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Insights into the abundance, expression and diversity of key denitrification genes in an ecologically managed greenhouse agricultural soil

R. Hernández Maqueda^{1,2*} , I. Ballesteros³ , D. Meca⁴, R. Linacero³ and F. del Moral¹

Abstract

Understanding the bacteria associated with nitrification and denitrification is crucial for comprehending the processes that lead to nitrous oxide emissions in agricultural greenhouse soils. Therefore, it is important to determine their abundance and expression to gain insight into these processes.

The aim of this study was to explore the bacterial communities associated with denitrification in a greenhouse agricultural soil amended with crop residues and manure for six years. For this purpose, we proceeded to detect and quantify the genes *nirK* and *nirS* and the gene *nosZ* through clone library construction, sequencing, phylogenetic analysis, and quantitative polymerase chain reaction (qPCR). Sequence analysis based on the clone library revealed that many of the *nirS* or *nirK* genes detected were not closely related to known denitrifier bacteria, but some of the *nosZ* sequences were related to the genera such as *Pseudomonas*, *Halomonas*, and *Marinobacter*. Furthermore, the qPCR revealed a high abundance of DNA copies in *nirK*, $6.08 \times 10^9 \pm 1.16 \times 10^9$, while *nirS* and *nosZ* showed lower values, $9.05 \times 10^6 \pm 1.65 \times 10^6$ and $8.71 \times 10^6 \pm 1.44 \times 10^6$, respectively. However, the highest expression rate was observed for *nirS* (mRNA/DNA ratio = 3.10×10^{-3}), while *nirK* and *nosZ* showed 10-fold lower expression rates (4.4×10^{-4} and 3.5×10^{-4} , respectively). The results of this work provide a preliminary overview of the diversity, abundance and expression of key genes associated with the denitrification process in this type of soil and are a starting point for further studies to understand how this type of soil management can influence the denitrification process.

Keywords Denitrifying genes, Nitrous oxide, *nirK*, *nirS*, *nosZ*

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Introduction

The denitrification process involves the progressive reduction of soluble nitrogen in the form of chemical ions, nitrate (NO₃) or nitrite (NO₂) to the gases nitric oxide (NO), nitrous oxide (N₂O), and molecular nitrogen (N₂). In agricultural soils, denitrification is considered to be one of the main sources of nitrogen loss [1], especially in intensive vegetable production systems in greenhouses [2]. This loss of nitrogen not only reduces crop efficiency and profitability but also contributes to global warming, as N₂O is considered the third most important greenhouse gas [3, 4].

One of the main approaches to understanding the denitrification process is to study the microbial dynamics involved in this pathway. To this end, one of the most widely employed approaches is based on the analysis of the diversity and abundance of functional genes involved in denitrification. Among the genes involved, *nirK*, *nirS*, and *nosZ* are the gene markers most studied [5–8]. The reduction of NO₂ to NO is carried out by two nonhomologous enzymes named nitrite reductases (NIR), encoded by *nirK* (Cu-NIR) and *nirS* (cd1-NIR). Organisms harboring these enzymes often coexist, although in different ratios depending on the environment [9]. In contrast, nitrous oxide reductase (NOS) is the only known enzyme responsible for the reduction of nitrous oxide to N₂ and is present in a wide range of soil microorganisms [8].

Studies conducted in agricultural soils in recent years have shown that the abundance and diversity of *nirK*, *nirS*, and *nosZ* are influenced by N fertilizer additions [10], type of fertilization (organic vs. mineral) [1, 11–13], management practices (tillage vs. no-till farming) [14], and soil physicochemical properties, particularly pH [15].

Despite these advances, many studies provide results that contradict each other, even for soils with similar characteristics, as shown by Ouyang et al. [9], reflecting that we are still far from understanding all the mechanisms that trigger this process.

In the southern Iberian Peninsula, intensive agriculture is predominant and characterized by the use of agrochemicals, which have contributed to higher productivity but caused a loss of soil quality [16], as well as a loss of biodiversity [17, 18]. To reverse this process, and in order to generate efficient farming systems in which soil biodiversity is not affected, many attempts have been made to replace mineral fertilizers with others based on ecological assumptions, such as the application of crop residues and manure [16, 19, 20]. These studies showed that the application of these organic amendments had a positive effect on several soil quality parameters, but they did not analyze whether these amendments had an effect on the denitrification process.

To address this lack of knowledge, in this study we analyze the denitrification process, through the analysis of

the functional genes *nirK*, *nirS* and *nosZ*, in an agricultural soil under greenhouse conditions to which organic amendments based on the application of crop residues and manure have been applied for six years.

