Biochar application as a win-win strategy to mitigate soil nitrate pollution without compromising crop yields: a case study in a Mediterranean calcareous soil

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ABSTRACT

Purpose The environmental benefits of biochar application, ranging from improvements in crop yield to global change mitigation, have been extensively studied in the last decade. However, such benefits have not been profusely demonstrated under a Mediterranean climate and still less in combination with high pH soils. In our study, the short- to medium effects of biochar application on a soil-plant system under Mediterranean conditions in an alkaline soil were assessed.

Material and methods Barley plants were grown in field mesocosms during three agronomical years at three biochar addition rates (0, 5 and 30 t ha^{-1}). Related to soil, different physico-chemical parameters were analyzed as well as microbial respiration, biomass and functional diversity. In the plant domain, *in vivo* ecophysiology variables such as leaf transpiration rate, stomatal conductance, and photosynthesis rate were determined while photosynthetic pigment content and soluble protein concentrations were measured in the laboratory. Additionally, crop yield and nutrient composition were also analyzed. The soil-plant connection was investigated by the N content ratio in both fractions establishing the nitrogen efficiency in the system.

Results and discussion The highest rate of biochar amendment enhanced soil moisture and electrical conductivity combined with an increase of SO_4^{2-}, Cl^-, Mg^{2+} and K^+, and decrease of NO_3^- and HPO_4^-. Notable variations regarding nutrition and moisture were induced in this Mediterranean alkaline soil after biochar addition although pH remained stable. Contrastingly, there were no major effects on microbial activity, but a lower abundance of the *nosZ* functional gene was found. Similarly, plant parameters were unaffected regarding chemical composition and ecophysiology although biochar induced a higher efficiency in the plant nitrogen uptake without increasing crop yield.

Conclusions Biochar addition at the highest rate (30 t ha^{-1}) reduced soil soluble nitrate although N uptake by the plant remained invariable, in turn coupled to no effects on crop productivity. Our study showed that, in a Mediterranean agroecosystem, a wood biochar produced by gasification was unable to increase crop yield, but enhanced soil water retention, decreased the need for N fertilization, and decreased soil soluble nitrate concentrations, something that could help to mitigate the excessive nitrate levels associated with over-fertilization.

KEYWORDS: crop yield; gasification biochar; plant efficiency; plant nutrition; soil nutrition

1 INTRODUCTION

Biochar is a carbon-rich product produced by the thermal processing of biomass and intended to be applied to soil for environmental management instead of being used for energy storage (Lehmann and Joseph 2015). Research on the environmental benefits of biochar application has been an important topic in the last decade in the fields of agronomy, global change and pollution mitigation, waste management and clean energy production (Lehmann and Joseph 2015). Application of biochar may modify physicochemical and biological soil properties of soil such as pH, electrical conductivity, cation exchange capacity, nutrient concentration, porosity and microbial community (Blanco-Canqui 2017; El-Naggar et al. 2018; Shaaban et al. 2018a; Sheng and Zhu 2018; Shi et al. 2018; Li et al. 2019). Regarding the agronomical benefits, it has been suggested that biochar leads to increased yields by enhancing water and nutrient retention and liming, with greatest effects on fertility in acid soils and those with coarse to medium texture (Jeffery et al. 2011). This explains why yield increases in tropical soils, which are acidic and with low cation exchange capacity, are disproportionately high in comparison to temperate soils, where crop yields are often already near their maximum potential (Jeffery et al. 2017). The information available for biochar effects on Mediterranean soils under non-irrigated field conditions is very scarce, despite their peculiarities. As indicative of the few examples of that, Olmo et al. (2014) reported higher grain and aboveground biomass wheat yields in an alkaline soil amended with a biochar made from olive-tree prunings, and Vaccari et al. (2011) also observed increased wheat yield in an acid soil. However, Marks et al. (2016) failed to find effects on a barley crop in an alkaline soil using the same biochar as in this study but at different application rates. It is worth noticing that biochar application does not provide enough nutrients to cover crop demands, so concurrent application of mineral or organic fertilization is generally implemented in experimental applications.

Some meta-analyses have shown that the increases in crop yields with biochar addition are coupled to higher soil microbial biomass, total C, N, K, and P contents, water retention, pH, and rhizobia nodulation (Jeffery et al. 2011, Biederman and Harpole 2013; Liu et al. 2018). However, while some studies have shown a 300% increases in yield (Cornelissen et al. 2013), and most have observed lower enhancements (around 10%), others have reported no effects or even negative responses (Jeffery et al. 2011). Despite the limited number of available studies to issue strong statements, in a recent meta-analysis it has been shown that positive effects would be expected between 5 and 50 t ha^{-1} (Jeffery et al. 2015), although factors such as soil type, management, biochar type (feedstock and pyrolysis procedure), crop type and local climate could modulate this response in each scenario (Kavitha et al. 2018; Liu et al. 2018).

Several soil-plant mechanisms have been proposed to explain such positive effects on crop yield (Jeffery et al. 2015; Kammann and Graber 2015): i) by direct provision of nutrients, though limited in most biochars, which explains why manure biochars provide better results than wood biochars; ii) by the reduced nutrient losses due to biochar cation exchange capacity; iii) by liming in acidic soils, since most biochars have neutral to alkaline pH; iv) by increasing water retention capacity, though there is a paucity of evidence; v) by increasing soil temperature; vi) by adsorbing pollutants; vii) by bulk soil or rhizosphere biological effects (community structure or function shifts); viii) by phytohormonal signaling interference (e.g. ethylene); and ix) by the induction of pathogen resistance. Regarding the negative effects on crop yield, N immobilization, excessive pH increases, release of phytotoxic substances such as sulphur or salts, and a reduction in pesticide efficacy, have been proposed as mechanisms (Jeffery et al. 2015).

Our aim was to assess the effect of biochar on a cereal crop growing in alkaline soil under Mediterranean conditions. For this purpose, we analyzed the effects of the application of a pine wood gasification biochar on soil-plant system dynamics, by comprehensively assessing crop yield and plant ecophysiological parameters, soil nutrient status, and the microbial community (abundance of some microbial functional genes). The study was carried out in large mesocosms placed outdoors under Mediterranean conditions and cropped to barley, the main crop in the area. The biochar effects were monitored along three cereal seasons according to the Mediterranean agronomic calendar (October-June) following biochar application. This study is of interest as it is centered on a relatively understudied type of biochar, under Mediterranean field conditions and in an alkaline soil, and assessing short- to medium effects.

2 MATERIALS AND METHODS

2.1 Soil and biochar properties

The soil of this study corresponds to the top layer (20 cm) of a loamy Typic Calcixerept used as the experimental agricultural soil and located in the Autonomous University of Barcelona campus (Cerdanyola del Vallès, Catalonia, NE Spain) (41°29'55.1"N, 2°06'07.5"E). The physicochemical properties of the studied soil are available in Table 1. The soil had been formerly used for grapevine and grain production and no pesticides had been applied for at least 5 years.

The biochar in this study was produced by gasification from *Pinus pinaster* and *P. radiata* wood chips. Details on its main properties and production system are described in Table 2. The biochar had a pH of 10.4, an electrical conductivity of 1,100µs cm^{-1} at 25° C, and a dry matter and C, N, S content (in %) of 95.8, 86.9, 0.16, and 0.22%, respectively. It had a relatively low volatile matter (VM) (8%) due to its elevated production temperature (Enders et al. 2012), and a moderate organic matter content for a wood biochar (88% by loss on ignition (LOI) at 375° C), as well as a 0.73% content of soot and around 1% of carbonates according to LOI at 1,100°C, which partly explains the high pH of this biochar.

