Universitat Autònoma de Barcelona

# Role of biochar in N cycling in a Mediterranean agroecosystem: potential benefits and trade-offs

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Ph. D. Thesis

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*A Carlos,*

*mi piedra angular.*

"Parece que solo he sido como un niño jugando en la playa que se ha entretenido de vez en cuando encontrando un canto rodado más suave o una concha más bonita de lo habitual, mientras el gran oceáno de la verdad se extiende del todo desconocido frente a mí"

Isaac Newton

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## General Abstract

Anthropogenic inputs of reactive nitrogen (N) via the Haber-Bosch process (synthetic $N_2$ fixation for fertiliser production) and legume cultivation are responsible for over half of the input of N to terrestrial ecosystems. These excessive inputs from agroecosystems have resulted in enhanced fluxes of nitrous oxide ($N_2O$), ammonia ($NH_3$), and nitrate ($NO_3^-$), which induce far-reaching adverse effects, including the acceleration of global warming, promoting eutrophication, and depleting stratospheric ozone, among others.

Soil-application of biochar, the carbon(C)-rich solid produced by thermochemical conversion of biomass under oxygen-limited conditions, could be a valuable tool to cope with elevated N fluxes. However, broad-scale biochar implementation is hindered by the uncertainties concerning possible unintended consequences as well as its long-term effects on soil quality, given its persistence in soil.

In order to fill in some of these knowledge gaps, a pine gasification biochar that was found to reduce nitrate contents in soil solution in a previous study was assessed both under greenhouse conditions (Chapter 1) and under field conditions (Chapter 2). Chapter 1 was aimed at evaluating whether this particular biochar could induce a nitrate leaching mitigation response both in the short and long-term and at two contrasted application rates (12 and 50 t $ha^{-1}$). To do so, soil columns filled with either fresh biochar or 6-yr field-aged biochar were monitored for 8 months. At the end of the trial, a significant reduction of $NO_3^-$ in soil solution as well as other ions (including chloride, magnesium, sodium, and calcium) was found for both application rates in the fresh biochar-amended columns but not in the aged biochar scenario. The formation of an organo-mineral coating that entrapped nutrient-enriched water into biochar pores was ascribed as the most plausible explanation. Despite the alleviation of $NO_3^-$ in soil solution, its leaching was unaffected by biochar treatment, casting doubt about possible environmental effects and encouraging further research to unravel biochar's role in $NO_3^-$ leaching mitigation.

In Chapter 2, by monitoring a wide range of soil properties in 6-yr old field mesocosms (from where the soil-aged biochar mixtures of Chapter 1 were taken), a soil function-based quality assessment was conducted. The 50 t $ha^{-1}$ application rate was the most effective in sequestering C and presented enhanced water contents at some sampling dates. However, an important trade-

off emerged, since it exerted negative effects towards soil fauna communities (nematodes and collembolans), and also it boosted methane ($CH_4$) soil emissions. Conversely, the 12 t $ha^{-1}$ rate did not pose serious risks to soil faunal communities and soils acted as a $CH_4$ sink. On the other hand, this same treatment showed signs of promotion of recalcitrant carbon metabolism, which, if maintained over time, could affect biochar's C sequestration potential and reduce its expected persistence. Taking all into consideration, in our agroecosystem, the 12 t $ha^{-1}$ rate would be safer to apply, although the reduced carbon sequestering capacity compared to the 50 t $ha^{-1}$ treatment.

Finally, Chapter 3 addressed the potential agronomical benefits of including biochar into fertiliser formulation with the aim to improve nutrient release efficiency and plant uptake. Particularly, a slow pyrolysis biochar was included in three organo-mineral fertiliser formulations (NPK, NP, and K). Nutrient leaching was slowed down in the formulation combining biochar and NPK (NPK+B). This effect was plausibly attributed to the labile-C added with the slow-pyrolysis biochar and the concomitant provision of NPK, which could have promoted microbial growth and caused a temporary storage of nutrients in microbial biomass. While the NPK+B fertiliser only significantly enhanced barley straw biomass and not grain, all biochar-based fertilisers presented improved plant nutrient content and export (in relation to potassium, sulphur, calcium, and manganese). Both the improved nutrient release pattern in NPK+B and the enhanced crop nutrient status found for all biochar-based fertilisers indicate that the investigated formulations hold promise for further research and development of new generation fertilisers.

## Resumen general

Las aportaciones de nitrógeno reactivo (N) mediante el proceso Haber-Bosch (fijación industrial de $N_2$ para producir fertilizantes) y el cultivo de leguminosas son responsables de más de la mitad del input de N a los ecosistemas terrestres. Estos insumos excesivos provenientes de los agroecosistemas han aumentado los flujos de óxido nitroso ($N_2O$), amoníaco ($NH_3$) y nitrato ($NO_3^-$), provocando efectos tales como la aceleración del calentamiento global, la eutrofización y la reducción de la ozono estratosférico, entre otros.

La enmienda del suelo con biochar, el sólido rico en carbono (C) producido por la conversión termoquímica de biomasa en condiciones limitantes de oxígeno, podría ser una herramienta valiosa para hacer frente a los flujos elevados de N. Sin embargo, la implementación a gran escala del biochar se ve comprometida por la incertidumbre sobre posibles efectos adversos, así como por sus efectos a largo plazo sobre la calidad del suelo, debido a su persistencia en el suelo.

Para reducir estas brechas de conocimiento, se evaluó en condiciones de invernadero (Capítulo 1) y en condiciones de campo (Capítulo 2) un biochar de gasificación que demostró previamente poder reducir el nivel de $NO_3^-$ soluble del suelo. El Capítulo 1 examinó la posible mitigación de lixiviación de nitratos a corto y largo plazo en dos dosis de aplicación contrastadas (12 y 50 t $ha^{-1}$). Se examinaron columnas de suelo con biochar fresco y biochar envejecido durante 6 años en el campo. Al cabo de 8 meses, se encontró una reducción significativa de $NO_3^-$ en la solución del suelo, así como de otros iones (incluyendo cloruro, magnesio, sodio y calcio), para las dos dosis de aplicación de biochar fresco. Este fenómeno se atribuyó a la formación de un recubrimiento organo-mineral sobre los poros del biochar, que atraparía agua y nutrientes dentro de los poros del biochar. A pesar de la disminución de $NO_3^-$ en la solución del suelo, la lixiviación no se redujo, poniendo en duda la capacidad de mitigación del biochar.

En el Capítulo 2, se evaluó la calidad del suelo mediante el seguimiento de varias propiedades del suelo en mesocosmos de campo establecidos hace 6 años (de donde provienen las mezclas suelo-biochar envejecido utilizados en el Capítulo 1). La dosis de 50 t $ha^{-1}$ fue la más eficaz en el secuestro de C y mejoró el contenido de humedad en el suelo puntualmente. Sin embargo, esta dosis representó un trade-off, ya que impactó negativamente las comunidades de fauna del suelo (nematodos y colémbolos), y también aumentó las emisiones de metano ($CH_4$). Aunque

la dosis de 12 t $ha^{-1}$ no presentó estos efectos negativos, mostró indicios de metabolismo de compuestos recalcitrantes de C, que, si se mantuviera en el tiempo, podría afectar el potencial de secuestro de C del biochar. En conjunto, se puede concluir que en nuestro agroecosistema la dosis de 12 t $ha^{-1}$ representa un escenario con menos riesgos.

Finalmente, el Capítulo 3 abordó los posibles beneficios agronómicos incluir un biochar de pirólisis lenta en tres formulaciones de fertilizantes organo-minerales (NPK, NP y K). La lixiviación de nutrientes se ralentizó en la formulación con biochar y NPK (NPK + B). Probablemente, el C lábil incorporado con el biochar y el input simultáneo de varios nutrientes podría haber estimulado el crecimiento microbiano causando un almacenamiento temporal de nutrientes en la biomasa microbiana. Aunque el fertilizante NPK + B solo mejoró significativamente la biomasa de paja de cebada y no el grano, todos los fertilizantes formulados con biochar provocaron un mejor estado nutricional vegetal (en relación al potasio, azufre, calcio y manganeso). Estos resultados indican que las formulaciones investigadas son prometedoras para el desarrollo de fertilizantes de nueva generación.

## Resum general

Les aportacions de nitrogen reactiu (N) mitjançant el procés Haber-Bosch (fixació industrial de $N_2$ per produir fertilitzants) i el cultiu de lleguminoses són responsables de més de la meitat de l'aportació de N als ecosistemes terrestres. Aquests inputs excessius provinents dels agroecosistemes han augmentat els fluxos d'òxid nitrós ($N_2O$), amoníac ($NH_3$) i nitrat ($NO_3^-$), provocant efectes tals com l'acceleració de l'escalfament global, l'eutrofització i la reducció de l'ozó estratosfèric, entre d'altres.

L'esmena del sòl amb biochar, el sòlid ric en carboni (C) produït per la conversió termoquímica de biomassa en condicions limitants d'oxigen, podria ser una eina valuosa per fer front als fluxos elevats de N. No obstant, la implementació a gran escala de biochar es veu compromesa per les incerteses sobre possibles conseqüències adverses, així com pels seus efectes a llarg termini sobre la qualitat del sòl, atesa la seva persistència al sòl.

Per tal d'escurçar aquests buits de coneixement, es va avaluar en condicions d'hivernacle (Capítol 1) i en condicions de camp (Capítol 2) un biochar de gasificació que va demostrar prèviament poder reduir el nivell de $NO_3^-$ soluble al sòl. El Capítol 1 examinà la possible mitigació de la lixiviació de nitrats tant a curt com a llarg termini i a dues dosis d'aplicació contrastades (12 i 50 t $ha^{-1}$). Es van examinar columnes de sòl amb biochar fresc i biochar envellit 6 anys al camp. Al cap de 8 mesos, es va trobar una reducció significativa de $NO_3^-$ en la solució del sòl, així com d'altres ions (incloent clorur, magnesi, sodi i calci), per a les dues dosis d'aplicació de biochar fresc. Aquest fenòmen s'atribuí a la formació d'un recobriment organo-mineral sobre els porus del biochar, tot atrapant aigua i nutrients dins els porus del biochar. Tot i la disminució de $NO_3^-$ en la solució del sòl, la lixiviació no va veure's afectada pel biochar, posant en dubte la seva capacitat de mitigació.

Al Capítol 2, s'avaluà la qualitat del sòl mitjançant el seguiment de diverses propietats del sòl en mesocosmos de camp establerts fa 6 anys (d'on provenen les mescles sòl-biochar envellit utilitzats al Capítol 1). La dosi de 50 t $ha^{-1}$ fou la més eficaç en el segrest de C i va millorar el contingut d'humitat al sòl puntualment. No obstant, aquesta dosi representà un trade-off ja que va impactar negativament les comunitats de fauna del sòl (nematodes i col·lèmbols), i també va augmentar les emissions de metà ($CH_4$). Encara que la dosi de 12 t $ha^{-1}$ no presentà aquests efectes negatius, va mostrar indicis de metabolisme de compostos recalcitrants de C, que, si es mantingués en el temps, podria afectar el potencial de segrest de C del biochar. En conjunt, es

pot concloure que al nostre agroecosistema la dosi de 12 t $ha^{-1}$ representa un escenari amb menys riscos.

Finalment, el Capítol 3 va abordar els possibles beneficis agronòmics d'incloure un biochar de piròlisi lenta en tres formulacions de fertilitzants organo-minerals (NPK, NP i K). La lixiviació de nutrients es va alentir en la formulació amb biochar i NPK (NPK + B). Probablement, el C làbil incorporat amb el biochar i l'input simultani de diversos nutrients podria haver estimulat el creixement microbià causant un emmagatzematge temporal de nutrients a la biomassa microbiana. Tot i que el fertilitzant NPK + B només va millorar significativament la biomassa de palla d'ordi i no el gra, tots els fertilitzants formulats amb biochar van provocar un millor estat nutricional vegetal ( en relació al potassi, sofre, calci i manganès). Aquests resultats indiquen que les formulacions investigades són prometedores per al desenvolupament de fertilitzants de nova generació.

# GENERAL INTRODUCTION

## Biochar definition

Biochar is the porous, carbon-rich solid obtained from the thermochemical conversion of organic materials in oxygen-restricted conditions. Biochar is deliberately applied to soil for long-term sequestration of carbon (C) and, potentially, for soil properties improvement (Shackley and Sohi, 2010). Biochar and charcoal are physicochemically almost indistinguishable (both derive from biomass thermic degradation), but they differ in their end purpose: charcoal is intended to be used as a fuel, whereas biochar is produced specifically for application to soil as part of agronomic or wider environmental management (Wiedner and Glaser, 2015).

As defined above, biochar could be manufactured by thermochemical processing of any carbonaceous material. However, it is highly recommended that biochar does not imply competing interests of land use, especially those related to food production. Otherwise, feedstocks for biochar production should ideally derive from waste recycling and promote circular economy (IBI, 2021). Thus, preferred feedstocks include bio-wastes from agriculture and forestry, sewage sludge, and animal manures, among others. Nevertheless, not all waste residues are appropriate for biochar production for agricultural purposes since they can contain toxic elements such as heavy metals (Vithanage et al., 2016). In an effort to regulate these issues, as well as other sustainability concerns, some voluntary guidelines have been put forward, such as the International Biochar Initiative (IBI) Biochar Standards and the European Biochar Certificate (EBC) (EBC, 2012; IBI, 2015).

## Unearthing biochar origins

"la tierra adentro, a dos leguas, y más, y a menos, parecían muy grandes ciudades que estaban blanqueando, y demás de esto la tierra es tan buena, tan fértil y tan natural como la de Nuestra España, porque nosotros entramos en ella por San Juan y ya comenzaban los indios a quemar los campos" [the inland, two leagues apart, seemed to hold very large cities that were bleaching, moreover, the land is as good, as fertile and as natural as that of our Spain, because we arrived on Saint John and the Indians were already beginning to burn the fields]. These words were written ca. 480 years ago by the Dominican pater Gaspar de Carvajal (the chronicler of the Spanish conquistador Francisco de Orellana) and describe large cities and fertile lands along the Amazon basin. These findings were largely regarded as a legend until recent archaeological discoveries gave them credit for, i.e., settlement places (Heckenberger et al., 2003), and,

especially, patches of fertile Amazonian dark earths (ADEs) (locally known as *Terra Preta de Índio*) (Glaser et al., 2001; Sombroek, 1966).
Although not without controversy (Silva et al., 2021), the prevalent hypothesis is that ADEs are Anthrosols generated by pre-Columbian indigenous populations as far back as 7000 yr BP by adding to the soil large amounts of charred residues, organic wastes, excrements and bones; however, it has not yet been elucidated if this process was purposeful or as an unintended consequence of human settlement (Glaser et al., 2001; Lima et al., 2002). *Terra Preta* soils have on average three times higher soil organic matter (SOM) content, increased nutrient levels, and a better nutrient retention capacity than surrounding highly-weathered, infertile soils (mainly Oxisols and Ultisols) (Glaser, 2007).

The high contents of charred material in ADEs (70% more than in surrounding soils, on average $\simeq$ 50 t $ha^{-1}$; Glaser et al., 2001), which give them their characteristic colouration, has been identified as the key feature explaining their enduring fertility (Teixeira et al., 2004). For this reason, soil amendment with charcoal (i.e., biochar) has recently gathered a great deal of scientific interest with the main aims of mimicking ADEs' fertility benefits as well as sequestering carbon into the soil (Lehmann et al., 2006). However, it must be noted that fertility benefits emerge especially in tropical acid soils, where the liming effect of biochar improves nutrient availability and reduces aluminium toxicity (Jeffery et al., 2011; 2017).

Although *Terra Preta* represents the paradigm for biochar contemporary use, historical charcoal application to soil appears to have had an independent development as a traditional agricultural technique in several areas of the world (Wiedner and Glaser, 2015). To cite some examples, Ogawa and Okimori (2010) proposed that rice husk biochar has been used for several thousands of years in Asia, ever since the onset of rice cultivation in that region. In the Iberian Peninsula, there exist written records (s. XVIII, XIX, and mid-XX) of structures similar to small-scale charcoal kilns ('Formiguers' in Catalan; 'hormiguero' or 'horniguero' in Spanish) which were used to produce a material able to control weeds, pathogens and with fertiliser value (Miret, 2004; Olarieta et al., 2011).

## Biochar production technologies and resulting properties

Biochar production is based on two biomass thermochemical conversion techniques: pyrolysis (which can be further classified into slow, fast, intermediate, flash, and microwave; Mašek et al.,

2016), and gasification (Bridgwater, 2007). Whereas pyrolysis is conducted in the near or complete absence of oxygen, gasification requires partial oxidation conditions, however, less oxygen is present than that required stoichiometrically for complete combustion (i.e., equivalence ratio around 0.25) (Brewer, 2012). All these processes yield three end-products: solid (biochar and/or ash), liquid (bio-oil or tar) and gas (syngas, i.e., mainly carbon monoxide and hydrogen) whether its final proportion depends on both the feedstock and conditions of thermochemical processing (**Table 1**) (Tomczyk et al., 2020). The technology that is most frequently advocated for biochar production is slow pyrolysis since it is the most efficient in terms of yielding biochar (ca. 35%) (Brownsort, 2009). On the other hand, bio-oil production is favoured in fast pyrolysis, whereas syngas production is favoured in gasification systems (Tomczyk et al., 2020). Thus, the latter are interesting from the standpoint of bioenergy generation. Namely, syngas can be used to fuel the biomass thermochemical degradation and/or renewable energy generation whereas bio-oil can be used as a renewable fuel (Steiner, 2008).

**Table 1**. Conditions and percentage of end-products of the main thermochemical biomass conversion techniques (Tomczyk et al., 2020).

| Process | Temperature (ºC) | Pressure | Residence time | Proportion of end-products (%) | | |
|---|---|---|---|---|---|---|
| | | | | Bio-oil | Syngas | Biochar |
| Slow pyrolysis | 350-800 | Atmospheric | Seconds-hours | 30 | 35 | 35 |
| Fast pyrolysis | 400-600 | Vacuum-atmospheric | Seconds | 75 | 13 | 12 |
| Gasification | 700-1500 | Atmospheric-elevated | Seconds-minutes | 5 | 85 | 10 |

Biochar composition is also strongly determined by pyrolysis conditions and feedstock, and can be described as having two main fractions: the organic fraction and the inorganic ash fraction. The organic fraction of biochar has a high carbon content, specifically, as the pyrolysis reaction progresses, biochar becomes increasingly depleted of oxygen and hydrogen while remaining carbons form new aromatic carbon-carbon bonds (Enders et al., 2012). A common way to represent the extent of these reactions is through a Van Krevelen diagram, which plots the molar H/C ratio against the molar O/C ratio. Whereas the H/C ratio indicates the degree of aromatisation, the O/C ratio is a proxy of the presence of polar functional groups (reactivity or electrical charge) (**Fig. 1**). Temperature is a key factor governing these parameters, namely, with increasing temperatures aromaticity increases and polarity decreases (Lopez-Capel et al., 2016).

According to EBC, biochar should have an H/C ratio below 0.7 and an O/C ratio below 0.4 (EBC, 2012).

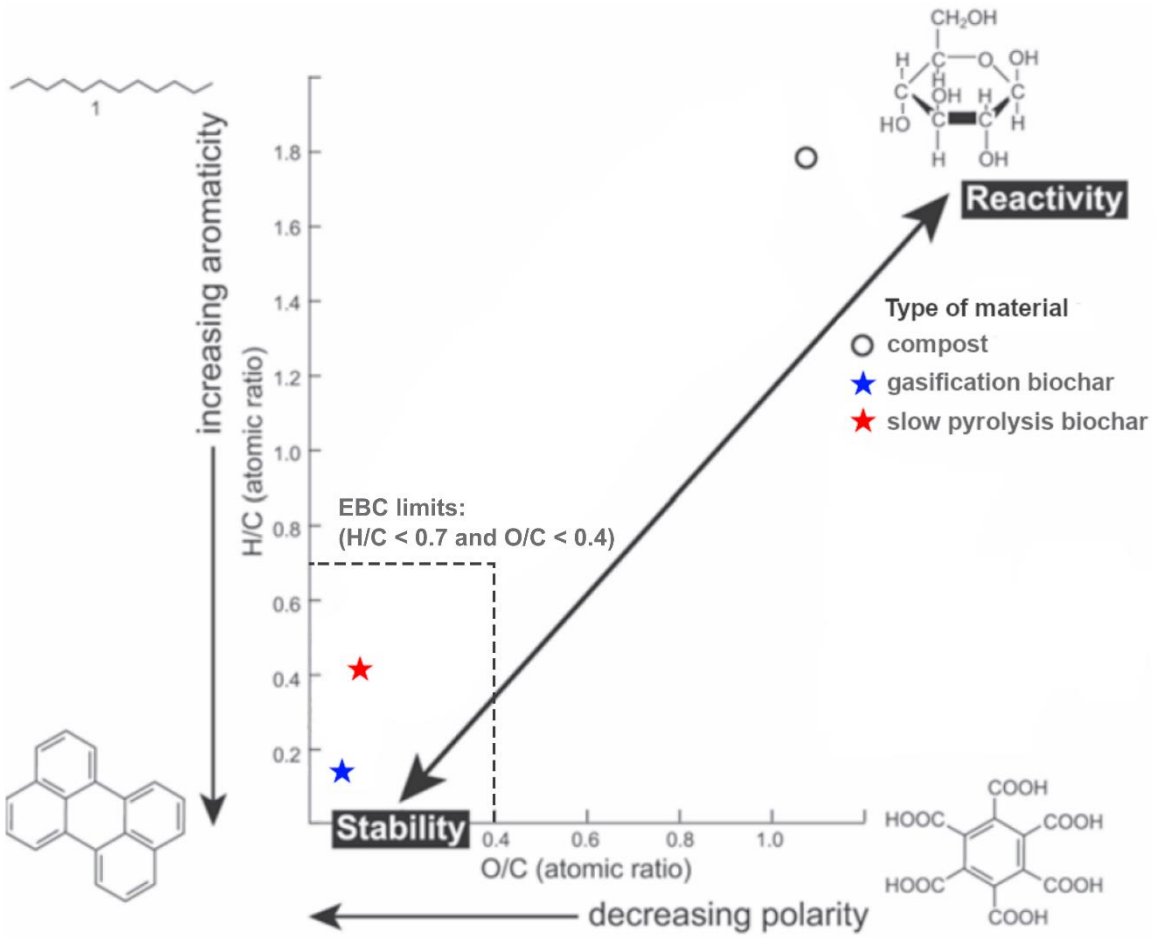



**Fig. 1.** Van Krevelen diagram showing the trade-off between reactivity and stability of biochar. The two biochars studied in the present thesis are plotted as a blue and a red star, both of them well below the EBC limits. Compost (not studied in this thesis) is also shown for comparability purposes (modified from an original by Bruno Glaser, In: Lopez-Capel et al., 2016).

The degree of aromatic condensation of biochar, i.e., the ordering of aromatic carbon sheets, is also highly affected by the production temperature. Precisely, biochars produced at lower temperatures (< 550 ºC) have a predominantly amorphous C structure (Joseph et al., 2010) while at higher temperatures turbostratic C and graphite-resembling structures are formed (Schimmelpfennig and Glaser, 2012; Tomczyk et al., 2020). These condensed aromatic structures explain biochar persistence and its potential as a C sequestration agent, as few

microorganisms have the suite of enzymes needed for decomposing such recalcitrant compounds (Brewer, 2012).

Thus, high-temperature biochars tend to have a greater C sequestration potential (Brewer, 2012; Ippolito et al., 2020; Kookana et al., 2011), conversely, as its O/C ratio is lower than in low-temperature biochars (i.e., functional groups are increasingly detached with temperature) its cation exchange capacity (CEC) tends to be lower (Zornoza et al., 2016). Nonetheless, CEC not only depends on surface functional groups but also on the surface area, and the latter is promoted at increased temperatures (up to a certain point where fine-pore structures are destroyed, at around 700-800 ºC; Chun et al., 2004). Hence, biochars with the highest CEC would be that produced at relatively low temperatures, with sufficient functional groups remaining but allowing for surface area to increase (Weber and Quicker, 2018). CEC will also depend on pH, which is highly interrelated to ash.

The inorganic ash fraction of biochar is the fraction most affected by feedstock properties as the non-volatile constituents present in the parent material become concentrated in the ash (Brewer, 2012). Increasing pyrolysis temperature also promotes hydroxide and carbonate phases to increase within the ash, thereby elevating pH values (Ippolito et al., 2020). For this reason, high-temperature biochars tend to have a higher liming value (Zornoza et al., 2016).

## Biochar-nitrogen interactions in agroecosystems

### *Nitrogen imbalances in agroecosystems*

Even though nitrogen (N) is the most plentiful element in the Earth's atmosphere, paradoxically, it is also the most limiting nutrient for plant growth in many ecosystems (Vitousek et al., 1997). This is because the largest pool of N (accounting for 99% of total N), consists of molecular nitrogen ($N_2$), which is unavailable to most organisms and has been described as "unreactive nitrogen". In contraposition, "reactive nitrogen" encompasses all N-containing molecules other than $N_2$, such as nitrate ($NO_3^-$) or ammonium ($NH_4^+$), which are biologically available but more mobile in the environment (Galloway et al., 2003; 2008).

Agroecosystems are particularly open ecosystems, i.e., they are subsidised ecosystems that rely upon high nutrient inputs and also generate large outputs (Brussaard et al., 2004). Among the inputs needed to sustain crop productivity, N-containing fertilisers are the fertiliser type added

in highest amounts (Ladha et al, 2005). Indeed, the anthropogenic inputs of industrially produced N fertilisers by the Haber-Bosch process (by artificial $N_2$ fixation) and the cultivation of $N_2$-fixing crops (biological fixation) currently exceed the natural N inputs to terrestrial systems (Galloway et al., 2008). Yet the N uptake by crops typically represents only about 50% or less of N applied with fertilisation (Davidson et al., 2011, Tilman et al., 2012). Consequently, all N that is not assimilated into plant tissues and exported at harvest is susceptible to undesirable losses. The main N loss pathways from cropping systems include: (i) leaching, predominantly in the $NO_3^-$ form, and, occasionally also as dissolved organic N (DON) and $NH_4^+$ forms, however, the latter is normally held in the cation exchange complex of soils; (ii) denitrification, provoking gaseous losses of nitrous oxide ($N_2O$), nitric oxide (NO), and $N_2$; and (iii) ammonia ($NH_3$) volatilisation (Cameron et al., 2013; Ladha et al., 2005).

These N losses entail diverse and cascading threats to the environment and human health (Galloway et al, 2003; Vitousek et al., 1997). Nitrate leaching to groundwater compromises drinking water resources. Both methemoglobinemia and the formation of carcinogenic N-nitroso compounds have been associated with the ingestion of water with high levels of nitrate. Nevertheless, more research is needed to clarify if methemoglobinemia is caused by nitrate or by a bacterial infection and to what extent nitrate ingestion induces endogenous nitrosation (Powlson et al., 2008; Ward et al., 2018). Furthermore, nitrate loading to surface waters causes eutrophication and low-oxygen conditions that endanger fish populations as well as other wildlife (Tilman et al., 2012). Perhaps, one of the most dramatic examples is the hypoxic zone formed annually in the Gulf of Mexico as a result of N leakage from grain farms along the Mississippi River drainage basin (Rabalais et al. 2002).

Regarding $N_2O$ (usually coming from denitrification but also from other processes such as nitrification and dissimilatory nitrate reduction; Baggs, 2011), it is a potent greenhouse gas (GHG) with a global warming potential 265 times greater than that of carbon dioxide for a 100-yr time scale (IPCC, 2014). Moreover, nitrogen oxides ($NO_x$) contribute to the depletion of stratospheric ozone ($O_3$), in fact, after chlorofluorocarbons being banned, $N_2O$ is nowadays the most important $O_3$-depleting agent (Ravishankara et al., 2009). Whereas in the troposphere, $NO_x$ could promote $O_3$ and smog formation (Chameides et al., 1994).
Finally, volatilised $NH_3$ is either returned to the earth's surface by deposition (through rainfall or attached in particulate matter) or transformed into aerosols (e.g., ammonium bisulfate or ammonium sulfate). Ammonium aerosols contribute to fine particulate matter and haze

concentrations, which may, in turn, have effects on radiative forcing. Once deposited, $NH_3$ might lead to acidification (Cameron et al., 2013; Galloway et al., 2003).

### *Potential role of biochar in reducing N losses in agroecosystems*

Biochar mechanisms for reducing N losses can be manifold partly due to the inherent complexity of soil N cycling. Taking as a reference the nitrogen cycle represented in **Fig. 2** we can broadly classify biochar N retention mechanisms as follows: i) increasing exchangeable N in detriment of soluble N; ii) microbial-related mechanisms: either promoting microbial biomass as an N sink or modifying microbial N cycling; iii) growing the soil organic N (SON) stock; and iv) boosting plant N uptake. All these mechanisms are related to the "storages" in **Fig. 2**, however, biochar has also the potential to increase the "inputs" as it can enhance biological N fixation (Rondon et al., 2007), as well as act as an N source itself, which will be discussed later. Regarding storage of N as exchangeable N, a widely proposed mechanism involves $NH_4^+$ adsorption onto biochar via its CEC (Glaser et al., 2002; Liang et al., 2006). On the other hand, although less commonly reported, anion exchange capacity (AEC) of biochar could also play a role and be a direct $NO_3^-$ retention mechanism (Lawrinenko and Laird, 2015). In addition, at the biochar pore level both physical adsorption and absorption can occur and result in $NH_4^+$, $NO_3^-$, $NH_3$, and DON entrapment (Downie et al., 2009; Lopez-Capel et al., 2016).

Moving to the microbial-related mechanisms, biochar could have a marked effect on microbial populations, which in turn, mediate N cycling in soil. The balances between mineralisation and immobilisation as well as between nitrification and denitrification would largely determine predominant N forms and N losses. A key parameter determining nitrification versus denitrification predominance is oxygen availability, which can be modified by biochar as it is a porous media affecting aeration and water retention (Hagemann et al., 2016). On the other hand, the net balance of mineralisation and immobilisation is mostly controlled by the C/N ratio of the material to be decomposed (Hagemann et al., 2016). In this sense, application of biochars that contain appropriate amounts of labile C (usually low-temperature biochars) might induce immobilisation of available N in soils (mostly $NH_4^+$ as is the preferred N form for microbial assimilation) (Bruun et al., 2012; Ippolito et al., 2012).

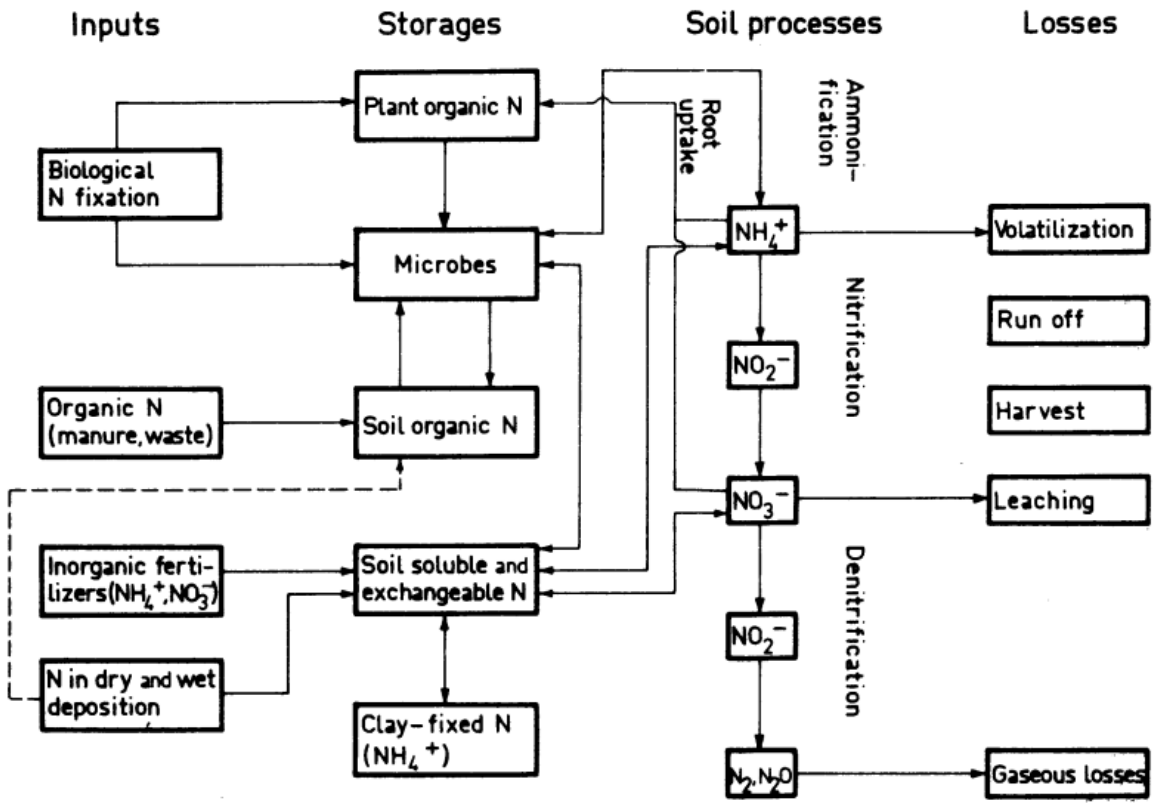



**Fig. 2.** Flow chart for nitrogen in soil with main reactions (soil processes) depicted (Rosswall, 1976).

Soil organic nitrogen is highly intertwined with microbial processes since N immobilised in microbial biomass could eventually be incorporated to this pool, and, conversely, microbial mineralisation diminishes its stock. As an example of a biochar impact on SON, after exposure of biochar to the soil environment, it can become occluded with organic matter or form aggregates with organo-mineral phases, thus, it has been suggested that N in these pools can be protected from microbial attack, and thus enlarge its residence time (DeLuca et al., 2009).

Regarding biochar's influence on plant organic N, numerous reports indicate biochar ability to enhance crop productivity and/or plant nitrogen use efficiency thereby improving crop N export (Chan and Xu, 2009). This could be achieved either directly or indirectly. The indirect mechanism is thought to be based on the nutrient retentive properties of biochar explained above, namely, nutrients retained in temporary storages such as CEC, AEC, or microbial biomass could become plant-available. For instance, Taghizadeh-Toosi et al. (2012) showed that $NH_3$ adsorbed onto biochar was available to plants. On the other hand, the direct mechanism of enhanced plant N retention is based on biochar acting as an N source itself. N availability from biochar vary widely depending on its feedstock and pyrolytic conditions (Joseph et al.,

2010). It is generally assumed that N is not readily available from biochar, as most N compounds volatilise above 200 ºC during the pyrolytic treatment (Kookana et al., 2011), and remaining N is found in a recalcitrant heterocyclic form (Knicker et al. 1996). Nonetheless, it has been demonstrated that this pool of N can also be assimilated by microorganisms (de la Rosa and Knicker, 2011). Moreover, some biochars, especially those derived from manures and bio-solids, could contain more available N forms such as amino acids (Hagemann et al., 2016).

Indeed, recent research is being devoted to optimising biochar as an N cycle regulator by including it as ingredient in new generation fertilisers (Joseph et al., 2013). Some promising results include the development of slow-release nutrient-enriched biochar fertilisers (Chen et al., 2018; Luo et al., 2021) or biochar fertilisers that promote plant nutrition through redox status improvement in the rhizosphere (Chew et al., 2020).

## Justification and structure of the thesis

The present thesis is underpinned by biochar effects on the nitrogen cycle in agroecosystems. Although major benefits have been found for biochar regarding N cycling (e.g., reduced $NO_3^-$ leaching), variegated results have been obtained, either positive, negative, or neutral (Clough et al., 2013; Hagemann et al., 2016). This is probably due to the wide variety of feedstocks and production technologies for biochar production which can deliver a wide spectrum of biochar properties. In addition, this inherent variability is magnified by the biochar interactions with the receiving soil-plant system, together with the climatic conditions. Finally, biochar ageing in soil and biochar application rate could further imply a source of variation (Joseph et al., 2010; Sorrenti et al., 2016) since nutrient retention properties or carbon storage itself can vary over time. Thus, there is a need for long-term field trials to help guide agronomic management decisions involving biochar. Likewise, an assessment of biochar's loading capacity, i.e, what biochar application rates are safe to apply, is also required (Verheijen et al., 2010).

Finally, if biochar is to be applied, it should be borne in mind that this material could impinge on a variety of soil functions. Biochar is an effective tool to store carbon in the soil for its high stability, and it is maybe the most consistent effect. In fact, a recent meta-analysis has shown that biochar application represents the most effective approach for increasing soil organic carbon (SOC) content in agroecosystems (by an average of 39%) compared to other management options, such as cover crops (6%) or conservation tillage (5%), which increased

SOC contents by a 6% and a 5%, respectively (Bai et al., 2019). In addition to the direct effect of carbon dioxide offsetting through C sequestration, biochar could also affect other GHGs such as $CH_4$ and $N_2O$. However, both mitigation and emission enhancement have been reported (Bruun et al., 2016; Cayuela et al., 2014). Similarly, the nutrient cycling function is sometimes adversely impacted thereby also affecting crop productivity, as an example, it has been reported that fresh biochar application could immobilise mineral N too tightly and prevent its plant uptake (Kookana et al., 2011). Also, soil food web functioning after biochar application has been mostly overlooked, and this is a knowledge gap that should be of concern since some biochars possess substances with ecotoxicological risks for soil biological communities (Domene, 2016; Lehmann et al., 2011; Godlewska et al., 2021).

Previous work from our research group (Marks et al., 2016), conducted in the same agroecosystem, testing a pine wood gasification biochar applied to a calcareous Fluventic Haploxerept soil cropped to barley, revealed a role for biochar in soil solution nitrate attenuation, but also biochar-mediated deleterious effects to both soil fauna (Marks et al., 2014a) and plants (Marks et al., 2014b) which was claimed to require further investigation. Chapters 1 and 2 build on these prior studies by using the same biochar-soil-crop system, and since the experimental plots of the cited works by Marks et al. were set up in 2011, the effect of natural ageing of biochar for 6 years was also addressed.

In detail, Chapter 1 deals with the possible mechanisms explaining topsoil nitrate reduction as well as the role of biochar ageing in soil in such effect. On the other hand, Chapter 2 intends to unravel biochar effects on a range of soil functions (C sequestration, water retention, GHG regulation, nutrient cycling, soil food web functioning, and food production) in an effort to evaluate if the detrimental effects found for biological endpoints in Marks et al. (2014a, 2014b) persisted over time and also to guarantee the long-term safety of amendment with this type of biochar on previously unassessed soil functions.

Additionally, in Chapter 3 the use of biochar as an additive in new generation fertilisers (organo-mineral fertilisers) to improve N use efficiency as well as that of other nutrients is assessed. This is of high interest since in temperate climates (as the one in our study system), where the direct addition of biochar agronomic benefits have not been so apparent as in tropical soils (Atkinson et al., 2010; Biederman and Harpole 2013), biochar optimisation through fertiliser blending could overcome the lack of effects.

The main parameters of the experimental design at each chapter could be observed in **Fig. 3**.

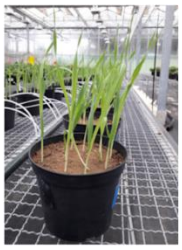
| | Chapter 1 | Chapter 2 | Chapter 3 |
|---|---|---|---|
| |  |  |  |
| **Experimental layout** | *Lysimeters* | *Field mesocosms* | *Pot experiment* |
| **Biochar type** | *Gasification* | *Gasification* | *Slow pyrolysis* |
| **Biochar ageing** | *Fresh and aged* | *Aged* | *Fresh* |
| **Biochar application form** | *Direct amendment* | *Direct amendment* | *Fertiliser ingredient* |
| **Biochar application rates** | *0, 12, 50 t $ha^{-1}$* | *0, 12, 50 t $ha^{-1}$* | *Subject to fertiliser formulation* |

**Fig. 3.** Summary of the main experimental parameters of the chapters of this thesis.

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# CHAPTER 1

## Fresh biochar application provokes a reduction of nitrate which is unexplained by conventional mechanisms

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## Abstract

Soil-applied biochar has been reported to possess the potential to mitigate nitrate leaching and thus, exert beneficial effects beyond carbon sequestration. The main objective of the present study is to confirm if a pine gasification biochar that has proven able to decrease soil-soluble nitrate in previous research can indeed exert such an effect and to determine by which mechanism. For this purpose, lysimeters containing soil-biochar mixtures at 0, 12 and 50 t biochar $ha^{-1}$ were investigated in two different scenarios: a fresh biochar scenario consisting of fresh biochar and a fallow-managed soil, and an aged biochar scenario with a 6-yr naturally aged biochar in a crop-managed soil. Soil columns were assessed under a mimicked Mediterranean ambient within a greenhouse setting during an 8-mo period which included a barley crop cycle. A set of parameters related to nitrogen cycling, and particularly to mechanisms that could directly or indirectly explain nitrate content reduction (i.e., sorption, leaching, microbially-mediated processes, volatilisation, plant uptake, and ecotoxicological effects), were assessed. Specific measurements included soil solution and leachate ionic composition, microbial biomass and activity, greenhouse gas (GHG) emissions, N and O isotopic composition of nitrate, crop yield and quality, and ecotoxicological endpoints, among others. Nitrate content reduction in soil solution was verified for the fresh biochar scenario in both 12 and 50 t $ha^{-1}$ treatments and was coupled to a significant reduction of chloride, sodium, calcium and magnesium. This effect was noticed only after eight months of biochar application thus suggesting a time-dependent process. All other mechanisms tested being discarded, the formation of an organo-mineral coating emerges as a plausible explanation for the ionic content decrease.

**Keywords:** gasification biochar; nitrate mitigation; ageing; lysimeters

## 1.1. Introduction

Anthropic activity has doubled the pool of reactive nitrogen (N) since pre-industrial times (Vitousek et al., 1997). Intensification of agriculture, and specifically the Haber-Bosch process (synthetic N fixation), and legume cultivation (biological N fixation) greatly contributed to enhanced N fluxes (Galloway et al., 2003). Although N is the main limiting nutrient in non-legume crops, it is estimated that approximately half of all nitrogen applied to boost agricultural production is not taken up by plants but lost to other environmental compartments (Davidson et al., 2011). The main N loss pathways from agroecosystems are: i) nitrate ($NO_3^-$) leaching, given that $NO_3^-$ is highly soluble in water and thus susceptible to leakage; ii) denitrification, mostly occurring under anaerobic conditions, where $NO_3^-$ is transitorily reduced to nitrite ($NO_2^-$), then to nitric oxide (NO), and finally to nitrous oxide ($N_2O$) or dinitrogen ($N_2$); and iii) ammonia ($NH_3$) volatilisation, mainly in alkaline soils after organic or $NH_4^+$-containing fertiliser application. These N losses might not only imply reduced yields but also pose a threat to environmental and human health e.g., high levels of $NO_3^-$ in water resources have been linked to sanitary problems such as cancer and methemoglobinemia (Powlson et al., 2008; Ward et al., 2018) and environmental adverse effects as eutrophication; $N_2O$ is a potent greenhouse gas (GHG) with 265 times the warming potential of carbon dioxide (IPCC, 2014); and $NH_3$ volatilisation can cause damage to sensitive crops (Pearson and Stewart, 1993), led to acidification (Cameron et al., 2013), and act as a secondary source of nitric and nitrous oxides (Bowman, 1990).

