Universitat de Girona

ORIGIN AND FATE OF NITRITE IN MODEL ECOSYSTEMS: CASE STUDIES IN GROUNDWATER AND CONSTRUCTED WETLANDS

Elena Hernández del Amo

Doctoral Thesis

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“Caminante, son tus huellas
el camino y nada más;
Caminante, no hay camino,
se hace camino al andar.**”**
Antonio Machado

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Sembla que ara sí, comença a acostar-se realment el final d'aquesta etapa, la tesi. Una etapa que sense vosaltres, la gent que m'envolta, no hagués estat possible, perquè d'una manera o altra tothom ha fet possible que aquesta tesi acabés agafant forma. Així que, què menys que dedicar-vos com a mínim un apartat, no?

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LIST OF PUBLICATIONS

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TABLE OF NON-CONVENTIONAL ABBREVIATIONS

Abbreviation	Description
16S rRNA	16S ribosomal ribonucleic acid
AMO	Ammonia monooxygenase
amoA	Ammonia monooxygenase subunit A
Anammox	Anaerobic ammonium oxidation
AOA	Ammonia Oxidizing Archaea
AOB	Ammonia Oxidizing Bacteria
AOM	Ammonia Oxidizing Microorganism
BLAST	Basic local alignment search tool
bp	Base pair
C/N	Carbon/Nitrogen ratio
cDNA	Complementary Deoxyribonucleic Acid
Chao1	Maximum richness index
Cq	Quantification cycles
CTAB	Cetyltrimethylammonium bromide
dbRDA	Distance-based redundancy analysis
DNA	Deoxyribonucleic acid
DNRA	Dissimilatory Nitrite Reduction to Ammonium
DO	Dissolved Oxygen
DW	Dry Weight
EC	Electrical Conductivity
FW	Fresh Weight
FWS-CW	Free Water Surface Constructed Wetland
H'	Shannon's diversity index
HAO	Hydroxylamine oxidoreductase
HGT	Horizontal Gene Transfer
IC	Inorganic Carbon
NAP	Free periplasmic nitrate reductase
napA	Free periplasmic nitrate reductase gene
NAR	Membrane-bound nitrate reductase
narG	Membrane-bound nitrate reductase gene
NAS	Assimilatory nitrate reductase

Abbreviation	Description
NCBI	National Center for Biotechnology Information
NirK or Cu-NIR	Periplasmic copper containing nitrite reductase
nirK	Periplasmic 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
NrfA	Cytochrome *c* nitrite reductase
nrfA	Cytochrome *c* nitrite reductase gene
OMZ	Oxygen Minimum Zone
OTU	Operational Taxonomic Unit
PCoA	Principal coordinate analysis
PD	PhyloDiversity index
PDNRA	Potential Nitrite Reduction to Ammonium
PNR	Potential Nitrite Reduction
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROL	Radial Oxygen Loss
Sobs	Observed richness
T	Temperature
TC	Total Carbon
TN	Total Nitrogen
TOC	Total Organic Carbon
WWTP	Waste Water Treatment Plant

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ABSTRACT

Ammonium and nitrate are among the most common pollutants in natural freshwater systems causing a tremendous impact on ecosystem functioning with potentially negative effects on human health. Removal of nitrogen from natural and constructed freshwater systems is mainly achieved through microbial processes. Microbial communities should be analysed accurately, studying their composition and potential activity, as well as their interaction with other players in the environment (i.e. plants), to understand their functioning. Main microorganisms implied in the effective nitrogen removal from water are nitrifiers and denitrifiers, producing a net elimination of ammonia to nitrogen gas. On the contrary, microorganisms that reduce nitrite back to ammonia as a secondary respiration process (DNRA) would eventually lead to the maintenance of soluble nitrogen forms in water.

Hot-spots for highly active N cycles are wetlands and groundwater. Constructed wetlands (CW) try to mimic natural systems to remove pollutants, and are environments of special interest for wastewater treatment. In these systems, vegetation has an important role, since the interaction between microorganisms and plants enhance microbial processes, specifically nitrification. One of the main plants used in CW is *Typha angustifolia*, which is also present in natural freshwater systems from Catalunya. Another environment of special scientific interest in Catalunya is groundwater, specifically in areas where serious nitrate accumulation has occurred in the past due to intensive agriculture practices. Natural attenuation processes, mainly denitrification, are known to relieve the impact of fertilization practices on groundwater resources. In this sense, to identify the occurrence of microbial processes that remove N from water has become a requirement for quality management in different systems.

In constructed wetlands, denitrification usually accounts for >60% of nitrogen removal and is supposedly affected by wetland management practices, such as dredging (and plant removal), which potentially cause an impact in sediment properties and microbiota. We have quantified the effects of a sediment dredging event on dissimilatory nitrite reduction activities in a model FWS-CW by analysing changes in the structure and activity of the microbial community. In the studied CW, potential rates for DNRA and denitrification were in accordance to changes in the physicochemical conditions of the wetland after sediment dredging (and plant removal) event. Denitrification was the predominant pathway and

eventually leaded to the complete nitrate removal in sediments. Significant decrease of 16S rRNA, *nirK* and *nirS* abundances was observed after sediment dredging, although q*nirS*+q*nirK*/q16S rRNA remained similar. The analysis of microbial community revealed the importance of the vegetation on microbial community structures, selecting specific phylotypes that potentially contribute to nitrogen cycle. In this sense, we could detect a high resilience of denitrifiers in the system that minimizes the effects of the impact leading to high recovery rates. These observations agree with the high functional redundancy among denitrifying species, leading to a situation in which several microorganisms with similar capacities are present at the same time and place.

The effect of plants is important for microbial communities implied in N cycle in roots and sediments, specifically for ammonia oxidation, which is highly dependent on the available oxygen. Emergent macrophytes increase oxygen concentrations in the rhizoplane due to continuous oxygen leakage from roots, thus generating an aerobic microenvironment, at the root surface that expands within the anaerobic sediment. We have quantified the potential effects of radial oxygen loss on the abundance and composition of ammonia oxidizers. *Typha angustifolia* roots collected from a CW and from an estuarine salinity gradient were divided into sections and analysed independently. The presence of vegetation affected the abundance of *amoA* gene as well as the nitrifier community structure. The abundance of ammonia oxidizing *Bacteria* was favoured in sediments in comparison to roots, which could be related to differences in the root microenvironment including the oxygen release rates. However, higher diversity of ammonia oxidizing archaea, which could be adapted to low oxygen concentrations, were mainly present in sediments, where the oxygen concentration is presumably lower than in roots. The oxygen release from roots was higher in the most mature sections of roots, though it seemed not to have a determinant effect on microbial communities. Nonetheless, we confirmed that radial oxygen loss is confined to tiny portions of *Typha* roots (<20% of the surface) having a limited effect on the nitrifying community.

Heterotrophic and autotrophic denitrification processes were analysed in a consolidated rock aquifer (limestone and marls), with a porosity related to fracture networks. Several approaches were used for this purpose, isotopic and microbiological methods, each of them providing distinct but complementary information about denitrification reactions, attenuation rates and their occurrence in the aquifer. We have investigated the contribution of both approaches to describe potential denitrification and microbial community structure.

Isotopic methods indicated the origin of nitrate (fertilization using manure) and that denitrification occurred, reaching a reduction of near 25% of the nitrate mass in groundwater. The studied area was divided into two zones with distinct agricultural pressures and, consequently, nitrate concentrations in groundwater. Denitrification occurred in both zones at different levels, indicating that attenuation processes took place all along the hydrogeological unit, and that the observed levels could be attributed to a larger flow path or, in a minor extent, to mixing processes that mask the actual denitrification rates. A correlation was observed between *nirS* and *nirK* genes and the isotopic composition. However, the groundwater microbiome and the distribution of potential denitrifiers did not reveal a major influence on denitrification level observed by isotopic methods. This focuses the interest of microbiological analysis to identify functional genes among the microbiome which should be complemented with additional, activity based, determinations.

Overall, the use of model environments and techniques allowed us to derive some key conclusions that can be generalized to some extent. First, we confirmed that mostly, changes on the microbial community structure were not necessarily linked to a variation of the potential nitrite + nitrate removal rate. Our results highlighted a high recovery of the functionality of an ecosystem service after a severe intervention, which was catalysed by different members of the microbial community. Second, we observed an important effect of vegetation on denitrifiers and ammonia oxidizers communities at different scales. The vegetation effect, mainly on the oxygen availability, could increase nitrite and enhance nitrite reducing activity, thus promoting N removal. Our results emphasize the importance of vegetation in freshwater systems to ensure a good ecosystem functioning. Finally, N attenuation on groundwater indicated that isotopic methods provide information of the overall denitrification ability of the hydrogeological unit, and that genomic data represent the processes actually acting nearby the well. A combination of both approaches is advised to support induced in situ attenuation actions in polluted sites. We are confident the results of the thesis will be considered in future management strategies in CWs as well as in natural freshwater systems to achieve a good water quality.

RESUM

L'amoni i el nitrat són dos dels contaminants més comuns en els sistemes d'aigua dolça, i causen un impacte considerable en el funcionament dels ecosistemes, a més de tenir efectes potencialment negatius en la salut humana. L'eliminació del nitrogen en sistemes d'aigua dolça naturals i construïts s'aconsegueix principalment a través de processos microbians. Per entendre el seu funcionament, les comunitats microbianes han de ser analitzades acuradament, a partir de l'estudi de la seva composició i activitat potencial, així com les interaccions amb altres elements de l'ambient com per exemple les plantes. Els principals microorganismes implicats en l'eliminació del nitrogen de l'aigua són els nitrificants i els desnitrificants. L'activitat conjunta d'aquests dos grups funcionals comporta una eliminació neta de l'amoni que és convertit a nitrogen gas. D'altra banda, els microorganismes implicats en processos alternatius, com per exemple el DNRA, podrien mantenir el nitrogen en forma soluble en l'aigua.

Els aiguamolls i l'aigua freàtica són ambients especialment interessants pel que fa a l'alteració del cicle del N. Els aiguamolls construïts (CW, acrònim de l'anglès *constructed wetlands*) intenten mimetitzar els sistemes naturals pel que fa a l'eliminació de contaminants, i són ambients d'especial interès pel tractaments d'aigües residuals. En aquests sistemes, la vegetació té un rol important ja que la interacció entre plantes i microorganismes és clau en la millora d'alguns processos microbians com la nitrificació. Una de les plantes més utilitzades en els CW és *Typha angustifolia*, la qual es troba present també en els sistemes naturals d'aigua dolça a Catalunya. Un altre ambient amb especial interès científic a Catalunya és l'aigua freàtica, concretament en aquelles àrees que es troben altament impactades degut a l'acumulació de nitrat provinent de l'agricultura intensiva. Com ja és sabut, els processos d'atenuació natural, principalment la desnitrificació, mitiguen l'impacte de les pràctiques de fertilització en els recursos d'aigua subterrània. En aquest sentit, la identificació dels processos microbians que eliminen el N de l'aigua han començat a ser un requeriment indispensable per una bona gestió de la qualitat de l'aigua en diferents sistemes.

En els aiguamolls construïts, la desnitrificació sovint representa més d'un 60% de l'eliminació del nitrogen i està afectada per pràctiques de gestió com per exemple el dragatge de sediment (i l'eliminació de vegetació), que poden causar un impacte en les propietats del sediment i en la microbiota. En aquest treball es va quantificar quin era l'efecte que causava

el dragatge en un CW de flux superficial sobre l'activitat de reducció desassimilatòria del nitrit. Aquests estudis es van realitzar a partir de l'anàlisi de l'estructura i l'activitat de la comunitat microbiana. En el CW Empuriabrava estudiat, les activitats potencials de desnitrificació i DNRA van anar en concordança amb els canvis en les condicions fisicoquímiques que es van produir després del dragatge i l'eliminació de la vegetació. El metabolisme principal que es donava al sediment, i que generalment conduïa a l'eliminació total del nitrat, era la desnitrificació. Es va observar una important disminució de les abundàncies dels gens 16S rRNA, *nirK* i *nirS* després del dragatge del sediment, tot i que les proporcions q*nirS*+q*nirK*/q16S rRNA es van mantenir. Les anàlisis realitzades a la comunitat microbiana van mostrar la importància de la vegetació en l'estructura d'aquestes comunitats, i van conduir a la selecció de filotips específics que podrien contribuït al cicle del nitrogen. En aquest sentit, es van detectar una gran resiliència dels desnitrificants en el sistema que minimitzava els efectes de l'impacte, donant lloc a una alta recuperació de les taxes de desnitrificació. Aquestes observacions es troben en concordança amb l'elevada redundància funcional entre els desnitrificants, una situació que comporta que molts microorganismes amb capacitats metabòliques similars es troben presents en el mateix lloc al mateix moment.

L'efecte de les plantes pot ser important per aquelles comunitats microbianes implicades en el cicle del N en les arrels i en els sediments, específicament per l'oxidació de l'amoni, la qual és altament depenent de la disponibilitat d'oxigen. Els macròfits emergents incrementen la concentració d'oxigen a la rizosfera degut a la pèrdua contínua d'oxigen a través de les arrels, el que genera un microambient aerobi a la superfície de l'arrel, que s'expandeix a través d'un sediment generalment anaerobi. En el capítol 4.2, es quantifiquen els efectes potencials de l'oxigen difós a través de les arrels a la comunitat microbiana, i específicament en els oxidadors d'amoni. Les arrels de *Typha angustifolia* es van obtenir d'un CW i de diferents llocs amb diferències de salinitat en l'estuari del riu Daró. Les arrels es van dividir en diferents seccions que es van analitzar independentment. L'abundància del gen *amoA*, així com l'estructura de la comunitat nitrificant, es va veure afectada per la vegetació. L'abundància dels bacteris oxidadors d'amoni es trobava afavorida en el sediment en comparació amb les arrels, el que podria està relacionat amb el microambient que es dona a l'arrel, en part degut a la difusió d'oxigen a la seva superfície. De tota manera, la major diversitat d'arqueus oxidadors d'amoni es trobava al sediment principalment, on la concentració d'oxigen és presumiblement inferior a la de les arrels. La difusió d'oxigen a través de les arrels fou major a les seccions més madures de l'arrel, tot i que això no va tenir

un efecte determinant en les comunitats microbianes. Tot i això, vam poder confirmar que la difusió d'oxigen a les arrels de *Typha* es dona en una zona molt reduïda de les arrels (<20% de la superfície), i té segurament un efecte limitat en la comunitat nitrificant a gran escala.

La desnitrificació autotròfica i heterotròfica es van analitzar en un aqüífer consolidat (roques calcàries i margues) amb una porositat relacionada amb la fracturació. Amb aquest objectiu, es van utilitzar dos mètodes, basats en tècniques isotòpiques i microbiològiques, que proporcionen informació diferent però alhora complementària de les reaccions de desnitrificació, taxes d'atenuació i la seva ocurrència en l'aqüífer. Es va estudiar la contribució d'ambdues aproximacions per tal de descriure la desnitrificació potencial i l'estructura de la comunitat microbiana. Els mètodes isotòpics indiquen que en els pous estudiats de la zona d'Osona l'origen del nitrat és bàsicament per purins i que la desnitrificació es dona en l'aqüífer i representa fins a un 25% de reducció del contingut del nitrat. L'àrea estudiada va ser dividida en dues zones amb diferents pressions agrícoles i, conseqüentment, diferents concentracions de nitrat en l'aigua. La desnitrificació era rellevant en ambdues zones en diferents nivells, indicant que els processos d'atenuació ocorrien al llarg de tota la unitat hidrogeològica i que els nivells observats podrien ser atribuïts al recorregut del flux d'aigua. En menor mesura, els processos de barreja que emmascararien, en part, les taxes reals de desnitrificació contribueixen també a la disminució del nitrat. Es va observar que l'abundància de gens desnitrificants i la composició isotòpica estaven correlacionades. Tot i això, el canvis en el microbioma de l'aigua subterrània i, més concretament en la distribució dels gèneres potencialment desnitrificants, no estaven del tot relacionats amb les diferències en els nivells de desnitrificació estimats per mètodes isotòpics. Això centra l'interès de les anàlisis microbiològiques per identificar els gens funcionals que es troben en el microbioma, que ha de ser complementat amb tècniques addicionals basades en la determinació de l'activitat.

En general, la utilització de dos ambients model (aiguamolls i aigua freàtica) i l'aplicació de diferents tècniques ens ha permès obtenir algunes conclusions claus que es podrien generalitzar fins a cert punt. En primer lloc, es confirma que alguns dels efectes observables en la comunitat microbiana no afecten de forma considerable a la taxa d'eliminació de nitrit i nitrat. Els resultats obtinguts suggereixen una alta recuperació de la funcionalitat dels ecosistemes després d'un impacte important. Hem pogut determinar l'efecte de la vegetació sobre les comunitats desnitrificants i oxidadores d'amoni a diferents

escales, proveint nitrit als reductors de nitrit i d'aquesta manera promovent l'eliminació de N de l'aigua. Així doncs, els nostres resultats emfatitzen la importància de la vegetació en els sistemes d'aigua dolça per assegurar el bon funcionament de l'ecosistema. Finalment, els estudis sobre l'atenuació del nitrogen en l'aigua subterrània ens indiquen que els mètodes isotòpics informen sobre la capacitat de desnitrificació general a la unitat hidrogeològica, mentre que les dades genòmiques informen de processos reals que es donen en un moment determinat i en un punt concret. Malgrat aquesta divergència aparent, la combinació d'ambdues tècniques ens permet disposar de dades complementàries per tal de gestionar millor accions d'atenuació en llocs contaminats. Confiem que els resultats d'aquesta tesi contribuiran al desenvolupament de futures estratègies de gestió de CW, així com de sistemes naturals, per tal de millorar la qualitat de l'aigua.

RESUMEN

El amonio y el nitrato son dos de los contaminantes más comunes en los sistemas de agua dulce. Son causantes de importantes impactos en el funcionamiento de estos ecosistemas, llegando a describirse efectos potencialmente negativos para la salud humana en casos extremos o continuados de contaminación. La eliminación del nitrógeno en sistemas de humedales de agua dulce naturales y construidos se consigue principalmente a través de procesos microbiológicos. Para entender su funcionamiento, las comunidades microbianas deben ser analizadas cuidadosamente, estudiando su composición y su actividad potencial, a la vez que las interacciones que se dan entre los microorganismos y otros componentes del ambiente como las plantas. Los principales microorganismos implicados en la eliminación efectiva del nitrógeno del agua son los nitrificantes y los desnitrificantes. La actividad combinada de ambos determina la eliminación neta de amonio hasta nitrógeno gaseoso. Por otro lado, procesos paralelos como la reducción desasimiladora a amonio (DNRA), podría ser contraproducente ya que representaría el mantenimiento del nitrógeno soluble en el agua.

Los humedales y el agua subterránea son *hot-spots* en referencia a la alteración del ciclo del N. Los humedales construidos (CW, acrónimo del inglés *constructed wetlands*) intentan mimetizar los sistemas naturales para eliminar contaminantes, siendo ambientes de especial interés para el tratamiento de aguas residuales. En estos sistemas, la vegetación tiene un rol importante, ya que la interacción entre plantas y microorganismos puede mejorar algunos procesos microbianos como la nitrificación. Una de las plantas más utilizadas en los CW es *Typha angustifolia*, la cual se encuentra también como planta dominante en muchos de los sistemas naturales de agua dulce en Catalunya. Otro ambiente con un relevante interés científico en Catalunya es el agua subterránea. En concreto, aquellas zonas que se encuentran altamente impactadas debido a la acumulación de nitrato debido a prácticas de agricultura intensiva, son de especial interés para científicos y gestores de los recursos hídricos. Como es sabido, los procesos de atenuación natural mitigan el impacto de las prácticas de fertilización en los recursos hídricos subterráneos. Por consiguiente, la identificación de los procesos microbianos que eliminan el N del agua ha empezado a ser un requisito indispensable para una buena gestión de la calidad del agua en distintos sistemas.

En los humedales construidos, la desnitrificación a menudo comprende más del 60% de la eliminación del nitrógeno y se ve afectada, supuestamente, por las prácticas de gestión como el dragado del sedimento (y la eliminación de la vegetación) que pueden causar un

impacto en las propiedades del sistema. En este trabajo se ha cuantificado cuál era el efecto que causaba en las actividades de reducción desasimiladora del nitrito un evento de dragado en un CW de flujo superficial. Para este estudio se ha analizado la estructura y la actividad de la comunidad microbiana. En el CW de Empuriabrava, la tasa potencial para la desnitrificación y para la DNRA fueron acorde con los cambios en las condiciones fisicoquímicas que se produjeron después del dragado y la eliminación de la vegetación. El metabolismo principal que se daba en el sedimento, y que representaba la eliminación total del nitrato, era la desnitrificación. Se observó una importante disminución en las abundancias de los genes 16S rRNA, *nirK* y *nirS* después del dragado del sedimento, aunque las proporciones de q*nirS*+q*nirK*/q16S rRNA se mantuvieron en valores similares. Los análisis realizados en la comunidad microbiana mostraron la importancia de la vegetación en estas comunidades, seleccionándose filotipos específicos que podrían contribuir en el ciclo del nitrógeno. En este sentido, se detectó una gran resiliencia de los desnitrificantes en el sistema de humedales, hecho que minimizaba los efectos del dragado conduciendo a una alta recuperación de las tasas de desnitrificación. Estas observaciones concuerdan con la alta redundancia funcional entre las bacterias desnitrificantes, sugiriendo que, en este caso particular, se da una situación en que diversas especies con capacidades metabólicas parecidas se encuentran presentes en el mismo lugar en el mismo momento.

El efecto de las plantas puede ser importante para aquellas comunidades microbianas implicadas en el ciclo del N en sedimentos en general y en particular asociadas a las raíces. Este efecto puede ser relevante para la oxidación del amonio, ya que es altamente dependiente de la disponibilidad de oxígeno. Los macrófitos emergentes aumentan la concentración de oxígeno en la rizosfera debido a la difusión de oxígeno a través de sus raíces, lo que genera un microambiente aerobio en la superficie de la raíz, que se expande a través de un sedimento generalmente anaerobio. En esta tesis se han cuantificado los efectos del oxígeno difundido a través de las raíces sobre la comunidad microbiana, y específicamente sobre las bacterias y arqueas oxidantes de amonio. Se utilizaron como modelo las raíces de *Typha angustifolia* que se obtuvieron de un sistema de humedales construidos y de diferentes localidades en el estuario del río Daró con diferente grado de salinidad. Las raíces se dividieron en tres secciones distintas que se analizaron independientemente. La abundancia del gen *amoA*, así como la estructura de la comunidad nitrificante, se vio afectada por la vegetación. La abundancia de bacterias oxidantes de amonio fue favorecida en el sedimento en comparación con las raíces, lo que podría estar relacionado con el microambiente que se

encuentra en la raíz, parcialmente debido a las pérdidas de oxígeno por ésta. Aun así, la mayor diversidad de arqueas oxidantes de amonio, capaces de adaptarse a bajas concentraciones de oxígeno, se encontraba en el sedimento principalmente, donde la concentración de oxígeno es presumiblemente más baja que en las raíces. La difusión de oxígeno a través de las raíces fue mayor en las secciones más maduras de la raíz, aunque ello no afectó de manera importante a las comunidades microbianas. De toda manera, pudimos confirmar que la pérdida radial de oxígeno se ve reducida a una parte muy pequeña de la raíz de *Typha* (<20% de la superficie), teniendo así un efecto limitado en la comunidad nitrificante a gran escala.

La desnitrificación autotrófica y heterotrófica se analizó en un acuífero consolidado (rocas calcáreas y margas), con una porosidad relacionada con la fracturación. Existen varios métodos que se utilizan con este objetivo, como las técnicas isotópicas y microbiológicas que proporcionan información diferente, pero a la vez complementaria referente a las reacciones de desnitrificación, tasas de atenuación y su ocurrencia en el acuífero. Se estudió la contribución de ambas aproximaciones metodológicas para describir la desnitrificación potencial y la estructura de la comunidad microbiana. Los métodos isotópicos indicaron el origen principal del nitrato, la fertilización usando purines. Además, los análisis isotópicos permitieron determinar que la desnitrificación ocurría en general en todos los pozos analizados, llegando a reducir un 25% el contenido del nitrato en el agua subterránea. El área estudiada se dividió en dos zonas con distintas presiones agrícolas y, consecuentemente, diferentes concentraciones de nitrato en el agua. La desnitrificación se daba en ambas zonas a distintos niveles, indicando que los procesos de atenuación ocurrían a lo largo de la unidad hidrogeológica y que los niveles observados se podrían atribuir al recorrido del flujo de agua o, en menor medida, a procesos de mezcla que podían enmascarar las tasas reales de desnitrificación. Se observó que la abundancia de genes desnitrificantes y la composición isotópica estaban correlacionados. Aun así, el microbioma del agua subterránea, y más concretamente la distribución de los géneros potencialmente desnitrificantes, no mostraban resultados análogos a los deducidos a partir de los métodos isotópicos en relación a los niveles de desnitrificación. Esto centra el interés de los análisis microbiológicos para identificar los genes funcionales que se encuentran en el microbioma, que a su vez ha de ser complementado con técnicas adicionales basadas en la determinación de la actividad.

En general, la utilización de ambientes modelo y distintas técnicas nos permitió obtener algunas conclusiones que se podrían generalizar hasta cierto punto. En primer lugar,

confirmamos que efectos significativos en la comunidad microbiana no afectaban a la tasa de eliminación de nitrito y nitrato. Los resultados obtenidos remarcaron una alta recuperación de la funcionalidad del ecosistema tras un importante impacto, que fue catalizada por diferentes miembros de la comunidad microbiana. Además, pudimos determinar el efecto de la vegetación en las comunidades desnitrificantes y oxidantes de amonio a diferentes escalas, suministrando nitrito para los reductores de nitrito y de esta manera promoviendo la eliminación de N del agua. Así pues, nuestros resultados enfatizan la importancia de la vegetación en los sistemas de agua dulce para asegurar un buen funcionamiento del ecosistema. Por último, se ha podido comprobar que el uso de métodos complementarios amplía la información disponible sobre un mismo problema. Por ejemplo, los métodos isotópicos proporcionan información sobre la capacidad de desnitrificación general en la unidad hidrogeológica, mientras que los datos genómicos tienden a representar los procesos reales que se dan en un momento determinado y un punto concreto. La combinación de ambas técnicas es importante para gestionar acciones de atenuación en lugares contaminados. Confiamos en que los resultados de esta tesis se tendrán en cuenta en futuras estrategias de gestión de CW, así como de sistemas naturales, para conseguir asegurar una buena calidad del agua.

1. INTRODUCTION

1.1. The nitrogen cycle in nature, a prokaryotic kingdom

3.1.1. Main steps in the nitrogen cycle

The growth of all organisms depends on the availability of mineral nutrients, being nitrogen (N) one of the most important: N is essential for life. In nature, N is found through various forms, which result from sequential chemical transformations, constituting de nitrogen cycle. Nitrogen is the fifth most abundant element in our solar system, over 78 percent by volume of the atmosphere is dinitrogen gas (N_2). Despite the importance of nitrogen and its overwhelming abundance in the atmosphere, nitrogen is often the nutrient that limits primary production in many ecosystems, since plants and animals are not able to fix nitrogen gas (Canfield et al., 2010). The biogeochemistry of nitrogen is almost entirely dependent on reduction-oxidation (redox) reactions, primarily mediated by microorganisms, and to a lesser extent on long-term recycling through the geosphere (Bernhard, 2010). Most relevant N transformation reactions fall into the following classifications: nitrogen fixation, nitrification, denitrification, dissimilatory nitrite reduction to ammonia (DNRA) and anammox (Figure 1.1). Moreover, organic matter mineralization (ammonification) and assimilation complete the nitrogen cycle through the biosphere.

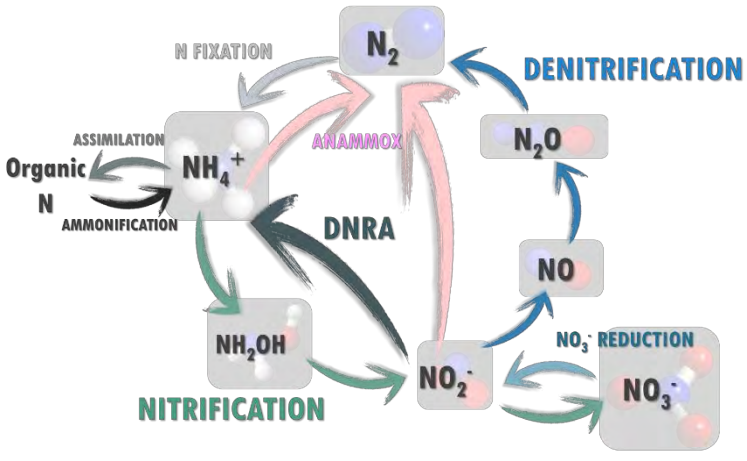

Figure 1.1. Nitrogen cycle. Nitrogen transformation reactions that constitute the nitrogen cycle. Arrow colours indicate main N-transforming processes. N_2: dinitrogen gas, N_2O: nitrous oxide, NO: nitric oxide, NO_2^-: nitrite, NO_3^-: nitrate, NH_2OH: hydroxylamine, NH_4^+: ammonia, Organic N: organic nitrogen.

Bacteria and archaea participate in all possible reactions in the nitrogen cycle and have a preponderant role in many of the nitrogen transformations (Zumft, 1997). Organisms that fix molecular nitrogen are named diazotrophs and many of them can also participate in other nitrogen transformations (Bernhard, 2010; Dixon and Kahn, 2004). Dinitrogen gas is the most abundant nitrogen form on Earth and can be fixed by free living or symbiotic bacteria and archaea by initially reducing it to NH_4^+, which is later assimilated in the form of organic nitrogen ($R\text{-}NH_2$). The nitrogen contained in organic matter ($R\text{-}NH_2$) can be further mobilized to more soluble forms after decomposition and mineralization. Ammonification (or mineralization) is the conversion of organic nitrogen into NH_4^+ in anaerobic conditions and is the main process in the solubilization of N back into water (Moir, 2011). Ammonia can be oxidized to nitrate (NO_3^-) by nitrification in two sequential steps carried out by Ammonia Oxidizing Bacteria and Archaea (AOB and AOA) and by Nitrite Oxidizing Bacteria (NOB), respectively (Dworkin and Falkow, 1992; Könneke et al., 2005). Nitrate can be reduced to N_2, either aerobically or anaerobically, by denitrification, a series of sequential enzymatic reactions catalysed by very different taxonomic groups of bacteria (Philippot and Hallin, 2005; Zumft, 1997). Nevertheless, denitrification is not the only pathway in which nitrite is reduced, there are other metabolisms, such as the dissimilatory nitrite reduction to ammonium (DNRA) or the anammox reaction, which contribute to nitrite reduction. DNRA bacteria reduce nitrite back to ammonia in anaerobic conditions (Burgin and Hamilton, 2007; Koop-Jakobsen and Giblin, 2010; Tiedje, 1988). Moreover, ANaerobic AMMonium OXidation (anammox) is an alternative set of reactions that can take part in dissimilatory nitrite reduction; converting nitrite and ammonia to nitrogen gas under autotrophic conditions (Jetten et al., 1999; Kartal et al., 2013). Overall, N_2 fixation, nitrification, denitrification, anammox and DNRA constitute a true nutrient cycle that governs most nitrogen conversions that occur in nature.

3.1.2. Nitrogen fixation

Atmospheric dinitrogen gas is the largest reservoir of freely accessible nitrogen, but it is biologically available only to prokaryotes. Some eukaryotes (e.g., legumes, termites and herbivores) also support nitrogen fixation, but only in symbiotic association with nitrogen-fixing prokaryotes. *Bacteria* and *Archaea* have the remarkable capacity to fix atmospheric nitrogen to ammonia under environmental conditions, a reaction only mimicked on an industrial scale by a chemical process that requires high temperatures, elevated pressure and

special catalysts (Dixon and Kahn, 2004). Nitrogen fixation is carried out by the nitrogenase, an enzyme composed of two metalloproteins. Depending of the metal cofactors present in the enzymes, nitrogenases can be classified as iron-iron (FeFe), vanadium-iron (VFe) and molybdenum-iron (MoFe) nitrogenases. N_2 fixation is encoded by *nifH*, which is used as a gene marker for the detection of nitrogen-fixing microorganisms (Kuypers et al., 2018; Zehr et al., 2003). Biological nitrogen fixation can be represented by the following equation, in which two moles of ammonia are produced from one mole of nitrogen gas at the expense of 16 moles of ATP and a supply of electrons and protons (hydrogen):

$$N_2 + 8H^+ + 8e- + 16\ ATP = 2NH_3 + H_2 + 16ADP + 16\ Pi$$

Nitrogenase genes are highly conserved but widely dispersed across many prokaryotic phyla suggesting that nitrogen fixation evolved by vertical inheritance and spread by horizontal gene transfer (Canfield et al., 2010). A wide range of *Bacteria* and *Archaea* contain nitrogenase genes providing them with a competitive advantage in environments that are depleted of bioavailable nitrogen.

The ability to fix nitrogen is found in many bacterial phyla, including green sulphur bacteria, *Firmibacteria*, *Actinobacteria*, *Cyanobacteria* and all subdivisions of the *Proteobacteria*. In *Archaea*, nitrogen fixation is mainly restricted to methanogens (Dixon and Kahn, 2004; Inoue et al., 2015). The ability of microorganisms to use nitrogen gas as the sole nitrogen source allow them to be found as free-living organisms in soils and water and also to stablish symbiotic relationships with plant hosts or in termites, conferring many ecological advantages. These relationships are not exempt of physiological constraints because nitrogen fixation is oxygen sensitive and energy dependent (Kuypers et al., 2018). Interactions between plants and associative nitrogen-fixing bacteria which are considered a subset of plant growth-promoting-rhizobacteria (Pii et al., 2015) are the simplest form of nitrogen-fixing symbiosis. Associative nitrogen fixing bacteria respond to root exudates via chemotaxis and colonize the rhizosphere of many plants but typically do not invade plant tissues (Santi et al., 2013). Many species of diazotrophic microorganisms have evolved beyond surface colonization to spread and multiply within plant tissues without causing damage and eliciting significant defence reactions. These bacteria are classified as endophytes due to their tight association with plant tissues (Pedraza, 2008). Endophytes are ubiquitous and their association can be obligate or facultative, exhibiting complex interactions with their hosts that range from mutualism to parasitism (Mus et al., 2016).

3.1.3. Ammonification

In soils and sediments, ammonium released from sedimented organic matter by ammonification can be assimilated as nitrogen source by the plant roots or used as electron source by nitrifying microorganisms. The deposition of organic matter, especially in aquatic ecosystems, can result from episodic eutrophication events such as the rapid sedimentation of annual phytoplankton and cyanobacterial blooms or, in shallower systems where macrophytes are dominant, from the deposition of dead material (Jensen et al., 1990). The quality of the deposited organic matter, i.e. whether it is labile or highly refractory, determines how rapidly it is mineralised (Enríquez et al., 1993). Organic components such as amino acids, purines, pyrimidines and urea are easily hydrolysed and ammonium is readily produced. The mineralisation of complex nitrogenous macromolecules in sediments and soils consists in an initial step where these complex polymers are hydrolysed to their monomeric components following by steps of deamination to release ammonium. Protease and urease, which catalyses the hydrolysis of proteins and urea to NH_3, respectively, affects ammonia dissolution to NH_4^+ and the subsequent volatilization to NH_3 (Hao and Benke, 2008; Herbert, 1999).

Many microorganisms have the ability to degrade organic matter and produce ammonium. *Proteobacteria* and *Firmicutes* are of extreme importance to the global N-cycle due to their high ammonifying activity (Jurado et al., 2014). There are several abiotic factors that impact ammonifiers, such as temperature, NH_4^+ concentration, DOC and pH, as well as the presence of fertilizers, which could lead to ammonification or N retention in soils and sediments (Gurlevik et al., 2004; Huang et al., 2019).

3.1.4. Nitrification

Nitrification is the sequential oxidation of ammonia to nitrate, carried out by *Bacteria* and *Archaea* in two steps: ammonia oxidation to nitrite (AOA and AOB), and later nitrite oxidation to nitrate (NOB). This process was discovered more than a century ago when Sergei Winogradsky isolated the first chemolithoautotrophic bacterium that grew by nitrification using ammonia or nitrite as energy source and electron donor (Winogradsky, 1892). Winogradsky stablished the fundamentals for chemoautotrophy, a "chlorophyll action without chlorophylls". Ammonia oxidation to nitrite, which transiently produces hydroxylamine as an intermediate step, is catalysed by the enzymes ammonia monooxygenase

(AMO) and hydroxylamine oxidoreductase in *Bacteria* (HAO) (Kowalchuk and Stephen, 2001; Lehtovirta-Morley, 2018; Prosser and Prosser I., 1989) (Figure 1.2). Many studies have analysed genes coding for the alpha subunit (*amoA*) of the multi enzymatic complex in order to study the distribution, abundance and activity of AOB and AOA in natural communities (E. Costa et al., 2006; Rotthauwe et al., 1997). Phylogenetically constrained groups of ammonia-oxidizing bacteria (AOB) and archaea (AOA) perform this reaction, and are known as ammonia oxidizing microorganisms (AOM). Specifically, AOB comprise members of the family *Nitrosomonadaceae* (Class *Betaproteobacteria*) and the genus *Nitrosococcus* (Kowalchuk and Stephen, 2001; Lehtovirta-Morley, 2018) within *Gammaproteobacteria*. In turn, AOA have been reassigned to the phylum *Thaumarchaeota* (Pester et al., 2012; Spang et al., 2010). Nitrite oxidation, which has been so far described only in *Bacteria*, is catalysed by the enzyme nitrite oxidoreductase (NXR), the *nxrA* and *nxrB* genes are the functional markers to detect NOB (Klotz and Stein, 2008). The known NOB belong to seven genera in four bacterial phyla: *Nitrobacter, Nitrotoga, Nitrococcus, Nitrospina, Nitrolancea, Nitrospira* and "*Candidatus* Nitromaritima" (Daims et al., 2016). All NOBs possess gram-negative cell envelopes except *Nitrolancea hollandica* which stains gram-positive and forms thick cell wall layers (Sorokin et al., 2012).

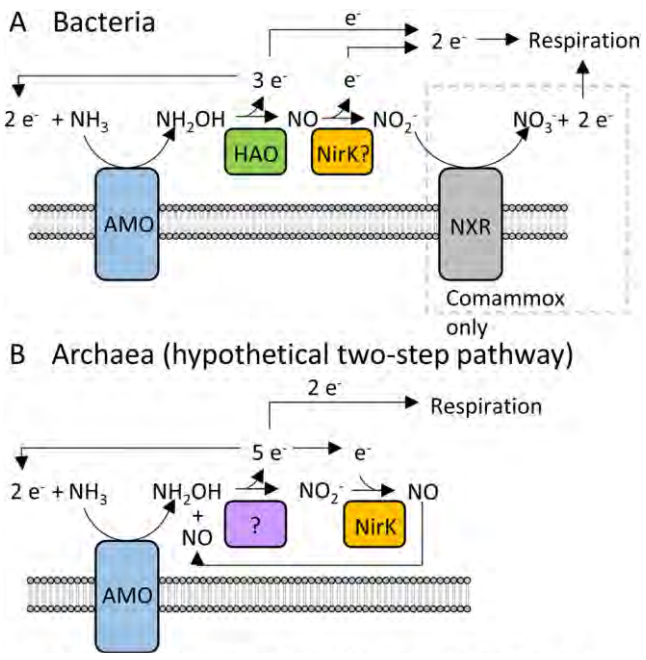

Figure 1.2. Nitrification process. Pathways of ammonia oxidation in bacteria and archaea. (A) AOB and comammox *Nitrospira*. (B) Hypothetical two-step model in AOA. The participation of NirK has not been confirmed in any of the models (adapted from Lehtovirta-Morley, 2018).

Until recently, all known AOM and NOB distributed in separate phyla and no isolate was found that could completely oxidize ammonium to nitrate. However, it has been demonstrated by metagenomic studies that *Nitrospira*-like bacteria are able to carry out a complete nitrification, including the two oxidation steps, a process that have been named as comammox (COMplete AMMonia OXidation) (Figure 1.2) (Daims et al., 2015; van Kessel et al., 2015). The evolutionary history of ammonia oxidation in *Nitrospira* is unknown. Based on their ammonia monooxygenase (AMO) and hydroxylamine dehydrogenase (HAO) sequences, beta-proteobacterial ammonia oxidizing bacteria (AOB) are the most reliable candidates to comammox differentiation (Daims et al., 2015; Palomo et al., 2018).

Ammonia Oxidizing Bacteria, Archaea and comammox usually coexist in most environments (Caliz et al., 2015; Xiao et al., 2017). Based on the distribution of *amoA* genes, it seems that AOA are ubiquitous on Earth. AOA are adapted to low ammonia concentrations, and their occurrence and activity in hot springs, in pristine environments, in deeper soil layers and in soils of low pH, suggest that many ammonia-oxidizing archaea are more adapted to extreme growth conditions and severe oligotrophy compared to AOB species, thus indicating a clear niche separation between them (Martens-Habbena et al., 2009; Prosser and Nicol, 2012; Schleper, 2010; Xiao et al., 2017). Moreover, AOA and AOB are differentially selected in the rhizosphere of plants thus showing a strong dependence to plant N-use strategy (Caliz et al., 2015; Thion et al., 2016; Trias et al., 2012). Similarly, comammox *Nitrospira* are adapted to environments with low ammonia concentration but show a competitive advantage over AOB and AOA in low oxygen environments and/or microaerophilic niches (Palomo et al., 2018).

3.1.5. The fate of nitrite, a N cycle keystone

Nitrate and nitrite produced by nitrifying microorganisms can be used as alternative electron acceptors for energy production in the absence of oxygen (Zumft, 1997). Nitrate is one of the major nitrogen sources for eukaryotes, *Bacteria* and *Archaea* that contain assimilatory nitrate reductases (NAS) (Kuypers et al., 2018). Nitrate can be dissimilatory reduced into nitrite by many *Bacteria* and *Archaea* (Philippot, 2005). Dissimilatory nitrate reduction is catalysed by two enzyme groups: the membrane-bound nitrate reductase (NAR) and the free periplasmic nitrate reductases (NAP). The two enzyme types are not exclusive and can occur concomitantly in the same organism (Richardson et al., 2001; Roussel-Delif et al., 2005). Further, nitrite can be reduced into other N compounds via denitrification,

dissimilatory nitrite reduction to ammonium (DNRA) or anaerobic ammonium oxidation (anammox). Dissimilatory nitrite reduction pathways consist in sequential enzymatic reactions (Jetten et al., 1999; Tiedje, 1988; Zumft, 1997). Actually, denitrification and DNRA pathways start reducing nitrate into nitrite through the reaction catalysed by *napA* and *narG* genes (Klotz and Stein, 2008; Zumft, 1997)), though most of DNRA or denitrifying organisms do not contain all genes necessary to complete the pathway (Jones et al., 2008; Welsh et al., 2014).

Denitrification consists in three sequential enzymatic reactions catalysed by metalloproteins that differ between organisms (Park and Yoo, 2009). First, nitrite reduction to nitric oxide (NO) is catalysed 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 cd1-NIR) (Figure 1.3). While the two enzymes perform the same reaction in the denitrification pathway, they are non-homologous and were thought to be mutually exclusive in denitrifying organisms (Jones et al., 2008). Recently, isolates containing the two nitrite reductases have been obtained (Graf et al., 2014; Wittorf et al., 2018a). The denitrification pathway is a widespread trait and the diversity of denitrifying bacteria is high, including very different taxonomic groups (Ligi et al., 2014a; Philippot and Hallin, 2005). Recent developments in the genome analysis of cultured and uncultured strains have shown that many diverse microorganisms possess *nirK* or *nirS*, including members of highly distant phyla, such as *Proteobacteria*, *Nitrospirae*, *Actinobacteria*, *Bacteroidetes*, *Spirochaetes*, *Chloroflexi* and *Firmicutes* within the bacteria, and *Euryarchaeota* and *Crenarchaeota* within the *Archaea* (Bartossek et al., 2010; Cantera and Stein, 2007; Mardanov et al., 2012; Moir, 2011; Nolan et al., 2009; Wei et al., 2015).

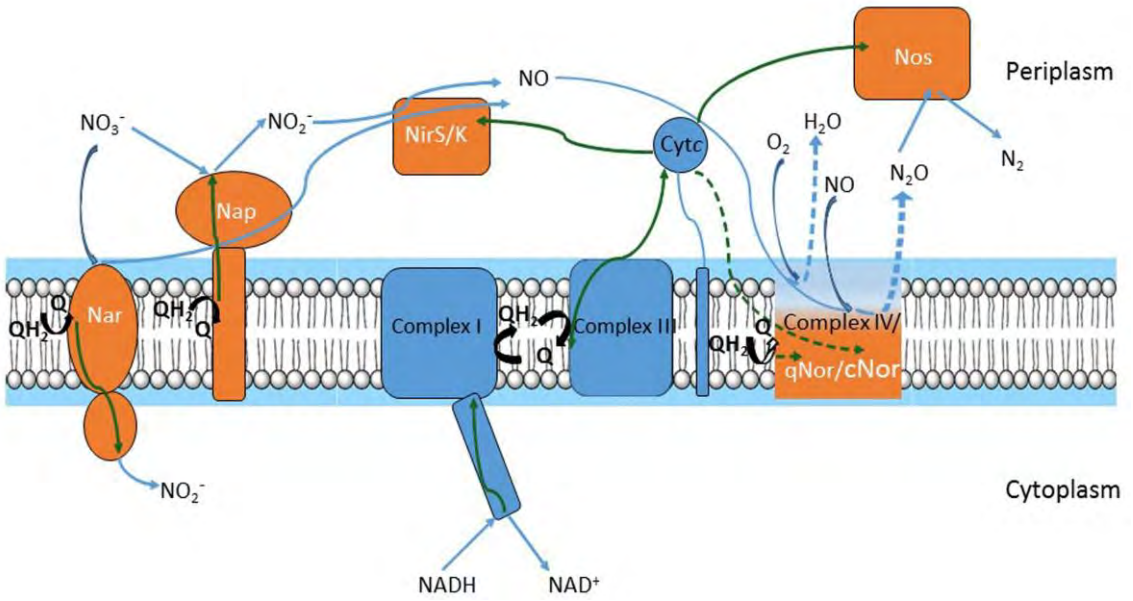

Figure 1.3. Denitrification pathway. Schematic representation of the most common metalloenzymes in the cell envelope. Nap, periplasmic nitrate reductase. Nap, membrane-bound nitrate reductase. NirS/K, periplasmic nitrite reductase copper containing and periplasmic nitrite reductase haem containing. qNor/cNor, membrane-bound nitric-oxide reductase. Nos, periplasmic nitrous oxide reductase (from Santana et al., 2017).

DNRA is another pathway that plays an important role on nitrite reduction depending on the environmental conditions. Nitrite reduction to ammonia has a significant relevance in the N cycle since it blocks the complete denitrification bypassing a true N elimination to a highly soluble compound, ammonia (Brunet and Garcia-Gil, 1996; Burgin and Hamilton, 2008; Koop-Jakobsen and Giblin, 2010; Tiedje, 1988). The different enzymes between DNRA and denitrification involve a cytochrome c nitrite reductase, the NrfA protein, which catalyses the reduction of nitrite to ammonia which is encoded in the *nrfA* gene (Simon, 2002; Welsh et al., 2014). NrfA shares evolutionary history with octaheme c-type cytochrome proteins like the hydroxylamine oxidoreductases and octaheme nitrite reductases (Klotz and Stein, 2008). What distinguishes NrfA from these other c-type cytochromes is a pentaheme structural core with an unusual CXXCK or CXXCH motifs in the first haeme binding domain (Klotz and Stein, 2008; Simon, 2002).

Both denitrification and DNRA, although competing for the same substrate, are mainly anaerobic processes using organic matter as electron donor and carbon source. However, the prevalence of any of the two processes mainly depends on nitrite availability

(Dong et al., 2009; Welsh et al., 2014). For this reason, DNRA bacteria, together with Nir-containing denitrifiers, are also known as nitrite reducers.

DNRA ability in bacteria is diverse due to the function of nitrite detoxification by *nrfA* gene, suggesting that this gene is transferable and was acquired independently by members of different taxonomic groups (Simon, 2002; Welsh et al., 2014)). DNRA activity is distributed among *Proteobacteria*, *Verrucomicrobia*, *Acidobacteria*, *Planctomycetes*, *Firmicutes* and *Chloroflexi* (Bu et al., 2017; Welsh et al., 2014). Tiedje (1988) listed several genera of soil DNRA bacteria, which are either obligate anaerobes (*Clostridium*), facultative anaerobes (*Citrobacter*, *Enterobacter*, *Erwinia*, *Escherichia*, *Klebsiella*) or aerobes (*Bacillus*, *Pseudomonas*), showing the ability to carry out this pathway at different conditions. Moreover, many bacteria capable of DNRA are found in the *Enterobacteriaceae,* which is a phylum in which known denitrifiers are less frequent (Guo et al., 2016; Zumft, 1997). DNRA is also found in *Archaea*, including *Thaumarchaeota*, *Euryarchaeota* and *Crenarchaeota* (Bu et al., 2017).

Despite the functional equivalence between NirK, NirS and NrfA nitrite reductases habitat selection for each nitrite reductase has been shown according to environmental parameters. There are many environmental factors which influence the competition between Nir and NrfA containing bacteria, including labile organic carbon, nitrate availability, the ratio of electron donor/ acceptor (carbon/nitrate), sulphide concentration, soil sand content, pH, microbial generation time, NO_3^-/NO_2^- and temperature (An and Gardner, 2002; Burgin and Hamilton, 2007; Dong et al., 2009; Friedl et al., 2018; Nizzoli et al., 2010; Papaspyrou et al., 2014). Moreover, many other parameters, such as salinity, pH, organic matter lability, nitrate content and redox potential, also affect the enrichment of one of either type of NIR-containing bacteria (Jones and Hallin, 2010; Lindemann et al., 2015).

Finally, the last pathway where nitrite can be used as an electron acceptor is anammox, in which ammonium and nitrite are converted to N_2 and nitrate favouring a net gas production (Jetten et al., 1999)). The first step of the reaction is the reduction of nitrite to nitric oxide by a typical denitrifying nitrite reductase (NirS or NirK). Membrane associated hydrazine synthase catalyse the second step, ammonium and NO are converted to hydrazine (N_2H_4). The last step consists in the oxidation of hydrazine to N_2, which is hypothesized to be catalysed by a variant of an already known enzyme, hydroxylamine oxidoreductase (HAO) (Kartal and Keltjens, 2016).

Anammox bacteria belong to the phylum *Planctomycetes* and, to date, there are five known *Candidatus* anammox genera: "*Candidatus* Kuenenia", "*Candidatus* Brocadia", "*Candidatus* Anammoxoglobus", "*Candidatus* Jettenia" and "*Candidatus* Scalindua" (Sonthiphand et al., 2014). Anammox bacteria were first discovered in WWTPs, but have shown to have a cosmopolitan distribution and are present in many environments. However, little is known about how environmental factors (i.e. salinity, ammonium, nitrite, organic content) control their spatial and temporal distribution (Humbert et al., 2010; Sonthiphand et al., 2014).

3.1.6. Down to gaseous compounds, ins and outs

Dissimilatory nitrite reduction pathways can produce soluble compounds as end products (i.e. DNRA) or gaseous compounds (denitrification and anammox). It is only the latter, the production of gaseous compounds, which is highly relevant in the environment since it may represent a net loss of N in a given system.

Nitric oxide, the main product of NIR, is reduced to nitrous oxide (N_2O), catalysed by nitric oxide reductases (NOR), which are generally membrane-bound and are highly heterogeneous among bacteria. Further, periplasmic nitrous oxide reductase (NOS) (Figure 1.3), encoded by genes *nosZ*I and *nosZ*II, catalyses the final step in the denitrification reaction and is responsible for the N_2 formation (Jones et al., 2013; Philippot, 2002; Throbäck et al., 2004). Denitrifying organisms presents many different subsets of denitrifying enzymes which can be found in bacterial species and, though a complete set of genes may exist in a denitrifying prokaryote, most of the denitrifying organisms isolated so far possess truncated pathways (Jones et al., 2013, 2008). This is a consequence of evolutionary driving forces acting on genes coding for the denitrification pathway, i.e. horizontal gene transfer (HGT), convergent evolution of different structural types and lineage sorting (Jones et al., 2008). Microorganisms lacking the last step in the denitrification process are of specific relevance for the environment since truncated denitrification is a prominent source of the greenhouse gas N_2O (Müller et al., 2014). However, other bacteria, which can exhibit a complete lack of NIR genes, participate exclusively in the reduction of nitrous oxide, thus diminishing the net production of green-house effect gases (Graf et al., 2014; Sanford et al., 2012) (Figure 1.4).

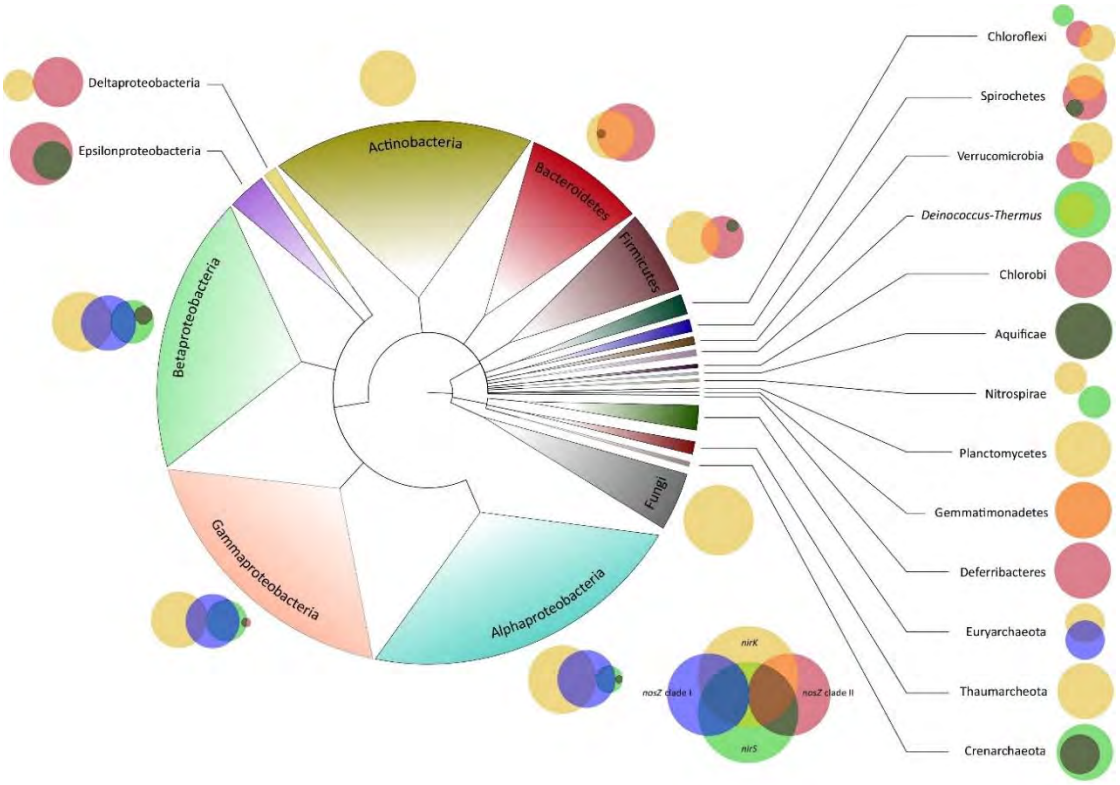

Figure 1.4. Taxonomic distribution of denitrifiers and presence of key genes within phyla. Genomes harbouring genes encoding the copper- and cytochrome cd1-type nitrite reductases (*nirK* and *nirS*, respectively) involved in denitrification, as well as the different variants of the *nosZ* gene encoding the N_2O reductase. The centre phylogram is based on NCBI taxonomic rankings, with clade size proportional to the number of genomes found within each phylum or, for the *Proteobacteria*, class grouping. Venn diagrams illustrate the frequency of *nir* and *nosZ* gene co-occurrences in the genomes of each taxonomic group, where circle size is proportional to the frequency of gene occurrence within each group, and overlap indicates degree of gene co-occurrences. Color-coding for each combination is shown at lower right (from Hallin et al., 2018).

