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REGULAR ARTICLE

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*

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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. 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 nitrate. Bacteria were more abundant than archaea in the bulk and rhizosphere soil. The abundance 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 gene copy number of denitrifiers, which were more abundant in the bulk soil.
Conclusions N-fertilisation decreases N_2O production due to increased abundance of the *nosZ* gene. The abundance of nitrification and denitrification genes in bulk and rhizosphere soils is dependent on the type of fertiliser. For both plant species, the ratio of the genes involved in production and reduction of N_2O by bulk and rhizosphere was similar.

Keywords Nitrogen fertiliser · Nitrification genes · Denitrification genes · qPCR · Cultivated soil

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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 represents 10–12% of the anthropogenic greenhouse gas emissions (IPCC 2013) and is of great concern due to its global warming potential 310 times greater than that of carbon dioxide (CO_2) (Ravishankara et al. 2009).

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 (for reviews see Bueno et al. 2012; Butterbach-Bahl et al. 2013).

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 the 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 was lower in the rhizosphere than in bulk soil of unfertilised rice, but 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 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 abundance 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. 2009).

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 or 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 carried out 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). In addition to their importance on human consumption, role in sustainable agriculture and economical interest for farmers, tomato and common bean were chosen because they are phylogenetically unrelated plants that could have a different impact on the nitrifying and denitrifying rhizosphere communities. 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 usually cultivated. The soil had been maintained under fallow conditions, without fertilisation and no irrigation during the last 10 years. It is a 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 × 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 × 21 × 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 × 4 replicates × 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 °C 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 (Metrohm) 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 100-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 (Qiagen) 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 °C until use. The total bacterial (16SB) and archaeal (16SA) communities were 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*I and *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 fourth 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 with the control soil. Also, the contents of NH_4^+-N, NO_3^--N and TN were lower in the rhizosphere than in the bulk 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 (Fig. 1a and b, 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).

Table 1 Physicochemical properties of the bulk and rhizosphere soil of tomato and common bean 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

Bulk soil					Rhizosphere soil			
	C	U	A	K	C	U	A	K
Tomato								
pH	7.1 ± 0.1b	8.3 ± 0.4d	7.4 ± 0.1c	7.4 ± 0.1c	6.7 ± 0.1a	7.6 ± 0.1c	7.2 ± 0.1b	7.0 ± 0.1b
NH_4^+ (mg N kg^{-1} dry soil)	3.5 ± 1.8a	29.5 ± 1.3e	23.2 ± 3.4d	7.9 ± 0.7b	2.4 ± 0.7a	20.7 ± 0.5d	15.5 ± 1.2c	5.3 ± 1.5b
NO_3^- (mg N kg^{-1} dry soil)	2.7 ± 0.9a	23.8 ± 0.5c	26.0 ± 0.8c	32.6 ± 1.5d	1.8 ± 0.6a	16.2 ± 0.1b	18.7 ± 0.7b	22.1 ± 0.5c
TOC (mg kg^{-1} dry soil)	1.8 ± 0.1a	3.0 ± 0.4b	2.8 ± 0.2b	2.8 ± 0.2b	2.2 ± 0.2a	3.6 ± 0.2c	3.4 ± 0.1c	3.4 ± 0.1c
TC (%)	2.2 ± 0.1a	3.8 ± 0.3b	3.4 ± 0.1b	3.5 ± 0.1b	2.8 ± 0.1a	4.8 ± 0.6c	4.3 ± 0.2c	4.4 ± 0.3c
TN (%)	0.1 ± 0.05a	0.4 ± 0.06c	0.4 ± 0.03c	0.4 ± 0.05c	0.1 ± 0.02a	0.2 ± 0.04b	0.2 ± 0.04b	0.3 ± 0.03b
Common bean								
pH	6.8 ± 0.1c	8.0 ± 0.2d	7.1 ± 0.1c	7.4 ± 0.2c	6.0 ± 0.1a	7.0 ± 0.2c	6.3 ± 0.1b	6.3 ± 0.1b
NH_4^+ (mg N kg^{-1} dry soil)	3.9 ± 1.5a	33.5 ± 0.1e	26.0 ± 0.5d	8.7 ± 2.1b	3.1 ± 1.5a	26.4 ± 0.5d	21.6 ± 1.9c	6.9 ± 1.1b
NO_3^- (mg N kg^{-1} dry soil)	2.9 ± 1.6a	28.5 ± 1.1c	29.2 ± 1.4c	38.7 ± 3.0d	2.4 ± 0.4a	21.2 ± 0.4b	24.8 ± 0.1b	29.2 ± 1.1c
TOC (mg kg^{-1} dry soil)	1.5 ± 0.2a	2.5 ± 0.3b	2.4 ± 0.2b	2.4 ± 0.2b	2.1 ± 0.2b	3.5 ± 0.1c	3.3 ± 0.2c	3.3 ± 0.1c
TC (%)	1.9 ± 0.1a	3.2 ± 0.1c	2.9 ± 0.1c	3.0 ± 0.1c	2.3 ± 0.2b	4.0 ± 0.2d	3.6 ± 0.2d	3.7 ± 0.1d
TN (%)	0.1 ± 0.03a	0.4 ± 0.06c	0.4 ± 0.06c	0.4 ± 0.06c	0.1 ± 0.02a	0.3 ± 0.05b	0.3 ± 0.05b	0.3 ± 0.05

