UNIVERSIDAD DE GRANADA

CSIC
CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

PROGRAMA DE DOCTORADO EN BIOLOGÍA FUNDAMENTAL Y DE SISTEMAS

Departamento de Microbiología, Facultad de Farmacia, Universidad de Granada

Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas

# EMISSION OF GREENHOUSE GASES AND MICROBIAL BIODIVERSITY IN SOILS OF AGRICULTURAL INTEREST. EFFECT OF NITROGEN FERTILISATION

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TESIS DOCTORAL, 2019

Editor: Universidad de Granada. Tesis Doctorales
Autor: Antonio Castellano Hinojosa
ISBN: 978-84-1306-212-9
URI: http://hdl.handle.net/10481/55892

Esta investigación forma parte de los Proyectos de Investigación "Emisión de óxido nitroso por suelos cultivados con leguminosas y hortalizas de interés agrícola", referencia Proyecto de Excelencia AGR-1968, de la Consejería de Economía, Innovación y Ciencia de la Junta de Andalucía, y RECUPERA 2020, "Emisión de óxido nitroso en suelos y biodiversidad bacteriana asociada a la fertilización nitrogenada", referencia 20134R070, financiado por el Ministerio de Economía y Competitividad

Esta investigación se ha llevado a cabo mayoritariamente en el Departamento de Microbiología del Suelo y Sistemas Simbióticos de la Estación Experimental del Zaidín del Consejo Superior de Investigaciones Científicas

La realización de esta tesis ha sido posible gracias a la beca FPU 2014/01633 del programa de Formación del Profesorado Universitario (FPU) del Ministerio de Educación, Cultura y Deporte del gobierno de España

*Lo que doy, me lo doy. Lo que no doy, me lo quito. Nada para mí que no sea para los demás.*

*Alejandro Jodorowsky*

# AGRADECIMIENTOS

## *AGRADECIMIENTOS*

Dedico estas líneas para agradecer a todas las personas que me han acompañado en este apasionante camino. Muchas personas han influido, directa o indirectamente, a lo largo de mi vida personal y académica en que hoy redacte estas líneas:

Agradezco a mis padres, a mis hermanas. Siempre un referente de valores y esfuerzo para mí. Gracias por apoyarme y acompañarme.

Gracias a mis directores de tesis: Dr. Eulogio J. Bedmar y Dr. Jesús González López. Largos y muchos han sido los días que hemos compartimos juntos. Os agradezco cada una de vuestras enseñanzas que me habéis transmitido. Vuestros valores personales y profesionales fueron, son y siempre serán un referente para mí. No puedo describir la inmensa alegría y orgullo que supone para mí que firméis esta tesis doctoral. Gracias por confiar en mí y haberme dado tantas posibilidades.

Mis dos estancias internacionales, en North Wyke (Rothamsted Research, Reino Unido) fueron una auténtica experiencia de vida para mí. Mi primera aventura fuera de España. No tengo palabras para agradecer a la Dra. Laura M. Cardenas y a su grupo de investigación. Fue un auténtico placer colaborar con una excelente persona e investigadora. Fueron tantos los detalles que tuvisteis conmigo que necesitaría varias páginas para describirlos. Gracias por vuestra confianza en mí. Os aprecio más de lo que pueda describir.

Mis estancias de doctorado en centros nacionales estuvieron llenas de buenos momentos personales y académicos. Gracias al Dr. Álvaro Peix y al Dr. Antonio Vallejo por abrirme las puertas de sus grupos de investigación en el IRNASA-CSIC (Salamanca) y ETSI-UPM (Madrid), respectivamente. Fue una auténtica suerte conocer y colaborar con investigadores de referencia como vosotros. Gracias por vuestros consejos.

Gracias a la Dra. Presentación Carrillo Lechuga y al Dr. Juan Manuel Medina Sánchez por vuestro cariño y apoyo siempre. Es una inmensa suerte haberos conocido, colaborar con vosotros y disfrutar de vuestra compañía. Gracias por creer en mí.

Muchos han sido los profesores que me han acompañado estos años y que han dejado su huella en mí. Gracias Dra. Belén Rodelas, Dra. Clementina Pozo, Dr. Miguel Quemada, Dr. Alejandro González-Martínez, Dra. Belén Juárez, Dra. Mª Jesús Delgado, Dra. Socorro Mesa, Dra. Concepción Calvo, Dra. Elisabeth Aranda, Dr. Maximino Manzanera y un largo etc. Asimismo, agradezco a todos de los profesores que me motivaron durante mi formación universitaria. Me siento muy afortunado de haber conocido a tan excelentes personas y docentes.

Gracias a todos mis compañeros/as de estancias en el IRNASA-CSIC, UPM y North Wyke: Zuleica, Jordana, Arancha, Mónica, Guillermo, Jaime, Alice, Kate, Nadine, Liz, Neil, Kira, Gemma y muchos otros. Vuestro cariño es el mejor de los recuerdos.

Gracias a todos mis compañeros/as del Departamento de Microbiología de la Universidad de Granada, del Departamento de Microbiología del Suelo y Sistemas Simbióticos de la Estación Experimental del Zaidín y del Instituto del Agua. Muchos buenos recuerdos compartimos juntos. No puedo olvidar a mis compañeros/as de Ecología.

Gracias a Virginia, Vicky y Ginés por sacarme siempre una sonrisa.

The stay with a lovely family in the village of North Tawton allowed me to know the customs of the Devon county and enjoy their hospitality and generosity. Many thanks, Jean and Alan.

Gracias especialmente al maestro D. Manolo García. Tu cariño siempre ha sido un regalo para mí.

## *IN MEMORIAM*

Dedico estas líneas para recordar a la profesora Dña. María Victoria Martínez Toledo; nuestra siempre querida "Mavi". Todo lo bueno que encuentren en mí es en gran medida gracias a ella.

Es difícil expresar en pocas palabras el gran impacto que Mavi tuvo en mi vida personal y académica. En segundo de carrera, apenas varias semanas después de comenzar la asignatura de Microbiología, le expresé mi deseo de realizar el trabajo fin de licenciatura en el Departamento de Microbiología. Recuerdo que me dijo "me alegro mucho, nuestro grupo es excelente y vas a aprender mucho". Tras finalizar el curso también le comenté mi deseo de realizar un doctorado en su grupo de investigación. Desde entonces, y durante cada uno de los siguientes 18 meses, recibía un email de su parte motivándome a sacar buenas notas. No lo podría creer. Una Catedrática de Universidad se acordaba cada mes de enviar un email a un estudiante que apenas conocía. "Antonio, tienes que sacar buenas notas para conseguir una beca y quedarte con nosotros, ánimo que puedes". No cabe duda de que ese breve email era una increíble motivación para mí.

Finalizada la carrera, conseguí la beca FPU del Ministerio. Lo celebramos y puedo asegurar que se alegró tanto o más que yo por la buena noticia. Lo que vino después, tristemente es conocido por todos. Sin embargo, ahora podrán entender el tamaño de mi gratitud hacia Mavi. Muchos son los recuerdos que guardo y que siempre me acompañaran. Sirvan estas líneas para recordarte de nuevo, como cada día. Gracias.

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## INDEX

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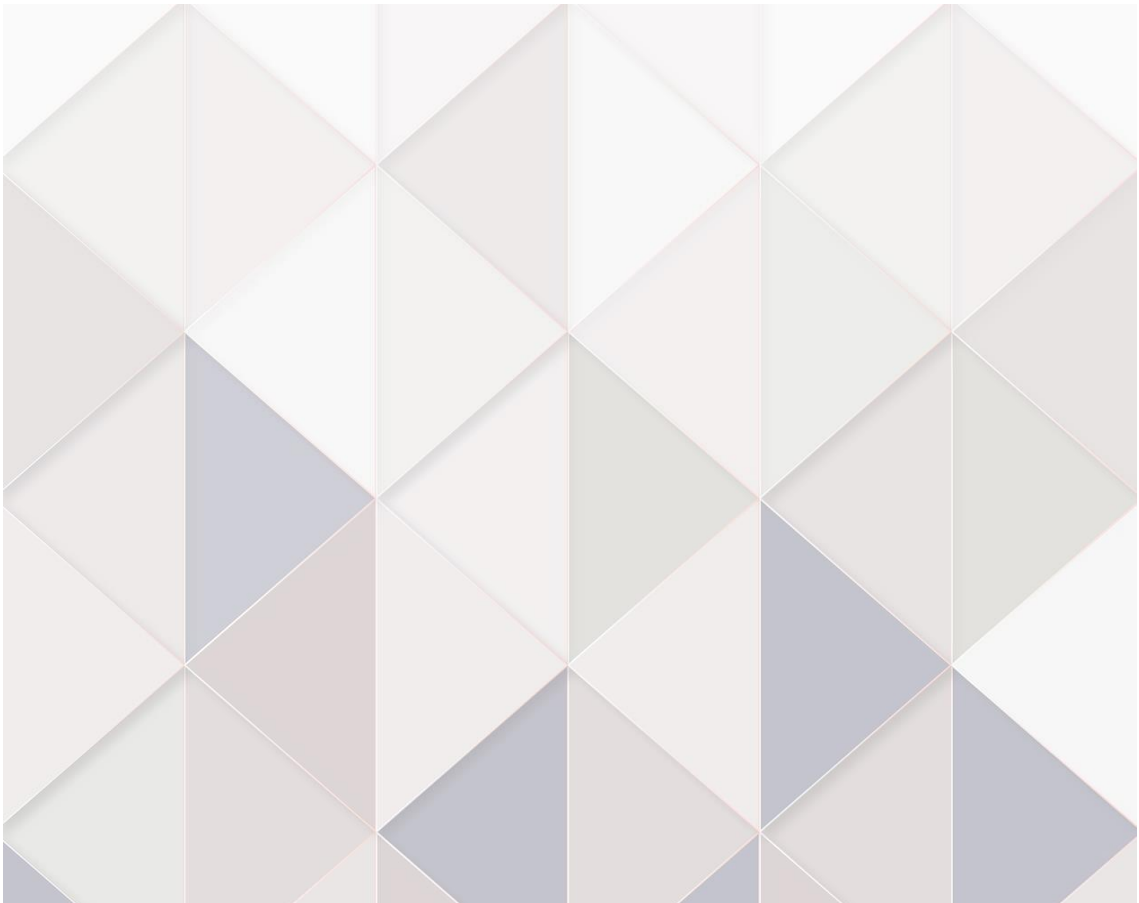


## SUMMARY

In agricultural soils, the application of inorganic nitrogen (N)-fertilisers leads to the interaction of multiple factors and processes which are mainly associated with changes in soil physicochemical properties, emission of greenhouse gases and microbial ecology. Although N is an essential nutrient for plant growth, increased application of N-fertilisers in agriculture has altered the natural N cycle, which result in many environmental, ecological and human health impacts. Among them, N-fertilisation may lead to an increase in the emission of greenhouse gases, acidic deposition and eutrophication.

After application of an N-fertiliser, the microbial processes of nitrification and denitrification are the main esponsible of the reactions driving the conversion of ammonia ($NH_4^+$) and nitrate ($NO_3^-$) to the release of the greenhouse gas nitrous oxide ($N_2O$) into the atmosphere, respectively. Combating the negative impacts of increasing $N_2O$ fluxes poses considerable challenges and will be ineffective without incorporating microbial regulated $N_2O$ processes into mitigation strategies. Although previous studies had shown individual relationships between N-fertilisation and soil biotic and abiotic parameters, an integrated study relating the form of the N-fertiliser with differences in $N_2O$ emission, changes in soil physicochemical properties, alterations in the abundance of the genes involved in $N_2O$ production and reduction, and effects on bacterial diversity had not been reported.

To address these questions, the N-fertilisers urea ($[CO(NH_2)_2]$ ammonia $(NH_4)_2SO_4$ and nitrate ($KNO_3$) were chosen to amend an agricultural Cambisol soil from Vega de Motril (Granada, Spain). Tomato (*Solanum lycopersicum*) and common bean (*Phaseolus vulgaris*) were used as representative vegetable plants. Soils, cultivated or not, were kept under greenhouse conditions and fertilised at an N rate of 260 kg N $ha^{-1}$. Unfertilised soil was used as a control. Uncultivated soils were incubated for 3 years and cultivated soils for 4 consecutive harvests of about 4 months each. During that time, the soils were watered once a week to reach 80% water filled pore space (WFPS). The concentration of the fertilisers was determined regularly and when required the soil was supplemented with the corresponding N-

fertiliser to reach the initial N-fertilisation rate. Soil abiotic variables pH, $NH_4^+$, $NO_3^-$, total carbon (TC), total N (TN) and total organic carbon (TOC) were determined by spot sampling during incubation. The production of $N_2O$ was regularly determined by gas chromatography. Soil samples were taken to determine the total abundance of a) soil bacteria (16SB) and archaea (16SA) by quantitative PCR (qPCR) of the 16S rRNA gene, respectively; b) ammonia oxidising (AO) bacteria (AOB) and archaea (AOA) by qPCR of the corresponding *amoA* gene, respectively, and c) denitrification genes by qPCR of the *napA*, *narG*, *nirK*, *nirS*, *norB*, *nosZ*I and *nosZ*II genes. Variations in the relative abundance of the bacterial OTUs were determined after pyrosequencing of the 16S rRNA gene.

To analyse the distinct effect of N-fertilisation and soil depth, urea, ammonium and nitrate were applied to the soil. $N_2O$ production and the abundance of N-cycling genes were determined along the 20-cm layer of the arable topsoil. Variations in $N_2O$ emissions along the soil profile were dependent on the type of N-fertiliser and the soil depth-related dissolved oxygen content. Also, N-gas emissions correlated with the abundance of the nitrifying and denitrifying communities in the soil. While $N_2O$ production by nitrification was dominant in the 0- to 10-cm soil horizon, denitrification was the main driver of N-gas production in the 10- to 20-cm depth. The *nosZ* gene was the most sensitive to soil depth-related dissolved oxygen content.

To determine the effect of N-fertilisation on the a) soil physicochemical characteristics, b) $N_2O$ emission, c) changes in the abundance of the bacterial and archaeal communities, d) abundance of the nitrifying and denitrifying guilds and e) variations in bacterial diversity, urea, ammonium and nitrate were applied to the soil cultivated or not with tomato and common bean. The study included the bulk and rhizosphere soil of the plants. Fluxes of $N_2O$ emission showed a peak about 2 weeks after N-fertilisation both in cultivated and uncultivated soils. The cumulative $N_2O$ emission was higher in cultivated than uncultivated soils, and higher in the soil cultivated with common bean. Regardless of the presence or the absence of the plants, on a yearly basis, urea produced the higher cumulative emission followed by ammonium and, finally, nitrate. Differences in $N_2O$ emissions were associated to a

distinct ratio of the genes involved in the production and reduction of $N_2O$. Simultaneous application of high water moisture content and inorganic N-fertiliser was required for maximum $N_2O$ production.

Decreases in $N_2O$ production during 3-year incubation for uncultivated soils and 4 consecutive harvests for cultivated soils were associated to increases in the *nosZ* gene abundance.

N-fertilisation decreased the abundance of the total bacterial and archaeal commnuties in uncultivated soils and increased their abundance in the cultivated soils. These results were associated to the soil carbon content.

The *amoA* AOA was more abundant than the *amoA* AOB gene in the rhizosphere soil and, on the contrary, the abundance of the *amoA* AOA was lower than that of the *amoA* AOB in the bulk soil. The denitrification genes were more abundant in the bulk soil. N-fertilisation decreased the number and the relative abundance of bacterial OTUs in soils cultivated or not with tomato and common bean plants; this effect was more severe in the rhizosphere soil. N availability mainly determined the changes in the structure of the bacterial community in bulk and even more in the rhizosphere soil, and the bacterial community became less diverse or dominated by a small group of OTUs. After N-fertilisation, dominant and rare OTUs decreased in the rhizosphere while only the rare OTUs vary in the bulk soil.

To explore the relative contribution of nitrification and denitrification to $N_2O$ production after 3-year fertilisation with ammonium or nitrate, the $^{15}N$ tracer technique was used. In the ammonium-treated soil, $N_2O$ originated from nitrification almost equally that from denitrification and emission from the nitrate-treated soil derived mostly from denitrification. The higher abundance of the *nosZ*I gene in the soil treated with nitrate was consistent with the highest $^{15}N_2$ enrichment.

To build a model to understand the relative importance of each analysed biotic and abiotic variables and bacterial biodiversity as drivers of $N_2O$ emission, combined random forest (RF) and structural equation modelling (SEM) analyses were run using the dataset derived from this study. The results show that $N_2O$ emissions were mainly controlled by the biotic (*amoA* AOA. *amoA* AOB, *napA*, *nirK*,

*norB*, *nosZ*I genes and a set of 16 bacterial OTUs) than the abiotic ($NH_4^+$ and $NO_3^-$ contents) variables.

To quantify the importance of bacterial and fungal denitrification post nitrate application, single and combined application of bacterial (streptomycin) and fungal (cycloheximide) growth inhibitors were used. Although bacteria and fungi almost equally contributed to soil $N_2O$ production, bacteria dominated over that of fungi during the first 2-3 days post nitrate application. After that time, the situation reversed and the production of $N_2O$ by fungi came to dominate that of bacteria.

Investigation of the effects of the single and combined application of the urease inhibitor N-(n-butyl) thiophosphoric triamide (NBPT) and the nitrification inhibitor 3,4 dimethylpyrazole phosphate (DMPP) on ammonia ($NH_3$) volatilisation and the abundance of the nitrifier and denitrifier communities have also pursued in this study. The application of the urease inhibitor NBPT reduced $NH_3$ volatilisation and did not affect the bacterial and archaeal abundance, nor that of the nitrifiers, but reduced the abundance of denitrifiers at 80% WFPS. DMPP, alone and in combination with NBPT, increases $NH_3$ volatilisation and the abundance of bacteria, archaea and nitrifiers in the soil. Regardless of the moisture conditions, DMPP and to a lower extent DMPP + NBPT, increases the gene copy number of the *norB*- and *nosZ*-bearing denitrifying communities, which indicates that DMPP, somehow, induces the expression of the, at least, the *norB* and *nosZ* denitrification genes.

## RESUMEN

En los suelos agrícolas, la aplicación de fertilizantes inorgánicos nitrogenados conduce a la interacción de múltiples factores y procesos que están asociados principalmente con cambios en las propiedades fisicoquímicas del suelo, la emisión de gases de efecto invernadero y la ecología microbiana. Aunque el nitrógeno (N) es un nutriente esencial para el crecimiento de las plantas, el aumento de la fertilización nitrogenada ha alterado el ciclo natural de N en la biosfera, lo que resulta en diversos impactos ambientales, ecológicos y sobre la salud humana. Entre ellos, el aumento de la emisión de gases de efecto invernadero, de la lluvia ácida y eutrofización.

Después de la aplicación de un fertilizante nitrogenado al suelo, los procesos microbianos de nitrificación y desnitrificación son los principales responsables de las reacciones que conducen a la conversión de amonio ($NH_4^+$) y nitrato ($NO_3^-$), respectivamente, a la liberación a la atmósfera del gas de efecto invernadero óxido nitroso ($N_2O$). Combatir los impactos negativos del aumento de los flujos de $N_2O$ plantea desafíos considerables y será ineficaz sin incorporar los procesos microbianos que intervienen en la emisión de $N_2O$ en las estrategias de mitigación. Aunque previos estudios han mostrado la existencia de relaciones individuales entre la fertilización con N y cambios en las propiedades bióticas y abióticas del suelo, un estudio integrado relacionando la forma del fertilizante N con diferencias en la emisión de $N_2O$, cambios en las propiedades fisicoquímicas del suelo, alteraciones en la abundancia de los genes involucrados en la producción y reducción de $N_2O$ y los efectos sobre la diversidad bacteriana no se ha realizado.

Para responder a estas preguntas se eligieron los fertilizantes nitrogenados urea ($[CO(NH_2)_2]$ amonio $(NH_4)_2SO_4$ y nitrato ($KNO_3$) para enmendar un suelo agrícola de textura franco-arenosa de tipo Cambisol procedente de Vega de Motril (Granada, España). Tomate (*Solanum lycopersicum*) y judía (*Phaseolus vulgaris*) se utilizaron como plantas representativas de la agricultura de la zona. Los suelos, cultivados o no, se mantuvieron en condiciones de invernadero y se fertilizaron con 260 kg N $ha^{-1}$. Como control se empleó suelo no fertilizado. Los suelos no cultivados se mantuvieron durante 3 años y los cultivados durante 4 cosechas consecutivas de,

aproximadamente, 4 meses cada una. Durante ese tiempo, los suelos se regaron una vez a la semana para alcanzar un 80% de WFPS (Water Filled Pore Space). La concentración de los fertilizantes se determinó regularmente y cuando fue necesario, el suelo se complementó con el fertilizante correspondiente para alcanzar la tasa inicial de fertilización nitrogenada. Las variables abióticas del suelo pH, $NH_4^+$, $NO_3^-$, carbono total (TC), nitrógeno total (TN) y carbono orgánico total (TOC) se determinaron mediante muestreos puntuales durante la incubación. La producción de $N_2O$ se determinó regularmente empleando cromatografía de gases. Se tomaron muestras de suelo para determinar la abundancia total de a) bacterias (16SB) y arqueas (16SA), b) bacterias (AOB) y arqueas (AOA) oxidantes de amonio, y c) genes de la desnitrificación. La estimación de la abundancia de los genes 16S rRNA, *amoA* AOA, *amoA* AOB, *napA*, *narG*, *nirK*, *nirS*, *norB*, *nosZ*I y *nosZ*II se llevó a cabo mediante PCR cuantitativa (qPCR). Las variaciones en la abundancia relativa de OTUs (Operational Taxonomic Units) se determinó mediante pirosecuenciación del gen 16S rRNA.

Para analizar el efecto de la fertilización con N y la profundidad del suelo se adicionó urea, amonio y nitrato al suelo. La producción de $N_2O$ y la abundancia de genes del ciclo del N se determinaron a lo largo de la capa cultivable (20 cm) del suelo. Las variaciones en las emisiones de $N_2O$ a lo largo del perfil dependieron del tipo de fertilizante y del contenido de oxígeno disuelto detectado a cada profundidad. Las emisiones de $N_2O$ se correlacionaron con la abundancia de las comunidades nitrificantes y desnitrificantes en el suelo. Si bien la producción de $N_2O$ por nitrificación fue dominante en el horizonte del suelo de 0 a 10 cm, la desnitrificación fue el principal impulsor de la producción de $N_2O$ en la profundidad de 10 a 20 cm. El gen *nosZ* fue el más sensible al contenido de oxígeno disuelto a lo largo del perfil del suelo.

Para determinar el efecto de la fertilización nitrogenada sobre a) las características fisicoquímicas del suelo, b) la emisión de $N_2O$, c) los cambios en la abundancia de las comunidades bacterianas y arqueas, d) la abundancia de las comunidades nitrificantes y desnitrificantes y e) las variaciones en la diversidad bacteriana, se aplicó al suelo urea, amonio o nitrato y se cultivó, o no, con tomate y

judía. El estudio incluyó tanto el suelo no rizosférico como el rizosférico de las plantas. Los flujos de emisión de $N_2O$ mostraron un pico máximo aproximadamente 2 semanas después de la fertilización con N, tanto en suelos cultivados como no cultivados. La emisión acumulada de $N_2O$ fue mayor en los suelos cultivados que en los no cultivados, y más elevada en el suelo cultivado con judía. Independientemente de la presencia o ausencia de las plantas, la emisión acumulada de $N_2O$ en el suelo tratado con urea fue mayor que el suelo con amonio y, a su vez, este mayor que el fertilizado con nitrato. Las diferencias en las emisiones de $N_2O$ se asociaron a un distinto porcentaje de los genes involucrados en la producción y reducción de $N_2O$. Se requiere, de forma simultánea, la aplicación de un fertilizante nitrogenado y condiciones de elevada humedad para la máxima producción de $N_2O$. Las disminuciones en la producción de $N_2O$ durante 3 años de incubación para suelos sin cultivar y 4 cosechas consecutivas para suelos cultivados se asociaron con aumentos en la abundancia del gen *nosZ*. La fertilización con N disminuyó la abundancia de bacterias y arqueas totales en suelos no cultivados y aumentó en los suelos cultivados. Estos resultados se asociaron al contenido de carbono del suelo.

El gen *amoA* AOA fue más abundante que el gen *amoA* AOB en el suelo de la rizosfera y, por el contrario, la abundancia del *amoA* AOA fue menor que la del *amoA* AOB en el suelo no rizosférico. Los genes de la desnitrificación fueron más abundantes en el suelo no rizosférico. La fertilización con N disminuyó el número y la abundancia relativa de las OTUs bacterianas en los suelos cultivados o no con plantas de tomate y judía; este efecto fue más severo en el suelo rizosférico. La disponibilidad de N determinó principalmente los cambios en la estructura de la comunidad bacteriana en el suelo no rizosférico y fue más intenso en el suelo rizosférico. Después de la fertilización, la comunidad bacteriana fue menos diversa o dominada por un menor grupo de OTUs. En los 3 suelos fertilizados, tanto las OTU dominantes y minoritarias disminuyeron en el suelo rizosférico, mientras que solo las OTU minoritarias disminuyeron en el suelo no rizosférico.

Para explorar la contribución relativa de la nitrificación y la desnitrificación en la producción de $N_2O$ después de una fertilización de 3 años con amonio o nitrato, se utilizó la técnica del marcado con $^{15}N$. En el suelo tratado con amonio, el $N_2O$ se

originó a partir de la nitrificación casi por igual que de la desnitrificación mientras que la emisión del suelo tratado con nitrato derivó principalmente de la desnitrificación. La mayor abundancia del gen *nosZ*I en el suelo tratado con nitrato fue consistente con el mayor enriquecimiento de $^{15}N_2$.

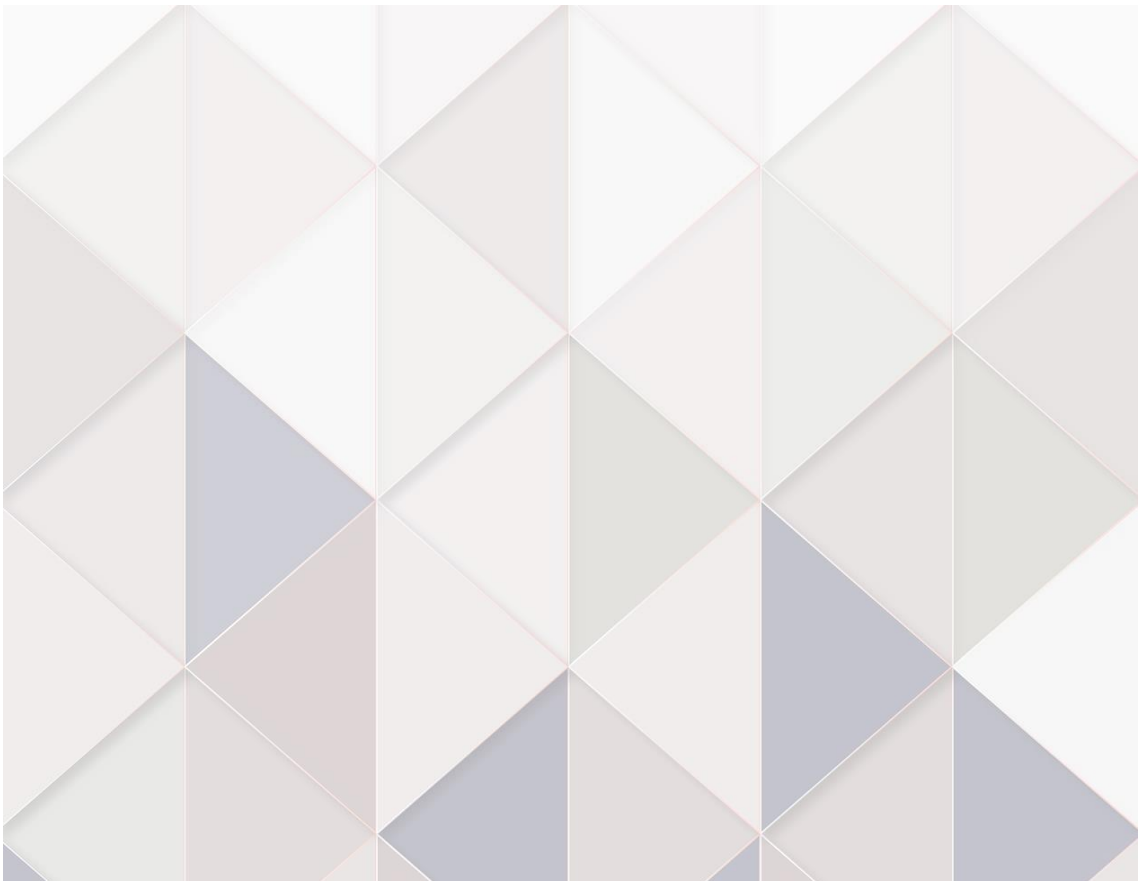
Para construir un modelo que explicara la importancia relativa de cada una de las variables bióticas y abióticas analizadas y la biodiversidad bacteriana como determinantes de la emisión de $N_2O$, se realizó un análisis combinado de modelos de ecuaciones estructurales y de "bosques aleatorios" utilizando el conjunto de datos derivado de este estudio. Los resultados mostraron que las emisiones de $N_2O$ estuvieron controladas principalmente por las variables bióticas (abundancia de los genes *amoA* AOA. *amoA* AOB, *napA*, *nirK*, *norB*, *nosZ*I y un conjunto de 16 OTUs bacterianas) más que por las variables abióticas (contenido de $NH_4^+$ y $NO_3^-$).

Para cuantificar la importancia de la desnitrificación bacteriana y fúngica después de la aplicación de nitrato, se utilizó la aplicación individual y combinada de inhibidores del crecimiento bacteriano (estreptomicina) y fúngico (cicloheximida). Aunque las bacterias y los hongos contribuyeron casi por igual a la producción de $N_2O$ en el suelo, las bacterias dominaron sobre las de los hongos durante los primeros 2-3 días posteriores a la aplicación de nitrato. Después de ese tiempo, la situación se revirtió y la producción de $N_2O$ por hongos llegó a dominar la de las bacterias.

La investigación de los efectos de la aplicación simple y combinada del inhibidor de la ureasa N-(n-butil) triamida tiofosfórica (NBPT) y del inhibidor de la nitrificación 3,4 dimetilpirazol fosfato (DMPP) sobre la volatilización de amoniaco ($NH_3$) y la abundancia de las comunidades nitrificantes y desnitrificantes también se abordó en este estudio. La aplicación de NBPT redujo la volatilización de $NH_3$ y no afectó la abundancia de bacterias y arqueas totales, ni la de los genes de la nitrificación, pero redujo la abundancia de genes de la desnitrificación al 80% de WFPS. El DMPP, solo y en combinación con NBPT, aumentó la volatilización del $NH_3$ y la abundancia de bacterias y arqueas totales así como de la comunidad nitrificante en el suelo. Independientemente de las condiciones de humedad, DMPP y, en menor medida, DMPP + NBPT, aumentaron el número de copias los genes *norB* y *nosZ*, lo

que indica que DMPP, de alguna manera, induce la expresión de, al menos, estos dos genes de la desnitrificación.

# INTRODUCTION



# 1. INTRODUCTION

## 1.1. Agriculture and nitrogen fertilisation

The human body requires approximately 2 kg N $yr^{-1}$ of protein to survive (Smil 2000). For thousands of years the collective human metabolic N requirement was met by the goods and services provided by unmanaged ecosystems, in essence using the nitrogen produced by naturally occurring biological nitrogen fixation (BNF). As populations increased and agriculture developed, natural sources of N had to be supplemented with additional sources. The so-called Green Revolution began at the end of the 1960s motivated by the need to feed an increasingly numerous population, consisted of a significant increase in agricultural productivity and, ultimately, in world food production (Galloway and Cowling 2002; Galloway et al. 2004; Kahiluoto et al. 2014). This was possible thanks to the use of improved plant varieties, especially cereals, and their increased production in a monoculture regime in response to an abundant application of water, pesticides and synthetic fertilisers, mainly N-fertilisers such as urea, ammonium or nitrate (Khush 2001) (Fig. 1).

According to the International Fertiliser Association of the total of fertilisers used in agricultural practices in 2016, more than 54.9%, 24.5% and 20.6% corresponds to nitrogen, phosphates and potassium, respectively (www.fertilizer.org). Data of the Organization for Food and Agriculture (FAO) of the World Health Organization show that the production of N-fertilisers worldwide has increased approximately 5 times from 1961 to 2018 (FAO 2015). The world N-fertiliser demand increased from 2010 at a growth rate of 1.5 percent and it is expected to be around 119,400,000 tonnes in 2018 (FAO 2015). Of the overall demand for N in 2018, 58% would be in Asia, 22% in the Americas, 11% in Europe, 8% in Africa and 1% in Oceania (FAO 2015).

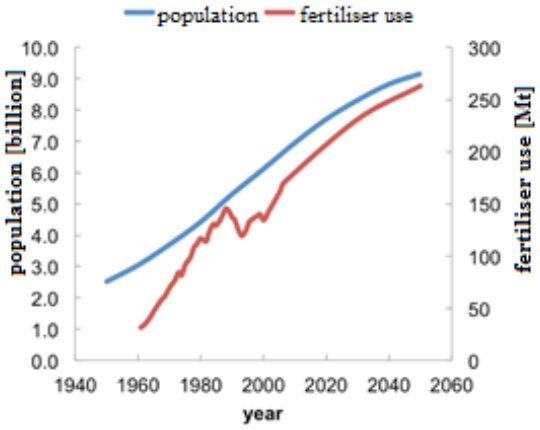



*Fig. 1. N-fertilisation consumption since the beginning of the so-called Green Revolution (www.fao.org).*

The compounds of N in nature can be divided into two large groups, N non-reactive (dinitrogen, $N_2$) and N reactive (Nr), which includes all biological compounds, photochemical and active radiates in the atmosphere or terrestrial biosphere such as reduced forms of inorganic N (ammonia, $NH_3$ and ammonium, $NH_4^+$), oxidized forms of inorganic N (NOx, nitrous oxide, $N_2O$, nitric acid, $HNO_3$ and nitrate, $NO_3^-$) and organic compounds (urea, amines, proteins and nucleic acids).

Until the emergence of the industrial processes of synthesis of N-fertilisers (Haber-Bosch and Wöhler processes), BNF and denitrification (the sequencial reduction of $NO_3$- to $N_2$) processes had similar yields, about 110 tons of $NH_4^+$ produced from $N_2$ compared to 108 tons of $NO_3^-$ eliminated as $N_2O$ or $N_2$ by denitrification (Gruber and Galloway 2008). While denitrification has not increased significantly, the total amount of Nr is close to 240-260 Tg N $yr^{-1}$ (Bouwman et al. 2013) and N-fertilisation represents between 100-121 N additional Tg N $yr^{-1}$, which double that $NH_4^+$ produced by the BNF (Galloway et al. 2008, Fowler et al. 2013). Other inorganic and organic nitrogenous compounds (livestock slurry, urban solid and liquid waste, industrial activities, etc.) are also deposited in soils, seas and oceans so the total annual contribution of Nr to the environment can reach 345 Tg N $yr^{-1}$ (Galloway et al. 2008; Bouwman et al. 2013). Consequently, denitrification can not eliminate the excess Nr produced (Galloway et al. 2008; Gruber and Galloway 2008; Nieder and Benbi 2008; Bouwman et al. 2013) (Fig. 2).

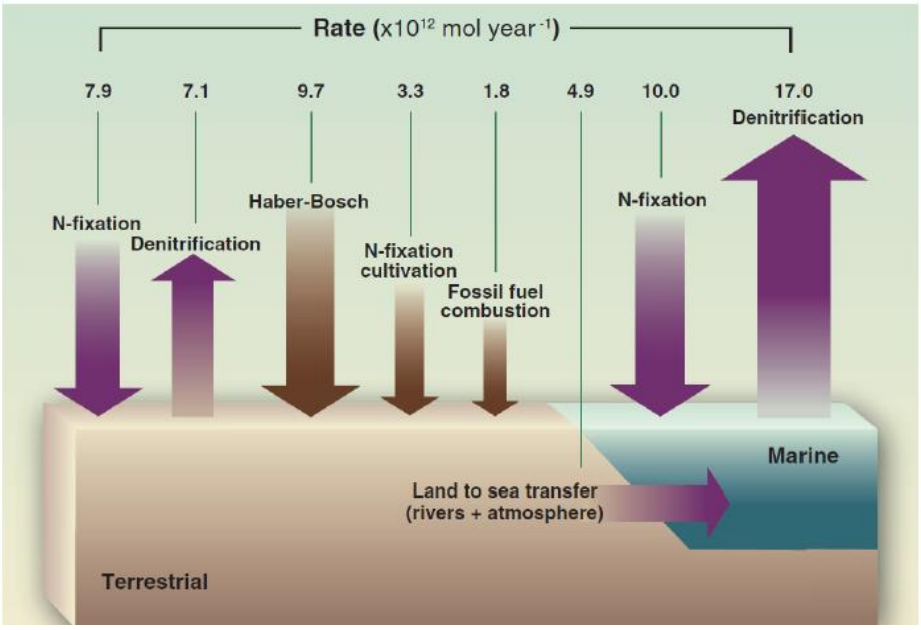



*Fig. 2. Rates of nitrogen flux in the nitrogen cycle. Arrow size shows the relative size of the flux and brown arrows represent anthropogenic inputs (Canfield et al. 2010).*

## 1.2. Nitrous oxide emissions

An estimated 40-70% of agriculturally applied N is available to plants during a growing season, while the rest is either converted into soil organic N or lost through $NH_3$ volatilisation, leaching, surface runoff, emission of greenhouse gases and soil erosion (Cameron and Moir 2013; Jones et al. 2014; Duran et al. 2016). The metabolism of most nitrogenous compounds commonly results in the formation of $NO_3^-$, which accumulates in soils, waters and sediments causing eutrophication, contamination of estuaries, surface and groundwater as well as human health problems (Erisman et al. 2015; Follet et al. 2010; Sutton et al. 2011).

Due to globalization, many problems of worldwide concern have been discussed in international state conferences during the last decades. $NO_3^-$ pollution has received great attention, as favours the emission of the greenhouse gas $N_2O$, related to global climate change (Sutton et al. 2011; Erisman et al. 2015). Agricultural is known to be the major anthropogenic source of $N_2O$ (Table 1.1) due to N-fertiliser use and manure management (6-7 Tg $N_2O$-N $yr^{-1}$) representing 56-70% of all global $N_2O$ sources (Syakila and Kroeze 2011: Butterbach-Bahl et al. 2013; Erisman et al. 2015). $N_2O$ emissions are of great importance because of its high global warming

potential: 298 times greater than $CO_2$ and considering 114 years lifetime in the atmosphere (Forster et al 2007). In addition, $N_2O$ is the single most important depleting substance of stratospheric ozone (Ravinshakara et al. 2009) and it is photolytically decomposed in the stratosphere. During the photolysis, NO is formed which leads to the destruction of ozone molecules (Crutzen 1981).

Nitrous oxide was discovered by Joseph Priestley in 1772. From a preindustrial value of about 270 ppb, the atmospheric concentration of nitrous oxide has been increasing to a concentration of 333 ppb in December 2018 (https://www.n2olevels.org/). The annual increase in atmospheric $N_2O$ concentration was 0.78 ppb during the last ten years (WMO 2017) and its contribution to the total anthropogenic greenhouse gas emission is about 7.9% (IPCC 2013).

*Table 1.1. Sources of nitrous oxide (IPCC 2013).*

| Source | $N_2O$ Tg N $yr^{-1}$ | Range |
|---|---|---|
| Anthropogenic sources | | |
| Fossil fuel combustion and industrial processes | 0.7 | 0.2 - 1.8 |
| Agriculture | 5.8 | 4.7 - 6.8 |
| Biomass and biofuel burning | 0.7 | 0.2 - 1.0 |
| Human excreta | 0.2 | 0.1 - 0.3 |
| Rivers, estuaries, coastal zones | 1.7 | 0.5 - 2.9 |
| Atmospheric deposition | 0.6 | 0.3 - 0.9 |
| Anthropogenic total | 9.7 | |
| Natural sources | | |
| Soils under natural vegetation | 6.6 | 3.3 - 9.0 |
| Oceans | 3.8 | 1.8 - 5.8 |
| Atmospheric chemistry | 0.6 | 0.3 - 1.2 |
| Natural total | 11.0 | |
| Total sources | 20.7 | 11.5 - 30.7 |

## 1.3. Production and consumption processes of nitrous oxide in soils

For the estimation of direct emissions from managed soils, the IPCC stablished a default value which is called emission factor 1 (EF1) (IPCC 2013). It assumes that 1.0% (range 0.3 - 3%) of the total N additions from mineral fertilisers, organic amendments and crop residues as well as N mineralized as a result of loss of soil carbon (C) are lost as direct $N_2O$ emissions (IPCC 2013).

Microbial nitrification and denitrification in managed and natural soils contribute approximately 70% of global $N_2O$ emissions. Nevertheless, there are other biotic and abiotic processes producing $N_2O$ in soils (see compressive reviews by Syakila and Kroeze 2011; Baggs and Philippot 2011; Butterbach-Bahl et al. 2013; Hu et al. 2015) (Fig. 3):

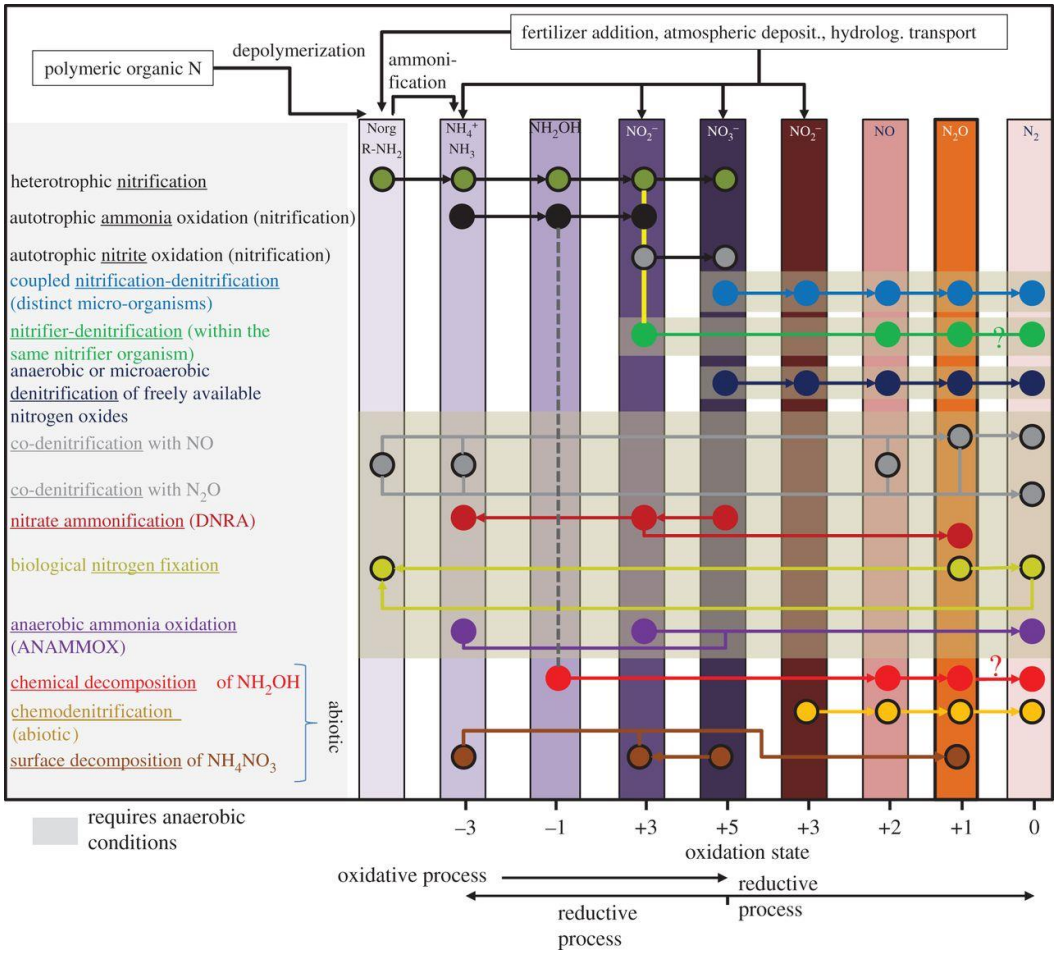



*Fig. 3. Biotic and abiotic processes of $N_2O$. Processes potentially leading to $N_2O$ formation and consumption, involved N compounds, their reaction pathways as well as their oxidation states are shown. According to current knowledge, anaerobic ammonia oxidation does not contribute to $N_2O$ formation or consumption. Processes predominantly requiring anaerobic (or micro-aerobic) conditions are underlined by grey illuminated segments. Norg/R-$NH_2$, monomeric organically bound N forms; $NH_4^+$, ammonium; $NH_3$, ammonia; $NH_2OH$, hydroxylamine; $NO_2^-$, nitrite; $NO_3^-$, nitrate; NO, nitric oxide; $N_2O$, nitrous oxide; $N_2$, molecular dinitrogen. DNRA, Dissimilatory Nitrate Reduction to Ammonium (Butterbach-Bahl et al. 2013).*

## 1.4. Nitrification

Nitrification was discovered and first described by the Russian microbiologist, Sergei Winogradsky at the end of the 19th century (Winogradsky 1892) and it is the oxidation of $NH_4^+$ to $NO_3^-$ with $NO_2^-$ as an intermediate under aerobic conditions. $NH_4^+$ is converted via $NH_2OH$ to $NO_2^-$. This step is performed by

chemolithoautotrophs microorganisms, which utilise $CO_2$ as C source, $NH_3$ as electron donor and oxygen ($O_2$) as electron acceptor. $NO_2^-$ is then further oxidized to $NO_3^-$. $N_2O$ can be formed by two biochemical pathways (Fig. 4a, b): Firstly, as a bio-product during the $NH_4^+$ oxidation, hydroxylamine is spontaneously decomposed to $N_2O$. This process is regarded as the main source of $N_2O$ from nitrification. Secondly, it can be formed by the so-called nitrifier denitrification (Kool et al. 2011; Wrage-Mönnig et al. 2018), where $N_2O$ is an intermediate of the reduction of $NO_2^-$ to $N_2$.

The most intensify studied groups of nitrifiers are the chemolitotrophic ammonium oxidising bacteria (AOB) and nitrite oxidising bacteria (NOB). The AOB are classified into three genera on the basis of their *rrs* gene sequences (Kowalchuk and Stephen 2001; Erguder et al. 2009): *Nitrosomonas*, *Nitrosospina* and *Nitrosococcus*. Much less studies have been done on the classification of NOB, which are classified into four genera: *Nitrobacter*, *Nitrospina*, *Nitrococcus* and *Nitrospira* (Daims et al. 2016).

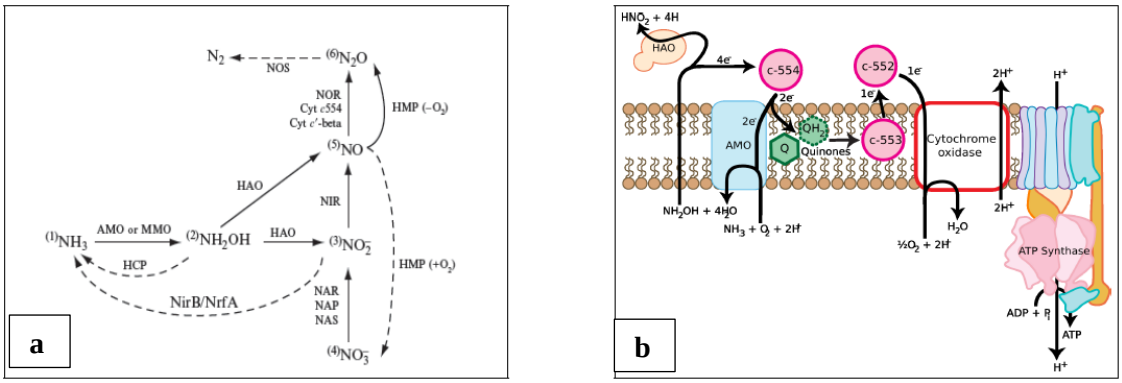



*Fig. 4.* ***a.*** *$N_2O$ production and consumption in bacteria. Solid lines: pathways that lead to $N_2O$ as an end product; dashed lines: pathways that consume $N_2O$ or remove a substrate for its production (AMO: ammonium monooxygenase, MMO methane monooxygenase, HAO hydroxylamine oxidoreductase).* ***b.*** *Enzymes of nitrification and energy generation in Nitrosomonas (AMO: ammonium monooxygenase, HAO hydroxylamine oxidoreductase, c: Cytochromes (Prosser 1989; Kowalchuk and Stephen 2001).*

Besides the chemolithotrophic bacteria, also some ammonia-oxidising archaea (AOA) can nitrify (Leininger et al. 2006; Stahl and de la Torre 2012; Hatzenpichler 2012; Prosser and Nicol 2012). The mechanisms by which AOA produces $N_2O$ remains unclear (Walker et al. 2010; Hink et al. 2017, 2018). Some authors suggest that $N_2O$ may be produced abiotically by oxidation of compounds such as $NH_2OH$, NO or $NO_2^-$ (Harper et al. 2015). Classified initially by *rrs* gene phylogeny as Crenarchaeota, comparative genomics and phylogeny of concatenated genes placed these microorganisms into the new archaeal phylum Thaumarchaeota (Hatzenpichler 2012; Prosser and Nicol 2012). Although being members of two different domains of life, AOB and AOA exploit homologous ammonia monooxygenases (*amoA* gene), that are members of the copper-containing membrane-bound monooxygenase (CuMMOs) enzyme family (Hatzenpichler 2012; Prosser and Nicol 2012).

Generally, AOA seem to dominate ammonia oxidation in soil under low N availability ($<$ 15 µg $NH_4^+$-N g dry $soil^{-1}$), whereas AOB become more competitive at higher N loads (Jia and Conrad 2009; Prosser and Nicol 2012). The nature of the $NH_4^+$ source might be of relevance for niche and physiological differentiation of archaeal and bacterial ammonia oxidisers (Hink et al. 2017, 2018). AOA activity was detected when $NH_4^+$ was supplied as mineralized organic N derived from composted manure or soil organic matter while AOB-dominated activity was measured with $NH_4^+$ originating from inorganic fertiliser (Schleper and Nicol 2010). In addition, (meta-) genome analyses (Martin-Cuadrado et al. 2008; Walker et al. 2010; Pester et al. 2012) and environmental studies (Herndl et al. 2005; Ingalls et al. 2006) indicate that AOA might be able to switch from autotrophic ammonia oxidation to a mixotrophic and possibly even heterotrophic lifestyle, a capacity that may contribute to their numerical dominance in soils.

### 1.5. Denitrification

Denitrification is a facultative respiratory pathway where $NO_3^-$ is stepwise reduced to $N_2O$ or $N_2$ via $NO_2^-$ and NO under oxygen-limiting conditions (Zumft 1997) (Fig. 5). Each step is coupled to the electron transport chain and electrons from

reductants can be passed on to different N oxides, allowing for the generation of a proton gradient across the membrane (Fig. 6).

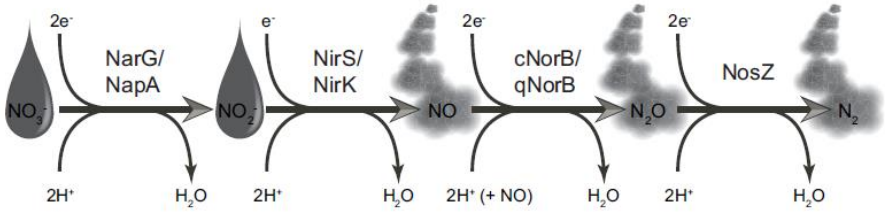



*Fig. 5. The denitrification pathway, with soluble and gaseous products indicated. Enzymes catalysing each step are listed above their respective function.*

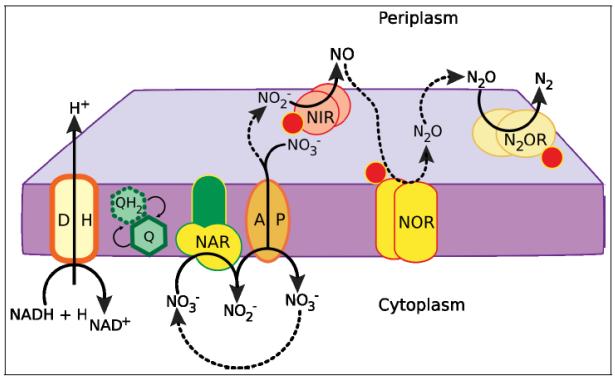



*Fig. 6. Formation of $N_2O$ during denitrification and involved enzymes (Moreno-Vivian et al. 1999).*

The complete denitrification pathway is catalysed by a series of different enzymes, some of them can be functionally redundant. The first step, the conversion of $NO_3^-$ to $NO_2^-$ is catalysed by either a membrane associated nitrate reductase, encoded by the *narG* gene, or its soluble periplasmic homologue encoded by the *napA* gene. The Nar enzyme is present in members of the phyla Proteobacteria, Firmicutes, Actinobacteria and the Archaea domain, while Nap is only present in Proteobacteria (Bru et al. 2007). Both types of enzymes have been found in the genome of Fungi (Shoun et al. 2012; Mothapo et al. 2015). Nar is an integral membrane enzyme composed of three subunits called NarGHI. The Nar proteins are encoded by the genes of the narGHJI operon. The Nap enzyme is composed of three subunits of which NapA and NapB are located in the periplasm and a third, called NapC, is an integral membrane protein. The enzyme is widely distributed in Proteobacteria (Simpson et al. 2010; Sparacino-Watkins et al. 2014).

The conversion of $NO_2^-$ to NO via nitrite reductases is considered the defining step in denitrification (Zumft 1997; Shapleigh 2006). This step is carried out by one of two different NO-forming $NO_2^-$ reductases, encoded by the *nirK* or *nirS* genes. Despite having similar functional roles and localization in the cell, each protein has completely different structural features. The NirK protein is a member of the multi-copper oxidase family, with copper ions as ligands within the catalytic centre. By contrast, the NirS protein contains two different heme ligands within the active centres of the enzyme. Previous studies has shown the functional redundancy of *nir* types in denitrifiers (Glockner et al. 1993). The *nirK* gene has been identified in both prokaryotes and in eukaryotes (Long et al. 2015), while the *nirS* gene has been identified only in Bacteria and Archaea (Mardanov et al. 2015). Although NirS and NirK are not related in evolutionary terms, the *nirK* gene prevails in Alphaproteobacteria, Firmicutes and Bacteroidetes, *nirS* it is more common in Betaproteobacteria and there are no differences in its abundance in the case of Gammaproteobacteria (Heylen et al. 2006).

The reduction of NO to $N_2O$ is carried out by nitric oxide reductase (Nor), a membrane associated protein- There are three types of enzymes Nor, one dependent on a citrochrome c or pseudoazurin (cNor), another that uses quinol (qNor) and the third, which is called qCuANor, is a qNor enzyme that contains a different active copper centre (de Vries et al. 2007; van Spanning et al. 2011; Shiro et al. 2012; Tosha and Shiro 2013). Although there is no clear prevalence between the *cnorB* and *qnorB* genes among the different phylogenetic groups, the Alphaproteobacteria only present the *cnorB* gene, while that the rest of the bacterial classes have one or another type of gene (Jones et al. 2008). The ability to reduce NO to $N_2O$ is not unique to denitrification, as NO is highly toxic and a powerful intracellular signalling compound, and thus microorganisms may possess Nor as a means of detoxification (Zumft 2005).

Finally, the conversion of $N_2O$ to $N_2$ is carried out by the *nosZ* gene product, thus closing the nitrogen cycle as $N_2$ can re-enter the biosphere through N-fixation. The operon of the genes is conserved in most of the microorganisms and usually includes the genes nosRZDFYLX (Wunsch et al. 2003, Pauleta et al. 2013). The *nosZ* gene

encodes the catalytic subunit of nitrous oxide reductase, an enzyme that contains two domains, one called CuA, which is involved in the transfer of electrons, and the other known as CuZ, which contains Cu and S, where the centre is located catalytic enzyme (Pauleta et al. 2013). The rest of the genes encode other proteins necessary for the transcription and assembly of the active centres of copper. Recently, a second clade of nitrous oxide reductase has been identified, herein named *nosZ*I (Sanford et al. 2012; Jones et al. 2013) which include a large fraction of nondenitrifying $N_2O$ reducers, which could be $N_2O$ sinks without major contribution to N2O formation (Hallin et al. 2018).

Physiologically, denitrification activity depends on the availability of $O_2$, N oxides, and suitable reductants to drive electron transport. In most denitrifying species, expression of denitrification genes is tightly regulated by $O_2$ levels due to its status as the preferred electron acceptor in most cases. However, the exact level of anoxia required for denitrification gene expression can differ substantially among organisms (Ollivier et al. 2011), and denitrification activity can persist in the presence of $O_2$ in environments that have shifted from anaerobic to aerobic (Morley et al. 2008; Ji et al. 2015).

### 1.6. Fungal denitrification

Although denitrification was thought over a long time to be restricted to prokaryotes, Shoun and Tanimoto (1991) showed that many fungi and yeasts (eukaryotes) also exhibit denitrification traits (Shoun et al. 2012; Shoun and Fushinobu 2017). The essential enzymes for catalysing denitrification by Fungi are nitrite reductase (*nirK* gene) and nitric oxide reductase cytochrome P450nor (*p450nor* gene) (Shoun and Fushinobu 2017).

Fungi can play an important role in $N_2O$ emissions from various ecosystems, such as vegetable fields under intensive, management grasslands, forests, croplands and wetlands (Crenshaw et al. 2008; Seo and DeLaune 2010; Rütting et al. 2013; Chen et al. 2014; Ma et al. 2017). Denitrifying fungal communities has a higher threshold oxygen demand (Zumft 1997; Zhou et al. 2001) and dominate bacteria under sub-anoxic conditions, and vice versa under strict anaerobic conditions (Seo and DeLaune 2010; Chen et al. 2015b). Also, fungi exhibit wider pH ranges for

optimal growth, prevailing at acidic pH and bacteria at neutral and alkaline pH (Herold et al. 2012, Chen et al. 2015b). However, the real role of fungi to produce $N_2O$ remains largely unknown (Maeda et al. 2015).

### 1.7. Estimation of $N_2O$ sources and $^{15}N$ tracer techniques

Attributing $N_2O$ production to different processes is a challenge as they may occur simultaneously in different micro-sites of the same soil (Robertson and Tiedje 1987). Advances in mass spectrometry, and particularly the ability to determine the isotope ratios of $^{14/15}N$ and $^{16/18}O$ in $N_2O$, potentially present a reliable means to source partition. This overcomes the problems associated with acetylene inhibition in which 10 Pa $C_2H_2$ is applied to inhibit nitrification, and 10 kPa $C_2H_2$ is applied to inhibit both nitrification and $N_2O$ reduction in denitrification (Klemedtsson et al. 1988). This can lead to an underestimation of denitrification as $C_2H_2$ can be decomposed and used as a substrate for denitrification if C is limiting (Stevens and Laughlin 1988; Groffman et al. 2006).

Enrichment approaches have been developed aimed at quantifying the individual sources of $N_2O$ in situ. To date, these have mostly focused on $^{15}N$, and mainly on distinguishing between nitrification and denitrification following application of $^{15}N$-labelled fertiliser. Application of $^{15}N$-labelled $NH_4^+$ and/or labelled $NO_3^-$ to soil and attribution of the $^{15}N$-$N_2O$ fluxes to nitrification or denitrification depending on the $^{15}N$ source applied negates the need for $C_2H_2$ inhibition, as nitrifier-, denitrifier-$N_2O$ and $^{15}N$-$N_2$ can be quantified (Baggs 2008). $^{15}N$ is usually applied at 1-10 atom % excess $^{15}N$, at which level isotopic fractionation appears to be independent of the isotopic enrichment (Mathieu et al. 2007). This ability to quantify $^{15}N$-$N_2O$ from each source provides a major advance over natural abundance techniques, and demonstrates the significance of nitrification and denitrification under certain environmental conditions.

### 1.8. Environmental controls of nitrous oxide fluxes

#### 1.8.1. Nitrogen and carbon contents

The form of N-fertiliser and its application rate influences the production of $N_2O$ since $NH_4^+$ and $NO_3^-$ are substrates for nitrification and denitrification, respectively.

They mainly stem from fertilisation, mineralization (e.g. from soil, N-rich residues) or atmospheric deposition. While $NH_4^+$ is usually bound to clays and humus through ion exchange, $NO_3^-$ is highly mobile. A linear increase of $N_2O$ emissions with increasing N-input has been observed (Stehfest and Bouwman 2006; Baggs and Philippot 2011; Butterbach-Bahl et al. 2013) and short-term peaks of $N_2O$ are normally measured after the application of organic and inorganic N-fertilisers (Syakila and Kroeze 2011; Baggs and Philippot 2011; Butterbach-Bahl et al. 2013).

Among the globally consumption of N products (137.7 Mt N $yr^{-1}$), 63% is urea and 10% is ammonium nitrate/calcium ammonium nitrate (AN/CAN) (Harty et al. 2016). Meta-analyses of fertiliser types indicated that there can be differences in $N_2O$ emissions between different fertiliser N forms (Bouwman et al. 2002), with AN/CAN exhibiting higher $N_2O$ emission factors (%) and quicker $N_2O$ loss compared to urea in high organic matter soils (Harty et al. 2016).

Increasing soil organic C enhances nitrification and denitrification reactions (Saggar et al. 2013), because it can stimulate microbial growth and activity, and also provide the organic C needed by soil denitrifiers (Cameron et al. 2013). It is normally accepted that increasing C supply decreases the $N_2O$:$N_2$ (Cameron et al. 2013; Saggar et al. 2013). Any process that influences the rate of C mineralization in soils (e.g., temperature, incorporation of crop residues, drying-wetting cycles, tillage, liming, organic or inorganic fertiliser input, root exudates) can have a large impact on denitrification rates and corresponding $N_2O$ emissions. Nitrogen transformations in soils include two important biological processes: immobilization (or assimilation), that is the uptake of N by microorganisms and its conversion into organic N, and mineralization (or ammonification), that is the conversion of organic N to $NH_3$. The balance between mineralization and immobilization depends on the soil C/N ratio and residues added. Soil and residues with a small C/N ratio (lower than 30/1) present a dominance of mineralization over the immobilization, and the available N can be absorbed by plants or used in microbial processes. The presence of high C/N ratio on the soil surface may increase the immobilization of the N-fertiliser applied (Baggs et al. 2000), and thus decrease the denitrification reactions and $N_2O$ emissions. When a small C/N ratio is present in the soil surface, the N

immobilization is reduced and more N will be available for nitrification and denitrification processes, thus increasing $N_2O$ emissions (Baggs et al. 2000).

### 1.8.2. Soil moisture content and oxygen availability

Oxygen concentration determines if the predominant N pathway in soils is anaerobic or aerobic (i.e denitrification and nitrification, respectively) through regulating the reactions of oxidation and reduction (Bollmann and Conrad 1998). The main factors determining $O_2$ concentration are the soil water content and $O_2$ consumption by plant roots and microorganisms (respiration). Soil moisture can directly or indirectly influence denitrification by providing a suitable environment for microbial growth and activity, preventing the supply of $O_2$ to microsites by filling soil pores, releasing available C and N substrates during wetting and drying cycles and through provision of a diffusion medium through which substrates and products are moved to and away from soil microorganisms. It has been shown that after rainfall and irrigation, denitrification rate increases due to decrease $O_2$ diffusion into the soil (Ruser et al. 2006). A closer relationship was found between water filled pore space (WFPS) and $N_2O$ emissions since this value also takes total pore space into account (Danielson and Sutherland 1986). $N_2O$ production from nitrification typically occur within the range of 30-60% WFPS and denitrification dominates in wet soils with WFPS >70% (Davidson 1991a; Bateman and Baggs 2005; Braker and Conrad 2011; Hu et al. 2015) (Fig. 7). However, it has been shown that in arable systems, this threshold value can also be higher (Ruser et al. 2006).

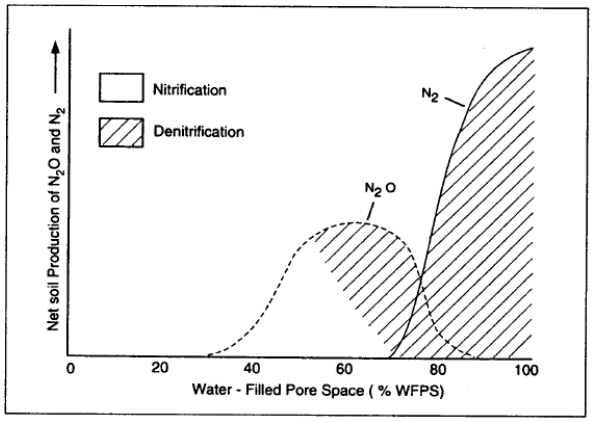



*Fig. 7. Relationship between water-filled pore space and net production of NO, $N_2O$ and $N_2$ (Davidson 1993).*

The more detailed description of $O_2$ diffusion and consumption processes in soils allows the estimation of the $O_2$ concentration in a given soil layer and its use as a proxy to divide the soil into aerobic and anaerobic microsites. This allows simultaneous simulation of nitrification and denitrification in a given soil layer.

### 1.8.3. pH

Both in a pure culture and in a natural environment nitrification and denitrification are closely linked at pH. Although the critical threshold for nitrification is 5, it has been shown to occur at a soil pH of 4.5 due to acid-adapted nitrifier strains (Bouwman 1990) which show that acidity also favours $N_2O$ production in soils (Martikainen and Boer 1993). It is fully accepted that an inhibition of denitrification reductases occurs at pH $\leq$ 7, especially nitrous oxide reductase (Knowles 1982). This inhibitory effect of reversible character (Glass and Silverstein 1998), seems to be originated by the formation of protonated species of nitrous acid ($HNO_2$), highly toxic (Glass and Silverstein 1999). Nevertheless, denitrification can still occur in acidic soils at pH values as low as 3.5 (Saggar et al. 2013).

### 1.8.4. Other factors

The $N_2O$ emitted from soils is also influenced by the soil type (Stevens and Laughlin 1998). Clayey soils tend to show greater $N_2O$ emissions than sandy soils (Brentrup et al. 2000), particularly in soils of fine texture (Chen et al. 2008a, Tan et al. 2009). In the clayey soil, the small amount of macropores would increase anaerobic microsites, also increasing $N_2O$ emissions. Neill et al. (2005) reported that emissions in sandy soils occur with greater soil moisture than that necessary for similar emissions in a clayey soil.

## 1.9. Ammonia volatilisation and urease and nitrification inhibitors

Emissions of $NH_3$ into the atmosphere have more than doubled since pre-industrial times due to increases in livestock numbers, fertiliser N use and combustion of fossil fuels (Erisman et al. 2015). Atmospheric $NH_3$ is an environmental concern for two reasons (Wang et al. 2015a; Xu et al. 2016): (1) formation of very fine particles in the air and (2) uncontrolled N deposition back to the soil and vegetation. When $NH_3$ (an alkaline compound) is released into the air, it clings to nearby surfaces and

significant amounts can be deposited. The remaining $NH_3$ can rapidly react with acidic compounds in the air (such as nitric acid or sulfuric acid) to form very small secondary aerosol particles. This fine particulate matter has a diameter of $< 2.5$ microns (referred to as PM 2.5), which is about 30 times smaller than a human hair. Some of these very small particles can persist in the air for several weeks and cause atmospheric haze. PM 2.5 particles are a health concern because they can be inhaled deeply into the lungs. Ammonia gas can be transported with the wind, redeposited as far as hundreds of miles from the original source. Ammonium deposited on the soil is generally converted to $NO_3^-$, with a release of acidity during nitrification. Widespread $NH_3$ fertilisation through atmospheric deposition can stimulate plant growth in pristine areas where N was previously limiting.

Volatilisation of $NH_3$ from fertilisers and animal wastes can be a major pathway of nutrient loss (UNECE 2001; Salazar et al. 2012). Emission of $NH_3$ from fertilised soils can range from near zero to up as much as 50% of the applied N (Francisco et al. 2011; Abalos et al. 2012). All fertilisers that contain or produce $NH_4^+$ are subject to volatile loss to some degree. Some $NH_4^+$-containing fertilisers such as ammonium nitrate or ammonium sulphate initially form a slightly acidic solution when they dissolve in the soil (pH between 4.5 and 5.5). In most circumstances, these acidic N sources do not lose significant amounts of $NH_3$ only up to few percent of the total N. Other N-fertilisers form alkaline conditions, which are more susceptible to $NH_3$ loss. For example when urea is applied to soil, it reacts rapidly with water and urease enzymes in a process called hydrolysis, producing ammonium carbonate, an unstable compound that quickly decomposes to release $NH_3$ gas.

A soil with greater pH buffering capacity (high clay or organic matter) generally has less volatile $NH_3$ loss than a poorly buffered soil (sandy texture). When urea or $NH_4^+$-based fertilisers are applied to wet soils, $NH_4^+$ adsorption to the particles in the soil matrix prevent its release into the atmosphere (Sanz-Cobeña et al. 2011; Yang et al. 2016a). On the other hand, if the soil is dry, there is a higher potential for $NH_3$ loss following surface application of fertiliser.

N losses due to $NH_3$ volatilisation can be reduced by applying urease inhibitors which depress urea hydrolysis (Francisco et al. 2011; Abalos et al. 2012) and

subsequent $NH_4^+$ concentration in the soil solution (Gill et al. 1999; Modolo et al. 2015). Phenylphosphorodiamidate (PPD/PPDA), hydroquinone and N-(n-butyl) thiophosphoric triamide (NBPT) are urease inhibitors effective both under laboratory (Carmona et al. 1990; Gill et al. 1999) and field conditions (Sanz-Cobeña et al. 2008; 2011; Zaman et al. 2009; Rodríguez-Soares et al. 2012). As an alternative to reduce $NH_3$ losses, nitrification inhibitors block the activity of the enzyme ammonia monooxygenase (Weiske et al. 2001; Zerulla et al. 2001), thus extending the period of permanence of $NH_4^+$ in soils and reducing the production of $N_2O$. However, the prolonged retention time of $NH_4^+$ in soil may increase ammonia emissions (Rodríguez-Soares et al. 2012; Qiao et al. 2015). Dicyandiamide (DCD) and 3,4 dimethylpyrazole phosphate (DMPP) are the most used nitrification inhibitors and they can effectively reduce nitrification rates under different laboratory (Gilsanz et al. 2016) and field conditions (Moir et al. 2012; Pfab et al. 2012). Although different meta-analysis have demonstrated that NBPT (Fan et al. 2018) and DMPP (Yang et al. 2016a) can be effective in reducing $N_2O$ emissions, its effect on N-cycling soil microbes is not well understood (Barrena et al. 2017; Montoya et al. 2018).

## 1.10. Biodiversity and abundance of microbial communities

### 1.10.1. Definition of biodiversity

The second article of the Convention on Biological Diversity (http://www.cbd.int/, Rio de Janeiro, 1992) which was derived from the United Nations Conference on Environment and Development (UNCED) defines biodiversity as the variability among living organisms from all sources including terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and ecosystems.

### 1.10.2 Analysis of microbial biodiversity

#### 10.10.2.1. Methods dependent on cell culture

It has been estimated that a gram of dry soil can contain between $1x10^9$-$1x10^{10}$ bacterial cells, $1x10^3$ and $1x10^6$ unique species of Bacteria and yield up to 50 μg DNA (Schloss and Handelsman 2004; Trevors 2010). Traditionally, the analysis of

bacterial diversity has been based on cell culture dependent methods that allow the growth of bacteria in a wide variety of culture media where different colony-forming units (CFUs) are obtained. However, it has been estimated that the number of bacteria that can be isolated from environmental samples do not exceed 10% of the total community (Stewart 2012). The simplest explanation to explain this finding is that microbiologists are failing to replicate essential aspects of the microbial environments (nutrients, pH, osmotic conditions or temperature). Once grown, the identification and taxonomic classification of the strains is done using phenotypic and genotypic techniques. The phenotypic characteristics of a bacterium do not reflect its phylogenetic relationship with other bacteria being the molecular analyses the most reliable.

#### 1.10.2.2. Independent methods of cell culture

The structure, composition and function of the microbial communities is influenced by several environmental factors. These circumstances cannot be analysed by the methods dependent on cell culture, so it has been necessary to develop techniques that allow the rapid and reproducible analysis of the microbial diversity in environmental samples. The polymerase chain reaction (Polymerase Chain Reaction, PCR) changed the knowledge of the microbial world since it has allowed the analysis of bacterial populations from DNA extracted directly from environmental samples. Developed by K. B. Mullis between 1983 and 1986, is characterized because it allows to obtain a large number of copies of a given fragment of DNA from a single copy of that DNA (Mullis 1990).

The term metagenomics was used for the first time by Handelsman et al. (1998) to refer to a methodology that sought to analyse a collection of genes sequenced from an environmental sample as a single genome. Handelsman (2004) and Riesenfeld et al. (2004) defined the metagenome like all the DNA that can be found in an environmental sample whatever the ecosystem. Thomas et al. (2012) defined metagenomics as the study of the metagenome -the collective genome of microorganisms from an environmental sample- to provide information on the microbial diversity and ecology of a specific environment.

Numerous molecular techniques have been developed to identify microorganisms from DNA of environmental samples such as (Rastogi and Sani 2011; Fakruddin and Mannan 2013): the denaturing gel electrophoresis (DGGE) (Muyzer et al. 1993) or of temperature gradient (TGGE) (Muyzer and Smalla 1998), the analysis of the restriction fragment length polymorphism (RFLP), and the terminal fragment polymorphism (T-RFLP) (Liu et al. 1997). Another methodology, the polymorphism analysis of the conformation of single strands of DNA (SSCP) (Schwieger and Tebbe 1998) of a given gene amplified provides excellent results in studies of biodiversity (Smalla et al. 2007).

The study of a functional community of microorganisms by TGGE, DGGE, RFLP, T-RFLP and SSCP present a serious limitation as it does not allow to determine the relative abundance of each population in the community. The construction of genomic libraries (Kim et al. 2004), another molecular method in microbial diversity studies, provided a reliable alternative but it is time-consuming and can be expensive. To solve this problem massive sequencing techniques were developed.

### 1.10.3. Massive sequencing techniques

The first steps in DNA sequencing occurred in the 70s by Sanger et al. (1977), who developed the enzymatic method of chain-terminating dideoxynucleotides, and Maxam and Gilbert (1977), who proposed the process of the chemical fragmentation. At the end of the 90s, the Sanger methodology was modified to allow the use of dideoxynucleotides labeled with fluorescence. Such nucleotides are analysed by capillary electrophoresis and produce an electropherogram from which deduce the sequence of such nucleotides. This allowed to improve, automate and increase the performance of the sequencing process which, in short, made it possible the development of automatic sequencers, resulting sequences between 500 and 1000 base pairs.

This first generation of automatic sequencers together with the development of Large-scale sequencing strategies (Whole Genome Shotgun Sequencing) allowed the assembly of the genomic sequences. Second-generation sequencers were developed looking for decreasing the costs of sequencing, capable of producing hundreds of thousands of sequences because of the possibility of carrying out

thousands of reactions (high throughput) by immobilizing the DNA on solid surfaces, which decreased the use of the reagents necessary for the sequencing process and, consequently, the cost of the process. In this way, novel DNA sequencing procedures were developed, called Next Generation Sequencing (NGS). These techniques allow to obtain a wider vision of the community microbial in terms of taxonomy and potential functioning (Steele and Streit 2005; Hugenholtz and Tyson 2008). The first approach to massive sequencing was the pyrosequencing of DNA (Nyrén 2001; Ronaghi 2001), which couples the synthesis of DNA to a chemiluminescent reaction. Since the first commercial model of pyrosequencer appeared in 2005 that was able to sequence up to 20 million bases in about 4 hours, this technology has been improved allowing the reading of up to one million fragments of about 400-700 base pairs (Buermans and den Dunnen 2014). During amplification, DNA from different samples is marked with specific labels that allows the simultaneous analysis of a targeted gene (Binladen et al. 2007; Parameswaran et al. 2007). Since then, other mass sequencing platforms have been developed (see comprensive reviews by Levy and Myers 2016; Goodwin et al. 2016). Due to the large amount of data generated by the use of these techniques, it is crucial the utilisation of specific tools that facilitate their analysis. The platforms metagenomics-RAST (Meyer et al. 2008), QIIME (Caporaso et al. 2010), MOTHUR (Schloss et al. 2009) and Ribosomal Database Project (RDP) (Cole et al. 2014) fulfil this function.

### 1.10.4. Bacterial diversity indices

The diversity of different ecosystems and the diversity of the same ecosystem over time can be estimated by the quantification of the number of species and their representativeness. While eukaryotic species contains members that can reproduce among them to give rise to a fertile offspring, in the domains Bacteria and Archaea there is no similar definition of species since they reproduce by binary partition, which does not require sexual compatibility. In addition, bacteria and archaea can transfer DNA to other strains which it does not have to be phylogenetically related. Due to the difficulty in defining the concept of bacterial species, the term Operative Taxonomic Unit (Operational Taxonomic Unit, OTU) was coined to establish the

taxonomic level that is conferred on an environmental sample, such as an individual, population, species, genus, strain, etc. This distinction is reached through the analysis of molecular marker genes, usually the 16S rRNA, and a percentage threshold of similarity that allows to classify them in the same or different OTU. To determine the bacterial diversity of an environmental sample from the sequences of the individuals that make up the population, it is accepted that each OTU corresponds to a group of sequences with at least 97% of similarity, a level that has traditionally been homologated to that of species (Schloss and Handelsman 2005). It is recognised that an ecosystem can be occupied by different communities, that each community is made up of different populations and that each population includes different individuals (genera, species, strains, OTUs). Similarly to the ecology of eukaryotes, three types of microbial diversity are distinguished: alpha, beta and gamma. The first refers to the species richness of the community and is determined by calculating species richness indices: Chao1, Jacknife, Margalef, Simpson, Shannon, etc (Lozupone and Knight 2009). The beta diversity indicates the rate of change in species of two communities and therefore reflects the difference in composition of the two communities and is usually quantified through the Alatalo, Morisita Horn, Jaccard, Sneatch , Sørensen and Sokal indices (Barwell et al. 2015). Gamma diversity makes reference to the species richness of the set of communities and integrates the alpha and beta components of diversity (Whittaker 1972). For a review of the determination of the alpha, beta and gamma biodiversity see Jost (2007), Lozupone and Knight (2009), Bunge et al. (2014) and Barwell et al. (2015).

### 1.10.5. Study of nitrifiers and denitrifiers communities

#### 1.10.5.1. Quantitative PCR

Although the elaboration of genomic libraries or mass sequencing techniques represented a significant advance to study the bacterial functional diversity, it does not inform about the occurrence of corresponding activity of the product that it encodes or allows to determine the number of copies (abundance) of the targeted gene. This problem was solved with the development of the real-time quantitative PCR (qPCR) technique (Heid et al. 1996). qPCR is based on the proportionality between the intensity of the fluorescence signal emitted during the phase

exponential of the PCR and the initial amount of the target DNA. The number of copies of the initial target DNA is determined by its comparison with the number of copies of a standard curve constructed with known initial concentrations of DNA. qPCR has been used to determine the number of copies of the nitrification and denitrification genes in many ecosystems such as agricultural soils, forest soils, rice sediments, soils dedicated to grazing, nests of nematodes, artificial soils and sediments of artificial wetlands (Philippot et al. 2002; Philippot 2006; Jones and Hallin, 2010; Hai et al. 2009; Levy-Booth et al. 2010, 2014; Zhang et al. 2013; Correa-Galeote et al. 2014; Chang et al. 2015). The disadvantages of this methodology are related with the limitation of its range and the obligation to make different dilutions (Smith and Osbron 2009).

There are two types of qPCR technologies that employ DNA probe with a fluorophore that binds specifically to the amplified DNA (TaqMan method) or a suspended fluorophore that also binds specifically to DNA during the amplification (SybrGreen method). The latter is a fluorescent dye that binds double-stranded DNA in a non-specific way. During the qPCR the intensity of the fluorescence is detected simultaneously with its emission, so that there is a logarithmic increase in the fluorescence emission until the substrates of the PCR reaction are limiting.

The activity of a particular gene in an environmental sample can be also analysed from its RNA using qPCR. This methodology is based on the direct extraction of RNA present in the sample that, once isolated and purified, is retro-transcribed to cDNA, which, in turn, is used as the target DNA of the amplification. Transcripts of nitrification and denitrification genes have been quantified by qPCR (see for example, Henderson et al. 2010; Dandie et al. 2011; Graham et al. 2011; Saleh-Lakha et al. 2011; Uchida et al. 2014; He et al. 2018).

# OBJETIVES

## 2. OBJETIVES

In agricultural soils, the application of inorganic N-fertilisers leads to the interaction of multiple factors and processes which are mainly associated with changes in soil physicochemical properties, emission of greenhouse gases and microbial ecology. Although N is an essential nutrient for plant growth, increased N-fertilisation in agriculture has altered the natural N cycle, which, in turns, results in environmental, ecological and human health effects. Among them, increases in the emission of greenhouse gases, nitrate contamination of waters and sediments, acidic deposition and eutrophication.

After application of an N-fertiliser, the microbial processes of nitrification and denitrification are responsible of the main reactions driving the conversion of ammonia ($NH_4^+$) and nitrate ($NO_3^-$) to the release of the greenhouse gas $N_2O$ into the atmosphere. As suggested by recent meta-analyses, combating the negative impacts of increasing $N_2O$ fluxes poses considerable challenges and will be ineffective without incorporating microbial regulated $N_2O$ processes into mitigation strategies. Although many studies have reported on the effect of N-fertilisation on soil microorganisms involved in the N-cycle many questions remains under debate.

Previous reports have shown that the way and extent N-fertilisers affect the emission and evolution of $N_2O$ and abundance of the nitrifier and denitrifier communities depend upon the N form and the biotic and abiotic properties of the soil. Although many of these studies have shown individual relationships between N-fertilisation and soil biotic and abiotic parameters, an integrated study relating the form of the N-fertiliser with differences in $N_2O$ emission, changes in soil physicochemical properties, alterations in the abundance of the genes involved in $N_2O$ production and reduction, and effects on bacterial diversity had not been reported. Moreover, the effect of the soil depth and type of N-fertilisation on $N_2O$ emission along the arable soil profile was unknown. Also, the controversy existed about the relative contribution of the aerobic nitrification and anoxic denitrification biochemical pathways to total $N_2O$ production. Although fungal denitrification had been reported, the input of the process to total $N_2O$ production associated to the form of the fertiliser had been scarcely reported. Finally, many question were raised

about the effect of the N-fertilisers on $N_2O$ release by the bulk and rhizosphere soil of cultivated plants.

To address these questions, the N-fertilisers urea ($[CO(NH_2)_2]$ ammonia $(NH_4)_2SO_4$ and nitrate ($KNO_3$) were chosen to amend an agricultural Cambisol soil from Vega de Motril. Tomato (*Solanum lycopersicum*) and common bean (*Phaseolus vulgaris*) were used as representative vegetable plants.

According to what has been mentioned above, the main objectives pursued in the study to achieve the PhD degree were:

1. To analyse the distinct effect of urea, ammonium and nitrate on $N_2O$ production and abundance of N-cycling genes along the 20-cm layer of the arable topsoil.
2. To determine the effect of N-fertilisation on the a) soil physicochemical characteristics, b) $N_2O$ emission, c) possible changes in the abundance of the bacterial and archaeal communities, d) abundance of the nitrifying and denitrifying guilds and e) variations in bacterial diversity. The study included the bulk and rhizosphere soil of tomato and common bean plants.
3. To estimate the relative contribution of the biological nitrification and denitrification processes to $N_2O$ production.
4. To build a model to understand the relative importance of N-fertilisation, each analysed biotic and abiotic variables and bacterial biodiversity as drivers of $N_2O$ emission.
5. To quantify the relative importance of bacterial and fungal denitrification post nitrate application.
6. To investigate the effects of the single and combined application of the urease inhibitor N-(n-butyl) thiophosphoric triamide (NBPT) and the nitrification inhibitor 3,4 dimethylpyrazole phosphate (DMPP) on $NH_3$ volatilisation and the abundance of the nitrifier and denitrifier communities.

# MATERIALS AND METHODS

# 3. MATERIALS AND METHODS

## 3.1. Site description and soil sampling

An Eutric Cambisol soil (30% clay, 12.5% silt, 57.5% sand, w/w; pH in water, 6.8; total C, 25 mg $kg^{-1}$; total N 1.02 mg $kg^{-1}$; $NO_3^-$ 6.8 mg $kg^{-1}$; exchangeable $NH_4^+$, not detected; $HCO_3^-$ 244 mg $kg^{-1}$) of the FAO series (FAO 2017) was collected from an agricultural area in the vicinity of Motril (Granada, Spain), (UTM coordinates 36° 43' 53.5" N, 3° 32' 56.2" W), that had been maintained under fallow conditions for more than 10 years without receiving any type of fertilisation (Fig. 1a, b).


*Fig. 1.* ***a.*** *Location of the agricultural area of Motril (Granada, Spain)* ***b.*** *Sampled area.*

Spade-squares (30 x 30 cm to a depth of 25 cm) were taken from 12 sites, freed of roots and plant residues, air dried to ~ 30% $H_2O$ (dry basis) and pooled together. Then, using a concrete mixer, the soil was independently mixed with either urea ($CON_2H_4$), ammonium sulphate [$(NH_4)_2SO_4$] or potassium nitrate ($KNO_3$) to a final concentration of 260 kg N $ha^{-1}$ (421.2 mg N $kg^{-1}$ dry soil) as recommended for horticultural crops and leguminous plants by the Spanish Ministry of Agriculture, Food and Environment (https://www.mapa.gob.es/es/agricultura/publicaciones/Publicaciones-fertilizantes.aspx). Unfertilised soil was used as a control.

### 3.2. Experimental setup

Soil was used to fill 20-kg capacity PVC pots (54 x 21 x 25 cm). All pots were maintained under greenhouse conditions with 16h light: 8h dark cycle. The soils were watered once a week to 80% WFPS. Three sets of experiments were arranged:

1. Effect of N-fertilisation and soil depth on $N_2O$ emissions and nitrifiers and denitrifiers abundance: urea, ammonium and nitrate treated soils (4 replicates per treatment) were maintained during 1 year under greenhouse conditions. A set of pots containing soil without fertilisation was used as a control. The concentration of extractable $NH_4^+$ and $NO_3^-$ was determined every 3 months and the soil further supplemented with the previously applied N-fertiliser to reach the initial fertilisation rate. For further details see Chapter I of the Result section.

2. Effect of N-fertilisation on $N_2O$ emissions and nitrifiers and denitrifiers abundance in uncultivated soils: urea, ammonium and nitrate treated soils (4 replicates per treatment) were maintained during 3 years under greenhouse conditions. A set of pots containing soil without fertilisation was used as a control. The concentration of extractable $NH_4^+$ and $NO_3^-$ was determined every 12 months and the soil further supplemented with the previously applied N-fertiliser to reach the initial fertilisation rate. For further details see Chapter II of the Result section.

3. Effect of N-fertilisation of on $N_2O$ emissions and nitrifiers and denitrifiers abundance in soils cultivated with tomato (*Solanum lycopersicum* var. Roma) and common-bean (*Phaseolus vulgaris* var. Kylie) plants: urea, ammonium and nitrate treated soils (8 replicates per treatment) were maintained during 4 consecutive crops of 4 months each under greenhouse conditions (Fig. 2a, b). A set of pots containing soil without fertilisation was used as a control. After appearance, the plants were trimmed to 3/container until harvest at 10% fructification about 4 months after sowing. Each pot contained three plants that were harvest at 10% of fructification (about 4 months); roots were also removed. The concentration of extractable $NH_4^+$ and $NO_3^-$ was determined after each crop and the soil supplemented with the previously applied N-fertiliser to reach the initial fertilisation rate. For further details see Chapters III.1 and III.2 of the Result section.


*Fig. 2.* ***a.*** *Pots used for the cultivation of tomato and bean plants* ***b.*** *Each pot contained three plants.*

4. Effect of N-fertilisation on the relative contribution of nitrification and denitrification to $N_2O$ production: uncultivated soil that had been treated for three years with ammonium (AS-3) and nitrate (PN-3) as well as the unfertilised control soil (NTC-3) was used. Cylindrical, closed-base plastic cores were packed with 30 g of air-dried, sieved to < 2 mm, NTC-3 soil (36 cores), AS-3 soil (36 cores), or PN-3 soil (36 cores) and placed into 500 ml Kilner jars (3 cores/jar). The cores containing the AS-3 soil received 6.2 µmol $NH_4^+$-N $g^{-1}$ (equivalent to 150 kg N $ha^{-1}$), of which one half (18 cores) was amended with $(^{15}NH_4)_2SO_4$ (AS-3_$^{15}$AS) and the other half with $K^{15}NO_3$ (AS-3_$^{15}$PN). Similarly, the cores with the PN-3 soil were each supplemented with 6.2 µmol $NO_3^-$-N $g^{-1}$ (equivalent to 150 kg N $ha^{-1}$) and then separated in two groups (18 cores/group) that received $(^{15}NH_4)_2SO_4$ (PN-3_$^{15}$AS) or $K^{15}NO_3$ (PN-3_$^{15}$PN). The $^{15}$N enriched ammonium and nitrate was mixed with natural abundance to produce $^{15}$N labelled treatments at 10 atom %. Labelled $^{15}$N-compounds were dissolved in distilled water and added to the soil to reach 80% WFPS. The jars containing the soil cores were kept in a cabinet at 22/16 ºC day/night temperature, on a 16h/8h light/dark cycle for 30 days after amendment. The cores into the jars were watered weekly to 80% WFPS by weight by adding distilled water from the top. Eighteen jars were used for determination of $N_2O$ emissions and other 18 served for destructive soil sampling. For further details see Chapter IV of the Result section.

5. Single and combined application of cycloheximide and streptomycin were used to selectively inhibit fungal and bacterial growth in the soil, respectively. Based on results from a a preliminary experiment, the optimal cycloheximide and

streptomycin optimal concentration to prevent fungal and bacterial growth was 2.0 mg $g^{-1}$ soil and inhibitors were effective during 10 days post N application. For the microcosm experiments 3 replicate cores of each cycloheximide, streptomycin, cycloheximide + streptomycin and control treatments were used. Treatments were diluted separately in sterile distilled water, mixed with 15 g soil samples within a plastic bag and then packed into cylindrical plastic cores. The cores (3/jar) were placed into 500 mL Kilner jars, the soil moisture adjusted to 50% WFPS and incubated overnight to allow diffusion into the soil pores. Then, $KNO_3$ was diluted in distilled water and added to the cores from the top to reach 80% WFPS and a final concentration of 260 kg equivalent N $ha^{-1}$. Control soil without the inhibitors also received $KNO_3$. The jars were kept in a cabinet at 22 °C/16 °C day/night; 16 h/8 h light/dark cycle for 10 d. For each treatment, 3 jars were used for determination of $N_2O$ emissions and 3 served for soil destructive sampling which started with a 2-day delay. For further details see Chapter VI of the Result section.

6. Effect of urease (NBPT) and nitrification (DMPP) inhibitors on ammonia volatilisation and nitrifiers and denitrifiers abundance: soil was used to fill 5-kg capacity PVC pots up to 5 cm from the rim. All pots were supplemented with urea at a final concentration of 260 kg N $ha^{-1}$. The experiment was arranged in a factorial randomised complete block design with six replications for each of the 4 treatments that were: urea, urea + NBPT, urea + DMPP, urea + NBPT + DMPP. NBPT and DMPP were added to give a proportion of inhibitor in the mixture of 0.25 and 0.8% on a weight basis, respectively. A set of pots containing soil without fertilisation was used as a control. Fertiliser and inhibitors were diluted in 100 ml water at the beginning of the experiment and applied to the pots from the top. Subsequently, half of the pots were adjusted to 50% WFPS and the other half to 80% WFPS and watered weekly to reach the corresponding WFPS. For each WFPS, half of the pots were used for $NH_3$ volatilisation measurements and the other half was used for soil sampling. All pots were maintained at 18 ºC for 60 days. For further details see Chapter VII of the Result section.

### 3.3. Soil physicochemical characteristics

Moisture was measured gravimetrically after drying of the soil for 24 h at 105 ºC. WFPS was calculated following equations (Danielson and Sutherland 1986):

Soil water content (g/g) = weight of moist soil - weight of oven-dried soil / weight of oven-dried soil

Soil bulk density ($g/cm^3$) = oven-dried weight of soil / volume of soil

Soil porosity (%) = soil bulk density / 2.65

Volumetric water content ($g/cm^3$) = soil water content × bulk density

WFPS (%) = volumetric water content × 100 / soil porosity

WFPS can be determined using the following equation:

WFPS = (soil water content × bulk density)/ [1 - bulk density / particle density × (2.65 g $m^{-3}$)]

An ionic chromatograph (Methohm) equipped with a Metrosep A supp-4-250 anion column and a Metrosep C2-150 cation column was used to determine $NO_2^-$, $NO_3^-$ and exchangeable $NH_4^+$ concentration, respectively, as indicated earlier (González-Martínez et al. 2016). Urease activity was determined as already reported which involves the incubation of soil with buffered urea solution, the extraction of exchangeable $NH_4^+$ with 1 N KCl and colorimetric $NH_4^+$ determination (Nannipieri et al. 1980). Dissolved oxygen concentration (DO) was monitored *in situ* using an oxygen sensor (Apogee Instruments) and pH was measured after water extraction (1:5, w/v) for 2 h. Total C (TC), total organic C (TOC) and total N (TN) were determined using a LECO TruSpec CN elemental analyser.

### 3.4. Ammonia emission analysis

Ammonia volatilisation was analysed using a gas flow-through system coupled to a chemiluminiscence ammonia analyser (Thermo Scientific, model 17i analyser) (Fig. 3) as previously described (Aneja et al. 2000; Walker et al. 2002).

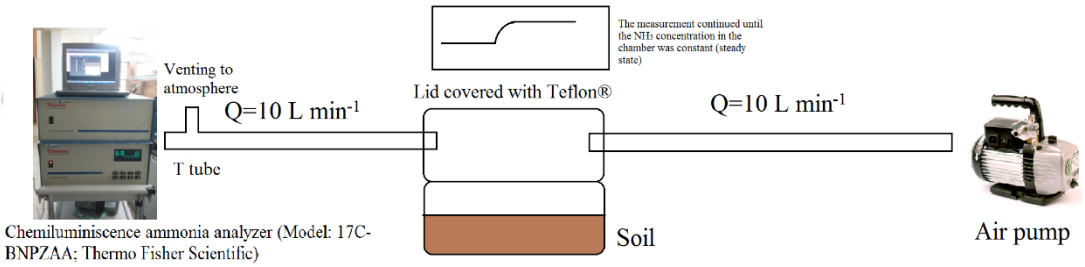



*Fig. 3. Schematic depict of the $NH_3$ volatilisation determination system.*

Briefly, to minimise $NH_3$ losses during the measurement, each container was closed with a Teflon®-covered lid provided with an inlet and with outlet holes. Air was pumped into the chamber through the inlet hole at a constant rate (Q = 10 L $min^{-1}$). Gas samples were transported through 3-m Teflon® tubing from the outlet hole to a T tube, with one part venting to the atmosphere and the remainder entering into the analyser at a flow rate of 0.5 L $min^{-1}$. Airflow samples were passed through a stainless steel converter, where $NH_3$ transforms to NO after reaction with ozone. A chamber coated with Teflon® was used as control. Under steady-state conditions, the change of concentration with respect to time is zero, so that the $NH_3$ flux was calculated as described by Kaplan et al. (1988). Cumulative $NH_3$ emissions were calculated by multiplying the length of time between two samplings by the average emissions rate for that period, and adding that amount to the previously gas accumulated. For further details see Chapter VII of the Result section.

### 3.5. Nitrous oxide emission

Soil cores were placed in glass bottles, sealed hermetically with rubber septa and evacuated with pure He to ensure $N_2$-free conditions. For estimation of $N_2$ emissions, 10% of the internal atmosphere of half of the bottles was removed and substituted by acetylene. Soil was incubated under greenhouse conditions and $N_2O$ assayed sequentially within times when gas emissions were linear using a Hewlett Packard 5890 gas chromatograph equipped with an electron capture detector as previously reported (Tortosa et al. 2011). Concentration of $N_2O$ was calculated using 2% (v/v) $N_2O$ standard (Air Liquide). Cumulative emissions were calculated after linear interpolation of the area between sampling points. $N_2$ production was estimated as the difference in $N_2O$ production in the presence and the absence of acetylene.

### 3.6. Isotopic analyses of soil mineral N and $N_2O$ and $N_2$

The $^{15}N$-enrichment of $NH_4^+$ and $NO_3^-$ was calculated after the conversion of $NO_3^-$ to NO by vanadium chloride ($V(III)Cl_3$) and the oxidation of $NH_4^+$ to $N_2$ by sodium hypobromite (NaOBr) as described by Laughlin et al. (1997) and Stevens and Laughlin (1998). $^{15}N$ enrichment of the resultant $N_2O$ and $N_2$ was measured using a TG2 trace gas analyser interfaced to a Sercon 20-22 isotope ratio mass spectrometer (Loick et al. 2016). The $^{15}N$ enrichment of $N_2O$ and $N_2$ was measured using a TG2 trace gas analyser interfaced to a Sercon 20-22 isotope ratio mass spectrometer as reported by Loick et al. (2016). The amount of $N_2O$ derived from the $^{15}N$-fertiliser amendment was calculated according to Senbayram et al. (2009):

$$N_2O\ N_{amend} = N_2O\_N_{total}\ (^{15}Nat\%ex_{sample} / {}^{15}Nat\%ex_{fert})$$

where $N_2O\_N_{total}$ = total emissions of $N_2O$ from the soil; $^{15}Nat\%\ ex_{sample}$ is the $^{15}N$ atom % excess of the emitted $N_2O$ ($^{15}N$ atom % of the measured sample minus the mean natural abundance $^{15}N$ of background $N_2O$ obtained); and $^{15}Nat\ \%ex_{fert}$ is the $^{15}N$ atom % excess of the applied amendment solution.

The percentages of $N_2O$ originating from nitrification and denitrification were determined according to Stevens et al. (1997), considering that the fraction of $N_2O$ derived from the denitrification (d) and nitrification (1 − d) pool can be calculated as:

$$d = (a_m - a_n) / (a_d - a_n)\ (\text{with } a_d \neq a_n)$$

where $a_m$, $a_d$ and $a_n$ are the average of the $^{15}N$ atom enrichment of the $N_2O$ mixture and the $NO_3^-$ and the $NH_4^+$ pools, respectively

### 3.7. DNA extraction and quantification of nitrification and denitrification genes

Soil DNA was extracted from 0.5 g samples as indicated earlier (Correa-Galeote et al. 2014), purified using GeneClean (MP Bio) spin columns and stored at -20 ºC until use. Essentially, the method of DNA extraction is as follows:

(1) Weight 0.25 g equivalent dry weight aliquots in a 2-ml microtube and keep frozen at -80 °C until use.

(2) Thaw the samples. Add 0.5 g of $10^6$-µm glass beads, 2 beads of 2mm diameter, and 1 ml of homogenization buffer extemporaneously prepared (100 µl 1M Tris-HCl (pH 8.0), 200 µl 0.5M EDTA (pH 8.0), 100 µl 1M NaCl, 50 µl 20% PVP 40 T, 100 µl 20% SDS, 450 µl MQ water).

(3) Homogenize the mixture by using a mini bead beater system ($1.600^{-1}$ shaking frequency/min for 30 s). Use a shaking flask previously kept at -20 °C. Incubate for 10 min at 70 °C then centrifuge at 14.000×g for 1 min at 4 °C.

(4) Transfer the supernatant to a new 2-ml microtube. Add 1:10 (v/v) 5M sodium acetate (pH 5.5) and mix by vortexing. Incubate on ice for 10 min then centrifuge at 14.000×g for 5min at 4 °C.

(5) Transfer the supernatant to a new 1.5-ml microtube. Add 1:1 (v/v) prechilled (-20 °C) isopropanol. Mix well by manual inversion. Incubate for at least 15min at −20 °C then centrifuge at 14.000×g for 30 min at 4 °C.

(6) Remove the supernatant. Wash the pellet (containing the nucleic acids) with prechilled (-20 °C) 70% ethanol with precaution to avoid pellet resuspension. Centrifuge for 15 min at 14.000×g at 4 °C.

(7) Discard the supernatant and dry the pellet for 15 min at 37 °C.

(8) Resuspend the pellet in 50 µl MQ water and store at -20 °C until use.

DNA concentration was measured using the Qubit® ssDNA assay kit (Molecular Probes). The size of the nitrifier community was estimated by qPCR of the *amoA* gene from ammonia-oxidizing Bacteria (*amoA* AOB) and Archaea (*amoA* AOA) and that of denitrifiers by qPCR of the *napA*, *narG*, *nirK*, *nirS*, *norB*, *nosZ* clade I (*nosZ*I) and *nosZ* clade II (*nosZ*II) genes using primers and thermal conditions described in Table 2.1A, B. The total bacterial (16SB), archaeal (16SA) and fungal (ITS Fungi) community was quantified using the corresponding 16S rRNA and ITS genes, respectively.

The reaction mixture for qPCR consisted of: 10-50 ng template DNA; 1.5 µl of each primer (10 m µM); 0.5 µl of bacteriophage T4 gene 32 protein (T4 Gp32, 500 ng/µL); 7.5 µl SYBR Green PCR buffer 2X (containing HotStar Taq polymerase, buffer

and dNTPs) (Sigma Aldrich, St. Louis, MO, USA); MQ/ultrapure water up to 15 µl. Assays for qPCR were carried out using a Bio-Rad iCycler iQ5 Thermocycler (Bio-Rad Laboratories, USA). Absolute quantifications were achieved by construction of standard curves with serial tenfold dilutions ($10^{-1}$-$10^{-12}$) of linearized plasmids (pGEM-T Easy vector, Promega, Madison, WI, USA) harbouring PCR-amplified inserts of the targeted genes. Amplicons were generated from P*seudomonas putida* NCB 957 (quantification of total Bacteria), DNA from the genomic clone 29i4 (quantification of total Archaea), *Pythium intermedium* ATCC 36445(quantification of ITS Fungi), *Nitrosospira multiformis* ATCC 25196 (quantification of *amoA* AOB), DNA from the fosmid clone 54d9 (quantification of *amoA* AOA), *P. aeruginosa* PAO1 (quantification of *napA* and *narG*), *Ensifer meliloti* 1021 (quantification of *nirK*), *P. fluorescens* C7R12 (quantification of *nirS*), *P. stutzeri* ATCC 14405 (quantification of *norB*) and *Bradyrhizobium japonicum* USDA110 (quantification of *nosZ*I), *P. denitrificans* PD1222 (quantification of *nosZ*II). Plasmid quality and concentration were measured on 1% (w/v) agarose gels and by Qubit® ssDNA assay.

Presence of PCR inhibitors in DNA extracted from soil was estimated by 1) diluting soil DNA extract and 2) mixing a known amount of standard DNA to soil DNA extract prior to qPCR. In all cases, inhibition was not detected. PCR efficiency for the different assays ranged between 90% and 99%. The quality of all qPCR amplifications was verified by electrophoresis in agarose (1% at 90V for 30 min) and by melting curve analysis.

**Table 2.1.** **a.** Primers used for quantification of total abundance of Bacteria, Archaea and Fungi (16SB, 16SA and ITS Fungi respectively), nitrifiers (*amoA* AOB and *amoA* AOA) and denitrifiers (*napA*, *narG*, *nirK*, *nirS*, *norB*, *nosZ*I and *nosZ*II) by qPCR. **b.** qPCR conditions for the quantification of each of the target genes.

**a.**

| Primer | Primer sequence (5´-3´) | Target gene | Amplicon size (base pair, bp) | Reference |
|---|---|---|---|---|
| 341F | CCTACGGGAGGCAGCAG | 16S rRNA Bacteria | 194 | Muyzer et al. (1993) |
| 534R | ATTACCGCGGCTGCTGGCA | | | |
| 771F | ACGGTGAGGGATGAAAGCT | 16S rRNA Archaea | 226 | Ochsenreiter et al. (2003) |
| 957R | CGGCGTTGACTCCAATTG | | | |
| ITS1 | TCCGTAGGTGAACCTGCGG | ITS Fungi | 146 | Gardes and Bruns (1993) |
| 5.8S | CGCTGCGTTCTTCATCG | | | Vilgalys and Hester (1990) |
| AmoA1F | GGGGTTTCTACTGGTGGT | *amoA* Bacteria | 490 | Rotthauwe et al. (1997) |
| AmoA2R | CCCCTCKGSAAAGCCTTCTTC | | | |
| Crenamo A23F | ATGGTCTGGCTWAGACG | *amoA* Archaea | 624 | Tourna et al. (2008) |
| Crenamo A616R | GCCATCCATCTGTATGTCCA | | | |
| narG-f | TCGCCSATYCCGGCSATGTC | *narG* | 174 | Bru et al. (2007) |
| narG-r | GAGTTGTACCAGTCRGCSGAYTCSG | | | |
| nap3F | TGGACVATGGGYTTYAAYC | *napA* | 152 | Bru et al. (2007) |
| napA4R | ACYTCRCGHGCVGTRCCRCA | | | |
| nirK876F | ATYGGCGGVAYGGCGA | *nirK* | 173 | Henry et al. (2004) |
| nirK1040R | GCCTCGATCAGRTTRTGGTT | | | |
| nirS4QF | AACGYSAAGGARACSGG | *nirS* | 425 | Throbäck et al. (2004) |
| nirS6QR | GASTTCGGRTGSGTCTTSAYGAA | | | |
| cnorB2F | GACAAGNNNTACTGGTGGT | *norB* | 389 | Braker and Tiedje (2003) |
| cnorB6R | GAANCCCCANACNCCNGC | | | |
| nosZ1840F | CGCRACGGCAASAAGGTSMSSGT | *nosZ*I | 267 | Henry et al. (2006) |
| nosZ2090R | CAKRTGCAKSGCRTGGCAGAA | | | |
| nosZIIF | CTIGGICCIYTKCAYAC | *nosZ*II | 690 | Jones et al. (2013) |
| nosZIIR | GCIGARCARAAITCBGTRC | | | |

**b.**

| | 16SB | 16SA | ITS Fungi | *amoA* AOA | *amoA* AOB | *narG*, *nirK*, *nirS* | *napA* | *nosZ*I | *nosZ*II |
|---|---|---|---|---|---|---|---|---|---|
| Stage 1: 1 cycle | 10 min at 95 ºC | 10 min at 95 ºC | 10 min at 95 ºC | 10 min at 95 ºC | 10 min at 95 ºC | 10 min at 95 ºC | 10 min at 95 ºC | 10 min at 95 ºC | 10 min at 95 ºC |
| Stage 2: 6 cycles with 1 ºC decrease by cycle | | | | | | 15s at 95 ºC | 15s at 95 ºC | 15s at 95 ºC | |
| | | | | | | 30s at 63 ºC | 30s at 61 ºC | 30s at 65 ºC | |
| | | | | | | 30s at 72 ºC | 30s at 72 ºC | 30s at 72 ºC | |
| Stage 3: 35 cycles | 15s at 95 ºC | 30s at 95 ºC | 15s at 95 ºC | 30s at 95 ºC | 30s at 95 ºC | 15s at 95 ºC | 15s at 95 ºC | 15s at 95 ºC | 30s at 95 ºC |
| | 30s at 60ºC | 30s at 60 ºC | 60s at 60ºC | 30s at 65 ºC | 30s at 52 ºC | 30s at 58 ºC | 30s at 56 ºC | 30s at 60 ºC | 60s at 48-63 ºC |
| | 30s at 72 ºC | 30s at 72 ºC | 30s at 72 ºC | 30s at 72 ºC | 30s at 72 ºC | 30s at 72 ºC | 30s at 72 ºC | 30s at 72 ºC | 60s at 72 ºC |
| Stage 4: 1 cycle | 10 min at 72 ºC | 10 min at 72 ºC | 10 min at 72 ºC | 10 min at 72 ºC | 10 min at 72 ºC | 10 min at 72 ºC | 10 min at 72 ºC | 10 min at 72 ºC | 10 min at 72 ºC |

### 3.8. Bacterial diversity study and pyrosequencing analysis

PCR amplification of the hypervariable V4-V5 regions of the 16S rRNA gene was performed using universal primers U519F and U926R (Baker et al. 2003) joined to a multiplex identifier sequence (Binladen et al. 2007; Parameswaran et al. 2007). For each sample, amplicons were generated in several replicate PCRs. The reaction mixture for qPCR consisted of: 1-10 ng template DNA; 1 µl of each primer (10 µM); 5 µl of Taq Enhanced; 2.5 µl of 10 x Taq Buffer; 1 µl $MgCl_2$ (10 mM); 1 µl de dNTPs (10 mM); 0.15 µl de Taq Master (5 Prime, USA, 5 U/µl); MQ/ultrapure water up to 25 µL. The PCR program consisted of an initial denaturation step at 94°C for 3 min, 25 cycles of denaturation at 94°C for 15 s, primer annealing at 55°C for 45 s and extension at 72°C for 1 min, followed by a final step of heating at 72°C for 8 min. Amplicons of the same treatment were pooled to reduce per-PCR variability and purified using GeneClean (MP Bio) spin columns. The final PCR product was quantified by Qubit® ssDNA assay kit (Molecular Probes) and visualized by agarose electrophoresis. Samples were combined in equimolar amounts and pyrosequenced in a Roche Genome Sequencer FLX system using 454 Titanium chemistry at Life Sequencing S.L. (Valencia, Spain).

