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Long-term effects of contrasting tillage on soil organic carbon, nitrous oxide and ammonia emissions in a Mediterranean Vertisol under different crop sequences

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Abstract

This 2-year study aimed to verify whether the continuous application of no tillage (NT) for over 20 years, in comparison with conventional tillage (CT), affects nitrous oxide (N_2O) and ammonia (NH_3) emissions from a Vertisol and, if so, whether such an effect varies with crop sequence (continuous wheat, WW and wheat after faba bean, FW). To shed light on the mechanisms involved in determining N-gas emissions, soil bulk density, water filled pore space (WFPS), some carbon (C) and nitrogen (N) pools, denitrifying enzyme activity (DEA), and nitrous oxide reductase gene abundance (nosZ gene) were also assessed at 0–15 and 15–30 cm soil depth. Tillage system had no significant effect on total NH_3 emissions. On average, total N_2O emissions were higher under NT (2.45 kg N_2O-N ha^{-1}) than CT (1.72 kg N_2O-N ha^{-1}), being the differences between the two tillage systems greater in FW than WW. The higher N_2O emissions in NT treatments were ascribed to the increased bulk density, WFPS, and extractable organic C under NT compared to CT, all factors that generally promote the production of N_2O. Moreover, compared to CT, NT enhanced the potential DEA (114 vs 16 μg N kg^{-1} h^{-1}) and nosZ gene abundance (116 vs 69 copy number mg^{-1} dry soil) in the topsoil. Finally, NT compared to CT led to an average annual increase in C stock of 0.70 Mg C ha^{-1} $year^{-1}$. Though NT can increase the amount os soil organic matter so storing CO_2 into soil, some criticisms related to the increase of N_2O emission arise, thereby suggesting the need for defining management strategies to mitigate such a negative effect.

Keywords No tillage; Greenhouse gas emissions; Carbon stock; nosZ gene; Mediterranean environment; Wheat

Abbreviations NT, no tillage; CT, conventional tillage;, WW, wheat grown after wheat; FW, wheat grown after faba bean; TOC, total organic carbon; EOC, extractable organic carbon; TN, total nitrogen; WFPS, water filled pore space; BD, bulk density; DEA, denitrifying enzyme activity

1. Introduction

Agricultural activities are considered as the primary source of nitrous oxide (N_2O) and ammonia (NH_3) emissions (IPCC, 2007; EEA, 2009). Nitrous oxide emissions are linked to many soil microbial processes: 1) dissimilatory reduction of nitrates and nitrites to N_2 when O_2 concentrations is decreasing (denitrification); 2) nitrification (oxidation of NH_3 to NH_2OH and then to N_2O); and 3) by nitrifiers paradoxically denitrifying (reduction of nitrites to N_2O) (Čuhel et al. 2010; Abalos et al. 2017; Kool et al. 2017). The emission of N_2O from soil is influenced by O_2 partial pressure, moisture and temperature, pH, organic C and nitrates availability (Čuhel et al. 2010; Stevenson et al. 2011). In a recent meta-analysis, Cayuela et al. (2017) indicated water regime, crop type, and N fertilizer management as the most important factors controlling the magnitude of N_2O emissions from Mediterranean agricultural lands. Similarly to N_2O, NH_3 volatilization is positively related to soil organic C and microbial biomass, soil pH, moisture, temperature, NH_4^+ concentration in the soil solution, and negatively related to soil cation exchange capacity (Cameron et al. 2013). Due to the plurality of factors influencing N gases emissions, it is not surprising that the estimates for both N_2O and NH_3 emissions from agricultural soils are extremely variable.

Tillage system, by influencing soil aeration and temperature, water content, total and labile organic C, and the supply of N (Martin-Lammerding et al. 2011; Laudicina et al. 2014), may affect the soil microbial structure and activity and hence the N gas emissions (Mutegi et al. 2010; García-Marco et al. 2016). However, knowledge of the effects of tillage system on N gas emissions from soils is still limited and often controversial, at least for the Mediterranean areas. Comparing no tillage (NT) to conventional tillage (CT; usually based on moldboard plowing), Plaza-Bonilla et al. (2014) observed, under rainfed Mediterranean conditions, higher N_2O emissions under NT in the short-term (<4 years) but similar N_2O fluxes between NT and CT in the long term (>10 years). Similar results are reported in many studies conducted in temperate, humid or sub-humid environments (Baggs et al. 2003; Six et al. 2004; Rochette 2008; Beare et al. 2009; Kong et al. 2009; Bayer et al.

2015), where the authors ascribed these effects to the poorer water drainage and aeration, and to the resulting lower availability of O_2 and the minor diffusion of gases through the soil under NT compared to CT conditions. On the contrary, other studies reported lower N_2O emissions in NT than in CT soils, both in temperate (Chatskikh and Olesen 2007; Omonode et al. 2011; van Kessel et al. 2013) and Mediterranean areas (García-Marco et al. 2016).

Tillage system can also have an important role in determining the amount of N lost as NH_3 via volatilization. Several authors observed higher NH_3 emissions in NT than CT soils, especially when crop residues left on soil surface are abundant and N fertilizer used is urea (Palma et al. 1998; Rochette et al. 2009). In NT systems, in fact, the lack of mixing the N fertilizer into the soil and conversely the direct contact of the N fertilizer granules with the crop residues present on soil surface increases the risk of NH_3 losses. Crop rotations that include N_2-fixing legume species within the crop sequence have been often reported as a valuable N gas emission mitigation strategy (as reviewed by Jensen et al. 2012 and Sanz-Cobena et al. 2017), mainly due to less mineral N fertilizers applied to soil. On the other hand, other authors have highlighted that, since denitrification rate is positively related to the concentration of soil nitrates (Wagner-Riddle and Thurtell 1998), legumes can increase N_2O emissions as a result of N released from decomposition of the N-rich crop residues (Rochette and Janzen 2005; Tellez-Rio et al. 2015a) and/or due to their poor efficiency in recovering the plant-available soil mineral N (Jensen et al. 2012; Saia et al. 2016).

Therefore, we performed a 2-year study in a typical Mediterranean environment: to verify i) whether the long-term (over 20 years) NT affects the C and N pools, and the emissions of N_2O and NH_3 from soil and, if so, ii) whether such effect varies when crop sequence varies, and iii) to gain a better insight into how agricultural management may affect N gas emissions through changes on soil physical and chemical properties. Durum wheat (*Triticum durum* Desf.) was used as focal crop.

2. Material and methods

97

98 *2.1. Experimental site*

99

100 The field crop trial was carried out under rainfed conditions at the experimental Pietranera farm of
101 the University of Palermo. The farm is located about 30 km north of Agrigento (Sicily, Italy, 37°30′
102 N, 13°31′ E; 178 m a.s.l.). The soil was classified as Chromic Haploxerert (Vertisol) and its
103 characteristics, determined at the beginning of the experiment (year 1991) and referring to the 0–40
104 cm top layer, were 525 g kg^{-1} clay, 216 g kg^{-1} silt, 259 g kg^{-1} sand, pH 8.1 (in water), 14 g kg^{-1}
105 total organic C, 1.29 g kg^{-1} total N, 36 mg kg^{-1} available P (Olsen).
106 The climate at the experimental site is semiarid Mediterranean, with a mean annual rainfall of 572
107 mm (1995 to 2015), concentrated mostly during the autumn–winter period (September–February;
108 76%), and spring (March–May; 19%). The dry period occurs from May to September. Mean air
109 temperature is 15.9°C in autumn, 9.7°C in winter, and 16.5°C in spring. Climatic data from
110 September 2013 to July 2015 was collected from the nearest weather station located 500 m far from
111 the experimental site.

