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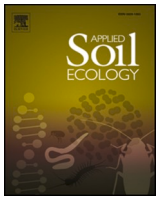

Influence of green manuring on soil properties, abundance and expression of key denitrification genes in a greenhouse Anthrosol

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ABSTRACT

Intercropping with green manure is a valuable agronomic practice for promoting sustainability agricultural systems. The aim of this study was to evaluate the effects of green manure (GM) – based on a mixture of vetch (*Vicia sativa* L.), forage turnip (*Brassica rapa* L. subsp. *rapa*), and oats (*Avena sativa* L.) – on the physicochemical properties of a greenhouse Anthrosol and its impact on the abundance and expression of denitrifying genes (*nirK*, *nirS*, and *nosZ* (clade I and II)) using real-time PCR. The cultivated crop throughout the three-year experiment was eggplant (*Solanum melongena* L. subs. *Telma*) fertilized with manure and crop residues from the previous growing season. A linear mixed model (LMM) was used to assess the effect of GM on both the physicochemical variables and the target-gene and transcript copy number in the soil. After three years of GM coverage, moderate changes in soil physicochemical properties were observed, with the exception of a significant decrease in soil temperature (<6 %) and ammonium ion concentration (<14 %) with GM application. Gene copy numbers remained largely unchanged between treatments, however transcripts levels decreased for all target genes under GM, with particularly pronounced reduction for *nirK* ($p < 0.0001$) and *nosZ II* ($p < 0.05$), suggesting partial suppression of gene transcription within the denitrification pathway. In addition, potassium (K^+) and soil moisture were found to correlate with DNA and RNA abundances, indicating a complex interaction between salinity and soil moisture in the regulation of the denitrification process.

1. Introduction

Nitrous oxide (N_2O) is considered the third most important greenhouse gas, with a global warming potential 265 times higher than that of CO_2 (IPCC, 2021). Agriculture is recognized as the main anthropogenic source contributing to the emissions of N_2O (Fowler et al., 2015; Tian et al., 2020), largely due to inefficiencies in nitrogen fertilizer application for crop nutrition (Seitzinger and Phillips, 2017; Cao et al., 2018; Scherbak et al., 2014). The need to optimize crop nutrition, enhance its efficiency, and mitigate N_2O emissions is therefore one of the main challenges for agriculture in the twenty-first century (UNEP, 2013; Ogle et al., 2019).

From this viewpoint, the concept of ecological intensification emerges as a strategy aimed at sustaining crop yields while mitigating environmental impacts to the greatest possible extent (Bommarco et al., 2018; Kleijn et al., 2019). This approach entails various strategies, including the progressive substitution of inorganic fertilizers with the integration of organic matter, such as crop residues, and the implementation of cover crops like green manure. Although these practices increase microbial abundance and diversity (Kim et al., 2020), assessing their efficacy in N_2O reduction implies an examination of nitrification and denitrification processes since these biological processes are the main contributors to N_2O emissions in soil (Butterbach-Bahl et al., 2013; Duan et al., 2019; Dong et al., 2023).

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To this end, in recent years, there has been several studies focused on understanding the diversity and abundance of soil microorganisms based on the analysis of functional genes associated with both nitrification and denitrification processes (Kuypers et al., 2018). In the case of denitrification, the most studied genes were *nirK* and *nirS*, which encode non-homologous nitrite reductase enzymes, and *nosZ*, which encodes nitrous oxide reductase, the only known enzyme capable of reducing N_2O to N_2 (Zumft, 1997; Braker et al., 1998; Kuypers et al., 2018). Two phylogenetically distinct clades of this enzyme (*nosZ I* and *nosZ II*) are currently recognized (Jones et al., 2013).

Numerous recent studies, as highlighted in the meta-analysis by You et al. (2022) have focused on examining the impact of fertilization on denitrifying genes. These studies suggest that nitrogen fertilization increase the abundance of *nirK*, *nirS*, and *nosZ* genes (Ouyang et al., 2018; You et al., 2022). Additionally, factors such as pH (Bowen et al., 2020) and soluble organic carbon (SOC) levels also influence the abundance of these genes (Ouyang et al., 2018; Xiao et al., 2021). However, considering this general pattern, it must be taken into account that there are conflicting results, as reflected by Xiao et al. (2021) and Sun and Jiang (2022). These inconsistencies may arise due to multiple factors, such as the heterogeneity of soil types, variations in experimental designs, the limited coverage of primers used to detect the diversity and abundance of microorganisms (Jones et al., 2013; Luo et al., 2021; Sun and Jiang, 2022), or the fact that DNA detection does not distinguish between active and inactive cells, which may sometimes fail to accurately reflect the activity of the metabolic process under study (Steven et al., 2017). Therefore, studies that analyze RNA abundance of these genes have increased in recent years as a measure to better understand the activity of the denitrifiers (Zhang et al., 2021; Duan et al., 2019; Dong et al., 2023).

Aligned with the principles of ecological intensification and the pursuit of fertilization alternatives to mitigate denitrification losses, we recently undertook a study into greenhouse agricultural soil. We compared conventional soil management reliant on inorganic fertilization with soil enriched with crop residues and manure (ecological intensification management) (Hernández Maqueda et al., 2024a).

To expand on the findings of previous studies, the present research was designed as a three-year experiment aimed at evaluating the effects of the annual incorporation of green manure (GM) in an agricultural soil (Anthrosol) managed under the principles of ecological intensification, using eggplant as the main crop. Green manuring is widely recognized for its ability to improve soil health by increasing root surface area, thereby enhancing nutrient accessibility. Additionally, it contributes to improved soil fertility and nutrient retention (Ma et al., 2021; Xu et al., 2023). Upon incorporation into the soil, the nitrogen contained in green manure plant tissues is gradually released through microbial decomposition, allowing for more efficient uptake by subsequent crops and reducing the risk of nitrogen losses through leaching or denitrification (Dabney et al., 2001).

