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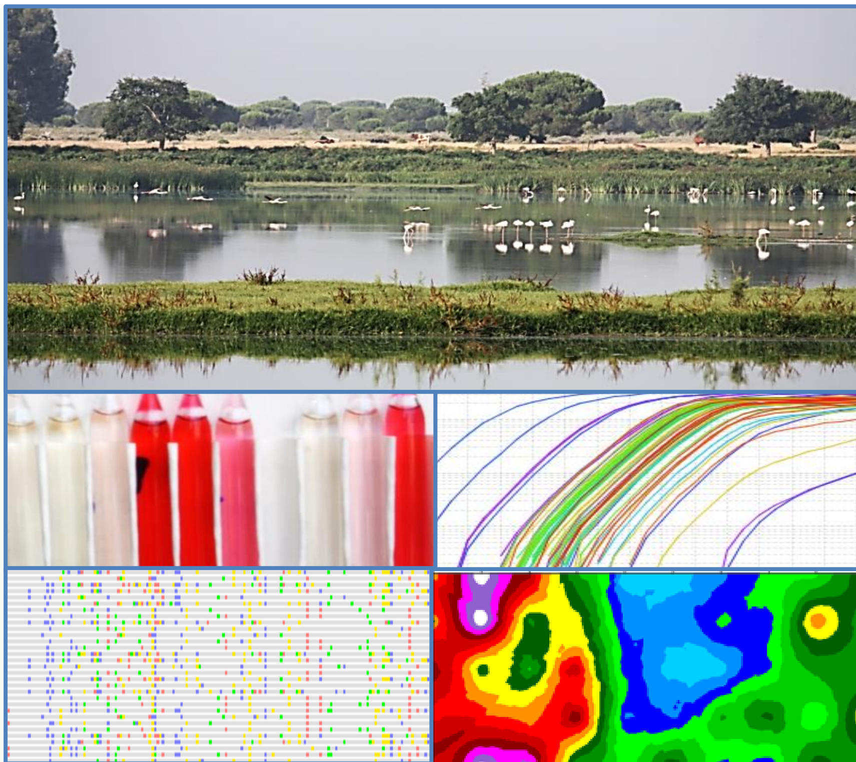
UNIVERSIDAD DE GRANADA
PROGRAMA OFICIAL DE POSGRADO EN MICROBIOLOGÍA

CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS
ESTACIÓN EXPERIMENTAL DEL ZAIDÍN

BIODIVERSIDAD Y ECOLOGÍA FUNCIONAL DE BACTERIAS DESNITRIFICANTES



TESIS DOCTORAL

DAVID CORREA GALEOTE

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DAVID CORREA GALEOTE
GRANADA, 2016

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Memoria presentada por David Correa Galeote,
licenciado en Farmacia, para optar al grado de doctor

Fdo: David Correa Galeote

Vº Bº del director de tesis

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Doctor en Ciencias Biológicas
Profesor de Investigación del CSIC

Granada, enero de 2016

Imagine Carl Linnaeus in Alice's shoes, shrinking to only 10 micrometers high.
Afforded the opportunity to investigate biological diversity at this spatial scale,
would Linnaeus have remained committed to plant exploration,
or would he have turned his attention to microbial life?

Jessica L. Green, Brendan J. M. Bohannan and Rachel J. Whitaker
(Microbial Biogeography: From Taxonomy to Traits. Science 320, 1039 2008)

A mi padre y a mi madre, gracias.

AGRADECIMIENTOS

Una vez más darles las gracias a mis padres por todo lo que hacen por mí cada día.

También darle las gracias al resto de mi familia,

mis hermanos y mis sobrinos. A mis abuelos Paco y Ángeles.

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y por ayudarme a que por fin pueda estar escribiendo estas líneas.

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otros aspectos del ciclo del N.

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El apartado 4 del artículo 18 del capítulo 1 de las Normas Reguladoras de las Enseñanzas Oficiales de Doctorado y del Título de Doctor por la Universidad de Granada establece que: “Una tesis doctoral puede también consistir en el reagrupamiento en una memoria de trabajos de investigación publicados por el doctorando en medios científicos relevantes en su ámbito de conocimiento”. De acuerdo con la normativa mencionada, esta Memoria de Doctorado se presenta como reagrupamiento de la investigación realizada en trabajos de investigación.

Para concurrir a la mención internacional de la tesis, la Memoria de Doctorado cumple el artículo 19 del mencionado capítulo 1 de las Normas Reguladoras de las Enseñanzas Oficiales de Doctorado y del Título de Doctor por la Universidad de Granada. De acuerdo con ello, se presenta la siguiente documentación:

1. Resumen. En castellano
2. Resumen. En inglés
3. Introducción. En castellano
4. Objetivos. En castellano
5. Trabajos publicados.
6. Discusión general. En castellano
6. Conclusiones. En castellano
7. Conclusiones. En inglés
8. Bibliografía general.

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RESUMEN

La contaminación por nitratos (NO_3^-) en el medio ambiente es cada vez más frecuente e intensa. El nitrógeno (N) es el elemento más abundante en la atmósfera aunque no puede utilizarse directamente por las plantas y animales. Las bacterias fijadoras de dinitrógeno (N_2) atmosférico son los únicos seres vivos capaces de reducirlo a amonio, lo que inicia su uso en la naturaleza. Desde la revolución verde a finales de los años 60, el ciclo del N se ha alterado gravemente por el exceso de NO_3^- procedente de los fertilizantes nitrogenados que, junto con el procedente de los purines del ganado y las aguas residuales e industriales, se acumula en la corteza terrestre. Consecuentemente, se produce una contaminación por nitratos de suelos, sedimentos y aguas, lo que supone una seria amenaza para la salud animal y humana. La desnitrificación es el único proceso biológico por el que los nitratos pueden eliminarse, evitando así su acumulación. Este proceso es un mecanismo alternativo de respiración por el que, en condiciones limitantes de oxígeno, el nitrato se reduce secuencialmente a nitrito (NO_2^-), óxido nítrico (NO), óxido nitroso (N_2O) y, finalmente, a dinitrógeno molecular (N_2), que se libera de nuevo a la atmósfera completándose así el ciclo del N. Estas reacciones son catalizadas por las enzimas nitrato reductasa, nitrito reductasa, óxido nítrico reductasa y óxido nitroso reductasa, codificadas por los genes *narG*/*napA*, *nirK*/*nirS*, c-*nor*/q-*nor* y *nosZ*, respectivamente. Paradójicamente, en este proceso se producen los intermediarios NO y N_2O que son importantes gases de efecto invernadero, por lo que su formación durante la desnitrificación supone una fuente de contaminación atmosférica.

Mientras que los aspectos fisiológicos, bioquímicos y genéticos de la desnitrificación se conocen en profundidad, apenas existe información sobre la abundancia o escasez de las distintas poblaciones desnitrificantes en diferentes hábitats, las bacterias desnitrificantes predominantes en un ecosistema concreto, y cómo la actividad funcional de la comunidad varía en el tiempo. Igualmente, se desconoce el efecto del contenido en nitratos sobre la abundancia, biodiversidad, distribución espacial y actividad funcional de la comunidad desnitrificante. Para ello, utilizando como modelo el Espacio Natural de Doñana (END), se analizó el contenido en nitratos, actividad desnitrificante, propiedades físico-químicas, abundancia relativa y biodiversidad de las poblaciones desnitrificantes. El estudio se prolongó en los años

2008, 2009 y 2010 y se tomaron muestras en los meses de abril y octubre que corresponden a las fechas de menor y mayor pluviosidad, respectivamente.

Los resultados de esta Memoria de Doctorado indican la existencia de contaminación por nitratos en los sedimentos del END, con valores de concentración superiores a los máximos establecidos por las autoridades españolas y europeas. Está contaminación presenta variaciones espacio-temporales que podrían deberse a cambios en la dinámica hídrica de los sedimentos del END. El estudio isotópico de los nitratos reveló su origen antrópico y podría deberse a las prácticas agrícolas que se realizan en el ecotono del END.

La abundancia relativa de las distintas poblaciones desnitrificantes se estimó en la laguna del Acebrón y en el arroyo de la Cañada, sitios con menor y mayor contenido en nitratos, respectivamente. Para ello, se determinó el número de copias de los genes *narG*, *napA*, *nirK*, *nirS* y *nosZ* mediante qPCR. En general, las poblaciones desnitrificantes fueron más abundantes en el sitio con mayor contenido en nitratos y fueron superiores en el mes de octubre. Se puede concluir, por tanto, la existencia de variaciones espacio-temporales en la comunidad desnitrificante producida por variaciones en la concentración de nitratos.

Para analizar el efecto del nitrato en la biodiversidad de la comunidad desnitrificante se construyeron genotecas del gen *nosZ* a partir del ADN aislado de los sedimentos. La biodiversidad, analizada como número de OTUs fue mayor en los sedimentos de octubre del arroyo de la Cañada. Junto a un aumento en la diversidad, se observa también una mayor homogeneidad en el número de individuos que conforman los distintos OTUs.

La actividad desnitrificante en la laguna del Acebrón fue similar entre las fechas de muestreo y, por el contrario, en el arroyo de la Cañada fue superior en el mes de abril. Aunque la concentración de nitratos en el mes de octubre en arroyo de la Cañada fue superior a la del mes de abril, la actividad desnitrificante fue menor. Es posible que este desajuste entre contenido en nitratos y actividad desnitrificante se deba a la disminución del caudal del arroyo, lo que resultaría en un aumento de la tensión de oxígeno en los sedimentos, y daría lugar, a su vez, a la inhibición de esta actividad.

Se ha demostrado la existencia de correlación entre el contenido en nitratos y la abundancia relativa de las poblaciones desnitrificantes. El análisis estadístico realizado indica que el gen *nosZ* es el que presenta una correlación más fuerte con el contenido en nitratos. Por otra parte, se ha puesto en evidencia la relación entre la diversidad de bacterias desnitrificantes de las muestras de sedimentos y el contenido en nitratos.

En contraste con otros estudios, nuestros datos indican que la actividad desnitrificante es independiente del contenido en nitratos y de la abundancia de la comunidad desnitrificante.

Finalmente se llevó a cabo un estudio sobre la distribución espacial de las poblaciones desnitrificantes. Se ha utilizado para ello el humedal artificial de los Guayules, balsa que se construyó con la finalidad de depurar las aguas contaminadas con nitratos que procedentes de las zonas agrícolas que rodean el END llegan a la marisma del Rocío. Este trabajo fue pionero en el análisis de la distribución espacial de comunidades desnitrificantes en humedales de agua dulce y puso de manifiesto que su análisis debe realizarse a espacios inferiores a los 5 m que se emplearon en nuestro estudio, como así lo mostraron los mapas de Krige que se elaboraron para las variables contenido en nitratos, actividad desnitrificante y abundancia relativa de las genes *narG*, *napA*, *nirK*, *nirS*, y *nosZ*. Los valores de autocorrelación obtenidos mediante el análisis de semivarianza de cada uno de los genes de la desnitrificación demostraron que el gen *nosZ* es el que mejor ajuste estadístico posee y podría explicar de forma efectiva la distribución de la comunidad desnitrificante.

Considerados el conjunto de resultados obtenidos, se propone el gen *nosZ* como marcador molecular para el estudio metagenómico y de la ecología funcional de la comunidad desnitrificante en sedimentos contaminados con nitratos.

SUMMARY

The contamination due to nitrates (NO_3^-) in the environment is becoming more frequent and intense. The nitrogen (N) is the more abundant element in the atmosphere but it cannot be used directly by plants and animals. The bacteria able to fix dinitrogen (N_2) are the only organism that can reduce it to ammonium which starts their use in the environment. Since the green revolution in the late 60s, the N-cycle has been seriously disrupted by the excessive NO_3^- from nitrogen fertilizers, together with manure from livestock, waste and industrial waters, which accumulates in the Earth's crust. Consequently, nitrate contamination occurs in soils, sediments and water that represent a serious threat to animal and human health. Denitrification is the only biological process where nitrates can be removed preventing their accumulation. This process is an alternative mechanism of respiration where under limited oxygen conditions the nitrates are sequentially reduced to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O) and finally, to molecular dinitrogen (N_2), that is release to the atmosphere completing the N-cycle. These reactions are catalysed by the nitrate-, nitrite-, nitric oxide and nitrous oxide enzymes which are codify by the *narG*/*napA*, *nirK*/*nirS*, c-*nor*/q-*nor* and *nosZ* genes, respectively. Paradoxically, in this process the two intermediaries NO and N_2O are important greenhouse gases that are produced so their formation during denitrification is a source of air pollution.

The physiological, biochemical and genetic aspects of denitrification are well known. However information about the abundance or shortage of different denitrifying populations in different habitats, dominant denitrifying bacteria in a particular ecosystem and how functional activity community varies over time is scarce. Similarly, the effect of nitrate content on the abundance, biodiversity, spatial distribution and functional activity of the denitrifying community is unknown. For that using as a model the Doñana Natural Area (END) the nitrate content, denitrifying activity, physicochemical properties, relative abundance and biodiversity of denitrifying populations were analyzed. The study was made in the years 2008, 2009 and 2010 and samples were taken in the months of April and October which respectively corresponding to lower and higher rainfall season.

The results of this thesis indicate the existence of nitrate contamination in sediments from the END, in concentrations greater than the maximum concentration established by the Spanish and European authorities. This contamination presents

spatial and temporal variations that could be produced by changes in water dynamics of the END sediments. The isotopic study of nitrates shows an anthropogenic origin of them which could be due to agricultural practices carried out in the ecotono of the END.

The relative abundance of the different denitrifying populations were determined in the Acebrón lagoon and Cañada creek, sampling sites with lower and higher nitrate content, respectively. For that the copy numbers of the genes *narG*, *napA*, *nirK*, *nirS* and *nosZ* was estimated by qPCR. In general, the denitrifying populations were more abundant in the site with higher nitrate content and it also was higher in October. Therefore there are spatial and temporal variations in the denitrifying community that could be caused by variations in nitrate concentrations.

Eight clone libraries of *nosZ* gene were constructed from DNA isolated from the sediments for analyze the effect of nitrate in the biodiversity of the denitrifying community. Biodiversity, analyzed as OTUs number, was higher in the sediments taken in October at the Cañada creek. A greater uniformity in the number of sequences within the OTUs obtained was also observed in these clone libraries.

The denitrifying activity in the Acebrón lagoon was similar between sampling moths, however a higher activity was observed in April at Cañada creek. Although the nitrate contents in October were higher than that for April at Cañada creek, the denitrifying activity was lower. It is possible that this mismatch between nitrate content and denitrifying activity could be due to the decrease in the stream flow observed in October that increased the oxygen concentration in the sediments that would produce an inhibition of this activity.

It has been demonstrated a significant correlation between the nitrate content and relative abundance of denitrifying populations. According to the statistical analysis indicates that the *nosZ* gene has a stronger correlation with nitrate content than the other genes. Also a relationship between diversity of denitrifying bacteria in the sediment samples and nitrate content were observed.

Although other studies indicates a relationship between denitrifying activity and, both nitrate content and abundance of the denitrifying community the values of

denitrifying activity were not correlated with the nitrate content neither with the relative abundance of the denitrification genes.

Finally, a study on the spatial distribution of denitrifying populations was made in the constructed wetland of los Guayules. This wetland was built for purify nitrate contaminated waters, that from agricultural areas surrounding the END, could reach the Rocío marsch. This work was pioneered in the analysis of the spatial distribution of denitrifying communities in freshwater wetlands and revealed that this analysis should be made using lower spaces than 5 m, spaces that were used in our study, as is showed by the Krige maps constructed from the variables of nitrate content, denitrifying activity and relative abundance *narG*, *napA*, *nirK*, *nirS* y *nosZ* gene. The autocorrelation values obtained by the analysis of semi-variance of each of the denitrification genes showed that the *nosZ* gene is the gene with the best statistical fit and could explain more effectively the distribution of the denitrifying community.

Taken together our results, the *nosZ* gene is proposed as molecular marker to study metagenomics and functional ecology of denitrifying community in sediments contaminated with nitrates.

INTRODUCCIÓN

1. El ciclo del N en la naturaleza

El nitrógeno (N) es un elemento esencial para todos los seres vivos porque forma parte de compuestos esenciales como son proteínas, ácidos nucleicos, hormonas, etc. Es, además, el cuarto elemento más abundante en la biomasa después del carbono, el hidrógeno y el oxígeno. Aunque es el elemento más abundante en la atmósfera, ya que está presente en un 78%, se encuentra en un estado químico biológicamente inerte para los seres eucariotas y para muchos procariotas. De ahí, que sea el nutriente limitante más importante para el crecimiento vegetal y, de hecho, la producción primaria en las cadenas tróficas suele estar limitada por su disponibilidad (Munch y Velthof, 2006).

El ciclo biogeoquímico del N se inicia con la transformación del nitrógeno molecular (N_2) hasta amonio biodisponible (NH_4^+) (Figura 1). Este proceso se lleva a cabo por los llamados microorganismos diazotrofos, principalmente bacterias, y se le denomina fijación biológica del nitrógeno (FBN). Los diazotrofos contienen en su genoma los genes *nif* que codifican la nitrogenasa, una enzima capaz de romper el triple enlace covalente del N_2. Las bacterias diazotrofas pueden reducir el N_2 tanto en vida libre como en simbiosis más o menos estrictas con plantas superiores mediante la formación de nódulos en las raíces, a veces en los tallos y hojas, de las plantas que infectan (ver revisiones de Liu et al. 2010; Reed et al. 2011; Ramírez-Bahena et al. 2013).

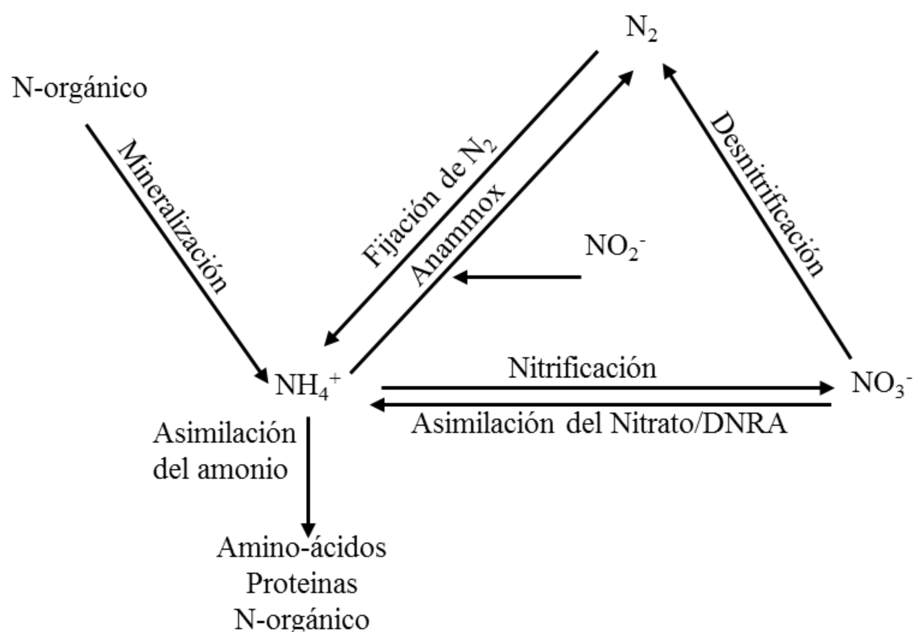

Figura 1. El ciclo biológico del N. Adaptado de Correa-Galeote et al. (2014a).

El amonio procedente de la FBN se incorpora, posteriormente, a la biomasa celular mediante su asimilación (Figura 1), que se produce, de forma mayoritaria, mediante la vía glutamina sintetasa (GS)-glutamato sintasa (GOGAT). Alternativamente, cuando la concentración de amonio es elevada, la enzima glutamato deshidrogenasa (GDH) interviene en la asimilación aeróbica del amonio (ver revisión de Moreno-Vivián et al. 2011). Además de su asimilación, el amonio puede oxidarse a nitrato (NO_3^-) mediante un proceso de dos etapas denominado nitrificación (Figura 1). En la primera etapa, el amonio se oxida a nitrito (NO_2^-), vía hidroxilamina (NO_2OH) por la enzima amonio monooxigenasa (Amo). Esta enzima está presente en géneros del dominio Bacteria (ammonia oxidizing bacteria, AOB), como *Nitrosomonas* y *Nitrosococcus*, y del dominio Archaea (ammonia oxidizing archaea, AOA), como *Nitrosopumilus*, *Nitrososphaera* y *Crenarchaeum*. En la segunda etapa, el nitrito formado se reduce a nitrato por la nitrito oxidoreductasa (nitrito oxidasa), enzima presente en géneros bacterianos como *Nitrobacter*, *Nitrococcus*, *Nitrospina* y *Nitrospira* (Mota et al. 2005). Para profundizar en los aspectos bioquímicos de la nitrificación se pueden consultar las revisiones de Ferguson et al. (2007) y Ward et al. (2011).

El nitrato procedente de la nitrificación puede ser reducido por la enzima nitrato reductasa asimilatoria (Nas) a NO_2^- que a su vez, puede convertirse por la enzima nitrito reductasa (Nir) en NH_4^+ que, posteriormente, es asimilado mediante la vía GS-GOGAT (Figura 1). Este proceso está muy extendido en la naturaleza y se lleva a cabo por plantas, hongos, algas y bacterias que utilizan el nitrato como fuente de N para su crecimiento (ver revisiones de Braker y Conrad, 2011; Baggs y Philippot, 2011). Además de la asimilación del nitrato, el proceso denominado amonificación del nitrato o reducción desasimilatoria del nitrato (DNRA) puede, a su vez, convertir el NO_3^- en NO_2^- mediante una de las enzimas nitrato reductasa respiratorias (Nar/Nap); posteriormente el NO_2^- se transforma en NH_4^+ por la acción de la enzima citocromo *c* nitrito reductasa (Nrf) (Mohan y Cole 2007). Finalmente, la desnitrificación es el proceso por el cual el NO_3^- se reduce a N_2, el cual retorna a la atmósfera, cerrándose así el ciclo del N en la naturaleza (ver más abajo).

Además de la desnitrificación, la oxidación anaeróbica del amonio en condiciones anóxicas transforma el NH_4^+ en N_2 empleando NO_2^- como aceptor de electrones. A este proceso se le denomina anammox (anaerobic ammonia oxidation) y las bacterias que lo realizan pertenecen, hasta la fecha, al phylum *Planctomycetes*, entre ellas los géneros *candidatus* Brocadia anammoxidans (Strous et al. 1999), *candidatus* Scalindua sorokinii (Kuypers et al. 2003), *candidatus* Kuenenia stuttgartiensis (Schmid et al. 2000), *canditatus*

Anammoxoglobus propionicus (Kartal et al. 2007) y *candidatus* Jettenia asiática (Quan et al. 2008). Para una revisión sobre anammox ver van Niftrik y Jetten (2012) y Ding et al. (2013).

La secuenciación del genoma de diferentes microorganismos que intervienen en el ciclo del N ha demostrado la existencia de bacterias que emplean nitrito para oxidar metano en condiciones anaeróbicas (Raghoebarsing et al. 2006; Hu et al. 2014), de bacterias capaces de desnitrificar empleando azufre (S) como donador de electrones (Bezbaruah y Zhang, 2003; Chen et al. 2014) y de arqueas hipertermófilas productoras de metano que fijan N_2 (Mehta y Baross, 2006). Estos ejemplos indican la existencia de una amplia biodiversidad y de nuevas capacidades metabólicas dentro del ciclo del N (Jetten, 2008).

Juntos, todos estos procesos conforman el ciclo global del N en la biosfera, en el que los microorganismos son esenciales para el mantenimiento del balance entre las formas reducidas y oxidadas del N (Richardson, 2011).

Para una revisión sobre la fisiología, bioquímica, genética y ecología de los microorganismos que intervienen en el ciclo del N se pueden consultar los trabajos incluidos en los libros Biology of the nitrogen cycle (editado por H. Bothe, S. J. Ferguson y W. E. Newton, 2007) y Nitrogen cycling in bacteria: molecular analysis (editado por J. W. B. Moir, 2011).

2. Desnitrificación

La desnitrificación es una forma alternativa de respiración en condiciones limitantes de oxígeno por la que los microorganismos reducen de forma secuencial el nitrato (NO_3^-) y/o nitrito (NO_2^-) a N_2 vía la producción como intermediarios el óxido nítrico (NO) y óxido nitroso (NO_2), de acuerdo a la siguiente secuencia de reacciones:

$$NO_3^- \rightarrow NO_2 \rightarrow {}^-NO \rightarrow N_2O \rightarrow N_2$$

La respiración del nitrato produce ATP ya que la nitrato reductasa y la óxido nítrico reductasa son enzimas integrales de membrana que acoplan la translocación de protones a la reducción del nitrato y del óxido nítrico (Figura 2). No obstante, la desnitrificación rinde menos ATP que la respiración oxigénica, pero es suficiente para permitir el crecimiento de las bacteria que lo realizan (Zumft, 1997; Simon et al. 2008).

Aunque durante más de 100 años se creyó que la desnitrificación solo se podía realizar por los miembros del dominio Bacteria, se ha demostrado que algunas arqueas (Philippot, 2002, Hayatsu et al. 2008), hongos (Takaya, 2002, Prendergast-Miller et al. 2011; Maeda et

al. 2015), protistas de la clase *Foraminifera* (Risgaard-Petersen et al. 2006) y amebas del género *Gromia* (Piña-Ochoa et al. 2010) son también capaces de desnitrificar. Igualmente, se ha observado que algunos microorganismos nitrificantes presentan en su genoma genes relacionados con la desnitrificación (Cebron y Garnier, 2005; Shaw et al. 2006). Las modernas técnicas de secuenciación, junto con las nuevas herramientas bioinformáticas, han permitido demostrar la presencia de genes de la desnitrificación en más de 60 microorganismos, lo que ha aumentado sustancialmente el número de especies capaces de desnitrificar, bien sea de forma total o parcial (Sanford et al. 2012; Mao et al. 2013).

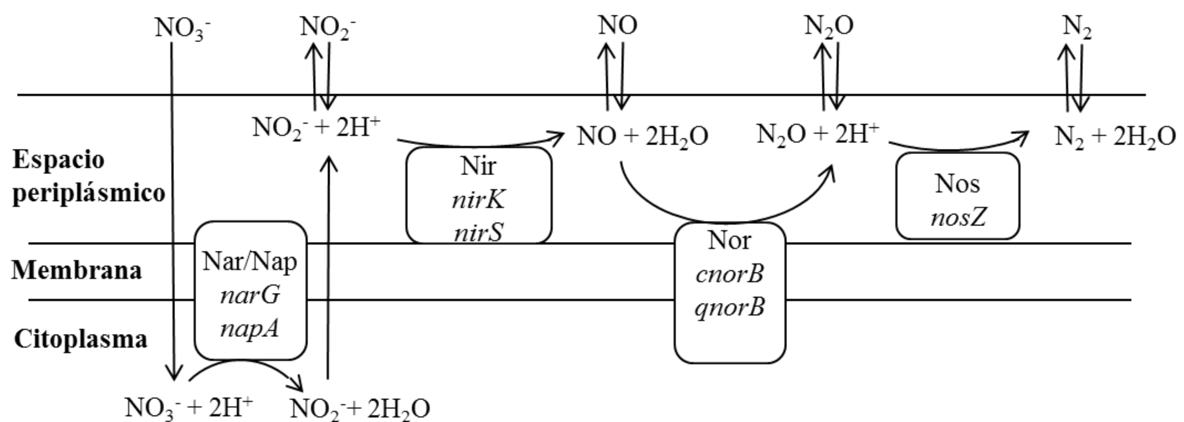

Fig. 2. Ruta metabólica de la desnitrificación. Se indica la localización relativa en la membrana citoplasmática de las enzimas implicados. Adaptado de Saggar et al. (2013).

2.1. Genes y enzimas de la desnitrificación

2.1.1. Nitrato reductasas

La primera etapa de la desnitrificación consiste en la reducción del nitrato a nitrito, proceso que puede catalizarse por dos enzimas bioquímicamente diferentes denominadas nitrato reductasa de membrana (Nar) y nitrato reductasa periplásmica (Nap), respectivamente. La enzima Nar está presente en miembros de los phyla Proteobacteria, Firmicutes, Actinobacterias y del dominio Archaea, mientras que Nap solo está presente en Proteobacteria (Bru et al. 2007). Ambos tipos de enzimas se encuentran en el genoma de diferentes hongos (Shoun et al. 2012; Mothapo et al 2015).

La Nar es una enzima integral de membrana compuesta por tres subunidades denominadas NarGHI. Las proteínas Nar están codificadas por los genes del operón *narGHJI*. Mientras que *narGHI* codifican las subunidades estructurales, *narJ* codifica una chaperona

necesaria para la maduración de la enzima y su inserción en la membrana. La organización de este operón está conservada en la mayoría de las especies que expresan actividad Nar y se ha estudiado ampliamente en *Escherichia coli* y *Paracoccus denitrificans*. Para una revisión ver los trabajo de Potter et al. (2001); Richardson et al. (2001, 2007); van Spanning et al. (2005); González et al. (2006); Richardson (2011); Sparacino-Watkins et al. (2014).

E. coli posee un duplicado funcional del operón *narGHJI* al que se denomina *narZYWV*, cuyos productos intervienen en la respuesta fisiológica frente al estrés más que en la respiración anaerobia (Blasco et al. 1990; Spector et al. 1999). Las subunidades NarG y NarH de algunas arqueas y bacterias se localizan en la parte exterior de la membrana citoplasmática en vez de en la interior, orientación que se ha propuesto como precursor evolutivo del sistema Nar (Martínez-Espinosa et al. 2007).

La enzima Nap está compuesta por tres subunidades de las que NapA y NapB se localizan en el periplasma y una tercera, denominada NapC, es una proteína integral de membrana. La enzima está ampliamente distribuida en todas las clases de proteobacterias y se ha estudiado, sobre todo, en *P. pantotrophus*, *E. coli*, *Rhodobacter sphaeroides* y *Desulfovibrio desfuromonas*.

Se han identificado ocho genes como componentes de los operones que codifican la enzima Nap en diferentes microorganismos (Richardson et al. 2001). La mayoría de las bacterias estudiadas presentan en común los genes *napABC*. El resto de genes del operón codifican proteínas no esenciales que no intervienen directamente en la reducción del nitrato a nitrito. Para revisiones ver Richardson et al. (2001, 2007), van Spanning et al. (2005), González et al. (2006), Jepson et al. (2007), Simpson et al. (2010), Richardson (2011) y Sparacino-Watkins et al. (2014).

2.1.2. Nitrito reductasas

La segunda etapa de la desnitrificación consiste en la reducción del nitrito a NO, primer intermediario gaseoso de la desnitrificación. Esta reacción puede llevarse a cabo por dos tipos de enzimas nitrito reductasa (Nir), una que contiene Cu (NirK) y otra un citocromo de tipo cd_1 (NirS), respectivamente, en su centro activo (van Spanning et al. 2005, 2007; Rinaldo y Cutruzzolà, 2007, Rinaldo et al. 2008; van Spanning, 2011). Ambas enzimas se localizan en el espacio periplásmico y ninguna de ellas es electrogénica.

La nitrito reductasa NirS es una enzima homodimérica con citocromos c y d_1 que está codificada por un complejo sistema de al menos 10 genes organizados de acuerdo a la secuencia *nirSECFDLGHJN* (van Spanning, 2011).

La enzima NirK es un complejo homotrimérico que contiene Cu como único metal con actividad redox (Godden et al. 1991). A diferencia de la compleja organización de los genes que catalizan NirS, la enzima está codificada por un único gen denominado *nirK* (Rinaldo y Cutruzzolà, 2007).

Aunque ambas enzimas están ampliamente distribuidas entre los microorganismos desnitrificantes, no se han encontrado evidencias de que una especie bacteriana contenga en su genoma la información para codificar, a la vez, los dos tipos de enzimas Nir. El gen *nirK* se ha identificado tanto en procariotas (Bacteria y Archaea) como en eucariotas (hongos) (Long et al. 2014), mientras que el gen *nirS* se ha identificado únicamente en Bacteria y Archaea (Mardanov et al. 2015). Aunque NirS y NirK no están relacionadas en términos evolutivos, el gen *nirK* prevalece en Alphaproteobacteria*,* Firmicutes *y* Bacteroidetes, *nirS* abunda más en Betaproteobacteria y no hay diferencias en su abundancia en el caso de Gammaproteobacteria (Heylen et al. 2006).

2.1.3. Óxido nítrico reductasas

La tercera reacción de la desnitrificación consiste en la reducción del óxido nítrico a óxido nitroso mediante la enzima óxido nítrico reductasa (Nor). Existen tres tipos de enzimas Nor, una dependiente de un citrocromo *c* o de la pseudoazurina (cNor), otra que utiliza quinol (qNor) y la tercera, a la que se denomina qCu_ANor, es una enzima qNor que contiene un centro activo de cobre diferente (para revisiones ver Zumft 2005; de Vries et al. 2007; van Spanning et al. 2005, 2007, 2011; Shiro et al. 2012; Spiro, 2012; Tosha y Shiro, 2013).

La enzima cNor es una proteína integral de membrana codificada por el operón *norCBQD*. La enzima qNor está formada por una única subunidad que recibe electrones tanto de la hidroquinona como de la menaquinona y está codificada por el gen *qnorB*. Aunque presenta dominios similares a los de NorC, su secuencia aminoacídica tiene elevada identidad con NorB (Matsumoto et al. 2012). Esta enzima está presente en desnitrificantes de los dominios Bacteria y Archaea, así como en algunos microorganismos patógenos no desnitrificantes (Hendriks et al. 2000; de Vries et al. 2003). La enzima qNor se ha propuesto como la antecesora de las restantes enzimas óxido nítrico reductasas (de Vries y Schröder, 2002).

La enzima qCu_ANor se ha descrito únicamente en la bacteria Gram positiva *Bacillus azotoformans* (Suharti et al. 2001). Esta enzima es un dímero al igual que NorCB, pero carece de grupos hemo *c* y, a diferencia de cNor, contiene cobre en forma de centro Cu_A. Esta enzima es dependiente de menaquinol y, además, acepta electrones procedentes del citocromo

c_{551}. Se ha sugerido que la acción mediada por el menaquinol es activa en la detoxificación del NO, mientras que la debida al citocromo tiene funciones bioenergéticas. Los genes que codifican la enzima qCu_ANor no se han identificado aún.

Aunque no existe un prevalencia clara entre los genes *cnorB* y *qnorB* entre los distintos grupos filogenéticos, las Alphaproteobacteria solo presentan el gen *cnorB*, mientras que el resto de clases bacterianas presentan o uno u otro tipo de gen (Jones et al. 2008). En general los genes *nor* no son co-redundantes, si bien la cepa R-25208 de *Pseudomonas* sp. contiene en su genoma los genes *cnorB* y *qnorB* (Heylen et al. 2007).

En hongos, la reducción de NO a N_2O se realiza mediante el citocromo P450nor (Shoun et al. 2012; Mothapo. et al 2015). Este citocromo se ha estudiado en *Fusarium oxysporum* y *Cylindrocarpon tonkenense* (Morozkina y Kurakov, 2007; Zhang y Shoun, 2008; Shoun et al., 2012).

2.1.4. Óxido nitroso reductasa

El último paso de la desnitrificación consiste en la reducción del óxido nitroso a nitrógeno molecular mediante la enzima de localización periplásmica denominada óxido nitroso reductasa (Nos). Se trata de una proteína dimérica codificada por el gen *nosZ* (para revisiones ver van Spanning et al. 2005, 2007; Zumft y Kroneck, 2006; van Spanning, 2011; Spiro, 2012; Pauleta et al. 2013). El operón de los genes *nos* está conservado en la mayoría de los microorganismos y suele incluir los genes *nosRZDFYLX* (Wunsch et al. 2003; Pauleta et al. 2013). El gen *nosZ* codifica la subunidad catalítica de la óxido nitroso reductasa, enzima que contiene dos dominios, uno denominado Cu_A, que está implicado en la transferencia de electrones, y el otro conocido como Cu_Z, que contiene Cu y S, en donde se localiza el centro catalítico de la enzima (Pauleta et al. 2013). El resto de genes codifican otras proteínas necesarias para la transcripción y el ensamblaje de los centros activos de cobre.

El análisis de las secuencias del gen *nosZ* indica la presencia de un péptido señal en el extremo N-terminal que contiene el motivo consenso de "dos argininas gemelas" (twin arginine motif, SRRXF/L). Este motivo es reconocido por el sistema de translocación denominado TAT (Twin Arginine Transporter) necesario para el transporte de la proteína NosZ en estado plegado desde la membrana interna citoplasmática hasta el espacio periplásmico, donde ocurre la reducción del óxido nitroso. Los genes *nosZ* que contienen este motivo se incluyen en el denominado clado nosZI que está integrado exclusivamente por arqueas, alfa-, beta- y gamma-proteobacterias (Jones et al. 2012).

La épsilon-proteobacteria *Wolinella succinogenes* representa un caso especial ya que es capaz de reducir el N_2O a N_2 pero incapaz de crecer anaeróbicamente con nitrato (Simon et al. 2004). El gen *nosZ* de esta bacteria carece del motivo de dos argininas gemelas y contiene 200 nucleótidos adicionales en su extremo carboxilo terminal. Estos nucleótidos codifican el motivo canónico de unión a hemo tipo *c* que reconoce el sistema de secreción denominado Sec, necesario para la translocación de NosZ (Pauleta et al. 2013). Secuencias similares al gen *nosZ* de *W. succinogenes* se han encontrado en bacterias de los géneros *Campylobacter*, *Sulfurimonas* y *Denitrovibrio*. Los genes *nosZ* con estas características en su secuencia forman parte del denominado clado nosZII que contiene bacterias pertenecientes a las clases delta- y épsilon-Proteobacteria del domino Bacteria, y a las clases Bacteroidetes, Firmicutes, Verrucomicrobia, Aquificae, Gemmatimonadetes, Spirochaetes y Deferribacteres, además de miembros pertenecientes al dominio Archaea (Jones et al. 2012, Sanford et al. 2012). Como excepción, los genes *nosZ* de las bacterias de la clase *Chloroflexi* son homólogos a los del clado nosZII aunque poseen la señal TAT en vez de la señal Sec (Jones et al. 2012).

3. Alteraciones en el ciclo del N

A finales de los años 60 se inició la llamada Revolución Verde que, motivada por la necesidad de alimentar a una población cada vez más numerosa, consistió en un importante incremento de la productividad agrícola y, en definitiva, de la producción mundial de alimentos. Ello fue posible gracias al empleo de variedades vegetales mejoradas, sobre todo de cereales, y a su mayor producción en régimen de monocultivo en respuesta a una abundante aplicación de agua, plaguicidas y fertilizantes sintéticos, principalmente los nitrogenados como la urea, el amonio y el nitrato (Khush 1999).

Según los datos de la Organización para la Alimentación y la Agricultura (FAO) de la Organización Mundial de la Salud, la producción de fertilizantes nitrogenados a nivel mundial se ha incrementado en más de 4 veces desde 1961 hasta 2013 (www.faostat3.fao.org; Heffer y Prud'homme 2015) (Figura 3). En la actualidad, del total de fertilizantes que se emplean en las prácticas agrícolas, más del 60% corresponde a los nitrogenados, el 25% a los fosfatados y el resto, un 15%, a los que contienen potasio (www.fertilizer.org; Heffer y Prud'homme, 2015).

Delwiche (1970) y Burns y Hardy (1975) estimaron que el total de N fijado a escala global era de 100 a 175 Tg/año (teragramo; 1 Tg = 1 x 10^{12} g) y Burris (1980) consideró que esta cantidad era de 122 Tg/año. Estas estimaciones se basaron en el empleo del método de reducción del acetileno y abarcaban tanto los sistemas agrícolas como naturales, incluyendo

los ecosistemas marinos. Posteriormente, Herridge et al. (2008) basándose en el contenido en N de los cultivos de leguminosas, de los arrozales y de los cereales, aunque sin considerar los ecosistemas naturales, establecieron que la FBN aporta al suelo entre 50 y 70 Tg de N al año (Herridge et al. 2008). Por tanto, si se considera válido el valor de 122 Tg/año calculado por Burris (1980), la FBN debida a los sistemas naturales varía de 52 a 72 Tg/año.

Los compuestos de N en la naturaleza se pueden dividir en dos grandes grupos, N no reactivo (N_2) y N reactivo (Nr), que incluye todos los compuestos biológicos, fotoquímicos y radiativos activos en la atmósfera y en la biosfera terrestre. El grupo Nr, por tanto, incluye, formas reducidas de N inorgánico (amonio y amoníaco), formas oxidadas de N inorgánico (NOx, N_2O, HNO_3, NO_3^-) y compuestos orgánicos (urea, aminas, proteínas y ácidos nucleicos). La concentración de Nr incrementó de 15 Tg N/año en 1860 a 187 Tg N/año en 2005 debido, sobre todo, a la aplicación de amonio y de urea producidos mediante los procesos de Haber-Bosch y Wöhler, respectivamente (Galloway et al. 2008; FAO Statistical Database 2006; http://faostat3.fao.org). En definitiva, además del N que se incorpora al suelo mediante la FBN, la fertilización nitrogenada representa entre 100 y 121 Tg N/año adicionales, lo que duplicaría el amonio producido por la FBN (Galloway et al. 2008; Fowler et al. 2013).

Hasta la aparición de los procesos industriales de síntesis de fertilizantes nitrogenados, la FBN y la desnitrificación tenían rendimientos similares, unas 110 toneladas de amonio producido a partir del N_2 frente a las 108 de nitratos eliminados como N_2O o N_2 mediante desnitrificación (Ayres et al. 1994; Gruber y Galloway 2008). Mientras que la desnitrificación no ha aumentado significativamente, la cantidad total de Nr se aproxima a los 240-260 Tg N/año (Bouwman et al. 2013). Si, además, se consideran otros compuestos nitrogenados tanto inorgánicos (nitrato, urea, etc.) como orgánicos (purines del ganado, residuos urbanos líquidos y sólidos, actividades industriales, etc.), así como la deposición de amonio en suelos, mares y océanos, el aporte anual total de Nr al medio ambiente puede alcanzar los 345 Tg N/año (Galloway et al. 2008; Bouwman et al. 2013). Consecuentemente, la desnitrificación no puede eliminar el exceso de Nr que se produce (Ayres et al. 1994; Galloway et al. 2008, Gruber y Galloway 2008; Nieder y Benbi 2008; Bouwman et al. 2013).

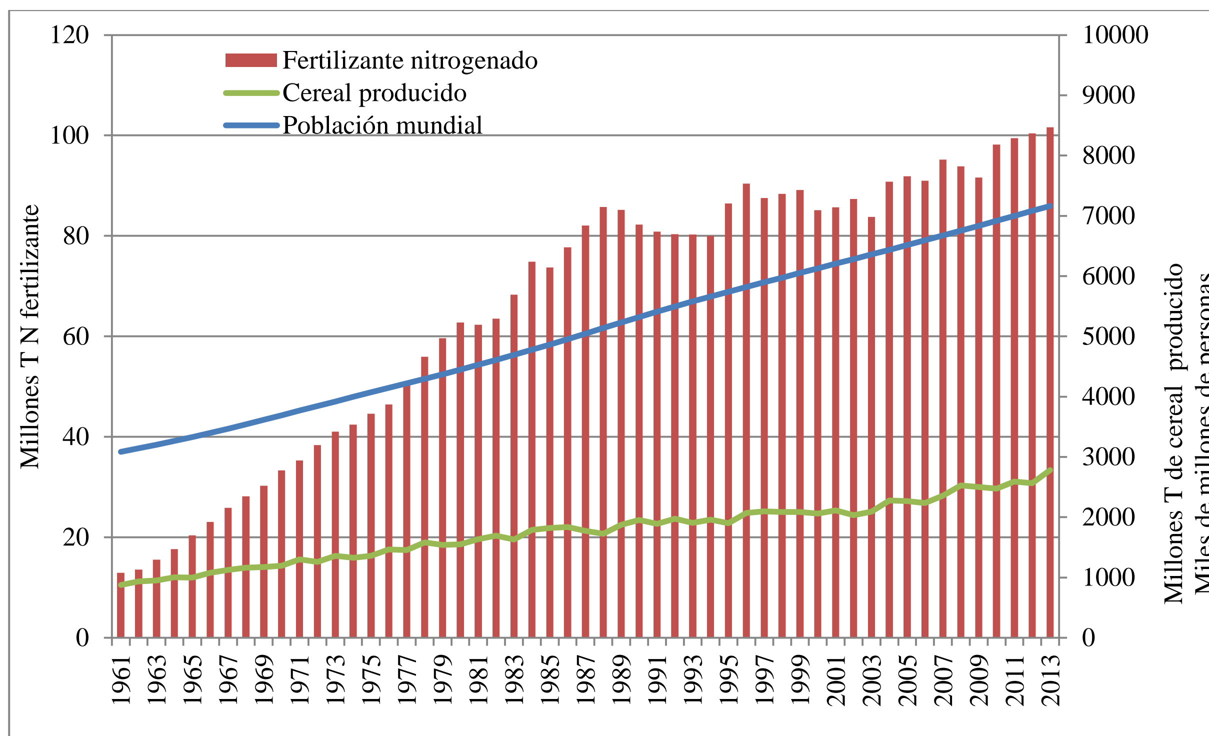

Figura 3. Producción de fertilizante nitrogenados y producción de cereal (millones de toneladas) al año en el mundo. Fuente: FAO (www.faostat3.fao.org).

Del total de N que se aporta al suelo cada año, más del 50% no se utiliza en la producción agrícola sino que se pierde debido a procesos de erosión, de escorrentía o lixiviación del fertilizante aplicado por las aguas de lluvia o de riego, emisión a la atmósfera de gases de nitrógeno, etc. (Van der Hoek, 1998; Goulding et al. 2004). El metabolismo de la mayoría de los compuestos nitrogenados resulta en la formación de nitrato, por lo que este anión es el que, de forma principal, se acumula en suelos, aguas y sedimentos. Este superávit de N en el medio ambiente origina graves daños a escala local, regional y global dando lugar tanto a pérdidas económicas como a problemas medioambientales y para la salud humana (Cowling et al. 2001; Galloway y Cowling 2002; Boyer et al. 2002; Galloway et al. 2003; 2008; Mosier et al. 2004; Krupnik et al. 2004; Erisman et al. 2007; Follet et al. 2010).

4. Contaminación de suelos, aguas y sedimentos por nitratos

El origen de la contaminación por nitratos de suelos, aguas y sedimentos es muy diverso, aunque cabe destacar la producida por el empleo de fertilizantes nitrogenados durante las prácticas agrícolas, los sistemas sépticos, diferentes procesos industriales y el almacenamiento y la aplicación de purines del ganado (Fields 2004). La lluvia y las aguas de riego producen la lixiviación de los nitratos formados, lo que origina un enriquecimiento progresivo de las aguas superficiales y subterráneas y, consecuentemente, la eutrofización de los embalses, estuarios y aguas litorales.

La Organización Mundial de la Salud (World Health Organization, WHO, 2011a) ha establecido que las aguas destinadas a consumo público no deben contener más de 50 mg de NO_3^- por L, concentración que también regula la directiva 91/676/CEE de la UE y el real decreto 261/1996 del Consejo de Ministros del Gobierno de España. El nitrito es otra forma reactiva de N más tóxica que el nitrato, de manera que su concentración máxima en aguas destinadas a consumo humano se ha establecido en 3 mg/L (WHO 2011a). En general, en aguas potables, su concentración no suele exceder de 0.3 mg/L (WHO 2011b).

La contaminación por nitratos es una seria amenaza para la salud humana y animal, aumenta la eutrofización de las aguas y favorece la emisión a la atmósfera de gases de efecto invernadero como el N_2O, relacionado con el cambio climático global. Así, el exceso de nitratos afecta tanto a los ecosistemas terrestres y marinos como a la atmósfera (Díaz y Rosenberg 2008; Sutton et al. 2011).

4.1. Efectos sobre la salud humana

Aunque el nitrato no es tóxico por sí mismo puede transformase en nitrito por las bacterias gastrointestinales, forma que sí es tóxica. Esta toxicidad se debe a que la hemoglobina de los glóbulos rojos tiene mayor afinidad por el nitrito que por el oxígeno, por lo que reacciona con él para formar metahemoglobina. Esta forma oxidada de hemoglobina dificulta, y llega a impedir, el adecuado transporte del oxígeno a los tejidos, lo que produce hipoxia. Este trastorno se conoce como metahemoglobinemia y es el responsable del síndrome del niño azul, así llamado por ser especialmente grave en bebés y niños menores de 6 años. En estos casos, la presencia de hemoglobina fetal agrava el proceso por ser fácilmente oxidable a metahemoglobina (Avery 1999). Además, la ingesta relativa de nitrato/nitrito, respecto al peso, es mayor en bebés frente a una misma dosis en un adulto, y la reducción del nitrato a nitrito por las bacterias gástricas puede verse favorecida debido a la menor producción de ácido gástrico en el bebé (FAO/WHO 1996). En condiciones de pH ácido y en

presencia de aminas y amidas, la nitrosación de los nitratos y nitritos originan nitrosaminas y nitrosamidas, respectivamente, compuestos a los que se ha implicado en la aparición de cánceres de esófago, estómago y colon (Ward et al. 2005).

Los productos E-249, nitrito potásico, y E-250, nitrito sódico, E-251, nitrato sódico, y E-252, nitrato potásico, se utilizan como conservantes de alimentos, en especial la carne y embutidos. Su empleo está autorizado por la Unión Europea siempre que su concentración no sobrepase los límites legalmente establecidos (Directiva 95/2/EC, modificada por las directivas 96/85/EC y 98/72/EC).

4.2. Efectos sobre la eutrofización de las aguas

Aproximadamente 60 Tg de formas reactivas de N llegan a los mares y océanos mediante su transporte por las aguas subterráneas y superficiales de ríos y arroyos (Boyer et al. 2006). A nivel global, este transporte ha producido durante las últimas décadas un considerable incremento de la intensidad, extensión y duración de las floraciones de algas (eutrofización) lo que, a su vez, ha originado situaciones de hipoxia que han acarreado a) la degradación del hábitat, b) alteraciones de la cadena alimentaria y c) pérdida de la biodiversidad del ecosistema (Díaz y Rosenberg 2008; Howarth 2008; Sutton et al. 2011).

4.3. Efectos sobre la contaminación atmosférica

Las bacterias capaces de realizar una desnitrificación completa, es decir reducir el nitrato hasta N_2, son escasas en la naturaleza. Muchas de las bacterias desnitrificantes no poseen, o no expresan, todas las enzimas necesarias para llevar a cabo cada una de las reducciones individuales que integran el proceso (Zumft 1997; Jones et al. 2008). Así, la ausencia de la enzima óxido nítrico reductasa originaría la formación de NO, y la de la enzima óxido nitroso reductasa la de N_2O. Resulta una paradoja que siendo la desnitrificación el único proceso biológico conocido para disminuir el exceso de nitratos que contaminan el medio ambiente sea, a su vez, un mecanismo por el que se liberan a la atmósfera NO y N_2O, dos potentes gases invernadero.

Según el protocolo de Kioto (Convention on Climate Change of the United Nations, CMNUCC¸ United Nations 1998), los gases de efecto invernadero (GEIs) más peligrosos son el dióxido de carbono (CO_2), el metano (CH_4), el óxido nitroso (N_2O), el hexafluoruro de azufre (SF_6) y los compuestos hidrofluorocarbonados (HFC) y perfluorocarbonados (PFC). Se estima que los tres primeros contribuyen en un 50%, 18% y 10%, respectivamente, al calentamiento global derivado de las actividades antropogénicas (Bates et al. 2008; Panel

Intergubernamental sobre Cambio Climático; Intergovernmental Panel for Climatic Change, IPCC 2007). Los países adscritos al protocolo de Kioto se han comprometido a reducir las emisiones de estos gases tras aceptar un conjunto de normas entre los que se encuentran a) adoptar medidas para mejorar la calidad de los datos que se tienen sobre las emisiones de óxido nitroso, b) organizar programas nacionales de mitigación de la producción de óxido nitroso, c) promover la transferencia de tecnologías ambientalmente sanas, d) cooperar en la investigación científica y en las redes internacionales de observación del clima y e) respaldar las iniciativas de educación, formación y sensibilización pública.

Entre los GEIs, la concentración de N_2O en la atmósfera ha incrementado en 50 partes por billón desde la era pre-industrial, pasando de 270 µg/L de aire en 1750 a 320 µg/L en 2005 (IPPC 2007, 2013), y aumenta a un ritmo del 0,2-0,3% por año (Richardson et al. 2009; Tian et al. 2015). La emisión de N_2O por fuentes de origen natural es de 11 Tg de N por año, de los cuales 6,6 Tg se emite por ecosistemas terrestres, 3,8 por ecosistemas marinos y 0,6 Tg tienen un origen atmosférico; además, 5,5 Tg se deben a fuentes antropogénicas (Ciais et al. 2013). Aunque solo contribuye en un 10% a la emisión global de GEIs, el N_2O tiene un potencial de calentamiento global casi 300 veces mayor que el del CO_2 y una vida media en la atmósfera de 150 años (Lassey y Harvey 2007; IPCC 2007, 2013), lo que convierte a este gas en el tercero en importancia entre los de efecto invernadero (Braker y Conrad 2011).

