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**Abstract**

Fertilization with micronutrients (e.g., zinc, Zn) is essential in order to overcome the global nutritional problems associated with human micronutrient deficiencies. However, little is known about the effect of micronutrient fertilizers and their interaction with nitrogen (N) on greenhouse gas (GHG) emissions and soil microbial processes involved in nitrous oxide (N2O) fluxes. In this context, a one-year field experiment was carried out using a winter wheat (Triticum aestivum L.) crop in Central Spain. Winter wheat was treated with different Zn sources (Zn-sulphate, Zn-lignosulphonate, Zn with a mixture of synthetic chelating compounds DTPA-HEDTA-EDTA and Zn-humic/fulvic acids) and N rates (0, 120 and 180 kg N ha-1). Zn sources were applied at 10 kg Zn ha-1 for Zn-sulphate and 0.36 kg Zn ha-1 for the rest of treatments. Nitrous oxide, methane (CH4) and respiration fluxes were measured (two-three times per week during the first month after each fertilization and thereafter with decreasing frequency), as were the total abundances of soil Bacteria and Archaea, ammonia-oxidizing Bacteria and Archaea, and denitrifying bacteria. The DTPA-HEDTA-EDTA reduced cumulative N2O losses by 21.4% and respiration fluxes by 24.4% from those of the no Zn application. The chelating of metal co-factors (mainly copper, Cu) of the enzymes involved in the nitrification and denitrification steps was the probable mechanism for the reduction of N2O emissions as bacterial amoA, nirK, nirS and norB gene abundances, as well as the extractable Cu content, decreased in this treatment. Unexpectedly, the DTPA-HEDTA-EDTA increased the copy number of nosZ by 31.2% over that of the no Zn application. The Zn applied together with the humic/fulvic acids mixture caused significant increases of total bacterial abundance and nitrifier and denitrifier communities, particularly the norB gene, thereby leading to the highest N2O emissions. The optimum N rate was 120 kg N ha-1 since it resulted in the lowest yield-scaled N2O losses and N surplus. The application of synthetic Zn chelates can be recommended as a win-win mitigation and adaptation strategy aimed at reducing yield-scaled GHG emissions and at the enhancement of Zn biofortification.

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| **Keywords** | Global warming potential; Nitrification inhibitors; Nitrogen cycling; Calcareous soil; Fertilizer rate; Micronutrient chelate. |
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**Highlights**

- Several Zn sources and N rates were tested in a rainfed wheat crop.
- The synthetic Zn chelate decreased $N_2O$ losses and the abundance of most of related genes.
- The chelating of Cu was the probable mechanism for the reduction of $N_2O$ emissions.
- The Zn applied with humic and fulvic stimulated nitrifiying and denitrifying abundances and $N_2O$ fluxes.
- A single dressing application of 120 kg N $ha^{-1}$ was the optimum N fertilization option.

# Zinc fertilizers influence greenhouse gas emissions and nitrifying and denitrifying communities in a non-irrigated arable cropland

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**Abstract**

Fertilization with micronutrients (e.g., zinc, Zn) is essential in order to overcome the global nutritional problems associated with human micronutrient deficiencies. However, little is known about the effect of micronutrient fertilizers and their interaction with nitrogen (N) on greenhouse gas (GHG) emissions and soil microbial processes involved in nitrous oxide ($N_2O$) fluxes. In this context, a one-year field experiment was carried out using a winter wheat (*Triticum aestivum* L.) crop in Central Spain. Winter wheat was treated with different Zn sources (Zn-sulphate, Zn-lignosulphonate, Zn with a mixture of synthetic chelating compounds DTPA-HEDTA-EDTA and Zn-humic/fulvic acids) and N rates (0, 120 and 180 kg N $ha^{-1}$). Zn sources were applied at 10 kg Zn $ha^{-1}$ for Zn-sulphate and 0.36 kg Zn $ha^{-1}$ for the rest of treatments. Nitrous oxide, methane ($CH_4$) and respiration fluxes were measured (two-three times per week during the first month after each fertilization and thereafter with decreasing frequency), as were the total abundances of soil Bacteria and Archaea, ammonia-oxidizing Bacteria and Archaea, and denitrifying bacteria. The DTPA-HEDTA-EDTA reduced cumulative $N_2O$ losses by 21.4% and respiration fluxes by 24.4% from those of the no Zn application. The chelating of metal co-factors (mainly copper, Cu) of the enzymes involved in the nitrification and denitrification steps was the probable mechanism for the reduction of $N_2O$ emissions as bacterial *amoA, nirK, nirS* and *norB* gene abundances, as well as the extractable Cu content, decreased in this treatment. Unexpectedly, the DTPA-HEDTA-EDTA increased the copy number of *nosZ* by 31.2% over that of the no Zn application. The Zn applied together with the humic/fulvic acids mixture caused significant increases of total bacterial abundance and nitrifier and denitrifier communities, particularly the *norB* gene, thereby leading to the highest $N_2O$ emissions. The optimum N rate was 120 kg N $ha^{-1}$ since it resulted in the

lowest yield-scaled $N_2O$ losses and N surplus. The application of synthetic Zn chelates can be recommended as a win-win mitigation and adaptation strategy aimed at reducing yield-scaled GHG emissions and at the enhancement of Zn biofortification.

**Keywords:** global warming potential; nitrification inhibitors; nitrogen cycling; calcareous soil; fertilizer rate; micronutrient chelate.

## 1. Introduction

Agriculture is a substantial contributor to climate change via the emissions of three greenhouse gases (GHGs) to the atmosphere: carbon dioxide ($CO_2$), methane ($CH_4$) and nitrous oxide ($N_2O$) (IPCC, 2014). Reducing GHG emissions from agriculture is essential in order to avoid increases in the mitigation cost in other sectors and to guarantee the achievement of the objective to limit warming to 2°C above pre-industrial levels (Wollenberg et al., 2016). Major efforts in direct GHG mitigation from agriculture are focused on $N_2O$, due to its calculated high cumulative forcing of 265 times that of $CO_2$ over a 100 year time horizon (IPCC, 2014) and because of its role in the depletion of stratospheric ozone ($O_3$) (Ravishankara et al., 2009). Regarding $CH_4$ fluxes, non-flooded agricultural soils are generally sinks of this GHG by its oxidation (Snyder et al., 2009).

It is critical to reduce environmental pollution without compromising food security in the context of an increasing worldwide population (Braun et al., 2007; Frank et al., 2017). Mitigation practices should therefore achieve the reduction of yield-scaled emissions, the maintenance or increase of crop yields and the improvement of net gross margins for farmers via increasing N use efficiency or crop quality (e.g., increased protein or micronutrient concentrations), while also addressing climate change

adaptation (Billen et al., 2015). Several mitigation strategies based on N fertilization, (e.g., management of N source including inhibitors or controlled-release fertilizers, placement, time of application or N rate) can reduce area-scaled emissions (Xia et al., 2017). However, the influence of micronutrients and the interaction with N on $N_2O$ emissions still remains poorly understood.

Nitrous oxide is released from agricultural soils mainly through autotrophic nitrification and heterotrophic denitrification (Ussiri and Lal, 2013). The critical step of nitrification, the oxidation of ammonia ($NH_3$) to hydroxylamine ($NH_2OH$), is carried out by ammonia-oxidizing bacteria (AOB) or archaea (AOA) which produce the enzyme ammonia monooxygenase encoded by the *amoA* gene. Denitrification, the dominant process in wet soils, is initiated with the reduction of nitrate ($NO_3^-$) to nitrite ($NO_2^-$) by nitrate reductase enzymes (encoded by the *narG*/*napA* genes), followed by $NO_2^-$ reduction by nitrite reductase enzymes (encoded by the *nirS* and *nirK* genes), nitric oxide (NO) reduction by nitric oxide reductase (encoded by *norB/norC* gene) and finally, the conversion of $N_2O$ by nitrous oxide reductase enzymes (encoded by *nosZ* genes), leading to the generation of $N_2$ as an end-product (Bueno et al., 2012). Enzymes that catalyse these stepwise biochemical processes contain trace metals as co-factors such as copper (Cu), zinc (Zn) or iron (Fe) (Glass and Orphan, 2012). The same accounts for $CH_4$ emission and consumption. As a consequence, previous studies have found an effect of Zn on $N_2O$ and $CH_4$ fluxes (Chen et al., 2014; Pramanik and Kim, 2017). However, so far it has not been clarified whether if this effect is micronutrient-dependent (Ruyters et al., 2010; De Brouwere et al., 2007) or source-dependent. Widespread products which inhibit nitrification, such as dicyandiamide (DCD) or 3,4-dimethylpyrazole phosphate (DMPP) act as metal chelators (Ruser and Schulz, 2015). Specifically, Duncan et al. 2017 showed that the chelation of Cu as the co-factor of the

ammonia monooxygenase was the inhibition mechanism for DMPP and nitrapyrin in low-Cu content soils. The influence of Cu availability on ammonium ($NH_4^+$) oxidation has been demonstrated in several studies (e.g. Bédard and Knowles, 1989 or Singh and Verma, 2007). Therefore, chelated agents as ethylenediaminetetraacetic acid (EDTA), which are used to apply micronutrients with enhanced efficacy (Alvarez, 2010), have been shown to inhibit nitrification (Hu et al., 2003). However, studying the specific inhibition mechanism, as well as the effect on other metal-dependent biochemical processes such as denitrification, is still needed.

Both the management of macronutrients fertilization (right time, right source, right place and right rate) (Venterea et al., 2016) and the study of its interactions with micronutrients (e.g., Zn, Cu, Fe) need to be considered to achieve an adequate nutrient balance. Micronutrients supply is essential to avoid severe penalties in growth, yield and crop quality associated to their deficiencies (Singh, 1996), and also for sustaining human and animal health (Kumar et al. 2016). Zinc has been recognized as one of the main target micronutrients, since its supplementation is associated with reductions of the incidence of infectious diseases such as pneumonia, particularly among children in areas with insufficient Zn supply (Gibson, 2012). A high incidence of Zn deficiency in humans has been associated with regions with soils with low Zn phytoavailability (Cakmak et al., 2016). Usually, calcareous and/or alkaline soils are associated with deficiencies of P or micronutrients (Singh, 1996; Deb et al. 2009). Besides, it is necessary to find synergistic effects between nutrients (Fageria and Baligar, 2005; Hamnér et al., 2017), with the aim to improve nutrient use efficiencies (e.g. N, thus reducing potential pollution impacts) and also crop quality through biofortification in deficient soils (Cakmak et al., 2016).

So far, the effect of Zn fertilizers on $N_2O$ and $CH_4$ emissions has not been studied in non-flooded crops, and an improved understanding of how these molecules modulate soil nitrifying and denitrifying microbiota is still needed. To that end, the main objective of this experiment was to study the effect of several Zn sources (conventional, synthetic chelates and natural chelates) and N fertilization rates on GHG emissions and yield-scaled emissions, quantifying also the microbial populations involved in $N_2O$ fluxes. We explored the hypothesis that $N_2O$ emissions (from both nitrification and denitrification processes) could be directly affected by Zn sources (particularly chelates).