In this context, the objectives of this study were (i) to assess the effects of sowing and subsequent incorporation of a green manure mixture—comprising vetch (*Vicia sativa* L.), forage turnip (*Brassica rapa* L. subsp. *rapa*), and oats (*Avena sativa* L.)—on the soil's physicochemical properties, and (ii) to quantify the abundance and expression of the *nirK*, *nirS*, and *nosZ* (clades I and II) genes in soils subjected to three consecutive years of green manure treatment, compared to an untreated control.

We hypothesized that green manure application could enhance nitrogen use efficiency, which would be reflected in changes in the abundance and expression of key genes involved in the denitrification process.

2. Material and methods

2.1. Experimental design and soil sampling

The experiment was carried out in a greenhouse covering an area of 841.25 m^2 at Las Palmerillas Experimental Station (Cajamar Foundation, Almería, Spain), located at 36° 48′ N latitude, 2° 43′ W longitude, and 155 m above sea level.

Since 2015, the soil of the greenhouse under study, an Hortic Anthrosol (IUSS working group WRB, 2022), has been managed according to the principles of ecological intensification, which, in terms of fertilization, involved minimal use of external fertilizers and instead incorporated organic amendments such as crop residues and manure. Initially, the soil was deeply plowed with chisel plows to improve its physical properties, as described by Salinas et al. (2020). Subsequently, organic amendments were added at a rate of 2 kg x m^2 (fresh weight) of chopped crop residues from previous seasons, contributing 307.20 ± 19 g x kg dry weight $soil^{-1}$ of total organic carbon (TOC) and 13.90 ± 0.20 g x kg dry weight $soil^{-1}$ of total nitrogen (TN). Additionally, 2.5 kg x m^2 (fresh weight) of manure was incorporated, providing 241.70 ± 14.15 g x kg dry weight $soil^{-1}$ of TOC and 20 ± 0.18 g x kg dry weight $soil^{-1}$ of TN. Further details on the management practices, the physicochemical properties of the base soil, and the composition of the amendments used can be found in Hernández Maqueda et al. (2024a).

To evaluate the effect of GM on soil properties, a mixture of vetch (*Vicia sativa* L.), forage turnip (*Brassica rapa* L. subsp. *rapa*), and oats (*Avena sativa* L.) (40 kg ha^{-1}, 40 kg ha^{-1}, and 20 kg h^{-1}, respectively) was sown in four randomly selected rows (crop lines) of the greenhouse. According to information provided by the commercial supplier (Biosemillas S.Coop.And), the carbon to nitrogen ratio at mowing time is approximately 10–15 for vetch, 25–27 for forage turnip and 25–29 for oats. The sowing was carried out in the month of November in the seasons of 2018–2019, 2019–2020 and 2020–2021.

After three years of incorporating GM, soil samples were analyzed to assess its impact on soil physicochemical properties, and on the abundance and expression of *nirK*, *nirS*, and *nosZ* (I and II) genes. To this end, a composite soil sample was taken from each of the rows where GM was applied (n = 4) and compared with four other samples randomly selected from the rows where no GM was applied (control). Each sample consisted of three soil subsamples taken at 0–20 cm depth and 15 cm from the central axis of the selected plants. The subsamples were then thoroughly mixed to form the final sample. Fifty grams of these samples were immediately frozen in liquid nitrogen and stored at −80 °C for subsequent DNA and RNA isolation (Fig. 1).

Samples were collected twice: the first time 15 days after the green manure was sown (t1 = November 2020), and the second time during

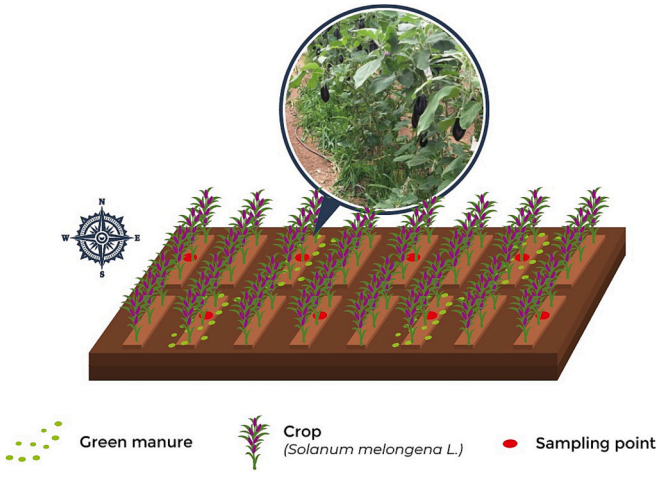

Fig. 1. Experimental design.

the full development of the green manure (t2 = March 2021). The crop in production at the time of sampling was eggplant (*Solanum melongena* L. var. Telma), planted in September 2020.

2.2. Soil properties

Total organic carbon (TOC) was measured in fine ground soil by the wet oxidation method according to Mingorance et al. (2007). Electrical conductivity (EC, dSm^{-1}) was measured in a 1:5 soil: water suspension (v/v), with a Crison 522 conductivity meter (UNE 77308:2001), and pH was measured in a 1:5 soil:water suspension (v/v) with a Crison basic 20 pH-meter (UNE-ISO 10390:2012). Total Nitrogen (TN) was measured using an Elementar Rapid N exceed elemental analyzer. Soil ammonium (NH_4^+) and nitrate (NO_3^-) contents were extracted with 2 mol L^{-1} KCl according to Mulvaney (1996). Dissolved cations were measured in 1:5 soil water suspension (v/v): Ca^{++} and Mg^{++} by atomic absorption spectroscopy. Na^+ and K^+ by flame photometry. Soil temperature (T) and volumetric water content (VWC) were measured by means of a Decagon Teros 12 sensor. Finally, the percentage of resistant soil aggregates (RA) was calculated using a Wet Sieving Apparatus (Eijkelkamp).

2.3. Nucleic acid extraction

RNeasy PowerSoil® Total RNA Isolation and RNeasy PowerSoil® DNA Elution Kit (Qiagen®, Valencia, CA) were used for total RNA and total DNA extraction, respectively. One gram of soil was used in duplicate for each sample according to the manufacturer's protocol.