2.2 Experimental setup

Twenty four field soil mesocosms were placed outdoors in the Autonomous University of Barcelona Campus in March 2011, each consisting of a 160 liter polypropylene box (53, 40.5 and 73 cm of inner height, width and length, respectively). The climate of the area is warm temperate, with dry and hot summers (Csa of the Köppen-Geiger climate classification system, Kottek et al. 2006) (Fig. S1). The bottom of the box had six holes (5 cm-diameter) and was covered by a 2-mm plastic mesh to allow water drainage and reduce soil losses, respectively. Each box was filled with a 20 cm soil layer mimicking a B horizon, and then an Ap horizon consisting of a 23 cm soil layer with or without biochar was added, to an initial soil volume of 127 liters. Due to the lower density of biochar compared to soil, the Ap layer corresponded to 87, 85, and 77 kg (dw) of soil or soil-biochar mixture containing 0, 0.216 and 1.4305 kg of biochar, respectively, and equivalent to a 0, 5, and 30 t ha^{-1} biochar addition rates. Eight mesocosms were prepared for each biochar application rate. The mesocosms were positioned in two rows to enhance their thermal isolation, and west-to-east oriented to ensure similar sunlight exposure. After their construction, a feed barley (*Hordeum vulgare* L. Graphic variety) purchased at RAGT (Palencia, Spain), was annually seeded at a density of 300 seeds m^{-2} (116 seeds per mesocosm), and cropped in June or July depending on the year. A pig slurry was also added annually as fertilizer at a 100 kg N ha^{-1} $year^{-1}$ rate, the usual practice in the area, estimated based on its hydrolysable (labile) N content (see Table S1). Annual fertilization (100 kg N ha^{-1} applications) was split into two: one in early March together with seeding and the other in late April to promote seedling growth. This corresponded to a total annual application of 37.5 g of pig slurry per mesocosm.

2.3 Soil sampling and analysis

Soil samplings were carried out on the 11th of March 2011 (when biochar was supplemented), at mesocosms setup, and on the 12th June 2011, 28th March and 5th June 2012, and on the 11th March and 4th June 2013 (Fig. S1). The sampling was carried out by collecting a single soil core 5.5 cm diameter x 7 cm height, which was then air dried in the laboratory and sieved to 2 mm.

Soil samples (50 g) were water-saturated for 2 h and then drained for 24 h at room temperature. Moisture was calculated as the weight loss after drying at 105ºC overnight as follows: moisture (%) = ((FW – DW)/DW) x 100), where FW=fresh weight and DR=dry weight.

A 1:5 (w/v) aqueous extract was prepared by adding 75 ml of deionized water to 15 g of soil and then vertically agitating them in 150 ml polyethylene cups for 2 h at 60 rev min^{-1}. Then, the extract was subsequently centrifuged and the supernatant was filtered through Whatman #42 paper filters. pH and conductivity were immediately determined in those extracts, while a 10 ml aliquot was taken and diluted to 1:10 and then stored frozen for the determination of ion content in all the samples at the end of the experiment. In the last case, soluble Ca^{2+}, K^+, Mg^{2+}, Na^+, and NH_4^+ were assessed with a CS12A Dionex cation column on a Dionex ICS-1100 ion chromatograph (Dionex, Sunnyvale, USA), while Cl^-, NO_2^-, NO_3^-, HPO_4^-, and SO_4^{2-} were measured in a AS4A-SC Dionex anion column on a Dionex DX-100 ion chromatograph (Dionex, Sunnyvale, USA).

The exchangeable NH_4^+ in soil (10 g) was extracted by shaking with 50 ml of 2 M KCl (Maynard et al. 2007) and the ammonium content determined by the colorimetric method in Forster (1995).

2.4 Soil microbial respiration, biomass and functional diversity

All soil microbial parameters were analyzed 3, 12, 18, 24 and 30 months after the biochar addition, as detailed in the previous section.

Soil microbial basal respiration and microbial biomass carbon were determined using an aliquot of 30 g soil samples stored at 4ºC. The soil basal respiration (BAS) was evaluated in gas traps following the protocol of Pell et al. (2006). The same sample was then used to estimate microbial biomass by the fumigation-extraction method according to protocol of Brookes and Joergensen (2006). Microbial biomass carbon (MB) was calculated as MB = E / 0.38, where E is the difference between organic carbon extracted from fumigated soil and from non-fumigated soil, and 0.38 is the conversion factor from E into microbial biomass carbon. Finally, the metabolic quotient (qCO_2) was calculated as qCO_2 = (µg CO_2-C g soil^{-1} hour^{-1} / µg MB_c-C g soil^{-1}) (Anderson and Domsch 1990).

The functional diversity was analyzed from soil aliquots stored at -20ºC. Total DNA of soil samples was extracted using the MoBio ultraclean DNA soil kit (MoBio, Laboratories Inc., CA, USA) according to the manufacturer's instructions. DNA concentration and quality were spectrophotometrically controlled by NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA), and visually by agarose gel electrophoresis. Quantitative polymerase chain reaction (qPCR) was performed to assess the abundance of the following genes: *amoA* for the ammonia-oxidizing bacteria (AOB) and archaea (AOA); *nxrB* for the beta sub-unit of nitrite-oxidase of *Nictrobacter* sp.; *nirK* and *nirS* for nitrite reducers to gaseous nitric oxide carrying a nitrite reductase enzyme; *nosZ* for denitrifiers carrying the nitrous oxide reductase enzyme and; *nifH* for N_2-fixing microbes to reduce it to NH_4^+. Representative genes of the microbial nitrogen cycle are detailed in Hagemann et al. (2016). All the qPCR were conducted in 96 well plates using 7900HT Fast Real-Time PCR System (Applied Biosystems, CA, USA). The specific primer combination, qPCR conditions and source of standard used for each gene are shown in Table S2. Single PCR reactions were prepared in 20 µl of final volume containing SYBR Green qPCR Master Mix (Biotools B&M Labs S.A., Madrid, Spain), forward and reverse primer (10 µM, 0.5 µl each) (Metabion International AG, Planegg-Martiensried, Germany); dimethyl sulfoxide, DMSO (0.5 µl), (Sigma-Aldrich, MI, USA); H_2O, and 5 µl template DNA (4 ng µl^{-1}). Specificity of the fluorescence signal was confirmed by the melting curve analysis of the PCR products at the end of each run. The correct size of amplicons was also checked by agarose gel (2%). Amplification efficiencies, slope and R^2 of each qPCR assay are reported in Table S3.

2.5 Plant sampling and analysis

A single annual sampling was carried out for the germination, physiological, and yield measurements, each carried out at different stages of barley development. Both the laboratory and the field physiological parameters were determined together, at a similar crop stage, when plants had completely emerged ears and fully developed flag leaves, which, depending on the climatic conditions each year, corresponded to late April to early June. Plant germination was assessed around March, between the development of the first shoot and the growth of the first tiller.

Regarding the field measurements, the chlorophyll activity (F_v / F_m ratio), a measurement of the maximum potential quantum efficiency of Photosystem II, was assessed using a PAM-210 Chlorophyll Fluorometer

(Heinz Walz GmbH, Germany). Three leaves from three different plants per mesocosm were selected in each mesocosm. For each leaf, around 5 cm of the central part of the leaf was wrapped with aluminum foil for 20 min to provide dark conditions and to stop photosynthesis. Thereafter, the leaf's upper side was placed in the fluorometer without removing the aluminum foil, to prevent the exposure to light, and only then removed in order to measure the initial (F_0) and the maximum (F_m) fluorescence. Then, the fluorescence variation (F_v) was calculated as [$F_v = F_m - F_0$], required for the calculation of the F_v / F_m ratio. The leaf transpiration rate (E), the stomatal conductance (g_s), and the photosynthesis rate (A), were measured with an LCpro portable infrared gas analyzer (ADC BioScientificLTd, Hoddesdon, EN, UK). To integrate the rate of CO_2 assimilation and the water lost by transpiration, the intrinsic water use efficiency (iWUE) was calculated by the A/g_s ratio. The measurements were carried out in early June 2011, late April 2012 and early May 2013, when plants had completely emerged ears and fully developed flag leaves. The flag leaf of three to four plants per mesocosms were measured at each sampling. The records for E, g_s, and A in May 2013 had to be discarded due to technical problems during data acquisition.