Raw sequences were processed through the Ribosomal Database Project (RDP) pyrosequencing pipeline1 release 11 (Cole et al. 2014). Sequences were trimmed for primers, filtered and assigned to their tags. Sequences shorter than 150 base pair, with quality scores $< 20$ or containing any unresolved nucleotides were removed from the dataset. Chimeras were identified using the Uchime tool from FunGene Database (Edgar et al. 2011) and removed from the dataset. Sequences were aligned using the Infernal alignment tool in RDP (Nawrocki et al. 2009). Aligned sequences were clustered into OTUs defined at 97% similarity cutoff using Complete Linkage Clustering RDP tool and their relative abundances calculated.

The bacterial OTU richness and Good´s coverage indices were calculated using PAST software (v3.14) (Hammer et al. 2001). Shannon and Simpson indices were calculated using the Vegan package v.2.0 of the statistical software R-Project v.2.15.1. Heat maps were generated including all the bacterial OTUs with at least $>$ 1% relative abundance in at least one of the samples. A phylogeny-dependent

cluster analysis based on the relative abundance of the bacterial OTUs was calculated using the software Fast UniFrac (Wood and Salzberg 2014) and the UniFrac tutorial (http://unifrac.colorado.edu/). The different samples were grouped after the 70% similarity in the cluster analysis, stating that samples belonged to the same group if they were clustered together past the 0.7 benchmark (Zhang et al. (2012). The Morisita-Horn and symmetric indices were used for the estimation of β-diversity among pairs of control and N-fertiliser samples (Barwell et al. 2015) using the packages vegetarian and vegan v2.0 implemented in R-Project.

# RESULTS

# Chapter I

## Distinct effect of nitrogen fertilisation and soil depth on nitrous oxide emissions and nitrifiers and denitrifiers abundance

**Published at Castellano-Hinojosa A, González-López J, Bedmar EJ. 2018. Biol Fertil Soils 54: 829-840. https://doi.org/10.1007/s00374-018-1310-9.**

Abstract

The nitrous oxide and molecular N emissions from 5 cm-length subsamples taken from 20 cm-length sample corers containing Eutric Cambisol soil fertilised either with urea, ammonium or nitrate for 1 year have been examined using gas chromatography. At the beginning of the incubation, the same N rate (260 kg N/ ha) was added to the soil and kept constant during the experiment. The total abundance of the soil Bacteria and Archaea and that of nitrifiers and denitrifiers was estimated by quantitative PCR of the corresponding biotic variables 16S rRNA, *amoA* and *napA*, *narG*, *nirK*, *nirS*, *norB*, *nosZ*I and *nosZ*II genes. The abiotic variables dissolved oxygen, pH, exchangeable $NH_4^+$-N and $NO_3^-$-N contents and total C and total N were also analysed. None of the three fertilisers affected the total abundance of Bacteria and Archaea and nitrification was the main driver of nitrous oxide production in the 0- to 5 cm and 5- to 10-cm soil layers while denitrification was in the 10- to 15cm and 15- to 20-cm soil horizons. Parallel to the reduction in the content of dissolved oxygen along the soil profile there was a decrease in the total and relative abundance of the bacterial and archaeal *amoA* gene and an increase in the abundances of the denitrification genes, mainly in the 10- to 15cm and 15- to 20-cm soil layers. A non-metric multidimensional scaling plot comparing the biotic and abiotic variables examined in each of the four 5-cm soil subsamples and the whole 20-cm sample showed a disparate effect of N-fertilisation on N-gas emissions and abundance of nitrifiers and denitrifiers bacterial and archaeal communities.

Keywords: arable top soil, N-fertilisers, N-gas emissions, nitrification, denitrification, qPCR, gene abundance

## Introduction

Nitrous oxide ($N_2O$) is the third most important greenhouse gas because of its global warming potential 310 times greater than that of carbon dioxide ($CO_2$) (Ravishankara et al. 2009), contributing about 8% to the total of anthropogenic greenhouse gases (Erisman et al. 2015). In the stratosphere, $N_2O$ can react with excited oxygen atoms formed in ozone ($O_3$) photolysis to form nitric oxide (NO), which, in turns, catalyzes the decomposition of $O_3$. After phasing out chlorofluorocarbons, $N_2O$ is considered today the major ozone-depleting compound (Ravishankara et al. 2009).

Globally considered, terrestrial ecosystems are responsible for about 70% of total $N_2O$ atmospheric emissions, of which around 45% can be attributed to microbial cycling of N in agriculture (Syakila and Kroeze 2011). Due to the intensified use of N-fertiliser in agriculture, atmospheric concentration of $N_2O$ is increasing at a rate of nearly 0.8 ppb per year (Hofmann et al. 2006). Human alteration of the N cycle has created serious air and water pollution leading to health, climate and environmental consequences including eutrophication, soil acidification and the loss of biodiversity (Galloway et al. 2008; Erisman et al. 2015).

Microbial nitrification and denitrification are main dominant sources of $N_2O$ in agricultural soils, the extent of which depends on moisture content as they are considered to occur under oxic and oxygen-limited conditions, respectively (Butterbach-Bahl et al. 2013 and references therein). Both processes rarely take place in isolation and more likely they occur simultaneously at aerobic and anaerobic microsites within the soil matrix (Baggs and Philippot 2011; Hallin et al. 2018).

Because of the growing interest of $N_2O$ as an influential greenhouse gas, understanding microbial nitrogenous emissions is fundamental for determination of the role soil environmental conditions play in the nitrification and denitrification processes. Measurements of $N_2O$ emissions from natural and agricultural soils have shown that the highest rates of denitrification occurred in the upper soil horizon (Clement et al. 2002; Cosandey et al. 2003; Kustermann et al. 2010), but other research revealed significant denitrification activity in patches of organic rich (Hill

et al. 2004) and in urine-treated subsoils (Dixon et al. 2010). Other works dealing with subsoil denitrification have been published (Casey et al. 2001; Dhondt et al. 2004; Groffman et al. 2009; Khalil and Richards 2011), but data on N-gas, $N_2O$ and $N_2$, emissions along the vertical profile through subsurface soil environments is limited (Jahangir et al. 2012; Barret et al. 2016; Loick et al. 2016).

Previous reports have shown that the way and extent N-fertilisers affect the emission and evolution of $N_2O$, as well as the abundance of nitrifier and denitrifier communities, depend upon the N form, the soil moisture and the soil type (De Rosa et al. 2016; Wang et al. 2017). The contribution, however, of different N-fertilisers available to nitrifying and denitrifying microorganisms has not been analysed for $N_2O$ and $N_2$ emissions through subsurface soil environments. Since nitrification can produce $N_2O$ even under high moisture conditions (Liu et al. 2017), it is still no clear whether or not nitrification, rather than denitrification, dominate $N_2O$ production in the first layers of the soil. Knowledge on the effect of N-fertilisation on the abundance of the soil nitrifier and denitrifier guilds and their link to the availability of ammonium and nitrate along the soil profile is crucial to establish mitigation strategies aimed to reduce the emissions of $N_2O$. Thus, the objectives of this study were: a) to determine the effect of soil depth and type of N-fertilisation on $N_2O$ and $N_2$ emissions along a 20 cm agricultural soil vertical profile; b) to study the relationships between N-gas ($N_2O$ and $N_2$) emissions and the abundance of nitrifiers and denitrifiers communities in the soil, and c) the links between soil depth, N-gas emissions and gene abundance.

## Materials and methods

### Experimental setup

An Eutric Cambisol (30% clay, 12.5% silt, 57.5% sand, w/w; pH in water, 6.8; total C, 25 mg $kg^{-1}$; total N 1.02 mg $kg^{-1}$; $NO_3^-$ 6.8 mg $kg^{-1}$; exchangeable $NH_4^+$, not detected; $HCO_3^-$ 244 mg $kg^{-1}$) of the FAO series (FAO 2017) was collected from an agricultural area in the vicinity of Motril (Granada, Spain), (UTM coordinates 36° 43' 53.5" N, 3° 32' 56.2" W), that had been maintained under fallow conditions for more than 10 years without receiving any type of fertilisation. Spade-squares (30 x 30 cm to a depth of 25 cm) were taken from 12 sites, freed of roots and plant residues, air

dried to ~ 30% $H_2O$ (dry basis) and pooled together. Soil was then supplemented independently either with urea (treatment T2), ammonium sulphate (treatment T3) or potassium nitrate (treatment T4) and mixed with a concrete mixer. Final concentration was of 260 kg of equivalent N $ha^{-1}$ as recommended for horticultural crops and leguminous plants by the Spanish Ministry of Agriculture, Food and Environment. A set of pots containing soil without fertilisation was used as a control (treatment T1). The soils were used to fill 20-kg capacity containers (54 x 21 x 25 cm, 4/treatment) and watered once a week to about 80% water filled pore space (WFPS). Lixiviated water was removed from the dishes supporting the containers. To ensure soil stabilization and the effect of fertilisers on microbial activities, the pots were maintained during 1 year under greenhouse conditions previously described (Tortosa et al. 2015). The concentration of each fertiliser was determined every 3 months and the soil was supplemented with the corresponding N-fertiliser to reach the initial fertilisation rate.

### Soil core preparation and soil physicochemical characteristics

After 1 year, stainless steel cylindrical core samplers (5 cm x 20 cm) were manually inserted into the ground to take soil from the different treatments (10 core samplers/treatment). To analyse the soil physicochemical properties, the soil from 4 core samplers was cut into 5-cm length subsamples that, together with the whole 20-cm core, were homogenized for 30 s at 1600 rpm using a minibead beater cell disrupter (Mikro-DismembratorS; B. Braun Biotech International). The undisturbed soil from the remaining 4 core samplers were used to determine $N_2O$ production. Moisture was measured gravimetrically after drying of the soil for 24 h at 105 ºC. WFPS was calculated according to Danielson and Sutherland (1986). An ionic chromatograph (Methohm) equipped with a Metrosep A supp-4-250 anion column and a Metrosep C2-150 cation column was used to determine $NO_2^-$, $NO_3^-$ and exchangeable $NH_4^+$ concentration, respectively, as indicated earlier (González-Martínez et al. 2016). Dissolved oxygen concentration (DO) was monitored *in situ* at the midpoint of each depth using an oxygen sensor (Apogee Instruments) and pH was measured after water extraction (1:5, w/v) for 2 h. Total C (TC) and total N (TN) were determined using a LECO TruSpec CN elemental analyser.

### Nitrous oxide emission analysis

For estimation of $N_2O$, the undisturbed soil from each of the four-5-cm subsamples and the 20-cm whole sample were half-cut longitudinally, placed in 30-ml glass bottles, sealed hermetically with rubber septa and evacuated with pure He to ensure $N_2$-free conditions. Then, a 10% of the internal atmosphere of half of the bottles was removed and substituted by acetylene. Soil was incubated under greenhouse conditions and $N_2O$ assayed sequentially within times when gas emissions were linear using a Hewlett Packard 5890 gas chromatograph equipped with an electron capture detector as previously reported (Tortosa et al. 2011). Concentration of $N_2O$ was calculated using 2% (v/v) $N_2O$ standard (Air Liquid). $N_2$ production was estimated as the difference in $N_2O$ production in the presence and the absence of acetylene.

### DNA extraction and quantification of nitrification and denitrification genes

Soil DNA was extracted from 0.5 g samples as indicated earlier (Correa-Galeote et al. 2014), purified using GeneClean (MP Bio) spin columns and stored at -20 ºC until use. DNA concentration was measured using the Qubit® ssDNA assay kit (Molecular Probes). The size of the nitrifier community was estimated by quantitative PCR (qPCR) of the *amoA* gene from ammonia-oxidizing Bacteria (*amoA* AOB) and Archaea (*amoA* AOA) and that of denitrifiers by qPCR of the *napA*, *narG*, *nirK*, *nirS*, *norB*, *nosZ* clade I (*nosZ*I) and *nosZ* clade II (*nosZ*II) genes using primers and thermal conditions described earlier (Correa-Galeote et al. 2014; Barrena et al. 2017; see also supplementary Tables S1A, B. The total bacterial (16SB) and archaeal (16SA) community was quantified using the corresponding 16S rRNA gene as a molecular marker. Assays for qPCR were carried out using a Bio-Rad iCycler iQ5 Thermocycler (Bio-Rad Laboratories, USA) with SYBR Green as the detection system. Presence of PCR inhibitors in DNA extracted from soil was estimated by 1) diluting soil DNA extract and 2) mixing a known amount of standard DNA to soil DNA extract prior to qPCR. In all cases, inhibition was not detected. PCR efficiency for the different assays ranged between 90% and 99%. The quality of all qPCR amplifications was verified by electrophoresis in agarose and by melting curve analysis. Gene abundances were analysed as absolute or relative abundances (gene

copy number/16S rRNA Bacteria (16SB) or 16S rRNA Archaea (16SA) gene copy number). As the number of 16S rRNA gene operon per cells is variable (Klappenbach et al. 2001), we did not convert the 16S rRNA gene copy data into cells numbers and we expressed our results as gene copy numbers per nanogram of DNA. Calculation of the gene copy number per nanogram of DNA instead of gram of soil minimized any bias related to soil DNA extraction efficiency.

### Statistical analyses

Measured variables in this study were first explored using the Shapiro-Wilk test and the Bartlett's test to check whether they meet the normality and homoscedasticity assumptions, respectively. Since most data set failed to fit a normal distribution, the Kruskal-Wallis and Conover-Iman tests were chosen for multiple comparisons among samples. BIO-ENV analysis using Primer software (PRIMER-E v. 6.0, Plymouth, UK) was performed to evaluate the influence of the abiotic variables (pH, DO, TC, TN, exchangeable $NH_4^+$, $NO_3^-$, $N_2O$ and $N_2$) on total abundance of the 16S rRNA, *amoA*, *napA*, *narG*, *nirK*, *nirS*, *norB*, *nosZ*I and *nosZ*II genes. Firstly, all the variables were standardized by sample-resemblance matrices generated by using the Bray-Curtis similarity coefficient. The abiotic data sets (except DO and pH) were transformed to log (x + 1) and normalized. Next, vectors representing the biotic and abiotic variables related to the samples were overlaid over a non-metric multidimensional scaling (NMDS) plot to illustrate potential monotonic correlations between them (Wos-Oxley et al. 2010). The stress level of the NMDS plots indicates how well the variables' data fit into two-dimensional spaces, with value $< 0.1$ showing that the NMDS ordination gives a valid bidimensional representation of the biotic data distribution (Clarke and Warwick 2001). The direction of each vector shows the sign of the relationship between the variable and the orientation of the samples in the NMDS ordination, while the vector length is proportional to the strength of the correlation. Additionally, the Pearson's product moment correlation coefficients (r) among vectors representing biotic and abiotic variables were calculated. Finally, a BEST analysis was carried out (Clarke and Warwick 2001), designating the subset of abiotic variables that best matched the similarities of the

biotic data between samples. The statistical significance of vectors and BEST values was tested by a global permutation test (499 permutations).

## Results

### Soil characteristics

Physicochemical properties of the soil used in this study are shown in Table 1. With an initial pH of 6.82, treatment of the soil with urea increased the pH of the three deeper subsamples (7.21-7.25) as compared to that determined in the 0- to 5-cm soil layer (6.97), an effect that was not found when the soil was amended with ammonium. Nitrate fertilisation also increased the pH to reach values higher than those produced by urea (7.30-7.45). Bulk soil density was similar along the 20-cm soil profile with values that varied between 1.45 and 1.5 g/ $cm^3$, which corresponds to a sandy-loam texture. There was a clear decrease in DO along the soil depth, ranging, on average, from 1.5 mg $l^{-1}$ determined in the 0-5 layer to 0.3 mg $l^{-1}$ found in the 15- to 20-cm layer; differences among treatments were not observed. Exchangeable ammonium was detected neither in the control soil nor in the soil treated with nitrate; its concentration was similar in soils fertilised with either urea (6.6-30.2 mg $kg^{-1}$) or ammonium (4.1-28.2 mg $kg^{-1}$) and decreased with depth until complete disappearance in the deepest soil layer. The nitrate content varied among treatments with the highest values determined in the nitrate-fertilised soil (46.2-98.2 mg $kg^{-1}$); regardless of the fertiliser, the content of nitrate diminished with the soil depth. Nitrate concentration in the control soil was not affected by depth. Only 13.1, 14.0 or 19.7% of the applied N remained in soils treated with either urea, ammonium or nitrate after 1-year treatment. Differences in TC values were not found among treatments and soil depths (2.78-3.03%). TN from N-fertilisation was always slightly higher in nitrate-fertilised soils (0.15-0.45%) than in soils treated with either urea (0.09-0.28%) or ammonium (0.10-0.35%) and significantly decreased along the soil profile. Nitrite content was below the detection limit (4 µg $l^{-1}$) in all samples analysed.

## Nitrous oxide emissions

The emissions of $N_2O$ varied with the type of N-fertiliser and the soil depth (Table 2). The production by the unfertilised soil was detected in the 4 horizons, of which the two deeper were significantly higher (0.59 and 0.67 nmol $N_2O$ g soil$^{-1}$ h$^{-1}$, respectively) producers than the two upper horizons (0.19 and 0.22 nmol $N_2O$ g soil$^{-1}$ h$^{-1}$, respectively). The treatment with urea or ammonium increased $N_2O$ emissions that were higher in the 0- to 5-cm layer (1.95 and 2.15 nmol $N_2O$ g soil$^{-1}$ h$^{-1}$, respectively) than in the remaining deeper layers. The fertilisation with nitrate also increased gas emissions from the 0- to 5-cm horizon downwards to reach the highest values in the deeper soil layer (0.35-1.60 nmol $N_2O$ g soil$^{-1}$ h$^{-1}$). When soils were incubated with acetylene, $N_2$ fluxes gradually increased with the soil depth and the soil treated with nitrate showed higher $N_2$ fluxes (0.05-1.22 nmol $N_2$ g soil$^{-1}$ h$^{-1}$) than the soils amended with either urea (0.03-0.52 nmol $N_2$ g soil$^{-1}$ h$^{-1}$) or ammonium (0.03-0.63 nmol $N_2$ g soil$^{-1}$ h$^{-1}$). Regardless of the horizon, the highest $N_2O/N_2$ ratios were detected in soils treated with urea (78.3 in the 0 to 5-cm layer) or ammonium (71.3 in the 0 to 5-cm layer), and those fertilised with nitrate showed the lowest values (1.3-7.0). Values of $N_2O/N_2$ ratios decreased with soil depth.

## Quantification of the nitrification- and denitrification-associated microbial communities

The total abundance of the bacterial and archaeal 16S rRNA, the *amoA* AOB and *amoA* AOA and of the *napA, narG, nirK, nirS, norB, nosZ*I and *nosZ*II genes along the soil profile is shown in Fig. 1 (see also supplementary Table S2). Members of the domains Bacteria and Archaea in the 4 soil subsamples were statistically more abundant in unfertilised than in N-fertilised soils, and the type of N-fertiliser produced no significant differences in their biomass. Under all conditions examined, the 16SB gene copy number was higher than that of the 16SA gene. The *amoA* AOA and *amoA* AOB genes were detected only in soils treated with either urea or ammonium. Differences in their abundance were not found between treatments and the copy number of the genes decreased with depth along the soil profile so that no *amoA* was found in the 15- to 20-cm horizon. For a given soil layer the copy number of the *amoA* AOB was statistically higher than that of the *amoA* AOA.

The *napA* and *narG* genes were found in unfertilised soils and the addition of any N-fertiliser increased their copy numbers in each of the 4 layers. Regardless of the treatment, the abundance of the genes was similar in the 0- to 5 and 5- to 10-cm layers and increased downwards to reach the highest values in the two deeper horizons. In the 4 soil layers, the abundance of the *napA* was greater than that of *narG*. The addition of any of the fertilisers also increased the abundance of the *nirK* and *nirS* genes in a way similar to that of the *napA* and *narG* genes so that fertilisation with either ammonium or nitrate increased the copy number of the genes in the 10- to 15 and 15- to 20-cm layers. The abundance of *nirK* was greater than that of *nirS* in all four layers. Increases in the abundance of *norB* after fertilisation were similar among treatments and increased with the soil depth from the 5-10 cm layer downwards, mainly in soils amended with nitrate. The *nosZ*I gene was not found in the upper layer of the unfertilised soil but appeared in the remaining deeper layers. The treatment either with urea or ammonium produced similar increments in the gene copy number along the soil profile and amendment with nitrate further increased its abundance mainly in the 10- to 15 and 15- to 20-cm horizons. The *nosZ*II gene was not found in unfertilised soils at any depth. Like *nosZ*I, the copy numbers of *nosZ*II increased after treatment either with urea or ammonium but nitrate produced the highest increases in the gene abundance among treatments and soil depths. Abundance of *nosZ*II was lower than that of *nosZ*I under all conditions examined.

Relative abundance of nitrification and denitrification genes along the soil profile is presented in Fig. 2 (see also supplementary Table S3). That of the *amoA* AOA and *amoA* AOB genes decreased with soil depth, in average, from 0.46% and 0.27% in the 0- to 5-cm layer to 0.03% and 0.01% in the 10- to 15-cm horizons from soils treated with urea or ammonium, respectively. On the other hand, the relative abundance of the denitrification genes was negligible in the upper layer and significantly increased after N-fertilisation, mainly with nitrate. Considering only the two deeper layers, *napA* and *norB* were the most abundant followed by *nirK*, *narG*, *nirS* and the less abundant *nosZ*I and *nosZ*II. In these two horizons,

fertilisation with nitrate produced higher increases in the relative abundance of the denitrification genes than the treatments with urea or ammonium.

### Linking soil depth with biotic and abiotic variables

NMDS plots based on Pearson correlation coefficients (Supplementary Tables S4-S8) are shown in Fig. 3. The NMDS analysis including the abiotic variables (pH, DO, exchangeable $NH_4^+$-N, $NO_3^-$-N, TC, TN, $N_2O$ and $N_2$) showed that DO, exchangeable $NH_4^+$-N and $N_2O$ best explained (94.3%) (Fig. 3a) the changes in the abundance of 16SA, 16SB, *amoA* AOA, *amoA* AOB, *napA*, *narG*, *nirK*, *nirS, norB, nosZ*I and *nosZ*II genes at the 0- to 5-cm soil depth (Fig. 3a). The analysis also revealed that the 16SB and 16SA genes display opposite ordinations and that the *amoA* AOA and *amoA* AOB genes clustered together and so did the denitrification genes. (Fig. 3a). The abundance of 16SA gene was positively related to DO and the abundance of 16SB with pH. While abundances of *amoA* AOA and *amoA* AOB genes correlated positively with DO, exchangeable $NH_4^+$-N content and $N_2O$ emissions the abundance of denitrification genes correlated positively with $NO_3^-$-N and TN contents and $N_2$. For the 5- to 10-cm horizon, ordination of the biotic variables were strongly dependent (71.4%) of the exchangeable $NH_4^+$-N and $N_2O$ variables (Fig. 3b). The correlation and ordination between the abiotic variables and the abundance of genes were similar to those found in the 0- to 5-cm soil layer (Fig. 3b), albeit the correlation between the $N_2O$ and the *amoA* AOA and *amoA* AOB total abundances were lower than those calculated for the 0- to 5-cm soil depth. The DO, $NO_3^-$-N content and $N_2O$ were the variables that best (88.6%) explained ordination of the abundance of genes in the 10- to 15-cm subsample (Fig. 3c). In this layer, the 16SA and 16SB genes showed no correlation and the nitrification and denitrification genes clustered together, respectively (Fig. 3c). A positive correlation was found between the *amoA* gene abundances and exchangeable $NH_4^+$-N and no correlation with $N_2O$ production. The abundance of the denitrification genes showed a strong positive correlation with TN and $NO_3^-$-N contents, $N_2O$, $N_2$ and pH, and negatively with DO. Exchangeable $NH_4^+$-N and the *amoA* AOB and *amoA* AOA genes were not detected in the 15- to 20-cm soil depth (supplementary Table S2) and, accordingly, they were not included in the NMDS analysis corresponding to that soil layer. At this depth, DO,

TN and $NO_3^-$-N contents and $N_2O$ were the abiotic variables that best explained (91.6%) the ordination of the gene abundances (Fig. 3d). No correlation was found between the 16SA and 16SB genes (Fig. 3d). The denitrification genes did not show a clear ordination, albeit the total abundance of the *norB* and *nosZ* genes positively correlated with $N_2O$ and $N_2$, respectively (Fig. 3d).

A joint NMDS analysis including the abiotic variables corresponding to the four soil layers revealed that DO, exchangeable $NH_4^+$-N, $NO_3^-$-N, $N_2O$ and $N_2$ were the abiotic variables best explaining (89.5%) ordination of the gene abundances (Fig. 3e). The vectors representing the total abundance of the 16SA and 16SB genes showed opposite direction, those involved in nitrification grouped together and so did the denitrification genes (Fig. 3e). The abundances of *amoA* AOA and *amoA* AOB genes correlated positively with DO and exchangeable $NH_4^+$-N contents. Although each of the denitrification genes correlated positively with TN and $NO_3^-$-N and $N_2O$ and $N_2$, the strongest correlations were found between the abundances of *napA* and *narG* with $N_2O$, *nirK* and *nirS* with $NO_3^-$-N content and $N_2O$, *norB* with $N_2O$, and *nosZ*I and *nosZ*II with $N_2$ (Fig. 3e). The NMDS plots also showed that of the denitrification genes with equivalent function, *napA*, *nirK*, and *nosZ*I strongly influenced the ordination with vector lengths $> 0.6$, while the contribution of *narG*, *nirS* and *nosZ*II was negligible as indicated by vector lengths $< 0.2$) (Fig. 3). The contribution of *amoA* AOB and *amoA* AOA genes was similar through the ordination (vector lengths $> 0.6$) (Fig. 3). Also, regardless of the depth, soil samples corresponding to treatments with urea and ammonium clustered together (Fig. 3).

## Discussion

In this study we examined depth-related emissions of $N_2O$ and the abundance of the nitrifying and denitrifying communities in response to varying N-fertilisers amendments along the 20-cm layer of the arable topsoil. Nitrification and denitrification are N-cycle biological processes considered to be the major sources for $N_2O$ release into the atmosphere from agricultural soils due to N-fertilisation. While $NH_4^+$ is the substrate for aerobic nitrification, nitrate, or a derived nitrogen oxide, and $O_2$-limiting conditions are required for denitrification. When a N source is available, N-gas ($N_2$ and $N_2O$) emissions are influenced by the oxygen

concentration in the soil matrix which, in turn, is dependent on the moisture content of the soil; in this way, the potential for $N_2O$ emissions have been related to WFPS, being generally accepted that nitrification in the main source of $N_2O$ in the range 30-60% WFPS whereas denitrification dominates at 50-90% WFPS (Davidson 1991b; Bateman and Baggs 2005).

In our mesocosm study, 24 h after watering the pots, the oxygen concentration at 2.5-cm below the soil surface was, on average, 3.34% (Table 1) and a calculated WFPS of 65%. In that layer, the soil containing ammonium-fertilisers emitted more $N_2O$ than that containing only nitrate (Table 2), which suggests the existence of active nitrification. Confirmation of nitrification in the 0- to 5-cm soil layer comes from the presence of the *amoA* AOA and *amoA* AOB genes in that layer, while no *amoA* genes were found in the control and the nitrate-treated-soils (Fig. 1, Table S2). In this sense, Liu et al. (2017) demonstrated that nitrification contributed to 87, 80 and 53% of total $N_2O$ production at 50, 70 and 85% WFPS, respectively, and Pan et al. (2018) found that nitrification dominated $N_2O$ production in urine-treated soils, and was correlated with the bacterial *amoA* gene abundance. Previous studies have also reported the existence of nitrification under similar oxygen conditions (Philips et al. 2002; Geets et al. 2006; Arnaldos et al. 2013), but whether or not $N_2O$ emissions were due to nitrifier denitrification (Wrage et al. 2001; Zhu et al. 2013) and/or aerobic denitrification (Takaya et al. 2003; Gao et al. 2010; Ji et al. 2015) cannot be ruled out. In the 0- to 5-cm soil horizon, partial amounts of $N_2O$ emissions were exclusively due to denitrification (treatment T4), albeit they were statistically lower than those from nitrification (treatments T1 and T2). The abundance of each of the denitrification gene was similar among treatments and statistically higher than the control soil (Fig. 1, Table S2), which indicates that urea (treatment T2) and ammonium (treatment T3) were metabolized to form nitrate, the molecule responsible for induction of the denitrification genes. Members of the domains Bacteria and Archaea appear to contribute to $N_2O$ emissions (Table 2) and the total abundance of *amoA* AOB was higher than that of the *amoA* AOA (Fig. 1, Table S2); the relative abundance, however, of *amoA* AOA was greater than that of *amoA* AOB (Fig. 2 and Table S3). Within the denitrifiers there were differences in the total and

relative abundances of each of the populations bearing the *narG*, *nirK*, *nirS*, *norB*, *nosZ*I and *nosZ*II genes, and *norB* was the gene with the highest relative abundance and that of *nosZ* was negligible (Fig. 2 and Table S3).

In the 5- to 10-cm soil horizon, at 7.5-cm below the surface, the content of dissolved oxygen was 2.45% (Table 1) and a calculated 70% WFPS. In this layer, $N_2O$ production was due mainly to denitrification because gas emissions decreased in soils treated with urea or ammonia while increased in the soil fertilised with nitrate (Table 2). The reduction in $N_2O$ production was most likely due to a decline in the total abundance of the *amoA* AOA and *amoA* AOB genes because the relative abundances of the *amoA* genes in this layer were statistically lower than those in the 0- to 5-cm horizon (Fig. 2, Table S3); on the other hand, changes in the total abundance of any of the denitrification genes were not observed, with the exception of *nosZ*I and *nosZ*II which showed a significant increment in comparison with the values found in the 0- to 5-cm layer. Conversely to nitrification, there were significant increases in the relative abundances of each of the denitrification genes and, again, *norB* was the most abundant gene (Fig. 2, Table S3).

The dissolved oxygen content of about 1.1% and WFPS of 75% in the 10- to 15-cm soil depth indicates an oxygen-limiting environment where nitrifying Bacteria and Archaea were still present (Fig. 1, Table S2), but more likely not active. If nitrification was functional in this layer, $N_2O$ emissions by soils treated with urea or ammonium should emit more gas than those treated only with nitrate since urea and ammonium are substrates for nitrification and, following nitrification, nitrate is also available for denitrification (Table 1). A lack of substrate availability to explain the lower $N_2O$ emissions can be ruled out because nitrate and ammonia were present in the 10- to 15-cm soil horizon (Table 1). However, there were statistically significant decreases in the total abundance of the *amoA* genes, both from Archaea and Bacteria (Fig. 1, Table S2), so that the relative abundances of the nitrifiers were much lower, almost null, than those in the two 5- to 10 and 0- to 5-cm upper soil layers (Fig. 2, Table S3). The reduction in the total abundances of the nitrifiers was accompanied by clear increments in the biomass of the denitrifiers (Fig. 1, Table S2), which suggests that the $N_2O$ emitted in the 10- to 15-cm soil horizon can be

attributed to denitrification. Like in the 5- to 10-cm soil layer, the gene pair *nar*G/*napA* and *norB* were the most abundant, followed by *nirK*/*nirS* and, finally, the *nosZI*/*nosZ*I genes.

The highest rates of $N_2O$ emissions were detected in the deepest 15- to 20-cm soil horizon (Table 2), where no *amoA* AOA and *amoA* AOB genes were found (Fig. 1, Table S2). While no increases in the total abundance of the denitrification genes were detected, their relative abundance showed significant increments (Fig. 2, Table S3). Increases in the relative abundance of the denitrification genes without parallel rises in their total abundance could be due to the reduction of bacterial and archaeal species capable of denitrification under conditions of very low oxygen content; other authors have also shown that N-fertilisation decreased microbial abundance after medium and long-term treatments (Wessén et al. 2011; Fierer et al. 2012; Kearns et al. 2015), more likely due to to the expansion of nitrophilous species and competitive exclusion (Yan et al. 2017). Otherwise, high $NO_3^-$-N content in this layer was associated with a higher $N_2O$ reductase activity (Table 2), which agrees with demostration that excess N-fertilisation decreases $N_2O$ emission by increasing $N_2O$ reductase activity (Qin et al. 2017).

The *norB* and *nosZ* genes appear to have different response to dissolved oxygen content; this is based in that the relative abundances of *norB* were similar or even higher than those of the remaining denitrification genes in the four soil horizons analysed, while the relative abundance of *nosZ* was irrelevant in the two upper soil horizons where the dissolved oxygen content is higher. The *nosZ* gene increased along the soil profile, mainly in the 15- to 20-cm horizon (Fig. 1, Table S2); this agrees with the suggestion that *nosZ* was induced at soil depths where the dissolved oxygen content was close or below 1.0%, which, in turn, resulted in lower $N_2O/N_2$ ratios (Table 2). The higher oxygen sensitivity of the *nosZ* gene relative to *norB* has been reported (Zumft 1997); accordingly, because the *norB* and *nosZ* genes have been used as molecular markers to study the abundance and diversity of denitrifiers, their $O_2$-dependent expression should be considered to define the precise gene to be used for modelling purposes. Because DNA provides evidence of gene existence but not of gene expression, whether or not changes in $N_2O$ emissions were due to

variations in the activity of nitrification/denitrification enzymes or to variations in the pool of enzymes cannot be elicited from the present results. Wertz et al. (2016) have shown that $N_2O$ emission rates were not correlated with the number of *nirS* or *nirK* transcripts, which suggests that changes in $N_2O$ emissions were mainly due to variations in the activity of the denitrification enzymes. The acetylene reduction inhibition method has been used to estimate $N_2$ production. Although run under $O_2$-limiting contiditions, problems associated with that methology cannot be ruled out, albeit the technnique is widely used to assay desnitrification because of its low coswt and ease of usage.

The NMDS plots carried out using the analysed biotic and abiotic variables for each individual soil layer revealed that $N_2O$ emissions were mainly due to nitrification driven by exchangeable $NH_4^+$-N and dissolved oxygen content in the 0- to 10-cm soil depth whereas emissions in the 10- to 20-cm soil horizon originated from denitrification controlled by DO, $NO_3^-$-N content and TN (Fig. 3). When the NMDS analysis was done using the abiotic and biotic parameters calculated for the whole 20-cm soil profile, the $N_2O$ emissions were mainly due to denitrification (Fig. 3). Thus, the 20-cm soil profile only gave a partial description of the process involved in $N_2O$ emissions.

Evidence accumulates to suggest that fungal denitrification also contributes to $N_2O$ soil emissions, and that Fungi dominate Bacteria in $N_2O$ production under sub-anoxic soil conditions (Shoun and Fushinobu 2017), contributing with up to a 18% of the potential denitrification in a soil treated with bacterial and fungal growth inhibitors (Herold et al. 2012). Thus, it is likely that a proportion of the $N_2O$ detected in our study comes from fungal denitrification.

## Conclusions

Variations in N-gas, $N_2O$ and $N_2$, emissions by 5-cm length soil subsamples taken from 20-cm-length samples containing an Eutric Cambisol soil were dependent on the type of N-fertiliser, whether urea, ammonium or nitrate, and the soil depth-related dissolved oxygen content. Also, N-gas emissions correlated with the abundance of the nitrifying and denitrifying communities in the soil. While $N_2O$ production by nitrification was dominant in the first two upper layers spanning the

0- to 10-cm soil horizon, denitrification was the main driver of N-gas production in the two deeper layers. Accordingly, the absolute and relative abundance of the ammonia-oxidizing bacteria and the ammonia oxidizing archaea decreased along the soil profile; on the contrary, abundances of the denitrification genes increased with depth to reach maximal values in the deepest soil layer. The *norB* and *nosZ* genes differed in their response to the concentration of dissolved oxygen and *norB* was less sensitive to $O_2$ than *nosZ*. Because of the disparate effect of N-fertilisers and soil depth on N-gas emissions and abundance of nitrifiers and denitrifiers, it is possible the obtaining of partial descriptions when individual soil layers are considered. Further studies including combined DNA-RNA approaches together with a detailed information on the spatio-temporal $O_2$ availability along the soil profile would result in a more accurate information to link the microbial community's structure and its metabolic ability requirements to produce N-gas in soils.

## Acknowledgments

This study was supported by the ERDF-cofinanced grant PEAGR2012-1968 from Consejería de Economía, Innovación y Ciencia (Junta de Andalucía, Spain) and the MINECO-CSIC Agreement RECUPERA 2020. ACH is recipient of a grant of MECD (FPU 2014/01633).

*Table 1. Physicochemical properties of 5 cm-length subsamples taken from 20 cm-length core samplers containing soil not treated (T1) or fertilised with urea (T2), ammonium sulfate (T3) or potassium nitrate (T4). For each soil depth and each treatment within a depth values followed by the same uppercase or lowercase letter are not statistically different according to the Kruskal-Wallis and Conover-Iman tests ($p < 0.05$; $n = 9$), respectively. DO, dissolved oxygen; in brackets is the percentage of DO; TC, total C; TN, total N. nd, not detected.*

| Soil depth (cm) | Treatment | pH | DO (mg $L^{-1}$; %) | $NH_4^+$-N (mg $kg^{-1}$) | $NO_3^-$-N (mg $kg^{-1}$) | TC (%) | TN (%) |
|---|---|---|---|---|---|---|---|
| 0-5 | T1 | 6.82 ± 0.12aA | 1.5 ± 0.2aD (3.4) | nd | 3.6 ± 1.0aA | 3.01 ± 0.11aA | 0.05 ± 0.02aA |
| | T2 | 6.97 ± 0.10 aA | 1.3 ± 0.2aD (3.2) | 30.2 ± 2.3aC | 48.6 ± 5.4bD | 3.03 ± 0.12aA | 0.28 ± 0.03bC |
| | T3 | 6.82 ± 0.11aA | 1.4 ± 0.2aD (3.0) | 28.2 ± 2.9aC | 54.8 ± 6.3bD | 2.82 ± 0.08aA | 0.35 ± 0.03bC |
| | T4 | 7.30 ± 0.14bA | 1.6 ± 0.2aD (3.7) | nd | 98.2 ± 5.3cD | 2.85 ± 0.07aA | 0.45 ± 0.05dC |
| 5-10 | T1 | 6.85 ± 0.10aA | 1.1 ± 0.1aC (2.5) | nd | 4.6 ± 1.1aA | 2.85 ± 0.10aA | 0.06 ± 0.02aA |
| | T2 | 7.24 ± 0.12bB | 1.1 ± 0.1aC (2.5) | 23.8 ± 3.0aB | 39.8 ± 8.3bC | 2.90 ± 0.06aA | 0.17 ± 0.03bB |
| | T3 | 6.89 ± 0.08aA | 1.0 ± 0.1aC (2.3) | 25.6 ± 3.5aB | 56.8 ± 9.3bC | 2.70 ± 0.08aA | 0.19 ± 0.03bB |
| | T4 | 7.45 ± 0.15bA | 1.1 ± 0.1aC (2.5) | nd | 79.2 ± 2.3cC | 2.84 ± 0.10aA | 0.29 ± 0.04cB |
| 10-15 | T1 | 6.87 ± 0.06aA | 0.8 ± 0.1aB (1.8) | nd | 3.9 ± 0.5aA | 2.88 ± 0.12aA | 0.05 ± 0.02aA |
| | T2 | 7.21 ± 0.06bB | 0.6 ± 0.1aB (1.4) | 6.6 ± 1.3aA | 26.8 ± 4.3bB | 2.97 ± 0.06aA | 0.13 ± 0.03bA |
| | T3 | 6.86 ± 0.05aA | 0.7 ± 0.1aB (1.6) | 4.1 ± 1.8aA | 20.2 ± 3.2bB | 2.88 ± 0.08aA | 0.14 ± 0.03bA |
| | T4 | 7.42 ± 0.10cA | 0.7 ± 0.1aB (1.6) | nd | 58.2 ± 5.3cB | 2.84 ± 0.06aA | 0.25 ± 0.05cB |
| 15-20 | T1 | 6.88 ± 0.06aA | 0.4 ± 0.1aA (0.9) | nd | 2.9 ± 2.3aA | 2.89 ± 0.04aA | 0.04 ± 0.03aA |
| | T2 | 7.25 ± 0.11bB | 0.3 ± 0.1aA (0.7) | nd | 12.6 ± 2.1bA | 2.79 ± 0.06aA | 0.09 ± 0.04aA |
| | T3 | 7.07 ± 0.11aA | 0.3 ± 0.1aA (0.7) | nd | 11.1 ± 1.6bA | 2.70 ± 0.07aA | 0.10 ± 0.02aA |
| | T4 | 7.33 ± 0.05bA | 0.3 ± 0.1aA (0.7) | nd | 46.2 ± 3.3cA | 2.78 ± 0.06aA | 0.15 ± 0.02bA |

*Table 2. $N_2O$ and $N_2$ production by 5 cm-length subsamples taken from 20 cm-length core samplers containing soil not treated (T1) or fertilised urea (T2), ammonium sulfate (T3) or potassium nitrate (T4). For each soil depth, and each treatment within a depth, values followed by the same uppercase, or lowercase, letter are not statistically different according to the Kruskal-Wallis and Conover-Iman tests ($p < 0.05$; $n = 9$), respectively. $N_2$ production was estimated as the difference in $N_2O$ production in the presence and in the absence of acetylene.*

| Soil depth (cm) | Treatment | $N_2O$ emissions (nmol $N_2O$ g $soil^{-1}$ $h^{-1}$) | $N_2$ emissions (nmol $N_2$ g $soil^{-1}$ $h^{-1}$) | $N_2O/N_2$ ratio |
|---|---|---|---|---|
| 0-5 | T1 | 0.19 ± 0.08aA | 0.03 ± 0.01aA | 6.3 ± 0.4a |
| | T2 | 1.95 ± 0.12cD | 0.03 ± 0.01aA | 78.3 ± 4.2b |
| | T3 | 2.15 ± 0.14cD | 0.03 ± 0.01aA | 71.3 ± 3.1b |
| | T4 | 0.35 ± 0.10aA | 0.05 ± 0.01bA | 7.0 ± 0.4a |
| 5-10 | T1 | 0.22 ± 0.05aA | 0.03 ± 0.01aA | 7.3 ± 0.5a |
| | T2 | 0.64 ± 0.08bA | 0.03 ± 0.02aA | 21.3 ± 1.4b |
| | T3 | 0.69 ± 0.10bA | 0.03 ± 0.02aA | 23.0 ± 1.2b |
| | T4 | 0.68 ± 0.11bA | 0.11 ± 0.02bB | 6.2 ± 0.4a |
| 10-15 | T1 | 0.59 ± 0.09aB | 0.02 ± 0.01aB | 6.5 ± 0.6b |
| | T2 | 0.72 ± 0.12bB | 0.11 ± 0.02bB | 8.4 ± 0.5c |
| | T3 | 0.76 ± 0.11bB | 0.10 ± 0.02bB | 9.3 ± 0.6c |
| | T4 | 0.98 ± 0.15bB | 0.47 ± 0.05cC | 1.7 ± 0.3a |
| 15-20 | T1 | 0.67 ± 0.08aB | 0.13 ± 0.03aC | 5.1 ± 2.1c |
| | T2 | 1.31 ± 0.10bC | 0.52 ± 0.05bC | 2.5 ± 0.4b |
| | T3 | 1.21 ± 0.08bC | 0.63 ± 0.06bC | 1.9 ± 0.4a |
| | T4 | 1.60 ± 0.12cC | 1.22 ± 0.12cD | 1.3 ± 0.3a |

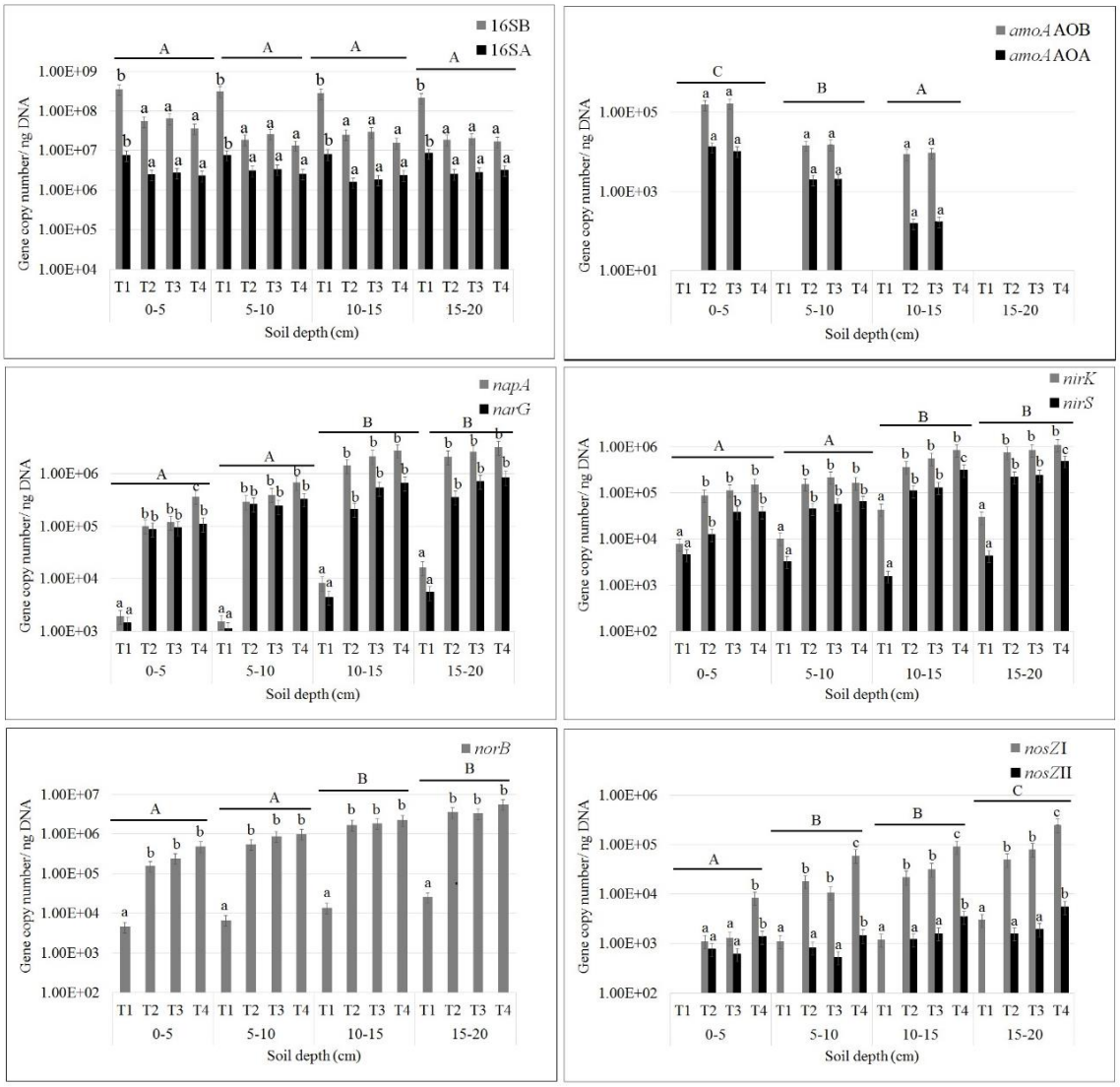



*Fig. 1. Total abundance of the 16SB, 16SA, amoA AOB, amoA AOA, napA, narG, nirK, nirS, norB, nosZI and nosZII genes in 5 cm-length subsamples taken from 20 cm-length core samplers containing soil not treated (T1) or fertilised with urea (T2), ammonium sulfate (T3) or potassium nitrate (T4). Values are expressed as gene copy number per ng soil DNA. Rectangles and horizontal lines with the same lowercase or uppercase letter, respectively, are not statistically different according to the Kruskal-Wallis and Conover-Iman tests ($n = 6$; $p < 0.05$). Bars represent standard errors.*

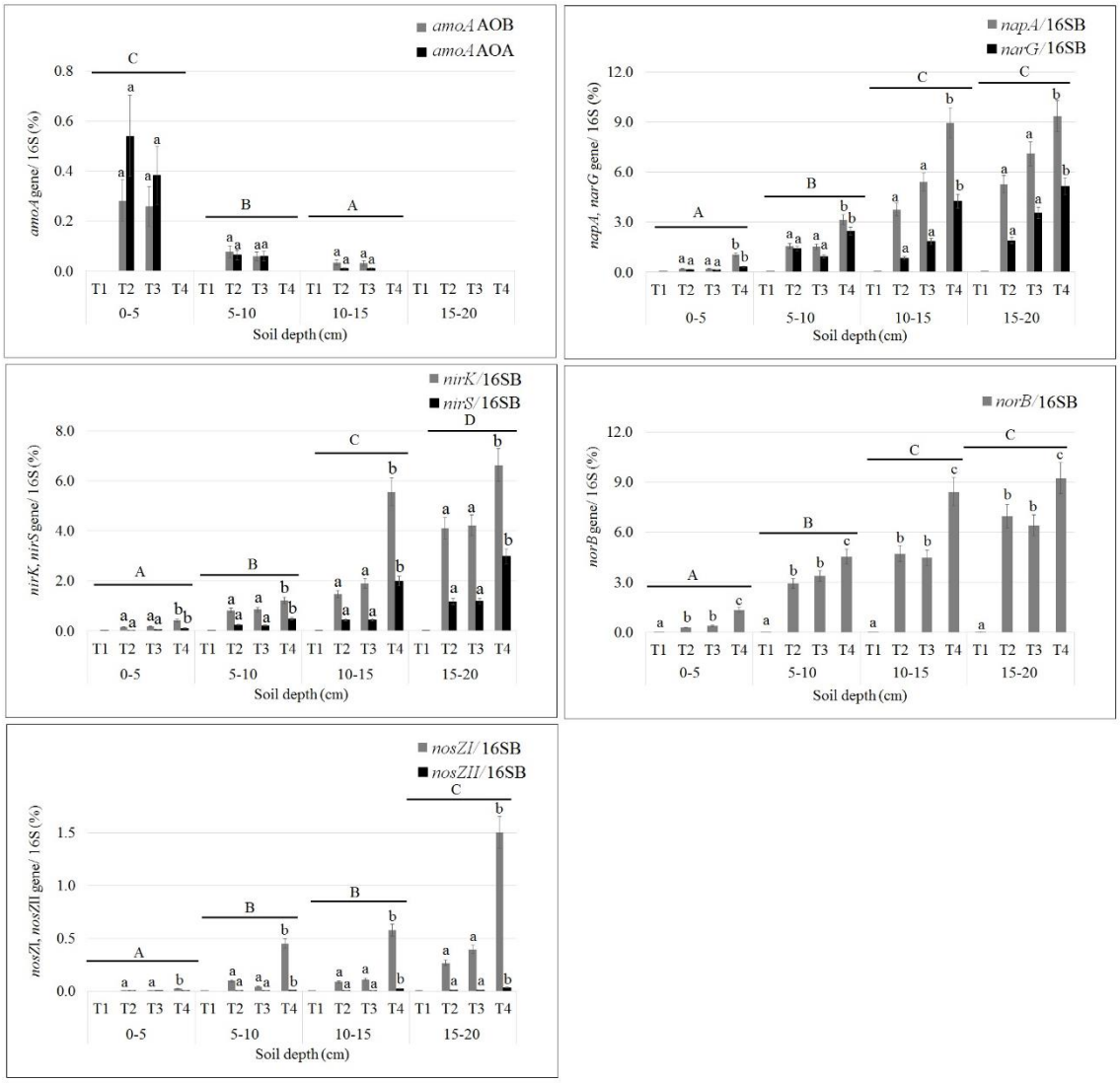



*Fig. 2. Relative abundance of the amoA AOB, amoA AOA, napA, narG, nirK, nirS, norB, nosZI and nosZII genes in 5 cm-length subsamples taken from 20 cm-length core samplers containing soil not treated (T1) or fertilised with urea (T2), ammonium sulfate (T3) or potassium nitrate (T4). Values (%) are expressed as gene copy number/16S rRNA Bacteria (16SB) or Archaea (16SA). Rectangles and horizontal lines with the same lowercase or uppercase letter, respectively, are not statistically different according to the Kruskal-Wallis and Conover-Iman tests ($n = 6$; $p < 0.05$). Error bars are standard errors.*

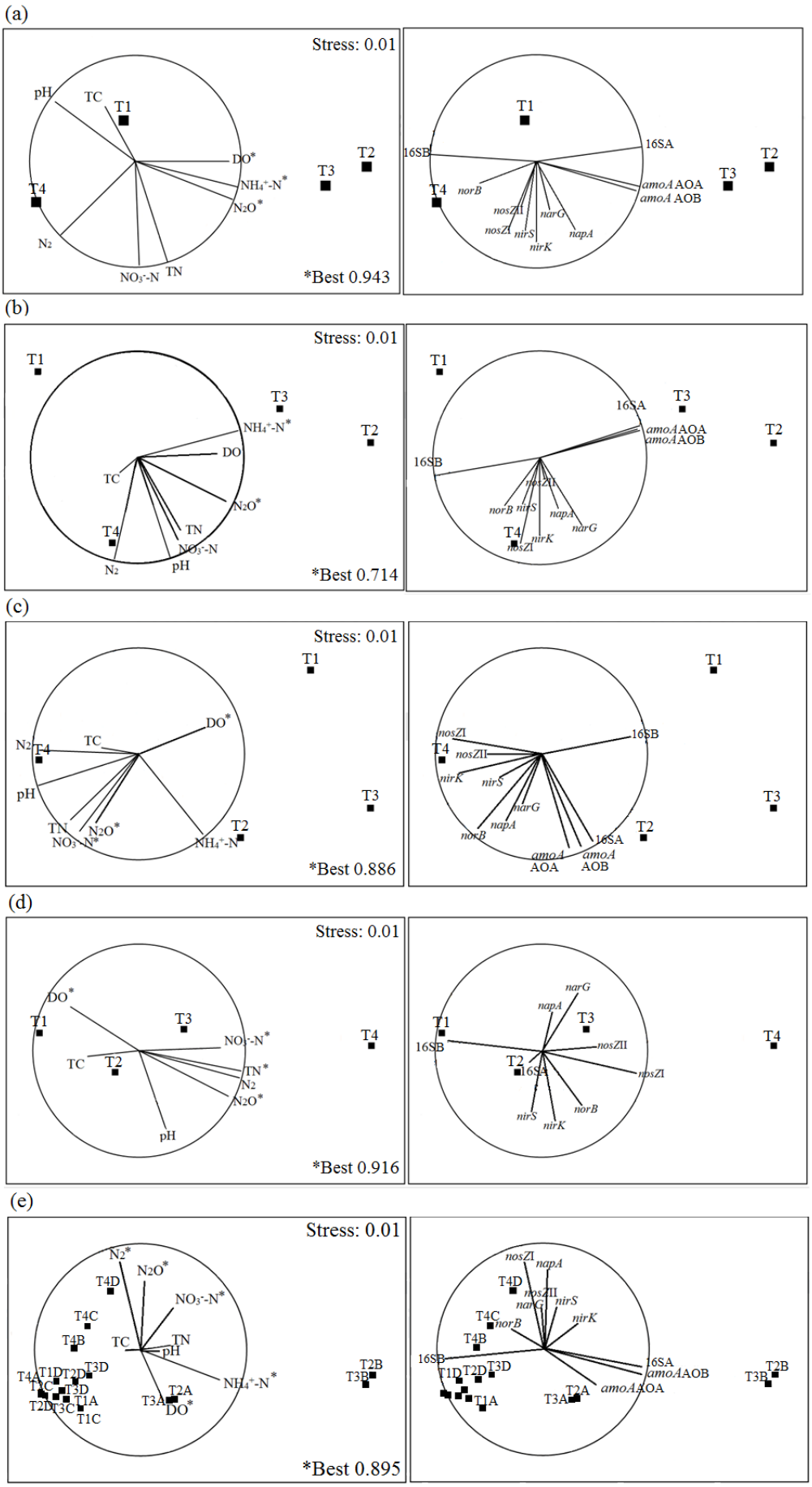

(a)
Stress: 0.01
pH
TC
T1
DO*
NH4+-N*
N2O*
T4
N2
NO3--N
TN
T2
T3
*Best 0.943
T1
16SB
16SA
T2
T3
T4
norB
amoA AOA
amoA AOB
nosZII
narG
nosZI
nirS
napA
nirK
(b)
Stress: 0.01
T1
T3
NH4+-N*
T2
DO
TC
N2O*
T4
TN
NO3--N
N2
pH
*Best 0.714
T1
16SA
T3
amoAAOA
amoAAOB
T2
16SB
nosZII
norB
nirS
napA
T4
narG
nirK
nosZI
(c)
Stress: 0.01
T1
DO*
N2
T4
TC
pH
T1
N2O*
NO3--N*
T2
NH4+-N
T3
*Best 0.886
T1
nosZI
16SB
T4
nosZII
nirK
nirS
narG
napA
T2
norB
16SA
amoA
AOA
amoA
AOB
T3
(d)
Stress: 0.01
DO*
T1
T3
NO3--N*
T4
TC
T2
TN*
N2
N2O*
pH
*Best 0.916
narG
napA
T1
T3
16SB
nosZII
T4
T2
16SA
nosZI
nirS
norB
nirK
(e)
Stress: 0.01
N2*
N2O*
T4D
NO3--N*
T4C
T4B
TC
TN
pH
T4A
T1D
T2D
T3D
T2C
T3C
T3A
T2A
T1A
T1C
DO*
NH4+-N*
T2B
T3B
*Best 0.895
nosZI
napA
T4D
nosZII
narG
nirS
T4C
norB
nirK
T4B
16SB
T1D
T2D
T3D
16SA
amoAAOB
T2B
T3B
T1A
T3A
T2A
amoAAOA

*Fig. 3. Non-metric multidimensional scaling (NMDS) plots illustrating the ordinations between the soil abiotic variables (pH, DO, TC, TN, $NH_4^+$, $NO_3^-$, $N_2O$ and $N_2$) and the total abundance of 16SA, 16SB, amoA AOA, amoA AOB, napA, narG, nirK, nirS, norB, nosZI and nosZII genes retrieved from 5 cm-length subsamples taken from 20 cm-length core samplers containing soil not treated (T1) or fertilised with urea (T2), ammonium sulfate (T3) or potassium nitrate (T4). Soil depths were 0-5* **(a)**, *5-10* **(b)**, *10-15* **(c)**, *15-20* **(d)** *and 0-20* **(e)** *cm. For each depth, the vectors in the plots illustrate the direction and strength of the relationships between the biotic (1) or abiotic (2) variables. Treatments are represented by squares. The variables which best explained the distributions of the biological data according to BIO-ENV analysis are marked with an asterisk (*).*

## Supplementary Material for Chapter I

# Distinct effect of nitrogen fertilisation and soil depth on nitrous oxide emissions and nitrifiers and denitrifiers abundance

**Published at Castellano-Hinojosa A, González-López J, Bedmar EJ. 2018. Biol Fertil Soils 54: 829-840. https://doi.org/10.1007/s00374-018-1310-9.**

*Table S1. A. Primers used for quantification of total abundance of Bacteria and Archaea (16SB and 16SA, respectively), nitrifiers (amoA AOB and amoA AOA) and denitrifiers (napA, narG, nirK, nirS, norB, nosZI and nosZII) by qPCR. B. qPCR conditions for the quantification of each of the target genes.*

**A.**

| Primer | Primer sequence (5´-3´) | Target gene | Amplicon size (base pair, bp) | Reference |
|---|---|---|---|---|
| 341F | CCTACGGGAGGCAGCAG | 16S rRNA Bacteria | 194 | Muyzer et al. (1993) |
| 534R | ATTACCGCGGCTGCTGGCA | | | |
| 771F | ACGGTGAGGGATGAAAGCT | 16S rRNA Archaea | 226 | Ochsenreiter et al. (2003) |
| 957R | CGGCGTTGACTCCAATTG | | | |
| AmoA1F | GGGGTTTCTACTGGTGGT | *amoA* Bacteria | 490 | Rotthauwe et al. (1997) |
| AmoA2R | CCCCTCKGSAAAGCCTTCTTC | | | |
| Crenamo A23F | ATGGTCTGGCTWAGACG | *amoA* Archaea | 624 | Tourna et al. (2008) |
| Crenamo A616R | GCCATCCATCTGTATGTCCA | | | |
| narG-f | TCGCCSATYCCGGCSATGTC | *narG* | 174 | Bru et al. (2007) |
| narG-r | GAGTTGTACCAGTCRGCSGAYTCSG | | | |
| nap3F | TGGACVATGGGYTTYAAYC | *napA* | 152 | Bru et al. (2007) |
| napA4R | ACYTCRCGHGCVGTRCCRCA | | | |
| nirK876F | ATYGGCGGVAYGGCGA | *nirK* | 173 | Henry et al. (2004) |
| nirK1040R | GCCTCGATCAGRTTRTGGTT | | | |
| nirS4QF | AACGYSAAGGARACSGG | *nirS* | 425 | Throbäck et al. (2004) |
| nirS6QR | GASTTCGGRTGSGTCTTSAYGAA | | | |
| cnorB2F | GACAAGNNNTACTGGTGGT | *norB* | 389 | Braker and Tiedje (2003) |
| cnorB6R | GAANCCCCANACNCCNGC | | | |
| nosZ1840F | CGCRACGGCAASAAGGTSMSSGT | *nosZ* Clado I | 267 | Henry et al. (2006) |
| nosZ2090R | CAKRTGCAKSGCRTGGCAGAA | | | |
| nosZIIF | CTIGGICCIYTKCAYAC | *nosZ* Clado II | 690-720 | Jones et al. (2013) |
| nosZIIR | GCIGARCARAAITCBGTRC | | | |

B.

| | 16SB | 16SA | *amoA* AOA | *amoA* AOB | *narG, nirK, nirS* | *napA* | *nosZ*I | *nosZ*II |
|---|---|---|---|---|---|---|---|---|
| Stage 1: 1 cycle | 10 min at 95 ºC | 10 min at 95 ºC | 10 min at 95 ºC | 10 min at 95 ºC | 10 min at 95 ºC | 10 min at 95 ºC | 10 min at 95 ºC | 10 min at 95 ºC |
| Stage 2: 6 cycles with 1 ºC decrease by cycle | | | | | 15s at 95 ºC | 15s at 95 ºC | 15s at 95 ºC | |
| | | | | | 30s at 63 ºC | 30s at 61 ºC | 30s at 65 ºC | |
| | | | | | 30s at 72 ºC | 30s at 72 ºC | 30s at 72 ºC | |
| Stage 3: 35 cycles | 15s at 95 ºC | 30s at 95 ºC | 30s at 95 ºC | 30s at 95 ºC | 15s at 95 ºC | 15s at 95 ºC | 15s at 95 ºC | 30s at 95 ºC |
| | 30s at 60ºC | 30s at 60 ºC | 30s at 65 ºC | 30s at 52 ºC | 30s at 58 ºC | 30s at 56 ºC | 30s at 60 ºC | 60s at 48-63 ºC |
| | 30s at 72 ºC | 30s at 72 ºC | 30s at 72 ºC | 30s at 72 ºC | 30s at 72 ºC | 30s at 72 ºC | 30s at 72 ºC | 60s at 72 ºC |
| Stage 4: 1 cycle | 10 min at 72 ºC | 10 min at 72 ºC | 10 min at 72 ºC | 10 min at 72 ºC | 10 min at 72 ºC | 10 min at 72 ºC | 10 min at 72 ºC | 10 min at 72 ºC |

*Table S2. Total abundance of the 16SB, 16SA, amoA AOB, amoA AOA napA, narG, nirK, nirS, norB, nosZI and nosZII genes in 5 cm subsamples from 20-cm core samplers containing soil not treated (T1) or fertilised with urea (T2), ammonium sulfate (T3) or potassium nitrate (T4). Statistical differences and standard erros are presented in Fig. 1.*

| Soil depth (cm) | Treatment | 16SB | 16SA | *amoA* AOB | *amoA* AOA | *napA* | *narG* | *nirK* | *nirS* | *norB* | *nosZ*I | *nosZ*II |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0-5 | T1 | $3.50\ 10^{8}$ | $7.44\ 10^{6}$ | nd | nd | $1.91\ 10^{3}$ | $1.43\ 10^{3}$ | $7.82\ 10^{3}$ | $4.56\ 10^{3}$ | $4.56\ 10^{3}$ | nd | nd |
| | T2 | $5.46\ 10^{7}$ | $2.45\ 10^{6}$ | $1.54\ 10^{5}$ | $1.32\ 10^{4}$ | $1.02\ 10^{4}$ | $8.85\ 10^{4}$ | $8.89\ 10^{4}$ | $1.25\ 10^{4}$ | $1.56\ 10^{5}$ | $1.12\ 10^{3}$ | $7.69\ 10^{2}$ |
| | T3 | $6.45\ 10^{7}$ | $2.67\ 10^{6}$ | $1.67\ 10^{5}$ | $1.02\ 10^{4}$ | $1.21\ 10^{4}$ | $9.41\ 10^{4}$ | $1.15\ 10^{5}$ | $3.78\ 10^{4}$ | $2.45\ 10^{5}$ | $1.29\ 10^{3}$ | $6.20\ 10^{2}$ |
| | T4 | $3.59\ 10^{7}$ | $2.28\ 10^{6}$ | nd | nd | $3.73\ 10^{5}$ | $1.11\ 10^{5}$ | $1.52\ 10^{5}$ | $3.89\ 10^{4}$ | $4.85\ 10^{5}$ | $8.35\ 10^{3}$ | $1.37\ 10^{3}$ |
| 5-10 | T1 | $3.09\ 10^{8}$ | $7.60\ 10^{6}$ | nd | nd | $1.53\ 10^{3}$ | $1.12\ 10^{3}$ | $1.02\ 10^{4}$ | $3.22\ 10^{3}$ | $6.69\ 10^{3}$ | $1.11\ 10^{3}$ | nd |
| | T2 | $1.89\ 10^{7}$ | $3.11\ 10^{6}$ | $1.45\ 10^{4}$ | $1.99\ 10^{3}$ | $2.96\ 10^{5}$ | $2.65\ 10^{5}$ | $1.56\ 10^{5}$ | $4.56\ 10^{4}$ | $5.56\ 10^{5}$ | $1.81\ 10^{4}$ | $8.25\ 10^{2}$ |
| | T3 | $2.61\ 10^{7}$ | $3.35\ 10^{6}$ | $1.52\ 10^{4}$ | $2.02\ 10^{3}$ | $4.02\ 10^{5}$ | $2.43\ 10^{5}$ | $2.22\ 10^{5}$ | $5.56\ 10^{4}$ | $8.81\ 10^{5}$ | $1.06\ 10^{4}$ | $5.28\ 10^{2}$ |
| | T4 | $1.34\ 10^{7}$ | $2.57\ 10^{6}$ | nd | nd | $6.89\ 10^{5}$ | $3.29\ 10^{5}$ | $1.64\ 10^{5}$ | $6.56\ 10^{4}$ | $1.01\ 10^{6}$ | $6.00\ 10^{4}$ | $1.45\ 10^{3}$ |
| 10-15 | T1 | $2.75\ 10^{8}$ | $8.10\ 10^{6}$ | nd | nd | $8.30\ 10^{3}$ | $4.47\ 10^{3}$ | $4.36\ 10^{4}$ | $1.56\ 10^{3}$ | $1.38\ 10^{4}$ | $1.19\ 10^{3}$ | nd |
| | T2 | $2.52\ 10^{7}$ | $1.57\ 10^{6}$ | $8.75\ 10^{3}$ | $1.56\ 10^{2}$ | $1.45\ 10^{6}$ | $2.12\ 10^{5}$ | $3.69\ 10^{5}$ | $1.12\ 10^{5}$ | $1.69\ 10^{6}$ | $2.20\ 10^{4}$ | $1.20\ 10^{2}$ |
| | T3 | $2.91\ 10^{7}$ | $1.80\ 10^{6}$ | $9.35\ 10^{3}$ | $1.73\ 10^{2}$ | $2.15\ 10^{6}$ | $5.37\ 10^{5}$ | $5.56\ 10^{5}$ | $1.30\ 10^{5}$ | $1.88\ 10^{6}$ | $3.20\ 10^{4}$ | $1.59\ 10^{2}$ |
| | T4 | $1.56\ 10^{7}$ | $2.37\ 10^{6}$ | nd | nd | $2.80\ 10^{6}$ | $6.64\ 10^{5}$ | $8.66\ 10^{5}$ | $3.11\ 10^{5}$ | $2.25\ 10^{6}$ | $9.00\ 10^{4}$ | $3.45\ 10^{3}$ |
| 15-20 | T1 | $2.10\ 10^{8}$ | $8.44\ 10^{6}$ | nd | nd | $1.63\ 10^{4}$ | $5.49\ 10^{3}$ | $2.97\ 10^{4}$ | $4.32\ 10^{3}$ | $2.56\ 10^{4}$ | $3.01\ 10^{3}$ | nd |
| | T2 | $1.88\ 10^{7}$ | $2.55\ 10^{6}$ | nd | nd | $2.12\ 10^{6}$ | $3.56\ 10^{5}$ | $7.71\ 10^{5}$ | $2.21\ 10^{5}$ | $3.56\ 10^{6}$ | $5.05\ 10^{4}$ | $1.60\ 10^{3}$ |
| | T3 | $2.03\ 10^{7}$ | $2.79\ 10^{6}$ | nd | nd | $2.66\ 10^{6}$ | $7.21\ 10^{5}$ | $8.55\ 10^{5}$ | $2.42\ 10^{5}$ | $3.33\ 10^{6}$ | $8.12\ 10^{4}$ | $1.95\ 10^{2}$ |
| | T4 | $1.66\ 10^{7}$ | $3.21\ 10^{6}$ | nd | nd | $3.21\ 10^{6}$ | $8.56\ 10^{5}$ | $1.10\ 10^{6}$ | $4.95\ 10^{5}$ | $5.59\ 10^{6}$ | $9.50\ 10^{4}$ | $5.45\ 10^{3}$ |

*nd: not detected

*Table S3. Relative abundance of the 16SB, 16SA, amoA AOB, amoA AOA, napA, narG, nirK, nirS, norB, nosZI and nosZII genes in 5 cm subsamples from 20-cm core samplers containing soil not treated (T1) or fertilised with urea (T2), ammonium sulfate (T3) or potassium nitrate (T4). Values are expressed in percentage as gene copy number/16S rRNA Bacteria (16SB) or Archaea (16SA). Statistical differences and standard erros are presented in Fig. 2.*

| Soil depth (cm) | Treatment | *amoA* AOB/ 16SB | *amoA* AOA/ 16SA | *napA*/ 16SB | *narG*/ 16SB | *nirK*/ 16SB | *nirS*/ 16SB | *norB*/ 16SB | *nosZ*I/ 16SB | *nosZ*II/ 16SB |
|---|---|---|---|---|---|---|---|---|---|---|
| 0-5 | T1 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | T2 | 0.28 | 0.54 | 0.19 | 0.16 | 0.16 | 0.02 | 0.29 | 0.00 | 0.00 |
| | T3 | 0.26 | 0.38 | 0.19 | 0.15 | 0.18 | 0.06 | 0.38 | 0.00 | 0.00 |
| | T4 | 0.00 | 0.00 | 1.04 | 0.31 | 0.42 | 0.11 | 1.35 | 0.02 | 0.00 |
| 5-10 | T1 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | T2 | 0.08 | 0.06 | 1.57 | 1.40 | 0.83 | 0.24 | 2.94 | 0.05 | 0.00 |
| | T3 | 0.06 | 0.06 | 1.54 | 0.93 | 0.85 | 0.22 | 3.38 | 0.04 | 0.00 |
| | T4 | 0.00 | 0.00 | 3.14 | 2.46 | 1.22 | 0.49 | 4.54 | 0.45 | 0.01 |
| 10-15 | T1 | 0.00 | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.01 | 0.00 | 0.00 |
| | T2 | 0.03 | 0.01 | 3.75 | 0.84 | 1.46 | 0.44 | 4.71 | 0.09 | 0.00 |
| | T3 | 0.03 | 0.01 | 5.39 | 1.85 | 1.91 | 0.45 | 4.46 | 0.11 | 0.01 |
| | T4 | 0.00 | 0.00 | 8.95 | 4.26 | 5.55 | 1.99 | 8.42 | 0.58 | 0.02 |
| 15-20 | T1 | 0.00 | 0.00 | 0.01 | 0.00 | 0.01 | 0.00 | 0.01 | 0.00 | 0.00 |
| | T2 | 0.00 | 0.00 | 5.28 | 1.89 | 4.10 | 1.18 | 6.94 | 0.27 | 0.01 |
| | T3 | 0.00 | 0.00 | 7.10 | 3.55 | 4.21 | 1.19 | 6.40 | 0.39 | 0.01 |
| | T4 | 0.00 | 0.00 | 9.34 | 5.16 | 6.63 | 2.98 | 9.23 | 1.51 | 0.03 |

*nd: no detected

*Table S4. Pearson correlation coefficients between the total abundance of the 16SB, 16SA, amoA AOB, amoA AOA napA, narG, nirK, nirS, norB, nosZI and nosZII genes and the physicochemical properties of the 0-5 cm subsample from 20-cm soil core samplers containing soil not treated (T1) or fertilised with urea (T2), ammonium sulfate (T3) or potassium nitrate (T4). Asterisks denote significant differences ($P < 0.05$); ns, non significant.*

| | DO | pH | $NH_4^+$-N | $NO_3^-$-N | TC | TN | $N_2O$ | $N_2$ |
|---|---|---|---|---|---|---|---|---|
| 16SB | -0.75* | 0.51 ns | -0.80* | -0.52 ns | 0.31 ns | -0.20 ns | -0.80* | 0.49 ns |
| 16SA | 0.85* | -0.14 ns | 0.86* | -0.30 ns | -0.14 ns | 0.16 ns | 0.82* | -0.54 ns |
| *amoA* AOB | 0.89* | -0.19 ns | 0.97* | 0.15 ns | -0.18 ns | 0.15 ns | 0.98* | -0.17 ns |
| *amoA* AOA | 0.91* | -0.08 ns | 0.95* | 0.21 ns | 0.11 ns | 0.19 ns | 0.95* | -0.16 ns |
| *napA* | 0.56 ns | 0.33 ns | 0.35 ns | 0.75* | -0.45 ns | 0.72* | 0.39 ns | 0.59 ns |
| *narG* | 0.51 ns | 0.19 ns | 0.15 ns | 0.79* | -0.39 ns | 0.71* | 0.22 ns | 0.52 ns |
| *nirK* | 0.46 ns | 0.29 ns | 0.22 ns | 0.89* | -0.56 ns | 0.85* | 0.26 ns | 0.56 ns |
| *nirS* | 0.42 ns | 0.35 ns | 0.30 ns | 0.88* | -0.59 ns | 0.82* | 0.29 ns | 0.59 ns |
| *norB* | 0.85* | 0.28 ns | 0.25 ns | 0.73* | -0.56 ns | 0.76* | 0.26 ns | 0.76* |
| *nosZ*I | 0.69 ns | 0.12 ns | 0.52 ns | 0.89* | -0.55 ns | 0.77* | 0.55 ns | 0.74* |
| *nosZ*II | 0.66 ns | 0.21 ns | 0.55 ns | 0.85* | -0.55 ns | 0.74* | 0.51 ns | 0.71* |

DO, dissolved oxygen; TC, total carbon; TN, total nitrogen

***Table S5.*** *Pearson correlation coefficients between the total abundance of 16SB, 16SA, amoA AOB, amoA AOA napA, narG, nirK, nirS, norB, nosZI and nosZII genes and the physicochemical properties of the 5-10 cm subsample from 20-cm soil core samplers containing soil not treated (T1) or fertilised with urea (T2), ammonium sulfate (T3) or potassium nitrate (T4). Asterisks denote significant differences ($P < 0.05$); ns, non significant.*

| | DO | pH | $NH_4^+$-N | $NO_3^-$-N | TC | TN | $N_2O$ | $N_2$ |
|---|---|---|---|---|---|---|---|---|
| 16SB | -0.85* | -0.49 ns | -0.89* | -0.58 ns | 0.31 ns | -0.50 ns | -0.75* | 0.52 ns |
| 16SA | 0.85* | -0.14 ns | 0.92* | -0.47 ns | -0.14 ns | 0.56 ns | 0.79* | -0.34 ns |
| *amoA* AOB | 0.91* | 0.19 ns | 0.97* | 0.55 ns | -0.18 ns | 0.50 ns | 0.85* | -0.25 ns |
| *amoA* AOA | 0.90* | 0.18 ns | 0.95* | 0.51 ns | -0.11 ns | 0.49 ns | 0.82* | -0.24 ns |
| *napA* | 0.38 ns | 0.88* | 0.13 ns | 0.85* | 0.66 ns | 0.90* | 0.76* | 0.72* |
| *narG* | 0.35 ns | 0.85* | 0.15 ns | 0.86* | 0.52 ns | 0.92* | 0.72* | 0.75* |
| *nirK* | 0.32 ns | 0.91* | 0.12 ns | 0.82* | 0.55 ns | 0.86* | 0.56 ns | 0.79* |
| *nirS* | 0.37 ns | 0.89* | 0.25 ns | 0.87* | 0.61 ns | 0.85* | 0.66 ns | 0.80* |
| *norB* | 0.72* | 0.80* | 0.62 ns | 0.82* | 0.53 ns | 0.82* | 0.61 ns | 0.81* |
| *nosZ*I | 0.80* | 0.80* | 0.48 ns | 0.88* | 0.05 ns | 0.75* | 0.59 ns | 0.98* |
| *nosZ*II | 0.80* | 0.92* | 0.55 ns | 0.95* | 0.15 ns | 0.84* | 0.51 ns | 0.75* |

DO, dissolved oxygen; TC, total carbon; TN, total nitrogen

*Table S6. Pearson correlation coefficients between the total abundance of 16SB, 16SA, amoA AOB, amoA AOA napA, narG, nirK, nirS, norB, nosZI and nosZII genes and the physicochemical properties of the 10-15 cm subsample from 20-cm soil core samplers containing soil not treated (T1) or fertilised with urea (T2), ammonium sulfate (T3) or potassium nitrate (T4). Asterisks denote significant differences ($P < 0.05$); ns, non significant.*

| | DO | pH | $NH_4^+$-N | $NO_3^-$-N | TC | TN | $N_2O$ | $N_2$ |
|---|---|---|---|---|---|---|---|---|
| 16SB | 0.61 ns | -0.89* | 0.55 ns | -0.62 ns | -0.61 ns | -0.65 ns | -0.55 ns | -0.75* |
| 16SA | 0.62 ns | -0.34 ns | 0.90* | 0.48 ns | -0.44 ns | -0.56 ns | -0.49 ns | -0.70* |
| *amoA* AOB | 0.55 ns | -0.44 ns | 0.97* | 0.55 ns | -0.48 ns | -0.50 ns | -0.55 ns | -0.75* |
| *amoA* AOA | 0.51 ns | -0.48 ns | 0.95* | 0.51 ns | -0.31 ns | -0.39 ns | -0.52 ns | -0.74* |
| *napA* | -0.81* | 0.77* | -0.34 ns | 0.86* | 0.65 ns | 0.85* | 0.81* | 0.78* |
| *narG* | -0.86* | 0.78* | -0.25 ns | 0.85* | 0.61 ns | 0.87* | 0.82* | 0.79* |
| *nirK* | -0.84* | 0.85* | -0.22 ns | 0.82* | 0.55 ns | 0.78* | 0.79* | 0.88* |
| *nirS* | -0.82* | 0.86* | -0.31 ns | 0.80* | 0.56 ns | 0.80* | 0.77* | 0.86* |
| *norB* | -0.79* | 0.81* | -0.23 ns | 0.92* | 0.62 ns | 0.95* | 0.90* | 0.73* |
| *nosZ*I | -0.75* | 0.85* | -0.52 ns | 0.78* | 0.61 ns | 0.81* | 0.70* | 0.88* |
| *nosZ*II | -0.70* | 0.90* | -0.50 ns | 0.81* | 0.60 ns | 0.90* | 0.72* | 0.92* |

DO, dissolved oxygen; TC, total carbon; TN, total nitrogen

*Table S7. Pearson correlation coefficients between the total abundance of 16SB, 16SA, amoA AOB, amoA AOA napA, narG, nirK, nirS, norB, nosZI and nosZII genes and the physicochemical properties of the 15-20 cm length subsample from 20-cm soil core samplers containing soil not treated (T1) or fertilised with urea (T2), ammonium sulfate (T3) or potassium nitrate (T4). Asterisks denote significant differences ($P < 0.05$); ns, non significant.*

| | DO | pH | $NO_3^-$-N | TC | TN | $N_2O$ | $N_2$ |
|---|---|---|---|---|---|---|---|
| 16SB | 0.88* | -0.58 ns | -0.76* | 0.65 ns | -0.78* | -0.85* | -0.82* |
| 16SA | 0.59 ns | -0.39 ns | -0.78* | 0.84* | -0.76* | -0.79* | -0.77* |
| *napA* | -0.72* | 0.33 ns | 0.66 ns | -0.51 ns | 0.59 ns | 0.64 ns | 0.65 ns |
| *narG* | -0.75* | 0.44 ns | 0.68 ns | -0.54 ns | 0.60 ns | 0.60 ns | 0.64 ns |
| *nirK* | -0.85* | 0.87* | 0.64 ns | -0.50 ns | 0.63 ns | 0.65 ns | 0.62 ns |
| *nirS* | -0.86* | 0.85* | 0.66 ns | -0.54 ns | 0.64 ns | 0.58 ns | 0.61 ns |
| *norB* | -0.89* | 0.77* | 0.78* | -0.66 ns | 0.90* | 0.92* | 0.81* |
| *nosZ*I | -0.85* | 0.56 ns | 0.93* | -0.78* | 0.92* | 0.95* | 0.96* |
| *nosZ*II | -0.82* | 0.52 ns | 0.91* | -0.82* | 0.91* | 0.94* | 0.94* |

DO, dissolved oxygen; TC, total carbon; TN, total nitrogen

*Table S8. Pearson correlation coefficients between the total abundance of 16SB, 16SA, amoA, napA, narG, nirK, nirS, norB, nosZI and nosZII genes and the physicochemical properties of a 20-cm length soil core not treated (T1) or fertilised with urea (T2), ammonium sulfate (T3) or potassium nitrate (T4). Asterisks denote significant differences ($P < 0.05$); ns, non significant.*

| | DO* | pH | $NH_4^+$-N | $NO_3^-$-N | TC | TN | $N_2O$ | $N_2$ |
|---|---|---|---|---|---|---|---|---|
| 16SB | - 0.25 ns | - 0.31 ns | - 0.59 ns | - 0.37 ns | 0.75* | -0.72* | -0.75* | 0.50 ns |
| 16S SA | 0.72* | 0.14 ns | 0.80* | 0.30 ns | -0.14 ns | 0.46 ns | 0.71* | -0.04 ns |
| *amoA* AOB | 0.89* | 0.19 ns | 0.88* | 0.55 ns | -0.18 ns | 0.58 ns | 0.70* | -0.70* |
| *amoA* AOA | 0.85* | 0.08 ns | 0.85* | 0.62 ns | -0.11 ns | 0.60 ns | 0.68* | -0.81* |
| *napA* | - 0.73* | 0.25 ns | - 0.52 ns | 0.55 ns | 0.80* | 0.38 ns | 0.71* | 0.68ns |
| *narG* | - 0.74* | 0.22 ns | - 0.66n s | 0.60 ns | 0.70* | 0.40 ns | 0.74* | 0.66ns |
| *nirK* | - 0.80* | 0.75* | - 0.49 ns | 0.82* | 0.76* | 0.85* | 0.80* | 0.69ns |
| *nirS* | - 0.82* | 0.33 ns | - 0.34 ns | 0.80* | 0.77* | 0.82* | 0.85* | 0.66* |
| *norB* | - 0.88* | 0.15 ns | - 0.40 ns | 0.70* | 0.75* | 0.77* | 0.95* | 0.90* |
| *nosZ*I | - 0.92* | 0.85* | - 0.52 ns | 0.82* | -0.50 ns | 0.78* | 0.89* | 0.96* |
| *nosZ*II | - 0.88* | 0.87* | - 0.56 ns | 0.80* | -0.55 ns | 0.76* | 0.85* | 0.94* |

DO, dissolved oxygen; TC, total carbon; TN, total nitrogen

# Chapter II

## Effect of nitrogen fertilisers on nitrous oxide emission, nitrifiers and denitrifiers abundance and bacterial diversity

**Submitted for publication**

Abstract

Application of inorganic N-fertilisers to agricultural soils increases the emission of the greenhouse gas nitrous oxide ($N_2O$), produces changes in soil physicochemical properties, alters the abundance of nitrifier and denitrifier guilds and modifies the structure and composition of the bacterial communities. Few studies, however, have integrated all these abiotic and biotic variables in a single analysis. In a 3-year study we examined the $N_2O$ emission from an agricultural sandy-loam soil amended with urea, ammonium sulphate or potassium nitrate. Soils were kept under greenhouse conditions and maintained at 70-80% water filled pore space. Soil samples were taken every year to determine a) soil physicochemical properties, b) the total abundance of bacteria and archaea, nitrifier and denitrifier communities, and c) changes in the structure and composition of the bacterial community. Gene abundance and biodiversity were estimated using quantitative PCR and pyrosequencing, respectively. Soils treated with the ammonium-based fertilisers significantly emitted more $N_2O$ than those amended with nitrate. N-fertilisation increased the abundance of bacteria and decreased that of archaea. The treatment with the ammonium-based fertilisers produced yearly increases in the abundance of the nitrification genes. Regardless of the form of the N-fertiliser, the abundance of the denitrification genes gradually increased during the experimental period. Calculation of the ratio between genes involved in $N_2O$ production and reduction decreased on a yearly basis in all three N-fertilised soils, which was related with a concomitant decreased in $N_2O$ emission. A non-metric multidimensional scaling plot showed that $N_2O$ emission was mainly positively related with the abundance of the *norB* gene and negatively with that of the *nosZ* gene. The Shannon diversity index indicated that N-fertilisation reduced the number of OTUs, mainly in the urea-treated soil. After N-fertilisation the bacterial community became less diverse, or dominated by a small group of OTUs as suggested by the Simpson index. Regarding the composition of the bacterial community, the N-fertilisation mainly reduced the number of those OTUs whose relative abundance was lower than 1%, and scarcely affected those with a higher relative abundance.

Keywords: N-fertilisation, nitrous oxide, bacterial diversity, denitrification, qPCR, pyrosequencing

## Introduction

Anthropogenic nitrogen (N) inputs into the earth crust have increased three to five-fold over the past century to meet the need of feeding an increasing world population; however, excessive and repeated load of N is leading to unprecedented increases in nitrate ($NO_3^-$) leaching and production of reactive N (Nr) species, which results in adverse severe environmental and human health impacts (Robertson and Vitousek 2009; Fowler et al. 2013; Erisman et al. 2015; IPCC 2017). Agricultural practices, through the application of nitrogen fertilisers, contribute with around 60% (3.5 Tg N year $^{-1}$) to emissions of nitrous oxide ($N_2O$) (IPCC 2013; Smith 2017), a gas with a global warming potential 300 times higher that of carbon dioxide, also contributing to the ozone layer depletion (Ravishankara et al. 2009; Portmann et al. 2012). During the biogeochemical N cycle, $N_2O$ can be produced by different microbial pathways, but is particularly dominated by nitrification and denitrification (Reay et al. 2012; Butterbach-Bahl et al. 2013; Hu et al. 2015; Hallin et al. 2018). It is generally agreed that nitrification, this is the oxidation of ammonium ($NH_4^+$) to $NO_3^-$ by the enzyme ammonia monooxygenase encoded by the bacterial and archaeal *amoA* gene, is favoured in aerated soils with moisture contents lower than 60% of water filled pore space (WFPS); on the other hand, denitrification, the process by which $NO_3^-$ is sequentially reduced to nitrite ($NO_2^-$), nitric oxide (NO), $N_2O$ and, finally, molecular nitrogen ($N_2$) by the nitrate-, nitrite-, nitric oxide-, and nitrous oxide-reductase enzymes encoded by the *napA*/*narG*, *nirK*/*nirS*, *norB* and *nosZ* genes, respectively (Butterbach-Bahl et al., 2013; Hallin et al. 2018) occurs in wet soils with > 70% WFPS (Bateman and Baggs 2005; Braker and Conrad 2011; Hu et al. 2015).

Soil texture and moisture content, pH, C and N availability and other soil physicochemical properties, as well as the climatic conditions and agricultural management practices, regulate the relative contribution of nitrification and denitrification to $N_2O$ emission (Reay et al. 2012; Butterbach-Bahl et al. 2013; Hu et al. 2015); also, the application of N-fertilisers is recognised as another important factor influencing $N_2O$ emission from soils (Bell et al. 2015). A number of studies have reported the effect of N-fertilisation on microbial biomass or/and community

composition, and this has been compiled in different meta-analyses studies (Geisseler and Scow 2014; Carey et al. 2016; Zhou et al. 2017b; Wang et al. 2018b; Ouyang et al. 2018). The meta-analysis conducted by Zhou et al. (2017b) included 454 field N-addition experiments and the results showed that the effect of fertilisers application on the total microbial abundance varied depending on biome types, methodologies used and N-addition rates. Across a total of 55 published papers, Wang et al. (2018b) found that N-application decreased soil microbial diversity and that changes in their abundance were dependent on the ecosystem analysed, the initial soil properties, the duration of the treatment, the N-addition rates and the changes in soil organic C. Based on 47 field studies, Ouyang et al. (2018) concluded that N-fertilisation significantly increased the *amoA* AOA, *amoA* AOB, *nirK*, *nirS* and *nosZ* genes and that the fertiliser form and duration, crop rotation and soil pH were main factors regulating the response of the N-cycling genes. Results from the 33 studies included in the meta-analysis carried out by Carey et al. (2016) indicated that N-fertilisers increased the abundance of the ammonia oxidising (AO) Bacteria (AOB) and Archaea (AOA), that AOB populations were more dynamic when faced to enhanced N supply, and that responses of AOB varied with the ecosystem, the fertiliser type and the soil pH. Previously, a review by Geisseler and Scow (2014) on 64 long-term trials from around the world revealed that increasing mineral N-fertilisation decreases microbial biomass and that the magnitude of the effect is pH dependent and affected by the duration of the experiment. Based on results from meta-analyses studies, it could be concluded that N-fertilisation induces changes in soil microbial biomass and community composition, albeit the response varies widely in direction and magnitude of change. However, the effect of different forms of N-fertiliser on $N_2O$ emission and on changes in the composition and structure of nitrifiers and denitrifiers communities has not been well explored.

In a 3-year microcosm study we examined $N_2O$ emission from an agricultural sandy-loam soil amended with urea, ammonium sulphate or potassium nitrate. We aimed to address the following questions: a) Does the N-fertiliser type determine $N_2O$ emissions linked to variations in the abundance of nitrification and denitrification genes and what factors are involved in the response of N-fertilisation

to N-genes abundance? b) Does N-fertilisation produce changes in soil structure and composition and what factors are involved in the response of biodiversity to N-fertilisation? The effect of N input on the abundance of the nitrifying and denitrifying guilds and on the bacterial diversity were studied using qPCR and 454-pyrosequencing, respectively. A non-metric multidimensional scaling (NMDS) analysis was also performed to evaluate the relationships between soil characteristics, $N_2O$ emissions, the abundance of the nitrifying and denitrifying communities, and the bacterial diversity.

## Materials and methods

### Soil preparation and soil physicochemical characteristics

A sandy-loam Eutric Cambisol soil of the FAO series (FAO 2017) from an agricultural area (36° 43' 53.5" N, 3° 32' 56.2" W) located in the vicinity of Motril (Granada, Spain) has been used in this study. The soil has a sandy-loam texture (30% clay, 12.5% silt, 57.5% sand, w/w); pH in water 6.8; total C 25 mg $kg^{-1}$; total N 1.02 mg $kg^{-1}$; $NO_3^-$ 6.8 mg $kg^{-1}$; exchangeable $NH_4^+$, not detected) and has been maintained without fertilisation and no irrigation for at least 10 years. Spade-squares (30 x 30 cm to a depth of 25 cm) were taken from 12 locations, freed of roots and plant residues, air dried and pooled together. Then, using a concrete mixer, the soil was independently mixed with either urea ($CON_2H_4$, soil UR), ammonium sulphate [$(NH_4)_2SO_4$, soil AS] or potassium nitrate ($KNO_3$, soil PN) to a final concentration of 260 kg equivalent N $ha^{-1}$ (421.2 mg N $kg^{-1}$ dry soil) as recommended by the Spanish Ministry of Agriculture, Food and Environment for horticultural crops and leguminous plants. A set of pots containing soil without fertilisation was used as a control (soil NT). Fertilised soils were used to fill 20-kg capacity pots (54 x 21 x 25 cm, long, wide and depth, respectively) and placed under greenhouse conditions previously reported (Tortosa et al. 2015) for 3 years (soils NT1-NT3, UR1-UR3, AS1-AS3 and PN1-PN3, respectively). The soils were watered weekly to reach 80% WFPS. The experiment was arranged in a factorial randomised complete block design with four replications for each soil. The content of exchangeable $NH_4^+$ and $NO_3^-$ was determined at the end of each year, and the soil was then supplemented with the corresponding N-fertiliser to reach the initial fertilisation rate.

### Soil analyses

After 1, 2 and 3 years from the setting of the experiment, stainless steel cylindrical core samplers (5 cm × 20 cm) were manually inserted into the soils to take 3 samples from the pots containing the control and each of N-fertilised soils; additionally, 4 samples of the NT soil were also used. The concentration of exchangeable $NH_4^+$-N and $NO_3^-$-N was measured by ionic chromatography (Metrohm) as indicated earlier (González-Martínez et al. 2016). The pH was measured after water extraction (1:5, w/v) for 2 h. Total carbon (TC) and total nitrogen (TN) were determined using a LECO TruSpec CN elemental analyser. WFPS was determined according to Danielson and Sutherland (1986).

### Nitrous oxide emission

$N_2O$ emission was estimated weekly 24 h after watering the pots as reported earlier (Castellano-Hinojosa et al. 2018). Essentially, undisturbed soil samples (50 g) from the core sampler were placed in 125-ml glass bottles, sealed hermetically with rubber septa, and evacuated with pure He ensure $N_2$-free conditions. The soils were kept under greenhouse conditions and $N_2O$ assayed sequentially within times when gas emission was linear using a Hewlett Packard 5890 gas chromatograph equipped with an electron capture detector as previously reported (Tortosa et al. 2011). Concentration of $N_2O$ was calculated using 2% (v/v) $N_2O$ standard (Air Liquide). The cumulative emission was calculated after linear interpolation between sampling points.

### DNA extraction and quantification of 16S rRNA, nitrification and denitrification genes

Total DNA was extracted from 500 mg soil samples as previously indicated (Correa-Galeote et al. 2014; Castellano-Hinojosa et al. 2018) and purified using GeneClean (MP Bio) spin columns. Quality and size of DNA were checked by electrophoresis on 1% agarose and DNA concentration was measured using the Qubit® ssDNA assay kit (Molecular Probes). DNA was stored at -80 ºC until use. The total bacterial (16SB) and archaeal (16SA) community was estimated by qPCR of the corresponding 16S rRNA gene as a molecular markers; the abundance of the nitrifiers was quantified after amplification of the *amoA* gene from Bacteria (*amoA* AOB) and Archaea (*amoA*

AOA) and that of denitrifiers by qPCR of the *napA/narG*, *nirK/nirS*, *norB* and *nosZI/nosZII* genes. Primers and thermal conditions for amplification have been published elsewhere (Correa-Galeote et al. 2014; Castellano-Hinojosa et al. 2018). PCR reactions were carried out in an ABI Prism 7900 Sequence Detection System (Applied Biosystems) and quantification was based on the fluorescence intensity of the SYBR Green dye during amplification. The presence of PCR inhibitors in DNA extracted from soil and the preparation of standard curves were carried out as indicated earlier (Correa-Galeote et al. 2014; Castellano-Hinojosa et al. 2018). PCR efficiency for the different assays ranged between 90 and 99%. As the number of 16S rRNA gene operon per cells is variable (Klappenbach et al. 2001), we did not convert the 16S rRNA gene copy data into cells numbers and we expressed our results as gene copy numbers per nanogram of DNA. Calculation of the gene copy number per nanogram of DNA instead of gram of soil minimised any bias related to soil.

### Bacterial diversity study and pyrosequencing analysis

PCR amplification of the hypervariable V4-V5 regions of the 16S rRNA gene was performed over each individual DNA extraction using universal primers U519F and U926R (Baker et al. 2003) joined to a multiplex identifier sequence (Binladen et al. 2007; Parameswaran et al. 2007). For each DNA sample, amplicons were generated in several replicate PCRs as described earlier (Correa-Galeote et al. 2016). The final PCR product was quantified by Qubit® ssDNA assay kit (Molecular Probes) and visualized by agarose electrophoresis. Samples were combined in equimolar amounts and pyrosequenced in a Roche Genome Sequencer FLX system using 454-Titanium chemistry at Life Sequencing S.L. (Valencia, Spain).

Raw sequences were processed through the Ribosomal Database Project (RDP) pyrosequencing pipeline1 release 11 (Cole et al. 2014). Sequences were trimmed for primers, filtered and assigned to their tags. Sequences shorter than 150 base pair, with quality scores $< 20$ or containing any unresolved nucleotides were removed from the dataset. Chimeras were identified using the Uchime tool from FunGene Database (Edgar et al. 2011) and removed from the dataset. Sequences were aligned using the Infernal alignment tool in RDP (Nawrocki et al. 2009).

Aligned sequences were clustered into operational taxonomic units (OTUs) defined at 97% similarity cutoff using Complete Linkage Clustering RDP tool and their relative abundances calculated.

### Diversity indices, heat maps and cluster analysis

The bacterial OTU richness and Good´s coverage indices were calculated using PAST software (v3.14) (Hammer et al. 2001). Shannon and Simpson indices were calculated using the Vegan package v.2.0 of the statistical software R-Project v.2.15.1. Heat maps were generated including all the bacterial OTUs with at least >1% relative abundance in at least one of the samples. A phylogeny-dependent cluster analysis based on the relative abundance of the bacterial OTUs was conducted in R project using the Bray-Curtis dissimilarity coefficient of the Fast UniFrac software (Zhang et al. 2012). Bacterial OTUs were grouped at cut off of 70% similarity. The cluster analysis was incorporated into the heat maps.

### Statistical analyses

Because of the absence of normality and homoscedasticity in the abiotic (pH, exchangeable $NH_4^+$-N, $NO_3^-$-N, TN, TC and $N_2O$) and biotic variables (total abundance of the 16SA, 16SB, *amoA* AOA, *amoA* AOB, *napA*, *narG*, *nirK*, *nirS*, *norB*, *nosZ*I and *nosZ*II), the Kruskal-Wallis and Conover-Iman tests were chosen for multiple comparisons among samples. A BIO-ENV analysis using Primer software (PRIMER-E v. 6.0, Plymouth, UK) was performed to evaluate the influence of the abiotic on the biotic variables. Firstly, all the variables were standardized by sample-resemblance matrices generated by using the Bray-Curtis similarity coefficient. The abiotic data sets (except pH) were transformed to log (x + 1) and normalized. Correlations between abiotic variables were analysed using Draftsman's plot, and data set with a mutual ratio of more than 0.9 were replaced by a single representative. Next, vectors representing the biotic and abiotic variables related to the samples were overlaid over a non-metric multidimensional scaling (NMDS) plot to illustrate potential monotonic correlations between them. A stress level < 0.2 of the NMDS plots indicates a good fit of the bidimensional representation of the biotic data distribution (Clarke and Warwick 2001). Vectors of very short length (< 0.2) were not displayed on the plots, since they indicate negligible links with the

ordination. The Pearson's product moment correlation coefficients (r) among vectors representing biotic and abiotic variables were calculated. Finally, a BEST analysis was carried out (Clarke and Warwick 2001), designating the subset of abiotic variables that best matched the similarities of the biotic data between samples. The non-parametric analysis ANOSIM (Clarke 1993) in the PRIMER-E v.6.0 software (Plymouth, UK) based on the relative abundance of the bacterial OTUs was used to examine similarities among N treatments and between the N treatments and the control. R values close to 1 indicate dissimilarity between treatments. A stepwise multiple regression analysis in the SPSS software (IBM Corp, USA) was performed to assess the abiotic variables most affecting the bacterial OTU richness.

## Results

### Soil characteristics

Physicochemical properties of the soil used in this study are shown in Table 1. With an initial value of 6.8, the pH in the NT soil remained almost constant (~ 7.0) during the 3-year long experimental time. The addition of urea to the soil increased the pH up to yearly values of 7.8, 7.9 and 8.0, respectively; the amendment with ammonium reached values of 7.4, 7.5 and 7.6, respectively, and the addition of nitrate produced increases up to 7.1, 7.2 and 7.2, respectively. Exchangeable $NH_4^+$-N was not detected in the NT or PN soils. In the soil treated with urea, the exchangeable $NH_4^+$-N content declined from 91.0 to 53.0 and to 26.4 mg N $kg^{-1}$ respectively, and from 80.2 to 39.2 and to 21.0 mg N $kg^{-1}$ in the AS soil after incubation for 1, 2 and 3 years, respectively. The $NO_3^-$-N concentration in the NT soil was 6.9 and 7.1, 6.8 and 6.6 mg N $kg^{-1}$ after 1, 2 and 3 years, respectively. The yearly fertilisation with urea increased the $NO_3^-$-N content to 48.6, 83.5 and to 114.6 mg N $kg^{-1}$, respectively and to 61.5, 92.1 and 92.1 mg N $kg^{-1}$ when the soil was amended with ammonium, respectively; the $NO_3^-$-N content, however, decreased from 300.6 to 255.6 and to 210.6 in the PN soil.

With a TC (%) value of 3.08 in the NT soil, the treatment with urea significantly increased that value up to 3.53, 3.69 and 3.80 for each of the 3 following years, respectively. The amendment with ammonium or nitrate did not result in significant changes in the TC values. The ammonium-based fertilisers increased the TN (%) from 0.04, 0.03 and 0.03 to 0.46, 0.42 and 0.40% in the UR soils, respectively, to 0.49,

0.44 and 0.40% in the AS soils, respectively, and to 0.51, 0.46 and 0.42% for the PN soils, respectively, after each of the 3-year treatment.

### $N_2O$ fluxes and cumulative emissions

$N_2O$ fluxes from the different soils are shown in Fig. 1. The emission reached the maximum values of 6.2 (at day 14), 4.9 (at day 7) and 3.1 (at day 7) kg N $ha^{-1}$ $h^{-1}$ after addition of urea, ammonium and potassium nitrate, respectively; then decreased to reach a basal level on day 60 of 0.4 kg N $ha^{-1}$ $h^{-1}$, which was maintained until the end of the first 1-year incubation time. During that time, the weekly watering of the soils to reach 80% WFPS did not increase $N_2O$ emissions (data not shown). After 1 year, the simultaneous addition to the soil of any of the N-fertiliser and the watering of the pots to reach 80% WFPS increased the $N_2O$ fluxes, albeit the maximum values were lower than those calculated for the first year. Similar patterns of $N_2O$ emissions were found when any of the water-diluted N-fertiliser was applied to the soil. The yearly calculations of cumulative emissions for the 3 year experiment showed that urea, ammonium and nitrate emitted 33.8, 26.3 and 15.9 kg N $ha^{-1}$, respectively (Fig. 1, inset). Fluxes of $N_2O$ were negligible in the NT soil and the cumulative emission of $N_2O$ after 3-year incubation was 1.6 kg N $ha^{-1}$ (Fig. 1, inset)

### Total abundance of nitrifying and denitrifying communities

The total abundance of the 16SA and 16SB, the *amoA* AOB and *amoA* AOA, and of the *napA*, *narG*, *nirK*, *nirS*, *norB*, *nosZ*I and *nosZ*II genes is shown in Table 2. The abundance of bacteria in the control soil remained without significant changes during the 3-year experimental time and significantly decreased from 1.5 x $10^9$ in NT3 to 1.9 x $10^8$, 2.3 x $10^8$ and to 2.1 x $10^8$ gene copy number x $ng^{-1}$ DNA in UR3, AS3 and PN3, respectively. Statistical differences were not found among treatments UR, AS and PN. Regardless of the treatment, bacteria were more abundant than archaea, whose abundance increased during the 3-year incubation period from 2.5 x$10^6$ in NT3 to 1.9 x$10^7$, 2.6 x $10^7$ and 2.9 x$10^7$ gene copy number x $ng^{-1}$ DNA in UR3, AS3 and PN3, respectively. As for bacteria, significant differences due to the type of fertiliser were not detected.

The bacterial *amoA* was more abundant that the archaeal *amoA* gene, and the addition of nitrate did not affect neither the abundance of the *amoA* AOB ($6.0 \times 10^4$ vs. $6.2 \times 10^4$ gene copy number x $ng^{-1}$ DNA in NT3 and PN3, respectively) nor the *amoA* AOA gene ($1.8 \times 10^3$ in NT3 vs. $1.2 \times 10^3$ gene copy number x $ng^{-1}$ DNA in PN3). Ammonium-based fertilisers increased the abundance of the *amoA* AOB gene during incubation to reach $1.2 \times 10^7$ and $9.2 \times 10^6$ gene copy number x $ng^{-1}$ DNA in UR3 and AS3, respectively; the treatment with urea or ammonium also increased the biomass of the *amoA* AOA gene up to $1.1 \times 10^6$ and $1.6 \times 10^6$ gene copy number x $ng^{-1}$ DNA in UR3 and AS3, respectively.

The abundance of the targeted denitrification genes in fertilised soils increased during incubation as compared to the control soil (values expressed as gene copy number x $ng^{-1}$ DNA): from $6.6 \times 10^3$ in NT3 to $4.6 \times 10^5$, $4.4 \times 10^5$ and $8.2 \times 10^5$ for the *napA* gene in UR3, AS3 and PN3, respectively; from $1.6 \times 10^4$ to in NT3 to $2.6 \times 10^6$, $3.9 \times 10^6$ and $7.2 \times 10^6$ for the *narG* gene in UR3, AS3 and PN3, respectively; from $6.9 \times 10^4$ in NT3 to $5.2 \times 10^6$, $5.7 \times 10^6$ and $9.4 \times 10^6$ for the *nirK* gene in UR3, AS3 and PN3, respectively; from $5.0 \times 10^4$ to $2.2 \times 10^5$, $2.4 \times 10^5$ and $5.6 \times 10^6$ for the *nirS* gene in UR3, AS3 and PN3, respectively; from $3.3 \times 10^4$ to $8.7 \times 10^6$, $4.9 \times 10^6$ and $8.9 \times 10^6$ for the *norB* gene in UR3, AS3 and PN3, respectively; from $1.5 \times 10^3$, $6.6 \times 10^5$, $8.7 \times 10^5$ and $9.2 \times 10^6$ for the *nosZ*I gene in UR3, AS3 and PN3, respectively; and from $1.5 \times 10^3$ to $2.8 \times 10^4$, $3.1 \times 10^4$ and $1.4 \times 10^5$ for the *nosZ*II gene in UR3, AS3 and PN3, respectively.

### Linking $N_2O$ emission and nitrification and denitrification gene abundance

An NMDS plot based on Pearson correlation coefficients between the biotic and abiotic variables measured during the 3-year study (supplementary Table S1) is shown in Fig. 2. The NMDS analysis showed that exchangeable $NH_4^+$-N, $NO_3^-$-N and $N_2O$ best explained (82.8%) the changes in the biotic variables. The analysis also revealed that the 16SB and 16SA genes displayed opposite ordination, that the *amoA* AOB and *amoA* AOA genes clustered together and that, except for *nosZII* and *nosZ*I, the remaining denitrification genes also grouped together. The 16SA gene was positively related to exchangeable $NH_4^+$-N ($r = 0.86$) and the 16SB gene with TC ($r = 0.84$). While the *amoA* AOB and *amoA* AOA genes correlated positively with the

exchangeable $NH_4^+$-N content ($r = 0.88$ and $r = 0.93$, respectively) the abundance of the denitrification genes, except the *nosZ*I/*nosZ*II couple, correlated positively with the $NO_3^-$-N content ($r > 0.73$) and TN ($r > 0.71$). The *nirK*, *nirS* and *norB* genes showed a significant positive correlation with $N_2O$ emissions ($r > 0.76$). The Fig. 2 also shows that the UR and AS soil samples clustered together, while those of the NT and PN soils grouped independently.