Además, en la atmósfera, el óxido nitroso puede convertirse en ácido nítrico, que es uno de los componentes que forman la denominada lluvia ácida (Anderson y Levine 1986; Rivett et al. 2008). También puede transformase en NO al que se ha implicado en la destrucción de la capa de ozono de la estratosfera que protege a la tierra de los efectos perjudiciales de la luz ultravioleta (Ravishankara et al. 2009).

Sin lugar a dudas, la perturbación de origen antropogénico del ciclo del N debido al incremento en la producción de fertilizantes nitrogenados y de las formas reactivas de nitrógeno oxidadas derivadas de la quema de los combustibles fósiles han conducido a una enorme acumulación de óxidos de nitrógeno (principalmente N_2O) en la biosfera (Erisman et al. 2008). Sin embargo, hay evidencias que sugieren que la mayoría del N_2O se produce a partir de la desnitrificación microbiana en suelos, aguas y sedimentos contaminados con nitratos. Como ya se ha indicado, más de la mitad del fertilizante que se aplica al suelo termina en ríos, lagos, mares y océanos contribuyendo a la eutrofización y al crecimiento masivo de algas en las plataformas continentales (Díaz y Rosenberg 2008). Y de ahí, que el exceso de nitratos afecte no solo a los ecosistemas terrestres y marítimos, sino que también

contribuya a la liberación a la atmósfera de gases de efecto invernadero implicados en el cambio climático global (Sutton et al. 2011).

5. Procesos que producen óxido nitroso

Como se ha indicado, la principal fuente de N_2O en la mayoría de los ecosistemas corresponde a la desnitrificación (Baggs 2008, 2011; Baggs y Philippot, 2011; Braker y Conrad 2011). Los procesos microbianos que, junto a la desnitrificación, también producen N_2O son la nitrificación, tanto autótrofa como heterótrofa, la codesnitrificación, la reducción desasimilatoria del nitrato a amonio (DNRA), la desnitrificación nitrificante y la desnitrificación acoplada a la nitrificación (Baggs y Philippot 2011). Además de estos procesos microbianos, la asimilación de NO_3^- por las plantas libera N_2O a la atmósfera (Smart y Bloom 2001). La descomposición química de la hidroxilamina (NH_2OH) y del nitrato amónico (NH_4NO_3) y la quimiodesnitrificación son procesos abióticos que también producen emisión de N_2O a la atmósfera (Butterbach-Bahl et al. 2013). En la figura 4 se muestran las diferentes vías metabólicas que producen de N_2O.

Estos procesos raramente ocurren de forma aislada ya que las distintas comunidades de microorganismos pueden llevar a cabo diferentes actividades del ciclo del N o, incluso, competir entre ellas en caso de limitación de los sustratos disponibles. Además, existe la posibilidad de transferencia de N_2O, o de otros productos intermediarios, de unos procesos a otros dependiendo de las condiciones medioambientales predominantes en un momento determinado, entre las que destacan la estructura de la comunidad microbiana y su localización dentro de la matriz del suelo. En definitiva, el N_2O producido en diferentes procesos pasaría a formar parte de un conjunto que podría reducirse a N_2 durante la última etapa de la desnitrificación (Baggs 2008, 2011; Baggs y Philippot 2011).

6. Factores que afectan la desnitrificación

Los requerimientos generales para que la desnitrificación ocurra son a) presencia de bacterias con la capacidad metabólica para llevarla a cabo, b) disponibilidad de donadores de electrones, como son los compuestos de carbono orgánicos, c) condiciones de limitación de oxígeno y d) presencia de un óxido de nitrógeno (NOx; NO_3^-, NO_2^- , NO o N_2O) como aceptor final de electrones. En términos generales, por tanto, la desnitrificación requiere condiciones limitantes de oxígeno, la presencia de nitrato, o de un óxido de nitrógeno derivado de él, y de una fuente de C asimilable.

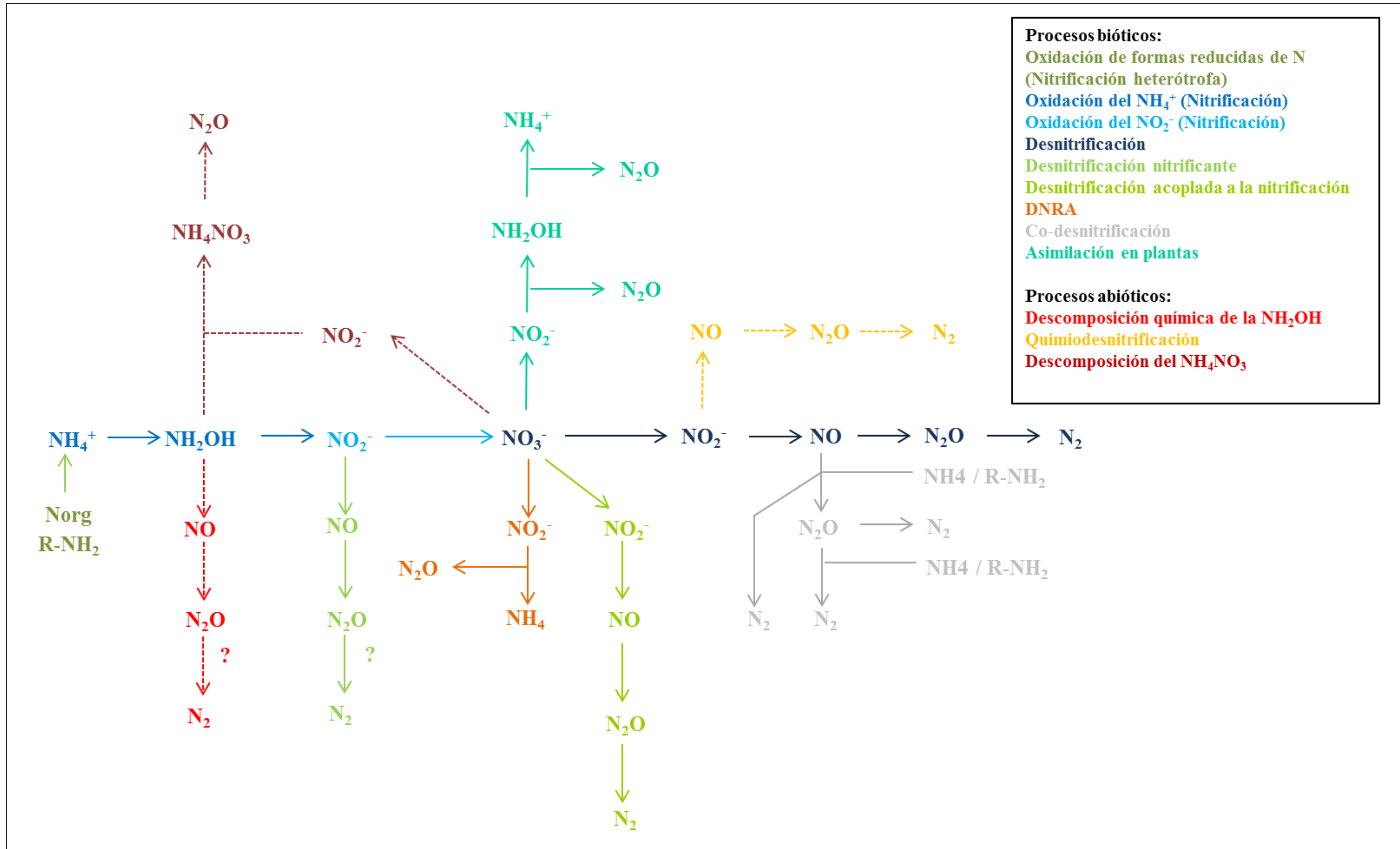

Figura 4. Vías metabólicas bióticas y abióticas que producen N_2O. Adaptado de Baggs y Philippot (2011) y Butterbach-Bahl et al. (2013)

La desnitrificación en el suelo ocurre irregularmente tanto en el tiempo como en el espacio debido a las condiciones climáticas, la heterogeneidad de las características del suelo y las prácticas agrícolas que se desarrollen en él (Braker y Conrad 2011; Butterbach-Bahl et al. 2013). Se ha sugerido que, en la naturaleza, la desnitrificación se realiza en los denominados puntos y tiempos calientes, por lo solo zonas concretas de un ecosistema serían las responsables de la desnitrificación en un determinado momento (Parkin 1987; Groffamn et al. 2006).

Los principales factores que afectan la desnitrificación en el suelo son el contenido en NO_3^-, la humedad, la temperatura, la relación C/N, el pH, la textura del suelo, la presencia o ausencia de plantas, los ciclos frio/calor o congelación/descongelación, la actividad microbiana, etc. (Signor y Cerri 2013; Medinets et al. 2015).

También la ganadería es responsable de la producción de N_2O, lo que se debe a la excreción de N como excremento o como orina, bien directamente, o como resultado de la aplicación al suelo del estiércol o como efluente procedente de la estabulación del ganado. El pastoreo también interviene en la producción de N_2O, ya que las zonas con abundantes pastos suelen estar compactados debido al tráfico del ganado, lo que origina una disminución del oxígeno produciéndose las condiciones adecuadas para que ocurra la desnitrificación.

7. Biodiversidad y abundancia de comunidades microbianas

7.1. Definición de biodiversidad

El artículo 2 del Convenio sobre Diversidad Biológica (http://www.cbd.int/) define la biodiversidad como la variabilidad de organismos vivos de cualquier fuente, incluidos los ecosistemas terrestres y marinos, otros ecosistemas acuáticos y los complejos ecológicos de los que forman parte. Comprende la diversidad dentro de cada especie, entre las especies y de los ecosistemas. Este convenio se materializó durante la conferencia de las Naciones Unidas sobre Medio Ambiente y Desarrollo que se celebró en Rio de Janeiro en 1992. España ratificó el convenio el 21 de diciembre de 1993.

7.2. Análisis de la biodiversidad microbiana

7.2.1. Métodos dependientes del cultivo celular

El método tradicional para el análisis de la diversidad bacteriana es el que se basa en métodos dependientes de cultivo celular. Para ello, se emplean diversos medios de laboratorio que permitan el crecimiento de las bacterias. Se ha formulado una amplia variedad de medios de cultivo, de manera que se pueda obtener el mayor número posible de diferentes unidades formadoras de colonias (UFCs). Una vez crecidas, la identificación y clasificación taxonómica de las bacterias crecidas se realiza empleando diversas técnicas, tanto fenotípicas como genotípicas.

Las características fenotípicas de una bacteria no aseguran su reproducibilidad biológica y, en la mayoría de los casos, tampoco refleja la relación filogenética con otras bacterias.

En general, la diversidad biológica de las poblaciones bacterianas es enorme. Un gramo de suelo puede contener entre 1 x 10^3 y 1 x 10^6 especies únicas de bacterias (Torsvik et al. 2002; Gans et al. 2005; Tringe et al. 2005a; Fierer et al. 2007). A pesar de ello, se ha estimado que el número de bacterias que pueden aislarse de muestras medioambientales en medios de cultivo no supera el 10% de las que, en realidad, pueden ocurrir en ese hábitat (Torsvik y Øvreås 2002; Singh et al. 2008; Peix et al. 2012). Estas limitaciones se han asociado a la dificultad de reproducir, en condiciones de laboratorio, los requerimientos nutricionales y las condiciones fisicoquímicas que ocurren en condiciones naturales. La posible toxicidad del medio, la auxotrofia producida por pérdida de nutrientes, supresión de las bacterias de lento crecimiento por aquellas de crecimiento más rápido, dificultad de reproducción en condiciones de laboratorio por la ausencia de interacciones inter e intraespecíficas que ocurren en la naturaleza, entre otras, también se han considerado como factores que limitan el cultivo bacteriano en medios de laboratorio (Schink 2002; Water y Bassler 2005). No obstante, a pesar de sus limitaciones, el empleo de medios de cultivo para el aislamiento de bacterias es una herramienta útil y complementaria de los métodos independiente de cultivo para estudios de fisiología, bioquímica y genética microbiana (Ben-Dov et al. 2009).

Estos métodos sirven también para confirmar los datos derivados de la observación de procesos ecológicos y para evaluar hipótesis originadas a partir de estudios de genómica y metagenómica (Giovannoni et al. 2007). En condiciones medioambientales, las comunidades bacterianas han evolucionado a lo largo de millones

de años de selección natural y han establecido, a través de múltiples interacciones, diversos consorcios microbianos difíciles de simular de manera artificial. En definitiva, si una bacteria no se puede cultivar en el laboratorio no es accesible para su posterior análisis.

Para resolver estos problemas nacieron los métodos independientes del cultivo bacteriano, por lo que el desarrollo de los métodos genotípicos (moleculares) de identificación de la biodiversidad representa un avance complementario y necesario para el estudio riguroso de la diversidad microbiana.

7.2.2. Métodos independientes del cultivo celular

Puesto que la biodiversidad microbiana es esencial para el funcionamiento de los ecosistemas, existe la necesidad de entender la variabilidad en la estructura y en las funciones de la comunidad microbiana en respuesta a los factores medioambientales que puedan modificarla. Estas circunstancias no pueden analizarse mediante los métodos dependientes de cultivo celular, por lo que ha sido necesario el desarrollo de técnicas que permitan el análisis rápido y reproducible de múltiples muestras medioambientales.

La reacción en cadena de la polimerasa (Polymerase Chain Reaction, PCR) ha cambiado radicalmente la comprensión del mundo microbiano ya que ha permitido el análisis de las poblaciones bacterianas a partir del ADN extraído de muestras medioambientales, sin necesidad de aislarlas ni cultivarlas. Desarrollada por K. B. Mullis entre 1983 y 1986, se caracteriza porque permite obtener un gran número de copias de un fragmento determinado de ADN partiendo, en teoría, de una única copia de ese ADN (Mullis 1990).

El término metagenómica se utilizó por primera vez por Handelsman et al. (1998) para referirse a una metodología que pretendía analizar una colección de genes secuenciados de una muestra ambiental como si se tratara de un único genoma. Posteriormente, Handelsman (2004) y Riesenfeld et al. (2004) definieron el metagenoma como todo el ADN que se puede encontrar en una muestra medioambiental sea cual sea el ecosistema. Chen y Pachter (2005) definieron la metagenómica como la aplicación de técnicas genómicas modernas para el estudio directo de comunidades de microorganismos en su entorno natural, evitando la necesidad de aislar y cultivar cada una de las especies que componen la comunidad.

Un problema que presenta el estudio del metagenoma es el aislamiento de ADN de las muestras medioambientales. Ello se debe a la existencia de compuestos que

pueden inhibir la hibridación de los cebadores al ADN molde, la actividad de la polimerasa, etc. Entre ellos, los ácidos húmicos, metales pesados y nucleasas se deben eliminar durante el proceso de aislamiento y purificación. Para ello, se han desarrollado diversos protocolos que permiten la obtención de ADN empleando, en ocasiones, kits comerciales. La utilización de polimerasas que minimicen los fallos en la lectura del ADN o que solo amplifiquen cuando se activan a elevadas temperaturas es recomendable.

En las últimas décadas se han desarrollado una serie de técnicas moleculares de identificación de microorganismos a partir del ADN de muestras medioambientales, entre ellas, la electroforesis en geles desnaturalizantes (DGGE) (Muyzer et al. 1993) o de gradiente de temperatura (TGGE) (Muyzer y Smalla 1998), el análisis del polimorfismo de la longitud de los fragmentos de restricción (RFLP), y del polimorfismo de fragmento terminal (T-RFLP) (Liu et al. 1997). Otra metodología, el análisis del polimorfismo de la conformación de las cadenas sencillas de ADN (SSCP) (Schwieger y Tebbe 1998) de un gen determinado amplificado mediante la reacción en cadena de la polimerasa (PCR) proporciona excelentes resultados en los estudios de biodiversidad (Smalla et al. 2007). La sensibilidad del SSCP aumenta cuando se emplea MDE^{T} (Mutation Detection Enhancement), un gel preparado a partir de una matriz de poliacrilamida (Sentinelli et al. 2000), que permite separar ADNs de acuerdo a su conformación y tamaño (Soto y Sukumar 1992), lo que aumenta la sensibilidad en comparación con los geles normales de poliacrilamida. Para una revisión sobre estas técnicas ver Rastogi y Sani (2011) y Fakruddin y Mannan (2013).

El estudio de una comunidad funcional de microorganismos mediante TGGE, DGGE, RFLP, T-RFLP y SSCP presenta una seria limitación ya que no permite determinar la abundancia relativa de cada población en la comunidad. Por tanto, estudiar la biodiversidad de una comunidad funcional es solo de valor limitado si los resultados no pueden cuantificarse. Para ello, se desarrolló la técnica de construcción de librerías genómicas, otro método molecular característico en los estudios de diversidad microbiana, que consiste en la inserción del ADN amplificado en vectores de clonación tales como pGEM-T Easy (Promega), TOPO TA y pCR-2.1 TOPO (Life Technologies). La secuenciación posterior del ADN clonado resultaría en la construcción de librerías génicas que darían información sobre la identidad de los fragmentos de ADN amplificados y permitiría el análisis filogenético detallado de tales genes. Aunque la obtención de librerías genómicas es un método adecuado para estudios de

biodiversidad, consume tiempo y puede resultar caro, si bien la información que aporta esta técnica es mayor que la producida por las mencionadas anteriormente.

7.2.3. Biodiversidad de bacterias desnitrificantes

Como se ha indicado, el gen 16S rARN es un marcador filogenético universal que se emplea en los estudios de identificación y filogenia bacteriana. Sin embargo, puesto que la capacidad de desnitrificar no está asociada a ningún grupo taxonómico específico, los métodos basados en el análisis del gen 16S rARN no son estrictamente válidos para el estudio de las comunidades de bacterias desnitrificantes. De ahí, que haya sido necesario el desarrollo de técnicas basadas en el empleo de genes funcionales como marcadores para identificar y cuantificar las poblaciones que las integran (Philippot 2006; Philippot y Hallin 2006; Hallin et al. 2007; Correa-Galeote et al. 2013a, 2014a). De esta manera, la biodiversidad de bacterias desnitrificantes en muestras medioambientales se ha estudiado mediante electroforesis en geles desnaturalizantes del gen *narG* (Alcántara-Hernández et al. 2009; Magalhães et al. 2011; Pastorelli et al. 2013), *napA* (Sharma et al. 2006; Alcántara-Hernández et al. 2009), *nirK* (Thröback et al. 2004; Sharma et al. 2005; Dandie et al. 2011; Hussain et al. 2011; Boulêtreau et al. 2014), *nirS* (Thröback et al. 2004; Sharma et al. 2005; Desnues et al. 2007) y *nosZ* (Enwall et al. 2005, 2009; Siciliano et al. 2007; Magalhães et al. 2011).

Mediante RFLP se han analizado los genes *narG* (Philippot et al. 2002; Chèneby et al. 2003; 2009; Mounier et al. 2004; Deiglmayr et al. 2004, 2006; Patra et al. 2005; Reyna et al. 2010), *napA* (Chèneby et al. 2009; Auclair et al. 2012) *nirK* (Braker et al. 2000; Priemé et al. 2002; Yan et al. 2003; Sharma et al. 2005; Jayakumar et al. 2009; Auclair et al. 2012), *nirS* (Braker et al. 2000; Priemé et al. 2002; Yan et al. 2003; Sharma et al 2005; Jayakumar et al. 2009; Auclair et al. 2012), *cnorB* (Auclair et al. 2012) y *nosZ* (Stres et al. 2004; Mounier et al. 2004; Dambreville et al. 2006; Horn et al. 2006; Ruiz-Rueda et al. 2009; Ma et al. 2011).

La técnica de T-RFLP también se ha empleado para estudiar los genes *narG* (Enwall et al. 2005; Bougon et al. 2009; Liu et al. 2012a), *nirK* (Thröback et al. 2004; Sharma et al. 2005; Chen et al. 2010), *nirS* (Enwall et al. 2005; Chen et al. 2010), *qnorB* (Chen et al. 2012a) y *nosZ* (Rich et al. 2003, 2004; Enwall y Hallin 2005; Wallenstein et al. 2006; Stres et al. 2008; Chen et al. 2012b). En la revisión bibliográfica realizada, no

se ha encontrado el empleo de la técnica SSCP para estudiar la biodiversidad de los genes de la desnitrificación en muestras ambientales.

Chèneby (1998, 2000) fue pionera en analizar la diversidad de bacterias desnitrificantes mediante la elaboración de librerías genómicas de los genes *narG* y *napA*, metodología que después se ha utilizado por otros autores (Smith et al. 2007; Henry et al. 2008; Alcántara-Hernández et al. 2009; Kofoed et al. 2012; Yu et al. 2014). Igualmente, se han construido librerías de los genes *nirK* (Henry et al. 2004; Sharma et al. 2005; Yoshida et al. 2009, 2012; Auclair et al. 2010; Yu et al. 2014; Gao et al. 2015), *nirS* (Sharma et al. 2005; Heylen et al. 2006; Desnues et al. 2007; Yoshida et al. 2009, 2012; Yu et al. 2014; Fan et al. 2015), *cnorB* (Auclair et al. 2010; Yu et al. 20014) y *nosZ* (Henry et al. 2006, 2008; Palmer et al. 2009; Auclair et al. 2010; Chen et al. 2012b; Ishii et al. 2011; Jung et al. 2013; Yu et al. 2014).

Como en la mayoría de las reacciones basadas en la PCR el desarrollo de cebadores para amplificar genes funcionales es una necesidad. Los primeros genes de la desnitrificación que se amplificaron fueron *nirK*, *nirS* y *nosZ* (Braker et al. 1998; Hallin y Lindgren 1999; Scala y Kerkhof 1999; Michotey et al. 2000; Yan et al. 2003) y, posteriormente, se desarrollaron los oligonucleótidos para la amplificación de los genes *narG*, *napA* y *norB* (Flanagan et al. 1999; Gregory et al. 2000; Philippot et al. 2002; Braker y Tiedje 2003; Chèneby et al. 2003). El aumento del número de secuencias de genes de la desnitrificación depositadas en las bases de datos permitió, por otra parte, el diseño y desarrollo de nuevos cebadores cuyo empleo resultó en la amplificación de genes de la desnitrificación a partir del genoma de otras bacterias (Henry et al. 2004, 2006; López-Gutiérrez et al. 2004; Thröback et al. 2004; Bru et al. 2007; Jones et al. 2013).

7.3. Abundancia de comunidades microbianas

7.3.1. Técnicas dependientes de la PCR

Aunque la elaboración de librerías genómicas representó un avance significativo para conocer la diversidad funcional bacteriana, no informa de la ocurrencia de la correspondiente actividad del producto que codifica ni permite determinar el número de copias (abundancia) del gen. Este problema se resolvió con el desarrollo de la PCR cuantitativa a tiempo real (qPCR) que emplea sondas fluorescentes (Nitsche et al. 1999) o colorantes (Morrison et al. 1999) para cuantificar el número de copias del ADN de un gen presente en muestras medioambientales. Esta técnica es de gran especificidad,

necesaria para la discriminación entre diferentes miembros de una familia génica, y sensibilidad, lo que se requiere para la identificación de transcritos poco abundantes.

Una vez diseñados y sintetizados los cebadores adecuados, se han descrito dos metodologías para determinar la abundancia de un determinado gen, la PCR competitiva (cPCR) y la PCR cuantitativa a tiempo real (qPCR). Siguiendo la normativa MIQE (Bustin et al. 2009), en esta Memoria de Doctorado se utilizan las siglas qPCR para referirnos a la PCR cuantitativa a tiempo real, dejando las iniciales RT-qPCR para indicar la transcripción reversa mediante PCR cuantitativa. cPCR y qPCR se basan en la proporcionalidad entre la intensidad de la señal de fluorescencia emitida durante la fase exponencial de la PCR y la cantidad inicial del ADN diana. El número de copias del ADN diana inicial se determina mediante su comparación con el número de copias de una curva estándar construida con concentraciones iniciales conocidas de ADN.

Ambas técnicas, cPCR y qPCR, se han empleado para determinar el número de copias de los genes de la desnitrificación. Hay que tener en cuenta que la mayoría de estos genes están presentes en una copia única en el genoma de una determinada bacteria, aunque los genes *narG* y *nosZ* pueden estar presentes en más de una copia (Philippot et al. 2002; Jones y Hallin, 2010).

Mediante cPCR se ha cuantificado la presencia del gen *nirS* en muestras marinas (Michotey et al. 2000), la de *nirK* en suelos y sedimentos de ríos (Qiu et al. 2004) y la de ambos, *nirK* y *nirS*, en muestras de biopelículas formadas en biorreactores de membrana aireada a diferentes profundidades (Cole et al. 2004). No obstante, que entre los inconvenientes de esta metodología destaquen la limitación de su rango dinámico, la obligación de efectuar diferentes diluciones y la necesidad de realizar múltiples electroforesis ha hecho que su aplicación sea muy limitada.

Existen dos tipos de tecnologías qPCR que emplean o una sonda de ADN con un fluoróforo que se une específicamente al ADN amplificado (método TaqMan) o un fluoróforo en suspensión que también se liga específicamente al ADN durante la amplificación (método SybrGreen). La amplificación mediante qPCR del gen *nirS* de *P. stutzeri* se realizó por primera vez utilizando la tecnología TaqMan (Grüntzig et al. 2001). Debido a la elevada especificidad del ADN que se utilizó como cebador, el oligonucleótido solo permite amplificar el gen *nirS* de *P. stutzeri* o secuencias génicas con elevada identidad, por lo que la utilidad de la técnica es limitada. Su ventaja, por otra parte, es que a partir de un mismo ADN, el empleo de sondas marcadas con diferentes fluoróforos permite amplificar más de un gen.

El SybrGreen es un colorante fluorescente que se une al ADN de doble cadena de forma no específica. Durante la qPCR la intensidad de la fluorescencia se detecta de forma simultánea a su emisión, por lo que se produce un incremento logarítmico en la emisión de fluorescencia hasta que los sustratos de la reacción de PCR son limitantes. La qPCR no necesita electroforesis iniciales, es muy reproducible y sensible y es más económica, menos laboriosa y necesita menos tiempo para su realización. La presencia de un gen determinado en una muestra medioambiental se puede analizar también a partir de su ARN mediante qPCR. Esta metodología se basa en la extracción directa del ARN presente en la muestra que, una vez aislado y purificado, se retro-transcribe a cADN que, a su vez, se emplea como ADN diana de la amplificación. Después de retro-transcribirse, los genes *narG*, *napA*, *nirK*, *nirS* y *nosZ* se han cuantificado mediante qPCR (Nogales et al. 2002; Henderson et al. 2010; Dandie et al. 2011).

Actualmente, la qPCR es la técnica más utilizada para cuantificar el número de copias de un determinado gen en muestras medioambientales, incluidos los genes de la desnitrificación, que se ha realizado a partir de suelos agrícolas (Baudoin et al. 2009; Morales et al. 2010; Attard et al. 2011; Marhan et al. 2011), suelos de bosques (Bárta et al. 2010; Brandt et al. 2014), sedimentos de arrozales (Yoshida et al. 2009; Chen et al. 2012a,b), suelos dedicados al pastoreo (Philippot et al. 2009; Legay et al. 2014), nidos de nematodos (Djigal et al. 2009), suelos artificiales (Hafeez et al. 2012) y sedimentos de humedales artificiales (Correa-Galeote et al. 2013b; Peralta et al. 2013).

Para revisiones sobre los métodos moleculares utilizados para el aislamiento e identificación de genes de la desnitrificación, el lector puede referirse a Philippot (2006), Zhang y Fang (2006), Wallenstein et al. (2006), Hallin et al. (2007), Smith y Osborn (2009), Fang et al. (2010), Brankatschk et al. (2012), Correa-Galeote et al. (2013a, 2014a), Pabinger et al. (2014), Lu et al. (2014) y Levy-Booth et al. (2014).

7.3.2. Distribución espacial y mapas de Krige

Mientras que la qPCR permite la cuantificación del número de copias de un gen determinado, como es el caso de los genes de la desnitrificación, el conocimiento de su abundancia no es válido para establecer su distribución espacial en las muestras de las que se aísla.

Los primeros estudios que analizaron la variabilidad espacial de diferentes procesos relacionados con el ciclo del N en un área de muestreo se deben a Huchinson y Mosier (1981), Linn y Doran (1984), Davidson y Swank (1986) y Zak et al. (1986),

quienes asumieron la independencia del valor de la variable medida en cada uno de los diferentes puntos de muestreo respecto al resto de valores de la misma variable en cada uno de los diferentes puntos muestreados. Posteriormente, Robertson et al. (1987) indicaron que la igualdad entre los valores de una variable medioambiental determinada en puntos de muestreo próximos en el área de estudio es mayor que la de los obtenidos en muestras más distantes. En consecuencia, el valor de una variable medioambiental en un punto determinado de muestreo está influenciado por los valores de dicha variable en los puntos de muestreo que la rodean. A esta propiedad se denominó autocorrelación. Se planteó así el uso de técnicas geoestadísticas para caracterizar la estructura espacial de una variable medioambiental, entre ellas la distribución de un determinado género bacteriano (Robertson et al. 1987). A este efecto fue fundamental el empleo de las técnicas geoestadísticas desarrolladas por Krige (1951) para estimar la abundancia de oro en una mina mediante el empleo de mapas de relieve elaborados a partir de los valores de contenido en mineral obtenidos a partir de un conjunto de catas.

La geoestadística ha permitido dibujar los correspondientes mapas de Krige después de la cuantificación de diferentes genes relacionados con el ciclo del N. Así, se determinó la distribución espacial de los genes 16S rARN, *nosZ*, y $cnorB_P$ (el gen *norB* de *Pseudomonas mandelii* y de especies relacionadas) en suelos de praderas y en suelos agrícolas (Miller et al. 2009) y de los genes *narG, napA*, *nirS*, *nirK*, *nosZ* y del gen 16S rARN en un pastizal con 3 formas de pastoreo diferentes (Philippot et al. 2009). Posteriormente, Bru et al. (2011) realizaron los mapas de Krige de la abundancia de los genes *narG, napA*, *nirS*, *nirK*, *nosZ* y *amoA* de una superficie de 35.000 km^2 de la región francesa de la Borgoña. También se han realizado los mapas de Krige de los genes *nirK* y *nir*S en una finca agrícola con dos regímenes, orgánico y químico, de fertilización (Enwall et al. 2010). Keil et al. (2011) investigaron la influencia de la fertilización química en la distribución espacial de los genes *narG, napA*, *nirS*, *nirK*, *nosZ* y *amoA*, y Abell et al. (2013) determinaron los cambios en la distribución espacial y temporal de los genes *amoA*, *nir*S y 16S rARN en sedimentos de un estuario de Tasmania. Recientemente, se ha evaluado la distribución espacial de los genes *amoA*, *nosZ* y 16S rARN del gén*ero Nitrospira* en sedimentos de un humedal artificial en China (Zhi et al. 2015).

7.3.3. Métodos independientes de la PCR

Además de la qPCR, existen otros dos métodos para determinar el contenido del ADN de un gen en muestras medioambientales. El primero se basa en la utilización de anticuerpos producidos como respuesta inmunológica a las proteínas codificadas por cualquiera de los genes de la desnitrificación, entre ellas NirK y NirS (Coyne et al. 1989; Ward et al. 1993; Metz et al. 2003). Tras la separación de las células marcadas mediante citometría de flujo, la afiliación taxonómica de la población puede determinarse mediante secuenciación del gen 16S rARN. Se ha propuesto al gen *narG*, que codifica la enzima nitrato reductasa de membrana (NarG), como diana para la cuantificación de las microorganismos aislados del suelo mediante esta técnica (Maron et al. 2004).

El segundo método, la técnica de los microarrays de ADN de cuantificación de comunidades bacterianas desnitrificantes en muestras medioambientales, se basa en la hibridación ADN-ADN. Para ello, se han utilizado microarrays construidos con fragmentos de ADN de distinto tamaño que han permitido conocer la diversidad y distribución de los genes *narG*, *nirK*, *nirS* y *nosZ* (Wu et al. 2001; Mergel et al. 2001; Cho y Tiedje 2002; Taroncher-Oldernburg et al. 2003; Tiquia et al. 2004). También se han empleado microarrays basados en el genoma completo para identificar bacterias desnitrificantes (Wu et al. 2004). Un microarray construido con genes funcionales medioambientales también fue útil para reconocer las propiedades funcionales de las comunidades microbianas (McGrath et al. 2010).

7.4. Nuevas técnicas de secuenciación masiva

Los primeros pasos en la secuenciación del ADN ocurrieron en los años 70 y se deben a Sanger et al. (1977), quienes desarrollaron el método enzimático de terminación de cadenas (método de los dideoxinucleótidos), y a Maxam y Gilbert (1977), quienes propusieron el proceso de fragmentación química. A finales de los años 90 se iniciaron los proyectos de secuenciación y la metodología de Sanger se modificó para permitir el empleo de dideoxinucleótidos marcados con fluorescencia. Tales nucleótidos se podían analizar mediante electroforesis capilar y producir un electroferograma a partir del cual deducir la secuencia de tales nucleótidos. Esto permitió mejorar, automatizar y aumentar el rendimiento del proceso de secuenciación lo que, en definitiva, posibilitó el desarrollo de los secuenciadores automáticos. De esta manera fue posible manejar hasta cerca de 100 muestras de ADN dando lugar a secuencias de entre 500 y 1000 bases.

Esta primera generación de secuenciadores automáticos junto con el desarrollo de estrategias de secuenciación a gran escala (Whole Genome Shotgun Sequencing) permitió el ensamblaje de las secuencias genómicas y facilitó la puesta en marcha del Proyecto Genoma Humano. En 1995 se publicó el genoma de *Haemophilus influenzae* y en 2001 apareció el primer borrador del genoma humano.

En búsqueda de soluciones que permitieran abaratar los costes de secuenciación se desarrollaron los secuenciadores de segunda generación, capaces de producir cientos de miles de secuencias por la posibilidad de llevarse a cabo miles de reacciones de secuenciación (alto rendimiento, high-throughput) mediante la inmovilización del ADN en superficies sólidas, que disminuía el empleo de los reactivos necesarios para el proceso de secuenciación y, consecuentemente, el coste del proceso. De esta manera se han desarrollado otros procedimientos de secuenciación de ADN a los que se denomina, en general, Next Generation Sequencing (NGS), que permiten la lectura más barata y eficiente de miles de secuencias de ADN, lo que las hace de gran interés en estudios de biodiversidad. Estas técnicas permiten obtener una visión más amplia de la comunidad microbiana en cuanto a taxonomía y funcionamiento potencial (Steele y Streit 2005; Hugenholtz y Tyson 2008). La primera aproximación a la secuenciación masiva fue la pirosecuenciación del ADN (Ronaghi et al. 1996, 1998; Nyrén 2001; Ronaghi 2001), que acopla la síntesis de ADN a una reacción quimioluminiscente. Desde el primer modelo comercial de pirosecuenciador aparecido en 2005 que era capaz de secuenciar hasta 20 millones de bases en unas 4 horas, se ha mejorado esta tecnología permitiendo la lectura de hasta un millón de fragmentos de 1000 pares de bases. Durante su amplificación, el marcaje con etiquetas específicas permite el análisis simultáneo de un gen aislado de diferentes muestras (Binladen et al. 2007; Parameswaran et al. 2007).

Al mismo tiempo que Roche/454 Life Sciences, las compañías Illumina/Solexa y SOLiD System desarrollaron tecnologías para la secuenciación masiva en paralelo del ADN. Illumina/Solexa utiliza una tecnología basada en el concepto de secuenciación por síntesis. Este proceso consiste en la incorporación de un nucleótido marcado con fluorescencia y protegido a la cadena del ADN naciente impidiendo la unión de nuevos nucleótidos. Tras detectarse la señal fluorescente, se elimina el grupo protector pudiéndose incorporar un nucleótido marcado, con lo que se empieza de nuevo el ciclo. Desde 2006, su optimización permite generar hasta 600 Gb de bases por ensayo. La tecnología SOLiD (Sequencing by Oligonucleotide Ligation and Detection) se basa en la secuenciación por unión de octámeros marcados de secuencia conocida a la cadena de

ADN, con la posterior detección de la señal fluorescente emitida tras cada ligación. Comercialmente introducido en 2007, utiliza un único proceso de secuenciación catalizado por una ADN ligasa.

Aunque las tecnologías NGS utilizan diferentes procedimientos, todas tienen interés por abaratar aún más los costes de secuenciación y aumentar la fiabilidad de las secuencias resultantes, lo que ha resultado en el desarrollo de los denominados secuenciadores de tercera generación basados en la secuenciación de una única molécula de ADN (Single Molecule Real Time Sequencing, SMRTS). El primer secuenciador de tercera generación lo fabricó Helicos BioSciences y se basa en la secuenciación a tiempo real de miles de millones de pequeñas moléculas únicas de ADN adheridas a una superficie sólida. La tecnología desarrollada por Pacific Bioscience emplea un enfoque diferente al resto de técnicas de secuenciación masiva ya que la ADN polimerasa se ancla a los nanoporos de una superficie sólida. El tamaño del nanoporo es tal que a través de él solo puede pasar una hebra de ADN. Al aplicar una corriente de iones a través del nanoporo, las características eléctricas de cada uno de los nucleótidos que constituyen el ADN generan una firma eléctrica distintiva.

Hasta la aparición de las tecnologías de cuarta generación, todos los procedimientos se basan en la detección óptica de la incorporación de un nuevo nucleótido. La tecnología Ion Torrent usa un chip semiconductor con más de un millón de micropocillos que contiene un ADN molde y una ADN polimerasa. El sistema registra los cambios de pH que se producen al liberarse un protón cada vez que un nuevo nucleótido se une al ADN molde. Otra tecnología, encuadrada en los secuenciadores de cuarta generación, es la desarrollada por ZS Genetics, que utiliza la microscopia electrónica y permite leer la secuencia de ADN directamente sobre una imagen electrónica. La lectura de la secuencia requiere la replicación previa de una hebra molde de ADN para poder marcarla con bases modificadas con yodo, bromo o triclorometilo antes de analizarlas.

Debido a la gran cantidad de datos que genera el empleo de estas técnicas es necesaria la utilización de herramientas específicas que faciliten su análisis. Las plataformas metagenomics-RAST (Meyer et al. 2008), QIIME (Caporaso et al. 2010), MOTHUR (Schloss et al. 2011), Ribosomal Database Project (RDP) (Cole et al. 2013) cumplen esta función. Para más información sobre las tecnologías NGS, así como las diferentes herramientas para el análisis de los datos, el lector puede consultar las

revisiones de Shokralla et al. (2012), Liu et al. (2012b), Mardis (2013), Salipante et al. (2014), Anders et al. (2014) y Blankenberg et al. (2015).

Desde la aparición de las técnicas NGS se han realizado múltiples análisis metagenómicos en ecosistemas acuáticos (DeLong et al. 2006; Quaiser et al. 2011; Yau et al. 2013), suelos (Tringe et al. 2005b; DeAngelis, 2010; Pacchioni et al. 2014) y ambientes extremos (Simon et al. 2009; Bodaker et al. 2010; Hamilton et al. 2014). En cuanto a los genes de la desnitrificación, Saarenheimo et al. (2015a) amplificaron mediante pirosecuenciación los genes *nirS*, *nirK* y *nosZ* de los sedimentos de diferentes lagos boreales. También mediante pirosecuenciación Mackelprang et al. (2011) y Andreote et al. (2012) analizaron la presencia de genes relacionados con el ciclo del N en sedimentos de un permafrost de Alaska y en manglares de Brasil, respectivamente, y Cobo-Díaz et al. (2015) estudiaron los genes implicados en el metabolismo del N en suelos de un encinar quemado. Otros autores han utilizado la plataforma Illumina para analizar la diversidad del gen *nosZ* en suelos agrícolas de USA (Orellana et al. (2014) y el metagenoma y el metatranscriptoma de los microorganismos implicados en el ciclo biogeoquímico del N en sedimentos del golfo de Méjico (Bristow et al. 2015).

7.5. Índices para estudiar la biodiversidad microbiana

La mayoría de los datos sobre diversidad microbiana se basan en los conocimientos adquiridos durante los estudios de biodiversidad realizados con organismos eucariotas y, como en ellos, se utilizan parámetros que permiten cuantificar el número de especies y su representatividad, de manera que sea posible comparar, y comprobar de forma estadística, la diversidad de distintos ecosistemas o la diversidad de un mismo ecosistema a través del tiempo.

Mientras que en los seres eucariotas una especie contiene individuos que pueden reproducirse entre ellos para dar lugar a una descendencia fértil, en los dominios Bacteria y Archaea no existe una definición similar de especie ya que se reproducen por partición binaria, que no requiere compatibilidad sexual. Además, Bacteria y Archaea pueden transferir ADN a otros bacterias con los que no están filogenéticamente relacionados, por lo que no existe indicación de una clasificación ordenada. Debido a la dificultad para definir el propio concepto de especie bacteriana se acuñó el término Unidad Taxonómica Operativa (Operational Taxonomic Unit, OTU) para establecer el nivel taxonómico que se confiere a una muestra en estudio, tal como individuo, población, especie, género, cepa, etc. A esta distinción se llega mediante análisis de

genes marcadores, habitualmente el 16S rARN, y un porcentaje umbral de semejanza que permite clasificarlos en el mismo o diferente OTU. Cuando se quiere determinar la diversidad bacteriana de una muestra medioambiental a partir de las secuencias de los individuos que componen la población se acepta que cada OTU corresponde a un grupo de secuencias con al menos el 97% de similitud, nivel que tradicionalmente se ha homologado al de especie (Schloss y Handelsman 2005).

Si se acepta que un ecosistema puede estar ocupado por distintas comunidades, que cada comunidad está formada por distintas poblaciones y que cada población incluye distintos individuos (géneros, especies, cepas, OTUs), al igual que en Ecología de eucariotas, se distinguen tres tipos de diversidad microbiana: alfa, beta y gamma. La primera se refiere a la riqueza de especies de la comunidad y se determina, generalmente, mediante el cálculo de los índices de riqueza de especies, Margalef, Jacknife, Chao1, Simpson, Shannon, Pielou, etc. La diversidad beta indica la tasa de cambio en especies de dos comunidades y refleja, por tanto, la diferencia de composición de las dos comunidades y, habitualmente, se cuantifica mediante los índices de Jaccard, Alatalo, Sørensen, Sokal y Sneatch, etc. La diversidad gamma hace referencia a la riqueza de especies del conjunto de comunidades e integra los componentes alfa y beta de la diversidad (Whittaker 1972). Para una revisión sobre determinación de la biodiversidad alfa, beta y gamma se recomienda los trabajos publicados por Johnsen et al. (2001), Moreno (2001), Hill et al. (2003), Hughes y Bohannan (2004), Magurran (2004), Chao et al. (2005), Lemos et al. (2011), Bohmann et al. (2014) y Bunge et al. (2014).

8. El Espacio Natural de Doñana

Doñana es uno de los más grandes e importantes humedales de Europa. Consiste en un amplio complejo de marismas costeras en la depresión del bajo Guadalquivir separada del océano Atlántico por un sistema de dunas tanto activas como inactivas. El Espacio Natural de Doñana (END) engloba un área de 104.970 ha, de las que 54.251 pertenecen al Parque Nacional y 53.835 al Parque Natural.

Debido a la variedad de ecosistemas y al alto número de especies que alberga, Doñana se declaró Parque Natural en 1969 y Reserva de la Biosfera por la Unesco (superficie de 77.260 hectáreas) en 1980. En 1994 la UNESCO lo declaró patrimonio de la Humanidad y en 2012 se aprobó la ampliación a más de 255.000 hectáreas como reserva de la biosfera. De esta forma se añade una zona de transición en la que se

incluyen los términos municipales que engloban la comarca de Doñana. También se ha declarado área de especial protección para las aves por la UE y humedal de especial interés internacional por parte de la convención Ramsar. Las marismas de Doñana se alimentan del agua del arroyo de la Rocina y del caño del Guadiamar, de la de lluvia que cae, sobre todo durante el otoño y el invierno, y de las inundaciones ocasionales que provoca el océano Atlántico.

La riqueza de la flora y fauna de Doñana es una de las más altas de Europa, con 875 plantas, 24 especies de peces, 12 de anfibios, 21 de reptiles, 226 de pájaros y 37 de mamíferos, especies todas con alto valor ecológico. Algunas de estas especies están amenazadas como son los casos del lince, la mangosta o el águila imperial.

El espacio natural contiene tanto aguas frescas como salobres, que incluyen marismas permanentes y temporales, lagos y dunas. Las marismas se alimentan principalmente de las lluvias de otoño e invierno y por los ríos, y por tanto presentan diferencias estacionales en su extensión. En Doñana existe un gradiente de salinidad que se incrementa de norte a sur y de oeste a este, el relieve es en general plano con ligeras variaciones. Las marismas de Doñana se consideran de importancia excepcional y hoy día cubren una superficie de 27.000 ha. Las dunas estabilizadas forman un paisaje ondulante cubierto con una vegetación que varía según la altura sobre el nivel freático. La zona sur está cubierta por bosques bajos de la especie *Pinus pinea*. Los matorrales de los arenales dependen de la alta o baja humedad. Entre los matorrales existen pequeñas manchas de árboles que incluyen robles, madroños, sabinas, mirtos y acebuches. La vegetación acuática está distribuida acorde al gradiente de salinidad además de si la zona está siempre cubierta de agua o está temporalmente cubierta.

El clima es mediterráneo con influencia atlántica, con veranos calientes y secos e inviernos fríos y húmedos. La media de temperatura es de 17 ºC y la precipitación media anual es de 580 mm (Espinar y Serrano, 2009). Julio y agosto son los meses más secos y las lluvias se concentran en invierno, alcanzando un máximo de 90 mm en diciembre.

El END es propiedad del estado español y está dedicado principalmente a la conservación de la naturaleza. Se permiten actividades agrícolas y ganaderas compatibles entre las que se encuentran la producción de corcho, la apicultura, la recogida de madera de pinos, recolección de piñones, la pesca y el pastoreo extensivo. La cría de ganado, principalmente vacas y caballos es una actividad tradicional. En el ecotono y las zonas limítrofes del END la principal actividad agrícola es el cultivo del

arroz al que se dedican más de 38.000 ha. Además, hay zonas de intenso turismo en el litoral y el turismo rural es cada vez más frecuente.

En los últimos años ha crecido la preocupación sobre el impacto medioambiental que puede producir el turismo de masas y, sobre todo, el ocasionado por las prácticas agrícolas que emplean exceso de fertilizantes químicos, entre ellos los nitrogenados. La orden conjunta de 7 de julio de 2009 de las consejerías de Agricultura y Pesca y de Medio Ambiente de la Junta de Andalucía modificó la consideración de zonas vulnerables a la contaminación por nitratos de origen agrario e incluyó a Doñana entre ellas por su especial importancia e interés.

La figura 5 corresponde a la imagen área dela Red de Información Ambiental de Andalucía (http://laboratoriorediam.cica.es/VisorGenerico/). En ella se indica la localización a lo largo del arroyo de la Rocina de la laguna de Acebrón y del arroyo de la Cañada, de la marisma del Rocío y del humedal de los Guayules, que han sido los principales sitios donde se tomaron las muestras de aguas y sedimentos que se han utilizado en esta Memoria de Doctorado.

Figura 5. Localización geográfica en el Espacio Natural de Doñana de la laguna del Acebrón (S1), arroyo de la Cañada (S2), vado de la Canariega (S3), marisma del Rocío (S4) y humedal de los Guayules.

OBJETIVOS

El grupo del Metabolismo del Nitrógeno del departamento de Microbiología del Suelo y Sistemas Simbióticos de la Estación Experimental del Zaidín, de la Agencia Estatal Consejo Superior de Investigaciones Científicas, ha sido pionero en la identificación y caracterización de los genes y enzimas de la desnitrificación en la bacteria modelo *Bradyrhizobium japonicum*, y ha revelado parte de los mecanismos moleculares por los que el oxígeno y el nitrato regulan el proceso (Bedmar et al. 2005, 2012, 2013; Bueno et al. 2012).

Como se ha indicado, mientras que los aspectos fisiológicos, bioquímicos y genéticos de la desnitrificación se saben con cierto detalle, sus aspectos ecológicos son menos conocidos. De hecho, apenas existe información sobre la abundancia o escasez de microorganismos desnitrificantes en diferentes hábitats, sobre la comunidad desnitrificante predominante en un momento determinado en un ecosistema concreto, y cómo la actividad funcional de la población desnitrificante podría variar en el tiempo y en el espacio. Igualmente, se desconoce el efecto que la contaminación por nitratos ejerce sobre la actividad, abundancia y distribución espacial de las poblaciones desnitrificantes en el suelo.

La vida microbiana en la mayoría de los ecosistemas terrestres y acuáticos es desconocida, aunque son los microorganismos los que llevan a cabo multitud de transformaciones de unos compuestos en otros, alterando su composición y propiedades, y participando directamente en la productividad de los mismos. Puesto que la biodiversidad microbiana es fundamental para el funcionamiento de tales ecosistemas, existe la necesidad de entender la variabilidad en la estructura y en las funciones de la comunidad microbiana en respuesta a los factores medioambientales que puedan modificarla. Esto es de capital interés en el ciclo biogeoquímico del N en la biosfera ya que los microorganismos son los únicos seres vivos que llevan a cabo las reacciones de óxido-reducción que lo conforman. La contaminación por nitratos de aguas, sedimentos, suelos y atmósfera es cada vez más frecuente e intensa, a lo que contribuye, en gran medida, la fuerte presión antropogénica que se ejerce sobre el medio ambiente. Siendo la desnitrificación el único proceso biológico por el que el nitrato se reduce a N_2 que se libera de nuevo a la atmósfera, son muy escasos los datos sobre la biodiversidad, estructura y dinámica de la comunidad de microorganismos desnitrificantes que ocurren en el medio ambiente, y apenas se conocen las diferencias de biodiversidad entre las comunidades desnitrificantes en zonas no contaminadas y contaminadas con nitratos. El exceso de nitratos en suelos y agua resulta en el

incremento de la producción de gases invernadero, sobre todo óxido nitroso, que se libera a la atmósfera durante la desnitrificación. La cuantificación de la producción de óxidos de nitrógeno permitiría estudiar la contribución de zonas contaminadas o no con nitratos a la emisión de tales gases.

Para la realización de este trabajo se eligió el Espacio Natural de Doñana como sistema modelo por la especial protección y conservación que se realiza de sus marismas y humedales. La relativamente escasa presión antropogénica a la que el parque está sometido hace pensar que los resultados obtenidos se deban a causas naturales y no a las ocasionadas por influencia humana.

De acuerdo con lo expuesto, los objetivos de esta Memoria de Doctorado fueron:

1. Determinar la emisión de gases de efecto invernadero, dióxido de carbono (CO_2), óxido nitroso (N_2O) y metano (CH_4), por los sedimentos de aguas contaminadas con nitratos. Determinar el origen de los nitratos contaminantes. Analizar las propiedades físico-químicas y la actividad biológica de los sedimentos.

2. Determinar el impacto de la contaminación por nitratos sobre la abundancia y la distribución espacial de las poblaciones de bacterias desnitrificantes.

3. Estudiar la biodiversidad de bacterias desnitrificantes en sedimentos con diferente concentración de nitratos.

ARTÍCULOS

Artículo I

Título:

Nitrate contamination, physicochemical properties and biological activities in surface waters and sediments of la Rocina stream (Doñana National Park, SW Spain): Greenhouse gas emissions and denitrification

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***G. Tortosa y D. Correa contribuyeron de igual forma a este artículo.**

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Este artículo contiene los resultados relativos al objetivo 1 de esta Memoria de Doctorado.

Resumen

La contaminación de los ecosistemas acuáticos por nitratos, sobre todo los que proceden de las prácticas agrícolas, es cada vez más frecuente e intensa, y puede originar efectos no deseados sobre la salud humana y animal, el medio ambiente y los servicios de los ecosistemas. Para conocer el nivel de contaminación del arroyo de la Rocina, que irriga la marisma del Rocío en el Espacio Nacional Doñana, se analizó el contenido en nitratos en más de 25 sitios a lo largo de su curso. Se seleccionaron cuatro puntos de muestreo con diferente grado de contaminación por nitratos y se tomaron muestras de las aguas y sedimentos del arroyo en los meses de octubre de 2008 y enero, abril y julio de 2009. La conductividad eléctrica, el pH, el contenido en macro- y micro-elementos y en C y N orgánico total presentaron variaciones en cada uno de las fechas y sitios de muestro debido a los cambios en las condiciones climáticas e hidrológicas que se producen a lo largo del año. Estos cambios estacionales también afectaron las actividades biológicas de los sedimentos, entre ellas la actividad deshidrogenasa, β-glucosidasa, aril-sulfatasa, fosfatasa y ureasa. La emisión de gases de efecto invernadero, especialmente la emisión de N_2O, se correlacionó con el contenido en nitratos, y se observó un aumento en la producción de gases en la época seca, fecha en la que la concentración de nitratos era más elevada.

El sitio de muestreo con mayor contenido en nitrato fue el arroyo de la Cañada donde se determinaron entre 61.6 y 106.6 mg L^{-1}. El análisis isotópico del $\delta^{15}N\text{-}NO_3^-$ indico que este nitrato fue de origen inorgánico, probablemente debido al empleo de fertilizantes sintéticos.

