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POBLACIONES DE MICROORGANISMOS OXIDADORES DE AMONIO Y DESNITRIFICANTES EN CULTIVO DE ARROZ

por

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RESUMEN

El arroz es un importante alimento a nivel mundial, su cultivo es potencialmente emisor de gases de efecto invernadero, tales como el CH_4 y el N_2O. Algunas estrategias empleadas para mitigar la emisión de estos gases incluyen el manejo de la inundación del cultivo o la selección del cultivar de arroz. Asimismo, suelos con características fisicoquímicas diferentes pueden tener potenciales distintos de emisión. El N_2O es biológicamente generado en el suelo principalmente por dos grupos microbianos, los microorganismos oxidadores de amonio y los microorganismos desnitrificantes. En este trabajo, se determinó el efecto del manejo del agua, el cultivar de arroz y el tipo de suelo sobre la abundancia y la estructura poblacional de ambos grupos microbianos. La abundancia de estos microorganismos se determinó mediante qPCR, mientras que las estructuras poblacionales se determinaron mediante T-RFLP. En el caso de los microorganismos oxidadores de amonio, se empleó como gen blanco el gen de la subunidad α de la enzima amonio monooxigenasa *amoA*; los genes *amoA* bacteriano y arqueano se analizaron por separado. Por otra parte, los genes *nirS* y *nirK*, que codifican para dos variedades de la enzima nitrito reductasa, fueron empleados como genes blanco para estudiar las poblaciones de microorganismos desnitrificantes. Se establecieron dos ensayos de invernáculo en dos años consecutivos, en el primer ensayo se evaluaron dos cultivares de arroz y dos manejos del agua, mientras que en el segundo se evaluaron dos tipos de suelo y dos momentos de establecimiento de la inundación. Los resultados mostraron que todos los factores evaluados tuvieron algún efecto sobre las poblaciones microbianas, aunque los efectos no fueron iguales para todas las poblaciones. El tipo de suelo fue el factor que mayor incidencia tuvo sobre las poblaciones, particularmente sobre las arqueas oxidadoras de amonio.

Palabras clave: arroz, N_2O, desnitrificantes, oxidadores de amonio

AMMONIA-OXIDIZING AND DENITRFYING MICROORGANISMS POPULATIONS IN RICE CROP

SUMMARY

Worldwide, rice is an important food source. Its farming is potentially a source of greenhouse effect gases (GHG), such as CH_4 and N_2O. Water management and or rice cultivar selection are some of the management strategies employed to mitigate these gases. In addition, soils with different physicochemical characteristics can differ in their tendency to emit GHG. In soils N_2O is biologically produced, mainly by two microbial guilds, ammonia-oxidizing microorganisms, and denitrifying microorganisms. In this thesis, it was determined the effect of water management, rice cultivar and soil type on the abundance and population structure of both guilds. These microorganisms abundance were determined by qPCR, while their population structures were determined by T-RFLP. For the ammonia-oxidizing microorganisms the *amoA* gene that encodes for the ammonia monooxygenase was used as the target gene; archeal and bacterial *amoA* gene were analyzed separately. On the other hand, *nirS* and *nirK* genes, which encode for the two kinds of the enzyme nitrite reductase, were used as the target genes to study the denitrifying microorganisms. Two greenhouse experiments were set up in two consecutive years. In the first experiment, two rice cultivars and two water managements were evaluated, while in the second experiment two soil types and two times of flooding were evaluated. Results showed that all the factors evaluated had some effect on the microbial populations, however not all the factors affected the populations equally. The soil type was the strongest factor shaping microbial populations, and had a particularly strong effect on the ammonia-oxidizing archaea.

Keywords: rice, N_2O, denitrifiers, ammonia-oxidizing microorganism

1. CAPÍTULO 1: INTRODUCCIÓN

1.1. EL CULTIVO DE ARROZ EN URUGUAY

El arroz es el alimento base para más de la mitad de la población mundial (Long-ping, 2014). Uruguay es uno de los principales exportadores mundiales de arroz, por lo que es un cultivo económicamente importante. La exportación de arroz en el primer semestre de 2014 se situó en el tercer lugar, después de las de soja y de carne bovina congelada.

El área sembrada de arroz alcanzó un record histórico de 205.000 ha en la zafra 1998/99, y actualmente registra un promedio de 175.000 ha sembradas por año en las últimas 5 zafras (Asociación Cultivadores de Arroz), de un total cercano a las 1.200.000 ha de cultivos de verano en el país (Hoffman et al., 2013).

En Uruguay, el arroz se siembra entre setiembre y noviembre y se cosecha entre febrero y abril. Durante su ciclo de cultivo, las plantas permanecen bajo inundación artificial entre el 55 y el 70% del tiempo. El resto del tiempo, el arroz crece bajo agricultura de secano intercalando baños o riegos rápidos. Alrededor de veinte días antes de la fecha prevista de cosecha, se realiza un drenaje final.

Existen tres regiones geográficas de cultivo de arroz en Uruguay. Las regiones Este y Norte/Litoral Oeste constituyen el 92% de la superficie total dedicada a la producción de este cultivo. En la zona Este, el cultivo se realiza en suelos de zonas bajas de la cuenca de la Laguna Merín. En la zona Norte/Litoral Oeste (de aquí en adelante zona Norte) el arroz se cultiva también en zonas bajas, aunque en esta zona presenta laderas de mayor pendiente con respecto a la zona Este, lo que resulta en un mayor drenaje y menor tendencia a la anegación.

1.2. GASES DE EFECTO INVERNADERO PRODUCIDOS POR LA AGRICULTURA: EL PAPEL DEL N_2O

El metano (CH_4) y el óxido nitroso (N_2O) son los principales gases de efecto invernadero (GEI) producidos por el sector agropecuario. Estos dos gases son, además, los dos "GEI no-CO_2" más importantes según el protocolo de Kioto sobre el cambio climático (De Cara et al., 2005). Si bien la concentración atmosférica actual

del N_2O es alrededor de 5 veces menor que la del CH_4 (Tarasova et al., 2014), su potencial invernadero es casi 15 veces mayor, en una escala de tiempo de 100 años, al del metano y 298 veces mayor al del CO_2 (Pelster et al., 2012).

Actualmente se estima que el N_2O es responsable del 7% de la fuerza de radiación ocasionada por los GEI (Gallaher et al., 2006). El aumento en la aplicación de fertilizantes nitrogenados tiene como consecuencia un incremento en la concentración de N_2O atmosférico, lo que determina que el porcentaje de la fuerza de radiación generada por éste tienda a aumentar en el futuro. Se estima que las emisiones de N_2O crecerán entre un 35-60% para el 2030 (Liu et al., 2010).

En problema ambiental adicional que genera el N_2O, es que una vez en la estratosfera, se reduce a NO. El NO reacciona con el O_3 formando O_2, lo que disminuye la capa de ozono. A pesar de ello, el Protocolo de Montreal, cuyo cometido es restringir la emisión de sustancias reductoras de la capa de ozono, excluye al N_2O entre las sustancias cuya emisión debe ser controlada. Esto puede determinar que el N_2O se convierta en el principal responsable del adelgazamiento de la capa de ozono en el siglo XXI (Ravishankara et al., 2009). La prolongada vida media de este gas en la atmósfera, determina que los efectos de su emisión trascienden a las generaciones actuales. Según estimaciones, se necesitaría disminuir la emisión de N_2O en un 50% para estabilizar su concentración atmosférica (IPCC, 2006).

Se estima que el 99% del total de las emisiones de N_2O generadas en Uruguay son producidas por la actividad agropecuaria (Baethgen y Martino, 2000). Generalmente, la cantidad de N_2O emitida en arrozales inundados es menor que la de CH_4 (Irisarri et al., 2012). Sin embargo, dado que tiene un potencial invernadero mayor que el del CH_4, el aporte al efecto invernadero del N_2O producido en arrozales no es desestimable.

1.3. EMISIÓN DE N_2O EN SUELOS

La emisión de N_2O ocurre en condiciones óxicas o levemente anóxicas. Las condiciones óxicas favorecen la nitrificación, que es un proceso aerobio, cuando la

provisión de oxígeno es limitada, la nitrificación genera N_2O como sub-producto. La desnitrificación, por otra parte, se activa cuando el oxígeno ha sido consumido y se generan microambientes anóxicos dentro del perfil del suelo (Smith et al., 2003). Estos procesos, nitrificación y desnitrificación, son los dos mecanismos biológicos mediante los cuales se genera N_2O en el suelo.

1.3.1. Microorganismos nitrificantes

Dentro del dominio Bacteria, la nitrificación autótrofa es un proceso de dos pasos en el que se ven involucrados dos grupos bacterianos, las bacterias oxidadoras de amonio (AOB) y las bacterias oxidadoras de nitrito. El primer paso de la nitrificación, realizados por las AOB, consiste en la oxidación del NH_4^+ a NO_2^-. Esta reacción es catalizada secuencialmente por dos enzimas, en primer lugar el amonio se oxida a hirdroxilamina (NH_2OH) por la enzima amonio monooxigenasa (AMO) presente en las AOB. Posteriormente, la enzima hidroxilamina oxidorreductasa (HAO) cataliza la oxidación de NH_2OH a NO_2^-. Se ha propuesto que en esta última reacción el N_2O es generado como subproducto (Otte et al., 1999). Por otra parte, en la desnitrificación nitrificante, los nitrificantes reducen NO_2^- vía N_2O a N_2, un proceso que recientemente se ha considerado como importante contribuyente a la emisión de N_2O, principalmente cuando las condiciones no son óptimas para la desnitrificación (Kool et al., 2011).

Las AOB autotróficas más conocidas se encuentran dentro de las clases Betaproteobacteria y Gammaproteobacteria, e incluyen géneros tales como *Nitrosomonas* y *Nitrosospira* (Purkhold et al., 2000). Se ha observado que la abundancia y estructura de las poblaciones de AOB son sensibles a cambios en el uso del suelo, por ello pueden ser un buen indicador del impacto de las prácticas agrícolas sobre los microorganismos del suelo (Patra et al., 2006). Los suelos inundados cultivados con arroz son considerados uno de los principales hábitats de las AOB; la aplicación de fertilizantes basados en NH_4^+ y la provisión de O_2 que realiza la planta a través de sus raíces, generan condiciones óptimas para el desarrollo de las AOB (Nicolaisen et al., 2004).

No solo las AOB pueden oxidar el amonio, recientemente se ha descubierto que esta reacción también puede ser efectuada por microrganismos del domino Archaea. Las arqueas oxidadoras de amonio (AOA) son microorganismos pertenecientes a la porción no termófila del phylum Crenarchaeota (Leininger et al., 2006). El avance en las técnicas empleadas para el conocimiento de las comunidades microbianas, especialmente aquellas que son independientes del cultivo, ha permitido comprender mejor el papel de las AOA en la nitrificación. Desde su descubrimiento, las poblaciones de AOA de diferentes ambientes han sido objeto de estudios y su abundancia y actividad en relación a las AOB ha sido contrastada. Particularmente, en los suelos de arroz inundados se ha sugerido que éstas podrían tener una actividad nitrificante más importante que la de las bacterias (Chen et al., 2008).

1.3.2 Microorganismos desnitrificantes

Los microorganismos desnitrificantes, en su mayoría heterótrofos, son anaerobios facultativos capaces de usar óxidos de nitrógeno (NO_3^-, NO_2^- o N_2O) como aceptor final de electrones en ambientes donde el O_2 es escaso. Estos microorganismos constituyen un grupo muy diverso con representantes en varias ramas filogenéticas, aunque se encuentran con mayor frecuencia entre las clases Alphaproteobacteria y Betaproteobacteria, aunque también existen arqueas y hongos desnitrificantes. La diversidad metabólica de los desnitrificantes es tal que entre ellos se encuentran fotótrofos, litótrofos, organótrofos, fermentadores, anaerobios facultativos, aerobios, diazótrofos, psicrófilos, termófilos e incluso microoganismos patógenos (Zumft, 1997).

La ruta completa de desnitrificación comprende la reducción de NO_3^- en N_2 (figura 1.1). Esta reducción ocurre en etapas discretas con los siguientes intermediarios: NO_2^-, NO y N_2O. No todas las reacciones de reducción de nitrato son parte de la desnitrificación. Sin embargo, en el paso en que el NO_2^- es reducido a NO la ruta la reducción del NO_3^- queda comprometida exclusivamente al objetivo de conservación de la energía, consecuentemente las enzimas nitrito reductasas han sido consideradas como las enzimas distintivas de la desnitrificación (Braker et al., 2000).

Existen dos tipos de nitrito reductasas, una que contiene cobre y otra con citocromo cd1, codificadas por los genes *nirK* y *nirS*, respectivamente. Aunque existen excepciones descritas recientemente (Graf et al., 2014), ambas enzimas son excluyentes y no coexisten en un mismo microorganismo (Jones et al., 2008). De esta manera, los microorganismos desnitrificantes se pueden dividir en desnitrificantes tipo nirK y tipo nirS, según la modalidad de nitrito reductasa que presentan (Jones et al., 2014). Entre los desnitrificantes pertenecientes al dominio Archaea y al reino Fungi solamente ha sido encontrado *nirK*.

Los últimos pasos de la ruta de desnitrificación son realizados por las enzimas óxido nítrico reductasa y óxido nitroso reductasa. La primera cataliza la reducción de NO a N_2O, mientras que la segunda reduce el N_2O a N_2. La óxido nítrico reductasa es una enzima heterodimérica codificada por los genes *norB* y *norC* (Hino et al., 2010), mientras que la óxido nitroso reductasa es codificada por el gen *nosZ* (Orellana et al., 2014).

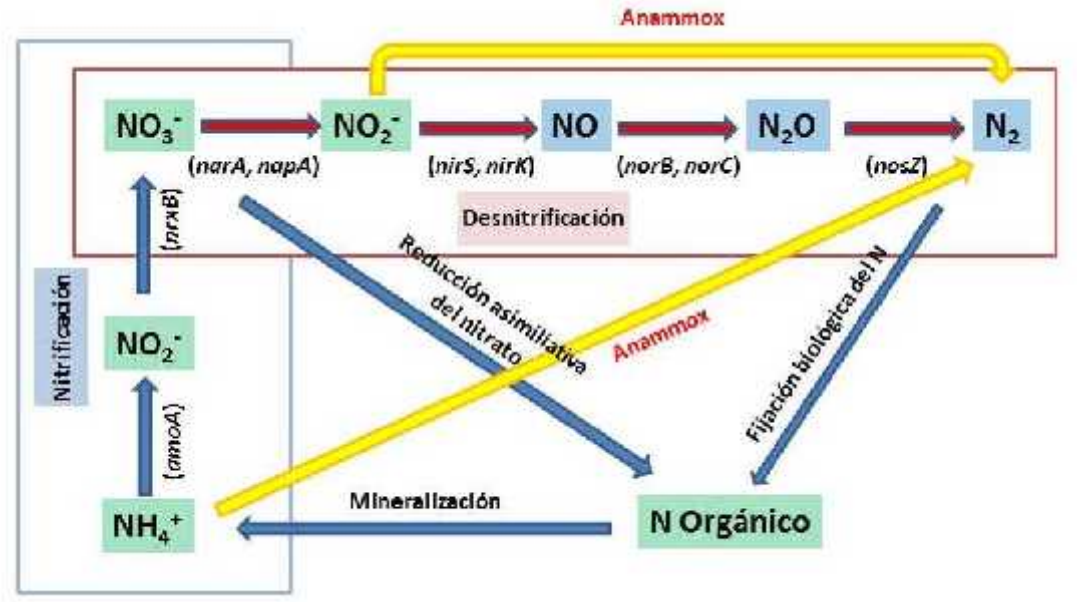

Figura 1.1. Esquema del ciclo biológico del nitrógeno. La desnitrificación está enmarcada en el cuadrante rojo, mientras que la nitrificación se enmarca en cuadrante celeste. Entre paréntesis se presentan los nombres de los genes que codifican las enzimas o sitios activos responsables en cada paso. Modificado de Azziz et al., 2016.

Según el conjunto de genes presente en un determinado microorganismo, los desnitrificantes pueden ser asignados a dos grandes grupos: aquellos con un gen funcional para cada paso de la vía de desnitrificación, capaces de realizar la desnitrificación completa y otros que carecen de alguno de los genes, pero que aún son capaces de utilizar óxidos de nitrógeno como aceptor de electrones. Entre estos últimos se encuentran microorganismos cuyo único gen de la ruta de desnitrificación es el gen *nosZ* (Sanford et al., 2012), u otros que carecen de este gen, pero contienen los genes que intervienen en pasos anteriores de la vía (Philippot et al., 2011).

La producción de N_2O como consecuencia de la desnitrificación se debe tanto a la expresión diferencial de los distintos genes involucrados, como al balance en las actividades desnitrificantes de microorganismos con diferentes conjuntos de genes desnitrificantes. Así, mientras los microorganismos que solamente tienen el gen *nosZ* son netamente consumidores de N_2O, los microorganismos desnitrificantes que carecen de él realizan desnitrificación incompleta y producen N_2O en lugar de N_2 como producto final.

Según estudios recientes, la contribución relativa de los procesos de nitrificación y desnitrificación en la emisión de N_2O depende de la concentración de O_2 del ambiente, lo cual, en el suelo, está ligado a la saturación del agua. Como regla general la generación de N_2O en suelos anegados es principalmente debida a la desnitrificación, mientras que la nitrificación tiene un papel más importante en la emisión en suelos con menos de 60% de saturación de agua (Bateman y Baggs, 2005).

1.4. EL CULTIVO DE ARROZ COMO FUENTE DE N_2O

El cultivo de arroz es considerado una fuente importante de emisiones de CH_4 y N_2O, por tanto, su contribución al cambio climático global es significativa (Johnson-Beebout et al., 2009).

Mientras el cultivo de arroz permanece inundado, solo el 0,1% del nitrógeno aplicado como fertilizante es emitido como N_2O (Mosier, 1998). Las emisiones de N_2O ocurren principalmente durante las etapas de secano (Xing et al., 2002), y su magnitud se relaciona más con los días en los que efectivamente el suelo permanece

seco (Towprayoon et al., 2005). Esta relación entre las emisiones de N_2O y el período de secano implica que diseñar adecuadamente el manejo del agua en los arrozales es clave para controlar estas emisiones (Zou et al., 2005).

Hasta la fecha, existen pocos artículos publicados acerca de la emisión de GEI en arrozales uruguayos. En el marco de un proyecto en el que se realizaron mediciones de GEI en la rotación arroz-pastura en el Este uruguayo, se compararon distintos tipos de cobertura invernal previa a la siembra de arroz en función de su efecto sobre las emisiones de CH_4 y N_2O. También se evaluó la incidencia de la fertilización nitrogenada y el momento de inundación. Los resultados corroboraron la predicción de que las emisiones de CH_4 y N_2O tienen un comportamiento opuesto durante el ciclo de cultivo. Mientras las emisiones de N_2O fueron máximas al comienzo de la inundación o en etapas de riego previas a ésta, las de CH_4 alcanzaron mayores concentraciones en las últimas etapas de suelo inundado. El agregado de nitrógeno al suelo no fue un determinante de la emisión de CH_4. En general, la emisión de N_2O se caracterizó por una dependencia aparente con el contenido de agua en suelo y la fertilización nitrogenada (Pereyra, 2009).

De los estudios enfocados a determinar la emisión de GEI en arrozales uruguayos, solamente una fracción de ellos analiza las poblaciones microbianas. Ferrando y Tarlera (2009) evaluaron por primera vez en Uruguay la diversidad de metanótrofos en arrozales inundados, sembrados con la variedad INIA Tacuarí. Los resultados revelaron una alta diversidad de las comunidades metanótrofas, consumidoras de CH_4, en los arrozales evaluados, tanto en la rizósfera de las plantas de arroz, como en la interfase suelo/agua.

También las bacterias desnitrificantes han sido objeto de estudio en otro caso nacional (Fernández Scavino et al., 2010). Los autores determinaron que la estructura de las poblaciones de microorganismos desnitrificantes varía significativamente entre la inundación y la cosecha y esta variación es dependiente de la presencia de arroz. En ese estudio sólo se consideró la fracción cultivable de los microorganismos desnitrificantes.

Recientemente, un estudio de campo realizado en el departamento de Treinta y Tres, con la variedad El Paso 144, ha concluido que el manejo del agua es un factor

determinante en la reducción de emisiones de GEI. Los autores encontraron que un sistema de manejo de agua alternativo al más utilizado actualmente ocasiona una disminución en la emisión de GEI, aunque puede provocar también disminuciones en el rendimiento del cultivo (Tarlera et al., 2016).

1.5. IMPACTO DE ALGUNAS PRÁCTICAS AGRÍCOLAS SOBRE LAS EMISIONES DE N_2O EN LOS ARROZALES Y SOBRE LOS MICROORGANISMOS RESPONSABLES

1.5.1 Manejo del agua

En Uruguay el arroz se siembra en suelo drenado y el cultivo se inunda unos 40 días después de la emergencia (Pittelkow et al., 2016), por lo que al inicio del ciclo del cultivo el suelo es predominantemente aerobio. Al establecerse la inundación el ambiente del suelo se vuelve más reductor. Esta dinámica tiene importantes efectos tanto en las poblaciones de los microorganismos del suelo como en la disponibilidad de nutrientes (Yang et al., 2005).

El manejo del agua representa uno de los principales factores a considerar en las estrategias de mitigación de emisiones de GEI. Sin embargo, una de las principales restricciones al empleo de diferentes manejos con ese fin es que la producción de los dos principales gases emitidos por los arrozales (CH_4 y N_2O) es promovida o restringida por condiciones ambientales opuestas (Johnson-Beebout et al., 2009).

La dinámica impuesta por el régimen hídrico controla, principalmente, el potencial redox (Eh) del suelo. La ausencia o escasez de oxígeno en el suelo que provoca la inundación, determina condiciones favorables para el desarrollo de microorganismos anaerobios estrictos o facultativos, los que utilizan como aceptor final de electrones alguna molécula oxidada distinta del O_2. Por tanto, los microorganismos desnitrificantes, en su mayoría anaerobios facultativos, que pueden utilizar óxidos de nitrógeno como aceptor final de electrones (Zumft, 1997), pueden ser favorecidos en estas condiciones.

Hay evidencias de que las distintas prácticas de manejo de la inundación pueden ocasionar diferencias en la cantidad de N_2O emitido a lo largo de un cultivo de arroz (Liu et al., 2010); los máximos valores de N_2O emitido ocurren durante las etapas de

drenaje de la inundación. La cantidad de N_2O que se emite se correlaciona con el tiempo en que el cultivo permanece sin inundación, independientemente de la cantidad de períodos o etapas en los que se drena (Towprayoon et al., 2005). Por el contrario, el CH_4 es emitido principalmente en los momentos en que el arroz permanece inundado (Said-Pullicino et al., 2015). Recientemente, se ha determinado que incluso una diferencia en el espesor de la capa de agua sobre el suelo ocasiona diferencias en las emisiones de GEIs, siendo éstas mayores y menores para CH_4 y N_2O, respectivamente, cuando la capa de agua es mayor (Ahn et al., 2014).

Una alternativa prometedora para mitigar las emisiones de N_2O ha surgido al evaluar conjuntamente el manejo de la inundación con la fertilización nitrogenada. Debido a que el fertilizante provee los compuestos nitrogenados que pueden servir como sustratos de los microorganismos desnitrificantes y nitrificantes, fertilizar en momentos en los que el suelo esté inundado y permanezca así durante unos días más disminuye la cantidad de N_2O emitido en comparación con la fertilización en suelo drenado (Peng et al., 2011).

Si bien existe abundante literatura respecto a la incidencia del manejo del agua sobre las emisiones de GEIs, los estudios que se centran en el efecto de este manejo sobre las poblaciones microbianas son relativamente más escasos. Sin embargo, la evidencia de la influencia de parámetros tales como la concentración de O_2 en suelo de arrozal sobre la comunidad total de microorganismos (Lüdemann et al., 2000), hace más plausible la hipótesis de que el manejo del agua afecta distintas poblaciones microbianas.

1.5.2. La planta de arroz

La planta de arroz (*Oryza sativa*) está especialmente adaptada a vivir en condiciones de inundación, por lo que se la considera una planta higrófita (Wassmann y Aulakh, 2000). Una de las principales adaptaciones a este modo de vida es el desarrollo del aerénquima, un tejido que permite el transporte de gases y cuya principal función es la de proveer O_2 a las raíces. Es también capaz de transportar otros gases, incluso en la dirección opuesta, desde la raíz hacia las hojas, tal como se ha observado que ocurre con los dos principales GEI vinculados con el arroz, el CH_4 y el N_2O. Se

estima que entre el 80-90% de CH_4 producido en un arrozal es emitido a través de este tejido (Setyanto et al., 2004).

Las plantas también proveen al suelo materia orgánica a través de la producción de exudados por la raíz. Este consiste un sustrato que promueve el crecimiento de muchos tipos de microorganismos y puede sustentar el crecimiento y actividad de microorganismos involucrados en la producción de CH_4 y N_2O. La cantidad y características de los exudados producidos dependen tanto de factores ambientales como de factores fisiológicos de la planta (Wassmann y Aulakh, 2000). Entre los principales factores fisiológicos que influyen en la producción de exudados se encuentran: estado fenológico, vigor y cultivar (Aulakh et al., 2001).

La mayoría de las investigaciones que han evaluado el papel de las variedades de arroz en la emisión de GEIs, se han enfocado en el análisis de las diferencias en la emisión de CH_4. Hay antecedentes que demuestran que existen diferencias entre distintos cultivares de arroz con respecto al potencial emisor de CH_4; estas diferencias se explican, según estos estudios, por diferencias en la biomasa de la raíz, el número de macollos, el número de hojas, y la superficie foliar (Baruah et al., 2010; Kerdchoechuen, 2005; Setyanto et al., 2004). Las variedades de alto rendimiento han resultado ser potencialmente menos emisoras de CH_4 que las tradicionales, como consecuencia principalmente de una menor biomasa radicular y de tallo (Baruah et al., 2010).

En cuanto a las emisiones de N_2O, existen antecedentes de diferencias cuantitativas entre las emisiones provocadas por cultivares híbridos y tradicionales (Ma et al., 2012). También se encontraron diferencias en las emisiones de N_2O de dos variedades de arroz cultivadas en un sistema de producción mixto que incluye piscicultura (Datta et al., 2009).

El efecto que las deposiciones radiculares ejercen sobre las características del suelo y sobre las poblaciones microbianas es conocido desde hace tiempo y se denomina «efecto rizósfera» (Bhuvaneswari y Subba-Rao, 1957). Si bien esto se ha estudiado ampliamente en varios tipos de plantas, los estudios cuyo objetivo fue comparar el efecto de distintos cultivares de arroz son escasos. La dependencia de las comunidades microbianas en los exudados producidos por la planta, junto a la

diferencia de exudados entre cultivares, sugiere que distintos cultivares pueden favorecer el desarrollo de poblaciones microbianas diferentes. Recientemente, se han comparado distintos cultivares de las dos subespecies existentes (indica y japónica) de arroz y se ha comprobado que la subespecie a la que pertenece el cultivar tiene efecto sobre la estructura global de la población bacteriana (Hardoim et al., 2011).

1.5.3. El tipo de suelo

El cultivo de arroz en Uruguay se realiza en tres grandes áreas, Norte, Centro y Este. Las zonas Norte y Este conjuntamente representan el 88% del área total de cultivo de arroz del país. La zona Norte incluye los departamentos de Artigas, Salto y Paysandú, mientras que la zona Este incluye los departamentos de Cerro Largo, Treinta y Tres, Rocha y Lavalleja (Pittelkow et al., 2016). Estas dos zonas presentan características edáficas y topográficas contrastantes entre sí.

En la zona Este la mayor parte de la producción se realiza sobre suelos de zonas bajas en la cuenca de la Laguna Merín. Estos se caracterizan por tener una gran diferenciación textural, con un horizonte subsuperficial pesado y un horizonte superficial de textura media o liviana. Por otra parte, en la zona Norte el terreno donde se siembra el arroz presenta frecuentemente laderas de mayor pendiente con respecto a la zona Este, los suelos predominantes son desarrollados sobre materiales derivados del basalto, con una textura pesada y menor diferenciación textural que los del Este. Asimismo, una diferencia sustancial entre los suelos de las dos zonas es el contenido de materia orgánica, el cual es mayor en los suelos de la zona Norte (Hernández y Berger, 2003). Debido a las diferencias topográficas, estas zonas tienen distintas tendencias al anegamiento y drenaje.

El tipo de suelo es uno de los factores más relevantes a considerar cuando se evalúan los parámetros que influencian las comunidades microbianas (Girvan et al., 2003). Se ha demostrado cierta relación entre parámetros del suelo tales como contenido de C y relación C/N con la abundancia de algunos grupos microbianos involucrados en la emisiones de CH_4 y N_2O presentes en distintos suelos de arrozales (Kravchenko y Yu, 2006). Si bien los tipos y fracciones de materia orgánica en el suelo juegan un papel importante en la actividad microbiana, la cantidad de esta también puede ser un

factor determinante. Por ejemplo, se ha encontrado que la cantidad de CH_4 producido aumenta en relación a la dosis de materia orgánica que se agrega al suelo (Nishiwaki et al., 2015).

La mayoría de los estudios que analizan el efecto de la materia orgánica en la emisión de GEIs están enfocados a buscar la relación entre el contenido de materia orgánica (ya sea contenido del suelo o agregado mediante fertilización) y la producción de CH_4; esta correlación ha sido verificada en suelos de arrozal de diversas partes del mundo (Yao et al., 1999). Esto tiene sentido bajo el concepto de que el CH_4 es el producto final de la degradación anaerobia de la materia orgánica; sin embargo, el vínculo entre la cantidad de materia orgánica y emisiones de N_2O ha sido menos explorado, a pesar de que los microorganismos desnitrificantes son heterótrofos (Zumft, 1997) y algunas arqueas oxidadoras de amonio (AOA) son mixótrofas (Daebeler et al., 2014), y por tanto su actividad depende de la disponibilidad de C orgánico. Existen antecedentes que muestran que el tipo de fertilizante orgánico utilizado tiene efecto sobre la cantidad de N_2O emitido (Zou et al., 2005). Las fuentes de C más disponibles pueden generar un consumo acentuado del O_2 como producto de la acción de los microorganismos que la descomponen, generando un ambiente más anóxico, lo que favorece la reducción de N_2O a N_2.

1.6. ESTRATEGIAS DE MITIGACIÓN DE LAS EMISIONES DE GEI EN ARROZALES

Las condiciones ambientales que favorecen la producción de los dos principales GEIs producidos por los arrozales determinan que encontrar estrategias que minimicen la emisión de ambos sea un objetivo difícil de alcanzar, o si acaso imposible (Johnson-Beebout et al., 2009). Minimizar la emisión de uno puede ocasionar un incremento en la producción del otro. Desde el punto de vista de la sustentabilidad de las estrategias de mitigación, estas no deberían comprometer el rendimiento del cultivo. Al considerar la fertilización nitrogenada como precursor de las emisiones de N_2O, se hace necesario optimizar su aplicación con el objetivo de mitigar las emisiones sin disminuir el aporte nutricional a la planta. El tipo de fertilizante a emplear, la técnica de aplicación, el laboreo del suelo y la rotación con

diferentes cultivos de invierno son parámetros cuyas variables se pueden considerar para mitigar las emisiones (Snyder et al., 2014).

