Soil management effects on greenhouse gases production at the macroaggregate scale

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Abstract

Agricultural management practices play an important role in greenhouse gases (GHG) emissions due to their impact on the soil microenvironment. In this study, two experiments were performed to investigate the influence of tillage and N fertilization on GHG production at the macroaggregate scale. In the first experiment, soil macroaggregates collected from a field experiment comparing various soil management systems (CT, conventional tillage; NT, no-tillage) and N fertilization types (a control treatment without N and mineral N and organic N with pig slurry treatments both at 150 kg N ha^{-1}) were incubated for 35 days. Methane (CH_4), carbon dioxide (CO_2) and nitrous oxide (N_2O) production was quantified at regular time intervals by gas chromatography. In the second experiment, the effects of fertilization type and soil moisture on the relative importance of nitrification and denitrification processes in N_2O emission from soil macroaggregates were quantified. Nitrate ammonium, macroaggregate-C concentration, macroaggregate water-stability, microbial biomass-C and N (MBC and MBN, respectively) and water-soluble C (WSC) were determined. While NT macroaggregates showed methanotrophic activity, CT macroaggregates acted as net CH_4 producers. However, no significant differences were found between tillage systems on the fluxes and cumulative emissions of CO_2 and N_2O. Greatest cumulative CO_2 emissions, macroaggregate-C concentration and WSC were found in the organic N fertilization treatment and the lowest in the control treatment. Moreover, a tillage and N fertilization interactive effect was found in macroaggregate CO_2 production: while the different types of N fertilizers had no effects on the emission of CO_2 in the NT macroaggregates, a greater CO_2 production in the CT macroaggregates was observed for the organic fertilization treatment compared with the mineral and control treatments. The highest N_2O losses due to nitrification were found in the mineral N treatment while

denitrification was the main factor affecting N_2O losses in the organic N treatment. Our results suggest that agricultural management practices such as tillage and N fertilization regulate GHG production in macroaggregates through changes in the proportion of C and N substrates and in microbial activity.

Keywords

Carbon dioxide, denitrification, fertilization, macroaggregate, methane, nitrification, nitrous oxide, tillage.

Introduction

The production and consumption of soil greenhouse gases (GHG) is mediated by several microbial processes (Conrad, 1996). For instance, soil carbon dioxide (CO_2) emissions are the result of microbial heterotrophic respiration while methane (CH_4) is normally oxidized by methanotrophic bacteria in aerobic soils (Goulding et al. 1995). Furthermore, soil nitrous oxide (N_2O) production is the result of nitrification and denitrification processes (Blackmer et al., 1980; Firestone et al., 1980; Poth and Focht, 1985). Those microbial processes are regulated by the physical protective capacity of aggregates that limit decomposition of organic C and N compounds (Elliott, 1986). Soil aggregates not only protect C and N, but they also regulate both the structure and the activity of the soil microbial community (Gupta and Germida, 1988; Miller et al., 2009). The intra-aggregate distribution of pores plays a major role in microbial access to oxygen, substrates and water. As Young and Ritz (2000) pointed out, soil structure regulates oxygen diffusion to habitat sites, depending on the connectivity and tortuosity of pore pathways. The aggregate architecture also controls the distribution of water films within soil matrix, affecting microbial microhabitats. Thus, the diffusion of oxygen to the center of aggregates will depend on the spatial arrangement of water films (Young and Ritz, 2000). The last factors affect the importance of denitrification and respiration activities and demonstrate the role played by soil aggregates regulating them (Beare et al., 1994; Estavillo et al., 2002). Moreover, due to their physical protective capacity, soil aggregates also regulate the microbial accessibility to substrates.

In a recent experiment, Lenka and Lal (2013) have suggested that the aggregate hierarchy theory of Tisdall and Oades (1982) could be extended to describe the effect of soil aggregation on GHG emission from soil. That theory postulates that the nature of the organic binding agents (transient, temporary and persistent) regulates different

hierarchical stages of aggregation. Microaggregates are formed by the joining of primary particles and silt-sized aggregates and persistent organic binding agents, while these microaggregates are bound together into macroaggregates by temporary and transient organic binding agents. These organic materials are protected by the heterogeneity of the soil microenvironment which limits the access of decomposers and their enzymes (Schmidt et al., 2011; Ananyeva et al., 2013).

The agricultural practices play an important role in GHG emissions due to their effects on the soil microenvironment. Tillage breaks soil aggregates leading to enhanced organic matter decomposition (Álvaro-Fuentes et al., 2008; Beare et al., 1994) and reduced C and N concentration (Plaza-Bonilla et al., 2010). Contrarily, the use and maintenance of no-tillage (NT) increases the stability of soil macroaggregates (Plaza-Bonilla et al, 2013b), a fact that could lead to a reduction in heterotrophic respiration due to a greater substrate protection, thus limiting the emissions of CO_2. Likewise, CH_4 production is also affected by tillage management. For instance, in a wheat-fallow rotation, Kessavalou et al. (1998) reported higher CH_4 uptake rates under NT when compared with a plough treatment. Also, Hütsch (1998a) reported 4.5-11 times greater CH_4 oxidation rates under NT than under conventional tillage (CT). Ball et al. (1999) hypothesized that the reduction in CH_4 oxidation usually found when tillage is performed could be due to the disturbance of the methanotrophic microbes by tillage, the changes in gas diffusivity or a long-term damage to methanotrophs due to disruption of soil structure. Tillage also has an impact on N_2O emissions. Estavillo et al. (2002), studying the effects of ploughing a permanent pasture on the emissions of this gas, observed an increase in both soil organic N mineralization and N_2O production rates from nitrification and denitrification processes after the breakage of soil aggregates by tillage.

Nitrogen fertilization has a strong impact on soil aggregation and C and N protection. The application of organic fertilizers such as pig slurry enhances the proportion of easily-decomposable C fractions (Morvan and Nicolardot, 2009) that could act as substrates for the denitrification process and the concomitant soil N_2O emissions to the atmosphere (Burford and Bremnner, 1975). Sexstone et al. (1985) quantified the diffusion of oxygen within soil aggregates establishing a relationship between their size and their potential to act as denitrifying microsites within soil. Nitrogen fertilization also plays a major role in methane oxidation. Different authors (Hütsch et al. 1993; Mosier et al. 1991; Steudler et al. 1989), working with incubated soil cores from agricultural, grassland and forest experiments, observed a decrease in CH_4 uptake when applying inorganic N to soil. Contrarily, recent findings suggest that ammonium-based fertilizers could stimulate the activity of methanotrophs (Bodelier and Landbroek, 2004).

In recent years, different experiments have been performed to analyze the effects of aggregate size on CO_2, CH_4 and N_2O production (Diba et al., 2011; Drury et al., 2004; Kimura et al., 2012). However, inconsistent results have been observed in the literature due to the simultaneous diverse microbial processes that soil aggregates can hold (Sey et al., 2008). For instance, Parkin (1987) related the spatial heterogeneity in the N_2O emissions usually observed in most experiments with the presence of particulate organic matter within soil aggregates. Those studies demonstrate that different aggregate attributes such as size or C fractions within them regulate GHG production processes. However, few experiments have studied the effects of agricultural management practices on soil GHG production at the aggregate scale.

Thus, the objectives of this study were: (i) to analyze the effect of the use of different types of tillage and N fertilization on the production of GHG by soil macroaggregates

and, (ii) to quantify the relative importance of the nitrification and denitrification processes on the macroaggregate emissions of N_2O depending on the type of fertilizer used. We hypothesized that (i) CT macroaggregates would emit a greater amount of GHG due to their lower protection of the organic C and N compounds when compared to NT macroaggregates and (ii) the application of pig slurry and mineral N would result in different rates of GHG production provided by soil macroaggregates.

Materials and Methods

Soil samples were collected from an experimental field established in 2010 in Senés de Alcubierre, NE Spain (41º 54' 12'' N, 0º 30' 15'' W), in an area with a temperate continental Mediterranean climate. This field experiment has a randomized block design with three replications comparing different tillage systems and N fertilization treatments. Two tillage systems (CT, conventional tillage with disk ploughing and NT, no-tillage) and two types of N fertilizers (mineral N with ammonium nitrate and ammonium sulphate and organic N with pig slurry), with three N doses (0, 75 and 150 kg N ha^{-1}), were compared. Each year, in the CT treatment, tillage is performed right before the seeding of barley (*Hordeum vulgare L.*) with one pass of a disk plough to 20 cm depth in October, after the application of organic and mineral fertilizers. The NT treatment consisted of a total herbicide application (1.5 L 36% glyphosate per hectare) for controlling weeds before sowing. Mineral N fertilizer was manually applied. The treatment with 150 kg N ha^{-1} was split into two applications: half of the dose before tillage as ammonium sulphate (21% N) and the other half at the beginning of tillering, in February, as ammonium nitrate (33.5% N). For the 75 kg N ha^{-1} treatment the entire dose was applied at tillering as ammonium nitrate. Equally, in the treatments with organic fertilization, the 75 kg N ha^{-1} rate was applied entirely at tillering and the 150 kg N ha^{-1} one was split into two applications, one half before tillage and the other half at tillering. The organic fertilization treatment consisted of the application of pig (*Sus scrofa*) slurry from a commercial farm in the area. The slurry was conventionally surface-spread using a commercial vacuum tanker fitted with a splashplate. The machinery was previously calibrated to apply the precise dose after analyzing the pig slurry. The main edaphoclimatic characteristics of the experimental site are listed in Table 1. Prior to the establishment of the experiment the field was conventionally tilled

and fertilized with mineral N for four decades until 2008. Then, the whole field was transformed to no-tillage. Finally, as commented before, when the experiment started in 2010, the CT plots were added. The cropping system is a continuous barley monoculture.

Experiment 1: GHG production from soil macroaggregates under different tillage and N fertilization treatments.