### Effect of the N-fertilisation on the structure of the bacterial community

A total of 672290 sequences were obtained from the 52 16S rDNA samples sent to pyrosequencing, of which 269.312 were retained after filtering and removing chimeras. The mean number of total retained sequences per library was 9.290, ranging from 7.050 to 1.1615. The average length of the retained sequences was 400 $\pm$ 5 base pair (mean $\pm$ SD). The values of the Good´s coverage index were higher than 86% at 90% confidence interval (Table 3). The number of OTUs was 2.250 in the NT soil and of 2.300, 2.320 and 2.350 in the NT1, NT2 and NT3 soils, respectively. The amendment with any of the N-fertiliser increased the number of OTUs at the end of the first year and gradually decreased during the remaining 2-year incubation. Values were 2.780, 1.880 and 1.380 in UR1, UR2, and UR3, respectively; 2.750, 1.930 and 1.640 in AS1, AS2 and AS3, respectively, and 2.700, 2.030 and 1.720 in PN1, PN2 and PN3, respectively (Table 3). The Shannon index of 2.44 for the OTUs in the NT soil was similar to the index in NT1 (2.48), NT2 (2.47) and NT3 (2.50). Regardless of the fertiliser form, the Shannon index (2.52, 2.50 and 2.49 for UR1, AS1 and PN1, respectively) was similar to that in the NT soils, decreased after incubation for 2 years (2.02, 2.03 and 2.17 for UR2, AS2 and PN2, respectively) and even more after incubation for 3 years (1.61, 1.73 and 1.79 for UR3, AS3 and PN3, respectively). While differences in the Simpson index were not found in the NT soil during incubation (0.6402, 0.6688, 0.6578 and 0.6562 for NT, NT1, NT2 and NT3, respectively), the fertilisation with urea decreased the values of the index to 0.5202, 0.4568 and 0.4518 for UR1, UR2 and UR3, respectively, to 0.5884, 0.4384 and 0.4175 for AS1, AS2 and AS3, respectively, and to 0.5741, 0.4378 and 0.4110 for PN1, PN2 and PN3, respectively (Table 3).

At the family taxonomic level, hierarchical clustering of the 46 bacterial OTUs with relative abundance > 1% showed a clear structuring effect of treatment origin (Table 3, Fig. 3). Taking 0.7 as a benchmark for differentiation, the OTUs clustered in 3 groups, of which the group I contained those from the NT soils, the group II was composed by the OTUs from the UR and AS soils and the group III was made of the OTUs from samples of the PN soil. An ANOSIM analysis showed that differences in the OTUs from the UR ($R = 0.956$), AS ($R = 0.916$) and PN ($R = 0.894$) soils were significantly different from the NT soil (Table S2); the UR and AS soils had a low $R$ value ($R = 0.212$), which suggests that they share a similar OTUs structure (Table S2). The structure of the PN soil fell apart of those in the UR and AS with R values of 0.466 and 0.386, respectively.

A stepwise regression analysis (Table 4) revealed that, after 3-year treatment, the content of the exchangeable $NH_4^+$-N in the UR soils explained 49.2% of the changes in the number of OTUs and 45.6% in the AS soils; also that the content of $NO_3^-$-N was responsible for the 42.3 and 40.9% of the changes in the UR and AS soils, respectively, and that $NO_3^-$-N explained the 84.6% in the PN soils.

### Effect of the N-fertilisation on the composition of the bacterial community

The heat map depicted in Fig. 4 shows the yearly changes in the relative abundance of the OTUs retrieved from the control and N-fertilised soils. In UR1 and AS1 there was an increase > 2% of the relative abundance of the Nocardioidaceae and Chromatiaceae and a decrease > 2% of the Flavobacteriaceae, Chitinophagaceae and Sphingomonadaceae; however, the OTUs Planococaceae and Pseudomonadaceae increased > 2% and Flavobacteriaceae, Gemmatimodaceae and Sphingomonadaceae decreased > 2% in the PN1 soil. After 2-year fertilisation with urea or ammonium the relative abundance of the Bacillaceace, Gemmatimodaceae and Chromatiaceae increased > 5% and that of Flavobacteriaceae, Chitinophagaceae and Planctomycetaceae decreased > 5%. In the PN soil, the abundance of the Planococaceae and Pseudomonadaceae increased > 5% and > 2%, respectively, and decreased the abundance of Flavobacteriaceae > 5% and that of Chitinophagaceae and Anaerolineaceae (> 2%).

Also, there were significant changes in the composition of the bacterial OTUs after treatment of the soil for 3 years. The addition of urea or ammonium increased the relative abundance of the OTUs Trueperaceae and Paenibacillaceae > 5%, that of Bacillaceae 1, Sphingomonadacaeae, Burkholderiaceae, Nitrosomonadaceae, Thiobacilliaceae, Pseudomonadaceae and Chromatiaceae > 2%, while decreased that of Flavobacteriaceae and Chitinophagaceae > 5% and Anaerolineaceae and Plantomycetaceae > 2%. At the end of the third year fertilisation with nitrate, the OTUs Trueperaceae, Alyciclobacillaceae, Bacillaceae 1, Planococaceae, Hyphomicrobiaceae, Comamonadaceae, Burkholderiaceae, Thiobacilliaceae and Pseudomonadaceae increased > 2%, while decreased the abundance of the Flavobacteriaceae and Chitinophagaceae > 5% and Gemmatimonadaceae and Planctomycetaceae > 2%.

Also, a heat map was built to compare the changes in the relative abundance of the OTUs retrieved from N-fertilised soils (Fig. S1). The heat map showed that after 1 year of N-fertilisation the OTUs composition of soils UR, AS and PN was similar; after two and three years, the composition of the soils UR and AS were also similar and clearly different from the PN soil.

## Discussion

Previous studies have shown that N-fertilisers, as well as other biotic and abiotic soil factors, can alter $N_2O$ emission and produce changes in the abundance and diversity of the microbial communities (Butterbach-Bahl et al. 2013; Zhou et al. 2017b; Wang et al. 2018b; Ouyang et al. 2018). Results in this paper extend those findings through an integrated research relating the form of the N-fertiliser with $N_2O$ emission, the abundance of the total bacterial and archaeal communities, the changes produced in the abundance of N-cycling genes and the variations occurred in the diversity of the bacterial communities during a 3-year microcosm study. We used an Eutric Cambisol soil supplemented with urea, ammonium or nitrate. The soil was maintained at WFPS varying weekly from ~70.0 to 80% and kept under greenhouse conditions.

The watering of the soil together with the addition of any of the N-fertilisers produced the highest values of $N_2O$ emission from 7 to 14 days depending on the

fertiliser (Fig. 1), which suggests that the simultaneous presence of nitrate and $O_2$-limiting conditions are required for fully $N_2O$ emission. It is possible that the retard in the occurrence of the $N_2O$ peaks is due to the fact that denitrification involves a series of reduction processes that would delay the emission of the gas in comparison with nitrification (Firestone at al. 1980; Well and Flessa 2009). After three consecutive years, the highest cumulative $N_2O$ emission was emitted by the UR soil, followed by the AS and PN soils (Fig. 1, inset). Nitrification and denitrification can occur in the UR and AS soils because of the presence of ammonium and nitrate, and only denitrification takes place in the PN soil. The contribution of nitrification to $N_2O$ production could explain the highest emissions in the UR and AS soils. Although the WFPS of the soils was above 70%, nitrification under anoxic conditions has been detected (Arnaldos et al. 2013; Liu et al. 2017; Castellano-Hinojosa et al. 2018; Pan et al. 2018). Higher emissions of $N_2O$ from soils treated with nitrate than with ammonium-based fertilisers have been published by other authors, particularly in high organic matter soils (Harty et al. 2016 and references therein).

The abundance of bacteria, estimated as the gene copy number of the 16SB gene, decreased after addition of any of the fertilisers, an effect that was already observed after 1 year treatment, and gradually diminished during the remaining two years (Table 2). Long-term experiments by other authors reported decreases (Hallin et al. 2009; Chan et al. 2013; Ouyang et al. 2018) and increases (Chen et al. 2017; Yang et al. 2017b) in 16SB abundance after addition of N-fertilisers. In contrast to bacteria, the abundance of 16SA increased in all three N-treated soils (Table 2). Similar results were reported in a long-term field experiment by Wang et al. (2018a) who found that increasing doses of urea led to an increase in the abundance of the archaeal community. Inselsbacher et al. (2010) suggested that application of N to agricultural soils enhances microbial competition for nutrients, which, in turn, leads to changes in their communities. The NMDS plot (Fig. 2) shows that bacteria were strongly influenced by the TC content of the soils, while archaea were more sensitive to changes in the $NH_4^+$-N content.

In all soils analysed, the *amoA* AOB was more abundant than the *amoA* AOA gene and yearly increases in their abundance were detected in the UR and AS soils (Table

2). Microcosm studies indicate greater growth and activity of AOB in soils treated with high levels of inorganic ammonium (Jia and Conrad 2009; Pratscher et al. 2011), which together with the given greater sensitivity to ammonia inhibition by AOA (Prosser and Nicol 2012), would result in AOB domination in the soil. This was confirmed by the BIO-ENV analysis which indicated a strong correlation between the abundance of the *amoA* AOA and *amoA* AOB genes and the $NH_4^+$ availability, but that the length of the vector in the NMDS plot corresponding to *amoA* AOB was higher than the *amoA* AOA gene (Fig. 2). In the meta-analysis carried out by Ouyang et al. (2018), inorganic N-fertilisation significantly increased archaeal (31%) and bacterial (313%), *amoA* gene.

Regardless of the form of the N-fertiliser, the total abundance of the denitrification genes gradually increased during the experimental period (Table 2). After the first year, and during the two following years, regardless of the form of the N-fertiliser, *narG* was more abundant than *napA*, *nirK* than *nirS* and *nosZ*I than *nosZ*II. Different denitrification genes have been considered as molecular targets for denitrification (Philippot et al. 2011; Levy-Booth et al. 2014; Hu et al. 2015; Hallin et al. 2018), and clear consensus does not exist. According to the BIO-ENV analysis (Fig. 2), the $N_2O$ emission was mainly related with the abundance of the *norB* gene (Table S1), and this was observed for the three N-fertilisers (Table 2). These results lend support to *norB* as a key gene in studies on $N_2O$ production. Contrary to the *norB* gene, there was a negative relationship between the $N_2O$ emission and the abundance of the *nosZ* gene (Fig. 2), so that the increases in the *nosZ* abundance were associated with concomitant decreases in the $N_2O$ emissions. From data in Table 2, calculation of the ratio between genes involved in $N_2O$ production (*amoA* AOB + *amoA* AOA + *nirK* + *nirS* + *norB*) and reduction (*nosZ*I + *nosZ*II) did not change in the NT soil during incubation and decreased on a yearly basis in all three N-fertilised soils. This could explain the decreases in the maximum and cumulative $N_2O$ emission found during incubation (Fig. 1).

Previous studies have shown that N-fertilisation alters or not the structure and composition of the bacterial community (Ramirez et al. 2010; Zhou et al. 2017b; Wang et al. 2018b). The yearly decreases in the number of OTUs found in this study,

suggest that all the three N-fertilisers affected the structure of the bacterial community (Table 3). The number of OTUs and the Shannon index were similar in the N-fertilised soils during the first two years; in UR soils, however, there was a significant reduction in biodiversity after 3-year treatment as compared with the values found in the AS and PN soils, between which differences were not found (Table 3). That the bacterial community became less diverse, or dominated by a small group of OTUs, after N-fertilisation is also supported by the yearly decreases in the Simpson index (Table 3) and, as indicated by the stepwise multiple regression analysis, by the availability of $NH_4^+$-N and/or $NO_3^-$N content (Table 4). These results agree with previous reports showing decreases in the number of OTUs after N-fertilisation (Wang et al. 2015; Yu et al. 2016; Zeng et al. 2016; Zhou et al. 2017a).

Regarding the composition of the bacterial community, the N-fertilisation mainly reduced the number of the OTUs whose relative abundance was lower than 1% and scarcely affected those with a higher relative abundance (Table 3). The heat maps in Figs. 3 and 4 show, on one hand, that the three fertilisers produced similar decreases in the OTUs with relative abundance higher than 1% and, on the other hand, that, if any, the increases produced in the relative abundance of the OTUs were dependent on the type of the N-fertiliser. It is interesting to mention that among OTUs which showed relative increases in their abundance after N-fertilisation, the families Bacillaceae 1 (Sun et al. 2016), Nitrosomonadaceae (Lourenço et al. 2018), Hyphomicrobiaceae (Martineau et al. 2015), Sphingomodacaeae, Pseudomonadaceae and Burkholderiaceae (Fan et al. 2018) contain members with the capability to produce $N_2O$.

During the 3-year incubation, the bacterial community composition was similar in soils treated with urea or ammonium and clearly differed from that in the soil treated with nitrate, as suggested by the phylogeny-dependent cluster analysis (Fig. 3), the ANOSIM analysis (Table S2) and the heat map in Fig. 4. These results agree with those which show that previously reported after application of urea (Yu et al. 2016; Zhou et al. 2017a) and ammonium nitrate (Zeng et al. 2016).

Taken together, the results on the structure and composition of the bacterial community indicate that N-fertilisation decreases soil biodiversity and that its response depends on the type of the N-fertiliser.

## Acknowledgments

This study was supported by the ERDF-cofinanced grant PEAGR2012–1968 from Consejería de Economía, Innovación y Ciencia (Junta de Andalucía, Spain). Authors also thank Sirena Network (AGL2015-68881-REDT for financial support. ACH is recipient of a grant of MECD (FPU 2014/01633).

*Table 1. Physicochemical properties of a soil not treated (NT) or supplemented with urea (UR), ammonium sulphate (AS) or potassium nitrate (PN) and kept under greenhouse conditions for 3 years (1, 2, 3). For each row, values followed by the same letter are not statistically different according to the Kruskal-Wallis and Conover-Iman tests ($p < 0.05$; $n = 4$). TC, total carbon; TN, total nitrogen; n.d: not detected.*

| | NT | NT1 | NT2 | NT3 | UR1 | UR2 | UR3 | AS1 | AS2 | AS3 | PN1 | PN2 | PN3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pH | 6.8a | 7.0a | 7.0a | 7.0a | 7.8c | 7.9c | 8.0c | 7.4b | 7.5b | 7.6b | 7.1a | 7.2a | 7.2a |
| Exchangeable $NH_4^+$-N (mg N $kg^{-1}$) | n.d | n.d | n.d | n.d | 91.0c | 53.0b | 26.4a | 80.2c | 39.2b | 21.0a | n.d | n.d | n.d |
| $NO_3^-$-N (mg N $kg^{-1}$) | 6.9a | 7.1a | 6.8a | 6.6a | 48.6b | 83.5c | 114.6d | 61.5b | 92.1c | 127.8d | 300.6g | 255.6f | 210.6e |
| TC (%) | 3.08a | 3.11a | 3.15a | 3.06a | 3.53b | 3.69b | 3.80b | 3.11a | 3.13a | 3.19a | 3.07a | 3.11a | 3.16a |
| TN (%) | 0.05a | 0.04a | 0.03a | 0.03a | 0.46b | 0.42b | 0.40b | 0.49b | 0.44b | 0.40b | 0.51b | 0.46b | 0.42b |

*Table 2. Total abundance of the 16SA, 6SSB, amoA AOB, amoA AOA, napA, narG, nirK, nirS, norB, nosZI and nosZII genes in a soil not treated (NT) or fertilised with urea (UR), ammonium sulphate (AS) or potassium nitrate (PN) and kept under greenhouse conditions for 3 years (1, 2, 3). For each column, values followed by the same letter are not statistically different according to the Kruskal-Wallis and Conover-Iman tests ($p < 0.05$; $n = 4$).*

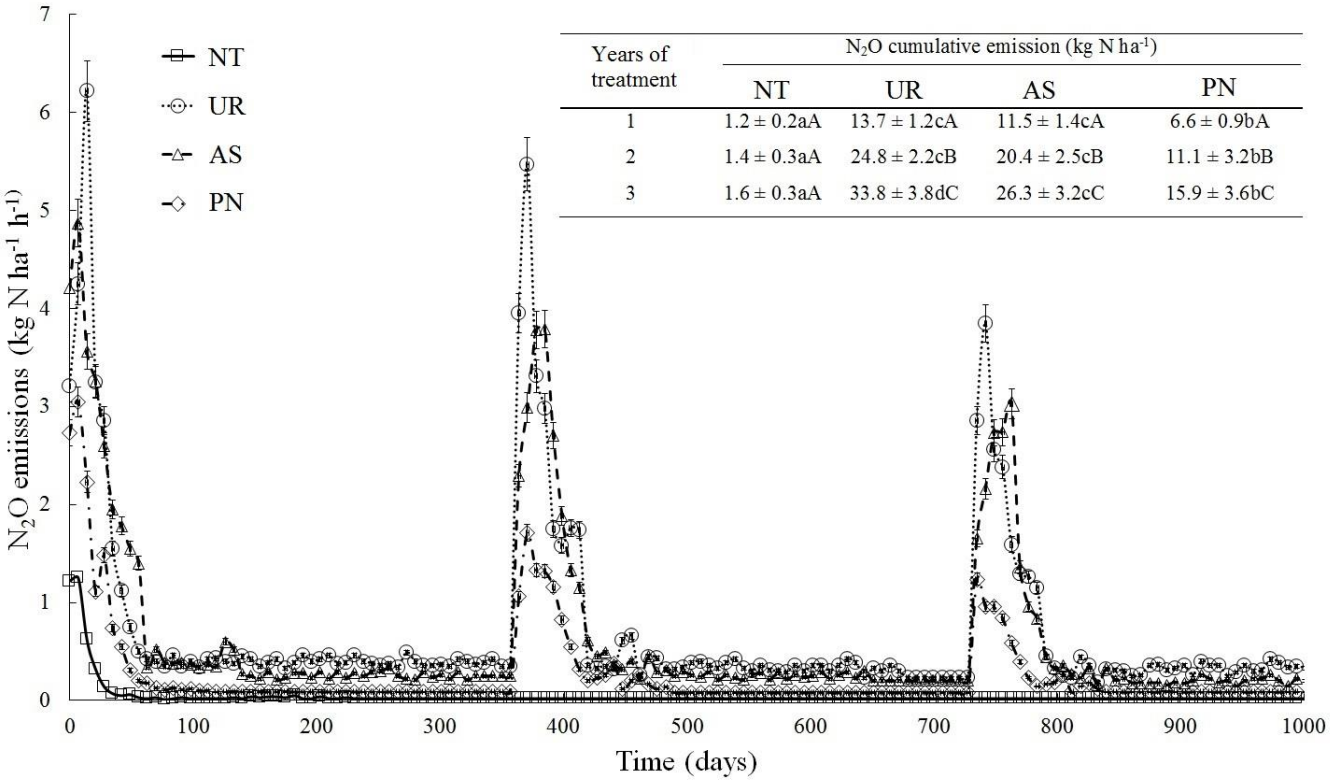
| | Gene copy number x $ng^{-1}$ DNA | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | 16SB | 16SA | *amoA* AOB | *amoA* AOA | *napA* | *narG* | *nirK* | *nirS* | *norB* | *nosZ*I | *nosZ*II |
| NT | $1.0x10^{9}$e | $1.6x10^{6}$a | $5.6x10^{4}$a | $1.2x10^{3}$a | $6.9x10^{3}$a | $1.9x10^{4}$a | $7.1x10^{4}$a | $5.1x10^{4}$a | $3.6x10^{4}$a | $1.2x10^{3}$a | $1.6x10^{3}$a |
| NT1 | $2.2x10^{9}$e | $2.5x10^{6}$a | $6.1x10^{4}$a | $2.0x10^{3}$a | $5.6x10^{3}$a | $1.6x10^{4}$a | $7.4x10^{4}$a | $5.2x10^{4}$a | $3.2x10^{4}$a | $1.1x10^{3}$a | $2.1x10^{3}$a |
| NT2 | $2.1x10^{9}$e | $2.4x10^{6}$a | $5.8x10^{4}$a | $2.1x10^{3}$a | $6.5x10^{3}$a | $1.5x10^{4}$a | $7.5x10^{4}$a | $5.3x10^{4}$a | $3.1x10^{4}$a | $1.2x10^{3}$a | $2.0x10^{3}$a |
| NT3 | $1.5x10^{9}$e | $2.5x10^{6}$a | $6.0x10^{4}$a | $1.8x10^{3}$a | $6.6x10^{3}$a | $1.6x10^{4}$a | $6.9x10^{4}$a | $5.0x10^{4}$a | $3.3x10^{4}$a | $1.5x10^{3}$a | $1.5x10^{3}$a |
| UR1 | $5.9x10^{8}$c | $4.1x10^{6}$b | $4.7x10^{6}$b | $3.2x10^{5}$b | $3.2x10^{5}$b | $1.8x10^{6}$b | $4.1x10^{6}$b | $1.4x10^{5}$b | $7.9x10^{6}$c | $2.3x10^{5}$b | $1.9x10^{4}$b |
| UR2 | $3.4x10^{8}$b | $9.3x10^{6}$c | $6.8x10^{6}$c | $5.8x10^{5}$c | $4.2x10^{5}$b | $2.2x10^{6}$b | $4.6x10^{6}$b | $2.0x10^{5}$b | $8.4x10^{6}$c | $3.7x10^{5}$c | $2.4x10^{4}$b |
| UR3 | $1.9x10^{8}$a | $1.9x10^{7}$d | $1.2x10^{7}$d | $1.1x10^{6}$d | $4.6x10^{5}$b | $2.6x10^{6}$b | $5.2x10^{6}$b | $2.2x10^{5}$b | $8.7x10^{6}$c | $6.6x10^{5}$d | $2.8x10^{4}$b |
| AS1 | $7.6x10^{8}$d | $3.2x10^{6}$b | $5.4x10^{6}$b | $4.1x10^{5}$b | $3.5x10^{5}$b | $2.5x10^{6}$b | $3.5x10^{6}$b | $1.6x10^{5}$b | $3.0x10^{6}$b | $4.0x10^{5}$c | $2.6x10^{4}$b |
| AS2 | $5.9x10^{8}$c | $5.6x10^{6}$c | $7.1x10^{6}$c | $6.4x10^{5}$c | $3.9x10^{5}$c | $3.5x10^{6}$c | $4.9x10^{6}$b | $1.8x10^{5}$b | $4.3x10^{6}$b | $7.2x10^{5}$d | $2.7x10^{4}$b |
| AS3 | $2.3x10^{8}$a | $2.6x10^{7}$d | $9.2x10^{6}$d | $1.6x10^{6}$d | $4.4x10^{5}$c | $3.9x10^{6}$c | $5.7x10^{6}$b | $2.4x10^{5}$b | $4.9x10^{6}$b | $8.7x10^{5}$e | $3.1x10^{4}$b |
| PN1 | $8.9x10^{8}$d | $6.1x10^{6}$c | $5.6x10^{4}$a | $2.0x10^{3}$a | $7.1x10^{5}$d | $6.4x10^{6}$d | $5.4x10^{6}$c | $2.2x10^{6}$b | $7.8x10^{6}$c | $5.1x10^{6}$f | $8.9x10^{4}$c |
| PN2 | $3.5x10^{8}$b | $1.4x10^{7}$d | $5.9x10^{4}$a | $2.2x10^{3}$a | $7.9x10^{5}$d | $7.0x10^{6}$d | $7.2x10^{6}$d | $4.5x10^{6}$c | $8.5x10^{6}$c | $7.2x10^{6}$g | $1.2x10^{5}$d |
| PN3 | $2.1x10^{8}$a | $2.9x10^{7}$e | $6.2x10^{4}$a | $1.2x10^{3}$a | $8.2x10^{5}$d | $7.2x10^{6}$d | $9.4x10^{6}$e | $5.6x10^{6}$d | $8.9x10^{6}$c | $9.2x10^{6}$h | $1.4x10^{5}$e |

*Table 3. Number of OTUs, values of Good's coverage index, Shannon and Simpson biodiversity indices and family numbers with >1 relative abundance in soil samples taken from a soil not treated (NT) or fertilised with urea (UR), ammonium sulphate (AS) or potassium nitrate (PN) and kept under greenhouse conditions for 3 years (1, 2, 3). For each row, values followed by the same letter are not statistically different according to the Kruskal-Wallis and Conover-Iman tests ($p < 0.05$; $n = 4$).*

| Diversity parameters | NT | NT1 | NT2 | NT3 | UR1 | UR2 | UR3 | AS1 | AS2 | AS3 | PN1 | PN2 | PN3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Number of OTUs | 2250d | 2300d | 2320d | 2350d | 2780e | 1880c | 1380a | 2750e | 1930c | 1640b | 2700e | 2030c | 1720b |
| Good´s coverage (%) | 91.1a | 87.8a | 89.2a | 87.2a | 88.2a | 89.2a | 90.2a | 86.2a | 88.5a | 89.5a | 87.9a | 87.8a | 90.2a |
| Shannon | 2.44c | 2.48c | 2.47c | 2.50c | 2.52c | 2.02b | 1.61a | 2.50c | 2.03b | 1.73a | 2.49c | 2.17b | 1.79a |
| Simpson | 0.6402a | 0.6688a | 0.6578a | 0.6562a | 0.5202b | 0.4568b | 0.4518d | 0.5884a | 0.4384 | 0.4175d | 0.5741a | 0.4378b | 0.4110c |
| Families with > 1% relative abundance | 43b | 43b | 43b | 43b | 44b | 37a | 37a | 43b | 37a | 37a | 44b | 38a | 37a |

*Table 4. Multiple stepwise regression analysis between the independent (abiotic) (exchangeable $NH_4^+$-N, $NO_3^-$-N, pH, TC and TN) and dependent (biotic) variables for a soil not treated (NT) or fertilised with urea (UR), ammonium sulphate (AS) or potassium nitrate (PN) and kept under greenhouse conditions for 3 years. Abiotic variables with $P > 0.1$ are not included in the table. β. Standardized regression coefficient; Multiple $R^2$. Coefficient of multiple determination; $R^2$ change. Change in multiple $R^2$ caused by entering a new variable in a single step.*

| Dependent (biotic) variables | Treatment | Independent (abiotic) variables | β | Multiple $R^2$ | $R^2$ change | *P* |
|---|---|---|---|---|---|---|
| Number of OTUs | UR | Exchangeable $NH_4^+$-N | 0.81 | 0.492 | 0.492 | 0.001 |
| | | $NO_3^-$-N | 0.71 | 0.915 | 0.423 | 0.002 |
| | AS | Exchangeable $NH_4^+$-N | 0.61 | 0.456 | 0.456 | 0.001 |
| | | $NO_3^-$-N | 0.59 | 0.865 | 0.409 | 0.002 |
| | PN | $NO_3^-$-N | 0.84 | 0.846 | 0.846 | 0.001 |




| Years of treatment | N2O cumulative emission (kg N ha-1) | | | |
|---|---|---|---|---|
| | NT | UR | AS | PN |
| 1 | 1.2 ± 0.2aA | 13.7 ± 1.2cA | 11.5 ± 1.4cA | 6.6 ± 0.9bA |
| 2 | 1.4 ± 0.3aA | 24.8 ± 2.2cB | 20.4 ± 2.5cB | 11.1 ± 3.2bB |
| 3 | 1.6 ± 0.3aA | 33.8 ± 3.8dC | 26.3 ± 3.2cC | 15.9 ± 3.6bC |

*Fig. 1. $N_2O$ emission fluxes (kg N $ha^{-1}$ $h^{-1}$) by a soil not treated (NT) or fertilised with urea (UR), ammonium sulphate (AS) and potassium nitrate (PN) for 3 years (1, 2, 3). Cumulative $N_2O$ emissions (kg N $ha^{-1}$) are shown in the inset. For each year, values in a row followed by the same lowercase letter are not statistically different among treatments. For each treatment, values in a column followed by the same uppercase letter are not statistically different among years. A Kruskal-Wallis and Conover was done ($p < 0.05$; $n = 4$).*

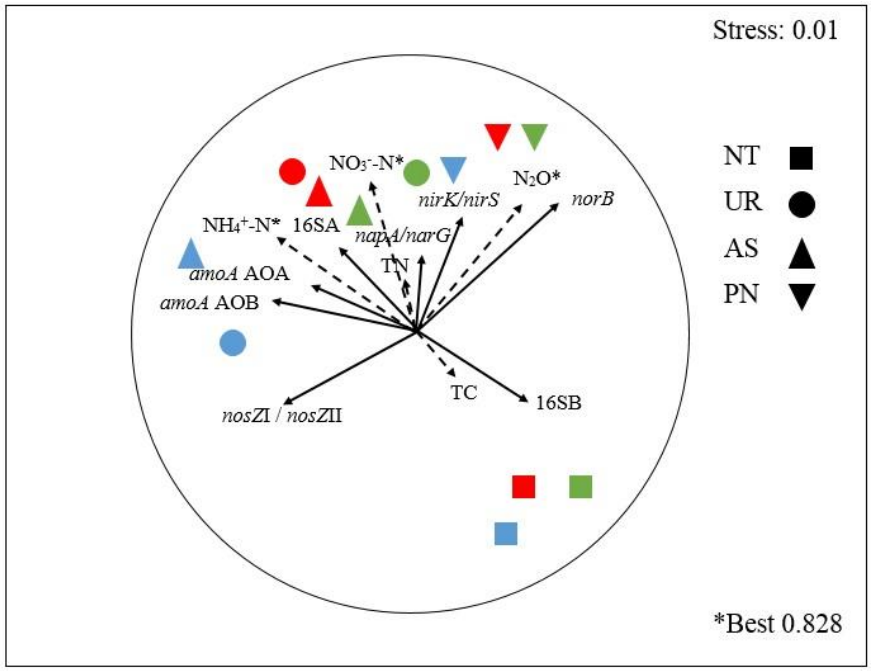



*Fig. 2. Non-metric multidimensional scaling (NMDS) plot illustrating the ordinations between the soil abiotic (pH, exchangeable $NH_4^+$-N, $NO_3^-$-N, TN, TC and $N_2O$) and biotic variables (total abundance of 16SA, 16SB, amoA AOA, amoA AOB, napA, narG, nirK, nirS, norB, nosZI and nosZII genes) retrieved from a soil not treated (NT, □) or fertilised with urea (UR, O), ammonium sulphate (AS, Δ) and potassium nitrate (PN, ∇). Years 1, 2 and 3 are highlighted in blue, red and green colour, respectively. Abiotic and biotic variables are represented by dashed and solid lines, respectively. The variables which best explained the distributions of the biological data according to BIO-ENV analysis are marked with an asterisk (*).*


*Fig. 3. Phylogeny-dependent cluster analysis (above) and heat map (below) at family level built with the OTUs showing relative abundance > 1% in at least one of the samples. OTUs were retrieved from soil samples not treated (NT) or fertilised with urea (UR), ammonium sulphate (AS) or potassium nitrate (PN) for 3 years (1, 2, 3). The benchmark used for clustering group definition is marked with a red line in the phylogenetic tree.*

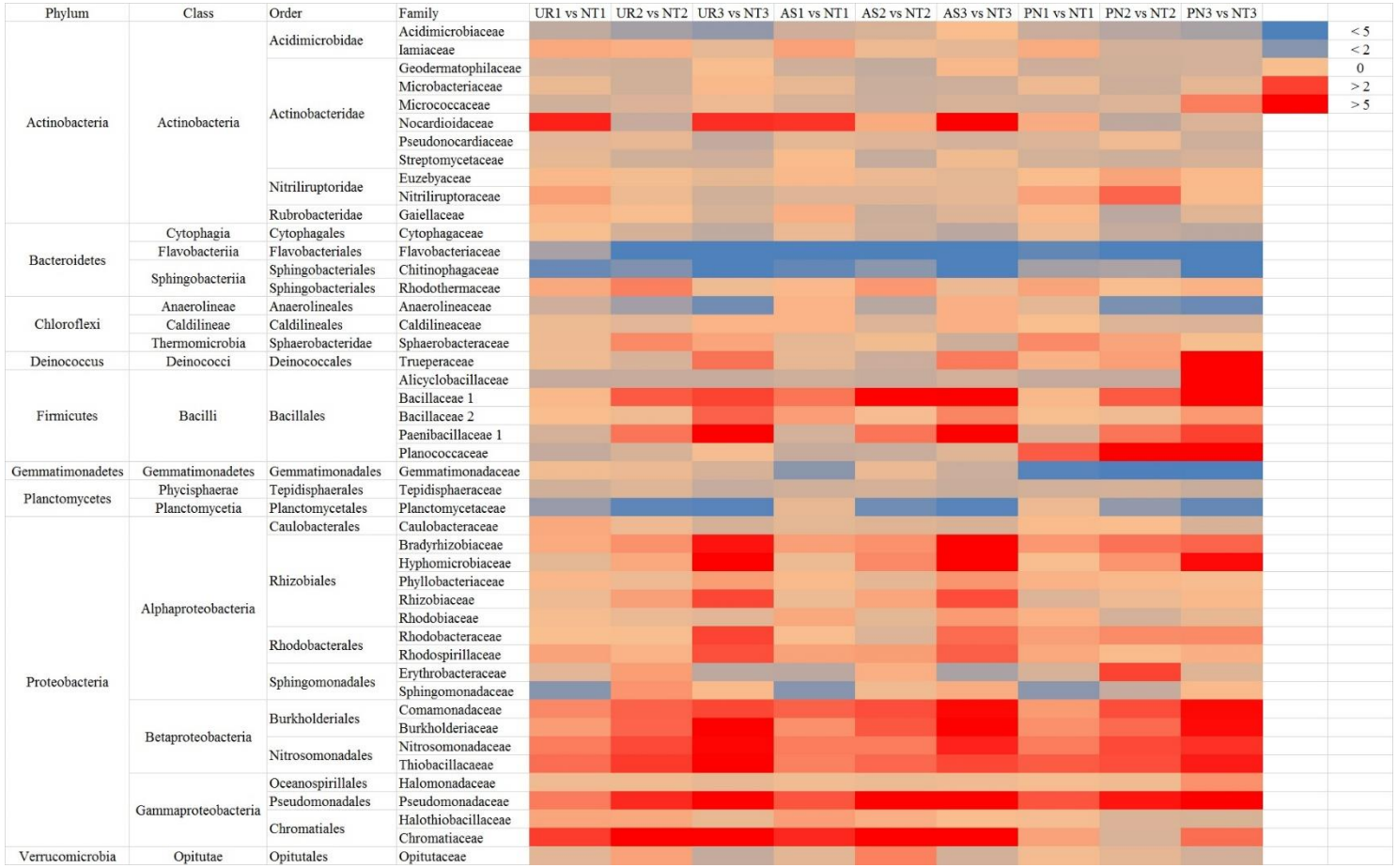



*Fig. 4. Heat map at family level showing changes in the relative abundance of the OTUs retrieved from soil samples not treated (NT) or fertilised with urea (UR), ammonium sulphate (AS) or potassium nitrate (PN) for 3 years (1, 2, 3). Only OTUs with >1% relative abundance in at least one sample are shown.*

# Supplementary material for Chapter II

## Effect of nitrogen fertilisers on nitrous oxide emission, nitrifiers and denitrifiers abundance and bacterial diversity

**Submitted for publication**

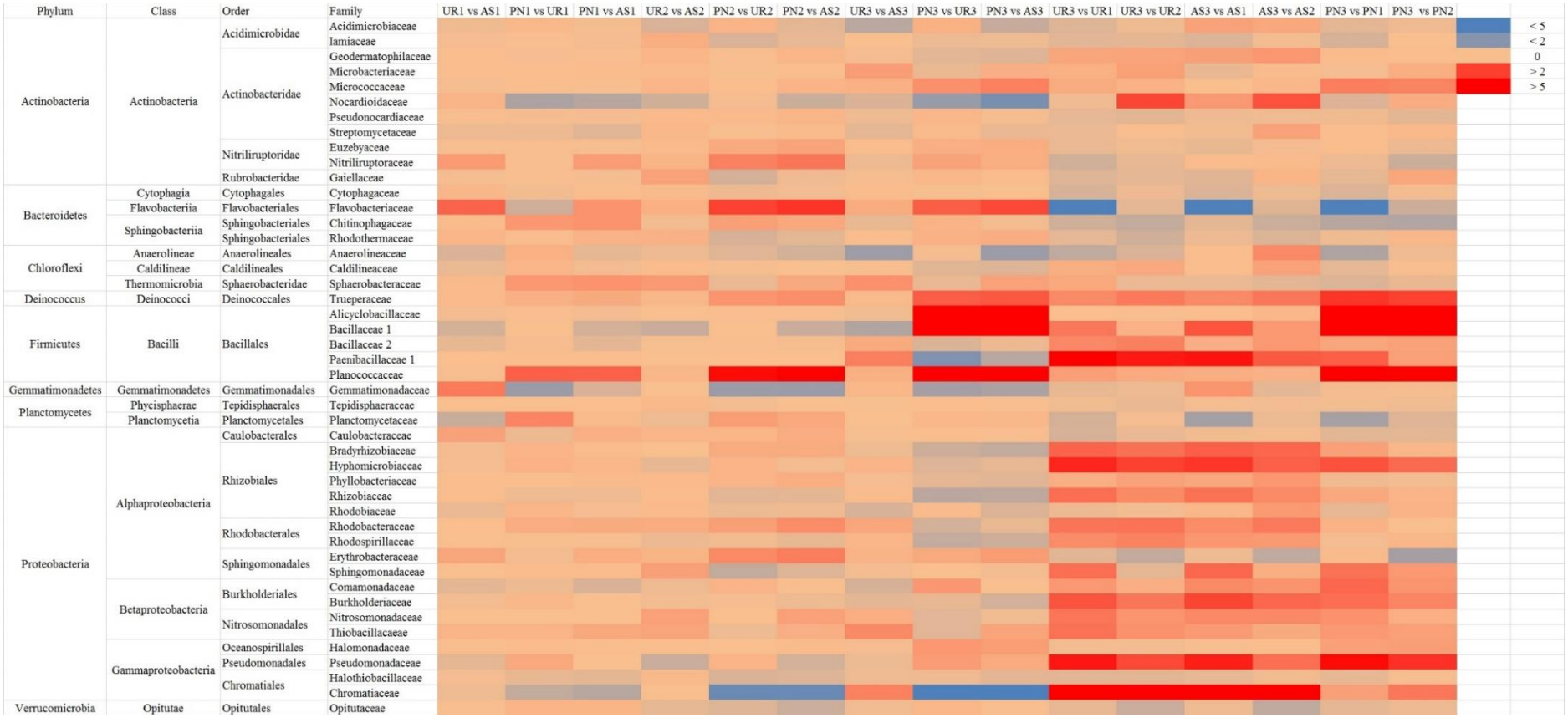
*Table S1. Pearson correlation coefficient between the total abundance of the 16SB, 16SA, amoA AOB, amoA AOA napA, narG, nirK, nirS, norB, nosZI and nosZII genes and the physicochemical properties of a soil not treated (NT) or fertilised with urea (UR), ammonium sulphate (AS) or potassium nitrate (PN) for 3 years (1, 2, 3). Asterisks denote significant differences ($P < 0.05$); ns, not significant. TC, total carbon; TN, total nitrogen.*

| | pH | Exchangeable $NH_4^+$-N | $NO_3^-$-N | TC | TN | $N_2O$ |
|---|---|---|---|---|---|---|
| 16SB | 0.50 ns | -0.80* | -0.52 ns | 0.84* | -0.50 ns | -0.50 ns |
| 16SA | -0.12 ns | 0.86* | 0.30 ns | -0.74* | 0.32 ns | 0.40 ns |
| *amoA* AOB | -0.17 ns | 0.88* | 0.25 ns | -0.58 ns | 0.28 ns | 0.15 ns |
| *amoA* AOA | -0.09 ns | 0.93* | 0.41 ns | -0.51 ns | 0.40 ns | 0.10 ns |
| *napA* | 0.34 ns | 0.35 ns | 0.85* | -0.45 ns | 0.80* | 0.29 ns |
| *narG* | 0.12 ns | 0.15 ns | 0.89* | -0.37 ns | 0.82* | 0.38 ns |
| *nirK* | 0.27 ns | 0.22 ns | 0.79* | -0.38 ns | 0.77* | 0.76* |
| *nirS* | 0.36 ns | 0.30 ns | 0.78* | -0.49 ns | 0.78* | 0.78* |
| *norB* | 0.27 ns | 0.24 ns | 0.73* | -0.46 ns | 0.71* | 0.90* |
| *nosZ*I | 0.15 ns | 0.50 ns | 0.19 ns | 0.35 ns | 0.15 ns | -0.84* |
| *nosZ*II | 0.23 ns | 0.50 ns | 0.15 ns | 0.45 ns | 0.10 ns | -0.82* |

*Table S2. Significance and similarity based on changes in the relative abundance of OTUs in a soil not treated (NT) or fertilised with urea (UR), ammonium sulphate (AS) or potassium nitrate (PN) for 3 years (1, 2, 3) using the non-parametric multivariate ANOSIM statistical method. Numbers in bold indicate significant effect at $p < 0.05$. R values close to 1 indicate dissimilarity between treatments.*

| OTUs | ANOSIM | |
|---|---|---|
| | *R* | *p* |
| UR1 + UR2 + UR3 vs NT1 + NT2 + NT3 | **0.956** | 0.006 |
| AS1 + AS2 + AS3 vs NT1 + NT2 + NT3 | **0.916** | 0.009 |
| PN1 + PN2 + PN3 vs NT1 + NT2 + NT3 | **0.894** | 0.011 |
| UR1 + UR2 + UR3 vs AS1 + AS2 + AS3 | 0.212 | 0.155 |
| UR1 + UR2 + UR3 vs PN1 + PN2 + PN3 | 0.466 | 0.115 |
| AS1 + AS2 + AS3 vs PN1 + PN2 + PN3 | 0.386 | 0.064 |

*Fig. S1. Heat map at family level showing changes in the relative abundance of the OTUs retrieved from soil samples fertilised with urea (UR), ammonium sulphate (AS) or potassium nitrate (PN) for 3 years (1, 2, 3). Only OTUs with >1% relative abundance in at least one sample are shown.*



# Chapter III.1

## Effect of nitrogen fertilisation on nitrous oxide emission and the abundance of microbial nitrifiers and denitrifiers in the bulk and rhizosphere soil of *Solanum lycopersicum* and *Phaseolus vulgaris*

**Submitted for publication**

Abstract

*Aims*

To determine the effect of three N-fertilisers on $N_2O$ emission and abundance of nitrification and denitrification genes in bulk and rhizosphere soil of tomato and common bean, two vegetable crops representative of main horticultural crops in South Spain.

*Methods*

Four consecutive harvests of tomato and common bean fertilised with urea, ammonium or nitrate were carried out under greenhouse conditions. The total abundance of Bacteria, Archaea, nitrifiers and denitrifiers was estimated by quantitative PCR. Soil physicochemical properties and $N_2O$ emission were also determined.

*Results*

Regardless of the plant species, the highest $N_2O$ emission was produced by the soil treated with urea, followed by ammonium and, finally, nitrate. Bacteria were more abundant than Archaea in the bulk and rhizosphere soil. The biomass of the ammonia-oxidising Archaea was greater than the ammonia-oxidising Bacteria in the rhizosphere, but lower in the bulk soil. N-fertilisation increased the biomass of denitrifiers, which were more abundant in the bulk soil.

*Conclusions*

Joint application of high water moisture content and inorganic N-fertiliser is required for maximum $N_2O$ production. Long-term N-fertilisation decreases $N_2O$ production due mainly to increasing abundance of the *nosZ* gene. Nitrification contributes to $N_2O$ production. The abundance of targeted N-cycle genes in bulk and rhizosphere soils is dependent on the type of the fertiliser.

Keywords: nitrogen fertiliser, nitrification, denitrification, nitrous oxide; qPCR

## Introduction

Nitrogen availability is a major worldwide limiting factor for plant growth (Ågren et al. 2012; Gojon 2017) and more than 80 million metric tons of N-fertilisers are applied yearly to increase crop production (Edgerton 2009; Lu and Tian 2017). Agricultural crops retain up to 70% of the applied N (Tilman et al. 2002; Sebilo et al. 2013), the remaining being lost mainly via ammonia volatilisation, nitrate ($NO_3^-$) leaching and N-gas [nitric oxide (NO) and nitrous oxide ($N_2O$)] production, these leading to negative environmental consequences (Galloway et al. 2008; Erisman et al. 2015). Particularly, $N_2O$ is of great concern due to its global warming potential 310 times greater than that of carbon dioxide ($CO_2$) (Ravishankara et al. 2009), and to that it already represents 10-12% of the anthropogenic greenhouse gas emissions (IPCC 2013).

Within the N cycle, the biological processes of nitrification and denitrification are considered to be the predominant sources of $N_2O$ in agricultural soils. Nitrification is the aerobic oxidation of ammonia ($NH_4^+$) to $NO_3^-$ by the enzyme ammonia monooxygenase encoded by the *amoA* gene of the ammonia-oxidising Archaea (AOA) and Bacteria (AOB), respectively. Denitrification is the sequential reduction of $NO_3^-$ to molecular nitrogen ($N_2$) via the formation of nitrite ($NO_2^-$), NO and $N_2O$, by the nitrate-, nitrite, nitric oxide- and nitrous oxide reductase enzymes encoded by the *napA*/*narG*, *nirK*/*nirS*, *norB* and *nosZ* genes, respectively, under $O_2$-limiting conditions (Butterbach-Bahl et al. 2013; Hallin et al. 2018 and references therein).

Root exudates increase N availability in the plant rhizosphere (Coskun et al. 2017; Meier et al. 2017). Plant species, and even cultivars, differ in the types and rates of root exudation and, consequently, have different impacts on the composition and activity of their rhizosphere microbial communities (Rengel and Marschner 2005; Philippot et al. 2009, 2013; Mommer et al. 2016). Previous reports have shown that the emission and evolution of $N_2O$, as well as the abundance of nitrifier and denitrifier communities in the soil, depend upon the N form, the soil moisture and the soil type (Butterbach-Bahl et al. 2013; Hu et al. 2015). A long-term field trial comparing unfertilised cropped and bare soils showed that maize

stimulated nitrification (Enwall et al. 2007) and other studies reported that nitrification is negatively affected in the rhizosphere of *Brachiaria*, *Sorghum*, *Pennisetum*, *Arachis* and *Leymus* (Philippot et al. 2009, 2013; Richardson et al. 2009; Subbarao et al. 2015).

By using quantitative PCR (qPCR), Hai et al. (2009) and Ke et al. (2013) found that regardless of the form and concentration of the N-fertiliser applied, the abundance of AOA was higher than that of AOB in rhizosphere soil from sorghum and rice plants, respectively, but a comparison with bulk soil was not reported. Later, Nie et al. (2014) published that the abundance of AOA and AOB were lower in the rhizosphere than in bulk soil of unfertilised rice; in contrast, Thion et al. (2016) found no differences in the abundance of AOA and AOB in the rhizosphere and bulk soil of 20 unfertilised grassland plants.

The stimulatory effect of plants on denitrification activity has been widely reported in soils and root exudates are considered to be potential determinants for enhanced denitrification activity in the rhizosphere (Philippot et al. 2009, 2013; Richardson et al. 2009; Guyonnet et al. 2017). Interestingly, there are even plants that are able to inhibit bacterial denitrification by secreting phytochemicals from their roots into the soil (Subbarao et al 2015). However, conflicting reports have been published regarding the abundance of the denitrification genes when the plants are treated with N-fertilisers. Bárta et al. (2010) reported that the *nirS* denitrifiers were primarily located in the rhizosphere soil, while the *nirK* were more abundant in the bulk soil of an acidified forest soil. Hamonts et al. (2013) published that the *nirK*, *nirS* and *nosZ* genes were less abundant in the bulk than in the rhizosphere soil of wheat plants treated with $KNO_3$, and Nie et al. (2014) found that the biomass of *nosZ* was lower in the rhizosphere than in the bulk soil of rice. Also, qPCR determination of the *nirK* and *nirS* revealed that gene abundance in the rhizosphere soil of sorghum fertilised with urea was higher than in soil treated with organic fertilisers (Hai et al. 2016).

We hypothesised that inorganic N-fertilisers differentially affect $N_2O$ emission and the abundance of genes involved in the nitrification and denitrification processes in bulk and rhizosphere soil. Here we show the effect of the application of

urea, ammonium sulphate and potassium nitrate to tomato (*Solanum lycopersicum*) and common bean (*Phaseolus vulgaris*) on the abundance of the nitrification (*amoA* AOA and *amoA* AOB) and denitrification (*napA*, *narG*, *nirK*, *nirS*, *norB*, *nosZ*I and *nosZ*II) genes during four consecutive harvests under greenhouse conditions. Urea is the most widely used N-fertiliser all over the world and ammonium nitrate is commonly used in Western Europe (Harty et al. 2016). Tomato and common bean were chosen because of their importance on human consumption, role in sustainable agriculture and economical interest for farmers. As a legume, common beans are also of great interest because of its ability to establish $N_2$-fixing symbioses with soil bacteria best known as rhizobia (Martínez-Romero 2003). The physicochemical properties of the bulk and rhizosphere soil as well as of the $N_2O$ fluxes and cumulative emissions were also recorded during plant growth.

## Materials and methods

### Sampling site, experimental setup and soil preparation

Soil samples were taken from an extensive land agricultural area (36° 43' 53.5" N, 3° 32' 56.2" W) located in the vicinity of Motril (Granada, Spain) where tomato and common bean are routinely cultivated. The soil had been maintained under fallow conditions, without fertilisation and no irrigation, during the last 10 years. It is an Eutric Cambisol (30% clay, 12.5% silt, 57.5% sand; pH in water, 7.1; total carbon, 25.0 mg $kg^{-1}$; total nitrogen, 1.0 mg $kg^{-1}$; $NO_3^-$, 6.8 mg $kg^{-1}$; $NH_4^+$, not detected; $HCO_3^-$, 244.0 mg $kg^{-1}$) of the FAO series (FAO 2017). Spade-squares (30 x 30 cm to a depth of 25 cm) were taken from 12 locations, roots and plant residues removed, air dried and independently mixed in a concrete mixer with either urea ($CON_2H_4$), ammonium sulphate [$(NH_4)_2SO_4$] or potassium nitrate ($KNO_3$) to a final concentration of 260 kg equivalent N $ha^{-1}$ (421.2 mg N $kg^{-1}$ dry soil) as recommended for horticultural crops and leguminous plants by the Spanish Ministry of Agriculture, Food and Environment. The soils were used to fill 20-kg capacity PVC containers (54 x 21 x 25 cm, long, wide and depth, respectively), placed under greenhouse conditions previously described (Tortosa et al. 2015) and watered to reach 80% water-filled pore space (WFPS). After 3 days, half of the containers were sown with tomato (*S. lycopersicum* var. Roma) and the other half

with common bean (*P. vulgaris* var. Kylie). Soil cultivated with tomato or common bean without fertilisation was used as a control. The experiment was arranged in a factorial randomised complete block design with four replications for each soil, accounting for a total of 32 containers (4 soils x 4 replicates x 2 plants). After appearance, the plants were trimmed to 3/container until harvest at 10% fructification about 4 months after sowing. During that time, the soils were watered once a week to reach 80% WFPS. Four consecutive harvests of each plant species were carried out. The concentration of the fertilisers was determined after each harvest and the soil was supplemented with the corresponding N-fertiliser to reach the initial fertilisation rate.

At the end of each harvest, samples of bulk soil were taken by using stainless steel cylindrical core samplers (5 cm × 20 cm) which were manually inserted into the different soils. For the rhizosphere soil, the roots were taken from the plants, the bulk soil removed, and the roots with the remaining adhering soil immersed in tubes containing sterile saline solution. Tubes were shaken in a vortex for 60 s and centrifuged at 6000 rpm for 1 min in a microfuge. The pelleted rhizosphere and the bulk soil were oven dried at 60 ºC for 24 h and used for soil analyses and DNA extraction.

### Soil and plant analyses

Concentrations of exchangeable $NH_4^+$-N and $NO_3^-$-N were determined using an ionic chromatograph (Methohm) equipped with a Metrosep A supp-4-250 anion column and a Metrosep C2-150 cation column as indicated earlier (González-Martínez et al. 2016). Total carbon (TC), total organic carbon (TOC) and total nitrogen (TN) were determined by using a LECO TruSpec CN elemental analyser. The pH was measured after water extraction (1:5, w/v) for 2 h. The WFPS was calculated according to Danielson and Sutherland (1986). Plant dry weight was determined on samples that had been dried at 60 °C for 48 h.

### Nitrous oxide emission

$N_2O$ emissions were measured routinely 24 h after watering the pots as previously published (Castellano-Hinojosa et al. 2018) with minor modifications as acetylene

was not used. Briefly, undisturbed soil samples (4/container) taken with the core sampler mentioned above (5 cm × 20 cm) were placed in 125-ml glass bottles, sealed hermetically with rubber septa, evacuated with pure He to ensure $N_2$-free conditions and incubated under greenhouse conditions. $N_2O$ was assayed sequentially within times when gas emission was linear using a Hewlett Packard 5890 gas chromatograph equipped with an electron capture detector. Concentration of $N_2O$ was calculated using 2% (v/v) $N_2O$ standard (Air Liquide). Cumulative $N_2O$ emissions were calculated by linear interpolation between gas sampling periods. The soil taken from the containers to determine $N_2O$ production was returned to the corresponding container.

### DNA extraction and quantification of nitrification and denitrification genes

Soil DNA was extracted from 0.5 g samples as indicated earlier (Correa-Galeote et al. 2014; Castellano-Hinojosa et al. 2018). After purification using GeneClean (Quiagen) spin columns the DNA quality and concentration were checked by electrophoresis on 1% agarose and using the Qubit® ssDNA assay kit (Molecular Probes), respectively. DNA was stored at - 80 ºC until use. The total bacterial (16SB) and archaeal (16SA) community was quantified using the corresponding 16S rRNA gene as a molecular marker. The size of the nitrifier community was estimated by qPCR of the *amoA* gene from ammonia-oxidising bacteria (*amoA* AOB) and archaea (*amoA* AOA) and that of denitrifiers by qPCR of the *napA*, *narG*, *nirK*, *nirS*, *norB*, *nosZ* clade I (*nosZ*I) and *nosZ* clade II (*nosZ*II) genes using primers and thermal conditions described earlier (Correa-Galeote et al. 2014; Castellano-Hinojosa et al. 2018). Assays for qPCR were carried out using a Bio-Rad iCycler iQ5 Thermocycler (Bio-Rad Laboratories, USA) with SYBR Green as the detection system. The presence of PCR inhibitors in DNA extracted from soil was estimated as reported earlier (Correa-Galeote et al. 2014; Castellano-Hinojosa et al. 2018). The quality of all qPCR amplifications was verified by electrophoresis in agarose and by melting curve analysis. The presence of PCR inhibitors in DNA extracted from soil was estimated by (1) diluting soil DNA extract and (2) mixing a known amount of standard DNA to soil DNA extract prior to qPCR. In all cases, inhibition was not detected. PCR efficiency for the different assays ranged between 90 and 99%.

### Statistical analyses

The abiotic variables (pH, $NH_4^+$-N, $NO_3^-$-N, TOC, TN, TC and $N_2O$) were first explored using the Shapiro-Wilk test and the Bartlett's test to check whether they meet the normality and homoscedasticity assumptions, respectively. Subsequently, Kruskal-Wallis and Conover-Iman tests were chosen to search for significant differences in gene abundance between a) bulk and rhizosphere soil and b) among harvests. The same tests were used to search for differences in the cumulative $N_2O$ emission among treatments. A 95% significance level ($P < 0.05$) was selected. Stepwise multiple regression analyses were performed to assess the abiotic variables (except $N_2O$) that significantly affected the abundance of the total 16S rRNA (16SA + 16SB genes), nitrification (*amoA* AOA + *amoA* AOB genes) and denitrification (*narG* + *napA* + *nirK* + *nirS* + *norB* + *nosZ*I + *nosZ*II genes) communities in bulk and rhizosphere soil of tomato and common bean plants. The probability criteria of $P < 0.05$ to accept and $P > 0.1$ to remove an abiotic variable of the analysis were applied. All abiotic data sets (except pH) were transformed to log (x+1) to normalise the distributions.

## Results

### Properties of the rhizosphere and bulk soil

Main physicochemical properties of the bulk and rhizosphere soil from tomato and common bean plants after 4 consecutive harvests are presented in Table 1. The physicochemical data of the soils after each harvest are presented in supplementary Tables S1 and S2. After the 4$^{th}$ harvest, urea significantly increased the pH of the bulk and rhizosphere soil or both plant species, while ammonium and nitrate produced lower increases as compared to the control soil. Also, the content of $NH_4^+$-N, $NO_3^-$-N and TN were lower in the rhizosphere than in the bulk of the three N-fertilised soils. The TOC and TC values, however, were significantly higher in the rhizosphere than in the bulk soil of the tomato and common bean plants. Regardless of the plant species and number of harvests, the dry weight of the plants was significantly higher in the soils treated with nitrate, followed by those amended with urea or ammonium and finally the control soil (Table S3).

### $N_2O$ fluxes and cumulative emissions

The $N_2O$ fluxes from soil cultivated with tomato for the first time increased during 2 weeks to reach the maximum values of 5.8, 4.1 and 1.2 nmol $N_2O$ g dry soil$^{-1}$ h$^{-1}$ after addition of urea, ammonium or nitrate, respectively (Fig. 1a). Then, $N_2O$ emission gradually decreased to a basal level of 0.4 nmol $N_2O$ g dry soil$^{-1}$ h$^{-1}$ after approximately 150 days. In soils cultivated with common bean, the $N_2O$ fluxes also peaked after about 2 weeks, albeit the maximum values were 7.4, 5.1 and 1.5 nmol $N_2O$ g dry soil$^{-1}$ h$^{-1}$ after addition of urea, ammonium or nitrate, respectively (Fig. 1b). Regardless of the plant species, the $N_2O$ emissions during the second, third and fourth harvest showed profiles that were similar to that of the first harvest (Figs 1a an 1b, respectively). It is to note that the values of the maximum emission peaks diminished one harvest after another of each tomato and common bean.

After 4 consecutive harvests, calculations of cumulative $N_2O$ emission showed that tomato-cultivated soil supplemented with urea, ammonium or nitrate emitted 54.9, 41.3 and 29.5 nmol $N_2O$ g dry soil$^{-1}$, respectively (Fig. 1a, inset) and that, for the same treatments, the soil cultivated with common bean produced 59.7, 50.1 and 36.5 nmol $N_2O$ g dry soil$^{-1}$, respectively (Fig. 1b, inset). Cumulative emissions of $N_2O$ during each consecutive harvest are shown in supplementary Table S4. Fluxes of $N_2O$ produced by control soil cultivated with tomato or common bean were low (Fig. 1a and 1b), though cumulative emissions after the 4$^{th}$ harvest were significant, 1.8 (Fig. 1a, inset) and 2.2 nmol $N_2O$ g dry soil$^{-1}$ (Fig. 1b, inset), respectively. After the 4 consecutive harvests, cumulative $N_2O$ emissions produced by soils cultivated with common bean treated with urea, ammonium and nitrate were 8.7, 21.3 and 23.7% higher than those emitted by tomato, respectively (Fig. 1a and 1b, insets; Table S3).

### Total abundance of the bacterial and archaeal communities

Data on the total abundance of the 16SA and 16SB genes corresponding to tomato (Fig. 2a) and common bean (Fig. 2b) were similar for each of the 4 consecutive harvests. Here, we present the results corresponding to the 4$^{th}$ harvest. For each plant species, the abundance of the bacterial and archaeal communities increased after N-fertilisation. The copy number of the 16SA gene in bulk and rhizosphere soil of tomato plants increased from 6.5 and 7.1 in the control soil to 7.4 and 8.0, 7.4 and

8.1, and to 7.2 and 7.9 log gene copy number x $g^{-1}$ dry soil after fertilisation with urea, ammonium or nitrate, respectively (Fig. 2a). For the 16SB gene, the copy number changed from 8.2 and 8.8 in the control soil to 9.0 and 9.7, 8.9 and 9.5, and to 8.9 and 9.6 log gene copy number x $g^{-1}$ dry soil after fertilisation with urea, ammonium or nitrate, respectively (Fig. 2a).

In bulk and rhizosphere soil of common bean, the biomass of the 16SA gene increased from 7.0 and 7.5 to 7.8 and 8.3, 7.6 and 8.4, and to 7.5 and 8.3 log gene copy number x $g^{-1}$ dry soil after fertilisation with urea, ammonium or nitrate, respectively (Fig. 2b). The calculated increases for the 16SB gene were from 8.8 and 9.1 to 9.3 and 9.8, 9.2 and 9.8, and to 9.1 and 9.9 log gene copy number x $g^{-1}$ dry soil after fertilisation with urea, ammonium or nitrate, respectively (Fig. 2b). A stepwise multiple regression analysis (Table 2) showed that the variance of the 16SA + 16SB gene abundance in soils cultivated with tomato and common bean was explained mainly by TC in the bulk soil (42-51%) and by TOC in the rhizosphere soil (46-56%).

### Total abundance of the nitrifying communities

Like the data on the 16SA and 16SB genes, here we present the results corresponding to the 4$^{th}$ harvest. It is of note, however, that while the abundance of the *amoA* AOB gene did not significantly change during the 4 consecutive harvests, the abundance of the *amoA* AOA gradually increased harvest after harvest (Fig. 3). Urea and ammonium increased the *amoA* AOA and *amoA* AOB gene copy number in tomato (Fig. 3a) and common bean (Fig. 3b) bulk and rhizosphere soils, and nitrate did not change the biomass of the *amoA* genes. When cultivated with tomato, the biomass of the *amoA* AOA gene varied from 4.9 and 5.9 in control soil to 6.5 and 7.5, 5.8 and 7.6, and to 5.1 and 6.1 log gene copy number x $g^{-1}$ dry soil after fertilisation with urea, ammonium or nitrate, respectively. The abundance of the *amoA* AOB gene ranged from 5.2 and 4.7 in control soil to 6.5 and 5.9, 6.2 and 6.0, and to 5.4 and 4.9 log gene copy number x $g^{-1}$ dry soil after amendment with urea, ammonium or nitrate, respectively (Fig. 3a). A similar pattern was found for common bean as the abundance of the *amoA* AOA in bulk and rhizosphere soil varied from 5.6 and 6.6 in the control soil to 7.2 and 8.3, 6.5 and 8.3, and to 5.8 and 6.8 log gene copy number x $g^{-1}$ dry soil fertilised with urea, ammonium and nitrate, respectively (Fig. 3b). Also,

the biomass of the *amoA* AOB gene changed from 6.0 and 5.4 in control soil to 7.3 and 6.5, 6.7 and 6.6, and to 6.2 and 5.5 log gene copy number x $g^{-1}$ dry soil treated with urea, ammonium or nitrate, respectively (Fig. 3b).

Regardless of the plant species, the *amoA* AOA gene was more abundant than the *amoA* AOB in the rhizosphere soil (Fig. 3a) and the biomass of the *amoA* AOA was lower than that of the *amoA* AOB in the bulk soil (Fig. 3b). A stepwise multiple regression analysis revealed that changes in the *amoA* AOA + *amoA* AOB genes were mainly explained by the $NH_4^+$-N (42-56%) and the TN (25-30%) content in the bulk soil and that pH (44-54%) and $NH_4^+$-N (31-33%) controlled the abundance of nitrifiers in the rhizosphere soil.

### Total abundance of the denitrifying communities

Similar to the total abundance of the 16SA, 16SB, *amoA* AOA and *amoA* AOB genes, here we present the data on the biomass of the denitrifying communities estimated for the 4th harvest. The addition of any of the N-fertilisers increased the abundance of the *napA*, *narG*, *nirK, nirS*, *norB* and *nosZ*I and *nosZ*II genes in the bulk and rhizosphere soil of tomato (Fig. 4a) and common bean (Fig. 4b). In bulk control soil cultivated with tomato, the abundance of the *napA* + *narG*, *nirK* + *nirS*, *norB* and *nosZ*I + *nosZ*II genes was 6.0, 6.6, 6.1 and 5.8 log gene copy number x $g^{-1}$ dry soil, respectively, and 4.9, 5.2, 5.0 and 5.1 log gene copy number x $g^{-1}$ dry soil in the rhizosphere control soil, respectively (Fig. 4a). In the bulk soil, considering the 3 fertilisers together, the biomass of the *napA* + *narG* ranged from 6.8 to 7.0, the *nirK* + *nirS* from 6.9 to 7.5, the *norB* from 6.6 to 7.2, and the *nosZ*I + *nosZ*II from 7.0 to 7.8 log gene copy number x $g^{-1}$ dry soil. For the rhizosphere soil, the abundance of the *napA* + *narG*, *nirK* + *nirS*, *norB* and *nosZ*I + *nosZ*II genes varied between 5.6 to 5.8, 5.7 to 6.6, 5.6 to 6.3, and 5.4 to 6.0 log gene copy number x $g^{-1}$ dry soil, respectively (Fig. 4a).

The gene copy number of the *napA* + *narG*, *nirK* + *nirS*, *norB* and *nosZ*I + *nosZ*II genes in the bulk and rhizosphere control soil of common bean was 6.3 and 5.9, 6.7 and 6.1, 6.3 and 5.8, and 6.3 and 5.7 log gene copy number x $g^{-1}$ dry soil, respectively (Fig. 4b). After N-fertilisation, the abundance of the *napA* + *narG*, *nirK* + *nirS*, *norB* and *nosZ*I + *nosZ*II genes in bulk soil varied between 7.2 and 7.4, 7.7 and 8.3, 7.4 and

8.1, and 7.3 and 8.1 log gene copy number x $g^{-1}$ dry soil, respectively (Fig. 4b). In the rhizosphere soil, the abundance of the genes ranged between 6.4 and 6.5, 6.9 and 7.7, 6.4 and 7.5, and between 6.4 and 7.4 log gene copy number x $g^{-1}$ dry soil, respectively (Fig. 4b). A stepwise multiple regression analysis revealed that changes in the abundance of the denitrification genes were controlled mainly by the content of $NO_3^-$-N in the bulk (37-50%) and the rhizosphere (51-59%) soil (Table 2).

## Discussion

### Properties of the bulk and rhizosphere soils

In this study we examined the physicochemical properties, $N_2O$ emissions and abundance of nitrifiers and denitrifiers in bulk and rhizosphere soil of two vegetable crops in response to the amendment with urea, ammonium and nitrate during 4 consecutive harvests. The amendment with urea increased the pH of the bulk soil (Table 1) compared to the remaining treatments, an effect most likely due to soil alkalinisation by urea hydrolysis (Sigurdarson et al. 2018). The pH of the control soil and of those fertilised with nitrate, ammonium or urea was lower in the rhizosphere than in the bulk soil (Tables 2a and 2b). Root exudation and respiration contribute to the decrease of the pH in the plant rhizosphere either by the release of hydrogen ions during ammonia oxidation or through exchange of $NH_4^+$ for $H^+$ during plant N uptake (Richardson et al. 2009).

Under the conditions used in this study, the $NH_4^+$-N content was higher in the bulk than in the rhizosphere soil (Table 1), this most likely due to ammonium consumption for plant growth as reported earlier (Philippot et al. 2009, 2013; Richardson et al. 2009). In addition to $NH_4^+$ taken by the plants, ammonium oxidation by nitrification and nitrate reduction by denitrification also contribute to its decrease in soils (Butterbach-Bahl et al. 2013; Philippot et al. 2013). As for the $NH_4^+$, the lower $NO_3^-$-N content in the rhizosphere soil of tomato and common bean (Table 1) is most probably due to its uptake by plants (de Vries et al. 2015; de Vries and Bardgett 2016). Albeit to a lesser extent, denitrification could contribute to the decrease of the $NO_3^-$-N content in rhizosphere soils (Philippot et al. 2009; Giles et al. 2012).

After 4 consecutive harvests, the values of TOC and TC were lower in the bulk than in rhizosphere soil of tomato and common bean, an effect that has been associated to the release of plant exudates (Richardson et al. 2009; Philippot et al. 2009). Because the plants were removed from the containers after each harvest, incorporation of organic C from the plant debris to the soil, if any, was negligible.

### $N_2O$ fluxes and cumulative emissions

Regardless of the number of the harvest, the maximum values of $N_2O$ emissions by the soil cultivated with tomato or common bean was reached after the combined addition of water (up to 80% WFPS) with any of the N-fertilisers (Figs. 1a and 1b). This indicates that both the existence of high moisture content, leading to $O_2$-limiting conditions, and the presence of nitrate, or a nitrogen oxide derived from it, are required to achieve maximal denitrification activity. Previously, other authors have shown similar results (Butterbach-Bahl et al. 2013; Hu et al. 2015, and references therein). Although the temporal profiles of the $N_2O$ emission were maintained during the 4 consecutive harvests, the maximum values of activity decreased one harvest after another (Figs. 1a and 1b) (see below).

The weekly watering of the soil without N-fertilisation did not produce further increases of the $N_2O$ fluxes during plant growth (Figs. 1a and 1b). However, when the soil was amended with any of the water-containing N-fertiliser a gradual increase in the $N_2O$ emission was observed and again reached a maximum after about 2 weeks (data not shown). These results show that high moisture conditions alone did not stimulate $N_2O$ emissions by soils already containing a N-source and that induction of denitrification activity, measured as $N_2O$ production, was achieved only after simultaneous watering to obtain a high moisture content (~ 70-80% WFPS) and amendment with newly added fertiliser.

The $N_2O$ fluxes were higher in soils containing urea and ammonium than in those supplemented with nitrate (Figs. 1a and 1b), which suggests that nitrification also contributed to $N_2O$ production. These results agree with those previously published which show that $N_2O$ emission by nitrifiers occurs under $O_2$-limiting conditions, thus contributing to the increase of $N_2O$ fluxes (Arnaldos et al. 2013; Liu et al. 2017; Castellano-Hinojosa et al. 2018; Pan et al. 2018).

Under the conditions used in this study, urea was the N-fertiliser with the highest potential for the release of $N_2O$ into the atmosphere, followed by ammonium and then nitrate. Nevertheless, other authors have reported that $N_2O$ emissions in soils amended with nitrate were higher than those treated with ammonium-based fertilisers, particularly under WFPS > 70% (Harty et al. 2016 and references therein). The cumulative $N_2O$ produced by the soils cultivated with common bean was higher than that emitted by tomato (Figs. 1a and 1b, insets). These differences could be due not only to variations in N uptake and assimilation, as reported for other plant species (Richardson et al. 2009; Philippot et al. 2009), but also to changes in the abundance of the genes or activity of the enzymes involved in $N_2O$ production and reduction (see below). Moreover, some plants are able to reduce ammonia volatilisation by secreting urease inhibitors so that more nitrogen is available for plant uptake (Subbarao et al. 2015). The production of urease inhibitors by tomato and common bean has not been reported.

### Abundance of the total community and of the nitrifier and denitrifier guilds

Fertilisation with urea, ammonium or nitrate increased the biomass of the total community as estimated by quantification of the bacterial 16SB and the archaeal 16SA genes (Fig. 2). The data also show that members of Bacteria were more abundant than those of Archaea in the bulk and rhizosphere fractions of unfertilised and N-fertilised soils (Fig. 2). Other authors also found that the 16SB gene in bulk and rhizosphere soil was more abundant than the 16SA gene in unfertilised (Nie e tal. 2014) and urea-fertilised paddy soils (Zhai et al. 2018). As indicated by the stepwise multiple regression analysis (Table 2), the increases in the 16S gene abundance in soils cultivated with tomato and common bean were explained mainly by TC in the bulk and by TOC in the rhizosphere soil. This not surprising as it is expected that organic C from the root exudates enhances growth of the microbial populations, especially those inhabiting the rhizosphere soil (Giles et al. 2012; Coskun et al. 2017; Meier et al. 2017).

Regardless of the plant species, the *amoA* AOA gene was more abundant than the *amoA* AOB in the rhizosphere soil (Fig. 3a) and, on the contrary, the biomass of the *amoA* AOA was lower than the *amoA* AOB in the bulk soil (Fig. 3b). Exudates

from the plant rhizosphere stimulate growth of both AOA and AOB, but the former are considered to prefer lower ammonia concentration (Prosser and Nicol 2012), so that ammonia uptake by plants may favour AOA. After determination of the AOA and AOB *amoA* gene abundances in the rhizosphere and bulk soil of 20 grassland plants, Thion et al. (2016) also found that AOA were more abundant in the rhizosphere. The *amoA* AOA also dominated the *amoA* AOB gene in the rhizosphere of sorghum (Hai et al. 2009) and rice (Hussain et al. 2011; Ke et al. 2013) treated with ammonium-based fertilisers. Other authors, however, have reported that *amoA* AOB was more abundant than *amoA* AOA in the rhizosphere soil from sorghum (Hai et al. 2009), barley (Glaser et al. 2010), common floating aquatic macrophytes (Wei et al. 2011; Trias et al. 2012) plants. Although many studies have demonstrated the ability of certain plant roots to produce and release nitrification inhibitors that suppress soil-nitrifier activity (Subbarao et al. 2015), there are no reported studies showing this effect in tomatoes and common bean plants.

With the exception of *napA* and *narG*, the remaining denitrification genes were more abundant in the bulk and rhizosphere fractions of the soil treated with nitrate than with urea or ammonium, and the genes in the bulk dominated over those in the rhizosphere soil (Fig. 4). After application of urea to the soil, Hai et al. (2009) found that the biomass of the *nirK* and *nirS* genes increased in the rhizosphere soil of sorghum. Our results also agree with those by Hussain et al. (2011) who found higher abundance of the *nirK* gene in the rhizosphere than in the bulk soil of urea-treated rice, and with those by Nie et al. (2014) who reported that the abundance of the *nosZ*I gene in the rhizosphere soil was lower than in the bulk soil of unfertilised rice. The finding that the denitrification genes were more abundant in the bulk than in the rhizosphere soil (Fig. 4) may be explained if one considers that the lower $NO_3^-$-N content in the rhizosphere of tomato and common bean (Table 1) may result in a strong competition for N, which, in turn, would affect growth of denitrifiers.

The abundance of the *nosZ*I + *nosZ*II gene pair gradually increased with the number of harvests both in the bulk and rhizosphere soil (Fig. 4). The *nosZ* genes code for the synthesis of nitrous oxide reductase, the enzyme involved in the reduction of $N_2O$ to $N_2$. The increments in the abundance of the *nosZ* genes might

explain the decreases in the maximum values of the $N_2O$ emission found at the beginning of each consecutive harvest (Fig. 1). Because *nosZ* was significantly more abundant in the nitrate-treated soil (Fig. 4), the lowest $N_2O$ emission in that soil could be also associated to greater reduction of $N_2O$ to $N_2$. Accordingly, the highest $N_2O$ emission in soils treated with urea or ammonium cannot be fully adscribed to nitrification but also to a lower potential of $N_2O$ reduction (Fig. 4).

From data in Table S5c, regardless of the treatment, after four consecutive harvests, calculation of the ratio between genes involved in $N_2O$ production (*amoA* AOB + *amoA* AOA + *nirK* + *nirS* + *norB*) and reduction (*nosZ*I + *nosZ*II) showed no differences among plants. These results suggest that differences in cumulative $N_2O$ emission from soils cultivated with common bean or tomato (Fig. 1, insets; Table S4) cannot be adscribed to variations in the abundance of the genes. Whether or not these differences could be due to changes in denitrification activity in the soils cannot be elicited from the present results

For tomato and common bean, nitrate was the fertiliser producing the lowest $N_2O$ emission and the highest plant dry weight. Nitrate increased dry weight of tomato and common bean and induced expression of $N_2O$-reducers leading to lower $N_2O$ emission. Maybe the higher price of nitrate compared to other N-fertilisers may prevent its use in agricultural practices. It is interesting to note that despite tomato and common bean are phylogenetically unrelated plants, they had a similar effect on the abundance of nitrifier and denitrifier guilds in bulk and rhizosphere soil. This suggests that fertilisation surpasses the plant in driving the variations in the N-cycling gene abundance.

## Acknowledgments

This study was supported by the ERDF-cofinanced grant PEAGR2012-1968 from Consejería de Economía, Innovación y Ciencia (Junta de Andalucía, Spain) and the MINECO-CSIC Agreement RECUPERA 2020. ACH is the recipient of a grant of MECD (FPU 2014/01633).

*Table 1. Physicochemical properties of the bulk and rhizosphere soil of tomato **(a)** and common bean **(b)** after 4 consecutive harvests. Soil samples were taken at the end of the harvest. The soil was fertilised with urea (U), ammonium sulphate (A) or potassium nitrate (K). Unfertilised soil was used as a control (C). For each row, values followed by the same letter are not statistically different according to the Kruskal-Wallis and Conover-Iman tests ($P < 0.05$; $n = 4$). TOC, total organic C; TC, total C; TN, total N.*

**a.**

| | Tomato | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Bulk soil | | | | Rhizosphere soil | | | |
| | C | U | A | K | C | U | A | K |
| pH | 7.1b | 8.3d | 7.4c | 7.4c | 6.7a | 7.6c | 7.2b | 7.0b |
| $NH_4^+$ (mg N $kg^{-1}$ dry soil) | 3.5a | 29.5e | 23.2d | 7.9b | 2.4a | 20.7d | 15.5c | 5.3b |
| $NO_3^-$ (mg N $kg^{-1}$ dry soil) | 2.7a | 23.8c | 26.0c | 32.6d | 1.8a | 16.2b | 18.7b | 22.1c |
| TOC (mg $kg^{-1}$ dry soil) | 1.8a | 3.0b | 2.8b | 2.8b | 2.2a | 3.6c | 3.4c | 3.4c |
| TC (%) | 2.2a | 3.8b | 3.4b | 3.5b | 2.8a | 4.8c | 4.3c | 4.4c |
| TN (%) | 0.1a | 0.4c | 0.4c | 0.4c | 0.1a | 0.2b | 0.2b | 0.3b |

**b.**

| Common bean | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Bulk soil | | | | Rhizosphere soil | | | |
| | C | U | A | K | C | U | A | K |
| pH | 6.8c | 8.0d | 7.1c | 7.1c | 6.0a | 7.0c | 6.3b | 6.3b |
| $NH_4^+$ (mg N $kg^{-1}$ dry soil) | 3.9a | 33.5e | 26.0d | 8.7b | 3.1a | 26.4d | 21.6c | 6.9b |
| $NO_3^-$ (mg N $kg^{-1}$ dry soil) | 2.9a | 28.5c | 29.2c | 38.7d | 2.4a | 21.2b | 24.8b | 29.2c |
| TOC (mg $kg^{-1}$ dry soil) | 1.5a | 2.5b | 2.4b | 2.4b | 2.1b | 3.5c | 3.3c | 3.3c |
| TC (%) | 1.9a | 3.2c | 2.9c | 3.0c | 2.3b | 4.0d | 3.6d | 3.7d |
| TN (%) | 0.1a | 0.4c | 0.4c | 0.4c | 0.1a | 0.3b | 0.3b | 0.3b |

*Table 2. Multiple stepwise regression analysis between biotic dependent, including the total (16SA + 16SB), nitrification (amoA AOA + amoA AOB) and denitrification (narG + napA + nirK +nirS + norB + nosZI +nosZII) genes and the abiotic independent (pH, $NH_4^+$-N, $NO_3^-$-N, TOC, TC and TN) variables determined in the bulk and rhizosphere soil of tomato and common bean. Only independent variables with a significant effect are included ($P < 0.05$). $R^2$ change, change in multiple $R^2$ caused by entering a new variable in a single step; P indicates the significant effect on the considered variable.*

| Plant specie | Type of soil | Dependent variable | Independent variable | $R^2$ change | *P* | Plant specie | Type of soil | Dependent variable | Independent variable | $R^2$ change | *P* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Tomato | Bulk | Total 16S rRNA | TC | 0.51 | 0.005 | Common bean | Bulk | Total 16S rRNA | TC | 0.42 | 0.012 |
| | | Nitrification genes | $NH_4^+$ | 0.42 | 0.004 | | | Nitrification genes | $NH_4^+$ | 0.56 | 0.003 |
| | | | TN | 0.25 | 0.005 | | | | TN | 0.30 | 0.019 |
| | | Denitrification genes | $NO_3^-$ | 0.37 | 0.002 | | | Denitrification genes | $NO_3^-$ | 0.50 | 0.002 |
| | | | pH | 0.31 | 0.008 | | | | pH | 0.29 | 0.011 |
| | Rhizosphere | Total 16S rRNA | TOC | 0.56 | 0.004 | | Rhizosphere | Total 16S rRNA | TOC | 0.46 | 0.005 |
| | | Nitrification genes | pH | 0.44 | 0.005 | | | Nitrification genes | pH | 0.54 | 0.003 |
| | | | $NH_4^+$ | 0.33 | 0.012 | | | | $NH_4^+$ | 0.31 | 0.020 |
| | | Denitrification genes | $NO_3^-$ | 0.51 | 0.002 | | | Denitrification genes | $NO_3^-$ | 0.59 | 0.003 |
| | | | TN | 0.22 | 0.010 | | | | TN | 0.29 | 0.023 |

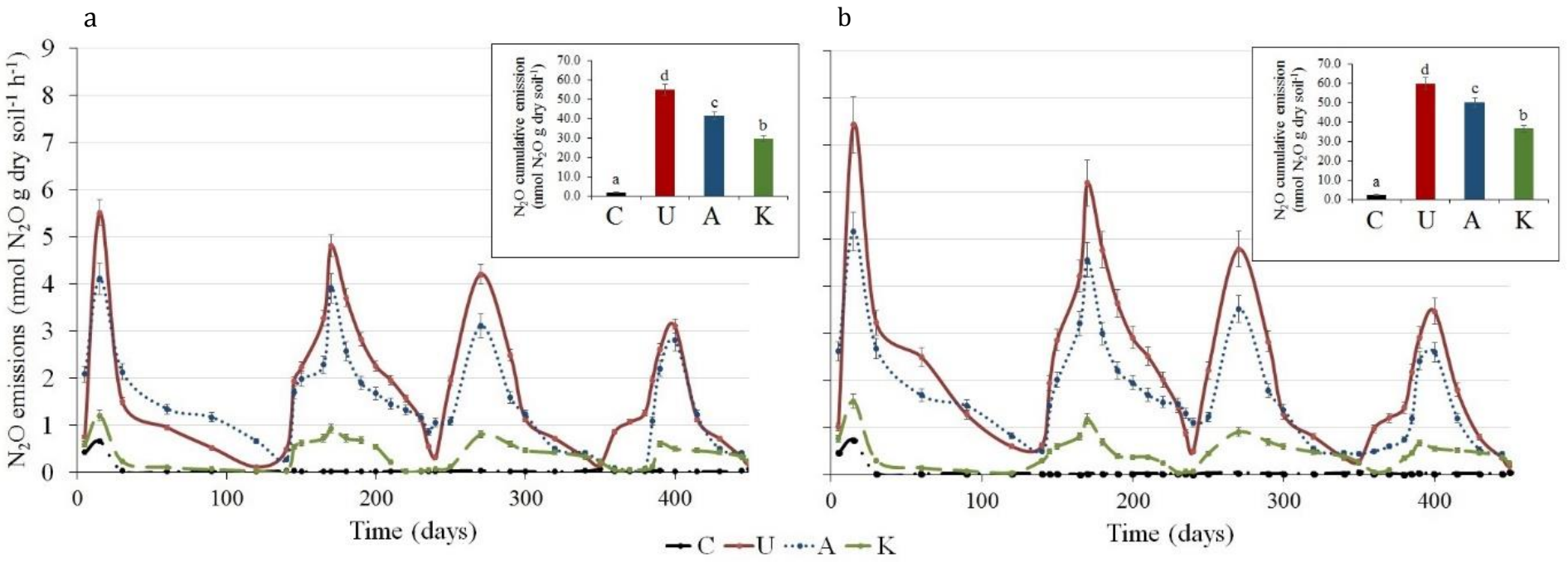



*Fig. 1. Nitrous oxide production by a soil cultivated with tomato **(a)** or common bean **(b)** during 4 consecutive harvests. The soil was fertilised with urea (U), ammonium sulphate (A) or potassium nitrate (K). Unfertilised soil was used as a control (C). Values are expressed as nmol $N_2O$ g dry $soil^{-1}$. The insets show the $N_2O$ cumulative emission after 4 consecutive harvests where, for each treatment, bars with the same letter are not statistically different according to the Kruskal-Wallis and Conover-Iman tests ($P < 0.05$). Error bars represent standard errors ($n = 4$).*

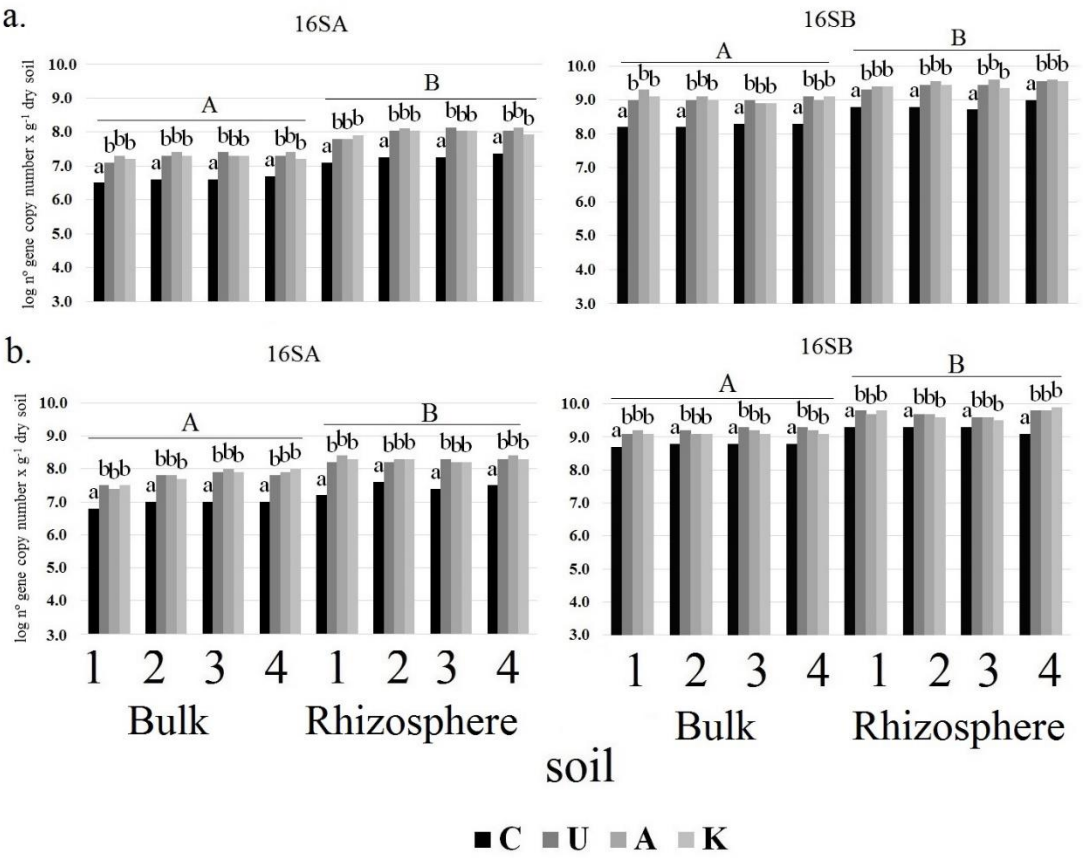



*Fig. 2. Total abundance of the 16SA and 16SB genes in the bulk and rhizosphere soil of tomato (a) and common bean (b) during 4 consecutive harvests. The soil was fertilised with urea (U), ammonium sulphate (A) or potassium nitrate (K). Unfertilised soil was used as a control (C). Values are expressed as log gene copy number x $g^{-1}$ dry soil. In each bulk and rhizosphere soil, rectangles with the same lowercase letter are not statistically different among harvests. Horizontal lines with the same uppercase letter are not statistically different between bulk and rhizosphere soils. A Kruskal-Wallis and Conover-Iman tests was done ($P < 0.05$; $n = 4$).*

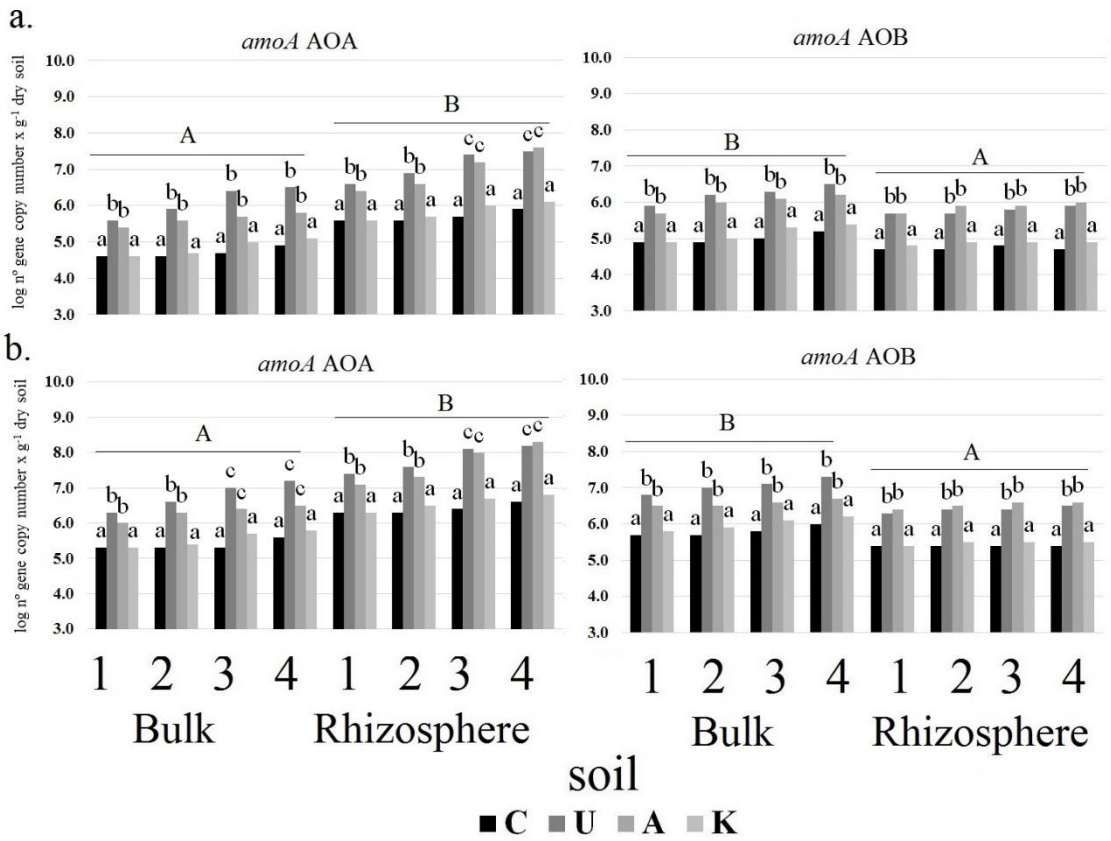



*Fig. 3. Total abundance of the amoA AOA and amoA AOB genes in the bulk and rhizosphere soil of tomato **(a)** and common bean **(b)** during 4 consecutive harvests. The soil was fertilised with urea (U), ammonium sulphate (A) or potassium nitrate (K). Unfertilised soil was used as a control (C). Values are expressed as log gene copy number x $g^{-1}$ dry soil. In each bulk and rhizosphere soil, rectangles with the same lowercase letter are not statistically different among harvests. Horizontal lines with the same uppercase letter are not statistically different between bulk and rhizosphere soils. A Kruskal-Wallis and Conover-Iman tests was done ($P < 0.05$; $n = 4$).*

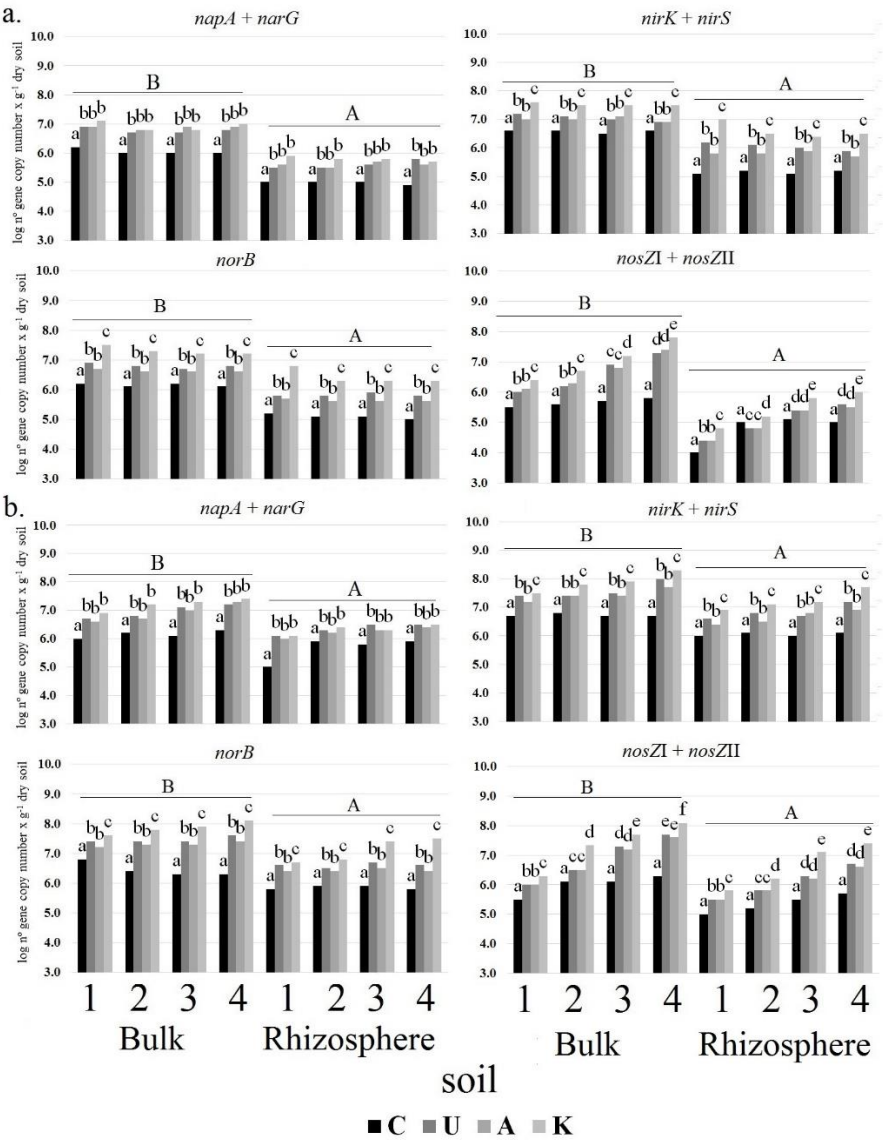



*Fig. 4. Total abundance of the napA + narG, nirK + nirS, norB and nosZI + nosZII genes in the bulk and rhizosphere soil of tomato* ***(a)*** *and common bean* ***(b)*** *during 4 consecutive harvests. The soil was fertilised with urea (U), ammonium sulphate (A) or potassium nitrate (K). Unfertilised soil was used as a control (C). Values are expressed as log gene copy number x g$^{-1}$ dry soil. In each bulk and rhizosphere soil, rectangles with the same lowercase letter are not statistically different among harvests. Horizontal lines with the same uppercase letter are not statistically different between bulk and rhizosphere soils. A Kruskal-Wallis and Conover-Iman tests was done ($P < 0.05$; $n = 4$).*

## Supplementary data for Chapter III.1

# Effect of nitrogen fertilisation on nitrous oxide emission and the abundance of microbial nitrifiers and denitrifiers in the bulk and rhizosphere soil of *Solanum lycopersicum* and *Phaseolus vulgaris*

**Submitted for publication**

*Table S1. Physicochemical properties of bulk soil of tomato **(a)** and common bean **(b)**. Four harvests were made. Soil samples were taken at the end of each harvest. The soil was fertilised with urea (U), ammonium sulphate (A) or potassium nitrate (K). Unfertilised soil was used as a control (C). For each row, values followed by the same letter are not statistically different according to the Kruskal-Wallis and Conover-Iman tests ($P < 0.05$; $n = 4$). TOC, total organic C; TC, total C; TN, total N.*

**a.**

| | Tomato | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C1 | C2 | C3 | C4 | U1 | U2 | U3 | U4 | A1 | A2 | A3 | A4 | K1 | K2 | K3 | K4 |
| pH | 7.1a | 7.0a | 7.2a | 7.1a | 7.9c | 8.1c | 8.2c | 8.3c | 7.4b | 7.3b | 7.5b | 7.4b | 7.5b | 7.6b | 7.4b | 7.4b |
| $NH_4^+$ (mg N $kg^{-1}$) | 1.6a | 2.5a | 3.0a | 3.5a | 42.0f | 38.4e | 34.5e | 29.5d | 35.8e | 27.1d | 26.9c | 23.2c | 3.4a | 5.4b | 5.9b | 7.9b |
| $NO_3^-$ (mg N $kg^{-1}$) | 3.0a | 3.4a | 2.5a | 2.7a | 14.5b | 18.4c | 20.2c | 23.8d | 13.6b | 20.7c | 25.2d | 26.0d | 45.9f | 40.3f | 34.4e | 30.6e |
| TOC (mg $kg^{-1}$) | 1.4a | 1.5a | 1.6a | 1.8a | 2.1b | 2.2b | 2.5c | 3.0c | 2.2b | 2.5c | 2.6c | 2.8c | 2.3b | 2.5c | 2.8c | 2.8c |
| TC (%) | 1.9a | 2.1a | 2.1a | 2.2a | 2.5b | 2.7b | 3.5c | 3.8c | 2.8b | 3.1b | 3.3c | 3.4c | 2.9b | 3.2b | 3.2b | 3.5c |
| TN (%) | 0.05a | 0.09a | 0.1a | 0.1a | 0.6c | 0.5b | 0.4b | 0.4b | 0.6c | 0.4b | 0.4b | 0.4b | 0.5b | 0.5b | 0.4b | 0.4b |

**b.**

| | Common bean | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C1 | C2 | C3 | C4 | U1 | U2 | U3 | U4 | A1 | A2 | A3 | A4 | K1 | K2 | K3 | K4 |
| pH | 6.8a | 6.8a | 6.9a | 6.8a | 7.6c | 7.8c | 7.9c | 8.0c | 7.1a | 7.0a | 7.2a | 7.1a | 7.2a | 7.3b | 7.1a | 7.1a |
| $NH_4^+$ (mg N $kg^{-1}$) | 1.7a | 2.8a | 3.3a | 3.9a | 46.5f | 40.1e | 35.4d | 33.5d | 38.8e | 31.9d | 29.2d | 26.0c | 3.7a | 5.9b | 6.5b | 8.7b |
| $NO_3^-$ (mg N $kg^{-1}$) | 3.3a | 3.7a | 2.8a | 2.9a | 16.3b | 20.1c | 22.2c | 26.5d | 15.4b | 22.8c | 27.2d | 29.2d | 50.1f | 46.8f | 42.1e | 38.7e |
| TOC (mg $kg^{-1}$) | 1.2a | 1.3a | 1.3a | 1.5a | 1.8a | 1.8a | 2.1b | 2.5b | 1.8a | 2.1b | 2.2b | 2.4b | 1.9a | 2.1b | 2.4b | 2.4b |
| TC (%) | 1.6a | 1.8a | 1.8a | 1.9a | 2.1b | 2.3b | 3.0c | 3.2c | 2.4b | 2.6b | 2.7b | 2.9c | 2.5b | 2.7b | 2.7b | 3.0c |
| TN (%) | 0.1a | 0.1a | 0.1a | 0.1a | 0.6c | 0.6c | 0.5b | 0.4b | 0.6c | 0.5b | 0.5b | 0.4b | 0.6c | 0.6c | 0.5b | 0.4b |

*Table S2. Physicochemical properties of rhizosphere soil of tomato **(a)** and common bean **(b)**. Four harvests were made. Soil samples were taken at the end of each harvest. The soil was fertilised with urea (U), ammonium sulphate (A) or potassium nitrate (K). Unfertilised soil was used as a control (C). For each row, values followed by the same letter are not statistically different according to the Kruskal-Wallis and Conover-Iman tests ($P < 0.05$; $n = 4$). TOC, total organic C; TC, total C; TN, total N.*

**a.**

| | Tomato | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C1 | C2 | C3 | C4 | U1 | U2 | U3 | U4 | A1 | A2 | A3 | A4 | K1 | K2 | K3 | K4 |
| pH | 6.7a | 6.6a | 6.8a | 6.7a | 7.4c | 7.6c | 7.7c | 7.6c | 7.0b | 7.2b | 7.1b | 7.2b | 7.1b | 7.1b | 7.0b | 7.0b |
| $NH_4^+$ (mg N $kg^{-1}$) | 1.6a | 1.7a | 2.0a | 2.4a | 28.7f | 25.9e | 22.7e | 20.7d | 23.5e | 19.7d | 17.3c | 15.5c | 2.3a | 3.6b | 3.9b | 5.3b |
| $NO_3^-$ (mg N $kg^{-1}$) | 2.0a | 2.3a | 1.7a | 1.8a | 19.0d | 12.9c | 14.8c | 16.2d | 9.4b | 13.2c | 16.5d | 18.7d | 30.3f | 27.9f | 24.9e | 22.1e |
| TOC (mg $kg^{-1}$) | 1.7a | 1.8a | 1.9a | 2.2a | 2.5b | 2.6b | 3.0b | 3.6c | 2.6b | 3.0b | 3.1b | 3.4c | 2.8b | 3.0b | 3.4c | 3.4c |
| TC (%) | 2.4a | 2.6a | 2.6a | 2.8a | 3.1b | 3.4b | 4.4c | 4.8c | 3.5b | 3.9c | 4.0c | 4.3c | 3.6b | 4.0c | 4.0c | 4.4c |
| TN (%) | 0.1a | 0.1a | 0.1a | 0.1a | 0.4c | 0.4c | 0.3b | 0.2b | 0.4c | 0.3b | 0.3b | 0.2b | 0.4c | 0.4c | 0.3b | 0.3b |

**b.**

| | Common bean | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C1 | C2 | C3 | C4 | U1 | U2 | U3 | U4 | A1 | A2 | A3 | A4 | K1 | K2 | K3 | K4 |
| pH | 6.0a | 5.9a | 6.1a | 6.0a | 6.7c | 6.8c | 6.9c | 7.0c | 6.3b | 6.2b | 6.3b | 6.3b | 6.3b | 6.4b | 6.3b | 6.3b |
| $NH_4^+$ (mg N $kg^{-1}$) | 1.4a | 2.2a | 2.6a | 3.1a | 37.8f | 33.3f | 30.3e | 26.4d | 30.4e | 26.1d | 23.5c | 21.6c | 3.0a | 4.7b | 5.2b | 6.9b |
| $NO_3^-$ (mg N $kg^{-1}$) | 2.6a | 3.0a | 2.2a | 2.4a | 25.1d | 16.9c | 18.4c | 21.2d | 12.3b | 17.9c | 22.2d | 24.8d | 40.9f | 35.1f | 31.3e | 29.2e |
| TOC (mg $kg^{-1}$) | 1.6a | 1.8a | 1.9a | 2.1a | 2.5b | 2.6b | 2.9b | 3.5c | 2.6b | 2.9b | 3.1c | 3.3c | 2.7b | 2.9b | 3.3c | 3.3c |
| TC (%) | 2.0a | 2.2a | 2.2a | 2.3a | 2.7b | 2.9b | 3.7c | 4.0c | 3.0b | 3.2b | 3.4c | 3.6c | 3.1b | 3.4c | 3.4c | 3.7c |
| TN (%) | 0.1a | 0.1a | 0.1a | 0.1a | 0.5c | 0.5c | 0.4b | 0.3b | 0.5c | 0.4b | 0.4b | 0.3b | 0.5c | 0.5c | 0.4b | 0.3b |

*Table S3. Dry weight of tomato **(a)** and common bean **(b)** plants for each of the 4 consecutive harvests. The soil was fertilised with urea (U), ammonium sulphate (A) or potassium nitrate (K). Unfertilised soil was used as a control (C). For each row, values followed by the same letter are not statistically different according to the Kruskal-Wallis and Conover-Iman tests ($P < 0.05$; $n = 4$).*

**a.**

| Harvest | Tomato | | | |
|---|---|---|---|---|
| | Dry weight (g) | | | |
| | C | U | A | K |
| 1 | 20.1 ± 3.7a | 49.1 ± 4.9b | 47.4 ± 3.2b | 57.4 ± 4.5c |
| 2 | 19.2 ± 4.0a | 45.3 ± 7.5b | 49.9 ± 5.8b | 61.2 ± 5.6c |
| 3 | 16.5 ± 4.1a | 52.3 ± 6.9b | 46.4 ± 7.2b | 63.4 ± 6.5c |
| 4 | 18.5 ± 3.9a | 47.4 ± 4.9b | 44.4 ± 4.2b | 60.4 ± 5.3c |

**b.**

| Harvest | Common bean | | | |
|---|---|---|---|---|
| | Dry weight (g) | | | |
| | C | U | A | K |
| 1 | 2.1 ± 0.6a | 6.1 ± 1.1b | 7.4 ± 1.5b | 10.4 ± 2.5c |
| 2 | 1.9 ± 0.5a | 7.5 ± 2.0b | 8.9 ± 2.3b | 12.5 ± 3.5c |
| 3 | 1.9 ± 0.6a | 8.2 ± 1.5b | 8.4 ± 2.5b | 14.2 ± 3.0c |
| 4 | 2.0 ± 0.4a | 8.4 ± 1.1b | 9.2 ± 2.4b | 13.2 ± 2.0c |

*Table S4. Cumulative $N_2O$-N emissions by soil cultivated with tomato **(a)** and common bean **(b)** for each of the 4 consecutive harvests. The soil was fertilised with urea (U), ammonium sulphate (A) or potassium nitrate (K). Unfertilised soil was used as a control (C). Values are expressed as kg N $ha^{-1}$. For each row, values followed by the same letter are not statistically different according to the Kruskal-Wallis and Conover-Iman tests ($P < 0.05$; $n = 4$).*

**a.**

| Crop | Tomato | | | |
|---|---|---|---|---|
| | $N_2O$ cumulative emission (nmol $N_2O$ g dry $soil^{-1}$) | | | |
| | C | U | A | K |
| 1 | 1.2a | 18.6c | 13.4c | 8.0b |
| 2 | 0.2a | 15.1c | 13.0c | 7.8b |
| 3 | 0.2a | 13.1d | 8.8c | 7.4b |
| 4 | 0.2a | 8.1d | 6.1b | 6.3b |

**b.**

| Crop | Common bean | | | |
|---|---|---|---|---|
| | $N_2O$ cumulative emission (nmol $N_2O$ g dry $soil^{-1}$) | | | |
| | C | U | A | K |
| 1 | 1.3a | 20.1c | 14.4c | 5.7b |
| 2 | 0.2a | 16.7d | 16.4c | 11.8b |
| 3 | 0.3a | 13.9d | 10.7c | 11.2b |
| 4 | 0.4a | 9.0c | 8.6c | 7.8b |

*Table S5. Averaged total abundance of the amoA AOA, amoA AOB, nirK + nirS, norB and nosZI + nosZII genes in the bulk and rhizosphere soil of tomato* ***(a)*** *and common bean* ***(b)*** *after 4 consecutive harvests. The ratio (amoA AOA + amoA AOB + nirK + nirS + norB ) / nosZI + nosZII is presented in* ***(c)****. The soil was fertilised with urea (U), ammonium sulphate (A) or potassium nitrate (K). Unfertilised soil was used as a control (C).*

**a.**

| Tomato | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| log nº gene copy number x $g^{-1}$ dry soil | | | | | | | | |
| | Bulk soil | | | | Rhizosphere soil | | | |
| | C | U | A | K | C | U | A | K |
| *amoA* AOA | 5.0 | 6.2 | 6.0 | 5.2 | 4.7 | 5.8 | 5.9 | 4.9 |
| *amoA* AOB | 4.7 | 6.1 | 5.6 | 4.9 | 5.7 | 7.1 | 7.0 | 5.9 |
| *nirK* + *nirS* | 6.6 | 7.1 | 7.0 | 7.5 | 5.2 | 6.1 | 5.8 | 6.6 |
| *norB* | 6.2 | 6.8 | 6.6 | 7.3 | 5.1 | 5.8 | 5.6 | 6.4 |
| *nosZ*I + *nosZ*II | 5.7 | 6.6 | 6.7 | 7.0 | 4.8 | 5.1 | 5.0 | 5.5 |

**b.**

| Common bean | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| log nº gene copy number x $g^{-1}$ dry soil | | | | | | | | |
| | Bulk soil | | | | Rhizosphere soil | | | |
| | C | U | A | K | C | U | A | K |
| *amoA* AOA | 5.8 | 7.1 | 6.6 | 6.0 | 5.4 | 6.4 | 6.5 | 5.5 |
| *amoA* AOB | 5.4 | 6.8 | 6.3 | 5.6 | 6.4 | 7.8 | 7.7 | 6.6 |
| *nirK* + *nirS* | 6.7 | 7.6 | 7.4 | 7.9 | 6.1 | 6.8 | 6.7 | 7.2 |
| *norB* | 6.5 | 7.5 | 7.3 | 7.9 | 5.9 | 6.6 | 6.4 | 7.1 |
| *nosZ*I + *nosZ*II | 6.0 | 6.9 | 6.8 | 7.4 | 5.4 | 6.1 | 6.0 | 6.6 |

**c.**

| *Ratio [(amoA AOA + amoA AOB + nirK + nirS + norB ) / (nosZI + nosZII)]* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Bulk soil | | | | Rhizosphere soil | | | |
| | C | U | A | K | C | U | A | K |
| Tomato | 4.0a | 4.0a | 3.8a | 3.5a | 4.3a | 4.9b | 4.8b | 4.4b |
| Common bean | 4.1a | 4.2b | 4.1b | 3.7b | 4.4a | 4.6a | 4.5a | 4.0a |

# Chapter III.2

## Effect of nitrogen fertilisation on the structure and composition of the bacterial community in the bulk and rhizosphere soil of *Solanum lycopersicum* and *Phaseolus vulgaris*

Abstract

In Chapter III.1, the effect of N-fertilisation on $N_2O$ emission, total abundance of bacteria and archaea and nitrification and denitrification genes after fertilisation with urea, ammonium and nitrate has been reported. Here we extend those findings by analysing the structure and composition of the bacterial community in the bulk and rhizosphere soil of the tomato (*Solanum lycopersicum*) and common bean (*Phaseolus vulgaris*) used in the previous chapter. Although four consecutive harvests were carried out, this study was performed on soil samples taken at the end of the first and fourth harvests. Bacterial biodiversity was estimated using pyrosequencing of the 16S rRNA gene. The Shannon diversity index showed that application of urea, ammonium and nitrate decreased the number of OTUs in the bulk and, even more, in the rhizosphere soil. After N-fertilisation the bacterial community became less diverse, or dominated by a small group of OTUs, as suggested by the Simpson index. A stepwise multiple regression analysis showed that the content of exchangeable ammonium ($NH_4^+$-N) and nitrate ($NO_3^-$N) were the main drivers of the changes in the number of OTUs in bulk and rhizosphere soil. The study of the beta diversity indices between the control and each of the N-treated soil showed that dominant and rare OTUs were different in the bulk and rhizosphere soil after the fourth harvest. The effect of N-fertilisation on the structure and composition of the bacterial community in bulk and rhizosphere soil was similar among tomato and common bean plants.