El manejo de las condiciones de inundación del cultivo tiene incidencia sobre el Eh del suelo. Cuando el Eh se mantiene entre -100mV y +200mV la producción de CH_4 se ve impedida por condiciones oxidantes, mientras que se favorece que la desnitrificación prosiga hasta N_2 (Hou et al., 2000). De esta manera, la emisión de CH_4 puede reducirse por manejos de agua que disminuyan el período total de inundación, tales como el retiro de agua en las etapas tempranas del cultivo o un drenaje intermitente. Según un modelo predictivo de la emisión de gases, el cambio de un sistema de inundación continua a un sistema que incluye un drenaje en el medio del ciclo de cultivo disminuiría las sensiblemente las emisiones de CH_4, aunque tendría un efecto contrario, aunque de menor dimensión, sobre las emisiones de N_2O (Li et al., 2005).

Se ha sugerido que un drenaje de pocos días durante la etapa de floración podría disminuir las emisiones de CH_4 sin aumentar demasiado las emisiones de N_2O, y sin afectar significativamente el rendimiento del cultivo (Towprayoon et al., 2005).

Una selección apropiada de cultivares representa una alternativa de fácil implementación para mitigar la emisión de GEIs. Distintos cultivares pueden presentar diferencias en los exudados producidos y en la estructura de su aerénquima, dos factores que determinan la potencialidad de emisión. Sin embargo, el estado actual del conocimiento de los cultivares no permite categorizarlos en cultivares de alto y bajo potencial emisor (Wassmann et al., 2000). Por tanto, para seleccionar cultivares es necesario evaluar las emisiones producidas por ellos bajo las condiciones particulares en las que se los utilizará.

1.7 OBJETIVOS E HIPÓTESIS

El objetivo fue evaluar la relación entre prácticas agrícolas utilizadas y la producción de arroz sobre poblaciones microbianas involucradas en la producción de N_2O.

Los grupos microbianos analizados fueron los siguientes:

1. Arqueas oxidadoras de amonio (AOA)

2. Bacterias oxidadoras de amonio (AOB)
3. Bacterias desnitrificantes tipo nirK
4. Bacterias desnitrificantes tipo nirS

De cada grupo microbiano analizados se determinó su abundancia y su estructura poblacional. Para tal fin se emplearon las técnicas q-PCR y T-RFLP, respectivamente.

Las prácticas agrícolas evaluadas fueron las siguientes:

1. Cultivar de arroz empleado: se evaluaron los cultivares INIA Tacuarí y El Paso 144.
2. Manejo del agua: retiro o mantenimiento de la inundación en la etapa de primordio floral, en adelante llamados tratamientos «drenado» o «no drenado».
3. Tipo de suelo: se evaluaron dos suelos, uno representativo de la región Norte y otro de la región Este.
4. Momento de la inundación: se evaluaron dos momentos, «inundación temprana» donde la inundación se instaló al macollaje, e «inundación tardía» donde la inundación se instaló en el estadio de primordio floral.

Los objetivos específicos fueron determinar la incidencia de cada una de las prácticas agrícolas antes enumeradas sobre la abundancia y estructura poblacional de cada uno de los grupos microbianos analizados.

La hipótesis del trabajo fue que las prácticas agrícolas tienen efecto sobre los parámetros biológicos de las poblaciones microbianas responsables de la producción de N_2O.

1.8 ESQUEMA GENERAL DE LA TESIS

Los capítulos de la tesis están conformados de la siguiente manera:

- En el **capítulo 2** se detalla la comprobación de especificidad de los cebadores que serían utilizados en las técnicas de q-PCR y T-RFLP. Además incluye el análisis de las secuencias obtenidas de la amplificación con dichos cebadores

y el análisis de las digestiones *in silico* cuyo objetivo fue seleccionar las enzimas de restricción que se usarían en los T-RFLP.

- El **capítulo 3** comprende a la publicación que trata sobre los efectos de prácticas de manejo en las poblaciones de arqueas y bacterias oxidadoras de amonio. Azziz, G., Trasante, T., Monza, J., e Irisarri, P. (2016). The effect of soil type, rice cultivar and water management on ammonia-oxidizing archaea and bacteria populations. *Applied Soil Ecology*, 100, 8-17.
- El **capítulo 4** comprende la publicación sobre las los efectos de prácticas de manejo sobre las poblaciones de bacterias desnitrificantes. Azziz, G., Monza, J., Etchebehere, C., e Irisarri, P. (2017). nirS and nirK type denitrifier communities are differentially affected by soil type, rice cultivar and water management. *European Journal of Soil Biology*, 78, 20-28.
- El **capítulo 5** corresponde al capítulo de libro «Heterotrophic denitrification and *Paracoccus* spp. as tools and agents for bioremediation purposes» el cual se redactó en el marco de esta tesis y se publicará en el libro «Microbes for Sustainability - Microbial Models: from environmental to industrial sustainability» editado por Susana Castro-Sowinski

2. CAPÍTULO 2: ELECCIÓN DE CEBADORES A SER UTILIZADOS EN EL ESTUDIO DE MICROORGANISMOS OXIDADORES DE AMONIO Y DESNITRIFICANTES

2.1. ANTECEDENTES

Clásicamente, la microbiología se ha basado en métodos de estudio que incluyen el cultivo de los microorganismos de interés. Sin embargo, desde el advenimiento de técnicas independientes de cultivo, ha quedado de manifiesto que solamente una pequeña proporción de los microorganismos presentes en el ambiente es apto de ser cultivado (Pham y Kim, 2012). Por ello, para el estudio de poblaciones microbianas, el uso de técnicas independientes de cultivo se hace cada vez más necesario. Las técnicas independientes de cultivo permiten tener acceso a una porción de la población microbiana previamente inaccesible, la cual puede representar una porción mayoritaria de la población, sobre todo en ambientes complejos como lo es el suelo (Hirsch et al., 2010).

En las técnicas basadas en PCR (*Polymerase Chain Reaction*) una secuencia de ADN presente en las bacterias se puede amplificar y analizar de distintas maneras para conocer aspectos de las comunidades microbianas tales como la abundancia y la diversidad. Cuando el objetivo es la población bacteriana total de una muestra, el gen usado como blanco es un gen universal como el ARNr 16S, que se encuentra presente en todas la bacterias (Case et al., 2007). Por otro lado, cuando la población objetivo es un gremio o grupo funcional microbiano, se busca un gen «marcador» el cual debe indicar la pertenencia del microorganismo al gremio.

En el caso de los microorganismos oxidadores de amonio, tanto arqueas como bacterias, el gen *amoA* que codifica para la subunidad A de la enzima amonio monooxigenasa (AMO), ha sido empleado como marcador de estas poblaciones (Rotthauwe et al., 1997). Debido a las diferencias entre los genes *amoA* bacterianos y arqueanos, los cebadores utilizados para amplificar los genes *amoA* de los microorganismos ambos reinos son diferentes (Francis et al., 2005). De tal modo, es posible conocer parámetros de estos dos grupos microbianos por separado.

Las poblaciones de microorganismos desnitrificantes, por su parte, han sido estudiadas mediante el uso de los genes involucrados en la cadena respiratoria desnitrificante como marcadores. Los genes *nir* y el gen *nosZ* son los más utilizados (Throbäck et al., 2004).

Si bien se han desarrollado cebadores específicos para el estudio de microorganimos oxidadores de amonio y desnitrificantes, también se ha demostrado que es difícil contar con un juego de cebadores capaz de amplificar exitosamente el gen blanco desde distintos suelos (Throbäck et al., 2004). Tanto las características del suelo como las de las poblaciones microbianas presentes en él pueden determinar que la amplificación no sea posible en algunas situaciones, o que ésta se dé de manera inespecífica, es decir que se amplifiquen genes no deseados. De esta manera, un requisito previo a utilizar estos cebadores para nuestro objetivo fue comprobar su eficiencia y especificidad en amplificar los genes presentes en las poblaciones con las que íbamos a trabajar.

El objetivo de esta actividad fue verificar la especificidad de los cebadores presentados en el cuadro 2.1 en el ADN extraído de nuestras muestras de suelo. La estrategia consistió en construir genotecas de los amplicones de cada uno de los genes y obtener las secuencias amplificadas.

2.2. MATERIALES Y MÉTODOS

2.2.1. Muestras de suelo

Las muestras se colectaron tal como se detalla en los capítulos 3 y 4 se tomaron en el momento de macollaje del tratamiento sembrado con la variedad INIA Tacuarí (*Oryza sativa* spp. *japonica*).

2.2.2. Extracción de ADN del suelo

Para extraer ADN de los suelos se utilizó el kit «Power Soil ® DNA Isolation Kit» de la empresa MoBio (San Diego, California). Se partió de 0,25 g de suelo fresco y se siguieron las instrucciones del fabricante para extracción con centrífuga. La cantidad e integridad del ADN obtenido fue chequeado mediante electroforesis en

gel de agarosa 0,8% (p/v) y cuantificado espectrofotométricamente mediante NanoDrop ® 2000c.

Cuadro 2.1.- Cebadores utilizados en esta tesis para amplificar los genes blanco de poblaciones microbianas oxidadoras de amonio y desnitrificantes.

gen blanco	nombre	Secuencia	sentido	referencia
amoA **bacteriano**	amoA-1F	5'-GGG GTT TCT ACT GGT GGT-3'	directo	(Rotthauwe et al., 1997)
amoA **bacteriano**	amoA-2Rs[1]	5'-CCT CKG SAA AGC CTT CTT C-3'	reverso	(Rotthauwe et al., 1997)
amoA **arqueano**	Arch-amoAF	5'-STA ATG GTC TGG CTT AGA CG-3'	directo	(Francis et al., 2005)
amoA **arqueano**	Arch-amoAR	5'-GCG GCC ATC CAT CTG TAT GT-3'	reverso	(Francis et al., 2005)
nirS	Cd3aF	5'-GTS AAC GTS AAG GAR ACS GG-3'	directo	(Throbäck et al., 2004)
nirS	R3Cd	5'-GAS TTC GGR TGS GTC TTG A-3'	reverso	(Throbäck et al., 2004)
nirK	nirK1F	5'-GGM ATG GTK CCS TGG CA-3'	directo	(Braker et al., 1998)
nirK	1040R	5'-GCC TCG ATC AGR TTR TGG TT-3'	reverso	(Hamonts et al., 2013)

[1] Este cebador incluye una modificación al original publicado por Rotthauwe et al. (1997). La modificación consiste en la remoción, de dos bases (CC) en el inicio del cebador para evitar la formación de dímeros de cebadores y mejorar la amplificación.

2.2.3. Amplificación de ADN

2.2.3.1 Gen *amoA* arqueano

La mezcla de reacción del PCR del gen *amoA* arqueano contuvo 25 µl de 10× Taq buffer, 1,5 mM de $MgCl_2$, 0,2 mM de dNTPs, 0,5 µM de cada cebador específico, DMSO 2,5% (v/v), BSA 0,004% (p/v), 1 U de Taq DNA polymerase (recombinante,

Thermo Scientific) y 2 µl de molde (3-7 ng de ADN). El volumen total de la reacción fue de 50 µl.

El ciclado térmico consistió en un paso de desnaturalización inicial de 3 min a 95ºC, seguido por 30 ciclos de: 45 s a 95ºC, 45 s a 55ºC, y 45 s a 72ºC, y un paso de extensión final de 10 min a 72ºC. Los productos de PCR fueron chequeados mediante electroforesis en gel de agarosa 1,2% (p/v). Las condiciones de amplificación tales como concentraciones de reactivos y parámetros del ciclado fueron optimizadas para obtener productos con bandas de un solo tamaño. Los productos de PCR obtenidos fueron purificados con el kit AxyPrep PCR Clean-up Kit (Axygen Biosciences) siguiendo las instrucciones del fabricante.

2.2.3.2 Gen *amoA* bacteriano

La mezcla de reacción del PCR del gen *amoA* bacteriano contuvo 25 µl de 10× DreamTaq buffer, 0,2 mM de dNTPs, 0,3 µM de cada cebador específico, DMSO 2,5% (v/v), BSA 0,004% (p/v), 1 U de DreamTaq DNA polymerase (recombinante, Thermo Scientific) y 2 µl de molde (3-7 ng de ADN). El volumen total de la reacción fue de 50 µl. El buffer de esta enzima contiene 20 mM de $MgCl_2$.

El ciclado térmico consistió en un paso de desnaturalización inicial de 5 min a 95ºC, seguido por 40 ciclos de: 30 s a 95ºC, 30 s a 58ºC, y 45 s a 72ºC, y un paso de extensión final de 10 min a 72ºC. Los productos de PCR fueron chequeados mediante electroforesis en gel de agarosa 1,2% (p/v). Las condiciones de amplificación tales como concentraciones de reactivos y parámetros del ciclado fueron optimizadas para obtener productos con bandas de un solo tamaño. En el caso de este gen, no fue posible obtener una banda única del tamaño esperado (ca. 490 pb). Por ello, el amplicón de tamaño esperado fue purificado desde gel de agarosa con el kit GeneJET PCR Purification Kit (Thermo Scientific) siguiendo las instrucciones del fabricante.

2.2.3.3 Gen *nirS*

La mezcla de reacción del PCR del gen *nirS* contuvo 25 µl de 10× Taq buffer, 3 mM de $MgCl_2$, 0,2 mM de dNTPs, 1,0 µM de cada cebador específico, DMSO 2,5 %

(v/v), BSA 0,004 % (p/v), 1 U de Taq DNA polymerase (recombinante, Thermo Scientific) y 2 µl de molde (3-7 ng de ADN). El volumen total de la reacción fue de 50 µl.

El ciclado térmico consistió en un paso de desnaturalización inicial de 5 min a 95ºC, seguido por 35 ciclos de: 45 s a 95ºC, 45 s a 58ºC, y 45 s a 72ºC, y un paso de extensión final de 10 min a 72ºC. Los productos de PCR fueron chequeados mediante electroforesis en gel de agarosa 1,2 % (p/v). Las condiciones de amplificación tales como concentraciones de reactivos y parámetros del ciclado fueron optimizadas para obtener productos con bandas de un solo tamaño. Los productos de PCR obtenidos fueron purificados con el kit AxyPrep PCR Clean-up Kit (Axygen Biosciences) siguiendo las instrucciones del fabricante.

2.2.3.4 Gen *nirK*

Para la amplificación del gen *nirK*, la mezcla de reacción consistió en 25 µl de 10× Taq buffer, 3mM de $MgCl_2$, 0,2 mM de dNTPs, 0,4 µM de cada cebador específico, DMSO 2,5 % (v/v), BSA 0.004 % (p/v), 1 U de Taq DNA polymerase (recombinante, Thermo Scientific) y 2 µl de molde (3-7 ng de ADN). El volumen total de la reacción fue de 50 µl.

El ciclado térmico consistió en un paso de desnaturalización inicial de 5 min a 95ºC, seguido por 40 ciclos de: 45 s a 95ºC, 45 s a 60ºC, y 45 s a 72ºC, y un paso de extensión final de 10 min a 72ºC. Los productos de PCR fueron chequeados mediante electroforesis en gel de agarosa 1,2 % (p/v). Las condiciones de amplificación tales como concentraciones de reactivos y parámetros del ciclado fueron optimizadas para obtener productos con bandas de un solo tamaño. Los productos de PCR obtenidos fueron purificados con el kit AxyPrep PCR Clean-up Kit (Axygen Biosciences) siguiendo las instrucciones del fabricante.

Luego de la purificación, todos los productos de PCR se cuantificaron con un espectrofotómetro NanoDrop 2000 (ThermoScientific).

2.2.4 Construcción de las genotecas y clonado

Los productos de PCR purificados fueron ligados al vector «pJET1.2 blunt» siguiendo las instrucciones del kit utilizado (CloneJET PCR Cloning Kit, ThermoFisher Scientific). Alrededor de 25 ng de los productos de PCR se incubaron durante 5 min a 70°C con la enzima «DNA blunting enzime»; luego se agregó 1 µl del vector (50 ng) y 5 U de ADN ligasa T4, esta mezcla se incubó a temperatura ambiente durante 10 minutos.

La mezcla de ligación se purificó mediante el uso del kit AxyPrep PCR Clean-up Kit (Axygen Biosciences) siguiendo las instrucciones del fabricante.

La transformación de células se hizo usando 1 µl de cada una de las mezclas de ligación purificadas. Células de *Escherichia coli* TOP10 se transformaron mediante electroporación (1.8 kV, 25 µF, 2,5 ms) y se sembraron en placas con medio LB amp^{50} (suplementado con ampicilina (50µg/ml), se incubaron durante 24 h a 37°C. De cada transformación se repicaron 30 colonias a tubos de ensayo con 5 ml de medio LB amp^{50}. Durante este repique se ultilizó el ansa para inocular 50 µl de H_2O ultrapura estéril en un microtubo de 2 µl con cada una de las colonias. Los tubos se incubaron durante 24 h a 37°C, al cabo de esta incubación se transfirieron 750 µl de cultivo a crioviales conteniendo 750 µl de glicerol 50 % (v/v). La genoteca generada se conservó a -20 °C.

Para la amplificación de los insertos de las distintas células transformadas se utilizó como molde la inoculación de la célula en H_2O ultrapura descrita anteriormente. Se utilizaron los cebadores pJET1.2:

- forward (5'-CGACTCACTATAGGGAGAGCGGC-3')
- reverse (5'-AAGAACATCGATTTTCCATGGCAG-3').

La mezcla de reacción consistió en 12,5 µl de 10× Taq buffer, 1,5mM de $MgCl_2$, 0,2 mM de dNTPs, 0,2 µM de cada cebador específico, DMSO 2.5% (v/v), BSA 0,004% (p/v), 1 U de Taq DNA polymerase (recombinante, Thermo Scientific) y 4 µl de molde. El volumen total de la reacción fue de 25 µl. Por cada célula transformante la reacción se realizó por duplicado. El ciclado térmico consistió en un paso de desnaturalización inicial de 3 min a 95ºC, seguido por 25 ciclos de: 30 s a 94ºC, 30 s a 60ºC, y 30 s a 72ºC, y un paso de extensión final de 10 min a 72ºC. Los productos

de PCR fueron chequeados mediante electroforesis en gel de agarosa 1,2 % (p/v). Las bacterias transformantes cuyos productos de PCR no resultaron del tamaño esperado fueron descartadas. Los productos de PCR cuyo tamaño coincidió con el esperado (118 pb del vector más el tamaño del gen en cuestión) fueron purificados mediante el uso del kit AxyPrep PCR Clean-up Kit (Axygen Biosciences) y enviados a la empresa Macrogen (Seúl, Corea del Sur) para su secuenciación.

2.2.5 Análisis de secuencias

Las secuencias obtenidas fueron visualizadas utilizando el software gratuito Chromas 2.6 (Technelisyum Pty Ltd), disponible en http://technelysium.com.au/wp/chromas/. Las secuencias fueron depuradas cortando los extremos, se eliminaron los tramos extremos que contenían bases con valor de calidad menor a 20. Las secuencias de cada gen se alinearon usando el software gratuito MEGA 7 mediante el algoritmo MUSCLE. El software se encuentra disponible en http://www.megasoftware.net/. Una vez alineadas, los tamaños de las secuencias se uniformaron cortando los extremos de manera que todas las bases de las secuencias se alinearon en su totalidad.

Posteriormente se construyeron árboles filogenéticos con las secuencias obtenidas de cada gen usando el software MEGA 7, y mediante el algoritmo «neighbour joining».

Las secuencias se depositaron en el banco de datos GeneBank del NCBI (National Center for Biotechnology Information).

2.2.5.1 Restricción *in silico*

Las secuencias obtenidas se sometieron a una digestión virtual o *in silico* mediante la cual se obtuvieron todos los sitios de corte de las enzimas de restricción comercialmente disponibles. Esto se realizó con la herramienta web NEBcutter V2.0, disponible en http://nc2.neb.com/NEBcutter2/. De cada enzima de interés se registró el sitio de corte proximal al extremo 5’.

2.3 RESULTADOS Y DISCUSIÓN

2.3.1 Gen *amoA* arqueano

Los productos de PCR obtenidos con los cebadores empleados y en las condiciones de ciclado utilizadas fueron de un tamaño cercano al esperado (635 pb) en todos los insertos evaluados (30). Se seleccionaron 21 de ellos para su secuenciación.

Las secuencias obtenidas y depuradas se encuentran en el Apéndice 1. Las secuencias fueron depositadas en el GeneBank del NCBI, los números de acceso, el tamaño de cada secuencia, así como el tamaño de los fragmentos de restricción teóricos se muestra en el cuadro 2.2. Todas las secuencias analizadas tuvieron un máximo de similitud con secuencias del gen *amoA* de arqueas. El promedio de similitud fue de 81%, mientras que la mínima similitud obtenida fue 73% y la máxima 86%.

Las secuencias se afiliaron con mayor similitud a tres géneros arqueanos. Once secuencias presentaron alta similitud con el género *Nitrososphaera*, 9 de ellas al género *Nitrosoarcheaum* y una de ellas al género *Nitrosopumilus*. El género *Nitrosophaera* pertenece a la clase Nitrososphaeria, mientras que los géneros *Nitrosoarcheaum* y *Nitrosopumilus* pertenecen a la clase Nitrosopumilales, por tanto se obtuvieron 11 y 10 secuencias de genes *amoA* emparentadas con representantes de cada una de estas clases, respectivamente.

Los resultados obtenidos al comparar nuestras secuencias con las presentes en la base de datos de genomas de referencia del NCBI permiten confirmar la especificidad de los cebadores utilizados. Asimismo, las digestiones *in silico* realizadas con las enzimas *AluI* y *HaeIII* resultaron en 5 fragmentos terminales, para ambas enzimas, del total de 21 secuencias analizadas. Por otra parte, las digestiones realizadas con la enzima *MboI* resultaron en 8 fragmentos terminales diferentes.

Cuadro 2.2- Resultados de la comparación de las secuencias obtenidas para el gen *amoA* arqueano con la base de datos del NCBI. Se detallan los números de acceso del GeneBank y el tamaño de la secuencia. Se muestra el nombre del organismo contra cuyo gen *amoA* se obtuvo el máximo de similitud en el BLAST. Se muestran los resultados de la digestión *in silico* de los amplicones según los sitios de corte de las enzimas *HaeIII*, *AluI* y *MboI*.

Gen	Clon	GeneBank Acc.	Tamaño (pb)	organismo con máxima similitud	% de similitud	tamaño del fragmento 5' proximal		
						HaeIII	AluI	MboI
amoA arqueano	H1	KX079956	635	*Nitrososphaera gargensis*	80%	398	635	332
amoA arqueano	H2	KX079957	635	*Nitrsoarcheaum limnia*	80%	631	287	373
amoA arqueano	H3	KX079958	635	*N. gargensis*	86%	398	586	139
amoA arqueano	H4	KX079959	635	*N. gargensis*	86%	398	586	139
amoA arqueano	H5	KX079960	635	*N. limnia*	80%	631	287	373
amoA arqueano	H6	KX079961	635	*N. gargensis*	79%	631	635	424
amoA arqueano	H7	KX079966	635	*N. limnia*	80%	631	287	373
amoA arqueano	H8	KX079962	635	*N. limnia*	80%	631	287	373
amoA arqueano	H9	KX079963	635	*N. limnia*	80%	631	287	373
amoA arqueano	H10	KX079964	635	*N. gargensis*	85%	631	208	310
amoA arqueano	H11	KX079965	632	*N. gargensis*	79%	628	635	424
amoA arqueano	H14	KX079967	635	*N. limnia*	80%	631	287	373
amoA arqueano	H15	KX079968	635	*N. limnia*	80%	631	287	373
amoA arqueano	H16	KX079969	635	*N. gargensis*	79%	208	169	635
amoA arqueano	H17	KX079970	635	*N. limnia*	80%	631	287	373
amoA arqueano	H18	KX079971	635	*N. gargensis*	86%	398	586	139
amoA arqueano	H19	KX079972	635	*Nitrosopumilus maritimus*	73%	631	208	421
amoA arqueano	H20	KX079973	635	*N. limnia*	76%	477	586	394
amoA arqueano	H22	KX079974	635	*N. gargensis*	79%	398	635	332
amoA arqueano	H25	KX079975	635	*N. gargensis*	85%	631	208	310
amoA arqueano	H26	KX079976	635	*N. gargensis*	78%	631	635	424

El árbol filogenético construido con las secuencias de los amplicones de *amoA* arqueano (figura 2.1) muestra que la información derivada de los sitios de corte generados *in silico* con la enzima *MboI* es coherente filogenéticamente.

A partir de estos resultados se puede concluir que los cebadores y las condiciones de ciclado usados, permiten la amplificación de genes *amoA* de origen arqueano con una especificidad muy alta. Además, teniendo en cuenta la información generada con las digestiones *in silico*, se seleccionó a la enzima *MboI* para generar los perfiles de T-RFLP de este gen.

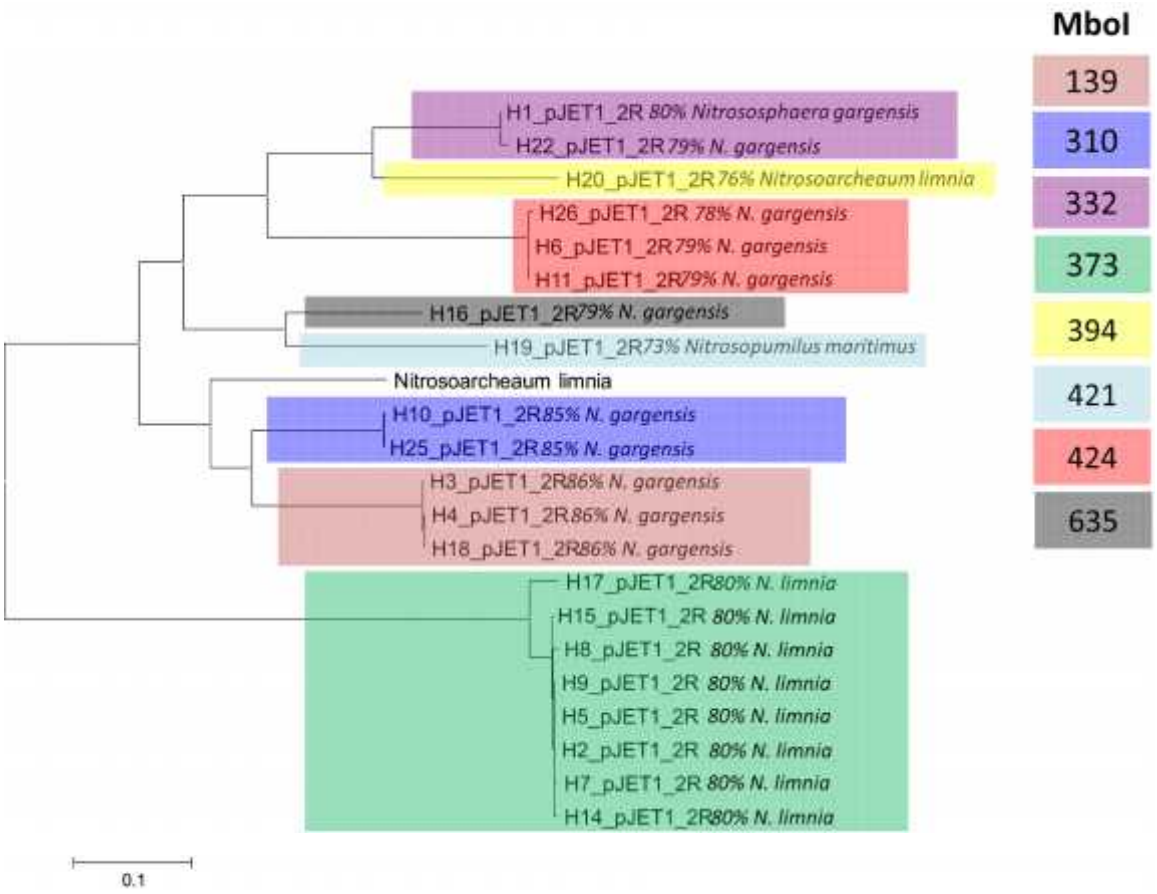

Figura 2.1.- Árbol filogenético construido con las secuencias de *amoA* arqueano mediante el algoritmo neighbour joining. Los cuadros de colores muestran los distintos sitios 5' proximales de corte teóricos que resultan de la digestión *in silico* con la enzima *MboI*.

2.3.2 Gen *amoA* bacteriano

Los productos de PCR obtenidos con los cebadores empleados y en las condiciones de ciclado utilizadas mostraron una fuerte banda de un tamaño cercano al esperado (490 pb) en todos los insertos evaluados. Sin embargo, también se obtuvieron bandas correspondientes a productos de PCR de pocos pares de bases (ca. 50) que pueden haber sido producidas debido a la formación de dímeros de cebadores. Por tanto, el ADN presente en la banda del tamaño esperado para el gen *amoA* en los 20 productos de PCR se purificó y secuenció.

Las secuencias obtenidas y depuradas se encuentran en el Apéndice 2. Las secuencias fueron depositadas en el GeneBank del NCBI, los números de acceso, el tamaño de cada secuencia, así como el tamaño de los fragmentos de restricción

teóricos se muestra en el cuadro 2.3. Todas las secuencias analizadas tuvieron un máximo de similitud con secuencias del gen *amoA* de bacterias. El promedio de similitud fue de 80 %, la mínima similitud fue 78 % y la máxima 91 %.

Las 20 secuencias se afiliaron con mayor similitud a dos de los cinco géneros de bacterias oxidadoras de amonio conocidas, *Nitrosomonas* y *Nitrosospira*. La mayoría (17) de las secuencias presentaron alta similitud con el género *Nitrosomonas*, mientras que 3 de ellas al género *Nitrosospira*. Ambos géneros pertenecen a la clase Betaproteobacteria, al igual que los géneros *Nitrosolobus* y *Nitrosovibrio*. Ninguna de las secuencias obtenidas presentó máxima similitud con el gen *amoA* de una bacteria del género *Nitrosococcus* el cual es el único género de bacterias oxidadoras de amonio que está incluido en la clase Gammaproteobacteria.

Cuadro 2.3.- Resultados de la comparación de las secuencias obtenidas para el gen *amoA* bacteriano con la base de datos del NCBI. Se detallan los números de acceso del GeneBank y el tamaño de la secuencia. Se muestra el nombre del organismo contra cuyo gen *amoA* se obtuvo el máximo de similitud en el BLAST. Se muestran los resultados de la digestión *in silico* de los amplicones según los sitios de corte de las enzimas *HaeIII*, *AluI* y *MboI*.