Regarding the laboratory measurements, the photosynthetic pigment content and the soluble protein concentrations were assessed in the flag leave of three randomly selected barley plants per mesocosm. The samples were collected on the same dates in which the field measurements were performed. In each flag leaf, four 1 cm-leaf disks were cut using a cork-borer. Two of the disks were immediately immersed in liquid nitrogen and then stored at -80°C for further analysis. The other two were dehydrated at 60°C for3 days for the assessment of leaf dry weight. The frozen discs were homogenized on 1 ml of bicine buffer (pH 8) (Lawlor et al. 1989) with a mixer. The whole process was carried out on ice and under soft light to avoid degradation of pigments and proteins. Chlorophyll a and b (Chla + b), and carotenoids were determined according to Lichtenthaler and Welburn (1983). A sample of 100 µl of the homogenate was mixed with 900 µl of absolute ethanol. After 10 min on ice and in the dark, the mix was centrifuged at 12,000 g for 2 min. The absorbance of supernatant was measured at 470, 649 and 665 nm for pigment concentration (mg l^{-1}). Soluble proteins were measured following the method described by Bradford (1976). After centrifugation of the resting volume (900 µl) at 12,000 g for 2 min, a sample of 20 µl of the supernatant was mixed with 4 ml of Bradford reagent (1:5) (Bio-Rad Laboratories GmbH, Munich, Germany). Absorbance was measured at 595 nm after 5 min and protein concentration (mg/l) was calculated by comparison to a standard curve from 0 µg up to 100 µg of bovine albumin (BSA) (Amresco, Ohio, USA). Pigment and protein contents were transformed based on dry weight to avoid the complications of changing water content.

The yield was assessed at barley harvesting between mid-June and early July, when plants were totally developed and senescent. All the aerial biomass was collected, and in the laboratory, the straw and the ears were manually separated, dried at 70ºC for 48 h, and weighed. The number of seedlings and ears per plot were also determined. The average ear weight and straw weight per plot were estimated by dividing their weight by their numbers in each mesocosm. The number of grains per ear was assessed by counting them in 20 randomly selected ears from each mesocosm. The quantitative yield results in 2012 were discarded due to the biasing effect of predation by wild boars in some of the microcosms.

The nutrient uptake was assessed by grinding the straw and the ears to 1 mm and then analyzing their macro and micronutrient content by near infrared reflectance spectroscopy (NIRS), by scanning the ground samples from 1,100 to 2,500 nm using a NIRSystems 5000 scanning monochromator (FOSS, Hilleröd, Denmark). Reflectance was recorded in 2 nm steps, which gave 692 data points for each sample, as log (1/R), where R represents reflected energy. The samples were scanned in duplicate using closed ring cup cells and the mean spectrum was calculated for each sample. The calibration process was performed according to the procedure described by Foskolos et al. (2015). A more detailed description of this procedure can be found in the Supplementary Material (Tables S4 and S5). A random subset of samples (34 straw and grain ground samples) was used for NIRS calibration, analyzed by the following reference methods: N by the Kjeldahl method, and Ca, Fe, K, Mg, Mn, P, S, and Zn by ICP-OES in an Optima 3200 R (Perkin-Elmer, Norwalk, CT, USA). The N efficiency of the plant-soil system was evaluated using three different parameters. The first one was defined as the percentage of water-soluble N-NO_3^- concentration in soil related to the plant N content (in %). The other two parameters were the nitrogen-accumulation efficiency (NAE) and the nitrogen-use efficiency (NUE) according to the definition of Sembiring et al. (1998). The nitrogen-accumulation efficiency was calculated as NAE = (Ns – Nsc) / (Nc - Ncc); where Ns was the total N-NH_4^+ and N-NO_3^- accumulated in soil profile of the biochar applied plots, Nsc was the total of N-NH_4^+ and N-NO_3^- accumulated in soil of non-amended plots, Nc was the N removed in crop of fertilized plots and Ncc the N removed in crop of non-amended plots. The nitrogen-use efficiency was calculated as NUE = (Nc - Ncc) + (Ns - Nsc) using the same nomenclature above. The nitrogen efficiency refers to the whole plant (straw and grain).

2.6 Statistics

The experiment was conducted in a field soil mesocosm where three biochar addition rates (0, 5 and 30 t ha^{-1}) were applied. Eight replicates per treatment were setup randomly assigned. Finally, a set of twenty four data was statistically analyzed for all the parameters. Physiological factors were measured in three plants as technical replicates but the mean per plot was calculated to reduce pseudoreplication. Data were analyzed by the software Statistica 7.0 (Stat Soft, Inc. OK, USA). Normal distribution was checked by the Kolmogorov-Smirnov test. Data that did not conform to a normal distribution were transformed with logarithm corrections before applying parametrical tests. To check the statistical differences among groups, a one-way ANOVA with repeated measures was used to compare the biochar treatments along the experiment, and using the mesocosm identity as subject. The post-hoc test of Bonferroni was used for pairwise comparisons between treatments within each sampling. Differences at $p < 0.05$ were considered significant. Statistical results of the one-way ANOVA with repeated measures are detailed in Tables S6-S17. Differences of the post-hoc test of Bonferroni are shown with different letters in the graphics.

3 RESULTS

3.1 Biochar effect on soil

The physical and chemical soil parameters are shown in Fig. 1 and Fig. 2. The application of pine wood chips biochar to this soil at the two rates tested (5 and 30 t ha^{-1}) raised the moisture compared to non-amended control soil (Fig. 1a). This effect was present at the highest rate (30 t ha^{-1}) in most samplings, although this was generally not observed in the summer samplings. The electrical conductivity was also higher in amended soils but the response was only associated with the highest application rate and restricted to the three months following the application (Fig. 1b). Before the biochar addition, the pH of the experimental soil was already basic (8.3, Table 1) and remained globally stable regardless of the quantity of added biochar (Fig. 1c), despite the 11.4 pH of this pine-gasified wood (Marks et al. 2014). A significant difference was only observed at the low dose of biochar three months after the application. However, this effect reverted in the following measurements.

The ionic content of the biochar-applied soils was analyzed along 2011-2013 and some significant differences among treatments were observed (Fig. 2). Namely, the supplementation of high amounts of biochar on this soil (30 t ha^{-1}) induced a statistically significant reduction of NO_3^- and HPO_4^- three months after the addition, in a trend that disappeared after one year of the application (Fig. 2a, b). On the contrary, SO_4^{2-} and Mg^{2+} increased their levels in soil after high biochar application (Fig. 2c, d), while this was also observed for K^+ and Cl^- (Fig. 2e, f), but with increases persisting slightly over a year. Other mineral components (Ca^{2+}, Na^+, NH_4^+ and NO_2^-) were non-significantly different between biochar treatments along the three years of the study (Fig. S2).

3.2 Biochar effect to the soil-microbial parameters

In Fig. 3, the effect of biochar addition three months after amendment is shown (11th June 2011), the only sampling with statistically significant differences.

The microbial biomass carbon and microbial activity based on the soil basal respiration remained unaltered three months after biochar application (Fig. 3a, b). The ecophysiological state of microbial biomass represented by the metabolic quotient was also stable regardless of the amount of applied biochar (Fig. 3c). However, the diversity of functional groups changed significantly three months after soil application of high amounts of biochar (Fig. 3d). Specifically, the copy number of the *nosZ* gene, mediating the last step of denitrification process, was reduced in our experiment. The second step of denitrification, nitrite reduction to gaseous nitric oxide (NO) catalyzed by *nirS* or *nirK* was also marginally reduced ($p < 0.1$). A decrease of *nirK* gene copies was detected in plots with 30 t $ha^{-1}$of biochar. In fact, there was a generalized tendency to reduce the microbial transformation processes of the N cycle when soil was amended with the highest quantity of biochar.

3.3 Biochar effect on crop yield and quality

3.3.1 Crop physiology

None of the studied physiological parameters revealed that plant growth on biochar-amended soils were significantly affected (Fig. 4).The concentration of key components of the primary plant metabolism, such us chlorophyll and proteins, showed similar values in biochar-applied and control soils (Fig. 4a, b). The activity of chlorophylls, analyzed by the F_v / F_m ratio, was also stable regardless of the quantity of biochar added to soil (Fig. 4c). Finally, the intrinsic water use efficiency, ratio of the photosynthesis rate (A), and the stomatal conductance (g_s), confirmed the lack of a biochar effect on the basic physiological functioning of barley plants with biochar addition in our plots (Fig. 4d).

