Primer name	Primer sequence
T7	5′-TAATACGACTCACTATAGGG-3′
SP6	5′-ATTTAGGTGACACTATAG-3′

The relative contributions of the functional genes (*narG*, *napA*, *nirS*, *nirK* and *nosZ*) compared with the16S rRNA gene were calculated as a proxy for denitrifying bacteria abundance.

The gene abundances and gene ratios were log transformed to ensure a normal distribution of the data. The normality was assessed for all variables, except for the abundances of the *narG* and *nirK* genes, using the Shapiro-Wilk tests. One-way ANOVA and post hoc tests (Tukey) were used with log-transformed data to characterise the effects of feeding regimes applied to the cathode on the abundance of different genes and ratios when equal variance of data was observed. Alternatively, non-parametric analyses (Kruskal-Wallis test) were used. All statistical analyses were performed using SPSS for Windows 15.0 (SPSS, Inc).

3.3.7 PCR-DGGE diversity and characterization of enrichments

The bacterial community composition of Mfc4 during shift conditions was characterized using 16S rRNA gene (chapter 4.1). PCR reactions were executed using primers 357F and 907R (Table 8), following the PCR conditions previously described (Lane 1991, Prat *et al.,* 2009) with minor modifications. To perform the DGGE, a reverse primer with GC clamp attached to the 5' end was used (Muyzer *et al.,* 1993):

5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC-3'

The composition of denitrifying communities of different MFCs (chapter 4.2) and the screening of enrichments of chemolithoautotrophic denitrifiers were performed by PCR-DGGE analysis of the gene coding for the nitrous oxide reductase (*nosZ*). PCR reactions were done with primers nosZ-F and nosZ1622R (Table 8). The same GC-clamp above was attached to the 5' end of the reverse primer for DGGE analysis. The PCR reactions were performed in a final volume of 50 µL, following the PCR conditions previously published (Throback *et al.,* 2004) with minor modifications (Table 9).

All chemicals and reagents were provided by Qiagen®, the PCR amplification reactions were done in a Gene Amp® 2700 thermal cycler (Applied Biosystems) as stated previously by Prat *et al.* (16S rRNA) and Enwall *et al.* (*nosZ*) (Enwall *et al.,* 2005, Prat *et al.,* 2009). PCR products were checked by electrophoresis in agarose gels prior to DGGE analysis.

Approximately, 100 ng of PCR amplified DNA was loaded on 6% (V/V) acrylamide–bis-acrylamide gels with a urea–formamide denaturing gradient. The urea–formamide gradient changed according to the amplified genes (Table 13). 100% denaturing urea-formamide solution contained 42.0 g urea and 40 mL formamide.

Table 13. DGGE conditions. Denaturing conditions in urea-formamide gradient.

Gene	Gradient	Reference
nosZ	40-70%	(Enwall *et al.,* 2005)
16S rRNA	30-80%	(Muyzer *et al.,* 1993)

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 on gels and used as a ruler for comparison of band migration properties.

Electrophoreses were run for 14 hours at 160 V and 60 ºC. Gels were stained with SYBR® Gold (Invitrogen, molecular Probes) for 45 minutes and visualized in a Herolab UVT-20M. Images were documented using ProgRes CapturePro 2.7 program. Representative bands of every position were excised using a sterile scalpel. The DNA was recovered by elution in 35 µl Tris/HCl (pH 8.0) at 65 ºC during 1 hour and re-amplified with either *nosZ* or 16S rRNA gene specific primers as described above.

The re-amplified DGGE-bands were sequenced in both directions, at the Macrogen service (Macrogen, Korea). The sequences were manually edited to check for nucleotide ambiguities using the BioEdit Alignment Editor v7.0 (Hall 1999). Sequences alignments were performed using the CLUSTALW software (European Bioinformatics Institute, http://www.ebi.ac.uk). The sequences were compared with those deposited in the GeneBank (NCBI) database using the BLASTN software (http://www.ncbi.nlm.nih.gov/BLAST/).

The *nosZ* gene sequences (chapter 4.2) have been submitted to the GenBank database under the accession numbers HQ621696 to HQ621729 (sequences from DGGE bands) and HQ630075 to HQ630210 (sequences obtained from enrichment cultures).

Digital images of DGGE gels were analysed using the GELCompar II© v.6.1 software package (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Comparison between samples loaded on different DGGE gels was completed using normalized values derived from standard samples. Band classes according to % migration in the gel lane, were fixed at a tolerance of 0.5 %. Pairwise sample similarities (Dice based correlation coefficients with fixed values of 0.5%) were calculated according to band position and used to cluster similar samples using the unweighted pair-group method with arithmetic averages (UPGMA). The enrichments grouped together containing bands at desired positions fixed after band class definition were selected for isolation.

3.3.8 Cloning of functional genes and phylogenetic analysis

The PCR products of *narG*, *napA, nirS*, *nirK* and *nosZ* genes were purified using the QIAquick PCR purification kit (Qiagen, Germany) and cloned using the TOPO TA Cloning® Kit for Sequencing (Invitrogen, Eugene, OR) according to manufacturer's instructions. At least 300 clones per gene were individually selected and screened by PCR using the primers M13 (Table 14). Amplicons of the expected size were sequenced at Macrogen service (Macrogen, the Netherlands).

Table 14. Primer sequences used to amplify cloned gene fragments.

Primer name	Primer sequence
M13F-20	5'-GTAAAACGACGGCCAG-3'
M13R	5'-CAGGAAACAGCTATGAC-3'

The sequences were examined for the presence of chimeras using the UCHIME algorithm (Edgar *et al.,* 2011) and manually refined using the BioEdit Alignment Editor v7.0. Reference sequences for the *narG*, *napA*, *nirS*, *nirK* and *nosZ* genes were obtained from completely sequenced genomes in the GenBank database, aligned using CLUSTALW (Larkin *et al.,* 2007) and used as a template file to define Operational Taxonomic Units (OTUs) using mothur v.1.22.1 (Schloss *et al.,* 2009). The OTUs were defined at threshold values of 33% (*narG*), 18% (*nirS*), 17% (*nirK*) and 20% (*nosZ*), as previously determined (Palmer *et al.,* 2009, Palmer *et al.,* 2012). The threshold cut-off values for *napA* were set at 21%. This value was estimated as the species level cut-off value according to pair-wise comparisons of *napA* and 16SrRNA gene sequences of 21 completed genomes deposited in the GenBank database (Table 15).

Table 15. Sequences of bacteria containing *napA* gene. 16S rRNA gene and *napA* gene sequences from bacteria with complete genomes available in NCBI database used in this study.

Bacterial specie	Accession number
Candidatus "Accumulibacter phosphatisclade"	CP001715
Leptothrix cholodnii	CP001013
Thauera sp. MZ1T	CP001281
Dechloromonas aromatica	CP000089
Magnetospirillum magneticum	NC007626
Cupriavidus necator	CP002878
Bordetella petrii	NC010170
Rhodobacter sphaeroides	CP000662
Bradyrhizobium sp. ORS278	NC009445
Pseudoxanthomonas suwonensis	CP002446
Pseudomonas stutzeri	CP002881
Bradyrhizobium japonicum	NC00944
Rhodopseudomonas palustris	CP000301
Ralstonia eutropha	CP000091
Edwardsiella tarda	CP002154
Pseudomonas mendocina	CP000680
Sinorhizobium fredii	CP001389
Pseudomonas sp.G-179	AF040988.1
Agrobacterium tumefaciens	AE007870
Escherichia coli	CP000946
Salmonella enterica serovar *Typhimurium*	NC016810.1

These sequences were analysed following the previously described computational procedure by Palmer and co-workers (Palmer *et al.,* 2009). Pair-wise similarities of both

napA and 16S rRNA genes were calculated and plotted. A consensus for species identification using 16S rRNA gene sequences similarity is a 97% cutoff. According to the distribution of all available combinations in the *napA* similarity *vs.* 16S rRNA similarity plot, a value of 79% was fixed to ensure species level differenciation using *napA* sequences. This values was used as a threshold value to defined OTUs at the species level.

Defined OTUs were used to calculate rarefaction curves and to estimate the maximum richness (Chao1) and diversity indices (Shannon). The deduced amino acid sequences were obtained for representative sequences of each OTU and functional gene, and aligned using the ClustalW algorithm in MEGA v.5.0 (Tamura *et al.,* 2011). Phylogenetic trees were reconstructed by neighbour-joining using the pair-wise deletion and *p*-distance methods. The tree topology was evaluated using bootstrap analysis with 10,000 replicates. Differences in the community composition based on the phylogeny of the *narG*, *napA*, *nirS*, *nirK* and *nosZ* genes were analysed from the tree topologies using the weighted Unifrac test (Lozupone *et al.,* 2006).

The sequence data derived from the cloning approach (chapter 4.1) have been submitted to the GenBank database under the accession numbers JX236709 - JX236736 (*napA* gene), JX236737 - JX236898 (*narG* gene), JX237055 - JX237212 (*nirS* gene), JX236899 - X237054 (*nirK* gene) and JX237213 - JX237355 (*nosZ* gene).

3.3.9 Sequencing of *nosZ* and 16S rRNA genes of isolated bacteria

Bacterial isolates containing *nosZ* genes were sequenced for both *nosZ* and 16S rRNA genes. The PCR products were purified by PCR reaction cleanup ExoSAP ® (Applied Biosystems®) with the addition of 0.03 µl of exonuclease, 0.3 µl of FastAP and 1.67 µl of miliQ water into 10 µL of PCR product and incubating at 37ºC for 60 minutes followed by heat inactivation at 85ºC for 15 minutes.

Sequencing reactions were performed using BigDye® Terminator method (Applied Biosystems®), following the manufacturer's instructions. The dNTPs precipitation were performed by adding 2 µL of sodium acetate (3 M), 50 µL ethanol (100%) and 2µL EDTA (125 mM), incubated in the dark for 15 minutes at room temperature. Supernatants were discarded after centrifugation at 2,000 rpm for 30 minutes. PCR products were further cleaned up with 70 µL of ethanol (70%) and centrifuged again for 15 minutes. Supernatants were discarded and amplification products dried at 60ºC for 15 minutes.

DNA samples were re-hydrated with 10 µL of formamide HI-DI (highly deionized) and sequenced by 3130 Genetic Analyzer (Applied Biosystems). Sequences obtained were manually checked using the BioEdit Alignment Editor v7.0 and aligned using CLUSTALW algorithm (European Bioinformatics Institute) (Hall 1999), together with reference *nosZ* sequences obtained from bacterial genomes. Sequences were compared with those deposited in the GeneBank (NCBI) database using the BLASTN software. Phylogenetic analyses were done using either the nucleotide or the deduced amino acid sequences with the MEGA v.5.0 software (Tamura *et al.,* 2011). Neighbor-joining trees were reconstructed with the pair-wise deletion method using kimura2 or amino-Poisson corrections for nucleotide or aminoacid sequences, respectively. Tree topology was evaluated by bootstrap analysis using 1,000 replicates.

3.4 Physiological characterization of bacterial isolates

Six bacterial strains, five isolates and a type strain were characterized physiologically and electrochemically. All bacterial isolates were checked for, 1) the use of alternative electron donors, thiosulfate, hydrogen and acetate; 2) their denitrification capacity using different electron donors and acceptors, and 3) their electrotrophic behaviour.

3.4.1 Growth rate

Bacterial isolates were physiologically characterised under heterotrophic and autotrophic conditions using supplemented BMM (Table 4). BMM solutions were boiled for 10 minutes and then cooled down to room temperature under nitrogen gas flow to ensure anaerobic conditions. The pH was adjusted at 6.9 ± 0.1. Prepared medium solutions were distributed anaerobically (under a N_2 stream) into hungate tubes (12 mL), sealed and autoclaved.

All tubes were supplemented individually with different electron donors according to the treatment, using stock solutions. Electron donor solutions were sterilized by filtration with 0.22 μm Millex-GP Millipore (Merck, KGaA, Darmstadt, Germany). Heterotrophic conditions were accomplished using a mixture of simple carbon compounds: ammonium acetate (CH_3COONH_4), sodium propionate ($C_3H_5NaO_2$) and ethanol (C_2H_6O) at proportion of 5:3:2 moles (final concentration, 0.373 $g \cdot L^{-1}$ of carbon). Autotrophic conditions were achieved using two different electron donors: thiosulphate (5 $g \cdot L^{-1}$), and hydrogen. Hydrogen was supplemented injecting 10 mL of the as a gas mixture of 32% CO, 32% H_2, 8% CO_2 and 28% N_2 into each tube. In all conditions nitrate was supplemented at 0.155 $g \cdot L^{-1}$. All conditions were analyzed in triplicates.

Tubes were incubated at 30ºC in a horizontal position to favour gas-liquid exchange. Bacterial strains were inoculated at 10% v/v into tubes from cell suspensions obtained from agar plate grown colonies. Negative controls (no inoculation of bacteria) and blank treatments (inoculated bacteria without the addition of any electron donor) were also prepared in every incubation experiment.

Growth was monitored for 10 days after inoculation by measuring the optical density at 600 nm every 24 hours (Cecil spectrofotometer CE 1021, Cecil Instruments Limited, Cambridge, GB). Growth rates were calculated during the exponential growth phase. Differences in growth rates according to experimental conditions or isolated bacteria were analysed statistically. The normality and the homogeneity of variances were

calculated using Shapiro-Wilk and Levene tests, respectively. When both assumptions were true, one-way ANOVA and post hoc test (Tukey) were used. Alternatively, non-parametric analysis (Kruskal-Wallis test) and post hoc (Games-Howell) were used. All statistical analyses were performed using SPSS for Windows 15.0 (SPSS, Inc).

3.4.2 Determination of NOx reducing and N_2O production

Nitrate and nitrite reduction capacity was evaluated in triplicate in the presence of hydrogen, thiosulphate or organic matter. Medium formulations and Hungate tubes preparation was made exactly as described in section 3.4.1. Nitrate was added at 0.177 $g \cdot L^{-1}$, final concentration. Due to the toxicity of nitrite for some bacteria, nitrite reducing tests were performed either at 6.5 $mg \cdot L^{-1}$ or 3.2 $mg \cdot L^{-1}$ (final concentrations in samples). The anaerobic incubations were done in a final volume of 16 mL of medium and with 9 mL of gas head-space.

The acetylene blockage (10% v/v) method was used when nitrite was added as electron acceptor to measure nitrous oxide accumulation. Comparison of nitrous oxide accumulation in the presence of acetylene with the one observed in the absence of acetylene, were used to estimate complete denitrification capacity (Knowles 1990).

All experiments were performed at 30ºC and maintained for at least 10 days. Concentrations of nitrate, nitrite and nitrous oxide were measured at the beginning (initial sample) and the end (final sample) of the incubation period. The amount of nitrate and nitrite was quantified by Ion Chromatography using a Waters IC-Pak™A column (Waters). Sodium Borate/Gluconate eluent was used as the mobile phase (Table 16). The eluent solution was filter through a 0.22 μm GNWP04700 nylon membrane filters (Milipore, Merck KGaA, Darmstadt, Germany).

Table 16. Composition of mobile phase in HPLC method. Composition of Sodium Borate/Gluconate of concentrate solution and eluent composition are detailed.

Sodium Borate/Gluconate concentrate		Sodium Borate/Gluconate eluent	
Compound	**($g \cdot L^{-1}$)**	**Compound**	**$mL \cdot L^{-1}$**
Sodium gluconate	16	Borate/Gluconate concentrate	20
Boric acid	18	n-butanol	20
Tetraborate decahydrate	25	acetonitrile	120
Glycerin	250 $mL \cdot L^{-1}$		

Nitrite and nitrate peaks were identified from retention times of known standards, at 6.0 ± 0.2 and 10.0 ± 0.3 minutes. Peak areas were used for quantification. Standard curves of NO_3^- and NO_2^- ranged from 0.4 to 10 $mg \cdot L^{-1}$. When necessary, samples were diluted with distilled water to ensure a proper quantification within the standard curve range.

Nitrous oxide (N_2O) concentration was quantified at the end of the incubation period in treatments with and without acetylene addition. Concentrations were measured at the liquid compartment using a calibrated N_2O minisensor 500 µm (Unisense, www.unisense.com) (Andersen *et al.,* 2001). Electrode was polarized at -800 mV and calibrated with a saturated N_2O (27.05 mM). Nitrous oxide in the gas phase was inferred from the partial gas pressure measured and Henry's Law constant for N_2O at 30 ºC (K_H = 4315 $Pa \cdot m^3 \cdot mole^{-1}$) using equation 2 and the ideal gas law (Equation 3).

$$P_{N_2O} = K_H \cdot [N_2O]_{liquid} \qquad \text{Equation (2)}$$

$$PV = nRT \qquad \text{Equation (3)}$$

P_{N_2O} is the partial pressure of nitrous oxide in the gas phase, K_H is the Henry's constant and $[N_2O]_{liquid}$ is the N_2O concentration in the liquid phase ($mole \cdot m^{-3}$). The addition of nitrous oxide measured in the two compartments accounted for the total N_2O production.

The normal distribution of samples was assessed using Shapiro-Wilk and homogeneity of variances using Levene tests. To compare rates of nitrate and nitrite reduction, and nitrous oxide accumulation, using different electron donors or the effect of acetylene gas on these activities, one-way ANOVA and post hoc tests (Tukey) were used. Alternatively, non-parametric analysis (Kruskal-Wallis test) and post hoc (Games-Howell) were used if normality of data distribution was not assured. All statistical analyses were performed using SPSS for Windows 15.0 (SPSS, Inc).

3.4.3 Biofilms development for electrochemical characterization

Six bacterial strains, C2S229.1, C2T108.3, C1S131/132.1, C1S131/132.2, C1S119.2 and *Oligotropha carboxydovorans* OM5^T, were forced to grow on graphite rods and to form monospecific biofilms. These biofilms were used for the electrochemical characterization of isolates.

Previous to incubation in the presence of bacteria, graphite rods were rinsed in an acidic solution HCl (1 M) over night, rods were later rinsed in a basic solution NaOH (1 M) o/n to activate the graphite surface electrochemically. Rods were immersed in anaerobic organic matter rich medium (TSB 6 g·L^{-1} supplemented with 0.15 g·L^{-1} of nitrate).

Ten mL of bacterial suspensions obtained from grown agar plates were aseptically inoculated in bottles containing activated graphite rods. Cell suspensions were stirred using a vortex and magnetic bar to enhance biofilm formation (Kalivoda *et al.,* 2008). Incubation temperature was 25ºC and suspensions were maintained for at least 60 days. Liquid samples of 2 mL were collected from the bottles immediately after inoculation and at the end of the incubation period. Nitrate and nitrite concentrations were measured by IC as described above. Agar plates with BMM supplemented with organic matter solution (sodium acetate, propionic acid and ethanol (100%), 5:3:2 moles) were also plated with liquid medium to evaluate for the purity of the bacterial suspension. Bacterial suspensions were further checked by contrast-phase optical microscopy to determine cell morphology and aggregation.

Once biofilms were formed and a thin whitish layer could be observed on graphite rods, the organic matter rich medium was replaced with inorganic BMM to provide autotrophic conditions. This was done after rinsing graphite rods, for at least three times, with freshly prepared organic matter free BMM. All transfers of graphite rods were done in anaerobic conditions. Vitamin supplemented BMM was used in the last transfer. Bottles containing colonized graphite rods were flushed for 10 minutes with gas mixture composed by 5% of H_2, 5% of CO_2 and 90% of N_2. Rods were maintained in anaerobic autotrophic conditions for 10 days for acclimatization of the bacterial biofilms to organic matter free conditions prior to electrochemical characterization. Samples were obtained at the beginning and at the end of the incubation period to measure the concentration of nitrate and nitrite.

3.4.4 Electrochemical characterization by cyclic voltammetry

A 250mL H-type cell was used as a reactor to characterize the graphite rods with biofilm attached. A cation exchange membrane was used as chamber separator (CMI-7000, Membranes Int., United States). The reactors were assembled following a three-electrode arrangement. The graphite rod with biofilm attached was used as working electrode (WE). An Ag/AgCl reference electrode (+197mV vs. Standard hydrogen electrode (SHE), model RE-5B BASi, United States) was positioned near to the WE electrode. An abiotic graphite rod was used as the counter electrode (CE).

The electroactivity of the biofilm was tested under different media containing nitrate, nitrite or nitrous oxide. All media contained: 2.64 $g \cdot L^{-1}$ KH_2PO_4, 4.32 $g \cdot L^{-1}$ Na_2HPO_4, 0.13 $g \cdot L^{-1}$ KCl, 0.02 $g \cdot L^{-1}$ NH_4Cl, 1.40 $g \cdot L^{-1}$ $NaHCO_3$ and 0.1 $mL \cdot L^{-1}$ of a micronutrients solution (Carmona-Martinez *et al.,* 2013). In addition, nitrate-, nitrite- or nitrous oxide- medium were amended with 0.18 $g \cdot L^{-1}$ $NaNO_3$ (30 $mg \cdot L^{-1}$ $N\text{-}NO_3^-$), 0.15 g·L-1 $NaNO_2$ (30 $mg \cdot L^{-1}$ $N\text{-}NO_2^-$) or 30 $mg \cdot L^{-1}$ $N\text{-}N_2O$. All media were sparged with N_2 gas during 15 minutes to remove dissolved oxygen. For nitrous oxide-medium, a volume of 40 mL of saturated N_2O-solution was spiked after N_2 flushing.

The electroactivity of the biofilm under each medium was tested during seven days under potentiostatic control (model SP-50, Bio-logic, France). Chronologically, nitrate, nitrite and nitrous oxide media were tested (turnover conditions). At the end, the activity under non-turnover conditions (*i.e.* no presence of oxidized forms of nitrogen) was evaluated. Abiotic electrochemical response of each graphite rod, after the removal of the biofilm attached, was evaluated under the different media to ensure that the electrochemical response was related to bacterial activity.

At all times during electrochemical characterization, the biofilm containing electrode (WE) was polarized at -123 mV *vs* SHE) based on previous results (Pous *et al.,* 2015). The activity under each media was electrochemically characterized by cyclic voltammetry (CV). CVs were performed from -553 to +297 mV *vs* SHE at scan rate of 1 $mV \cdot s^{-1}$. Three cycles were recorded, but only the third is represented in this study. Peak potentials observed in CVs were calculated using SOAS software (Fourmond *et al.,* 2009).

4.1 Denitrifying bacteria affect current production and nitrous oxide accumulation in Microbial Fuel Cell

Nitrate reduction has been previously demonstrated using biocathodic bioelectrochemical systems. These systems are promising technologies currently used to partially suppress the carbon dependence of denitrification processes (Clauwaert *et al.*, 2007, Gregory *et al.*, 2004, Park *et al.*, 2006, Puig *et al.*, 2011, Virdis *et al.*, 2008). Process for biocathodic removal of nitrate contaminated subsurface waters with low amounts of organic matter has been recently patented (WO 2014/082989 A1), proving the reliability of the method.