Gen	Clon	GeneBank Acc.	Tamaño (pb)	organismo con máxima similitud	% de similitud	tamaño del fragmento 5' proximal		
						HaeIII	AluI	MboI
amoA bacteriano	amoA2	KX079936	491	*Nitrosomonas eutropha*	78%	167	225	30
amoA bacteriano	amoA3	KX079937	491	*N. eutropha*	78%	167	225	30
amoA bacteriano	amoA5	KX079938	491	*N. eutropha*	78%	167	225	30
amoA bacteriano	amoA6	KX079939	491	*N. eutropha*	78%	167	225	30
amoA bacteriano	amoA7	KX079940	491	*N. eutropha*	78%	167	225	30
amoA bacteriano	amoA8	KX079941	491	*N. eutropha*	78%	167	225	30
amoA bacteriano	amoA9	KX079942	491	*N. eutropha*	78%	167	225	30
amoA bacteriano	amoA11	KX079943	491	*N. eutropha*	78%	167	225	30
amoA bacteriano	amoA13	KX079944	491	*N. eutropha*	78%	167	225	30
amoA bacteriano	amoA14	KX079945	491	*N. eutropha*	78%	167	225	30
amoA bacteriano	amoA15	KX079946	491	*N. eutropha*	78%	167	225	30
amoA bacteriano	amoA16	KX079947	491	*Nitrosospira multiformis*	87%	142	201	111
amoA bacteriano	amoA17	KX079948	491	*N. multiformis*	91%	142	201	111
amoA bacteriano	amoA18	KX079949	491	*N. eutropha*	78%	167	225	30
amoA bacteriano	amoA19	KX079950	491	*N. eutropha*	78%	167	225	30
amoA bacteriano	amoA20	KX079951	491	*N. eutropha*	78%	167	225	30
amoA bacteriano	amoA21	KX079952	491	*N. eutropha*	78%	167	225	30
amoA bacteriano	amoA23	KX079953	490	*N. multiformis*	87%	167	201	111
amoA bacteriano	amoA24	KX079954	491	*N. eutropha*	78%	167	225	30
amoA bacteriano	amoA25	KX079955	491	*N. eutropha*	78%	167	225	30

El árbol filogenético (figura 2.2) construido con las secuencias de los amplicones de *amoA* bacteriano, y los respectivos sitios de corte generados *in silico* con las enzimas

AluI y *HaeIII*, muestra la información derivada de los cortes de cualquiera de estas enzimas guarda un preciso grado de coherencia con la similitud de las secuencias y con la asignación filogenética de cada una de ellas. La enzima *MboI* fue descartada como opción para generar los fragmentos debido a que la mayoría de los fragmentos terminales tendría un tamaño muy pequeño para su apreciación en los perfiles de T-RFLP (30 pb).

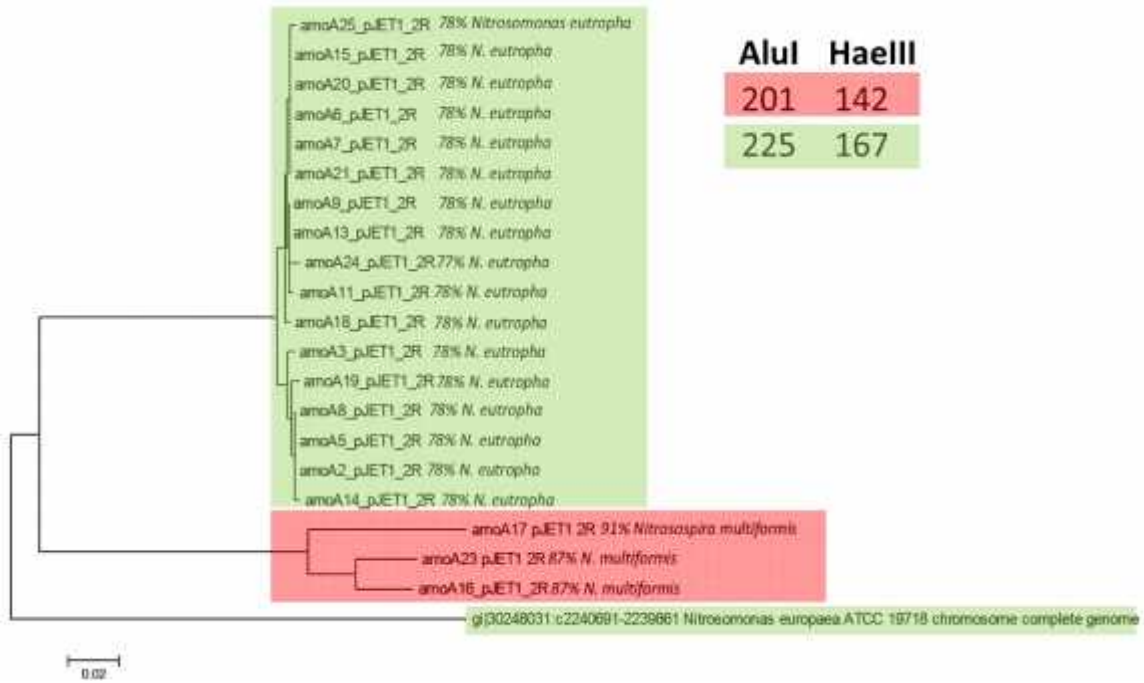

Figura 2.2.- Árbol filogenético construido con las secuencias de *amoA* bacteriano mediante el algoritmo neighbour joining. Los cuadros de colores muestran los distintos sitios 5' proximales de corte teóricos que resultan de la digestión *in silico* con las enzimas *AluI* y *HaeIII*.

Todas las secuencias obtenidas afiliadas a *Nitrosomonas eutropha* son cortadas por *AluI* en la base 225 desde el extremo 5', mientras que la digestión por *HaeIII* provocaría un fragmento terminal de 167 pb en todas ellas. Por otra parte, las 3 secuencias afiliadas a *Nitrosospira multiformis* son digeridas en la posición 142 y 201 por la enzimas *HaeIII* y *AluI*, respectivamente.

Los cebadores y las condiciones de ciclado usados, permiten la amplificación de genes *amoA* bacteriano con una especificidad muy alta. Sin embargo, se requirió purificar el producto específico desde el gel, debido a la presencia de amplicones de

tamaño distinto al esperado, los cuales se mantuvieron a pesar de las condiciones de ciclado optimizadas que se ensayaron. La información generada con las digestiones *in silico*, permiten verificar que ambas enzimas de restricción candidatas, *HaeIII* y *AluI* generarían perfiles de T-RFLP informativos para conocer la estructura poblacional del gen *amoA* bacteriano.

2.3.3 Gen *nirS*

La amplificación del gen *nirS* con condiciones óptimas del PCR resultó en un producto de tamaño único de todas las muestras. El tamaño fue el esperado para este gen, ca. 410 pb. Luego de la ligación del producto y transformación de las células se obtuvieron alrededor de 30 clones. El inserto de 20 de estos clones, previa confirmación del tamaño del inserto, fue amplificado y enviado para su secuenciación.

Las secuencias obtenidas y depuradas se encuentran en el Apéndice 3. Las secuencias fueron depositadas en el GeneBank del NCBI, los números de acceso, el tamaño de cada secuencia, así como el tamaño de los fragmentos de restricción teóricos se muestra en el cuadro 2.4. Cinco de las secuencias obtenidas tuvieron máxima similitud con genes *nirS* de bacterias del género *Bradyrhizobium*, que pertenece a la clase Alphaproteobacteria. El resto de las secuencias fueron altamente similares a secuencias del gen *nirS* de bacterias de géneros incluidos en la clase Betaproteobacteria.

Los resultados obtenidos al contrastar nuestras secuencias con la base de datos de genomas de referencia del NCBI permiten confirmar la especificidad de los cebadores utilizados. Asimismo, las digestiones *in silico* realizadas con las enzimas *AluI* y *HaeIII* resultaron en 10 y 11 fragmentos terminales, respectivamente, del total de 20 secuencias analizadas. Esto indica que la digestión de las secuencias de *nirS* con cualquiera de las dos enzimas es potencialmente informativa a efectos de analizar la estructura de la población de este gen. La enzima *MboI* fue descartada debido a que varias secuencias fueron cortadas por esta enzima en sitios muy proximales al extremo 5’ lo que generaría fragmentos muy pequeños para ser

detectados en el análisis de fragmentos; cinco de las secuencias son cortadas en el sitio 25.

Cuadro 2.4.- Resultados de la comparación de las secuencias obtenidas para el gen *nirS* con la base de datos del NCBI. Se detallan los números de acceso del GeneBank y el tamaño de la secuencia. Se muestra el nombre del organismo contra cuyo gen *nirS* se obtuvo el máximo de similitud en el BLAST. Se muestran los resultados de la digestión *in silico* de los amplicones según los sitios de corte de las enzimas *HaeIII, AluI* y *MboI*.

Gen	Clon	GeneBank Acc. Number	Tamaño (pb)	organismo con máxima similitud	% de similitud	tamaño del fragmento 5'		
						HaeIII	AluI	MboI
nirS	S3	KX079920	414	*Bradyrhizobium oligotrophicum*	86%	79	38	73
nirS	S4	KX079928	409	*Cupriavidus taiwanensis*	81%	115	224	112
nirS	S5	KX079921	414	*B. oligotrophicum*	94%	232	226	28
nirS	S6	KX079922	410	*Rubrivivax gelatinosus*	77%	139	224	93
nirS	S7	KX079929	371	*B. oligotrophicum*	86%	n.d.	n.d.	n.d.
nirS	S8	KX079909	406	*Thiobacillus denitrificans*	79%	139	221	109
nirS	S9	KX079930	413	*B. oligotrophicum*	89%	115	227	93
nirS	S10	KX079931	411	*C. taiwanensis*	81%	261	186	25
nirS	S11	KX079932	410	*R. gelatinosus*	77%	135	224	93
nirS	S12	KX079933	410	*R. gelatinosus*	78%	135	224	93
nirS	S13	KX079919	390	*Ralstonia eutropha*	78%	119	38	25
nirS	S14	KX079934	409	*Cupriavidus metallidurans*	80%	226	50	93
nirS	S15	KX079911	409	*T. denitrificans*	79%	135	50	93
nirS	S16	KX079923	411	*T. denitrificans*	82%	226	186	25
nirS	S17	KX079935	404	*R. gelatinosus*	77%	135	186	25
nirS	S18	KX079910	409	*Cupriavidus necator*	80%	103	no corta	24
nirS	S19	KX079924	409	*R. gelatinosus*	81%	271	338	93
nirS	S20	KX079925	410	*Azoarcus aromaticum*	79%	135	186	73
nirS	S21	KX079926	413	*B. oligotrophicum*	86%	79	38	73
nirS	S22	KX079927	410	*R. gelatinosus*	78%	261	186	25
nirS	estándar		415	*Pseudomonas stutzeri*	98%	147	239	99

La información generada con *HaeIII* representaría mejor la clasificación filogenética de estas secuencias que la generada con *AluI*. El árbol filogenético (figura 2.3) construido con las secuencias de los amplicones de *nirS*, y los respectivos sitios de corte generados *in silico* con la enzima *HaeIII*, muestra la información derivada de los cortes de esta enzima guarda un grado de coherencia aceptable con la similitud de las secuencias y con la asignación filogenética de cada una de ellas. Existen en el árbol cuatro grupos cuyo sitio de corte con *HaeIII* es idéntico (sitios de corte: 79, 135, 139 y 226). Por otra parte, el grupo conformado por las secuencias S8 y S6 en

generaría fragmentos de 221 y 224 pb, respectivamente, si es digerido por *AluI*. Las secuencias S11, S12, son cortadas por *AluI* en un mismo sitio (224), pero las secuencias S15 y S20, que forman el mismo grupo y que son digeridas por *HaeIII* en el mismo sitio que S11 y S12, son cortadas en los sitios 50 y 186 por *AluI*, respectivamente. Finalmente, el grupo formado por S14 y S16 es cortado por *AluI* en los sitios 50 y 186, respectivamente.

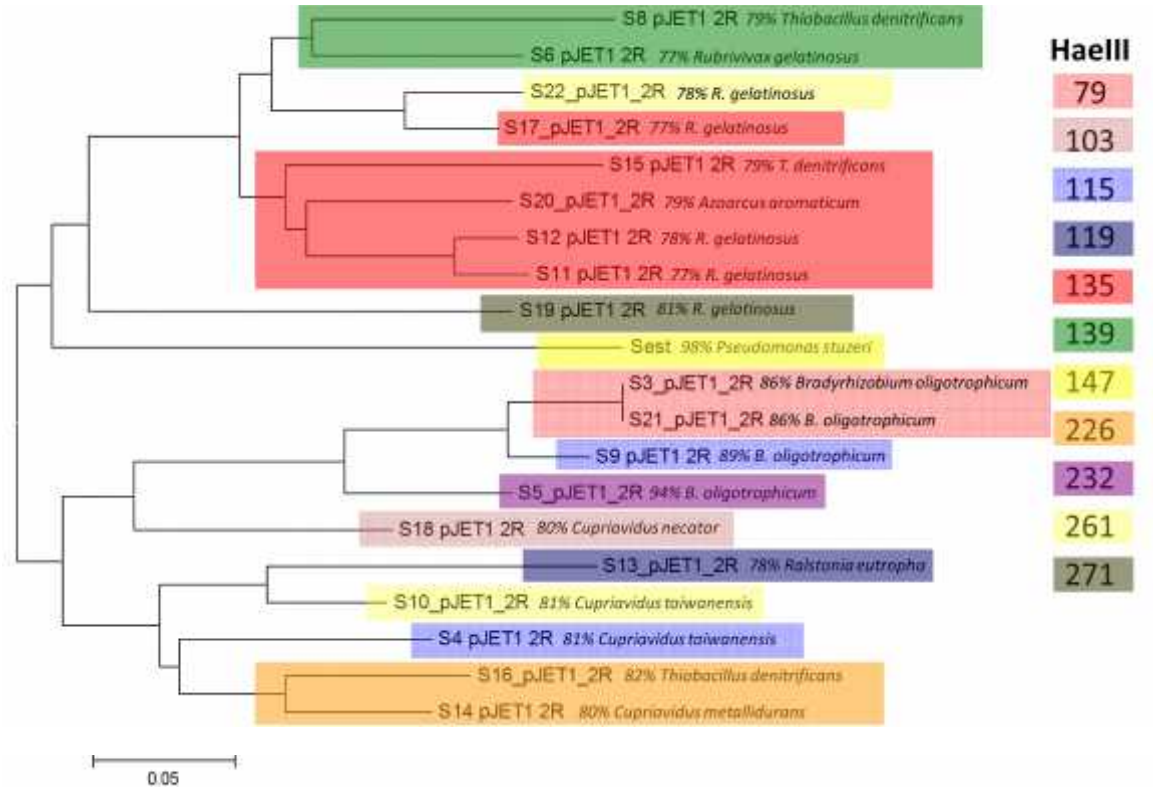

Figura 2.3.- Árbol filogenético construido con las secuencias de *nirS* mediante el algoritmo neighbour joining. Los cuadros de colores muestran los distintos sitios 5' proximales de corte teóricos que resultan de la digestión *in silico* con la enzima *HaeIII*.

A partir de estos resultados se puede concluir que los cebadores y las condiciones de ciclado usadas permiten la amplificación de genes *nirS* con una especificidad muy alta. Además, teniendo en cuenta la información generada con las digestiones *in silico*, se selecciona como enzima de corte para generar los perfiles de T-RFLP de *nirS* a la enzima *HaeIII*.

2.3.4 Gen *nirK*

La amplificación del gen *nirK* con condiciones óptimas del PCR resultó en un producto de tamaño único de todas las muestras. El tamaño fue el esperado para este gen, ca. 515 pb. Luego de la ligación del producto y transformación de las células se obtuvieron solamente 8 clones. El inserto de estos clones, previa confirmación de su tamaño, fue amplificado y enviado para su secuenciación.

Las secuencias obtenidas y depuradas se encuentran en el Apéndice 4. Las secuencias fueron depositadas en el GeneBank del NCBI, los números de acceso, el tamaño de cada secuencia, así como el tamaño de los fragmentos de restricción teóricos se muestra en el cuadro 2.5. Solamente una secuencia de las obtenidas tuvo máxima similitud con genes *nirK* de bacterias pertenecientes a la clase Betaproteobacteria, específicamente con el género *Achromobacter*. El resto de las secuencias fueron altamente similares a secuencias del gen *nirK* de bacterias del género *Bradyrhizobium* que pertenece a la clase Alphaproteobacteria.

Cuadro 2.5.- Resultados de la comparación de las secuencias obtenidas para el gen *nirK* con la base de datos del NCBI. Se detallan los números de acceso del GeneBank y el tamaño de la secuencia. Se muestra el nombre del organismo contra cuyo gen *nirK* se obtuvo el máximo de similitud en el BLAST. Se muestran los resultados de la digestión *in silico* de los amplicones según los sitios de corte de las enzimas *HaeIII, AluI* y *MboI*.

Gen	Clon	GeneBank Acc.	Tamaño (pb)	organismo con máxima similitud	% de similitud	tamaño del fragmento 5' proximal		
						HaeIII	AluI	MboI
nirK	KB	KX079912	515	*Bradyrhizobium elkanii*	85%	248	219	128
nirK	KC	KX079913	515	*Bradyrhizobium paxllaeri*	87%	65	515	74
nirK	KD	KX079914	515	*B. paxllaeri*	87%	65	515	74
nirK	K6	KX079915	515	*B. paxllaeri*	87%	65	105	128
nirK	K8	KX079916	404	*B. elkanii*	85%	342	105	310
nirK	K10	KX079917	515	*Achromobacter arsenitoxydans*	80%	65	188	352
nirK	K11	KX079908	514	*Bradyrhizobium lablabi*	86%	64	218	105
nirK	K13	KX079918	515	*B. paxllaeri*	84%	65	219	128
nirK	estándar		514	*Sinorhizobium meliloti*	100%	324	514	42

Los resultados obtenidos al contrastar nuestras secuencias con la base de datos de genomas de referencia del NCBI permiten confirmar la especificidad de los cebadores utilizados.

Al analizar las digestiones *in silico* con las enzimas candidatas, se descartó la enzima *AluI* ya que no generaba cortes en las secuencias KC ni KD. Por otra parte, las enzimas *HaeIII* y *MboI* generarían fragmentos de 4 y 6 tamaños diferentes, respectivamente (incluyendo en el análisis la secuencia de referencia). Debido a que la enzima *MboI* generaría fragmentos de tamaños distintos en secuencias muy similares, se seleccionó la enzima *HaeIII* para generar los fragmentos del T-RFLP del gen *nirK*.

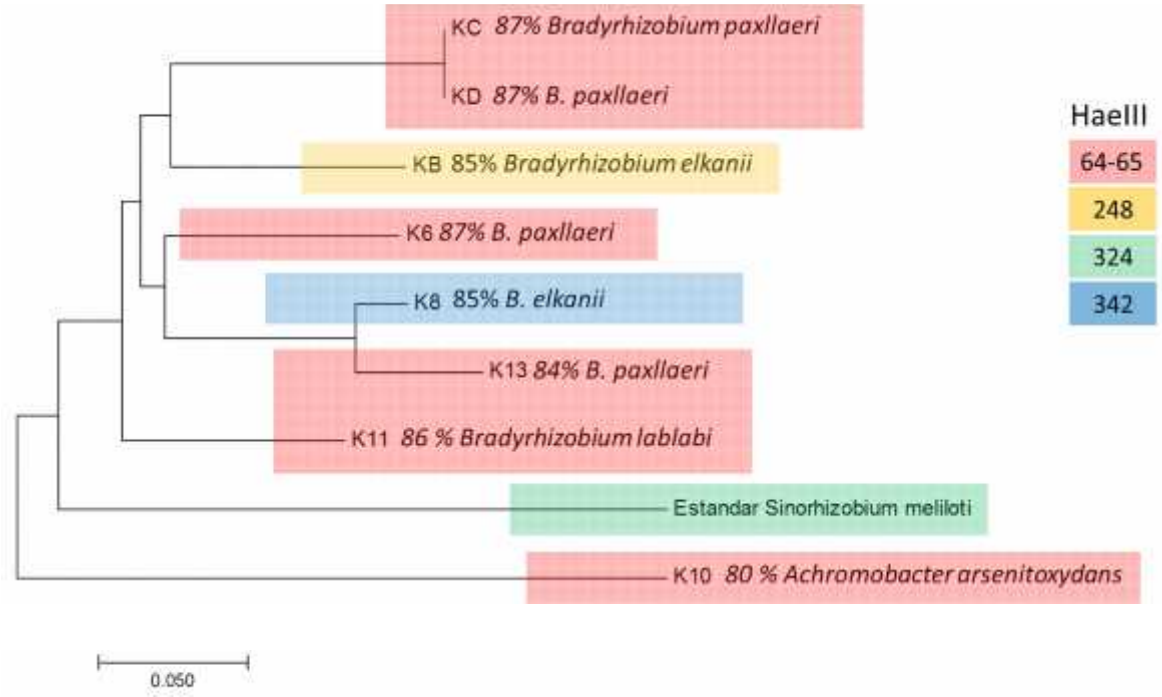

Figura 2.4.- Árbol filogenético construido con las secuencias de *nirK* mediante el algoritmo neighbour joining. Los cuadros de colores muestran los distintos sitios 5' proximales de corte teóricos que resultan de la digestión *in silico* con la enzima *HaeIII*.

3. THE EFFECT OF SOIL TYPE, RICE CULTIVAR AND WATER MANAGEMENT ON AMMONIA-OXIDIZING ARCHAEA AND BACTERIA POPULATIONS

3.1 SUMMARY

Ammonia-oxidizing microorganisms are partly responsible for the production of N_2O, a potent greenhouse gas. Rice paddies provide a habitat where ammonia oxidizing microorganisms can be active. The influence of different agricultural practices on these microorganisms, particularly on archaeal ammonia oxidizers, is an active field of research. In this work, we conducted two greenhouse experiments where we analyzed the influence of two soil types with different organic matter contents, two rice cultivars and water management on both archaeal (AOA) and bacterial (AOB) ammonia oxidizers. We determined the AOA and AOB abundance and population structure by q-PCR and T-RFLP, respectively. The archaeal and bacterial ammonia monooxygenase subunit A gene was used as the PCR target. The AOA and AOB copy numbers were affected by sampling time in both experiments. AOA abundance was also influenced by the time of flooding. The population structure of AOA was more variable than that of AOB and was strongly determined by soil type. Changes in AOB population structure were observed mainly according to sampling time.

3.2 RESUMEN

Los microorganismos oxidadores de amonio son parcialmente responsables por la producción de N_2O, un potente gas de efecto invernadero. Los arrozales proveen un hábitat en el cual los microorganismos oxidadores de amonio pueden estar activos. La influencia que tienen diferentes prácticas agrícolas sobre estos microorganismos, particularmente sobre las arqueas oxidadoras de amonio, es activo campo de investigación. En este trabajo, llevamos a cabo dos experimentos en invernáculo donde analizamos la influencia de dos tipos de suelo con diferente contenido de materia orgánica, dos cultivares de arroz y el manejo del agua sobre las arqueas (AOA) y bacterias (AOB) oxidadoras de amonio. Determinamos la abundancia y

estructura poblacional de AOA y AOB mediante q-PCR y T-RFLP, respectivamente. El gen de la subunidad A de la amonio monooxigenasa arqueana y bacteriana fue utilizado como blanco de los PCRs. Los número de copia de los genes de AOA y AOB fueron afectados por el momento de muestreo en ambos experimentos. La abundancia de AOA también fue influenciada por el momento de inundación. La estructura de la población de AOA fue más variable que la de AOB y fue fuertemente determinada por el tipo de suelo. Los cambios en la estructura de la población de AOB fueron observados principalmente según el momento de muestreo.

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The effect of soil type, rice cultivar and water management on ammonia-oxidizing archaea and bacteria populations

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ABSTRACT

Ammonia-oxidizing microorganisms are partly responsible for the production of N_2O, a potent greenhouse gas. Rice paddies provide a habitat where ammonia oxidizing microorganisms can be active. The influence of different agricultural practices on these microorganisms, particularly on archaeal ammonia oxidizers, is an active field of research. In this work, we conducted two greenhouse experiments where we analyzed the influence of two soil types with different organic matter contents, two rice cultivars and water management on both archaeal (AOA) and bacterial (AOB) ammonia oxidizers. We determined the AOA and AOB abundance and population structure by q-PCR and T-RFLP, respectively. The archaeal and bacterial ammonia monooxygenase subunit A gene was used as the PCR target. The AOA and AOB copy numbers were affected by sampling time in both experiments. AOA abundance was also influenced by the time of flooding. The population structure of AOA was more variable than that of AOB and was strongly determined by soil type. Changes in AOB population structure were observed mainly according to sampling time.

1. Introduction

Rice is the staple food for more than 3 billion people worldwide (FAO, 2007); it is cultivated on arable lands in tropical and subtropical areas covering approximately 20% of the global cultivated land (Peng et al., 2011).

Cultivated soil is the main anthropogenic source of N_2O, contributing 1.7–4.8 Tg of N_2O-N yr^{-1} (IPCC, 2007). Paddy rice is a significant source of CH_4, whereas N_2O emissions from paddy fields are thought of as marginal. Although most of the rice-originated N_2O is emitted by upland rice, efforts are being made to understand the dynamics of N_2O emissions in paddy rice to reduce this emission (Zou et al., 2009). N_2O has a 100-year global warming potential, 298 times that of CO_2 (IPCC, 2007), and an atmospheric lifetime of more than 100 years (Ko et al., 1991); it also degrades stratospheric ozone via NO_x.

Nitrous oxide is biologically produced in soil through the processes of denitrification and nitrification. In denitrification, a wide group of microorganisms, which include mainly prokaryotes and some eukaryotes, use NO_x as an electron acceptor in anaerobic respiration. Thus, this process takes place mainly in anoxic or microaerophilic environments (Zumft, 1997). The final product of denitrification can be either NO, N_2O or N_2, depending on the microorganisms that are involved and on the environmental conditions (Bakken et al., 2012).

Nitrification is the biological conversion of ammonia (NH_3) to nitrate (NO_3^-); it occurs in two steps by the action of two distinct groups of microorganisms. In the first step, ammonia serves as energy source to a group of chemoautotrophic microorganisms that are collectively called ammonia oxidizers and convert ammonia to nitrite (Egli et al., 2003). Ammonia-oxidizing bacteria (AOB), notably *Nitrosomonas* spp., were first described in the late nineteenth century (Winogradsky, 1891), while the first report of an archaeon that was able to grow by aerobically oxidizing ammonium was published in 2005 (Könneke et al., 2005). Thus, it is not surprising that the interest in and the knowledge about the ecological significance of ammonia oxidizing archaea (AOA) have increased exponentially in recent years. Currently, the relative importance of AOA and AOB in different environments is being questioned (Di et al., 2009; Leininger et al., 2006; Zhang et al., 2011). Although the mechanisms through which N_2O is formed during nitrification are not completely understood, it is clear that ammonia oxidation can account for up to 80% of the total emitted N_2O, depending on the environmental conditions and soil type (Gödde and Conrad, 1999).

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Ammonia-oxidizing microorganisms are slow growing (Kowalchuk and Stephen, 2001), and their cultivation is possible for a modest proportion (Smith et al., 2001). Culture independent techniques, such as terminal-restriction fragment length polymorphism (T-RFLP) (Osborn et al., 2000), have been extensively used to study both AOA and AOB (Glaser et al., 2010) using the ammonia monooxygenase subunit A (amoA) gene as a target (Francis et al., 2005; Rotthauwe et al., 1997).

Several studies have indicated the influence of plant genotype in shaping the communities of plant-associated bacteria (Germida and Siciliano, 2001). This influence is modulated by soil type and changes during the growing season (Reichardt et al., 2001). Briones et al. (2003) reported different nitrification activity and AOB populations associated with roots of different rice cultivars. In addition, different rice cultivars can have different CH_4 emission rates; therefore, the use of more-efficient cultivars is a promising strategy to mitigate greenhouse gas emissions (Setyanto et al., 2004).

Several reports have shown that alterations in water management, such as mid-season drainage, are effective options for CH_4 mitigation in irrigated rice fields (Itoh et al., 2011). However, this practice of alternate anaerobic and aerobic cycling can stimulate N_2O emission.

The main objective of the present study was to analyze the dynamics in AOA and AOB abundance and community structure in simulated paddy rice fields throughout the crop cycle. We used two rice cultivars (one *indica* and one *japonica*), two water regimes, and two contrasting soil types according to their organic matter content. We hypothesized that these factors might affect AOA and AOB in different ways. We found that only the soil type influenced the AOA community structure.

2. Materials and methods

2.1. Greenhouse experiments

Soils were collected from paddy fields in two consecutive years from the top layer (15 cm in depth) two months before rice sowing. Two sites were selected, representing the main geographical zones where rice is cultivated in Uruguay. Soil 1 was collected at the Instituto Nacional de Investigación Agropecuaria (INIA) (33°16′10.00″ S; 54°10′04.00″ W), and soil 2 was collected from a private paddy field (31°22′10″ S; 57°27′45″ W). Soil 1, referred to hereafter as "lower OM" (lower organic matter), is a Typic Argiudoll, with pH 5.7, 3.4% organic matter, 13 μg/g P Bray I, and 10 μg/g NO_3-N. Soil 2, referred to hereafter as "higher OM" (higher organic matter), is a Typic Hapludert, with pH 5.6, 5.8% organic matter, 10 μg/g P Bray I and 15 μg/g NO_3-N.

In two consecutive years, two greenhouse experiments were carried out. The experimental unit consisted of 12 plastic boxes (40 × 60 × 35 cm), each containing approximately 60 kg of sieved soil. The boxes were set in a randomized block design, comprising three replicates of each treatment. The rice cultivars that were used were El Paso 144 (*Oryza sativa* ssp. *indica*) and Tacuari (*O. sativa* spp. *japonica*). Seeds were sowed in two parallel lines by box at a density equal to field conditions. At sowing, ammonium nitrate was applied at the equivalent of 100 kg ha^{-1}. Subsequent fertilization was applied as urea at the equivalent of 20 kg ha^{-1}, at tillering and at the flower primordium stage.

The first experiment consisted of a 2 × 2 factorial design. The factors were rice cultivar and water management. The soil that was used in this experiment was from the INIA site (lower OM). Flooding was established at tillering in all treatments. In the drained treatment, boxes were drained at the beginning of the flowering stage and flooded again 16 days after draining. In the not-drained treatment, flooding was maintained until one week before harvest. The treatments were as follows: Tacuari, drained (TD); Tacuari, not drained (TnD); El Paso 144, drained (EPD); and El Paso 144, not drained (EpnD). Each treatment had three replicates. The definitive draining for all boxes was performed one week before harvest.

Soil samples were taken at tillering, at the flower primordium stage, five days after draining (draining), and at harvest. From each plastic box, six randomly collected soil cores (2 × 5 cm) were taken and mixed together.

The second experiment consisted of a 2 × 2 factorial design. The factors were soil type and time of flooding. The soils that were used were "lower OM" (LOM) and "higher OM" (HOM). The water regime consisted of an early flooding (at the tillering stage) and late flooding (after flower primordium). The treatments were as follows: lower OM, early flooding (LOMEF); lower OM, late flooding (LOMLF); higher OM, early flooding (HOMEF); and higher OM, late flooding (HOMLF). Each treatment had three replicates.

The final draining was performed one week before harvest. Soil samples were taken one week after sowing, one week after tillering, two days after the flower primordium stage, and at harvest.

2.2. Nucleic acid extraction

Soil samples were air-dried at 26 °C and 40% relative humidity for 48 h prior to DNA extraction. DNA was extracted from 0.25 g (dry weight) of soil using the MoBio Power Soil® DNA Isolation Kit (San Diego, CA, USA). The extracted DNA was checked on a 0.8% agarose gel. The DNA concentration and purity were determined with NanoDrop® 2000c UV–vis spectrophotometry (USA).

2.3. q-PCR analysis

The abundance of AOA and AOB was determined by real-time PCR using a LineGene K Thermocycler (Bioer Technology) using the fluorescent dye SYBR-Green I. All of the samples and standards were quantified in triplicate.

