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Long-term no-tillage application increases soil organic carbon, nitrous oxide emissions and faba bean (*Vicia faba* L.) yields under rain-fed Mediterranean conditions

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Abstract

The introduction of legumes into crop sequences and the reduction of tillage intensity are both proposed as agronomic practices to mitigate the soil degradation and negative impact of agriculture on the environment. However, the joint effects of these practices on nitrous oxide (N_2O) and ammonia (NH_3) emissions from soil remain unclear, particularly concerning semiarid Mediterranean areas. In the frame of a long-term field experiment (23 years), a 2-year study was performed on the faba bean (*Vicia faba* L.) to evaluate the effects of the long-term use of no tillage (NT) compared to conventional tillage (CT) on yield and N_2O and NH_3 emissions from a Vertisol in a semiarid Mediterranean environment. Changes induced by the tillage system in soil bulk density, water filled pore space (WFPS), organic carbon (TOC) and total nitrogen (TN), denitrifying enzyme activity (DEA), and bacterial gene (16S, amoA, and nosZ) abundance were measured as parameters potentially affecting N gas emissions. No tillage, compared with CT, increased the faba bean grain yield by 23%. The tillage system had no significant effect on soil NH_3 emissions. Total N_2O emissions, averaged over two cropping seasons, were higher in NT than those in CT plots (2.58 vs 1.71 kg N_2O-N ha^{-1}, respectively). In addition, DEA was higher in NT compared to that in CT (74.6 vs 18.6 μg N_2O-N kg^{-1} h^{-1}). The higher N_2O emissions in NT plots were ascribed to the increase of soil bulk density and WFPS, bacteria (16S abundance was 96% higher in NT than that in CT) and N cycle genes (amoA and nosZ abundances were respectively 154% and 84% higher in NT than that in CT). The total N_2O emissions in faba bean (not N-fertilized crop) were similar to those measured in other N-fertilized crops. Therefore, the benefits of no tillage (e.g. increase of soil organic carbon and faba bean yield) in contrast to conventional tillage have to be evaluated in view of the potential impacts of greater N_2O emissions.

1. Introduction

Nitrous oxide (N_2O) and ammonia (NH_3) are two gases whose emissions to the atmosphere are undesirable because of their environmental harmfulness (Cameron et al., 2013). Indeed, N_2O is a major stratospheric ozone layer depleting compound and a powerful greenhouse gas with a global warming potential of 265 times higher than that of carbon dioxide (CO_2) (Revell et at., 2015). Ammonia contributes to acid rains, and its deposition onto soils and surface waters may lead to acidification and eutrophication (Erisman et al., 2007).

Agricultural activities account for approximately 60% and 90% of the global anthropogenic emissions of N_2O and NH_3 (EEA, 2009; IPCC, 2014). Soil NH_3 volatilization is a complex process related to soil temperature, water content, pH and carbon (C) and nitrogen (N) pools and availability (Cameron et al., 2013). Nitrous oxide in soil is produced under oxic and anoxic conditions as a result of several microbial processes (nitrification, denitrification, heterotrophic nitrification with denitrification, coupled nitrification–denitrification, and nitrifier denitrification) (Khalil et al., 2004); the contribution of each of these processes to N_2O production varies in relation to availability and forms of N, organic C content, and O_2 partial pressure (Wrage et al., 2001; Cameron et al., 2013). Estimates of N gas emissions are greatly variable due to the great amount of factors affecting them among which climatic conditions and agronomic management. Regarding the latter, the tillage system, by affecting soil physical and chemical properties, in turn, affects N gas emissions (Mutegi et al., 2010; Martin-Lammerding et al., 2011; García-Marco et al., 2016).

No-tillage (NT) is widely considered an environmentally friendly soil management technique. However, despite ecological benefits potentially derived from NT use, such as the mitigation of soil erosion, reduction of energy use, increase of C sequestration, and improvement of soil microbiota (Uri et al., 1999; Baggs et al., 2003), several authors have found that NT, compared to conventional tillage (CT), increases N_2O emissions (Rochette et al., 2008; Plaza-Bonilla et al., 2014; Bayer et al., 2015; Badagliacca et al., 2018). These authors attributed this effect to the changes induced by NT application in some soil properties, with negative repercussions on soil gas diffusion, water drainage, and detritusphere in the soil surface. In contrast, other works found opposite results (van Kessel et al., 2013; García-Marco et al., 2016) or no differences by the tillage system (Tellez-Rio et al., 2015b). Such discrepancies may be due to differences in soil type, climatic conditions, agronomic management, and the duration of the experiment (van Kessel et al., 2013). In relation to the latter factor, it has been shown that in short term experiment N_2O emissions are higher in NT than in CT, whereas in experiments longer than 10 years, N_2O emissions are similar between the two tillage systems (Plaza-Bonilla et al., 2014). Such results emphasize the importance of long-term studies in providing evidence of the environmental friendliness of NT.

Studies aimed at comparing the N_2O emissions among tillage systems have been generally carried out in temperate or humid zones, whereas few studies have been performed in arid and semiarid environments, likely because it is often assumed for such areas that N losses as N_2O have low or insignificant magnitudes (Aguilera et al., 2013; Sanz-Cobena et al., 2017). In contrast, the risks of N losses via NH_3 volatilization are potentially consistent in arid and semiarid environments (Sanz-Cobena et al., 2008; Ferrara et al., 2014). The extent of such losses has often been estimated in relation to the amount and type of N fertilizer applied, whereas few studies have examined these losses in relation to tillage systems.

Additionally, crop type affects soil N gas emissions (Tellez-Rio et al., 2015a; Bayer et al., 2015; Guardia et al., 2016). Studies on soil N emissions have been performed in cereal crops, whereas few studies have considered such emissions in the cropped soils of grain legumes, particularly legumes typical of arid and semiarid environments (chickpea, faba bean, lentil, and lupin). Including N_2-fixing legume species within the crop sequence is reported as a valuable direct and indirect N gas emission mitigation strategy due to less mineral N fertilizers applied to soil (Jensen et al., 2012; Sanz-Cobena et al., 2017) and their use as feedstocks thus decreasing N emissions associated to their production and transport (Tongwane et al., 2016). However, since microbial processes that lead to N_2O production are positively associated with soil nitrates (Wagner-Riddle and Thurtell, 1998), legumes can increase N_2O emissions as a result of their poor efficiency in recovering the plant-available mineral N (Jensen et al., 2012; Saia et al., 2016; Ruisi et al., 2017). The aim of this study, therefore, was to evaluate the impact of long-term NT and CT, in affecting the grain and biomass yield of faba beans and the emissions of N_2O and NH_3 from a Vertisol under typical Mediterranean conditions. Indeed, tillage management, through the modification induced on the physical and chemical properties of the soil in turn affects bacteria abundance and N cycle genes and, consequently, N gas emissions..

2. Material and Methods

2.1 Experimental site

The field experiment was performed at Pietranera, an experimental farm of the University of Palermo located approximately 30 km north of Agrigento (Sicily, Italy, 37°30′ N, 13°31′ E; 178 m a.s.l.). The soil is a Chromic Haploxerert (Soil Survey Staff, 2010) and plots had a constant slope of 7%. The following soil characteristics, determined at the beginning of the experiment (year 1991) and referring to the 0–40 cm top layer, were observed: 525 g kg^{-1} clay, 216 g kg^{-1} silt, 259 g kg^{-1} sand, pH 8.1 (in water), 14 g kg^{-1} total organic C, 1.29 g kg^{-1} total N, 36 mg kg^{-1} available P (Olsen). The climate at the experimental site is semiarid Mediterranean, with a mean annual rainfall of 572 mm (period of observation from 1995 to 2015), concentrated mostly during the autumn–winter period (September–February; 76%), and in the spring (March–May; 19%). The dry period occurs from May to September. Mean air temperatures are 15.9°C in the fall, 9.7°C in the winter, and 16.5°C in the spring. The average minimum and maximum annual temperatures are 10.0°C and 23.3°C, respectively. Climatic trends from September 2013 to July 2015 were collected from the nearest weather station located 500 m from the experimental site (Figure S1).