Previous studies have shown that cathodes of MFCs harbour complex bacterial communities, and include members of the *Proteobacteria*, *Firmicutes* and *Chloroflexi*, as the most abundant representative species (Chen *et al.,* 2008, He *et al.,* 2009, Wrighton *et al.,* 2010). Wrighton and co-workers (Wrighton *et al.,* 2010) showed that the observed changes in the dominant members of the bacterial community did not correspond with the changes in reactor functioning. This observation potentially reflects the fact that most of the observed phylotypes did not exhibit relevant denitrification activity, suggesting that information about functional groups is required for a better understanding of the process.

Our goal was to identify the relevant players of nitrate, nitrite and nitrous oxide reductions as key metabolic steps in the denitrification process occurring in biofilms developed in the cathode of a MFC. Different operating conditions were used to assess the effect of different electron donors (electron transfer between the electrode or organic matter) and electron acceptors (nitrate or nitrite) in the microbial community. We examined the community composition and abundance of nitrate, nitrite and nitrous oxide reducing bacteria using five functional genes of the denitrification pathway. Abundances of functional genes were analysed through quantitative PCR, and the structure of the nitrate reducer, nitrite reducer and nitrous oxide reducer bacterial communities were assessed using a cloning-sequencing approach. MFC performances, in terms of nitrogen removal, power density generation and nitrous oxide accumulation, were compared for the different electron acceptors and donors used.

4.1.1 Denitrifying cathode performances under different feeding conditions

During one year, an MFC was operated under different cathodic feeding conditions: autotrophic with nitrate (Period 1), heterotrophic with nitrate (Period 2) and autotrophic with nitrite (Period 3). The different performances of the MFC during those operational conditions are summarized in table 1. During the different experimental conditions studied, either nitrate or nitrite was removed in the cathode and current was subsequently produced. The highest current production (15 $A \cdot m^{-3}$ NCC) and cathode Coulombic Efficiencies (CE, 85%) were achieved when nitrate was used as the electron acceptor under strictly autotrophic conditions (Period 1). When organic matter was also introduced into the cathode (Period 2), electrotrophy was affected, decreasing the current production (11 $A \cdot m^{-3}$ NCC) and cathode CE (58%). This decrease could be due to the partially removal of nitrate via conventional heterotrophic denitrification. The use of nitrite as an electron acceptor without the presence of organic matter resulted in an increase in current production to values similar to those found for nitrate (14.1 $A \cdot m^{-3}$ NCC), but cathode CE was decreased (41%).

Table 17. Effluent characteristics at different operational periods. Effect of different feeding conditions on the effluent characteristics and the MFC performance. Represented values are the means and standard deviation (n=5) at steady state conditions.

	Period 1	Period 2	Period 3
Flow ($L \cdot d^{-1}$)	2.0±0.3	1.4±0.5	1.2±0.2
COD (ppm COD)	n.d.	31±22	n.d.
NO_3^- (ppm N-NO_3^-)	22.6±0.3	20.7±4.8	0.0±0.2
NO_2^- (ppm N-NO_2)	0.2±0.1	2.1±3.4	4.6±4.8
N_2O (ppm N-N_2O)	0.6±0.2	4.0±1.9	13.0±5.9
N_2 (ppm N-N_2)	3.5±1.2	3.8±1.8	5.6±2.6
Cd ($A \cdot m^{-3}$ NCC)	15.0±2.3	11.0±7.0	14.1±8.2
CE (%)	85±11	58±17	41±17

* Concentrations of N_2O and N_2 were estimated on the basis of the electron balance at the cathode according to the method proposed of Virdis *et al.* (Virdis *et al.,* 2009).
COD: chemical oxygen demand; **NO_3^-**: nitrate; **NO_2^-**: nitrite; **N_2O**: nitrous oxide; **N_2**: dinitrogen gas; **Cd**: current density; **CE**: coulombic efficiency.
Period 1: Autotrophic with NO_3^-, **Period 2**: Heterotrophic with NO_3^- and **Period 3**: Autotrophic with NO_2^-.

The decrease of the CE suggested the accumulation of denitrification intermediates (nitrite or nitrous oxide). The concentration of intermediates in the cathode effluent showed higher rates of nitrous oxide during period 3 (Table 17). In this period, up to 70% of the nitrogen removed was converted into nitrous oxide. In contrast, when nitrate was fed during period 1, nitrous oxide level accounted for only 14% of the nitrogen removed. Nitrous oxide accumulation was significantly corelated to the decrease of CE (p = 0.033, ANOVA, n=3).

The power production and efficiency of nitrogen removal were influenced by the three cathodic influents (nitrate, nitrate plus organic matter and nitrite). The highest current production (15 $A \cdot m^{-3}$ NCC) was achieved when nitrate was used as the electron acceptor. In contrast, when organic matter was added, heterotrophic denitrification was kinetically favoured over autotrophic denitrification, and current production was reduced to 11 $A \cdot m^{-3}$ NCC. The use of nitrite as the initial electron acceptor without the presence of organic matter increased the current production to 14.1 $A \cdot m^{-3}$ NCC. Nitrate reduction has a higher potential ($E^{\circ\prime}$= +0.433 V *vs.* standard hydrogen electrode, SHE) when compared with that for nitrite ($E^{\circ\prime}$= +0.350 V *vs.* SHE) (Clauwaert *et al.,* 2007), therefore cathodic denitrification from nitrate is thermodynamically more favourable under autotrophic conditions. The amount of energy available for the bacteria to grow in the cathode compartment using nitrate is estimated to be an 11% higher than using nitrite.

The use of nitrate was not only beneficial for energy production, but also for the minimisation of nitrous oxide production, and for the increase of CE. Apart from N_2 production, N_2O represents a major sink during nitrate removal in the MFC (Table 1).

The reasons for the different potentials for N_2O emissions in the three conditions tested are multifactorial and include differences in the prevailing physicochemical conditions (*i.e.* temperature, pH and C/N ratio), the nature of the main electron acceptor and the composition of the denitrifying community (Čuhel *et al.,* 2010, Jones *et al.,* 2013, van Cleemput 1998). Under conditions of low electron availability (*i.e.* in the presence of organic matter), a lower affinity of the N_2O reductase towards the electron donor facilitates the accumulation of this intermediate (Pous *et al.,* 2013). In addition, other studies suggest that the carbon source significantly impacts on the net N_2O emission but exhibits a relatively minor effect on N_2O production mechanism (i.e. nitrate ammonification when glucose was used, or heterotrophic denitrification occurs with acetate) which are probably affected by community composition (Hu *et al.,* 2013).

When nitrite was used as an electron acceptor in the denitrifying MFC, the Coulombic Efficiency decreased to a 41%, compared to nitrate, and the N_2O concentration in the effluent increased to 13 mg $N\text{-}N_2O \cdot L^{-1}$ (a 70% of produced gases). It was proven that the use of nitrite as an electron acceptor and its accumulation in biological wastewater treatments cause an increase of NO and N_2O emissions during denitrification, agreeing with the results obtained in the MFC (Kampschreur *et al.,* 2009, Wunderlin *et al.,* 2012).

4.1.2 Quantification of the *narG*, *napA*, *nirK*, *nirS*, *nosZ* and 16S rRNA genes

The abundance of the 16S rRNA gene varied between 1.89×10^6 and 5.07×10^6 copies/ng DNA and was significantly higher ($p < 0.001$) than most of the denitrifying functional genes analysed (Figure 15). During the heterotrophic period, the abundance of 16S rRNA gene was significantly lower ($p < 0.026$) compared to the autotrophic periods with either nitrate or nitrite. The abundance of all functional genes varied between $0.3 \pm 0.0 \times 10^4$ and $2.2 \pm 0.1 \times 10^4$ gene copies/ng of DNA, except for *nirS* and *nosZ*, which were detected at higher concentrations. The amount of *nirS* varied from $1.71 \pm 0.5 \times 10^6$ to $2.65 \pm 0.3 \times 10^6$ gene copies/ng of DNA, but no significant differences were observed between periods. On the contrary, the abundance of *narG*, *napA*, *nirK* and *nosZ* showed significant differences ($p < 0.005$) according to the operating conditions. A significant decrease on the amount of *narG* and *napA* genes was observed during heterophic period in comparison to autotrophic period with nitrate. Also, the amount of *nosZ* significantly decreased during heterotrophic period in comparison to both autotrophic periods.

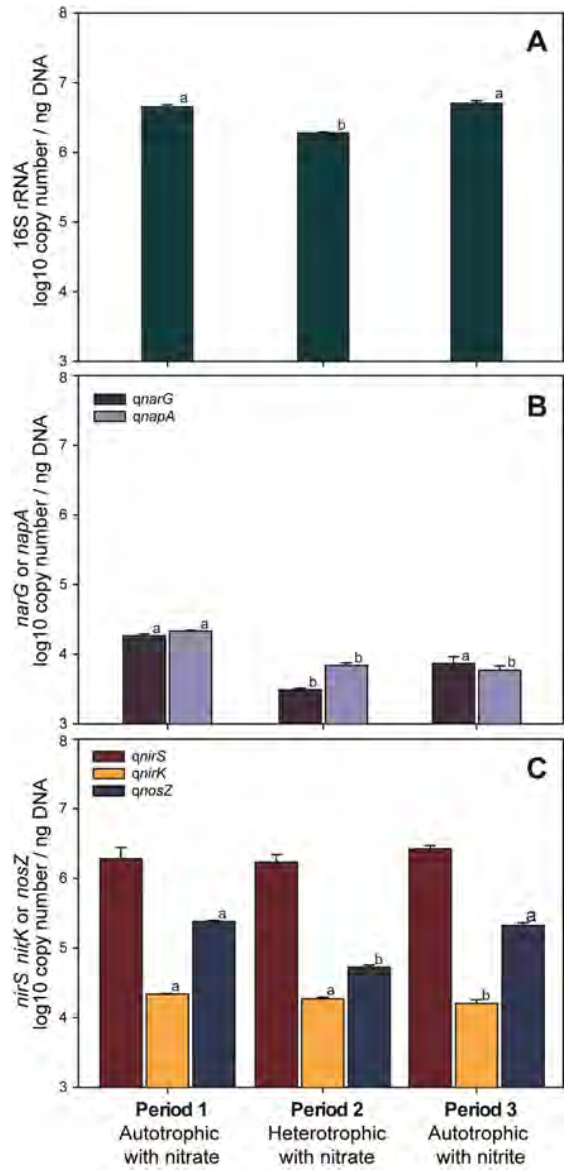


Figure 15. Abundances of the 16S rRNA and functional genes for the three periods studied. Mean values of the number of copies of the bacterial 16S rRNA **(A)** and the functional genes, *narG* and *napA* **(B)**, *nirS*, *nirK* and *nosZ* **(C)** are represented. Standard errors of the mean are indicated (n=2). Different letters above bars indicate significant differences ($p < 0.05$) between the three periods for the corresponding gene.

Gene ratios were used to evaluate the capacity of the system to reduce nitrate to nitrogen gas during the sequential process of denitrification. The ratios of (q*narG*+q*napA*)/q*nosZ* and (q*narG*+q*napA*)/(q*nirS*+q*nirK*) were consistently below a value of one, indicating a lower amount of nitrate reductases (*narG* and *napA*) compared with the amount of other genes implied in other steps in the denitrification pathway such as nitrite or nitrous oxide reductases (Table 18). Significant differences of both ratios were observed in relation to feeding conditions. The (q*nirS*+q*nirK*)/q*nosZ* ratio varied from 7.99 to 32.45, indicating a great potential of the system for the accumulation of intermediate gases, especially under heterotrophic conditions.

Table 18. Functional gene ratios. Relationship between the functional gene copy numbers, the 16SrRNA gene copy and the ratios between the genes.

Gene ratio	Period 1	Period 2	Period 3	
q*narG*/*q*16S rRNA (x100)	0.4±0.1	0.2±0.0	0.1±0.0	ns
q*napA*/*q*16S rRNA (x100)	0.5±0.1	0.4±0.0	0.1±0.0	**
q*nirS*/*q*16S rRNA (x100)	42.0±15.9	90.0±23.9	52.2±3.4	*
q*nirK*/*q*16S rRNA (x100)	0.5±0.0	1.0±0.0	0.3±0.0	***
q*nosZ*/*q*16S rRNA (x100)	5.4±0.2	2.8±0.3	4.2±0.7	**
(q*narG*+q*napA*)/q*nosZ*	0.17±0.01	0.19±0.01	0.06±0.01	***
(q*narG*+q*napA*)/(q*nirS*+q*nirK*)	0.02±0.01	0.01±0.00	0.01±0.00	*
(q*nirK*+q*nirS*)/q*nosZ*	7.99±3.29	32.45±8.88	12.80±2.10	**

The values show the mean and standard deviation (SD). The statistical significances between the treatments were calculated using one-way ANOVA or Kruskal-Wallis test depending on the normality of the data. ns, not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Denitrification is considered the main source of N_2O accumulation at a global scale (Maltais-Landry *et al.,* 2009, Morales *et al.,* 2010, Palmer and Horn 2012, Philippot *et al.,* 2011, Søvik and Kløve 2007). However, no consensus exists whether to consider either the *nirS* or the *nirK*-containing bacterial community as the main responsible for N_2O accumulation, since the abundance of these two type denitrifiers can vary significantly from environment to environment (Abell *et al.,* 2009, García-Lledó *et al.,* 2011, Jones and Hallin 2010, Philippot *et al.,* 2011). In the cathode of the MFC, NirS-type denitrifiers outnumbered NirK-type denitrifiers by two orders of magnitude at all working conditions. High q*nirS*/16S rRNA values were observed, suggesting a clear implication of the *nirS*-type denitrifiers in the denitrification potential and the accumulation of N_2O.

In order to ensure the quantification of nitrite reductases, the dominant members of the bacterial community on the cathode of the MFC were estimated by a PCR-DGGE approach using the 16S rRNA gene as a target (Figure 16). DGGE fingerprints varied significantly when the three periods were compared. In general, the richness of the community was rather low (from 8 to 12 DGGE Bands). The low number of bands, together with the fact that most changes affected high intensity bands, indicated that bacterial communities were dominated by a relatively low number of phylotypes and were selected according to enrichment conditions in the cathode.

Sequences from eighteen of the most prominent DGGE bands were used for identification to detect for putative denitrifiers containing either cytochrome *cd* nitrite (*nirS*) or copper containing nitrite reductases (*nirK*). A densitometric analysis of DGGE

gel images was used to estimate the relative abundance of *nirS* containing bacteria. During the autotrophic periods with nitrate and nitrite, DGGE bands corresponding to *nirS*-containing bacteria, accounted for 88 and 65% of overall band intensity, respectively. These values decreased to 44% during the heterotrophic period with nitrate. The results revealed that a high amount of the bacteria identified using *Bacteria* universal primers could belongs to *nirS*-containing bacteria which are in agreement with the high *nirS* gene copy numbers found using *q*PCR.

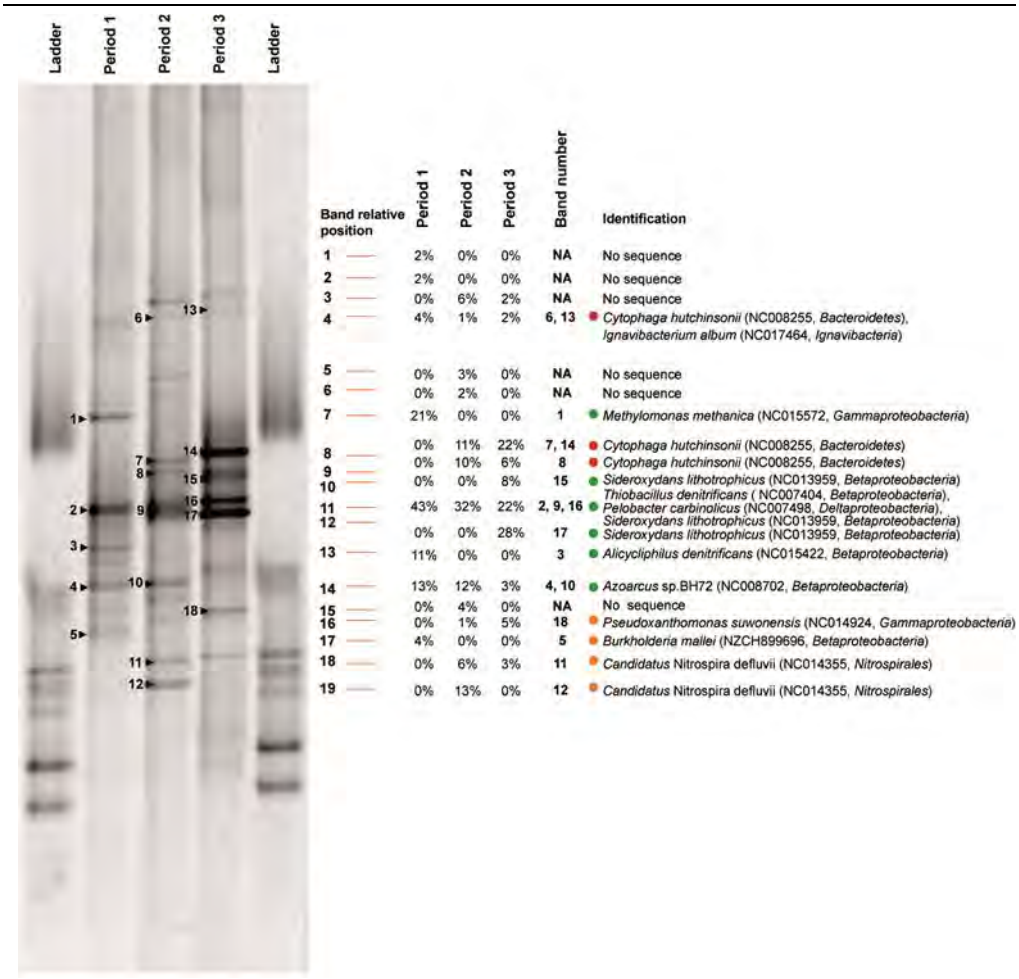


Figure 16. DGGE banding profiles of 16S rRNA genes from the three different studied periods. Period 1: Autotrophic conditions with nitrate; Period 2: Heterotrophic conditions with nitrate; Period 3: Autotrophic conditions with nitrite. Sequenced DGGE bands are indicated with an arrow head and identified with number. Predicted band positions are indicated at the right side of the DGGE gel. Estimated relative abundances (%) obtained from densitometric curves are show for each band and period. Highest similarity hits (BLAST search) to sequenced DGGE bands are indicated on the right. Putative NirS-containing denitrifiers (green dots) and NirK-containing denitrifiers (orange) are indicted. Red dots highligth those bacteria species for which no known nitrite reductase gene is found.

Different alternatives were considered to explain the high q*nirS*/q16S rRNA ratio found. First, the presence of multiple copies of *nirS* gene in a single genome, *i.e. Thauera* sp., *Thiobacillus denitrificans*, *Dechloromonas aromatica* or *Magnetospirillum magneticum* (Etchebehere and Tiedje 2005, Jones *et al.,* 2008), was evaluated. Second, an overestimation of the *nirS* abundance due to *q*PCR bias was considered. However, this possibility was excluded after the examination of dissociation curves and the cloning of random *q*PCR products, which led us to confirm the specificity of the reaction. The observed prevalence of *nirS* over *nirK* denitrifiers may be the result of a selective enrichment of the former due to a putative enhanced capacity of electron harvesting by cytochrome *c* family mediators. This feature has been confirmed in *Geobacter sulfurreducens* ATCC 51573 by the analysis of a GSU3274 deletion mutant. GSU3274 is a gene coding for a putative cytochrome *c* family protein (Strycharz *et al.,* 2011).

Despite the occurrence of some limitations, such as the presence of multiple gene copies per genome and differences in specific activity (Jones *et al.,* 2008, Philippot 2002), *q*PCR analyses of functional genes provide significant data to infer community dynamics (Enwall *et al.,* 2010, Kandeler *et al.,* 2006). The ratio between the abundance of nitrite reductases and nitrous oxide reductase allowed us to estimate the potential to reduce completely nitrite to N_2. In the MFC and at the working conditions used in this study, the estimated N_2O accumulation significantly correlated (r^2=0.992) with the (q*nirK*+q*nirS*)/q*nosZ* ratio. Higher accumulations of N_2O were observed when nitrite was used as the electron acceptor, similarly to what has been described in other environments (Palmer *et al.,* 2012, Wunderlin *et al.,* 2012).

4.1.3 Community structure of denitrifying bacteria

A total of 619 valid sequences, 162 for *narG*, 158 for *nirS*, 156 for *nirK* and 143 for *nosZ*, were obtained from the cloning assay and used in the present study. Additionally, 28 high-quality sequences could be obtained for the *napA* gene. Although the cloning effort included the screening of 490 clones, the number of *napA* valid sequences was too low to obtain an adequate analysis of the *napA*-containing community, thus the results on this gene have only been included for comparison with other genes.

We are aware that primers used in this study are biased towards detecting mainly *Proteobacteria* (*nirK* and *nirS*) and Clade I *nosZ* gene, thus underestimating the actual diversity and abundance of nitrite and nitrous oxide reducers (Green *et al.,* 2012, Jones *et al.,* 2008, Jones *et al.,* 2013). However, previous works analysing the bacterial diversity on MFC cathodes have shown *Proteobacteria* as being particularly dominant in the biofilm

community thus minimizing the impact of primer biases (Chen *et al.,* 2008, Wrighton *et al.,* 2010).

The diversity and phylogenetic analyses were conducted on the basis of operational taxonomic units (OTUs). Rarefaction curves were constructed to visualise the saturation of the bacterial diversity (Figure 17). The curves obtained for *napA* and *nirS* genes did not approach saturation, which indicates that a large fraction of the species diversity remains to be analyzed. On the other hand, rarefaction curves for *nirK*, *narG* and *nosZ* approximate to a saturation indicating a reasonable description of the gene diversity was obtained.