112

113 *2.2. Experimental design and crop management*

114

115 The experiment was set up in fall 1991 as a strip-plot design with two replications, where three soil
116 tillage systems (conventional, reduced, and no tillage) acted as vertical treatments and three crop
117 sequences (wheat–wheat, wheat–faba bean, and wheat–berseem clover) as horizontal ones. More
118 details are reported in Giambalvo et al. (2012) and Amato et al. (2013). The experimental factors
119 tested here were tillage system (conventional tillage, CT, and no tillage, NT) and crop sequence
120 (continuous wheat, WW, and wheat after faba bean, FW). Conventional tillage consisted of one
121 moldboard plowing to a depth of 30 cm in the summer, followed by one or two shallow harrowing
122 (0–15 cm) operations before planting. No tillage consisted of sowing by direct drilling. Plot area

size was 370 m^2 (18.5 × 20.0 m). In NT plots, weeds were controlled before planting with glyphosate at a dose of 533 to 1,066 g acid equivalent ha^{-1}, depending on the development of weeds. Every year, WW and FW plots were broadcast fertilized with 69 kg ha^{-1} of P_2O_5 just before planting. Nitrogen fertilizer was broadcast on the soil surface at 120 kg N ha^{-1} in WW plots and 80 kg N ha^{-1} in FW plots. The total amount of N fertilizer was broadcasted as follows: 50% applied immediately before planting (as diammonium phosphate and urea) and 50% applied at mid-tillering (end of March; during this experiment, it was just before the 2nd soil sampling) as ammonium nitrate. Crop planting was always in December 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). Durum wheat (cv. Anco Marzio) was planted in rows spaced 16 cm apart at 350 viable seeds m^{-2}. In WW and FW plots, weeds were controlled by applying post-emergence herbicides at the early growth stage of the crop. Wheat was harvested in late June or beginning of July and stubble (about 20–25 cm from the soil surface) was left standing. Wheat straw was baled and removed from the field. The soil surface covered by mulch in the NT treatments was always >30%. Faba bean (*Vicia faba* L., cv. Gemini) in rotation with wheat was managed as follows: broadcast fertilized with 46 kg ha^{-1} P_2O_5 before planting; sown at 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; harvested in late June or beginning of July, leaving standing straw and uniformly spreading crop residues.

2.3. Soil sampling and analyses

During the cropping season 2013–2014, in each plot, two soil samples (each composed by 3 mixed subsamples) were collected from the 0–15 cm and 15–30 cm soil layers in December 2013 (before sowing), April 2014 (wheat heading), and July 2014 (wheat harvest) for a total of 96 soil samples, which were gently sieved to pass through a 2 mm mesh sieve. Total organic carbon (TOC) was

149 determined by the Walkley–Black procedure whereas total nitrogen (TN) by the Kjeldhal method.
150 Extractable organic carbon (EOC) and nitrates were determined on soil extracts prepared by
151 shaking 25 g of soil with 100 mL 0.5 M K_2SO_4 (1:4 w/v) for 45 min on a horizontal shaker (70
152 rpm). Soil suspensions were filtered through Whatman 42 paper and extracts analysed by acid
153 dichromate oxidation method (Vance et al. 1987) to determine organic C and by the chromotropic
154 acid method (Sims and Jackson 1971) for nitrate concentration. The concentration of EOC was used
155 as a reliable indicator of available C (Laudicina et al. 2013). Carbon (C) inputs per each treatment
156 were calculated on the basis of the mean biomass productions of the crops since the beginning of
157 the experiment (Giambalvo et al. 2012; Amato et al. 2013). As regards wheat, stubble was
158 estimated as corresponding to the 30% of total straws. For both wheat and faba bean, root biomass
159 was considered as 30% of the total epigeic biomass according to Zanatta et al. (2007). The C
160 content of both root and shoot residues was considered in the proportion of 43% for both crops
161 (Kong et al. 2005). Thus, C inputs were calculated as follows:

$$C\ input\ wheat = [(TEB - G) \times 0.3 + (\mathrm{TEB} \times 0.3)] \times 0.43$$

162 and

$$C\ input\ faba\ bean = [(TEB - G) + (\mathrm{TEB} \times 0.3)] \times 0.43$$

163 where TEB and G are total epigeic biomass and grain yield of wheat (or faba bean), respectively.
164 At each N_2O field measurement, gravimetric water content of soil at 0–15 cm and at 15–30 cm
165 depth was determined by weight difference between fresh and dried (24 h at 105°C) sample while
166 WFPS was calculated as follows:

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

167 where SWC is the gravimetric soil water content, BD is the soil bulk density and PD is the soil
168 particle density (2.65 g cm^{-3}). Soil BD was determined by core method (Grossman and Reinsch
169 2002).
170

171 *2.4. Ammonia and nitrous oxide emissions*

172

173 Ammonia emissions were monitored after each fertilization, i.e. at sowing and at tillering, in two
174 cropping cycles, in 2013–2014 (from 18.12.2013 to 02.01.2014 and from 24.03.2014 to 09.04.2014)
175 and in 2014–2015 (from 23.12.2014 to 09.01.2015 and from 13.04.2015 to 28.04.2015). Soil NH_3
176 volatilization was evaluated using the Conway's microdiffusion-incubation method adapted for soil
177 by Bremner and Krogmeier (1989) according to Qi et al. (2012). Briefly, a plastic jar of 100 mL of
178 volume (5.0 cm of diameter) containing 20 mL of 3% (w/v) of boric acid solution was suspended
179 above the ground. Then, an airproof chamber with a diameter of 16.0 cm (6,032 cm^3 of volume)
180 was put on the soil surface covering the plastic jar. The chamber was anchored to the ground
181 through small wire arches in order to avoid gas leakages from the system and the chamber
182 movement caused by wind. Three chambers per plot were placed for a total of 24 chambers. The
183 NH_3 volatilized from soil was trapped by boric acid solution and determined by titration with 0.005
184 M H_2SO_4 back to the original pH (3.4~3.5). The boric acid solution was replaced each day during
185 the first week and then every two days until the NH_3 emission was negligible (generally after 20
186 days since fertilization). Total NH_3 volatilization was calculated as the sum of the NH_3 volatilized
187 during the observation period. Also soil N_2O fluxes were measured over two cropping cycles, from
188 sowing to harvest, in 2013–2014 and in 2014–2015. Greenhouse gas fluxes were sampled using the
189 closed chamber technique (Hutchinson and Mosier 1981; Baker et al. 2003). Three polyvinyl
190 chloride opaque chambers, with a diameter of 31.5 cm and height of 30.0 cm, were placed in each
191 plot. The chambers were fitted in a polyvinyl chloride frame inserted into the soil to a depth of 5 cm
192 in order to minimize the later diffusion of gases and avoid the soil disturbance. The frames were
193 placed at the beginning of each sampling year, after the plant emergence, enclosing two plant rows,
194 and were removed at the end of the crop cycle. During crop growth, the chambers height was
195 progressively increased to accommodate the plants, using appropriate extension of the same
196 diameter of the chamber, reaching a maximum height of 90.0 cm. At the top of each chamber, a

197 rubber stopper with a three-way stopcock was placed in order to take gas samples. Gas samples (10
198 mL in volume) were taken at 0, 30 and 60 minutes after the chamber closure from the headspace of
199 each chamber using a 10 mL syringes, fitted on the three-way stopcock, connected with a needle in
200 order to store the samples in a 7 mL pre-evacuated Exetainer® (Labco Limited, Buckinghamshire,
201 UK). Before gas sampling the air inside the chamber was mixed by suctioning and pumping using
202 the 10 mL syringe. Air samples were taken simultaneously for each crop in both tillage systems. All
203 gas samples were taken always at the same interval (7–10 a.m.). After air sampling, the chambers
204 were immediately removed from the frames to minimize enclosure effects on soil environmental
205 conditions and plant growth. Eight gas samples per plot were taken at regular interval from January
206 10^{th} to June 6^{th} 2014 as well as from January 8^{th} to June 17^{th} 2015. Concentration of N_2O in the gas
207 samples were determined by GC-ECD (TRACE-GC, Thermo Scientific, Milan, Italy) as described
208 below for the denitrifying enzyme activity (detection limit < 50 ppb). Flux rates were calculated
209 from the N_2O concentration increase during the 60 minutes chamber closure period by applying the
210 equation of Jantalia et al. (2008):

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

211 where $\Delta C/\Delta t$ is the change in N_2O concentration in the chamber during the closing time Δt, V and A
212 are respectively the volume of the chamber and the area of the soil covered by the chamber, V_m is
213 the molar volume corrected for the air temperature at the sampling time and m is the molecular
214 weight of N_2O. The seasonal amount of N_2O emissions were accumulated from the emission rates
215 between every two consecutive days of the measurements by following equation according with Cai
216 et al. (2012):

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

217

218 *2.5. Denitrifying enzyme activity (DEA)*

Denitrifying enzyme activity was determined on soil samples collected on 10.12.0.2013, 01.04.2014 and 09.07.2014 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, were added. Flasks were sealed with butyl rubber stoppers, evacuated and flushed four times with 99.999% helium, thus equilibrating the internal pressure to the atmospheric one. Each evacuation and/or flushing lasted for 2 min. Then, using a 15 mL syringe, 10 mL 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). After which, the flasks were then shaken horizontally at 70 rpm. After 30 and 60 min from the addition of acetylene, 1 mL samples of headspace atmosphere were taken with a gas-tight syringe and N_2O concentration was measured by a gas chromatograph (TRACE-GC, Thermo Scientific, Milan, Italy) equipped with a 80–100 mesh stainless-steel column packed with Poropak Q and an electron capture detector. DEA was calculated from the N_2O increase during half an hour incubation (60–30 min).