Denitrification is not the only pathway that produces nitrous oxide (Hallin et al., 2018). In nitrification, the intermediate of ammonium oxidation (hydroxylamine, NH_2OH) can be abiotically oxidized, leading to N_2O formation (Bremner et al., 1980; Heil et al., 2016). However, the majority of N_2O emitted by ammonia oxidation is attributed to nitrifier denitrification, which could occur during O_2 limitation and produce nitrous oxide as a final product (Kool et al., 2011; Shaw et al., 2006; Wrage-Mönnig et al., 2018). Moreover, some DNRA bacteria can also produce N_2O, and even link N_2O reduction to N_2 with energy

conservation (Jurado et al., 2017; Mania et al., 2014). The contribution of dissimilatory nitrite reduction to overall N_2O budgets is uncertain, although most studies conclude that this pathway have less contribution to N_2O emissions compared to denitrification and ammonia oxidation (Butterbach-Bahl et al., 2013; Jurado et al., 2017; Sun et al., 2018). Then, greenhouse gas emission via N cycle is common in different pathways. The ability to reduce N_2O is a taxonomically widespread trait, and *nosZ* gene was found in approximately 12% of sequenced microbial genomes, including 12 phyla (Hallin et al., 2018). However, N_2O reduction is highly dependent on environmental conditions, such as NH_4^+, NO_x^- and O_2 concentrations, pH, salinity, etc. (Domeignoz-Horta et al., 2018; Hallin et al., 2018; Jurado et al., 2017; Kuypers et al., 2018).

1.2. Altered Nitrogen cycles

3.1.7. N contamination due to anthropogenic activities

Human activities are changing the balance of nitrogen on Earth. Burning fossil fuels for energy, intensive agriculture and disposal of organic wastes have an effect on the N cycle. This intensive practices has mainly negative consequences to the ecosystem functioning and need to be considered to manage N (Seelig and Nowatzki, 2017). Groundwater contamination by N is commonly related to the conditions and activities around water wells, as livestock, fertilizer storage and deposition or the presence of septic systems. In most crops, regular applications of N fertilizer are used to meet plant requirements for optimum yield. In fact, surface water contamination by N has been shown to be more prevalent in agricultural areas compared to other land-uses. It has been estimated that approximately 60 % of fertilizer nitrogen (N) used in agriculture is never incorporated into plants, and instead washes out of the soil into rivers or ground- waters, primarily as nitrate. Moreover, contamination of streams with ammonia (NH_3) from municipal sewage treatment is common downstream of urban areas (Canfield et al., 2010; Domeignoz-Horta et al., 2018; Seelig and Nowatzki, 2017; Stuart and Lapworth, 2016). Anthropogenic activity has increased contamination by nitrogen compounds in soils and water bodies, having implications at the global scale, such as the increase on greenhouse gases emission, specifically N_2O (Butterbach-Bahl et al., 2013; Domeignoz-Horta et al., 2018). The increase of anthropogenic discharge coupled to the high stability and solubility of ammonia and nitrate have led to their accumulation in freshwater bodies since decades ago (Benedict et al., 1998; Mockler et al.,

2017), limiting the use of natural water sources for human consumption (Park and Yoo, 2009; Shrimali and Singh, 2001).

Nitrate not only impacts on water quality, but it is accumulated in vegetables and fruits (Katan, 2009). Nitrate has been pointed as a hazard for human health implicated in methaemoglobinaemia or the "blue baby syndrome" (Knobeloch et al., 2000), though this disease is associated to other factors as inheritance or induction by chemical compounds (Fewtrell, 2004). Additionally, in different parts of the body (e.g. oral cavity, stomach, bladder, or intestines) nitrate-reducing bacteria can produce nitrosamines, which have been potentially related to non-Hodgkin's lymphoma and gastric cancers (Chang and Parsonnet, 2010; Winneberger and J.H.T., 1981). Nitrate *per se* has not been shown to produce a carcinogenic effect, but when converted into nitrite, it may react with blood amines and amides to produce nitrosamines that have carcinogenic potential (Garcia and Teixeira, 2017; Magkos et al., 2006). Environmental concerns are also associated to nitrite accumulation because, together with ammonia, nitrite is potentially toxic and has been implicated in cases of massive fish mortality and eventual losses of the aquatic plant beds or coral reefs, among other problems (Carpenter et al., 1998; Mcisaac, 2003; Murphy, 1991).

3.1.8. Effects on groundwater

Nitrate pollution of groundwater is increasing substantially in areas of intensive livestock. Groundwater nitrate pollution, as a general worldwide issue, is a current topic in scientific research and water planning forums (e.g. (Galloway et al., 2008; Sutton et al., 2011)). In Europe, maximum nitrate concentration for drinking water is set to 50 mg NO_3^-/L (Directives 91/676/EC and 98/83/EC), which is breached at many aquifers located in areas with high population densities, preventing the use of the underground water for domestic facilities. The European Water Framework Directive (2000/60/EC), which aims to provide a management context for all European water bodies, recognizes this fact and lists nitrate as one of the main contaminants that could hamper the achievement of the goals of the directive. In Catalonia (NE of Spain) nine areas were declared as vulnerable to nitrate pollution from agricultural sources according to the European nitrate directive EEC-1991 (DOGC, 1998).

Since groundwater resources are intensively used for potable water supply, their contamination with reactive N can have negative impact on communities that depend on

this resource. Mostly originated by the intensive use of fertilizers produced by livestock rising (Stark and Richards, 2008), nitrate consequences on human health (World Health Organization 2011) as well as those to the environment (Mason, 2002; Vitousek et al., 1997; Wilson et al., 1999) have been broadly exposed. However, the first N compound that arrives to the subsurface is ammonia and organic N, which are normally rapidly attenuated by mineralization, ammonification and sorption (Nikolenko et al., 2018). In front of the resilience and persistence of nitrate in groundwater (Böhlke et al., 2002), the occurrence of natural attenuation processes due to denitrification (Rivett et al., 2008) and to DNRA (Herrmann et al., 2017) must be identified and the conditions for which they can be enhanced, preserved (Figure 1.5). Therefore, denitrification field studies gain importance as a means to manage nitrate pollution.

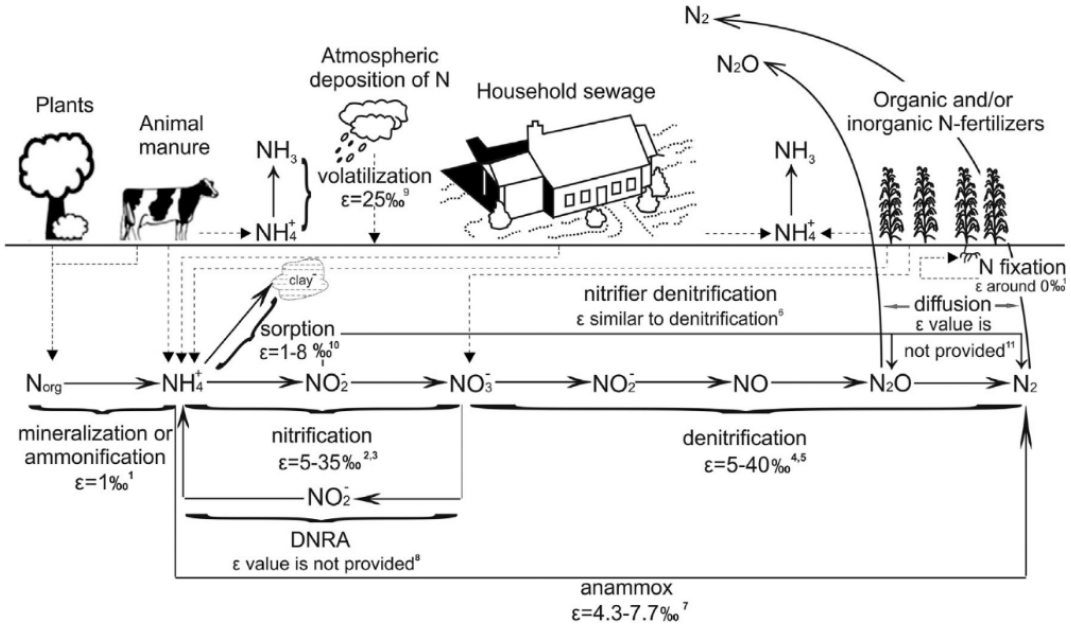

Figure 1.5. N sources and transformation processes that affect N species in the subsurface. The enrichment values ($^{15}N\text{-}NO_3^-$, $^{15}N\text{-}NH_4^+$) of N transformation processes are provided through the isotopic fractionation (ε) (from Nikolenko et al. 2018).

Nitrate mass removal in groundwater can occur by autotrophic or heterotrophic denitrification. Whether one or the other occur in preference will depend on the availability of organic matter in the environment and the presence of reduced inorganic compounds (sulphur, hydrogen, iron and others), which act as electron donors and redox conditions. Groundwater environments are usually characterized by low organic matter content,

especially in consolidated rock aquifers. Therefore, nitrate attenuation is usually achieved by autotrophic denitrification (Jahangir et al., 2014). For autotrophic denitrification to occur, alternative electron donors are needed as substitutes of the organic matter. According to the electron donor used autotrophic bacteria have been divided into hydrogen-based and sulphur-based denitrifiers (Zhang and Lampe, 1999). Other electron donors can be used alternatively, such as iron II (Fe^{2+}) and other metals (Straub et al., 1996; Weber et al., 2006). There are evidences that, reduction of nitrate by Fe^{2+} can occur either biotically or abiotically in groundwater. Biotic nitrate reduction in the presence of Fe^{2+} is mainly catalysed by denitrification coupled to microbial oxidation of pyrite, which can also been carried out by DNRA bacteria. This has been widely studied in groundwater and lab-scale fermenters and, in most cases, pyrite dependent denitrification can be catalysed by different *Proteobacteria*, *Bacteroidetes*, *Firmicutes* and *Verrucomicrobia* bacteria (Pu et al., 2014; Rivett et al., 2008; Torrentó et al., 2011; Vidal-Gavilan et al., 2013). Accordingly, the identification of relevant microorganisms within groundwater microbial communities that perform nitrate removal by autotrophic or heterotrophic denitrification is essential to promote their activity in groundwater nitrate management.

3.1.9. Effects on freshwater environments

Release of nitrogen to lakes, rivers and coastal areas constitutes the main risk for reduced water quality and increase eutrophication. To determine the eutrophication level of water, chlorophyll and depth of Secchi disk are used as indicators, since the rise of chlorophyll is consequence of a high level of nutrients as N (Council Directive 91/676/EEC). Anthropogenic sources of N in surface freshwater systems are mainly municipal wastewater, runoff from agricultural areas, landfill leachate and loads from the industry. Wetlands play an integral role in preventing the degradation of freshwater ecosystem health due to their ability to mitigate nutrient concentration via biological, chemical and physical processes (Clairmont et al., 2019; Morrissey et al., 2013). Wetland plants together with microbial community play a vital role in the ability of the systems to improve water quality (Brix, 1997; Vymazal, 2007). In fact, changes in water quality of the wetland cause changes in sediment microbial communities compared to those associated to plants; disturbing biogeochemical cycles of the wetland, including N cycle (Clairmont et al., 2019).

Denitrification coupled to nitrification is one of the key processes for nitrogen removal in wetlands (Peralta et al., 2013; Saunders and Kalff, 2001). In a broad sense, the presence of wetland vegetation has been shown to impact the balance between the two processes. Plants stimulate N removal in sediments by providing both aerobic conditions for nitrification and organic matter mainly for denitrifying organisms but also for DNRA bacteria (Rütting et al., 2011; Shelef et al., 2013). The presence of emergent macrophytes has been shown to consistently favour higher nitrogen removal rates and usually differences in the efficiency of nitrogen removal can be measured when different plant species are used (Bañeras et al., 2012; García-Lledó et al., 2011a). Moreover, seasonal variations in the nitrification and denitrification capacity have been found, which could be attributed mainly to changes in both the quality and quantity of carbon exudates on the surface of the leaves and roots, as well as to differences in water residence time and nutrient load (Bastviken et al., 2005; García-Lledó et al., 2011a).

The high variability of wetland microenvironments, mainly associated to the presence of plants, enables a high microbial diversity, which is related to high ecosystem functioning (i.e. the sum of all processes provided by a given ecosystem) (Ansola et al., 2014; Shelef et al., 2013). However, there are many factors controlling ecosystem functioning, including environmental factors as pH, oxygen, temperature, presence of metals, etc. (Ansola et al., 2014; Choudhury et al., 2018). Nitrifying microorganisms are not highly diverse, since little specific groups are able to oxidize ammonium and/or nitrite (Daims et al., 2016; Lehtovirta-Morley, 2018; Pester et al., 2012). Niche differentiation of ammonia oxidizers ensure a widespread distribution of this microbial metabolism in different environments, including in microhabitats of constructed wetlands (Abell et al., 2014; Hink et al., 2018; Trias et al., 2012). On the other hand, DNRA and *nir*-containing microorganisms, are phylogenetically diverse and widely distributed in different environments with various environmental conditions (Bonilla-Rosso et al., 2016; Bu et al., 2017; Graf et al., 2014; Rütting et al., 2011; Song et al., 2014; Wei et al., 2015). Accordingly, the identification of relevant microorganisms within freshwater microbial communities that perform nitrogen removal from water by nitrification, denitrification and DNRA is essential to promote their activity in freshwater systems nitrate management.

1.3. Relevant aspects on the study of microbial activities

3.1.10. Potential *vs* Actual activities

Microbial community studies need to include activity data to understand the function of microbial communities in the ecosystem. Determining metabolic rates under controlled laboratory conditions provides an estimate of the inherent activity of a given sample, if performed under the ideal conditions for the activity (Seitzinger et al., 1993). Although in most cases laboratory experiments do not directly reflect the actual activity in the field, they provide necessary data to evaluate the individual effects of different environmental parameters or to compare potential activities in completely different simulated situations. Experimental activity analysis allows for a precise comparison of samples without the side effects of uncontrolled environmental variables and so may be useful to determine which are the best conditions for different microbial N cycle pathways (Ruiz Rueda, 2008).

Potential activities can be measured by mass-balance calculations. This can be easily done in laboratory microcosms carried out to mimic conditions of the studied samples, as anaerobic conditions and with addition of organic matter and NO_3^- at similar C/N ratio than the studied environment. The loss of NO_3^- and NO_2^- can be assumed to occur by nitrate and nitrite reducers (including denitrifying, DNRA and anammox organisms); while NH_4^+ production can be assumed to be due to DNRA pathway (Caffrey et al., 2019; H. Kim et al., 2016; Ruiz-Rueda et al., 2009; Song et al., 2014). Moreover, the acetylene blockage method can be used to measure complete denitrification, since N_2O is accumulated due to inhibition. The nitrous oxide reductase activity is inhibited under an atmosphere containing near to 10% of acetylene (Balderston et al., 1976; Tiedje, 1988). The method has limitations, such as inhibitory effects on other processes, i.e. nitrification and anammox, which can lead to erroneous estimates of the potential rates (Seitzinger et al., 1993).

On the other hand, *in situ* activity measurements reflect a more accurate estimation of actual microbial activities. These kinds of measurements are usually obtained by using specifically designed incubation chambers that are installed and operated in the study site. In most cases, nutrients are added to speed up the processes, in others, evolution of N chemical species is followed without further intervention (Christensen et al., 1990; García-Lledó et al., 2011a; Horváth et al., 2006; Qin et al., 2017)).

From a methodological point of view, *in situ* experiments are far more complex and often entail the use of specially designed material, though data provided by these methods are closely reflecting actual conditions of the environment. However, laboratory measurements allow stablishing different conditions to study the activity of microbial community. Then, both methods are useful and could be complementary to measure microbial activity of different pathways.

3.1.11. Isotopic methods

Microbial activity related to nitrogen cycle can be measured easily by mass balance equations as explained above. However, other methods are available for estimating N transformations, such as using stable N isotopes, both in laboratory incubations and field measurements (H. Kim et al., 2016; Song et al., 2014). The use of these methods involves adding ^{15}N labelled compounds in the form of NO_3^- or NH_4^+, and the subsequent quantification by mass spectrometry of the ^{15}N labelled generated gas compounds. There are different isotopic methods to determine microbial activity, though the most used is the isotopic pairing technique (Nielsen, 1992).

The isotopic pairing technique relies on the natural abundance of stable nitrogen isotopes, which theoretically are found in 99.64% ^{14}N and 0.36% ^{15}N (Nielsen, 1992). Experiments based on this method consist in the addition of a known $\delta^{15}NO_3^-$ concentration (minimum 98% ^{15}N) to the water column in an experimental system where $\delta^{15}NO_3^-$ can subsequently diffuse into the sediment over time and be directly reduced, or $\delta^{15}NO_3^-$ which can mix it with ambient $^{14}NO_3^-$ molecules found within the sediment interstitial water and be reduced. The result from these two pathways is denitrification which can produce N_2 molecules with atomic molecular masses $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ (Figure 1.6) (Lenaker, 2009; Steingruber et al., 2001). When denitrification occurs and nitrate concentration decreases, the $\delta^{15}N$ value of the residual NO_3^- increases since microorganisms select ^{14}N than ^{15}N (Fukada et al., 2003).

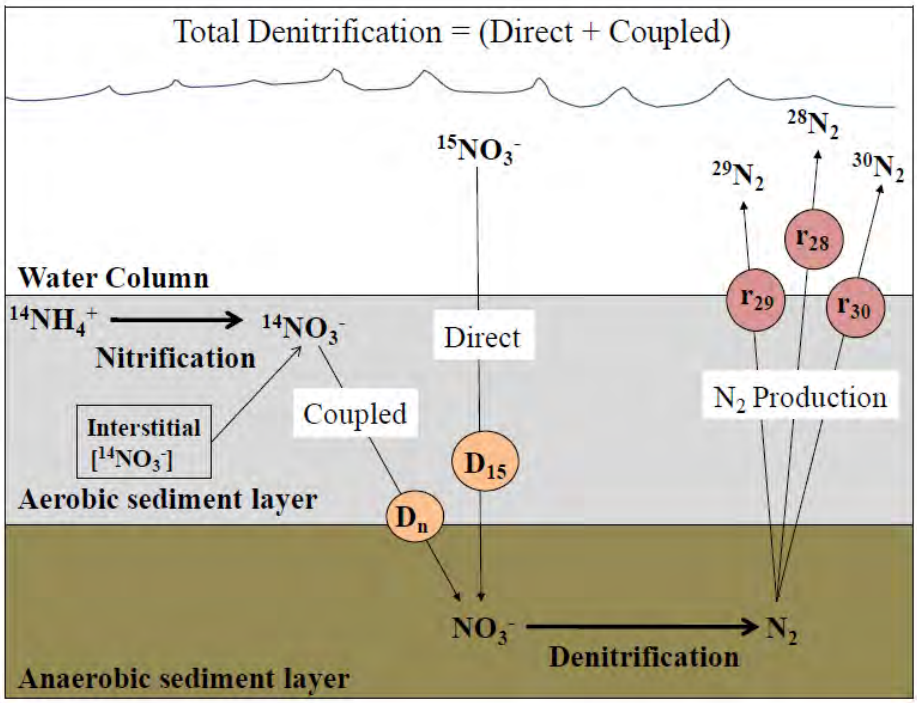

Figure 1.6. Isotope pairing technique conceptual model. Diagram illustrates contribution of ^{15}N and ^{14}N via direct and coupled denitrification to labelled $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ products (from Lenaker, 2009).

As a further advance of the isotopic approach, the dual isotope method has also been used. The dual isotope method consists of the simultaneous measurement of $\delta^{15}N$ and $\delta^{18}O$ in NO_3^-. Similarly to what occurs for $\delta^{15}N$, fractionation during denitrification causes the $\delta^{18}O$ value of the residual NO_3^- to increase as nitrate concentrations decrease ($^{14}NO_3^-$) (Böttcher et al., 1990). Hence, the dual isotope method produces a distinctive isotopic signature on a $\delta^{15}N$ versus $\delta^{18}O$ cross-plot and overcomes the ambiguity associated with using only $\delta^{15}N$ data where the isotopic enrichment can be confused with human and animal waste sources of nitrogen that typically have heavy $\delta^{15}N$ signatures in excess of 10% (Heaton, 1986). The initial values of NO_3^- isotope composition ($\delta^{15}N$ and $\delta^{18}O$) differ between nitrate sources (Aravena and Mayer, 2009; Fukada et al., 2003; Puig et al., 2017), so they are useful to determine the origin of nitrate pollution in the environment (Table 1.1). When plotting data as $\delta^{15}N$ or $\delta^{18}O$ versus the natural logarithm of the residual NO_3^-, the data matches to a regression line if denitrification is occurring (Fukada et al., 2003). Then, denitrification causes an increase of the $\delta^{15}N$ and $\delta^{18}O$ values of the residual nitrate by means of isotopic fractionation (Kendall, 1998), which is represented by ε, with a $\varepsilon N/\varepsilon O$ ratio that ranges from 1.3 to 2.1 (Böttcher et al., 1990; Fukada et al., 2003). Moreover, denitrification can be

detected by the complementary use of other isotopes according to the characteristics of the studied environment. Autotrophic denitrification in which pyrite (FeS_2) act as main electron donor in carbon-limited systems can also be traced with the use of sulphur and oxygen isotopes (Moncaster et al., 2000; Otero et al., 2009; Vitòria et al., 2008). Otherwise, in dissolved organic carbon rich environments, heterotrophic denitrification can be identified by nitrate isotopes and carbon isotopes in bicarbonate derived from the oxidation of organic matter (Aravena and Robertson, 1998); yet other sources of alkalinity may mask the imprint of denitrification in the carbon isotopic signature (Puig et al., 2017).

Table 1.1. Isotope ratio values according to the origin of NO_3^- sources. Ranges of nitrate isotope compositions of the main potential sources of NO_3^- (adapted from Puig et al., 2017).

NO_3^- source **Isotope ratio (‰)**	Pig manure	Mineral fertilizers	Sewage	Soil
$\delta^{15}N$	+8 to +16	-4 to +8	+5 to +20	+3 to +8
$\delta^{18}O$	+3.4 to +4.6	+ 17 to + 25	+ 3.4 to + 4.6	+3.4 to + 4.6

3.1.12. Molecular methods

Microbial community studies focused on analysing the activity of different pathways are complemented by knowledge on the composition and abundance of genes involved in those pathways. This is mainly achieved by the use of molecular methods based on the analysis of the DNA extracted from environmental samples which provides information about the diversity and structure of microbial communities (Smith and Osborn, 2009). The successful application of molecular methods relies on the nucleic acid recovery from environmental samples, efficiently representing the actual microbial community (Hurt et al., 2001). Generally, microbial community analyses by molecular methods are focussed on analysing the highly conserved 16S rRNA gene, providing insights into the composition and structure of communities in different environments (Philippot and Hallin, 2005). Molecular markers for 16S rRNA gene targeting bacteria and archaea have been designed (Caporaso et al., 2012; Kemnitz et al., 2007; López-Gutiérrez et al., 2004). The use of theses markers allows to have an approach of total abundance of *Bacteria* or *Archaea* using qPCR, as well as to determine the structure of microbial community composition by different next generation sequencing techniques (Schuster, 2008).

However, when the microbial N cycle is the target of the study, the 16S rRNA gene is not reliable due to a clear lack of monophyly in most N transforming bacteria. Consequently, molecular markers targeting different functional genes need to be used to

study composition and structure of microbial communities related to N cycle, specifically for nitrifiers, nitrate and nitrite reducers and denitrifiers (Jones et al., 2008; Philippot, 2002; Rotthauwe et al., 1997). Among these molecular markers, genes coding for ammonia monooxygenase (*amoA*), nitrite oxidoreductase (*nxrB*), hydrazine synthase of anammox (*hzs*) nitrate reductases (*narG* and *napA*), dissimilatory nitrite reductases (*nrfA*, *nirS* and *nirK*), nitric oxide reductases (c*norB* and q*norB*), and nitrous oxide reductases (*nosZ* types I and II) (Bonilla-Rosso et al., 2016; Graf et al., 2014; Harhangi et al., 2012; Jones et al., 2013; Mohan et al., 2004; Pester et al., 2014; Philippot, 2002; Rotthauwe et al., 1997; Welsh et al., 2014; Zumft, 1997), have been widely used in many environments and sample types to study nitrifying, denitrifying, DNRA and anammox communities.

1.4. Microbes meet Plants. Impacts on N cycle

3.1.13. Constructed Wetlands, a model environment for N cycling

The increased concentration of N-pollutants such as NH_4^+, NO_3^- and NO_2^- in water resources and the direct and indirect effects of these compounds on the 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 from urban wastewater can be accomplished by different techniques. Chemical and physical methods 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). Other methods, such as chemical precipitation with magnesium, rhodium, palladium and copper, produce pollutant intermediates or end products, such as ammonium, limiting the overall nitrogen removal efficiency from water (Dozier et al., 2008; Shrimali and Singh, 2001; Till et al., 1998).

As an alternative, biologically driven processes are the desired choice for large-scale applications because minimize operational costs (Shrimali and Singh, 2001). Biological nitrification and denitrification are therefore used to finally remove nitrogen from water in most European countries. Process control of biological nitrification-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., 1998; Haugen et al., 2002; Schnobrich et al., 2007; Sunger and Bose, 2009). The combination of both processes

promotes the net loss of nitrogen from the system, and is similar to what occurs in natural environments (Rivett et al., 2008; Vymazal, 2007; Zumft, 1997).

Conventional water treatment technologies are routinely applied to ensure minimum standards of quality before water is discharged to the environment (European Directive 91/271/CEE). Conventional Wastewater Treatment Plants (WWTP), perform satisfactorily but their construction, operation and maintenance entail substantial capital and energy costs, which raises the need for more sustainable alternatives. Treatment strategies based on constructed wetlands (CWs) are classified within these cost-effective treatment systems (Lee et al., 2009).

Wetlands have traditionally been considered as water polishing systems (Kadlec and Wallace, 2009; Vymazal, 2007), but over the past three decades they have gained a lot of popularity and have become widely implemented (Brix, 1994; Haberl et al., 1995) in urban settlements as engineered water treatment ecosystems. CWs are good alternatives for wastewater treatment for small and medium scale industrial or rural exploitations, and small villages. Generally, CWs constitute a practical and sustainable wastewater treatment option to ensure nitrogen removal.

Constructed wetlands basically mimic the functioning of natural systems, but the processes for improving water quality are regulated and forced to occur at a higher speed. There are different types of CW, which can be classified first according to the type of macrophyte growth and further based on the water regime (Figure 1.7). The most used types of CW are generally Free Water Surface (FWS), Horizontal Sub-Surface Flow (HSSF) and Vertical Sub-Surface Flow (VSSF) (Figure 1.8). Constructed wetlands have some limitations, such as low phosphorus removal, which must be taken into consideration when treatment technology is selected. However, constructed wetlands are a viable alternative in wastewater treatment technology and there are tens of thousands of applications in all parts of the world (Vymazal, 2011, 2007). Critical parameters influencing the removal rate of contaminants include the water flow or load, the preferred direction of flow, the hydraulic retention time and the mean depth. All of these are controlled in a way that larger loads of pollutants can be treated in a more predictable manner. Briefly, the treatment is carried out by a complex series of physical, chemical and biological processes in which the water, sediments, plants and microbes inhabiting the different compartments interact. The presence of vegetation in CWs guarantees successful performance, since plants exhibit both structural and biochemical

properties upon which many of the mechanisms involved in the removal of pollutants rely (Brix, 1994). As a result, the organization of plant masses and their composition within the wetlands is generally included as a key component in initial wetland design.

In CWs, nitrogen removal is achieved through a combination of microbial activities (coupled nitrification and denitrification) and plant assimilation processes (Vymazal, 2007). True N elimination steps from water occur via dissimilatory nitrite reduction to gaseous compounds, which mainly occurred by denitrification activity. However, DNRA and anammox, may also play important roles in CWs (Burgin and Hamilton, 2008; Koop-Jakobsen and Giblin, 2010; Tiedje, 1988).

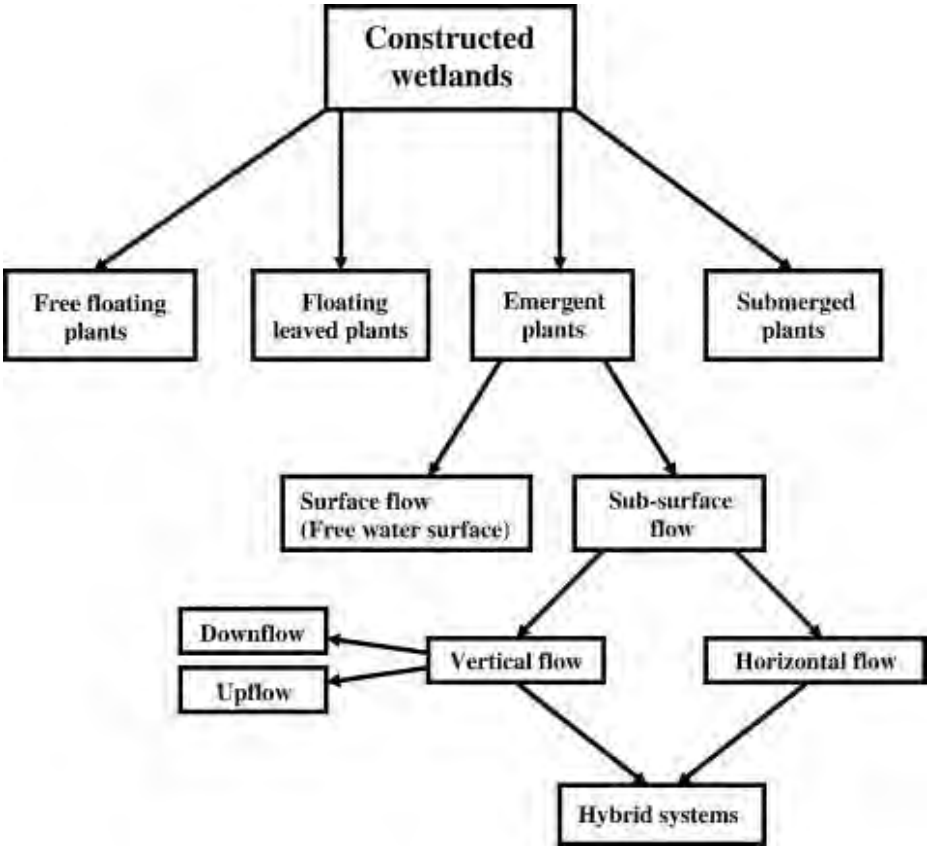

Figure 1.7. Classification of constructed wetlands for wastewater treatment. The first classification is centred on the type of vegetation. Water flow is latter considered for wetlands harbouring emergent plants (from Vymazal, 2007).

García-Lledó et al. showed that DNRA accounted for approximately 10% of the total nitrate reduction in the Empuriabrava free water surface constructed wetlands (FWS-CW)

(García-Lledó et al., 2011a). Other studies, have also pointed to a similar importance of DNRA activity, approximately the 5-10% of total NO_3^- removal in constructed wetlands receiving different nitrate concentrations (Scott et al., 2008; van Oostrom and Russell, 1994). Anammox activity can also take part in dissimilatory nitrite reduction, accounting from 2% in rivers, and up to 30% in freshwater wetlands (H. Kim et al., 2016; Wenk et al., 2013). In most CWs, microbial N-reduction processes account for 60-80% of the total nitrogen removal and clearly surpass the plants uptake contribution (Jahangir et al., 2014; Lee et al., 2009). Aquatic vegetation has been proven to have a significant effect on the composition of microbial communities compared to non-vegetated areas in CWs, and consequently in N removal (Vymazal, 2011). In particular, emergent macrophytes have multiple effects on the sediment where they pump oxygen and organic molecules, such as carbohydrates and amino acids, through the roots into the anaerobic sediment, which stimulate microbial growth and activities. Moreover, at a larger scale, plants contribute to a net increase of the available organic matter at the sediment surface as a result of decaying plant debris (Srivastava et al., 2017). Therefore, the distribution of vegetated and non-vegetated plots and which plant species are planted are crucial in determining the N-removal efficiency in most CWs (García-Lledó et al., 2011a; Koop-Jakobsen and Giblin, 2010; Lin et al., 2012; Stottmeister et al., 2003; Truu et al., 2009).

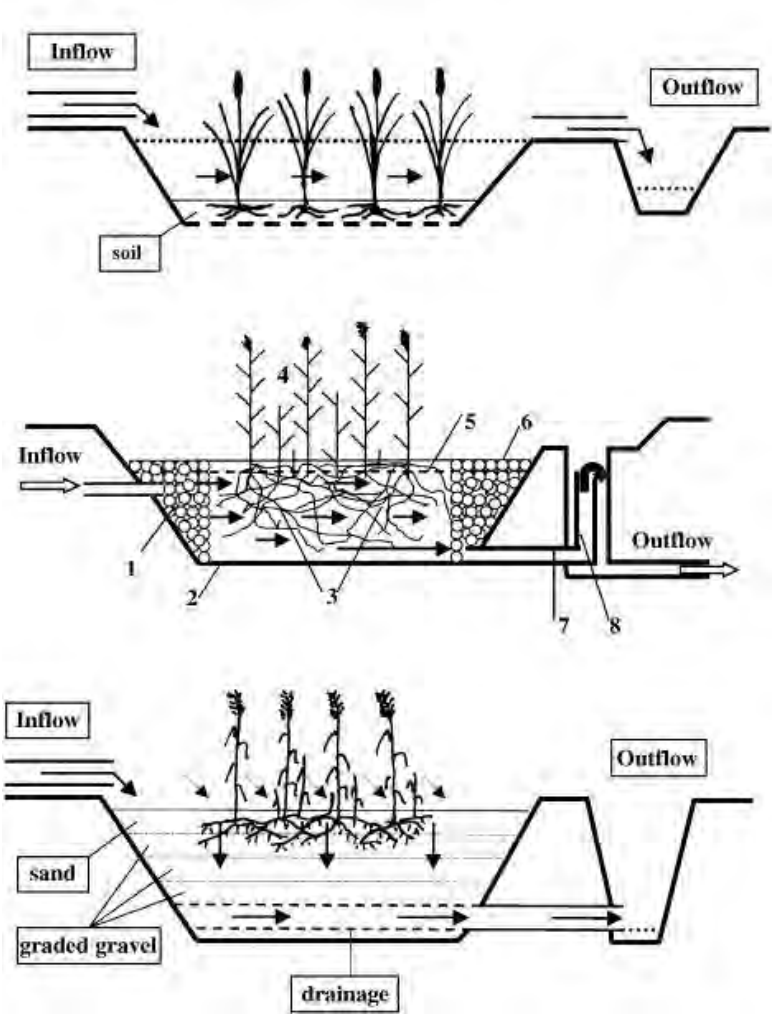

Figure 1.8. Constructed wetlands for wastewater treatment. From top to bottom: CW with free water surface and emergent macrophytes (FWS), CW with horizontal sub-surface flow showing the flow of water by numbers (HSSF, HF), CW with vertical sub-surface flow (VSSF, VF) (from Vymazal, 2007).

3.1.14. Nitrifiers and denitrifiers meet the plant root

Typical vegetation of wetlands are aquatic macrophytes, including members of four different groups: emergent (e.g. *Typha angustifolia*), floating leaved (e.g., *Hydrilla* spp.), free floating (e.g., *Pistia stratiotes*) and submerged macrophytes (e.g., *Chara* spp.). Macrophytes can stablish relationships with microorganisms in different ways. Briefly, there are two types of symbiotic relationship between microorganisms and plants namely endophytic and ectophytic (Figure 1.9) (Weyens et al., 2009). Within the endophytic relationships, N_2 fixing diazotrophs (Nielsen et al., 2001) and arbuscular mycorrhizal fungi (Šraj-Kržič et al., 2006) are well-known examples. Ammonia-oxidizing bacteria (Wei et al., 2011) and methanotrophic bacteria (Sorrell et al., 2002), are among the most common microorganisms

present in ectophytic relationships with plants. Ectophytic interactions involving both roots and leaves are important plant–microbe interactions as several biochemical reactions occurring at the interactive surface influence the elemental cycles in aquatic ecosystem (Laanbroek, 2010). The most active part of macrophytes is the rhizoplane (the part of root remaining in contact with water or soil), due to the interactions with various microbial communities. Roots of aquatic plants provide extended surface for the benthic microbial community to rest and act as a customized niche for each microbe ensuring the continuous supply of nutrients and organic carbon, while macrophytes obtain mineral nutrients and defensive immunity from microorganisms, forming firm interrelationships among them (Shelef et al., 2013; Srivastava et al., 2017). Moreover, macrophytes provide an additional oxygen source for microorganisms growing in the rhizoplane, mainly due to the radial oxygen loss (ROL) (Colmer, 2003; Kotula and Steudle, 2009). The ROL depends largely on plant species (Brix, 1997; Stottmeister et al., 2003) and on water redox potential (Wießner et al., 2002) accounting for 90 % of oxygen in the rhizosphere stimulating the growth of aerobic nitrifying microorganisms (Brix, 1997; Reddy et al., 1989) and the aerobic decomposition of organic matter present as plant exudates by heterotrophic bacteria. Oxygen is utilized mostly as a primary electron acceptor for energy generation (Bodelier, 2003) and to carry out a number of beneficial oxidation processes (Laanbroek, 2010).

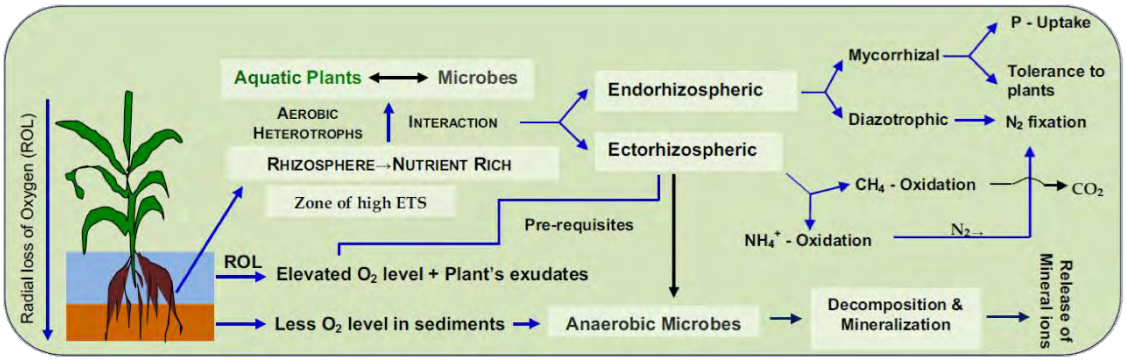

Figure 1.9. Plant–microbe interactions at rhizoplane in a fresh water ecosystem (from Srivastava et al., 2017).

Coupled microbial nitrification and denitrification are relevant for nitrogen removal in wetlands, and may account for the 60-80 % of total N reduction in the system, depending on some environmental factors influenced by plants such as temperature and aeration (Faulwetter et al., 2009; Wu et al., 2014). The sequential oxidation of ammonia to nitrate (nitrification) is an aerobic autotrophic process, whereas denitrification, the step-wise conversion of nitrate to nitrogen gas, is an anaerobic respiration process (Zumft, 1997). This

a priori opposite conditions favouring nitrification and denitrification can co-occur at the root surface of emergent macrophytes, in which ROL provides microniches for nitrification to occur in an anaerobic environment (García-Lledó et al., 2011a; Risgaard-Petersen and Jensen, 1997). Then, the focus of the study of the N cycle on wetlands must include the study of microbe–macrophytes interactions, to have a complete overview of ecosystem functioning.

2. AIMS

Nitrogen removal from natural and constructed freshwater systems is a key process to ensure a good water quality. This N elimination is mainly carried out by microorganisms. To increase the comprehension of microbial players in the nitrogen cycle of different ecosystems, such as surface and groundwater, it is important to analyse microbial communities and their activities in the N cycle using different methodological perspectives, and their relationship to environmental and physicochemical variables. Accordingly, the main goal for this thesis was to study the microbial community participating in the N cycle and their potential activities in different environments, concretely based on NO_2^- production and removal since it is an important compound determining the N removal from water, in order to determine some key factors to manage systems (constructed or natural) polluted by N compounds.

The following specific objectives were defined:

1. To study possible changes in the potential activity and the community structure of nitrite reducers after sediment dredging and plant removal in a Constructed Wetland. Microbial community is essential in CW to ensure an efficient nutrient removal and then return water in better conditions to natural environment. Generally, microbial community of CW is stable, though it could be altered by different perturbations caused from the management of the environment. We studied the effect on microbial community structure on basis of 16S rRNA and using quantification of different genes, as well as the changes on N removal and nitrite reduction potential activities before and after important perturbation (complete dredge of the sediment and removal of vegetation) in Empuriabrava FWS-CW system. We hypothesized changes in microbial community structure and their potential activities removing nitrite from the system, since changes in the environment could alter completely microbial metabolisms.
2. **To measure how radial oxygen loss and environment determine ammonia oxidizers community on *Typha angustifolia*.** Nitrifiers, and concretely ammonia oxidizers (AOA, AOB and Comammox), are the first microorganisms responsible for start N removal from water. Nitrifying microorganisms are highly dependent of oxygen availability. Differential radial oxygen loss (ROL) along the roots could determine niche differentiation between nitrifying groups, and affect their

nitrification activity. We studied the effect of ROL along roots in total microbial community structure and specifically in nitrifying genera, as well as in the abundance of the gene *amoA*. The studied roots were from *Typha angustifolia*, obtained from Empuriabrava FWS-CW and nearly natural area, which showed a conductivity gradient and allowed us to compare constructed and natural systems. This plant species was selected since it is one of the most used plant species in CW and is also highly abundant in natural environments of the studied region. We hypothesized that areas with higher ROL should select for specific nitrifying organisms, and could show higher abundance of these microorganisms.

3. **To test natural attenuation of nitrate in Osona's groundwater using isotopes to microbiome data** Nitrate pollution in groundwater is a problem in many regions. Different methodological approaches, benefitting from complementary scientific disciplines, have been used to ascertain the contribution of microorganisms in the overall N cycle in different environments. We studied microbial community structure, and potential denitrifying activity, from N polluted groundwater in the Osona's region, using two techniques: molecular methods and isotope-based approach. We hypothesized that similar results in terms of potential denitrification would be obtained from both methods, which could be used to confirm the results and the complementarity of techniques.

3. MATERIALS AND METHODS

3.2. Study sites

3.2.1. Empuriabrava FWS-CW

The Empuriabrava free water surface constructed wetland (FWS-CW) system is located 50 km to the north-east of Girona (42°14'40.4"N 3°06'15.1"E, NE Spain) (Figure 3.1). The system works as a tertiary treatment to increase the water quality of the effluent of the Empuriabrava wastewater treatment plant (WWTP). The wetland is included in the natural preserved area of *Els Aiguamolls de l'Empordà* (https://aiguamollsdelemporda.cat/) and was designed to provide additional water to avoid excessive desiccation of the protected area in summer. The system is sparsely covered with reed (*Phragmites australis*), cattail (*Typha latifolia*) and narrowleaf cattail (*T. angustifolia*), as the main emergent macrophytes. On January 2013, a large part of vegetation was removed and sediment was dredged in about 70% of the surface area of the Europa lagoon (Figure 3.2). This action is regularly done to prevent cattail accumulation and softening of the lagoon shore that would lead to a collapse of the CW lagoon. After dredging, *Typha angustifolia* remained as the predominant plant species in the lagoon (30% surface). Dredged and non-dredged plots were defined and were sampled before (23th July 2012, BD samples) and after (11th July 2013, AD samples) sediment dredging, in order to study the effect of this impact in microbial community (specific objective 1). Five sampling locations, four in vegetated areas (V, locations named BM12, PH12, TL12 and TA12), and one in a non-vegetated area (NV, named RS12) were defined for BD sites (Figure 3.2). Seven sampling locations were defined for 2013, three located in a vegetated area (AD-V, named TA131, TA132 and TA133), and four from the dredged zone, which were located in a non-vegetated area (AD-NV sampling points named RS131, RS132, RS133 and RS134). In summary, to analyse the effect of sediment dredging on the distribution and activity of denitrifiers in the Empuriabrava FWS-CW four groups of samples were defined: BD-V (before dredging and vegetated area, 4 sampling locations), BD-NV (before dredging and non-vegetated area, 1 location), AD-V (after dredging and vegetated area, 3 locations) and AD-NV (after dredging and non-vegetated area, 4 locations) analysed.

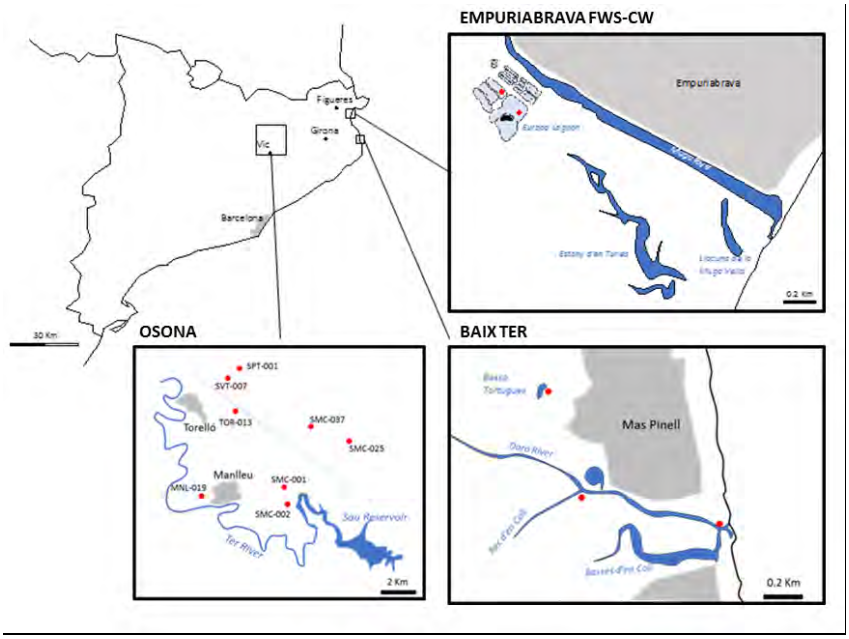

Figure 3.1. Location of the three study sites of the thesis. Map of the top-left is Catalonia, all the study sites are located at the north-east of this region. In Osona, dot-line differentiate between studied areas. Grey- large urban areas. Red dots- location of sampling spots.

On the other hand, the presence of *Typha angustifolia* could have an important effect on microbial community, especially on nitrifiers. In order to study the effect of oxygen release by roots of *T. angustifolia* on the microbial community in different aquatic environments and to compare it with the adjacent sediment, plant and sediment samples were collected from FWS-CW (specific objective 2). For this purpose, three different sites from the FWS-CW were selected and sampled on July 2014: two from treatment cells and one from a final polishing lagoon (Europa Lagoon), named Cell-1, Cell-2 and EE respectively (Figure 3.3).

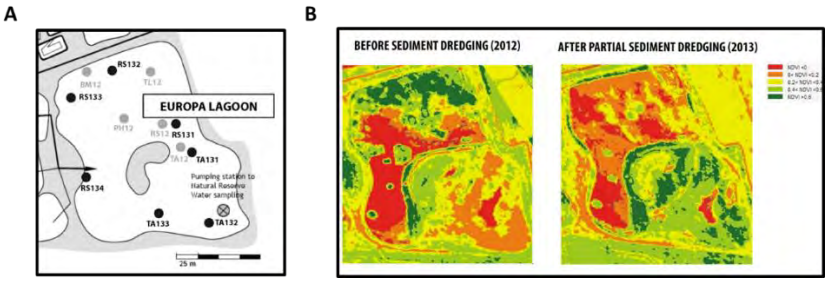

Figure 3.2. Sampling points and state of the lagoon before and after sediment dredging event. A. Position of sampling plots. Grey - of samples collected before dredging event. Black - of samples collected after dredging event. B. Images of the normalized difference vegetation index (NDVI) in the Europa lagoon before and after sediment dredging action. Dredged areas (devoid of vegetation) appear orange to red in the NDVI images. Source: www.icc.cat.

3.1.1. River Daró estuary

Typha angustifolia is one of the most abundant macrophytes in freshwater natural systems of Catalonia dealing with different salinity conditions and therefore not only being exclusive of CW. Accordingly, the River Daró estuary together with the Empuriabrava FWS-CW was chosen as model environments with a high density of *Typha* plants following a salinity gradient. Selected spots in those areas were used in order to determine tight root-microbe interactions for key players in the N-cycle, such as the nitrifying bacteria and archaea (specific objective 2). River Daró estuary belongs to a naturally preserved area, the *Parc Natural del Montgrí, Illes Medes i Baix Ter* (42°00'33.3"N 3°11'09.8"E). In the area, three sampling positions were selected; the Daró River mouth (DM), at the site an irrigation channel discharges to the river (Rec Coll, 300 meters up the river mouth, RC), and at an intermittent lagoon located in the northern side of the river bed (Bassa de les Tortugues, BT) (Figure 3.1). In all sites, cattail (*Typha angustifolia*) was the dominant macrophyte. Samples were obtained on the third week of July 2015.

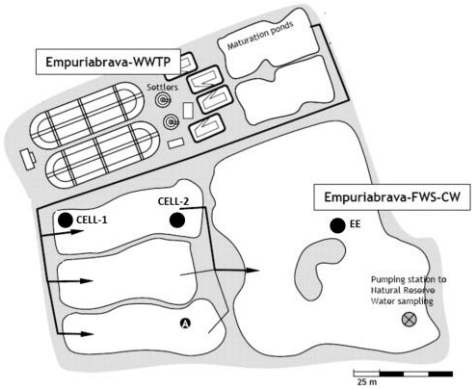

Figure 3.3. Sampling points of *T. angustifolia* roots in the FWS-CW. Samples for the effect of radial oxygen loss on nitrifying bacteria and archaea were collected in the Europa Lagoon (EE) and on the treatment cells (Cell-1 and Cell-2). In all cases *Typha angustifolia* roots were harvested.

3.1.2. Osona

The Osona region is located approximately 60 km to the north of Barcelona (NE Spain), in the internal basins of Catalonia (Figure 3.1). It constitutes a geomorphological basin surrounded by ranges that attain 1,300 m asl in its north-eastern and eastern limit. The basin is drained by the Ter River that frames the study area in its west and south boundaries (see inset in Figure 3.2). With an area of 1,260 km^2 and a total population of 154,000 inhabitants, it constitutes an intensive agricultural and livestock production area with more

than 740,000 head of hogs, and 65,000 head of cattle (Menció et al., 2016). Slurry and manure produced by husbandry activities is used as the main fertilizer for crops, although synthetic fertilizers are also applied. Because of Osona's orography the arable land is located at the basin rather than in the surrounding ranges. Consequently, a major application of manure is expected at low altitude. In consequence, Osona has been classified as Nitrate Vulnerable Zone, because of the Nitrate Directive (Directive 91/676/EC).

The geological setting of the Osona region consists of a sequence of Paleogene sedimentary layers with a total thickness of approximately 1,500 m, which overlies the igneous and metamorphic rocks of the Hercynian basement (Figure 3.4). The study area is situated at the north-eastern part of Osona, where the sedimentary formations are constituted by a thick (≈500 m) basal level of conglomerates, overlaid by an alternation of carbonate formations, with calcareous, marls and carbonate sandstone layers (≈1,000 m; see stratigraphic column and geologic cross-section in Abad García, 2001 and Menció et al., 2011a). In particular, the wells chosen for this study exploit productive levels constituted by silt, sandstones and marls layers. Joints and fractures affect most of the sedimentary rocks and they constitute the main porosity of these layers. Mean annual rainfall is about 585 m, and actual evapotranspiration reaches 480 mm.

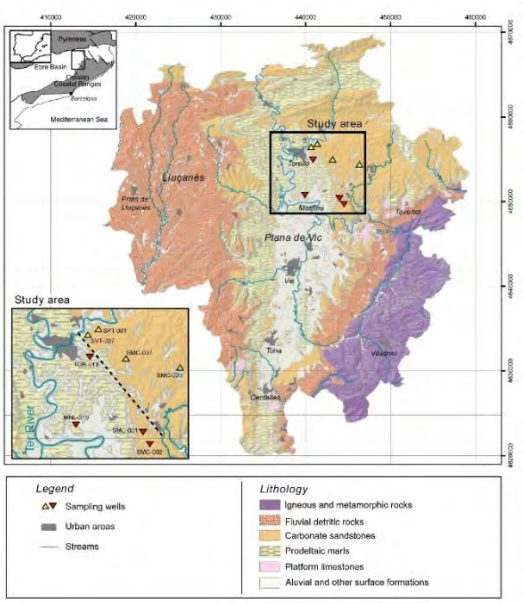

Figure 3.4. Geographic and geologic setting of the study area. Geological map from the Institut Cartogràfic i Geològic de Catalunya (2017). Dashed line in the zoomed image indicates separation between NE and SW wells.