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 b), though cumulative emissions after the fourth 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 b, 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 fourth harvest. For each plant species, the abundance of the bacterial and archaeal communities is greater in the rhizosphere than in the bulk soil. 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 × 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 × g^{-1} dry soil after fertilisation with urea, ammonium or nitrate, respectively (Fig. 2a).

In bulk and rhizosphere soil of common bean, the abundance 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 × 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 × 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%).

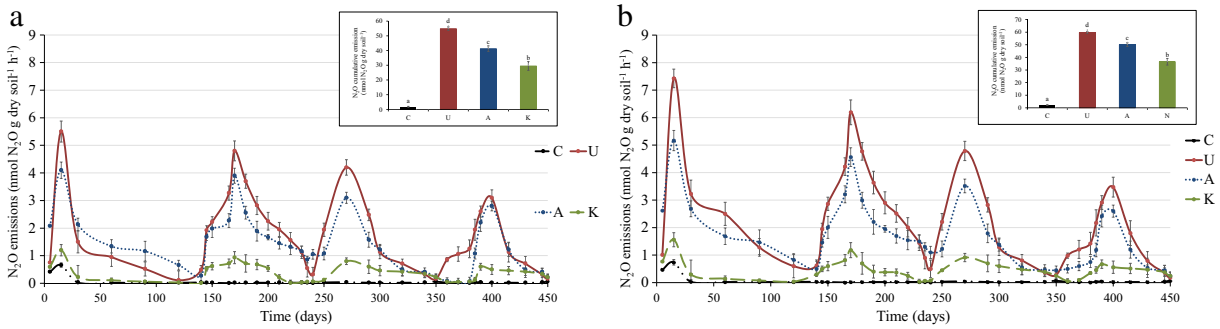

Fig. 1 Nitrous oxide production by a soil cultivated with tomato (**a**) or common bean (**b**) during 4 consecutive harvests. The soil was fertilised with urea (U), ammonium sulphate (A) or potassium nitrate (K). Unfertilised soil was used as a control (C). Values are expressed as nmol N_2O g dry $soil^{-1}$. 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 deviations ($n = 4$)

Total abundance of the nitrifying community

Like the data on the 16SA and 16SB genes, here we present the results corresponding to the fourth harvest. It is to note, however, that while the abundance of the *amoA* AOB gene did not significantly change during the 4 consecutive harvests, that 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) in bulk and rhizosphere soil, and nitrate did not change the abundance of the *amoA* genes. When cultivated with tomato, the abundance 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 × 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 × 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 × g^{-1} dry soil fertilised with urea, ammonium and nitrate, respectively (Fig. 3b). Also, the abundance 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 × 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 abundance 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 community

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 fourth 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 × g^{-1} dry soil, respectively, and 4.9, 5.2, 5.0 and 5.1 log gene copy number × 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