3.3.2 Crop performance

The highest input of biochar increased the number of seedlings per plot in the first year (Fig. 5a). However, this higher germination rate was not observed in the other two analyzed years. Similarly, the crop performance or productivity, measured as the ear total weight per plot (Fig. 5b), did not suffer from variations along the studied period. Three extra parameters related to crop production (ear number per plot, number of grains per ear, and straw weight per plot) were also analyzed and no yield differences were observed among treatments (Fig. S3).

3.3.3 Crop nutrient content and uptake efficiency

The nutrient analysis of barley (either for straw and grain) revealed no differences in plants growing at 0, 5 and 30 t biochar ha^{-1}, neither for macronutrients nor for micronutrients. The levels of nitrogen in mature plants (straw and grain) (Fig. 6a) showed a similar content among biochar-applied soils and non-amended soil. Minimal differences, in no case significant, were observed for the rest of elements (Fig. S4-S5). However, the soil-to-plant N content ratio was estimated, herein referred as nitrogen efficiency, indicating that nitrogen uptake was hindered, as lower values were observed in the first and second year in the 30 t ha^{-1} treatment (Fig. 6b). These trends disappeared in the third year following the biochar application. The N efficiency of the plant and soil system was further investigated using the Sembiring et al. (1998) coefficients of the nitrogen-accumulation efficiency (NAE) and nitrogen-use efficiency (NUE). The addition of biochar triggered a small and negative NAE ratio for the three studied years (Fig. 6c). The lower amount of NO_3^- in amended plots (5 and 30 t ha^{-1}) compared to control plots was the main cause of the negative response for this ratio. However, the nitrogen-use efficiency showed variable values along the time period with negative percentages for the first and third year but positive for the second one (Fig. 6d). The lower amount of N in soil amended plots compared to control plots was balanced by the N content in the crop.

4 DISCUSSION

In this work, the effect of biochar amendment to a barley-cultivated soil in a Mediterranean ambient was investigated along three agronomical seasons. After the biochar addition, physical and chemical variables of the soil were studied and its effect on the soil biota and the crop yield and quality. All the analyzed parameters were marked by important inter-annual variability, mostly explained by the rainfall differences among years (Fig. S1). The Mediterranean climate regions are characterized by a high inter-annual variability in precipitation that influences the capacity to sustain the biotic systems in this biome (Cid et al. 2017) potentially explaining the contrasting effects of biochar among years. An example of that was in 2011, when higher emerged seedling rates on plots amended at the higher biochar addition rate were recorded, while the remaining crop parameters were unaffected (Fig. 5a). This result was coupled with a high precipitation episode (around 80 mm) registered after the seed sowing that year (Fig. S1).

The biochar in this study was produced by gasification of pine wood chips at high production temperatures, which yields a very stable material with moderate organic carbon content and that is highly alkaline. One of the described soil impacts induced by the addition of biochar is the alkalization of soil pH (Atkinson et al. 2010; Yuan et al. 2011; Shi et al. 2018), but the soil in this study has an already basic pH (8.3) and hence was globally unaffected by the addition of biochar. However, other described changes such as the increase of moisture and electrical conductivity were registered in biochar amended soils at least 3 months after the application, in agreement with other studies (Singh et al. 2010; Karhu et al. 2011; Saarnio et al. 2013; Blanco-Canqui 2017).

Similarly, biochar supplementation altered the soluble ionic content of the receiving soils by increasing the levels of SO_4^{2-}, Cl^-, Mg^{2+} and K^+, and decreasing the concentration of NO_3^- and HPO_4^- (Fig. 2). The increment on some mineral elements can be directly linked to the contents of these components in the biochar or the feedstock (Atkinson et al. 2010), while the decreases in some elements might be explained by different mechanisms, involving increased soil inorganic N assimilation, accelerated losses by NH_3 volatilization and/or increased plant N uptake (Liu et al. 2018). Another plausible mechanism could be the enhanced retention of cations (and anions) based on the highly porous nature of biochar. Porosity combined with the small particle size in most part of biochars provides a large surface area for the direct or indirect retention of anions and cations, respectively (the last by bridging) (Joseph et al. 2010; Lehmann and Joseph 2015). This could be in turn influenced by or associated with biochar aging in the specific case of soluble N forms (Singh et al. 2010; Wang et al. 2012). As an example, it has been suggested that nitrate could be retained through bridge-bonding with divalent cations or trivalent metals associated with the biochar surface (Mizuta et al. 2004; Tsukagoshi et al. 2010). Ventura et al. (2013), who also found reduced nitrate contents in biochar plots, hypothesized that the main mechanism is ammonia volatilization in the strong alkaline environment generated around the biochar, which would compete with nitrate production by nitrification. Phosphate, that also dropped in our experiment with biochar adition, has been observed to be strongly absorbed by biochar due to their natural Mg and Ca content (Gunther et al. 2018). Bridge-bonding with divalent cations is a plausible explanation as the Mg content is highly increased on the 30 t ha $^{-1}$ treatment (Fig. 2d). Contrastingly, a recent meta-analysis associated biochar application with a significant enhancement (45%) of the soil available P, with the C:N ratio and biochar feedstock being the key factors related to this positive effect (Gao et al. 2019). This divergent result, far of the scope of this study, will deserve a deeper analysis in further experiments to fully understand the drop of HPO_4^- in this biochar-soil system.

Our results confirmed that the addition of biochar globally affects the physicochemical properties of the soil. However, the biological response is not in agreement with those changes, as shown by the lack of effects on microbial biomass and activity of the microbiome (Fig. 3a-c). Biochar has been often associated with modifications of the microbial community (Noyce et al. 2015; Mierzwa-Hersztek et al. 2017; Sheng and Zhu 2018) although the absence of microbial effects under field conditions has also been widely reported (Castaldi et al. 2011; Scheer et al. 2011; Zhang et al. 2012; Ameloot et al. 2013). In a similar study (Marks et al. 2016), carried out in analogous Mediterranean conditions, using the same biochar and a similar alkaline soil, these authors failed to find significant effects on soil microbial biomass, respiration, and metabolic coefficient. In our study, the only apparent effect was observed at the higher biochar application rate, which induced a significantly lower abundance of the *nosZ* functional gene in the bulk soil, responsible for the last step of the denitrification process where N_2O is reduced to N_2 (Fig. 3d). This functional gene pertains to the denitrifier soil microorganisms functional group, that catalyze the stepwise reduction of NO_3^- to N_2 by the functional genes *narG* and *napA* (nitrate reductases), *nirK* and *nirS* (nitrite reductases), *norB* (nitric oxide reductase), and *nosZ* (nitrous oxide reductase), respectively (Philippot et al. 2007; Harter et al. 2017; Kuypers et al. 2018). Since the abundance of *nirK* /*nirS* genes in bulk soil was not significantly affected along the 96 days after the biochar application in our study, the lower abundance of the *nosZ* functional gene in June 2011 would ~~be~~ unlikely be associated with a decreased catalysis of N_2O to N_2. This situation would suggest a higher accumulation of N_2O, similar to the results of Sanchez-Garcia et al. (2014), although numerous studies have related biochar with the mitigation of nitrous oxide emissions via denitrification (Cayuela et al. 2014; Ameloot et al. 2016; Harter et al. 2017; Liu et al. 2018). The emission rates of gases (N_2O and also CO_2) after biochar application is also highly influenced by crop, soil type, biochar type used and N fertilization (Shaaban et al 2018b; Sun et al. 2018; Yoo et al. 208; Yu et al. 2018). Moreover, greenhouse gas measurements carried out in June 2012 on the same mesocosm (Ribas et al. 2019) showed negative N_2O emission rates in the higher biochar application rate compared to the lower application rate and the control. Alternatively, the difference in *nosZ*, the last functional group of the denitrification process, could be explained by the lower nitrate levels, which is also supported by our finding of decreased NO_3^- concentration at the high biochar dose (Fig. 2a). Moreover, a decrease of NO_2^- was observed in the experiment of Ribas et al. (2019) supporting the idea that the lower content of both substrates could influence the rhythm of the denitrification process.