Soil samples were obtained from both tillage treatments (CT and NT) and the lower (0 kg N ha^{-1}, Control) and the higher (150 kg mineral N ha^{-1}, Mineral, and 150 kg organic N ha^{-} with pig slurry, Organic) fertilization treatments of the field experiment. Soil sampling was performed in March 2012 during the late tillering stage of the crop, three weeks after the top-dressing application of fertilizers. In each plot (i.e., tillage and N fertilization treatments), soil samples were collected from four areas that correspond to the four replications. From each sampling area, a composite sample of approximately 500 g was taken from the 0-5 cm soil depth using a flat spade and outside the wheel tracks areas. Afterwards, the samples were stored in crush-resistant airtight plastic containers for 3-4 hours. Once in the laboratory, the samples were gently passed through an 8mm sieve and air-dried at room temperature. Soil macroaggregates (0.250-8 mm) were obtained placing the 8-mm soil sieved sample on the top of a 0.250 mm sieve in an electromagnetic sieve apparatus (Filtra FTL-0200, Badalona, Spain). A sieving time of 1 min and the lowest power program of the device were used to avoid macroaggregate breakage. The dry-sieved macroaggregates (0.250-8 mm) obtained were stored in aluminium trays taking care to avoid any breakage until further analyses.

Samples of 40-g each of dry-sieved macroaggregates (0.250-8 mm) were placed in 500 ml Mason jars. Four jars were built for each tillage and N fertilization combination. A

stainless steel fitting turned to accommodate two silicon-Teflon septa was inserted in the lid of each jar to ensure air tightness. A volume of 12.8 ml of distilled water was added to each macroaggregate sample using a micropipette in order to avoid the breakage of the macroaggregates when adding the water, and also to obtain a gravimetric moisture content of about 32%. This value corresponds to the field capacity of the bulk soil of our experiment according to Saxton and Rawls (2006). All the jars were covered with a layer of parafilm which was pinpricked to ensure air exchange and avoid sample desiccation during the incubation process. The weight of the jars with the wet macroaggregate samples was recorded and then every 48 hours to check for water evaporation. Distilled water was added when needed. Air samples were withdrawn at 0(0), 4(0.17), 12(0.5), 24(1), 48(2), 72(3), 192(8), 384(16), 504(21), 672(28) and 840(35) hours(days) after the beginning of the incubation process. The parafilm layer of each jar was removed 15 min prior to each gas sampling. Then all the lids were tightly closed and a 15 ml headspace gas sample was withdrawn with the use of a gas-tight syringe, pumping twice before the extraction to ensure a total mixing of the gas in the jar (0 min sampling). Afterwards, 15 ml of ambient air were injected in the jars to compensate for the volume previously withdrawn. A second gas sampling was performed 60 min later. The gas samples obtained were injected in 12 ml Exetainer borosilicate glass vials (model 038W, Labco, High Wycombe, UK) until their analysis. Once the samplings were made (i.e., after 60 min) the lids were opened and the jars were covered with a parafilm layer until the next sampling event. Also, the jars were covered during the incubation to avoid light exposure. As explained in the next section, the difference in GHG concentration between 0 and 60 min samplings was used to calculate the GHG fluxes.

Gas and soil analysis

205 The gas samples were analyzed with an Agilent 7890A gas chromatography system
206 equipped with an electron capture detector (ECD) and a flame ionization detector (FID)
207 plus methanizer, and three automated valves to obtain the three gases of interest (i.e.,
208 CH_4, CO_2 and N_2O) for each gas sample injection. A HP-Plot Q column (30 m long,
209 0.32 mm of section and 20 µm) was used along with a 15 m long pre-column of the
210 same material. The injector and the oven temperature were set to 50ºC. The temperature
211 of the FID and ECD detectors was set to 250ºC and 300ºC, respectively. The methanizer
212 temperature was set to 375ºC. For the FID detector, H_2 was used as a carrier gas and N_2
213 as a make-up gas at 35 and 25 ml min^{-1}, respectively. In the case of the ECD detector,
214 5% methane in Argon was used as a make-up gas at 30 ml min^{-1}. The volume of sample
215 injected was 1 ml. The system was calibrated using analytical grade standards (Carburos
216 Metálicos, Barcelona, Spain). Soil CH_4, CO_2 and N_2O production in the jar headspace
217 was calculated according to Holland et al. (1999). Gas concentrations (ppm) obtained
218 with the chromatography system were converted to mass units with the ideal gas
219 equation:

220 $$C_m = (C_v \times M \times P)/(R \times T)$$

221 where C_m is the mass/volume concentration (e.g., mg CO_2-C m^{-3} incubation jar
222 headspace), C_v is the volume/volume concentration (ppm of each GHG obtained with
223 the chromatography system), M is the molecular weight of each GHG (e.g., 12 g CO_2-C
224 mol^{-1} or 28 g N_2O-N mol^{-1}), P is atmospheric pressure, R is the universal gas constant
225 and T is the incubation temperature (298 K). C_m was multiplied by the headspace
226 volume of the incubation jars (5×10^{-4} m^{-3}) to obtain the mass of CH_4-C, CO_2-C or
227 N_2O-N accumulated during the incubation. Thus, the mass of GHG produced (e.g., mg
228 CH_4-C kg^{-1} macroaggregates h^{-1}) is calculated as follows:

229 $f = ((C_1-C_0)/(m \times t)) \times 1000$

230 where f is the mass of gas produced per unit of time, C_1 and C_0 are the mass of C or N
231 produced at the end and at the beginning of two consecutive samplings, respectively, m
232 is the mass of air-dried macroaggregates in each jar (0.04 kg) and t is the incubation
233 period (1 h). Finally, the cumulative production of CH_4-C, CO_2-C and N_2O-N was
234 calculated using the trapezoid rule by linear interpolation between two consecutive
235 samplings.

236 Additionally, the initial mineral N (i.e., nitrate and ammonia), the C concentration, and
237 the proportion of water-stable macroaggregates were quantified for each experimental
238 unit. Once the incubation was finished, the microbial biomass-C and microbial biomass-
239 N (MBC and MBN, respectively), the nitrate and ammonia content, the water-soluble C
240 (WSC) and the C concentration of each 40 g macroaggregates sample were also
241 quantified. Soil nitrate (NO_3^-) and ammonium (NH_4^+) were determined extracting 10 g
242 of macroaggregates with 80 ml of 1 M KCl and using a continuous flow autoanalizer
243 (Seal Autoanalyzer 3). The macroaggregate-C concentration was quantified by the wet
244 oxidation method of Walkley-Black described by Nelson and Sommers (1996), with a
245 modification to increase the digestion of soil organic carbon (SOC), which consisted in
246 boiling the sample and the extraction solution at 150ºC for 30 min (Mebius, 1960). The
247 proportion of water-stable macroaggregates and their sand content were determined
248 following a modification of the method of Elliott (1986) as described in Plaza-Bonilla et
249 al. (2013a). The microbial biomass-C and microbial biomass-N were determined with
250 the chloroform-fumigation and direct extraction method of Vance et al. (1987). The
251 extracts were analyzed for organic C and N with a multi C/N TOC-TNB analyzer 3100
252 (Analytik Jena, Jena, Germany). The extraction coefficient applied for both C and N
253 was 0.38 (Sparling and Zhu, 1993; Vance et al., 1987). The WSC was extracted by

shaking 10 g of macroaggregates in 40 ml of distilled water with 0.5 g potassium sulphate in a centrifuge tube for 30 min, centrifuging for 5 min at 5000 rpm and filtering all supernatant solution through a Whatman no.42 filter. The organic C in the filtrate was determined by the same device used for the MBC-MBN determination.

Data analysis

Cumulative GHG data were log-transformed and analyzed using the SAS statistical software (SAS Institute Inc., 1990). To compare the effects of tillage, fertilizer treatments and sampling time on cumulative GHG emissions, a repeated measures analysis of variance for a bifactorial design was performed for each gas. When significant, differences among treatments were identified at the 0.05 probability level of significance using an LSD test. For each sampling time, the linear relationship between CO_2 and N_2O production in the CT and NT macroaggregates was determined with the statistical package JMP 10 (SAS Institute Inc, 2012). To analyse the relationship between CO_2 production, proportion of water-stable macroaggregates and their C concentration, a stepwise regression was performed using the statistical package JMP 10 (SAS Institute Inc, 2012).

Experiment 2: Relative importance of nitrification and denitrification in N_2O production from soil macroaggregates under different N fertilization types.

Soil samples from the 0-5 cm soil depth were collected in the same fertilization treatments as in Experiment 1 (i.e., 0 and 150 kg N ha^{-1} as mineral N and pig slurry), only under NT. However, for this experiment soil sampling was performed on December 2012, three weeks after the pre-seeding fertilization of the crop. In each plot

277 (i.e., N treatment), six areas that would correspond to the six replications of the
278 experiment were defined.

279 From each area, a 500 g composite soil sample was taken from the 0-5 cm soil depth
280 using a flat spade, taking care to avoid the wheel track areas. Dry-sieved
281 macroaggregates fractionation was analogous to Experiment 1. The experimental set up
282 consisted of three N fertilization types (0 kg N ha^{-1}, mineral N at 150 kg ha^{-1} and
283 organic N with pig slurry at 150 kg ha^{-1}), two soil moisture treatments (15% and 30 %
284 gravimetric water content) and three levels of acetylene (0%, 0.01% and 5%, v v^{-1}).
285 Each combination of the three factors was repeated six times according to the
286 experimental replications. Therefore, the total number of observations was 108. To
287 achieve this number of observations, the dry-sieved macroaggregates from each
288 experimental replication was divided in six subsamples of 40 g that were placed in
289 Mason jars. These six subsamples were divided in two groups. In the first group, three
290 subsamples were moistened with distilled water to 15% gravimetric water content. The
291 other three subsamples were moistened to 30% gravimetric water content. The lids of
292 the jars were closed and a 15 ml headspace gas sample was taken for every jar (0 min
293 sampling). Afterwards, for each soil moisture treatment three acetylene (C_2H_2)
294 treatments were applied: 0%, 0.01% and 5% (v v^{-1}) corresponding to partial pressures of
295 0 Pa, 10 Pa and 5000 Pa, respectively) following the method proposed by Klemedtsson
296 et al. (1988) to differentiate the relative contribution of the nitrification and
297 denitrification processes in N_2O emissions. Different drawbacks of the method have
298 been reported in the literature. Among them, Baggs (2008) enumerates (i) a possible
299 underestimation of denitrification by preventing the supply of nitrifier-NO_3^-, mainly in
300 aquatic systems (Groffman et al., 2006), (ii) acetylene could be used as a C-substrate for
301 denitrification, and (iii) a limited diffusion of acetylene into fine textured soils.