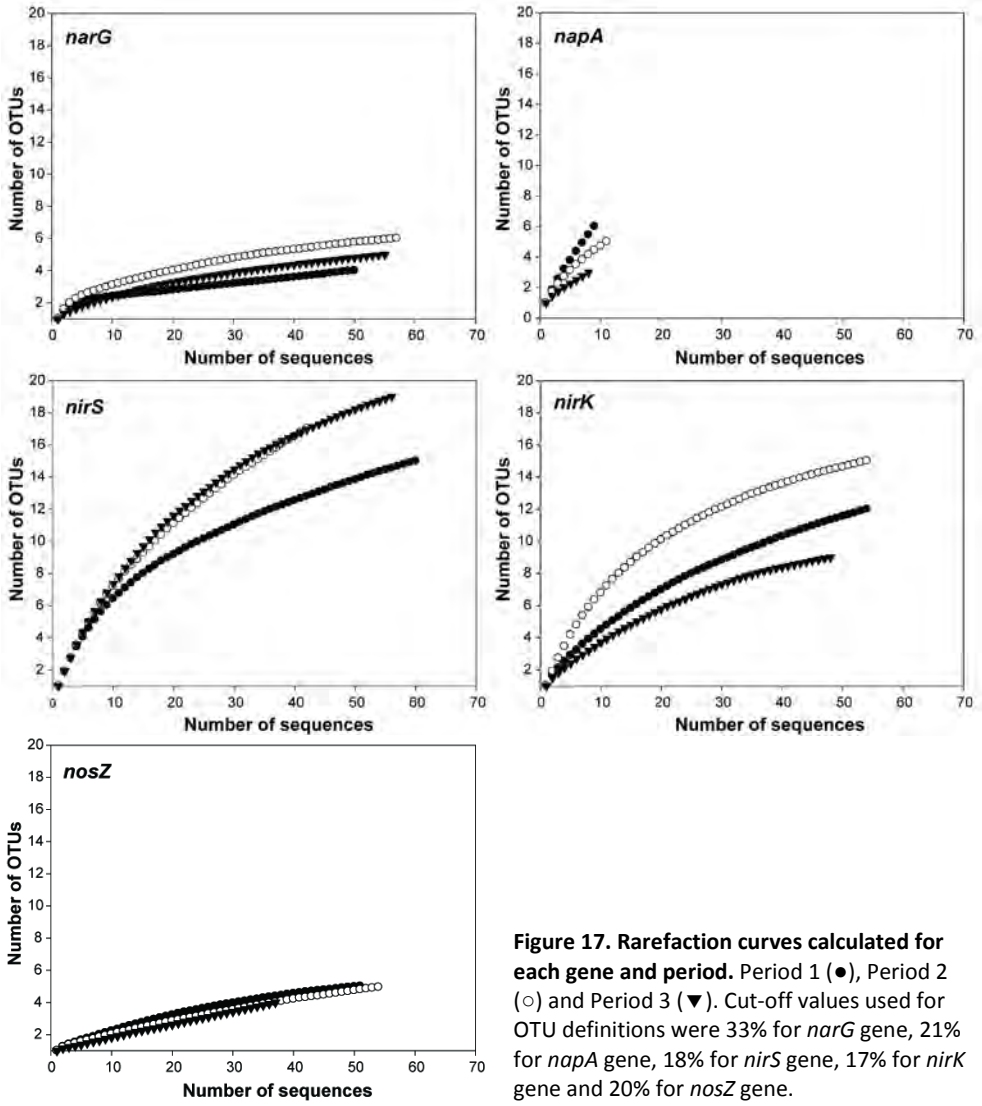


Figure 17. Rarefaction curves calculated for each gene and period. Period 1 (●), Period 2 (○) and Period 3 (▼). Cut-off values used for OTU definitions were 33% for *narG* gene, 21% for *napA* gene, 18% for *nirS* gene, 17% for *nirK* gene and 20% for *nosZ* gene.

Except for the *napA* gene, the coverage values for all samples were higher than 90%, (Table 19). The maximum richness (number of OTUs) was estimated according to Chao1 index and varied from 5 to 34 OTUs. Maximum values of expected richness were identified for *nirS*-containing denitrifiers. The higher complexity of the *nirS*-containing community was confirmed from estimates of the Shannon diversity index. In contrast, the lowest diversity was observed for nitrous oxide reductase (*nosZ*) under all conditions that were analysed.

Table 19. Alpha diversity estimates for each gene analyzed in the three periods. Mean values and SD are given for Shannon's diversity Index (H') according to rarefied samples.

Gene	**Period 1** (autotrophic with NO_3^-)				
	n	**S_{obs}**	**S_{Chao1}**	**C (%)**	**H'**
narG	50	4	5	96	0.84±0.19
napA	9	6	16	88.9	1.58±0.57
nirS	60	15	25.5	93.3	2.29±0.22
nirK	54	12	17	93.6	1.57±0.36
nosZ	51	5	5.5	98.0	0.57±0.30
Gene	**Period 2** (heterotrophic with NO_3^-)				
	n	**S_{obs}**	**S_{Chao1}**	**C (%)**	**H'**
narG	57	6	6.5	96.5	1.11±0.24
napA	11	5	6.5	90.9	1.29±0.53
nirS	42	16	34	92.9	2.45±0.26
nirK	54	15	18.3	96.3	2.40±0.22
nosZ	54	5	8	96.4	0.48±0.28
	Period 3 (autotrophic with NO_2^-)				
	n	**S_{obs}**	**S_{Chao1}**	**C (%)**	**H'**
narG	55	5	5.5	96.4	0.67±0.28
napA	8	3	4	75	0.74±0.59
nirS	56	19	22.5	96.6	2.63±0.23
nirK	48	9	9.6	100	1.19±0.38
nosZ	37	4	7	97.3	0.37±0.32

n: number of sequences used; **S_{obs}**,:observed richness; **S_{Chao1}**:expected richness; **C**: coverage; **H'**: Shannon diversity index.

narG gene sequences grouped into 11 different OTUs. OTU 1 and 2 were the most abundant, which comprised 76 and 53 sequences, respectively. OTU 1 was almost exclusively found during the autotrophic periods, whereas OTU 2 was predominantly found during the heterotrophic period (Figure 18).

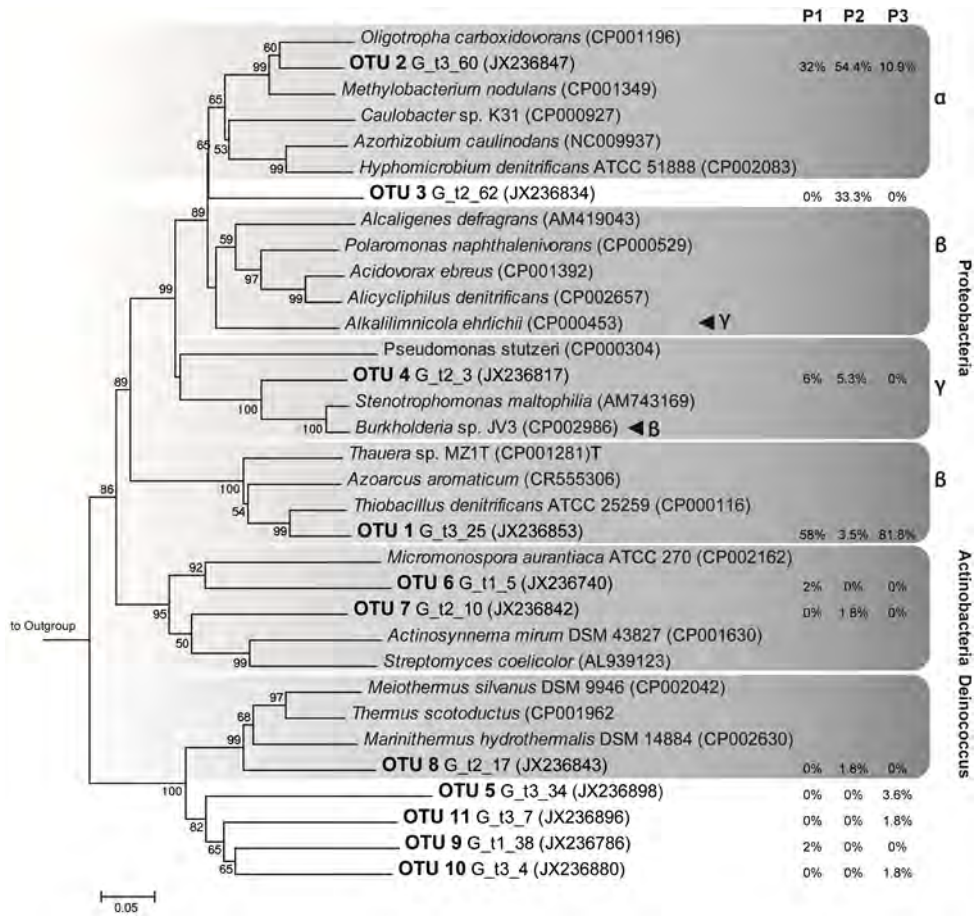


Figure 18. Neighbor-joining phylogenetic tree of amino acid deduced *narG* sequences. The representative sequences of each OTU and accession numbers of deposited sequences are shown. The percentage of sequences from the three conditions analysed are indicated (P1, autotrophic with nitrate; P2, heterotrophic with nitrate; P3, autotrophic with nitrite). The bootstrap values higher than 50% are shown at the nodes of the tree (10,000 replicates). *narG* gene of *Haloarcula marismortui* ATCC 43049 (NC006397) was used as outgroup.

Representative sequences from OTU 1 and 2 were approximately 81% similar to the betaproteobacterium *Thiobacillus denitrificans* and the alphaproteobacterium *Methylobacterium nodulans*, respectively. OTU 3 (19 sequences) was exclusively observed during the heterotrophic period and showed a low sequence similarity with most cultivated bacteria. Maximum similarities (73%) were observed with *Polaromonas naphthalenivorans* (Figure 18).

Sequences of the periplasmic nitrate reductase (*napA*) were distributed into 10 different OTUs (Figure 19). The most abundant OTU (10 sequences) was shared between autotrophic and heterotrophic periods and showed the highest sequence similarity (85%)

to *Dechlorosoma suillum*. OTU 2 was similar to *Sinorhizobium fredii* (78%) and was exclusively observed during the autotrophic period with nitrite.

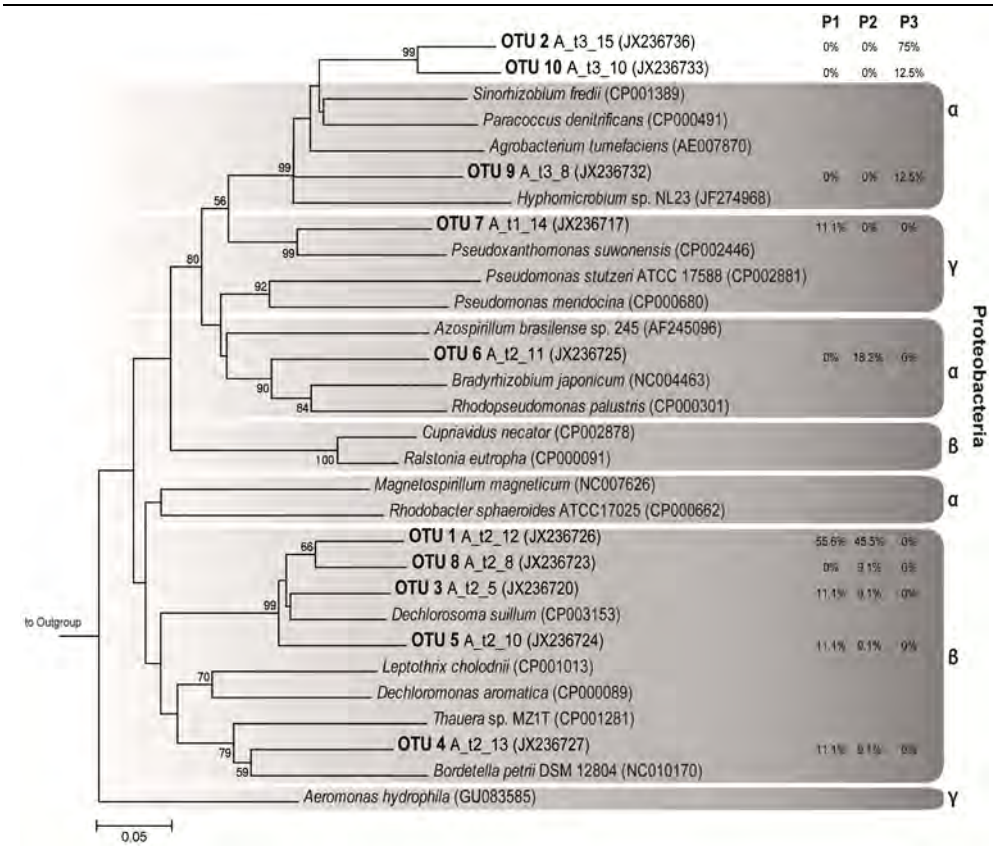


Figure 19. Neighbor-joining phylogenetic tree of amino acid deduced *napA* sequences. The representative sequences of each OTU and accession numbers of deposited sequences are shown. The percentage of sequences from the three conditions analysed are indicated next to each OTU, (P1, autotrophic with nitrate; P2, heterotrophic with nitrate; P3, autotrophic with nitrite). The bootstrap values higher than 50% are shown at the nodes of the tree (10,000 replicates). *napA* gene of *Escherichia coli* ATCC8739 (CP000946) was used as outgroup.

nirS sequences were assigned to 31 different OTUs without a clear dominance. The most abundant OTUs, 1, 2 and 3, were affiliated with *Betaproteobacteria* and showed relatively high similarities (>84%) with *Dechlorosomonas* sp., *Thauera* sp. and *Cupriavidus pauculus*, respectively. OTU 1 was exclusively observed under nitrate feeding (Figure 20) whereas OTUs 2 and 3 were found at all operating conditions. OTUs 4, 8 and 11 were observed almost exclusively during nitrite feeding conditions. According to BLAST searches with reference genomic sequences database, the highest similarities of these sequences were found with alphaproteobacterium *Paracoccus denitrificans* (OTU 8) and gammaproteobacterium *Rhodanobacter* sp. (OTUs 4 and 11).

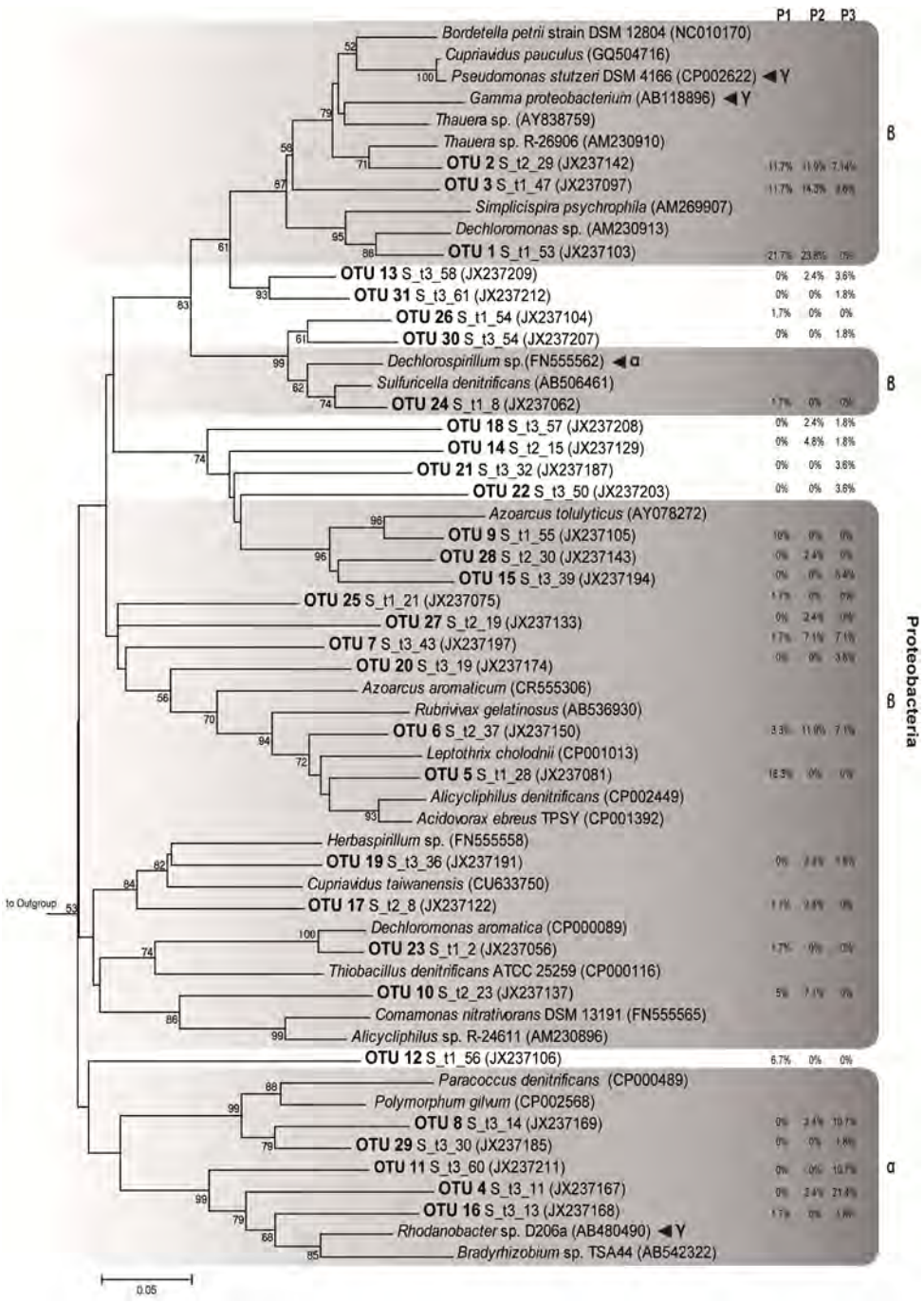


Figure 20. Neighbor-joining phylogenetic tree of amino acid deduced *nirS* sequences. The representative sequences of each OTU and accession numbers of deposited sequences are shown. The percentage of sequences from the three conditions analysed are indicated next to each OTU (P1, autotrophic with nitrate; P2, heterotrophic with nitrate; P3, autotrophic with nitrite). The bootstrap values higher than 50% at the nodes of the tree (10,000 replicates). *nirS* gene of *Rhodothermus marinus* DSM4252 (NC013501) was used as outgroup.

The gene encoding the copper-containing nitrite reductase, *nirK,* showed a different distribution between samples. Two out of a total of 22 OTUs were clearly dominant (up to 70 sequences) during autotrophic periods supplemented with either nitrate or nitrite.

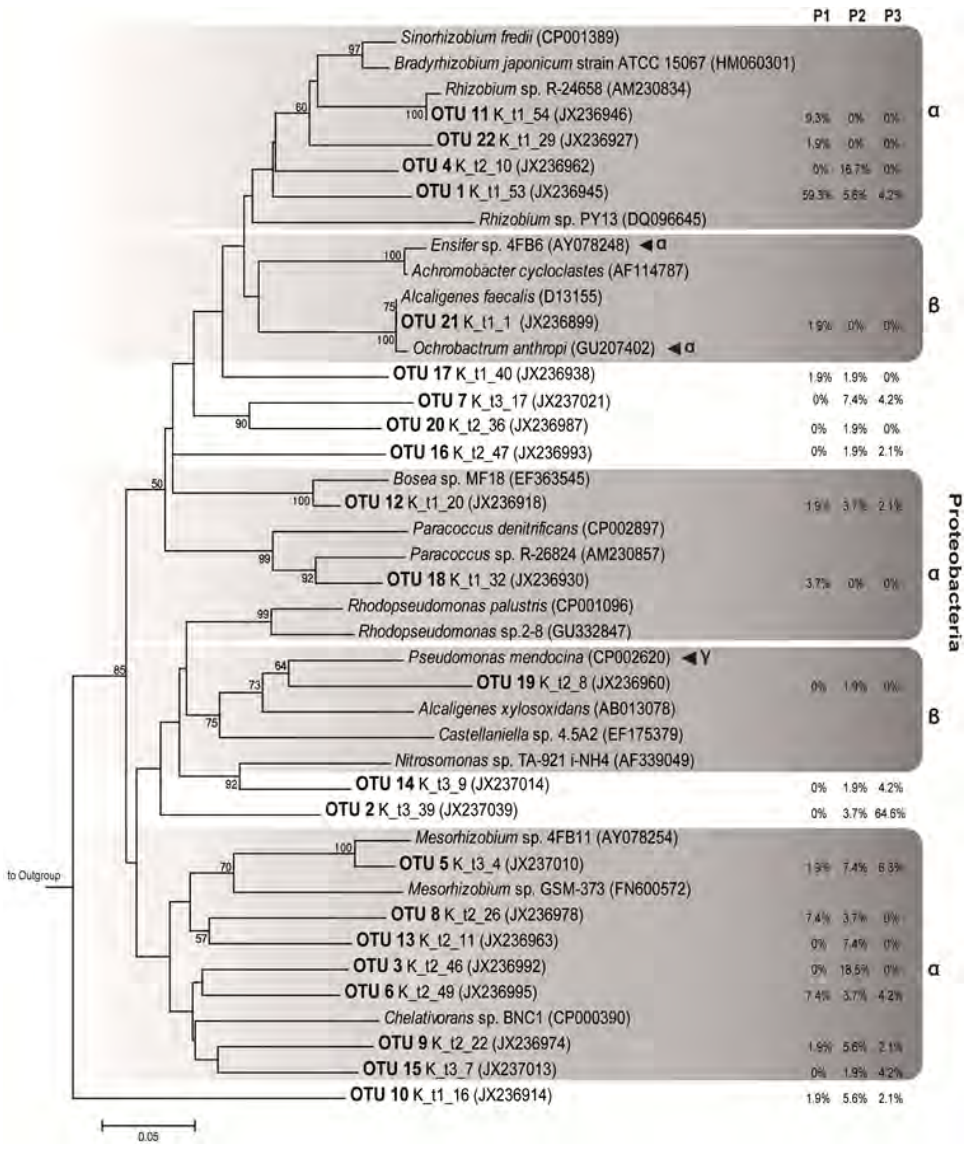


Figure 21. Neighbor-joining phylogenetic tree of amino acid deduced *nirK* sequences. The representative sequences of each OTU and accession numbers of deposited sequences are shown. The percentage of sequences from the three conditions analysed are indicated next to each OTU (P1, autotrophic with nitrate; P2, heterotrophic with nitrate; P3, autotrophic with nitrite). The bootstrap values higher than 50% are shown at the nodes of the tree (10,000 replicates). *nirK* gene of *Nitrosomonas* sp. C-56 (AF339044) was used as outgroup.

The representative sequences of OTUs 1 and 2 were similar to *Sinorhizobium fredii* (84%) and *Rhodopseudomonas palustris* (85%), respectively (Figure 21). In contrast,

during the heterotrophic period, *nirK* sequences distributed into 18 different OTUs. The most abundant OTU (82% similar to *Mesorhizobium* sp. 4FB11) comprised only 10 sequences.

The OTU distribution of *nosZ* genes revealed a relatively homogenous community for all periods. Almost 90% of sequences were grouped into a single OTU with a relatively high similarity (85%) to the predicted nitrous oxide reductase gene of *Oligotropha carboxidovorans* (Figure 22).