2.6. NosZ gene abundance

In the first week of May 2014, when the soil and plants likely reached the maximum biological activity, four soil samples (each composed by mixing 3 subsamples) were collected from the superficial layer (0–15 cm) of all plots and stored at –20°C until analyses. Immediately before starting 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 PowerSoil® DNA Isolation Kit (MoBio, Carlsbad, CA, USA) following the manufacturer's instructions. Then, DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and amplified by PCR using specific primers for nosZ genes, i.e. typical of denitrifying bacterial community; more precisely nosZ-1840F and nosZ-2090R primers (267 bp). PCR conditions

consisted of an initial denaturation 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 at concentration of 30 μM for both nosZ primers, 2 μL of 0.2mM dNTPs, 0.15 μL of 5 U Taq polymerase (Bioline, London, UK), 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® (Biotium, Fremont, CA, USA). The PCR results for each gene were used in order to choose the best DNA PCR concentration for qPCR. Real time PCR (qPCR) was performed on BioRad iQ 5 QPCR. 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. NosZ standard curve was 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 replications per qPCR.

2.7. Statistical analyses

Before performing parametric statistical analyses, normal distribution and variance homogeneity of the data were checked by Kolmogorov–Smirnoff goodness-of-fit and Levene's tests, respectively. Following the strip-plot procedure, two-way ANOVA was performed with tillage (CT, conventional tillage, and NT, no tillage) and crop sequence (WW, continuous wheat, and FW wheat after faba-bean) as factors for total NH_3 and N_2O emissions (field measures averaged for two consecutive years and cumulated with regard to crop stages) and nosZ, while with repeated measures (soil sampled in three occasions per cropping year) for TOC, TN, EOC, N-NO_3^-, DEA. Treatment means

were compared using Tukey's HSD test at the 5% probability level. Residual maximum likelihood variance components were also performed to determine which of the two factors or their interaction accounted for the majority of the variation in each of the measured variable. Statistical analyses were carried out with SAS statistical package (SAS 2009). Reported data, expressed on oven-dry basis (105°C) of soil, are the arithmetic means ± standard deviation.

3. Results

3.1. Weather conditions

Total rainfall in 2013–2014 was 603 mm, with a homogenous rain distribution during the crop cycle (Fig S1; supporting information). Mean year temperature was 15.2°C. In the 2014–2015 the total rainfall was 660 mm. Rainfall distribution showed an opposite trend during the crop cycle; the period from September to January had low rainfall while from February to July the rainfall was high. Mean year temperature was 15.8°C.

3.2. Soil characteristics and carbon inputs

Bulk density was affected only by tillage and was higher in NT than CT (Table 1). In the 0–15 cm soil layer, TOC ranged from 13.3 g kg^{-1} in WW-CT to 18.8 g kg^{-1} in FW-NT and was affected only by tillage. In the deeper soil layer (15–30 cm), TOC was not affected by any treatment and it was on average 14.0 g kg^{-1} (Table 1). Carbon inputs showed significant differences by crop sequence (on average 1.81 and 2.44 t ha^{-1} y^{-1} in WW and in FW rotation, respectively) whereas no differences were observed due to tillage system (Figure 1). At the 0–30 cm soil layer, C stock was 58 and 41 t C ha^{-1} in NT and CT, respectively. Thus, the continuous application of NT for over 20 years determined in the 0–30 cm soil layer, relative to CT, an average annual increase of about 0.70 t C

297 ha^{-1} year^{-1}. Extractable organic C in the 0–15 cm soil layer ranged from 53.9 mg kg^{-1} to 78.9 mg
298 kg^{-1}. On average, NT plots had an EOC content 42% higher than CT ones. On the contrary, in the
299 15–30 cm soil layer, EOC concentration was higher in CT plots than NT ones (+25% on average;
300 Table 1). Total nitrogen (TN) content in the 0–15 cm soil layer paralleled that of TOC and was
301 affected only by tillage . On average, TN was 0.55 g kg^{-1} higher in NT (+42%) than in CT. In the
302 15–30 soil layer, tillage system and crop sequence had no significant effect on TN. Soil nitrate
303 concentration in the topsoil and in the subsoil was not affected by the experimental factors (Table
304 1).
305
306 *3.3. Ammonia emissions*
307
308 Crop sequence markedly affected soil NH_3 emissions at both sowing and tillering. Tillage system
309 had no significant effect on this gaseous emission. After the fertilization at tillering time, NH_3
310 emissions, on average during the two experimental years, ranged from 7.4 kg N ha^{-1} with FW to
311 12.9 kg N ha^{-1} with WW (Figure 2). Total NH_3 emissions, as sum of NH_3-N emissions after sowing
312 and tillering, were about 10.0 kg N ha^{-1} to 16.0 kg N ha^{-1}, respectively, under wheat after faba and
313 continuous wheat, regardless of tillage (Figure 2). Total NH_3 emissions accounted for
314 approximately 13% of the N added with fertilizer, whose rate varied in relation to the previous crop
315 (80 and 120 kg N ha^{-1} respectively in FW and WW).
316
317 *3.4. Nitrous oxide emissions*
318
319 Nitrous oxide fluxes, at the beginning of the measurement period (crop cycle 2013–2014, January
320 10th), on average, was 17.6 μg N_2O-N m^{-2} h^{-1}, with NT slightly but significantly higher than CT
321 (Figure 3a). Nitrous oxide emission rate increased during the experimental period reaching the
322 maximum at the third measurement occasion (February14th) with NT-WF having an emission rate

184.7 μg N_2O-N m^{-2} h^{-1}; at this time, the widest and significant differences in nitrous oxide emission rate (up to 64.0 μg N_2O-N m^{-2} h^{-1}) between CT and NT plots were recorded. After the third measurement, N_2O emission rate drastically decreased, with a new slight increase recorded from April to half May. Thereafter, at the end of May, the rate returned back almost to the values recorded at the beginning of the measurements. On average, NT plots showed higher emission rates compared to CT ones, whereas the trend was less clear when comparing crop sequences within the same tillage system. During the second year of observation (cropping season 2014–2015, Figure 3b), N_2O emission rate from soil ranged from 8.8 μg N_2O-N m^{-2} h^{-1} to 179.4 μg N_2O-N m^{-2} h^{-1}. At the beginning of the measurements (January 8th), the emission rate showed similar values to those recorded during the previous cropping season. Then, the emission rate started to highly increase so reaching the maximum values during the third measurement occasion (February 12th). Thereafter, it quickly decreased reaching almost the initial values. On April 30th, N_2O emission rate started again to remarkably increase so showing values ranging from 76.4 to 146.6 μg N_2O-N m^{-2} h^{-1}. At the end of the cropping season, finally, emission rate again decreased but not reaching the initial values for all treatments. Also for the cropping season 2014–2015, on average, NT plots showed higher emission rates compared to CT ones, although such trend appeared less pronounced compared to the previous cropping season. Again, no clear trend was evident by comparing crop sequences within the same tillage system. Total N_2O emissions, on average between the two cropping seasons, ranged from 170.8 mg N_2O-N m^{-2} to 260.5 mg N_2O-N m^{-2} (Figure 4) and were significantly affected by both experimental factors (tillage system and crop sequence) and by their interaction. In particular, tillage had a greater effect on N_2O emissions while the effect of crop sequence was narrow although significant, similarly to factor interaction. NT plots, in both years, on average showed 43% more N_2O emission than CT plots (2.45 and 1.72 kg N_2O-N ha^{-1} in NT and CT respectively). In CT plots, no difference was observed among crop sequences, while in NT, FW plots showed higher emissions than WW plots (2.61 and 2.29 kg N_2O-N ha^{-1} in NT and CT respectively, corresponding to 1.9% and 3.3% of the total N applied with the fertilizer).

3.5. Denitrifying Enzyme Activity (DEA)

Denitrification enzyme activity (average of the three samplings) ranged from 15.2 to 128.4 µg N kg^{-1} h^{-1}, and from 12.7 µg N kg^{-1} h^{-1} to 17.4 µg N kg^{-1} h^{-1}, in the 0–15 cm and in the 15–30 cm soil samples, respectively (Table 2). In the 0–15 cm soil samples, DEA was mainly affected by tillage whereas in the 15–30 cm layer DEA was, on average, 15.0 µg N kg^{-1} h^{-1}, with no treatment effect. In the topsoil samples, within the same crop sequence, DEA was much higher in NT compared to CT. In CT plots, DEA did not show significant differences between crop sequences, whereas in NT continuous wheat, DEA was 128.3 µg N kg^{-1} h^{-1} against 99.3 µg N kg^{-1} h^{-1} in wheat after faba bean (+29%, Table 2).