However, acetylene-based methods still have a role in systems with high NO_3^- concentrations (Groffman et al., 2006), such as the agricultural soil of our experiment, and are useful for comparative purposes between different treatments (Estavillo et al., 2002). According to each treatment, different volumes of ambient air were injected to equilibrate the pressure into the jars. The jars with the macroaggregates were incubated at 25ºC for 24 hours. After that, another 15 ml gas sample was withdrawn to calculate the accumulation of N_2O in the 24 hours period for each jar. Air gas samples were stored and analyzed following the same methodology as in Experiment 1. It was assumed that the N_2O measured in the treatment without acetylene (i.e., 0% C_2H_2,) corresponded to the N_2O produced by the nitrification and denitrification processes. In turn, the N_2O measured in the treatment with 0.01% C_2H_2 corresponded only to that produced during the denitrification process (Davidson et al., 1986) and, finally, the N_2O measured in the treatment with a C_2H_2 concentration of 5% corresponded to the N_2O produced due to a complete denitrification (Yoshinari et al., 1977). The production of N_2O by the nitrification process was calculated from the difference between the N_2O measured in the 0% and the 0.01% C_2H_2 treatments, while the production of N_2O by the denitrification process corresponded to the amount of N_2O measured in the 0.01% C_2H_2 treatment, and complete denitrification (i.e., N_2O that would be reduced to N_2) was calculated as the difference between the N_2O measured in the 5% and the 0.01% C_2H_2 treatments. The gas samples were analyzed with an Agilent 7890A gas chromatography system equipped with an ECD detector with the same parameters as in Experiment 1. Moreover, the mineral N content as nitrate and ammonium and the WSC were also determined prior to the incubation following the methodology described above.

Data analysis

326 The N_2O production data were transformed using the Box-Cox procedure and analyzed
327 using the SAS statistical software (SAS Institute Inc., 1990). To compare the effects of
328 fertilizer treatments and soil moisture on N_2O production an analysis of variance was
329 performed. When significant, differences among treatments were identified at the 0.1
330 probability level of significance using an LSD test. Furthermore, the linear relationship
331 between WSC and N_2O production was determined with the statistical package JMP 10
332 (SAS Institute Inc., 2012)

Results

Experiment 1: GHG production from soil macroaggregates under different tillage and N fertilization treatments.

Tillage significantly affected the fluxes of CH_4 produced by soil macroaggregates. As an average of all the samplings performed during the incubation period, macroaggregates of the CT treatment acted as emitters of CH_4 while those under NT acted as a CH_4 sink (Table 2). Also, significant differences on cumulative CH_4 fluxes were observed between CT and NT (Fig. 1a). According to the data, the methanotrophic activity in the NT treatment began after the first 72 hours of macroaggregate incubation (Fig. 1a). In contrast to CH_4, no significant differences were found between tillage systems on neither the fluxes nor the cumulative emissions of CO_2 and N_2O (Table 2, Fig. 1b and c).

Nitrogen fertilization treatments did not affect the fluxes of CH_4 and N_2O (Table 2). Also, cumulative emissions of CH_4 and N_2O did not differ between N fertilization treatments (Fig. 2a and c). CO_2 followed a different trend, with greater average fluxes in the organic treatment (1669.4 µg CO_2-C kg macroaggregates^{-1} h^{-1}) when compared with the control (1217.5 µg CO_2-C kg macroaggregates^{-1} h^{-1}) and the mineral (1199.4 µg CO_2-C kg macroaggregates^{-1} h^{-1}) treatments (Table 2). Also, cumulative CO_2 emissions were the greatest under the organic fertilization treatment in the first 48 hours of the incubation, without differences between the control and mineral treatments (Fig 2b). When the incubation was finished (i.e., after 840 hours), the organic treatment presented a greater cumulative CO_2 emission when compared with the mineral treatment, while the control presented intermediate values (Fig. 2b).

The interaction between tillage and N fertilization significantly affected the fluxes of CO_2 (Table 2). The different N fertilization treatments did not show different CO_2 fluxes for the NT macroaggregates, whereas the CT macroaggregates under organic fertilization emitted greater amount of CO_2 (1824.5 µg CO_2-C kg macroaggregates^{-1} h^{-1}) compared with the control (1021.4 µg CO_2-C kg macroaggregates^{-1} h^{-1}) and mineral (1155.1 µg CO_2-C kg macroaggregates^{-1} h^{-1}) fertilization treatments (Table 2).

No differences between tillage systems were found in the organic carbon (OC) concentration of dry-sieved macroaggregates before or after 840 hour incubation (Table 3). However, different results arose when analyzing the OC concentration in the soil macroaggregates under different N fertilization treatments. In this case, greater macroaggregate-C concentration was found in the organic fertilization treatment both before and after the incubation period when compared with the control and mineral treatments (Table 3). Nevertheless, the decrease in the OC concentration during the incubation was not statistically different between N fertilization treatments. Significant differences between tillage and N fertilization treatments were found on the initial NO_3^- concentration of the macroaggregates (Table 3). A greater initial NO_3^- concentration was found in the CT treatment than in the NT treatment, with 110.4 and 92.6 mg NO_3^--N kg^{-1} dry-sieved macroaggregates, respectively. In the case of N fertilization, the mineral treatment showed the greatest initial NO_3^- concentration while the control presented the smallest one and the organic treatment intermediate values. After the incubation period (i.e., 840 hours) the mineral and organic fertilization treatments showed greater NO_3^- concentration when compared with the control. Also, a significant interaction between tillage and N fertilization was found on this variable: while the macroaggregates of the control and organic fertilization treatments presented no significant differences between CT and NT, the macroaggregates of the CT treatment

fertilized with mineral N presented a greater amount of initial NO_3^- when compared to the ones of the NT treatment (Table 3). Significant differences between tillage and N fertilization treatments were also found on the NO_3^- concentration variation (0 vs. 840 hours). In this case, the NT-control treatment presented the greatest increase in the NO_3^- concentration in the macroaggregates, followed by the CT-control treatment (Table 3).

Differences between N fertilization treatments were also found on the initial NH_4^+-N concentration and its variation during the incubation process. The organic treatment presented the greatest values, followed by the mineral and the control treatments (Table 3). The reduction of the NH_4^+-N concentration during the incubation period was higher in the fertilized treatments (about 87% and 93% reduction in the NH_4^+ concentration in the mineral and the organic treatments, respectively) compared with the control (about 32% reduction) (Table 3). Furthermore, no differences between treatments were found on the MBC content. However, a greater MBN content was found in the organic treatment compared with the mineral and the control treatments (Table 3). The WSC content after the incubation process was significantly affected by both tillage and N fertilization treatments. Thus, a greater WSC content was found under NT than under CT and in the organic N treatment compared with the mineral and control ones (Table 3).

A highly significant polynomial relationship (r^2= 0.72; $p<0.001$) was observed between the initial NH_4^+ concentration in the macroaggregates and the cumulative N_2O-N emission during the first 48 hours of incubation (Fig. 3). Furthermore, significant linear relationships were observed between CO_2 and N_2O production in six samplings in CT and in three samplings in NT (Table 4).

At the end of the incubation period, a greater proportion of water-stable macroaggregates was quantified under NT compared with CT (Fig. 4). Moreover, a significant interaction ($P<0.05$) between tillage and N fertilization was found on the water-stability of macroaggregates. While under NT no differences between fertilization treatments were observed in the proportion of water-stable macroaggregates, under CT a greater proportion of water-stable aggregates was found in the organic treatment when compared with the mineral treatment, with intermediate values in the control (Fig. 4).

Experiment 2: Relative importance of nitrification and denitrification on N_2O production from soil macroaggregates under different N fertilization types.

No differences between N fertilization treatments were found on NH_4^+ concentration of the macroaggregates before the incubation (Table 5). In contrast, before the incubation process, the fertilized treatments (mineral and organic) presented a greater NO_3^- concentration in the macroaggregates when compared with the control (Table 5). Moreover, significant differences between N fertilization treatments were found on the WSC concentration with greater values in the organic fertilization treatment when compared with the mineral and the control ones (Table 5).

At the 15% moisture level, total losses of N as N_2O and N_2 during the incubation of the macroaggregates resulted in 173, 254 and 139 mg N kg^{-1} h^{-1} for the control, mineral and organic treatments, respectively (Table 6). In turn, at the 30% moisture level, N losses reached 4751, 5552 and 4922 mg N kg^{-1} h^{-1} for the control, mineral and organic treatments, respectively (Table 6). Both N fertilization and soil moisture content significantly affected the amount of N_2O-N produced due to the nitrification and denitrification processes (Table 6). The production of N_2O due to the nitrification process was greater in the mineral treatment when compared with the control, with

intermediate values in the organic treatment. Different results were obtained in the production of N_2O due to the denitrification process. In this case, the organic treatment showed greater values than the control, while intermediate values were found in the mineral treatment (Table 6). Nevertheless, the production of N_2 due to a complete denitrification process was only affected by soil moisture, with the greatest values in the 30% moisture treatment when compared with the 15% moisture treatment (Table 6). Moreover, the production of N_2O due to nitrification and denitrification processes was 4.3 and 7.3 times greater in the 30% than in the 15% moisture treatment, respectively (Table 6). No significant relationship was found between WSC and the amount of N_2O produced during the denitrification process (data not shown).

Discussion

Effects of tillage and N fertilization on GHG production from soil macroaggregates

CH_4 was the only greenhouse gas produced by the macroaggregates that presented significant differences between tillage treatments. In the CT treatment macroaggregates acted as CH_4 producers, whereas macroaggregates of the NT treatment oxidized CH_4 mainly from the first 72 hours until the end of the incubation. Methanotrophic activity is reduced by anoxic conditions. In our experiment, an equal amount of water was added to the macroaggregates of both tillage treatments to bring them to the field capacity of undisturbed soil in our field experiment. Therefore, it could be hypothesized that differences in the intra-aggregate pore architecture and connectivity could have maintained a higher amount of aerobic microsites within the NT macroaggregates, thus facilitating the oxidation of CH_4. This hypothesis is in line with the findings of Kravchenko et al. (2013) who studied the effects of tillage on the intra-aggregate porosity of macroaggregates and observed higher intra-aggregate porosity >100 μm in NT macroaggregates when compared with CT macroaggregates. Another hypothesis could be the influence of the different types of tillage on the diversity of microorganisms within macroaggregates, which could have maintained a greater amount of methanotrophs in the NT treatment.