Therefore, the main objective of this work was to characterize the abundance and expression of the functional genes *nirK*, *nirS* and *nosZ*, to understand the dynamics related to the denitrification process, as well as to make a first approach to determine the associated bacterial population.

Materials and methods

Soil sampling

Soil sampling was performed on an agricultural soil managed according to the principles of ecological intensification defined by Bommarco et al. [21], Kleijn et al. [22], which consists in maximizing production while reducing environmental impacts by reducing anthropogenic inputs such as inorganic fertilizers and pesticides. In the case of the greenhouse under study, inorganic fertilizers have been replaced by crop residue and manure-based fertilization over the last six years. The greenhouse is located at the Palmerillas Experimental Station (Cajamar Foundation) in the Spanish province of Almería (36° 48' N; 2° 3' W longitude and 155 m altitude). Soil samples were collected during the 2020–2021 season, in which the crop in production was eggplant (*Solanum melongena* L. var. Telma). Three crop lines were randomly selected; within each line, three soil subsamples were collected at a depth of 0–15 cm, and 15 cm from the center line of randomly selected plants. The subsamples were thoroughly mixed to produce a composite sample. Fifty grams of each composite sample was immediately snap-frozen in liquid nitrogen. The samples were stored at -80 °C for DNA/RNA extraction.

Soil properties

The following physicochemical characteristics were analyzed: Soil Moisture (SM), Temperature (T), Electrical Conductivity (EC), Total Organic Carbon (TOC), Total Nitrogen (TN), Carbon to Nitrogen ratio (C: N), and pH.

SM, soil temperature, and electrical conductivity (EC, dSm-1) were measured with a soil sensor (Teros 12, Meter Group), TOC was measured on fine ground soil using the wet oxidation method according to [23], TN was measured with an elemental analyzer ELEMENTAR Rapid N, and finally pH was measured in a 1:5 soil: water suspension (v/v) with a Crison basic 20 pH meter (UNE-ISO 10390:2012).

Nucleic acid extraction

Genomic DNA and total RNA were isolated from 1 g of each soil sample by employing the RNeasy PowerSoil® Total RNA Isolation and RNeasy PowerSoil® DNA

Elution Kit (Qiagen®, Valencia, CA), respectively, according to the manufacturer's instructions. Each extraction was carried out in duplicate. Extracted RNA and DNA were quantified using a Nanodrop ND-1000 spectrophotometer (ThermoFisher Scientific Inc., United States) and stored at -80°C. The absence of DNA carryovers in the RNA samples was verified by PCR without reverse transcription, and the remaining DNA was removed using NZY DNase I (Nzytech, Portugal). Replicate DNA and RNA samples were pooled. The Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific Inc., United States) was used to obtain complementary DNA (cDNA) suitable for qPCR analyses. Briefly, 10 µL of RNA was used in a 20 µL reverse transcription reaction using random primers.

Conventional polymerase chain reaction (PCR)

In order to optimize amplification conditions and detect the presence of genes, samples were primarily amplified by conventional PCR. The primers used for amplification and their sequences are listed in Table 1.

Conventional PCR assays were performed in a SensoQuest LabCycler (Progen Scientific Ltd, Germany) for a final volume of 20 µL, containing 5 µL of 10-fold diluted DNA template, 0.2 µM reverse and forward primers, 0.01% BSA (bovine serum albumin), and 1x DNA AmpliTools Master Mix (Biotools, Loganholme, Australia). The PCR program included an initial step at 95 °C, 2 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s (*nirK* and *nirS* primers), or at 62 °C for 30 s (*nosZ* primers), elongation at 72 °C for 45 s, and a final step at 72 °C for 5 min.

Clone libraries, sequencing, and phylogenetics analysis

Clones of the PCR product were obtained using the TOPO® TA Cloning® Kit with pCR™4 vector and transformed into *Escherichia coli* DH5α™ competent cells. Plasmids were purified with SPEEDTOOLS Plasmid DNA Purification kit (Biotools, Loganholme, Australia). Sequencing was performed in an ABI PRISM 3700

sequencer (Applied Biosystems, Foster City, CA, USA) at the Genomics Unit (Universidad Complutense de Madrid).

Sequences were edited and manually aligned using PhyDE® [26] and then compared with those in the GenBank nonredundant nucleotide (nr/nt) database using BLASTN on the NCBI's homepage (<http://www.ncbi.nlm.nih.gov/blast>). All the sequences were deposited in the GenBank with accession numbers as follows OQ344290-OQ344309 (*nirS*), OQ360659-OQ360682 (*nirK*), and OQ344310-OQ344329 (*nosZ*). The sequences obtained were aligned with the closest matches of the target genes published in the databases.