The isolated RNA and DNA were diluted in 70 μL of the SR7 solution (included in both kits) and quantified using a Nanodrop ND-1000 spectrophotometer (ThermoFisher Scientific Inc., United States). 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.

2.4. Quantitative PCR

The quantitative polymerase chain reaction (qPCR) assay was conducted to quantify the *nirK*, *nirS*, *nosZ1*, and *nosZ2* genes and their corresponding transcripts using the Applied Biosystems® 7900HT Fast Real-Time PCR (ThermoFisher Scientific Inc., United States).

Purified RNA was reverse transcribed using Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific Inc., United States) 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.

The primers used for amplification were nirkC2F/nirkC2R for *nirK* (Wei et al., 2015), nirSC1F/nirSC1R for *nirS* (Wei et al., 2015), nosZ1F/nosZ1R for *nosZ1* (clade I) (Henry et al., 2006), and nosZ2F/nosZ2R for *nosZ2* (clade II) (Jones et al., 2013). Each qPCR reaction was performed with 1× Applied Biosystems™ SYBR™ Green PCR Master Mix, 0,2 μM forward and reverse primers, 0.01 % BSA (bovine serum albumin), and a diluted template in a final volume of 15 μL. For DNA quantification, 3 μL of 10-fold diluted template was used, while for cDNA 5 μL of a 3-fold diluted template was employed. Thermal cycling conditions for the functional genes were as follows: an initial denaturation at 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C (*nirK* and *nirS*) or 62 °C (*nosZ1* and *nosZ2*) for 1 min; and a final melt curve stage at 95 °C for 15 s. Samples and no-template controls were run in duplicate. At least, two independent quantitative qPCR assays were performed for each treatment. Amplification specificity was confirmed through melting curves analysis for each qPCR reaction. Gene or transcript copy numbers were determined from standard curves, created from serial dilutions of plasmid DNA containing specific gene fragments ranging from 10^{10} to 10^2 copies (Hernández Maqueda et al., 2024b). The PCR efficiency derived from standard curves ranged from 86 to 106 %, with an R^2 value higher than 0.995. Results were expressed as gene or transcript copies per gram dry soil.

2.5. Statistical analysis

To test the assumptions of parametric statistical analyses, all response variables were transformed to a natural logarithm, when necessary, to achieve normality and homogeneity of variance, and then examined using the Shapiro-Wilk (S—W) and Bartlett's tests, respectively. Each variable was treated independently. Extreme outliers were excluded from the analysis.

The relations between the DNA abundances of the investigated genes were evaluated using the Pearson product-moment correlation coefficient. Meanwhile, the associations between the abundances of RNA and between DNA and RNA were assessed using Spearman's rank correlation coefficient. For relationships with physicochemical variables, the choice between the two coefficients depended on their distribution (normal or non-normal).

A linear mixed model (LMM) was employed to examine the influence of GM on both the physicochemical variables and the abundances of DNA and RNA present in the soil. The model, following the "REML" specification, utilized residual (restricted) maximum likelihood estimation, as outlined by McCulloch and Searle (2001) and West et al. (2015). The fixed effects included GM application versus control (no application), orientation (north versus south), and line (representing the crop line from which the sample was obtained, with options of 3, 4, 8, and 12). The intercept functioned as a random effect (RE), capturing random deviations for each sample from the overall fixed intercept. The observation was nested within the fixed effect level of GM. Additionally, crop stage was considered a fixed and repeated factor in the analysis. The two-way interactions between fixed factors were also included as such in the model, but only the results for which we reject the null hypothesis of equality of means are shown. The goodness of fit of the best model was assessed using the Bayesian Information Criterion (BIC). The degrees of freedom (df) for the RE required for Wald or F tests or Akaike's information criterion (AIC) should be between 1 and N - 1 (where N is the number of RE levels). Because the data set is small and, in some cases, has unequal variances, the Satterthwaite approximation was used to calculate the df and adjust the standard errors (Satterthwaite, 1946). The overview of the model proposed by Satterthwaite is explained in Supplementary material (S1).

The comparison of the levels of the fixed factor "line" was performed using Tukey's test. If any of the two-way interactions between fixed factors showed a significant effect, the means were compared using a *t*-test if the variable was normally distributed or a u-test if not (Agresti, 2019).

For the DNA abundance of the *nirK* and RNA nosZ2 genes, a general linear model (GLM) with repeated measures was created (Kuehl, 2000). For this second case, RNA differences between the north and south orientations were evaluated using a paired t-test.

The statistical analyses were carried out with the free software R version 4.2.3 (R Core Team, 2022), with the following packages: *readxl* version 1.3.2. for reading the tables, *car* version 3.1–2, *carData* version 3.0–5, and *stats* version 4.1.3 for the exploratory analysis of the variables and the analyses of normality and correlations. Graphs were created using the package *ggplot2* version 3.4.1. Linear mixed models were made with the package *lme4* version 1.1–35.1. Finally, repeated-measures analyses of variance were performed with the package *ez* version 4.4–0.

3. Results

3.1. Effect of green manuring on soil physicochemical properties

Sowing and the subsequent incorporation of green manure for three years produced moderate changes in soil physicochemical properties

Table 1
Least square means of the physicochemical variables analyzed according to treatment level: GM application (Yes and Control); and crop stage (t1 and t2); with the significance of its effect on the variable.