Keywords: N-fertilisation, bacterial diversity, pyrosequencing, tomato, common bean, OTUs

## Introduction

In Chapter III.1 we determined the effect of the N-fertilisers urea, ammonium and nitrate on $N_2O$ emission and abundance of nitrification and denitrification genes in bulk and rhizosphere soil of tomato (*Solanum lycopersicum*) and common bean (*Phaseolus vulgaris*) plants during four consecutive harvests.

Studies simultaneously analysing in bulk and rhizosphere soil the diversity of the bacterial community after inorganic N-fertilisation are scarce and they all were carried out in maize plants (Peiffer et al. 2013; García-Salamanca et al. 2013; Yang et al. 2017a). This is why we analysed the variations in the structure and composition of the bacterial community in bulk and rhizosphere soil of tomato and common bean use on previous studies described in Chapter III.1.

## Materials and methods

### Experimental setup, bacterial diversity and statistical analysis

The experimental setup of this study has been described in Chapter III.1. Samples were taken from the bulk and rhizosphere soil as described in Chapter III.1. The bacterial diversity analysis, alpha diversity indices and heat maps were as described in Chapter II. Bulk (B) and rhizosphere (R) samples were obtained only after the first (1) and fourth (4) harvest in soils cultivated with tomato and common bean treated with urea (UR), ammonium sulphate (AS) or potassium nitrate (PN) fertilisers as indicated in Chapter III.1. Soil cultivated with tomato or common bean without fertilisation was used as a control (NT). Biodiversity of the original soil is dealt with in Chapter II.

The Morisita-Horn and symmetric indices were used to estimate the β-diversity between pairs of control and N-fertiliser samples (Barwell et al. 2015), using the packages vegetarian and vegan v2.0 implemented in R-Project.

Because of the absence of normality and homoscedasticity in exchangeable ammonium ($NH_4^+$-N), nitrate ($NO_3^-$-N), pH, total organic C (TOC), total C (TC) and total N (TN) abiotic variables, the Kruskal-Wallis and Conover-Iman tests were chosen for multiple comparisons among samples. Principal Coordinates Analysis (PCoA) including all the OTUs retrieved from bulk and rhizosphere soil samples was

performed using R (vegan package v.2.0). A stepwise multiple regression analysis in the SPSS software (IBM Corp, USA) was performed to assess the abiotic variables most affecting the relative abundance of the bacterial operational taxonomic units (OTUs).

## Results

### Effect of N-fertilisation on the structure of the bacterial community

A total of 1655800 sequences were obtained from the 128 16S rDNA samples sent to pyrosequencing, of which 620125 were retained after filtering and removing chimeras. The mean number of total retained sequences per library was 9820, ranging from 8650 to 12102. Average length of the retained sequences was 400 $\pm$ 5 base pair (mean $\pm$ SD). Good´s coverage analysis was higher than 92.25% at 90% confidence interval (Table 1).

The number of OTUs in NTB and NTR soils of tomato corresponding to the 1$^{st}$ harvest was 2299 and 2712, respectively, and 2280 and 2688 after the 4$^{th}$ harvest, respectively (Table 1A). The amendment with any of the N-fertilisers decreased the number of OTUs both in bulk and rhizosphere soil after the 1$^{st}$ and 4$^{th}$ harvests to 1910, 1454, 2080 and 1320 in UR1B, UR4B, UR1R and UR4R, respectively, to 2080, 1735, 2120 and 1405 in AS1B, AS4B, AS1R and AS4R, respectively, and to 2010, 1790, 2090 and 1580 in PN1B, PN4B, PN1B and PN4B, respectively (Table 1A). The Shannon and Simpson indices after each harvest are presented in Table 1A. In the N-treated soils, significant decreases in each index were found after the 1$^{st}$ harvest, which were even lower after the 4$^{th}$. Urea produced the highest losses of biodiversity, followed by ammonium and nitrate, among which differences were not observed, in both bulk and rhizosphere soil.

The number of OTUs in NTB and NTR soils of common beans corresponding to the 1$^{st}$ harvest was 2358 and 2851, respectively, and 2320 and 2837 after the 4$^{th}$ harvest, respectively (Table 1B). Fertilisation with any of the 3-fertilisers decreased the number of OTUs both in bulk and rhizosphere soil after 1 and 4 harvests to 2050, 1695, 2256 and 1458 in UR1B, UR4B, UR1R and UR4R, respectively, to 2110, 1781, 2118 and 1525 in AS1B, AS4B, AS1R and AS4R, respectively, and to 2150, 1859,

2269 and 1638 in NT1B, NT4B, NT1B and NT4B, respectively (Table 1B). Also, as above, the Shannon and Simpson diversity indices decreased after the 1st harvest and even more after the 4th harvest in the N-treated soils (Table 1B). Both in bulk and rhizosphere soil, urea produced the highest losses of biodiversity, followed by ammonium and nitrate, among which differences were not observed.

Values of the Morisita-Horn and symmetric indices for the pair of samples analysed after the 1st and 4th harvests are shown in Table 2. In the bulk soil of tomato and common bean, after the 1st harvest, the dominant OTUs of the bacterial community in the control and N-treated soils were similar as the values of Morisita-Horn were 0.185686 and 0.231245, respectively. Changes in the dominant OTUs were observed after the 4th harvest with values higher than 0.845656 and 0.858566, respectively. In the rhizosphere soil of tomato and common bean, the dominant OTUs were different both after the 1st and 4th harvests, with values higher than 0.885622 and 0.822356, respectively. In N-treated soils, the symmetric index indicated that rare OTUs in bulk and rhizosphere soils differed from those in the control soil after the 1st and 4th harvests, with values higher than 0.606565 and 0.623232 for tomato and common bean, respectively (Table 2).

The PCoA analysis showed that the soil samples retrieved from bulk and rhizosphere soils treated with urea, ammonium or nitrate clustered separately (Fig. 1). Samples from the ammonium-treated soils clustered together, and those from the soils amended with nitrate and the control soil fell in two separated groups (Fig. 1). A stepwise regression analysis (Table 3) revealed that, regardless of the plant species, after the 4th harvest, the content of the exchangeable $NH_4^+$-N in the urea-treated bulk soil explained 26.1% of the changes in the number of OTUs and 25.6% in the soil amended with ammonium. The analysis also showed that the content of $NO_3^-$-N was responsible for 36.2, 33.9 and 74.6% of the changes in soils treated with urea, ammonium and nitrate, respectively. In the rhizosphere soil, the exchangeable $NH_4^+$-N content contributed with 32.2 and 40.3% of the variations in the number of OTUS in the urea- and ammonium-treated soil and the content of $NO_3^-$-N was responsible for 31.6, 41.9 and 82.1% of the changes in soils treated with urea, ammonium and nitrate, respectively.

### Effect of N-fertilisation on the composition of the bacterial community in the bulk soil

The relative abundance of the bacterial OTUs in the analysed soil samples is shown in Fig. S1. The heat map depicted in Fig. 2 shows the changes in the relative abundance of the OTUs retrieved from the control and N-treated bulk soil after the 1st and 4th harvests of tomato and common bean. Because the similarity between the results from tomato and common bean, only those corresponding to tomato are described below.

The amendment of the soil with any of the ammonium-based fertilisers after the 1st harvest increased > 5% the relative abundance of Nocardioidaceae and > 2% that of Bacillaceae 1 and Chromatiaceae and decreased > 2% that of Flavobacteriaceae, Anaerolineaceae, Sphingomonadaceae, Halomonadaceae, Moraxellaceae and Sinobacteraceae. In the nitrate treated soil, the relative abundance of Thiobacillacaeae and Pseudomonadaceae increased > 5% and that of Intrasporangiaceae, Flavobacteriaceae, Sphingomonadaceae, Halomonadaceae, Moraxellaceae and Sinobacteraceae decreased > 2%.

After the 4th harvest, the application of urea or ammonium increased > 5% the relative abundance of Nocardioidaceae, > 2% that of Bacillaceae 1, Bacillaceae, Paenibacillaceae 1, Bradyrhizobiaceae, Hyphomicrobiaceae, Rhizobiaceae, Rhodobacteraceae, Rhodospirillaceae, Comamonadaceae, Burkholderiaceae, Nitrosomonadaceae, Thiobacillacaeae, Pseudomonadaceae and Chromatiaceae and decreased > 5% that of Flavobacteriaceae and Anaerolineaceae and > 2% that of Acidimicrobiaceae, Intrasporangiaceae, Chitinophagaceae, Gemmatimonadaceae, Planctomycetaceae, Halomonadaceae, Moraxellaceae and Sinobacteraceae.

The fertilisation with nitrate increased > 5% the abundance of Bacillaceae 1, Paenibacillaceae 1, Hyphomicrobiaceae, Comamonadaceae, Burkholderiaceae, Thiobacillacaeae and Pseudomonadaceae, > 2% that of Trueperaceae, Bacillaceae 2, Planococcaceae, Bradyrhizobiaceae and Nitrosomonadaceae and decreased > 5% Flavobacteriaceae, Anaerolineaceae and > 2% that of Intrasporangiaceae, Chitinophagaceae, Gemmatimonadaceae, Planctomycetaceae, Moraxellaceae and Sinobacteraceae.

## Effect of N-fertilisation on the composition of the bacterial community in the rhizosphere soil

The relative abundance of the bacterial OTUs in the analysed soil samples is presented in Fig. S2. The heat map in Fig. 3 show the changes in the relative abundance of the OTUs retrieved from the control and N-fertilised rhizosphere soils after the 1st and 4th harvests of tomato and common bean. Because the similarity between the results from tomato and common bean, only those corresponding to tomato are described below

After the 1st harvest, clear differences in the relative abundance of the OTUs from the control and N-fertilised soils were not detected. After the 4th harvest, the application of urea or ammonium increased > 5% the relative abundance of Paenibacillaceae 1, Bradyrhizobiaceae, Hyphomicrobiaceae, Pseudomonadaceae, Chromatiaceae and > 2% that of Nocardioidaceae, Bacillaceae 1, Comamonadaceae, Burkholderiaceae, Nitrosomonadaceae and Thiobacillacaeae, and decreased > 2% the relative abundance of 30 OTUs (see Fig. 3). In nitrate-treated soils, the relative abundance of the Bacillaceae 1, Comamonadaceae, Burkholderiaceae, Pseudomonadaceae increased > 5% and Paenibacillaceae 1, Hyphomicrobiaceae, Nitrosomonadaceae and Thiobacillacaeae increased > 2%. Addition of nitrate decreased > 2% the relative abundance of 31 OTUs after the 4th harvest (Fig. 3).

## Discussion

Although previous studies have shown that N-fertilisers can alter he structure and composition of the soil bacterial community (Philippot et al. 2009, 2011, 2013; Geisseler and Scow 2014; Zhou et al. 2017b; Wang et al. 2018b), their effects on the plant rhizosphere is largely unknown. Results in this paper show that after the 1st and 4th harvests the addition to the soil of urea, ammonium or nitrate decreased the number of OTUs in samples from tomato (Table 1A) and common bean (Table 1B). As suggested by the Shannon index, the highest losses of diversity were detected in the rhizosphere soil cultivated with tomato or common bean that were fertilised with urea, followed by fertilisation with ammonium and, finally, after addition of nitrate (Table 1A, 1B). That the bacterial community became less diverse in bulk and rhizosphere soil, or dominated by a small group of OTUs after N-fertilisation, is

also supported by the decreases in the Simpson index (Table 1A, 1B). Also, the Morisita-Horn and symmetric indices used for the estimation of the β-diversity showed that dominant and rare OTUs were significantly different in the rhizosphere soil of the N-treated soils both in the 1st and 4th harvests. Changes in the dominant OTUs of the bulk soil were detected only after the 4th harvest, while differences in the rare OTUs were found in the 1st and 4th harvests (Table 2). Taking together, the α- and β-diversity results, suggest that despite the lower $NH_4^+$-N and $NO_3^-$-N content in the rhizosphere than in the bulk soil (Tables S1 and S2), the loss of diversity was significantly higher in the rizosphere soil, which results in changes in the structure of the bacterial community as confirmed by the PCoA analysis (Fig. 1). Similar results were found in bulk and rhizosphere soil of maize treated with ammonium and nitrate for 12 weeks (Peiffer et al. 2013).

A stepwise multiple regression analysis, which included the number of OTUS from the samples retrieved from bulk and rhizosphere soil of tomato and common bean, showed that the exchangeable $NH_4^+$-N and $NO_3^-$-N contents were involved in the diversity losses (Table 3). Because the regression values in Table 3 were higher in the rhizosphere soil, the results suggest that N availability is a more crucial driver of bacterial diversity in the rhizosphere than the bulk soil.

The heat maps in Figs. 2 and 3 show, on one hand, that urea, ammonium and nitrate produced similar decreases in the OTUs with a relative abundance higher than 1% and, on the other hand, that, if any, the increases in the relative abundance of the OTUs were dependent on the type of the N-fertiliser. It is interesting to mention that among OTUs whose relative abundance increased more than 2% after N-fertilisation, the families Bacillaceae, Bradyrhizobiaceae, Burkholderiaceae, Chromatiaceae, Comamonadaceae, Hyphomicrobiaceae, Nitrosomonadaceae, Pseudomonadaceae, Rhizobiaceae and Thiobacillacaeae contain member with nitrifying and denitrifying capabilities as reported by other authors (Daims et al. 2016; Zhang et al. 2016; Zhou et al. 2017b; Wang et al. 2018a, b). Changes in the composition of the bacterial community composition is clearly dependent of the form of the fertiliser, this is ammonium or nitrate, changes that were observed in the bulk (Fig. 2) and rhizosphere (Fig. 3) soil. Changes in the composition of the

bacterial community have been previously reported after soil fertilisation with urea (Yu et al. 2016; Zhou et al. 2017a) or ammonium nitrate (Zeng et al. 2016).

Taken together, our results indicate that N-fertilisers decreased soil biodiversity and that variations in the structure and composition of the bacterial community differ between bulk and rhizosphere soil depending on the form of the N-fertiliser. Because very similar variations were found in bulk and rhizosphere soil of tomato and common bean, changes in the soil bacterial diversity are mainly due to N-fertilisation rather than to the plant.

## Acknowledgments

This study was supported by the ERDF-cofinanced grant PEAGR2012–1968 from Consejería de Economía, Innovación y Ciencia (Junta de Andalucía, Spain). ACH is recipient of a grant of MECD (FPU 2014/01633).

*Table 1. Number of OTUs, values of Good's coverage index and Shannon and Simpson biodiversity indices in bulk (B) and rhizosphere (R) soil of tomato **(a)** and common bean **(b)**. Soil samples correspond to the first (1) and fourth (4) harvests carried out in Chapter III.1. The soil was fertilised with urea (UR), ammonium sulphate (AS) or potassium nitrate (PN). Unfertilised soil was used as a control (NT). For each row, values followed by the same letter are not statistically different according to the Kruskal-Wallis and Conover-Iman tests ($P < 0.05$; $n = 4$).*

**a.**

| | Bulk soil | | | | | | | | Rhizosphere soil | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | NT1B | NT4B | UR1B | UR4B | AS1B | AS4B | PNB1 | PN4B | NT1R | NT4R | UR1R | UR4R | AS1R | AS4R | PN1R | PN4R |
| Number of OTUs | 2299d | 2280d | 1910d | 1454a | 2080d | 1735c | 2010d | 1790c | 2712e | 2688e | 2080d | 1320a | 2120d | 1405a | 2090d | 1580b |
| Shannon | 2.58e | 2.68e | 2.44d | 1.67b | 2.40c | 1.88c | 2.39d | 1.90c | 3.23g | 3.17g | 2.89f | 1.60a | 2.92f | 1.74b | 2.90g | 1.72b |
| Simpson | 0.746f | 0.758f | 0.602e | 0.526c | 0.612e | 0.572d | 0.614e | 0.576d | 0.824g | 0.839g | 0.618e | 0.406a | 0.628e | 0.455b | 0.621e | 0.465b |
| Good´s coverage (%) | 92.25 | 92.84 | 92.90 | 93.76 | 93.10 | 92.58 | 92.46 | 92.56 | 94.56 | 98.56 | 94.56 | 97.56 | 98.12 | 94.65 | 96.23 | 94.55 |

**b.**

| | Bulk soil | | | | | | | | Rhizosphere soil | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | NT1B | NT4B | UR1B | UR4B | AS1B | AS4B | PN1B | PN4B | NT1R | NT4R | UR1R | UR4R | AS1R | AS4R | PN1R | PN4R |
| Number of OTUs | 2358c | 2320c | 2050c | 1695b | 2110c | 1781b | 2150c | 1859c | 2851d | 2837d | 2256c | 1458a | 2118c | 1525a | 2269c | 1638b |
| Shannon | 2.65e | 2.62e | 2.49d | 1.82b | 2.42d | 1.95c | 2.44d | 1.99c | 3.30f | 3.26f | 2.50d | 1.78a | 2.42d | 1.86b | 2.45d | 1.90b |
| Simpson | 0.784f | 0.76bf | 0.621e | 0.502c | 0.631e | 0.562d | 0.632e | 0.582d | 0.832g | 0.825g | 0.642e | 0.415a | 0.612e | 0.462b | 0.646e | 0.465b |
| Good´s coverage (%) | 93.56 | 93.12 | 92.56 | 94.11 | 93.18 | 92.56 | 94.56 | 95.66 | 94.58 | 96.65 | 97.56 | 94.56 | 96.56 | 98.56 | 94.56 | 96.55 |

*Table 2. Morisita-Horn and symmetric β-diversity indices of each pair of sequencing samples in bulk (B) and rhizosphere (R) soil of tomato and common bean. Soil samples correspond to the first (1) and fourth (4) harvests carried out in Chapter III.1. The soil was fertilised with urea (UR), ammonium sulphate (AS) or potassium nitrate (PN). Unfertilised soil was used as a control (NT).*

| | Tomato | | Common bean | |
|---|---|---|---|---|
| | Morisita-Horn | Symmetric | Morisita-Horn | Symmetric |
| | Bulk soil | | | |
| NT1 vs UR1 | 0.147152 | 0.606565 | 0.174235 | 0.623232 |
| NT1 vs AS1 | 0.162739 | 0.612325 | 0.178875 | 0.645612 |
| NT1 vs PN1 | 0.179360 | 0.653265 | 0.167535 | 0.625584 |
| NT4 vs UR4 | 0.856145 | 0.689873 | 0.875656 | 0.651666 |
| NT4 vs AS4 | 0.853245 | 0.691139 | 0.858566 | 0.664304 |
| NT4 vs PN4 | 0.845656 | 0.689563 | 0.859566 | 0.656842 |
| | Rhizosphere soil | | | |
| NT1 vs UR1 | 0.885622 | 0.656559 | 0.822356 | 0.662512 |
| NT1 vs AS1 | 0.875555 | 0.645565 | 0.828456 | 0.651362 |
| NT1 vs PN1 | 0.885656 | 0.632592 | 0.835689 | 0.682656 |
| NT4 vs UR4 | 0.951204 | 0.896723 | 0.931245 | 0.877477 |
| NT4 vs AS4 | 0.961335 | 0.834375 | 0.944828 | 0.886747 |
| NT4 vs PN4 | 0.955686 | 0.886666 | 0.948457 | 0.856627 |

*Table 3. Multiple stepwise regression analysis in bulk (B) and rhizosphere (R) soil of tomato* ***(a)*** *and common bean* ***(b)*** *between the independent abiotic (exchangeable $NH_4^+$-N, $NO_3^-$-N, pH, TOC, TC and TN) and dependent biotic (number of OTUs) variables for a soil not treated (NT) or fertilised with urea (UR), ammonium sulphate (AS) or potassium nitrate (PN). Abiotic variables with $P > 0.1$ are not included in the table. β, standardized regression coefficient; multiple $R^2$, coefficient of multiple determination; $R^2$ change, change in multiple $R^2$ caused by entering a new variable in a single step. TOC, total organic C; TC, total C; TN, total N.*

**a.**

| Dependent variable | Treatment | Independent variable | β | Multiple $R^2$ | $R^2$ change | *P* |
|---|---|---|---|---|---|---|
| Number of OTUs | UR | Exchangeable $NH_4^+$-N | 0.62 | 0.261 | 0.261 | 0.008 |
| | | $NO_3^-$-N | 0.51 | 0.623 | 0.362 | 0.002 |
| | AS | Exchangeable $NH_4^+$-N | 0.55 | 0.256 | 0.256 | 0.010 |
| | | $NO_3^-$-N | 0.56 | 0.595 | 0.339 | 0.003 |
| | PN | $NO_3^-$-N | 0.72 | 0.746 | 0.746 | 0.001 |

**b.**

| Dependent variable | Treatment | Independent variable | β | Multiple $R^2$ | $R^2$ change | *P* |
|---|---|---|---|---|---|---|
| Number of OTUs | UR | Exchangeable $NH_4^+$-N | 0.68 | 0.322 | 0.322 | 0.006 |
| | | $NO_3^-$-N | 0.54 | 0.725 | 0.403 | 0.003 |
| | AS | Exchangeable $NH_4^+$-N | 0.66 | 0.316 | 0.316 | 0.009 |
| | | $NO_3^-$-N | 0.70 | 0.735 | 0.419 | 0.003 |
| | PN | $NO_3^-$-N | 0.86 | 0.821 | 0.821 | 0.001 |

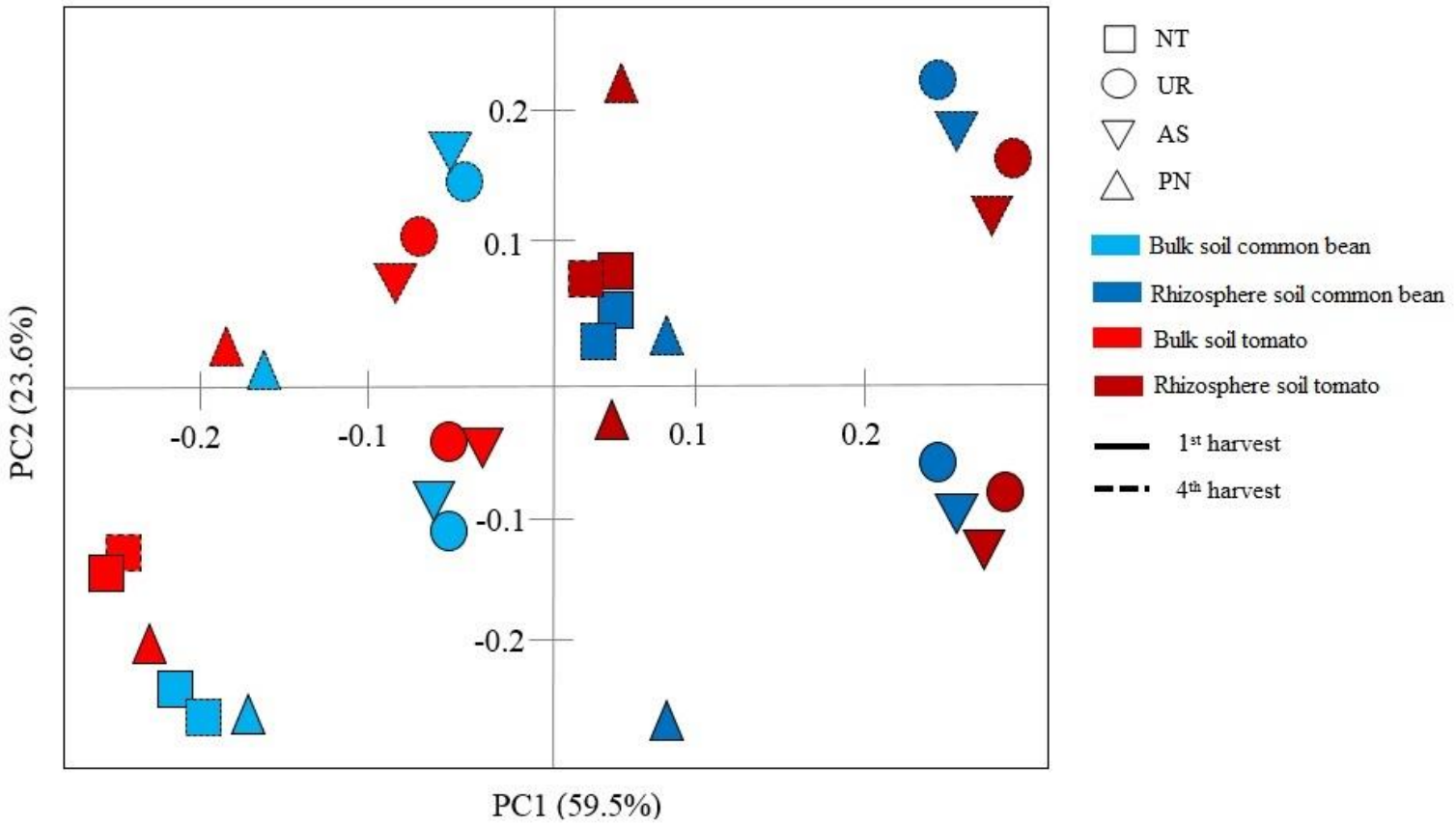



*Fig. 1. Principal coordinates analysis (PCoA) for the OTUs retrieved from soil samples taken in bulk and rhizosphere soil of tomato and common bean Soil samples correspond to the first (1) and fourth (4) harvests carried out in Chapter III.1. The soil was fertilised with urea (UR), ammonium sulphate (AS) or potassium nitrate (PN). Unfertilised soil was used as a control (NT).*

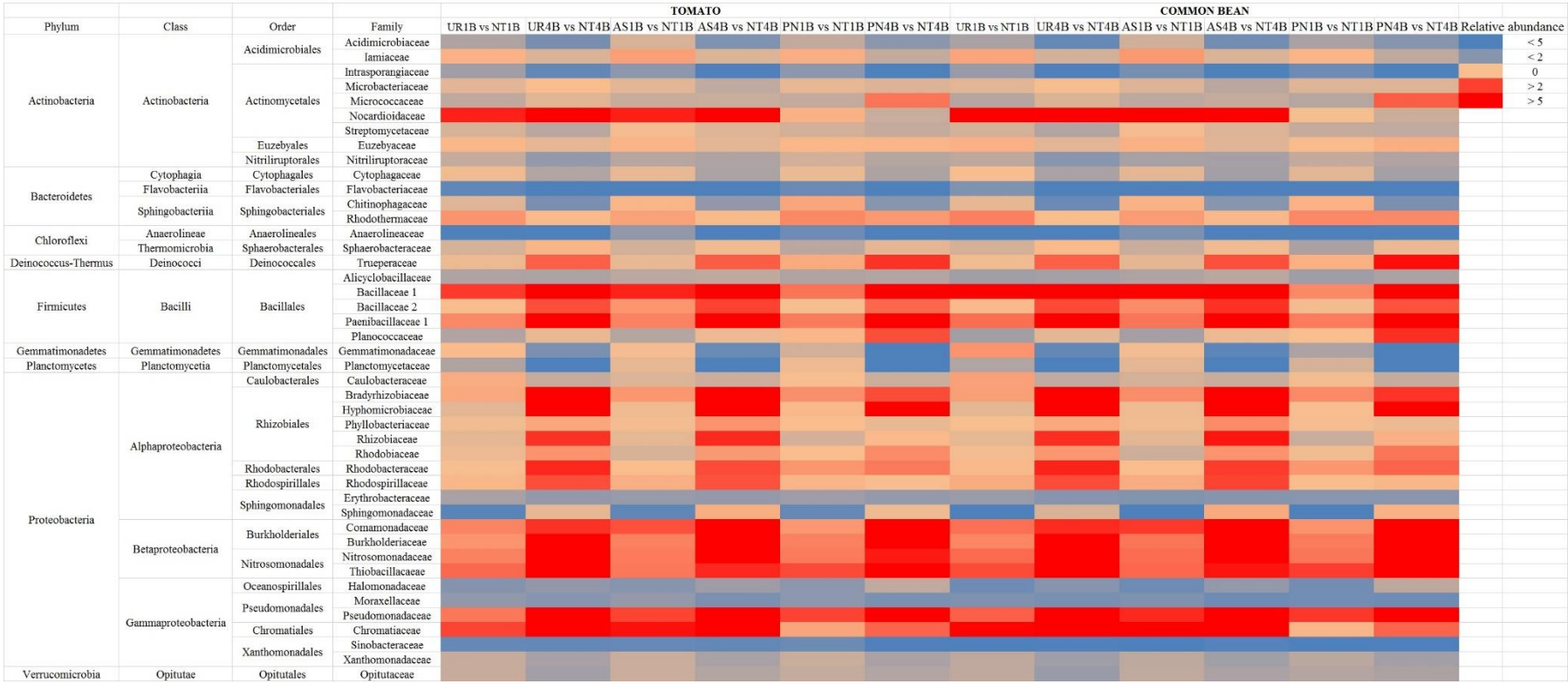



*Fig. 2. Heat map showing changes in the relative abundance of the OTUs retrieved from bulk soil samples not treated (NT) or fertilised with urea (UR), ammonium sulphate (AS) or potassium nitrate (PN). OTUs correspond to the family level. Soil samples correspond to the first (1) and fourth (4) harvests carried out in Chapter III.1. Only OTUs with >1% relative abundance in at least one sample were included in this analysis.*

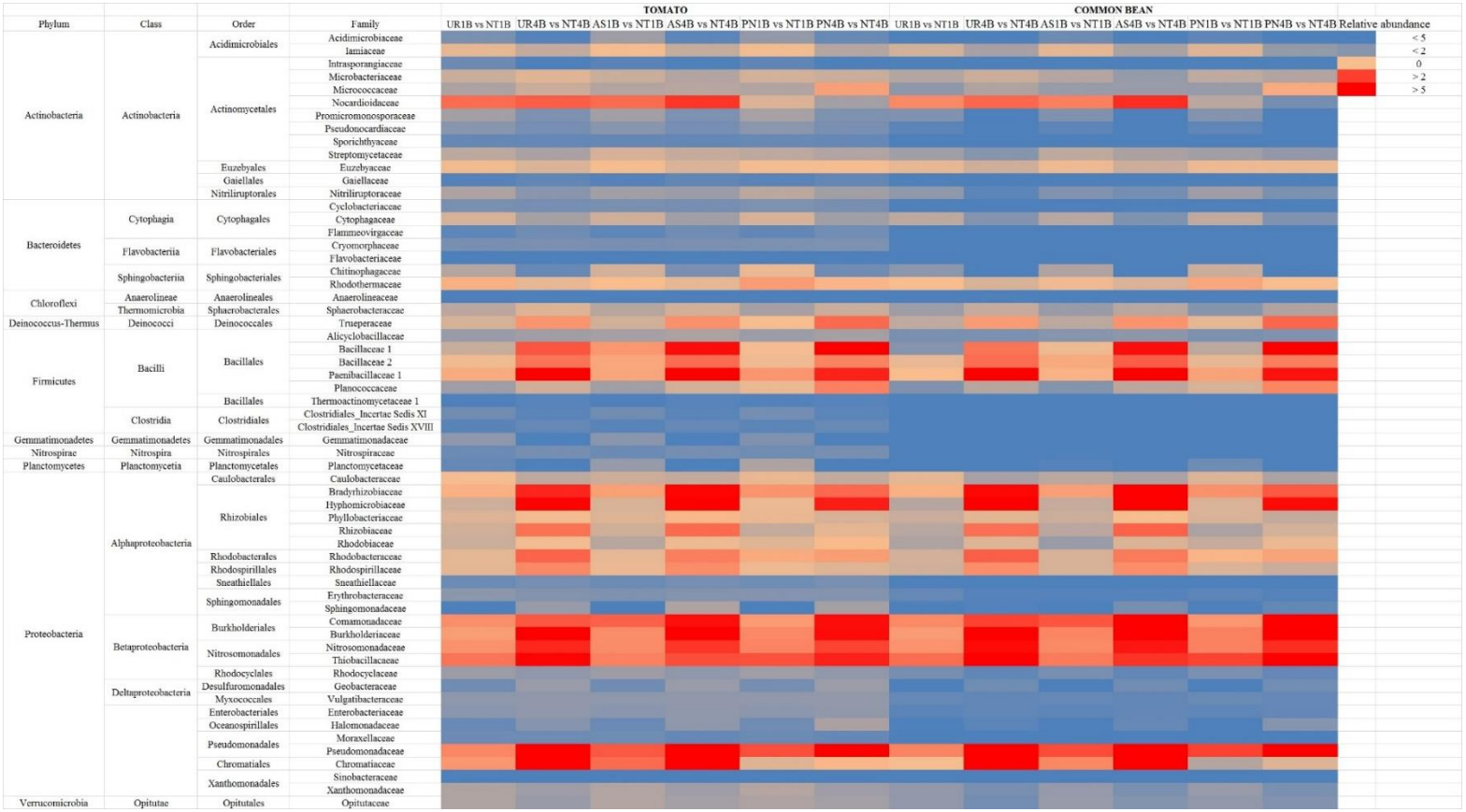



*Fig. 3. Heat map showing changes in the relative abundance of the OTUs retrieved from rhizosphere soil samples not treated (NT) or fertilised with urea (UR), ammonium sulphate (AS) or potassium nitrate (PN). OTUs correspond to the family level. Soil samples correspond to the first (1) and fourth (4) harvests carried out in Chapter III.1. Only OTUs with >1% relative abundance in at least one sample were included in this analysis.*

## Supplementary material for Chapter III.2

# Effect of nitrogen fertilisation on the structure and composition of the bacterial community in the bulk and rhizosphere soil of *Solanum lycopersicum* and *Phaseolus vulgaris*

*Table S1. Physicochemical properties of bulk (B) soil of tomato* ***(a)*** *and common bean* ***(b).*** *Soil samples correspond to the first (1) and fourth (4) harvests carried out in Chapter III.1. The soil was fertilised with urea (UR), ammonium sulphate (AS) or potassium nitrate (PN). Unfertilised soil was used as a control (NT). For each row, values followed by the same letter are not statistically different according to the Kruskal-Wallis and Conover-Iman tests ($P < 0.05$; $n = 4$). TOC, total organic C; TC, total C; TN, total N.*

**a.**

| | Tomato | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | NT1B | NT4B | UR1B | UR4B | AS1B | AS4B | PN1B | PN4B |
| pH | 7.1a | 7.1a | 7.9c | 8.3c | 7.4b | 7.4b | 7.5b | 7.4b |
| $NH_4^+$ (mg N $kg^{-1}$) | 1.6a | 3.5a | 42.0d | 29.5c | 35.8d | 23.2c | 3.4a | 7.9b |
| $NO_3^-$ (mg N $kg^{-1}$) | 3.0a | 2.7a | 14.5b | 23.8c | 13.6b | 26.0c | 45.9d | 30.6c |
| TOC (mg $kg^{-1}$) | 1.4a | 1.8a | 2.1b | 3.0c | 2.2b | 2.8c | 2.3b | 2.8c |
| TC (%) | 1.9a | 2.2a | 2.5b | 3.8c | 2.8b | 3.4c | 2.9b | 3.5c |
| TN (%) | 0.05a | 0.1a | 0.6c | 0.4b | 0.6c | 0.4b | 0.5b | 0.4b |

**b.**

| | Common bean | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | NT1B | NT4B | UR1B | UR4B | AS1B | AS4B | PN1B | PN4B |
| pH | 6.8a | 6.8a | 7.6b | 8.0b | 7.1a | 7.1a | 7.2a | 7.1a |
| $NH_4^+$ (mg N $kg^{-1}$) | 1.7a | 3.9a | 46.5d | 33.5c | 38.8cd | 26.0c | 3.7a | 8.7b |
| $NO_3^-$ (mg N $kg^{-1}$) | 3.3a | 2.9a | 16.3b | 26.5c | 15.4b | 29.2c | 50.1e | 38.7d |
| TOC (mg $kg^{-1}$) | 1.2a | 1.5a | 1.8a | 2.5b | 1.8a | 2.4b | 1.9a | 2.4b |
| TC (%) | 1.6a | 1.9a | 2.1b | 3.2c | 2.4b | 2.9c | 2.5b | 3.0c |
| TN (%) | 0.1a | 0.1a | 0.6c | 0.4b | 0.6c | 0.4b | 0.6c | 0.4b |

*Table S2. Physicochemical properties of rhizosphere (R) soil of tomato **(a)** and common bean **(b)**. Soil samples correspond to the first (1) and fourth (4) harvests carried out in Chapter III.1. The soil was fertilised with urea (UR), ammonium sulphate (AS) or potassium nitrate (PN). Unfertilised soil was used as a control (NT). For each row, values followed by the same letter are not statistically different according to the Kruskal-Wallis and Conover-Iman tests ($P < 0.05$; $n = 4$). TOC, total organic C; TC, total C; TN, total N.*

**a.**

| | Tomato | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | NT1R | NT4R | UR1R | UR4R | AS1R | AS4R | PN1R | PN4R |
| pH | 6.7a | 6.7a | 7.4c | 7.8c | 7.0b | 7.2b | 7.1b | 7.0b |
| $NH_4^+$ (mg N $kg^{-1}$) | 1.6a | 2.4a | 28.7d | 20.7c | 23.5c | 15.5c | 2.3a | 5.3b |
| $NO_3^-$ (mg N $kg^{-1}$) | 2.0a | 1.8a | 19.0c | 16.2c | 9.4b | 18.7c | 30.3d | 22.1c |
| TOC (mg $kg^{-1}$) | 1.7a | 2.2a | 2.5b | 3.6c | 2.6b | 3.4c | 2.8b | 3.4c |
| TC (%) | 2.4a | 2.8a | 3.1b | 4.8c | 3.5b | 4.3c | 3.6b | 4.4c |
| TN (%) | 0.1a | 0.1a | 0.4c | 0.2b | 0.4c | 0.2b | 0.4c | 0.3b |

**b.**

| | Common bean | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | NT1R | NT4R | UR1R | UR4R | AS1R | AS4R | PN1R | PN4R |
| pH | 6.0a | 6.0a | 6.7b | 7.0b | 6.3a | 6.3a | 6.3a | 6.3a |
| $NH_4^+$ (mg N $kg^{-1}$) | 1.4a | 3.1a | 37.8d | 26.4c | 30.4d | 21.6c | 3.0a | 6.9b |
| $NO_3^-$ (mg N $kg^{-1}$) | 2.6a | 2.4a | 25.1c | 21.2c | 12.3b | 24.8c | 40.9e | 29.2d |
| TOC (mg $kg^{-1}$) | 1.6a | 2.1a | 2.5b | 3.5c | 2.6b | 3.3c | 2.7b | 3.3c |
| TC (%) | 2.0a | 2.3a | 2.7b | 4.0c | 3.0b | 3.6c | 3.1b | 3.7c |
| TN (%) | 0.1a | 0.1a | 0.5c | 0.3b | 0.5c | 0.3b | 0.5c | 0.3b |

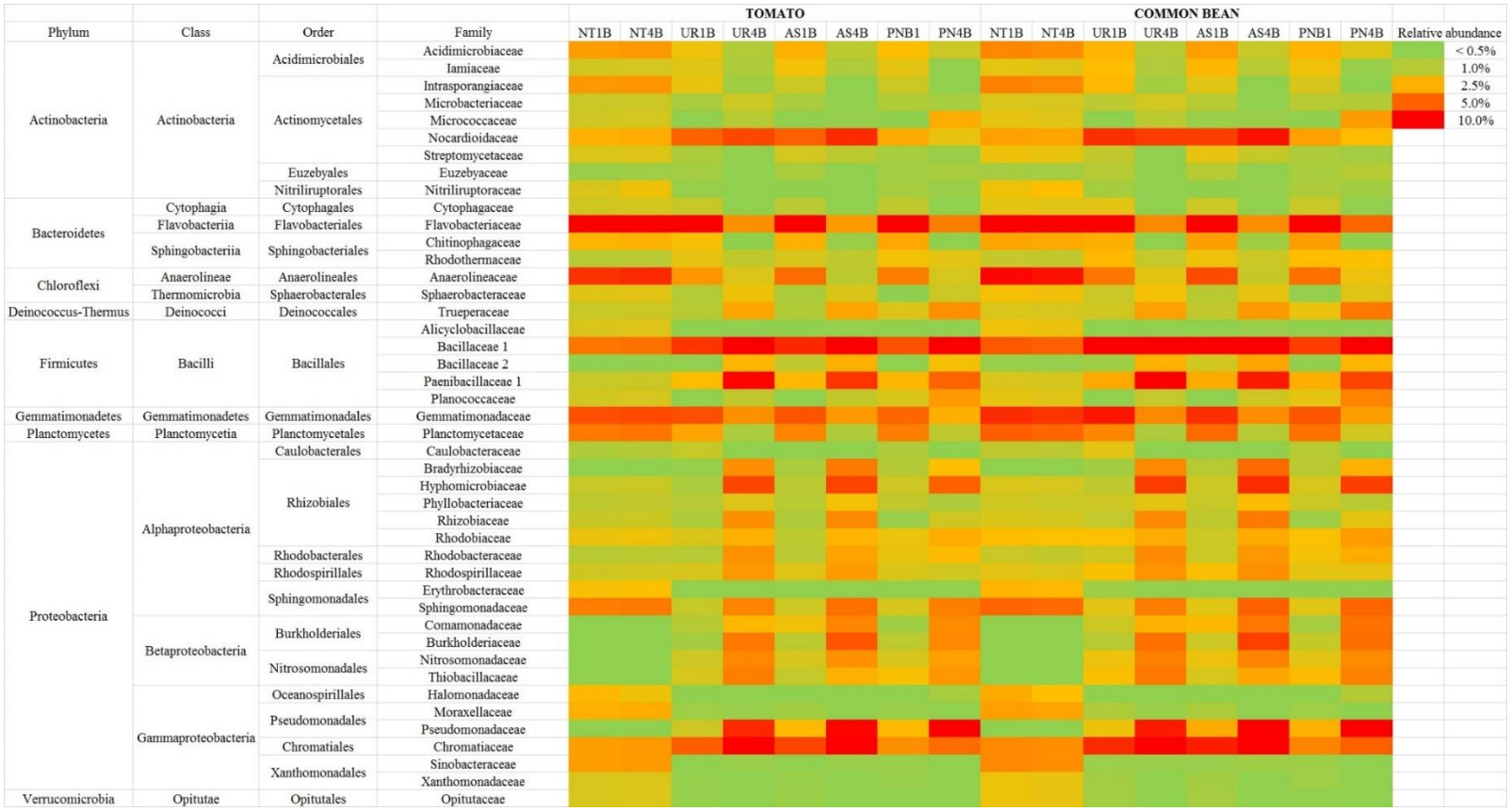



***Fig. S1.*** *Heat map showing the relative abundance of the OTUs retrieved from bulk soil samples not treated (NT) or fertilised with urea (UR), ammonium sulphate (AS) or potassium nitrate (PN). OTUs correspond to the family level. Soil samples correspond to the first (1) and fourth (4) harvests carried out in Chapter III.1. Only OTUs with >1% relative abundance in at least one sample were included in this analysis.*

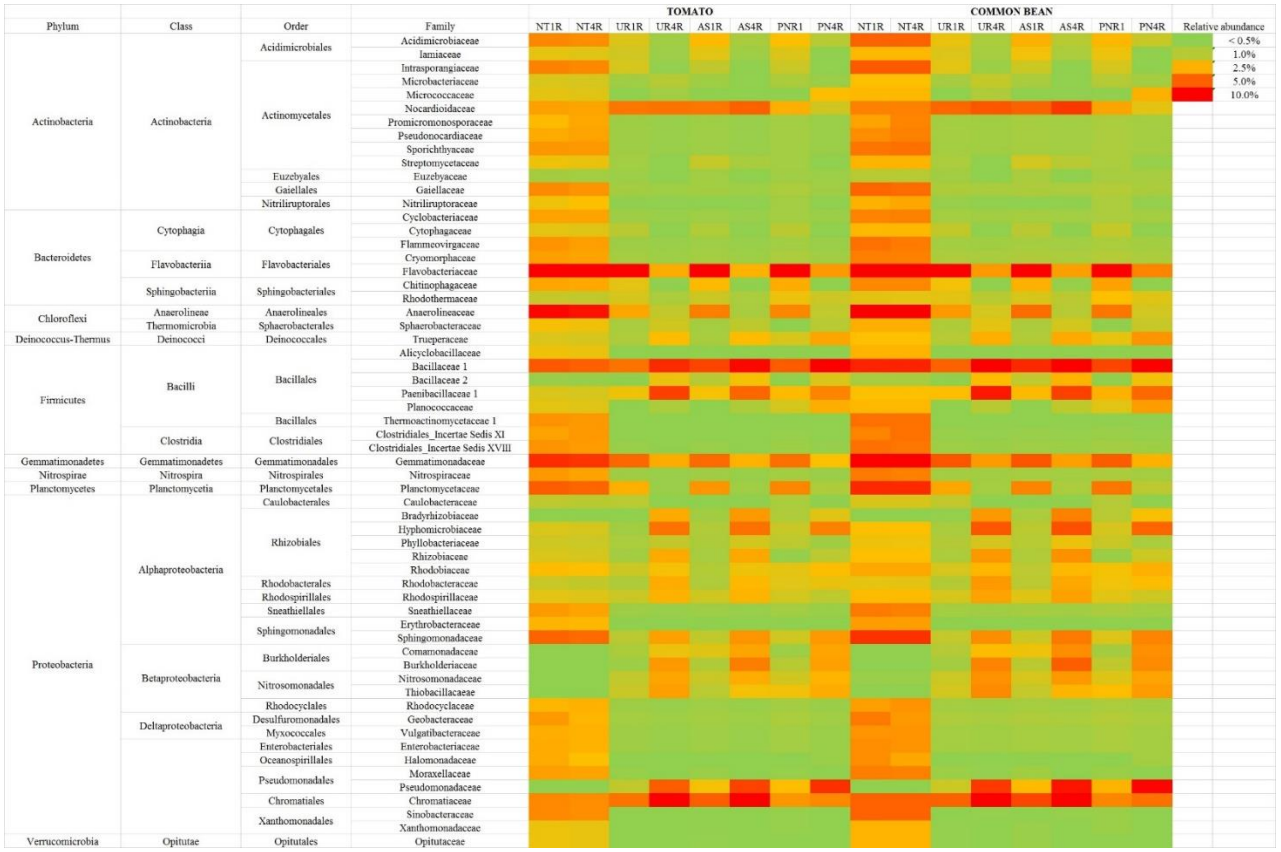



*Fig. S2. Heat map showing the relative abundance of the OTUs retrieved from bulk soil samples not treated (NT) or fertilised with urea (UR), ammonium sulphate (AS) or potassium nitrate (PN). OTUs correspond to the family level. Soil samples correspond to the first (1) and fourth (4) harvests carried out in Chapter III.1. Only OTUs with >1% relative abundance in at least one sample were included in this analysi*

# Chapter IV

## Occurrence and $^{15}N$-quantification of simultaneous nitrification and denitrification in N-fertilised soils incubated under oxygen-limiting conditions

**Submitted for publication**

## Abstract

Nitrification and denitrification are known to co-occur in soils, but information on the relative source partitioning of the $N_2O$ produced in different soils and conditions is still lacking. Here, we have used the $^{15}N$ tracer technique to explore the relative contribution of nitrification and denitrification to $N_2O$ production by soils treated with $(NH_4)_2SO_4$ or $KNO_3$ for 3 years. After that time, the soils were amended with $(^{15}NH_4)_2SO_4$ or $K^{15}NO_3$ and incubated at 80% water filled pore space for 30 days. $N_2O$ emissions, $NH_4^+$ and $NO_3^-$ and their corresponding $^{15}N$-enrichment were determined. The effect of N addition on N transformation rates was also estimated. The total abundance of nitrifiers was estimated by quantitative PCR (qPCR) of the *amoA* gene from bacteria and archaea, and that of denitrifiers by using the *nirK*, *nirS*, *norB* and *nosZ*I genes as molecular targets. In the ammonium-treated soil, the $N_2O$ originated by nitrification and denitrification during incubation ranged from 49.0 to 58.0% and from 42.0 to 51.0%, respectively. The production of $N_2O$ was accompanied by a decrease in the $NH_4^+$ content and a parallel increase in the concentration of $NO_3^-$, both labelled and non-labelled. Also, the abundance of the bacterial and archaeal *amoA* gene increased during incubation. On the other hand, in the soil treated with nitrate, the $^{15}N$-tracer technique showed that denitrification contributed with 84.0 to 99.0% to the total $N_2O$ produced. Decreases in labelled and unlabelled $NO_3^-$ paralleled the increase in $^{15}N$ enrichment of $N_2O$ and the abundance of the *nirK*, *nirS*, *norB* and *nosZ*I genes. The results also showed that values of $^{15}N_2$ enrichment were significantly higher in the nitrate-treated soil, which agrees with the higher abundance of the *nosZ*I gene. A study on the N transformation rates indicated that autotrophic and heterotrophic nitrification were responsible for $N_2O$ production in the ammonium-treated soil and that denitrification was the most important $N_2O$ source in the soil treated with nitrate.

Keywords: nitrous oxide, nitrification, denitrification, isotopes, qPCR

## Introduction

Anthropogenic sources of the greenhouse gas nitrous oxide ($N_2O$) are primarily generated from agricultural soils amended with nitrogen (N) fertilisers, representing up to 70% of the annual global fluxes emitted to the atmosphere (Erisman et al. 2015; Smith 2017). Nitrous oxide contributes ~6% of the radiative forcing by long-lived greenhouse gases and it is the third most important individual contributor to the combined forcing (WMO 2017). $N_2O$ production in soils results predominantly from a range of microbial pathways, but two N transformations, namely nitrification and denitrification, are generally considered to be the main $N_2O$ producing processes (Reay et al. 2012; Butterbach-Bahl et al. 2013; Hu et al. 2015). The emissions of $N_2O$ are influenced by several biotic and abiotic factors, of which an N source is fundamental (Reay et al. 2012; Butterbach-Bahl et al. 2013; Hu et al. 2015). In addition, the favourable conditions for $N_2O$ production from nitrification typically occur within the range of 30-60% water-filled pore space (WFPS) and denitrification dominates in wet soils with WFPS >70% (Davidson 1991b; Bateman and Baggs 2005; Braker and Conrad 2011; Hu et al. 2015). Recently, Castellano-Hinojosa et al. (2018) reported the occurrence of $N_2O$ down a soil profile with decreasing dissolved oxygen concentrations; nitrification was the main driver of $N_2O$ production in the first 0 to 10 cm of the arable topsoil layer, while denitrification was the main process from 10 to 20 cm depth. Although nitrification is an aerobic process and denitrification is carried out under oxygen-limiting conditions, both microbial pathways can simultaneously take place within soil aggregates, for example, in oxic microsites under water-saturated environments and in anoxic surroundings in well-aerated soils (Barnard et al. 2005; Butterbach-Bahl et al. 2013; Zhu et al. 2013). Nitrification activity was not supressed by low oxygen concentrations in paddy soils (Yang et al. 2016b) and active nitrification was found under moisture conditions >70% WFPS in soils treated with ammonium ($NH_4^+$)-based fertilisers (Hao et al. 2003; Meng et al. 2005; Ding et al. 2010; Huang et al. 2014), for example. The existence of denitrification under oxic conditions has also been reported (Takaya et al. 2003; Gao et al. 2009; Ji et al. 2015).

Attributing $N_2O$ emissions specifically to nitrification or denitrification is difficult due to the different phylogenetic groups of the soil nitrifier and denitrifier guilds involved in the processes leading to the production of $N_2O$ (Levy-Booth et al. 2014; Hallin et al. 2018), as well as to the difficulty of absolutely assigning $N_2O$ produced to a particular process with certainty. The acetylene inhibition assay (Baggs 2008; 2011) and the $^{15}N$-tracing technique (Arah 1977; Stevens et al. 1997; Müller et al. 2004, 2007, 2014) were developed to identify $N_2O$ sources and other N transformations occurring in the soil following the application of $^{15}N$-labelled fertilisers. The latter technique has been used to estimate $N_2O$ emissions from nitrification and denitrification under $O_2$-limiting conditions (Mathieu et al. 2006; Morse and Bernhardt 2013; Huang et al. 2014; Han et al. 2018). This approach is based on the application of $^{15}N$-labelled ammonium ($NH_4^+$) and/or labelled nitrate ($NO_3^-$) to soil where the attribution of the $^{15}N$-$N_2O$ fluxes to nitrification or denitrification depend on the $^{15}N$ source applied, as nitrifier-, denitrifier-$N_2O$ and $^{15}N$-dinitrogen ($N_2$) can be quantified. $^{15}N$ is usually applied at $>$ 1 atom % excess $^{15}N$, at which level isotopic fractionation appears to be independent of the isotopic enrichment (Booth et al. 2005; Baggs 2008).

During studies on the effect of the application of ammonium sulfate [$(NH_4)_2SO_4$] and potassium nitrate ($KNO_3$) on $N_2O$ emissions by an Eutric Cambisol soil, the observation was made that under high moisture conditions the $NO_3^-$ content in soils treated with [$(NH_4)_2SO_4$] increased over a 3-year incubation period. Hence, we hypothesised that simultaneous nitrification and denitrification occurs in the soil and that both processes could act as $N_2O$ source. Here, we have used the $^{15}N$ tracer technique to explore the relative contribution of nitrification and denitrification to $N_2O$ production by the N-fertilised soils. The soils were amended with $^{15}N$-labelled substrates and incubated at 80% WFPS for 30 days. The effect of N-fertilisation on N transformation rates was also estimated. $N_2O$ emissions and their $^{15}N$-enrichements were determined by spot sampling during the incubation period. The abundances of the *amoA* gene from ammonia-oxidising Bacteria and Archaea and of the genes involved in the synthesis of the enzymes responsible for the sequential

reduction of $NO_3^-$ to nitrite ($NO_2$-; *napA*, *narG*), nitric oxide (NO; *nirK*, *nirS*), $N_2O$ (*norB*) and $N_2$ (*nosZI*) were also determined.

## Materials and methods

### Soil preparation

The main physicochemical properties of the soil used in this study have already been published (Castellano-Hinojosa et al. 2018). Briefly, the soil was a sandy-loam Eutric Cambisol (pH in water 6.8; total C 25 mg $kg^{-1}$; total N 1.02 mg $kg^{-1}$; $NO_3^-$ 6.8 mg $kg^{-1}$; exchangeable $NH_4^+$ not detected) maintained without fertilization and no irrigation for at least 10 years (UTM coordinates 36° 43′ 53.5″ N, 3° 32′ 56.2″ W). Arable top soil (25 cm) samples were taken from 12 different locations, freed of roots and plant residues and mixed together to obtain a composite sample. The composite sample was then divided into three and maintained as a no treatment control (NTC), or had either $(NH_4)_2SO_4$ or $KNO_3$ added (henceforth identified as the AS and PN soils, respectively) at a rate of 260 kg N $ha^{-1}$. Even distribution of the fertiliser treatments was achieved by mixing with a concrete mixer. The soils were used to fill 20-kg capacity containers (54 × 21 × 25 cm, long, wide and depth, respectively) and kept for 3 years under controlled environmental conditions (Tortosa et al. 2015). Pots were watered once a week to 80% water-filled pore space (WFPS) and varied from 62 to 80% during the week. The concentration of extractable $NH_4^+$ and $NO_3^-$ was determined every 12 months and the soil supplemented with the previously applied N-fertiliser to 260 kg N $ha^{-1}$. This resulted in three soil pools that had been treated (fertilised) consistently differently for three years (NTC-3, AS-3 and PN-3).

### Experimental setup

Cylindrical, closed-base plastic cores (6.3 cm diameter, 10 cm height) were packed to a bulk density of 0.8 g $cm^{-3}$ with 30 g of air-dried, sieved to < 2 mm, NTC-3 soil (36 cores), AS-3 soil (36 cores), or PN-3 soil (36 cores) and placed into 500 ml Kilner jars (3 cores/jar). The cores containing the AS-3 soil received 6.2 μmol $NH_4^+$-N $g^{-1}$ (equivalent to 150 kg N $ha^{-1}$), of which one half (18 cores) was amended with $(^{15}NH_4)_2SO_4$ (AS-3_$^{15}$AS) and the other half with $K^{15}NO_3$ (AS-3_$^{15}$PN). Similarly, the cores with the PN-3 soil were each supplemented with 6.2 μmol $NO_3^-$-N $g^{-1}$

(equivalent to 150 kg N $ha^{-1}$) and then separated in two groups (18 cores/group) that received $(^{15}NH_4)_2SO_4$ (PN-3_$^{15}$AS) or $K^{15}NO_3$ (PN-3_$^{15}$PN). The $^{15}N$ enriched ammonium and nitrate was mixed with natural abundance to produce $^{15}N$ labelled treatments at 10 atom %. Before the treatments were applied, to avoid the pulse of respiration associated with wetting (Kieft et al. 1987) the soils were watered with distilled water to reach 40% WFPS and incubated for 2 days. Labelled $^{15}N$-compounds were dissolved in distilled water and added to the soil to reach 80% WFPS. The 18 cores containing NTC soil did not receive any N amendment, were watered to reach 40% WFPS, incubated for 2 days and finally re-watered with distilled water to reach 80% WFPS.

The jars containing the soil cores were kept in a cabinet at 22/16 ºC day/night temperature, on a 16h/8h light/dark cycle for 30 days after amendment. During incubation, the jars remained open to maintain aerobic conditions in the headspace. The cores into the jars were watered weekly to 80% WFPS by weight by adding distilled water from the top. The WFPS varied from 79.1 $\pm$ 1.1% to 64.2 $\pm$ 1.6% after and before watering the pots, respectively. Eighteen jars were used for determination of $N_2O$ emissions and other 18 served for destructive soil sampling.

### Nitrous oxide analyses

Based on previous experiments, it was decided what frequency of sampling to follow: $N_2O$ emissions were sampled after incubation for 0, 1, 4, 8, 11, 15, 18, 22, 26 and 30 days after addition of the amendments. Sampling was conducted by sealing the jars and withdrawing 5 ml headspace gas with a syringe at 0, 20 and 40 mins after lid closure and transferring the sample to 12 ml pre-evacuated vials. $N_2O$ concentrations were analysed by gas chromatography using a Perkin Elmer Clarus 500 gas chromatograph (Perkin Elmer Instruments, Beaconsfield, UK) equipped with an electron capture detector. Cumulative emissions of $N_2O$ were calculated from the area under the curve after linear interpolation between sampling points.

### Isotopic analyses of $N_2O$ and $N_2$

For $^{15}N$ analyses, 15 ml headspace samples were taken after sealing the jars for 40 mins, and injected into pre-flushed and evacuated 12 ml exetainers (Labco).

Sampling for headspace gas $^{15}N$-enrichments was conducted in parallel with that for $N_2O$ determinations; once the $N_2O$ emissions were known, only the samples obtained after incubation for 1, 4, 8, 11 and 30 days were selected for $^{15}N$ analysis. $^{15}N$ enrichment of $N_2O$ and $N_2$ was measured using a TG2 trace gas analyser interfaced to a Sercon 20-22 isotope ratio mass spectrometer as reported by Loick et al. (2016).

### Soil analyses

The extractable $NH_4^+$ and $NO_3^-$ concentrations in soil samples taken at 8, 15, 22 and 30 days of incubation were analysed by automated colorimetry from 2 M KCl soil extracts using a Skalar SANPLUS Analyser (Skalar Analytical B.V., Breda, Netherlands) (Searle, 1984). The $^{15}N$-enrichment of $NH_4^+$ and $NO_3^-$ was calculated after the conversion of $NO_3^-$ to NO by vanadium chloride ($V(III)Cl_3$) and the oxidation of $NH_4^+$ to $N_2$ by sodium hypobromite (NaOBr) as described by Laughlin et al. (1997) and Stevens and Laughlin (1998). $^{15}N$ enrichment of the resultant $N_2O$ and $N_2$ was measured as above using a TG2 trace gas analyser interfaced to a Sercon 20-22 isotope ratio mass spectrometer (Loick et al. 2016).

### Nitrification and denitrification contribution to $N_2O$ emission

The amount of $N_2O$ derived from the $^{15}N$-fertiliser amendment was calculated according to Senbayram et al. (2009):

$$N_2O\ N_{amend} = N_2O\_N_{total}\ (^{15}Nat\%ex_{sample}/\ ^{15}Nat\%ex_{fert})\ \text{(Equation 1)}$$

where $N_2O\_N_{total}$ = total emissions of $N_2O$ from the soil; $^{15}Nat\%\ ex_{sample}$ is the $^{15}N$ atom % excess of the emitted $N_2O$ ($^{15}N$ atom % of the measured sample minus the mean natural abundance $^{15}N$ of background $N_2O$ obtained in our experiment, 0.386 atom %); and $^{15}Nat\ \%ex_{fert}$ is the $^{15}N$ atom % excess of the applied amendment solution.

The percentages of $N_2O$ originating from nitrification and denitrification were determined according to Stevens et al. (1997), considering that the fraction of $N_2O$ derived from the denitrification (d) and nitrification ($1 - d$) pool can be calculated as:

$d = (a_m - a_n)/ (a_d - a_n)$ (with $a_d \neq a_n$) (Equation 2)

where $a_m$, $a_d$ and $a_n$ are the average of the $^{15}N$ atom enrichment of the $N_2O$ mixture and the $NO_3^-$ and the $NH_4^+$ pools, respectively.

### Quantification of N transformation rates

The $^{15}N$ tracing model was used to quantify the simultaneously occurring gross N transformation rates in the soil (Müller et al. 2004; 2007, 2014). The concentration of $NH_4^+$-N and $NO_3^-$-N and their $^{15}N$ enrichments (average ± standard error) were used as the input data set for the model. The Akaike information criterion was used to select the most appropriate model (Rütting and Müller, 2007; Rütting et al. 2008). The final model considered five N pools, i.e., $NH_4^+$, $NO_3^-$, labile ($N_{lab}$) and recalcitrant ($N_{rec}$) organic N, and adsorbed $NH_4^+$ ($NH_4^+{}_{ads}$), and eight N transformation processes including: 1) mineralization of $N_{lab}$ to $NH_4^+$ ($M_{Nlab}$); 2) mineralization of $N_{rec}$ to $NH_4^+$ ($M_{Nrec}$); 3) immobilization of $NH_4^+$ to $N_{lab}$ ($I_{NH4\text{-}Nlab}$); 4) immobilization of $NH_4^+$ to $N_{rec}$ ($I_{NH4\text{-}Nrec}$); 5) oxidation of $NH_4^+$ to $NO_3^-$ ($O_{NH4}$, autotrophic nitrification); 6) oxidation of $N_{rec}$ to $NO_3^-$ ($O_{Nrec}$, heterotrophic nitrification); 7) immobilisation of $NO_3^-$ to $N_{rec}$ ($I_{NO3}$); 8) dissimilatory $NO_3^-$ reduction to $NH_4^+$ (DNRA, $D_{NO3}$). The $^{15}N$ tracing model allows the calculation of N transformation rates based on zero-order, first-order or Michaelis-Menten kinetics enabling more realistic simulation of N dynamics.

### DNA extraction and quantification of 16S rRNA, nitrification and denitrification genes

Soil samples for DNA extraction were taken 30 d after addition of the amendments, including the control soil. Total DNA was extracted and purified from 500 mg of soil as described earlier (Correa-Galeote et al. 2014; Castellano-Hinojosa et al. 2018) and the concentration measured using the Qubit® ssDNA assay kit (Molecular Probes). The total bacterial (16SB) and archaeal (16SA) community was quantified by qPCR using the corresponding 16S rRNA gene as molecular marker. The size of the nitrifier community was estimated by qPCR of the *amoA* gene from Bacteria (*amoA* AOB) and Archaea (*amoA* AOA), and that of the denitrifier community was calculated using the *nirK/nirS*, *norB* and *nosZ*I genes. Primers and thermal conditions for qPCR were described previously (Correa-Galeote et al. 2014;

Castellano-Hinojosa et al. 2018). qPCR efficiency for the different assays ranged from 90% to 99%. The quality of qPCR amplification was verified by electrophoresis in agarose and by a melting curve analysis. Gene abundances were also analysed as relative abundances (gene copy number/16S rRNA gene Bacteria or Archaea copy number).

### Statistical analysis

Statistical analysis was performed using Statgraphics Centurion XVII software (StatPoint Technologies Inc., Warrenton, VA, USA). The measured variables were first explored using the Shapiro-Wilk test and the Bartlett´s test to check whether they meet the normality and homoscedasticity assumptions, respectively. Differences in the measured variables between treatments or sampling times were assessed by ANOVA at $p < 0.05$ with a Tukey test for post-hoc comparisons.

## Results

### $N_2O$ fluxes and cumulative emissions

$N_2O$ fluxes from the different soils are shown in Fig. 1. Emissions from the AS-3_15AS soil reached the maximum value after incubation for 4 d (0.35 kg N $ha^{-1}$ $d^{-1}$), then decreased, reaching a basal level on day 15 (0.10 kg N $ha^{-1}$ $d^{-1}$) which was maintained until the end of the incubation. In the AS-3_$^{15}$PN soil, $N_2O$ fluxes also peaked on day 4 (0.42 kg N $ha^{-1}$ $d^{-1}$) and then declined to a basal level by day 15 (0.10 kg N $ha^{-1}$ $d^{-1}$). In the PN-3_$^{15}$AS soil, maximum $N_2O$ emissions were observed on the first day (0.19 kg N $ha^{-1}$ $d^{-1}$) and then gradually decreased until a basal level of 0.09 kg N $ha^{-1}$ $d^{-1}$ was reached on day 15. The highest $N_2O$ emission from the PN-3_$^{15}$PN soil was detected on day 8 (0.20 kg N $ha^{-1}$ $d^{-1}$) and was followed by a decrease to a basal level by day 15 (0.09 kg N $ha^{-1}$ $d^{-1}$). Cumulative emissions of $N_2O$ were significantly greater in the AS-3_$^{15}$PN treatment (4.7 kg N $ha^{-1}$) than in the AS-3_$^{15}$AS treatment (3.1 kg N $ha^{-1}$). Differences in the cumulative emission of $N_2O$ between the PN-3_$^{15}$AS and PN-3_$^{15}$PN treatments were not found (1.1 kg and 1.0 kg N $ha^{-1}$, respectively). The NTC soil showed a basal level of $N_2O$ emissions within the range of 0.02-0.04 kg N $ha^{-1}$ $d^{-1}$ over the experimental period and cumulative emissions of $N_2O$ accounted for less than 0.1 kg N $ha^{-1}$ after 30 d (data not shown). After

incubation for 30 days, a calculated 2.1, 3.1, 0.7 and 0.6% of the applied N was emitted as $N_2O$ in the AS-3_$^{15}$AS, AS-3_$^{15}$PN, PN-3_$^{15}$AS and PN-3_$^{15}$PN treatments, respectively.

### Soil mineral N

Extractable $NH_4^+$-N concentrations decreased in the AS-3_$^{15}$AS and AS-3_$^{15}$PN treatments from day 8 (3.7 and 4.6 μg N $g^{-1}$ dry soil, respectively) until day 30 (1.1 and 3.0 μg N $g^{-1}$ dry soil, respectively) (Fig. 2A). This decrease was accompanied by an increase in extractable $NO_3^-$-N concentrations (1.9 and 0.9 μg N $g^{-1}$ dry soil, respectively on day 8 to 3.4 and 1.9 μg N $g^{-1}$ dry soil, respectively on day 30) (Fig. 2B).

The concentration of $NH_4^+$-N was negligible in the PN-3_$^{15}$AS and PN-3_$^{15}$PN treatments, with values lower than 0.02 μg $g^{-1}$ dry soil at all sampling dates (Fig. 2A). Nitrate concentrations in the PN-3_$^{15}$AS and PN-3_$^{15}$PN treatments decreased from day 8 (5.2 and 4.6 μg $g^{-1}$ dry soil, respectively) to day 30 (4.2 and 3.2 μg $g^{-1}$ dry soil, respectively) (Fig. 2B). The average concentrations of $NH_4^+$-N and $NO_3^-$-N were approximately 0.02 μg N $g^{-1}$ dry soil in the control soil during the experimental period (data not shown).

### $^{15}$N enrichment

The $^{15}$N enrichment of $NH_4^+$ in the AS-3_$^{15}$AS and PN-3_$^{15}$AS treatments was 9.475 and 9.578 atom %, respectively, on day 8, and 5.645 and 7.357 atom %, respectively, by day 30 (Fig. 3A). These decreases were accompanied by increases in the $^{15}NO_3^-$ content, with enrichments varying from 0.853 and 0.708 atom % on day 8 to 4.235 and 2.573 atom % on day 30, (AS-3_$^{15}$AS and PN-3_$^{15}$AS, respectively) (Fig. 3B). The $^{15}$N enrichment of $NH_4^+$ found in the AS-3_$^{15}$PN and PN-3_$^{15}$PN treatments did not change much during the incubation, remaining within the ranges of 1.096-1.327 and 0.759-0.999 atom %, respectively (Fig. 3A). In these treatments, the $^{15}$N enrichment of $NO_3^-$ decreased from 9.206 and 7.314 atom % on day 8 to 7.125 and 1.316 atom % on day 30 (AS-3_$^{15}$PN and PN-3_$^{15}$PN, respectively) (Fig. 3B).

In the AS-3_$^{15}$AS treatment, the $^{15}$N enrichment of $N_2O$ diminished from 3.915 atom % on day 8 to 2.055 atom % on day 11 and afterwards increased to 4.324 atom

% by day 30 (Fig. 3C). The $^{15}N$ atom % of $N_2O$ from the AS-3_$^{15}$PN treatment was 1.685 atom % on day 4, it then decreased to 1.005 atom % on day 11 and appeared to remain at this level until the end of the experiment (Fig. 3C). In the PN-3_$^{15}$AS and PN-3_$^{15}$PN treatments, the $^{15}N$ enrichment of $N_2O$ gradually increased to 0.725 atom % and 1.475 atom %, respectively, on day 11 and significant changes were not detected afterwards (Fig. 3C). In all treatments, $N_2$ $^{15}N$-enrichments rose until day 11 (to maximal values of 1.184, 0.704, 0.642 and 0.427 atom % for the PN-3_$^{15}$PN, PN-3_$^{15}$AS, AS-3_$^{15}$PN and AS-3_$^{15}$AS treatments, respectively) and appeared to remain at these levels until the end of the experiment (Fig. 3D).

### Effect of the N source on $N_2O$ production pathways

Approximately 90% of the $N_2O$ emitted during the 30-d incubation originated from the applied $^{15}N$-fertiliser, and around 10% was derived from native soil $NO_3^-$ (data from Equation 1). The analysis of the $^{15}N$ enrichment in $N_2O$ and mineral N pools showed that 49.0-58.0% of the $N_2O$ in the AS soil originated from nitrification (AS-3_$^{15}$AS treatment) and 42.0-51.0% derived from denitrification (AS-3_$^{15}$PN treatment) (data from Equation 2, Fig. 4). In the PN soil, 84.0-99.0% of the $N_2O$ resulted from denitrification (PN-3_$^{15}$PN treatment) and 1.0-16.0% came from nitrification (PN-3_$^{15}$AS treatment) (Fig. 4).

### Gross N transformation rates

Gross nitrogen transformation rates over the incubation, calculated using Müller et al.'s (2004, 2007, 2014) $^{15}N$ tracing model, are shown in Table 1. The total mineralization rate ($M_{Nlab} + M_{Nrec}$) was much higher in the AS-3 soil (0.614 μg N $g^{-1}$ $d^{-1}$) than in the PN-3 soil (0.012 μg N $g^{-1}$ $d^{-1}$), and $M_{Nrec}$ was the main contributor to mineralisation in the AS-3 soil. In this soil, the rate of $I_{NH4\text{-}Nlab}$ was 1.123 μg N $g^{-1}$ $d^{-1}$ and 0.383 μg N $g^{-1}$ $d^{-1}$ that of $I_{NH4\text{-}Nrec}$, but, $NH_4^+$ immobilisation was barely detected in the PN-3 soil. $O_{NH4}$ and $O_{Nrec}$ were negligible in the PN-3 soil, but the major pathways of $NH_4^+$ oxidation in the AS-3 soil (0.709 and 0.576 μg N $g^{-1}$ $d^{-1}$, respectively). Nitrification capacity, which is the ratio of $O_{NH4}$ to total mineralization, was 1.15 in the AS-3 soil and almost null in the PN-3 soil. Nitrate consumption was mostly due to immobilisation of $NO_3^-$ to $N_{rec}$ and only occurred to any great extent

in the PN-3 soil, as a rate of $I_{NO3}$ of 1.420 $\mu g\ N\ g^{-1}\ d^{-1}$ was estimated in the PN-3 soil, and was negligible in the AS-3 soil. Finally, $D_{NO3}$ rates were low in both soils.

### Quantification of 16S rRNA, nitrification and denitrification genes

The abundance of the bacterial communities significantly decreased and, conversely, the biomass of the archaeal communities increased in the AS-3_$^{15}$AS, AS-3_$^{15}$PN, PN-3_$^{15}$AS, and PN-3_$^{15}$PN soils compared to the NTC soil (Table 2). Differences in the abundance of the bacterial and archaeal communities were not found between the treatments (Table 2). The total and relative abundances of the *amoA* AOB and *amoA* AOA genes in the PN-3_$^{15}$AS and PN-3_$^{15}$PN soils were similar to those in the NTC soil, while higher abundances were found in the AS-3_$^{15}$AS and AS-3_$^{15}$PN soils (Table 2).

The total and relative abundances of the denitrification genes significantly increased in the AS-3_$^{15}$AS, AS-3_$^{15}$PN, PN-3_$^{15}$AS and PN-3_$^{15}$PN soils compared to the NTC soil (Table 2). While differences in the *nirK*, *nirS* and *norB* abundances were not found, the biomass of the *nosZI* was significantly greater in the PN-3_$^{15}$AS and PN-3_$^{15}$PN soils (Table 2).

### Discussion

Nitrification and denitrification are considered the main biological processes involved in $N_2O$ production in soils. Despite the nitrification enzymes being $O_2$-dependent and those involved in denitrification requiring $O_2$-limiting conditions, $N_2O$ production by nitrifiers in suboxic environments and by denitrification under oxic conditions have been reported (Hao et al. 2003; Meng et al. 2005; Ding et al. 2010; Huang et al. 2014; Ji et al. 2015; Yang et al. 2016b). The debate, however, about the amount of $N_2O$ produced by either process regardless of the content of the dissolved oxygen in soils still persists.

Here, in a mesocosm study, using the $^{15}$N-tracer technique we show the existence of simultaneous nitrification and denitrification in an Eutric Cambisol soil fertilised with ammonium sulphate for 3 years. After incubation of the soil for 30 d at 64.2-79.1% WFPS, the $N_2O$ originated by nitrification and denitrification was 49.0-58.0% and 42.0-51.0%, respectively (Fig. 4). Under high moisture contents,

nitrification is likely to occur in oxic microsites within the soil aggregates (Barnard et al. 2005; Butterbach-Bahl et al. 2013; Zhu et al. 2013). The production of $N_2O$ was accompanied by a decrease in the $NH_4^+$ content (Fig 2A) and a parallel increase in the concentration of $NO_3^-$ (Fig. 2B), a pattern that was also observed for the evolution of $^{15}NH_4^+$ (Fig. 3A) and of $^{15}NO_3^-$ (Fig. 3B) during incubation, respectively. In addition, there were significant increments in the abundance of the *amoA* AOB and *amoA* AOA genes (Table 2). All this shows the occurrence of nitrification activity under those conditions. In fact, using the $^{15}N$-tracer technique, nitrification has been involved in $N_2O$ production under elevated soil moisture conditions, with contributions ranging from 0.13-2.32% (Mathieu et. al (2006), 13.0-31.0% (Morse and Bernhardt 2013); 35.0-53.0% (Huang et al. 2014), and 18.4% (Han et al. 2018). These differences could be due to intrinsic biotic and abiotic site-dependent variables.

The total abundance of the *amoA* AOB gene (Table 2) was one order of magnitude greater than that of the *amoA* AOA; assuming similar ammonia monooxygenase activity rates, members of Bacteria would contribute, at least, 10 times more than Archaea to total $N_2O$ production. The *nor* bacterial operon contains the genes responsible for the synthesis of the $N_2O$-forming nitric oxide reductase enzyme; because the total abundance of *norB* was similar to that of the bacterial plus archaeal *amoA* genes, ammonia monooxygenase and nitric oxide reductase equally contributed to $N_2O$ production.

When the soil was fertilised with potassium nitrate during 3 years, the $^{15}N$-tracer technique showed that denitrification was the main source of nitrous oxide, contributing with 84.0-99.0% to the total $N_2O$ produced (Fig. 4). It also revealed that it was not $^{15}NH_4^+$ but $^{15}NO_3^-$ which produced the highest $^{15}N$ enrichment of the $N_2O$ released by the soil (Fig. 3C). The consumption of nitrate for denitrification over the incubation period (Fig. 2B) and the increase in the total abundance of each of the denitrification genes lend support to the existence of active denitrification activity in the PN soil (Table 2). Data in Fig. 3D show that values of $^{15}N_2$ enrichment in the PN-3 soil was significantly higher than that in the AS-3 soil, which is consistent with the higher relative and total abundance of the *nosZI* gene in the PN soil (Table 2).

Nitrous oxide production by Fungi under high moisture conditions has been reported (Shoun and Fushinobu 2017 and references therein); accordingly, the possibility that a proportion of the $N_2O$ detected in this study may originate from fungal nitrification/denitrification cannot be ruled out.

Considering the gross N transformation rates, autotrophic and heterotrophic nitrification were responsible for $N_2O$ production in the AS-3 soil (Table 1) and denitrification was the most important $N_2O$ source in the PN-3 soils as indicated by the $I_{NO3}$ transformation rates. Although DNRA has been shown to produce $N_2O$ (Baggs 2011; Butterbach-Bahl et al. 2013), rates of $D_{NO3}$ were almost null (Table 1).

## Acknowledgments

This study was supported by the ERDF-cofinanced grant PEAGR2012-1968 from Consejería de Economía, Innovación y Ciencia (Junta de Andalucía, Spain) and the MINECO-CSIC Agreement RECUPERA 2020. ACH is recipient of a grant of MECD (FPU 2014/01633).

*Table 1. Model parameters, their description, kinetics and gross rates of N transformations in AS-3 and PN-3 soils after incubation for 30 d with $^{15}N$-labelled fertiliser. For each soil, values of N transformation rates followed by the same lowercase letter in a column are not statistically different. For each model parameter, values followed by the same uppercase letter in a row are not statistically different between soils. ANOVA at $p < 0.05$ was used for statistical analyses ($n = 3$).*

| Model parameter | Transformation | Kinetics* | Soil N transformation rate (μg N $g^{-1}day^{-1}$) | |
|---|---|---|---|---|
| | | | AS-3 soil | PN-3 soil |
| $M_{Nlab}$ | Mineralisation of $N_{lab}$ to $NH_4^+$ | 1 | 0.059 ± 0.043bB | 0.004 ± 0.002bA |
| $M_{Nrec}$ | Mineralisation of $N_{rec}$ to $NH_4^+$ | 0 | 0.555 ± 0.024cB | 0.008 ± 0.004bA |
| $I_{NH4\text{-}Nlab}$ | Immobilisation of $NH_4^+$ to $N_{lab}$ | 1 | 1.123 ± 0.270eB | 0.000 ± 0.000aA |
| $I_{NH4\text{-}Nrec}$ | Immobilisation of $NH_4^+$ to $N_{rec}$ | 1 | 0.383 ± 0.262cB | 0.007 ± 0.003bA |
| $O_{NH4}$ | Oxidation of $NH_4^+$ to $NO_3^-$ | 1 | 0.709 ± 0.030dB | 0.000 ± 0.000aA |
| $O_{Nrec}$ | Oxidation of $N_{rec}$ to $NO_3^-$ | 0 | 0.576 ± 0.034cA | 0.677 ± 0.011cB |
| $I_{NO3}$ | Inmobilisation of $NO_3^-$ to $N_{rec}$ | 1 | 0.001 ± 0.001aA | 1.420 ± 0.070dB |
| $D_{NO3}$ | Dissimilatory reduction of $NO_3^-$ to $NH_4^+$ | 0 | 0.070 ± 0.005aA | 0.020 ± 0.000aA |

*0, zero-order kinetics; 1, first-order kinetics

*Table 2. Total abundance of the 16SB and 16SA, amoA AOB, amoA AOA, nirK, nirS, norB and nosZI genes in NTC, AS-3_$^{15}$AS, AS-3_$^{15}$PN, PN-3_$^{15}$AS and PN-3_$^{15}$PN soils after incubation for 30 d at 80% WFPS. Relative abundance (%) of the targeted genes is shown in brackets. For each column, values followed by different letters indicate significant differences according to ANOVA at $p < 0.05$ ($n = 3$).*

| Soil | Gene copy number x g$^{-1}$ dry soil | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 16SB | 16SA | *amoA* AOB | *amoA* AOA | *nirK* | *nirS* | *norB* | *nosZI* |
| NTC | 1.4 x $10^9$b | 3.3 x $10^6$a | 6.1 x $10^4$a (< 0.01) | 3.5 x $10^3$a (< 0.1) | 6.2 x $10^4$a (< 0.01) | 6.4 x $10^4$a (< 0.1) | 3.3 x $10^4$a (< 0.01) | 4.7 x $10^3$a (< 0.01) |
| AS-3_$^{15}$AS | 3.4 x $10^8$a | 2.7 x $10^7$b | 3.5 x $10^7$c (10.3) | 1.6 x $10^6$c (5.9) | 2.1 x $10^6$b (0.6) | 1.2 x $10^6$b (0.4) | 8.6 x $10^6$b (2.5) | 5.6 x $10^6$b (1.6) |
| AS-3_$^{15}$PN | 3.3 x $10^8$a | 2.6 x $10^7$b | 2.5 x $10^7$c (7.6) | 1.1 x $10^6$c (4.2) | 2.8 x $10^6$b (0.8) | 2.1 x $10^6$b (0.6) | 8.9 x $10^6$b (2.7) | 5.8 x $10^6$b (1.8) |
| PN-3_$^{15}$AS | 2.4 x $10^8$a | 2.7 x $10^7$b | 8.1 x $10^4$b (< 0.1) | 9.6 x $10^3$b (< 0.1) | 3.2 x $10^6$b (1.3) | 1.6 x $10^6$b (0.7) | 9.3 x $10^6$b (3.9) | 1.2 x $10^7$c (5.0) |
| PN-3_$^{15}$PN | 2.2 x $10^8$a | 2.3 x $10^7$b | 6.3 x $10^4$a (< 0.1) | 5.6 x $10^3$a (< 0.1) | 4.0 x $10^6$b (1.8) | 1.8 x $10^6$b (0.8) | 9.8 x $10^6$b (4.4) | 2.0 x $10^7$c (9.0) |

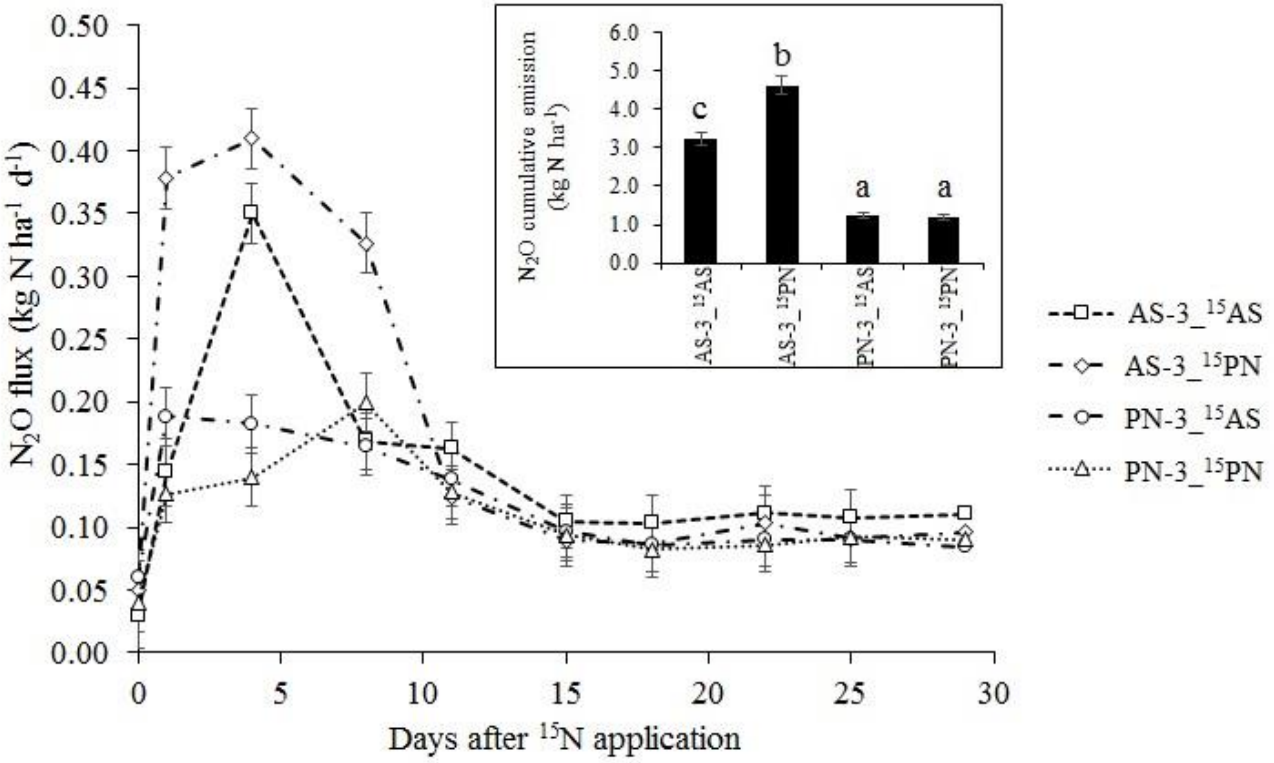



*Fig. 1. Nitrous oxide emissions from the AS-3_$^{15}$AS, AS-3_$^{15}$PN, PN-3_$^{15}$AS and PN-3_$^{15}$PN treatments over the course of a 30-d incubation. Cumulative $N_2O$ emissions for each treatment are shown in the inset. Rectangles with the same letter are not statistically different according to ANOVA at $p < 0.05$. Error bars indicate standard errors ($n = 3$).*

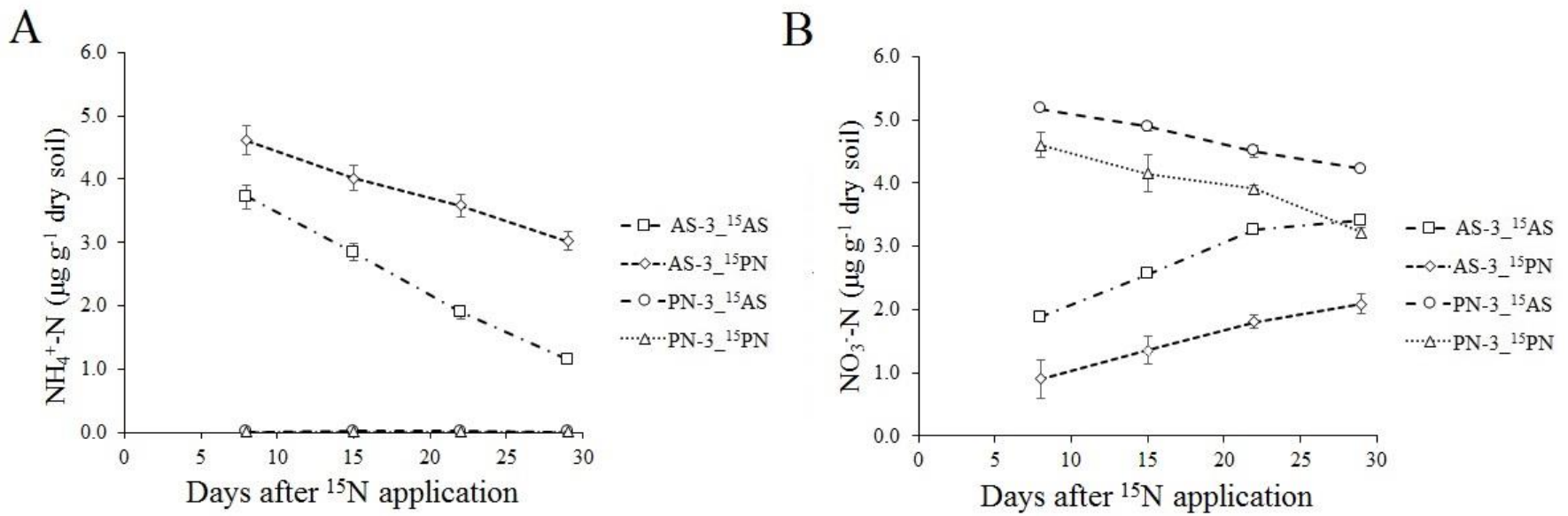



*Fig. 2. Concentrations of extractable soil $NH_4^+$ **(A)** and $NO_3^-$ **(B)** in AS-3_$^{15}$AS, AS-3_$^{15}$PN, PN-3_$^{15}$AS and PN-3_$^{15}$PN treatments over the course of a 30-d incubation. Error bars indicate standard errors (n = 3).*

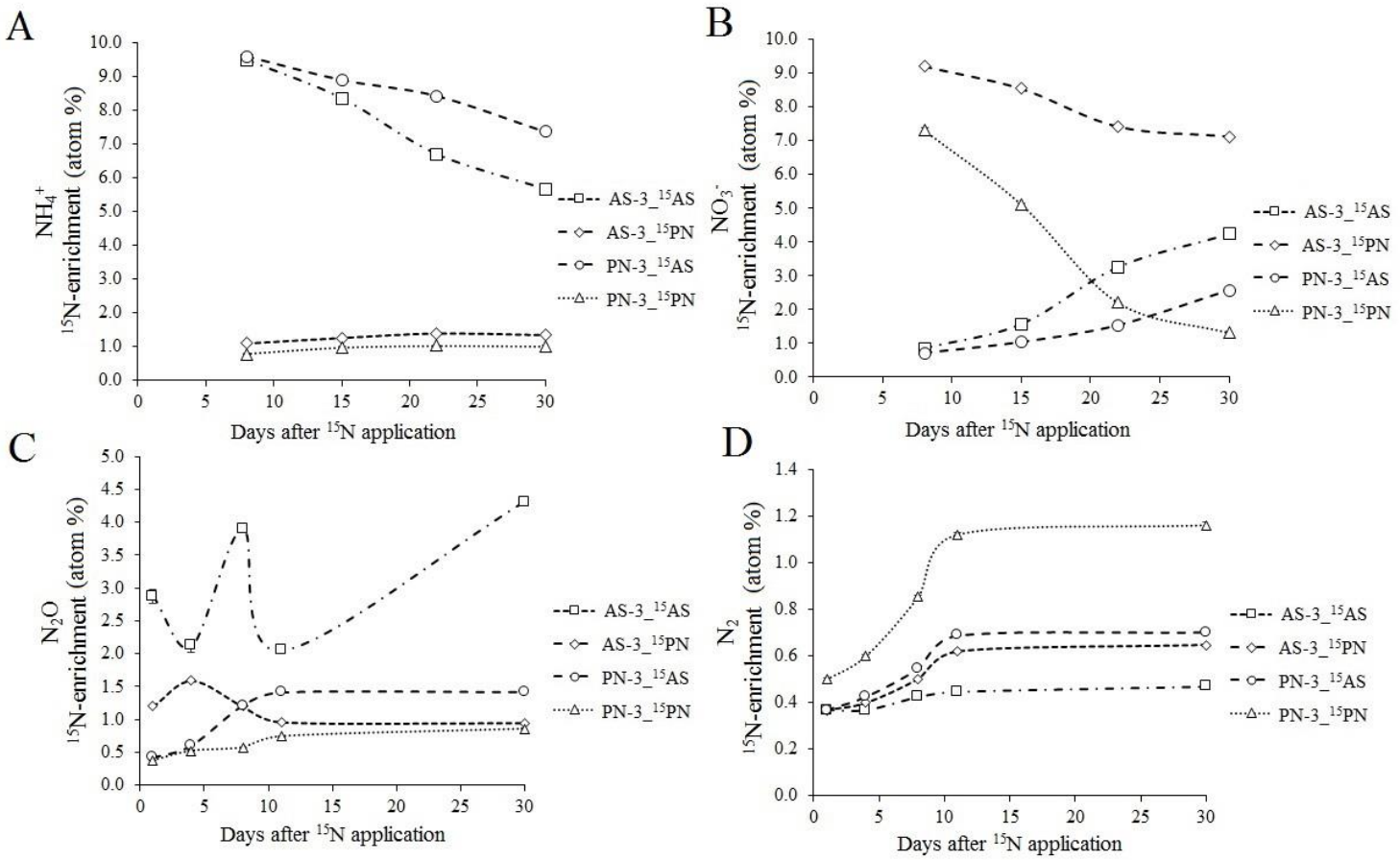



*Fig. 3.* *$^{15}$N-enrichments of $NH_4^+$ **(A)**, $NO_3^-$ **(B)**, $N_2O$ **(C)** and $N_2$ **(D)** in the AS-3_$^{15}$AS, AS-3_$^{15}$PN, PN-3_$^{15}$AS and PN-3_$^{15}$PN treatments over the course of a 30-d incubation. Error bars indicate standard errors (n = 3).*

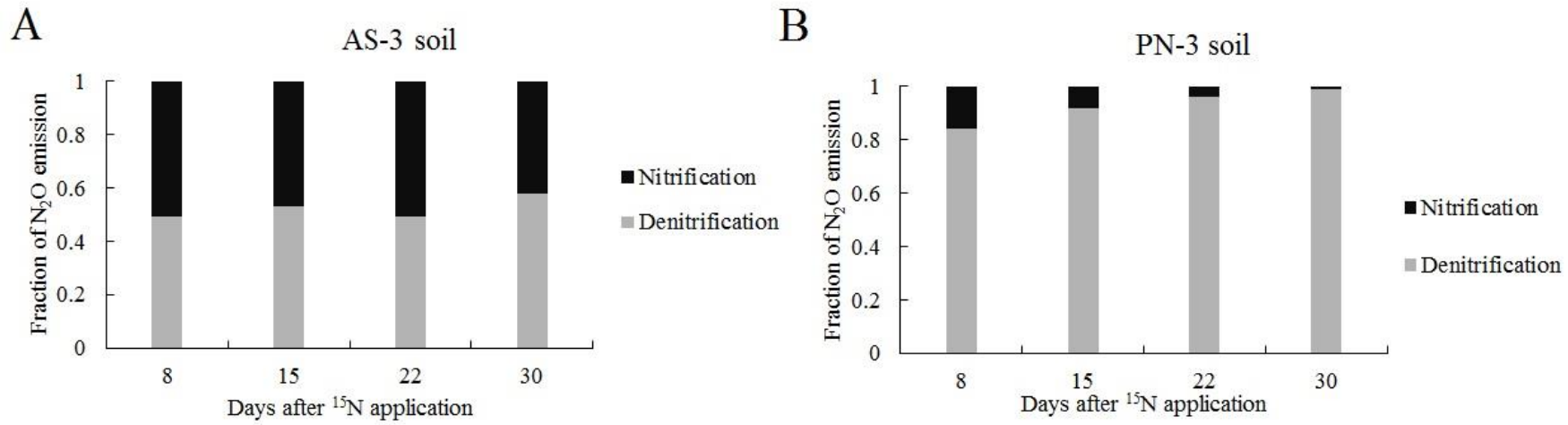



*Fig. 4. Fraction of the $N_2O$ emission from nitrification and denitrification in AS-3* ***(A)*** *and PN-3* ***(B)*** *soils during incubation for 30 d with $^{15}N$-fertiliser.*

# Chapter V

## Relative importance of soil properties, N-cycling genes and bacterial diversity for nitrous oxide emissions in uncultivated and cultivated soils

Abstract

Soil physicochemical properties, N-cycling gene abundance and bacterial diversity are abiotic and biotic drivers of nitrous oxide ($N_2O$) emission in soils. However, how they are related to each other and their individual significance on the $N_2O$ emissions are not well understood. Using a dataset containing results from previous studies on $N_2O$ emission by uncultivated and cultivated soils treated with the N-fertilisers urea, ammonium sulphate or potassium nitrate, a combined random forest (RF) and structural equation modelling (SEM) analyses was used to evaluate the relative importance and the direct and indirect effects of soil properties (pH, $NH_4^+$, $NO_3^-$, TOC, TC and TN), N-cycling gene abundance (*amoA*, *napA*, *narG*, *nirK*, *nirS*, *norB* and *nosZ*) and the relative abundance of bacterial OTUs (16S rRNA gene) as drivers for $N_2O$ emissions. The RF analysis showed that the $NH_4^+$ and $NO_3^-$ content, the *napA*, *nirK*, *norB*, *nosZ*I genes and a set of 16 bacterial OTUs were the main predictors of $N_2O$ emissions in uncultivated and cultivated soil. The SEM model showed that $N_2O$ emission was indirectly (positive) driven by the nitrifiers *amoA* gene and the denitrifiers *napA*, *narG*, *nirK*, *nirS*, *norB* gene and indirectly (negative) driven by the *nosZ* genes. The SEM model also indicated that $N_2O$ was directly controlled by the relative abundance of 16 bacterial OTUs, of which 3 belonged to the nitrifier guild and 13 were well-known denitrifiers. The $NH_4^+$ and $NO_3^-$ contents indirectly (positive) controlled the relative abundance of the bacterial OTUs. Overall, this study highlights that $N_2O$ are mainly controlled by biotic rather than abiotic factors and point to *norB* and *nosZI* genes and OTUs *Bacillus*, *Bradyrhizobium*, *Flavobacterium, Hyphomicrobium* and *Pseudomonas* as the best correlated variables for the study of $N_2O$ emission.

Keywords: N-fertilisation, nitrous oxide, nitrification, denitrification, gene abundance, bacterial diversity, RF and SEM models

## Introduction

In agricultural soils, the effect of the application of different nitrogen (N) fertilisers involve interactions of multiple factors and processes which are mainly associated with changes in soil physicochemical properties, emission of greenhouse gases and microbial ecology (Butterbach-Bahl et al. 2013; Hallin et al. 2018). It is well established that agricultural practices, through the application of N-fertilisers, contribute about 70% to the global production of the greenhouse gas nitrous oxide ($N_2O$), mainly due to the contribution of the microbial processes of nitrification and denitrification (Butterbach-Bahl et al. 2013; Hu et al. 2015; Smith 2017). The occurrence and influence of these processes is largely dependent of soil characteristics (i.e. texture, pH, available C and N and organic matter), climatic conditions (i.e. temperature and moisture) and agricultural management practices (i.e. type of cultivation, tillage and crop rotation) (Butterbach-Bahl et al. 2013; Hu et al. 2015). Moreover, the importance of each of the denoted factors can vary over time (Hallin et al. 2009), space (Regan et al. 2017), vertical soil profile (Castellano-Hinojosa et al. 2018) and with the type of the N-fertiliser applied (Burger and Rodney 2011).

Many studies have linked the production of $N_2O$ with the abundance of soil N-cycling genes using quantitative PCR (qPCR) (Hu et al. 2015; Hallin et al. 2018). In this sense, the abundance of the nitrifying community is estimated by qPCR of the *amoA* gene from Bacteria (*amoA* AOB) and Archaea (*amoA* AOA) (Hu et al. 2015), whereas the size of the denitrifying community is quantified using the genes coding for the enzymes nitrate- (*napA* and *narG*), nitrite- (*nirS* and *nirK*), nitric oxide- (*norB*) and nitrous oxide- reductases (*nosZ*I and *nosZ*II), respectively (Hallin et al. 2018). Based on results from meta-analyses studies, Ouyang et al. (2018) concluded that N application to agricultural ecosystems significantly increased the *amoA* AOA, *amoA* AOB, *nirK*, *nirS* and *nosZ* genes and that N-fertilisation form and duration, crop rotation and soil pH were main factors regulating the response of targeted N-cycling genes. Also, Carey et al. (2016) indicated that N-fertilisers increased the abundance of the nitrifiers, that AOB populations were more dynamic when faced to

enhanced N supply and that responses of AOB varied with the ecosystem, fertiliser type and soil pH.

N enrichment may result in indirect changes in soil properties which can lead to different bacterial diversity effects (Eilers et al. 2012; Vos et al. 2013; Zeng et al. 2016). Studies carried out to describe the effect of N-fertilisation on bacterial diversity have shown a frequently mixed and inconsistent effect associated with a high site-dependence (Ramirez et al. 2010; Wessén et al. 2010; Zeng et al. 2016; Regan et al. 2017). Moreover, the role of the different bacterial operational taxonomic units (OTUs) in driving $N_2O$ emissions have been poorly studied (Butterbach-Bahl et al. 2013; Powell et al. 2015; Delgado-Baquerizo et al. 2016).

Although efforts have improved our understanding of the relationships between the abundance of nitrifiers and denitrifiers and N losses from agroecosystems, a fundamental question remains widely debated: Are soil properties more important than N-cycling gene abundance and bacterial diversity as drivers of $N_2O$ emission? Or is one the cause of the other, therefore stimulation of a particular property generates a chain of events that results in increase in emissions? In particular, many studies have contributed data on specific microbial traits, which mean it is difficult to establish a general consensus since microbial communities' traits above and beyond that of environmental factors. However, despite the theoretical importance of microbial communities for the production of $N_2O$, a large body of the literature assumes that soil functions are driven mainly by soil properties with a minimal control by microbial communities (Powell et al. 2015; Delgado-Baquerizo et al. 2016). Recent studies provide evidence on the importance of specialised microbial communities for denitrification (Philippot et al. 2013a, b; Powell et al. 2015), due to its high dependency on particular physiological pathways; however, despite this, $N_2O$ emissions have been commonly reported to be more sensitive to resource availability than to microbial community (Robertson and Groffman 2007; Wang et al. 2013; Castaldelli et al. 2013; Graham et al. 2014). N-fertilisation in uncultivated and cultivated soils alters both soil properties and microbial communities in terrestrial ecosystems since roots sense and respond to changes in N inputs, which includes the regulation of gene expression, metabolism, and further N and C uptake

and assimilation (Richardson et al. 2009). Thus, the study of the distinct influences of soil properties, N-cycling genes and bacterial OTUs on $N_2O$ emission provides the opportunity to identify critical abiotic and biotic variables to accurately assess the response of ecosystem functioning as a source of $N_2O$.

In this study we have collected the data from previous studies on $N_2O$ emission, abundance of nitrification and denitrification genes and bacterial diversity from uncultivated and cultivated soils treated with urea, ammonium sulphate or potassium nitrate (Castellano-Hinojosa et al. 2018; Chapters II, III.1 and III.2). We used a combined random forest (RF) and structural equation modelling (SEM) analyses to evaluate as a whole: (1) whether $N_2O$ emission was primarily explained by changes in soil physicochemical characteristics, N-cycling gene abundance or bacterial diversity, (2) the relative importance of the N-cycling genes and bacterial OTUs as drivers of $N_2O$ emission, and (3) which N-cycling genes or bacterial OTUs could be used as a molecular marker for $N_2O$ production.

## Materials and methods

### Experimental setup and statistical analysis

The experimental setup of this study has been reported (Castellano-Hinojosa et al. 2018; Chapters II, III.1 and III.2). The dataset used in this study can be found in Annex I. Before conducting the analyses, all data were log-transformed to achieve normality. Two-way ANOVAs in IBM SPSS Statistics (SPSS Inc, Chicago, IL, USA) were used to evaluate the changes in soil properties [pH, ammonium ($NH_4^+$) and nitrate ($NO_3^-$) contents, total organic carbon content (TOC), total carbon (%TC) and total nitrogen (%TN), N-cycling gene abundance (*amoA* AOB, *amoA* AOA, *napA*, *narG*, *nirK*, *nirS*, *norB*, *nosZ*I and *nosZ*II) and the relative abundance of bacterial OTUs retrieved from uncultivated and cultivated soils. Only OTUs at genus level with ≥ 0.5% relative abundance in at least one of the samples were included in the analyses.

The Spearman's correlation analysis was used to evaluate: (1) the relationships between soil properties and N-cycling gene abundance with $N_2O$ emission and (2) the relationships between soil properties and bacterial OTUs with $N_2O$ emission. We

then evaluated which soil properties, N-cycling gene abundance or bacterial OTUs were the most important predictor for $N_2O$ emission using RF analysis (Breiman 2001). The soil properties, gene abundance and bacterial OTUs were selected as predictor variables and the $N_2O$ emission as the response variable. The analyses were conducted using the RF package included in the R statistical software (R Development Core Team 2008). The significance of the RFs and the cross-validated $R^2$ were assessed with 5000 permutations of the response variable using the A3 package included in the R statistical software. Similarly, the significance of each predictor was assessed using the rfPermute package included in the R statistical software. Finally, a structural equation modelling (SEM, Grace 2006) was conducted to evaluate how soil properties, N-cycling gene abundance and bacterial OTUs directly or indirectly determined $N_2O$ emission. Data from the control soils were selected as the baseline condition for the building of the structural equation models. Because the number of data on bacterial diversity was lower than those of the soil properties and N-cycling gene abundance, two different models were built, one including the soil properties and the N-cycling gene abundance and the other containing the soil properties and the bacterial OTUs. The genes with an equivalent function were included as a composite variable. The use of composite variables does not alter the underlying assumptions for the construction of the structural equation models; it also collapses the effects of multiple conceptually related variables into a single composite effect, aiding the interpretation of the model results (Shipley 2001). *A priori* model was designed (Fig. S1) and its overall goodness-of-fit evaluated using the Chi-square test ($\chi^2$; the model has a good fit when $0 \leq \chi^2 \leq 2$ and $0.05 < P \leq 1.00$) and the root mean square error of approximation test (RMSEA; the model has a good fit when $0 \leq \text{RMSEA} \leq 0.05$ and $0.10 < P \leq 1.00$ (Schermelleh-Engel et al. 2003). According to the metric of the tests, the *a priori* model had a satisfactory fit to the data, and thus no *post hoc* alterations were made. Finally, the total standardized effects, this is the sum of the direct and indirect effects, were calculated for each SEM model. SEM analyses were conducted using AMOS 20.0 (IBM SPSS, Chicago, USA).

## Results

### Soil properties versus N-cycling gene abundance as predictor of $N_2O$ emission

Among soil properties, only the content of $NH_4^+$ ($P = 0.022$) and $NO_3^-$ ($P < 0.001$) was positively related to $N_2O$ emission in uncultivated soils (Table 1). Likewise, the $NH_4^+$ ($P = 0.010$), $NO_3^-$ ($P < 0.001$) and TOC ($P < 0.010$) content were highly related to $N_2O$ emission in cultivated soils (Table 1). The *amoA* AOB ($P < 0.039$), *amoA* AOA ($P < 0.001$), *napA* ($P = 0.029$), *nirK* ($P < 0.015$) and *norB* ($P < 0.010$) gene abundance was positively related to $N_2O$ emission both in uncultivated and cultivated soils (Table 1). On the contrary, the *nosZ*I gene abundance ($P < 0.001$) was negatively related to $N_2O$ in both soils (Table 1).

The RF models identified both soil properties and N-cycling gene abundance as the main predictors of $N_2O$ emission in uncultivated (Fig. 1A) and cultivated soils (Fig. 1B), and the content of $NH_4^+$, $NO_3^-$ and TOC ($P < 0.01$) was a more important predictor than gene abundance ($P < 0.05$).

The SEM model showed that $N_2O$ emission is, albeit indirect, positively driven by the ammonia oxidising genes (*amoA* AOB + *amoA* AOA) and the denitrification genes (*napA* + *narG*, *nirK* + *nirS*, and *norB*) and negatively driven by the *nosZ*I + *nosZ*II genes (Fig. 2A). Overall, the model explained 91% of the variance in $N_2O$ emission. The standardised effect of the $NH_4^+$ content on nitrifiers and of the $NO_3^-$ content on denitrifiers, except the *napA* + *narG*-containing guilds, were the dominant predictors of $N_2O$ emission (Fig. 2B). When the direct effect was considered, the *nosZ* genes showed the most positive response to the $NO_3^-$ content followed by the *norB*, then *nirK* + *nirS* and, finally, the *napA* + *narG* genes. On the other hand, our model suggests that the TOC content directly controlled the abundance of the nitrifier and denitrifier communities (Fig. 2A). The pH had neither direct nor indirect effect on $N_2O$ emission (Fig. 2).