## 2. Materials and Methods

### *2.1 Site description*

The field experiment was located in the National Center of Irrigation Technology, "CENTER" (latitude 40º25'1.31''N, longitude 3º29'45.07''W) in the Madrid region (Spain). According to the Soil Taxonomy of the USDA, the soil is a *Typic xerofluvent* (Soil Survey Staff, 2014) with a silt loam texture (10% clay, 59.5% silt, and 30.5% sand) on the upper horizon (0-20 cm). The major physicochemical properties of the topsoil, measured by conventional methods, were: organic matter (Walkley-Balck), 20.7 g $kg^{-1}$; total Nitrogen, 1.64 g $kg^{-1}$; bulk density, 1.27 Mg $m^{-3}$; water pH, 8.2; $CaCO_3$, 8.16 g $kg^{-1}$; extractable P (Olsen), 28.4 mg $kg^{-1}$; total K, 3.14 g $kg^{-1}$; exchangeable K, 0.51 g $kg^{-1}$; DTPA-extractable Cu, 1.56 mg $kg^{-1}$, DTPA-extractable Zn 0.81 mg $kg^{-1}$ (values below 1 mg $kg^{-1}$ indicate Zn deficiency, Brennan et al., 1993); and DTPA-extractable Fe, 4.41 mg $kg^{-1}$. According to data from the meteorological station placed in the field, the mean annual average temperature and rainfall during the last 10 years were 14.1°C and 393 mm, respectively. Data of rainfall

143 and air and soil temperatures (at 10 cm depth) were obtained daily from the
144 meteorological station located at the field site.
145 *2.2 Experimental design and management*
146 A field experiment was carried out from October 2015 to July 2016. A total of
147 45 plots (4 m x 5 m) were arranged in a three-replicate randomized block design. Each
148 plot was a result of a factorial combination of three N rates (0, 120 and 120+60 kg N ha-
149 $^{1}$) with five Zn sources: control without Zn application (Zn0); Zn sulphate (S, $ZnSO_4$
150 35% Zn; w/w); Zn-lignosulphonate (LS, 7.5% Zn; w/w); Zn applied with a mixture of
151 chelating compounds (Ch, Zn-DTPA-HEDTA-EDTA 7% Zn; w/w); and Zn-
152 humic/fulvic acids (HuFu, 7% Zn; w/w). Zn sources were applied in two dressing
153 applications. At each time, the application was 0 kg Zn $ha^{-1}$ for Zn0; 0.18 kg Zn $ha^{-1}$ for
154 LS, Ch and HuFu; and 5 kg Zn $ha^{-1}$ for S (Martens and Westermann, 1991; Mortvedt
155 and Gilkes, 1993). Therefore, total Zn rate was 10 kg Zn $ha^{-1}$ for S and 0.36 kg Zn $ha^{-1}$
156 for the rest of treatments.
157 The field was seeded on October 27$^{th}$ with winter wheat (*Triticum aestivum* L.
158 ´Ingenio`) at 200 kg $ha^{-1}$. No fertilizers (N, P, or K) were applied at seeding according
159 to the previous soil analysis. Nitrogen and Zn-based fertilizers were split into two
160 dressing applications, at the beginning and the end of the stem elongation stage (Z30
161 and Z39, Zadocks et al., 1974). In the case of N, the second dressing fertilization (60 kg
162 N $ha^{-1}$) was only carried out in the N120+60 treatment, while the plots corresponding to
163 both N-fertilized treatments (N120 and N120+60) received 120 kg N $ha^{-1}$ at the first
164 dressing application. All of the N was applied through urea (46% N; EuroChem Agro).
165 Both N and Zn sources were applied through liquid solutions which were sprayed
166 homogeneously through a knapsack sprayer (foliar-soil application). The wheat was

167 harvested on June 21st with a research plot combine (Wintersteiger Inc.). The field was
168 kept free of weeds, pests and diseases following local practices.
169 *2.3 GHG sampling and analyses*
170 During the first month after fertilization, gas and soil samples were collected 2-3
171 times per week, considering it to be the most critical period of high gas emissions.
172 Afterwards, the frequency of sampling was decreased progressively, increasing after
173 rainfall events. The GHG ($N_2O$, $CH_4$ and $CO_2$) fluxes were measured using the closed
174 chamber technique. Opaque cylindrical static chambers of 19.3 L were placed in each
175 plot and fitted into stainless steel rings which had been inserted into the soil to a depth
176 of 10 cm at the beginning of the experiment in order to avoid the lateral diffusion of
177 gases and disturbance of the gas fluxes (Hutchinson and Livingston, 2001).
178 Measurements of $N_2O$, $CO_2$ and $CH_4$ emissions were made at 0, 30 and 60 min to test
179 the linearity of gas accumulation in each chamber. The increases in $N_2O$, $CH_4$ and $CO_2$
180 concentrations within the chamber headspace were generally linear (> 90% of cases,
181 particularly when highest fluxes or emission peaks were reported, $R^2 > 0.90$) during the
182 sampling period (1 h). In the case of nonlinear fluxes, linear regressions were
183 performed, since it has been described as the recommended option for three sampling
184 points (Venterea et al., 2012).
185 The concentrations of $N_2O$, $CO_2$ and $CH_4$ were determined by gas
186 chromatography, using an HP-6890 gas chromatograph (GC) equipped with a
187 headspace autoanalyser (HT3), both of which were from Agilent Technologies
188 (Barcelona, Spain). HP Plot-Q capillary columns transported the gas samples to a $^{63}Ni$
189 electron-capture detector (ECD) to determine the $N_2O$ concentrations with a limit of
190 detection of 50 ppb; and to a flame-ionization detector (FID) fitted with a methanizer
191 for the $CH_4$ and $CO_2$ concentrations (with a limit of detection of 850 ppb).

192 *2.4 Soil and crop sampling and analyses*

193 To relate gas emissions to soil properties, soil samples were collected at 0-10 cm
194 depth during the growing season on almost all gas-sampling occasions, particularly after
195 each fertilization event. In the whole experimental period, soil samples were collected in
196 4 of each 5 times in which GHGs were sampled. Three soil cores (2.5 cm diameter and
197 12 cm length) were randomly collected close to the ring in each plot, and then mixed
198 and homogenized in the laboratory. Soil ammonium ($NH_4^+$) and nitrate ($NO_3^-$)
199 concentrations were determined using 8 g of soil extracted with 50 mL of KCl (1 M),
200 and measured by automated colorimetric determination using a flow injection analyser
201 (FIAS 400 Perkin Elmer) equipped with a UV-Vis spectrophotometric detector. Soil
202 dissolved organic carbon (DOC) was determined by extracting 8 g of homogeneously
203 mixed soil with 50 mL of deionized water and then analysing the resulting solution with
204 a total organic C analyser (multi N/C 3100 Analityk Jena) equipped with an IR detector.
205 The water-filled pore space (WFPS) was calculated by dividing the volumetric water
206 content by total soil porosity, assuming a particle density of 2.65 g $cm^{-3}$. Gravimetric
207 water content was determined by oven-drying soil samples at 105°C with a MA30
208 Sartorius ®. The method DTPA-TEA (DTPA 5 mM + $CaCl_2$ 10 mM + TEA 0.1 M
209 adjusted to pH 7) (Lindsay and Norvell, 1978) was used to determine the soil
210 concentrations of micronutrients available for plants. This procedure consisted of
211 weighing 10 g of the sieved soil into a 125 mL conical glass flask and extracting the
212 micronutrients with 20 mL of the DTPA-TEA extraction solution and shaking for 2 h.
213 The suspension was filtered through Whatman no. 42 filter paper and the filtrate was
214 measured by flame atomic absorption spectroscopy (FAAS) (Perkin-Elmer AAnalyst
215 700).

Wheat was harvested on June 21st with a research plot combine (Wintersteiger Inc.). Previously, the plants of one row had been harvested and air-dried to determine the total N and Zn contents of grain and straw. The N content was determined using a TruMac CN Leco elemental analyser. Total Zn in the dry plant matter was extracted by wet acidic digestion in a digester (SPB 50-24 with an SPB digital, Perkin-Elmer). First, air-dried samples were sieved at 2 mm, except for the flour samples which were sieved at 1 mm. Then, 1 g of dried and sieved sample was weighed and 10 mL of $HNO_3$ (65%), 3 mL of HF (48%) [except for the flour which received 10 mL HCL (37%)] and 10 mL of deionized water was added. This mixture was left overnight and was then placed in a digester for 2 h at 140ºC. After that, the sample was filtered through Whatman no. 41 filter paper into a 50 mL graduated flask and the concentrations of Zn and other micronutrients were determined by FAAS.

*2.5 DNA extraction and quantification of nitrifying and denitrifying microbial communities*

Soil samples for DNA purposes were taken after each fertilization event, during the period between fertilizations, and at the end of the field experiment. Total DNA was extracted from 500 mg of soil using the commercial PowerSoil® DNA isolation kit (Qiagen). Briefly, the DNA extraction method involved the physicochemical lysis of microbial cells with gentle bead-beating bound to a silica spin filter, washed and finally eluted in Milli-Q® water. Quality and size of DNA were checked by electrophoresis on 1% agarose, and DNA concentration was measured using the Qubit® ssDNA assay kit (Molecular Probes). DNA was stored at -80ºC until use.

The size of nitrifying communities was estimated by quantitative PCR (qPCR) of the *amoA* gene from ammonia-oxidizing Bacteria (AOB) and Archaea (AOA) (Rotthauwe et al., 1997; Tourna et al., 2008, respectively). Similarly, denitrifying communities were

241 estimated by qPCR of the *nirK*, *nirS*, *norB* and *nosZ* genes using primers and thermal
242 conditions described previously (Henry et al., 2004; Throbäck et al., 2004; Braker and
243 Tiedje, 2003; Henry et al., 2006, respectively). The total bacterial and archaeal
244 communities were quantified using the 16S rRNA gene as a molecular marker (Lopez-
245 Gutiérrez et al., 2004; Ochsenreiter et al., 2003, respectively). qPCR was performed in
246 an ABI Prism 7900 Sequence Detection System (Applied Biosystems) employing the
247 fluorophore SYBR Green to quantify the total abundance of targeted genes. Standard
248 curves were obtained using serial dilutions (ranging from $10^8$ down to $10^2$ copies $\mu L^{-1}$)
249 of linearized plasmids containing clones in the targeted genes. Amplicons were
250 generated from *Pseudomonas putida* NCB 957 (quantification of total Bacteria), DNA
251 from the genomic clone 29i4 (quantification of total Archaea), *Nitrosospira multiformis*
252 ATCC 25196 (quantification of AOB), DNA from the fosmid clone 54d9
253 (quantification of AOA, Leininger et al., 2006), *Pseudomonas stutzeri* ATCC 14405
254 (quantification of *nirK*, *nirS*, *norB* and *nosZ*, Nogales et al., 2002). PCR efficiency for
255 the different assays ranged between 90% and 99%. The quality of qPCR amplification
256 was verified by electrophoresis in agarose and by a melting curve analysis. We
257 expressed the abundance of targeted genes as gene copy number per nanogram of DNA
258 instead of gram of soil in order to minimize any bias related to soil DNA extraction
259 efficiency (Klappenbach et al., 2001).