Abstract

Climatic influence (global warming and decreased rainfall) could lead to an increase in the ecological and toxicological effects of the pollution in aquatic ecosystems, especially contamination from agricultural nitrate (NO_3^-) fertilizers. Physicochemical properties of the surface waters and sediments of four selected sites varying in NO_3^- concentration along la Rocina Stream, which feeds Marisma del Rocio in Doñana National Park (South West, Spain), were studied. Electrical conductivity, pH, content in macro and microelements, total organic carbon and nitrogen, and dissolved carbon and nitrogen were affected by each sampling site and sampling time. Contaminant NO_3^- in surface water at the site with the highest NO_3^- concentration (ranged in 61.6-106.6 mg L^{-1}) was of inorganic origin, most probably from chemical fertilizers, as determined chemically (90% of the total dissolved nitrogen from NO_3^-) and by isotopic analysis of ^{15}N-NO_3^-. Changes in seasonal weather conditions and hydrological effects at the sampling sites were also responsible for variations in some biological activities (dehydrogenase, β-glucosidase, arylsulphatase, acid phosphatase and urease) in sediments, as well as in the production of the greenhouse gases CO_2, CH_4 and N_2O. Both organic matter and NO_3^- contents influenced rates of gas production. Increased NO_3^- concentration also resulted in enhanced levels of potential denitrification measured as N_2O production. The denitrification process was affected by NO_3^- contamination and the rainfall regimen, increasing the greenhouse gases emissions (CO_2, CH_4 and especially N_2O) during the driest season in all sampling sites studied.

Keywords

Doñana National Park, Surface waters and sediments, Nitrate contamination, Greenhouse gases, Biological activities, Denitrification

Introduction

Anthropogenic influence on the biogeochemical N cycle can produce important alterations of the cycle leading to concomitant environmental risks such as increased concentration of greenhouse gases, acidification of soils, streams and lakes, transfer of nitrogen through rivers to estuaries and coastal oceans, accelerated losses of biological diversity and human health and economy problems (Vitousek et al., 1997, Galloway et al., 2008 and Mulholland et al., 2008). In aquatic ecosystems, water acidification, eutrophization, including occurrence of toxic algae, and toxicity of ammonia (NH_3), nitrite (NO_2^-), and nitrate (NO_3^-) are the three major environmental problems due to inorganic nitrogen pollution (Camargo and Alonso, 2006). Furthermore, increasing global warming and decreased rainfall in some continental areas may increase ecological and toxicological effects of this type of environmental contamination (Camargo and Alonso, 2006). Abuse in utilization of nitrogenous chemical fertilizers has been shown to enhance emission of carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) greenhouse gases (Thornton and Valante, 1996, Merbach et al., 1996, Merbach et al., 2001,Davidson and Verchot, 2000 and Liu and Greaver, 2009). In addition to chemical fertilizers, release of greenhouse gases to the atmosphere can be induced by changes in precipitations, temperature, seasons, drought, regional deforestation, global warming, and El Niño events (Christensen et al., 1990, Smith et al., 2003 and Davidson et al., 2004).

Wetlands are among the most important ecosystems on Earth because of their role in regulating global biogeochemical cycles. Climate change and anthropogenic effects may have significant impacts on coastal and inland wetlands (Mitsch and Gosselink, 2007 and Olías et al., 2008). Accordingly, physicochemical and biological monitoring is needed for assessment of ecological risks due to freshwater pollution and to provide maximal information for adequate protection of aquatic ecosystems (Camargo, 1994). Several authors and reports have shown that NO_3^- contamination of soils and surface and groundwater is becoming more intense and frequent due to the great consume of inorganic nitrogen, mainly nitrate and ammonium salts from agrochemicals, of the intensive farming (Spalding and Exner, 1993 and European Commission, 2002). Denitrification is the biological process by which NO_3^- can be transformed into molecular nitrogen (N_2) via formation of NO_2^- and nitric oxide (NO). Thus, it represents the major pathway by which NO_3^- can be removed from soils and

waters to avoid NO_3^- accumulation and contamination. And yet, incomplete denitrification results in the production of the greenhouse gases NO and N_2O (Aulakh et al., 1992, Conrad, 1996 and Groffman et al., 2006).

The European directive 91/676/CEE concerning NO_3^- contamination from agricultural sources defines the so-called “nitrate vulnerable zones” as reference areas of special environmental protection to prevent soil and water nitrate contaminations. An example is Doñana National Park (DNP), one of the most important wetlands in Europe covering an area around 60,000 ha in a marshy area of SW Spain, in the estuary of the Guadalquivir River. These water flows are susceptible of NO_3^- contamination from small urban areas in the surrounding of the park and agricultural practices allowed in the ecotone, where organic farming of strawberries and rice is common. This area is the most fertile and productive zone of Doñana as a result of its permanent humidity and of the fertilization it receives from the animals either living there or crossing it (Suso and Llamas, 1993).

Several authors have noted that surface and groundwater of DNP wetland are becoming polluted during the last 20 years. Suso and Llamas (1993) remarked that some wetlands and small streams could be depleted by groundwater extraction for agricultural reclamation, affecting negatively the quality of surface and groundwater. Olías et al. (2008) evaluated the water quality of the Almonte-Marismas aquifer (upon which DNP is located) and showed that it was affected by pollution of both agricultural and urban origins. They detected some shallow points located in the agricultural zones with high concentrations of NO_3^- and sulphates (SO_4^{2-}) from fertilizer pollution. Finally, Serrano et al. (2006) reviewed the aquatic systems of DNP and they focused on processes affecting water quality. They noted that there has been a considerable increase of NO_3^- concentration in the water flows of La Rocina and El Partido Streams during the past decade, probably due to the increase in cultivated land and fertilizer applications. They advise that the influence of this pollution on the eutrophication of the nearby marshes should not be overlooked.

Our research aim was to evaluate the anthropogenic (especially from agriculture) and seasonal influence in La Rocina Stream and how it could be affected by the physicochemical and biological characteristics of the surface water and its aquatic sediments, focusing in the NO_3^- contamination and its influence on the greenhouse gas production (CO_2, CH_4 and N_2O) and the denitrification process.

Materials and methods

Description and selection of sampling sites

In 1982, DNP was declared a Reserve of the Biosphere by UNESCO and a Wetland of International Interest per RAMSAR Convention. DNP wetland has three important surface water inputs (Arambarri et al., 1996 and Serrano et al., 2006). Two natural streams called La Rocina and El Partido, in the north edge of the park, and the Guadiamar River, which represents the main water input of the wetlands and suffered the Aznalcollar mine spill in 1998 (Cabrera et al., 1999, Grimalt et al., 1999, Sierra et al., 2003 and Olías et al., 2005).

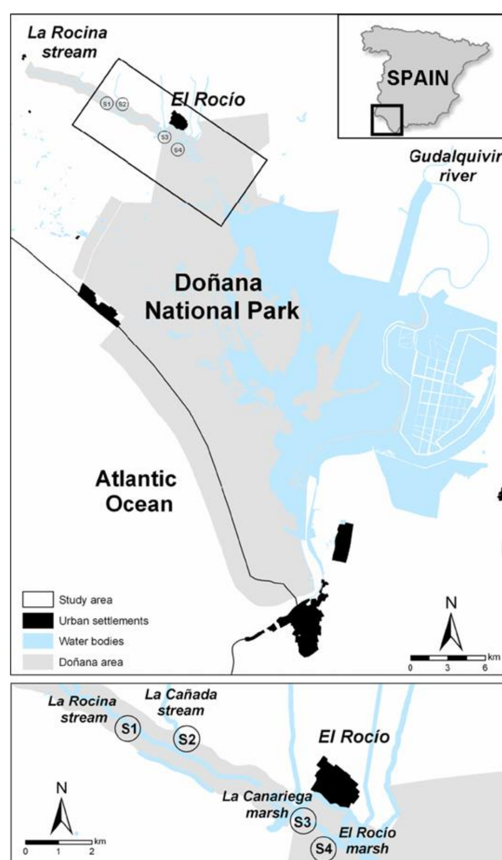

Fig. 1. Geographical situation of Doñana National Park (DNP) and La Rocina Stream. Sampling sites: Palacio del Acebrón (S1), Arroyo de la Ca˜nada (S2), Vado de la Canariega (S3) and Marisma del Rocío (S4).

The study was performed on La Rocina Stream, located in the north of the DNP wetland, which is one of the main natural streams feeding El Rocío marsh (Fig. 1).

Selection of sampling sites was based on their NO_3^- content (in situ measurements using a Nitrate Test Kit, CHEMetrics Inc.) after screening of more than

25 points along the course of La Rocina basin (462 km^2) at the different sampling times. Four sampling sites along the course of La Rocina stream differing in their NO_3^- concentration were selected (Fig. 1). The Universal Transverse Mercator (UTM) coordinates for the sites were as follows: 29S 0718632, 4114294 for the lagoon of Palacio del Acebrón (S1); 29S 0717797, 4113881 for the small stream Arroyo de la Cañada (S2); 29S 0722653, 4111704 for the junction between the stream and the marsh called Vado de la Canariega (S3); and 29S 0723654, 4111088 for the El Rocío marsh (S4). S1 and S2 had the lowest and the highest NO_3^- concentration, respectively, whereas the values detected in S3 and S4 were between those detected in S1 and S2. Also, differences on hydrological morphology (S1: a small stream, S2: a lagoon, S3: the union between a stream and a marsh, S4: a marsh) and in riparian vegetation (not present in S3 and S4) were noted.

Samples were taken in October 2008 (T1) and January (T2), April (T3) and July (T4) 2009 in order to represent the pluvial regimen (dry and wet). Rainfall, relative humidity and air temperature were collected from the Manecorro RM1 meteorological station, which belongs to the Singular Scientific and Technological Installation (http://icts.ebd.csic.es/) of Doñana National Park located about 200 m away from S3, and from Estación Manual Palacio de Doñana (EM05, http://www-rbd.ebd.csic.es).

Physicochemical properties

Four replicates of the surface waters (approximately 1–2 m from the shore in streams and 3–4 m in lagoons for each replicate) and semi-disturbed sediments (0–10 cm from the upper layer using an EIJKELKAMP Peat sampler) were taken at each sampling site, placed in a portable fridge and processed in the laboratory within 24 h of sample collection. Subsets of samples from the sediments were lyophilized and kept frozen at 20º C until use.

In water samples, pH and electrical conductivity (EC) were analyzed using a Basic 20 Crison pHmeter and a Basic 525 Crison conductimeter at the laboratory, respectively. After filtration through 0.45 µm filters, dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were determined using an automatic Shimadzu TOC-VCSN analyzer. NO_3^- and NO_2^- concentrations were estimated by ion chromatography (HPLC) using an IC-Pac anion HC (Waters) column at the facilities of Servicio de Instrumentación (EEZ-CSIC). NH_4^+ was determined by a colorimetric method based on Berthelot's reaction (Kempers and Zweers, 1986 and Sommers et al.,

1992), adding sodium citrate to complex divalent cations. Macro and microelements (P, K, Ca, Mg, Na, S, Fe, Cu, Mn, and Zn) were determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) using an IRIS Intrepid II XDL (Thermo Fisher Scientific Inc.).

Texture of the sediments was determined in fresh samples according to the Spanish Official Methods for Soils and Waters (MAPA, 1974). NH_4^+, after 2 h extraction 1:20 (w/v) with 2 N KCl, and water extracted (1:20, w/v) NO_3^- and NO_2^- were also analyzed in fresh samples as indicated above. All other assays were performed in ground samples (0.2 mm) after lyophilization. pH and EC were measured after water extraction (1:5, w/v) for 2 h. Total organic carbon (TOC) and total nitrogen (TN) were determined using a LECO TruSpec CN Elemental Analyzer. DOC and TDN were obtained after 2 h water extraction 1:20 (w/v) and estimated as indicated for surface waters. Macro and microelements were analyzed by ICP-OES after microwave digestion with a mixture (1:1) HF:HCl. Organic nitrogen (N_{ORG}) was calculated as the difference between either TDN in surface waters or TN in sediments, and the content in inorganic nitrogen (N_{INORG}), considering N_{INORG} as $N\text{-}NO_3^- + N\text{-}NO_2^- + N\text{-}NH_4^+$.

Enzymatic analysis in sediments

A selection of some enzymatic activities related to the metabolic activity and the main biogeochemical cycles were measured in the freeze-dried sediments. Dehydrogenase was used as an estimation of overall microbial activity, β-glucosidase as the enzyme that catalyses the hydrolysis of disaccharides (C cycle), arylsulphatase as a measure of the enzymes catalyzing the hydrolysis of organic sulphate esters (S cycle), acid phosphatase as a measure of the enzymes responsible for the hydrolysis of phosphate esters (P cycle), and urease which catalyses the hydrolysis of urea to CO_2 and NH_3 (N cycle). Dehydrogenase was determined according to García et al. (1997), β-glucosidase, arylsulphatase and acid phosphatase, were determined as described by Tabatabai (1982) and urease activity was determined according to Kandeler and Gerber (1988). Briefly, these techniques were based on a controlled incubation of the sediments after adding the initial substrate (INT: 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-tetrazolium for dehydrogenase, *p*NG: 4-nitrophenyl-beta-D-glucopyranoside for β-glucosidase, *p*NPS:*p*-nitrophenyl sulphate for arylsulphatase, *p*NPP: 4-nitrophenyl phosphate for acid phosphatase and urea for urease activity, respectively) and measuring

the ending product of each enzyme reaction colorimetrically (INTF: iodonitrotetrazolium formazan for dehydrogenase, *p*NP: *p*-nitrophenol for β-glucosidase, arylsulphatase, acid phosphatase and NH_4^+, measured as described above for water and sediment samples, for urease activity).

Gas emission (CO_2, CH_4 and N_2O) and denitrification potential of the sediments

The emission of CO_2, CH_4 and N_2O were measured after 24 h aerobic incubation (25 °C) of 20–30 g of the freshly collected sediments in 125 mL glass bottles. Gas concentrations were analyzed in the headspace by a Varian 4900 Gas Chromatograph with a PoraPlot Q column (10 m length, 0.15 mm internal diameter) and thermal conductivity detector (TCD). Denitrifying Enzyme Activity (DEA) and Denitrification Potential (DP) were also determined in the fresh sediments using an acetylene inhibition technique adapted fromSimek et al. (2004). DEA is a measure of denitrifying enzymes in the sediment and reflects whether the environmental conditions of the sediments at the moment of sampling would induce the activity of the denitrifying bacteria, whereas DP represents a long-term denitrification potential, allowing the maximum regrowth of denitrifying bacteria (Tiedje, 1994). DEA was determined using an anerobic slurry prepared by mixing 25 g moist sediment and 25 ml of a solution containing 1 mM glucose, 1 mM KNO_3 and 1 g L^{-1}chloramphenicol (to prevent protein synthesis and growth) in a 125 mL glass bottle. The headspace was evacuated and flushed four times with He and 10 ml of acetylene were added. The samples were shaken at 25 °C and the concentration of N_2O was measured in the headspace after 30 and 60 min of incubation by gas chromatography, as previously described. DEA was calculated from the N_2O increase during a half an hour incubation (60–30 min) and using the Bunsen coefficient for the N_2O dissolved in water. DP was determined by mixing 6 g moist sediment with 5 mL of a solution containing 1 mM $KNO_3$and 1 mM glucose in a 125 mL glass bottle. After evacuating and flushing the headspace four times with He, 10 ml of acetylene were added and the samples were incubated at 25 °C during 48 h. DP was calculated from the N_2O increase in the headspace after the second day of incubation and using the Bunsen coefficient for the N_2O dissolved in water.

Isotope measurements

$\delta^{15}N$ of NO_3^- was determined following the methodology described by Silva et al. (2000)with modifications. Water samples (10–30 l) were first filtered through

Whatman filter paper and then passed through 0.45 μm filters (High Capacity GWV, Groundwater Sample Filter). Possible interferences from sulphate and phosphate in the samples were eliminated by adding an excess of $BaCl_2$, and dissolved CO_2 was removed by adding HCl and gentle heating. Water samples were then eluted through a cation exchange resin (AG 50W X8 100–200 mesh, Bio-Rad) to remove dissolved organic matter and the excess of Ba^{2+}, and passed through an anion exchange resin (AG1 X8 100–200 mesh, Bio-Rad) to retain NO_3^-. Finally, nitrate was eluted from the column by adding 1 N HCl, and the solution containing HNO_3 and HCl was neutralized with Ag_2O (Merck). The resulting AgCl precipitate was removed by filtration (0.45 mm membrane filter) leaving only Ag^+ and NO_3^- in solution. The solutions were freeze-dried yielding a pure, dry $AgNO_3$ precipitate.

Nitrogen isotope ratios and total nitrogen contents of $AgNO_3$ precipitates were determined by thermal de-composition in a Carlo Elba NC1500 elemental analyzer on line with a Delta Plus XL (ThermoQuest) mass spectrometer (EA-IRMS). The overall precision of analyses was ±0.1‰ for $\delta^{15}N$. The stable composition is reported as δvalues per mil: $\delta = (R_{sample}/R_{standard} - 1) \times 1000$, where $R = {}^{15}N/{}^{14}N$ for $\delta^{15}N$. Nitrogen contents of the $AgNO_3$ samples were typically ~8%, indicating that no major contaminants were present in the precipitate. Commercial N_2 was used as the internal standard for the nitrogen isotopic analyses, contrasted with the international standard.$\delta^{15}N$ values for all samples were normalized against internationally accepted reference materials (IAEA N1, $\delta^{15}N$ = +0.4‰, IAEA N2, $\delta^{15}N$ = +20.3‰). The nitrogen isotope ratios of $AgNO_3$ generated from dissolved IAEA-NO-3 potassium nitrate were within +4.65‰ (n = 12), similar to the accepted value. Duplicate nitrogen isotope ratio determinations on $AgNO_3$ from laboratory nitrate samples were performed with a precision generally better than ±0.2‰. $\delta^{15}N$ values are reported with respect to air.

Statistical analysis

Differences between the different physicochemical parameters were checked out using the analysis of variance (ANOVA) and the Tukey post hoc test at $p < 0.05$. Pearson coefficients were calculated to obtained correlation between variables using the SSPS 17.0 program for Windows XP. A principal component analysis (PCA) was performed to analyze relationships among parameters concerning physicochemical characterization of the sediments, their enzymatic activities and gas production.

Table 1a. Physicochemical properties of the surface waters at the sampling sites.

Sampling Site	Times	pH	[1]EC	[2]DOC	[2]NO_3^-	[2]NO_2^-	[2]NH_4^+	[2]P	[2]K	[2]Ca	[2]Mg	[2]Na	[2]S	[2]Fe	[2]Cu	[2]Mn	[2]Zn
S1	T1	6.62b	0.63a	17.7a	0.2c	nd	0.1	<0.6	9.0a	49.3a	20.9a	66.8a	61.2a	0.0d	nd	0.9	<0.01
	T2	6.57b	0.26c	16.7b	0.3b	0.6	nd	<0.6	4.8c	12.6b	6.0c	43.8b	7.0c	0.3a	0.1	<0.01	0.3
	T3	6.99b	0.12d	5.7d	0.6a	nd	nd	<0.6	2.0d	7.2c	3.3d	16.8c	2.4d	0.1c	0.1	<0.01	0.2
	T4	7.60a	0.33b	8.9c	nd	nd	nd	<0.6	5.2b	3.4d	8.6b	8.6d	46.6b	0.2b	<0.01	<0.01	0.2
S2	T1	7.75c	0.52c	28.3a	61.6d	0.1c	0.1	<0.6	10.9c	38.5b	6.8d	20.5c	16.2c	nd	nd	<0.01	<0.01
	T2	7.15d	0.57b	3.6c	106.6a	nd	nd	1.9a	16.0b	36.5c	11.2c	28.7b	18.5c	<0.01	0.1	0.1	<0.01
	T3	8.89a	0.62a	6.7b	101.6b	0.5b	0.1	1.2b	27.7a	56.9a	20.3b	51.6a	30.0b	<0.01	0.1	0.1	<0.01
	T4	7.96b	0.50d	3.1c	68.8c	1.2a	nd	0.6c	14.4b	15.1d	58.7a	16.6c	46.2a	0.1	<0.01	0.1	0.1
S3	T1	8.03b	1.22b	68.1b	1.9	0.5	0.3b	<0.6	24.3b	71.2a	25.3b	144.2a	84.1b	nd	<0.01	<0.01	nd
	T2	7.30c	0.44c	12.3c	3.0	0.4	nd	<0.6	5.5d	24.2d	7.9b	37.9c	14.9b	nd	0.1	<0.01	0.1
	T3	8.38a	0.34d	21.0c	nd	nd	0.2b	<0.6	9.7c	28.8c	12.2b	53.1b	9.2b	0.52	0.3	<0.01	0.5
	T4	8.03b	3.11a	135.2a	nd	nd	4.7a	2.3	41.3a	43.0b	30.1a	30.2d	581.3a	0.22	<0.01	<0.01	<0.01
S4	T1	7.95a	0.90a	77.7a	1.1c	0.1a	0.8b	<0.6	21.2a	70.3a	25.6b	103.4a	42.9b	<0.01	<0.01	0.2b	nd
	T2	7.54b	0.34d	6.8d	3.6b	0.2a	0.3d	<0.6	4.5d	23.0c	6.3d	25.4c	9.6d	<0.01	<0.01	0.2b	<0.01
	T3	8.32a	0.60c	12.9c	5.7a	nd	0.1c	<0.6	13.7c	51.4b	21.6c	79.4b	24.7c	<0.01	<0.01	<0.01	<0.01
	T4	7.51b	0.83b	16.3b	nd	nd	0.9a	<0.6	17.3b	14.1d	59.2a	26.0c	105.3a	<0.01	<0.01	1.2a	0.1
S		*	*	*	*	-	*	-	*	*	*	*	*	-	-	-	-
T		*	*	*	*	-	*	-	*	*	*	*	*	-	-	-	-
S x T		*	*	*	*	-	*	-	*	*	*	*	*	-	-	-	-

Sampling sites: Palacio del Acebrón (S1), Arroyo de la Cañada (S2), Vado de la Canariega (S3) and Marisma del Rocío (S4). Sampling times: October 2008 (T1), January 2009 (T2), April 2009 (T3) and July 2009 (T4). For each variable, at a given sampling site, values followed by the same letter are not statistically different according to Tukey´s test at $p \leq 0.05$. [1]Values of electrical conductivity (EC) are expressed in dS cm^{-1}.

[2]Values of dissolved organic carbon (DOC), NO_3^-, NO_2^- and NH_4^+ are expressed in mg L^{-1}. nd: no detected. *: $p \leq 0.05$.

Results

Meteorological data

Total rainfall at DNP during the period of study was 299.9 mm, a value which is lower than that of 477.5 mm, which represents the mean rainfall for the previous 5 years. Main rainfall was registered in October 2008 (119.25 mm) and March–April 2009 (49.49 and 30.90 mm), respectively, coinciding with T1 and T3 sampling times. T2 (January 2009) presented 9.3 mm and T4 (July 2009) 0.11 mm, being the driest season. This pluvial regimen affected water dynamic in the sampling places studied especially at T4, transforming the stream and lagoon waters sampled in swamps (especially in S3 that presented a high eutrophization rate). Relative humidity was higher in autumn and winter seasons (T1: 73.79% and T2: 79.69%) than in spring and summer seasons (T3: 65.38% and T4: 48.68%), decreasing with air temperature (T1: 18.04 °C, T2: 9.73 °C, T3: 14.55 °C and T4: 25.31 °C).

Table 1b. Dissolved nitrogen (DN), dissolved organic carbon (DOC) and organic nitrogen (N_{ORG}) in the surface waters at the sampling sites.

Sampling Site	Times	DN (mg L^-	DOC/DN	N_{ORG}	N-NO_3^-	N-NO_2^-	N-NH_4^+
S1	T1	0.6b	29.5	79.5	7.5	-	13.0
	T2	0.7a	23.9	64.2	9.7	26.1	-
	T3	0.3d	19.0	54.8	45.2	-	-
	T4	0.4c	22.3	100.0	-	-	-
S2	T1	15.6b	1.8	10.1	89.2	0.2	0.5
	T2	15.3b	0.1	3.7	96.3	-	-
	T3	21.6a	0.3	4.2	94.7	0.7	0.4
	T4	9.4c	0.2	8.6	89.3	2.1	-
S3	T1	4.4b	15.5	81.5	9.8	3.5	5.3
	T2	1.1b	11.2	27.3	61.6	11.1	-
	T3	1.2b	17.5	87.0	-	-	13.0
	T4	18.1a	7.5	79.8	-	-	20.2
S4	T1	3.6a	21.6	75.0	6.9	0.8	17.3
	T2	1.1d	6.2	20.6	73.9	5.5	-
	T3	2.1c	6.1	35.0	61.3	-	3.7
	T4	2.3b	7.1	69.6	-	-	30.4

Sampling sites: Palacio del Acebrón (S1), Arroyo de la Cañada (S2), Vado de la Canariega (S3) and Marisma del Rocío (S4). Sampling times: October 2008 (T1), January 2009 (T2), April 2009 (T3) and July 2009 (T4). For DN, at a given sampling site, values followed by the same letter are not statistically different according to Tukey´s test at $p \leq 0.05$. N_{ORG} = DN – N_{INORG}, where N_{INORG} (inorganic nitrogen) = N-NO_3^- + N-NO_2^- + N-NH_4^+. nd, no detected.

Surface waters: physicochemical characterization and isotopic analysis

Surface water showed in general slightly basic pH values, especially in S2, S3 and S4 (average values of 7.94 and 7.83 in S2, S3 and S4, respectively, Table 1a). EC was related directly to total K, Ca, Mg, Na and S concentration in waters, and also to the sampling season. EC values typically varied within the range from 0.12 and 1.22 dS cm^{-1}depending on the sampling season. The highest EC values were generally recorded during rainy the season (T1), with the exception of the large EC value (3.11 dS cm^{-1}) registered in S3 during the driest season (T4), due to the eutrophization caused for the swamp water. In general, soluble organic matter was high at T1 (S1: 17.7, S2: 28.3 and S4: 77.7 mg L^{-1} of DOC) for all sampling sites studied with the exception of S3, that presented 135.2 mg L^{-1} of DOC at T4, due to the high water eutrophication (the P concentration at this location was the highest value for all sampling sites and seasons) which produced an elevated suspended algae content (green water colour by visual observation).

S2 presented higher TDN concentrations (in the range 21.6 and 9.4 mg L^{-1}) than the other three locations S1, S3 and S4 that presented an overall of 0.5, 6.2 and 2.3 mg L^{-1}, respectively (Table 1b). In S3 at the driest season (T4), TDN showed a value of 18.1 mg L^{-1} especially due to the high NH_4^+ content (Table 1a and Table 1b). S2 presented a large NO_3^- concentration (61.6, 106.6, 101.6 and 68.8 mg L^{-1} at T1, T2, T3 and T4, respectively) respect to the other sampling sites studied (less that 6 mg L^{-1}, Table 1a). These NO_3^- concentrations represented between 90 and 97% of the TDN of the surface waters (Table 1b). Also, this fact was noticed in DOC/TDN ratio values, being smaller in S2 (average of 0.6) than in S1, S3 and S4 (23.7, 12.9 and 10.2, respectively). These sampling sites presented an important organic nitrogen fraction (average of 74.6, 68.9 and 50.1%, respectively), not in S2 that was predominantly inorganic (average of 92.4% of NO_3^- respect to TDN content).

With the procedure used in this study, isotopic analysis of N-NO_3^- could be carried out only in S2, the site with the highest NO_3^- concentrations. Values of $\delta^{15}N$ ranged from −1.6 to +6‰ (AIR) with an average of −0.78‰ (AIR). This relatively low value, closed to that of the atmosphere air, indicates that contaminant NO_3^- was of inorganic origin because atmospheric air is used for their synthesis (Vitoria et al., 2004). Moreover, since mean average values of $\delta^{15}N$ for most inorganic Spanish fertilizers vary between −1 and +2‰ (AIR), being the total range between −4 and +6‰

(Otero et al., 2005), the stable isotopes of nitrogen indicate an origin related with fertilizers used in agricultural practices.

Sediments: physicochemical characterization, enzymatic activities and gas production

Sand constituted more than 85% of the components of the sediments. According to the corresponding contents in clay and silt, S1 and S2 were classified as loamy sand sediments, those from S3 as sand, and sandy loam for S4. Similar to surface waters, values of pH, EC and content in macro and microelements in sediments from the four sampling sites varied both among the sites and with the sampling time (Table 2a). Despite these differences, the values of TOC and TN were always higher in S1 than in the remaining sampling sites (Table 2b). Regardless of the sampling sites and sampling times, more than 90% of the nitrogen content in the sediments was of organic origin and, accordingly, the greatest values of DOC and TDN were also found in S1 (Table 2b). Similarly, β-glucosidase, dehydrogenase, urease acid phosphatase and arylsulphatase activities varied greatly with both the sampling sites and the sampling times (Fig. 2). Whereas S4 showed the highest values of dehydrogenase (mean average value of 10.26 μg INTF g^{-1} h^{-1}), acid phophatase (mean average value of 23.5 μg *p*NP g^{-1} h^{-1}), urease (mean average value of 600 μg *p*NP g^{-1} h^{-1}) and arylsulphatase (mean average value of 400 μg *p*NP g^{-1} h^{-1}) activities, S1 was the site with the greatest values of β-glucosidase activity (mean average value of 90 μg *p*NP g^{-1} h^{-1}) (Fig. 2).

Table 2a. Physicochemical properties of the sediments at the sampling sites.

Sampling Site	Times	pH (1:5)	[1]EC	[2]P	[2]K	[2]Ca	[2]Mg	[2]Na	[2]S	[2]Fe	[3]Cu	[3]Mn	[3]Zn
S1	T1	5.44	860	0.12d	5.07c	7.28a	3.09b	0.71b	7.75b	16.39a	11c	252a	25b
	T2	5.51	390	0.45b	5.51b	5.89b	2.88b	0.71b	3.34d	15.40b	25a	163c	27b
	T3	3.98	565	0.83a	4.43d	5.28c	1.96c	0.55c	9.66a	13.47c	11c	98d	44a
	T4	5.90	178	0.32c	6.71a	6.89a	3.40a	0.82a	5.61c	13.42c	11c	189b	22b
S2	T1	5.05d	628a	0.12d	2.92b	5.04bc	1.72a	0.28a	3.80a	10.32b	8a	136a	16b
	T2	7.13c	82c	0.45a	3.21a	3.86c	1.79a	0.24b	0.65b	12.26a	4b	136a	17b
	T3	7.59b	153b	0.21c	1.92c	5.75b	0.92c	0.21bc	0.48c	5.06d	10a	114b	16b
	T4	8.26a	113bc	0.27b	2.02c	36.83a	1.33b	0.21c	0.52c	5.81c	9a	107b	19a
S3	T1	8.58a	90b	0.12b	0.68b	0.44c	0.44bc	0.10b	0.16a	2.57ab	5a	58a	7c
	T2	7.72c	84c	0.45a	0.91b	0.96a	0.62b	0.10b	0.15a	4.11a	4a	82a	11a
	T3	6.75d	39d	0.02c	0.57b	0.25c	0.28c	0.11b	0.14a	2.07b	2a	65a	17b
	T4	8.20b	101a	0.04c	1.29a	0.68b	0.76a	0.25a	0.12a	3.61ab	2a	69a	4d
S4	T1	7.36c	117c	<0.01	1.92d	1.32d	1.08c	0.23a	0.20a	5.66d	3d	140c	13d
	T2	7.55b	165b	<0.01	8.72b	5.58a	5.19a	0.48b	0.67a	26.60a	26a	313a	56a
	T3	6.81d	115c	0.17b	7.61c	2.78c	3.51b	0.49b	0.59a	13.67c	19c	150c	34c
	T4	7.68a	221a	0.33a	10.95a	4.37b	5.12a	0.80a	0.65a	19.37b	23b	256b	46b
S		*	*	*	*	*	*	*	*	*	*	*	*
T		*	*	*	*	*	*	*	*	*	*	*	*
S x T		*	*	*	*	*	*	*	*	*	*	*	*

Sampling sites: Palacio del Acebrón (S1), Arroyo de la Cañada (S2), Vado de la Canariega (S3) and Marisma del Rocío (S4). Sampling times: October 2008 (T1), January 2009 (T2), April 2009 (T3) and July 2009 (T4). For each variable, at a given sampling site, values followed by the same letter are not statistically different according to Tukey´s test at $p \leq 0.05$. [1]Values of EC (electrical conductivity) are expressed in µS cm^{-1}. [2]For each nutrient, values are expressed in g Kg^{-1} (sediment dry weight). [3]For each nutrient, values are expressed in mg Kg^{-1} (sediment dry weight). *: $p \leq 0.05$.

Table 2b. Total organic carbon (TOC), total nitrogen (TN), organic nitrogen (N_{ORG}), dissolved organic carbon (DOC) and dissolved nitrogen (DN) in sediments at the sampling sites.

Sampling	Season	[1]TOC	[1]TN	TOC/TN	N_{ORG} (%)	[2]NO_3^-	[2]NO_2^-	[2]NH_4^+	[2]DOC	[2]DN
S1	T1	183.4a	5.7b	31.9	99.4	nd	nd	41b	1137c	70b
	T2	154.2b	9.0a	17.1	99.5	34	nd	44b	2580a	144a
	T3	99.8c	3.7c	27.3	99.3	7	nd	30c	332d	30c
	T4	206.0a	6.9b	29.8	99.1	nd	nd	79a	1934b	138a
S2	T1	78.4a	3.1a	25.5	99.6	32	nd	6c	280b	22bc
	T2	18.3b	1.0c	18.8	97.5	54	nd	16b	258b	23b
	T3	38.4b	1.6bc	23.7	97.8	3	nd	45a	174c	16c
	T4	22.7b	0.5c	42.9	96.8	nd	nd	20b	412a	42a
S3	T1	3.1a	0.3a	11.7	98.5	32a	nd	6c	163b	15b
	T2	3.4a	0.3a	12.2	97.8	4b	nd	7c	147b	17b
	T3	1.8b	0.2a	8.3	92.3	3b	nd	20b	25c	3c
	T4	1.7b	0.2a	11.0	89.9	nd	nd	26a	206a	33a
S4	T1	5.4d	0.4c	10.8	97.3	nd	nd	14c	310c	27c
	T2	16.6c	1.5b	10.9	98.6	4	nd	25b	382b	43b
	T3	25.2a	1.9a	12.6	97.7	nd	nd	55a	196d	19c
	T4	20.1b	1.9a	10.5	98.8	nd	nd	30b	669a	104a
S		*	*	*	*	-	-	*	*	*
T		*	*	*	*	-	-	*	*	*
S x T		*	*	*	*	-	-	*	*	*

Sampling sites: Palacio del Acebrón (S1), Arroyo de la Cañada (S2), Vado de la Canariega (S3) and Marisma del Rocío (S4). Sampling times: October 2008 (T1), January 2009 (T2), April 2009 (T3) and July 2009 (T4). For each variable, at a given sampling site, values followed by the same letter are not statistically different according to Tukey´s test at $P \leq 0.05$. [1]Values of TOC and TN are expressed in g Kg^{-1} (sediment dry weight). [2]Values of NO_3^-, NO_2^-, NH_4^+, DOC and DN are expressed in mg Kg^{-1} (sediment dry weight). DOC and DN were obtained after 2 h water extraction (1:20, w:v) of the lyophilized sediments. N_{ORG} = TN – N_{INORG}, where N_{INORG} (inorganic nitrogen) = N-NO_3^- + N-NO_2^- + N-NH_4^+. nd, no detected. *: $p \leq 0.05$.

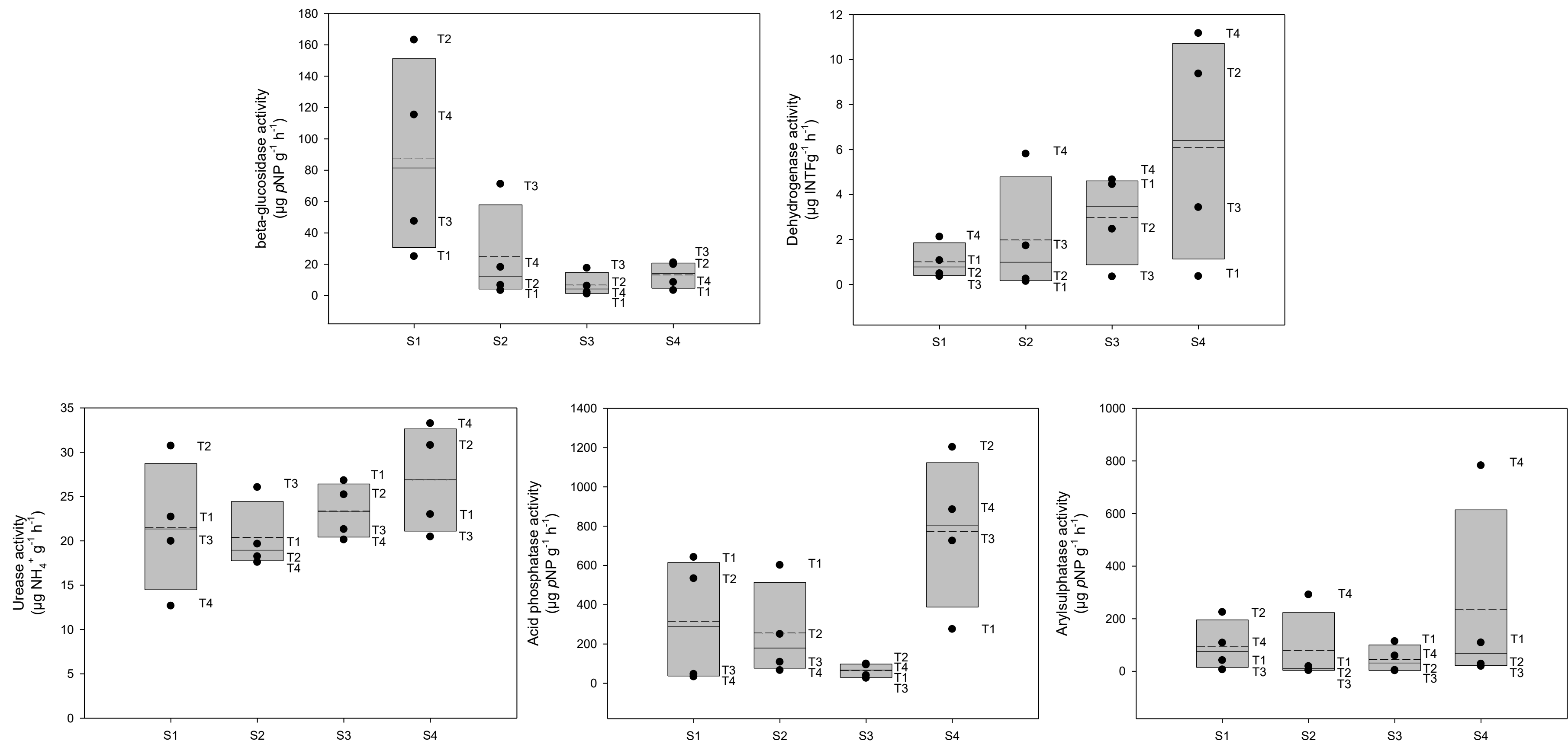

Fig 2. β-Glucosidase, dehydrogenase, urease, acid phosphatase and arylsulphatase activities in sediments. Vertical boxes show the median (dash line), mean (solid line) and the 5th/95th percentiles. Sampling sites: Palacio del Acebrón (S1), Arroyo de la Cañada (S2), Vado de la Canariega (S3) and Marisma del Rocío (S4). Sampling times: October 2008 (T1), January 2009 (T2), April 2009 (T3) and July 2009 (T4).

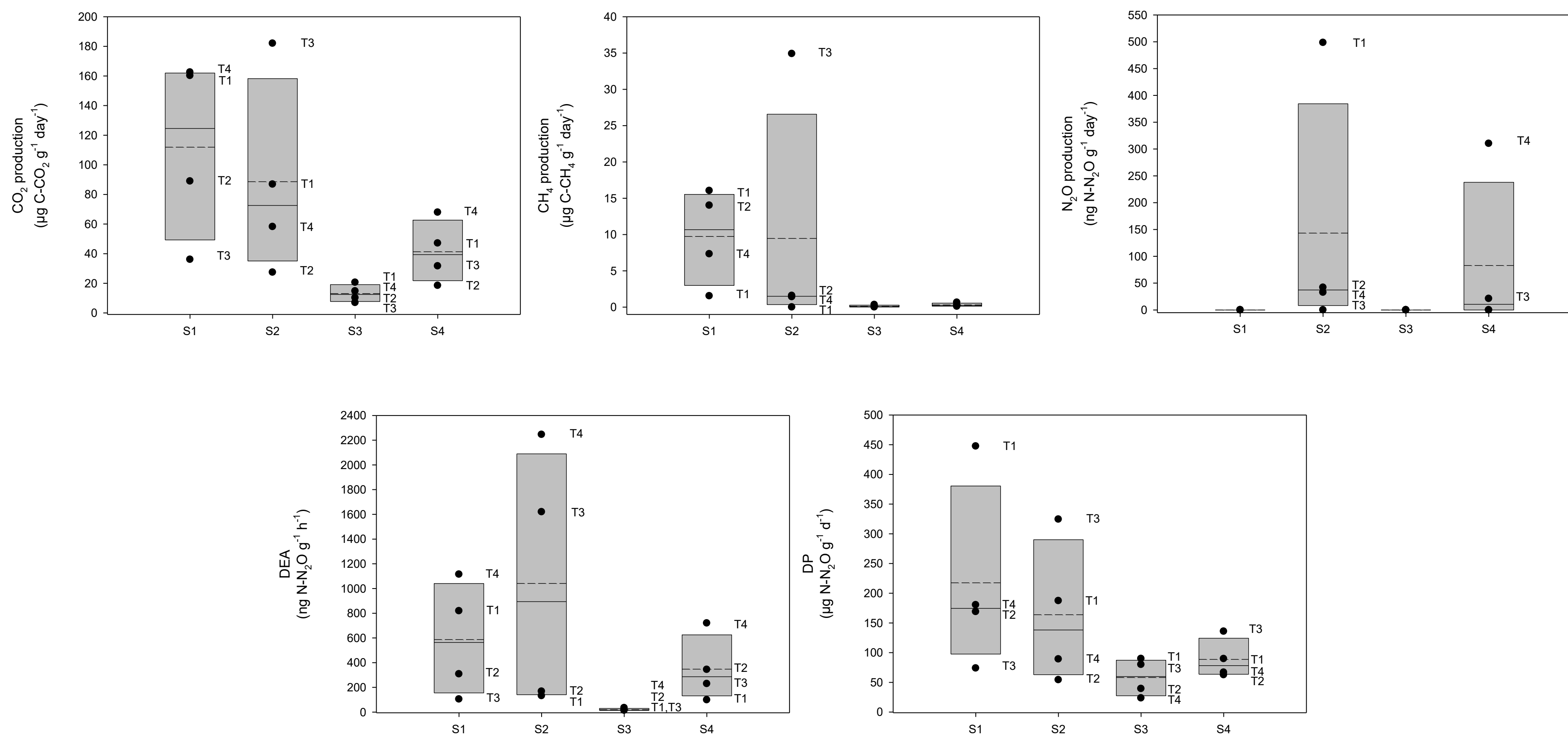

Fig 3. CO_2, CH_4 and N_2O emissions, denitrifying enzymatic activity (DEA) and denitrification potential (DP) in sediments. The vertical boxes show the median (dash line), mean (solid line) and the 5th/95th percentiles. Sampling sites: Palacio del Acebrón (S1), Arroyo de la Cañada (S2), Vado de la Canariega (S3) and Marisma del Rocío (S4). Sampling times: October 2008 (T1), January 2009 (T2), April 2009 (T3) and July 2009 (T4).

The mean average value of CO_2 produced at sampling sites S1, S2, S3 and S4 were 112, 89, 13, and 41 µg C-CO_2 g^{-1} day^{-1}, respectively (Fig. 3). Methane production from the sediments was also higher in S1 (mean average value of 9.7 µg C-CH_4 g^{-1} day^{-1}) than in S3 and S4 (mean average values of 0.2 and 0.3 µg C-CH_4 g^{-1} day^{-1}, respectively). Methane production occurred in S2 was in general low. Unexpectedly, values for S2 detected at T3 were the highest for all samples analyzed (34.9 µg C-CH_4 g^{-1} day^{-1}) (Fig. 3). S2, the site with the highest NO_3^- contents in its surface water, and S4 showed maximal values of N_2O production with mean average values of 500 and 310 ng N-N_2O g^{-1} day^{-1}, respectively. Potential denitrification as assayed by DEA and DP showed that S1 and S2 have the highest potential for denitrification compared to S3 and S4. The highest values for DEA were obtained during the driest season (T4) with 1115, 2246 and 719 ng N-N_2O g^{-1} h^{-1} in S1, S2 and S4, respectively. Despite fluctuations at the sampling times, S1 and S2 also showed maximal values of DP with mean average values of 218, 164, 58 and 89 µg N-N_2O g^{-1} d^{-1} for S1, S2, S3 and S4, respectively.

Statistical analysis

Pearson correlation matrix revealed that TOC, TN, NH_4^+, DOC and TDN were positive and significantly ($p \leq 0.01$) correlated with CO_2 production and with β-glucosidase activity (Table 3). After PCA analysis, except for NO_3^- and pH, the remaining parameters analyzed clustered in 3 main groups (Fig. 4a). The first cluster contained most of the parameters related with the organic fraction of the sediments (TOC, TN, DOC, TDN, NH_4^+, CO_2, CH_4, DP and β-glucosidase activity), the second cluster included the inorganic components (K, Ca, Mg, Fe, Cu, Mn and Zn), and the third cluster was composed of the enzymatic activities dehydrogenase, acid phophatase, urease and arylsulphatase. The first principal component (PC1) explained 36.79% of the total variance of the data, whereas the second principal component (PC2) was responsible for 20.34%. According to these two axes, the sampling sites were ordered as a function of the three clusters mentioned above (Fig. 4b). Consequently, S1 showed the highest values for the organic fraction and S4 for the enzymatic fraction. Nevertheless, sizes of the clusters indicated that seasonal variation affected much more to S1 and S4 than to S2 and S3.

Table 3. Pearson correlation matrix (n=16) between the physicochemical properties, enzymatic activities and greenhouse gas emissions in sediments at four sampling sites along La Rocina stream.

	pH	EC	TOC	TN	NH_4^+	DOC	DN	DH	GC	AS	AP	UR	CO_2	CH_4	DEA	DP
pH																
EC	-0.770**															
TOC	-0.720**	0.676**														
TN	-0.698**	0.601*	0.932**													
NH_4^+	NS	NS	0.663**	0.644**												
DOC	NS	NS	0.815**	0.992**	0.612*											
DN	NS	NS	0.718**	0.815**	0.622*	0.932**										
DH	0.549*	NS	NS	NS	NS	NS	NS									
GC	NS	NS	0.707**	0.839**	0.689**	0.861**	0.736**	NS								
AS	NS	NS	NS	NS	NS	NS	0.521*	0.654**	NS							
AP	NS	NS	NS	NS	NS	NS	NS	0.509*	NS	NS						
UR	NS	NS	NS	NS	NS	NS	NS	0.499*	NS	NS	0.554*					
CO_2	NS	NS	0.714**	0.605**	0.625**	0.516*	NS	NS	0.542*	NS	NS	NS				
CH_4	NS	NS	NS	NS	NS	NS	NS	NS	0.539*	NS	NS	NS	0.804**			
DEA	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.606*	0.506*		
DP	NS	0.641**	0.632**	0.513**	NS	NS	NS	NS	NS	NS	NS	NS	0.854**	0.765**	NS	

**, *: significant at $p < 0.01$ and 0.05, respectively, NS: not significant. EC: Electrical conductivity, TOC: total organic carbon, TN: total nitrogen, DOC: dissolved organic carbon, DN: dissolved nitrogen, DH: dehydrogenase activity; GC, β-glucosidase activity; AS, arylsulphatase activity; AP, acid phosphatase activity; UR: urease activity; DEA: denitrifying enzymatic activity; and DP, denitrification potential.

Discussion

Although to a different extent depending on the sampling site, physicochemical properties of the surface waters sampled along La Rocina Stream were influenced by seasonal variation (especially rainfall regime). These results agree with those of Espinar and Serrano (2009) which indicate that development of temporary wetlands in DNP are influenced by climate and geology of the region. This is especially important in wetlands located in semiarid areas such as the South of Spain, where the climate is unpredictable and produces a wide range of hydrological conditions (Serrano et al., 2006). Thus, it is possible that rainfall, evaporation, groundwater discharge, biogeochemical interactions at the sediment–water interface affected chemical composition of the surface waters along the course of La Rocina Stream. Hydrological dynamic at each sampling site was affected by the sampling time. That was clearly visible in S3 at T4, the driest sampling time, where stream waters were transformed into swampy waters, and in S4 where desiccation almost emptied the El Rocio marsh.

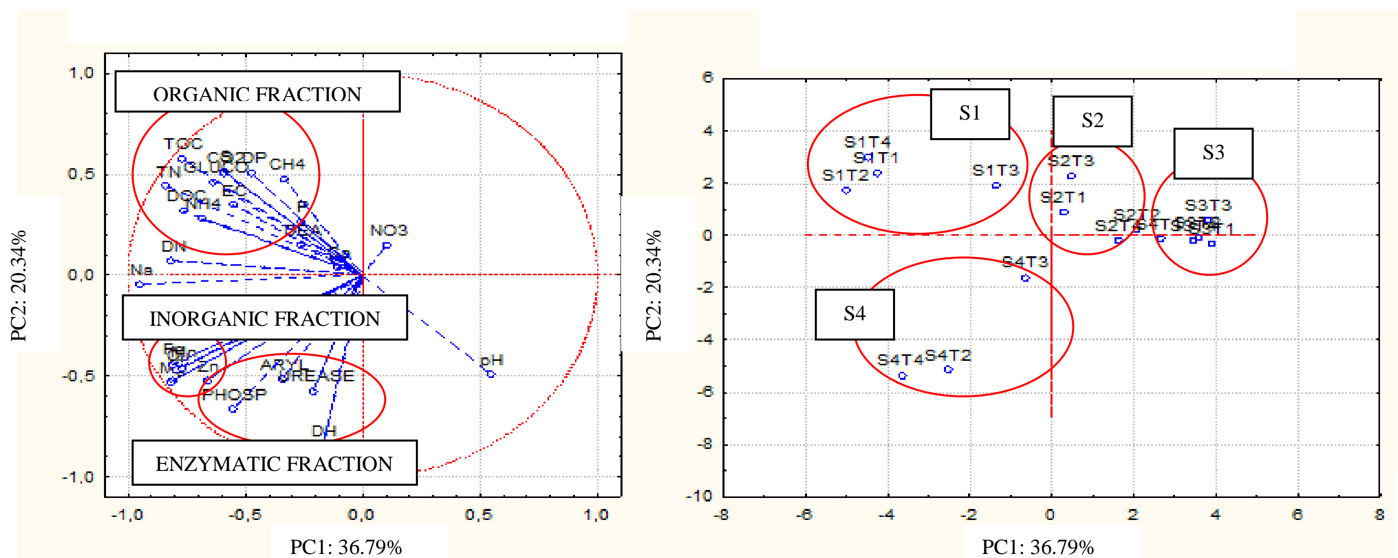

Fig 4. a. Principal Component Analysis performed on the whole set of measured sediments properties and b., the spatial and seasonal distribution of the parameters. Sampling sites: Palacio del Acebrón (S1), Arroyo de la Cañada (S2), Vado de la Canariega (S3) and Marisma del Rocío (S4). Sampling times: October 2008 (T1), January 2009 (T2), April 2009 (T3) and July 2009 (T4).

Several authors have reported continuous increases in pollution (Suso and Llamas, 1993 and Olías et al., 2008), and more precisely in NO_3^- content, in surface- and groundwater of DNP during the last two decades (González-Quesada et al., 1987 and Serrano et al., 2006). Recently, contamination due to NO_3^- and SO_4^{2-} in the Doñana aquifer has been linked to utilization of agrochemicals during the agricultural practices that take place in the ecotone of the Park (Olías et al., 2008). Values of NO_3^- content in S2 were higher than the 50 mg L^{-1} defined by the European directive 91/676/CEE as the upper limit for NO_3^- contamination from agricultural sources (European Commission, 1991). At that site, concentration of inorganic N represented more than 90% of the TDN. In this study, based on isotopic analysis of the contaminant NO_3^- in surface waters of La Rocina Stream, we show that, at least in S2, they were of inorganic origin, more probably from chemical fertilizers.

The interpretation of the nutrients dynamic in aquatic ecosystems could be biased by the strong effects of hydrology on physicochemical (Espinar and Serrano, 2009). For that, microbiological processes involved in the principal biogeochemical cycles are needed (Faulwetter et al., 2009). Soil microorganisms mediate many processes that are of particular interest in freshwater wetland ecosystems where nutrient cycling is highly responsive to fluctuating hydrology and nutrients and soil gas releases may be sensitive to climate warming (Gutknecht et al., 2006). Determination of enzymatic activities in sediments of La Rocina Stream varied both among sampling sites and among sampling times. Although determinations of enzymatic activities in sediments are relatively scarce, previous analyses have shown they vary widely across the different wetland ecosystems examined (Gutknecht et al., 2006). In our study, dryness and temperature positively affected dehydrogenase, as values of activity were always greater at T4. Similarly, β-glucosidase activity correlated significantly with the content of the organic matter fraction, as the highest values of activity were detected in S1 and S2, the sites with the highest TOC concentrations. Similar results were reported by Williams and Jochem (2006) who showed that, despite the complex relationships between biological and environmental parameters, the kinetic of several ectoenzymes, among them β-glucosidase, were controlled by organic matter availability.