2.2 Experimental design and crop management

The experiment was set up in fall 1991 as a strip-plot design with two replications, where three soil tillage systems (conventional, reduced, and no-tillage) served as vertical treatments and three crop sequences (wheat–wheat, wheat–faba bean, and wheat–berseem clover) served as horizontal treatments (Giambalvo et al., 2012; Amato et al., 2013). The present study was performed in the frame of this long-term field experiment. The experimental factor tested was tillage system: CT vs NT. The data were collected only in faba bean plots. Conventional tillage consisted of one mouldboard ploughing to a depth of 30 cm in the summer, followed by one or two shallow harrowing (0–15 cm) operations before planting. No-tillage consisted of sowing by direct drilling. The plot area size was 370 m^2 (18.5 × 20.0 m). In NT plots, the weeds were controlled before planting with glyphosate at a dose of 533 to 1066 g acid equivalent ha^{-1}, depending on the development of weeds. The faba bean crops were broadcast fertilized with 46 kg ha^{-1} P_2O_5 before planting and received no N fertilizer. Faba bean (cv. Gemini) planting was consistently performed in December by using a no-till seed drill with hoe openers under both CT and NT, making the appropriate sowing depth adjustments to ensure a homogeneous planting depth (3–5 cm) and applying a density of 40 viable seeds m^{-2} with an inter-row spacing of 75 cm. No rhizobial inocula were applied before planting because soil has a native rhizobial population. The weeds were mechanically controlled by shallow hoeing (with minimum soil disturbance) when the plants were at the third-leaf stage; when necessary, the operation was repeated at the seventh-leaf stage. Each year, at faba bean maturity (on average on late June), three sample areas of 9 m^2 each were identified within each plot; the faba bean plants in these sample areas were counted and their aboveground biomass and grain yield were recorded. The remainder of the plot was then mechanically harvested, leaving standing straw and uniformly spreading crop residues. The soil surface covered by mulch in the NT treatments was consistently >30%.

2.3 Soil sampling and analyses

During the cropping season 2013–2014, two soil samples per plot (each composed by 3 mixed subsamples of 1.5 kg) were collected from the 0–15 cm and 15–30 cm soil layers in December (before sowing), April (at full flowering), and late June (at harvest) for a total of 48 soil samples. The samples were air-dried at room temperature until constant weight was reached (it takes no more than one week), gently passed through a 2-mm mesh sieve, and stored in sealed polyethylene bottles at 4°C prior to physical and chemical characterization.

Total nitrogen (TN) was determined by the Kjeldhal method and total organic carbon (TOC) by the Walkley–Black procedure (Nelson and Sommers 1996). Extractable organic carbon (EOC) and nitrates were determined on 0.5 M K_2SO_4 soil extracts (1:4 v/w) by the acid dichromate oxidation method (Vance et al., 1987) and the chromotropic acid method (Sims and Jackson, 1971), respectively. The concentration of EOC was used as an indicator of available C (Laudicina et al., 2013).

At each N_2O field measurement, the gravimetric water content of soil at 0–15 cm depth was determined by the weight difference between the fresh and dried (24 h at 105°C) samples, while the water-filled pore space (WFPS) was calculated by using the following equation:

$$WFPS = \frac{SWC \times BD}{(1 - BD/PD)} \times 100$$

where SWC is the gravimetric soil water content, BD is the soil bulk density and PD is the soil particle density (2.65 g cm^{-3}). The soil BD was determined by the core method (Grossman and Reinsch, 2002).

2.4 16S, amoA and nosZ genes abundance

In the first week of May 2014, when the soil and plants likely reached the maximum biological activity, 4 soil samples (each composed by mixing 3 subsamples) were collected from the superficial layer (0–15 cm) of both NT and CT plots, and stored at –20°C until analyses. Immediately before starting the analyses, soil samples were thawed and gently sieved at 2 mm mesh size.

DNA was extracted and purified from 2 g aliquots of soil samples using the RNA PowerSoil® Total Isolation Kit (MoBio, USA) following the manufacturer's instructions. Then, DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA) and amplified by PCR. The primers used were F341 and R907 (550 pb), amoA-1F and amoA-2R (491 bp), nosZ-1840F and nosZ-2090R (267 bp) for 16S, amoA (Rotthauwe et al., 1997) and nosZ (Henry et al., 2006), respectively. For 16S gene the PCR program was initiated by a hot start of 5 min at 94°C; after 9 min of initial denaturation at 95°C, a touchdown thermal profile protocol was used, and the annealing temperature was decreased by 1°C per cycle from 65°C to 55°C; then 20 additional cycles at 55°C were performed. Amplification was carried out with 1 min of denaturation at 94 °C, 1 min of primer annealing, and 1.5 min of primer extension at 72°C, followed by 10 min of final primer extension. For amoA, a nitrifying bacterial gene, PCR program was performed with an initial denaturation at 94°C for 90 s and then 20 cycles of denaturation at 94°C for 40 s, annealing at 53°C for 30 s, and extension at 72°C for 40 s. Finally, for nosZ, a denitrifying bacterial gene, PCR conditions consisted of an initial denaturating step of 95°C for 15 min, followed by 30 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 30° s and a final step of 72°C for 8 min.

Reaction mixture of PCR consisted of 25 μL with the following ingredients: soil DNA dilution (from 1:10 to 1:5), 1 μL of both primers, front and rear, at concentration 2.5 μL at concentration of 30 μM for nosZ primers, 2 μL of 0.2 mM dNTPs, 0.15 μL of 5 U Taq polymerase (Bioline), 2.5 μL of 10X PCR buffer, 0.75 μL of 1.5 mM $MgCl_2$ and sterile Milli-Q water to a final volume of 25 μL. Sterile water was used as a negative control to replace DNA in PCR reactions. PCR products were analysed by electrophoresis in 2% agarose gels stained with GelRed®. The PCR results for each gene were used in order to choose the best DNA PCR concentration for qPCR. Quantification of the DNA copy number was performed on an iQ5 thermocycler using iQ5-Cycler software (Bio-Rad, Munich, Germany). Amplification was performed in 20 µL reaction mixtures composed by 10.5 µL of SyberGreen 2X, 0.84 µL of both primers, and sterile Milli-Q water to a final volume of 20 µL. Primers and qPCR conditions were the same of PCR amplification described above.

16S, amoA and nosZ standard curves were constructed using plasmid relating Ct (cycle threshold) to the added mass of linearized plasmid DNA and the number of gene copies. The amount of template DNA was calculated by interpolating the cycle threshold with the standard curve, determined by the Bio-Rad iQ5 software program. All reactions were carried out in triplicate with four replication per qPCR. The potential presence of qPCR inhibitors was tested by mixing 1 µl (4–8 ng) of soil DNA extracts with a known amount of recombinant plasmid DNA (pCR®2.1, Invitrogen, Carlsbad, CA, USA) with the appropriate primers. Controls, where DNA templates were replaced by filter-sterilized milliQ water, were carried out simultaneously. Ct values were not significantly different between the DNA extracts and the controls.