Despite the fact that *nosZ* gene abundance was statistically lower, a decreased N cycle in terms of functional genes abundance (nitrification, denitrification and fixation) was observed at the higher biochar concentrations (Fig. 3d). The *nosZ* gene has been revealed as the key to decreasing the emissions of N_2O and different studies have related biochar addition with an enhanced activity of nitrous oxide reducers (Harter et al. 2014; Harter et al. 2017). Biochar has also been related to a higher activity of the ammonia-oxidizer groups (AOA and AOB) from the nitrification process (Prommer et al. 2014). These contrasting results deserve a further study based on the activity of the nitrogen-cycling network functional groups for this specific biochar-soil system.

Regarding the effect of biochar on plant physiology, no strong general effects were observed. Namely, the physiological parameters were unaffected by biochar treatments, nor was there any biochar addition rate-dependent response observed in our experimental data (Fig. 4). The effect of biochar on plant physiology has not been exhaustively investigated and the primary metabolism components such as chlorophyll and protein content have received even less attention. Positive effects of biochar application were recorded on physiological parameters of wheat and rice (Rehman et al. 2017), and maize (Haider et al. 2015). On these three monocots, biochar improved the soil-plant water relations and photosynthesis. In another study on wheat, no significant effect was observed in the F_v / F_m ratio but a significant positive linear relationship was demonstrated between biochar addition and the photosynthetic rate and stomatal conductance (Akhtar et al. 2015). On the other hand, Rehman et al. (2017) also failed to find any relationship between biochar addition and chlorophyll content in wheat and rice. However, it is important to remark that the studies are not fully comparable with our study, since they were not developed under Mediterranean conditions or alkaline soil, nor using the type of biochar in this study, and moreover, were carried out under stress conditions of salinity, drought or heavy metal toxicity.

Regarding plant growth and yield, many studies have been developed and results of two meta-analyses reported benefits in aboveground and crop productivity after adding biochar to soils (Jeffrey et al. 2011; Biederman and Harpole 2013). Focused on cereals, between 7 to 60% yield increases have been reported (Rogovska et al. 2014; Agegnetu et al. 2016; Si et al. 2018). This contrasts with our observations, where crop yield or straw/grain productivity were not affected by biochar amendment (Fig. 5 and S3) but, however, agree with other studies (Marks et al. 2016; Hansen et al. 2017). Marks et al. (2016) used the same biochar in a different alkaline soil and described no crop improvements in the first three agronomical seasons following the application. These findings seem to support the conclusions by Jeffrey et al. (2017), whose global-scale meta-analysis found no effect of biochar on crop yield in temperate latitudes whereas a 25% average increase is observed in the tropics.

Concerning the crop composition, plant nitrogen content remained invariable regardless of the quantity of biochar in soil (Fig. 6a). Plants absorb nitrogen from the soil mainly in the form of NO_3^-, and our results revealed that plants were able to cope with the reduced soluble NO_3^-content at the high biochar dose and not vary their total N content. Similarly, a tendency was also shown for plant P content (Fig. S4) and the lower soil soluble HPO_4^- (Fig. 2b). The explanation could be that there is a higher N and P uptake efficiency of plants at high biochar dose. By calculating integrative indexes relating the N in soil to that in plants (N efficiency, NAE and NUE) general decreased ratios in biochar were revealed (Fig. 6b-d), which are interpreted as an increased N uptake efficiency. This means that plants amended with biochar were (or had to be) more efficient in N acquisition compared to control plants. These results are consistent with other authors' observations who have claimed that biochar has the capacity to improve N fertilizer use efficiency in plants (Chan et al. 2007; Ding et al. 2010; Zhen et al. 2013; Haider et al. 2015; Wang et al. 2017). Barley plants in this biochar-amended soil were able to uptake, mobilize and load the same amounts of N and P than control plants with lower soluble contents and equal crop yields, something that might be of environmental interest for a decreased availability/leaching of nitrates in term of groundwater protection. Since agrochemical fertilizers are the major contributors of water pollution (Addiscott et al. 1991) biochar amendments might ameliorate this effect (Liu et al. 2018).

Furthermore, the higher water retention in biochar plots in our study compared to other soil organic amendments (Sombroek et al. 2003; Liang et al. 2006; Amonette and Joseph 2009; Novak et al. 2012) and associated with the biochar high porosity is of interest. The plant available water fraction rather than the total moisture content is the true measure of water availability (Baronti et al. 2013). Despite the fact that we lack this information, the lack of effects of biochar on the intrinsic water use efficiency of barley plants suggest that this higher moisture content does not provide higher water availability or that the water provision is optimum in all the treatments. In the barley crop in this study both explanations might be plausible, since barley cropping is carried out in the rainy period between late-winter and springtime. In spite of that fact, higher soil moisture content might be of interest for other Mediterranean crops, including those growing in summer, the most challenging season in this climate, characterized by scarce, short and heavy rains and high temperatures.

Benefits of biochar are often limited to specific conditions and the effects on real applications should be determined on a case-by-case basis. Our results are of interest as i) they provide information on a relatively understudied type of biochar (gasified-wood biochar) under field conditions of calcareous soils and Mediterranean climate, and assessing short to medium effects, ii) it is a comprehensive study on the effects of biochar on soil chemical, physical and biological properties and its effects on plant physiology, nutrition and crop yield, iii) they highlight the increased water retention and reduced soluble nitrate contents induced by biochar without affecting crop productivity but associated with greater plant nutritional efficiency.

5 CONCLUSIONS

- Biochar addition to a Mediterranean agroecosystem and an alkaline soil did not cause any effect on plant nutrient uptake, crop yields or plant physiology.
- The lower soluble content of some soil macronutrients (N and P) associated with the addition of high rates of biochar (30 t ha^{-1}) were not translated to lower N and P plant contents indicating a higher uptake efficiency of plants.
- Our results confirm that biochar is a suitable soil amendment in Mediterranean agroecosystems in which N fertilizer application might be moderated whenever yields are unaffected, potentially allowing the mitigation of nitrate pollution and an increase in soil water retention.
- The decreased abundance of denitrifiers suggests a hampered denitrification process in our system with a tendency to decrease fixation and nitrification.

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TABLES

Table 1 Physicochemical properties of the soil used for the mesocosms construction

Parameter	Units	Value
Ph	-	8.3
EC	µS/m (25ºC 1:5 w/v)	200
Sand	%	36.4
Silt	%	44.9
Clay	%	18.7
C	%	2.63
N	%	0.18
C/N	%	14.6
CEC	cmol(+)/kg	13.9
Cd	mg/kg	<0.1
Cu	mg/kg	121
Cr	mg/kg	25
Ni	mg/kg	19
Pb	mg/kg	35
Zn	mg/kg	104

Table 2 Characterization of the used biochar

Feedstock	Production method	Production temperature
Pinus pinaster & *P. radiata* wood chip	Gasification	600 – 900 °C
Parameter	**Units (method)**	**Value**
pH	(H_2O, 1:10)	10.4
EC	μSm^{-1} (25ºC, 1:5 w/v)	1,100
C	% (elemental analyzer)	86.9
N	% (elemental analyzer)	0.16
S	% (ICP-OES)	0.22
Carbonats	% (ASTM D4373)	2.75
Dry matter	% (Gravimetry)	95.8

FIGURE CAPTIONS

Fig. 1 Moisture (a), electrical conductivity (EC) (b), and pH (c) in soils amended with three biochar concentrations (0, 5 and 30 t ha^{-1}) at 6 different samplings along three years of *Hordeum vulgare* cropping.