### Bacterial diversity as predictor of $N_2O$ emission

Out of the 70 different bacterial OTUs with ≥ 0.5% relative abundance in at least one of the samples analysed in this study, only 16 were positively correlated to $N_2O$ emission ($P < 0.05$) in uncultivated and cultivated soils (Table 2). Those OTUs were

represented by well-known nitrifiers and denitrifiers guilds, among which *Bacillus*, *Bradyrhizobium*, *Flavobacterium, Hyphomicrobium* and *Pseudomonas* were the most related to $N_2O$ emission ($P < 0.001$) (Table 2). On the other hand, the RF model revealed the bacterial OTUs over the soil properties as the main predictor of $N_2O$ emission in uncultivated (Fig. 3A) and cultivated soils (Fig. 3B).

Overall, the SEM model explained 84% of the variance of the $N_2O$ emission which were direct and positively driven by *Bacillus*, *Bradyrhizobium*, *Comamonas*, *Cupriavidus*, *Ensifer*, *Flavobacterium*, *Hyphomicrobium*, *Mycobacterium*, *Nitrosococcus*, *Nitrosomonas*, *Nitrosospira*, *Paracoccus*, *Pseudomonas*, *Rhodococcus Sphingomonas* and *Thiobacillus* (Fig. 4A). The $NH_4^+$ content, albeit indirect, had a positive effect on *Nitrosomonas, Nitrosospira*, *Nitrosococcus*; also the $NO_3^-$ content, though indirect, positively controlled the denitrifying OTUs (Fig. 4A). Our model also pointed to the TOC content having an indirect but positive effect on the nitrifying OTUs *Nitrosomonas, Nitrosospira* and *Nitrosococcus* (Fig. 4A). Soil pH and TN content had negligible effects on nitrifying and denitrifying OTUs (Fig. 4A). Based on the total standardized effects, *Bacillus*, *Bradyrhizobium*, *Flavobacterium, Hyphomicrobium* and *Pseudomonas* were the most important drivers of $N_2O$ emission (Fig. 4B). The TC content had neither direct nor indirect effect on $N_2O$ emission (Fig. 4 A,B).

## Discussion

In this study, a combined RF and SEM analysis was used to test the importance of soil properties, N-cycling gene abundance and bacterial diversity as drivers of $N_2O$ emission in uncultivated and cultivated soils treated with the N-fertilisers urea, ammonium sulphate or potassium nitrate. Previous studies have demonstrated that $N_2O$ emission is influenced by both abiotic (e.g. soil physicochemical properties and climatic factors) and biotic (e.g. N-cycling gene abundance, microbial OTUs diversity and microbial biomass) variables (Powell et al. 2015; Delgado-Baquerizo et al. 2016; Martins et al. 2017), but very few have dealt with the integration of the obtained data in a single analysis to better describe their relative importance and simultaneous relationships using RF and SEM approaches (Delgado-Baquerizo et al. 2016; Martins et al. 2017).

### Effects of soil properties and N-cycling gene abundance on regulation of $N_2O$ emission

Results in Table 1 and Fig. 1 provide evidence that out of the soil properties analysed in this study, the content of $NH_4^+$ and $NO_3^-$ was the main $N_2O$ emission determinants in the uncultivated and cultivated soils; also, the content of TOC could be considered a key predictor for $N_2O$ emission, but only for the cultivated soil. Because $N_2O$ emission is mainly originated during the microbial processes of nitrification and denitrification (Butterbach-Bahl et al. 2013; Hu et al. 2015), it is expected that $NH_4^+$ and $NO_3$ are good predictor for $N_2O$ emission. Other authors have also pointed out at $NH_4^+$ and $NO_3$ as key factors to explain $N_2O$ emission (Firestone and Davidson 1989; Butterbach-Bahl et al. 2013; Hu et al. 2015). Because the higher C content in the soil results in higher $N_2O$ emissions (Delgado-Vaquerizo et al. 2016 and references therein), it is not surprising that, after RF analysis, the TOC content resulted in an important predictor for the $N_2O$ emission from the cultivated soil (Table 1, Fig. 1). Higher TOC content in cultivated soils has been associated with root exudation (Richardson et al. 2009) and litter inputs from plants (Schlesinger and Bernhardt 2013), both resulting in higher availability of organic matter for microbial growth.

Among the genes included in this study, the RF analysis also revealed that the *amoA* AOB, *amoA* AOA, *napA*, *nirK*, *norB* and *nosZ*I genes were the most important predictors of $N_2O$ emission from uncultivated (Fig. 1A) and cultivated (Fig. 1B) soils. This is not surprising since nitrification and denitrification processes were involved in the emission of $N_2O$ (Chapter IV)

Considering the 3 variables above, the output of the SEM model showed that the soil properties, mainly the $NH_4^+$ and $NO_3$ content, directly influenced the N-cycling gene abundance and that $N_2O$ emission was under the indirect control of the targeted genes (Fig. 2). Based on the highest regression weight and size effect (Fig. 2A, B), the $N_2O$ emission was positively controlled by *norB* and negatively regulated by *nosZ*. The presence of these two genes has been shown in 83% of the 300 genomes analysed from denitrifying bacterial genomes (Hallin et al. 2018). The output also revealed that *amoA* AOA + *amoA* AOB had an indirect but positive effect on the $N_2O$ emission (Fig. 2A, B). On the other hand, the SEM model indicated that

the *amoA* genes had the lowest regression weight and size effect of all the targeted N-cycling genes. This may be related to the 80% WFPS of the studied soils, a condition which favours denitrification over nitrification (Butterbach-Bahl et al. 2013; Hu et al. 2015). The SEM model also showed that the $NO_3^-$ content differently affected the denitrification genes as indicated by their regression weight and size effect, from highest to lowest, *nosZ*I + *nosZ*II, *norB*, *nirK* + *nirS* and *napA* + *narG* (Fig. 2A).

### Effects of soil properties and bacterial diversity on regulation of $N_2O$ emission

Out of the 70 OTUs in the studied soils (Annex I), 16 showed significant correlation with the $N_2O$ emission (Table 2), of which *Nitrosospira*, *Nitrosomonas* and *Nitrosococcus* contain well-known nitrifying species (Daims et al. 2016) and the remaining 13 OTUs have been reported as denitrifying bacteria (Zhang et al. 2016). The RF analysis identified the relative abundance of the bacterial OTUs as a better predictor for $N_2O$ emission than the soil properties both for uncultivated (Fig. 3A) and cultivated soils (Fig. 3B). This can be explained after consideration that $N_2O$ production during nitrification and denitrification relies on specific groups of microorganisms whose abundance increase after N application (Schimel et al. 2005; Philippot et al. 2013a, b; Powell et al. 2015). Using RF analysis, other authors have reported conflicting results during studies on the role of microbial diversity and soil properties in controlling $N_2O$ production (Graham et al. 2014; Powell et al. 2015). According to data in Fig. 3, among the 13 denitrifiers, the OTUs *Bacillus*, *Bradyrhizobium*, *Flavobacterium*, *Hyphomicrobium* and *Pseudomonas* had the greatest importance as $N_2O$ predictors.

The SEM analysis revealed that $N_2O$ emission was directly and positively influenced by the relative abundance of the bacterial OTUs, that the $NH_4^+$ content indirectly (positive) affected the nitrifying OTUs (Fig. 4A) and that the $NO_3^-$ content also indirectly (positive) regulated the relative abundance of the denitrifying OTUs (Fig. 4B). TOC and pH have been reported as driving factor for $N_2O$ production (Butterbach-Bahl et al. 2013; Delgado-Baquerizo et al. 2016; Martins et al. 2017); the SEM output, however, showed no clear effect of those soil properties on $N_2O$ emission (Fig. 3).

We acknowledge that our interpretations, which are based on observational data, have limitations since the models evaluated are approximations of the true system. However, we believe that this study provides a useful summary of existing relationships among soil properties, N-cycling gene abundance and relative abundance of bacterial OTUs, and also a strong framework toward further advancing our understanding of the abiotic and biotic drivers of $N_2O$ in soils.

## Acknowledgments

This study was supported by the ERDF-cofinanced grant PEAGR2012-1968 from Consejería de Economía, Innovación y Ciencia (Junta de Andalucía, Spain) and the MINECO-CSIC Agreement RECUPERA 2020. The authors would like to thank Jess Evans for advice on statistical analysis. ACH is recipient of MECD (FPU 2014/01633) and FEMS (FEMS-RG-2017-0067) grants.

*Table 1. Spearman correlation coefficient (ρ) between soil properties (pH, $NH_4^+$, $NO_3^-$, TOC, TC and TN) and N-cycling gene abundance (amoA AOB, amoA AOA, napA, narG, nirK, nirS, norB, nosZI and nosZII) with $N_2O$ emissions. Correlations with $P \leq 0.05$ are in bold and denote significant differences. TOC, total organic C; TC, total C; TN, total N.*

| | | Uncultivated soil | Cultivated soil | |
|---|---|---|---|---|
| | | | Tomato | Common bean |
| | | $N_2O$ ($\rho$, *P*-value) | | |
| Soil property | pH | 0.020, 0.922 | -0.041, 0.621 | 0.085, 0.845 |
| | $NH_4^+$ | **0.415, 0.022** | **0.514, 0.010** | **0.577, <0.001** |
| | $NO_3^-$ | **0.715, <0.001** | **0.715, <0.001** | **0.845, <0.001** |
| | TOC | 0.125, 0.452 | **0.421, 0.025** | **0.487, 0.010** |
| | TC | 0.063, 0.641 | 0.245, 0.068 | 0.321, 0.055 |
| | TN | 0.345, 0.085 | 0.324, 0.066 | 0.339, 0.080 |
| N-cycling gene abundance | *amoA* AOB | **0.357, 0.033** | **0.380, 0.039** | **0.398, 0.034** |
| | *amoA* AOA | **0.485, <0.001** | **0.515, <0.001** | **0.477, <0.001** |
| | *napA* | **0.556, 0.025** | **0.588, 0.029** | **0.556, 0.025** |
| | *narG* | 0.156, 0.126 | 0.266, 0.388 | 0.288, 0.145 |
| | *nirK* | **0.715, 0.015** | **0.740, 0.014** | **0.777, 0.010** |
| | *nirS* | 0.212, 0.152 | 0.312, 0.880 | 0.294, 0.110 |
| | *norB* | **0.651, 0.010** | **0.688, 0.005** | **0.612, 0.008** |
| | *nosZ*I | **-0.885, <0.001** | **-0.721, <0.001** | **-0.750, <0.001** |
| | *nosZ*II | -0.251, 0.122 | -0.320, 0.082 | -0.364, 0.088 |

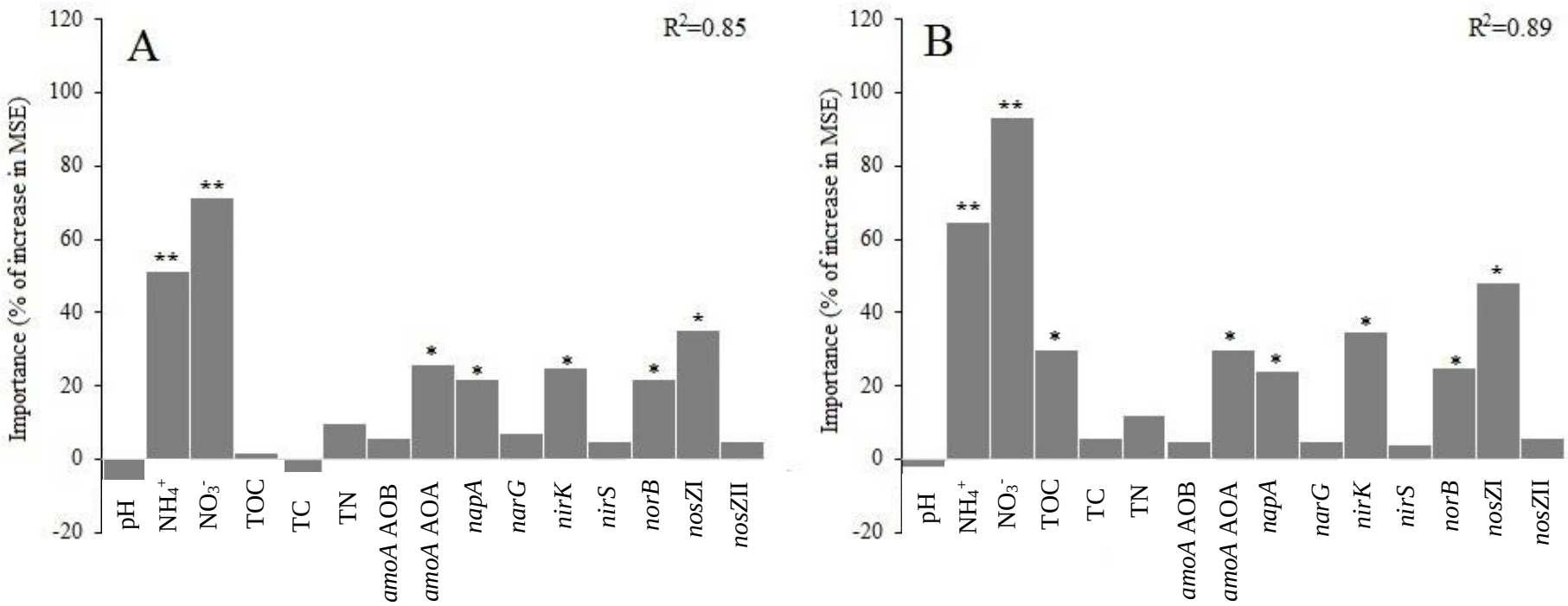



*Fig. 1. Random forest mean predictor importance (% of increase in the mean square error, MSE) of the soil properties pH, $NH_4^+$, $NO_3^-$, TOC, TC and TN and the N-cycling amoA AOB, amoA AOA, napA, narG, nirK, nirS, norB, nosZI and nosZII gene abundance. Data were taken from uncultivated **(A)** or cultivated soils **(B)** Significance levels are: *P < 0.05; ** P < 0.01. TOC, total organic C; TC, total C; TN, total N.*

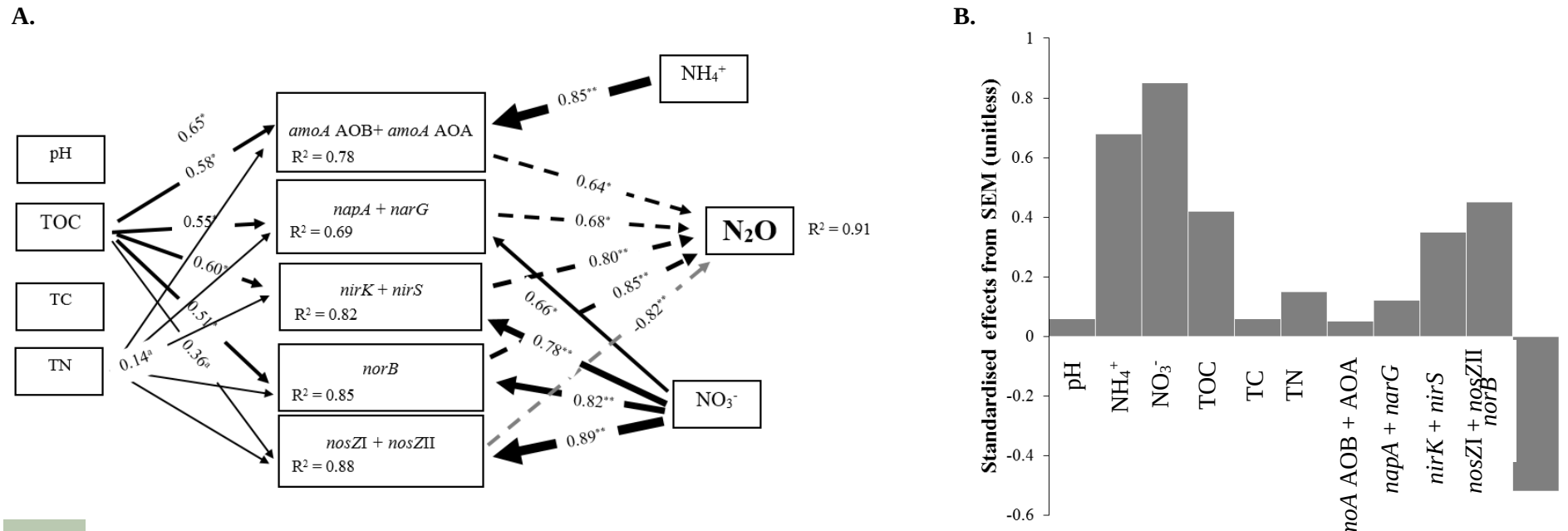



*Fig. 2.* ***A)*** *Structural equation model (SEM) based on the effect of the soil properties pH, $NH_4^+$, $NO_3^-$, TOC, TC and TN together with the ammonia oxidising amoA AOB + amoA AOA genes, the napA + narG, nirK + nirS, norB and nosZI + nosZII denitrification gene abundance on $N_2O$ emissions. Numbers within the arrows are standardized path coefficients showing the size of the effect on the relationship between variables. Continuous and dashed arrows indicate direct and indirect relationships, respectively. Black and grey arrows indicate positive and negative relationships, respectively. The width of the arrows is proportional to the strength of the path coefficients. $R^2$ indicates the proportion of the variance explained and is shown above every response variable. The model was satisfactorily fitted to the data ($\chi^2$ = 1.46, P = 0.23, d. f. = 1; RMSEA = 0.13, P = 0.24). Significance levels are: $^a$P < 0.10; *P < 0.05, **P < 0.01 and ***P < 0.001. TOC, total organic C; TC, total C; TN, total N. The pH variable has been omitted because significant relationships were not found.* ***(B)*** *Standardized total effects (direct plus indirect effects) derived from the corresponding structural equation model.*

*Table 2. Spearman correlation coefficient (ρ) between bacterial OTUs and $N_2O$ emission. Correlations with $P \leq 0.05$ are in bold and denote significant differences. Only bacterial OTUs with $P \leq 0.05$ are shown.*

| OTU | Uncultivated soil | Cultivated soil | |
|---|---|---|---|
| | | Tomato | Common bean |
| | | $N_2O$ ($\rho$, *P*-value) | |
| *Bacillus* | **0.755, <0.001** | **0.833, <0.001** | **0.777, <0.001** |
| *Bradyrhizobium* | **0.625, <0.001** | **0.611, <0.001** | **0.644, <0.001** |
| *Comamonas* | **0.325, 0.033** | **0.211, 0.042** | **0.201, 0.038** |
| *Cupriavidus* | **0.254, 0.044** | **0.321, 0.040** | **0.338, 0.045** |
| *Ensifer* | **0.356, 0.028** | **0.358, 0.029** | **0.367, 0.038** |
| *Flavobacterium* | **0.725, <0.001** | **0.845, <0.001** | **0.784, <0.001** |
| *Hyphomicrobium* | **0.821, <0.001** | **0.843, <0.001** | **0.854, <0.001** |
| *Mycobacterium* | **0.368, 0.029** | **0.301, 0.038** | **0.284, 0.041** |
| *Nitrosococcus* | **0.284, 0.035** | **0.310, 0.025** | **0.345, 0.010** |
| *Nitrosomonas* | **0.289, 0.040** | **0.333, 0.033** | **0.340, 0.012** |
| *Nitrosospira* | **0.295, 0.036** | **0.365, 0.025** | **0.333, 0.025** |
| *Paracoccus* | **0.394, 0.020** | **0.388, 0.029** | **0.377, 0.033** |
| *Pseudomonas* | **0.788, <0.001** | **0.791, <0.001** | **0.766, <0.001** |
| *Rhodococcus* | **0.412, 0.018** | **0.321, 0.018** | **0.340, 0.029** |
| *Sphingomonas* | **0.403, 0.020** | **0.310, 0.031** | **0.340, 0.042** |
| *Thiobacillus* | **0.355, 0.036** | **0.385, 0.045** | **0.318, 0.038** |

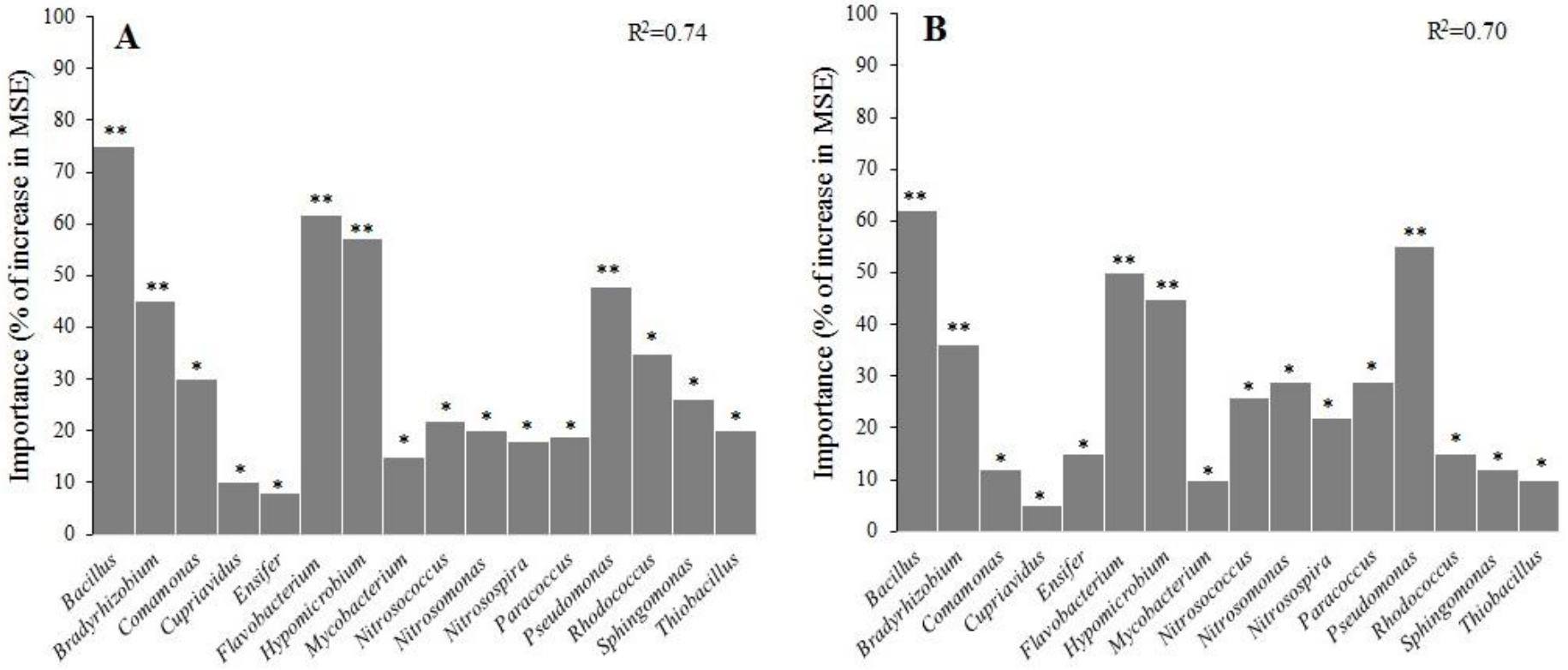



*Fig. 3. Random forest mean predictor importance (% of increase in the mean square error, MSE) of soil properties pH, $NH_4^+$, $NO_3^-$, TOC, TC and TN and the bacterial OTUs relative abundance. Data were taken from uncultivated* ***(A)*** *or cultivated soils* **(B)** *Significance levels are: *P < 0.05; ** P < 0.01. Soil properties showed significance levels higher than 0.05 and, accordingly, are not included in the figure.*

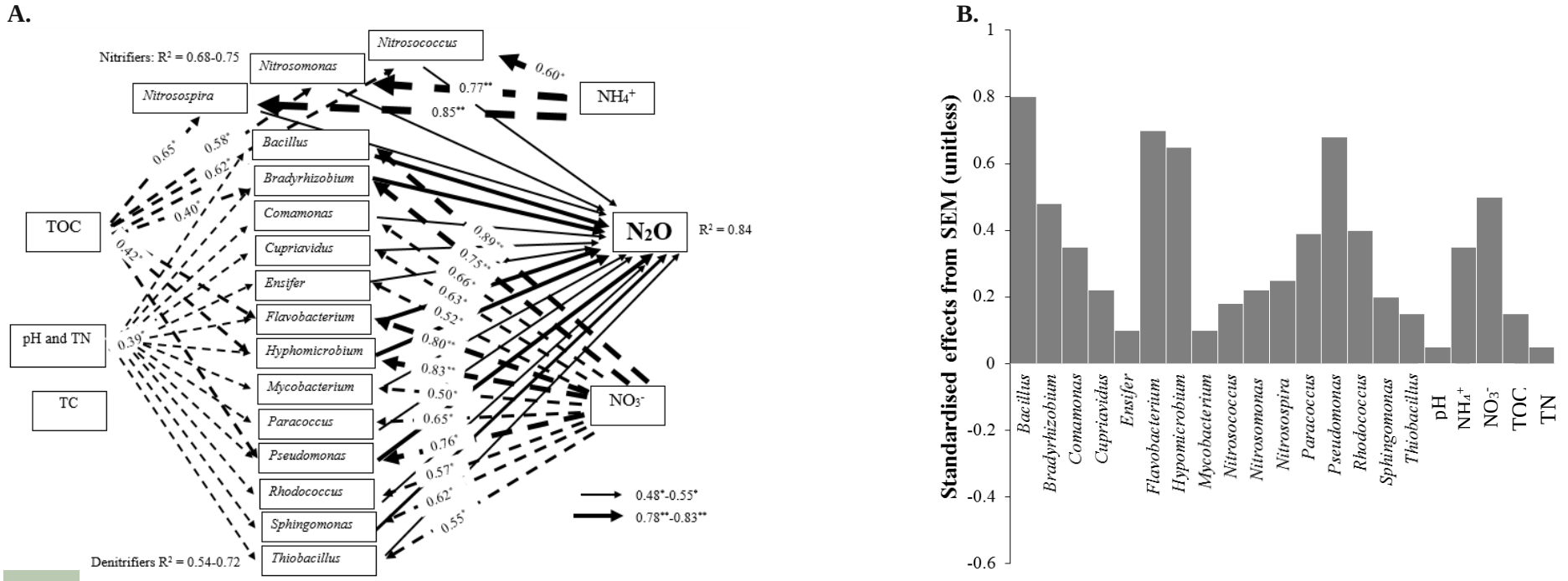



*Fig. 4.* ***(A)*** *Structural equation model (SEM) based on the effect of the soil properties pH, $NH_4^+$, $NO_3^-$, TOC, TC and TN together with the bacterial OTUs relative abundance on $N_2O$ emissions. Numbers within the arrows are standardized path coefficients showing the size of the effect on the relationship between variables. Continuous and dashed arrows indicate direct and indirect relationships, respectively. Black and grey arrows indicate positive and negative relationships, respectively. The width of the arrows is proportional to the strength of the path coefficients. $R^2$ indicates the proportion of the variance explained and is shown above every response variable. The model was satisfactorily fitted to the data ($\chi^2 = 1.46$, $P = 0.23$, d. f. = 1; RMSEA = 0.13, $P = 0.24$). Significance levels are: $^aP < 0.10$; $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. TOC, total organic C; TC, total C; TN, total N. The TC variable has been omitted because significant relationships were not found.* ***(B)*** *Standardized total effects (direct plus indirect effects) derived from the corresponding structural equation model.*

## Supplementary material for Chapter V

# Relative importance of soil properties, N-cycling genes and bacterial diversity for nitrous oxide emissions in uncultivated and cultivated soils

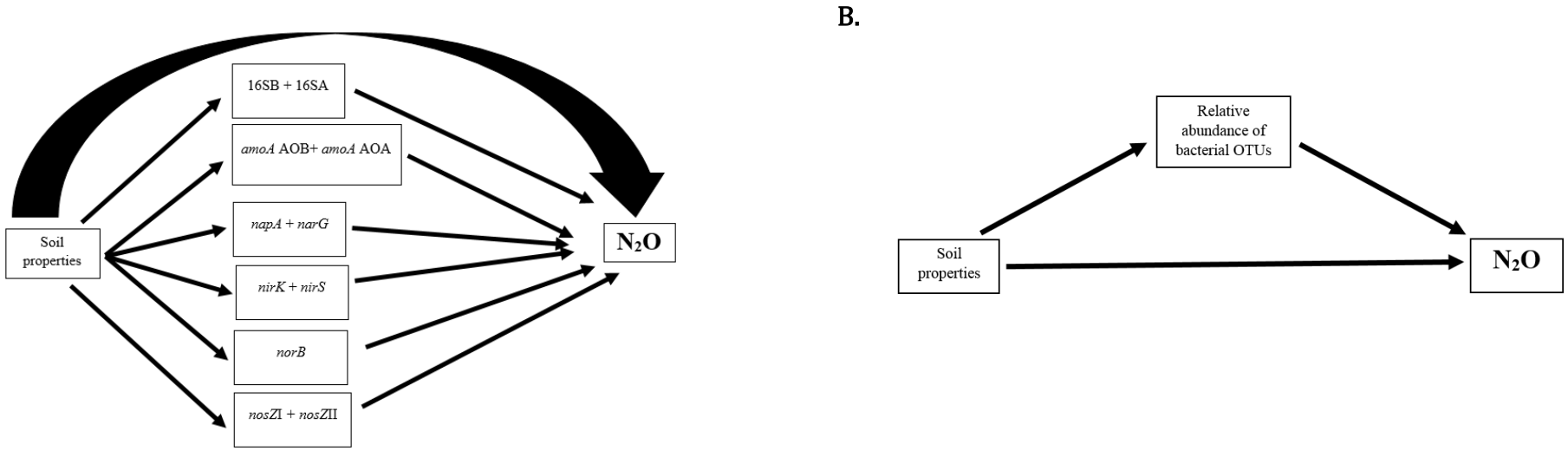



*Fig. S1. A priori generic structural equation models (SEM) used in this study.* ***(A)*** *Soil properties, N-cycling gene abundance and $N_2O$ emissions;* ***(B)*** *soil properties, bacterial diversity relative abundance and $N_2O$ emissions.*

# Chapter VI

## Relative contributions of bacteria and fungi to nitrous oxide emissions in four contrasting soils following nitrate application

**Submitted for publication**

Abstract

Bacteria and fungi have been shown to produce nitrous oxide ($N_2O$) during denitrification, but their contribution after nitrate ($NO_3^-$) application to soil is not clearly established. In a microcosm experiment we studied the relative contribution of bacteria and fungi to $N_2O$ production by four contrasting soils after $KNO_3$ addition. The soils were wetted to 80% water-filled pore space (WFPS) and kept under greenhouse conditions for 10 days. The fungicide cycloheximide and the bactericide streptomycin were used to determine the microbial origin of the $N_2O$ emissions. The abundance of the bacterial and fungal communities was estimated by quantitative PCR (qPCR) of the bacterial 16S rRNA and the fungal ITS genes, respectively. The size of denitrifiers was calculated after quantification of the *nirK*, *nirS*, *norB*, *nosZ*I and *nosZ*II genes. After 10 d, bacteria and fungi made comparable contributions to cumulative $N_2O$ emissions, an effect that occurred in the four soils analysed. There was, however, a crossing point during incubation as bacteria dominated $N_2O$ production during the first 48-96 h and fungi contributed more afterwards. After 240 h incubation, the fungal to bacterial ratio of $N_2O$ emissions were 1.26, 1.30, 1.31 and 2.02, which suggests that fungal denitrification contributed more than bacteria overall. The total abundance of 16S rRNA and ITS genes and that of denitrification genes indicated that the antibiotics used to separate the relative contributions of bacteria and fungi to soil $N_2O$ emissions were effective.

Keywords: Nitrate, nitrous oxide, antibiotics, fungi, bacteria, qPCR

## Introduction

Nitrous oxide ($N_2O$) is the third most important individual contributor to the combined radiative forcing by long-lived greenhouse gases (WMO 2017). Nitrogen (N) fertilised agricultural soils are the main anthropogenic source of $N_2O$ and account for approximately 70% of annual global fluxes to the atmosphere (Erisman et al. 2015; Smith 2017). The emission of $N_2O$ from soils is the result of multiple biological and non-biological processes, of which nitrification and denitrification are considered the main $N_2O$-producing biological pathways. Denitrification is responsible for the anoxic reduction of nitrate ($NO_3^-$) and nitrite ($NO_2^-$) to molecular nitrogen ($N_2$) via the formation of nitric oxide (NO) and $N_2O$ under oxygen- ($O_2$) limited conditions, i.e., > 70% water-filled soil pore space (WFPS) (Braker and Conrad 2011; Butterbach-Bahl et al. 2013; Hu et al. 2015). For over a century denitrification was exclusively attributed to the anaerobic respiratory function of members of the Bacteria and Archaea domains (Zumft and Kroneck 2007; Philippot et al. 2007), but it is now widely accepted that $N_2O$ production is a widespread trait in fungi (Maeda et al. 2015) playing an important role in $N_2O$ emissions from various ecosystems, such as grasslands, croplands and wetlands (Crenshaw et al. 2008; Seo and DeLaune 2010; Rütting et al. 2013; Chen et al. 2014; Ma et al. 2017). Other studies have shown that fungi grow at wider pH ranges than bacteria, have the capability to use complex organic compounds (van der Wal et al. 2006; Rousk et al. 2010) and contribute more than bacteria to soil $N_2O$ production under low $O_2$ concentrations (Seo and DeLaune 2010; Shoun et al. 2012; Mothapo et al. 2015; Chen et al. 2015; Shoun and Fushinobu 2017).

It is well established that many bacterial denitrifiers biosynthesise nitrous oxide reductase (Nos), the enzyme responsible for the reduction of $N_2O$ to $N_2$ (Hallin et al. 2017 and references therein). This enzyme has not been found in fungi, however, where the production of $N_2O$ is carried out by the enzyme P450Nor (see Shoun and Fushinobu 2017 for a review). Both bacterial and fungal denitrifiers can use copper- (Cu) containing nitrite reductase (NirK) to reduce $NO_2^-$ to NO, so that the gaseous emission by fungi is $N_2O$ rather than $N_2$ (Baggs 2011).

Due to high yield requirements, agricultural soils are often intensively managed. The effect of carbon (C) substrate addition on the relative contributions of fungi and bacteria to $N_2O$ emissions from different soils maintained at high moisture contents has been studied (e.g. Chen et al. 2014; 2015; Zhou et al. 2016; Ma et al. 2017). Their relative contributions to $N_2O$ emissions following N application without C addition (which is a common agricultural practice – i.e. inorganic N-fertiliser application) remains largely unknown. Herein, in a microcosm experiment, we studied the effect of $KNO_3$ fertilisation on the relative contributions of bacteria and fungi to $N_2O$ production from four contrasting soils during a 10 d incubation at 80% WFPS. The fungicide cycloheximide and the bactericide streptomycin were used to distinguish the microbial origins of emitted $N_2O$. The abundance of the bacterial and fungal communities and that of bacterial denitrifiers was estimated by quantitative PCR (qPCR) of the 16S rRNA of bacteria, ITS of fungi and *nirK*, *nirS*, *norB*, *nosZ*I and *nosZ*II genes, respectively.

## Materials and methods

### Soil sampling

Soils from: 1) a permanent pasture (*Lolium perenne* cv. Aber magic), which had not been ploughed for at least 15 years, located on the North Wyke Farm Platform (NWFP) (Rothamsted Research, United Kingdom, 50°46'10"N, 3°54'5"W) (soil PP); 2) a pasture sown with a mixture of grass (*L. perenne* cv. Aber magic) and clover (*Trifolium repens* cv. Aberherald) also located on the NWFP (soil WCmix); 3) a semi-improved, enclosed upland grassland (60% bracken, *Pteridium aquilinum,* and 39% semi-improved grassland, with minor areas of marsh/wet flush and gorse, *Ulex europaeus*) at Henfaes Research Station Abergwyngregyn, North Wales (270 m a.s.l., 53°13'13''N, 4°0'34''W); (soil Upl); and 4) an agricultural soil in the vicinity of Motril (Granada, Spain) maintained under fallow conditions for at least 10 years (soil Fal) were used in this study. The PP and WCmix, are Stagni-vertic Cambisol soils, the Upl is an Orthic Podzol and the Fal is an Eutric Cambisol soil (FAO 2017), respectively. The main physicochemical characteristics of the soils are shown in Table 1. For each soil, spade-squares (20 × 20 cm to a depth of 10 cm) were taken

from 12 locations along a 'W' line across each field. After collection, the soil was air dried to ca. 30% gravimetric moisture content, sieved to < 2 mm and stored at 4 °C.

### Experimental setup

Single and combined applications of cycloheximide and streptomycin were used to selectively inhibit fungal and bacterial growth, respectively in the soil. A preliminary experiment was performed to assess: a) the optimal concentration of the inhibitors, b) the duration of its effect under the experimental conditions, and c) the effect of the inhibitors on non-target microorganisms. For this purpose, cycloheximide and streptomycin in the range 1.0 to 3.0 mg $g^{-1}$ soil were diluted separately in sterile distilled water (SDW), mixed with 15 g soil samples in a plastic bag and then packed to a bulk density of 0.8 g $cm^{-3}$ into cylindrical plastic cores (6.3 cm diameter, 10 cm height). The cores (3/jar) were placed into 500 ml Kilner jars, the soil moisture adjusted to 50% WFPS and incubated overnight to allow diffusion into the soil pores. Then, $KNO_3$ was diluted in SDW and added to the cores from the top to reach 80% WFPS and a final concentration of 421.2 mg N $kg^{-1}$ dry soil (equivalent to 260 kg N $ha^{-1}$). Control soil without the inhibitors also received $KNO_3$. The jars were kept in a cabinet at 22 °C/16 °C day/night; 16 h/8 h light/dark cycle for 15 d. Soil moisture was maintained daily by adding SDW water. Every 2 days, soil samples (1 g) were taken, serially diluted using sterile saline solution and used (100 µl) for inoculation of Petri dishes containing either tryptone soybean agar (TSA) medium or potato dextrose agar (PDA) medium. Plates were maintained at 30 °C for 5 d. Results showed that: the optimal cycloheximide and streptomycin concentrations to prevent fungal and bacterial growth were 2.0 mg $g^{-1}$ soil; the inhibitors were effective throughout the 10 days following N application; and the inhibitors did not affect the growth of non-target microorganisms.

For the main microcosm experiments three replicate cores of each the cycloheximide, streptomycin, cycloheximide + streptomycin and control treatments of soils PP, WCmix, Upl and Fal were prepared and maintained as indicated above for 10 d. During incubation, the lids of the jars remained open to maintain aerobic conditions in the headspace. For each treatment, three replicate jars were used for the determination of $N_2O$ emissions and three served for soil destructive sampling

(treatments to these jars lagged those to gas sampling jars by 2 days to enable destructive sampling at peak $N_2O$ emissions).

### Gas analyses

Jar headspaces were first sampled 3 h after N addition to avoid the pulse of respiration associated with wetting (Kieft et al. 1987) and then daily for 10 d. The jars were sealed, 25 mL gas samples taken with a syringe at 0, 20 and 40 minutes and then transferred to 20 mL pre-evacuated vials. Headspace $N_2O$ concentrations were analysed by gas chromatography using a Perkin Elmer Clarus 500 gas chromatograph (Perkin Elmer Instruments, Beaconsfield, UK) equipped with an electron capture detector.

### Soil analyses

Soil samples (15 g) were taken at 3, 48, 96 and 240 h after N addition. The concentration of $NO_3^-$ was determined by automated colorimetry from 2 M KCl soil extracts using a Skalar SANPLUS Analyser (Skalar Analytical B.V., Breda, Netherlands). WFPS was monitored over the experimental period and calculated from soil moisture contents by drying soil subsamples (5 g) at 105 ºC overnight. The pH was measured after water extraction (1:5, w/v) for 2 h. Total C (TC) and total N (TN) were determined using a LECO TruSpec CN elemental analyser.

### DNA extraction and quantification of 16S rRNA, ITS and denitrification genes

Soil DNA was extracted from 0.5 g samples taken 240 h post N treatment as indicated earlier (Correa-Galeote et al. 2014; Castellano-Hinojosa et al. 2018), purified using Powersoil DNA isolation kit (MP Bio) spin columns and stored at -20 ºC until use. DNA concentration was measured using the Qubit® ssDNA assay kit (Molecular Probes). The abundance of bacteria was estimated by qPCR of the 16S rRNA (16SB) gene using primers and conditions previously published (Castellano-Hinojosa et al. 2018) and that of the fungi by quantification of the internal transcribed space (ITS) gene (Gardes and Bruns 1993; Vilgalys and Hester 1990). The size of the denitrifying community was calculated using the *nirK*, *nirS, norB*, *nosZ*I and *nosZ*II genes as molecular markers for qPCR (Castellano-Hinojosa et al. 2018). Assays for qPCR were carried out using a Bio-Rad iCycler iQ5 Thermocycler

(Bio-Rad Laboratories, USA) with SYBR Green as the detection system. In all cases, inhibition was not detected. PCR efficiency for the different assays ranged between 90% and 100%. The quality of all qPCR amplifications was verified by electrophoresis in agarose and by melting curve analysis.

### Statistical analysis

Statistical analyses were performed using Statgraphics Centurion XVI software (StatPoint Technologies Inc, Warrenton, USA). Prior to the statistical tests all data were analysed to test their normal distribution (Kolmogorov-Smirnov test) and equality of variance (Levene test). Cumulative emission of $N_2O$ was calculated from the area under the curve after linear interpolation between sampling points. Differences in soil characteristics between treatments as well as differences in the total abundance of targeted genes were assessed by ANOVA at $P < 0.05$. Where treatment effects proved to be significant, Fisher's Least Significant Test (LSD) was used as a post hoc test to ascertain differences among treatment levels.

Fungal and bacterial contributions to soil $N_2O$ fluxes were estimated by the equation described by Chen et al. (2015): $100 \times (A-B)/A-D$, where A: $N_2O$ flux from the control soil; B: $N_2O$ flux from the soil treated with either cycloheximide to obtain the bacterial contribution or streptomycin to reveal the fungal contribution; D: $N_2O$ flux from the soil treated with cycloheximide and streptomycin. The inhibitor additivity ratio (IAR) was used to evaluate non-target effects following the equation by Beare et al. (1990): $(A-B) + (A-C)/(A-D)$, where A, B, C and D represent $N_2O$ flux from the control-, cycloheximide-, streptomycin- and (cycloheximide + streptomycin)-treated soil, respectively. An IAR of ~1.0 indicated that inhibitory effects of the cycloheximide or streptomycin were not confounded. The ratio of fungal to bacterial contributions (F:B) was calculated by dividing the fungal by the bacterial contribution to soil $N_2O$ fluxes.

## Results

### Soil properties

With initial values of 6.2, 6.1, 5.0 and 7.3 for PP, WCmix, Upl and Fal, respectively, the pH of the soils decreased during incubation for 48 h after application of the

treatments and increased afterwards to levels similar to those of the untreated soil (Fig. S1). Regardless of the soil type, differences amongst treatments were not found through the experimental period. After fertilisation with $KNO_3$, the content of $NO_3^-$-N increased from 1.8, 29.5, 46.4 and 7.1 mg $kg^{-1}$ dry soil to 422.3, 450.4, 467.2 and 428.2 mg $kg^{-1}$ dry soil in PP, WCmix, Upl and Fal (Fig. S2). Except for the cycloheximide + streptomycin treatment, the $NO_3^-$-N content decreased throughout the experimental period, and after incubation for 240 h the $NO_3^-$-N contents were higher in the streptomycin-treated soils followed by cycloheximide and finally the control treatments (Fig. S2). For PP, WCmix, Upl and Fal, the initial percentages of 0.43, 0.70, 0.72 and 0.16% for TN (Fig. S3) and of 3.65, 6.69, 7.42 and 2.69% for TC (Fig. S4) did not statistically change during the incubation period.

### Fungal and bacterial contributions to $N_2O$ emissions

The effect of the single and combined application of cycloheximide and streptomycin on $N_2O$ production by the soils used in this study is shown in Fig. 1. The control soils showed the highest $N_2O$ fluxes of 0.83, 0.87 and 1.34 kg N $ha^{-1}$ $h^{-1}$ for PP, WCmix, and Fal, respectively, at 48 h, and of 0.58 kg N $ha^{-1}$ $h^{-1}$ after 72 h for Upl. The $N_2O$ fluxes gradually decreased afterwards to reach the lowest emissions by the end of the experimental period. When cycloheximide was used, the fluxes from PP, WCmix, Upl and Fal reached maximum values of 0.51, 0.54, 0.34 and 0.85 kg N $ha^{-1}$ $h^{-1}$, respectively, after 48 h incubation. Then, the $N_2O$ emissions gradually decreased to basal values that were not higher than 0.2 kg N $ha^{-1}$ $h^{-1}$ after incubation for 240 h. The application of streptomycin produced a peak of 0.36 kg N $ha^{-1}$ $h^{-1}$ at 48 h in PP and of 0.34 at 72 h kg N $ha^{-1}$ $h^{-1}$ in Upl; two peaks of 0.39 and 0.38 kg N $ha^{-1}$ $h^{-1}$ were observed at 48 and 96 h for WCmix, respectively, and of 0.72 and 0.55 kg N $ha^{-1}$ $h^{-1}$ at 72 and 120 h for Fal, respectively. After incubation for 240 h, the cumulative $N_2O$ emissions of soils treated with cycloheximide or streptomycin were significantly lower than those from the corresponding control soils, and the combined application of both inhibitors further decreased $N_2O$ production (Table 2).

During the first 48 to 96 h of incubation, the contribution of bacteria to $N_2O$ emission was greater than that of fungi averaging 58, 79, 65 and 55% of the total

emissions for PP, WCmix, Upl and Fal, respectively (Fig. 2). The situation reversed afterwards and the contribution of fungal denitrifiers to total $N_2O$ emissions from 96 to 240 h averaged 58, 63, 71 and 60%, for PP, WCmix, Upl and Fal, respectively. The average F:B ratios of $N_2O$ emissions over the course of the incubation were 1.30, 1.26, 2.22 and 1.31 for PP, WCmix, Upl and Fal, respectively (Fig. 3). However, F:B ratios during the first 96 h averaged 0.89, 0.36, 0.56 and 0.90, while from 96 h until the end of the incubation, F:B ratios were 1.43, 1.91, 3.14 and 1.46 for PP, WCmix, Upl and Fal, respectively (Fig. 3). The IAR values were similar among the four soils varying from 0.95 to 1.02 (Fig. 3).

### Total abundance of bacteria and fungi

After incubation for 240 h, the abundance of the 16SB gene in PP, WCmix and Fal was significantly higher in the control soils ($2.4 \times 10^9$, $5.9 \times 10^9$ and $3.8 \times 10^8$ gene copy number x $g^{-1}$ dry soil, respectively) and the soils treated with cycloheximide ($2.1 \times 10^9$, $7.5 \times 10^9$ and $9.0 \times 10^8$ gene copy number x $g^{-1}$ dry soil, respectively) than in the soils treated with streptomycin- ($6.0 \times 10^8$, $1.4 \times 10^9$ and $3.0 \times 10^8$ gene copy number x $g^{-1}$ dry soil, respectively) or cycloheximide + streptomycin ($5.3 \times 10^8$, $1.6 \times 10^9$ and $3.4 \times 10^8$ gene copy number x $g^{-1}$ dry soil, respectively) (Fig. 4). Significant differences were not found between treatments in Upl (Fig. 4). The abundance of the fungal ITS gene in PP, WCmix, Upl and Fal was statistically higher in the control soils ($1.8 \times 10^7$, $1.7 \times 10^7$, $3.0 \times 10^7$ and $4.3 \times 10^7$ gene copy number x $g^{-1}$ dry soil, respectively) and the streptomycin-treated soils ($1.7 \times 10^7$, $1.5 \times 10^7$, $2.2 \times 10^7$ and $3.7 \times 10^7$ gene copy number x $g^{-1}$ dry soil, respectively) than in the soils amended with cycloheximide ($3.9 \times 10^6$, $6.6 \times 10^6$, $2.7 \times 10^6$ and $2.6 \times 10^6$ gene copy number x $g^{-1}$ dry soil, respectively) or the soils treated with cycloheximide + streptomycin ($4.9 \times 10^6$, $9.4 \times 10^6$, $2.5 \times 10^6$ and $3.8 \times 10^6$ gene copy number x $g^{-1}$ dry soil, respectively) (Fig. 4).

### Total abundance of denitrifiers

The copy number of the *nirK, nirS, norB* and *nosZ*I genes in all four soils were generally significantly higher in the control and the cycloheximide-treated soils compared to the soils treated with streptomycin or cycloheximide + streptomycin (Fig. 4, Table S1). As an exception, there were no differences in the copy number of

the *nirS* gene in the PP and WCmix soils between treatments (Fig. 4, Table S1). Regardless of the soil type, significant differences in the abundance of the *nosZ*I gene were not found between treatments (Fig. 4, Table S1).

## Discussion

The bacterial inhibitor streptomycin and the fungal inhibitor cycloheximide were used in this microcosm study to analyse the relative contribution of fungi and bacteria to $N_2O$ emissions by four different soils amended with $KNO_3$ and incubated under $O_2$-limiting conditions. Cycloheximide and streptomycin inhibit protein synthesis, and thus soil fungal and bacterial activity, respectively (Anderson and Domsch 1973). Because the antibiotics used may exert impacts on non-target organisms if their concentrations are inappropriate (Badalucco et al. 1994), a preliminary experiment was conducted to assess the optimal antibiotic concentrations and to determine their IAR values during the incubation time. According to Beare et al. (1990), calculations of IAR showed that the sum of the separate effects was comparable to the combined effect of streptomycin and cycloheximide for soil $N_2O$ emissions suggesting that, at the concentrations used in this study, the two antibiotics were fully selective for the organisms that were cultured during our testing (Fig. 3).

With the aid of the antibiotic selective inhibition, our results showed that bacteria and fungi contributed almost equally to soil $N_2O$ production during incubation for 240 h, and that this effect was similar for the four soils under study (Table 2). After incubation, more than 50% reduction in soil cumulative $N_2O$ emission was made by the activity of soil fungi or bacteria, a percentage that is similar to that found by Chen et al. (2014) across five different ecosystems and lower than the values found in other studies (Chen et al. 2015; Ma et al. 2017) dealing mainly with the effect of C sources on differential $N_2O$ production by bacteria and fungi. The results also showed that $N_2O$ production by bacteria dominated over that of fungi during the first 48-96 h of incubation, after which time, the situation reversed and the production of $N_2O$ by fungi dominated (Fig. 2). Since a pattern similar to that found in $N_2O$ emissions was not observed for the pH, $NO_3^-$-N content, %TN and %TC parameters analysed in this study (Figs. S1-S4, respectively), it is

possible that changes in the contribution of bacteria and fungi to $N_2O$ emissions during incubation could be due to differences in the rates of nitrate utilisation. In fact, nitrate utilisation was faster by bacteria than fungi (Fig. S2). Using $^{15}N$-labeled $KNO_3$, Myrold and Posavatz (2007) showed that bacteria, not fungi, had the greatest potential for assimilating, or immobilising, $NO_3^-$ in many soils, particularly in relatively undisturbed soils. On the other hand, nitrite reductase (NirK) and P450nor are essential enzymes of the fungal denitrification system, which are the minimum pair to ensure denitrification; P450nor receives electrons directly from NAD(P)H, which indicates that the enzyme is not associated with the respiratory chain, functioning preferentially as an electron sink over energy production efficiency under anoxic conditions (Shoun and Fushinobu 2017 and references therein). All this is consistent with denitrification being more rapidly induced in bacteria than in fungi after nitrate addition, and would explain the higher fungal to bacterial ratio during incubation for the first 96 h (Fig. 2).

The F:B ratio of $N_2O$ emissions in PP, WCmix, Upl and Fal averaged 1.30, 1.26, 2.02 and 1.31, respectively, which suggests that fungal denitrification dominated bacterial denitrification after incubation for 240 h (Fig. 3). These values are within the range of 0.5-4.0 estimated for woodlands, temperate and semiarid grasslands, wetlands, and tropical peatlands (Laughlin and Stevens 2002; McLain and Martens 2006; Spokas et al. 2006; Yanai et al. 2007; Ma et al. 2008; Laughlin et al. 2009; Chen et al. 2014). Fungal contribution to soil $N_2O$ production was more pronounced in Upl (Figs. 2 and 3), the soil with the lowest pH and highest organic matter (Table 1). Fungi have been documented to be able to grow over a broader range of pH than bacteria (Wheeler et al. 1991; van der Wal et al. 2006; Rousk et al. 2010), which together with the observations that the product ratio of denitrification is strongly affected by pH (Firestone et al. 1980; Simek and Cooper 2002) and that low pH hinders the synthesis of a functional $N_2O$ reductase enzyme (Bakken et al. 2012), could explain the higher fungal $N_2O$ emissions by Upl. In fact, fungal-to-bacterial biomass ratio has been found to increase with reduced soil pH (Bååth and Anderson 2003; Rousk et al. 2009, 2010). Also, it has been demonstrated that fungal denitrification can be the dominant $N_2O$-emitting process in soils with high content

of organic matter (Rütting et al. 2013; Ma et al. 2017) or amended with organic fertilisers (Wei et al. 2014).

The bacterial biomass, determined as the abundance of the 16SB gene, was similar in the control and cycloheximide-treated soils and lower in the soils treated with streptomycin (Fig. 4). On the contrary, the abundance of fungi, estimated after qPCR determination of the ITS gene, was similar in the control and streptomycin-treated soils and significantly lower in the soils treated with cycloheximide (Fig. 4). The relationship between community abundance the bacterial and fungal contributions to $N_2O$ emissions should be considered with caution as the gene copy numbers do not fully equate the population density because bacterial and fungal genomes may contain variable numbers of the 16S rRNA (Klappenbach et al. 2000) and ITS (Bellemain et al. 2010) regions. Unlike 16S rRNA and ITS regions, genes involved in bacterial denitrification are generally single copies per genome (Kandeler et al. 2006, Jones et al. 2008). In this sense, the results show that, in parallel with the lower abundance of the 16SB gene, the *nirK*, *nirS*, *norB* and *nosZ*I denitrification genes were also less abundant in the soils treated with streptomycin (Fig. 4). Moreover, in Upl, because of the acid pH, not only the abundance of the 16SB gene, but also that of the *nirK*, *nirS*, *norB* and *nosZ*I genes was lower compared with those estimated in the other three soils, and *nosZ*II was even not detected (Fig. 4) which correlates with the lowest cumulative $N_2O$ emission detected in Upl (Fig. 1). Taken together, all the data lend support to the results obtained on the relative contribution of bacteria and fungi to $N_2O$ production after addition of nitrate to four different soils. Further studies are needed to elucidate the significance of denitrification in fungi and its consequences for $N_2O$ emissions under field conditions. The effect of the amount and type of N-fertilisers on fungal $N_2O$ emissions should be explored further. Characterisation and development of molecular probes targeting genes in the fungal denitrification pathway may prove useful in gaining further understanding of the abundance of fungal denitrifiers in soils.

## Acknowledgments

This study was supported by the ERDF-cofinanced grant PEAGR2012–1968 from Consejería de Economía, Innovación y Ciencia (Junta de Andalucía, Spain). ACH thanks the Federation of European Microbiological Societies (FEMS) Research Grant 2017-2 program (FEMS-RG-2017-0067) and Ministerio de Educación Cultura y Deporte (MECD) for grant FPU 2014/01633. The authors would also like to acknowledge the UK Biotechnology and Biological Sciences Research Council (BBSRC) funded projects BBS/E/C/000I0310, BBS/E/C/000I0320 and BBS/E/C/000J0100 and UK Natural Environment Research Council (NERC) funded projects NE/M013847/1 and NE/M015351/1.

*Table 1. Physicochemical properties of the permanent pasture (PP), mixture of grass and clover (WCmix), upland (Upl) and fallow (Fal) soils used in this study. Values represent the mean ± standard errors (n = 3). TN, total N; TC, total C.*

| Soil type | pH | $NO_3^-$-N (mg $kg^{-1}$ dry soil) | TN (%) | TC (%) |
|---|---|---|---|---|
| PP | 6.16 ± 0.03 | 1.8 ± 0.1 | 0.43 ± 0.03 | 3.65 ± 0.03 |
| WCmix | 6.09 ± 0.05 | 29.5 ± 1.8 | 0.70 ± 0.05 | 6.69 ± 0.05 |
| Upl | 4.96 ± 0.05 | 46.4 ± 2.3 | 0.72 ± 0.05 | 7.42 ± 0.05 |
| Fal | 7.35 ± 0.05 | 7.1 ± 0.3 | 0.16 ± 0.05 | 2.69 ± 0.05 |

*Table 2. Cumulative $N_2O$ emissions (kg N $ha^{-1}$) from the permanent pasture (PP), mixture of grass and clover (WCmix), upland (Upl) and fallow (Fal) soils not treated (NT) or supplemented with cycloheximide (CHX), streptomycin (Sm) or both (CHX + Sm). Soils were incubated for 240 h under greenhouse conditions after N addition. Values represent the mean ± standard error (n = 3). For each soil type and gas emission, values followed by different letters indicate significant differences among treatments according to ANOVA and Fisher's least significant tests (p < 0.05; n = 3). Percentages of inhibition (%) are shown in brackets.*

| | | Soil type | | | |
|---|---|---|---|---|---|
| Gas | Treatment | PP | WCmix | Upl | Fal |
| $N_2O$ | NT | 3.82 ± 0.15a | 5.53 ± 0.14a | 3.24 ± 0.17a | 6.28 ± 0.16a |
| | CHX | 2.15 ± 0.14b (56%) | 3.17 ± 0.12b (57%) | 1.79 ± 0.12b (55%) | 3.32 ± 0.12b (53%) |
| | Sm | 2.17 ± 0.10b (57%) | 3.19 ± 0.09c (58%) | 1.81 ± 0.10b (56%) | 3.32 ± 0.15b (53%) |
| | CHX +Sm | 0.38 ± 0.05c (10%) | 0.38 ± 0.06d (7%) | 0.49 ± 0.06c (15%) | 0.36 ± 0.05c (6%) |

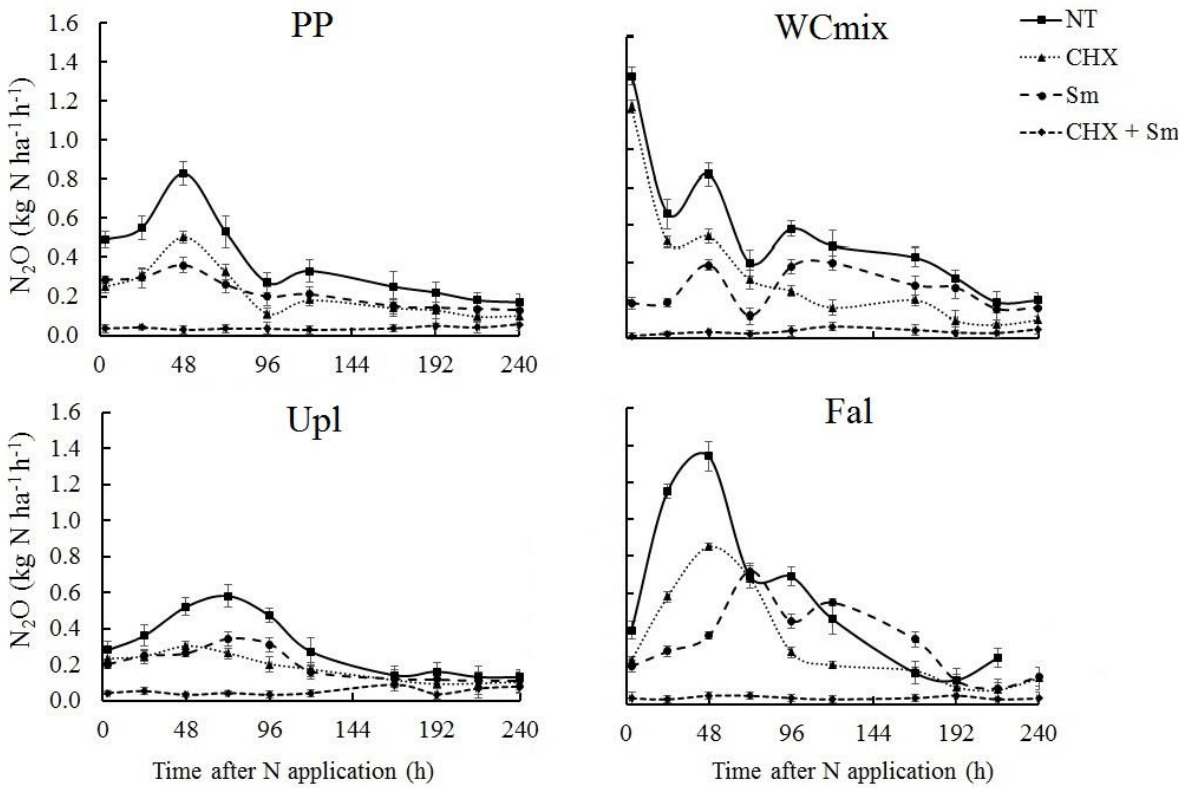



*Fig. 1. $N_2O$ fluxes (kg N $ha^{-1}$ $h^{-1}$) from a permanent pasture (PP), a mixture of grass and clover (WCmix), an upland (Upl) and a fallow (Fal) soil not treated (NT) or supplemented with cycloheximide (CHX), streptomycin (Sm) or both (CHX + Sm). Soils were incubated for 240 h under greenhouse conditions after N addition. Values represent the mean ± standard error (n = 3).*

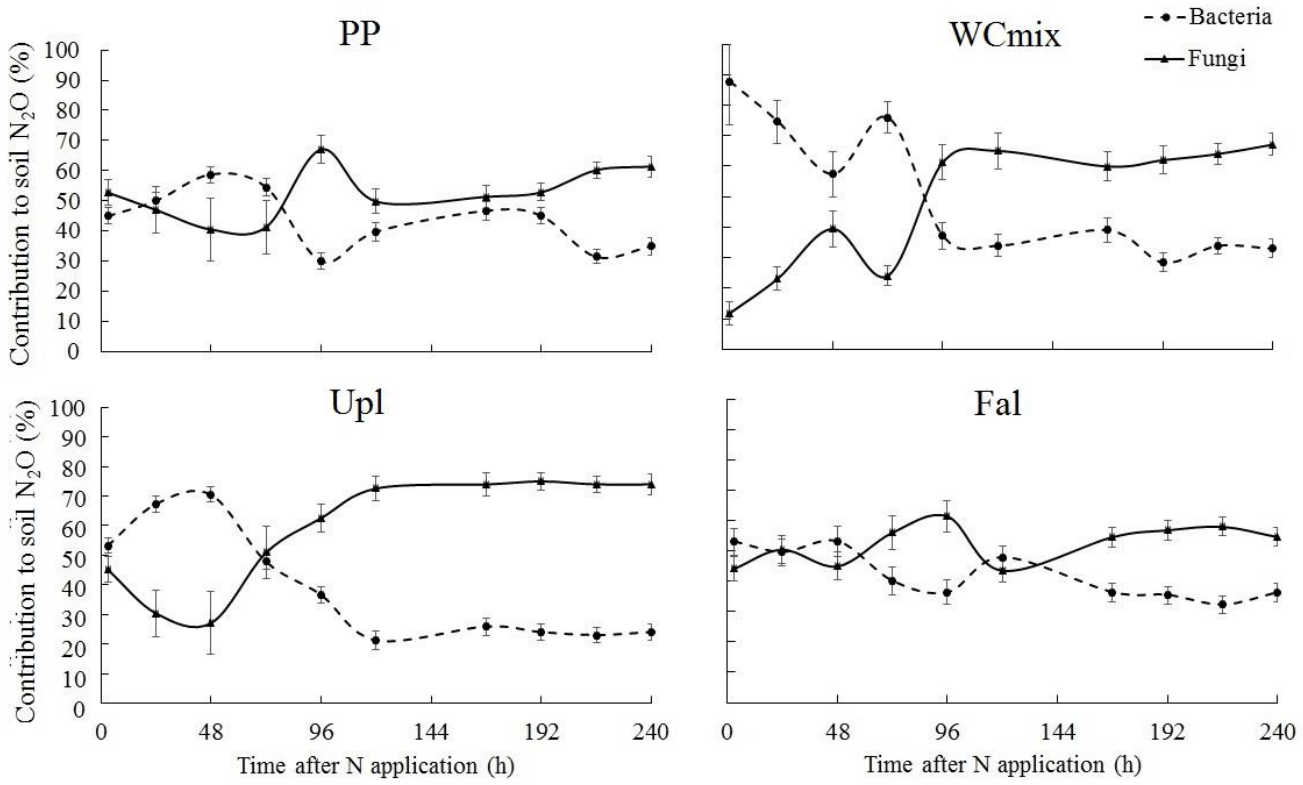



*Fig. 2. Contribution (%) of bacteria and fungi to $N_2O$ production from a permanent pasture (PP), a mixture of grass and clover (WCmix), an upland (Upl) and a fallow (Fal) soil not treated (NT) or supplemented with cycloheximide (CHX), streptomycin (Sm) or both (CHX + Sm). Soils were incubated for 240 h under greenhouse addition after N addition. Values represent the mean ± standard error (n = 3).*

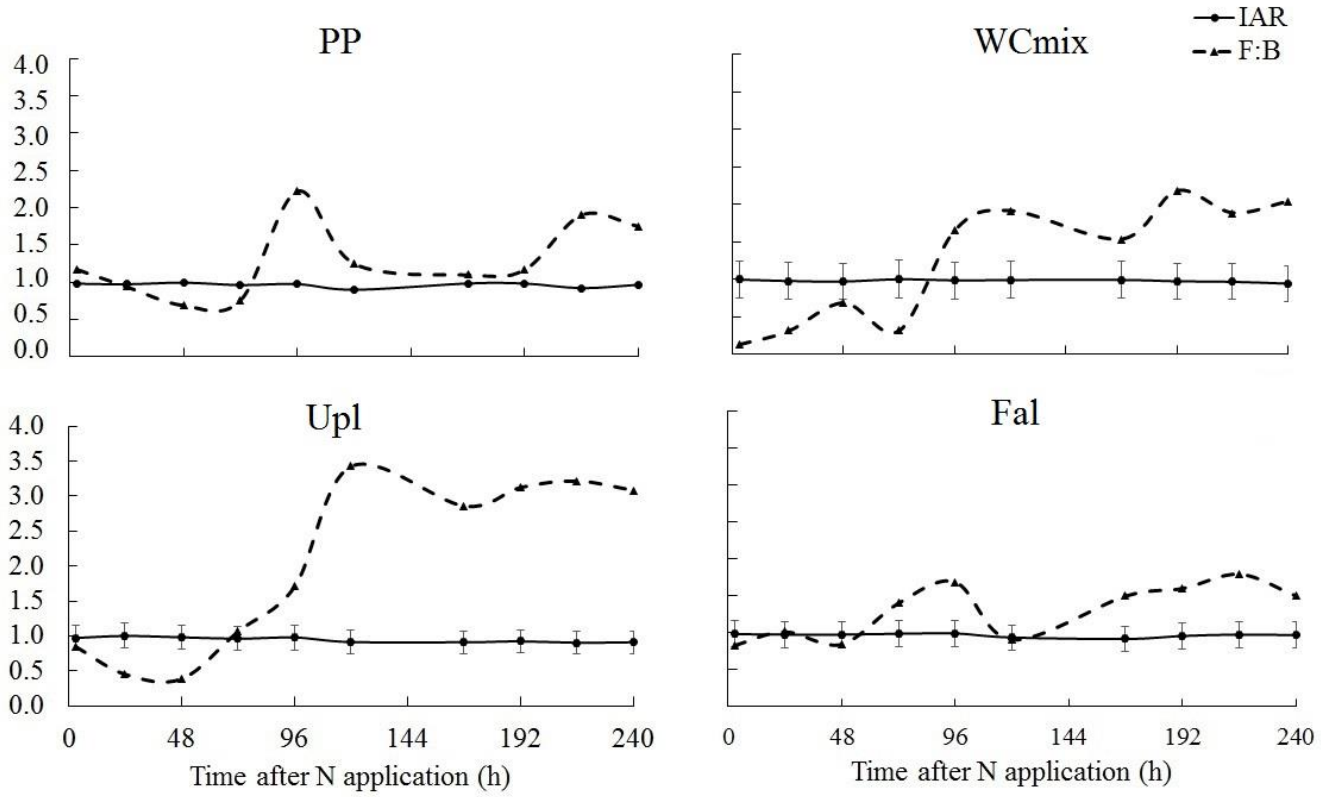



*Fig. 3. Fungal to bacterial contribution ratios (F:B) and inhibitor additivity ratios (IAR) of $N_2O$ emissions from a permanent pasture (PP), a mixture of grass and clover (WCmix), an upland (Upl) and a fallow (Fal) soil not treated (NT) or supplemented with cycloheximide (CHX), streptomycin (Sm) or both (CHX + Sm). Soils were incubated for 240 h under greenhouse conditions after N addition. Values represent the mean ± standard error (n = 3).*

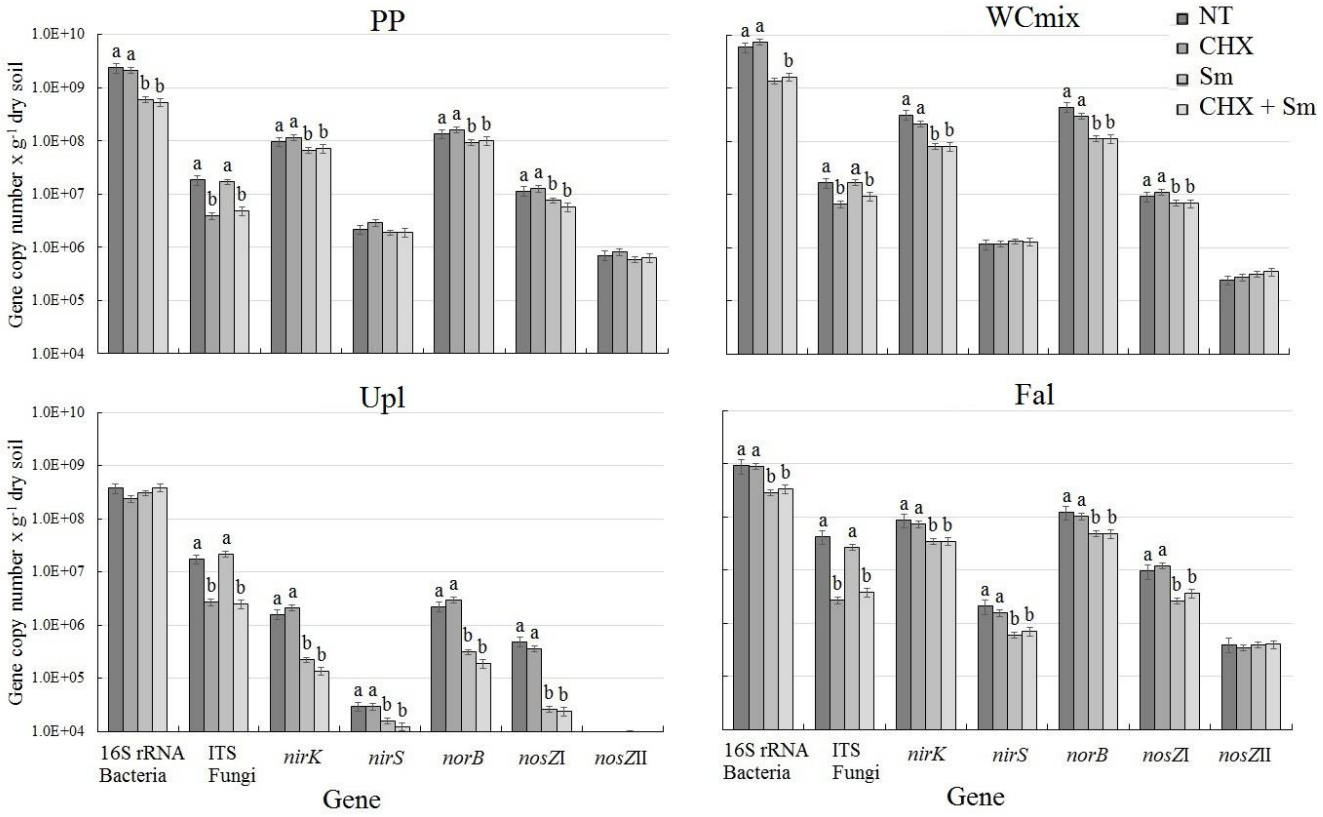



*Fig. 4. Total abundance of the 16S rRNA of bacteria, ITS of fungi, nirK, nirS, norB, nosZI and nosZII genes in a permanent pasture (PP), a mixture of grass and clover (WCmix), an upland (Upl) and a fallow (Fal) soil not treated (NT) or supplemented with cycloheximide (CHX), streptomycin (Sm) or both (CHX + Sm). Soils were incubated for 240 h under greenhouse conditions after N addition. Values represent the mean ± standard error (n = 3). For each gene, bars with the same letter are not statistically different according to the ANOVA and Fisher's Least Significant tests ($p < 0.05$; n = 3).*

## Supplementary data for Chapter VI

# Relative contributions of bacteria and fungi to nitrous oxide emissions in four contrasting soils following nitrate application

**Submitted for publication**

*Table S1. Total abundance of the nirK, nirS, norB, nosZI and nosZII genes in PP, WCmix, Upl and Fal soils not treated (NT) or supplemented with cycloheximide (CHX), streptomycin (Sm) or both (CHX + Sm). Soils were incubated for 240 h under greenhouse conditions after N addition. Values are expressed as gene copy number x $g^{-1}$ dry soil. For each column, values followed by the same letter are not statistically different according to ANOVA and Fisher's Least Significant tests ($p < 0.05$; $n = 3$).*

| Soil type | Treatment | Gene | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 16S Bacteria | ITS Fungi | *nirK* | *nirS* | *norB* | *nosZ*I | *nosZ*II |
| PP | NT | $2.4 \pm 1.2 \times 10^9$ | $1.8 \pm 1.4 \times 10^7$ | $9.6 \pm 1.1 \times 10^7$ | $2.2 \pm 1.0 \times 10^6$ | $1.3 \pm 1.0 \times 10^8$ | $1.1 \pm 0.5 \times 10^7$ | $7.1 \pm 0.5 \times 10^5$ |
| | CHX | $2.1 \pm 1.5 \times 10^9$ | $3.9 \pm 1.0 \times 10^6$ | $1.2 \pm 0.8 \times 10^8$ | $2.9 \pm 0.6 \times 10^6$ | $1.6 \pm 1.0 \times 10^8$ | $1.3 \pm 0.6 \times 10^7$ | $8.2 \pm 0.6 \times 10^5$ |
| | Sm | $6.0 \pm 1.5 \times 10^8$ | $1.7 \pm 1.1 \times 10^7$ | $6.7 \pm 1.2 \times 10^7$ | $1.9 \pm 0.8 \times 10^6$ | $9.4 \pm 0.8 \times 10^7$ | $7.6 \pm 0.9 \times 10^6$ | $6.0 \pm 0.8 \times 10^5$ |
| | CHX + Sm | $5.3 \pm 1.9 \times 10^8$ | $4.9 \pm 1.0 \times 10^6$ | $7.2 \pm 1.4 \times 10^7$ | $1.9 \pm 1.0 \times 10^6$ | $1.0 \pm 0.6 \times 108$ | $5.8 \pm 1.0 \times 10^6$ | $6.4 \pm 0.5 \times 10^5$ |
| WCmix | NT | $5.9 \pm 1.1 \times 10^9$ | $1.7 \pm 0.4 \times 10^7$ | $3.2 \pm 1.0 \times 10^8$ | $1.2 \pm 1.1 \times 10^6$ | $4.4 \pm 0.5 \times 10^8$ | $9.2 \pm 1.2 \times 10^6$ | $2.4 \pm 0.6 \times 10^5$ |
| | CHX | $7.5 \pm 1.0 \times 10^9$ | $6.6 \pm 0.6 \times 10^6$ | $2.2 \pm 1.1 \times 10^8$ | $1.2 \pm 1.0 \times 10^6$ | $3.0 \pm 0.9 \times 10^8$ | $1.1 \pm 1.1 \times 10^7$ | $2.8 \pm 0.5 \times 10^5$ |
| | Sm | $1.4 \pm 0.8 \times 10^9$ | $1.5 \pm 1.2 \times 10^7$ | $8.2 \pm 0.5 \times 10^7$ | $1.3 \pm 0.9 \times 10^6$ | $1.1 \pm 0.5 \times 10^8$ | $7.0 \pm 1.0 \times 10^6$ | $3.2 \pm 0.6 \times 10^5$ |
| | CHX + Sm | $1.6 \pm 1.1 \times 10^9$ | $9.4 \pm 1.0 \times 10^6$ | $8.1 \pm 0.6 \times 10^7$ | $1.3 \pm 0.8 \times 10^6$ | $1.1 \pm 0.6 \times 10^8$ | $6.8 \pm 1.3 \times 10^6$ | $2.5 \pm 0.8 \times 10^5$ |
| Upl | NT | $3.8 \pm 0.6 \times 10^8$ | $3.0 \pm 1.1 \times 10^7$ | $1.6 \pm 1.2 \times 10^6$ | $3.0 \pm 1.0 \times 10^4$ | $2.2 \pm 0.8 \times 10^6$ | $4.9 \pm 1.1 \times 10^5$ | $7.7 \pm 0.9 \times 10^3$ |
| | CHX | $2.4 \pm 1.2 \times 10^8$ | $2.7 \pm 1.4 \times 10^6$ | $2.1 \pm 0.5 \times 10^6$ | $2.9 \pm 0.6 \times 10^4$ | $3.0 \pm 1.1 \times 10^6$ | $3.6 \pm 0.8 \times 10^5$ | $1.4 \pm 0.8 \times 10^3$ |
| | Sm | $3.0 \pm 1.1 \times 10^8$ | $2.2 \pm 0.5 \times 10^7$ | $2.2 \pm 0.9 \times 10^5$ | $1.6 \pm 0.9 \times 10^4$ | $3.1 \pm 1.0 \times 10^5$ | $2.6 \pm 0.9 \times 10^4$ | $9.1 \pm 1.1 \times 10^3$ |
| | CHX + Sm | $3.9 \pm 1.4 \times 10^8$ | $2.5 \pm 1.2 \times 10^6$ | $1.4 \pm 1.1 \times 10^5$ | $1.2 \pm 1.0 \times 10^4$ | $1.9 \pm 1.3 \times 10^5$ | $2.4 \pm 1.3 \times 10^4$ | $6.0 \pm 1.0 \times 10^2$ |
| Fal | NT | $9.2 \pm 1.5 \times 10^8$ | $4.3 \pm 1.0 \times 10^7$ | $8.8 \pm 1.0 \times 10^7$ | $2.1 \pm 1.0 \times 10^6$ | $1.2 \pm 1.0 \times 10^8$ | $9.5 \pm 1.0 \times 10^6$ | $4.0 \pm 1.0 \times 10^5$ |
| | CHX | $9.0 \pm 1.0 \times 10^8$ | $2.5 \pm 1.1 \times 10^6$ | $7.3 \pm 1.0 \times 10^7$ | $1.6 \pm 0.6 \times 10^6$ | $1.0 \pm 0.9 \times 10^8$ | $1.2 \pm 1.3 \times 10^7$ | $3.5 \pm 1.1 \times 10^5$ |
| | Sm | $2.9 \pm 0.3 \times 10^8$ | $3.7 \pm 0.6 \times 10^7$ | $3.5 \pm 0.8 \times 10^7$ | $6.0 \pm 0.8 \times 10^5$ | $4.8 \pm 0.8 \times 10^7$ | $2.6 \pm 1.0 \times 10^6$ | $3.9 \pm 0.9 \times 10^5$ |
| | CHX + Sm | $3.4 \pm 0.5 \times 10^8$ | $3.8 \pm 1.0 \times 10^6$ | $3.5 \pm 1.2 \times 10^7$ | $7.0 \pm 0.9 \times 10^5$ | $4.9 \pm 0.9 \times 10^7$ | $3.7 \pm 0.6 \times 10^6$ | $4.0 \pm 0.8 \times 10^5$ |

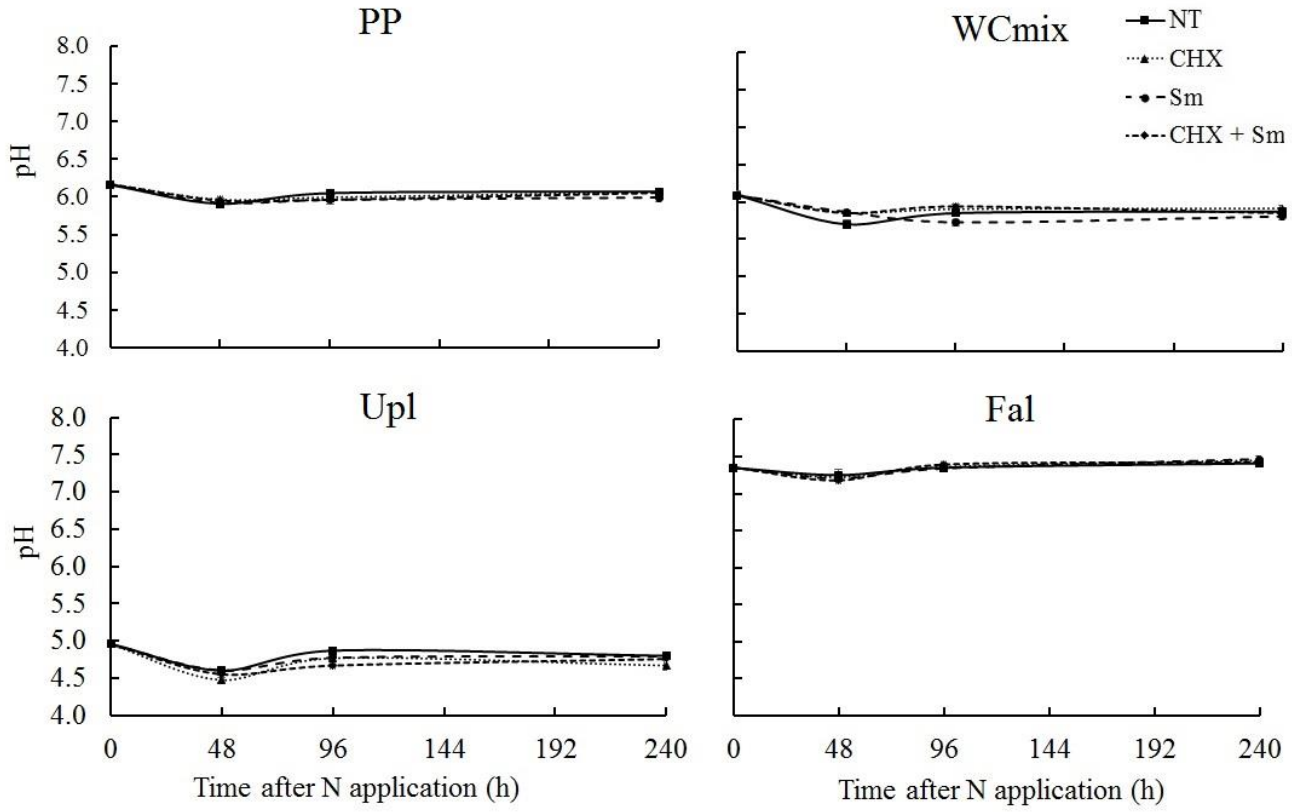



*Fig. S1. pH values of PP, WCmix, Upl and Fal soils not treated (NT) or supplemented with cycloheximide (CHX), streptomycin (Sm) or both (CHX + Sm). Soils were incubated for 240 h under greenhouse conditions after N addition. Values represent the mean ± standard error (n = 3).*

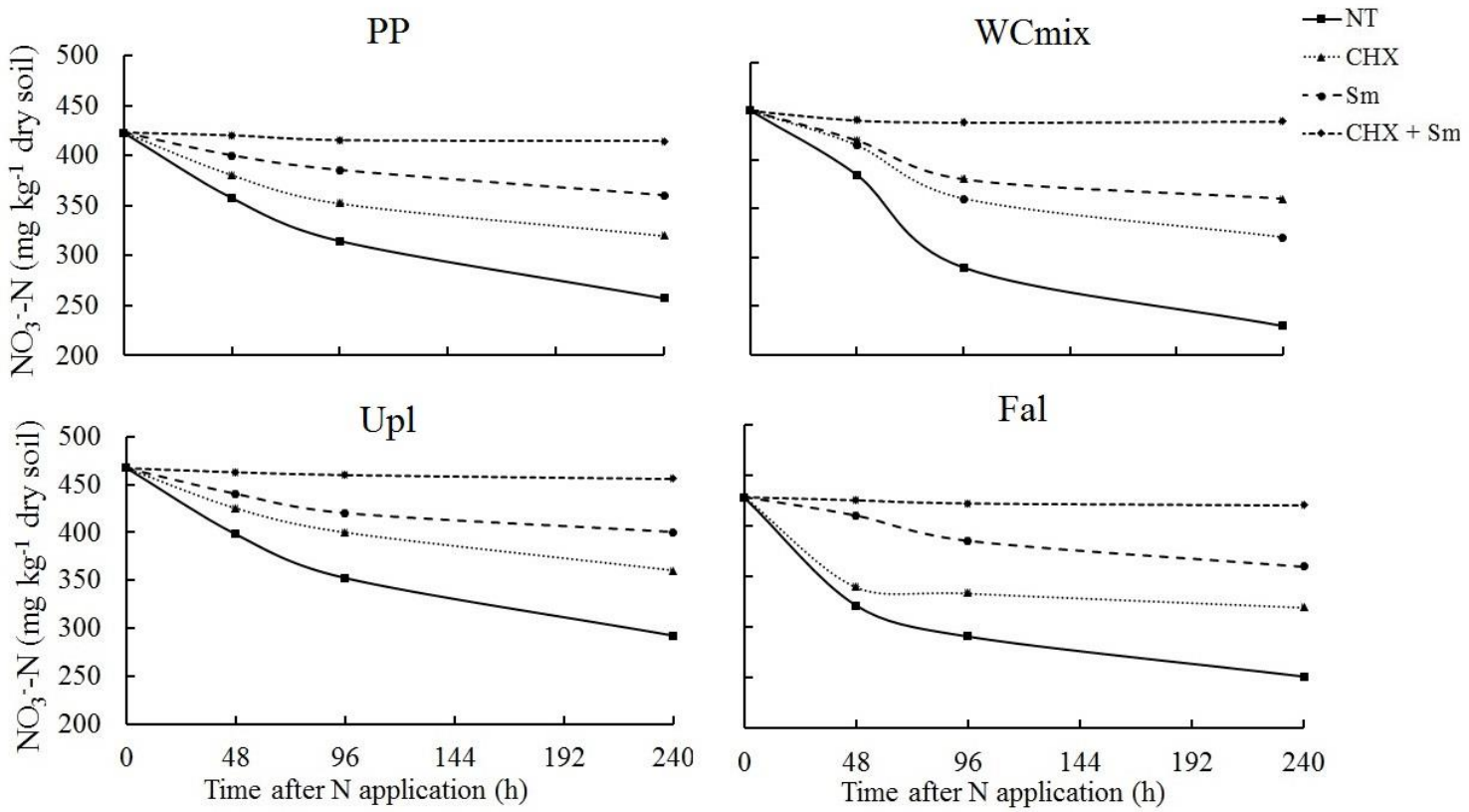



*Fig. S2. $NO_3^-$-N content (mg kg$^{-1}$ dry soil) of PP, WCmix, Upl and Fal soils not treated (NT) or supplemented with cycloheximide (CHX), streptomycin (Sm) or both (CHX + Sm). Soils were incubated for 240 h under greenhouse conditions after N addition. Values represent the mean ± standard error (n = 3).*

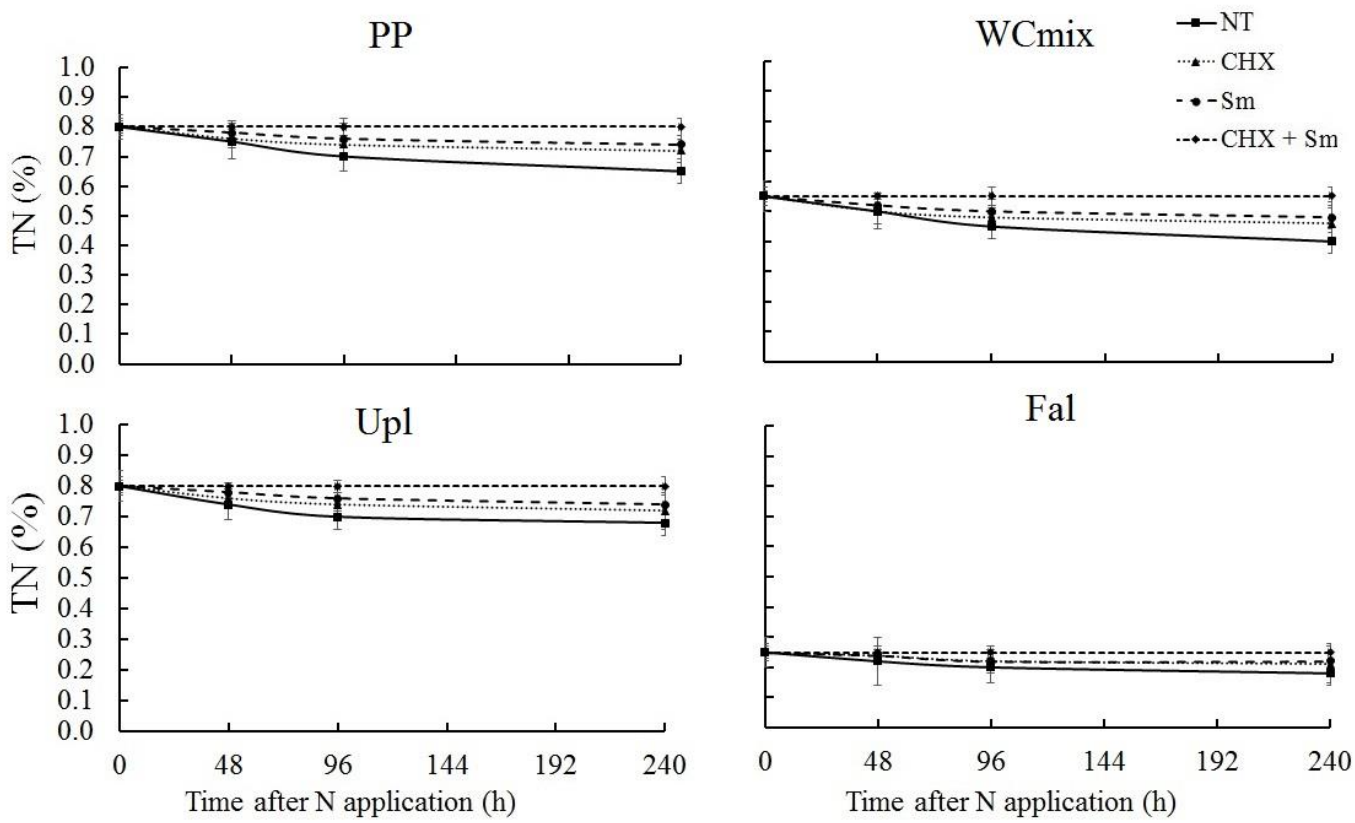



*Fig. S3. TN content (%) of PP, WCmix, Upl and Fal soils not treated (NT) or supplemented with cycloheximide (CHX), streptomycin (Sm) or both (CHX + Sm). Soils were incubated for 240 h under greenhouse conditions after N addition. Values represent the mean ± standard error (n = 3).*

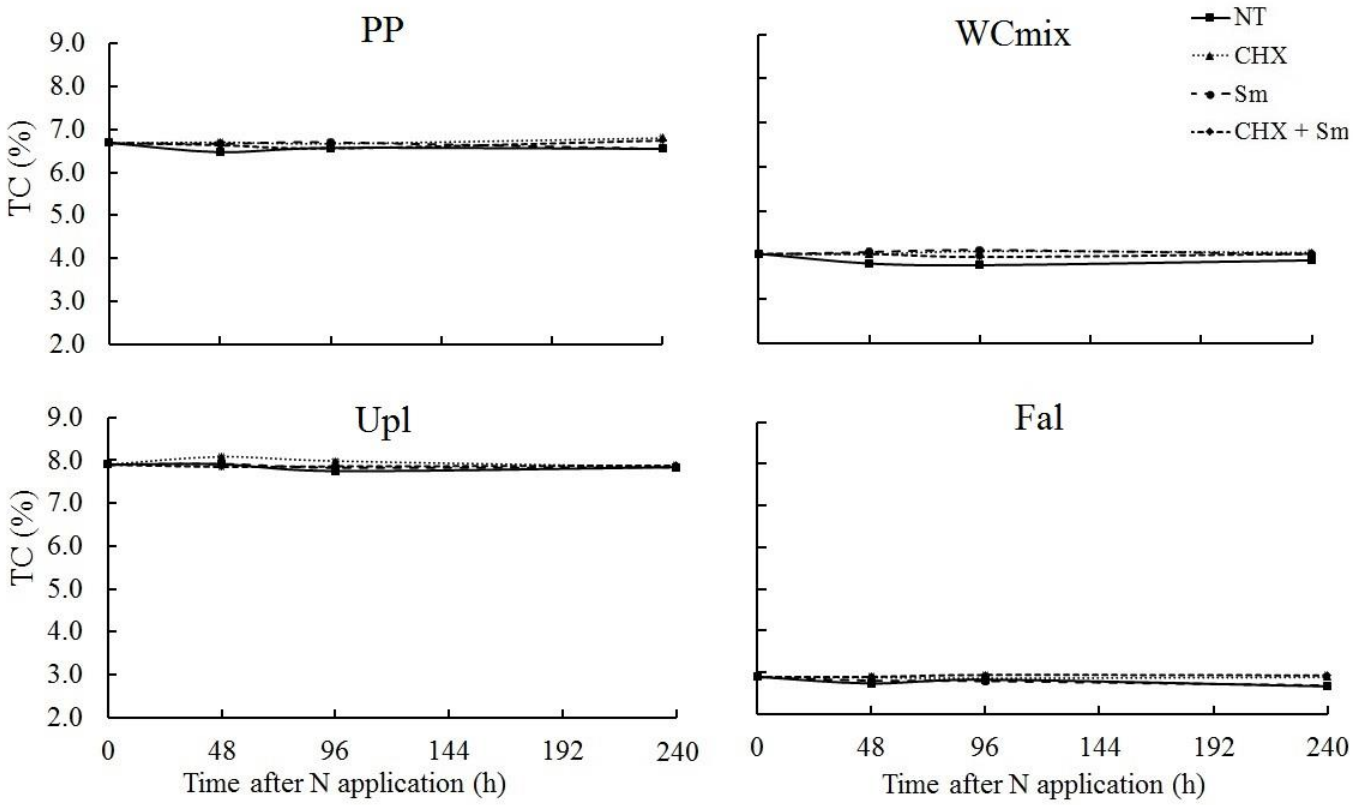



*Fig. S4. TC content (%) of PP, WCmix, Upl and Fal soils not treated (NT) or supplemented with cycloheximide (CHX), streptomycin (Sm) or both (CHX + Sm). Soils were incubated for 240 h under greenhouse conditions after N addition. Values represent the mean ± standard error (n = 3).*

# Chapter VII

## Effect of urease and nitrification inhibitors on ammonia volatilisation and abundance of N-cycling genes

**Submitted for publication**

Abstract

The effect of the combined application of urease and nitrification inhibitors on ammonia volatilisation and the abundance of nitrifiers and denitrifiers communities are largely unknown. Here, in a mesocosm experiment, ammonia volatilisation was monitored in a soil treated with urea and with the single or combined application of the urease inhibitor N-(n-butyl) thiophosphoric triamide (NBPT) and the nitrification inhibitor 3,4 dimethylpyrazole phosphate (DMPP) under 50 and 80% water-filled pore space (WFPS). The effect of the treatments on the abundance of Bacteria and Archaea was estimated by quantitative PCR (qPCR) of their corresponding 16S rRNA gene, that of nitrifiers using *amoA* genes and that of denitrifiers by qPCR of the *norB* and *nosZ*I denitrification genes. After urea treatment, N losses due to $NH_3$ volatilisation accounted for 23.0 and 9.2% at 50 and 80% WFPS, respectively. NBPT reduced $NH_3$ volatilisation to 2.0 and 2.4% whereas DMPP increased N losses up to 36.8 and 26.0% at 50 and 80% WFPS, respectively. The combined application of NBPT and DMPP also increased $NH_3$ emissions, albeit to a lesser extent than that of DMPP alone. As compared to an unfertilised control soil, both at 50 and 80% WFPS, NBPT did not affect neither the abundance of Bacteria and Archaea nor of the nitrifiers, and decreased that of denitrifiers at 80% WFPS. Regardless of the moisture conditions, application of DMPP increased the biomass of denitrifiers.

Keywords: ammonia volatilisation, 3,4-dimethylpyrazole phosphate (DMPP), N-(n-butyl) thiophosphoric triamide (NBPT), nitrifiers, denitrifiers, qPCR

## Introduction

Most agricultural practices rely on external nitrogen (N) inputs to maintain plant growth and yields. Excess N applied to soils as synthetic or organic fertilisers leads to reduced use efficiency mainly due to nitrate ($NO_3^-$) leaching losses, ammonia ($NH_3$) volatilisation and N-gas production of nitric oxide (NO), nitrous oxide ($N_2O$) and dinitrogen ($N_2$) (Erisman et al. 2015). Urea is the N-fertiliser most widely applied in modern agriculture, and its use has increased 100-fold in the last 50 years mainly due to its low cost (Thompson and Meisinger 2004; Glibert et al. 2006; Heffer and Prud'homme 2011). Among the management strategies to improve the N use efficiency of fertilisers and to mitigate gas emissions, urease and nitrification inhibitors are commonly used. Urease is the enzyme responsible for the conversion of urea into carbon dioxide ($CO_2$) and $NH_3$. When urea is applied to the soil, from 10 to 40% of urea-N is directly lost as $NH_3$ volatilized into the atmosphere (UNECE 2001; Salazar et al. 2012; Cantarella et al. 2018) and this loss can be reduced by applying urease inhibitors which depress urea hydrolysis (Francisco et al. 2011; Abalos et al. 2012) and subsequent ammonium ($NH_4^+$) concentration in the soil solution (Gill et al. 1999; Modolo et al. 2015). Phenylphosphorodiamidate (PPD/PPDA), hydroquinone and N-(n-butyl) thiophosphoric triamide (NBPT) are urease inhibitors with the latter being generally the most used and the most effective both under laboratory (Carmona et al. 1990; Gill et al. 1999) and field conditions (Sanz-Cobeña et al. 2008; 2011; Zaman et al. 2009; Rodríguez-Soares et al. 2012; Tao et al. 2018). However, the effectiveness of urease inhibitors is still uncertain because soil characteristics, environmental and weather conditions as well as types and concentrations of inhibitors can affect $NH_3$ emissions (Sanz-Cobeña et al. 2011).

Nitrification inhibitors block the activity of the enzyme ammonia monooxygenase, encoded by ammonia monooxygenase gene (*amoA*) (Weiske et al. 2001; Zerulla et al. 2001), which is responsible for the first step of nitrification, thus extending the period of permanence of changeable ammonium in soils and reducing the production of $N_2O$. However, the prolonged retention time of $NH_4^+$ in soil may increase ammonia emissions (Rodríguez-Soares et al. 2012; Qiao et al. 2015).

Dicyandiamide (DCD) and 3,4 dimethylpyrazole phosphate (DMPP) are the most used nitrification inhibitors and they can effectively reduce nitrification rates under different laboratory and field conditions (Moir et al. 2012; Pfab et al. 2012; Yu et al. 2015; Gilsanz et al. 2016; Lan et al. 2018). Despite being an efficient commercially available nitrification inhibitor, experiments designed to evaluate $NH_3$ volatilisation after DMPP application are relatively scarce, and conclusive information does not exist (Menéndez et al. 2009; Yang et al. 2016a).

Both urease and nitrification inhibitors can affect the activity and composition of microbial populations. The addition of NBPT did not affect (Giovannini et al. 2009) or decreased the activity and the abundance of the bacterial and archaeal *amoA* gene (Shi et al. 2017). The application of DMPP inhibited the abundance of the *amoA* gene of ammonia-oxidising bacteria (Li et al. 2008; Beltran-Rendon et al. 2011; Di and Cameron 2011; Yang et al. 2013; Ruser and Schulz 2015; Duan et al. 2016) and ammonia-oxidising archaea (Kleineidam et al. 2011; Shi et al. 2016a, b). Further, urease and nitrification inhibitors can also affect denitrification, the process by which $NO_3^-$ is sequentially reduced to nitrite ($NO_2^-$), NO, $N_2O$ and, finally, $N_2$ by the nitrate-, nitrite-, nitric oxide-, and nitrous oxide-reductase enzymes encoded by the *napA*/*narG*, *nirK*/*nirS*, *norB* and *nosZ* genes, respectively (Butterbach-Bahl et al. 2013; Hallin et al. 2018). Under field conditions, repeated applications of NBPT decreased the abundance of the *narG* and *nirK* genes (Shi et al. 2017), and the addition of DMPP increased the abundance of the *narG*, *nirK* and *nosZ* genes in a soil with 80% of water-filled pore space (WFPS) under greenhouse conditions (Barrena et al. 2017).

The combined application of urease and nitrification inhibitors during urea application could result in the reduction of gaseous $NH_3$ as well as $N_2O$ and $NO_3^-$ leaching losses. Information on the effects of the simultaneous amendment of urease and nitrification inhibitors on ammonia volatilisation and abundances of nitrifiers and denitrifiers is scarce. In this context, the objective of this study was to investigate the effects of the single and combined application of the urease inhibitor NBPT and the nitrification inhibitor DMPP on $NH_3$ volatilisation and on the abundance of nitrifying and denitrifying soil communities under 50 and 80% WFPS.

## Materials and methods

### Soil sampling and experimental design

An Eutric Cambisol (30% clay, 12.5% silt, 57.5% sand, w/w; pH in water, 7.1; organic C, 4%; total N 0.2%; $NO_3^-$ 6.8 mg $kg^{-1}$; exchangeable $NH_4^+$, not detected; $HCO_3^-$ 244 mg $kg^{-1}$) of the FAO series (FAO 2017) was collected from an agricultural soil (UTM coordinates 36° 43' 53.5" N, 3° 32' 56.2" W) in the vicinity of Motril (Granada, Spain). The soil had been maintained without fertilisation and irrigation for more than 10 years. Spade-squares (30 x 30 cm to a depth of 25 cm) were taken from 12 sites. After sampling, roots and plant residues were removed, air-dried to ~ 30% $H_2O$ (dry basis) and pooled to obtain a composite sample.

In a greenhouse at the farm of the Technical University of Madrid, Spain, 96 PVC pots (26 cm diameter, 15 cm height) were filled each with 5 kg of soil up to 5 cm from the rim. All pots were supplemented with urea at a final concentration of 260 kg N $ha^{-1}$ (1.51 g N $kg^{-1}$) as recommended for horticultural crops by the Spanish Ministry of Agriculture, Food and Environment. The experiment was arranged in a factorial randomized complete block design with six replications for each of the 4 treatments that were: urea, urea + NBPT, urea + DMPP, urea + NBPT + DMPP. NBPT and DMPP were added to give a proportion of inhibitor in the mixture of 0.25 and 0.8% on a weight basis, respectively. A set of pots containing soil without fertilisation was used as a control. Fertiliser and inhibitors were diluted in 100 mL water at the beginning of the experiment and applied to the pots from the top. Subsequently, half of the pots were adjusted to 50% WFPS and the other half to 80% WFPS and watered weekly to reach the corresponding WFPS. For each WFPS, half of the pots were used for $NH_3$ volatilisation measurements and the other half was used for soil sampling. All pots were maintained at 18 ºC for 60 days.

### Soil analyses

Concentrations of $NO_3^-$ and exchangeable $NH_4^+$, urease activity, pH and moisture content were determined in soil cores collected at a depth of 0-5 cm from the rim of the pots at 1, 5, 15, 30 and 60 days after the start of the experiment. An ionic chromatograph (Methohm) equipped with a Metrosep A supp-4-250 anion column

and a Metrosep C2-150 cation column were used to determine $NO_3^-$-N and exchangeable $NH_4^+$-N concentrations, respectively, as indicated earlier (González-Martínez et al. 2016). Urease activity was determined as already reported which involves the incubation of soil with buffered urea solution, the extraction of exchangeable $NH_4^+$ with 1 N KCl and colorimetric $NH_4^+$ determination (Nannipieri et al. 1980). The pH was measured after water extraction (1:5, w/v) for 2 h. Moisture was measured gravimetrically after drying of the soil for 24 h at 105 ºC. The WFPS was calculated according to Danielson and Sutherland (1986).

### $NH_3$ emission measurement

Ammonia volatilisation was analyzed using a gas flow-through system coupled to a chemiluminiscence ammonia analyzer (Thermo Scientific, model 17i analyzer) (Fig. S1) as previously described (Aneja et al. 2000; Walker et al. 2002). To minimize $NH_3$ losses during the measurement, each container was closed with a Teflon®-covered lid provided with an inlet and with outlet holes. Air was pumped into the chamber through the inlet hole at a constant rate (Q = 10 L $min^{-1}$). Gas samples were transported through 3-m Teflon® tubing from the outlet hole to a T tube, with one part venting to the atmosphere and the remainder entering into the analyzer at a flow rate of 0.5 L $min^{-1}$. The excess air was vented though another outlet hole to minimize pressure differences between the chamber and the atmosphere. Airflow samples were passed through a stainless steel converter, where $NH_3$ transforms to nitric oxide (NO) after reaction with ozone. The lower detectable limit was 1.0 ppb (v/v). The steady-state concentration of $NH_3$ in the chamber was reached after 20 minutes. A chamber coated with Teflon® was used as control. The air flow rate was checked daily with a flowmeter.

Under steady-state conditions, the change of concentration with respect to time is zero, so that the $NH_3$ flux was calculated following the equation proposed for reactive gases by Kaplan et al. (1988):

$$J/h = \left(L/h + q/V\right) C_{eq} - q\left(C_{air}/V\right) \quad \text{(equation 1)}$$

where h is the internal height of the chamber (m), J is the emission flux per unit area (mg N $m^{-2}$ $s^{-1}$), q is the flow rate through the chamber ($m^3$ $s^{-1}$), V is the volume of the chamber ($m^3$), $C_{eq}$ is the concentration measured at the chamber outlet (mg N $m^{-3}$), $C_{air}$ is the $NH_3$ concentration in the air from the pump passing through the control chamber mentioned above and L is the sum of the loss of $NH_3$ through reactions with the chamber walls (m $s^{-1}$). The value of L was 5 x $10^{-4}$ m $s^{-1}$, which agrees with that reported by Walker et al. (2002). Cumulative $NH_3$ emissions were calculated by multiplying the length of time between two samplings by the average emissions rate for that period, and adding that amount to the previously gas accumulated. $NH_3$ fluxes were determined daily during the first 15 days and again every 5 days until the end of the 60 days-experimental time. $NH_3$-N losses after incubation for 60 days were estimated by dividing the cumulative $NH_3$ emissions by to the total N applied.

### DNA extraction and quantification of nitrifying and denitrifying bacterial genes

Total soil DNA was extracted from 0.5-g samples taken at 1 and 60 days after the start of the experiment, purified using GeneClean (MP Bio) spin columns and stored at -20 ºC until use as reported earlier (Castellano-Hinojosa et al. 2018). The DNA concentration was measured using the Qubit® ssDNA assay kit (Molecular Probes). Total bacterial and archaeal communities were quantified using the 16S rRNA gene from Bacteria (16SB) and Archaea (16SA), respectively, as molecular markers. The size of the nitrifier community was estimated by amplification of the *amoA* gene from ammonia-oxidizing Bacteria (*amoA* AOB) and Archaea (*amoA* AOA) while that of the denitrifier community was assessed via the amplification of the *norB* and *nosZI* genes, using primers and thermal conditions previously described (Castellano-Hinojosa et al. 2018). Quantitative PCR (qPCR) assays were carried out using a Bio-Rad iCycler iQ5 Thermocycler (Bio-Rad Laboratories, USA) with SYBR Green as the detection system. PCR efficiency for the different assays ranged between 90 and 99%. The quality of all qPCR amplifications was verified by electrophoresis in agarose and by melting curve analysis.

### Statistical analyses

The measured variables were first explored using the Shapiro-Wilk test and the Bartlett's test to check whether they meet the normality and homoscedasticity

assumptions, respectively. Since most data sets failed to fit the normal distribution, the Kruskal-Wallis test was chosen to search for significant differences, and the Conover-Iman test was used for multiple comparisons among samples. These tests were performed with the aid of the software package Statgraphics Centurion XVII (StatPoint Technologies Inc, Warrenton, USA). A 95% significance level ($p < 0.05$) was selected.

Redundancy analysis (RDA) was performed to verify the relationship between the biotic variables (abundance of 16SA, 16SB, *amoA* AOA, *amoA* AOB, *norB* and *nosZ*I genes) and the abiotic variables (pH, $NH_3$, exchangeable $NH_4^+$-N, $NO_3^-$-N) (*Leps and Smilauer* 2003). All variables were tested for multicollinearity, and the distance between any two sample points reflects their relative similarity. Collinear variables were considered with variance inflation factors (VIFs) > 10 and tolerances < 0.1. The statistical significance of each variable was assessed by the Monte Carlo permutation test (unrestricted permutations, reduced model, 999 permutations (Leps and Smilauer 2003), using the software package CANOCO 4.5 (Biometris, Wageningen, Netherlands).