2.5 Denitrifying enzyme activity (DEA)

Denitrifying enzyme activity was determined on soil samples using the anaerobic slurry technique as described by Šimek et al. (2004). Briefly, 20 g of soil were weighted in a 125 mL flask and 20 mL of a solution 1mM in glucose, 1mM in KNO_3 and containing 1 g L^{-1} of chloramphenicol was added. Flasks were sealed with butyl rubber stoppers, evacuated and flushed four times with 99.999% helium equilibrating the internal pressure to the atmospheric one. Each evacuation and/or flushing lasted for 2 min. After that, using a 15 mL syringe, ten millilitres of internal atmosphere was removed and replaced with pure acetylene in order to block the conversion of N_2O to N_2 (Smith and Tiedje, 1979). The flasks were then shaken on a horizontal shaker at 70 rpm. After 30 and 60 min from the addition of acetylene, 1 mL sample of headspace atmosphere were taken with a gas-tight syringe and N_2O concentration was measured by a gas chromatograph (TRACE-GC, Thermo Scientific, Milano, Italia) equipped with a 80–100 mesh stainless-steel column packed with Poropak

Q and an electron capture detector (ECD). DEA was calculated from the N_2O increase during a half an hour incubation (60–30 min).

2.6 Ammonia and nitrous oxide emissions

Field measurements of NH_3 emissions were performed during two periods in two cropping cycles, after sowing and at full flowering (in 2013-2014 from 18.12.2013 to 02.01.2014 and from 24.03.2014 to 09.04.2014, and in 2014–2015 from 23.12.2014 to 09.01.2015 and from 13.04.2015 to 28.04.2015). The soil NH_3 volatilization was monitored by Conway's microdiffusion-incubation method (Bremner and Krogmeier, 1989; Qi et al., 2012) a simple and low-cost closed chamber technique that can lead to reliable comparison among treatments (Miola et al., 2014; Shigaki and Dell, 2015). Soil N_2O emissions were measured during the 2013–2014 and 2014–2015 cropping cycles, from sowing to harvest. Greenhouse gas fluxes were sampled using the closed chamber technique (Hutchinson and Mosier, 1981; Baker et al., 2003). Soil N_2O gas fluxes were measured eight times per cropping year at regular intervals (from 10/01/2014 to 06/06/2014 and from 08/01/2015 to 17/06/2015). At each sampling time, six field measurements per treatment were done for NH_3 or for N_2O. More details about field gas sampling for NH_3 and N_2O emissions are reported in Badagliacca et al. (2018). The concentration of N_2O in the gas samples was assessed by a gas chromatograph equipped with an electron capture detector as described above to determine the denitrifying enzyme activity. Flux rates were calculated from the N_2O concentration increase during the 60-minute chamber closure period by the following equation according to Jantalia et al. (2012):

$$f = \frac{\Delta C}{\Delta t} \times \frac{V}{A} \times \frac{m}{V_m}$$

where $\Delta C/\Delta t$ is the change in N_2O concentration in the chamber during the closing time Δt, V and A are respectively the volume of the chamber and the area of the soil covered by the chamber, Vm is the molar volume corrected for the air temperature at the sampling time and m is the molecular weight of N_2O.

The seasonal amount of N_2O emissions were accumulated from the emission rates between every two consecutive days of the measurements by the following equation according to Cheng et al. (2012):

$$Cumulative\ N_2O\ emissions\ = \sum_{i=1}^{n} (F_i + F_{i+1})/2 \times (t_{i+1} - t_i) \times 24$$

2.7 Statistical analyses

The normal distribution and variance homogeneity of the data were assessed by Kolmogorov–Smirnoff goodness-of-fit and Levene's tests, respectively. Following the strip-plot procedure, one-way ANOVA was performed with tillage (CT and NT) as factor for total NH_3 and N_2O emissions (field measures averaged for two consecutive years and cumulated with regard to crop stages), 16S, amoA and nosZ gene abundance, and with repeated measures (soil sampled in three occasions per cropping year) for TOC, TN, EOC, N-NO_3^-, and DEA. The treatment means were compared using Fisher's protected least significant difference (LSD) test at $P<0.05$. Statistical analyses were performed with SAS statistical package (SAS, 2009). Reported data, expressed on a soil oven-dry basis (105°C), are the arithmetic means.

3. Results

3.1 Climatic conditions

The total rainfall in 2013–2014 and 2014-2015 cropping seasons was almost similar (603 and 660 mm, respectively). Rainfall was homogenously distributed during the 2013-2014 crop cycle (Figure S1), whereas it was mainly concentrated in February-March during the 2014-2015 crop cycle. Also the mean year temperature was similar (15.2°C and 15.8 °C).

3.2 Soil properties

Results on the soil properties for the 0–15 and 15–30 cm soil layers are reported in Table 1. Bulk density was significantly higher under NT than that under CT (+10% and +12% respectively for the 0–15 cm and the 15–30 cm soil layers). In the superficial soil layer, long-term NT application, compared to CT, increased ($P<0.05$) TOC by 4.2 g kg^{-1} (+32%). In the deeper soil layer (15–30 cm), TOC was not affected by tillage system. Thus, considering the 0–30 cm soil layer, NT compared to CT led to an average annual increase in C stock of 0.56 Mg C ha^{-1} year^{-1}. Total N in the 0–15 cm soil layer was 46% higher in NT than CT. As for TOC, TN was not affected by tillage in the deeper soil layer. Extractable organic C and nitrates were not affected by the tillage system in the superficial soil layer (0–15 cm), whereas in the deeper soil layer (15–30 cm), CT showed higher values of both properties than NT. In particular, CT plots, on average, showed 18.3 mg C kg^{-1} (+39%) and 0.19 mg N kg^{-1} (+17%) more than NT plots.

3.3 16S, amoA and nosZ genes abundance

The results regarding abundance are reported in the Table 2. The abundance of all three studied genes (16S, amoA and nosZ genes) was affected by tillage system and showed a higher number of gene copies in NT than in CT. NT application increased bacterial 16S gene copies by +96%, showing 71.2 c.n. mg^{-1} more dry soil than in CT. NT increased the number of amoA and nosZ genes copies by +154% and +84%, respectively, showing 90.2 c.n. mg^{-1} and 48.3 c.n. mg^{-1} more copies than CT. Finally, compared to CT, NT application increased the amoA/16S and amoA/nosZ ratio but not the nosZ/16S ratio.

3.4 Ammonia emissions

Ammonia emissions, as an average of the two cropping seasons, ranged from 30.2 to 32.1 mg m^{-2} in the first sampling period (after sowing) and from 17.0 to 19.4 mg m^{-2} in the second sampling period (at full flowering; Figure 1). Therefore, the amount of N lost as NH_3-N during the monitoring period was approximately 49.3 mg m^{-2}. Tillage system had no effect on soil NH_3 emission.

3.5 Denitrifying enzyme activity (DEA)

Soil denitrifying enzyme activity was affected by tillage system only in the superficial soil layer, while no significant differences were observed in the deepest soil layer. No tillage increased denitrification activity by +301% in the 0–15 cm soil layer, showing, on average, a rate of denitrification of 74.6 μg N kg^{-1} h^{-1} vs 18.6 μg N kg^{-1} h^{-1} of CT (Figure 2).