Phylogenetic analysis of the cloned sequences of each gene was carried out, along with similar sequences retrieved from the GenBank, by a maximum likelihood method using MEGA11 software [27]. The model was selected according to the Akaike Information Criterion (AIC). Tree topologies and branch lengths were computed with 500 bootstrap replicates. To estimate the number of groups that could be assigned to different OTUs (operational taxonomic units) within the cloned sequences, we used the assemble species by automatic partitioning (ASAP) method [28]. The method was applied through an online tool (<https://bioinfo.mnhn.fr/abi/public/asap>, accessed on April 27th, 2023, with default parameters and a p-distance substitution model).

Quantitative PCR

A quantitative polymerase chain reaction (qPCR) assay was used to quantify the *nirK*, *nirS*, and *nosZ* genes and transcripts using an Applied Biosystems® 7900HT Fast Real-Time PCR (ThermoFisher Scientific Inc., United States). The primers used in the qPCR assay were the same as those used in conventional PCR tests.

Purified DNA was diluted 10-fold and cDNA was diluted 3-fold. We used 3 µL of diluted template for DNA quantification and 5 µL of diluted template for cDNA quantification. Reactions were performed with diluted template, 1x Applied Biosystems™ SYBR™ Green PCR Master Mix, 0.2 µM reverse and forward primers, 0.01% BSA, and molecular biology grade water for a final volume of 15 µL. Given that we used a standardized nucleic acid extraction kit, we chose a fixed volume of template rather than a fixed concentration to avoid bias in DNA/RNA quantification. To avoid the influence of inhibitors in our PCR tests, we determined the optimal template concentration.

Thermal cycling conditions for functional genes were as follows: 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C (*nirK* and *nirS*) or 62 °C (*nosZ*) for 1 min; and one cycle (melting curve stage) at 95 °C for 15 s.

For each qPCR test, the specificity of the amplification was checked using melting curves. Gene or transcript

Table 1 Primers used in this study. Sequences for each primer, annealing temperature and reference authors are indicated

Primer name	Sequence 5'-3'	Annealing Temp (°C)	Reference author
nirKC2F	TGC ACA TCG CCA ACG GNA TGT WYG G	54	[5, 24]
nirKC2R	GGC GCG GAA GAT GSH RTG RTC NAC	54	
nirSC1F	ATC GTC AAC GTC AAR GAR ACV GG	54	
nirSC1R	TTC GGG TGC GTC GTC TTS ABG AAS AG	54	
NosZ1F	ATG TCG ATC ARC TGV K CR TTY TC	56	[14,
NosZ1R	WCS YTG TTC MTC GAC AGC CAG	56	25]

copy numbers were calculated using standard curves generated from serial 10-fold dilutions of cloned plasmids containing specific gene fragments. Results were expressed as gene copies or transcripts per gram of dry soil. PCR efficiency ranged from 86 to 106% with an R^2 value greater than 0.995.

Statistical analysis

Statistical analyses were conducted using the free software R, version 4.2.3 [29]. The primary packages utilized were *readxl* version 1.3.2 for spreadsheet reading and *WRS2* version 1.1-4 for computing the mean and standard deviation of DNA copy numbers and transcripts for each gene, based on three composite samples with two nucleic acid extractions per sample ($n=6$). Graphics were generated using the *ggplot2* package, version 3.4.1.

Results and discussion

Physicochemical soil properties

The analysis of basic soil properties revealed the following results: the volumetric water content (SM) was 0.137 ± 0.02 , the temperature ranged from 17.2 to 18.2, EC was 4.13 ± 2.63 dSm⁻¹, TOC was 30.8 ± 0.42 g kg⁻¹ soil, TN was 9.8 ± 0.23 g kg⁻¹ soil, the C: N ratio was 3.14 ± 0.79 , and finally, the pH was 7.9 ± 0.08 .

Through exhaustive analyses of different case studies concerning the influence of soil physical properties on the abundance and composition of denitrifying microorganisms [15], and later [9] showed that pH and temperature are determinant aspects of the denitrification process in soils. Specifically, the pH should be slightly alkaline (between 7 and 8) and the temperature should be above 10 °C (preferably around 25°C). In addition, they showed that the C: N ratio also influences the presence of this type of microorganisms, with the optimal ratio being around 5.