Eight selected wells from a wider database from previous studies from Osona region were sampled in summer 2014 to analyse the denitrification processes, as an example of autotrophic denitrification in a fractured aquifer. Eight wells were named according to previous studies and are encoded with a three letters code (location) and a consecutive numbering: SPT-001, SVT-007, TOR-013, SMC-037, SMC-025, SMC-002, SMC-001 and MNL-019. Due to its high sensitivity to nitrate contamination of groundwater in the area, we checked a combination of isotope determinations and molecular microbiology approaches in order to obtain a complete picture of potential denitrification of the selected wells and to determine relevant environmental factors shaping complete or incomplete denitrification activities.

3.2. Sample collection and chemical analyses

3.2.1. Groundwater

Groundwater for hydrochemical, isotopic and microbiological examination was taken by pumping and aliquots were distributed according to the analyses. For microbial analyses, 2 L were collected in a sterilized glass bottle and transported at 4°C in a dark environment. All samples were filtered (0.22 μm pore-size isopore polycarbonated membrane filters; Merck Millipore, Darmstadt, Germany). Filters for microbiological analysis were stored at -20 ºC until the DNA extraction. Physicochemical parameters (pH, Eh, electrical conductivity (EC), dissolved oxygen (DO) and temperature) were measured in situ using a flow cell to avoid contact of water with the atmosphere. In the laboratory, alkalinity was determined using Gran titration; concentrations of Br^-, F^-, SO_4^{2-}, Cl^-, Ca^{2+}, Na^+, Mg^{2+}, K^+ and $N\text{-}NH_4^+$ were measured by ionic chromatography; TN and TOC were determined by catalytic oxidation; and $N\text{-}NO_2^-$, $N\text{-}NO_3^-$, $P\text{-}PO_4^{2-}$ and PT were determined by spectrophotometry. All the samples had an ionic mass balance error <1%. For sulphide (H_2S) analysis, 10 mL of water were collected in sterile screw-capped glass tubes, alkalinized by adding NaOH (0.1 M final concentration) and zinc acetate at 0.1 M final concentration was added to fix sulphide as ZnS. H_2S was analysed by the leucomethylene-blue method (Trüper and Schlegel, 1964).

3.2.2. Surface water samples

In Empuriabrava FWS-CW and River Daró estuary, physical and chemical monitoring of water was done at the different sampling sites. Temperature, conductivity, oxygen and pH were measured with a portable multiparametric probe (Yellow Spring

Instruments 650MDS). Additionally, water samples (20 mL) were collected and analysed using ion chromatography (IC) for nitrate, nitrite and ammonium concentration as described previously (García-Lledó et al., 2011a).

Physical and chemical monitoring of water from FWS-CW was done routinely every 2 weeks from January 2012 to December 2013. Samples were collected at the influent and effluent of the Europa Lagoon and analysed for the concentration total inorganic nitrogen, nitrate, nitrite and ammonium by experienced personnel at the WWTP and gently provided by the Consorci de la Costa Brava (www.ccbgi.org).

3.2.3. Sediment sampling

In all cases, sediments were collected at random in an area of a square meter. Three sediment samples were collected from each plot. Sediment cores were obtained using a 5 cm diameter methacrylate tube mounted in a manual sampler. Large vegetal debris, observed at naked eye, was removed from the surface of the sediment core. In areas containing vegetation, roots were removed from the upper part of the core, from 0 to 3 cm depth, using sterile forceps. The upper 3 cm of the sediment cores were aseptically transferred to a sterile container and chilled on ice for transportation. Samples to be used for RNA analysis were kept in liquid nitrogen. Once in the laboratory, sediment was completely homogenized and 2 g aliquots were stored at -20 ºC and -80 ºC for DNA and RNA analyses, respectively, until processed.

Concentration of nitrogen compounds (NO_3^-, NO_2^- and NH_4^+), total nitrogen (TN), total organic carbon (TOC), and pH were also measured in the sediment. The content of NO_3^-, NO_2^-, NH_4^+, TN and TOC were analysed from 1/10 (dry weight/volume) suspensions as previously described (García-Lledó et al., 2011; Noguerola et al 2015). Sediment pH was measured in a stabilized 1/5 (dw/vol.) suspension. Duplicates were always performed for all chemical determinations.

3.2.4. Collection of *Typha angustifolia* roots

In each location, three plant shoots were selected randomly in one square meter area covered exclusively by *Typha angustipholia*. In all sampling plots, plant densities were higher than ten individuals per m^2. Plants were manually harvested taking special care in maintaining the roots intact (Figure 3.5). Immediately after collection, roots were thoroughly rinsed with water from the same sampling site to remove all loosely attached sediment. Non-senescent and integer roots in which tips have not been damaged were selected, cut using sterile forceps

and scissors, and rinsed twice in sterile isotonic solution. Intact roots (> 6 cm in length) were separated for the oxygen diffusion analyses, which were performed within 4 hours after collection. For molecular analyses, selected roots were selectively cut from different sections along the root longitudinal axis. Root tips were defined from 0 to 1.5 cm from the tip. Middle root section was defined from 1.5 to 4.5 cm. Basal root sections included all segments collected at distances higher than 4.5 cm from the root tip. Aliquot samples of the three sections were distributed in sterile plastic bags, chilled to 4 ºC for transportation, and finally stored at -20 ºC.

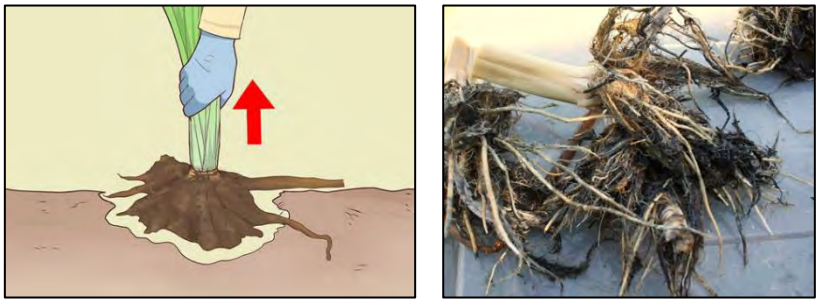

Figure 3.5. Sampling of *T. angustifolia* roots. Plant harvesting procedure and example image of roots obtained after sampling and mild root rinsing (source of images: www.wikihow.com and Sara Ramió-Pujol).

3.3. Scanning Electron Microscopy

Scanning electron microscopy was used in order to ensure that microorganisms were adhered to roots after cleaning of *Typha angustifolia* roots. Images were only collected for the study related to objective 2 (Chapter 4.2). For microscopy observations, root samples were fixed with 2.5% [wt/vol] glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, washed and dehydrated successively in ethanol. Finally, roots were dried at the critical point, and carbon evaporated. Examinations were performed in a scanning electron microscope FE-SEM S-4100 (Hitachi, Tokyo, Japan) at the Serveis Tècnics de Recerca (STR, Universitat de Girona). Digital images were collected and processed using the Quartz PCI measurement software (Quartz Imaging Corporation, Vancouver, Canada).

3.4. Molecular methods

3.4.1. DNA extraction and quantification

Total DNA was extracted from filtered groundwater (1 L per filter), roots and sediment using a combination of enzymatic cell lysis in the presence of lysozyme (final concentration 1 mg/mL, at 37 °C for 45 min) and proteinase K (final concentration 0.2 mg/mL, at 55 °C for 1 h), followed by a modified CTAB (cetyltrimethylammonium bromide) extraction protocol (Llirós et al., 2008). For root and sediment samples, up to 1 g of root sections and sediment was used. In some samples less than 0.5 g of root tips were used for the extraction due to the difficulty of sampling intact tips sections. Moreover, the little number of tips that could be obtained did not allow generate three replicates of this section in any sample. Dried DNA pellets were finally rehydrated in 50 μL of 10 mM Tris-HCl buffer (pH 7.4).

DNA extraction from FWS-CW sediment sampled in July 2012 and 2013 was done using the FastDNA® SPIN Kit for soil (MP, Biomedicals) following the manufacturer's instructions with minor modifications. In all cases, DNA concentration was determined using Qubit® 2.0 fluorometer (Invitrogen, Molecular Probes Inc., Oslo, Norway). DNA extracts were stored at −20 ºC.

3.4.2. RNA extraction and cDNA synthesis

RNA was extracted using the RNA PowerSoil® Total RNA Isolation Kit (MoBio Laboratories) supplemented with the RNA PowerSoil® DNA Elution Accessory Kit (MoBio Laboratories) to elute as well the DNA present in the same sample. Aliquots of 60 μl of the extracted RNA were digested with RTS DNase Kit (MoBio Laboratories) according to the manufacturer's instructions. To ensure that RNA samples were DNA-free, a control PCR was carried out on the RNA samples using bacterial 16S rRNA gene primers 27F/1492R (Weisburg et al., 1991). cDNA synthesis was achieved by SuperScript® III First-Strand Synthesis System for RT-PCR, following the manufacturer's instructions (Invitrogen, Thermofisher). The obtained cDNA extracts were quantified using a Qubit® 2.0 Fluorometer (Invitrogen, Thermofisher) and stored at -80 ºC.

3.4.3. Quantitative PCR (qPCR)

Gene abundances were determined using quantitative PCR (qPCR). The qPCR amplification was performed for the functional genes *amoA* (AOA and AOB), *nirS*, *nirK*,

nrfA, *nosZ*I and *nosZ*II, as well as 16S rRNA from anammox. Additionally, the bacterial and archaeal 16S rRNA gene was also quantified and used as a proxy for total abundance. All reactions were performed in a Lightcycler 96 Real-Time PCR system using the LightCycler® 480 SYBR Green I Master (Roche Life Science, Basel, Switzerland). The reactions were performed with a final volume of 20 µL containing 1× LightCycler® 480 SYBR Green I Master, up to 10 ng of DNA, and 1 µM of each primer. Primers and thermal cycling conditions used for each target gene were used according to Hallin et al. for *amoA* (AOA and AOB), *nirS*, *nirK*, *nosZ*I and 16S rRNA bacterial genes (Hallin et al., 2009), to Jones et al. for *nosZ*II (Jones et al., 2013), to Welsh et al. for *nrfA* gene (Welsh et al., 2014), to Humbert et al. for anammox bacteria (Humbert et al 2012), and to Kemnitz et al. for 16S rRNA *Archaea* (Kemnitz et al 2007) with minor modifications (Table 3.1). In all cases, qPCR primers were obtained from Biomers (Ulm, Germany).

The standard curves were generated using serial dilutions (from 10^2 to 10^8 copies per reaction) of plasmids containing known sequences of the target genes. For each gene, a clone containing the gene sequence without any mismatch in its priming sequence was used to perform the standard curve (Figure 3.6). Additionally, to check for the specificity of the qPCR reaction, melting curves were analysed to ensure that all of them produce a single dissociation peak (Figure 3.7). The qPCR efficiencies for all analysed genes are listed in table 3.2. The negative controls resulted in undetectable values in all qPCR reactions.

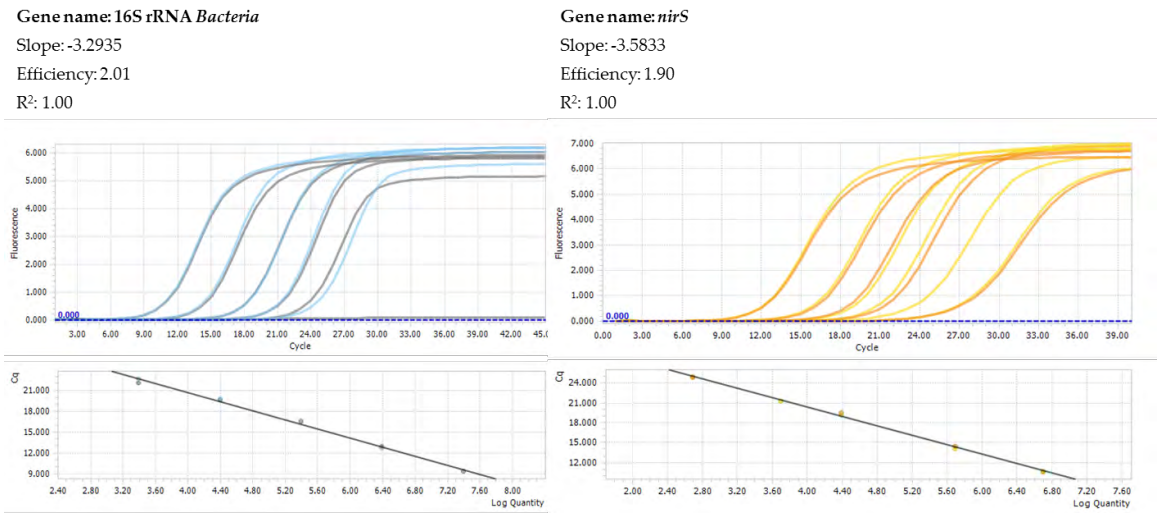

Figure 3.6. Standard curves of qPCR. Examples of the amplification and standard curves obtained for two of the studied genes (16S rRNA *Bacteria* and *nirS*) by qPCR.

Table 3.1. Primers and thermal conditions used for qPCR amplifications of 16S rRNA and functional genes. In all cases, the first step of thermal program (DNA denaturation) consisted in one cycle of 10 minutes at 95 ºC.

Primers and amplicon size	**Sequence (5' – 3')**	Thermal conditions	Reference
16S rRNA *Bacteria* 341F/534R	CCT ACG GGA GGC AGC AG ATT ACC GCG GCT GCT GGC A	95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, 35 cycles	(Lopez-Gutierrez, et al. 2004)
16S rRNA *Archaea* Arch364aF/ArchA934b	CGG GGY GCA SCA GGC GCG AA GTG CTC CCC CGC CAA TTC CT	95 °C for 15 s, 66 °C for 30 s, 72 °C for 30 s, 35 cycles	(Kemnitz, et al. 2007)
nirK nirK876/nirKR3Cu	ATY GGC GGV CAY GGC GA GCC TCG ATC AGG TTR TGG TT	95 °C for 15 s, 63 to 58 °C for 30 s (-1 °C by cycle), 72 °C for 30 s, 6 cycles 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, 40 cycles	(Henry, et al. 2004)
nirS nirSCd3aFm/nirSR3cdm	AAC GYS AAG GAR ACS GG GAS TTC GGR TGS GTC TTS AYG AA	95 °C for 15 s, 65 to 60 °C for 30 s (-1°C by cycle), 72 °C for 1 min, 6 cycles 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, 40 cycles	(Throback, et al. 2004)
*nosZ*I nosZ2F/nosZ2R	CGC RAC GGC AAS AAG GTS MSS GT CAK RTG CAK SGC RTG GCA GAA	95 °C for 15 s, 65 to 60 °C for 30 s (-1 °C by cycle), 72 °C for 30 s, 6 cycles 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, 40 cycles	(Henry, et al. 2006)
*nosZ*II nosZIIF/nosZIIR´	CTI GGI CCI YTK CAY AC GCI GAR CAR AAI TCB GTR C	95 °C for 15 s, 54 °C for 30 s, 72 °C for 30 s, 40 cycles	(Jones et al 2013)
amoA (AOB) amoA-1F/amoA-2R	GGG GTT TCT ACT GGT GGT CCC CTC KGS AAA GCC TTC TTC	94 °C for 30 s, 55 °C for 45 s, 72 °C for 30 s, 35 cycles	(Rotthauwe, et al. 1997)
amoA (AOA) CrenamoA23F/CrenamoA616r	ATG GTC TGG CTW AGA CG GCC ATC CAT CTGTATGTCCA	95 °C for 15 s, 55 °C for 30 s, 72 °C for 30 s, 35 cycles	(Tourna, et al. 2008)
16S rRNA anammox A438f/A694r	GTC RGG AGT TAD GAA ATG ACC AGA AGT TCC ACT CTC	95 °C for 30 s, 55.5 °C for 15 s, 72 °C for 35 s, 40 cycles	(Humbert et al., 2012)
nrfA nrfAf2aw/nrfA7R1	CAR TGY CAY GTB GAR TA TWN GGC ATR TGR CAR TC	95 °C for 30 s, 53 °C for 30 s, 72 °C for 35 s, 50 cycles	(Welsh et al., 2014)

Inhibition tests for every sample were performed independently before qPCR assays were done. For inhibition tests a known number of copies of the plasmid DNA (pGEM-TEasy, Promega, Madison, WI), were added to the extracted DNA samples at a concentration of 5 ng/μL. This concentration is considered to be sufficient to detect significant increase in cycle thresholds (Ct) in case of PCR inhibition since generally it corresponds to a 1:10 or 1:100 dilutions (Hallin et al., 2009). Independently of the plasmid solution containing a known number of copies supplemented to the sample, Ct quantifications were done in parallel using plasmid specific PCR primers (M13 universal primers). When needed, DNA extracts were diluted until no difference in the measured threshold cycle (Ct) values compared to those obtained with an internal standard, were detected.

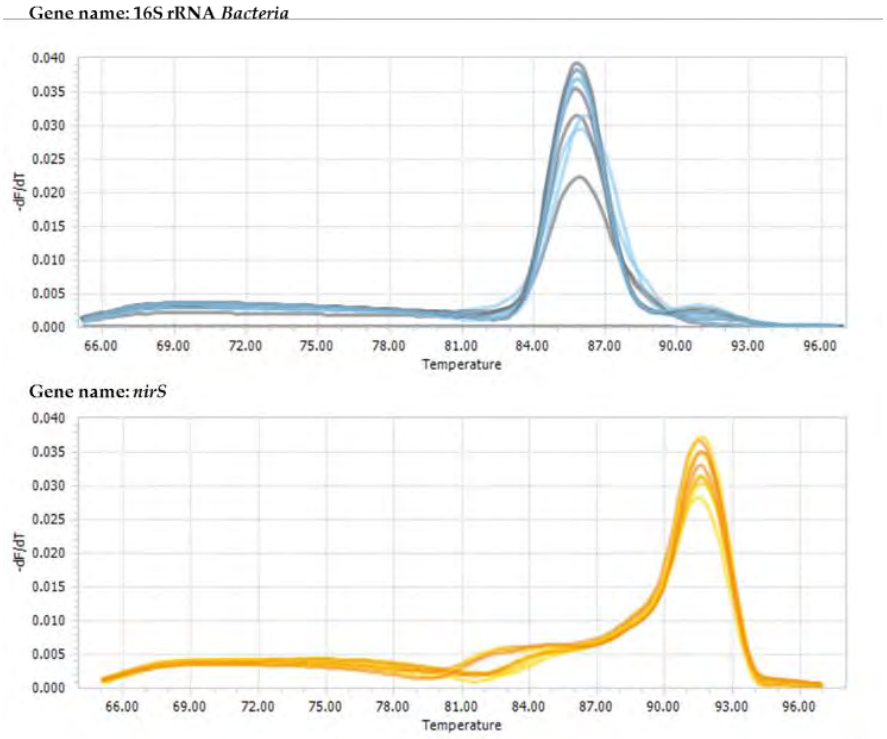

Figure 3.7. Dissociation peaks of qPCR. Examples of the melting peaks obtained by qPCR for two of the studied genes (16S rRNA *Bacteria* and *nirS*) by qPCR.

The relative contributions of the functional genes (*amoA Bacteria, amoA Archaea*, *nirS*, *nirK*, *nrfA*, *nos*ZI and *nos*ZII) compared with the 16S rRNA gene were calculated as a proxy for nitrifying, denitrifying and DNRA microorganisms abundance.

Table 3.2. Efficiency of qPCR standard curves. Values of efficiency (%) and the lineal adjustment of the standard curves (R^2) for all genes analysed by qPCR.

Target gene	N	qPCR efficiency (%)	R^2
16S rRNA *Bacteria*	5	89.6 ± 7.5	1.00 ± 0.00
16S rRNA *Archaea*	2	78.5 ± 5.0	1.00 ± 0.00
amoA Bacteria	2	88.0 ± 1.4	0.99 ± 0.00
amoA Archaea	2	90.0 ± 1.4	1.00 ± 0.01
nirK	3	87.3 ± 8.5	0.99 ± 0.00
nirS	3	85.3 ± 4.0	0.99 ± 0.01
nrfA	3	92.6 ± 12.2	0.98 ± 0.02
*nosZ*I	1	80.0	0.99
*nosZ*II	1	83.0	0.99
16S rRNA anammox	1	82.0	0.98

3.4.4. Barcoded Amplicon Massive Sequencing

For all samples, sequencing of 16S rRNA genes was performed at MSU Genomics Core (Michigan, USA) using an Illumina MiSeq platform (Mardis, 2008). The bacterial 16S rRNA V3-V4 region was amplified using dual indexed Illumina compatible primers F515/R806 (Caporaso et al., 2010) for sediment and root samples, and Pro341F/Pro806R as described previously (Takahashi et al., 2014) for water samples. Dual indexed, Illumina compatible ends were added to the primary PCR products by secondary PCR with primers directed at the Fluidigm CS1/CS2 oligo ends. Each set of amplicon libraries were batch normalized using Invitrogen SequalPrep DNA Normalization Plates and normalized products pooled. Sequencing was performed in a 2x250bp paired end format using a v2 500 cycle reagent cartridge. Base calling was done by Illumina Real Time Analysis (RTA) v1.18.54 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.19.0.

Quality of raw reads was initially checked using the FastQC application (www.bioinformatics.babraham.ac.uk). Raw sequences were demultiplexed, joined paired reads, quality-filtered, chimera checked and clustered into operational taxonomic units (OTUs) (97% cut-off) using Usearch v.9.1 (Edgar, 2016). Sequences were quality-filtered using a maximum expected error of 0.25 and a minimum sequence length of 250 bp. Due to a drop of the sequence quality at the end of reads, no effective merging of forward and reverse sequences (< 10% total raw reads) could be obtained for the expected amplicon size in water samples (464bp). Therefore, forward (R1) and reverse (R2) sequences were analysed separately. These sequences were quality-filtered using a minimum score of 28 and a

minimum sequence length of 200 bp. Singletons and doubletons, OTUs containing one or two sequences, were removed to avoid spurious diversity. Paired-end sequences were aligned and classified using Mothur v1.39 (Schloss, 2008). Taxonomic classification of the OTU representative sequences was done using the SILVA release 123 (water samples) and 128 (roots and sediment samples) reference alignment and taxonomy database. Sequences yielding < 50 % bootstrap or unclassified genera with the SILVA database were taxonomically identified by Blast (NCBI). Moreover, in two samples from FWS-CW after the sediment dredging event, *nirK* and *nirS* genes were also analysed by Illumina Miseq platform. Similar procedure as 16S rRNA gene was done, though it was analysed by FunGene Pipeline and sequences were classified according to FunGene Repository (Fish et al., 2013).

To deeply analyse microbial community, alpha-diversity indicators of richness (Observed richness and Chao1) and diversity (Shannon and phylodiversity indices) were calculated in Mothur after normalization of the number of sequences in each sample by randomly selecting a subset corresponding to the lowest amount of sequences found in a sample. In the case of water samples, no significant differences were found between R1 and R2 datasets, for alphadiversity indices (Mann-Whitney, $p > 0.05$) (Table 3.3). Consequently, only R1 dataset was used for the subsequent analyses since it contained a larger number of sequences that passed quality filtering. This was done to minimize possible biases of diversity indices due to different sampling efforts. Similarity between samples was checked by the analysis of beta-diversity for 16S rRNA. Unweighted and/or weighted Unifrac distance was calculated and samples clustered in a PCoA using Mothur and primer-e v6 (Clarke and Warwick, 2001). Differences between sample groups were tested with ANalysis Of SIMilarity (ANOSIM) and/or PERMutational Analysis Of Variance (PERMANOVA) tests using primer-e v6

Table 3.3. Filtered sequences and alphadiversity in groundwater. Filtered sequences and alphadiversity indices average±standard deviation, for R1 and R2 analysed separately in groundwater samples. Filtered sequences in red colour are samples that were discarded due to the low number of reads obtained. Sobs: observed richness; Chao1: Chao1 index for richness; H': Shannon index.

	Filtered sequences R1	Filtered sequences R2	Sobs R1	Sobs R2	Chao1 R1	Chao1 R2	H' R1	H' R2
SMC-025A	2220	2246	280.420 ± 6.155	244.275 ± 5.040	387.185 ± 26.284	324.791 ± 20.563	4.888 ± 0.031	4.517 ± 0.028
SMC-025B	2856	2666	280.765 ± 7.318	237.083 ± 5.515	421.031 ± 33.023	331.041 ± 25.286	4.744 ± 0.039	4.423 ± 0.033
SMC-037A	4444	2477	341.471 ± 8.006	256.440 ± 5.513	475.959 ± 30.326	341.737 ± 22.983	4.952 ± 0.047	4.342 ± 0.038
SMC-037B	8262	4681	375.898 ± 8.942	293.855 ± 6.668	534.413 ± 33.800	382.685 ± 24.063	5.182 ± 0.048	4.648 ± 0.043
SPT-001A	3551	2758	266.808 ± 7.227	222.355 ± 5.330	413.236 ± 35.217	308.776 ± 23.990	4.708 ± 0.039	4.387 ± 0.033
SPT-001B	3445	2709	281.752 ± 7.736	248.248 ± 6.507	450.138 ± 39.898	367.856 ± 30.082	4.790 ± 0.039	4.469 ± 0.034
SVT-007A	2556	1656	309.999 ± 6.165	240.669 ± 3.111	390.492 ± 20.802	292.174 ± 12.713	5.370 ± 0.023	5.060 ± 0.012
SVT-007B	3387	1871	344.086 ± 7.282	286.273 ± 4.188	461.683 ± 28.012	343.429 ± 14.487	5.450 ± 0.026	5.216 ± 0.015
SMC-001A	6542	3805	232.178 ± 6.957	183.612 ± 4.729	313.540 ± 25.685	230.450 ± 17.067	4.700 ± 0.038	4.318 ± 0.031
SMC-001B	4049	1492	247.888 ± 7.300	199.709 ± 2.494	356.301 ± 28.317	279.449 ± 14.100	4.716 ± 0.038	4.291 ± 0.014
SMC-002A	8526	7473	137.620 ± 6.242	131.461 ± 5.576	222.139 ± 30.788	195.166 ± 24.515	3.142 ± 0.054	3.275 ± 0.044
SMC-002B	6811	6328	108.748 ± 5.079	116.735 ± 4.839	169.441 ± 26.055	158.080 ± 17.590	3.107 ± 0.045	3.392 ± 0.040
MNL-019A	7661	6497	178.633 ± 6.999	142.627 ± 5.771	270.799 ± 26.927	211.080 ± 24.027	3.446 ± 0.061	2.885 ± 0.052
MNL-019B	3408	2663	184.263 ± 5.509	139.849 ± 3.923	243.404 ± 19.204	175.506 ± 13.855	3.898 ± 0.045	3.288 ± 0.038
TOR-013A	1249	409	377.111 ± 3.127		486.445 ± 12.802		5.498 ± 0.010	
TOR-013B	125	77						

3.5. Microbial activity approaches

3.5.1. Isotopic characterization

The oxygen and hydrogen isotopic characterization ($\delta^{18}O$ and δD) of water samples was obtained by CO_2 and H_2 equilibrium, respectively, and isotope ratio mass spectrometry (IRMS). Notation is expressed in terms of the ‰ deviation of the isotope ratio of the sample relative to that of the V-SMOW standard. Reproducibility of the samples calculated from standards systematically interspersed in the analytical batches was ±0.06‰ for $\delta^{18}O$ and ±0.7‰ for δD.

For the $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ analysis, dissolved NO_3^- was concentrated using anion-exchange columns Bio Rad® AG 1-X8 (Cl^-) 100–200 mesh resin after extracting the SO_4^{2-} and PO_4^{3-} by precipitation with $BaCl_2 \cdot 2H_2O$ and filtration. Next, the dissolved NO_3^- was eluted with HCl and converted to $AgNO_3$ by adding Ag_2O. The $AgNO_3$ solution was then freeze-dried to purify the $AgNO_3$ for analysis (collection and purification procedures modified from Silva et al., 2000). The $\delta^{15}N_{NO3}$ was determined in a Carlo Erba Elemental Analyzer (EA) coupled in continuous flow to a Finnigan Delta C IRMS. The $\delta^{18}O_{NO3}$ was determined in duplicate with a ThermoQuest TC/EA (high Temperature Conversion/Elemental Analyzer) unit coupled with a Finnigan Matt Delta C IRMS. Notation is expressed in terms of δ (‰) relative to that of AIR (atmospheric N_2) and V-SMOW (Vienna Standard Meand Oceanic Water) as the international standards for $\delta^{15}N$ and $\delta^{18}O$, respectively (Otero et al., 2009). Precision ($\equiv 1\sigma$) of the samples calculated from international and internal standards systematically interspersed in the analytical batches was ±0.3‰ and ±0.4‰, for $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$, respectively.

3.5.2. Potential denitrification and DNRA activity assays

Potential nitrate+nitrite reduction (NR) and dissimilatory nitrite reduction to ammonia (DNRA) rates were determined according to Ruiz-Rueda et al. (2009) with minor modifications. Briefly, 2.5 g of fresh sediment were diluted in 45 mL in a sterile isotonic solution (Ringer solution, Scharlab, Barcelona, Spain), homogenized and filled in 125 mL serum sealed glass bottles (Figure 3.8). Headspace was exchanged after five vacuum-filling cycles with filtered pure N_2 (99.5%) to ensure anaerobic conditions. After twelve hours in agitation, sediment slurries were supplemented with KNO_3 (1,680 μg $N\text{-}NO_3^-$/L final concentration) and glucose (1,440 μg $C\text{-}C_6H_{12}O_6$/L), at a C:N ratio near to 1:1. Samples of

the liquid phase were taken every 2 h on the first 8 h of incubation. A final sample was collected after 24 h. The experiments were conducted at 25ºC with continuous agitation (170 rpm) in a rotary shaker. Controls with autoclaved sediment slurries were also included. In order to discriminate between DNRA and denitrification, the acetylene blocking technique was used. Pure acetylene (up to a 0.1 atm. partial pressure) was added shortly before nitrate amendment.

Figure 3.8. Example of slurries prepared to determine potential denitrification and DNRA activities in sediment of Empuriabrava FWS-CW.

For chemical determinations, 2 mL samples of the liquid compartment were collected, centrifuged for 2 min at 10,300 rpm and filtered through a 0.22 μm pore size membrane filter (Jet Biofil, Guangzhou, China). Samples were kept at -20 ºC until analysed. Nitrate and nitrite concentrations were analysed by ion chromatography (Waters Corporation, Barcelona, Spain) using a 4.6 × 200 mm Waters Spherisorb® 5 μM SAX as analytical column. Ammonium concentration was determined by the salicylate sodium-nitroprusside colorimetric method (APHA, AWWA, WEF 2012). Nitrous oxide concentration in the liquid phase was directly measured in the incubation bottle at the end of the experiment (24 h), using a selective N_2O electrode (Unisense, Aarhus, Denmark). Total accumulated N_2O (liquid + gas phases) was calculated according to Henry's law.

Potential nitrate+nitrite reduction (NR) and dissimilatory nitrate reduction to ammonia (DNRA) rates were estimated as the slope of a linear correlation of nitrite + nitrate reduction and ammonium increase concentrations through the eight first hours of experiment, respectively. N_2O and ammonium concentrations at the end of the incubation period were used to estimate the proportion of nitrate removed through denitrification and DNRA processes, respectively.

3.5.3. Estimation of radial oxygen diffusion

Intact roots from *Typha angustifolia* were fixed at the bottom of a methacrylate container and immersed in a deoxygenated 0.15% agar solution. Roots were slightly bended in a way the base of the root could be exposed to the air, while tip remained immersed (Figure 3.9). Low melting agar was used to minimize oxygen diffusion at the surface of the chamber and to prevent convective diffusion (Meeren et al., 2001). Agar solution was boiled for 20 min and bubbled with pure nitrogen gas while cooling (Kotula and Steudle, 2009). In addition, 5 mL of an exponential baker's yeast culture (*Saccharomyces cerevisiae*) was added to ensure oxygen consumption and to force oxygen to diffuse from the root. Roots were deepened about 3 to 3.5 cm in the agar solution. Maximum duration of measurements was three hours using the same agar solution. After this time, new solutions were prepared and degassed.

In order to estimate diffusion at different positions along the root longitudinal axis, oxygen concentrations were measured orthogonally to the root surface at different distances. Oxygen measurements were obtained with an oxygen microsensor (tip diameter 50 μm, Unisense, Denmark) and a micromanipulator. Potential oxygen diffusion rates (pOD, μmol O_2/L/μm) were calculated as the tangent at the root surface of O_2 concentration *vs* distance curves (Kotula & Steudle, 2009).

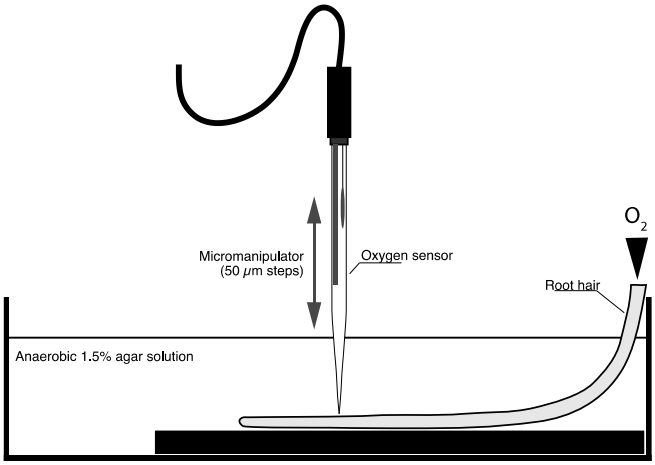

Figure 3.9. Schematic drawing of the set-up for measuring oxygen diffusion. Diagram showing the set-up used to monitor oxygen profiles at different positions along the root surface. Micromanipulator was moved in the xy axis to locate the oxygen sensor at the desired position over the root. Once positioned, it was moved on the z axis at 50 μm steps.

3.6. Statistical analyses

Differences in hydrochemical and isotopic data were assessed using independent samples T-tests for normally distributed data. Non-parametric tests were used (Mann-Whitney test) for non-normally distributed data. Gene abundance data were log transformed prior to any statistical test. Despite this transformation, no normal distribution of data was achieved (Kolmogorov Smirnov test, $p < 0.05$), and non-parametric tests were used. Differences in gene abundances were tested among individual samples or sample groups (see results for details) using either Kruskal-Wallis or Mann-Whitney tests, respectively. Differences between genes coding for enzymes catalysing the same reaction (i.e. *nirK vs nirS* and *nosZ*I *vs nosZ*II) were also analysed using Mann-Whitney tests or by paired sample Wilcoxon tests. Pair-wise correlations analysis of gene abundances and physicochemical parameters and activity or potential rates was performed using Spearman's correlation test. Correlation analysis of gene and relative abundances of operational taxonomic units (OTUs) with physicochemical variables was performed using non-parametric Spearman's correlation test. In all cases, the significance level for all tests was set at 0.05. All analyses were performed using SPSS 23.0 (IBM SPSS, Inc).

In the study of the possible changes in the potential activity and the community structure of nitrite reducers after sediment dredging and plant removal in a CW (Chapter 4.1), differences in gene abundances were tested for the effects of sampling location, dredging (and plant removal) and vegetation by Kruskal-Wallis (and Dunn's test with Bonferroni correction) and U Mann-Whitney tests. Kruskal-Wallis test was used when more than two groups were compared, while U Mann-Whitney test was chosen to determine differences between two groups. Differences between genes coding for enzymes catalysing the same reaction (*nirK* and *nirS* vs *nrfA*) were tested by paired sample Wilcoxon test. Correlation analysis of gene and OTUs abundances with physicochemical variables from water and sediment was performed using non-parametric Spearman's correlation test.

Regarding to the study of how radial oxygen loss and environment determine ammonia oxidizers community on *Typha angustifolia* (Chapter 4.2), Wilcoxon test for paired samples was conducted to identify differences in oxygen diffusion rates among roots sections. Differences in gene abundances were tested between samples grouped according to their location and root section (Mann-Whitney test). Correlations of gene abundances, estimated oxygen diffusion rates, and physicochemical parameters were performed using Spearman's correlation test.

Finally, in the study of natural attenuation of nitrate in Osona's groundwater using isotopes to microbiome data (Chapter 4.3), when hydrochemical and isotopic data were normally distributed, independent samples T-test was conducted to identify relationships among samples; otherwise, non-parametric tests were used (Mann-Whitney test). Regarding to gene abundances, differences between samples (Kruskal Wallis test), and samples grouped according to their denitrification level and to the area of wells (Mann-Whitney test) were tested. Differences between genes coding for enzymes catalysing the same reaction (i.e. *nirK vs nirS* and *nosZI vs nosZII*) at the same sample or grouping according to potential denitrification level was also analysed using Mann-Whitney test. Correlation analysis of gene abundances between them and with physicochemical parameters of groundwater was performed using Spearman's correlation test.

4. RESULTS AND DISCUSSION

4.1. Changes in the potential activity and the community structure of nitrite reducers after sediment dredging and plant removal in a Constructed Wetland

Microbial community is essential in CW to ensure an efficient nutrient removal and then return water in better conditions to natural environment. Generally, microbial community of CW is stable, though it could be altered by different perturbations caused from the management of the environment. Among the various management activities regularly developed in wetlands, plant harvesting and sediment dredging to avoid clogging of the CW, are among the ones generating a larger impact (Griffiths and Philippot, 2013; Thullen et al., 2002). Different studies indicated a high sensitivity albeit short recovery times, of microbial communities to different disturbances in CW (Ligi et al., 2014a; Smith and Ogram, 2008).

In this chapter the effect of a scheduled episode of sediment dredging and vegetation clearance (~70% of the wetland area) in the Empuriabrava FWS-CW (NE, Spain) is assessed. The Empuriabrava FWS-CW is set as a tertiary treatment to reduce the nitrogen load before water is discharged to the environmentally protected area of *Els Aiguamolls de l'Empordà*. Potential activities of denitrification and DNRA were determined six months before and after sediment dredging. Vegetated and non-vegetated areas were sampled in the two periods (for more information see section 3.1.2). The structure of microbial community was analysed as a mean to estimate the abundance of nitrite reducers and to identify bacteria potentially participating in nitrite transformation pathways. Although differences in the microbial community were found, our results point to a high recovery of the potential nitrite reduction rates in the sediment and thus reflecting a high recovery rate of the system and functional redundancy of the N-cycling populations in the wetland.

4.1.1. Physicochemical characterization of the Europa Lagoon

The annual hydraulic load to the Empuriabrava CW had a marked seasonality with minimum values between November and February (average 2,724 ± 656 m^3/h), and maximum loads on August and September (4,420 ± 1,259 m^3 h). The water inflow to the system during 2012

and 2013 showed NO_3^- concentrations between 98 and 7,000 µg N- NO_3^-/L, while NH_4^+ concentration values ranged from 294 to 7,294 µg N-NH_4^+/L. The inflow of NO_2^- was almost undetectable during the whole period (Figure 4.1). The output of N compounds was always lower than the input reflecting the capacity of the system for nitrogen removal. On average, concentrations at the outflow of Europa Lagoon were 420 ± 329 µg N-NH_4^+/L, 528 ± 511 µg N- NO_3^-/L and 6 ± 21 µg N-NO_2^-/L, representing between 15% and 91% of NH_4^+ removal, and 50% to 97% in the case of nitrite + nitrate.

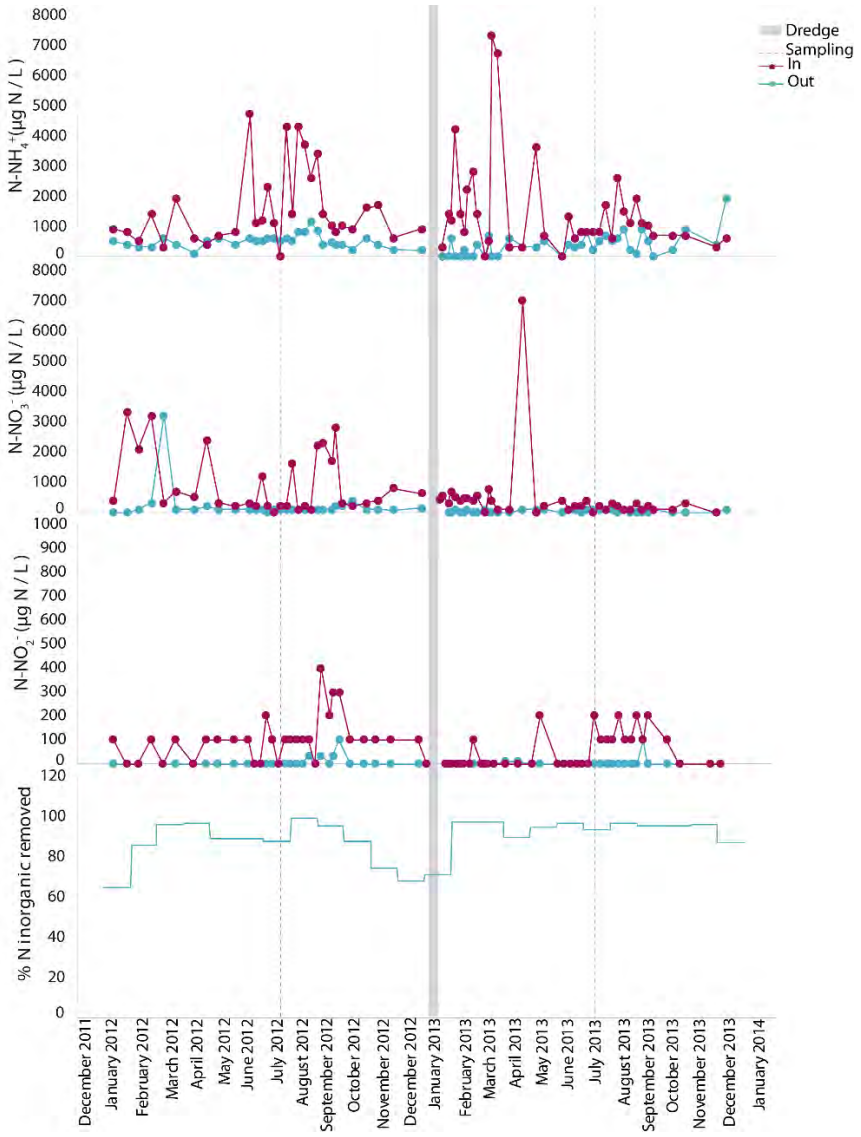

Figure 4.1. Nitrogen compounds in water of Empuriabrava FWS-CW. Concentration of ammonia and nitrate at the inlet and outlet of the Empuriabrava FWS-CW during 2012 and 2013 and % of total nitrogen removed in the lagoon. Sampling dates are indicated by dashed lines. Light grey bar indicates the sediment dredging works in the Constructed Wetland.

Sampling locations were contained within four different areas, vegetated zones that were sampled before (BD-V) and after dredging (AD-V), and bare sediments that were sample before (BD-NV) and after dredging (AD-NV). Samples contained in all four groups exhibited homogenous water physicochemical characteristics (Kruskal-Wallis test, $p > 0.05$). However, significant differences in temperature, redox potential and pH were observed between the two sampling dates (Mann-Whitney test, $p < 0.05$), temperature being slightly higher in July 2013 (after dredging) compared to July 2012, and redox and pH_w values being higher in 2012 (Table 4.1). Before the dredging period NO_2^- was not detected in any of the sediments collected whereas NO_3^- varied between 0.14 and 0.21 μg N/g DW sediment, and NH_4^+ between 0.56 and 16.66 μg N/g DW showing no significant differences between sampling sites (Kruskal-Wallis test, $p > 0.05$). After dredging, significant differences (Mann-Whitney test, $p < 0.05$) were found between V and NV, despite the year of collection, for pH (higher in NV) (Table 4.1). Comparisons of physicochemical parameters of the sediment between BD and AD samples showed that, after sediment dredging, TN and NO_3^- concentrations were significantly higher (2-fold and 10-fold, respectively) (Mann-Whitney test, $p < 0.05$), although these differences cannot entirely attributed to the dredging effect but to a significant change in the quality of the influent to the system. Since similar differences in the N content were found in dredged and non-dredged locations (AD-NV and AD-V samples), these changes were partially attributable to fluctuations on nutrient content in the influent water.

Table 4.1. Physicochemical parameters of studied points in FWS-CW. Physicochemical characterization of water and sediment in different studied areas at the sampling moment. w: water, s: sediment. T: temperature, DO: dissolved oxygen, TN: total nitrogen, TOC: total organic carbon. a indicates significant differences (Mann-Whitney test, $p < 0.05$) between sampling dates, b indicates significant differences Mann-Whitney test, $p < 0.05$ between sampling groups in the same date. BD refers to the samples before the sediment dredging (2012) and AD after sediment dredging (2013).

Group	Sample	pH_w	T_w (ºC)	DO_w (%)	$Redox_w$ (mV)	pH_s	NO_2^-s (µg N/g DW)	NO_3^-s (µg N/g DW)	NH_4^+s (µg N/g DW)	TN_s (µg N/g DW)	TOC_s (µg C/g DW)
BD-NV	RS12	9.21[a]	22.53[a]	144.47	253.30[a]	8.22	0.00	0.21[a]	2.50	34.41[a]	142.47
BD-V	BM12	8.64	26.70	84.70	245.70	7.89	0.00	0.39	10.18	21.05	105.94
	PH12	8.89	20.09	53.70	237.00	8.17	0.00	0.25	16.67	70.73	545.84
	TA12	9.02	22.10	100.47	257.70	8.29	0.00	0.20	0.60	5.48	58.89
	TL12	8.63	20.45	29.40	253.30	7.97	0.00	0.22	6.31	10.38	75.90
	Mean values	8.85 ± 0.20[a]	20.88 ± 1.07[a]	61.19 ± 36.12	249.33 ± 10.91[a]	8.14 ± 0.16	0.00 ± 0.00	0.22 ± 0.03[a]	7.86 ± 8.15	28.86 ± 36.34[a]	226.88 ± 276.36
AD-NV	RS131	8.20	29.23	90.60	98.00	8.20	0.00	6.14	10.18	78.24	177.29
	RS132	8.09	31.14	95.70	119.00	8.09	0.00	4.80	1.97	34.99	919.98
	RS133	8.08	30.30	91.20	121.00	8.08	0.00	3.00	2.17	23.38	59.14
	RS134	8.22	29.25	168.00	135.00	8.22	0.00	4.23	2.57	52.26	94.60
	Mean values	8.15 ± 0.07[a]	29.98 ± 0.92[a]	111.38 ± 37.82	118.25 ± 15.26[a]	8.15 ± 0.07[b]	0.00 ± 0.00	4.54 ± 1.30[a]	4.21 ± 3.88	47.22 ± 23.84[a]	312.75 ± 407.83
AD-V	TA131	7.63	29.00	44.30	125.00	7.63	0.00	4.70	2.74	29.59	79.26
	TA132	7.00	29.45	102.00	145.00	7.00	0.04	11.03	6.88	78.35	352.19
	TA133	7.84	29.43	79.60	117.00	7.84	0.00	8.06	5.62	51.76	190.24
	Mean values	7.49 ± 0.44[a]	29.29 ± 0.25[a]	75.30 ± 29.09	129.00 ± 14.42[a]	7.49 ± 0.44[b]	0.01 ± 0.02	7.93 ± 3.17[a]	5.08 ± 2.12	53.23 ± 24.41[a]	207.23 ± 137.26

4.1.2. Microbial community structure of the sediment

Microbial communities were studied on the basis of the 16S rRNA gene sequences. A total of 1,626,891 sequences passed quality filtering. On average, 43,554 sequences were obtained per sample (ranging from 26,433 to 68,038). A subset of 26,000 sequences per sample was randomly obtained and used for diversity analyses. At this defined sequencing depth, rarefaction curves revealed a reasonable coverage of bacterial richness and phylodiversity (Figure 4.2). Significant differences were observed for phylodiversity (PD) index (Kruskal-Wallis test and Dunn's test with Bonferroni correction, $p < 0.05$) between BD-NV (87.40 ± 3.08) and the groups AD-NV (116.28 ± 12.58) and AD-V (117.66 ± 14.21), suggesting significant changes in the microbial community structure of years 2012 and 2013 (Figure 4.3). The highest increase in the PD was in non-vegetated areas, probably as a response to the 10-fold increase of nitrate concentration in the sediment. However, in disagreement with the later possibility, Van der Zaan et al. showed that the enrichment with inorganic nutrients normally lead to the opposite results, that is, a decrease in biodiversity (Van Der Zaan et al., 2010). Sediment dredging changed both environmental variables and nutrient resources availability, which could lead to greater microbial diversity and the enrichment of taxa that are more resistant to changes. Shifts in microbial community structure after an impact have not been always attributed to a direct disturbance effect but rather to changes in the physicochemical habitat and complex ecological factors, such as interspecies interaction with newly recruited organisms (Griffiths and Philippot, 2013). It is important to notice that, in this study, the lowest diversity values were found in areas which were never planted, in agreement with previous results found in sediments from other CWs (Li et al., 2016; Jian Zhang et al., 2015).

Before sediment dredging, observed richness (number of OTU) varied between $2,025 \pm 14$ and $3,648 \pm 21$, being significantly lower in the TA12 sample (areas covered with *Typha angustifolia*) compared to the BM12 samples (*Bolboschoenus maritimus*, Kruskal-Wallis test and Dunn's test with Bonferroni correction, $p < 0.05$, data not shown). This observation suggested a variation in the microbial community composition according to plant species, similar to previous studies in the same FWS-CW (García-Lledó et al., 2011a; Ruiz-Rueda et al., 2009). Shannon (H') and PD diversity indices varied from 6.15 to 7.14 and from 71.14 to 125.12, respectively (Figure 4.3). Again, significant differences in diversity were found between samples BM12 and TA12 (Kruskal-Wallis test and Dunn's test with Bonferroni correction, $p < 0.05$), being higher in BM12 (Table 4.2).

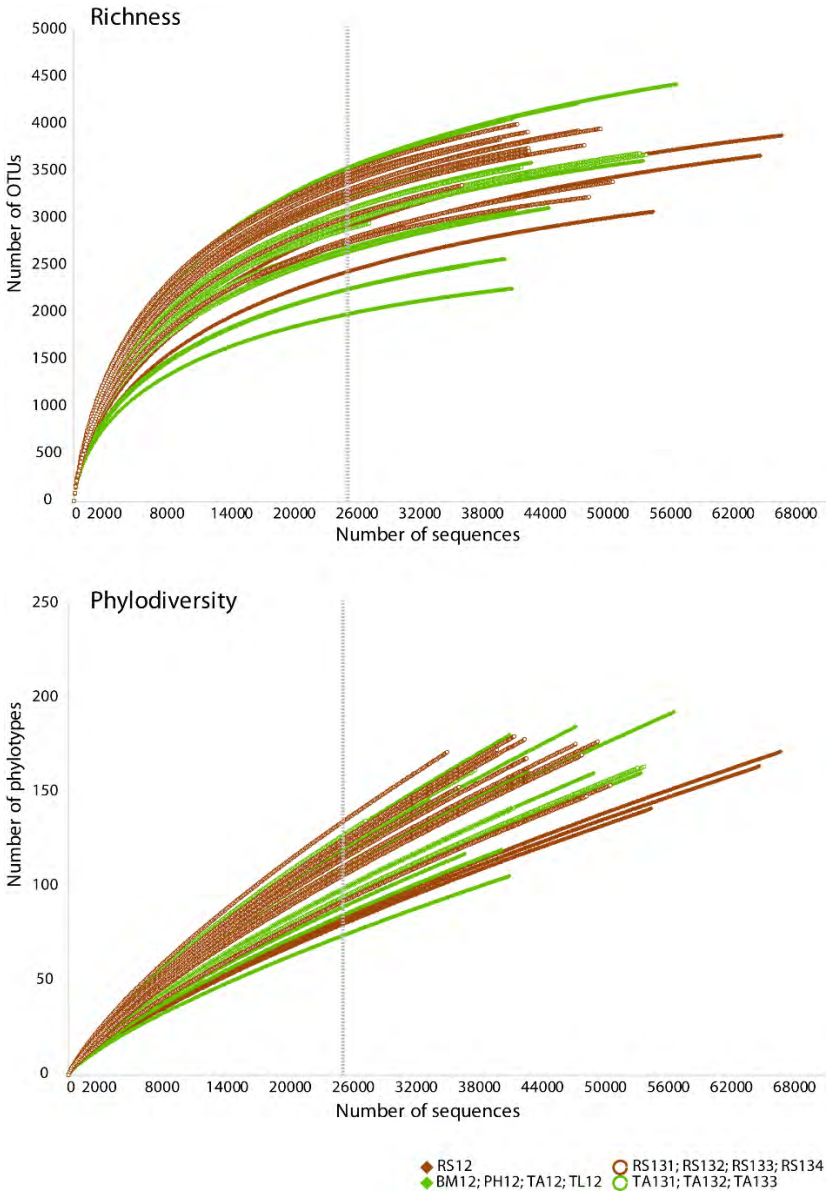

Figure 4.2. Rarefaction curves of samples analysed before and after sediment dredging. A subsample of 26,000 sequences was used to analyse alphadiversity (dot line). Different markers indicate the studied groups (BD-V, BD-NV, AD-V and AD-NV). Above: rarefaction curve according to the observed richness. Below: rarefaction curve according to the phylodiversity index.

After sediment dredging, observed richness varied between 2,768 ± 17 and 3,576 ± 19 while H' and PD indices ranged from 6.50 to 7.16, and from 94.01 to 138.69, in vegetated and non-vegetated areas, respectively (Figure 4.3). No significant differences (Kruskal-Wallis test $p > 0.05$) were found for any of the indicators. When samples were grouped according to their position in dredged or non-dredged areas, significant differences were detected for richness (Kruskal-Wallis test and Dunn's test with Bonferroni correction, $p < 0.05$) being higher in the dredged area (3,307 ± 258 OTUs) compared to the non-dredged (3,056 ± 200 OTUs). This evidenced a higher tendency for recruiting new bacterial species from the

adjacent water in non-vegetated sediments. However, the high microbial diversity in the sediment of Empuriabrava CW, independently of the sample group, may indicate enhanced ecosystem functions and a greater stability of the microbial community, thus reflecting a mature environment, similar to a natural wetland (Ansola et al., 2014; Röske et al., 2012; Wang et al., 2012).