3.6 Water filled pore space (WFPS) and nitrous oxide emissions in field

During the experiment, soil WFPS ranged from 0.39 to 0.79 m^3 m^{-3} during the 2013–2014 cropping season and from 0.37 to 0.78 m^3 m^{-3} during the 2014–15 cropping season (Figure 3). During both cycles, NT showed higher WFPS values than CT (+18%, on average between cycles). On average, during both cropping cycles, WFPS values were 0.58 m^3 m^{-3} in CT and 0.68 m^3 m^{-3} in NT, showing values higher than 0.60 m^3 m^{-3} for a period of 90 days in CT and 130 days in NT.

Soil N_2O fluxes measured in the field ranged from 11.0 to 141.0 μg N_2O-N m^{-2} h^{-1} in the 2013–2014 cropping season and from 11.0 to 130.4 μg N_2O-N m^{-2} h^{-1} in the 2014–2015 cropping season (Figure 3). During both experimental years, NT plots showed higher emission fluxes than CT plots, reaching the maximum fluxes in the third measurement epoch and continuing with a smooth trend in the first experimental year, with a second late peak in May of the second experimental year (Figure 3). Total N_2O emissions were affected by tillage system (Figure 4). The total N_2O emitted was of 258.5 mg N-N_2O m^{-2} in NT and of 170.8 mg N-N_2O m^{-2} in CT. Therefore, NT application increased N_2O emissions approximately +51% (Figure 4).

3.7 Faba bean biomass and grain yields

The grain yield of faba bean was 55% higher in NT than CT in 2013–2014 (2.44 vs 1.57 Mg ha^{-1}, respectively), whereas no difference by tillage was observed in 2014–2015 (1.85 Mg ha^{-1}, on average; Figure 5A). Considering the 1992–2013 period, faba bean produced 23% more grain under NT than CT (on average 2.18 vs 1.77 Mg ha^{-1}, respectively). Biomass yield was 20% higher in NT than in CT in 2013–2014 (6.95 vs 5.80 Mg ha^{-1}, respectively), but the opposite results were observed in 2014–2015 (3.54 vs 4.33 Mg ha^{-1}, respectively; Figure 5B). The average biomass yield of faba bean over the 1992–2013 period was 5.47 Mg ha^{-1} with no difference by tillage.

4. Discussions

4.1 Changes in soil properties by tillage system

The higher total organic C and total N content only in the 0–15 cm soil layer of NT compared to that in CT is consistent with the finding of previous studies performed in similar semiarid Mediterranean environments (López-Bellido et al., 2010; Parras-Alcántara and Lozano-García, 2014). Such results may be ascribed to the soil organic matter protection by macro-aggregates. Six et al. (2000) argued that the application of NT avoids the breakdown of soil macro-aggregates and favours the formation of C-enriched soil micro-aggregates that physically protect soil organic matter from degradation. In contrast, by destroying soil aggregate and increasing soil aeration, CT promotes soil organic matter decomposition by exposing previous protected organic matter to oxidation processes (Plaza-Bonilla et al., 2013; Laudicina et al., 2016, 2017).

Moreover, whereas the incorporation of crop residues into the soil favours their decomposition in CT, crop residues accumulate on soil surface in NT, making these crops less attackable by soil microorganisms, reducing their decomposition and thus increasing soil organic matter content in the upper soil layer (Dungait et al., 2012). The continuous application of NT for over 20 years, compared to CT, led to an average annual increase in C stock of 0.56 Mg C ha^{-1} year^{-1} in the top 0–30 cm of soil. This value is slightly higher than that reported by Mazzoncini et al. (2016) who observed, after 28 years of NT management under Mediterranean environment, a mean annual C sequestration rate in the 0–30 cm soil layer of 0.40 Mg ha^{-1} year^{-1}. The effect of tillage system on the decomposition of soil organic matter was also confirmed by the availability of organic C. The effect of crop residue incorporation by tillage was evident in the deeper soil layer (15–30 cm), where conventionally tilled soil had higher EOC and nitrate contents than those in NT soil due to both the higher amount of crop residues and the higher decomposition rate. In the 0–15 cm soil layer, no differences were observed between CT and NT for EOC concentration; here the differences in TOC values (with higher values in NT than CT) were likely counteracted by the lower decomposition rate. Similarly, Zhang et al. (2011) did not find significant differences for water extractable organic C between CT and NT in the 0–15 cm soil layer.

4.2 16S, amoA, and nosZ genes abundance

Long-term NT application increased the abundance of 16S, amoA and nosZ bacterial genes. It is likely that NT, determining organic C and N stratification, and increasing soil BD, water retention, and lowering fluctuations of moisture and temperature, promoted favourable conditions for heterotrophic microorganisms according to the findings of previous studies (Cui et al., 2012; Pastorelli et al., 2013; Kaurin et al., 2015). Moreover, long-term NT application promoted not only an increase of the soil microbial community (as demonstrated by the higher values of 16S gene observed in NT compared to CT) but also a shift in its structure and function (as highlighted by the variation of the amoA/16S and the amoA/nosZ genes ratios). Such changes in the microbial population have an important effect on nutrient cycling and N_2O gas emission, such as nitrification and denitrification. Indeed, several studies have highlighted a direct relation between N_2O emission and the abundance of such genes into the soil (Hallin et al., 2009; Smith et al., 2010). The consistent increase of ammonia-oxidizing bacteria in NT was consistent with the findings of Li et al. (2015) and Krauss et al. (2017). AmoA genes encode ammonia monooxygenase, the common enzyme of nitrifying bacteria, which oxidizes ammonia to the intermediate hydroxylamine (Wood, 1986). This gene could be involved in N_2O production during the decomposition of intermediates, such as

NH_2OH, or via the nitrifier denitrification under conditions of N limitation or high NO_2^- concentration (Wrage et al., 2001). A positive correlation between N_2O fluxes and amoA transcripts was observed under low O_2 availability and increased NH_4^+ concentration by Theodorakopoulos et al. (2017) and Zhu et al. (2013), indicating the strategic importance of the enzyme encoded by this gene on N_2O emissions.

In NT, the increase of soil BD and WFPS, with the consequent reduction of the O_2 availability, promoted the growth of bacteria capable of using alternative electron acceptors, such as NO_3^--N, in the denitrification pathway, as highlighted by the nosZ gene copy numbers. This result confirms the findings of other authors who observed an increase of denitrifier abundance in NT or minimum tillage compared to CT (Melero et al., 2011; Tellez-Rio et al., 2015a). As the last enzyme of the denitrification pathway, nitrous oxide reductase (encoded by the nosZ gene) catalyses the reduction of N_2O to N_2. This enzyme is not present in all microorganisms involved in the denitrification process. In fact, some microorganisms can perform the complete pathway with all denitrification enzymes, while other microorganisms lack the nosZ gene and produce only N_2O as the denitrification end-product (Philippot et al., 2011), and still, other microorganisms have the nosZ gene but are only able to use N_2O as an electron acceptor, thus consuming the free N_2O in the soil produced from other microorganisms (Sanford et al., 2012).

4.3 Ammonia emissions

The method used to estimate NH_3 emissions could lead to underestimation, and therefore, in absolute terms, the quantity of N lost as volatilized ammonia may have been greater (Miola et al., 2014; Shigaki and Dell, 2015). However, the method used in this study was the same of that previously used by Badagliacca et al. (2018) so that the results are comparable. Regardless the tillage system, the amount of NH_3 was approximately 0.49 kg NH_3-N ha^{-1} lower than that emitted by N-fertilized wheat grown in the same study area during the same experimental period (13.2 kg NH_3-N ha^{-1}; Badagliacca et al., 2018). This finding is further confirmation that N fertilization is the predominant factor in determining the magnitude of NH_3 losses from soil (Saggar et al., 2013). On the other hand, the tillage system had no significant effect on soil NH_3 emission. This result is in contrast to the findings of previous studies in which the N losses via NH_3 volatilization were significantly higher in NT compared to CT (Palma et al., 1998; Sommer et al., 2004). However, it is to note that differences in NH_3 emissions between the two tillage systems have been mainly ascribed to the different fate of the N fertilizer applied and not to the changes in the soil physical and chemical characteristics induced by tillage. In fact, when N fertilizer is buried following tillage, NH_3 losses are reduced because of the increased resistance to the upward diffusion of NH_3 present in the liquid and gaseous phases and the increased adsorption of NH_4^+ on soil particles (Sommer et al., 2004). Since in this study N fertilizers were not applied, no differences occurred between the two tillage systems.