Biochar was amended once in March of 2011. Error bars correspond to the standard deviation (n=8). Different letters indicate statistically significant differences among treatments for a specific sampling

Fig. 2 Ionomic composition of NO_3^- (a), HPO_4^- (b), SO_4^{2-} (c), Mg^{2+} (d), K^+ (e), and Cl^- (f) in soils amended with three biochar concentrations (0, 5 and 30 t ha^{-1}) at 6 different samplings along three years of *H. vulgare* crop. Biochar was amended once in March of 2011. Error bars correspond to the standard deviation (n=8). Different letters indicate statistically significant differences among treatments for a specific sampling

Fig. 3 Soil basal respiration (a), microbial biomass carbon (b), microbial metabolic quotient (c) and, copy number of genes encoding for enzymes that catalyze process of the microbial transformation processes (nitrification, denitrification and fixation) of the nitrogen cycle (d). Soils were amended with three biochar concentrations (0, 5 and 30 t ha^{-1}) and graphics represent the sampling data of June 2011, three months later the biochar was added. Error bars correspond to the standard deviation (n = 8). Different letters indicate statistically significant differences among treatments

Fig. 4 Chlorophyll (a + b) concentration (a), soluble proteins concentration (b), chlorophyll fluorescence (c), and intrinsic water use efficiency (iWUE) (d) in *H. vulgare* leaves. Plants were cultivated in amended soils with different biochar concentrations (0, 5 and 30 t ha^{-1}) along three years. Biochar was amended once in March of 2011. iWUE data from 2013 were not acceptable due to technical problems. Error bars correspond to the standard deviation (n=8)

Fig. 5 Effects of the biochar application rates (0, 5 and 30 t ha^{-1}) along the experiment (2011-2013) on germination rate (as the number of *H. vulgare* seedlings per plot) (a) and average of ear weigth (b). Biochar applied once on March 2011. The yield in 2012 was not assessed due to the impact of predation by wild boars. Error bars correspond to the standard deviation (n = 8). Different letters indicate statistically significant differences among treatments for the corresponding year

Fig. 6 Nitrogen content in mature plants of *H. vulgare* (g Kg^{-1}) (a), N efficiency as the to N total in soil (mg of $N\text{-}NO_3$ and $N\text{-}NH_4$ Kg^{-1}) compared to N total in plant (mg N Kg^{-1}) (b), Nitrogen accumulation efficiency (NAE) in % (d) and Nitrogen use efficiency (NUE) in % (d). Barley plants were cultivated in amended soils with different biochar concentrations (0, 5 and 30 t ha^{-1}) along three years. Biochar was amended once in March of 2011. Error bars correspond to the standard deviation (n=8). Different letters indicate statistically significant differences among treatments for a specific year

Figure 1

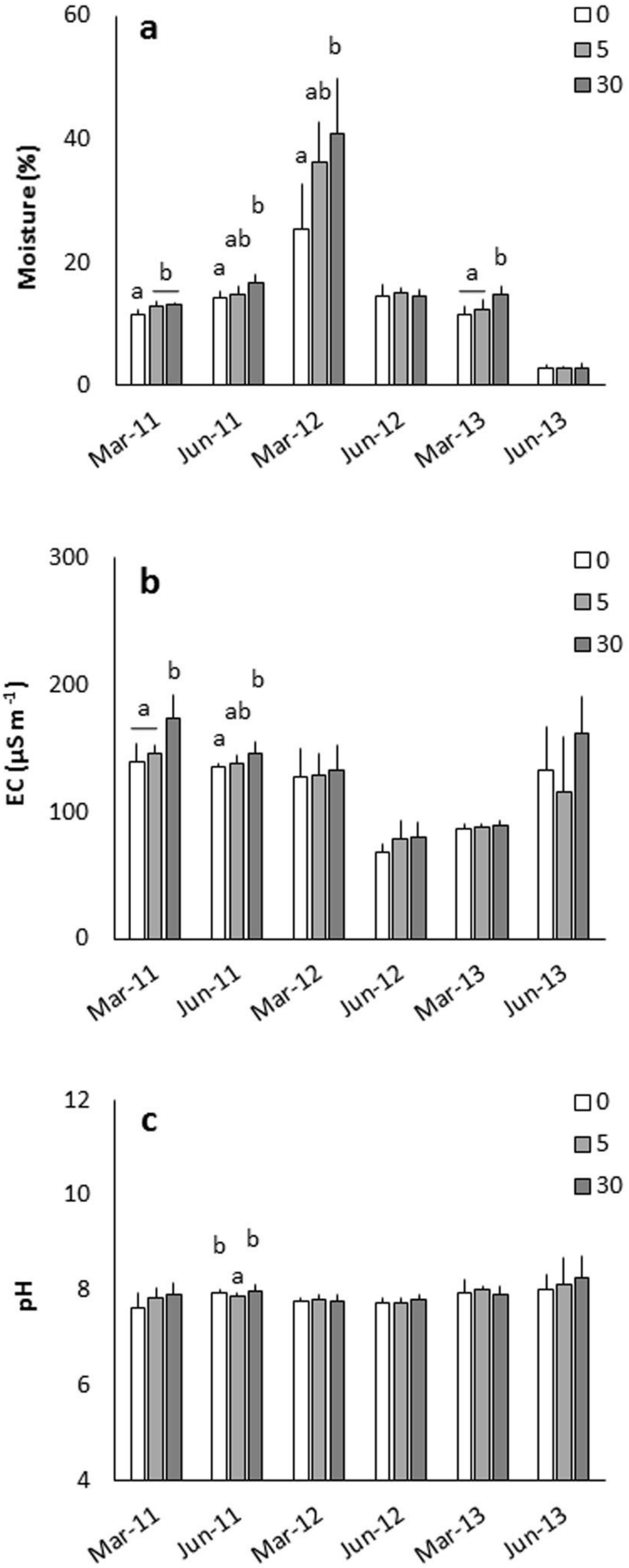

a
Moisture (%)
0
20
40
60
□0
5
30
a
b
ab
Mar-11
Jun-11
Mar-12
Jun-12
Mar-13
Jun-13
b
EC (µS m⁻¹)
0
100
200
300
c
pH
4
6
8
10
12