The reaction mixture for AOA (12.5 μl) contained 6.25 μl of 2× Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific Inc.), 10 μg of bovine serum albumin, 2.5% (v/v) dimethyl sulfoxide (DMSO) and 0.5 μM each primer: Arch-amoAF (5′-STA ATG GTC TGG CTT AGA CG-3′) and Arch-amoAR (5′-GCG GCC ATC CAT CTG TAT GT-3′) (Francis et al., 2005). Thermal cycling was as follows: 95 °C for 10 min and 40 × (95 °C, 45 s; 53 °C, 45 s; 72 °C, 45 s; and 79 °C, 15 s for data collection), and the program ended with a melt curve from 65 °C to 90 °C. Standard curves were generated by amplifying 10-fold dilutions of a mix of linearized pJET1.2/blunt plasmids containing five different archaeal amoA genes. The PCR efficiency ranged from 85.39% to 101.35%, averaging 94.36%, and the correlation coefficient ranged from 0.977 to 0.998, averaging 0.990.

The reaction mixture for AOB (12.5 μl) contained 6.25 μl of 2× SYBR® Premix Ex TaqTM (TaKaRa Bio, Inc.), 10 μg of bovine serum albumin, 2.5% (v/v) dimethyl sulfoxide (DMSO), 1× ROX reference dye and 0.4 μM each primer: amoA-1F (5′-GGG GTT TCT ACT GGT GGT-3′) (Rotthauwe et al., 1997) and amoA-2Rs (5′-CCT CKG SAA AGC CTT CTT C-3′) modified from those originally published by Rotthauwe et al. (1997) to minimize dimmer formation. Thermal cycling was as follows: 95 °C for 10 min and 40 × (95 °C, 30 s; 58 °C, 30 s; 72 °C, 45 s; and 80 °C, 15 s for data collection), and the program ended with a melt curve from 72 °C to 90 °C. Standard curves were generated by amplifying 10-fold dilutions of linearized pJET1.2/blunt plasmid containing *amoA* gene from *Nitrosomonas europaea*. The PCR efficiency ranged from 90.60% to 105.82%, averaging 98.55%, and the correlation coefficient ranged from 0.986 to 1, averaging 0.994.

Gene abundances were standardized by the mass of DNA that was extracted per gram of dry soil and log10 transformed before analysis.

2.4. Terminal restriction fragment length polymorphism (T-RFLP) analysis

The population structure of ammonium-oxidizing microorganisms was determined through T-RFLP analysis. Archaeal amoA amplification was performed using the primers Arch-amoAF and Arch-amoAR; the forward primer was labeled with 6-carboxyfluorescein (FAM). The PCR reaction mixtures contained 25 μl of 10× Taq buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM each primer, 2.5% DMSO, 0.004% BSA, 1 U of Taq DNA polymerase (recombinant, Thermo Scientific) and 2 μl of template (3–7 ng of DNA) in a total volume of 50 μl. The PCR cycle for AOA consisted of an initial denaturation step at 95 °C for 3 min, followed by 30 cycles of 95 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s, and a final elongation step of 72 °C for 10 min. The PCR products were checked on a 1.2% agarose gel, and only single bands products of the correct size (ca. 635 bp) were collected for further analysis. The PCR products were purified with the AxyPrep PCR Clean-up Kit (Axygen Biosciences). The purified products were quantified with NanoDrop® 2000c UV–vis spectrophotometry. Aliquots containing 100 ng of purified PCR product were digested with the tetrameric restriction enzyme MboI (recognition site: AG/CT) at 37 °C for 4 h, after which the enzyme was inactivated at 80 °C for 20 min. To reduce bias, three PCR products were pooled for each digestion.

Bacterial *amoA* amplification was performed using the primers amoA-1F and amoA-2Rs; the forward primer was labeled with 6-carboxyfluorescein (FAM). The PCR reaction mixtures contained 25 μl of 10× DreamTaq buffer, 0.2 mM dNTPs, 0.3 μM each primer, 2.5% DMSO, 0.004% BSA, 1 U of DreamTaq DNA polymerase (recombinant, Thermo Scientific) and 2 μl of template (1/10 dilution of the q-PCR product) in a total volume of 50 μl. A dilution of the q-PCR product was used as a template because the amplification of bacterial *amoA* directly from the soil DNA was difficult. PCR products were checked on a 1.2% agarose gel. The expected size bands (ca. 490 bp) were excised from the gel and purified with the GeneJET PCR Purification Kit (Thermo Scientific). The purified products were quantified with NanoDrop® 2000c UV–vis spectrophotometry. To reduce bias, three PCR products were pooled for each digestion. Aliquots containing 100 ng of purified PCR product were digested with the tetrameric restriction enzyme HaeIII (recognition site: GG/CC) at 37 °C for 4 h, after which the enzyme was inactivated at 80 °C for 20 min.

The restriction enzymes were chosen based on the *in silico* testing of 20 cloned sequences that were previously amplified from our samples. Restriction fragments were sent to Macrogen, Inc., where they were analyzed by an ABI 3730XL DNA analyzer (Applied Biosystem). The size of each terminal restriction fragment (T-RF) was determined comparing them to a fluorescently labeled size standard (GeneScanTM 1200 LIZ® for archaeal *amoA* and GeneScanTM 500 LIZTM for bacterial amoA) using the Peak ScannerTM Software v1.0 (Applied Biosystems).

The relative abundance of individual T-RFs was calculated as the percentage of total peak area in a given T-RFLP profile. Only those T-RFs with a relative abundance of greater than 1% were considered. For archaeal amoA, T-RFs that were smaller than 120 bp were omitted to avoid dimmer signal. For bacterial amoA, T-RFs that were smaller than 30 bp were omitted.

2.5. Statistical analysis

The number of OTUs per sample, Shannon index (Shannon and Waever, 1949) and abundance of gene copies were analyzed using a factorial analysis of variance (ANOVA) with Tukey's HSD post-hoc analysis to determine the significance of sampling time, water management, soil type and rice cultivar within each experiment. The normality and variances homogeneity were checked with Shapiro–Wilk and Levene's tests, respectively. The software package STATISTICA 8.0 (StatSoft, Inc.) was used.

T-RFLP were transformed into numerical data using the software Peak ScannerTM 1.0 (Applied Biosystems), binned and normalized with the software T-REX (Culman et al., 2009) and exported to an excel spreadsheet. The non-metric multidimensional scaling (NMS) of the T-RFLP data matrix was performed using the "vegan" package (Oksanen et al., 2014) within the R Statistical software (R Core Team, 2014). Multiresponse permutation procedures (MRPP) were used to test the statistical significance of the community composition between different treatments using the "vegan" package. Indicator species analysis was performed using the "indicspecies" package (De Cáceres and Legendre, 2009) within the R Statistical software.

3. Results

3.1. Archaeal ammonia oxidizing populations

3.1.1. Abundance of AOA

In the first experiment, the archaeal *amoA* gene copy number ranged from 3.4×10^4 to 2.3×10^6 per gram of dry weight soil. The gene copy number was not affected either by rice cultivar nor water management (Table 1). Statistically significant differences were observed between the sampling times (Fig. 1A). A higher copy number of the *amoA* gene was detected at "draining" and harvest compared to tillering. There was a significant interaction effect between the sampling time and rice cultivar. The cultivar "El Paso" at tillering had the lowest number of *amoA* copies. Although there were significant differences between abundances at harvest and tillering, the abundance of amoA in "El Paso" at harvest, although high, was not significantly different than that of "Tacuarí" at tillering.

In the second experiment, the archaeal *amoA* gene copy number ranged from 8.2×10^4 to 1.6×10^6 per gram of dry weight soil. The gene copy number was affected by the time of flooding and the sampling time but not by the soil type (Fig. 1B and Table 2). The interactions between soil type and sampling time and between the time of flooding and sampling time were significant; "early flooding" had a significantly higher copy number than did "late flooding" at the flower primordium stage. "Late flooding" treatment harbored higher numbers of the archaeal *amoA* gene compared to "early flooding" ($p < 0.05$; Tukey's HSD) (Table 2). Regarding sampling time, the abundance of the archaeal *amoA* gene was significantly lower at harvest ($p < 0.05$; Tukey's HSD).

3.1.2. Archaeal amoA population structure

Every different terminal restriction fragment (T-RF) that was observed was considered an operational taxonomic unit (OTU). OTUs were named after the size in base pairs of the T-RFs. Basic

Table 1
Archaeal and bacterial *amoA* gene copy number according to rice cultivar or water management. Values were $\log_{10}$ transformed and correspond to averages of all samples of each variable (standard deviation). Asterisks indicate statistically significant differences between treatments (Tukey's HSD, $p < 0.05$).

	Cultivar		Water management	
	El Paso ($n = 24$)	Tacuarí ($n = 24$)	Drained ($n = 2$)	Not drained ($n = 12$)
Archaeal *amoA*	5.73 (0.46)	5.78 (0.28)	6.04 (0.24)	5.93 (0.25)
Bacterial *amoA*	6.29 (0.41)*	6.18 (0.23)*	6.23 (0.34)*	6.42 (0.47)*

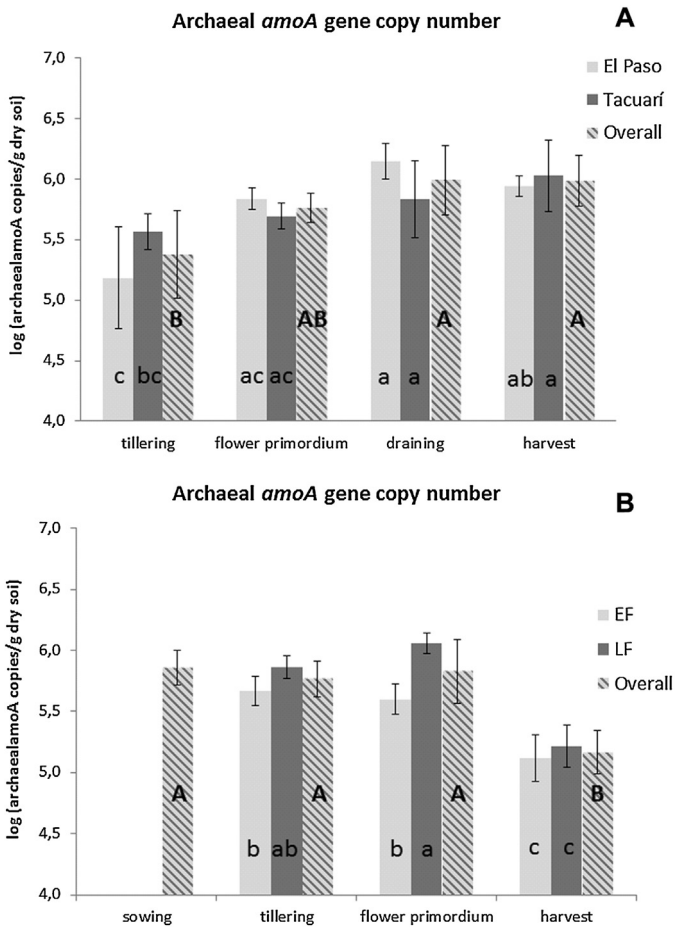

Fig. 1. Abundance of archaeal *amoA* genes. (A) According to sampling time and to the rice cultivar. Light gray bars represent abundance on "El Paso" cultivar and dark gray on "Tacuarí" cultivar. Striped bars represent the overall abundance. (B) According to sampling time and time of flooding. Light gray bars represent the abundance in "Late flooding" treatment and dark gray in "Early flooding". Different uppercase letters on striped bars indicate significant differences (Tukey's test $p < 0.05$) at different sampling times. Different lowercase letters on gray bars indicate significant differences according to the sampling time × rice cultivar interaction (1A) or differences according to the sampling time × time of flooding interaction (1B) (Tukey's test $p < 0.05$). Gray bars represent means ($n = 3$)±SDs. Striped bars represent means ($n = 6$) ± SDs.

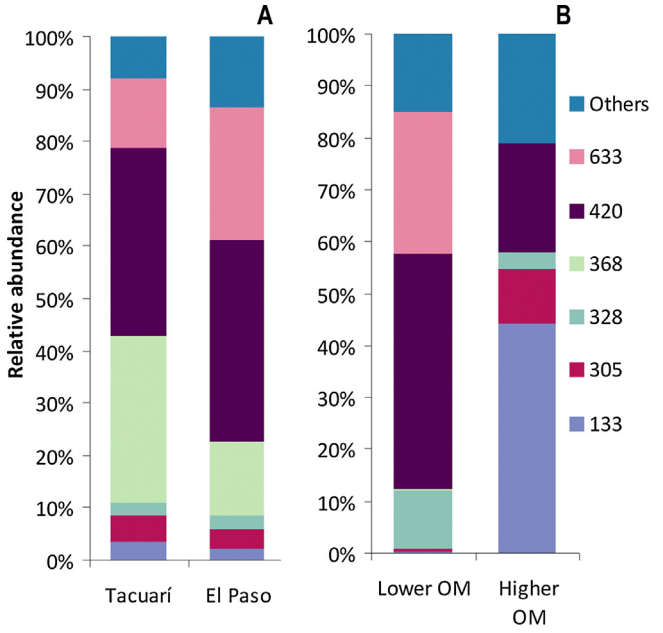

Fig. 2. The relative abundances of T-RFs that were obtained from T-RFLP profiles of archaeal *amoA*. (A) Average relative abundance for the two rice cultivars (El Paso, Tacuarí). (B) Average relative abundance for the different soil types (lower organic matter, higher organic matter). OTUs named "others" represent all OTUs with maximum relative abundances below 10%. Others included OTUs 125, 143, 210, 229, 232, 263, 267, 289, 316, 326, 345, 350, 390, 393, 398, 414, 434, 442, 571, 581, 591, 594, 610, 621 and 630.

analysis from T-RFLP data from the 48 samples showed that populations harbored a maximum of 10 OTUs and a minimum of 3 OTUs (three and one sample, respectively). Most of the samples (15) contained 6 OTUs, and the average number of OTUs per sample was 7.3. A total of 27 different OTUs were obtained, most of them (17) occasional because they were present in less than three samples. OTUs 368, 420 and 633 were the more abundant and had an average relative abundance greater than 10% (Fig. 2A). In addition, OTUs 420 and 633 were the only ones that were present in every sample.

The sampling time had a significant effect on the number of OTUs that were observed (*N*) (Table 3). In addition, the cultivar effect was significant; "El Paso" had an average *N* of 8.0 (s.d. 1.5), while the average *N* in "Tacuarí" samples was 6.5 (s.d. 1.6). According to the sampling time, the populations at tillering had a lower *N* than did the rest of the samples.

The Shannon diversity index (*H*) showed the same tendency as *N*. The rice cultivar and sampling time had significant effects on *H*. Samples of "El Paso" had a higher *H* ($H = 0.67$; s.d. 0.09) than did those of "Tacuarí" ($H = 0.60$; s.d. 0.13).

The population structure of archaeal *amoA* can be slightly distinguished according to cultivar treatment in the NMS scatter plot. Samples from the "El Paso" treatment tended to cluster right from "Tacuarí" samples (Fig. 3A). Among samples from "Tacuarí", only those that were collected at flowering primordium clustered among "El Paso" samples, while in samples from "El Paso", only those that were collected at tillering were located near "Tacuarí" samples. Samples that were taken at tillering and at flower primordium were clustered and separated from each other. This pattern indicates an effect of sampling time on community

Table 2
Archaeal and bacterial *amoA* gene copy number according to soil type or time of flooding. Values were $\log_{10}$ transformed and correspond to averages of all samples of each variable (standard deviation). Asterisks indicate statistically significant differences between treatments (Tukey's HSD, $p < 0.05$).

	Soil		Time of flooding	
	Higher OM ($n = 24$)	Lower OM ($n = 24$)	Late flooding ($n = 18$)	Early flooding ($n = 18$)
Archaeal *amoA*	5.66 (0.29)	5.65 (0.39)	5.74 (0.34)*	5.57 (0.33)*
Bacterial *amoA*	5.49 (0.39)*	5.67 (0.48)*	5.41 (0.29)	5.38 (0.38)

Table 3
Archaeal and bacterial amoA number of OTUs (*N*) and Shannon diversity index (*H*) according to sampling date. Values correspond to averages of all samples on each date (standard deviation). Top and bottom table represent first (cultivar and water management) and second (soil type and time of flooding) experiment, respectively. In the same row, different letters indicate statistically significant differences (Tukey's HSD, $p < 0.05$).

1st assay		Tillering	Flower primordium	Draining	Harvest
Archaeal	*N*	5.5 (1.1) b	7.7 (1.6) a	8.0 (1.1) a	7.9 (1.6) a
amoA	*H*	0.51 (0.11) b	0.66 (0.08) a	0.72 (0.06) a	0.66 (0.08) a
Bacterial	*N*	3.2 (1.3) b	5.8 (1.9) a	5.4 (1.0) a	6.3 (1.2) a
amoA	*H*	0.34 (0.14) b	0.60 (0.09) a	0.60 (0.07) a	0.59 (0.05) a

2nd assay		Sowing	Tillering	Flower primordium	Harvest
Archaeal	*N*	5.6 (1.4) a	6.7 (2.6) a	6.3 (2.2) a	5.7 (1.6) a
amoA	*H*	0.61 (0.09) a	0.63 (0.15) a	0.62 (0.11) a	0.58 (0.10) a
Bacterial	*N*	4.1 (0.8) c	5.6 (0.9) ab	7.8 (2.7) a	5.0 (1.0) bc
amoA	*H*	0.44 (0.09) b	0.46 (0.08) b	0.59 (0.14) a	0.48 (0.09) b

composition, with populations from different cultivars separating from each other as the plant grows. The results from MRPP comparing "El Paso" and "Tacuarí" showed a slight ($A = 0.09542$) but statistically significant ($p < 0.001$) difference between the two treatments. Indicator species analysis identified OTU 398 as associated with cultivar "El Paso" (stat 0.8; $p < 0.001$). OTU 442 was associated with all of the sampling times except for tillering (stat 0.866; $p < 0.001$). In this experiment, the time of flooding had no effect on the population structure of archaeal *amoA*.

In the second experiment, as in the first, we observed a number of OTUs per sample that ranged from 3 to 10 (four and two samples, respectively). Most of the samples (10) harbored 5 OTUs, and the average number of OTUs per sample was 6. A total of 16 different OTUs were observed; only 4 of them were present in fewer than 3 samples. Thus, the difference between the two experiments in the number of OTUs that were observed is due to the number of occasional OTUs that were present in the previous experiment. Interestingly, OTUs 350 and 368 were not present in any sample, although 368 was one of the three most abundant OTUs in the previous experiment. OTUs 125 and 326 were not present in the previous experiment and were only present in higher OM samples (13 and 24 samples, respectively). Likewise, OTU 633 was present in 22 out of 24 lower OM samples but was absent in all of the higher OM samples. The most abundant OTUs were 420, 133 and 633, with an average relative abundance of 33, 23 and 13%, respectively (Fig. 2B). Noticeably, although OTU 633 was absent from the higher OM samples, it was one of the most abundant in the experiment due to its high abundance in lower OM samples (average 27.2% relative abundance in lower OM samples). Only OTU 420 was present in every sample.

The soil type had a significant effect on *N*; samples from higher OM soil had a higher *N* (7.5; s.d. 1.6) than did samples from lower OM soil (4.6; s.d. 1.1). In addition, there was an interaction between sampling time and soil type; higher OM samples at tillering had the highest value that was significantly higher than all of the lower OM samples and higher OM samples at sowing and at harvest.

Only the soil type had a significant effect on *H*. Populations from higher OM soil were slightly but significantly more diverse ($H = 0.69$, s.d. 0.08) than those from lower OM soil ($H = 0.52$; s.d. 0.07).

The results from NMS showed that soil had a strong effect on the AOA population structure. All of the higher OM samples were grouped at the left end of the two-dimensional space, while all of the lower OM samples grouped at the right end (Fig. 3B). The *A* statistic that was obtained from the MRPP between soil treatments

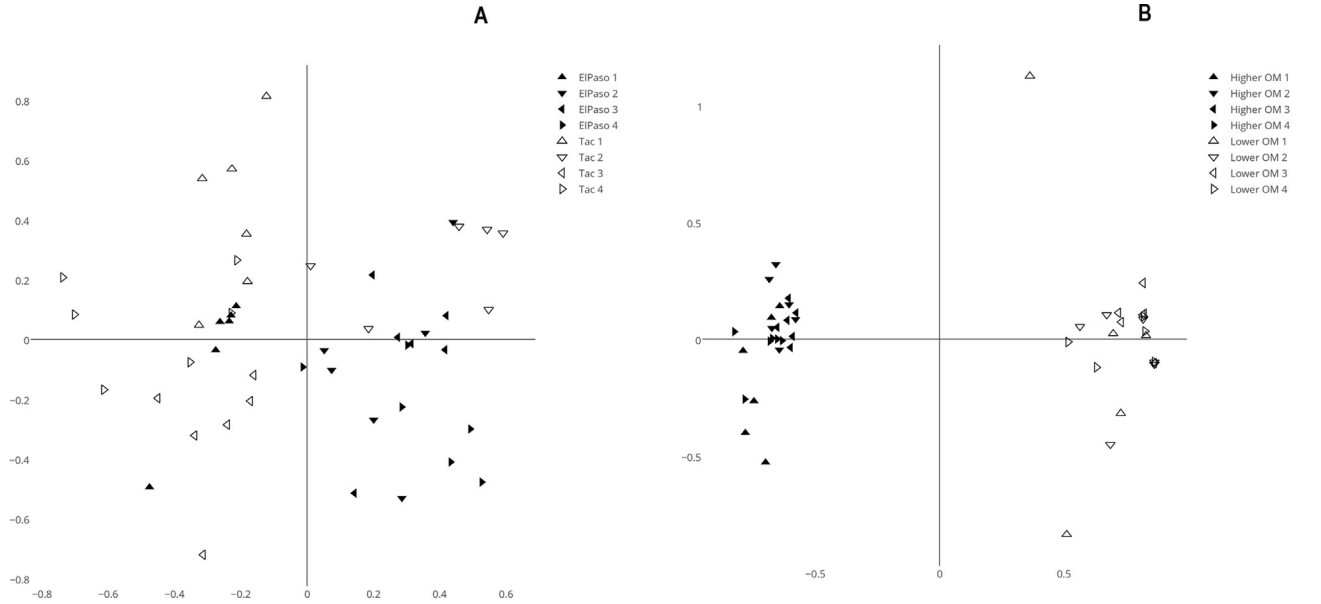

Fig. 3. Non-metric multidimensional scaling (NMS) ordination of T-RFLP profiles of archaeal *amoA* populations. (A) According to the cultivar and sampling time. El Paso samples are symbolized with filled triangles and Tacuarí (Tac) with open triangles. Sampling times are 1, tillering; 2, flower primordium; 3, draining; and 4, harvest (stress = 0.1120506). (B) According to the soil type and sampling time. Higher organic matter soil samples are represented with filled triangles and lower organic matter soil samples with open triangles. Sampling times are 1, sowing; 2, tillering; 3, flower primordium; and 4, harvest (stress = 0.05063612).

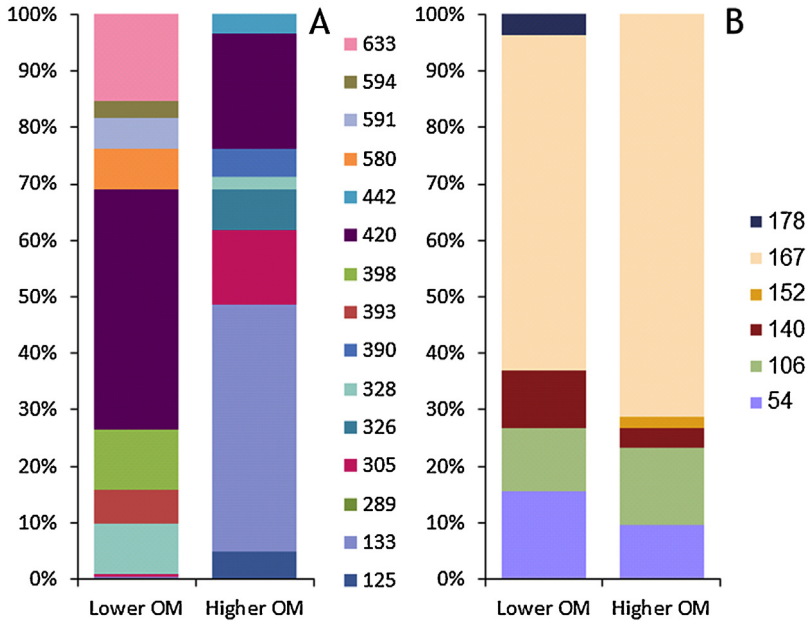

Fig. 4. Relative abundances of T-RFs that were obtained from the T-RFLP profiles of archaeal (A) and bacterial (B) *amoA* from the sowing sample from the second experiment. The average relative abundances for the different soil types are shown.

was 0.51, with a significance of 0.001. Thus, population structures of archaeal ammonia oxidizers were markedly different according to soil type. This difference was observed from the first sample, which was taken at sowing when the experiment was set up (Fig. 4A). Due to the strong effect of the soil type, the time of flooding and sampling time were analyzed separately from the soil type. In both higher OM and lower OM samples, no effects on population structure were found for the time of flooding or sampling time (data not shown). Indicator species analysis identified OTUs 326 (1.00; $p < 0.001$), 133 (0.996; $p < 0.001$), 305 (0.980; $p < 0.001$), 390 (0.947; $p < 0.01$) and 442 (0.906; $p < 0.001$) as significantly associated with higher OM populations. OTU 633 (0.977; $p < 0.001$) was significantly associated with lower OM samples.

3.2. Bacterial ammonia oxidizing populations

3.2.1. Abundance of AOB

In the first experiment, the bacterial *amoA* gene copy number ranged from 3.9×10^5 to 2.1×10^7 per gram of dry weight soil. All three of the studied independent variables had significant effects on the bacterial *amoA* gene copy number. Cultivar "El Paso" had significantly higher numbers of *amoA* than did "Tacuarí" (Fig. 5A, Table 1), in contrast to that seen for archaeal *amoA* at tillering. With respect to water management, the abundance of bacterial *amoA* was slightly but significantly higher in the treatment "not drained" (Table 1). The sampling time also had a significant effect, with gene copy numbers being significantly higher at draining and lower at harvest (Fig. 4A). The interaction between cultivar and sampling time was significant. At draining, bacterial *amoA* gene numbers were higher in "El Paso" than in "Tacuarí". In fact, the bacterial *amoA* copy number in "El Paso" at draining was significantly higher than in any other cultivar × sampling time condition.

In the second experiment, the bacterial *amoA* gene copy number ranged from 9.4×10^4 to 3.9×10^6 per gram of dry weight soil. Both the soil type and sampling time had an effect on bacterial *amoA* abundance (Fig. 5B), while the time of flooding had no significant effect. Samples from lower OM had significantly higher numbers of bacterial *amoA* than did those from higher OM (Table 2). Regarding sampling time, the highest numbers of *amoA* were obtained at sowing and the lowest at tillering (Fig. 5B). The soil type and sampling time also showed significant interaction; samples of lower OM soil at sowing were significantly higher than higher OM samples at the same moment.

3.2.2. Bacterial amoA population structure

The number of OTUs per sample ranged from one to 9, and only one sample contained one and 9 OTUs. The average number of OTUs per sample was 5.1; most samples (11) contained 5 OTUs. Only one OTU (167) was present in all of the samples, whereas OTU 140 was absent from only one. A total of 15 OTUs were observed, 6 of which were present in 3 or fewer samples (Fig. 6A). The most abundant OTUs were 167, 140, 178 and 106, with an average relative abundance of 49, 26, 10 and 8%, respectively.

Only the sampling time had a significant effect on N. Samples at tillering had a significantly lower N than did those from other sampling times. The same effect of sampling time was observed with *H* (Table 3).

The results from the NMS and MRPP showed that neither cultivar nor water regime had a significant effect on the AOB community structure (Fig. 7A). Samples that were collected at tillering grouped together regardless of cultivar. MRPP showed that the population structure varied according to the sampling time ($A = 0.1729$, $p < 0.001$). Accordingly, indicator species analysis found no OTU as significantly associated with any cultivar, but found OTU 178 as associated with all of the sampling times except for tillering (0.931, $p < 0.001$). Thus, OTU 178 probably depends on changes in the soil environment induced by the rice plant, regardless of the cultivar.

In the second experiment, the number of OTUs that were obtained ranged from three to 14. As in the first experiment, most of the samples (17) contained 5 OTUs; the average number of OTUs per sample was 5.6. OTU 167 was present in all of the samples as in the first experiment. OTU 106 was absent from only one sample, and OTU 140 was missing from 4 samples: three samples at sowing and one at tillering. A total of 23 OTUs were observed (Fig. 6B), 13 of which were present in 3 or fewer samples. The most abundant OTUs were 167, 140, 106 and 178, with an average relative

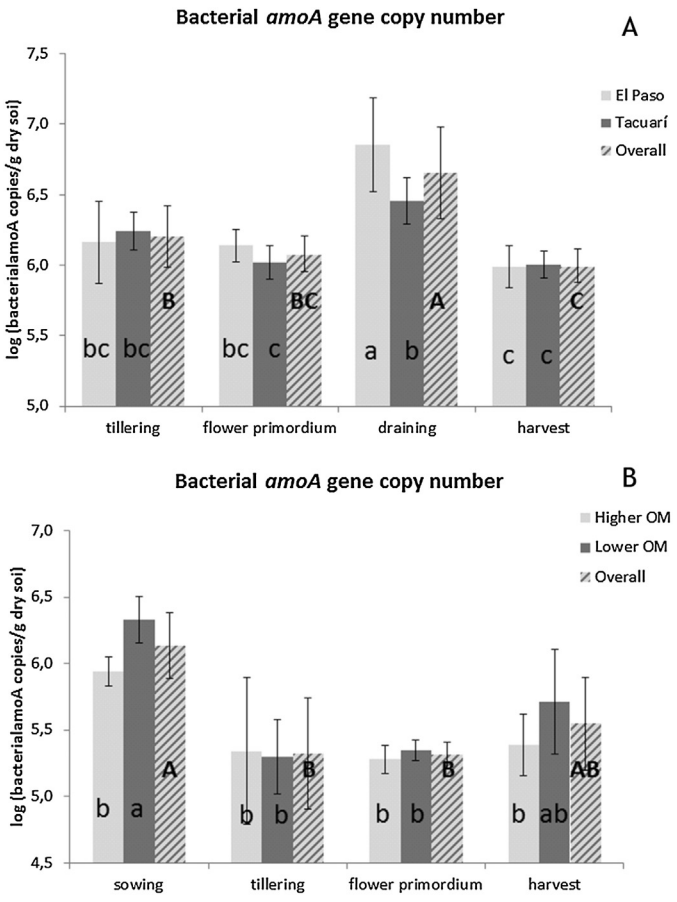

Fig. 5. Abundance of bacterial *amoA* genes. (A) According to the sampling time and to the rice cultivar. Light gray bars represent the abundance on "El Paso" cultivar and dark gray on "Tacuarí" cultivar. Striped bars represent the overall abundance. (B) According to the sampling time and soil type. Light gray bars represent abundance on higher organic matter soil and dark gray on lower organic matter soil. Striped bars represent the overall abundance. Different uppercase letters on striped bars indicate significant differences (Tukey's test $p < 0.05$) at different sampling times. Different lowercase letters inside gray bars indicate significant differences according to the sampling time × rice cultivar interaction (4A) or differences according to the sampling time × soil type interaction (4B) (Tukey's test $p < 0.05$). Gray bars represent means ($n = 3$) ± SDs. Striped bars represent means ($n = 6$) ± SDs.

abundance of 59, 16, 9 and 7%, respectively. OTU 195 was only present in 9 samples, all from higher OM soil. In the sowing sample, which represents the starting point of the experiment, OTU 178 was absent from higher OM samples, while OTU 152 was found only in higher OM soil (Fig. 4B).