4.5 Field nitrous oxide emissions and denitrifying enzyme activity

Total N_2O emissions (2.14 kg N_2O-N ha^{-1}; as average of the two cropping seasons) were higher than the mean value reported by Aguilera et al. (2013) for legumes grown under Mediterranean conditions (0.7 kg N_2O-N ha^{-1}). Moreover, total N_2O emissions measured in faba bean were similar to those measured in the same study area and experimental period for N-fertilized wheat (2.08 kg N_2O-N ha^{-1} on average; Badagliacca et al., 2018). This finding suggests that cumulative N_2O emissions in a grain legume crop grown in the Mediterranean environment could be similar or even higher than those of other N-fertilized crops grown in the same environment. Evidently, the low efficiency in the use of mineral N by the grain legume (compared to cereal crops) and the N release in root exudates and decaying root nodules determines the increase of N available for nitrification or denitrification with a consequent increase in N_2O emissions (Yang and Cai 2005; Sanz-Cobena et al., 2014; Tellez-Rio et al., 2015).

The higher total N_2O emissions in NT than in CT are in contrast with the findings of Volpi et al. (2018), who noted how the reduction in tillage intensity (specifically the application of minimum tillage, MT, in place of CT) mitigated soil N_2O emissions in faba bean in a Mediterranean environment. These authors suggested that the incorporation of crop residues into the soil by CT, favouring their decomposition, provoked an increase of N available for N_2O emissions. These authors also observed that the application of MT increased N demand and N uptake from soil compared to that of CT, thereby lowering the soil N available for nitrification and denitrification. This latter hypothesis was not confirmed in this experiment, in which no differences in the faba bean N uptake between CT and NT were found since the values between the two tillage systems for both biomass production and N concentration were similar (N concentration data not shown).

The higher N_2O emissions observed in NT than that in CT can be ascribed to several co-concurrent physical, chemical and microbiological factors: i) bulk density was increased by long-term NT application (1143 vs 1029 kg m^{-3} in NT and CT on average for the 0–30 cm soil layer, respectively) thereby increasing the incidence of soil anoxic microsites (Tellez-Rio et al., 2015b; Balaine et al., 2016); ii) WFPS was increased by NT having often values higher than 60%, which are considered above the critical threshold to promote denitrification (Regina and Alakukku, 2010) by enhancing denitrifier activity (Gregorich et al., 2005; Rochette, 2008); iii) the less soil disturbance in NT than in CT, the greater the amount of C and N pools that in turn enhance microbial biomass. This combination of factors in NT increased the total soil bacteria community (16S gene), including the nitrifiers (amoA gene, +155% than CT) and denitrifiers (nosZ gene, +84% than CT) in the N cycle. Overall, the analysis of N cycle functional gene abundance suggests a more active nitrogen-cycling bacterial community in NT, and the size of both nitrifying and denitrifying communities may be correlated with denitrification potential and N_2O in the field (Morales et al., 2010; Krauss et al., 2017). Thus, the increase of amoA gene copies in NT indicates an important contribution of nitrification to global N_2O soil emissions, consistent with Sanz-Cobena et al. (2017), in rain-fed Mediterranean cropping systems.

Denitrifying enzyme activity resembled field N_2O emissions, with markedly higher soil denitrification potential in NT topsoil than in CT (+301%). The difference between the two tillage systems was amplified by DEA since it was determined under laboratory optimal conditions with unlimited carbon and nitrate availability and with the reduction of N_2O to N_2 blocked by C_2H_2. This last step in the denitrification chain is performed by the nitrous oxide reductase enzyme encoded by the nosZ gene. Therefore, the greater amount of bacteria containing the nosZ gene plays an important role in reducing the environmental impact of denitrification on the N_2O:N_2 ratio. The higher amount of nosZ retrieved in NT than in CT is consistent with the findings of Baudoin et al. (2009) and Melero et al. (2011) and might be, in part, responsible for the lower N_2O magnitude observed in field than in DEA.

4.5 Faba bean biomass and grain yields

The faba bean average grain yield during the period from 1992–2013 (i.e., from the beginning of the tillage experiment to the 2014–2015 cropping season) was significantly higher in NT than that in CT (+23%), thus showing that the long-term application of NT is advantageous for this species in rain-fed Mediterranean cropping systems, as previously reported by Giambalvo et al. (2012). These results are consistent with those of López-Bellido et al. (2011) and Muñoz-Romero et al. (2011), who observed higher grain yields of faba beans in NT than that in CT under the same conditions. Considering that in Mediterranean environments, water scarcity during the spring is often the main factor limiting the growth and productivity of rain-fed crops (Lampurlanés et al., 2002; Ruisi et al., 2014), the advantage of NT over CT is often attributable to the higher water available for the crop during grain filling, mainly due to reduced soil water evaporation (Lampurlanés and Cantero-Martínez, 2006) and deeper soil water storage (Lampurlanés et al., 2016) under the NT system.

Focusing on the results obtained in the two cropping seasons, we found a higher faba bean grain yield in NT than that in CT in 2013–2014, which is consistent with the long-term average data (i.e., the 1992–2013 period), and no difference between the two tillage systems in 2014–2015. Considering that in the second year, water availability was not a limiting factor for the growth of faba bean, the lack of differences between NT and CT for grain yield was not attributable to differences in water availability between the two tillage systems but rather to other factors, such as the different intensities in weed infestation, that were markedly higher in NT than that in CT (data not shown); this difference could have consequently cancelled out the advantage of NT over CT for faba bean grain yield in the second cropping season.

5. Conclusion

The long-term continuous application of NT, compared to CT, increases faba bean grain yield, bulk density, WFPS, and TOC and TN, with marked repercussions on both the size and structure of the soil microbial community. These benefits were, however, counteracted by an increase of the N_2O emissions from soil in NT (approximately +50% compared to CT, on average). These findings highlight the importance of accurately defining management strategies to mitigate this negative effect. On the whole, total N_2O emissions during the faba bean-growing season were particularly high for Mediterranean environments and comparable to those measured on N-fertilized crops (e.g., winter cereals) grown in the same study area. These findings also suggest that grain legumes, traditionally considered as environmentally friendly crops, have some weaknesses from the ecological viewpoint that must be carefully addressed when planning agronomic strategies.

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Figure captions

Figure S1 Rainfall (blue columns) and daily mean air temperature (yellow line) at the experimental site during 2013–2014 and 2014–2015 growing seasons (from September 2013 to July 2015).

Figure 1. Ammonia (NH_3) emissions from soil under conventional (CT) and no tillage (NT). The bottom rectangles represent the average NH_3 emitted at sowing time, whereas upper rectangles represent the average NH_3 emitted at full tillering time. Each full column (the sum of previous two rectangles) represents the total NH_3 emitted from each treatment averaged on the two growing seasons. Reported values are means (n=6) ± SE (bars). Different letters indicate significant differences among treatments at $P \leq 0.05$.