## Results

### Soil physicochemical properties

With an initial value of 7.1 in the control soil and about 7.7 in the urea-treated soil, the pH remained largely unchanged during the 60-days experimental period, both at 50 and 80% of the WFPS (Table S1). The presence of NBPT, both in single and combined application with the DMPP, delayed and decreased the urease activity during the experimental period (Table S2). Regardless of the treatment, the exchangeable $NH_4^+$-N concentration declined with time, and decreases were higher at 50% WFPS (Table S3). After 60 days, the content of exchangeable $NH_4^+$-N in the soil treated with urea + NBPT (120 and 130 mg $NH_4^+$-N $kg^{-1}$ at 50 and 80% WFPS, respectively) was higher than the amounts found in soils amended with urea (36.7 and 52.7 mg $NH_4^+$-N $kg^{-1}$ at 50 and 80% WFPS, respectively), urea + DMPP (10.1 and 23.4 mg $NH_4^+$-N $kg^{-1}$ at 50 and 80% WFPS, respectively) and urea + NBPT + DMPP (38.1 and 48.2 mg $NH_4^+$-N $kg^{-1}$ at 50 and 80% WFPS, respectively). The combined addition of DMPP and NBPT presented lower ammonium content that the

single application of NBPT, showing a similar ammonium content than urea treatment (Table S3). On the other hand, soil $NO_3^-$-N increased with time so that values found after 60 days at 50 and 80% WFPS were 76.1 and 39.8, 9.5 and 6.6, 38.2 and 29.5, 35.4 and 25.1 mg $NO_3^-$-N $kg^{-1}$ in soils treated with urea, urea + NBPT, urea + DMPP, and urea + NBPT + DMPP, respectively (Table S4). The urease activity and the exchangeable $NH_4^+$-N were negligible in the control soil during the experimental period whereas $NO_3^-$-N content remained in a range around 7 mg $NO_3^-$-N $kg^{-1}$ (Table S4).

### Ammonia emissions

After application of any of the treatments, at 50% WFPS, the $NH_3$ fluxes reached maximal values of 1.02 kg N $ha^{-1}$ $h^{-1}$ for urea, 0.15 kg N $ha^{-1}$ $h^{-1}$ for urea + NBPT, 1.42 kg N $ha^{-1}$ $h^{-1}$ for urea + DMPP and 1.22 kg N $ha^{-1}$ $h^{-1}$ for urea + NBPT + DMPP after incubation for 2 d (Table 1), and then diminished to similar basal levels (0.05-0.10 kg N $ha^{-1}$ $h^{-1}$) that remained until the end of the experimental period. At 80% WFPS, the highest $NH_3$ fluxes obtained after the treatment with urea, urea + NBPT, urea + DMPP, and urea + NBPT + DMPP were 0.58, 0.08, 0.92 and 0.72 kg N $ha^{-1}$ $h^{-1}$, respectively, values that were reached 3, 8, 6 and 8 d after application, respectively; similar to 50% WFPS, $NH_3$ emission decreased afterwards to basal levels of about 0.04-0.08 kg N $ha^{-1}$ $h^{-1}$ (Table 1). The cumulative $NH_3$ emissions during the experimental period are presented in Table 1. The data show that the addition of urea, urea + NBPT, urea + DMPP and urea + NBPT + DMPP at 50 and 80% WFPS resulted in N losses of 23.0 *versus* 9.2%, 2.0 *versus* 2.4%, 36.8 *versus* 26.0% and 29.0 *versus* 14.8% of the applied N, respectively. Ammonia volatilisation was not detected in the control soil during the experimental period.

### Abundance of the bacterial and archaeal communities

The abundance of the bacterial (16SB) and the archaeal (16SA) communities in soil samples at 50 and 80% WFPS taken from the control and amended soils at 1 and 60 days after application of the treatments are shown in Fig. 1. The changes in the abundance of the genes are referred to the control soil. After incubation for 1 day, the 16SB and 16SA gene abundances in the control soil at 50% WFPS were $3.1x10^8$ and $1.4x10^7$ gene copy number x $g^{-1}$ dry soil, respectively, and $3.2x10^8$ and $1.5x10^7$

at 80% WFPS, respectively. After incubation for 60 days, significant differences in the gene abundances were not found (Fig. 1). In the urea-treated soil, after 1 day incubation at 50% WFPS, the abundance of the 16SB gene increased to $1.1x10^9$ gene copy number x $g^{-1}$ dry soil and $1.8x10^9$ after incubation for 60 days. At 80% WFPS, the gene copy number increased to $1.1x10^9$ and $3.0x10^9$ gene copy number x $g^{-1}$ dry soil after 1 and 60 days of incubation, respectively. The abundance of the 16SA gene at 50 and 80% WFPS increased after 1 day to $4.8x10^7$ and $4.9x10^7$ gene copy number x $g^{-1}$ dry soil, respectively, and to $8.3x10^7$ and $1.3x10^8$, respectively, after 60 days incubation.

The application of urea + NBPT did not affect the copy number of the 16SB and 16SA genes neither at 50 or 80% WFPS nor after incubation for 1 or 60 days. When the soil was supplemented with urea + DMPP, at 50 and 80% WFPS, the abundance of the 16SB gene after 1 day increased to $7.6x10^8$ and $7.8x10^8$ gene copy number x $g^{-1}$ dry soil, respectively, and to $1.3x10^9$ and $2.1x10^9$ after 60 days incubation, respectively. The biomass of the 16SA gene at 50 and 80% WFPS also increased to $3.5x10^7$ and $3.6x10^7$ gene copy number x $g^{-1}$ dry soil after 1 day, respectively, and to $6.0x10^7$ and $9.7x10^7$ at the end of the incubation time. The abundance of the 16SB gene in soils treated with urea + NBPT + DMPP after 1 day at 50 and 80% WFPS increased to $6.5x10^8$ and $6.6x10^8$ gene copy number x $g^{-1}$ dry soil, respectively and to $1.1x10^9$ and $1.8x10^9$ at d 60, respectively. At 50 and 80% WFPS the 16SA gene abundance increased to $3.0x10^7$ and $3.1x10^7$ gene copy number x $g^{-1}$ dry soil after 1 day, and to $5.1x10^7$ and $8.2x10^7$ after 60 days, respectively.

### Abundance of the nitrifying communities

Data on the abundance of the nitrification *amoA* AOB and *amoA* AOA genes are shown in Fig. 2. The changes in the biomass of the genes are referred to the control soil. In this soil, the *amoA* AOB gene abundance at 50 and 80% WFPS was $1.1x10^5$ and $1.2x10^5$ gene copy number x $g^{-1}$ dry soil at d 1, respectively, these values were similar to those calculated after incubation for 60 days. At d 1, the abundance of the *amoA* AOA gene at 50 and 80% WFPS was $3.8x10^4$ and $3.7\ x10^4$ gene copy number x $g^{-1}$ dry soil, respectively and changes were not detected after 60 days. In the urea-treated soil, at 50% WFPS, the abundance of the *amoA* AOB gene at d 1 increased to

$1.2x10^6$ gene copy number x $g^{-1}$ dry soil and $7.8x10^6$ at d 60. At 80% WFPS the abundance increased to $4.7x10^5$ at d 1 and $7.9x10^5$ at d 60, respectively. The biomass of the *amoA* AOA gene at 50 and 80% WFPS increased to $3.9x10^5$ and $1.2x10^5$ gene copy number x $g^{-1}$ dry soil at d 1, respectively, and to $2.4x10^6$ and $4.1x10^5$ at d 60, respectively. The application of urea + NBPT at 50 and 80% WFPS did not affect the copy number of the *amoA* AOB and *amoA* AOA genes each after treatment for 1 and 60 days. When the soil was supplemented with urea + DMPP, at 50 and 80% WFPS, the abundance of the *amoA* AOB gene increased after treatment for 1 day to $8.9x10^5$ and $4.3x10^5$ gene copy number x $g^{-1}$ dry soil, respectively, and to $4.0x10^6$ and $6.5x10^5$ after incubation for 60 days, respectively. The copy number x $g^{-1}$ dry soil of the *amoA* AOA gene at 50 and 80% WFPS also increased to $2.9x10^5$ and $1.2x10^5$ at day 1, respectively, and to $4.8x10^5$ and $2.3x10^5$ at the end of the incubation time. The biomass of the *amoA* AOB gene in the soil treated with urea + NBPT + DMPP at 50 and 80% WFPS increased to $4.5x10^5$ and $2.2x10^5$ gene copy number x $g^{-1}$ dry soil at day 1, respectively, and to $2.0x10^6$ and $3.3x10^5$ at day 60, respectively. A similar effect was observed at 50 and 80% WFPS for the *amoA* AOA gene whose abundance increased to $1.6x10^5$ and $5.9x10^4$ gene copy number x $g^{-1}$ dry soil after 1 day, respectively, and to $2.0x10^5$ gene and $1.3x10^5$ after 60 days, respectively.

### Abundance of the denitrifying communities

Fig. 3 shows the abundance of the *norB* and *nosZI* denitrification genes. The variations in the biomass of the genes are referred to the control soil. In this soil, the abundance of the *norB* gene after incubation for 1 day was $1.6x10^6$ gene copy number x $g^{-1}$ dry soil at 50% WFPS and $2.3x10^6$ at 80% WFPS. Incubation for 60 days did not alter the copy number of the *norB* gene. Like *norB*, the biomass of the *nosZI* gene at day 1 was $7.2x10^5$ and $1.0x10^6$ gene copy number x $g^{-1}$ dry soil at 50 and 80% WFPS, respectively, and increase in the biomass of the gene was not observed at day 60. The addition of urea at 50% WFPS did not affect the abundance of the *norB* gene at day 1 ($1.8x10^6$ gene copy number x $g^{-1}$ dry soil) and increased to $2.7x10^6$ after incubation for 60 days. A similar situation was found to happen at 80% WFPS as the abundance of the *norB* gene did not change after 1 day ($2.5x10^6$ gene copy number x $g^{-1}$ dry soil) and increased to $1.1x10^7$ after 60 days. At 50 and 80%

WFPS, the abundance of the *nosZ*I gene remained unchanged after 1 day ($7.9x10^5$ and $1.2x10^6$ gene copy number x $g^{-1}$ dry soil, respectively) and increased to $1.2x10^6$ and $4.9x10^6$ after incubation for 60 days, respectively. The application of urea + NBPT at 50% WFPS did not affect the copy number of the *norB* gene after incubation for 1 and 60 days. At 80% WFPS, the abundance of the *norB* gene decreased to $1.8x10^6$ and $7.5x10^5$ gene copy number x $g^{-1}$ dry soil after 1 and 60 days, respectively. The biomass of the *nosZ*I was not affected after incubation for 1 and 60 days at 50% WFPS and decreased to $7.5x10^5$ and $6.8x10^5$ gene copy number x $g^{-1}$ dry soil at 80% WFPS. When the soil was supplemented with urea + DMPP at 50 and 80% WFPS the copy number of the *norB* gene increased to $2.1x10^6$ and $2.2x10^6$ gene copy number x $g^{-1}$ dry soil after incubation for 1 day, respectively, and to $5.2x10^6$ and $1.3x10^7$ after 60 days, respectively. The treatment of the soil with urea + DMPP at 50 and 80% WFPS also increased the abundance of the *nosZ*I gene to $9.3x10^5$ and $2.9x10^6$ gene copy number x $g^{-1}$ dry soil after 1 day, respectively, and to $1.6x10^6$ and $6.0x10^6$ at day 60, respectively. The abundance of the *norB* gene in the soil treated with urea + NBPT + DMPP increased at 50 and 80% WFPS to $2.1x10^6$ and $2.8x10^6$ gene copy number x $g^{-1}$ dry soil at day 1, respectively, and to $2.8x10^6$ and $3.8x10^6$ after incubation for 60 days, respectively. A similar effect was observed at 50 and 80% WFPS for the *nosZ*I gene whose abundance increased to $8.3x10^5$ and $2.4x10^6$ gene copy number x $g^{-1}$ dry soil after 1 d, respectively, and to $9.7x10^5$ and $3.0x10^6$ at day 60, respectively.

### Linking $NH_3$ fluxes, soil physicochemical properties and biological variables

An RDA analysis showed significant relationships between the biotic (abundance of 16SA, 16SB, *amoA* AOA, *amoA* AOB, *norB* and *nosZ*I genes) and the abiotic (pH, $NH_3$, exchangeable $NH_4^+$-N and $NO_3^-$-N) variables, both at 50 and 80% WFPS (Monte Carlo test, $p = 0.001$) (Fig. 4). Goodness of the analysis is indicated by the fact that the sum of the two axes explained 86.2 and 78.2% of the total variability at 50 (Fig. 4A) and 80% WFPS (Fig. 4B), respectively. At 50% WFPS, the exchangeable $NH_4^+$-N concentration was positively related with the abundance of the *amoA* AOA and *amoA* AOB genes, except for the urea + NBPT treatment. As indicated by the length of the vectors this correlation was lower at 80% WFPS (Fig. 4A). The analysis also

showed that, except for the soil treated with urea + NBPT, there was a positive correlation between the $NO_3^-$-N concentration and the abundance of the *norB* and *nosZ*I genes when urea, urea + DMPP or urea + NBPT + DMPP were applied to the soil each at 50 (Fig. 4A) and 80% WFPS (Fig. 4B).

## Discussion

### Effect of NBPT and DMPP on $NH_3$ emissions

Previous studies have established that the urease inhibitor NBPT (Carmona et al. 1990; Gill et al. 1997; Sanz-Cobeña et al. 2008; 2011; Zaman et al. 2009) and the nitrification inhibitor DMPP (Kim et al. 2012; Yang et al. 2016a) reduce or increase ammonia emissions, respectively. This study extends those findings by showing the effects of the single and combined application of NBPT and DMPP on $NH_3$ volatilisation and the abundance of nitrifying and denitrifying communities in an Eutric Cambisol soil under 50 and 80% WFPS. Different authors have reported values of urea-dependent ammonia volatilisation ranging from 5 to 50% of the applied N, these differences most probably due to effects of soil factors and fertilisation management (Yang et al. 2016a and references therein). In this mesocosm study, the amount of N lost through $NH_3$ volatilisation during 60 days after urea application accounted for 23.0 and 9.2% at 50 and 80% WFPS of the N applied to the soil, respectively (Table 1). The length of the vector in the RDA analysis corresponding to $NH_3$ volatilisation was longer at 50% than at 80% WFPS (Fig. 4A and 4B), which indicates that 50% WFPS favoured $NH_3$ volatilisation. This agrees with the idea that high water contents facilitate $NH_4^+$ adsorption to the particles in the soil matrix thus preventing its release into the atmosphere (Sanz-Cobeña et al. 2011; Yang et al. 2016a and references therein).

Whereas the amendment of the soil with urea + NBPT drastically reduced volatilisation to a final N loss of about 2 and 2.4% at 50 and 80% WFPS, respectively, the addition of urea + DMPP clearly increased $NH_3$ emissions (Table 1). These results can be explained assuming that DMPP prolonged the retention time of ammonium in the soil, thus annulling the positive effect of the reduction of the urease activity in the days following its application, producing emissions higher than those of the urea without the inhibitor; the combined application, however, of NBPT

and DMPP alleviated the inhibitory effect of the treatment with NBPT alone, with a corresponding increase in the amount of $NH_3$ released into the atmosphere of 29% at 50% WFPS and 14.8% at 80% WFPS (Table 1). In fact, the inclusion of NBPT delayed the urease hydrolysis (Table S2) leaving less $NH_4^+$ available (Table S3) for $NH_3$ volatilisation. Similar results on $NH_3$ emissions after application to the soils of urea + NBPT + DCD have been published (Cantarella et al. 2018 and references therein).

### Effect of NBPT and DMPP on the abundance of bacterial and archaeal communities

NBPT did not affect the 16SB and 16SA abundance under the conditions used in this study (Fig. 1). These results agree with those reported by Shi et al. (2017) who found no significant effect of NBPT on the 16SB gene. The application of DMPP, however, in single and in combination with NBPT, increased the gene abundances, albeit to a lower extent than those produced by the treatment with urea (Fig. 1), which suggests a positive effect of DMPP on the bacterial and archaeal biomass. Using ammonium sulfate nitrate as a fertiliser, Barrena et al (2017) have shown that the addition of DMPP to a grassland soil did not affect the 16SB gene abundance both at 40 and 80% WFPS. It is to note that amendment with NBPT, DMPP and NBPT + DMPP reduced the abundance of Bacteria and Archaea compared with the single application of urea.

### Effect of NBPT and DMPP on nitrifiers

NBPT did not affect the copy number of the *amoA* AOB and *amoA* AOA genes at 50 and 80% WFPS as compared to the control soil (Fig. 2). The formation of a tridentate ligand between NBPT and urease reducing the activity of the enzyme has been reported, which would result in the reduction of the $NH_3$ available for nitrification (Manunza et al. 1999; Chen et al. 2008b). The $NH_4^+$ content in the soil amended with urea + NBPT slightly diminished during the incubation time (Table S3), thus showing the effectivity of NBPT on urease inhibition.

Although DMPP increased the abundance of the nitrifiers at 50 and 80% WFPS (Fig. 2), the inhibitor has been shown to act as a chelating compound, reducing the availability of Cu, a cofactor required for activity of the ammonia monooxygenase

enzyme (Ruser and Schulz 2015). It is possible that the prolonged retention time of $NH_4^+$ in the soil could induce the expression of the *amoA* genes with a concomitant increase in their abundances, but not in the activity of the enzyme. A temporal effect of DMPP on nitrification can be assumed as the nitrate content in the soil treated with urea + DMPP increased during the incubation time. Alleviation by DMPP of the inhibitory effect of NBPT after combined application of the inhibitors cannot be elicited from the present results.

Although nitrification is considered to be carried out under aerobic conditions (Sahrawat 2008; Butterbach-Bahl et al. 2013 and references therein), in our study, increases in the *amoA* AOA and *amoA* AOB genes (Fig. 2), and in the nitrate content (Table S4), suggest the existence of nitrifying activity under oxygen-limiting conditions (80% WFPS). Furthermore, the RDA analysis showed a positive correlation between the $NH_4^+$ content and the *amoA* genes from nitrifiers, mainly at 50% (Fig. 4A), and to a lesser extent, also at 80% (Fig. 4B).

### Effect of NBPT and DMPP on denitrifiers

Regardless of the moisture content, the abundance of the *norB* and *nosZI* genes increased in the urea-treated soil (Fig. 3). While effect of NBPT on denitrifiers was not detected at 50% WFPS, the inhibitor reduced 1.4 times the abundance of the genes after 60 days at 80% WFPS (Fig. 3). This effect could be explained considering that NBPT diminished the $NH_4^+$ availability, thus leading to a shortage of nitrate available for denitrification. Previously, Shi et al. (2017) showed that NBPT reduces the abundance of the *narG* and *nirK* genes under 60% WFPS.

The treatment with urea + DMPP increased 2.5-fold at 50% WFPS and 5.5-fold at 80% WFPS the copy numbers of the *norB* and *nosZ* genes (Fig. 3). Based on *nosZI* gene analyses, Duan et al. (2016) and Barrena et al. (2017) reported significant increases in the abundance of denitrifying bacteria as a consequence of DMPP application to soils maintained at 80% WFPS. Torralbo et al. (2017) demonstrated that DMPP induced both *nosZI* gene expression and nitrous oxide reductase activity under nitrifying conditions. The mechanism by which DMPP increases the abundance of denitrifiers is unknown, albeit induction of gene expression or enzyme activity would result in higher abundance of the denitrification genes.

The observation that the abundance of the *norB* and *nosZ* genes increased at 50% WFPS suggests they can be induced under oxygenic environments. This is supported by data in Fig. 1 which showed an increase in the 16SB biomass at 50% WFPS. The existence of aerobic denitrification in soils has been reported (Ji et al. 2015; Marchant et al. 2017). Increases in the gene copy numbers of *norB* and n*osZ* from day 1 to 60 of incubation were not observed in soils treated with urea + NBPT, which suggest an effective temporal effect of NBPT on denitrifiers.

## Conclusions

Application to soil of urease (NBPT) and nitrification (DMPP) inhibitors are among the management strategies to reduce N losses in soils, thus improving the N use efficiency during agricultural practices. In a mesocosm study using an Eutric Cambisol soil, our results show that NBPT, as expected, reduces $NH_3$ volatilisation and that the addition of DMPP or DMPP + NBPT increases $NH_3$ volatilisation. The moisture conditions affected fluxes of $NH_3$ as they were higher at 50 than at 80% WFPS.

NBPT does not affect the bacterial and archaeal biomass, nor that of the nitrifiers at 50 and 80% WFPS, but reduced the abundance of denitrifiers at 80% WFPS. DMPP, alone and in combination with NBPT, increases the abundance of Bacteria, Archaea and nitrifiers in the soil, as determined using qPCR. DMPP, and to a lower extent DMPP + NBPT, increases the gene copy number of the *norB*- and *nosZ*-bearing denitrifying communities, which indicates that DMPP, somehow, induces the expression of the, at least, the *norB* and *nosZ* denitrification genes. This effect is independent of the moisture content of the soil.

Nitrification can be carried out under oxygen-limiting conditions (80% WFPS) as suggested by the increases in the abundance of the bacterial and archaeal *amoA* genes under those conditions. Conversely, based on the increases in the biomass of denitrifiers at 50% WFPS, it is possible that denitrification, at least nitric oxide and nitrous oxide reduction, takes place.

## Acknowledgments

This study was supported by the ERDF-cofinanced grants PEAGR2012-1968 from Consejería de Economía, Innovación y Ciencia (Junta de Andalucía, Spain), AGL2015-64582-C3-3-R from MINECO, the MINECO-CSIC Agreement RECUPERA 2020 and the Autonomous Community of Madrid (P2013/ABI-2717). Authors also thank Sirena Network (AGL2015-68881-REDT) for financial support. ACH is recipient of a grant of MECD (FPU 2014/01633). This paper is dedicated to M. V. Martínez Toledo, for her dedication to Science and endless support to her students.

*Table 1. Ammonia maximal fluxes, cumulative $NH_3$ emissions, $NH_3$-N loss and $NH_3$-basal emission in soils treated with urea, urea + NBPT, urea + DMPP and urea + NBPT + DMPP at 50 and 80% WFPS. Urea was added at 260 kg N $ha^{-1}$. Soil without fertilisation was used as a control. Incubation time was 60 days. Values followed by the same lowercase letter in a column are not statistically different among treatments. For each treatment and $NH_3$ calculation at 50 and 80% WFPS, values followed by the same uppercase letter in a row are not statistically different according to the Kruskal-Wallis and Conover-Iman tests ($p < 0.05$; $n = 3$). nd, not detected.*

| | $NH_3$ calculation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $NH_3$ maximal emission (kg N $ha^{-1}$ $h^{-1}$) | | $NH_3$ cumulative emission (kg N $ha^{-1}$) | | $NH_3$-N loss (%) | | $NH_3$-basal emission (kg N $ha^{-1}$ $h^{-1}$) | |
| | WFPS (%) | | WFPS (%) | | WFPS (%) | | WFPS (%) | |
| Treatment | 50 | 80 | 50 | 80 | 50 | 80 | 50 | 80 |
| Control | nd | | | | | | | |
| Urea | 1.02 ± 0.09bB | 0.58 ± 0.06bA | 59.9 ± 2.9bB | 24.8 ± 1.8bA | 23.0 ± 1.1bB | 9.2 ± 1.1bA | 0.08 ± 0.05aA | 0.07 ± 0.04aA |
| Urea + NBPT | 0.15 ± 0.04aA | 0.08 ± 0.03aA | 5.2 ± 1.0aA | 6.7 ± 1.1aA | 2.0 ± 0.4aA | 2.4 ± 0.6bA | 0.05 ± 0.03aA | 0.04 ± 0.03aA |
| Urea + DMPP | 1.42 ± 0.11dB | 0.92 ± 0.08dA | 95.6 ± 3.6dB | 74.1 ± 2.6dA | 36.8 ± 1.6dB | 26.0 ± 1.4dA | 0.10 ± 0.04aA | 0.08 ± 0.05aA |
| Urea + NBPT + DMPP | 1.22 ± 0.08cB | 0.72 ± 0.07cA | 75.3 ± 2.8cB | 43.7 ± 3.5cA | 29.0 ± 1.2cB | 14.4 ± 1.1cA | 0.09 ± 0.04aA | 0.07 ± 0.04aA |

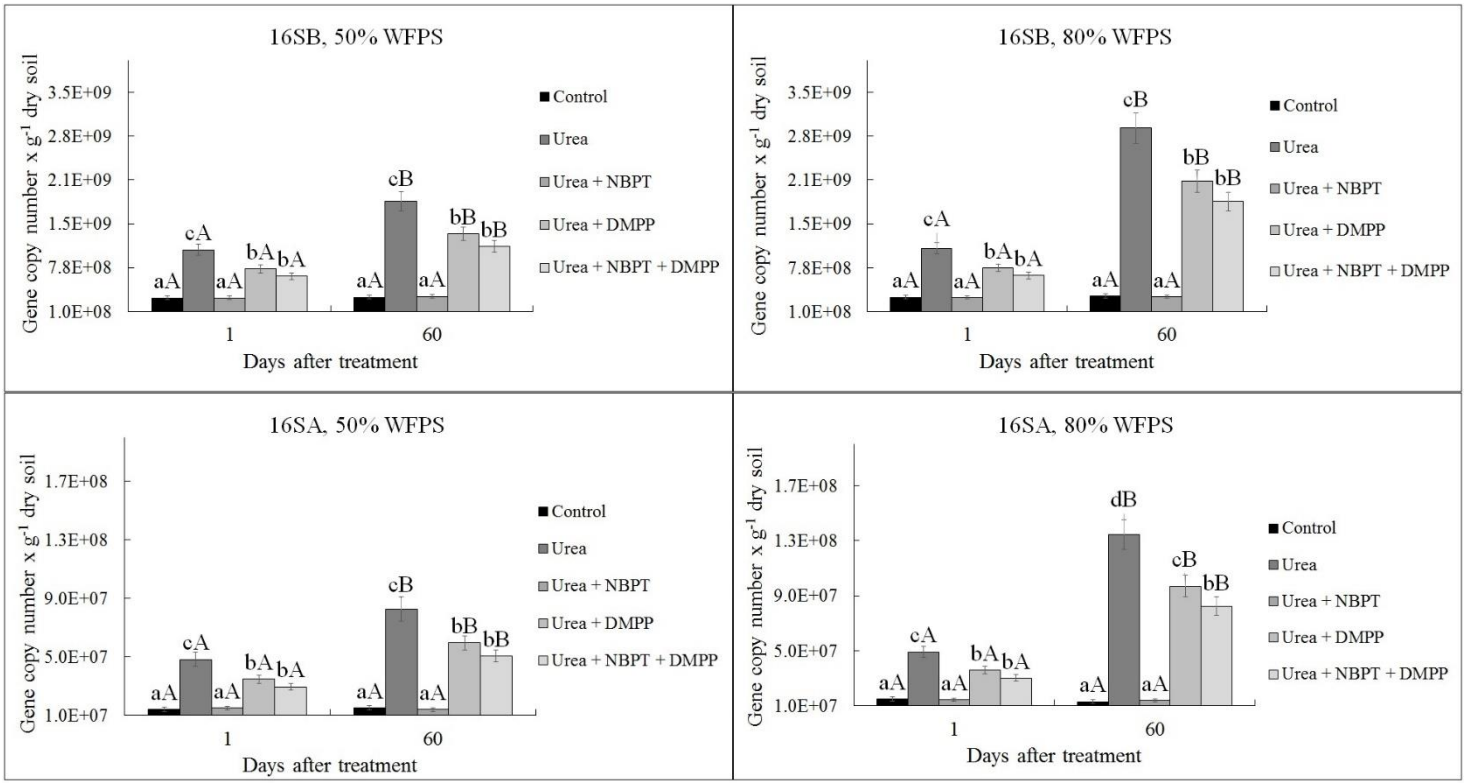



*Fig. 1. Total abundance of the 16SB and 16SA genes in soils treated with urea, urea + NBPT, urea + DMPP and urea + NBPT + DMPP at 50 and 80% WFPS during 60 days. Soil without fertilisation was used as a control. Samples were taken at 1 and 60 days after treatment. For each day, different lowercase letters indicate statistical differences among treatments; for each treatment, different uppercase letters indicate statistical differences among sampling days, according to the Kruskal-Wallis and Conover-Iman tests were performed ($p < 0.05$; $n = 3$). Bars represent standard errors.*

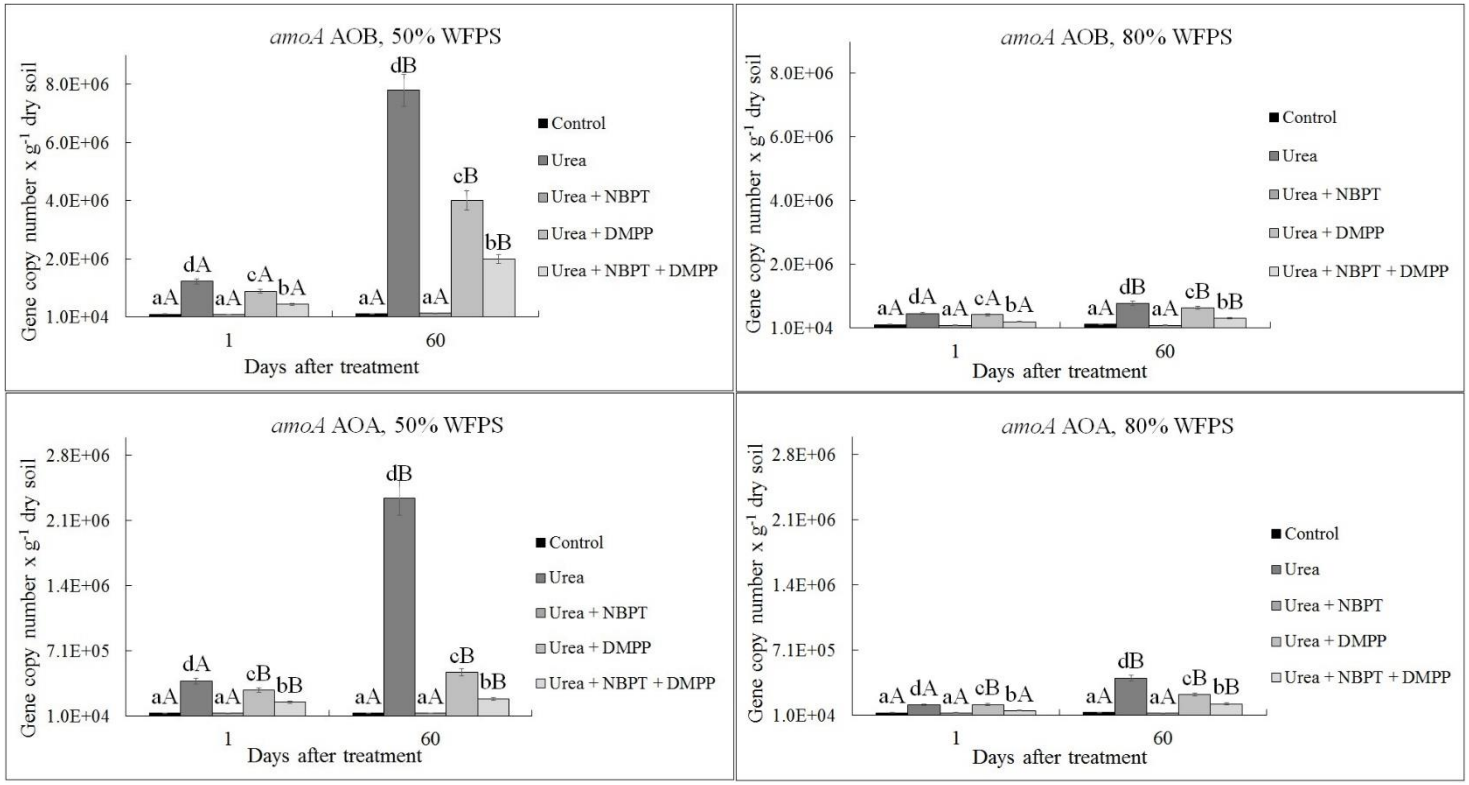



*Fig. 2. Total abundance of the amoA AOB and amoA AOA genes in soils treated with urea, urea + NBPT, urea + DMPP and urea + NBPT + DMPP at 50 and 80% WFPS during 60 days. Soil without fertilisation was used as a control. Samples were taken at 1 and 60 days after treatment. For each day, different lowercase letters indicate statistical differences among treatments; for each treatment, different uppercase letters indicate statistical differences among sampling days, according to the Kruskal-Wallis and Conover-Iman tests were performed ($p < 0.05$; $n = 3$). Bars represent standard errors.*

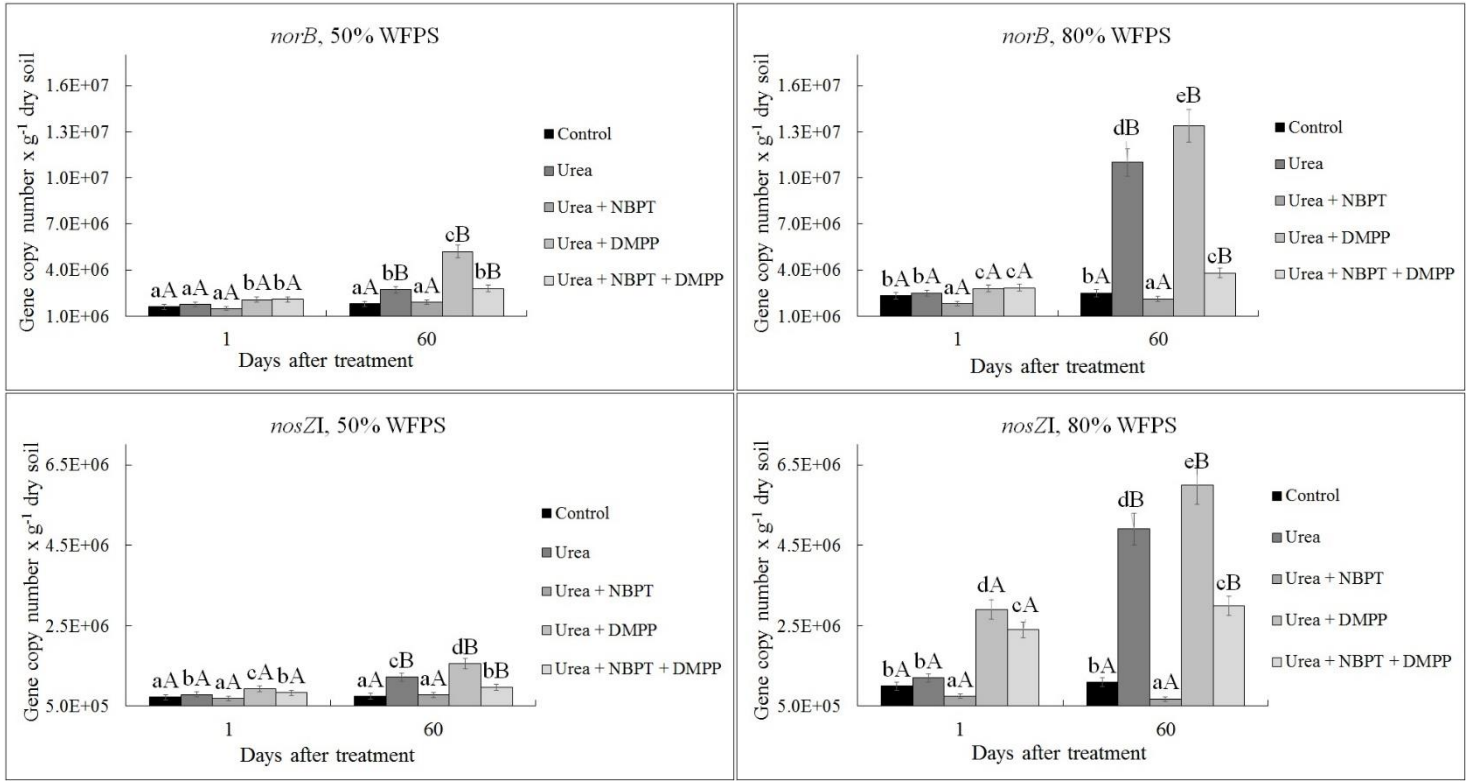



*Fig. 3. Total abundance of the norB and nosZI genes in soils treated with urea, urea + NBPT, urea + DMPP and urea + NBPT + DMPP at 50 and 80% WFPS during 60 days. Soil without fertilisation was used as a control. Samples were taken at 1 and 60 days after treatment. For each day, different lowercase letters indicate statistical differences among treatments; for each treatment, different uppercase letters indicate statistical differences among sampling days, according to the Kruskal-Wallis and Conover-Iman tests were performed ($p < 0.05$; $n = 3$). Bars represent standard errors.*

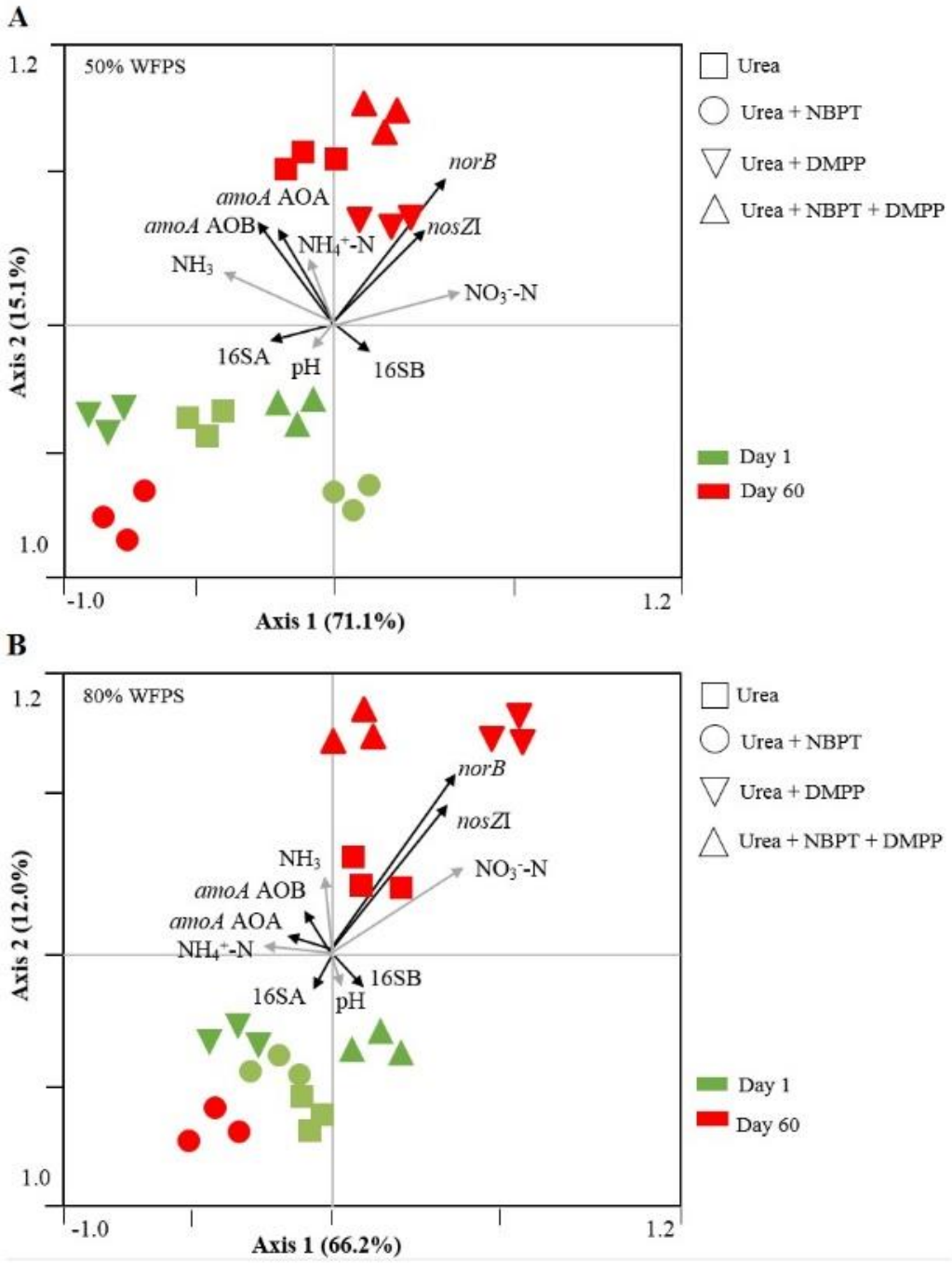



*Fig. 4. Redundancy analysis (RDA, triplot) including the biotic variables (total abundance of the 16SB, 16SA, amoA AOB, amoA AOA, norB and nosZI genes), the abiotic variables ($NH_4^+$-N $NO_3^-$-N, $NH_3$ flux and pH) and the treatments with urea, urea + NBPT, urea + DMPP and urea + NBPT + DMPP. Biotic and abiotic variables and treatments are represented by black and grey arrows and by the □, O, ∇, Δ symbols, respectively. Green and red colours represent 1 and 60 sampling days, respectively (n = 3).* ***A.*** *50% WFPS;* ***B.*** *80% WFPS.*

## Supplementary data for Chapter VII

# Effect of urease and nitrification inhibitors on ammonia volatilisation and abundance of N-cycling genes

**Submitted for publication**

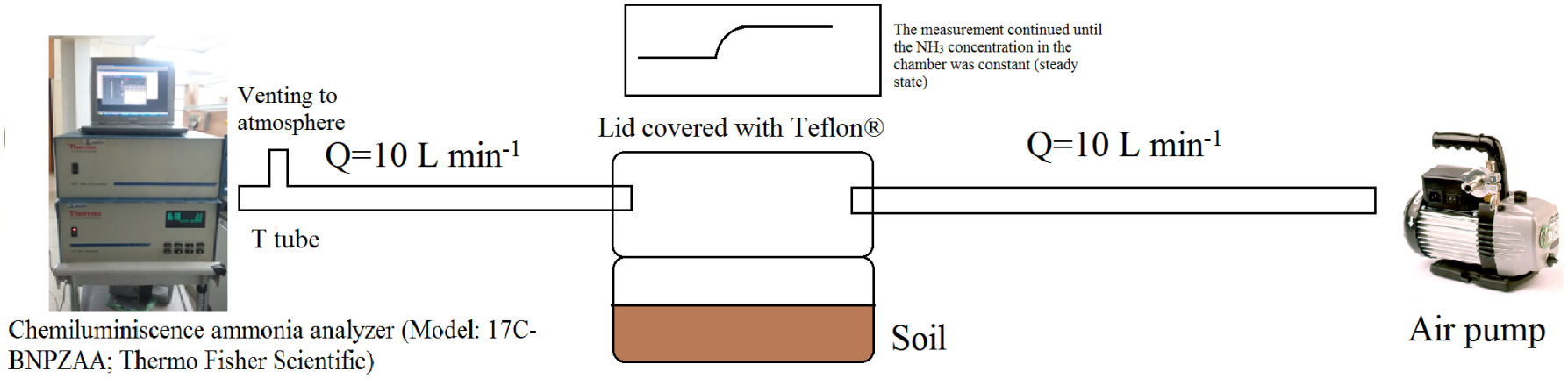



*Fig. S1. Schematic depict of the $NH_3$ volatilisation determination system.*

*Table S1. pH values in soils treated urea, urea + NBPT, urea + DMPP and urea + NBPT + DMPP at 50 and 80% WFPS during 60 days. Soil without fertilisation was used as a control. Samples were taken 1, 5, 15, 30 and 60 days after treatment. For each time, values followed by the same lowercase letter in a row are not statistically different among treatments. For each treatment and WFPS, values followed by the same uppercase letter in a column are not statistically different among samplings. Kruskal-Wallis and Conover-Iman tests were performed ($p < 0.05$; $n = 3$).*

| | | pH | | | | |
|---|---|---|---|---|---|---|
| | | Treatments | | | | |
| Time (d) | WFPS (%) | Control | Urea | Urea + NBPT | Urea + DMPP | Urea + NBPT + DMPP |
| 1 | 50 | 7.0 ± 0.1aA | 7.7 ± 0.2bB | 7.3 ± 0.2abA | 7.8 ± 0.2bA | 7.6 ± 0.2bA |
| | 80 | 7.1 ± 0.1aA | 8.1 ± 0.2bB | 7.4 ± 0.2aA | 8.2 ± 0.3bA | 7.9 ± 0.2bA |
| 5 | 50 | 7.1 ± 0.2aA | 7.7 ± 0.2bB | 7.2 ± 0.2aA | 7.9 ± 0.2bA | 7.4± 0.2abA |
| | 80 | 7.2 ± 0.1aA | 7.9 ± 0.2bB | 7.2 ± 0.2aA | 8.2 ± 0.2bA | 7.6 ± 0.2abA |
| 15 | 50 | 7.1 ± 0.1aA | 7.3 ± 0.2aA | 7.2 ± 0.2aA | 7.8 ± 0.2bA | 7.5 ± 0.2abA |
| | 80 | 7.0 ± 0.1aA | 7.7 ± 0.2bB | 7.2 ± 0.2aA | 8.0 ± 0.3bA | 7.6 ± 0.2abA |
| 30 | 50 | 7.1 ± 0.1aA | 7.2 ± 0.2aA | 7.1 ± 0.2aA | 7.8 ± 0.2bA | 7.4 ± 0.2abA |
| | 80 | 7.1 ± 0.1aA | 7.3 ± 0.2aA | 7.2 ± 0.2aA | 7.9 ± 0.2bA | 7.5 ± 0.2abA |
| 60 | 50 | 7.2 ± 0.1aA | 7.2 ± 0.2aA | 7.1 ± 0.2aA | 7.7 ± 0.2bA | 7.4 ± 0.2abA |
| | 80 | 7.1 ± 0.1aA | 7.2 ± 0.2aA | 7.2 ± 0.2aA | 7.9 ± 0.2bA | 7.5 ± 0.2abA |

*Table S2. Urease activity in soils treated urea, urea + NBPT, urea + DMPP and urea + NBPT + DMPP at 50 and 80% WFPS during 60 days. Soil without fertilisation was used as a control. Samples were taken 1, 5, 15, 30 and 60 days after treatment. For each time, values followed by the same lowercase letter in a row are not statistically different among treatments. For each treatment and WFPS, values followed by the same uppercase letter in a column are not statistically different among samplings. Kruskal-Wallis and Conover-Iman tests were performed ($p < 0.05$; $n = 3$). nd not detected.*

| | | Urease activity (μmol $NH_3$ $g^{-1}$ $h^{-1}$) | | | | |
|---|---|---|---|---|---|---|
| | | Treatment | | | | |
| Time (d) | WFPS (%) | Control | Urea | Urea + NBPT | Urea + DMPP | Urea + NBPT + DMPP |
| 1 | 50 | nd | 15.7 ± 3.0bC | 4.8 ± 0.6aA | 15.9 ± 2.0bC | 4.5 ± 0.6aA |
| | 80 | | 19.8 ± 2.1bC | 6.8 ± 1.2aA | 20.1 ± 2.2bC | 6.6 ± 1.5aA |
| 5 | 50 | | 9.7 ± 1.2bB | 5.3 ± 1.1aA | 9.5 ± 1.6bB | 5.6 ± 1.1aA |
| | 80 | | 11.6 ± 1.2bB | 8.2 ± 1.5aAB | 12.1 ± 1.8bB | 10.4 ± 0.4bB |
| 15 | 50 | | 8.5 ± 1.3aB | 6.2 ± 1.6aA | 7.5 ± 1.1aB | 5.9 ± 1.3aA |
| | 80 | | 9.6 ± 1.1aB | 8.5 ± 1.5aAB | 9.7 ± 1.0aB | 7.6 ± 1.4aAB |
| 30 | 50 | | 5.8 ± 1.1aA | 10.5 ± 1.2bB | 5.9 ± 1.6aA | 11.1 ± 1.4bB |
| | 80 | | 6.6 ± 0.9aA | 9.8 ± 0.9bB | 6.8 ± 0.8aA | 8.1 ± 0.5aAB |
| 60 | 50 | | 4.9 ± 0.7aA | 9.5 ± 1.1bB | 4.5 ± 0.9aA | 9.1 ± 1.0bB |
| | 80 | | 5.7 ± 0.6aA | 8.9 ± 1.2bAB | 6.2 ± 0.7aA | 8.9 ± 1.1bAB |

*Table S3. Exchangeable $NH_4^+$-N concentration in soils treated urea, urea + NBPT, urea + DMPP and urea + NBPT + DMPP at 50 and 80% WFPS during 60 days. Soil without fertilisation was used as a control. Samples were taken 1, 5, 15, 30 and 60 days after treatment. For each time, values followed by the same lowercase letter in a row are not statistically different among treatments. For each treatment and WFPS, values followed by the same uppercase letter in a column are not statistically different among samplings. Kruskal-Wallis and Conover-Iman tests were performed ($p < 0.05$; $n = 3$). nd not detected.*

| | | Exchangeable $NH_4^+$-N (mg $NH_4^+$-N $kg^{-1}$) | | | | |
|---|---|---|---|---|---|---|
| | | Treatment | | | | |
| Time (d) | WFPS (%) | Control | Urea | Urea + NBPT | Urea + DMPP | Urea + NBPT + DMPP |
| 1 | 50 | nd | 106.7 ± 7.2bE | 167.4 ± 7.9dC | 84.7 ± 5.4aF | 123.4 ± 5.8cE |
| | 80 | | 136.1 ± 8.9aD | 170.1 ± 5.1bD | 129.4 ± 6.2aE | 136.8 ± 5.1aD |
| 5 | 50 | | 82.1 ± 5.5bD | 163.5 ± 5.5dC | 66.7 ± 5.9aD | 101.7 ± 5.2cD |
| | 80 | | 104.2 ± 4.5aC | 168.2 ± 3.9cD | 96.2 ± 4.8aD | 121.5 ± 4.4bD |
| 15 | 50 | | 69.2 ± 6.8bC | 153.4 ± 6.6dC | 50.5 ± 5.1aC | 88.3 ± 6.5cC |
| | 80 | | 97.2 ± 3.9bC | 157.9 ± 3.6cC | 70.5 ± 4.9aC | 107.2 ± 4.7bC |
| 30 | 50 | | 52.3 ± 4.6bB | 136.9 ± 4.5cB | 32.9 ± 5.6aB | 56.9 ± 4.3bB |
| | 80 | | 71.3 ± 4.9bB | 143.3 ± 4.5cB | 52.7 ± 5.8aB | 75.4 ± 4.1bB |
| 60 | 50 | | 36.7 ± 5.1bA | 120.1 ± 2.7cA | 10.1 ± 3.0aA | 38.1 ± 3.2bA |
| | 80 | | 52.7 ± 5.6bA | 130.1 ± 3.8cA | 23.4 ± 3.9aA | 48.2 ± 3.5bA |

***Table S4.*** *$NO_3^-$-N concentration in soils treated urea, urea + NBPT, urea + DMPP and urea + NBPT + DMPP at 50 and 80% WFPS during 60 days. Soil without fertilisation was used as a control. Samples were taken 1, 5, 15, 30 and 60 days after treatment. For each time, values followed by the same lowercase letter in a row are not statistically different among treatments. For each treatment and WFPS, values followed by the same uppercase letter in a column are not statistically different among samplings. Kruskal-Wallis and Conover-Iman tests were performed ($p < 0.05$; $n = 3$).*

| | | $NO_3^-$-N (mg $NO_3^-$-N $kg^{-1}$) | | | | |
|---|---|---|---|---|---|---|
| | | Treatment | | | | |
| Time (d) | WFPS (%) | Control | Urea | Urea + NBPT | Urea + DMPP | Urea + NBPT + DMPP |
| 1 | 50 | 6.9 ± 0.2bA | 20.2 ± 1.5cA | 2.5 ± 0.5aA | 8.0 ± 1.0bA | 7.7 ± 1.2bA |
| | 80 | 7.1 ± 0.3bA | 11.2 ± 1.6cA | 1.5 ± 0.6aA | 7.2 ± 1.2bA | 7.1 ± 1.3bA |
| 5 | 50 | 6.8 ± 0.3bA | 35.2 ± 1.9dB | 4.5 ± 0.5aB | 15.5 ± 1.6cB | 14.3 ± 1.8cB |
| | 80 | 7.1 ± 0.2bA | 22.9 ± 2.6dB | 3.1 ± 0.7aB | 11.9 ± 1.2cB | 9.1 ± 1.0cA |
| 15 | 50 | 7.0 ± 0.3bA | 48.4 ± 2.2dC | 5.9 ± 0.7aC | 21.6 ± 1.4cC | 19.6 ± 1.6cC |
| | 80 | 6.8 ± 0.3bA | 28.2 ± 2.6dC | 4.1 ± 0.8aB | 14.3 ± 1.5cB | 11.3 ± 1.5cB |
| 30 | 50 | 6.8 ± 0.3aA | 62.5 ± 2.1dD | 6.6 ± 0.9aC | 29.4 ± 1.3cD | 25.7 ± 2.1cD |
| | 80 | 6.6 ± 0.3aA | 36.2 ± 2.2dD | 5.8 ± 1.0aB | 19.2 ± 1.4cC | 16.5 ± 1.3cC |
| 60 | 50 | 6.9 ± 0.3aA | 76.1 ± 1.6dE | 9.5 ± 1.0bD | 38.2 ± 2.0cE | 35.4 ± 1.2cE |
| | 80 | 6.6 ± 0.3aA | 39.8 ± 4.9dD | 6.6 ± 1.0aC | 29.5 ± 1.2cD | 25.1 ± 1.4bD |

# DISCUSSION

## 5. DISCUSSION

Although previous reports had shown individual relationhips between N-fertilisation and soil biotic and abiotic parameters, an integrated study relating the form of the N-fertiliser with differences in $N_2O$ emission, changes in soil physicochemical properties, alterations in the abundance of the genes involved in $N_2O$ production and reduction, and effects on bacterial diversity had not been reported. Moreover, the effect of the soil depth and type of N-fertilisation on $N_2O$ emission along the arable soil profile was unknown. Also, the controversy existed about the relative contribution of the aerobic nitrification and anoxic denitrification biochemical pathways to total $N_2O$ production. Although fungal denitrification had been reported, the input of the process to total $N_2O$ production associated to the form of the fertiliser had been scarcely reported. Finally, many question were raised about the effect of the N-fertilisers on $N_2O$ release by the bulk and rhizosphere soil of cultivated plants.

To address these questions, a soil of agricultural interest was chosen, fertilised with either urea, ammonium sulphate or potassium nitrate and maintained under greenhouse conditions. The effect that N-fertilisation exert on soil properties, $N_2O$ emissions and nitrifiers and denitrifiers abundance a) along the 20 cm layer of the arable topsoil (Chapter I), b) in uncultivated soils treated during 3 years (Chapter II) and c) in bulk and rhizosphere soil of tomato and common bean during 4 consecutive harvest (Chapters III.1 and III.2) was studied. Changes in the relative abundance of bacterial OTUs in uncultivated (Chapter II) and cultivated (Chapter III.2) soils were also considered.

The source of $N_2O$ emission from the soils was studied by using the $^{15}N$-tracer technique (Chapter IV). Taking together the dataset obtained in Chapters II, III.1 and III.2, we built a model to understand the relative importance of N-fertilisation, each analysed biotic and abiotic variables and bacterial biodiversity as drivers of $N_2O$ emission (Chapter V). The relative importance of bacterial fungal denitrifiers to produce $N_2O$ emission in soils post nitrate application was also addressed (Chapter VI). Finally, we studied the effect of urease and nitrification inhibitors, which are

widely use to decrease N losses in soils, on ammonia volatilisation and abundance of N-cycling genes (Chapter VII).

## Chapter I

In this Chapter, we determined the effect of the type of N-fertilisation (urea, ammonium or nitrate) and soil depth on N-gas emissions ($N_2O$ and $N_2$) and abundance of nitrifiers and denitrifiers communities along the 20-cm layer of the arable topsoil after 1 year of incubation. Twenty-four hours after watering the post, we observed a clear reduction in the content of dissolved oxygen along the soil profile from oxygen concentrations of 3.34% (WFPS of 65%) at 2.5-cm to 0.70% (WFPS of 80%) at 17.5-cm soil depth. In the first 0- to 5-cm, the soil containing ammonium-fertilisers emitted more $N_2O$ than that containing only nitrate, which was related to a higher total and relative abundance of the *amoA* AOA and *amoA* AOB genes in that layer. Previous studies have also reported the existence of nitrification under similar oxygen conditions (Philips et al. 2002; Geets et al. 2006; Arnaldos et al. 2013). Partial amounts of $N_2O$ emissions were exclusively due to denitrification, as observed in the nitrate-treated soil, albeit they were significantly lower than those from nitrification.

In the 5- to 10-cm soil horizon, it was observed a reduction in $N_2O$ production as compared to the above layer due mainly to a decline in the total and relative abundance of the *amoA* AOA and *amoA* AOB genes. On the other hand, there were significant increases in the relative abundances of each of the denitrification genes. In the 10- to 15-cm and 15- to 20-cm soil layers, a dissolved oxygen content lower than 1.1% (WFPS $\geqq$ 75%) indicates an oxygen-limiting environment. The reduction in the total abundances of the nitrifiers was accompanied by clear increments in the abundance of the denitrifiers, and *norB* was the most abundant, followed by *nirK*/*nirS* and, finally, the *nosZI*/*nosZ*II genes. The highest rates of $N_2O$ emissions were detected in the deepest 15- to 20-cm soil horizon. Although no increases in the total abundance of the denitrification genes was observed, their relative abundance showed significant increments which may be related to the reduction of bacterial and archaeal species capable of denitrification under conditions of very low $O_2$ content. Other authors have also shown that N-fertilisation decreased microbial

abundance (Wessén et al. 2011; Fierer et al. 2012; Kearns et al. 2015), more likely due to the expansion of nitrophilous species and competitive exclusion (Yan et al. 2017).

It is to note that the *norB* gene was less sensitive to $O_2$ than *nosZ* gene since the relative abundances of *norB* was similar or even higher than those of the remaining denitrification genes in the four soil horizons analysed. On the contrary, the relative abundance of *nosZ* was negligible in the 0- to 10-cm upper soil horizons but increased along the soil profile, mainly in the 15-20 cm horizon where the dissolved oxygen content was close or below 1.0%, which, in turn, resulted in lower $N_2O/N_2$ ratios. The higher oxygen sensitivity of the *nosZ* gene relative to *norB* has been reported (Zumft 1997).

The NMDS plots carried out to analyse the biotic and abiotic variables most influencing the $N_2O$ emissions for each individual soil layer revealed that $N_2O$ production was mainly due to nitrification which was controlled by exchangeable $NH_4^+$-N and dissolved oxygen content in the 0- to 10-cm soil depth whereas emissions in the 10- to 20-cm soil horizon originated from denitrification controlled by dissolved oxygen, $NO_3^-$-N content and TN. When the NMDS analysis was done for the whole 20-cm soil profile, the $N_2O$ emissions were mainly due to denitrification. Thus, the 20-cm soil profile only gave a partial description of the process involved in $N_2O$ emissions.

## Chapter II

In this Chapter we carried out an integrated research relating the form of the N-fertiliser (urea, ammonium or nitrate) with $N_2O$ emission, the abundance of the total bacterial and archaeal communities, the changes produced in the abundance of N-cycling genes and the variations occurred in the diversity of the bacterial communities during a 3-year microcosm study.

The watering of the soil together with the addition of any of the N-fertilisers produced the highest values of $N_2O$ emission which suggests that the simultaneous presence of nitrate and $O_2$-limiting conditions are required for fully $N_2O$ emission. After three consecutive years, the highest cumulative $N_2O$ emission was emitted by

the soils treated with urea, followed by ammonium and finally nitrate. The higher $N_2O$ emission in the ammonium-treated soils may be associated to a simultaneous contribution of nitrification and denitrification because of the presence of ammonium and nitrate, and only denitrification takes place in the soil amended with nitrate. Although the WFPS of the soils was above 70%, nitrification under anoxic conditions has been detected (Arnaldos et al. 2013; Liu et al. 2017; Castellano-Hinojosa et al. 2018; Pan et al. 2018).

After addition of any of the fertilisers, the abundance of bacteria decreased during the experimental period. In contrast to bacteria, the abundance of archaea increased in all three N-treated soils. The NMDS plot showed that bacteria were strongly influenced by the TC content of the soils, while archaea were more sensitive to changes in the $NH_4^+$-N content. Inselsbacher et al. (2010) suggested that application of N to agricultural soils enhances microbial competition for nutrients, which, in turn, leads to changes in their communities. After N-fertilisation, other authors reported decreases (Hallin et al. 2009; Chan et al. 2013; Ouyang et al. 2018) in the abundance of bacteria and increases in that of bacteria (Chen et al. 2017; Yang et al. 2017b) and archaea (Wang et al. 2018a).

In all soils analysed, the *amoA* AOB was more abundant than the *amoA* AOA gene and yearly increases in their abundance were detected in the ammonium-treated soils. Previous studies reported a greater growth and activity of AOB in soils treated with high levels of inorganic ammonium (Jia and Conrad 2009; Pratscher et al. 2011), which together with the given greater sensitivity to ammonia inhibition by AOA (Prosser and Nicol 2012), would result in AOB domination in the soil. This was confirmed by the BIO-ENV analysis which indicated a stronger correlation between the abundance of the *amoA amoA* AOB gene and the $NH_4^+$ availability.

Regardless of the form of the N-fertiliser, the total abundance of the denitrification genes gradually increased during the experimental period. According to the BIO-ENV analysis, the $N_2O$ emission was mainly positively related with the abundance of the *norB* gene, and this was observed for the three N-fertilisers. Contrary to the *norB* gene, there was a negative relationship between the $N_2O$ emission and the abundance of the *nosZ* gene, so that the increases in the *nosZ*

abundance were associated with concomitant decreases in the $N_2O$ emissions. Calculation of the ratio between genes involved in $N_2O$ production (*amoA* AOB + *amoA* AOA + *nirK* + *nirS* + *norB*) and reduction (*nosZ*I + *nosZ*II) did not change in the control soil during incubation and decreased on a yearly basis in all three N-fertilised soils. This could explain the decreases in the maximum and cumulative $N_2O$ emission found during incubation. Values of the ratio were the highest and the lowest in the urea- and nitrate- treated soils, respectively, which may explained the variations in the fluxes and cumulative $N_2O$ emission.

Previous studies have shown that N-fertilisation alters or not the structure and composition of the bacterial community (Ramirez et al. 2010; Zhou et al. 2017b; Wang et al. 2018b). We found that N-fertilisation affected the structure of the bacterial community as decreases in the number of OTUs were observed. The Shannon diversity index revealed that fertilisation with urea produce the highest reduction in biodiversity after 3-year treatment as compared with the values found in the soils fertilised with ammonium or nitrate, between which differences were not found. The Simpson index showed that the bacterial community became less diverse, or dominated by a small group of OTUs after N-fertilisation mainly explained by the availability of $NH_4^+$-N and/or $NO_3^-$N content, as indicated by the stepwise multiple regression analysis.

Regarding the composition of the bacterial community, the three fertilisers mainly reduced the number of the OTUs whose relative abundance was lower than 1% and scarcely affected those with a higher relative abundance. Interestingly, OTUs which showed relative increases in their abundance after N-fertilisation (i.e. Bacillaceae 1, Nitrosomonadaceae, Hyphomicrobiaceae, Sphingomodacaeae, Pseudomonadaceae and Burkholderiaceae) contain members with the capability to produce $N_2O$. During the 3-year incubation, the bacterial community composition was similar in soils treated with urea or ammonium and clearly differed from that in the soil treated with nitrate, as suggested by the phylogeny-dependent cluster analysis and the ANOSIM analysis.

Chapter III.1

In this Chapter, we aimed at studying the effect of the application of either urea, ammonium sulphate or potassium nitrate on soil physicochemical properties, $N_2O$ emissions and abundance of nitrification and denitrification genes in the bulk and rhizosphere soil of tomato and common-bean crops maintained under greenhouse conditions for 4 consecutive harvests.

Regardless of the number of the harvest, combined addition of water (up to 80% WFPS) with any of the N-fertilisers produced the maximum values of $N_2O$ as observed in the uncultivated soils (Chapter II). The $N_2O$ fluxes were higher in soils amended with ammonium-based fertilisers than in those supplemented with nitrate, which suggests that nitrification also contributed to $N_2O$ production. These results agree with those previously reported in Chapters I and II which showed that $N_2O$ emission by nitrifiers can occur even under $O_2$-limiting conditions, thus contributing to the increase of $N_2O$ fluxes (Arnaldos et al. 2013; Liu et al. 2017; Castellano-Hinojosa et al. 2018; Pan et al. 2018).

Members of bacteria were more abundant than those of archaea in the bulk and rhizosphere fractions of unfertilised and N-fertilised soils. Other authors also found similar results in unfertilised (Nie e tal. 2014) and urea-fertilised paddy soils (Zhai et al. 2018). Whereas application of any of the N-fertilisers decreased the abundance of total bacteria and archaea in uncultivated soils (Chapter II), the effect was opposite when the soil was cultivated with tomato and common bean plants. This pattern might be related to the higher values of TC and TOC in the cultivated soils as the stepwise multiple regression analysis showed that the increases in the 16S rRNA gene abundance in cultivated soils were explained mainly by TC in the bulk and by TOC in the rhizosphere soil.

Regardless of the plant species, the *amoA* AOA gene was more abundant than the *amoA* AOB in the rhizosphere soil and, the opposite pattern was detected in the bulk and in the uncultivated soils (Chapter II). This behaviour has been previously associated to the preference of AOA, rather than AOB, to low ammonia concentration (Prosser and Nicol 2012), so that ammonia uptake by plants may favour AOA. Other authors have found similar results in unfertilised grassland plants (Thion et al.

(2016) and in the rhizosphere of sorghum (Hai et al. 2009) and rice (Hussain et al. 2011; Ke et al. 2013) treated with ammonium-based fertilisers.

The *nirK* + *nirS*, *norB* and *nosZ*I + *nosZ*II denitrification genes were more abundant in the bulk over those in the rhizosphere soil, especially in the nitrate-treated soil, which agrees with other authors (Hussain et al. 2011; Nie et al. 2014). This result may be explained if one considers that the lower $NO_3^-$-N content in the rhizosphere of tomato and common bean may result in a strong competition for N, which, in turn, would affect growth of denitrifiers. The abundance of the *nosZ*I + *nosZ*II gene pair gradually increased with the number of harvests both in the bulk and rhizosphere soil and may explained the decreases in the maximum values of the $N_2O$ emissions found at the beginning of each consecutive harvest, a pattern also observed in uncultivated soils (Chapter II).

Regardless of the N-fertiliser form, calculation of the ratio between genes involved in $N_2O$ production and reduction did not vary between plant species and do not explained the highest cumulative $N_2O$ emissions in soils cultivated with common bean than tomato. However, regardless of the N-form, comparison of the ratio between uncultivated (Chapter II) and cultivated soils, may explained the highest $N_2O$ emission in the latter. As observed in uncultivated soils (Chapter II), values of the ratio were the highest in the soil treated with urea and the lowest in the soil amended with nitrate, which may explained the variations in the fluxes and cumulative $N_2O$ emission.

## Chapter III.2

In this Chapter we examined the effect of the application of urea, ammonium sulphate or potassium nitrate on the structure and composition of the bacterial community in bulk and rhizosphere soil of two tomato and common bean. The application of any of the N-fertilisers clearly decreased the number of OTUs in the bulk, and even more in the rhizosphere soil from the first to the fourth harvest both in tomato and common bean. As observed in uncultivated soils (Chapter II), urea determined the highest losses of bacterial diversity (Shannon index) both in bulk and rhizosphere soils. Similar results have been previously reported in agricultural soils fertilised with urea or ammonium nitrate (Wang et al. 2015b; Yu et al. 2016;

Zeng et al. 2016 Zhou et al. 2017a) but here we extend those findings to the rhizosphere. Regardless of the plant species, application of any of the N-fertilisers decreased the values of Simpson index respect to the control bulk soil which suggest that the structure of the bacterial community was dominated by a smaller group of OTUs, a pattern more severe in the rhizosphere.

A stepwise multiple analysis showed that both in bulk and rhizosphere soils the availability of exchangeable $NH_4^+$-N and $NO_3^-$-N mainly determined the changes in the number of OTUs with higher values of regression for the rhizosphere. This may suggest a more sensitive effect of N availability on rhizosphere microbes even if lower $NH_4^+$-N and $NO_3^-$-N contents were detected (Chapter III. 1). The β-diversity analysis showed that both dominant and rare OTUs largely changed in the rhizosphere of N-treated soils respect to the control while dominant OTUs remain more stable in the bulk soil. Similar results were found when compared bulk and rhizosphere soil of maize plants grown under N-fertilisation for 12 weeks (Peiffer et al. 2013).

The three fertilisers produced similar decreases in the OTUs with relative abundance higher than 1% and, on the other hand, that, if any, the increases produced in the relative abundance of the OTUs were dependent on the type of the N-fertiliser. It is interesting to note that N-fertilisation favoured OTUs that contain nitrifying and denitrifying members (Daims et al. 2016; Zhang et al. 2016; Zhou et al. 2017b; Wang et al. 2018a, b) such as Bacillaceae, Bradyrhizobiaceae, Burkholderiaceae, Chromatiaceae, Comamonadaceae, Hyphomicrobiaceae, Nitrosomonadaceae, Pseudomonadaceae, Rhizobiaceae and Thiobacillacaeae.

After 4 consecutive harvest, the bacterial community composition was similar in bulk and rhizosphere soils treated with urea or ammonium and clearly differed from that in the soil treated with nitrate. Similar results were observed in the uncultivated treated soils (Chapter II). Because results were similar among tomato and common bean plants it may be concluded that the changes in the soil bacterial diversity are not controlled by the plants.

Chapter IV

The results obtained in Chapters I, II and III.1 suggested that, even under the high moisture conditions used in this study, both nitrification and denitrification may contribute to the production of $N_2O$ in the ammonium-treated soils. To solve this question, in this Chapter we used the $^{15}N$-tracer technique to determine the origin of the $N_2O$ emission in the soils fertilised with ammonium sulphate or potassium nitrate for 3 years. We showed the existence of simultaneous nitrification and denitrification in the ammonium-treated soil where the $N_2O$ originated by nitrification (49.0-58.0%) and denitrification (42.0-51.0%). Under high moisture contents, it is possible that nitrification may occur in oxic microsites within the soil aggregates (Barnard et al. 2005; Butterbach-Bahl et al. 2013; Zhu et al. 2013). Using the $^{15}N$-tracer technique, nitrification has been involved in $N_2O$ production under elevated soil moisture conditions (Mathieu et. al 2006; Morse and Bernhardt 2013; Huang et al. 2014; Han et al. 2018). In the soil treated with ammonium, the production of $N_2O$ was accompanied by a decrease in the $NH_4^+$ content and a parallel increase in the concentration of $NO_3^-$, a pattern that was also observed for the evolution of $^{15}NH_4^+$ and of $^{15}NO_3^-$ during incubation, respectively. In addition, there were significant increments in the abundance of the *amoA* AOB and *amoA* AOA genes. All this shows the occurrence of nitrification activity under those conditions which agrees the results obtained in Chapters I, II and III.1.

When the soil was fertilised with potassium nitrate during 3 years, the $^{15}N$-tracer technique showed that denitrification was the main source of nitrous oxide, contributing with 84.0-99.0% to the total $N_2O$ produced. The consumption of nitrate over the incubation period and the increase in the total abundance of each of the denitrification genes lend support to the existence of active denitrification activity in this soil. Also, the $^{15}NO_3^-$ produced the highest $^{15}N$ enrichment of the $N_2O$ released by the soil. The values of $^{15}N_2$ enrichment in the nitrate-treated soil was significantly higher than that in the ammonium soil, which is consistent with the higher relative and total abundance of the *nosZI* gene in the nitrate soil as also observed in Chapters I, II and III.1.

## Chapter V

In this Chapter, a combined RF and SEM analysis was used to study the relative importance of soil properties, N-cycling gene abundance and bacterial diversity as drivers of $N_2O$ emission in the uncultivated and cultivated soils treated with the N-fertilisers urea, ammonium or nitrate.

Among the soil properties, the content of $NH_4^+$ and $NO_3^-$ were the main determinants for $N_2O$ emission in the uncultivated and cultivated soils. Also, the content of TOC could be considered a key predictor for $N_2O$ emission, but only for the cultivated soil. Because in our soils, the $N_2O$ emission was mainly originated during the microbial processes of nitrification and denitrification (Chapter I-IV), it is expected that $NH_4^+$ and $NO_3$ are good predictor for $N_2O$ emission. A higher TOC content in the cultivated soils (Chapter III.1) was associated with root exudation (Richardson et al. 2009) which together with a higher ratio of genes involved in $N_2O$ production and reduction in the cultivated than uncultivated soils (Chapter II), may explained its importance as drivers for $N_2O$ emission.

Among the genes included in this study, the RF showed that the *amoA* AOB and *amoA* AOA had similar importance regarding their goodness as $N_2O$ predictors. It is to note that despite the higher total abundance of the *amoA* AOB gene in the studied soils, the relative abundance of the *amoA* AOA gene overpassed that of the *amoA* AOB gene (Chapters II and III.1). This would explain the similar importance that each gene showed in the RF analysis. According to this analysis, the *napA* gene had more significance than the *narG* gene. However, the total and relative abundance of the *narG* gene in the studied soils was higher than those of *napA* (Chapters II and III.1). The dominance of *narG* over *napA* has been reported in other studies (Philippot 2005; Bru et al. 2007), but *napA* appears to function as a better predictor for estimation of $N_2O$ emissions. The *nirK* gene was a better predictor than *nirS* according to the RF analysis. This finding could be explained considering the highest total and relative abundance of *nirK* detected in the soils used in this study (Chapters II and III.1) together with the observation that *nirK* is commonly dominant in soils (Levy-Booth et al. 2010). The *norB* gene was also a good predictor for $N_2O$ emission. This gene has been considered as a good molecular marker during

denitrification studies (Chen et al. 2015; Kearns et al. 2015; Castellano-Hinojosa et al. 2018). *nosZ*I was a more significant predictor for $N_2O$ emission than *nosZ*II as indicated by the RF analysis. It is known that *nosZ*I is more abundant than *nosZ*II in most ecosystems and that 51% of the *nos*ZII-bearer denitrifiers appear to be non-denitrifying $N_2O$-reducers (Hallin et al. 2018; Chapters II and III.1).

The output of the SEM model showed that the $NH_4^+$ and $NO_3$ content directly influenced the N-cycling gene abundance and that $N_2O$ emission was under the indirect control of the targeted genes. $N_2O$ emission was positively controlled by *norB* and negatively regulated by *nosZ*. The output also revealed that *amoA* AOA + *amoA* AOB had an indirect but positive effect on the $N_2O$ emission but had a lower regression weight and size effect than those of the denitrification genes. This may be related to the 80% WFPS of the studied soils, a condition which favours denitrification over nitrification (Butterbach-Bahl et al. 2013; Hu et al. 2015). Also, data in Chapter IV indicate that $N_2O$ emission from ammonium-treated soil was almost equally originated from nitrification and denitrification while 84-99% of the $N_2O$ emission from nitrate-treated soil derived from denitrification. In this sense, it is possible that the results in Chapter IV gave a partial description of the processes involved in $N_2O$ emission.

Sixteen OTUs showed significant correlation with the $N_2O$ emission, of which *Nitrosospira*, *Nitrosomonas* and *Nitrosococcus* contain well-known nitrifying species (Daims et al. 2016) and the remaining 13 OTUs have been reported as denitrifying bacteria (Zhang et al. 2016). The RF analysis identified the relative abundance of the bacterial OTUs as a better predictor for $N_2O$ emission than the soil properties both for uncultivated and cultivated soils. This can be explained after consideration that $N_2O$ production during nitrification and denitrification relies on specific groups of microorganisms whose abundance increase after N application (Schimel et al. 2005; Philippot et al. 2013a, b; Powell et al. 2015).

### Chapter VI

In this Chapter it was studied the relative importance of fungal and bacterial denitrification as producers of $N_2O$. The bacterial inhibitor streptomycin and the fungal inhibitor cycloheximide were successfully used to distinguish the relative

contribution of fungi and bacteria to $N_2O$ emission by four different soils amended with $KNO_3$ and kept under $O_2$-limiting conditions.

The results showed that both bacteria and fungi almost equally contributed to soil $N_2O$ production during incubation for 240 h, and that this effect was similar for the four studied soils. The results also showed that $N_2O$ production by bacteria dominated over that of fungi during the first 48-96 h of incubation and that, after that time, the situation reversed and the production of $N_2O$ by fungi came to dominate that of bacteria. Because a pattern similar to that found in $N_2O$ emissions was not observed for the pH, $NO_3^-$-N content, TN and TC soil properties analysed in this study, it is possible that changes in the contribution of bacteria and fungi to $N_2O$ emissions during incubation could be due to differences in the rates of nitrate utilisation. In this sense, Myrold and Posavatz (2007) showed that bacteria, not fungi, had the greatest potential for assimilating, or immobilizing, $NO_3^-$ in many soils. On the other hand, nitrite reductase (NirK) and P450nor are essential enzymes of the fungal denitrification system, which are the minimum pair to ensure denitrification; P450nor receives electrons directly from NAD(P)H, which indicates that the enzyme is not associated with the respiratory chain, functioning preferentially as an electron sink over energy production efficiency under anoxic conditions (Shoun and Fushinobu 2017 and references therein). All this is consistent with denitrification being more rapidly induced in bacteria than in fungi after nitrate addition, and would explain the higher fungal to bacterial ratio during incubation for the first 96 h.

The qPCR determination of the 16SB, ITS and *nirK*, *nirS*, *norB* and *nosZI* denitrification genes genes also confirmed that inhibitors were selective studying the bacterial and fungal origin of the $N_2O$ emission.

## Chapter VII

The application of the urease inhibitor NBPT and the nitrification inhibitor DMPP are among the management strategies to reduce N loss, thus increasing the N use efficiency. In this Chapter the effect of the single and combined application of the inhibitors together with urea on $NH_3$ volatilisation and the abundance of N-cycling genes was studied.

$NH_3$ volatilisation was favoured under aerated conditions which agrees with the idea that high water contents facilitate $NH_4^+$ adsorption to the particles in the soil matrix thus preventing its release into the atmosphere (Sanz-Cobeña et al. 2011; Yang et al. 2016a and references therein). The urea + NBPT drastically reduced volatilisation both at 50 and 80% WFPS, while the addition of urea + DMPP clearly increased $NH_3$ emissions even higher than those of the urea treatment alone. The combined urea + NBPT + DMPP showed an intermediate volatilisation potential. These results can be explained assuming that DMPP prolonged the retention time of ammonium in the soil, thus annulling the positive effect of the reduction of the urease activity in the days following its application, producing emissions higher than those of the urea without the inhibitor. Previous studies have established that the urease inhibitor NBPT (Carmona et al. 1990; Gill et al. 1997; Sanz-Cobeña et al. 2008; 2011; Zaman et al. 2009) and the nitrification inhibitor DMPP (Kim et al. 2012; Yang et al. 2016a) reduce or increase ammonia emissions, respectively.

As compared to unfertilised control soil, NBPT did not affect the abundance of the 16SB and 16SA, *amoA* AOB and *amoA* AOA genes at 50 and 80% WFPS. While effect of NBPT on denitrifiers was not detected at 50% WFPS, the inhibitor reduced the abundance of the genes after 60 days at 80% WFPS. This effect could be explained considering that NBPT diminished the $NH_4^+$ availability, thus leading to a shortage of nitrate available for denitrification. Previously, Shi et al. (2017) showed that NBPT reduces the abundance of the *narG* and *nirK* genes under 60% WFPS.

The application of DMPP, however, in single and in combination with NBPT, increased the 16SB and 16SA gene abundances, albeit to a lower extent than those produced by the treatment with urea, which suggests a positive effect of DMPP on the bacterial and archaeal biomass. Using ammonium sulfate nitrate as a fertiliser, Barrena et al (2017) reported that the addition of DMPP to a grassland soil did not affect the 16SB gene abundance both at 40 and 80% WFPS. Although DMPP increased the abundance of the nitrifiers at 50 and 80% WFPS, the inhibitor has been shown to act as a chelating compound, reducing the availability of Cu, a cofactor required for activity of the ammonia monooxygenase enzyme (Ruser and Schulz 2015). It is possible that the prolonged retention time of $NH_4^+$ in the soil

could induce the expression of the *amoA* genes with a concomitant increase in their abundances, but not in the activity of the enzyme. The treatment with urea + DMPP increased 2.5-fold at 50% WFPS and 5.5-fold at 80% WFPS the copy numbers of the *norB* and *nosZ* genes. Based on *nosZ*I gene analyses, Duan et al. (2016) and Barrena et al. (2017) reported significant increases in the abundance of denitrifying bacteria as a consequence of DMPP application to soils maintained at 80% WFPS. Torralbo et al. (2017) demonstrated that DMPP induced both *nosZ*I gene expression and nitrous oxide reductase activity under nitrifying conditions. The mechanism by which DMPP increases the abundance of denitrifiers is unknown, albeit induction of gene expression or enzyme activity would result in higher abundance of the denitrification genes.

# CONCLUSIONS - CONCLUSIONES

## 6. CONCLUSIONS

1. Nitrous oxide ($N_2O$) and dinitrogen ($N_2$) emissions along the soil profile are controlled by the type of the N-fertiliser and the soil depth-related dissolved oxygen content. $N_2O$ production by nitrification is dominant in the 0-10 cm soil horizon, while denitrification is the main driver of N-gas production in the 10-20 cm depth. The *nosZ* gene is the most sensitive to soil depth-related dissolved oxygen content.

2. Maximum $N_2O$ production requires simultaneous application of an N-fertiliser and high water moisture content. The cumulative nitrous oxide emission is higher in cultivated than uncultivated soils, and higher in the soil cultivated with common bean. Regardless of the presence or the absence of the plants, on a yearly basis, urea produced the higher cumulative emission followed by ammonium and, finally, nitrate. The ratios between genes involved in $N_2O$ production and reduction support differences in $N_2O$ emission under all conditions examined.

3. Yearly decreases in $N_2O$ cumulative emissions can be associated to increases in the abundance of the *nosZ* genes.

4. Cultivated and uncultivated soils differentially affect the abundance of the bacterial and archaeal communities whose variations could be associated to the soil carbon content.

5. Ammonium-based fertilisers increase the abundance of the nitrifying and denitrifying communities, which, in turn, results in higher $N_2O$ emissions.

6. The plant rhizosphere favours the abundance of the *amoA* gene of the ammonia oxidising archaea while ammonia oxidising bacteria are dominant in the bulk soil. The targeted denitrification genes are more abundant in the bulk soil. These effects cannot be fully explained from the present results.

7. N-fertilisation decreases bacterial biodiversity in uncultivated and cultivated soils. This effect is more severe in the rhizosphere soil. Changes in the structure of the bacterial community are related to N availability. After N-fertilisation, dominant and rare operational taxonomic units decrease in the rhizosphere, while only the rare operational taxonomic units diminise in the bulk soil.

8. $N_2O$ emission almost equally originates from nitrification and denitrification in the ammonium-treated soil while derived from denitrification in the soil treated with nitrate.

9. Modellisation of the data determined in this study shows that $N_2O$ emission is mainly controlled by the biotic than abiotic soil variables analysed.

10. Bacteria and fungi almost equally contribute to $N_2O$ production in soils amended with nitrate that were kept under oxygen-limiting conditions.

11. The urease inhibitor N-(n-butyl) thiophosphoric triamide (NBPT) reduces ammonia volatilisation and does not affect the bacterial, archaeal and nitrifier communities. NBPT also reduces the abundance of denitrifiers at high water moisture content. The nitrification inhibitor 3,4 dimethylpyrazole phosphate (DMPP) increases ammomia volatilisation and the abundance of bacteria, archaea, nitrifiers and denitrifiers.

## 6. CONCLUSIONES

1. Las emisiones de óxido nitroso ($N_2O$) y dinitrógeno ($N_2$) a lo largo del perfil del suelo están controladas por el tipo de fertilizante nitrogenado aplicado y el contenido de oxígeno disuelto existente en cada profundidad del suelo. La producción de $N_2O$ por nitrificación es dominante en el horizonte del suelo de 0 a 10 cm, mientras que la desnitrificación es el principal impulsor de la producción de $N_2O$ en la profundidad de 10 a 20 cm. El gen *nosZ* es el más sensible al contenido de oxígeno disuelto encontrado en cada profundidad del suelo.

2. La producción máxima de $N_2O$ requiere la aplicación simultánea de un fertilizante nitrogenado y alto contenido de humedad. La emisión acumulada de $N_2O$ es mayor en los suelos cultivados que en los no cultivados, y más alta en el suelo cultivado con frijol común. Independientemente de la presencia o ausencia de las plantas, la urea produce una mayor emisión acumulada, seguida por el amonio y, finalmente, el nitrato. Las relaciones entre los genes implicados en la producción y reducción de $N_2O$ apoyan las diferencias en la emisión de $N_2O$ en todas las condiciones examinadas.

3. Las disminuciones anuales en las emisiones acumuladas de $N_2O$ se pueden asociar a aumentos en la abundancia de los genes *nosZ*.

4. Los suelos cultivados y no cultivados afectan diferencialmente la abundancia de las comunidades de bacterias y arqueas totales, cuyas variaciones podrían estar asociadas al contenido de carbono del suelo.

5. Los fertilizantes de base amoniacal aumentan la abundancia de las comunidades nitrificantes y desnitrificantes, lo que a su vez produce mayores emisiones de $N_2O$.

6. La rizosfera de las plantas favorece la abundancia del gen *amoA* de arqueas oxidadoras del amonio, mientras que las bacterias oxidadoras del amonio son dominantes en el suelo no rizosférico. Los genes de desnitrificación estudiados son más abundantes en el suelo no rizosférico. Estos efectos no pueden explicarse totalmente a partir de los resultados obtenidos.

7. La fertilización con nitrógeno disminuye la biodiversidad bacteriana en suelos no cultivados y cultivados. Este efecto es más severo en el suelo rizosférico. Los

cambios en la estructura de la comunidad bacteriana están relacionados con la disponibilidad de nitrógeno. Después de la fertilización, las unidades taxonómicas operativas bacterianas dominantes y minoritarias disminuyen en el suelo rizosférico, mientras que solo las unidades taxonómicas operacionales minoritarias disminuyen en el suelo no rizosférico.

8. La emisión de $N_2O$ se originan casi por igual a partir de la nitrificación y desnitrificación en suelos tratados con amonio, mientras que derivan principalmente de la desnitrificación en suelos tratados con nitrato.

9. La modernización de los datos determinados en este estudio muestra que la emisión de $N_2O$ está controlada principalmente por las variables bióticas más que las abióticas del suelo.

10. Las bacterias y los hongos contribuyen de manera casi igual a la producción de $N_2O$ en suelos tratados con nitrato y mantenidos bajo condiciones limitantes de oxígeno.

11. El inhibidor de la ureasa N- (n-butil) tiofosfórico triamida (NBPT) reduce la volatilización del amoníaco y no afecta a las comunidades de bacterias y arqueas totales asi como de nitrificantes. El NBPT también reduce la abundancia de genes de la desnitrificación bajo condiciones de alta humedad. El inhibidor de la nitrificación 3,4 dimetilpirazol fosfato (DMPP) aumenta la volatilización de la amoniaco y la abundancia de bacterias y arqueas totales y genes de la nitrificación y de la desnitrificación.

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# ANNEX I

## ANNEX I

Soil physicochemical properties, cumulative $N_2O$-N emissions, total abundance of the 16SA, 16SB, *amoA* AOB, *amoA* AOA, *napA, narG, nirK, nirS, norB, nosZ*I and *nosZ*II genes and relative abundance of bacterial OTUs at Genus taxonomic level (> 0.5% of relative abundance in at least one of the samples) in soil samples taken from uncultivated and cultivated soils not treated (NT) or supplemented with urea (UR), ammonium sulphate (AS) or potassium nitrate (PN). Uncultivated soils were kept under greenhouse conditions for 3 years (1, 2, 3) and cultivated soils for 4 consecutive harvests (1, 2, 3 and 4). TOC, total organic C; TC, total C; TN, total N; n.d: not detected; R, replicate.

| | | Uncultivated soil | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Soil property | Replicate | NT | NT1 | NT2 | NT3 | UR1 | UR2 | UR3 | AS1 | AS2 | AS3 | PN1 | PN2 | PN3 |
| pH | R1 | 6.87 | 7.07 | 7.05 | 7.02 | 7.88 | 7.95 | 8.08 | 7.31 | 7.42 | 7.53 | 7.01 | 7.13 | 7.11 |
| | R2 | 6.80 | 7.00 | 7.02 | 7.03 | 7.80 | 7.90 | 8.00 | 7.40 | 7.50 | 7.60 | 7.10 | 7.20 | 7.20 |
| | R3 | 6.72 | 6.92 | 6.94 | 6.95 | 7.71 | 7.81 | 7.90 | 7.45 | 7.54 | 7.66 | 7.171 | 7.26 | 7.24 |
| Exchangeable $NH_4^+$-N (mg N $kg^{-1}$) | R1 | 0.00 | 0.00 | 0.00 | 0.00 | 98.28 | 57.24 | 25.48 | 77.39 | 37.83 | 20.27 | 0.00 | 0.00 | 0.00 |
| | R2 | 0.00 | 0.00 | 0.00 | 0.00 | 91.00 | 53.00 | 26.40 | 80.20 | 39.20 | 21.00 | 0.00 | 0.00 | 0.00 |
| | R3 | 0.00 | 0.00 | 0.00 | 0.00 | 87.82 | 51.15 | 28.51 | 86.62 | 42.34 | 22.68 | 0.00 | 0.00 | 0.00 |
| $NO_3^-$-N (mg N $kg^{-1}$) | R1 | 6.66 | 6.86 | 6.57 | 6.37 | 46.93 | 80.63 | 128.70 | 69.06 | 103.43 | 143.52 | 337.57 | 287.04 | 236.50 |
| | R2 | 6.90 | 7.10 | 6.80 | 6.60 | 48.60 | 83.50 | 114.60 | 61.50 | 92.10 | 127.80 | 300.60 | 255.60 | 210.60 |
| | R3 | 7.75 | 7.97 | 7.64 | 7.41 | 54.58 | 93.77 | 110.66 | 59.38 | 88.93 | 123.40 | 290.26 | 246.81 | 203.36 |
| TC (%) | R1 | 3.15 | 3.18 | 3.22 | 3.13 | 3.61 | 3.78 | 3.89 | 3.18 | 3.08 | 3.14 | 3.02 | 3.06 | 3.11 |
| | R2 | 3.08 | 3.11 | 3.15 | 3.06 | 3.53 | 3.69 | 3.80 | 3.11 | 3.13 | 3.19 | 3.07 | 3.11 | 3.16 |
| | R3 | 3.03 | 3.06 | 3.10 | 3.01 | 3.48 | 3.63 | 3.74 | 3.06 | 3.20 | 3.26 | 3.14 | 3.18 | 3.23 |
| TN (%) | R1 | 0.06 | 0.04 | 0.03 | 0.03 | 0.47 | 0.42 | 0.41 | 0.50 | 0.45 | 0.41 | 0.52 | 0.47 | 0.43 |
| | R2 | 0.05 | 0.04 | 0.03 | 0.03 | 0.45 | 0.41 | 0.39 | 0.48 | 0.43 | 0.39 | 0.50 | 0.45 | 0.41 |
| | R3 | 0.05 | 0.04 | 0.03 | 0.03 | 0.46 | 0.42 | 0.40 | 0.49 | 0.44 | 0.40 | 0.51 | 0.46 | 0.42 |

| Cultivated soil; Bulk | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tomato | | | | | | | | | | | | | | | | | |
| Soil property | Replicate | NT1 | NT2 | NT3 | NT4 | UR1 | UR2 | UR3 | UR4 | AS1 | AS2 | AS3 | AS4 | PN1 | PN2 | PN3 | PN4 |
| pH | R1 | 7.10 | 7.00 | 7.20 | 7.10 | 7.90 | 8.10 | 8.20 | 8.30 | 7.36 | 7.23 | 7.46 | 7.32 | 7.42 | 7.50 | 7.33 | 7.33 |
| | R2 | 7.19 | 7.09 | 7.30 | 7.19 | 8.00 | 8.21 | 8.31 | 8.41 | 7.46 | 7.33 | 7.56 | 7.42 | 7.52 | 7.60 | 7.43 | 7.43 |
| | R3 | 7.00 | 6.91 | 7.10 | 7.03 | 7.85 | 8.03 | 8.09 | 8.19 | 7.40 | 7.3 | 7.56 | 7.42 | 7.5 | 7.6 | 7.4 | 7.4 |
| Exchangeable $NH_4^+$-N (mg N $kg^{-1}$) | R1 | 1.73 | 2.70 | 3.24 | 3.78 | 45.36 | 41.47 | 37.26 | 29.50 | 35.80 | 27.10 | 26.90 | 23.20 | 3.40 | 5.40 | 5.90 | 7.90 |
| | R2 | 1.65 | 2.58 | 3.10 | 3.61 | 43.35 | 39.63 | 35.61 | 28.19 | 34.21 | 25.90 | 25.71 | 22.17 | 3.25 | 5.16 | 5.64 | 7.55 |
| | R3 | 1.60 | 2.50 | 3.00 | 3.50 | 42.00 | 38.40 | 34.50 | 31.86 | 38.66 | 29.27 | 29.05 | 25.06 | 3.67 | 5.83 | 6.37 | 8.53 |
| $NO_3^-$-N (mg N $kg^{-1}$) | R1 | 3.00 | 3.40 | 2.50 | 2.70 | 14.50 | 18.40 | 20.20 | 23.80 | 13.60 | 20.70 | 25.20 | 26.00 | 45.90 | 40.30 | 34.40 | 30.60 |
| | R2 | 2.86 | 3.24 | 2.39 | 2.58 | 13.83 | 17.55 | 19.27 | 22.71 | 12.97 | 19.75 | 24.04 | 24.80 | 43.79 | 38.45 | 32.82 | 29.19 |
| | R3 | 3.15 | 3.57 | 2.63 | 2.84 | 15.23 | 19.32 | 21.21 | 24.99 | 14.28 | 21.74 | 26.46 | 27.30 | 48.20 | 42.32 | 36.12 | 32.13 |
| TOC (mg N $kg^{-1}$) | R1 | 1.40 | 1.50 | 1.60 | 1.80 | 2.10 | 2.20 | 2.50 | 3.00 | 2.20 | 2.50 | 2.60 | 2.80 | 2.30 | 2.50 | 2.80 | 2.80 |
| | R2 | 1.35 | 1.45 | 1.55 | 1.74 | 2.03 | 2.12 | 2.41 | 2.90 | 2.12 | 2.41 | 2.51 | 2.70 | 2.22 | 2.41 | 2.70 | 2.70 |
| | R3 | 1.32 | 1.41 | 1.50 | 1.69 | 1.97 | 2.07 | 2.35 | 2.82 | 2.07 | 2.35 | 2.44 | 2.63 | 2.16 | 2.35 | 2.63 | 2.63 |
| TC (%) | R1 | 1.83 | 2.03 | 2.03 | 2.12 | 2.41 | 2.61 | 3.38 | 3.67 | 2.70 | 2.99 | 3.19 | 3.28 | 2.80 | 3.09 | 3.09 | 3.38 |
| | R2 | 1.90 | 2.10 | 2.10 | 2.20 | 2.50 | 2.70 | 3.50 | 3.80 | 2.80 | 3.10 | 3.30 | 3.40 | 2.90 | 3.20 | 3.20 | 3.50 |
| | R3 | 1.91 | 2.11 | 2.11 | 2.21 | 2.51 | 2.71 | 3.52 | 3.82 | 2.82 | 3.12 | 3.32 | 3.42 | 2.92 | 3.22 | 3.22 | 3.52 |
| TN (%) | R1 | 0.05 | 0.09 | 0.10 | 0.11 | 0.58 | 0.48 | 0.39 | 0.39 | 0.58 | 0.39 | 0.37 | 0.39 | 0.48 | 0.48 | 0.39 | 0.36 |
| | R2 | 0.06 | 0.11 | 0.12 | 0.13 | 0.72 | 0.60 | 0.48 | 0.48 | 0.72 | 0.48 | 0.47 | 0.48 | 0.60 | 0.60 | 0.48 | 0.46 |
| | R3 | 0.05 | 0.09 | 0.10 | 0.10 | 0.60 | 0.50 | 0.40 | 0.40 | 0.60 | 0.40 | 0.40 | 0.40 | 0.50 | 0.50 | 0.40 | 0.40 |

| Cultivated soil; Bulk | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Common bean | | | | | | | | | | | | | | | |
| Soil property | Replicate | NT1 | NT2 | NT3 | NT4 | UR1 | UR2 | UR3 | UR4 | AS1 | AS2 | AS3 | AS4 | PN1 | PN2 | PN3 | PN4 |
| pH | R1 | 6.98 | 6.98 | 7.08 | 6.98 | 7.80 | 8.00 | 8.10 | 8.21 | 7.10 | 7.00 | 7.20 | 7.10 | 7.20 | 7.30 | 7.10 | 7.10 |
| | R2 | 6.70 | 6.70 | 6.80 | 6.70 | 7.49 | 7.69 | 7.79 | 7.89 | 7.18 | 7.08 | 7.28 | 7.18 | 7.28 | 7.38 | 7.18 | 7.18 |
| | R3 | 6.80 | 6.80 | 6.90 | 6.80 | 7.60 | 7.80 | 7.90 | 8.00 | 7.28 | 7.18 | 7.39 | 7.28 | 7.39 | 7.49 | 7.28 | 7.28 |
| Exchangeable $NH_4^+$-N (mg N $kg^{-1}$) | R1 | 1.80 | 2.97 | 3.50 | 4.13 | 49.29 | 42.51 | 37.52 | 35.51 | 41.13 | 33.81 | 30.95 | 27.56 | 3.92 | 6.25 | 6.89 | 9.22 |
| | R2 | 1.70 | 2.80 | 3.30 | 3.90 | 46.50 | 40.10 | 35.40 | 33.50 | 38.80 | 31.90 | 29.20 | 26.00 | 3.70 | 5.90 | 6.50 | 8.70 |
| | R3 | 1.62 | 2.67 | 3.15 | 3.72 | 44.38 | 38.27 | 33.79 | 31.97 | 37.03 | 30.45 | 27.87 | 24.81 | 3.53 | 5.63 | 6.20 | 8.30 |
| $NO_3^-$-N (mg N $kg^{-1}$) | R1 | 3.34 | 3.74 | 2.83 | 2.93 | 16.48 | 20.32 | 22.44 | 26.79 | 15.57 | 23.05 | 27.50 | 29.52 | 50.65 | 47.31 | 42.56 | 39.12 |
| | R2 | 3.05 | 3.42 | 2.59 | 2.68 | 15.09 | 18.60 | 20.55 | 24.53 | 14.25 | 21.10 | 25.18 | 27.03 | 46.37 | 43.32 | 38.97 | 35.82 |
| | R3 | 3.30 | 3.70 | 2.80 | 2.90 | 16.30 | 20.10 | 22.20 | 26.50 | 15.40 | 22.80 | 27.20 | 29.20 | 50.10 | 46.80 | 42.10 | 38.70 |
| TOC (mg N $kg^{-1}$) | R1 | 1.20 | 1.30 | 1.30 | 1.50 | 1.80 | 1.80 | 2.10 | 2.44 | 1.76 | 2.05 | 2.15 | 2.34 | 1.85 | 2.05 | 2.34 | 2.34 |
| | R2 | 1.21 | 1.36 | 1.32 | 1.51 | 1.86 | 1.81 | 2.11 | 2.58 | 1.85 | 2.16 | 2.28 | 2.47 | 1.95 | 2.16 | 2.45 | 2.47 |
| | R3 | 1.17 | 1.27 | 1.28 | 1.46 | 1.76 | 1.76 | 2.05 | 2.50 | 1.80 | 2.10 | 2.20 | 2.40 | 1.90 | 2.10 | 2.40 | 2.40 |
| TC (%) | R1 | 1.62 | 1.82 | 1.82 | 1.92 | 2.12 | 2.32 | 3.03 | 3.23 | 2.43 | 2.63 | 2.73 | 2.93 | 2.53 | 2.73 | 2.73 | 3.03 |
| | R2 | 1.60 | 1.80 | 1.80 | 1.90 | 2.10 | 2.30 | 3.00 | 3.20 | 2.40 | 2.60 | 2.70 | 2.90 | 2.50 | 2.70 | 2.70 | 3.00 |
| | R3 | 1.53 | 1.72 | 1.72 | 1.82 | 2.01 | 2.20 | 2.87 | 3.06 | 2.30 | 2.49 | 2.58 | 2.77 | 2.39 | 2.58 | 2.58 | 2.87 |
| TN (%) | R1 | 0.10 | 0.11 | 0.11 | 0.10 | 0.58 | 0.58 | 0.48 | 0.39 | 0.58 | 0.48 | 0.48 | 0.39 | 0.58 | 0.58 | 0.48 | 0.39 |
| | R2 | 0.10 | 0.11 | 0.10 | 0.10 | 0.62 | 0.62 | 0.52 | 0.41 | 0.62 | 0.52 | 0.52 | 0.41 | 0.62 | 0.62 | 0.52 | 0.41 |
| | R3 | 0.10 | 0.11 | 0.10 | 0.10 | 0.60 | 0.60 | 0.50 | 0.40 | 0.60 | 0.50 | 0.50 | 0.40 | 0.60 | 0.60 | 0.50 | 0.40 |

| Cultivated soil; Rhizosphere | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tomato | | | | | | | | | | | | | | | | | |
| Soil property | Replicate | NT1 | NT2 | NT3 | NT4 | UR1 | UR2 | UR3 | UR4 | AS1 | AS2 | AS3 | AS4 | PN1 | PN2 | PN3 | PN4 |
| pH | R1 | 6.70 | 6.60 | 6.80 | 6.70 | 7.40 | 7.60 | 7.70 | 7.60 | 7.00 | 7.20 | 7.10 | 7.20 | 7.10 | 7.10 | 7.00 | 7.00 |
| | R2 | 6.66 | 6.56 | 6.76 | 6.66 | 7.36 | 7.55 | 7.65 | 7.55 | 6.96 | 7.16 | 7.06 | 7.16 | 7.06 | 7.06 | 6.96 | 6.96 |
| | R3 | 6.88 | 6.78 | 6.98 | 6.88 | 7.60 | 7.80 | 7.91 | 7.80 | 7.19 | 7.39 | 7.29 | 7.39 | 7.29 | 7.29 | 7.19 | 7.19 |
| Exchangeable $NH_4^+$-N (mg N $kg^{-1}$) | R1 | 1.69 | 1.80 | 2.12 | 2.54 | 30.39 | 27.42 | 24.03 | 21.92 | 23.50 | 19.70 | 17.30 | 15.50 | 2.30 | 3.60 | 3.90 | 5.30 |
| | R2 | 1.57 | 1.66 | 1.96 | 2.35 | 28.11 | 25.37 | 22.23 | 20.27 | 21.74 | 18.22 | 16.00 | 14.34 | 2.13 | 3.33 | 3.61 | 4.90 |
| | R3 | 1.60 | 1.70 | 2.00 | 2.40 | 28.70 | 25.90 | 22.70 | 20.70 | 24.88 | 20.86 | 18.32 | 16.41 | 2.44 | 3.81 | 4.13 | 5.61 |
| $NO_3^-$-N (mg N $kg^{-1}$) | R1 | 2.24 | 2.58 | 1.90 | 2.02 | 21.28 | 14.45 | 16.58 | 18.14 | 10.53 | 14.78 | 18.48 | 20.94 | 33.94 | 31.25 | 27.89 | 24.75 |
| | R2 | 2.00 | 2.30 | 1.70 | 1.80 | 19.00 | 12.90 | 14.80 | 16.20 | 9.40 | 13.20 | 16.50 | 18.70 | 30.30 | 27.90 | 24.90 | 22.10 |
| | R3 | 1.91 | 2.20 | 1.63 | 1.72 | 18.19 | 12.35 | 14.17 | 15.51 | 9.00 | 12.63 | 15.79 | 17.90 | 29.00 | 26.71 | 23.83 | 21.15 |
| TOC (mg N $kg^{-1}$) | R1 | 1.70 | 1.80 | 1.90 | 2.20 | 2.50 | 2.60 | 3.00 | 3.60 | 2.47 | 2.85 | 2.95 | 3.23 | 2.66 | 2.85 | 3.23 | 3.23 |
| | R2 | 1.81 | 1.92 | 2.02 | 2.34 | 2.66 | 2.77 | 3.19 | 3.83 | 2.91 | 3.36 | 3.47 | 3.81 | 3.14 | 3.36 | 3.81 | 3.81 |
| | R3 | 1.62 | 1.71 | 1.81 | 2.09 | 2.38 | 2.47 | 2.85 | 3.42 | 2.60 | 3.00 | 3.10 | 3.40 | 2.80 | 3.00 | 3.40 | 3.40 |
| TC (%) | R1 | 2.28 | 2.47 | 2.47 | 2.66 | 2.95 | 3.23 | 4.18 | 4.56 | 3.33 | 3.71 | 3.80 | 4.09 | 3.42 | 3.80 | 3.80 | 4.18 |
| | R2 | 2.50 | 2.70 | 2.70 | 2.91 | 3.22 | 3.54 | 4.58 | 4.99 | 3.64 | 4.06 | 4.16 | 4.47 | 3.74 | 4.16 | 4.16 | 4.58 |
| | R3 | 2.40 | 2.60 | 2.60 | 2.80 | 3.10 | 3.40 | 4.40 | 4.80 | 3.50 | 3.90 | 4.00 | 4.30 | 3.60 | 4.00 | 4.00 | 4.40 |
| TN (%) | R1 | 0.10 | 0.11 | 0.10 | 0.10 | 0.38 | 0.39 | 0.29 | 0.19 | 0.38 | 0.29 | 0.29 | 0.19 | 0.38 | 0.38 | 0.30 | 0.29 |
| | R2 | 0.11 | 0.11 | 0.11 | 0.11 | 0.42 | 0.42 | 0.32 | 0.21 | 0.42 | 0.32 | 0.32 | 0.21 | 0.42 | 0.42 | 0.32 | 0.32 |
| | R3 | 0.10 | 0.10 | 0.10 | 0.10 | 0.40 | 0.40 | 0.30 | 0.20 | 0.40 | 0.30 | 0.30 | 0.20 | 0.40 | 0.40 | 0.30 | 0.30 |

| Cultivated soil; Rhizosphere | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Common bean | | | | | | | | | | | | | | | | | |
| Soil property | Replicate | NT1 | NT2 | NT3 | NT4 | UR1 | UR2 | UR3 | UR4 | AS1 | AS2 | AS3 | AS4 | PN1 | PN2 | PN3 | PN4 |
| pH | R1 | 5.94 | 5.84 | 6.04 | 5.94 | 6.63 | 6.73 | 6.83 | 7.21 | 6.49 | 6.39 | 6.49 | 6.49 | 6.49 | 6.59 | 6.49 | 6.49 |
| | R2 | 6.00 | 5.90 | 6.10 | 6.00 | 6.70 | 6.80 | 6.90 | 7.00 | 6.30 | 6.20 | 6.30 | 6.30 | 6.30 | 6.40 | 6.30 | 6.30 |
| | R3 | 6.18 | 6.08 | 6.28 | 6.18 | 6.90 | 7.00 | 7.11 | 6.93 | 6.24 | 6.14 | 6.24 | 6.24 | 6.24 | 6.34 | 6.24 | 6.24 |
| Exchangeable $NH_4^+$-N (mg N $kg^{-1}$) | R1 | 1.40 | 2.20 | 2.60 | 3.10 | 37.80 | 33.30 | 30.30 | 26.40 | 30.40 | 26.10 | 23.50 | 21.60 | 3.00 | 4.70 | 5.20 | 6.90 |
| | R2 | 1.57 | 2.46 | 2.91 | 3.47 | 42.34 | 37.30 | 33.94 | 29.57 | 34.05 | 29.23 | 26.32 | 24.19 | 3.36 | 5.26 | 5.82 | 7.73 |
| | R3 | 1.32 | 2.07 | 2.45 | 2.92 | 35.56 | 31.33 | 28.51 | 24.84 | 28.60 | 24.55 | 22.11 | 20.32 | 2.82 | 4.42 | 4.89 | 6.49 |
| $NO_3^-$-N (mg N $kg^{-1}$) | R1 | 2.47 | 2.85 | 2.09 | 2.28 | 23.85 | 16.06 | 17.48 | 20.14 | 14.02 | 20.41 | 25.31 | 28.27 | 46.63 | 40.01 | 35.68 | 33.29 |
| | R2 | 2.60 | 3.00 | 2.20 | 2.40 | 25.10 | 16.90 | 18.40 | 21.20 | 12.30 | 17.90 | 22.20 | 24.80 | 40.90 | 35.10 | 31.30 | 29.20 |
| | R3 | 2.96 | 3.42 | 2.51 | 2.74 | 28.61 | 19.27 | 20.98 | 24.17 | 11.69 | 17.01 | 21.09 | 23.56 | 38.86 | 33.35 | 29.74 | 27.74 |
| TOC (mg N $kg^{-1}$) | R1 | 1.84 | 2.07 | 2.19 | 2.42 | 2.88 | 2.99 | 3.34 | 4.03 | 2.99 | 3.34 | 3.57 | 3.80 | 3.11 | 3.34 | 3.80 | 3.80 |
| | R2 | 1.49 | 1.67 | 1.77 | 1.95 | 2.33 | 2.42 | 2.70 | 3.26 | 2.42 | 2.70 | 2.88 | 3.07 | 2.51 | 2.70 | 3.07 | 3.07 |
| | R3 | 1.60 | 1.80 | 1.90 | 2.10 | 2.50 | 2.60 | 2.90 | 3.50 | 2.60 | 2.90 | 3.10 | 3.30 | 2.70 | 2.90 | 3.30 | 3.30 |
| TC (%) | R1 | 2.10 | 2.31 | 2.31 | 2.42 | 2.84 | 3.05 | 3.89 | 4.20 | 3.15 | 3.36 | 3.57 | 3.78 | 3.26 | 3.57 | 3.57 | 3.89 |
| | R2 | 1.93 | 2.13 | 2.13 | 2.22 | 2.61 | 2.80 | 3.58 | 3.87 | 2.90 | 3.09 | 3.29 | 3.48 | 3.00 | 3.29 | 3.29 | 3.58 |
| | R3 | 2.00 | 2.20 | 2.20 | 2.30 | 2.70 | 2.90 | 3.70 | 4.00 | 3.00 | 3.20 | 3.40 | 3.60 | 3.10 | 3.40 | 3.40 | 3.70 |
| TN (%) | R1 | 0.10 | 0.10 | 0.10 | 0.10 | 0.50 | 0.50 | 0.40 | 0.30 | 0.50 | 0.40 | 0.40 | 0.30 | 0.52 | 0.50 | 0.40 | 0.31 |
| | R2 | 0.10 | 0.10 | 0.10 | 0.10 | 0.52 | 0.52 | 0.41 | 0.31 | 0.52 | 0.41 | 0.41 | 0.31 | 0.51 | 0.52 | 0.41 | 0.31 |
| | R3 | 0.10 | 0.10 | 0.10 | 0.10 | 0.50 | 0.50 | 0.40 | 0.30 | 0.50 | 0.40 | 0.40 | 0.30 | 0.50 | 0.50 | 0.40 | 0.30 |