Sequencing and phylogenetic analysis

Since there have been no previous studies on the diversity of *nirK*, *nirS*, and *nosZ* in the studied soil environment, an initial approach by clone library analysis was performed to investigate the presence of different groups in the denitrifying community prior to a high throughput sequencing study. Twenty-four selected isolates with *nirK* (*nirK*-1 to *nirK*-24), and twenty with *nirS* (*nirS*-1 to 20), and *nosZ* (*nosZ*-1 to *nosZ*-20) showed similarity to bacterial sequences of the target genes when compared with the EMBL/GenBank/DDDBJ databases. The mean distance calculated using the Tamura 3-parameter model between the clone sequences was 0.36 for *nirK*, 0.28 for *nirS*, and 0.26 for *nosZ* fragments.

According to the best partition score, *nirK* isolates can be split into 17 OTUs, *nirS* isolates into 11 OTUs, and

nosZ isolates into 13 OTUs at a distance threshold of 0.043, 0.035, and 0.048, respectively.

Phylogenetic analysis of the *nirK* sequences showed that some of them could not be grouped into clusters with 80% similarity (Fig. 1). Four of the isolated sequences were grouped in one cluster together with known sequences from related genera of *Thermomonas*, *Lysobacter*, *Pseudoxanthomonas*, and *Stenotrophomonas*. The other sequences obtained do not have a close similarity with bacterial *nirK* gene sequences deposited in the databases.

The majority of amplified *nirS* denitrifier genes are grouped into a main cluster (Fig. 2). All *nirS* isolates were related to uncultured bacteria except one sequence associated with the *Pseudomonas* genus.

The *nosZ* sequences were grouped into three main clusters with 80% similarity within them (Fig. 3). Cluster I comprised uncultured bacteria, *Bradyrhizobium oligotrophicum*, *Thalassobaculum fulvum*, *Paracoccus* sp., *Agrobacterium tumefaciens*, and *Aureimonas* sp.; Cluster II was related to the *nosZ* of the *Pseudomonas* genus; and cluster III to the *Marinobacter* and *Halomonas* genera.

The comparison of sequences with those available in the databases allows us to identify the presence of Alpha- and Gamma-Proteobacteria related to the main denitrifying genera such as *Agrobacterium*, *Pseudomonas*, *Marinobacter*, and *Halomonas* [15, 30]. However, most of the *nirK* and *nirS* clones belong to groups of denitrifying bacteria that have not yet been isolated, similar to that reported in paddy soil by Yoshida et al. [31]. This could be due to the limitations of culture-dependent analysis of denitrifying bacteria present in soils [32].

Although we analyzed a small number of clones, the number of OTUs identified and the structure of the phylogenetic trees suggest that the community of nitrifying bacteria in the soil under study is not simple. In this sense, according to Ouyang et al. [9], to characterize the diversity of functional groups associated with the denitrification process, it is advisable to design clade-specific primers that can cover the existing diversity. Following these recommendations, a more thorough study is underway with the aim of characterizing in depth the composition of the bacterial community associated with the denitrification process.

Abundance and expression of the *nirK*, *nirS*, and *nosZ* genes

Gene copy numbers for *nirK*, *nirS*, and *nosZ* were $6.08 \times 10^9 \pm 1.16 \times 10^9$, $9.05 \times 10^6 \pm 1.65 \times 10^6$, and $8.71 \times 10^6 \pm 1.44 \times 10^6$, respectively (Fig. 4A).

Denitrifying bacteria carrying *nirK* and *nirS* often coexist in the soil environment, although *nirK* and *nirS* encode non-homologous enzymes (copper-dependent and cytochrome-cd1, respectively). In order to