The sampling time was the only factor that significantly affected N (Table 3). Populations at flower primordium had a significantly higher *N* than did populations at sowing and at harvest. On the other hand, *H* was affected by sampling time and by soil type. The highest values of the diversity index were observed during flower primordium. According to soil type, lower OM soil populations were more diverse ($H = 0.69$; s.d. 0.08) than were those in higher OM soil ($H = 0.52$; s.d. 0.07).

Populations from different soil type treatments were partially separated (Fig. 7B). The results from MRPP comparing higher OM with lower OM soils showed a slight but statistically significant ($A = 0.1583$; $p < 0.001$) difference between the two treatments. Samples from higher OM soil had a delta value of 17.5 compared to the expected delta of 27.22, while lower OM soil samples had a delta value of 28.31. Thus, the higher OM *amoA* bacterial population structure was less divergent that that in lower OM soil. The time of flooding had no significant effect on the bacterial *amoA* gene population structure. The sampling time significantly affected the population structure. In contrast to results from archaeal *amoA*, samples from the first sampling time were clustered together regardless of soil type. The *A* statistic that was obtained from the MRPP between sampling time was 0.1519, with a significance of 0.001. Indicator species analysis identified OTU 178 (0.894; $p < 0.001$) as significantly associated with lower OM soil populations. OTU 54 was associated with sowing samples (0.962; $p < 0.001$), while OTU 158 was associated with flower primordium samples (0.913; $p < 0.001$). On the other hand, OTU 115 was associated with all of the samples except for those that were taken at sowing (0.913; $p < 0.001$), indicating dependence on the rice root regardless of the soil type.

4. Discussion

In this study, we air dried the soil samples prior to DNA extraction to reduce bias occasioned by soil differential moisture. This bias could have been significant in our samples because they were taken from flooded and non-flooded soils; this is not the most used practice, but as reported by Lauber et al. (2010), the exposure of soil to this temperature for 48 h should not have a significant effect on the microbial community.

The relative abundance of AOA and AOB in different environments has been a subject of recent study (Leininger et al., 2006). There seems to be no straightforward way to predict if any of these populations would be more abundant given certain environmental conditions. To what extent abundance also means functional predominance and which population is responsible for the N_2O emissions remain open questions. Some authors have found that nitrifying potential depends on AOB abundance (Wang et al., 2011), while others found no correlation of AOA or AOB abundances with nitrifying potential (Boyle-Yarwood et al., 2008). Our results indicate that AOB populations are more abundant in most samples, which is in contrast with previous results from other works in paddy soils (Chen et al., 2011; Jiang et al., 2013; Wang et al., 2014). Chen et al. (2011), in determining population abundances with the same molecular approach that we used, found AOA populations between 400 and 2400 times higher than those of AOB. The average copy number of archaeal AOA in their work was on the order of 10^8 gene copy number per gram of soil dry weight: approximately two orders of magnitude greater than ours. Wang et al. (2014) found AOA to be 10 times more abundant than AOB in paddy soils, with AOB was more abundant in the 20–25-cm depth layer. Acidic conditions have a positive effect con AOA abundances and no effect on AOB populations (Nicol et al., 2008); thus, pH can be a factor explaining the differences in AOA abundance in our samples. These cited studies used DNA extracted directly from the field site samples, which is an important difference from our study. Therefore, the influence of the manipulation of soil to set the microcosms on AOA populations cannot be excluded. On the other hand, Wang et al. (2011) found AOB as more abundant than AOA in N-rich sediments from wetlands that included paddy samples. In their samples, AOB had a maximum value of 1.7×10^8 per gram of soil dry weight compared to 2.08×10^7, the maximum that we observed. The physicochemical heterogeneity of paddy soils can be responsible for the observed discrepancies in AOA and AOB abundance. It appears that no general trend can be observed regarding AOA/AOB abundance from paddy soil samples. These population abundances may be subject to case-specific conditions.

Most studies have encountered that the archaeal and bacterial *amoA* gene copy numbers are relatively stable and resilient to environmental changes. Florio et al. (2014), in studying the effects of a nitrification inhibitor in a microcosm experiment, found an effect on the transcript of the bacterial *amoA* gene but not on the *amoA* gene copy number. Others have obtained similar trends and

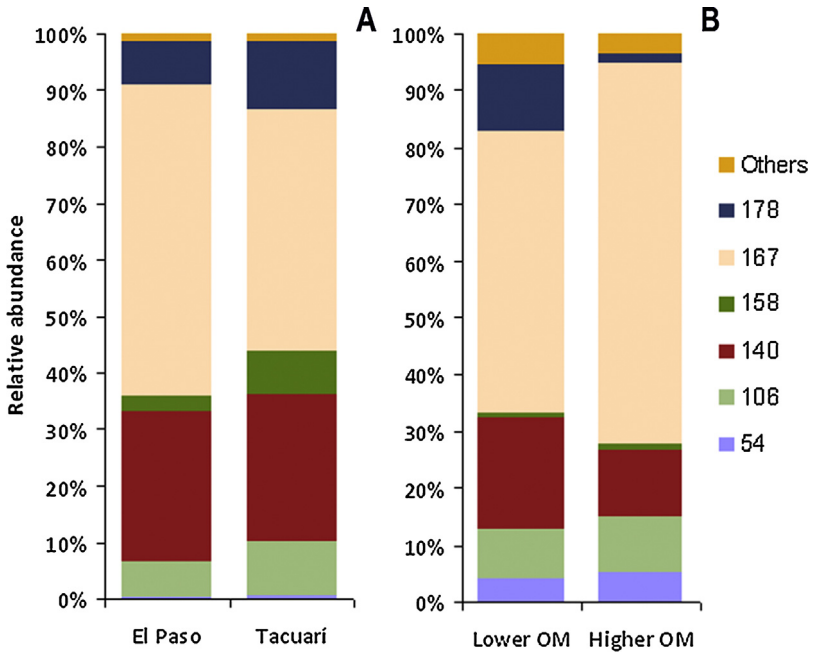

Fig. 6. Relative abundances of T-RFs that were obtained from the T-RFLP profiles of bacterial amoA according to rice cultivar (A) and soil type (B). OTUs named "others" represent all of the OTUs with maximum relative abundances below 5%. Others included OTUs 40, 42, 47, 56, 100, 115, 133, 136, 141, 152, 161, 165, 172, 186, 195, 204, 431 and 486.

observed that AOA were even more stable than were AOB (Nardi et al., 2013). Due to the slow growth rate of ammonia-oxidizing microorganisms, an increase in the *amoA* copy number can be perceivable just 21 days after NH_4^+ concentration increase through fertilizer application (Glaser et al., 2010). Urea application at flowering primordium and draining can explain the higher AOA and AOB populations at draining in the first experiment. In addition, the low populations of AOB at harvest in both experiments and of AOA in the second experiment may be the result of competition for NH_4^+ with the rice plant.

Water management only had effect on the archaeal *amoA* gene copy number in the second experiment. Flooding diminished O_2 diffusion rates, providing conditions for the generation of anaerobic zones in soil. Although ammonia oxidation is an aerobic process that is regulated by the concentrations of O_2 and NH_4^+, many ammonia-oxidizing microorganisms have ancillary

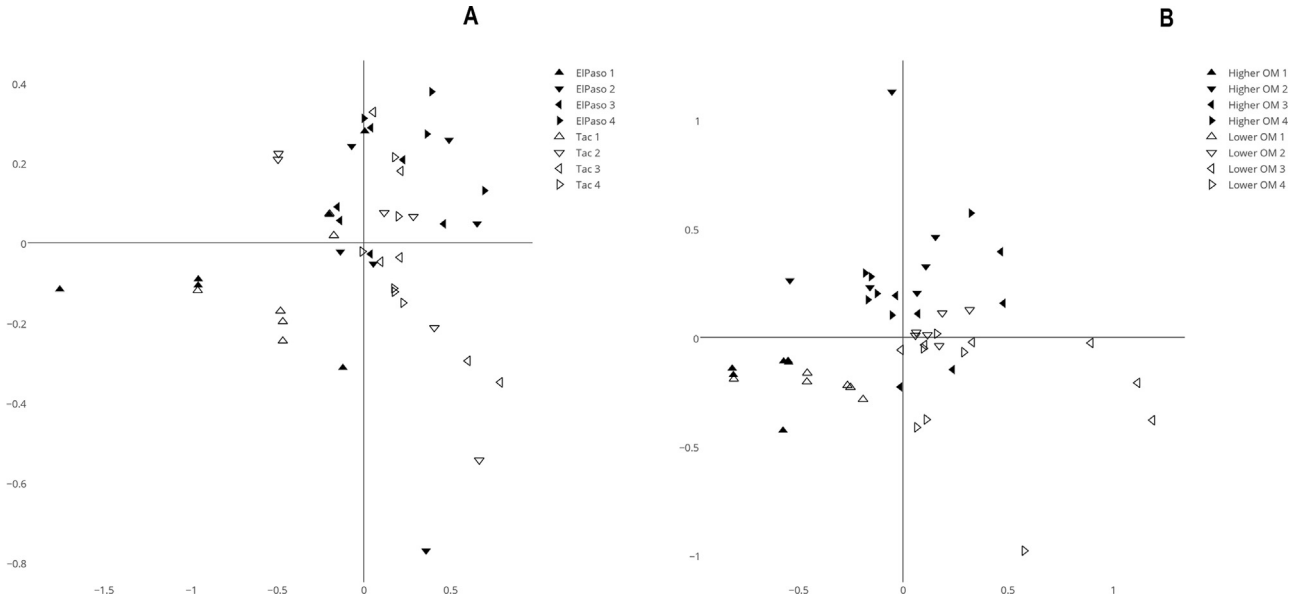

Fig. 7. Non-metric multidimensional scaling (NMS) ordination of T-RFLP profiles of bacterial *amoA* populations. (A) According to the cultivar and sampling time. El Paso samples are symbolized with filled triangles and Tacuarí with open triangles. Sampling times are 1, tillering; 2, flower primordium; 3, draining; and 4, harvest (stress = 0.1132538). (B) According to soil type and sampling time. Higher OM samples are represented with filled triangles and Lower OM samples with open triangles. Sampling times are 1, sowing; 2, tillering; 3, flower primordium; and 4, harvest (stress = 0.1361672).

metabolisms that help them thrive in anoxic environments (Schmidt et al., 2004). Previous studies have found that both AOA and AOB decrease after flooding (Fujii et al., 2010) whereas in others, the opposite tendency has been found (Wang et al., 2014)

The population structure of the AOA and AOB *amoA* gene has recently been studied in many environments, such as forest soil (Boyle-Yarwood et al., 2008), wetland sediments (Wang et al., 2011), compost (Yamada et al., 2013), mangrove (Li and Gu, 2013), ocean (Francis et al., 2005) and paddy soils (Chen et al., 2011). Different approaches have been used to assess these microbial communities, with the most commonly used being DGGE, T-RFLP, clone library, and, more recently, high-throughput sequencing (Huang et al., 2014).

We used T-RFLP of both the archaeal and bacterial *amoA* gene to determine the effects of water management, rice cultivar and soil type on AOA and AOB population structure. The soil type had the strongest effect on population structure. The AOA population was more sensitive than AOB, comprising two very different populations according to soil type. This same result has been previously obtained in studies where the influence of tree species (Boyle-Yarwood et al., 2008), fertilization (Chen et al., 2011), and mycorrhizal colonization (Chen et al., 2013) on AOA and AOB population structure was evaluated. On the other hand, AOB population is more sensitive than the AOA population due to changes in land use (Zhao et al., 2015), fertilization (Wang et al., 2009) and Zn concentration (Vasieladis et al., 2012). Therefore, differential responses of AOA and AOB population structures to environmental changes seem to be case specific, and no prediction can be made about which population is more sensitive.

With the increasing amount of data regarding AOA, it is becoming apparent that, in some soils, almost all archaea carry the *amoA* gene (Leininger et al., 2006). In contrast, AOB is a phylogenetically restricted group, with only 5 genera described thus far (Fiencke et al., 2005). Thus, it is not surprising that AOA population structure is more variable than AOB regarding the geographical origin of the soil. It is possible that the total number of AOA OTUs is higher than that of AOB.

Different genera of ammonia-oxidizing bacteria have different N_2O production rates (Shaw et al., 2006). Thus, the information that is obtained through population structure can be relevant to potential N_2O emissions. We conducted a series of *in silico* digestion using NCBI bacterial *amoA* nucleotides sequences. Sequences belonging to *Nitrosomonas* are often digested at position 167, while sequences belonging to *Nitrosospira* are cleaved at position 140. Nevertheless, in some cases, the digestions were exactly the opposite. Therefore, to assign either OTU 140 or 167 to a specific genus of AOB would be misleading.

The sampling time was the only factor that influenced AOB population structure. Plant and root development may play a significant role in shaping the AOB population, probably by providing surfaces for attaching and nutrients by exudates. Although we examined bulk soil, previous studies have shown that plants influence microbial communities, as apparent in both bulk soil and the rhizosphere (Chen et al., 2008).

Despite both archaeal and bacteria being able to oxidize ammonium, these microorganisms comprise two ecologically very different groups; their responses to changes in the environment are therefore not linked. As they do not share many crucial intermediates in their ammonia oxidation pathway, such as hydroxylamine, their contribution to N_2O emissions may not be equivalent.

From our results, it is apparent that the AOB population structure is more stable than that of AOA, but the opposite occurs regarding gene copy number. One possible explanation is the relative diversity of these two groups of microorganisms. While AOB comprise a phylogenetically restricted group, ammonia oxidation appears to be widespread among archaea. Thus, as environmental conditions influence the abundance of certain AOA OTUs, their total number remains stable, while the opposite occurs to AOB.

Because we used DNA-based strategies to assess changes in the number and populations structure of AOA and AOB, no conclusion about how the active fraction of these populations reacts to the factors analyzed can be drawn. Whether different groups of AOA or AOB increase or decrease their activity in different soils with different water management or different rice cultivars remains an open question.

To know the impact of different agricultural practices on AOA and AOB, populations can be helpful to design practices that are aimed at mitigating greenhouse gas emissions. Therefore, to establish relationships between population structure and N_2O emissions and to determine the microorganisms that are responsible for such emissions needs to be addressed in the short term.

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4. *NIRS* AND *NIRK*-TYPE DENITRIFIER COMMUNITIES ARE DIFFERENTIALLY AFFECTED BY SOIL TYPE, RICE CULTIVAR AND WATER MANAGEMENT

4.1 SUMMARY

Denitrification is one of the main biological sources of N_2O, a greenhouse gas. In paddies, water flooding provides anaerobic soil conditions that can boost denitrification. In this work, the influences of rice cultivar, water management and soil type on the abundance and community structure of denitrifying bacteria were evaluated; *nirS* and *nirK*-type denitrifiers were considered separately. Two microcosm experiments were performed, and the abundance and community structure for both nitrite reductase genes were assessed using qPCR and T-RFLP, respectively. In the first experiment, two rice cultivars and two water managements were evaluated; in the second experiment, two soils, which mainly differed in their organic matter content, and two flooding times were evaluated. The results show that sampling time was the main factor driving *nirS* and *nirK* abundances. The *nirK*:*nirS* ratio, recently proposed as an indicator of the soil N_2O emission potential, was higher in samples taken at the flower primordium stage of rice in both experiments, suggesting that this stage could be a hot moment for N_2O emissions. Soil type was the main factor influencing the *nirS* community structure, while it had no effect on *nirK* communities. Although the *nirK* community structure was not affected by cultivar or soil type, statistical analysis showed that some T-RFs were associated with each cultivar or soil type, indicating that these factors exerted effects on certain subsets of the denitrifying population rather than the whole community. The community structure of *nirS*-type denitrifiers was more sensitive to the analyzed factors than the *nirK*-type, and soil type was the main factor that influenced this community structure.

4.2 RESUMEN

La desnitrificación es una de las principales fuentes biológicas de N_2O, el cual es un gas de efecto invernadero. En los arrozales, la inundación provee condiciones anaeróbicas en el suelo que pueden potenciar la desnitrificación. En este trabajo, se evaluó la influencia del cultivar de arroz, el manejo del agua y el tipo de suelo sobre la abundancia y la estructura de la comunidad de las bacterias desnitrificantes; los desnitrificantes tipo *nirS* y tipo *nirK* fueron considerados por separado. Se realizaron dos experimentos en microcosmos, la abundancia y la estructura de la comunidad de ambos genes de nitrito reductasa fueron evaluadas mediante qPCR y T-RFLP, respectivamente. En el primer experimento, se evaluaron dos cultivares de arroz y dos manejos del agua; en el segundo experimento, se evaluaron dos suelos, que diferían principalmente en el contenido de materia orgánica, y dos tiempos de inundación. Los resultados muestran que el momento de muestreo fue el factor que más incide sobre las abundancias de *nirS* y *nirK*. En ambos experimentos, la relación *nirK*:*nirS*, recientemente propuesta como indicador del potencial emisor de N_2O del suelo, fue mayor en las muestras tomadas en la etapa de primordio floral, lo que sugiere que esta etapa puede constituir un «hot moment» de emisión de N_2O. El tipo de suelo fue el factor con mayor influencia sobre la estructura de la comunidad de *nirS*, mientras que no tuvo efecto sobre la comunidad de *nirK*. A pesar de que la comunidad de *nirK* no fue afectada por el cultivar de arroz, ni por el tipo de suelo, los análisis estadísticos mostraron que algunos T-RFs estaban asociados con determinado cultivar o tipo de suelo, lo que indica que estos factores ejercen efecto sobre un subconjunto de la población desnitrificante y no sobre la comunidad total. La estructura de la comunidad de los desnitrificantes tipo *nirS* fue más sensible a los factores analizados que la de los desnitrificantes tipo *nirK*, el suelo fue el factor que más influenció la estructura de esta comunidad.

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Original article

nirS- and *nirK*-type denitrifier communities are differentially affected by soil type, rice cultivar and water management

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ABSTRACT

Denitrification is one of the main biological sources of N_2O, a greenhouse gas. In paddies, water flooding provides anaerobic soil conditions that can boost denitrification. In this work, the influences of rice cultivar, water management and soil type on the abundance and community structure of denitrifying bacteria were evaluated; *nirS* and *nirK*-type denitrifiers were considered separately. Two microcosm experiments were performed, and the abundance and community structure for both nitrite reductase genes were assessed using qPCR and T-RFLP, respectively. In the first experiment, two rice cultivars and two water managements were evaluated; in the second experiment, two soils, which mainly differed in their organic matter content, and two flooding times were evaluated. The results show that sampling time was the main factor driving *nirS* and *nirK* abundances. The *nirK*:*nirS* ratio, recently proposed as an indicator of the soil N_2O emission potential, was higher in samples taken at the flower primordium stage of rice in both experiments, suggesting that this stage could be a hot moment for N_2O emissions. Soil type was the main factor influencing the *nirS* community structure, while it had no effect on *nirK* communities. Although the *nirK* community structure was not affected by cultivar or soil type, statistical analysis showed that some T-RFs were associated with each cultivar or soil type, indicating that these factors exerted effects on certain subsets of the denitrifying population rather than the whole community. The community structure of *nirS*-type denitrifiers was more sensitive to the analyzed factors than the *nirK*-type, and soil type was the main factor that influenced this community structure.

1. Introduction

Rice is one of the most consumed staple foods worldwide and is cultivated on nearly 155 million ha [1]. Most rice is cultivated in flooded lowlands called paddy fields. Irrigated rice cultivation often includes water regimes that alternate between flooded and drained conditions throughout the crop cycle. Rice is cultivated mainly in tropical and subtropical regions. Uruguay is among the top 12 rice exporters worldwide and contains the southernmost areas in the world where irrigated rice is cultivated [2].

Rice farming in irrigated paddies is responsible for significant amounts of CH_4 emissions, which are the end product of anaerobic organic matter degradation [3]. Rice systems also emit N_2O, and its emission tends to increase when management practices such as the use of mid-season drainages are implemented to reduce CH_4 emissions [4]. The greenhouse potential of nitrous oxide is approximately 300-fold higher than that of CO_2 [5]. Nitrous oxide has an average lifetime of approximately 120 years in the stratosphere and is predicted to be the most important ozone-depleting substance in the future [6]. In soils, N_2O is biologically produced mainly through processes of nitrification and denitrification [7].

Denitrification is an anaerobic respiration in which oxidized forms of nitrogen are used as electron acceptors that are sequentially reduced to gaseous forms [8]. The nitrite reductase enzyme reduces NO_2^- to NO and is present in all denitrifiers. There are two known classes of this enzyme: a copper-containing nitrite reductase, and a cytochrome cd1-containing nitrite reductase, which are encoded by the *nirK* and *nirS* genes, respectively [9]. Recently both genes have been found in the same genome [10]. This is considered an exception, and it has not been confirmed that both enzymes are functional in the same cell.

The complete denitrification pathway leads to N_2 production.

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However, not all denitrifiers have the complete set of denitrifying genes. For instance, many denitrifiers lack the nitrous oxide reductase gene *nosZ*, which reduces N_2O to N_2 [11], and simply emit N_2O as the product of denitrification. Another way that N_2O can be produced by denitrification is through the differential regulation of genes in the denitrifying pathway. Many abiotic factors, including O_2 concentration, modulate these genes expression [12]. In soil where microniches provide environmental heterogeneity and the bacterial community is diverse, a mixture of NO, N_2O and N_2 is produced. The exact ratio of this gas mixture depends on many factors and has been found to vary according to soil type in paddies [13]. In particular, as most denitrifiers depend on carbon compounds as electron donors, soil organic matter content affects denitrification rates [14]. Temporary water-logging and drying/rewetting events are described as "hot moments" for denitrification [15].

Among the environmental factors that shape the denitrifier communities in soil, carbon availability, soil moisture, temperature and pH are considered the most significant [16]. Recent studies also show that *nirS* and *nirK*-type denitrifying communities respond differently to environmental parameters such as nitrate and oxygen concentrations in rice fields [17].

In this work, the effect of different agricultural practices on the abundance and community structure of the *nirK* and *nirS*-type denitrifiers in soil microcosms under rice cultivation was analyzed. Two greenhouse experiments were conducted in two consecutive years mimicking paddy conditions and agricultural practices. In the first experiment the factors tested were rice cultivar and water management (mid-season drainage vs. continuous flooding). In the second experiment, two soil types, each from the two main geographical rice-growing areas in Uruguay, and two times of flooding establishment were evaluated. The rice cultivar and soil types employed are characteristic of Uruguay.

2. Materials and methods

2.1. Greenhouse experiments

Two different greenhouse experiments were conducted. Each experiment consisted of a 2 × 2 factorial design with three replicates; 12 microcosms were set in a randomized block design. Each microcosm was constructed by pouring approximately 60 kg of soil into an impermeable plastic box (40 × 60 × 35 cm). The soil used was collected from paddy fields approximately eight weeks before rice was sowed; only the top 15-cm layer was collected. Soils were obtained from two different paddy fields. The sites represent the two main geographical zones of rice cultivation in Uruguay. Soil 1, referred to henceforth as Eastern soil, is a Typic Argiudoll, with pH = 5.7, 3.4% organic matter, 13 μg g^{-1} P Bray I, 10 μg $g^{-1}NO_3$-N, 0.42 cmol kg^{-1} K, 3.3 cmol kg^{-1} Mg, 5.4 cmol kg^{-1} Ca and 0.83 cmol kg^{-1} Na; it was collected from the Instituto Nacional de Investigación Agropecuaria (INIA) (33°16'10.00″ S; 54°10'04.00″ W). Soil 2, referred to henceforth as Northern soil is a Typic Hapludert, with pH = 5.6, 5.8% organic matter, 10 μg g^{-1} P Bray I, 15 μg $g^{-1}NO_3$-N, 0.62 cmol kg^{-1} K, 9.4 cmol kg^{-1} Mg, 25.5 cmol kg^{-1} Ca and 0.64 cmol kg^{-1} Na; it was collected from a private field (31°22'10″ S; 57°27'45″W). Seeds were sowed in two parallel lines by microcosm at a density that mimics field conditions (equivalent to 170 kg of seeds ha^{-1}). Microcosms were fertilized at sowing with pellets on dry soil (ammonium phosphate (18–46), 25 kg ha^{-1}), at tillering and at flower primordium stage (urea 20 kg ha^{-1}, applied as a 0.2% (w/v) solution); quantities and timing mimicked field conditions. Flooding was established by watering the boxes until a 5-cm layer of water formed above the soil.

2.1.1. First experiment (rice cultivar and water management)

The factors analyzed in the first experiment were rice cultivar and water management (Table 1). The cultivars evaluated were: El Paso 144 (*Oryza sativa* ssp. *indica*) and INIA Tacuari (*O. sativa* spp. *japonica*), hereafter Tacuarí; seeds were provided by INIA. In this experiment only Eastern soil was used. The two water management treatments are referred to as "drained" and "not drained" treatments. Flooding was established at tillering in all treatments and was maintained by watering the boxes periodically; to drain the boxes in the drained treatments, watering was interrupted for 3–4 days. In the drained treatments, microcosms were drained at the flower primordium stage and re-flooded 16 days later; boxes were eventually drained one week before harvest. In the "not drained" treatment, flooding was maintained until one week before harvest. The four treatments were: Tacuarí, drained; Tacuarí, not drained; ElPaso144, drained and ElPaso144, not drained. Each treatment had three replicates.

2.1.2. Second experiment (soil type and time of flooding)

The factors analyzed in the second experiment were soil type and time of flooding (Table 1). In this experiment only cultivar El Paso 144 was planted. Early flooding treatment consisted of flooding of the microcosms at the tillering stage; in the late flooding treatment, microcosms were flooded five days after the flower primordium stage. In both treatments flooding was maintained until one week before harvest. The four treatments were: Eastern soil, early flooding; Eastern soil, late flooding; Northern soil, early flooding and Northern soil, late flooding. Each treatment consisted of three replicates.

Table 1 shows the sampling times of the experiments. They were chosen according to the phenological stage of the plants and corresponded to fertilization and water manipulation times. Each sample consisted of six randomly collected bulk soil cores (2 × 5 cm) mixed together.

2.2. Nucleic acid extraction

DNA was extracted from 0.25 g (dry weight) of soil using the MoBio Power Soil® DNA Isolation Kit (San Diego, CA, USA) following the manufacturer's instructions. The extracted DNA was evaluated for size and yield on a 0.8% agarose gel.

2.3. Quantitative PCR (qPCR) analysis

The abundance of *nirS* and *nirK* genes was determined by real-time PCR using a Line Gene K thermocycler (Bioer Technology) using the fluorescent dye SYBR-Green I. All samples and standards were quantified in triplicate.

The reaction mixture for *nirK* (12.5 μl) contained 6.25 μl of 2 × Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific Inc.), 10 μg of bovine serum albumin, 2.5% (v/v) dimethyl sulfoxide (DMSO) and 0.5 μM each primer: 876F (5'-ATY GGC GGV CAY GGC GA-3') and 1040R (5'-GCC TCG ATC AGR TTR TGG TT-3') [18]. Thermal cycling was as follows: 95 °C for 10 min; six touchdown cycles (95 °C, 15 s; 63 °C (decreasing 1 °C per cycle), 30 s; 72 °C, 30 s); 34 cycles (95 °C, 15 s; 58 °C, 30 s; 72 °C, 30 s and 80 °C, 15 s for data collection), and the program ended with a melt curve from 65 °C to 90 °C [19]. Standard curves were generated by amplifying 10-fold dilutions of a mix of linearized pJET1.2/blunt plasmids containing the *Sinorhizobium meliloti* 1021 *nirK* gene. The PCR efficiency ranged from 92.0% to 99.6%, and the correlation coefficient ranged from 0.985 to 1.

The reaction mixture for *nirS* (12.5 μl) contained 6.25 μl of

Table 1
Factors analyzed and sampling times in the first and second experiment.

	Rice cultivar	Soil type	Water regime	Sampling times
First experiment	El Paso/Tacuarí	Eastern soil	Drained/not drained	tillering/flower primordium/drainage/harvest
Second experiment	El Paso	Eastern soil/Northern soil	Early flooding/Late flooding	sowing/tillering/flower primordium/harvest

2 × Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific Inc.), 10 μg of bovine serum albumin, 2.5% (v/v) dimethyl sulfoxide (DMSO) and 0.3 μM each primer: Cd3aF (5'-GTS AAC GTS AAG GAR ACS GG-3') and R3Cd (5'-GAS TTC GGR TGS GTC TTG A-3') [20]. Thermal cycling was as follows: 95 °C for 10 min, 40× (95 °C, 60 s; 51 °C, 60 s; and 60 °C, 60 s for data collection), and the program ended with a melt curve from 60 °C to 90 °C. Standard curves were generated by amplifying 10-fold dilutions of linearized pJET1.2/blunt plasmid containing the *Pseudomonas stutzeri nirS* gene. The PCR efficiency ranged from 94.9% to 107.2%, and the correlation coefficient ranged from 0.997 to 1. A series of two-fold dilutions of the soil DNA samples were run to check for inhibitors.

Gene abundances were standardized by the mass of DNA that was extracted per gram of dry soil and $\log_{10}$ transformed before analysis.

2.4. Terminal restriction fragment length polymorphism (T-RFLP) analysis

The community structure of denitrifiers was determined through T-RFLP analysis. Nitrite reductase genes were amplified using the primers nirK1F (5'-GGM ATG GTK CCS TGG CA-3') [9] and 1040R for *nirK*; and Cd3aF and R3Cd for *nirS*. Both forward primers were labeled with 6-carboxyfluorescein (FAM). The PCR reaction mixtures contained: 25 μl 10 × Taqbuffer, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM each *nirK* primer or 1.0 μM each *nirS* primer, 2.5% DMSO, 0.004% BSA, 1U of Taq DNA polymerase (recombinant, Thermo Scientific) and 2 μl of template (2–5 ng of DNA) in a total volume of 25 μl. The PCR cycle for *nirK* consisted of an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of 95 °C for 45 s, 60 °C for 45 s, 72 °C for 45 s, and a final elongation step of 72 °C for 10 min. The PCR cycle for *nirS* consisted of an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of 95 °C for 45 s, 58 °C for 45 s, 72 °C for 45 s, and a final elongation step of 72 °C for 10 min. The PCR products were checked on a 1.2% agarose gel, and only single-band products of the expected size were collected for further analysis. The PCR products were purified with the AxyPrep PCR Clean-up Kit (Axygen Biosciences). The purified products were quantified with Nanodrop® 2000c UV–vis spectrophotometry. To reduce bias, three PCR products were pooled prior to purification. Aliquots containing 100 ng of purified PCR product were digested with the tetrameric restriction enzyme *Hae*III (Thermo Fisher Scientific) (recognition site: 5'-GG/CC-3') at 37 °C for 4 h, after which the enzyme was inactivated at 80 °C for 20 min.

Restriction fragments were sent to Macrogen Inc. (Seoul, Republic of Korea) where they were analyzed using an ABI 3730XL DNA analyzer (Applied Biosystems). The size of each terminal restriction fragment (T-RF) was determined by comparison to a fluorescently labeled size standard (GeneScan TM 500 LIZ TM) using the Peak Scanner TM Software v1.0 (Applied Biosystems).

The relative abundance of individual T-RFs was calculated as the percentage of total peak area in a given T-RFLP profile. Only those T-RFs with a relative abundance greater than 1% were considered. Terminal fragments that were smaller than 30 bp were omitted. T-RFs were designed with a K or an S for the *nirK* and *nirS* gene fragments, respectively, followed by the size in base pairs of the corresponding fragment. The number of T-RFs (N), and Shannon (*H'*) and Pielou (*J'*) indexes [21] were calculated for each sample.