3.6. Denitrifying bacterial community gene (NosZ)

Denitrifying bacterial community gene (NosZ) abundance, determined on 0–15 cm soil, ranged from 62.2 to 126.1 copy number mg^{-1} of dry soil (Figure 5). NosZ abundance was mainly affected by tillage and, marginally, by the interaction Tillage × Crop. NosZ was, on average, 43% higher in NT than CT plots. Within the same tillage systems, significant differences among crop sequences occurred only in NT plots, with WW plots having NosZ abundance 20% higher than FW plots (Figure 5).

4. Discussion

4.1. C and N pools

374 Long-term NT played a significant role in affecting C and N pools. After over 20 years, both TOC
375 and TN concentrations were significantly higher in NT than CT, but this was true only in the upper
376 soil layer (0–15 cm); no differences for these two parameters were found between CT and NT in the
377 15–30 cm soil layer. These results agree with those of previous studies carried out in environments
378 similar to (López-Bellido et al. 2010), or different from (Conceiçao et al. 2013; Liu et al. 2014), that
379 of the present experiment. Many studies have highlighted the importance of considering also deeper
380 soil horizons to compare TOC values in soils under different tillage systems. Most of these studies
381 have shown that the higher TOC concentration generally observed in the upper soil layer under NT
382 compared to CT is often counteracted by an opposite effect in the deeper soil layer (VandenBygaart
383 and Angers 2006; Luo et al. 2010). These findings do not seem to be confirmed by our study where
384 an average annual increase in C stock of about 0.70 t C ha^{-1} $year^{-1}$ in the top 30 cm of soil was
385 observed in NT compared to CT. Considering that the C input was similar between the two soil
386 tillage systems, the different TOC concentration between CT and NT could be linked to the effects
387 that these two tillage techniques had on the fate of crop residues and, consequently, on the labile
388 soil organic C pools. In CT soils, crop residues are mixed into the soil so favouring their
389 accessibility to microorganisms (Dungait et al. 2012), thereby speeding up their mineralization.
390 Intensive soil cultivation increases degradation of the soil organic matter, through both the increase
391 of aeration, that favors the oxidation processes, and the increase of soil aggregates disruption, that
392 reduces the physical protection of the soil organic matter from microbial attack (Six et al. 2000;
393 Plaza-Bonilla et al. 2013; Laudicina et al. 2014; 2016). EOC showed higher values under NT than
394 CT in the upper soil layer (0–15 cm) and the opposite pattern in the deeper soil layer (15–30 cm).
395 This result can be ascribed to the different stratification of the crop residues induced by the different
396 tillage systems rather than to differences in C transfer between upper and lower soil layers.
397 Although a higher concentration of nitrates was expected in CT than NT soils, due to the higher
398 mineralization of the organic matter under CT (Ruisi et al. 2016), no differences between the two
399 soil tillage systems were detected for this parameter. Several factors can have contributed to this

400 result, such as possible differences by tillage system in the environmental releases of N. Anyway,
401 an important role has been played by the differences in the N uptake in wheat grown under CT and
402 NT. It is well known that wheat is a crop with high N requirements and able to effectively utilize N
403 when it becomes available. So the different amount of N taken up by wheat grown under CT and
404 NT could have canceled the differences in the soil nitrate availability between the two tillage
405 systems.
406 Although C inputs were greatly different between the two crop sequences, no differences were
407 observed between WW and FW for C and N pools probably because of the different quality of the
408 crop residues returned to the soil. Due to the lower C/N ratio, the faba bean residues are more easily
409 mineralizable compared to wheat residues (Nguyen and Marschner 2016) so the greater C inputs in
410 FW is counteracted by their more easily mineralization.
411
412 *4.2. NH_3 emissions*
413
414 The total amount of NH_3 emitted was on average of 13.2 kg N ha^{-1}, which is comparable with the
415 values of other authors (Gioacchini et al. 2002; Engel et al. 2011;). On average, NH_3 losses
416 represented the 13% of the total N applied in the field. Such percentage value is similar to that
417 reported in other studies conducted in other environments (Ferm 1998; Palma et al. 1998), lower
418 than that observed by de Morais et al. (2013) and higher than that observed by Bouwman et al.
419 (2002) and Jantalia et al. (2012). Such data discrepancy may be due to different doses and types of
420 the N-fertilizers applied, soil types, and crops (Bouwman et al. 2002). In the present study, 76% of
421 the total NH_3-N emitted were observed after the N fertilization carried out at tillering (while the
422 remaining 24% occurred after the N fertilization carried out at sowing. This result can be explained
423 taking into account that, in both CT and NT soils and regardless to the crop sequence, the N
424 fertilizer was buried, even if partially, into the soil during the sowing operations, whereas it was
425 applied on the soil surface at tillering time. Indeed, NH_3 losses are reduced by burying N fertilizers

426 into the soil because of the increased resistance to the upward diffusion of ammoniacal N present in
427 the liquid and gaseous phases and the increased adsorption of NH_4^+ on soil particles when urea is
428 incorporated at a depth (Sommer et al. 2004; Rochette et al. 2013).
429 Total soil NH_3 emissions were not affected by tillage. This result disagrees with the findings of
430 many authors that usually report higher soil NH_3 losses in NT soils compared to CT ones (Palma et
431 al. 1998; Sommer et al. 2004; Rochette et al. 2009). Such differences for NH_3 emissions are
432 generally attributed to the higher urease activity in NT soils compared to CT soils (Roscoe et al.
433 2000; Jian-She et al. 2011), the presence of surface residues in NT that reduces the contact of N
434 fertilizer granules with soil (McInnes et al. 1986; Rochette et al. 2009), and the more abundant
435 presence of shallow cracks on the soil surface in CT soils compared to NT soils that favors the
436 penetration of N fertilizer granules into the soil profile (Rochette et al. 2009). Probably, in our
437 experiment, two factors may have contributed to determine a similar response between CT and NT
438 for NH_3 emissions: the type of N fertilizer applied (ammonium nitrate that we have used at tillering
439 time versus urea as often used in the experiments mentioned above) and the occurrence of rains in
440 the period immediately following the top-dressing N fertilization, which has certainly favored the
441 dragging of the N fertilizer along soil profile. Total soil NH_3 losses were strongly affected by crop
442 sequences, being significantly higher under WW than WF. This result is attributable to the different
443 amounts of N fertilizer applied to wheat in the two crop sequences (120 kg N ha^{-1} in wheat grown
444 after wheat and 80 kg N ha^{-1} in wheat grown after faba bean), so much so that the percentage of
445 fertilizer N applied that was emitted as NH_3 accounted for 13% in both FW and WW crop
446 sequences; it is well known that, generally, the higher the amount of N-fertilizer applied the higher
447 the NH_3 emissions (Saggar et al. 2013).
448
449 *4.3. N_2O emissions, DEA, nosZ abundance*
450

451 Total N_2O losses measured from sowing to harvest were, on average, 2.08 kg N_2O-N ha^{-1}, which
452 are close to the mean value (2.8 kg N_2O-N ha^{-1}) reported by Cayuela et al. (2017) in a recent meta-
453 analysis that included data from 53 field studies performed in Mediterranean areas. Specifically, for
454 winter cereals, the authors reported average cumulative emissions much lower than those observed
455 in the present experiment (0.7 versus 2.1 kg N_2O-N ha^{-1}) but, at the same time, they observed
456 higher values (2.3 kg N_2O-N ha^{-1} on average) in environments with a mean annual precipitation
457 >450 mm, that is the condition under which our experiment has been conducted.
458 Besides the differences in magnitude among treatments, daily N_2O emission rates varied during the
459 crop cycle showing a first peak in February and a second peak in May. Also Mutegi et al. (2010)
460 observed two peaks: the first one in autumn, which was attributed to the soil disturbance during
461 seedbed preparation and crop sowing under wet and warm weather conditions; and a second peak in
462 spring, as a result of the rapid transformations in soil of the N applied as fertilizer or slurry as also
463 observed by other authors (Dobbie and Smith 2003; Chatskikh et al. 2005). However, the two peaks
464 observed in the present study did not occur in concomitance or immediately afterwards wheat
465 sowing or N-fertilization. The peak observed in February was likely favored by the occurrence of
466 abundant rains, which led to a much lower availability of O_2 in the soil, associated to a concomitant
467 availability of the N residual from the fertilization done at sowing time. The second peak (in May)
468 was favored by rainfall events and also by the increase of the temperatures in the spring, both
469 factors that can have stimulated the activity of denitrifying bacteria, associated to an adequate
470 availability of nutrients (mainly C and N) from both the N residual from the fertilization done at
471 tillering (top-dressing) and the mineralization of the soil organic matter.
472 In the present research, rates of N_2O emissions were higher in NT soils than CT soils, similarly to
473 many other studies (Liu et al. 2007; Oorts et al. 2007; Rochette et al. 2008). This result has to be
474 ascribed to the co-occurrence of at least three main factors. Firstly, long-term NT increased bulk
475 density, and, as a consequence, the incidence of soil anaerobic microsites (Tellez-Rio et al. 2015b);
476 this condition certainly have enhanced the activity of denitrifiers and the emission of N_2O.