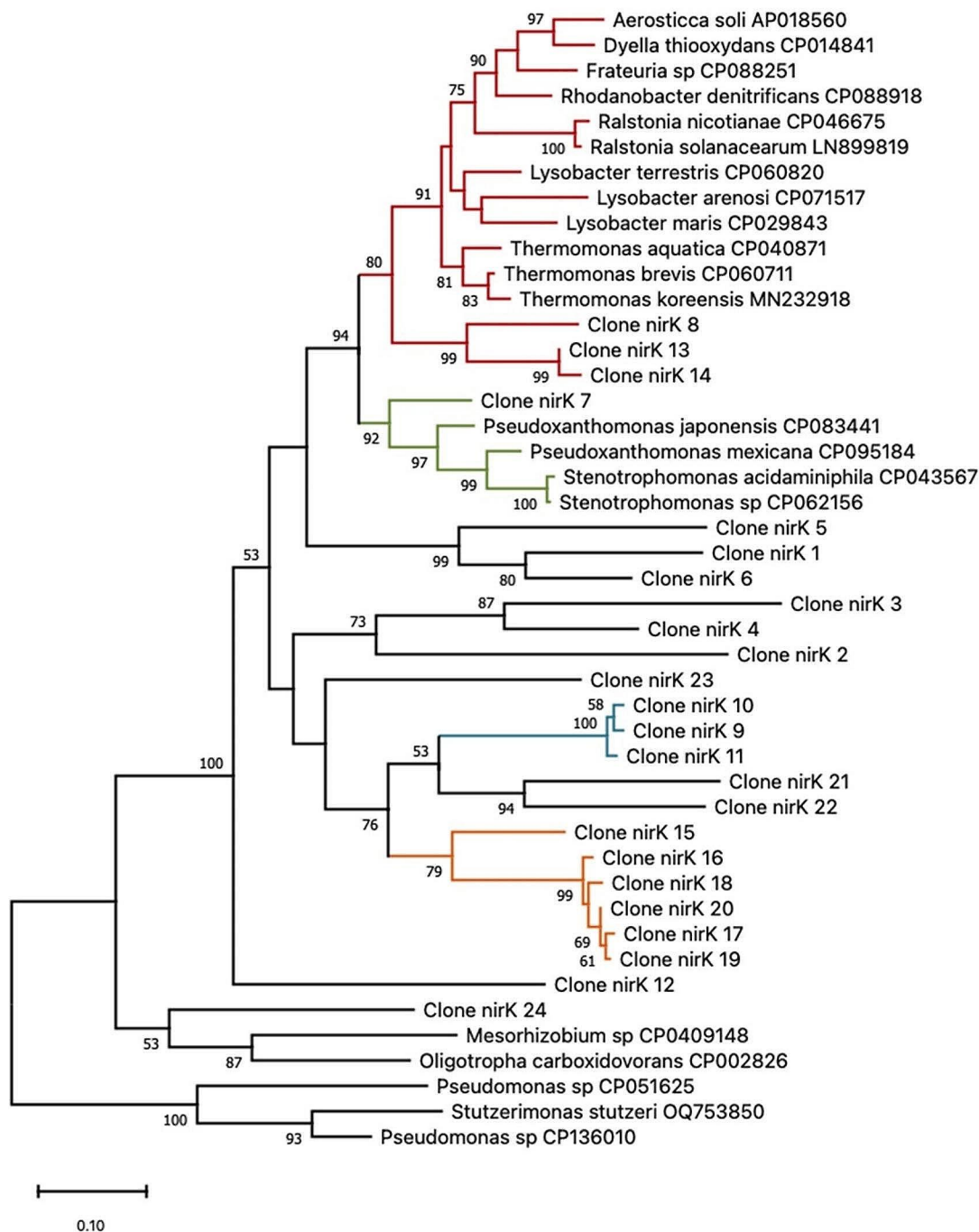


Fig. 1 Maximum likelihood phylogenetic tree based on *nirK* sequences and the General Time Reversible model. Bootstrap values higher than 50% are indicated above branches. Colors represent clusters with 80% similarity

understand the co-occurrence relationships between bacteria harboring *nirK* and *nirS*, the ratio between the abundance of these two genes has been analyzed in many studies, showing that the abundance of both genes increases with soil fertilization [9, 13, 33], but *nirK* seems to be slightly favored by the addition of nitrogen in either

mineral or organic form [10] and by the duration of fertilization time [9, 10]. In line with what these authors assert, the use of organic fertilizers for six years in the soil analyzed in the present research could explain the higher abundance of *nirK* found in this work.