2.5. Statistical analysis

The number of T-RFs per sample, Shannon and Pielou indexes and gene abundance were analyzed using a factorial analysis of variance (ANOVA) with Tukey's HSD post-hoc analysis to determine the significance of sampling time, water management, soil type and rice cultivar within each experiment. The normality and variance homogeneity were checked with Shapiro-Wilk and Levene's tests, respectively. The software package STATISTICA 8.0 (StatSoft, Inc.) was used.

T-RFLP were transformed into numerical data using the software Peak Scanner TM 1.0 (Applied Biosystems), binned and normalized with the software T-REX [22] and exported to an excel spreadsheet. The non-metric multidimensional scaling (NMS) of the T-RFLP data matrix was performed using the "vegan" package [23] within the R Statistical software (R Core Team 2014). Multi-response permutation procedures (MRPP) were used to test the statistical significance of community composition between different treatments, using the "vegan" package. Indicator species analysis was performed using the "indicspecies" package [24] within the R Statistical software. When multiple comparisons were performed, the p-values were corrected using the Benjamini-Hochberg procedure [25].

3. Results

3.1. Rice cultivar and water management (first experiment)

3.1.1. Abundance of nitrite reductase genes

The results of the first experiment showed that cultivar had a significant effect on the abundance of the *nirS* gene. Higher numbers were observed in the Tacuarí treatment compared with El Paso treatment (Table 2). According to the sampling time, the highest *nirS* numbers were observed at harvest while the lowest numbers were observed at tillering and at flower primordium (Fig. 1A). Overall, the *nirS* abundance showed a tendency to increase throughout the experiment, regardless of the water management condition. There was also a significant interaction between the sampling time and rice cultivar. The cultivar El Paso at tillering had the lowest number of *nirS* compared to any other cultivar × sampling time condition (Fig. 1A).

The *nirK* abundance was not affected by water management and rice cultivar; differences were observed according to sampling time (Fig. 1B, Table 2). Samples taken at harvest had the highest *nirK* number, while the lowest gene number was observed at tillering. Similar to *nirS*, the *nirK* gene showed an increase in gene number throughout the experiment.

In all of the samples, the *nirK* gene was more abundant than *nirS*. In the first experiment, the cultivar and sampling time had significant effects on the *nirK*:*nirS* ratio. Samples from the El Paso cultivar had a significantly higher ratio than samples from Tacuarí (1.90 ± 0.35 and 1.75 ± 0.32; El Paso and Tacuarí, respectively). According to the sampling time, the highest ratio was observed at

Table 2
Effect of rice cultivar or water management on *nirS* and *nirK* gene abundances. Values were $\log_{10}$ transformed and correspond to averages of all samples of each variable (standard deviation). Asterisks indicate statistically significant differences between treatments (Tukey's HSD, $p < 0.05$).

	Cultivar		Water management	
	El Paso (n = 24)	Tacuarí (n = 24)	Drained (n = 12)	Not drained (n = 12)
nirS	7.44 (0.65)*	7.61 (0.49)*	8.02 (0.40)	7.84 (0.47)
nirK	9.46 (0.27)	9.39 (0.32)	9.60 (0.35)	9.46 (0.27)

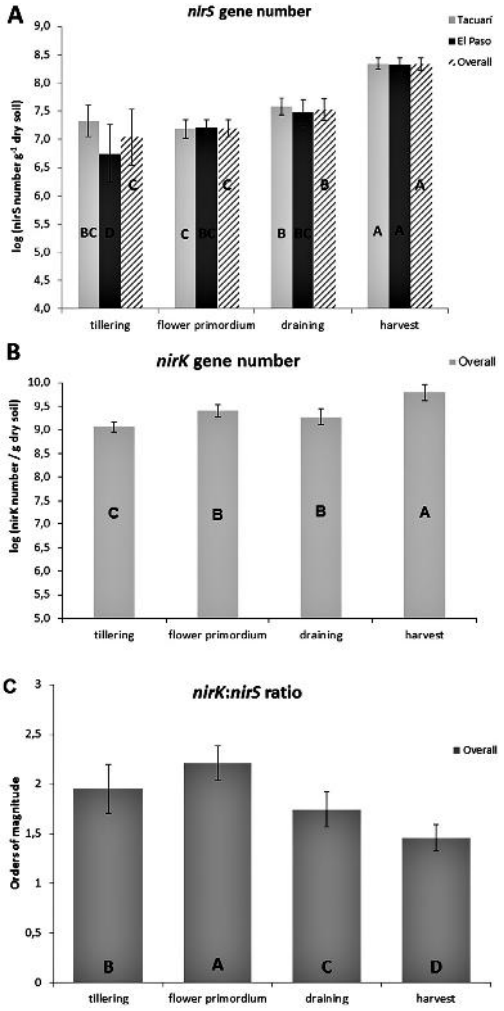

Fig. 1. Abundance of denitrifying genes in the first experiment. (A) *nirS* abundance according to sampling date and to rice cultivar. Light gray bars represent abundance on "Tacuarí" cultivar and dark gray on "El Paso" cultivar. Striped bars represent overall abundance. (B) *nirK* abundance according to sampling date. (C) *nirK*:*nirS* ratio according to sampling date. Different letters on striped bars (A) indicate significant differences (Tukey's test $p < 0.05$) in different sampling date. Different letters on gray bars indicate significant differences according to sampling date x rice cultivar interaction (A) or differences according to sampling date (B, C) (Tukey's test $p < 0.05$). Gray bars represent means (n = 3) ± SDs. Striped bars represent means (n = 6) ± SDs.

flower primordium, which was significantly higher than that observed at any other sampling time (Fig. 1C).

3.1.2. Nitrite reductase gene community structure

3.1.2.1. nirS-type denitrifiers community structure. In the first experiment, a total of 31 different T-RFs were obtained; eight were present in less than three samples. The most abundant *nirS* T-RF was S133 irrespective of the treatment (Fig. S1A). Indicator species analysis identified four *nirS* T-RFs as associated with different sampling times (Table 3). One T-RF was associated with the first three sampling times and three T-RFs with the last three sampling times. This analysis also found one T-RF (227) to be significantly associated with samples from the El Paso cultivar. No T-RF was associated with any water management treatment.

Sampling time had a significant effect on N, and *H′* and *J′* indexes. Shannon and Pielou indexes were significantly higher in the flower primordium samples than at any other sampling time (Table S1). Tillering exhibited the lowest N, *H′* and *J′* values. An interaction between sampling time and rice cultivar was observed for N and *H′*. For instance, at draining N was higher in El Paso than in Tacuarí samples.

The samples ordinated in the NMS scatter plot clustered together mainly according to sampling time (Fig. 2A). Samples from the tillering stage clustered at the left end of the ordination space, while those from flower primordium clustered at the upper right quadrant. There was a separation of the samples according to cultivar only for the samples taken at drainage and harvest. This separation was clearer in the samples collected at drainage. To test the statistical significance of differences in community structures, the MRPP test was performed. The results showed that communities were significantly different according to sampling time ($A = 0.1924$, $p < 0.001$); the only sampling time with a delta value higher than expected was drainage, which can be explained by the cultivar effect at this sampling time. The MRPP results also showed only a small difference in the *nirS* community structure of the samples from different cultivars, ($A = 0.03324$, $p = 0.007$). No significant difference was observed between the communities from samples of different water managements ($A = 0.01044$, $p = 0.12$). Nevertheless, when analyzing the effect of rice cultivar for each sampling time, it was found that the *nirS* community structure from different cultivars differed significantly in the drainage samples (see "TAC C" and "EP C" convex hulls in Fig. 2A) ($A = 0.271$, $p = 0.006$).

3.1.2.2. nirK-type denitrifiers community structure. A total of 96 different T-RFs were obtained; 45 of these were present in less than three samples. The two most abundant *nirK* T-RFs of the first experiment were K63 and K59, irrespective of the treatment (Fig. S1B). The indicator species analysis showed an effect of the cultivar and the sampling time on certain T-RFs: one T-RF associated with El Paso and two T-RFs associated with Tacuarí treatments (Table 3). Two T-RFs were associated with the tillering samples, and one each with the flower primordium and harvest samples. K73 was associated with the first three sampling times and K511 was associated with the last three.

There was no effect of any of the independent variables on N. However, sampling time × cultivar interaction was significant. The number of *nirK* T-RFs in the samples from Tacuarí at tillering was significantly higher than in any other sampling time × cultivar condition (Table S2). Shannon index was influenced by the sampling time and by sampling time × cultivar interaction. Samples

Table 3
List of all terminal restriction fragments (T-RFs) found by the indicator species analysis as associated with each rice cultivar, or sampling time on the first experiment. Only those variables for which associated T-RFs were found are shown. Values on each treatment column are the statistic given by the analysis; numbers closer to 1 signify a stronger association between the T-RF and the treatment.

nirS/*nirK*	T-RF	Tacuarí	El Paso	Tillering	Flower primordium	Drainage	Harvest
nirS	S270			0.889**	0.889**	0.889**	
nirS	S78				0.941***	0.941***	0.941***
nirS	S259				0.867**	0.867**	0.867**
nirS	S188				0.828***	0.828***	0.828***
nirS	S227		0.725***				
nirK	K43	0.500*		0.707***			
nirK	K69			0.707***			
nirK	K37				0.61*		
nirK	K74						0.816***
nirK	K73			0.816***	0.816***	0.816***	
nirK	K511				0.782**	0.782**	0.782**
nirK	K283		0.570*				
nirK	K56	0.500*					

Significance codes: *p < 0.05; **p < 0.01; ***p < 0.001 (permutation test).

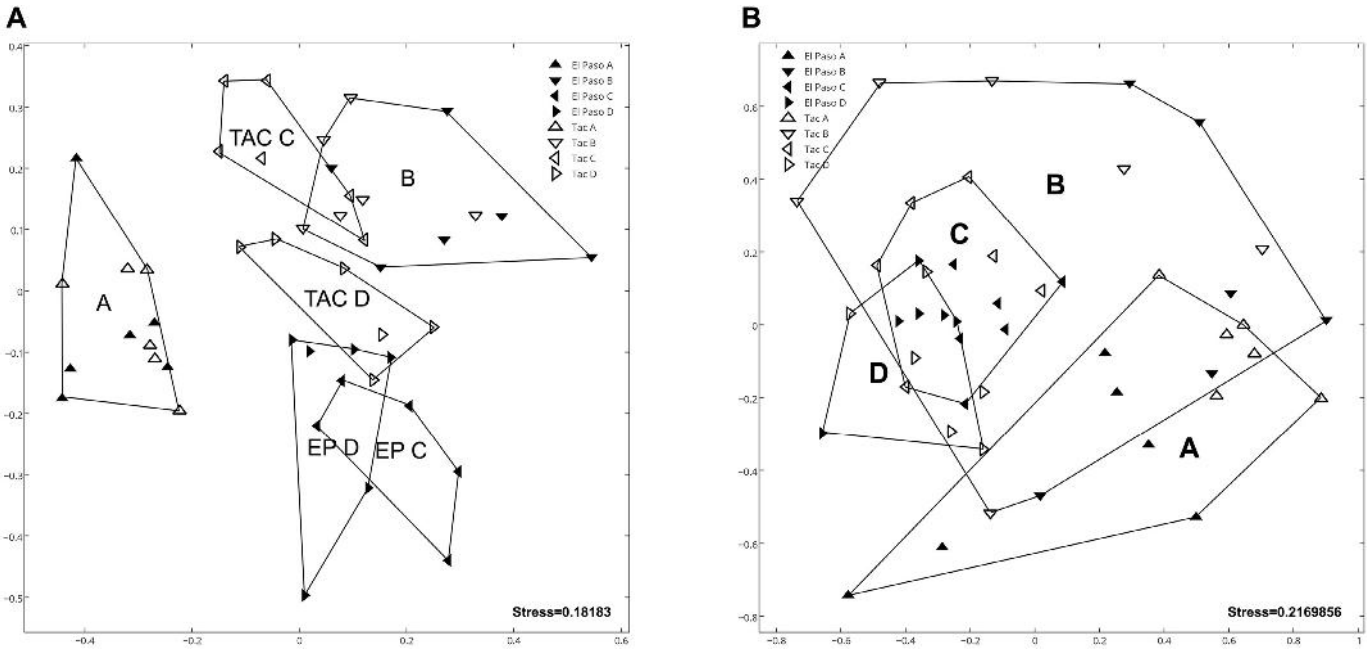

Fig. 2. Non-metric multidimensional scaling (NMS) ordination of terminal restriction fragment length polymorphism (T-RFLP) profiles of denitrifying genes communities in the first experiment. *nirS* (A) and *nirK* (B) communities according to the rice cultivar and sampling time. El Paso samples are symbolized with filled triangles and Tacuarí (Tac) with open triangles. Sampling times are A, tillering; B, flower primordium; C, drainage; and D, harvest. Convex hulls were constructed according to sampling time (A and B) and to sampling time x rice cultivar (TAC C, TAC D, EP C and EP D).

taken at harvest had the lowest *H'* values observed. In addition, *H'* was significantly higher in Tacuarí at tillering than in El Paso at tillering. The Pielou index showed the same trend as mentioned for N and *H'* and was significantly lower in the harvest samples (Table S2).

In the NMS space, no clustering of the samples according to the cultivar was evident (Fig. 2B). This is in agreement with the MRPP results between cultivars (A = −0.0001343, p = 0.396), which showed no effect of rice cultivar on community structure. No effect of water management was detected with the MRPP and no T-RF was found associated with any of the water management treatments. However, sampling time had a significant effect on the *nirK* community structure according to the MRPP results (A = 0.1331, p = 0.001). Only samples from the flower primordium stage did not group together significantly (46.19 and 37.2; observed and expected delta, respectively).

3.2. Soil type and time of flooding (second experiment)

3.2.1. Abundance of nitrite reductase genes

In the second experiment, all three independent variables (soil type, time of flooding, and sampling time) had significant effects on *nirS* gene abundance. Late flooding treatment had higher *nirS* average gene number than early flooding (Table 4). Soil type affected *nirS* abundance, which was significantly higher in the Northern soil (Table 4). According to sampling time, sowing samples had the highest gene number, while the lowest was observed at flower primordium (Fig. 3A). There was a significant interaction between sampling time and soil type and also between sampling time and time of flooding. Differences in the *nirS* abundances between soil types were observed only at tillering and at flower primordium (Fig. 3A), while differences between the time of flooding treatments were observed only at tillering and at flower primordium (Fig. S2).

Table 4
Denitrifying genes number according to soil type or time of flooding in the second experiment. Values were $\log_{10}$ transformed and correspond to averages of all samples of each variable (standard deviation). Asterisks indicate statistically significant differences between treatments (Tukey's HSD, $p < 0.05$).

	Soil type		Time of flooding	
	Northern soil (n = 24)	Eastern soil (n = 24)	Late flooding (n = 18)	Early flooding (n = 18)
nirS	7.07 (0.37)*	6.91 (0.54)*	6.96 (0.22)*	6.62 (0.37)*
nirK	9.01 (0.28)*	8.79 (0.19)*	9.00 (0.26)*	8.79 (0.26)*

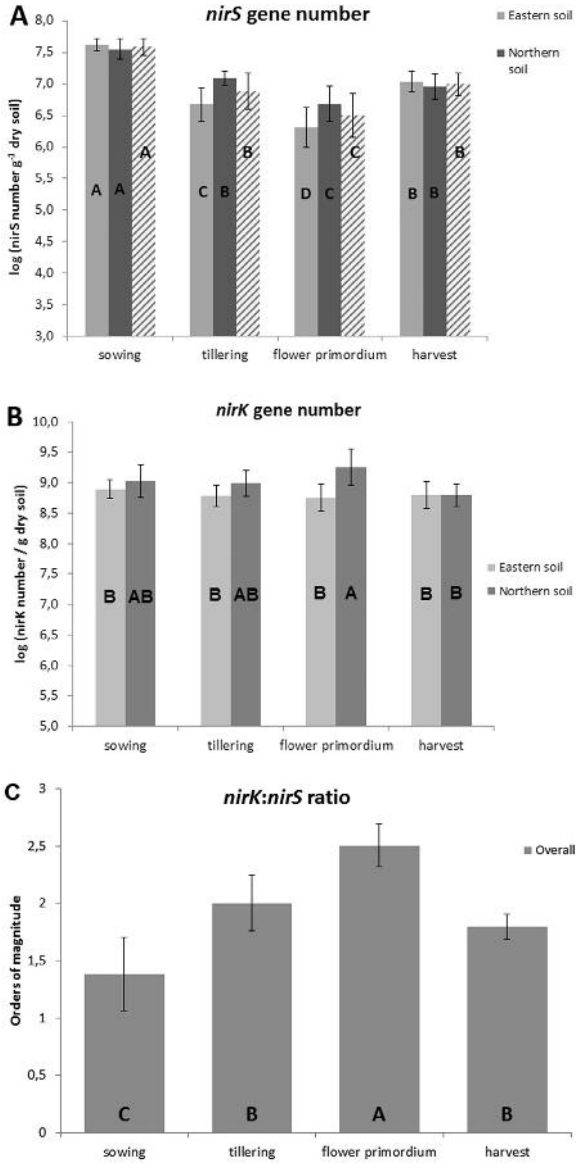

Fig. 3. Abundance of denitrifying genes in the second experiment. (A) *nirS* abundance according to sampling date and to soil type. Light gray bars represent abundance on "Eastern" soil and dark gray on "Northern" soil. Striped bars represent overall abundance. (B) *nirK* abundance according to sampling date and to soil type. Light gray bars represent abundance on "Eastern" soil and dark gray on "Northern" soil. (C) *nirK*:*nirS* ratio according to sampling date. Different letters on striped bars (A) indicate significant differences (Tukey's test $p < 0.05$) in different sampling date. Different letters on gray bars indicate significant differences according to sampling date x soil type interaction (A, B) or differences according to sampling date (C) (Tukey's test $p < 0.05$). Gray bars represent means (n = 3) ± SDs. Striped bars represent means (n = 6) ± SDs.

Soil type and flooding time significantly affected *nirK* abundance (Table 4), while sampling date had no effect on the abundance of this gene. Late flooding led to significantly higher *nirK* numbers. If the tillering sample is not accounted for, the average gene number of *nirK* per gram of dry soil weight for late and early flooding were 1.1×10^9 and 5.6×10^8, respectively, which indicates that this difference was accentuated in the late stages of the experiment. As observed for *nirS*, Northern soil harbored higher numbers of the *nirK* gene than Eastern soil (Table 4). There was also a significant interaction between sampling time and soil type. The Northern soil samples taken at flower primordium had a significantly higher number of *nirK* genes than the Eastern soil samples at any sampling time (Fig. 3B).

Only sampling time had a significant effect on the *nirK*:*nirS* ratio. The abundance of the *nirK* gene relative to the *nirS* gene number was highest in the flower primordium samples (Fig. 3C).

3.2.2. Nitrite reductase genes community structures

3.2.2.1. nirS-type denitrifiers community structure. In the second experiment, the total number of T-RFs observed was 36, ten of which were present in less than three samples. The most abundant *nirS* T-RF was S75, which was prominently the most abundant in the Northern soil treatment (Fig. S3A). Indicator species analysis identified two *nirS* T-RFs associated with Northern soil samples and three with Eastern soil samples (Table 5). In Northern soil samples, two T-RFs were associated with flower primordium samples, and one with harvest samples. S187 was associated with the first two sampling times. In Eastern soil samples, S43 was associated with the flower primordium samples and S78 with all sampling times excluding sowing.

Sampling time had a significant effect on N and *J'* but no effect on the *H'* index. The number of T-RFs observed was highest at flower primordium, but *J'* index in this sampling time was the lowest (Table S1). An interaction between sampling time and soil type was observed for N and *H'*. The number of T-RFs at sowing was lower than at flower primordium only for the Northern soil samples. Samples from Northern soil at sowing had the lowest value for *H'* and were significantly lower than for any other soil type × sampling time combination.

In the NMS, the samples were clearly separated according to soil type. Samples from the Eastern soil were located at right, while those from the Northern soil were located on the left of the ordination space (Fig. 4A). Accordingly, the MRPP test showed that samples from different soils harbored *nirS* communities with different structures ($A = 0.1956$, $p = 0.001$). Due to this strong effect of soil type, time of flooding and sampling time effects were analyzed separately for each soil treatment. MRPP showed that communities from both Northern and Eastern soils were affected by sampling time ($A = 0.3018$, $p = 0.001$; $A = 0.2327$, $p = 0.001$, respectively). The time of flooding had no effect on the *nirS* community structure of Northern or Eastern soil samples.

3.2.2.2. nirK-type denitrifiers community structure. In the second experiment, the total number of T-RFs observed was 105. Forty-seven T-RFs were present in less than three samples. The most abundant *nirK* T-RFs were K63 in the Northern soil samples and K54 in the Eastern soil samples (Fig. S3B). Indicator species analysis found two and three T-RFs associated with Northern and Eastern

Table 5
List of all terminal restriction fragments (T-RFs) found by the indicator species analysis as associated with each soil type, or sampling time on the second experiment. Only those variables for which associated T-RFs were found are shown. Values on each treatment column are the statistic given by the analysis; numbers closer to 1 signify a stronger association between the T-RF and the treatment.

nirK/*nirS*	T-RF	Northern soil	Eastern soil	Sowing	Tillering	Flower primordium	Harvest
nirS	S362	0.816***					
nirS	S270		0.890***				
nirS	S78		0.791***				
nirS	S211		0.791***				
nirS	S114	0.612**				0.852[a]**	
nirS	S224					0.850[a]**	
nirS	S43					0.816[b]**	0.913[a]***
nirS	S187			0.913[a]***	0.913[a]***		
nirS	S78				0.913[b]**	0.913[b]**	0.913[b]**
nirK	K319	0.677***					
nirK	K101	0.636*					
nirK	K61		0.687**				
nirK	K66		0.681**				
nirK	K154		0.536*				

Significance codes: *p < 0.05; **p < 0.01; ***p < 0.001 (permutation test).
[a] Associated to the indicated sampling date only in "Northern soil" samples.
[b] Associated to the indicated samplings date only in "Eastern soil" samples.

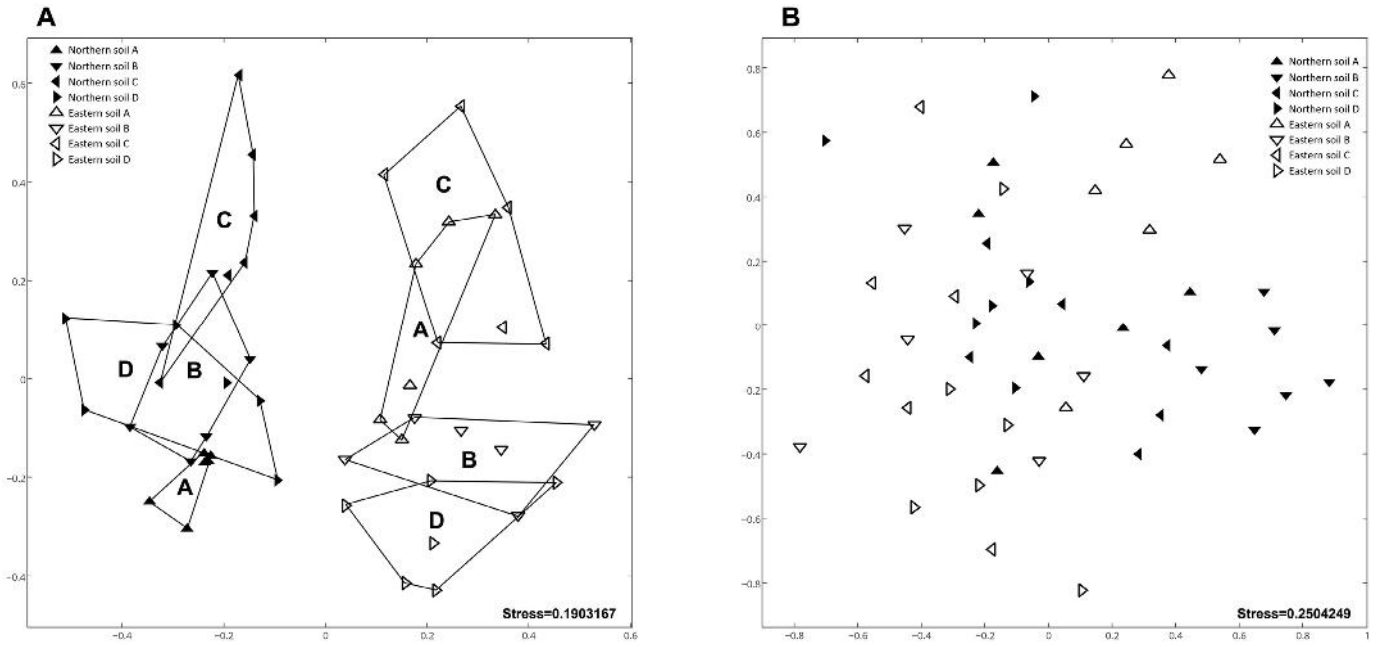

Fig. 4. Non-metric multidimensional scaling (NMS) ordination of terminal restriction fragment length polymorphism (T-RFLP) profiles of denitrifying genes communities in the second experiment. *nirS* (A) and *nirK* (B) communities according to the soil type and sampling time. "Northern soil" samples are symbolized with filled triangles and "Eastern soil" samples with open triangles. Sampling times are A, sowing; B, tillering; C, flower primordium; and D, harvest. Convex hulls were constructed according to sampling time x soil type (A).

soil samples, respectively (Table 5). In this experiment, there was no effect of any of the independent variables analyzed on N, *H'* or *J'* (Table S2). In examining the ordination of the samples in the NMS space, no arrangement due to soil type or sampling time was clear (Fig. 4B). In coincidence with this, the MRPP results showed that soil type and sampling time had a slight influence on the *nirK* communities (A = 0.03241, p = 0.001; and A = 0.03554, p = 0.001, respectively). The MRPP results of flooding time showed no significant effect of these variables on the *nirK* community composition (A = −0.003636, p = 0.667).

4. Discussion

Denitrification is primarily affected by availability of C sources, nitrate concentration, O_2 availability, and temperature. However, as concluded by many studies, denitrification rates and abundance of denitrifying microorganisms are not always correlated [26]. Therefore, it is not surprising that the abundance of the denitrifying genes did not follow some predictions based on denitrification optimum parameters, such as anaerobic environments or soil with high organic matter content. In our case, and in accordance with the mentioned predictions, Northern soil samples presented higher *nir* genes abundance than samples from Eastern soil. However, late flooding treatment resulted in higher nitrite reductase gene number than early flooding treatment, suggesting that anaerobic conditions sustained for longer times did not increase denitrifier communities. On the other hand, as most denitrifying microorganisms are heterotrophs, the composition of the plant exudates

can affect the denitrifying abundance, as shown by Henry et al. [27] in an experiment with artificial exudates. Although the effect of rice cultivar on *nirS*-type denitrifier abundance was less than half an order of magnitude, it was statistically significant, and can be partially attributed to the difference in the exudates of both cultivars.

Sampling time was one of the main factors affecting *nir* genes abundance. The higher number observed at the end of the first experiment can be partially explained by the rhizosphere effect. Although we did not sample rhizosphere soil specifically, plant growth determined that at late sampling times most of the sampled soil was in contact with rice roots. The rhizosphere has been shown to increase denitrifier abundance, although the results also show that *nirK* and *nirS* abundances do not respond equally to the rhizosphere effect [28]. In the second experiment, the high levels of *nirS* gene number observed at the sowing samples were probably a consequence of native communities of the soil collected to set up the experiment, since these samples were taken immediately after the experiment began.

The abundance of denitrifying genes observed in our samples were high compared to some previous studies [29,30]. In particular, in the case of *nirK*, they reached a maximum of 1.7×10^{10} genes per gram of dry soil, which is near the maximum obtained in some studies for bacterial 16S rRNA gene numbers [31]. However, similar numbers had been reported previously for the *nirK* gene in a riparian soil using the same molecular approach that we used [32]. Using qPCR, *nirS* numbers per gram of soil above 10^9 have been observed in constructed wetlands [33]. In some soils, *narG* has been found to be as abundant as the 16S rRNA gene, with a maximum gene number of 1.12×10^{11} per gram of dry soil [34].

In light of genomic data that shows that *nirS*-type denitrifiers tend to have a complete denitrification pathway compared to the *nirK*-type [11], the *nirS*:*nirK* ratio has been recently proposed as a significant indicator of the N_2O sink capacity of a soil [35]. In our samples, *nirK* was more abundant than *nirS* by an average of two orders of magnitude. Significantly, this parameter was higher at the flower primordium stage in both experiments, indicating that this stage can be relevant in N_2O emission potential from rice paddies and may constitute a hot moment for denitrifier-produced N_2O. N_2O peaks have been detected at approximately this stage of the crop in a rice field where soils for this assay were collected [36]. The *nirK* gene has been found to be more abundant than *nirS* in paddy soils in a chronosequence that dates back 2000 years [37]. Yoshida et al. [30] also found higher *nirK* numbers in paddies throughout the growing season, but the *nirK*:*nirS* ratio decreased after flooding. The decrease in *nirK*:*nirS* ratio after flooding was expected because *nirS* denitrifiers are alleged to prefer more constant anaerobic conditions [33].

With respect to the diversity of the nitrite reductase population, the number of T-RFs obtained was higher for *nirK* than for *nirS* gene in both experiments. However, these two genes were amplified by two different sets of primers, and the extent to which each pair of primers introduce bias is unknown. This issue has been observed in previous studies in different environments, such as peat soil [38], eutrophic lakes [39] and agricultural soils [40]. For instance, it is known that the *nirS* primers used in this study are biased against five phyla [41], while the *nirK* primers employed fail to amplify archaeal *nirK* and bacterial class I and II *nirK* [41]. Therefore, a comparison of the results between these two genes must be considered cautiously. Similarly, two different sets of primers were employed to obtain data for *nirK* abundance and population structure, and it is probable that the amplification biases imposed by these primers are different.

Among the factors analyzed, soil type exerted the strongest influence on the denitrifying community, particularly on the *nirS*-type denitrifiers. In a previous study conducted with the same samples, soil type was the major factor driving the ammonia oxidizing community structure [42]. This differential influence of environmental factors on *nirS* and *nirK* communities has been previously observed. Dandie et al. [32] compared riparian and agricultural soils using DGGE and found that *nirK*, but not *nirS*, communities were different in all three months sampled. Conversely, *nirK* and *nirS* communities were equally influenced by plant species in coastal wetlands [43]. Whether these two denitrifying groups are ecologically equivalent is a fundamental question that must be studied; the differential responses of *nirK* and *nirS*-type denitrifiers to the factors analyzed in this study showed that they are not ecologically redundant.

The only factor that had a clear effect on communities of *nirK*-type denitrifiers in the first experiment was sampling time. The time points for sampling represented different rice development stages. The effect of sampling time on *nirK* communities associated with plants has been observed previously [44] indicating that in some cases these communities can be sensitive to plant development; plant species affected the *nirK* community, while the functional group of the plant (grass or forb) did not. Sampling time has previously been observed as a main factor influencing denitrifier communities; Wolsing and Priemé [45] found that seasonal variation better explained community structure variation than fertilization source and dosage. Although MRPP found no influence of the factors studied on the *nirK* community structure, the indicator species analysis identified different *nirK* T-RFs associated with each rice cultivar or soil type evaluated. Thus, although rice cultivar and soil type did not have an effect that impacted the community structure of *nirK*, certain T-RFs were affected. This is in agreement with the broad phylogenetic and metabolic spectrum that denitrifiers comprise. Other techniques such as next generation DNA sequencing that avoid some of the drawbacks of T-RFLP may be used to deepen the knowledge of these communities and their response to environmental factors and agricultural practices.