According to our results, no differences between tillage systems were found on the amount of macroaggregate-C mineralized as CO_2. That result could be related to the absence of differences in macroaggregate-C concentration prior and after the incubation. Different results were obtained by Fernández et al. (2010) when using soil of a long-term (14-yr) experiment. These authors observed higher production of CO_2 by NT macroaggregates when compared with CT macroaggregates and related this finding

462 to the higher amount of organic C in the NT macroaggregates. Thus, in our experiment,
463 the similar macroaggregate-C concentration found in the CT and NT treatments would
464 have influenced the lack of differences in CO_2 production by macroaggregates.

465 Similarly to CO_2 production, no differences between tillage treatments were found on
466 the fluxes and cumulative emissions of N_2O by soil macroaggregates. Although a higher
467 initial NO_3^- concentration susceptible to denitrification and a greater reduction in the
468 concentration of NH_4^+ during the incubation were found under CT, no greater MBN
469 content was found in this treatment when compared with NT. For that reason, the
470 hypothesis of a greater N immobilization under CT was not supported by our data.
471 Another hypothesis could be a greater or complete denitrification under CT, in which
472 the mineral N would be emitted as N_2. It is known that the $N_2/(N_2 + N_2O)$ ratio
473 increases with decreasing O_2 concentration (Tiedje, 1988). That hypothesis would be in
474 line with the smaller intra-aggregate porosity described above and the related higher
475 anaerobic conditions in CT macroaggregates.

476 Wrage et al. (2001) suggested that a greater soil organic matter content and better
477 aggregate structure could facilitate O_2 diffusion, thus reducing the production of N_2O in
478 NT soils. In our experiment, we found a greater water-stability of macroaggregates
479 under the NT treatment when compared with the CT treatment as it has also been
480 observed in other studies in the Mediterranean area (Álvaro-Fuentes et al., 2009; Plaza-
481 Bonilla 2010, 2013b). However, the greater macroaggregate water-stability under NT
482 was not followed by a lower production of N_2O as suggested by Wrage et al. (2001).
483 Extrapolating our results to a structured soil, the greater macroaggregate water-stability
484 found under NT could imply a more interconnected porous space in the soil matrix. This
485 could lead to a greater aeration and reduced N_2O emissions in NT when compared with
486 CT.

Macroaggregate CO_2 emissions were influenced by fertilization type: when the incubation was finished (i.e., after 840 hours), the macroaggregate CO_2 losses under the organic fertilization treatment were higher than under the mineral fertilization treatment. Moreover, the interaction between tillage and N fertilization types also affected the CO_2 produced by macroaggregates. Thus, under CT the CO_2 emissions were greater with the use of organic fertilizer compared with either the use of mineral fertilizers or in absence of fertilization. The application of pig slurry usually enhances the amount of readily decomposable C compounds in the soil (Arcara et al., 1999; Sánchez-Martín et al., 2008; Yang et al., 2003). This fact was observed in our experiment, in which a higher WSC content was measured in the macroaggregates of the pig slurry treatment. A similar trend was observed in macroaggregate water-stability. In this case, unlike the CT treatment, the NT treatment did not show an interaction with the type of fertilizer on macroaggregate water-stability. Contrarily, the application of organic fertilizer under CT led to greater proportion of water-stable macroaggregates than the control treatment. These findings suggest that the use of NT buffers the effects of the application of organic fertilizers on the increase of macroaggregate stability (Plaza-Bonilla et al., 2013a).

We found significant linear relationships between the cumulative CO_2 production and (i) the macroaggregate-C concentration (R^2: 0.21; *P*: 0.016) and (ii) the proportion of water-stable macroaggregates (R^2: 0.19; *P*: 0.021). However, when both variables (i.e., macroaggregate-C concentration and proportion of water-stable macroaggregates) were included in a stepwise procedure in order to analyze their relationship with CO_2 production, no statistical significance was found. This finding suggests that the relationship found between CO_2 production and macroaggregate stability was due to a

511 greater C concentration in those macroaggregates that are more water-stable resulting in
512 a greater production of CO_2.

513 In contrast to tillage, the different N fertilization treatments had no significant effects on
514 CH_4 emission. However, a trend (not significant) to CH_4 uptake could be observed in
515 Figure 2a in the control treatment and near zero emissions in the organic treatment.
516 Ammonium has been reported to be a competitive inhibitor of CH_4 oxidation
517 (Whittenbury et al., 1970). Interestingly, the uptake of CH_4 that we observed in the NT
518 and control treatments began after the first 72 hours of incubation and coincided with
519 the reduction in the rate of N_2O emissions. Hütsch (1998b) pointed out that CH_4
520 metabolism only begins when the nitrification process is almost completed. That
521 conclusion would explain the time-lapse that we found until the CH_4 uptake began in
522 the NT and control treatments.

523 In our experiment, fertilization type did not lead to differences in the N_2O produced by
524 soil macroaggregates. However, a trend to lower emissions under the control treatment
525 and higher emissions under the organic fertilization with pig slurry was observed (Fig.
526 2c). It is already known that the denitrification process is intensified under the presence
527 of easily decomposable C fractions such as WSC (Arcara et al., 1999). Thus, the
528 application of organic wastes, such as animal manure, usually enhances N_2O emissions
529 when compared with inorganic fertilizers (Heller et al., 2010) due to their easily
530 decomposable C content and sufficient mineral N to activate the population of
531 denitrifiers in soil (Johnson et al., 2007; Sánchez-Martín et al., 2008). Our results show
532 the relationship between the initial ammonium concentration in soil macroaggregates
533 and their N_2O production. That relationship could be explained by the role played by the
534 NH_4^+ ion in the nitrification and denitrification processes. Ammonium oxidation is the

first step in the nitrification process that produces NO_3^-, which in turn is the most important ion involved in the denitrification process.

N_2O production by soil macroaggregates as affected by the type of N fertilization

At 15% soil moisture, the nitrification process was the predominant N_2O producer, while at 30% soil moisture the denitrification process emitted nine times more N (as the sum of N_2O and N_2) than nitrification. These results agree with the conceptual model developed by Bouwman (1998) about N_2O emissions fractionation from nitrification and denitrification processes as a function of water-filled pore space. Although in small amounts, the denitrification process lead to N_2O production in the macroaggregates incubated at 15% soil moisture content. This could be related with the presence of anaerobic microsites within the macroaggregates. Sexstone et al. (1985) quantified oxygen profiles in wet aggregates and found anaerobic centers in all the aggregates that denitrified. However, aerobic denitrification could have also occurred. As in other studies (Bandibas et al., 1994; Diba et al., 2011; Liu et al., 2007), we observed an important increase of the N_2O evolved when doubling soil moisture. This finding demonstrates the role played by the absence of oxygen as electron acceptor on nitrification and denitrification processes (Bouwman, 1998).

The combination of higher NO_3^- concentration and greater WSC in the organic N treatment would explain the greater amount of N_2O evolved due to the denitrification process when compared to the control treatment (Burford and Bremner, 1975; Mulvaney et al., 1997). Contrarily, the greater N_2O loss from nitrification in the mineral N treatment cannot be explained by a higher NH_4^+ concentration before the incubation, a fact that could be related to a greater organic N mineralization during the incubation that could have increased the amount of mineral N susceptible of being nitrified.

559 Different authors have observed a greater mineralization in N fertilized soils compared
560 with soils without N fertilization (Hatch et al., 2000; Zhang et al., 2012). Another
561 hypothesis could be a more efficient nitrification process in the control treatment in
562 comparison with the mineral treatment that would explain the lower N_2O emissions
563 found in the control. Nemergut et al. (2008) and Ramirez et al. (2012) found changes in
564 soil microbial community when using repeated application of mineral fertilizer when
565 compared to unfertilized soils. Thus, it could be hypothesized that the mineral fertilizer
566 applications in our field experiment could have led to changes in the microbial
567 community structure with higher nitrification efficiency in the unfertilized treatment.
568 Furthermore, our data shows a similar N_2O emission in the mineral and the organic
569 fertilization treatments. This suggests that readily decomposable C was not a limiting
570 factor to denitrification in the mineral treatment. The lack of differences between
571 fertilization treatments on macroaggregate N_2O production after the first 24 hours of
572 incubation in Experiment 1 corroborates this hypothesis.

Conclusions

Tillage and N fertilization treatments affected the production of GHG at the soil macroaggregate scale due to changes in C and N substrates within macroaggregates. Moreover, the different methanogenic and methanotrophic activities found in the tillage treatments suggest changes in porosity and anaerobic conditions within soil macroaggregates when either conventional tillage or no-tillage are used. Easily decomposable C compounds associated with the organic fertilization together with the presence of nitrate stimulated the denitrifying activity. The use of mineral and organic fertilizers leads to differences in the relative importance of the nitrification and the denitrification processes in the production of N_2O by soil macroaggregates: while N_2O losses due to the nitrification process were preponderant in the mineral fertilization treatment, denitrification N_2O losses had a higher importance under organic fertilization due to a higher presence of C-labile compounds. A significant effect of the interaction between tillage and N fertilization treatments on CO_2 production, with higher emissions under CT when applying organic fertilizers and no differences between types of fertilizers on CO_2 emissions under NT, demonstrated the capacity of NT aggregates to protect C. Our study shows that tillage and N fertilization and their interaction play a major role in GHG production from soil macroaggregates due to their impact on the soil mineral and organic substrates that regulate the microbial activity.

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601 **References**

602 Álvaro-Fuentes, J., Arrúe, J.L., Cantero-Martínez, C., López, M.V., 2008. Aggregate
603 breakdown during tillage in a Mediterranean loamy soil. Soil & Tillage Research 101,
604 62-68.

605 Álvaro-Fuentes, J., Cantero-Martínez, C., López, M.V., Paustian, K., Denef, K.,
606 Stewart, C., Arrúe, J.L., 2009. Soil aggregation and soil organic carbon stabilization:
607 effects of management in semiarid Mediterranean agroecosystems. Soil Science Society
608 of America Journal 73, 1519-1529.

609 Ananyeva, K., Wang, W., Smucker, A.J.M., Rivers, M.L., Kravchenko, A.N., 2013.
610 Can intra-aggregate pore structures affect the aggregate's effectiveness in protecting
611 carbon? Soil Biology & Biochemistry 57, 868-875.

612 Arcara, P.G., Gamba, C., Bidini, D., Marchetti, R., 1999. The effect of urea and pig
613 slurry on denitrification, direct nitrous oxide emission, volatile fatty acids, water-soluble
614 and anthrone-reactive carbon in maize-cropped soil from the Po plain (Modena, Italy).
615 Biology and Fertility of Soils 29, 270-276.