Variable	Green manure				Crop stage				σ_b^2	σ^2	BIC
	Yes	Control	SED	p	t1	t2	SED	p			
Total organic carbon (g kg^{-1} soil)	27.4981	32.5650	2.3768	0.2792	30.8129	29.2503	1.6702	0.3924	0.8673	9.5642	42.376
Total nitrogen (g kg^{-1} soil)	4.0613	3.6263	0.3481	0.2580	3.7488	3.9388	0.2659	0.5017		0.2424	18.213
C/N ratio	7.2192	9.4764	1.3582	0.2384	8.6413	8.0544	0.8257	0.5039	0.4813	2.7271	44.503
Ammonium (mg kg^{-1} soil)	25.3365	28.8505	1.8398	0.1047	29.9073	24.2798	1.4051	***0.0071***		6.7694	38.19
Nitrate (mg kg^{-1} soil)	10.2755	12.1707	2.1293	0.4077	9.3952	13.0511	1.6263	***0.0656***		9.0677	39.944
Ca (mmol L^{-1} soil)	10.6719	11.2969	2.0747	0.7709	8.8438	13.1250	1.4670	***0.0193***		8.6088	51.017
K (mmol L^{-1} soil)	17.9348	20.7481	2.0095	0.1991	12.4361	26.2468	1.4209	***<0.001***		8.0759	50.505
Na (mmol L^{-1} soil)	12.8804	12.2283	6.5777	0.9258	11.4674	13.6413	2.8482	0.4878		21.6328	30.292
pH	7.7938	7.7937	0.0472	0.821	7.7500	7.8375	0.0334	0.1015		0.0045	
Electrical conductivity (dS m^{-1})	5.0762	5.3288	0.9697	0.8033	4.1783	6.2267	0.7406	***0.0326***		1.8805	30.505
Temperature (°C)	15.7819	16.7849	0.1498	***0.0216***	16.0786	16.4882	0.0911	***0.0041***	0.00583	0.0332	9.2383
Volumetric water content (m^3/m^3)	0.3099	0.3219	0.0131	0.6714	0.3068	0.3250	0.0131	***0.0007***			
RA (%)	73.2665	71.4698	2.8216	0.6390	69.8989	74.8374	1.8425	***0.0438***	2.1420	11.6389	43.701

σ_b^2 = Intercept variance (random effect); σ^2 = error variance; SED = standard error of the differences of means; BIC = Bayesian information criterion. Bold + italic values indicate significant differences in soil properties ($p < 0.05$) according to the LMM. Ca: calcium; K: potassium; Na: sodium; RA: Resistant aggregates.

(Table 1).

The LMM analysis showed that the application of GM caused a significant decrease ($p < 0.05$) in soil temperature from 16.8 to 15.8 °C on average. TOC, Carbon to Nitrogen ratio (C/N ratio), ion ammonium (NH_4^+), nitrate (NO_3^-), Calcium (Ca^{++}), K^+, EC, and VWC also tended to decrease with GM application. In contrast, pH and sodium (Na^+) remained unchanged, and TN and percentage of RA tended to increase, although no significant differences were observed for any of these variables. Only NH_4^+ values were close to being statistically significant ($p < 0.1$).

The analysis of the fixed effects of crop stage on physicochemical variables showed that NH_4^+ decreased significantly from t1 to t2 ($p < 0.05$). By contrast, NO_3-, Ca^{++} and K^+ cations, EC, VWC, and RA increased significantly (Table 1). Na^+ also tended to increase from t1 to t2, although no significant differences were observed. TOC, TN, C/N ratio, and pH did not show significant changes between the two times analyzed.

The interaction of GM application and crop stage analyzed in the LMM showed significant differences ($p < 0.05$) for both NH_4^+ (Supplementary Table S2) and soil temperature (Supplementary Table S3).

3.2. Abundance of nirK, nirS, nosZ1, and nosZ2 genes

In this soil, regardless of the type of management (control vs. GM), a higher number of copies of the copper-dependent nitrite reductase gene (*nirK*) versus the cd1-dependent nitrite reductase gene (*nirS*) were detected, with differences of about three orders of magnitude: around 10^{10} copies for *nirK* versus 10^7 copies for *nirS*. Similarly, regarding the gene encoding nitrous oxide reductase, *nosZ2* showed a higher copy number compared to *nosZ1*, with approximately 10^{10} copies of *nosZ2* versus 10^7 copies of *nosZ1*.

The LMM analysis (Table 2) indicated that, in general terms, there was no significant change in the amount of *nirK* and *nirS* DNA copies, either due to the effect of GM or the crop stage. However, when examining the effect of crop stage, a significant decrease ($p < 0.05$) in the levels of *nosZ1* and *nosZ2* DNA copies was observed between t1 and t2. The interaction between GM application and crop stage is significant for *nirS* and *nosZ2*. For *nirK* and *nosZ1*, the interaction of both factors is close to being significant, with p-values close to 0.05 (0.087 and 0.086, respectively).

The analysis of the gene copy number variation between the management systems at both t1 and t2 (Fig. 2), showed that at t1, no significant differences were observed for any of the genes analyzed based on management type (control vs. GM) (*nirK*: 1.36×10^{10} vs. 5.23×10^9, *nirS*: 1.98×10^7 vs. 1.53×10^7, *nosZ1*: 1.75×10^7 vs. 1.35×10^7, and *nosZ2*: 1.92×10^{10} vs. 1.38×10^{10}). In contrast, at full GM development (t2), an increase in target copies was observed in the GM lines compared to the control. This increase was statistically significant ($p < 0.05$) for *nirK* (3.79×10^9 vs. 1.86×10^{10}, $p = 0.005$) and *nirS* (3.63×10^6 vs. 3.23×10^7, $p = 0.003$). The abundance of *nosZ1* (2.48×10^4 vs. 1.19×10^5) and *nosZ2* (2.54×10^9 versus 9.84×10^9) also showed an increase under GM conditions compared to control in t2 but without statistical significance.

Finally, the ratio of nitrous oxide reductase to nitrite reductase genes (*nosZ1* + *nosZ2*)/(*nirk* + *nirS*) varied according to sampling time and GM application. After GM sowing (t1), the ratio (*nosZ1* + *nosZ2*)/(*nirk* + *nirS*) was 1.41 for control samples and 2.62 for GM samples. When GM was in full development (t2), the ratio (*nosZ1* + *nosZ2*)/(*nirk* + *nirS*) decreased for both control and GM samples (0.67 and 0.53, respectively).

3.3. Transcripts of nirK, nirS, nosZ1, and nosZ2 genes

The analysis of the LMM (Table 3) showed that GM tended to

Table 2
Least square means of the treatment levels: Green manure and the temporal variable: crop stage, with the significance of their effect on the gene copies abundance.