Figure 2. Denitrifying enzyme activity (DEA) ($\mu g\ N_2O\text{-}N\ kg^{-1}h^{-1}$) determined on soil samples collected at 0–15 cm and 15–30 cm soil layers under conventional (CT) and no tillage (NT). Reported values are means (n = 4) ± SE (bars). Different letters indicate significant differences among treatments at $P \leq 0.05$.

Figure 3. Nitrous oxide (N_2O) emission fluxes ($\mu g\ m^{-2}\ h^{-1}$) and water filled pore space (WFPS; $m^3\ m^{-3}$) from soil under conventional (CT) and no tillage (NT) during the 2013–2014 [A] and 2014–2015 [B] growing seasons. Reported values are means (n=6) ± SE (bars).

Figure 4. Total nitrous oxide (N_2O) emission (mg N_2O-N m^{-2}) from soil under conventional (CT) and no tillage (NT). Reported values are means (n = 6) ± SE (bars). Different letters indicate significant differences among treatments at $P \leq 0.05$.

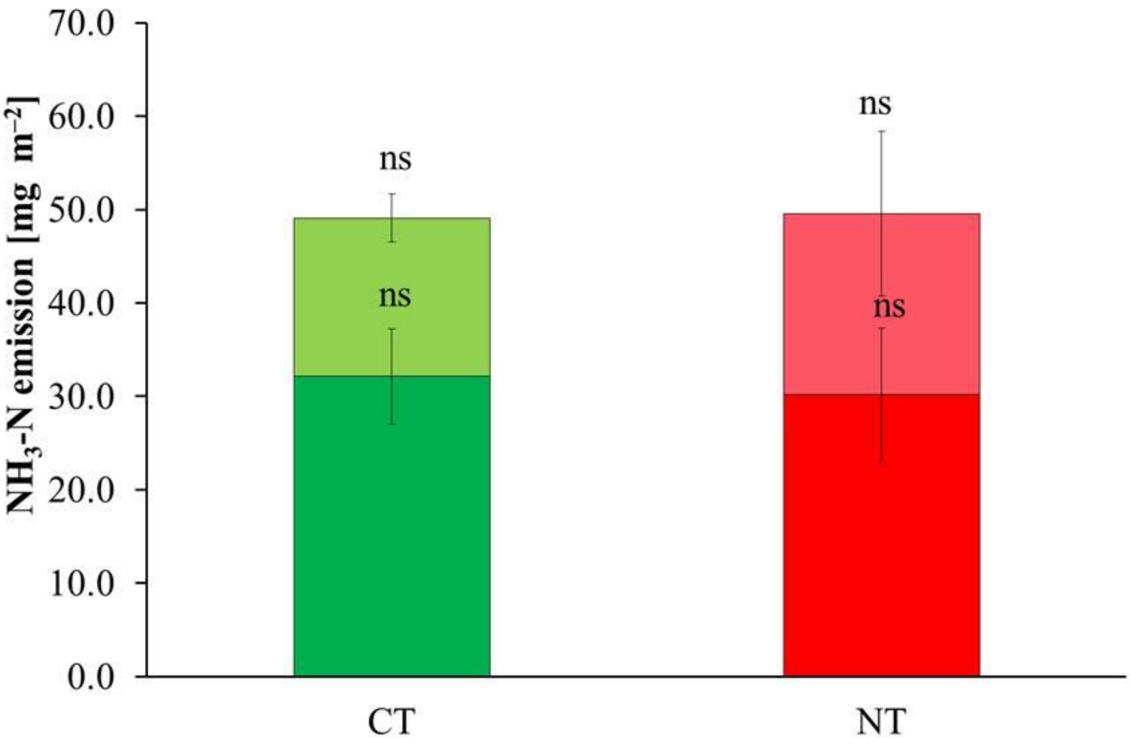
Figure 5. Grain [A] and biomass [B] yields of faba bean as affected by conventional (CT) and no tillage (NT) in 2013–2014 and 2014–2015 and in the 1992–2013 period. For both grain and biomass yield, reported values are means (n=2 for both the 2013–2014 and 2014–2015 growing seasons, and n=42 for the 1992–2013 period) + SE (bars). Different letters at the top of the histograms indicate significant differences by tillage system at $P \leq 0.05$.

Table 1. Physical and chemical properties of soil cropped with faba bean after 23 years of conventional tillage (CT) and no tillage (NT) application. Lower case letters indicate significant differences ($P<0.05$) between the two tillage system. NS, not significant.

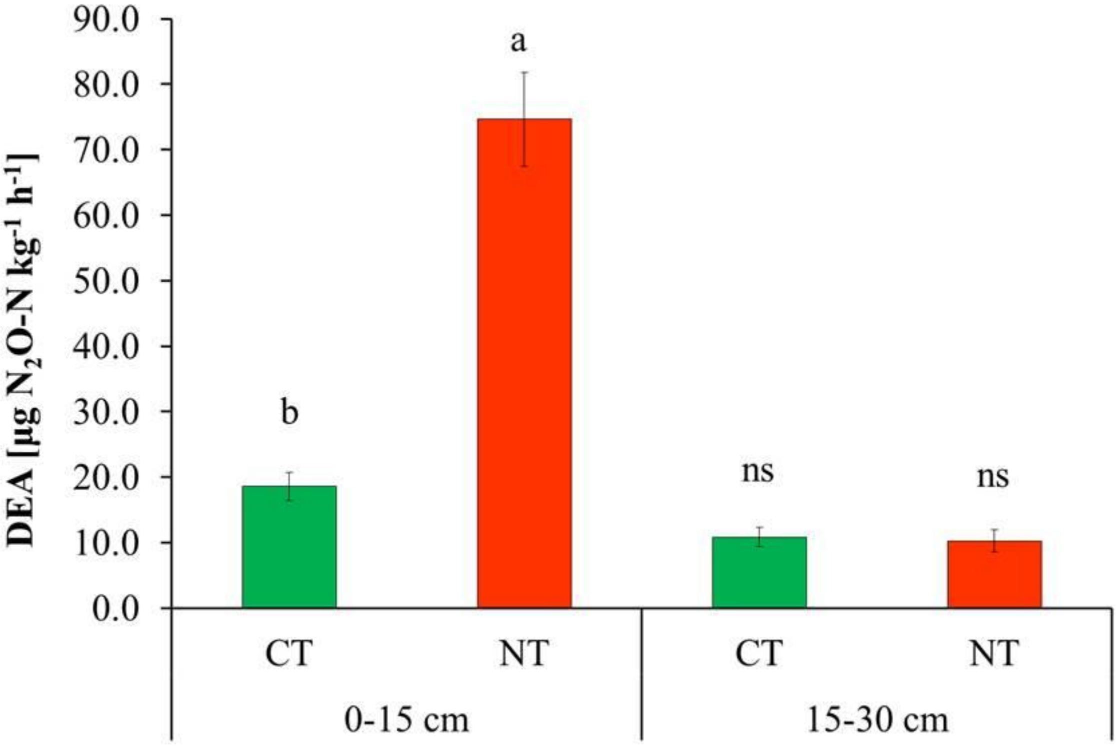
Tillage system	Bulk density	Total organic C	Total N	Extractable organic C	NO_3^--N
	kg m^{-3}	g kg^{-1}	g kg^{-1}	mg kg^{-1}	mg kg^{-1}
0-15 cm soil layer					
CT	1007 b	13.1 b	1.3 b	60.4	1.40
NT	1108 a	17.3 a	1.9 a	65.5	1.40
15-30 cm soil layer					
CT	1052 b	13.2	1.4	64.9 a	1.34 a
NT	1178 a	14.0	1.3	46.6 b	1.15 b