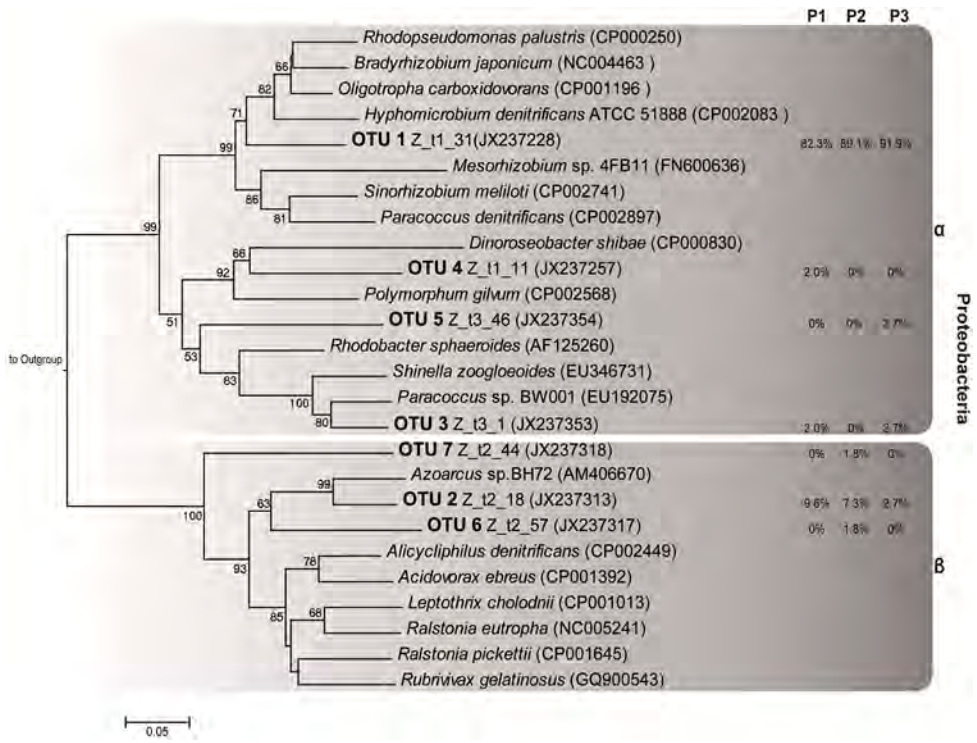


Figure 22. Neighbor-joining phylogenetic tree of amino acid deduced *nosZ* sequences. The representative sequences of each OTU and accession numbers of deposited sequences are shown. The percentage of sequences from the three conditions analysed are indicated next to each OTU (P1, autotrophic with nitrate; P2, heterotrophic with nitrate; P3, autotrophic with nitrite). The bootstrap values higher than 50% are shown at the nodes of the tree (10,000 replicates). *nosZ* gene of *Haloarcula marismortui* ATCC 43049 (AY596297) was used as outgroup.

The MFC set-up provided excellent conditions to assess how changes in the main electron acceptors (nitrate *vs.* nitrite) and donors (cathode *vs.* organic matter) affected the composition of the denitrifier community and how it was related to the MFC performance. These changes were observed into the OTU classifications identification, revealing differences into community composition according to the operational conditions applied (Figure 23).

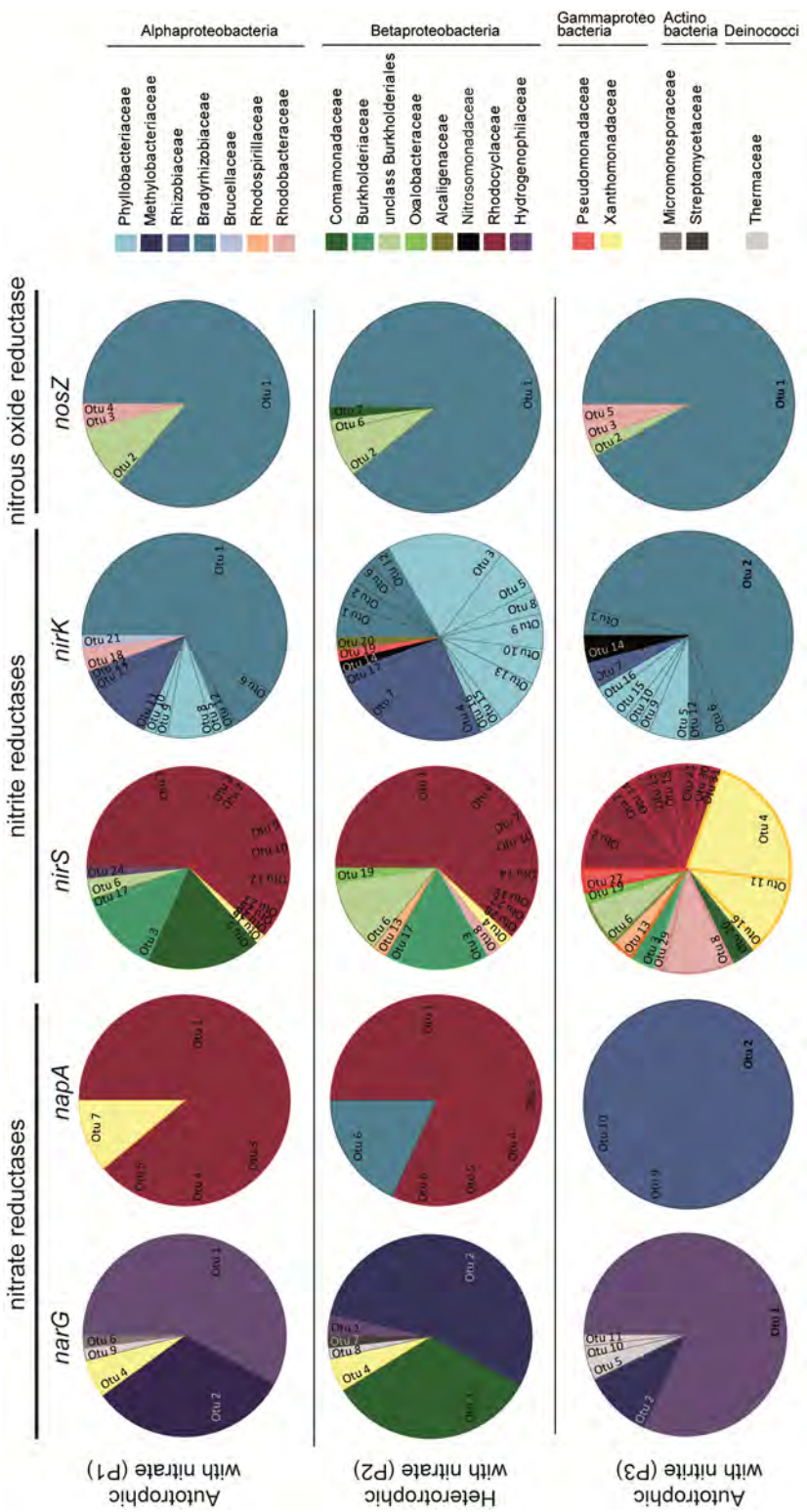


Figure 23. Pie charts showing the community composition of denitrifiers according to functional gene similarities. Five denitrification genes, *narG*, *napA*, *nirS*, *nirK* and *nosZ*, at three different feeding periods in the cathode of a MFC are indicated. To facilitate the comparison between OTU distributions of bacterial communities, colors have been used as an indication of bacterial families and orders. Assignations have been made according to sequence similarities and may not reflect the exact phylogeny of the bacteria present.

The *narG*-containing community composition was highly affected by the presence of organic matter, changing the dominance of *Hydrogenophilaceae* family in autotrophic conditions for a community dominated by members of *Methylobacteriaceae* and *Comamonadaceae* families. In contrast, periplasmatic nitrate reductase (*napA*) bacterial community composition was more affected by the electron acceptor (nitrate or nitrite) than the type of metabolism, autotrophic or heterotrophic. The feeding of nitrite, produced a radical change in the composition of the community, which changed from a community composed basically by *Rhodocyclaceae* members to a community completely dominated by *Rhizobiaceae*. However, these results probably show only a minor part of the community complexity since the number of *napA* sequences obtained in this study was rather low.

The abundance of *narG* and *napA* containing nitrate reducers increased during nitrate addition thus showing the importance of these communities in the first reduction step. Changes in the abundance were accompanied by changes in the OTU composition. A large number of the retrieved *narG* sequences during autotrophic treatments supplemented with either nitrate or nitrite showed a high similarity to *narG* of the obligate chemolithoautotrophic bacterium *Thiobacillus denitrificans*. *T. denitrificans* has an optimal pH for growth around 7.5-8.0 and high denitrification rates (0.78 g NO_3^- g $cell^{-1} \cdot h^{-1}$) (Claus and Kutzner 1985), which fall in the same range of those estimated in the MFC according to bacterial abundances. During the heterotrophic treatment, in contrast, analyses of the *narG* containing community revealed a higher relative abundance of sequences related to *Methylobacterium nodulans*, a bacterium able to grow using one carbon compounds and reducing nitrate to nitrite (Jourand *et al.,* 2004), and *Polaromonas naphthalenivorans,* a facultative chemolitotroph isolated from polluted habitats (Yagi *et al.,* 2009). These two bacteria partially substituted obligate autotrophs during heterotrophic conditions.

A similar trend was observed for both nitrite reductases (*nirS* and *nirK*) community compositions. The composition of *nirS* community was more affected by the electron acceptor used contrasting *nirK*-containing community, which changed according to the presence of organic matter. During the period in which nitrite was used as an electron acceptor, *Xanthomonadaceae* members were enriched in detriment to dominant *Rhodocyclaceae* members. In a similar way, for the *nirK* community, the presence of organic matter favoured the development of *Phyllobacteraceae* which became more abundant in contrast to autotrophic periods dominated by *Bradyrhizobiaceae*.

The *nirS*-containing community showed highest similarities when nitrate was used as an electron acceptor despite the addition of organic matter. *nirS* sequences similar to those

found in members of the family *Rodocyclaceae* were the most abundant. *Rodocyclaceae* have been found as the dominant bacterial population in industrial WWTPs (Heylen *et al.,* 2006b). OTU 6, with a high similarity to *Rubrivivax gelatinosus nirS* gene, accumulated during heterotrophic conditions. *Rubrivivax gelatinosus* has been described as an obligate nitrite reducer able to use different carbon sources (Nagashima *et al.,* 2012). In contrast, when nitrite was used as the first electron acceptor, *nirS* sequences similar to those found in *Gammaproteobacteria*, in particular *Rhodanobacter* sp., were the most abundant. A recent analysis of complete genome sequences of six *Rhodanobacter* strains isolated from soils have revealed that at least three of them lack the ability to reduce nitrate (Kostka *et al.,* 2012). Similarly to what has been observed for the *nirS* gene, bacteria enriched when nitrite was fed in MFC, suggest the exclusive use of nitrite as electron acceptor. This is the case for OTU 2 (85% similar to *nirK* sequence of *Rhodopseudomonas palustris*). *Rhodopseudomonas palustris* lacks an ortholog of a dissimilatory nitrate reductase in its genome, suggesting that nitrate reduction cannot be done in this bacterium (Lee *et al.,* 2002). The addition of nitrite as initial electron acceptor impacted the composition of *nirS*- and *nirK*-type denitrifiers in the MFC, and possibly caused an enrichment of selected obligate nitrite reducers in view of sequence similarities with the detected functional genes.

Contrasting to the previous genes, the *nosZ*-containing community remained almost invariable during all conditions, which were dominated in all conditions by members of the same family, *Bradyrhizobiaceae*. The community composition obtained for this gene, was the only one that remained stable during all the conditions tested. Sequences with a high similarity to the *nosZ* gene of *Oligotropha carboxidovorans*, a carboxidotrophic bacterium (Volland *et al.,* 2011), clearly dominated the *nosZ* community. The presence of *Oligotropha* like *nosZ* sequences has also been detected as major components of the nitrous oxide reducing communities in samples of acidic peat soils (Palmer *et al.,* 2012) and in the cathode of a denitrifying MFC (Puig *et al.,* 2011). Moreover, gene abundances during autotrophic conditions supported the idea of *nosZ* community minimally affected by the initial electron acceptor.

The significance of the observed differences between the microbial communities under different operating conditions in the MFC, was analysed using pair-wise weighted UniFrac analysis for the 5 molecular markers (Table 20).

Table 20. UniFrac distance scores and *p* values. Denitrifier communities were analyzed according to different functional genes and the three periods analysed in the MFC.

Gene	Period 1 *vs*. Period 2	Period 1 *vs*. Period 3	Period 2 *vs*. Period 3
narG	0.25 ns	0.13 ns	0.36 ns
napA	0.07 ns	0.41 *	0.40 *
nirS	0.10 ns	0.25 *	0.22 ***
nirK	0.18 ns	0.22 ns	0.16 ns
nosZ	0.02 ns	0.04 ns	0.04 ns

The Unifrac values were calculated using amino acid deduced sequences and phylogenetic trees.
ns: not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The UniFrac values confirmed the observed differences in the community composition, although statistically significant differences were only observed for *napA* and *nirS* containing communities. In both cases, significant differences were observed for the community of the autotrophic with nitrite period (period 3) compared to the other two treatments. Low UniFrac values were obtained in all pair-wise comparisons for the *nosZ* community indicating a highly similar and stable community was present in all operating periods.

The community composition of the different denitrifying genes revealed differences at the family level, indicating that the presence of a single bacterial species containing all the denitrifying genes was not likely to occur. Members of the same family, *Rhodocyclaceae*, were identified as dominant for *napA* and *nirS* genes, these genes are responsible of nitrate and nitrite reduction, respectively. Both communities were also affected by the presence of nitrite as an electron acceptor favouring the development of other bacterial families under this condition. Members of *Bradyrhizobiaceae* were identified as dominant in *nirK* and *nosZ* communities, these genes are responsible of nitrite and nitrous oxide reduction, respectively. Although the dominant populations for *narG* and *nirK* genes were not the same, both were affected in a similar way.

In conclusion, the cathodic biofilm of the MFC was dominated by *nirS*-type denitrifiers at all conditions tested and its abundance relative to nitrous oxide reducers highly correlated with N_2O emissions. The denitrifying bacterial communities identified affected the electrochemical performance increasing the current density for about 25% in autotrophic conditions. Also the suspected relevant players in nitrate and nitrite reduction have been identified on the basis of functional gene similarities. Their relative dominance at each period was highly affected by the changes of the electron acceptor or electron donors. Contrarily, the *nosZ* community remained almost invariable during all periods tested. Most *nosZ* sequences showed a high similarity to nosZ gene of *Oligotropha carboxidovorans*, suggesting that they may have an active and preponderant role in electron harvesting in the cathode surface. This may raise new questions, such as

which mechanisms are involved in electron transfer and what the location of *O. carboxidovorans*-like bacteria in the biofilm is, that will be investigated in the near future.

4.2 Enrichment and isolation of *nosZ*-containing bacteria from Microbial Fuel Cells

Autotrophic denitrifiers are supposed to enrich in cathodes of denitrifying MFCs when nitrate is supplied as the solely electron acceptor and no organic matter is added (Wrighton *et al.,* 2010, Xing *et al.,* 2010). Our main goal was to obtain isolates with *nosZ* sequences similar to those found in the dominant *nosZ* communitiy identified in denitrifying biocathodes (chapter 4.1), and characterize them physiologically. The use of culture-dependent methods are mandatory, and are essential to test hypotheses formulated from culture independent methods (Donachie *et al.,* 2007). Studies with pure cultures will help in establishing the relationship between function and structure of microbial communities, which is essential to postulate new hypotheses in microbial ecology (Dahllof 2002, Nichols 2007). In this study we aimed at deciphering the electric capacities of some members of the denitrifying community isolated in pure culture.

Samples obtained from anodes and cathodes of different MFCs were characterized by PCR-DGGE of the *nosZ* gene, and samples selected to start enrichment of autotrophic denitrifiers. Autotrophic conditions with nitrate were used in the enrichment process. Denitrifiers in a denitrifying cathode are supposed to be able to use electrode-derived electrons to catalyze nitrate reduction (Virdis *et al.,* 2008). However, in the absence of an electrode supplying electrons for growth, inorganic electron donors for chemolithoautotrophic growth need to be used.

Different inorganic compounds can be used as a source of energy by autotrophic denitrifiers, some can use hydrogen, iron (II) or sulphur reduced forms (e.g. thiosulphate, sulphide or sulphur) as electron donors (Weber *et al.,* 2006). The autotrophic denitrification can be conducted by two different types of denitrifiers, referred to as hydrogen or sulphur-based denitrifiers, although no phylogenetic relationship exists within each group (Park and Yoo 2009). No specific mechanism for extracellular electron transfer (EET) has been described for electrotrophic bacteria yet, although the use of *c*-type cytochromes and copper containing oxidoreductases similar of those involved in the respiration of iron (III) or sulphur compounds have been proposed to mediate the entrance of electrons inside the cell (Holmes *et al*., 2008, Yamanaka and Fukumori 1995, Yarzábal *et al*., 2004). Moreover, periplasmic hydrogenases have been speculated to participate in the creation of networks interconnecting different electron active enzymes in outer membrane to capture the electrons (Van Ommen Kloeke *et al.,* 1995). In the present study we used sulphide, thiosulphate and hydrogen as inorganic electron donors,

not only for the suggested relation of these compounds with EET mechanism, but also for their high diffusivity in the liquid media. Additionally, long incubation times were used to favour the selection of microorganisms with lower growth rates like autotrophic bacteria (Kaeberlein *et al.,* 2002).

4.2.1 Community structure of *nosZ*-containing bacteria in anodes and cathodes of different MFCs

The community composition of denitrifying bacteria in the anode and cathode of different MFCs was analyzed by PCR-DGGE targeting the nitrous oxide reductase coding gene (*nosZ*). DGGE fingerprints varied not only according to sample origin but also to operational conditions. DGGE profiles showed a rather low complexity, and changes between samples were limited to variations in a few numbers of bands (Figure 24). As expected, profiles showed differences between anode and cathode compartments. Samples from the anode of Mfc1, Mfc2 and Mfc4 showed a greater number of bands (i.e. species richness) compared to the cathodes of Mfc3 and Mfc4, in which a single band was obtained. The cathode samples presented a much simpler *nosZ*-containing bacterial community, which can be related to the use of strictly autotrophic conditions with nitrate, making the conditions more restrictive and leading to the enrichment of a dominant population (Park *et al.,* 2006). It is interesting to note that *nosZ* containing bacteria were detected at both electrode compartments despite the use of specific conditions for denitrification (limited oxygen availability and presence of nitrate). Most denitrifiers are facultative microorganisms able to grow using different metabolisms such as aerobic respiration and/or fermentation (Knowles 1982).

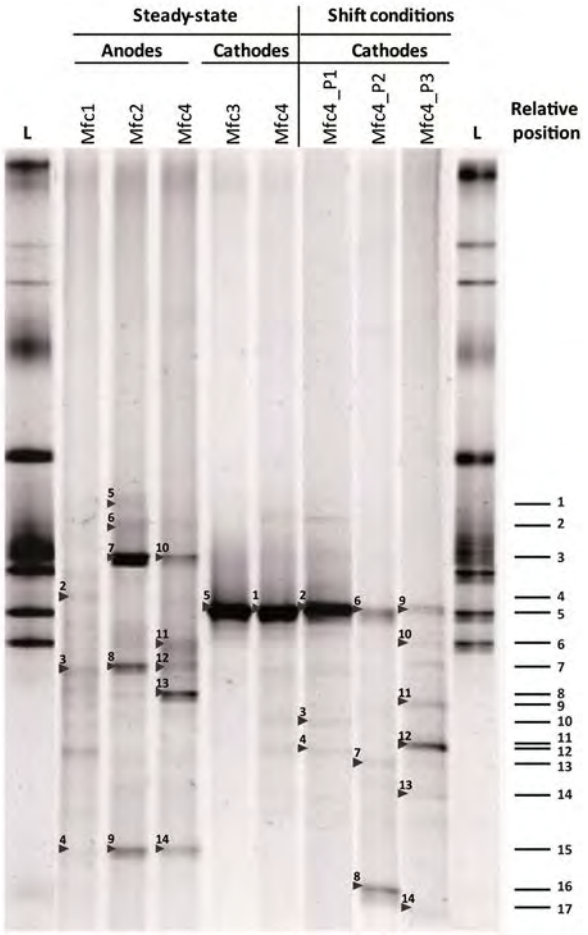


Figure 24. **DGGE patterns of *nosZ* gene from biofilm samples.** Samples from steady-state conditions of Mfc1, Mfc2 and Mfc4 anodes, and Mfc3 and Mfc4 cathodes are on the left. Samples from the two shift experiments in which feed solution was changed: Mfc4_P1, autotrophic conditions with nitrate; Mfc4_P2, heterotrophic conditions with nitrate; and Mfc4_P3, autotrophic conditions with nitrite, are on the right handside of the image. Sequenced bands are indicated with an arrow head and number. Band relative positions are indicated on the right side.

In addition, DGGE patterns showed some differences on the intensity of some bands. Although no direct relationship can be established between band intensity and the relative bacterial abundance in the original sample (Muyzer *et al.,* 1993), it is generally assumed that most intense bands derive from most abundant bacteria.

Changes in the community composition in the cathodes could be related to the different feeding conditions applied to the MFC. The PCR-DGGE characterization of samples from shift experiments (chapter 4.1) allowed the evaluation of how trophic conditions (heterotrophic or autotrophic) and electron acceptors (nitrate or nitrite) affected microbial communities based on the *nosZ* gene. In the first shift experiment (Period 2), feed was changed from a nitrate enriched synthetic wastewater to a partially treated urban wastewater coming from the Mfc1 anode effluent. The main difference in the latter conditions is the presence of organic matter. Under these operational conditions,

band Ca_3 (Mfc4_P1) decreased in intensity and two new bands appeared (Ca_7 and Ca_8 in Mfc4_P2).

In the second shift experiment (Period 3), nitrate was replaced by nitrite as the major electron acceptor. DGGE profiles changed significantly, leading to a more complex community in the case of nitrite (Mfc4_P3). Changes in the community structure of cathode denitrifiers reflected an effect of the feeding regime, suggesting its active role in electron utilization.

Twenty-eight bands from seventeen positions were excised from DGGE gels, re-amplified by PCR and *nosZ* genes were sequenced. Most sequences yielded low similarity values with previously published sequences (from 81% to 92) (Table 21). All sequences were classified as *Alphaproteobacteria* except those retrieved from bands An_2, An_4, An_9, An_14, Ca_3 and Ca_4 that showed a closer similarity to *Betaproteobacteria*. The presence of *Proteobacteria* and their predominance in the cathodes of denitrifying MFCs has been detected in previous studies (Knowles 1982, Park *et al.,* 2006, Wrighton *et al.,* 2010).

Sequences similar to those found in *Rhodopseudomonas palustris*, *Oligotropha carboxidovorans* strain OM5 (CP002826) and *Alicycliphilus denitrificans* BC (CP002449) were the only ones found simultaneously both in anodes and cathodes. *Oligotropha carboxidovorans* and *Alicycliphilus denitrificans*, have been shown to grow using different organic compounds as energy sources (Mechichi *et al.,* 2003, Oosterkamp *et al.,* 2011, Paul *et al.,* 2008). Additionally, *O. carboxidovorans* is able to use CO, CO_2 and H_2 as the sole carbon and reducing equivalents source for chemolithoautotrophic growth (Paul *et al.,* 2008). In turn, *Rhodopseudomonas palustris* is a versatile photosynthetic bacterium which can growth using phototrophic or chemotrophic energy, combined with either autotrophic or heterotrophic lifestyles (Larimer *et al.,* 2004). Additionally, *Rhodopseudomonas palustris* has been described as a electrogenic microorganism and implicated in electricity production via anaerobic respiration (Xing *et al.*, 2008).

All other identified phylotypes, i.e. *Hyphomicrobium nitrativorans*, *Mesorhizobium* sp. *Azoarcus* sp. and *Thiobacillus denitrificans*, were specifically found in either the anode or the cathode samples, showing some specificity.