Gene copies	Green manure (GM)				Crop stage (CS)				σ_b^2	σ^2	BIC	GM × CS
	Yes	Control	SED	p	t1	t2	SED	p				p
nirK[a] (×10^9)	11.35	9.27	1.51	0.489	9.42	11.2	2.47	0.503	–	–	–	0.087
nirS (×10^5)	23.24	12.36	5.77	0.09	17.65	17.95	4.08	0.94		66.628	67.38	**0.0024**
nosZ1 (×10^6)	1.324	1.629	0.52	0.61	2.21	0.75	0.36	**0.006**	0.009	0.5133	30.61	0.081
nosZ2 (×10^9)	9.99	12.45	3.35	0.48	16.25	6.19	2.37	**0.003**		2.24 (×10^7)	169.2	**0.029**

σ_b^2 = Intercept variance (random effect); σ^2 = error variance; SED = standard error of the differences of means; BIC = Bayesian information criterion. Bold values indicate significant differences in soil properties ($p < 0.05$) according to the LMM test for *nirS*, *nosZ1* and *nosZ2*.

[a] A general linear model (GLM) with repeated measures was used for *nirK*. Thus, for *nirK*, the p-values correspond to the GLM test for repeated samples.

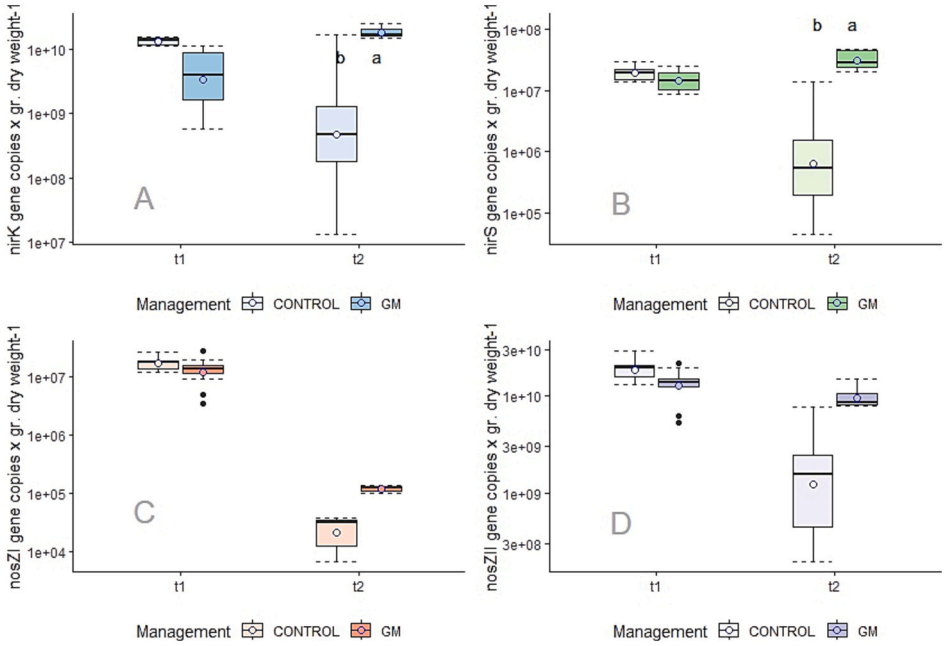

Fig. 2. Copy number of each of the genes analyzed after sowing the green manure (t1) and with GM in full development (t2).

Table 3
Least square means of the treatment levels: Green manure and the temporal variable: crop stage, with the significance of their effect on the transcript copies of the genes analyzed.

Transcript copies	Green manure (GM)				Crop stage (CS)				σ_b^2	σ^2	BIC	GM × CS
	Yes	Control	SED	p	t1	t2	SED	p				p
nirK (×10^6)	1.81	4.60	0.29	**<0.0001**	0.92	5.48	0.20	**<0.0001**		0.16	19.30	**<0.0001**
nirS (×10^4)	1.54	3.19	1.08	0.27	1.54	3.19	0.70	0.057	0.17	1.98	41.68	**0.0138**
nosZ1 (×10^3)	1.92	1.99	0.71	0.93	1.29	2.62	0.50	**0.029**		1.01	33.87	**0.0021**
nosZ2[a] (×10^6)	58.7	71.6	4.48	**0.031**	58.83	71.49	7.40	**0.029**	–	–	–	0.148

σ_b^2 = Intercept variance (random effect); σ^2 = error variance; SED = standard error of the differences of means; BIC = Bayesian information criterion. Bold values indicate significant differences in soil properties ($p < 0.05$) according to the LMM test for *nirK*, *nirS* and *nosZ1*.

[a] A general linear model (GLM) with repeated measures was used for *nosZ2*. Thus, for *nosZ2* the p-value corresponds to the GLM test for repeated samples.

decrease the abundance of RNA copies of all genes, although only with statistically significance for *nirK* ($p < 0.0001$) and for *nosZ2* ($p < 0.05$). Conversely, gene expression from t1 to t2 tended to significantly increase (*nirK*, $p < 0.0001$; *nirS* p-0.05; *nosZ1*, $p < 0.05$; and *nosZ2*, $p < 0.05$). The interactions between GM and crop stage showed significant differences for *nirK*, *nirS*, and *nosZ1*.

When the two types of management (control versus GM) were compared at t1 and t2 (Fig. 3) according to the interactions detected by the LMM, it was found that there were no significant differences between either system at t1, nor in *nirK* (6.1×10^5 versus 1.2×10^6), *nirS* (9.7×10^3 versus 1.5×10^4), or *nosZ2* (5.6×10^7 versus 6.1×10^7). However, *nosZ1* varied from 1.4×10^2 in the samples control to 2.4×10^3 in the samples with GM ($p < 0.05$). In contrast, the copies of transcripts decreased for all genes in GM samples compared to the control at t2. Thus, *nirK* decreased from 9.1×10^6 to 1.9×10^6 ($p < 0.001$), *nirS* from 4.5×10^4 to 9.7×10^3 ($p = 0.037$), *nosZ1* from 3.7×10^3 to 1.5×10^3 ($p = 0.028$), and *nosZ2* from 8.3×10^7 to 6.1×10^7 ($p = 0.029$).