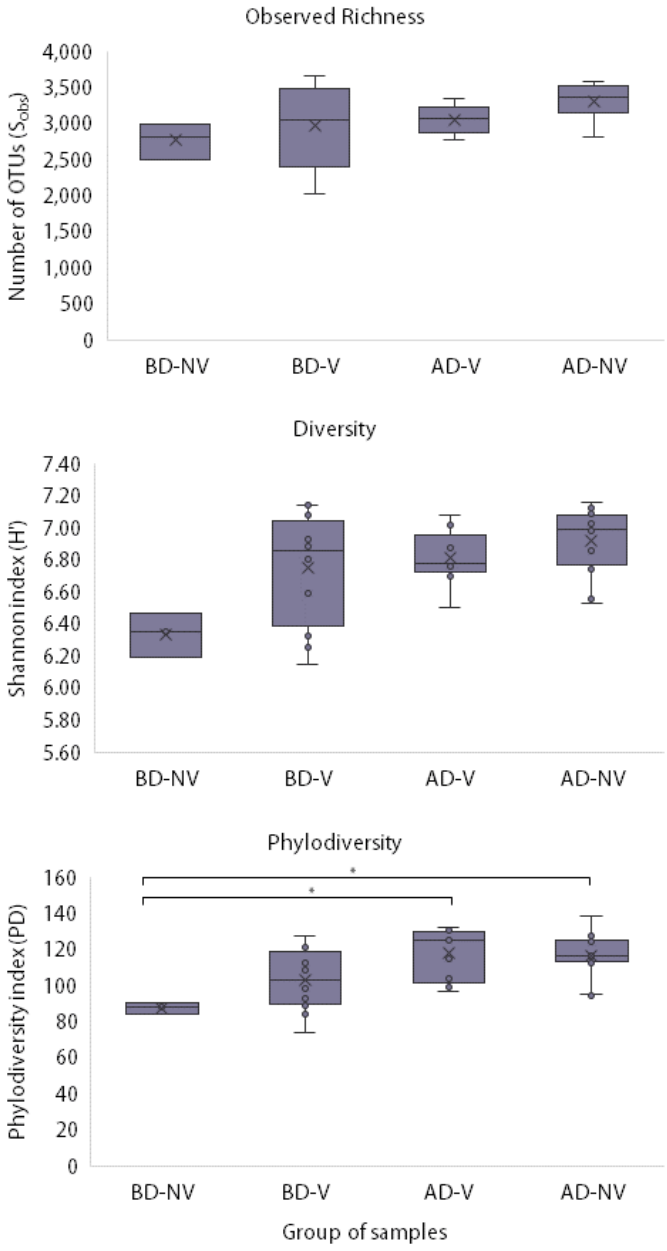

Figure 4.3. Alphadiversity before and after sediment dredging. Box plots indicating calculated richness and diversity indices at the studied areas before and after sediment dredging according to the presence of vegetation. Edges in the boxes show median of each sample group, while crosses indicate mean values and whiskers the observed variance. * significant differences for Kruskal-Wallis test and Dunn's test with Bonferroni correction ($p < 0.05$). BD before dredging, AD after dredging, V vegetated, NV unvegetated.

Differences in the structure of microbial communities were analysed with a Principal Coordinates Analysis (PCoA), based on the Unifrac unweighted distance matrix. The reason to use Unifrac unweighted instead of Unifrac weighted was because our interest was in microbial composition, not in relative abundance of each taxon. The PCoA showed samples distributed clearly into two clusters, mainly according to the presence of vegetation (Figure 4.4). Samples collected in areas covered with *Typha angustifolia* before sediment dredging (TA12) clustered apart from other vegetated samples, reflecting an effect of changing environmental conditions in those samples (high pH and low NH_4^+) compared to the rest.

Table 4.2. Results obtained from the analyses of alphadiversity in FWS-CW samples. Letters a, b and c indicate the replicates analysed for each sample. Sobs refers to observed richness, H' is Shannon index.BD: Before dredging, AD: After dredging, NV: non-vegetated, V: vegetated.

Group	Sample	#seqs	Sobs	**Diversity (H')**	phyloDiversity
BD-NV	RS12-a	68,038	2,980.77 ± 22.89	6.46 ± 0.01	90.37
	RS12-b	55,610	2,492.43 ± 19.44	6.19 ± 0.01	84.22
	RS12-c	65,955	2,816.22 ± 2.05	6.35 ± 0.01	87.62
BD-V	BM12-a	57,946	3,629.25 ± 21.85	7.14 ± 0.01	110.33
	BM12-b	48,555	3,648.32 ± 21.79	7.14 ± 0.01	121.93
	BM12-c	42,218	3,580.10 ± 19.60	7.08 ± 0.01	127.49
	PH12-a	34,504	3,030.34 ± 14.00	6.88 ± 0.01	121.16
	PH12-b	45,596	2,690.50 ± 17.20	6.59 ± 0.01	92.67
	PH12-c	50,144	3,065.16 ± 20.25	6.83 ± 0.01	98.23
	TA12-a	41,344	2,297.05 ± 15.05	6.25 ± 0.01	84.31
	TA12-b	37,830	2,300.95 ± 13.73	6.32 ± 0.01	88.84
	TA12-c	42,036	2,025.01 ± 14.19	6.15 ± 0.01	73.73
	TL12-a	54,733	3,030.47 ± 20.13	6.80 ± 0.01	94.85
	TL12-b	42,200	3,074.96 ± 17.22	6.89 ± 0.01	108.28
	TL12-c	43,994	3,170.89 ± 18.26	6.93 ± 0.01	112.69
AD-NV	RS131-a	55,053	3,026.99 ± 21.51	6.69 ± 0.01	96.94
	RS131-b	28,384	2,939.09 ± 8.10	6.76 ± 0.00	130.76
	RS131-c	54,408	3,066.22 ± 20.61	6.76 ± 0.01	99.07
	RS132-a	42,491	2,768.87 ± 16.95	6.50 ± 0.01	104.03
	RS132-b	39,004	3,335.02 ± 16.88	7.02 ± 0.01	124.92
	RS132-c	35,971	3,294.82 ± 15.72	7.08 ± 0.01	127.75
	RS133-a	33,065	3,117.38 ± 13.49	6.88 ± 0.01	132.32
	RS133-b	43,114	3,167.95 ± 17.87	6.87 ± 0.01	115.08
	RS133-c	26,433	2,786.92 ± 3.50	6.78 ± 0.00	128.04
	RS134-a	40,966	3,460.39 ± 18.70	7.08 ± 0.01	124.11
	RS134-b	43,749	3,342.53 ± 19.12	7.00 ± 0.01	113.58
	RS134-c	43,751	3,331.24 ± 17.32	7.02 ± 0.01	113.65
AD-V	TA131-a	36,314	3,560.52 ± 15.33	7.16 ± 0.01	138.69
	TA131-b	42,673	3,576.53 ± 18.76	7.04 ± 0.01	127.50
	TA131-c	43,749	3,548.19 ± 18.87	7.12 ± 0.01	125.12
	TA132-a	49,115	3,274.04 ± 19.74	6.88 ± 0.01	112.73
	TA132-b	48,526	3,390.98 ± 21.00	6.86 ± 0.01	115.97
	TA132-c	50,774	3,430.35 ± 19.69	6.98 ± 0.01	115.98
	TA133-a	51,800	2,852.34 ± 19.12	6.53 ± 0.01	95.21
	TA133-b	49,474	2,812.27 ± 17.64	6.56 ± 0.01	94.01
	TA133-c	37,374	3,107.50 ± 16.04	6.74 ± 0.01	118.82

The contribution of vegetation and dredging event (sampling date) to community composition was analysed using Dist-LM. Significant contributions were found for both variables, showing a Pseudo-F value of 3.718 for vegetation and 2.153 in the case of sampling date (p-value 0.001). Permutational analysis of variance (PERMANOVA) tests confirmed significant differences between vegetated and non-vegetated samples (Pseudo-F value 3.734, p-value 0.001), showing the importance of vegetation in microbial community composition (Table 4.3). When testing for the statistical differences among the four sample groups initially defined (BD-NV, BD-V, AD-NV, AD-V), highly significant differences were detected when non-vegetated samples were compared to vegetated samples, independently of the sampling year. These results showed that relative abundance of each taxon is dependent of more specific sampling site conditions. In addition, the contribution of sediment main physicochemical parameters to sample distribution on the PCoA was analysed from a correlation test. Concentrations of NH_4^+ and NO_3^-, and pH values resulted in high correlation values (Spearman's test, $R^2 > 0.6$), suggesting a significant influence on the variance of microbial community composition and structure (Figure 4.4) in the Empuriabrava CW, thus reinforcing a combined effect of management strategies (dredging) and prevalent environmental conditions. pH and N content were previously found as environmental drivers for the microbial community distribution in wetland sediments (Ansola et al., 2014; Ligi et al., 2014b; Zhou et al., 2017). Moreover, main microbial communities in the sediment of the Empuriabrava CW distributed according to the presence of vegetation revealing an effect of plants in the rhizosphere, most probably due to the root exudation of organic molecules (Shelef et al., 2013).

Table 4.3. Pair-wise PERMANOVA tests in FWS-CW. Comparisons between sample groups according to Unweighted Unifrac matrix. BD: Before Dredging; AD: After Dredging; V: Vegetated; NV: Non-Vegetated. N=36

Compared groups	Pseudo-F	p-value
Vegetated, non-vegetated	3.774	0.001
2012, 2013	2.364	0.001
BD-NV , BD-V	1.507	0.005
BD-NV, AD-V	1.813	0.005
BD-NV, AD-NV	1.378	0.002
BD-V, AD-V	1.489	0.001
BD-V, AD-NV	1.716	0.001
AD-NV, AD-V	1.842	0.001

The number of sequences assigned to *Bacteria* was much greater than those assigned to *Archaea* in the Empuriabrava FWS-CW sediment. Taxonomy of 16S rRNA gene at the phylum level did not show differences between clusters in the PCoA, at the phylum level.

The main represented phyla in all samples were *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi* and *Firmicutes*, in a descending order (Figure 4.4). *Proteobacteria* accounted for the majority of bacteria in the Empuriabrava FWS-CW sediment (up to 43 % of sequences), similar to what has been previously found in natural systems and in wastewater treatment plants (Andreote et al., 2012; Miao and Liu, 2018). Differences between sampling sites at the taxonomic level were related to the presence of vegetation and dredging. *Chloroflexi* and *Planctomycetes* were mainly detected in samples from vegetated areas. *Chloroflexi* has been found in wastewater treatment plants and in wetlands, being related to nitrogen cycle in different environments (Guo et al., 2015; He et al., 2015; Rivière et al., 2009), and has been previously associated to the rhizosphere of *T. angustifolia* (Gao and Shi, 2018; Guo et al., 2015). *Planctomycetes*, a group that includes anammox bacteria, were also related to the vegetated areas, although at lower densities (below 0.05 %). Anammox bacteria have been observed in association with *Chloroflexi* in different biological reactors (Akaboci et al., 2018; Duarte Pereira et al., 2014). In this sense, the most represented OTU correlated to vegetation (Spearman's test, $R^2 > 0.9$) was assigned to *Levilinea* (2.04% of sequences from vegetated samples), a genus previously reported to play an important role in carbohydrates metabolism and organic acids synthesis, leading to an intensification of denitrification in CW using newspaper as a carbon source (Si et al., 2018). In addition, *Streptomyces* and *Racemicystis* (< 1% of sequences) was a genus that also showed a correlation (Spearman's test, $R^2 > 0.9$) with the presence of vegetation. Some *Streptomyces* are known to be denitrifiers associated with the rhizoplane of *T. angustifolia* in wetlands (Rifaat et al., 2000; Shoun et al., 1998).

On the contrary, non-vegetated areas were characterized by the presence of *Bacteroidetes* and *Verrucomicrobia*. The former are potential opportunists and usually their abundance could be related to an enrichment of organic matter (Grießmeier et al., 2017; Mohit et al., 2015). Although no significant differences were detected in this study, an increase of TOC in AD-NV compared to BD-V pointed to optimal conditions for the development of representatives of this phylum. *Bacteroidetes* members are involved in the nitrogen cycle (Heylen et al., 2006a) and have been previously detected in different natural and human made environments, including CWs, wastewater treatment plants and biological reactors treating wastewater (Ansola et al., 2014; Gabarró et al., 2013; Mohit et al., 2015; Jian Zhang et al., 2015). *Verrucomicrobia*, common sediment bacteria, were also characteristic for samples without vegetation. However, the most representative OTU of non-vegetated areas (Spearman's test, $R^2 > 0.9$) was classified as *Herbaspirillum* (1.13% of sequences from NV samples), a genus within *Burkholderiales*. *Herbaspirillum* species have been previously found in

freshwater sediment lakes and some strains are capable for nitrate respiration and denitrification (Ishii et al., 2009; Tamaki et al., 2005). *Bacillus, Tropicimonas* and *Oryzobacter* (< 1% of sequences) were also correlated to non-vegetated samples. *Bacillus* was previously reported as a denitrifying and also DNRA genus (Hoffmann et al., 1998; Verbaendert et al., 2014). Interestingly, all specific genera associated to either vegetated or unvegetated sediments have links to nitrogen cycle, showing its importance in the sediment of the Empuriabrava FWS-CW.

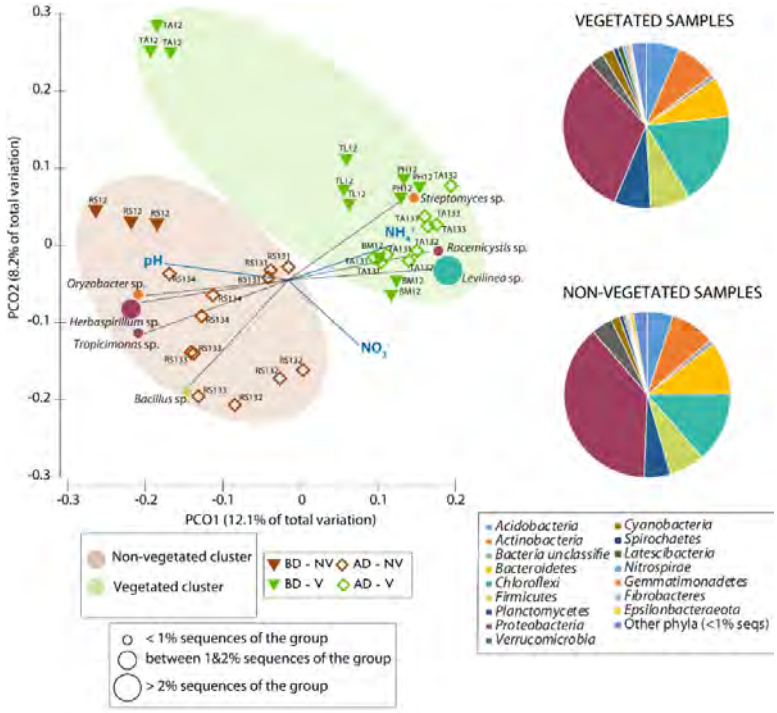

Figure 4.4. Distribution of samples from FWS-CW in the PCoA and main phyla of V and NV samples. PCoA distribution of OTU based microbial community structure in sampling locations (BD before dredging, AD after dredging, V vegetated, NV unvegetated). Sample distribution in the PCoA are correlated to environmental variables (blue lines, Spearman's test, $R^2 > 0.6$), or representative OTUs (black dashed lines, Spearman's test $R^2 > 0.9$). Colour and size of dots indicate corresponding phyla and relative abundance, respectively. Pie plots on the right-hand side show the relative contribution of microbial groups (phylum level) to the average microbial community for samples collected in vegetated and unvegetated areas.

4.1.3. Abundance of 16S rRNA and nitrite reductase genes

Total abundance of 16S rRNA Bacteria, *nirK* and *nirS* showed significant differences according to dredging (Kruskal-Wallis test and Dunn's test with Bonferroni correction, $p < 0.05$). In all cases, higher abundances were found in samples collected before dredging, though for 16S rRNA and *nirK* no differences were detected between BD-V and AD-NV

(Figure 4.5). Quantifications for *nrfA* and 16S rRNA anammox genes could not be performed for BD samples. q*nirK* was always higher than q*nirS*, showing a dominance of *nirK*-type over *nirS*-type bacteria, in Empuriabrava CWs, which has been previously observed in this and other systems (García-Lledó et al., 2011b; Paranychianakis et al., 2016), though *nirS* gene is more abundant than *nirK* in most environments. Relative abundance of denitrifiers (q*nirK*+q*nirS*/q16S rRNA) was similar in all samples except for TA12 and RS12, which significantly showed the lowest (0.053 ± 0.009) and highest (0.116 ± 0.010) q*nirK*+q*nirS*/q16S rRNA values, respectively (Figure 4.6). When samples were grouped according to sample groups, significant differences were detected between AD-V and all others (Kruskal-Wallis test and Dunn's test with Bonferroni correction, $p < 0.05$).

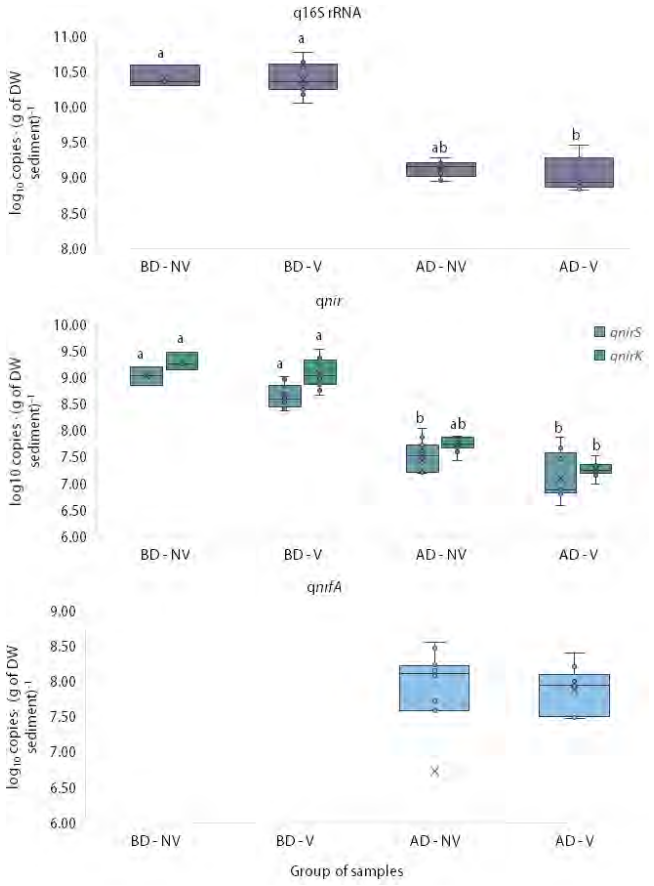

Figure 4.5. Abundances of 16S rRNA, *nirS*, *nirK* and *nrfA* genes before and after sediment dredging. Box plots showing the abundances of 16S rRNA, *nirS*, *nirK* and *nrfA* according to the presence of vegetation and dredging event (BD before dredging, AD after dredging, V vegetated, NV unvegetated). Edges in the boxes show median of each sample group, while crosses indicate mean and whiskers the observed variance. Letters above the boxes denote sample groups in which no significant differences were found (Kruskal-Wallis test and post-Hoc Dunn's test with Bonferroni correction).

qPCR results revealed that all gene abundances decreased after dredging. More precisely, 16S rRNA Bacteria abundance decreased from $2.39 \pm 0.98 \cdot 10^{10}$ to $1.33 \pm 0.55 \cdot 10^{9}$ copies/g of DW sediment, q*nirS* gene from $6.30 \pm 3.85 \cdot 10^{8}$ to $3.87 \pm 2.84 \cdot 10^{7}$ copies/g of DW sediment and q*nirK* from $1.53 \pm 0.90 \cdot 10^{9}$ to $4.17 \pm 2.37 \cdot 10^{7}$ copies/g of DW sediment. *nrfA* gene ranged from $2.99 \cdot 10^{7}$ to $3.59 \cdot 10^{8}$ copies/g of DW sediment. Interestingly, relative abundances of *nrfA* to total bacteria (q*nrfA*/q16S rRNA) were higher than q*nirS*/q16S rRNA and q*nirK*/q16S rRNA (paired sample Wilcoxon test, $p < 0.05$). However, when both denitrifying nitrite reductases were considered together (q*nirS*+q*nirK*/q16S rRNA) no significant differences were detected to the q*nrfA*/q16S rRNA ratio (paired sample Wilcoxon test, $p > 0.05$). Despite some differences in the abundance of key genes were observed before and after dredging, the relative amount of nitrite reductase genes (q*nirK*+q*nirS*/q16S rRNA) remained constant in the dredged area. However, *nirS* abundance was significantly affected by dredging in non-vegetated areas, and *nirK* occurred preferentially at higher levels in vegetated areas. Even though the two nitrite reductases are functionally equivalent, denitrifiers harbouring either nitrite reductase seem to show a preference for certain environments and have differential niche preferences (Jones and Hallin, 2010). The *nirS* gene has been shown to be widely distributed among bacterial phylotypes, while the *nirK* gene is less spread (Lindemann et al., 2015). *nirS* denitrifying species seemed to be more affected by the impact, and changed its abundance in all sampling sites after the dredging of the sediment. The different response of *nirK*- and *nirS*-type containing denitrifiers may be explained by their intrinsic physiological and genetic differences of the two groups (Heylen et al., 2006a), such as a better adaptation to dried events of *nirK*-type compared to *nirS*-type denitrifiers (Penton et al., 2015). It should be mentioned that the lagoon was completely drained before the sediment dredging, agreeing with a higher resistance of the *nirK*-type denitrifiers. Samples were also tested for the abundance of anammox based on 16S rRNA gene quantifications. Results indicated that the abundance was rather low, ranging from $1.94 \cdot 10^{3}$ to $1.41 \cdot 10^{4}$ copies/g of DW sediment, and was not consistently found in all samples, suggesting a low incidence of anammox reaction in nitrite reduction in the Empuriabrava FWS-CW. It is consistent with the low presence of *Planctomycetes* sequences found in all samples.

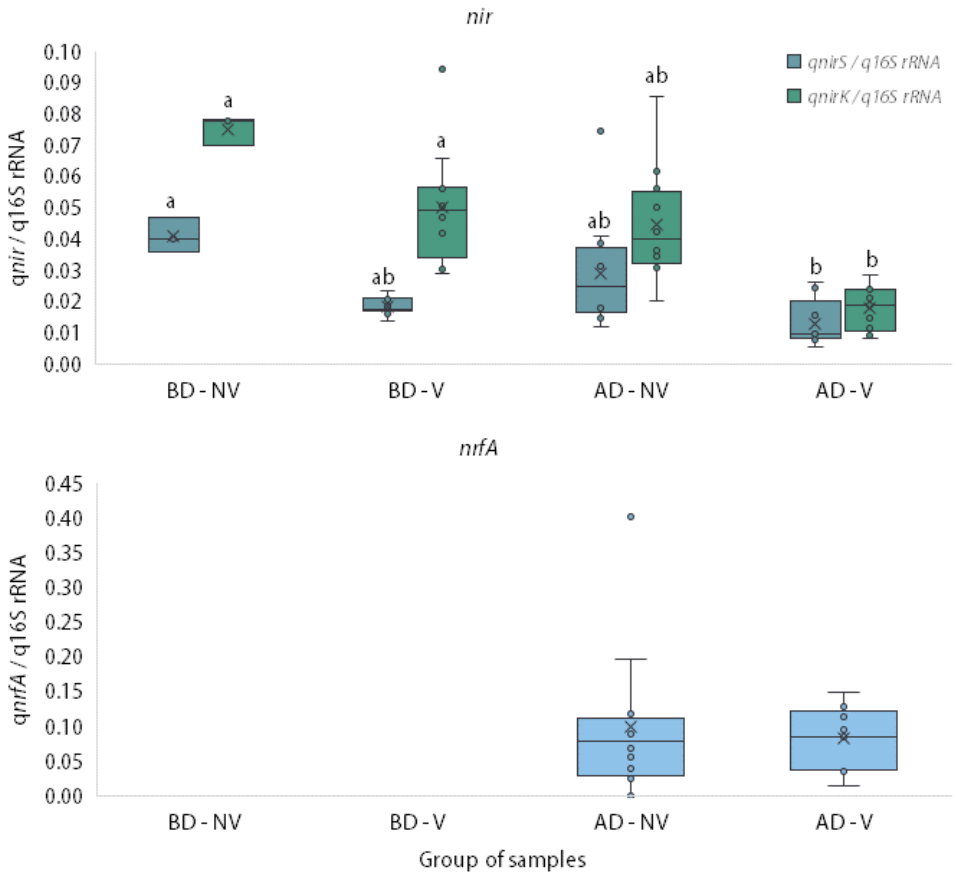

Figure 4.6. Relative abundances of *nir* (q*nirS*+q*nirK*/q16S rRNA) and *nrfA* (q*nrfA*/q16S rRNA). Edges in the boxes show median of each sample group, while crosses indicate mean and whiskers the observed variance. Letters above the boxes denote sample groups in which no significant differences were found (Kruskal-Wallis test and post-Hoc Dunn's test with Bonferroni correction). BD before dredging, AD after dredging, V vegetated, NV unvegetated.

4.1.4. Detection of active denitrifying bacteria using RT-*q*PCR

The levels of gene expression for studied nitrite reductases after the impact were also studied in selected samples. Only two samples were selected for this analysis since all samples from the same area showed the same physicochemical conditions, similar microbial community composition and gene abundances. Gene transcripts were detected and quantified for *nrfA*, *nirS* and *nirK*. Values obtained were significantly lower than the DNA ones, for the genes coding for nitrite reductases (paired sample Wilcoxon test, $p < 0.05$), while *Bacteria* 16S rRNA were higher for cDNA compared to DNA (paired sample Wilcoxon test, $p < 0.05$) (Table 4.4). The ratio cDNA/DNA was between 4.26 ± 3.66 and 14.1 ± 11.2 for 16S rRNA. Functional genes ratios were from $8.57 \cdot 10^{-6} \pm 2.08 \cdot 10^{-6}$ to $8.60 \cdot 10^{-4} \pm 2.81 \cdot 10^{-4}$. Similar results were found in previous studies, indicating the importance to analyse

transcripts in order to determine the gene expression of nitrite reducers (Smith et al., 2007; Wei et al., 2015). No significant differences were found between sampling groups for relative transcript copy numbers (ratio cDNA/DNA), which would show similar proportion of cells potentially active in the populations analysed (U Mann-Whitney test $p > 0.05$).

Table 4.4. Gene abundances of studied genes in active and resident communities. Abundances of 16S rRNA and nitrite reductases genes and transcripts (copies/g of DW sediment). Significant differences were found in all cases between DNA and cDNA (paired sample Wilcoxon test, $p < 0.05$). BD before dredging, AD after dredging, V vegetated, NV unvegetated.

Sample group	DNA or cDNA	q16S rRNA	q*nirK*	q*nirS*	q*nrfA*
AD-V	DNA	$2.55 \cdot 10^{10} \pm 7.78 \cdot 10^{9}$	$3.35 \cdot 10^{8} \pm 1.77 \cdot 10^{8}$	$4.11 \cdot 10^{8} \pm 1.07 \cdot 10^{8}$	$6.19 \cdot 10^{8} \pm 4.90 \cdot 10^{8}$
	cDNA	$9.93 \cdot 10^{10} \pm 8.18 \cdot 10^{9}$	$2.22 \cdot 10^{5} \pm 1.16 \cdot 10^{5}$	$3.46 \cdot 10^{3} \pm 8.25 \cdot 10^{2}$	$8.76 \cdot 10^{4} \pm 4.82 \cdot 10^{4}$
AD-NV	DNA	$2.48 \cdot 10^{10} \pm 1.14 \cdot 10^{9}$	$6.64 \cdot 10^{8} \pm 4.18 \cdot 10^{8}$	$6.39 \cdot 10^{8} \pm 1.63 \cdot 10^{8}$	$4.58 \cdot 10^{8} \pm 4.16 \cdot 10^{8}$
	cDNA	$2.98 \cdot 10^{11} \pm 2.94 \cdot 10^{11}$	$5.27 \cdot 10^{5} \pm 2.73 \cdot 10^{5}$	$2.92 \cdot 10^{4} \pm 4.37 \cdot 10^{3}$	$1.24 \cdot 10^{5} \pm 3.65 \cdot 10^{4}$

In order to analyse differences in gene abundances according to water physicochemical variables, pair-wise correlation tests were performed. NO_3^- and TN correlated negatively (Spearman's test, $p < 0.05$) to 16S rRNA abundance, qnirK, qnirS, qnrfA and qnirK+qnirS/q16S rRNA ratio (Spearman's test, $p < 0.01$) to NH4+ (Table 4.5). pH and redox potential were positive correlated to q16S rRNA, qnirK and qnirS genes, while temperature showed a negative correlation to all them. Then, changes in pH and nitrogen concentration in sediments were related to the decrease of gene abundances, consistently with the results of other studies in soils and wetlands (Fierer and Jackson, 2006; Hallin et al., 2009; Ligi et al., 2014a), stating the pH as the most important factor determining the abundance of nitrite reductases. Moreover, Ligi et al (2014) showed that nitrate increase affected negatively the relative abundance of *nirK*, which is consistent with the negative correlation to q*nirK*+q*nirS*/q16S rRNA found in the Empuriabrava CW (Table 4.5). The difference in water temperature between sampling dates had a negative effect on *nirK* and *nirS* abundances. Lower temperatures typically decrease the denitrification rate (Faulwetter et al., 2009; Philippot et al., 2007) and recent studies have shown a possible negative impact of changes in *nirK* containing bacteria due to temperature (Jiang et al., 2017; Penton et al., 2015). Contrarily, high temperatures favour nitrification (Faulwetter et al., 2009), which could lead to a transient nitrate accumulation in sediments. Although *nirS* gene is more abundant than *nirK* in most environments, q*nirK*/q*nirS* ratio showed a dominance for *nirK*-type

denitrifiers in Empuriabrava CWs, which has been previously observed in this and other systems (García-Lledó et al., 2011b; Paranychianakis et al., 2016).

Table 4.5. Correlation between gene abundances and physicochemical parameters in FWS-CW samples. Spearman's correlation coefficients between physicochemical parameters from sediment and water, potential activities and 16S rRNA and nitrite reductases abundances. R^2 coefficient is showed only in significant correlations (*: $p < 0.05$; **: $p < 0.01$). w: water, s: sediment. T: temperature, DO: dissolved oxygen, TN: total nitrogen, TOC: total organic carbon, n.s.: not significative.

	pH_s	NO_{3^-s}	NO_{2^-s}	NH_{4^+s}	TN_s	TOC_s	pH_w	T_w	DO_w	$Redox_w$	PNRR	PDNRAR
q16S rRNA	0.380*	-0.809**	n.s.	n.s.	-0.501**	n.s.	0.799**	-0.840**	n.s.	0.684**	0.652**	-0,577**
q*nirK*	0.519**	-0.825**	n.s.	n.s.	-0.509**	n.s.	0.892**	-0.684**	n.s.	0.667**	0.707**	-0,499**
q*nirS*	0.433**	-0.834**	n.s.	n.s.	-0.521**	n.s.	0.830**	-0.782**	n.s.	0.682**	0.666**	-0,602**
q*nrfA*	n.s.	-0.632**	n.s.	-0.615**	-0.531*	n.s.	n.s.	n.s.	n.s.	n.s.	-0.579**	n.s.
q*nirK*+q*nirS*/q16S rRNA	0.445**	-0.519**	n.s.	n.s.	-0.347*	n.s.	0.565**	n.s.	n.s.	n.s.	n.s.	n.s.
q*nirK*/q*nirS*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

4.1.5. Potential dissimilatory nitrite reduction activity

Nitrate and nitrite were reduced concomitantly to ammonium production in all samples. Before sediment dredging, potential nitrate+nitrite reduction rate (PNRR) varied between 6.16 μg N/h/(g of DW sediment) (sample BM12) and 49.7 μg N/h/(g of DW sediment) (PA12). After the perturbation (2013 samples), PNRR decreased to minimum and maximum of 1.68 μg N/h/(g of DW sediment) (RS133) and 9.1 μg N/h/(g of DW sediment) (RS131), respectively. An opposite behaviour was observed for potential dissimilatory nitrite reduction to ammonia (PDNRA) rates, which tended to increase after sediment dredging. PNDRAR values ranged from 0.14 μg N/h/(g of DW sediment) (sample RS12) to 1.96 μg N/h/(g of DW sediment) (BM12) before the perturbation, and between 0.14 μg N/h/(g of DW sediment) (TA131) and 4.34 μg N/h/(g of DW sediment) (RS132) after the perturbation. Despite these variations, no significant differences in PNRR and PDNRAR were observed all over the lagoon when samples before and after dredging (Kruskal-Wallis test, $p > 0.05$) were considered separately. However, considering the four defined sample groups, significant differences were detected between BD-V and after dredge (AD-V and AD-NV) for PNRR, showing a higher nitrogen removal rates coupled to vegetated areas (Kruskal-Wallis test and Dunn's test with Bonferroni correction, $p < 0.05$). The highest PNRR in BD-V samples suggested a higher nitrification potential on vegetated areas due to active aeration of the sediment through the plant aerenchyma (Shelef et al., 2013). The nitrite

and nitrate produced by nitrifiers and the excretion of carbon on the root surface results in an increased denitrification, as has been previously observed in constructed wetlands (Lin et al., 2012), a flooded agroecosystem (C. Ryan Penton et al., 2013), and river sediments (Kim et al., 2016). Moreover, species-specific effect of plants on the bacterial community composition and their activities of both sediments and rhizospheres of CW have been previously reported (Ruiz-Rueda et al., 2009). On the other hand, PDNRAR showed significant differences between AD-NV and BD-V and BD-NV (Kruskal-Wallis test and Dunn's test with Bonferroni correction, $p < 0.05$), with the highest values after the sediment dredging (Figure 4.7). The ratio PDNRAR/PNRR averaged 0.02 ± 0.01 in BD-NV and 0.04 ± 0.02 in BD-V samples, whereas increased to 0.72 ± 0.46 in AD-NV and 0.40 ± 0.26 in AD-V, after dredging.

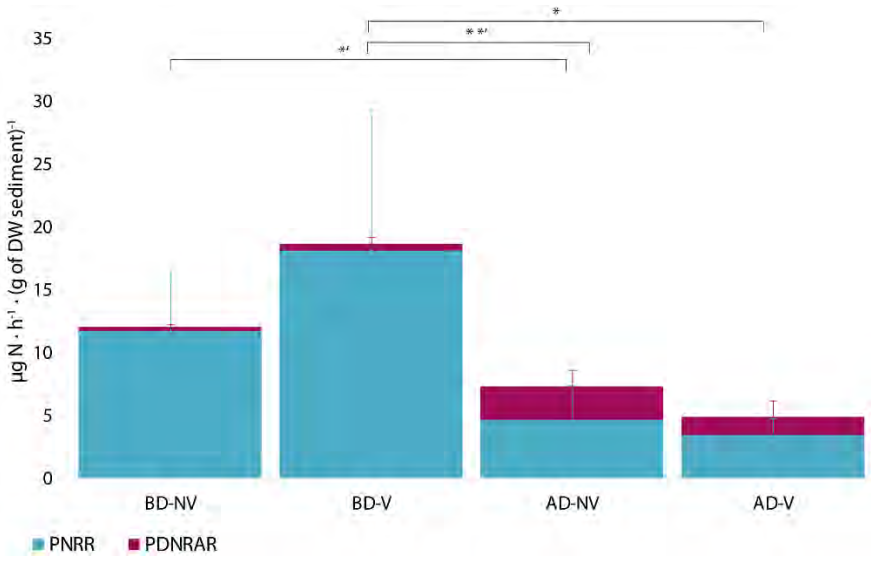

Figure 4.7. PNRR and PDNRAR in the studied groups. Rates of potential nitrate+nitrite reduction (PNRR) and DNRA (PDNRAR) according to the presence of vegetation and dredging event (BD before dredging, AD after dredging, V vegetated, NV unvegetated). * indicates significant differences for PNRR and *' for PDNRAR (Kruskal-Wallis test and Dunn's test with Bonferroni correction, $p < 0.05$).

Despite the observed differences in the measured potential rates, nitrate was completely removed in all samples. Denitrification accounted for the majority of nitrite reduction activity in the Empuriabrava sediments, whereas DNRA occurred at a much less proportion, especially before sediment dredging. DNRA and denitrification compete for nitrogen oxides in many environments, such as wetlands, estuaries, and groundwater (Dong et al., 2009; Hernández-del Amo et al., 2018; Scott et al., 2008). One of the critical factors promoting DNRA in anoxic environments is an increased ratio of organic carbon (electron donors) to NO_3^- concentrations (Burgin and Hamilton, 2007; Tiedje, 1988). We have shown

that the Empuriabrava FWS-CW have a high TOC-C/NO_3^--N mole ratio (from 20 to 1,000 C/N w/w), but DNRA activities remained at less than a half compared to denitrification. The lack of true anaerobic conditions in the wetland (positive redox potentials), might be a reason for the dominance of facultative anaerobic denitrifiers activity over DNRA, since the latter group is primarily comprised of obligatory fermentative or sulfide oxidizing bacteria (Kim et al., 2016; Rütting et al., 2011). Moreover, TOC may include a significant amount of complex organic carbon, which is reluctant to the utilization by DNRA bacteria (Gabarró et al., 2013).

Potential nitrite+nitrate reduction rate was highly positively correlated to q16S rRNA, q*nirK* and q*nirS* abundances, and negatively to q*nrfA* (Spearman's test, $p < 0.01$), indicating the relevance of denitrifying bacteria in the Empuriabrava CW. Contrarily, potential ammonium production rate showed highly negatively correlated to q16S rRNA, q*nirK* and q*nirS* (Spearman's test, $p < 0.01$). Similar correlations between gene abundances and nitrogen removal rates were found in a reactor treating leachate (Gabarró et al., 2013), and in river sediments (Kim et al., 2016). In summary, our results indicate that, although some changes in the microbial community structure could be observed, the potential for nitrogen removal in Empuriabrava FWS-CW remained almost unaffected after a sediment-dredging event, thus indicating a high resilience of the microbial community involved in denitrification.

4.1.6. *nirK* and *nirS* analysis reveals a low number of genera being active denitrifying bacteria

nirK and *nirS* Illumina Miseq allowed to define the phylogenetic structure of DNA and cDNA-based denitrifying community after sediment dredging, the main nitrite reduction pathway in Empuriabrava FWS-CW. A total of 98,382 sequences for *nirK* and 35,103 for *nirS* passed quality filtering. Unfortunately, no reliable sequences could be obtained from the cDNA-based community of *nirS* gene in AD-NV group. To avoid biased results at diversity analyses, subsample of sequences was used for both genes. For *nirK* a subset of 4,300 sequences per sample was randomly obtained, while for *nirS* 2,800 sequences per sample were used (Table 4.6). The number of OTUs for *nirK* was clearly higher in samples from the DNA fraction (resident community), which were between 96 and 121, while for the cDNA fraction (active community) ranged between 2 and 15 OTUs. Resident community showed usual diversity values for Shannon index (2.90 and 3.00), while active community showed a lower diversity (1.78 and 0.48). Similarly, phylodiversity values for the cDNA fraction (2.03

and 0.11) were lower than for the DNA fraction (2.73. and 4.52), showing the highest values in the later (Table 4.6). Similar results were obtained for *nirS* alphadiversity. The number of OTUs for the resident community was between 105 and 113, while only 7 OTUs were detected for the active community. Shannon diversity for the resident community was similar in both areas, with values of 2.96 and 2.76, while active community showed lower diversity (H' = 0.93). The same pattern was observed at phylodiversity index, with values of 7.53 at DNA communities and 0.77 at cDNA (Table 4.6).

Table 4.6. Alphadiversity of denitrifying nitrite reductases. *nirK* and *nirS* alphadiversity values of studied areas for DNA and cDNA communities. #sequences: total number of sequences obtained; Sobs: number of OTUs observed, H': Shannon diversity index; phyloDiversity: phylogenetic diversity index. BD before dredging, AD after dredging, V vegetated, NV unvegetated.

Gene	Sample group	DNA or cDNA	#sequences	Sobs	H'	phyloDiversity
nirK	AD-V	DNA	52,938	121 ± 5	2.90 ± 0.02	2.73
		cDNA	5,587	16 ± 0	1.78 ± 0.01	2.03
	AD-NV	DNA	35,503	96 ± 4	3.00 ± 0.02	4.52
		cDNA	4,352	2 ± 0	0.48 ± 0.00	0.11
nirS	AD-V	DNA	20,362	113 ± 4	2.96 ± 0.03	7.53
		cDNA	2,812	7 ± 0	0.93 ± 0.00	0.77
	AD-NV	DNA	11,929	105 ± 3	2.76 ± 0.03	7.53

For *nirK*, sequences assigned to 38 OTUs represented more than 92% of the total community, while for *nirS*, 35 OTUs accounted for 88% of total sequences, for DNA based analyses. Putatively active NirK-containing communities were represented by 15 OTUs (only 2 in the dredged area) accounting for all sequences in the study. Active NirS-containing communities were also very specific, and only 5 OTU representative sequences were obtained accounting for more than 99% of all sequence signatures.

Between 25% and 44% of total sequences from *nirK* recovered from total communities were associated to unclassified *Proteobacteria* after sediment dredging in vegetated and unvegetated areas, respectively (Figure 4.8a). The most represented genus in AD-V area for DNA community was *Rhizobium* (40.5% of total sequences), while it was less represented in the active community of this area (29% of total sequences). In the presence of plants, several *nirK* genes of denitrifiers were affiliated with *Rhizobium* in various environments (Bremer et al., 2007; Costa et al., 2006; Gómez-Hernández et al., 2011). *Ochrobactrum* was the genus with the highest relative abundance at cDNA community (48.3% of total sequences), and it was less of 0.1% at DNA. In AD-NV, *Ochrobactrum* was also the most represented genus in the active community (81.2% of total sequences), while in the

resident community it represented less of 0.05% of total sequences. *Ochrobactrum* was previously found in water and sediments as a *nirK*-containing denitrifiers (Helen et al., 2016; Junier et al., 2008; Wei et al., 2015). Species of this genus have shown multiple copies of *nirK*, providing adaptive advantages to the denitrifiers in changing environments, and high resistance to salinity, without a destabilization of NIR enzyme, which could be according to high conductivity of the Empuriabrava FWS-CW (Causey et al., 2006; Helen et al., 2016). The other representative genus of the cDNA fraction at AD-NV was *Defluviimonas*, which was the most represented at the DNA fraction (32.5% of total sequences) (Figure 4.8a). This *nirK*-containing genus was previously found in groundwater, exposed rock material in aquifer and biofilters of marine aquaculture, showing a high resistance and ability of reduce nitrite at different physicochemical conditions (Foesel et al., 2011; Herrmann et al., 2017). Both, *Ochrobactrum* and *Defluviimonas*, were not detected in microbial community analysed by 16S rRNA gene. It could be consequence of a very low representation of these genera in total community, since the orders of these genera, *Rhizobiales* and *Rhodobacterales* respectively, represented between 2 and 3% of total sequences. However, the high percentages of sequences related to *nirK*-containing *Ochrobactrum* and *Defluviimonas* could also be related to the horizontal gene transfer (HGT), which is normally in denitrifying genes (Philippot and Hallin, 2005).

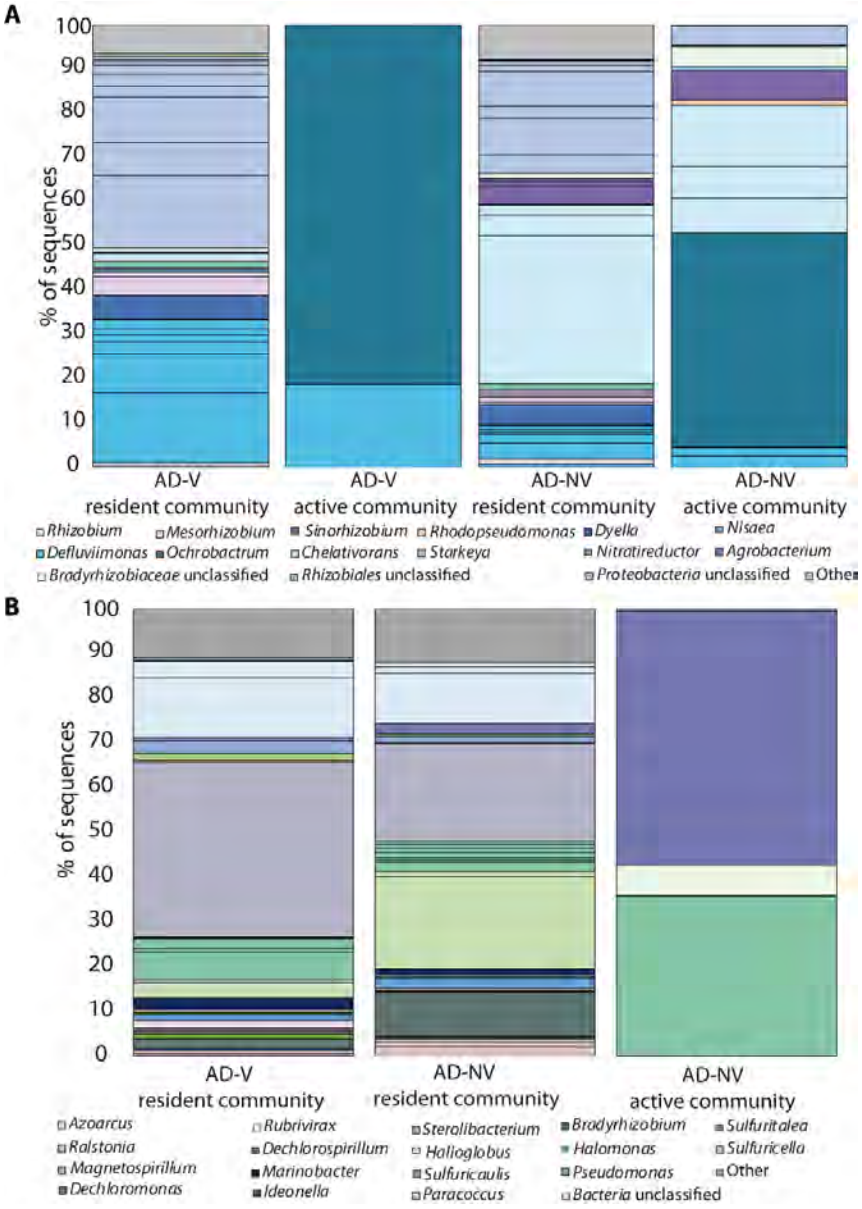

Figure 4.8. Taxonomy of *nirK* and *nirS* resident and active communities. Relative abundances of denitrifying *nirK* (A) and *nirS* (B) communities at the genus level in two samples from the different studied groups after sediment dredging (AD-V and AD-NV). Lines in the bars indicate different OTUs associated to each genus. AD after dredging, V vegetated, NV unvegetated.

Denitrifying communities characterised by *nirS* gene were composed of bacteria belonging to four different genera identified in the resident community (based on DNA analyses), together with unclassified *Bacteria* (which were between 13.8 and 17.6% of total sequences) (Figure 4.8b). AD-NV was basically represented by *Ralstonia* (38.9% of total sequences) and *Pseudomonas* (9.6% of total sequences), while other genera had less than 6% of total sequences. *Ralstonia* and *Paracoccus* were the most abundant genus at AD-V (21.75% of total sequences each one) similarly to other studies in sediment of wetlands and

agricultural soil (Chen et al., 2014; Yunfu et al., 2017). Moreover, *Ralstonia* was previously identified in the same FWS-CW as one of the most represented *nirS*-containing genus (Ruiz-rueda et al., 2007). Contrarily, the active community from AD-V was mainly represented by *Sulfuritalea* (56.7% of total sequences) and *Pseudomonas* (35.6% of total sequences), while in the resident community these genera represented only between 2.2% and 6.6% of the total sequences. *Sulfuritalea* has been shown as a genus affected by impacts in the organic matter load in different studies (Miao and Liu, 2018; Remmas et al., 2016). However, this genus was shown as an active denitrifier treating wastewater (McIlroy et al., 2016), and able to grow in different environments with low C/N as groundwater or WWTP (Herrmann et al., 2017; Li et al., 2017). Then, *Sulfuritalea* could be considered an important genus in the denitrifying community of FWS-CW, which may be affected by the dredging of the sediment. *Pseudomonas* was identified as a *nirS*-containing denitrifier in cultured experiments (Glockner et al., 1993; Härtig and Zumft, 1999) and different environments as agricultural soils and WWTP (Etchebehere and Tiedje, 2005; Henderson et al., 2010; Yunfu et al., 2017). Moreover, some *Pseudomonas* are able to show high level of *nirS* expression in different conditions of C, N and oxygen, and even in soils with high copper concentrations (Black et al., 2016; Saleh-Lakha et al., 2009; Wittorf et al., 2018b), being an important denitrifying genus in the N removal in Empuriabrava FWS-CW. Similar to the most represented *nirK*-containing genera from active community, *Sulfuritalea* was not detected in total microbial community, while *Pseudomonas* represented 0.16 % of total sequences analysed by 16S rRNA gene. These results suggest that HGT is highly present in denitrifying communities and then any correlation could be found between 16S rRNA and functional genes. Moreover, taking into account the low ratios of gene quantification between active and resident denitrifiers, together with PNRR analysed and the low representation of *nir*-containing genera in microbial community it could suggest that, if optimal conditions are found, denitrifying community can have high yield and ensure the correct functioning of FWS-CW.

4.1.7. Final remark

Results obtained in this chapter indicated that changes in the microbial community were rather limited, pointing to a high resilience of the bacteria involved in nitrate and nitrite reduction. Different studies have highlighted high recovery rates (at different time-scale ranges) of bacterial communities in soils and sediments after different disturbances (Griffiths and Philippot, 2013; McKew et al., 2011; Mohit et al., 2015). Sediment dredging and plant removal enhanced the overall N-removal capacity of the system at the whole environment scale despite potential denitrification rates were reduced in favour to dissimilatory reduction

to ammonia when analysed in the laboratory. Although those changes were not evident at the large scale, transient changes in the microbial community composition and activity due to large management practices should be further investigated in order to predict and evaluate the occurrence of undesirable activities, such as DNRA, in ecosystem services. In this sense, the study of microbial community after a perturbation in systems to treat wastewater (i.e. CW) could be essential to ensure an optimal functioning of the ecosystem.

4.2. Radial oxygen loss and environment determine ammonia oxidizers community on *Typha angustifolia*

Microorganisms implied in N cycle are highly affected by the presence of vegetation. Nitrifiers, and concretely ammonia oxidizers (AOA, AOB and Comammox), are the first microorganisms responsible for start N removal from water, since normally N arrives to water as NH_4^+ form (Canfield et al., 2010; Seelig and Nowatzki, 2017). The positive effect of vegetation to nitrifying community has been previously studied in different environments (Caliz et al., 2015; Ruiz-Rueda et al., 2009; Trias et al., 2012; Xiao et al., 2017). Specifically, in the root surface and the rhizosphere, AOB, AOA and Comammox populations could be affected by plant functional traits, such as some phytochemicals exuded by roots (Thion et al., 2016). Furthermore, differential radial oxygen loss (ROL) along the roots; due to the differential presence of apoplastic barriers composed of suberin, lignin, carbohydrates, and structural cell wall proteins; could determine niche differentiation between the three groups, and affect their nitrification activity. However, there are no clear evidences of how ROL influence the structure and function of ammonia oxidation microorganisms on the rhizoplane.

The aim of this chapter was to quantify the effect of ROL on the spatial distribution of ammonia oxidizing archaea (AOA) and bacteria (AOB) on the root surface (rhizoplane) of the narrowleaf cattail (*Typha angustifolia*.). We hypothesized that areas with higher ROL should select for specific nitrifying organisms. With this aim, we analysed individuals collected from Empuriabrava FWS-CW and from Baix Ter, along River Daró estuary (for more information see sections 3.1.2 and 3.1.3) in order to test for generalized effects at environments of different physicochemical conditions, emphasizing the effect of conductivity gradient. *T. angustifolia* was chosen since it is one of the most used plant species in CW (García-Lledó et al., 2011a; Vymazal, 2011), and is also highly abundant in natural environments of the studied region.

4.2.1. Physicochemical characterization of the sampling points

The sampled environments were chosen since they have different water physicochemical conditions and are distributed in a relatively small geographical area, thus a rather small effect of weather differences was expected. Water temperatures were around 26 ºC, being slightly lower in the Bassa de les Tortugues and higher in the Daró river mouth (Table 4.7). When samples were grouped according to location (i.e. FWS-CW, and Baix Ter),

no significant differences of the measured temperature were observed (U Mann-Whitney test, $p > 0.05$). Sampled environments spanned along a salinity gradient, ranging from slightly saline (conductivity values of 11.95 mS/cm), such as Bassa de les Tortugues, to low salinity fresh water, such as Rec Coll (0.823 mS/cm). Water in the Empuriabrava FWS-CW showed typical conductivity values for the system in summer (García-Lledó et al., 2011b).

Oxygen concentration, pH and Redox values showed significant differences between the two geographical locations (U Mann-Whitney test, $p < 0.05$). The samples from the Empuriabrava FWS-CW were characterized by lower oxygen concentration (0.36 ± 0.06 ppm), higher pH (8.85 ± 0.21), and variable Redox values (from -92 to 70 mV). In contrast, samples from the Baix Ter exhibited a higher oxygenation of water (on average 11.64 ± 1.89 ppm O_2), relatively lower pH (7.34 ± 0.17), and higher positive Redox values (Table 4.7.).

Table 4.7. Water physicochemical characterization in FWS-CW and Baix Ter. Main water physicochemical parameters in the studied sites at the sampling time.

	Temperature (ºC)	Conductivity (mS/cm)	Redox (mV)	Oxygen (ppm)	pH
Empuriabrava Constructed Wetlands					
Europa Lagoon	27.6	3.84	23.7	0.42	8.6
Treatment Cell 1	26.0	3.22	-92.7	0.37	8.95
Treatment Cell 2	24.4	3.38	70.0	0.29	8.99
Baix Ter					
Bassa Tortugues	22.8	11.96	170.5	9.51	7.18
River Daró Mouth	28.7	1.13	90.5	13.05	7.31
Rec Coll	28.0	0.82	123.0	12.37	7.52

4.2.2. Radial oxygen diffusion

Oxygen concentrations were measured orthogonally to the longitudinal axis of the root at different positions, from the tip to the base and were used to estimate potential radial oxygen loss (ROL). Ten different roots from *Typha angustifolia*, five from each of the two geographical sites, were analysed. In all cases, oxygen concentration tended to increase at the root surface confirming diffusion from the root although a high variation of oxygen profiles was observed between roots (Figure 4.9). Moreover, in the majority of the roots (all analysed except one root from DM and one from RC, Figure 4.9), oxygen leakage was higher in the middle section (from 1.5 to 4.5 cm from the tip) although estimated diffusions were highly variable, from 0.003 to 0.316 μmol O_2/L/μm. Oxygen diffusion at the root tip was highly variable and ranged from no detectable diffusion to values similar to those obtained in the middle section (from $3x10^{-5}$ to 0.196 μmol O_2/L/μm). Basal portions (next to the plant junction) always showed lower estimated diffusion values, ranging from 0.002 to 0.038 μmol

O_2/L/µm (Table 4.8). As a mean value for the ten measurements, diffusion in the middle section was between 2 and 20 times higher compared to the root tip.