616 Baggs, E.M., 2008. A review of stable isotope techniques for N_2O source partitioning in
617 soils: recent progress, remaining challenges and future considerations. Rapid
618 Communications in Mass Spectrometry 22, 1664-1672.

619 Ball, B.C., Scott, A., Parker, J.P., 1999. Field N(2)O, CO(2) and CH(4) fluxes in
620 relation to tillage, compaction and soil quality in Scotland. Soil & Tillage Research 53,
621 29-39.

622 Bandibas, J., Vermoesen, A., Degroot, C.J., Vancleemput, O., 1994. The effect of
623 different moisture regimes and soil characteristics on nitrous-oxide emission and
624 consumption by different soils. Soil Science 158, 106-114.

625 Beare, M.H., Cabrera, M.L., Hendrix, P.F., Coleman, D.C., 1994. Aggregate-protecte
626 and unprotected organic-matter pools in conventional-tillage and no-tillage soils. Soil
627 Science Society of America Journal 58, 787-795.

628 Blackmer, A.M., Bremner, J.M., Schmidt, E.L., 1980. Production of N_2O by ammonia-
629 oxidizing chemoautotrophic microorganisms in soil. Applied and Environmental
630 Microbiology 40, 1060-1066.

631 Bodelier, P.L.E., Laanbroek, H.J., 2004. Nitrogen as a regulatory factor of methane
632 oxidation in soils and sediments. FEMS Microbiology Ecology 47, 265-277.

633 Bouwman, A.F., 1998. Environmetal science – Nitrogen oxides and tropical agriculture.
634 Nature 392, 866-867.

635 Burford, J.R., Bremner, J.M., 1975. Relationships between denitrification capacities of
636 soils and total, water-soluble and readily decomposable soil organic-matter. Soil
637 Biology & Biochemistry 7, 389-394.

638 Conrad, R., 1996. Soil microorganisms as controllers of atmospheric trace gases (H_2,
639 CO, CH_4, OCS, N_2O, and NO). Microbiological Reviews 60, 609-640.

640 Davidson, E.A., Swank, W.T., Perry, T.O., 1986. Distinguishing between nitrification
641 and denitrification as sources of gaseous nitrogen production in soil. Applied
642 Environmental Microbiology 52, 1280-1286.

643 Diba, F., Shimizu, M., Hatano, R., 2011. Effects of soil aggregate size, moisture content
644 and fertilizer management on nitrous oxide production in a volcanic ash soil. Soil
645 Science and Plant Nutrition 57, 733-747.

646 Drury, C.F., Yang, X.M., Reynolds, W.D., Tan, C.S., 2004. Influence of crop rotation
647 and aggregate size on carbon dioxide production and denitrification. Soil & Tillage
648 Research 79, 87-100.

649 Elliott, E.T., 1986. Aggregate structure and carbon, nitrogen and phosphorus in native
650 and cultivated soils. Soil Science of America Journal 50, 627-633.

651 Estavillo, J.M., Merino, P., Pinto, M., Yumulki, S., Gebauer, G., Sapek, A., Corré, W.,
652 2002. Short term effect of ploughing a permanent pasture on N_2O production from
653 nitrification and denitrification. Plant and Soil 239, 253-265.

654 Fernández, R., Quiroga, A., Zorati, C., Noellemeyer, E., 2010. Carbon contents and
655 respiration rates of aggregate size fractions under no-till and conventional tillage. Soil &
656 Tillage Research 109, 103-109.

657 Firestone, M.K., Firestone, R.B., Tiedje, J.M., 1980. Nitrous oxide from soil
658 denitrification: factors controlling its biological production. Science 208, 749-751.

659 Goulding, K.E.T., Hütsch, B.W., Webster, C.P., Willison, T.W., Powlson, D.S., 1995.
660 The effects of agriculture on methane oxidation in the soil. Philosophical Transactions
661 of the Royal Society A-Mathematical Physical and Engineering Sciences 351, 313-325.

662 Groffman, P.M., Altabet, M.A., Bohlke, J.K., Butterbach-Bahl, K., David, M.B.,
663 Firestone, M.K., Giblin, A.E., Kana, T.M., Nielsen, L.P., Voytek, M.A., 2006. Methods
664 for measuring denitrification: Diverse approaches to a difficult problem. Ecological
665 Applications 16, 2091-2122.

Gupta, V.V.S.R., Germida, J.J., 1988. Distribution of microbial biomass and its activity in different soil aggregate size classes as affected by cultivation. Soil Biology & Biochemistry 20, 777-786.

Hatch, D.J., Lovell, R.D., Antil, R.S., Jarvis, S.C., Owen, P.M., 2000. Nitrogen mineralization and microbial activity in permanent pastures amended with nitrogen fertilizer or dung. Biology and fertility of Soils 30, 288-293.

Heller, H., Bar-Tal, A., Tamir, G., Bloom, P., Venterea, R.T., Chen, D., Zhang, Y., Clapp, C.E., Fine, P., 2010. Effects of manure and cultivation on carbon dioxide and nitrous oxide emissions from a corn field under Mediterranean conditions. Journal of Environmental Quality 39, 437-448.

Holland, E.A., Robertson, G.P., Greenberg, J., Groffman, P.M., Boone, R.D., Gosz, J.R., 1999. Soil CO2, N2O and CH4 exchange. In: Roberston, G.P., Coleman, D.C., Bledsoe, C.S., Sollins, P. (Eds.) Standard soil methods for long-term ecological research. Oxford University Press, New York. pp. 185-201.

Hütsch, B.W., 1998a. Tillage and land use effects on methane oxidation rates and their vertical profiles in soil. Biology and Fertility of Soils 27, 284-292.

Hütsch, B.W., 1998b. Methane oxidation in arable soil as inhibited by ammonium, nitrite, and organic manure with respect to soil pH. Biology and Fertility of Soils 28, 27-35.

Hütsch, B.W., Webster, C.P., Powlson, D.S., 1993. Long-term effects on nitrogen fertilization on methane oxidation in soil of the Broadbalk wheat experiment. Soil Biology & Biochemistry 25, 1307-1315.

688 Johnson, J.M.F., Franzluebbers, A.J., Weyers, S.L., Reicosky, D.C., 2007. Agricultural
689 opportunities to mitigate greenhouse gas emissions. Environmental Pollution 150, 107-
690 124.

691 Kessavalou, A., Doran, J.W., Mosier, A.R., Drijber, R.A. 1998. Greenhouse gas fluxes
692 following tillage and wetting in a wheat-fallow cropping system. Journal of
693 Environmental Quality 27, 1105-1116.

694 Kimura, S.D., Melling, L., Goh, K.J., 2012. Influence of soil aggregate size on
695 greenhouse gas emission and uptake rate from tropical peat soil in forest and different
696 oil palm development years. Geoderma 185, 1-5.

697 Klemedtsson, L., Svensson, B.H., Rosswall, T., 1988. A method of selective inhibition
698 to distinguish between nitrification and denitrification as sources of nitrous oxide in
699 soil. Biology and Fertility of Soils 6, 112-119.

700 Kravchenko, A.,, Chun, H.C., Mazer, M., Wang, W., Rose, J.B., Smucker, A., Rivers,
701 M., 2013. Relationships between intra-aggregate pore structures and distributions of
702 Escherichia coli within soil macroaggregates. Applied Soil Ecology 63, 134-142.

703 Lenka, N.K., Lal, R., 2013. Soil aggregation and greenhouse flux after 15 years of
704 wheat straw and fertilizer management in a no-till system. Soil & Tillage Research 126,
705 78-89.

706 Liu, X.J., Mosier, A.R., Halvorson, A.D., Reule, C.A., Zhang, F.S., 2007. Dinitrogen
707 and N_2O emissions in arable soils: Effects of tillage, N source and soil moisture. Soil
708 Biology & Biochemistry 39, 2362-2370.

709 Mebius, L.J., 1960. A rapid method for the determination of organic carbon in soil.
710 Analytica Chimica Acta 22, 120-124.

711 Miller, M.N., Zebarth, B.J., Dandie, C.E., Burton, D.L., Goyer, C., Trevors, J.T., 2009.
712 Denitrifier community dynamics in soil aggregates under permanent grassland and
713 arable cropping systems. Soil Science Society of America Journal 73, 1843-1851.

714 Morvan, T., Nicolardot, B., 2009. Role of organic fractions on C decomposition and N
715 mineralization of animal wastes in soil. Biology and Fertility of Soils 45, 477-486.

716 Mosier, A., Schimel, D., Valentine, D., Bronson, K., Parton, W., 1991. Methane and
717 nitrous oxide fluxes in native, fertilized and cultivated grasslands. Nature 350, 330-332.

718 Mulvaney, R.L., Khan, S.A., Mulvaney, C.S., 1997. Nitrogen fertilizers promote
719 denitrification. Biology and Fertility of Soils 24, 211-220.

720 Nelson, D.W., Sommers, L.E., 1996. Total carbon, organic carbon and organic matter.
721 In: Methods of soil analysis. Part 3. Chemical methods. ASA and SSSA, Madison, WI.
722 P. 961-1010.

723 Nemergut, D.R., Townsend, A.R., Sattin, S.R., Freeman, K.R., Fierer, N., Neff, J.C.,
724 Bowman, W.D., Schadt, C.W., Weintrub, M.N., Schmidt, S.K., 2008. The effects of
725 chronic nitrogen fertilization on alpine tundra soil microbial communities: implications
726 for carbon and nitrogen cycling. Environmental Microbiology 10, 3093-3105.

727 Parkin, T.B., 1987. Soil microsites as a source of denitrification variability. Soil Science
728 Society of America Journal 51, 1194-1199.

729 Plaza-Bonilla, D., Cantero-Martínez, C., Álvaro-Fuentes, J., 2010. Tillage effects on
730 soil aggregation and soil organic carbon profile distribution under Mediterranean semi-
731 arid conditions. Soil Use and Management 26, 465-474.

732 Plaza-Bonilla, D., Álvaro-Fuentes, J, Cantero-Martínez, C., 2013a. Soil aggregate
733 stability as affected by fertilization type under semiarid no-tillage conditions. Soil
734 Science Society of America Journal 77, 284-292.