Wetlands play an important role in carbon cycle and in global climate change. The emission of greenhouse gases, especially CO_2, and CH_4, shows a large spatial and temporal variation due to the complex interactions between environmental variables and

the microbiological processes leading to gas production. The carbon flux is related to many external factors, including soil environment, hydrological conditions, vegetation type and exogenous nitrogen (Ma and Lu, 2008). As revealed by Pearson correlation matrix (Table 3), CO_2 and CH_4 fluxes showed a strong seasonal influence, especially at S1 and S2, the sampling sites with the highest TOC concentrations. There is to note, however, that NO_3^- contamination increased production not only of N_2O, but also of $CO_2$and CH_4. These results agree with those which show that alterations in the biogeochemical cycles in nature may lead to altered biogenic fluxes of CO_2, CH_4 and N_2O, the three main gases contributing to global warming (Liu and Greaver, 2009).

In addition to NO_3^-, denitrification correlated positively with the content of organic matter in the sediments. Accordingly, increased potential denitrification was observed at sites with the highest TOC values. Similar results were obtained during studies on denitrification and its relationship with organic carbon quality in three coastal wetland soils (Dodla et al., 2008). Also, Sirivedhin and Gray (2006) found that the sediment denitrification potential showed a positive relationship with the biodegradable organic carbon concentration produced by the periphytic algae in wetlands. Denitrification was also affected by the pluvial regime, as the highest values of DEA were registered during the driest season at each sampling time. Hernández and Mitsch (2007) founded that soil temperature, flood frequency and nitrate availability were important factors controlling denitrification in created wetlands. Davidson (1991) observed an increase N_2O production in dry season, and especially during drying and wetting cycles, caused by a temporal accumulation of mineral nitrogen into soil surface, which will become rapidly available to microbial biomass when dry soil is wetted.

Conclusions

The surface water of La Rocina Stream showed NO_3^- contamination, probably to agricultural sources. This contamination decreased along La Rocina basin and apparently, the superficial water body of DNP wetland was not affected. More research is needed to evaluate how the NO_3^- pollution could affect DNP groundwater. The environmental conditions such as precipitation rate, hydrological morphology and organic matter content greatly influenced the physicochemical characteristics of the surface waters of DNP wetland. The biological activity and greenhouse gas production in their aquatic sediments were also affected by these environmental parameters,

especially the hydrology which had a major effect during the driest season. The denitrification process was affected by anthropogenic activity (nitrate contamination from agricultural practices) and the rainfall regimen, increasing the GHG emissions (CO_2, CH_4 and especially N_2O) during the driest season in all sampling sites studied.

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Artículo II

Título:

Activity, abundance and diversity of denitrifier communities in sediments differing in nitrate content from la Rocina stream.

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En preparación

Este artículo contiene los resultados relativos a los objetivos 2 y 3 de esta Memoria de Doctorado.

Resumen

Se ha estudiado la abundancia relativa, la biodiversidad y la actividad desnitrificante de las comunidades bacterianas en sedimentos de dos sitios del cauce del arroyo de la Rocina, el palacio del Acebrón (S1) y el arroyo de la Cañada (S2). Ambos se localizan en el Espacio Natural de Doñana y se diferencian en su contenido en nitrato siendo la concentración en S2 mayor que en S1. La toma de muestras se realizó en los meses de abril y octubre de los años 2008, 2009 y 2010. La relación entre el contenido en nitratos, la actividad desnitrificante, medida como la producción de N_2O, y la abundancia relativa de los genes de la desnitrificación *narG*, *napA*, *nirK*, *nirS* y *nosZ*, analizada mediante qPCR, se determinó mediante el test de Spearman. El contenido en nitratos presentó el mayor grado de correlación con la abundancia relativa del gen *nosZ* ($r = 0,86$). Un análisis principal de componentes (PCA) mostró una nula correlación entre el contenido en nitrato y la emisión de N_2O y entre la abundancia relativa de los genes de la desnitrificación y la emisión de N_2O. Igualmente, reveló una estrecha relación entre el contenido en nitrato y la abundancia relativa de los genes de la desnitrificación, siendo el gen *nosZ* la variable que mejor explica la variabilidad de las restantes propiedades. La construcción de ocho librerías genómicas utilizando el gen *nosZ* como marcador molecular demostró que, como indica el incremento en el número de unidades taxonómicas operativas (operational taxonomic units, OTUs), la biodiversidad de las comunidades microbianas que contenían el gen *nosZ* era mayor en el sitio con mayor contenido en nitratos. De las 504 secuencias analizadas, más del 21% de ellas correspondió a OTUs sin clasificar. Las familias Bradryhizobiaceae y Rhodocyclaceae fueron las más abundantes, independientemente del contenido en nitratos de los sedimentos. Las familias Rhizobiaceae y Beijerinckiaceae fueron exclusivas del sitio con mayor contenido en nitratos. Un análisis de correspondencia canónica (canonical correspondence analysis, CCA) demostró que el contenido en nitratos afecta la biodiversidad de las comunidades que presentan el gen *nosZ* en su genoma.

Abstract

Denitrification activity and relative abundance and diversity of denitrifier communities were examined in sediments taken from two sites along la Rocina stream differing in nitrate concentration. During a 3-year study, samples were taken in April and October representing the wet and dry seasons, respectively. Nitrate contents did not affect denitrification activity but the size of denitrifier communities was higher in the site with higher nitrate content. A Spearman test including as variables the nitrate content, the denitrification activity, measured as N_2O production, and the relative abundance of each the *narG*, *napA*, *nirK*, *nirS* and *nosZ* denitrification genes, determined as the ratio between a given gene copy number and the 16S rRNA gene copy number after qPCR, showed that the content of nitrate has the strongest correlation ($r = 0.86$) with the relative abundance of the *nosZ* gene. A PCA analysis showed a null correlation between nitrate content and N_2O emissions and between N_2O emissions and denitrification gene abundances, but a strong assemblage between the content of nitrate and the *narG*, *napA*, *nirK, nirS* and *nosZ* denitrification genes relative abundances, the *nosZ* gene being the variable that better explains the variability of the remaining properties. Construction of eight genomic libraries using the *nosZ* gene as a molecular marker revealed that, as indicated by the increase in the number of operational taxonomic units (OTUs), biodiversity of the *nosZ*-bearing communities was higher in the site with higher nitrate content. Out of the 504 clone sequences analyzed, more than 21% of them corresponded to unclassified OTUs. Regardless of the nitrate content in the sediments, the Bradryhizobiaceae and Rhodocyclaceae were the most abundant families. A CCA analysis showed that nitrate concentration affect the biodiversity of *nosZ*-bearing communities of the sediments. At the site with higher nitrate content the families Rhizobiaceae and Beijerinckiaceae showed up that were not present in sediments with lower nitrate content.

Key words

Nnitrate contamination, realative abundance of denitrification genes, *nosZ* biodiversity, qPCR, clone libraries.

Introduction

Denitrification is the biological process in the biogeochemical nitrogen (N) cycle by which nitrate (NO_3^-) is sequentially reduced to dinitrogen gas (N_2) via the intermediate compounds nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O) when oxygen concentrations are limiting. The respiratory electron transfer from reducing equivalents to N oxides is coupled to proton translocation and energy conservation, which permits cells growth when they face to a shortage of oxygen. Comprehensive reviews on the physiology, biochemistry and genetics of the denitrification enzymes have been published elsewhere (Zumft, 1997; van Spanning et al. 2007; Richardson, 2011; Kraft et al. 2011; Sánchez et al. 2011; Bedmar et al. 2013).

Denitrifiers constitute a taxonomically diverse group of microorganisms included in more than 60 genera of bacteria and some archaea (Philippot, 2002; Hayatsu et al. 2008), fungi (Takaya, 2002; Prendergast-Miller et al. 2011), Foraminifera (Risgaard-Petersen et al. 2006) and the ameboid *Gromia* (Piña-Ochoa et al. 2010). Some nitrifiers also have genes involved in denitrification (Cebron and Garnier, 2005; Shaw et al. 2006). The density of denitrifiers in soils can be up to 10^9 cells per g of soil (Babic et al. 2008; Dandie et al. 2008; Henry et al. 2008), and both cultivation-independent and -dependent methods have shown that the proportion of denitrifiers represent up to 5% of the total soil microbial community (Tiedje, 1988; Henry et al. 2006; Jones et al. 2013).

Since the ability to denitrify is sporadically distributed both within and between different genera and cannot be associated with any specific taxonomic group, a 16S rRNA phylogeny-based approach is not possible to study denitrifiers. Therefore, existing techniques to study the ecology of this bacterial community are based on the use of functional genes in the denitrification pathway, or their transcripts, as molecular markers (reviewed in Philippot, 2006; Philipot and Halllin, 2006; Hallin et al. 2007; Correa-Galeote et al. 2013a). Respiratory nitrate reduction is carried out by two biochemically different enzymes, a membrane-bound nitrate reductase (Nar) or a periplasmic nitrate reductase (Nap) encoded by genes of the *narGHJI* or *napABCDEFKL* operons, respectively (Richardson et al. 2001, 2007; González et al. 2006; Richardson, 2011). Two types of respiratory nitrite reductases (Nir) have been described in denitrifying bacteria, NirK and NirS, encoded by *nirK* or genes of the more complex *nirS* gene clusters, respectively (Rinaldo and Cutruzzolà, 2007; van Spanning, 2011). The conversion of NO into N_2O can be carried out by three types of nitric oxide

reductases (Nor), cNor, qNor, and qCuANor, of which cNor is an integral membrane enzyme encoded by the *norCBQD* operon (Zumft, 2005; de Vries et al. 2007). The final step in denitrification is the reduction of N_2O to N_2, a reaction catalyzed by the nitrous oxide reductase (Nos); the *nos* gene clusters often comprise the *nosRZDFYLX* genes, of which the *nosZ* gene encodes the monomers of Nos (Wunsch et al. 2003; Zumft and Kroneck, 2007; Pauleta et al. 2013).

Several studies have used *narG*/*napA*, *nirK*/*nirS*, *norB* and *nosZ* as molecular markers of denitrifying bacteria to study their abundance and diversity in various environments, including fresh (Gregory et al. 2003) and marine waters (Braker et al. 2003), glacier forelands (Kandeler et al. 2006), creek sediments (Rich and Myrold 2004), estuarine (Smith et al. 2007; Magalhães et al. 2008; Chon et al. 2011) and marine sediments (Liu et al. 2003), constructed (Ruiz-Rueda et al. 2009; García-Lledó et al. 2011; Correa-Galeote et al. 2013b; Ligi et al. 2013a,b) and ephemeral wetlands (Ma et al. 2008; Carrino-Kyker et al. 2012), paddy soils (Yoshida et al. 2009; Chen et al. 2010, 2012a,b), grassland and agricultural soils (Henry et al. 2006; Enwall et al. 2007; Ramírez et al. 2010), subtropical soils (Yu et al. 2014), turfgrass (Dell et al. 2010), activated sludge (Srinandan et al. 2011), riparian zones (Dandie et al. 2011), constructed technosols (Hafeez et al. 2012), streams (Baxter et al. 2013), soil crusts (Abed et al. 2013), etc.

A major factor controlling denitrification activity is nitrate availability. A study on the impact of long-term N-fertilization in nitrate-reducing microorganisms revealed no changes in the composition of the nitrate-reducing community, except if pH changes were associated with the application of different nitrate-containing fertilizers (Enwall et al. 2005). Amendment of repacked grassland soils cores with different nitrate concentrations for 14 days revealed that the community structure of nitrate-reducing microorganisms remained constant over the experimental period indicating that the community showed a high resistance towards fluctuating nitrate concentrations (Deiglmayr et al. 2006). In a microcosm study, treatment of forest vernal pools with nitrate for two days increased denitrification, but associated changes in diversity of denitrifying communities were not observed (Carrino-Kyker et al. 2012).

Despite the pivotal role of denitrification in the N cycle, very few studies have dealt with the effect of nitrate concentration on the structure and biodiversity of denitrifiers in natural environments with null or very scarce anthropogenic effects. In a previous study (Tortosa et al. 2011), we analyzed the biological and physicochemical

properties of la Rocina stream, a main natural creek feeding el Rocio marsh within Doñana National Park, a wetland covering around 60.000 ha in a marshy area of SW Spain, in the estuary of the Guadalquivir River. Screening of more than 25 points along the course of la Rocina stream (36 km) revealed differences in nitrate concentration in its sediments, most probably due to contamination from agricultural practices allowed in the ecotone of the Park, as no urban areas are located nearby. Thus, la Rocina stream provides a unique model system to study the effect of nitrate content on abundance and biodiversity of denitrifying communities in sediments as the long term effect related to nitrate content could influence community abundance, composition and activity. In this study, we determined the size of the denitrifier communities and the total bacterial population by quantifying genes encoding some of the enzymes catalyzing reaction in the N cycle and 16S rRNA genes, respectively. Denitrification activity was examined as N_2O production and biodiversity was analyzed by using the *nosZ* gene as a molecular marker for construction of genomic libraries. The effect of nitrate on denitrification activity, genes abundance and biodiversity, was explored by integrated statistical analysis.

Materials and methods

In a previous study, the physicochemical properties of the surface waters and sediments of selected sites varying in NO_3^- content along la Rocina stream, which feeds marisma del Rocío in Doñana National Park, were determined (Tortosa et al. 2011). From those data, two sites, el Acebrón lagoon (S1, UTM coordinates 29S 0718632, 4114294) and la Cañada creek (S2, UTM coordinates 29S 0722653, 4111704), representing the sites with the lowest and highest nitrate concentration, respectively, were selected for sampling. Sediment samples were taken as indicated earlier (Tortosa et al. 2011) in April and October years 2008, 2009 and 2010 in order to represent the wet and dry pluvial regimes, respectively. Samples were placed on ice while returned to the laboratory and then stored at -80 ºC until use.

Denitrification activity

Denitrification activity was measured as N_2O emission by the sediments. Denitrifying enzyme activity was carried out as previously describe earlier (Šimek and Hopkins, 1999; Šimek et al. 2004). Essentially, 25 g of sediment was placed in 125-ml glass bottles containing 25 ml of a solution made of 1 mM glucose, 1 mM KNO_3 and 1

g/l chloramphenicol. The bottles were closed with serum caps and acetylene (10% (v/v) was injected into each bottle to inhibit nitrous oxide reductase (Yoshinari and Knowles, 1976). After incubation for at least 1 h at 25 ºC, gas samples (500 µl) were withdrawn from the headspace and injected in a gas chromatograph equipped with an electron capture detector (ECD) and a Porapak Q-packed stainless-steel column (180 x 0.32 cm) (Agilent Technologies, S.L., Madrid, Spain). N_2 at 20 ml/min served as a carrier gas. Oven, detector and injector temperature were 60, 375 and 125 ºC, respectively. Concentrations of nitrous oxide in each sample were calculated from standards of pure nitrous oxide. The Bunsen coefficient for the N_2O dissolved in water was considered during calculations.

DNA extraction

DNA was extracted from 250 mg of each subsample stored at -80 °C according to the ISO standard 11063 'Soil quality-Method to directly extract DNA from soil samples' (Petrić et al. 2011). Briefly, samples were homogenized in 1 ml of extraction buffer (1 M Tris-HCl, 0.5 M EDTA, 1 M NaCl, 20% PVP 40, 20% SDS) for 30 s at 1600 rpm in a minibead beater cell disrupter (Mikro-DismembratorS; B. Braun Biotech International, Germany). Soil and cell debris were removed by centrifugation (14000 x *g* for 1 min at 4 °C). After precipitation with ice-cold isopropanol, nucleic acids were purified using both PVPP and GeneClean Turbo Kit (MP Bio, USA) spin columns. Quality and size of soil DNAs were checked by electrophoresis on 1% agarose. DNA was also quantified by spectrophotometry at 260 nm using a BioPhotometer (Eppendorf, Germany).

Quantification of the denitrification-associated microbial community

The size of the denitrifier community was estimated by quantitative, real-time PCR (qPCR) of *narG*, *napA*, *nirK*, *nirS* and *nosZ* gene fragments using reaction mixtures, primers and thermal cycling conditions described previously (Correa-Galeote et al. 2013a,b). The total bacterial community was quantified using 16S rRNA gene as molecular marker as described by Correa-Galeote et al. (2013a,b). Reactions were carried out in an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Quantification was based on the fluorescence intensity of the SYBR Green dye during amplification. Two independent qPCR assays were performed for each gene. Standard curves were obtained using serial dilutions of linearized plasmids containing cloned

narG, *napA*, *nirK*, *nirS*, *nosZ* and 16S rRNA genes amplified from bacterial strains. PCR efficiency for the different assays ranged between 90% and 99%. No template controls gave null or negligible values. Presence of PCR inhibitors in DNA extracted from sediments was estimated by (i) diluting soil DNA extract and (ii) mixing a known amount of standard DNA to sediment DNA extract prior to qPCR. In all cases, inhibition was not detected. Methodological evaluation of the real-time PCR assays showed a good reproducibility of 95.0 ± 12% between two runs.

Gene abundances were analyzed as absolute and relative abundances (gene copy number/16S rRNA gene Bacteria copy number). As the number of 16S rRNA gene operon per cells is variable (Klappenbach et al. 2001), we did not convert the 16S rRNA gene copy data into cells numbers and we expressed our results as gene copy numbers per g of soil.

Clone library construction and DNA sequencing

nosZ amplicons were purified using the QIAquick PCR purification kit (Qiagen, Germany) and cloned using the pGEM-T Easy cloning kit according to the manusfacturer's instructions (Promega, USA). The recombinant *Escherichia coli* JM109 cells were inoculated onto solid Luria Bertani (LB) medium (Miller 1972) containing ampicillin and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and grown overnight at 37 ºC. White colonies were screened by PCR using the vector primers Sp6 and T7 (Invitrogen). Purity of amplified products was checked by observation of a unique band of the expected size in a 1% agarose gel stained with GelRed as indicated by the manufacturer's (Biotium Inc., USA). Nucleotide sequences of clones containing inserts of the expected size were determined by sequencing with the vector primer Sp6 and the BigDye terminator cycle kit v3.1 (Applied Biosystems, USA) according to the manufacturer's instructions, followed by electrophoresis on an ABI 3100 genetic analyser (Applied Biosystems, USA) at the sequencing facilities of Estación Experimental del Zaidín, CSIC, Granada, Spain.

Phylogenetic analysis

The DNA sequences of *nosZ* gene fragments were aligned by using the ClustalW program available in the Geneious software package (version 6.0.3, Biomatters, New Zealand). Vector sequence was removed and discrepancies in alignment verified manually. The obtained sequences were compared against database

sequences using the BLASTN program in Geneious and those showing similarity higher than 80% of those previously deposited for *nosZ* were selected as positives. A distance matrix was calculated according to Kimura's two-parameter model (Kimura, 1980) using the dnadist Phylip-3.68 package software (University of Washington, USA).

Estimation of the richness (S) as operational taxonomic units (OTUs) and Chao1, Shannon-Weaber (H´) and Simpson (D) diversity indexes were calculated using the MOTHUR program (Schloss et al. 2009). . In this study, 3% sequence divergence was used to define OTUs and compare libraries. The Good´s coverage index was calculated according to Magurran (2004). A phylogenetic tree was constructed from a matrix of pairwise genetic distances by using the neighbor-joining method available in Geneious. Bootstrap analysis was based on 1000 resamplings.

Statistical analyses

Measured variables in this study were first explored using the Shapiro-Wilk test to check whether they meet the normality assumptions. We used the Mann-Whitney test to compare data between sampling sites and times of sampling, and the Kruskal-Wallis and Conover-Iman combined tests for comparisons among sampling years. A Spearman correlation matrix was made to study relations between measured variables. Statistical analyses were carried out by the XLSTAT 2012.4.02 version software (Addinsoft). A principal component analysis (PCA) was performed to analyze relationships among parameters concerning nitrate content, denitrification acitivity and denitrification genes relative abundance. Similarly, a canonical correspondence analysis (CCA) was made to determine the effect of the nitrate content in the structure of the *nosZ*-bearing communities. Multivariate analyses were carried out by the PC-ORD 6.08 version software (MJM). The analysis of molecular variance (AMOVA) to determine population-specific differences among clone libraries was run using MOTHUR (Schloss et al. 2009).

Nucleotide sequence accession numbers

The nucleotide sequences of *nosZ* reported in this study have been deposited in GeneBank under the accession numbers KC936294 to KC936797.

Results

Nitrate content in sediments

For the 3-year study, nitrate content in sediments from site S1 varied between 0.13-0.25 mg NO_3^-/kg dry weight, and between 25.37-47.11 mg NO_3^-/kg dry weight from those taken at site S2 (Table 1). According to the Mann-Whitney test, nitrate content in S1 was lower than that in S2, regardless of the sampling season and year. Also, the content of nitrate in October was always higher than that found in April for each sampling site, except for year 2008 at S1 when no differences were detected. A Kruskal-Wallis and Conover-Iman combined tests revealed statistical differences among years.

Table 1. Nitrate content and denitrification activity in sediments from la Rocina stream.

Year	Sampling month	Sampling site	Nitrate content (mg NO_3^-/Kg dry sediment)	Denitrification activity (ng N-N_2O/g dry sediment x h)
2008	April	S1	0.20 ± 0.02 (y, a, B)	164 ± 8.72 (x, a, B)
		S2	25.37 ± 0.29 (y, b, A)	1393 ± 121 (xy, a, A)
	October	S1	0.17 ± 0.01 (y, a, B)	114 ± 8.08 (x, a, A)
		S2	32.36 ± 0.38 (y, a, A)	130 ± 16.46 (x, b, A)
2009	April	S1	0.22 ± 0.01 (x, a, B)	164 ± 7.28 (x, a, B)
		S2	31.09 ± 1.07 (x, b, A)	1616 ± 122 (x, a, A)
	October	S1	0.13 ± 0.01 (z, b, B)	126 ± 9.74 (x, a, A)
		S2	47.11 ± 0.86 (x, a, A)	137 ± 9.38 (x, b, A)
2010	April	S1	0.18 ± 0.01 (y, b, B)	194 ± 17.48(x, a, B)
		S2	26.60 ± 1.05 (y, b, A)	1134 ± 44.91 (y, a, A)
	October	S1	0.25 ± 0.01 (x, a, B)	113 ± 9.88(x, a, A)
		S2	34.12 ± 0.80 (y, a, A)	134 ± 6.96 (x, b, A)

Values of nitrate concentration (n = 4 ± SE) are expressed as mg NO_3^-/kg dry sediment. Values of activity (n = 4 ± SE) are expressed as ng N-N_2O/g dry sediment x h. Based on the Kruskal-Wallis and Conover-Iman combined tests ($\alpha = 0.05$), letters x, y and z indicate significant differences among years for a given sampling site and sampling month. According to the Mann-Whitney test ($\alpha = 0.05$), letters a and b indicate significant differences between sampling months for a given sampling site and year, and letters A and B show significant differences between sampling sites for a given sampling month and year.

Denitrification activity

Emission of N_2O in sediments varied between 113-194 ng N-N_2O/g dry sediment x h and 130-1616 ng N-N_2O/g dry sediment x h in sediments from S1 and S2, respectively (Table 1). For the 3-year study, N_2O production in S2 was statistically higher than that in S1 for the samples taken in April, and no differences were found in samples taken during October. At S1, N_2O emissions detected in April were always similar that those found in October, regardless of the year. At S2, however, the N_2O emission was higher in April than in October. A Kruskal-Wallis and Conover-Iman combined tests revealed that whereas no differences in N_2O emission were found at sites S1 for April and October 2008, 2009 and 2010 and at S2 for October 2008, 2009 and 2010, denitrification activity in sediments taken in April at S2 were statistically different among sampling years.

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

Sediment samples contained amounts of 16S rRNA target molecules ranging from 7.38 x 10^6 to 2.91 x 10^9 copies/kg dry sediment (supplementary material Table 1S). According to the Mann-Whitney test, the content of target DNA was similar in samples taken from each site regardless of the year and sampling season, except for samples taken in October 2010, when the copy number of the 16S rRNA at S1 was higher than that at S2. At S1, the number of target molecules was higher in October than in April, except for year 2009 when they had similar values. At S2, however, no differences in the 16S rRNA copy number were detected for each year, except for October 2009 when the number of target genes was higher than in April 2009. A Kruskal-Wallis and Conover-Iman combined tests revealed statistical differences among years.

The copy number of *narG* ranged from 2.19 x 10^6 to 3.53 x 10^8 copies/kg dry sediment (supplementary material Table 1S). According to the Mann-Whitney test, the copy number of *narG* in April was similar between S1 and S2 for each sampling year; S2, however, the abundance of *narG* in October was always higher than that at S1, with the exception of year 2010, when differences between sampling sites were not detected. The number of target molecules was similar between April and October for the two sites, except for years 2008 at S2 and 2010 at S1, when the copy number of *narG* was higher in October.

The copy number of *napA* varied between 1.57 x 10^6 and 3.84 x 10^7 copies/kg dry sediment (supplementary material Table 1S). Based on the Mann-Whitney test, the copy number of *napA* at S1 and S2 was similar, regardless of the sampling year; an exception was found as abundance of *napA* in October 2009 was higher at S2. No differences in the copy numbers of *napA* were detected between the sampling months for both sampling sites, except at S2 in 2008 and S1 in 2010, when the abundance of *napA* was higher in October than in April.

The copy number of *nirK* ranged from 1.17 x 10^5 to 2.22 x 10^7 copies/kg dry sediment (supplementary material Table 1S). According to the Mann-Whitney test, the content of target DNA was similar in samples taken from each site regardless of the year and sampling seasons, except for samples taken in April 2008, when the copy number of *nirK* was higher at S1 than at S2. Similarly, no differences were found between seasons for each year and each sampling site, except for years 2008 at S2 and 2009 at S1, when values were higher in October.

The copy number of *nirS* ranged from 3.91 x 10^5 to 2.72 x 10^8 copies/kg dry sediment (supplementary material Table 1S). According to the Mann-Whitney test, the copy number of *nirS* at S1 was lower than that at S2 for both years 2008 and 2009, but no differences were found between sites for each season in year 2010. For each sampling site and year, the abundance of *nirS* in October was higher than that in April; the content of *nirS*, however, in April and October 2009 was similar.

The copy numbers of *nosZ* oscillated from 1.67 x 10^4 to 4.67 x 10^6 copies/kg of dry sediment (supplementary material Table 1S). According to the Mann-Whitney test, the content of target DNA at S2 was higher than that at S1, regardless of the year and sampling season, except for samples taken in April 2008 and October 2010 that had similar *nosZ* gene copy number.

A Kruskal-Wallis and Conover-Iman combined tests revealed statistical differences among years for the copy number of the *narG*, *napA*, *nirS*, *nirK* and *nosZ* *nosZ* denitrification genes.

Relative abundance of the narG, napA, nirS, nirK and nosZ denitrification genes

The relative abundance of the *narG*, *napA*, *nirS*, *nirK* and *nosZ* denitrification genes are shown in Table 2. Although the relative abundance of each *narG*, *napA*, *nirS* and *nosZ* genes in sediments taken at S2 was higher than that in sediments sampled at S1, some exceptions for the *narG*, *napA* and *nirS* genes were detected among years.

This pattern was not observed for *nirK*, as no clear differences in relative abundance were found between sampling sites.

Whereas no differences in relative abundance of *narG* were observed between sampling months at S1, values at S2 were similar in October 2008, but higher in October 2009 and 2010. Similarly, relative abundance of *napA* at S1 was similar for the 2 sampling months during the 3-year study, except for April 2009, the sampling time with the lowest relative abundance. Regarding S2, relative abundance of *napA* was higher in October than in April 2009 and 2010 and similar for the 2 sampling months in 2008.

In general, the sampling month did not affect the relative abundance of the *nirK* gene, though two exceptions were found, one at S2 in 2009 and the other at S1 in 2010. Relative abundance of *nirS* was always higher in October, except for year 2008 at both sampling sites, when similar relative abundances were found between sampling months. The pattern of relative abundance of the *nosZ* genes followed that of the *narG* gene as, for the 3-year study, values found at S1 in April were similar to those determined in October. Similarly, relative abundance of *nosZ* at S2 was always higher in October than in April regardless of the year.

Based on a Kruskal-Wallis and Conover-Iman combined tests, the relative abundance of *narG* was similar among years, except for April 2009 at S1, the sampling time with the lowest relative abundance. In contrast, the relative abundance of the *napA* and *nirS* denitrification genes was different for each one of the 3 years, except for October at S2. No differences in relative abundance of the *nirK* and *nosZ* genes were observed at S2 regardless of the year.

For the 3-year study, the mean gene relative abundance of each *narG* (8.09%), *napA* (4.79%), *nirS* (6.16%), *nirK* (1.38%), and *nosZ* (0.10%) genes at S1 for the month of April was similar to that for the month of October (9.56%, 5.50%, 6.46% 4.61% and 0.12%, respectively). In contrast, the relative abundance of the *narG* (13.21%), *napA* (7.44%), *nirS* (5.61%), *nirK* (3.54%) and *nosZ* (0.28%) genes at S2 for the month of April was lower than that for the month of October (31.14%, 17.87%, 9.22%, 13.36% and 0.57%, respectively).

Table 2. Relative abundance of *narG*, *napA*, *nirS*, *nirK* and *nosZ* denitrification genes in sediments from la Rocina stream.

Year	Sampling month	Sampling site	Gene relative abundance (%)				
			narG	*napA*	*nirK*	*nirS*	*nosZ*
2008	April	S1	9.71 ± 1.10 (x, a, B)	6.11 ± 0.56 (x, a, B)	8.29 ± 0.32 (x, a, A)	1.78 ± 0.18 (x, b, B)	0.11 ± 0.01 (x, a B)
		S2	17.92 ± 1.93 (x, a, A)	12.46 ± 1.22 (x, a, A)	7.81 ± 1.13 (x, a, A)	5.70 ± 0.61 (x, b, A)	0.25 ± 0.01 (x, b, A)
	October	S1	9.08 ± 0.44 (x, a, B)	8.19 ± 0.79 (x, a, B)	8.82 ± 0.52 (x, a, A)	3.23 ± 0.28 (y, a, B)	0.14 ± 0.01 (x, a, B)
		S2	34.79 ± 4.73 (x, a, A)	21.48 ± 2.38 (x, a, A)	9.33 ± 0.76 (x, a, A)	9.93 ± 1.45 (x, a, A)	0.57 ± 0.07 (x, a, A)
2009	April	S1	5.25 ± 0.06 (y, a, B)	1.92 ± 0.12 (z, b, B)	4.69 ± 0.47 (y, a, B)	0.40 ± 0.04 (y, b, B)	0.04 ± 0.01 (y, a, B)
		S2	10.59 ± 1.24 (x, b, A)	6.46 ± 0.58 (y, b, A)	3.22 ± 0.22 (x, a, A)	2.47 ± 0.34 (y, b, A)	0.29 ± 0.02 (x, b, A)
	October	S1	8.35 ± 1.38 (x, a, B)	5.74 ± 0.93 (y, a, B)	3.28 ± 0.20 (z, a, B)	1.42 ± 0.24 (z, a, B)	0.05 ± 0.01 (y, a, B)
		S2	36.16 ± 6.17 (x, a, A)	17.05 ± 0.94 (x, a, A)	8.57 ± 1.35 (x, b, A)	12.94 ± 1.13 (x, a, A)	0.53 ± 0.07 (x, a, A)
2010	April	S1	9.31 ± 0.85 (x, a, B)	3.41 ± 0.27 (y, a, A)	5.51 ± 0.28 (y, b, A)	1.96 ± 0.03 (x, b, A)	0.15 ± 0.01 (x, a, B)
		S2	11.26 ± 2.24 (x, b, A)	6.35 ± 1.13 (x, b, A)	5.81 ± 0.85 (x, a, A)	2.45 ± 0.35 (y, b, A)	0.29 ± 0.02 (x, b, A)
	October	S1	11.26 ± 0.77 (x, a, B)	2.57 ± 0.53 (z, a, B)	7.28 ± 0.26 (y, a, B)	9.18 ± 0.70 (x, a, B)	0.17 ± 0.02 (x, a, B)
		S2	22.47 ± 0.51 (x, a, A)	15.08 ± 3.60 (x, a, A)	9.75 ± 0.31 (x, a, A)	17.20 ± 1.57 (x, a, A)	0.51 ± 0.09 (x, a, A)

Values (n = 4 ± SE) are expressed as percentage of the ratio between a given denitrification gene copy number and the 16S rRNA gene copy number. Based on a Kruskal-Wallis and Conover-Iman combined tests (α = 0.05), letters x, y and z indicate significant differences among years for a given sampling site and sampling month. According to the Mann-Whitney test (α = 0.05), letters a and b show significant differences between sampling months for a given sampling site and year, and letters A and B represent significant differences between sampling sites for a given sampling month and year.

Correlation tests and multivariate analysis

A Spearman test showed that correlation between nitrate content and abundance of each denitrification gene was very weak (supplementary material Table 2S). In contrast, there was a strong correlation between the content of nitrate and the relative abundance of each *narG*, *napA*, *nirS*, and *nosZ* genes, the highest strength of correlation observed between nitrate concentration and the relative abundance of *nosZ* (Table 2). The pairwise analysis also showed that there was a strong correlation among abundances of each denitrification gene as well as among the relative abundance of each *narG*, *napA*, *nirS*, *nirK*, and *nosZ* gene, the highest values corresponding to the correlation between *nosZ* and the remaining denitrification genes (Table 2). Correlation was not found between denitrification activity and either nitrate content or any of the denitrification genes (Table 2 and supplementary material Table 2S).

Table 3. Spearman coefficient values between nitrate content, relative abundance of the *narG*, *napA*, *nirS*, *nirK* and *nosZ* denitrification genes and denitrification activity in sediments from la Rocina stream.

	Nitrate content	*narG*	*napA*	*nirK*	*nirS*	*nosZ*
narG	0.677*					
napA	0.564*	0.765*				
nirK	0.278	0.627*	0.406*			
nirS	0.668*	0.784*	0.634*	0.585*		
nosZ	0.856*	0.817*	0.690*	0.483*	0.793*	
Denitrification activity	0.019	-0.030	0.050	-0.127	-0.038	0.134

Values followed by asterisk (*) are statistically significant (P-value < 0.05).

PCA analysis including the variables nitrate concentration, denitrification activity and relative abundance of each denitrification gene resulted in two new factors (Fig. 1). Factor 1 accounted for 61.11% of the total variation in the properties of the samples. The relative abundances of *narG* (r = 0.927) and *nosZ* (r = 0.929) variables weighed the most heavily in forming factor 1, and were positively related with it. Factor 2 accounted for an additional 15.62% of the variation of the analyzed variables and is described exclusively by the denitrification activity variable (r = 0.927). No correlation was found between denitrification activity and the other six variables (supplementary

material Table 3S). The nitrate concentration and the relative abundance of each denitrification gene separate the samples taken at sites S1 and S2, regardless of the sampling year. Samples from S1 and S2 ranked highly negatively and positively for factor 1, respectively. Samples from S2 were also separated according to the season sampling, the samples taken in April placed in between S1 and those taken in October. While factor 2 significantly separated season-dependent samples from S2, it did not explain variations in samples from S1.

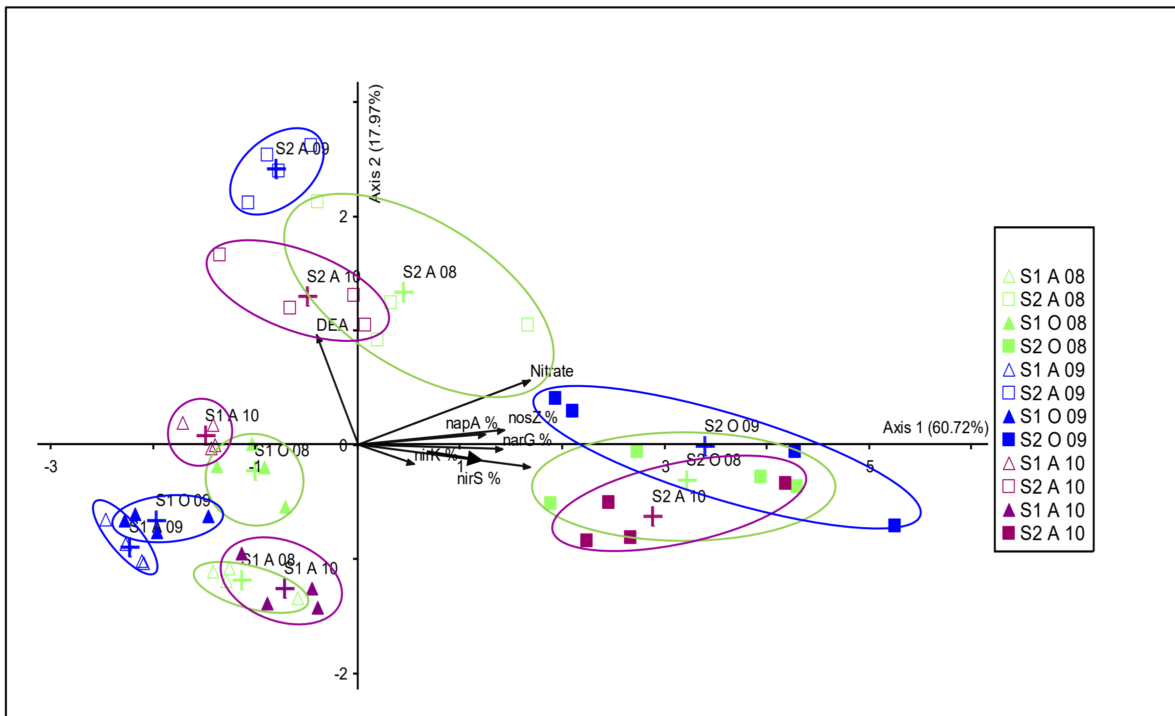

Fig. 1. Principal components analysis (PCA) of nitrate content, relative abundance of the *narG*, *napA*, *nirS*, *nirK* and *nosZ* denitrification genes and denitrification activity and ranking of the samples on PCA factors 1 and 2. Sediments were taken in April (A) and October (O) 2009 (09) and 2010 (10) at El Acebrón lagoon (S1) and la Cañada creek (S2).

Analysis of clone libraries

The construction of genomic libraries for each April and October sampling months and for each S1 and S2 sampling sites was limited to years 2009 and 2010. The 8 *nosZ* libraries contained 504 clones grouped in 109 OTUs (supplementary material Table 4S). At S1, 65 and 63 clones were obtained in April 2009 and 2010, respectively, and 61 and 60 in October 2009 and 2010, respectively. Whereas 58 clones were obtained at S2 for April 2009 and 2010, 70 and 69 clones were procured in October 2009 and 2010, respectively. Six libraries contained between 25 and 29 OTUs and the remaining two libraries, which corresponded to October 2009 and 2010, the sampling months with the highest number of clones, included 35 and 34 OTUs, respectively (Table 4 and supplementary material Table 4S).

The Good´s coverage index for each library (Table 4) was higher than 75%, which indicates that the sampling effort was enough to permit extrapolations for analysis of total *nosZ* biodiversity in the samples. The lower and upper confidence intervals of each Chao1 and Shannon-Weaber indexes overlapped in all cases (data not shown), which suggests that there were no differences in biodiversity among the 8 *nosZ* clone libraries. The Simpson index (Table 4) varied between 0.031 and 0.055, which clearly indicates the high diversity of each one of the 8 libraries.

Table 4. Diversity indexes of *nosZ* clone libraries from la Rocina stream sediments as estimated with the Simpson index and Shannon-Weaber and Chao 1 richness estimators computed using Mothur.

Year	Sampling month	Sampling site	Number of clones	Number of OTUs	Good's coverage	Chao1	Shannon-Weaber	Simpson
2009	April	S1	65	29	75.38	53.0	3.07	0.047
		S2	58	25	77.59	38.0	2.92	0.053
	October	S1	61	27	78.69	36.7	3.02	0.050
		S2	70	35	75.29	48.9	3.15	0.034
2010	April	S1	63	29	76.19	44.0	3.10	0.045
		S2	58	26	79.31	33.3	2.96	0.055
	October	S1	60	29	76.66	31.1	3.11	0.044
		S2	69	34	75.36	45.3	3.29	0.031

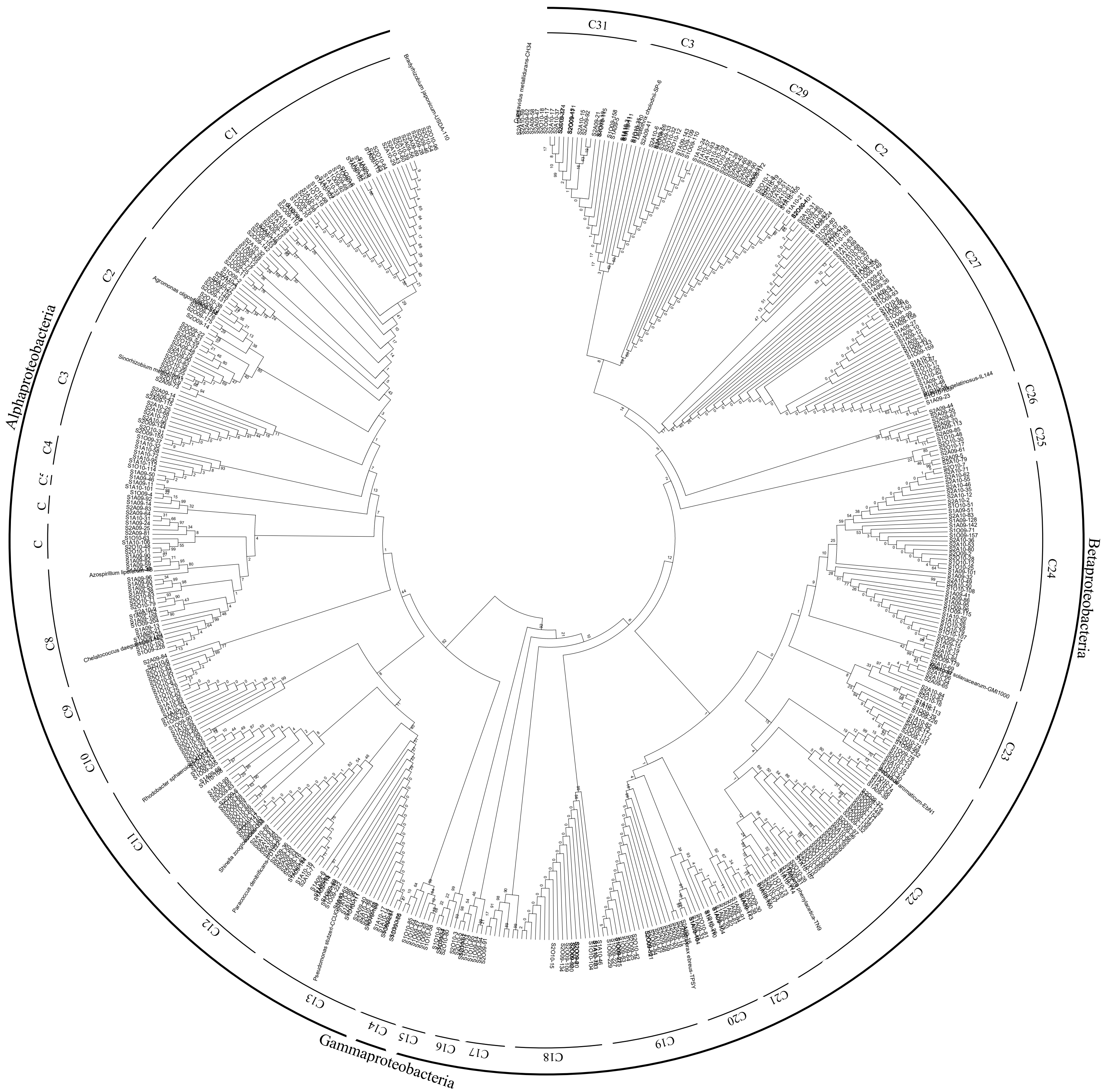

Fig. 2. Neighbor-joining phylogenetic tree based on 504 *nosZ* DNA sequences cloned from la Rocina stream sediments and other cultured bacteria. Sediments were taken in April (A) and October (O) 2009 (09) and 2010 (10) at El Acebrón lagoon (S1) and la Cañada creek (S2). The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets.

Construction of a phylogenetic tree based on the 504 *nosZ* sequences showed they distributed into 31 clusters (Fig. 2). Overall, members of the Betaproteobacteria class were the most abundant (59.1%) followed by those of the Alphaproteobacteria (39.5%) and the Gammaproteobacteria (1.4%).

Clusters C4, C5, C6, C7 and C10 within the Alphaproteobacteria and clusters C15, C16, C17, C18, C20, C24, C25, C27, C28 and C30 included in the Betaproteobacteria contained clones showing homology with unclassified *nosZ* gene sequences deposited in GenBank (supplementary material Table 5S). Out of the 504 clones analyzed, 7.34% and 32.73% of the Alphaproteobacteria and Betaproteobacteria clones represented unclassified bacteria, respectively. At site S1, 52.31% and 49.21% of the clones in the April 2009 and 2010 libraries as well as 45.90% and 63.33% of those in October 2009 and 2010 libraries were also unclassified sequences, respectively. The number of unclassified clones was clearly lower in libraries from site S2, with 20.69% and 34.48% for April 2009 and 2010, respectively, and 11.43% and 18.84% for October 2009 and 2010, respectively.

The family Bradyrhizobiaceae in cluster C1 (52 clones) and Rhodocyclaceae in cluster C22 (44 clones) together with the unclassified clusters C24 (43 clones) and C27 (40 clones) contained the highest numbers of clones sequenced (supplementary material Table 4S). Only 14 clones were members of the family Pseudomonadaceae within the Gammaproteobacteria and they all were found at site S1 (supplementary material Table 4S).

Fig. 3 shows that, at S1, 14 clusters were present in April of both the 2009 (16 clusters) and 2010 (19 clusters) gene libraries. The October 2009 (18 clusters) and 2010 (21 clusters) libraries shared 17 clusters. At S2, the April 2009 (18 clones) and 2010 (16 clones) gene libraries had 13 common clusters. The October 2009 gene library contained 17 clusters, all of which were present in the October 2010 library (22 clusters). The number of clones in each cluster, however, varied between years. In 2009, at S1, the April and October gene libraries shared 12 clusters. In 2010, the April and October libraries had 16 common clusters. The unclassified clusters C4, C5 and C10 from Alphaproteobacteria, the C14 Gammaproteobacteria cluster and the unclassified clusters C15 and C27 from Betaproteobacteria were found only in S1. The C2 Bradyrhziobiaceae cluster, the C3 and C12 Rhizobiaceae clusters and the C9 Beijereinckiaceae of the Alphaproteobacteria and the C26 unclassified Burkholderiales and C31 Comamonadaceae of the Betaproteobacteria were present only in S2.

The *nosZ* sequences of clones in clusters C1 and C2 related to the Bradyrhizobiaceae family, C3 and C12, considered as Rhizobiaceae family, C19 and C31, belonging to the Comamonadaceae family, and C21 and C22, included in the Burkholderiaceae family, clustered separately (Fig. 2), suggesting they were not phylogenetically related.

AMOVA of the 504 *nosZ* sequences indicated that total sequence variation was 3.62% among libraries and 96.38% within the clone libraries (supplementary material Table 6S), which indicates the existence of a high randomized diversity. At S1, pairwise alignments revealed that sequences from April 2009 and 2010 and October 2009 were statistically the same population, and that those from October 2010 were significantly different (Table 5). On the contrary, at S2, sequences in the clone libraries from April 2009 and 2010 and October 2009 were statistically different populations, but no differences however, were found between sequences in the October 2009 and 2010 clone libraries (Table 5).

Table 5. Pairwise dissimilarity indexes (Fst) from AMOVA of *nosZ* clone libraries.

	Clone library			
Clone library	A 09	O 09	A 10	O 10
A 09		**1.53**	**1.99**	**4.01***
O 09	2.66*		**1.25**	**3.02***
A 10	2.60*	3.16*		**1.23**
O 10	2.79*	1.55	3.98*	

Clones from S1 are shown in boldface. An asterisk indicates a *P*-value < 0.05 observed Fst value compared to Fst value from 1000 randomizations of the sequences. A and O stand for the months of April and October, respectively.

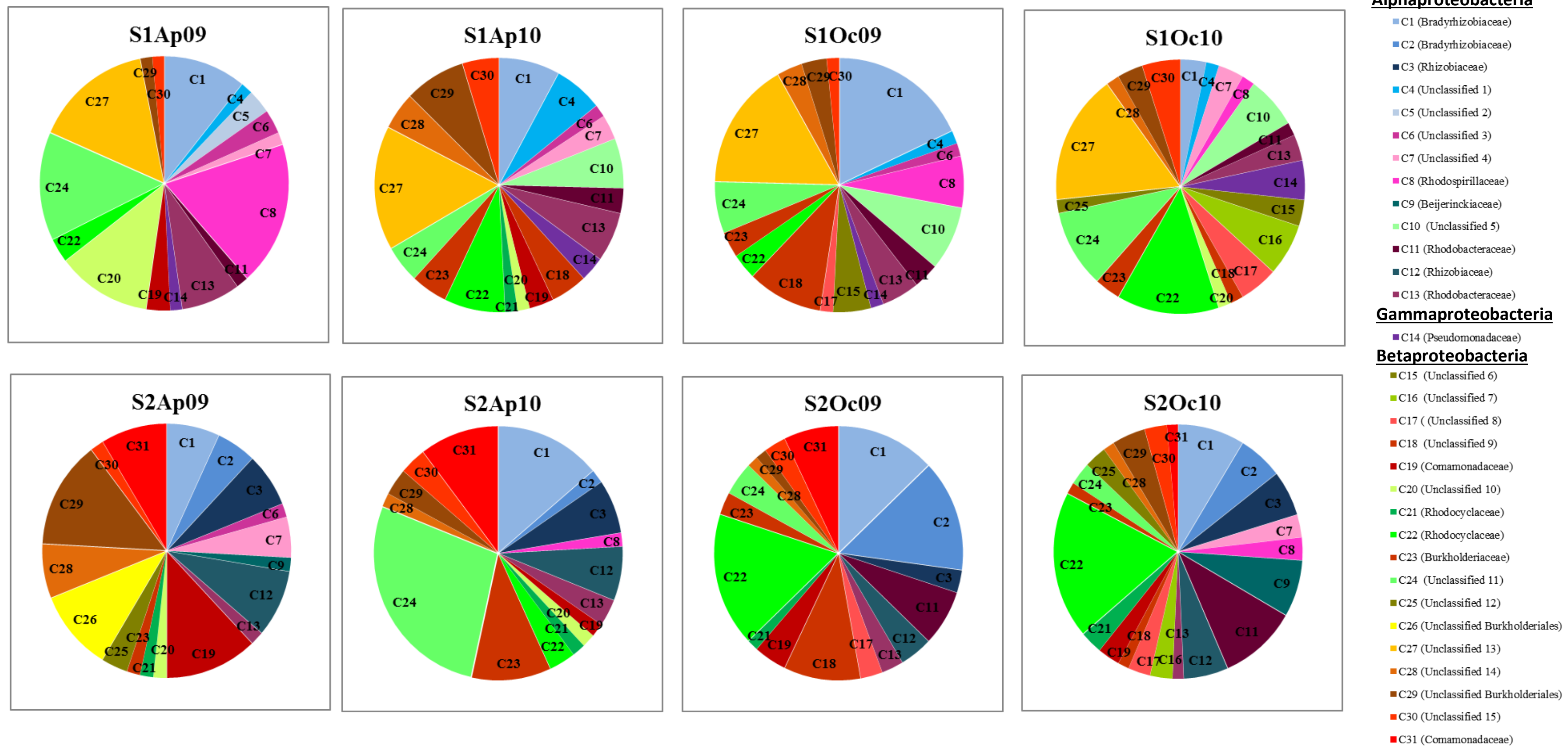

Fig. 3. Pie charts comparing the *nosZ* communities composition of sediments from la Rocina stream. To facilitate the comparison between clusters color has been used as an indication of bacterial families and unidentified groups.

A CCA sample ordination based on the relative abundance of the sequences found in the *nosZ* gene clusters within each clone library showed that the eight samples distributed in two clearly separated groups (Fig. 4). The two CCA axes explained 42.2% of the total variance and revealed that nitrate concentration of the sediments was responsible for the grouping of the clone libraries along the 2 axes, whose canonical coefficients were 1.01 and -0.024 1 for axes 1and 2, respectively.