Figure 2

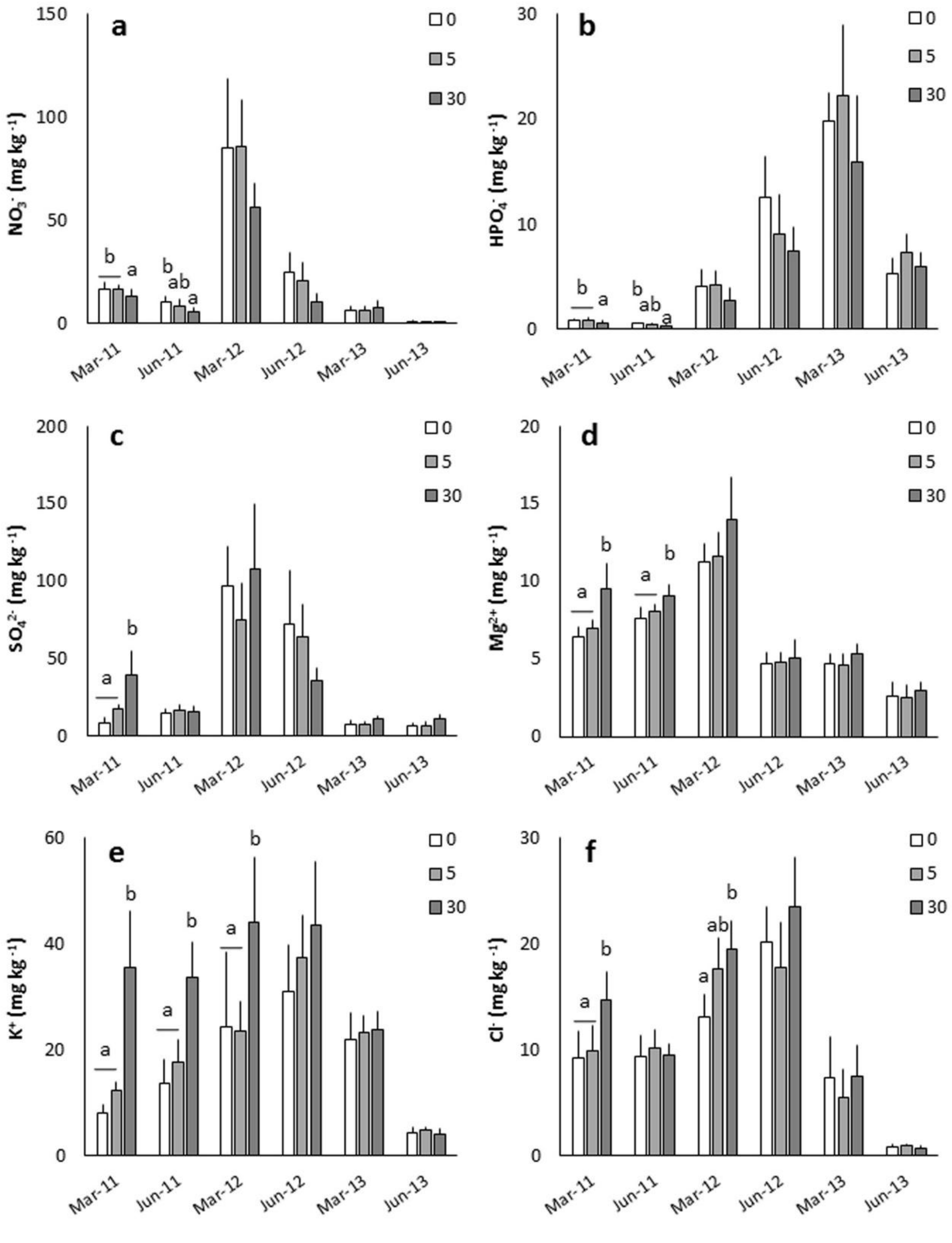

a
b
c
d
e
f
NO3- (mg kg-1)
HPO4- (mg kg-1)
SO42- (mg kg-1)
Mg2+ (mg kg-1)
K+ (mg kg-1)
Cl- (mg kg-1)
0
5
30
Mar-11
Jun-11
Mar-12
Jun-12
Mar-13
Jun-13

Figure 3

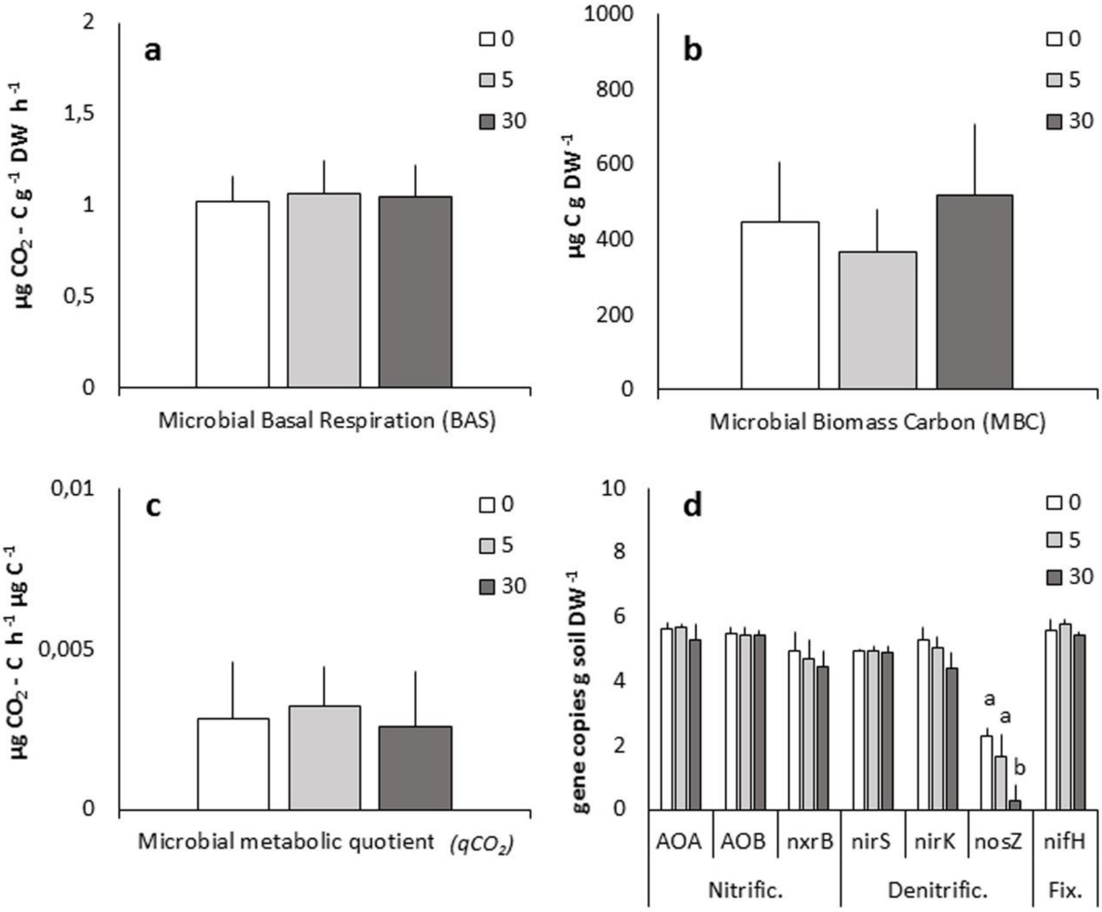
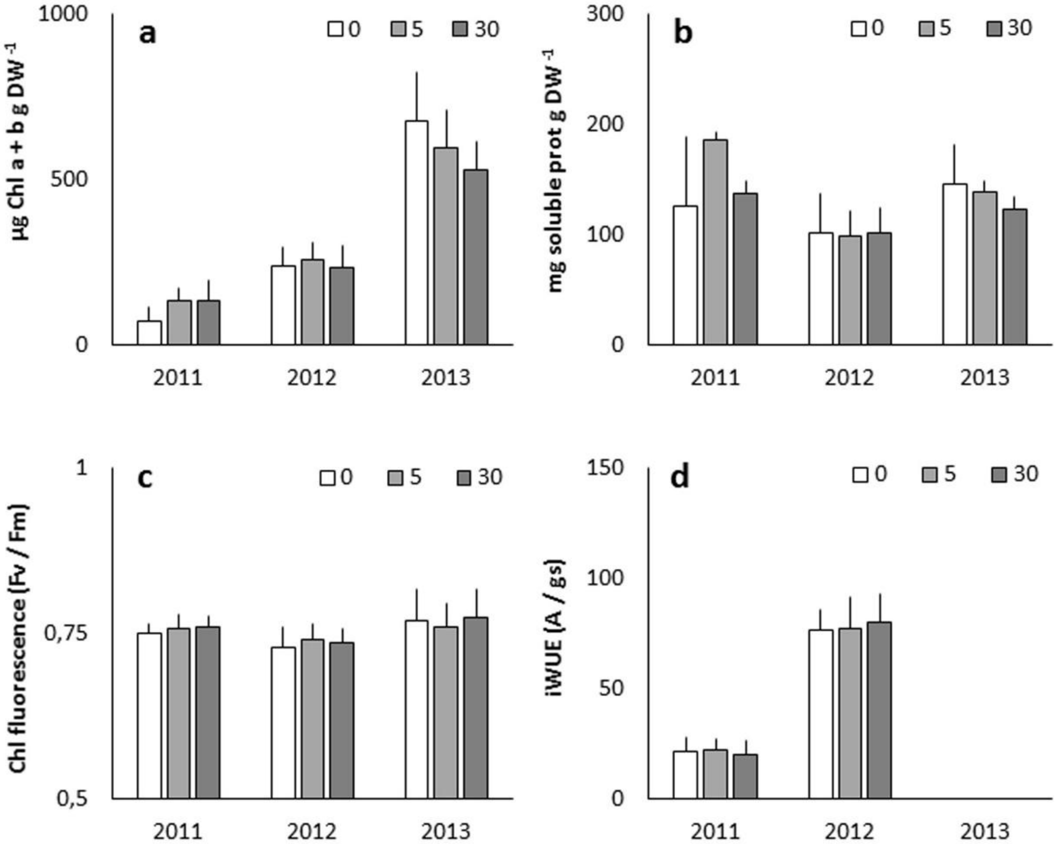

a
b
c
d
0
5
30
μg CO2 - C g-1 DW h-1
Microbial Basal Respiration (BAS)
μg C g DW-1
Microbial Biomass Carbon (MBC)
μg CO2 - C h-1 μg C-1
Microbial metabolic quotient (qCO2)
gene copies g soil DW-1
AOA
AOB
nxrB
nirS
nirK
nosZ
nifH
Nitrific.
Denitrific.
Fix.
a
a
b