Therefore, there is an urge to develop mitigation strategies to cope with elevated N fluxes, and biochar amendment to soil has arisen as a valuable option. Biochar is the solid by-product of biomass pyrolysis or gasification i.e., thermal decomposition in zero or very low oxygen conditions (Sohi et al., 2010). Biochar is characterised by its polycondensed aromatic carbon backbone, a high surface area provided by its porous structure, and the abundance of reactive functional groups on its surface. Those properties have been reported to translate into a high C stability (and therefore C sequestration potential), and an increased nutrient and water retention capacity (Glaser et al., 2016). Physicochemical properties of biochar are highly dependent on the biomass feedstock and the pyrolysis procedure used (especially temperature), and its practical effects in the field can further vary as a result of application rates, climate conditions, soil properties, crop type, and residence time in soil (Joseph et al., 2010; Nguyen et al., 2017). Furthermore, the inner complexity of the soil nitrogen cycle leads to a variety of mechanisms in which biochar

addition can alter N transformations. This is why many inconsistencies on the biochar effect on N fluxes are found in the literature, either increasing or diminishing them, as well as having no effects at all (Clough et al., 2013). Despite the aforementioned disparity, numerous studies have pointed out biochar's ability to reduce $NO_3^-$ leaching (Dempster et al., 2012; Kammann et al., 2015; Ventura et al., 2013; Yao et al., 2012). The principal suggested mechanisms comprehend sorption, microbial N-cycling shifts (including immobilisation, mineralisation, nitrification and denitrification), and $NH_3$ volatilisation among others, described hereafter.

Sorption of $NH_4^+$ through biochar's cation exchange capacity (CEC) is a classic proposed mechanism to explain nitrogen retention (Lehmann et al., 2003; Liang et al., 2006; Nelissen et al., 2012), which is expected to intensify over time leading to a larger nutrient retention in aged as opposed to fresh biochar (Kookana et al., 2011). Conversely, $NO_3^-$ sorption by anion exchange capacity (AEC) is restricted to few examples (Lawrinenko and Laird, 2015), but other mechanisms such as bridge bonding (Mukherjee et al., 2011), non-conventional ion-water bonding and non-conventional hydrogen bonding (Conte et al., 2014; Kammann et al., 2015) have been suggested to explain direct $NO_3^-$ retention.

Regarding microbial N-cycling, biochar could alter it in multifarious pathways. It has been reported that the labile C pool present in fresh biochars can cause a transitory increase in soil microbial biomass shortly after being applied, leading to a simultaneous C and N retention in microbial biomass (Ippolito et al., 2012). N mineralisation and nitrification can decrease with biochar addition as a result of toxic effects (Clough et al., 2010), or as inorganic N is retained and excluded from metabolic routes (Pal, 2016). Biochar can also affect whether denitrification is favoured, as this process is stimulated in anaerobic conditions, and biochar can influence water-filled pore space, and, in turn, oxygen supply (Hagemann et al., 2016).

Finally, $NH_3$ volatilisation, associated to the liming effect of some biochars, is a proposed N loss path (Schomberg et al., 2012) whereas $NH_3$ adsorption onto biochar is also possible (Asada et al., 2006; Doydora et al., 2011), which can indeed lead to enhanced plant N uptake (Mandal et al., 2016; Taghizadeh-Toosi et al., 2012).

Previous studies of our research group have pointed out a reduction of the soluble $NO_3^-$ topsoil content in outdoor biochar-amended mesocosms under Mediterranean conditions fifteen months following the application (Marks et al., 2016) but the mechanism responsible for that

reduction was not ascertained. Therefore, the aims of this study were to: i) prove that biochar is effective in reducing nitrate concentrations at short- and long-term, and ii) determine which one of the above explained mechanisms is mainly operating. For this purpose, N-pools were monitored for 8 months in greenhouse lysimeters mimicking a plant-soil system using two biochar supplementation scenarios (freshly added biochar and biochar naturally aged in soil for 6 years), applied at three addition rates (0, 12 and 50 t $ha^{-1}$).

## 1.2. Materials and methods

### 1.2.1. Lysimeter setup

A lysimeter system was set up in a greenhouse setting at the IRTA Torre Marimón experimental station (Caldes de Montbui, NE Spain) to simulate the effects of biochar agricultural amendment at increasing application rates (0, 12 and 50 t $ha^{-1}$, which corresponded to 0, 37.7, and 157.1 g of biochar per lysimeter), and at two contrasted ageing scenarios: just after the biochar application (fresh), and 6 years after natural ageing of biochar in outdoor soil mesocosms (aged). The biochar used in this experiment was produced from *Pinus pinaster* and *P. radiata* wood chips within a gasification reactor (600-900ºC) with a residence time of 10 s (see biochar physicochemical characterisation summarised in **Table 1**). For a more detailed description on the biochar preparation refer to Marks et al. (2014a).

**Table 1.** Characteristics of the biochar used in the experiment.

| Parameter | Unit | Value | Method |
|---|---|---|---|
| C | g $kg^{-1}$ | 782 | elemental analysis |
| N | g $kg^{-1}$ | 2.10 | elemental analysis |
| H | g $kg^{-1}$ | 8.81 | elemental analysis |
| S | g $kg^{-1}$ | 0.34 | elemental analysis |
| O | g $kg^{-1}$ | 70.68 | difference of sum of elemental analysis and ash |
| $O/C_{org}$ | | 0.07 | |
| $H/C_{org}$ | | 0.14 | |
| Ash | % | 13.61 | difference of fixed carbon and volatile matter |
| Volatile matter | % | 2.8 | gravimetrically (mass loss between 150ºC-600ºC) |
| P | g $kg^{-1}$ | 1.34 | |
| Na | g $kg^{-1}$ | 0.48 | |
| K | g $kg^{-1}$ | 9.36 | |
| Ca | g $kg^{-1}$ | 20.52 | |
| Mg | g $kg^{-1}$ | 2.10 | |
| $CaCO_3$ | g $kg^{-1}$ | 33.4 ± 0.62 | calcimetry |
| $C\text{-}CO_3$ | g $kg^{-1}$ | 4 ± 0.62 | calcimetry |
| PAH (16 congeners) | mg $kg^{-1}$ | 438 | 1:1 acetone:hexane extraction, gas chromatography-mass spectrometry |
| pH ($H_2O$, 1:20) | - | 11.14 ± 0.13 | |
| EC (25ºC, 1:20) | dS $m^{-1}$ | 0.3 ± 0.01 | |
| CEC | $mmol_c$ $kg^{-1}$ | 3.62 ± 0.11 | ISO 23470, 2007 |
| $\delta^{15}N$ | ‰ | -0.9 | elemental analysis-isotope ratio mass spectrometry |
| Brunauer-Emmett-Teller (BET) surface area | $m^2$ $g^{-1}$ | 19.77 | $N_2$ adsorption isotherm, 77K |
| Porosity | % | 80.56 | Hg porosimetry |
| Mean porus size | nm | 1220 | Hg porosimetry |
| Micropore (ø < 2 nm) volume | $cm^3$ $g^{-1}$ | 0.0034 | |
| Mesopore (2 nm ≤ ø ≤ 50 nm) volume | $cm^3$ $g^{-1}$ | 0.0196 | |
| Macropore (ø > 50 nm) volume | $cm^3$ $g^{-1}$ | 2.82 | |

Each lysimeter consisted of a polyvinyl chloride (PVC) tube (48 cm height x 20 cm diameter) with a perforated lid in the bottom, which was covered with a 2mm-mesh gauze and a 2 cm quartz sand layer to ensure proper drainage without substantial soil loss. Then, two 20 cm layers of soil (6.7 kg each) were added to mimic B and Ap horizons, the former consisting of only soil, and the latter of soil-biochar mixtures. The soil used for the lysimeters construction corresponded to a Fluventic Haploxerept (Soil Survey Staff, 2010) described in detail in Marks et al. (2016), but two differentially managed soils within the same field were used according to the different biochar ageing scenarios. A soil portion that has been under fallow since 2011 was either used as B-horizon for all the lysimeters and also to prepare soil-biochar mixtures used as Ap-horizon in the lysimeters of the fresh biochar scenario. Instead, for the aged biochar scenario, the Ap horizon corresponded to the topsoil (20 cm) of outdoor mesocosms of the experiment described in Marks et al. (2016). The mesocosms were set up in March 2011 and therefore contained biochar aged for six years. Also, the mesocosms had been fertilised with a thermally dried pig slurry at a 50 kg N $ha^{-1}$ $year^{-1}$ rate and cropped to barley all over this period. In summary, two different biochar scenarios, fresh (F) and aged (A), and three addition rates (0, 12 and 50 t $ha^{-1}$) were tested, yielding a total of six treatments hereafter designated as $A_0$, $A_{12}$, $A_{50}$, $F_0$, $F_{12}$, and $F_{50}$, assigned in a fully replicated (n = 5) randomised design.

The lysimeters were set up on 23rd March 2017 and left to stabilise for 11 days after an initial watering. On 3rd April fifteen barley seeds (*Hordeum vulgare*) were sown (later thinned to only 1 plant per lysimeter), and each lysimeter was fertilised with 7.3 g of a thermally dried pig slurry which corresponded to a 100 kg N $ha^{-1}$ $_{\text{addition rate}}$ based on the available N (see pig slurry characterisation in **Supplementary Table S1**). A drip irrigation system was installed on each lysimeter to keep moisture around 50% of the maximum water holding capacity (i.e., 16.5% moisture w/w) during barley growth. After harvest (on 3rd July) a drought period was simulated in order to mimic the Mediterranean climate. Only three spaced irrigation events were performed during summer and early fall, which coupled to the high temperatures in the greenhouse, led to dry soil conditions during most of the period. Drought conditions were suppressed to some extent shortly before the final sampling, with a fourth irrigation event coupled to lower temperatures (records of greenhouse temperature and lysimeters moisture are shown in the **Supplementary Fig. S1**).

Soil physicochemical, microbial, and isotopic parameters were assessed at five samplings along 2017, each corresponding to relevant stages in terms of fertilisation and plant development:

pre-fertilisation (3rd April); post-fertilisation (5th April); developed plant (7th June); harvest (5th July) and bare soil (4th December) (**Supplementary Fig. S1**). GHG soil emissions were assessed at the same dates except for the pre-fertilisation sampling, carried out at 30th March instead of 3rd April, and that of the developed plant stage, which was substituted by an earlier one (12th April) taken as additional post-fertilisation sampling in order to cover the possible gas emission peaks after fertilisation.

### 1.2.2. Soil physicochemical analyses

#### 1.2.2.1. Soil extract analyses: water-soluble and exchangeable ions, pH, moisture and electrical conductivity

A 5.5x7 cm core was used to collect soil samples in each lysimeter, then soil was manually homogenised in a plastic bag. KCl extracts were immediately prepared in the greenhouse on a 1:5 w/v ratio by mixing 20 g of fresh soil with 100 ml of 2 M KCl. Once in the laboratory, 1:5 ratio (w/v) water extracts were prepared by mixing 40 g of soil with 200 ml of distilled water and by shaking for 1 h in a vertical agitator (120 rpm) whereas KCl extracts were shaken for 30 min (ISO/TS 14256-1: 2003). In parallel, 10 g of soil were used for moisture determination. Both KCl and water extracts were centrifuged for 5 min at 8000 rpm, filtered in Whatman #42 filter paper, and frozen at -20 °C for later determination of ion content. Before freezing, a portion of the water extracts was used for pH and electrical conductivity (EC) measurement.

Water-soluble ionic concentrations were determined by liquid chromatography on a Dionex ICS-1100 ion chromatograph (Dionex, Sunnyvale, USA) using a AS4A-SC Dionex anion column for $Cl^-$, $NO_2^-$, $NO_3^-$, $HPO_4^{2-}$ and $SO_4^{2-}$ determination and a CS12A Dionex cation column for $Na^+$, $K^+$, $Mg^{2+}$, and $Ca^{2+}$ determination. All the ion concentrations were estimated using linear calibration except for $SO_4^{2-}$, $Mg^{2+}$, and $Ca^{2+}$ in which quadratic regression substantially increased fitting ($R^2$). Detection limit (LOD) estimation was stipulated as three times the standard deviation of five blank values.

Exchangeable N-$NH_4^+$ was assessed by subtracting water extractable concentrations to KCl extractable concentrations. For comparability purposes, both KCl and water extractable N-$NH_4^+$ were measured using the salicylate method (Willis et al., 1996), in a Spectronic 20 Genesys 4001/4 spectrophotometer. To validate the possibility of nitrate bridge bonding mechanisms

later discussed, KCl-extractable $NO_3^-$ was determined following Matsumura et al. (1999), but only for the bare soil sampling. It was found that increasing the volume sample up to 1 ml (instead of the recommended 0.1 ml in Willis et al. (1996)) for N-$NH_4^+$ determination in KCl and water extracts increased sensitivity without interferences, and for N-$NH_4^+$ determination after Kjeldahl digestions of soil and $K_2SO_4$ extracts (see below) sample volumes were set at 0.3 and 0.5 ml, respectively.

#### 1.2.2.2. Soil total Kjeldahl nitrogen and organic carbon

A portion of the collected soil was air-dried and finely ground (ø < 0.2 mm) in order to assess Kjeldahl nitrogen and organic carbon. Total Kjeldahl nitrogen (TKN) was assessed using the micro-Kjeldahl method by Bremner (1965) with the following modifications: after digestion was finished, digestates were diluted with distilled water to make up a volume of 100 ml and N-$NH_4^+$ was measured by the salicylate method. Organic carbon was determined by the Walkley–Black $K_2Cr_2O_7$-$H_2SO_4$ oxidation method (Nelson and Sommers, 1983).

#### 1.2.2.3. Leachates

After soil sampling, an irrigation-induced leaching was carried out by placing each lysimeter on a glass tray but suspended 1.3 cm above its surface to allow drainage. The water addition needed to produce a leachate volume of c.a. 200 ml was estimated by measuring lysimeters water content gravimetrically, and taking into account trials before the lysimeters setup that enabled us to estimate the water holding capacity around 24%. This procedure allowed the calculation of the total volume of water in the system (soil water content + water added to provoke leaching) required to express leachate analysis on a dry basis. In the laboratory, the obtained leachates were filtered and analysed by liquid chromatography as described for water extracts (except for ammonium measurement, which was also undergone by chromatography instead of the salicylate method).

### 1.2.3. Soil microbial analyses

A subsample of the fresh soil batch previously described was used to determine microbiological endpoints. Soil basal respiration (BAS) was assessed with $CO_2$ traps according to Pell et al. (2006). Microbial biomass-carbon ($C_{mic}$) and nitrogen ($N_{mic}$) were obtained using the chloroform fumigation-extraction method (Vance et al., 1987). In detail, 10 g of soil fresh weight (FW)

corresponding to the fumigated samples were exposed to a chloroform-saturated atmosphere (fumigated) by placing the samples in a hermetically closed desiccator and by boiling 50 ml of chloroform placed in a 100 ml beaker under vacuum for 2 minutes and then closing the desiccator seal for 24 h. Both the unfumigated and fumigated samples were then mixed with 0.5 M $K_2SO_4$ at a 1:4 soil:solution ratio, vertically shaken for 120 minutes, and the extracts filtered through Whatman #42 filter paper.

$C_{mic}$ was determined by wet oxidation of an extract aliquot with potassium dichromate followed by titration with Mohr's salt, and estimated as the difference in C concentration between fumigated and non-fumigated soil divided by $K_{EC}$ = 0.38 (Vance et al., 1987).

$N_{mic}$ was assessed by a Kjeldahl digestion of an extract aliquot coupled to salicylate $N\text{-}NH_4^+$ determination explained in Cabrera and Beare (1993). The difference in the Kjeldhal N concentration in between fumigated and non-fumigated soil, divided by $K_{EN}$ = 0.5 (Voroney et al., 2008), was used to calculate $N_{mic}$. Although $N_{mic}$ is generally estimated using the total nitrogen in $K_2SO_4$ extracts (therefore including $N\text{-}NH_4^+$ but also $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$ concentration), we used Kjeldahl nitrogen instead (organic nitrogen plus inorganic $N\text{-}NH_4^+$) since nearly all the nitrogen in microorganisms is organic. $N_{mic}$ of the post-fertilisation sampling was not considered due to the negative values found in many lysimeters which are plausibly an artefact related to the elevated and heterogeneous concentrations of inorganic $NH_4^+$ in both fumigated and unfumigated samples, sampled two days after the dried pig slurry application. The calculation of $N_{mic}$ for the $F_0$ treatment at the bare soil sampling was also elusive as the high nitrate levels in the $F_0$ treatment interfered with Kjeldahl measurement, since $NO_3^-$ can undergo reaction with $NH_4^+$ to form $N_2O$ during digestion. Even when assessed with the pre-treatment proposed by Wyland et al. (1994) such interference persisted, as shown by values below the analytical blanks. For this reason, $N_{mic}$ of the mentioned treatment was also measured with the ninhydrin method (Brookes and Joergensen, 2006).

The organic carbon and Kjeldahl nitrogen in $K_2SO_4$ extracts of the unfumigated samples were taken as dissolved organic C and N (DOC and DON[1]).

[1] Dissolved organic carbon and nitrogen (DOC and DON), and extractable organic carbon and nitrogen (EOC and EON) are used interchangeably throughout this thesis.

### 1.2.4. Gas sampling: $N_2O$, $CO_2$, $NH_3$

Trace gas emissions of $N_2O$ and $CO_2$ were evaluated employing non-flow-through, non-steady-state chambers. The gases were collected according to the methodology of Collier et al. (2014) and using static chambers (21.5 cm high, 21 cm diameter) with a vent to prevent pressure gradients influencing gas exchange. For emission rates estimation, gases were accumulated in the chamber and air samples were collected at three time points: one taken immediately after chamber closure (t= 0), and after 10 and 20 min or after 15 and 30 min, as chamber deployment duration was prolonged in samplings when air temperature was cooler. It is recommended (De Klein and Harvey, 2015) that chamber height (cm) to deployment time (h) ratio should be ≥40 cm $h^{-1}$, in our case it was 64.5 cm $h^{-1}$ for 20 min deployment duration and 43 cm $h^{-1}$ for 30 min deployment duration. Gas samples were extracted from the static chambers using a plastic syringe (20 ml) and injected into a 12 ml vial (Exetainers®, Labco Ltd., Ceredigion, UK), and then analysed by gas chromatography (Agilent 7890A) coupled to ECD and TCD. The detection limits of the GC are 10 ppmV and 20 ppbV for $CO_2$ and $N_2O$, respectively. Quality of analysis was checked using standards of known gas concentrations (250 and 1003 ppmV for $CO_2$ and 175 and 600 ppbV for $N_2O$). Fluxes were calculated from the slope of the linear regression between the concentration of each GHG and the accumulation time inside the chamber, subsequently corrected by the air temperature, the atmospheric pressure, and the surface-volume ratio of the chamber, as described in detail by Barton et al. (2008). The squared Pearson's correlation coefficient ($r^2$) corresponding to the concentration of $CO_2$ accumulated in a linear and increasing manner was used as an indicator that the system was functioning properly. This is why the $N_2O$ fluxes were only considered when the $CO_2$ fluxes had an $r^2 \geq 0.80$.

$NH_3$ emissions were measured by chemical traps, which consisted of 10 ml of a 0.5% (w / v) boric acid solution, placed in 50 ml plastic cups, containing 3 drops of indicator (0.099 g of bromocresol green and 0.066 g of methyl red dissolved in 100 ml of 96% ethanol). A trap was placed at each lysimeter soil surface and then the lysimeter sealed with a polyethylene sheet to allow $NH_3$ accumulation and its capture in the traps. Cups were only collected when the indicator colour changed from pink to green and the time registered. At collection, each trap was closed with a lid and transported to the lab for its titration with 1 mM HCl for the $NH_4^+$ concentration estimation. These measurements were only carried out around the fertilisation event, with non-detectable $NH_3$ concentrations in a pre-fertilisation accumulation period of 91

h, and detectable levels only in the 9 days following fertilisation as represented by four samplings with accumulation times ranging between 19 and 46 h.

### 1.2.5. Isotopic composition analyses

Isotopic composition analyses were performed in lysimeters' soil KCl extracts (for treatments $A_0$, $A_{50}$, $F_0$, and $F_{50}$) and leachates (for treatments $F_0$ and $F_{50}$). Ancillary measurements included the determination of the $\delta^{15}N$ and $\delta^{18}O$ of dissolved $NO_3^-$ from irrigation water at two different dates (3rd April 2017 and 4th December 2017), and of the bulk $\delta^{15}N$ of soil ($F_0$, $F_{50}$, and $A_{50}$), harvested barley stem and leaves ($F_0$, $F_{50}$), biochar, and pig slurry. The $\delta^{15}N$ and $\delta^{18}O$ of dissolved $NO_3^-$ were determined using a modified cadmium and azide reduction method (McIlvin and Altabet, 2005; Ryabenko et al., 2009) followed by a simultaneous $\delta^{15}N$ and $\delta^{18}O$ analysis of resultant $N_2O$ using a Pre-Con (Thermo Scientific) coupled to a Finnigan MAT-253 Isotope Ratio Mass Spectrometer (Thermo Scientific). The bulk $\delta^{15}N$ of soil, plant, biochar, and pig slurry samples was determined in a Carbo Erba EA-Finnigan Delta C IRMS. Following Coplen (2011), several international and laboratory (UB) standards were interspersed among samples for the normalisation of the isotope results i.e., USGS-32, USGS-34, USGS-35, UB-$IWS_{NO3}$ ($\delta^{15}N$ = +16.9 ‰, $\delta^{18}O$ = +28.5 ‰) for the $\delta^{15}N$ and $\delta^{18}O$ of dissolved $NO_3^-$; and USGS-40, IAEA-N1, IAEA-N2 and UCGEMA-P, for the $\delta^{15}N_{bulk}$ of solid materials. The standard deviation reproducibility of the samples was ±1.0 ‰ for $\delta^{15}N$ of dissolved $NO_3^-$; ±1.5 ‰ for $\delta^{18}O$ of dissolved $NO_3^-$; and ±0.2 ‰ for $\delta^{15}N_{bulk}$ of solid materials. Values of $\delta^{15}N$ are reported relative to Atmospheric (AIR), and $\delta^{18}O$ values are reported relative to Vienna Standard Mean Ocean Water (V-SMOW) in per mill (‰) as defined by equations a and b:

$$\delta^{15}N_{NO_3} = \left[\frac{\left(\frac{^{15}N}{^{14}N}\right)_{sample} - \left(\frac{^{15}N}{^{14}N}\right)_{AIR}}{\left(\frac{^{15}N}{^{14}N}\right)_{AIR}}\right] \qquad (a)$$

$$\delta^{18}O_{NO_3} = \left[\frac{\left(\frac{^{18}O}{^{16}O}\right)_{sample} - \left(\frac{^{18}O}{^{16}O}\right)_{VSMOW}}{\left(\frac{^{18}O}{^{16}O}\right)_{VSMOW}}\right] \qquad (b)$$

### 1.2.6. Plant yield and nutrient uptake

Above-ground barley biomass was harvested at the end of its life cycle in early summer (3rd July, 2017) and dried at 60ºC for 48 h. Growth parameters assessed were straw and grain weight, ear count, and mean number of grains per ear. After straw and grain grinding in a ball-mill, nutrient content (N, P, K, Ca, Mg, S, Mn, and Zn) was obtained by near infrared spectrometry (NIRS) by scanning the ground samples in duplicate from 1100 to 2500 nm using a NIRSystems 5000 scanning monochromator (FOSS, Hilleröd, Denmark) employing the calibrations developed in a previous study (Martos et al., 2020).

### 1.2.7. Ecotoxicological characterisation

Subsamples from each soil and soil-biochar mixtures were taken before lysimeters setup to be used for the ecotoxicity assessment. On the one hand, the collembolan *Folsomia candida* was used as a proxy of toxicity to soil organisms using the survival and reproduction test of the ISO Guideline 11267 (ISO 1999).

On the other hand, elutriates from the soil and soil-biochar mixtures were prepared to assess aquatic toxicity, mimicking the potential exposure of aquatic organisms to runoff. For this purpose, growth inhibition of the algae *Raphidocelis subcapitata* (SAG 61.81, Inst. Plant Physiology U. Göttingen) was tested following OECD 201 (2011). Specifically, yield inhibition rate (72 h) was assessed at four elutriate dilutions (81.6, 51, 30.6, and 10.2 % v/v) of an initial elutriate prepared as follows: a 1:10 (w/v) soil-water mixture suspension (25 g of air-dried soil: 250 ml of water) was prepared, stirred for 12 h in a vertical agitator (120 rpm), and centrifuged 20 minutes at 10000 rpm. Then, centrifuge tubes were decanted to collect the supernatants, kept refrigerated until testing before 24 h. The used method is a modification of the DIN 38414_S4 (1984), since a higher centrifugation speed was used to reduce the turbidity caused by biochar particles in the suspension (from 4500 to 10000 rpm).

Finally, potential impacts on nitrogen-related microbial functional groups were assessed at the 12th April 2017 sampling (9 days after fertilisation), when a microbial activity peak was expected, so as to detect any ecotoxicological effects. The target functional genes assessed were: amoA for the ammonia-oxidizing bacteria (AOB) and archaea (AOA); nxrB for the beta subunit of nitrite oxidase of *Nitrobacter* sp.; nirK and nirS for $NO_2^-$ 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 $NH_4^+$. Fresh soil samples stored at -80 °C were used for simultaneous extraction of DNA and RNA following the protocol described in Griffiths et al. (2000) with the modifications provided by Töwe et al. (2011). Nucleic acids were quantified with the Qubit 3.0 Fluorimeter (Life Technologies) as instructed by manufacturer. Retro RNA transcription was performed using All-in-One cDNA Synthesis SuperMix (Bimake) following the manufacturer's protocol. The real-time PCR (quantitative PCR indicated as qPCR) was carried out in the UAB Campus Agrogenomic Service, with a LightCycler® 480 System (Roche). **Supplementary Table S2** shows more details of the qPCRs of the quantified functional genes. All the samples and standards were analysed in duplicate and each plate contained 6 negative control replicates. The amplification efficiency was calculated as: $E = [10^{(-1/slope)} -1]*100$, was and the results were: nifH: 87-93%; bacterial amoA: 88-93%; archaeal amoA: 87-93%; nxrB: 97-99%; nirK: 97-99%; nosZ: 86-90% and nirS: 82-84%. These efficiency values are consistent with those reported in the literature by similar studies (Töwe et al., 2010; Harter et al., 2014).

### 1.2.8. Statistical tests

The statistical treatment of the experimental data was carried out using R software v. 3.6.1 (R Core Team, 2019), and its visualisation using the packages *ggplot2* (Wickham, 2016) and *ggpubr* v 0.2.3 (Kassambara, 2019a). Fresh and aged-biochar treatments were always tested separately since their corresponding controls were found to differ significantly in key properties such as organic carbon and Kjeldahl nitrogen, as expected by the different starting points of each scenario (six years of fallow in the fresh biochar scenario and continuous cropping in the aged biochar scenario).

Longitudinal data (i.e., variables for which exist a between-subjects factor = biochar addition rate, and a within-subjects factor = different sampling dates) were analysed using two-way mixed ANOVAs, which were computed with the *rstatix* package v0.2.0 (Kassambara, 2019b). Shapiro-Wilk and Levene tests were used to ensure normal distribution and homogeneous variances, respectively. When these assumptions were not met, the test was run on the $log_{10}$-transformed variable. The assumption of sphericity was checked using the Mauchly's test and when violated the Greenhouse-Geisser correction was applied. Finally, homogeneity of covariances was tested by Box's M. Statistical results of the mixed ANOVA are shown in **Supplementary Table S3**

**(S3.1.-S3.36.)**. Pairwise comparisons were tested with t test with Bonferroni adjustment, and the significance level was set at $p < 0.05$.

By contrast, parameters analysed at a single sampling date were assessed by means of the Kruskal–Wallis test followed by pairwise comparisons with the Wilcoxon's test with Bonferroni adjustment (*rstatix* package v 0.2.0 (Kassambara, 2019b)) since the low n values resulted insufficient to ensure that requirements for parametric tests were being met. It should be noted that when mixed ANOVA analysis of longitudinal data was not significant, differences between treatments at each sampling date were also checked using this approach. Finally, for tests that only involved two independent groups, the Mann-Whitney-Wilcoxon test with Bonferroni adjustment was used. In the provided graphs, significant differences between biochar-amended and control lysimeters are indicated by lower case letters. Hereafter within the text, all values are reported as mean ± standard error (SE).

## 1.3. Results

### 1.3.1. Soil physicochemical parameters

Results for moisture, EC, pH, Total Kjeldahl Nitrogen (TKN), and $C_{org}$ are presented in **Fig. 1**. Biochar application significantly increased $C_{org}$ in both fresh and aged biochar scenarios throughout the entire experiment. While in the fresh biochar scenario the increase of $C_{org}$ was proportional to biochar application rate, in the aged biochar scenario the difference between 12 and 50 t $ha^{-1}$ was less marked. Regarding the remainder parameters, significant biochar effects were only found in the fresh biochar scenario: i) moisture levels were significantly enhanced at two sampling dates (5th April and 5th July), with a non-significant increase at 4th December, the effect being more pronounced in the $F_{50}$ treatment than in $F_{12}$; ii) EC was higher in the $F_{50}$ treatment compared to $F_0$ at the pre-fertilisation sampling (3rd April), whereas this trend reverted at the bare soil sampling (4th December), being $F_0$ the treatment with highest values; iii) $F_{50}$ treatment lead to significant higher TKN at one sampling date (7th June) with respect to control.

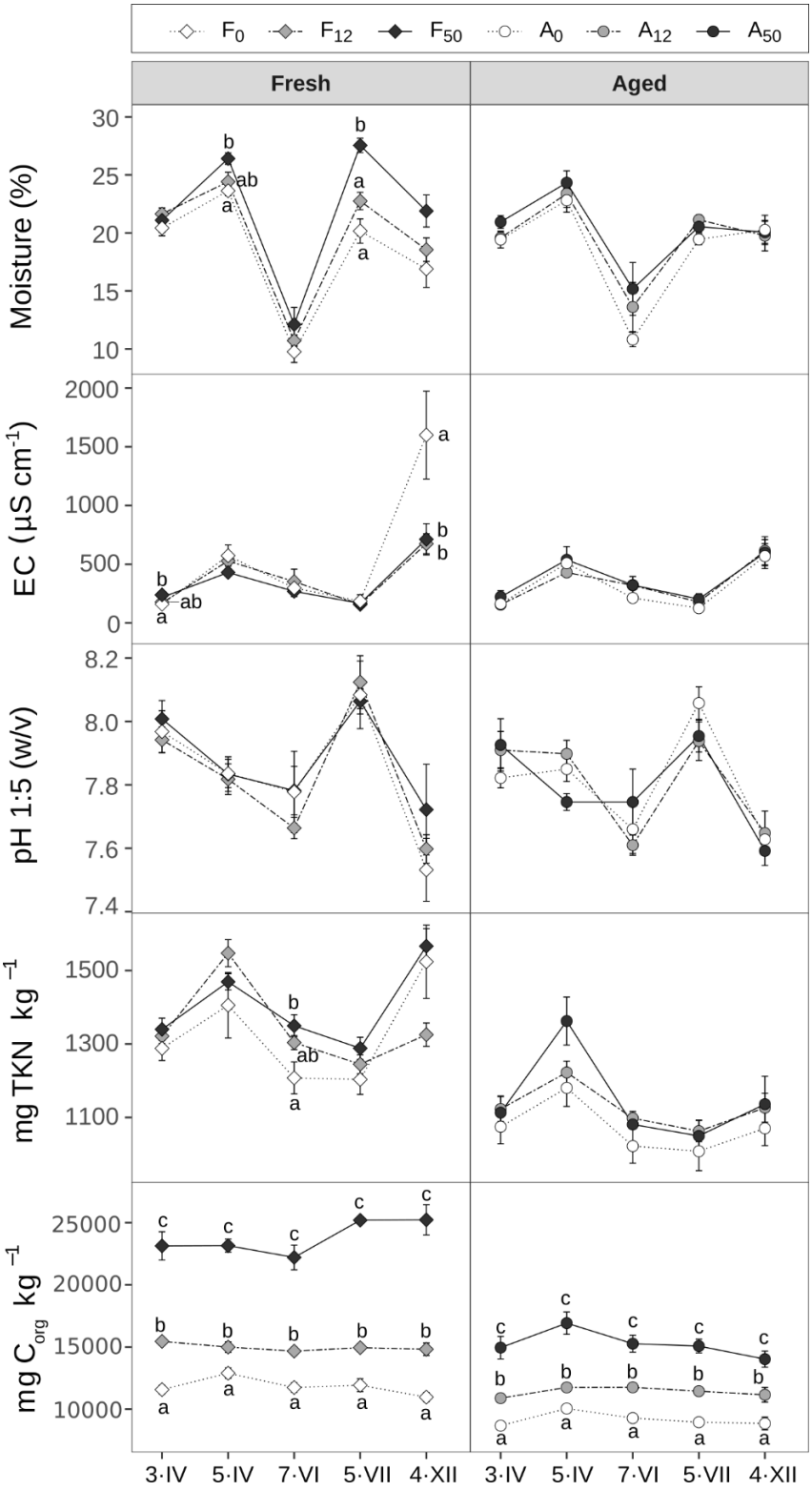


**Fig. 1**. Moisture (%), EC (µS $cm^{-1}$), pH 1:5 (w/v), Total Kjeldahl Nitrogen (TKN) (mg $kg^{-1}$ DW soil) and Corg (mg $kg^{-1}$ DW soil) along five samplings (3·IV = 3rd April; 5·IV = 5th April; 7·VI = 7th June; 5·VII = 5th July and 4·XII = 4th December). Abbreviations for the biochar treatments correspond to: $F_0$ = fresh 0 t $ha^{-1}$; $F_{12}$ = fresh 12 t $ha^{-1}$; $F_{50}$ = fresh 50 t $ha^{-1}$; $A_0$ = aged 0 t $ha^{-1}$; $A_{12}$ = aged 12 t $ha^{-1}$; $A_{50}$ = aged 50 t $ha^{-1}$. Symbols represent the mean values, and bars represent the corresponding standard error (n = 5). Different letters indicate statistically significant differences between treatments within a particular sampling.

It has to be pointed out that TKN measured at 4th December (a date with high levels of N-$NO_3^-$) is misleading since we observed important inconsistencies between total nitrogen (measured by combustion) and the sum of TKN plus ($NO_3^-$ +$NO_2^-$)-N in $F_0$ and $F_{50}$ treatments (data not shown). As Bremner and Mulvaney (1983) reported, soils with significant amounts of $NO_3^-$ and $NO_2^-$ present unexpected problematics in total N analysis, since the usually employed Kjeldahl methods do not quantitatively recover N-$NO_3^-$ and N-$NO_2^-$, but they usually include some of this N. Finally, regarding soil pH, it was unaffected by any of the treatments.

Concerning N species, concentrations of N-$NO_2^-$ and N-$NH_4^+$ were below detection limits in all the leachates, as also found for N-$NO_2^-$ in all the water extracts except in the post-fertilisation sampling. N-$NO_3^-$ was the dominant inorganic N form in soil extracts and leachates along the different sampling dates with exception of the post-fertilisation sampling (5th April), where total (soluble + exchangeable) N-$NH_4^+$ outnumbered N-$NO_3^-$ in soil extracts (**Supplementary Fig. S2**). N-$NO_3^-$ concentration in soil extracts only differed significantly as affected by biochar addition at the bare soil sampling in fresh biochar treatments. Namely, a significant decrease in N-$NO_3^-$ content in $F_{12}$ (-69 %) and $F_{50}$ (-64 %) with respect to control was observed (**Fig. 2**). In order to confirm this result, a Kruskal-Wallis H test was performed excluding one extreme outlier present in the $F_0$ treatment. Statistical significance remained ($\chi^2_{(2, N = 14)} = 6.53$, $p = 0.04$), but results of the Bonferroni post hoc test were not sufficient to make statements about pair-wise differences. Since N-$NO_3^-$ availability reduction still showed an important magnitude effect for both $F_{12}$ and $F_{50}$, -57 % and -51 % respectively, it was concluded that the effect is consistent.

Furthermore, this trend was also shown for another anion ($Cl^-$), several cations ($Ca^{2+}$, $Mg^{2+}$, $Na^+$), and DOC (**Fig. 3**), with significant differences among treatments shown in **Supplementary Fig. S3** for ionic species, and in the **Supplementary Fig. S4** for DOC. We found that those concentration reductions are more robust in the $F_{12}$ treatment for some ionic species and that the differences in DOC, although following the same tendency, are not significant. Other ions such as $K^+$, $SO_4^{2+}$, N-$NO_2^-$ and N-$NH_4^+$ didn't show this trend. $HPO_4^{2-}$ is not shown as its signal-to-noise ratio in the chromatogram didn't exceed the set value of 3.

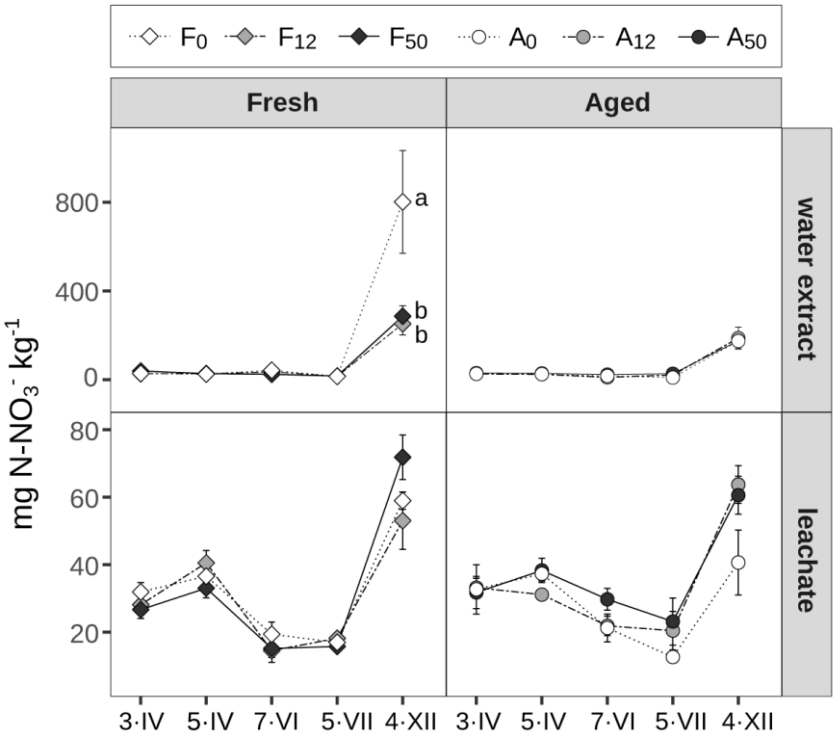


**Fig. 2.** N-$NO_3^-$ (mg $kg^{-1}$ DW soil) evolution in water extracts (1st row) and leachates (2nd row) along five samplings (3·IV = 3rd April; 5·IV = 5th April; 7·VI = 7th June; 5·VII = 5th July and 4·XII = 4th December). Abbreviations for the biochar treatments correspond to: $F_0$ = fresh 0 t $ha^{-1}$; $F_{12}$ = fresh 12 t $ha^{-1}$; $F_{50}$ = fresh 50 t $ha^{-1}$; $A_0$ = aged 0 t $ha^{-1}$; $A_{12}$ = aged 12 t $ha^{-1}$; $A_{50}$ = aged 50 t $ha^{-1}$. Symbols represent the mean values, and bars represent the corresponding standard error (n = 5). Different letters indicate statistically significant differences between treatments within a particular sampling.

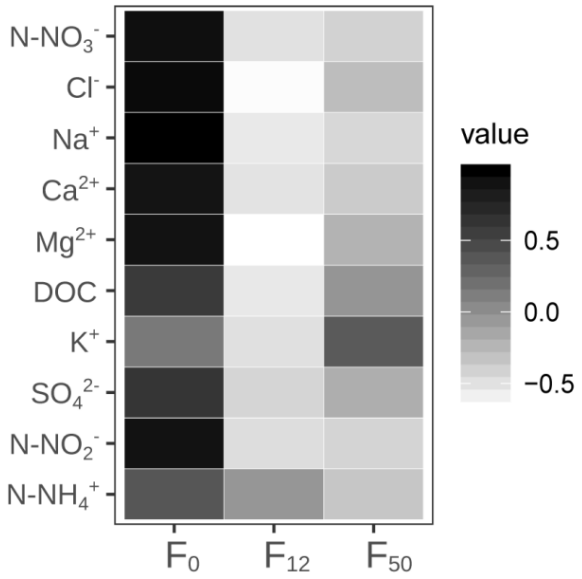


**Fig. 3.** Heatmap of ionic and DOC concentrations in soil solution (mg $kg^{-1}$ DW soil) at the bare soil sampling (4th December) for the fresh biochar scenario. Abbreviations for the biochar treatments correspond to: $F_0$ = fresh 0 t $ha^{-1}$; $F_{12}$ = fresh 12 t $ha^{-1}$; $F_{50}$ = fresh 50 t $ha^{-1}$; $A_0$ = aged 0 t $ha^{-1}$; $A_{12}$ = aged 12 t $ha^{-1}$; $A_{50}$ = aged 50 t $ha^{-1}$. The range of values [-0.5 - 1.5] which is used for heatmap colouring refers to standardised variables (mean subtracted and divided by standard deviation) (n = 5).

It is also worth noting that although $N\text{-}NO_3^-$ increased its concentration at the bare soil sampling, the proportion of losses as leaching decreased at this sampling, especially for the $F_0$ treatment. Specifically, $N\text{-}NO_3^-$ leachate content for $F_0$ treatment represented a 10% of the $N\text{-}NO_3^-$ soil solution content while for $F_{12}$ a 20% and for $F_{50}$ a 25% (**Supplementary Fig. S2**). N-forms in leachates did not show significant differences between treatments (**Fig. 2**, **Supplementary Fig. S3**) although a trend to increased ionic content can be observed for the $F_{50}$ treatment.

In relation to exchangeable N forms, $N\text{-}NH_4^+$ only exhibited an important peak at the post-fertilisation sampling (5th April) (**Supplementary Fig. S5**). At this date there were no significant differences due to treatment although an inverse trend was observed between ageing scenarios: while in the fresh biochar scenario biochar treatments surpass control in exchangeable $N\text{-}NH_4^+$ concentrations, the opposite is true in the aged biochar scenario. At the 5th July sampling $F_{50}$ had significantly larger concentrations ($2.53 \pm 0.31$ mg $kg^{-1}$) than $F_{12}$ ($1 \pm 0.2$ mg $kg^{-1}$). KCl-extractable $N\text{-}NO_3^-$ didn't significantly exceed water-soluble $N\text{-}NO_3^-$ (**Supplementary Fig. S6**) and showed a significant reduction in $F_{12}$ treatment ($278 \pm 27.6$ mg $kg^{-1}$) and a non-significant reduction in $F_{50}$ treatment ($315 \pm 60.5$ mg $kg^{-1}$) compared to $F_0$ ($752 \pm 217.4$ mg $kg^{-1}$) after a Kruskal Wallis H test ($\chi^2_{(2, N = 15)} = 7.98$, $p = 0.02$).

Finally, DON (**Supplementary Fig. S4**) is not discussed due to the methodological issues previously explained in section 3.2 that hindered its estimation for the $F_0$ treatment at the bare soil sampling.