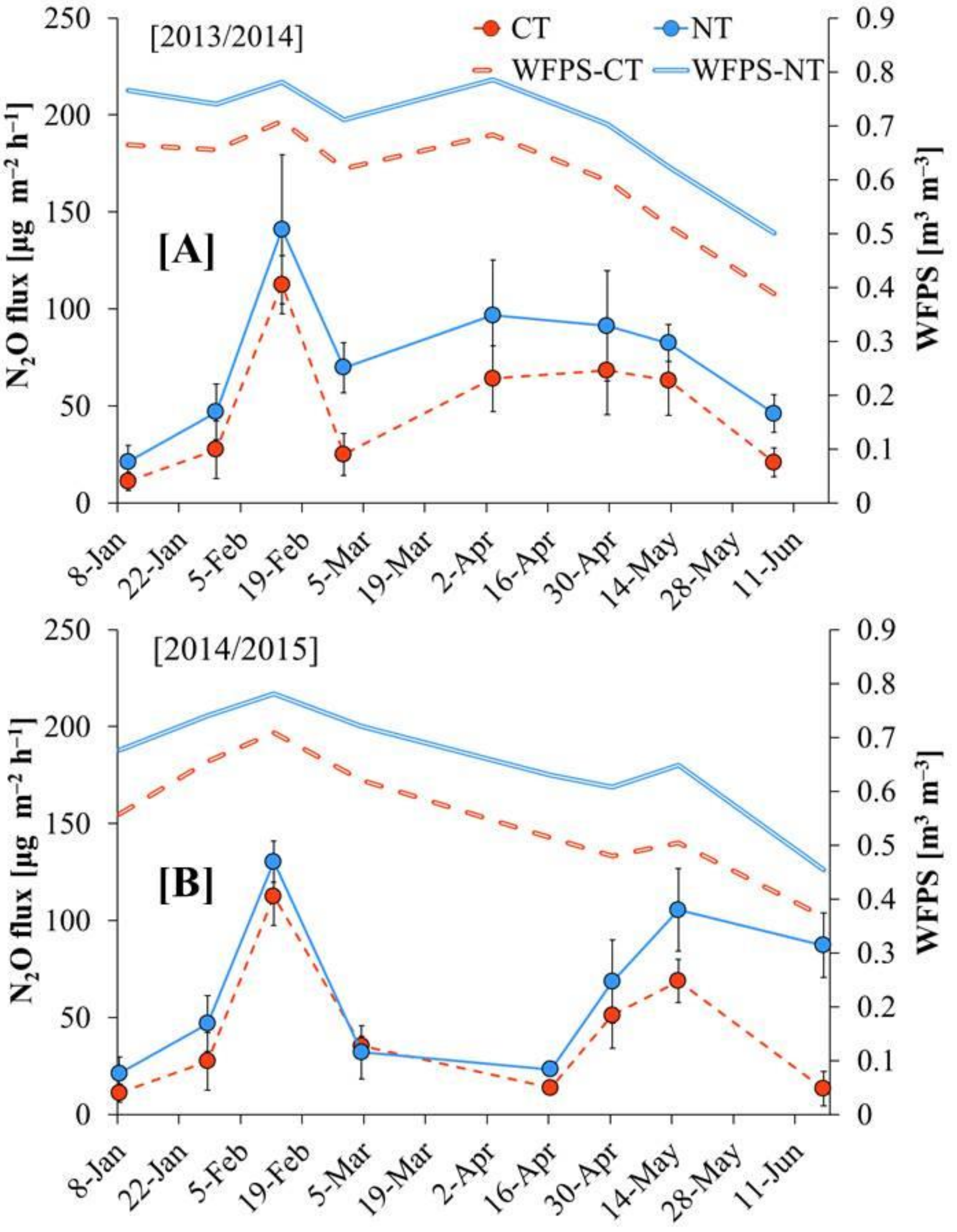
Table 2. Gene copy abundance (c. n. mg^{-1} d.s.) and their ratios in the 0-15 cm layer of soil cropped with faba bean after 25 years of conventional tillage (CT) and no tillage (NT) application. Lower case letters indicate significant differences (P<0.05) between the two tillage system. NS, not significant.

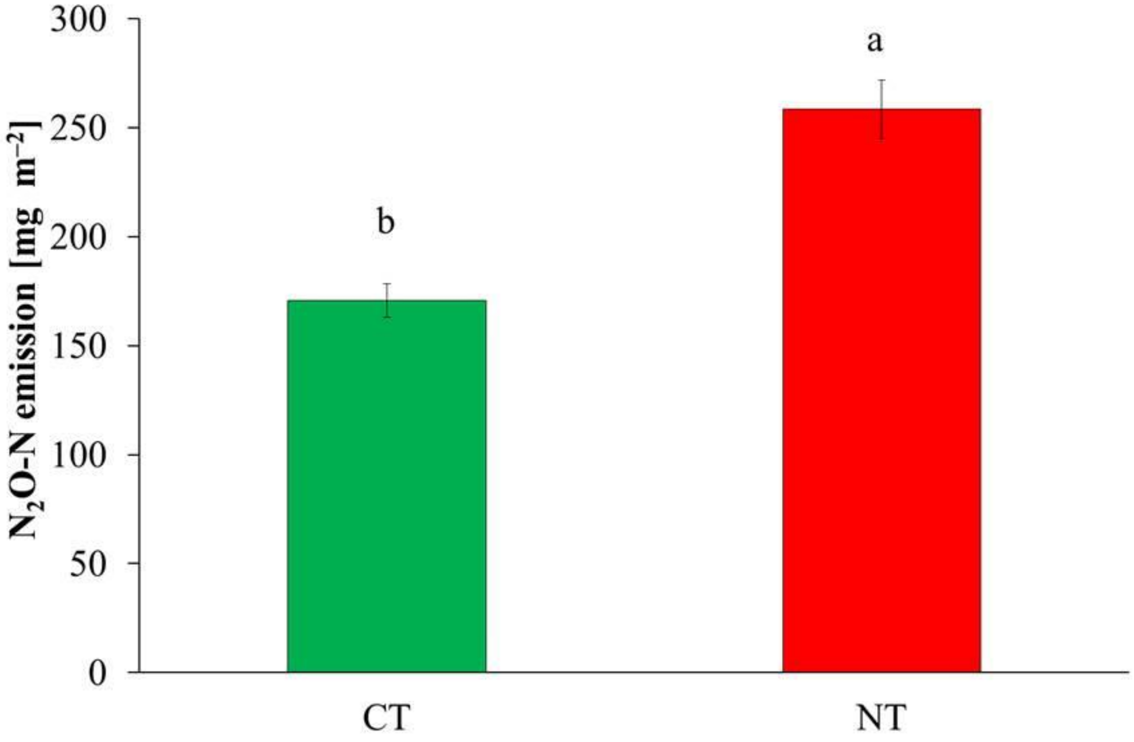
Tillage system	16S	amoA	nosZ	amoA/16S	nosZ/16S	amoA/nosZ
CT	74.3 b	58.4 b	57.7 b	0.77	0.81	1.09
NT	145.5 a	148.6 a	106.0 a	1.04	0.74	1.45