Secondly, due to the increase of bulk density, also WFPS values were higher in NT than in CT and, for most of the time, higher than 60%, that is commonly considered the critical threshold above which denitrification is promoted (Linn and Doran 1984; Regina and Alakukku 2010). Thirdly, as NT increased the amount of labile C (EOC) but apparently not nitrates, it is likely that, with the lower oxygen availability and in the presence of readily decomposable organic substrates, classical denitrifier microorganisms were constrained to use nitrates as final acceptors of electrons. This explains why nitrates could not accumulate. Laboratory measurements confirmed higher soil denitrification potential in topsoil under NT than under CT, according to Baudoin et al. (2009). However, an increase in DEA may not necessarily result in a proportional increase in the N_2O emissions as these latter also depend on the molar $N_2O/(N_2O+N_2)$ ratio. In fact, the DEA assay provides a measure of the potential denitrification since it is performed under laboratory optimal conditions. Moreover, during the DEA assay the further reduction of N_2O to N_2 is inhibited, thus leading to N_2O accumulation. The N_2O reduction to N_2 is catalyzed by the nitrous oxide reductase enzyme that is the last enzyme of the denitrification pathway. Therefore, a greater abundance of N_2O-reducers is an important factor for soil N_2O sink capacity and our data seem to highlight that the adoption of NT can drive microbial communities favoring these microorganisms. In fact, the nosZ gene was significantly higher under NT than CT conditions and this may have at least partly offset the drastic increase in DEA by increasing the proportion of total N losses as N_2, in other words by reducing the $N_2O/(N_2O+N_2)$ ratio. This might also explain why lower N_2O emissions in the field were observed in the NT-WW compared to NT-FW system; in fact, despite the upper soil layer of NT-WW showed higher values of DEA compared to NT-FW (mainly due to the greater amount of N-fertilizer applied), it also showed a greater abundance of nosZ gene that, as a result, determined lower N_2O emissions in the field in comparison to NT-FW. From another perspective, despite the minor total denitrification potential in NT-FW compared to NT-WW (–23% of DEA value, on average), the lower abundance of nosZ gene determined a lower conversion from N_2O to N_2, and thus higher N_2O emissions in the field. Therefore, in NT, the inclusion of faba bean in

503 rotation with wheat, diversifying crop residues and hence substrate availability for microbial
504 community, affected the composition of denitrifier community and the soil potential to reduce the
505 N_2O emissions and the $N_2O/(N_2O+N_2)$ product ratio (Domeignoz-Horta et al. 2015; Tatti et al.
506 2015; Giles et al. 2017). This circumstance can explain the Tillage × Crop interaction observed in
507 the field and the slight but significant differences between the two crop sequences under NT for the
508 N_2O emissions.
509
510 **5. Conclusions**
511
512 The continuous use of NT, applied for over 20 years to a Vertisol in a semiarid Mediterranean
513 environment, determined relevant changes in the soil physical and chemical properties, with an
514 increase, compared to CT, in the soil organic matter content but only in the uppermost soil layer (0–
515 15 cm). The different organic carbon stratification between NT and CT soils has considerable
516 implications from an agronomic point of view. In fact, the uppermost soil layer is undoubtedly the
517 most fragile, being exposed to the atmospheric agents. In the semiarid Mediterranean environments,
518 where soil erosion often represents a major issue, the maintenance of crop residues on soil surface
519 coupled with the increase of organic C in the uppermost soil layer can contribute to significantly
520 reduce such a phenomenon. It must be noted that effects of NT on the physical and chemical
521 properties of soil did not vary with crop sequence. The latter had little or no influence on the soil
522 parameters although C inputs varied considerably (being higher in FW than in WW), highlighting
523 how the quality of crop residues, by influencing their own fate, is as important as their amount.
524 No tillage showed contrasting effects on N gas emissions from soil. No differences were observed
525 between CT and NT as regard NH_3 emissions, whereas NT determined higher N_2O emissions. After
526 all, NT determined a considerable increase of the potential denitrifying enzyme activity in the
527 uppermost soil layer, favored by the increase of organic matter (and in particular of its labile
528 fractions) and by the increase of the water filled pore space. Such effects were counterbalanced only

529 partially by the increase of the nitrous oxide reductase activity (as evidenced by the higher values of
530 nosZ gene abundance observed in NT than in CT soils), responsible of the N_2O reduction to N_2.
531 In conclusion, though the long-term application of NT offers some agronomic and environmental
532 benefits in semiarid Mediterranean environment at the same time it presents some critical points
533 related to the increase in nitrous oxide emissions. This highlights the importance of defining
534 particularly accurate management strategies that can mitigate these negative effects.

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FIGURE CAPTIONS

Fig. 1. Carbon inputs to the soil under different management practices (CT, conventional tillage; NT, no tillage; WW, wheat after wheat; FW, wheat after faba bean). Reported values are means (n=17) ± SE (bars). Different letters indicate significant differences among treatments at $P \leq 0.05$.

Fig. 2. Ammonia (NH_3) emission from soil under different management practices (CT, conventional tillage; NT, no tillage; WW, wheat after wheat; FW, wheat after faba bean). Red rectangles (bottom) represent the NH_3 emitted after the fertilization at sowing, while grey rectangles represent the NH_3 emitted after the fertilization at tillering. Each full column (the sum of previous two rectangles) represent 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 $P \leq 0.05$ for total NH_3 emissions.

Fig. 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 different management practices (CT, conventional tillage; NT, no tillage; WW, wheat after wheat; FW, wheat after faba bean) during the 2013–2014 [a] and 2014–2015 [b] growing seasons. Reported values are means (n=6) ± SE (bars). F and black arrows indicate the time of N top-dressing.

Fig. 4. Total nitrous oxide (N_2O) emission from soil under different management practices (CT, conventional tillage; NT, no tillage; WW, wheat after wheat; FW, wheat after faba bean). Reported values are means (n=6) ± SE (bars). Different letters indicate significant differences among treatments at $P \leq 0.05$.

Fig. 5. Abundances of nosZ gene (expressed as gene copy number mg^{-1} of dry soil) in the 0–15 cm soil layer under different management practices (CT, conventional tillage; NT, no tillage; WW, wheat after wheat; FW, wheat after faba bean). Reported values are means (n=4) ± SE (bars). Different letters indicate significant differences among treatments at $P \leq 0.05$.

TABLES

Table 1

Bulk density, total organic carbon (TOC), total nitrogen (TN), extractable organic carbon (EOC), and nitrates (NO_3^--N) determined on samples collected at 0–15 cm and 15–30 cm soil layers under different management practices (CT, conventional tillage; NT, no tillage; WW, wheat after wheat; FW, wheat after faba bean). For each parameter, the proportion of variance explained by Tillage, Crop, and Tillage × Crop interaction is also reported.

Treatment		Bulk density kg m^{-3}	TOC g C kg^{-1}	TN g N kg^{-1}	EOC mg C kg^{-1}	NO_3^--N mg N kg^{-1}
0–15 cm						
CT	WW	987	13.3	1.3	53.9	1.6
	FW	950	14.2	1.3	54.8	1.6
NT	WW	1174	17.4	1.9	75.8	1.8
	FW	1128	18.8	1.8	78.9	1.6
Tillage (%)		93.2***	89.9**	96.2***	85.3**	ns
Crop (%)		ns	ns	ns	ns	ns
Tillage × Crop (%)		ns	ns	ns	ns	ns
15–30 cm						
CT	WW	1040	13.7	1.3	55.8	1.5
	FW	1047	14.2	1.4	62.4	1.6
NT	WW	1344	13.6	1.4	47.2	1.4
	FW	1260	14.3	1.5	47.5	1.3
Tillage (%)		93.6***	ns	ns	68.3**	ns
Crop (%)		ns	ns	ns	ns	ns
Tillage × Crop (%)		ns	ns	ns	ns	ns

*, **, and *** significant at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively; ns, not significant.