Table 21. Phylogenetic identification of partial *nosZ* sequences from DGGE bands. The blast search was restricted to *nosZ* sequences from previously cultivated microorganisms (*ref. genomic database* in NCBIBlast).

Bands	Bands position	Nearest relative type strains or cultured representatives	Sequence similarity
		Alphaproteobacteria	
Ca_1, 2, 5, 6 and 9	5	*Hyphomicrobium nitrativorans* NL23 (CP006912)	88%
Ca_7	13	*Mesorhizobium* sp. 4FB11 (FN600636)	90%
Ca_8 and 14	16 and 17	*Mesorhizobium* sp. 4FB11 (FN600636)	92%
Ca_13	14	*Mesorhizobium* sp. 4FB11 (FN600636)	91%
An_3	7	*Oligotropha carboxidovorans* OM5 (CP002826)	92%
An_11, 12 and 13	6, 7 and 8	*Oligotropha carboxidovorans* OM5 (CP002826)	90%
Ca_10	6	*Oligotropha carboxidovorans* OM5 (CP002826)	90%
Ca_11	9	*Oligotropha carboxidovorans* OM5 (CP002826)	89%
An_5, 6, 7 and 10	1, 2 and 3	*Rhodopseudomonas palustris* DX-1(CP002826)	99%
An_8	7	*Rhodopseudomonas palustris* HaA2 (CP000250)	91%
Ca_12	11	*Rhodopseudomonas palustris* BisA53 (CP000463)	92%
		Betaproteobacteria	
An_2	4	*Alicycliphilus denitrificans* BC (CP002449)	81%
Ca_3	10	*Alicycliphilus denitrificans* BC (CP002449)	89%
An_9	15	*Azoarcus* sp. KH32C (AP012304)	86%
An_4 and 14	15	*Thiobacillus denitrificans* ATCC 25259 (EU346731)	86%
Ca_4	12	*Rhodoferax ferrireducens* T118 (CP000267)	88%

The sequence retrieved from most intense band found in all cathode samples (position 5, bands Ca_1, Ca_2, Ca_5, Ca_6 and Ca_9) clusters to *Hyphomicrobium nitrativorans* (88% according to sequence similarity), although almost similar blast search indicators are found with *Oligotropha carboxidovorans* OM5 (CP002826) *nosZ* sequence. Before the publication of the complete genome of *Hyphomicrobium nitrativorans* (Martineau *et al.,* 2013), the most prominent nitrous oxide reducer found in cathodes was identified as *Oligotropha carboxidovorans* (Puig *et al.,* 2011, Vilar-Sanz *et al.,* 2013), although according to the updated comparisons made here, this assignation needs revision.

The analysis of *nosZ* genes highlighted the relevant bacterial groups involved in the last step of the denitrification process. For most identified species, almost identical sequences were retrieved both from anode and cathode samples revealing the facultative trait of denitrifiers present in MFCs. Despite the presence of *nosZ*-containing denitrifiers in the anode of MFC we cannot be sure if these bacteria participate in current production as expected in normal MFC functioning (Logan 2009). Electrogenesis, or the ability to release electrons by anode-respiring bacteria (ARB), is a physiological trait observed in several functional groups, such as metal-reducers, sulphate-reducers, and nitrate-reducers, but is highly dependent on the species (or even the strain) considered (Rabaey *et al.,* 2004). The use of wastewater in the anodes promotes the presence not only of ARB

but also of fermentative bacteria, not necessarily involved in electrogenesis (Park *et al.,* 2001, Zhang *et al.,* 2006). Besides, complex and highly divergent bacterial communities integrated with members harboring different metabolic capacities can be established as a result of inter-species competition for the utilization of organic substrates or other complex cell-to-cell synergistic relationships (Jung and Regan 2007, Parameswaran *et al.,* 2009, Xing *et al.,* 2009).

The presence of similar denitrifying bacteria in both anodes and cathodes of the studied MFCs provided a good source to obtain enrichments for further isolation of potentially autotrophic denitrifiers.

4.2.2 Enrichment of chemolithoautotrophic bacteria from MFCs

Enrichments of autotrophic denitrifying bacteria were performed using two different samples, the biofilm sample from Mfc3 cathode (denitrifying MFC), and detached bacterial biofilm from Mfc1 anode. Samples were chosen to increase the possibility to obtain pure cultures of denitrifying bacteria from any of the two compartments. Samples were serially diluted and plated onto mineral medium. All plates were incubated using anaerobic conditions and three electron donors were used, sulphide, thiosulphate and hydrogen. Viable bacteria in the used media ranged from 2.85 to 1.78 x 10^4 cfu·mL^{-1} and from 9.4 to 7.91 x 10^3 cfu·mL^{-1} in the Mfc1 anode or the Mfc3 cathode, respectively.

A molecular inspection of the types of bacteria present on agar plates was done using plate wash PCR (PW-PCR) and DGGE fingerprinting (Stevenson *et al.,* 2004). As predicted from the results of the original bacterial communities, PWPCR-DGGE fingerprints of the *nosZ* gene showed that a higher number of bands were obtained from Mfc1 anode compared to Mfc3 cathode (Figure 2).

The maximum number of bands obtained from cathode enrichments occurred in the plates where sulphide was used as the electron donor. Differences in the number and positions of DGGE-bands occurred in relation to the electron donor for the Mfc1 anode and only two of them (positions 12 and 17) were found in all samples independently of the electron donor used. Similar numbers of bands were obtained when hydrogen (11 bands) and sulphide (12) were used as electron donors, but only one band (position 2) was common for the two treatments. The number of DGGE bands increased to 19 if thiosulphate was used as an electron donor. Lower number of bands was obtained for the Mfc3 cathode enrichments, two of them were common in the three electron donors used (positions 9 and 27). Two bands were found at both anode and cathode enrichments (positions 2 and 27), although not with all the electron donors used. These results

indicate that the origin of the inoculum had a more pronounced effect on the bacterial richness than the electron donor used.

None of DGGE bands found in cathode enrichments corresponded to the most abundant band found in the original sample. It could be due to the fact that culture-depend methods may produce a bias on microorganisms that grow on agar plates due to the selective character of media and cultivation conditions used (Ellis *et al.,* 2003, Madigan *et al.,* 2004). Nevertheless, bands at the same position, 12, as the most abundant DGGE-band identified in cathodes were retrieved from anode enrichments.

Different electron donors such as sulphide, thiosulphate and hydrogen were chosen to mimic the electron supply at the electrode surface. Although autotrophic denitrifiers have been shown to be able to use a variety of electron donors, there are two types widely studied: Hydrogen- and Sulphur-based dependent denitrifiers, composed by different bacterial groups which are able to conduct each type of metabolism (Park and Yoo 2009). Hydrogen oxidizing bacteria (HOB) use hydrogen, one of the most thermodynamically favourable electron donors for nitrate-based respiration (Benedict *et al.,* 1997, Park and Yoo 2009). Alternatively, sulphur-based autotrophic denitrification is catalyzed by common soil bacteria, which use inorganic reduced sulphur compounds for nitrate reduction (Koenig and Liu 2001, Park and Yoo 2009, Sengupta and Ergas 2006, Zhang and Lampe 1999).

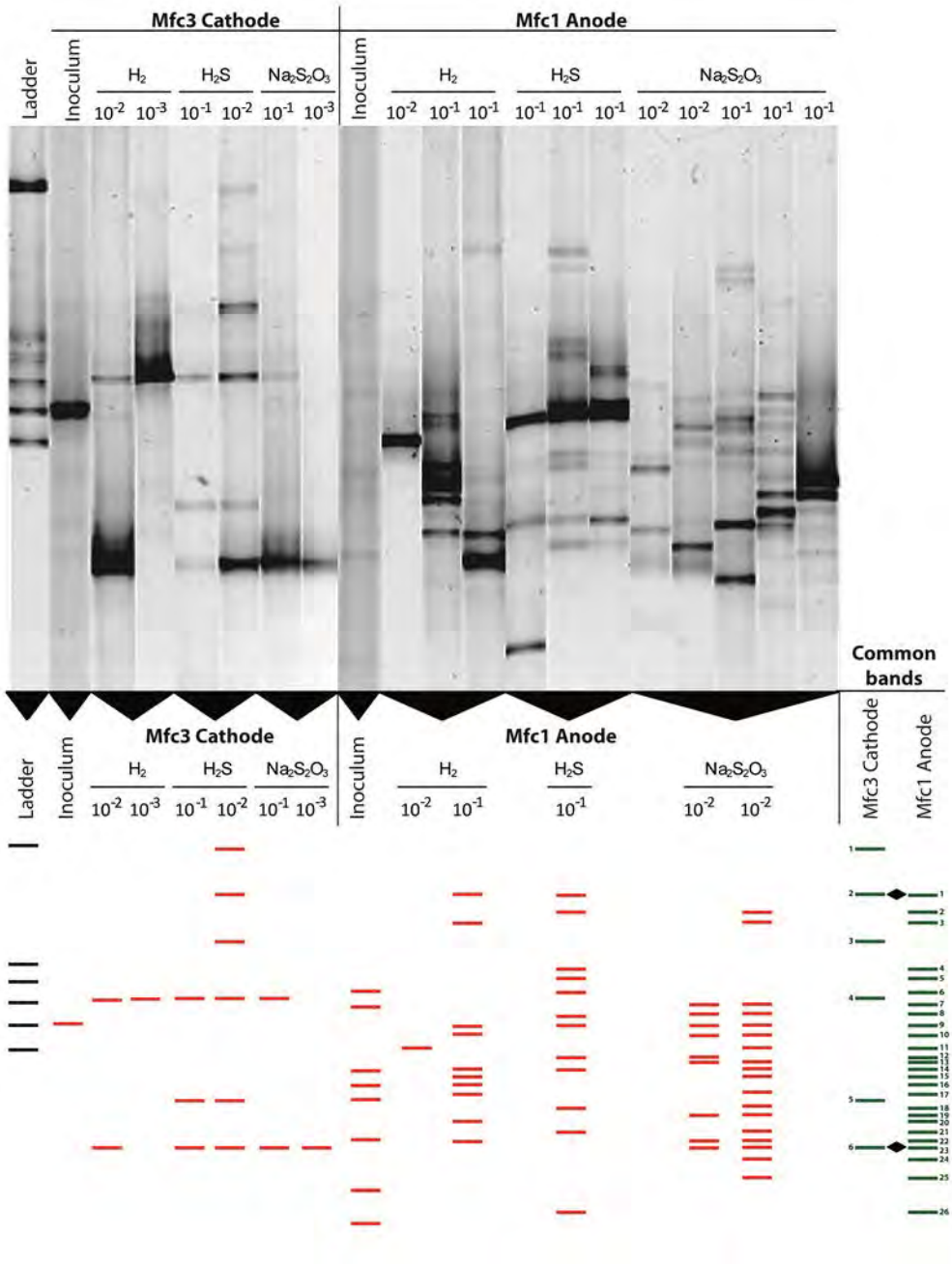


Figure 25. DGGE fingerprint of PWPCR of *nosZ* gene. Samples from different dilutions and enriched with different electron donors are indicated at each lane. Relative position of the different bands identified is represented (down right) as green lines and numbers. Bands found both in agar plates and inoculum samples, are indicated with the black diamonds.

A selective enrichment from the above analysed agar plates was continued using 96-well plates and selected inorganic liquid media. A total of 991 colonies were chosen according to morphological differences and abundance and were re-inoculated (Table 22).

Table 22. Number of colonies picked into enrichment wells. Colonies inoculated into 96-well plates with a fresh liquid medium containing different electron donors.

Electron donors	Inoculums from Mfc1			Inoculums from Mfc3		
	H_2S	$Na_2S_2O_3$	H_2	H_2S	$Na_2S_2O_3$	H_2
Dilution 10^{-1}	251	185	165	25	59	-
Dilution 10^{-2}	33	87	19	26	32	66
Dilution 10^{-3}	4	4	3	3	4	25

Mfc1: Air cathode MFC, **Mfc3**: Denitrifying MFC
H_2S: sulphide, **$Na_2S_2O_3$**: thiosulphate, **H_2**: hydrogen

Microwell plates were incubated for 30 days at room temperature, reinoculated in fresh media and incubated for an additional 30 days period. After this period, 731 out of 991 wells showed dense bacterial accumulations at the bottom of wells were screened for the presence of the *nosZ* gene by PCR. Positive *nosZ* amplification was obtained in 240 (34.2%) samples, 197 from the anode compartment and 53 from the cathode (Table 23). In the anode, similar percentages of positive *nosZ*-containing enrichments were observed for the different electron donors and, in this sense, no relevant differences between the incubation conditions were detected. These results agree with previous studies that showed that both, soluble sulphur compounds and hydrogen can sustain autotrophic denitrification (Batchelor and Lawrence 1978, Batchelor and Lawrence 1986, Park and Yoo 2009, Sengupta and Ergas 2006).

Table 23. Enrichments screened looking for *nosZ*-containing bacteria. Number of liquid enrichments screened for the presence of *nosZ* gene and the number of positive enrichments.

Electron donors	Inoculum origins	Screened enrichments	*nosZ* positives
Hydrogen	Mf1 Anode	187	64
	Mfc3 Cathode	39	11
Sulphide	Mfc1 Anode	288	89
	Mfc3 Cathode	45	33
Thiosulfate	Mfc1 Anode	133	44
	Mfc3 Cathode	39	9

From cathode enrichments, 43.1% of the screened wells resulted in positive *nosZ* PCR products. A higher amount (73.3%) of putative denitrifiers was obtained when sulphide was used as electron donor in enrichments obtained from cathode inoculum, in contrast to thiosulphate (23.1%) and hydrogen (28.2%). Energetically, the nitrate reduction using sulphide or thiosulphate is the most attractive process for chemoautotrophs (Shao *et al.,* 2010). Five moles of electrons are produced from the oxidation of 0.42 moles of sulphide

during autotrophic denitrification, whereas 0.84 and 3.03 moles of thiosulphate and hydrogen are needed to obtain the same amount of electrons, respectively (Park and Yoo 2009).

In addition, differences according to the inoculum origin were observed, higher percentage of *nosZ* positive enrichments were obtained from Mfc3 cathode (43.1%) compared to Mfc1 Anode (31.1%). In this case, although a lower amount of colonies were obtained, these resulted in higher percentage of *nosZ*-containing enrichments.

4.2.3 Phylogenetic analysis of enriched chemolithoautotrophic denitrifiers

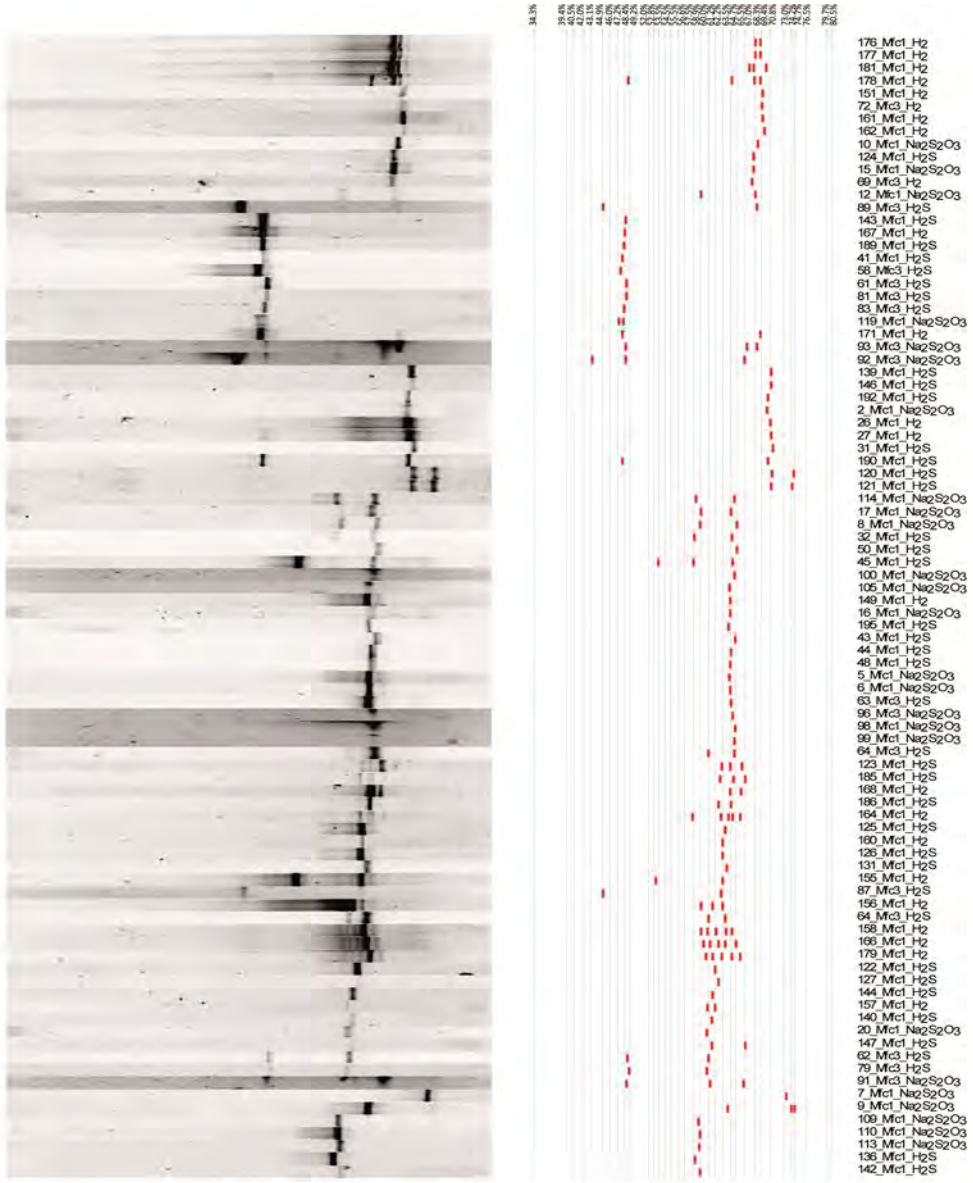
PCR positive wells obtained during the selective enrichment process, were screened with DGGE to confirm the presence of a single band sequence. Almost 60% of samples showed a single band in the DGGE profile, 27% showed two distinct DGGE bands and only 13% of the enrichments appeared to have 3 or more bands in their profiles. At least theorethically, the presence of a single band in the fingerprint is an indication of the presence of a single *nosZ*-containing bacteria in the enrichment culture, and probably the presence of a single bacterial species. However, this may not be the case due to combinations of different bacteria with similar or identical *nosZ* genes, or, alternatively, the simultaneous presence of a *nosZ* containing and a *nosZ* lacking bacteria in the same enrichment. Therefore, culture purity must be assured also in those wells presenting a single DGGE band by re-isolation of bacterial colonies.

The fingerprints obtained from these enrichments, were processed using GelCompare® software to determine band classes according to migration in DGGE gels (Figure 26). A total number of 34 different positions were defined.

However, several factors must be considered in order to evaluate DGGE profiles as a screening method for isolates. DGGE is a technique based on the separation of DNA sequences according to the GC content, and consequently, to its melting behaviour. According to this, different bacterial species might yield PCR products with similar melting behaviours, resuting in bands at the same position on the gel. Additionally, the identification of the same species at different band positions might be due to the fact that the identification was done according to the closest cultured bacteria available on databases and the percentage of similarity.

Few bacterial species, *Hyphomicrobium denitrificans*, *Sinorhizobium meliloti*, and *Shinella zoogloides*, were identified as exclusive species found at certain band positions

with a high number of enrichments (Table 24). The identification of different bacterial species at each band position showed that the species identified were closely related (positions 8, 27 or 28, as an example). Nevertheless, there were also some band positions in which sequences retrieved could be assigned to either *Alpha-* or *Gammaproteobacteria* (examples in positions 9, 16, 25 or 27). Additionally, sequences retrieved from different positions were almost identical.



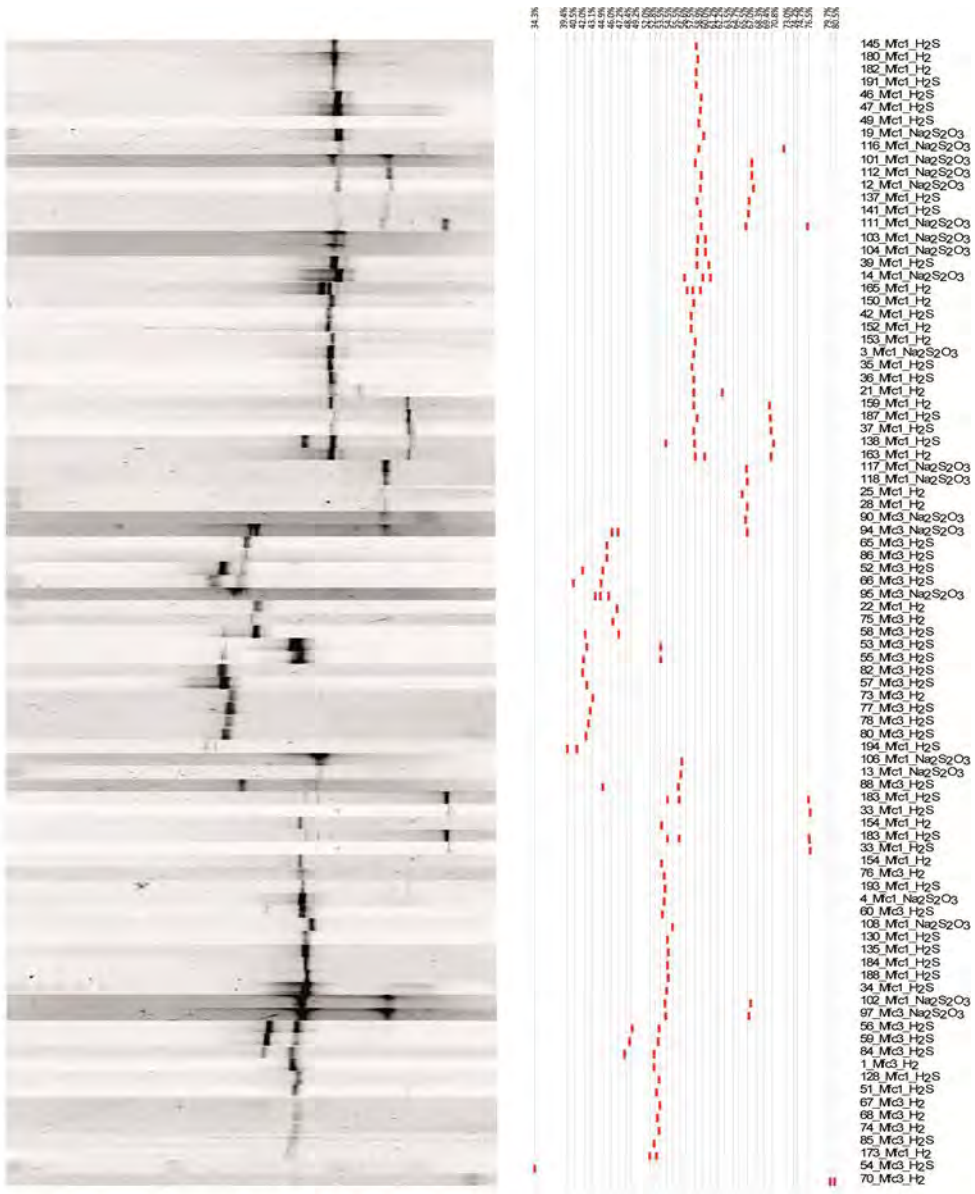


Figure 26. (◄▲)Band class determination of positive *nosZ*-containing bacteria defined according to Dice band based analysis with GelCompare®. The bands used are indicated in red, and its relative position are indicated as migration distances (percentatge). Enrichments are named as Mfc1 (anode sample) or Mfc3 (cathode) followed by the electron donor used in the enrichment: H_2, H_2S or $Na_2S_2O_3$.