How denitrifier communities relate to N_2O emissions potential is still not fully understood [29,46]. DNA studies provide information about the presence of these microorganisms, but give no data about their metabolic activity. As denitrifying microorganisms are very diverse metabolically and denitrification is an opportunistic growth mechanism, they can be active and grow by relying on other electron acceptors. Even so, DNA-based approaches offer relevant data about the effects of environmental factors on microbial communities and represent a first approach to this knowledge.

In conclusion, we found that in these microcosms, the *nirK* and *nirS* gene abundance changed with the rice growth cycle. In our experiments, *nirK* was more abundant than *nirS*. The flower primordium stage had the higher *niK*:*nirS* ratio, suggesting that this stage may be a hot moment for N_2O emissions. Soil type mostly differed with the organic matter content and strongly influenced the *nirS* community structure. We found that *nirK* and *nirS* denitrifiers were differentially affected by the factors analyzed, supporting the hypothesis that they belong to two distinct guilds and have different ecological significance.

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POS_2011_1_3380].

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejsobi.2016.11.003.

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5. HETEROTROPHIC DENITRIFICATION AND PARACOCCUS SPP. AS TOOLS FOR BIOREMEDIATION

5.1 SUMMARY

Denitrifiers comprise a metabolically diverse group of microbes used as a resource for environmental engineering, due to their ability to perform anaerobic respiration. The main use of denitrification in environmental sustainability is the removal of nitrate and nitrite in water treatment plants.

Heterotrophic denitrifiers are those that use organic molecules as C sources, including pollutants, a trait that makes them as a potential tool for many bioremediation processes. A notorious advantage of denitrifiers over other microorganisms is that they are able to degrade pollutants in anaerobic environments, which extend their potential usefulness.

In this chapter, recent advances regarding the use of heterotrophic denitrifiers in environmental sustainability will be discussed. We end the chapter discussing the singularities of denitrifying strains of the genus *Paracoccus* and their potentiality in environmental sustainability.

5.2 RESUMEN

Los desnitrificantes comprenden un grupo de microbios metabólicamente diverso, y debido a su capacidad de realizar una respiración anaeróbica son un recurso utilizado en la ingeniería ambiental. El principal uso de la desnitrificación en la sustentabilidad ambiental es la remoción de nitrato y nitrito llevada a cabo en plantas de tratamiento de aguas.

Los desnitrificantes heterótrofos son aquellos que usan moléculas orgánicas como fuente de C, incluso moléculas contaminantes, atributo que los convierte en herramientas potenciales para muchos procesos de biorremediación. Una ventaja notoria de los desnitrificantes sobre otros microorganismos es que son capaces de degradar compuestos contaminantes en ambientes anaeróbicos, lo que aumenta su utilidad potencial.

En este capítulo, se discutirán los avances recientes relacionados con el uso de desnitrificantes heterótrofos en la sustentabilidad ambiental. El capítulo finaliza discutiendo las singularidades de ciertas cepas del género *Paracoccus* y su potencialidad en la sustentabilidad ambiental.

Heterotrophic Denitrification and *Paracoccus* spp. as Tools for Bioremediation

10

Gastón Azziz, Gabriela Illarze, and Pilar Irisarri

Abstract

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10.1 Introduction

Denitrification is a biological process where nitrogen oxides (i.e., NO_3^-, NO_2^-) are converted to reduced forms. The main products of denitrification are N_2O and N_2, which are in gaseous state in all environmental conditions. Microorganisms capable of performing denitrification are collectively called denitrifying microorganisms or denitrifiers. These microorganisms comprise a phylogenetically broad and diverse

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group, with representatives in the domains Bacteria, Archaea, and Eukarya. Most well-known and characterized denitrifiers, however, belong to the Bacteria domain (Long et al. 2015). In the Eukarya domain, a few fungi species are able to denitrify (Shoun et al. 1992).

Not every biological N oxide reduction is considered to be denitrification. The term denitrification is restricted to the use of N oxides as electron acceptors in a respiratory chain, coupled with ATP synthesis for energy conservation. Most denitrifying microorganisms are facultative anaerobes and therefore can use either O_2 or certain N oxides as electron acceptors (Zumft 1997). With a few exceptions, such as *Bacillus azotoformans* (Nielsen et al. 2015) that is unable to use O_2 as electron acceptor, no obligate denitrifiers are known. Thus, denitrification is a process that is favored in anaerobic or microaerobic environments.

As a very diverse group of microorganisms, denitrifiers can use different compounds as electron sources. Regarding their preferred electron source, denitrifiers can be separated into heterotrophic and autotrophic denitrifiers. Heterotrophic denitrifiers use organic compounds as electron source and comprise a metabolically diverse group. The process by which organic molecules serve as electron donors in a denitrification process is called heterotrophic denitrification. On the other hand, autotrophic denitrifiers use inorganic molecules as electron donors. Methane can also be used as electron donor by a group of denitrifiers called denitrifying methanotrophs (He et al. 2015). Autotrophic denitrification is widely used in environmental sustainability; however, to discuss such examples is beyond the scope of this chapter.

The complete denitrification pathway comprises the conversion of NO_3^- to N_2 (Fig. 10.1). This reduction occurs in a stepwise manner with the following intermediates: NO_2^-, NO, and N_2O:

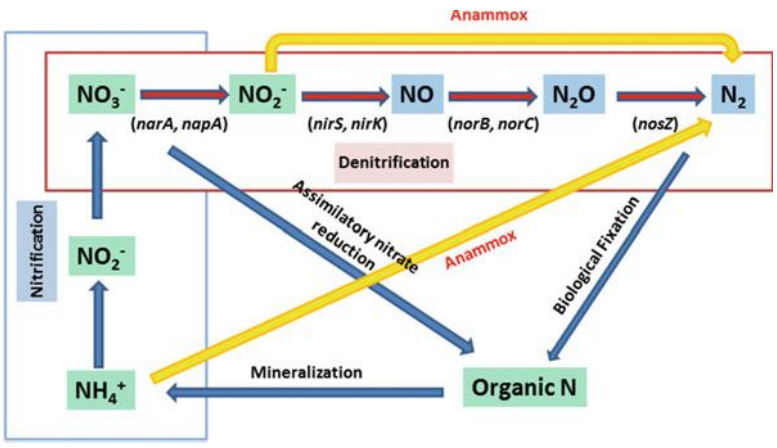

Fig. 10.1 Overview of the microbiological nitrogen cycle. The denitrification pathway is outlined in a *red square* and the names of the genes that code for the different enzymes active sites are included. Gaseous products are depicted as *blue boxes*

(i) The conversion of nitrate to nitrite (NO_2^-) is catalyzed by either a periplasmic or a membrane-bound respiratory nitrate reductase (Bell et al. 1990), whose catalytic subunits are encoded by the *napA* and *narG* genes, respectively. Interestingly, *narG* has also been found in non-denitrifying microorganisms such as *Escherichia coli* (Philippot 2002).
(ii) Nitrite is then reduced to nitric oxide (NO) by a nitrite reductase. Nitrite reductases have long been considered the step-committed enzymes of the denitrification processes, due to the fact that in this step the reduction of N oxides becomes exclusively committed to energy conservation. There are two kinds of nitrite reductases, a copper and a cytochrome cd1-containing nitrite reductase, encoded by the *nirK* and *nirS* genes, respectively. Most information support that *nirK* and *nirS* genes do not coexist in the same organism (Jones et al. 2008), but a few exceptions have been found recently (Graf et al. 2014). Denitrifying microorganisms can be classified into *nirK*- and *nirS*-type denitrifiers (Jones et al. 2014).
(iii) Nitric oxide is reduced to nitrous oxide (N_2O) by the nitric oxide reductase, a heterodimeric enzyme encoded by the *norB* and *norC* genes (Hino et al. 2010).
(iv) Finally, N_2O is converted to N_2 by the nitrous oxide reductase, encoded by the *nosZ* gene (Orellana et al. 2014).

According to the set of genes present in the microorganism, denitrifiers can be divided into two groups: (1) microorganisms with a functional entire denitrifying pathway (complete denitrifiers) and are therefore able to perform the stepwise reduction of NO_3^- or NO_2^- all the way to N_2 and (2) microorganisms lacking some of the genes involved in the reduction pathway to N_2, but that still use N oxides as electron acceptors in a respiratory chain. Examples of these incomplete denitrifiers are those that have either *nirK* or *nirS* genes but lack *nosZ*; consequently their denitrification product is N_2O rather than N_2. After the analysis of many denitrifier genomes, the following observation was done: *nirK*-type denitrifiers are more prone to lack the *nosZ* gene than *nirS*-type denitrifiers (Jones et al. 2008). A few microorganisms carry only the *nosZ* gene (Sanford et al. 2012), suggesting that they just reduce N_2O to N_2.

Denitrifiers are practically present on every habitable environment on earth, and denitrification occurs in a myriad of environments (anaerobic and microaerobic milieus). Extremophilic denitrifiers have been recently described, and most of them belong to the Archaea domain (Offre et al. 2013). The denitrification in extreme environments opens up new possibilities and challenges for the study of this process in novel conditions and environments.

Most denitrifying organisms are neutrophilic, but also acidophilic (Huang et al. 2014a) and alkaliphilic (Sorokin et al. 2001) denitrifiers have been reported, with a prevalence of N_2O over N_2 production as final product at low pH (Huang et al. 2014b).

The temperature also plays an important role in the regulation of denitrification rates. Although denitrification activity increases with temperature, it can still occur at near 0 °C, with optimal activity in soil at the mesophilic range (Palmer et al. 2010).

Thermophilic denitrification (over 50 °C) is carried out mainly by spore-forming gram-positive bacteria and archaea, with a few known thermophilic gram-negative bacteria (Courtens et al. 2014).

The aim of this chapter is to analyze the current knowledge on denitrification and denitrifying organisms, with focus in heterotrophic denitrification processes and their use as biological tools to improve environmental sustainability, mainly as mediators of bioremediation. Current uses, pilot-scale experiences, and perspective of future applications, as well as limitations of the different cases, will be discussed. The chapter ends with a section dedicated to the genus *Paracoccus*, which has gained attention recently due to its metabolic versatility.

10.2 Heterotrophic Denitrification in Bioremediation Strategies

10.2.1 Nitrate and Nitrite Contamination of Waters

Nitrate and nitrite produce several environmental and human health risks (methemoglobinemia and gastrointestinal cancer; Powlson et al. 2008). The excess of these molecules in water bodies can trigger an accelerated growth of certain microorganisms. This often results in algae or cyanobacterial blooms with an excessive biochemical oxygen demand (BOD), which can be harmful for the resident normal biota. Besides, some cyanobacterial strains release toxins that can be detrimental to human health (Sivonen 1996).

The WHO guideline values of NO_3^- and NO_2^- in drinking water are 50 mg/l and 3 mg/l, respectively (World Health Organization 2011), and the "Nitrates Directive" of the European Commission states that all surface freshwater or groundwater with more than 50 mg/l of nitrate is considered polluted or at risk of pollution (European Commission).

Nitrate pollution derives mainly from agricultural practices and from sewage. As the worldwide food demand continuously increases, the usage of fertilizer in agriculture is not foreseeable to decrease; therefore the development of environmental friendly techniques to efficiently remove N from water is becoming necessary.

The use of denitrification to remove NO_3^- and NO_2^- from sewage and water in treatment plants certainly was the first use of denitrifying microorganisms in environmental sustainability and arguably is among the first experiences in the use of microorganisms as bioremediation agents. The idea of designing an anoxic zone to enhance denitrification in an activated sludge with the aim of N-removal from water dates back to 1962 (Lofrano and Brown 2010). In the following subsections, we summarize the current knowledge and recent innovations of water N-removal technologies that use denitrification and denitrifying organisms, with emphasis in the N-removal performed by heterotrophic denitrification.

Due to its applicability in many different scenarios and the need to improve wastewater treatments, N-removal by heterotrophic denitrifying microorganisms is a dynamic field of research. Denitrification is mainly used to remove excess nitrate or nitrite from groundwater or from wastewater, as described in the following sections.

10.2.1.1 N-Removal from Groundwater

Groundwater contamination with nitrate and nitrite has different sources. Although the most common cause of nitrate contamination is agricultural practices and inadequate wastewater management, other sources such as mining, aquaculture, landfill leachate, and industrial wastes (Wakida and Lerner 2005) also play an important role on groundwater N-pollution.

As part of a natural biogeochemical cycle, naturally occurring denitrification should be sufficient to reduce nitrate and nitrite levels in a pristine groundwater. However, anthropogenic activities have often led to increased concentrations of these molecules, and usually the native resident microbiota of aquifers is not sufficient to remediate pollution. As explained earlier, denitrification rates are subject to environmental conditions, mainly to oxygen and nutrient concentrations (Rivett et al. 2008). As heterotrophic denitrifiers use organic molecules as source of energy and these organic molecules are far from an optimum concentration for denitrification in natural conditions, usually the denitrification rates are slow, even when denitrifying microorganisms are present in the groundwater.

Strategies to alleviate nitrate or nitrite contamination in groundwater include the modification of the environmental parameters to enhance the denitrification process by the resident microbiota by the addition of organic molecules or by the inoculation of the groundwater with denitrifying microorganisms.

In groundwater, the most limiting factor for denitrification is often the energy source which in the case of heterotrophic denitrification is organic molecules. One way to tackle this limitation is to add organic material to the environment. For example, as the efficient denitrification takes place in riparian buffer zones (Burt et al. 1999), water purification systems have been designed to simulate these zones as shown in Fig. 10.2. A denitrification wall (a permeable reactive barrier or matrix, composed of organic material that has been dug in a trench to the groundwater) is enriched in resident bacteria, and as the groundwater passes through this wall, the denitrification process takes place, and the nitrate levels are reduced. Denitrification walls are relatively inexpensive and require little maintenance. It has been shown that a wall constructed with sawdust as C source may remove NO_3^- through denitrification after 14 years of operation (Long et al. 2011). In a field trial conducted to demonstrate the utility of sawdust as amendment in a denitrification wall, the nitrate concentrations were tenfold reduced (from 5 to 16 mg of N L^{-1} in the incoming side to 0.6 and 2 mg of N L^{-1} in the outgoing site) (Schipper and Vojvodić-Vuković 1998), even after a year of operation without subsequent C-source additions.

A critical point to consider in the implementation of denitrification walls is the permeability of the matrix. Depending on the subsoil quality of the aquifer, some materials used to construct the wall can force the water to flow underneath the wall (Schipper et al. 2004), avoiding the purification process. Different flow rates through the wall can also influence the nitrate removal rate; therefore, the porosity of the material should be considered. In addition, the microorganisms that proliferate on the matrix can alter its porosity, either by forming biofilms or by degrading the matrix that decrease or increase the porosity, respectively (Scherer et al. 2000). The material used in the construction of the matrix, which provides physical support and acts as C source, has also to be evaluated. Many materials show good nitrate removal

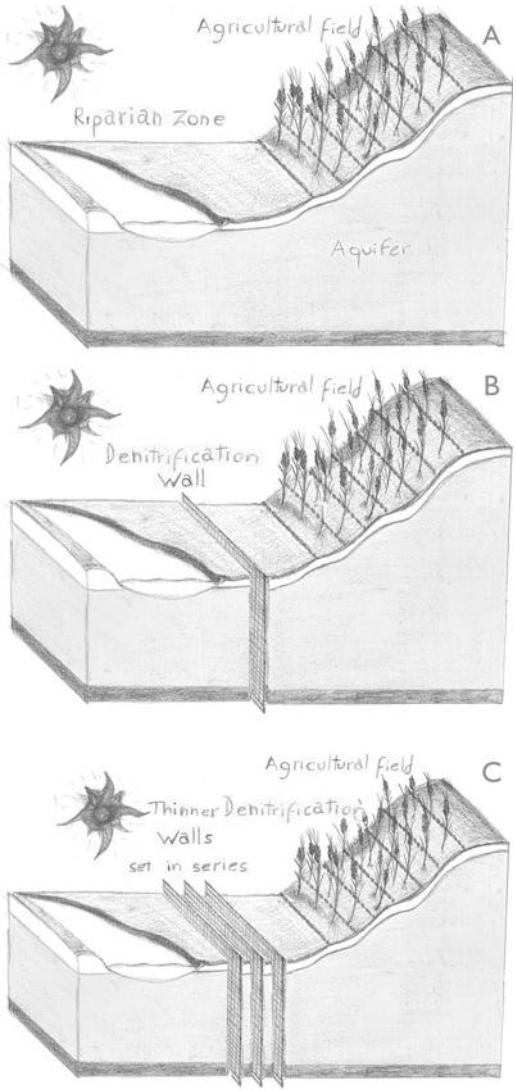

Fig. 10.2 Riparian zones (**a**) provide a natural way to remove nitrate and nitrite from groundwater. To improve and enhance this natural process, as well as to mimic them, nitrification walls can be constructed (**b**). A series of thinner denitrification walls can be more efficient than a single but broader one (**c**) (Drawing by Juan José Marizcurrena)

rates, such as softwood (Gibert et al. 2008) or dried cornstalks, cardboard, wood chips saturated with soybean oil, and wood chips (Greenan et al. 2006). Currently, the installation of a series of walls over the groundwater, instead of a single but broader wall, is recommended (Schmidt and Clark 2012) (Fig. 10.2c).

The addition of organic substrates to groundwater by direct injection also improves the denitrification process. A natural attenuation of nitrate contamination in a uranium mining site in Arizona was observed in microcosm experiments, but the addition of ethanol or methanol increased the nitrate removal rate over an order of magnitude (Borden et al. 2012; Carroll et al. 2009). Moreover, the nitrate

concentration in the groundwater remained low even months after the injection. Ethanol may have served as a primer for denitrifiers proliferation; meanwhile, the decay of the microbial population may have provided nutrients for the following generations of denitrifiers, eliminating the need of further ethanol applications.

The addition of glucose and acetate in a lab-scale study to enhance natural denitrification of an aquifer in Spain showed that both molecules increased nitrate removal rates, but the addition of acetate produced nitrite accumulation (Calderer et al. 2010). Under anaerobic conditions, most glucose (99 %) was used for energy source in denitrification, suggesting that the addition of this sugar is a promising strategy to enhance in situ denitrification of aquifers, but optimum parameters in a full-scale operation have still to be set up.

The inoculation of groundwater or the use of submerged filters embedded with microorganisms (a single strain or a consortium of microorganisms) may increase the denitrification rates. Classically, submerged filters are inoculated with activated sludge that typically consists in a diverse and uncharacterized population of microorganisms, but usually these microbes (denitrifiers and other ones) form biofilm that occludes the filter (Gómez et al. 2000). The inoculation with axenic cultures may circumvent this disadvantage. A study comparing the effect of the inoculation with activated sludge or *Hydrogenophaga pseudoflava* showed the use of *H. pseudoflava* gives a better quality of the treated water (Moreno et al. 2005).

Biofilm-based filters used to treat groundwater have a limited efficiency regarding removal of nitrates. Membrane reactors, for instance, are prone to fouling; aeration can be used to control fouling, but it disrupts the anaerobic environment needed for denitrification. Currently, many efforts have been made to decrease fouling by using different polymers in the construction of the filters (Yamato et al. 2006). Submerged filters are usually primed with activated sludge and are limited by the number of bacteria they can sustain (de la Rúa et al. 2008). One variant of biofilm filters are fiber-based biofilms, in which fiber threads are used to construct the matrix of the biofilms. Lab-scale studies that evaluated the performance of a fiber-based biofilm reactor found that nitrate removal rates were above 99 % with nitrate loads of 148.66 ± 10.64 mg L^{-1} of NO_3^- (Wang et al. 2009). In the first runs, nitrite was present in the effluent, but the problem could be solved by adjusting retention times.

The use of constructed wetlands for pollution control is yet another strategy to remove nitrate from groundwater. This depends on both plant uptake and denitrification to remove nitrate and other contaminants from groundwater. Although at first it was thought that plant uptake was the main driver of nitrate removal in constructed wetlands, an experiment evaluating different macrophyte species (Lin et al. 2002) found that between 89 and 96 % of the nitrate was removed by denitrification.

Denitrifying microorganisms have played a paramount role on nitrate and nitrite removal from groundwater. These microorganisms constitute the natural way in which nitrogen is returned to the atmosphere. As showed above, the use of denitrifiers represents a promising and environmentally sound alternative to remove nitrate and nitrite from groundwater, either as inoculants or by enhancing the denitrifying activity of resident populations.

10.2.1.2 N-Removal from Wastewater

Industrial and municipal wastewaters often contain high levels of nitrates that must be diminished before discharge to the environment, and the approaches used to promote denitrification in groundwater can also be applied to wastewater treatment. However, due to the different nature of both environments, different bioremediation strategies have to be performed.

Sequencing batch reactors (SBRs) are the most used approach to treat wastewater. They have been used since the 1920s, and for a long time, they were employed without full understanding of the biological processes that occurred. These systems are used to remove both C and N pollutants, and many variants have been developed (Wang and Li 2009). Usually the tanks containing the wastewater are filled, at the bottom, with sludge containing microbes that can use pollutants as nutrients, and as wastewater comes in contact with the sludge, the pollutants start being removed. The rationale behind this operation is to alternate aerobic and anaerobic conditions in such a way that couples nitrification with denitrification to remove both ammonium and nitrate. Ammonium removal can also be anaerobically done due to the activity of anaerobic ammonium-oxidizing (Anammox) microorganisms, which in turn produce nitrate further used by denitrifiers. The main difference between a SBR and a typical activated sludge is that SBR occurs following sequential steps in a single tank.

In this subsection we will focus on the recent uses and research on the role of the heterotrophic denitrification process and heterotrophic denitrifying microorganisms in SBR treatment of wastewater.

Using the SBR technology, Lyles et al. (2008) treated shrimp aquaculture wastewater, obtaining fully reusable water. Authors showed that removal of ammonia, nitrate, and nitrite was achieved after 7 days of treatment in the pilot-scale SBR and confirmed that the sequential processes of nitrification and denitrification were responsible for N-removal. However, the key microorganisms were not identified, and the population responsible for such performance was not characterized.

An alternative to the classic nitrification-denitrification approach is the process called nitrification-denitrification via nitrite accumulation (Ruiz et al. 2006). It consists of the sequential process of nitritation (conversion of ammonium to nitrite, carried out by autotrophic ammonia-oxidizing microorganisms) and denitritation (reduction of nitrite to N_2) that creates a shortcut in the metabolic pathway in the N cycle (Fig. 10.1), which allows the oxidation of ammonia to nitrite and further denitrification (Fig. 10.3). The main advantage of this alternative is that it spares aeration costs for ammonia oxidation and reduces C input needs for the heterotrophic nitrate reduction. However, during this process, it is important to maintain a low O_2 pressure during nitrification and denitrification. Interestingly, it was found that once the reactor is stabilized, increasing aeration does not restore nitrite oxidation to nitrate (Yongzhen et al. 2007).

This system has been used mostly to treat wastewaters with relatively low nitrogen levels such as domestic wastewaters. The nitrification-denitrification process via nitrite accumulation is commonly influenced by environmental factors. At temperatures above 20 °C, the development of ammonia-oxidizing bacteria (AOB) over

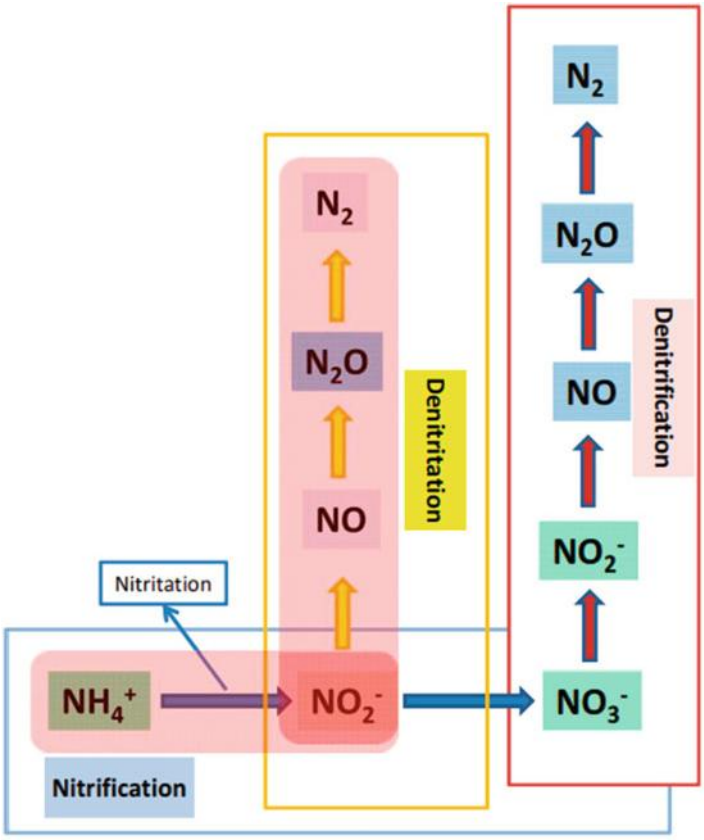

Fig. 10.3 Nitrification-denitrification via nitrite accumulation couples the processes of nitritation and denitritation (outlined in a *yellow square*) and creates a shortcut in the nitrification-denitrification process. Nitrification-denitrification via nitrite accumulation is highlighted in *pink*

nitrite-oxidizing bacteria (NOB) guarantees sufficient nitrite accumulation (Wu et al. 2007). Aeration also increases the development of AOB over NOB in temperate temperature ranges (Blackburne et al. 2008). Unfortunately, this strategy is unaffordable in cold environments, but the enrichment and posterior acclimation with AOB, together with NOB washout by aeration control, have shown promising results (Guo et al. 2010).

The use of SBRs for piggery wastewater treatment has operational limitations to many farmers, but Shipin et al. (2007) developed a system that takes the advantages of the nitritation-denitritation coupled process and works on separate tanks containing floating filters.

Contamination with both nitrate and ammonium is a common situation in wastewater. The SBR systems embody the most ancient use of denitrifiers in environmental sustainability and one of the most studied and improved N-removal strategies. However, the use of SBR systems has many limitations. The development of

systems that take advantage of nitrification-denitrification via nitrite accumulation can represent a leap forward in optimizing wastewater treatment systems. It is expectable that future researches would be focused in improving these systems.

10.2.2 Polyaromatic Hydrocarbon (PAH) Degradation

PAHs are polyaromatic hydrocarbon molecules (Wilcke 2000) with potential carcinogenic effects. They occur naturally as a consequence of wildfires and volcanic activity, but are also produced by plants (Edwards 1983). However, anthropogenic sources are the main matter of concern. Bioremediation is currently the most promising strategy to solve contamination by PAHs, in terms of feasibility. Soil is the main sink of this pollutant and the target of most bioremediation strategies. In the present subsection, we will focus on the use of denitrifying microorganisms as bioremediation agents to treat PAH pollution.

PAHs are naturally degraded by many microorganisms mainly through mineralization. However, naturally occurring degradation is insufficient to cope with the amounts of PAHs at polluted sites. Therefore, different strategies that involve the utilization of PAH-degrading microorganisms are being developed.

Bioaugmentation is the inoculation to polluted sites with microorganisms capable of pollutant degradation. It has been shown that this is a good strategy to remediate PAH-contaminated areas. Aerobic degradation of PAHs is a widespread trait among microorganisms (Habe and Omori 2003). However, if in situ bioremediation is intended for environments where O_2 availability is spatially or temporally scarce, denitrifying microorganisms provide a promising resource for the degradation of PAHs.

The degradation of three PAHs present in polluted aquifers was successful (Durant et al. 1995), but it was limited by the environmental conditions. The authors evaluated whether nitrate amendment could enhance PAHs degradation in anaerobic conditions. It was found that nitrate and phosphorus were limiting the anaerobic degradation. This study was one of the first to show that indigenous heterotrophic denitrifying bacteria present in polluted aquifers were able to degrade PAHs, but this degradation was limited by the environmental conditions of the aquifer. It also opened the possibility of in situ enhancement of anaerobic degradation through the addition of limiting nutrients.

Following the rationale of providing nutrients to optimize denitrifying activity, Wang et al. (2012) conducted a microcosm experiment in an anthracene-contaminated aquifer sediments amended with nitrate (80 days of anaerobic incubation at 25 °C) showing that only 1.2 % of anthracene remained, probably due to the decontaminating activity of *Paracoccus*, *Herbaspirillum*, *Azotobacter*, and *Rhodococcus* strains. These results clearly show that given appropriate conditions, populations of anaerobic PAH-degrading microorganisms can be enriched, and nitrate addition is a feasible strategy to provide those conditions. Also mangrove forest sediments supplemented with phenanthrene showed an enrichment of PAH-degrading bacteria, including *Paracoccus versutus* (Guo et al. 2005). Therefore, mangrove sediments are an auspicious source of PAH-degrading microorganisms, particularly PAH-degrading denitrifiers.

Denitrification is an interesting alternative to bioremediate PAH-polluted sites. Particularly, denitrifying organisms may play a crucial role in degrading PAHs in these environments where aerobic conditions are not strict. Soil is the most common environment where PAH contamination occurs, and most soils have fluctuating oxygen dynamics. Thus, the development of systems that take advantage of denitrifiers to degrade PAHs in situ is probably one of the most promising approaches to solve this problem.

10.2.3 Bioremediation of Other Organic Pollutants

Heterotrophic denitrifying microorganisms have been used to decontaminate environments polluted with different organic molecules and are the main alternative of bioremediation when aerobic degradation of the pollutant is impracticable.

Among recalcitrant products, it has been shown the degradation of petroleum hydrocarbons (Hasinger et al. 2012), toluene (Martínez et al. 2007), ethylbenzene, and m-xylene (Kao and Borden 1997), by denitrification. In most cases of groundwater contamination with benzene, toluene, ethylbenzene, and xylene (BTEX), nitrate is a limiting factor for denitrification, but nitrate injection did enhance BTEX degradation in the aquifer as shown by da Silva and Corseuil (2012). Probably, nitrate alleviates the BOD which resulted in the conservation of microaerophilic niches that supported the activity of aerobic degraders that may have contributed with the increase in BTEX degradation. Recently, a strain of *Pseudomonas thivervalensis* was found to degrade BTEX under denitrifying conditions at 10 °C, showing their potential use at low temperatures (Qu et al. 2015).

10.3 *Paracoccus*

Bacteria of the genus *Paracoccus* are gram-negative Alphaproteobacteria that exhibit a great range of metabolic flexibility. The number of species classified as *Paracoccus* has been rapidly growing. In 1991 only two species were recognized, but by the year 2006, 14 species were proposed (Kelly et al. 2006). Currently, 51 different species of *Paracoccus* are registered into the taxonomy browser of the NCBI, being *P. denitrificans* the type specie, a denitrifying bacterium. Due to their metabolic diversity, many researchers analyzed the potential of *Paracoccus* strains in environmental sustainability, mainly by the use of their denitrifying abilities. The following examples reflect the diversity of potential uses that encompass the genus.