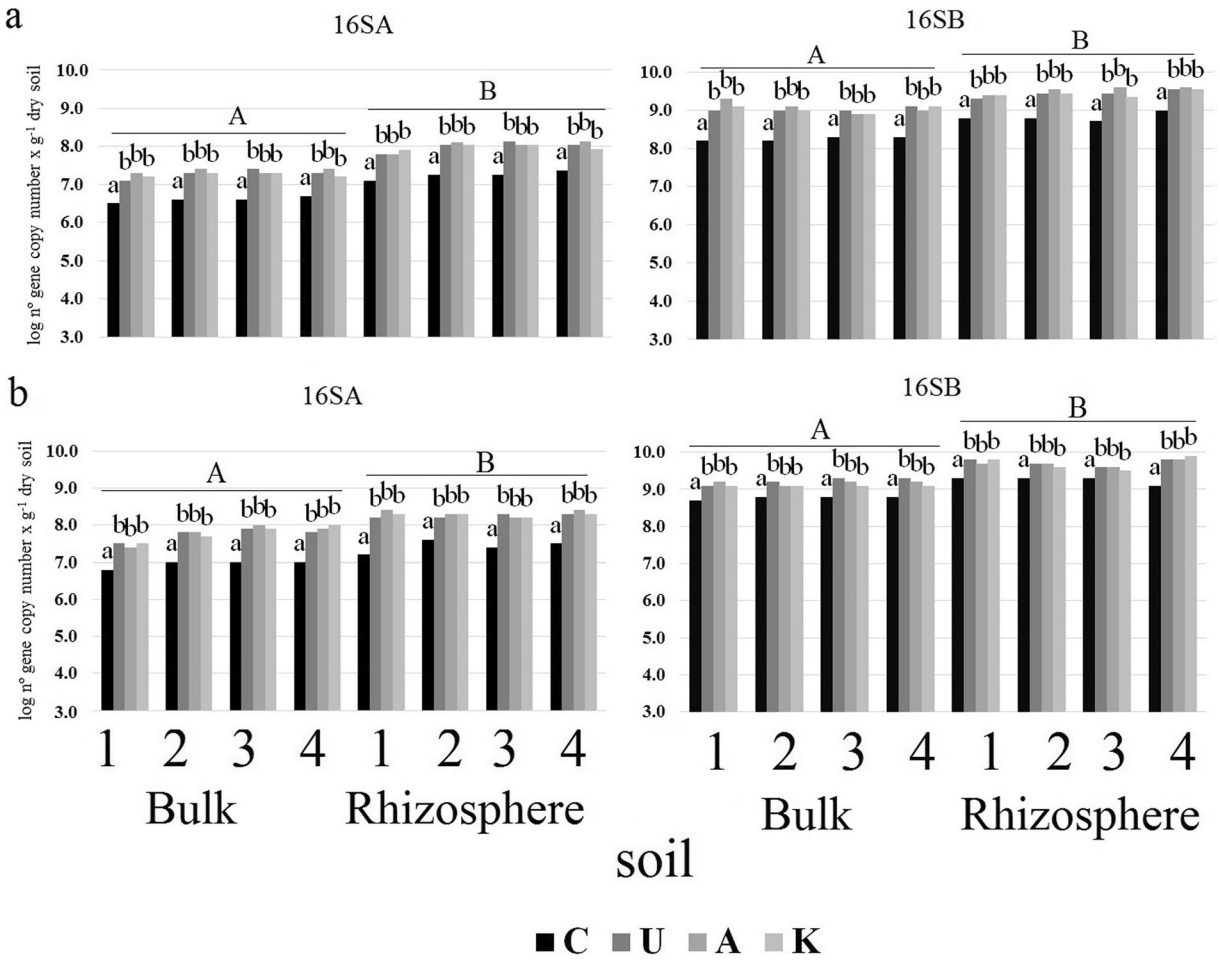

Fig. 2 Total abundance of the 16SA and 16SB genes in the bulk and rhizosphere soil of tomato (**a**) and common bean (**b**) during 4 consecutive harvests. The soil was fertilised with urea (U), ammonium sulphate (A) or potassium nitrate (K). Unfertilised soil was used as a control (C). Values are expressed as log gene copy number × 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. Kruskal-Wallis and Conover-Iman tests were done ($P < 0.05$; $n = 4$)

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 × 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 × 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 × 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 × 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 × 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).

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

Discussion

Properties of the bulk and rhizosphere soil

In this study, we examined the physicochemical properties, N_2O emission and abundance of nitrifiers and denitrifiers in bulk and rhizosphere soil of two vegetable crops in response to the amendment with urea, ammonium or nitrate during 4 consecutive harvests. The amendment with urea increased the pH of the bulk soil (Table 1) compared with 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 (Table 1). 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 exchangeable NH_4^+-N, 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.