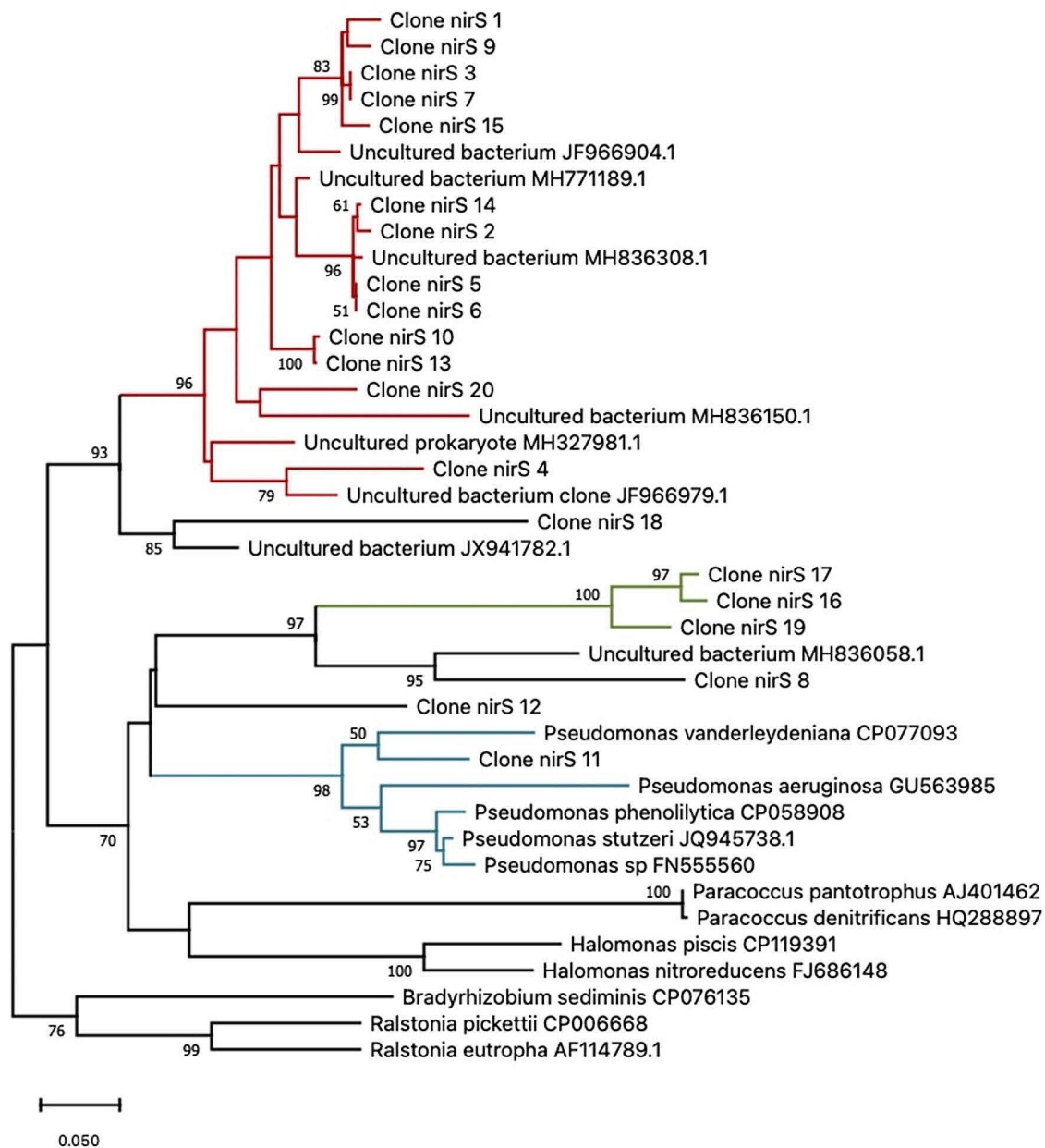


Fig. 2 Maximum likelihood phylogenetic tree based on *nirS* sequences and the General Time Reversible model. Bootstrap values higher than 50% are indicated above branches. Colors represent clusters with 80% similarity

On the other hand, the abundance of *nosZ*/*(nirK+nirS)* has been proposed as a metric for indirectly estimating potential N_2O emissions to the atmosphere [34]. In this study, the ratio is less than one (1×10^{-3}), indicating a deficiency of nitrous oxide reductase (NOS) activity capable of reducing the N_2O produced as a co-product in previous steps of the denitrification process. This fact might suggest that fertilization based on adding crop residues and manure could favor the presence of bacteria with partial denitrifying activity, mainly carrying

NIR genes (primarily *nirK*) as opposed to bacteria carrying NOS genes. However, it should be noted that the diversity of bacteria carrying genes encoding the enzyme nitrous oxide reductase seems to be more complex than previously thought [35], so the number of gene copies reflected in the present work may underestimate the true abundance of this type of bacteria.