735 Plaza-Bonilla, D., Cantero-Martínez, C., Viñas, P., Álvaro-Fuentes, J., 2013b. Soil
736 aggregation and organic carbon protection in a no-tillage chronosequence under
737 Mediterranean conditions. Geoderma 193-194, 76-82.

738 Poth, M., Focht, D.D., 1985. ^{15}N kinetic analysis of N_2O production by *Nitrosomonas*
739 *europaea*: an examination of nitrifier denitrification. Applied and Environmental
740 Microbiology 49, 1134-1141.

741 Ramirez, K.S., Craine, J.M., Fierer, N., 2012. Consistent effects of nitrogen
742 amendments on soil microbial communities and processes across biomes. Global
743 Change Biology 18, 1918-1927.

744 Sánchez-Martín, L., Vallejo, A., Dick, J., Skiba, U.M., 2008. The influence of soluble
745 carbon and fertilizer nitrogen on nitric oxide and nitrous oxide emissions from two
746 contrasting agricultural soils. Soil Biology & Biochemistry 40, 142-151.

747 SAS Institute Inc., 1990. SAS user's guide, statistics, 6th edn. Vol. 2. SAS Insititute,
748 Cary, NC.

749 SAS Institute Inc., 2012. Using JMP 10. SAS Insititute, Cary, NC.

750 Saxton, K.E., Rawls, W.J., 2006. Soil water characteristic estimates by texture and
751 organic matter for hydrologic solutions. Soil Science Society of America Journal 70,
752 1569-1578.

753 Sexstone, A.J., Revsbech, N.P., Parkin, T.B., Tiedje, J.M., 1985. Direct measurement of
754 oxygen profiles and denitrification rates in soil aggregates. Soil Science Society of
755 America Journal 49, 645-651.

756 Sey, B.K., Manceur, A.M., Whalen, J.K., Gregorich, E.G., Rochette, P., 2008. Small-
757 scale heterogeneity in carbon dioxide, nitrous oxide and methane production from
758 aggregates of a cultivated sandy-loam soil. Soil Biology & Biochemistry 40, 2468-
759 2473.

760 Shmidt, M.W.I., Torn, M.S., Abiven, S., Dittmar, T., Guggenberger, G., Jannssens, I.A.,
761 Kleber, M., Knögel-Knabner, I., Lehmann, J., Manning, D.A.C., Nannipieri, P., Rasse,
762 D.P., Weiner, S., Trumbore, S.E., 2011. Persistence of soil organic matter as an
763 ecosystem property. Nature 478, 49-56.

764 Soil Survey Stuff, 1994. Keys to Soil Taxonomy. United States Department of
765 Agriculture, Soil Conservation Service, Washington, USA. (306 pp).

766 Sparling, G., Zhu, C.Y., 1993. Evaluation and calibration of biochemical methods to
767 measure microbial biomass-C and biomass-N in soils from Western Australia. Soil
768 Biology & Biochemistry 25, 1793-1801.

769 Steudler, P.A., Bowden, R.D., Melillo, J.M., Aber, J.D., 1989. Influence of nitrogen
770 fertilization on methane uptake in forest soils. Nature 341, 314-316.

771 Tiedje, J.M., 1988. Ecology of denitrification and dissimilatory nitrate reduction to
772 ammonium. In: Zehnder, A,J.B. (ed.) Biology of Anaerobic Microorganisms. New
773 York. Wiley 179-244.

774 Tisdall, J.M., Oades, J.M., 1982. Organic-matter and water-stable aggregates in soils.
775 1982. Journal of Soil Science 33, 141-163.

776 Vance, E.D., Brookes, P.D., Jenkinson, D.S., 1987. An extraction method for measuring
777 soil microbial biomass-C. Soil Biology & Biochemistry 19, 703-707.

778 Whittenbury, R., Phillips, K.C., Wilkinson, J.K., 1970. Enrichment, isolation and some
779 properties of methane utilizing bacteria. Journal of General Microbiology 61, 205-218.

780 Wrage, N., Velthof, G.L, van Beusichem, M.L., Oenema, O., 2001. Role of nitrifier
781 denitrification in the production of nitrous oxide. Soil Biology & Biochemistry 33,
782 1723-1732.

783 Yang, X.M., Drury, C.F., Reynolds, W.D., Tan, C.S., McKenney, D.J., 2003.
784 Interactive effects of composts and liquid pig manure with added nitrate on soil carbon
785 dioxide and nitrous oxide emissions from soil under aerobic and anaerobic conditions.
786 Canadian Journal of Soil Science 83, 343-352.

787 Yoshinari, T., Haynes, R., Knowles, R., 1977. Acetylene inhibition of nitrous oxide
788 reduction and measurement of denitrification and nitrogen fixation in soil. Soil Biology
789 & Biochemistry 9, 177-183.

790 Young, I.M., Ritz, K., 2000. Tillage, habitat space and function of soil microbes. Soil &
791 Tillage Research 53, 201-213.

792 Zhang, J., Cai, Z., Yang, W., Zhu, T., Yu, Y., Yan, X., Jia, Z., 2012. Long-term field
793 fertilization affects soil nitrogen transformations in a rice-wheat-rotation cropping
794 system. Journal of Plant Nutrition and Soil Science 175, 939-946.

795 **Figure captions**

796 **Fig. 1** Cumulative CH_4 (A), CO_2 (B) and N_2O (C) production from dry-sieved
797 macroaggregates (0.250-8 mm) as affected by tillage (CT, conventional tillage; NT, no-
798 tillage). * For each sampling time, values are significantly different at $P<0.05$.

799 **Fig. 2** Cumulative CH_4 (A), CO_2 (B) and N_2O (C) production from dry-sieved
800 macroaggregates (0.250-8 mm) as affected by N fertilization (0, control; mineral N at
801 150 kg N ha^{-1} and organic N with pig slurry at 150 kg N ha^{-1}). For each sampling time
802 different letters indicate significant differences between N fertilization treatments at
803 $P<0.05$.

804 **Fig. 3** Regression analysis between the initial concentration of NH_4^+-N and the
805 cumulative N_2O-N emissions after 48 hours of incubation of dry-sieved
806 macroaggregates (0.250-8 mm).

807 **Fig. 4** Proportion of sand-free water-stable macroaggregates (0.250-8 mm) as affected
808 by tillage (CT, conventional tillage; NT, no-tillage) and N fertilization treatments
809 (control without fertilization; mineral at 150 kg N ha^{-1}; organic at 150 kg N ha^{-1}) at the
810 end of the incubation period (after 840 hours). Different letters indicate significant
811 differences between tillage and fertilization treatments at $P<0.05$. * Indicate significant
812 differences between tillage treatments at $P<0.05$.

Table 1 General characteristics of the experimental site. Soil properties were measured in the Ap horizon (0-30 cm depth) at the beginning of the experiment.

Elevation (masl)	395
Mean air temperature (ºC)	13.4
Annual precipitation (mm)	327
Annual ETo (mm)	1197
Soil classification†	Typic calcixerept
pH (H_2O, 1:2.5)	8.0
Organic C (g kg^{-1})	15.6
Organic N (g kg^{-1})	1.4
EC 1:5 (dS m^{-1})	1.0
CaCO3 eq. (%)	28.9
Particle size distribution (%)	
Sand (2000-50 µm)	6.2
Silt (50-2 µm)	63.3
Clay (<2 µm)	30.5

†According to the USDA classification (Soil Survey Staff, 1994).

817 **Table 2** Analysis of variance of the fluxes of CH_4, CO_2 and N_2O from dry-sieved
818 macroaggregates (μg CH_4-C, CO_2-C and N_2O-N kg^{-1} macroaggregates h^{-1}, respectively)
819 as affected by tillage (CT, conventional tillage; NT, no-tillage), N fertilization
820 treatments (0, control; mineral N at 150 kg N ha^{-1} and organic N with pig slurry at 150
821 kg N ha^{-1}), sampling time, and their interactions. Values are the means of all samplings
822 (0, 4, 12, 24, 48, 72, 192, 384, 504, 672 and 840 hours after the beginning of the
823 incubation).

	CH_4 fluxes	CO_2 fluxes	N_2O fluxes
Tillage (T)	*	n.s.	n.s.
CT	0.073 a¶	1333.67	0.754
NT	-0.207 b	1406.19	0.919
N fertilization (N)	n.s.	*	n.s.
Control	-0.175	1217.45 b	0.556
Mineral	0.074	1199.36 b	0.866
Organic	-0.096	1669.39 a	1.042
Sampling time (t)	**	***	***
T x N	n.s.	*	n.s.
CT-Control	0.046	1021.40 d	0.458
CT-Mineral	0.214	1155.13 dc	0.676
CT-Organic	-0.040	1824.48 a	1.127
NT-Control	-0.470	1478.84 ab	0.686
NT-Mineral	-0.066	1243.58 bcd	1.056
NT-Organic	-0.153	1514.30 abc	0.956
T x t	***	n.s.	n.s.
N x t	n.s.	*	n.s.
N x T x t	n.s.	n.s.	n.s.

824

825 n.s.: not significant; *P<0.05; **P<0.01; *** P<0.001

826 ¶ For each gas and treatment, different letters indicate significant differences between
827 treatments at P<0.05.

Table 3 Organic C concentration (OC, g kg^{-1}) and mineral N content (nitrate, NO_3^-, and ammonium, NH_4^+, in mg kg^{-1}) of dry-sieved macroaggregates (0.250-8 mm) before (0 hours) and after (840 hours) incubation, microbial biomass C and N (MBC and MBN, respectively; mg C or N kg^{-1}) and water-soluble C (WSC; mg C kg^{-1}) after the incubation (840 hours), and % of variation of C, nitrate and ammonium during the incubation as affected by tillage (CT, conventional tillage; NT, no-tillage) and N fertilization treatments (0, control; mineral N at 150 kg N ha^{-1}; and organic N with pig slurry at 150 kg N ha^{-1}), and their interaction.