260 *2.6 Calculations and statistical analysis*

261 Cumulative gas emissions during the experimental period were calculated by
262 multiplying the average flux of two successive determinations by the length of the
263 period between sampling and adding that amount to the previous cumulative total. The
264 global-warming potential (GWP) of $N_2O$ and $CH_4$ emissions was calculated in units of
265 $CO_2$ equivalents over a 100-year time horizon. A radiative forcing potential relative to

266 $CO_2$ of 265 was used for $N_2O$ and 28 for $CH_4$ (IPCC, 2014). Yield-scaled $N_2O$
267 emissions were calculated as the N-$N_2O$ cumulative emissions to aboveground N uptake
268 ratio. The N surplus was estimated as the N application minus above-ground N uptake
269 (van Groenigen et al., 2010).

270 The analysis of data was performed using the *Statgraphics Plus 5.1* program.
271 Two-way ANOVAs were performed for mineral N, GHG emissions, yield-scaled $N_2O$
272 emissions, N surplus and the total abundance of targeted genes. Data distribution
273 normality and variance uniformity were previously assessed by the Shapiro-Wilk test
274 and Levene´s statistic, respectively and were log10 transformed when necessary. Means
275 were separated by an LSD test at $P < 0.05$. For non-normally distributed data, the
276 Kruskal–Wallis test was used on non-transformed data to evaluate differences at $P <$
277 0.05. Simple Linear Regression analyses were performed to determine the relationships
278 between GHG fluxes and soil $NH_4^+$-N, $NO_3^-$-N, WFPS and temperature. Redundancy
279 analyses (RDA) were performed to validate the relationship between response variables
280 (total abundance of target genes) and explanatory variables ($NH_4^+$-N, $NO_3^-$-N, $CO_2$,
281 $NH_4$ and $N_2O$) (Leps and Smilauer, 2003).

282

283 **3. Results**

284 *3.1 Environmental conditions*

285 The average of daily mean soil temperature during the wheat cropping cycle was
286 11.6°C (9.4°C the first month after N fertilization) (Fig. 1a), and the minimum and
287 maximum air temperatures were 35.0 and -6.7°C, respectively (data not shown). Total
288 precipitation over the wheat cropping cycle was 340 mm (92 mm in the first 45 days
289 after N fertilization). After fertilization, soil WFPS (Fig. 1b) ranged from 10% to 65%
290 (Fig. 1b).

291 *3.2 Mineral N, DOC and trace metals*

292 Mineral N concentrations peaked after both fertilization events (data not shown).
293 From the beginning of May to the end of the experiment, soil $NH_4^+$ concentrations were
294 below 2 mg N kg soil$^{-1}$, while $NO_3^-$ decreased below 15 mg N kg$^{-1}$ after mid-May to the
295 end of wheat cropping cycle. Average $NH_4^+$ contents after first fertilizer application
296 (Fig. 2a) were significantly higher in the N-fertilized plots, with respect to C ($P$ =
297 0.000). After the second fertilization, $NH_4^+$ contents increased in the order N0 < 120 <
298 N120+60 ($P$ = 0.000), regardless of Zn source. Average $NO_3^-$ concentrations responded
299 similarly to those of $NH_4^+$ after each fertilizer application (Fig. 2b, $P$ = 0.001). The Zn
300 source did not significantly affect the average $NH_4^+$ ($P$ = 0.522) or $NO_3^-$ concentrations
301 ($P$ = 0.332, Fig. 2a, b). The `Zn source x N rate´ interaction was not significant neither
302 for $NH_4^+$ ($P$ = 0.634) nor for $NO_3^-$ ($P$ = 0.659) contents.

303 Average DOC content was 156 mg C kg soil$^{-1}$, and no significant effect of N rate
304 or Zn source was found. Only on March 23$^{rd}$ (first fertilization), DOC contents were
305 increased in the HuFu treatment with respect to the other Zn sources (by 19%, $P$ <
306 0.05), for all N rates. Zn-DTPA in soil ranged from 1.0 to 7.5 mg Zn kg soil$^{-1}$ and small
307 differences were observed between treatments, except for S (which usually resulted in
308 higher extractable Zn contents, data not shown). Cu-DTPA in soil ranged from 1.5 to
309 2.6 mg Cu kg$^{-1}$ (Fig. 3). The concentrations of extractable Cu were generally lower in
310 the Ch treatment compared with the other Zn sources. That was statistically significant
311 for the 120 kg N ha$^{-1}$ rate at both fertilizations. No significant differences were observed
312 for extractable Fe (which ranged from 4.1 to 6.1 mg Fe kg soil$^{-1}$, data not shown).

313 *3.3 $N_2O$ emissions*

314 To improve the visualization of daily GHG fluxes, only the emissions
315 corresponding to the fertilizer application period (March-June) have been represented in

316 Fig. 4. After wheat harvest and before fertilization, fluxes were low (< 0.1 mg mg N $m^{-2}$
317 $d^{-1}$). Since no significant differences were observed between N0 treatments, an average
318 of $N_2O$ emissions of all these treatments (N0-Zn0, N0-S, N0-LS, N0-Ch and N0-HuFu)
319 has been represented in Fig. 4a. In addition, only the fluxes corresponding to N180 are
320 shown, since after the first fertilization, $N_2O$ losses were statistically similar to those
321 from N120, and after the second fertilizer application, the fluxes from N120 treatments
322 did not differ significantly from those of N0. Daily $N_2O$ emissions ranged from -0.16 to
323 2.17 mg N $m^{-2}$ $d^{-1}$ (Fig. 4a). Nitrous oxide fluxes peaked immediately after both
324 fertilizer applications, and both $N_2O$ peaks were short-lived. Cumulative $N_2O$ emissions
325 (Table 1) were significantly higher in N fertilized treatments (N120 and N120+60) than
326 in N0, but no significant differences were found between both N rates. Concerning Zn
327 treatments, cumulative losses decreased in the order HuFu > S = Zn0 > Ch, showing LS
328 intermediate emissions between HuFu and S/Zn0. Nitrous oxide emissions significantly
329 correlated with $NO_3^-$ ($P$ = 0.001, $n$=28, $r$=0.87) and $NH_4^+$ contents ($P$ = 0.049, $n$=28,
330 $r$=0.61), as well as with soil respiration ($P$ = 0.004, $n$=28, $r$=0.72).
331 *3.4 $CH_4$ oxidation and soil respiration*
332 Methane fluxes were generally negative, ranging from -0.8 to 1.2 mg C $m^{-2}$ $d^{-1}$
333 (Fig. 4b). No significant differences between Zn sources or N rates were found in
334 cumulative $CH_4$ uptake (Table 1), although HuFu and Ch resulted in the numerically
335 lower oxidation rates. Respiration fluxes ranged from 0.2 to 8.2 g C $m^{-2}$ $d^{-1}$ (Fig. 4c).
336 Two remarkable peaks were observed after both N fertilization events. The Ch
337 treatment resulted in the lowest cumulative respiration fluxes (Table 1), which were
338 significantly lower than those of Zn0 treatment. The net GWP exhibited a similar
339 pattern as $N_2O$ emissions, and Ch and HuFu resulted in the lowest and highest $CO_2$
340 equivalent emissions, respectively.

341 *3.5 Yield-scaled $N_2O$ emissions, N surplus and Zn*

342 Yield-scaled $N_2O$ emissions (Table 1) were significantly lower in the case of the
343 Ch treatment, in comparison to the other Zn sources or Zn0. Regarding N rate, the
344 amount of N emitted per kilogram of aboveground N uptake decreased in the order N0
345 > N120+60 > N120. A significant interaction between both factors was found (Table 1,
346 Fig. S1). This interaction showed that HuFu was the Zn source leading to higher yield-
347 scaled $N_2O$ emissions, except for Zn0. Yield-scaled losses from S were also highly
348 influenced by N rate, being highest for N0 and low for N120 and N120+60. The N
349 surplus was only affected by N rate (Table 1), increasing in the order N0 < N120 <
350 N120+60 ($P < 0.05$). All Zn sources significantly increased Zn content in wheat grain
351 (by 23% on average, and by 21% in the case of Ch) (data not shown) with respect to
352 Zn0.

353 *3.6 Total abundances of nitrifying and denitrifying communities*

354 Total abundance of Bacteria and Archaea, as determined by their 16S rRNA
355 gene copy numbers, that of nitrifiers, estimated as the abundance of bacterial and
356 archaeal *amoA* gene and that of denitrifiers, estimated as the abundance of the *nirK,*
357 *nirS, norB* and *nosZ* genes were only quantified in the Zn0, Ch and HuFu treatments
358 combined with N0 (Table S1) and N120 treatment (Fig. 5, Table S1) because significant
359 differences were found between those treatments and $N_2O$ emissions. Total abundance
360 of Bacteria (16SB) increased by 37.7% during the fertilization period in N120+HuFu
361 treatment respect to N120+Zn0 treatment, while N120+Ch fertilizer did not affect the
362 copy number of 16SB during the experiment compared with N120+Zn0 treatment (Fig.
363 5a). In contrast, the total abundance of Archaea (16SA) increased by a 35.72% in
364 N120+Ch compared with N120+Zn0 treatment throughout the experimental period;
365 however, significant changes were not observed in the copy number of 16SA in

366 N120+HuFu treatment in comparison to N120+Zn0 during the experimental period
367 (Fig. 5a).

368 Regardless of the sampling date, the total abundance of the *amoA* gene from
369 AOB in the N120+HuFu treatment was higher than those found in the N120+Ch and
370 N120+Zn0 treatments, but AOA significant differences compared with the N120+Zn0
371 treatment were only obtained after the second fertilization event and before harvest (Fig.
372 5b). However, AOB decreased by more than 42% in N120+Ch treatment in comparison
373 to N120+Zn0 throughout the experiment, while AOA did not show significant changes
374 among these treatments (decrease of 2.52% on average during the experiment period),
375 (Fig. 5b).

376 Regarding denitrification genes, addition of N120+Ch to the soil decreased the
377 abundance of the *nirK*, *nirS* and *norB* genes compared to the remaining treatments.
378 Interestingly, a significant increase was found in the *nosZ* gene along the experimental
379 period in the N120+Ch (Fig. 5c and 5d). The copy number of *nirK, nirS, norB* and *nosZ*
380 genes increased with the application of N120+HuFu fertilizer in compared to the
381 N120+Zn0 treatment, highlighting significant increases of *norB* gene, by 50.18% and
382 42.15% compared to Ch and Zn0, respectively, regardless of the sampling date (Fig. 5c
383 and 5d).