A total of 137 DGGE-bands were excised, reamplified and sequenced. For all sequences, similarities above 82% with sequences available in databases were obtained (Table 24). The sequences were classified into *Alphaproteobacteria* (65.7%), *Betaproteobacteria* (16.8%) and *Gammaproteobacteria* (17.5%). No representatives of other phylogenetic

groups could be obtained. Nonetheless, the majority and the more widely distributed denitrifier representatives are contained within the *Proteobacteria* (Shao *et al.,* 2010).

Table 24. DGGE bands sequences from enrichments identified according to Blastn search (NCBI database). The closest cultivated bacteria and the percentage of similarity are indicated. Number of sequences identified to each bacterium are indicated.

Band position		Number of bands	Number of sequences	Most Probable Identification	
34.3%	**1**	1	1	*Ochrobactrum anthropi* (AB490237)	99%
39.4%	**2**	1	0	Not identified	-
40.5%	**3**	2	1	*Hyphomicrobium denitrificans* (CP002083)	98%
42.0%	**4**	7	4	*Hyphomicrobium denitrificans* (CP002083)	98%
			2	*Rhodopseudomonas palustris* HaA2 (CP000250)	91%
43.1%	**5**	5	1	*Hyphomicrobium denitrificans* (CP002083)	98%
			1	*Pseudomonas stutzeri* (HE814032)	92%
44.9%	**6**	8	7	*Hyphomicrobium denitrificans* (CP002083)	98%
46.0%	**7**	3	1	Shinella zoogloeoides (EU346731)	92%
47.2%	**8**	4	1	*Hyphomicrobium denitrificans* (CP002083)	98%
			1	*Sinorhizobium* sp. PD 12 (DQ377784)	87%
48.4%	**9**	15	2	*Pseudomonas stutzeri* (EU346731)	92%
			4	*Sinorhizobium meliloti* (CP004138)	86%
			2	*Hyphomicrobium denitrificans* (CP002083)	98%
49.2%	**10**	5	2	*Sinorhizobium meliloti* (CP004138)	86%
52.0%	**11**	1	0	Not identified	-
52.8%	**12**	5	3	*Hyphomicrobium nitrativorans* (CP006912)	88%
			1	*Paracoccus* sp. BW001 (EU192075)	82%
53.5%	**13**	12	1	*Paracoccus* sp. BW001 (EU192075)	82%
			1	*Rhodobacter sphaeroides* (AF125260)	84%
			2	*Hyphomicrobium nitrativorans* (CP006912)	88%
			1	*Rhodopseudomonas palustris* (CP000250)	93%
54.5%	**14**	12	3	*Rhodobacter sphaeroides* (AF125260)	84%
55.5%	**15**	1	0	Not identified	-
56.6%	**16**	4	1	Sinorhizobium meliloti (CP004138)	86%
			1	Oligotropha carboxidovorans (CP002826)	87%
			1	Pseudomonas stutzeri (JQ513867)	86%
57.5%	**17**	2	1	Mesorhizobium sp. D206b (AB480511)	88%
58.9%	**18**	29	2	Sinorhizobium fredii USDA 257 (CP003563)	92%
			5	*Oligotropha carboxidovorans* (CP002826)	92%
			2	*Aeromonas media* (CP007567)	99%
			1	*Shinella zoogloeoides* (EU346731)	92%
			1	*Paracoccus denitrificans* PD1222 (CP000490)	89%
60.0%	**19**	24	1	*Sinorhizobium fredii* USDA 257 (CP003563)	83%
			3	*Oligotropha carboxidovorans* (CP002826)	93%
			4	*Mesorhizobium* sp. D206b (AB480511)	88%
			1	*Rhodobacter sphaeroides* (CP000662)	82%
			1	*Aeromonas media* (CP007567)	99%
61.2%	**20**	11	0	*Sinorhizobium fredii* USDA 257 (CP003563)	83%
62.2%	**21**	11	1	*Mesorhizobium* sp. 4FB11 (FN600636)	88%
63.5%	**22**	15	2	*Shinella zoogloeoides* (EU346731)	91%
			1	*Achromobacter xylosoxidans* (CP002287)	95%
			1	*Rhodopseudomonas palustris* BisA53 (CP000463)	92%
			1	*Chelatococcus daeguensis* (JX394219)	86%

			2	*Sinorhizobium fredii* USDA 257 (CP003563)	91%
64.7%	**23**	22	5	*Shinella zoogloeoides* (EU346731)	92%
			1	*Mesorhizobium* sp. 4FB11 (FN600636)	89%
			3	*Sinorhizobium fredii* USDA 257 (CP003563)	91%
65.5%	**24**	11	3	*Shinella zoogloeoides* (EU346731)	92%
67.0%	**25**	20	3	*Shinella zoogloeoides* (EU346731)	93%
			5	*Pseudomonas stutzeri* (HE814032)	93%
68.3%	**26**	11	2	*Shinella zoogloeoides* (EU346731)	93%
			1	*Pseudomonas stutzeri* (HE814032)	93%
			1	*Achromobacter xylosoxidans* (CP002287)	100%
			1	*Azospirillum brasilense* (CP007796)	98%
			1	*Mesorhizobium* sp. 4FB11 (FN600636)	88%
69.4%	**27**	9	1	*Oligotropha carboxidovorans* (CP002826)	86%
			1	*Pseudomonas stutzeri* (HE814032)	93%
			1	*Sinorhizobium fredii* USDA 257 (CP003563)	86%
70.8%	**28**	16	3	*Mesorhizobium* sp. D206b (AB480511)	87%
			2	*Hyphomicrobium denitrificans* (CP002083)	98%
			2	*Rhodopseudomonas palustris* HaA2 (CP000250)	88%
73.0%	**29**	2	1	*Shinella zoogloeoides* (EU346731)	90%
			1	*Alicycliphilus denitrificans* (CP002657)	85%
74.2%	**30**	2	2	*Mesorhizobium* sp. D206b (AB480511)	88%
74.7%	**31**	2	1	*Mesorhizobium* sp. D206b (AB480511)	88%
76.5%	**32**	3	1	*Mesorhizobium* sp. 4FB11 (FN600636)	91%
79.7%	**32**	1	0	Not identified	-
80.5%	**32**	1	0	Not identified	-

Thirteen different species of *Alphaproteobacteria* were obtained. Sequences highly similar to *Hyphomicrobium denitrificans nosZ* gene (18 sequences) could be retrieved from enrichments of both, cathode and anode samples, and from all electron donors. The same number of sequences were found for *Mesorhizobium* sp. and *Sinorhizobium* sp., although inoculum-specific differences in species among this two genera were observed. Eleven sequences were identified as *Oligotropha carboxidovorans*, and were obtained exclusively from the anode sample, and using sulphide or thiosulphate as the electron donor. When sulphide was used, *Rhodopseudomonas palustris* (7 sequences) and *Rhodobacter sphaeroides* (6 sequences) could be enriched from cathode and anode samples. Sequences from *Hyphomicrobium nitrativorans* were obtained from enrichments from the cathode using hydrogen and sulphide as electron donors. *nosZ* sequences similar to those of *Paracoccus* sp. were obtained from anode enrichments using sulphide.

Three different species were classified within the *Betaproteobacteria*, *Shinella zoogloides* (15 sequences from anode samples), *Alicycliphilus denitrificans* (6 sequences from anode samples) and *Achromobacter xylosoxidans* (2 sequences obtained from the cathode).

Only two different species of *Gammaproteobacteria* were detected. However, 21 sequences were classified as *Pseudomonas* spp. being the genus most frequently found.

The different *Pseudomonas* species found were related to the origin of the inoculum and the electron donor used. *Aeromonas media* was enriched from the anode using hydrogen and sulphide.

Representatives of all members of the bacterial community identified in the inoculuated samples were recovered in the enrichments with the only exception of *Thiobacillus denitrificans*. Despite this absence in the enrichment cultures, many other species not previously detected in the original sample could be obtained, revealing that the use of different enrichment conditions (electron donors) could improve selection of less abundant microbes. *Hyphomicrobium denitrificans*, *Sinorhizobium fredii*, *Alicycliphilus denitrificans* and *Shinella zoogloeoides* were obtained independently of the electron donor used. However, six bacterial species were found exclusively when sulphide was used (i.e. *Oligotropha carboxidovorans*, *Rhodopseudomonas palustris*, *Chelatococcus daeguensis*, *Achromobacter xylosoxidans*, *Rhodobacter sphaeroides* and *Paracoccus* sp.BW001), and *Azospirillim brasilense* was found exclusively in thiosulphate enrichments. Any of the identified bacteria was enriched exclusively when hydrogen was used.

A graphical distribution of enriched sequences was done according to the source of the enrichment (anode or cathode), the electron donor used (H_2, H_2S or $Na_2S_2O_3$) and their taxonomical classification (Figure 27). Most of the sequences, 57.7%, were grouped into the *Rhizobiales*, including members of the *Bradyrhizobiaceae*, *Hyphomicrobiaceae*, *Rhizobiaceae*, *Phyllobacteriaceae*, *Beijerinckiaceae* and *Brucellaceae*. Their occurrence in the enriched samples was found independently of the inoculum source and the electron donor. Sequences identified as *Rhodobacteraceae*, *Rhodospirillaceae* and *Pseudomonadaceae* were also found in the two sources.

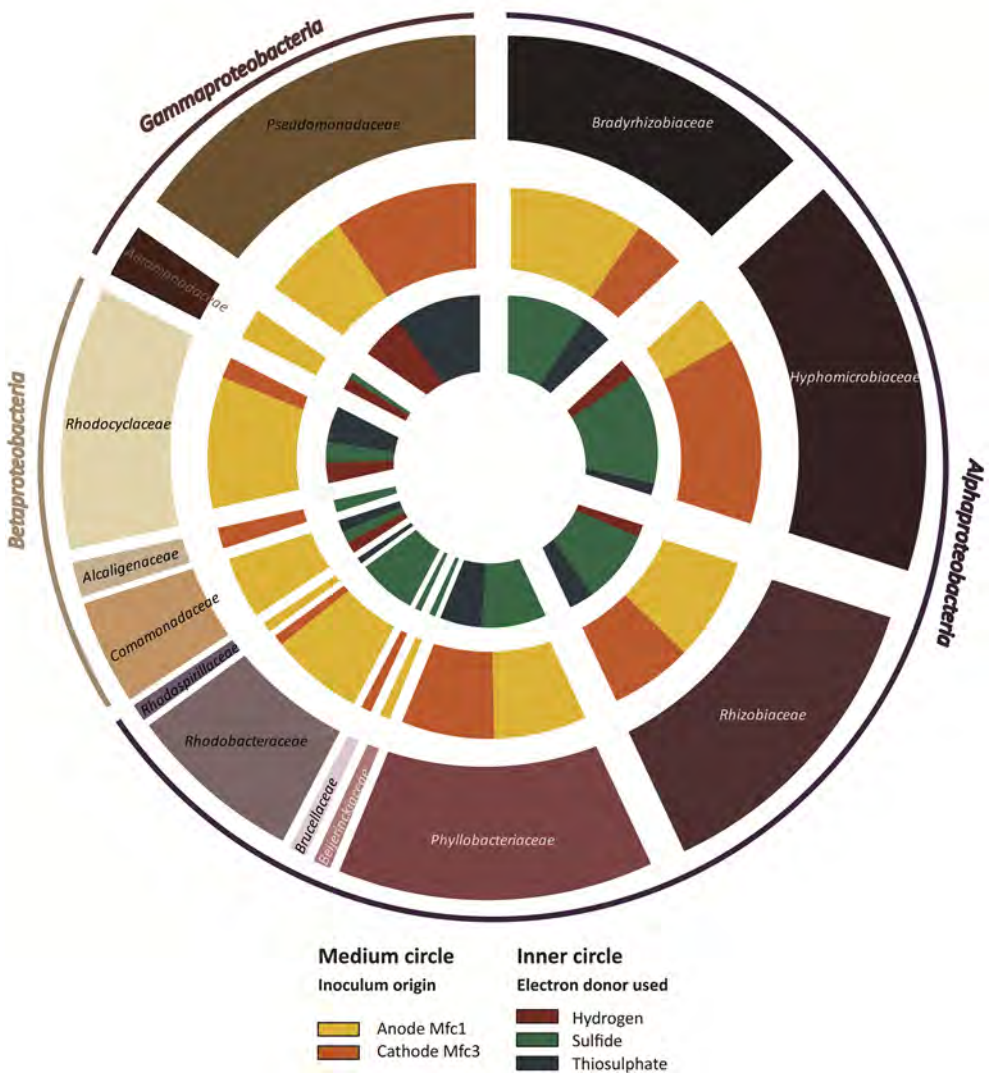


Figure 27. Graphical distribution of enriched sequences. Percentage of enrichments obtained according to the electron donor used (inner ring), the origin of inoculum (middle ring) and the phylogenetic affiliation using *nosZ* gene according to NCBI database, classification are showed at order level (external ring).

Comamonadaceae and *Aeromonadaceae* were only detected in the enrichments obtained from the Mfc1 anode, and *Alcaligenaceae* only found into cathode enrichments.

All the obtained enrichments belonged to the *Proteobacteria* and some of them have been recognized as autotrophic denitrifiers. Wrighton *et al.* (Wrighton *et al.*, 2010) found that *Gammaproteobacteria* was the most represented group in a sample from non-loop BES, with separated anodic and cathodic streams. The previous results contrast our findings, in which *Alphaproteobacteria* accounted for 65.7% of the obtained sequences.

Although different methods were used, the relative abundance of *Alphaproteobacteria* found when the *nosZ* gene was targeted, suggests a more pronounced implication of this group in the last denitrification step in biocathodes. Or, alternatively, a strong PCR bias of the used *nosZ* primers towards this bacterial group as already mentioned in previous sections.

The enrichments containing *Alphaproteobacteria* were mainly obtained with sulphide, accounting for 48.2% of the total. Although the ability to use sulphur-reduced compounds has been poorly studied, it is likely that *Rhodobacter* sp. and *Sinorhizobium* sp. (frequently found in our samples) are able to denitrify using elemental sulphur (Koenig *et al.,* 2005). In contrast, *Gammaproteobacteria* were mainly enriched using thiosulphate (10.2%) or hydrogen (6.6%). The hydrogenotrophic denitrification is a highly selective process in which *Pseudomonas* species were observed in many reactors were hydrogen was used to stimulate denitrification (Szekeres *et al.,* 2002). Other species, like *Aeromonas* sp. were isolated from hydrogen dependent denitrification reactors (Liessens *et al.,* 1992). On the contrary, *Betaproteobacteria* did not show preferences for any of the the electron donors used.

A total of 20 species were identified according to their similarity to *nosZ* gene. The goal of the enrichments was to isolate bacterial species with *nosZ* sequences which had the same phylogenetic affiliation to the unique *nosZ* band found in the cathodes. Therefore, the enrichments of *nosZ*-containing bacteria identified as members of *Hyphomicrobiaceae* and *Bradyrhizobiaceae* families were of interest for further isolation. A total of eleven sequences retrieved from re-amplified DGGE bands were identified as *Oligotropha carboxidovorans* (positions 16, 18, 19 and 27) and five as *Hyphomicrobium nitrativorans* (positions 12 and 13). To select isolates representatives of these species, a cluster analysis based on Dice coefficient of DGGE fingerprints were used to detect common bands with a tolerance of 1.5%, all the enrichments grouped in these clusters were selected (Figure 28).

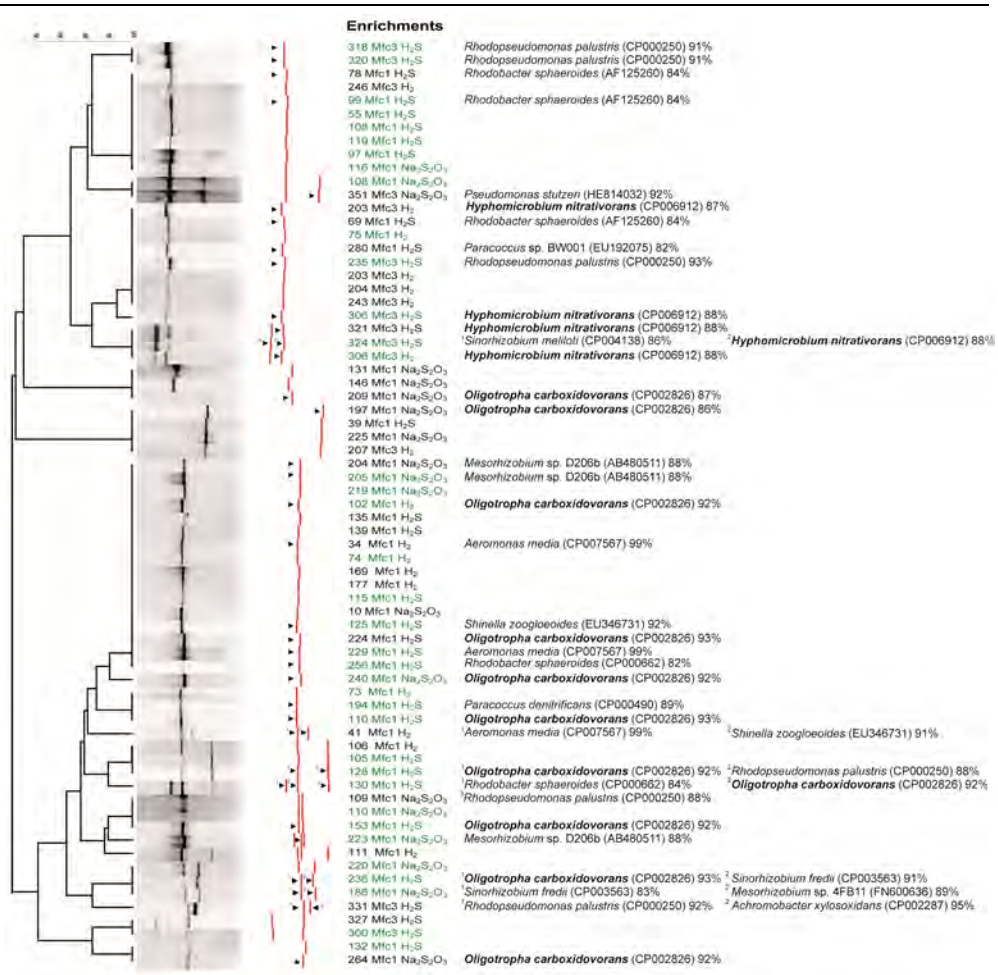


Figure 28. DGGE fingerprints from enrichments containing *nosZ* sequences belonging to the cluster identified as *Hyphomicrobium nitrativorans* or *Oligotropha carboxidovorans*. Cluster analysis based on Dice coefficient, at a band postion tolerance of 1.5%. Groups have been made by using a UPGMA method. Relative band positions are indicated with red lines, enrichment codes highlighted green are selected for further isolation. On the left column, the most closely cultivated bacteria and percentage of similarity to DGGE band sequences according to blastn search (NCBI database) are shown.

The *nosZ*-like *Oligotropha carboxidovorans* enrichments were grouped in three clusters according the relative position of bands on the DGGE gel. However, the same sequence was not retrieved from all the sequenced DGGE bands from the same position. In this sense, 3 sequences identified as *Aeromonas media* and other 3 as *Mesorhizobium sp.* were obtained. Additionally, sequences corresponding to *Shinella zoogloides*, *Rhodobacter sphareoides*, *Paracoccus denitrificans*, *Sinorhizobium fredii* and *Rhodopseudomonas palustris* were also identified. Enrichments with *nosZ*-like *Hyphomicrobium nitrativorans* were identified in DGGE bands located in distinct relative position in the

gel. In this case, also different sequences were obtained from sequencing bands at similar position, 3 sequences of each *Rhodopseudomonas palustris* and *Rhodobacter sphaeroides*, and only one sequence of *Paracoccus* sp. BW001. Co-migration of fragments with different sequences may arise, which make impossible to identify inequivocally a DGGE band position (Ruiz-Rueda 2008).

Thirty-seven out of 68 enrichments of the desired *Oligotropha-Hyphomicrobium* group exhibited higher growth rates in liquid cultures and were selected for isolation in pure cultures.

4.2.4 Isolation and phylogenetic characterization of *Hyphomicrobium-Oligotropha nosZ*-like bacteria

Samples of 37 liquid enrichments were spread on agar plates. After the growth period, only morphological distinctly colonies were selected for isolation purposes. A total of 125 isolates were obtained growing under autotrophic conditions. Additionally, all isolates were transferred to a medium with organic matter to determine their ability to growth in heterotrophic conditions. All isolates, except six, were able to grow in the presence of organic matter. The corresponding 119 isolates were further characterized for the presence of *nosZ* genes (Table 25). Strict autotrophs were discarded due to their slow growth in liquid cultures.

The isolates were screened for the presence of *nosZ* gene by PCR. Forty-five out of 119 isolates were positive. PCR products were sequenced and 37 resulted in good quality unambiguous sequences, which were used to reconstruct a phylogenetic tree (Figure 29). The other 8 *nosZ* sequences were discarded due to the presence of stop codons in the coding region (C1S97.1, C1S99.2, C1S128.3, C2T223.2 and C2T219.4), or due to their low quality that resulted in shorter amino acid sequences (C2T116.1, C2T240.3 and C2T240.10).

Despite the isolation directed to members of the *Alphaproteobacteria* class, *Betaproteobacteria* and *Gammaproteobacteria* were also detected revealing the presence of mixed cultures in the micro-well enrichments. Additionally, the analysis of sequences retrieved from the isolates show that some of them corresponded to clonal isolated colonies.

Table 25. Isolates obtained from enrichments able to growth autotrophic and heterotrophically. Electron donors used, and inoculum origin are indicated. Enrichment well origin, number of isolates obtained from the well and *nosZ* postive isolates are indicated.