Figure 4



a
b
c
d
0
5
30
µg Chl a + b g DW -1
mg soluble prot g DW -1
Chl fluorescence (Fv / Fm)
iWUE (A / gs)
1000
500
300
200
100
1
0,75
0,5
150
50
2011
2012
2013

Figure 5

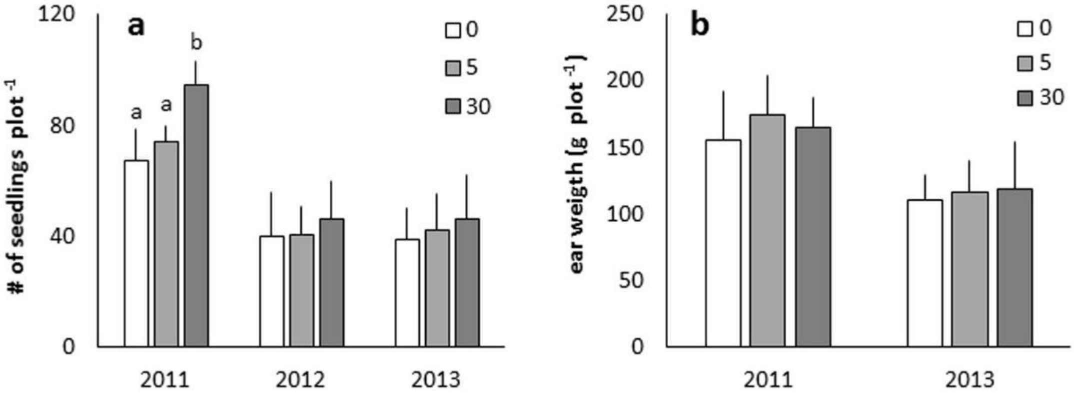
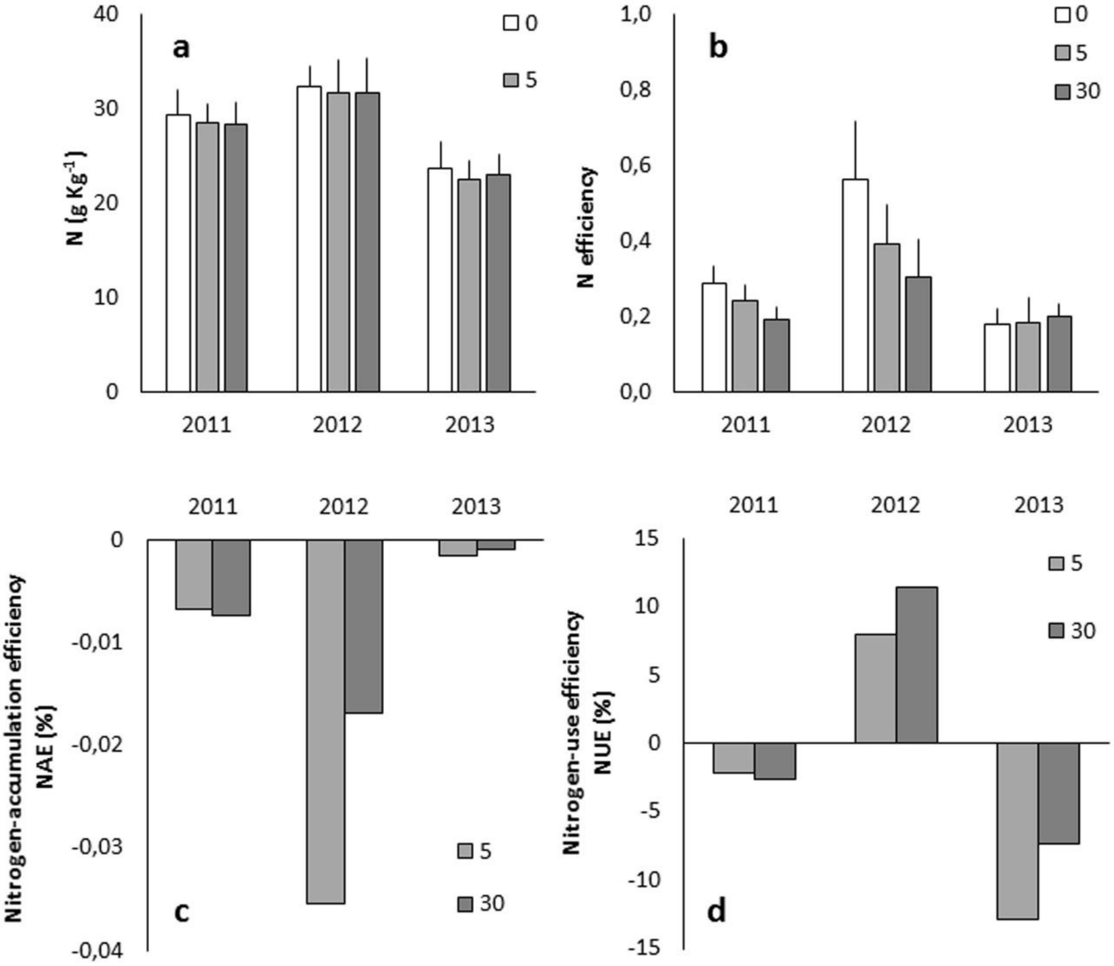

a
120
80
40
0
# of seedlings plot -1
a
a
b
0
5
30
2011
2012
2013
b
250
200
150
100
50
0
ear weigth (g plot -1)
0
5
30
2011
2013

Figure 6


a
b
c
d
N (g Kg^{-1})
N efficiency
Nitrogen-accumulation efficiency NAE (%)
Nitrogen-use efficiency NUE (%)
0
5
30
2011
2012
2013
40
30
20
10
0
1,0
0,8
0,6
0,4
0,2
0,0
-0,01
-0,02
-0,03
-0,04
15
-5
-10
-15

SUPPLEMENTARY MATERIAL

1. SUPPLEMENTARY TABLES

Table S1 Characterization of the pig slurry

Table S2 Source of the standard for each bacterial *strains* and thermal profiles for qPCR of the different target genes

Table S3 Results of the amplification efficiencies for the different genes

Information about the Near Infrared Reflectance Spectroscopy (NIRS) calibrations

Table S4 Population statistics of calibration data set used for the estimation of chemical composition values from the near-infrared measurements

Table S5 Calibration and cross-validation statistics for the determination of chemical composition parameters by near-infrared analysis

2. SUPPLEMENTARY FIGURES

Fig. S1 Mean daily and maximum daily temperature (white and filled dots, respectively), and 24-hours accumulated precipitation (grey bars) along the experimental period (2011-2013) in the Cerdanyola del Vallès weather station, 8.5 km far from the experimental field. Data provided by Meteorological Service of Catalonia (Meteocat). Arrows indicate the plant and soil measurement events

Fig. S2 Ionomic composition of NO_2^-, NH_4^+, Na^+, and Ca^{2+} in soils amended with three biochar concentrations (0, 5 and 30 t ha^{-1}) at 6 different samplings along three years of *H. vulgare* crop. Biochar was amended in March of 2011. Error bars correspond to the standard deviation (n=8)

Fig. S3 Effects of the biochar application rates (0, 5 and 30 t ha^{-1}) along the experiment (2011-2013) on quantitative effects on yield, measured as the number of *H. vulgare* ears per mesocosm; the number of grains per ear per mesocosm; and the average of straw weigth. Biochar applied once on March 2011. The yield in 2012 was not assessed due to the impact of predation by wild boars. Error bars correspond to the standard deviation (n = 8). Different letters indicate statistically significant differences among treatments for the corresponding year

Fig. S4 Percentage of macronutrients (P, S, Ca, Mg and K) in plants (grain and straw) of *H. vulgare*. Plants were cultivated on amended soils with different biochar concentrations (0, 5 and 30 t ha^{-1}) along three years. Biochar was amended once in March of 2011. Error bars correspond to the standard deviation (n=8)

Fig. S5 Percentage of micronutrient (Fe, Mn and Zn) in plants (grain and straw) of *H. vulgare*. Plants were cultivated on amended soils with different biochar concentrations (0, 5 and 30 t ha^{-1}) along three years. Biochar was amended once in March of 2011. Error bars correspond to the standard deviation (n=8)