Treatment	0 hours			840 hours						% variation 840-0 hours		
	OC	NO_3^-	NH_4^+	OC	NO_3^-	NH_4^+	MBC	MBN	WSC	OC	NO_3^-	NH_4^+
CT	6.07 (0.8)	110.4 (83.1) a¶	20.1 (15.1)	5.78 (0.8)	147.9 (72.7)	1.7 (0.7)	865.8 (232.4)	228.2 (115.6)	197.2 (43.1) b	-6.41 (3.7)	59.0 (68.9) b	-79.0 (28.9) b
NT	6.16 (0.9)	92.6 (56.0) b	13.3 (12.4)	5.72 (0.8)	141.9 (58.3)	2.0 (0.4)	954.4 (236.9)	236.5 (142.5)	234.9 (71.9) a	-7.78 (5.6)	100.8 (92.5) a	-62.3 (35.9) a
Control	5.54 (0.4) b	24.1 (4.5) c	3.0 (0.7) c	5.21 (0.6) b	87.3 (56.9) b	2.0 (0.8)	893.0 (278.4)	177.0 (70.98) b	199.5 (16.7) b	-6.07 (5.1)	182.2 (58.8) a	-32.5 (30.6) a
Mineral	5.94 (0.9) b	180.0 (43.4) a	19.0 (11.5) b	5.46 (0.5) b	183.6 (66.3) a	1.8 (0.5)	977.9 (213.1)	182.6 (96.5) b	174.3 (33.3) b	-7.61 (5.3)	7.1 (37.9) c	-86.8 (10.4) b
Organic	6.87 (0.5) a	100.4 (16.4) b	28.1 (11.7) a	6.60 (0.6) a	163.6 (12.3) a	1.7 (0.4)	859.4 (219.9)	337.5 (140.1) a	274.3 (68.5) a	-8.05 (3.8)	65.9 (22.9) b	-92.7 (4.4) b
CT-Control	5.51 (0.2)	26.0 (5.5) d	3.4 (0.8)	5.19 (0.2)	104.3 (82.2)	1.9 (1.3)	688.3 (81.3)	123.2 (44.7)	191.7 (19.0)	-5.87 (3.3)	132.2 (30.2) b	-49.2 (35.7)
CT-Mineral	5.62 (0.3)	214.4 (28.7) a	19.5 (4.2)	5.29 (0.1)	179.5 (92.4)	1.3 (0.2)	1023.2 (272.6)	208.9 (50.9)	156.5 (18.4)	-5.61 (4.7)	-13.8 (45.8) e	-93.0 (1.1)
CT-Organic	7.08 (0.4)	90.9 (8.3) c	37.4 (6.4)	6.86 (0.5)	159.9 (6.1)	1.9 (0.2)	885.9 (203.6)	352.6 (92.6)	243.4 (31.7)	-9.08 (2.2)	77.0 (16.6) c	-94.8 (0.8)
NT-Control	5.56 (0.6)	22.2 (2.6) d	2.5 (0.3)	5.23 (0.8)	70.4 (5.8)	2.1 (0.1)	1097.8 (249.9)	230.8 (45.1)	207.3 (11.3)	-6.27 (7.0)	219.7 (43.9) a	-15.7 (13.0)
NT-Mineral	6.27 (1.2)	145.7 (20.7) b	18.6 (17.0)	5.62 (0.8)	187.8 (40.7)	2.3 (0.1)	932.5 (161.6)	156.2 (131.5)	192.1 (37.5)	-9.61 (5.6)	28.1 (10.1) de	-80.5 (12.1)
NT-Organic	6.66 (0.6)	109.9 (17.8) c	18.7 (6.5)	6.34 (0.6)	167.4 (16.7)	1.6 (0.5)	832.9 (263.6)	322.5 (191.4)	305.2 (86.0)	-7.36 (5.0)	54.7 (24.8) cd	-90.5 (5.6)

¶ For each variable, different letters indicate significant differences between treatments at $P<0.05$. Values between parentheses are the standard deviations of the mean.

835 **Table 4** R^2 coefficients of the linear relationships between carbon dioxide (CO_2) and nitrous oxide (N_2O) production in conventional tillage (CT)
836 and no-tillage (NT) macroaggregates at different times of the incubation period.

Tillage system	Sampling (hours)										
	0	4	12	24	48	72	192	384	504	672	840
CT	n.s.	0.71***	0.56**	0.61**	0.76***	0.33*	0.40*	n.s.	n.s.	n.s.	n.s.
NT	n.s.	0.72***	n.s.	n.s.	n.s.	n.s.	0.62**	n.s.	n.s.	n.s.	0.78***

837

838 n.s.: not significant; *P<0.05; **P<0.01; ***P<0.001

839 **Table 5** Mineral N content (nitrate, NO_3^-, and ammonium, NH_4^+, in mg kg^{-1}) and water-
840 soluble C (WSC; mg C kg^{-1}) of dry-sieved macroaggregates (0.250-8 mm) before
841 incubation, as affected by N fertilization treatments (0, control; mineral N at 150 kg N
842 ha^{-1}, and organic N with pig slurry at 150 kg N ha^{-1}).

Treatments	NH_4^+	NO_3^-	WSC
Control	1.88	20.19 b¶	90.08 b
Mineral	2.13	85.22 a	92.45 b
Organic	2.58	88.96 a	114.47 a

843

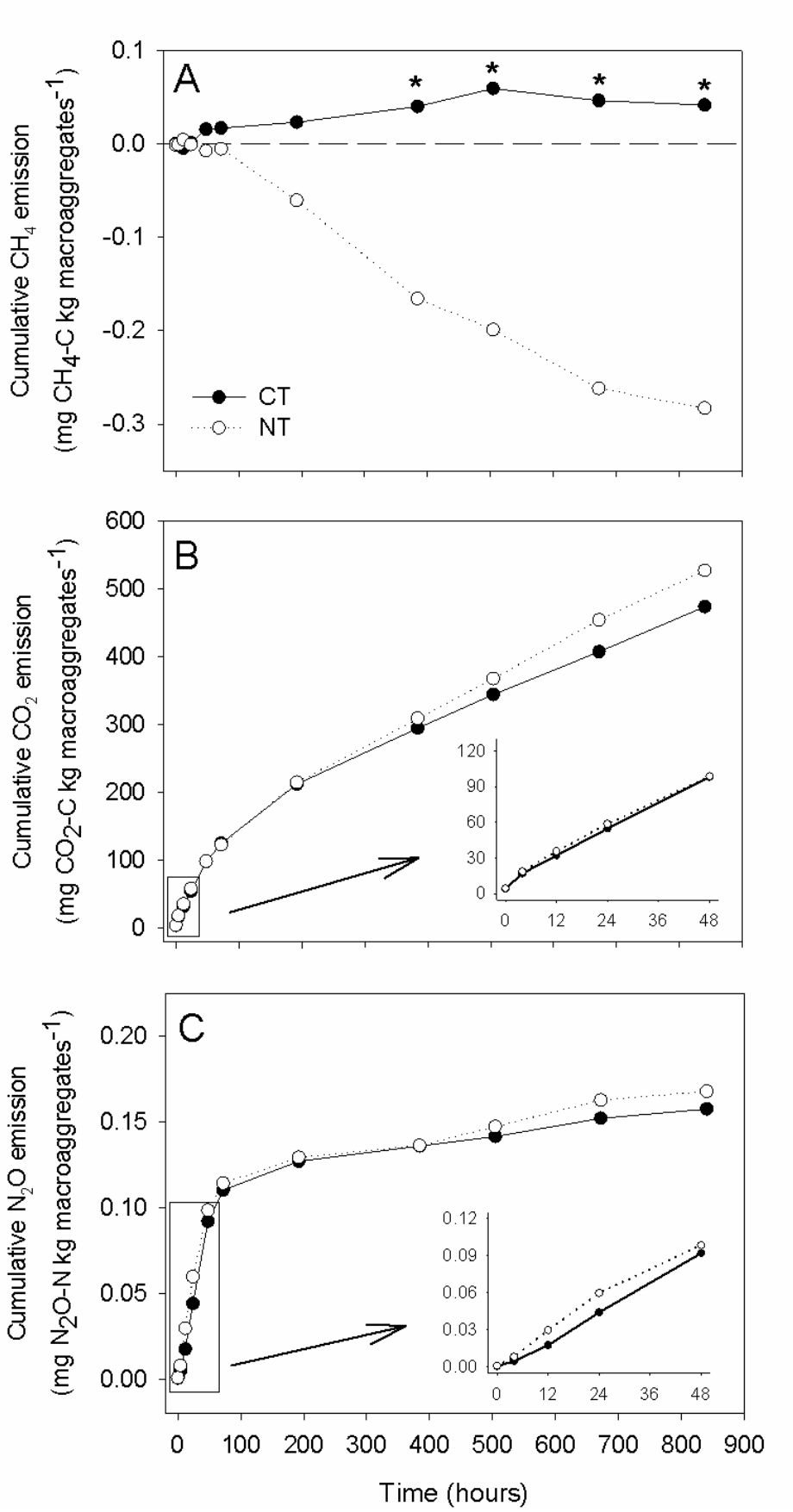
844 ¶ For each variable, different letters indicate significant differences between N fertilization
845 treatments at $P<0.05$.

846 **Table 6** Analysis of variance of the production of N_2O (mg N_2O-N kg^{-1}
847 macroaggregates h^{-1}) from nitrification and denitrification processes and N_2 from
848 denitrification process of dry-sieved macroaggregates as affected by N fertilization
849 treatments (0, control; mineral N at 150 kg N ha^{-1}, and organic N with pig slurry at 150
850 kg N ha^{-1}), soil moisture (15 and 30% gravimetric water content), and their interactions.