### 1.3.2. Soil microbial analyses

None of the microbial parameters was significantly affected by any biochar treatment (**Table 2**) with the exception of an increased BAS value in the $A_{50}$ treatment in comparison with $A_0$ at the harvest sampling date (7th June). Unfortunately, we could not properly measure $N_{mic}$ for $F_0$ treatment at the bare soil sampling (4th December) by Kjeldahl means due to the methodological issues explained in section 2.3. Although ninhydrin method has been proved useful to determine the presence of N in this treatment, the obtained value ($24.1 \pm 2.35$ mg $kg^{-1}$) is not shown in the table as this method underestimates $N_{mic}$ compared to Kjeldahl method (Hedqvist and Udén, 2006).

**Table 2.** Average values of Cmic, Nmic (mg kg-1 DW soil) and BAS (mg C-CO2 kg-1 h-1) per treatment ± standard errors (n = 5) along five sampling dates (3·IV = 3rd April; 5·IV = 5th April; 7·VI = 7th June; 5·VII = 5th July and 4·XII = 4th December). Abbreviations for the biochar treatments correspond to: F0 = fresh 0 t ha-1; F12 = fresh 12 t ha-1; F50 = fresh 50 t ha-1; A0 = aged 0 t ha-1; A12 = aged 12 t ha-1; A50 = aged 50 t ha-1. Letters in bold indicate significant differences between treatments for a specific sampling date and n/a = not available.

| Parameter | Sampling date | Treatment | | | | | |
|---|---|---|---|---|---|---|---|
| | | **$F_0$** | **$F_{12}$** | **$F_{50}$** | **$A_0$** | **$A_{12}$** | **$A_{50}$** |
| **$C_{mic}$** (mg $kg^{-1}$) | 3·IV | 357 ± 76.4 | 398 ± 32.4 | 358 ± 33.6 | 243 ± 31.0 | 261 ± 32.6 | 202 ± 35.5 |
| | 5·IV | 337 ± 61.1 | 355 ± 39.1 | 423 ± 51.8 | 207 ± 22.9 | 268 ± 29.9 | 182 ± 11.7 |
| | 7·VI | 275 ± 20.8 | 271 ± 19.0 | 278 ± 22.7 | 150 ± 27.1 | 205 ± 27.7 | 216 ± 33.8 |
| | 5·VII | 277 ± 10.0 | 263 ± 16.1 | 240 ± 12.0 | 191 ± 21.7 | 183 ± 7.7 | 173 ± 22.7 |
| | 4·XII | 291 ± 33.6 | 273 ± 19.7 | 255 ± 18.7 | 199 ± 14.6 | 225 ± 12.7 | 238 ± 8.4 |
| **$N_{mic}$** (mg $kg^{-1}$) | 3·IV | 75.1 ± 4.9 | 89.3 ± 10.3 | 77.5 ± 3.5 | 44.3 ± 4.2 | 53.7 ± 3.6 | 51.6 ± 3.0 |
| | 5·IV | n/a | n/a | n/a | n/a | n/a | n/a |
| | 7·VI | 55.4 ± 6.1 | 66.7 ± 3.4 | 65.3 ± 6.0 | 44.6 ± 5.3 | 46.0 ± 3.9 | 47.5 ± 5.0 |
| | 5·VII | 79.5 ± 2.0 | 80.3 ± 2.2 | 73.0 ± 3.8 | 40.3 ± 3.1 | 47.4 ± 1.4 | 41.8 ± 2.3 |
| | 4·XII | n/a | 61.6 ± 3.4 | 53.5± 11.8 | 37.4 ± 3.7 | 34.7 ± 3.3 | 41.1 ± 4.4 |
| **BAS** (mg $C\text{-}CO_2$ $kg^{-1}$ $h^{-1}$) | 3·IV | 0.9 ± 0.0 | 1.1 ± 0.0 | 1.1 ± 0.1 | 0.8 ± 0.1 | 0.8 ± 0.0 | 0.8 ± 0.1 |
| | 5·IV | 0.9 ± 0.1 | 0.9 ± 0.1 | 0.9 ± 0.0 | 0.8 ± 0.1 | 0.7 ± 0.0 | 0.9 ± 0.1 |
| | 7·VI | 0.8 ± 0.0 | 0.7 ± 0.1 | 0.9 ± 0.1 | 0.7 ± 0.0 **a** | 0.9 ± 0.1 **ab** | 1.0 ± 0.1 **b** |
| | 5·VII | 0.7 ± 0.1 | 0.8 ± 0.1 | 1.0 ± 0.0 | 0.5 ± 0.1 | 0.6 ± 0.0 | 0.5 ± 0.1 |
| | 4·XII | 0.6 ± 0.1 | 0.9 ± 0.1 | 0.8 ± 0.1 | 0.5 ± 0.1 | 0.7 ± 0.1 | 0.6 ± 0.1 |

### 1.3.3. Gas emission rates: $N_2O$, $NH_3$, and $CO_2$

Gas emission rates results are summarised in **Fig. 4**. Regarding the mixed ANOVA results of $N\text{-}N_2O$ emissions, there were no significant main effects of treatment although a significant interaction of treatment with time occurred in both fresh and aged biochar scenarios (**Supplementary Table S3.30**). Nevertheless, t-tests between treatments within the different dates were not sufficient to make statements about pair-wise differences. However, some inverse non-significant trends can be observed. Namely, at the 12th April sampling, when emissions peaked, there was a trend to increased emissions for 12 t $ha^{-1}$ and less markedly for 50 t $ha^{-1}$ compared to 0 t $ha^{-1}$ in both fresh and aged biochar scenarios, whereas at 5th April, only 7 days before, the control surpassed biochar treatments in both scenarios.

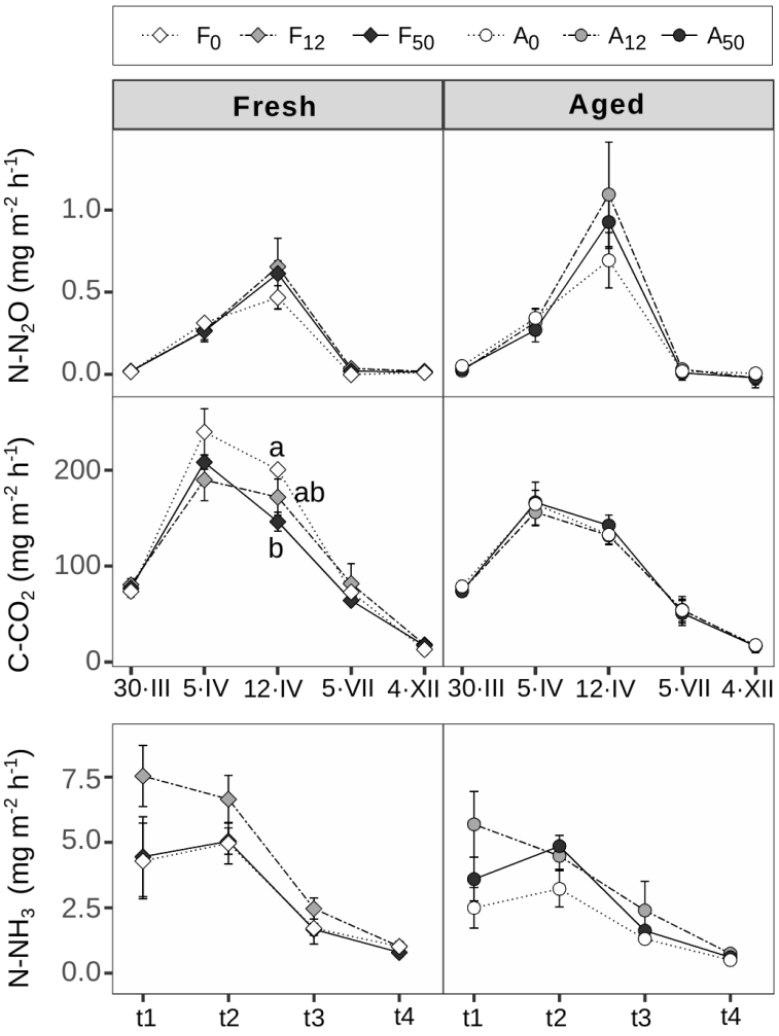


**Fig. 4.** Emission rates of $N\text{-}N_2O$, $C\text{-}CO_2$, and $N\text{-}NH_3$ (mg $m^{-2}$ $h^{-1}$). Abbreviations for the biochar treatments correspond to: $F_0$ = fresh 0 t $ha^{-1}$; $F_{12}$ = fresh 12 t $ha^{-1}$; $F_{50}$ = fresh 50 t $ha^{-1}$; $A_0$ = aged 0 t $ha^{-1}$; $A_{12}$ = aged 12 t $ha^{-1}$; $A_{50}$ = aged 50 t $ha^{-1}$. Different letters indicate statistically significant differences between treatments within a particular sampling. $N\text{-}N_2O$ and $C\text{-}CO_2$ were measured along five different samplings (30·III = 30th March; 5·IV = 5th April; 12·IV = 12th April; 5·VII = 5th July and 4·XII = 4th December), symbols represent the mean values, and bars represent the corresponding standard error, n is ≤ 5 as values were filtered (see 2.4 section in methodology). $N\text{-}NH_3$ was measured along 4 sampling periods after the fertilization with pig slurry (**t1** = 3/4/17-5/4/17; **t2** = 5/4/17-6/4/17; **t3** = 6/4/17-7/4/17; **t4** = 10/4/17-12/4/17), symbols represent the mean values, and bars represent the corresponding standard error (n = 5).

$NH_3$ was only detectable during a 9-day period after the fertilisation event. Despite the lack of statistical significance on N-$NH_3$ emissions between treatments, it can be observed a sustained tendency for higher emissions in $F_{12}$ than $F_0$ and $F_{50}$, while in the aged biochar scenario this trend is less marked.

Finally, soil C-$CO_2$ emissions were significantly reduced by the $F_{50}$ treatment with respect to control at the post-fertilisation 12th April sampling date, while in the previous sampling date (5th April), which is also described as post-fertilisation, $F_0$ also shows a non-significant trend to highest C-$CO_2$ emission rates.

### 1.3.4. N and O isotopic composition of $NO_3^-$ in KCl extracts and leachates in the fresh biochar scenario

Concerning N and O isotopic composition of $NO_3^-$ in KCl extracts, there was a lack of significant variations due to biochar treatment. Nevertheless, some temporal trends can be observed. In the pre-fertilisation sampling (3rd April), $\delta^{15}$N-$NO_3$ values of control and biochar lysimeters fell between the observed range of soil N (+5 to +6.8 ‰) (**Fig. 5**). After fertilisation (5th April), the value of $\delta^{15}$N-$NO_3$ slightly decreased. Later, in summer (7th June and 5th July), values of $\delta^{15}$N-$NO_3$ increased in all treatments, and in the $F_0$ treatment this was coupled to an increase in $\delta^{18}$O-$NO_3$ (in the $F_{50}$ treatment, the $\delta^{18}$O-$NO_3$ also increased on 7th June but not on 5th July). Finally, on 4th December, $\delta^{15}$N-$NO_3$ values evolved towards to the pre-fertilisation values. **Supplementary Fig. S7** shows the isotopic composition of aged KCl extracts, which presented a similar temporal pattern to that of the fresh ones.

Regarding leachates, they presented lower variability in $\delta^{15}$N-$NO_3$ than KCl extracts but a similar temporal trend. $\delta^{18}$O-$NO_3$ showed inverse tendencies with respect to KCl extracts between treatments: whereas in KCl extracts the $\delta^{18}$O-$NO_3$ was lower in the $F_0$ compared to the $F_{50}$ treatment on 3rd April, 5th April , 7th June and 4th December, for the leachates the opposite is true. There was a remarkable effect of biochar treatment in $\delta^{18}$O-$NO_3$ of leachates, namely $F_0$ treatment showed significant higher $\delta^{18}$O-$NO_3$ values than $F_{50}$ throughout all the sampling dates. Maybe the influence of $\delta^{18}$O-$NO_3$ of the irrigation water, which was quite high (+6.6 to +7.2 ‰), was higher in $F_0$, raising its values. Conversely, since $F_{50}$ possessed an enhanced water content, the effect of irrigation water could be diluted.

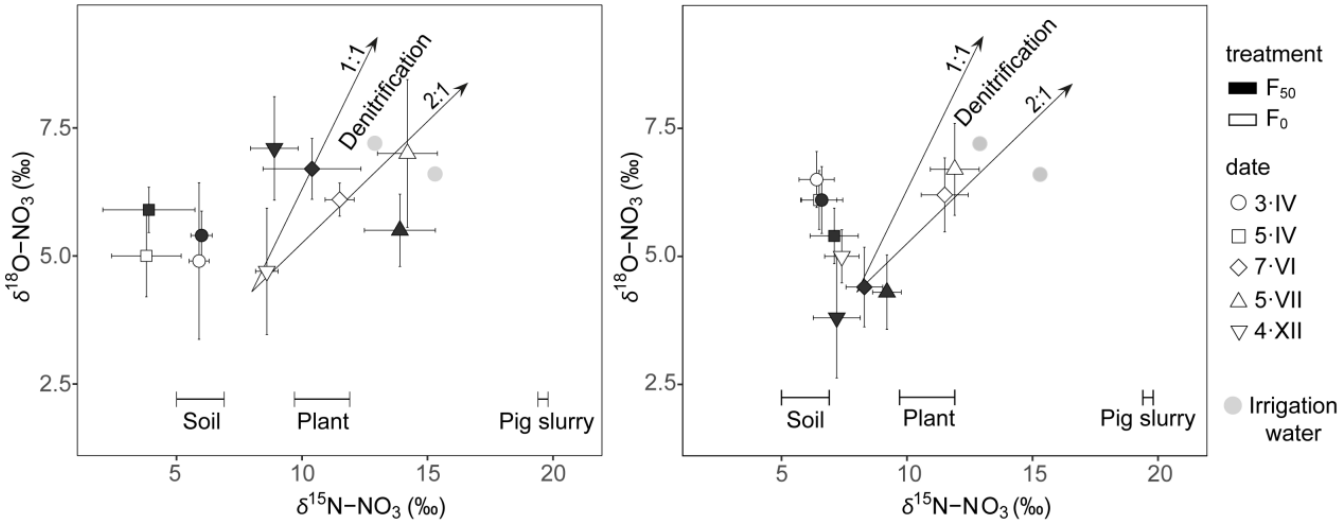


**Fig. 5.** $\delta^{18}O$ and $\delta^{15}N$ of nitrate measured in KCl extracts (left graph) and leachates (right) for the fresh biochar scenario along five different samplings (3·IV = 3rd April; 5·IV = 5th April; 7·VI = 7th June; 5·VII = 5th July and 4·XII = 4th December). Symbols with error bars represent the mean values and standard error (n = 5) respectively. The two arrows indicate typical expected slopes for values resulting from denitrification. Abbreviations for the biochar treatments correspond to: $F_0$ = fresh 0 t ha$^{-1}$; $F_{50}$ = fresh 50 t ha$^{-1}$. $\delta^{15}N$ of soil, harvested plants and pig slurry, and also $\delta^{15}N$ vs $\delta^{18}O$ of dissolved $NO_3^-$ from irrigation water are shown.

### 1.3.5. Plant nitrogen export and growth parameters

Neither growth parameters nor N plant export showed any significant difference due to biochar treatments. **Table 3** shows these endpoints including three lysimeters that presented underdeveloped plants: two replicates of the $A_{50}$ treatment and one of the $A_{12}$ treatment, although we were unable to identify if this was due to treatment effects or other factors. At the 5th July sampling, those lysimeters showed a three-fold increase in $NO_3^-$ concentrations in water extracts and leachates compared to the ones with well-developed plants, which were attributed to a decreased plant uptake. Since nitrate content differences between underdeveloped and well-developed plants did not represent statistically significant differences, the underdeveloped plant $NO_3^-$ data were not excluded from the soil extracts and leachate analyses. In addition, the uptake of other nutrients besides N was also studied (**Supplementary Table S4**) again without any remarkable biochar effect.

**Table 3.** Average values of different growth parameters and N plant uptake per treatment ± standard errors (n = 5) at harvest (3rd July). Export of N in straw and grain was calculated as total N concentration in straw/grain per straw/grain biomass). Abbreviations for the biochar treatments correspond to: $F_0$ = fresh 0 t ha$^{-1}$; $F_{12}$ = fresh 12 t ha$^{-1}$; $F_{50}$ = fresh 50 t ha$^{-1}$; $A_0$ = aged 0 t ha$^{-1}$; $A_{12}$ = aged 12 t ha$^{-1}$; $A_{50}$ = aged 50 t ha$^{-1}$. The absence of letters indicates that the observed differences were not significant.

| Parameter | Treatment | | | | | |
|---|---|---|---|---|---|---|
| | **$F_0$** | **$F_{12}$** | **$F_{50}$** | **$A_0$** | **$A_{12}$** | **$A_{50}$** |
| **Straw weight (g)** | 14.0 ± 0.8 | 14.8 ± 0.5 | 15.0 ± 0.8 | 12.7 ± 0.3 | 10.1 ± 1.7 | 7.4 ± 2.8 |
| **Grain weight (g)** | 6.2 ± 0.9 | 6.5 ± 0.6 | 7.4 ± 0.8 | 7.5 ± 0.2 | 6.6 ± 1.2 | 4.5 ± 1.8 |
| **Ear count** | 13.0 ± 1.5 | 17.0 ± 1.7 | 16.4 ± 1.5 | 12.4 ± 1.4 | 8.4 ± 2.0 | 7.0 ± 2.8 |
| **Grains per ear (mean)** | 12.9 ± 0.5 | 11.0 ± 0.8 | 12.0 ± 1.5 | 15.2 ± 0.9 | 20.2 ± 3.1 | 11.1 ± 3.7 |
| **Straw N exported (g)** | 0.17 ± 0.02 | 0.14 ± 0.02 | 0.09 ± 0.03 | 0.10 ± 0.01 | 0.09 ± 0.01 | 0.05 ± 0.02 |
| **Grain N exported (g)** | 0.17 ± 0.02 | 0.19 ± 0.01 | 0.20 ± 0.02 | 0.20 ± 0.01 | 0.17 ± 0.03 | 0.11 ± 0.05 |

### 1.3.6. Ecotoxicological endpoints

Regarding the ecotoxicity of soil and soil elutriates of the soil-biochar mixtures collected before lysimeters setup, and despite a slight trend indicating toxicity for $F_{50}$ treatment, no significant effect on adult survival and reproduction were found neither for *Folsomia candida* (**Supplementary Table S5**), nor in the growth inhibition test with *Raphidocelis subcapitata* (**Supplementary Table S6**).

Concerning the impacts on microbial functional groups related to N-cycle shortly after fertilisation (12th April), not a single significant difference was obtained, although the number of gene transcripts involved in the nitrification process, i.e., bacterial amoA (AOB), archeal amoA gene (AOA) and nxrB, showed a trend to decrease in $F_{12}$ treatment (**Supplementary Fig. S8**).

## 1.4. Discussion

### 1.4.1. Fresh biochar treatments mitigated nitrate and other ion concentrations at the bare soil sampling

Considering both controls ($F_0$, $A_0$) and biochar treatments ($F_{12}$, $F_{50}$, $A_{12}$, $A_{50}$), two different dynamics in soluble ion concentrations could be distinguished. Namely, $K^+$, $SO_4^{2-}$, $N\text{-}NO_2^-$ and $N\text{-}NH_4^+$ showed highest concentrations following fertilisation, with $N\text{-}NO_2^-$ and $N\text{-}NH_4^+$ having negligible concentrations throughout the rest of the samplings, while $N\text{-}NO_3^-$, $Cl^-$, $Ca^{2+}$, $Mg^{2+}$, and $Na^+$, reached their highest concentrations at the bare soil sampling, 8 months after fertilisation (especially for the $F_0$ treatment). The ion peak found in the bare soil sampling might be attributed to two main factors: i) the interruption of both nutrient uptake by plants, as they were not present since harvest (3·VII), and ion leaching (ceased during a 5-mo period, from the harvest sampling to the bare soil sampling); ii) the release of nutrients from barley belowground biomass decomposition, which in turn, could have induced native soil organic matter mineralisation. Other studies support these assumptions as it is well established that in croplands a maximum of mineral N accumulation and potential leaching occurs after harvest (Harmsen and Schreven, 1955; Macdonald et al., 1989). Furthermore, the intermittent drought simulation conducted in our experiment, which coincided with the post-harvest period, could also have boosted mineralisation (Appel, 1998; Sparling et al., 1995).

Remarkably, and as a main research interest of this study, the addition of both rates of fresh biochar significantly reduced $NO_3^-$ concentrations in soil solution at the bare soil sampling compared to its controls ($F_0$). Conversely, in the aged biochar scenario none of the ions studied were affected by the biochar treatments. This is partly in agreement with a previous study of our research group using the same biochar, the same biochar application rates, and monitoring the same outdoor mesocosms used for the collection of soil with aged biochar for lysimeters construction. Specifically, a reduced nitrate content in water extracts after 15 months was found for the 50 t $ha^{-1}$ treatment (Marks et al., 2016). But while in the mentioned study the reduction was only found for nitrate, in the present study fresh biochar also provoked a concurrent reduction in soluble $Cl^-$, $Ca^{2+}$, $Mg^{2+}$and $Na^+$ contents, which was more consistent for the $F_{12}$ than for the $F_{50}$ treatment. In contrast, other ions were not significantly affected by any of the biochar treatments at the bare soil sampling (i.e., $K^+$, $SO_4^{2-}$, N-$NO_2^-$ and N-$NH_4^+$). Previous studies with the same gasification biochar used in this experiment repeatedly reported an increase of $K^+$ and $SO_4^{2-}$ availability, which was mainly attributed to direct release from biochar over time (Marks et al., 2016; Martos et al., 2020; Ribas et al., 2019). Thus, the increased provision of $K^+$ and $SO_4^{2-}$ by biochar could have counteracted or diluted the reduction effect seen for these ions. Regarding N-$NO_2^-$ and N-$NH_4^+$, the lack of significant changes could be ascribed to its minimal concentrations at the bare soil sampling.

### 1.4.2. Nitrate mitigation as affected by fresh biochar addition: appraisal of mechanisms

Several mechanisms behind the nitrate mitigation induced by biochar have been proposed by previous literature and thereafter discussed in this section. The different mechanisms are examined by one or several measurements carried out for this purpose, and grouped as sorption, leaching, microbially-mediated processes, volatilisation, plant uptake, and ecotoxicological effects on key biological groups. Finally, the role of biochar ageing is also explored.

#### 1.4.2.1. Sorption related mechanisms

One long-accepted mechanism to explain nitrogen retention onto biochar is its capacity to improve soil CEC. Precisely, negatively-charged acid functional groups present in biochar's surface (such as carboxyl or hydroxyl groups similar to those of soil humic acids) are able to electrostatically attract cations such as $NH_4^+$, preventing them to enter the nitrification pathway (Pal, 2016) or to be easily leached. However, the same biochar used in this experiment was

demonstrated to be unable to enhance soil CEC 15 months after its application in outdoor mesocosms in a previous study (Marks et al., 2016). This result was attributed to the high degree of aromaticity of this gasification biochar, and its concomitant low abundance of surface functional groups as measured by Fourier transform infrared spectroscopy analysis (Marks et al., 2014a). The absence of significant differences in exchangeable ammonium in the biochar treatments at the post-fertilisation sampling in our study further supports this idea, as it was the only sampling with relatively important $NH_4^+$ concentrations. Another mechanism related to negatively charged biochar surfaces is bridge bonding. $NO_3^-$ retention might occur in biochar by means of polivalent cations, such as $Ca^{2+}$ or $Mg^{2+}$, acting as bridge bonds between nitrate and the functional groups responsible for CEC (Mukherjee et al., 2011). However, given the lack of differences in KCl and water-extractable N-$NO_3^-$, and taking into account that $NH_4^+$ was at minimal concentrations at the bare soil sampling, neither $NH_4^+$ retention through enhanced CEC nor $NO_3^-$ bridge bonding are regarded as important mechanisms to explain $NO_3^-$ retention at that sampling.

On the other hand, even though biochar is reported to mainly possess a net negative surface charge (Harvey et al., 2012, Novak et al., 2009a), mechanisms for direct $NO_3^-$ retention involving positive charges have also been suggested. Nevertheless, reports of mechanisms of direct $NO_3^-$ sorption, such as biochar's anion exchange capacity (Lawrinenko and Laird, 2015), non-conventional hydrogen bonding (Conte et al., 2014; Fang et al., 2014) or counter-ion displacement (i.e., $NO_3^-$ occupying $Cl^-$ exchange sites) (Fidel et al., 2018) are mainly pH-dependent and favoured in acidic conditions, and therefore not expected in our alkaline soil-biochar system. It has also been suggested that $NO_3^-$ retention on biochar can be due to base functional groups present on biochar's surface pyrolysed at high temperatures (Kameyama al., 2012). However, although the gasification biochar of this study was produced at high temperatures (600-900 ºC), as stated earlier, lack of surface functionality casts doubt on any $NO_3^-$ retention in such functional groups.

Biochar properties able to cause nutrient physical adsorption (physisorption as defined in Rouquerol et al., 2014), such as surface area and porosity, are highly interrelated with surface functionality-mediated retention. Namely, as biochar surface increases, the potential number of functional groups able to adsorb nutrients can also increase, hindering the evaluation of these two mechanisms separately. The biochar in our study presented a relatively low Brunauer–Emmett–Teller (BET) surface area (19.77 $m^2\ g^{-1}$). Instead, total porosity was high (80.6 %), and

the pore size distribution showed the following volumes (in $cm^3$ $g^{-1}$): macropores = 2.82; mesopores = 0.02; micropores = 0.003 (**Table 1**). The pore volumes values were measured by $N_2$ sorption and mercury porosimetry, but we lack information about $CO_2$ adsorption. This latter method enables the characterisation of sub-micropores, covering the smaller range of pores that $N_2$ sorption doesn't encompass (Brewer et al., 2014). Thus, although we cannot exhaustively describe biochar's porosity, it seems clear that macropores, probably derived from pine-wood cell structures, are the dominant pore size class. Since micropores are the main contributor to the biochar physisorption capacity (Downie et al., 2009), the low volume of micropores and BET surface area lead us to disregard physisorption as an important process in our biochar. On the other hand, macroporosity is relevant to soil hydrology and it is expected that biochars with a high volume of macropores with diameters of greater than 50 nm can have a high degree of water-holding capacity (Joseph et al., 2009). Therefore, the high degree of macroporosity of this biochar is probably behind the higher moisture contents in soil-biochar mixtures at some sampling dates, especially for the $F_{50}$ treatment.

It is important to note that at the biochar pore-level not only adsorption can take place but also absorption (Lopez-Capel et al., 2016). In relation to this, Major et al., (2009) stated that biochar porosity can contribute to nutrient sorption through the entrapment of nutrient-containing water within its pores through capillary forces. However, pore-related sorption was discarded to explain the mitigation of $NO_3^-$ in soil solution at the bare soil sampling, since if this mechanism is to be acting, we would expect to have found differences between control and biochar treatments in previous sampling dates.
To sum up, both adsorption (via surface functionality bonding and physical means) and nutrient entrapment in biochar pores can be mostly rejected to explain the ionic content decrease at the bare soil sampling.

#### 1.4.2.2. Leaching

Some authors have reported a decrease in $NO_3^-$ leaching after biochar addition, although explaining this effect through a variety of mechanisms (Ippolito et al., 2012; Jassal et al., 2015; Knowles et al., 2011; Yao et al., 2012). It has been suggested that at biochar application rates >10 t $ha^{-1}$, which are able to increase available water (Blanco-Canqui, 2017), leaching might be reduced. However, biochar supplementation could also enhance hydraulic conductivity or preferential flow around larger particles resulting in greater leaching and nutrient losses (Clough

et al., 2013). For instance, Kameyama et al. (2012) reported that saturated hydraulic conductivity increased when higher rates (≥ 5% w/w) of biochar were applied. In our study, biochar treatments did not cause a significant change in leachate concentrations in any of the studied sampling dates, thus, leaching was not considered as an important escape route in our system that could explain differences in soil solution at the bare soil sampling.

#### 1.4.2.3. Microbially mediated mechanisms

##### *1.4.2.3.1. Organic matter mineralisation vs immobilisation*

If mineralisation of residual plant debris and/or soil organic matter was effectively reduced in the fresh biochar treatments in the period from harvest to the bare soil sampling, the observed multiple reduction in ionic content at the bare soil sampling could be explained. Supporting this idea, Marks et al. (2016), in a study using the same biochar and soil as ours, showed that in some of the incubation periods studied, N mineralisation as $NO_3^-$ was reduced for both the 12 and 50 t $ha^{-1}$ biochar treatments after 6 and 12 months of biochar supplementation in soil, with the effect being more pronounced for the 50 t $ha^{-1}$ treatment.

However, in our study we lack direct nitrogen mineralisation rates measurements, thus, the mineralisation process is approached as carbon mineralisation rates (Hart et al., 1994; Kätterer and Andrén, 2001), which were measured as $CO_2$ emission rates using the static chamber methodology and soil basal respiration (BAS). There were no biochar induced differences in BAS or $CO_2$ chamber-measured emission rates at the bare soil sampling and, indeed, $CO_2$ chamber-measured emissions were very low in all treatments. By contrast, the $CO_2$ emission suppression found at the 12th April post-fertilisation sampling for the $F_{50}$ treatment suggests that mineralisation might be reduced (negative priming) at high biochar doses. The cause of $CO_2$ reduction is out of the scope of this study but possible explanations comprehend not only negative priming (thoroughly reviewed in Whitman et al., 2015) but also mechanisms not related to changes in mineralisation, as direct $CO_2$ adsorption onto biochar (Madzaki et al., 2016; Sethupathi et al., 2017) or biochar acting as an alkaline trap of $CO_2$ by promoting its precipitation in the form of carbonates (Fornes et al., 2015). Importantly, even if the negative priming was effectively acting at the post-fertilisation sampling, it could also only have been transitory and did not exert effects at the bare soil sampling. As an example, Naisse et al. (2015), in a study also testing a gasification biochar, reported a negative priming effect that only lasted

a few weeks after its application. Furthermore, N and O isotopic composition of nitrate in KCl extracts reinforce this notion as they reveal that both $F_0$ and $F_{50}$ presented a very similar (not significantly different) $\delta^{15}$N-$NO_3$ value at the bare soil sampling, which was comprised between the values of soil organic matter and plant debris, hence, indicating a similar extent of mineralisation. Therefore, a possible lowered mineralisation in fresh biochar treatments at the bare soil sampling is not regarded as an important mechanism to explain the differential ion content in soil solution.

Regarding a potential role of microbial immobilisation in the last sampling (bare soil) to explain the multiple ion reduction, despite the lack of $N_{mic}$ measurement at that sampling, immobilisation seems an unlikely explanation given that a variety of other ions apart from nitrate were also reduced and not following the known microbial stoichiometry. As an example, $Ca^{2+}$ was reduced ca. 2000 mg $kg^{-1}$ in biochar treatments compared to $F_0$, while for N-$NO_3^-$ the difference was of only ca. 300 mg $kg^{-1}$ despite being a macronutrient. Moreover, $C_{mic}$ didn't show differences as a function of biochar treatment at this sampling. As a result, the reduction of ionic content in the fresh biochar treatments as explained by microbial immobilisation is discarded.

*1.4.2.3.2. Nitrification vs denitrification*

N-$NO_3^-$ was the dominant N form in soil solution throughout the entire experiment except for a short period after fertilisation, when N-$NH_4^+$ gained importance. This pattern is found in most agricultural soils, as nitrification normally converts $NH_4^+$ into $NO_3^-$ within 2-3 weeks after fertiliser application resulting in $NO_3^-$ accumulation in the soil (Norton, 2008). The rapid onset of nitrification is also supported by isotopic composition analyses of KCl extracts since at the post-fertilisation sampling (5th April) $\delta^{15}$N-$NO_3$ slightly decreased with respect to the pre-fertilisation sampling. This is indicative of the nitrification onset given that the generated $NO_3^-$ through nitrification is depleted in $\delta^{15}$N with respect to the substrate, especially at the beginning of the reaction (Kendall and Aravena, 2000).

Although nitrification seems to be a major process in this experimental system, denitrification could also be operating. Therefore, in order to gain insight into whether denitrification was an important process, we examined the N and O isotopic composition of dissolved nitrate, as denitrification has a distinct and predictable effect on $\delta^{15}$N-$NO_3$ and $\delta^{18}$O-$NO_3$ (Kendall et al.,

2008). Namely, denitrification causes a coupled enrichment in $\delta^{15}$N-$NO_3$ and $\delta^{18}$O-$NO_3$ of the residual nitrate, leading a ratio of isotopic fractionation $\varepsilon^{15}$N /$\varepsilon^{18}$O between 2:1 and 1:1 depending on the tested conditions (Böttcher et al., 1990; Fukada et al., 2003; Granger et al., 2008; Wunderlich et al., 2013).

None of the investigated lysimeters showed a clear denitrification trend except for the $F_0$ treatment at 7th June and 5th July samplings (both in KCl extracts and leachates) and also for the $F_{50}$ KCl extract at 7th June. However, isotopic composition analyses interpretation is not straightforward since other processes could also have risen $\delta^{15}$N-$NO_3$ and $\delta^{18}$O-$NO_3$ separately resulting in the same output as denitrification. On the one hand, $\delta^{15}$N-$NO_3$ could have risen due to the input of N-$NO_3^-$ derived from nitrification of the highly $^{15}$N enriched pig slurry, microbial immobilisation (Kendall et al., 2008) or plant uptake (Craine et al., 2015). On the other hand, $\delta^{18}$O-$NO_3$ values could have increased due to a major vapour evaporation in summer months, a process which depletes soil water in the lighter oxygen isotope (Briand et al., 2017) since two atoms of oxygen in $NO_3^-$ are assumed to come from water during nitrification (Hollocher, 1984). Such an effect would be more pronounced in $F_0$ due to its lower water content (more evaporation expected to have taken place). Despite the uncertainty about the processes that caused $\delta^{15}$N-$NO_3$ and $\delta^{18}$O-$NO_3$ enrichment in summer, its punctual occurrence points to denitrification not being an important process. Furthermore, the observed narrow range of $\delta^{18}$O-$NO_3$ underpins this notion (Nikolenko et al., 2018).

All things considered, nitrification appears to be the key process in our system, while denitrification would not represent a major force for the nitrate losses observed. In addition, denitrification could not explain the concurrent reduction of the other ionic species besides nitrate.

#### 1.4.2.4. Ammonia volatilisation

The ammonia volatilisation, assessed shortly after fertilisation, when maximum emissions rates were expected, was not significantly affected by biochar amendment though we found a tendency to higher emission rates in $F_{12}$ treatment. Volatilisation of soil nitrogen as ammonia is promoted in alkaline soils (Rao and Batra 1983), and therefore, biochars with liming capacity might potentially displace the equilibrium between $NH_4^+$ and $NH_3$ and promote $NH_3$ production and volatilisation (Nelissen et al., 2012; Novak et al., 2009b; Taghizadeh-Toosi et al., 2011).

However, this is not likely in our soil, since although the biochar was highly alkaline ($pH_{1:20}$ 11.14), it was unable to cause further increases of pH in the already alkaline tested soil ($pH_{1:2.5}$ 8.2) (Marks et al., 2016).

We cannot totally discard this mechanism, since we lack ammonia measurements in the samplings after 9 days of the fertilisation event, and mineralisation of barley roots could also have promoted ammonification and hence ammonia volatilisation leading later to lower nitrate levels. However, the lack of significant differences shortly after fertilisation and the fact that volatilisation is unable to explain the concurrent reduction of the other ions seem to discard this mechanism.

#### 1.4.2.5. Plant nitrogen export

The biochar used in this study has been shown to exert contrasting effects on crop performance. While Marks et al. (2014a) showed an inhibitory effect on barley growth attributed to low P availability (as biochar plausibly promoted its precipitation) in laboratory plant tests, Martos et al. (2020) reported a higher N efficiency uptake but no effects on crop yield in field mesocosms when the same biochar was applied at lower and similar rates in an alkaline soil. Similarly, Marks et al. (2016) revealed no biochar-mediated effects on barley responses the first three years after the application in the same mesocosms where soil with aged biochar was obtained for lysimeters construction, and therefore the same application rate. In agreement with the findings of Martos et al. (2020) and Marks et al. (2016), in our study nitrogen export was unaffected by biochar treatments, therefore preventing this mechanism as an explanation of the reduced ionic availability at the bare soil sampling.

#### 1.4.2.6. Ecotoxicological effects on key soil biological groups

Biochar has been proven to contain toxic compounds for microbial communities and other biological groups such as volatile organic compounds, acetaldehyde, aldehydes, and ethylene (Nguyen et al., 2017), conversely, biochar can in turn reduce the bioavailability of toxic chemicals present in soil (Ahmad et al., 2014), so the impact of biochar in soils is hard to predict. The gasification pine-biochar of this study had large quantities of PAH (438 mg $kg^{-1}$) ten times higher than the maximum values reported for another gasification biochar by Hale et al. (2012), and had a high pH (11.14). Therefore, it has the potential to provoke toxic impacts to soil organisms. However, we failed to find any effect on collembolans or algae performance, nor in

N-cycle functional microbial groups. This is in agreement with previous laboratory studies using the same fresh gasification biochar, that only found negative effects on collembolans at higher concentrations than the ones on this experiment, but with effects on enchytraeids at relatively close concentrations (Marks et al., 2014b), and mainly attributed to the increasing pH with increasing dose, something that was not found in our study. However, this biochar was shown to decrease faunal feeding activities the three years after biochar addition in the soil mesocosms where the soil with aged biochar was collected (Marks et al., 2016) without any detectable increase in soil pH, but we did not found this effect after six years in the same plots (unpublished results).

Regarding N-cycle microbial groups, biochar has been linked to nitrification inhibition of the nitrifier *Nitrosomonas* by the release of α-pinene in a pine-derived biochar similar to ours (Clough et al., 2010). In addition, since PAH can exert toxic effects on nitrifiers and denitrifiers (Guo et al., 2011; Sverdrup et al., 2002) some inhibiting effects could be expected in this biochar with high values of this compound. However, microbial functional diversity of N-cycle microorganisms at 12th April sampling did not show significant effects of biochar either when measured as gene copies or transcripts. Although a slight non-significant reduction in nitrifiers was noted for the $F_{12}$ treatment, any ecotoxicological effect seems unlikely because a similar or higher reduction could be expected in $F_{50}$. In summary, and despite the high PAH load and pH value of this biochar, ecotoxicological effects on N cycling via soil organisms seem to be limited.

#### 1.4.2.7. On the potential role of ageing: is biochar pore occlusion by organo-mineral layers behind the reduction of soil nitrate?

As biochar ages in soil, fragmentation and changes on the surface of biochar particles including redox reactions, solubilisation and interactions with microbes and organic matter, can alter its properties, which can, in turn, influence biochar effects on soil properties (Blanco-Canqui, 2017; Joseph et al., 2010). Namely, it has been recurrently claimed that CEC increases with biochar ageing through oxidative reactions on biochar surfaces as well as through sorption of organic matter, both processes leading to an increase in surface functionality (Kookana et al., 2011; Liang et al., 2006). By contrast, Hagemann et al. (2017) proposed that the main mechanism involved in biochar ageing is not surface oxidation but the formation of an organo-mineral coating which has been proved in co-composted biochar (Kammann et al., 2015) but also in soil-aged biochar. This could have a collateral consequence, which is the occlusion of nutrient-

loaded water within pores, first retained by capillarity forces, and then trapped due to the organo-mineral plaque obstructing the pore (Joseph et al., 2018). Importantly, other studies of soil aged biochar particles have also reported the formation of porous agglomerates on the surfaces of the biochar, which in some cases implied the formation of organo-mineral associations (Archanjo et al., 2017).

This mechanism could be the one behind the concurrent reduction of nitrates along with other cations and anions in fresh biochar lysimeters in the last sampling of this study, only observed after 8 months of biochar application. Notably, this mechanism could explain why in other studies some biochar effects upon nutrient availability are only found long after its application. As an example, Ventura et al. (2013) only noted a reduction in $NO_3^-$ leaching after 13-mo of biochar addition. Nevertheless, the formation of organo-mineral coatings in soil-aged biochar particles cited in the study of Hagemann et al. (2017) had been described after 2.5 years of ageing in soil. By contrast, in our study, the reduced ionic content was observed in a shorter timeframe, so it is difficult to ascertain whether this time period is sufficient for this occlusion to occur. Some examples concerning the timing of the process in field aged biochar include Lin et al. (2012), that revealed that soil mineral phases attachment onto the biochar surfaces occurred within the first year (c.a. 4 months) of incubation, while Mukherjee et al. (2014) observed the formation of organic matter coatings within 15 months of biochar ageing, and de la Rosa et al. (2018) reported coatings of soil organic matter and microbial mats onto biochar after 24 months.

In the study of Joseph et al. (2018), it is also hypothesised that the concentration gradient emerging from drying in the composting process could boost ion movement into biochar pores (which are subsequently trapped in the pores), therefore it is plausible that our drought simulation exerted a similar effect. By contrast, a possible drawback for the organo-mineral coating mechanism explanation in our system might be the lack of a linear effect of biochar addition in ionic reduction since the effect is more apparent in $F_{12}$ than in $F_{50}$. However, this might be explained by the findings of Teixidó et al. (2013), who found a larger loss of biochar surface area in the 1% than in the 2% biochar-soil mixtures after an artificial ageing process. This effect was attributed to a better foulant coverage of organic matter when biochar is more diluted in soil. In this regard, differential organic matter fouling and/or microbial colonisation could explain the lack of linearity in this study.

The organo-mineral coating hypothesis might also explain the general lack of significant results in the biochar aged scenario, since once biochar pores are occluded, its capacity to interact with water, nutrients and microorganisms might be limited (Mukherjee et al., 2011). For instance, the lack of moisture content enhancement in the aged biochar scenario is consistent with pore clogging, as observed by Sorrenti et al. (2016).

Our results highlight the importance of long-term studies to validate the observed biochar effects in the short-term, which is mandatory considering biochar long residence time in soil, in order to prevent contrary or unintended effects than the ones motivating their use in soil as a result of ageing processes. The ageing mechanism indicated in this study, suggested as plausible by default of other mechanisms, has been only recently reported in the literature and require further research for its validation. Nanoscale analysis of biochar surfaces by means of microscopy and spectroscopic techniques is therefore needed to gain further insight onto biochar evolution over time and, specifically, on organo-mineral coating formation.

## 1.5. Conclusions

As expected by previous research with the same pine gasification biochar, a significant decrease of nitrate in soil solution was confirmed. However, this result was only true for the fresh biochar scenario and not for the aged one. In the present study, both biochar application rates (12 and 50 t $ha^{-1}$) in the fresh biochar scenario reduced nitrate levels as well as other ions (chloride, sodium, magnesium and calcium) at the bare soil sampling, the effect being more apparent for the 12 t $ha^{-1}$ treatment. However, the ionic content reduction was only found for soil solution and not in leachates, therefore, bringing into question biochar's ability to mitigate nitrate aquifer pollution.

Sorption, leaching, microbial mineralisation and immobilisation, ammonia volatilisation, plant export, and ecotoxicological effects on biological groups regulating N-cycle were discarded as explanatory mechanisms for the observed ionic content reduction. Notably, this reduction was only detected after 8 months of biochar application, presumably indicating the need for biochar to be in contact with soil in order to provoke effects. By contrast, in the aged biochar scenario, after 6 years of contact with soil, no effects were found. In this sense, the formation of an organo-mineral coating trapping nutrient-rich water could explain the punctual and concurrent reduction of the different ionic species in the fresh biochar scenario but may also be the cause

of the lack of effects in the aged biochar scenario, since once the pores are clogged by this coating its retentive properties could be lost. Nevertheless, our data does not allow us to demonstrate this mechanism and thus more studies are needed to support this hypothesis.