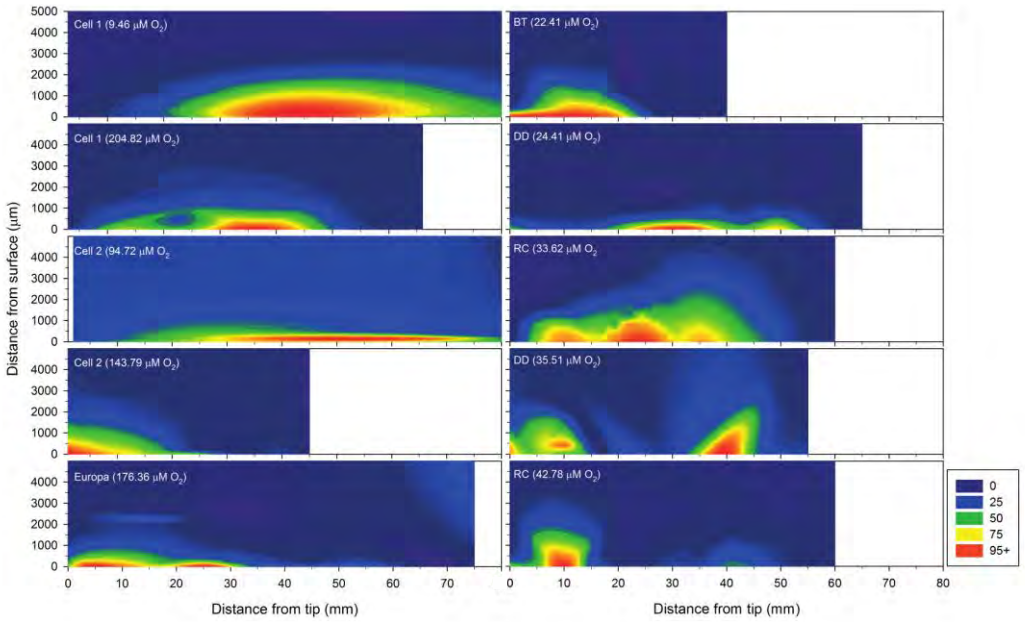

Figure 4.9. Radial oxygen loss at different roots. Contour plots showing the potential radial oxygen loss by plotting the relative concentration of oxygen (%) of 10 different roots at different points along the root longitudinal axis. Left- roots collected in the Empuriabrava FWS-CW. Right- roots collected in the Daró estuary. In each graph we indicate the maximum measured O_2 concentration. Cell 1 and 2- Sampling points 1 and 2 and the treatment cells of the CW, Europa- Europa Lagoon at the CW, BT- Bassa de les Tortugues, DM- Daró river mouth, RC- Rec Coll.

For the two geographical areas analysed, significant differences of potential oxygen diffusion rates were found between the middle section compared to either the tip or the basal sections (paired sample Wilcoxon test, $p < 0.05$). Most likely, these diffusion differences are due to changes in the metabolic activity of the root system. Aerenchyma is the main channel by which oxygen is transported and delivered into the different parts of the macrophyte and therefore O_2 molecules within the root aerenchyma will either be consumed by cells in adjacent tissues, diffuse towards the root apex, or diffuse radially to the rhizosphere (ROL) and be consumed in the soil (Armstrong, 1980). ROL is determined by the concentration gradient, the physical resistance to O_2 diffusion in the radial direction, and consumption of O_2 by cells along this radial diffusion path (Armstrong, 1980; Armstrong and Beckett, 1987). If the metabolic activities of the root cells consume abundant oxygen, oxygen transport to the rhizosphere will be diminished (Colmer, 2003), as is expected in the actively growing root tip which use O_2 for their development metabolism. Moreover, the release of oxygen is also

limited by the root barrier, which is affected by the environment in which the root system is located. When the oxygen is transported to the root, the oxygen is first used for respiration, and the remaining oxygen is released through the intercellular space of the root tip and the lateral root. Therefore, during oxygen transport, the release rate to is affected by the adjacent sediment pressure, which is essential in relation to the number of nearly roots, length, and porosity of the root system (Wang et al., 2018). Adventitious roots of many wetland species contain a barrier to ROL in the basal zones (Armstrong, 1971; McDonald et al., 2002; Seago et al., 1999; Smits et al., 1990). A barrier to ROL in basal root zones can enhance longitudinal O_2 diffusion towards the apex, by diminishing losses to the rhizosphere (Colmer, 2003). However, the proportion of O_2 lost from different regions of the root system can differ markedly, depending on the formation of ROL barriers. In *Typha*, there appears to be a close relationship between aerenchyma formation and maturation of the exodermal bands and suberin lamellae (ROL barriers). Further, *Typha* has extensive casparian bands and suberin lamellae in all walls of the exodermis, which would act as a series of resistances to gaseous exchanges (Seago et al., 1999).

Table 4.8. Oxygen diffusion rates in the studied roots. Average rates of estimated oxygen diffusion rates at different root sections grouped according to the geographical area. Mean values and SE are given. Statistically significant differences for homogeneous variance groups for each geographical area are indicated in the last column by letter (Kruskal-Wallis test and Dunn's test with Bonferroni correction, $p < 0.05$).

	Diffusion rate (nmol O_2/L/**µm**)	Relative diffusion[a]	
Empuriabrava Constructed Wetlands			
Root Tip (<15 mm, n=6)	12.5±14.3	1	a
Middle section (15 to 45 mm, n=6)	51.5±57.3	27.16±49.17	b
Basal section (>45 mm, n=5)	8.4±6.6	14.47±22.93	ab
Baix Ter			
Root Tip (<15 mm, n=6)	10.4±6.4	0.95±0.12	AB
Middle section (15 to 45 mm, n=8)	16.5±7.2	2.63±2.05	B
Basal section (>45 mm, n=5)	7.4±11.0	0.29±0.17	A

[a] Relative to the potential diffusion rate measured at the tip

4.2.3. Abundance of 16S rRNA and ammonia oxidizers

Bacterial cells tightly attached to the root surface were clearly observed in all sections of the root, albeit at relatively low densities (Figure 4.10). In order to determine changes on cell distribution along the root longitudinal axis, qPCR analyses of 16S rRNA and *amoA* genes were used. Bacterial 16S rRNA gene abundances varied between $5.79 \cdot 10^5$ and $1.09 \cdot$

10^9 copies/g of fresh weight (FW) in rhizoplane (root samples), whereas archaeal 16S rRNA genes were found at lower densities, from $1.91 \cdot 10^3$ to $2.49 \cdot 10^8$ copies/g FW (Figure 4.11). On the contrary, abundances of *amoA* genes were rather low. Values varied between $1.26 \cdot 10^4$ and $8.06 \cdot 10^5$ copies/g FW, and from $2.25 \cdot 10^1$ to $4.03 \cdot 10^5$ copies/g FW for AOB and AOA, respectively. Higher abundances of *Bacteria* over *Archaea* were previously found in the *Typha* rhizosphere of similar environments (Trias et al., 2012; Wei et al., 2011). In contrast, *amoA* from AOA was more abundant than AOB in roots and rhizosphere of macrophytes as *Typha* spp., *Paspalum distichum*, *Phragmites australis* and *Ruppia* spp. (Trias et al., 2012; Zhou et al., 2016), rhizosphere of agricultural plants (*Ipomoea batatas* and *Zea mays*) (C Ryan Penton et al., 2013), and even in grassland soils (Sterngren et al., 2015). However, Sterngren et al (2015) showed a higher activity of nitrification by AOB, and Huang et al (2016) found higher AOB densities compared to AOA in sediments and rhizospheres from *Ceratophyllum demersum* and *Potamogeton malainus* in Lake Taihu. Generally, the highest abundance of all genes was observed in the middle section, and the lowest in the base, although observed differences were not significant. This fact supports that oxygenation by roots have a significant impact on microbial community, including increase of aerobic niches and aerobic degradation. A positive correlation between increased O_2 and heavy-metal sedimentation or nitrification activity, has been previously observed (Shelef et al., 2013). Regarding to sediment samples, similar values of abundances were observed for total *Bacteria* and *Archaea* in FWS-CW samples while in Baix Ter locations *Bacteria* was more abundant, 16S rRNA *Bacteria* ranging from $1.71 \cdot 10^5$ and $4.36 \cdot 10^9$ copies/g FW sediment, and *Archaea* from $3.57 \cdot 10^2$ and $1.74 \cdot 10^9$ copies/g FW (Figure 4.11).

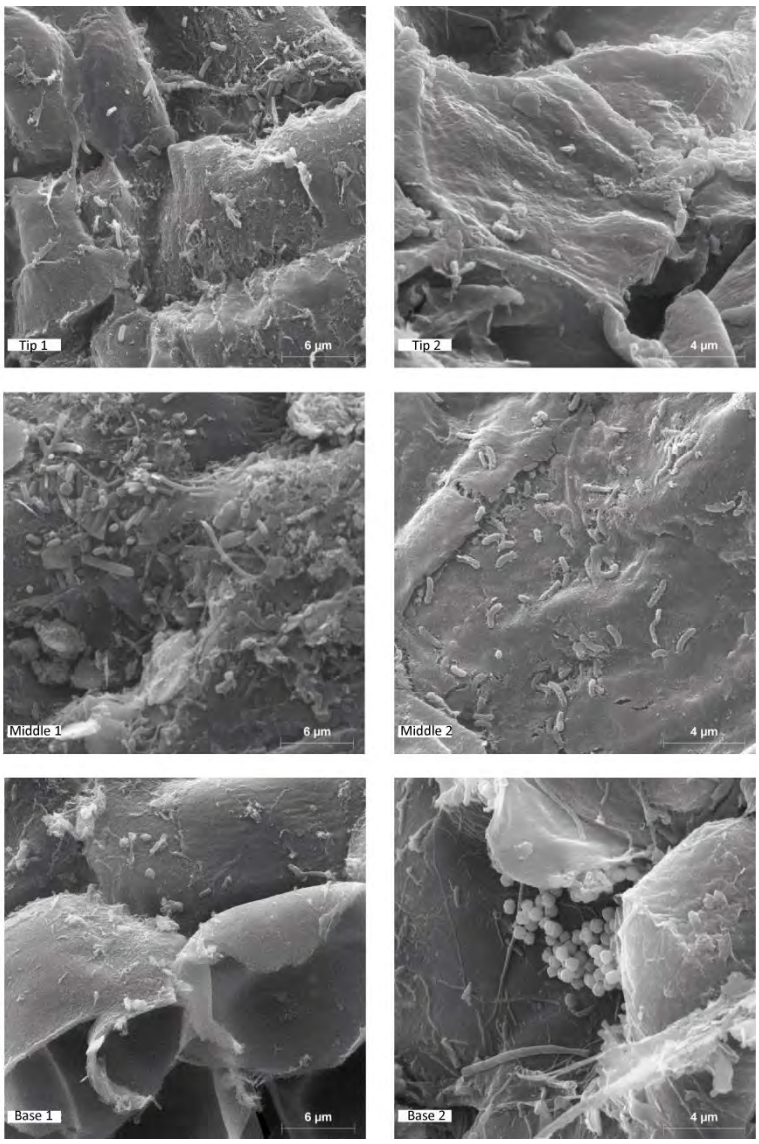

Figure 4.10. Composition of SEM images of root surfaces. Different parts of the roots are shown from the tip (upper images), to the basal section (lower images). The scale is indicated in each image, to have an approach of the size of the observed samples.

To determine differences in gene abundances between root sections and sediment, each sampling site was analysed separately. For constructed wetlands, abundances of 16S rRNA *Bacteria* and *amoA Archaea* showed significant differences between middle and tip sections, being both genes more abundant in the middle. 16S rRNA *Bacteria* was significantly more abundant in middle than in basal section (Mann- Whitney test, $p < 0.05$). These results suggest important effects of oxygen and root exudates on the microbial community. Oxygenation by roots has been shown to have a significant impact on important mechanisms of wastewater treatment in CWs, including enhancement of microbial activity and promoting biofilm formation, and the excretion of carbon by roots has been reported to increase

denitrification (Shelef et al., 2013; Ueckert et al., 1990; Vymazal, 2011). Moreover, the ratio q*amoA*_A/q*amoA*_B showed significant differences between all root sections and sediment (Mann-Whitney test, $p < 0.05$), with the lowest values in sediment (Figure 4.12). Probably, sediment had different physicochemical variables compared to water, which could affect microbial communities in different way than roots, favouring AOB over AOA in sediment. AOA were described to be more sensible to salinity and to pH than AOB in microcosms and estuarine sediments (Santos et al., 2018; Thion et al., 2016); though it depends on the ecotype, archaea are able to survive in a wider range of salinity and oxygen concentration (Erguder et al., 2009).

In the saline lagoon Bassa de les Tortugues, 16S rRNA bacteria and *amoA* Archaea were found at significantly higher abundances in the middle than compared to the basal section of roots (Mann-Whitney test, $p < 0.05$) (Figure 4.11). In Daró River sediment, higher q*amoA* values for archaea were found in the middle section compared to the basal section of the root (Mann-Whitney test, $p < 0.05$). Finally, for Rec Coll samples, significantly higher abundances of all four studied genes were found in the middle compared to the basal section (Mann-Whitney test, $p < 0.05$). These results indicate an important effect of ROL on total microbial community and also on ammonia oxidizers, especially in *Archaea*. The presence of archaeal *amoA* was reported in activated sludge bioreactors with low DO concentrations operating under oxic–anoxic conditions as well as in oxygen minimum zones (OMZs) in the ocean (Bristow et al., 2016; Erguder et al., 2009) indicating that AOA are adapted to low oxygen concentrations. However, Santoro et al. (2008) retrieved almost constant archaeal *amoA* gene copies in aerobic subterranean aquifer sediments with pore water at DO levels of 0.1–0.2 mM. Qin et al (2017) showing that growth rates of AOA isolates increase when the oxygen supply was higher, though some strains were negatively affected when DO increased to saturated values. Könneke et al. (2005) reported the fully aerobic growth of *Nitrosopumilus maritimus* during cultivation and near-stoichiometric conversion of ammonium to nitrite.

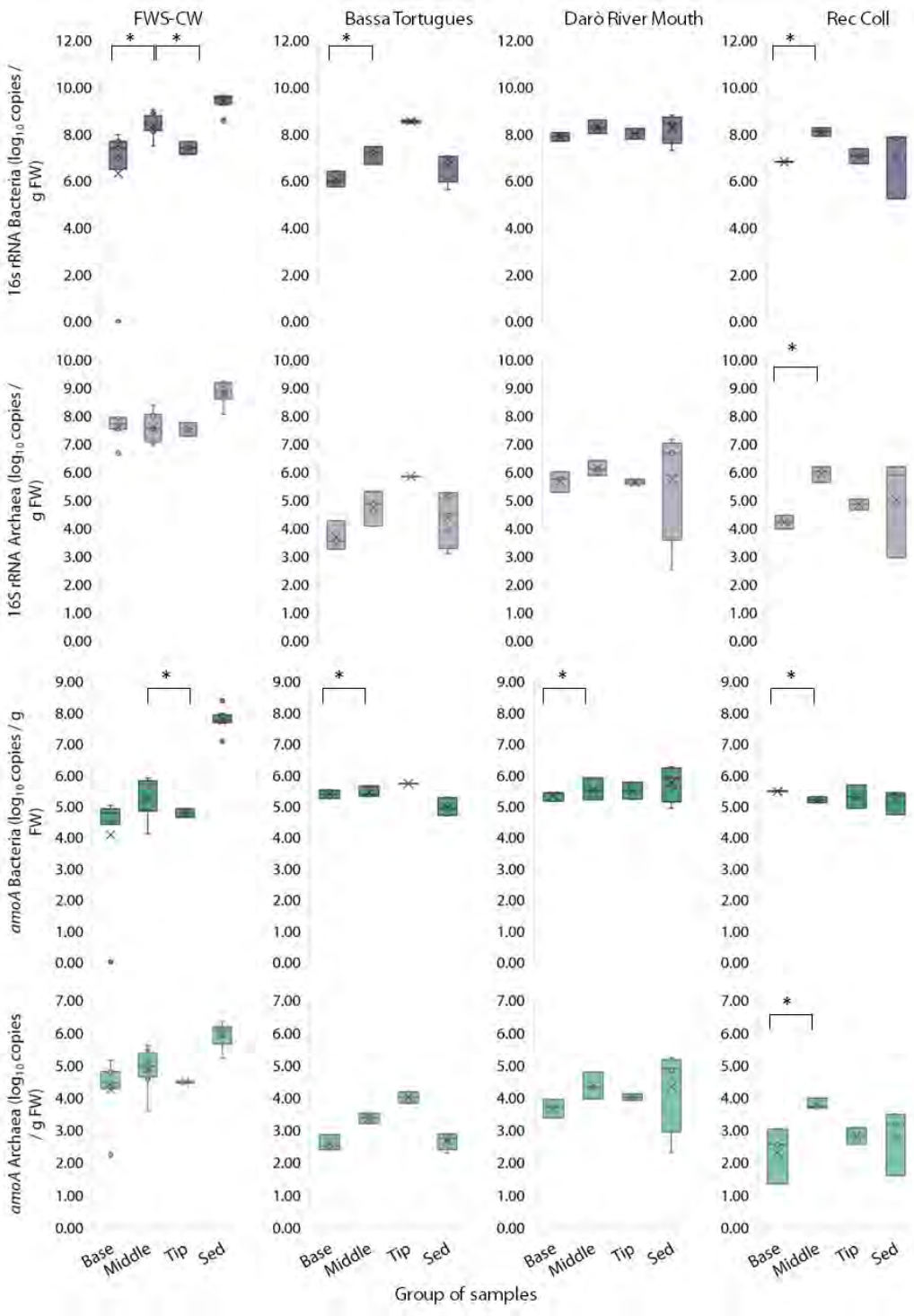

Figure 4.11. Abundances of 16S rRNA and *amoA* genes in roots and sediments. Box plots showing the abundance (copies/gFW) of 16S rRNA and *amoA* genes according to the sampled environments and sections of the root. Abundance of genes in sediments is also shown in each of the sampled environments for comparison. * significant differences for Kruskal-Wallis test and Dunn's test with Bonferroni correction ($p < 0.05$) between root sections. Tips- 0 to 15 mm from the tip, Middle- 15 to 45 mm from the tip, Base- Basal section > 45 mm from the root tip, Sed- sediment adjacent to the root.

In order to predict a potential selection effect of *Typha angustifolia* roots over ammonia oxidizing archaea or bacteria the q*amoA*_Archaea/q*amoA*_Bacteria ratio was calculated for root sections and compared to the values obtained for sediments. Both, root sections and sediment, showed a dominance of AOB over AOA, except in the wetland with ratios of q*amoA*_Archaea/qa*moA*_Bacteria ranging from 0.001 and 0.747 for roots, and from 0.001 to 0.119 in sediments (Figure 4.12). Thion et al. (2016) remarked the importance of pH in the dominance of AOB over AOA, selecting AOB in alkaline media, which was also shown in coastal lagoons (Trias et al., 2012). In general, significant differences of this ratio could be measured between roots and sediments, and in two of the studied ecosystems, i.e. RC and DM, this ratio was significantly higher in the middle section compared to the basal (Mann-Whitney test, $p < 0.05$) (Figure 4.12). Different studies have been shown the higher positive effect of oxygen on AOB over AOA community in rice field soil, sediment and water from an estuary or in river sediment (Abell et al., 2014; Ke et al., 2015; Santoro et al., 2008). However, these clear differences between high and low oxygen concentration were not appreciable when roots were considered (Ke et al., 2015), probably because not different root sections and consequently microenvironments of root were taking into account.

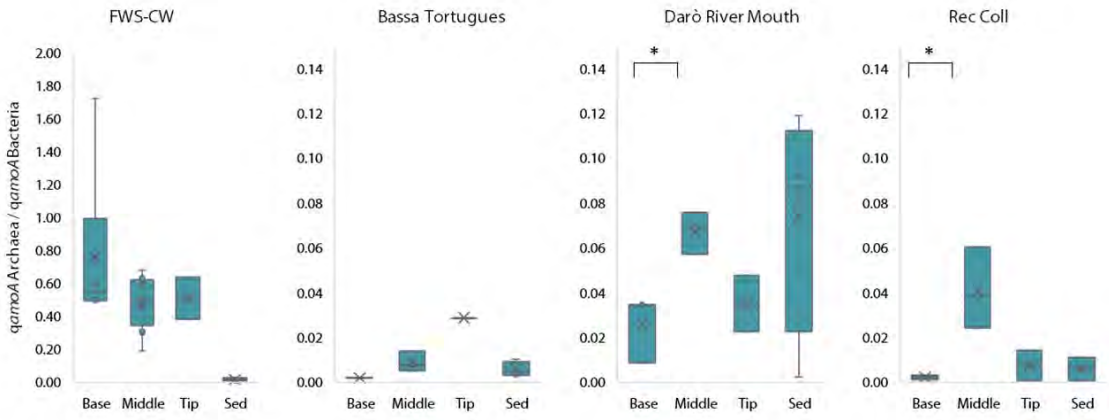

Figure 4.12. Ratio between ammonia oxidizing *Archaea* and ammonia oxidizing *Bacteria* in roots and sediments. Box plot showing the ratio between ammonia oxidizing *Archaea* and ammonia oxidizing *Bacteria* (q*amoA Archaea*/q*amoA Bacteria*) in the four studied environments according to the root section. Ratios in sediment samples are included for comparison. Tips- 0 to 15 mm from the tip, Middle- 15 to 45 mm from the tip, Base- Basal section > 45 mm from the root tip, Sed- sediment adjacent to the root.

4.2.4. Microbial community structure

Microbial communities at different root sections were studied on the basis of the 16S rRNA gene sequence. A total of 3,836,951 sequences passed quality filtering. On average, 66,154 sequences were obtained per sample (ranging from 23,001 to 142,783). Four replicates (i.e. two replicates of the middle section, Cell 2 and Europa Lagoon, one replicate from the basal section, Bassa de les Tortugues, and one from sediment, Rec Coll) were discarded from the analysis due to low sequencing depth. For comparisons between samples, a subset of 22,500 sequences per sample was randomly selected and used for alpha and beta diversity analyses. Observed richness (S_{obs}, number of OTUs) varied between 158 and 3,530 (Table 4.9). No significant differences (Kruskal-Wallis test $p > 0.05$) were detected between root sections and sediment in any of the studied sampling locations. Diversity indices varied from 3.65 to 7.11 (Shannon's H') and from 10.85 to 152.42 (Phylodiversity, PD). Despite the large variations in diversity, no significant differences were found between root sections and sediment in of the analysed sites (Kruskal-Wallis test $p > 0.05$).

According to the number of sequences reads, bacteria were clearly dominant over archaea in all samples. The latter accounted from 0.005% of the total sequences in the sediment of the Daró River Mouth, to 8% in the sediment of the Empuriabrava CW treatment cell. The phylum *Proteobacteria* was clearly dominant in all samples and accounted for more than 50% of all sequences (Figure 4.13), similarly to what has been previously found in natural systems and in wastewater treatment plants (Andreote et al., 2012; Miao and Liu, 2018). *Bacteroidetes*, *Chloroflexi* (mainly in CW) and *Firmicutes* were also abundant. Taxonomy pattern at the phylum level was quite similar in all samples of the same sampling point. Microbial composition of all three sampling sites at the Empuriabrava CW were highly similar and could were considered equal for further analyses.

Putative ammonia oxidizers were analysed separately (Table 4.10). AOB was generally more represented than AOA, according to the relative abundance of sequences, which was in concordance to qPCR results. AOB were represented by *Nitrosomonadaceae*, with some sequences of genus *Nitrosomonas* and uncultured members (Figure 4.13). *Nitrospira* spp. were detected in all samples (0.1 to 2.7% of sequences) being more abundant in Daró River Mouth. On the other hand, AOA were represented by members of the AK59 group, the Marine Group I, the Soil Crenarchaeotic group, and "*Candidatus* Nitrosoarchaeum" (mainly in CW), "*Candidatus* Nitrosopumilus" and "*Candidatus* Nitrososphaera". *Nitrosomonas* is the AOB genus typically found in freshwater, while other genera, as *Nitrosospira*, are more

common in soils. Comammox *Nitrospira* is widely distributed in different environments. Regarding to AOA, *Nitrosopumilus* is generally found in marine environments, while "*Candidatus* Nitrosoarchaeum" is typically found in freshwater (Lehtovirta-Morley, 2018), being the most abundant AOA in all the studied samples.

Table 4.9. Alphadiversity in root samples. Number of total sequences obtained per sample and alpha diversity indices of each sample. S_{obs} = number of OTUs observed, H' = Shannon index, PD = PhyloDiversity index. Mean and SE of triplicates are shown except when indicated.

	Section	#filtered sequences	S_{obs}	H'	PD
Empuriabrava Constructed Wetlands					
Europa Lagoon	Base	90,300	2,832±482	6.95±0.18	97.69±18.35
	Middle	69,898	2,036±1,598	6.51±0.62	82.12±41.8
	Sediment	108,318	3,450±108	6.98±0.04	107±0.61
Cell 1	Base	105,443	1,639±57	5.24±0.16	63.04±6.4
	Middle	135,780	1,452±67	4.86±0.15	53.46±11.25
	Tip (n=1)	32,492	3,530	7.11	152.42
	Sediment	140,984	3,123±87	6.86±0.2	104.28±9.82
Cell 2	Base	147,922	2,145±234	5.94±0.28	75.02±5.6
	Middle	115,823	1,768±706	5.2±0.66	63.58±17.32
	Tip (n=1)	24,457	773	5	42.09
	Sediment	113,304	2,356±1,212	5.92±1.36	91.93±40.77
Baix Ter					
Bassa Tortugues	Base	98,799	158±32	3.65±0.43	10.85±3.18
	Middle	295,797	897±379	4.82±0.4	20.43±5.54
	Tip	86,359	932±83	4.29±0.01	36.22±11.73
	Sediment	368,587	1,180±718	4.69±1.19	30.79±17.19
Daró River Mouth	Base	271,082	2,440±1,004	6.43±0.59	54.75±23.45
	Middle	222,485	2,498±812	6.4±0.13	63.96±16.56
	Tip	170,788	2,653±216	6.5±0.26	60.24±15.18
	Sediment	375,224	2,815±1,857	5.52±3.32	58.11±39.68
Rec Coll	Base	233,031	985±696	4.8±0.53	26.36±14.61
	Middle	240,514	1,979±113	5.4±0.51	50.73±12.02
	Tip	96,659	385±336	4.28±0.67	12.75±7.11
	Sediment	292,905	1,731±1324	5.72±1.78	40.91±36.51

Differences in the structure of microbial communities were analysed with a Principal Coordinates Analysis (PCoA), based on the Unifrac weighted distance matrix. Two clusters were observed in PCoA samples distribution according to the two studied geographical sites (Figure 4.14), which was confirmed by PERMANOVA (pseudo-F=10.716, p=0.001). Redox and pH were correlated to the distribution of samples (Spearman's test, $r^2>0.8$). The two parameters shown to affect microbial community structure in sediment of constructed and natural coastal wetlands, as well as on root surface of different plant species, showing for

redox potential a reverse trend in comparison to pH (Ligi et al., 2014b; Nunan et al., 2005; Zhou et al., 2017). Gradient of conductivity found between the studied sites seems to not affect microbial community composition. There were some phyla associated to samples distribution. In Empuriabrava CW *Proteobacteria* was correlated to root samples cluster. This phylum was previously found as representative and differential between rhizoplane and rhizosphere in macrophytes (Pietrangelo et al., 2018), and in crop plants, increasing the relative abundance on root surface compared to the sediment (Bulgarelli et al., 2012; Edwards et al., 2015). On the other hand, *Planctomycetes, Acidobacteria, Nitrospirae, Bacteroidetes, Gemmatimonadetes* and *Chloroflexi* were mainly relevant for sediment samples. *Chloroflexi* and *Planctomycetes* have been previously detected in the rhizosphere of *T. angustifolia* and *Bacteroidetes* in the bulk sediment on the same environment, since all phyla could be related to wastewater treatment. Moreover, *Chloroflexi* has been found in wastewater treatment plants and in wetlands, being related to nitrogen cycle in different environments (Guo et al., 2015; He et al., 2015; Rivière et al., 2009), and has been previously associated to the rhizosphere of *T. angustifolia*, as well as the phylum *Nitrospirae* (Gao and Shi, 2018; Guo et al., 2015). *Planctomycetes* includes anammox bacteria, which were previously related to *Chloroflexi* in different biological reactors due to their co-occurrence (Akaboci et al., 2018; Duarte Pereira et al., 2014), were also detected in different rhizospheres more than in the rhizoplane (Edwards et al., 2015; Peiffer et al., 2013). *Bacteroidetes* members are involved in the nitrogen cycle (Heylen et al., 2006b) and have been previously detected in different natural and human-made environments, including CWs, wastewater treatment plants and biological reactors treating wastewater (Ansola et al., 2014; Gabarró et al., 2013; Mohit et al., 2015; Jinping Zhang et al., 2015). Positive correlation of *Acidobacteria* and *Gemmatimonadetes* with sediment samples was consistent to studies of agricultural plant roots-microbiome interactions (Edwards et al., 2015; Zhang et al., 2017), but contrary to Pietrangelo et al. (2018), who found *Acidobacteria* as a central core of the rhizoplane microbiota of *Typha latifolia* from natural wetlands, suggesting an important effect of sediment on microbial community of the rhizosphere.

Table 4.10. Genera selected as putative ammonia oxidizing microorganisms.

Genus	Ammonia oxidizer group	Reference
AK59	AOA	Yang et al 2016
Group C3	AOA	Gubry-Rangin, et al 2014
Marine Group I	AOA	Pester et al 2011
"*Candidatus* Nitrosoarchaeum"	AOA	Kim et al 2011
"*Candidatus* Nitrosopumilus"	AOA	Mosier et al 2012
Soil Crenarchaeotic Group (SCG)	AOA	Caliz et al 2015
"*Candidatus* Nitrososphaera"	AOA	Spang et al 2012
Nitrosomonas	AOB	Purkhold et al 2000
Nitrospira	Comammox	Daims et al 2015

Regarding to Baix Ter samples distribution, there were two phyla associated to the PCoA distribution, specifically to sediment samples (rhizosphere), *Planctomycetes and Acidobacteria*. These results are accordingly with that found in rhizosphere in Empuriabrava CW, where *Planctomycetes* and *Acidobacteria* are representative from sediment and not from root surfaces, similar to other studies (Zhang et al., 2017; Edwards et al., 2015). Interestingly, the same phyla as in CW were correlated to roots (*Proteobacteria* in Rec Coll) and sediment (*Acidobacteria* in Rec Coll and Bassa de les Tortugues, and *Chloroflexi* and *Nitrospirae* in Bassa de les Tortugues). Summarizing, total microbial community structure depends on geographical site and its physicochemical parameters, but in the same site, microbial community differs between rhizoplane and rhizosphere. The later showed that the biggest differences were between the more mature sections of the root and sediment, showing an establishment of microbial community accordingly to the properties of different microhabitats, either the sediment or the root. The plant effect is strongest directly at the root surface, and differences between rhizoplane (root surface) and rhizosphere soil microbial communities may be observed, showing different niches properties between them (Ofek-Lalzar et al., 2014). These results suggest the ability of some microorganisms to colonize the rhizoplane, showing a stable microbial community in old root surface sections different from the sediment, as was previously found in other studies (Pietrangelo et al., 2018; Shelef et al., 2013).

AOB, AOA and Comammox genera sequences were selected as putative ammonia oxidizers (Table 4.10), and were used to determine differences in the structure of ammonia oxidizing communities according to a PCoA, based on the Unifrac weighted distance matrix.

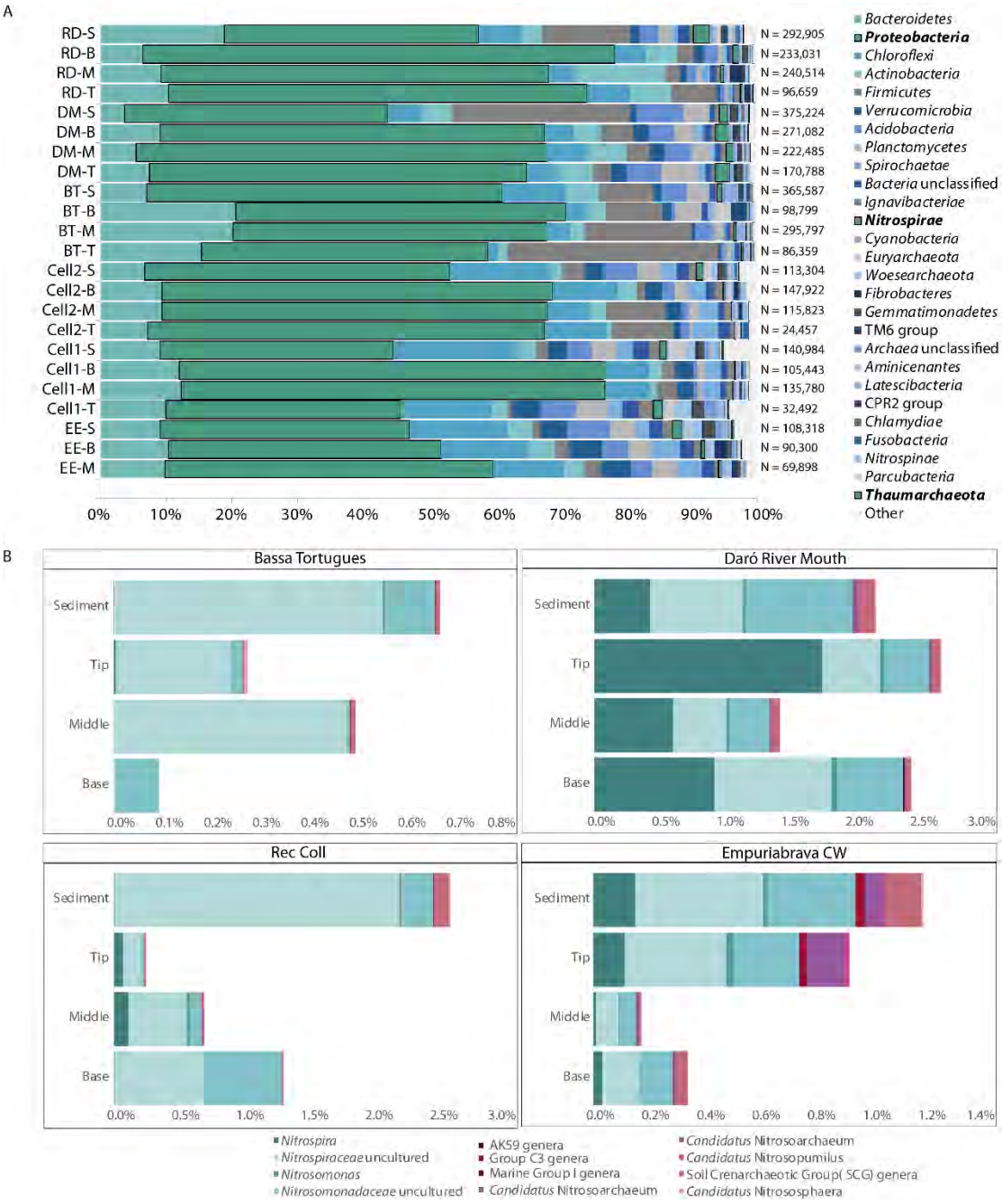

Figure 4.13. Main phyla of 16S rRNA and genera of ammonia putative oxidizers. A. Taxonomy at phylum level on the studied samples and relative abundance of each. Red colour indicates phyla that specifically belong to ammonia oxidizers or comammox. “Other” refers to phyla that represented < 1% of total sequences in all samples. At each column, N indicates the total number of sequences analysed to determine taxonomy of microbial community. B. Taxonomy of putative ammonia oxidizers at genus level on the studied samples and relative abundance of each genus. “Blues” refers to AOB, while “Purples” corresponds to AOA.

Table 4.11. PERMANOVA comparisons between studied sites and root sections. Main test and pair-wise PERMANOVA tests comparing sample groups according to putative ammonia oxidizers weighted unifrac matrix.

	Compared groups	Statistic		p-value
		Pseudo-F	t	
	Empuriabrava FWS-CW – Baix Ter	15.727		0.001
Empuriabrava FWS-CW				
	Root section	3.444		0.005
	Base - Middle		1.454	0.117
	Base - Sediment		2.213	0.031
	Base - Tip		0.772	0.448
	Middle – Sediment		2.740	0.002
	Middle – Tip		1.217	0.147
	Tip - Sediment		0.871	0.579

Similar to total microbial community structure, ammonia oxidizers grouped according to the geographical site (Figure 4.15) (PERMANOVA pseudo-F = 15.727, p = 0.001). PCoA distribution of samples was correlated to redox, oxygen and temperature of water (Spearman's test, r^2>0.6), and not correlation was found to conductivity though it was significantly different between sampling sites. The effect of temperature on ammonia-oxidizing microorganisms is mainly manifested in the effect on the activity of ammonia monooxygenase (Khangembam, 2016). Described AOB are mainly mesophilic, while the range of adaptation temperature of AOA is larger (Yin et al., 2018). However, Zeng et al (2014) showed that in environments between 15 ºC and 35 ºC, the diversity of AOB was higher than that of AOA, especially at 25 ºC. Dissolved oxygen of water had a direct advantageous effect on ammonia oxidizing community (Wei et al., 2011). Due to the difference in the affinity of nitrifying microbes for oxygen (AOA>AOB>NOB (nitrite-oxidizing bacteria, including *Nitrospira*)) (Yin et al., 2018), the oxygen concentration will affect the nitrification process. High oxygen affinity makes AOA more competitive than AOB in hypoxic environments (Erguder et al., 2009; Martens-Habbena et al., 2009; Santoro et al., 2008). In fact, *Nitrosomonadaceae* and *Nitrospiraceae*, which had a low affinity for oxygen (Yin et al., 2018), were the most represented taxons in Daró River Mouth and Rec Coll, where the highest oxygen concentrations were found (Figure 4.13, Table 4.7). No clear effects are described for redox potential on ammonia oxidizers, though an increase of redox potential by oxygen release from the root could stimulate oxidation processes as ammonia oxidation (Bodelier et al., 1996).

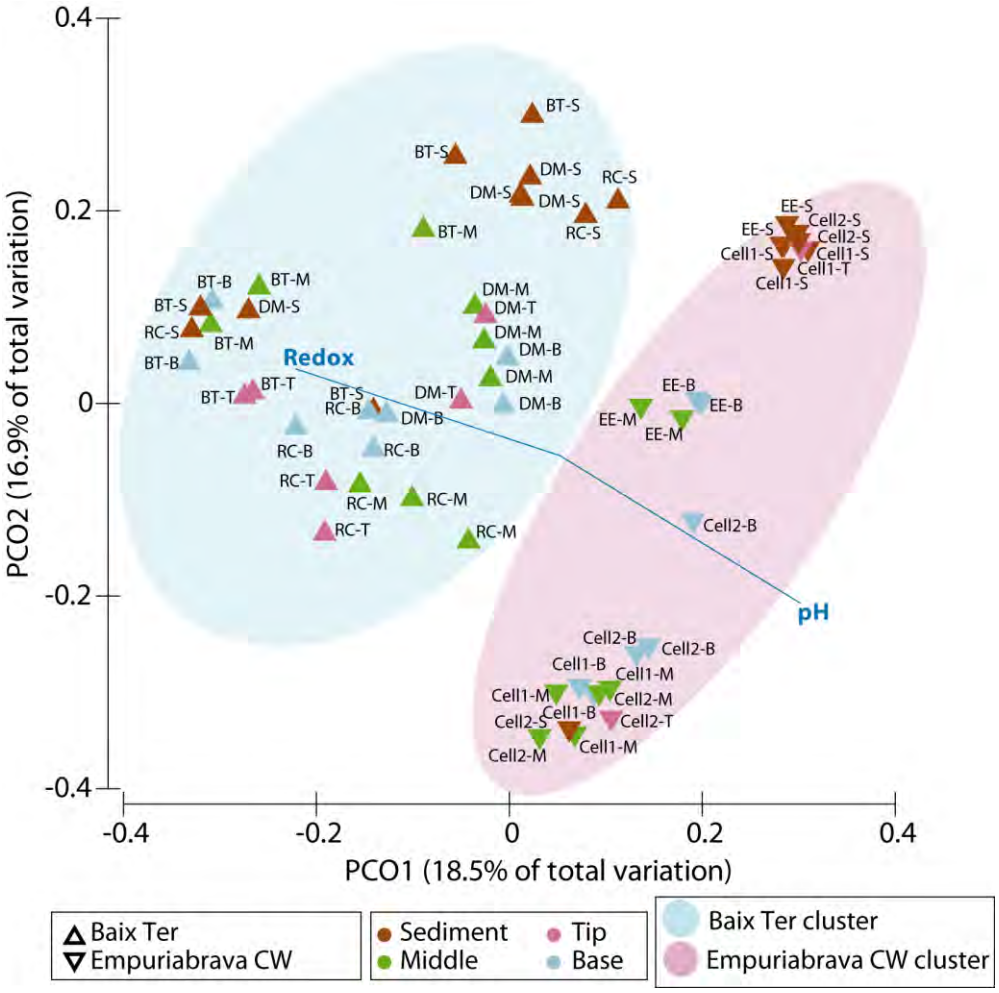

Figure 4.14. Distribution of root samples in a PCoA. PCoA distribution of samples according to microbial community composition in roots of *Typha angustifolia* and adjacent sediment of the studied samples determined by Unifrac weighted matrix. Blue vectors indicate physicochemical variables correlated with microbial community composition.

Empuriabrava CW samples distribution on the PCoA grouped according to sample type (Figure 4.15) (root or sediment), showing significant differences between middle section and sediment and base and sediment (PERMANOVA pseudo-F = 3.44, $p < 0.01$) (Table 4.11). The largest differences were observed between the more mature root section and the sediment, showing stable ammonia oxidizers communities accordingly to the properties of different microhabitats, either the sediment or the root (Shelef et al., 2013; Pietrangelo et al., 2018). In CW, "*Candidatus* Nitrososphaera" was positively correlated to sediment samples. *Thaumarchaeota* Group C3 in Bassa de les Tortugues, and "*Candidatus* Nitrosoarchaeum" and *Thaumarchaeota* Marine Group I in Daró River Mouth, were correlated to sediment samples. In contrast, *Nitrosomonadaceae* in Rec Coll *Nitrospira* in Daró River Mouth and Rec Coll were

positively correlated to root samples (Figure 4.15). Consistently with other studies, AOA were clearly favoured in the most hypoxic samples (rhizosphere), and AOB in the rhizoplane, suggesting a clear effect of ROL (Shelef et al., 2013; Wei et al., 2011; Bodelier et al., 1996; Yin et al., 2018).

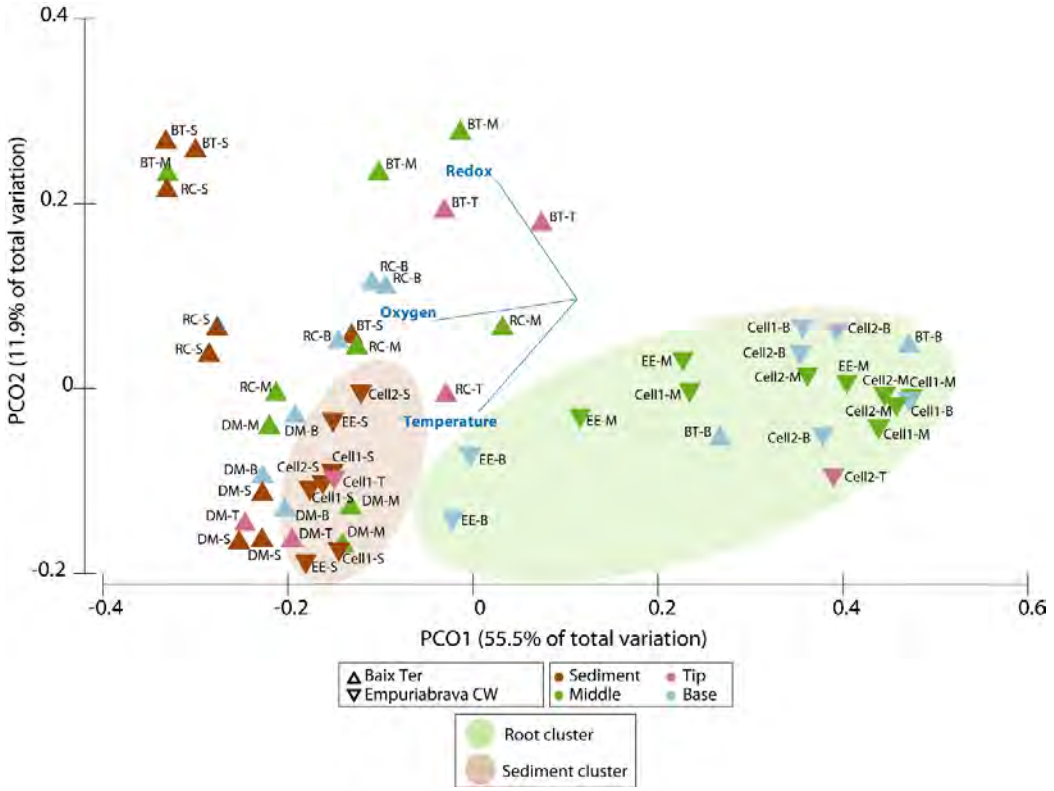

Figure 4.15. Distribution of root samples in a PCoA according to OTUs related to AOM. PCoA distribution of samples according to putative ammonia oxidizers community composition in roots of *Typha angustifolia* and adjacent sediment of the studied samples determined by Unifrac weighted matrix. Blue vectors indicate physicochemical variables correlated with microbial community composition.

4.2.5. Final remark

Results obtained in this chapter suggested an important role of oxygen released by roots on the abundance and distribution of ammonia-oxidizing microorganisms. Experiments were conducted considering only the rizhoplane, i.e. the sediment area in closest contact to the root, in order to avoid interferences with the adjacent sediment. The effect of radial oxygen loss (ROL) favoured the presence of AOB in the rhizoplane whereas AOA occurred at higher relative densities in the adjacent sediment. This fact was in clear coincidence with a higher affinity of AOA for low O_2 concentration, which has been previously described in the literature (Yin et al., 2018; Zhou et al., 2016). In terms of

abundance AOB are predominant in all study sites. Unfortunately, differential abundance of AOA or AOB in the different root sections could not be confirmed statistically. Due to the low number of cells present on the root surface, fragments from different root hairs had to be pooled in a single sample for molecular analysis and paired statistic tests could not be applied. Nevertheless, lower AOM abundances and higher AOA/AOB ratios tended to concentrate at the basal section of roots. However, further analyses of microbial activity and active microbial community should be done to confirm these differences. Environmental variables (i.e. pH and redox potential) were shown to affect microbial community and more specifically ammonia-oxidizers, in agreement with other surveys of rhizospheres and rhizoplanes (Ligi et al., 2014b; Nunan et al., 2005). Although being one of the most claimed effects of vegetation on wetlands performance, oxygenation of sediment layers, at the micrometric scale this effect has to be re-considered, at least for *Typha* plants. A vegetation effect on sediment bacteria is clear and was measured in all studied ecosystems in this thesis. However, a direct implication of ROL on the first step on N elimination in CW, albeit being essential in the management of wetlands, could not be confirmed.

4.3. Natural attenuation of nitrate in Osona: from isotopes to microbiome data

Different methodological approaches, benefitting from complementary scientific disciplines, have been used to ascertain the contribution of microorganisms in the overall nitrogen cycle. Due to the complexity of the latter, it is common that most experimental works rely on combinations of different methodological perspectives to have a complete picture of how oxidation-reduction reactions in the N cycle occur in a given environment. The goal of this chapter was to elucidate the complementarity between isotopic and microbiological information; that is, to show how these data mutually supply each other's lack, and contribute to characterize the denitrification potential in a highly sensitive environment, a groundwater flow path. In this sense, data from an isotope-based approach are combined with the microbiological information for which key molecular markers (genes) for bacterial denitrification are quantified. Furthermore, the structure of the microbial community is also analysed as a means to identify the bacteria potentially participating in the regional nitrate transformation pathways. According to the authors' knowledge, there are very few scientific reports combining these two approaches in groundwater research (e.g., Kim et al*.,* 2015). For this purpose, a highly sensitive area to N contamination in Catalonia has been chosen as a model area for this study. The study is based in eight wells of the Osona area (for more information see section 3.1.1), which have previously monitored in other studies (Boy-Roura et al., 2013b; Menció et al., 2011b; Otero et al., 2009) and were suspected to contain specific bacteria for pyrite based autotrophic denitrification. Hence, an accurate approximation to the nitrate reduction potential in this area is proposed.

4.3.1. Hydrogeological dynamics and hydrochemistry

Samples have been grouped in two distinct sets according to their geographical location: a first set included wells located in the NE area (SMC-025, SMC-037, SPT-001, SVT-007), and the second, wells in the SW area (MNL-019, SMC-001, SMC-002, TOR-013). Well depths ranged from 60 to 115 m (Table 4.12).

The NE area is located on carbonate sandstone formations, while the wells of the SW were drilled in prodeltaic marls. Alluvial formations, and therefore agricultural land uses, are more extended in the SW area. Wells in the NE area are located in a hilly formation which delimits the surrounding ranges of the Osona basin. In a broad sense, NE area constitutes the up gradient of the regional flow field, where a general flow path NE-SW can be drawn.

Local orography and the influence of the Ter River in the south-western boundary of the sampled area control local scale flow systems that might affect the capture zone of the shallowest wells (60 m depth).

Given the similar mineralogy of both carbonate sandstones and prodeltaic marls, a comparable hydrochemical composition of all samples should be expected. Accordingly, the major hydrochemical facies is calcium-bicarbonate, yet samples from the SW zone show a significant increase of sulphate (TOR-013 and MNL-19; Figure 4.16, Table 4.12), and two samples (SMC-025 and MNL-019), one from each zone, present a larger percentage of sodium. Despite hydrochemical similarity, small differences among samples are consistent with the general flow field; and more importantly, both groups of samples largely differ on their nitrate content, which can be initially attributed to a larger proportion of arable land in the SW zone. Mean nitrate concentration in the NE zone wells is of 23±2 mg/L, and in the SW zone wells of 71±7 mg/L.

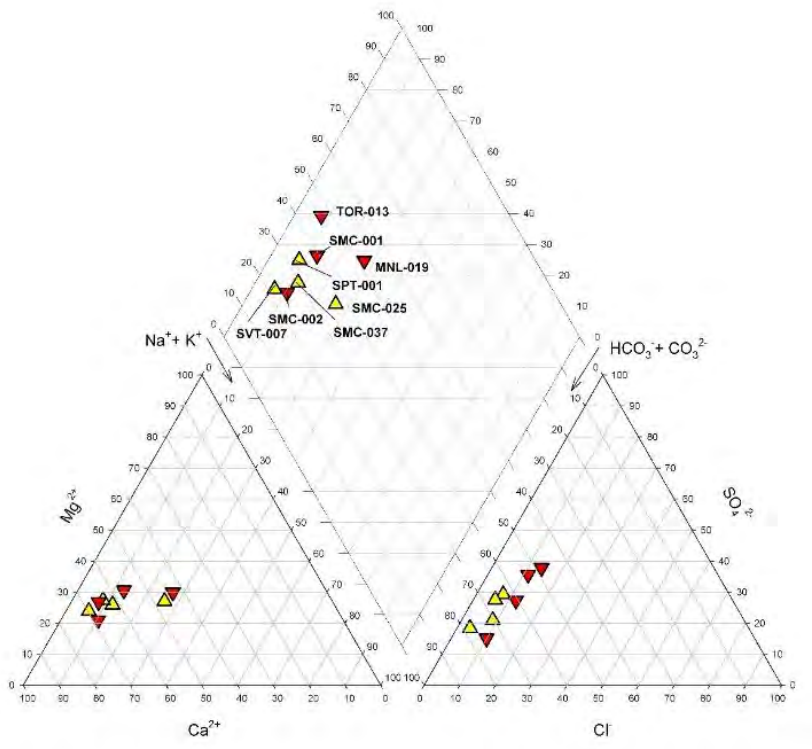

Figure 4.16. Piper diagram of July 2014 campaign. Legend: Yellow triangles, wells located in the NE area; and Red upside down triangles, wells located in the SW area.

DO and potential redox values are relevant since they are related to the occurrence of denitrification processes. While DO shows varying values at the NE zone (mean: 4.2±2.0 mg/L), samples of the SW zone are considerably depleted in oxygen (mean: 1.3±0.7 mg/L). pE values, as indicators of the redox potential of groundwater, do not show a neat difference between zones, ranging from 5.08 to 7.61 (Table 4.12). These values are representative of groundwater where dissolved oxygen has been consumed heterotrophically, but SO_4^{2-} is not yet reduced (e.g., Stumm and Morgan, 1996). Conductivity EC mean values increase from 818±61 to 1029±71 µS/cm from the NE to SW zones (Table 4.12). A similar pattern was observed for Ca^{2+}, Mg^{2+}, SO_4^{2-}, Cl^- and NO_3^- concentrations with lowest values present in the NE zone wells (ranges of 118.5-136.5 mg Ca^{2+}/L, 25.6-41.5 mg Mg^{2+}/L, 33.9-98.4 mg SO_4^{2-}/L, 11.3-32.8 mg Cl^-/L, and 16.6-26.3 mg NO_3^-/L), and the highest in SW zone samples (ranges of 133.6-176.9 mg Ca^{2+}/L, 29.1-56.2 mg Mg^{2+}/L, 107.7-213.3 mg SO_4^{2-}/L, 35.9-71.1 mg Cl^-/L, and 54.6-89.17 mg NO_3^-/L; Table 4.12).

In the studied area, samples with the highest Na^+ concentrations (MNL-019 and SMC-025) revealed the occurrence of cation exchange (Figure 4.17a). In addition, there is a displacement from the origin of most of groundwater samples with ratios ≈0:1, meaning that the larger proportion of Ca^{2+}+Mg^{2+} with respect to HCO_3^-+SO_4^{2-} is not related to sulphate or carbonate. Similarly, Figure 4.17b indicates that such unexplained Ca^{2+} increase can be attributed to nitrate pollution, which affects the overall hydrochemical composition of the water (Böhlke et al., 2002; Menció et al., 2016).