827 **Table 2**

828 Average potential denitrifying enzyme activity (DEA) determined on soil samples collected at 0–15
829 cm and 15–30 cm soil layers under different management practices (CT, conventional tillage; NT,
830 no tillage; WW, wheat after wheat; FW, wheat after faba bean). The proportion of variance
831 explained by Tillage, Crop, and Tillage × Crop interaction is also reported.

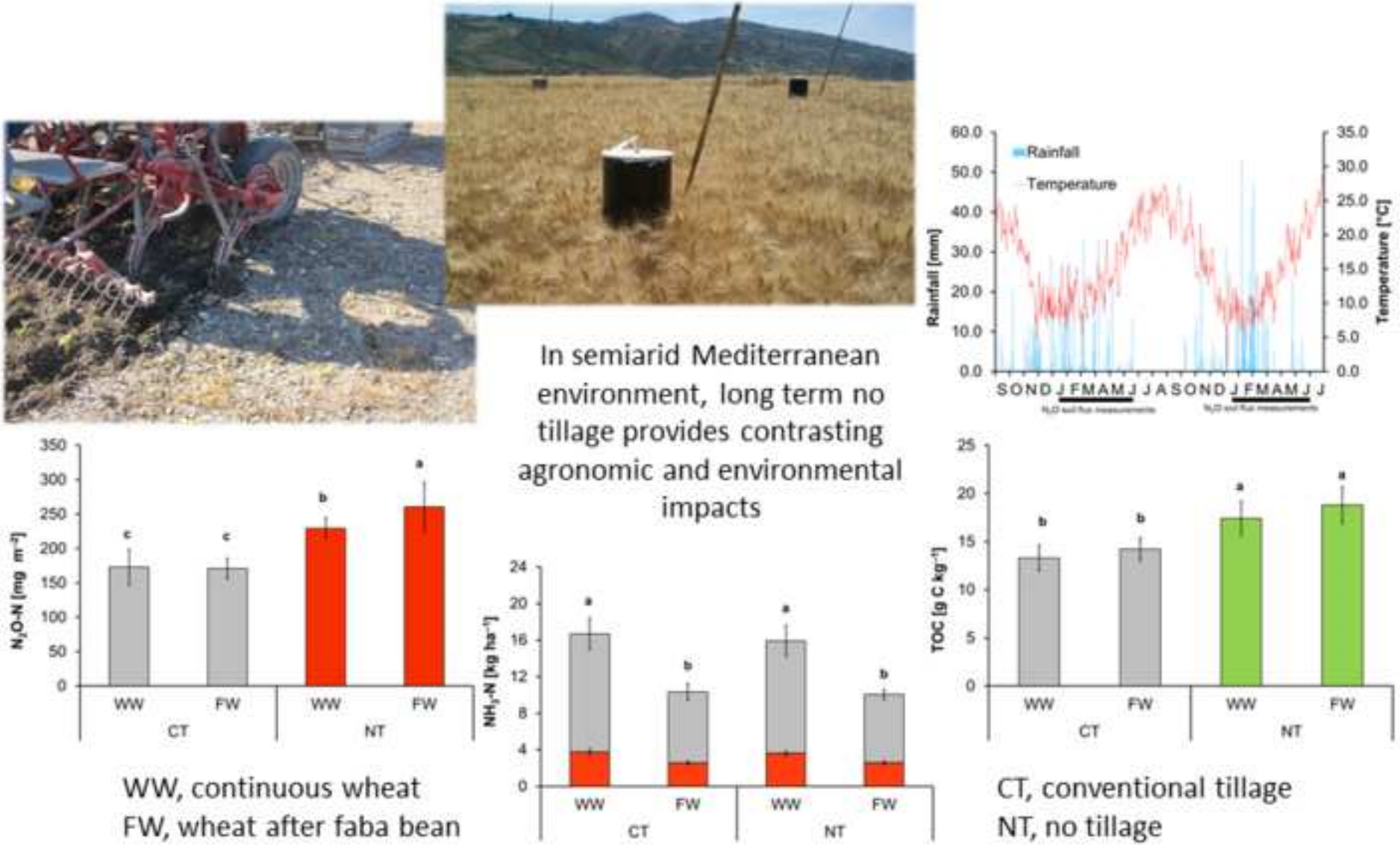
832

Treatment		DEA ($\mu g\ N_2O\text{-}N\ kg^{-1}\ h^{-1}$)	
		0–15 cm	*15–30 cm*
CT	WW	16.5 c	17.0
	FW	15.2 c	17.4
NT	WW	128.3 a	12.7
	FW	99.3 b	12.8
Tillage (%)		93.7***	ns
Crop (%)		2.2**	ns
Tillage × Crop (%)		3.6*	ns

833 Where the Tillage × Crop interaction is significant, different letters indicate significant differences
834 among treatments at $P \leq 0.05$.

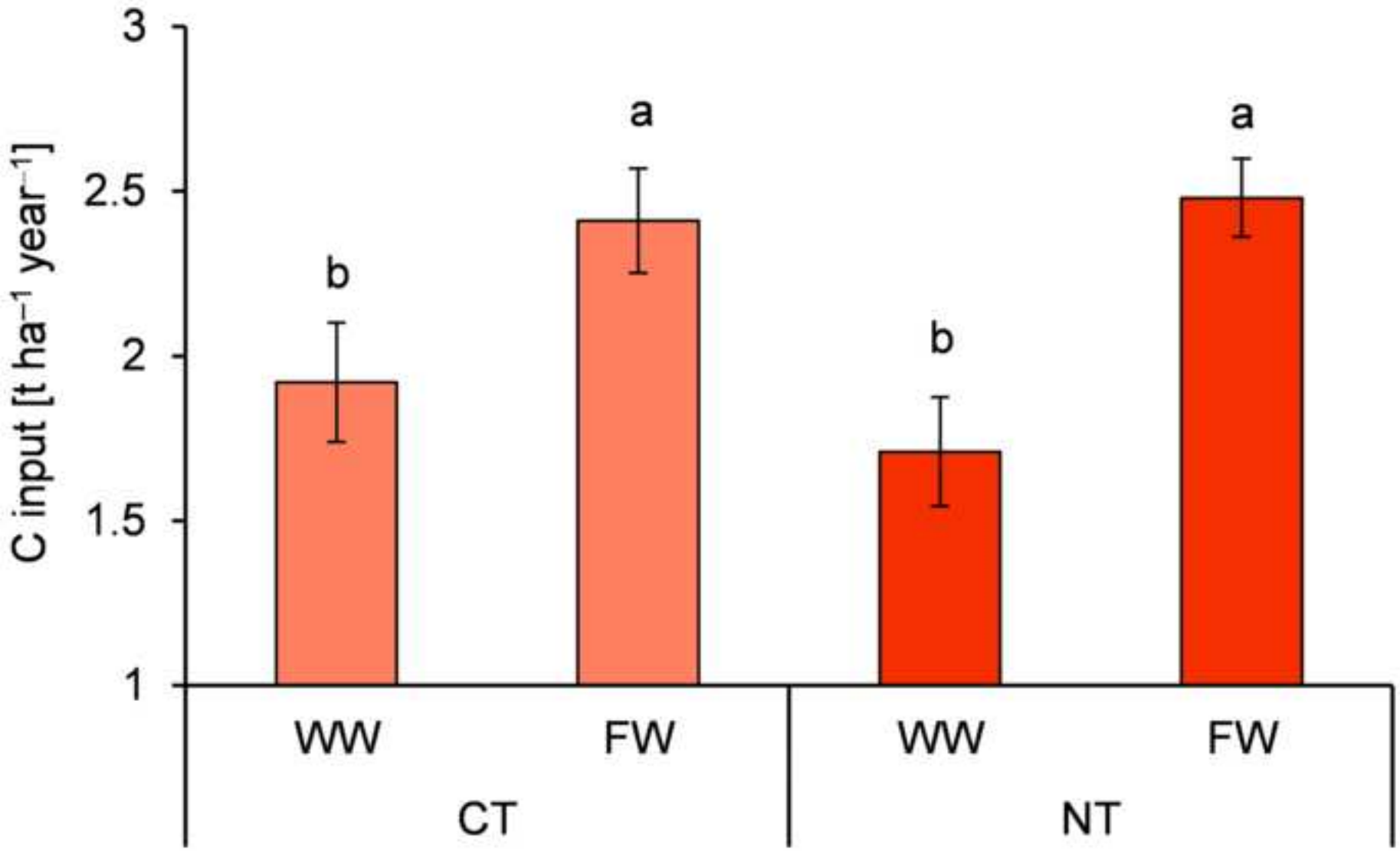
835 *, **, and *** significant at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively; ns, not significant.