Organophosphate insecticides are widely used in agriculture due to its effectiveness in battling economically important plagues. However, they are highly toxic compounds that persist in the environment and commonly reach groundwater through lixiviation. During the isolation of monocrotophos-degrading microbes (a kind of organophosphate), Jia et al. (2006) found a *Paracoccus* strain with promising traits as organophosphate degrader. *Paracoccus* sp. M-1 degrades monocrotophos both aerobically and anaerobically, and interestingly, the anaerobic degradation

occurred via denitrification. Thus, these abilities point out this microbe as an excellent candidate for in situ bioremediation of soils, where O_2 concentration varies greatly. Remarkably, it was also found that M-1 may degrade a variety of monocrotophos-related compounds, a useful trait that may be exploited for the bioremediation of co-contaminated soils.

Paracoccus sp. T231, a strain isolated from activated sludge samples collected in a wastewater treatment plant, showed the ability to perform the anaerobic degradation of trimethylamine, at a rate that was found to be dependent on nitrate concentration (Kim et al. 2001). This trait supports the hypothesis that denitrification was responsible for the anaerobic degradation.

According to the NCBI genome database, the genomes of three strains of *Paracoccus denitrificans* are currently available. The genome analysis of *P. denitrificans* strain SD1, an isolate that uses *N,N*-dimethylformamide (DMF) as sole carbon and nitrogen source, showed the presence of nitrate and nitrite reductase genes and also coding sequences for enzymes probably involved in DMF degradation (Siddavattam et al. 2011). The analysis of the complete genome of *P. denitrificans* strain TRP has shown the presence of at least 25 coding sequences for the degradation of xenobiotic compounds (Li et al. 2011).

As describe above (Sect. 10.2.1.2), the classic biological approach to remove both nitrate and ammonium from water bodies is coupling the nitrification and denitrification processes, typically in SBRs. A recent alternative to SBR has been brought by the discovery of microorganisms that can perform both nitrification and denitrification under similar environmental conditions. Contrasting to conventional denitrification, which takes place in anaerobic environments, aerobic denitrification occurs in the presence of O_2, a condition that promotes nitrification. *P. denitrificans* (Ludwig et al. 1993) (formerly *Thiosphaera pantotropha*; Robertson et al. 1988) was the first bacterium described as an heterotrophic nitrifier and aerobic denitrifier (HNAD) microbe. The HNAD process is also performed by *P. versutus* strain LYM that simultaneously removes ammonium and nitrate under aerobic conditions (Shi et al. 2013). Contrary to many anaerobic denitrifiers, the end product of HNAD was mainly N_2 (Takaya et al. 2003).

Due to their ability to denitrify under aerobic conditions, it is not surprising that *Paracoccus* spp. are found as dominant bacterial population in environments where O_2 concentrations fluctuate. This hypothesis was proved by Neef et al. (1996). The authors, using DNA probes targeting *Paracoccus*, found that *Paracoccus* spp. were the dominant genus in the sand bed of a methanol feed reactor, in a wastewater treatment system. In this system, aeration was used to prevent clogging by the sludge, but aeration is a tricky solution. Low aeration would allow clogging, but excessive aeration may inhibit the denitrification process. Thus, *Paracoccus* strains could be used during the decontamination of N-polluted environments and avoid aeration-derived problems.

The members of the genus *Paracoccus* give insight to a myriad of opportunities for different bioremediation prospects. In Table 10.1, examples of *Paracoccus* strains and the results in the degradation of pollutants are shown. Besides denitrifying strains, non-denitrifiers *Paracoccus* are also included in the table.

Table 10.1 List of *Paracoccus* spp. and their use during the degradation of environmental pollutants

Strain	Pollutant degraded	Potential environments where it can be used	Denitrifying conditions	References
Paracoccus sp. BW001	Pyridine	Activated sludge/wastewater	Yes	Bai et al. (2008)
Paracoccus sp. TOH	Piperazine	Activated sludge/wastewater	No	Cai et al. (2013)
Paracoccus sp. TRP	Chlorpyrifos/3,5,6-trichloro-2-pyridinol/pyridine/methyl parathion/carbofuran	Activated sludge/wastewater/soil	No	Xu et al. (2008)
Paracoccus denitrificans M-1	Pyrene	Sediments	Yes	Yang et al. (2013)
P. denitrificans M-1	Monocrotophos	Activated sludge/wastewater	Yes	Jia et al. (2006)
Paracoccus sp. SKG	Acetonitrile	Wastewater	No	Santoshkumar et al. (2011)
Paracoccus versutus LYM	Nitrate/ammonium	Water treatment plants	No	Shi et al. (2013)
P. denitrificans SD1	*N,N*-dimethylformamide	Wastewater/coal mine	Yes	Siddavattam et al. (2011)
Paracoccus sp. XF-3	Chlorothalonil	Soil	No	Yue et al. (2015)
Paracoccus sp. MKU1 and MKU2	*N,N*-dimethylformamide	Activated sludge/wastewater	No	Nisha et al. (2015)
P. denitrificans GH3	Isopropanol	Soil	No	Geng et al. (2015)
Paracoccus pantotrophus SAG_1	Melanoidin	Soil/wastewater	No	Santal et al. (2016)

10.4 Final Considerations

The potential use of denitrifying microorganisms as pollutant removal agents is mainly based on its metabolic diversity. These microorganisms are active at both anaerobic and aerobic environments, simultaneously removing N oxides and organic or inorganic molecules by their use as electron acceptors and donors, respectively.

A single strain or a community of denitrifying microorganisms could be used to remove the pollutant. Contaminant removal techniques that rely on a single microorganism face the challenge of the removal efficiency. Some isolates that effectively remove a contaminant in certain environmental conditions are unable to do so in the prevailing or fluctuating conditions of a more complex contaminated site. Moreover, many contaminated sites present a mixture of contaminants rather than a single pollutant. Often, to find a microorganism that efficiently removes all pollutants present at certain sites is certainly impossible. However, advances in metabolic engineering have opened the possibility of improving the bioremediation abilities of bacterial isolates (Singh et al. 2008). Thus, specific enzymatic activities or pathways for the degradation of many pollutants can be optimized or simply introduced into other bacteria by genomic engineering.

Alternatively, the use of microbial communities can circumvent some of the drawbacks. Usually there is a lack of information about the composition and identity of the microbial community, but next-generation sequencing approaches may allow the full description of the communities, giving insight to the metabolic pathways involved in the degradation of pollutants. To understand the phylogenetic diversity and the metabolic profile of a community that efficiently remove pollutants may help to design the novel strategies of N-removal. In this scenario, *Paracoccus* strains may play a relevant role.

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6. DISCUSIÓN GENERAL

Si bien el trabajo de la tesis fue divido en dos publicaciones, cada una de ellas enfocada a un grupo particular de microorganismos involucrados en la emisión de N_2O, el análisis de los resultados en conjunto permite realizar algunas apreciaciones generales.

El estudio de las comunidades de microorganismos oxidadores de amonio y desnitrificantes fue realizado mediante el análisis por separado de los dos subgrupos que componen cada población. Entre los oxidadores de amonio se analizaron las arqueas oxidadoras de amonio (AOA) y las bacterias oxidadoras de amonio (AOB), mientras que entre los desnitrificantes se estudiaron los de tipo nirK y tipo nirS.

Las cuatro poblaciones definidas para este estudio no tuvieron la misma respuesta a los factores evaluados en el estudio. Sin embargo, se pueden hacer algunas consideraciones generales. En cuanto a la abundancia de las poblaciones, el factor que más las influenció fue el tiempo de muestreo. El tiempo de muestreo afectó la abundancia de las poblaciones de AOA y AOB en los dos ensayos realizados, mientras que ambas poblaciones de desnitrificantes variaron con el tiempo de muestreo en el primer experimento y los desnitrificantes nirS variaron exclusivamente en el segundo.

Los cambios en el número de microorganismos están vinculados a condiciones bióticas y abióticas del ambiente en el que se encuentran. La disponibilidad de nutrientes, temperatura, tensión de O_2 y pH son los factores más determinantes para el crecimiento microbiano. Las poblaciones bacterianas presentes en las muestras obtenidas durante distintos momentos de nuestros experimentos estuvieron sometidas por distintos lapsos de tiempos a condiciones variables en el suelo. El desarrollo de la planta de arroz, la aplicación de fertilizantes y el riego ocasionaron cambios en los factores de crecimiento bacteriano que influenciaron la abundancia de todos los grupos microbianos estudiados.

El análisis de la abundancia de genes universales para arqueas y bacterias, tal como el gen del ARNr 16S arqueano y bacteriano (Banning et al., 2015), arroja información acerca del efecto sobre toda la comunidad bacteriana y permite el

cálculo de la proporción de cada grupo de interés sobre la abundancia total de microorganismos. Esto último es muy valioso para determinar si algún parámetro influenció diferencialmente algún grupo microbiano en particular.

En cuanto al manejo del agua y el tipo de suelo, la abundancia de oxidadores de amonio y de desnitrificantes no siempre fue afectada en el sentido que sugerían las predicciones más evidentes. Así, los períodos inundados del cultivo, favorecen la generación de ambientes anaerobios, algo que en principio beneficiaría a los microorganismos desnitrificantes mientras que perjudicaría a los oxidadores de amonio. Concordantemente con esto, las poblaciones de AOA fueron mayores en el tratamiento «inundación tardía» en el segundo experimento. Sin embargo, las poblaciones desnitrificantes también fueron mayores en ese tratamiento con respecto al tratamiento «inundación temprana». Esta aparente discrepancia puede explicarse en parte por el método utilizado para evaluar la abundancia de microorganismos. El qPCR basado en ADN como molde de la amplificación da como resultado la cantidad del gen blanco presente en el ambiente, pero con esa estimación no es posible determinar la actividad de los microorganismos que albergan dicho gen en el momento de muestreo. En el caso de las bacterias desnitrificantes, la vía de desnitrificación es, generalmente, una vía alternativa a la respiración aerobia (Zumft, 1997), lo que implica que muchos microorganismos desnitrificantes pueden proliferar en ambientes aerobios usando vías de conservación de energía alternativas a la desnitrificación.

Para conocer si la actividad de estos microorganismos cambia en respuesta a los factores evaluados y, particularmente si los genes estudiados son transcriptos en esos momentos, es necesario realizar un RT-qPCR cuyo blanco es el ARN extraído del ambiente (Leininger et al., 2006).

En cuanto al efecto de los factores sobre las estructuras poblacionales de los distintos genes, tanto en oxidadores de amonio como en desnitrificantes se observaron efectos del tiempo de muestreo y del cultivar, pero no del manejo del agua. En los casos en los que se observó efecto del cultivar de arroz (AOA y *nirS*), éste fue acentuado en las muestras colectadas en las últimas etapas del cultivo, lo que puede ser resultado de un efecto rizosférico mayor en esas etapas.

Al igual que en el primer ensayo, en el segundo el momento de muestreo afectó significativamente la estructura de las poblaciones de todos los genes excepto *nirK*. Esto indica que existe un efecto de sucesión en estas comunidades bacterianas a lo largo de los dos ensayos, probablemente dirigido por la planta de arroz y los cambios ambientales ocasionados por las aplicaciones de fertilizantes y el riego.

La hipótesis de que la planta de arroz ocasiona cambios en la comunidad es plausible ya que se conoce el efecto de los exudados de las raíces sobre las comunidades microbianas del suelo (Wieland et al., 2001). Sin embargo, para determinar la magnitud del efecto sería necesario realizar un ensayo similar al llevado a cabo en este trabajo, e incluir un tratamiento sin la planta de arroz, de manera de no confundir el efecto ocasionado por la planta con aquellos generados por otros cambios ambientales y determinar si existe una sucesión de las poblaciones microbianas en ausencia de la planta de arroz.

En el segundo ensayo, el suelo fue el factor que más influyó en la comunidad bacteriana con un efecto significativo sobre la estructura poblacional de todos los genes analizados excepto el gen *nirK*. Particularmente, las poblaciones de AOA fueron marcadamente diferentes entre los distintos suelos.

Los dos suelos usados en el segundo experimento difieren en sus características fisicoquímicas, pero también fueron extraídos de dos zonas diferentes de nuestro país. Aquellas poblaciones de genes para las cuales se encontraron diferencias en su estructura según el tipo de suelo, fueron diferentes desde el primer momento de muestreo, que en el caso del segundo ensayo fue en la siembra. Esto implica que las poblaciones diferían antes de empezar el experimento. Hasta qué punto estas diferencias son producto de las distintas características del suelo y hasta cuál son producto de la historia ecológica de los sitios y de la biogeografía es una pregunta interesante, pero que no se puede responder con los experimentos realizados. Por ejemplo, la composición microbiana del suelo de cada zona puede estar tan influenciada por la historia reciente del manejo del suelo como por sus características fisicoquímicas. La biogeografía de los microorganismos en una disciplina muy poco explorada en comparación con la de los macroorganismos (Martiny et al., 2006) por

lo que poco se sabe acerca de las distribuciones geográficas de los distintos grupos de microorganismos y las causas de ellas.

Una pequeña fracción de los microorganismos es cultivable mediante los métodos de cultivo conocidos (Torsvik y Øvreås, 2002), sin embargo las metodologías empleadas en este trabajo para determinar la abundancia y la estructura poblacional de los microorganismos estudiados permiten evitar la limitación que implica el cultivo de microorganismos.

Particularmente, el cultivo de microorganismos oxidadores de amonio es complejo y en muchos casos imposible. A pesar de ello, los métodos empleados no están exentos de sesgos y limitaciones. La utilización de cebadores que implica el PCR es, probablemente, el sesgo más claro que imponen las técnicas basadas en PCR (Bergmann et al., 2011). Si bien los cebadores utilizados están diseñados para complementar con secuencias conservadas de un gen, y tienen bases degeneradas que amplían el espectro de secuencias a las que se pueden hibridar, éstos no tienen la misma especificidad con todas las secuencias blanco, por eso el resultado obtenido puede estar sesgado hacia un subgrupo particular de genes (Shimomura et al., 2012). Este efecto se ha evidenciado con el descubrimiento de nuevos grupos de microorganismos oxidadores de amonio o desnitrificantes, los cuales contienen genes *amoA* o *nir* que no son amplificables con los cebadores tradicionalmente usados (Jones et al., 2014; Pester et al., 2012).

El desarrollo de técnicas de secuenciación masiva y su aplicación al estudio de comunidades microbianas presentan una alternativa que permite eludir el sesgo introducido por el uso de cebadores (Quail et al., 2012). La secuenciación masiva, permite obtener secuencias de genes no amplificables y también contar con las secuencias de una gran cantidad de los genes presentes en el ambiente. De esta manera, una vez obtenidos los metagenomas, se pueden analizar las poblaciones de cualquier gen, por ejemplo, se podrían buscar las poblaciones de los otros genes en la vía de desnitrificación, así como poblaciones de cualquier gen de interés.

La relación entre los cambios en las poblaciones de microorganismos oxidadores de amonio y desnitrificantes y el potencial emisor de N_2O del suelo no ha sido establecido claramente. Correlacionar datos de la abundancia y estructura

poblacional de estos microorganismos con medidas de emisión de N_2O puede aportar información útil para establecer la relación entre estas poblaciones y la emisión de N_2O.

Por otra parte, conocer cómo se ven afectadas estas poblaciones en condiciones de campo por las distintas prácticas agrícolas empleadas en las diferentes zonas del país, es un paso crucial para elaborar un plan de manejo adecuado enfocado a mitigar las emisiones de gases con efecto invernadero en el cultivo de arroz en Uruguay.

6.1 CONCLUSIÓN

Los resultados de este trabajo permiten concluir que existe efecto de los factores analizados sobre las poblaciones de microorganismos responsables de las emisiones de N_2O en arroz. Todos los factores influenciaron en mayor o menor medida la abundancia y la estructura poblacional de estos microorganismos.

El análisis de la abundancia los microorganismos desnitrificantes tipo *nirK* y tipo *nirS* aporta una prueba más a la hipótesis de que durante el estadio de primordio floral del cultivo el potencial emisor de N_2O por parte de los desnitrificantes es muy alto, algo que se conoce como «hot moment».

Por otra parte, el efecto diferencial que tuvieron los factores estudiados sobre las poblaciones de desnitrificantes tipo *nirS* y *nirK* sugiere que estos dos grupos de microorganismos son ecológicamente no redundantes.

En cuanto a las poblaciones de microorganismos oxidadores de amonio, los dos grupos analizados, arqueas y bacterias, fueron afectados de manera diferencial por los factores. En el caso de las arqueas y bacterias oxidadoras de amonio, la diferencia ecológica de los grupos es más evidente y conocida que en los microorganismos desnitrificantes. La estructura poblacional de las arqueas oxidadoras de amonio fue marcadamente diferente según el tipo de suelo, de tal manera que es posible considerar a estos microorganismos como potenciales indicadores en suelos de arrozales de distintos orígenes georgráficos.

En algunos casos, particularmente al analizar el efecto del manejo del agua, no se detectaron efectos sobre la estructura poblacional de los microorganismos, pero sí se encontraron efectos sobre algunos T-RFs, esto indica que cierto subgrupo de la

población se ve afectado aunque la estructura global de la población no sea afectada. La técnica utilizada no permite concluir si estos grupos sensibles son emisores relevantes de N_2O, sin embargo estos resultados abren la posibilidad de que ciertos grupos importantes estén influenciados por factores como el manejo del agua, aunque la estructura poblacional no lo esté.

Globalmente, los resultados indican, inequívocamente, que las prácticas agrícolas en el cultivo de arroz, manejo de la inundación, cultivar de arroz y tipo de suelo, tienen incidencia sobre las poblaciones microbianas emisoras de N_2O. Por tanto, el diseño de prácticas de manejo para este cultivo es una herramienta fundamental para la generación de estrategias de mitigación de emisión de gases de efecto invernadero.

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8. ANEXO Material suplementario del capítulo 4

Supplementary table 1. *nirS* number of terminal restriction fragments (**N**), and Shannon (*H*) and Pielou (*J*) diversity indexes according to sampling time. Values correspond to averages of all samples on each date (standard deviation). Top and bottom table represent first (cultivar and water management) and second (soil type and time of flooding) experiment, respectively. In the same group (rows), different letters indicate statistically significant differences (Tukey's HSD, $p<0.05$). *Interaction not statistically significant ($p<0.05$).

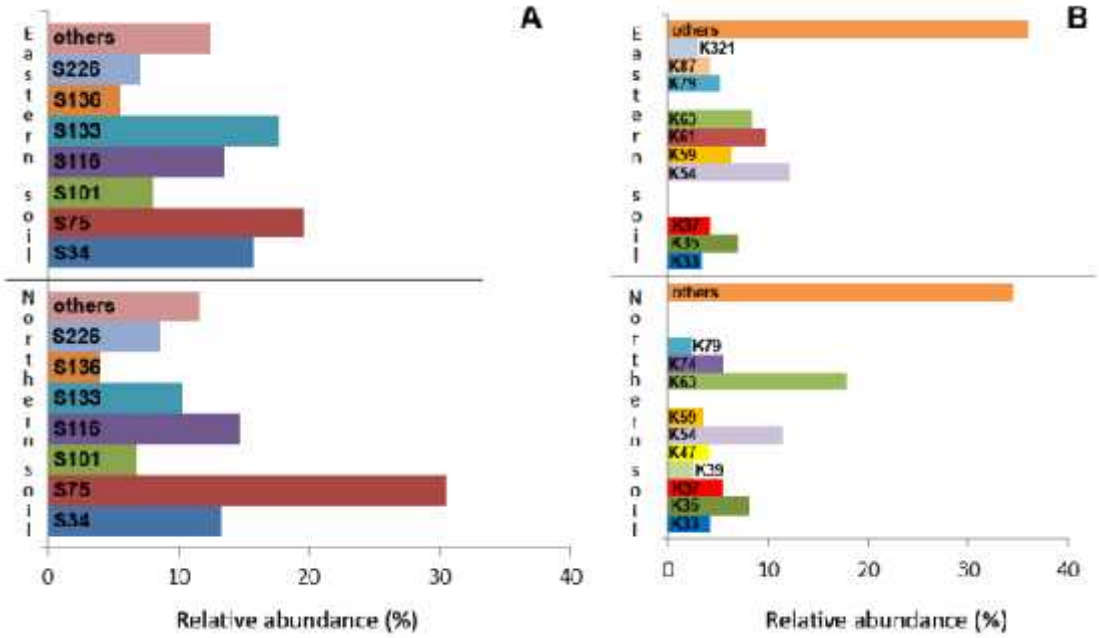
1st experiment		tillering	flower primordium	draining	harvest
N	Tacuarí	12.7 (1.0) C	14.0 (1.3) AC	13.7 (0.5) BC	13.7 (1.4) BC
	El Paso	13.2 (1.3) C	15.5 (1.2) AB	16.0 (1.2) A	14.3 (1.4) AC
	Overall	12.9 (1.2) B	14.8 (1.4) A	14.7 (1.5) A	14.0 (1.3) AB
H'	Tacuarí	1.99 (0.11) CD	2.21 (0.06) AB	2.11 (0.12) BC	2.13 (0.07) BC
	El Paso	1.93 (0.12) D	2.36 (0.08) A	2.26 (0.09) AB	2.21 (0.07) AB
	Overall	1.96 (0.17) C	2.29 (0.11) A	2.18 (0.13) B	2.17 (0.08) B
J'	Tacuarí	0.78 (0.03)*	0.84 (0.03)*	0.81 (0.03)*	0.82 (0.03)*
	El Paso	0.75 (0.06)*	0.86 (0.01)*	0.82 (0.03)*	0.83 (0.02)*
	Overall	0.77 (0.05) C	0.85 (0.02) A	0.81 (0.03) B	0.82 (0.03) AB

2nd experiment		sowing	tillering	flower primordium	harvest
N	Northern soil	10.0 (0.63) B	12.0 (1.67) AB	12.8 (1.17) A	11.5 (1.87) AB
	Eastern soil	12.0 (1.3) AB	11.8 (1.5) AB	13.7 (1.6) A	11.7 (1.8) AB
	Overall	11.0 (1.4) B	11.9 (1.5) AB	13.3 (1.4) A	11.6 (1.7) B
H'	Northern soil	1.98 (0.05) B	2.05 (0.07) A	2.06 (0.12) A	2.14 (0.10) A
	Eastern soil	2.17 (0.05) A	2.17 (0.08) A	2.14 (0.08) A	2.14 (0.08) A
	Overall	2.07 (0.11) A	2.11 (0.09) A	2.10 (0.11) A	2.14 (0.09) A
J'	Northern soil	0.86 (0.02)*	0.83 (0.03)*	0.81 (0.03)*	0.88 (0.03)*
	Eastern soil	0.88 (0.02)*	0.88 (0.03)*	0.82 (0.02)*	0.88 (0.04)*
	Overall	0.87 (0.02) A	0.86 (0.04) A	0.81 (0.02) B	0.88 (0.03) A

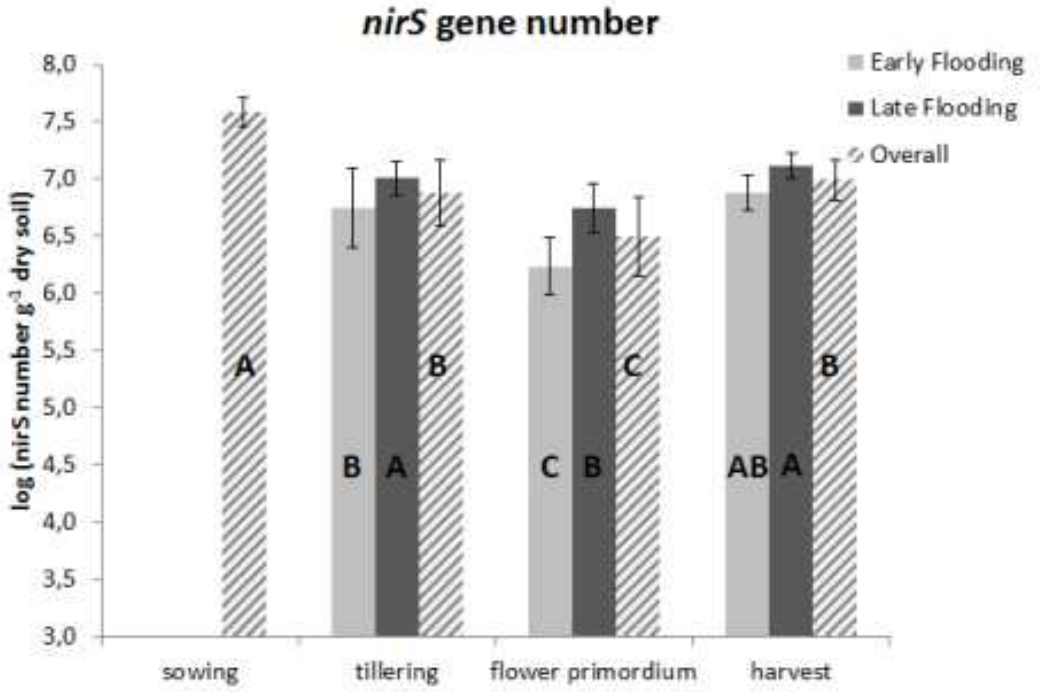
Supplementary table 2. *nirK* number of terminal restriction fragments (**N**), and Shannon (*H*) and Pielou (*J*) diversity indexes according to sampling time. Values correspond to averages of all samples on each date (standard deviation). Top and bottom table represent first (cultivar and water management) and second (soil type and time of flooding) experiment, respectively. In the same group (rows), different letters indicate statistically significant differences (Tukey's HSD, $p<0.05$). *Interaction not statistically significant ($p<0.05$).

1st experiment		tillering	flower primordium	draining	harvest
N	Tacuarí	17.2 (2.3) A	15.0 (5.0) B	13.2 (1.2) B	12.8 (1.5) B
	El Paso	11.7 (3.5) B	15.5 (4.4) B	13.5 (2.0) B	16.3 (2.3) B
	Overall	14.4 (4.0) A	15.3 (4.5) A	13.3 (1.6) A	14.6 (2.6) A
H'	Tacuarí	2.5 (0.3) A	2.2 (0.4) AC	2.0 (0.1) BC	1.7 (0.1) C
	El Paso	1.9 (0.3) BC	2.2 (0.3) AB	2.0 (0.2) AC	2.1 (0.3) AC
	Overall	2.2 (0.4) A	2.2 (0.4) A	2.0 (0.2) AB	1.9 (0.3) B
J'	Tacuarí	0.86 (0.07)*	0.82 (0.07)*	0.77 (0.04)*	0.68 (0.04)*
	El Paso	0.80 (0.06)*	0.83 (0.05)*	0.79 (0.06)*	0.74 (0.07)*
	Overall	0.83 (0.07) A	0.82 (0.06) A	0.78 (0.05) A	0.71 (0.06) B

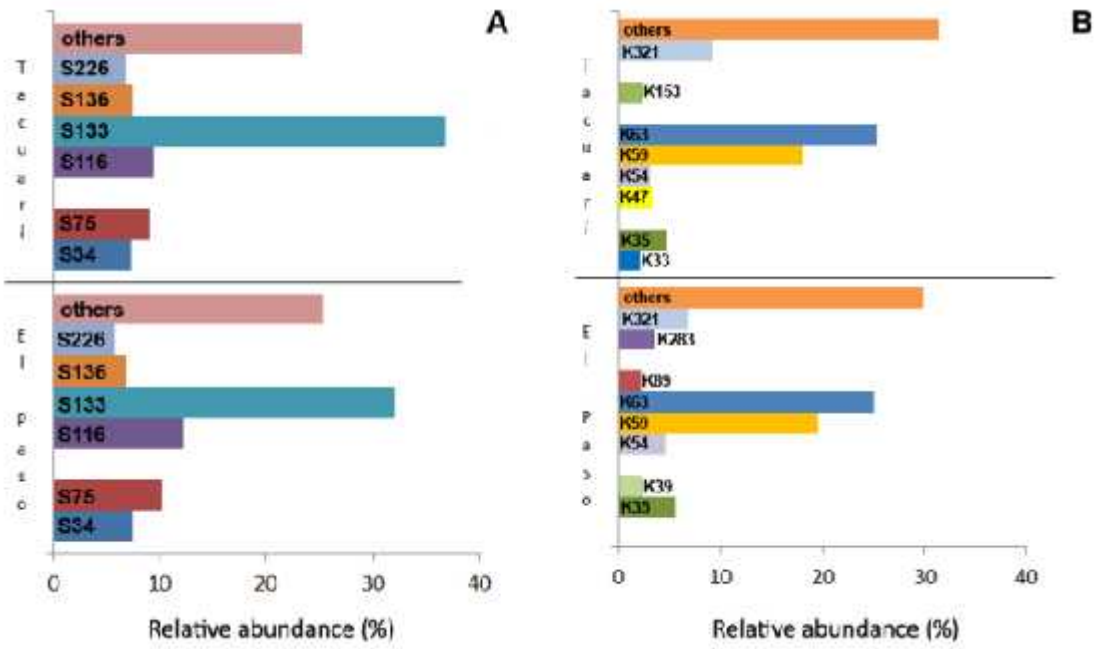
2nd experiment		Sowing	tillering	flower primordium	harvest
N	Northern soil	13.7 (3.0) *	15.7 (1.6) *	17.3 (1.4) *	17.2 (4.8) *
	Eastern soil	16.2 (3.6) *	17.0 (3.5) *	16.8 (3.2) *	15.5 (4.2) *
	Overall	14.9 (3.6) A	16.3 (2.7) A	17.1 (2.4) A	16.3 (4.4) A
H'	Northern soil	2.3 (0.3) *	2.4 (0.2) *	2.4 (0.2) *	2.4 (0.3) *
	Eastern soil	2.3 (0.3) *	2.5 (0.3) *	2.4 (0.2) *	2.2 (0.3) *
	Overall	2.3 (0.3) A	2.4 (0.2) A	2.4 (0.2) A	2.3 (0.3) A
J'	Northern soil	0.88 (0.03) *	0.86 (0.05) *	0.84 (0.06) *	0.87 (0.04) *
	Eastern soil	0.83 (0.07) *	0.87 (0.04) *	0.87 (0.03) *	0.83 (0.07) *
	Overall	0.85 (0.06) A	0.87 (0.05) A	0.86 (0.05) A	0.85 (0.06) A



Supplementary figure 1. Relative abundances of the terminal restriction fragments (T-RFs) that were obtained from the terminal restriction fragment length polymorphism profiles of denitrifying genes in the first experiment. *nirS* (A) and *nirK* (B) genes T-RFs are arranged according to rice cultivar (n=24). T-RFs names are depicted inside or nearby bars. T-RFs named “others” represent all of the T-RFs with maximum relative abundances below 5% (*nirS*) or below 2.5% (*nirK*). Others included 25 and 85 T-RFs for *nirS* and *nirK*, respectively.



Supplementary figure 2. Abundance of *nirS* gene in the second experiment according to sampling date and to time of flooding. Light gray bars represent abundance on "early flooding" and dark gray on "late flooding" treatment. Striped bars represent overall abundance. Different letters on striped bars indicate significant differences (Tukey's test $p<0.05$) in different sampling date. Different letters on gray bars indicate significant differences according to time of flooding x soil type interaction (Tukey's test $p<0.05$). Gray bars represent means (n=3) ± SDs. Striped bars represent means (n=6) ± SDs.



Supplementary figure 3. Relative abundances of the terminal restriction fragments (T-RFs) that were obtained from the terminal restriction fragment length polymorphism profiles of denitrifying genes in the second experiment. *nirS* (A) and *nirK* (B) genes T-RFs are arranged according to soil type (n=24). T-RFs names are depicted inside or nearby bars. T-RFs named "others" represent all of the T-RFs with maximum relative abundances below 5% (*nirS*) or below 2.5% (*nirK*). Others included 29 and 92 T-RFs for *nirS* and *nirK*, respectively.