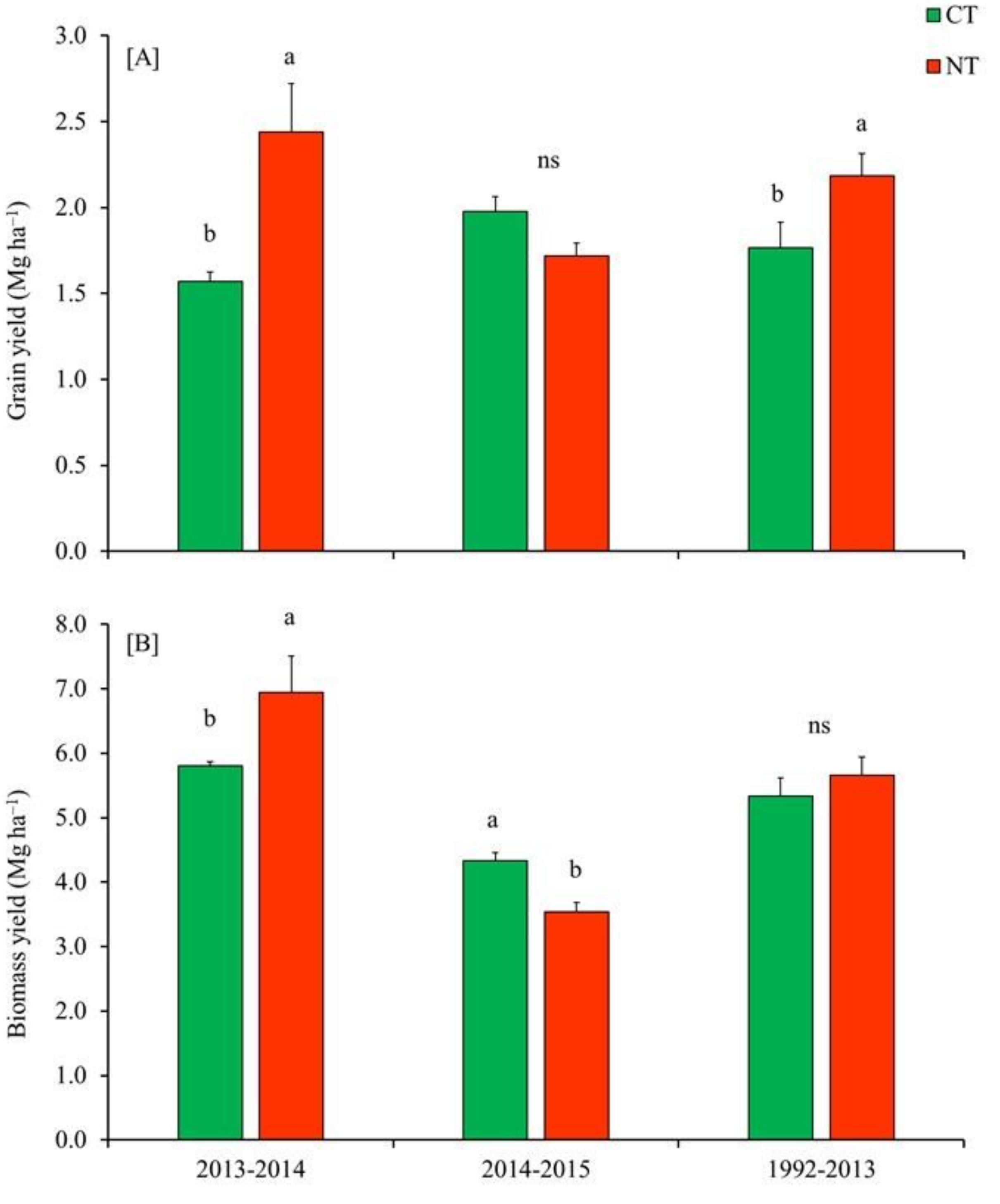


NH_3-N emission [mg m^{-2}]
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
ns
ns
ns
ns
CT
NT

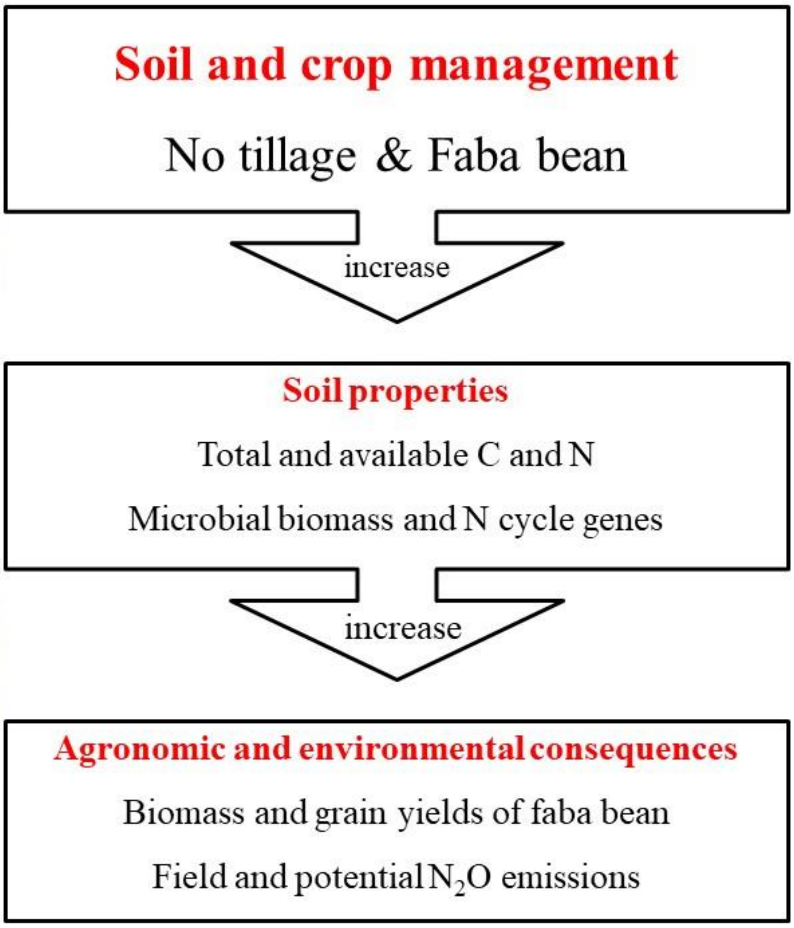

DEA [µg N₂O-N kg⁻¹ h⁻¹]
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
a
b
ns
ns
CT
NT
CT
NT
0-15 cm
15-30 cm


[2013/2014]
CT
NT
WFPS-CT
WFPS-NT
[A]
N$_2$O flux [µg m^{-2} h^{-1}]
WFPS [m^3 m^{-3}]
0
50
100
150
200
250
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
8-Jan
22-Jan
5-Feb
19-Feb
5-Mar
19-Mar
2-Apr
16-Apr
30-Apr
14-May
28-May
11-Jun
[2014/2015]
[B]
N$_2$O flux [µg m^{-2} h^{-1}]
WFPS [m^3 m^{-3}]
0
50
100
150
200
250
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
8-Jan
22-Jan
5-Feb
19-Feb
5-Mar
19-Mar
2-Apr
16-Apr
30-Apr
14-May
28-May
11-Jun


N2O-N emission [mg m−2]
300
250
200
150
100
50
0
a
b
CT
NT


CT
NT
[A]
Grain yield (Mg ha^{-1})
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
ns
a
b
[B]
Biomass yield (Mg ha^{-1})
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
a
b
ns
a
b
2013-2014
2014-2015
1992-2013

820



Soil and crop management

No tillage & Faba bean

increase

Soil properties

Total and available C and N

Microbial biomass and N cycle genes

increase

Agronomic and environmental consequences

Biomass and grain yields of faba bean

Field and potential N_2O emissions