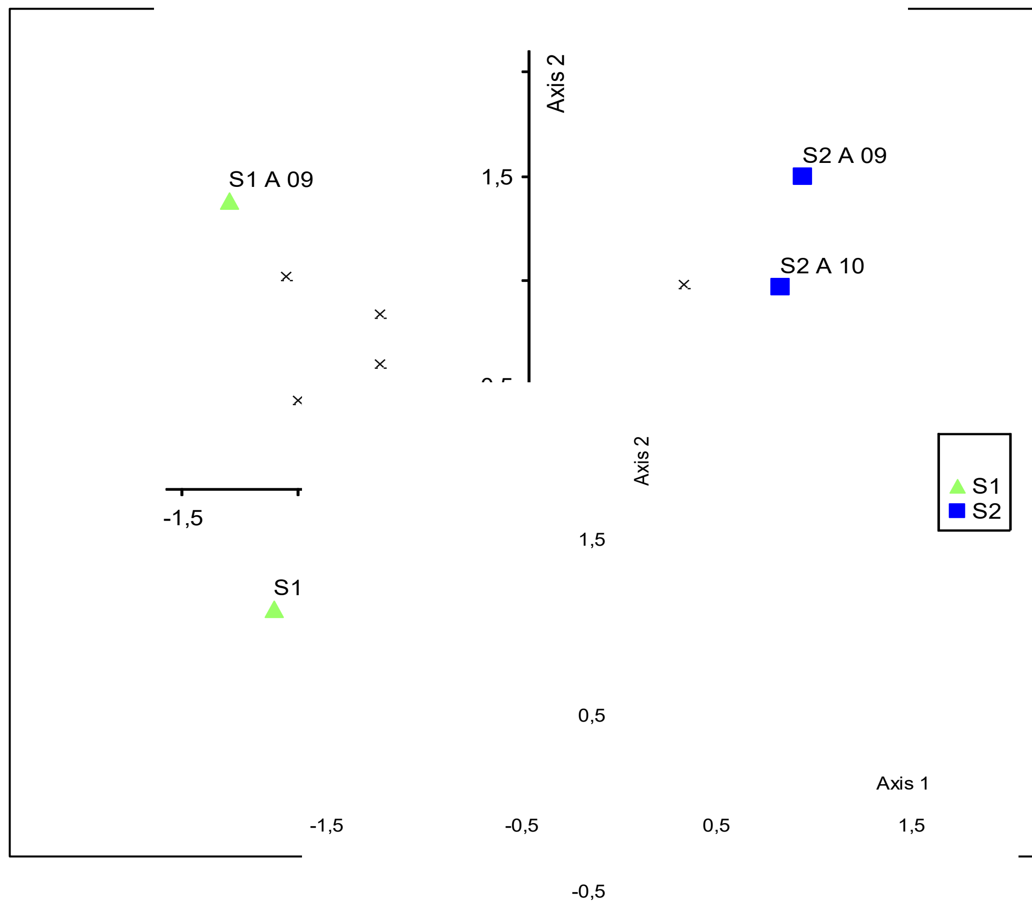

Fig. 4. Canonical correspondence a *osZ* clone libraries. Crosses represent gles represent the axes 1 and 2 scores f (09) and 2010 (10) at e l Acebrón lagoo ctor for the nitrate concentration of the sediments.

Discussion

The *narG*, *napA*, *nirK*, *nirS* and *nosZ* denitrification genes have been used to study the size of denitrifier communities in different environmental conditions (for reviews see Correa-Galeote et al. 2013a; Saggar et al. 2013, and references therein). In this paper, relative abundances of those genes were determined using qPCR to examine the size of denitrifiers in sediments from la Rocina stream taken at sites with relatively low (S1) and high (S2) nitrate concentration, and to analyze their correlation with the nitrate content and denitrification activity of the sediments. Due to its consideration as a national park, Doñana is subjected to special regulations and any anthropogenic effect derives mainly from agricultural practices allowed in the ecotone of the park, where farming of rice and strawberries is common. Sediment samples were taken in April and October in order to represent the wet and dry seasons, respectively. During the 3-year study, hydrological dynamic at each sampling site was affected by the sampling date, which was clearly visible at S2 in October, where stream waters were transformed into swampy waters, and finally in dry sediments. With slight differences, the content of nitrate in S1 was similar for the two sampling seasons and lower than that in S2, where nitrate content in October was always higher than that in April. All those values were smaller than the 50 mg/L defined by the European directive 91/676/CEE as the upper limit for NO_3^--contamination from agricultural sources (European Commission 1991) and similar to those found in sediments from other lakes (Wang et al. 2013) and rivers (Reyna et al. 2010; Chon et al. 2011).

Values of N_2O emission by sediments were relatively low and remained constant at S1, but those found at S2 were greater and highly variable, and no clear relation was found between the content of nitrate and N_2O production. Shifts in N_2O release could be due to changes in water content, mainly at S2, at the end of the dry season, when the water flow is scarce or even null as compared with that in April. Woodward et al. (2009) proposed that oxygenic conditions remaining in sediments after a drought period would result in inhibition of denitrification activity, thus explaining shifts is N_2O emission at the end of the dry season. Previously, Tortosa et al (2011) showed that the pluvial regime affected denitrification rates as the lowest values of activity, measured as N_2O production, were registered at the end of the dry season. Temporal variations in N_2O release have been reported in creek sediments (Rich and Myrold 2004) and agricultural (Dandie et al. 2008) and riparian soils (Deslippe et al. 2014).

The copy number of the 16S rRNA gene fell within the ranges determined by other authors that used the same primers for DNA amplification (Dandie et al. 2007; Bárta et al. 2010; García-Lledó et al. 2011; Keil et al. 2011). Similarly, using primers reported in this study, abundance of denitrification genes in sediments from la Rocina stream were similar to those found for *narG* (Smith et al. 2007; Lindsay et al. 2010), *napA* (Marhan et al. 2011), *nirK* (Henry et al. 2006; Dandie et al. 2008; Su et al. 2010; Attard et al. 2011), *nirS* (Yoshida et al. 2009; Attard et al. 2011; Deslippe et al. 2014) and *nosZ* (Torrentó et al. 2011; Ma et al. 2011; Deslippe et al. 2014) genes from soils and sediments under different environmental conditions. In general, whereas abundance of *napA*, *nirK* and *narG* remained relatively constant regardless of the sampling dates and the sampling sites, the *nirS* and *nosZ* genes showed higher seasonal variation.

Because amounts of DNA and efficiency levels in qPCRs to quantify 16S rRNA and *narG*, *napA*, *nirK*, *nirS* and *nosZ* denitrification genes were similar (Torrentó et al. 2011), it is possible to determine the relative abundance (ratio between a given gene copy number and the 16S rRNA gene copy number) of the denitrification genes. Relative abundance of the *narG*, *napA*, *nirS* and *nosZ* genes increased significantly in S2 as compared with the values found in S1. As expected, seasonal variations were not observed for S1; at S2, however, relative abundances in October were higher than those in April. The relative abundance of *nirK*, however, did not show significant differences either between sampling sites or samplings dates.

Relative abundances of denitrification genes in sediments from la Rocina stream are within the range of those reported for other environmental samples, e.g. *narG* (Henry et al. 2006; Čuhel et al. 2010), *nap*A (Kandeler et al. 2009; Bru et al. 2011; Wieder et al. 2013), *nirK* (Chen et al. 2012a; Palmer et al. 2012), *nirS* (Chen et al. 2012a; Chon et al. 2011; Ligi et al. 2013a,b) and *nosZ* (Chen et al. 2012a, Ligi et al. 2013a,b) gene. No differences in the relative abundance of the *nirS* and *nirK* genes were found in sediments from la Rocina stream, which agrees with results by Enwall et al. (2010) during studies on the spatial distribution of those genes. Nevertheless, dominance of the *nirK* (Henry et al. 2004; Chen et al. 2010) or the *nirS* (Kandeler et al. 2006) genes in environmental samples has been reported. Under all conditions examined in this study, relative abundances of *narG*/*napA* were always higher than those of *nirK*/*nirS* which, in turn, widely exceed those of *nosZ*. All these data which suggests that incomplete denitrifiers are more abundant than those able to carry out the

complete denitrification process in sediments from la Rocina stream. Similar results were reported for constructed wetlands (García-Lledó et al. 2011), aquifer waters and sediments (Torrentó et al. 2011), paddy (Chen et al. 2012a) and riparian soils (Deslippe et al. 2014). It is to note, however, that *narG* gene can be present in more than one copy (Flanagan et al. 1999) and that *narG* and *napA* can coexist in the same bacteria (Sias et al 1980; Warnecke-Eberz and Friedrich, 1993.

Given that nitrate is a major factor involved in expression of denitrification genes, its presence in sediments is expected to increase their relative abundances and, concomitantly, the denitrification activity. A Spearman test including the 13 variables analyzed in this study revealed that nitrate content correlated best with relative abundance of the *narG*, *napA*, *nirS* and *nosZ*, the highest positive correlation found between the content of nitrate and *nosZ* relative abundance. In contrast, the relative size of *nosZ* did not correlate with nitrate content in a constructed wetland (García-Lledó et al. 2011) and in grassland soils (Keil et al. 2011). The content of nitrate did not affect the abundance of the total bacteria community as the copy numbers of the 16S rRNA genes in sediments from S1 and S2 were similar and no correlation were found between them. The test also showed the absence of correlation between the content of nitrate and N_2O emissions as well as between N_2O emissions and gene abundances. These results agree with those found in sediments (Ma et al. 2008) and soils samples (Henderson et al. 2010; Dandie et al. 2011). Other authors, however, reported a link between nitrate content and N_2O emissions (Luo et al. 1999, Zechmeister-Boltenstern et al. 2002, Hefting et al. 2003, Magalhães et al. 2008) and between N_2O emissions and abundances of the *nirK* (Brankatschk et al. 2010; Enwall et al. 2010; Attard et al. 2011), *nirS* (Brankatschk et al. 2010; Enwall et al. 2010) and *nosZ* (Petersen et al. 2012) genes, respectively. As indicated above, the presence of oxygen in dry soils may inhibit denitrification activity (Tiedje, 1988; Woodward et al. 2009) which, in turn, would result in the decrease of N_2O emission. It is also possible that nutrient limitation during the dry season due to water shortage could also limit denitrification activity (Smith et al. 2010).

A PCA analysis showed a strong assemblage between nitrate content and the *narG*, *napA*, *nirK, nirS* and *nosZ* denitrification genes relative abundances which cluster together in factor 1, and reflect the idea that nitrate clearly influences the denitrifier communities as relative abundances of the denitrification genes change with changes in

the nitrate content. In agreement with the Spearman's test, the PCA confirmed the null correlation between nitrate content and N_2O emissions and between N_2O emissions and denitrification gene abundances that were comprised independently in PCA factor 1. Regardless of the year, samples from a given sampling site and month grouped together, which suggests the absence of temporal variation during the 3-year study.

Taken together, our data suggest that *nosZ* could be used as a molecular marker for characterization of denitrifier communities. Similar proposals have been suggested previously (Chen et al. 2012b; Deslippe et al. 2014) as *nosZ* is commonly used to investigate denitrifier communities in environmental samples (Rösch et al. 2002; Rich and Myrold, 2004; Throbäck et al. 2004; Henry et al. 2006; Horn et al. 2006; Palmer et al. 2009; Dell et al. 2010; Chon et al. 2011; Ishii et al. 2011; Correa-Galeote et al. 2013b; Ligi et al 2013a,b). Moreover, *nosZ* has been shown to be more stable under different environmental conditions than other N-cycle genes (Wallenstein et al. 2006; Chroňáková et al. 2009; Rasche et al. 2010). Also, despite some divergences (Ishii et al. 2011), bacterial phylogeny based on the 16S rRNA is more congruent with that of the *nosZ* than with those based on any other denitrification gene (Jones et al. 2008; Srinandan et al. 2011). In our study, *nosZ* showed a strong correlation with the content of nitrate in sediments and, in addition, as revealed by the PCA, it was the variable that better assume the variability of the remaining properties. Accordingly, the *nosZ* gene was used as a molecular marker to analyze diversity of bacterial denitrifiers in the sediment samples. Because of the scarce temporal variation of denitrification genes discussed above, the study was limited to years 2009 and 2010.

The richness (S), estimated as the number of OTUs, was similar among libraries, except for that corresponding to samples taken in October 2009 and 2010 at S2 that were higher than those found in the remaining libraries. It is possible that the higher nitrate content in the samples could result in a higher *nosZ*-richness in the sediments. Considering the 504 clones isolated in our study, the richness in sediments was higher than that previously reported in soils and sediments (Rich and Myrold 2004; Enwall et al. 2005), a waste sludge (Srinandan et al. 2011) and in a eutrophic lake (Wang et al. 2013), and more similar to those obtained by Chen et al. (2012a) in a paddy soil under different fertilization regimes, though different primers for *nosZ* amplification were used in those studies. Similar to the increase in richness at S2, the presence of nitrate also produced a higher richness of the *nirK*/*nirS*-bearing (Santoro et al. 2006) and *narG*-

targeted (Reyna et al. 2010) communities in marine and river sediments, respectively. However, other studies on diversity of denitrifiers have shown no community structure changes in response to nitrate content (Wolsing and Priemé, 2004; Carrino-Kyker et al. 2012). Whereas no differences in the diversity indexes Chao1 and Shannon-Weaber were detected among libraries, the Simpson index of the libraries corresponding to October 2009 and 2010 at S2 was lower than those of the other libraries, which suggests that the appearance of new OTUs and the increase in the population evenness was due to the presence of nitrate. Based on *nosZ* denitrification gene, other authors have reported that nitrate did not affect richness of the denitrifier communities (Zhou et al. 2011, Chen et al. 2012b, Vilar-Sanz et al. 2013).

The AMOVA test revealed that whereas structure of denitrifier communities at S1 remained relatively constant during the 2-year study, the structure of the communities at S2 changed with the sampling date and sampling year, suggesting that nitrate is a driving force for community dynamics.

Betaproteobacteria in la Rocina sediments dominated over the Alphaproteobacteria, which agrees with reports by Srinandan et al. (2011) in inactivated sludges and Chen et al. (2012b) in paddy soils, and differ from those by Henry et al. (2006) and Magalhães et al. (2008) found in soils and sediments from a river estuary, respectively. The presence of Gammaproteobacteria was much lower than the other two bacterial classes and was restricted to S1, suggesting that this population might be circumscribed to specific sites as previously reported for paddy soils (Chen et al. 2012a).

There is a substantial diversity among the *nosZ*-bearing communities in the 504 clones that is not represented in culture collections. Similar results were found in marine sediments by Scala and Kherkoff (1999) when analyzing *nosZ* denitrifiers and Chen et al. (2010) and Smith and Ogram (2008) during bacterial diversity studies based on the *nirK* and *nirS* genes communities in soil and sediments. The biological meaning of the difference in the number of unclassified bacteria between S1 (53%) and S2 (21%) cannot be elicited from the present results.

Although scarce differences were observed in the numbers of clusters and their composition among libraries, the CCA in Fig. 4 shows that nitrate content affected the *nosZ* diversity. This lends support to the use of *nosZ* diversity as a useful tool to evaluate the effect of nitrate content in denitrifying communities. Reyna et al. (2010)

found a high diversity in the sediments with the higher nitrate content when they analyzed the diversity of the *narG*-bearing communities in sediments with different nitrate content. Santoro et al. (2006) also found that diversity of the *nirS* and *nirK* communities in marine sediments with low nitrate levels where lower than that for sediments with higher nitrate content.

The dominant clusters C1, C22, C24 and C27 found in this study were also reported as dominant groups in different environments. Thus, the C1 cluster (Bradyrhizobiaceae family) was the main group in eutrophic lake sediments (Wang et al. 2013) and ephemeral wetland soil (Ma et al. 2011), cluster C22 (Rhodocyclaceae family) in wastewater treatment plants (Chon et al. 2010), unclassified cluster C24 in paddy soils (Ishii et al. 2011), and cluster C27 in a activated sludge (Srinandan et al. 2011). Nevertheless, members of the Rhizobiaceae (Dell et al. 2010) and Rhodospirillaceae (Wang et al. 2013) and Burkholderiaceae (Chen et al. 2012b) families were the most abundant groups in different environmental samples.

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Artículo III

Título:

Spatial distribution of N-cycling microbial communities showed complex patterns in constructed wetland sediments

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Este artículo contiene los resultados relativos al objetivo 2 de esta Memoria de Doctorado.

Resumen

Los humedales artificiales (wetlands) se utilizan para el tratamiento biológico de las aguas residuales procedentes de tierras agrícolas que transportan compuestos contaminantes como los nitratos. La eliminación de nitrógeno en los humedales se produce mediante asimilación directa por las plantas y por medio de los procesos microbianos conocidos como nitrificación y desnitrificación. En este trabajo se ha investigado los genes implicados en los procesos de nitrificación (*amoA*) y desnitrificación (*napA*, *narG*, *nirK* y *nosZ*) y así como la distribución espacial de las comunidades microbianas implicadas en sedimentos de un humedal irrigado con aguas contaminadas con nitratos. Para caracterizar las comunidades microbianas se utilizó la PCR cuantitativa a tiempo real (qPCR) y se empleó el análisis de la varianza geoestadística para relacionarlas con la vegetación de cubierta y las propiedades fisico-químicas de los sedimentos del humedal. Para los genes implicados en nitrificación y desnitrificación, los datos de autocorrelación basados en el análisis de la semivarianza indicaron la tendencia a una distribución en parche para escalas de 10 m. Las propiedades físico-químicas de los sedimentos, sin embargo, mostraron diferentes tipos de distribución ya que se detectó distribución en parches para el contenido en C, nitrato y amonio pero no para el pH y la humedad. La actividad desnitrificante y la producción potencial de N_2O mostraron una distribución espacial común que, a su vez, fue diferente a la de la razón $N_2O/(N_2O + N_2)$.

Abstract

Constructed wetlands are used for biological treatment of wastewater from agricultural lands carrying pollutants such as nitrates. Nitrogen removal in wetlands occurs from direct assimilation by plants and through microbial nitrification and denitrification. We investigated the spatial distribution of N-cycling microbial communities and genes involved in nitrification and denitrification in constructed wetland sediments receiving irrigation water. We used quantitative real-time PCR (qPCR) to characterize microbial communities. Geostatistical variance analysis was used to relate them with vegetation cover and biogeochemical sediment properties. The spatial distribution of the N-cycling microbial communities of sediments was heterogeneous and complex. Total communities of bacteria and crenarchaea showed different spatial distributions. Analysis of autocorrelation patterns through semivariance indicated a tendency towards a patchy distribution over scales around 10 m for genes involved in the nitrification and denitrification processes. In contrast, biogeochemical sediment properties showed diverse spatial distributions. While almost no patchiness was found for pH and moisture, patchiness at scales between 8 to 10 m was detected for carbon, nitrate and ammonia. Denitrification variables showed spatial autocorrelation at scales comparable to genes. However, denitrifying enzyme activity and potential N_2O production showed a common spatial pattern, different from that of the $N_2O/(N_2O + N_2)$.

Keywords

Constructed wetlands, N-cycling microbes, spatial patterns

Introduction

Nitrogen (N) is one of the most important plant nutrients in terrestrial ecosystems but excess use of reactive nitrogen threatens the quality of air, soil and water (Rockström, 2009). Thus, more than 11 million tonnes of N fertilizers are used in EU agricultural and much of this added N is lost in the environment (Sutton et al., 2011). For example, the nitrate leaching from crops in Spain may reach to 150-300 N kg ha-1 (Ramos et al., 2002). When nitrate ends up in water, it contributes to eutrophication, the excessive growth of algae which causes the death of other organisms such as fishes. High levels of nitrate in drinking water are also of human health concerns since it can poison infants by provoking methemoglobinemia (Greer & Shannon, 2005). In addition, nitrate can be transformed in the digestive tract in nitrosamines, which are carcinogenic (Craddock & Henderson, 1986). Constructed wetlands have been extensively developed in the last decades as alternatives to on-site treatment methods for diffuse or nonpoint nitrogen pollution of water, and are used for biological treatment of wastewater from agricultural lands, industries or medium size urban settlements (Bruland et al. 2006; Leonard & Swanson, 2001). Nitrogen removal in constructed wetlands varied between 250 and 603 g N m-2 year-1 (Vymazal, 2008). Apart from direct assimilation by plants, removal of N is achieved through microbial nitrification (Purkhold et al., 2000; Treusch et al., 2005) and denitrification activities (Hey et al., 2012). These two N-cycling processes are mainly associated with the subsurface sediment (Kallner Bastviken et al., 2003). For a better management of constructed wetlands, the role of plant species, pH, nutrient flow and organic carbon availability have mostly been investigated (Bachand & Horne, 2000; Park et al., 2008, Peralta et al., 2010).

Despite their crucial role in N-removal, only few studies have focused on the microorganisms performing the nitrification and denitrification processes in constructed wetlands (Chon et al. 2011; Song et al, 2010; 2012). Analysis of the potential activity and the diversity of nitrifying and denitrifying communities in constructed wetlands show that plants species influence both the functioning and structure of these N-cycling guilds (Ruiz-Rueda et al., 2008). Similarly, the presence and type of plants was related to the abundances of denitrifiers in the same wetland (Garcia-Lledó et al., 2011). Differences in the denitrifier community structure were also reported between the different areas of the wetland sediment by Kjellin et al. (2007). The microbial community structure patterns were related to the water flow with increased diversity

with decreasing nutrient levels and increasing water residence times. The water residence times also best explained spatial variations of potential denitrification in the wetland (Kjellin et al. 2007). While the spatial distribution of N-cycling communities have been investigated in arable soil for a better understanding of N-processes in soil (Philippot et al. 2009a; Enwall et al., 2010), our knowledge the spatial distribution of the different microbial guilds in constructed wetlands in scarce. However, understanding the spatial heterogeneity of the nitrifying and denitrifying communities and of their activities across these engineered systems in relation to biogeochemical sediment properties and vegetation cover is of importance for wetland construction and optimal N-removal.

In this work, we investigated the spatial distribution of the abundance and the activity of N-cycling microbial guilds in constructed wetland sediments receiving irrigation water from orchard crops. Since the irrigation water course through the constructed wetland was expected to cause spatial variation in nitrogenated leachates, we hypothesized that key environmental variables, potential denitrification, potential N_2O emissions and communities of microbial denitrifiers and ammonia-oxidizers would show a defined spatial structure. Microbial communities were quantified by real time PCR and, along with environmental and denitrification measurements were analysed using geostatistical methods.

Material and Methods

Experimental site

The experimental site was a constructed wetland called Los Guayules (UTM coordinates 29S 0721735, 4108590) located near El Rocio marsh within Doñana National Park (South West Spain). The wetland receives water from irrigation of nearby fruit orchards. The water regime is seasonal, flooding in winter and partially drying during summer. Vegetation is represented by a perennial community of aquatic and water-associated plants dominated by *Typha* spp., *Imperata cylindrica*, *Juncus effusus*, *Scirpus holoschoenus* and *S. maritimus* (Fig. S1a).

Sampling design

To detect the spatial variation of environmental variables, denitrifying genes and associated activity, a regular design of 50 sampling points in a 25 m x 50 m grid with 5 m separation distance was used. The grid was oriented following the irrigation water

course through the constructed wetland (Fig. S1b). Sampling was carried out during the dry season. The dry season was chosen for sampling since it was the time when a higher abundance of denitrifiers was found, determined by qPCR in preliminary surveys. The wetland sediments remain with high moisture content even during the dry season. Vegetation cover was estimated by recording the percentage of cover using a 25 cm x 25 cm square placed over each sampling point. Cover percentage was coded as 0 (no vegetation cover), 1 (1-50 % cover), 2 (51-75 % cover) and 3 (75-100 %) cover.

Soil analyses

Texture of the site sediments was determined in samples according to the Spanish Official Methods for Soils and Waters (MAPA, 1974). It was classified as a sandy clay loam sediment, and contains 55% sand, 22.5% clay, and 22.5% silt.

The top sediment layer (0-20 cm) was collected from the 50 sampling points. Samples were kept refrigerated during transport to the laboratory. Samples were appropriately fractionated and either immediately treated or stored under appropriate conditions depending on the analyses to be performed. Sediment moisture was determined gravimetrically by over-drying the sample at 105 °C for 24 h. In fresh samples, NH_4^+ (after 2 h extraction 1:20 w/v with 2N KCl), NO_3^- and NO_2^- (water-extracted 1:20 w/v) and pH (after water extraction 1:5 w/v for 2 h), total organic carbon (TOC) and total nitrogen (TN) were determined as indicated earlier (Tortosa et al., 2011).

Denitrification activity measurements

Potential denitrifying enzyme activity (DEA) was determined in fresh sediment samples using an acetylene inhibition technique as previously described (Ryden & Dawson, 1982). Briefly, an anaerobic slurry was prepared by mixing 25 g soil and 25 ml of a solution containing 1 mM glucose, 1 mM KNO_3 and 1 g l-1 chloramphenicol in a 125 ml glass bottle. The headspace was evacuated and flushed four times with He and 10 ml of acetylene were added. The samples were shaken at 25ºC and the concentration of N_2O was measured in the headspace after 30 and 60 min of incubation by gas chromatography as previously described (Tortosa et al., 2011). DEA was calculated from the N_2O increase during incubation using the Bunsen coefficient for the N_2O dissolved in water. Potential N_2O production was determined by incubating parallel sediment samples without acetylene.

DNA extraction

DNA was extracted from 250 mg of each sub-sample stored at -80 °C according to the ISO standard 11063 'Soil quality-Method to directly extract DNA from soil samples' (Petric et al., 2011). Briefly, samples were homogenized in 1 ml of extraction buffer (1M Tris-HCl, 0,5 M EDTA, 1M NaCl, 20% PVP 40, 20% SDS) for 30 s at 1.600 r.p.m. in a minibead beater cell disrupter (Mikro-DismembratorS; B. Braun Biotech International). Soil and cell debris were removed by centrifugation (14.000 x g for 1 min at 4 °C). After precipitation with ice cold isopropanol, nucleic acids were purified using both PVPP (Biorad) and GeneClean (MP Bio) spin columns. Quality and size of soil DNAs were checked by electrophoresis on 1% agarose. DNA was also quantified by spectrophotometry at 260 nm using a BioPhotometer (Eppendorf, Hamburg, Germany).

Quantification of the N-cycle associated microbial community

The size of the nitrifier community was estimated by quantitative PCR (qPCR) of *amoA* from ammonia-oxidizing bacteria (AOB) and archaea (AOA) (Wessén et al., 2011) and that of the denitrifier community by qPCR of *narG*, *napA*, *nirK*, *nirS* and *nosZ* gene fragments using reaction mixtures, primers and thermal cycling conditions described previously (Henry et al., 2004; 2006; Kandeler et al.; 2006; Bru et al., 2007; Philippot et al., 2009a, b). The total bacterial and crenarchaeal community was quantified using 16S rRNA as molecular marker as described by López-Gutierrez et al. (2004) and Ochsenreiter et al. (2003), respectively. Reactions were carried out in an ABI Prism 7900 Sequence Detection System (Applied Biosystems, USA). Quantification was based on the fluorescence intensity of the SYBR Green dye during amplification. Two independent qPCR assays were performed for each gene. Standard curves were obtained using serial dilutions of linearized plasmids containing cloned *amoA*, *narG*, *napA*, *nirK*, *nirS*, *nosZ* and 16S rRNA genes amplified from bacterial strains. PCR efficiency for the different assays ranged between 90% and 99%. No template controls gave null or negligible values. Presence of PCR inhibitors in DNA extracted from soil was estimated by (a) diluting soil DNA extract and (ii) mixing a known amount of standard DNA to soil DNA extract prior to qPCR. In all cases, inhibition was not detected. Methodological evaluation of the real-time PCR assays showed a good reproducibility of 95.0 ± 12% between two runs.

Statistical analysis

Variables includes in the study were explored using standard statistical techniques. Many of the variables did not meet the normality assumptions and thus several transformation procedures were applied. Correlations (either parametric or non-parametric) were performed on transformed variables. Statistical analyses were performed using SPSS 18 (IBM). Spatial analyses (Cressie, 1991) were performed with transformed variables using semivariograms models from GS+ 9 (Gamma Design Software). Semivariance is a statistic measuring the degree of autocorrelation between spatial samples at different lag distances, in other words, it calculates the degree of similarity between points on a surface. Spatial analyses (Cressie, 1991) were performed with transformed variables using semivariograms models from GS+ 9 (Gamma Design Software). Semivariance is a statistic measuring the degree of autocorrelation between spatial samples at different lag distances:

$$\gamma (h) = [\ 1/2N(h)\ ]\ \Sigma\ [\ z_i - z_{i+h}]^2$$

where

γ (h) = Semivariance for interval distance class *h*;

zi = measured sample value at point *i*;

z_{i+h} = measured sample value at point *i*+*h*; and

N(*h*) = total number of sample couples for the lag interval *h*.

Semivariograms are characterized by three model parameters: Nugget variance: model y intercept; Sill: model asymptote; Range: distance over which spatial correlation is apparent. For linear semivariograms there is no sill and no effective range, since spatial autocorrelation occurs throughout the entire range sampled and there is no characteristic spatial scale for variation. For Exponential and Gaussian models the range is the distance at which the sill (C + C0) is within 5% of the asymptote (the sill never meets the asymptote in these models). Semivariance fit: Residual Sum of Squares (RSS), [$C/(C_0 + C)$]: proportion of sample variance (C_0 + C) that is explained by spatially structured variance C (0 = pure nugget effect). Semivariograms were calculated with the field data and fitted to any of the following models: linear, exponential, spherical or Gaussian, either isotropic or anisotropic, using the statistics (RSS, the Residual Sums of Squares and $C_0/(C_0 + C)$, the proportion of sample variance (C_0 +C) that is explained by spatially structured variance (C) provided by the software. RSS provides an exact measure of how well the model fits the data; the lower RSS, the

better the model fits. Thus, from different possible models the one with lower RSS is chosen. $C_0/(C_0+C)$ value will be 1.0 for a variogram with no nugget variance (where the curve passes through the origin); conversely, it will be 0 where there is no spatially dependent variation at the range specified, i.e. where there is a pure nugget effect. This pure nugget effect should be interpreted with caution since it may be the result of a lack of resolution at small spatial scales. Fractal variograms were also calculated to explore if the measured variables showed fractal or self-similarity properties, indicated by the Hausdorff-Besicovitch statistic D (Burrough 1981). D is close to 1 for linear dimensions and 2 for plane dimensions.

Interpolation to estimate values in an area for points not actually sampled was done by ordinary kriging over the whole sampled field. Cross-validation analysis was used to evaluate kriging fit. In cross-validation analysis each measured point in the spatial domain is individually removed from the domain and its value estimated as though it were never there. Then the point is replaced and the next point is removed and estimated, and so on. In this way a regression of estimated vs. actual values for each sample location in the domain is calculated. The regression coefficient represents a measure of the goodness of fit for the least-squares model describing the linear regression equation. A perfect 1:1 fit would have a regression coefficient (slope) of 1.00. For more details on geostatistical methods refer to the Appendix in Supplemental Material.

Gene abundances were analysed as absolute or relative abundances (gene copy number/16S rRNA Bacteria copy number). Since the number of 16S rRNA operon per cells is variable (Klappenbach et al., 2001), we did not convert the 16S rRNA gene copy data into cells numbers and we expressed our results as gene copy numbers per nanogram of DNA.

Calculation of the gene copy number per nanogram of DNA instead of gram of soil minimized any bias related to soil DNA extraction efficiency.

Results

Environmental variables

The sampled area was almost totally covered with vegetation, although with different percentages (Fig. S2). The surveyed sediment was acid, with pH values ranging from 4.47 to 6.42. Percentage of sediment moisture varied from 3.53 to 22.46. Total organic carbon (TOC) varied from 2.91 to 23.35 g kg^{-1} dry sediment. Ammonium

and NO_3^- content varied widely, up to 8.73 and 168 mg kg^{-1} dry sediment respectively, depending on the vicinity to the source of suspected contaminated water. Total nitrogen (TN) content varied less, ranging from 0.24 to 1.89 g kg^{-1} dry sediment. Expectedly, several sediment variables were cross-correlated (Table S1). NO_3^- was correlated with TN content and TOC. TN showed a high correlation with TOC, a lower one with sediment moisture and an inverse correlation with pH. Sediment moisture was also correlated with TOC and inversely correlated with pH. Only pH was significantly correlated (negatively) with vegetation cover and ammonia.

Fitted semivariance models revealed that sediment pH (Fig. 1, Table S2) and moisture (Table S2), showed spatial dependence over almost all the range considered (ranges about 31 m). In contrast, TOC (Table S2), NO_3^- and ammonia (Table S2 and Fig. 1) did not show spatial dependence over medium and larger scales (ranges between 8 to 10 m). As TN was fitted by a linear semivariance model, no spatial dependence was observed at the scale considered in the study (pure nugget effect) (Table S2 and Fig. 1).

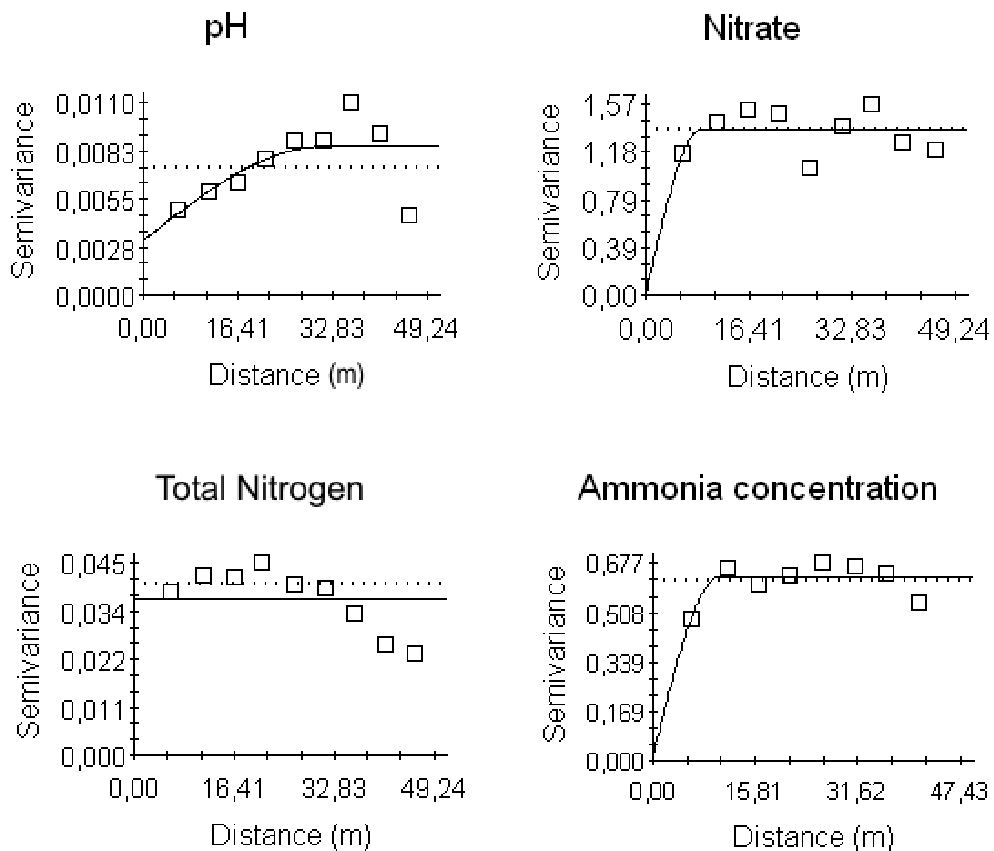

Fig. 1. Semivariograms of some environmental variables. Semivariance models and parameters for all the environmental variables are given in Table S2.

Fractal variograms showed values of the Hausdorff-Besicovitch statistic *D* close to 2, indicating a plane distribution of self-similar, repetitive variation of the environmental variables over the sampled area, but only sediment moisture and ammonia showed good fit (R2 = 0.88 and 0.56, respectively, Table S3). In contrast, nitrate concentration showed a *D* value closer to 1.5 (R2 = 0.49), indicating a repetitive but more linear distribution (Table S3). Interpolated (kriged) maps of environmental variables showed a good fit, except for nitrate (Table S3). Spatial distribution of environmental variables is shown in Fig. 2 and Fig. S3.

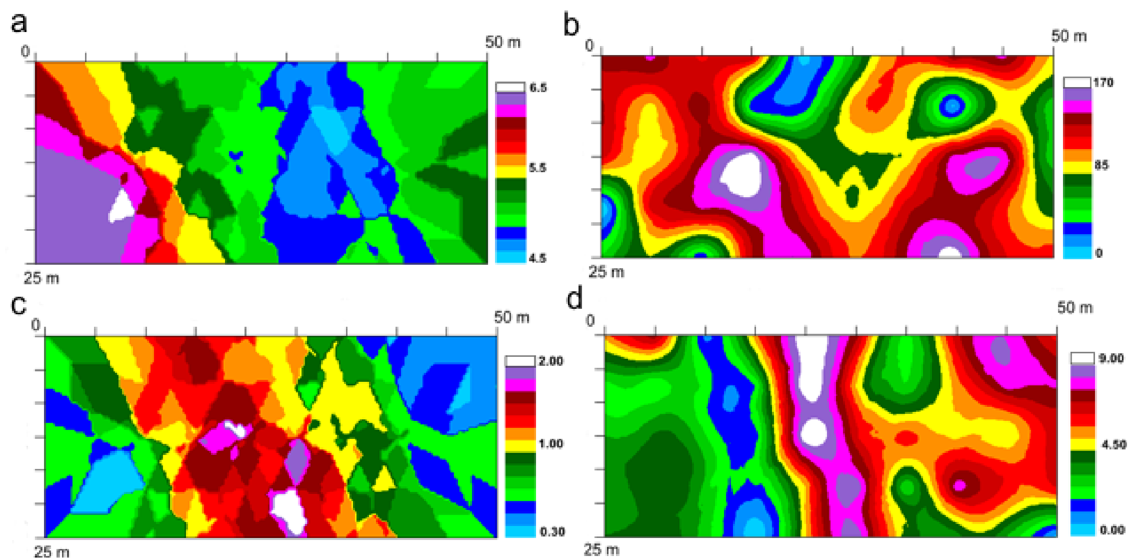

Fig. 2. Interpolated maps for environmental variables. (a) pH, (b) nitrate (mg/Kg sediment), (c) total nitrogen (g/Kg sediment) and (d) ammonia (mg/Kg sediment). Colour scales indicate extrapolated values by kriging. Maps are shown in the same orientation as in Fig. S1b (sampling area).

Spatial distribution of microbial communities

Gene abundances were analysed as absolute (hereafter abundance) or relative abundances (gene copy numbers/16S rRNA Bacteria copy numbers, hereafter relative abundance). Abundances of both total bacterial and crenarchaeal community (16S rRNA of Bacteria and crenarchaea) and denitrification genes (*narG*, *napA*, *nirS*, *nirK*, *nosZ*) were highly variable, ranging several magnitude orders. Absolute abundance of 16S rRNA of Bacteria ranged between 360 to 360000 copies, one order of magnitude greater than the number of copies of 16S rRNA of crenarchaea. The *narG* absolute

abundance varied between 30 to 5700 copies, one order of magnitude lower than *napA*. *nirK* absolute abundance ranged between 4 to 3700 copies, one order of magnitudes lower than *nirS*. *nosZ* varied between 10 to 1700 copies. In general, abundances of *narG*, *napA*, *nirS* *nirK* were highly correlated with total bacterial community (as determined by the 16Sr RNA gene) (Spearman´s rho values between 0.85 and 0.96, p = 0.000), although *nosZ* showed a lower correlation (0.52, p =0.000) (Fig. S4a). Abundances of *narG* and *napA* were highly correlated (Spearman´s rho = 0.92, p = 0.000), as well as abundances of *nirS* and *nirK* (Spearman´s rho = 0.94, p = 0.000). However, relative abundances of *narG* and *napA* were negatively correlated (Spearman´s rho = -0.55, p = 0.000) (Fig. S4b). AOB and AOA (the ammonia-oxidizing bacteria and archaea harbouring the *amoA* gene) showed very sparse distributions. Although AOB was a magnitude order more abundant (up to 1700 copies) than AOA, their abundances were moderately correlated (Spearman´s rho = 0.50, p = 0.000). Few significant relationships involving environmental variables and genes distribution were found (Table S4). The few significant correlations involved complex relationships. For example, the relative abundance of *nosZ* was significantly, although negatively, correlated with pH, and not correlated with TN (Fig. S4c), even when TN which was in turn negatively correlated with pH (Table S1). Other significant correlations showed negative low values, like those between pH and abundances of 16S rRNA Bacteria and Archaea, *narG*, *napA*, *nirS* and *nirK*, and the relative abundance of *nirK*. pH also showed a significant and positive correlation with the abundance ratio *nosZ*/*narG*. Nitrate was significantly although low correlated with the abundance ratio *nirS*/*nirK* (Fig. S4d). TN was correlated with the relative abundance of *nirK*. Neither soil moisture nor TOC showed significant correlations with genes distribution. For ammonia-oxidizing communities, only *amoA* from AOA showed a significant correlation with ammonia (Spearman´s rho = 0.41, p = 0.02). Vegetation cover showed low but significant correlations with total bacterial and crenarchaeal communities, and with absolute abundances of denitrifier genes *narA*, *napA*, *nirS* and *nirK*, but not with *nosZ*. However, the ratio between abundances of *nosZ*/*narG* showed a significant correlation. Only the relative abundance of *nirS* showed significant correlations with vegetation cover.

Total bacteria community determined by the 16S rRNA gene did not show a characteristic spatial dependence (Table S2 and Fig. S5a). In contrast, the abundance of the crenarchaeal community showed a characteristic spatial dependence, although over

a scale around 10 m (Table S2). Relative abundances of *narG*, *napA*, and *nirS*, as well as the ratio *nirS*/*nirK*, showed spatial dependence at 8-11 m while it ranged near the limit of the sampled area (32 m) for the relative abundance of *nosZ* (Fig. 3 and Table S2). In contrast, the relative abundance of *nirK* showed no spatial dependence over the sampled area (linear model). Similarly, *nosZ* showed no characteristic spatial dependence (linear model) over the sampled area, while its relative abundance (*nosZ*/16S rRNA bacteria) showed spatial dependence at ranges near the limit of the sampled area (32 m). As *amoA* from bacteria and crenarchaea showed very sparse distributions, semivariograms could not be calculated.

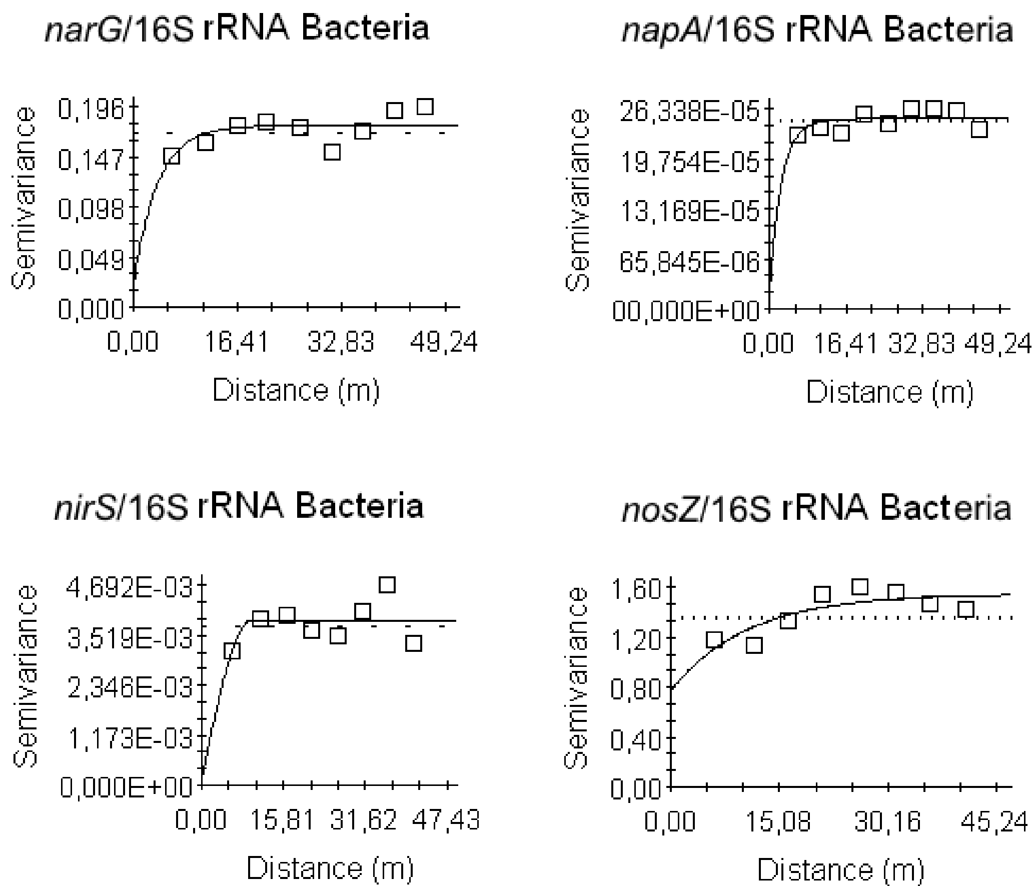

Fig. 3. Semivariograms of some denitrification genes (relative abundances to total bacterial community). Semivariance models and parameters for all the studied genes are given in Table S2.

Fractal variograms of abundances and relative abundances of genes showed a general trend towards repetitive plane distributions, but only abundance of *nirK*, relative abundances of *nirS*, *nirK*, *nosZ* and the ratio *nirS*/*nirK* showed a good fit (*D* between 1.68 and 1.93, R2 between 0.57 and 0.89, Table S3)

Interpolated (kriged) maps of genes distribution showed in general a poor fit, except for the total crenarchaeal community and the relative abundance of *nosZ* (Table S3). Spatial distribution of genes is shown in Fig. 4 and Fig. S6. As *amoA* from bacteria and Crenarchaea showed very sparse distributions, kriged maps could not be fitted. A quantile post diagram is shown for AOA (Fig. S7a), together with the kriged map for ammonia distribution (Fig. S7b).

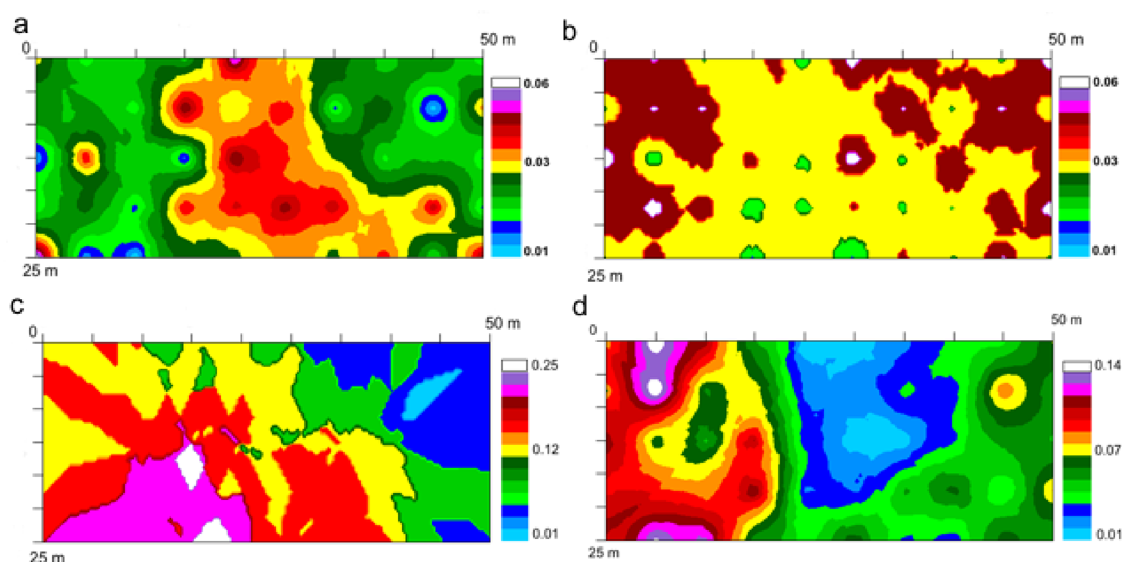

Fig. 4. Interpolated maps for some gene relative abundances distributions. (a) *narG*, (b) *napA*, (c) *nirS* and (d) *nosZ* . Colour scales indicate extrapolated values by kriging. Maps are shown in the same orientation as in Fig. S1b (sampling area).

Denitrification activity and products

Potential denitrification activity (DEA), potential N_2O production, and the proportion of terminal N_2O produced as terminal product of denitrification [$N_2O/(N_2O+N_2)$] showed highly heterogonous distributions over the sampled area. DEA varied from sampling sites with no activity to 600 ng N- N_2O g^{-1} dry soil h^{-1}. Potential N_2O production also varied widely, from no production to 22.41 ng N- N_2O g^{-1} dry soil h^{-1}.

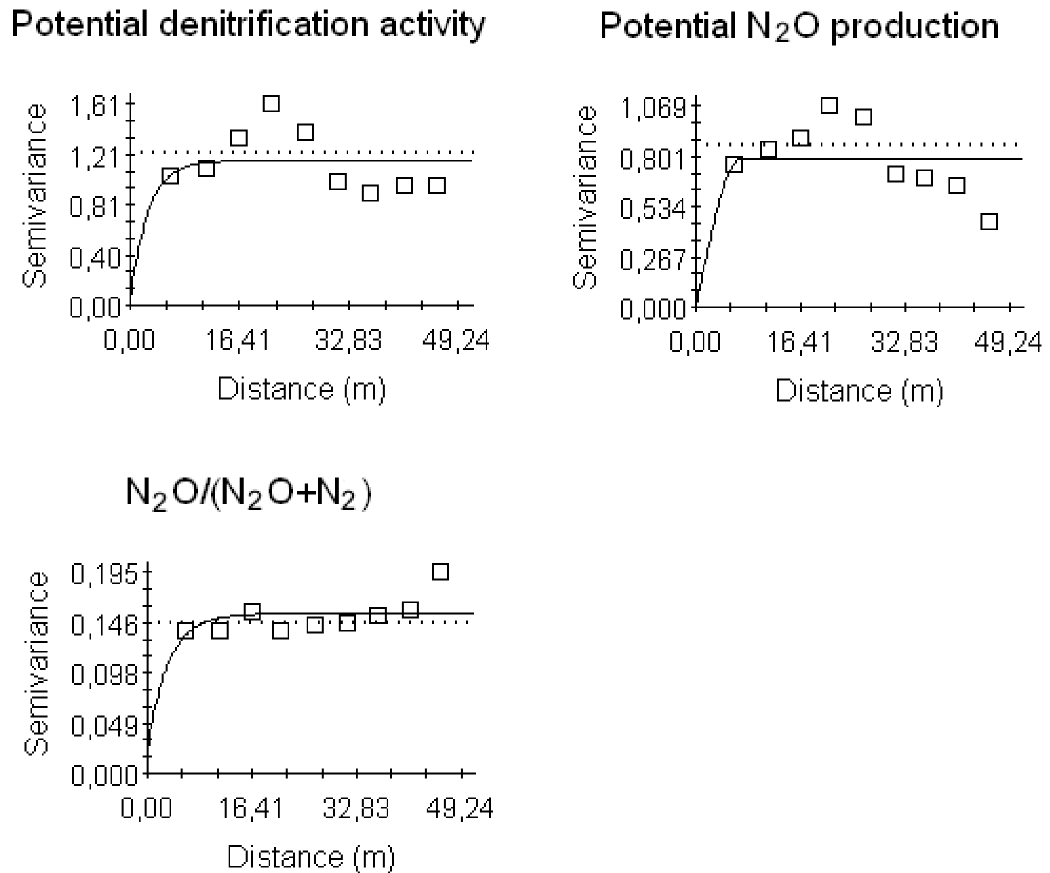

Fig. 5. Semivariograms of denitrification variables. Semivariance models and parameters are given in Table S2.

As expected, denitrification variables were significantly correlated between them and also showed correlations with some environmental variables in a complex way (Table S1). DEA was negatively correlated with pH and positively correlated with TOC and TN, although not with nitrate. It was also correlated with potential N_2O production and negatively correlated with $N_2O/(N_2O+N_2)$. Potential N_2O production showed a similar correlation pattern with other variables as DEA. Denitrification activity variables (DEA and potential N_2O production) were similarly correlated although with relatively low values with vegetation cover. As showed in Table S4, DEA and potential N_2O production were similarly correlated although with relatively low values with total bacterial and crenarchaeal communities, and with abundances of *narG*, *napA*, *nirK*, *nir*S/*nirK*. However, DEA and potential N_2O production showed no correlations with *nos*Z. Relative abundances of *nirS* and *nos*Z showed similar significant correlations with DEA, while relative abundances of *nirS* and *narG* similarly correlated with

potential N_2O production. In contrast, the $N_2O/(N_2O+N_2)$ ratio did not show correlation with any absolute or relative gene abundance. However, DEA and potential N_2O production showed a peak in spatial dependence around 24 m, not present in the $N_2O/(N_2O+N_2)$ ratio (Fig. 5).

Fractal variograms showed *D* values close to 2, indicating a plane distribution of self-similar, repetitive variation of the denitrification variables over the sampled area, but with relatively low fit (R^2 from 0.33 to 0.60, Table S3). Interpolated (kriged) maps of denitrification variables distribution showed a good fit only for DEA (Fig. 6, Table S3).