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*Supplementary Material Chapter 1*

**Table S1.** Pig slurry characterisation. * Kjeldahl N = (organic N) + ($N\text{-}NH_4$); **Available N = (Kjeldahl N) - (non-hidrolisable N). w.w. stands for wet weight and d.w. for dry weight.

| Parameter | Units | Value |
|---|---|---|
| Dry matter | % (w.w.) | 86.3 |
| pH | water, 1:5 (v/v) | 6.35 |
| Electrical conductivity | dS/m, 25ºC | 51.7 |
| Organic matter | % (w.w.) | 58.3 |
| Kjeldahl N* | % (w.w.) | 5.92 |
| Organic N | % (w.w.) | 2.44 |
| $N\text{-}NH_4$ | % (w.w.) | 3.48 |
| Non-hidrolisable N | % (w.w.) | 0.99 |
| Available N** | % (w.w.) | 4.9 |
| C/N ratio (based on organic N) | | 11.9 |
| C/N ratio (based on Kjeldahl N) | | 4.9 |
| P | $g\ kg^{-1}$ (d.w.) | 29 |
| K | $g\ kg^{-1}$ (d.w.) | 38.5 |
| Ca | $g\ kg^{-1}$ (d.w.) | 27.5 |
| Mg | $g\ kg^{-1}$ (d.w.) | 10.6 |
| Fe | $g\ kg^{-1}$ (d.w.) | 4 |

**Table S2.** Primers and thermal profiles used for real-time PCR quantification of the different target genes.

| Target gene | Primers | Thermal profile | Number of cycles | Reference |
|---|---|---|---|---|
| *nifH* | nifHF<br>nifHR | 98 °C – 45 s/ 55 °C – 45 s/ 72 °C – 45 s | 40 | Harter et al., 2014 |
| *amoA* AOA | amo19F<br>CrenamoA16r48x | 94°C, 45 s / 55°C, 45 s / 72°C, 45 s | 40 | Töwe et al., 2014 |
| *amoA* AOB | amoA1F<br>amoA2R | 94 °C – 30 s/ 58.5 °C – 30 s/ 72 °C – 30 s | 40 | Harter et al., 2014 |
| *nirK* | nirK876C<br>nirK1040 | 95 °C – 15 s/63 °C – 30 s/72 °C – 30 s 95 °C – 15 s/58 °C – 30 s/72 °C – 30 s | 6a<br>40 | Harter et al., 2014 |
| *nosZ* | nosZ2F<br>nosZ2R | 95 °C – 15 s/63-58 °C – 30 s/72 °C – 30 s<br>95 °C – 15 s/60 °C – 58 s/72 °C – 30 s | 6a | Harter et al., 2014 |
| *nirS* | nirScd3aF<br>nirSR3cd | 95 °C –15 s/ 57 °C – 30 s/ 60 °C – 15 s | 40 | Töwe et al., 2014 |
| *nxrB* | A189<br>A682 | 94 °C –30 s/58.5 °C – 30 s/72 °C – 30 s | 40 | Vanparys et al., 2006 |

**[a] touchdown -1ºC for cycle**

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**Table S3.** Summary of the results of two-way mixed ANOVAs on different variables, with treatment (biochar application rate) as between-subjects factor, and time (sampling dates) as within-subjects factor. Mixed ANOVA was conducted separately for the fresh and the aged biochar scenarios. Degrees of freedom (df) are shown as: (degrees of freedom numerator, degrees of freedom denominator); the effect size is reported as generalised eta squared ($\eta_G^2$), and significant p-values ($p < .05$) are marked in bold.

**Table S3.1.** Moisture

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (2, 12) | 11.90 | **0.001** | 0.38 | (2, 11) | 1.48 | 0.27 | 0.07 |
| time | (4, 48) | 114.93 | **< .001** | 0.87 | (1.9, 20.3) | 38.50 | **< .001** | 0.72 |
| treat. x time | (8, 48) | 2.40 | **0.029** | 0.22 | (3.7, 20.3) | 0.53 | 0.70 | 0.07 |

**Table S3.2.** Electrical conductivity (EC)

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (2, 12) | 1.75 | 0.22 | 0.06 | (2, 12) | 0.94 | 0.42 | 0.03 |
| time | (4, 48) | 66.13 | **< .001** | 0.81 | (2.0, 23.5) | 23.49 | **< .001** | 0.61 |
| treat. x time | (8, 48) | 2.37 | **0.03** | 0.24 | (3.9, 23.5) | 0.31 | 0.86 | 0.04 |

**Table S3.3.** pH

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (2, 12) | 0.30 | 0.74 | 0.02 | (2, 12) | 0.05 | 0.96 | 0.002 |
| time | (4, 48) | 23.26 | **< .001** | 0.53 | (2.6, 31.4) | 20.67 | **< .001** | 0.58 |
| treat. x time | (8, 48) | 0.67 | 0.72 | 0.06 | (5.2, 31.4) | 1.44 | 0.24 | 0.16 |

**Table S3.4.** Soil total Kjeldahl nitrogen (TKN)

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (2, 12) | 2.50 | 0.12 | 0.11 | (2, 12) | 1.54 | 0.26 | 0.11 |
| time | (2.3, 27.9) | 18.37 | **< .001** | 0.52 | (4, 48) | 16.34 | **< .001** | 0.40 |
| treat. x time | (4.7, 27.9) | 2.87 | **0.035** | 0.25 | (8, 48) | 1.07 | 0.40 | 0.08 |

**Table S3.5.** Soil organic carbon ($C_{org}$)

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (2, 12) | 248.76 | **< .001** | 0.94 | (2, 12) | 54.25 | **< .001** | 0.87 |
| time | (2.7, 31.8) | 2.04 | 0.14 | 0.09 | (4, 48) | 14.21 | **< .001** | 0.24 |
| treat. x time | (5.3, 31.8) | 3.59 | **0.01** | 0.25 | (8, 48) | 1.49 | 0.18 | 0.06 |

**Table S3.6.** N-$NO_3^-$ in water extracts

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (2, 12) | 1.97 | 0.18 | 0.08 | (2, 12) | 0.097 | 0.91 | 0.004 |
| time | (1.9, 23.1) | 116.14 | **< .001** | 0.88 | (1.6, 13.8) | 43.93 | **< .001** | 0.73 |
| treat. x time | (3.8, 23.1) | 2.96 | **< .001** | 0.27 | (2.3, 13.8) | 0.10 | 0.93 | 0.01 |

**Table S3.7.** N-$NH_4^+$ in water extracts

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (2, 12) | 0.72 | 0.51 | 0.02 | (2, 12) | 0.15 | 0.86 | 0.005 |
| time | (1, 12.04) | 31.50 | **< .001** | 0.68 | (1.5, 17.9) | 92.09 | **< .001** | 0.86 |
| treat. x time | (2, 12.04) | 0.63 | 0.55 | 0.08 | (2.9, 17.9) | 0.19 | 0.90 | 0.02 |

**Table S3.8.** Exchangeable N-$NH_4^+$ (N-$NH_4^+$ in KCl extracts – N-$NH_4^+$ in water extracts)

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (2, 12) | 0.93 | 0.42 | 0.03 | (2, 12) | 0.38 | 0.69 | 0.01 |
| time | (1, 12.02) | 24.08 | **< .001** | 0.62 | (1, 12.1) | 34.58 | **< .001** | 0.70 |
| treat. x time | (2, 12.02) | 1.05 | 0.38 | 0.12 | (2, 12.1) | 0.45 | 0.65 | 0.06 |

**Table S3.9.** $N\text{-}NO_2^-$ in water extracts

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (2, 12) | 1.13 | 0.31 | 0.05 | (2, 12) | 0.12 | 0.89 | 0.002 |
| time | (4, 48) | 30.97 | **< .001** | 0.67 | (4, 48) | 45.33 | **< .001** | 0.77 |
| treat. x time | (8, 48) | 1.57 | 0.16 | 0.17 | (8, 48) | 0.29 | 0.97 | 0.04 |

**Table S3.10.** $Na^+$ in water extracts

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (2, 12) | 2.76 | 0.10 | 0.12 | (2, 12) | 0.64 | 0.54 | 0.03 |
| time | (4, 48) | 109.9 | **< .001** | 0.87 | (1.3, 15.9) | 39.25 | **< .001** | 0.71 |
| treat. x time | (8, 48) | 2.51 | **0.02** | 0.23 | (2.7, 15.9) | 0.68 | 0.56 | 0.08 |

**Table S3.11.** $Cl^-$ in water extracts

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (2, 12) | 1.83 | 0.20 | 0.06 | (2, 12) | 0.22 | 0.81 | 0.006 |
| time | (2.6, 31.5) | 130.99 | **< .001** | 0.90 | (1.3, 15.1) | 40.86 | **< .001** | 0.74 |
| treat. x time | (5.2, 31.5) | 3.91 | **0.007** | 0.34 | (2.5, 15.1) | 0.47 | 0.68 | 0.06 |

**Table S3.12.** $K^+$ in water extracts

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (2, 12) | 1.38 | 0.29 | 0.07 | (2, 12) | 2.58 | 0.12 | 0.08 |
| time | (1.6, 18.7) | 54.53 | **< .001** | 0.76 | (1.2, 14.8) | 49.81 | **< .001** | 0.77 |
| treat. x time | (3.1, 18.7) | 0.52 | 0.68 | 0.06 | (2.5, 14.8) | 1.76 | 0.20 | 0.19 |

**Table S3.13.** $Ca^{2+}$ in water extracts

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta^2_G$ | **df** | **F** | **p** | $\eta^2_G$ |
| treatment | (2, 12) | 0.70 | 0.52 | 0.03 | (2, 12) | 0.97 | 0.41 | 0.04 |
| time | (2.4, 28.6) | 70.40 | **< .001** | 0.81 | (1.6, 19.7) | 23.10 | **< .001** | 0.58 |
| treat. x time | (4.8, 28.6) | 2.24 | 0.08 | 0.21 | (3.3, 19.7) | 0.41 | 0.76 | 0.05 |

**Table S3.14.** $Mg^{2+}$ in water extracts

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta^2_G$ | **df** | **F** | **p** | $\eta^2_G$ |
| treatment | (2, 12) | 0.69 | 0.52 | 0.03 | (2, 12) | 1.58 | 0.25 | 0.04 |
| time | (2.4, 28.3) | 92.89 | **< .001** | 0.85 | (1.3, 15.7) | 39.26 | **< .001** | 0.74 |
| treat. x time | (4.7, 28.3) | 2.10 | 0.098 | 0.20 | (2.6, 15.7) | 0.34 | 0.78 | 0.04 |

**Table S3.15.** $SO_4^{2-}$ in water extracts

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta^2_G$ | **df** | **F** | **p** | $\eta^2_G$ |
| treatment | (2, 12) | 0.04 | 0.96 | 0.00 | (2, 12) | 1.78 | 0.21 | 0.07 |
| time | (2.2, 26.0) | 63.48 | **< .001** | 0.79 | (2.3, 27.1) | 25.74 | **< .001** | 0.62 |
| treat. x time | (4.3, 26.0) | 0.47 | 0.77 | 0.05 | (4.5, 27.1) | 1.09 | 0.39 | 0.12 |

**Table S3.16.** $N\text{-}NO_3^-$ in leachates

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta^2_G$ | **df** | **F** | **p** | $\eta^2_G$ |
| treatment | (2, 9) | 0.21 | 0.81 | 0.01 | (2, 11) | 3.08 | 0.09 | 0.11 |
| time | (1.7, 15.4) | 50.96 | **< .001** | 0.83 | (4, 44) | 22.93 | **< .001** | 0.62 |
| treat. x time | (3.4, 15.4) | 0.92 | 0.47 | 0.15 | (8, 44) | 1.71 | 0.12 | 0.19 |

**Table S3.17.** $N\text{-}NH_4^+$ in leachates

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (2, 9) | 0.82 | 0.47 | 0.12 | (2, 11) | 3.12 | 0.09 | 0.12 |
| time | (1.6, 14.6) | 28.63 | **< .001** | 0.66 | (4, 44) | 29.24 | **< .001** | 0.67 |
| treat. x time | (3.3, 14.6) | 1.35 | 0.30 | 0.22 | (8, 44) | 0.59 | 0.78 | 0.07 |

**Table S3.18.** $N\text{-}NO_2^-$ in leachates

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (2, 9) | 0.20 | 0.83 | 0.01 | (2, 11) | 0.18 | 0.84 | 0.01 |
| time | (1.7, 15.5) | 9.08 | **0.003** | 0.46 | (1.4, 15.2) | 9.93 | **0.004** | 0.41 |
| treat. x time | (3.5, 15.5) | 0.64 | 0.62 | 0.11 | (2.8, 15.2) | 0.30 | 0.81 | 0.04 |

**Table S3.19.** $Na^+$ in leachates

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (2, 9) | 0.52 | 0.61 | 0.03 | (2, 11) | 0.98 | 0.41 | 0.04 |
| time | (2.0, 18.3) | 43.86 | **< .001** | 0.78 | (2.1, 23.0) | 18.68 | **< .001** | 0.56 |
| treat. x time | (4.1, 18.3) | 1.08 | 0.40 | 0.15 | (4.2, 23.0) | 1.82 | 0.16 | 0.20 |

**Table S3.20.** $Cl^-$ in leachates

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (2, 9) | 0.61 | 0.56 | 0.04 | (2, 11) | 1.31 | 0.31 | 0.08 |
| time | (4, 36) | 61.03 | **< .001** | 0.83 | (4, 44) | 15.74 | **< .001** | 0.48 |
| treat. x time | (8, 36) | 2.00 | 0.07 | 0.24 | (8, 44) | 1.77 | 0.11 | 0.17 |

**Table S3.21.** $K^+$ in leachates

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta^2_G$ | **df** | **F** | **p** | $\eta^2_G$ |
| treatment | (2, 9) | 2.79 | 0.11 | 0.12 | (2, 11) | 2.06 | 0.17 | 0.10 |
| time | (4, 36) | 22.40 | **< .001** | 0.66 | (4, 44) | 19.21 | **< .001** | 0.56 |
| treat. x time | (8, 36) | 1.60 | 0.16 | 0.22 | (8, 44) | 1.92 | 0.08 | 0.20 |

**Table S3.22.** $Ca^{2+}$ in leachates

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta^2_G$ | **df** | **F** | **p** | $\eta^2_G$ |
| treatment | (2, 9) | 0.38 | 0.70 | 0.01 | (2, 11) | 0.61 | 0.56 | 0.03 |
| time | (4, 36) | 11.45 | **< .001** | 0.51 | (4, 44) | 6.07 | **< .001** | 0.28 |
| treat. x time | (8, 36) | 1.85 | 0.099 | 0.26 | (8, 44) | 0.77 | 0.63 | 0.09 |

**Table S3.23.** $Mg^{2+}$ in leachates

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta^2_G$ | **df** | **F** | **p** | $\eta^2_G$ |
| treatment | (2, 9) | 2.78 | 0.11 | 0.12 | (2, 11) | 4.59 | **0.035** | 0.12 |
| time | (4, 36) | 22.40 | **< .001** | 0.66 | (4, 44) | 16.38 | **< .001** | 0.56 |
| treat. x time | (8, 36) | 1.60 | 0.16 | 0.22 | (8, 44) | 2.89 | **0.01** | 0.31 |

**Table S3.24.** $SO_4^{2-}$ in leachates

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta^2_G$ | **df** | **F** | **p** | $\eta^2_G$ |
| treatment | (2, 9) | 0.23 | 0.80 | 0.01 | (2, 11) | 3.29 | 0.07 | 0.11 |
| time | (1.8, 15.8) | 6.12 | **0.01** | 0.35 | (1.5, 16.0) | 14.27 | **< .001** | 0.51 |
| treat. x time | (3.5, 15.8) | 1.00 | 0.43 | 0.15 | (2.9, 16.0) | 1.88 | 0.17 | 0.21 |

**Table S3.25.** Dissolved organic carbon in $K_2SO_4$ extracts (DOC)

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (2, 12) | 0.58 | 0.57 | 0.01 | (2, 12) | 0.22 | 0.80 | 0.01 |
| time | (1.7, 20.1) | 7.41 | **0.006** | 0.35 | (4, 48) | 5.34 | **0.001** | 0.27 |
| treat. x time | (3.4, 20.1) | 1.24 | 0.32 | 0.15 | (8, 48) | 1.15 | 0.35 | 0.14 |

**Table S3.26.** Dissolved organic nitrogen in $K_2SO_4$ extracts (DON)

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (2, 12) | 0.15 | 0.86 | 0.01 | (2, 12) | 0.36 | 0.70 | 0.01 |
| time | (2, 24) | 27.92 | **< .001** | 0.57 | (1.4, 17.3) | 39.10 | **< .001** | 0.72 |
| treat. x time | (4, 24) | 1.32 | 0.29 | 0.11 | (2.9, 17.3) | 0.39 | 0.76 | 0.05 |

**Table S3.27.** Microbial biomass carbon ($C_{mic}$)

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (2, 12) | 0.02 | 0.98 | 0.00 | (2, 12) | 2.56 | 0.12 | 0.07 |
| time | (1.9, 22.5) | 7.21 | **0.004** | 0.33 | (2.5, 30.4) | 2.44 | 0.09 | 0.14 |
| treat. x time | (3.7, 22.5) | 0.62 | 0.64 | 0.08 | (5.1, 30.4) | 1.31 | 0.28 | 0.15 |

**Table S3.28.** Microbial biomass nitrogen ($N_{mic}$)

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (2, 12) | 2.19 | 0.16 | 0.11 | (2, 12) | 1.15 | 0.35 | 0.06 |
| time | (2, 24) | 10.35 | **< .001** | 0.36 | (3, 36) | 6.00 | **0.002** | 0.26 |
| treat. x time | (4, 24) | 0.87 | 0.50 | 0.09 | (6, 36) | 0.81 | 0.57 | 0.09 |

**Table S3.29.** Soil basal respiration (BAS)

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta^2_G$ | **df** | **F** | **p** | $\eta^2_G$ |
| treatment | (2, 12) | 3.75 | 0.054 | 0.14 | (2, 12) | 1.23 | 0.33 | 0.04 |
| time | (4, 48) | 7.97 | **< .001** | 0.33 | (4, 48) | 13.68 | **< .001** | 0.47 |
| treat. x time | (8, 48) | 1.69 | 0.13 | 0.17 | (8, 48) | 1.66 | 0.13 | 0.18 |

**Table S3.30.** N-$N_2O$ emission rate

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta^2_G$ | **df** | **F** | **p** | $\eta^2_G$ |
| treatment | (2, 2) | 4.47 | 0.18 | 0.62 | (2, 2) | 13.46 | 0.06 | 0.66 |
| time | (4, 8) | 257.01 | **< .001** | 0.99 | (4, 8) | 43.09 | **< .001** | 0.95 |
| treat. x time | (8, 8) | 9.72 | **0.002** | 0.86 | (8, 8) | 5.47 | **0.01** | 0.82 |

**Table S3.31.** C-$CO_2$ emission rate

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta^2_G$ | **df** | **F** | **p** | $\eta^2_G$ |
| treatment | (2, 2) | 6.39 | 0.14 | 0.56 | (2, 2) | 0.39 | 0.72 | 0.05 |
| time | (4, 8) | 131.96 | **< .001** | 0.98 | (4, 8) | 12.83 | **0.001** | 0.85 |
| treat. x time | (8, 8) | 4.46 | **0.025** | 0.78 | (8, 8) | 0.09 | 1.00 | 0.07 |

**Table S3.32.** N-$NH_3$ emission rate

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta^2_G$ | **df** | **F** | **p** | $\eta^2_G$ |
| treatment | (2, 12) | 2.87 | 0.096 | 0.14 | (2, 12) | 2.70 | 0.10 | 0.17 |
| time | (1.5, 17.5) | 28.40 | **< .001** | 0.61 | (3, 36) | 28.93 | **< .001** | 0.57 |
| treat. x time | (2.9, 17.5) | 0.99 | 0.42 | 0.10 | (6, 36) | 1.73 | 0.14 | 0.14 |

**Table S3.33.** $\delta^{15}$N-$NO_3^-$ in soil KCl extracts

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (1, 8) | 0.03 | 0.86 | 0.00 | (1, 8) | 1.00 | 0.35 | 0.02 |
| time | (4, 32) | 30.72 | **< .001** | 0.69 | (4, 32) | 29.97 | **< .001** | 0.77 |
| treat. x time | (4, 32) | 0.16 | 0.96 | 0.01 | (4, 32) | 1.41 | 0.25 | 0.13 |

**Table S3.34.** $\delta^{18}$O-$NO_3^-$ in soil KCl extracts

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (1, 8) | 0.44 | 0.53 | 0.02 | (1, 8) | 1.99 | 0.20 | 0.06 |
| time | (4, 32) | 0.88 | 0.49 | 0.06 | (4, 32) | 1.94 | 0.13 | 0.15 |
| treat. x time | (4, 32) | 1.56 | 0.21 | 0.1 | (4, 32) | 0.35 | 0.84 | 0.03 |

**Table S3.35.** $\delta^{15}$N-$NO_3^-$ in leachates

| | Fresh | | | |
|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (1, 6) | 1.21 | 0.31 | 0.10 |
| time | (4, 24) | 13.82 | **< .001** | 0.52 |
| treat. x time | (4, 24) | 2.15 | 0.11 | 0.15 |

**Table S3.36.** $\delta^{18}$O-$NO_3^-$ in leachates

| | Fresh | | | |
|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (1, 6) | 7.53 | **0.034** | 0.22 |
| time | (4, 24) | 1.30 | 0.29 | 0.15 |
| treat. x time | (4, 24) | 0.66 | 0.63 | 0.08 |

**Table S4.** Nutrient content in grain and straw of harvested barley. Reported values are mean ± standard errors (n = 5). Abbreviations for the biochar treatments correspond to: $F_0$ = fresh 0 t ha$^{-1}$; $F_{12}$ = fresh 12 t ha$^{-1}$; $F_{50}$ = fresh 50 t ha$^{-1}$; $A_0$ = aged 0 t ha$^{-1}$; $A_{12}$ = aged 12 t ha$^{-1}$; $A_{50}$ = aged 50 t ha$^{-1}$. The absence of letters indicates that there were no significant differences.

| | Nutrient | Treatment | | | | | |
|---|---|---|---|---|---|---|---|
| | | **$F_0$** | **$F_{12}$** | **$F_{50}$** | **$A_0$** | **$A_{12}$** | **$A_{50}$** |
| **Grain** | P (%) | 0.83 ± 0.0 | 0.86 ± 0.0 | 0.83 ± 0.0 | 0.83 ± 0.0 | 0.81 ± 0.0 | 0.84 ± 0.0 |
| | K (%) | 1.07 ± 0.1 | 1.13 ± 0.1 | 1.05 ± 0.0 | 1.06 ± 0.1 | 1.10 ± 0.1 | 1.05 ± 0.1 |
| | Ca (%) | 0.12 ± 0.0 | 0.10 ± 0.0 | 0.10 ± 0.0 | 0.09 ± 0.0 | 0.06 ± 0.0 | 0.09 ± 0.0 |
| | Mg (%) | 0.13 ± 0.0 | 0.14 ± 0.0 | 0.13 ± 0.0 | 0.14 ± 0.0 | 0.14 ± 0.0 | 0.14 ± 0.0 |
| | S (%) | 0.06 ± 0.0 | 0.07 ± 0.0 | 0.07 ± 0.0 | 0.05 ± 0.0 | 0.04 ± 0.0 | 0.05 ± 0.0 |
| | Mn (mg kg$^{-1}$) | 2.00 ± 0.0 | 1.87 ± 0.1 | 1.82 ± 0.1 | 1.90 ± 0.1 | 1.84 ± 0.1 | 1.87 ± 0.1 |
| | Zn (mg kg$^{-1}$) | 6.11 ± 0.1 | 6.54 ± 0.4 | 6.10 ± 0.1 | 6.05 ± 0.2 | 6.02 ± 0.2 | 5.88 ± 0.1 |
| **Straw** | P (%) | 0.05 ± 0.0 | 0.07 ± 0.0 | 0.05 ± 0.0 | 0.07 ± 0.0 | 0.17 ± 0.1 | 0.30 ± 0.1 |
| | K (%) | 1.18 ± 0.2 | 1.35 ± 0.3 | 1.36 ± 0.1 | 1.61 ± 0.3 | 1.63 ± 0.4 | 1.88 ± 0.5 |
| | Ca (%) | 0.28 ± 0.1 | 0.31 ± 0.0 | 0.29 ± 0.0 | 0.29 ± 0.1 | 0.31 ± 0.1 | 0.19 ± 0.0 |
| | Mg (%) | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.1 | 0.0 ± 0.1 |
| | S (%) | 0.30 ± 0.0 | 0.29 ± 0.0 | 0.28 ± 0.0 | 0.29 ± 0.0 | 0.31 ± 0.0 | 0.32 ± 0.0 |
| | Mn (mg kg$^{-1}$) | 4.22 ± 0.5 | 4.22 ± 0.2 | 3.98 ± 0.4 | 3.45 ± 0.1 | 3.76 ± 0.2 | 3.87 ± 0.4 |
| | Zn (mg kg$^{-1}$) | 3.34 ± 0.1 | 3.60 ± 0.0 | 3.53 ± 0.1 | 3.50± 0.2 | 3.63 ± 0.2 | 4.19 ± 0.5 |

**Table S5.** Mean values of *Folsomia candida* adult survival (%) and juvenile number per treatment ± standard errors (n = 5) at the onset of the experiment. Abbreviations for the biochar treatments correspond to: $F_0$ = fresh 0 t ha$^{-1}$; $F_{12}$ = fresh 12 t ha$^{-1}$; $F_{50}$ = fresh 50 t ha$^{-1}$; $A_0$ = aged 0 t ha$^{-1}$; $A_{12}$ = aged 12 t ha$^{-1}$; $A_{50}$ = aged 50 t ha$^{-1}$. The absence of letters indicates that the observed differences were not significant.

| Parameter | Treatment | | | | | |
|---|---|---|---|---|---|---|
| | **$F_0$** | **$F_{12}$** | **$F_{50}$** | **$A_0$** | **$A_{12}$** | **$A_{50}$** |
| **Adult survival (%)** | 86 ± 5.1 | 86 ± 10.3 | 76 ± 10.3 | 88 ± 4.9 | 94 ± 4.0 | 90 ± 4.5 |
| **Juvenile number** | 574.6 ± 97.5 | 517 ± 22.0 | 438 ± 45.0 | 552.8 ± 59.9 | 512.4 ± 52.0 | 540.2 ± 29.2 |

**Table S6.** *Raphidocelis subcapitata* yield inhibition (%) at four elutriate concentrations (C): 81.6, 51, 30.6, and 10.2%. Abbreviations for the biochar treatments correspond to: $F_0$ = fresh 0 t ha$^{-1}$; $F_{12}$ = fresh 12 t ha$^{-1}$; $F_{50}$ = fresh 50 t ha$^{-1}$; $A_0$ = aged 0 t ha$^{-1}$; $A_{12}$ = aged 12 t ha$^{-1}$; $A_{50}$ = aged 50 t ha$^{-1}$. Values are averages per treatment ± standard errors (n = 5) at the onset of the experiment. The absence of letters indicates that the observed differences were not significant.

| Parameter | C% elutriate | Treatment | | | | | |
|---|---|---|---|---|---|---|---|
| | | **$F_0$** | **$F_{12}$** | **$F_{50}$** | **$A_0$** | **$A_{12}$** | **$A_{50}$** |
| **Yield inhibition (%)** | 10.2 | -1.3 ± 1 | 0.4 ± 0.6 | 8.1 ± 9 | 11.6 ± 7.6 | -1.6 ± 0.5 | 7.2 ± 6.1 |
| | 30.6 | -4.8 ± 3.3 | -4 ± 1 | -4 ± 1.3 | 1.9 ± 6.3 | -4.4 ± 2.1 | -4.9 ± 1.1 |
| | 51 | -5.8 ± 0.4 | -3.6 ± 1.4 | -5.4 ± 0.2 | 1.8 ± 6.1 | -2.4 ± 1.7 | -2.4 ± 1 |
| | 81.6 | -2.2 ± 0.5 | -2.9 ± 1.3 | 5.7 ± 9.4 | -3.4 ± 1.1 | -2.6 ± 1.9 | -2 ± 0.7 |

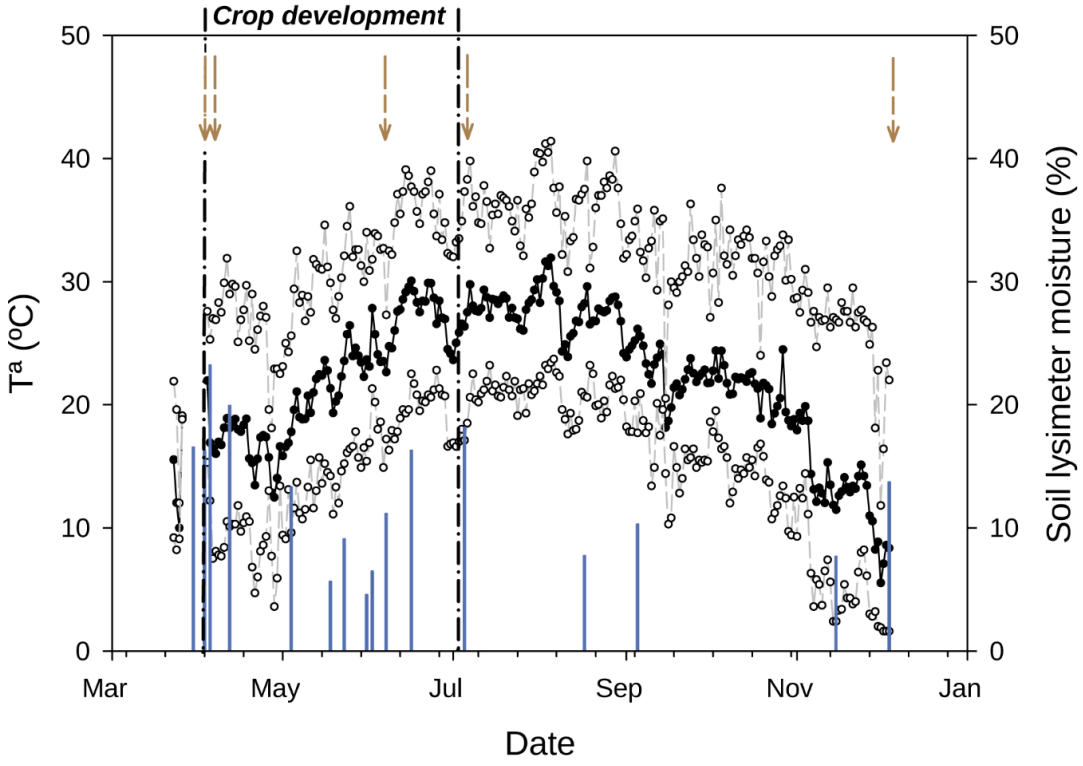


**Fig. S1.** Record of greenhouse's mean daily temperature (filled dots), maximum and minimum daily temperature (empty dots), and soil lysimeters moisture (%) measured gravimetrically (blue bars). The brown arrows indicate the sampling dates and dash-dotted lines indicate the period ranging from fertilisation and sowing events (first line) and harvest (second line), thus covering the period of barley crop development. Temperature data has been provided by IRTA-Torre Marimón.

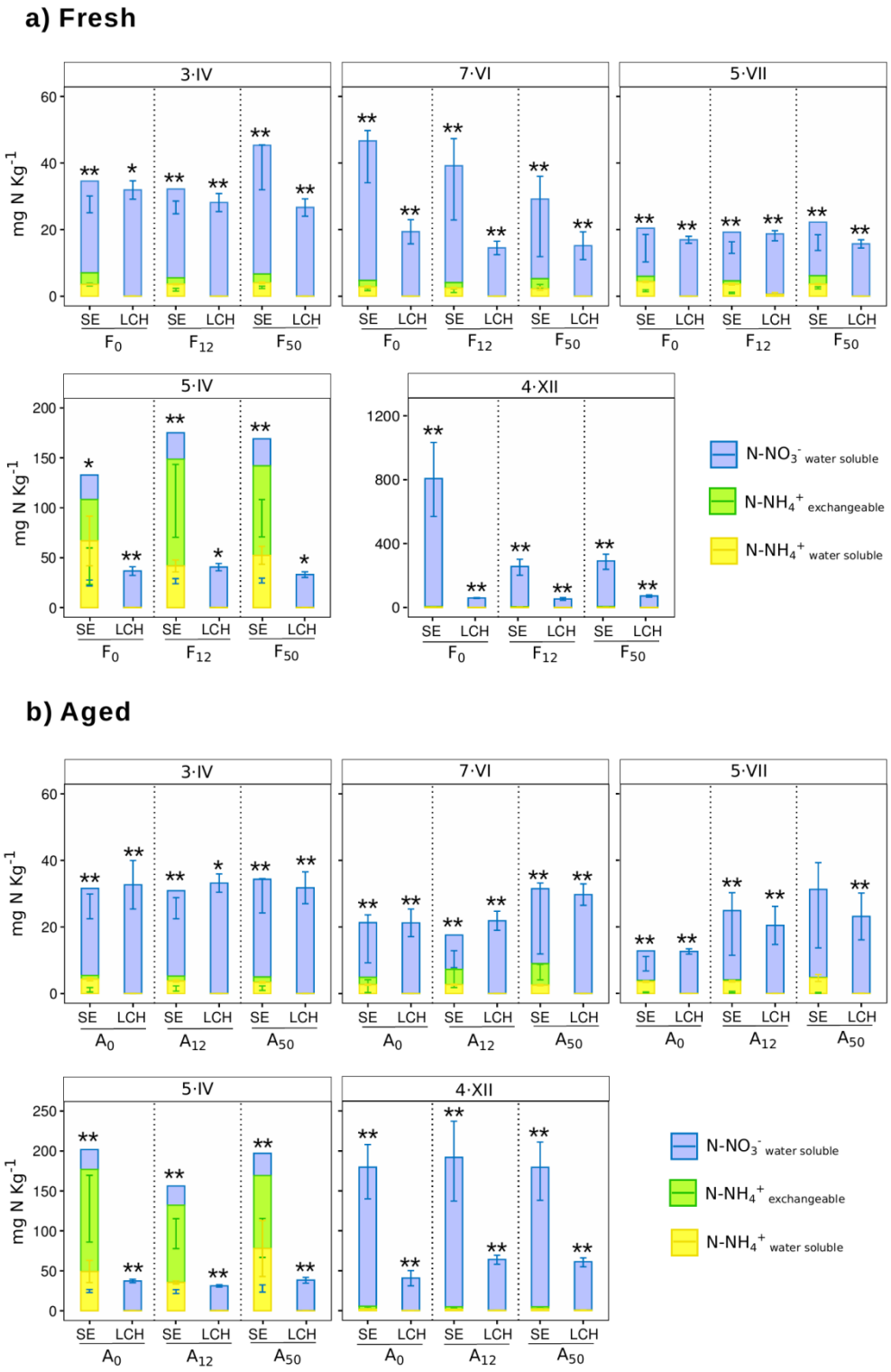


**Fig. S2.** $N\text{-}NO_3^-$ and $N\text{-}NH_4^+$ (mg $kg^{-1}$ DW soil) evolution in soil extracts (SE) and leachates (LCH) along five different samplings (3·IV = 3rd April; 5·IV = 5th April; 7·VI = 7th June; 5·VII = 5th July and 4·XII = 4th December) in a) fresh biochar scenario and b) aged biochar scenario. Abbreviations for the biochar treatments correspond to: $F_0$ = fresh 0 t $ha^{-1}$; $F_{12}$ = fresh 12 t $ha^{-1}$; $F_{50}$ = fresh 50 t $ha^{-1}$; $A_0$ = aged 0 t $ha^{-1}$; $A_{12}$ = aged 12 t $ha^{-1}$; $A_{50}$ = aged 50 t $ha^{-1}$. Bars correspond to mean values and error bars represent standard error (n = 5). Asterisks indicate statistically significant differences between $N\text{-}NO_3^-$ and $N\text{-}NH_4^+$ within each soil extract or leachate (* = $p < 0.05$, ** = $p < 0.01$). For soil extracts comparisons were made between $N\text{-}NO_3^-$ (water soluble) and total $N\text{-}NH_4^+$ (exchangeable + water soluble) while in leachates water soluble concentrations of $N\text{-}NO_3^-$ and $N\text{-}NH_4^+$ were compared.

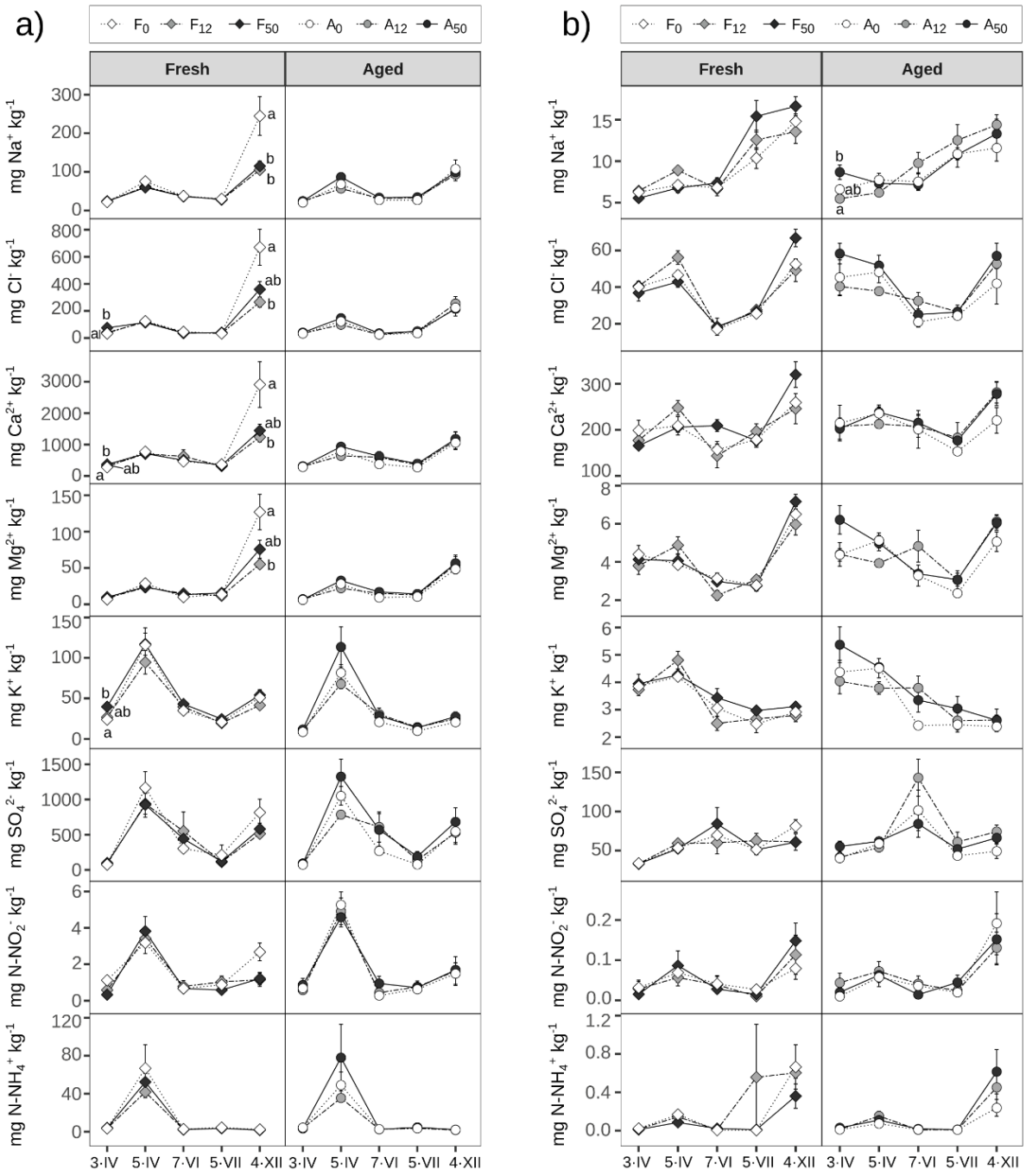


**Fig. S3.** Ionic concentrations (mg $kg^{-1}$ DW soil) evolution in water extracts (a) and leachates (b) along five different samplings (3·IV = 3rd April; 5·IV = 5th April; 7·VI = 7th June; 5·VII = 5th July and 4·XII = 4th December). Abbreviations for the biochar treatments correspond to: $F_0$ = fresh 0 t $ha^{-1}$; $F_{12}$ = fresh 12 t $ha^{-1}$; $F_{50}$ = fresh 50 t $ha^{-1}$; $A_0$ = aged 0 t $ha^{-1}$; $A_{12}$ = aged 12 t $ha^{-1}$; $A_{50}$ = aged 50 t $ha^{-1}$. Symbols represent the mean values, and bars represent the corresponding standard error (n = 5). Different letters indicate statistically significant differences between treatments within a particular sampling.

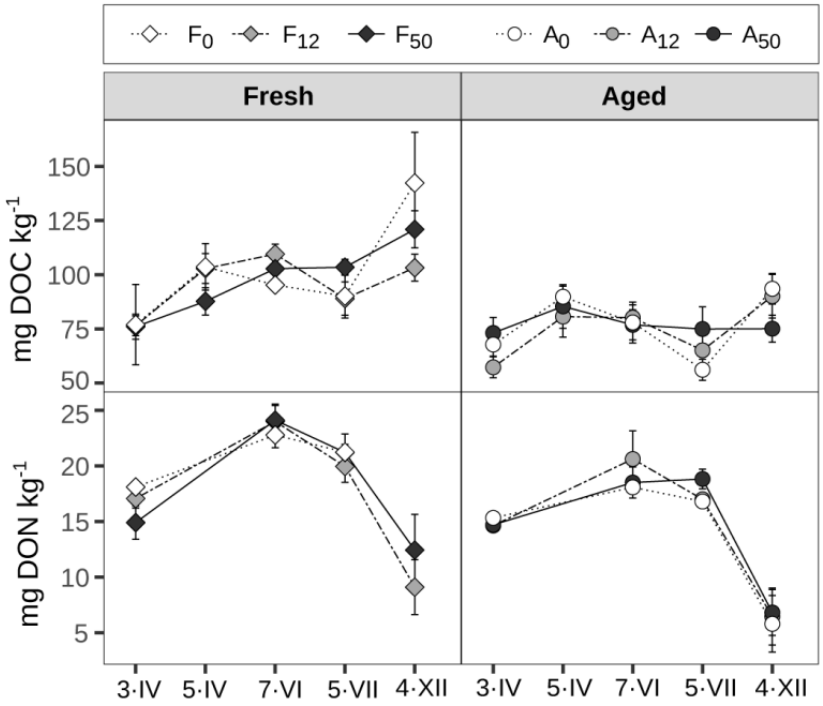


**Fig. S4.** DOC and DON concentrations (mg $kg^{-1}$ DW soil) evolution in water extracts along five different samplings (3·IV = 3rd April; 5·IV = 5th April; 7·VI = 7th June; 5·VII = 5th July and 4·XII = 4th December). Note that DON for the 5·IV sampling (both fresh and aged scenarios) and for $F_0$ at 4·XII is not shown as its calculation was not possible. Abbreviations for the biochar treatments correspond to: $F_0$ = fresh 0 t $ha^{-1}$; $F_{12}$ = fresh 12 t $ha^{-1}$; $F_{50}$ = fresh 50 t $ha^{-1}$; $A_0$ = aged 0 t $ha^{-1}$; $A_{12}$ = aged 12 t $ha^{-1}$; $A_{50}$ = aged 50 t $ha^{-1}$. Symbols represent the mean values, and bars represent the corresponding standard error (n = 5). The absence of letters indicates that the observed differences were not significant.