384 After the experimental time, soil fertilized with N120 and amended with HuFu
385 and Ch fertilizers showed higher copy numbers of 16SB and 16SA than those of
386 N0+HuFu and N0+Ch, respectively. With the 120 kg N $ha^{-1}$ application, independently
387 of Zn source, the *amoA* gen from AOA and AOB, *nirK, nirS* and *norB* genes obtained a
388 higher proportion than the same treatments with N0 (Table S1). In the same way, HuFu
389 and Ch fertilizers combined with N120 increased the *nosZ* gene respect to those

treatments with N0 but was not observed for N120+Zn0 treatment in comparison to N0+Zn0.

Nitrous oxide emissions were positively correlated with the abundances of *norB*, *nirK* and *amoA* genes, and all of these variables showed a close relationship with HuFu treatment (Fig. S2). The opposite was observed for Ch, which was related with the *nosZ* gene.

## 4. Discussion

### *4.1 Effect of Zn source on $N_2O$ emissions*

Our results suggested that $N_2O$ emissions and related gene abundances were affected by Zn source. In comparison to the treatment which did not receive any Zn source (Zn0), only Hufu and Ch showed significant effects on $N_2O$ emissions (Table 1), increasing and decreasing the cumulative losses, respectively. Ruser and Schulz (2015) noted that one of the most likely mechanisms of commercial nitrification inhibitors (e.g., DCD, and, DMPP) for retarding the oxidation of $NH_4^+$ was the removal of co-factors of ammonia monooxygenase (involved in the first step of nitrification). Therefore, the addition to soil of strong chelating compounds (e.g., the DTPA-HEDTA-EDTA mixture) could also affect the availability of trace metals (e.g., Cu) for microorganisms, to a higher extent than other fertilizers (LS or HuFu). That was actually observed for the N120 treatment (Fig. 3), indicating that the chelation of Cu could be a consistent hypothesis for the inhibition of nitrification and the reduction of $N_2O$ losses from the Ch treatment (Duncan et al., 2017). Copper is the main trace metal involved in soil processes affecting the emission of $N_2O$, e.g., $NH_4^+$ oxidation (first step of nitrification) and in the reduction of nitrite ($NO_2^-$, second step of denitrification) and $N_2O$ (last step of denitrification) (Glass and Orphan, 2012). That was in agreement with the observed reductions in the abundances of AOB and *nirK* gene, and with the

415 increases in the copy numbers of *nosZ* gene. In addition, we also detected a reduction of
416 AOA and *nirS* gene abundances, though the relevance of these microbial populations
417 was lower from a quantitative viewpoint, as observed in calcareous soils (Tellez-Rio et
418 al., 2015; Zhong et al., 2016; Tao et al., 2017). Our results demonstrate an indubitable
419 effect of Ch on nitrifying (despite the fact that we did not observe any differences in soil
420 mineral N contents, Fig. 2) and denitrifying communities. Considering that the chelation
421 of metal co-factors is the most likely mode of action of nitrification inhibitors, previous
422 findings also noted a reduction in the copy numbers of *amoA* (Di and Cameron., 2011;
423 Akiyama et al., 2013) and *nirK* gene (Barrena et al., 2017) due to the application of
424 nitrification inhibitors, while the increments in *nosZ* gene were observed by Torralbo et
425 al. (2017) after the use of a pyrazole-based inhibitor. In spite of the finding that a
426 limitation of Cu availability could affect negatively the *nosZ* abundance (Sullivan et al.,
427 2013), Torralbo et al. (2017) attributed the increases of the *nosZ* gene expression to i) a
428 transient induction of these microbial population in response to the effect of nitrification
429 inhibitors on other denitrifying microorganisms; or ii) the use of $N_2O$ as an alternative
430 electron acceptor due to the reduction of nitrification activity and therefore $NO_3^-$
431 availability. In addition, a reduction of redox potential after application of synthetic
432 chelating agents (e.g., EDTA) as reported by Almendros et al. (2015), could also have
433 favoured the reduction of $N_2O$ to $N_2$, since the highest soil moisture contents were
434 recorded at the fertilization dates (Fig. 1b). Our results therefore confirmed an effect of
435 chelating compounds as DTPA-HEDTA-EDTA on nitrifying microorganisms (Hu et al.,
436 2003), and also on denitrifying communities. Further research is needed under a variety
437 soil moisture conditions (i.e., more favourable for denitrification or complete
438 denitrification), since our results were not in agreement with Pramanik and Kim (2017),

who reported an increase in $N_2O$ losses after the addition of EDTA in a submerged paddy crop.

The mitigation efficacy obtained with the Ch application (21.4%) in comparison to Zn0 was lower than that achieved with common nitrification inhibitors, such as DCD (average 42.3%) or DMPP (40.2%) (Gilsanz et al., 2016). This could be a result of the short-term effect of Ch application on $N_2O$ emissions (1-4 days after application, see Fig. 3a), which is even shorter than that of nitrification inhibitors (Benckiser et al., 2013; Chaves et al., 2006).

The possible side-effect on non-target microorganisms must also be considered before recommending the application of synthetic chelating agents as a sustainable GHG mitigation practice. In addition to the effect of chelating compounds on nitrifiers/denitrifiers through the removal of the co-factors of metalloenzymes, adverse effects on soil microbiota have been associated with synthetic chelates (Grčman et al., 2001; Epelde et al., 2008), which are products with low biodegradability (Bucheli-Witscheli and Egli, 2001), high solubility in soil and long persistence (Alkorta et al., 2003). This effect of the Ch treatment on soil microbiota may help to explain the reduction of cumulative $CO_2$ fluxes with respect to the no Zn application (Zn0). However, no effect of Ch on total abundance of 16SB in soil was found. In addition to this effect, a potential polluting effect of chelated fertilizers through metal leaching to groundwater has been observed (Gonzalez et al., 2015). This problem should be further considered, particularly under favourable conditions for leaching (i.e. coarse-textured soils and high rainfall amount) and for highly soluble metal-chelate compounds (Alkorta et al., 2004).

The significant increase in $N_2O$ losses after the addition of the HuFu occurred in both emission peaks (Fig. 4a). The increase in the total abundance of denitrifying

communities (i.e., *nirK*, *nirS*, *norB* and *nosZ*) indicated that this fertilizer provided a C-labile source to heterotrophic denitrifiers, thereby stimulating their activity. Surprisingly, we also observed that the HuFu enhanced the AOA, AOB and 16SB total abundances. Although nitrifying microorganisms are autotrophs, our results support the findings of Valdrighi et al. (1996) and Vallini et al. (1997), who showed that chemolitotrophic bacteria reacted positively to the addition of humic acids to the soil, possibly as a result of improved nutrition due to enhanced microbial membrane permeability. Since the fertilizers were applied as liquid solutions containing N, the soil moisture at fertilization events, expressed as WFPS, was adequate for both nitrification and denitrification processes (60-70%, Fig. 1b) (Pilegaard, 2013). The relevant contribution of an incomplete denitrification to $N_2O$ losses from HuFu fertilizer was corroborated by the disproportional increase in *norB* (i.e., the reduction of NO to $N_2O$) and *nosZ* (i.e., the reduction of $N_2O$ to $N_2$) gene abundances (Fig. 5). The significant correlation of $N_2O$ losses with both $NH_4^+$ and $NO_3^-$ concentrations, along with the close linkage between $N_2O$ emissions and both AOB and denitrifier (i.e., *norB*, *nirK*) abundances (Fig. S2) demonstrate the importance of both biochemical processes for the release of $N_2O$. This interaction between available C-labile and mineral N sources explained the fact that HuFu treatment resulted in the highest yield-scaled $N_2O$ losses in N-fertilized treatments, but not in the N0 treatment.

*4.2 Effect of N rate on $N_2O$ emissions*

As expected, the addition of synthetic N significantly increased $N_2O$ losses (Table 1) compared to the unfertilized N0 treatment. Moreover, the expressions of all measured genes encoding nitrifying and denitrifying microorganisms (except *nosZ* gene) were increased after the application of N and Zn fertilizers (Table S1), which is

consistent with the results of Tian et al. (2014) and Sun et al. (2015). Regarding N rate, even though the N120+60 treatments increased the cumulative $N_2O$ losses in comparison to the single application of 120 kg N $ha^{-1}$, this was not statistically significant. The low N rate added (60 kg N $ha^{-1}$) and the scarce rainfall which occurred from two weeks after the second fertilizer application (Fig. 1a) may explain these results. Regarding the yield-scaled $N_2O$ emissions, our results showed the beneficial effect of N synthetic fertilization to decrease $N_2O$ losses per kilogram of aboveground N uptake (Table 1). Comparing both N fertilization strategies, the single application of 120 kg N $ha^{-1}$ resulted in similar (Zn0, LS) or lower (S, Ch and HuFu) yield-scaled $N_2O$ emissions with respect to N120+60 (Fig. S1). Our findings were similar to those of Lebender et al. (2014), who reported the lowest yield-scaled $N_2O$ emissions occurring in winter wheat at intermediate N fertilizer rates (127-150 kg N $ha^{-1}$). 120 kg N $ha^{-1}$ can be recommended as the most advisable rate in our field experiment from both economic and environmental viewpoints, since it involves savings in synthetic N fertilizer (in comparison to 120+60), thus reducing $CO_2$ emissions associated with its transport and manufacturing (Lal, 2004). In addition, the highest N rate (120+60) significantly increased N surplus, as found by Rasmussen et al. (2015). This residual N in soil can result in N losses after rainfall events in summer or early autumn due to leaching (Gabriel et al., 2012) and $N_2O$ (pulsing effect, Sanchez-Martin et al., 2010; Liang et al., 2016).

*4.3 Methane uptake*

Several studies have noted that mineral N concentrations influence $CH_4$ aerobic oxidation (Tate, 2015). The meta-analysis of Aronson and Helliker (2010) reported that small amounts (<100 kg N $ha^{-1}$) of N tended to increase $CH_4$ oxidation, while larger amounts tended to inhibit methanotrophy. Indeed, we observed that fertilized plots

514 (N120 and 120+60) led to the highest rates of $CH_4$ uptake, but the differences were not
515 statistically significant.

516 Pramanik and Kim (2017) reported a reduction of $CH_4$ emissions as a result of
517 EDTA application in a submerged paddy field. They argued that the last enzyme-
518 mediated step of methanogenesis (reduction of methyl-CO-M to $CH_4$) was suppressed
519 as a result of the EDTA application. Conversely, non-flooded agricultural soils are net
520 $CH_4$ sinks since this GHG is oxidized by methane oxidizing bacteria (Stein et al., 2012).
521 In our experiment, which was performed under rainfed aerobic conditions, there was no
522 significant effect of Zn treatments on $CH_4$ fluxes. The aerobic oxidation of $CH_4$ depends
523 on several metal-containing enzymes (mainly Cu and Fe) (Glass and Orphan, 2012).
524 Therefore, it could be hypothesized that the addition of strong synthetic chelating
525 compounds could affect temporary $CH_4$ uptake due to the reduction of trace metals
526 available to methanotrophs. In this sense, two treatments, Ch and HuFu tended to
527 reduce cumulative $CH_4$ oxidation (Table 1). In the case of HuFu, the addition of small
528 amounts of a C source could explain this result, resulting in an increased methanogenic
529 activity (Le Mer and Roger, 2001) which was too small to cause significant differences.
530 Although not significant, this tendency affected (with respect to area-scaled $N_2O$ losses)
531 the obtained GWP, and the Ch treatment only significantly reduced GHG losses
532 compared to HuFu, S and LS (Table 1).