Electron donor	Inoculum origin	Enrichment well	Number of isolates	Number of *nosZ* positive isolates	Code of isolates
Hydrogen	Mfc1	74	2	0	-
	Mfc3	306	2	2	C1H306.1 and C1H306.2
Sulphide	Mfc1	55	2	0	-
		97	3	2	C1S97.1 and C1S97.2
		99	2	1	C1S99.2
		105	5	5	C1S105.1, C1S105.2, C1S105.3, C1S105.4 and C1S105.5
		108	6	0	-
		110	2	0	-
		115	4	3	C1S115.1, C1S115.2 and C1S115.3
		119	8	2	C1S119.2 and C1S119.7
		125	3	3	C1S125.1, C1S125.2 and C1S125.3
		128	4	2	C1S128.1 and C1S128.3
		130	2	0	-
		131/132	4	3	C1S131/132.1, C1S131/132.2 and C1S131/132.3
		153	3	0	-
		194	2	0	-
		229	2	2	C2S229.1 and C2S229.2
		256	2	0	-
	Mfc3	300	1	0	-
		306	2	1	C1S306.1
		318	2	2	C2S318.1 and C2S318.2
		320	2	1	C2S320.1
Thiosulphate	Mfc1	108	7	2	C2T108.3 and C2T108.5
		110	2	2	C2T110.1 and C2T110.2
		116	7	3	C2T116.1, C2T116.6 and C2T116.7
		186	5	1	C2T186.1
		205	4	2	C2T205.2 and C2T205.4
		219	11	1	C2T219.4
		220	4	0	-
		223	4	3	C2T223.1, C2T223.2 and C2T223.4
		240	10	2	C2T240.3 and C2T240.10

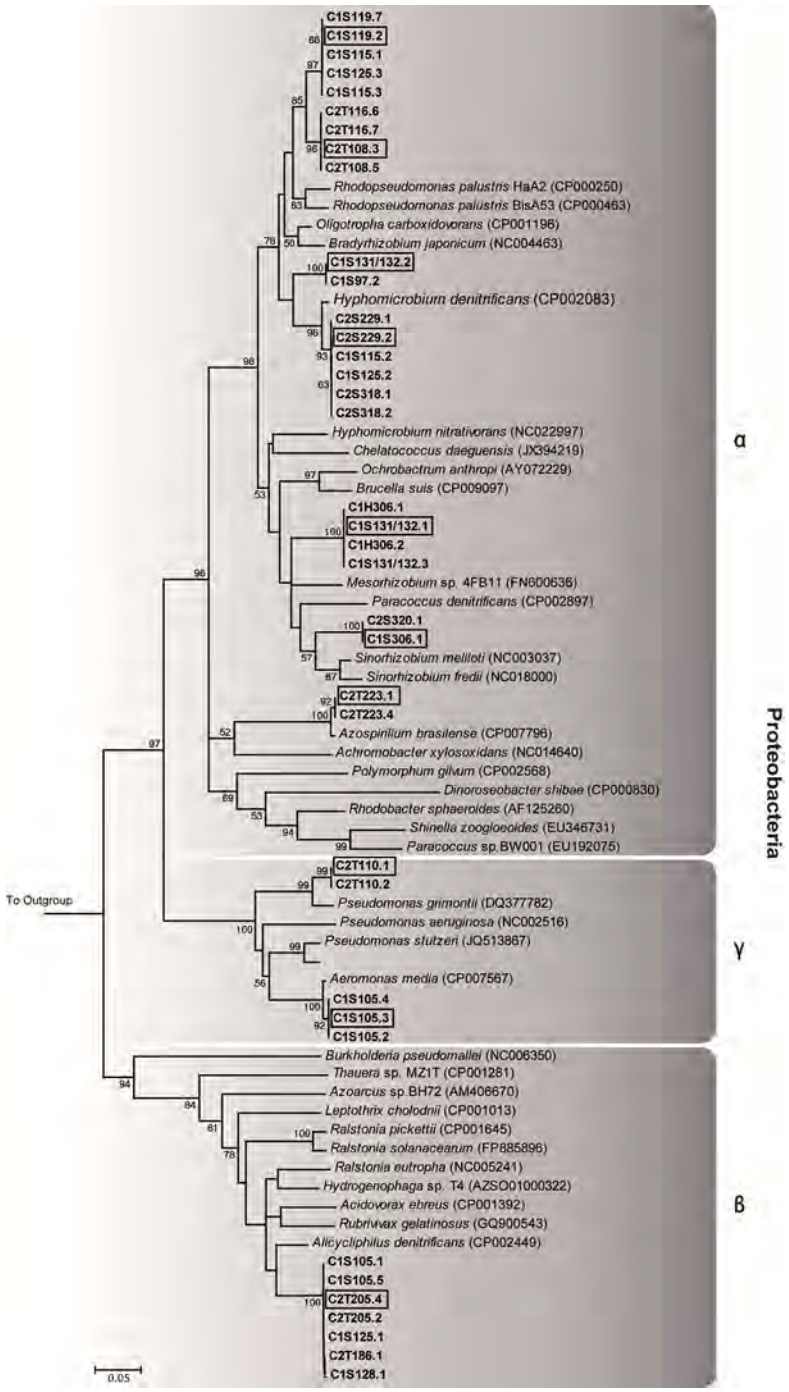


Figure 29. Neighbor-joining phylogenetic tree of amino acid deduced *nosZ* sequences of isolates. The name of isolates are indicated in bold and are grouped according to their similarity. The reference sequences were retrieved from GenBank and are added for comparison. The bootstrap values higher than 50% are shown at the node of the tree (1000 replicates). *nosZ* gene of *Haloarcula marismortui* ATCC 43049 (NC006397) was used as the outgroup. Clonal isolates sequences are indicated as groups, the representative isolate sequence are framed.

Sequences C1S119.2 and C2T108.3 (and other similar sequences) were identified as *Rhodopseudomonas palustris* with 93% of similarity, although they split into two different groups. Only two sequences, grouped with the representative C1S131/132.2, were identified as *Oligotropha carboxidovorans* with 92% of similarity. Sequences highly related to *Hyphomicrobium denitrificans* were grouped with sequence C2S229.1. Additionally, other *Alphaproteobacteria* were identified and grouped into three different clades, the representative sequences being C1S131/132.1, C1S306.1 and C2T223.1. C1S131/132.1 comprised four isolates which were identified as *Sinorhizobium fredii* USDA 257 with 88% of similarity but the relative position in the phylogenetic tree is closer to *Mesorhizobium* sp. 4FB11. Two groups (C2T110.1 and C1S105.3) contained sequences identified as the gammaproteobacterium *Pseudomonas grimontii* and *Aeromonas media*. Only one group with seven sequences were identified as *Betaproteobacteria*, more specifically as *Alicycliphilus denitrificans*.

All 37 isolates, except C2T223.1 and C2T223.4, were also characterized for the 16S rRNA gene sequence (Figure 30). After analysing 16S rRNA gene sequences and comparing the phylogeny with that obtained with the analysis of *nosZ* genes, some inconsistencies were found. For instance, group C1S131/132.2, previously identified as *Oligotropha carboxidovorans* for *nosZ*, showed a higher similarity to *Bradyrhizobium japonicum* (99%) when the 16S rRNA sequence was used. Similarly, groups C1H306.2 and C1S131/132.1, were related to different species when either *nosZ* or 16S rRNA sequences were used. This misclassification within the *Alphaproteobacteria* class reveals a higher micro-diversity within *nosZ* harbouring microorganisms, which was not detected when higher taxonomic levels are considered.

All results obtained for *Gammaproteobacteria* and for both genes were in complete agreement. However, the most unexpected result was that sequences grouped with C2T205.4, according to *nosZ* gene, clustered to *Hydrogenophaga pseudoflava* (C2T205.4), or within the *Alphaproteobacteria* (C2T205.2), and were specifically identified as *Rhizobium* sp.. It is known that horizontal gene transfer (HGT) events may play a role in the observed misclassifications. In fact, *nosZ* is a likely candidate for HGT modifications in bacterial species because it has been commonly found in plasmids and other mobile genetic elements (Jones *et al.,* 2008). HGT events were observed in some *nosZ* genes from *Betaproteobacteria*, although they were mainly clustered in a well-supported clade, some examples such as *Dechlorosomonas aromatica* grouped with *Magnetospirillum* in the same clade as *Epsilonproteobacteria*, and all *Achromobacter* isolates were inserted into the *Alphaproteobacteria* clade (Jones *et al.,* 2008).

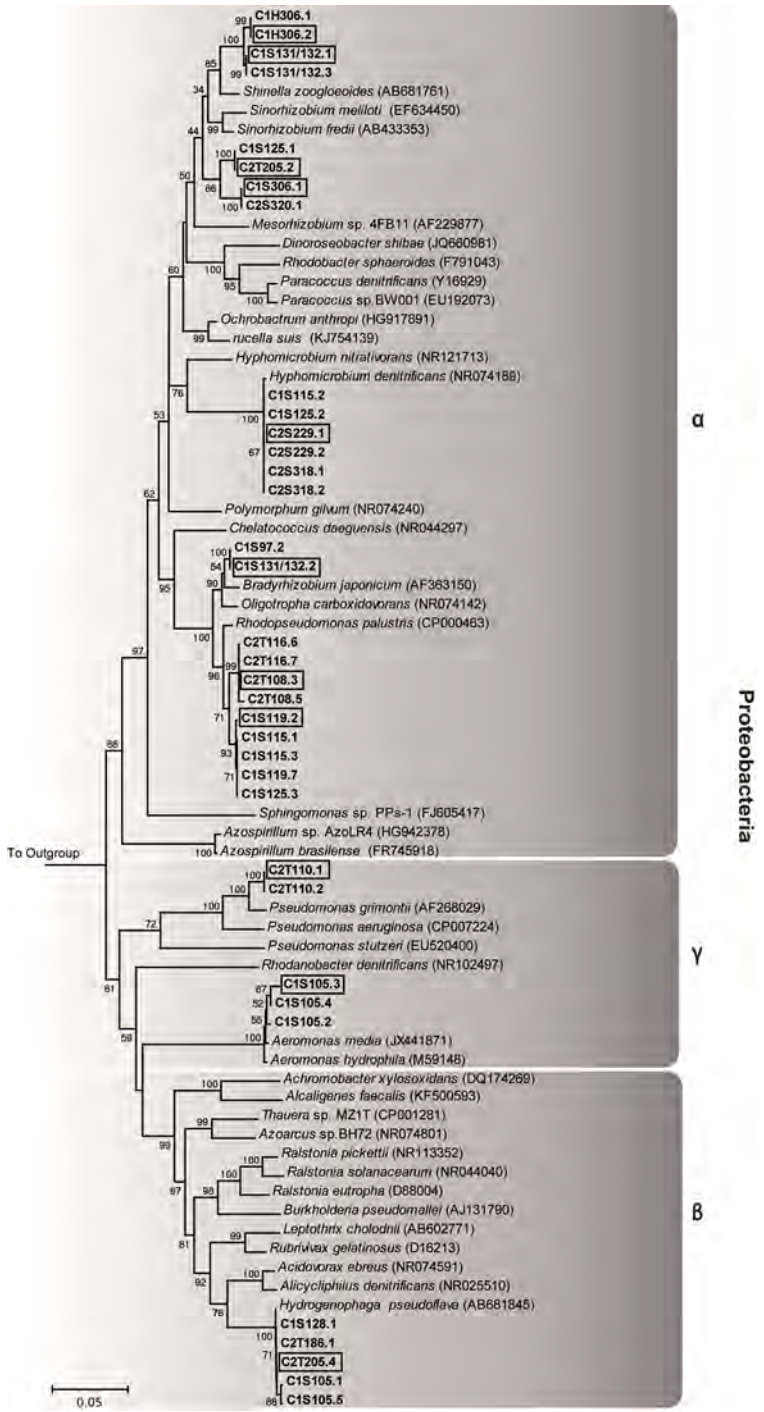


Figure 30. Neighbor-joining phylogenetic tree of 16S rRNA sequences of isolates. The name of isolates are indicated in bold and are grouped according their similarity. The reference sequences were retrieved from GenBank and are added for comparison. The bootstrap values higher than 50% are shown at the node of the tree (1000 replicates). 16S rRNA gene of *Haloarcula marismortui* ATCC43049 (AY596297) was used as the outgroup. Clonal isolates sequences are indicated as groups, the representative isolate sequence are framed.

Ten different groups were defined according to nosZ tree topology. However, our goal was to ensure that the sequences from the bacterial isolates were the same of those from the cathode community, and therefore we performed an OTU based approach similar to that of chapter 4.1. OTUs were defined at a cut-off of 0.2 and compared to sequences from the cloning assay (Figure 31). Twenty one isolates were classified into OTU 1, and representative sequences were defined from *nosZ* gene phylogeny (Figure 29). According to the observed subgroups within OTU 1, five isolates were selected as representative members of the group.

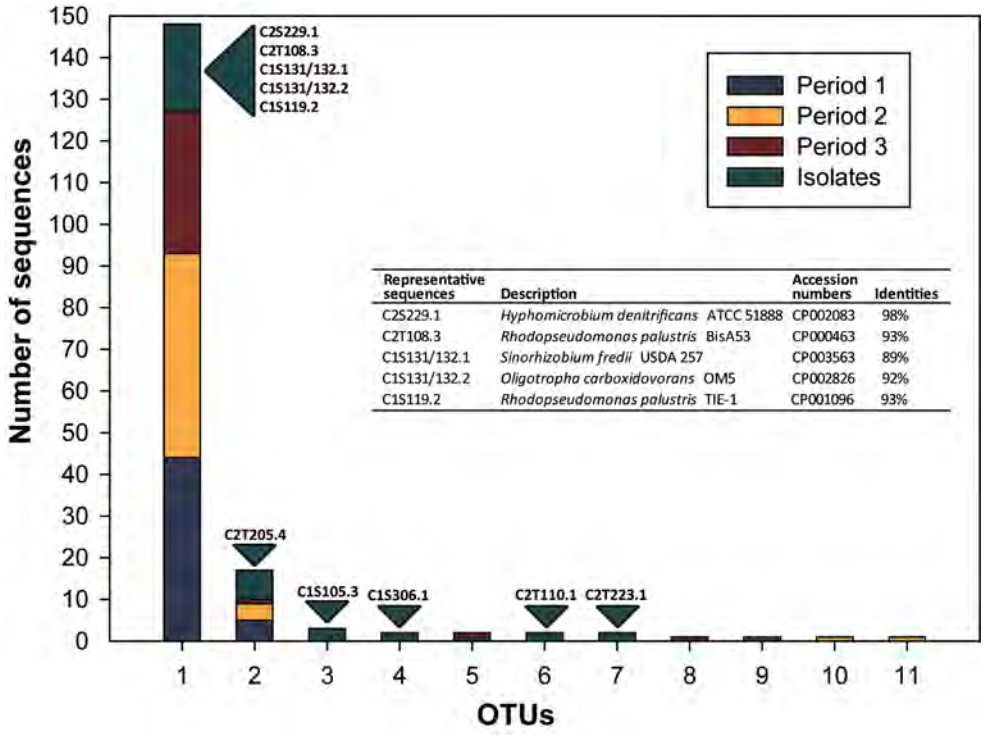


Figure 31. OTU-based classification of *nosZ* clone sequences from chapter 4.1 and from isolates. Sequences were grouped at a cut-off level of 0.2. Different colours indicate the different operational conditions: Period 1 (autotrophic conditions with nitrate), Period 2 (heterotrophic conditions with nitrate) and Period 3 (autotrophic conditions with nitrite). The sequences obtained from isolates are represented as green stacked bars. Representative sequences, identified according to phylogenetic tree topology, are indicated in each OTU. Isolates selected for physiology experiments are indicated in the inserted table.

All of the sequences classified in these OTUs belong to the *Rhizobiales* order, this order was the most represented and occurred as a single population on the cathode. *Rhizobium* related genera form a complex and heterogeneous group that includes strains with different metabolic profiles. The use of nitrogen oxides (nitrate and nitrite) as electron acceptors is a common trait in this group, but the end products of their metabolism vary

significantly in different isolates of the same species (Bedmar *et al.,* 2005, Knowles 1982, Sameshima-Saito *et al.,* 2004). The abundance of this group in our enrichments and community characterization imply a role in the denitrification of MFC.

These representative *nosZ*-containing bacteria were characterized by sequencing this gene and the 16S rRNA gene. The sequences were compared with bacterial genomes available in NCBI databases using BLAST-N tool (Table 26). All the sequences were identified as belonging to the same bacteria with the exception of the isolate C1S131/132.1.

Table 26. Phylogenetic identification and percentage of similarity of the retrieved partial *nosZ* and 16S rRNA sequences. The blast search was restricted to the previously cultivated microorganisms (NCBI database).

Isolates	nosZ gene Most closely related bacterium	Identities
C2S229.1	*Hyphomicrobium denitrificans* (CP002083)	98%
C2T108.3	*Rhodopseudomonas palustris* BisA53 (CP000463)	93%
C1S131/132.1	*Sinorhizobium fredii* USDA 257 (CP003563)	88%
C1S131/132.2	*Oligotropha carboxidovorans* OM5 (CP002826)	92%
C1S119.2	*Rhodopseudomonas palustris* TIE1 (CP001096)	93%
Isolates	**16S rRNA** **Most closely related bacterium**	**Identities**
C2S229.1	*Hyphomicrobium denitrificans* (CP005587)	99%
C2T108.3	*Rhodopseudomonas palustris* (KJ776424)	99%
C1S131/132.1	*Rhizobium selenitireducens* (NR044216)	100%
C1S131/132.2	*Oligotropha carboxidovorans* (KJ676724)	99%
C1S119.2	*Rhodopseudomonas palustris* (KJ776425)	100%

The presence of genes coding for key enzymes in the denitrification pathway was tested for all isolates. Genes analyzed were the nitrate reductases *napA* and *narG* and the nitrite reductases *nirK* and *nirS*, using conventional molecular methods and previously described PCR primers. The presence of these genes was used as an approximation of the potential denitrifying capacities of each isolate. Although the presence of denitrifying genes not necessarily indicates the functionality of the enzyme, it can indicate a potential ability to perform the reductive reaction. *Oligotropha carboxidovorans* OM5^T was chosen as a type strain and included in the analysis for comparison. This strain was chosen as the closest cultured representative to the most abundant OTU available in culture collections.

A positive amplification for at least one of the genes implicated in the denitrification process was obtained for all isolates (Table 27). Three of the isolates presented a putatively complete denitrification pathway, whereas two of them (C2T108.3 and C1S119.2), were negative for the nitrate reductases (*napA* or *narG*) with the primers

used. These isolates were identified as *Rhodopseudomonas palustris* strains TIE-1 and BisA53, respectively. The complete genome sequences of this species (http://www.genome.jp/kegg/) reveal that nitrate reductases are lacking in agreement with the results obtained here.

All five isolates contained a copper containing nitrite reductase (*nirK*-type). The presence of this gene, together with *nosZ*, indicates that all isolates have the potential to reduce nitrite to nitrogen gas.

Table 27. PCR detection of different denitrifying functional genes for each isolate. The presence or absence of different functional genes is indicated as (+) in affirmative cases and with (-) when amplification was not obtained.

	Functional denitrifying genes			
Isolates code	***napA***	***narG***	***nirS***	***nirK***
C2S229.1	-	+	-	+
C2T108.3	-	-	-	+
C1S131/132.1	-	+	-	+
C1S131/132.2	+	-	-	+
C1S119.2	-	-	-	+
O. carboxidovorans OM5^T	+	-	-	+

The characterization of these sequences revealed differences in their ability to reduce nitrate. This trait has not been considered as essential in denitrification because nitrate reduction can also be carried out by nitrate-reducers which are not denitrifiers. All of our isolates contain two genes implied in this process, indicating that they presumably are able to completely reduce nitrite to nitrogen gas.

The fact that all the bacterial isolates also contain *nirK* genes, lead us to suspect that according to community composition retrieved in chapter 4.1 the same bacteria are carrying these two genes, *nirK* and *nosZ*. This indicates that the bacterial strains retrieved are representative of bacterial diversity found in the cathode. However, the available primers targeting mainly bacterial isolates form *Alpha-* and *Gammaproteobacteria* grouped within NirK-type I, whereas the NirK-type II of nitrite reducers had highly variety of sequences including *Cytophaga- Flavobacterium-Bacteroidetes* and *Betaproteobacteria* (Braker *et al.*, 1998, Jones *et al.*, 2008, Priemé *et al.*, 2002).

4.3 Denitrifiers isolated from biocathodes showed different electrotrophic capacities

Microbial community analyses of biofilms in cathodes were performed with either the 16S rRNA gene based markers (Chen *et al.*, 2008; He *et al.*, 2009; Kondaveeti *et al.*, 2014; Wrighton *et al.*, 2010) or functional genes as molecular markers of the denitrification pathway (Vilar-Sanz *et al.*, 2013). The use of different functional genes in a case-study (Chapter 4.1) has revealed that differences between relevant species participating in different steps of the denitrification pathway exist according to changes in electron donors and acceptors. The diversity of nitrate and nitrite reducers was significantly impacted by the presence of organic matter or the use of nitrate or nitrite as the electron acceptor. Contrarily, nitrous oxide reducers showed a high homogeneity despite the conditions used and 85% of *nosZ* sequences clustered in a single group. Sequences showed a high similarity to denitrifiers, such as *Oligotropha carboxidovorans* (Vilar-Sanz *et al.*, 2013) and *Hyphomicrobium nitrativorans*. However, according to these results, we could only hypothesize about the active role of the dominant *nosZ* containing bacteria as true electrotrophs. In this section we aim at deciphering experimentally this possibility using some selected isolates belonging to the most abundant *nosZ* gene cluster found in a dMFC. Electroactivity will be assayed using cyclic voltammetry.

The electrochemical capacity of purified denitrifying enzymes has been analyzed for many components of respiratory pathways. *i. e.* nitrate reductases (Anderson *et al.*, 2001), nitrite reductases (Serra *et al.*, 2011), nitrous oxide reductases (Dell'Acqua *et al.*, 2010) and cytochromes (Correia dos Santos *et al.*, 2003). In most, if not all of these studies, isolated proteins were used in direct contact with the electrode without the presence of other cell components. In another set of experiments, cathode biofilms were electrochemically characterized for denitrification using identical methodology (Gregoire *et al.,* 2014, Pous *et al.,* 2014), showing the suitability of such methods to be applied in living bacteria. As expected, the use of living cells, and in particular complex biofilm communities, may cause undesired interactions with the electrode and eventually interfere in the electrochemical characterization (Harnisch and Freguia 2012). This can be minimized and the electrotrophic activities of defined bacteria can be elucidated, if artificial biofilms composed of a single bacterial species are used.

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5.1 Interest of autotrophic denitrifiers in MFC research

Performance of MFCs depends on the ability of cathodic bacteria to accept electrons from an electrode, generating a pool of electrons that are transferred from the anode to the cathode. Ideally, the greater the demand for electrons on the cathode is, the higher the amount of energy that is produced. Conventional MFCs only depend on the bacterial metabolism to generate the flow of electrons between both chambers. Variations on this set-up configuration include the use of a potentiostat, which is used to fix the potential to a certain independently of the electrons demand by bacteria. This normally generates an increase of the current production (Bond and Lovley 2003, Lovley 2006).