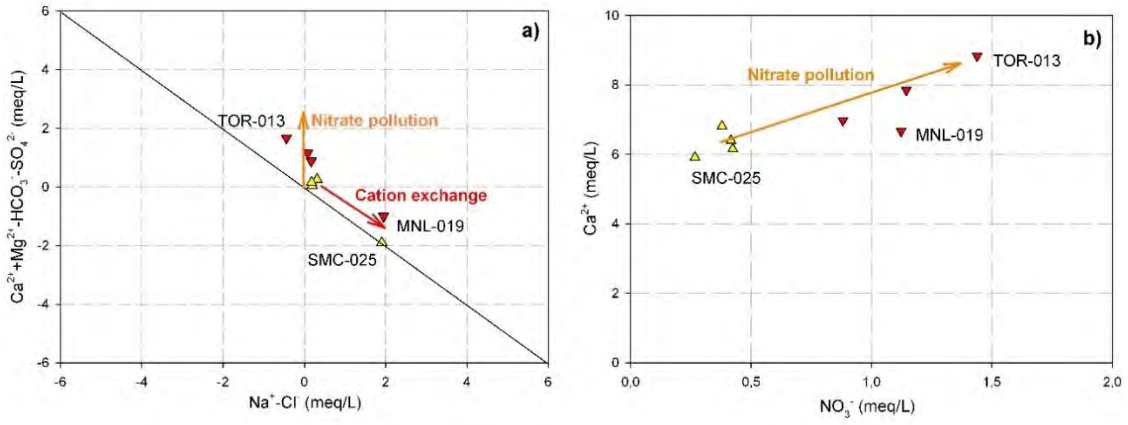

Figure 4.17. Bivariate relationships of cations and anions. a) Na^+-Cl^- *vs* Ca^{2+}+Mg^{2+} -HCO^{3-} -SO_4^{2-}; b) NO_3^- *vs* Ca^{2+}. Legend: Yellow triangles, wells located in the NE area. Red upside-down triangles, wells located in the SW area.

Table 4.12. Physicochemical characterization of the studied wells. Hydrochemical values of sampling points obtained in July 2014. Legend: Mean values, mean±standard error; *, parameter with significant difference between studied areas (p-value< 0.05).

Area	NE area					SW area				
Sampling point	SMC-025	SMC-037	SPT-001	SVT-007	Mean values	SMC-001	SMC-002	MNL-019	TOR-013	Mean values
Well depth (m)	80	67	100	90		110	115	60	70	
EC (µS/cm)	968	802	832	668	818±61.5*	949	889	1215	1061	1,029±71.7*
pH	7.25	7.26	7.20	7.52	7.31±0.07*	7.09	7.03	7.23	7.16	7.13±0.04*
pE	6.09	5.76	6.41	5.72	6.00±0.16	6.76	7.29	5.08	7.61	6.69±0.56
O_2 (mg/L)	6.60	1.65	0.26	8.42	4.23±1.95	1.04	0.81	0.07	3.13	1.26±0.66
T (ºC)	16.0	16.4	18.0	18.2	17.2±0.6	17.1	18.0	16.2	15.3	16.7±0.6
HCO_3^- (mg/L)	468.5	414.8	417.2	400.2	425.2±14.9	414.8	461.2	419.7	392.9	422.2±14.3
Cl^- (mg/L)	32.8	30.4	22.8	11.3	24.3±4.8*	48.2	35.9	71.1	48.8	51.0±7.3*
SO_4^{2-} (mg/L)	98.4	91.2	68.4	33.9	73.0±14.5	144.6	107.7	213.3	146.4	153.0±22.0
Na^+ (mg/L)	65.1	26.8	19.2	11.3	30.6±11.9	35.1	25.1	90.9	21.4	43.1±16.2
K^+ (mg/L)	17.1	2.7	3.4	2.8	6.5±3.5	2.2	4.1	8.0	4.1	4.6±1.2
Mg^{2+} (mg/L)	41.5	32.6	35.4	25.6	33.8±3.3*	45.9	29.1	56.2	44.0	43.8±5.6*
Ca^{2+} (mg/L)	118.5	128.2	136.5	123.2	126.6±3.8*	139.8	157.3	133.6	176.9	151.9±9.7*
IC (mg/L)	104.1	92.2	110.1	88.0	98.6±5.1	92.2	102.5	93.3	86.2	93.6±3.4
TOC (mg/L)	6.8	2.8	1.3	2.1	3.3±1.2	1.9	1.8	1.9	2.4	2.0±0.1
TC (mg/L)	110.9	95.0	111.4	90.1	101.9±5.5	94.1	104.3	95.2	88.6	95.6±3.3
NH_4^+ (mg/L)	0.007	0.006	0.038	0.034	0.021±0.009	0.007	0.007	0.007	0.090	0.028±0.021
NO_2^- (mg/L)	<0.004	<0.004	<0.004	0.010	0.010	<0.004	0.025	0.226	0.004	0.085±0.061
NO_3^- (mg/L)	16.6	25.8	23.5	26.3	23.1±2.2*	54.6	71	69.6	89.17	71.09±7.08*
TN (mg/L)	3.8	6	5.5	6.1	5.4±0.5*	13.1	17.1	16.8	21.6	17.2±1.7*
P-PO_4^{2-} (mg/L)	0.032	<0.003	0.005	0.003	0.013±0.008	<0.003	<0.003	<0.003	0.003	0.003
TOC:TN	29.38	15.88	20.37	14.74	20.09±3.33	7.19	6.10	5.66	4.10	5.76±0.64
H_2S (mg/L)	0.040	0.007	0.006	0.023	0.019±0.008	0.009	0.028	0.020	0.023	0.020±0.004

4.3.2. Denitrification level according to isotopic fractionation

As regards to isotopic data (Table 4.13), samples showed light values of $\delta^{18}O_{H2O}$ and δD, ranging from -6.8 to -6.37‰ and from -44.77 to -41.99‰, respectively, being representative of high altitude recharge areas in the Osona region (Menció et al., 2011a). Sample SMC-037 exhibited heavier values probably caused by evaporation (almost 5%, according to Gonfiantini's (1986) formula) originated by a recent modification of the well withdrawal mechanism. This result contrasts with those from the preceding sampling campaigns conducted in 2005, 2006 and 2010, when this well showed similar values to the rest of the samples in this area (Boy-Roura et al., 2013b; Menció et al., 2011a). From a regional perspective, water stable isotopes indicate a rainfall recharge from the surrounding ranges and, more importantly, wells located at the SW zone, also present a large contribution from this recharge origin. Nevertheless, their higher nitrate content indicates that local infiltration from nearby arable land must also contribute to the sampled groundwater. In consequence, groundwater samples, especially those from the SW zone are constituted by a mixture of regional flow paths intercepted at the deeper parts of the uncased wells, and local infiltration from the upper levels. Given that stable isotope data mostly correspond to high altitude recharge, it can be inferred that regional flows are the dominant resource that dilutes a local high-nitrate recharge. Mean nitrate values around 70 mg/L in the SW zones (Table 4.12) are considerably lower than the high nitrate concentrations registered in shallow wells and natural springs (Boy-Roura et al., 2013b, 2013a; Menció et al., 2011a), supporting the above mentioned dilution process.

The $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ isotopic signatures of NO_3^- in groundwater provide evidence of denitrification. In the studied wells, $\delta^{15}N_{NO3}$ ranged between +10.4 to +26.9‰, and $\delta^{18}O_{NO3}$ between +3.8 to +12.3 ‰, with nitrate concentrations between 16.5 and 89.2 mg/L. Isotopic values of dissolved NO_3^- in groundwater, plotted in Figure 4.18 together with the isotopic ranges of the main NO_3^- sources, confirmed the manure and/or sewage origin of the nitrogen. Given the intense agricultural and husbandry activity in the region, manure application as fertilizer stands as the main nitrogen source in groundwater. The lowest $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values are found in SVT-007 (with +10.4‰ and +3.8‰, respectively). The rest of samples showed heavier $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values displayed along a positive trend, consistent with denitrification isotopic enrichment paths that have their origins in the range of manure nitrogen source.

In Figure 4.18 the percentage of natural denitrification has also been represented, according to the N and O isotopic enrichment factors (ε_N of -26.3 ±1.8‰, and ε_O of -20.4±1.3‰) determined by Torrentó *et al.* (2011) in lab experiments using Osona rock cores and groundwater. For instance, according to the $\delta^{18}O_{NO3}$ enrichment factor, samples SPT-001, TOR-013 and SMC-001 present a degree of denitrification lower than 10%; SMC-002, approximately of 15%; and SMC-037, SMC-025 and MNL-019, a degree that can be even higher than 25%. Such denitrification percentages are more conservative than those using the enrichment factors estimated by Otero et al. (2011), which would give a rank between 12 and 50%.

Furthermore, the plot between $\delta^{15}N_{NO3}$ and $[NO_3^-]$ differentiates between each of the two geographical zones, being their samples properly aligned following a denitrification trend of slope close to that of the nitrogen enrichment factor (ε_N=-26.3‰; Figure 4.18b). This result is particularly interesting from the hydrodynamic perspective as it distinguishes two distinct nitrate source areas: the NE area, located in the hills with a lower nitrate input, and the SW area, located in the basin with a larger proportion of arable land and, therefore, a larger manure input that results in higher nitrate concentrations. As derived from the geological setting and the water stable isotopes, nitrate concentration is a mixing of the distinct aquifer levels intercepted by the boreholes, especially in the SW zone. Indeed, Figure 4.18b demonstrates that wells from each zone have a common hydrogeological setting related to recharge areas and the mixing of flow lines, which finally determine their hydrochemical composition and denitrification potential expressed by isotopic values. The dispersion from an expected lineal enrichment trend shown by samples from the two zones can be attributed to the effect of mixing flow lines in the sampling borehole. It would then be erroneous, from a hydrogeological perspective, drawing a single flow path according to an increasing denitrification level. The unsolved question so far is whether such denitrification level is the consequence of a large flow distance, equivalent to a long residence time within the aquifer, or to the specific conditions within the capture zone of each well (including mixing of flow lines) that will enhance denitrification up to distinct degrees.

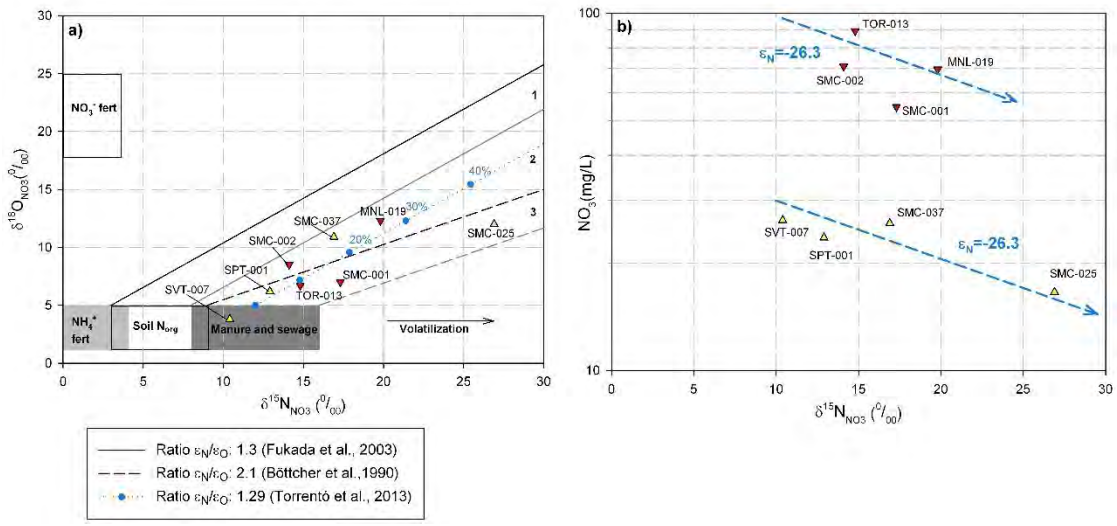

Figure 4.18. Relationship between isotopic data. a) $\delta^{15}N_{NO3^-}$ and $\delta^{18}O_{NO3^-}$ for the nitrate in groundwater samples with estimated isotopic enrichment factors of Böttcher et al. (1990) and Fukada et al. (2003); ranges of local potential NO_3^- sources from Puig et al. (2013), Vitòria et al. (2004) and Vitòria et al. (2008); and percentage of natural denitrification quantified according to Torrentó et al. (2011). Legend: 1, denitrified samples from soil N_{org}; 2, denitrified samples from both sources; and 3, denitrified samples from manure and sewage N, considering a 1:3 slope for the minimum $\delta^{15}N_{NO3}$ value and a 2:1 slope for the maximum $\delta^{15}N_{NO3}$ described for each source; b) $\delta^{15}N_{NO3}$ and NO_3^-, showing the denitrification trends based on the enrichment factor estimated by Torrentó et al. (2011). Note that a logarithm scale is used for the Y axis. Yellow triangles, wells located in the NE area. Red upside-down triangles, wells located in the SW area.

In synthesis, wells are classified in two generic groups (NE and SW zones) according to isotopic analyses, which are as well consistent with geographical location, hydrogeological features of the aquifer system, and land use spatial distribution. However, since we focus on the denitrification process, its occurrence and its extent, we also grouped samples as those with low (SPT-001, TOR-013, SMC-001, SVT-007 and SMC-002, denitrification level < 20%) and high denitrification levels (SMC-037, SMC-025 and MNL-019, denitrification level >20%; Figure 4.18b), naming them as Low-$DL_{isotope}$ and High-$DL_{isotope}$. Such sorting based on the percentage of nitrogen mass removal summarizes the contribution of the isotopic data to the estimate of natural attenuation levels of nitrate in groundwater, regardless mixing effects. Denitrification levels, as defined here, will later on be used to contrast isotopic with microbiological information.

Table 4.13. Isotopic data of sampling points obtained in July 2014. Legend: Mean values, mean±standard error; *, parameter with significant difference between areas (p-value< 0.05).

Area	NE area					SW area				
Sampling point	SMC-025	SMC-037	SPT-001	SVT-007	Mean values	SMC-001	SMC-002	MNL-019	TOR-013	Mean values
$\delta^{18}O_{H2O}$ (‰ VSMOW)	-6.70	-4.76	-6.46	-6.80	-6.18±0.48	-6.45	-6.37	-6.50	-6.45	-6.44±0.03
δ D (‰ VSMOW)	-43.88	-35.74	-42.95	-44.77	-41.84±2.07	-43.18	-42.61	-42.26	-41.99	-42.51±0.26
δ-excess (‰ VSMOW)	9.72	2.38	8.74	9.65	7.62±1.76	8.40	8.39	9.76	9.57	9.03±0.37
$\delta\ ^{18}O_{NO3}$ (‰ VSMOW)	12.00	10.90	6.20	3.80	8.23±1.94	7.00	8.50	12.30	6.70	8.63±1.29
$\delta\ ^{15}N_{NO3}$ (‰ AIR)	26.90	16.90	12.90	10.40	16.78±3.63	17.30	14.10	19.80	14.80	16.50±1.30

4.3.3. Abundance of 16S rRNA and denitrification genes

The concentration of bacterial 16S rRNA gene ranged from $5.03 \cdot 10^3$ to $3.12 \cdot 10^5$ copies/ng DNA (Table 4.14). As predicted, 16S rRNA gene abundance was always higher than any other functional gene. There were no significant differences in the abundance of total bacteria between the studied wells (Kruskal-Wallis test, $p>0.05$). All studied functional genes, albeit at different relative abundances, were found in all groundwater samples. Abundance of *nirS* and *nirK*, genes implied in nitrite reduction, was highly variable and ranged between $1.41 \cdot 10^2$ and $1.54 \cdot 10^5$ copies/ng DNA, and from $5.12 \cdot 10^1$ to $7.57 \cdot 10^4$ copies/ng DNA, respectively. Previous surveys in groundwater also detected high and significant abundances of *nirS* compared to *nirK* (Barrett et al., 2013). *nrfA* gene, responsible of the DNRA process, occurred at low abundance, with values ranging from $2.90 \cdot 10^1$ to $7.26 \cdot 10^3$ gene copies/ng DNA. Nitrous oxide reductases *nosZ*I and *nosZ*II ranged from $3.96 \cdot 10^2$ to $1.80 \cdot 10^4$ copies/ng DNA, and from $2.46 \cdot 10^2$ to $1.30 \cdot 10^5$ copies/ng DNA, respectively. In wells SMC-001, SMC-002, SPT-001 and MNL-019, *nosZ*II was not detected (<20 copies/ng DNA). However, in samples where *nosZ*II was present, this gene appeared at higher concentrations than *nosZ*I (Mann Whitney test, $p<0.05$). In all wells, gene copy abundance values were similar to those found in other groundwater studies (Barrett et al., 2013; Herrmann et al., 2017), and lower than those found in other environments characterized by higher concentrations of organic carbon such as constructed wetlands, estuaries and agricultural soils (García-Lledó et al., 2011b; Hallin et al., 2009; Lindemann et al., 2015).

In general, 16S rRNA gene correlated positively (Spearman's correlation, $p<0.01$) with all functional genes except *nirK*, indicating that the relative abundance of potential denitrifiers remained constant in the studied wells (Table 4.15). This hypothesis was confirmed by the positive pair-wise correlations between functional genes. *nrfA* abundance appears positively correlated to *nirS* (Spearman's correlation, $p<0.01$), but not to *nirK*, pointing to a selection of bacteria harbouring either nitrite reductase for specific environmental conditions in groundwater. *nosZ* gene abundance (*nosZ*I+*nosZ*II) correlated positively to *nirS*, and negatively to *nirK* (Spearman's correlation $p<0.01$).

In order to analyse differences in gene abundances according to physicochemical characteristics of the water, pair-wise correlation tests were performed (Table 4.16). H_2S correlated positively with *nrfA* and *nosZ* genes (Spearman's test, $p<0.01$), and negatively with *nirK* (Spearman's test, $p<0.05$). Brunet and Garcia-Gil (1996) showed that denitrification can be inhibited by the presence of H_2S, which provokes an accumulation of ammonia due to

DNRA. In this sense, the higher correlation between *nrfA* and H_2S ($R=0.811$, $p<0.01$) suggested that DNRA might have contributed to the nitrate reduction in some wells, as it was previously observed in limestone aquifer (Herrmann et al., 2017). NO_3^- was not correlated to any studied gene, while NO_2^- was positively correlated to *nrfA*. Abundances of *nirS* and *nirK* genes were positively correlated to $\delta^{15}N$ and $\delta^{18}O$ (Spearman's test, $p<0.05$), which might indicate a relationship between denitrification level ($DL_{isotope}$) and gene abundances.

Table 4.14. Abundances of total bacteria, denitrification and DNRA genes in the studied wells. Mean abundance values and standard deviation of studied genes (number of copies ($x10^3$)/ng of DNA extracted) at the studied wells. DL means Denitrification Level according to isotopic analyses. Values were obtained from two replicates for each sample, except for SMC-001, MNL-019 and TOR-013.

Area	NE area				SW area			
Sampling point	SMC-025	SMC-037	SPT-001	SVT-007	SMC-001	SMC-002	MNL-019	TOR-013
DL	High	High	Low	Low	Low	Low	High	Low
q16S rRNA	213.31 ± 99.04	14.76 ± 9.73	37.41 ± 24.21	69.47 ± 0.31	111.93	88.83 ± 18.00	165.43	74.46
q*nirS*	17.64 ± 9.99	1.45 ± 1.31	0.65 ± 0.01	4.94 ±1.93	153.74	28.76 ± 5.45	42.63	1.4
q*nirK*	0.21 ± 0.16	7.41 ± 3.27	3.55 ± 2.81	0.36 ± 0.18	4.06	1.50 ± 0.47	1.13	5.29
q*nrfA*	2.11 ± 0.79	0.10 ± 0.03	0.05 ± 0.02	1.04 ± 0.11	0.59	6.55 ± 0.71	3.52	0.32
q*nosZ*I	13.04 ± 4.99	0.50 ± 0.11	4.03 ± 0.82	4.85 ± 2.36	9.88	13.84 ± 2.83	8.62	1.44
q*nosZ*II	100.13 ± 30.11	0.72 ± 0.47	<0.02	24.15 ± 20.19	<0.02	<0.02	<0.02	3.52

Despite the changes in abundance of denitrification genes and the existing correlation between them, no significant differences were detected when samples were grouped according to the denitrification level based on isotopic analyses (Mann-Whitney test, $p>0.05$; Table 4.14). There is then a lack of consistency between molecular and isotopic analyses, used as proxies for potential denitrification. Although it was reasonable to think that the presence of denitrifying bacteria and denitrification reactions could be concurrently detected (Kim et al., 2015), actual results in Osona brings in the need of a thoughtful discussion on the hydrodynamic aspects of these observations. Nitrate, as a dissolved compound, flows at the pore water velocity. Conversely, bacteria, being a particulate material, possibly remain attached to surfaces and form biofilms, or occur as aggregates having a lower mobility (Griebler and Lueders, 2009; Williamson et al., 2012). Unfortunately, biofilm sampling was not possible due to methodological constraints. Previous experiments comparing attached

and free-living bacteria in groundwater revealed significant differences in the microbial community, probably affecting denitrifying bacteria (Herrmann et al., 2017). Lastly, significant differences in denitrifiers may occur between wells, since bacteria with this metabolism are well scattered in the phylogenetic tree (Philippot and Hallin, 2005). Most of denitrifying bacteria do not contain all genes necessary for complete denitrification (Jones et al., 2013, 2008) and, in addition, the same set of genes in two different bacteria may lead to considerable changes in activity. According to this, it is reasonable to think that information derived from isotopic signatures and abundance of genes may not coincide in reporting denitrification reactions (Mann-Whitney test, $p>0.05$). Both results derived from isotopic and microbial data corroborate the occurrence and extent of denitrification, yet they fail to indicate at which location/moment along the flow path these reactions had taken place

Table 4.15. Correlation between studied genes in groundwater. Spearman correlation coefficients between 16S rRNA and functional genes abundances. n.s., not significant; *p < 0.05; **p < 0.01.

	q16S rRNA	q*nirS*	q*nirK*	q*nrfA*	q*nosZ*I	q*nosZ*II	q*nirS*+q*nirK*	q*nosZ*I+q*nosZ*II
q16S rRNA	1.000							
q*nirS*	0.819 **	1.000						
q*nirK*	n.s.	n.s.	1.000					
q*nrfA*	0.698 **	0.747 **	n.s.	1.000				
q*nosZ*I	0.703 **	0.813 **	n.s.	0.764 **	1.000			
q*nosZ*II	0.893 **	0.929 **	-0.857 *	0.893 **	0.964 **	1.000		
q*nirS*+q*nirK*	0.571 *	0.775 **	n.s.	0.582 *	0.670 *	n.s.	1.000	
q*nosZ*I+q*nosZ*II	0.791 **	0.692 **	-0.742 **	0.747 **	0.797 **	1.000 **	n.s.	1.000

Table 4.16. Pairwise correlation between gene abundances and physicochemical parameters in groundwater. Spearman correlation coefficients between physicochemical parameters in water and analysed genes abundances. n.s., not significant; *p < 0.05; **p < 0.01.

	EC	pH	O_2	HCO_3^-	S-SO_4^{2-}	N-NH_4^+	N-NO_2^-	N-NO_3^-	H_2S	$\delta^{15}N$	$\delta^{18}O$
q16S rRNA	0.777 **	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.711 **	0.611 **	n.s.
q*nirS*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
q*nirK*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-0.617 *	n.s.	n.s.
q*nrfA*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.695 **	n.s.	0.811 **	n.s.	n.s.
q*nosZ*I	n.s.	n.s.	n.s.	0.700 **	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
q*nosZ*II	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.945 **	n.s.	n.s.
q*nirS*+q*nirK*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.573 *	0.578 *
q*nosZ*I+q*nosZ*II	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.856 **	n.s.	n.s.
nir/*nos*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

4.3.4. The groundwater microbiome

In order to infer the main taxonomical groups responsible for denitrification in Osona wells, microbial communities were studied on the basis of the 16S rRNA gene sequence. A total of 69,092 sequences passed quality filtering. Roughly on average 4,600 sequences were obtained per sample (ranging from 1,249 to 8,526). For one of the replicates from TOR-013 no sufficient number of sequences was obtained and was removed from the study. A subset of 1,150 sequences per sample was randomly obtained and used for diversity analysis. Despite the lower number of sequences used, rarefaction curves revealed a reasonable coverage of bacterial richness (Figure 4.19). Observed richness (Sobs, number of OTUs) varied between 109±5 and 377±3 (Table 4.17). Diversity of microbial community was estimated by Shannon and phylodiversity indices (H' and PD), which varied from 3.12 to 5.45 and from 3.97 to 11.22, respectively. No significant differences (Mann-Whitney test $p<0.05$) were found between samples grouped according to $DL_{isotope}$ for alpha diversity indicators.

Table 4.17. Alphadiversity values and potential denitrification level of studied wells. Values were obtained from two replicates for each sample, except in TOR-013 where only for one of the replicates had optimal results. #sequences: number of sequences obtained; H': Shannon diversity index; phyloDiversity: phylogenetic diversity index, Sobs: number of OTUs observed.

Area	NE area				SW area			
Sampling point	SMC-025	SMC-037	SPT-001	SVT-007	SMC-001	SMC-002	MNL-019	TOR-013
DL	High	High	Low	Low	Low	Low	High	Low
#sequences	5,076	12,706	6,996	12706	10,591	15,337	11,069	1,249
H'	4.82 ± 0.10	5.07 ± 0.16	4.75 ± 0.06	5.41 ± 0.06	4.71 ± 0.01	3.12 ± 0.02	3.67 ± 0.32	5.45
phyloDiversity	9.37 ± 0.64	9.20 ± 2.86	7.40 ± 0.10	7.28 ± 4.69	6.47 ± 1.17	5.71 ± 0.05	6.51 ± 01.17	3.97
Sobs	280.59 ± 0.24	358.68 ± 24.34	274.28 ± 10.57	327.04 ± 24.10	240.03 ± 11.11	123.18 ± 20.42	181.45 ± 3.98	344.09

Bacteria were clearly dominant over *Archaea*, which ranged from 0.1% (SMC-002) to 6% (MNL-019) of the total sequences (Figure 4.20). Two bacterial phyla (*Proteobacteria* and *Parcubacteria*) accounted for almost 40% of all sequences in all samples, except those retrieved from SMC-002. In most of the wells, 5 to 20% of the sequences remained as unclassified *Bacteria*. A closer inspection of these sequences, using updated NCBI nucleotide collection database for cultured bacteria, yielded high similarity values to "*Candidatus* Parcubacteria", "*Candidatus* Gracilibacteria" and "*Candidatus* Saccharibacteria". These phyla, unknown until recently, have been described in many environments, including groundwater (Kindaichi et al., 2016; Kutvonen et al., 2015). Some members of these phyla have been also reported as potential denitrifiers according to whole genome sequences (Albertsen et al., 2013).

Groundwater microbiome varied notably in three of the wells, revealing a higher relative abundance of specific phyla. In particular, *Firmicutes* (mainly *Clostridium* sp.) accounted for approximately 25% of total sequences in SPT-001. Secondly, SMC-002 was characterized by high abundance of *Nitrospirae* (about 40%) and *Acidobacteria* (about 15%) which were less represented in other wells. Finally, MNL-019 had a high relative abundance of *Bacteroidetes*. In relation to denitrification capacity, all these four phyla contain denitrifying members (Heylen et al., 2006b; Lücker et al., 2010; Pu et al., 2014; Rösch et al., 2002).

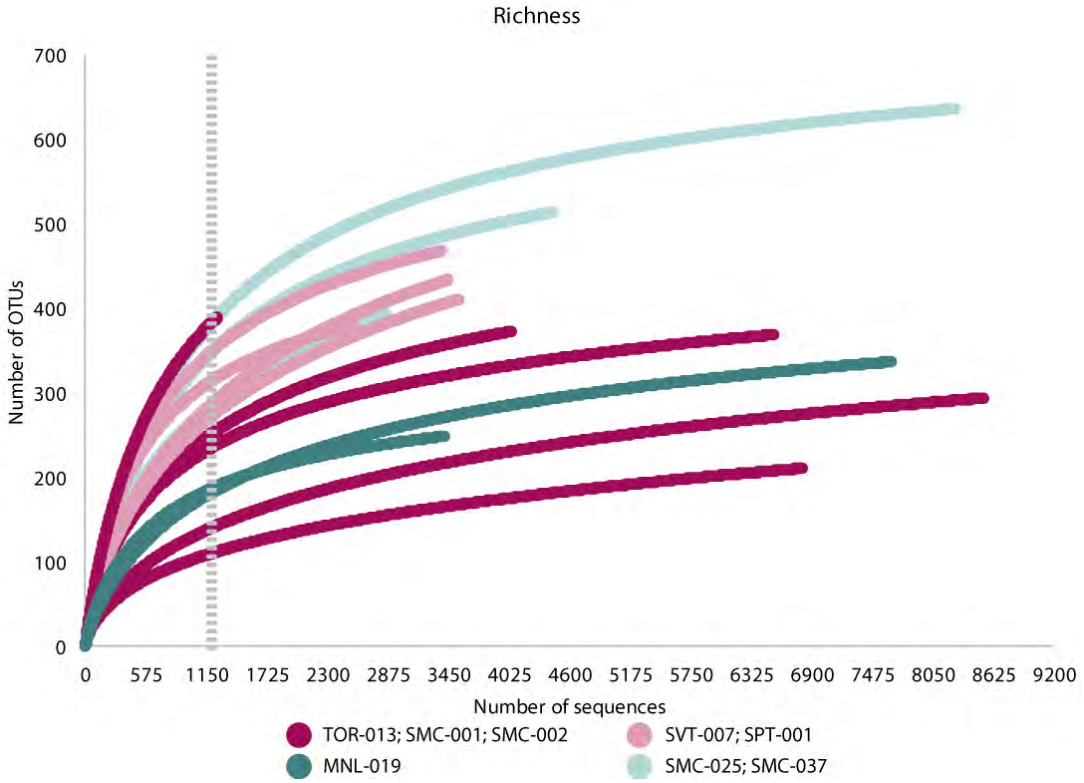

Figure 4.19. Rarefaction curves of microbial richness in groundwater samples analysed. Rarefaction curves of samples analysed, using a subsample of 1,150 sequences (dot line). Different markers showed samples grouping according to area (SW or NE) and denitrification level (high or low).

Statistical correlation between microbial communities was analysed using a Principal Coordinates Analysis (PCoA), based on the Unifrac weighted distance matrix. Distances were calculated from OTU distributions accounting for their relative abundance and their phylogenetic relationships (Figure 4.21). PCoA clustering confirmed the different microbial community composition of well SMC-002. Significantly, the position of this sample in the PCoA correlated positively (Spearman's correlation, R^2>0.6) with redox potential (Eh). All other samples clustered in two groups: a first one correlated with pH, O_2 (SMC-037, SMC-025; TOR-013, and SVT-007; Spearman's correlation, R^2>0.6) and a second group correlated with S-SO_4^{2-} (SMC-001, MNL-019, and SPT-001; Spearman's correlation, R^2>0.6). Oxygen and pH have previously been shown to be key drivers of denitrification in different environments (García-Lledó et al., 2011b; Graham et al., 2010; Jurado et al., 2017). Correlation with SO_4^{2-} may be interesting in Osona wells, since this compound is produced as the result of the oxidation of sulphides, appearing in the form of pyrite in the region, by autotrophic denitrifiers (Otero et al., 2009; Pauwels et al., 2000).

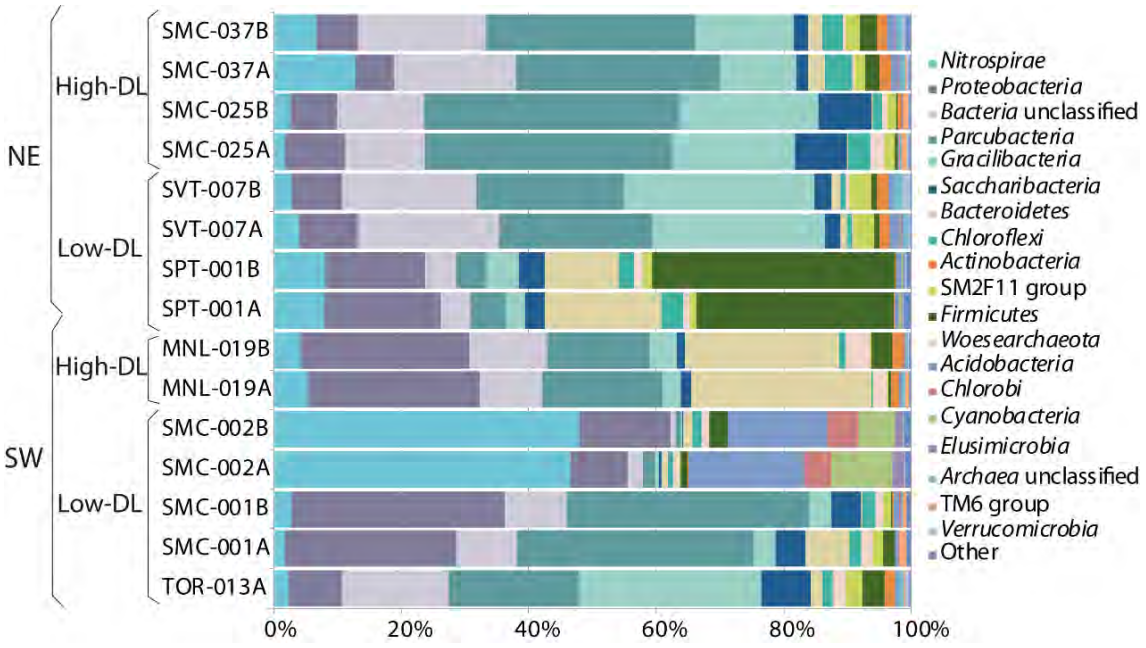

Figure 4.20. Taxonomy of the main phyla in groundwater. Relative abundances of main phyla (found > 1% of sequences) in each replicate in the studied wells. Bars are grouped according to the location of the sampled well (NE and SW) and the Potential denitrification level according to isotopic fractionation (High-DL and Low-DL). "Other" refers to phyla that represented < 1% of total sequences in all samples.

Analyses of similarities (ANOSIM) test is a distribution-free analogue of one-way ANOVA, used to test spatial differences in community's structure (Clarke, 1993). At the studied wells, results show that the distribution of microbial communities resulting from PCoA is not related to the denitrification level ($DL_{isotope}$), as defined according to isotopic analyses (ANOSIM R-value 0.011, p-value 0.293), as samples were not clustered according to this factor. Next, we tested if the hydrogeological areas (NE or SW) were a determinant factor defining the "well" microbial community. In this case, the ANOSIM value was higher (R-value 0.159) and slightly significant (p-value 0.049; Figure 4.21). Statistical results suggest that microbial communities are shaped by the hydrogeological features of the aquifer; whereas the $DL_{isotope}$ activity, as a denitrification proxy, integrates all geochemical reactions/processes occurred along the flow path. Moreover, supporting this hypothesis, the pattern showed by the abundance of denitrifying genes (*nirS*, *nirK*, *nosZ*I and *nosZ*II) in the distribution on the PCoA coincided with the zones. q*nirS*+*qnirK* showed a significantly higher presence in wells located in the SW area (Mann-Whitney test, $p<0.05$), in the discharge zone, specifically due to the high abundance of *nirS*. Generally *NirS*-type denitrifiers are predominant compared to *NirK*-type (Barrett et al., 2013; Lindemann et al., 2015), which could explain the predominance of *nirS* over *nirK*. Even though the two nitrite reductases are

functionally equivalent, denitrifiers harbouring either nitrite reductase seem to show a preference for certain environments (García-Lledó et al., 2011b; Jones and Hallin, 2010). Distinct groundwater physico-chemical properties could determine the presence of the two types of nitrite reductase in each zone (NE and SW), probably due to differential niche preferences. More precisely, NO_3^- and TOC:TN ratio were greater in SW wells than in NE, and this suggests a positive selection of *nirS*-type denitrifiers (Gao et al., 2016; Herrmann et al., 2017). Moreover, wells with higher *nir* abundance were negatively correlated to oxygen concentration and pH which agrees with several studies at different environments including groundwater (García-Lledó et al., 2011; Graham et al., 2010; Jurado, 2017).

Similarly to what has been observed for q*nirS*+q*nirK*, the ratio (q*nirS*+q*nirK*)/(q*nosZ*I+q*nosZ*II), a proxy of N_2O accumulation (García-Lledó et al., 2011b; Saarenheimo et al., 2015b), was higher in the SW zone wells (Figure 4.21). In wells located in the NE zone, *nosZ*II gene was significantly more abundant compared to the SW (Mann-Whitney test, $p<0.05$), whereas *nosZ*I remained at similar abundance in both areas. This was in agreement with the fact that the majority of the typical *nosZ*I-type containing bacteria have the complete set of denitrification genes, while the latter occurs in less than half of the known *nosZ*II-carrying microorganisms. In fact, *nosZ*II-type reductase was referred to a "non-denitrifier nitrous oxide reductase" (Sanford et al., 2012). The lack of nitrous oxide reductase in some denitrifying bacteria, which results in high (q*nirS*+q*nirK*)/(q*nosZ*I+q*nosZ*II) ratios, was reported more than a decade ago when the *Agrobacterium tumefaciens* genome was sequenced (Wood, 2001). A later survey of bacterial genomes confirmed that approximately 1/3 of denitrifying bacterial isolates have a truncated pathway (Jones et al., 2008). Similarly to *nir* genes, the relative importance of *nosZ* genes seems to systematically differ between habitats and with environmental conditions (Jones et al., 2013), yet the exact controls that modulate their relative abundance in nature are uncertain. At wells with high (q*nirS*+q*nirK*)/(q*nosZ*I+q*nosZ*II) ratios, nitrate concentration was generally high, leading to a possible N_2O accumulation. This has been previously shown in groundwater (Jahangir et al., 2013; Jurado et al., 2017). The dominance of *nirS* over *nirK* and *nosZ* in SW wells, can also be due to changes in trace metals rather than other variables frequently considered to alter denitrifiers abundance and activity, i.e. nitrate, organic carbon, pH and oxygen. For instance, the bioavailability of copper (Cu) and iron (Fe) is hypothesized to control the expression and activity of nitrite and nitrous oxide reductases and was defined as a selection variable explaining the dominance of *nirS* denitrifiers in Cu limited boreal lakes (Saarenheimo et al., 2015a).

4.3.5. Distribution of potential denitrifying genera

Between 68.7 and 93.7% of representative sequences could be classified at the genus level (Figure 4.22), and between 13.4% and 67.4% of the total analysed sequences belonged to genera containing putative denitrifying bacteria. In low organic matter environments as Osona, autotrophic dissimilatory nitrate reduction is achieved by oxidation of pyrite, using it as an electron donor which in turn is oxidized to SO_4^{2-}. In the studied wells, SO_4^{2-} was predominantly found in the SW area. Coupling of autotrophic denitrification to sulphide or iron oxidation has been known since at least twenty years ago, and it has been proven in many isolates and pure cultures (Straub et al., 1996; Weber et al., 2006). According to the microbial community composition, *Acidiferrobacter* and *Sideroxydans* were proportionally more abundant in the SW area (Figure 4.22). *Acidiferrobacter* has been described as an anaerobic iron- and sulphur-oxidizer able to reduce nitrate autotrophically to nitrogen gas (Niu et al., 2016). However, this bacterium is acidophilic and remains uncertain if the described reactions can be performed at pH values ranging from 7.03 to 7.52 (Table 4.12). *Sideroxydans* is a well-known iron dependent nitrate reducer (Blöthe and Roden, 2009). Collectively these results suggest that at least for these specified genera a relationship between pyrite and denitrification must exist in the studied zone, as already proved by Vitòria *et al.* (2008) and Otero *et al.* (2009) based on a multi-isotopic approach. Moreover, also related to pyrite oxidation, *Clostridium* was found in some studied wells, but it was mainly abundant in a single well of NE zone (SPT-001). This genus is able to reduce nitrate using sulphur as electron donor and it was previously found in groundwater (Pu et al., 2014).

Nitrospira species were found almost exclusively in a single well (SMC-002; 48% of total sequences) revealing a higher niche selection for certain bacteria due to changes in the hydrochemical properties. Considering the NH_4^+ and O_2 concentrations found in SW zone (Table 4.12), nitrification reactions are not expected and *Nitrospira* could be related to denitrification. Other denitrifying genera as *Cytophaga* were found in MNL-019, located in the SW zone, and *Clostridium* related species appeared to be more abundant in SPT-001, in the NE zone. Interestingly, the presence of these potentially denitrifying genera is a determinant factor explaining the sample distribution in the PCoA. Despite the changes in relative abundance observed for denitrifying genera in some wells, no significant differences in the relative abundances of any of the identified potentially denitrifiers could be detected between NE and SW zones (Mann-Whitney test, $p>0.05$; Figure 4.22). The later indicates that, at least for the selected genera, enrichment is promoted by local conditions.

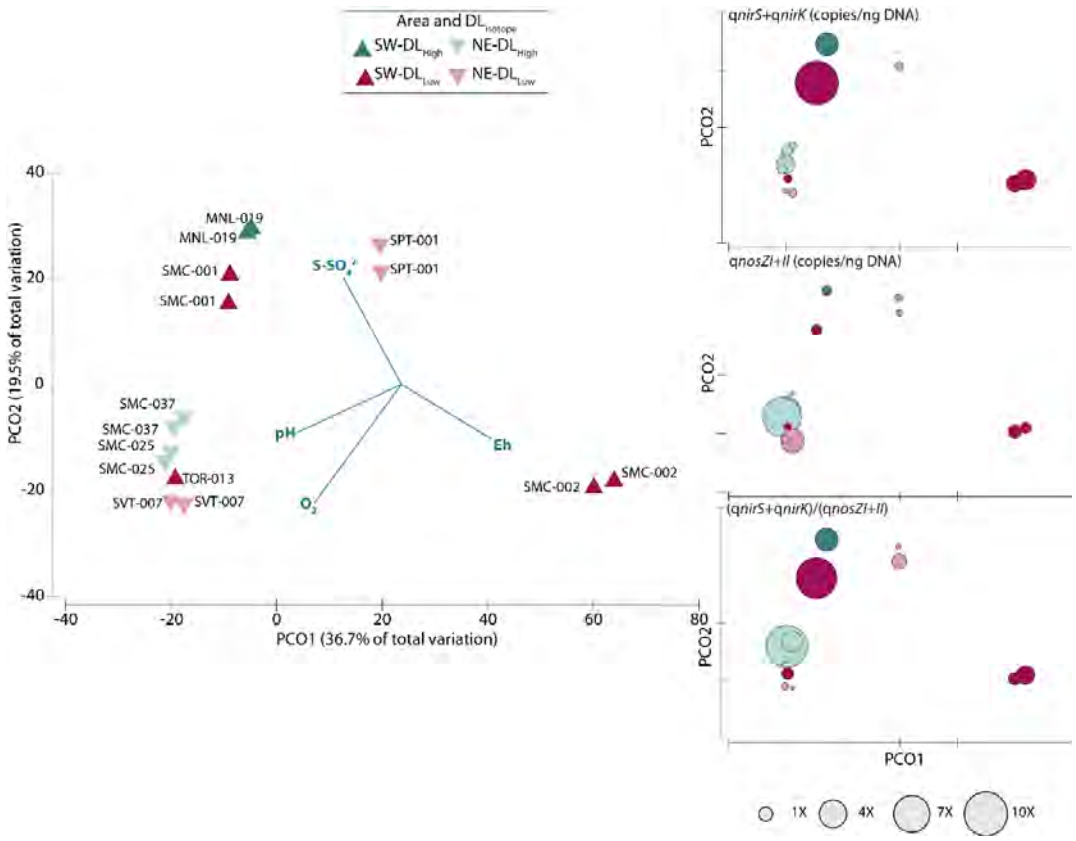

Figure 4.21. Distribution of groundwater samples in PCoA. Left: PCoA distribution of OTUs microbial community and environmental parameters (vectors) correlated with this distribution (Spearman's correlation, R>0.6). Bold lines indicate high $DL_{isotope}$. Right: Distribution of abundances of *nirK*+*nirS* and *nosZ*I+*nosZ*II, and (q*nirS*+q*nirK*)/(q*nosZ*I+q*nosZ*II) ratio according to PCoA of microbial community. In the legend, 1X refers to $2 \cdot 10^4$ (copies/ng) or 2 (ratio).

Moreover, it is remarkable that less abundant but well-known denitrifying genera, such as *Geobacillus*, *Solitalea*, *Campylobacter* and "*Candidatus* Saccharimonas" were detected mainly in wells with High-$DL_{isotope}$. This is in agreement with previous works in which members of these genera were found in groundwater (Albertsen et al., 2013; Kuppardt et al., 2014; Stanley et al., 1998). Unfortunately, we do not have enough experimental evidence to relate the presence of these species to the higher denitrification levels measured by isotopic fractionation. The relationship between taxonomic and isotopic data in groundwater has been recently shown in an agricultural area in Korea (Kim et al., 2015). This relationship was not so clear in the selected area of the Osona basin. However, in the studied region, nitrate attenuation by bacteria denitrification seems to be highly correlated with specific conditions of the wells and water. In this sense, disturbances in hydrogeological parameters could affect groundwater microbial stability and denitrification processes (Baho et al., 2012). Additional experimental measurements, such as sequencing of *nirS* and *nirK* genes and implementing

methods directed to analyses of active bacteria (mRNA determinations), would successfully help in linking the identification of microbial processes that produce denitrification, and providing a better link between isotopic and microbiological information.

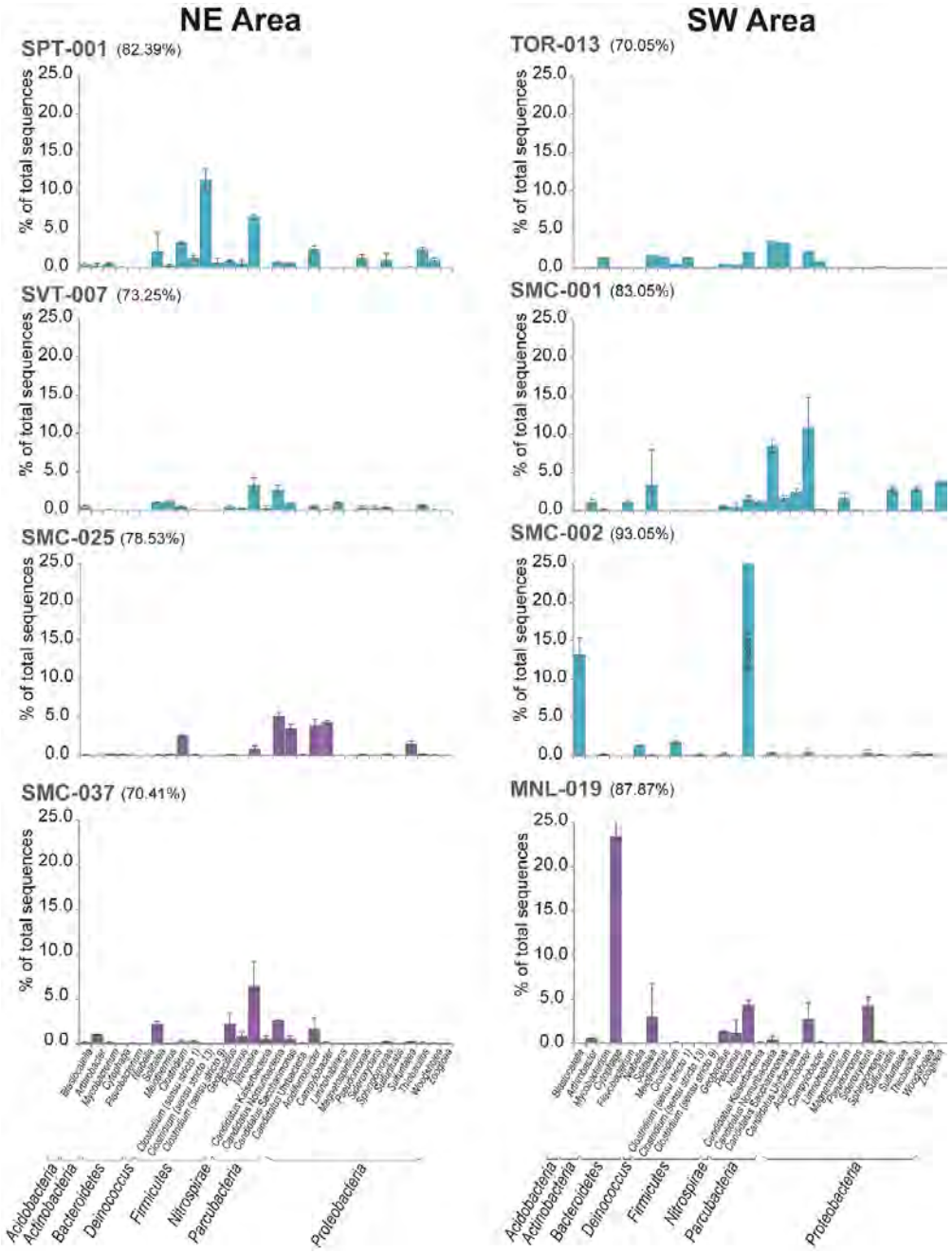

Figure 4.22. Potential denitrifying genera found in groundwater samples. Relative abundances of sequences belonging to suspected denitrifying bacteria at the genus level. Genus are grouped according to their phylum. Mean abundances (bars) and SD (whiskers) of two replicates are shown for each well. Wells were organized according to their location (NE and SW). Percentage between brackets refers to % of representative sequences classified at genus level in each sample. Blue bars, wells with Low-DL. Purple bars, wells with High-DL.

4.3.6. Final remark

Results obtained in this chapter allowed us to identify denitrification processes using isotopic and microbiological data. Hydrogeological data (geology, hydrochemistry and water stable isotopes) confirmed a common hydrogeological framework for the study area. Nitrate stable isotopes were used to identify organic manure mineralization as the main source of nitrate in the area, and could be used to estimate the degree of denitrification in the selected wells. Interestingly, different locations of the zones were classified as presenting a low or a high denitrification level ($DL_{isotope}$). This finding points out that, 1) ideal denitrification conditions (mainly due to autotrophic denitrification as shown by previous research; Otero et al., 2009) could be found all along the studied area and may not reflect the intensity of agriculture practices in close vicinity to the well; and 2) the degree of denitrification is more likely determined by either the residence time (or length of the flow path) or the mixing proportions of the distinct flow lines intercepted by the well capturing depths. Gene abundances and information on microbial species composition provided complementary evidences of the occurrence of denitrification in groundwater. The abundance of denitrifying nitrite reductases, *nirK* and *nirS*, were statistically correlated with isotopic data, though these gene abundances were not statistically different based on the classification of denitrification level ($DL_{isotope}$) areas. This is attributed to the fact that many different bacterial genera share similar genes and, consequently, they are able to strengthen denitrification processes. From a practical point of view, combined isotopic and microbiome data are essential to ascertain the ability and success of induced in-situ attenuation methods designed to reduce groundwater nitrate content at polluted sites.

5. GENERAL DISCUSSION

5.1. Environmental relevance of ammonia oxidizers and nitrite reducers

The ubiquitous presence of nitrogenous forms in aquatic ecosystems is combined with a highly active (and complex) cycle, involving many conversion steps that pose some difficulties in understanding N cycling in nature. Ammonia in water is generally resulting from ammonification of organic nitrogen or from the drainage of animal manure and urban sewage (Moir, 2011). In aerobic conditions, ammonia is rapidly oxidized to hydroxylamine and then to nitrite by ammonia oxidizers. Nitrite is further oxidized to nitrate by nitrite oxidizers. The two reactions are known as nitrification process (Dworkin and Falkow, 1992; Könneke et al., 2005). In most situations, ammonia oxidizers are the first microbial group responsible to start nitrogen removal from water. This group includes ammonia oxidizing archaea (AOA), bacteria (AOB) and comammox microorganisms. Ammonia oxidation is catalysed by the ammonia monooxygenase (AMO) enzyme. The catalytic subunit of the enzyme is encoded in the *amoA* gene and it is an excellent molecular proxy for the study of ammonia oxidizers diversity and activity (Klotz and Stein, 2008). Being the ammonia oxidation an aerobic process, its relation to the presence of oxygen in the environment is a key parameter to be considered in order to infer the potential capacity to promote nitrification (Shelef et al., 2013). In this sense, the analyses of *amoA* in plant roots (*Typha angustifolia* as a model example in the present work) suggested an importance of oxygen release by roots for nitrifying microorganisms. In fact, ammonia-oxidizing community was observed to be different between roots and sediment, which could be caused by an effective oxygen release from the root (see chapter 4.2). *Typha* sp. is a common emergent macrophyte in temperate climates and its presence and abundance in coastal wetlands is crucial for an effective removal of nitrogen in the land-to-sea transition. The results obtained here from plants collected in a salinity gradient (coastal transition areas), pointed to an effect in the aeration capacity of such plants, which promoted an effective selection of AOB on the rhizoplane. The observed selection effect revealed a tight plant-bacteria interaction beyond the aeration effect that deserves a closer attention in further analyses.

Nitrate resulting from nitrification can be reduced into nitrite by a wide range of *Bacteria* and *Archaea*. Nitrate reductases either membrane associated (NAR) or periplasmic (NAP) are responsible for the reduction step (Cabello et al., 2004; Philippot, 2005; Richardson et al., 2001; Roussel-Delif et al., 2005). Nitrite, either originated from ammonia oxidation or nitrate reduction, can be used as an alternative electron acceptor in anaerobic conditions by nitrite reducers. The majority of nitrite reducers are included in three groups, true denitrifiers (those that produce gaseous compounds), DNRA bacteria (those that promote a dissimilatory reduction of nitrite and produce ammonia), and anammox (which couple nitrite reduction to ammonia oxidation with the production of nitrate and nitrogen gas). The reduction of nitrite to nitric oxide in denitrifiers and anammox bacteria is catalysed by nitrite reductases NirK and/or NirS, encoded by *nirK* and *nirS* genes (Kartal and Keltjens, 2016; Zumft, 1997). On the other hand, nitrite reductase of DNRA bacteria is NrfA (encoded by *nrfA*), which catalyses the reduction of nitrite to ammonia, keeping the N in soluble form (Simon, 2002; Welsh et al., 2014). There are many environmental factors which influence the competition between Nir and Nrf containing bacteria, including labile organic carbon, nitrate concentration, the ratio of electron donor/acceptor (carbon/nitrate), the sulphide concentration, the pH, the redox potential, the NO_3^-/NO_2^- ratio, and the temperature (An and Gardner, 2002; Burgin and Hamilton, 2007; Dong et al., 2009; Friedl et al., 2018; Nizzoli et al., 2010; Papaspyrou et al., 2014). Nir and Nrf containing microorganisms include a wide range of phyla which could be highly distant (Bu et al., 2017; Guo et al., 2016; Moir, 2011; Wei et al., 2015). Moreover, several events of horizontal transfer of nitrite reductase genes have been reported in the literature and have been thought to be common (Jones et al., 2008). According to these, the results presented in chapter 4.1.6 showed a clear difference in taxa detected by 16S rRNA or by genes coding for nitrite reductases. Due to the absence of monophyletism within denitrifiers, to compare the dominance of one or other process in a given environment specific analyses of functional genes (nitrite or nitric oxide reductases) are preferred to phylogenetic determinations involving 16S rRNA gene. Accordingly, two different environments studied in this thesis (groundwater and FWS-CW) with favourable conditions for denitrification and DNRA, showed the simultaneous presence of denitrifiers and DNRA bacteria, as well as similar abundances of *Nir* and *Nrf* genes. Nitrite reductases were correlated to specific environmental factors, i.e. the presence of H_2S and pH, as it was previously shown in similar environments (Herrmann et al., 2017; Ligi et al., 2014b), which could determine the fate of NO_2^- towards gaseous or soluble compounds.