In recent years, numerous studies have been conducted to develop primers with the highest possible coverage to detect what has recently been termed the atypical *nosZ*



Fig. 3 Maximum likelihood phylogenetic tree based on *nosZ* sequences and the Tamura 3-parameter model. Bootstrap values higher than 50% are indicated above branches. Colors represent clusters with 80% similarity

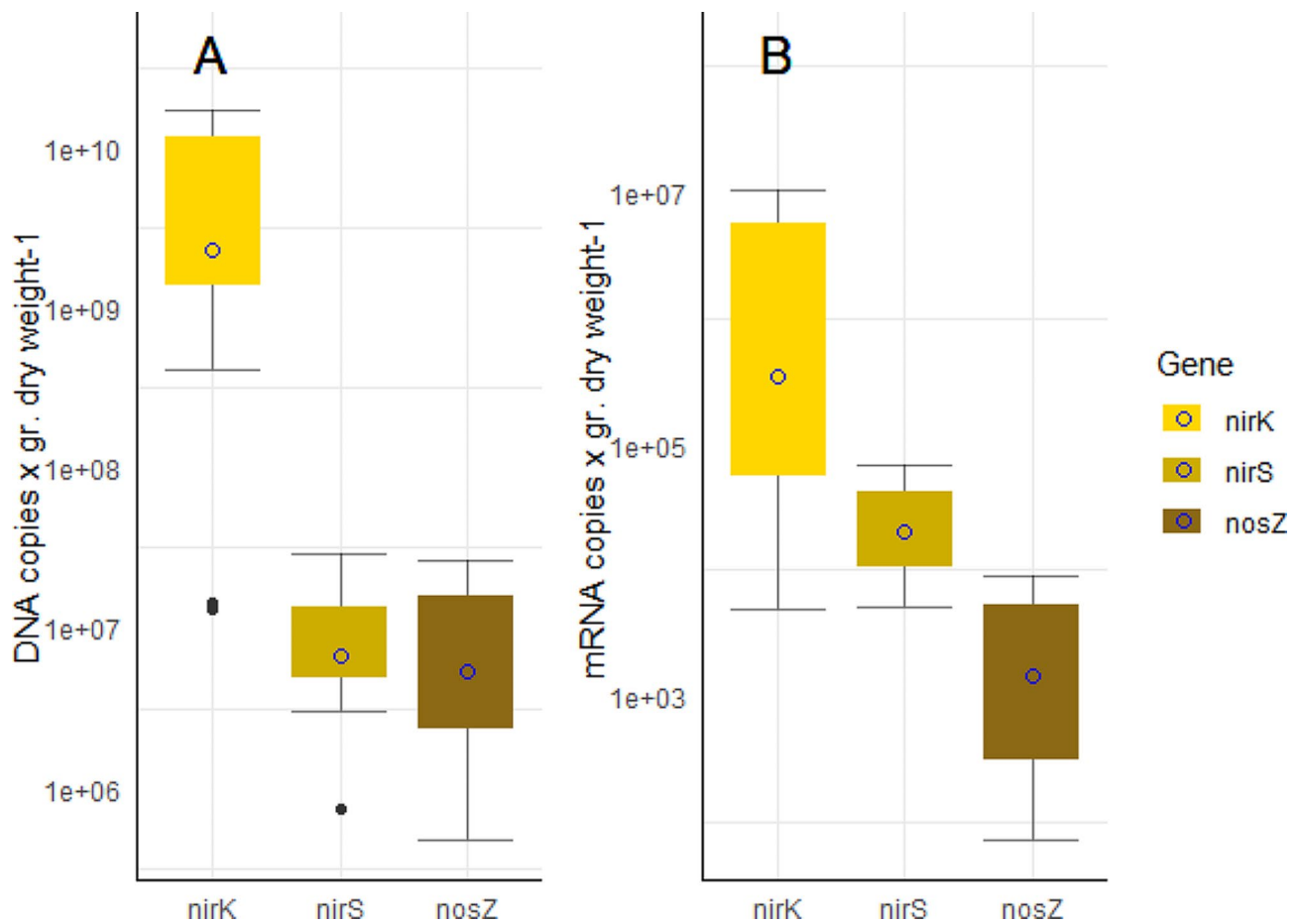


Fig. 4 Abundance of the *nirK*, *nirS*, and *nosZ* genes. (A): DNA copies; (B): mRNA transcripts

clade II [36]. Therefore, for future studies, it is advisable to analyze different pairs of *nosZ* primers capable of collecting the maximum diversity so that we can verify whether what was observed in this study is due to a limitation of the coverage of the selected primers or, on the contrary, to the influence of the type of soil management on the abundance of the different genes.

By analyzing mRNA transcripts, the values for *nirK*, *nirS*, and *nosZ* were $2.70 \times 10^6 \pm 8.60 \times 10^5$, $2.74 \times 10^4 \pm 5.92 \times 10^3$, and $3.09 \times 10^3 \pm 6.60 \times 10^2$, respectively (Fig. 4B).

There is limited research available in the literature on mRNA abundance in environmental samples, mainly due to the difficulty of extracting RNA, the difficulty of designing primers that assemble the existing diversity, and the limitations of correlating gene expression and function, as discussed by Saleh-Lakha et al. [37].