Denitrification in cathodes is of interest in MFC research since nitrogen removal can be acomplished without the addition of organic matter, and functioning exclusively with electrons released from the cathode. Several studies have been focused in the performance of denitrifying Microbial Fuel Cells (dMFCs) in which the efficiency of nitrate removal, the current production and the bacterial communities are characterized (Chen *et al.,* 2008, He *et al.,* 2009, Van Doan *et al.,* 2013, Wrighton *et al.,* 2010). Operational conditions in the MFC have been shown to affect the bacterial community composition, the relative abundance of particular phylotypes, and the denitrifying

activity (Van Doan *et al.,* 2013, Vilar-Sanz *et al.,* 2013, Wrighton *et al.,* 2010). Although the communities in denitrifying biocathodes are complex, some groups (*Betaproteobacteria*) are commonly found independently of working conditions (Chen *et al.,* 2008, Gregoire *et al.,* 2014, He *et al.,* 2009, Wrighton *et al.,* 2010), indicating the importance of these species in the performance of the dMFCs. Also according to this statement, convergent communities evolved in different reactors inoculated with different wastewater sources (Yates *et al.,* 2012).

The present study focuses on characterizing the denitrifying bacterial community from biocathodes in different operational conditions. Operational conditions affected the bacterial community composition and their ability to denitrify completely to nitrogen gas. Accumulation of nitrous oxide due to truncated denitrification pathways occurred only when nitrite was used as an electron acceptor, which may be a matter of concern for the accumulation of greenhouse gases. In order to avoid the production of non desirable gases, the knowledge of mechanisms involved in this process, as well as the bacteria responsible of that reaction, must be understood.

Electrotrophy in denitrifying biocathodes have been proven experimentally several times (Clauwaert *et al.,* 2007, Pous *et al.,* 2014, Puig *et al.,* 2011, Virdis *et al.,* 2008), however the direct implication of denitrifiying bacteria in this process have not been completely revealed. Gregory and co-workers (Gregory *et al.,* 2004), showed that *Geobacter metallireducens* could use electrons to reduce nitrate. Moreover, a community highly enriched in *Thiobacillus denitrificans* was able to reduce nitrate to nitrite in a biocathode without any additional electron source (Pous *et al.,* 2014). Despite these examples, the exact mechanism for electron harvesting in denitrifying bacteria remains unknown. Due to the presence of a complex microbial community in most studies, it is possible that some denitrifiers are not real electrotrophs and other accompanying species harvest the electrons from the cathode that are later used by denitrifiers, establishing complex syntrophic relationships similar to those described in methanogenic aggregates (Morita *et al.,* 2011).

The bacteria capable of electron transfer and the mechanisms underlying electrotrophic denitrification is of great research significance since it will be a key factor to optimize dMFCs performance.

5.2 Insights into denitrifier communities: What makes the difference?

The community structure of three denitrifying biocathodes was characterized with five functional genes (the nitrate reductases *narG* and *napA*, the nitrite reductases *nirS* and *nirK*, and the nitrous oxide reductase *nosZ*) involved in the denitrification process. Differences on the community composition as well as the abundance of these genes appeared to be highly affected by the operational conditions. In the presence of organic matter, the amount of *nosZ* copies decreased while the *nirS* and *nirK* increased when compared to autotrophic conditions (in the complete absence of organic matter). As shown in chapter 4.1 not only the abundance of denitrifying bacteria but also the community composition were affected by the presence of organic matter. Under these conditions, *nirK*-containing denitrifiers where enriched on *Phylobacteriaceae*, whereas *Bradyrhizobiaceae* dominated the *nirK*-containing community under autotrophic conditions. A similar variation was observed for *narG*-containing bacteria, heterotrophic conditions caused the partial disappearance of *Hydrogenophillaceae* in front of *Methylobacteriaceae*.

Contrarily to the differences observed in the nitrite and nitrate reductase genes, *nosZ*-containing communities appeared to be fairly stable in all assayed conditions and only differences in abundance were recorded. It should be mentioned that the primer set used here has been shown to bias *nosZ* detection to the recently named *nosZ* clade I bacteria, leaving clade II uncovered (Jones *et al.,* 2013). This limitation means, in practice, that a part of the *nosZ*-containing bacteria are not covered with our analysis, and could account for some unobserved differentiation of the nitrous oxide reductase community. NosZ clade I is composed mainly of members of *Alpha*-, *Beta*- and *Gammaproteobacteria* and a few *Archaea*. Whereas in clade II, a greater number of different taxonomic groups are present including *Epsilon*- and *Deltaproteobacteria* or *Bacteroidetes*, among others (Jones *et al.,* 2013). However, previous studies have shown that *Proteobacteria* accounted for 80% of bacteria in biocathodes, and more precisely, classes *Alpha*-, *Beta*- and *Gammaproteobacteria* accounted for about 90% of the *Proteobacteria*, revealing that *nosZ* clade II bacteria may not be abundant at these conditions (Chen *et al.,* 2010, Gregoire *et al.,* 2014, He *et al.,* 2009, Wrighton *et al.,* 2010).

In future studies, it could be interesting to explore new techniques to determine the bacterial diversity. Pitfalls associated to primer set selection, especially for the functional genes used here, have been previously detected. Commonly, denitrifiers are

underestimated in pure cultures due to ambiguous assessment of the ability to denitrify in strain characterization experiments (Verbaendert *et al.*, 2011). In some cases, the presence of denitrification genes is detected by conventional genome sequencing methods, but no physiological evidences exist for denitrification activities To avoid constrains of targeting a specific organism, metabolic pathways can be detected by reverse transcriptase PCR (RT-PCR) using short non directed (random) sequences as primers (Singh *et al.*, 2009). To obtain a higher detail of members of the denitrifier community, analysis of metagenomes may provide a good opportunity, both for genetic potentials and transcriptional profiles (Cardenas and Tiedje 2008, Schneider and Riedel 2010). These are powerful tools to investigate structural, evolutionary and metabolic properties of complex microbial communities that might be used in future studies to have a widely understand of denitrifying communities in MFCs.

In the MFCs analyzed here, the NosZ-containing community was dominated by a single OTU (more than 88% of sequences), indicating a fairly low diversity independent of the applied conditions. This fact poses some interesting questions. What is relevant for electrotrophy in our MFC cathodes? Are those bacteria that occur specifically at certain conditions the key to electrotrophy? Or, conversely, are those who remain unchanged the ones determining the MFC performance? In chapter 4.2 and 4.3 we directed our research to these common phylotypes containing *nosZ* genes.

The presence of *nosZ* genes in sequenced bacterial genomes is rather unpredicteable. Both bacteria having an almost complete denitrification pathway but lacking the last step (coded by *nosZ* gene), and some species having exclusively the last step of denitrification, can be found. The abundance of genomes lacking *nosZ* genes seems to indicate an evolutionary tendency to eliminate this metabolic step, probably because it contributes poorly to the overall bioenergetic requirements of the cells (Graf *et al.,* 2014, Jones *et al.,* 2008). In MFC research, the substitution of nitrate for nitrous oxide as an electron acceptor in a biocathode resulted in the decrease on bacterial diversity, revealing a higher specialization of *nosZ*-containing bacteria (Desloover *et al.,* 2011).

The stability of the community according to *nosZ* gene, and its higher abundance during autotrophic conditions, could suggest an active role of these bacteria in electrotrophy. Under the two autotrophic conditions tested similar gene abundances were found, whereas the presence of organic matter caused a decrease in *nosZ* abundance. The presence of organic matter allowed the growth of heterotrophic bacteria which could outcompete electrotrophic bacteria by using a more energetically favourable metabolism (Park and Yoo 2009).

According to the community composition of the different denitrifying genes, differences at family level might indicate that the presence of single bacterial species containing all enzymes was not likely to occur. Similar patterns were observed with couples of genes involved in consecutive steps of denitrification. Bacterial communities based on *napA* and *nirS* genes were mainly composed of members of the *Rhodocyclaceae* family probably indicating that nitrate reduction to nitric oxide could be performed by the same bacterial species. This fact is reinforced because for both genes the relative abundance of *Rhodocyclaceae* decreased significantly in the presence of nitrite as an alternative electron acceptor favouring the development of other bacteria. Dominance of *Rhodocyclales* (46%) and *Burkholderiales* (15%) in high current density denitrifying cathode communities has been reported (Gregoire *et al.,* 2014). On the other hand, members of the same family, *Bradyrhizobiaceae*, were identified as dominant in *nirK* and *nosZ* communities as the genes responsible for nitrite and nitrous oxide reduction, respectively. *nirS*+*nosZ* denitrifiers are mainly represented by the *Burkholdiareales* and *Rhodocyclales*, whereas the *nirK*+*nosZ* denitrifiers are overrepresentented within the *Rhizobiales* (Jones *et al.,* 2013, Sanford *et al.,* 2012). Another interesting observation is that dominant families identified in the *narG* community that were exclusively found when this gene was analyzed, indicating that the first denitrification step occurs independently. The high variability in the community indicates that the complete reduction of nitrate to nitrogen gas, must be performed by mixed bacterial species, revealing a possible syntrophic relationship between different members of the community. A similar behaviour has been observed in soils, in which bacteria containing exclusively *nosZ* genes mitigate N_2O emissions due to incomplete nitrate reductions (Philippot *et al.,* 2011).

In the three conditions tested, the *nirS*-type nitrite reducers outnumbered *nirK*-type nitrite reducers by at least an order of magnitude in cathodic biofilm, according quantitative PCR results. The high abundance of *nirS*-containing bacteria was an unexpected result since in many environmental studies, as well in wastewater treatment plants, the *nirK*-type denitrifiers exceeded *nirS*-type denitrifiers (García-Lledó *et al.,* 2011, Hallin *et al.,* 2009, Philippot *et al.,* 2009, Van Doan *et al.,* 2013). The presence of duplicate copies of *nirS* gene has been identified in different bacterial strains including *Thiobacillus denitrificans*, *Dechloromonas aromaticum*, or *Thauera* species, and *Magnetospirillum magneticum* had up to three gene copies (Jones *et al.,* 2008). A *nirS*-containing community dominated by bacteria lacking the *nosZ* gene could explain the difference in family affiliations between *nirS* and *nosZ* gene communities. This occurs in

some *Thaurea* species, a member of the *Rhodocyclales*, which is the most abundant *nirS* bacteria in our samples (Liu *et al.,* 2013).

Differences on electrochemical performance of MFC under different conditions could be used to reveal the role of each electron acceptor. Differences in current production between autotrophic and heterotrophic conditions indicate that the nitrate is partially removed via heterotrophic denitrification. On the other hand, the accumulation of denitrifying intermediates can be estimated by differences in coulumbic efficiency. During autotrophic conditions with nitrate almost 83% of nitrogen removed was in form of nitrogen gas, whereas nitrous oxide accumulation was observed during the heterotrophic period with nitrate (51%) and autotrophic period with nitrite (70%). These differences were not completely explained by changes in the NIR/NOS ratio. Gene quantifications were performed with DNA extracts and may not reflect activity. Additionally, differences on enzyme kinetics may be affected by the operational conditions under low electron availability (*i.e.* in the presence of organic matter), a lower affinity of the N_2O reductase towards the electron donor facilitates the accumulation of this intermediate (Pous *et al.,* 2013). The highest amount of nitrous oxide accumulation was reported when nitrite was used as an electron acceptor in an MFC. It has been proven that the use of this electron acceptor produces an increase of nitrous oxide emissions during denitrification (Kampschreur *et al.,* 2009, Wunderlin *et al.,* 2012).

Dominance of *nirS* bacteria lead us to speculate about the possible role of cytochrome cd_1 nitrite reductases in gathering electrons directly from an electrode. In this sense, proposed mechanisms for EET in biocathodes include cytochromes as components of the electron transport chain playing a key role in electrode-cell transfer (Rosenbaum *et al.,* 2011). Additionally, although it is known that exoelectrogenesis and electrotrophy may have some structural and mechanistic differences, cytochrome mediated mechanisms have been proposed to participate in the two processes (Bond and Lovley 2003, Logan 2009, Lovley 2006). However, electrotrophic behaviour must be affected by the presence of organic matter, and the composition of the *nirS* community structure was significantly affected by the change of electron acceptor and not by the trophic regime.

A reductive point of view of the results obtained in the analysis of bacterial communities, led us to study in deeper detail some of the bacteria present in biocathodes. We directed our screening to the most stable of the bacterial communities, *i.e. nosZ*-containing bacteria.

5.3 Isolation of autotrophic denitrifiers: Looking for a true electrotroph

The community composition revealed a dominance of *Alpha*- and *Betaproteobacteria* bacteria in biocathodes. However, the enrichment and isolation procedure we used led to the enrichment of bacteria from the *Alpha* subgroup (about a 64 % of sequences), despite different electron donors were used (Chapter 4.2), since we directed our screening to *nosZ* gene. Enrichment of *Betaproteobacteria* occurred less frequently (16.8%). Surprisingly, all isolates contained the *nirK* gene even though *nirS* abundance was significantly higher in cathode communities, which is a consequence of directing our enrichment to *nosZ*-containing bacteria. Of course, this bias limits the conclusions of our research.

Enrichment and isolation were conducted using nitrate as electron acceptor and the presence of *nosZ* genes and their similarity to the observed OTU 1 (chapter 4.1) were used as the screening method. Surprisingly, two of the isolates selected lacked the ability to reduce nitrate. The two bacterial strains were identified as *Rhodopseudomonas palustris*, a purple non-sulphur bacterium able to grow photoautotrophically, this metabolism also could allow the growth of this bacterium under autotrophic conditions with hydrogen in the presence of light (Jiao *et al.,* 2005).

Electrochemical characterization of the selected bacterial isolates revealed differences on its electrocatalytic activity (chapter 4.3). Although all the bacterial strains were phylogenetically related, and contained the *nirK* gene, only four out of six isolates had catalytic activity using nitrite as an electron acceptor. Although, our purpose was to determine the role of *nosZ*-containing bacteria in the use of electrons, the results indicated that, at least for the tested isolates, bioelectrochemical activity was mediated by NirK. In fact, the community composition (chapter 4.1) indicated not only the stability of *nosZ*-containing bacteria during all the conditions tested, but also similar patterns were observed for *nirK* community during strictly autotrophic conditions. According to our data, copper-containing nitrite reductases were able to use electrons released from an electrode to electrochemically reduce nitrite. The role of this enzyme seems clear because only the presence of nitrite produces an electrochemical response. In this sense, and although we did not analyze the enzyme structure, a proposed mechanism can be envisioned from literature review. Copper-containing nitrite reductases can be classified according to different properties. An interesting type for the present work is represented by those enzymes that contain an additional *c*-type domain at the C-terminal (Ellis *et al.,*

2007, Nojiri *et al.,* 2007). This domain acts as an electron acceptor for further transference of electrons to T1Cu site (Ellis *et al.,* 2007). The electrochemical mediated activity of this enzyme is not surprising taking into the account the presence of metal atoms in the active sites of the enzyme. Additionally, the presence of the *c*-type domain as an insertion on the gene structure could be the responsible of the physiological differences among closely related bacteria. In our case, two isolates identified as *Rhodopseudomonas palustris* showed different electrochemical nitrite reduction capacity. We can speculate this variation may be due to the referred insercion on the gene sequence. Unfortunately we have no experimental evidences (gene sequences) to prove this hypothesis.

5.4 Electrically derived electrons may not drive all reductive steps in denitrification

All the characterized isolates revealed the ability to use electrons released from electrode, although not all them in the same metabolic reaction. Two of them have been proven to have the ability to produce hydrogen electrochemically (chapter 4.3), whereas the other four showed electrochemical nitrite reduction. While the cathode community was able to completely reduce nitrate to nitrogen gas, selected isolates were only capable of nitrite reduction electrotophically, despite having the ability to denitrify completely in the presence of organic matter. This fact lead us to question ourselves about synergistic relationships in the cathode community that would ensure a complete denitrification reaction though electrochemically independent. Of course, the presence of other bacteria that may perform a complete denitrification electrochemically cannot be ruled out.

However, despite the few number of isolates characterized, we were able to detect the presence of hydrogen producers, which may contribute to the denitrification process by providing hydrogen as a reducing power source. This finding could indicate that the complete nitrate reduction that occurs in the biocathode could be mediated by a combination of both electrotrophic and hydrogenotrophic bacteria. Hydrogenotrophic bacteria could completely reduce nitrate to nitrogen using hydrogen as an electron donor (Karanasios *et al.,* 2010). According to this, although our isolates were not able to reduce nitrous oxide electrochemically, this reaction could be accomplish in the biocathode due to the presence of hydrogen. In fact, most of the bacterial families identified in the cathodes can use hydrogen for its autotrophic growth (Park and Yoo 2009, Robertson and Kuenen 1990).

A model about possible relationships between bacteria present in the biocathode that might lead to the complete nitrate reduction can be proposed (Figure 46). Only nitrite reduction seems to be electrotrophically mediated and although the bacterial strains contain *nosZ* gene, no nitrous oxide reduction was observed. However, two strains showed the ability to produce hydrogen electrochemically which could be sustaining the complete denitrification.

Complete denitrification has been reported in denitrifying biocathodes, and although different bacterial strains with complete denitrification pathways were characterized, no nitrate or nitrous oxide reduction electrochemically-mediated have been reported. These findings sustain the hypothesis that the complete denitrification in biocathodes is performed by different bacterial species that cooperate to reduce nitrate to nitrogen gas.

Additionally, the characterization of hydrogen producers could indicate that not all denitrifiers are electrotrophically active and some of them could be exclusively hydrogenotrophic.

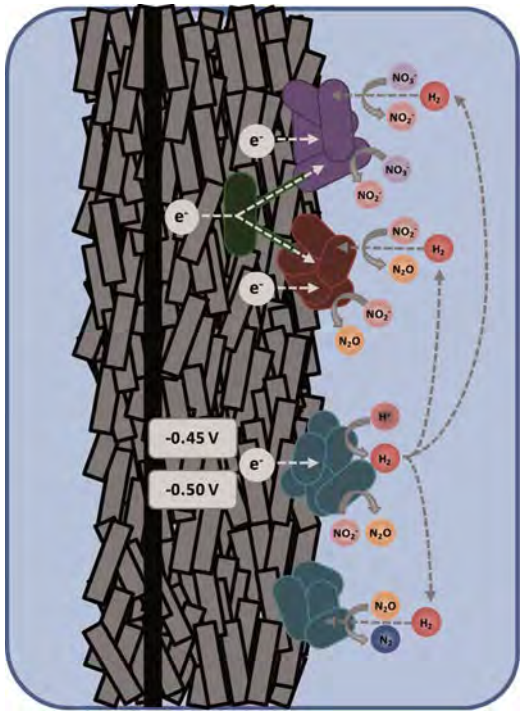

Figure 46. Proposed model of denitrification reactions which could occur in the cathode. Demonstrate electrotrophic nitrite reduction which occurs at -0.50 V and hydrogen porduction at -0.45V (*vs*. SHE). The model proposes that hydrogen produced could mediate nitrate, nitrite and nitrous oxide reduction. Additionally for *nirS*-containing bacteria and nitrate reducers, electrotrophic processes can not be ruled out. The electron might be directly accepted by bacteria or bacterial mediated.

Electrochemically active nitrate reduction was measured in cathodic biofilm dominated by *Thiobacillus denitrificans*, indicating its preponderant role in harvesting electrons from electrode (Pous *et al.,* 2014). This bacterial species was identified as the most abundant member of the *narG* community in our biocathodes. Although the *Thiobacillus denitrificans* ability to denitrify completely has been reported (Beller *et al.,* 2006), revealing the presence of a complete denitrification pathway, we could not detect significant amounts of *Thiobacillus denitrificans* when genes other than *narG* were analyzed.

The bacterial community of biocathode was dominated by *nirS*-containing bacteria which were not characterized electrochemically in the present study. Nevertheless, the bacterial community characterized from high-current producing biofilms was dominated by *Rhodocyclales* and *Burkholderiales*, identified as dominant bacterial species in *nirS*-containing communities (Gregoire *et al.,* 2014). However, no reports with pure cultures exist so far.

Little is known on the EET mechanisms which occur on denitrifying biocathodes. This lack of knowledge is mainly due to challenging and time consuming methods that need to be applied to isolate cathode microorganisms (Gregoire *et al.,* 2014). Most studies on EET mechanisms rely in the analysis of highly enrich complex communities understimating the effects of minor populations that also occur in the cathode. We have focused our efforts on cultivating and characterizing electrochemically denitrifying strains to contribute filling this gap. The EET ability has been demonstrated through conductive minerals in different bacterial species, *Geobacter* sp., *Thiobacillus* sp. and more recently *Mariprofundus ferrooxydans* (Gregoire *et al.,* 2014, Pous *et al.,* 2014). The ability of oxidize metals, often insoluble in the environment, required EET mechanism to obtain electrons in a similar way as when cathode is used as electron source, being an indicator of electrotroph activity (Summers *et al.,* 2013). In our isolates, different mechanisms might occur because our findings on electrochemical behavior are limited to the denitrifying genes. If other non-denitrifying bacteria are implied in the process of using electrons is not elucidated here. It is possible that more complex reactions occur in the cathode. Nevertheless, our work contributes to core knowledge on the relationships of electrotrophic, nitrite respiring bacteria, although more work is required to determine the enzymatic mechanism for the process of capturing electrons.

1) Relevant players in nitrate, nitrite and nitrous oxide reduction in MFC biocathodes have been identified on the basis of gene sequence similarities. Community composition analyses revealed the presence of different species cooperating for a common goal, nitrate elimination from water.

2) The electrochemical performance of MFCs were related to the presence of specific bacterial types. Current density increased for about 25% in autotrophic conditions when nitrate was used as electron acceptor. This corresponded to the selection of characteristic nitrate and nitrite reducers.

3) Cathode biofilms were dominated by *nirS*-type denitrifiers. *nirS*-type containing populations were highly affected by the use of nitrate or nitrite as the initial electron acceptor. On the contrary, *nirK*-type containing bacterial populations were mainly affected by the presence of organic matter in the feed. As expected NIR/NOS ratio was correlated to N_2O emissions.

4) *nosZ*-containing bacteria remained almost invariable during all periods tested. Most *nosZ* sequences clustered in a single group with a high similarity to *nosZ* genes of *Oligotropha carboxidovorans* and *Hyphomicrobium nitrativorans*.

5) Bacteria belonging to the major *nosZ* group in cathodes could be effectively enriched and isolated using inorganic electron donors, such as thiosulphate, sulphide and hydrogen. Five isolates were selected and temptatively identified: C2S229.1, C2T108.3, C1S131/132.1, C1S131/132.2, C1S119.2.

6) All the bacterial isolates, except C2T108.3 and C1S119.2, probably lacking a nitrate reductase gene, were able to reduce completely nitrate to nitrogen gas, under autotrophic and heterotrophic conditions.

7) Electrotrophic behaviour was confirmed for C2T108.3, C1S131/132.1, and quite possibly for C2S229.1, and electrochemical nitrite reduction was observed. Mid-point potentials were measured at -500 mV. Isolates C1S131/132.2 and C1S119.2 had mid-point potentials around -450 mV which indicated their ability to produce hydrogen electrochemically.

8) The obtained results confirm that biocatalyzed electrochemical hydrogen production may play a role in driving nitrate reduction in autotrophic conditions although are not likely to occur in the used cathode potentials. However, this observation reinforces the need for a complex cooperative bacterial net to be developed in the MFC to increase nitrogen removal efficiences.

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