533 **Conclusions**

534 The key role of the management of N fertilization (rate, timing, source and
535 placement) for the reduction of $N_2O$ emissions has been broadly demonstrated so far.
536 However, our study also indicated that the application of micronutrient fertilizers can
537 also modify soil GHG fluxes. The application of some of these sources (i.e. synthetic
538 chelates) can be considered as a win-win strategy leading to $N_2O$ mitigation and Zn

biofortification. The chelating of metal co-factors (mainly Cu) of the enzymes involved in the nitrification and denitrification steps was the probable mechanism for the reduction of $N_2O$ emissions as bacterial *amoA, nirK, nirS* and *norB* gene abundances, as well as the extractable Cu content, decreased in this treatment. Accordingly, the abundance of *nosZ* gene, which encodes the reduction of $N_2O$ to $N_2$, increased in the presence of the synthetic Zn chelate. On the other hand, the Zn applied with humic and fulvic stimulated nitrifiying and denitrifying microbiota thereby increasing $N_2O$ fluxes. Our results indicated that the effect of Zn treatments on GHG emissions was source-dependent rather than Zn availability dependent. We demonstrated that the micronutrient fertilizer source can be used as a $N_2O$ mitigation strategy. The application of Zn via synthetic chelates and intermediate N rates (i.e. 120 kg N $ha^{-1}$) can result in an optimum balance between yield-scaled $N_2O$ mitigation and grain biofortification in soils with Zn deficiencies.

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**Figure captions**

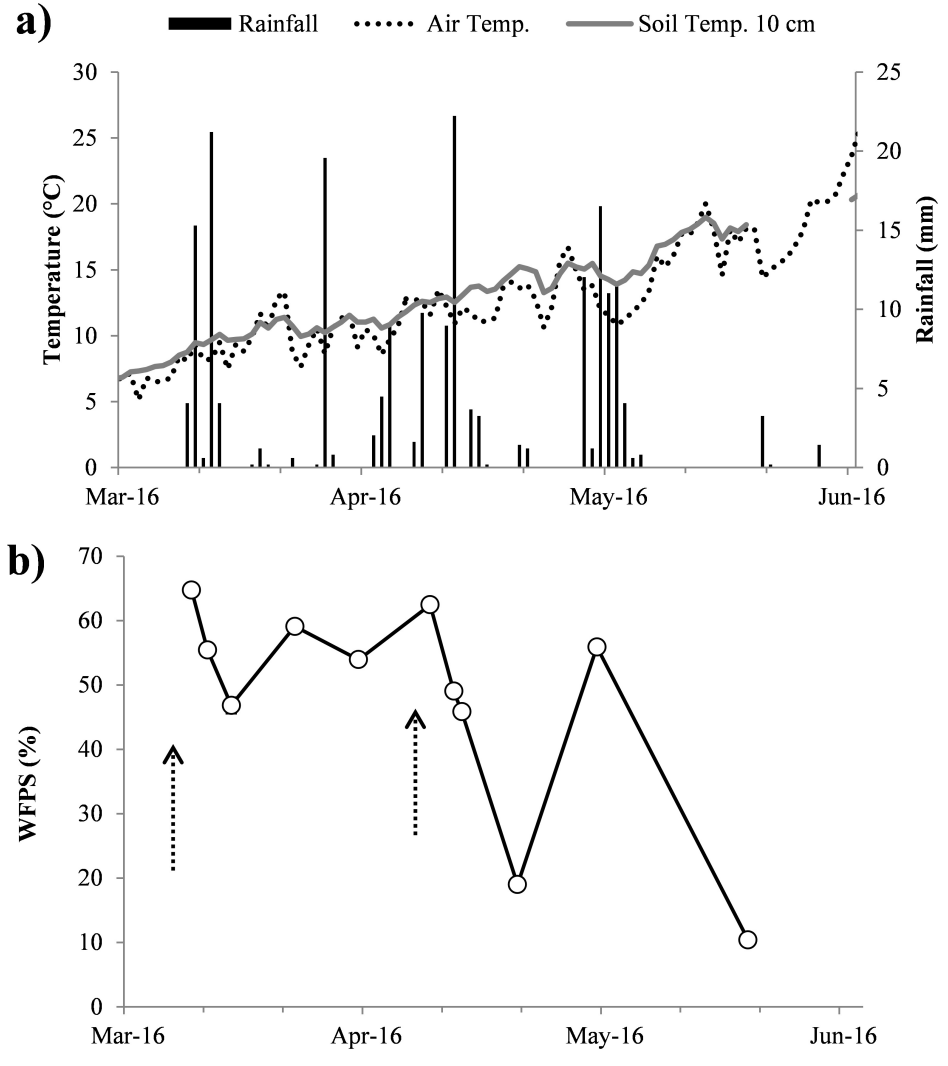
**Fig. 1** Daily mean air and soil temperatures (ºC) and rainfall (mm) (a), and soil water-filled pore space (WFPS, %) (b), during the experimental period. The dotted arrows indicate the dates of the fertilization events (23$^{rd}$ March and 22$^{nd}$ April).

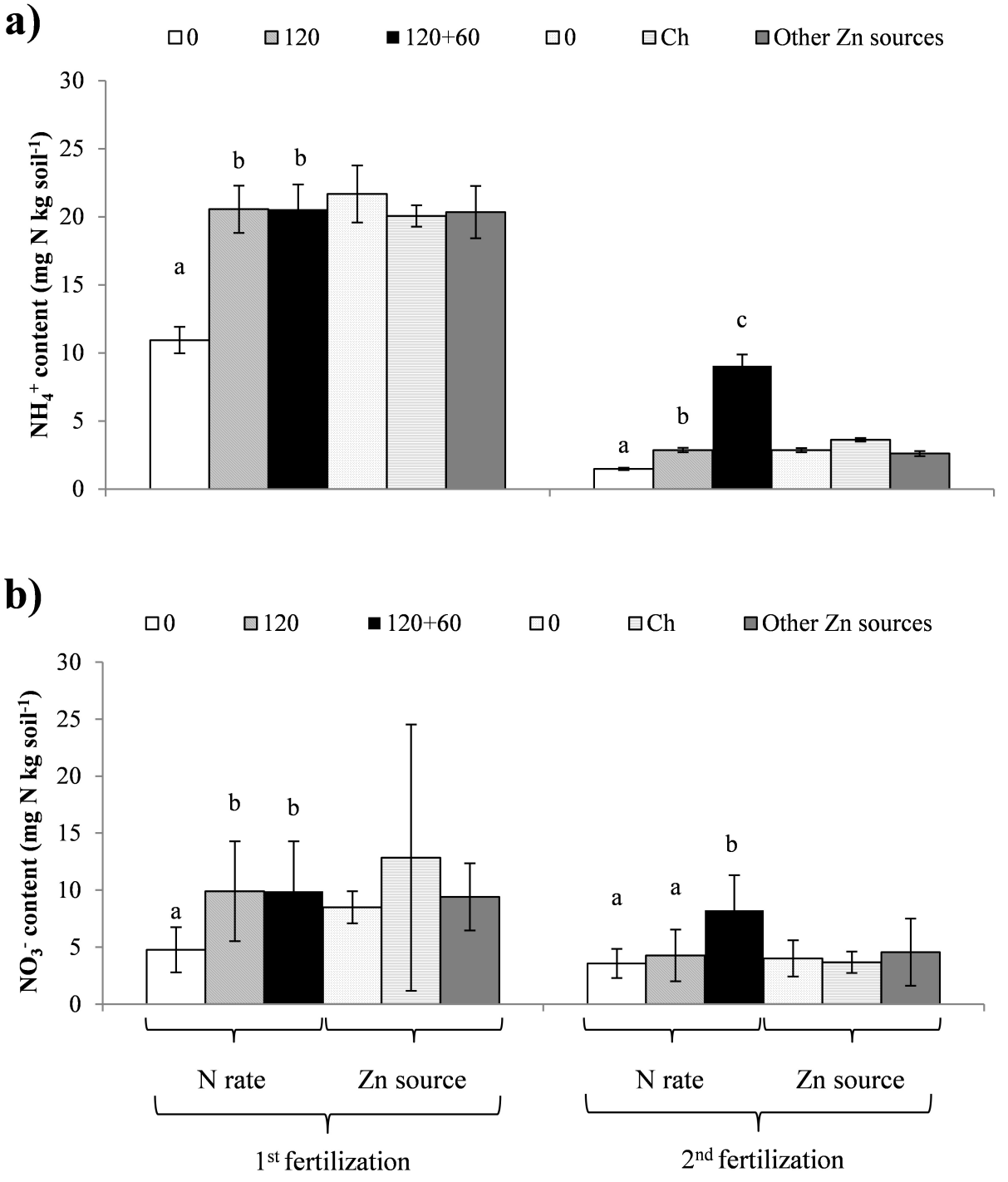
**Fig. 2** Average $NH_4^+$-N (a) and $NO_3^-$-N (b) concentrations in the 0–10 cm soil layer during the experimental period for the N application rates (0 kg N ha$^{-1}$, N0, 120 kg N ha$^{-1}$, N120, 180 kg N ha$^{-1}$, N120+60) and the Zn sources (control without Zn, 0, synthetic chelate, Ch, and other Zn sources, i.e. average of Zn-sulphate, Zn-lignosulphonate, and Zn-Humic/Fulvic acids). Statistical differences at $P < 0.05$ (LSD test) are presented by different letters. Vertical lines indicate standard errors.