5.2. Ammonia oxidizers and nitrite reducers: cosmopolitan, resilient and resistant microbial communities

Putative ammonia oxidizers and nitrite reducers, were found in all the studied environments in this thesis at reasonable abundances, according to metagenomic and quantitative PCR analyses. Nitrite reducers were detected as free-living microorganisms suspended in groundwater and also attached to sediment particles and plant roots. These results agree with recent studies, in which nitrite reducers were found in water from aquifers (Herrmann et al., 2017; Kuppardt et al., 2014), urban lakes (Zhang et al., 2018), sediment of natural and constructed wetlands (Ansola et al., 2014), hypoxic estuarine sediments (Caffrey et al., 2019), pasture soils (Friedl et al., 2018) and activated sludge (van der Berg et al., 2017). Despite being widely spread in nature, genera that were associated to putative denitrifiers or DNRA microorganisms were essentially different when groundwater and FWS-CW sediment samples were compared (see chapters 4.1.2 and 4.3.4). Nitrite reducers found in groundwater matched those found in environments characterized by poor organic matter content, as copper mines (Liu et al., 2016), or in enriched mineral medium with Fe(II) (Blöthe and Roden, 2009). Contrarily, genera associated to the sediment of FWS-CW were most commonly found in environments with a higher availability of C compounds (Ansola et al., 2014; Friedl et al., 2018; Mohit et al., 2015; Jinping Zhang et al., 2015). Nonetheless, nitrite reducers are highly widespread taxonomically, and many of them are able to grow in oligotrophic conditions carrying out autotrophic denitrification in the presence of an alternative source of reducing equivalents, such as sulphide, reduced iron or hydrogen (Miao and Liu, 2018).

Regarding to ammonia oxidizers, they are not as widespread taxonomically as nitrite reducers, so their identification at genus level is, at some extent, easier. In this thesis, nine genera of ammonia oxidizers were detected in the rhizoplane and rhizosphere of *Typha angustifolia* (see chapter 4.2.4). Seven of them were archaea, and two of them bacteria (one belonging to the Comammox group). In fact, putative Comammox bacteria, mainly related to the genus *Nitrospira* (Daims et al., 2015; van Kessel et al., 2015), were found in all the environments studied in this thesis (groundwater, sediment from wetlands and estuary, and roots of *Typha angustifolia*). In all cases, there was a positive correlation among the presence of *Nitrospira* (or *Nitrospiraceae*) and the redox potential and oxygen concentration, showing the importance of aerobic conditions for nitrification process. However, *Nitrospira* related species were found in both aerobic and anaerobic environments due to their ability to use

formate and H_2, in the presence or not of nitrite (Gruber-Dorninger et al., 2015; Koch et al., 2019).

The effect of radial oxygen loss (ROL) on ammonia oxidizers was also related to samples with higher oxygen content, which would occur on the rhizoplane better than on the sediment. High oxygen affinity makes AOA more competitive than AOB in hypoxic environments such as deep oceans, deep soils, and sediments (Yin et al., 2018; Zhou et al., 2016). The results for *Typha* supported that ROL affected selectively on ammonia oxidizers although differences along sections of the root, albeit observed, were not statistically significant. In fact, ROL could be the factor that determined the diversity of ammonia oxidizers that were associated to plant species (Trias et al., 2012; Clairmont et al., 2019; Wang et al., 2018). Besides ROL, roots may also act as a selective force for microorganisms by releasing organic exudates, which could increase heterotrophic metabolisms, such as denitrification and DNRA (Shelef et al., 2013). In this sense, the presence or not of vegetation impacted on the microbial community in a broader sense, similar to what was observed as the result of a large perturbation of the system (i.e. sediment dredging and plant removal). After sediment dredging, microbial community of the area where vegetation was removed recovered rapidly (six months). These results not only showed the importance of vegetation on microorganisms, but also a high resilience of the sediment microbial community that maintained although physicochemical parameters differed between the two periods. Considering that potential for nitrate, nitrite and ammonium removal remained almost unchanged, it could be extrapolated that nitrite reducers and ammonia oxidizers were highly resilient in this environment.

Not only the presence of vegetation or the organic matter content are key factors to determine microbial communities in different environments. There were other physicochemical variables that determined the structure of the microbial community in the studied environments. Among those variables that were studied here, pH and redox potential were the most prominent. pH has been previously found as an important environmental driver for the microbial community distribution in wetland sediments, rhizospheres, rhizoplanes and groundwater (Jurado et al., 2017; Ligi et al., 2014b; Nunan et al., 2005). The optimal pH range for nitrite reduction is between 5.5 and 8.0, while for ammonia oxidation ranges between 6.0 and 8.0 (Jahangir et al., 2014; Rust et al., 2000). Redox potential has previously shown to affect microbial community structure in sediment of constructed and natural wetlands, as well as on root surface of different plant species and in groundwater, showing generally a reverse trend in comparison to pH (Jurado et al., 2017; Ligi et al., 2014b;

Zhou et al., 2017). Water pH and redox potential were also correlated to gene abundances, specifically to 16S rRNA and to *nir* genes in FWS-CW, while in groundwater nitrite reductases (*nir* and *nrfA* genes) were correlated to H_2S (see chapters 4.1.4 and 4.3.3). In the case of nitrite reductases, redox potential and H_2S were important factors determining which metabolism (denitrification or DNRA) occurred in the sediment as the main nitrite reduction step, since high redox potential could inhibit DNRA metabolism, while high H_2S concentration could inhibit denitrification (Brunet and Garcia-Gil, 1996; Friedl et al., 2018).

5.3. Analyses of microbial processes from diferent perspectives: complementarity of methods

Microbial community studies need to be complemented with activity data in order to understand the function of microbial communities in the ecosystem. In this thesis two techniques have been used to determine potential activities of nitrate + nitrite reduction. On the one hand, mass balance calculations combined to acetylene blockage method was used to analyse nitrate removal through denitrification. On the other hand, dual isotope partitioning method was used to determine the relevance of autotrophic denitrification in groundwater. The $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ isotopic signature of NO_3^- in groundwater provide evidence of denitrification (concretely nitrate reduction) at the moment of sampling, and specifically takes into account the actual conditions of water. In this sense, when the presence of isotopes was analysed, the transformations of the N species by microorganisms contained in the groundwater and also attached at the sediment of the aquifer were considered. As shown in chapter 4.3, those data do not match completely to the organisms present in the water but rather complement them. The most plausible reason for the observed results is that nitrogen species (dissolved) and microorganisms (particles) show a completely different dynamics in the interstitial water. Mass balance was used to estimate the potential activity for different processes using laboratory microcosms and could be used to complement indirect methods. Nonetheless, both methods (isotopic and microcosm determinations) are useful and may provide interesting information according to the aim of the study in each case, allowing the determination of potential microbial activities (Aravena and Mayer, 2009; Caffrey et al., 2019; Fukada et al., 2003; H. Kim et al., 2016; Puig et al., 2017; Ruiz-Rueda et al., 2009; Song et al., 2014). In this thesis, these techniques were used to know potential activity of denitrifying and DNRA bacteria and archaea, but were not used to determine

potential activity of ammonia oxidizers, though both techniques could be used (Davidson et al., 1991; H. Kim et al., 2016; Salk et al., 2018).

The obtained results from dual isotope technique led us to hypothesize that denitrifying and DNRA abundances and microbial community structures would be grouped according to denitrification level in groundwater (see chapter 4.3.2). Similarly, with the obtained results from potential nitrate + nitrite reduction and DNRA activities from FWS-CW after sediment dredging, we hypothesized that no significant changes in the structure of the microbial community occurred after a significant perturbation (sediment dredging), since the potential ability to remove nitrogen was similar before and after the impact (see chapter 4.1.5). However, we could not confirm our hypotheses by molecular methods related to total resident community of each environment. First, regarding to the relationship between isotopic measurements and molecular methods, nitrate, as a dissolved compound, flows at the pore water velocity. Conversely, bacteria, being a particulate material, possibly remain attached to surfaces and form biofilms, or occur as aggregates, having a lower mobility (Griebler and Lueders, 2009; Williamson et al., 2012). Second, most of denitrifying bacteria do not contain all genes necessary for a complete denitrification (Jones et al., 2013, 2008) and, in addition, the same set of genes in two different bacteria may lead to considerable differences in activity. According to this, it is reasonable to think that information derived from isotopic signatures or from mass balance and abundance of functional genes may not coincide. This is the case when the microbial community was analysed by 16S rRNA gene sequencing since microorganisms involved in denitrification or DNRA harbour functional genes transferred by HGT from different phylogenetically distant microorganisms based on 16S rRNA gene similarities (Jones et al., 2008; Philippot and Hallin, 2005). Finally, when the resident community and not the active part of it (mRNA determination) was analysed, gene abundance and activity do not necessarily match. In fact, the analyses of complementary DNA (cDNA) obtained from rRNA (phylogenetic marker) and mRNA (functional genes) revealed a large difference in the active members of the community in the sediment of FWS-CW after sediment dredging when compared to the resident community (DNA based community analyses). Similar results have been previously shown in other studies of nitrite and nitrate reducers in sediments (Smith et al 2007) and denitrifiers in soils (Thompson et al., 2018).

In the case of ammonia oxidizers, we specifically analysed the effect ROL at different root positions on the structure of the community. We hypothesized that changes on ROL rates would cause changes on the root microbial community, and especially on nitrifiers.

Previous results have shown that oxygen release affect nitrifying microorganisms (Shelef et al., 2013, Wang et al., 2018). Gene abundances confirmed in some cases an effect of ROL on total bacteria and specifically on ammonia oxidizers in different root sections (see chapter 4.2.3). However, the analysis of the microbial community did not reveal a clear effect according to root sections in terms of diversity. Conversely, a more general effect of roots was evidenced and significant differences in the microbial community structure of roots were found in comparison to the sediment. Oxygen and other components released from the root were relevant for the selection of ammonia oxidizers (Srivastava et al., 2017; Yin et al., 2018). In the same way as for nitrite reducers community analyses, it would have been interesting analyse the active community and not only the resident one, in order to check if ROL affected differently the active ammonia oxidizers.

Despite some improvements that could be done in the different studies performed in this thesis, the use of different methods to study microbial communities has allowed us to use different techniques that complement each other and to obtain more information of microbial community functioning in diverse environments. Moreover, the study of different environments has allowed us to determine common factors that could affect microbial communities implied in the N cycle. Altogether, the results are of interest for management of both natural and constructed systems since vegetation and environmental factors determine the prevalence of some microbial groups, such as denitrifiers, DNRA and ammonia oxidizing *Archaea* or *Bacteria*. Moreover, microbial communities revealed to be resilient to perturbations caused by sediment dredging in constructed wetlands, or increased nitrate pollution in groundwater, highlighting the adaptive capacity of natural (or naturalized) ecosystems in terms of N decontamination.

5.4. Implication of the results in system management

Microorganisms have not been traditionally considered as good biological indicators of environment health except in cases of extreme degradation. However, in this thesis we provide case studies evidence of their response to changes in ecosystem parameters, such as nutrient loading, physicochemical properties, or habitat alterations. These evidences add to different studies that confirmed a correlation between microbiome of the environment and pollution (Wright et al., 2009; Zhang et al., 2013; Zheng et al., 2018). New methodological methods (basically molecular methods) are becoming more and more common and straightforward and provide more insightful information about the microorganisms

inhabiting ecosystems, what their activities are, and how do they change to environmental stressors. These technical improvements enabled highly specific studies, centred to a limited number of microorganisms or metabolic reactions, which are changing our perspective of microorganisms as ecological indicators.

We now can assure that, in the case of environments polluted with N, ammonia oxidizers are key players in the system, usually revealing its potentiality for N removal. In this thesis, we described how oxygen and redox potential affected ammonia oxidizers in wetlands. AOA have been demonstrated to have more adaptability to extreme environments, with low oxygen, high salinity or low pH (Kim et al., 2016; Lehtovirta-Morley, 2018). However, AOB are more able to grow in polluted environments than AOA (Urakawa and Bernhard, 2017). In terms of application, in surface freshwater ecosystems with high ammonium input, as could happen in the effluent of a WWTP, is important to ensure an oxygenated area (i.e. presence of vegetation) to promote the growth of AOB. According to our results, an initial vegetation belt with a high oxygenation capacity of the sediment will promote ammonia transformation to nitrite and its further conversion to nitrogen gas.

On the other hand, nitrite produced by ammonia oxidizers is still an N soluble compound that should be removed. In this sense, sediments with high organic matter (or alternative electron donors in oligotrophic environments), and anoxic conditions provide the ideal environment for nitrite reducers and denitrification (Ligi et al., 2014; Jurado et al., 2017), and not surprisingly our studies have indicated a high correlation between nitrite reductase abundances and redox and pH. However, these two factors could also favour DNRA over denitrification, mainly if redox potential decreases to a certain level (Friedl et al., 2018). Considering these facts, in the management of the wetland systems, it is important to ensure an alternation of oxic and anoxic areas, which in FWS-CW can be achieved with modifying the extension and localization of planted and unplanted areas. Vegetation management significantly changes microenvironment conditions specifically at the rhizosphere, and microbial community diversity and richness improves, potentially enhancing N removal. Summarizing, to manage different ecosystems mainly wetlands and potentially groundwater in terms of N removal, is important to study microbial communities to know what their activity and composition are. Accordingly, the indirect control of environmental parameters, such as the presence or not of vegetation (and which type of vegetation), as well as the regulation of physicochemical variables could enhance N removal.

6. CONCLUDING REMARKS

1. True denitrification and DNRA occur simultaneously in the sediment of Empuriabrava FWS-CW. Microbes potentially implied in the two processes were identified on the basis of 16S rRNA sequence similarity. Resident communities (DNA based) differed from active communities (mRNA based), for both *nirS* and *nirK* containing bacteria.
2. Microbial community of wetland sediments were highly affected by the presence of vegetation. Plant removal and sediment dredging caused a relevant impact in the abundance and composition of nitrite reducers in the Empuriabrava FWS-CW. DNRA pathway was favored over denitrification. At the ecosystem scale, nitrogen removing capacity was not affected, suggesting a highly resilient nitrite-reducing community in the FWS-CW.
3. Radial oxygen loss played an important role in shaping the ammonia oxidizing community. Oxygen leakage affected differentially at the rhizoplane compared to the sediment on ammonia oxidizing *Archaea* and *Bacteria* in terms of abundances and diversity
4. Genes related to true denitrification and DNRA were detected in the Osona region, with a dominance of nitrite reductases implied in denitrification pathway.
5. The presence of potential nitrite reducers clearly differed in environments low C:N ratio (groundwater) and high C:N ratio (wetlands).
6. pH and redox (i.e. presence of H_2S) appeared as important parameters determining microbial community structure in the studied environments. Those variables correlated positively to nitrite reductases abundance in sediment and groundwater, thus shaping the abundance of denitrifiers and DNRA bacteria as relevant players in nitrite reduction.
7. The combination of different methodologies such as potential activity measurements (isotope ratios) and molecular methods provided different and complementary information and allowed an in deep analysis of microbial communities and activity, which could be of interest to design induced attenuation actions.

7. REFERENCES

Abad García, A., 2001. Paleotaxodonta y pteriomorphia del Eoceno del margen sur de la depresión Central Catalana. PhD Diss. Univ. Autònoma Barcelona. Universitat Autònoma de Barcelona.

Abell, G.C.J., Ross, D.J., Keane, J., Holmes, B.H., Robert, S.S., Keough, M.J., Eyre, B.D., Volkman, J.K., 2014. Niche differentiation of Ammonia-Oxidising *Archaea* (AOA) and *Bacteria* (AOB) in response to paper and pulp mill effluent. Microb. Ecol. 67, 758–768. doi:10.1007/s00248-014-0376-7

Akaboci, T.R.V., Gich, F., Ruscalleda, M., Balaguer, M.D., Colprim, J., 2018. Assessment of operational conditions towards mainstream partial nitritation-anammox stability at moderate to low temperature: Reactor performance and bacterial community. Chem. Eng. J. 350, 192–200. doi:10.1016/J.CEJ.2018.05.115

Albertsen, M., Hugenholtz, P., Skarshewski, A., Nielsen, K.L., Tyson, G.W., Nielsen, P.H., 2013. Genome sequences of rare, uncultured bacteria obtained by differential coverage binning of multiple metagenomes. Nat. Biotechnol. 31, 533–538. doi:10.1038/nbt.2579

An, S., Gardner, W., 2002. Dissimilatory nitrate reduction to ammonium (DNRA) as a nitrogen link, versus denitrification as a sink in a shallow estuary (Laguna Madre/Baffin Bay, Texas). Mar. Ecol. Prog. Ser. 237, 41–50. doi:10.3354/meps237041

Andreote, F.D., Jiménez, D.J., Chaves, D., Dias, A.C.F., Luvizotto, D.M., Dini-Andreote, F., Fasanella, C.C., Lopez, M.V., Baena, S., Taketani, R.G., de Melo, I.S., 2012. The microbiome of brazilian mangrove sediments as revealed by metagenomics. PLoS One 7, e38600. doi:10.1371/journal.pone.0038600

Ansola, G., Arroyo, P., Sáenz de Miera, L.E., 2014. Characterisation of the soil bacterial community structure and composition of natural and constructed wetlands. Sci. Total Environ. 473, 63–71. doi:10.1016/j.scitotenv.2013.11.125

Aravena, R., Mayer, B., 2009. Isotopes and processes in the nitrogen and sulfur cycles. Environ. Isot. Biodegrad. anda Bioremediation 203–246. doi:doi:10.1201/9781420012613.ch7

Aravena, R., Robertson, W.D., 1998. Use of multiple isotope tracers to evaluate denitrification in ground water: Study of nitrate from a large-flux septic system plume. Ground Water. doi:10.1111/j.1745-6584.1998.tb02104.x

Armstrong, W., 1980. Aeration in higher plants. Adv. Bot. Res. 7, 225–332. doi:10.1016/S0065-2296(08)60089-0

Armstrong, W., 1971. Radial Oxygen Losses from intact rice roots as affected by distance from the apex, respiration and waterlogging. Physiol. Plant. 25, 192–197. doi:10.1111/j.1399-3054.1971.tb01427.x

Armstrong, W., Beckett, P.M., 1987. Internal aeration and development of stela anoxia in submerged roots: a multishelled mathem mathematical model combining axial diffusion of oxygen in the cortex with radial losses to the stele, the wall layers and the rhizosphere. New Phytol. 105, 221–245. doi:10.1111/j.1469-8137.1987.tb00860.x

Baho, D.L., Peter, H., Tranvik, L.J., 2012. Resistance and resilience of microbial communities - Temporal and spatial insurance against perturbations. Environ. Microbiol. 14, 2283–2292. doi:10.1111/j.1462-2920.2012.02754.x

Balderston, W.L., Sherr, B., Payne, W.J., 1976. Blockage by acetylene of nitrous oxide reduction in *Pseudomonas perfectomarinus*. Appl. Environ. Microbiol. 31, 504–8.

Bañeras, L., Ruiz-Rueda, O., López-Flores, R., Quintana, X.D., Hallin, S., 2012. The role of plant type and salinity in the selection for the denitrifying community structure in the rhizosphere of wetland vegetation. Int. Microbiol. 15, 89–99. doi:10.2436/20.1501.01.162

Barrett, M., Jahangir, M.M.R., Lee, C., Smith, C.J., Bhreathnach, N., Collins, G., Richards, K.G., O'Flaherty, V., 2013. Abundance of denitrification genes under different peizometer depths in four Irish agricultural groundwater sites. Environ. Sci. Pollut. Res. 20, 6646–6657. doi:10.1007/s11356-013-1729-3

Bartossek, R., Nicol, G.W., Lanzen, A., Klenk, H.P., Schleper, C., 2010. Homologues of nitrite reductases in ammonia-oxidizing archaea: Diversity and genomic context. Environ. Microbiol. doi:10.1111/j.1462-2920.2010.02153.x

Bastviken, S.K., Eriksson, P.G., Premrov, a., Tonderski, K., 2005. Potential denitrification in wetland sediments with different plant species detritus. Ecol. Eng. 25, 183–190. doi:10.1016/j.ecoleng.2005.04.013

Benedict, S.W., Ahmed, T., Jahan, K., 1998. Autotrophic denitrification using hydrogen oxidizing bacteria in continuous flow biofilm reactor. Toxicol. Environ. Chem. 67, 197–214. doi:10.1080/02772249809358614

Bernhard, A., 2010. The nitrogen cycle: processes, players, and human impact. Nat. Educ. Knowl. 3, 25.

Black, A., Hsu, P.-C.L., Hamonts, K.E., Clough, T.J., Condron, L.M., 2016. Influence of copper on expression of *nirS* , *norB* and *nosZ* and the transcription and activity of NIR, NOR and N $_2$ OR in the denitrifying soil bacteria *Pseudomonas stutzeri*. Microb. Biotechnol. 9, 381–388. doi:10.1111/1751-7915.12352

Blöthe, M., Roden, E.E., 2009. Composition and activity of an autotrophic Fe(II)-oxidizing, nitrate-reducing enrichment culture. Appl. Environ. Microbiol. 75, 6937–40. doi:10.1128/AEM.01742-09

Bodelier, P., Libochant, J.A., Blom, C., Laanbroek, H.J., 1996. Dynamics of nitrification and denitrification in root-oxygenated sediments and adaptation of ammonia-oxidizing bacteria to low-oxygen or anoxic habitats. Appl. Environ. Microbiol. 62, 4100–7. doi:10.1128/aem.02806-08

Bodelier, P.L.E., 2003. Interactions Between oxygen-releasing roots and microbial processes in flooded soils and sediments. Springer, Berlin, Heidelberg, pp. 331–362. doi:10.1007/978-3-662-09784-7_13

Böhlke, J.K., Wanty, R., Tuttle, M., Delin, G., Landon, M., 2002. Denitrification in the recharge area and discharge area of a transient agricultural nitrate plume in a glacial outwash sand aquifer, Minnesota. Water Resour. Res. 38, 10-1-10–26. doi:10.1029/2001WR000663

Bonilla-Rosso, G., Wittorf, L., Jones, C.M., Hallin, S., 2016. Design and evaluation of primers targeting genes encoding NO-forming nitrite reductases: implications for ecological

inference of denitrifying communities. Sci. Rep. 6, 39208. doi:10.1038/srep39208

Böttcher, J., Strebel, O., Voerkelius, S., Schmidt, H.L., 1990. Using isotope fractionation of nitrate-nitrogen and nitrate-oxygen for evaluation of microbial denitrification in a sandy aquifer. J. Hydrol. doi:10.1016/0022-1694(90)90068-9

Boy-Roura, M., Menció, A., Mas-Pla, J., 2013a. Temporal analysis of spring water data to assess nitrate inputs to groundwater in an agricultural area (Osona, NE Spain). Sci. Total Environ. doi:10.1016/j.scitotenv.2013.02.065

Boy-Roura, M., Nolan, B.T., Menció, A., Mas-Pla, J., 2013b. Regression model for aquifer vulnerability assessment of nitrate pollution in the Osona region (NE Spain). J. Hydrol. 505, 150–162. doi:10.1016/j.jhydrol.2013.09.048

Bremer, C., Braker, G., Matthies, D., Reuter, A., Engels, C., Conrad, R., 2007. Impact of plant functional group, plant species, and sampling time on the composition of nirK-type denitrifier communities in soil. Appl. Environ. Microbiol. 73, 6876–6884. doi:10.1128/AEM.01536-07

Bremner, J.M., Blackmer, A.M., Waring, S.A., 1980. Formation of nitrous oxide and dinitrogen by chemical decomposition of hydroxylamine in soils. Soil Biol. Biochem. doi:10.1016/0038-0717(80)90072-3

Bristow, L., Tage, D., Tiano, L., Mills, D.B., Bertagnolli, A.D., Wright, J.J., Hallam, S.J., Ulloa, O., Canfield, D.E., Revsbe, Thamdrup, B., 2016. Ammonium and nitrite oxidation at nanomolar oxygen concentrations in oxygen minimum zone waters. Proc. Natl. Acad. Sci. 113, 10601–10606. doi:10.1073/pnas.1600359113

Brix, H., 1997. Do macrophytes play a role in Constructed Treatment Wetlands? Water Sci. Technol. 35, 11–17.

Brix, H., 1994. Use of constructed wetlands in water pollution control: historical development, present status, and future perspectives. Water Sci. Technol. 30, 209–223.

Brunet, R.C., Garcia-Gil, L.J., 1996. Sulfide-induced dissimilatory nitrate reduction to ammonia in anaerobic freshwater sediments. FEMS Microbiol. Ecol. 21, 131–138.

Bu, C., Wang, Y., Ge, C., Ahmad, H.A., Gao, B., Ni, S.-Q., 2017. Dissimilatory nitrate reduction to ammonium in the Yellow River Estuary: rates, abundance, and community diversity. Sci. Rep. 7, 6830. doi:10.1038/s41598-017-06404-8

Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren van Themaat, E., Ahmadinejad, N., Assenza, F., Rauf, P., Huettel, B., Reinhardt, R., Schmelzer, E., Peplies, J., Gloeckner, F.O., Amann, R., Eickhorst, T., Schulze-Lefert, P., 2012. Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. Nature 488, 91–95. doi:10.1038/nature11336

Burgin, A.J., Hamilton, S.K., 2008. NO3 −-Driven SO4 2− production in freshwater rcosystems: implications for N and S cycling. Ecosystems 11, 908–922. doi:10.1007/s10021-008-9169-5

Burgin, A.J., Hamilton, S.K., 2007. Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways. Front. Ecol. Environ. 5, 89–96. doi:10.1890/1540-9295(2007)5[89:HWOTRO]2.0.CO;2

Butterbach-Bahl, K., Baggs, E.M., Dannenmann, M., Kiese, R., Zechmeister-Boltenstern, S., 2013. Nitrous oxide emissions from soils: how well do we understand the processes

and their controls? Philos. Trans. R. Soc. B Biol. Sci. 368, 20130122–20130122. doi:10.1098/rstb.2013.0122

Cabello, P., Roldán, M.D., Moreno-Vivián, C., 2004. Nitrate reduction and the nitrogen cycle in archaea. Microbiology 150, 3527–3546. doi:10.1099/mic.0.27303-0

Caffrey, J.M., Bonaglia, S., Conley, D.J., 2019. Short exposure to oxygen and sulfide alter nitrification, denitrification, and DNRA activity in seasonally hypoxic estuarine sediments. FEMS Microbiol. Lett. 366. doi:10.1093/femsle/fny288

Caliz, J., Montes-Borrego, M., Triadó-Margarit, X., Metsis, M., Landa, B.B., Casamayor, E.O., 2015. Influence of edaphic, climatic, and agronomic factors on the composition and abundance of nitrifying microorganisms in the rhizosphere of commercial olive crops. PLoS One 10, e0125787. doi:10.1371/journal.pone.0125787

Canfield, D.E., Glazer, A.N., Falkowski, P.G., 2010. The evolution and future of Earth's nitrogen cycle. Science 330, 192–196. doi:10.1126/science.1186120

Cantera, J.J.L., Stein, L.Y., 2007. Molecular diversity of nitrite reductase genes (nirK) in nitrifying bacteria. Environ. Microbiol. doi:10.1111/j.1462-2920.2006.01198.x

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336. doi:10.1038/nmeth.f.303

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G., Knight, R., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. 6, 1621–1624. doi:10.1038/ismej.2012.8

Carpenter, S.R., Caraco, N.F., Correll, D.L., Howarth, R.W., Sharpley, A.N., Smith, V.H., 1998. Nonpoint pollution of surface waters with phosphorus and nitrogen. Ecol. Appl. doi:10.1890/1051-0761(1998)008[0559:NPOSWW]2.0.CO;2

Causey, M.B., Beane, K.N., Wolf, J.R., 2006. The effects of salinity and other factors on nitrite reduction by Ochrobactrum anthropi 49187. J. Basic Microbiol. 46, 10–21. doi:10.1002/jobm.200510588

Chang, A.H., Parsonnet, J., 2010. Role of bacteria in oncogenesis. Clin. Microbiol. Rev. doi:10.1128/CMR.00012-10

Chen, Y., Zhou, W., Li, Y., Zhang, J., Zeng, G., Huang, A., Huang, J., 2014. Nitrite reductase genes as functional markers to investigate diversity of denitrifying bacteria during agricultural waste composting. Appl. Microbiol. Biotechnol. 98, 4233–43. doi:10.1007/s00253-014-5514-0

Choudhury, M.I., McKie, B.G., Hallin, S., Ecke, F., 2018. Mixtures of macrophyte growth forms promote nitrogen cycling in wetlands. Sci. Total Environ. 635, 1436–1443. doi:10.1016/J.SCITOTENV.2018.04.193

Christensen, S., Simkins, S., Tiedje, J.M., 1990. Spatial variation in denitrification: dependency of activity centers on the soil environment. Soil Sci. Soc. Am. J. 54, 1608. doi:10.2136/sssaj1990.03615995005400060016x

Clairmont, L.K., Stevens, K.J., Slawson, R.M., 2019. Site-specific differences in microbial community structure and function within the rhizosphere and rhizoplane of wetland plants is plant species dependent. Rhizosphere 9, 56–68. doi:10.1016/J.RHISPH.2018.11.006

Clarke, K.R., 1993. Non-parametric multivariate analyses of changes in community structure. Aust. J. Ecol. 18, 117–143.

Clarke, K.R., Warwick, R.M., 2001. Change in marine communities. An approach to statistical analysis and interpretation., 2nd ed, Natural Environment Research Council. Plymouth Marine Laboratory, UK, Plymouth (United Kingdom).

Colmer, T.D., 2003. Long-distance transport of gases in plants: A perspective on internal aeration and radial oxygen loss from roots. Plant, Cell Environ. 26, 17–36. doi:10.1046/j.1365-3040.2003.00846.x

Costa, E., Pérez, J., Kreft, J.-U., 2006. Why is metabolic labour divided in nitrification? Trends Microbiol. 14, 213–219. doi:10.1016/J.TIM.2006.03.006

Costa, R., Götz, M., Mrotzek, N., Lottmann, J., Berg, G., Smalla, K., 2006. Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. FEMS Microbiol. Ecol. 56, 236–249. doi:10.1111/j.1574-6941.2005.00026.x

Daims, H., Lebedeva, E.V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinszky, M., Vierheilig, J., Bulaev, A., Kirkegaard, R.H., von Bergen, M., Rattei, T., Bendinger, B., Nielsen, P.H., Wagner, M., 2015. Complete nitrification by Nitrospira bacteria. Nature 528, 504–509. doi:10.1038/nature16461

Daims, H., Lücker, S., Wagner, M., 2016. A new perspective on microbes formerly known as Nitrite-Oxidizing Bacteria. Trends Microbiol. doi:10.1016/j.tim.2016.05.004

Davidson, E.A., Hart, S.C., Shanks, C.A., Firestone, M.K., 1991. Measuring gross nitrogen mineralization, and nitrification by 15 N isotopic pool dilution in intact soil cores. J. Soil Sci. 42, 335–349. doi:10.1111/j.1365-2389.1991.tb00413.x

Dixon, R., Kahn, D., 2004. Genetic regulation of biological nitrogen fixation. Nat. Rev. Microbiol. 2, 621–631. doi:10.1038/nrmicro954

Domeignoz-Horta, L.A., Philippot, L., Peyrard, C., Bru, D., Breuil, M.-C., Bizouard, F., Justes, E., Mary, B., Léonard, J., Spor, A., 2018. Peaks of in situ N $_2$ O emissions are influenced by N $_2$ O-producing and reducing microbial communities across arable soils. Glob. Chang. Biol. 24, 360–370. doi:10.1111/gcb.13853

Dong, L.F., Smith, C.J., Papaspyrou, S., Stott, A., Osborn, a M., Nedwell, D.B., 2009. Changes in benthic denitrification, nitrate ammonification, and anammox process rates and nitrate and nitrite reductase gene abundances along an estuarine nutrient gradient (the Colne estuary, United Kingdom). Appl. Environ. Microbiol. 75, 3171–9. doi:10.1128/AEM.02511-08

Dozier, M.C., Melton, Rebecca H., Hare, M.F., Hopkins, J., Lesikar, B.J., 2008. Drinking water problems: nitrates. Texas A&M Univ. Syst. AgriLife Commun. B-6184 3-0.

Duarte Pereira, A., Dutra Leal, C., França Dias, M., Etchebehere, C., Augusto Chernicharo, C.L., Calabria de Araújo, J., 2014. Effect of phenol on the nitrogen removal performance and microbial community structure and composition of an anammox reactor. Bioresour. Technol. 166, 103–111. doi:10.1016/j.biortech.2014.05.043

Dworkin, M., Falkow, S., 1992. The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications, 2nd ed. Springer-Verlag New York. doi:10.1007/978-1-4757-2191-1

Edgar, R.C., 2016. UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. bioRxiv. doi:10.1101/081257

Edwards, J., Johnson, C., Santos-Medellín, C., Lurie, E., Podishetty, N.K., Bhatnagar, S., Eisen, J.A., Sundaresan, V., 2015. Structure, variation, and assembly of the root-associated microbiomes of rice. Proc. Natl. Acad. Sci. U. S. A. 112, E911-20. doi:10.1073/pnas.1414592112

Enríquez, S., Duarte, C.M., Sand-Jensen, K., 1993. Patterns in decomposition rates among photosynthetic organisms: the importance of detritus C:N:P content. Oecologia 94, 457–471. doi:10.1007/BF00566960

Erguder, T.H., Boon, N., Wittebolle, L., Marzorati, M., Verstraete, W., 2009. Environmental factors shaping the ecological niches of ammonia-oxidizing archaea. FEMS Microbiol. Rev. 33, 855–869. doi:10.1111/j.1574-6976.2009.00179.x

Etchebehere, C., Tiedje, J., 2005. Presence of Two Different Active nirS Nitrite Reductase Genes in a Denitrifying Thauera sp. from a High-Nitrate-Removal-Rate Reactor. Appl. Environ. Microbiol. 71, 5642–5645. doi:10.1128/AEM.71.9.5642-5645.2005

Faulwetter, J.L., Gagnon, V., Sundberg, C., Chazarenc, F., Burr, M.D., Brisson, J., Camper, A.K., Stein, O.R., 2009. Microbial processes influencing performance of treatment wetlands: A review. Ecol. Eng. 35, 987–1004. doi:10.1016/j.ecoleng.2008.12.030

Fewtrell, L., 2004. Drinking-water nitrate, methemoglobinemia, and global burden of disease: A discussion. Environ. Health Perspect. 112, 1371–1374. doi:10.1289/ehp.7216

Fierer, N., Jackson, R.B., 2006. The diversity and biogeography of soil bacterial communities. Proc. Natl. Acad. Sci. U. S. A. 103, 626–31. doi:10.1073/pnas.0507535103

Fish, J. a., Chai, B., Wang, Q., Sun, Y., Brown, C.T., Tiedje, J.M., Cole, J.R., 2013. FunGene: The functional gene pipeline and repository. Front. Microbiol. 4, 1–14. doi:10.3389/fmicb.2013.00291

Foesel, B.U., Drake, H.L., Schramm, A., 2011. Defluviimonas denitrificans gen. nov., sp. nov., and Pararhodobacter aggregans gen. nov., sp. nov., non-phototrophic Rhodobacteraceae from the biofilter of a marine aquaculture. Syst. Appl. Microbiol. 34, 498–502. doi:10.1016/J.SYAPM.2011.08.006

Friedl, J., De Rosa, D., Rowlings, D.W., Grace, P.R., Müller, C., Scheer, C., 2018. Dissimilatory nitrate reduction to ammonium (DNRA), not denitrification dominates nitrate reduction in subtropical pasture soils upon rewetting. Soil Biol. Biochem. 125, 340–349. doi:10.1016/J.SOILBIO.2018.07.024

Fukada, T., Hiscock, K.M., Dennis, P.F., Grischek, T., 2003. A dual isotope approach to identify denitrification in groundwater at a river-bank infiltration site. Water Res. 37, 3070–3078. doi:10.1016/S0043-1354(03)00176-3

Gabarró, J., Hernández-Del Amo, E., Gich, F., Ruscalleda, M., Balaguer, M.D., Colprim, J., 2013. Nitrous oxide reduction genetic potential from the microbial community of an intermittently aerated partial nitritation SBR treating mature landfill leachate. Water Res. 47, 7066–7077. doi:10.1016/j.watres.2013.07.057

Galloway, J.N., Townsend, A.R., Erisman, J.W., Bekunda, M., Cai, Z., Freney, J.R., Martinelli, L.A., Seitzinger, S.P., Sutton, M.A., 2008. Transformation of the nitrogen cycle: Recent trends, questions, and potential solutions. Science (80-.). doi:10.1126/science.1136674

Gao, J., Xie, Y., Jin, H., Liu, Y., Bai, X., Ma, D., Zhu, Y., Wang, C., Guo, T., 2016. Nitrous oxide emission and denitrifier abundance in two agricultural soils amended with crop residues and urea in the north China plain. PLoS One 11, e0154773. doi:10.1371/journal.pone.0154773

Gao, T., Shi, X.-Y., 2018. Taxonomic structure and function of seed-inhabiting bacterial microbiota from common reed (Phragmites australis) and narrowleaf cattail (Typha angustifolia L.). Arch. Microbiol. 200, 869–876. doi:10.1007/s00203-018-1493-3

García-Lledó, A., Ruiz-Rueda, O., Vilar-Sanz, Sala, L., Bañeras, L., 2011a. Nitrogen removal efficiencies in a free water surface constructed wetland in relation to plant coverage. Ecol. Eng. 37, 678–684.

García-Lledó, A., Vilar-Sanz, A., Trias, R., Hallin, S., Bañeras, L., 2011b. Genetic potential for N2O emissions from the sediment of a free water surface constructed wetland. Water Res. 45, 5621–32. doi:10.1016/j.watres.2011.08.025

Garcia, J.M., Teixeira, P., 2017. Organic versus conventional food: A comparison regarding food safety. Food Rev. Int. 33, 424–446. doi:10.1080/87559129.2016.1196490

Glockner, A.B., Jüngst, A., Zumft, W.G., 1993. Copper-containing nitrite reductase from Pseudomonas aureofaciens is functional in a mutationally cytochrome cd1-free background (NirS) of Pseudomonas stutzeri. Arch. Microbiol. 160, 18–26. doi:10.1007/bf00258141

Gómez-Hernández, N., Reyes-González, A., Sánchez, C., Mora, Y., Delgado, M.J., Girard, L., 2011. Regulation and symbiotic role of nirK and norC Expression in Rhizobium etli. Mol. Plant-Microbe Interact. 24, 233–245. doi:10.1094/MPMI-07-10-0173

Gonfiantini, R., 1986. Environmental isotopes in lake studies, in: The Terrestrial Environment, B. pp. 113–168. doi:10.1016/B978-0-444-42225-5.50008-5

Graf, D.R.H., Jones, C.M., Hallin, S., 2014. Intergenomic comparisons highlight modularity of the denitrification pathway and underpin the importance of community structure for N2O emissions. PLoS One 9, e114118. doi:10.1371/journal.pone.0114118

Graham, D.W., Trippett, C., Dodds, W.K., O'Brien, J.M., Banner, E.B.K., Head, I.M., Smith, M.S., Yang, R.K., Knapp, C.W., 2010. Correlations between in situ denitrification activity and nir-gene abundances in pristine and impacted prairie streams. Environ. Pollut. 158, 3225–3229. doi:10.1016/j.envpol.2010.07.010

Griebler, C., Lueders, T., 2009. Microbial biodiversity in groundwater ecosystems. Freshw. Biol. 54, 649–677. doi:10.1111/j.1365-2427.2008.02013.x

Grießmeier, V., Bremges, A., McHardy, A.C., Gescher, J., 2017. Investigation of different nitrogen reduction routes and their key microbial players in wood chip-driven denitrification beds. Sci. Rep. 7, 17028. doi:10.1038/s41598-017-17312-2

Griffiths, B.S., Philippot, L., 2013. Insights into the resistance and resilience of the soil microbial community. FEMS Microbiol. Rev. 37, 112–29. doi:10.1111/j.1574-6976.2012.00343.x

Gruber-Dorninger, C., Pester, M., Kitzinger, K., Savio, D.F., Loy, A., Rattei, T., Wagner, M.,

Daims, H., 2015. Functionally relevant diversity of closely related Nitrospira in activated sludge. ISME J. 9, 643–55. doi:10.1038/ismej.2014.156

Guo, L.J., Zhao, B., An, Q., Tian, M., 2016. Characteristics of a novel aerobic denitrifying bacterium, Enterobacter cloacae strain HNR. Appl. Biochem. Biotechnol. 178, 947–959. doi:10.1007/s12010-015-1920-8

Guo, Y., Gong, H., Guo, X., 2015. Rhizosphere bacterial community of Typha angustifolia L. and water quality in a river wetland supplied with reclaimed water. Appl. Microbiol. Biotechnol. 99, 2883–2893. doi:10.1007/s00253-014-6182-9

Gurlevik, N., Kelting, D.L., Allen, H.L., 2004. Nitrogen mineralization following vegetation control and fertilization in a 14-year-old Loblolly pine plantation. Soil Sci. Soc. Am. J. 68, 272. doi:10.2136/sssaj2004.2720

Haberl, R., Perfler, R., Mayer, H., 1995. Constructed wetlands in Europe. Water Sci. Technol. doi:10.1016/0273-1223(95)00631-1

Hallin, S., Jones, C.M., Schloter, M., Philippot, L., 2009. Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment. ISME J. 3, 597–605. doi:10.1038/ismej.2008.128

Hallin, S., Philippot, L., Löffler, F.E., Sanford, R.A., Jones, C.M., 2018. Genomics and ecology of novel N2O-reducing microorganisms. Trends Microbiol. 26, 43–55. doi:10.1016/j.tim.2017.07.003

Hao, X., Benke, M.B., 2008. Nitrogen transformation and losses during composting and mitigation strategies., in: Compost I. Dynamic Soil, Dynamic Plant 2 (Special Issue 1). pp. 10–18.

Harhangi, H.R., Le Roy, M., van Alen, T., Hu, B.-L., Groen, J., Kartal, B., Tringe, S.G., Quan, Z.-X., Jetten, M.S.M., Op den Camp, H.J.M., 2012. Hydrazine synthase, a unique phylomarker with which to study the presence and biodiversity of anammox bacteria. Appl. Environ. Microbiol. 78, 752–8. doi:10.1128/AEM.07113-11

Härtig, E., Zumft, W., 1999. Kinetics of nirS expression (cytochrome cd1 nitrite reductase) in Pseudomonas stutzeri during the transition from aerobic respiration to denitrification: evidence for a denitrification-specific nitrate- and nitrite-responsive regulatory system. J. Bacteriol. 172, 7057–7064.

Haugen, K.S., Semmens, M.J., Novak, P.J., 2002. A novel in situ technology for the treatment of nitrate contaminated groundwater. Water Res. doi:10.1016/S0043-1354(02)00043-X

He, S., Malfatti, S.A., McFarland, J.W., Anderson, F.E., Pati, A., Huntemann, M., Tremblay, J., Glavina del Rio, T., Waldrop, M.P., Windham-Myers, L., Tringe, S.G., 2015. Patterns in wetland microbial community composition and functional gene repertoire associated with methane emissions. MBio 6, e00066-15. doi:10.1128/mBio.00066-15

Heaton, T.H.., 1986. Isotopic studies of nitrogen pollution in the hydrosphere and atmosphere: A review. Chem. Geol. Isot. Geosci. Sect. 59, 87–102. doi:10.1016/0168-9622(86)90059-X

Heil, J., Vereecken, H., Brüggemann, N., 2016. A review of chemical reactions of nitrification intermediates and their role in nitrogen cycling and nitrogen trace gas formation in soil. Eur. J. Soil Sci. 67, 23–39. doi:10.1111/ejss.12306

Helen, D., Kim, H., Tytgat, B., Anne, W., 2016. Highly diverse nirK genes comprise two

major clades that harbour ammonium-producing denitrifiers. BMC Genomics 17, 155. doi:10.1186/s12864-016-2465-0

Henderson, S.L., Dandie, C.E., Patten, C.L., Zebarth, B.J., Burton, D.L., Trevors, J.T., Goyer, C., 2010. Changes in denitrifier abundance, denitrification gene mRNA levels, nitrous oxide emissions, and denitrification in anoxic soil microcosms amended with glucose and plant residues. Appl. Environ. Microbiol. 76, 2155–2164. doi:10.1128/AEM.02993-09

Herbert, R.A., 1999. Nitrogen cycling in coastal marine ecosystems. FEMS Microbiol. Rev. 23, 563–590. doi:10.1111/j.1574-6976.1999.tb00414.x

Hernández-del Amo, E., Menció, A., Gich, F., Mas-Pla, J., Bañeras, L., 2018. Isotope and microbiome data provide complementary information to identify natural nitrate attenuation processes in groundwater. Sci. Total Environ. 613–614, 579–591. doi:10.1016/j.scitotenv.2017.09.018

Herrmann, M., Opitz, S., Harzer, R., Totsche, K., Küsel, K., 2017. Attached and suspended denitrifier communities in pristine limestone aquifers harbor high fractions of potential autotrophs oxidizing reduced iron and sulfur compounds. Microb. Ecol. doi:10.1007/s00248-017-0950-x

Heylen, K., Gevers, D., Vanparys, B., Wittebolle, L., Geets, J., Boon, N., De Vos, P., 2006a. The incidence of nirS and nirK and their genetic heterogeneity in cultivated denitrifiers. Environ. Microbiol. 8, 2012–2021. doi:10.1111/j.1462-2920.2006.01081.x

Heylen, K., Vanparys, B., Wittebolle, L., Boon, N., Vos, P. De, Verstraete, W., 2006b. Cultivation of denitrifying bacteria : optimization of isolation conditions and diversity study. Appl. Environ. Microbiol. 72, 2637–2647. doi:10.1128/AEM.72.4.2637

Hink, L., Gubry-Rangin, C., Nicol, G.W., Prosser, J.I., 2018. The consequences of niche and physiological differentiation of archaeal and bacterial ammonia oxidisers for nitrous oxide emissions. ISME J. 12, 1084. doi:10.1038/s41396-017-0025-5

Hoffmann, T., Frankenberg, N., Marino, M., Jahn, D., 1998. Ammonification in Bacillus subtilis utilizing dissimilatory nitrite reductase is dependent on resDE. J. Bacteriol. 180, 186–9.

Horváth, L., Führer, E., Lajtha, K., 2006. Nitric oxide and nitrous oxide emission from Hungarian forest soils; linked with atmospheric N-deposition. Atmos. Environ. 40, 7786–7795. doi:10.1016/J.ATMOSENV.2006.07.029

Huang, Y., Li, D., Wang, L., Yong, C., Sun, E., Jin, H., Huang, H., 2019. Decreased enzyme activities, ammonification rate and ammonifiers contribute to higher nitrogen retention in hyperthermophilic pretreatment composting. Bioresour. Technol. 272, 521–528. doi:10.1016/J.BIORTECH.2018.10.070

Humbert, S., Tarnawski, S., Fromin, N., Mallet, M.-P., Aragno, M., Zopfi, J., 2010. Molecular detection of anammox bacteria in terrestrial ecosystems: distribution and diversity. ISME J. 4, 450–4. doi:10.1038/ismej.2009.125

Humbert, S., Zopfi, J., Tarnawski, S.-E., 2012. Abundance of anammox bacteria in different wetland soils. Environ. Microbiol. Rep. 4, 484–490. doi:10.1111/j.1758-2229.2012.00347.x

Hurt, R.A., Qiu, X., Wu, L., Roh, Y., Tiedje, J.M., Zhou, J., Roh, Y.U.L., Palumbo, A. V, 2001. Simultaneous recovery of RNA and DNA from soils and sediments 67.

doi:10.1128/AEM.67.10.4495

Inoue, J., Oshima, K., Suda, W., Sakamoto, M., Iino, T., Noda, S., Hongoh, Y., Hattori, M., Ohkuma, M., 2015. Distribution and evolution of nitrogen fixation genes in the phylum Bacteroidetes. Microbes Environ. Environ. 30, 44–50. doi:10.1264/jsme2.me14142

Ishii, S., Yamamoto, M., Kikuchi, M., Oshima, K., Hattori, M., Otsuka, S., Senoo, K., 2009. Microbial populations responsive to denitrification-inducing conditions in rice paddy soil, as revealed by comparative 16S rRNA gene analysis. Appl. Environ. Microbiol. 75, 7070–8. doi:10.1128/AEM.01481-09

Jahangir, M.M.R., Fenton, O., Gill, L., Müller, C., Johnston, P., Richards, K.G., 2014. Carbon and nitrogen dynamics and greenhouse gases emissions in constructed wetlands: a review. Hydrol. Earth Syst. Sci. Discuss. 11, 7615–7657. doi:10.5194/hessd-11-7615-2014

Jahangir, M.M.R., Johnston, P., Addy, K., Khalil, M.I., Groffman, P.M., Richards, K.G., 2013. Quantification of in situ denitrification rates in groundwater below an arable and a grassland system. Water. Air. Soil Pollut. 224. doi:10.1007/s11270-013-1693-z

Jensen, M.H., Lomstein, E., Ssrensen, J., 1990. Benthic NHT and NO3- flux following sedimentation of a spring phytoplankton bloom in Aarhus Bight, Denmark.

Jetten, M.S.M., Strous, M., Pas-schoonen, K.T. Van De, Schalk, J., Dongen, U.G.J.M. Van, Graaf, A.A. Van De, Logemann, S., Y, G.M.I., Loosdrecht, M.C.M. Van, Kuenen, J.G., 1999. The anaerobic oxidation of ammonium. FEMS Microbiol. Rev. 22, 421–437.

Jiang, X., Yao, L., Guo, L., Liu, G., Liu, W., 2017. Multi-scale factors affecting composition, diversity, and abundance of sediment denitrifying microorganisms in Yangtze lakes. Appl. Microbiol. Biotechnol. 101, 8015–8027. doi:10.1007/s00253-017-8537-5

Jones, C.M., Graf, D.R.H., Bru, D., Philippot, L., Hallin, S., 2013. The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. ISME J. 7, 417–26. doi:10.1038/ismej.2012.125

Jones, C.M., Hallin, S., 2010. Ecological and evolutionary factors underlying global and local assembly of denitrifier communities. ISME J. 4, 633–41. doi:10.1038/ismej.2009.152

Jones, C.M., Stres, B., Rosenquist, M., Hallin, S., 2008. Phylogenetic analysis of nitrite, nitric oxide, and nitrous oxide respiratory enzymes reveal a complex evolutionary history for denitrification. Mol. Biol. Evol. 25, 1955–66. doi:10.1093/molbev/msn146

Junier, P., Kim, O., Witzel, K., Imhoff, J., Hadas, O., 2008. Habitat partitioning of denitrifying bacterial communities carrying nirS or nirK genes in the stratified water column of Lake Kinneret, Israel. Aquat. Microb. Ecol. 51, 129–140. doi:10.3354/ame01186

Jurado, A., Borges, A. V., Brouyère, S., 2017. Dynamics and emissions of N_2O in groundwater: A review. Sci. Total Environ. 585, 207–218. doi:10.1016/j.scitotenv.2017.01.127

Jurado, M., López, M.J., Suárez-Estrella, F., Vargas-García, M.C., López-González, J.A., Moreno, J., 2014. Exploiting composting biodiversity: Study of the persistent and biotechnologically relevant microorganisms from lignocellulose-based composting. Bioresour. Technol. 162, 283–293. doi:10.1016/J.BIORTECH.2014.03.145

Kadlec, R.H., Wallace, S.D., 2009. Treatment Wetlands, Second Edition. Taylor & Francis

Group, LLC. doi:10.1201/9781420012514

Kartal, B., de Almeida, N.M., Maalcke, W.J., Op den Camp, H.J.M., Jetten, M.S.M., Keltjens, J.T., 2013. How to make a living from anaerobic ammonium oxidation. FEMS Microbiol. Rev. 37, 428–61. doi:10.1111/1574-6976.12014

Kartal, B., Keltjens, J.T., 2016. Anammox biochemistry: a tale of heme c proteins. Trends Biochem. Sci. 41, 998–1011. doi:10.1016/j.tibs.2016.08.015

Katan, M.B., 2009. Nitrate in foods: harmful or healthy? Am. J. Clin. Nutr. 90, 11–12. doi:10.3945/ajcn.2009.28014

Ke, X., Lu, W., Conrad, R., 2015. High oxygen concentration increases the abundance and activity of bacterial rather than archaeal nitrifiers in rice field soil. Microb. Ecol. 70, 961–970. doi:10.1007/s00248-015-0633-4

Kemnitz, D., Kolb, S., Conrad, R., 2007. High abundance of Crenarchaeota in a temperate acidic forest soil. FEMS Microbiol. Ecol. 60, 442–8. doi:10.1111/j.1574-6941.2007.00310.x

Kendall, C., 1998. Tracing nitrogen sources and cycling in catchments, in: Isotope Tracers in Catchment Hydrology. Elsevier, pp. 519–576. doi:10.1016/B978-0-444-81546-0.50023-9

Khangembam, C.D., 2016. Effect of temperature on nitrifying microbes , emphasizing on ammonia oxidizing archaea and bacteria. J. Biol. Sci. Med. 2, 7–14.