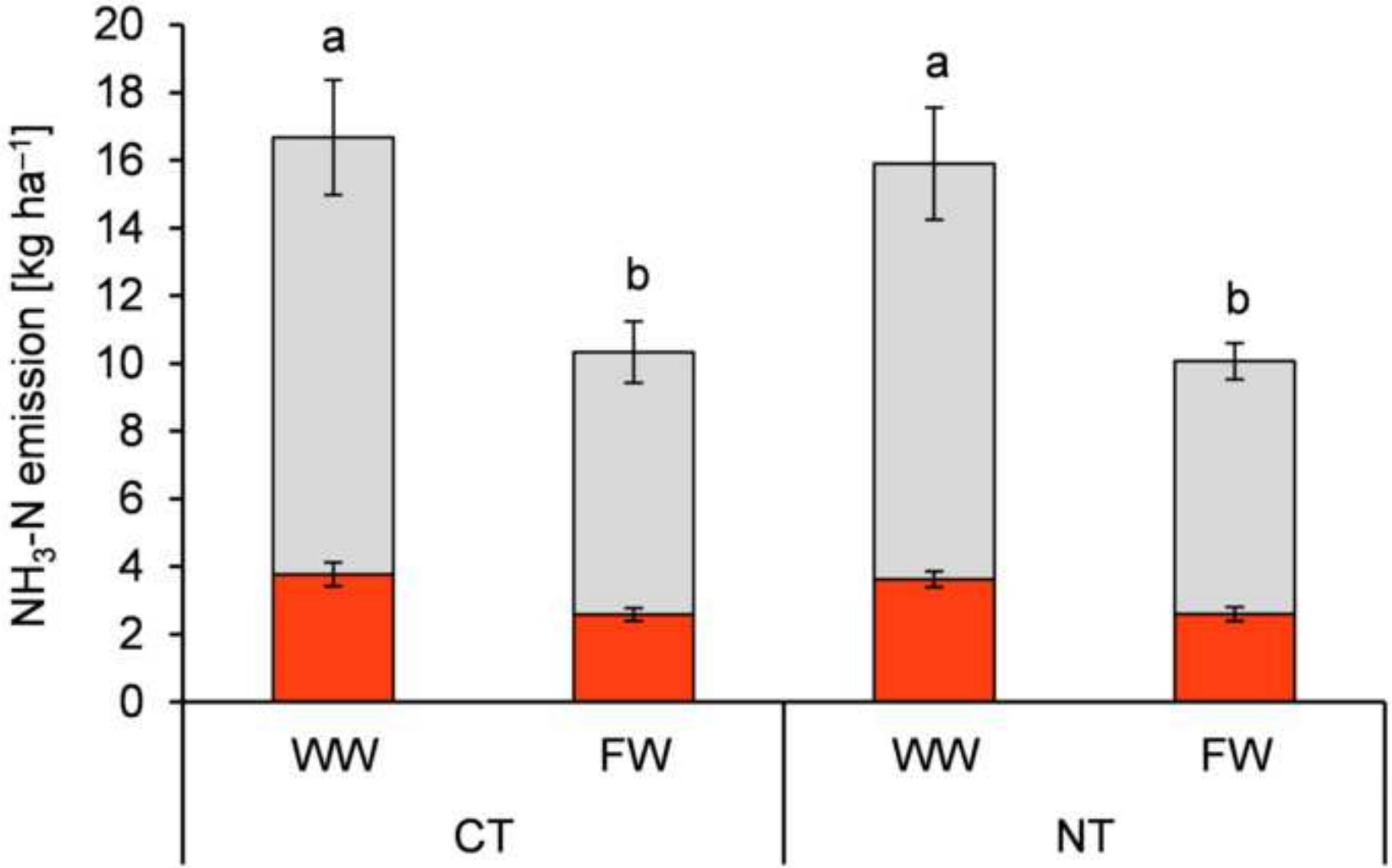
*Graphical Abstract

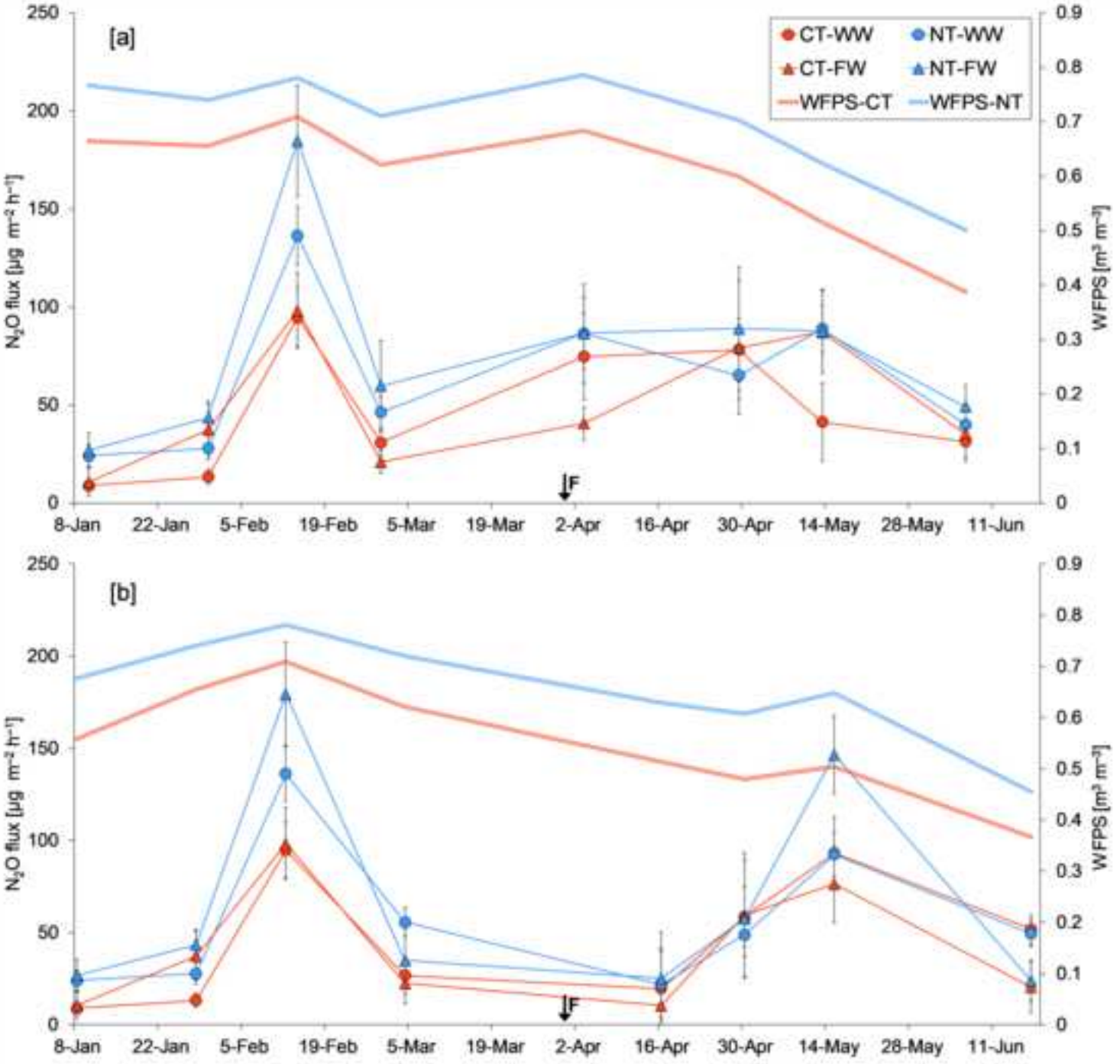


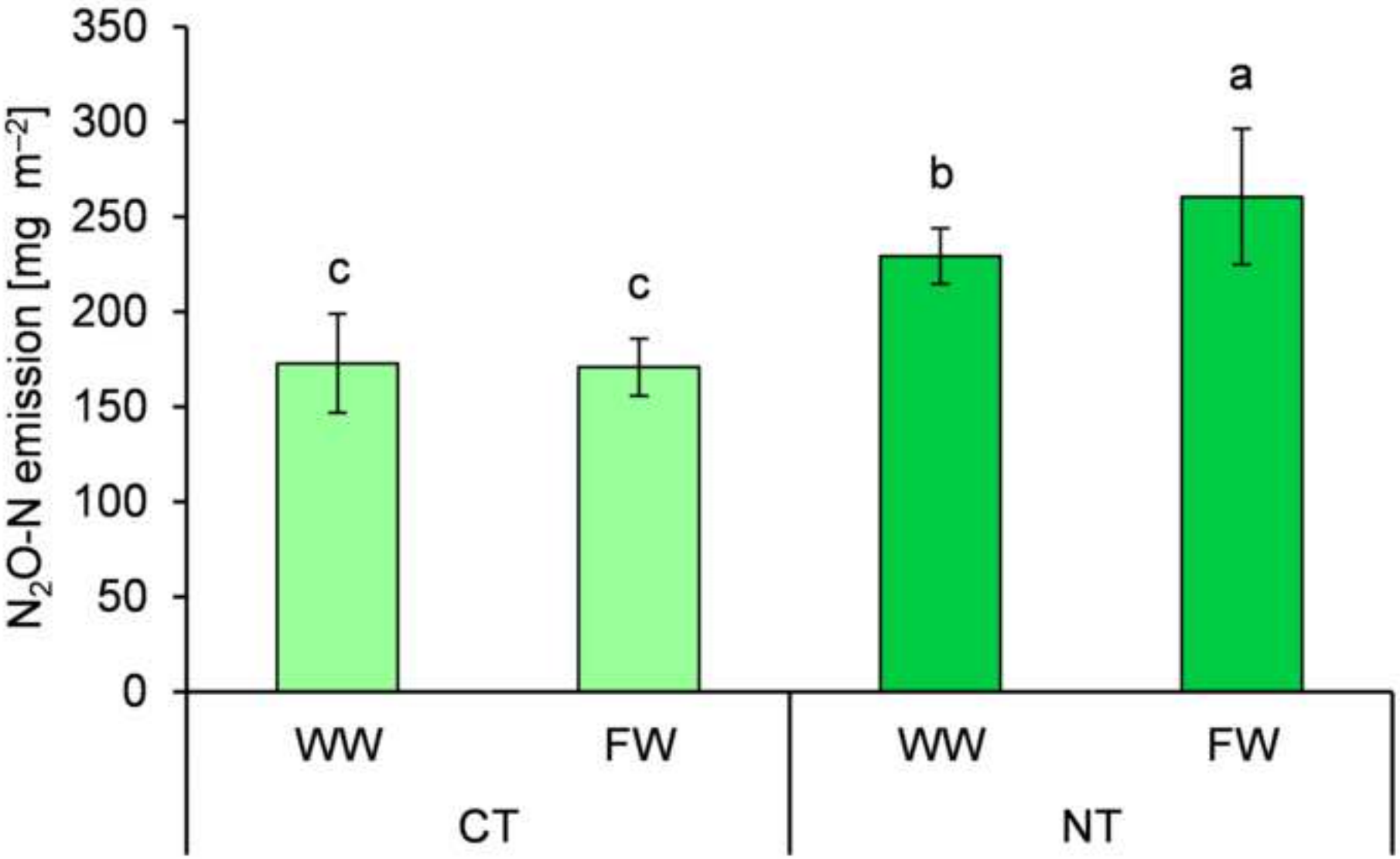
Highlights

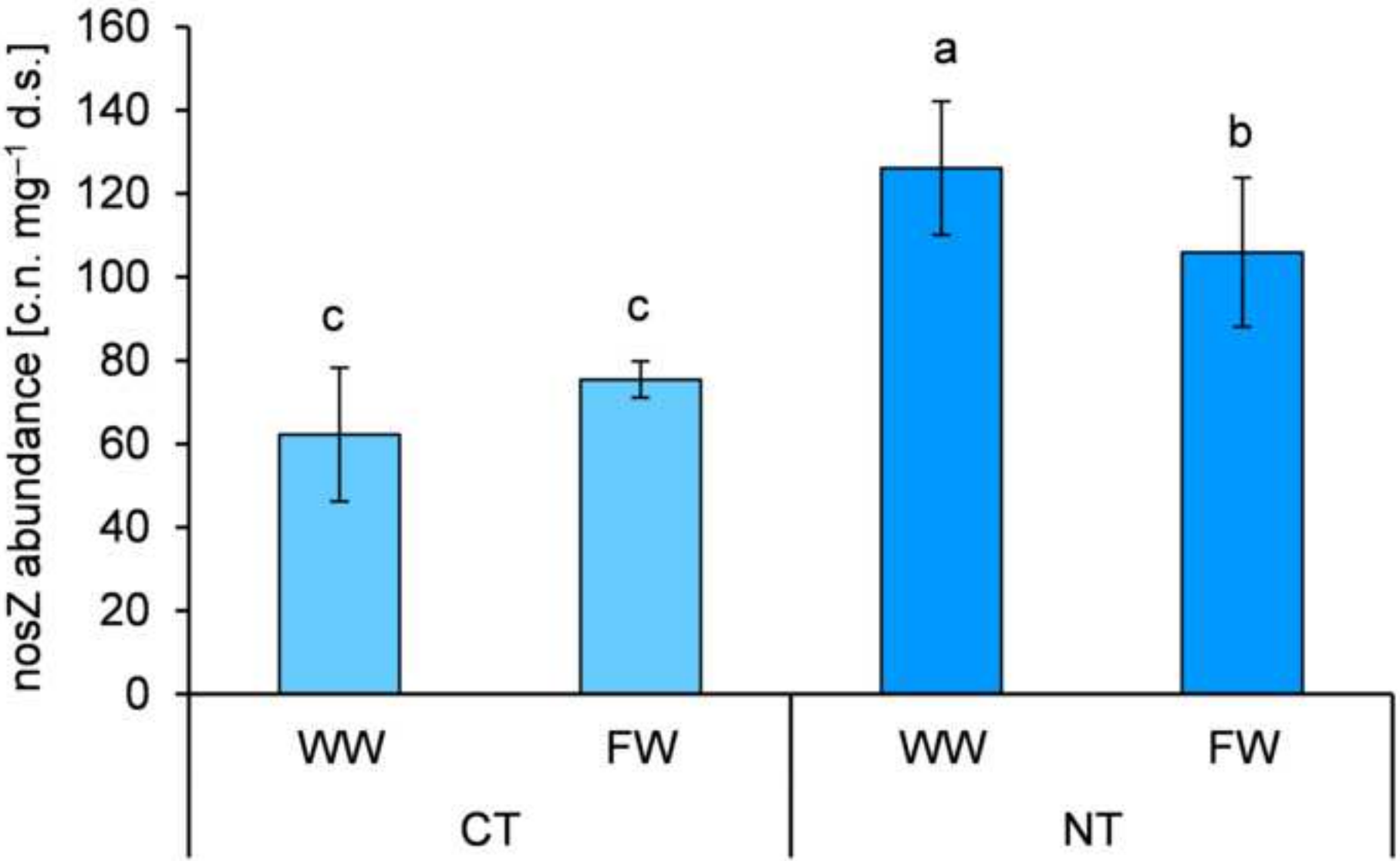
- Tillage effects on soil organic C and N gases emissions were assessed in two crop sequences
- N_2O emissions, but not total NH_3 emissions, were higher in NT than CT
- Continuous application of NT for 23 years increased bulk density, WFPS, TOC and EOC in the topsoil compared to CT
- Denitrifying enzyme activity and nosZ gene were enhanced by long term NT


C input [t ha⁻¹ year⁻¹]
3
2.5
2
1.5
1
b
a
b
a
WW
FW
WW
FW
CT
NT


20
18
16
14
12
10
8
6
4
2
0
NH3-N emission [kg ha−1]
a
b
a
b
WW
FW
WW
FW
CT
NT


[a]
CT-WW
NT-WW
CT-FW
NT-FW
WFPS-CT
WFPS-NT
N2O flux [µg m-2 h-1]
WFPS [m3 m-3]
F
8-Jan
22-Jan
5-Feb
19-Feb
5-Mar
19-Mar
2-Apr
16-Apr
30-Apr
14-May
28-May
11-Jun
[b]


350
300
250
200
150
100
50
0
N2O-N emission [mg m−2]
c
c
b
a
WW
FW
WW
FW
CT
NT


nosZ abundance [c.n. mg^{-1} d.s.]
160
140
120
100
80
60
40
20
0
c
c
a
b
WW
FW
WW
FW
CT
NT