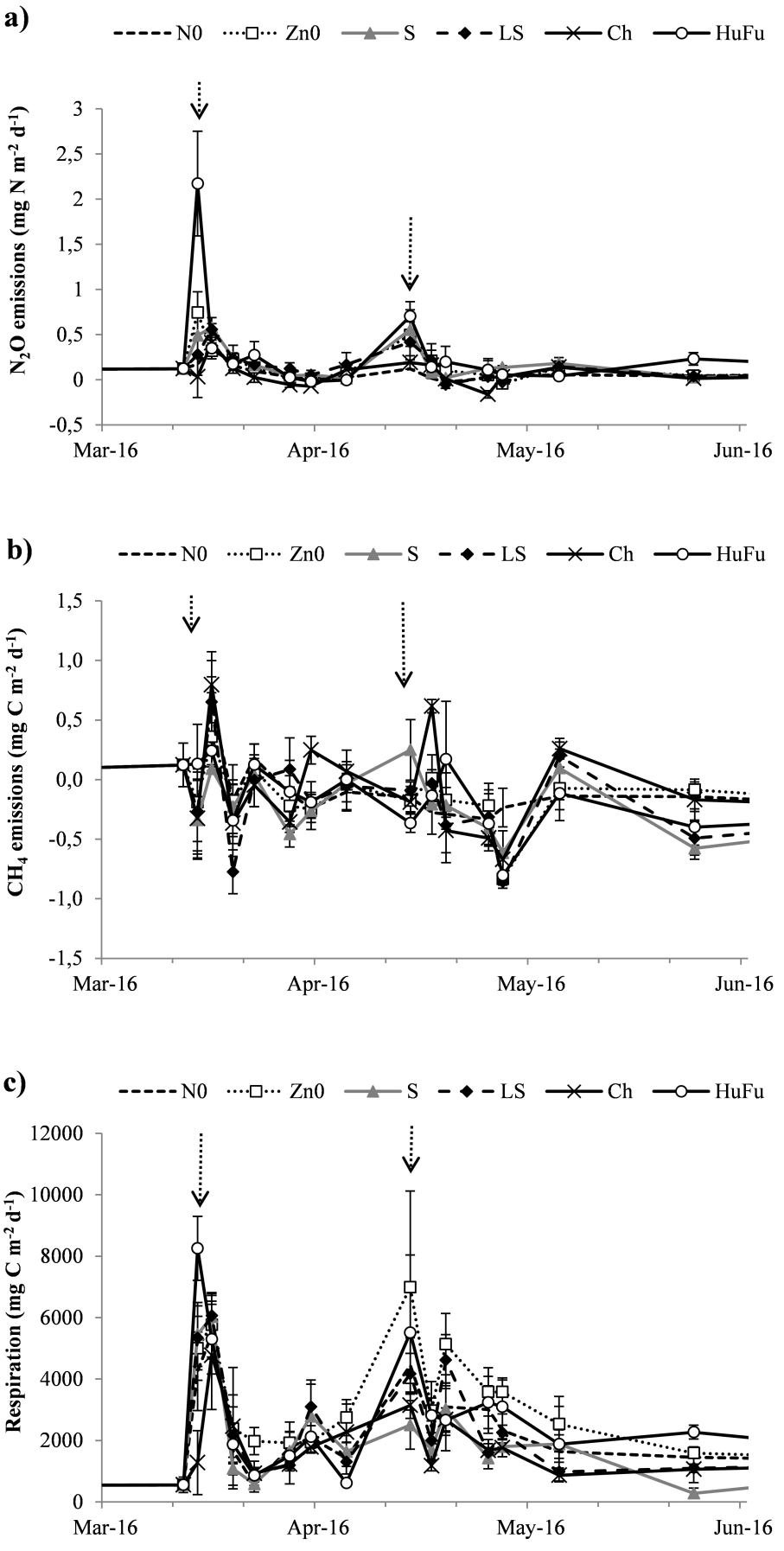
**Fig. 3** DTPA-extractable Cu (mg kg soil$^{-1}$) in soil with several different Zn fertilizers (control without Zn, Z0, Zn-sulphate, S, Zn-lignosulphonate, LS, Zn- DTPA-HEDTA-EDTA, Ch, Zn-Humic/Fulvic acids, HuFu) and different N application rates (0, 120 and 180 kg N ha$^{-1}$). Statistical differences at $P < 0.05$ (LSD test) are presented by different letters. Vertical lines indicate the standard deviation from the mean.

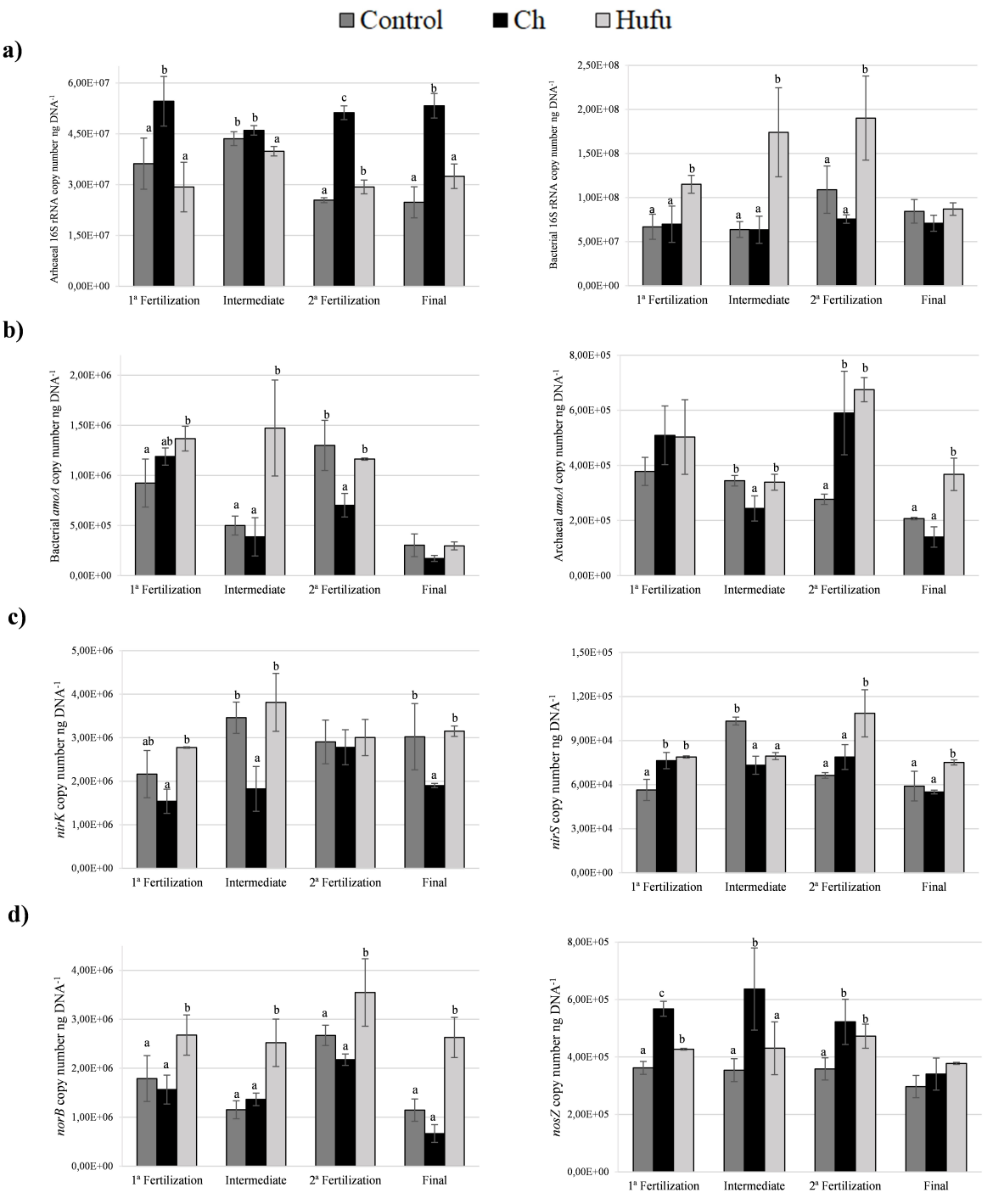
**Fig. 4** Daily $N_2O$ (a), $CH_4$ (b) and soil respiration (c) fluxes for N application rate of 180 kg N ha$^{-1}$ (N120+60) combined with different Zn sources (control without Zn, Z0, Zn-sulphate, S, Zn-lignosulphonate, LS, Zn- DTPA-HEDTA-EDTA, Ch, Zn-Humic/Fulvic acids, HuFu) and N0 treatment (0 kg N ha$^{-1}$). The dotted arrows indicate

889 the dates of the fertilization events (23rd March and 22nd April). Vertical lines indicate
890 standard errors.
891 **Fig. 5** Copy numbers of 16SB, 16SA (a), the *amoA* gene from AOB and AOA (b), *nirK,*
892 *nirS* (c) *norB* and *nosZ* (d) genes at both fertilization events, in the period between
893 fertilizations and before harvest for the N rate of 120 kg N $ha^{-1}$ and different Zn sources
894 (control without Zn, Zn0, Zn-DTPA-HEDTA-EDTA, Ch, Zn-Humic/Fulvic acids,
895 HuFu). Statistical differences at $P < 0.05$ (LSD test) are indicated by different letters.
896 Vertical lines indicate the standard deviation from the mean. The scale of the Y-axes
897 has been adapted in each case to improve the visualization of the data.
898 **Fig. S1** Significant ´Zn source x N rate´ interaction for Yield-scaled $N_2O$ cumulative
899 emissions for each N rate (0 kg N $ha^{-1}$, N0, 120 kg N $ha^{-1}$, N120, 180 kg N $ha^{-1}$,
900 N120+60) and Zn source (control without Zn, Z0, Zn-sulphate, S, Zn-lignosulphonate,
901 LS, Zn- DTPA-HEDTA-EDTA, Ch, Zn-Humic/Fulvic acids, HuFu) combinations.
902 Vertical lines indicate the standard error of the mean.
903 **Fig. S2.** Redundancy analysis (triplot) between the response variables: total abundance
904 of nitrifiers (AOB and AOA) and denitrifiers (*nirK*, *nirS*, *norB*, *nosZ*) genes represented
905 by black arrows and the explanatory variables: $NH_4^+$-N $NO_3^-$-N, $CO_2$, $CH_4$ and $N_2O$
906 emissions represented by grey arrows. A multicollinearity test was used to evaluate all
907 predictor and explanatory variables. Collinear variables were considered with variance
908 inflation factors (VIFs) > 10 and tolerances < 0.1. A Monte Carlo permutation test was
909 used to assess the statistical significance of each variable using CANOCO software
910 version 4.5 (Biometris, Wageninger, Netherlands).


a)
Rainfall
Air Temp.
Soil Temp. 10 cm
Temperature (°C)
Rainfall (mm)
30
25
20
15
10
5
0
25
20
15
10
5
0
Mar-16
Apr-16
May-16
Jun-16
b)
WFPS (%)
70
60
50
40
30
20
10
0
Mar-16
Apr-16
May-16
Jun-16


a)
0
120
120+60
0
Ch
Other Zn sources
$NH_4^+$ content (mg N kg soil$^{-1}$)
30
25
20
15
10
5
0
a
b
b
a
b
c
b)
0
120
120+60
0
Ch
Other Zn sources
$NO_3^-$ content (mg N kg soil$^{-1}$)
30
25
20
15
10
5
0
a
b
b
a
a
b
N rate
Zn source
N rate
Zn source
1st fertilization
2nd fertilization


a)
N0
Zn0
S
LS
Ch
HuFu
N2O emissions (mg N m-2 d-1)
3
2,5
2
1,5
1
0,5
0
-0,5
Mar-16
Apr-16
May-16
Jun-16
b)
N0
Zn0
S
LS
Ch
HuFu
CH4 emissions (mg C m-2 d-1)
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
Mar-16
Apr-16
May-16
Jun-16
c)
N0
Zn0
S
LS
Ch
HuFu
Respiration (mg C m-2 d-1)
12000
10000
8000
6000
4000
2000
0
Mar-16
Apr-16
May-16
Jun-16