Kim, H., Bae, H.-S., Reddy, K.R., Ogram, A., 2016. Distributions, abundances and activities of microbes associated with the nitrogen cycle in riparian and stream sediments of a river tributary. Water Res. 106, 51–61. doi:10.1016/J.WATRES.2016.09.048

Kim, H., Kaown, D., Mayer, B., Lee, J.Y., Hyun, Y., Lee, K.K., 2015. Identifying the sources of nitrate contamination of groundwater in an agricultural area (Haean basin, Korea) using isotope and microbial community analyses. Sci. Total Environ. 533, 566–575. doi:10.1016/j.scitotenv.2015.06.080

Kim, J.J., Comstock, J., Ryan, P., Heindel, C., Koenigsberger, S., 2016. Denitrification and dilution along fracture flowpaths influence the recovery of a bedrock aquifer from nitrate contamination. Sci. Total Environ. 569, 450–468. doi:10.1016/j.scitotenv.2016.06.091

Kindaichi, T., Yamaoka, S., Uehara, R., Ozaki, N., Ohashi, A., Albertsen, M., Nielsen, P.H., Nielsen, J.L., 2016. Phylogenetic diversity and ecophysiology of Candidate phylum Saccharibacteria in activated sludge. FEMS Microbiol. Ecol. 92, fiw078. doi:10.1093/femsec/fiw078

Klotz, M.G., Stein, L.Y., 2008. Nitrifier genomics and evolution of the nitrogen cycle. FEMS Microbiol. Lett. 278, 146–156. doi:10.1111/j.1574-6968.2007.00970.x

Knobeloch, L., Salna, B., Hogan, A., Postle, J., Anderson, H., 2000. Blue babies and nitrate-contaminated well water. Environ. Health Perspect. doi:10.1289/ehp.00108675

Koch, H., van Kessel, M.A.H.J., Lücker, S., 2019. Complete nitrification: insights into the ecophysiology of comammox *Nitrospira*. Appl. Microbiol. Biotechnol. 103, 177–189. doi:10.1007/s00253-018-9486-3

Könneke, M., Bernhard, A.E., de la Torre, J.R., Walker, C.B., Waterbury, J.B., Stahl, D.A.,

2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature 437, 543–546. doi:10.1038/nature03911

Kool, D.M., Dolfing, J., Wrage, N., Van Groenigen, J.W., 2011. Nitrifier denitrification as a distinct and significant source of nitrous oxide from soil. Soil Biol. Biochem. 43, 174–178. doi:10.1016/j.soilbio.2010.09.030

Koop-Jakobsen, K., Giblin, A.E., 2010. The effect of increased nitrate loading on nitrate reduction via denitrification and DNRA in salt marsh sediments. Limnol. Oceanogr. 55, 789–802. doi:10.4319/lo.2009.55.2.0789

Kotula, L., Steudle, E., 2009. Measurements of oxygen permeability coefficients of rice (Oryza sativa L.) roots using a new perfusion technique. J. Exp. Bot. 60, 567–580. doi:10.1093/jxb/ern300

Kowalchuk, G.A., Stephen, J.R., 2001. Ammonia-Oxidizing Bacteria: A model for molecular microbial ecology. Annu. Rev. Microbiol. 55, 485–529. doi:10.1146/annurev.micro.55.1.485

Kuppardt, A., Kleinsteuber, S., Vogt, C., Lüders, T., Harms, H., Chatzinotas, A., 2014. Phylogenetic and Functional Diversity Within Toluene-Degrading, Sulphate-Reducing Consortia Enriched from a Contaminated Aquifer. Microb. Ecol. 68, 222–234. doi:10.1007/s00248-014-0403-8

Kutvonen, H., Rajala, P., Carpén, L., Bomberg, M., 2015. Nitrate and ammonia as nitrogen sources for deep subsurface microorganisms. Front. Microbiol. 6, 1079. doi:10.3389/fmicb.2015.01079

Kuypers, M.M.M., Marchant, H.K., Kartal, B., 2018. The microbial nitrogen-cycling network. Nat. Rev. Microbiol. 16, 263–276. doi:10.1038/nrmicro.2018.9

Laanbroek, H.J., 2010. Methane emission from natural wetlands: Interplay between emergent macrophytes and soil microbial processes. A mini-review. Ann. Bot. 105, 141–153. doi:10.1093/aob/mcp201

Lee, C., Fletcher, T.D., Sun, G., 2009. Nitrogen removal in constructed wetland systems. Eng. Life Sci. 9, 11–22. doi:10.1002/elsc.200800049

Lehtovirta-Morley, L.E., 2018. Ammonia oxidation: Ecology, physiology, biochemistry and why they must all come together. FEMS Microbiol. Lett. 365, 1–9. doi:10.1093/femsle/fny058

Lenaker, P.L., 2009. Applying the isotope pairing technique to evaluate how water temperature and habitat type influence denitrification estimates in Breton Sound. Louisiana State University.

Li, J., Li, D., Cui, Y., Xing, W., Deng, S., 2017. Micro-electrolysis/retinervus luffae-based simultaneous autotrophic and heterotrophic denitrification for low C/N wastewater treatment. Environ. Sci. Pollut. Res. 24, 16651–16658. doi:10.1007/s11356-017-9179-y

Li, Y., Wu, B., Zhu, G., Liu, Y., Ng, W.J., Appan, A., Tan, S.K., 2016. High-throughput pyrosequencing analysis of bacteria relevant to cometabolic and metabolic degradation of ibuprofen in horizontal subsurface flow constructed wetlands. Sci. Total Environ. 562, 604–613. doi:10.1016/J.SCITOTENV.2016.04.020

Ligi, T., Oopkaup, K., Truu, M., Preem, J.-K., Nõlvak, H., Mitsch, W.J., Mander, Ü., Truu, J., 2014a. Characterization of bacterial communities in soil and sediment of a created

riverine wetland complex using high-throughput 16S rRNA amplicon sequencing. Ecol. Eng. 72, 56–66. doi:10.1016/j.ecoleng.2013.09.007

Ligi, T., Truu, M., Truu, J., Nõlvak, H., Kaasik, A., Mitsch, W.J., Mander, Ü., 2014b. Effects of soil chemical characteristics and water regime on denitrification genes (nirS, nirK, and nosZ) abundances in a created riverine wetland complex. Ecol. Eng. 72, 47–55. doi:10.1016/j.ecoleng.2013.07.015

Lin, X., McKinley, J., Resch, C.T., Kaluzny, R., Lauber, C.L., Fredrickson, J., Knight, R., Konopka, A., 2012. Spatial and temporal dynamics of the microbial community in the Hanford unconfined aquifer. ISME J. 6, 1665–76. doi:10.1038/ismej.2012.26

Lindemann, S., Zarnoch, C.B., Castignetti, D., Hoellein, T.J., 2015. Effect of eastern oysters (Crassostrea virginica) and seasonality on nitrite reductase gene abundance (nirS, nirK, nrfA) in an urban estuary. Estuaries and Coasts 39, 218–232. doi:10.1007/s12237-015-9989-4

Liu, Y.-H., Guo, J.-W., Salam, N., Li, L., Zhang, Y.-G., Han, J., Mohamad, O.A., Li, W.-J., 2016. Culturable endophytic bacteria associated with medicinal plant Ferula songorica: molecular phylogeny, distribution and screening for industrially important traits. 3 Biotech 6, 209. doi:10.1007/s13205-016-0522-7

Llirós, M., Casamayor, E.O., Borrego, C., 2008. High archaeal richness in the water column of a freshwater sulfurous karstic lake along an interannual study. FEMS Microbiol. Ecol. 66, 331–342. doi:10.1111/j.1574-6941.2008.00583.x

López-Gutiérrez, J.C., Henry, S., Hallet, S., Martin-Laurent, F., Catroux, G., Philippot, L., 2004. Quantification of a novel group of nitrate-reducing bacteria in the environment by real-time PCR. J. Microbiol. Methods 57, 399–407. doi:10.1016/j.mimet.2004.02.009

Lücker, S., Wagner, M., Maixner, F., Pelletier, E., Koch, H., Vacherie, B., Rattei, T., Damsté, J.S.S., Spieck, E., Le Paslier, D., Daims, H., 2010. A Nitrospira metagenome illuminates the physiology and evolution of globally important nitrite-oxidizing bacteria. Proc. Natl. Acad. Sci. U. S. A. 107, 13479–84. doi:10.1073/pnas.1003860107

Magkos, F., Arvaniti, F., Zampelas, A., 2006. Organic food: buying more safety or just peace of mind? A critical review of the literature. Crit. Rev. Food Sci. Nutr. 46, 23–56. doi:10.1080/10408690490911846

Mania, D., Heylen, K., van Spanning, R.J.M., Frostegård, Å., 2014. The nitrate-ammonifying and *nosZ* -carrying bacterium *Bacillus vireti* is a potent source and sink for nitric and nitrous oxide under high nitrate conditions. Environ. Microbiol. 16, 3196–3210. doi:10.1111/1462-2920.12478

Mardanov, A. V., Gumerov, V.M., Slobodkina, G.B., Beletsky, A. V., Bonch-Osmolovskaya, E.A., Ravin, N. V., Skryabin, K.G., 2012. Complete genome sequence of strain 1860, a crenarchaeon of the genus *Pyrobaculum* able to grow with various electron acceptors. J. Bacteriol. doi:10.1128/JB.06465-11

Mardis, E.R., 2008. Next-generation DNA sequencing methods. Annu. Rev. Genomics Hum. Genet. 9, 387–402. doi:10.1146/annurev.genom.9.081307.164359

Martens-Habbena, W., Berube, P.M., Urakawa, H., de la Torre, J.R., Stahl, D.A., 2009. Ammonia oxidation kinetics determine niche separation of nitrifying *Archaea* and *Bacteria*. Nature 461, 976–979. doi:10.1038/nature08465

Mason, C.F., 2002. Biology of freshwater pollution. Prentice Hall.

McDonald, M.P., Galwey, N.W., Colmer, T.D., 2002. Similarity and diversity in adventitious root anatomy as related to root aeration among a range of wetland and dryland grass species. Plant, Cell Environ. doi:10.1046/j.0016-8025.2001.00817.x

McIlroy, S.J., Starnawska, A., Starnawski, P., Saunders, A.M., Nierychlo, M., Nielsen, P.H., Nielsen, J.L., 2016. Identification of active denitrifiers in full-scale nutrient removal wastewater treatment systems. Environ. Microbiol. 18, 50–64. doi:10.1111/1462-2920.12614

Mcisaac, G., 2003. Surface water pollution by nitrogen fertilizers, in: Encyclopedia of Water Science. Marcel Dekker, Inc., pp. 950–955. doi:10.1081/E-EWS

McKew, B.A., Taylor, J.D., McGenity, T.J., Underwood, G.J.C., 2011. Resistance and resilience of benthic biofilm communities from a temperate saltmarsh to desiccation and rewetting. ISME J. 5, 30–41. doi:10.1038/ismej.2010.91

Meeren, P. Van der, Vleeschauwer, D. De, Debergh, P., 2001. Determination of oxygen profiles in agar-based gelled in vitro plant tissue culture media. Plant Cell. Tissue Organ Cult. 65, 239–245. doi:10.1023/A:1010698225362

Menció, A., Boy, M., Mas-Pla, J., 2011a. Analysis of vulnerability factors that control nitrate occurrence in natural springs (Osona Region, NE Spain). Sci. Total Environ. doi:10.1016/j.scitotenv.2011.04.048

Menció, A., Mas-Pla, J., Otero, N., Regàs, O., Boy-Roura, M., Puig, R., Bach, J., Domènech, C., Zamorano, M., Brusi, D., Folch, A., 2016. Nitrate pollution of groundwater; all right…, but nothing else? Sci. Total Environ. 539, 241–251. doi:10.1016/j.scitotenv.2015.08.151

Menció, A., Mas-Pla, J., Otero, N., Soler, A., 2011b. Nitrate as a tracer of groundwater flow in a fractured multilayered aquifer. Hydrol. Sci. J. 56, 108–122. doi:10.1080/02626667.2010.543086

Miao, L., Liu, Z., 2018. Microbiome analysis and -omics studies of microbial denitrification processes in wastewater treatment: recent advances. Sci. China Life Sci. 61, 753–761. doi:10.1007/s11427-017-9228-2

Mitsch, W.J., Gosselink, J.G., 2015. Wetlands, 5th ed, Wetlands. John & Wiley Sons. doi:10.1108/rr-09-2015-0230

Mockler, E.M., Deakin, J., Archbold, M., Gill, L., Daly, D., Bruen, M., 2017. Sources of nitrogen and phosphorus emissions to Irish rivers and coastal waters: Estimates from a nutrient load apportionment framework. Sci. Total Environ. 601–602, 326–339. doi:10.1016/J.SCITOTENV.2017.05.186

Mohan, S.B., Schmid, M., Jetten, M., Cole, J., 2004. Detection and widespread distribution of the nrfA gene encoding nitrite reduction to ammonia, a short circuit in the biological nitrogen cycle that competes with denitrification. FEMS Microbiol. Ecol. 49, 433–43. doi:10.1016/j.femsec.2004.04.012

Mohit, V., Archambault, P., Lovejoy, C., 2015. Resilience and adjustments of surface sediment bacterial communities in an enclosed shallow coastal lagoon, Magdalen Islands, Gulf of St. Lawrence, Canada. FEMS Microbiol. Ecol. 91, fiv038. doi:10.1093/femsec/fiv038

Moir, J.W.B., 2011. Nitrogen cycling in bacteria : molecular analysis. Caister Academic Press.

Moncaster, S.J., Bottrell, S.H., Tellam, J.H., Lloyd, J.W., Konhauser, K.O., 2000. Migration and attenuation of agrochemical pollutants: Insights from isotopic analysis of groundwater sulphate. J. Contam. Hydrol. doi:10.1016/S0169-7722(99)00104-7

Morrissey, E.M., Jenkins, A.S., Brown, B.L., Franklin, R.B., 2013. Resource availability effects on nitrate-reducing microbial communities in a freshwater wetland. Wetlands 33, 301–310. doi:10.1007/s13157-013-0384-2

Müller, C., Laughlin, R.J., Spott, O., Rütting, T., 2014. Quantification of N_2O emission pathways via a ^{15}N tracing model. Soil Biol. Biochem. 72, 44–54. doi:10.1016/j.soilbio.2014.01.013

Murphy, A.P., 1991. Chemical removal of nitrate from water. Nature. doi:10.1038/350223a0

Mus, F., Crook, M.B., Garcia, K., Costas, A.G., Geddes, B.A., Kouri, E.D., Paramasivan, P., Ryu, M.-H., Oldroyd, G.E.D., Poole, P.S., Udvardi, M.K., Voigt, C.A., Ané, J.-M., Peters, J.W., 2016. Symbiotic nitrogen fixation and the challenges to its extension to nonlegumes. Appl. Environ. Microbiol. 82, 3698–3710. doi:10.1128/AEM.01055-16

Nielsen, L.B., Finster, K., Welsh, D.T., Donelly, A., Herbert, R.A., De Wit, R., Lomstein, B.A.A., 2001. Sulphate reduction and nitrogen fixation rates associated with roots, rhizomes and sediments from *Zostera noltii* and *Spartina maritima* meadows. Environ. Microbiol. doi:10.1046/j.1462-2920.2001.00160.x

Nielsen, L.P., 1992. Denitrification in sediment determined from nitrogen isotope pairing. FEMS Microbiol. Ecol. 9, 357–361. doi:10.1111/j.1574-6941.1992.tb01771.x

Nikolenko, O., Jurado, A., Borges, A. V., Knöller, K., Brouyère, S., 2018. Isotopic composition of nitrogen species in groundwater under agricultural areas: A review. Sci. Total Environ. 621, 1415–1432. doi:10.1016/j.scitotenv.2017.10.086

Niu, J., Deng, J., Xiao, Y., He, Z., Zhang, X., Van Nostrand, J.D., Liang, Y., Deng, Y., Liu, X., Yin, H., 2016. The shift of microbial communities and their roles in sulfur and iron cycling in a copper ore bioleaching system. Sci. Rep. 6, 34744. doi:10.1038/srep34744

Nizzoli, D., Carraro, E., Nigro, V., Viaroli, P., 2010. Effect of organic enrichment and thermal regime on denitrification and dissimilatory nitrate reduction to ammonium (DNRA) in hypolimnetic sediments of two lowland lakes. Water Res. 44, 2715–24. doi:10.1016/j.watres.2010.02.002

Noguerola, I., Picazo, A., Llirós, M., Camacho, A., Borrego, C.M., 2015. Diversity of freshwater *Epsilonproteobacteria* and dark inorganic carbon fixation in the sulphidic redoxcline of a meromictic karstic lake. FEMS Microbiol. Ecol. 91, fiv086. doi:10.1093/femsec/fiv086

Nolan, M., Tindall, B.J., Pomrenke, H., Lapidus, A., Copeland, A., del Rio, T.G., Lucas, S., Chen, F., Tice, H., Cheng, J.F., Saunders, E., Han, C., Bruce, D., Goodwin, L., Chain, P., Pitluck, S., Ovchinikova, G., Pati, A., Ivanova, N., Mavromatis, K., Chen, A., Palaniappan, K., Land, M., Hauser, L., Chang, Y.J., Jeffries, C.D., Brettin, T., Göker, M., Bristow, J., Eisen, J.A., Markowitz, V., Hugenholtz, P., Kyrpides, N.C., Klenk, H.P., Detter, J.C., 2009. Complete genome sequence of *Rhodothermus marinus* type strain (R-10 T). Stand. Genomic Sci. doi:10.4056/sigs.46736

Nunan, N., Daniell, T.J., Singh, B.K., Papert, A., McNicol, J.W., Prosser, J.I., 2005. Links between plant and rhizoplane bacterial communities in grassland soils, characterized using molecular techniques. Appl. Environ. Microbiol. 71, 6784–92.

doi:10.1128/AEM.71.11.6784-6792.2005

Ofek-Lalzar, M., Sela, N., Goldman-Voronov, M., Green, S.J., Hadar, Y., Minz, D., 2014. Niche and host-associated functional signatures of the root surface microbiome. Nat. Commun. 5, 4950. doi:10.1038/ncomms5950

Otero, N., Torrentó, C., Soler, A., Menció, A., Mas-Pla, J., 2009. Monitoring groundwater nitrate attenuation in a regional system coupling hydrogeology with multi-isotopic methods: The case of Plana de Vic (Osona, Spain). Agric. Ecosyst. Environ. 133, 103–113. doi:10.1016/j.agee.2009.05.007

Palomo, A., Pedersen, A.G., Fowler, S.J., Dechesne, A., Sicheritz-Pontén, T., Smets, B.F., 2018. Comparative genomics sheds light on niche differentiation and the evolutionary history of comammox *Nitrospira*. ISME J. 12, 1779–1793. doi:10.1038/s41396-018-0083-3

Papaspyrou, S., Smith, C.J., Dong, L.F., Whitby, C., Dumbrell, A.J., Nedwell, D.B., 2014. Nitrate reduction functional genes and nitrate reduction potentials persist in deeper estuarine sediments. Why? PLoS One 9, e94111. doi:10.1371/journal.pone.0094111

Paranychianakis, N. V., Tsiknia, M., Kalogerakis, N., 2016. Pathways regulating the removal of nitrogen in planted and unplanted subsurface flow constructed wetlands. Water Res. 102, 321–329. doi:10.1016/J.WATRES.2016.06.048

Park, J.Y., Yoo, Y.J., 2009. Biological nitrate removal in industrial wastewater treatment: which electron donor we can choose. Appl. Microbiol. Biotechnol. 82, 415–429. doi:10.1007/s00253-008-1799-1

Pauwels, H., Foucher, J.-C., Kloppmann, W., 2000. Denitrification and mixing in a schist aquifer: influence on water chemistry and isotopes. Chem. Geol. 168, 307–324. doi:10.1016/S0009-2541(00)00201-1

Pedraza, R.O., 2008. Recent advances in nitrogen-fixing acetic acid bacteria. Int. J. Food Microbiol. 125, 25–35. doi:10.1016/J.IJFOODMICRO.2007.11.079

Peiffer, J.A., Spor, A., Koren, O., Jin, Z., Tringe, S.G., Dangl, J.L., Buckler, E.S., Ley, R.E., 2013. Diversity and heritability of the maize rhizosphere microbiome under field conditions. Proc. Natl. Acad. Sci. U. S. A. 110, 6548–53. doi:10.1073/pnas.1302837110

Penton, C.R., Deenik, J.L., Popp, B.N., Bruland, G.L., Engstrom, P., St. Louis, D., Tiedje, J., 2013. Importance of sub-surface rhizosphere-mediated coupled nitrification–denitrification in a flooded agroecosystem in Hawaii. Soil Biol. Biochem. 57, 362–373. doi:10.1016/J.SOILBIO.2012.10.018

Penton, C.R., Johnson, T. a, Quensen, J.F., Iwai, S., Cole, J.R., Tiedje, J.M., 2013. Functional genes to assess nitrogen cycling and aromatic hydrocarbon degradation: primers and processing matter. Front. Microbiol. 4, 279. doi:10.3389/fmicb.2013.00279

Penton, C.R., St Louis, D., Pham, A., Cole, J.R., Wu, L., Luo, Y., Schuur, E.A.G., Zhou, J., Tiedje, J.M., 2015. Denitrifying and diazotrophic community responses to artificial warming in permafrost and tallgrass prairie soils. Front. Microbiol. 6, 746. doi:10.3389/fmicb.2015.00746

Peralta, R.M., Ahn, C., Voytek, M.A., Kirshtein, J.D., 2013. Bacterial community structure of nirK-bearing denitrifiers and the development of properties of soils in created mitigation wetlands. Appl. Soil Ecol. 70, 70–77. doi:10.1016/j.apsoil.2013.04.008

Pester, M., Maixner, F., Berry, D., Rattei, T., Koch, H., Lücker, S., Nowka, B., Richter, A., Spieck, E., Lebedeva, E., Loy, A., Wagner, M., Daims, H., 2014. *NxrB* encoding the beta subunit of nitrite oxidoreductase as functional and phylogenetic marker for nitrite-oxidizing *Nitrospira*. Environ. Microbiol. 16, 3055–3071. doi:10.1111/1462-2920.12300

Pester, M., Rattei, T., Flechl, S., Gröngröft, A., Richter, A., Overmann, J., Reinhold-Hurek, B., Loy, A., Wagner, M., 2012. *amoA*-based consensus phylogeny of ammonia-oxidizing archaea and deep sequencing of *amoA* genes from soils of four different geographic regions. Environ. Microbiol. 14, 525–539. doi:10.1111/j.1462-2920.2011.02666.x

Philippot, L., 2005. Tracking nitrate reducers and denitrifiers in the environment. Biochem. Soc. Trans. 33, 200–4. doi:10.1042/BST0330200

Philippot, L., 2002. Denitrifying genes in bacterial and Archaeal genomes. Biochim. Biophys. Acta 1577, 355–76.

Philippot, L., Hallin, S., 2005. Finding the missing link between diversity and activity using denitrifying bacteria as a model functional community. Curr. Opin. Microbiol. 8, 234–9. doi:10.1016/j.mib.2005.04.003

Philippot, L., Hallin, S., Schloter, M., 2007. Ecology of denitrifying prokaryotes in agricultural soil, in: Advances in Agronomy. Elsevier Inc., pp. 249–305. doi:10.1016/S0065-2113(07)96003-4

Pietrangelo, L., Bucci, A., Maiuro, L., Bulgarelli, D., Naclerio, G., 2018. Unraveling the composition of the root-associated bacterial microbiota of *Phragmites australis* and *Typha latifolia*. Front. Microbiol. 9, 1650. doi:10.3389/fmicb.2018.01650

Pii, Y., Mimmo, T., Tomasi, N., Terzano, R., Cesco, S., Crecchio, C., 2015. Microbial interactions in the rhizosphere: beneficial influences of plant growth-promoting rhizobacteria on nutrient acquisition process. A review. Biol. Fertil. Soils 51, 403–415. doi:10.1007/s00374-015-0996-1

Prosser, J.I., Nicol, G.W., 2012. Archaeal and bacterial ammonia-oxidisers in soil: The quest for niche specialisation and differentiation. Trends Microbiol. 20, 523–531. doi:10.1016/j.tim.2012.08.001

Prosser, J.I., Prosser I., J., 1989. Autotrophic nitrification in bacteria, Advances in microbial physiology. doi:10.1016/S0065-2911(08)60112-5

Pu, J., Feng, C., Liu, Y., Li, R., Kong, Z., Chen, N., Tong, S., Hao, C., Liu, Y., 2014. Pyrite-based autotrophic denitrification for remediation of nitrate contaminated groundwater. Bioresour. Technol. 173, 117–123. doi:10.1016/j.biortech.2014.09.092

Puig, R., Soler, A., Widory, D., Mas-Pla, J., Domènech, C., Otero, N., 2017. Characterizing sources and natural attenuation of nitrate contamination in the Baix Ter aquifer system (NE Spain) using a multi-isotope approach. Sci. Total Environ. doi:10.1016/j.scitotenv.2016.11.206

Qin, S., Clough, T., Luo, J., Wrage-Mönnig, N., Oenema, O., Zhang, Y., Hu, C., 2017. Perturbation-free measurement of in situ di-nitrogen emissions from denitrification in nitrate-rich aquatic ecosystems. Water Res. 109, 94–101. doi:10.1016/J.WATRES.2016.11.035

Reddy, K.R., Patrick, W.H., Lindau, C.W., 1989. Nitrification-denitrification at the plant root-sediment interface in wetlands. Limnol. Oceanogr. 34, 1004–1013. doi:10.4319/lo.1989.34.6.1004

Remmas, N., Melidis, P., Katsioupi, E., Ntougias, S., 2016. Effects of high organic load on amoA and nirS gene diversity of an intermittently aerated and fed membrane bioreactor treating landfill leachate. Bioresour. Technol. 220, 557–565. doi:10.1016/J.BIORTECH.2016.09.009

Richardson, D.J., Berks, B.C., Russell, D.A., Spiro, S., Taylor, C.J., 2001. Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. Cell. Mol. Life Sci. 58, 165–178. doi:10.1007/PL00000845

Rifaat, H.M., Márialigeti, K., Kovács, G., 2000. Investigation on rhizoplane actinomycete communities of cattail (*Typha angustifolia*) from a Hungarian wetland. SUO 51, 197–203.

Risgaard-Petersen, N., Jensen, K., 1997. Nitrification and denitrification in the rhizosphere of the aquatic macrophyte Lobelia dortmanna L. Limnol. Ocean. 42, 529–537.

Rivett, M.O., Buss, S.R., Morgan, P., Smith, J.W.N., Bemment, C.D., 2008. Nitrate attenuation in groundwater: a review of biogeochemical controlling processes. Water Res. 42, 4215–32. doi:10.1016/j.watres.2008.07.020

Rivière, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., Li, T., Camacho, P., Sghir, A., 2009. Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. ISME J. 3, 700–714. doi:10.1038/ismej.2009.2

Rösch, C., Mergel, A., Bothe, H., 2002. Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. Appl. Environ. Microbiol. 68, 3818–29. doi:10.1128/AEM.68.8.3818-3829.2002

Röske, K., Sachse, R., Scheerer, C., Röske, I., 2012. Microbial diversity and composition of the sediment in the drinking water reservoir Saidenbach (Saxonia, Germany). Syst. Appl. Microbiol. 35, 35–44. doi:10.1016/J.SYAPM.2011.09.002

Rotthauwe, J.H., Witzel, K.P., Liesack, W., 1997. The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. 63, 4704–12.

Roussel-Delif, L., Tarnawski, S., Hamelin, J., Philippot, L., Aragno, M., Fromin, N., 2005. Frequency and diversity of nitrate reductase genes among nitrate-dissimilating *Pseudomonas* in the rhizosphere of perennial grasses grown in field conditions. Microb. Ecol. 49, 63–72. doi:10.1007/s00248-003-0228-3

Ruiz-Rueda, O., Hallin, S., Bañeras, L., 2009. Structure and function of denitrifying and nitrifying bacterial communities in relation to the plant species in a constructed wetland. FEMS Microbiol. Ecol. 67, 308–19. doi:10.1111/j.1574-6941.2008.00615.x

Ruiz-rueda, O., Trias, R., Garcia-gil, L.J., Bañeras, L., 2007. Diversity of the nitrite reductase gene nirS in the sediment of a free-water surface constructed wetland. Int. Microbiol. 10, 253–260. doi:10.2436/20.1501.01.34

Ruiz Rueda, O., 2008. Nitrifying and denitrifying bacterial communities in the sediment and rhizosphere of a free water surface constructed wetland. Universitat de Girona.

Rust, C.M., Aelion, C.M., Flora, J.R.V., 2000. Control of pH during denitrification in subsurface sediment microcosms using encapsulated phosphate buffer. Water Res. 34, 1447–1454. doi:10.1016/S0043-1354(99)00287-0

Rütting, T., Boeckx, P., Müller, C., Klemedtsson, L., 2011. Assessment of the importance of

dissimilatory nitrate reduction to ammonium for the terrestrial nitrogen cycle. Biogeosciences 8, 1779–1791. doi:10.5194/bg-8-1779-2011

Saarenheimo, J., Rissanen, A.J., Arvola, L., Nykänen, H., Lehmann, M.F., Tiirola, M., 2015a. Genetic and environmental controls on nitrous oxide accumulation in lakes. PLoS One. doi:10.1371/journal.pone.0121201

Saarenheimo, J., Tiirola, M.A., Rissanen, A.J., 2015b. Functional gene pyrosequencing reveals core proteobacterial denitrifiers in boreal lakes. Front. Microbiol. 6, 674. doi:10.3389/fmicb.2015.00674

Saleh-Lakha, S., Shannon, K.E., Henderson, S.L., Zebarth, B.J., Burton, D.L., Goyer, C., Trevors, J.T., 2009. Effect of nitrate and acetylene on *nirS*, *cnorB*, and *nosZ* expression and denitrification activity in *Pseudomonas mandelii*. Appl. Environ. Microbiol. 75, 5082–5087. doi:10.1128/AEM.00777-09

Salk, K.R., Steinman, A.D., Ostrom, N.E., 2018. Wetland restoration and hydrologic reconnection result in enhanced watershed nitrogen retention and removal. Wetlands 38, 349–359. doi:10.1007/s13157-017-0972-7

Sanford, R. a, Wagner, D.D., Wu, Q., Chee-Sanford, J.C., Thomas, S.H., Cruz-García, C., Rodríguez, G., Massol-Deyá, A., Krishnani, K.K., Ritalahti, K.M., Nissen, S., Konstantinidis, K.T., Löffler, F.E., 2012. Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. Proc. Natl. Acad. Sci. U. S. A. 109, 19709–14. doi:10.1073/pnas.1211238109

Santana, M.M., Gonzalez, J.M., Cruz, C., 2017. Nitric oxide accumulation: The evolutionary trigger for phytopathogenesis. Front. Microbiol. 8, 1947. doi:10.3389/fmicb.2017.01947

Santi, C., Bogusz, D., Franche, C., 2013. Biological nitrogen fixation in non-legume plants. Ann. Bot. 111, 743–767. doi:10.1093/aob/mct048

Santoro, A.E., Francis, C.A., De Sieyes, N.R., Boehm, A.B., 2008. Shifts in the relative abundance of ammonia-oxidizing bacteria and archaea across physicochemical gradients in a subterranean estuary. Environ. Microbiol. 10, 1068–1079. doi:10.1111/j.1462-2920.2007.01547.x

Santos, J.P., Mendes, D., Monteiro, M., Ribeiro, H., Baptista, M.S., Borges, M.T., Magalhães, C., 2018. Salinity impact on ammonia oxidizers activity and *amoA* expression in estuarine sediments. Estuar. Coast. Shelf Sci. 211, 177–187. doi:10.1016/J.ECSS.2017.09.001

Saunders, D.L., Kalff, J., 2001. Nitrogen retention in wetlands, lakes and rivers. Hydrobiologia 443, 205–212. doi:10.1023/A:1017506914063

Schleper, C., 2010. Ammonia oxidation: different niches for bacteria and archaea? ISME J. 4, 1092–1094. doi:10.1038/ismej.2010.111

Schloss, P.D., 2008. Evaluating different approaches that test whether microbial communities have the same structure. ISME J. 2, 265–275. doi:10.1038/ismej.2008.5

Schnobrich, M.R., Chaplin, B.P., Semmens, M.J., Novak, P.J., 2007. Stimulating hydrogenotrophic denitrification in simulated groundwater containing high dissolved oxygen and nitrate concentrations. Water Res. doi:10.1016/j.watres.2007.01.044

Schuster, S.C., 2008. Next-generation sequencing transforms today's biology. Nat. Methods

5, 16–18. doi:10.1038/nmeth1156

Scott, J.T., McCarthy, M.J., Gardner, W.S., Doyle, R.D., 2008. Denitrification, dissimilatory nitrate reduction to ammonium, and nitrogen fixation along a nitrate concentration gradient in a created freshwater wetland. Biogeochemistry 87, 99–111. doi:10.1007/s10533-007-9171-6

Seago, J.L., Peterson, C.A., Enstone, D.E., Scholey, C.A., 1999. Development of the endodermis and hypodermis of *Typha glauca* Godr. and *Typha angustifolia* L. roots. Can. J. Bot. Can. Bot. 77, 122–134. doi:10.1139/cjb-77-1-122

Seelig, B., Nowatzki, J., 2017. Our effect on nitrogen in the environment. North Dakota State University.

Seitzinger, S.P., Nielsen, L.P., Caffrey, J., Christensen, P.B., 1993. Denitrification measurements in aquatic sediments: A comparison of three methods. Biogeochemistry 23, 147–167. doi:10.1007/BF00023750

Shaw, L.J., Nicol, G.W., Smith, Z., Fear, J., Prosser, J.I., Baggs, E.M., 2006. *Nitrosospira* spp. can produce nitrous oxide via a nitrifier denitrification pathway. Environ. Microbiol. 8, 214–222. doi:10.1111/j.1462-2920.2005.00882.x

Shelef, O., Gross, A., Rachmilevitch, S., 2013. Role of plants in a constructed wetland: current and new perspectives. Water (Switzerland) 5, 405–419. doi:10.3390/w5020405

Shoun, H., Kano, M., Baba, I., Takaya, N., Matsuo, M., 1998. Denitrification by actinomycetes and purification of dissimilatory nitrite reductase and azurin from Streptomyces thioluteus. J. Bacteriol. 180, 4413–5.

Shrimali, M., Singh, K.., 2001. New methods of nitrate removal from water. Environ. Pollut. doi:10.1016/S0269-7491(00)00147-0

Si, Z., Song, X., Wang, Y., Cao, X., Zhao, Y., Wang, B., Chen, Y., Arefe, A., 2018. Intensified heterotrophic denitrification in constructed wetlands using four solid carbon sources: Denitrification efficiency and bacterial community structure. Bioresour. Technol. 267, 416–425. doi:10.1016/J.BIORTECH.2018.07.029

Simon, J., 2002. Enzymology and bioenergetics of respiratory nitrite ammonification. FEMS Microbiol. Rev. 26, 285–309.

Smith, C.J., Nedwell, D.B., Dong, L.F., Osborn, a M., 2007. Diversity and abundance of nitrate reductase genes (*narG* and *napA*), nitrite reductase genes (*nirS* and *nrfA*), and their transcripts in estuarine sediments. Appl. Environ. Microbiol. 73, 3612–22. doi:10.1128/AEM.02894-06

Smith, C.J., Osborn, a M., 2009. Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. FEMS Microbiol. Ecol. 67, 6–20. doi:10.1111/j.1574-6941.2008.00629.x

Smith, J.M., Ogram, A., 2008. Genetic and functional variation in denitrifier populations along a short-term restoration chronosequence. Appl. Environ. Microbiol. 74, 5615–5620. doi:10.1128/AEM.00349-08

Smits, A.J.M., Laan, P., Thier, R.H., van der Velde, G., 1990. Root aerenchyma, oxygen leakage patterns and alcoholic fermentation ability of the roots of some nymphaeid and isoetid macrophytes in relation to the sediment type of their habitat. Aquat. Bot. doi:10.1016/0304-3770(90)90095-3

Song, B., Lisa, J., Tobias, C., 2014. Linking DNRA community structure and activity in a shallow lagoonal estuarine system. Front. Microbiol. 5:460. doi:10.3389/fmicb.2014.00460

Sonthiphand, P., Hall, M.W., Neufeld, J.D., 2014. Biogeography of anaerobic ammonia-oxidizing (anammox) bacteria. Front. Microbiol. 5, 399. doi:10.3389/fmicb.2014.00399

Sorokin, D.Y., Lücker, S., Vejmelkova, D., Kostrikina, N.A., Kleerebezem, R., Rijpstra, W.I.C., Damsté, J.S.S., Le Paslier, D., Muyzer, G., Wagner, M., van Loosdrecht, M.C.M., Daims, H., 2012. Nitrification expanded: discovery, physiology and genomics of a nitrite-oxidizing bacterium from the phylum *Chloroflexi*. ISME J. 6, 2245–56. doi:10.1038/ismej.2012.70

Sorrell, B.K., Downes, M.T., Stanger, C.L., 2002. Methanotrophic bacteria and their activity on submerged aquatic macrophytes. Aquat. Bot. doi:10.1016/S0304-3770(01)00215-7

Spang, A., Hatzenpichler, R., Brochier-Armanet, C., Rattei, T., Tischler, P., Spieck, E., Streit, W., Stahl, D.A., Wagner, M., Schleper, C., 2010. Distinct gene set in two different lineages of ammonia-oxidizing archaea supports the phylum *Thaumarchaeota*. Trends Microbiol. 18, 331–340. doi:10.1016/J.TIM.2010.06.003

Šraj-Kržič, N., Pongrac, P., Klemenc, M., Kladnik, A., Regvar, M., Gaberščik, A., 2006. Mycorrhizal colonisation in plants from intermittent aquatic habitats. Aquat. Bot. doi:10.1016/j.aquabot.2006.07.001

Srivastava, J.K., Chandra, H., Kalra, S.J.S., Mishra, P., Khan, H., Yadav, P., 2017. Plant–microbe interaction in aquatic system and their role in the management of water quality: a review. Appl. Water Sci. 7, 1079–1090. doi:10.1007/s13201-016-0415-2

Stanley, Cunningham, Jones, 1998. Isolation of *Campylobacter jejuni* from groundwater. J. Appl. Microbiol. 85, 187–191. doi:10.1046/j.1365-2672.1998.00494.x

Stark, C.H., Richards, K.G., 2008. The continuing challenge of agricultural nitrogen loss to the environment in the context of global change and advancing research. Dyn. Soil, Dyn. Plant 2, 1–12.

Steingruber, S.M., Friedrich, J., Gächter, R., Wehrli, B., 2001. Measurement of denitrification in sediments with the 15N isotope pairing technique. Appl. Environ. Microbiol. 67, 3771–8. doi:10.1128/AEM.67.9.3771-3778.2001

Sterngren, A.E., Hallin, S., Bengtson, P., 2015. Archaeal ammonia oxidizers dominate in numbers, but *Bacteria* drive gross nitrification in N-amended grassland soil. Front. Microbiol. 6, 1350. doi:10.3389/fmicb.2015.01350

Stottmeister, U., Wießner, a., Kuschk, P., Kappelmeyer, U., Kästner, M., Bederski, O., Müller, R. a., Moormann, H., 2003. Effects of plants and microorganisms in constructed wetlands for wastewater treatment. Biotechnol. Adv. 22, 93–117. doi:10.1016/j.biotechadv.2003.08.010

Straub, K.L., Benz, M., Schink, B., Widdel, F., 1996. Anaerobic, nitrate-dependent microbial oxidation of ferrous iron. Appl. Environ. Microbiol. 62, 1458–60.

Stuart, M.E., Lapworth, D.J., 2016. Macronutrient status of UK groundwater: Nitrogen, phosphorus and organic carbon. Sci. Total Environ. 572, 1543–1560. doi:10.1016/J.SCITOTENV.2016.02.181

Sun, Y., De Vos, P., Willems, A., 2018. Influence of nitrate and nitrite concentration on N2

O production via dissimilatory nitrate/nitrite reduction to ammonium in *Bacillus paralicheniformis* LMG 6934. Microbiologyopen 7, e00592. doi:10.1002/mbo3.592

Sunger, N., Bose, P., 2009. Autotrophic denitrification using hydrogen generated from metallic iron corrosion. Bioresour. Technol. doi:10.1016/j.biortech.2009.03.008

Sutton, M., Howard, C., Erisman, J., 2011. The European nitrogen assessment: sources, effects and policy perspectives. Cambridge Univ. Press. doi:10.1017/CBO9780511976988

Takahashi, S., Tomita, J., Nishioka, K., Hisada, T., Nishijima, M., 2014. Development of a prokaryotic universal primer for simultaneous analysis of *Bacteria* and *Archaea* using next-generation sequencing. PLoS One 9, e105592. doi:10.1371/journal.pone.0105592

Tamaki, H., Sekiguchi, Y., Hanada, S., Nakamura, K., Nomura, N., Matsumura, M., Kamagata, Y., 2005. Comparative analysis of bacterial diversity in freshwater sediment of a shallow eutrophic lake by molecular and improved cultivation-based techniques. Appl. Environ. Microbiol. 71, 2162–9. doi:10.1128/AEM.71.4.2162-2169.2005

Thion, C.E., Poirel, J.D., Cornulier, T., De Vries, F.T., Bardgett, R.D., Prosser, J.I., 2016. Plant nitrogen-use strategy as a driver of rhizosphere archaeal and bacterial ammonia oxidiser abundance. FEMS Microbiol. Ecol. 92, 265–75. doi:10.1093/femsec/fiw091

Thompson, K.A., Deen, B., Dunfield, K.E., 2018. Impacts of surface-applied residues on N-cycling soil microbial communities in miscanthus and switchgrass cropping systems. Appl. Soil Ecol. 130, 79–83. doi:10.1016/J.APSOIL.2018.06.005

Throbäck, I.N., Enwall, K., Jarvis, A., Hallin, S., 2004. Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiol. Ecol. 49, 401–17. doi:10.1016/j.femsec.2004.04.011

Thullen, J.S., Sartoris, J.J., Walton, W.E., 2002. Effects of vegetation management in constructed wetland treatment cells on water quality and mosquito production. Ecol. Eng. 18, 441–457. doi:10.1016/S0925-8574(01)00105-7

Tiedje, J.M., 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium. Environ. Microbiol. Anaerobes 179–244.

Till, B.A., Weathers, L.J., Alvarez, P.J.J., 1998. Fe(0)-Supported autotrophic denitrification. Environ. Sci. Technol. 32, 634–639. doi:10.1021/ES9707769

Torrentó, C., Urmeneta, J., Otero, N., Soler, A., Viñas, M., Cama, J., 2011. Enhanced denitrification in groundwater and sediments from a nitrate-contaminated aquifer after addition of pyrite. Chem. Geol. 287, 90–101. doi:10.1016/j.chemgeo.2011.06.002

Trias, R., Ruiz-Rueda, O., Garcia-Lledo, A., Vilar-Sanz, A., Lopez-Flores, R., Quintana, X.D., Hallin, S., Baneras, L., 2012. Emergent macrophytes act selectively on ammonia-oxidizing Bacteria and Archaea. Appl. Environ. Microbiol. 78, 6352–6356.

Trüper, H.G., Schlegel, H.G., 1964. Sulphur metabolism in Thiorhodaceae I. Quantitative measurements on growing cells of *Chromatium okenii*. Antonie Van Leeuwenhoek 30, 225–238. doi:10.1007/BF02046728

Truu, M., Juhanson, J., Truu, J., 2009. Microbial biomass, activity and community composition in constructed wetlands. Sci. Total Environ. 407, 3958–71. doi:10.1016/j.scitotenv.2008.11.036

Ueckert, J., Hurek, T., Fendrik, I., Niemann, E.G., 1990. Radial gas diffusion from roots of rice (*Oryza sativa* L.) and Kallar grass (*Leptochloa fusca* L. Kunth), and effects of inoculation with *Azospirillum brasilense*. Plant Soil. doi:10.1007/BF02851910

Urakawa, H., Bernhard, A.E., 2017. Wetland management using microbial indicators. Ecol. Eng. 108, 456–476. doi:10.1016/j.ecoleng.2017.07.022

Van Der Zaan, B., Smidt, H., De Vos, W.M., Rijnaarts, H., Gerritse, J., 2010. Stability of the total and functional microbial communities in river sediment mesocosms exposed to anthropogenic disturbances. FEMS Microbiol. Ecol. 74, 72–82. doi:10.1111/j.1574-6941.2010.00931.x

van Kessel, M.A.H.J., Speth, D.R., Albertsen, M., Nielsen, P.H., Op den Camp, H.J.M., Kartal, B., Jetten, M.S.M., Lücker, S., 2015. Complete nitrification by a single microorganism. Nature 528, 555. doi:10.1038/nature16459

van Oostrom, A.J., Russell, J.M., 1994. Denitrification in constructed wastewater wetlands receiving high concentrations of nitrate. Water Sci. Technol. 29, 7–14.

Verbaendert, I., Hoefman, S., Boeckx, P., Boon, N., De Vos, P., 2014. Primers for overlooked nirK, qnorB, and nosZ genes of thermophilic Gram-positive denitrifiers. FEMS Microbiol. Ecol. 89, 162–180. doi:10.1111/1574-6941.12346

Vidal-Gavilan, G., Folch, a., Otero, N., Solanas, a. M., Soler, a., 2013. Isotope characterization of an in situ biodenitrification pilot-test in a fractured aquifer. Appl. Geochemistry 32, 153–163. doi:10.1016/j.apgeochem.2012.10.033

Vitòria, L., Soler, A., Canals, À., Otero, N., 2008. Environmental isotopes (N, S, C, O, D) to determine natural attenuation processes in nitrate contaminated waters: Example of Osona (NE Spain). Appl. Geochemistry. doi:10.1016/j.apgeochem.2008.07.018

Vitousek, P.M., Aber, J.D., Howarth, R.W., Likens, G.E., Matson, P.A., Schindler, D.W., Schlesinger, W.H., Tilman, D.G., 1997. Human alteration of the global nitrogen cycle: Sources and consequences. Ecol. Appl. doi:10.1890/1051-0761(1997)007[0737:HAOTGN]2.0.CO;2

Vymazal, J., 2011. Constructed wetlands for wastewater treatment: five decades of experience. Environ. Sci. Technol. 45, 61–69. doi:10.1021/es101403q

Vymazal, J., 2007. Removal of nutrients in various types of constructed wetlands. Sci. Total Environ. 380, 48–65. doi:10.1016/j.scitotenv.2006.09.014

Wang, Q., Hu, Y., Xie, H., Yang, Z., Wang, Q., Hu, Y., Xie, H., Yang, Z., 2018. Constructed wetlands: a review on the role of Radial Oxygen Loss in the rhizosphere by macrophytes. Water 10, 678. doi:10.3390/w10060678

Wang, Y., Sheng, H.-F., He, Y., Wu, J.-Y., Jiang, Y.-X., Tam, N.F.-Y., Zhou, H.-W., 2012. Comparison of the levels of bacterial diversity in freshwater, intertidal wetland, and marine sediments by using millions of illumina tags. Appl. Environ. Microbiol. 78, 8264–71. doi:10.1128/AEM.01821-12

Weber, K. a, Pollock, J., Cole, K. a, Connor, S.M.O., Achenbach, L. a, Coates, J.D., 2006. Anaerobic nitrate-dependent iron (II) bio-oxidation by a novel lithoautotrophic betaproteobacterium, strain 2002. Society 72, 686–694. doi:10.1128/AEM.72.1.686

Wei, B., Yu, X., Zhang, S., Gu, L., 2011. Comparison of the community structures of ammonia-oxidizing bacteria and archaea in rhizoplanes of floating aquatic macrophytes.

Microbiol. Res. 166, 468–474. doi:10.1016/J.MICRES.2010.09.001

Wei, W., Isobe, K., Nishizawa, T., Zhu, L., Shiratori, Y., Ohte, N., Koba, K., Otsuka, S., Senoo, K., 2015. Higher diversity and abundance of denitrifying microorganisms in environments than considered previously. ISME J. 9, 1954–1965. doi:10.1038/ismej.2015.9

Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697–703.

Welsh, A., Chee-Sanford, J.C., Connor, L.M., Löffler, F.E., Sanford, R. a, 2014. Refined NrfA phylogeny improves PCR-based *nrfA* gene detection. Appl. Environ. Microbiol. 80, 2110–9. doi:10.1128/AEM.03443-13

Wenk, C.B., Blees, J., Zopfi, J., Veronesi, M., Bourbonnais, A., Schubert, C.J., Niemann, H., Lehmann, M.F., 2013. Anaerobic ammonium oxidation (anammox) bacteria and sulfide-dependent denitrifiers coexist in the water column of a meromictic south-alpine lake. Limnol. Oceanogr. 58, 1–12. doi:10.4319/lo.2013.58.1.0001

Weyens, N., van der Lelie, D., Taghavi, S., Vangronsveld, J., 2009. Phytoremediation: plant-endophyte partnerships take the challenge. Curr. Opin. Biotechnol.

Wießner, A., Kuschk, P., Kästner, M., Stottmeister, U., 2002. Abilities of helophyte species to release oxygen into rhizospheres with varying redox conditions in laboratory-scale hydroponic systems. Int. J. Phytoremediation. doi:10.1080/15226510208500069

Williamson, W.M., Close, M.E., Leonard, M.M., Webber, J.B., Lin, S., 2012. Groundwater biofilm dynamics grown in situ along a nutrient gradient. Ground Water. doi:10.1111/j.1745-6584.2011.00904.x

Wilson, W.S., Ball, A.S., Hinton, R.H., 1999. Managing risks of nitrates to humans and the environment. Royal Society of Chemistry.

Winneberger, J.H.T., 1981. Nitrogen, public health, and the environment: some tools for critical thought, in: National Individual Onsite Wastewater Systems Conference. Ann Arbor Science Publishers,Ann Arbor, MI, USA, United States.

Winogradsky, S., 1892. Contributions à la morphologie des organismes de la nitrification. Arch. Sci. Biol. 1, 88–137.

Wittorf, L., Jones, C.M., Bonilla-Rosso, G., Hallin, S., 2018a. Expression of nirK and nirS genes in two strains of Pseudomonas stutzeri harbouring both types of NO-forming nitrite reductases. Res. Microbiol. 169, 343–347. doi:10.1016/J.RESMIC.2018.04.010

Wittorf, L., Jones, C.M., Bonilla-Rosso, G., Hallin, S., 2018b. Expression of *nirK* and *nirS* genes in two strains of *Pseudomonas stutzeri* harbouring both types of NO-forming nitrite reductases. Res. Microbiol. 169, 343–347. doi:10.1016/J.RESMIC.2018.04.010

Wood, D.W., 2001. The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. Science (80-.). 294, 2317–2323. doi:10.1126/science.1066804

Wrage-Mönnig, N., Horn, M.A., Well, R., Müller, C., Velthof, G., Oenema, O., 2018. The role of nitrifier denitrification in the production of nitrous oxide revisited. Soil Biol. Biochem. 123, A3–A16. doi:10.1016/J.SOILBIO.2018.03.020

Wright, A.L., Ramesh Reddy, K., Newman, S., 2009. Microbial Indicators of Eutrophication in Everglades Wetlands. Soil Sci. Soc. Am. J. doi:10.2136/sssaj2009.0083

Wu, S., Kuschk, P., Brix, H., Vymazal, J., Dong, R., 2014. Development of constructed wetlands in performance intensifications for wastewater treatment: A nitrogen and organic matter targeted review. Water Res. 57, 40–55. doi:10.1016/J.WATRES.2014.03.020

Xiao, H., Schaefer, D.A., Yang, X., 2017. pH drives ammonia oxidizing bacteria rather than archaea thereby stimulate nitrification under *Ageratina adenophora* colonization. Soil Biol. Biochem. 114, 12–19. doi:10.1016/J.SOILBIO.2017.06.024

Yin, Z., Bi, X., Xu, C., 2018. Ammonia-Oxidizing *Archaea* (AOA) play with Ammonia-Oxidizing *Bacteria* (AOB) in nitrogen removal from wastewater. Archaea 2018, 1–9. doi:10.1155/2018/8429145

Yunfu, G., Wang, Y., Xiang, Q., Yu, X., Zhao, K., Zhang, X., Lindström, K., Yufu, H., Liu, S., 2017. Implications of wetland degradation for the potential denitrifying activity and bacterial populations with *nirS* genes as found in a succession in Qinghai-Tibet plateau, China. Eur. J. Soil Biol. 80, 19–26. doi:10.1016/J.EJSOBI.2017.03.005

Zehr, J.P., Jenkins, B.D., Short, S.M., Steward, G.F., 2003. Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Environ. Microbiol. 5, 539–54.

Zhang, J., Liu, B., Zhou, X., Chu, J., Li, Y., Wang, M., 2015. Effects of emergent aquatic plants on abundance and community structure of ammonia-oxidising microorganisms. Ecol. Eng. 81, 504–513. doi:10.1016/J.ECOLENG.2015.04.029

Zhang, J., Wang, Q., Fan, J., Xie, H., Liu, C., Liang, S., Hu, Z., Yang, Z., Zhao, C., 2015. Comparisons of microbial abundance and community among different plant species in constructed wetlands in summer. Ecol. Eng. 82, 376–380.

Zhang, T.C., Lampe, D.G., 1999. Sulfur:limestone autotrophic denitrification processes for treatment of nitrate-contaminated water: Batch experiments. Water Res. doi:10.1016/S0043-1354(98)00281-4

Zhang, W., Wu, X., Liu, G., Chen, T., Zhang, G., Dong, Z., Yang, X., Hu, P., 2013. Pyrosequencing Reveals Bacterial Diversity in the Rhizosphere of Three Phragmites australis Ecotypes. Geomicrobiol. J. doi:10.1080/01490451.2012.740145

Zhang, Y., Xu, J., Riera, N., Jin, T., Li, J., Wang, N., 2017. Huanglongbing impairs the rhizosphere-to-rhizoplane enrichment process of the citrus root-associated microbiome. Microbiome 5, 97. doi:10.1186/s40168-017-0304-4

Zheng, Y., Dzakpasu, M., Wang, X., Zhang, L., Ngo, H.H., Guo, W., Zhao, Y., 2018. Molecular characterization of long-term impacts of macrophytes harvest management in constructed wetlands. Bioresour. Technol. 268, 514–522. doi:10.1016/j.biortech.2018.08.030

Zhou, X., Zhang, J., Li, Y., Liu, B., Chu, J., Wang, M., He, Z., 2016. Distribution characteristics of ammonia oxidizing microorganisms in rhizosphere sediments of cattail. Ecol. Eng. 88, 99–111. doi:10.1016/J.ECOLENG.2015.12.023

Zhou, Z., Meng, H., Liu, Y., Gu, J.-D., Li, M., 2017. Stratified bacterial and archaeal community in mangrove and intertidal wetland mudflats revealed by high throughput 16S rRNA gene sequencing. Front. Microbiol. 8, 2148. doi:10.3389/fmicb.2017.02148

Zumft, W.G., 1997. Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. 61, 533–582.

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