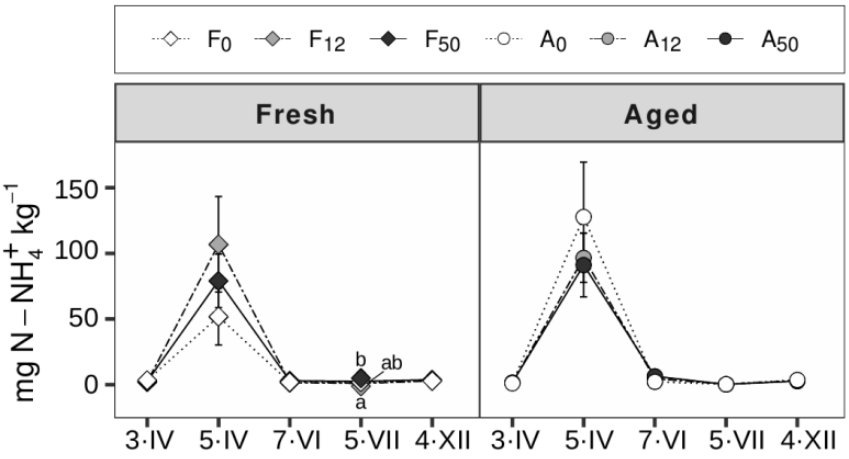


**Fig. S5.** Exchangeable N-$NH_4^+$ (mg $kg^{-1}$ DW soil), measured as KCl extractable N-$NH_4^+$ concentrations minus soluble N-$NH_4^+$ concentrations, along five different samplings (3·IV = 3rd April; 5·IV = 5th April; 7·VI = 7th June; 5·VII = 5th July and 4·XII = 4th December). Abbreviations for the biochar treatments correspond to: $F_0$ = fresh 0 t $ha^{-1}$; $F_{12}$ = fresh 12 t $ha^{-1}$; $F_{50}$ = fresh 50 t $ha^{-1}$; $A_0$ = aged 0 t $ha^{-1}$; $A_{12}$ = aged 12 t $ha^{-1}$; $A_{50}$ = aged 50 t $ha^{-1}$. Symbols represent the mean values, and bars represent the corresponding standard error (n = 5). Different letters indicate statistically significant differences among treatments for a specific sampling. Means ± SE at 5·VII for the fresh biochar scenario were: $F_0$ = 1.63 ± 0.29 mg $kg^{-1}$, $F_{12}$ = 1.00 ± 0.20 mg $kg^{-1}$, and $F_{50}$ = 2.53 ± 0.31 mg $kg^{-1}$.

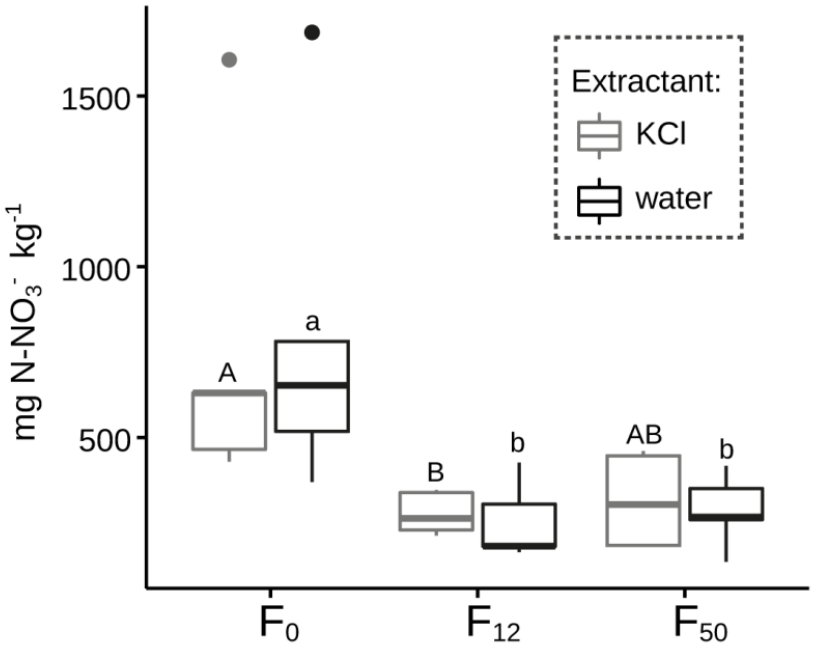


**Fig. S6.** KCl-extractable and soluble (water extract) $N\text{-}NO_3^-$ content (mg $kg^{-1}$ DW soil) at the 4th December, 2017 sampling date (bare soil sampling). Abbreviations for the biochar treatments correspond to: $F_0$ = fresh 0 t $ha^{-1}$; $F_{12}$ = fresh 12 t $ha^{-1}$; $F_{50}$ = fresh 50 t $ha^{-1}$. Different uppercase letters indicate statistical significance between treatments ($F_0$, $F_{12}$ and $F_{50}$) within the KCl extracts, while lowercase letters indicate differences within the water extracts ($p<0.05$). There were no statistical differences between KCl and water extracts on each treatment.

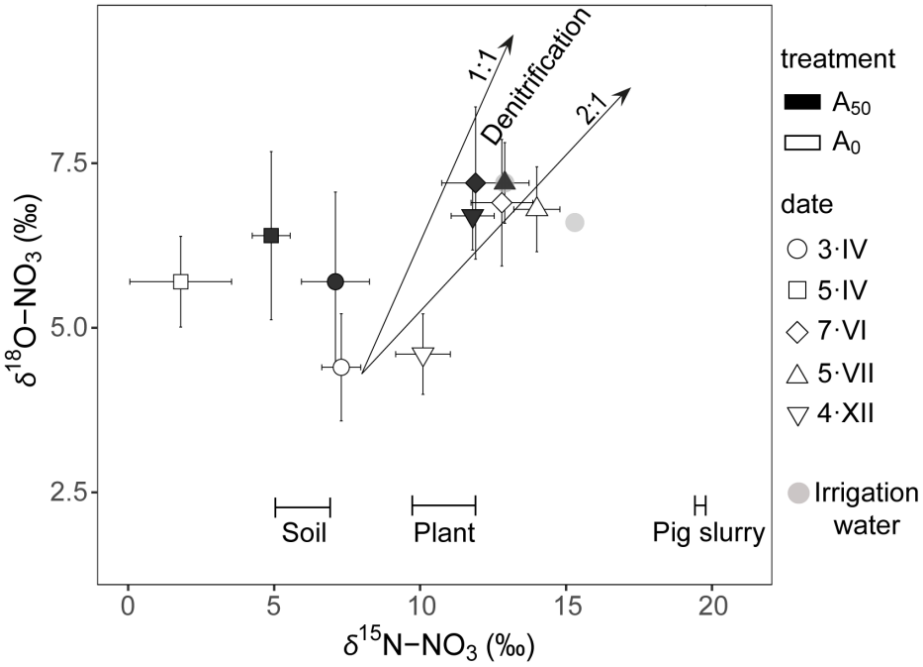


**Fig. S7.** $\delta^{18}O$ and $\delta^{15}N$ of nitrate measured in KCl extracts for the aged scenario along five different samplings (3·IV = 3rd April; 5·IV = 5th April; 7·VI = 7th June; 5·VII = 5th July and 4·XII = 4th December). Symbols with error bars represent the mean values and standard error (n = 5) respectively. The two arrows indicate typical expected slopes for values resulting from denitrification. Abbreviations for the biochar treatments correspond to: $A_0$ = aged 0 t $ha^{-1}$; $A_{50}$ = aged 50 t $ha^{-1}$. $\delta^{15}N$ of soil, harvested plants (from fresh scenario) and pig slurry, and also $\delta^{15}N$ vs $\delta^{18}O$ of dissolved $NO_3^-$ from irrigation water are shown.

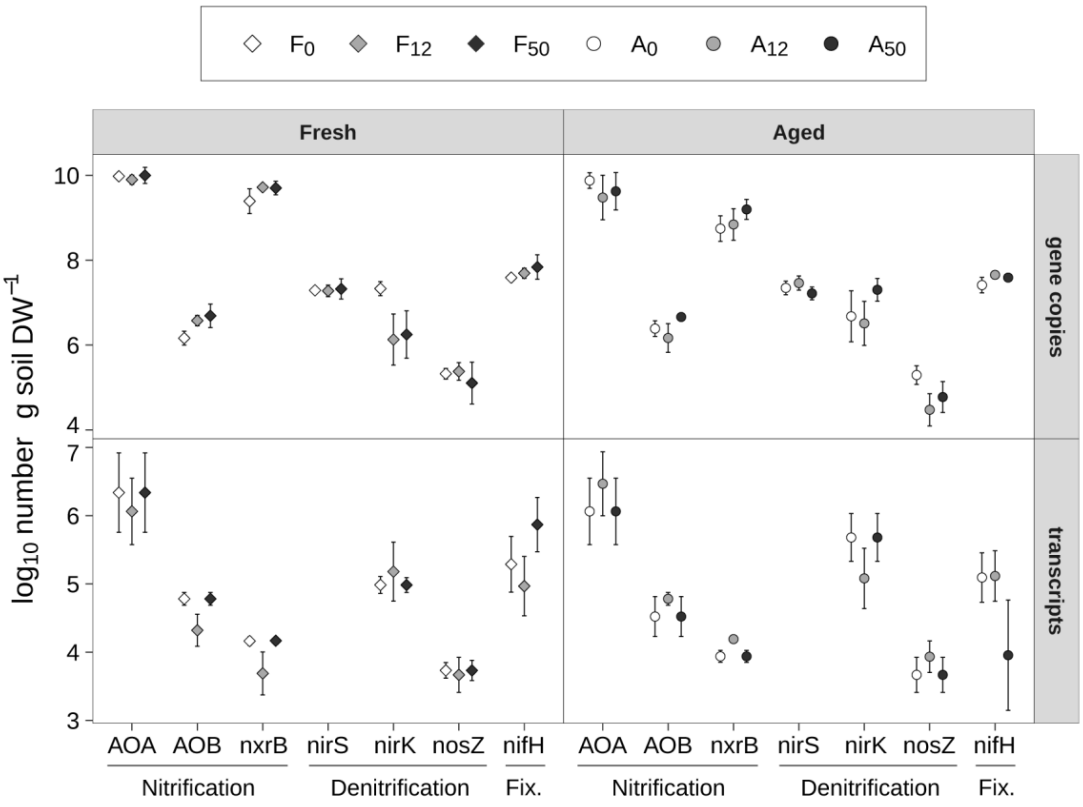


**Fig. S8.** Gene copies and transcripts for enzymes that catalyse processes of the nitrogen cycle (nitrification, denitrification, and fixation) on 12th April, 2017 (9 days after fertilisation). nirS transcripts were not detectable and thus are not shown. Abbreviations for the biochar treatments correspond to: $F_0$ = fresh 0 t ha$^{-1}$; $F_{12}$ = fresh 12 t ha$^{-1}$; $F_{50}$ = fresh 50 t ha$^{-1}$; $A_0$ = aged 0 t ha$^{-1}$; $A_{12}$ = aged 12 t ha$^{-1}$; $A_{50}$ = aged 50 t ha$^{-1}$. Symbols represent the mean values, and bars represent the corresponding standard error (n = 5). The absence of letters indicates that the observed differences were not significant.

# CHAPTER 2

## Long-term effects of gasification biochar application on soil functions in a Mediterranean agroecosystem: higher addition rates sequester more carbon but pose a risk to soil faunal communities

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## Abstract

Biochar applications can have important implications for many of the soil functions upon which agroecosystems rely, particularly regarding organic carbon (C) storage. This study evaluated the impacts of adding a highly aromatic gasification biochar at different rates (0, 12 and 50 t $ha^{-1}$) to a barley crop on the provision of crucial soil functions (carbon sequestration, water retention, greenhouse gas (GHG) emissions, nutrient cycling, soil food web functioning, and food production). After natural ageing in the field for six years, a wide range of soil properties representative of the studied soil functions were measured and integrated into a soil quality index. Results showed that C sequestration increased with biochar rate (23 and 68% higher than in the control for the 12 and 50 t biochar $ha^{-1}$ treatments, respectively). Water retention was enhanced at the 50 t $ha^{-1}$ treatment depending on the sampling date. Despite biochar additions neither abating nor increasing $CO_2$ equivalent emissions (carbon dioxide plus nitrous oxide and methane), the system shifted from being a methane sink (-0.017 ± 0.01 mg $m^{-2}$ $h^{-1}$ at the 12 t $ha^{-1}$ treatment), to a net source (0.025 ± 0.02 mg $m^{-2}$ $h^{-1}$ at the 50 t $ha^{-1}$ treatment). In addition, biochar ageing provoked a loss of nitrate mitigation potential, and indeed ammonium production was stimulated at the 50 t $ha^{-1}$ rate. The 50 t $ha^{-1}$ treatment also adversely affected nematode and collembolan functional diversity. Lastly, biochar did not affect barley yield. The results of the soil quality index indicated that no biochar treatment provided more benefits to our agricultural soil, and, although the 50 t $ha^{-1}$ treatment increased C sequestration, this was potentially offset by its harmful effects on soil faunal communities. Therefore, application of this biochar at high rates should be avoided to prevent risks to soil biological communities.

**Keywords:** gasification biochar; soil functions; ageing; soil food web; greenhouse emissions; nutrient cycling

## 2.1. Introduction

Biochar is the solid C-rich fraction obtained from thermal decomposition of biomass under limited oxygen supply and it is manufactured to act as a soil conditioner (Shackley et al., 2016). Biochar has attracted a great deal of research interest due to its beneficial effects on soil functioning (C sequestration, water retention, GHG emissions mitigation, etc.), representing a win-win scenario to achieve more sustainable managed agroecosystems (Laird, 2008; Sohi et al., 2009).

Biochar has the potential to increase C storage in soil and act as long-term C sink due to its high stability (Lehmann, 2006; Swift et al., 2001; Zavalloni et al., 2011). The polycondensed aromatic structures that conform biochar materials are responsible for its high resistance to microbial attack and its long residence times, ranging from centuries to millennia (Schmidt and Noack, 2000). Indeed, biochar has been suggested to be a climate change mitigation tool in the latest report of the Intergovernmental Panel on Climate Change (Smith et al., 2014).

Biochar has the potential to influence hydrological properties of soils, including water retention, infiltration rates, and soil hydrophobicity (Major et al., 2009; Sohi et al., 2009), mainly resulting from biochar porosity, surface chemistry and its effects on soil aggregation (Downie et al., 2009). In an extensive review by Blanco-Canqui et al. (2017) as much as a 90% of studied biochar-amended soils were found to increase their ability to retain water.

In terms of GHG emissions regulation, in addition to the direct impact on C cycle by carbon storage in soil, biochar-amended soils can alter $CO_2$ emissions by either positive or negative priming (Whitman et al., 2015), increasing C utilisation efficiency by microbes (Lehmann et al., 2011), or by changes in physico-chemical processes such as direct adsorption onto biochar surfaces or induction of carbonate-precipitation (Fornes et al., 2015; Madzaki et al., 2016). Furthermore, the emissions of other important GHGs such as nitrous oxide ($N_2O$) and methane ($CH_4$), could also be mitigated after biochar additions, although these effects have shown to be more consistent for $N_2O$ than for $CH_4$ (Bruun et al., 2016; Cayuela et al., 2014: Kammann et al., 2017). Biochar potential for the net removal of GHGs from the atmosphere has been globally estimated to account for 1-1.8 Pg C-$CO_2$-eq $yr^{-1}$ (Woolf et al., 2010).

Biochar additions can affect nutrient cycling through three main mechanisms (DeLuca et al., 2015): i) by acting as a direct source of nutrients, ii) by stimulating microbial activity that accelerates decomposition rates, and iii) by preventing nutrient losses. Remarkably, many authors have noted that biochar can reduce nutrient losses through leaching (Laird et al., 2010; Lehmann et al., 2003; Yao et al., 2012), particularly by increasing the retention of cationic nutrients (Lehmann et al., 2003; Liang et al., 2006).

Biochar can also induce shifts in the physico-chemical environment such as changes in soil pH and EC values and in the availability of toxic substances (such as polycyclic aromatic hydrocarbons (PAH), heavy metals, or dioxins), which will indirectly affect plant growth and soil biota communities (Domene, 2016; Lehmann et al., 2011; Godlewska et al., 2021). Biochar additions have been linked to increased crop yields due to enhanced water and nutrient retention, with more apparent effects in acid soils where the observed pH buffering effect resulted in increased nutrient availability and decreased aluminium toxicity (Jeffery et al. 2017; Shetty et al., 2021), but also in plant growth impairment through N immobilisation (Kookana et al., 2011). Furthermore, by altering the availability of different C fractions to bacteria and fungi, biochar could have an impact on soil food web interactions, e.g. by altering either top-down or bottom-up regulation (McCormack et al, 2013). However, the impacts of biochar on soil food web functioning have not been fully investigated (Lehmann et al., 2011). Nevertheless, the available evidence indicates that microorganisms appear to thrive in the porous structure of biochar, which may offer some protection from desiccation and shelter against grazers (Pietikäinen et al., 2000; Zackrisson et al., 1996), but also due to increased water, labile C or nutrient availability (Luo et al., 2013; Smith et al., 2010). By contrast, effects on higher trophic levels are less clear, as either positive, negative or neutral effects have been reported for microfauna (nematodes) (Liu et al., 2020; Zhang et al., 2013), mesofauna (collembolans and mites) (Briones et al., 2020; Gruss et al., 2019; Marks et al., 2014), and macrofauna (earthworms) (Briones et al., 2020; Tammeorg et al., 2014).

Due to its long-term residence time in soil, a comprehensive assessment of biochar effects on soil processes and functioning is required. Nevertheless, there is still a paucity of long-term trials to reliably elucidate biochar effects on soils, with most studies being conducted in short-term incubations and under laboratory conditions. Furthermore, determining the maximum amounts of biochar C that can be safely added to the soil without compromising other soil

functions, i.e., the so-called biochar loading capacity (Verheijen et al., 2010) is also urgently needed.

In order to fill some of these knowledge gaps, the main aims of this study were to: (i) unravel biochar long-term effects on soil-based ecosystem functions (C sequestration, water retention, GHG regulation, nutrient cycling, soil food web functioning, and food production) (ii) integrate the impacts on these soil functions in a soil quality index that could be used to compare conventional agricultural management (0 t biochar $ha^{-1}$) with two contrasted biochar application scenarios (12 and 50 t biochar $ha^{-1}$). An additional aim of this research was to corroborate if the soil solution nitrate alleviation found for this same biochar at the short-term (Marks et al., 2016; Llovet et al., 2021) was maintained at the long-term in field conditions. For these purposes, the study was carried out in outdoor mesocosms cropped to barley on an annual basis and containing a naturally aged biochar (applied six years before the study). Provided that the gasification procedure to obtain the biochar used in this study yielded a highly aromatic product with high concentrations of PAHs, we hypothesised that although this biochar might have a high C storage potential, it would have limited positive effects on soil communities due to its low labile organic matter content and potential toxic effects, more so at higher addition rates.

## 2.2. Materials and methods

### 2.2.1. Biochar properties

The biochar in this study was produced from a mixture of *Pinus pinaster* and *P. radiata* wood chips at the Guascor Group gasification facility (Vitoria, Northern Spain). Gasification reactor temperatures ranged between 600 and 900 °C and the residence time was of 10 s. The resulting biochar had a pH ($H_2O$, 1:20) of 11.14, an electrical conductivity (EC) of 0.3 dS $m^{-1}$ at 25 °C, a CEC value of 3.62 $mmol_c$ $kg^{-1}$, and a C, N, P and S content of 782, 2.1, 1.34 and 0.34 g $kg^{-1}$, respectively. The $O/C_{org}$ (0.07) and $H/C_{org}$ (0.14) ratios were low, thus rendering this biochar as highly aromatic (Lehmann et al., 2015). Also, this biochar was described as having very low surface functionality (Marks et al., 2016), and low surface area (19.77 $m^2$ $g^{-1}$) (Llovet et al., 2021). Further physiochemical details of the biochar can be found in Llovet et al. (2021).

### 2.2.2. Experimental setup

The study site was located at the IRTA Torre Marimón experimental farm in Caldes de Montbui (Catalonia, NE Spain, 41º36'35" N, 2º10'17" E). The area has a sub-humid coastal Mediterranean climate with average annual minimum and maximum temperatures of 7.9 ºC and 20.4 ºC, respectively, and a mean annual rainfall of 641 mm (1950–2017) (METEOCAT, 2020). The meteorological records of the study site were obtained from the nearest weather station (Caldes de Montbui, station X9, Network of Automatic Weather Stations, Generalitat de Catalunya; 41º36'45" N, 2º10'6" E) and is shown in **Fig. 1** along with the main sampling dates and fertilisation events. The year of the sampling (2017) was relatively drier (478 mm annual mean), and slightly warmer than average (mean minimum and maximum temperatures were 8.5 and 21.8 ºC).

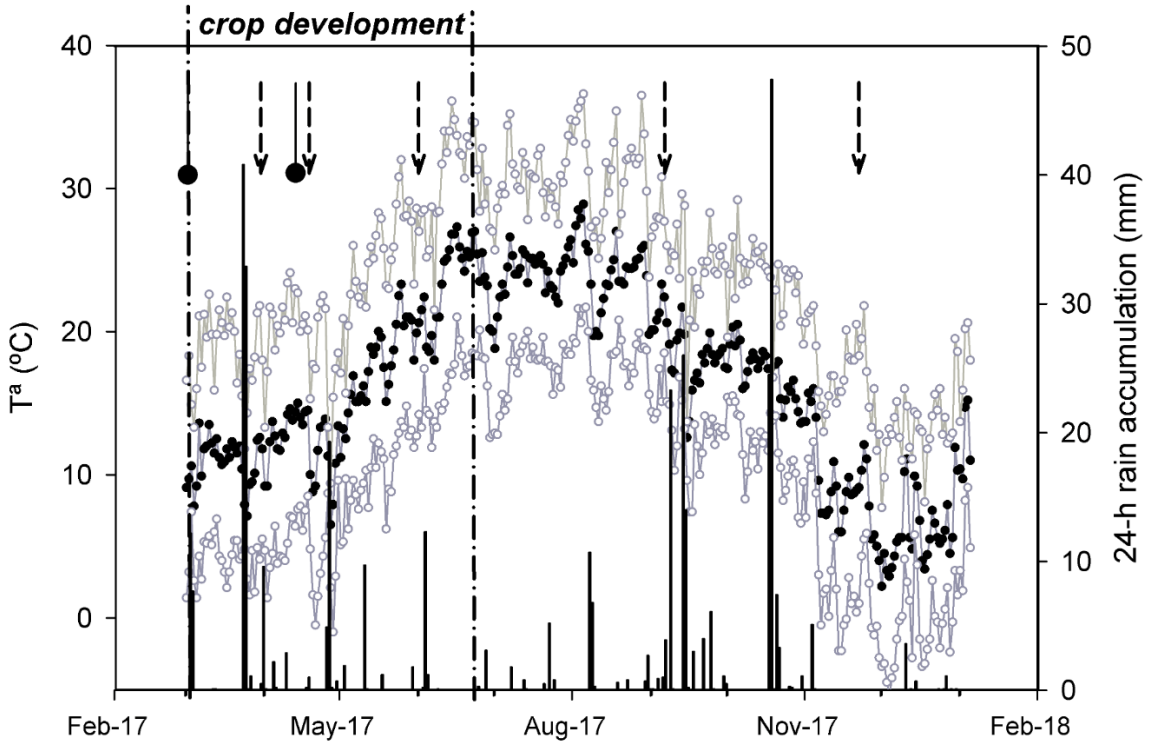


**Fig. 1.** Mean daily temperature (filled dots), together with the maximum and minimum daily temperatures (empty dots), and the 24-h accumulated precipitation (bars) during the study period. The main sampling dates are indicated as arrows in the top. Agronomic management is also shown: the two events of fertilisation (top big black dots) and crop development period from seeding to harvesting (vertical dashed lines). Data has been provided by the Meteorological Service of Catalonia (XEMEC) and correspond to the Caldes de Montbui station, the closest to the experimental site (distance of 421 m).

The field trial was carried out in mesocosms established in 2011 (for a thorough description of the mesocosms installation along with soil properties refer to Marks et al., 2016). Briefly, eighteen squared mesocosms (1 $m^2$), each delimited by four stainless steel plates inserted to 30 cm depth, were constructed by excavating the topsoil (20 cm) of a deep calcareous sandy-loam alluvial soil (Fluventic Haploxerept following Soil Survey Staff, 2010). Three biochar treatments, i.e., 0, 12, and 50 t $ha^{-1}$ were assigned in a completely randomised block design, with 6 replicates per treatment. Excavated soil was thoroughly mixed with biochar before refilling the mesocosms, and the soil in the control treatments was also excavated, mixed, and refilled. The mesocosms were planted with barley and fertilised on an annual basis since their establishment in 2011. In brief, a malting barley (*Hordeum vulgare* L. Graphic variety) was sown at a density of 250 seeds $m^2$ and an oven-dried pig slurry added at a rate of 50 kg N $ha^{-1}$ $year^{-1}$, based on its hydrolysable (labile) N content (see Llovet et al., 2021). Fertilisation was split into two: at the time of seeding and after 1.5 months, when seedlings started to emerge. Agronomic management also involved a minimum tillage to a 20 cm depth with a flat shovel, removal of matured grain and stover and no application of pesticides. In the year of the study, tillage, seeding and the first fertiliser application were conducted on 1st March, the second fertilisation on 12th April, and the harvest on 22nd June (see also **Supplementary Table 1**).

### 2.2.3 Sampling scheme

Most of the measured parameters were assessed at five samplings along 2017, covering the entire barley growing season and post-harvest: 1) inter-fertilisation period (i.e., between the two fertilisation events) (29th March); 2) post-fertilisation (18th April); 3) maximum crop growth (31st May); 4) post-harvest summer bare soil (5th September), and 5) post-harvest autumn bare soil (22nd November). The full time schedule for the different soil analyses (including GHG emissions and crop measurements) is described in **Supplementary Table S1**. With the exception of soil faunal analyses, described in section 2.4.5., soil sampling proceeded as follows: five soil cores (0-20 cm depth, Ø 2.5 cm) were randomly collected from each mesocosm and pooled to obtain a composite sample. In the laboratory, soil was homogenised and sieved to 5 mm and immediately processed (for microbial analyses) or stored at -20°C or -80°C depending on analytical requirements. A 20 g subsample of the collected soil was air-dried for two weeks and then finely grinded (<0.2mm) in order to assess organic C and Kjeldahl N.

### 2.2.4. Soil functions assessment

In order to evaluate potential impacts of biochar additions on six soil functions (carbon sequestration, water retention, GHG emissions, nutrient cycling, soil food web functioning and food production), relevant soil properties were measured and used as indicators (Ferrarini et al., 2018; Rinot et al., 2019; Zornoza et al., 2015).

#### 2.2.4.1. Carbon sequestration

Soil organic carbon content (SOC) was used as a proxy for potential carbon sequestration (Powlson et al., 2011). In our study, SOC was measured by means of the $K_2Cr_2O_7$-$H_2SO_4$ wet oxidation method (Nelson and Sommers, 1983). Although this methodology does not oxidise all biochar C (Rumpel et al., 2006) previous studies using the same biochar but in a different soil (Raya-Moreno et al. 2017) have reported that approximately 90% of the total C (soil plus biochar) can be recovered with this method.

#### 2.2.4.2. Water retention

Soil water retention was measured as soil gravimetric water content. This parameter was determined by drying 10 g of field-moist soil at 105 °C for 12 h.

#### 2.2.4.3. GHG emissions

Trace gas exchange rates of $CO_2$, $N_2O$ and $CH_4$ were measured using a polyvinyl chloride (PVC) paired 'base-chamber' design, i.e. the chamber (47.4 to 57.4 cm high and 24.1 cm diameter) was fitted to a ring inserted into the soil. Ring installation took place 29 days before the first measurements to avoid artefacts due to soil disturbance. Gas samples were collected immediately after chamber closure (t= 0), and after 20 and 40 mins using a plastic syringe (20 ml) and then injected into a 12 ml vial (Exetainers®, Labco Ltd., Ceredigion, UK). Gas concentrations were analysed by gas chromatography (GC) (Agilent 7890A) coupled to a thermal conductivity detector (TCD) for $CO_2$, to an electron capture detector (ECD) for $N_2O$, and to a flame ionisation detector (FID) for $CH_4$. The detection limits of the GC were 10 ppmV, 20 ppbV and 100 ppbV for $CO_2$, $N_2O$ and $CH_4$, respectively. The quality of the measurements was checked using standards of known gas concentrations (250 and 1003 ppmV for $CO_2$, 175 and 600 ppbV for $N_2O$ and 0.5 and 2.5 ppmV for $CH_4$). Fluxes (mg $m^{-2}$ $h^{-1}$) were calculated from the slope of the linear regression between the concentration of each GHG and the

accumulation time, subsequently corrected by the air temperature, atmospheric pressure, and the surface-volume ratio of the chamber, according to Collier et al. (2014). The squared Pearson's correlation coefficient ($r^2$) for the linear regression of $CO_2$ was used to check the goodness fit of the flux rate values (De Klein and Harvey, 2015), and accordingly, flux rates of $CO_2$, $N_2O$ and $CH_4$ were discarded from further analysis if $r^2 < 0.80$. Lastly, the $CO_2$ equivalent ($CO_2$-eq) emissions for each gas were calculated by multiplying each flux value by its associated global warming potential (GWP) over 100 years (1, 265 and 28 for $CO_2$, $N_2O$ and $CH_4$ respectively) (IPCC, 2014), and finally, the total GHG emissions from each treatment were obtained by adding the $CO_2$-eq emissions of each gas (Myhre et al., 2013).

In addition, unpublished results of C-$CH_4$ exchange rates from a previous study (Llovet et al., 2021) were also used in the present study to get a better understanding of C-$CH_4$ responses to biochar additions (see **Supplementary Fig. S2**).

#### 2.2.4.4 Nutrient cycling

Nutrient cycling was assessed by measuring proxies for mineralisation and decomposition. Mineralisation was estimated as the soluble nutrient status, together with pH and electrical conductivity measures. In addition, different N compartments were evaluated because of the expected biochar impact. The soil enzymatic activity profile was used as indicator of both organic matter decomposition (e.g., β-glucosidase) and mineralisation (e.g., urease, phosphatases, arylsulfatase). Lastly, tea bag mass loss and bait lamina were used as proxies for decomposition and detritivore fauna activity, respectively.

*2.2.4.4.1. Soil ionic content, N pools, pH, and electrical conductivity (EC)*

Water-soluble ionic concentrations were determined in 1:5 ratio (w/v) extracts prepared by mixing 40 g of soil with 200 ml of distilled water and shaking for 1 h in a vertical agitator (120 rpm). Then, extracts were centrifuged for 5 min at 8000 rpm, filtered in Whatman #42 filter paper, and frozen at -20 °C. Before freezing, a subsample of the water extracts was used for pH and EC measurements. Ionic content was quantified by liquid chromatography on a Dionex ICS-1100 ion chromatograph (Dionex, Sunnyvale, USA) using a AS4A-SC Dionex anion column for $Cl^-$, $NO_2^-$, $NO_3^-$, $HPO_4^{2-}$ and $SO_4^{2-}$ determinations and a CS12A Dionex cation column for $Na^+$, $K^+$, $Mg^{2+}$, and $Ca^{2+}$ determinations. All ion concentrations were estimated using a linear calibration except for $SO_4^{2-}$, $Mg^{2+}$, and $Ca^{2+}$ in which a quadratic regression significantly

increased the fitting ($R^2$). Lower limit of detection (LOD) was estimated to be three times the standard deviation of five blank values. Signal-to-noise ratio was also checked and as a result, $HPO_4^{2-}$ was discarded from analyses as its signal-to-noise ratio in the chromatogram did not exceed the set value of 3.

Total Kjeldahl nitrogen (TKN) was assessed using the micro-Kjeldahl method by Bremner (1965) but modified as follows: after digestion was finished, digestates were diluted with distilled water to make up a volume of 100 ml and $N\text{-}NH_4^+$ was measured by the salicylate method (Willis et al., 1996).

Exchangeable $N\text{-}NH_4^+$ was assessed by subtracting the amounts of water extractable $N\text{-}NH_4^+$ from those of the KCl-extractable $N\text{-}NH_4^+$. KCl extracts were prepared by mixing 20 g of fresh soil with 100 ml of 2 M KCl (1:5 w/v ratio). Extracts were shaken for 30 min (ISO/TS 14256-1: 2003), centrifuged for 5 min at 8000 rpm, filtered in Whatman #42 filter paper, and frozen at -20 °C before analysis. $N\text{-}NH_4^+$ concentration in KCl extracts was assessed using the salicylate method (Willis et al., 1996) in a Spectronic 20 Genesys 4001/4 spectrophotometer. Due to a cooling system failure, the batch of KCl extracts corresponding to the sampling date 5th September was discarded. Water-soluble $N\text{-}NH_4^+$ was assessed by the salicylate method to allow for direct comparison with KCl extractable $N\text{-}NH_4^+$.

Microbial nitrogen ($N_{mic}$) was measured using the chloroform fumigation-extraction method (Brookes and Joergensen, 2006) followed by a Kjeldahl digestion (without including $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$ concentration) and salicylate $N\text{-}NH_4^+$ determination as explained in Cabrera and Beare (1993). The Kjeldahl nitrogen of the non-fumigated samples from the fumigation–extraction method was taken as a measure of extractable organic N (EON).

*2.2.4.4.2. Soil enzymatic activity profile*

The activities of key enzymes such as dehydrogenase, β-glucosidase, urease, alkaline phosphomonoesterase, phosphodiesterase, and arylsulfatase were determined. Urease, β-glucosidase, and arylsulfatase activities were determined as explained in Paz-Ferreiro et al. (2014) and references therein; urease activity was expressed as μmol $NH_4^+$ $g^{-1}$ $h^{-1}$ while β-glucosidase and arylsulfatase as μmol p-nitrophenol $g^{-1}$ $h^{-1}$. Alkaline phosphomonoesterase activity was assessed according to Tabatabai and Bremner (1969) and phosphodiesterase following Bowman

and Tabatabai (1978), and both expressed as μmol p-nitrophenol $g^{-1}$ $h^{-1}$. Lastly, dehydrogenase activity was assessed using the method described by Camiña et al. (1998) with the results being expressed as μmol iodonitrotetrazolium formazan $g^{-1}$ $h^{-1}$.

*2.2.4.4.3. Tea bag decomposition*

The tea bag method, initially described by Keuskamp et al. (2013), uses commercially available tea bags of green tea (*Camellia sinensis*) and rooibos tea (*Aspalathus linearis*) as a standardised surrogate for the conventional litterbag method. The weight loss of tea leaves after a three-month incubation period buried in soil is used to calculate the decomposition constant k and the stabilisation factor S. At each plot, three tea bag pairs, consisting of a green and a rooibos tea bag, were randomly buried at a depth of 8 cm in two different periods: spring (8th March 2017-7th June 2017) and autumn (2nd October 2017-5th January 2018). It should be mentioned that Lipton, the tea bag manufacturer, changed the mesh materials of the tea bags from woven nylon mesh (0.25 mm mesh size) to polypropylene nonwoven mesh (non-uniform size, but finer than 0.25 mm) in 2017, and this led to a change in the tea bag material used in this experiment (from woven bags in spring to non-woven in autumn). Although this could affect the comparability of data derived from the use of two different mesh materials, some recent evidence suggests that equivalent results are yielded (Mori et al., 2020). However, the data resulting from the spring sampling precluded the calculation of the decomposition rate k as rooibos weight mass loss was higher than that expected with the method assumptions. To avoid potential methodological flaws, neither k decomposition rate nor S stabilisation factor were calculated, and instead, results were expressed as percentage of weight mass lost. We also found that a few teabags (19 out of 216) had been perforated, probably by macrofauna activity (Eggleton et al., 2020), and therefore they were excluded from analysis (this was done by removing the two paired bags if one of them had to be discarded).

*2.2.4.4.4. Bait lamina test*

The bait lamina test visually assesses soil detritivore feeding rates of a bait substrate placed in plastic strips inserted in soil (Von Törne, 1990), a method widely used to assess the role of fauna in nutrient cycling (Jänsch et al., 2017). Unlike the tea bag method, which mainly reflects microbial metabolism (although some mesofaunal contribution is also possible) (Keuskamp et al., 2013), the bait lamina test is assumed to be directly associated to the feeding activity of soil invertebrates, even if microbial processes may play a minor role (Kratz, 1998). Each bait-lamina

consisted of a PVC strip (160 mm length, 6 mm width and 1 mm thickness) with 16 holes spaced by 5 mm and filled with a mixture of cellulose, wheat flour and activated charcoal in a 7:2.5:0.5 (w/w/w) ratio. At each plot, eight bait laminae were placed (10 cm distance between them). In addition, two additional bait laminae were inserted at each control plot to periodically check and decide whether the test should be finished (namely when half of the lamina holes were punctured). Total feeding rates (0-8 cm depth), as well as the depth-specific rates (0-3, 3-6, 6-8 cm), were determined. Quantitative feeding activity was reported as in Vorobeichik and Bergman (2020) i.e., a five-point scale in which points corresponded to the approximate proportion of the area of bait consumed was used: 0 – untouched; 0.25 – consumed about 25%; 0.50 – about 50%; 0.75 – about 75%; and 1 – totally eaten. Two samplings were undergone one in spring (2nd June 2017-22nd June 2017), and another in autumn (22nd November 2017-5th January 2018). Thus, bait laminae were exposed for 20 days in spring and for 44 days (due to low feeding activity) in autumn. A few bait laminae were lost at the spring sampling: 8 bait laminae (the entire set) in one mesocosm of the 12 t $ha^{-1}$ treatment, and 4 bait laminae in one control mesocosm and one lamina of the 50 t $ha^{-1}$ treatment.

#### 2.2.4.5. Soil food web functioning

Microorganism, nematode, microarthropod and earthworm communities were selected as key soil groups to assess biochar impacts on soil food web functioning.

##### *2.2.4.5.1. Microorganisms: microbial biomass content, activity, efficiency and fungal:bacterial ratio*

Microbial biomass C content was assessed by the chloroform fumigation–extraction method (Brookes and Joergensen, 2006), with the organic C of the non-fumigated samples being taken as a measure of extractable organic C (EOC). Soil basal respiration (BAS) was assessed with NaOH traps according to Pell et al. (2006). Finally, the metabolic quotient ($qCO_2$), was calculated as the BAS/$C_{mic}$ ratio, which has been suggested as a measure of the C resources use efficiency by microbial biomass (Wardle and Ghani, 1995).

Quantitative PCR (qPCR) was used to determine total bacterial and fungal abundance. Soil samples stored at −80 °C were used for DNA extraction following the protocol described by Griffiths et al. (2000) with the modifications suggested by Töwe et al. (2011). Nucleic acids were quantified with the Qubit 3.0 Fluorimeter (Life Technologies) as instructed by the manufacturer. The target genes were bacterial 16S rRNA and fungal 18S rRNA. Real-time quantitative PCR

was carried out using a LightCycler® 480 System (Roche). **Supplementary Table S2** shows further details of the qPCRs analytical procedures. All the samples and standards were analysed in duplicate and each plate contained 6 negative control replicates. The amplification efficiency was calculated as follows: $E = [10^{(-1 / slope)} - 1] * 100$, and the results were 97-102% for 16S and 89-94% for 18S. Fungal 18S rRNA gene copy number/bacterial 16S rRNA gene copy number (F:B) ratio was calculated on the basis of the log-transformed copy number values.

*2.2.4.5.2. Nematodes and microarthropods*

Nematodes and microarthropods were extracted from fresh soil samples taken in spring (31st May 2017) and in autumn (22nd November 2017). On each sampling occasion, two soil cores (5 cm Ø, 15 cm depth) were collected from each mesocosm and pooled into a composite sample for nematode assessment whereas three cores were taken and pooled for microarthropod extraction.

In the case of nematodes, soil samples were divided into two subsamples: 50 g of fresh soil from each sample were weighed and air-dried to calculate soil moisture, and around 300 g were used for nematodes extraction using the Baermann funnel method (Barker et al., 1985). All nematodes were counted under a dissecting microscope, and at least 100 nematodes from each sample were identified to genus or family. Based on their mouth parts, nematodes were classified as bacterial feeders, fungal feeders, plant parasites/herbivores, omnivores, and predators (Yeates et al., 1993). Nematode families were also classified along the colonizer-persister (cp) scale into five groups, from microbial feeders with short life cycles and high reproduction rates (cp-1 and cp-2), to predators and omnivores with long life cycles, low reproduction rates and very sensitive to environmental perturbations (cp-4 and 5), while cp-3 corresponds to an intermediate level of resistance to perturbation (Bongers and Bongers, 1998; Ferris et al., 2001). Nematode-based ecological indices (i.e., Maturity Index 2-5 (MI2-5) and nematode metabolic footprints of herbivores, fungivores, bacterivores, predators, and omnivores) were calculated using the NINJA web application (Sieriebriennikov et al., 2014), with dauer larvae exclusion from analyses as recommended in Sánchez-Moreno and Ferris (2018). The MI2-5, based on the abundances of c-p groups (2-5) of free-living nematodes (i.e. non-plant parasites), is an indicator of the condition of the soil food web along ecological succession. The exclusion of group cp-1 from this index rules out any transient effect of the opportunistic bacterial-feeding nematodes and leads to values attributable to longer-lasting environmental effects. On the other hand, metabolic

footprints are based on the biomass and metabolic activity of each nematode taxon, and provide information about the contribution of each feeding guild to ecosystem function based on their utilization of carbon and energy. The underlying assumption is that the greater the amount of carbon and energy processed by any given taxa, the greater the magnitude of their ecosystem function (Ferris, 2010; Sánchez-Moreno and Ferris, 2018).

Microarthropods were extracted using Berlese funnels, in the dark for 5 days followed by 2 days under light and heat conditions to force the downward movement of the organisms, which were collected in flasks filled with 75% ethanol. Two different eco-morphological indices, both based on the assumption that euedaphic groups decrease as soil quality is impaired, were assessed, i.e., the Soil Biological Quality-arthropod index (QBS-ar) proposed by Parisi et al. (2005) and collembolan life-form traits (LFT) described in da Silva et al. (2016). For the latter, five features are observed and qualitatively scored: length of the antennae, size of furca, presence of ocelli, pigmentation, and the presence of scales or dense hairs along the body. The final life-form score of an individual is the sum of all the partial scores divided by the maximum value possible (20) so that it ranges from 0 to 1, with 1 being associated to more euedaphic traits and 0 to epigeic ones.

*2.2.4.5.3. Earthworms*

Earthworms were collected on 1st March 2017 during the tillage of the plots and hand-sorted. Due to their low density, the individuals from plots with the same treatment were pooled. Earthworms were counted, weighed and fixed in ethanol: formaldehyde solution (1:1 v/v) and then stored in a 10% formaldehyde solution prior to their identification to species level.