3.4. Interaction between gene abundances, RNA transcripts and soil properties

In general, the following was observed (Fig.4): (i) The copy numbers of the four genes analyzed correlated positively with each other. A positive and significant correlation was found between *nosZ1* and *nosZ2* ($r = 0.959$; $p < 0.001$) and between *nirK* and *nirS* ($r = 0.820$; $p < 0.001$). The rest of the relationships were positive but not significant, with a moderate value for *nirS* and *nosZ2* ($r = 0.413$; $p = 0.112$). (ii) The transcript copy numbers of the four genes also correlated positively with each other. A positive and significant correlation was observed between *nirK* and *nosZ1* (Rho = 0.624; $p = 0.010$), between *nirS* and *nosZ1* (Rho = 0.506; $p = 0.046$), and between *nirK* and *nirS* (Rho = 0.503; $p = 0.047$). The remaining correlations were positive but not significant. (iii) Gene copy number correlated negatively with trasncript copy number so that when one increased, the other decreased (Supplementary Table S4). (iv) The only physicochemical variables that showed any correlation with gene abundance or expression were NH_4^+, K, t°, and VWC.

Regarding the correlation of gene copies with physicochemical variables, NH_4^+ was inversely correlated with *nirS* (Rho = −0.540, $p = 0.038$). It is also noteworthy that K levels were inversely and significantly correlated with the amount of DNA of *nosZ1* (Rho = −0.583, $p = 0.018$) and nosZ2 (Rho = −0. 618, $p = 0.011$); similarly, VWC was inversely and significantly correlated with the amount of DNA of *nosZ1* (Rho = −0.581, $p = 0.018$) and *nosZ2* (Rho = −0.511, $p = 0.043$).

Finally, regarding the correlation of transcript copies with physicochemical variables, a positive and significant correlation was observed between soil K content and transcript content of *nirK* (Rho = 0.790, $p < 0.001$) and *nirS* (Rho = 0.500, $p = 0.048$). Similarly, a positive and significant correlation was observed between VWC and the amount of *nirK* transcript copies (Rho = 0.658, $p = 0.006$). A close to positive significance ($p \leq 0.05$) is appreciable between T values and *nirK*

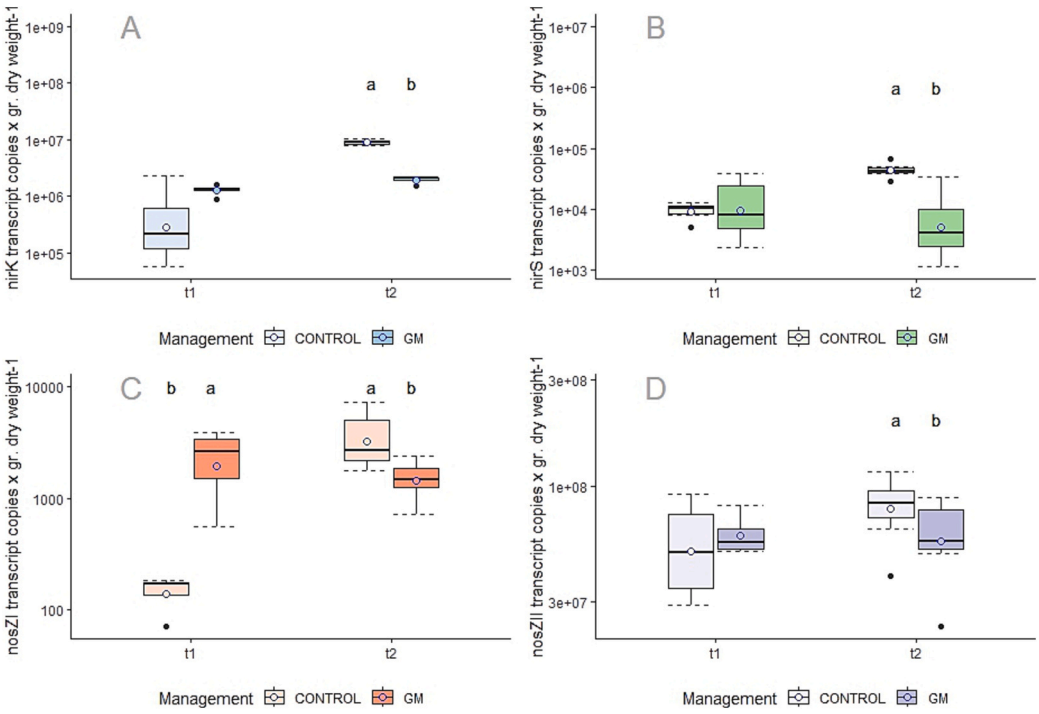

Fig. 3. Copies of transcript for each of the genes analyzed after sowing the green manure (t1) and with GM in full development (t2). CAMBIAR FIG RNA Y DNA.

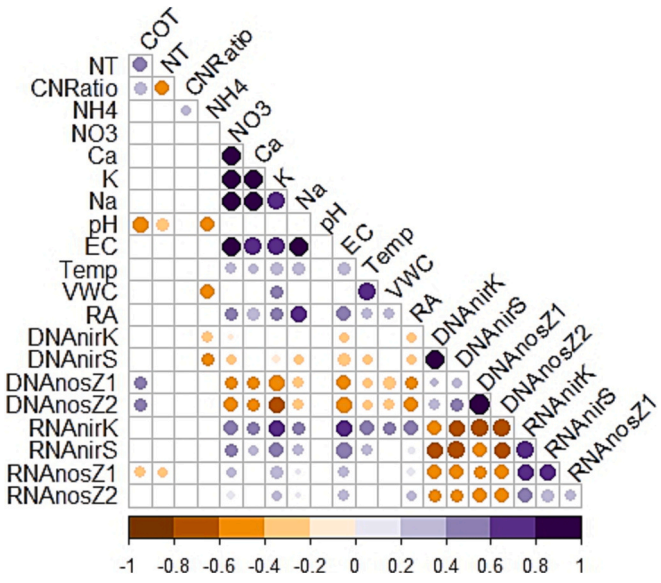

Fig. 4. Correlation matrix of the variables analyzed based on Spearman's Rho nonparametric test.

transcript copies (Rho = 0.481, p = 0.059).