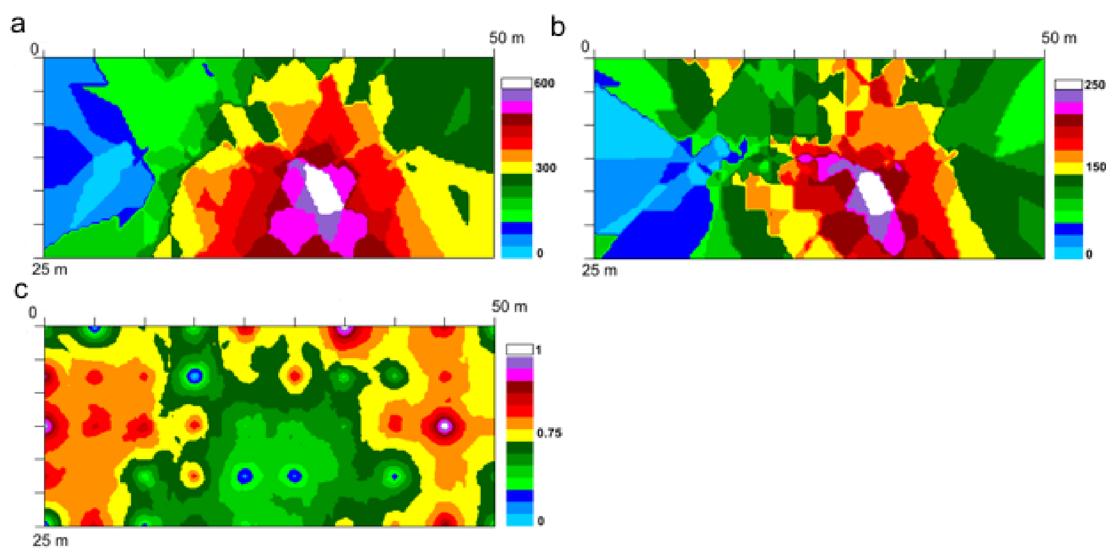

Fig. 6. Interpolated maps for denitrification variables distributions. (a) Potential denitrification activity (DEA), (b) potential N_2O production, (c) $N_2O/(N_2O+N_2)$. Colour scales indicate extrapolated values by kriging. Maps are shown in the same orientation as in Fig. S1b (sampling area).

Discussion

In this work, we studied the spatial pattern of N-cycle processes and communities in a constructed wetland with a seasonal flooding water regime, in relation with environmental variables (sediment biogeochemical properties and vegetation cover). Biogeochemical properties of the sediments showed contrasted spatial distributions. Both pH and sediment moisture showed autocorrelation patterns over larger scales (around 30 m) compared with total organic carbon, nitrate and ammonia contents (around 8 to 10 m). Total nitrogen content showed autocorrelation over the entire sampled area. Thus, over the scale considered in the study, almost no patchiness

was found for pH and sediment moisture, while smaller scale patchiness was found for carbon, nitrate and ammonia.

Both DEA and potential N_2O production had a common spatial pattern with lower rates north-west of the wetland and highest rates in the central area. This pattern was markedly different to that of the $N_2O/(N_2O+N_2)$ ratio, which showed the highest ratios in the middle of the wetland. This was supported by the significant negative correlation between DEA and the $N_2O/(N_2O+N_2)$ ratio, which suggests that N_2O is more reduced in areas where the potential denitrification is higher. Similar results were reported in a pasture soil by Philippot et al. (2009a), who showed that the spatial patterns of soil properties, which were strongly affected by presence of cattle, imposed significant control on potential denitrification activity and potential N_2O production.

It is well-known that denitrification highly depends on soil variables like pH, water content, N amount and forms present, and available carbon (Groffman et al., 1988). However, we found low or no correlations of denitrification with pH, sediment moisture and nitrate, and only low correlations with organic carbon, nitrogen and vegetation cover. Although in the wetland system studied the potential denitrification activity and the potential N_2O production weakly depended on some environmental properties and microbial communities, the proportion of the final products of denitrification, either N_2 or N_2O, did not depend on the variables studied, although indeed showed a defined spatial pattern. An inverse relationship between pH, the relative abundance of *nosZ* and the proportion N_2O as terminal product of denitrification was found by Philippot et al. (2009a). We found a negative relationship between pH and the potential N_2O production but not relationship between pH and the relative abundance of *nosZ*, nor with the proportion N_2O as terminal product of denitrification. It is known that a low pH increases N_2O production from denitrification (Šimek & Hopkins, 1999) through a decrease in N_2O reduction (van den Heuvel et al., 2011), and thus our result is difficult to explain.

The spatial distribution of N-cycling microbial communities of constructed wetland sediments from crop irrigation waters was heterogeneous and complex. Total communities of bacteria and crenarchaea showed different spatial distributions, with no characteristic spatial dependence over the sampled area for the 16S rRNA gene of

bacteria, but with spatial autocorrelation at small scales for the 16S rRNA gene of crenarchaea. Genes involved in the nitrification and denitrification processes were distributed following spatial patterns with different degrees of autocorrelation but in general confined to small distances, around 10 m. Analysis of autocorrelation patterns through semivariance and fractal variograms indicated a tendency to a patchy distribution over small scales.

Given the limited availability of comparable published works on N-cycling genes distribution focusing constructed wetlands and on small spatial scales, discussion of our results is somewhat limited to previous works made on soils, mainly grasslands and farms. Philippot et al. (2009a) reported in grazed grasslands a non-random distribution pattern of the size of the denitrifier community estimated by quantification of the denitrification genes copy numbers with a scale spatial dependence (6–16 m) similar to that found in our work. Keil et al. (2011) found, also in grasslands, that soil properties were affected by management practices and showed spatial heterogeneity on greater scales compared with gene distributions. The discrepancy we found between the spatial distributions of environmental variables such as pH and sediment moisture and genes could be responsible for the few associations found between their respective spatial patterns. However, even in the case of sediment biogeochemical properties showing spatial organization at smaller scales (TOC, nitrate and ammonia) comparable with that of the genes, a consistent pattern of relationship with genes did not appear in our study. A few more correlations appeared between denitrification variables (DEA and potential N_2O production) and some gene distributions. Vegetation cover correlated with pH and almost all absolute gene abundances, only with relative abundances of *nirS* and *nosZ*, and with DEA and potential N_2O production. However, all the correlations found with vegetation showed relatively low values. This is unexpected since the fluxes of N_2O in a Danish wetland were influenced by gas transport mediated by macrophytes (Jørgensen et al., 2012). These findings configure a scenario of complex relationships between spatial distributions of biogeochemical properties of the sediments, genes and denitrification activities and products, dominated more by specific correlations than by a general pattern. This general decoupling of spatial organization of habitat variables and genes was shown also by the mentioned comparable studies. Philippot et al. (2009a) found that the spatial patterns of soil properties did not influence the size of the denitrifier community. Keil et al. (2011) found that spatial heterogeneity decreased with higher grassland use including fertilization for soil biogeochemical properties, but

increased for N-cycling microorganisms, allowing the authors to think that factors not considered in the study were driving the microbial distribution found. Even when sampling was performed using a nested scale approach, from cm to m, only a few significant correlations were found (Keil et al., 2011). Thus, reducing the sampling scale to cm did not render further strong evidence of correlation between spatial patterns of soil properties and N-cycling microbial communities. Regarding the spatial distribution of microbial communities probably the adequate scale could be even smaller. Nunan et al. (2002) found aggregated pattern of microbial communities in an arable soil using geostatistics, with lengths of spatial autocorrelation varying between 240 and 1,560 µm in the topsoil and 0–990 µm in the subsoil. It is also possible to think that a scale issue may be involved, not only related with two dimensional but also with the three dimensional distribution of microbial communities in soil (wetland sediment in our work). For example, Dechesne et al. (2003) studied the spatial distributions of ammonia oxidizers and 2,4-D degraders microbial communities using a 3D experimental and modelling approach considering different volume scales (from 50 µm3) and found different 3D aggregated spatial distributions of the two microbial groups. This differential distribution might have been related with the distribution of the substrate (ammonia or 2,4-D) through the complex 3D pattern of soil pores (Dechesne et al., 2007).

Although in our work a general pattern of spatial correspondence between sediment properties and N-cycle associated microbial communities did not appear at the sampling scale used, clearly the distribution of the microbial communities associated to the N-cycle showed a heterogeneous, patchy pattern that in some cases suggests a differential utilization of the sediment habitat by microbial groups harbouring different genes implicated in similar paths of the denitrification process. For example, although both *narG* and *napA* genes [coding for membrane-bound and periplasmic nitrate reductase, respectively, harboured by the same or different bacteria (Deiglmayr et al., 2004)] code for the same step in denitrification, their relative abundances were negatively correlated. Moreover, the relative abundance of these two genes showed larger patches for *narG* (higher range of autocorrelation length), as shown in the interpolated maps. Thus, microbial groups harbouring *narG* or *napA* occupy differential habitat locations perhaps reflecting past competitive exclusion. Relative abundances of *nirS* and *nirK* genes [coding for cytochrome *cd*1 or copper nitrite reductase,

respectively, also carried by different bacteria (Jones et al. 2008)] were not correlated, in spite of the apparently similar distributions of absolute abundances of *nirS* and *nirK* shown by the interpolated maps. This may be due to their very different absolute abundance ranges (*nirS* was an order of magnitude more abundant than *nirK*). The distribution of the relative abundances of these two genes indicates that bacterial groups harboring *nirS* are much more abundant than those carrying *nirK* and that they may use the habitat differentially but not exerting competitive exclusion. Our findings agree in general with results from other works studying the spatial distribution of *narG*, *napA*, *nirS* and *nirK* genes, showing differential habitat use by bacteria harboring genes coding for the same denitrification step (Philippot et al., 2009a; Hallin et al., 2009; Enwall et al., 2010; Keil et al., 2011). Since genes in both functionally-redundant pairs (*narG-napA* and *nirS-nirK*) showed the same correlation pattern with the environmental variables considered in this study, it is clear that some other dimension of the ecological niche may be explaining the different spatial distribution of bacteria carrying these genes. Among other relevant niche components, spatial distribution of nutrients and water adsorbed in the three-dimensional soil micro-structure, as well as interactions with other bacteria and predators (especially protozoa), are important factors in determining the spatial distribution of soil bacteria (Dechesne et al., 2007).

In contrast to the pairs *narG-napA* and *nirS-nirK*, the genes encoding the first step of nitrification, *amoA*, from bacteria and archaea showed a moderate correlation, indicating that this nitrification step may be performed simultaneously by bacteria and archaea in the same habitat location. However, as spatial distribution of both AOA and AOB were highly sparse further interpretations in terms of possible ecological niche variables involved is difficult to make. Our results contrast with those found for the same gene by Wessén et al. (2011) in soils at farm scale, where spatial distributions of both communities did not overlap and were related with soil pH and clay content. In our study AOB abundance was higher than AOA abundance, as shown also by Tada et al. (2011) and by Limpiyakorn et al. (2011) in constructed wetlands for wastewater treatment. However, we found that only AOA showed a significant correlation with ammonia in spatial locations of joint occurrence with ammonia. This may be explained by the finding of Limpiyakorn et al. (2011) that AOA can outcompete AOB under low ammonium levels, comparable to those found in our study.

Coming back to our formulated hypothesis, we found a mixed support to it, since the spatial distribution of N-cycle associated microbial communities and genes, and their functional activities, showed a complex relationship with wetland sediment properties and environmental variables. Microbial genetic background is an important factor shaping the microbial niche, which may or not allow a bacterial group to exploit its microhabitat and even modify it (Marco, 2008). However, further studies involving a range of spatial scales, other potentially relevant niche dimensions like O2 soil diffusion and also incorporating a three dimensional approach could be useful to explain the seeming uncoupling between spatial distributions of environmental properties and those of microbial communities.

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Artículo IV

Título:

Determination of denitrification genes abundance in environmental samples

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Este artículo contiene los protocolos y procedimientos para resultados relativos a los objetivos 2 y 3 de esta Memoria de Doctorado 2 y 3 de esta Memoria de Doctorado.

Resumen

La diversidad de los microorganismos que intervienen en el ciclo biogeoquímico del N es de interés fundamental en el estudio de la ecología microbiana. El proceso de desnitrificación, una etapa clave de este ciclo, consiste en la reducción del nitrato a dinitrógeno molecular vía la formación de los productos intermediarios nitrito, óxido nítrico y óxido nitroso. Este proceso se realiza de forma secuencial por las enzimas nitrato-, nitrito-, óxido nítrico- y óxido nitroso-reductasa, respectivamente. La cuantificación de ácidos nucleicos en muestras medioambientales se realiza habitualmente mediante la reacción en cadena de la polimerasa a tiempo real (qPCR). En este trabajo se revisan, actualizan y discuten diversos protocolos ampliamente utilizados para a) la extracción de ADN de muestras medioambientales, b) las condiciones de qPCR para la determinación de la abundancia del gen 16S rRNA y de los genes de la desnitrificación *narG*/*napA*, *nirK*/*nirS*, c-*nor*/q-*nor* y *nosZ*; c) los cebadores que se emplean; y d) la metodología para la construcción de las rectas de calibrado. El método ISO 11063 y la directriz MIQUE se consideran en este trabajo con la finalidad de mejorar la transparencia experimental de estos protocolos.

Abstract

Diversity of microorganisms involved in the biogeochemical N cycle is of fundamental interest in microbial ecology. Denitrification is a key step in the cycle by which nitrate is reduced to dinitrogen gas via the soluble nitrite and the gaseous compounds nitric oxide and nitrous oxide. The process is carried out by the sequential activity of the nitrate-, nitrite-, nitric oxide-, and nitrous oxide-reductase enzyme, respectively. The fluorescence-based quantitative real-time polymerase chain reaction (qPCR) is widely used for quantification of nucleic acids in samples obtained from numerous, diverse sources. Here, we provide a well proven methodology for isolation of DNA from environmental samples, and describe relevant experimental conditions for utilization of qPCR to assay the 16S rRNA and *nar*/*nap*, *nirK*/*nirS*, c-*nor*/q-*nor* and *nos* denitrification genes that encode synthesis of denitrifying enzymes. The ISO 11063 standard method and MIQUE guidelines are considered with the aim to increase experimental transparency.

Key words

Environmental samples; denitrifier communities; DNA extraction; DNA purification; DNA quantification; real-time PCR; relative abundance.

Introduction

When faced with a shortage of oxygen (O_2), many bacterial species are able to switch from O_2-respiration to using nitrate or its derived-nitrogen oxides to support respiration in a process known as denitrification. During this process the water-soluble nitrate is converted into gaseous nitrogen-containing gases. These are the a) cytotoxic and ozone-depleting nitric oxide (NO), b) potent and long-lived greenhouse gas nitrous oxide (N_2O), and c) the relatively inert dinitrogen gas (N_2). Because denitrification is performed by more than 60 bacterial genera, it was believed that the process would be performed exclusively by bacteria. Now, there are evidences that some fungi [111, 89], archaea [118] and some Foraminifera and Gromiida [98, 86] are also able to denitrify. Moreover, nitrifiers also have genes involved in denitrification [14, 104]. A list of archaeal, bacterial and fungal genera for which at least one denitrifying gene has been reported [84].

Genes and enzymes involved in denitrification

Reduction of nitrate to dinitrogen gas is carried out by the sequential activity of the enzymes nitrate-, nitrite-, nitric oxide-, and nitrous oxide-reductase, encoded by the *nar*/*nap*, *nirK*/*nirS*, c-*nor*/q-*nor* and *nos* genes, respectively.

Respiratory nitrate reductases

The first reaction of denitrification, the conversion of nitrate to nitrite, is catalyzed by two biochemically different enzymes, a membrane-bound nitrate reductase (Nar), or a periplasmic nitrate reductase (Nap) [reviewed in 93, 94, 121, 123, 35, 95]. Nar is a 3-subunit enzyme composed of NarGHI, where the catalytic subunit NarG and the one [3Fe-4S] and three [4Fe-4S] NarH subunit are located in the cytoplasm and associate with NarI whose N-terminus faces the periplasm. Nar proteins are encoded by genes of a *narGHJI* operon. *narGHI* genes encode the structural subunits, and *narJ* codes for a cognate chaperone required for maturation and membrane insertion of Nar. In some archaea and bacteria the NarGH subunits are on the outside rather than the inside of the cytoplasmic membrane. The Nar enzyme couples quinol oxidation with proton translocation and energy conservation, which permits cell growth under oxygen-limiting conditions [137, 105].

Nap is a 2-subunit enzyme composed of the NapAB complex located in the periplasm and a transmembrane NapC component. NapA is the catalytic subunit, NapB

is a diheme cytochrome c_{552}, and NapC is a c-type tetra-heme membrane-anchored protein involved in the electron transfer from the quinol pool to NapAB [reviewed in 88, 93, 94, 121, 123, 35, 95]. Up to eight different genes have been identified as components for operons that encode perisplasmic nitrate reductases in different organisms. Most bacteria studied thus far have the *napABC* genes in common. The remaining *napDEFKL* genes encode for different proteins that are not directly involved in the nitrate reduction, but in functions required for proper functioning of the enzyme. Although Nap is also linked to quinol oxidation, it does not synthesize ATP [105]. Physiological functions for Nap systems include the disposal of reducing equivalents during aerobic growth on reduced carbon substrates and anaerobic nitrate respiration as a part of bacterial ammonification or denitrification pathways [88]. *Escherichia coli* has a functional duplicate of the *narGHJI* operon named *narZYWV*, which physiologically has a function during stress response rather than anaerobic respiration.

Respiratory nitrite reductases

Two types of respiratory nitrite reductases (Nir) have been described in denitrifying bacteria, NirS and NirK [96, 97 121, 122]. Both are located in the periplasmic space, catalyze the one-electron reduction of nitrite to nitric oxide and neither of the enzymes is electrogenic. The best-characterized *nirS* genes clusters are those from *Pseudomonas aeruginosa* (*nirSMCFDLGHJEN*), *P. denitrificans* (*nirXISECFDLGHJN*) and *P. stutzeri* (*nirSTBMCFDLGH* and *nirJEN*). The NirK enzymes contain type I and II copper centers in the active site and is encoded by the *nirK* gene [96]. Both Nir enzymes are widespread among denitrifiers, but no evidence exists that the same specie could have both enzymes.

Respiratory nitric oxide reductases

Three types of nitric oxide reductases (Nor) have been characterized, cNor, qNor, and qCu_ANor [reviewed in 138, 27, 121, 122, 123]. The cNor is an integral membrane enzyme composed of two subunits, the heme *c* containing-NorC and NorB, which use cytochrome bc_1 complex and a soluble cytochrome *c* or pseudoazurin as electron donors. The qNor uses quinol or menaquinol as electron donors. The enzyme has been found not only in denitrifying archaea and soil bacteria, but also in pathogenic microorganisms that do not denitrify [26] and in the Gram-positive bacterium *Bacillus azotoformans* [110]. This enzyme is bifunctional using both menahydroquinone

(MKH2) and a specific c-type cytochrome c_{551} as electron donor. It was suggested that the MKH2-linked activity of qCu_ANor serves detoxification and the c_{551} pathway has a bioenergetics function. The cNor is encoded by the *norCBQD* operon. The *norC* and *norB* genes encode subunit II and subunit I, respectively and the *norQ* and *norD* genes encode proteins essential for activation of cNor. Some denitrifiers have additional *norEF* genes, the products of which are involved in maturation and/or stability of Nor activity [43]. As a unique case, the Nor of *Roseobacter denitrificans* is similar to cNor, but differs in that it contains copper [66].

Respiratory nitrous oxide reductase

The final step in denitrification consists of the two-electron reduction of nitrous oxide to N_2, a reaction catalysed by the nitrous oxide reductase (Nos) located in the periplasmic space [reviewed in 121, 122, 123, 139]. Nos is a homodimer of a 65 kDa copper-containing subunit, where each monomer is made up of the Cu_A and Cu_Z domains. The *nos* gene clusters often comprise the *nosRZDFYLX* genes. The *nosZ* gene encodes the monomers of Nos. The *nosDFYL* genes encode proteins that are apparently required for copper assemblage into Nos, although their specific role still remains unknown. The NosRX proteins have roles in transcription regulation, activation, and Cu assemblage of Nos [139].

Molecular markers for denitrifying bacteria

Cultivation-dependent and -independent methods have shown that denitrifiers in soils represents up to 5% of the total soil microbial community [114, 46], reaching a density of up to 10^9 cells/g of soil [2, 24, 47]. Although the diversity of denitrifiers was studied by isolating bacterial strains [33], the culture-dependent isolation techniques are limited because of the fact that only a fraction of the bacterial community is cultivable. Application of molecular methods to study microbial diversity in the environment without cultivation was also used to assess the composition of denitrifier communities in environmental samples, mainly soils, waters and sediments. However, since the ability to denitrify is sporadically distributed both within and between different genera and cannot be associated with any specific taxonomic group, a 16S rRNA phylogeny-based approach is not possible to study denitrifiers. Therefore, existing techniques to study the ecology of this bacterial community are based on the use of functional genes in the denitrification pathway, or their transcripts, as molecular markers [reviewed in

82, 83, 42]. Accordingly, DNA extraction followed by PCR amplification of denitrification genes is currently the most common way to quantify the denitrifier communities. For this purpose, utilization of reliable primers that allow amplification of the target genes is required. The *nirK*, *nirS* and *nosZ* genes were the first denitrification genes to be partially amplified [7, 101, 41, 70, 133], and primers were also developed for amplification of the *narG*, *napA* and *nosB* genes [31, 37, 81, 17, 8]. With the exponential increase in the databases of the amount of DNA sequences corresponding to denitrification genes, new primers were developed with broader amplification range, including *nirK* [45], *nirS* [113], *nosZ* [46], *narG* [59] and *napA* [10]. A list of commonly used PCR primers for denitrification genes has been published [42]. It is expected that genome sequencing and metagenomic projects will provide new denitrification gene sequences which could aid in designing new primers primers as recently demonstrated [53].

PCR-independent analyses of denitrifier communities

Immunological assays

The structure and abundance of active denitrifiers can be determined by targeting proteins encoded by any of the denitrification genes. Antibodies have been used to detect NirK and NirS reductases in isolated denitrifiers [22, 69, 127]. After removal of the antibody-labelled cells using flow cytometry, the phylogenetic affiliation of the population could be determined with 16S rRNA oligonucleotide probes. The membrane-bound NarG nitrate reductase was also proposed as a target for quantification of cells isolated from soils [63]. Although the presence of a denitrifying enzyme indicates the presence of the corresponding activity, such an activity can vary among the different species of denitrifiers. Stability of denitrifying enzymes in environmental samples is not well known as it is the time an enzyme may be detected after disappearance of its substrate.

DNA microarrays

Quantification of microbial communities can be approached using DNA microarrays based on DNA-DNA hybridization. For denitrifying bacteria, oligomer microarrays of different molecular sizes have been developed for assessing *narG*, *nirK*, *nirS* and *nosZ* diversity and distribution [18, 68, 112, 115, 131]. Microarray-based whole-genome hybridization has also been used as a technique to detect and identify

microorganisms in environmental samples [132]. An environmental functional gene microarray to profile microbial gene transcripts was useful for assessing functional attributes of microbial communities [67].

PCR-dependent analyses of denitrifier communities

Fingerprinting of denitrifier communities

Several techniques have been described to resolve PCR-amplified denitrification genes. Cloning and sequencing of the PCR amplicons offer detailed information, but more rapid analysis can be achieved using fingerprinting methods. PCR-restriction fragment length polymorphism (PCR-RFLP), terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) have been used to obtain information on the predominant populations in the denitrifier communities. All these techniques separate PCR amplicons of the same size on the basis of their nucleotide-sequence polymorphism. Comprehensive reviews on molecular methods to assess diversity of denitrifying bacteria have been published [83, 42, 103]. Based on the number of peaks or bands and on their relative intensity, these techniques can give estimates of both richness and evenness, but estimation of the total number of denitrifiers is neglected. To circumvent this problem, competitive PCR (cPCR) and quantitative real-time PCR (qPCR) can be used. According to MIQUE guidelines [12], the initials RT-qPCR should be used for reverse transcription-qPCR.

Quantification by PCR of denitrifier communities: cPCR and qPCR

PCR can be used for enumeration of denitrifiers using denitrifying genes as molecular markers. Both cPCR and qPCR technologies rely on the direct proportionality between the intensity of the fluorescent signal measured during the exponential phase of the PCR reaction and the initial amount of target DNA. The copy number of initial target DNA is thereby determined by comparison to a standard curve constructed using target DNA of a known concentration. Although most denitrification genes so far studied are present in single copies within bacterial genomes, *narG* and nosZ can be present in more than one copy [52, 81].

Competitive PCR (cPCR) is based on the simultaneous amplification of the target DNA and a control DNA with a known concentration, the so-called competitor. They compete for the primers during amplifications. Because the method assumes that

both DNAs have the same amplification efficiency, the mass ratio between the two amplicons can be used to determine the initial amount of target DNA. This ratio is estimated by agarose gel analysis of PCRs of multiple dilutions of the competitor with the target DNA. This method has been used for quantification of cytochrome *cd*$_1$-denitrifying bacteria in environmental marine samples [70]. cPCR has also been used to quantify the *nirK* gene in soil and stream-sediment samples [90] and the *nirK* and *nirS* genes in membrane-aerated biofilms at different depths [20]. Some drawbacks of this method are its limited dynamic range, the need to screen multiple dilutions and the requirement for a gel migration step.

Amplification of the *nirS* gene by quantitative PCR (qPCR) was first shown in 2001 [39]. In their experiments they used the TaqMan technology and designed primers for the *Pseudomonas stutzeri nirS* gene. Due to the high specificity of this system, the primer-probe set was specific for *nirS* sequences that correspond only to *P. stutzeri* and, therefore, was of limited utility. SYBR Green is a fluorescent dye that binds non-specifically to double-stranded DNA. During PCR, the intensity of the fluorescence is detected, which results in a logarithmic increase in emission of fluorescence until the reagent become limiting. The cycle number of the PCR at which the fluorescent signal crosses the threshold receives different names, threshold cycle (C_t), take-off point (TOP), crossing point (C_p) and quantification cycle (C_q). The MIQE Guidelines [12] propose the use of the latter name for describing the fractional PCR cycle used for quantification. Because there is a positive correlation between the log of the initial DNA template concentration and the corresponding C_q, if one knows the starting amount of target DNA, a standard curve can be constructed by plotting C_q as a function of the log of the copy number of the target DNA. The gene copy number in the DNA sample can then be determined based on its C_q. The PCR efficiency of the reaction, as indicated by the slope of the curve as well as the lack of PCR inhibition has to be checked. Dilutions of extracted DNA or addition of a given amount of control DNA to environmental DNA can be used for verification.

qPCR does not require a gel migration step, is highly reproducible and sensitive, and is less expensive, laborious and time-consuming than cPCR. Reviews dealing with the advantages and limitations of qPCR have been published [135, 107, 9]. Accordingly, qPCR is currently the main method used for quantification of environmental samples, including the denitrifier communities.

Because RNA provides evidence of gene expression, qPCR techniques based on RNA extracted directly from environmental samples can be retro-transcribed to cDNA and used for qPCR. After reverse transcription, the *narG*, *napA*, *nirK*, *nirS* and *nosZ* genes were quantified by standard qPCR [74]. Quantification of the *nosZ* was also achieved using cDNA, but *nirS* and *nirK* genes could not be amplified [25]. Using cDNA from an agricultural soil, the *nosZ* and *nirSp* (*nirS* from *Pseudomonas mandelii*) genes were quantified by qPCR, but amplification of the *nirK* and *nirS* from the total soil community, or their transcripts, was not obtained even when different primers, PCR conditions and cycling parameters were used [44]. As for all PCR-based techniques, qPCRs are subjected to well-known biases introduced by, e.g. DNA extraction procedures, primer selection, and PCR conditions.

DNA extraction from environmental samples

A conventional approach to evaluate the abundances of denitrifiers in environmental samples include: a) DNA extraction and purification, b) PCR's inhibition tests, c) Target gene quantification by qPCR, and d) analysis of the obtained results. In this review, the term environmental sample refers mainly to soil and sediment samples.

Initial attempts for DNA isolation

Pioneer methods for soil DNA isolation used *ex situ* methodologies that included long incubations of soils in a solution made of sodium cholate and Chelex 100 resin, followed by centrifugation and passage through a Percoll gradient to separate the most dense soil particles from the floating organic matter and microorganisms [48, 117]. Further breakage of the cells by sonication and differential centrifugation allowed DNA extraction. Later, it was shown that the DNA samples obtained by this methodology were not representative of the entire DNA in the soil samples [56, 108].

First approaches to total soil DNA isolation from environmental samples were developed by several authors using mechanical and enzymatic lysis, followed by cleaning of the crude extract and DNA precipitation. Accordingly, soils were treated with a high salt concentration-extraction buffer containing hexadecyl-trimethyl ammonium bromide (CTAB) and proteinase K. Samples were further incubated with sodium dodecyl sulfate (SDS), mixed with a mixture of clorophorm/isoamyl alcohol (24:1), precipitated with isopropanol, washed with 70% ethanol and, finally, resuspended in milliQ (MQ) water [136]. This methodology was improved to

simultaneously recover RNA and DNA from soils and sediments by homogenization of the samples in a high salt concentration-extraction buffer containing CTAB and SDS, frozen in liquid nitrogen and ground until thawed [49]. Other authors homogenized the soil samples in an extraction buffer containing PVPP and Chelex 100 resin, extracted the DNA with the same buffer supplemented with SDS, proteinase K and 10% Sarkosyl, and purified it with a mixture of phenol/clorophorm/isoamyl alcohol (25:24:1) [54]. Cell lysis was also achieved using a long treatment at 68 ºC in the presence of SDS and guanidine isothiocyanate followed by precipitation with polyethylene glycol (PEG-8000) and purification with CTAB, chloroform and ammonium acetate [30, 87].

The ISO standard 11063 Soil quality- method to directly extract DNA from soil samples

During evaluation of the effectiveness of nine DNA extraction procedures, homogenization of the samples using a bead beater disrupter and SDS in the extraction buffer gave the best results [71]. Based on these data, a method was developed and published that has been shown to provide good quantity and quality DNA [64]. In addition to good reproducibility, the method provided results for DNA extraction from diverse environmental samples, including soils from a range of origins and different physical and chemical characteristics [17, 59, 65, 80, 81]. Accordingly, this method was proposed in 2006 by the Agence Française pour la Normalisation (AFNOR) to the International Organization for Standardization (ISO). Because an ISO standard would give information on the identity and quality of each compound in the protocol, it would also provide a complete quality control for users, thus avoiding the risks associated to commercial kits. After recognition of the need for an international standard for soil DNA extraction, an action was formally agreed and the method was evaluated by 15 independent European laboratories, 6 from France and 9 from other countries including Finland, Germany, Italy, Spain and Sweden. The amount of DNA extracted from 12 different soils was compared to evaluate both the reproducibility of the standardized method and the abundance and genetic structure of the total bacterial community. Quantification of the 16S rRNA gene abundances by quantitative PCR (qPCR) and analysis of the total bacterial community structure by automated ribosomal intergenic spacer analysis (A-RISA) showed acceptable to good levels of reproducibility. The method has been unanimously approved by the ISO as an international standard method (ISO standard 11063) [79]. The method has also been used to extract DNA from river sediments and agricultural soil, waters, biofilms and glacier soils [10, 11],

polychlorinated biphenyls-contaminated sites [80], constructed wetlands [21] and technosols [40].

Essentially, the method is as follows:

1. Sieve samples to <2 mm. Weight 0.25 g equivalent dry weight aliquots in a 2-ml microtube and keep frozen at - 80 ºC until use.
2. Thaw the samples. Add 0.5 g of 106 µm glass beads, 2 beads of 2 mm diameter and 1 ml of homogenization buffer extemporaneously prepared (100 µl 1 M Tris HCl (pH 8.0), 200 µl 0.5 M EDTA (pH 8.0), 100 µl 1 M NaCl, 50 µl 20% PVP 40T, 100 µl 20% SDS, 450 µl MQ water).
3. Homogenize the mixture by using a mini bead beater system (1.600^{-1} shaking frequency/min for 30 s). Use a shaking flask previously kept at -20 ºC. Incubate for 10 min at 70 ºC, then centrifuge at 14.000 x *g* for 1 min at 4 ºC.
4. Transfer the supernatant to a new 2-ml microtube. Add 1:10 (v/v) 5 M sodium acetate (pH 5.5) and mix by vortexing. Incubate on ice for 10 min, then centrifuge at 14.000 x *g* for 5 min. at 4 ºC
5. Transfer the supernatant to a new 1.5-ml microtube. Add 1:1 (v/v) prechilled (-20 ºC) isopropanol. Mix well by manual inversion. Incubate for at least 15 min at -20 ºC, then centrifuge at 14.000 x *g* for 30 min at 4ºC.
6. Remove the supernatant. Wash the pellet (containing the nucleic acids) with prechilled (-20 ºC) 70% ethanol with precaution to avoid pellet resuspension. Centrifuge for 15 min at 14.000 x *g* at 4 ºC.
7. Discard the supernatant and dry the pellet for 15 min at 37 ºC.
8. Resuspend the pellet in 50 µl MQ water
9. Prepare sample aliquots and store at -20 ºC until use.

DNA purification

Because of the presence of PCR-inhibitory compounds in the environmental samples, DNA purification is recommended. There is to note, however, that DNA purification is not part of the ISO standard 11063.

DNA purification through PVPP and sepharose 4B columns

As a first step of purification, prepare the PVPP column as follows:

1. Fill about 1.2 cm of an empty micro-spin chromatography column with PVPP powder (about 95 mg) and add 400 µl of MQ water.
2. Place the column in a 1.5-ml tube and centrifuge for 2 min at 1000 x *g*. Discard the eluate. Add 400 µl of MQ water to the column and centrifuge for 2 min at 1000 x *g*. At this moment, the column can be kept at 4 ºC.
3. Add the 50 µl DNA sample to the column and place it in ice for 5 min.
4. Place the column into a new tube and centrifuge at 1.000 x *g* for 4 min at 10 ºC to recover the DNA sample. Quantify the final volume of the sample.

Further DNA purification can be obtained by using sepharose 4B columns as indicated earlier [64, 79]:
1. Fill an empty micro-spin chromatography column with 1 ml of sepharose 4B solution.
2. Place the column in a 2-ml tube and centrifuge for 2 min at 1.100 x *g* at 10 ºC. Discard the eluate.
3. Add 500 µl TE buffer (10 mM Tris and 1 mM EDTA). At this moment, the column can be kept at 4 ºC.
4. Centrifuge at 1.100 x *g* for 2 min at 10 ºC. Discard the eluate.
5. Place the column in a new 2-ml tube. Add the DNA sample and centrifuge at 1.400 x *g* for 5 min at 10 ºC. Quantify the final volume of the sample

After PVPP cleaning, alternatively to utilization of sepharose 4B columns, commercial kits can be used for DNA purification. Here, we described cleaning of DNA samples using the Geneclean® turbo kit (GLASSMILK®-embedded membrane, MP Bio). Following manufacturer's instructions, the procedure allows purification of DNA fragments of sizes from 0.1 kb to 300 kb.

Essentially,
1. To the DNA sample (V) add 5 volumes of salt solution and mix well by vortexing.
2. Add the mixture to a Geneclean® cartridge and centrifuge at 14.000 x *g* until it all has passed through the filter.
3. Add 500 µl of the ethanol-containing Geneclean® washing solution to the cartridge and centrifuge at 14.000 x *g* for 5 s. Discard the eluate. Recentrifuge the cartridge at 14.000 x *g* for an additional 4 min and discard the eluate.

4. Place the cartridge into a new 1.5-ml tube.
5. Add 50 µl Geneclean® elution solution directly onto the GLASSMILK®-embedded membrane and incubate at room temperature 5 min.
6. Centrifuge at 14.000 x *g* for 1 min. Recover the eluate.

Other extraction and purification methods

The following methods are used to extract environmental DNA: a) cation-exchange [51], b) nitrogen-grinding [125], c) microwave-based rupture [77], d) Nycodenz gradient separation [6], e) solvent-based beating [15], f) aluminum-based extraction [78], and g) calcium chloride [58].

In many laboratories, utilization of commercial kits for DNA isolation is also frequent. Among them, MoBIO PowerSoil DNA kit (MoBIO) [23, 19, 62], ultra clean soil DNA kit (Ozyme, MoBIO) [61, 99, 5], fast DNA spin kit for soil (BIO 101/Q-Biogene) [29, 50, 55, 102].

A combination of hand-made and kit methodologies have also been used [38, 57, 109]. This method used a Bio-101 Multimix 2 matrix tube in combination with the fast-prep FP120 bead beater disruptor. DNA extraction was performed with addition of hexadecyl-trimethyl ammonium bromide (CTAB) to the commercial extraction buffer.

In addition to PVPP, sepharose 4B and Geneclean® turbo kit DNA, DNA purification can be achieved using: a) phenol [119], b) elutip-d and sephadex G-200 columns [120], c) cesium chloride, glassmilk and spearmine [106], d) PVPP and Microcon-100 columns, microconcentrators [129], e) agarose gel electrophoresis [136], f) HR S400 spin columns fast DNA purification kit and elution through Qiagen Mini column [91], g) Wizard DNA Clean-Up System [28], h) AllPrep DNA/RNA mini kit [34].

Although most protocols were originally designed for DNA extraction from soils and sediments, they have also been used to isolate and purify DNA from diverse environmental samples, such as the rizosphere of plants [99], biofilms formed on estuarine rocks colonized by algae [61], the vermicompost from a fresh olive waste [124], soil-feeding mounds of nematodes or termites [28, 30] and a bioreactor's biofilm [13]. Isolation and purification of DNA from aqueous samples usually requires concentration of the bacterial biomass by filtering the samples through 0.22 µm membranes and further homogenization of the filters [116]. Thermal shocks do not usually increase DNA yield and, in turn, may release humic material. This treatment,

however, resulted in extraction of DNA from the Gram-positive actinomycete *Micromonospora* [32].

Checking quantity and quality of the purified DNA

Quantity and quality of the DNA throughout an extraction process can be estimated by electrophoresis on 1% agarose gels in 0.5X TBE buffer [100] at 80 V. Samples (4 µl) can be supplemented with 1 µl loading buffer (40% sucrose and 0.25% bromophenol blue) before loading the samples on the gel. After electrophoresis, DNA can be visualized by staining with ethidium bromide, GelRed, Sybr Green I, etc. Alternatively, quantification of purified DNA can be determined by spectrometry at 260 nm. Quality of the samples can be checked by measuring absorbance of the sample at 230 nm (shows the presence of organic solvents), 280 nm (indicates the presence of proteins), and 400 nm (suggests the presence of humic acid).

Preparation of standard DNAs for qPCR

Standard DNAs were obtained after amplification from genomic DNA of the 16S rRNA, *narG* and *napA* genomic DNA from *P. aeruginosa* PAO1, *nirS* from *P. fluorescens* C7R12, *nirK* from *Ensifer meliloti* 1021, and *nosZ* from *Bradyrhizobium japonicum* USDA110. Primers used for amplification are presented in Table 1. Those primers have been used for amplification of the 16S rRNA and denitrification genes from agricultural soils [1, 5, 24, 62, 72], forest soils [4], containers filled with wood by-products [128], rice paddy field soils [134], grassland pasture soils [85], soils from the Burgundy region [11], soil-feeding mounds of nematodes [28], technosols [40] and constructed wetlands [21].

Primers, reaction mixture and thermocycler conditions used for PCR amplification of bacterial standard 16S rRNA and denitrification genes:

The primers used for the amplification of the bacterial standard for 16S rRNA and *narG*, *napA*, *nirK*, *nirS* and *nosZ* denitrification genes are shown in Table 1.

Reaction mixture for PCR-amplification of standard 16S rRNA and denitrification genes:

1. 1 to 5 ng template DNA (either 16S rRNA, *narG*, napA *nirS*, *nirK* or *nosZ*).
2. 0.6 µmol forward primer for each gene
3. 0.6 reverse primer for each gene

4. PCR buffer 1X (2.5 µl)

5. 1.5 mmol $MgCl_2$

6. 0.4 mM dNTPs

7. 0.04 U high fidelity Taq polymerase

8. Add up to 25 µl MQ/ultrapure water

Table 1. Primers used for PCR amplification of bacterial standard 16S rRNA and denitrification genes

Primer	Primer sequence (5´-3´)	Target gene	Size of the amplicon (base pair, bp)	Reference
T7	TAATACGCATCACTATAGGG		150	Promega Corp.
Sp6	GATTTAGGTGACACTATAG			
341F	CCTACGGGAGGCAGCAG	16S rRNA*	194	[73]
534R	ATTACCGCGGCTGCTGGCA			
narG-f	TCGCCSATYCCGGCSATGTC	*narG*	174	[10]
narG-r	GAGTTGTACCAGTCRGCSGAYTCSG			
nap3F	TGGACVATGGGYTTYAAYC	*napA*	152	[10]
napA4R	ACYTCRCGHGCVGTRCCRCA			
nirK876F	ATYGGCGGVAYGGCGA	*nirK*	173	[45]
nirK1040R	GCCTCGATCAGRTTRTGGTT			
nirS4QF	AACGYSAAGGARACSGG	*nirS*	425	[113]
nirS6QR	GASTTCGGRTGSGTCTTSAYGAA			
nosZ1840F	CGCRACGGCAASAAGGTSMSSGT	*nosZ*	267	[46]
nosZ2090R	CAKRTGCAKSGCRTGGCAGAA			

*In addition to the abundance, quantification of the 16S rRNA gene allows calculation of the relative abundance of a denitrification gene as the ratio between the abundance of any denitrification gene and the abundance of the 16S rRNA gene.

The thermocycler conditions for PCR-amplification of denitrification genes are shown in Table 2.

After amplification, the PCR products were electrophoresed on agarose gels to check size and purity of amplicons, purified using any appropriate commercial kit, and cloned in pGEM-T Easy vector (Promega) following manufacturer’s instructions. Plasmids were used to transform *Escherichia coli* JM109. The presence of the insert in the plasmid can be verified by PCR using T7 and Sp6 primers and further sequencing of the corresponding DNA fragments. DNA sequences will 100%-match the corresponding sequences of each denitrification gene. Care should be taken to remove

the DNA sequences corresponding to the polylinker regions of the plasmid which are also amplified.

Calculation of the copy number of standard DNA R

Recombinant pGEM-T easy plasmid containing insert DNA can be linearized using the restriction enzyme SalI. Because other enzymes can be used to linearize the pGEMT Easy vector, the existence of a unique cutting site in the DNA sequence can be checked by using the web site http://www.bioinformatics.org/sms2/rest digest.html in the Sequence Manipulation Suite Program. After digestion, use any appropriate commercial kit to purify DNA. Then determine DNA concentration (ng/µL) by spectrophotometry as indicated above. To calculate the molecular weight of a DNA fragment, use the formula MW (ng/mol) = bp number ×660 g/mol × 109 ng/g, where MW is the DNA molecular weight, bp is the number of base pairs (nucleotides) in the double stranded DNA, and 660 is the molecular weight of 1 base pair. Then the molarity (M) of standard DNAs can be calculated as M (mole/µL) = DNA concentration (ng/µL)/MW (ng/mol). Since 1 mol of any DNA contains 6.023×1023 molecules (Avogadro's number), then the DNA copy number can be calculated as follow: copy number/µL = M (mol/µL)×6.023×1023 copies/mol. It is recommended to prepare a stock of standard DNAs in 25-µL aliquots containing 0.5 × 108 copies/µL. Keep them at −20 °C until use. Avoid repeated freezing/thawing of the aliquots.

Table 2. Thermocycler conditions for amplification of standard 16S rRNA and denitrification genes by PCR

	Gene			
	narG, nirK and *nirS*	*napA*	*nosZ*	16S rRNA
Stage1: 1 cycle	10 min at 95 ºC	10 min at 95 ºC	10 min at 95ºC	10 min at 95ºC
Stage 2: 6 cycles with 1 ºC decrease by cycle	15 s at 95ºC	15 s at 95 ºC	15 s at 95 ºC	
	30 s at 63 ºC	30 s at 61 ºC	30 s at 65 ºC	
	30 s at 72 ºC	30 s at 72 ºC	30 s at 72 ºC	
Stage 3: 35 cycles	15 s at 95 ºC	15 s at 95 ºC	15 s at 95 ºC	15 s at 95 ºC
	30 s at 58 ºC	30 s at 56 ºC	30 s at 60 ºC	30 s at 60 ºC
	30 s at 72 ºC	30 s at 72 ºC	30 s at 72 ºC	30 s at 72 ºC
Stage 4: 1 cycle	10 min at 72 ºC	10 min at 72 ºC	10 min at 72 ºC	10 min at 72 ºC

It is recommended to prepare a stock of standard DNAs in 25 µl-aliquots containing 0.5 x 10^8 copies/µl. Keep them at -20 ºC until use. Avoid repeated freezing/thawing of the aliquots.

Inhibition test

During environmental DNA extraction, humic acids, organic and phenolic compounds, glycogen, fats, Ca^{2+} ions, heavy metals, detergents, antibiotics and constituents of bacterial cells can be co-extracted [130]. Because quality of the template DNA is one of the most important determinants of the sensitivity, accuracy and reliability of any PCR [75], care should be taken to avoid the presence of inhibitory compounds in the extracted DNA solution. Three potential mechanisms could inhibit PCRs: binding of the inhibitor to the polymerase, interaction of the inhibitor with the DNA, and interaction with the polymerase during primer extension. As a result, significant reductions in the sensitivity and kinetics of PCR assays can be produced. In addition, for statistical comparisons among samples, a similar PCR efficiency is required [3].

qPCR is currently the method of choice to test the presence of inhibitory compounds in DNA from an environmental sample [76]. During qPCR, inhibition can be detected by changes in a) the efficiency of the reaction, b) the melting curve due to modifications of the PCR product, c) the relative amounts of the PCR product due to the different inhibition levels.

Moreover, bacteriophage T4 gene 32 protein (T4gp32) can be used to limit the PCR inhibition and enhance the PCR amplification by stabilization of the single stranded DNA.

The absence of PCR inhibitors in the soil DNA extracts can be analyzed by mixing a known amount of standard DNA. Because this standard DNA is supposed not to be targeted in the environmental sample, the linearized *Sca*I pGEM-T easy vector without insert can be used for this purpose with environmental DNA. Then, the standard DNA can be amplified by qPCR using universal primers SP6 and T7 as described earlier [46]. A typical inhibition test can be run as follows:

Reaction mixture for inhibition test

1. 2 ng template (environmental) DNA
2. 1 x 10^7 copies of *Sca*I-digested pGEM-T Easy vector (without insert)

3. 1 µM T7 primer
4. 1 µM Sp6 primer
5. 250 ng T4 Gp32
6. 7.5 µl SYBR Green PCR buffer 2X (containing HotStar Taq polymerase and dNTPs)
7. Add MQ/ultrapure water up to 15 µl

In separate wells, add a) standard DNA and template DNA, b) standard DNA without template DNA, c) qPCR control without any DNA. Add eventually mastermix. Utilization of SYBR Green PCR buffer is recommended over preparation of a mixture containing each reaction component prepared independently. Keep at 4 °C until use (according to manufacturer's instructions). Mix the plate, then centrifuge before qPCR.

qPCR conditions for inhibition test

Thermocycler conditions are shown in Table 3.

Table 3. Thermocycler conditions for qPCR inhibition test	
Stage1*: 1 cycle	10 min at 95ºC
Stage* 2: 35 cycles	15 s at 95 ºC
	30 s at 55 ºC
	30 s at 72 ºC
	30 s at 80 ºC (data collection step)
Stage 3**: Dissociation stage (melting curve: 30 cycles with 0.5 ºC increase by cycle)	15 s at 95 ºC
	15 s at 80 ºC
	15 s at 95 ºC

*Times and temperatures should be set according to the manufacturer's instructions. Values in table 3 have been employed successfully with different buffers.

**Dissociation curves can be established by each laboratory. Values in table 3 are widely used.

Gene quantification by qPCR

Reaction mixture for qPCR

1. 2 ng template (environmental) DNA
2. 1 µM forward primer for 16SrRNA and 2 µM for denitrification genes
3. 1 µM forward primer for 16SrRNA and 2 µM for denitrification genes
4. 250 ng T4 Gp32
5. 7.5 µl SYBR Green PCR buffer 2X (containing HotStar Taq polymerase, buffer and dNTPs)

6. Add MQ/ultrapure water up to 15 µl

Table 4. Thermocycler conditions for quantification of 16S rRNA and denitrification genes by qPCR

	Genes			
	narG, nirK and *nirS*	*napA*	*nosZ*	16S rRNA
Stage1*: 1 cycle	10 min at 95 ºC	10 min at 95 ºC	10 min at 95ºC	10 min at 95ºC
Stage 2*: 6 cycles with 1 ºC decrease by cycle	15 s at 95ºC	15 s at 95 ºC	15 s at 95 ºC	
	30 s at 63 ºC	30 s at 61 ºC	30 s at 65 ºC	
	30 s at 72 ºC	30 s at 72 ºC	30 s at 72 ºC	
	30 s at 80 ºC (data adquisition)	30 s at 80 ºC (data adquisition)	30 s at 80 ºC (data adquisition)	
Stage 3*: 35 cycles	15 s at 95 ºC	15 s at 95 ºC	15 s at 95 ºC	15 s at 95 ºC
	30 s at 58 ºC	30 s at 56 ºC	30 s at 60 ºC	30 s at 60 ºC
	30 s at 72 ºC	30 s at 72 ºC	30 s at 72 ºC	30 s at 72 ºC
	30 s at 80 ºC (data adquisition)	30 s at 80 ºC (data adquisition)	30 s at 80 ºC (data adquisition)	30 s at 80 ºC (data adquisition)
Stage 4**: Dissociation stage (melting curve: 30 cycles with 0.5 ºC increase by cycle)	15 s at 95 ºC	15 s at 95 ºC	15 s at 95 ºC	15 s at 95 ºC
	15 s at 80ºC	15 s at 80 ºC	15 s at 80 ºC	15 s at 80 ºC
	15 s at 95ºC	15 s at 95 ºC	15 s at 95 ºC	15 s at 95 ºC

*Times and temperatures should be set according to the manufacturer's instructions. Values in table 4 have been employed successfully with different buffers.

**Dissociation curves can be established by each laboratory. Values in table 4 are widely used.

Preparation of a standard DNA curve

For quantification of environmental DNA, construction of a standard curve is required. For that purpose, prepare serial decimal dilutions ranging from 0.5×10^7 copies/µl to 0.5×10^2 copies/µl from the stock of standard DNAs samples. Take 2 µl from each of the 6 DNA dilutions and use them independently as template DNA to run qPCRs. Final DNA copy numbers for each run should go from 1×10^7 copies to 1×10^2 copies. In separate wells, add reaction mixture without any DNAs.

Table 5. Other primers used for PCR amplification of bacterial standard 16S rRNA and denitrification genes			
Primer	Primer sequence (5´-3´)	Target gene	Reference
519F	GWATTACCGCGGCKGCTG	16S rRNA	[116]
907R	CCGTCAATTCMTTTRAGTTT		
1055f	ATGGCTGTCGTCAGCT	16S rRNA	[19]
1392r	ACGGGCGGTGTGTAC		
1960m2f	TAYGTSGGGCAGGARAAACTG	*narG*	[59]
2050m2r	CGTAGAAGAAGCTGGTGCTGT		
narG328f	GACAAACTTCGCAGCGG	*narG*	[92]
narG497r	TCACCCAGGACGCTGTTC		
V16	GCNCCNTGYMGNTTYTGYGG	*napA*	[126]
V17	RTGYTGRTTRAANCCCATNGTCCA		
F1aCu	ATCATGGTSCTGCCGCG	*nirK*	[29]
R3Cu	TTGGTGTTRGACTAGCTCCG		
nirK517F	TTYGTSTAYCACTGCGCVCC	*nirK*	[16]
nirK1055R	GCYTCGATCAGRTTRTGGTT		
nirS263F	TGCGYAARGGGGCANCBGGCAA	*nirS*	[16]
nirS950R	GCBACRCGSGGYTCSGGATG		
nirS2F	TACCACCCSGARCCGCGCGT	*nirS*	[19]
nirS3R	GCCGCCGTCRTGVAGGAA		
nirSsh2F	ACCGCCGCCAACAACTCCAACA	$nirS_{Pm}$[1]	[44]
nirSsh4R	CCGCCCTGGCCCTGGAGC		
forward	ACAAGGAGCACAACTGGAAGG T	$nirS_{Ps}$[2]	[39]
reverse	CGCGTCGGCCCAGA		
$cnorB_P$F	CATGGCGCTGATAACGGG	$cnorB_P$[3]	[23]
$cnorB_P$R	CTTIACCATGCTGAAGGCG)		
$cnorB_B$F	AIGTGGTCGAGAAGTGGCTCT	$cnorB_B$[4]	[23]
$cnorB_B$R	TCTGIACGGTGAAGATCACC		
nirS263F	TGCGYAARGGGGCANCBGGCAA	*nirS*	[16]
nirS950R	GCBACRCGSGGYTCSGGATG		
nosZ1F	WCSYTGTTCMTCGACAGCCAG	*nosZ*	[44]
nosZ1R	ATGTCGATCARCTGVKCRTTYTC		
Forward	AGAACGACCAGCTGATCGACA	*nosZ*	[19]
Reverse	TCCATGGTGACGCCGTGGTTG		
nosZ-F-1181	CGCTGTTCITCGACAGYCAG	*nosZ*	[60]
nosZ-R-1880	ATGTGCAKIGCRTGGCAGAA		

[1]*nirS* gene from populations of *P. mandelii* and related species ($nirS_{Pm}$-bearing communities).

[2]*nirS* gene from populations of *P. stutzeri* and related species ($nirS_{Ps}$-bearing communities).

[3]*norB* gene for populations of *P. mandelii* and closely related strains ($cnorB_P$-bearing communities).

[4]*norB* gene for populations of *Bosea*, *Bradyrhizobium*, and *Ensifer* spp. ($cnorB_B$-bearing communities).