From the few studies on the abundance of RNA transcripts of denitrifying genes, it can be concluded that the expression of *nosZ* is more dependent on environmental factors than that of *nirS* and *nirK*. Henderson et al. (2010) and Németh et al. (2014) [38, 39], showed how *nosZ* transcripts vary with the addition of crop residues, while *nirS*

transcripts remain nearly unchanged. Similar results were highlighted by Dandie et al. [25], who found greater variation in *nosZ* transcripts than in *nirK* or *nirS* when comparing agricultural soil to riparian soil.

It is not possible to determine whether fertilization management has affected the regulation of gene activity in this study, as the initial situation of the greenhouse soil has not been characterized. However, the detection of mRNA indicates that the genes were actively expressed at the time of sampling.

The use of mRNA/DNA ratios to infer the activity status of environmental bacterial populations is generally accepted, as the difference between mRNA and DNA copies could indicate dead or dormant bacteria [40, 41]. As can be observed in Fig. 5 *nirS* has the highest mRNA/DNA ratio (3.10×10^{-3}), while *nirK* and *nosZ* showed 10-fold lower expression rates (4.4×10^{-4} and 3.5×10^{-4} , respectively), which may indicate that bacteria carrying the *nirS* gene had a higher level of transcriptional activity at the time of sample collection. Though, copy numbers of gene transcripts expressed as the ratio of mRNA/DNA for each gene were very low, never exceeding one gene transcript per gene copy.

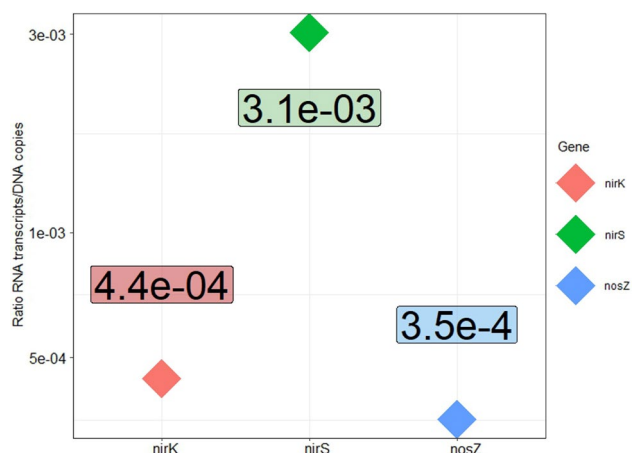


Fig. 5 Ratio of mRNA transcripts / DNA copies by functional gene

However, it should be taken into account that expression rates vary not only by soil management type, as mentioned above, but also by seasonality, as pointed out by [14]. Therefore, to better understand the expression activity of the genes involved in the denitrification process, it would be advisable to focus efforts on improving the coverage and specificity of primers designed to amplify denitrifying genes, as suggested by Ouyang et al. [9], Tatti et al. [14], and Luo et al. [33].

The sequencing of *nirK*, *nirS*, and *nosZ* clones allowed us to briefly characterize for the first time the population of denitrifying organisms in an ecologically managed greenhouse soil in the semi-arid southeast of the Iberian Peninsula. Furthermore, this study shows that gene abundance and expression are significantly higher in *nirK* than in *nirS* and *nosZ*. The highest ratio of RNA transcripts/DNA copies occurs in the *nirS* gene. On the other hand, the low copies found in *nosZ* (both DNA and mRNA) shows an imbalance with respect to the copies found in *nirK*, highlighting the need to use different primers that allow us to capture as much diversity as possible. The results shown in this work are relevant as a starting point for more comprehensive studies, based on metagenomics and metatranscriptomics, to understand the dynamics of bacterial composition associated with the denitrification process. Finally, it is also advisable to carry out comparative studies between different types of soil management to verify their influence on the abundance and expression of these genes.

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Author contributions

All authors contributed to the conceptualization and design of the study. Material preparation and data collection were performed by FM, DM, and

RHM; DNA/RNA extraction and the subsequent analysis were carried out by IB, RL, and RHM. The analysis of the physicochemical properties of the soil was performed by FM and DM. The first draft of the manuscript was written by RHM, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability

All DNA sequences are available in genbank repository with accession numbers as follows OQ344290-OQ344309 (*nirS*), OQ360659-OQ360682 (*nirK*), and OQ344310-OQ344329 (*nosZ*). Additional data used in this study are available from the corresponding author by request.

Declarations

Conflict of interest

The authors declare no competing interests.

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