Effects	Nitrification-N_2O	Denitrification-N_2O	Denitrification-N_2
N fertilization (N)	*	*	n.s.
Control	122.51 b¶	180.06b	1949.91
Mineral	455.25 a	303.12 ab	1436.76
Organic	247.90 ab	381.07 a	1752.38
Soil moisture (SM)	***	***	***
15%	115.06 b	69.68 b	5.32 b
30%	500.84 a	506.49 a	4120.01 a
NxSM	n.s.	n.s.	n.s.
Control – 15%	87.15	56.94	29.33
Control – 30%	193.22	303.18	4254.60
Mineral – 15%	159.37	73.39	21.0
Mineral – 30%	751.14	532.85	4268.27
Organic – 15%	60.13	78.70	0
Organic – 30 %	341.78	683.44	3896.46

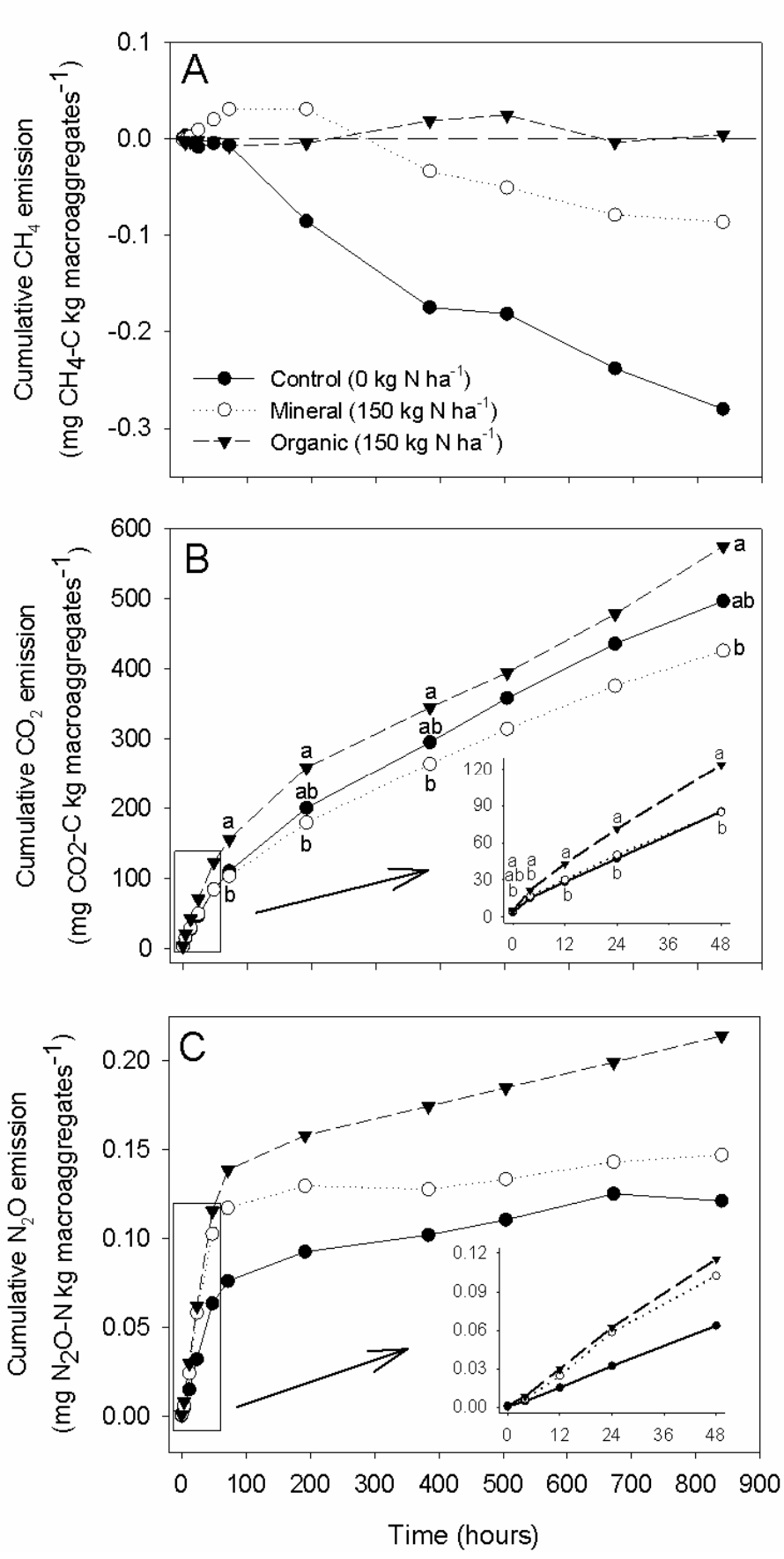
851 n.s.: not significant; *P<0.1; **P<0.01; *** P<0.001

852 ¶ For each process, different letters indicate significant differences between N fertilization or
853 moisture treatments at P<0.1.


A
B
C
Cumulative CH4 emission
(mg CH4-C kg macroaggregates-1)
Cumulative CO2 emission
(mg CO2-C kg macroaggregates-1)
Cumulative N2O emission
(mg N2O-N kg macroaggregates-1)
CT
NT
Time (hours)
*

854

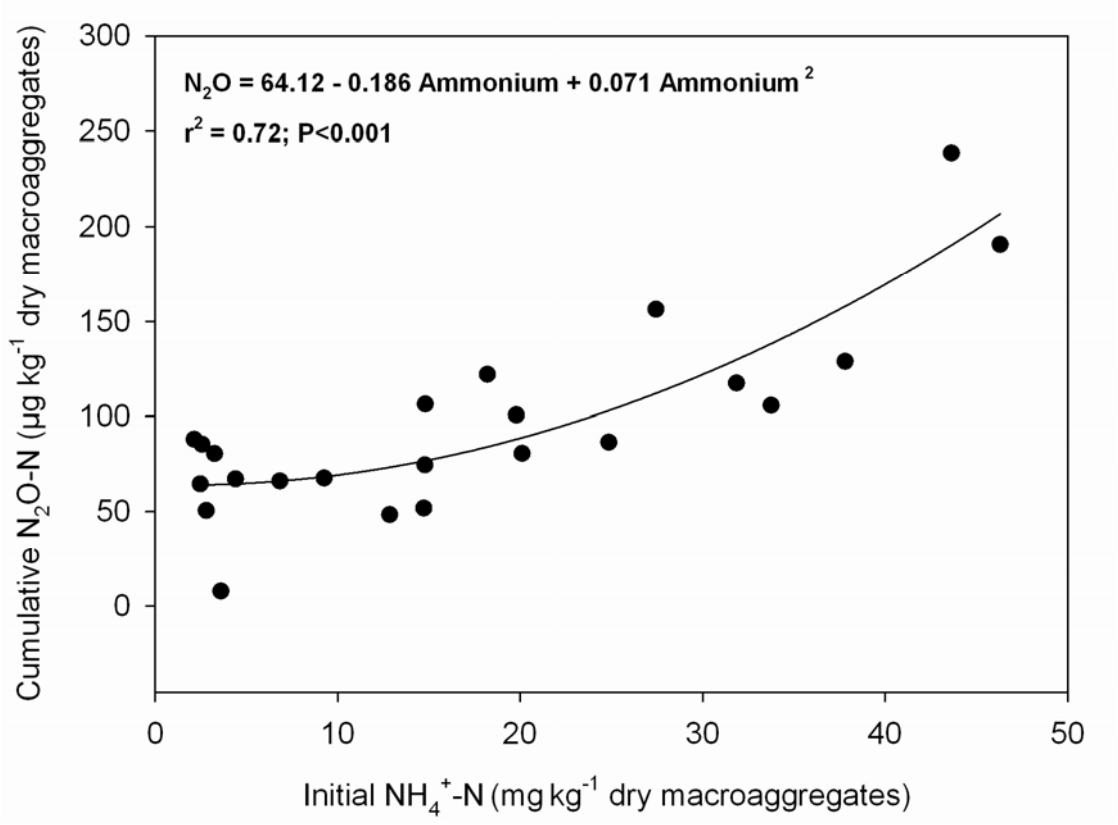
855 **Fig 1.**


A
B
C
Cumulative CH4 emission
(mg CH4-C kg macroaggregates-1)
Cumulative CO2 emission
(mg CO2-C kg macroaggregates-1)
Cumulative N2O emission
(mg N2O-N kg macroaggregates-1)
Control (0 kg N ha-1)
Mineral (150 kg N ha-1)
Organic (150 kg N ha-1)
Time (hours)

856

857 **Fig. 2**

858



859

860 **Fig. 3**

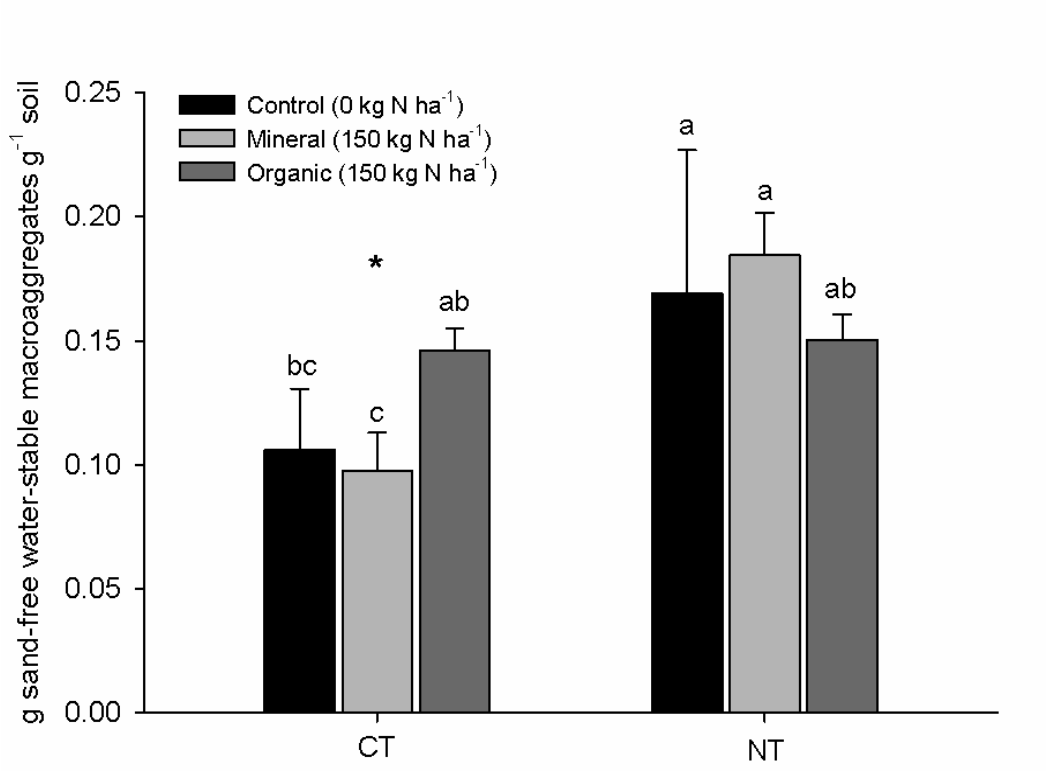

Control (0 kg N ha-1)
Mineral (150 kg N ha-1)
Organic (150 kg N ha-1)
g sand-free water-stable macroaggregates g-1 soil
0.25
0.20
0.15
0.10
0.05
0.00
*
bc
c
ab
a
a
ab
CT
NT

Fig. 4