External DNA controls

In qPCR, external DNA controls can be genomic DNA isolated from denitrifying bacteria. External DNA for the 16S rRNA, *narG*, *napA* and *nirS* genes have been isolated from *P. aeruginosa* PAO1, *nirK* and 16S rRNA from *E. meliloti* 1021, and 16S rRNA, *napA* and *nosZ* genes from *B. japonicum* USDA110. Since those genomes were completely sequenced, their size and the copy number of the targeted gene per genome are known. These data allow determining the expected copy number of the targeted gene per unit of weight (ex: copy number per ng of genomic DNA). These samples can then be used to assess the reliability of the assay. A usual copy number for external DNA controls is 1 x 10^7.

Thermocycler conditions for bacterial 16S rRNA and denitrification genes

Thermocycler conditions for bacterial 16S rRNA and *narG*, *napA*, *nirK*, *nirS* and *nosZ* denitrification genes are presented in Table 4.

Utilization of SYBR Green PCR buffer is recommended over preparation of a mixture containing each reaction component prepared independently. Keep at 4 °C until use (according to the manufacturer's instructions). Mix the plate, then centrifuge before qPCR. New standard dilutions should be prepared for each reaction curve.

After qPCR, for each sample, the software of the q-PCR thermocycler will retrieve values of fluorescence intensity throughout the amplification cycles. At a certain cycle, the fluorescence intensity crosses over a level where the amplification enters a logarithmic growth phase. This cycle is called the quantitative (C_q). This value is inversely proportional to the log value of the initial DNA concentration in the reaction mixture. During qPCR, keep track on the background, exponential amplification, linear amplification and plateau of each curve. Finally, a standard curve is drawn by plotting the C_q value of each standard DNA against the tenth log of the DNA initial copy numbers in each reaction mixture. Because data values involved in the construction of the curve contribute to the final quantification of the environmental sample, the following descriptors of the curve should be reported: the amplification efficiency (E), the linear regression coefficient (r^2) and the y-intercept. Once the standard curve has been obtained, the copy numbers of each DNA sample can be calculated by interpolation of the C_q values in the standard curve. Export the data set to a spread sheet application and run appropriate statistical analyses.

When running inhibition tests, absence of inhibition is considered when differences in C_q values are ± 1 cycle. Should inhibition be detected, re-purification of the sample DNA is required.

Other primers for qPCR

Several research groups have developed different sets of primers for qPCR amplification of the bacterial 16S rRNA and denitrifying genes (Table 5). Accumulation in the databank of complete sequences from bacterial genome projects and from newly isolated denitrifying bacteria will help to design and increase sensitivity or new denitrification primers.

Using the new set of primers, nosZ-II-F (CTIGGICCIYTKCAYAC) and nosZ-II-R (GCIGARCARAAITCBGTRC) [53], a much larger diversity than that previously reported for bacterial and archaeal populations carrying a *nosZ* gene has been detected [36, 52]. Quantification of the new nosZ-II population in different environmental samples revealed that its relative abundance is similar to that found when the conventional *nosZ* primers (Table 1) were used.

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Artículo V

Título:

Microbial nitrogen cycle: Determination of microbial functional activities and related N-compounds in environmental samples

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Este artículo contiene los protocolos y procedimientos para estimar la actividad de las enzimas implicadas en los procesos de óxido-reducción que intervienen en el ciclo del N y que en esta Memoria de Doctorado se han empleado para la consecución del objetivo 1.

Resumen

El N forma parte de compuestos biológicos esenciales como aminoácidos, ácidos nucleicos, proteínas, hormonas, etc. El N es el elemento mayoritario de los que componen la atmósfera terrestre, si bien no se encuentra en una forma biodisponible para las plantas y los animales. El ciclo del N se inicia mediante la reducción del dinitrógeno atmosférico (N_2) a amonio (NH_4^+) biodisponible, proceso que realiza la enzima nitrogenasa. Posteriormente, parte de este amonio se oxida a nitrato mediante la nitrificación, proceso que ocurre en dos etapas, la primera catalizada por la enzima amonio monooxigenasa y, la segunda, por la nitrito oxidoreductasa. Finalmente el nitrato se reduce a N_2 por los microorganismos desnitrificantes y se emite a la atmósfera, cerrándose así el ciclo del N en la biosfera. La desnitrificación se lleva a cabo por la actividad secuencial de las enzimas nitrato-, nitrito-, óxido nítrico- y óxido nitroso-reductasa. El amonio, procedente bien de la fijación o de la adición al suelo de fertilizante sintéticos nitrogenados, que no se desnitrifica puede incorporar a la biomasa celular para formar aminoácidos y otros compuestos nitrogenados mediante a) la enzima glutamato deshidrogenasa; b) la actuación secuencial de las enzimas glutamina sintetasa/glutamato sintasa. Tras la muerte celular, el nitrógeno orgánico se libera al medio externo y se mineraliza por los microorganismos.

Este trabajo es un compendio de los distintos protocolos que actualmente se emplean para determinar las actividades funcionales del ciclo del N en muestras medioambientales y de los procedimientos para analizar los compuestos nitrogenados que se producen durante las reacciones de óxido-reducción que componen el ciclo. Las nuevas metodologías desarrolladas con el fin de mejorar el conocimiento del ciclo del N también se incluyen en este trabajo.

Esta revisión fue solicitada por la Dra. E. M. Marco, que actuó como editora, para formar parte del libro Metagenomics of the Microbial Nitrogen Cycle: Theory, Methods and Applications, que es el segundo de una serie que con el nombre genérico de Metagenomics publica Caister Academic Press.

Abstract

Nitrogen (N) is part of essential compounds such as proteins, nucleic acids, hormones, etc. Although N makes up to about 80 per cent of the Earth's atmosphere, it is not readily available for plant and animal consumption. Free-living and symbiotic microbes contain the enzyme nitrogenase which initiates the N-cycle in the biosphere by reducing dinitrogen gas to bio-available ammonia, a process called nitrogen fixation. Ammonia is subsequently oxidized to nitrate by nitrification, a two-step aerobic pathway during which ammonia is oxidized to nitrate and nitrite by the enzymes ammonia monooxygenase and nitrite oxidoreductase, respectively. Finally, nitrate is reduced to dinitrogen gas by denitrifying microorganisms, thereby closing the N cycle. Denitrification is carried out by the sequential activity of the enzymes nitrate-, nitrite, nitric oxide and nitrous oxide-reductase, respectively. Ammonia can also be incorporated into cellular biomass via the glutamine synthetase-glutamate synthase and glutamate dehydrogenase pathways to form amino acids and other nitrogen compounds. After cellular death, organic nitrogen compounds are released to the environment to be mineralized by microbial activities. Widely-used procedures for determination of microbial functional activities of the nitrogen cycling microorganisms and of N-compounds produced during the redox reactions of the cycle will be addressed. In addition, we will consider new methodologies being developed for further understanding of the N-cycle.

Introduction

Most of the N in the earth's atmosphere is found as dinitrogen gas (N_2), a form which is inaccessible to eukaryotes and many bacteria. Diazotrophic microorganisms, mainly bacteria, contain the enzyme nitrogenase, which converts bio-unavailable N_2 gas to bio-available ammonium (NH_4^+). This process is called biological nitrogen fixation and initiates the N cycle in the biosphere (Figure 1). Ammonium is subsequently incorporated into cellular biomass mainly via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway. Alternatively, glutamate dehydrogenase (GDH) may also be involved in aerobic ammonium assimilation.

Besides its incorporation into organic nitrogen compounds, ammonium can be oxidized to nitrate (NO_3^-) by nitrifying bacteria in a process called nitrification. During nitrification the enzymes ammonia monooxygenase, hydroxylamine oxidoreductase and nitrite oxidoreductase (nitrite oxidase) oxidize ammonium to hydroxylamine (NH_2OH), nitrite (NO_2^-) and nitrate, respectively. Nitrate can be reduced to ammonia through the nitrate assimilation process by the assimilatory nitrate reductase and nitrite reductase enzymes.

Under oxygen-limiting conditions, nitrate can be reduced to N_2 via the formation of nitrite, nitric oxide (NO) and nitrous oxide (N_2O), the so called denitrification pathway. Denitrification converts nitrate to N_2, which returns to the atmosphere, thus closing the N cycle in the biosphere.

In addition to denitrification, anaerobic ammonium oxidation (anammox) converts nitrite and ammonium directly into N_2, thus largely contributing to production of N_2. Genome sequencing of several N-cycle organisms, the nitrite-dependent anaerobic methane oxidation (Raghoebarsing et al., 2006) and hypertermophilic N_2-fixing methane producing archaea (Mehta and Baross, 2006) are examples of the biodiversity and metabolic capacity of new nitrogen conversions within the N cycle (Jetten, 2008; van Nifrik and Jetten, 2012; Kartal et al. 2012). Together these processes form the global N cycle and microorganisms are essential for maintaining the balance between reduced and oxidized forms of nitrogen (van Spanning, 2011).

Until appearance of metagenomics in the last decade, the direct determination of N-compounds and the microbial functional activities have been used as classical approaches to characterize the environmental N-cycle processes shown in Figure 1. Although several methods for the study of N-cycle compounds are currently available,

other new techniques are being developed due to advances in analytical technologies that are being continuously improved.

In this chapter, we present some widely-used analytical procedures for the practical study of the main N-cycle processes occurring under environmental conditions, aiding researchers to better understanding the most important reactions of the N-cycle.

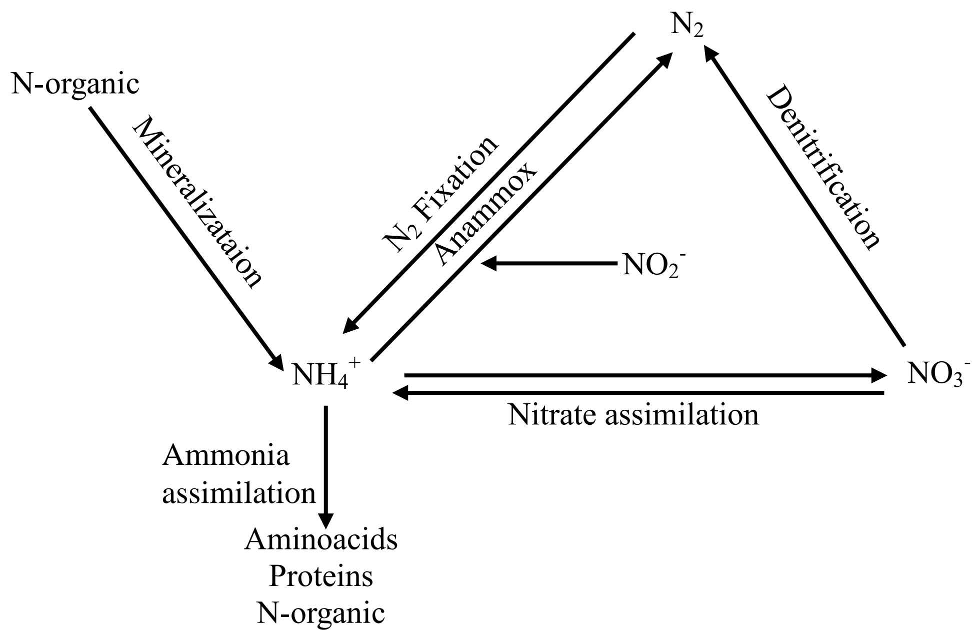

Fig 1. A scheme of the N-cycle

Microbial functional activities of the N-cycle

Biological nitrogen fixation

Nitrogen fixation is the reduction of dinitrogen gas (N_2) to ammonium (NH_4^+), a process carried out by the enzyme nitrogenase. Nitrogenase activity can be determined according to the following protocols.

The acetylene-dependent ethylene production technique

Besides N_2 to NH_4^+, the nitrogenase complex also reduces acetylene, azide, cyanide, nitrous oxide and protons. Activity of nitrogenase can be readily detected in environmental samples using the acetylene-dependent ethylene production (acetylene reduction activity, ARA) assay (Hardy et al., 1973).

Procedure

1. Weigh 25 g fresh sample (soils, sediments, etc.) and place it within a 100 mL bottle. Bottles have to be hermetically closed (e.g. by using rubber septa). Weights of the samples and volume of the bottles may vary to optimize the assay. Septa must allow injection and sampling of the internal atmosphere of the bottles. As a blank include bottles without sample.
2. Close the bottle-containing sample ($n \geq 4$). Flush the headspace for 5 min with N_2, or the more expensive Ar or He, to create anoxic conditions.
3. Replace 10% of the internal atmosphere of the bottles by the same volume of acetylene (C_2H_2). Mix well by shaking. Commercial acetylene, or that obtained by mixing calcium carbide (CaC_2) and water (1:15 w/v), can be used.
4. Incubate the samples at 25 ºC. Incubation time depend of the acetylene reduction activity of the samples.
5. Take gas aliquots from the headspace of the bottle for injection onto the gas chromatograph. Aliquots from 100 to 1000 µL can be used.

Kinetic of ethylene (C_2H_4) production can be followed by taking samples over time. Because assays are run in closed systems, gas accumulation may cause feedback inhibition of the enzymatic activity. Accordingly, gas samples must be taken during the exponential phase of gas production. Long incubation times may result in spoiling of the samples.

When activity of the samples is low, nitrogenase activity can be determined after incubation of the samples under non-limiting N_2-fixing conditions, this is, in the presence of an excess carbon source, e.g. 10% glucose. It is to note that any available nitrogen source inhibits nitrogenase and, accordingly, nitrate concentration should be checked in the samples. Chloramphenicol can be used to prevent new protein synthesis and growth of N_2-fixing microorganisms. Under these conditions, nitrogenase activity can be detected within 0-48 h depending on ethylene production kinetic.

Ethylene can be determined by gas chromatography using a flame ionization detector (FID). The chromatograph is usually provided with as a carrier gas, and H_2 and synthetic air to make up the flame. Gas fluxes through the chromatograph as well as through the oven, injector and detector temperatures may vary depending on each chromatograph commercial brand and the type of column used for the chromatography.

Concentration of ethylene in each sample can be calculated from standards of pure ethylene. A correction for dissolved ethylene in water (Bunsen solubility coefficient) has to be considered when using sediments or soil slurries. Values are usually expressed as mol C_2H_4 produced x kg^{-1} (dry soil, dry sediment, etc.) x h^{-1}. Gas tight syringes should be used.

ARA can also be used to estimate rates of nitrogen fixation by pure cultures of free-living and symbiotic N_2-fixing microorganisms. For free-living cells, liquid and solid media have been defined which allow microbial growth for determination of nitrogenase activity. For symbiotic bacteria, nodules, either from roots, stems or leaves can be used.

Determination of ARA by the acetylene-dependent ethylene production technique has several disadvantages related to a) diffusion of acetylene in the soil, especially in wet or heavy-textured soils, b) degradation of acetylene by bacteria, c) inhibition of other processes, for example, nitrification, or d) disturbance of the soil structure when soil cores are taken. In addition, the acetylene-inhibition method does not provide information on field N_2 production rates.

Readers are referred to Burris (1974) and Hardy and Holsten (1977) to learn on general problems regarding determination of nitrogen fixation, and Vessey (1994) and Minchin et al. (1994) for concerns related to the use of ARA to assay nitrogenase activity in nodulated legumes. Previous methods describing the acetylene reduction assay can be found in Zechmeister-Boltenstern (1996a).

The isotope ^{15}N-labelled method

A direct method for determination of nitrogenase activity is based on the utilization of $^{15}N_2$. Samples are incubated with $^{15}N_2$ and O_2 for a long period of time in hermetic bottles. After the incubation, total nitrogen content and the ratios between ^{15}N and ^{14}N are determined.

Procedure

1. Weigh 10 g of environmental solid sample (soils, sediments, etc.) ($n \geq 4$) in a gas-tight bottle equipped for gases injection and withdrawal. Determine the remaining headspace volume.

2. Close the bottle. Flush the headspace with He for 5 min.

3. Substitute 50% of the headspace volume with enriched $^{15}N_2$ and 20% with O_2.

4. Seal the bottle and incubate at 25 ºC in the dark. Incubation time depends on nitrogenase activity, varying between 3 to more than 30 days.
5. After incubation, the ratio $^{15}N/^{14}N$ is determined in the solid environmental simple by mass spectrometry.
6. Total solid nitrogen (T_N) of the samples is assayed as indicated in "Total solid N".
7. As a blank use a set of samples incubated without enriched $^{15}N_2$.

The isotopic composition of a sample is reported as $\delta^{15}N$ (‰):
$\delta\ ^{15}N$ (‰) = ($R_{sample}/R_{standard}$ - 1) x 1000, where
R = $^{15}N/^{14}N$ ratio.

Commercial N_2 can be used as the standard for isotopic composition analyses. The $^{15}N/^{14}N$ ratio in the standard is calculated against a reference curve obtained by using internationally accepted reference materials (http://www.iaea.org).

The proportion of N derived from N_2 fixation ($\%N_{FIX}$) is calculated as:
$\%N_{FIX}$ = 100 x [1 - (A/B)], where
A = Atom% ^{15}N excess in samples incubated with enriched $^{15}N_2$.
B = Atom% ^{15}N excess in samples incubated without enriched $^{15}N_2$.
Atom% ^{15}N = $\delta\ ^{15}N$ (‰) x 100.

The fixed-nitrogen content (FN) is calculated as FN = ($\%N_{FIX}$ x T_N)/100

The advantage of this method is that it does not imply disturbance of the soil, that N_2 production can be determined, and that an N budget can be made. The method also has some disadvantages, the most important being that a) analysis of ^{15}N is only possible at specialised laboratories, and b) ^{15}N is not distributed homogeneously throughout the soil. ^{15}N -labelled material can be expensive and can only be used on small plots.

Other protocols for determination of nitrogenase activity have been published by Bergensen (1980), Weaver and Danso (1994), Zechmeister-Boltenstern (1996b) and Wilson et al. (2012).

Ammonification/mineralization

Protease activity

Proteases are involved in the progressive cleavage of proteins to polypetides or oligopeptides, and finally to amino acids. The activity of the different types of proteases in environmental samples can be determined using specific substrates (Ladd and Jackson, 1982), casein and N-α-benzoyl-l-argininamide being widely used.

Method based in utilization of casein as substrate

Originally developed by Ladd and Butler (1972), determination of protease activity is based on incubation of an environmental sample with excess casein as a substrate and trichloroacetic acid-soluble peptides determination using the Folin-Ciocalteu's phenol reagent.

Procedure

1. Weigh 1 g environmental solid sample (soils, sediments, etc.) ($n \geq 4$) and place in a 25 mL Erlenmeyer flask.
2. Add 2.5 mL solution A. As a control prepare at least 3 tubes adding 1.3 mL solution B. Mix the flasks briefly and close with screw caps.
3. Incubate at 50 ºC for 2 h on a rotatory shaker.
4. After incubation, add 1.2 mL solution A to the control.
5. Add 1 mL solution C to stop the reaction. Mix briefly
6. Centrifuge at 3.000 x *g*.
7. Take 1 mL supernatant into a glass tube. Add 7 mL solution D and 1 mL solution E. Mix briefly. Incubate at room temperature for 30 min.
8. Add 1 mL solution F. Preincubate 5 min at 37 ºC. Finally, incubate 30 min at room temperature.
9. Determine absorbance at 578 nm.

Solution A: 1.05 g casein in 50 mL 0.1 M Tris-HCl, pH 8.1. Prepare daily.

Solution B: 0.1 M Tris-HCl buffer. Dissolve 12.1 g Tris in 1 L distilled water in a volumetric flask. Adjust pH to 8.1 with 5 M HCl.

Solution C: Mix 800 mL 0.1 M Tris, pH 8.1, and 320 mL tetracholoracetic acid solution (175 g tetracholoracetic acid in 1 L distilled water).

Solution D: 3.7 g Na_2CO_3 in 100 mL distilled water.

Solution E: 0.06 g $CuSO_4$ in 100 mL distilled water.
Solution F: Folin-Ciocalteu solution. Mix 10 mL commercial Folin-Ciocalteu phenol reagent and 30 mL distilled water.

Solution C containing variable amounts of tyrosine (mg L^{-1}) can be used to prepare standard curves. The blue colour produced by the Folin-Ciocalteu reactive is stable at least 1.5 h.

Kinetic of peptide production can be followed by taking samples along time. Results are ussually expressd as mol NH_4^+x g^{-1} x h^{-1} of dry weight sample

Other protocols for determination of protease activity can be found in Ladd and Butler (1972), Kandeler (1996c), Nunnan et al. (2000), Bonmatí et al. (2003) and Rejsek et al. (2008).

Method based in utilization of benzoyl-l-argininamide as substrate

Determination of protease activity is based on production of NH_4^+ after controlled incubation of an environmental sample with an excess N-α-benzoyl-l-argininamide as substrate.

Procedure

1. Weigh 0.5 g solid sample (soils, sediments, etc.) ($n \geq 4$) and place it in a glass incubation tube (avoid cleaning of the tubes with phosphate-containing detergents). As a control, prepare at least 3 tubes without N-α-benzoyl-l-argininamide.
2. Add 2 mL phosphate buffer and 0.5 mL solution C.
3. Incubate samples in a shaking water bath at 39 ºC for 1.5 hour.
4. Add 0.4 mL HCl 5M to samples and controls to stop the reaction, and 7.1 mL distilled water to each tube (final volume 10 mL).
5. Centrifuge at 15.000 x *g* for 15 min.
6. Screen through qualitative filter paper.
7. Measure NH_4^+ content as described in “Ammonium determination”.

Phosphate buffer (0.1 M, pH: 7.1): Mix 39 mL solution A (15.60 g NaH_2PO_4 x $2H_2O$ in 1000 mL distilled water), 61 mL solution B (17.80 g Na_2HPO_4 x $2H_2O$ in 1000 mL distilled water) and 100 mL distilled water. Store at 4 ºC.
Solution C: 0.4977 g 30 mM N-α-benzoyl-l-argininamide in 50 mL phosphate buffer.

Buffer phosphate solutions containing variable amounts of NH_4^+ (mg L^{-1}) can be used to prepare standard curves. Results are usually expressed as mol NH_4^+ x g^{-1} x h^{-1} of dry weight sample. Sample weight should be checked for optimal results.

Other protocols for determination of protease activity can be found in Ladd and Butler (1972), Nannipieri et al. (1980), Bonmatí et al. (1991; 2003), Kandeler (1996c), Nunnan et al. (2000) and Rejsek et al. (2008).

Urease activity

Urease catalyses the hydrolysis of urea into ammonium and carbon dioxide (CO_2). The methodology described here is based on NH_4^+ production after controlled incubation of soils with an excess of urea as a substrate.

Procedure

1. Weigh 1 g solid sample (soils, sediments, etc.) ($n \geq 4$) and place it in a glass incubation tube (avoid cleaning of the tubes with phosphate-containing detergents). As a control, prepare at least 3 tubes without urea solution (add 0.5 mL distilled water instead).
2. Add 2 mL phosphate buffer and 0.5 mL solution C.
3. Incubate samples in a shaking water bath at 37 ºC for 2 h.
4. Add 7.5 mL distilled water to each tube (final volume 10 mL).
5. Centrifuge at 15.000 x *g* for 10 min.
6. Screen through qualitative filter paper.
7. Measure NH_4^+ content as described in "Ammonium determination".

Phosphate buffer (0.1 M, pH: 7.1): Mix 39 mL solution A (15.60 g NaH_2PO_4 x $2H_2O$ in 1000 mL distilled water), 61 mL solution B (17.80 g Na_2HPO_4 x $2H_2O$ in 1000 mL distilled water) and 100 mL distilled water. Store at 4 ºC.
Solution C: 6.4% urea (3.2 g urea in 50 mL phosphate buffer)

Aqueous solutions containing variable amounts of NH_4^+ (mg L^{-1}) can be used to prepare standard curves. Results are usually expressed as mol NH_4^+ x g^{-1} x h^{-1} of dry weight sample.

Weight of the sample should be checked for optimal results.

Other protocols for determination of urease activity can be found in Nannipieri et al. (1980), Tabatabai (1982), Kandeler (1996d) and Öhlinger (1996).

Nitrification

Nitrification is the oxidation of ammonium to nitrate. The first step of the process is the oxidation of ammonium to nitrite which, in turn, is oxidized to nitrate. Nitrification is usually determined by measuring nitrite accumulation after addition of $NaClO_3$, which inhibits nitrite oxidation to nitrate.

Short-term nitrification assay

This method is based on incubation of the samples for a short period of time, usually no longer than 6 h. The assay uses $(NH_4)_2SO_4$ as substrate. After extraction with KCl, nitrite content is determined. Sodium chlorate is used to inhibit nitrite oxidation.

Procedure

1. Weigh 5 g of environmental sample (soil, sediment, etc.) ($n \geq 4$) and place it within a 100 mL Erlenmeyer flask.
2. Add 20 mL 1 mM solution A and 0.1 mL solution B. Mix and close the flasks with caps.
3. Incubate at 25 ºC for 5 h on an orbital shaker. As a control, at least 3 replicates should be kept for 5 h at -20 ºC.
4. After incubation, thaw the control at room temperature.
5. Add 5 mL solution C to samples and controls.
6. Mix and screen through qualitative filter paper immediately. If required, keep the filtrates overnight at 4 ºC.
7. Use filtrates to determine nitrite concentration as indicated in "Ammonium determination".

Solution A (1 mM): 0.132 g $(NH_4)_2SO_4$ in 100 mL distilled water.
Solution B (1.5 M): 15.97 g $NaClO_3$ in 100 mL distilled water.
Solution C (2 M): 149.12 g KCl in 1000 mL distilled water in a volumetric flask.

Aqueous solutions containing variable amounts of either $NaNO_2$ or KNO_2 (mg L^{-1}) can be used to prepare standard curves. Nitrification is expressed as mg NO_2^- x g soil x h.

Since potential nitrification of soils with pH value below 5 is very low, this method is of limited value for acid soils.

Concentration of the inhibitor $NaClO_3$ has been optimized for soils with a humus content ranging from 1.5 to 3.5%. Inhibit concentration should be optimized depending on organic matter concentration of the soils.

Ammonium oxidation is inhibited by high amounts of ammonium. Optimum substrate concentration for each sample should be checked.

Other protocols for determination of nitrification activity have been published by Schmidt and Belser (1982), Berg and Rosswall (1985) and Kandeler (1996b).

Long-term nitrification assay

This method is based on incubation of the samples up to 3 weeks at 25 ºC using NH_4Cl as a substrate. After extraction of inorganic nitrogen compounds with KCl solution, ammonium and nitrate concentrations are determined. The oxidation of the substrate is equivalent to the nitrification dynamics, and is expressed as percentage of the added nitrogen (Beck, 1976; Kandeler, 1996b).

Procedure

1. Weigh 10 g of environmental sample (soil, sediment, etc.) ($n \geq 4$) and place it into a 100 mL flask.
2. Add dropwise 1 mL solution A.
3. Adjust soil moisture to 50-60% of water holding capacity with distilled water.
4. Close the flasks with caps and weigh them.

5. Incubate samples at room temperature for up to 3 weeks (a fixed temperature, e.g. 25 ºC can be used). As a control, at least 3 replicates should be kept the same time at -20 ºC.
6. Weigh the flasks once a week to control soil moisture content. When required, add distilled water to adjust the soil moisture content.
7. After incubation, add 50 mL KCl to both samples and controls. Shake the flasks for 30 min in a rotary shaker at about 125 rpm.
8. Screen flask contents through filter paper and measure ammonium and nitrate concentration as indicated in "Ammonium determination" and "Nitrate determination".

Solution A (75.7 mM): 1 g $(NH_4)_2SO_4$ in 100 mL distilled water in a volumetric flask.
Solution B (2 M): 149 g KCl in 1 L distilled water.

For calculation of the results there is to consider that inorganic nitrogen can be released from organic nitrogen (ammonification), and that both ammonium and nitrate can be immobilized by microorganisms during incubation.

To determine the correlation factor is used the following formula:
$Na = (N\text{-}NO_3^- + N\text{-}NH_4^+)_X - (c + N\text{-}NO_3^- + N\text{-}NH_4^+)_Y$
Where:
Na = correction factor for ammonification and immobilization during the incubation time.
$_X$ = amount of inorganic (NO_3^-+ NH_4^+) nitrogen after incubation.
$_Y$ = initial amount of inorganic (NO_3^- + NH_4^+) nitrogen.
c = amount of NH_4^+ added at the beginning of the experiment.

If Na < 0: higher ammonification than immobilization.
If Na > 0: higher immobilization than ammonification.

The nitrification turnover is equivalent to the amount of nitrogen which is released from the substrate per day and gram dry matter.

The results can be expressed as percentage loss of the initially added substrate according the following formula:

$\% \text{ N d}^{-1} = (NO_3^{-}\text{-}N_X - NO_3^{-}\text{-}N_Y) \times 100/(c + Na) \times n$

Where:

$_X$ = NO_3^- content after incubation.

$_Y$ = initial NO_3^- content.

c = amount of initially added NH_4^+.

Na = correction factor for ammonification and immobilization during incubation.

n = days of incubation time.

$\%N\ d^{-1}$ = nitrification turnover.

Because nitrification in soils with high amounts of easily decomposable carbon compounds proceeds very fast, the incubation time can be reduced.

It is not recommended to increase the substrate concentration because of the toxicity of high ammonia concentration is soils.

Since nitrification in air-dried and rewetted soils starts after a lag-phase, a pre-incubation time should be considered.

Ammonification and/or immobilization during incubation could not be considered. , some authors not consider these process and the results as expressed as g NO_3^- x g^{-1} of sample x h^{-1}.

Other protocols for determination of long-term nitrification activity have been published by Schmidt and Belser (1982), Berg and Rosswall (1985), Kandeler et al. (1996b), Hu et al. (2002) and Li et al. (2011).

Alternatively, nitrification activity can be studied by measuring rates of nitrite oxidation (Schmidt and Belser, 1982). For that purpose, varying concentrations of NO_2^- are added to soils slurries along with nitrapyrin to inhibit oxidation of NH_4^+. It is recommended to follow NO_2^- disappearance by taking samples over time to calculate results during exponential phase of NO_2^- oxidation.

Nitrate assimilation

Reduction of nitrate to ammonium is catalyzed by the sequential actuation of the assimilatory nitrate reductase and nitrite reductase enzymes.

Determination of nitrate disappearance

Procedure

1. Sieve the soil trough a 2 mm screen.
2. Mix 10 g sample with glucose (2.5 mg of C g^{-1} sample) and 30 mL distilled water in a flask ($n \geq 4$).
3. Incubate the samples in a rotary shaker at 125 rev min^{-1} (to maintain aerobic conditions) at 30 ºC for 16-24 h conditions.
4. After incubation, add 250 µg KNO_3 g^{-1} soil. As a control, at least 3 replicates should be included without addition of KNO_3.
5. Incubate at 30 ºC for 2 h.
6. Centrifuge10 min at 3000 x *g*.
7. To 5 mL supernatant add 15 mL 1.3 M KCl to extract NO_3^-.
8. Determine NO_3^- concentration as indicated in “Nitrate determination”.

Preincubation of the samples is recommended to stimulate microbial activity and assimilation of preexisting ammonium and nitrate.

Other protocols for determination of nitrate assimilation have been published by Rice and Tiedje (1989) and McCarty and Bremner (1992).

Determination of nitrite production

Procedure

1. Weigh 5 g environmental sample (soils, sediments, etc.) ($n \geq 4$) into a glass tube.
2. Add 4 mL solution A, 1 mL solution B and 5 mL distilled water into the tubes. Mix and close the tubes with screw caps.
3. Incubate the tubes for 24 h at 25 ºC (samples). As a control, at least 3 replicates should be kept for 5 h at -20 ºC.
4. After incubation thaw the control at room temperature.

5. Add 10 mL solution C to both samples and controls. Mix and immediately filter the samples and controls through qualitative filter papers.
6. Use filtrates to determine nitrite concentration as indicated in "Nitrite determination".

Solution A: 0.9 mM 2,4 dinitrophenol (DNP) solution
Solution B: 25 mM KNO_3 (2.53 g KNO_3 in 1 L distilled water in a volumetric flask.
Solution C: 4 M KCl (298.24 g KCl in 1 L distilled water in a volumetric flask.

Aqueous solutions containing variable amounts of either $NaNO_2$ or KNO_2 (mg L^{-1}) can be used to prepare standard curves. Activity is expressed as µg NO_2^- x g^{-1} x h^{-1}.

It is recommended to follow NO_2^- production kinetic by taking samples over time to calculate results during exponential phase of NO_2^- production.

To reduce the possible presence of lag phase, samples can be pre-incubated overnight with DNP.

Prior to any analysis, an estimation of the optimum amount of inhibitor should be tested as DNP concentration may vary from 5 to 300 µg DNP (Abdelmagid and Tabatabai, 1987).

Other protocols for determination of nitrate assimilation have been published by Kandeler et al. (1996a) and Deiglmayr et al. (2004).

Denitrification

Denitrification is associated with the production of N_2, N_2O and NO by environmental samples. The most frequently used measurement method to assay denitrification is the determination of N_2O production by gas chromatography. The ^{15}N-labelling technique can also be used to detect N_2O and N_2. NO can also be determined by using a chemiluminescent analyser.

Determination of N_2O production

Assessment of denitrification is hard to study because of the difficulties to quantify its gaseous end products (N_2O and N_2) and high spatial and temporal variability (Groffman et al., 2006; 2009; Philippot et al., 2009; Hallin et al., 2009; Bru et al., 2011; Keil et al., 2011). Although molecular methods (Philippot and Hallin, 2006) have contributed to understanding of denitrification, gene expression, denitrifier

community composition and enzyme activities could not be easily related with the simultaneous production and emission of denitrification products (Wallenstein et al., 2006; Čuhel et al., 2010). Methodological problems continue hampering our understanding of denitrification at site, from landscape to continental scale, as well as the controls and magnitude of net N_2O losses (Butterbach-Bahl et al., 2011).

This method is based on the inhibition of the nitrous oxide reductase, the enzyme reducing N_2O to N_2, by acetylene at 0.1%-10% concentration (Balderston et al., 1976; Yoshinari et al., 1977).

Procedure

1. Weigh 25 g fresh sample (soils, sediments, etc.) ($n \geq 4$) and place it within a 100 mL bottle. Bottles have to be hermetically closed (e.g by using rubber septum). Weights of the samples and volume of the bottles may vary to optimize the assay. Septa must allow injection and sampling of the internal atmosphere of the bottles. As a blank include a bottle without sample.
2. Close the bottle-containing sample. Then, evacuate and flush the headspace 4-5 times with N_2, or the more expensive Ar and He, to create anoxic conditions.
3. Replace 10% of the internal atmosphere by the same volume of acetylene. Commercial acetylene, or that obtained by mixing calcium carbide (CaC_2) and water (1:15 w/v), can be used. Mix well by shaking.
4. Incubate the samples at 25 ºC. Incubation time depend of the denitrification activity of the samples.
5. Take gas aliquots from the headspace of the bottle for injection onto the gas chromatograph. Aliquots from 100 to 1000 µL can be used. Gas tight syringes should be used.

Kinetic of N_2O production can be followed by taking samples along time. Because assays are run in closed systems, gas accumulation may cause feedback inhibition of the enzymatic activity. Accordingly, gas samples must be taken during the exponential phase of gas production. Long incubation times may result in spoiling of the samples.

When activity of the samples is low, denitrifying enzymatic activity can be determined after incubation of the samples under non-limiting denitrifying conditions,

this is, in the presence of an excess carbon and nitrogen sources. Under these conditions, addition of chloramphenicol to prevent new protein synthesis and, consequently, growth of denitrifying microorganisms can be used. The procedure is the same as indicated above, except that 25 mL of a sterile solution containing 1 mM KNO_3, 1 mM glucose and 1 g L^{-1} chloramphenicol is added to the samples. Under these conditions, N_2O production is usually detected within 24-48 h.

N_2O can readily be detected by gas chromatography, using either a thermal conductivity detector (TCD) or the more sensitive electron conductivity detector (ECD). The chromatograph is usually provided with N_2, Ar and He, as carrier gas. Carrier gas flux through the chromatographic column as well as oven, injector and detector temperatures may vary depending on the commercial brand of the chromatograph and of the type of column used for chromatography.

Concentration of N_2O can be calculated from standards of pure nitrous oxide. A correction for dissolved N_2O in water (Bunsen solubility coefficient 54.4% at 25º C) has to be considered. Values are usually expressed as mol N_2O produced x kg^{-1} (soil, sediment, etc.) x h^{-1}.

Other protocols for determination of N_2O production have been published by Smith and Tiedje (1979), Tiedje (1982) and Šimek et al. (2000; 2004).

Determination of N_2 production

In addition to N_2O formation, denitrification can be estimated analyzing production of N_2, the end product of the process.

Procedure

The procedure is the same as indicated above for N_2O production, except that acetylene is not added to the bottles where samples are incubated.

N_2 can be detected by gas chromatography, using a TCD, but N_2 cannot be used as the carrier gas. Concentration of N_2 can be calculated from standards of pure N_2. Values are usually expressed as mol N_2 produced x kg^{-1} (soil, sediment, etc.) x h^{-1}.

During studies on denitrification, N_2 production can be determined by analysing N_2O production in parallel environmental samples incubated with and without acetylene (Philippot et al., 2009). N_2 concentration is then estimated as the difference between the N_2O produced in the presence and in the absence of acetylene, respectively. In addition,

the ratio [$N_2O/(N_2O + N_2)$] represents an estimation of the nitrous oxide reductase activity in the samples.

$^{15}N_2$ isotope determination

^{15}N-labelled substrates such as nitrate and ammonium can be measured using mass spectrometry. The advantage of this method is that it does not imply disturbance of the soil, that N_2 production can be determined, and that an N budget can be made. The method also has some disadvantages, the most important being that a) analysis of ^{15}N is only possible at specialised laboratories, b) only denitrification from ^{15}N is measured, and c) ^{15}N is not distributed homogeneously throughout the soil. ^{15}N - labelled material can be expensive and can only be used on small plots. The readers are referred to Baggs (2008) for a comprehensive review on stable isotope techniques for determination of N_2O in soils.

Determination of NO production

This method is based on the determination of NO fluxes of an environmental sample incubated within a dynamic flow through chamber. After the incubation the NO concentration is determined by chemiluminiscence (Parrish et al., 1987; Pilegaard et al., 1999).

Procedure

1. Weigh 10 g of environmental sample (soil, sediment, etc.) ($n \geq 4$) and place it into a 100 mL flask.
2. Place the flask inside a stainless-steel chamber provided with a gas-tight lid, inlet and exhaust ports for gas flushing, and a sampling port.
3. The chamber can be introduced in a water bath to control the temperature and reduce potential gas diffusion into the chamber.
4. Replace the internal atmosphere of the chamber by thorough flushing with an N_2-free gas (either He or Ar) to create anoxic conditions. Flush with the N_2-free gas until the original sample atmosphere is replaced. Overpressure should be avoided.
5. Take 40 mL of the head space of the chamber into an airtight bag pre-filled with 2 L of pure N_2.

6. Determine the NO concentrations with a $NO-NO_2-NO_x$ chemiluminescent analyser. To obtain a well-represented NO concentration of the gas stored in the bag, a sample flow of 600 mL min^{-1} and 3 minutes of continuous measurement is required to get a stable output signal.

Kinetic of NO production can be followed by taking samples along time (Veldkamp and Keller, 1997). Incubation times as long as 3 weeks have been recommended (Pilegaard et al., 1999).

Concentration of NO can be calculated from standards of pure nitric oxide.

Values are usually expressed as flux of NO as follow:

$F = (V_{head} \times \Delta C_i^* \times M) / (M_{ds} \times MV \times 10^3) \times (273/273 + T)$

F = NO emissions in µg h^{-1} Kg^{-1}.

V = Volume of the headspace (mL).

ΔC_i^* = Change in NO concentration (mL x m^{-3}).

M = atomic wight of the N in NO (14 g mol^{-1}).

M_{ds} = dry weight of environmental sample.

MV = molar volume of NO at 273 K and 1013 hPa (L mol^{-1}).

T = incubation temperature.

Other protocols for determination of NO emission have been published by Parrish et al. (1987), Pilegaard et al. (1999) and Wang et al. (2011).

Determination of some N-compounds relative to the N-cycle

Total solid N

Total solid N (T_N) refers to each inorganic (NH_4^+, NO_3^-, NO_2^-) and organic N (amino acids, proteins and other organic compounds) contents in solid samples (soils, plants, sediments, etc.). The Kjeldahl (wet digestion) and the Dumas (dry digestion) methods are widely used for determination of T_N.

Determination of T_N: the Kjeldahl method

The Kjeldahl method is a two-step process by which organic N of a sample is digested (oxidized) into NH_4^+ by acidic digestion with H_2SO_4. Ammonium salts produced can be collected and dissolved with a strong alkali. The ammonium produced

can be distilled, dissolved in acid solution, and finally titrated with caustic soda to indirectly measure nitrogen. In his original method, Kjeldahl used KSO_4 to raise the boiling point of the acid and Hg as catalyst to speed the digestion. For the back titration process of the released ammonium, he used a solution of boric acid. According to the sample characteristics, diverse modifications of the original Kjeldahl method have been introduced in order to solve the recovery of refractory heterocyclic compounds or molecules containing N-N and N-O linkages (Du Preez and Bate, 1989; Bremner and Mulvaney, 1982; Watkins et al., 1987; Domini et al., 2009).

A protocol to carry out Kjeldahl method in the laboratory requires a Kjeldahl Steam Distillation system with a block digester (available commercially).

Procedure

1. Dry the sample at 60 ºC for 48 h.
2. Homogenize the sample by grinding to ≤ 0.5 mm.
3. Weigh 1 g sample and place it into the block digester tube.
4. Add about 5.0 g of catalyst mixture (K_2SO_4-$CuSO_4$ x 5 H_2O-Se, 100:10:1 w/w ratio) and 15 mL H_2SO_4, and swirl carefully.
5. Set temperature in the block digester at 370 ºC for 3-5 h until complete digestion.
6. Cool the sample at room temperature.
7. Add 15 mL distilled water and transfer the sample into a steam flask containing 10 mL 10 N NaOH.
8. Distilled the sample to recover ammonium released.
9. Determine ammonium as indicated in "Ammonium determination".

The Kjeldahl method is widely used in industrial, agricultural, and food analysis. A major disadvantage of the method is that it only converts organic-N (mainly proteins) and some NO_3^- into NH_4^+. It is difficult to automatize and consumes relatively high amounts of sample.

Determination of T_N: the Dumas method

This methodology is based on combustion of samples (900-1020 ºC) in the presence of oxygen to obtain N-compounds which are further reduced catalytically into N_2. Usually, the process is automatically run by the so called (macro/micro) Elemental Analysers, of which several brands are commercially available.

Procedure (for preparation of the samples for the Analyser)

1. Dry the sample at 60 ºC for 48 h.
2. Homogenise the sample by grinding to ≤ 0.5 mm.
3. Weigh (1-50 mg) the sample and place it into a tin capsule and close it.
4. Subject the sample to analysis in an Elemental Analyser.

Before use, calibrate the Elemental Analyser with standard N-compounds provided by the manufactures according to the expected N content of the samples to be analysed.

The Dumas method is fast and clean. It requires just a small amount of sample and causes no environmental hazards. It has been successfully used for T_N determination in soils, plants, and other proteinaceous samples (Kirsten and Hesselius, 1983; Simonne et al., 1997; Wiles et al. 1998; Jung et al. 2003).

In solid samples, $T_N = N_{INORGANIC} + N_{ORGANIC}$, where $N_{INORGANIC}$ is mainly $NH_4^+ + NO_3^- + NO_2^-$. $N_{ORGANIC}$ is calculated indirectly using this equation, and it refers to nitrogen of organic molecules, such as proteins, amino acids, etc.

Regardless of the methodology used, T_N can be expressed as % or as part per million of N in relation to the dry weight of the solid sample.

An indirect N determination of crude protein content can be done by multiplying $N_{ORGANIC}$ by 6.25, a value which refers the experimental average N content in proteins.

In addition to Kjeldahl and Dumas methods, the near infra-red (NIR) spectroscopy has been described as a promising technology for T_N determination in plants (Gitelson et al., 2003) and soils (Russell, 2003).

Determination of total dissolved N

Total dissolved nitrogen (T_{DN}) refers to N in liquid samples containing organic and inorganic N compounds. In addition to liquid samples, T_{DN} can be analysed in solid samples after extraction by mechanical means or piezometry (Randall et al., 1997;

Kalbitz et al., 2000; Jones and Willett, 2006; Krause et al., 2009; Hood-Nowotny et al., 2010).

Prior to determination, N-compounds in solid samples can be extracted as follows:
1. Mix the solid sample with water (1:20 w/v) and shake in a water bath for 2 hours at 25 ºC. 2. Centrifuge at 15.000 x *g* for 20 min.
3. Filter the supernatant through qualitative filter paper or 0.45 µm pore size appropriate membranes.

The weight to volume ratio can be modified to increase the content of dissolved N in the samples.

Liquid samples and liquid-extracted samples from solid materials can now be used for T_{DN} determination. For liquid samples, the Kjeldahl method is not adequate as urea cannot be determined properly (Solorzano and Sharp, 1980).

If the Dumas method is to be used, the liquid sample is added to an inert absorbent, e.g. diatomaceous earth, to avoid sample evaporation.

The most accurate analytical method for T_{DN} is based on a high-temperature catalytic oxidation (Merriam et al., 1996). Liquid samples are combusted, converted into NO which further reacts with ozone to form nitrogen dioxide (NO_2), which is detected with a nitrogen-specific chemiluminescence detector coupled to an automatic liquid Analyser. This technique is now accepted as the most precise and efficient technique for T_{DN} determination (Álvarez-Salgado and Miller, 1998).

Before use, calibrate the automatic liquid Analyser with standard N-compounds provided by the manufactures according to the expected N content of the samples to be analysed.

T_{DN} is usually expressed in mg x L^{-1} for liquid samples and in part per million of N in relation to the dry weight of the solid sample.

In liquid samples, $T_{DN} = D_{INORGANIC} + D_{ORGANIC}$, where $D_{INORGANIC}$ is mainly N-NH_4^+ + N-NO_3^- + N-NO_2^-. $D_{ORGANIC}$ can be calculated indirectly using this equation.

Determination of dissolved inorganic N

Dissolved inorganic nitrogen (D_{IN}) refers to NH_4^+, NO_3^- and NO_2^- found in either solid or liquid samples. After extraction, samples should be kept at -20 ºC to prevent NH_4^+, NO_3^- and NO_2^- assimilation or chemical modifications.

Ammonium determination

Indirect method

NH_4^+ can be readily determined using an indirect, colorimetric method based on Berthelot's reaction (Weatherfourn, 1967; Patton and Crouch, 1977).

Procedure

1. Add 1.6 mL reactive A, 0.8 mL reactive B, 0.8 mL reactive C (see below), and 6.4 mL distilled water to 0.4 mL liquid-extracted sample.
2. Mix well by vortexing or shaking. Keep at least 45 min in darkness until appearance of blue color.
3. Measure absorbance at 660 nm.

Reactive A: (freshly prepared): 7.81 g sodium salicylate and 25 mg sodium nitroprusside. Add distilled water to fill up to 100 mL in a volumetric flask.
Reactive B: 4.0 g NaOH and 0.5 g of sodium dichloride isocyanurate. Add distilled water to fill up to 100 mL in a volumetric flask (pH 13).
Reactive C: 9.33 g sodium citrate and add distilled water to fill up to 100 mL in a volumetric flask.

Aqueous solutions containing variable amounts of $(NH_4)_2SO_4$ (mg L^{-1}) can be used to prepare standard curves.

Direct methods

Ion chromatography (Michalski, 2006; Michalski and Kurzyca, 2006) and the use of an ammonium ion-selective electrode (Bakker, 2004; Bakker and Qin, 2006) are two alternatives for determination of NH_4^+ in most liquid samples. The former is a sensitive and accurate technique with good reproducibility and versatility (e.g. it offers the possibility of simultaneous detection of some other anions). A review on types of samples, columns and eluents has been published by Michalski and Kurzyca (2006). A

conductometric detector is usually employed for ion chromatography. The latter is fast and accurate, usually well-suited for portable field applications, with the disadvantage that the electrode may respond to other anions with similar physical properties.

Nitrate determination

Indirect methods

These assays are based on chemical reduction of NO_3^- to NO_2^-. Then nitrite is determined as indicated in "Nitrite determination". Several reducing agents have been investigated, and copperised Cd and Zn are widely used, with efficiencies for NO_3^- to NO_2^- conversion approaching 100% (Fanning, 2000). Commercial kits have been developed for *in situ* application during field determinations of nitrate content in liquid samples that are easy to use following manufacturers's instructions.

Under laboratory conditions, nitrate in liquid and liquid-extracted samples can also be determined after chemical reduction. Reduction columns were first described by Wood et al. (1967) and can be purchased or prepared in the laboratory.

A. Preparation of the column

1. Weigh 25 g 40-60 mesh commercial Cd granules and wash them with 6N HCl. Rinse thoroughly with distilled water.
2. Mix the granules with 100 ml 2% $CuSO_4$ solution until blue colour partially fades (5-10 minutes).
3. Decant the granules. Mix them with fresh $CuSO_4$ solution until a brown colloidal precipitate begins to develop.
4. Wash with distilled water to remove the precipitated Cu.
5. Insert a glass wool plug into the bottom of a glass column (15-20 cm long) and fill with water. Add copperised Cd granules to produce a column 13.0-18.0 cm. Pour distilled water until exceed the height of the granules to prevent the entrapment of air.
6. Wash the column with 200 mL solution A.
7. Activate the column by passing through it at least 100 mL solution C at a rate of 5-10 mL $minuto^{-1}$.

Solution A: Dissolve 13 g NH_4Cl and 1.7 g ethylene diaminetetraacetate (EDTA) in 750 mL distilled water in a volumetric flask, adjust to pH 8.5 with concentrated NH_4OH and

dilute to 1 L distilled water. Finally, dilute 300 mL solution to 500 mL with distilled water.

Solution B: Dissolve 7.21 mg KNO_3 in 1 L distilled water in a volumetric flask.

Solution C: Mix solution A and solution B in 3:1 proportion.

B. Sample reduction

Procedure

1. Extract nitrate from solid environmental samples as indicated in section "Direct methods" below.
2. Filter liquid samples through qualitative filter paper or 0.45 µm pore size appropriate membranes.
3. Add 75 ml solution C to 25 ml liquid or liquid-extracted samples. Mix gently and pour into the column.
4. Collect the eluate at a rate of 5-10 mL min^{-1}. Discard the first 25 ml. Collect the remaining 75 ml into a clean flask. Determine nitrite concentration as described "Nitrite determination" within 15 minutes after reduction.

Aqueous solutions containing variable amounts of either $NaNO_3$ or KNO_3 (mg L^{-1}) can be used to prepare standard curves. Reduce standards as described for samples. Compare at least one nitrite standard to a reduced nitrate standard at the same concentration to verify reduction column efficiency. Also determine any contaminant nitrite in the samples.

Nitrate concentration is expressed mg x L^{-1} (liquid samples) or mg x g^{-1} of dry sample (liquid-extracted samples).

There is no need to wash columns between samples. If columns are not to be reused for several hours or longer, pour 50 mL solution A on to the top of the column and let it pass through the system. Then, add 100 mL more, close the Cu-Cd column and store.

Crutchfield and Grove (2011) have described a Cd reduction microplate method for nitrate determination.

Other protocols for nitrate determination by the Cd reduction method have been published by Wood et al. (1967), Jones (1984), APHA Standard Methods (1995) and Gal et al. (2004).

Direct methods

High Performance Liquid Chromatography (HPLC) (Thayer and Huffaker, 1980) and ion chromatography (Stratford, 1999; Kissner and Koppenol 2005; Michalski and Kurzyca, 2006) are two alternatives for determination of nitrate and nitrite in most liquid samples. A comprehensive review on HPLC and ion chromatography applications, including types of columns, eluents, detectors and matrix samples has been previously published (Michalski and Kurzyca, 2006).

Prior to determination, NO_3^- in solid samples can be extracted as follows:
1. Mix the solid sample with water (1:20 w/v) and shake in a water bath for 2 hours at 25 ºC. 2. Centrifuge at 15.000 x *g* for 20 min.
3. Filter the supernatant through qualitative filter paper or 0.45 µm pore size appropriate membranes.

The weight to volume ratio can be modified to increase the NO_3^- content in the samples.

Procedure (for HPLC)
1. To prepare the eluent, dissolve 0.1049 g LiOH in 1000 mL Milli-Q water (2.5 mM) in a volumetric flask. Gas the eluent with N_2 for 10 min to avoid dissolved CO_2 interference. Alternatively, a CO_2 trapping agent such as ascarite can be used. A borate/gluconate buffer can also be used as an eluent.
2. Filter the eluent and the samples through 0.45 µm pore size appropriate membrane.
3. Aqueous solutions containing variable amounts of either $NaNO_3$ or KNO_3 (mg L^{-1}) can be used to prepare standard curves.
4. Subject the samples to HPLC. Absorbance can be determined at 220 nm.

Nitrate ion-selective electrodes have been developed that can be used for laboratory and field nitrate determinations. They are commercially available.

Nitrite determination

Indirect method

The classical method for nitrite identification and quantitative determination is the Griess reaction (Griess, 1864), which uses diazotation and coupling to form a purple dye by adding the sulphanilamide-naphthylethylene diamine dihydrochloride reagent (Snell and Snell 1949; Nicholas and Nason, 1957).

Procedure

1. Filter the sample through 0.45 µm pore size membrane.
2. Add 0.4 mL reactive A to 20 mL sample and mix well by gentle shaking for 5 minutes.
3. Add 0.4 mL reactive B to the mixture. Mix well by gentle shaking.
4. Keep in darkness, at least for 30 min, until color development.
5. Measure absorbance at 540 nm.