| Years of treatment | Uncultivated soil | | | | |
|---|---|---|---|---|---|
| | $N_2O$ cumulative emission (nmol $N_2O$ g dry soil$^{-1}$) | | | | |
| | Replicate | NT | UR | AS | PN |
| 1 | R1 | 1.2 | 13.7 | 11.5 | 6.6 |
| | R2 | 1.1 | 13.9 | 11.8 | 6.9 |
| | R3 | 1.3 | 13.5 | 11.2 | 6.3 |
| 2 | R1 | 0.2 | 11.1 | 8.9 | 4.5 |
| | R2 | 0.2 | 11.4 | 9.3 | 4.8 |
| | R3 | 0.2 | 10.8 | 8.6 | 4.2 |
| 3 | R1 | 0.2 | 9.0 | 5.9 | 4.8 |
| | R2 | 0.2 | 9.3 | 5.7 | 4.9 |
| | R3 | 0.2 | 8.8 | 6.3 | 4.6 |

| Cultivated soil | | | | | |
|---|---|---|---|---|---|
| Tomato | | | | | |
| Harvest | | $N_2O$ cumulative emission (nmol $N_2O$ g dry $soil^{-1}$) | | | |
| | Replicate | NT | UR | AS | PN |
| 1 | R1 | 1.2 | 18.6 | 13.4 | 8.0 |
| | R2 | 1.3 | 18.9 | 13.9 | 8.4 |
| | R3 | 1.1 | 18.5 | 13.2 | 7.6 |
| 2 | R1 | 0.2 | 15.1 | 13.0 | 7.8 |
| | R2 | 0.2 | 15.4 | 13.3 | 7.4 |
| | R3 | 0.2 | 14.8 | 12.6 | 7.9 |
| 3 | R1 | 0.2 | 13.1 | 8.8 | 7.4 |
| | R2 | 0.2 | 13.6 | 8.4 | 7.7 |
| | R3 | 0.2 | 12.8 | 9.3 | 7.2 |
| 4 | R1 | 0.2 | 8.6 | 6.4 | 6.0 |
| | R2 | 0.2 | 7.6 | 5.8 | 6.8 |
| | R3 | 0.2 | 8.1 | 6.1 | 6.3 |

| Cultivated soil | | | | | |
|---|---|---|---|---|---|
| Common bean | | | | | |
| Harvest | | $N_2O$ cumulative emission (nmol $N_2O$ g dry $soil^{-1}$) | | | |
| | Replicate | NT | UR | AS | PN |
| 1 | R1 | 1.5 | 20.9 | 14.0 | 5.9 |
| | R2 | 1.4 | 19.6 | 14.9 | 5.4 |
| | R3 | 1.3 | 20.1 | 14.4 | 5.7 |
| 2 | R1 | 0.2 | 16.2 | 15.9 | 12.3 |
| | R2 | 0.2 | 17.3 | 16.6 | 11.6 |
| | R3 | 0.2 | 16.7 | 16.4 | 11.8 |
| 3 | R1 | 0.3 | 13.9 | 10.9 | 11.5 |
| | R2 | 0.3 | 13.2 | 10.2 | 11.3 |
| | R3 | 0.3 | 13.9 | 10.7 | 11.2 |
| 4 | R1 | 0.5 | 9.6 | 8.9 | 7.9 |
| | R2 | 0.4 | 8.8 | 8.4 | 7.4 |
| | R3 | 0.4 | 9.0 | 8.6 | 7.8 |

| Uncultivated soil | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gene copy number x $ng^{-1}$ DNA | | | | | | | | | | | | |
| Treatment | Replicate | 16SB | 16SA | *amoA* AOB | *amoA* AOA | *napA* | *narG* | *nirK* | *nirS* | *norB* | *nosZ*I | *nosZ*II |
| NT | R1 | 1.12E+09 | 1.79E+06 | 6.27E+04 | 1.34E+03 | 7.73E+03 | 2.13E+04 | 7.95E+04 | 5.71E+04 | 4.03E+04 | 1.34E+03 | 1.79E+03 |
| | R2 | 9.96E+08 | 1.59E+06 | 5.58E+04 | 1.20E+03 | 6.88E+03 | 1.89E+04 | 7.07E+04 | 5.08E+04 | 3.59E+04 | 1.20E+03 | 1.59E+03 |
| | R3 | 1.00E+09 | 1.60E+06 | 5.60E+04 | 1.20E+03 | 6.90E+03 | 1.90E+04 | 7.10E+04 | 5.10E+04 | 3.60E+04 | 1.20E+03 | 1.60E+03 |
| NT1 | R1 | 2.42E+09 | 2.75E+06 | 6.71E+04 | 2.20E+03 | 6.16E+03 | 1.76E+04 | 8.14E+04 | 5.72E+04 | 3.52E+04 | 1.21E+03 | 2.31E+03 |
| | R2 | 2.10E+09 | 2.39E+06 | 5.83E+04 | 1.91E+03 | 5.36E+03 | 1.53E+04 | 7.08E+04 | 4.97E+04 | 3.06E+04 | 1.05E+03 | 2.01E+03 |
| | R3 | 2.20E+09 | 2.50E+06 | 6.10E+04 | 2.00E+03 | 5.60E+03 | 1.60E+04 | 7.40E+04 | 5.20E+04 | 3.20E+04 | 1.10E+03 | 2.10E+03 |
| NT2 | R1 | 2.03E+09 | 2.32E+06 | 5.60E+04 | 2.03E+03 | 6.28E+03 | 1.45E+04 | 7.24E+04 | 5.12E+04 | 2.99E+04 | 1.16E+03 | 1.93E+03 |
| | R2 | 2.36E+09 | 2.64E+06 | 6.51E+04 | 2.36E+03 | 7.29E+03 | 1.68E+04 | 8.41E+04 | 5.94E+04 | 3.48E+04 | 1.35E+03 | 2.24E+03 |
| | R3 | 2.10E+09 | 2.40E+06 | 5.80E+04 | 2.10E+03 | 6.50E+03 | 1.50E+04 | 7.50E+04 | 5.30E+04 | 3.10E+04 | 1.20E+03 | 2.00E+03 |
| NT3 | R1 | 1.64E+09 | 2.74E+06 | 6.57E+04 | 1.97E+03 | 7.23E+03 | 1.75E+04 | 7.56E+04 | 5.48E+04 | 3.61E+04 | 1.64E+03 | 1.64E+03 |
| | R2 | 1.43E+09 | 2.39E+06 | 5.74E+04 | 1.72E+03 | 6.31E+03 | 1.53E+04 | 6.60E+04 | 4.78E+04 | 3.16E+04 | 1.43E+03 | 1.43E+03 |
| | R3 | 1.50E+09 | 2.50E+06 | 6.00E+04 | 1.80E+03 | 6.60E+03 | 1.60E+04 | 6.90E+04 | 5.00E+04 | 3.30E+04 | 1.50E+03 | 1.50E+03 |
| UR1 | R1 | 6.50E+08 | 4.51E+06 | 5.18E+06 | 3.52E+05 | 3.52E+05 | 1.98E+06 | 4.51E+06 | 1.54E+05 | 8.70E+06 | 2.53E+05 | 2.09E+04 |
| | R2 | 5.81E+08 | 4.04E+06 | 4.63E+06 | 3.15E+05 | 3.15E+05 | 1.77E+06 | 4.04E+06 | 1.38E+05 | 7.78E+06 | 2.26E+05 | 1.87E+04 |
| | R3 | 5.90E+08 | 4.10E+06 | 4.70E+06 | 3.20E+05 | 3.20E+05 | 1.80E+06 | 4.10E+06 | 1.40E+05 | 7.90E+06 | 2.30E+05 | 1.90E+04 |
| UR2 | R1 | 3.61E+08 | 9.88E+06 | 7.22E+06 | 6.16E+05 | 4.46E+05 | 2.34E+06 | 4.89E+06 | 2.12E+05 | 8.92E+06 | 3.93E+05 | 2.55E+04 |
| | R2 | 3.22E+08 | 8.80E+06 | 6.43E+06 | 5.49E+05 | 3.97E+05 | 2.08E+06 | 4.35E+06 | 1.89E+05 | 7.95E+06 | 3.50E+05 | 2.27E+04 |
| | R3 | 3.40E+08 | 9.30E+06 | 6.80E+06 | 5.80E+05 | 4.20E+05 | 2.20E+06 | 4.60E+06 | 2.00E+05 | 8.40E+06 | 3.70E+05 | 2.40E+04 |
| UR3 | R1 | 2.09E+08 | 2.12E+07 | 1.32E+07 | 1.21E+06 | 5.06E+05 | 2.86E+06 | 5.72E+06 | 2.42E+05 | 9.57E+06 | 7.26E+05 | 3.08E+04 |
| | R2 | 1.79E+08 | 1.82E+07 | 1.13E+07 | 1.04E+06 | 4.34E+05 | 2.45E+06 | 4.90E+06 | 2.08E+05 | 8.21E+06 | 6.23E+05 | 2.64E+04 |
| | R3 | 1.90E+08 | 1.90E+07 | 1.20E+07 | 1.10E+06 | 4.60E+05 | 2.60E+06 | 5.20E+06 | 2.20E+05 | 8.70E+06 | 6.60E+05 | 2.80E+04 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AS1 | R1 | 7.27E+08 | 3.06E+06 | 5.17E+06 | 3.92E+05 | 3.35E+05 | 2.39E+06 | 3.35E+06 | 1.53E+05 | 2.87E+06 | 3.83E+05 | 2.49E+04 |
| | R2 | 8.37E+08 | 3.52E+06 | 5.95E+06 | 4.52E+05 | 3.86E+05 | 2.75E+06 | 3.86E+06 | 1.76E+05 | 3.30E+06 | 4.41E+05 | 2.86E+04 |
| | R3 | 7.60E+08 | 3.20E+06 | 5.40E+06 | 4.10E+05 | 3.50E+05 | 2.50E+06 | 3.50E+06 | 1.60E+05 | 3.00E+06 | 4.00E+05 | 2.60E+04 |
| AS2 | R1 | 5.58E+08 | 5.30E+06 | 6.71E+06 | 6.05E+05 | 3.69E+05 | 3.31E+06 | 4.63E+06 | 1.70E+05 | 4.07E+06 | 6.81E+05 | 2.55E+04 |
| | R2 | 6.61E+08 | 6.28E+06 | 7.96E+06 | 7.17E+05 | 4.37E+05 | 3.92E+06 | 5.49E+06 | 2.02E+05 | 4.82E+06 | 8.07E+05 | 3.03E+04 |
| | R3 | 5.90E+08 | 5.60E+06 | 7.10E+06 | 6.40E+05 | 3.90E+05 | 3.50E+06 | 4.90E+06 | 1.80E+05 | 4.30E+06 | 7.20E+05 | 2.70E+04 |
| AS3 | R1 | 2.56E+08 | 2.90E+07 | 1.02E+07 | 1.78E+06 | 4.90E+05 | 4.34E+06 | 6.35E+06 | 2.67E+05 | 5.46E+06 | 9.69E+05 | 3.45E+04 |
| | R2 | 2.20E+08 | 2.49E+07 | 8.80E+06 | 1.53E+06 | 4.21E+05 | 3.73E+06 | 5.45E+06 | 2.29E+05 | 4.68E+06 | 8.32E+05 | 2.96E+04 |
| | R3 | 2.30E+08 | 2.60E+07 | 9.20E+06 | 1.60E+06 | 4.40E+05 | 3.90E+06 | 5.70E+06 | 2.40E+05 | 4.90E+06 | 8.70E+05 | 3.10E+04 |
| PN1 | R1 | 9.48E+08 | 6.50E+06 | 5.97E+04 | 2.13E+03 | 7.57E+05 | 6.82E+06 | 5.75E+06 | 2.34E+06 | 8.31E+06 | 5.43E+06 | 9.48E+04 |
| | R2 | 8.51E+08 | 5.83E+06 | 5.36E+04 | 1.91E+03 | 6.79E+05 | 6.12E+06 | 5.17E+06 | 2.10E+06 | 7.46E+06 | 4.88E+06 | 8.51E+04 |
| | R3 | 8.90E+08 | 6.10E+06 | 5.60E+04 | 2.00E+03 | 7.10E+05 | 6.40E+06 | 5.40E+06 | 2.20E+06 | 7.80E+06 | 5.10E+06 | 8.90E+04 |
| PN2 | R1 | 3.73E+08 | 1.49E+07 | 6.29E+04 | 2.34E+03 | 8.42E+05 | 7.46E+06 | 7.67E+06 | 4.80E+06 | 9.06E+06 | 7.67E+06 | 1.28E+05 |
| | R2 | 3.34E+08 | 1.34E+07 | 5.64E+04 | 2.10E+03 | 7.55E+05 | 6.69E+06 | 6.88E+06 | 4.30E+06 | 8.12E+06 | 6.88E+06 | 1.15E+05 |
| | R3 | 3.50E+08 | 1.40E+07 | 5.90E+04 | 2.20E+03 | 7.90E+05 | 7.00E+06 | 7.20E+06 | 4.50E+06 | 8.50E+06 | 7.20E+06 | 1.20E+05 |
| PN3 | R1 | 2.24E+08 | 3.09E+07 | 6.61E+04 | 1.28E+03 | 8.74E+05 | 7.67E+06 | 1.00E+07 | 5.97E+06 | 9.48E+06 | 9.80E+06 | 1.49E+05 |
| | R2 | 2.01E+08 | 2.78E+07 | 5.94E+04 | 1.15E+03 | 7.85E+05 | 6.90E+06 | 9.00E+06 | 5.36E+06 | 8.53E+06 | 8.81E+06 | 1.34E+05 |
| | R3 | 2.10E+08 | 2.90E+07 | 6.20E+04 | 1.20E+03 | 8.20E+05 | 7.20E+06 | 9.40E+06 | 5.60E+06 | 8.90E+06 | 9.20E+06 | 1.40E+05 |

| Cultivated soils | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tomato; Bulk | | | | | | | | | | | | |
| Gene copy number x $ng^{-1}$ DNA | | | | | | | | | | | | |
| | Replicate | 16SB | 16SA | *amoA* AOB | *amoA* AOA | *napA* | *narG* | *nirK* | *nirS* | *norB* | *nosZ*I | *nosZ*II |
| NT1 | R1 | 1.04E+09 | 1.60E+07 | 7.80E+04 | 3.90E+04 | 1.89E+05 | 2.65E+05 | 5.16E+05 | 1.12E+05 | 3.59E+05 | 2.18E+05 | 1.04E+04 |
| | R2 | 9.67E+08 | 1.48E+07 | 7.23E+04 | 3.61E+04 | 1.75E+05 | 2.45E+05 | 4.78E+05 | 1.04E+05 | 3.32E+05 | 2.02E+05 | 9.59E+03 |
| | R3 | 1.53E+09 | 2.34E+07 | 1.14E+05 | 5.72E+04 | 2.77E+05 | 3.88E+05 | 7.56E+05 | 1.64E+05 | 5.26E+05 | 3.20E+05 | 1.52E+04 |
| NT2 | R1 | 5.17E+08 | 1.50E+07 | 8.20E+04 | 4.10E+04 | 1.74E+05 | 2.49E+05 | 4.85E+05 | 1.82E+05 | 3.36E+05 | 2.40E+05 | 9.94E+03 |
| | R2 | 4.79E+08 | 1.39E+07 | 7.60E+04 | 3.80E+04 | 1.61E+05 | 2.31E+05 | 4.50E+05 | 1.68E+05 | 3.11E+05 | 2.22E+05 | 9.21E+03 |
| | R3 | 7.58E+08 | 2.20E+07 | 1.20E+05 | 6.01E+04 | 2.54E+05 | 3.65E+05 | 7.11E+05 | 2.66E+05 | 4.92E+05 | 3.52E+05 | 1.46E+04 |
| NT3 | R1 | 9.52E+08 | 1.60E+07 | 8.70E+04 | 4.35E+04 | 1.85E+05 | 2.66E+05 | 5.20E+05 | 1.89E+05 | 3.61E+05 | 2.52E+05 | 1.03E+04 |
| | R2 | 1.13E+09 | 1.90E+07 | 1.03E+05 | 5.16E+04 | 2.19E+05 | 3.16E+05 | 6.16E+05 | 2.24E+05 | 4.28E+05 | 2.98E+05 | 1.22E+04 |
| | R3 | 1.01E+09 | 1.69E+07 | 9.20E+04 | 4.60E+04 | 1.96E+05 | 2.82E+05 | 5.50E+05 | 2.00E+05 | 3.81E+05 | 2.66E+05 | 1.09E+04 |
| NT4 | R1 | 9.38E+08 | 1.43E+07 | 1.40E+05 | 7.02E+04 | 1.64E+05 | 2.37E+05 | 4.56E+05 | 1.70E+05 | 3.17E+05 | 2.24E+05 | 9.63E+03 |
| | R2 | 1.06E+09 | 1.61E+07 | 1.58E+05 | 7.89E+04 | 1.85E+05 | 2.67E+05 | 5.13E+05 | 1.91E+05 | 3.56E+05 | 2.52E+05 | 1.08E+04 |
| | R3 | 1.00E+09 | 1.53E+07 | 1.50E+05 | 7.50E+04 | 1.76E+05 | 2.53E+05 | 4.87E+05 | 1.81E+05 | 3.38E+05 | 2.39E+05 | 1.03E+04 |
| UR1 | R1 | 8.65E+08 | 1.30E+07 | 8.42E+05 | 4.21E+05 | 1.86E+05 | 2.67E+05 | 5.13E+05 | 1.93E+05 | 3.59E+05 | 2.49E+05 | 1.04E+04 |
| | R2 | 1.02E+09 | 1.54E+07 | 9.98E+05 | 4.99E+05 | 2.21E+05 | 3.16E+05 | 6.08E+05 | 2.29E+05 | 4.25E+05 | 2.95E+05 | 1.23E+04 |
| | R3 | 9.14E+08 | 1.38E+07 | 8.90E+05 | 4.45E+05 | 1.97E+05 | 2.82E+05 | 5.42E+05 | 2.04E+05 | 3.80E+05 | 2.63E+05 | 1.10E+04 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| UR2 | R1 | 2.62E+09 | 1.99E+07 | 1.40E+06 | 7.02E+05 | 1.86E+05 | 2.67E+05 | 5.14E+05 | 1.92E+05 | 3.61E+05 | 2.46E+05 | 1.04E+04 |
| | R2 | 2.95E+09 | 2.24E+07 | 1.58E+06 | 7.89E+05 | 2.09E+05 | 3.01E+05 | 5.78E+05 | 2.16E+05 | 4.05E+05 | 2.77E+05 | 1.17E+04 |
| | R3 | 2.80E+09 | 2.13E+07 | 1.50E+06 | 7.50E+05 | 1.99E+05 | 2.86E+05 | 5.49E+05 | 2.05E+05 | 3.85E+05 | 2.63E+05 | 1.11E+04 |
| UR3 | R1 | 1.34E+09 | 2.39E+07 | 1.92E+06 | 2.06E+06 | 2.02E+05 | 2.89E+05 | 5.58E+05 | 2.12E+05 | 3.91E+05 | 2.78E+05 | 1.15E+04 |
| | R2 | 1.58E+09 | 2.82E+07 | 2.27E+06 | 2.43E+06 | 2.39E+05 | 3.42E+05 | 6.59E+05 | 2.51E+05 | 4.62E+05 | 3.28E+05 | 1.36E+04 |
| | R3 | 1.46E+09 | 2.61E+07 | 2.10E+06 | 2.25E+06 | 2.21E+05 | 3.16E+05 | 6.09E+05 | 2.32E+05 | 4.27E+05 | 3.03E+05 | 1.26E+04 |
| UR4 | R1 | 1.46E+09 | 2.75E+07 | 2.66E+06 | 2.98E+06 | 2.24E+05 | 3.25E+05 | 6.34E+05 | 2.30E+05 | 4.43E+05 | 3.06E+05 | 1.25E+04 |
| | R2 | 1.84E+09 | 3.44E+07 | 3.32E+06 | 3.72E+06 | 2.81E+05 | 4.06E+05 | 7.92E+05 | 2.87E+05 | 5.54E+05 | 3.82E+05 | 1.56E+04 |
| | R3 | 1.61E+09 | 3.00E+07 | 2.90E+06 | 3.25E+06 | 2.45E+05 | 3.55E+05 | 6.92E+05 | 2.51E+05 | 4.84E+05 | 3.34E+05 | 1.37E+04 |
| AS1 | R1 | 1.70E+09 | 2.95E+07 | 3.86E+05 | 1.93E+05 | 2.63E+05 | 3.77E+05 | 7.28E+05 | 2.71E+05 | 5.06E+05 | 3.52E+05 | 1.45E+04 |
| | R2 | 2.24E+09 | 3.90E+07 | 5.10E+05 | 2.55E+05 | 3.47E+05 | 4.98E+05 | 9.62E+05 | 3.58E+05 | 6.69E+05 | 4.65E+05 | 1.92E+04 |
| | R3 | 1.98E+09 | 3.45E+07 | 4.50E+05 | 2.25E+05 | 3.07E+05 | 4.39E+05 | 8.49E+05 | 3.16E+05 | 5.91E+05 | 4.10E+05 | 1.69E+04 |
| AS2 | R1 | 7.21E+08 | 1.06E+07 | 4.50E+05 | 3.68E+05 | 1.44E+05 | 4.71E+05 | 9.03E+05 | 3.37E+05 | 6.29E+05 | 4.82E+05 | 1.81E+04 |
| | R2 | 9.26E+08 | 1.36E+07 | 5.78E+05 | 4.73E+05 | 1.85E+05 | 6.06E+05 | 1.16E+06 | 4.33E+05 | 8.09E+05 | 6.20E+05 | 2.33E+04 |
| | R3 | 8.33E+08 | 1.22E+07 | 5.20E+05 | 4.25E+05 | 1.67E+05 | 5.45E+05 | 1.04E+06 | 3.89E+05 | 7.27E+05 | 5.57E+05 | 2.10E+04 |
| AS3 | R1 | 6.32E+08 | 9.70E+06 | 5.49E+05 | 4.39E+05 | 2.65E+05 | 8.74E+05 | 1.67E+06 | 6.19E+05 | 1.15E+06 | 9.70E+05 | 3.36E+04 |
| | R2 | 7.97E+08 | 1.22E+07 | 6.93E+05 | 5.54E+05 | 3.34E+05 | 1.10E+06 | 2.11E+06 | 7.80E+05 | 1.45E+06 | 1.22E+06 | 4.24E+04 |
| | R3 | 7.48E+08 | 1.15E+07 | 6.50E+05 | 5.20E+05 | 3.14E+05 | 1.03E+06 | 1.98E+06 | 7.32E+05 | 1.36E+06 | 1.15E+06 | 3.98E+04 |
| AS4 | R1 | 6.83E+08 | 1.04E+07 | 7.74E+05 | 6.30E+05 | 8.68E+05 | 2.83E+06 | 5.32E+06 | 1.98E+06 | 3.78E+06 | 3.25E+06 | 1.08E+05 |
| | R2 | 5.97E+08 | 9.11E+06 | 6.77E+05 | 5.51E+05 | 7.60E+05 | 2.47E+06 | 4.66E+06 | 1.73E+06 | 3.31E+06 | 2.84E+06 | 9.41E+04 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R3 | 6.61E+08 | 1.01E+07 | 7.50E+05 | 6.10E+05 | 8.41E+05 | 2.74E+06 | 5.16E+06 | 1.92E+06 | 3.66E+06 | 3.14E+06 | 1.04E+05 |
| PN1 | R1 | 4.33E+08 | 6.42E+06 | 7.73E+04 | 3.87E+04 | 1.66E+06 | 5.24E+06 | 9.88E+06 | 3.71E+06 | 7.05E+06 | 7.01E+06 | 2.03E+05 |
| | R2 | 5.35E+08 | 7.94E+06 | 9.56E+04 | 4.78E+04 | 2.05E+06 | 6.48E+06 | 1.22E+07 | 4.59E+06 | 8.72E+06 | 8.67E+06 | 2.51E+05 |
| | R3 | 4.81E+08 | 7.14E+06 | 8.60E+04 | 4.30E+04 | 1.85E+06 | 5.83E+06 | 1.10E+07 | 4.13E+06 | 7.84E+06 | 7.80E+06 | 2.26E+05 |
| PN2 | R1 | 7.76E+08 | 4.04E+07 | 9.52E+04 | 4.76E+04 | 2.00E+05 | 5.10E+05 | 9.33E+05 | 3.57E+05 | 6.83E+05 | 5.19E+05 | 1.97E+04 |
| | R2 | 9.97E+08 | 5.19E+07 | 1.22E+05 | 6.12E+04 | 2.57E+05 | 6.55E+05 | 1.20E+06 | 4.59E+05 | 8.77E+05 | 6.67E+05 | 2.52E+04 |
| | R3 | 8.97E+08 | 4.67E+07 | 1.10E+05 | 5.50E+04 | 2.31E+05 | 5.89E+05 | 1.08E+06 | 4.13E+05 | 7.89E+05 | 6.00E+05 | 2.27E+04 |
| PN3 | R1 | 7.64E+08 | 1.19E+07 | 1.74E+05 | 8.70E+04 | 6.00E+05 | 1.44E+06 | 2.92E+06 | 1.05E+06 | 2.04E+06 | 1.62E+06 | 5.78E+04 |
| | R2 | 9.66E+08 | 1.49E+07 | 2.17E+05 | 1.09E+05 | 7.50E+05 | 1.80E+06 | 3.66E+06 | 1.31E+06 | 2.55E+06 | 2.02E+06 | 7.23E+04 |
| | R3 | 8.44E+08 | 1.30E+07 | 1.90E+05 | 9.50E+04 | 6.56E+05 | 1.57E+06 | 3.19E+06 | 1.15E+06 | 2.23E+06 | 1.76E+06 | 6.32E+04 |
| PN4 | R1 | 6.65E+08 | 9.67E+06 | 2.29E+05 | 1.14E+05 | 1.80E+06 | 4.49E+06 | 8.70E+06 | 3.25E+06 | 6.04E+06 | 5.18E+06 | 1.77E+05 |
| | R2 | 7.85E+08 | 1.14E+07 | 2.71E+05 | 1.35E+05 | 2.12E+06 | 5.31E+06 | 1.03E+07 | 3.84E+06 | 7.14E+06 | 6.12E+06 | 2.09E+05 |
| | R3 | 7.26E+08 | 1.06E+07 | 2.50E+05 | 1.25E+05 | 1.96E+06 | 4.91E+06 | 9.50E+06 | 3.55E+06 | 6.60E+06 | 5.65E+06 | 1.94E+05 |

| Cultivated soil | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tomato; Rhizosphere | | | | | | | | | | | | |
| Gene copy number x $ng^{-1}$ DNA | | | | | | | | | | | | |
| | Replicate | 16SB | 16SA | *amoA* AOB | *amoA* AOA | *napA* | *narG* | *nirK* | *nirS* | *norB* | *nosZ*I | *nosZ*II |
| NT1 | R1 | 1.80E+09 | 2.78E+07 | 5.16E+04 | 3.65E+05 | 1.77E+06 | 2.48E+06 | 4.83E+06 | 1.05E+06 | 3.36E+06 | 2.04E+06 | 9.68E+04 |
| | R2 | 2.03E+09 | 3.13E+07 | 5.80E+04 | 4.10E+05 | 1.99E+06 | 2.79E+06 | 5.43E+06 | 1.18E+06 | 3.77E+06 | 2.29E+06 | 1.09E+05 |
| | R3 | 1.93E+09 | 2.97E+07 | 5.52E+04 | 3.90E+05 | 1.89E+06 | 2.65E+06 | 5.16E+06 | 1.12E+06 | 3.59E+06 | 2.18E+06 | 1.04E+05 |
| NT2 | R1 | 1.70E+09 | 3.07E+07 | 5.48E+04 | 4.23E+05 | 1.79E+06 | 2.57E+06 | 5.01E+06 | 1.87E+06 | 3.46E+06 | 2.48E+06 | 1.03E+05 |
| | R2 | 1.49E+09 | 2.69E+07 | 4.79E+04 | 3.70E+05 | 1.57E+06 | 2.25E+06 | 4.38E+06 | 1.64E+06 | 3.03E+06 | 2.17E+06 | 8.97E+04 |
| | R3 | 1.65E+09 | 2.98E+07 | 5.31E+04 | 4.10E+05 | 1.74E+06 | 2.49E+06 | 4.85E+06 | 1.82E+06 | 3.36E+06 | 2.40E+06 | 9.94E+04 |
| NT3 | R1 | 1.72E+09 | 3.14E+07 | 5.16E+04 | 3.98E+05 | 1.69E+06 | 2.44E+06 | 4.76E+06 | 1.73E+06 | 3.30E+06 | 2.30E+06 | 9.43E+04 |
| | R2 | 2.22E+09 | 4.03E+07 | 6.63E+04 | 5.12E+05 | 2.18E+06 | 3.13E+06 | 6.12E+06 | 2.23E+06 | 4.24E+06 | 2.96E+06 | 1.21E+05 |
| | R3 | 1.99E+09 | 3.62E+07 | 5.97E+04 | 4.60E+05 | 1.96E+06 | 2.82E+06 | 5.50E+06 | 2.00E+06 | 3.81E+06 | 2.66E+06 | 1.09E+05 |
| NT4 | R1 | 2.21E+09 | 4.07E+07 | 4.83E+04 | 6.87E+05 | 1.61E+06 | 2.32E+06 | 4.46E+06 | 1.66E+06 | 3.10E+06 | 2.19E+06 | 9.43E+04 |
| | R2 | 2.80E+09 | 5.08E+07 | 6.04E+04 | 8.58E+05 | 2.01E+06 | 2.90E+06 | 5.58E+06 | 2.08E+06 | 3.87E+06 | 2.74E+06 | 1.18E+05 |
| | R3 | 2.44E+09 | 4.44E+07 | 5.28E+04 | 7.50E+05 | 1.76E+06 | 2.53E+06 | 4.87E+06 | 1.81E+06 | 3.38E+06 | 2.39E+06 | 1.03E+05 |
| UR1 | R1 | 1.10E+09 | 1.68E+07 | 4.31E+05 | 4.00E+06 | 1.77E+06 | 2.54E+06 | 4.88E+06 | 1.84E+06 | 3.41E+06 | 2.36E+06 | 9.90E+04 |
| | R2 | 1.36E+09 | 2.08E+07 | 5.34E+05 | 4.95E+06 | 2.19E+06 | 3.14E+06 | 6.03E+06 | 2.27E+06 | 4.22E+06 | 2.92E+06 | 1.22E+05 |
| | R3 | 1.22E+09 | 1.87E+07 | 4.80E+05 | 4.45E+06 | 1.97E+06 | 2.82E+06 | 5.42E+06 | 2.04E+06 | 3.80E+06 | 2.63E+06 | 1.10E+05 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| UR2 | R1 | 9.57E+08 | 1.43E+07 | 4.46E+05 | 6.43E+06 | 1.70E+06 | 2.45E+06 | 4.71E+06 | 1.76E+06 | 3.30E+06 | 2.26E+06 | 9.54E+04 |
| | R2 | 1.26E+09 | 1.88E+07 | 5.89E+05 | 8.49E+06 | 2.25E+06 | 3.24E+06 | 6.22E+06 | 2.33E+06 | 4.36E+06 | 2.98E+06 | 1.26E+05 |
| | R3 | 1.12E+09 | 1.66E+07 | 5.20E+05 | 7.50E+06 | 1.99E+06 | 2.86E+06 | 5.49E+06 | 2.05E+06 | 3.85E+06 | 2.63E+06 | 1.11E+05 |
| UR3 | R1 | 8.01E+08 | 1.25E+07 | 6.10E+05 | 2.25E+07 | 2.21E+06 | 3.16E+06 | 6.09E+06 | 2.32E+06 | 4.27E+06 | 3.03E+06 | 1.26E+05 |
| | R2 | 6.62E+08 | 1.01E+07 | 6.74E+05 | 3.04E+07 | 2.29E+06 | 3.32E+06 | 6.48E+06 | 2.35E+06 | 4.53E+06 | 3.12E+06 | 1.28E+05 |
| | R3 | 7.45E+08 | 1.14E+07 | 7.58E+05 | 3.42E+07 | 2.58E+06 | 3.73E+06 | 7.28E+06 | 2.64E+06 | 5.09E+06 | 3.51E+06 | 1.44E+05 |
| UR4 | R1 | 7.08E+08 | 1.08E+07 | 7.20E+05 | 3.25E+07 | 2.45E+06 | 3.55E+06 | 6.92E+06 | 2.51E+06 | 4.84E+06 | 3.34E+06 | 1.37E+05 |
| | R2 | 6.06E+08 | 9.25E+06 | 6.17E+05 | 2.78E+07 | 2.10E+06 | 3.04E+06 | 5.93E+06 | 2.15E+06 | 4.14E+06 | 2.86E+06 | 1.17E+05 |
| | R3 | 7.33E+08 | 1.12E+07 | 7.46E+05 | 3.37E+07 | 2.54E+06 | 3.68E+06 | 7.17E+06 | 2.60E+06 | 5.01E+06 | 3.46E+06 | 1.42E+05 |
| AS1 | R1 | 1.11E+09 | 1.70E+07 | 5.20E+05 | 2.25E+06 | 3.07E+06 | 4.39E+06 | 8.49E+06 | 3.16E+06 | 5.91E+06 | 4.10E+06 | 1.69E+05 |
| | R2 | 1.22E+09 | 1.87E+07 | 5.70E+05 | 2.47E+06 | 3.36E+06 | 4.81E+06 | 9.31E+06 | 3.46E+06 | 6.47E+06 | 4.50E+06 | 1.85E+05 |
| | R3 | 1.03E+09 | 1.57E+07 | 4.80E+05 | 2.08E+06 | 2.83E+06 | 4.05E+06 | 7.83E+06 | 2.92E+06 | 5.45E+06 | 3.79E+06 | 1.56E+05 |
| AS2 | R1 | 9.37E+08 | 1.43E+07 | 7.50E+05 | 4.25E+06 | 1.67E+06 | 5.45E+06 | 1.04E+07 | 3.89E+06 | 7.27E+06 | 5.57E+06 | 2.10E+05 |
| | R2 | 8.11E+08 | 1.24E+07 | 6.49E+05 | 3.68E+06 | 1.44E+06 | 4.71E+06 | 9.03E+06 | 3.37E+06 | 6.29E+06 | 4.82E+06 | 1.81E+05 |
| | R3 | 1.03E+09 | 1.56E+07 | 8.22E+05 | 4.66E+06 | 1.83E+06 | 5.97E+06 | 1.14E+07 | 4.27E+06 | 7.97E+06 | 6.10E+06 | 2.30E+05 |
| AS3 | R1 | 7.74E+08 | 1.17E+07 | 8.30E+05 | 1.75E+07 | 3.14E+06 | 1.03E+07 | 1.98E+07 | 7.32E+06 | 1.36E+07 | 1.15E+07 | 3.98E+05 |
| | R2 | 8.39E+08 | 1.27E+07 | 9.00E+05 | 1.90E+07 | 3.40E+06 | 1.12E+07 | 2.14E+07 | 7.94E+06 | 1.47E+07 | 1.25E+07 | 4.32E+05 |
| | R3 | 7.24E+08 | 1.09E+07 | 7.77E+05 | 1.64E+07 | 2.94E+06 | 9.69E+06 | 1.85E+07 | 6.86E+06 | 1.27E+07 | 1.08E+07 | 3.73E+05 |
| AS4 | R1 | 7.49E+08 | 1.14E+07 | 9.10E+05 | 4.10E+07 | 8.41E+06 | 2.74E+07 | 5.16E+07 | 1.92E+07 | 3.66E+07 | 3.14E+07 | 1.04E+06 |
| | R2 | 8.20E+08 | 1.25E+07 | 9.97E+05 | 4.49E+07 | 9.22E+06 | 3.00E+07 | 5.65E+07 | 2.10E+07 | 4.01E+07 | 3.45E+07 | 1.14E+06 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R3 | 6.91E+08 | 1.05E+07 | 8.39E+05 | 3.78E+07 | 7.76E+06 | 2.53E+07 | 4.75E+07 | 1.77E+07 | 3.38E+07 | 2.90E+07 | 9.61E+05 |
| PN1 | R1 | 1.18E+09 | 1.83E+07 | 6.20E+04 | 4.30E+05 | 1.85E+07 | 5.83E+07 | 1.10E+08 | 4.13E+07 | 7.84E+07 | 7.80E+07 | 2.26E+06 |
| | R2 | 1.09E+09 | 1.70E+07 | 5.74E+04 | 3.98E+05 | 1.71E+07 | 5.40E+07 | 1.02E+08 | 3.83E+07 | 7.27E+07 | 7.23E+07 | 2.09E+06 |
| | R3 | 1.73E+09 | 2.69E+07 | 9.09E+04 | 6.30E+05 | 2.71E+07 | 8.54E+07 | 1.61E+08 | 6.05E+07 | 1.15E+08 | 1.14E+08 | 3.31E+06 |
| PN2 | R1 | 7.88E+08 | 1.18E+07 | 7.10E+04 | 5.50E+05 | 2.31E+06 | 5.89E+06 | 1.08E+07 | 4.13E+06 | 7.89E+06 | 6.00E+06 | 2.27E+05 |
| | R2 | 7.30E+08 | 1.09E+07 | 6.58E+04 | 5.10E+05 | 2.14E+06 | 5.46E+06 | 9.99E+06 | 3.82E+06 | 7.31E+06 | 5.56E+06 | 2.10E+05 |
| | R3 | 1.16E+09 | 1.73E+07 | 1.04E+05 | 8.06E+05 | 3.39E+06 | 8.63E+06 | 1.58E+07 | 6.05E+06 | 1.16E+07 | 8.80E+06 | 3.33E+05 |
| PN3 | R1 | 6.64E+08 | 1.05E+07 | 7.80E+04 | 9.50E+05 | 6.56E+06 | 1.57E+07 | 3.19E+07 | 1.15E+07 | 2.23E+07 | 1.76E+07 | 6.32E+05 |
| | R2 | 7.20E+08 | 1.14E+07 | 8.46E+04 | 1.03E+06 | 7.11E+06 | 1.70E+07 | 3.46E+07 | 1.25E+07 | 2.42E+07 | 1.91E+07 | 6.85E+05 |
| | R3 | 6.22E+08 | 9.81E+06 | 7.31E+04 | 8.90E+05 | 6.14E+06 | 1.47E+07 | 2.99E+07 | 1.08E+07 | 2.09E+07 | 1.65E+07 | 5.92E+05 |
| PN4 | R1 | 5.43E+08 | 8.36E+06 | 6.65E+04 | 1.12E+06 | 1.76E+07 | 4.41E+07 | 3.78E+07 | 3.19E+07 | 2.88E+07 | 5.08E+07 | 1.74E+06 |
| | R2 | 6.71E+08 | 1.03E+07 | 8.23E+04 | 1.39E+06 | 2.18E+07 | 5.46E+07 | 4.67E+07 | 3.95E+07 | 3.56E+07 | 6.29E+07 | 2.15E+06 |
| | R3 | 6.04E+08 | 9.30E+06 | 7.40E+04 | 1.25E+06 | 1.96E+07 | 4.91E+07 | 4.20E+07 | 3.55E+07 | 3.20E+07 | 5.65E+07 | 1.94E+06 |

| Cultivated soil | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Common bean; Bulk | | | | | | | | | | | | |
| Gene copy number x $ng^{-1}$ DNA | | | | | | | | | | | | |
| | Replicate | 16SB | 16SA | *amoA* AOB | *amoA* AOA | *napA* | *narG* | *nirK* | *nirS* | *norB* | *nosZ*I | *nosZ*II |
| NT | R1 | 1.32E+09 | 1.08E+08 | 4.89E+05 | 1.75E+05 | 7.24E+05 | 7.68E+05 | 2.51E+06 | 5.55E+05 | 2.05E+06 | 9.79E+05 | 1.84E+04 |
| | R2 | 1.48E+09 | 1.21E+08 | 5.50E+05 | 1.97E+05 | 8.14E+05 | 8.63E+05 | 2.82E+06 | 6.25E+05 | 2.30E+06 | 1.10E+06 | 2.07E+04 |
| | R3 | 1.41E+09 | 1.15E+08 | 5.23E+05 | 1.87E+05 | 7.74E+05 | 8.21E+05 | 2.68E+06 | 5.94E+05 | 2.19E+06 | 1.05E+06 | 1.97E+04 |
| NT1 | R1 | 6.39E+08 | 9.88E+07 | 5.03E+05 | 1.80E+05 | 6.51E+05 | 7.07E+05 | 2.31E+06 | 8.81E+05 | 1.88E+06 | 1.05E+06 | 1.73E+04 |
| | R2 | 7.55E+08 | 1.17E+08 | 5.95E+05 | 2.13E+05 | 7.70E+05 | 8.35E+05 | 2.73E+06 | 1.04E+06 | 2.22E+06 | 1.25E+06 | 2.04E+04 |
| | R3 | 6.98E+08 | 1.08E+08 | 5.49E+05 | 1.97E+05 | 7.11E+05 | 7.72E+05 | 2.52E+06 | 9.62E+05 | 2.05E+06 | 1.15E+06 | 1.89E+04 |
| NT2 | R1 | 1.15E+09 | 1.03E+08 | 5.21E+05 | 1.86E+05 | 6.78E+05 | 7.38E+05 | 2.41E+06 | 8.97E+05 | 1.96E+06 | 1.08E+06 | 1.75E+04 |
| | R2 | 1.45E+09 | 1.30E+08 | 6.57E+05 | 2.35E+05 | 8.55E+05 | 9.30E+05 | 3.05E+06 | 1.13E+06 | 2.48E+06 | 1.36E+06 | 2.21E+04 |
| | R3 | 1.36E+09 | 1.22E+08 | 6.16E+05 | 2.21E+05 | 8.02E+05 | 8.73E+05 | 2.86E+06 | 1.06E+06 | 2.33E+06 | 1.28E+06 | 2.07E+04 |
| NT3 | R1 | 1.23E+09 | 1.01E+08 | 9.20E+05 | 3.30E+05 | 6.60E+05 | 7.19E+05 | 2.32E+06 | 8.81E+05 | 1.89E+06 | 1.05E+06 | 1.79E+04 |
| | R2 | 1.55E+09 | 1.26E+08 | 1.15E+06 | 4.12E+05 | 8.24E+05 | 8.99E+05 | 2.90E+06 | 1.10E+06 | 2.36E+06 | 1.31E+06 | 2.24E+04 |
| | R3 | 1.35E+09 | 1.10E+08 | 1.01E+06 | 3.60E+05 | 7.20E+05 | 7.86E+05 | 2.53E+06 | 9.62E+05 | 2.06E+06 | 1.15E+06 | 1.96E+04 |
| UR1 | R1 | 1.23E+09 | 9.91E+07 | 5.96E+06 | 2.14E+06 | 8.08E+05 | 8.75E+05 | 2.82E+06 | 1.08E+06 | 2.32E+06 | 1.26E+06 | 2.09E+04 |
| | R2 | 1.35E+09 | 1.09E+08 | 6.53E+06 | 2.34E+06 | 8.85E+05 | 9.59E+05 | 3.09E+06 | 1.19E+06 | 2.54E+06 | 1.38E+06 | 2.29E+04 |
| | R3 | 1.14E+09 | 9.14E+07 | 5.50E+06 | 1.97E+06 | 7.45E+05 | 8.07E+05 | 2.60E+06 | 9.99E+05 | 2.14E+06 | 1.16E+06 | 1.93E+04 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| UR2 | R1 | 3.78E+09 | 1.53E+08 | 1.01E+07 | 3.60E+06 | 8.14E+05 | 8.86E+05 | 2.86E+06 | 1.09E+06 | 2.35E+06 | 1.26E+06 | 2.11E+04 |
| | R2 | 3.24E+09 | 1.31E+08 | 8.61E+06 | 3.08E+06 | 6.97E+05 | 7.59E+05 | 2.45E+06 | 9.32E+05 | 2.01E+06 | 1.08E+06 | 1.81E+04 |
| | R3 | 3.92E+09 | 1.59E+08 | 1.04E+07 | 3.73E+06 | 8.44E+05 | 9.18E+05 | 2.96E+06 | 1.13E+06 | 2.44E+06 | 1.31E+06 | 2.19E+04 |
| UR3 | R1 | 1.98E+09 | 1.88E+08 | 1.41E+07 | 1.08E+07 | 9.06E+05 | 9.79E+05 | 3.17E+06 | 1.23E+06 | 2.61E+06 | 1.46E+06 | 2.39E+04 |
| | R2 | 1.83E+09 | 1.74E+08 | 1.30E+07 | 1.00E+07 | 8.39E+05 | 9.07E+05 | 2.94E+06 | 1.14E+06 | 2.42E+06 | 1.35E+06 | 2.22E+04 |
| | R3 | 2.90E+09 | 2.75E+08 | 2.06E+07 | 1.58E+07 | 1.33E+06 | 1.44E+06 | 4.64E+06 | 1.80E+06 | 3.82E+06 | 2.13E+06 | 3.51E+04 |
| UR4 | R1 | 2.18E+09 | 2.16E+08 | 1.94E+07 | 1.56E+07 | 1.00E+06 | 1.10E+06 | 3.60E+06 | 1.33E+06 | 2.95E+06 | 1.60E+06 | 2.60E+04 |
| | R2 | 2.42E+09 | 2.40E+08 | 2.16E+07 | 1.74E+07 | 1.12E+06 | 1.22E+06 | 4.00E+06 | 1.48E+06 | 3.28E+06 | 1.78E+06 | 2.89E+04 |
| | R3 | 1.97E+09 | 1.96E+08 | 1.76E+07 | 1.41E+07 | 9.10E+05 | 9.96E+05 | 3.26E+06 | 1.20E+06 | 2.67E+06 | 1.45E+06 | 2.35E+04 |
| AS1 | R1 | 2.67E+09 | 2.48E+08 | 3.02E+06 | 1.08E+06 | 1.26E+06 | 1.36E+06 | 4.42E+06 | 1.68E+06 | 3.60E+06 | 1.97E+06 | 3.21E+04 |
| | R2 | 2.95E+09 | 2.74E+08 | 3.32E+06 | 1.19E+06 | 1.39E+06 | 1.50E+06 | 4.87E+06 | 1.85E+06 | 3.97E+06 | 2.17E+06 | 3.54E+04 |
| | R3 | 2.39E+09 | 2.22E+08 | 2.70E+06 | 9.67E+05 | 1.13E+06 | 1.22E+06 | 3.95E+06 | 1.50E+06 | 3.23E+06 | 1.76E+06 | 2.88E+04 |
| AS2 | R1 | 1.12E+09 | 8.80E+07 | 3.48E+06 | 2.04E+06 | 6.84E+05 | 1.69E+06 | 5.42E+06 | 2.06E+06 | 4.44E+06 | 2.67E+06 | 3.98E+04 |
| | R2 | 1.04E+09 | 8.15E+07 | 3.23E+06 | 1.89E+06 | 6.33E+05 | 1.56E+06 | 5.03E+06 | 1.91E+06 | 4.11E+06 | 2.48E+06 | 3.69E+04 |
| | R3 | 1.65E+09 | 1.29E+08 | 5.11E+06 | 2.99E+06 | 1.00E+06 | 2.47E+06 | 7.95E+06 | 3.02E+06 | 6.50E+06 | 3.92E+06 | 5.84E+04 |
| AS3 | R1 | 1.01E+09 | 8.27E+07 | 4.36E+06 | 2.50E+06 | 1.29E+06 | 3.21E+06 | 1.03E+07 | 3.88E+06 | 8.27E+06 | 5.51E+06 | 7.57E+04 |
| | R2 | 8.74E+08 | 7.16E+07 | 3.77E+06 | 2.16E+06 | 1.11E+06 | 2.78E+06 | 8.89E+06 | 3.36E+06 | 7.16E+06 | 4.77E+06 | 6.55E+04 |
| | R3 | 1.11E+09 | 9.06E+07 | 4.77E+06 | 2.73E+06 | 1.41E+06 | 3.52E+06 | 1.13E+07 | 4.25E+06 | 9.06E+06 | 6.04E+06 | 8.29E+04 |
| AS4 | R1 | 8.93E+08 | 7.26E+07 | 5.03E+06 | 2.93E+06 | 3.45E+06 | 8.49E+06 | 2.68E+07 | 1.02E+07 | 2.23E+07 | 1.51E+07 | 1.98E+05 |
| | R2 | 9.78E+08 | 7.96E+07 | 5.51E+06 | 3.21E+06 | 3.78E+06 | 9.30E+06 | 2.94E+07 | 1.11E+07 | 2.45E+07 | 1.65E+07 | 2.17E+05 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R3 | 8.24E+08 | 6.70E+07 | 4.63E+06 | 2.70E+06 | 3.18E+06 | 7.83E+06 | 2.47E+07 | 9.37E+06 | 2.06E+07 | 1.39E+07 | 1.83E+05 |
| PN1 | R1 | 6.50E+08 | 5.14E+07 | 5.76E+05 | 2.06E+05 | 7.57E+06 | 1.81E+07 | 5.72E+07 | 2.19E+07 | 4.79E+07 | 3.74E+07 | 4.29E+05 |
| | R2 | 7.23E+08 | 5.72E+07 | 6.41E+05 | 2.30E+05 | 8.42E+06 | 2.01E+07 | 6.36E+07 | 2.43E+07 | 5.32E+07 | 4.17E+07 | 4.77E+05 |
| | R3 | 5.88E+08 | 4.65E+07 | 5.22E+05 | 1.87E+05 | 6.85E+06 | 1.64E+07 | 5.17E+07 | 1.98E+07 | 4.33E+07 | 3.39E+07 | 3.88E+05 |
| PN2 | R1 | 1.21E+09 | 3.36E+08 | 7.37E+05 | 2.64E+05 | 9.48E+05 | 1.83E+06 | 5.60E+06 | 2.19E+06 | 4.81E+06 | 2.88E+06 | 4.31E+04 |
| | R2 | 1.04E+09 | 2.88E+08 | 6.31E+05 | 2.26E+05 | 8.12E+05 | 1.56E+06 | 4.80E+06 | 1.87E+06 | 4.12E+06 | 2.47E+06 | 3.69E+04 |
| | R3 | 1.25E+09 | 3.48E+08 | 7.64E+05 | 2.74E+05 | 9.83E+05 | 1.89E+06 | 5.81E+06 | 2.27E+06 | 4.99E+06 | 2.99E+06 | 4.47E+04 |
| PN3 | R1 | 1.14E+09 | 9.37E+07 | 1.27E+06 | 4.56E+05 | 2.69E+06 | 4.87E+06 | 1.66E+07 | 6.09E+06 | 1.36E+07 | 8.47E+06 | 1.20E+05 |
| | R2 | 1.06E+09 | 8.68E+07 | 1.18E+06 | 4.22E+05 | 2.49E+06 | 4.51E+06 | 1.54E+07 | 5.64E+06 | 1.26E+07 | 7.85E+06 | 1.11E+05 |
| | R3 | 1.67E+09 | 1.37E+08 | 1.87E+06 | 6.68E+05 | 3.94E+06 | 7.14E+06 | 2.43E+07 | 8.92E+06 | 1.99E+07 | 1.24E+07 | 1.76E+05 |
| PN4 | R1 | 9.80E+08 | 7.61E+07 | 1.68E+06 | 6.00E+05 | 8.04E+06 | 1.52E+07 | 4.94E+07 | 1.88E+07 | 4.02E+07 | 2.71E+07 | 3.68E+05 |
| | R2 | 1.06E+09 | 8.25E+07 | 1.82E+06 | 6.51E+05 | 8.72E+06 | 1.65E+07 | 5.36E+07 | 2.04E+07 | 4.36E+07 | 2.94E+07 | 3.99E+05 |
| | R3 | 9.18E+08 | 7.12E+07 | 1.57E+06 | 5.62E+05 | 7.53E+06 | 1.43E+07 | 4.63E+07 | 1.76E+07 | 3.77E+07 | 2.54E+07 | 3.44E+05 |

| Cultivated soils | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Common bean; Rhizosphere | | | | | | | | | | | | |
| Gene copy number x $ng^{-1}$ DNA | | | | | | | | | | | | |
| | Replicate | 16SB | 16SA | *amoA* AOB | *amoA* AOA | *napA* | *narG* | *nirK* | *nirS* | *norB* | *nosZ*I | *nosZ*II |
| NT | R1 | 8.72E+09 | 1.03E+08 | 2.27E+05 | 1.86E+06 | 3.63E+06 | 4.12E+06 | 1.32E+07 | 3.18E+06 | 1.25E+07 | 4.59E+06 | 1.33E+05 |
| | R2 | 1.10E+10 | 1.29E+08 | 2.84E+05 | 2.32E+06 | 4.54E+06 | 5.15E+06 | 1.65E+07 | 3.97E+06 | 1.56E+07 | 5.74E+06 | 1.66E+05 |
| | R3 | 9.63E+09 | 1.13E+08 | 2.48E+05 | 2.03E+06 | 3.96E+06 | 4.50E+06 | 1.44E+07 | 3.47E+06 | 1.36E+07 | 5.01E+06 | 1.45E+05 |
| NT1 | R1 | 7.55E+09 | 1.04E+08 | 2.19E+05 | 1.95E+06 | 3.34E+06 | 3.88E+06 | 1.24E+07 | 5.15E+06 | 1.17E+07 | 5.05E+06 | 1.27E+05 |
| | R2 | 8.92E+09 | 1.22E+08 | 2.58E+05 | 2.31E+06 | 3.94E+06 | 4.58E+06 | 1.47E+07 | 6.09E+06 | 1.38E+07 | 5.97E+06 | 1.51E+05 |
| | R3 | 8.24E+09 | 1.13E+08 | 2.39E+05 | 2.13E+06 | 3.64E+06 | 4.23E+06 | 1.36E+07 | 5.63E+06 | 1.28E+07 | 5.52E+06 | 1.39E+05 |
| NT2 | R1 | 9.32E+09 | 1.29E+08 | 2.51E+05 | 2.24E+06 | 3.84E+06 | 4.48E+06 | 1.44E+07 | 5.81E+06 | 1.36E+07 | 5.72E+06 | 1.43E+05 |
| | R2 | 1.05E+10 | 1.45E+08 | 2.82E+05 | 2.52E+06 | 4.32E+06 | 5.04E+06 | 1.62E+07 | 6.53E+06 | 1.52E+07 | 6.44E+06 | 1.61E+05 |
| | R3 | 9.96E+09 | 1.38E+08 | 2.68E+05 | 2.39E+06 | 4.11E+06 | 4.79E+06 | 1.54E+07 | 6.21E+06 | 1.45E+07 | 6.12E+06 | 1.53E+05 |
| NT3 | R1 | 1.05E+10 | 1.45E+08 | 2.03E+05 | 3.34E+06 | 3.16E+06 | 3.69E+06 | 1.17E+07 | 4.82E+06 | 1.10E+07 | 4.72E+06 | 1.24E+05 |
| | R2 | 1.38E+10 | 1.91E+08 | 2.69E+05 | 4.42E+06 | 4.18E+06 | 4.88E+06 | 1.54E+07 | 6.37E+06 | 1.46E+07 | 6.23E+06 | 1.63E+05 |
| | R3 | 1.22E+10 | 1.69E+08 | 2.37E+05 | 3.90E+06 | 3.69E+06 | 4.31E+06 | 1.36E+07 | 5.62E+06 | 1.29E+07 | 5.50E+06 | 1.44E+05 |
| UR1 | R1 | 5.16E+09 | 6.01E+07 | 1.82E+06 | 1.95E+07 | 3.49E+06 | 4.05E+06 | 1.28E+07 | 5.35E+06 | 1.22E+07 | 5.11E+06 | 1.30E+05 |
| | R2 | 6.51E+09 | 7.58E+07 | 2.30E+06 | 2.47E+07 | 4.41E+06 | 5.11E+06 | 1.62E+07 | 6.75E+06 | 1.54E+07 | 6.44E+06 | 1.64E+05 |
| | R3 | 6.11E+09 | 7.12E+07 | 2.16E+06 | 2.31E+07 | 4.14E+06 | 4.80E+06 | 1.52E+07 | 6.34E+06 | 1.44E+07 | 6.05E+06 | 1.54E+05 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| UR2 | R1 | 5.76E+09 | 6.52E+07 | 2.41E+06 | 4.02E+07 | 4.30E+06 | 5.01E+06 | 1.59E+07 | 6.57E+06 | 1.51E+07 | 6.25E+06 | 1.61E+05 |
| | R2 | 5.04E+09 | 5.71E+07 | 2.11E+06 | 3.52E+07 | 3.77E+06 | 4.39E+06 | 1.39E+07 | 5.75E+06 | 1.32E+07 | 5.47E+06 | 1.41E+05 |
| | R3 | 5.58E+09 | 6.32E+07 | 2.34E+06 | 3.90E+07 | 4.17E+06 | 4.86E+06 | 1.54E+07 | 6.37E+06 | 1.46E+07 | 6.06E+06 | 1.56E+05 |
| UR3 | R1 | 3.61E+09 | 4.27E+07 | 2.48E+06 | 1.06E+08 | 4.19E+06 | 4.85E+06 | 1.54E+07 | 6.49E+06 | 1.47E+07 | 6.29E+06 | 1.59E+05 |
| | R2 | 4.27E+09 | 5.05E+07 | 2.93E+06 | 1.25E+08 | 4.94E+06 | 5.72E+06 | 1.82E+07 | 7.66E+06 | 1.73E+07 | 7.43E+06 | 1.88E+05 |
| | R3 | 4.01E+09 | 4.74E+07 | 2.75E+06 | 1.17E+08 | 4.64E+06 | 5.37E+06 | 1.71E+07 | 7.19E+06 | 1.62E+07 | 6.98E+06 | 1.76E+05 |
| UR4 | R1 | 3.66E+09 | 4.25E+07 | 3.35E+06 | 1.75E+08 | 5.33E+06 | 6.25E+06 | 2.01E+07 | 8.05E+06 | 1.90E+07 | 7.95E+06 | 1.98E+05 |
| | R2 | 3.34E+09 | 3.88E+07 | 3.06E+06 | 1.60E+08 | 4.87E+06 | 5.70E+06 | 1.83E+07 | 7.35E+06 | 1.74E+07 | 7.26E+06 | 1.81E+05 |
| | R3 | 3.54E+09 | 4.10E+07 | 3.24E+06 | 1.69E+08 | 5.15E+06 | 6.03E+06 | 1.94E+07 | 7.78E+06 | 1.84E+07 | 7.68E+06 | 1.91E+05 |
| AS1 | R1 | 5.10E+09 | 5.93E+07 | 2.14E+06 | 1.07E+07 | 5.90E+06 | 6.84E+06 | 2.18E+07 | 8.97E+06 | 2.06E+07 | 8.64E+06 | 2.17E+05 |
| | R2 | 6.03E+09 | 7.01E+07 | 2.53E+06 | 1.27E+07 | 6.97E+06 | 8.08E+06 | 2.57E+07 | 1.06E+07 | 2.43E+07 | 1.02E+07 | 2.56E+05 |
| | R3 | 5.57E+09 | 6.48E+07 | 2.34E+06 | 1.17E+07 | 6.44E+06 | 7.47E+06 | 2.38E+07 | 9.80E+06 | 2.24E+07 | 9.44E+06 | 2.37E+05 |
| AS2 | R1 | 3.96E+09 | 4.58E+07 | 2.85E+06 | 1.87E+07 | 2.96E+06 | 7.82E+06 | 2.47E+07 | 1.02E+07 | 2.33E+07 | 1.08E+07 | 2.48E+05 |
| | R2 | 4.99E+09 | 5.78E+07 | 3.60E+06 | 2.36E+07 | 3.73E+06 | 9.87E+06 | 3.11E+07 | 1.29E+07 | 2.94E+07 | 1.37E+07 | 3.13E+05 |
| | R3 | 4.69E+09 | 5.43E+07 | 3.38E+06 | 2.21E+07 | 3.50E+06 | 9.26E+06 | 2.92E+07 | 1.21E+07 | 2.76E+07 | 1.28E+07 | 2.93E+05 |
| AS3 | R1 | 3.62E+09 | 4.15E+07 | 3.49E+06 | 8.51E+07 | 6.17E+06 | 1.65E+07 | 5.18E+07 | 2.12E+07 | 4.82E+07 | 2.47E+07 | 5.22E+05 |
| | R2 | 4.07E+09 | 4.67E+07 | 3.93E+06 | 9.57E+07 | 6.94E+06 | 1.85E+07 | 5.82E+07 | 2.39E+07 | 5.42E+07 | 2.78E+07 | 5.87E+05 |
| | R3 | 3.87E+09 | 4.44E+07 | 3.74E+06 | 9.10E+07 | 6.59E+06 | 1.76E+07 | 5.53E+07 | 2.27E+07 | 5.15E+07 | 2.64E+07 | 5.58E+05 |
| AS4 | R1 | 3.37E+09 | 3.90E+07 | 3.68E+06 | 1.92E+08 | 1.59E+07 | 4.18E+07 | 1.30E+08 | 5.34E+07 | 1.25E+08 | 6.50E+07 | 1.31E+06 |
| | R2 | 4.16E+09 | 4.83E+07 | 4.55E+06 | 2.37E+08 | 1.97E+07 | 5.18E+07 | 1.61E+08 | 6.61E+07 | 1.55E+08 | 8.04E+07 | 1.62E+06 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R3 | 3.74E+09 | 4.34E+07 | 4.10E+06 | 2.13E+08 | 1.77E+07 | 4.65E+07 | 1.44E+08 | 5.94E+07 | 1.39E+08 | 7.23E+07 | 1.46E+06 |
| PN1 | R1 | 6.08E+09 | 7.19E+07 | 2.88E+05 | 2.31E+06 | 4.00E+07 | 1.02E+08 | 3.18E+08 | 1.32E+08 | 3.08E+08 | 1.85E+08 | 3.26E+06 |
| | R2 | 5.32E+09 | 6.29E+07 | 2.52E+05 | 2.02E+06 | 3.50E+07 | 8.95E+07 | 2.78E+08 | 1.16E+08 | 2.69E+08 | 1.62E+08 | 2.86E+06 |
| | R3 | 5.89E+09 | 6.97E+07 | 2.79E+05 | 2.24E+06 | 3.88E+07 | 9.91E+07 | 3.08E+08 | 1.28E+08 | 2.98E+08 | 1.79E+08 | 3.16E+06 |
| PN2 | R1 | 3.57E+09 | 4.10E+07 | 2.93E+05 | 2.62E+06 | 4.45E+06 | 9.17E+06 | 2.76E+07 | 1.17E+07 | 2.74E+07 | 1.26E+07 | 2.91E+05 |
| | R2 | 4.51E+09 | 5.13E+07 | 3.66E+05 | 3.27E+06 | 5.56E+06 | 1.15E+07 | 3.45E+07 | 1.46E+07 | 3.43E+07 | 1.58E+07 | 3.64E+05 |
| | R3 | 3.94E+09 | 4.48E+07 | 3.20E+05 | 2.86E+06 | 4.86E+06 | 1.00E+07 | 3.02E+07 | 1.28E+07 | 3.00E+07 | 1.38E+07 | 3.18E+05 |
| PN3 | R1 | 2.87E+09 | 3.45E+07 | 3.04E+05 | 4.28E+06 | 1.19E+07 | 2.31E+07 | 7.74E+07 | 3.08E+07 | 7.33E+07 | 3.51E+07 | 7.65E+05 |
| | R2 | 3.69E+09 | 4.43E+07 | 3.90E+05 | 5.49E+06 | 1.53E+07 | 2.97E+07 | 9.95E+07 | 3.96E+07 | 9.41E+07 | 4.51E+07 | 9.83E+05 |
| | R3 | 3.32E+09 | 3.98E+07 | 3.51E+05 | 4.94E+06 | 1.38E+07 | 2.67E+07 | 8.94E+07 | 3.56E+07 | 8.46E+07 | 4.06E+07 | 8.84E+05 |
| PN4 | R1 | 2.59E+09 | 3.03E+07 | 2.85E+05 | 5.57E+06 | 3.53E+07 | 7.15E+07 | 1.01E+08 | 9.43E+07 | 1.04E+08 | 1.11E+08 | 2.32E+06 |
| | R2 | 3.42E+09 | 4.00E+07 | 3.77E+05 | 7.36E+06 | 4.67E+07 | 9.45E+07 | 1.33E+08 | 1.25E+08 | 1.38E+08 | 1.47E+08 | 3.07E+06 |
| | R3 | 3.02E+09 | 3.54E+07 | 3.33E+05 | 6.50E+06 | 4.12E+07 | 8.34E+07 | 1.18E+08 | 1.10E+08 | 1.22E+08 | 1.30E+08 | 2.71E+06 |

| | | Uncultivated soils | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Relative abundance (%) | | | | | | | | | | | | |
| Genus | Replicate | NT | NT1 | NT2 | NT3 | UR1 | UR2 | UR3 | AS1 | AS2 | AS3 | PN1 | PN2 | PN3 |
| Aciditerrimonas | R1 | 0.2 | 0.1 | 0.1 | 0.1 | 0.6 | 0.7 | 0.6 | 0.2 | 0.6 | 0.8 | 0.9 | 0.8 | 0.9 |
| | R2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.7 | 0.8 | 0.7 | 0.2 | 0.7 | 1.0 | 1.1 | 1.0 | 1.1 |
| | R3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.5 | 0.6 | 0.5 | 0.2 | 0.5 | 0.7 | 0.8 | 0.7 | 0.8 |
| Acidovorax | R1 | 0.1 | 0.2 | 0.1 | 0.2 | 0.9 | 1.1 | 1.0 | 1.2 | 1.2 | 0.9 | 1.3 | 1.4 | 1.0 |
| | R2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.8 | 1.0 | 0.9 | 1.1 | 1.1 | 0.8 | 1.2 | 1.3 | 0.9 |
| | R3 | 0.1 | 0.2 | 0.1 | 0.2 | 1.0 | 1.2 | 1.1 | 1.3 | 1.3 | 1.0 | 1.4 | 1.5 | 1.1 |
| Adhaeribacter | R1 | 0.2 | 0.1 | 0.2 | 0.1 | 0.9 | 0.5 | 0.6 | 0.9 | 1.2 | 1.0 | 0.6 | 1.2 | 1.2 |
| | R2 | 0.3 | 0.2 | 0.3 | 0.2 | 1.4 | 0.8 | 0.9 | 1.4 | 1.8 | 1.5 | 0.9 | 1.8 | 1.8 |
| | R3 | 0.2 | 0.1 | 0.2 | 0.1 | 0.7 | 0.4 | 0.5 | 0.7 | 1.0 | 0.8 | 0.5 | 1.0 | 1.0 |
| Allokutzneria | R1 | 0.3 | 0.2 | 0.1 | 0.1 | 0.3 | 0.5 | 0.4 | 0.5 | 0.2 | 0.3 | 0.3 | 0.4 | 0.2 |
| | R2 | 0.5 | 0.3 | 0.2 | 0.2 | 0.5 | 0.8 | 0.6 | 0.8 | 0.3 | 0.5 | 0.5 | 0.6 | 0.3 |
| | R3 | 0.2 | 0.2 | 0.1 | 0.1 | 0.2 | 0.4 | 0.3 | 0.4 | 0.2 | 0.2 | 0.2 | 0.3 | 0.2 |
| Amaricoccus | R1 | 0.5 | 0.1 | 0.3 | 0.6 | 0.2 | 0.3 | 0.2 | 0.2 | 0.3 | 0.1 | 0.2 | 0.3 | 0.1 |
| | R2 | 0.8 | 0.2 | 0.5 | 0.9 | 0.3 | 0.5 | 0.3 | 0.3 | 0.5 | 0.2 | 0.3 | 0.5 | 0.2 |
| | R3 | 0.4 | 0.1 | 0.2 | 0.5 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.1 | 0.2 | 0.2 | 0.1 |
| Arenimonas | R1 | 0.5 | 0.6 | 0.2 | 0.3 | 0.4 | 0.3 | 0.2 | 0.2 | 0.3 | 0.4 | 0.5 | 0.3 | 0.4 |
| | R2 | 0.8 | 0.9 | 0.3 | 0.5 | 0.6 | 0.5 | 0.3 | 0.3 | 0.5 | 0.6 | 0.8 | 0.5 | 0.6 |
| | R3 | 0.4 | 0.5 | 0.2 | 0.2 | 0.3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.3 | 0.4 | 0.2 | 0.3 |
| Arthrobacter | R1 | 0.6 | 0.4 | 0.8 | 0.5 | 0.9 | 1.1 | 1.0 | 0.8 | 0.9 | 0.5 | 0.6 | 0.7 | 0.8 |
| | R2 | 0.5 | 0.4 | 0.7 | 0.4 | 0.8 | 1.0 | 0.9 | 0.7 | 0.8 | 0.4 | 0.5 | 0.6 | 0.7 |
| | R3 | 0.7 | 0.5 | 1.0 | 0.6 | 1.1 | 1.3 | 1.2 | 1.0 | 1.1 | 0.6 | 0.7 | 0.8 | 1.0 |
| Azotobacter | R1 | 0.2 | 0.4 | 0.1 | 0.1 | 0.5 | 0.6 | 0.9 | 1.0 | 1.1 | 0.9 | 0.2 | 0.2 | 0.1 |
| | R2 | 0.3 | 0.5 | 0.1 | 0.1 | 0.7 | 0.8 | 1.2 | 1.3 | 1.4 | 1.2 | 0.3 | 0.3 | 0.1 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R3 | 0.2 | 0.3 | 0.1 | 0.1 | 0.4 | 0.5 | 0.7 | 0.8 | 0.9 | 0.7 | 0.2 | 0.2 | 0.1 |
| Bacillus | R1 | 0.5 | 0.7 | 0.7 | 0.6 | 9.0 | 7.8 | 8.6 | 6.5 | 6.5 | 8.5 | 7.5 | 8.6 | 6.5 |
| | R2 | 0.6 | 0.8 | 0.8 | 0.8 | 11.7 | 10.1 | 11.2 | 8.5 | 8.5 | 11.1 | 9.8 | 11.2 | 8.5 |
| | R3 | 0.3 | 0.5 | 0.5 | 0.4 | 6.3 | 5.5 | 6.0 | 4.6 | 4.6 | 6.0 | 5.3 | 6.0 | 4.6 |
| Bradyrhizobium | R1 | 0.6 | 0.5 | 0.5 | 0.4 | 6.8 | 8.5 | 8.2 | 7.2 | 7.0 | 7.6 | 7.8 | 8.0 | 7.4 |
| | R2 | 0.4 | 0.3 | 0.3 | 0.3 | 4.8 | 5.9 | 5.7 | 5.0 | 4.9 | 5.3 | 5.5 | 5.6 | 5.2 |
| | R3 | 0.7 | 0.6 | 0.6 | 0.4 | 7.6 | 9.5 | 9.2 | 8.1 | 7.8 | 8.5 | 8.7 | 9.0 | 8.3 |
| Brevundimonas | R1 | 0.1 | 0.0 | 0.0 | 0.1 | 0.2 | 0.4 | 0.5 | 0.4 | 0.3 | 0.4 | 0.2 | 0.2 | 0.1 |
| | R2 | 0.1 | 0.0 | 0.0 | 0.1 | 0.1 | 0.3 | 0.3 | 0.3 | 0.2 | 0.3 | 0.1 | 0.1 | 0.1 |
| | R3 | 0.1 | 0.0 | 0.0 | 0.1 | 0.3 | 0.5 | 0.7 | 0.5 | 0.4 | 0.5 | 0.3 | 0.3 | 0.1 |
| Caulobacter | R1 | 0.1 | 0.2 | 0.0 | 0.1 | 0.5 | 0.2 | 0.2 | 0.3 | 0.1 | 0.1 | 0.2 | 0.3 | 0.1 |
| | R2 | 0.1 | 0.2 | 0.0 | 0.1 | 0.6 | 0.2 | 0.2 | 0.4 | 0.1 | 0.1 | 0.2 | 0.4 | 0.1 |
| | R3 | 0.1 | 0.2 | 0.0 | 0.1 | 0.5 | 0.2 | 0.2 | 0.3 | 0.1 | 0.1 | 0.2 | 0.3 | 0.1 |
| Chryseobacterium | R1 | 0.1 | 0.5 | 0.2 | 0.4 | 0.3 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R2 | 0.1 | 0.7 | 0.3 | 0.5 | 0.4 | 0.4 | 0.3 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R3 | 0.1 | 0.4 | 0.1 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Comamonas | R1 | 0.4 | 0.5 | 0.5 | 0.2 | 3.4 | 3.2 | 2.5 | 2.9 | 3.6 | 3.4 | 2.9 | 3.2 | 3.6 |
| | R2 | 0.4 | 0.4 | 0.4 | 0.2 | 3.0 | 2.8 | 2.2 | 2.6 | 3.2 | 3.0 | 2.6 | 2.8 | 3.2 |
| | R3 | 0.5 | 0.6 | 0.6 | 0.2 | 4.1 | 3.8 | 3.0 | 3.5 | 4.3 | 4.1 | 3.5 | 3.8 | 4.3 |
| Cupriavidus | R1 | 0.5 | 0.9 | 0.2 | 0.5 | 4.0 | 4.2 | 2.4 | 2.9 | 2.8 | 3.5 | 3.4 | 2.8 | 3.8 |
| | R2 | 0.4 | 0.7 | 0.1 | 0.4 | 3.0 | 3.1 | 1.8 | 2.2 | 2.1 | 2.6 | 2.5 | 2.1 | 2.8 |
| | R3 | 0.6 | 1.1 | 0.2 | 0.6 | 4.9 | 5.1 | 2.9 | 3.5 | 3.4 | 4.3 | 4.1 | 3.4 | 4.6 |
| Devosia | R1 | 0.2 | 0.3 | 0.1 | 0.2 | 0.3 | 0.1 | 0.1 | 0.2 | 0.1 | 0.5 | 0.3 | 0.5 | 0.2 |
| | R2 | 0.2 | 0.3 | 0.1 | 0.2 | 0.3 | 0.1 | 0.1 | 0.2 | 0.1 | 0.4 | 0.3 | 0.4 | 0.2 |
| | R3 | 0.2 | 0.4 | 0.1 | 0.2 | 0.4 | 0.1 | 0.1 | 0.2 | 0.1 | 0.6 | 0.4 | 0.6 | 0.2 |
| Ensifer | R1 | 0.5 | 0.3 | 0.3 | 0.9 | 2.9 | 3.6 | 3.4 | 2.6 | 2.9 | 2.6 | 3.9 | 2.4 | 2.6 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R2 | 0.4 | 0.2 | 0.2 | 0.7 | 2.2 | 2.7 | 2.5 | 1.9 | 2.2 | 1.9 | 2.9 | 1.8 | 1.9 |
| | R3 | 0.6 | 0.4 | 0.4 | 1.1 | 3.5 | 4.4 | 4.1 | 3.2 | 3.5 | 3.2 | 4.8 | 2.9 | 3.2 |
| Flavisolibacter | R1 | 0.4 | 0.2 | 0.2 | 0.2 | 0.9 | 0.9 | 1.2 | 1.4 | 1.5 | 1.2 | 1.3 | 1.6 | 1.5 |
| | R2 | 0.3 | 0.1 | 0.1 | 0.1 | 0.7 | 0.7 | 0.9 | 1.0 | 1.1 | 0.9 | 1.0 | 1.2 | 1.1 |
| | R3 | 0.5 | 0.2 | 0.2 | 0.2 | 1.1 | 1.1 | 1.5 | 1.7 | 1.8 | 1.5 | 1.6 | 2.0 | 1.8 |
| Flavitalea | R1 | 0.5 | 0.5 | 0.3 | 0.3 | 0.4 | 0.2 | 0.1 | 0.2 | 0.3 | 0.3 | 0.1 | 0.2 | 0.2 |
| | R2 | 0.4 | 0.4 | 0.2 | 0.2 | 0.3 | 0.1 | 0.1 | 0.1 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 |
| | R3 | 0.6 | 0.6 | 0.4 | 0.4 | 0.5 | 0.2 | 0.1 | 0.2 | 0.4 | 0.4 | 0.1 | 0.2 | 0.2 |
| Flavobacterium | R1 | 0.5 | 0.3 | 0.2 | 0.2 | 5.5 | 5.9 | 6.0 | 7.0 | 7.5 | 6.8 | 7.9 | 7.1 | 7.0 |
| | R2 | 0.7 | 0.4 | 0.3 | 0.3 | 7.2 | 7.7 | 7.8 | 9.1 | 9.8 | 8.8 | 10.3 | 9.2 | 9.1 |
| | R3 | 0.4 | 0.2 | 0.2 | 0.2 | 4.3 | 4.6 | 4.7 | 5.5 | 5.9 | 5.3 | 6.2 | 5.5 | 5.5 |
| Fontibacillus | R1 | 0.2 | 0.2 | 0.2 | 0.2 | 0.9 | 1.5 | 1.5 | 1.6 | 1.2 | 1.5 | 1.3 | 1.4 | 1.5 |
| | R2 | 0.3 | 0.3 | 0.3 | 0.3 | 1.2 | 2.0 | 2.0 | 2.1 | 1.6 | 2.0 | 1.7 | 1.8 | 2.0 |
| | R3 | 0.1 | 0.1 | 0.1 | 0.1 | 0.6 | 1.1 | 1.1 | 1.1 | 0.8 | 1.1 | 0.9 | 1.0 | 1.1 |
| Gemmatimonas | R1 | 0.1 | 0.2 | 0.1 | 0.2 | 1.2 | 1.5 | 1.4 | 1.8 | 1.7 | 1.5 | 1.9 | 2.0 | 2.2 |
| | R2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.8 | 1.0 | 1.0 | 1.3 | 1.2 | 1.0 | 1.3 | 1.4 | 1.5 |
| | R3 | 0.1 | 0.2 | 0.1 | 0.2 | 1.3 | 1.7 | 1.6 | 2.0 | 1.9 | 1.7 | 2.1 | 2.2 | 2.5 |
| Gp10 | R1 | 0.2 | 0.1 | 0.2 | 0.1 | 0.0 | 0.5 | 0.6 | 0.4 | 0.5 | 0.6 | 0.8 | 0.5 | 0.6 |
| | R2 | 0.2 | 0.1 | 0.2 | 0.1 | 0.0 | 0.4 | 0.5 | 0.4 | 0.4 | 0.5 | 0.7 | 0.4 | 0.5 |
| | R3 | 0.2 | 0.1 | 0.2 | 0.1 | 0.0 | 0.6 | 0.7 | 0.5 | 0.6 | 0.7 | 1.0 | 0.6 | 0.7 |
| Gp4 | R1 | 0.0 | 0.1 | 0.1 | 0.2 | 0.5 | 0.6 | 0.8 | 0.6 | 0.8 | 0.7 | 1.2 | 1.0 | 0.8 |
| | R2 | 0.0 | 0.1 | 0.1 | 0.1 | 0.4 | 0.4 | 0.6 | 0.4 | 0.6 | 0.5 | 0.9 | 0.7 | 0.6 |
| | R3 | 0.0 | 0.1 | 0.1 | 0.2 | 0.6 | 0.7 | 1.0 | 0.7 | 1.0 | 0.9 | 1.5 | 1.2 | 1.0 |
| Gp6 | R1 | 0.2 | 0.1 | 0.1 | 0.0 | 0.9 | 0.5 | 0.6 | 0.7 | 0.8 | 1.0 | 0.6 | 0.7 | 0.8 |
| | R2 | 0.1 | 0.1 | 0.1 | 0.0 | 0.7 | 0.4 | 0.4 | 0.5 | 0.6 | 0.7 | 0.4 | 0.5 | 0.6 |
| | R3 | 0.2 | 0.1 | 0.1 | 0.0 | 1.1 | 0.6 | 0.7 | 0.9 | 1.0 | 1.2 | 0.7 | 0.9 | 1.0 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gp7 | R1 | 0.2 | 0.0 | 0.1 | 0.2 | 0.8 | 0.5 | 0.6 | 0.5 | 0.4 | 0.6 | 0.8 | 0.5 | 0.6 |
| | R2 | 0.1 | 0.0 | 0.1 | 0.1 | 0.6 | 0.4 | 0.4 | 0.4 | 0.3 | 0.4 | 0.6 | 0.4 | 0.4 |
| | R3 | 0.2 | 0.0 | 0.1 | 0.2 | 1.0 | 0.6 | 0.7 | 0.6 | 0.5 | 0.7 | 1.0 | 0.6 | 0.7 |
| Halomonas | R1 | 0.1 | 0.0 | 0.1 | 0.2 | 0.8 | 0.9 | 1.0 | 0.4 | 0.8 | 0.9 | 1.0 | 1.2 | 0.8 |
| | R2 | 0.1 | 0.0 | 0.1 | 0.3 | 1.0 | 1.2 | 1.3 | 0.5 | 1.0 | 1.2 | 1.3 | 1.6 | 1.0 |
| | R3 | 0.1 | 0.0 | 0.1 | 0.1 | 0.6 | 0.6 | 0.7 | 0.3 | 0.6 | 0.6 | 0.7 | 0.8 | 0.6 |
| Hyphomicrobium | R1 | 0.2 | 0.3 | 0.4 | 0.5 | 5.6 | 5.9 | 6.1 | 5.9 | 6.3 | 7.0 | 7.1 | 5.6 | 5.6 |
| | R2 | 0.3 | 0.4 | 0.5 | 0.7 | 7.3 | 7.7 | 7.9 | 7.7 | 8.2 | 9.1 | 9.2 | 7.3 | 7.3 |
| | R3 | 0.2 | 0.2 | 0.3 | 0.4 | 4.4 | 4.6 | 4.8 | 4.6 | 4.9 | 5.5 | 5.5 | 4.4 | 4.4 |
| Ilumatobacter | R1 | 0.0 | 0.2 | 0.2 | 0.1 | 0.8 | 0.5 | 0.5 | 0.4 | 0.4 | 0.3 | 0.6 | 0.4 | 0.5 |
| | R2 | 0.0 | 0.2 | 0.2 | 0.1 | 0.7 | 0.4 | 0.4 | 0.4 | 0.4 | 0.3 | 0.5 | 0.4 | 0.4 |
| | R3 | 0.0 | 0.2 | 0.2 | 0.1 | 1.0 | 0.6 | 0.6 | 0.5 | 0.5 | 0.4 | 0.7 | 0.5 | 0.6 |
| Litorilinea | R1 | 0.2 | 0.1 | 0.2 | 0.2 | 0.8 | 0.5 | 0.4 | 0.3 | 0.2 | 0.3 | 0.1 | 0.2 | 0.5 |
| | R2 | 0.3 | 0.1 | 0.3 | 0.3 | 1.0 | 0.7 | 0.5 | 0.4 | 0.3 | 0.4 | 0.1 | 0.3 | 0.7 |
| | R3 | 0.2 | 0.1 | 0.2 | 0.2 | 0.6 | 0.4 | 0.3 | 0.2 | 0.2 | 0.2 | 0.1 | 0.2 | 0.4 |
| Luteimonas | R1 | 0.0 | 0.1 | 0.0 | 0.1 | 0.5 | 0.4 | 0.6 | 0.4 | 0.5 | 0.6 | 0.4 | 0.3 | 0.5 |
| | R2 | 0.0 | 0.1 | 0.0 | 0.1 | 0.7 | 0.5 | 0.8 | 0.5 | 0.7 | 0.8 | 0.5 | 0.4 | 0.7 |
| | R3 | 0.0 | 0.1 | 0.0 | 0.1 | 0.4 | 0.3 | 0.4 | 0.3 | 0.4 | 0.4 | 0.3 | 0.2 | 0.4 |
| Lysobacter | R1 | 0.1 | 0.2 | 0.2 | 0.1 | 0.5 | 0.3 | 0.1 | 0.2 | 0.4 | 0.3 | 0.4 | 0.2 | 0.6 |
| | R2 | 0.1 | 0.2 | 0.2 | 0.1 | 0.4 | 0.3 | 0.1 | 0.2 | 0.3 | 0.3 | 0.3 | 0.2 | 0.5 |
| | R3 | 0.1 | 0.2 | 0.2 | 0.1 | 0.6 | 0.3 | 0.1 | 0.2 | 0.4 | 0.3 | 0.4 | 0.2 | 0.7 |
| Marmoricola | R1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.5 | 0.3 | 0.2 | 0.1 | 0.5 | 0.3 | 0.2 | 0.0 | 0.0 |
| | R2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.3 | 0.2 | 0.1 | 0.1 | 0.3 | 0.2 | 0.1 | 0.0 | 0.0 |
| | R3 | 0.1 | 0.1 | 0.1 | 0.1 | 0.6 | 0.3 | 0.2 | 0.1 | 0.6 | 0.3 | 0.2 | 0.0 | 0.0 |
| Massilia | R1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.3 | 0.5 | 0.4 | 0.2 | 0.3 | 0.2 | 0.1 | 0.2 | 0.2 |
| | R2 | 0.1 | 0.0 | 0.0 | 0.0 | 0.3 | 0.4 | 0.3 | 0.2 | 0.3 | 0.2 | 0.1 | 0.2 | 0.2 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R3 | 0.1 | 0.0 | 0.0 | 0.0 | 0.3 | 0.6 | 0.4 | 0.2 | 0.3 | 0.2 | 0.1 | 0.2 | 0.2 |
| Mesorhizobium | R1 | 0.5 | 0.4 | 0.3 | 0.4 | 0.6 | 0.9 | 1.1 | 1.2 | 1.0 | 0.5 | 0.6 | 0.4 | 0.5 |
| | R2 | 0.3 | 0.3 | 0.2 | 0.3 | 0.4 | 0.6 | 0.8 | 0.8 | 0.7 | 0.3 | 0.4 | 0.3 | 0.3 |
| | R3 | 0.6 | 0.4 | 0.3 | 0.4 | 0.7 | 1.0 | 1.2 | 1.3 | 1.1 | 0.6 | 0.7 | 0.4 | 0.6 |
| Methylohalomonas | R1 | 0.2 | 0.1 | 0.2 | 0.2 | 0.6 | 0.4 | 0.5 | 0.6 | 0.8 | 0.9 | 0.7 | 0.8 | 0.9 |
| | R2 | 0.2 | 0.1 | 0.2 | 0.2 | 0.5 | 0.4 | 0.4 | 0.5 | 0.7 | 0.8 | 0.6 | 0.7 | 0.8 |
| | R3 | 0.2 | 0.1 | 0.2 | 0.2 | 0.7 | 0.5 | 0.6 | 0.7 | 1.0 | 1.1 | 0.8 | 1.0 | 1.1 |
| Microbacterium | R1 | 0.2 | 0.1 | 0.2 | 0.2 | 0.6 | 0.4 | 0.5 | 0.6 | 0.8 | 0.9 | 0.7 | 0.8 | 0.9 |
| | R2 | 0.2 | 0.1 | 0.2 | 0.2 | 0.5 | 0.3 | 0.4 | 0.5 | 0.7 | 0.8 | 0.6 | 0.7 | 0.8 |
| | R3 | 0.2 | 0.1 | 0.2 | 0.2 | 0.7 | 0.5 | 0.6 | 0.7 | 0.9 | 1.0 | 0.8 | 0.9 | 1.0 |
| Microvirga | R1 | 0.4 | 0.5 | 0.4 | 0.2 | 0.1 | 0.0 | 0.2 | 0.2 | 0.1 | 0.1 | 0.2 | 0.2 | 0.2 |
| | R2 | 0.5 | 0.7 | 0.5 | 0.3 | 0.1 | 0.0 | 0.3 | 0.3 | 0.1 | 0.1 | 0.3 | 0.3 | 0.3 |
| | R3 | 0.3 | 0.4 | 0.3 | 0.1 | 0.1 | 0.0 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Mycobacterium | R1 | 0.2 | 0.1 | 0.2 | 0.2 | 1.8 | 2.2 | 3.2 | 3.4 | 2.4 | 2.1 | 2.9 | 3.1 | 3.3 |
| | R2 | 0.2 | 0.1 | 0.2 | 0.2 | 1.5 | 1.9 | 2.7 | 2.9 | 2.0 | 1.8 | 2.5 | 2.6 | 2.8 |
| | R3 | 0.2 | 0.1 | 0.2 | 0.2 | 2.0 | 2.5 | 3.6 | 3.8 | 2.7 | 2.4 | 3.2 | 3.5 | 3.7 |
| Nitriliruptor | R1 | 0.1 | 0.0 | 0.2 | 0.1 | 0.5 | 0.8 | 0.9 | 0.5 | 0.9 | 0.9 | 0.5 | 0.9 | 1.0 |
| | R2 | 0.1 | 0.0 | 0.2 | 0.1 | 0.4 | 0.7 | 0.8 | 0.4 | 0.8 | 0.8 | 0.4 | 0.8 | 0.8 |
| | R3 | 0.1 | 0.0 | 0.2 | 0.1 | 0.6 | 0.9 | 1.0 | 0.6 | 1.0 | 1.0 | 0.6 | 1.0 | 1.1 |
| Nitrosococcus | R1 | 0.2 | 0.5 | 0.2 | 0.3 | 1.4 | 2.5 | 2.0 | 3.0 | 3.5 | 3.4 | 3.5 | 2.2 | 2.6 |
| | R2 | 0.3 | 0.8 | 0.3 | 0.5 | 2.1 | 3.8 | 3.0 | 4.5 | 5.3 | 5.1 | 5.3 | 3.3 | 3.9 |
| | R3 | 0.2 | 0.4 | 0.2 | 0.2 | 1.1 | 2.0 | 1.6 | 2.4 | 2.8 | 2.7 | 2.8 | 1.8 | 2.1 |
| Nitrosomonas | R1 | 0.2 | 0.1 | 0.3 | 0.1 | 3.0 | 3.2 | 3.1 | 2.5 | 2.3 | 2.4 | 0.3 | 0.5 | 0.3 |
| | R2 | 0.2 | 0.1 | 0.3 | 0.1 | 2.5 | 2.7 | 2.6 | 2.1 | 1.9 | 2.0 | 0.3 | 0.4 | 0.3 |
| | R3 | 0.2 | 0.1 | 0.3 | 0.1 | 3.4 | 3.6 | 3.5 | 2.8 | 2.6 | 2.7 | 0.3 | 0.6 | 0.3 |
| Nitrosospira | R1 | 0.2 | 0.1 | 0.3 | 0.3 | 2.6 | 2.4 | 2.6 | 2.4 | 1.9 | 2.4 | 3.6 | 3.6 | 3.4 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R2 | 0.1 | 0.1 | 0.2 | 0.2 | 1.9 | 1.8 | 1.9 | 1.8 | 1.4 | 1.8 | 2.7 | 2.7 | 2.5 |
| | R3 | 0.2 | 0.1 | 0.4 | 0.4 | 3.2 | 2.9 | 3.2 | 2.9 | 2.3 | 2.9 | 4.4 | 4.4 | 4.1 |
| Nitrosovibrio | R1 | 0.1 | 0.2 | 0.1 | 0.2 | 0.8 | 0.7 | 0.9 | 0.8 | 0.9 | 0.6 | 0.8 | 0.5 | 0.6 |
| | R2 | 0.2 | 0.3 | 0.2 | 0.3 | 1.2 | 1.1 | 1.4 | 1.2 | 1.4 | 0.9 | 1.2 | 0.8 | 0.9 |
| | R3 | 0.1 | 0.2 | 0.1 | 0.2 | 0.6 | 0.6 | 0.7 | 0.6 | 0.7 | 0.5 | 0.6 | 0.4 | 0.5 |
| Nocardioides | R1 | 0.2 | 0.1 | 0.1 | 0.2 | 0.5 | 0.6 | 0.5 | 0.4 | 0.5 | 0.4 | 0.3 | 0.3 | 0.4 |
| | R2 | 0.2 | 0.1 | 0.1 | 0.2 | 0.4 | 0.5 | 0.4 | 0.3 | 0.4 | 0.3 | 0.3 | 0.3 | 0.3 |
| | R3 | 0.2 | 0.1 | 0.1 | 0.2 | 0.6 | 0.7 | 0.6 | 0.4 | 0.6 | 0.4 | 0.3 | 0.3 | 0.4 |
| Novosphingobium | R1 | 0.1 | 0.2 | 0.1 | 0.2 | 0.5 | 0.4 | 0.6 | 0.5 | 0.4 | 0.4 | 0.5 | 0.6 | 0.4 |
| | R2 | 0.2 | 0.3 | 0.2 | 0.3 | 0.8 | 0.6 | 0.9 | 0.8 | 0.6 | 0.6 | 0.8 | 0.9 | 0.6 |
| | R3 | 0.1 | 0.2 | 0.1 | 0.2 | 0.4 | 0.3 | 0.5 | 0.4 | 0.3 | 0.3 | 0.4 | 0.5 | 0.3 |
| Ohtaekwangia | R1 | 0.4 | 0.5 | 0.4 | 0.3 | 0.2 | 0.1 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R2 | 0.3 | 0.3 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R3 | 0.4 | 0.6 | 0.4 | 0.3 | 0.2 | 0.1 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Opitutus | R1 | 0.5 | 0.4 | 0.2 | 0.3 | 0.2 | 0.2 | 0.1 | 0.2 | 0.2 | 0.3 | 0.1 | 0.2 | 0.2 |
| | R2 | 0.4 | 0.3 | 0.2 | 0.3 | 0.2 | 0.2 | 0.1 | 0.2 | 0.2 | 0.3 | 0.1 | 0.2 | 0.2 |
| | R3 | 0.6 | 0.4 | 0.2 | 0.3 | 0.2 | 0.2 | 0.1 | 0.2 | 0.2 | 0.3 | 0.1 | 0.2 | 0.2 |
| Paenibacillus | R1 | 0.1 | 0.2 | 0.2 | 0.2 | 0.6 | 0.8 | 0.9 | 1.0 | 1.2 | 1.0 | 1.2 | 1.0 | 1.2 |
| | R2 | 0.1 | 0.2 | 0.2 | 0.2 | 0.5 | 0.7 | 0.8 | 0.9 | 1.1 | 0.9 | 1.1 | 0.9 | 1.1 |
| | R3 | 0.1 | 0.2 | 0.2 | 0.2 | 0.7 | 1.0 | 1.1 | 1.2 | 1.4 | 1.2 | 1.4 | 1.2 | 1.4 |
| Paracoccus | R1 | 0.3 | 0.2 | 0.3 | 0.1 | 2.4 | 2.6 | 2.9 | 3.5 | 3.4 | 3.3 | 4.0 | 3.5 | 3.4 |
| | R2 | 0.5 | 0.3 | 0.5 | 0.2 | 3.6 | 3.9 | 4.4 | 5.3 | 5.1 | 5.0 | 6.0 | 5.3 | 5.1 |
| | R3 | 0.2 | 0.2 | 0.3 | 0.1 | 1.9 | 2.1 | 2.3 | 2.8 | 2.7 | 2.6 | 3.2 | 2.8 | 2.7 |
| Pedobacter | R1 | 0.2 | 0.1 | 0.3 | 0.4 | 0.5 | 0.4 | 0.3 | 0.4 | 0.5 | 0.2 | 0.3 | 0.2 | 0.4 |
| | R2 | 0.3 | 0.1 | 0.4 | 0.5 | 0.7 | 0.5 | 0.4 | 0.5 | 0.7 | 0.3 | 0.4 | 0.3 | 0.5 |
| | R3 | 0.2 | 0.1 | 0.2 | 0.3 | 0.4 | 0.3 | 0.2 | 0.3 | 0.4 | 0.2 | 0.2 | 0.2 | 0.3 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Phenylobacterium | R1 | 0.2 | 0.1 | 0.2 | 0.1 | 0.3 | 0.3 | 0.5 | 0.4 | 0.2 | 0.6 | 0.5 | 0.4 | 0.5 |
| | R2 | 0.3 | 0.1 | 0.3 | 0.1 | 0.4 | 0.4 | 0.7 | 0.5 | 0.3 | 0.8 | 0.7 | 0.5 | 0.7 |
| | R3 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.2 | 0.4 | 0.3 | 0.2 | 0.5 | 0.4 | 0.3 | 0.4 |
| Pirellula | R1 | 0.4 | 0.5 | 0.3 | 0.4 | 0.4 | 0.5 | 0.5 | 0.4 | 0.3 | 0.5 | 0.5 | 0.4 | 0.6 |
| | R2 | 0.3 | 0.4 | 0.3 | 0.3 | 0.3 | 0.4 | 0.4 | 0.3 | 0.3 | 0.4 | 0.4 | 0.3 | 0.5 |
| | R3 | 0.4 | 0.6 | 0.3 | 0.4 | 0.4 | 0.6 | 0.6 | 0.4 | 0.3 | 0.6 | 0.6 | 0.4 | 0.7 |
| Pontibacter | R1 | 0.5 | 0.4 | 0.3 | 0.3 | 0.3 | 0.2 | 0.3 | 0.2 | 0.1 | 0.2 | 0.2 | 0.2 | 0.1 |
| | R2 | 0.4 | 0.4 | 0.3 | 0.3 | 0.3 | 0.2 | 0.3 | 0.2 | 0.1 | 0.2 | 0.2 | 0.2 | 0.1 |
| | R3 | 0.6 | 0.5 | 0.4 | 0.4 | 0.4 | 0.2 | 0.4 | 0.2 | 0.1 | 0.2 | 0.2 | 0.2 | 0.1 |
| Pseudomonas | R1 | 0.3 | 0.6 | 0.5 | 0.4 | 4.5 | 5.0 | 5.6 | 5.6 | 6.0 | 6.8 | 6.0 | 7.4 | 6.1 |
| | R2 | 0.3 | 0.5 | 0.4 | 0.3 | 3.8 | 4.2 | 4.7 | 4.7 | 5.1 | 5.7 | 5.1 | 6.3 | 5.2 |
| | R3 | 0.3 | 0.7 | 0.6 | 0.4 | 5.0 | 5.6 | 6.3 | 6.3 | 6.7 | 7.6 | 6.7 | 8.3 | 6.8 |
| Pseudoxanthomonas | R1 | 0.4 | 0.5 | 0.5 | 0.6 | 0.8 | 0.9 | 1.0 | 0.8 | 0.9 | 0.8 | 0.9 | 1.0 | 0.8 |
| | R2 | 0.3 | 0.3 | 0.3 | 0.4 | 0.6 | 0.6 | 0.7 | 0.6 | 0.6 | 0.6 | 0.6 | 0.7 | 0.6 |
| | R3 | 0.4 | 0.6 | 0.6 | 0.7 | 0.9 | 1.0 | 1.1 | 0.9 | 1.0 | 0.9 | 1.0 | 1.1 | 0.9 |
| Ramlibacter | R1 | 0.4 | 0.5 | 0.4 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R2 | 0.5 | 0.7 | 0.5 | 0.4 | 0.3 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R3 | 0.3 | 0.4 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Rhodococcus | R1 | 0.2 | 0.3 | 0.2 | 0.1 | 2.5 | 2.0 | 2.6 | 2.2 | 3.0 | 2.4 | 2.3 | 2.4 | 2.6 |
| | R2 | 0.3 | 0.5 | 0.3 | 0.2 | 3.8 | 3.0 | 3.9 | 3.3 | 4.5 | 3.6 | 3.5 | 3.6 | 3.9 |
| | R3 | 0.2 | 0.2 | 0.2 | 0.1 | 2.0 | 1.6 | 2.1 | 1.8 | 2.4 | 1.9 | 1.8 | 1.9 | 2.1 |
| Saccharibacteria | R1 | 0.4 | 0.5 | 0.2 | 0.4 | 0.2 | 0.4 | 0.5 | 0.4 | 0.3 | 0.4 | 0.4 | 0.6 | 0.4 |
| | R2 | 0.6 | 0.8 | 0.3 | 0.6 | 0.3 | 0.6 | 0.8 | 0.6 | 0.5 | 0.6 | 0.6 | 0.9 | 0.6 |
| | R3 | 0.3 | 0.4 | 0.2 | 0.3 | 0.2 | 0.3 | 0.4 | 0.3 | 0.2 | 0.3 | 0.3 | 0.5 | 0.3 |
| Salinibacillus | R1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.4 | 0.6 | 0.8 | 0.9 | 1.0 | 1.0 | 0.8 | 0.8 | 0.8 |
| | R2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.6 | 0.9 | 1.2 | 1.4 | 1.5 | 1.5 | 1.2 | 1.2 | 1.2 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R3 | 0.1 | 0.1 | 0.1 | 0.1 | 0.3 | 0.5 | 0.6 | 0.7 | 0.8 | 0.8 | 0.6 | 0.6 | 0.6 |
| Salinimicrobium | R1 | 0.1 | 0.2 | 0.1 | 0.1 | 0.4 | 0.5 | 0.5 | 0.5 | 0.4 | 0.5 | 0.5 | 0.5 | 0.4 |
| | R2 | 0.1 | 0.2 | 0.1 | 0.1 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 |
| | R3 | 0.1 | 0.2 | 0.1 | 0.1 | 0.5 | 0.6 | 0.6 | 0.6 | 0.5 | 0.6 | 0.6 | 0.6 | 0.5 |
| Salinisphaera | R1 | 0.1 | 0.1 | 0.0 | 0.0 | 0.4 | 0.5 | 0.6 | 0.4 | 0.5 | 0.4 | 0.9 | 0.9 | 0.8 |
| | R2 | 0.1 | 0.1 | 0.0 | 0.0 | 0.5 | 0.7 | 0.8 | 0.5 | 0.7 | 0.5 | 1.2 | 1.2 | 1.0 |
| | R3 | 0.1 | 0.1 | 0.0 | 0.0 | 0.3 | 0.4 | 0.5 | 0.3 | 0.4 | 0.3 | 0.7 | 0.7 | 0.6 |
| Sediminibacter | R1 | 0.4 | 0.5 | 0.6 | 0.8 | 0.7 | 0.9 | 0.4 | 0.9 | 0.9 | 0.9 | 0.7 | 0.9 | 0.9 |
| | R2 | 0.3 | 0.4 | 0.5 | 0.7 | 0.6 | 0.8 | 0.3 | 0.8 | 0.8 | 0.8 | 0.6 | 0.8 | 0.8 |
| | R3 | 0.4 | 0.6 | 0.7 | 0.9 | 0.8 | 1.0 | 0.4 | 1.0 | 1.0 | 1.0 | 0.8 | 1.0 | 1.0 |
| Sphaerobacter | R1 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.8 | 0.9 | 0.9 | 0.9 | 0.9 | 0.7 | 0.8 | 0.9 |
| | R2 | 0.2 | 0.2 | 0.0 | 0.0 | 0.0 | 1.2 | 1.4 | 1.4 | 1.4 | 1.4 | 1.1 | 1.2 | 1.4 |
| | R3 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.6 | 0.7 | 0.7 | 0.7 | 0.7 | 0.6 | 0.6 | 0.7 |
| Sphingomonas | R1 | 0.2 | 0.2 | 0.1 | 0.2 | 2.3 | 3.0 | 2.6 | 2.6 | 2.4 | 2.8 | 3.0 | 2.4 | 2.3 |
| | R2 | 0.1 | 0.1 | 0.1 | 0.1 | 1.6 | 2.1 | 1.8 | 1.8 | 1.7 | 2.0 | 2.1 | 1.7 | 1.6 |
| | R3 | 0.2 | 0.2 | 0.1 | 0.2 | 2.6 | 3.4 | 2.9 | 2.9 | 2.7 | 3.1 | 3.4 | 2.7 | 2.6 |
| Sphingopyxis | R1 | 0.2 | 0.2 | 0.1 | 0.1 | 0.4 | 0.5 | 0.4 | 0.3 | 0.4 | 0.5 | 0.5 | 0.9 | 0.4 |
| | R2 | 0.2 | 0.2 | 0.1 | 0.1 | 0.3 | 0.4 | 0.3 | 0.3 | 0.3 | 0.4 | 0.4 | 0.8 | 0.3 |
| | R3 | 0.2 | 0.2 | 0.1 | 0.1 | 0.4 | 0.6 | 0.4 | 0.3 | 0.4 | 0.6 | 0.6 | 1.0 | 0.4 |
| Sporosarcina | R1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 | 0.4 | 0.4 | 0.5 | 0.4 | 0.4 |
| | R2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.3 | 0.5 | 0.6 | 0.8 | 0.6 | 0.6 | 0.8 | 0.6 | 0.6 |
| | R3 | 0.1 | 0.1 | 0.1 | 0.1 | 0.2 | 0.2 | 0.3 | 0.4 | 0.3 | 0.3 | 0.4 | 0.3 | 0.3 |
| Terrimonas | R1 | 0.2 | 0.1 | 0.2 | 0.1 | 0.5 | 0.5 | 0.4 | 0.3 | 0.2 | 0.2 | 0.2 | 0.4 | 0.2 |
| | R2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.3 | 0.3 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.3 | 0.1 |
| | R3 | 0.2 | 0.1 | 0.2 | 0.1 | 0.6 | 0.6 | 0.4 | 0.3 | 0.2 | 0.2 | 0.2 | 0.4 | 0.2 |
| Thiobacillus | R1 | 0.2 | 0.2 | 0.2 | 0.1 | 2.3 | 2.4 | 2.8 | 2.4 | 3.0 | 2.6 | 2.4 | 2.6 | 2.8 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R2 | 0.3 | 0.3 | 0.3 | 0.1 | 3.0 | 3.1 | 3.6 | 3.1 | 3.9 | 3.4 | 3.1 | 3.4 | 3.6 |
| | R3 | 0.2 | 0.2 | 0.2 | 0.1 | 1.8 | 1.9 | 2.2 | 1.9 | 2.3 | 2.0 | 1.9 | 2.0 | 2.2 |
| Truepera | R1 | 0.1 | 0.2 | 0.1 | 0.2 | 0.5 | 0.8 | 0.9 | 0.8 | 1.0 | 1.2 | 1.4 | 1.4 | 1.6 |
| | R2 | 0.2 | 0.3 | 0.2 | 0.3 | 0.8 | 1.2 | 1.4 | 1.2 | 1.5 | 1.8 | 2.1 | 2.1 | 2.4 |
| | R3 | 0.1 | 0.2 | 0.1 | 0.2 | 0.4 | 0.6 | 0.7 | 0.6 | 0.8 | 1.0 | 1.1 | 1.1 | 1.3 |

| | | Cultivated soil; Rhizosphere | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Common bean | | | | | | | |
| | | Relative abundance (%) | | | | | | | |
| Genus | Replicate | NT1 | NT4 | UR1 | UR4 | AS1 | AS4 | PN1 | PN4 |
| Aciditerrimonas | R1 | 0.1 | 0.1 | 0.4 | 0.4 | 0.1 | 0.6 | 0.7 | 0.6 |
| | R2 | 0.1 | 0.1 | 0.5 | 0.4 | 0.4 | 0.6 | 0.8 | 0.7 |
| | R3 | 0.1 | 0.1 | 0.5 | 0.5 | 0.5 | 0.6 | 0.9 | 0.7 |
| Acidovorax | R1 | 0.1 | 0.1 | 0.6 | 0.6 | 0.8 | 0.7 | 1.0 | 0.9 |
| | R2 | 0.1 | 0.1 | 0.6 | 0.6 | 0.7 | 0.7 | 0.8 | 0.8 |
| | R3 | 0.1 | 0.1 | 0.8 | 0.7 | 0.8 | 0.9 | 0.8 | 0.8 |
| Adhaeribacter | R1 | 0.2 | 0.1 | 0.7 | 0.6 | 0.6 | 0.8 | 0.5 | 0.7 |
| | R2 | 0.2 | 0.1 | 0.6 | 0.5 | 0.6 | 0.6 | 0.4 | 0.5 |
| | R3 | 0.2 | 0.1 | 0.3 | 0.3 | 0.4 | 0.4 | 0.3 | 0.4 |
| Allokutzneria | R1 | 0.2 | 0.1 | 0.2 | 0.2 | 0.4 | 0.2 | 0.3 | 0.3 |
| | R2 | 0.3 | 0.2 | 0.2 | 0.2 | 0.3 | 0.2 | 0.2 | 0.2 |
| | R3 | 0.4 | 0.4 | 0.2 | 0.2 | 0.2 | 0.1 | 0.2 | 0.2 |
| Amaricoccus | R1 | 0.4 | 0.5 | 0.2 | 0.1 | 0.1 | 0.1 | 0.2 | 0.2 |
| | R2 | 0.4 | 0.4 | 0.2 | 0.2 | 0.1 | 0.2 | 0.3 | 0.2 |
| | R3 | 0.4 | 0.3 | 0.3 | 0.2 | 0.1 | 0.3 | 0.4 | 0.3 |
| Arenimonas | R1 | 0.4 | 0.2 | 0.3 | 0.3 | 0.1 | 0.3 | 0.4 | 0.3 |
| | R2 | 0.4 | 0.3 | 0.4 | 0.4 | 0.3 | 0.3 | 0.5 | 0.4 |
| | R3 | 0.4 | 0.3 | 0.5 | 0.4 | 0.4 | 0.3 | 0.4 | 0.3 |
| Arthrobacter | R1 | 0.4 | 0.4 | 0.7 | 0.6 | 0.5 | 0.4 | 0.5 | 0.4 |
| | R2 | 0.3 | 0.3 | 0.6 | 0.5 | 0.6 | 0.5 | 0.4 | 0.3 |
| | R3 | 0.3 | 0.2 | 0.5 | 0.5 | 0.7 | 0.7 | 0.3 | 0.3 |
| Azotobacter | R1 | 0.1 | 0.1 | 0.4 | 0.3 | 0.7 | 0.7 | 0.2 | 0.1 |
| | R2 | 0.2 | 0.2 | 2.4 | 2.1 | 1.9 | 2.5 | 2.1 | 1.9 |
| | R3 | 0.3 | 0.3 | 5.0 | 4.3 | 3.4 | 5.0 | 4.6 | 4.2 |
| Bacillus | R1 | 0.3 | 0.4 | 6.4 | 5.5 | 4.2 | 6.2 | 5.9 | 5.4 |
| | R2 | 0.4 | 0.4 | 5.9 | 5.1 | 4.4 | 6.0 | 6.0 | 5.4 |
| | R3 | 0.3 | 0.3 | 4.2 | 3.7 | 3.6 | 4.6 | 4.9 | 4.3 |
| Bradyrhizobium | R1 | 0.4 | 0.3 | 4.6 | 3.9 | 4.4 | 5.2 | 5.8 | 5.0 |
| | R2 | 0.3 | 0.2 | 3.0 | 2.6 | 2.9 | 3.5 | 3.8 | 3.3 |
| | R3 | 0.2 | 0.1 | 1.9 | 1.6 | 1.9 | 2.3 | 2.4 | 2.1 |
| Brevundimonas | R1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.3 | 0.3 | 0.2 | 0.1 |
| | R2 | 0.1 | 0.1 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.1 |
| | R3 | 0.1 | 0.1 | 0.3 | 0.3 | 0.3 | 0.2 | 0.2 | 0.2 |
| Caulobacter | R1 | 0.1 | 0.1 | 0.4 | 0.3 | 0.2 | 0.1 | 0.2 | 0.2 |
| | R2 | 0.1 | 0.1 | 0.3 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 |
| | R3 | 0.1 | 0.2 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 |
| Chryseobacterium | R1 | 0.1 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 |