### 2.2.4.6. Food production

The effects of biochar treatments on barley germination were assessed by counting the number of seedlings per mesocosm (i.e., 1 $m^2$) 16 days after sowing, whereas seedling development was assessed by counting the number of tillers per plant 67 days after sowing. Aboveground barley biomass was harvested in early summer (22nd June, 2017), when plants were fully developed and senescent. Aboveground biomass was dried at 60 ºC for 48 h, and then, straw and grain were weighed separately to determine yields (expressed as g $m^{-2}$). Thereafter, straw and grain were grinded using a ball-mill, and nutrient content (N, P, K, Ca, Mg, S, Fe, Mn, and Zn) was measured by near infrared spectrometry (NIRS) by scanning the milled samples in duplicate

from 1100 to 2500 nm using a NIRSystems 5000 scanning monochromator (FOSS, Hilleröd, Denmark) and the calibrations developed in a previous study (Martos et al., 2020). Protein content was estimated by multiplying the N content by a 6.25 nitrogen-to-protein conversion factor (Jones, 1931). Grain and straw nutrient export was calculated by multiplying the measured nutrient concentrations by the measured biomass.

#### 2.2.4.7. Soil quality index

To evaluate agroecosystem performance as influenced by each of the biochar addition treatments, the six measured soil functions were ascribed to an indicator and integrated into a soil quality index. Specifically, the selected indicators were: organic carbon (C sequestration); gravimetric water content (water retention); $CO_2$-eq emissions (GHG regulation); inorganic N, i.e., soluble ammonium and nitrate, at the growing season and at post-harvest (nutrient cycling); Maturity Index 2-5 (soil food web functioning); and grain yield (food production). Although several approaches had been proposed for soil quality index evaluation, they all include three general steps: (i) selection of a minimum data set of indicators, (ii) indicator transformation into combinable scores, and (iii) integration of transformed indicators into indices (Andrews et al., 2002).

For the first step, two main options are available: selecting the minimum dataset through expert opinion or statistical analyses (e.g., principal component analysis or ANOVAs). In our research a mixed approach was conducted, and statistical significance was used to select an indicator as suitable for representing a soil function; however, if the statistically significant indicator could not be associated with a clear interpretation scheme (e.g., temporal diverging patterns within the same treatment or difficulties in assigning scoring functions due to the lack of interpretation criteria), they were discarded, as recommended by Bünemann et al. (2018). Accordingly, enzymatic activity was discarded because when screened for seasonal stability the temporal diverging patterns criterion was not met (Dick 1994). Similarly, rooibos mass loss was also excluded from analyses because it was not clear whether the high rooibos decomposition rate observed in the 12 t $ha^{-1}$ treatment could result in better or worse soil functioning. Although faster mass loss could be linked to increased nutrient recycling, it could also lead to accelerated nutrient losses in soils. In addition, rapid degradation of lignin (a major constituent of rooibos) has been reported to adversely affect organic matter stabilisation (Gul et al., 2014).

The second step in indicator values transformation was undergone following the methodology described in Liebig et al. (2001). In brief, prior to transformation, indicators were averaged across all sampling dates with the exception of C sequestration, which was expressed as a cumulative value and thus, the final values were used; and inorganic N content, which was averaged into two different indicators (which were given an equal weight in the analyses) depending on crop presence/ absence. Accordingly, inorganic nitrogen content (soluble N-$NO_3^-$ and N-$NH_4^+$) measured during the barley growing season was used as an indicator of nutrient availability, whereas the same variable measured at post-harvest was selected as an indicator of potential N leaching and thus, environmental risk. Although the target ion to reduce environmental risk is nitrate and not ammonium, the latter was also included since in agricultural systems N-$NH_4^+$ is rapidly converted to N-$NO_3^-$ (Norton et al., 2008). In our studied system, inorganic nitrogen contents reached its maximum (up to 20.4 mg $kg^{-1}$ at a 20 cm soil depth) at the post-harvest period and potential risk of nitrate leaching has been reported to occur at about 40 mg N-$NO_3^-$ $kg^{-1}$ in the top 30 cm (Allan and Killorn, 1996; Pattison et al., 2010). Albeit sampling depths are not comparable, we assumed 40 mg N-$NO_3^-$ + N-$NH_4^+$ $kg^{-1}$ as the upper limit for inorganic nitrogen during the growing season.

Once averaged, each indicator was transformed using one of the following scoring functions (ranging from 0 to 1): i) 'more is better' (e.g., C sequestration); ii) 'less is better' (e.g., GHG emissions); and iii) 'optimum' (e.g., inorganic nitrogen at the growing season). For 'more is better' indicators, each observation was divided by the highest value observed across the entire dataset (so that the highest observed value scored 1); for 'less is better' indicators, the lowest observed value in the entire dataset was divided by each observation (so that the lowest observed value equates to 1); and 'optimum' parameters were scored according to a threshold value as 'more is better' if below the threshold limit value, and as 'less is better' if above the threshold value. An overview of selected indicators and its scoring functions is given in **Table 1**. The indicator values results were graphically represented as a radar chart, a suitable display to better visualise the contribution of each soil function (Bünemann et al., 2018).

Finally, for the last step, the six studied soil functions were integrated into an additive soil quality index (all soil functions were assigned an equal weight and summed so the index ranges from 0 to 6), as follows: Soil quality index = f {C sequestration, Water retention, Greenhouse gas emissions, Nutrient cycling, Soil food web functioning, Food production}.

**Table 1.** Soil functions with their selected indicators, scoring functions and references for their use.

| Soil function | Indicator | Scoring function | Reference |
|---|---|---|---|
| C sequestration | Soil organic carbon | More is better | Gugino et al. (2009) |
| Water retention | Gravimetric moisture content | More is better | Lowery et al. (1996); van Eekeren et al. (2010) |
| GHG emissions | Total $CO_2$-eq emissions ($CO_2$, $N_2O$ and $CH_4$ emissions) | Less is better | Costanza et al. (1997) |
| Nutrient cycling | Inorganic nitrogen (soluble N-$NO_3^-$ and N-$NH_4^+$) | Optimum (growing season) Less is better (post-harvest)[a] | van der Meulen et al. (2018) |
| Soil food web functioning | Maturity Index 2-5 | More is better | Andrews et al. (2004); Ferrarini et al. (2014) |
| Food production | Grain yield | More is better | Haefele et al. (2014); Johnson et al. (2002) |

[a] The final function for nutrient cycling was = (0.5 * inorganic N at the growing season) + (0.5 * inorganic N at post-harvest)

### 2.2.5. Statistical analyses

All the statistical analyses of the experimental data was carried out using R software v. 3.6.1 (R Core Team, 2019) and CANOCO software version 5.12 (Ter Braak and Šmilauer, 2012), and its visualisation using the packages *ggplot2* (Wickham, 2016) and *ggpubr* v 0.2.3 (Kassambara, 2019a). Longitudinal data (i.e., variables for which exist a between-subjects factor = biochar application rate, and a within-subjects factor = different sampling dates) was analysed using two-way mixed ANOVAs, which were computed with the *rstatix* package v0.2.0 (Kassambara, 2019b). Prior to statistical analyses, the Shapiro-Wilk and Levene tests were used to ensure that data had a normal distribution and homogeneous variances, respectively. When these assumptions were not met, the test was run on the $\log_{10}$-transformed variable. The assumption of sphericity was checked using the Mauchly's test and when violated the Greenhouse-Geisser correction was applied. Finally, homogeneity of covariances was tested by Box's M. Statistical results of the mixed ANOVA are shown in **Supplementary Table S3 (S3.1.-S3.54)**. Pairwise comparisons were tested with t test with Bonferroni adjustment, and the significance level was set at $p < 0.05$. Only treatment effects (main fixed factor and its interaction with time) are discussed.

For community data obtained on more than two sampling occasions (i.e., ionic content and enzymatic activities) the Principal Response Curves (PRC) method (Van den Brink and Ter Braak, 1999) was conducted using CANOCO 5.12. The PRC describes the trajectory over time

of the community response (expressed as coefficient of community response, i.e., the canonical coefficient $C_{dt}$) in each treatment group compared to the control one, which response is set to 0; whereas the weight ($b_k$) indicates the affinity of the response of each soil attribute (here ions or enzymes) relative to the overall community response. Positive $b_k$ values indicate attributes whose response pattern follows the PRC; by contrast, negative values indicate attributes whose response pattern follows an opposite trend to the overall PRC pattern. Near zero $b_k$ values represent weak responses or response patterns unrelated to the PRC. Data was log-transformed and then centred and standardised prior to conducting PRC. Significance of the first axis was checked using the Monte Carlo permutation test while significances at each time point were evaluated by performing redundancy analysis (RDA) on data subsets for each sampling date.

Finally, those parameters measured on a single sampling event as well as soil quality index statistical analyses were assessed by means of one-way ANOVA, Welch's ANOVA (if homoscedasticity assumption was not met) or the Kruskal–Wallis test (if normality assumption was not met) followed by t-test with Bonferroni correction, Games-Howell test and Dunn test with Bonferroni correction, respectively (*rstatix* package v 0.2.0 (Kassambara, 2019b)). It should be noted that when mixed ANOVA analysis of longitudinal data was not significant, differences between treatments at each sampling date were also checked using this approach. All values reported in the text are mean ± standard error (SE).

## 2.3. Results

### 2.3.1. Carbon sequestration

Biochar application caused a significant increase in soil organic C content after being six years in the field (**Fig. 2a**). According to the mixed ANOVA analysis, both biochar treatment and its interaction with time were significant (**Supplementary Table S3.1.**). As expected, higher biochar application rates resulted in major soil organic C contents, with an average increase in C contents of 22.62 and 67.94% than control for the 12 and 50 t biochar $ha^{-1}$ treatments, respectively. Except for the first sampling, these differences between the three treatments were observed at all sampling dates (**Fig. 2a**).

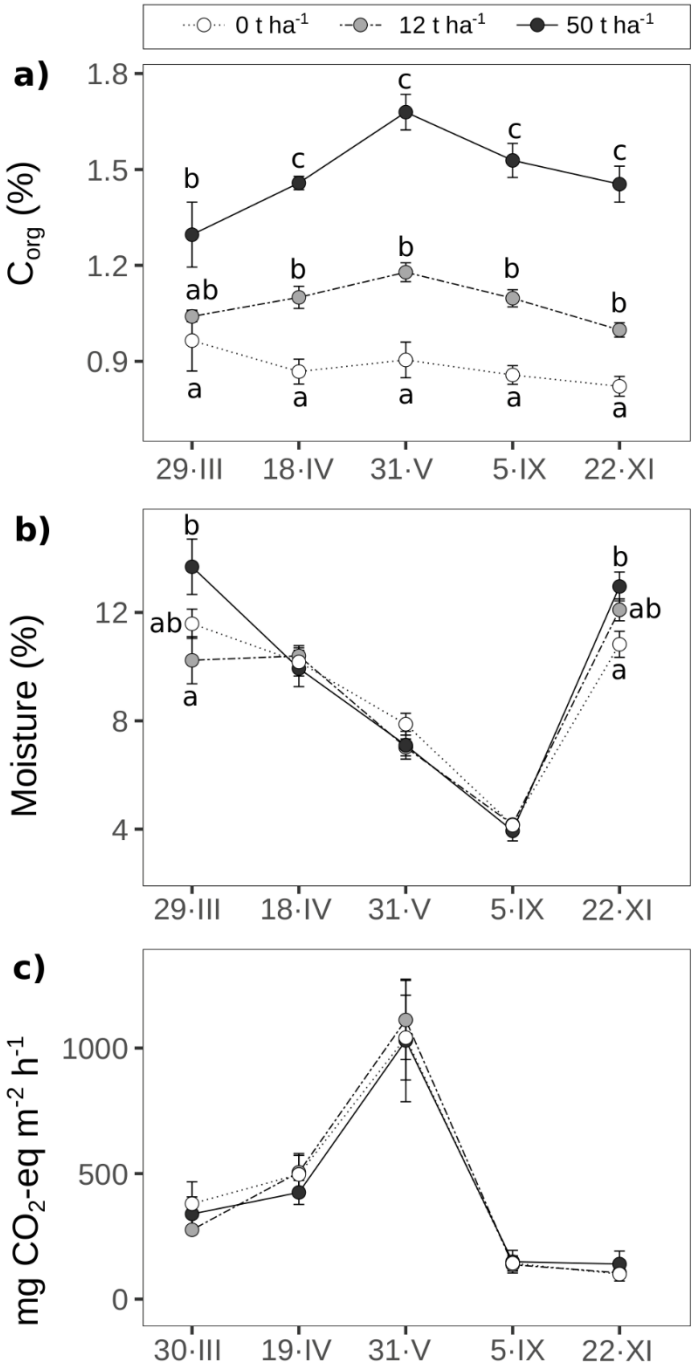


**Fig. 2.** Organic carbon ($C_{org}$) (%) (a) gravimetric moisture content (%) (b), and total $CO_2$-equivalent emissions (mg $m^{-2}$ $h^{-1}$) (c) along five samplings. For (a) and (b) samplings were: 29·III = 29th March; 18·IV = 18th April; 31·V = 31st April; 5·IX = 5th September and 22·XI = 22nd November; while for (c) corresponded to 30th March (30·III); 19th April (19·IV); 31st April (31·V); 5th September (5·IX) and 22nd November (22·XI). Symbols represent the mean values, and bars represent the corresponding standard error (n = 6). Different letters indicate statistically significant differences between treatments within a particular sampling.

### 2.3.2. Water retention

There was a significant interaction of treatment with time, i.e., increasing biochar application rate was associated to higher gravimetric water content (**Fig. 2b**) on two sampling occasions. In detail, the 50 t $ha^{-1}$ treatment enhanced moisture level with respect to the 12 t $ha^{-1}$ treatment at the 29th March sampling (33.71% increase) and with respect to control at the 22nd November sampling (19.73% increase).

### 2.3.3. GHG emissions

$CO_2$-equivalent emissions are summarised in **Fig. 2c**. On a quantitative basis, $CO_2$ was by far the major contributor to global $CO_2$-eq emissions and, when pooling treatments and sampling dates, emissions (as mg $CO_2$-eq $m^{-2}$ $h^{-1}$) averaged 433 ± 47 for $CO_2$, 5.89 ± 1.9 for $N_2O$, and -0.02 ± 0.2 for $CH_4$. Overall, there were no significant differences due to biochar treatment on $CO_2$-eq. When GHG exchange rates were analysed individually (**Supplementary Fig. S1**) there were no significant biochar-mediated effects regarding C-$CO_2$ and N-$N_2O$ exchange rates. However, biochar treatment was found to have a significant main effect on C-$CH_4$ exchange rates (**Supplementary Table S3.6**), with the 50 t $ha^{-1}$ treatment emitting significantly ($p = 0.02$) more methane (0.025 ± 0.02 mg $m^{-2}$ $h^{-1}$, $n = 24$) than the 12 t $ha^{-1}$ treatment (-0.017 ± 0.01 mg $m^{-2}$ $h^{-1}$, $n = 26$). It is also worth mentioning that while in the 50 t $ha^{-1}$ treatment there was a net emission of C-$CH_4$, in the 12 t $ha^{-1}$ treatment there was a mean net uptake (negative flux).

The **Supplementary Fig. S2** shows the C-$CH_4$ exchange rates from a lysimeter experiment by Llovet et al. (2021) (unpublished data) using the 6-yr aged biochar and soil from the mesocosms described in this study (aged biochar scenario) and also biochar freshly applied at the same application rates (fresh biochar scenario). While in the fresh biochar scenario there was also a main effect of biochar treatment on $CH_4$ emissions (**Supplementary Table S3.7**), i.e. the 50 t $ha^{-1}$ treatment emissions (0.01 ± 0.01 mg $m^{-2}$ $h^{-1}$, $n = 21$) were significantly higher than that of 12 t $ha^{-1}$ treatment (-0.018 ± 0.01 mg $m^{-2}$ $h^{-1}$; $n = 20$, $p = 0.01$) and the control (-0.013 ± 0.01 mg $m^{-2}$ $h^{-1}$; $n = 19$, $p = 0.04$), in the aged biochar scenario (equivalent to the one in this study), there was a significant interaction of treatment with time (**Supplementary Table S3.7**). Although different trends could be observed between the control C-$CH_4$ fluxes, which showed more steady values, and in biochar treatments, which were more fluctuating over time (**Supplementary Fig. S2**), t-tests between treatments within the different dates were not sufficient to make statements about pair-wise differences.

### 2.3.4. Nutrient cycling

#### 2.3.4.1. Soil ionic content, N pools, pH, and EC

The PRC analysis of the soil ionic content (**Fig. 3a**) was conducted without including $N\text{-}NO_2$ as it was below detection limits in nearly all samples. The results showed that 70.07% of the overall variance was explained by time and 3.87% by the biochar treatment. The first canonical axis of the PRC captured a significant proportion (65.06%) of the variance explained by biochar treatment (Monte Carlo permutation test, 499 permutations, $F = 0.7$, $p = 0.034$). However, significance tests carried out at each sampling time by means of RDA showed only marginally significant differences. The ionic species that contributed most to the accomplishment of the PRC was magnesium ($b_k = 2.03$) and the lesser nitrogen species. Individual trajectories of each ion can be seen at **Supplementary Fig. S3a** (magnesium, calcium, sulphate, chloride, sodium and potassium) and at **Supplementary Fig. S4** (nitrate, nitrite and ammonium). As expected by the PRC results, Mg showed significant effects due to an interaction between treatment and time, i.e., the 50 t $ha^{-1}$ treatment had greater Mg concentrations than the control on 5th September. By contrast, ammonium, which had a low weight in PRC, also showed significant results according to mixed ANOVA. This contrasting result is probably a consequence of a different temporal pattern of ammonium when compared to the other ions in the PRC. Specifically, according to mixed ANOVA (**Supplementary Table S3.9**) there was a significant treatment effect on soluble $N\text{-}NH_4^+$ content. Nonetheless, the t-tests between the different treatments (averaged across all sampling dates) showed no significant differences, probably because of the near-zero values obtained in the first three samplings (pre-harvest period). For this reason, statistical comparisons were carried out separately for those samplings performed at the pre-harvest period (29th March, 18th April and 31st May) and the post-harvest period (5th September and 22nd November), when $N\text{-}NH_4^+$ reached higher concentrations. At the post-harvest period the 50 t $ha^{-1}$ treatment had significantly higher $N\text{-}NH_4^+$ concentrations ($2.413 \pm 0.12$ mg $kg^{-1}$) than the 12 t $ha^{-1}$ treatment ($1.988 \pm 0.11$ mg $kg^{-1}$; $p = 0.034$) and the control ($1.991 \pm 0.11$ mg $kg^{-1}$; $p = 0.036$) whereas at the pre-harvest period no significant differences between treatments were found.

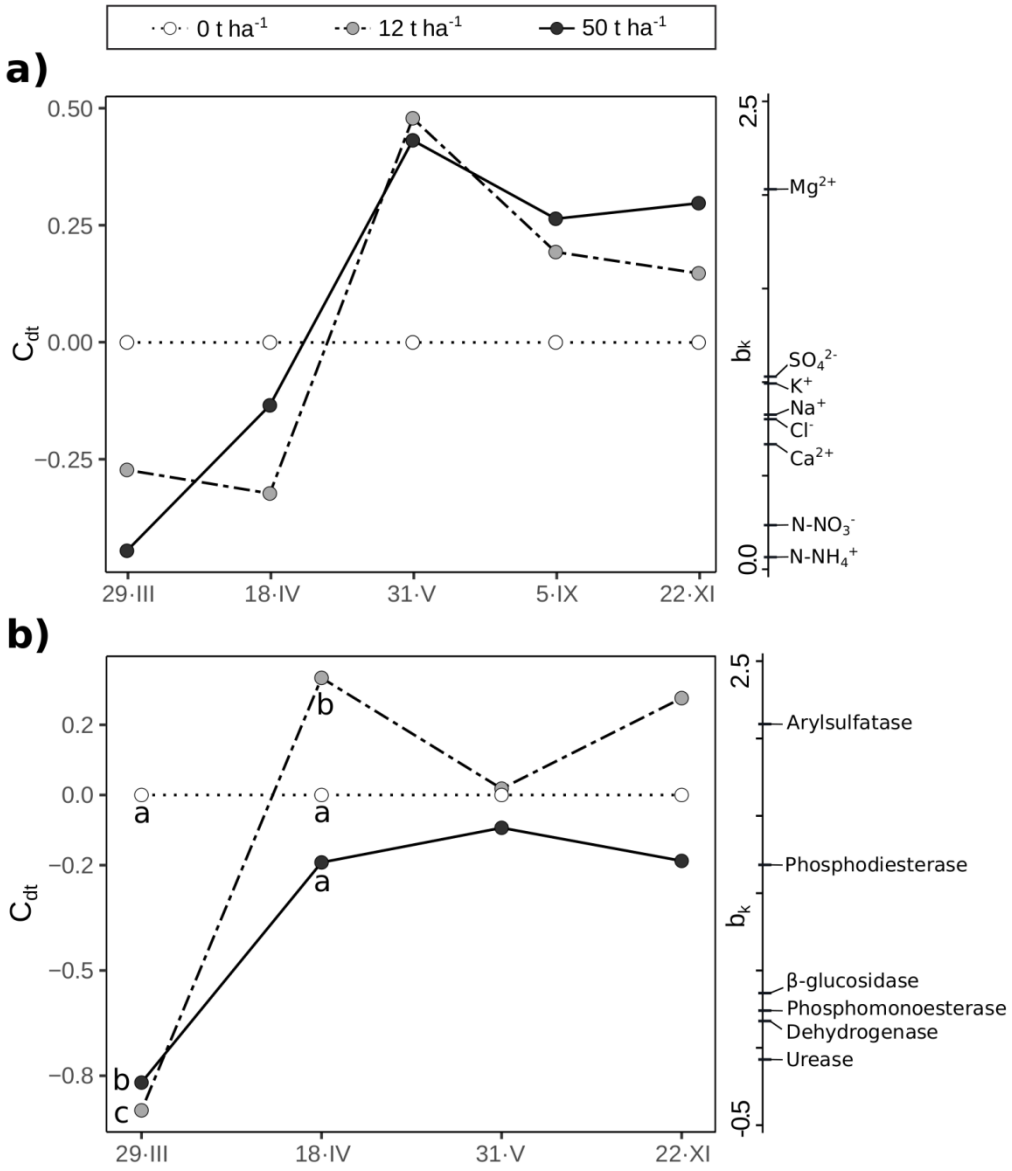


**Fig. 3.** PRC diagram (left) and weight ($b_k$) (right), for the first principal component of the soil ionic content (a) and enzymatic activities (b) showing shifts caused by biochar treatments (12 and 50 t $ha^{-1}$) along five and four sampling dates, respectively, in comparison to control. Different letters indicate statistically significant differences between treatments within a particular sampling time.

The other investigated nitrogen pools (soluble ionic content of $N\text{-}NO_3^-$, $N\text{-}NO_2^-$, exchangeable $N\text{-}NH_4^+$, total Kjeldahl nitrogen (TKN), microbial nitrogen, and extractable organic nitrogen) showed no significant response to biochar additions. However, similar to $N\text{-}NH_4^+$, they also seemed to be slightly higher at the 50 t biochar $ha^{-1}$ during the post-harvest samplings (**Supplementary Fig. S4**).

Regarding pH and EC, there was a lack of significant effects due to biochar treatment (**Supplementary Fig. S5**).

#### 2.3.4.2. Soil enzymatic profile

The PRC of enzymatic activities (**Fig. 3b**) revealed that 60.01% of the total variance was explained by time whereas 11.35 % was attributed to biochar treatment. A significant proportion of the variance (56.04%) was captured by the first canonical axis of the PRC (Monte Carlo permutation test, 499 permutations, $F = 1.4$, $p = 0.012$). According to RDA analyses performed on the data collected on each sampling time, there were contrasted and significant temporal variations in enzymatic responses to biochar addition. At the inter-fertilisation sampling time (29th March), the control treatment showed a higher enzymatic activity compared to the 12 t $ha^{-1}$ (pseudo-$F = 9.7$, $p = 0.01$) and the 50 t $ha^{-1}$ treatments (pseudo-$F = 9.2$, $p = 0.004$); and at the 12 t $ha^{-1}$ treatment was also significantly lower than that of the 50 t $ha^{-1}$ (pseudo-$F = 4.0$, $p = 0.004$)). However, at the post-fertilisation sampling (18th April) enzymatic activities significantly increased at the 12 t $ha^{-1}$ treatment relative to the control (pseudo-$F = 3.3$, $p = 0.026$) and the 50 t $ha^{-1}$ (pseudo-$F = 6.8$, $p = 0.004$) treatments. As indicated by $b_k$ values of the PRC, the most responsive enzyme was arylsulfatase ($b_k = 2.09$), followed by phosphodiesterase ($b_k = 1.18$). Individual trajectories of each enzyme are shown in **Supplementary Fig. S3b**.

#### 2.3.4.3. Tea bag decomposition

There was a significant ($p=0.009$) effect of biochar treatment on rooibos mass loss (**Supplementary Table S3.30.**). On average, the 12 t $ha^{-1}$ treatment induced a marginally significant greater mass loss of rooibos (28.3 ± 1.35 %) with respect to the control (24.4 ± 0.92 %) ($p = 0.07$), while no differences with the 50 t $ha^{-1}$ treatment were found (26.9 ± 1.13 %). This stimulatory effect was more apparent at the spring sampling (Kruskal-Wallis H test ($\chi^2_{(2, N = 18)} = 6.35$, $p = 0.04$)) (**Fig. 4**). There were no significant biochar effects on green tea mass losses.

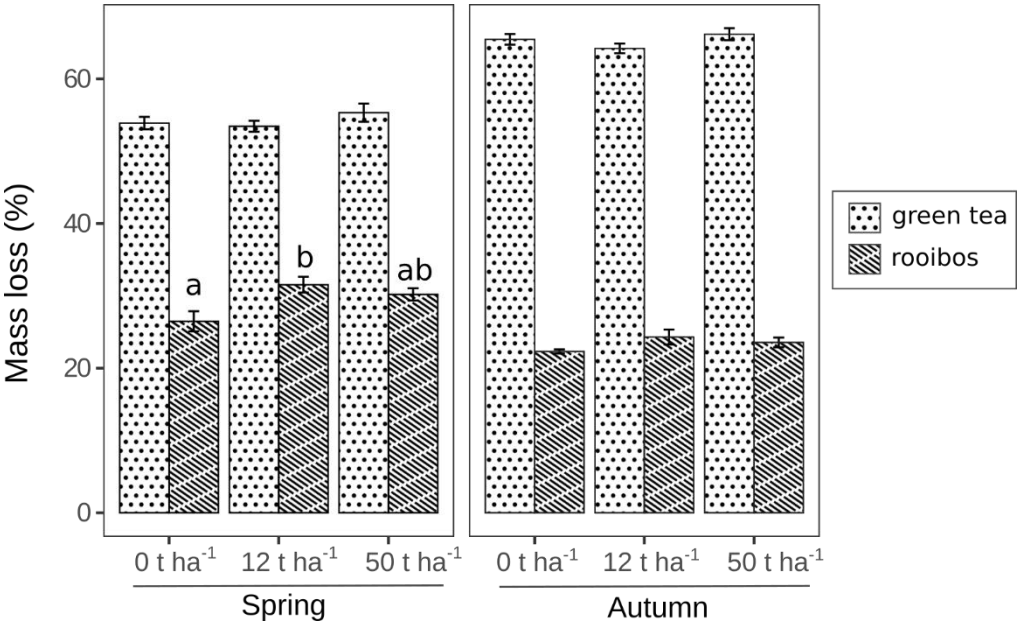


**Fig. 4.** Mean (± SE) green tea and rooibos mass loss (%) at the spring and autumn samplings. N is equal to 6 except from the 12 t $ha^{-1}$ treatment at the autumn sampling which had n = 5. Different letters indicate statistically significant differences between treatments within a particular sampling.

#### 2.3.4.4. Bait lamina

Feeding activity as evaluated by bait laminae is presented in **Supplementary Fig. S6**. No significant differences in bait consumption due to biochar treatment were detected on any sampling occasion, either in the total depth assessed (0–8 cm) or at any of the depth intervals. Nevertheless, a non-significant trend towards decreased feeding activity in response to biochar addition rates was observed at the spring sampling. In contrast, at the autumn sampling, a trend towards an increase in bait consumption, albeit non-significant, was observed at the 12 t $ha^{-1}$ treatment.

### 2.3.5. Soil food web functioning

#### 2.3.5.1. Microorganisms

Microbial parameters including microbial biomass, basal soil respiration and metabolic quotient, as well as EOC, were not significantly influenced by biochar additions (**Supplementary Fig. S7**). Similarly, qPCR-based microbial abundance (bacterial 16S and fungal 18S rRNA gene copy numbers) and the fungal:bacterial ratio were not significantly affected by biochar at any of the studied sampling dates (**Supplementary Fig. S8**).

#### 2.3.5.2. Nematodes

The results of the mixed ANOVA showed that the MI2-5 was significantly affected by biochar treatment. On average, the MI2-5 significantly decreased at the 50 t biochar $ha^{-1}$ treatment (2.14 ± 0.0) compared to the control one (2.54 ± 0.1; p = 0.01), but the differences with the 12 t biochar $ha^{-1}$ treatment (2.44 ± 0.1) were only marginally significant (p= 0.06) (**Supplementary Table S4**). The effects of biochar on metabolic footprints showed that fungivore and herbivore footprints were significantly higher at the 50 t biochar $ha^{-1}$ treatment than at the 12 t biochar $ha^{-1}$ and the control treatments, whereas the omnivore footprint followed the opposite pattern (**Supplementary Tables S3.43-47** and **Fig. 5**). When analysing the results for each sampling date separately, it can be seen that while the MI2-5 showed significant differences with biochar application rates at both spring and autumn samplings (**Supplementary Table S4**), in the case of the metabolic footprints significant differences were only observed during the autumn sampling (**Fig. 5**). Total nematode abundance and abundance per trophic guild are shown in **Supplementary Table S4**. In brief, spring and autumn combined results showed that total nematode abundance was significantly higher at the 50 t biochar $ha^{-1}$ treatment and, as already pointed out by metabolic footprint data, this increment in nematode abundance was mainly driven by the increase of hervibore and fungivore nematodes. In an opposite direction, omnivore nematode abundance was significantly decreased at the 50 t biochar $ha^{-1}$ treatment.

#### 2.3.5.3. Microarthropods

Results of microarthropods analyses are summarised in **Supplementary Fig. S9**. Because collembolans were only recorded in just two control plots in the spring sampling, it was not possible to test if the values of collembolan LFT met the normality criterion. Hence, mixed ANOVA was not conducted and this data was analysed separately for each sampling. Spring sampling results were significantly different between treatments according to a Kruskal-Wallis H test ($\chi^2_{(2, N = 13)} = 7.69$, p = 0.02) and the post-hoc pair-wise comparisons indicated that lower values of LFT (59 % decrease) were recorded at the 50 t $ha^{-1}$ (0.21 ± 0.04, n = 6) treatment compared to the control (0.51 ± 0.01; n = 2, p = 0.049). By contrast, no significant effects between treatments were found for the autumn sampling. Although the QBS-ar index showed a decreasing trend with increasing biochar application rate in spring (albeit not significant), in autumn this tendency disappeared.

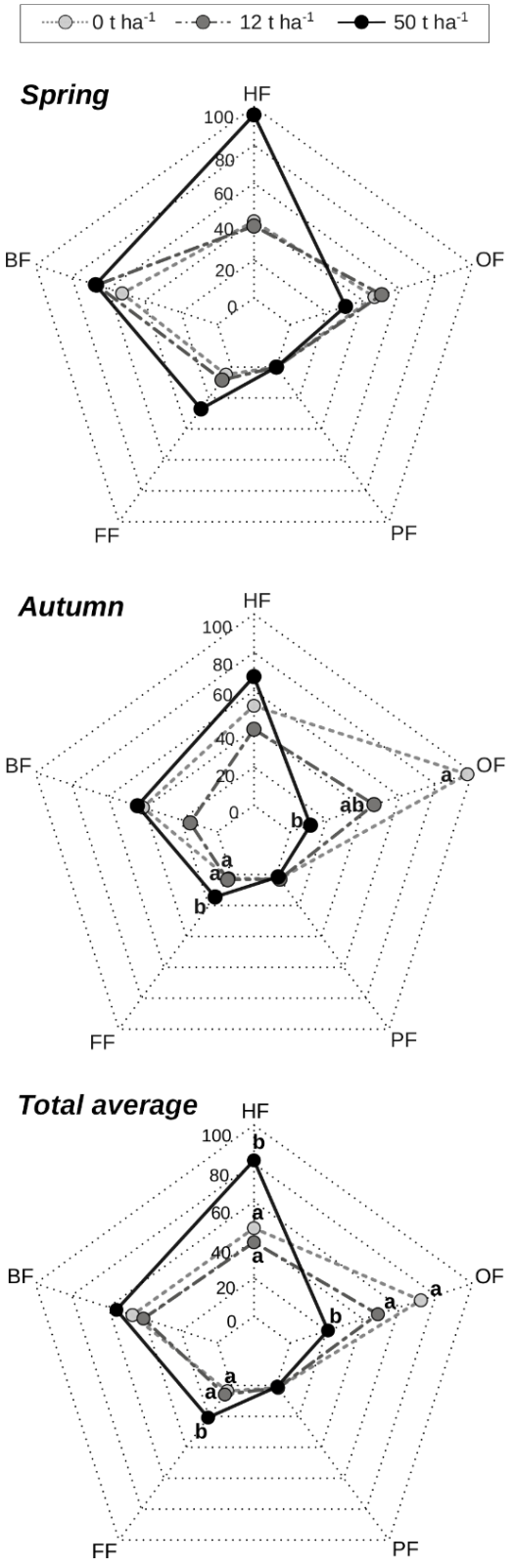


**Fig. 5.** Metabolic footprints (in µg C/ 100 g dry soil) of bacterivore (BF), fungivore (FF), herbivore (HF), omnivore (OF) and predator (PF) nematodes at the spring and autumn samplings (n = 6) as well as the mean values of the two seasons (total average) (n = 12). For spring and autumn samplings assessed separately, different letters indicate statistically significant differences between treatments according to ANOVA and Kruskal-Wallis tests whereas for the global mean assessment different letters indicate statistically significant differences between treatments according to main effects of treatment in mixed ANOVA.

#### 2.3.5.4. Earthworms

Only two earthworm species were recorded in the mesocosms which belonged to two different ecological groupings (Bouché, 1977): the endogeic species *Aporrectodea rosea,* and the anecic *Aporrectodea trapezoides*. Despite the fact that a slight decrease in earthworm biomass and abundance with increased biochar addition rate was observed (**Supplementary Fig. S10**), the paucity in the data did not allow for these differences to be statistically proven.

### 2.3.6. Food production

Barley seedling emergence and tiller count is shown in **Supplementary Fig. S11**. Although a slight increased trend in germination and tillering rate was observed with biochar addition rates, biochar had no significant effect on neither of these two parameters.

Grain and straw yields, and protein and nutrient contents are shown in **Table 2** while nutrient export is reported in **Supplementary Table S5**. Biochar additions did not exert any significant effect on grain and straw yields. Overall, there was a trend towards reduced grain nutrient content with increasing biochar rate, the only significant effect being a reduced grain Zn concentration in the 50 t $ha^{-1}$ treatment with respect to control (ANOVA: $F_{2, 15} = 3.69$, $p = 0.049$). However, Zn nutrient export in grain was not significantly affected by biochar treatments. No significant differences were observed in straw nutrient content but a slight increase ($p > 0.05$) in straw nutrient export was observed at the highest the biochar rates.

### 2.3.7. Soil quality index

The impact of the different biochar addition rates on soil functions are shown in **Fig. 6**, using the indicators described in **Table 1**. The additive soil quality index (derived from the sum of all soil functions) scored 3.82 ± 0.09 for the control, 3.80 ± 0.14 for the 12 t $ha^{-1}$ treatment and 3.96 ± 0.19 for the 50 t $ha^{-1}$ treatment and showed no statistical significant differences among treatments. However, when analysed individually, two soil functions showed significant differences between treatments. Specifically, the C sequestration function significantly increased proportionally to biochar rate (Welch ANOVA: $F_{2, 9.15} = 45.5$, $p = <0.001$). On the other hand, the soil food web functioning was significantly lower at the highest biochar rate (50 t $ha^{-1}$) when compared with the control (ANOVA: $F_{2, 15} = 5.89$, $p = 0.01$).

**Table 2.** Yield, protein content (PC), and nutrient content in grain and straw of harvested barley. Reported values are mean ± standard errors (n = 6). Different letters indicate statistically significant differences between treatments within a particular sampling.

| | **Grain** | | | **Straw** | | |
|---|---|---|---|---|---|---|
| | 0 t $ha^{-1}$ | 12 t $ha^{-1}$ | 50 t $ha^{-1}$ | 0 t $ha^{-1}$ | 12 t $ha^{-1}$ | 50 t $ha^{-1}$ |
| Yield (g $m^{-2}$) | 29.46 ± 8.4 | 17.78 ± 3.1 | 18.03 ± 6.6 | 89.96 ± 11.0 | 105.06 ± 6.2 | 127.76 ± 11.6 |
| PC (%) | 11.87 ± 0.4 | 11.06 ± 0.7 | 10.57 ± 0.5 | 8.69 ± 0.7 | 7.16 ± 0.8 | 7.03 ± 0.4 |
| N (g $kg^{-1}$) | 18.98 ± 0.7 | 17.70 ± 1.1 | 16.91 ± 0.8 | 13.90 ± 1.2 | 11.45 ± 1.3 | 11.25 ± 0.7 |
| P (g $kg^{-1}$) | 6.51 ± 0.2 | 6.00 ± 0.2 | 5.87 ± 0.2 | 2.56 ± 0.2 | 2.43 ± 0.2 | 2.40 ± 0.1 |
| K (g $kg^{-1}$) | 10.85 ± 0.2 | 10.62 ± 0.4 | 10.37 ± 0.3 | 17.21 ± 0.8 | 18.42 ± 0.2 | 18.09 ± 0.4 |
| S (g $kg^{-1}$) | 0.34 ± 0.0 | 0.35 ± 0.0 | 0.35 ± 0.0 | 2.00 ± 0.1 | 2.20 ± 0.1 | 2.09 ± 0.1 |
| Ca (g $kg^{-1}$) | 0.82 ± 0.1 | 0.83 ± 0.1 | 0.75 ± 0.1 | 5.44 ± 0.1 | 5.68 ± 0.2 | 5.20 ± 0.2 |
| Mg (g $kg^{-1}$) | 1.41 ± 0.0 | 1.34 ± 0.0 | 1.29 ± 0.0 | 1.40 ± 0.1 | 1.49 ± 0.0 | 1.42 ± 0.0 |
| Fe (mg $kg^{-1}$) | 12.09 ± 1.7 | 12.89 ± 3.3 | 10.00 ± 3.0 | 25.33 ± 2.1 | 22.39 ± 1.5 | 17.92 ± 2.5 |
| Mn (mg $kg^{-1}$) | 1.62 ± 0.0 | 1.54 ± 0.1 | 1.59 ± 0.0 | 3.06 ± 0.3 | 3.14 ± 0.2 | 2.97 ± 0.1 |
| Zn (mg $kg^{-1}$) | 5.43 ± 0.1 **a** | 5.20 ± 0.1 **ab** | 4.95 ± 0.1 **b** | 4.82 ± 0.2 | 4.52 ± 0.2 | 4.25 ± 0.2 |

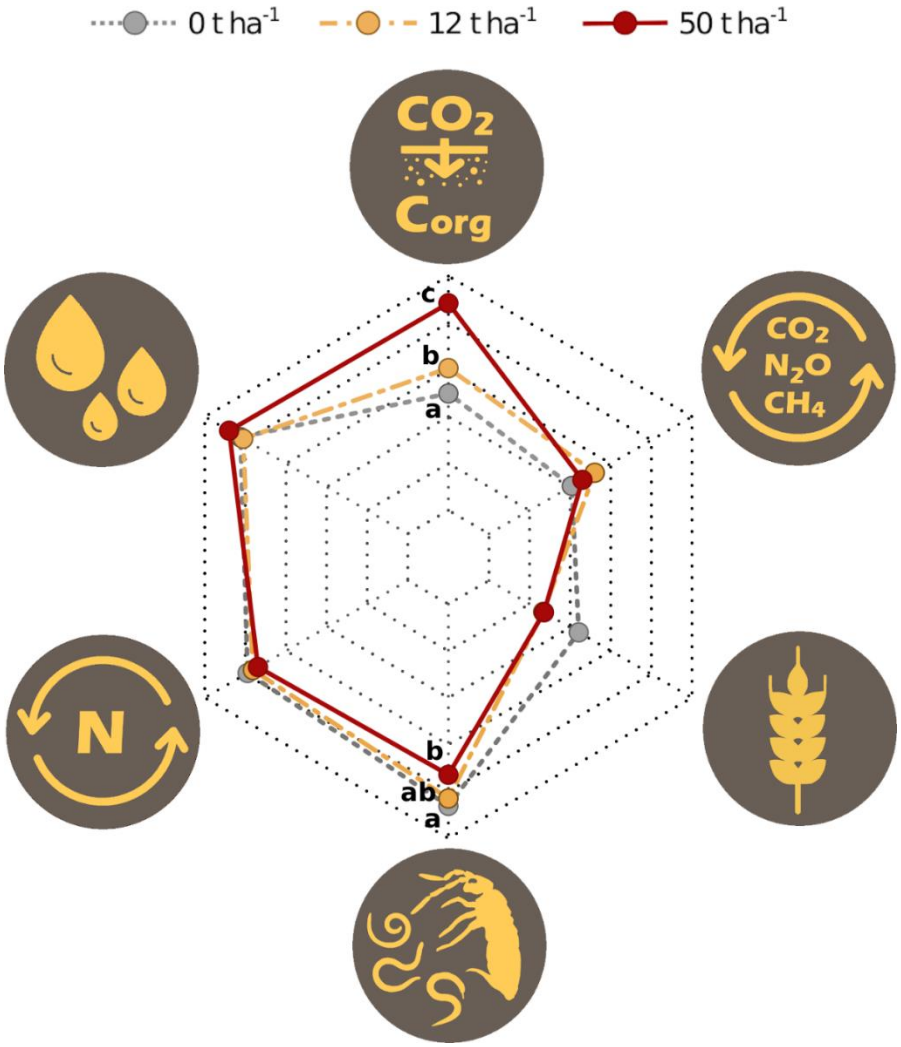


**Fig. 6.** Radar chart indicating the performance of the different biochar treatments (0, 12 and 50 t $ha^{-1}$) on (from the top to the left) C sequestration, water retention, nutrient cycling, soil food web functioning, food production, and GHG emissions. The displayed values are transformed scores to a 0-1 scale as explained in section 2.4.7. Different letters indicate statistically significant differences between treatments on a specific soil function.

## 2.4. Discussion

### 2.4.1. Carbon sequestration

Increased soil C sequestration has been the most commonly reported benefit of biochar amendments (Laird, 2008; Lehmann et al., 2006; Shackley et al., 2013). In agreement with this, our results also indicated an increase in soil C storage after 6 years of biochar application. This is in line with our initial hypotheses, and can be explained by the fact that our biochar has a very low $H/C_{org}$ ratio (0.14), which is a proxy of its resistance to microbial attack (Bruun et al., 2016). Another factor that could promote the long-term stability of biochar in soils is its occlusion in organo-mineral complexes, as suggested elsewhere (Glaser et al., 2002) and proved for this specific biochar in another study with a different soil (Raya-Moreno, 2018). Hence, C sequestration was increasingly promoted with biochar rate after 6-yr of its application, suggesting this gasification biochar as a good option to store C in soil in a relatively stable manner. However, when compared to the organic C data derived from the same mesocosms at the start of the experiment (4th April, 2011) (data not shown), the results showed that all treatments, including the control, reduced its SOC contents after 6 years: on average, SOC was reduced by a 26% at the control soil, by a 25.2% at the 12 t $ha^{-1}$ treatment, and by 22.7% at 50 t $ha^{-1}$ treatment. We surmise that this SOC loss is not related to biochar C loss, since both biochar treatments and the control soil lost SOC at a similar rate, probably as a result of the continuous cultivation with the removal of crop aboveground residues throughout the six years of this study. Clearly, further research is warranted to corroborate C persistence after biochar additions over longer time-frames.