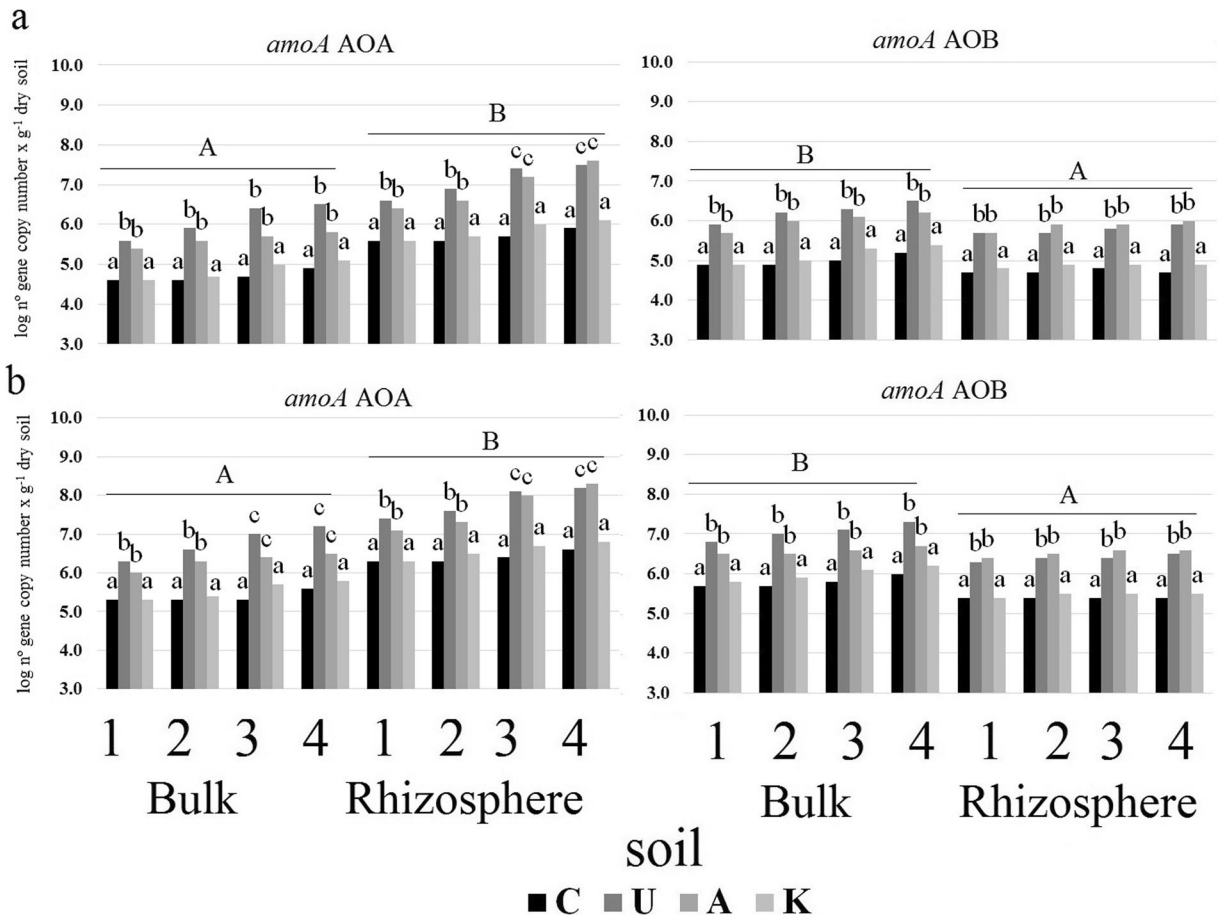

Fig. 3 Total abundance of the *amoA* AOA and *amoA* AOB genes in the bulk and rhizosphere soil of tomato (**a**) and common bean (**b**) during 4 consecutive harvests. The soil was fertilised with urea (U), ammonium sulphate (A) or potassium nitrate (K). Unfertilised soil was used as a control (C). Values are expressed as log gene copy number × 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. Kruskal-Wallis and Conover-Iman tests were done ($P < 0.05$; $n = 4$)

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 emission by the soil cultivated with tomato or common bean were reached after the combined addition of water (up to 80% WFPS) with any of the N-fertilisers (Fig. 1a and b). 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 (Fig. 1a and b) (see below).

The weekly watering of the soil without N-fertilisation did not produce further increases of the N_2O fluxes during plant growth (Fig. 1a and b). 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 (Fig. 1). These results show that high moisture conditions alone did not stimulate N_2O