Reactive A: 1 g sulfonamide ($C_6H_8N_2O_2S$) in 100 mL 10% HCl (10 mL of HCl in 90 mL distilled water).

Reactive B: 0.1 g N-(1-naphthyl)-ethylenediamine dihydrochloride ($C_{12}H_{14}N_2$, NNEDA) in 100 mL distilled water.

Aqueous solutions containing variable amounts of either $NaNO_2$ or KNO_2 (mg L^{-1}) can be used to prepare standard curves. Results are usually expressed as mg NO_2^- x L-1 (liquid samples) or mg x g^{-1} of dry sample (liquid-extracted samples).

Direct methods

Nitrite determination can be assayed by HPLC as described for nitrate detection in section “Nitrate determination-direct methods”. Prior to determination, NO_2^- in solid samples has to be extracted as indicated in section “Nitrate determination-direct methods”.

Aqueous solutions containing variable amounts of either $NaNO_2$ or KNO_2 (mg x L^{-1}) can be used to prepare standard curves.

Other spectroscopic methods, including UV/visible, chemiluminescence, fluorimetric, Infrared (IR), Raman and molecular cavity emission, have been reviewed

by Moorcroft et al. (2001). Also, examples of HPLC and ion chromatography applications for their determination, including type of columns, eluents, detectors and matrix samples are reviewed and discussed in Michalski and Kurzyca (2006).

Determination of gaseous N compounds

During the denitrification pathway N_2O and N_2 are produced whose determination can be accomplished by gas chromatography using thermal conductivity (TCD) and electron capture (ECD) detectors after separation of the samples through either packed or capillary chromatographic columns. Protocols for NO, N_2O and N_2 determination are explained in section 2.5.

Photoacoustic infrared spectroscopy has been developed for continuous measurement of NH_3 and N_2O in environmental samples (Osada et al., 1998). This methodology is based on the detection of acoustic waves that result from absorption of infrared radiation of the sample. It is, however, an expensive technology for routine analysis.

Mass spectrometry, mainly membrane inlet mass spectrometry (MIMS), allows detection of gases in liquid samples (Srinivasan et al., 1997), with the major advantage of being able to detect parts-per-trillion of NO, N_2O and N_2 (Lloyd et al., 1996; Kana et al., 1998; Kim et al., 1999), and the disadvantage of being an expensive technology.

Future trends and directions

Molecular methods have greatly contributed to the understanding of processes involved in the microbial N-cycle. On one side, targeting functional genes at the DNA level allows for their detection and analysis of diversity, providing inferences to which genes are functionally important in the environment. On the other side, analysis of gene expression targeting mRNA provides evidence of actual activity. However, measurement of microbial N-cycle-related activities and N-derived compounds in environmental samples is often hard to study because of the difficulties to quantify the end products resulting from the microbial activities. As an example, this is the situation when studying the assessment of denitrification in environmental samples. Maybe because of the problems to quantify its gaseous end products (N2O and N2) or to the high spatial and temporal variability of the process (Groffman et al., 2006, 2009; Philippot et al., 2009; Hallin et al., 2009; Bru et al., 2011; Keil et al., 2011), total N

losses from arable soils due to denitrification have been estimated to be in the range of 22-87 Tg N x year^{-1} (Hofstra and Bowman, 2005). Thus, methodological problems continue hampering our understanding of the N-cycle related pathways. Although available, many techniques are rather expensive and cannot be afforded by ordinary laboratories, and this without considering the special facilities and installation that complex apparatus and equipment require for their functioning. The advancement of scientific knowledge requires the development of new methodologies for simple, cheap and effective estimation of the parameters you wish to analyse.

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DISCUSIÓN GENERAL

Mientras que los aspectos fisiológicos, bioquímicos y genéticos de la desnitrificación se conocen en profundidad, apenas existe información sobre la abundancia o escasez de las distintas poblaciones desnitrificantes en diferentes hábitats, las bacterias desnitrificantes predominantes en un ecosistema concreto, y cómo la actividad funcional de la comunidad varía en el tiempo. Igualmente, se desconoce el efecto del contenido en nitratos sobre la abundancia, biodiversidad, distribución espacial y actividad funcional de la comunidad desnitrificante.

Para la realización de este trabajo se eligió el Espacio Natural de Doñana como sistema modelo por la especial protección y conservación que se realiza en el parque de sus marismas y humedales. La relativamente escasa presión antrópica a la que está sometido hace pensar que los resultados obtenidos se deban a causas naturales y no a las ocasionadas por influencias del honbre.

El Espacio Natural de Doñana (END) es un área de especial protección y conservación, aunque existen algunos efectos antrópicos derivados, principalmente, de las prácticas agrícolas que se realizan en su ecotono, donde es frecuente el cultivo de arroz y fresa. Según el real decreto 261/1996 del Consejo de Ministros sobre protección de las aguas contaminadas por nitratos procedentes de fuentes agrarias se debe, fundamentalmente, a la aplicación excesiva, o inadecuada, de fertilizantes nitrogenados. Siguiendo la directiva 676/96/CEE, la legislación española impone en el real decreto 261/1996 la obligación de detectar las aguas afectadas por esta contaminación.

Diferentes trabajos han detectado problemas de contaminación en el END. Uno de los primeros fue el artículo de Cabrera et al. (1984) que informaron sobre una concentración excesiva de metales pesados y de nitratos, además de los pesticidas ácido 2,4-diclorofenoxiacético y 2,4,5-ácido triclorofenoxiacético, en concentraciones cercanas a los límites establecidos. En este trabajo se indica por primera vez que es posible la contaminación del END a través de los distintos caños de agua que lo irrigan, esto es, que el END no se encuentra aislado de las zonas de agrícolas que lo rodean por lo que es susceptible de recibir contaminantes procedentes de su ecotono. Unos años después, Suso et al. (1993) detectaron niveles de nitrato que oscilaron entre 5,70 y 25 mg de NO_3^- por L y señalaron que los valores más elevados correspondían a zonas donde la agricultura de regadío es más intensa. En años siguientes, los valores de nitrato

detectados en el END por Serrano et al. (2006), Manzano et al. (2009) y Espinar y Serrano (2009) fueron similares a los descritos por Suso et al. (1993).

En el artículo I de esta Memoria de Doctorado se presenta parte de los datos de la investigación realizada en el END. Se determinó el contenido en nitratos en más de 20 sitios a lo largo del arroyo de la Rocina y se seleccionaron 4 puntos de acuerdo a su contenido: laguna del Acebrón (S1), arroyo de la Cañada (S2), vado de la Canariega (S3) y marisma del Rocío (S4). Después, se llevó a cabo la caracterización físico-química de las aguas y sedimentos de cada uno de los puntos de muestreo, se analizó la actividad biológica de los sedimentos, se determinó la emisión por los mismos de gases de efecto invernadero como el dióxido de carbono, el metano y el óxido nitroso y, finalmente, se comprobó el origen de los nitratos contaminantes. Este estudio se realizó en los meses de octubre de 2008, y enero, abril y julio de 2009.

En el artículo II se analizó la abundancia relativa de los genes de la desnitrificación *narG*, *napA*, *nirK*, *nirS* y *nosZ* en los sitios S1 y S2, que fueron los de menor y mayor contenido en nitratos, respectivamente, lo que se había determinado en el artículo I. En ambos sitios de muestreo se cuantificó tanto el contenido en nitratos como la actividad desnitrificante. Además se estudió la diversidad de bacterias desnitrificantes que presentaban el gen *nosZ* en su genoma. Las muestras se tomaron en abril y octubre de los años 2008, 2009 y 2010, que representan la época húmeda y seca, respectivamente.

En el artículo III de la Memoria se recogen los datos de abundancia relativa y de distribución espacial de los genes de la desnitrificación *narG*, *napA*, *nirK*, *nirS* y *nosZ* en sedimentos del humedal de los Guayules, que recoge aguas contaminadas con nitratos procedentes de los arroyos Soto Grande y Soto Chico (García-Novo y Marín Cabrera 2005). Esta balsa artificial se construyó en 2005 y se seleccionó a instancias del personal del END por la función que cumple en la descontaminación de las aguas antes de ser vertidas a la marisma del Rocío. Dentro del humedal se acotó un área de 25 x 50 m y se tomaron muestras de los sedimentos en 50 puntos separados entre sí por una distancia de 5 m. Además, se determinaron las propiedades físico-químicas y la actividad desnitrificante de los sedimentos de los 50 puntos.

El artículo IV cuenta la historia de cómo, a lo largo del tiempo, se diseñaron los cebadores que permitieron amplificar los genes de la desnitrificación y se desarrolló la metodología de reacción en cadena de la polimerasa a tiempo real (qPCR) para cuantificar el ADN a partir de muestras medioambientales. Se explican los procedimientos basados en técnicas comerciales y el denominado ISO 11063 que puede utilizarse como técnica general de laboratorio para la extracción de ADN ya que su resultado se ha contrastado por diversos laboratorios internacionales y refrendado por la oficina internacional de normalización (ISO). Además contiene la metodología para comprobar la ausencia o existencia de inhibidores de la PCR y se explica cómo construir las rectas de calibrado para la cuantificación del número de copias de un gen determinado.

El artículo V forma parte del libro "Metagenomics of the microbial nitrogen cycle: theory, methods and applications", editado por D.E. Marco y publicado por Caister Academic Press, Norwich (UK), UK. En éste capítulo se recogen las metodologías empleadas para analizar las distintas actividades y cuantificar los productos que se forman durante las reacciones de óxido-reducción que ocurren durante el ciclo del N.

Las determinaciones del contenido NO_3^- realizadas en los artículos I, II y III indican la existencia de contaminación por el mismo en el END. El contenido en nitratos varió de forma estacional y fue siempre superior en S2, respecto a los otros tres puntos de muestreo. En algunos casos, como los descritos en el artículo II en el arroyo de la Cañada y en el artículo III en el humedal de los Guayules, respectivamente, el contenido en nitratos superó el valor de 50 mg por litro que establece la directiva europea 91/676/CEE como límite superior para las aguas superficiales. Los resultados del artículo II indican que a lo largo de los 3 años de estudio, el contenido en nitrato en S1, fue similar en los meses de abril y octubre, pero en S2 el contenido en nitratos en octubre fue estadísticamente superior al de abril.

En general, el contenido en nitratos de los lugares analizados en el END son similares a los descritos por oros autores en sedimentos nitratos de lagos (Wang et al. 2013; Saarenheimo et al. 2015b) y ríos (Reyna et al. 2010; Chon et al. 2011; Shen et al. 2014).

Los datos meteorológicos de la Reserva Biológica de Doñana (http://icts.ebd.csic.es) indican que la pluviosidad de la zona varió entre los distintos años analizados. Los valores de precipitación fueron 425, 631 y 588 mm en los años 2008, 2009 y 2010, respectivamente, lo que indica la existencia de diferencias inter-anuales en la pluviosidad. También ocurren variaciones intra-anuales, ya que los valores de precipitaciones en los meses de junio y agosto de 2008, junio y julio de 2009 y enero de 2010 fueron nulos y, por el contrario, la precipitación fue abundante en octubre de 2008 (119 mm), diciembre de 2009 (295 mm) y febrero de 2010 (200 mm). De acuerdo con Espinar y Serrano (2009), el clima condiciona las propiedades hidrogeológicas especialmente en regiones semiáridas de clima mediterráneo, como es el caso del END, lo que se traduce en variaciones en las propiedades físico-químicas de sus aguas y sedimentos. A excepción de la laguna del Acebrón que mantuvo los niveles de agua, el resto de sitios de muestreo presentaron variaciones en su volumen de agua, pasando de ser lugares irrigados a zonas pantanosas cercanas a la desecación.

Artículo I

En este artículo se describen las propiedades físico-químicas y biológicas de las aguas superficiales y de los sedimentos de los 4 puntos de muestreo (S1-S4) que se seleccionaron a lo largo del arroyo de la Rocina. Estas propiedades variaron de forma estacional y se relacionaron, principalmente, con los cambios en la dinámica hídrica que experimenta el END. Tales variaciones fueron evidentes en S2, S3 y S4 y fueron de menor importancia en S1. Estas variaciones espacio-temporales coinciden con las descritas por Serrano et al. (2006) y Espinar y Serrano (2009) en muestras de agua de los arroyos de el Partido y la Rocina y en diversos humedales del END.

El contenido en nitratos en las muestras de agua y sedimentos varió entre sitios y fechas de muestreo. Independientemente de la fecha, el contenido en nitratos fue superior en S2 y, en general, se observó un incremento en el mes de julio. Por tanto, la contaminación por nitratos en el arroyo de la Rocina presenta claras variaciones espacio-temporales, si bien la contaminación es sostenida en el tiempo en algunos de los sitios de muestreo analizados. Además de las variaciones en la dinámica hídrica del END, es posible que el contenido en nitratos se afecte por los diferentes suministros

externos de fertilizantes nitrogenados procedentes de las zonas agrícolas situadas en el ecotono del END que en función de las prácticas agrícolas se realizan a lo largo del año.

Es por ello, que se decidió estudiar el origen de la contaminación por nitratos, lo que se realizó mediante análisis isotópico del ^{15}N del NO_3^- de los sedimentos de S2, lo que no se pudo realizar en S1, S3 y S4 por su escaso contenido en nitratos. Nuestros datos indican que los valores de $\delta^{15}N$ variaron entre -1.6 a + 6‰ (AIR) con un valor medio de -0.78‰. Este valor de $\delta^{15}N$ es cercano al del aire, lo que indica un origen inorgánico del nitrato ya que para su síntesis química se emplea aire atmosférico (Vitòria et al. 2004). Además, el valor de $\delta^{15}N$ coincide con los valores de la mayoría de los fertilizantes nitrogenados sintéticos empleados en España, que varía entre -4 y +6‰ (Otero et al. 2005; Pastén-Zapata et al. 2014). Todo ello indica que se trata de nitratos producidos mediante síntesis química, lo que coincide los datos de Olías et al. (2008) y los más recientes de Jiménez-Morillo et al. (2015) que concluyen que la contaminación por nitratos en Doñana se debe al empleo de fertilizantes nitrogenados en las zonas agrícolas adyacentes al END.

Los microorganismos tienen un papel fundamental en multitud de procesos biológicos que intervienen en los ciclos biogeoquímicos de los elementos y, por tanto, participan en el funcionamiento de los ecosistemas (Gutknecht et al. 2006). Estos procesos son de particular interés en ecosistemas formados por zonas húmedas, tales como el END, donde los ciclos de los nutrientes son muy sensibles a fluctuaciones en la hidrología. Además, las actividades enzimáticas del suelo responden de forma rápida frente a cambios en el ecosistema tanto de origen natural como antrópico (Gianfreda et al. 2005). Los valores de las actividades enzimáticas β-glucosidasa, deshidrogenasa, ureasa, fosfatasa ácida y arilsulfatasa variaron tanto entre sitios como entre fechas de muestreo. Chang et al. (2014) han descrito resultados similares en humedales artificiales de China cuando analizaron la actividad de las enzimas deshidrogenasa, diacetato hidrolasa, catalasa, ureasa y fosfatasa. Coincidiendo con los datos de Shi et al. (2013) en suelos cultivados en Quebec (Canadá), la actividad deshidrogenasa es la que más está influenciada por los cambios climáticos, si bien todas las actividades enzimáticas variaron de forma estacional. A pesar de las complejas relaciones que existen entre los parámetros físico-químicos y las actividades biológicas de los ecosistemas, uno de los principales factores que controlan la actividad biológica es el contenido en carbono orgánico total (Williams y Jochem, 2006). De hecho, se observó una estrecha

correlación entre los valores de actividad β-glucosidasa y el contenido en carbono orgánico total, relación que ya se ha descrito en zonas semiáridas de Irán (Nosrati et al. 2012) y en mesocosmos construidos a partir de muestras de un humedal (Salvato et al. 2012).

Durante las transformaciones que llevan a cabo los microorganismos en los ciclos de los elementos se producen efectos no deseados como puede ser la liberación a la atmósfera de gases de efecto invernadero tales como dióxido de carbono (CO_2), metano (CH_4) y óxido nitroso (N_2O) que contribuyen al calentamiento global (Baird 2001; Liu y Greaver 2009). El análisis de los gases de efecto invernadero indicó la existencia de variaciones espacio-temporales de estas emisiones, especialmente la de CO_2 y CH_4, con valores que fueron más elevados en la época seca. Estos datos coinciden con los resultados descritos en sedimentos de arroyos (Rich y Myrold 2004; Burgin y Groffman 2012) y de estuarios (Smith et al. 2015) y, en suelos agrícolas (Dandie et al. 2008) y de ribera (Deslippe et al. 2014). Las variaciones espacio-temporales pueden deberse al efecto que ejercen las variables ambientales sobre los procesos que resultan en la producción de tales gases (Christensen et al. 2012). Además de la de la relación con la humedad, el pH, la temperatura, etc., la formación de estos gases se agrava con el aporte de compuestos nitrogenados cuyo metabolismo resulte en la producción de nitrato (Liikanen et al. 2006; Wang et al. 2008; Song et al. 2014). De hecho, en este trabajo, el contenido en nitrato estimuló la emisión de N_2O, CO_2 y CH_4, resultados que concuerdan con los de Yao et al. (2013) en suelos de arroz tratados con nitratos de la provincia china de Jiangsu.

La relación entre las propiedades físico-químicas y de su variabilidad espacio-temporal se analizó mediante un análisis de componentes principales (PCA). Este análisis agrupó las variables en tres componentes a los que se denominó fracción orgánica, fracción inorgánica y fracción enzimática; otras dos variables, el pH y el contenido en nitratos, se agruparon de forma independiente respecto al resto de propiedades. No se observó relación entre el contenido en nitratos y la actividad desnitrificante (determinada como emisión de N_2O), cuando es conocido que, en términos generales, la presencia de nitratos en una muestra medioambiental produce un aumento en la producción de N_2O (Fulweiler y Heiss 2014). La emisión de N_2O se produce de forma irregular en los ecosistemas, siendo más activa en algunos de los

micrositios que lo componen a los que se denomina puntos calientes respecto a otros micrositios del ecosistema (McClain et al. 2003; Vidon et al. 2010; Butterbach-Bahl et al. 2013); aún más, para un sitio caliente existen momentos calientes, esto es, cuando la actividad desnitrificante es más intensa (Shapleigh 2013; Medinets et al. 2015). La existencia de sitios y momentos calientes puede resultar en el desacople entre el contenido en nitratos y la actividad desnitrificante en muestras medioambientales, lo que dificultaría su cuantificación con precisión y podría explicar los resultados obtenidos en este trabajo.

El análisis PCA indicó, además, que los valores de las variables analizadas se agruparon de forma independiente de acuerdo a los sitios de muestreo. Para un determinado sitio también se observaron diferencias entre las distintas fechas de muestreo. Se puede concluir, por tanto, que existe cierta variabilidad espacio-temporal en el conjunto de propiedades determinadas en los diferentes sitios y fechas de muestreo del arroyo de la Rocina.

Artículo II

Conocidas las propiedades biológicas y físico-químicas de los sedimentos del arroyo de la Rocina (Tortosa et al. 2011; artículo I de esta Memoria de Doctorado), se seleccionaron los sitios S1 (laguna del Acebrón) y S2 (arroyo de la Cañada), los puntos con mayor y menor contenido en nitratos, respectivamente, y la toma de muestras de sedimento se realizó durante los meses de abril y octubre de los años 2008, 2009 y 2010. En cada uno de las muestras de los sedimentos se determinó mediante qPCR la abundancia del gen 16S rRNA, que se empleó como marcador de la población bacteriana, y de los genes *narG*, *napA*, *nirK*, *nirS* y *nosZ*, que se utilizaron como indicadores de las poblaciones desnitrificantes. Además, se analizó el contenido en nitratos y la actividad desnitrificante de cada sitio. Las metodologías empleadas se describen en Correa-Galeote et al. (2013a, artículo IV; 2014b, artículo V).

Independientemente de las fechas de muestreo, el contenido en nitratos de S1 fue inferior al de S2. En S1 no se observaron diferencias entre los meses de toma de muestras, en S2 la concentración de nitratos fue superior en octubre. Es posible que estos resultados se deban a que las fluctuaciones hídricas en S1 son muy escasas ya que laguna del Acebrón mantiene un caudal de agua relativamente constante, mientras que

S2 está sujeto a las variaciones típicas de los arroyos mediterráneos. En S2, independientemente del año de estudio, el que se determine la concentración de nitratos fue mayor en octubre, mes que coincide con el período de mayor sequía, puede deberse a que en esas fechas el arroyo de la Cañada experimenta un nivel mínimo de agua, lo que resulta en el incremento de la concentración de nitratos.

En general, la actividad desnitrificante en S1 no varió entre fechas de muestreo; en S2, sin embargo, fue superior en abril y los valores de emisión de octubre fueron similares a los de S1. La disminución de la actividad desnitrificante de S2 en octubre podría deberse al escaso caudal de agua en el arroyo al final de la época seca lo que aumentaría la tensión de oxígeno en los sedimentos. Se ha demostrado que este hecho inhibe la actividad desnitrificante (Tiedje 1988; Woodward et al. 2009). También es posible que la disminución de nutrientes debida al menor caudal de agua resulte en una menor producción de N_2O (Smith et al. 2010).

El número de copias del gen 16S rRNA en S1 y S2 fue similar al descrito por otros autores cuando emplearon los mismos cebadores para amplificar ADN aislado de diferentes ecosistemas (Dandie et al. 2007; Bárta et al. 2010; García-Lledó et al. 2011; Keil et al. 2011, Zhou et al. 2014). En general, no se observaron diferencias espacio-temporales en la abundancia del gen 16S rRNA a lo largo de los 3 años de estudio. Estos resultados son similares a los descritos por Keil et al. (2011) en suelos de pradera de la montaña Schwäbische Alb localizada en el sureste de Alemania.

Por otra parte, el número de copias de los genes *narG*, *napA*, *nirK*, *nirS* y *nosZ*, y su abundancia relativa, son similares a los descritos en suelos y sedimentos que otros autores han estudiado empleando los mismos cebadores para la amplificación de tales genes (Henry et al. 2006, 2008; Smith et al. 2007; Dandie et al. 2008; Kandeler et al. 2009; Yoshida et al. 2009; Čuhel et al. 2010; Lindsay et al. 2010; Su et al. 2010; Attard et al. 2011; Bru et al. 2011; Chon et al. 2011; Torrentó et al. 2011; Marhan et al. 2011; Chen et al. 2012a; Palmer et al. 2012; Hamonts et al. 2013; Wieder et al. 2013; Deslippe et al. 2014; Ligi et al. 2014a,b; Zhang et al. 2015).

La abundancia relativa de los genes *narG/napA* fue siempre superior a la de los genes *nirK/nirS* y esta, a su vez, superior a la del gen *nosZ.* Este resultados coinciden con los descritos en humedales artificiales de Ampurias (Girona) (García-Lledó et al.

(2011), aguas y sedimentos del acuífero de Osona (Barcelona) (Torrentó et al. 2011), suelos de pradera de Taoyuan (China) (Chen et al 2012a) y suelos de ribera del río Manawatu (Nueva Zelanda) (Deslippe et al 2014) y sugieren que, en general, las poblaciones capaces de reducir el nitrato dominan sobre el resto de poblaciones desnitrificantes y que existe una fracción elevada de bacterias desnitrificantes que carecen de actividad óxido nitroso reductasa, posiblemente por carecer en su genoma del gen *nosZ*.

La abundancia relativa de los genes *narG, napA y nosZ* varió de forma estacional solo en S2, y fue estadísticamente superior en el mes de octubre. La mayor abundancia de genes desnitrificantes en octubre de S2 coincide con los valores máximos de contenido en nitratos, pero no en cuanto a la actividad desnitrificante se refiere, lo que, como se ha indicado anteriormente, podría deberse al aumento de la concentración de oxígeno en la matriz del suelo. Los genes *nirS* y *nirK* siguieron patrones espacio-temporales diferentes ya que, independientemente del sitio de muestreo, la abundancia relativa de *nirS* estuvo sujeta a variaciones estacionales, diferencias que no se observaron para el gen *nirK*.

En conjunto, estos resultados indican la existencia de variaciones espacio-temporales en la comunidad desnitrificante. Estas variaciones en las abundancias relativas de los genes *narG*, *napA* y *nirS* también ocurren a lo largo del estuario del río Colne (Colchester, UK) (Smith et al. 2015).

Puesto que se conoce el contenido en nitratos, la actividad desnitrificante y la abundancia de los genes de la desnitrificación en los sitios S1 y S2 del arroyo de la Rocina, nuestros datos permiten relacionar sustrato (nitratos) con la abundancia de los genes de la desnitrificación y su función (actividad desnitrificante).

Para estudiar estas relaciones se empleó, por una parte, el test de correlación de Spearman y, por otra, se realizó un análisis de componentes principales (PCA).

El primero demostró la existencia de correlación positiva entre el contenido en nitratos y la abundancia relativa de cada uno de los genes *narG*, *napA*, *nirS* y *nosZ*, y que la correlación más fuerte ocurrió con *nosZ*, esto es, que este gen es el más sensible a las variaciones espacio-temporales del contenido en nitratos. Esta correlación positiva entre el contenido en nitratos y la abundancia de las poblaciones desnitrificante también se ha descrito en sedimentos de río Suquía (Argentina) (Reyna et al. 2010) y del estuario del río Colne (UK) (Smith et al. 2015). Sin embargo, el contenido en nitratos

no se correlacionó con la abundancia total de bacterias de la comunidad determinada como el número de copias del gen 16S rRNA, lo que indica que la comunidad desnitrificante responde de forma específica al contenido en nitratos.

La actividad desnitrificante fue independiente del contenido en nitratos. Como ya se ha indicado es posible que esta ausencia de correlación se deba a la disminución de actividad desnitrificante que ocurre al reducirse el aporte de agua en S2 en el mes de octubre. Resultados similares se han descrito en humedales efímeros de Canadá, suelos cultivados con patata, suelos agrícolas y zonas húmedas de Canadá en los trabajo de Ma et al. (2008), Henderson et al. (2010) y Dandie et al. (2011), respectivamente, mientras que otros autores han indicado la existencia de correlación entre el contenido en nitratos y la emisión de N_2O en diversos ecosistemas (Luo et al. 1999; Zechmeister-Boltenstern et al. 2002; Hefting et al. 2003; Magalhães et al. 2008; Morales et al. 2015).

Tampoco se ha observado correlación entre la abundancia relativa de los genes de la desnitrificación y la actividad desnitrificante, posiblemente relacionada con la disminución de la actividad en la época seca. Esta ausencia de correlación se ha publicado para los genes *narG* y *napA* (Ikeda et al. 2009) y para los genes *nirK*, *nirS* y *nosZ* (Zhang et al. 2014). En otros casos, por el contrario, se ha descrito la existencia de correlación entre la actividad desnitrificante y la abundancia del gen *nirK* (Enwall et al. 2010; Attard et al. 2011; Brankatschk et al. 2011), *nirS* (Enwall et al. 2010; Brankatschk et al. 2011) y *nosZ* (Petersen et al. 2012). Además del ya indicado efecto debido a la hidrogeología de los sedimentos de la Rocina, se ha propuesto que el desacoplamiento entre la abundancia relativa y la emisión de N_2O puede deberse a que los posibles controles post-transcripcionales en la actividad desnitrificante sean diferentes a los que controlan la abundancia relativa de dichas poblaciones (Smith et al. 2015).

Puesto que en ninguno de los artículos que componen esta Memoria de Doctorado se han establecido vínculos entre el contenido en nitratos y la actividad desnitrificante, es necesario continuar con el análisis del efecto que los flujos de N producen en las poblaciones desnitrificantes y tratar de entender de forma global la ecología de estos microorganismos.

Finalmente, el test de Spearman reveló una estrecha correlación entre la abundancia relativa de cada uno de los genes de la desnitrificación, relación que fue mayor entre el gen *nosZ* y cada uno de los restantes genes. Si para un determinado ecosistema, como son los sedimentos del arroyo de la Rocina, existe correlación entre los genes desnitrificantes, es posible que las correspondientes poblaciones bacterianas

compartan el mismo hábitat (Enwall et al. 2010). Nuestro trabajo indica que, por tanto, un factor que altere una población desnitrificante puede, a su vez, alterar toda la comunidad. Este pudiera ser el caso del nitrato que, en general, estimula la comunidad desnitrificante en muestras de sedimento del arroyo de la Rocina.

El PCA confirmó la correlación positiva que el test de Spearman demostró entre el contenido en nitratos y abundancia relativa de las poblaciones desnitrificantes. Además este análisis indicó que los valores de las diferentes variables analizadas en este trabajo se agruparon de acuerdo a las fechas y sitios de muestreo, por lo que hay que aceptar la existencia de variabilidad espacio-temporal de tales variables en las muestras de los sedimentos del arroyo de la Rocina.

Como continuación del trabajo sobre el efecto de los nitratos sobre la comunidad desnitrificante, nos propusimos estudiar la diversidad biológica de las poblaciones desnitrificantes en cada uno de los sitios S1 y S2.

El gen *nosZ* se ha empleado como marcador molecular en estudios metagenómicos de comunidades desnitrificantes en diversas muestras medioambientales (Rösch et al. 2002; Rich y Myrold 2004; Throbäck et al. 2004; Henry et al. 2006; Horn et al. 2006; Palmer et al. 2009; Dell et al. 2010; Chon et al. 2011; Ishii et al. 2011; Chen et al 2012b; Ligi et al 2014a, b; Deslippe et al 2014). Este gen, además, es más estable frente a factores medioambientales que los otros genes del ciclo del N (Wallenstein et al. 2006; Chroňáková et al. 2009; Rasche et al. 2010) y, aunque existen ciertas inexactitudes en su filogenia (Ishii et al. 2011), las relaciones filogenéticas entre el gen 16S rRNA y el *nosZ* son más congruentes que las que ocurren entre las del gen 16S rRNA y el resto de genes de la desnitrificación (Jones et al. 2008; Srinandan et al. 2011).

En nuestro estudio los resultados del PCA no solo mostraron una fuerte correlación entre el gen *nosZ* y el contenido en nitratos de los sedimentos sino que, además, fue la propiedad que mejor resume la variabilidad del resto de propiedades analizadas.

Por todo ello, se decidió utilizar el gen *nosZ* como marcador de las comunidades desnitrificantes en los sitios S1 y S2. Se amplificó entonces dicho gen a partir del ADN aislado de las muestras de sedimentos y se construyeron 8 genotecas correspondientes a los meses de abril y octubre de 2009 y 2010 de cada sitio. Dada la ausencia de

diferencias en la abundancia relativa del gen *nosZ*, no se construyeron las genotecas correspondientes a las muestras del año 2008.

Las 8 librerías genómicas contenían un total de 504 clones cuyos insertos de ADN presentaban homología con secuencias del gen *nosZ* depositadas en las bases de datos. Estas secuencias se agruparon en 109 unidades taxonómicas operativas (OTUs), una riqueza superior a la previamente estimada en otros trabajos que emplearon muestras de sedimentos de ribera del lago Creek, Oregón, (USA) (Rich y Myrold 2004), suelos agrícolas de Suecia (Enwall et al. 2005), lodos residuales (Srinandan et al. 2011) y aguas eutrofizadas del lago Baiyangdian (China) (Wang et al. 2013), y similar a la obtenida por Chen et al. (2012a) en arrozales con distintos tipos de fertilización de la región de Taoyuan (China).

La construcción de un árbol filogenético distribuyó las 504 secuencias en 31 grupos filogenéticos. La clase Betaproteobacteria fue mayoritaria respecto a la Alphaproteobacteria, un resultado similar a los descritos por Srinandan et al. (2011) en lodos de depuradoras y por Chen et al. (2012b) en suelos de arrozales de Taoyuan (China). Nuestros resultados, sin embargo, contrastan con los publicados por Henry et al. (2006) en suelos agrícolas de diversos países, y por Magalhães et al. (2008) y Yang et al. (2014) en los sedimentos de los estuario del río Duero (Portugal) y Jiaolai (China), respectivamente. El número de secuencias del gen *nosZ* pertenecientes a la clase Gammaproteobacteria fue inferior al de individuos de las otras dos clases bacterianas y únicamente se encontraron en las muestras de los sedimentos procedentes de S1. Estos resultados sugieren que los miembros de esta clase están circunscritos a sitios específicos, lo que concuerda con lo descrito por Chen et al. (2012a) al analizar la diversidad del gen *nosZ* en suelos de arrozales situados en la región de Taoyuan (China).

Una elevada fracción de secuencias no pudo adscribirse a un determinado género, posiblemente por tratarse de bacterias no cultivables. Estos resultados coinciden con los descritos por Bellini et al. (2013) cuando analizaron la comunidad bacteriana del gen *nosZ* en un acuífero de Raigón (Uruguay) y por y Smith y Ogram (2008) y Chen et al. (2010) al estudiar la biodiversidad de las bacterias que presentaban en su genoma los genes *nirK* y *nirS* en muestras de suelos agrícolas de Florida (USA) y de la región de Taoyuan (China), respectivamente.

La riqueza en el número de OTUs fue similar en 6 de las 8 librerías genómicas. Las 2 restantes, correspondientes a los meses de octubre del sitio S2 contenían una mayor riqueza de OTUs. Es posible que el mayor contenido en nitrato en las muestras de octubre en S2 redunde en una mayor riqueza de bacterias con el gen *nosZ*. Aunque algunos autores han observado que un elevado contenido en nitratos produce un incremento de la diversidad del gen *narG* en sedimentos del río Suquía (Argentina) (Reyna et al. 2010), otros no encontraron cambios en la estructura de la comunidad desnitrificante en respuesta a alteraciones en el contenido en nitratos en suelos agrícolas de Rørrendegård (Dinamarca) (Wolsing y Priemé 2004) y en bosques de Ohio (USA) (Carrino-Kyker et al. 2012).

No se detectaron diferencias ni en el número (índice de Chao1) ni en la riqueza específica (índice de Shannon-Weaber) de secuencias con escasa representación en las 8 genotecas. Igualmente, no se observó la dominancia de un determinado OTUs en ninguna de las genotecas, si bien la equidad en la composición de los OTUs en las genotecas de los meses de octubre de S2 fue superior a las de S1. Cabe suponer que el contenido en nitratos fue responsable de los cambios en la riqueza y dominancia en las genotecas de octubre de S2.

De acuerdo a las variaciones nucleotídicas de las secuencias analizadas mediante AMOVA, la estructura de la comunidad desnitrificante se mantuvo relativamente constante en las genotecas de S1, mientras que se detectaron cambios en las secuencias del ADN del gen *nosZ* de los sedimentos de S2. Todos estos datos sugieren que las diferencias en el contenido en nitratos entre S1 y S2 es el factor que controla los cambios en las estructura de las poblaciones desnitrificantes.

El análisis de la correspondencia canónica (CCA) confirmó de manera estadística que el contenido en nitratos afecta la diversidad de las comunidades desnitrificantes que poseen el gen *nosZ* en su genoma. Por tanto, el análisis de la diversidad de este gen en muestras medioambientales es útil para conocer el efecto del contenido en nitratos sobre la comunidad desnitrificante. Además, el CCA reveló diferencias significativas en la composición de las genotecas entre los meses de abril y octubre en ambos sitios de muestreo, lo que indica variaciones espacio-temporales en la estructura de las comunidades desnitrificantes de los sedimentos. Tales diferencias estacionales se han descrito en suelos agrícolas de Rørrendegård (Dinamarca) (Wolsing

y Priemé 2004) y en suelos de Ontario (Canadá) (Smith et al. 2010) y en Truro (Canadá) (Tatti et al. 2015).

Tanto en S1 como en S2, los OTUs de la familia Bradyrhizobiaceae y Rhodocyclaceae, junto con otros dos OTUs no clasificados a nivel de género fueron los más abundantes. En S2 aparecieron nuevos OTUs, pero ninguno de ellos se presentó como dominante. Por ello, las diferencias en la estructura de las comunidades desnitrificantes en S1 y S2 no se deben a cambios específicos de un determinado grupo filogenético sino más bien a cambios en la proporcionalidad de las poblaciones, esto es diferencias en el número de individuos (secuencias) que componen los distintos grupos. OTUs de las familias Bradyrhizobiaceae y Rhodocyclaceae, junto con las menos abundantes Rhizobiaceae, Burkholderiaceae y Rhodospirillaceae identificadas en nuestro estudio se han caracterizado como grupos dominantes en sedimentos eutrofizados del lago Baiyangdian (China) (Wang et al. 2013), suelos de humedales efímeros Saskatchewan (Canadá) (Ma et al. 2011), lodos activos de depuradora (Srinandan et al. 2011), en campos de golf de Carolina (USA) (Dell et al. 2010) y en arrozales situados de Taoyuan (China) (Chen et al. 2012b).

Recientemente, se ha descrito una variante del gen *nosZ* que no puede ser amplificada con los cebadores tradicionalmente empleados (Sandford et al. 2012, Jones et al. 2013). Existen, por tanto, dos grupos de bacterias desnitrificantes que contienen el gen nosZ, uno el *nosZ* clásico y otro que incorpora este nuevo clado al que se ha denominado nosZII (Jones et al. 2013). No obstante, la suma de las bacterias desnitrificantes que contienen una cualquier de las variantes del gen *nosZ* es inferior a la suma de desnitrificantes que presentan en su genoma cualquiera de los genes *nirK* o *nirS* (Jones et al. 2013).

Del análisis de diferentes muestras medioambientales se ha establecido que ambos clados se distribuyen en proporciones similares (Ligi et al. 2015), por lo que hay que aceptar que en nuestro estudio se ha obviado la existencia de este nuevo grupo bacteriano que compone el clado II. Este hecho, sin embargo, no introduce sesgo alguno en los resultados obtenidos ya que existe un claro efecto de la concentración de nitratos sobre la abundancia y estructura de la comunidad desnitrificante general.

Artículo III

Una vez analizada las relaciones entre los genes de la desnitrificación y su función, determinada como actividad desnitrificante, se abordó la tarea de estudiar la distribución espacial de las poblaciones desnitrificantes.

Robertson et al. (1987) fueron los primeros en demostrar la distribución espacial de los procesos de mineralización del N, desnitrificación y desnitrificación. Posteriormente, otros trabajos han contribuido a estudiar la distribución espacial de los microorganismos que intervienen en el ciclo del N lo que, en la mayoría de los casos, se ha hecho a partir de la cuantificación del número de copias de genes funcionales relacionados con él (Philippot et al. 2009; Enwall et al. 2010; Bru et al. 2011, Keil et al. 2011). Sin embargo, hasta la fecha de publicación del artículo III no se había estudiado la distribución espacial de los genes de la desnitrificación en sedimentos contaminados con nitratos.

Aunque hubiera sido preferible utilizar para ello cualquiera de los sitios S1 y S2 ya estudiados, no fue posible llevarlo a término porque S1 es una laguna donde no se puede acotar una superficie de trabajo y porque S2 es una zona de pequeña superficie que impide establecer las adecuadas repeticiones de cuadrículas de trabajo.

Los humedales artificiales constituyen un método de tratamiento biológico alternativo para la depuración *in situ* de aguas contaminadas con nitratos procedentes de zonas urbanas de tamaño medio, industrias, zonas agrícolas, etc. Su eficacia y bajo coste han hecho que su empleo se haya extendido en las últimas décadas (Leonard y Swanson 2001; Bruland et al. 2006; Kadlec y Wallace 2008). Además de la desnitrificación y de la nitrificación, las plantas de los humedales también pueden asimilar nitrato, contribuyendo a su eliminación del medio ambiente (Salvato et al. 2012). En el END se han construido varios humedales con la finalidad de depurar las aguas de riego procedentes de las zonas agrícolas que lo rodean y que, en su recorrido, podrían alcanzar la marismas. Uno de estos humedales es el de los Guayules que protege la marisma del Rocío del aporte de aguas contaminadas con nitratos.

Para analizar la distribución espacial de las poblaciones desnitrificantes se determinó la abundancia del número de copias de los genes de la desnitrificación *narG*, *napA*, *nirK*, *nirS* y *nosZ* en cada uno de los 50 puntos de muestreo que se establecieron

en el humedal. Además se cuantificaron que contenían el gen *amoA*, las poblaciones de Bacterias (AOB) y Archaea (AOA). En cada punto se determinó también el número de copias del gen 16S rRNA, que se empleó como marcador molecular del total de las comunidades Bacteria y Archaea. La abundancia de genes se realizó mediante qPCR siguiendo la metodología descrita por Correa-Galeote et al. (2013a, artículo IV de esta Memoria). Finamente, se evaluaron las propiedades físico-químicas y la actividad desnitrificante en cada uno de los 50 puntos de muestreo de la manera descrita previamente por Correa-Galeote et al. (2014b, artículo V de esta Memoria de Doctorado).

Los estudios sobre distribución espacial de las comunidades microbianas se basan en el empleo de técnicas geoestadísticas desarrolladas en la industria minera para tratar de establecer la ubicación de la veta de mineral. En resumen, la geoestadística es una herramienta para determinar la variación espacial de una determinada propiedad y predecir los valores de los puntos no muestreados en el área de estudio. Este análisis asume que los puntos situados más cerca en el espacio comparten valores similares respecto a aquellos más lejanos en el espacio, propiedad a la que se ha denominado autocorrelación. Si existe autocorrelación espacial se puede construir un modelo matemático denominado semivariograma que describe la relación espacial entre los puntos de muestreo. A partir de este modelo se estima el valor del total del área muestreada y se elabora un mapa de su distribución al que se denomina mapa de Krige, por ser D. G. Krige quien desarrolló las técnicas geoestadísticas de análisis de la distribución espacial de una veta de mineral.

El semivariograma de la mayoría de las propiedades físico-químicas determinadas en cada uno de los 50 puntos de muestreo del humedal de los Guayules mostró que no hubo correlación para distancias menores de 5 m, la autocorrelación sí fue patente para escalas espaciales a partir de los 8-10 m. Puesto que los valores de autocorrelación de los semivariogramas del contenido en nitrógeno total fueron cercanos al área total de muestreo, y los de pH y humedad del suelo superiores a dicha área, no se encontró casi ninguna agregación en los correspondientes mapas de Krige.

Respecto a las comunidades de bacterias y de arqueas hay que indicar que en las primeras no existió autocorrelación entre los valores de abundancia, lo que indica la

ausencia de distribución espacial en el área de muestreo; sin embargo, las segundas se correlacionaron entre sí en escalas espaciales cercanas a los 50 m. Todo ello a pesar de que la abundancia de Bacteria fue un orden de magnitud superior al de Archaea, lo que también se ha descrito en los trabajos de Laverock et al. (2014) y Lee et al. (2015) en sedimentos marinos de Plymouth (UK) y suelos de arrozal de Sacheon (Corea del Sur), respectivamente.

La abundancia del número de copias del gen *amoA* en Bacteria fue superior a la correspondiente de Archaea y en ningún caso se observó autocorrelación en los valores de esta propiedad, por lo que no se pudieron construir los correspondientes mapas de Krige de este gen. No obstante, otros autores han publicado la existencia de correlación espacial en ambas comunidades y se han elaborado los correspondientes mapas de Krige (Fierer et al. 2009; Bru et al. 2011; Wessén et al. 2011).

Como indicó el test de Spearman, la abundancia de las comunidades AOA y AOB se correlacionaron entre sí, lo que indica que ambos tipos de microorganismos comparten el mismo hábitat y se regulan por los mismos factores medioambientales. Mientras que unos autores han descrito la existencia de correlación entre estas comunidades (Keil et al. 2011), otros no la han observado (Wessén et al. 2011), si bien los ecosistemas estudiados fueron diferentes, en suelos de montaña de Alemania en el primer caso y suelos agrícolas con diferente tipo de labranza de Suecia en el segundo.

Respecto a las poblaciones desnitrificantes hay que indicar que sus patrones de distribución fueron diferentes para cada uno de los genes *narG*, *napA*, *nirK*, *nirS* y *nosZ* y de ahí los diferentes mapas de Krige obtenidos. Mientras que los cuatro primeros presentaron valores de autocorrelación aproximados de unos 10 m, el del gen *nosZ* fue próximo al del área total de muestreo, esto es, 50 m.

Puesto que los mapas de Krige revelan diferentes distribuciones espaciales para cada uno de los genes de la desnitrificación hay que admitir que las distintas poblaciones desnitrificantes podrían afectarse de diferente forma por las variaciones que puedan ocurrir en el medio ambiente.

Nuestro trabajo sobre la distribución espacial de los genes de la desnitrificación ha sido pionero en el estudio de sedimentos de zonas húmedas de agua dulce ya que la mayoría de los trabajos realizados utilizaron suelos agrícolas o de pradera (Philippot et al. 2009; Enwall et al. 2011; Keil et al. 2011). En estos estudios la distribución espacial

de los genes *narG*, *napA*, *nirK*, *nirS* y *nosZ* es similar a la obtenida en nuestro trabajo ya que en ellos se establecieron valores de autocorrelación en torno a los 8-10 m.

Similar a la distribución de los genes de la desnitrificación fue la de la actividad desnitrificante, esto es, los valores de autocorrelación se agruparon sobre los 10 m. Esta distribución coincide con la publicada por Song et al. (2014) en un humedales del río Olentangy situado en Ohio (USA). La razón $N_2O/(N_2O + N_2)$ es cercana a 1, lo que indica que la producción de N_2 en los Guayules es prácticamente nula, lo que resultaría en una mayor emisión de N_2O. Estos resultados son similares a los descritos en los trabajos de Philippot et al. (2009) en suelos de praderas de Cěský Krumlov (República Checa) y Wang et al. (2013) en sedimentos eutrofizados del lago Baiyangdian localizado en la llanura de Hebei (China).

Dada la complejidad la compleja distribución espacial de las variables estudiadas y, por tanto, la dificultad de establecer comparaciones entre los diferentes mapas de Krige, se llevó un análisis de Spearman para conocer la relación, positiva o negativa, de unas propiedades con otras. En general, las propiedades físico-químicas no influenciaron en la distribución espacial de los genes de la desnitrificación.

Así, la abundancia relativa de los genes de la desnitrificación es independiente del contenido en nitratos. Esta ausencia de correlación se ha determinado en los trabajos de Keil et al. (2011), García-Lledó et al. (2011) y Ligi et al. (2015) en suelos de pradera del sureste alemán, en humedales artificiales de Ampurias (Girona), en sedimentos de marismas y meandros cercanos al río Olentangy, Ohio, (USA), respectivamente. El test de Spearman también reveló la no correlación entre el contenido en nitratos y actividad desnitrificante, resultado que coincide con los de los artículos I y II de esta Memoria de Doctorado.

Se ha propuesto que el pH es uno de los principales factores físico-químico que afecta la abundancia de las comunidades microbianas (Hallin et al 2009; Čuhel et al. 2010; Bru et al. 2011; Herold et al. 2012). En los sedimentos del humedal de los Guayules, el pH se correlacionó negativamente con la abundancia de la población bacteriana total y con la abundancia relativa de los genes *nirK* y *nosZ* y no existió correlación con la abundancia relativa del resto de genes.

Por otra parte, la actividad desnitrificante se correlacionó positivamente con la abundancia de las comunidades Bacteria y Archaea así como con la abundancia relativa

de los genes *nirS* y *nosZ*. Esta correlación fue similar a la observada entre el contenido en carbono orgánico total, nitrógeno total, grado de cobertura vegetal y la actividad desnitrificante.

Además, hay que destacar que el grado de cobertura vegetal se correlacionó positivamente con la comunidad total de Bacteria y Archaea y con la abundancia relativa de los genes *nirS* y *nosZ*. Aunque se sabe que la liberación de compuestos carbonados por las raíces de las plantas estimula las poblaciones microbianas del suelo (Bradford et al. 2013), en el humedal de los Guayules no existió correlación entre el carbono orgánico total y la abundancia relativa de los genes desnitrificantes, por lo que la relación positiva entre la cobertura vegetal y la abundancia de los genes *nirS* y *nosZ* podría deberse a otros mecanismos (Salvato et al. 2012). También se observó una correlación positiva entre el grado de cobertura vegetal y la emisión de N_2O, lo que contrasta con los resultados de Jørgensen et al. (2011) que describieron una correlación negativa entre ambas propiedades, lo que atribuyeron a que la emisión de N_2O que se produce a través de las plantas de *Phalaris arundinacea* resulta en una disminución del flujo de N_2O a través del suelo.

El empleo de diferentes escalas de muestreo, desde centímetros hasta metros, para explicar la distribución espacial de las comunidades desnitrificantes no aumentó ni el número ni la fuerza de las correlaciones entre los factores físico-químicos y las comunidades desnitrificantes en una pradera de la montaña Schwäbische Alb del sureste alemán (Keil et al. 2011). Por tanto, las determinaciones de las variables analizadas deberían realizarse en dimensiones espaciales más pequeñas que permitan concretar la ausencia o existencia de tales correlaciones.

Artículos IV y V

Los artículos IV y V son descripciones metodológicas que contienen la mayoría de las técnicas y procedimientos utilizados en la realización de esta Memoria de Doctorado.

En el artículo IV se describen las técnicas y metodologías que se han empleado para a) determinar la actividad desnitrificante como producción de N_2O; b) estimar el contenido en amonio, nitrato, N total y N disuelto y c) determinar el origen de los compuestos de nitrato contaminantes detectados en los sedimentos del arroyo de la Rocina. La utilización y calidad de tales técnicas está ampliamente contrastada.

El artículo V contiene información sobre diversas metodologías para la extracción y purificación de ADN de muestras medioambientales y se describe en profundidad la metodología ISO 11063 para la extracción de ADN (Petric et al. 2011). También se revisan, actualizan y discuten los diversos protocolos que se utilizan para cuantificación de ácidos nucleicos en muestras medioambientales mediante la reacción en cadena de la polimerasa a tiempo real (qPCR). También se describen las reacciones de PCR y condiciones de funcionamiento de los termocicladores para la determinación de la abundancia de los genes analizados en esta Memoria de Doctorado, incluyendo el 16S rRNA y los genes de la desnitrificación *narG*/*napA*, *nirK*/*nirS*, c-*nor*/q-*nor* y *nosZ*. Se debe indicar que el empleo del método estándar ISO 11063 y los principios descritos en la guía protocolo MIQUE (Bustin et al. 2009) se consideran con el objeto de incrementar la transparencia experimental.

CONCLUSIONES

1. A pesar de la especial protección con la que cuenta el Espacio Natural de Doñana, existe contaminación por nitratos en los sedimentos del arroyo de la Rocina y del humedal de los Guayules.

2. Los nitratos analizados en el arroyo de la Cañada proceden de los fertilizantes nitrogenados que se emplean en las prácticas agrícolas que se realizan en el ecotono de Doñana.

3. Existen variaciones espacio-temporales en el conjunto de los valores de las propiedades físico-químicas, incluido el contenido en nitratos, y biológicas de los sedimentos del arroyo de la Rocina, que se deben a cambios en la dinámica hídrica del arroyo.

4. La abundancia relativa y la biodiversidad de las poblaciones desnitrificantes de la laguna del Acebrón y del arroyo de la Cañada está sujeta a variaciones espacio-temporales.

5. El nitrato afecta positivamente la abundancia relativa y la biodiversidad de las poblaciones desnitrificantes en la laguna del Acebrón y en el arroyo de la Cañada.

6. La actividad desnitrificante es independiente tanto del contenido en nitratos como de la abundancia relativa de las poblaciones desnitrificantes.

7. La existencia de correlación positiva entre la abundancia relativa de todos y cada uno de los genes de la desnitrificación sugiere el funcionamiento conjunto de la comunidad desnitrificante en sedimentos del arroyo de la Cañada y de la laguna del Acebrón.

8. Los mapas de Krige del humedal de los Guayules indican que el estudio de la distribución espacial de las poblaciones desnitrificantes debe realizarse a escalas microscópicas, inferiores a las empleadas en este estudio.

9. Los análisis de abundancia relativa, biodiversidad y distribución espacial de las poblaciones desnitrificantes realizados en este estudio indican que el gen *nosZ* es un marcador molecular adecuado para el estudio de la metagenómica y ecología funcional de tales poblaciones.

CONCLUSSIONS

1. Despite the special protection of the Doñana Natural Area, there is a nitrate contamination in sediments from Rocina stream and wetland of los Guayules.

2. The nitrates observed in Cañada creek derives from nitrogen fertilizer used in agricultural practices carried out in the ecotone of Doñana.

3. There are spatial and temporal variations in the whole values of the physico-chemical properties, including the nitrate content, and biological of the sediments taken in Rocina stream, which are due to changes in the hydrodynamics of the stream.

4. Relative abundance and biodiversity of denitrifying populations of Acebrón lagoon and Cañada creek present spatial and temporal variations.

5. Nitrate positively affects the relative abundance and biodiversity of denitrifying populations in Acebrón lagoon and in Cañada creek.

6. The denitrifying activity is independent of both the nitrate content and the relative abundance of denitrifying populations.

7. The existence of positive correlation between the relative abundance of each and every one of the denitrification genes suggests the assembly function of the denitrifying community in sediments from Acebrón lagoon and Cañada creek.

8. Krige maps of Guayules wetland indicate that the study of the spatial distribution of denitrifying populations should be performed at microscopic scales lower than the used in this study.

9. The analysis of relative abundance, biodiversity and spatial distribution of denitrifying populations performed in this study indicate that the *nosZ* gene is a suitable molecular marker for the study of metagenomics and functional ecology of these populations.

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