The correlation coefficients (Pearson or Spearman) between gene and transcript copies with physicochemical properties, the significance tested on both sides of the distribution, and the number of observations used are presented in Supplementary Tables S5 and S6, respectively.

4. Discussion

4.1. Effect of green manuring on soil properties

After three years of GM cover, moderate changes in soil physicochemical properties were observed with the exception of a significant decrease in soil temperature (−6 %) and ammonium ion concentration (−14 %). The remaining analyzed soil properties remained largely unchanged, a trend also noted by Walker et al. (2022), Hu et al. (2023), and Yang et al. (2023). This may be attributed to the fact that significant changes in physical and chemical properties after GM application tend to manifest in the long term (> 10 years), as suggested by Blanco-Canqui (2022) and Das et al. (2022).

However, this study shows some valuable trends that are worth mentioning. Firstly, we observed a slight increase in resistant aggregates (> 2 %), and a decrease in volumetric water content (−3.8 %) in soil with GM compared to the control. According to Ma et al. (2021); Dabney et al. (2001), although GM may increase water infiltration into soil, they also use water to grow and can potentially reduce soil water content. Additionally, as previously noted, GM application also led to a decrease in soil temperature (−6 %), likely due to the mulching effect of the living cover crop (Dabney et al., 2001).

These results collectively suggest a trend towards improving soil physical properties, which aligns with findings from various studies, as reported in a meta-analysis of 104 case studies conducted by Ma et al. (2021) which indicated that GM practices enhance root surface area, improve soil hydraulic properties, and facilitate greater gas exchange. However, the lack of statistical support obtained in this study, prevents us from confirming this hypothesis highlighting the need for further studies to determine whether this trend persists.

On the other hand, there was a slight increase of 12 % in total nitrogen (TN) and a decrease of 23 % in the C/N ratio compared to the control treatment. The rise in nitrogen with green manuring, particularly in the presence of legumes, has been extensively discussed in scientific literature and is primarily attributed to the incorporation of decomposing organic matter and nitrogen fixation (Ma et al., 2021). The decline in the C/N ratio is also commonly observed with GM application, as highlighted by several authors (Yang et al., 2023; Hu et al., 2023; Li et al., 2022) and is attributed to the increase in nitrogen, coupled with the rise in organic carbon, predominantly after long periods (> 10 years) of GM application (Blanco-Canqui, 2022; Das et al., 2023). A key consideration in this study is that the carbon to nitrogen (C/N) ratio is <20, independently of the management implemented. According to

findings from Huang et al. (2004), this low C/N ratio typically promotes the rapid mineralization of organic matter, leading to the preferential release of nitrogen in its inorganic forms, namely ammonium and nitrate. This process enhances nutrient availability, potentially increasing plant uptake through direct absorption. The lower concentrations of NH_4^+ and NO_3^- observed in green-manured soils may indicate greater nutrient uptake by plants in these treatments. However, as highlighted by Huang et al. (2004), Xia et al. (2018), and Li et al. (2022), this scenario is also associated with an elevated risk of nitrous oxide (N_2O) emissions. This increased risk stems from the fact that readily available NH_4^+ promotes both nitrification and denitrification, key processes in the production of N_2O. Nevertheless, further investigation is required since the specific dynamics between mineralization, nitrification, and denitrification, and their impact on N_2O emissions, were not a central focus of this research. Future studies should therefore explore in more detail the interplay between these processes to understand more fully the effect of low C/N ratios on nitrogen transformations and N_2O release in this particular context.

4.2. Effects of GM on the abundance and expression of denitrifying genes

In general, GM application did not produce significant changes in the abundance of the studied genes with the exception of *nirS*, which increased by 88 %, albeit at the limit of statistical significance ($p < 0.09$). Recent studies focusing on the effect of N addition including green manure on *nirK*, *nirS*, and *nosZ* have proliferated, suggesting that fertilization increases the abundance of all genes, particularly *nirK* (You et al., 2022). Additionally, organic fertilization appears to have a more positive effect on the abundance of these genes compared to inorganic fertilization (Ouyang et al., 2018). However, there are fewer studies specifically examining the effect of GM on the abundance of these genes, and the results are contradictory. Thus, similar to our study, Xiao et al. (2021) found that GM fertilization increased the abundance of both *nirK* and *nirS* with *nirS* being particularly favored by this type of fertilization. Li et al. (2022) found comparable results, showing that *nirK* and *nirS* abundance increased with GM fertilization alone or mixed with chemical fertilization or Lyu et al. (2024) which demonstrated that no-tillage with green manure mulching based on common veth increased the transcription of nosZ genes. In contrast, Fang et al. (2020) and Hu et al. (2023) showed that GM application in maize crops decreased the abundance of *nirK*, *nirS* and *nosZ*, something also observed by Wang et al. (2022) in tea crops.

When examining the effect of GM on transcript copies, we observe a decrease in abundance for *nirK*, *nirS*, *nosZ1*, and *nosZ2* by 61 %, 52 %, 3.5 %, and 13 %, respectively. However, this decrease is statistically significant only for *nirK* and *nosZ2*. These results appear to be contradictory to recent studies showing increased transcription of these genes, particularly *nosZ*, following the application of certain types of GM. For example, Thompson et al. (2018) demonstrated that returning biomass residues resulted in significantly higher *nosZ* transcripts compared to soils with residues removed, although it did not affect *nirS* gene expression. Similarly, Linton et al. (2020) found that rotating maize and soybean crops increased the expression of the *nosZ2* (atypical) gene in farmland compared to monoculture, but only after the addition of urea-ammonium. More recently, Hu et al. (2023) showed that the application of Green Manure based on the legume *Vicia villosa* Roth (hairy vetch) resulted in increased transcripts of *nirK*, *nirS*, and *nosZ*.