Control
Ch
Hufu
a)
Archaeal 16S rRNA copy number ng DNA-1
6,00E+07
4,50E+07
3,00E+07
1,50E+07
0,00E+00
1ª Fertilization
Intermediate
2ª Fertilization
Final
Bacterial 16S rRNA copy number ng DNA-1
2,50E+08
2,00E+08
1,50E+08
1,00E+08
5,00E+07
0,00E+00
b)
Bacterial amoA copy number ng DNA-1
2,00E+06
1,50E+06
1,00E+06
5,00E+05
0,00E+00
Archaeal amoA copy number ng DNA-1
8,00E+05
6,00E+05
4,00E+05
2,00E+05
0,00E+00
c)
nirK copy number ng DNA-1
5,00E+06
4,00E+06
3,00E+06
2,00E+06
1,00E+06
0,00E+00
nirS copy number ng DNA-1
1,50E+05
1,20E+05
9,00E+04
6,00E+04
3,00E+04
0,00E+00
d)
norB copy number ng DNA-1
4,00E+06
3,00E+06
2,00E+06
1,00E+06
0,00E+00
nosZ copy number ng DNA-1
8,00E+05
6,00E+05
4,00E+05
2,00E+05
0,00E+00

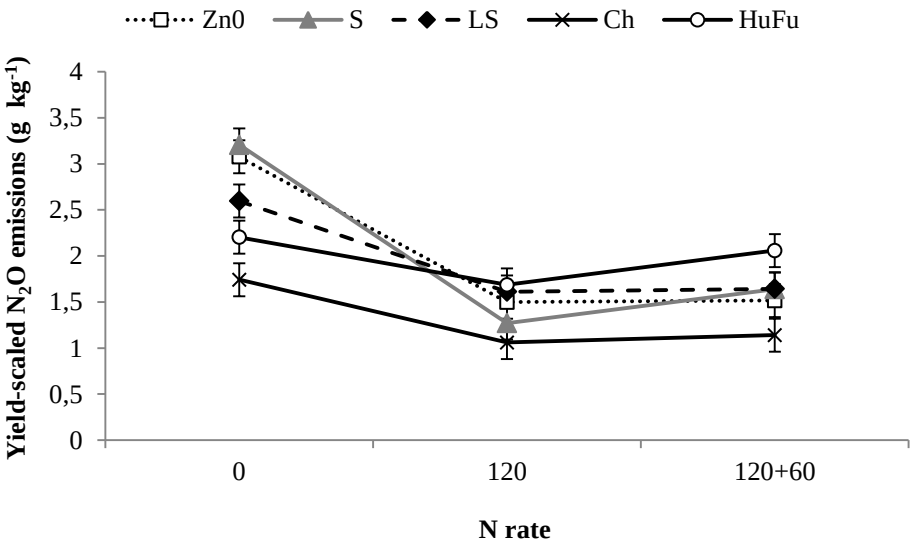



**Fig. S1.** Significant ´Zn source x N rate´ interaction for Yield-scaled $N_2O$ cumulative emissions for each N rate (0 kg N $ha^{-1}$, N0, 120 kg N $ha^{-1}$, N120, 180 kg N $ha^{-1}$, N120+60) and Zn source (control without Zn, Z0, Zn-sulphate, S, Zn-lignosulphonate, LS, Zn- DTPA-HEDTA-EDTA, Ch, Zn-Humic/Fulvic acids, HuFu) combinations. Vertical lines indicate the standard error of the mean.

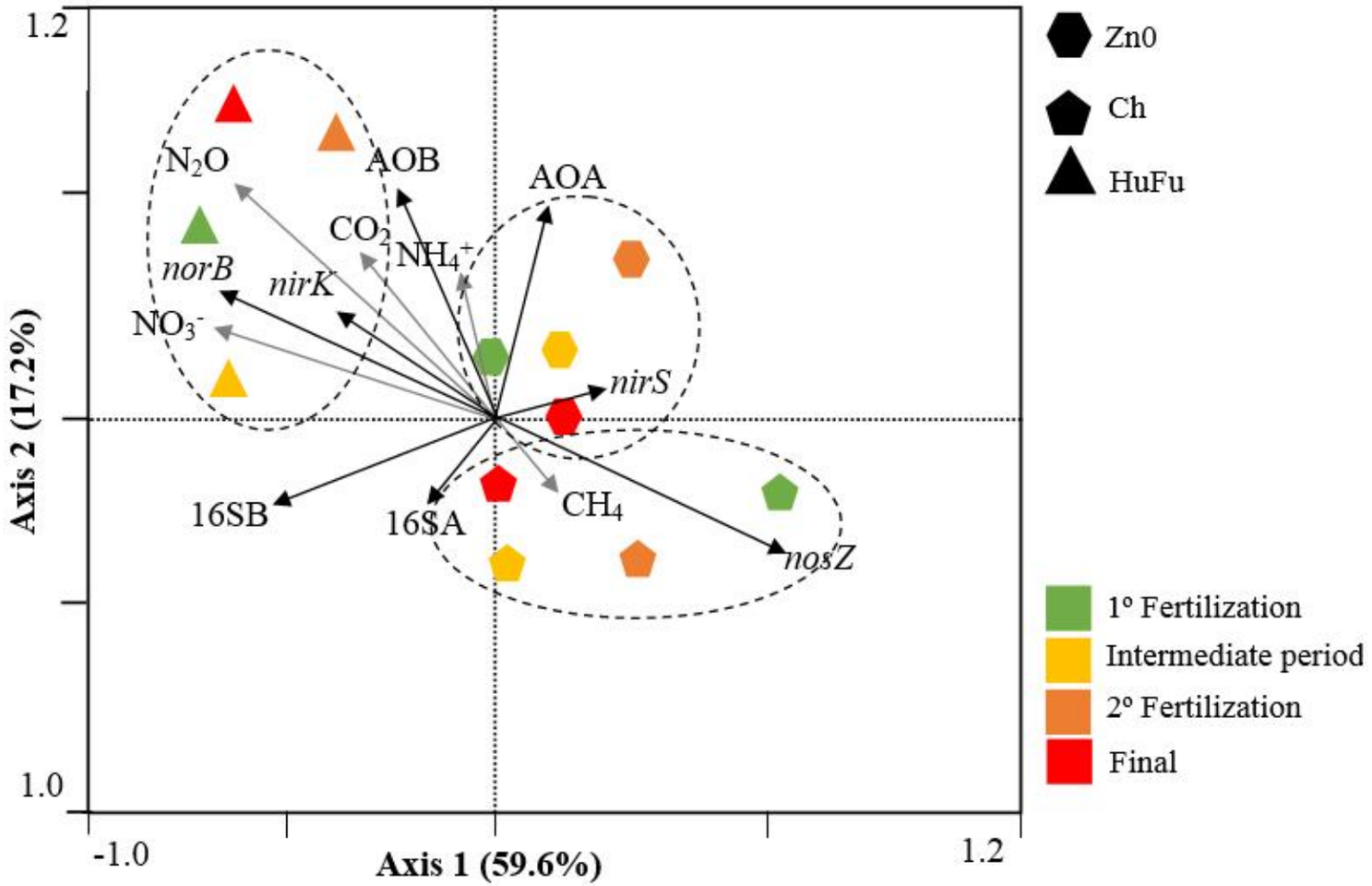



**Fig. S2.** Redundancy analysis (triplot) between the response variables: total abundance of nitrifiers (AOB and AOA) and denitrifiers (*nirK*, *nirS*, *norB*, *nosZ*) genes represented by black arrows and the explanatory variables: $NH_4^+$-N $NO_3^-$-N, $CO_2$, $CH_4$ and $N_2O$ emissions represented by grey arrows. A multicollinearity test was used to evaluate all predictor and explanatory variables. Collinear variables were considered with variance inflation factors (VIFs) > 10 and tolerances < 0.1. A Monte Carlo permutation test was used to assess the statistical significance of each variable using CANOCO software version 4.5 (Biometris, Wageninger, Netherlands).

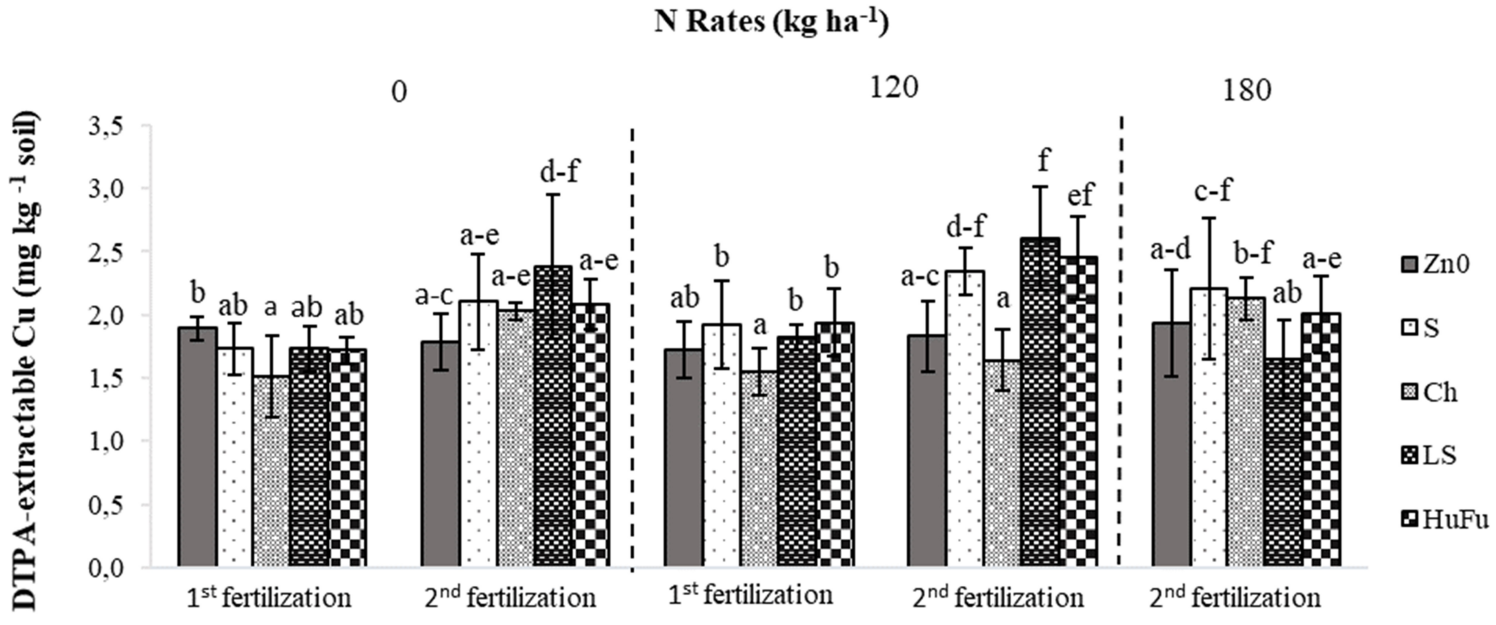

N Rates (kg ha-1)
0
120
180
DTPA-extractable Cu (mg kg -1 soil)
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
b ab a ab ab
a-c a-e a-e d-f a-e
ab b a b b
a-c d-f a f ef
a-d c-f b-f ab a-e
1st fertilization
2nd fertilization
1st fertilization
2nd fertilization
2nd fertilization
Zn0
S
Ch
LS
HuFu