### 2.4.2. Water retention

Our results obtained under field conditions showed a significant increase in soil moisture content at the 50 t $ha^{-1}$ biochar rate but only on two out of the five sampling dates. The fact that significant results were only found at the highest biochar rate is not surprising since it has been reported that large volumes of biochar are needed to be incorporated into soil before it can lead to measurable positive effects on water retention (Verheijen et al., 2010). Water retention amelioration could be explained by the high degree of porosity of this biochar (81%) (Llovet et al., 2021). However, in a previous study comparing the effects of biochar ageing, it was observed that the 6-yr field-aged biochar did not induce significant effects on moisture retention but the

fresh biochar did (Llovet et al., 2021), and this was attributed to a pore occlusion in biochar as a consequence of ageing in soil. Therefore, the positive effects on soil moisture observed in the current study with aged biochar might be at least partly related to increased soil aggregation, namely, better soil aggregation could offset the potential pore occlusion.

This influence of biochar on soil water retention properties has the potential to increase plant-available water (Razzaghi et al., 2020). However, water available to plants is estimated to be retained in macropores ranging from 10 μm to 80 μm (Joseph et al., 2009), and since the gasification biochar used in this study had a mean pore size of 1.2 μm, it is unlikely that plants were able to access to this water strongly held by capillary forces within the pores. In relation to this, Ojeda et al. (2015), in a study using the same biochar but added to the soil at a rate of 1% biochar C (ca. 33 t biochar $ha^{-1}$), observed that available water for plant uptake remained unchanged independently of biochar additions after one month and after one year of incubation.

### 2.4.3. GHG emissions

GHG emissions measured as $CO_2$-eq were unaffected by biochar treatment, however, some effects were found in relation to $CH_4$ exchange rates. $CH_4$ is mainly produced under anaerobic conditions where methanogenic archaea thrive, while it is consumed by methanotrophic bacteria in oxic soils. Thus, soil $CH_4$ exchange rates depend on the balance between methanogenic and methanotrophic communities, which are in turn underpinned by oxygen and substrate availabilities (Conrad, 2007). Well-aerated upland soils, including agricultural soils, are characterised by $CH_4$ consumption by methanotrophic bacteria. Hence, generally they possess $CH_4$ sink potential (up to 0.049 mg C-$CH_4$ $m^{-2}$ $h^{-1}$; Kamman et al., 2017).

Our results showed that the highest biochar rate (50 t $ha^{-1}$) represented a source of C-$CH_4$ (0.025 ± 0.02 mg $m^{-2}$ $h^{-1}$), and was significantly different from the 12 t $ha^{-1}$ treatment, which was a sink (-0.017 ± 0.01 mg $m^{-2}$ $h^{-1}$). The control was also a sink (-0.009 ± 0.01 mg $m^{-2}$ $h^{-1}$), but no significant differences were found with respect to biochar treatments. When compared with the results of the data arising from the study by Llovet et al. (2021) with the same biochar, similar effects were found for the freshly applied biochar, with the 50 t $ha^{-1}$ treatment presenting enhanced $CH_4$ emissions in comparison with both the control and the 12 t $ha^{-1}$ treatment. Remarkably, Ribas et al. (2019), utilising the same biochar as in this study, also found increased

$CH_4$ emissions with the highest application rate (30 t $ha^{-1}$) 16 months after its application. The increased $CH_4$ emissions were ascribed to the formation of biochar-induced anoxic microsites facilitated by higher temperatures at summer months causing higher bacterial activity and oxygen depletion within biochar micropores, which would have favoured methanogenic metabolism and also the last step of denitrification, thus, also repressing $N_2O$ emissions. Data from the lysimeter study by Llovet et al. (2021) also showed that fresh biochar presented the most apparent effect at the summer sampling, thus, supporting the mechanism involving anaerobic conditions in microsites. In the mesocosms of the current experiment, instead, results emerged as a main effect not restricted to seasonal temperature variations, but microbial activity and oxygen exhaustion could also have been triggered after fertilisation events or crop belowground residue decomposition (Hagemann et al., 2016). According to this, the differences between the two biochar rates could be explained by differential clogging of biochar pores as a result of its ageing, namely, if the 12 t $ha^{-1}$ biochar had a larger proportion of clogged pores, as suggested by Llovet et al. (2021), anoxic microsite formation would have been hindered.

Consistently with our results, in a recent meta-analysis by Ji et al. (2018), it was found that for upland soils, the response of soil $CH_4$ uptake rates presented a declined trend with biochar increasing rates, ranging from an insignificant increase effect (+9%) at the amended rates lower than 20 t $ha^{-1}$ to the largest decrease effect (- 171%) at a rate over 60 t $ha^{-1}$. This rate-dependent response was mainly attributed to toxic compounds which may affect methanotrophs and to differences in the availability of methane oxidation competitors, such as $NH_4$. In this regard, the increased levels of soluble $NH_4^+$ at the 50 t $ha^{-1}$ treatment could be responsible for $CH_4$ oxidation competition and thus, increased $CH_4$ emissions. It was not possible to conclude which of these mechanisms is ultimately responsible, particularly given the contrasting responses that can occur with biochar. Thus, biochar's stimulated production or inhibited consumption of methane both in the short and long-term requires further research.

### 2.4.4. Nutrient cycling

#### 2.4.4.1. Soil ionic content, N pools, pH and EC

In our study system, an agroecosystem, nitrate pollution mitigation is a major environmental goal and biochar has been found to reduce nitrate levels on these same mesocosms fifteen months following its application (Marks et al., 2016). However, our results indicate that, in the

long-term, this mitigation capacity could be lost. Indeed, tendencies to increased soluble nitrate and ammonium contents in the 50 t $ha^{-1}$ treatment were observed, which, although slight, were significant in the case of ammonium at the post-harvest period. The finding of lessened nitrate mitigation potential at the long-term is in line with a previous study using the same biochar in a lysimeter system (Llovet et al., 2021), where nitrate reductions were reported after eight months of fresh biochar application, but not in the six-year field-aged biochar scenario. As hypothesised by Llovet et al. (2021), this could be the consequence of pore clogging with biochar ageing, which prevents nitrate retention within biochar pores. Alternatively, lower nitrate mitigation potential can also be a consequence of changes in N transformation dynamics over time. Although it is commonly asumed that biochar ageing increases its cation adsorption capacity, and thus its $NH_4^+$ retention (Kookana et al., 2011; Liang et al., 2016), formation of organo-biochar-complexes after continued organic amendement can stimulate N mineralisation and increase $NH_4^+$ soluble content (Gao et al., 2019). Additionally, increased $NH_4^+$ production in arable soils owing to biochar additions has been shown in other studies (Nelissen et al. 2012; Prommer et al. 2014), and linked to increasing recalcitrant microbial metabolism and the promotion of soil ammonia-oxidiser populations, respectively. Another potential explanation could be the positive influence of fungivore nematodes on N mineralisation, since the 50 t $ha^{-1}$ treatment had a higher fungivore metabolic footprint, and some species of fungivore nematodes have been seen to increase N mineral content as they excrete the non-assimilated ammonium (Chen and Ferris, 1999).

Regarding the effects of biochar additions on other ions, magnesium was the most responsive ion in the PRC analysis. Despite not presenting a consistent behaviour over time, it was significantly enhanced at the highest biochar treatment on one sampling date. Increased magnesium contents after adding biochar have also been reported in studies using the same gasification biochar and were attributed to biochar acting as a source of this cation (Martos et al., 2020; Ribas et al., 2019). In contrast, in relation to EC and pH, which are respectively good integrators and regulators of nutrient availability (Moebius-Clune, 2010), no significant biochar effects were found.

#### 2.4.4.2. Enzymatic activity

Enzymatic activity showed significant differences in response to biochar additions that did not coincide either in treatment or in time so no trend was apparent, and the driving factors of these

responses remain elusive. There is little knowledge on the effects of biochar on enzymatic reactions, but an interplay of various factors has been suggested in the literature. The main source of variation seems to be related to biochar potentially affecting the target substrate and enzyme availability through sorption processes and, in turn, enzymes could be inactivated or its medium-life elongated depending on whether their interaction with biochar stabilises or denatures their active sites (in particular for extracellular enzymes, i.e., all examined enzymes in this study except from dehydrogenase, which is only found within viable cells; Dick, 1994). In addition, changes in microbial substrate utilisation driven by microbial community shifts due to biochar additions are also possible (Bailey et al., 2011; Lammirato et al., 2011; Zimmerman and Gao, 2013). It should also be considered that co-location of substrates and enzymes onto biochar surfaces might enhance enzymatic efficiency (Lehmann et al., 2011). In the case of arylsulfatase (the most responsive enzyme in PRC), a co-location of enzymes and substrate on biochar surfaces is likely since biochar has been reported to be rich in ester-bounded sulphur, the substrate of arylsulfatase (Churka Blum et al., 2013). Therefore, the decreased activity of this enzyme observed at the 50 t $ha^{-1}$ treatment could indicate a decrease in enzyme production as a result of a more efficient enzymatic activity. In any case, the lack of biochar effects on sulphate levels in soil solution and on sulphur export by the crop suggests that, at least, sulphur cycling was not impaired by the highest biochar rate.

#### 2.4.4.3. Detritivore feeding activity and microbial decomposition

The bait lamina test, which assesses soil invertebrate feeding activity, showed no significant biochar-mediated effects. Compared with the same mesocosms the first three years following biochar application (Marks et al., 2016), where reduced feeding activity in biochar treatments was noted, ageing of biochar could have reduced such inhibitory effects. For instance, PAH concentration or availability could have been alleviated with time (Hatzinger and Alexander, 1995).

In relation to microbial decomposition, the tea bag index can provide information of residue decomposition of contrasted quality since green tea and rooibos tea differ in their recalcitrance, the former being more labile and the latter with cellulose and lignin enriched contents (Keuskamp et al., 2013). We found increased decomposition rates of rooibos in the 12 t $ha^{-1}$ biochar treatment when compared to the control. This result suggests that biochar could stimulate the degradation of aromatic compounds, since both biochar and rooibos present

recalcitrant carbon structures. This is in line with the results of other studies (Hockaday et al., 2006; Wiednera and Glaser, 2013), which also reported the boost of fungal families more capable of using some recalcitrant biochar fractions. The findings of Wengel et al. (2006) and de la Rosa et al. (2018) further emphasised this suggestion, as they demonstrated aromatic structure degradation by *Schizophyllum commune* (Basidiomycota) and *Fusarium oxysporum* (Ascomycota), respectively, the proposed mechanism being the secretion of exoenzymes like peroxidases and laccases. Moreover, one study conducted with a gasification biochar, as the one in our study, also pointed out similar conclusions, namely, Imparato et al. (2016) found higher activity of phenol oxidase, an enzyme also linked to aromatic C degradation. In addition, other studies have noted an increased abundance of bacterial families able to decompose recalcitrant C compounds (including those of the phylum actinobacteria (Yanardağ et al., 2017) and proteobacteria), such as *Streptosporangiaceae*, *Thermomonosporaceae* and the genus *Rhodopseudomonas* (Anderson et al., 2011). In this regard, and although fungi (mainly white-rot Basidiomycota) are commonly regarded as main recalcitrant C decomposers, bacterial communities could also have played a role. The lack of biochar effects on the F:B ratio does not point to significant alterations of the relative contribution of bacteria and fungi in decomposition, and rather, changes in degradation capabilities might have arisen due to a species composition microbial shift.

However, the lack of significant effects on the highest biochar rate is not straightforward to understand. One explanation could be a top-down regulation by fungivore nematodes on fungal aromatic C decomposers, as in this study a significantly larger fungivore metabolic footprint was found at the highest biochar rate. Another potential mechanism could be a reduced enzymatic activity, as some signs of minor enzymatic activity were found for the 50 t $ha^{-1}$ biochar in contrast with the 12 t $ha^{-1}$. As an example, laccase, an important enzyme in aromatic C decomposition, was found to reduce its activity by 60-99% when was adsorbed to high-temperature biochars (Zimmerman and Gao, 2013).

Further implications of our results include the fate and persistence of biochar in soil, since if biochar-C degradation is found to be true for our biochar, this could imply a reduction in its C sequestration potential (Hockaday et al., 2016). By contrast, a major activity of oxidative enzymes (as phenol oxidase) could, in turn, induce the formation of negative charge and increase biochar's cation exchange capacity, thus resulting in beneficial impacts on nutrient retention (Imparato et al., 2016).

### 2.4.5. Soil food web functioning: microorganisms, nematodes, microarthropods and earthworms

In this study, nematodes (microfauna), microarthropods (mesofauna) and earthworms (macrofauna) appeared to respond negatively to increased biochar rate applications, whereas no significant effect was found for microbial-related parameters. Although biochar is expected to positively impact microbial communities by providing a favourable environment and resources to grow (Pietikäinen et al., 2000), long-term effects on microbial biomass and respiration are less likely to occur, since microbial stimulation effects are often related to the low and transient bioavailable C fractions in biochar (Ameloot et al., 2013; Jones et al., 2021). In addition, changes in pore connectivity and clogging with biochar ageing could diminish habitat suitability for microbes (Sorrenti et al., 2016). Moreover, Marks et al. (2016) also reported limited effects on microbial parameters of this same biochar even at the short-term, probably owing to its high degree of aromaticity.

In the case of nematodes, the lower values of the MI2-5 at the highest biochar addition rate compared to the control indicate a greater level of disturbance in this treatment, with the soil microfaunal community showing a degraded condition compared to the control as found in soils subjected to different types of perturbation (Coutinho et al., 2018; Šalamún et al., 2017). Furthermore, such high-dose biochar treatment showed a reduction in the omnivore footprint and a concomitant increase of the fungivore and herbivore footprints, indicating a loss of functionality of higher soil food web levels while the biomass and diversity of lower guilds are increased. In the higher biochar dose, our results showed that soil food web functions performed by high trophic guilds such as pest suppression (Carrascosa et al., 2014; Steel and Ferris, 2016) could be impaired, while the fungal-derived C and energy flow as well as pest pressure would be enhanced (Ferris, 2010). Biochar effects on nematodes are highly variable, depending on biochar and soil type. Some studies have found no adverse effects of biochar on plant-feeding nematodes (Mondal et al., 2021) while others reported negative effects (Arshad et al., 2020). Indeed, in a previous experiment in similar conditions to this one, low biochar rates (5 tons $ha^{-1}$) favoured predator and herbivore nematodes, and high addition rates (30 t $ha^{-1}$) favoured both bacterivores and fungivores (Domene et al., 2021).

The high levels of PAHs found in the gasification biochar used in this study (438 mg $kg^{-1}$; Llovet et al., 2021), exceed by far those recommended in biochar guidelines (EBC, 2012; IBI, 2015),

and could be at least partially responsible for the low values of both MI2-5 and the omnivore footprint at the highest biochar rate. This is in agreement with the findings of Blakely et al. (2002) who stated that soil nematodes are sensitive indicators of PAH pollution. By contrast, Erstfeld and Snow-Ashbrook (1999) found a positive relationship between PAH concentration and the relative abundance of omnivore/predator nematodes, the most sensitive groups to perturbation (Ferris et al., 2001). Additionally, toxic effects of N species, such as $NH_4^+$, especially on omnivore nematodes, have also been reported in the literature (Tenuta and Ferris, 2004; Wang et al., 2006), and therefore, the increased $NH_4^+$ levels measured at the highest biochar rate could have also contributed to this response. Indeed, $NH_4^+$ concentrations increased in autumn (post-harvest period), when the effects on metabolic footprints were more apparent.

In relation to microarthropod communities, the values of the QBS-ar index were consistent with those observed in agricultural soils, and in most cases, below 93.7, which corresponds to the threshold value proposed by Menta et al. (2018) to differentiate between low biological quality soils and high biological quality soils. While QBS-ar was unaffected by biochar treatment, the highest biochar addition rate (50 t $ha^{-1}$) significantly lowered collembolan LFT values in spring, while in autumn this negative effect was alleviated. Marks et al. (2014) also noted an inhibitory response of collembolans to this same gasification biochar, which was mainly ascribed to an alkalinisation effect. However, in this study, no biochar effects on soil pH were observed and ecotoxicological effects associated to PAH are the most likely responsible mechanism (Marks et al., 2014; Marks et al., 2016).

Similarly, macrofauna also showed negative responses to biochar additions and both earthworm abundance and biomass, decreased at the highest biochar addition rate. These results are in agreement with Briones et al. (2020), who reported significant negative effects of biochar on earthworm abundance and species richness, and with those of Gomez-Eyles et al. (2011) who observed a significant weight loss of earthworms that had been exposed to hardwood biochar.

### 2.4.6 Food production

Biochar has been shown to exert limited effects on crop yield and quality in already fertile soils from temperate regions (Jeffery et al., 2017; Jones et al., 2012). Our results are in agreement with this general trend as no major significant effects were found either in yield or crop quality.

It has to be emphasised that grain yield in the year of this trial (2017) was exceptionally low, ranging from 17.8 to 29.5 g $m^{-2}$, compared to previous years (271 to 353 g $m^{-2}$ in 2012-2013; Marks et al., 2016). These differences could be ascribed to the drier conditions in 2017, which could have in turn synergistically acted with the herbivore nematode pressure at the grain filling period (Dababat and Fourie, 2018).

Regarding the nutrient content of the grain, biochar significantly decreased Zn concentration at the highest application rate and similar (but non-significant) trends were found for other nutrients and protein contents. Although biochar has the potential to immobilise Zn (Beesley and Marmiroli, 2011), it seems unlikely in this study, where total (grain plus straw) Zn export tended to be greater at the highest biochar application rate. Since the 50 t $ha^{-1}$ treatment had the highest herbivore metabolic footprint, a greater herbivore nematode pressure during the grain fill period might have also been responsible for the barley lowered capacity to adsorb water and nutrients as a result of root damage due to plant parasitic nematode infection (Santana-Gomes et al., 2013). In agreement with this, Thompson et al. (2008) observed that Zn levels in wheat were also lowered when plants were infected by the herbivore nematode of the genus *Pratylenchus*. While lowered grain nutrient content should be of high concern if barley was intended for human or cattle consumption, malting barley poses different requirements. Interestingly, the optimum protein content for European malting barley is 10.7%, with acceptable range values between 9.5 and 11.5% (Söderström et al., 2010). According to this, the highest biochar rate showed the most optimal values (10.6 %), while the control was outside the range (11.9%) and the 12 t $ha^{-1}$ treatment was slightly less optimal than the highest biochar rate (11.1%).

### 2.4.7. Biochar impacts on soil quality index

The soil quality index showed that no biochar rate (0, 12 or 50 t $ha^{-1}$) significantly outcompeted the others. Although the 50 t $ha^{-1}$ treatment significantly stored more carbon, a trade-off emerged at this application rate, as it also posed risks for soil faunal communities. Specifically, the MI2-5 was reduced at this treatment indicating a more simplified nematode trophic web and a higher risk of pest outbreak (Sánchez-Moreno and Ferris, 2018), as evidenced by significant increases in the herbivore metabolic footprint. Since this index could also potentially indicate pollution-related disturbances (Korthals et al., 1996), ecotoxicological assessments should be performed before applying biochar to the field, to ensure its innocuousness. As stated earlier,

PAHs are the most frequently occurring contaminants in biochar but their bioavailability is usually very low (Godlewska et al., 2021), so further research is needed to determine PAH availability to soil fauna as well as of other potential pollutants in a wider range of soil types and environmental conditions.

Despite the higher water retention at some sampling events and enhanced $CH_4$ emission as a result of amending the soils with the highest biochar rate, they contributed little to the soil quality index since such differences were probably diluted when transformed to indicator values as a result of pooling sampling dates and summing the effect of all the GHGs as $CO_2$-eq, respectively.

In the case of the nutrient cycling function, it is important to mention that although slight increases in inorganic nitrogen content were measured at the highest biochar rate, they did not exceed the upper limits indicated in the literature (i.e., 40 mg $kg^{-1}$ $N\text{-}NO_3^-$) and therefore, it is unlikely that they could represent a threat to aquifer pollution in our system. However, some other environmental risks might arise from increased ammonium contents such as the inhibition of methanotrophs (thus enhancing $CH_4$ emissions), or toxic effects to beneficial soil fauna, such as omnivore nematodes, which are highly sensitive to nitrogenous species (Tenuta and Ferris, 2004; Wang et al., 2006).

Finally, regarding the food production function, the lack of significant effects in this study is not surprising. Although biochar amendment has been described as a win–win strategy as it could increase both soil C sequestration and crop yield (Laird, 2008), there can also be trade-offs between these two soil functions (Jeffery et al., 2015) since the high recalcitrance (high aromaticity) and lower capacity to retain nutrients (low surface functionality) of gasification biochars (You et al., 2017) make these materials suitable to optimise C sequestration but less accessible to plants.

## 2.5. Conclusions

Biochar long-term impacts on soil functions followed the predictions for a highly aromatic biochar. While C sequestration was increasingly promoted with biochar rate, crop production and microbial endpoints such as biomass and respiration were unaffected by biochar. However, biochar did induce some rate-dependent effects upon specific microbial communities. Namely,

increased $CH_4$ emissions at the highest biochar rate (50 t $ha^{-1}$) in contrast with the medium biochar rate (12 t $ha^{-1}$) evidenced methanotrophic and/or methanogenic communities' activity shifts. In addition, stimulation of lignin-enriched materials decomposition by microbes was observed at the medium biochar rate. Also consistent with our initial hypotheses, the highest application rate caused significant adverse effects on soil nematode and microarthropod communities, which could be due to the high PAH content of this biochar, however, the lack of information about its availability precludes the full interpretation of the causal factors and further research is needed. Ageing of biochar in soil should also be taken into account when considering biochar impacts on soils. In this direction, it was noted that the soil solution nitrate alleviation found for this same biochar in the short-term disappeared after 6 years of its application, possibly, as a result of pore clogging or changes in N dynamics with biochar exposure to the soil environment. Similarly, biochar's water retentive properties seem to have weakened over time.

When all soil functions were integrated into a soil quality index, no biochar addition rate resulted more beneficial. Nevertheless, the highest biochar rate seems to have exceeded the biochar loading capacity, i.e., it compromised the soil food web functioning, which could, in turn, have effects on other important functions as it regulates pest suppressiveness, nutrient cycling and thus, indirectly food production. Furthermore, the highest biochar rate could also represent a source of methane. In this sense, in our system, the 12 t $ha^{-1}$ rate would be safer to apply, although sequestering less carbon than the 50 t $ha^{-1}$. The ultimate selection of what biochar addition rate to add in soil will depend on the target management of the field and the local soil conditions.

## 2.6. Acknowledgments

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*Supplementary Material Chapter 2*

**Table S1**. Sampling schedule specified for each measured parameter. Two categories of sampling frequencies are distinguished: punctual sampling dates (coded as "Sampling date"), which all occurred during 2017; and over a certain period covering several months (coded as "Sampling period"), covering both 2017 and the beginning of 2018.

| **Parameter** | **Sampling date** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1·III | 17·III | 29·III | 30·III | 18·IV | 19·IV | 7·V | 31·V | 22·VI | 5·IX | 22·XI |
| Organic carbon | | | × | | × | | | × | | × | × |
| Gravimetric water | | | × | | × | | | × | | × | × |
| GHG emissions | | | | × | | × | | × | | × | × |
| Ionic content | | | × | | × | | | × | | × | × |
| pH | | | × | | × | | | × | | × | × |
| EC | | | × | | × | | | × | | × | × |
| Enzymes | | | × | | × | | | × | | | × |
| N pools[a] | | | × | | × | | | × | | × | × |
| Microbial parameters[b] | | | × | | × | | | × | | × | × |
| F:B ratio | | | × | | × | | | × | | | × |
| Microarthropods | | | | | | | | × | | | × |
| Nematodes | | | | | | | | × | | | × |
| Earthworms | × | | | | | | | | | | |
| Germination | | × | | | | | | | | | |
| Tillering | | | | | | | × | | | | |
| Crop yield and nutrient export | | | | | | | | | × | | |

| | **Sampling period** | | | |
|---|---|---|---|---|
| | 8·III·2017 - 7·VI·2017 | 2·X·2017 - 5·I·2018 | 2·VI·2017 - 22·VI·2017 | 22·XI·2017 - 5·I·2018 |
| Tea bag method | × | × | | |
| Bait lamina | | | × | × |

[a] N pools include exchangeable N-$NH_4^+$, total Kjeldahl nitrogen, microbial nitrogen, and extractable organic nitrogen.

[b] Microbial parameters include microbial biomass, respiration, and metabolic quotient

**Table S2.** Primers and thermal profiles used for real-time PCR quantification of the different target genes.

| Target gene | Primers | Thermal profile | Number of cycles | Reference |
|---|---|---|---|---|
| *16S rRNA gene* | EUB338<br>EUB518 | 98 °C – 45 s/ 55 °C – 45 s/ 72 °C – 45 s | 40 | Fierer et al., 2005 |
| *18S r RNA gene* | 5.8s ITS1f | 98°C, 45 s / 53°C, 45 s / 72°C, 45 s | 40 | Fierer et al., 2005 |

**Reference**

Fierer N., Jackson J.A., Vilgalys R., Jackson R.B., 2005. Assessment of soil microbial community structure by use of taxon-specific quantitative PCR Assays. Appl. Environ. Microbiol. 7: 4117–4120. https://doi.org/10.1128/AEM.71.7.4117-4120.2005.

**Table S3.** Summary of the results of two-way mixed ANOVAs on different variables, with treatment (biochar application rate) as between-subjects factor, and time (sampling dates) as within-subjects factor. Degrees of freedom (df) are shown as: (degrees of freedom numerator, degrees of freedom denominator); the effect size is reported as generalised eta squared ($\eta_G^2$), and significant p-values ($p < 0.05$) are marked in bold.

**Table S3.1.** Soil organic carbon ($C_{org}$)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 59.99 | **< .001** | 0.83 |
| time | (1.99, 29.91) | 9.78 | **< .001** | 0.21 |
| treatment x time | (3.99, 29.91) | 5.46 | **0.002** | 0.23 |

**Table S3.2.** Moisture

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.85 | 0.449 | 0.07 |
| time | (4, 60) | 251.67 | **< .001** | 0.86 |
| treatment x time | (8, 60) | 6.88 | **< .001** | 0.25 |

**Table S3.3.** $CO_2$ equivalents

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 4) | 0.39 | 0.701 | 0.04 |
| time | (1.10, 4.41) | 19.52 | **0.009** | 0.79 |
| treatment x time | (2.21, 4.41) | 0.44 | 0.687 | 0.14 |

**Table S3.4.** N-$N_2O$ exchange rate

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 4) | 1.55 | 0.318 | 0.07 |
| time | (4, 16) | 4.96 | **0.009** | 0.53 |
| treatment x time | (8, 16) | 0.88 | 0.553 | 0.29 |

**Table S3.5.** C-$CO_2$ exchange rate

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 4) | 0.41 | 0.691 | 0.05 |
| time | (1.09, 4.35) | 19.63 | **0.009** | 0.79 |
| treatment x time | (2.17, 4.35) | 0.43 | 0.691 | 0.14 |

**Table S3.6.** C-$CH_4$ exchange rate (field mesocosms)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 4) | 10.87 | **0.024** | 0.20 |
| time | (4, 16) | 0.93 | 0.471 | 0.18 |
| treatment x time | (8, 16) | 1.25 | 0.335 | 0.37 |

**Table S3.7.** C-$CH_4$ exchange rate in fresh and aged lysimeters (unpublished results from the study of Llovet et al. (2021))

| | Fresh | | | | Aged | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F** | **p** | $\eta_G^2$ | **df** | **F** | **p** | $\eta_G^2$ |
| treatment | (2, 2) | 39.40 | **0.025** | 0.69 | (2, 2) | 0.82 | 0.551 | 0.30 |
| time | (4, 8) | 2.38 | 0.138 | 0.53 | (4, 8) | 11.43 | **0.002** | 0.74 |
| treatment x time | (8, 8) | 3.09 | 0.066 | 0.75 | (8, 8) | 11.61 | **0.001** | 0.85 |

**Table S3.8.** N-$NO_3^-$

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.07 | 0.929 | 0.00 |
| time | (4, 60) | 61.36 | **< .001** | 0.71 |
| treatment x time | (8, 60) | 0.53 | 0.829 | 0.04 |

**Table S3.9.** N-$NH_4^+$ (soluble)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 3.91 | **0.043** | 0.10 |
| time | (1.95, 29.26) | 318.57 | **< .001** | 0.94 |
| treatment x time | (3.90, 29.26) | 1.78 | 0.161 | 0.16 |

**Table S3.10.** $N\text{-}NO_2^-$

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 1.04 | 0.379 | 0.03 |
| time | (2.63, 39.38) | 11.66 | **< .001** | 0.38 |
| treatment x time | (5.25, 39.38) | 1.00 | 0.436 | 0.10 |

**Table S3.11.** $Na^+$

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.09 | 0.918 | 0.00 |
| time | (3.03, 45.48) | 43.16 | **< .001** | 0.69 |
| treatment x time | (6.06, 45.48) | 1.61 | 0.165 | 0.13 |

**Table S3.12.** $Cl^-$

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.84 | 0.450 | 0.02 |
| time | (4, 60) | 39.35 | **< .001** | 0.69 |
| treatment x time | (8, 60) | 0.76 | 0.638 | 0.08 |

**Table S3.13.** $K^+$

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 1.12 | 0.351 | 0.05 |
| time | (2.51, 37.69) | 19.71 | **< .001** | 0.47 |
| treatment x time | (5.03, 37.69) | 0.98 | 0.443 | 0.08 |

**Table S3.14.** $Ca^{2+}$

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.13 | 0.875 | 0.01 |
| time | (4, 60) | 118.93 | **< .001** | 0.84 |
| treatment x time | (8, 60) | 1.48 | 0.182 | 0.12 |

**Table S3.15.** $Mg^{2+}$

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.02 | 0.982 | 0.00 |
| time | (2.05, 30.68) | 22.24 | **< .001** | 0.49 |
| treatment x time | (4.09, 30.68) | 3.21 | **0.025** | 0.21 |

**Table S3.16.** $SO_4^{2-}$

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.37 | 0.696 | 0.01 |
| time | (4, 60) | 69.68 | **< .001** | 0.78 |
| treatment x time | (8, 60) | 2.05 | 0.055 | 0.17 |

**Table S3.17.** pH

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.09 | 0.911 | 0.00 |
| time | (2.38, 35.73) | 12.37 | **< .001** | 0.41 |
| treatment x time | (4.76, 35.73) | 1.09 | 0.381 | 0.11 |

**Table S3.18.** Electrical conductivity (EC)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.08 | 0.924 | 0.00 |
| time | (4, 60) | 52.68 | **< .001** | 0.74 |
| treatment x time | (8, 60) | 1.12 | 0.363 | 0.11 |

**Table S3.19.** Soil total Kjeldahl nitrogen (TKN)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.60 | 0.561 | 0.07 |
| time | (4, 60) | 50.92 | **< .001** | 0.23 |
| treatment x time | (8, 60) | 1.43 | 0.204 | 0.02 |

**Table S3.20.** Exchangeable N-$NH_4^+$ (N-$NH_4^+$ in KCl extracts – N-$NH_4^+$ in water extracts)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 1.16 | 0.342 | 0.04 |
| time | (1.02, 15.23) | 390.75 | **< .001** | 0.95 |
| treatment x time | (2.03, 15.23) | 0.98 | 0.400 | 0.09 |

**Table S3.21.** Microbial biomass nitrogen ($N_{mic}$)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.54 | 0.594 | 0.02 |
| time | (4, 60) | 94.51 | **< .001** | 0.81 |
| treatment x time | (8, 60) | 0.94 | 0.489 | 0.08 |

**Table S3.22.** Extractable organic nitrogen (EON)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.45 | 0.648 | 0.02 |
| time | (4, 60) | 31.57 | **< .001** | 0.58 |
| treatment x time | (8, 60) | 0.34 | 0.947 | 0.03 |

**Table S3.23.** Dehydrogenase

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 4.20 | **0.036** | 0.14 |
| time | (3, 45) | 72.55 | **< .001** | 0.78 |
| treatment x time | (6, 45) | 0.78 | 0.589 | 0.07 |

**Table S3.24.** Phosphomonoesterase

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.59 | 0.568 | 0.02 |
| time | (3, 45) | 127.87 | **< .001** | 0.86 |
| treatment x time | (6, 45) | 5.65 | **< .001** | 0.34 |

**Table S3.25.** Phosphodiesterase

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.40 | 0.679 | 0.01 |
| time | (1.38, 20.65) | 1.58 | 0.229 | 0.07 |
| treatment x time | (2.75, 20.65) | 2.90 | 0.063 | 0.22 |

**Table S3.26.** β-glucosidase

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.27 | 0.771 | 0.00 |
| time | (2.3, 34.51) | 77.89 | **< .001** | 0.82 |
| treatment x time | (4.6, 34.51) | 4.08 | **0.006** | 0.32 |

**Table S3.27.** Arylsulphatase

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 10.17 | **0.002** | 0.29 |
| time | (3, 45) | 26.59 | **< .001** | 0.55 |
| treatment x time | (6, 45) | 6.53 | **< .001** | 0.38 |

**Table S3.28.** Urease

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.25 | 0.779 | 0.01 |
| time | (1.42, 21.32) | 159.14 | **< .001** | 0.88 |
| treatment x time | (2.84, 21.32) | 0.87 | 0.465 | 0.08 |

**Table S3.29.** Green tea mass loss

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 1.93 | 0.179 | 0.14 |
| time | (1, 15) | 318.22 | **< .001** | 0.89 |
| treatment x time | (2, 15) | 0.174 | 0.842 | 0.01 |

**Table S3.30.** Rooibos mass loss

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 14) | 6.64 | **0.009** | 0.31 |
| time | (1, 14) | 53.01 | **< .001** | 0.66 |
| treatment x time | (2, 14) | 1.18 | 0.336 | 0.08 |

**Table S3.31.** Bait lamina (total: 0-8 cm)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 14) | 1.11 | 0.357 | 0.10 |
| time | (1, 14) | 4.28 | 0.057 | 0.08 |
| treatment x time | (2, 14) | 0.61 | 0.557 | 0.02 |

**Table S3.32.** Bait lamina (0-3 cm)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 14) | 0.09 | 0.912 | 0.01 |
| time | (1, 14) | 8.57 | **0.011** | 0.21 |
| treatment x time | (2, 14) | 0.09 | 0.916 | 0.01 |

**Table S3.33.** Bait lamina (3-6 cm)

| Source | df | F | P | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 14) | 1.38 | 0.283 | 0.13 |
| time | (1, 14) | 5.11 | **0.040** | 0.08 |
| treatment x time | (2, 14) | 1.39 | 0.281 | 0.04 |

**Table S3.34.** Bait lamina (6-8 cm)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 14) | 2.33 | 0.133 | 0.19 |
| time | (1, 14) | 0.16 | 0.700 | 0.00 |
| treatment x time | (2, 14) | 0.95 | 0.411 | 0.04 |

**Table S3.35.** Microbial biomass carbon ($C_{mic}$)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 1.29 | 0.304 | 0.03 |
| time | (4, 60) | 10.63 | **< .001** | 0.36 |
| treatment x time | (8, 60) | 0.76 | 0.642 | 0.08 |

**Table S3.36.** Extractable organic carbon (EOC)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.42 | 0.663 | 0.02 |
| time | (4, 60) | 60.24 | **< .001** | 0.723 |
| treatment x time | (8, 60) | 1.30 | 0.262 | 0.101 |

**Table S3.37.** Soil basal respiration (BAS)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 2.50 | 0.116 | 0.06 |
| time | (2.00, 30.06) | 55.82 | **< .001** | 0.75 |
| treatment x time | (4.01, 30.06) | 1.21 | 0.326 | 0.12 |

**Table S3.38.** $qCO_2$

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.69 | 0.519 | 0.01 |
| time | (2.46, 36.96) | 14.35 | **< .001** | 0.46 |
| treatment x time | (4.93, 36.96) | 0.57 | 0.717 | 0.06 |

**Table S3.39.** Bacterial 16S rRNA gene copy number

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 13) | 1.39 | 0.283 | 0.06 |
| time | (1.52, 19.79) | 2.09 | 0.158 | 0.10 |
| treatment x time | (3.04, 19.79) | 0.22 | 0.887 | 0.02 |

**Table S3.40.** Fungal 18S rRNA gene copy number

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 9) | 0.85 | 0.459 | 0.06 |
| time | (3, 27) | 6.87 | **0.001** | 0.33 |
| treatment x time | (6, 27) | 2.14 | 0.082 | 0.24 |

**Table S3.41.** Ratio F:B

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 8) | 0.70 | 0.523 | 0.05 |
| time | (3, 24) | 0.37 | 0.779 | 0.03 |
| treatment x time | (6, 24) | 0.66 | 0.680 | 0.10 |

**Table S3.42.** MI2-5 index

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 5.89 | **0.013** | 0.33 |
| time | (1, 15) | 12.59 | **0.03** | 0.24 |
| treatment x time | (2, 15) | 2.15 | 0.15 | 0.10 |

**Table S3.43.** Fungivore metabolic footprint

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 7.54 | **0.005** | 0.42 |
| time | (1, 15) | 5.42 | **0.034** | 0.09 |
| treatment x time | (2, 15) | 1.32 | 0.296 | 0.05 |

**Table S3.44.** Herbivore metabolic footprint

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 8.92 | **0.003** | 0.36 |
| time | (1, 15) | 0.02 | 0.880 | 0.00 |
| treatment x time | (2, 15) | 0.52 | 0.604 | 0.04 |

**Table S3.45.** Bacterivore metabolic footprint

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.21 | 0.812 | 0.02 |
| time | (1, 15) | 9.21 | **0.008** | 0.11 |
| treatment x time | (2, 15) | 1.59 | 0.236 | 0.04 |

**Table S3.46.** Omnivore metabolic footprint

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 5.92 | **0.013** | 0.30 |
| time | (1, 15) | 0.76 | 0.398 | 0.02 |
| treatment x time | (2, 15) | 1.45 | 0.266 | 0.08 |

**Table S3.47.** Predator metabolic footprint

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.37 | 0.697 | 0.05 |
| time | (1, 15) | 9.95 | **0.007** | 0.40 |
| treatment x time | (2, 15) | 0.37 | 0.697 | 0.05 |

**Table S3.48.** Nematode total abundance

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 4.86 | **0.024** | 0.31 |
| time | (1, 15) | 4.65 | **0.048** | 0.09 |
| treatment x time | (2, 15) | 0.73 | 0.500 | 0.03 |

**Table S3.49.** Fungivore abundance

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 6.33 | **0.010** | 0.38 |
| time | (1, 15) | 7.99 | **0.013** | 0.13 |
| treatment x time | (2, 15) | 1.20 | 0.330 | 0.04 |

**Table S3.50.** Herbivore abundance

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 11.51 | **< .001** | 0.50 |
| time | (1, 15) | 1.32 | 0.268 | 0.03 |
| treatment x time | (2, 15) | 0.67 | 0.523 | 0.03 |

**Table S3.51.** Bacterivore abundance

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.07 | 0.935 | 0.01 |
| time | (1, 15) | 21.35 | **< .001** | 0.34 |
| treatment x time | (2, 15) | 0.53 | 0.600 | 0.03 |

**Table S3.52.** Omnivore abundance

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 6.28 | **0.010** | 0.33 |
| time | (1, 15) | 1.14 | 0.252 | 0.04 |
| treatment x time | (2, 15) | 0.51 | 0.609 | 0.03 |

**Table S3.53.** Predator abundance

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.48 | 0.631 | 0.06 |
| time | (1, 15) | 9.48 | **0.008** | 0.39 |
| treatment x time | (2, 15) | 0.48 | 0.631 | 0.06 |

**Table S3.54.** QBS-ar index

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (2, 15) | 0.54 | 0.592 | 0.02 |
| time | (1, 15) | 0.06 | 0.806 | 0.00 |
| treatment x time | (2, 15) | 1.20 | 0.328 | 0.10 |

**Table S4.** Maturity index 2-5 (MI2-5), total nematode abundance, and abundance per trophic guild (bacterivores, fungivores, herbivores, omnivores and predators) (individuals/ 100 g dry soil) at the spring and autumn samplings as well as the mean values of the two seasons (total average). Reported values are mean ± standard error. Different letters indicate statistically significant differences between treatments according to ANOVA and Kruskal-Wallis tests (seasonal results) and according mixed ANOVA (total average).

| | **Spring** (n = 6) | | | **Autumn** (n = 6) | | | **Total average** (n = 12) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 t $ha^{-1}$ | 12 t $ha^{-1}$ | 50 t $ha^{-1}$ | 0 t $ha^{-1}$ | 12 t $ha^{-1}$ | 50 t $ha^{-1}$ | 0 t $ha^{-1}$ | 12 t $ha^{-1}$ | 50 t $ha^{-1}$ |
| MI2-5 | 2.33 ± 0.1 **a** | 2.26 ± 0.0 **ab** | 2.12 ± 0.0 **b** | 2.74 ± 0.2 **a** | 2.62 ± 0.2 **ab** | 2.17 ± 0.0 **b** | 2.54 ± 0.1 **a** | 2.44 ± 0.1 **ab** | 2.14 ± 0.0 **b** |
| Total abundance | 945.9 ± 128 | 1104.7 ± 200 | 1936.2 ± 611 | 856.3 ± 65.9 **ab** | 729.3 ± 99.3 **a** | 1329.1 ± 164 **b** | 901.1 ± 70.4 **a** | 917.0 ± 120 **a** | 1632.6 ± 315 **b** |
| Bacterivores | 374.6 ± 98.9 | 467.1 ± 139 | 434.5 ± 89.6 | 163.9 ± 36.4 | 109.0 ± 20.4 | 175.6 ± 75.2 | 269.2 ± 59.4 | 288.0 ± 86.3 | 305.0 ± 68.1 |
| Fungivores | 53.38 ± 24.4 | 101.9 ± 31.6 | 344.0 ± 246 | 25.88 ± 9.9 **a** | 35.00 ± 17.1 **a** | 156.9 ± 46.9 **b** | 39.63 ± 13.2 **a** | 68.47 ± 19.9 **ab** | 250.41 ± 123 **b** |
| Herbivores | 456.5 ± 55.0 **a** | 470.2 ± 54.4 **a** | 1125.5 ± 287 **b** | 586.5 ± 70.7 **ab** | 542.9 ± 81.0 **a** | 978.9 ± 91.5 **b** | 521.5 ± 47.0 **a** | 506.5 ± 47.8 **a** | 1052.2 ± 145 **b** |
| Omnivores | 61.47 ± 13.3 | 65.48 ± 22.9 | 32.25 ± 10.8 | 74.80 ± 26.3 | 38.02 ± 5.5 | 15.51 ± 6.1 | 68.14 ± 14.2 **a** | 51.75 ± 12.0 **a** | 23.88 ± 6.4 **b** |
| Predators | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 5.22 ± 2.7 | 4.47 ± 1.7 | 2.25 ± 2.3 | 2.61 ± 1.5 | 2.24 ± 1.1 | 1.13 ± 1.1 |

**Table S5.** Nutrient export in grain and straw of harvested barley. Reported values are mean ± standard errors (n = 6). The absence of letters indicates that the observed differences were not significant.

| | **Grain** | | | **Straw** | | |
|---|---|---|---|---|---|---|
| | 0 t $ha^{-1}$ | 12 t $ha^{-1}$ | 50 t $ha^{-1}$ | 0 t $ha^{-1}$ | 12 t $ha^{-1}$ | 50 t $ha^{-1}$ |
| N (g) | 54.47 ± 14.9 | 32.28 ± 6.7 | 36.33 ± 12.9 | 120.57 ± 10.5 | 116.97 ± 10.5 | 139.97 ± 6.1 |
| P (g) | 19.01 ± 5.5 | 10.41 ± 2.0 | 12.19 ± 4.2 | 23.29 ± 3.2 | 25.27 ± 1.4 | 30.02 ± 1.6 |
| K (g) | 32.46 ± 9.7 | 18.93 ± 3.7 | 21.24 ± 7.3 | 158.12 ± 23.5 | 193.64 ± 12.1 | 230.08 ± 19.7 |
| S (g) | 1.02 ± 0.3 | 0.64 ± 0.1 | 0.72 ± 0.3 | 18.26 ± 2.6 | 23.31 ± 2.0 | 26.83 ± 2.8 |
| Ca (g) | 2.39 ± 0.7 | 1.58 ± 0.5 | 1.91 ± 0.7 | 48.83 ± 6.0 | 59.94 ± 4.8 | 66.01 ± 5.6 |
| Mg (g) | 4.21 ± 1.3 | 2.35 ± 0.5 | 2.70 ± 0.9 | 12.90 ± 2.0 | 15.66 ± 0.9 | 18.05 ± 1.5 |
| Fe (mg) | 39.53 ± 13.8 | 25.59 ± 9.3 | 27.93 ± 12.7 | 222.37 ± 25.7 | 231.06 ± 8.5 | 216.63 ± 19.2 |
| Mn (mg) | 4.85 ± 1.5 | 2.68 ± 0.5 | 3.27 ± 1.1 | 27.00 ± 3.4 | 33.44 ± 3.9 | 38.10 ± 4.0 |
| Zn (mg) | 15.84 ± 4.4 | 9.17 ± 1.8 | 10.48 ± 3.6 | 42.81 ± 4.6 | 47.14 ± 2.1 | 53.48 ± 3.7 |

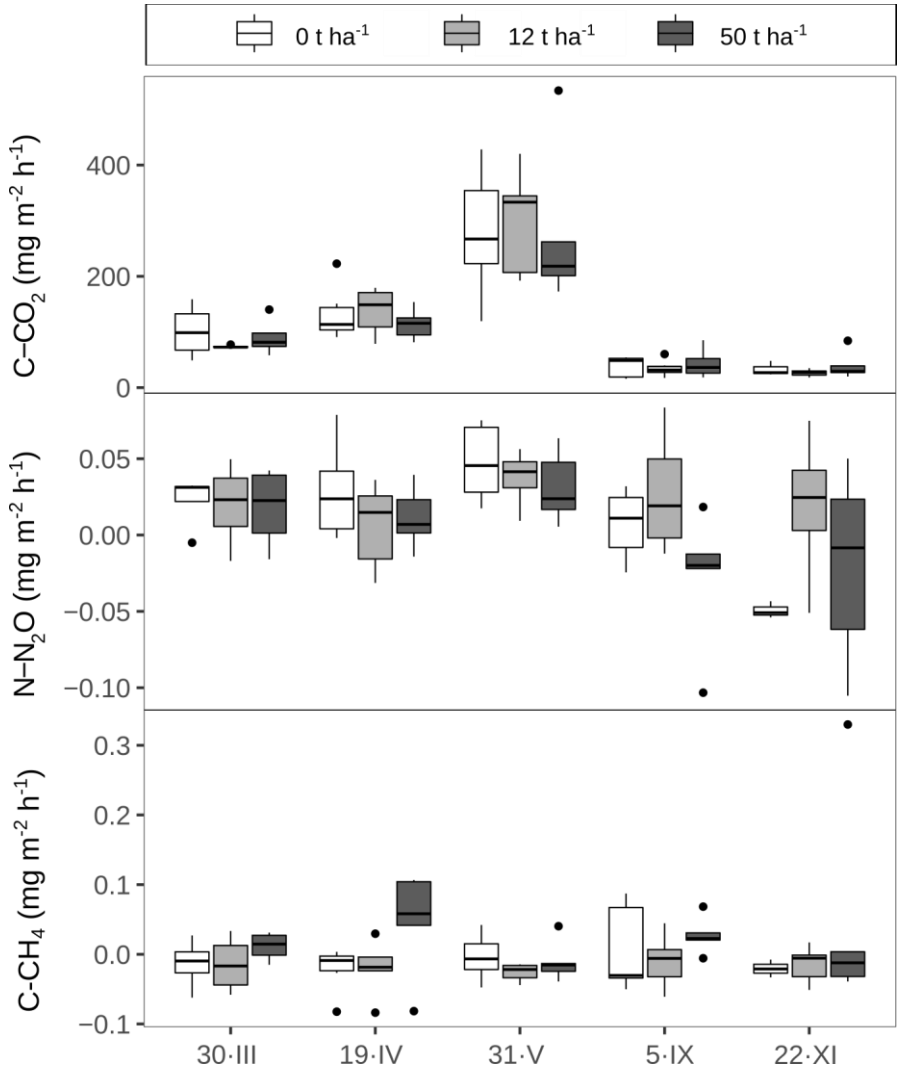


**Fig. S1.** Exchange rates of N-$N_2O$, C-$CO_2$, and C-$CH_4$ (mg $m^{-2}$ $h^{-1}$) measured along five different samplings (30·III = 30th March; 19·IV = 19th April; 31·V = 31st April; 5·IX = 5th September and 22·XI = 22nd November), n is ≤ 6 (see 2.4.3. section in methodology). The absence of letters indicates that the pairwise contrasts were not significant.