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|---|---|---|---|---|---|---|---|---|---|
| | R2 | 0.1 | 0.2 | 0.9 | 0.8 | 0.7 | 0.9 | 0.8 | 0.7 |
| | R3 | 0.2 | 0.2 | 1.6 | 1.4 | 1.2 | 1.6 | 1.5 | 1.3 |
| Comamonas | R1 | 0.3 | 0.1 | 2.5 | 2.1 | 1.9 | 2.6 | 2.4 | 2.1 |
| | R2 | 0.3 | 0.2 | 2.6 | 2.3 | 1.9 | 2.6 | 2.5 | 2.1 |
| | R3 | 0.3 | 0.3 | 2.6 | 2.3 | 1.9 | 2.5 | 2.5 | 2.0 |
| Cupriavidus | R1 | 0.4 | 0.3 | 2.8 | 2.4 | 1.9 | 2.5 | 2.7 | 2.1 |
| | R2 | 0.3 | 0.3 | 1.9 | 1.7 | 1.3 | 1.8 | 1.8 | 1.5 |
| | R3 | 0.2 | 0.2 | 1.3 | 1.1 | 0.9 | 1.3 | 1.2 | 1.0 |
| Devosia | R1 | 0.1 | 0.1 | 0.2 | 0.2 | 0.1 | 0.4 | 0.2 | 0.3 |
| | R2 | 0.2 | 0.3 | 0.8 | 0.7 | 0.7 | 0.9 | 1.2 | 0.9 |
| | R3 | 0.3 | 0.4 | 1.3 | 1.1 | 1.0 | 1.3 | 1.9 | 1.3 |
| Ensifer | R1 | 0.4 | 0.6 | 2.0 | 1.8 | 1.7 | 1.9 | 3.1 | 2.1 |
| | R2 | 0.3 | 0.5 | 1.6 | 1.4 | 1.4 | 1.5 | 2.4 | 1.7 |
| | R3 | 0.3 | 0.3 | 1.2 | 1.0 | 1.2 | 1.3 | 1.9 | 1.4 |
| Flavisolibacter | R1 | 0.3 | 0.1 | 0.6 | 0.5 | 0.9 | 0.9 | 1.0 | 1.0 |
| | R2 | 0.3 | 0.2 | 0.5 | 0.4 | 0.6 | 0.7 | 0.7 | 0.7 |
| | R3 | 0.3 | 0.2 | 0.4 | 0.4 | 0.4 | 0.5 | 0.5 | 0.5 |
| Flavitalea | R1 | 0.4 | 0.2 | 0.3 | 0.2 | 0.1 | 0.2 | 0.1 | 0.1 |
| | R2 | 0.4 | 0.2 | 1.5 | 1.3 | 1.6 | 1.8 | 2.1 | 1.7 |
| | R3 | 0.4 | 0.2 | 3.1 | 2.7 | 3.6 | 3.9 | 4.8 | 3.9 |
| Flavobacterium | R1 | 0.4 | 0.1 | 4.0 | 3.5 | 4.7 | 5.1 | 6.4 | 5.2 |
| | R2 | 0.3 | 0.1 | 2.9 | 2.5 | 3.5 | 3.8 | 4.7 | 3.8 |
| | R3 | 0.2 | 0.1 | 1.5 | 1.3 | 2.0 | 2.1 | 2.4 | 2.0 |
| Fontibacillus | R1 | 0.1 | 0.1 | 0.6 | 0.6 | 1.0 | 1.1 | 1.0 | 0.9 |
| | R2 | 0.1 | 0.1 | 0.7 | 0.6 | 1.1 | 1.1 | 1.2 | 1.0 |
| | R3 | 0.1 | 0.1 | 0.6 | 0.5 | 0.9 | 0.9 | 1.1 | 1.0 |
| Gemmatimonas | R1 | 0.1 | 0.1 | 0.8 | 0.7 | 1.1 | 1.0 | 1.4 | 1.2 |
| | R2 | 0.1 | 0.1 | 0.5 | 0.4 | 0.8 | 0.8 | 1.1 | 0.9 |
| | R3 | 0.1 | 0.1 | 0.3 | 0.3 | 0.6 | 0.7 | 1.0 | 0.8 |
| Gp10 | R1 | 0.1 | 0.1 | 0.0 | 0.0 | 0.3 | 0.5 | 0.7 | 0.4 |
| | R2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.3 | 0.5 | 0.8 | 0.5 |
| | R3 | 0.1 | 0.1 | 0.2 | 0.2 | 0.3 | 0.5 | 0.8 | 0.6 |
| Gp4 | R1 | 0.0 | 0.1 | 0.4 | 0.3 | 0.4 | 0.5 | 0.9 | 0.7 |
| | R2 | 0.0 | 0.1 | 0.4 | 0.4 | 0.4 | 0.6 | 0.8 | 0.6 |
| | R3 | 0.1 | 0.1 | 0.5 | 0.4 | 0.4 | 0.6 | 0.7 | 0.6 |
| Gp6 | R1 | 0.1 | 0.0 | 0.6 | 0.5 | 0.5 | 0.7 | 0.5 | 0.4 |
| | R2 | 0.1 | 0.0 | 0.6 | 0.5 | 0.4 | 0.6 | 0.5 | 0.4 |
| | R3 | 0.1 | 0.1 | 0.6 | 0.5 | 0.4 | 0.6 | 0.6 | 0.4 |
| Gp7 | R1 | 0.1 | 0.1 | 0.6 | 0.5 | 0.3 | 0.4 | 0.6 | 0.4 |
| | R2 | 0.1 | 0.1 | 0.6 | 0.5 | 0.3 | 0.5 | 0.7 | 0.5 |
| | R3 | 0.1 | 0.2 | 0.7 | 0.6 | 0.3 | 0.7 | 0.9 | 0.7 |

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|---|---|---|---|---|---|---|---|---|---|
| Halomonas | R1 | 0.1 | 0.1 | 0.6 | 0.5 | 0.3 | 0.7 | 0.8 | 0.7 |
| | R2 | 0.1 | 0.2 | 1.7 | 1.5 | 1.5 | 2.2 | 2.4 | 1.9 |
| | R3 | 0.1 | 0.3 | 3.2 | 2.8 | 3.0 | 4.1 | 4.5 | 3.4 |
| Hyphomicrobium | R1 | 0.1 | 0.4 | 4.1 | 3.5 | 4.0 | 5.3 | 5.8 | 4.4 |
| | R2 | 0.1 | 0.3 | 3.0 | 2.5 | 2.8 | 3.6 | 4.1 | 3.1 |
| | R3 | 0.0 | 0.1 | 1.4 | 1.2 | 1.2 | 1.5 | 1.8 | 1.3 |
| Ilumatobacter | R1 | 0.0 | 0.1 | 0.6 | 0.5 | 0.3 | 0.2 | 0.5 | 0.3 |
| | R2 | 0.0 | 0.1 | 0.6 | 0.5 | 0.2 | 0.2 | 0.4 | 0.3 |
| | R3 | 0.1 | 0.1 | 0.7 | 0.6 | 0.3 | 0.3 | 0.3 | 0.2 |
| Litorilinea | R1 | 0.1 | 0.1 | 0.6 | 0.5 | 0.2 | 0.2 | 0.1 | 0.1 |
| | R2 | 0.1 | 0.1 | 0.5 | 0.4 | 0.2 | 0.3 | 0.2 | 0.1 |
| | R3 | 0.0 | 0.1 | 0.4 | 0.4 | 0.3 | 0.4 | 0.3 | 0.2 |
| Luteimonas | R1 | 0.0 | 0.1 | 0.4 | 0.3 | 0.3 | 0.4 | 0.3 | 0.2 |
| | R2 | 0.0 | 0.1 | 0.4 | 0.3 | 0.2 | 0.4 | 0.3 | 0.2 |
| | R3 | 0.0 | 0.1 | 0.3 | 0.3 | 0.1 | 0.2 | 0.3 | 0.2 |
| Lysobacter | R1 | 0.1 | 0.1 | 0.4 | 0.3 | 0.1 | 0.2 | 0.3 | 0.2 |
| | R2 | 0.1 | 0.1 | 0.4 | 0.3 | 0.1 | 0.2 | 0.3 | 0.2 |
| | R3 | 0.1 | 0.1 | 0.3 | 0.3 | 0.1 | 0.2 | 0.2 | 0.1 |
| Marmoricola | R1 | 0.1 | 0.1 | 0.3 | 0.3 | 0.1 | 0.2 | 0.1 | 0.1 |
| | R2 | 0.1 | 0.0 | 0.3 | 0.2 | 0.1 | 0.2 | 0.1 | 0.1 |
| | R3 | 0.1 | 0.0 | 0.3 | 0.2 | 0.1 | 0.2 | 0.1 | 0.1 |
| Massilia | R1 | 0.1 | 0.0 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R2 | 0.2 | 0.1 | 0.3 | 0.2 | 0.3 | 0.2 | 0.2 | 0.2 |
| | R3 | 0.2 | 0.2 | 0.3 | 0.3 | 0.5 | 0.3 | 0.3 | 0.2 |
| Mesorhizobium | R1 | 0.3 | 0.3 | 0.4 | 0.3 | 0.7 | 0.3 | 0.4 | 0.3 |
| | R2 | 0.3 | 0.2 | 0.4 | 0.3 | 0.6 | 0.4 | 0.5 | 0.4 |
| | R3 | 0.2 | 0.2 | 0.4 | 0.4 | 0.5 | 0.6 | 0.5 | 0.4 |
| Methylohalomonas | R1 | 0.1 | 0.1 | 0.4 | 0.4 | 0.4 | 0.7 | 0.6 | 0.5 |
| | R2 | 0.1 | 0.1 | 0.4 | 0.4 | 0.4 | 0.7 | 0.6 | 0.5 |
| | R3 | 0.1 | 0.1 | 0.4 | 0.4 | 0.4 | 0.7 | 0.6 | 0.5 |
| Microbacterium | R1 | 0.1 | 0.1 | 0.4 | 0.4 | 0.4 | 0.7 | 0.6 | 0.5 |
| | R2 | 0.2 | 0.1 | 0.3 | 0.3 | 0.3 | 0.5 | 0.4 | 0.4 |
| | R3 | 0.3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.3 | 0.3 | 0.3 |
| Microvirga | R1 | 0.3 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.2 | 0.1 |
| | R2 | 0.2 | 0.1 | 0.5 | 0.4 | 0.8 | 0.6 | 0.9 | 0.8 |
| | R3 | 0.2 | 0.1 | 0.8 | 0.7 | 1.4 | 1.0 | 1.4 | 1.3 |
| Mycobacterium | R1 | 0.1 | 0.1 | 1.3 | 1.1 | 2.2 | 1.5 | 2.3 | 2.0 |
| | R2 | 0.1 | 0.1 | 1.0 | 0.8 | 1.6 | 1.2 | 1.6 | 1.5 |
| | R3 | 0.1 | 0.1 | 0.7 | 0.6 | 1.0 | 1.0 | 1.1 | 1.0 |
| Nitriliruptor | R1 | 0.1 | 0.1 | 0.4 | 0.3 | 0.3 | 0.7 | 0.4 | 0.5 |
| | R2 | 0.1 | 0.1 | 0.6 | 0.5 | 0.9 | 1.3 | 1.2 | 0.9 |

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|---|---|---|---|---|---|---|---|---|---|
| | R3 | 0.1 | 0.2 | 1.0 | 0.8 | 1.8 | 2.3 | 2.5 | 1.8 |
| Nitrosococcus | R1 | 0.2 | 0.2 | 1.1 | 0.9 | 2.2 | 2.7 | 3.0 | 2.1 |
| | R2 | 0.2 | 0.2 | 1.5 | 1.3 | 2.0 | 2.5 | 2.2 | 1.6 |
| | R3 | 0.1 | 0.1 | 1.6 | 1.4 | 1.5 | 1.7 | 0.9 | 0.7 |
| Nitrosomonas | R1 | 0.1 | 0.1 | 2.1 | 1.8 | 1.6 | 1.7 | 0.2 | 0.3 |
| | R2 | 0.1 | 0.1 | 2.0 | 1.7 | 1.6 | 1.7 | 1.1 | 1.0 |
| | R3 | 0.1 | 0.1 | 1.9 | 1.6 | 1.5 | 1.7 | 1.7 | 1.5 |
| Nitrosospira | R1 | 0.1 | 0.2 | 1.8 | 1.6 | 1.5 | 1.7 | 2.8 | 2.4 |
| | R2 | 0.1 | 0.2 | 1.4 | 1.2 | 1.2 | 1.3 | 2.1 | 1.7 |
| | R3 | 0.1 | 0.2 | 1.2 | 1.1 | 1.1 | 1.1 | 1.7 | 1.3 |
| Nitrosovibrio | R1 | 0.1 | 0.2 | 0.6 | 0.5 | 0.6 | 0.5 | 0.7 | 0.5 |
| | R2 | 0.1 | 0.2 | 0.6 | 0.5 | 0.5 | 0.4 | 0.6 | 0.4 |
| | R3 | 0.1 | 0.1 | 0.4 | 0.3 | 0.3 | 0.3 | 0.3 | 0.2 |
| Nocardioides | R1 | 0.1 | 0.1 | 0.4 | 0.3 | 0.3 | 0.3 | 0.2 | 0.2 |
| | R2 | 0.1 | 0.1 | 0.4 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| | R3 | 0.1 | 0.2 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 |
| Novosphingobium | R1 | 0.1 | 0.2 | 0.4 | 0.3 | 0.4 | 0.3 | 0.4 | 0.4 |
| | R2 | 0.2 | 0.2 | 0.3 | 0.3 | 0.3 | 0.2 | 0.3 | 0.3 |
| | R3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.1 | 0.1 | 0.2 | 0.1 |
| Ohtaekwangia | R1 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R2 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R3 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.2 | 0.1 | 0.1 |
| Opitutus | R1 | 0.4 | 0.2 | 0.1 | 0.1 | 0.1 | 0.2 | 0.1 | 0.1 |
| | R2 | 0.3 | 0.2 | 0.2 | 0.2 | 0.3 | 0.4 | 0.4 | 0.3 |
| | R3 | 0.2 | 0.2 | 0.3 | 0.3 | 0.5 | 0.5 | 0.6 | 0.5 |
| Paenibacillus | R1 | 0.1 | 0.1 | 0.4 | 0.4 | 0.7 | 0.8 | 1.0 | 0.8 |
| | R2 | 0.1 | 0.1 | 0.9 | 0.7 | 1.2 | 1.3 | 1.7 | 1.4 |
| | R3 | 0.2 | 0.1 | 1.6 | 1.4 | 2.2 | 2.3 | 3.0 | 2.4 |
| Paracoccus | R1 | 0.2 | 0.1 | 1.9 | 1.6 | 2.5 | 2.7 | 3.5 | 2.8 |
| | R2 | 0.2 | 0.1 | 1.4 | 1.2 | 1.8 | 1.9 | 2.5 | 2.0 |
| | R3 | 0.2 | 0.2 | 0.7 | 0.6 | 0.8 | 0.8 | 1.0 | 0.8 |
| Pedobacter | R1 | 0.1 | 0.3 | 0.4 | 0.3 | 0.3 | 0.2 | 0.2 | 0.2 |
| | R2 | 0.1 | 0.2 | 0.3 | 0.3 | 0.3 | 0.2 | 0.3 | 0.2 |
| | R3 | 0.1 | 0.1 | 0.3 | 0.2 | 0.3 | 0.4 | 0.4 | 0.3 |
| Phenylobacterium | R1 | 0.1 | 0.1 | 0.2 | 0.2 | 0.3 | 0.5 | 0.4 | 0.3 |
| | R2 | 0.2 | 0.1 | 0.2 | 0.2 | 0.3 | 0.4 | 0.4 | 0.3 |
| | R3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.3 | 0.3 | 0.3 |
| Pirellula | R1 | 0.3 | 0.3 | 0.3 | 0.2 | 0.3 | 0.4 | 0.4 | 0.3 |
| | R2 | 0.3 | 0.2 | 0.3 | 0.2 | 0.2 | 0.3 | 0.3 | 0.2 |
| | R3 | 0.3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Pontibacter | R1 | 0.4 | 0.2 | 0.2 | 0.2 | 0.1 | 0.2 | 0.2 | 0.1 |

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|---|---|---|---|---|---|---|---|---|---|
| | R2 | 0.3 | 0.2 | 1.2 | 1.0 | 1.3 | 1.8 | 1.7 | 1.6 |
| | R3 | 0.3 | 0.3 | 2.1 | 1.8 | 2.3 | 3.1 | 3.0 | 2.8 |
| Pseudomonas | R1 | 0.2 | 0.3 | 3.2 | 2.7 | 3.6 | 4.9 | 4.7 | 4.4 |
| | R2 | 0.2 | 0.3 | 2.3 | 2.0 | 2.6 | 3.5 | 3.4 | 3.2 |
| | R3 | 0.2 | 0.3 | 1.5 | 1.3 | 1.7 | 2.2 | 2.2 | 2.0 |
| Pseudoxanthomonas | R1 | 0.3 | 0.4 | 0.5 | 0.5 | 0.5 | 0.6 | 0.7 | 0.6 |
| | R2 | 0.3 | 0.3 | 0.4 | 0.3 | 0.3 | 0.4 | 0.5 | 0.4 |
| | R3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.2 | 0.3 | 0.3 | 0.3 |
| Ramlibacter | R1 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R2 | 0.2 | 0.2 | 0.7 | 0.6 | 0.5 | 0.6 | 0.7 | 0.6 |
| | R3 | 0.2 | 0.1 | 1.5 | 1.3 | 1.2 | 1.5 | 1.5 | 1.3 |
| Rhodococcus | R1 | 0.2 | 0.1 | 2.0 | 1.7 | 1.6 | 1.9 | 2.0 | 1.7 |
| | R2 | 0.2 | 0.1 | 1.4 | 1.2 | 1.2 | 1.4 | 1.5 | 1.3 |
| | R3 | 0.3 | 0.2 | 0.6 | 0.5 | 0.6 | 0.7 | 0.7 | 0.7 |
| Saccharibacteria | R1 | 0.3 | 0.3 | 0.2 | 0.1 | 0.3 | 0.3 | 0.3 | 0.4 |
| | R2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.4 | 0.5 | 0.5 | 0.4 |
| | R3 | 0.1 | 0.1 | 0.3 | 0.2 | 0.6 | 0.7 | 0.6 | 0.5 |
| Salinibacillus | R1 | 0.1 | 0.1 | 0.3 | 0.3 | 0.6 | 0.8 | 0.7 | 0.6 |
| | R2 | 0.1 | 0.1 | 0.3 | 0.3 | 0.6 | 0.7 | 0.6 | 0.5 |
| | R3 | 0.1 | 0.1 | 0.3 | 0.2 | 0.4 | 0.4 | 0.4 | 0.4 |
| Salinimicrobium | R1 | 0.1 | 0.1 | 0.3 | 0.3 | 0.3 | 0.4 | 0.4 | 0.3 |
| | R2 | 0.1 | 0.0 | 0.3 | 0.3 | 0.3 | 0.4 | 0.5 | 0.4 |
| | R3 | 0.1 | 0.0 | 0.3 | 0.3 | 0.3 | 0.4 | 0.7 | 0.6 |
| Salinisphaera | R1 | 0.1 | 0.0 | 0.3 | 0.3 | 0.3 | 0.3 | 0.7 | 0.6 |
| | R2 | 0.1 | 0.2 | 0.4 | 0.3 | 0.4 | 0.4 | 0.7 | 0.6 |
| | R3 | 0.2 | 0.3 | 0.4 | 0.3 | 0.4 | 0.5 | 0.5 | 0.5 |
| Sediminibacter | R1 | 0.3 | 0.5 | 0.5 | 0.4 | 0.6 | 0.7 | 0.5 | 0.5 |
| | R2 | 0.2 | 0.4 | 0.3 | 0.3 | 0.6 | 0.7 | 0.5 | 0.5 |
| | R3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.7 | 0.8 | 0.7 | 0.6 |
| Sphaerobacter | R1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.6 | 0.7 | 0.6 | 0.6 |
| | R2 | 0.1 | 0.0 | 0.5 | 0.5 | 1.0 | 1.2 | 1.2 | 1.0 |
| | R3 | 0.1 | 0.1 | 0.9 | 0.8 | 1.1 | 1.3 | 1.5 | 1.2 |
| Sphingomonas | R1 | 0.1 | 0.1 | 1.5 | 1.3 | 1.6 | 1.9 | 2.2 | 1.7 |
| | R2 | 0.1 | 0.1 | 1.1 | 0.9 | 1.1 | 1.4 | 1.6 | 1.3 |
| | R3 | 0.1 | 0.1 | 0.8 | 0.7 | 0.8 | 1.0 | 1.1 | 1.0 |
| Sphingopyxis | R1 | 0.1 | 0.1 | 0.3 | 0.2 | 0.2 | 0.4 | 0.4 | 0.5 |
| | R2 | 0.1 | 0.1 | 0.2 | 0.2 | 0.2 | 0.3 | 0.4 | 0.4 |
| | R3 | 0.1 | 0.1 | 0.2 | 0.2 | 0.3 | 0.4 | 0.5 | 0.4 |
| Sporosarcina | R1 | 0.1 | 0.1 | 0.2 | 0.1 | 0.4 | 0.3 | 0.4 | 0.3 |
| | R2 | 0.1 | 0.1 | 0.2 | 0.2 | 0.3 | 0.3 | 0.4 | 0.3 |
| | R3 | 0.1 | 0.1 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |

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|---|---|---|---|---|---|---|---|---|---|
| Terrimonas | R1 | 0.1 | 0.1 | 0.3 | 0.3 | 0.2 | 0.1 | 0.1 | 0.2 |
| | R2 | 0.1 | 0.1 | 0.8 | 0.7 | 0.6 | 0.7 | 0.7 | 0.7 |
| | R3 | 0.2 | 0.1 | 1.4 | 1.2 | 1.3 | 1.5 | 1.5 | 1.4 |
| Thiobacillus | R1 | 0.1 | 0.1 | 1.7 | 1.4 | 1.6 | 2.0 | 2.0 | 1.7 |
| | R2 | 0.1 | 0.1 | 1.3 | 1.1 | 1.3 | 1.6 | 1.7 | 1.5 |
| | R3 | 0.1 | 0.1 | 0.7 | 0.6 | 0.8 | 1.2 | 1.4 | 1.2 |
| Truepera | R1 | 0.1 | 0.2 | 0.4 | 0.3 | 0.6 | 1.0 | 1.2 | 1.0 |
| | R2 | 0.1 | 0.2 | 0.4 | 0.4 | 0.6 | 1.0 | 1.3 | 1.1 |
| | R3 | 0.1 | 0.1 | 0.3 | 0.2 | 0.4 | 0.7 | 0.9 | 0.8 |

| | | Cultivated soil; Bulk | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Common bean | | | | | | | |
| | | Relative abundance (%) | | | | | | | |
| Genus | Replicate | NT1 | NT4 | UR1 | UR4 | AS1 | AS4 | PN1 | PN4 |
| Aciditerrimonas | R1 | 0.2 | 0.1 | 0.5 | 0.4 | 0.2 | 0.7 | 0.8 | 0.7 |
| | R2 | 0.2 | 0.1 | 0.6 | 0.5 | 0.4 | 0.7 | 0.9 | 0.9 |
| | R3 | 0.1 | 0.1 | 0.6 | 0.5 | 0.6 | 0.7 | 0.9 | 0.9 |
| Acidovorax | R1 | 0.1 | 0.2 | 0.7 | 0.6 | 0.9 | 0.7 | 1.1 | 1.1 |
| | R2 | 0.1 | 0.1 | 0.7 | 0.6 | 0.8 | 0.8 | 0.9 | 1.0 |
| | R3 | 0.2 | 0.1 | 0.9 | 0.8 | 0.9 | 0.9 | 0.8 | 1.0 |
| Adhaeribacter | R1 | 0.2 | 0.1 | 0.8 | 0.7 | 0.7 | 0.9 | 0.6 | 0.8 |
| | R2 | 0.2 | 0.1 | 0.6 | 0.6 | 0.6 | 0.7 | 0.5 | 0.7 |
| | R3 | 0.3 | 0.1 | 0.4 | 0.3 | 0.5 | 0.4 | 0.3 | 0.4 |
| Allokutzneria | R1 | 0.3 | 0.1 | 0.3 | 0.2 | 0.4 | 0.3 | 0.3 | 0.3 |
| | R2 | 0.3 | 0.2 | 0.2 | 0.2 | 0.3 | 0.2 | 0.3 | 0.3 |
| | R3 | 0.4 | 0.5 | 0.2 | 0.2 | 0.2 | 0.1 | 0.2 | 0.3 |
| Amaricoccus | R1 | 0.5 | 0.6 | 0.2 | 0.2 | 0.2 | 0.1 | 0.2 | 0.2 |
| | R2 | 0.5 | 0.5 | 0.2 | 0.2 | 0.2 | 0.2 | 0.3 | 0.3 |
| | R3 | 0.5 | 0.4 | 0.3 | 0.3 | 0.2 | 0.3 | 0.4 | 0.3 |
| Arenimonas | R1 | 0.5 | 0.3 | 0.4 | 0.3 | 0.2 | 0.4 | 0.5 | 0.4 |
| | R2 | 0.5 | 0.3 | 0.5 | 0.4 | 0.3 | 0.4 | 0.5 | 0.4 |
| | R3 | 0.4 | 0.3 | 0.6 | 0.5 | 0.4 | 0.3 | 0.4 | 0.4 |
| Arthrobacter | R1 | 0.5 | 0.4 | 0.8 | 0.6 | 0.6 | 0.4 | 0.5 | 0.6 |
| | R2 | 0.4 | 0.3 | 0.7 | 0.6 | 0.7 | 0.5 | 0.4 | 0.4 |
| | R3 | 0.3 | 0.2 | 0.6 | 0.5 | 0.8 | 0.7 | 0.3 | 0.3 |
| Azotobacter | R1 | 0.2 | 0.1 | 0.4 | 0.4 | 0.8 | 0.7 | 0.2 | 0.2 |
| | R2 | 0.3 | 0.2 | 2.7 | 2.3 | 2.2 | 2.8 | 2.2 | 2.4 |
| | R3 | 0.3 | 0.4 | 5.8 | 4.9 | 4.0 | 5.5 | 4.9 | 5.2 |
| Bacillus | R1 | 0.4 | 0.5 | 7.4 | 6.3 | 4.9 | 6.9 | 6.3 | 6.8 |
| | R2 | 0.4 | 0.5 | 6.8 | 5.8 | 5.1 | 6.7 | 6.4 | 6.7 |
| | R3 | 0.4 | 0.3 | 4.9 | 4.2 | 4.2 | 5.1 | 5.2 | 5.3 |
| Bradyrhizobium | R1 | 0.5 | 0.3 | 5.2 | 4.5 | 5.1 | 5.8 | 6.2 | 6.2 |
| | R2 | 0.3 | 0.2 | 3.4 | 2.9 | 3.4 | 3.8 | 4.0 | 4.1 |
| | R3 | 0.2 | 0.2 | 2.2 | 1.9 | 2.2 | 2.5 | 2.6 | 2.6 |
| Brevundimonas | R1 | 0.1 | 0.1 | 0.2 | 0.1 | 0.3 | 0.3 | 0.2 | 0.2 |
| | R2 | 0.1 | 0.1 | 0.2 | 0.2 | 0.3 | 0.2 | 0.2 | 0.2 |
| | R3 | 0.1 | 0.1 | 0.4 | 0.3 | 0.3 | 0.2 | 0.2 | 0.2 |
| Caulobacter | R1 | 0.1 | 0.1 | 0.4 | 0.4 | 0.2 | 0.1 | 0.2 | 0.2 |
| | R2 | 0.1 | 0.2 | 0.4 | 0.3 | 0.2 | 0.1 | 0.1 | 0.2 |
| | R3 | 0.1 | 0.3 | 0.3 | 0.3 | 0.1 | 0.1 | 0.1 | 0.1 |
| Chryseobacterium | R1 | 0.1 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 |

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|---|---|---|---|---|---|---|---|---|---|
| | R2 | 0.2 | 0.3 | 1.1 | 0.9 | 0.8 | 1.0 | 0.9 | 0.9 |
| | R3 | 0.2 | 0.2 | 1.8 | 1.5 | 1.4 | 1.8 | 1.6 | 1.6 |
| Comamonas | R1 | 0.4 | 0.2 | 2.9 | 2.5 | 2.3 | 2.8 | 2.5 | 2.6 |
| | R2 | 0.4 | 0.3 | 3.0 | 2.6 | 2.3 | 2.9 | 2.7 | 2.7 |
| | R3 | 0.4 | 0.3 | 3.0 | 2.6 | 2.2 | 2.8 | 2.6 | 2.5 |
| Cupriavidus | R1 | 0.4 | 0.4 | 3.2 | 2.8 | 2.2 | 2.8 | 2.8 | 2.6 |
| | R2 | 0.3 | 0.3 | 2.2 | 1.9 | 1.5 | 2.0 | 2.0 | 1.8 |
| | R3 | 0.3 | 0.3 | 1.5 | 1.3 | 1.0 | 1.4 | 1.3 | 1.3 |
| Devosia | R1 | 0.2 | 0.2 | 0.3 | 0.2 | 0.2 | 0.4 | 0.3 | 0.3 |
| | R2 | 0.3 | 0.4 | 1.0 | 0.8 | 0.8 | 1.0 | 1.3 | 1.1 |
| | R3 | 0.3 | 0.5 | 1.5 | 1.3 | 1.2 | 1.4 | 2.0 | 1.7 |
| Ensifer | R1 | 0.4 | 0.8 | 2.3 | 2.0 | 1.9 | 2.1 | 3.3 | 2.6 |
| | R2 | 0.4 | 0.6 | 1.8 | 1.5 | 1.6 | 1.7 | 2.5 | 2.1 |
| | R3 | 0.4 | 0.4 | 1.4 | 1.2 | 1.4 | 1.4 | 2.0 | 1.8 |
| Flavisolibacter | R1 | 0.3 | 0.2 | 0.7 | 0.6 | 1.0 | 1.0 | 1.1 | 1.2 |
| | R2 | 0.4 | 0.2 | 0.6 | 0.5 | 0.7 | 0.7 | 0.7 | 0.8 |
| | R3 | 0.4 | 0.2 | 0.5 | 0.4 | 0.5 | 0.5 | 0.5 | 0.6 |
| Flavitalea | R1 | 0.4 | 0.3 | 0.3 | 0.3 | 0.1 | 0.2 | 0.1 | 0.1 |
| | R2 | 0.4 | 0.2 | 1.7 | 1.5 | 1.9 | 2.0 | 2.3 | 2.2 |
| | R3 | 0.5 | 0.2 | 3.6 | 3.1 | 4.1 | 4.3 | 5.1 | 4.9 |
| Flavobacterium | R1 | 0.4 | 0.2 | 4.6 | 4.0 | 5.4 | 5.7 | 6.8 | 6.5 |
| | R2 | 0.4 | 0.2 | 3.4 | 2.9 | 4.1 | 4.2 | 5.0 | 4.7 |
| | R3 | 0.2 | 0.2 | 1.7 | 1.5 | 2.3 | 2.4 | 2.6 | 2.5 |
| Fontibacillus | R1 | 0.2 | 0.2 | 0.7 | 0.6 | 1.2 | 1.2 | 1.1 | 1.1 |
| | R2 | 0.1 | 0.2 | 0.8 | 0.7 | 1.3 | 1.2 | 1.3 | 1.3 |
| | R3 | 0.1 | 0.1 | 0.7 | 0.6 | 1.1 | 1.0 | 1.2 | 1.2 |
| Gemmatimonas | R1 | 0.1 | 0.2 | 0.9 | 0.8 | 1.3 | 1.1 | 1.5 | 1.5 |
| | R2 | 0.1 | 0.1 | 0.6 | 0.5 | 0.9 | 0.9 | 1.2 | 1.2 |
| | R3 | 0.1 | 0.1 | 0.4 | 0.3 | 0.7 | 0.8 | 1.0 | 1.0 |
| Gp10 | R1 | 0.2 | 0.1 | 0.0 | 0.0 | 0.3 | 0.5 | 0.7 | 0.6 |
| | R2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.4 | 0.5 | 0.8 | 0.7 |
| | R3 | 0.1 | 0.1 | 0.2 | 0.2 | 0.4 | 0.5 | 0.9 | 0.8 |
| Gp4 | R1 | 0.0 | 0.2 | 0.4 | 0.3 | 0.4 | 0.6 | 1.0 | 0.9 |
| | R2 | 0.1 | 0.1 | 0.5 | 0.4 | 0.5 | 0.6 | 0.8 | 0.8 |
| | R3 | 0.1 | 0.1 | 0.6 | 0.5 | 0.5 | 0.7 | 0.7 | 0.7 |
| Gp6 | R1 | 0.2 | 0.0 | 0.7 | 0.6 | 0.5 | 0.8 | 0.5 | 0.5 |
| | R2 | 0.2 | 0.1 | 0.7 | 0.6 | 0.5 | 0.7 | 0.6 | 0.5 |
| | R3 | 0.2 | 0.1 | 0.7 | 0.6 | 0.4 | 0.6 | 0.6 | 0.5 |
| Gp7 | R1 | 0.2 | 0.2 | 0.6 | 0.6 | 0.4 | 0.5 | 0.7 | 0.5 |
| | R2 | 0.1 | 0.2 | 0.6 | 0.6 | 0.3 | 0.6 | 0.7 | 0.7 |
| | R3 | 0.1 | 0.2 | 0.8 | 0.7 | 0.4 | 0.8 | 0.9 | 0.9 |

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|---|---|---|---|---|---|---|---|---|---|
| Halomonas | R1 | 0.1 | 0.2 | 0.7 | 0.6 | 0.3 | 0.7 | 0.8 | 0.9 |
| | R2 | 0.1 | 0.3 | 2.0 | 1.7 | 1.7 | 2.4 | 2.6 | 2.4 |
| | R3 | 0.2 | 0.4 | 3.7 | 3.1 | 3.5 | 4.5 | 4.8 | 4.3 |
| Hyphomicrobium | R1 | 0.2 | 0.4 | 4.7 | 4.0 | 4.6 | 5.8 | 6.2 | 5.5 |
| | R2 | 0.1 | 0.3 | 3.4 | 2.9 | 3.2 | 4.0 | 4.3 | 3.8 |
| | R3 | 0.0 | 0.2 | 1.6 | 1.4 | 1.4 | 1.6 | 1.9 | 1.7 |
| Ilumatobacter | R1 | 0.0 | 0.1 | 0.7 | 0.6 | 0.3 | 0.3 | 0.5 | 0.4 |
| | R2 | 0.1 | 0.1 | 0.7 | 0.6 | 0.3 | 0.3 | 0.4 | 0.3 |
| | R3 | 0.1 | 0.2 | 0.8 | 0.7 | 0.3 | 0.3 | 0.3 | 0.3 |
| Litorilinea | R1 | 0.2 | 0.2 | 0.7 | 0.6 | 0.2 | 0.2 | 0.1 | 0.1 |
| | R2 | 0.1 | 0.1 | 0.6 | 0.5 | 0.3 | 0.3 | 0.2 | 0.2 |
| | R3 | 0.0 | 0.1 | 0.5 | 0.4 | 0.3 | 0.4 | 0.3 | 0.3 |
| Luteimonas | R1 | 0.0 | 0.1 | 0.4 | 0.4 | 0.3 | 0.5 | 0.3 | 0.3 |
| | R2 | 0.0 | 0.1 | 0.4 | 0.4 | 0.3 | 0.4 | 0.3 | 0.3 |
| | R3 | 0.1 | 0.1 | 0.3 | 0.3 | 0.2 | 0.3 | 0.3 | 0.2 |
| Lysobacter | R1 | 0.1 | 0.1 | 0.4 | 0.3 | 0.1 | 0.2 | 0.3 | 0.2 |
| | R2 | 0.1 | 0.1 | 0.4 | 0.3 | 0.1 | 0.2 | 0.3 | 0.2 |
| | R3 | 0.1 | 0.1 | 0.4 | 0.3 | 0.1 | 0.2 | 0.2 | 0.1 |
| Marmoricola | R1 | 0.1 | 0.1 | 0.4 | 0.3 | 0.1 | 0.2 | 0.2 | 0.1 |
| | R2 | 0.1 | 0.1 | 0.3 | 0.3 | 0.1 | 0.2 | 0.1 | 0.1 |
| | R3 | 0.1 | 0.0 | 0.3 | 0.3 | 0.1 | 0.2 | 0.1 | 0.1 |
| Massilia | R1 | 0.1 | 0.0 | 0.2 | 0.2 | 0.1 | 0.2 | 0.1 | 0.1 |
| | R2 | 0.2 | 0.1 | 0.3 | 0.3 | 0.4 | 0.2 | 0.2 | 0.2 |
| | R3 | 0.3 | 0.2 | 0.4 | 0.3 | 0.6 | 0.3 | 0.3 | 0.3 |
| Mesorhizobium | R1 | 0.4 | 0.3 | 0.5 | 0.4 | 0.9 | 0.4 | 0.5 | 0.4 |
| | R2 | 0.3 | 0.3 | 0.5 | 0.4 | 0.7 | 0.5 | 0.5 | 0.5 |
| | R3 | 0.3 | 0.2 | 0.5 | 0.4 | 0.6 | 0.6 | 0.6 | 0.6 |
| Methylohalomonas | R1 | 0.2 | 0.2 | 0.5 | 0.4 | 0.5 | 0.8 | 0.6 | 0.6 |
| | R2 | 0.2 | 0.2 | 0.5 | 0.4 | 0.5 | 0.8 | 0.6 | 0.7 |
| | R3 | 0.2 | 0.2 | 0.5 | 0.4 | 0.5 | 0.7 | 0.6 | 0.6 |
| Microbacterium | R1 | 0.2 | 0.2 | 0.5 | 0.4 | 0.5 | 0.7 | 0.6 | 0.6 |
| | R2 | 0.2 | 0.2 | 0.4 | 0.3 | 0.4 | 0.5 | 0.4 | 0.5 |
| | R3 | 0.3 | 0.2 | 0.2 | 0.2 | 0.3 | 0.3 | 0.4 | 0.4 |
| Microvirga | R1 | 0.3 | 0.2 | 0.1 | 0.1 | 0.2 | 0.1 | 0.2 | 0.2 |
| | R2 | 0.3 | 0.2 | 0.5 | 0.5 | 1.0 | 0.6 | 0.9 | 1.0 |
| | R3 | 0.2 | 0.1 | 0.9 | 0.8 | 1.6 | 1.1 | 1.5 | 1.6 |
| Mycobacterium | R1 | 0.2 | 0.2 | 1.5 | 1.2 | 2.5 | 1.7 | 2.4 | 2.5 |
| | R2 | 0.1 | 0.1 | 1.1 | 0.9 | 1.8 | 1.4 | 1.7 | 1.8 |
| | R3 | 0.1 | 0.1 | 0.8 | 0.7 | 1.2 | 1.1 | 1.2 | 1.3 |
| Nitriliruptor | R1 | 0.1 | 0.1 | 0.4 | 0.3 | 0.4 | 0.7 | 0.4 | 0.6 |
| | R2 | 0.1 | 0.1 | 0.7 | 0.6 | 1.0 | 1.4 | 1.3 | 1.2 |

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|---|---|---|---|---|---|---|---|---|---|
| | R3 | 0.2 | 0.3 | 1.1 | 0.9 | 2.0 | 2.6 | 2.6 | 2.2 |
| Nitrosococcus | R1 | 0.2 | 0.3 | 1.3 | 1.1 | 2.5 | 3.0 | 3.3 | 2.6 |
| | R2 | 0.2 | 0.2 | 1.7 | 1.5 | 2.4 | 2.8 | 2.4 | 1.9 |
| | R3 | 0.2 | 0.1 | 1.8 | 1.6 | 1.8 | 1.9 | 0.9 | 0.8 |
| Nitrosomonas | R1 | 0.2 | 0.1 | 2.4 | 2.1 | 1.9 | 1.9 | 0.3 | 0.3 |
| | R2 | 0.2 | 0.1 | 2.3 | 2.0 | 1.8 | 1.9 | 1.2 | 1.2 |
| | R3 | 0.2 | 0.2 | 2.2 | 1.8 | 1.8 | 1.9 | 1.9 | 1.9 |
| Nitrosospira | R1 | 0.2 | 0.3 | 2.1 | 1.8 | 1.8 | 1.9 | 3.0 | 3.0 |
| | R2 | 0.1 | 0.2 | 1.6 | 1.4 | 1.4 | 1.4 | 2.2 | 2.2 |
| | R3 | 0.1 | 0.3 | 1.4 | 1.2 | 1.2 | 1.2 | 1.8 | 1.7 |
| Nitrosovibrio | R1 | 0.1 | 0.2 | 0.7 | 0.6 | 0.7 | 0.5 | 0.7 | 0.6 |
| | R2 | 0.1 | 0.2 | 0.6 | 0.5 | 0.6 | 0.5 | 0.6 | 0.5 |
| | R3 | 0.1 | 0.2 | 0.4 | 0.4 | 0.3 | 0.3 | 0.3 | 0.3 |
| Nocardioides | R1 | 0.2 | 0.2 | 0.4 | 0.3 | 0.3 | 0.3 | 0.3 | 0.2 |
| | R2 | 0.1 | 0.2 | 0.4 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| | R3 | 0.1 | 0.2 | 0.5 | 0.4 | 0.4 | 0.4 | 0.4 | 0.5 |
| Novosphingobium | R1 | 0.1 | 0.2 | 0.5 | 0.4 | 0.4 | 0.4 | 0.5 | 0.5 |
| | R2 | 0.2 | 0.2 | 0.4 | 0.3 | 0.3 | 0.3 | 0.4 | 0.4 |
| | R3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.1 | 0.1 | 0.2 | 0.2 |
| Ohtaekwangia | R1 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R2 | 0.4 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R3 | 0.4 | 0.3 | 0.2 | 0.1 | 0.1 | 0.2 | 0.1 | 0.1 |
| Opitutus | R1 | 0.4 | 0.3 | 0.2 | 0.1 | 0.1 | 0.2 | 0.1 | 0.1 |
| | R2 | 0.3 | 0.2 | 0.3 | 0.2 | 0.4 | 0.4 | 0.4 | 0.4 |
| | R3 | 0.2 | 0.2 | 0.4 | 0.3 | 0.5 | 0.6 | 0.7 | 0.6 |
| Paenibacillus | R1 | 0.1 | 0.2 | 0.5 | 0.4 | 0.8 | 0.8 | 1.0 | 1.0 |
| | R2 | 0.1 | 0.2 | 1.0 | 0.9 | 1.4 | 1.5 | 1.8 | 1.7 |
| | R3 | 0.3 | 0.1 | 1.8 | 1.6 | 2.5 | 2.6 | 3.2 | 3.0 |
| Paracoccus | R1 | 0.3 | 0.1 | 2.2 | 1.8 | 2.9 | 2.9 | 3.7 | 3.5 |
| | R2 | 0.3 | 0.2 | 1.6 | 1.4 | 2.1 | 2.1 | 2.7 | 2.5 |
| | R3 | 0.2 | 0.3 | 0.8 | 0.7 | 0.9 | 0.8 | 1.1 | 1.0 |
| Pedobacter | R1 | 0.2 | 0.4 | 0.4 | 0.4 | 0.3 | 0.2 | 0.3 | 0.2 |
| | R2 | 0.2 | 0.3 | 0.4 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| | R3 | 0.2 | 0.2 | 0.3 | 0.3 | 0.3 | 0.4 | 0.4 | 0.3 |
| Phenylobacterium | R1 | 0.2 | 0.1 | 0.3 | 0.2 | 0.3 | 0.5 | 0.4 | 0.4 |
| | R2 | 0.2 | 0.2 | 0.3 | 0.2 | 0.3 | 0.5 | 0.4 | 0.4 |
| | R3 | 0.3 | 0.2 | 0.3 | 0.2 | 0.3 | 0.4 | 0.4 | 0.3 |
| Pirellula | R1 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.4 | 0.4 | 0.4 |
| | R2 | 0.4 | 0.3 | 0.3 | 0.3 | 0.2 | 0.3 | 0.3 | 0.3 |
| | R3 | 0.4 | 0.3 | 0.3 | 0.2 | 0.2 | 0.3 | 0.3 | 0.2 |
| Pontibacter | R1 | 0.4 | 0.3 | 0.3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |

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|---|---|---|---|---|---|---|---|---|---|
| | R2 | 0.4 | 0.3 | 1.4 | 1.2 | 1.5 | 2.0 | 1.8 | 2.0 |
| | R3 | 0.3 | 0.3 | 2.4 | 2.0 | 2.7 | 3.5 | 3.2 | 3.5 |
| Pseudomonas | R1 | 0.3 | 0.3 | 3.6 | 3.1 | 4.2 | 5.5 | 5.0 | 5.6 |
| | R2 | 0.3 | 0.4 | 2.6 | 2.3 | 3.0 | 3.8 | 3.6 | 4.0 |
| | R3 | 0.3 | 0.4 | 1.7 | 1.5 | 1.9 | 2.4 | 2.3 | 2.6 |
| Pseudoxanthomonas | R1 | 0.3 | 0.5 | 0.6 | 0.5 | 0.6 | 0.6 | 0.7 | 0.8 |
| | R2 | 0.3 | 0.4 | 0.5 | 0.4 | 0.4 | 0.4 | 0.5 | 0.5 |
| | R3 | 0.4 | 0.4 | 0.4 | 0.3 | 0.3 | 0.3 | 0.3 | 0.4 |
| Ramlibacter | R1 | 0.4 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R2 | 0.3 | 0.2 | 0.8 | 0.7 | 0.6 | 0.7 | 0.7 | 0.7 |
| | R3 | 0.2 | 0.1 | 1.8 | 1.5 | 1.4 | 1.6 | 1.6 | 1.7 |
| Rhodococcus | R1 | 0.2 | 0.1 | 2.3 | 1.9 | 1.8 | 2.1 | 2.1 | 2.2 |
| | R2 | 0.3 | 0.2 | 1.6 | 1.4 | 1.4 | 1.6 | 1.6 | 1.7 |
| | R3 | 0.3 | 0.3 | 0.7 | 0.6 | 0.7 | 0.8 | 0.8 | 0.9 |
| Saccharibacteria | R1 | 0.4 | 0.4 | 0.2 | 0.2 | 0.3 | 0.4 | 0.4 | 0.5 |
| | R2 | 0.3 | 0.3 | 0.2 | 0.2 | 0.5 | 0.5 | 0.5 | 0.5 |
| | R3 | 0.2 | 0.2 | 0.3 | 0.3 | 0.6 | 0.8 | 0.7 | 0.7 |
| Salinibacillus | R1 | 0.1 | 0.1 | 0.4 | 0.3 | 0.7 | 0.9 | 0.7 | 0.7 |
| | R2 | 0.1 | 0.1 | 0.4 | 0.3 | 0.6 | 0.8 | 0.7 | 0.7 |
| | R3 | 0.1 | 0.1 | 0.3 | 0.3 | 0.4 | 0.5 | 0.4 | 0.4 |
| Salinimicrobium | R1 | 0.1 | 0.1 | 0.3 | 0.3 | 0.4 | 0.4 | 0.4 | 0.4 |
| | R2 | 0.1 | 0.1 | 0.3 | 0.3 | 0.4 | 0.4 | 0.5 | 0.5 |
| | R3 | 0.1 | 0.0 | 0.4 | 0.3 | 0.4 | 0.4 | 0.8 | 0.7 |
| Salinisphaera | R1 | 0.1 | 0.0 | 0.3 | 0.3 | 0.3 | 0.3 | 0.8 | 0.8 |
| | R2 | 0.2 | 0.2 | 0.4 | 0.4 | 0.4 | 0.5 | 0.7 | 0.7 |
| | R3 | 0.2 | 0.4 | 0.4 | 0.4 | 0.5 | 0.5 | 0.6 | 0.6 |
| Sediminibacter | R1 | 0.3 | 0.7 | 0.6 | 0.5 | 0.7 | 0.7 | 0.6 | 0.7 |
| | R2 | 0.3 | 0.5 | 0.4 | 0.3 | 0.7 | 0.7 | 0.6 | 0.7 |
| | R3 | 0.2 | 0.3 | 0.2 | 0.2 | 0.8 | 0.9 | 0.7 | 0.8 |
| Sphaerobacter | R1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.7 | 0.8 | 0.7 | 0.7 |
| | R2 | 0.1 | 0.1 | 0.6 | 0.5 | 1.2 | 1.3 | 1.3 | 1.2 |
| | R3 | 0.1 | 0.1 | 1.1 | 0.9 | 1.3 | 1.5 | 1.6 | 1.5 |
| Sphingomonas | R1 | 0.2 | 0.2 | 1.8 | 1.5 | 1.8 | 2.1 | 2.4 | 2.1 |
| | R2 | 0.2 | 0.1 | 1.3 | 1.1 | 1.3 | 1.5 | 1.7 | 1.6 |
| | R3 | 0.2 | 0.1 | 0.9 | 0.8 | 0.9 | 1.1 | 1.2 | 1.2 |
| Sphingopyxis | R1 | 0.2 | 0.1 | 0.3 | 0.3 | 0.2 | 0.4 | 0.4 | 0.6 |
| | R2 | 0.1 | 0.1 | 0.3 | 0.2 | 0.3 | 0.4 | 0.4 | 0.5 |
| | R3 | 0.1 | 0.1 | 0.3 | 0.2 | 0.4 | 0.4 | 0.5 | 0.5 |
| Sporosarcina | R1 | 0.1 | 0.1 | 0.2 | 0.2 | 0.4 | 0.4 | 0.5 | 0.4 |
| | R2 | 0.1 | 0.1 | 0.3 | 0.2 | 0.4 | 0.3 | 0.4 | 0.4 |
| | R3 | 0.1 | 0.1 | 0.3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |

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|---|---|---|---|---|---|---|---|---|---|
| Terrimonas | R1 | 0.2 | 0.1 | 0.4 | 0.3 | 0.2 | 0.2 | 0.2 | 0.2 |
| | R2 | 0.2 | 0.1 | 0.9 | 0.7 | 0.7 | 0.8 | 0.8 | 0.9 |
| | R3 | 0.2 | 0.1 | 1.6 | 1.4 | 1.5 | 1.7 | 1.6 | 1.7 |
| Thiobacillus | R1 | 0.2 | 0.1 | 1.9 | 1.7 | 1.9 | 2.2 | 2.1 | 2.2 |
| | R2 | 0.2 | 0.1 | 1.4 | 1.2 | 1.5 | 1.8 | 1.8 | 1.9 |
| | R3 | 0.1 | 0.2 | 0.8 | 0.7 | 1.0 | 1.4 | 1.5 | 1.5 |
| Truepera | R1 | 0.1 | 0.2 | 0.5 | 0.4 | 0.7 | 1.1 | 1.3 | 1.3 |
| | R2 | 0.1 | 0.2 | 0.5 | 0.4 | 0.7 | 1.1 | 1.4 | 1.4 |
| | R3 | 0.1 | 0.1 | 0.3 | 0.3 | 0.5 | 0.8 | 0.9 | 0.9 |

| | | Cultivated soil; Rhizosphere | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Tomato | | | | | | | |
| | | Relative abundance (%) | | | | | | | |
| Genus | Replicate | NT1 | NT4 | UR1 | UR4 | AS1 | AS4 | PN1 | PN4 |
| Aciditerrimonas | R1 | 0.1 | 0.1 | 0.4 | 0.3 | 0.1 | 0.5 | 0.6 | 0.5 |
| | R2 | 0.1 | 0.1 | 0.5 | 0.3 | 0.3 | 0.5 | 0.7 | 0.6 |
| | R3 | 0.1 | 0.1 | 0.5 | 0.3 | 0.4 | 0.5 | 0.7 | 0.6 |
| Acidovorax | R1 | 0.1 | 0.1 | 0.6 | 0.4 | 0.6 | 0.5 | 0.9 | 0.7 |
| | R2 | 0.1 | 0.1 | 0.6 | 0.4 | 0.6 | 0.6 | 0.7 | 0.6 |
| | R3 | 0.1 | 0.1 | 0.7 | 0.5 | 0.6 | 0.7 | 0.7 | 0.7 |
| Adhaeribacter | R1 | 0.1 | 0.1 | 0.6 | 0.5 | 0.5 | 0.7 | 0.5 | 0.5 |
| | R2 | 0.2 | 0.1 | 0.5 | 0.4 | 0.4 | 0.5 | 0.4 | 0.4 |
| | R3 | 0.2 | 0.1 | 0.3 | 0.2 | 0.3 | 0.3 | 0.3 | 0.3 |
| Allokutzneria | R1 | 0.2 | 0.1 | 0.2 | 0.2 | 0.3 | 0.2 | 0.2 | 0.2 |
| | R2 | 0.2 | 0.2 | 0.2 | 0.1 | 0.2 | 0.2 | 0.2 | 0.2 |
| | R3 | 0.3 | 0.3 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.2 |
| Amaricoccus | R1 | 0.3 | 0.4 | 0.1 | 0.1 | 0.1 | 0.1 | 0.2 | 0.1 |
| | R2 | 0.3 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.2 | 0.2 |
| | R3 | 0.3 | 0.3 | 0.2 | 0.2 | 0.1 | 0.2 | 0.3 | 0.2 |
| Arenimonas | R1 | 0.3 | 0.2 | 0.3 | 0.2 | 0.1 | 0.3 | 0.4 | 0.2 |
| | R2 | 0.4 | 0.2 | 0.4 | 0.3 | 0.2 | 0.3 | 0.4 | 0.3 |
| | R3 | 0.3 | 0.2 | 0.4 | 0.3 | 0.3 | 0.2 | 0.4 | 0.3 |
| Arthrobacter | R1 | 0.4 | 0.3 | 0.6 | 0.4 | 0.4 | 0.3 | 0.4 | 0.4 |
| | R2 | 0.3 | 0.2 | 0.5 | 0.4 | 0.5 | 0.4 | 0.3 | 0.3 |
| | R3 | 0.2 | 0.2 | 0.5 | 0.3 | 0.6 | 0.5 | 0.3 | 0.2 |
| Azotobacter | R1 | 0.1 | 0.1 | 0.3 | 0.2 | 0.5 | 0.5 | 0.1 | 0.1 |
| | R2 | 0.2 | 0.2 | 2.2 | 1.6 | 1.5 | 2.0 | 1.8 | 1.5 |
| | R3 | 0.2 | 0.3 | 4.5 | 3.3 | 2.7 | 4.0 | 4.0 | 3.4 |
| Bacillus | R1 | 0.3 | 0.4 | 5.8 | 4.2 | 3.3 | 5.0 | 5.1 | 4.3 |
| | R2 | 0.3 | 0.3 | 5.3 | 3.8 | 3.4 | 4.8 | 5.2 | 4.3 |
| | R3 | 0.3 | 0.2 | 3.8 | 2.8 | 2.9 | 3.7 | 4.2 | 3.4 |
| Bradyrhizobium | R1 | 0.3 | 0.2 | 4.1 | 3.0 | 3.5 | 4.2 | 5.0 | 4.0 |
| | R2 | 0.2 | 0.2 | 2.7 | 1.9 | 2.3 | 2.8 | 3.3 | 2.6 |
| | R3 | 0.2 | 0.1 | 1.7 | 1.2 | 1.5 | 1.8 | 2.1 | 1.7 |
| Brevundimonas | R1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.2 | 0.2 | 0.1 | 0.1 |
| | R2 | 0.1 | 0.1 | 0.2 | 0.1 | 0.2 | 0.2 | 0.1 | 0.1 |
| | R3 | 0.1 | 0.1 | 0.3 | 0.2 | 0.2 | 0.1 | 0.2 | 0.1 |
| Caulobacter | R1 | 0.1 | 0.1 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 |
| | R2 | 0.1 | 0.1 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R3 | 0.1 | 0.2 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 |
| Chryseobacterium | R1 | 0.1 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

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| | R2 | 0.1 | 0.2 | 0.9 | 0.6 | 0.5 | 0.7 | 0.7 | 0.6 |
| | R3 | 0.2 | 0.1 | 1.4 | 1.0 | 0.9 | 1.3 | 1.3 | 1.0 |
| Comamonas | R1 | 0.3 | 0.1 | 2.3 | 1.6 | 1.5 | 2.1 | 2.0 | 1.7 |
| | R2 | 0.3 | 0.2 | 2.4 | 1.7 | 1.5 | 2.1 | 2.2 | 1.7 |
| | R3 | 0.3 | 0.2 | 2.4 | 1.7 | 1.5 | 2.0 | 2.2 | 1.6 |
| Cupriavidus | R1 | 0.3 | 0.3 | 2.5 | 1.8 | 1.5 | 2.0 | 2.3 | 1.7 |
| | R2 | 0.2 | 0.2 | 1.8 | 1.3 | 1.0 | 1.5 | 1.6 | 1.2 |
| | R3 | 0.2 | 0.2 | 1.2 | 0.8 | 0.7 | 1.0 | 1.1 | 0.8 |
| Devosia | R1 | 0.1 | 0.1 | 0.2 | 0.1 | 0.1 | 0.3 | 0.2 | 0.2 |
| | R2 | 0.2 | 0.3 | 0.8 | 0.5 | 0.5 | 0.7 | 1.0 | 0.7 |
| | R3 | 0.2 | 0.4 | 1.2 | 0.8 | 0.8 | 1.0 | 1.6 | 1.1 |
| Ensifer | R1 | 0.3 | 0.5 | 1.8 | 1.3 | 1.3 | 1.5 | 2.6 | 1.7 |
| | R2 | 0.3 | 0.4 | 1.4 | 1.0 | 1.1 | 1.2 | 2.0 | 1.4 |
| | R3 | 0.3 | 0.3 | 1.1 | 0.8 | 1.0 | 1.0 | 1.6 | 1.1 |
| Flavisolibacter | R1 | 0.2 | 0.1 | 0.6 | 0.4 | 0.7 | 0.7 | 0.9 | 0.8 |
| | R2 | 0.3 | 0.1 | 0.5 | 0.3 | 0.5 | 0.5 | 0.6 | 0.5 |
| | R3 | 0.3 | 0.2 | 0.4 | 0.3 | 0.4 | 0.4 | 0.4 | 0.4 |
| Flavitalea | R1 | 0.3 | 0.2 | 0.3 | 0.2 | 0.1 | 0.2 | 0.1 | 0.1 |
| | R2 | 0.3 | 0.2 | 1.4 | 1.0 | 1.3 | 1.5 | 1.8 | 1.4 |
| | R3 | 0.4 | 0.2 | 2.8 | 2.0 | 2.8 | 3.2 | 4.2 | 3.1 |
| Flavobacterium | R1 | 0.3 | 0.1 | 3.6 | 2.6 | 3.7 | 4.1 | 5.6 | 4.2 |
| | R2 | 0.3 | 0.1 | 2.7 | 1.9 | 2.8 | 3.1 | 4.1 | 3.0 |
| | R3 | 0.2 | 0.1 | 1.4 | 1.0 | 1.6 | 1.7 | 2.1 | 1.6 |
| Fontibacillus | R1 | 0.1 | 0.1 | 0.6 | 0.4 | 0.8 | 0.9 | 0.9 | 0.7 |
| | R2 | 0.1 | 0.1 | 0.6 | 0.5 | 0.9 | 0.9 | 1.0 | 0.8 |
| | R3 | 0.1 | 0.1 | 0.6 | 0.4 | 0.7 | 0.7 | 0.9 | 0.8 |
| Gemmatimonas | R1 | 0.1 | 0.1 | 0.7 | 0.5 | 0.9 | 0.8 | 1.2 | 1.0 |
| | R2 | 0.1 | 0.1 | 0.5 | 0.3 | 0.6 | 0.7 | 1.0 | 0.8 |
| | R3 | 0.1 | 0.1 | 0.3 | 0.2 | 0.5 | 0.6 | 0.8 | 0.6 |
| Gp10 | R1 | 0.1 | 0.1 | 0.0 | 0.0 | 0.2 | 0.4 | 0.6 | 0.4 |
| | R2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.2 | 0.4 | 0.7 | 0.4 |
| | R3 | 0.0 | 0.1 | 0.2 | 0.1 | 0.3 | 0.4 | 0.7 | 0.5 |
| Gp4 | R1 | 0.0 | 0.1 | 0.3 | 0.2 | 0.3 | 0.4 | 0.8 | 0.6 |
| | R2 | 0.0 | 0.1 | 0.4 | 0.3 | 0.3 | 0.5 | 0.7 | 0.5 |
| | R3 | 0.1 | 0.0 | 0.5 | 0.3 | 0.3 | 0.5 | 0.6 | 0.4 |
| Gp6 | R1 | 0.1 | 0.0 | 0.6 | 0.4 | 0.4 | 0.6 | 0.4 | 0.3 |
| | R2 | 0.1 | 0.0 | 0.6 | 0.4 | 0.3 | 0.5 | 0.5 | 0.3 |
| | R3 | 0.1 | 0.1 | 0.5 | 0.4 | 0.3 | 0.4 | 0.5 | 0.3 |
| Gp7 | R1 | 0.1 | 0.1 | 0.5 | 0.4 | 0.3 | 0.4 | 0.5 | 0.3 |
| | R2 | 0.1 | 0.1 | 0.5 | 0.4 | 0.2 | 0.4 | 0.6 | 0.4 |
| | R3 | 0.1 | 0.1 | 0.6 | 0.4 | 0.3 | 0.6 | 0.7 | 0.6 |

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| Halomonas | R1 | 0.1 | 0.1 | 0.5 | 0.4 | 0.2 | 0.5 | 0.7 | 0.6 |
| | R2 | 0.1 | 0.2 | 1.5 | 1.1 | 1.1 | 1.7 | 2.1 | 1.5 |
| | R3 | 0.1 | 0.3 | 2.9 | 2.1 | 2.4 | 3.3 | 3.9 | 2.8 |
| Hyphomicrobium | R1 | 0.1 | 0.3 | 3.7 | 2.7 | 3.1 | 4.2 | 5.0 | 3.5 |
| | R2 | 0.1 | 0.2 | 2.7 | 1.9 | 2.2 | 2.9 | 3.5 | 2.5 |
| | R3 | 0.0 | 0.1 | 1.3 | 0.9 | 0.9 | 1.2 | 1.5 | 1.1 |
| Ilumatobacter | R1 | 0.0 | 0.1 | 0.5 | 0.4 | 0.2 | 0.2 | 0.4 | 0.3 |
| | R2 | 0.0 | 0.1 | 0.5 | 0.4 | 0.2 | 0.2 | 0.3 | 0.2 |
| | R3 | 0.1 | 0.1 | 0.6 | 0.4 | 0.2 | 0.2 | 0.2 | 0.2 |
| Litorilinea | R1 | 0.1 | 0.1 | 0.5 | 0.4 | 0.2 | 0.2 | 0.1 | 0.1 |
| | R2 | 0.1 | 0.1 | 0.5 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 |
| | R3 | 0.0 | 0.1 | 0.4 | 0.3 | 0.2 | 0.3 | 0.2 | 0.2 |
| Luteimonas | R1 | 0.0 | 0.1 | 0.3 | 0.2 | 0.2 | 0.4 | 0.3 | 0.2 |
| | R2 | 0.0 | 0.1 | 0.3 | 0.2 | 0.2 | 0.3 | 0.3 | 0.2 |
| | R3 | 0.0 | 0.1 | 0.3 | 0.2 | 0.1 | 0.2 | 0.2 | 0.1 |
| Lysobacter | R1 | 0.1 | 0.1 | 0.3 | 0.2 | 0.1 | 0.2 | 0.3 | 0.2 |
| | R2 | 0.1 | 0.1 | 0.3 | 0.2 | 0.1 | 0.2 | 0.2 | 0.1 |
| | R3 | 0.1 | 0.1 | 0.3 | 0.2 | 0.1 | 0.2 | 0.2 | 0.1 |
| Marmoricola | R1 | 0.1 | 0.1 | 0.3 | 0.2 | 0.0 | 0.2 | 0.1 | 0.1 |
| | R2 | 0.1 | 0.0 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R3 | 0.1 | 0.0 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 |
| Massilia | R1 | 0.1 | 0.0 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R2 | 0.1 | 0.1 | 0.3 | 0.2 | 0.3 | 0.2 | 0.2 | 0.1 |
| | R3 | 0.2 | 0.1 | 0.3 | 0.2 | 0.4 | 0.2 | 0.3 | 0.2 |
| Mesorhizobium | R1 | 0.3 | 0.2 | 0.4 | 0.3 | 0.6 | 0.3 | 0.4 | 0.3 |
| | R2 | 0.2 | 0.2 | 0.4 | 0.3 | 0.5 | 0.4 | 0.4 | 0.3 |
| | R3 | 0.2 | 0.2 | 0.4 | 0.3 | 0.4 | 0.4 | 0.5 | 0.4 |
| Methylohalomonas | R1 | 0.1 | 0.1 | 0.4 | 0.3 | 0.3 | 0.5 | 0.5 | 0.4 |
| | R2 | 0.1 | 0.1 | 0.4 | 0.3 | 0.3 | 0.6 | 0.5 | 0.4 |
| | R3 | 0.1 | 0.1 | 0.4 | 0.3 | 0.3 | 0.5 | 0.5 | 0.4 |
| Microbacterium | R1 | 0.1 | 0.1 | 0.4 | 0.3 | 0.3 | 0.5 | 0.5 | 0.4 |
| | R2 | 0.2 | 0.1 | 0.3 | 0.2 | 0.2 | 0.4 | 0.4 | 0.3 |
| | R3 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.2 | 0.3 | 0.2 |
| Microvirga | R1 | 0.2 | 0.1 | 0.1 | 0.0 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R2 | 0.2 | 0.1 | 0.4 | 0.3 | 0.6 | 0.5 | 0.8 | 0.6 |
| | R3 | 0.1 | 0.1 | 0.7 | 0.5 | 1.1 | 0.8 | 1.3 | 1.0 |
| Mycobacterium | R1 | 0.1 | 0.1 | 1.1 | 0.8 | 1.7 | 1.2 | 2.0 | 1.6 |
| | R2 | 0.1 | 0.1 | 0.9 | 0.6 | 1.2 | 1.0 | 1.4 | 1.2 |
| | R3 | 0.1 | 0.1 | 0.6 | 0.5 | 0.8 | 0.8 | 1.0 | 0.8 |
| Nitriliruptor | R1 | 0.1 | 0.1 | 0.3 | 0.2 | 0.3 | 0.5 | 0.3 | 0.4 |
| | R2 | 0.1 | 0.1 | 0.5 | 0.4 | 0.7 | 1.0 | 1.0 | 0.8 |

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|---|---|---|---|---|---|---|---|---|---|
| | R3 | 0.1 | 0.2 | 0.9 | 0.6 | 1.4 | 1.9 | 2.1 | 1.4 |
| Nitrosococcus | R1 | 0.1 | 0.2 | 1.0 | 0.7 | 1.7 | 2.2 | 2.6 | 1.7 |
| | R2 | 0.1 | 0.2 | 1.3 | 1.0 | 1.6 | 2.0 | 1.9 | 1.2 |
| | R3 | 0.1 | 0.1 | 1.4 | 1.0 | 1.2 | 1.4 | 0.8 | 0.5 |
| Nitrosomonas | R1 | 0.1 | 0.1 | 1.9 | 1.4 | 1.3 | 1.4 | 0.2 | 0.2 |
| | R2 | 0.1 | 0.1 | 1.8 | 1.3 | 1.2 | 1.4 | 1.0 | 0.8 |
| | R3 | 0.1 | 0.1 | 1.7 | 1.2 | 1.2 | 1.4 | 1.5 | 1.2 |
| Nitrosospira | R1 | 0.1 | 0.2 | 1.7 | 1.2 | 1.2 | 1.4 | 2.4 | 1.9 |
| | R2 | 0.1 | 0.2 | 1.3 | 0.9 | 0.9 | 1.0 | 1.8 | 1.4 |
| | R3 | 0.1 | 0.2 | 1.1 | 0.8 | 0.8 | 0.9 | 1.5 | 1.1 |
| Nitrosovibrio | R1 | 0.1 | 0.1 | 0.6 | 0.4 | 0.5 | 0.4 | 0.6 | 0.4 |
| | R2 | 0.1 | 0.1 | 0.5 | 0.4 | 0.4 | 0.4 | 0.5 | 0.3 |
| | R3 | 0.1 | 0.1 | 0.3 | 0.2 | 0.2 | 0.2 | 0.3 | 0.2 |
| Nocardioides | R1 | 0.1 | 0.1 | 0.3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| | R2 | 0.1 | 0.1 | 0.3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| | R3 | 0.1 | 0.1 | 0.4 | 0.3 | 0.3 | 0.3 | 0.4 | 0.3 |
| Novosphingobium | R1 | 0.1 | 0.1 | 0.4 | 0.3 | 0.3 | 0.3 | 0.4 | 0.3 |
| | R2 | 0.1 | 0.2 | 0.3 | 0.2 | 0.2 | 0.2 | 0.3 | 0.2 |
| | R3 | 0.2 | 0.1 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Ohtaekwangia | R1 | 0.2 | 0.2 | 0.1 | 0.1 | 0.0 | 0.1 | 0.1 | 0.1 |
| | R2 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R3 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Opitutus | R1 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.2 | 0.1 | 0.1 |
| | R2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.3 | 0.3 | 0.3 |
| | R3 | 0.2 | 0.1 | 0.3 | 0.2 | 0.4 | 0.4 | 0.5 | 0.4 |
| Paenibacillus | R1 | 0.1 | 0.1 | 0.4 | 0.3 | 0.5 | 0.6 | 0.8 | 0.6 |
| | R2 | 0.1 | 0.1 | 0.8 | 0.6 | 1.0 | 1.1 | 1.5 | 1.1 |
| | R3 | 0.2 | 0.1 | 1.4 | 1.0 | 1.7 | 1.9 | 2.6 | 1.9 |
| Paracoccus | R1 | 0.2 | 0.1 | 1.7 | 1.2 | 2.0 | 2.1 | 3.0 | 2.2 |
| | R2 | 0.2 | 0.1 | 1.3 | 0.9 | 1.4 | 1.5 | 2.2 | 1.6 |
| | R3 | 0.1 | 0.2 | 0.7 | 0.5 | 0.6 | 0.6 | 0.9 | 0.6 |
| Pedobacter | R1 | 0.1 | 0.3 | 0.3 | 0.2 | 0.2 | 0.1 | 0.2 | 0.1 |
| | R2 | 0.1 | 0.2 | 0.3 | 0.2 | 0.2 | 0.2 | 0.3 | 0.2 |
| | R3 | 0.1 | 0.1 | 0.2 | 0.2 | 0.2 | 0.3 | 0.3 | 0.2 |
| Phenylobacterium | R1 | 0.1 | 0.1 | 0.2 | 0.1 | 0.2 | 0.4 | 0.4 | 0.2 |
| | R2 | 0.2 | 0.1 | 0.2 | 0.2 | 0.2 | 0.3 | 0.4 | 0.2 |
| | R3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.3 | 0.3 | 0.2 |
| Pirellula | R1 | 0.2 | 0.2 | 0.3 | 0.2 | 0.2 | 0.3 | 0.3 | 0.2 |
| | R2 | 0.3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.3 | 0.2 |
| | R3 | 0.3 | 0.2 | 0.2 | 0.2 | 0.1 | 0.2 | 0.2 | 0.2 |
| Pontibacter | R1 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

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|---|---|---|---|---|---|---|---|---|---|
| | R2 | 0.3 | 0.2 | 1.1 | 0.8 | 1.0 | 1.4 | 1.5 | 1.3 |
| | R3 | 0.2 | 0.2 | 1.9 | 1.3 | 1.8 | 2.5 | 2.6 | 2.3 |
| Pseudomonas | R1 | 0.2 | 0.2 | 2.9 | 2.1 | 2.8 | 4.0 | 4.1 | 3.6 |
| | R2 | 0.2 | 0.3 | 2.1 | 1.5 | 2.0 | 2.8 | 2.9 | 2.5 |
| | R3 | 0.2 | 0.3 | 1.4 | 1.0 | 1.3 | 1.8 | 1.9 | 1.6 |
| Pseudoxanthomonas | R1 | 0.2 | 0.3 | 0.5 | 0.3 | 0.4 | 0.4 | 0.6 | 0.5 |
| | R2 | 0.2 | 0.3 | 0.4 | 0.3 | 0.3 | 0.3 | 0.4 | 0.3 |
| | R3 | 0.3 | 0.3 | 0.3 | 0.2 | 0.2 | 0.2 | 0.3 | 0.2 |
| Ramlibacter | R1 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R2 | 0.2 | 0.1 | 0.6 | 0.5 | 0.4 | 0.5 | 0.6 | 0.5 |
| | R3 | 0.2 | 0.1 | 1.4 | 1.0 | 1.0 | 1.2 | 1.3 | 1.1 |
| Rhodococcus | R1 | 0.1 | 0.1 | 1.8 | 1.3 | 1.2 | 1.6 | 1.7 | 1.4 |
| | R2 | 0.2 | 0.1 | 1.3 | 0.9 | 0.9 | 1.2 | 1.3 | 1.1 |
| | R3 | 0.2 | 0.2 | 0.5 | 0.4 | 0.5 | 0.6 | 0.6 | 0.6 |
| Saccharibacteria | R1 | 0.3 | 0.3 | 0.1 | 0.1 | 0.2 | 0.3 | 0.3 | 0.3 |
| | R2 | 0.2 | 0.2 | 0.2 | 0.1 | 0.3 | 0.4 | 0.4 | 0.4 |
| | R3 | 0.1 | 0.1 | 0.2 | 0.2 | 0.4 | 0.6 | 0.5 | 0.4 |
| Salinibacillus | R1 | 0.1 | 0.1 | 0.3 | 0.2 | 0.5 | 0.7 | 0.6 | 0.5 |
| | R2 | 0.1 | 0.1 | 0.3 | 0.2 | 0.4 | 0.6 | 0.5 | 0.4 |
| | R3 | 0.1 | 0.1 | 0.2 | 0.2 | 0.3 | 0.3 | 0.4 | 0.3 |
| Salinimicrobium | R1 | 0.1 | 0.1 | 0.3 | 0.2 | 0.3 | 0.3 | 0.4 | 0.3 |
| | R2 | 0.1 | 0.0 | 0.3 | 0.2 | 0.2 | 0.3 | 0.4 | 0.3 |
| | R3 | 0.1 | 0.0 | 0.3 | 0.2 | 0.3 | 0.3 | 0.6 | 0.5 |
| Salinisphaera | R1 | 0.1 | 0.0 | 0.3 | 0.2 | 0.2 | 0.2 | 0.6 | 0.5 |
| | R2 | 0.1 | 0.2 | 0.3 | 0.2 | 0.3 | 0.3 | 0.6 | 0.5 |
| | R3 | 0.2 | 0.3 | 0.3 | 0.2 | 0.3 | 0.4 | 0.5 | 0.4 |
| Sediminibacter | R1 | 0.2 | 0.5 | 0.4 | 0.3 | 0.5 | 0.5 | 0.5 | 0.4 |
| | R2 | 0.2 | 0.3 | 0.3 | 0.2 | 0.5 | 0.5 | 0.5 | 0.4 |
| | R3 | 0.1 | 0.2 | 0.2 | 0.1 | 0.6 | 0.6 | 0.6 | 0.5 |
| Sphaerobacter | R1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.5 | 0.6 | 0.5 | 0.4 |
| | R2 | 0.1 | 0.0 | 0.5 | 0.4 | 0.8 | 1.0 | 1.1 | 0.8 |
| | R3 | 0.1 | 0.1 | 0.8 | 0.6 | 0.9 | 1.1 | 1.3 | 0.9 |
| Sphingomonas | R1 | 0.1 | 0.1 | 1.4 | 1.0 | 1.3 | 1.6 | 1.9 | 1.4 |
| | R2 | 0.1 | 0.1 | 1.0 | 0.7 | 0.9 | 1.1 | 1.4 | 1.0 |
| | R3 | 0.1 | 0.1 | 0.7 | 0.5 | 0.6 | 0.8 | 1.0 | 0.8 |
| Sphingopyxis | R1 | 0.1 | 0.1 | 0.3 | 0.2 | 0.2 | 0.3 | 0.3 | 0.4 |
| | R2 | 0.1 | 0.1 | 0.2 | 0.2 | 0.2 | 0.3 | 0.3 | 0.3 |
| | R3 | 0.1 | 0.1 | 0.2 | 0.1 | 0.3 | 0.3 | 0.4 | 0.3 |
| Sporosarcina | R1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.3 | 0.3 | 0.4 | 0.3 |
| | R2 | 0.1 | 0.1 | 0.2 | 0.1 | 0.2 | 0.2 | 0.3 | 0.2 |
| | R3 | 0.1 | 0.1 | 0.2 | 0.2 | 0.2 | 0.1 | 0.2 | 0.2 |

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|---|---|---|---|---|---|---|---|---|---|
| Terrimonas | R1 | 0.1 | 0.1 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.2 |
| | R2 | 0.1 | 0.1 | 0.7 | 0.5 | 0.5 | 0.6 | 0.6 | 0.5 |
| | R3 | 0.1 | 0.1 | 1.3 | 0.9 | 1.0 | 1.2 | 1.3 | 1.1 |
| Thiobacillus | R1 | 0.1 | 0.1 | 1.5 | 1.1 | 1.3 | 1.6 | 1.7 | 1.4 |
| | R2 | 0.1 | 0.1 | 1.1 | 0.8 | 1.0 | 1.3 | 1.5 | 1.2 |
| | R3 | 0.1 | 0.1 | 0.7 | 0.5 | 0.7 | 1.0 | 1.2 | 1.0 |
| Truepera | R1 | 0.1 | 0.1 | 0.4 | 0.3 | 0.5 | 0.8 | 1.1 | 0.8 |
| | R2 | 0.1 | 0.1 | 0.4 | 0.3 | 0.5 | 0.8 | 1.1 | 0.9 |
| | R3 | 0.0 | 0.1 | 0.3 | 0.2 | 0.3 | 0.6 | 0.8 | 0.6 |

| | | Cultivated soil; Bulk | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Tomato | | | | | | | |
| | | Relative abundance (%) | | | | | | | |
| Genus | Replicate | NT1 | NT4 | UR1 | UR4 | AS1 | AS4 | PN1 | PN4 |
| Aciditerrimonas | R1 | 0.2 | 0.1 | 0.5 | 0.4 | 0.1 | 0.6 | 0.8 | 0.6 |
| | R2 | 0.1 | 0.1 | 0.6 | 0.4 | 0.3 | 0.6 | 0.9 | 0.7 |
| | R3 | 0.1 | 0.1 | 0.6 | 0.4 | 0.5 | 0.5 | 0.9 | 0.8 |
| Acidovorax | R1 | 0.1 | 0.1 | 0.7 | 0.5 | 0.7 | 0.6 | 1.1 | 1.0 |
| | R2 | 0.1 | 0.1 | 0.7 | 0.5 | 0.7 | 0.6 | 0.9 | 0.9 |
| | R3 | 0.2 | 0.1 | 0.9 | 0.6 | 0.7 | 0.8 | 0.8 | 0.9 |
| Adhaeribacter | R1 | 0.2 | 0.1 | 0.8 | 0.6 | 0.6 | 0.7 | 0.6 | 0.7 |
| | R2 | 0.2 | 0.1 | 0.6 | 0.5 | 0.5 | 0.6 | 0.5 | 0.6 |
| | R3 | 0.2 | 0.1 | 0.4 | 0.3 | 0.4 | 0.3 | 0.3 | 0.4 |
| Allokutzneria | R1 | 0.3 | 0.1 | 0.3 | 0.2 | 0.3 | 0.2 | 0.3 | 0.3 |
| | R2 | 0.3 | 0.2 | 0.2 | 0.2 | 0.3 | 0.2 | 0.3 | 0.3 |
| | R3 | 0.4 | 0.4 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.2 |
| Amaricoccus | R1 | 0.5 | 0.5 | 0.2 | 0.1 | 0.1 | 0.1 | 0.2 | 0.2 |
| | R2 | 0.5 | 0.4 | 0.2 | 0.2 | 0.1 | 0.1 | 0.3 | 0.2 |
| | R3 | 0.5 | 0.3 | 0.3 | 0.2 | 0.1 | 0.2 | 0.4 | 0.3 |
| Arenimonas | R1 | 0.5 | 0.2 | 0.4 | 0.3 | 0.1 | 0.3 | 0.5 | 0.3 |
| | R2 | 0.5 | 0.3 | 0.5 | 0.3 | 0.3 | 0.3 | 0.5 | 0.4 |
| | R3 | 0.4 | 0.3 | 0.5 | 0.4 | 0.3 | 0.3 | 0.4 | 0.4 |
| Arthrobacter | R1 | 0.5 | 0.4 | 0.8 | 0.5 | 0.5 | 0.3 | 0.5 | 0.5 |
| | R2 | 0.4 | 0.3 | 0.6 | 0.5 | 0.5 | 0.4 | 0.4 | 0.4 |
| | R3 | 0.3 | 0.2 | 0.6 | 0.4 | 0.7 | 0.6 | 0.3 | 0.3 |
| Azotobacter | R1 | 0.2 | 0.1 | 0.4 | 0.3 | 0.6 | 0.6 | 0.2 | 0.1 |
| | R2 | 0.2 | 0.2 | 2.7 | 1.9 | 1.7 | 2.3 | 2.2 | 2.0 |
| | R3 | 0.3 | 0.4 | 5.7 | 4.0 | 3.2 | 4.5 | 4.9 | 4.4 |
| Bacillus | R1 | 0.4 | 0.4 | 7.3 | 5.2 | 4.0 | 5.7 | 6.4 | 5.7 |
| | R2 | 0.4 | 0.4 | 6.7 | 4.8 | 4.1 | 5.5 | 6.5 | 5.7 |
| | R3 | 0.4 | 0.3 | 4.8 | 3.4 | 3.4 | 4.2 | 5.2 | 4.5 |
| Bradyrhizobium | R1 | 0.5 | 0.3 | 5.2 | 3.7 | 4.1 | 4.8 | 6.2 | 5.3 |
| | R2 | 0.3 | 0.2 | 3.4 | 2.4 | 2.7 | 3.2 | 4.1 | 3.5 |
| | R3 | 0.2 | 0.1 | 2.2 | 1.5 | 1.8 | 2.1 | 2.6 | 2.2 |
| Brevundimonas | R1 | 0.1 | 0.1 | 0.2 | 0.1 | 0.2 | 0.3 | 0.2 | 0.1 |
| | R2 | 0.1 | 0.1 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| | R3 | 0.1 | 0.1 | 0.4 | 0.3 | 0.2 | 0.2 | 0.2 | 0.2 |
| Caulobacter | R1 | 0.1 | 0.1 | 0.4 | 0.3 | 0.2 | 0.1 | 0.2 | 0.2 |
| | R2 | 0.1 | 0.1 | 0.4 | 0.3 | 0.2 | 0.1 | 0.1 | 0.2 |
| | R3 | 0.1 | 0.2 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 |
| Chryseobacterium | R1 | 0.1 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 |

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| | R2 | 0.2 | 0.2 | 1.1 | 0.8 | 0.6 | 0.8 | 0.9 | 0.8 |
| | R3 | 0.2 | 0.2 | 1.8 | 1.3 | 1.1 | 1.5 | 1.6 | 1.4 |
| Comamonas | R1 | 0.3 | 0.1 | 2.8 | 2.0 | 1.8 | 2.4 | 2.5 | 2.2 |
| | R2 | 0.4 | 0.2 | 3.0 | 2.1 | 1.8 | 2.4 | 2.7 | 2.2 |
| | R3 | 0.4 | 0.3 | 3.0 | 2.1 | 1.7 | 2.3 | 2.7 | 2.2 |
| Cupriavidus | R1 | 0.4 | 0.3 | 3.2 | 2.3 | 1.7 | 2.3 | 2.9 | 2.2 |
| | R2 | 0.3 | 0.3 | 2.2 | 1.6 | 1.2 | 1.7 | 2.0 | 1.5 |
| | R3 | 0.3 | 0.2 | 1.5 | 1.0 | 0.8 | 1.2 | 1.3 | 1.1 |
| Devosia | R1 | 0.2 | 0.1 | 0.3 | 0.2 | 0.1 | 0.3 | 0.3 | 0.3 |
| | R2 | 0.3 | 0.3 | 1.0 | 0.7 | 0.6 | 0.8 | 1.3 | 0.9 |
| | R3 | 0.3 | 0.4 | 1.5 | 1.0 | 1.0 | 1.2 | 2.0 | 1.4 |
| Ensifer | R1 | 0.4 | 0.6 | 2.3 | 1.6 | 1.6 | 1.7 | 3.3 | 2.2 |
| | R2 | 0.4 | 0.5 | 1.8 | 1.3 | 1.3 | 1.4 | 2.5 | 1.8 |
| | R3 | 0.4 | 0.3 | 1.4 | 1.0 | 1.1 | 1.2 | 2.0 | 1.5 |
| Flavisolibacter | R1 | 0.3 | 0.1 | 0.7 | 0.5 | 0.8 | 0.8 | 1.1 | 1.0 |
| | R2 | 0.4 | 0.2 | 0.6 | 0.4 | 0.6 | 0.6 | 0.8 | 0.7 |
| | R3 | 0.4 | 0.2 | 0.5 | 0.3 | 0.4 | 0.4 | 0.5 | 0.5 |
| Flavitalea | R1 | 0.4 | 0.2 | 0.3 | 0.2 | 0.1 | 0.2 | 0.1 | 0.1 |
| | R2 | 0.4 | 0.2 | 1.7 | 1.2 | 1.5 | 1.7 | 2.3 | 1.9 |
| | R3 | 0.5 | 0.2 | 3.6 | 2.5 | 3.3 | 3.6 | 5.2 | 4.1 |
| Flavobacterium | R1 | 0.4 | 0.1 | 4.6 | 3.2 | 4.4 | 4.7 | 6.9 | 5.5 |
| | R2 | 0.3 | 0.1 | 3.3 | 2.4 | 3.3 | 3.5 | 5.0 | 4.0 |
| | R3 | 0.2 | 0.1 | 1.7 | 1.2 | 1.9 | 2.0 | 2.6 | 2.1 |
| Fontibacillus | R1 | 0.2 | 0.1 | 0.7 | 0.5 | 1.0 | 1.0 | 1.1 | 1.0 |
| | R2 | 0.1 | 0.1 | 0.8 | 0.6 | 1.0 | 1.0 | 1.3 | 1.1 |
| | R3 | 0.1 | 0.1 | 0.7 | 0.5 | 0.8 | 0.8 | 1.2 | 1.0 |
| Gemmatimonas | R1 | 0.1 | 0.1 | 0.9 | 0.6 | 1.0 | 0.9 | 1.5 | 1.3 |
| | R2 | 0.1 | 0.1 | 0.6 | 0.4 | 0.7 | 0.7 | 1.2 | 1.0 |
| | R3 | 0.1 | 0.1 | 0.4 | 0.3 | 0.6 | 0.6 | 1.0 | 0.8 |
| Gp10 | R1 | 0.2 | 0.1 | 0.0 | 0.0 | 0.3 | 0.4 | 0.7 | 0.5 |
| | R2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.3 | 0.4 | 0.8 | 0.6 |
| | R3 | 0.1 | 0.1 | 0.2 | 0.2 | 0.3 | 0.4 | 0.9 | 0.6 |
| Gp4 | R1 | 0.0 | 0.1 | 0.4 | 0.3 | 0.4 | 0.5 | 1.0 | 0.8 |
| | R2 | 0.1 | 0.1 | 0.5 | 0.4 | 0.4 | 0.5 | 0.8 | 0.7 |
| | R3 | 0.1 | 0.1 | 0.6 | 0.4 | 0.4 | 0.6 | 0.7 | 0.6 |
| Gp6 | R1 | 0.2 | 0.0 | 0.7 | 0.5 | 0.4 | 0.7 | 0.5 | 0.5 |
| | R2 | 0.2 | 0.0 | 0.7 | 0.5 | 0.4 | 0.6 | 0.6 | 0.5 |
| | R3 | 0.2 | 0.1 | 0.7 | 0.5 | 0.4 | 0.5 | 0.6 | 0.5 |
| Gp7 | R1 | 0.2 | 0.1 | 0.6 | 0.5 | 0.3 | 0.4 | 0.7 | 0.5 |
| | R2 | 0.1 | 0.1 | 0.6 | 0.5 | 0.3 | 0.5 | 0.7 | 0.6 |
| | R3 | 0.1 | 0.2 | 0.8 | 0.5 | 0.3 | 0.6 | 0.9 | 0.8 |

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| Halomonas | R1 | 0.1 | 0.1 | 0.6 | 0.5 | 0.2 | 0.6 | 0.8 | 0.8 |
| | R2 | 0.1 | 0.2 | 1.9 | 1.4 | 1.4 | 2.0 | 2.6 | 2.0 |
| | R3 | 0.1 | 0.3 | 3.6 | 2.6 | 2.8 | 3.8 | 4.8 | 3.6 |
| Hyphomicrobium | R1 | 0.2 | 0.4 | 4.7 | 3.3 | 3.7 | 4.8 | 6.2 | 4.6 |
| | R2 | 0.1 | 0.3 | 3.4 | 2.4 | 2.6 | 3.3 | 4.4 | 3.3 |
| | R3 | 0.0 | 0.1 | 1.6 | 1.1 | 1.1 | 1.4 | 1.9 | 1.4 |
| Ilumatobacter | R1 | 0.0 | 0.1 | 0.7 | 0.5 | 0.3 | 0.2 | 0.5 | 0.4 |
| | R2 | 0.1 | 0.1 | 0.7 | 0.5 | 0.2 | 0.2 | 0.4 | 0.3 |
| | R3 | 0.1 | 0.1 | 0.8 | 0.5 | 0.2 | 0.2 | 0.3 | 0.2 |
| Litorilinea | R1 | 0.2 | 0.1 | 0.7 | 0.5 | 0.2 | 0.2 | 0.1 | 0.1 |
| | R2 | 0.1 | 0.1 | 0.6 | 0.4 | 0.2 | 0.3 | 0.2 | 0.2 |
| | R3 | 0.0 | 0.1 | 0.5 | 0.3 | 0.2 | 0.4 | 0.3 | 0.2 |
| Luteimonas | R1 | 0.0 | 0.1 | 0.4 | 0.3 | 0.2 | 0.4 | 0.3 | 0.2 |
| | R2 | 0.0 | 0.1 | 0.4 | 0.3 | 0.2 | 0.3 | 0.3 | 0.2 |
| | R3 | 0.1 | 0.1 | 0.3 | 0.2 | 0.1 | 0.2 | 0.3 | 0.2 |
| Lysobacter | R1 | 0.1 | 0.1 | 0.4 | 0.3 | 0.1 | 0.2 | 0.3 | 0.2 |
| | R2 | 0.1 | 0.1 | 0.4 | 0.3 | 0.1 | 0.2 | 0.3 | 0.2 |
| | R3 | 0.1 | 0.1 | 0.4 | 0.3 | 0.1 | 0.2 | 0.2 | 0.1 |
| Marmoricola | R1 | 0.1 | 0.1 | 0.4 | 0.3 | 0.1 | 0.2 | 0.2 | 0.1 |
| | R2 | 0.1 | 0.0 | 0.3 | 0.2 | 0.1 | 0.2 | 0.1 | 0.1 |
| | R3 | 0.1 | 0.0 | 0.3 | 0.2 | 0.1 | 0.2 | 0.1 | 0.1 |
| Massilia | R1 | 0.1 | 0.0 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R2 | 0.2 | 0.1 | 0.3 | 0.2 | 0.3 | 0.2 | 0.2 | 0.2 |
| | R3 | 0.3 | 0.2 | 0.4 | 0.3 | 0.5 | 0.2 | 0.3 | 0.2 |
| Mesorhizobium | R1 | 0.4 | 0.3 | 0.5 | 0.3 | 0.7 | 0.3 | 0.5 | 0.3 |
| | R2 | 0.3 | 0.2 | 0.5 | 0.3 | 0.6 | 0.4 | 0.5 | 0.4 |
| | R3 | 0.3 | 0.2 | 0.5 | 0.3 | 0.5 | 0.5 | 0.6 | 0.5 |
| Methylohalomonas | R1 | 0.2 | 0.1 | 0.5 | 0.4 | 0.4 | 0.6 | 0.6 | 0.6 |
| | R2 | 0.2 | 0.1 | 0.5 | 0.4 | 0.4 | 0.6 | 0.6 | 0.6 |
| | R3 | 0.2 | 0.1 | 0.5 | 0.4 | 0.4 | 0.6 | 0.6 | 0.5 |
| Microbacterium | R1 | 0.2 | 0.1 | 0.5 | 0.3 | 0.4 | 0.6 | 0.6 | 0.5 |
| | R2 | 0.2 | 0.1 | 0.4 | 0.2 | 0.3 | 0.4 | 0.5 | 0.4 |
| | R3 | 0.3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.3 | 0.4 | 0.3 |
| Microvirga | R1 | 0.3 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.2 | 0.1 |
| | R2 | 0.3 | 0.1 | 0.5 | 0.4 | 0.8 | 0.5 | 0.9 | 0.8 |
| | R3 | 0.2 | 0.1 | 0.9 | 0.6 | 1.3 | 0.9 | 1.6 | 1.3 |
| Mycobacterium | R1 | 0.2 | 0.1 | 1.4 | 1.0 | 2.0 | 1.4 | 2.4 | 2.1 |
| | R2 | 0.1 | 0.1 | 1.1 | 0.8 | 1.5 | 1.1 | 1.8 | 1.6 |
| | R3 | 0.1 | 0.1 | 0.8 | 0.6 | 1.0 | 0.9 | 1.2 | 1.1 |
| Nitriliruptor | R1 | 0.1 | 0.1 | 0.4 | 0.3 | 0.3 | 0.6 | 0.4 | 0.5 |
| | R2 | 0.1 | 0.1 | 0.6 | 0.5 | 0.8 | 1.2 | 1.3 | 1.0 |

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| | R3 | 0.2 | 0.2 | 1.1 | 0.8 | 1.6 | 2.1 | 2.6 | 1.9 |
| Nitrosococcus | R1 | 0.2 | 0.2 | 1.3 | 0.9 | 2.0 | 2.5 | 3.3 | 2.2 |
| | R2 | 0.2 | 0.2 | 1.7 | 1.2 | 1.9 | 2.3 | 2.4 | 1.6 |
| | R3 | 0.1 | 0.1 | 1.8 | 1.3 | 1.4 | 1.6 | 0.9 | 0.7 |
| Nitrosomonas | R1 | 0.2 | 0.1 | 2.4 | 1.7 | 1.5 | 1.6 | 0.3 | 0.3 |
| | R2 | 0.2 | 0.1 | 2.3 | 1.6 | 1.5 | 1.6 | 1.2 | 1.0 |
| | R3 | 0.2 | 0.1 | 2.1 | 1.5 | 1.4 | 1.5 | 1.9 | 1.6 |
| Nitrosospira | R1 | 0.2 | 0.2 | 2.1 | 1.5 | 1.4 | 1.6 | 3.0 | 2.5 |
| | R2 | 0.1 | 0.2 | 1.6 | 1.1 | 1.1 | 1.2 | 2.2 | 1.8 |
| | R3 | 0.1 | 0.2 | 1.4 | 1.0 | 1.0 | 1.0 | 1.8 | 1.4 |
| Nitrosovibrio | R1 | 0.1 | 0.2 | 0.7 | 0.5 | 0.5 | 0.4 | 0.7 | 0.5 |
| | R2 | 0.1 | 0.2 | 0.6 | 0.4 | 0.5 | 0.4 | 0.6 | 0.4 |
| | R3 | 0.1 | 0.1 | 0.4 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| Nocardioides | R1 | 0.2 | 0.1 | 0.4 | 0.3 | 0.2 | 0.3 | 0.3 | 0.2 |
| | R2 | 0.1 | 0.1 | 0.4 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| | R3 | 0.1 | 0.2 | 0.5 | 0.3 | 0.3 | 0.3 | 0.4 | 0.4 |
| Novosphingobium | R1 | 0.1 | 0.2 | 0.4 | 0.3 | 0.3 | 0.3 | 0.5 | 0.4 |
| | R2 | 0.2 | 0.2 | 0.4 | 0.3 | 0.3 | 0.2 | 0.4 | 0.3 |
| | R3 | 0.2 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.2 | 0.1 |
| Ohtaekwangia | R1 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R2 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R3 | 0.4 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Opitutus | R1 | 0.4 | 0.2 | 0.2 | 0.1 | 0.1 | 0.2 | 0.1 | 0.1 |
| | R2 | 0.3 | 0.2 | 0.3 | 0.2 | 0.3 | 0.4 | 0.4 | 0.3 |
| | R3 | 0.2 | 0.2 | 0.4 | 0.3 | 0.4 | 0.5 | 0.7 | 0.5 |
| Paenibacillus | R1 | 0.1 | 0.1 | 0.5 | 0.4 | 0.6 | 0.7 | 1.0 | 0.8 |
| | R2 | 0.1 | 0.1 | 1.0 | 0.7 | 1.1 | 1.2 | 1.8 | 1.4 |
| | R3 | 0.2 | 0.1 | 1.8 | 1.3 | 2.0 | 2.1 | 3.2 | 2.5 |
| Paracoccus | R1 | 0.3 | 0.1 | 2.1 | 1.5 | 2.3 | 2.4 | 3.7 | 2.9 |
| | R2 | 0.2 | 0.1 | 1.6 | 1.2 | 1.7 | 1.8 | 2.7 | 2.1 |
| | R3 | 0.2 | 0.2 | 0.8 | 0.6 | 0.8 | 0.7 | 1.1 | 0.8 |
| Pedobacter | R1 | 0.2 | 0.3 | 0.4 | 0.3 | 0.3 | 0.1 | 0.3 | 0.2 |
| | R2 | 0.2 | 0.2 | 0.4 | 0.3 | 0.3 | 0.2 | 0.3 | 0.2 |
| | R3 | 0.2 | 0.1 | 0.3 | 0.2 | 0.3 | 0.3 | 0.4 | 0.3 |
| Phenylobacterium | R1 | 0.2 | 0.1 | 0.3 | 0.2 | 0.3 | 0.4 | 0.4 | 0.3 |
| | R2 | 0.2 | 0.1 | 0.3 | 0.2 | 0.3 | 0.4 | 0.4 | 0.3 |
| | R3 | 0.2 | 0.2 | 0.3 | 0.2 | 0.2 | 0.3 | 0.4 | 0.3 |
| Pirellula | R1 | 0.3 | 0.3 | 0.3 | 0.2 | 0.2 | 0.3 | 0.4 | 0.3 |
| | R2 | 0.4 | 0.3 | 0.3 | 0.2 | 0.2 | 0.3 | 0.3 | 0.3 |
| | R3 | 0.4 | 0.2 | 0.3 | 0.2 | 0.2 | 0.2 | 0.3 | 0.2 |
| Pontibacter | R1 | 0.4 | 0.2 | 0.3 | 0.2 | 0.1 | 0.1 | 0.2 | 0.1 |

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| | R2 | 0.4 | 0.2 | 1.4 | 1.0 | 1.2 | 1.6 | 1.8 | 1.7 |
| | R3 | 0.3 | 0.3 | 2.3 | 1.7 | 2.1 | 2.9 | 3.2 | 3.0 |
| Pseudomonas | R1 | 0.2 | 0.3 | 3.6 | 2.6 | 3.4 | 4.5 | 5.0 | 4.7 |
| | R2 | 0.3 | 0.3 | 2.6 | 1.8 | 2.4 | 3.2 | 3.6 | 3.4 |
| | R3 | 0.3 | 0.3 | 1.7 | 1.2 | 1.5 | 2.0 | 2.3 | 2.2 |
| Pseudoxanthomonas | R1 | 0.3 | 0.4 | 0.6 | 0.4 | 0.5 | 0.5 | 0.7 | 0.6 |
| | R2 | 0.3 | 0.3 | 0.4 | 0.3 | 0.3 | 0.3 | 0.5 | 0.4 |
| | R3 | 0.4 | 0.3 | 0.4 | 0.3 | 0.2 | 0.3 | 0.4 | 0.3 |
| Ramlibacter | R1 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| | R2 | 0.3 | 0.2 | 0.8 | 0.6 | 0.5 | 0.6 | 0.7 | 0.6 |
| | R3 | 0.2 | 0.1 | 1.7 | 1.2 | 1.1 | 1.4 | 1.7 | 1.4 |
| Rhodococcus | R1 | 0.2 | 0.1 | 2.2 | 1.6 | 1.5 | 1.8 | 2.1 | 1.8 |
| | R2 | 0.2 | 0.1 | 1.6 | 1.1 | 1.1 | 1.3 | 1.6 | 1.4 |
| | R3 | 0.3 | 0.3 | 0.7 | 0.5 | 0.6 | 0.7 | 0.8 | 0.7 |
| Saccharibacteria | R1 | 0.4 | 0.3 | 0.2 | 0.1 | 0.3 | 0.3 | 0.4 | 0.4 |
| | R2 | 0.3 | 0.2 | 0.2 | 0.2 | 0.4 | 0.4 | 0.5 | 0.5 |
| | R3 | 0.2 | 0.1 | 0.3 | 0.2 | 0.5 | 0.6 | 0.7 | 0.6 |
| Salinibacillus | R1 | 0.1 | 0.1 | 0.4 | 0.3 | 0.6 | 0.7 | 0.7 | 0.6 |
| | R2 | 0.1 | 0.1 | 0.4 | 0.3 | 0.5 | 0.6 | 0.7 | 0.6 |
| | R3 | 0.1 | 0.1 | 0.3 | 0.2 | 0.3 | 0.4 | 0.4 | 0.4 |
| Salinimicrobium | R1 | 0.1 | 0.1 | 0.3 | 0.2 | 0.3 | 0.3 | 0.4 | 0.4 |
| | R2 | 0.1 | 0.0 | 0.3 | 0.2 | 0.3 | 0.3 | 0.6 | 0.5 |
| | R3 | 0.1 | 0.0 | 0.4 | 0.3 | 0.3 | 0.3 | 0.8 | 0.6 |
| Salinisphaera | R1 | 0.1 | 0.0 | 0.3 | 0.2 | 0.3 | 0.3 | 0.8 | 0.7 |
| | R2 | 0.2 | 0.2 | 0.4 | 0.3 | 0.4 | 0.4 | 0.7 | 0.6 |
| | R3 | 0.2 | 0.3 | 0.4 | 0.3 | 0.4 | 0.4 | 0.6 | 0.5 |
| Sediminibacter | R1 | 0.3 | 0.6 | 0.6 | 0.4 | 0.5 | 0.6 | 0.6 | 0.6 |
| | R2 | 0.2 | 0.4 | 0.4 | 0.3 | 0.5 | 0.6 | 0.6 | 0.6 |
| | R3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.7 | 0.7 | 0.7 | 0.7 |
| Sphaerobacter | R1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.6 | 0.7 | 0.7 | 0.6 |
| | R2 | 0.1 | 0.0 | 0.6 | 0.4 | 0.9 | 1.1 | 1.3 | 1.1 |
| | R3 | 0.1 | 0.1 | 1.1 | 0.7 | 1.0 | 1.2 | 1.6 | 1.2 |
| Sphingomonas | R1 | 0.2 | 0.1 | 1.8 | 1.2 | 1.5 | 1.8 | 2.4 | 1.8 |
| | R2 | 0.2 | 0.1 | 1.2 | 0.9 | 1.0 | 1.3 | 1.7 | 1.3 |
| | R3 | 0.2 | 0.1 | 0.9 | 0.6 | 0.7 | 0.9 | 1.2 | 1.0 |
| Sphingopyxis | R1 | 0.2 | 0.1 | 0.3 | 0.2 | 0.2 | 0.3 | 0.4 | 0.5 |
| | R2 | 0.1 | 0.1 | 0.3 | 0.2 | 0.2 | 0.3 | 0.4 | 0.4 |
| | R3 | 0.1 | 0.1 | 0.3 | 0.2 | 0.3 | 0.4 | 0.5 | 0.5 |
| Sporosarcina | R1 | 0.1 | 0.1 | 0.2 | 0.1 | 0.3 | 0.3 | 0.5 | 0.4 |
| | R2 | 0.1 | 0.1 | 0.3 | 0.2 | 0.3 | 0.3 | 0.4 | 0.3 |
| | R3 | 0.1 | 0.1 | 0.3 | 0.2 | 0.2 | 0.1 | 0.2 | 0.2 |

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|---|---|---|---|---|---|---|---|---|---|
| Terrimonas | R1 | 0.2 | 0.1 | 0.4 | 0.3 | 0.2 | 0.1 | 0.2 | 0.2 |
| | R2 | 0.2 | 0.1 | 0.9 | 0.6 | 0.6 | 0.7 | 0.8 | 0.7 |
| | R3 | 0.2 | 0.1 | 1.6 | 1.1 | 1.2 | 1.4 | 1.6 | 1.4 |
| Thiobacillus | R1 | 0.2 | 0.1 | 1.9 | 1.4 | 1.5 | 1.8 | 2.1 | 1.8 |
| | R2 | 0.1 | 0.1 | 1.4 | 1.0 | 1.2 | 1.5 | 1.8 | 1.6 |
| | R3 | 0.1 | 0.1 | 0.8 | 0.6 | 0.8 | 1.1 | 1.5 | 1.3 |
| Truepera | R1 | 0.1 | 0.2 | 0.4 | 0.3 | 0.5 | 0.9 | 1.3 | 1.1 |
| | R2 | 0.1 | 0.2 | 0.5 | 0.3 | 0.6 | 0.9 | 1.4 | 1.1 |
| | R3 | 0.1 | 0.1 | 0.3 | 0.2 | 0.4 | 0.6 | 1.0 | 0.8 |