**Table 1.** Total cumulative $N_2O$-N, $CH_4$-C and respiration fluxes, yield-scaled $N_2O$ emissions (YSNE), global warming potential (GWP) and N surplus with the different Zn sources (control without Zn, Z0, Zn-sulphate, S, Zn-lignosulphonate, LS, Zn- DTPA-HEDTA-EDTA, Ch, Zn-Humic/Fulvic acids, HuFu) and in three N application rates (0 kg N $ha^{-1}$, N0, 120 kg N $ha^{-1}$, N120, 180 kg N $ha^{-1}$, N120+60).

| Effect | $N_2O$ cumulative emissions (g N-$N_2O$ $ha^{-1}$) | Yield-scaled $N_2O$ emissions (g N-$N_2O$ kg $Nup^{-1}$) | $CH_4$ cumulative emissions (g C-$CH_4$ $ha^{-1}$) | Cumulative respiration (Mg C-$CO_2$ $ha^{-1}$) | GWP (kg $CO_2$ $ha^{-1}$) | N surplus (kg N $ha^{-1}$) |
|---|---|---|---|---|---|---|
| N rate | $P = 0.001$ | $P = 0.000$ | $P = 0.466$ | $P = 0.995$ | $P = 0.001$ | $P = 0.000$ |
| N0 | 205.6 a | 2.56 c | -151.4 | 2.75 | 50.2 a | -84.4 a |
| N120 | 243.8 b | 1.43 a | -198.5 | 2.77 | 59.1 ab | -51.6 b |
| N120+60 | 271.1 b | 1.60 b | -194.4 | 2.76 | 66.4 b | 7.7 c |
| S.E. | 12.0 | 0.08 | 29.4 | 0.16 | 3.3 | 4.8 |
| Zinc source | $P = 0.000$ | $P = 0.000$ | $P = 0.418$ | $P = 0.108$ | $P = 0.000$ | $P = 0.340$ |
| Zn0 | 232.3 b | 2.03 b | -202.4 | 3.11 b | 55.9 ab | -33.3 |
| S | 241.1 b | 2.04 b | -214.7 | 2.58 ab | 57.9 b | -40.2 |
| LS | 253.4 bc | 1.95 b | -207.5 | 2.88 ab | 61.3 b | -41.9 |
| Ch | 182.5 a | 1.31 a | -155.2 | 2.35 a | 44.0 a | -48.4 |
| HuFu | 291.6 c | 1.98 b | -127.4 | 2.87 ab | 73.7 c | -50.1 |
| S.E. | 15.5 | 0.10 | 38.0 | 0.37 | 4.3 | 6.3 |
| N rate x Zinc source | $P = 0.448$ | $P = 0.018$ | $P = 0.346$ | $P = 0.756$ | $P = 0.369$ | $P = 0.434$ |

Different letters within columns indicate significant differences by applying the LSD test at $P < 0.05$. Standard Error (S.E.) and P values are given for each effect.

**Table S1.** Copy numbers of 16SB, 16SA (a), the *amoA* gene from AOB and AOA (b), *nirK, nirS* (c) *norB* and *nosZ* (d) genes at both fertilization events, in the period between fertilizations and before harvest in the treatments with two N application rates (0 kg N $ha^{-1}$, N0, 120 kg N $ha^{-1}$, N120) amended with three Zn sources (control without Zn, Zn0, Zn-DTPA-HEDTA-EDTA, Ch, Zn-Humic/Fulvic acids, HuFu). Statistical differences at $P < 0.05$ (LSD test) are indicated by different letters.

| | | Gene copy number ng $DNA^{-1}$ | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **TREATMENT** | **TIME** | **Archaeal 16S rRNA** | **Bacterial 16S rRNA** | **Archaeal *amoA*** | **Bacterial *amoA*** | ***nirK*** | ***nirS*** | ***norB*** | ***nosZ*** |
| N0Zn0 | **1st Fertilization** | $1.15x10^7$ a | $9.78x10^7$ ab | $5.17x10^4$ a | $6.24x10^5$ b | $1.40x10^6$ b | $5.18x10^4$ a | $6.36x10^5$ a | $4.21x10^5$ c |
| N0ZnCH | | $2.75x10^7$ b | $5.97x10^7$ a | $1.62x10^5$ a | $6.36x10^5$ b | $7.80x10^5$ a | $4.94x10^4$ a | $7.29x10^5$ a | $2.83x10^5$ a |
| N0ZnHUFU | | $1.15x10^7$ a | $1.29x10^8$ b | $1.28x10^5$ a | $3.04x10^5$ a | $8.70x10^5$ a | $5.01x10^4$ a | $1.14x10^6$ ab | $3.52x10^5$ b |
| N120xZn0 | | $3.62x10^7$ b | $6.69x10^7$ a | $3.78x10^5$ b | $9.24x10^5$ c | $2.16x10^6$ c | $5.64x10^4$ a | $1.79x10^6$ c | $3.62x10^5$ b |
| N120ZnCH | | $5.46x10^7$ c | $6.98x10^7$ a | $5.09x10^5$ b | $1.19x10^6$ d | $1.54x10^6$ b | $7.64x10^4$ b | $1.56x10^6$ bc | $5.68x10^5$ c |
| N120ZnHUFU | | $2.93x10^7$ b | $1.15x10^8$ b | $5.03x10^5$ b | $1.37x10^6$ d | $2.77x10^6$ d | $7.88x10^4$ b | $2.68x10^6$ d | $4.27x10^5$ d |
| N0Zn0 | **Intermediate** | $3.17x10^7$ a | $6.98x10^7$ ab | $3.70x10^5$ b | $4.44x10^5$ a | $1.25x10^6$ a | $7.75x10^4$ a | $3.76x10^5$ a | $5.04x10^5$ bc |
| N0ZnCH | | $6.47x10^7$ d | $9.10x10^7$ ab | $3.60x10^5$ b | $4.80x10^5$ a | $2.06x10^6$ ab | $8.15x10^4$ a | $7.47x10^5$ ab | $4.45x10^5$ ab |
| N0ZnHUFU | | $3.87x10^7$ ab | $9.94x10^7$ b | $7.13x10^5$ c | $2.93x10^5$ a | $2.28x10^6$ b | $7.57x10^4$ a | $8.92x10^5$ bc | $4.59x10^5$ b |
| N120xZn0 | | $4.36x10^7$ bc | $6.38x10^7$ a | $3.44x10^5$ b | $4.99x10^5$ a | $3.46x10^6$ c | $1.03x10^5$ b | $1.15x10^6$ cd | $3.54x10^5$ a |
| N120ZnCH | | $4.60x10^7$ c | $6.35x10^7$ ab | $2.44x10^5$ a | $3.87x10^5$ a | $1.83x10^6$ ab | $7.32x10^4$ a | $1.37x10^6$ d | $6.37x10^5$ c |
| N120ZnHUFU | | $3.99x10^7$ bc | $1.74x10^8$ c | $3.39x10^5$ b | $1.47x10^6$ b | $3.81x10^6$ c | $7.94x10^4$ a | $2.52x10^6$ e | $4.31x10^5$ ab |
| N0Zn0 | **2nd Fertilization** | $4.89x10^7$ c | $7.71x10^7$ a | $3.80x10^5$ a-c | $5.01x10^5$ ab | $2.79x10^6$ | $8.48x10^4$ b | $7.44x10^5$ a | $4.64x10^5$ |
| N0ZnCH | | $4.98x10^7$ c | $7.45x10^7$ a | $1.14x10^6$ bc | $3.22x10^5$ a | $2.41x10^6$ | $7.35x10^4$ ab | $1.21x10^6$ ab | $4.46x10^5$ |
| N0ZnHUFU | | $3.38x10^7$ b | $1.01x10^8$ a | $2.43x10^5$ a | $5.08x10^5$ ab | $3.33x10^6$ | $1.28x10^5$ d | $1.68x10^6$ bc | $5.25x10^5$ |
| N120xZn0 | | $2.54x10^7$ a | $1.09x10^8$ a | $2.77x10^5$ ab | $1.30x10^6$ c | $2.90x10^6$ | $6.62x10^4$ a | $2.67x10^6$ d | $3.59x10^5$ |
| N120ZnCH | | $5.12x10^7$ c | $7.57x10^7$ a | $5.90x10^5$ c | $7.02x10^5$ b | $2.78x10^6$ | $7.88x10^4$ ab | $2.17x10^6$ cd | $5.22x10^5$ |
| N120ZnHUFU | | $2.93x10^7$ ab | $1.90x10^8$ b | $6.75x10^5$ c | $1.16x10^6$ c | $3.00x10^6$ | $1.09x10^5$ c | $3.55x10^6$ e | $4.72x10^5$ |
| N0Zn0 | **Final** | $2.56x10^7$ bc | $5.05x10^7$ a | $2.89x10^5$ bc | $2.02x10^5$ ab | $2.99x10^6$ | $8.43x10^4$ c | $8.05x10^5$ ab | $3.96x10^5$ c |
| N0ZnCH | | $2.73x10^7$ bc | $7.13x10^7$ ab | $2.63x10^5$ bc | $1.84x10^5$ a | $3.12x10^6$ | $8.32x10^4$ bc | $5.77x10^5$ a | $5.49x10^5$ d |
| N0ZnHUFU | | $1.24x10^7$ a | $8.61x10^7$ bc | $5.69x10^5$ d | $2.66x10^5$ a-c | $4.24x10^6$ | $6.86x10^4$ ab | $8.52x10^5$ bc | $2.92x10^5$ a |
| N120xZn0 | | $2.48x10^7$ b | $8.44x10^7$ bc | $2.07x10^5$ ab | $3.03x10^5$ bc | $3.02x10^6$ | $5.90x10^4$ a | $1.15x10^6$ c | $2.97x10^5$ ab |
| N120ZnCH | | $5.33x10^7$ d | $7.09x10^7$ ab | $1.40x10^5$ a | $1.71x10^5$ a | $1.90x10^6$ | $5.50x10^4$ a | $6.67x10^5$ ab | $3.41x10^5$ a-c |
| N120ZnHUFU | | $3.25x10^7$ c | $1.01x10^8$ c | $3.68x10^5$ cd | $3.95x10^5$ c | $3.15x10^6$ | $7.51x10^4$ bc | $2.63x10^6$ d | $3.78x10^5$ bc |