However, it is important to note that the abundance of transcripts for these genes is influenced not only by the cropping system and type of fertilization but also by changes in environmental variables, as observed by Krause et al. (2017) for *nosZ* (both *nosZI* and the atypical *nosZII*). In this regard, most studies indicate that pH and total organic carbon (TOC) are the primary factors influencing bacterial abundance and composition in agricultural soils under different fertilization regimes (Ouyang et al., 2018; Xiao et al., 2021; Dong et al., 2022). In a recent study, we found a positive correlation between the abundance of *nirK* and *nosZ* and both pH and TOC when comparing soils similar to the control in the present study with soils under inorganic fertilization (Hernández Maqueda et al., 2024a). In contrast, no such correlations were observed in this work. A possible explanation is that GM, at least after the first three years of application, does not produce any change in any of these properties.

But in this study, we observed how soil potassium (K) and moisture correlate with the abundance and expression of *nirK*, *nirS*, and *nosZ* genes. According to most authors, soil moisture favors the denitrification process (Nadeau et al., 2019; Meng et al., 2020). However, the interaction between soil salinity and moisture appears to play a central role in denitrification regulation (Meng et al., 2020; Pan et al., 2023). According to Meng et al., 2020, this occurs because salinity affects the abundance, diversity and activity of denitrifying microorganisms. In our study, moisture, Ca, K, and EC increased during crop development, and these changes had differential effects on the functional genes analyzed. A positive correlation was observed between K, soil moisture, and the expression of *nirK* and *nirS*, whereas a negative effect was found on the abundance of *nosZ* I and *nosZ2* II genes. This suggests that the interaction between salinity and moisture induces distinct responses in the abundance and expression of the nitrito reductase genes compared to nitrous oxide reductase genes. Although *nirK*-type denitrifiers are generally more resistant to environmental fluctuation (Sun and Jiang, 2022), elevated salinity levels significantly reduce the overall abundance of denitrifying genes. As salinity increases, dominant microbial genera are progressively replaced by salt-adaptable microbes (Pan et al., 2023).

These results further highlight that the fluctuation in transcripts of *nirK*, *nirS*, and *nosZ* is not only related to the type of fertilization and nutrient availability but is also influenced by the dynamics of the crop cycle, as recently demonstrated by Maul et al. (2019), Han et al. (2020), Hu et al. (2023), and Hernández Maqueda et al. (2024a, 2024b). According to Hu et al. (2023), transcript abundance is more strongly influenced by the complex interactions between temporal dynamics and management practices. In line with these observations, this study, through linear mixed model (LMM), revealed significant interactions between green manure (GM) treatment and crop stage at both t1 and t2. For example, the application of green manure reduced the number of transcripts of *nirK*, *nirS*, and *nosZ* (I and II) during the growing phase of both crop and GM (t2), a period that coincides with the peak expression of these genes in this type of soils (Hernández Maqueda et al., 2024a).

These observations could suggest that the use of green manure may act as a nitrogen sink, and that could influence the abundance and gene expression of the denitrifiers, potentially reducing nitrogen losses through denitrification. However, further research is needed to confirm this hypothesis, given the complexity of the denitrification process and the involvement of numerous post-transcriptional regulatory mechanisms (Wang et al., 2018; Han et al., 2022). Therefore, a controlled laboratory study examining soil nitrogen cycling, nitrogen loss, and the transcriptional pathways of nitrification over a broader temporal sequence is needed to better understand the potential of GM as a strategy to avoid losses through denitrification pathway.

Lastly, in addition to the limitations discussed above, it is important to highlight a few additional aspects that were not addressed in this study but should be considered in future research. On one hand, nitrogen addition not only affects the abundance of denitrifying genes but also alters the diversity and composition of the bacterial communities harboring these genes. As indicated by Xiao et al. (2021) and Sun and Jiang (2022), these changes can influence nitrification-denitrification processes in ways that remain largely unexamined. Furthermore, future studies should also focus on the abundance, composition, and diversity of denitrifying fungi, as fungi-mediated denitrification appears to play a crucial role in agricultural soils, as noted by Aldossari and Ishii (2021) and Jiang et al. (2022).

5. Conclusions

From the analysis of the results obtained in this study, the following conclusions can be drawn:

After three years of GM application, moderate changes in soil physicochemical properties were observed, including significant decreases in soil temperature (−6 %) and NH_4^+ concentration (−14 %). However, most of the other soil properties analyzed remained largely unchanged, suggesting that significant changes tend to manifest over the long term.

The abundance and expression of the analyzed genes depend on a complex interaction between GM application, sampling time, and soil properties. Specifically, GM application does not produce significant changes in gene copy numbers, but GM reduces the abundance of transcript copy number, significantly for *nirK* and *nosZII*. On the other hand, the crop stage also influences the fluctuation of gene abundance and expression, leading to a decrease in abundance of genes involved in nitrous oxide reductase reduction and an increase in transcripts copies of all genes during the maximum development stage of the crop and GM. Regarding the analyzed soil properties, K^+ and soil moisture were found to affect the abundance and expression of denitrifying genes, suggesting that salinity and soil moisture are key in regulating denitrification.

In conclusion, this study highlights the intricate interactions between green manuring, soil properties, and the denitrification process. Future research should consider controlled laboratory experiments that explore soil nitrogen cycling, nitrogen loss, and the transcriptional pathways of denitrification over an extended time frame. Additionally, incorporating the analysis of bacterial and fungal composition and diversity through metagenomics and metatranscriptomics would offer deeper insights into the underlying mechanisms of denitrification in agricultural soils.

CRediT authorship contribution statement

R. Hernández Maqueda: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **I. Ballesteros:** Writing – review & editing, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **A.J. Aguirre:** Writing – review & editing, Methodology, Formal analysis, Data curation. **D. Meca:** Writing – review & editing, Resources, Investigation, Formal analysis, Data curation. **R. Linacero:** Writing – review & editing, Validation, Supervision, Resources. **F. del Moral:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2025.106216.

Data availability

Data will be made available on request.

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