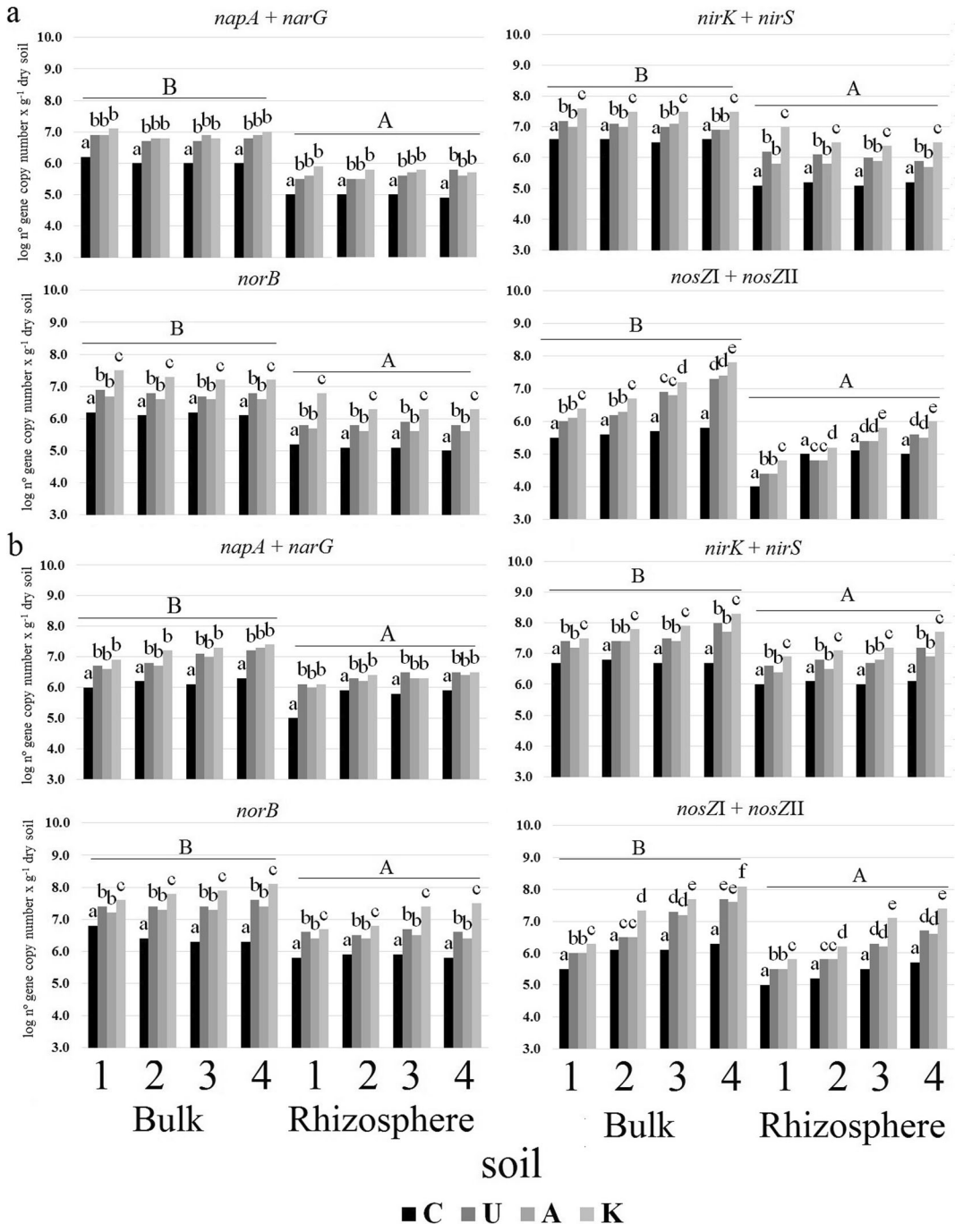

Fig. 4 Total abundance of the *napA* + *narG*, *nirK* + *nirS*, *norB* and *nosZ*I + *nosZ*II genes in the bulk and rhizosphere soil of tomato (**a**) and common bean (**b**) during 4 consecutive harvests. The soil was fertilised with urea (U), ammonium sulphate (A) or potassium nitrate (K). Unfertilised soil was used as a control (C). Values are expressed as log gene copy number × 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. Kruskal-Wallis and Conover-Iman tests were done ($P < 0.05$; $n = 4$)

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 (Fig. 1a and b), 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 (Fig. 1a and b, 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 abundance 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 et al. 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 the TC content in the bulk and by the TOC content 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 abundance 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) found that AOA were more abundant in the rhizosphere, and 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) and common floating aquatic macrophyte plants (Wei et al. 2011; Trias et al. 2012). 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 tomato and common bean.

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 abundance 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. In this sense, 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 ascribed to nitrification but also to a lower potential of N_2O reduction (Fig. 4).

From the data in Table S5c, regardless of the N-fertiliser, after four consecutive harvests, the 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 plant species. However, the total abundance of all of the abovementioned genes was higher in the soil cultivated with common bean (Table S5d), this more likely explaining the higher values of N_2O production by that soil (Fig. 1, insets; Table S4). The reasons why abundance of nitrifiers and denitrifiers is higher in soils cultivated with common bean compared with tomato 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. Maybe the higher price of nitrate compared with other N-fertilisers may prevent its use in agricultural practices.

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