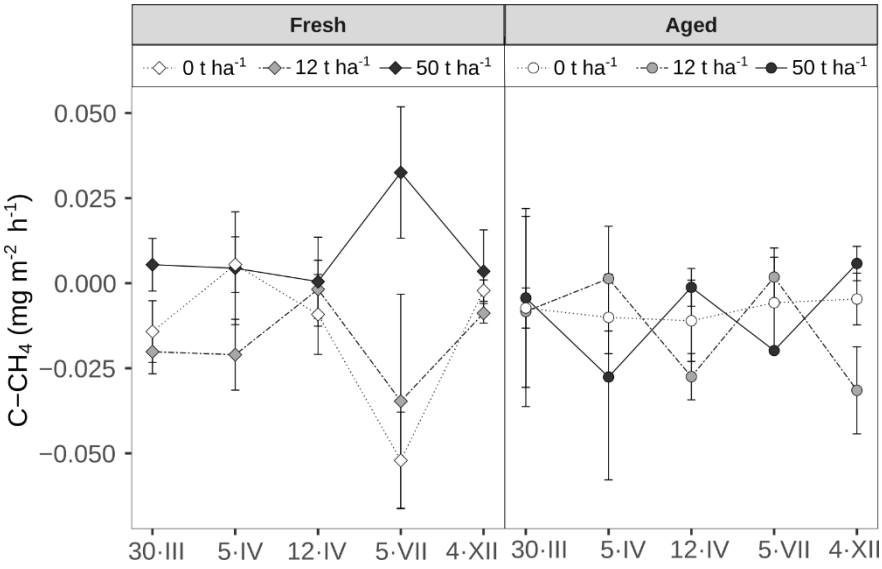


**Fig. S2.** Exchange rates of C-$CH_4$ (mg $m^{-2}$ $h^{-1}$) measured in the experiment described in Llovet et al. (2021), (unpublished data). $CH_4$ sampling was carried out as in Llovet et al. (2021) together with $CO_2$ and $N_2O$. Gas samples were collected along five different samplings (30·III = 30th March; 5·IV = 5th April; 12·IV = 12th April; 5·VII = 5th July and 4·XII = 4th December), and analysed with a flame ionisation detector (FID) for $CH_4$ quantification. Symbols represent the mean values, and bars represent the corresponding standard error ($n \leq 6$). The absence of letters indicates that the pairwise contrasts were not significant.

## Reference

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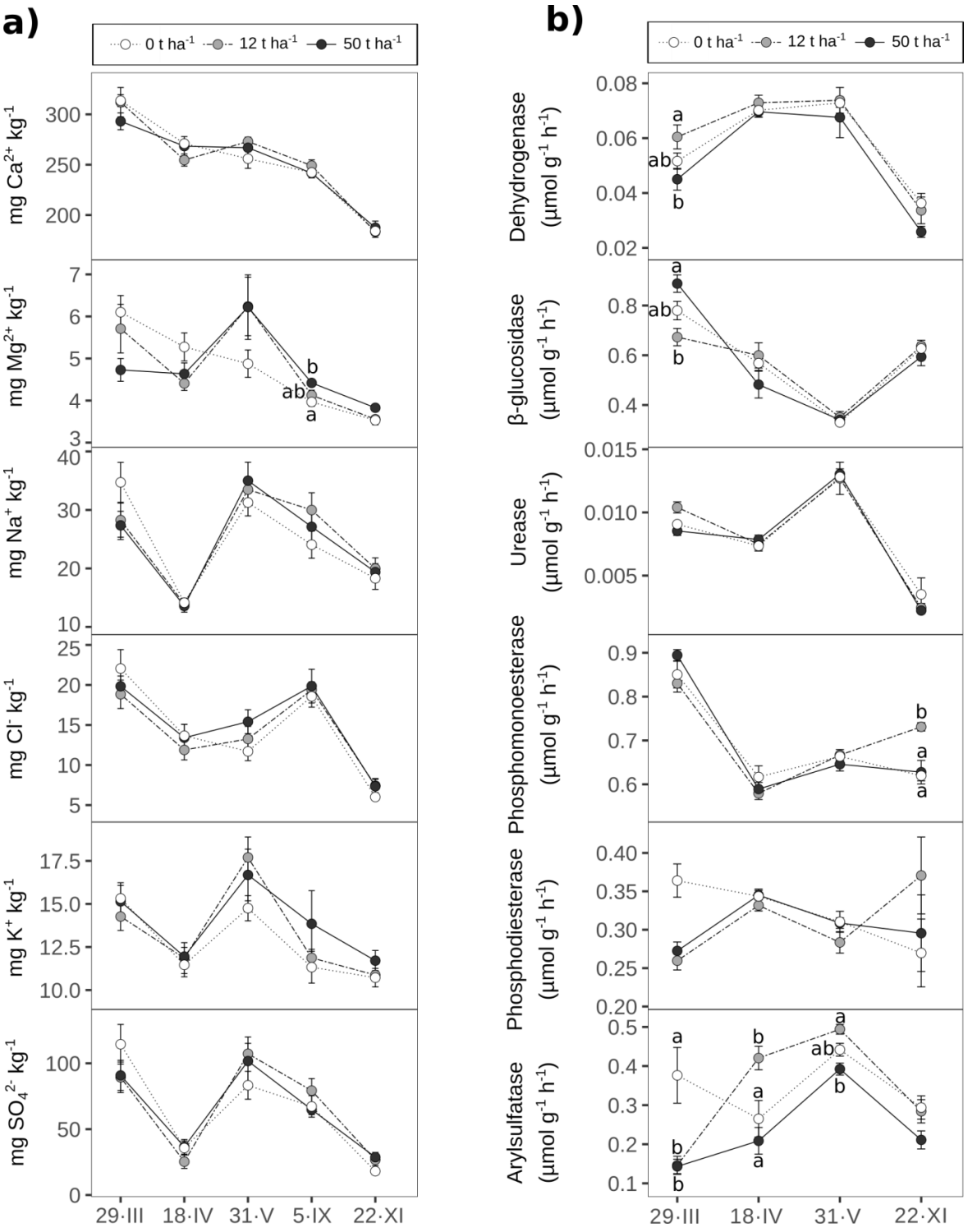


**Fig. S3.** Ionic concentrations (mg $kg^{-1}$ dry soil) (a) and enzymatic activities (µmol $g^{-1}$ dry soil $h^{-1}$) (b). Ions were monitored along five different samplings (29·III = 29th March; 18·IV = 18th April; 31·V = 31st April; 5·IX = 5th September and 22·XI = 22nd November) while enzymes along four samplings (29·III = 29th March; 18·IV = 18th April; 31·V = 31st April and 22·XI = 22nd November). Symbols represent the mean values, and bars represent the corresponding standard error (n = 6). Different letters indicate statistically significant differences between treatments within a particular sampling.

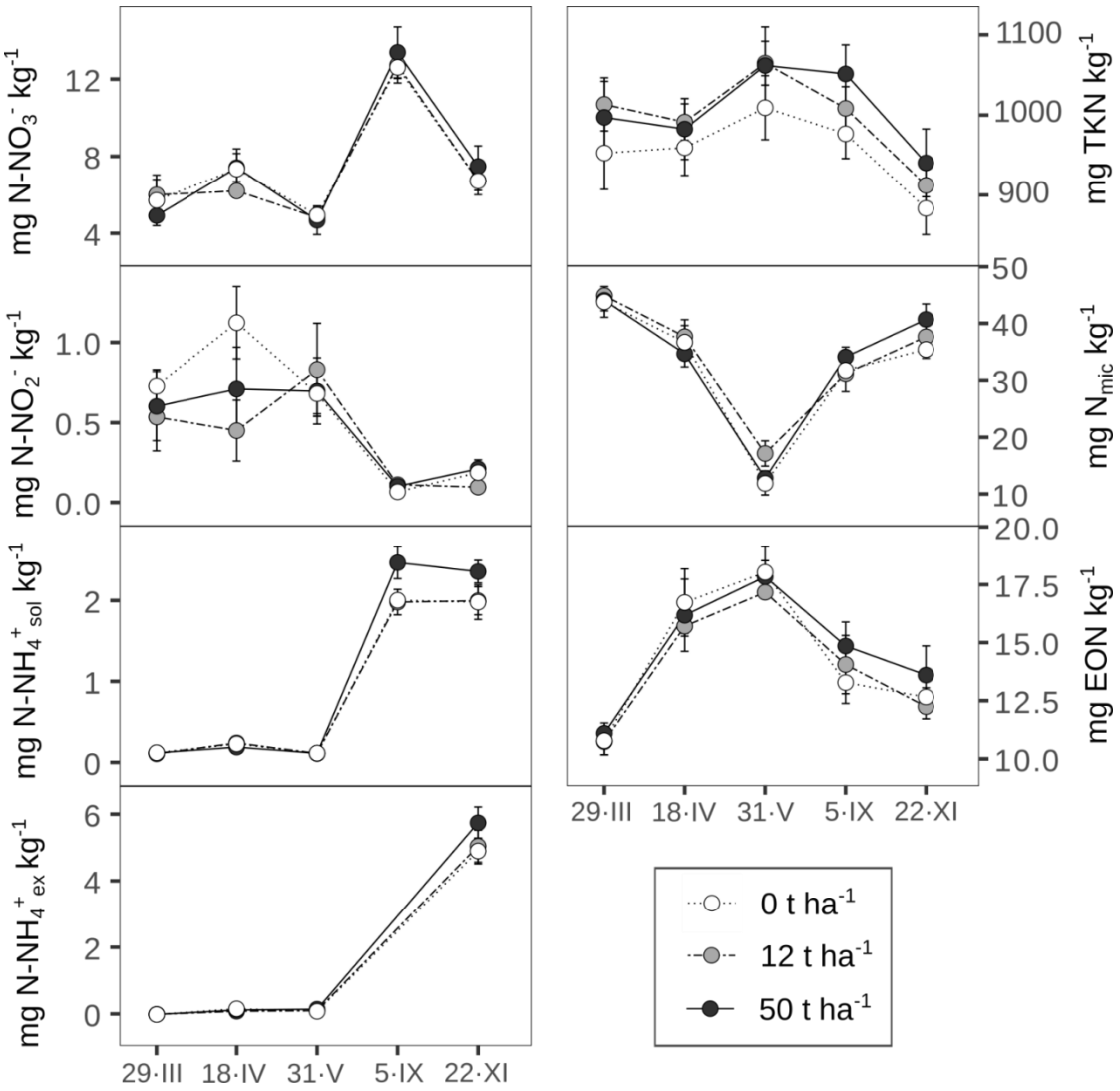


**Fig. S4.** Soluble ionic content of $N\text{-}NO_3^-$, $N\text{-}NO_2^-$ and $N\text{-}NH_4^+$, exchangeable $N\text{-}NH_4^+$, total Kjeldahl nitrogen (TKN), microbial nitrogen ($N_{mic}$), and extractable organic nitrogen (EON) (all in mg $kg^{-1}$ dry soil) along five different samplings (29·III = 29th March; 18·IV = 18th April; 31·V = 31st April; 5·IX = 5th September and 22·XI = 22nd November). Symbols represent the mean values, and bars represent the corresponding standard error (n = 6). The absence of letters indicates that the observed differences within different samplings were not significant.

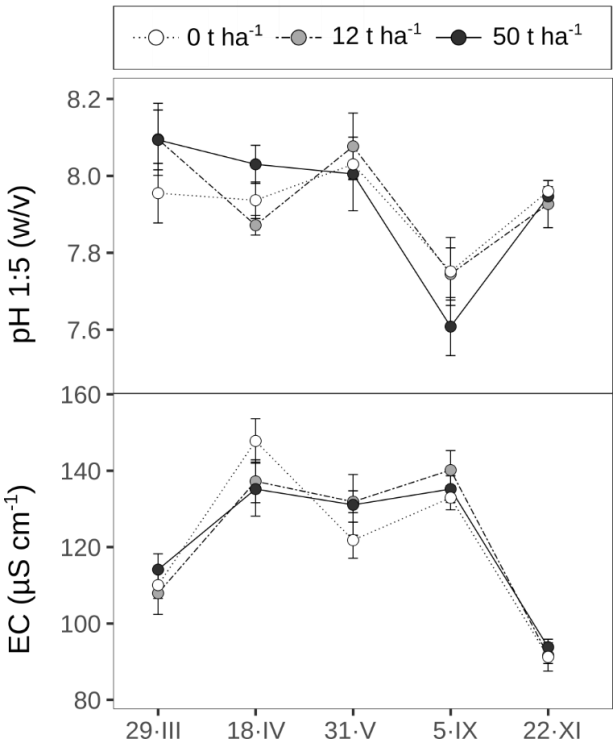


**Fig. S5.** pH 1:5 (w/v) and EC (µS $cm^{-1}$) along five different samplings (29·III = 29th March; 18·IV = 18th April; 31·V = 31st April; 5·IX = 5th September and 22·XI = 22nd November). Symbols represent the mean values, and bars represent the corresponding standard error (n = 6). The absence of letters indicates that the observed differences were not significant.

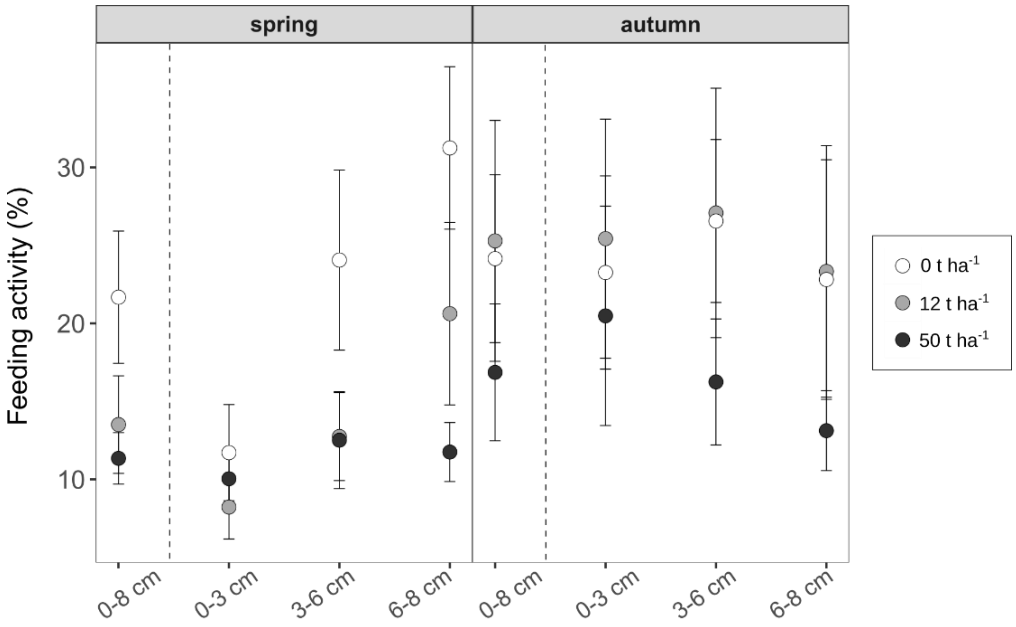


**Fig. S6.** Fauna feeding activity (percent bait lamina holes eaten) at the spring and autumn samplings. Results are expressed by total depth (0-8 cm) and three subsets of depth intervals (0-3 cm, 3-6 cm and 6-8 cm). Symbols represent the mean values, and bars represent the corresponding standard error, n is equal to 6 except from the 12 t $ha^{-1}$ treatment at the spring sampling which is n=5 (see the explanation in 2.4.4.2. section in methodology). The absence of letters indicates that the observed differences were not significant.

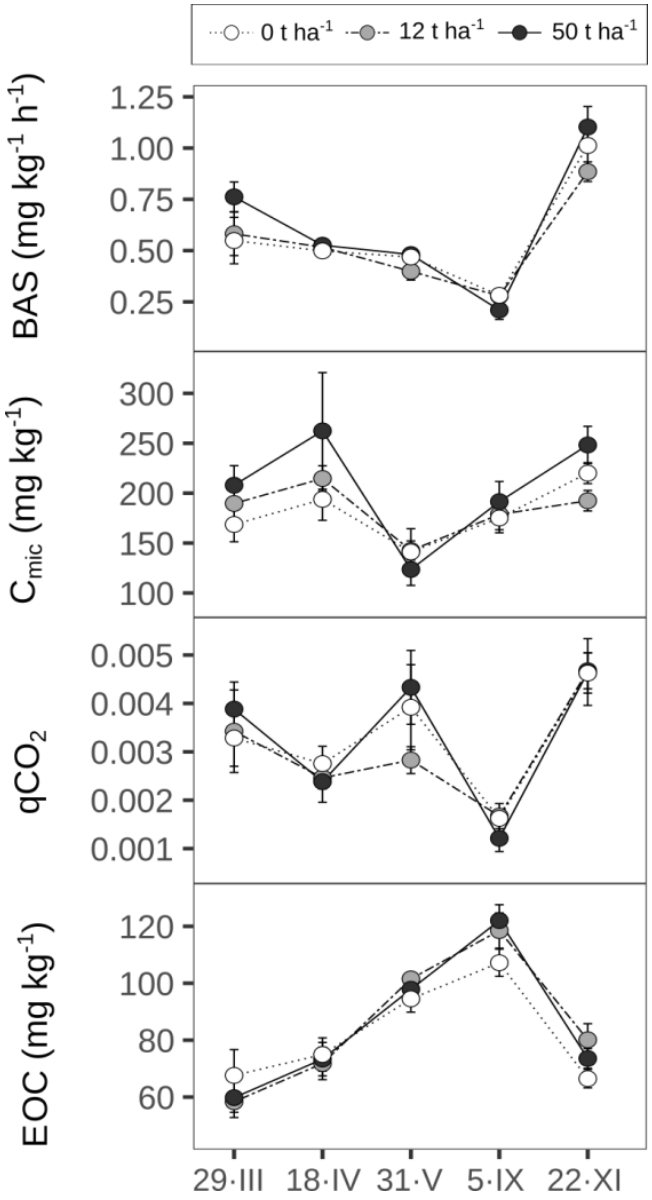


**Fig. S7.** Soil basal respiration (BAS) (μg C-$CO_2$ $g^{-1}$ dry soil $h^{-1}$); microbial carbon ($C_{mic}$) (μg C $g^{-1}$ dry soil); metabolic quotient ($qCO_2$) (μg C-$CO_2$ $g^{-1}$ dry soil $h^{-1}$/ μg $C_{mic}$ $g^{-1}$ dry soil) and extractable organic carbon (EOC) (mg $kg^{-1}$ dry soil) along five different samplings (29·III = 29th March; 18·IV = 18th April; 31·V = 31st April; 5·IX = 5th September and 22·XI = 22nd November). Symbols represent the mean values, and bars represent the corresponding standard error (n = 6). The absence of letters indicates that the observed differences were not significant.

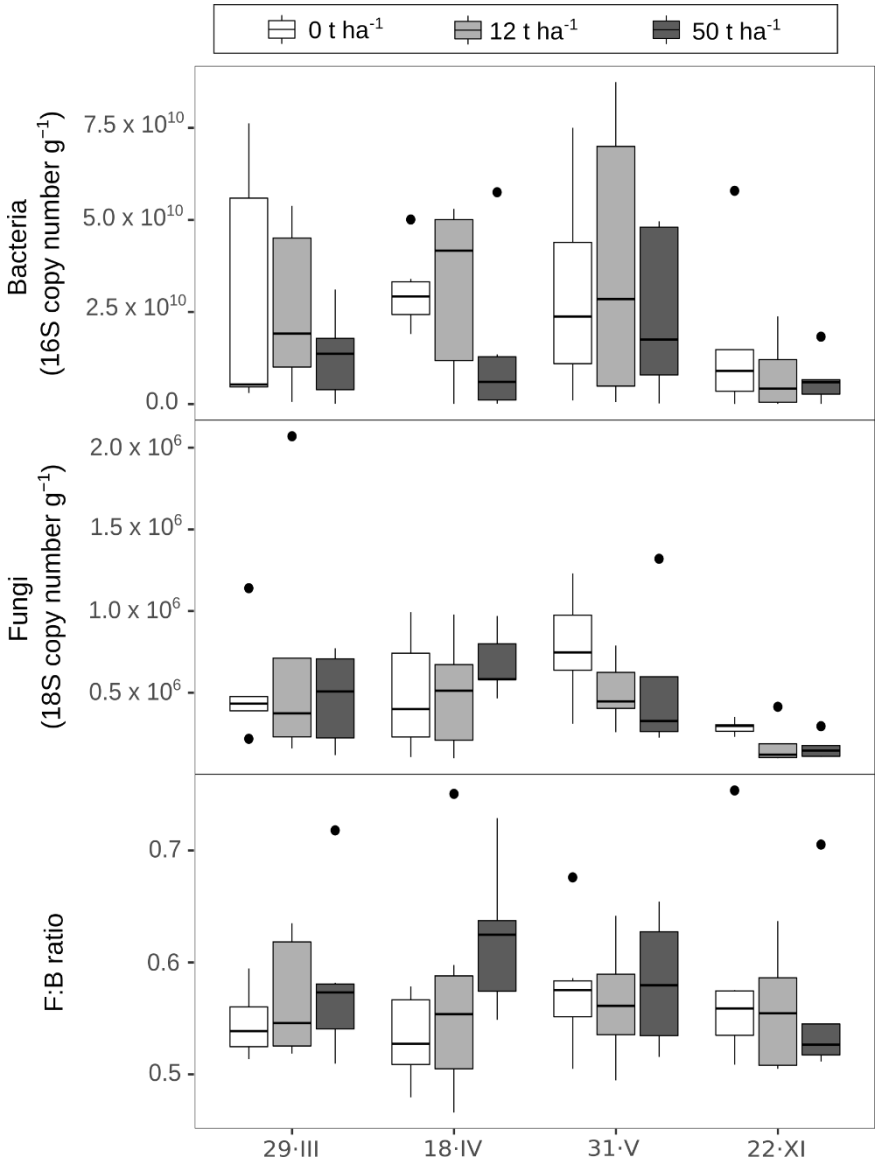


**Fig. S8.** Bacterial 16S rRNA gene copy number $g^{-1}$ dry soil; fungal 18S rRNA gene copy number $g^{-1}$ dry soil; and fungal 18S rRNA gene copy number/bacterial 16S rRNA gene copy number (F:B ratio) along five different samplings (29·III = 29th March; 18·IV = 18th April; 31·V = 31st April; 5·IX = 5th September and 22·XI = 22nd November). As in some mesocosms copy numbers were not detectable n is ≤ 6. The absence of letters indicates that the observed differences were not significant. The F:B ratio was calculated on the basis of the log-transformed copy number values.

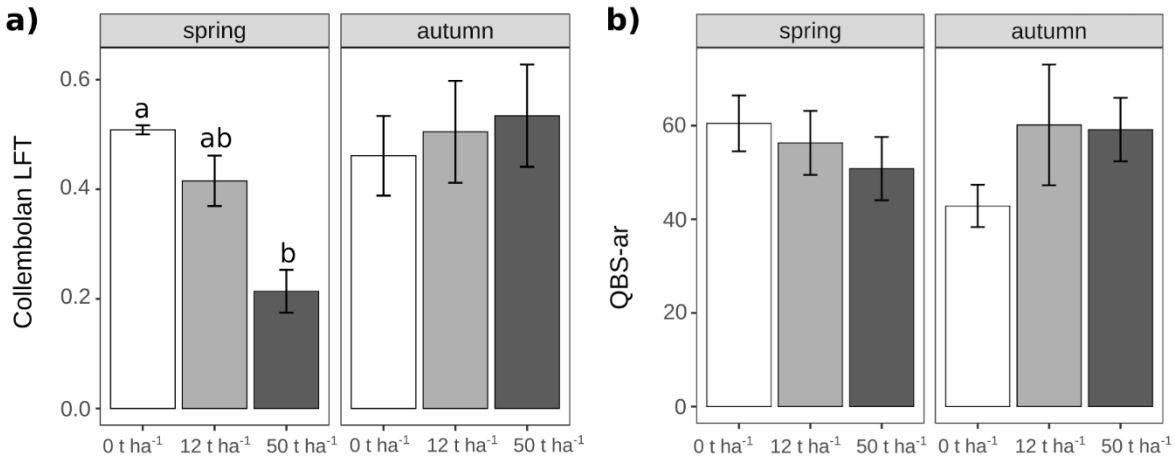


**Fig. S9.** Mean (± SE) collembolan life-form traits (LFT) index (a) and soil biological quality-arthropod index QBS-ar (b) at the spring and autumn samplings. All bars represent n = 6 except from collembolan LFT at the spring sampling where the 0 t ha$^{-1}$ is n =2 and the 12 t ha$^{-1}$ is n=5. Different letters indicate statistically significant differences between treatments.

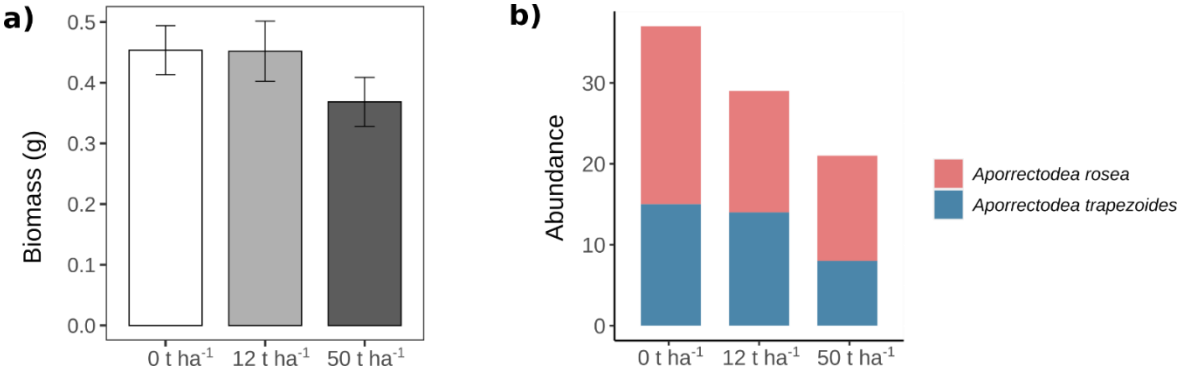


**Fig. S10.** Mean (± SE) total biomass (grams) of earthworms (a) and earthworm absolute abundance (expressed as a count) including species identification (b). Both measures are pooled per treatment (no replicates).

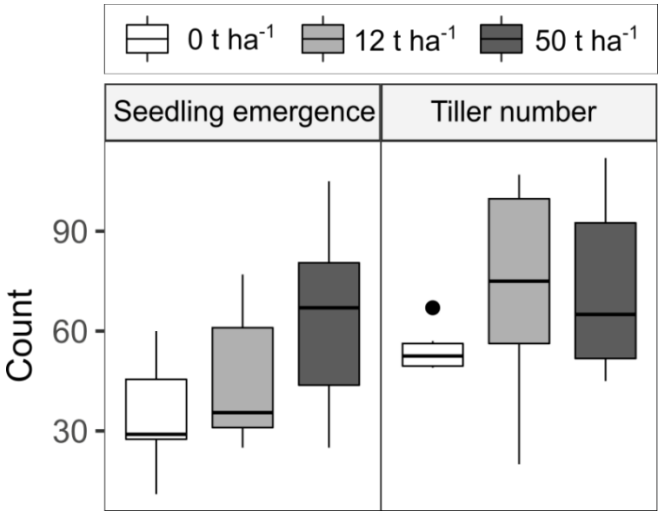


**Fig. S11.** Barley seedling emergence and tiller number assessed at 47 and 67 days following the seeding date, respectively (n=6). The absence of letters indicates that the observed differences were not significant.

# CHAPTER 3

# Effects of biochar addition to organo-mineral fertilisers on nutrient release and barley crop parameters

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*Manuscript in preparation*

## Abstract

There is an urge to develop new generation fertilisers able to minimise nutrient losses and increase crop yields, especially in light of the decreasing availability of mineral resources and the high energy demand for conventional fertilisers' production. Biochar is a rich recalcitrant carbon material produced from biomass pyrolysis which is able to non-permanently retain nutrients and therefore it could be interesting as ingredient in slow-release fertilisers. This capacity mainly stems from biochar's sorption properties, attributed to its porous structure and high surface area fostering reactive functional groups. Nevertheless, there is still a paucity of data regarding biochar performance as fertiliser ingredient. Therefore, the main objective of this study was to assess the possible use of biochar-based fertilisers to improve nutrient release efficiency, plant growth and nutrient uptake.

A greenhouse pot experiment using a Fluventic Haploxerept and barley (*Hordeum vulgare*) was set up to compare three commercial organo-mineral fertiliser formulations (NPK, NP and K) with the corresponding formulations containing biochar (NPK+B, NP+B and K+B). Nutrient (i.e., nitrate, ammonium, phosphate, potassium, sodium, calcium, manganese, chloride, and sulphate) leaching from the pots was monitored along a 106-day period, and plant growth parameters were analysed at the end of the plant cycle, together with plant nutrient uptake.

Nutrient leaching was slowed down in the NPK+B compared to the NPK fertiliser, but not in the other tested formulations. One plausible explanation would be microbial nutrient immobilisation mediated by the concurrent NPK and biochar labile C provision (or alternatively only by the biochar rate provision), i.e., nutrients could be temporary stored in microbial biomass and subsequently released upon microbial turnover. However, the inclusion of biochar in the formulations had limited effects on crop productivity with NPK+B only significantly enhancing straw biomass but not grain, whereas some benefits in nutrient content and export (involving potassium, sulphur, calcium and manganese) were found for all the biochar-based fertilisers, possibly indicating that biochar acted as a source of these nutrients.These results provide some evidence of the potential use of the studied biochar in fertiliser formulation to slowly release nutrients and to meet nutrient availability with plant demands. In addition, biochar might have a nutrient value per se, at least in the short term.

**Keywords:** Biochar, slow-release fertilisers, nutrient leaching, crop growth

## 3.1. Introduction

Nitrogen (N), phosphorus (P), and potassium (K) are the main limiting nutrients for crop growth, but due to the high solubility of traditional mineral fertiliser formulations, there is a poor synchrony between N, P, and K release rate and crop uptake needs (Pang et al., 2018). Apart from solubilisation and subsequent leaching losses, other processes such as $NH_3$ volatilisation and denitrification, in the case of N, (Cameron et al., 2013) or P retrogradation (Vanderdeelen, 1995), also contribute to low nutrient use efficiency by plants. As a matter of fact, plant nutrient uptake from mineral fertilisers has been reported to be as low as 33-50% for nitrogen (Hirel et al., 2011; Raun and Johnson, 1999) and 10- 25% for phosphorus (Syers et al., 2008), which results in economic costs for farmers and has environmental risks associated. Namely, N and P leaching and runoff to water bodies provokes eutrophication (Vitousek et al., 1997) and has been linked to human health risks, such as the carcinogenic effect of N-nitroso compounds formation after nitrate-polluted drinking water exposure (Ward et al., 2018).

Furthermore, conventional NPK fertiliser production involves a high amount of fossil fuels and energy for N industrial fixation (Haber-Bosch process) and exploitation of non-renewable resources (phosphate rock and potash) in the case of P and K (Blanco, 2011). This is why there is an urgent need to develop fertiliser formulations able to increase nutrient availability without further compromising natural resources. Strategies which might be used for this purpose include: preventing nutrient losses, e.g. by using slow-release fertilisers (Calabi-Floody et al., 2018); promoting waste's nutrient recycling, either by using organic waste combinations, hybrid fertilisers that combine organic wastes enriched with mineral components (Kominko et al., 2017), or by adding nutrient–solubilising microorganisms (Das and Pradhan, 2016); and using new nutrient sources such as struvite (Talboys et al., 2016). The use of biochar as fertiliser ingredient would mostly fit with the first strategy, for its known nutrient retention capacity.

Biochar, the solid co-product of pyrolysis, has been widely proposed as an effective tool to increase the refractory C pool in soils but also to enhance nutrient retention and reduce nutrient leaching out of the root zone (Laird, 2010a; Lehmann et al., 2003). Different mechanisms have been put forward to explain biochar's retentive capabilities, such as: i) electrostatic adsorption of nutrients onto biochar due to its micropore structure (physisorption) (Nguyen et al., 2017) or surface charge (chemisorption), the latter including both negative functional groups (Liang et al., 2006), and, although less commonly reported, positively charged functional groups

(Lawrinenko and Larid, 2015); ii) the retention of nutrient-enriched water in biochar's pores (Brockhoff et al., 2010; Lehmann et al., 2003); iii) biochar-induced pH alterations which can affect solubility and leachability of nutrients (Laird and Rogovska, 2015); and iv) by influencing nutrient cycling through biochar's interaction with soil microbes, e.g., by nutrient immobilisation in microbial biomass (Ippolito et al., 2012; Novak et al., 2010).

Despite the beneficial effects of some biochars when applied on its own, a great deal of research has demonstrated that the co-application of biochar with inorganic or organic fertilisers could exert synergistic effects on crop yields, and indeed, it is sometimes required to achieve any agronomic benefits (Alburquerque et al., 2013; Mete et al., 2015, Oladele et al., 2019; Pandit et al., 2019; Rafiq et al., 2020; Steiner et al., 2007). Consequently, the importance of optimising biochar application in the form of new-generation fertilisers, i.e., being able to reduce biochar application rates and concomitantly improve nutrient use efficiency, has been highlighted (Joseph et al., 2013). Although the development of biochar-based fertilisers is still on its infancy there are some examples with promising results (Chen et al., 2018; Chew et al., 2020; Liao et al., 2020; Luo et al., 2021). In this direction, the use of biochar in organic farming as a fertiliser or soil conditioner was approved in Europe in 2019, by amending the Annex I of Regulation (EC) No 889/2008 (European Parliament, 2019).

The main aim of this study was to elucidate if biochar inclusion in fertiliser formulation of three organo-mineral fertilisers could enhance nutrient retention and thus act as slow-release fertilisers, by reducing or at least delaying nutrient leaching losses, coupled to an improved crop nutrient uptake and growth. For this purpose, a pot experiment using barley as model crop was used to compare three conventional organo-mineral fertilisers (NPK, NP, and K) with the corresponding biochar-based fertilisers (NPK+B, NP+B, and K+B). Nutrient dynamics shifts and plant use efficiency were assessed by leachate nutrient content monitoring, plant nutrient uptake and biomass quantification.

## 3.2. Materials and methods

### 3.2.1. Experimental setup

The greenhouse pot experiment was conducted at the IRTA Torre Marimón experimental station in Caldes de Montbui (Barcelona, NE Spain)). Six organo-mineral fertilisers produced

by Desarrollos Agroquímicos S.A. DASA-ELFER (Menàrguens, Lleida, Spain) were tested in a fully randomised design with six-fold replication. Fertilisers were produced in a tablet form (see **Supplementary Fig. S1**), all of them contained maize flour in their formulation and differed in their macronutrient content (NPK, NP and K), and in the presence of biochar (+B). The six resulting combinations were designated as NPK, NP, K, NPK+B, NP+B, and K+B. The exact formulation is not shown for being subjected to business confidentiality but it should be noted that N was provided as a mixture of ammonium and organic sources while P and K were supplemented in an inorganic form.

The biochar used for NPK+B, NP+B, and K+B formulations was produced by slow pyrolysis (400-550 ºC, 120 min) from a mixed feedstock of *Quercus ilex*, *Quercus suber*, and *Eucaliptus* sp., and obtained by Corchos Oliva S.L. (Oliva de la Frontera, Badajoz, Spain). A detailed characterisation of the biochar is presented in **Table 1**.

The top soil layer (0–20 cm depth) of a deep calcareous sandy-loam alluvial soil (a Fluventic Haploxerept, according to Soil Survey Staff, 2010) was collected from the IRTA Torre Marimón experimental farm (41º36'47" N, 2º10'16" E) and used as soil matrix for the experiment (see Marks et al., 2016 for a detailed soil description). Pots with a height of 20 cm were filled with 1.8 kg of soil sieved to 5 mm. On the onset of the experiment, the amount of fertiliser tablets to provide 66.67 mg N $kg^{-1}$ (equivalent to 173.3 kg N $ha^{-1}$) for N-containing fertilisers, and equivalent K contents for K-containing fertilisers (**Table 2**) were added to each pot at a 5 cm depth. Biochar addition rates were not possible to even out (**Table 2**) since each fertiliser formulation differed in its biochar proportion for stability purposes. Aside from fertilisation, pots were watered to field capacity (around a 20 % moisture content) and fifteen barley seeds (*Hordeum vulgare* L. Graphic variety) were sown (thinned to three plants after a fortnight period). Plants were grown under a photoperiod of 14 h light:10h dark.

**Table 1.** Characteristics of the biochar used in the experiment.

| Parameter | Unit | Value | Method |
|---|---|---|---|
| C | g $kg^{-1}$ | 632.3 | elemental analysis |
| N | g $kg^{-1}$ | 4.5 | elemental analysis |
| H | g $kg^{-1}$ | 21.7 | elemental analysis |
| S | g $kg^{-1}$ | 0.8 | elemental analysis |
| O | g $kg^{-1}$ | 94.3 | difference of sum of elemental analysis and ash |
| $O/C_{org}$ | % | 0.14 | |
| $H/C_{org}$ | % | 0.41 | |
| Ash (540 °C) | % | 24.64 | |
| Volatile matter | % | 22.69 | |
| P | g $kg^{-1}$ | 1.02 | |
| Na | g $kg^{-1}$ | 0.48 | |
| K | g $kg^{-1}$ | 4.90 | |
| Ca | g $kg^{-1}$ | 41.00 | |
| Mg | g $kg^{-1}$ | 3.16 | |
| pH ($H_2O$, 1:20) | - | 8.5 ± 0.63 | |
| EC (25°C, 1:20) | dS $m^{-1}$ | 0.4 ± 0.003 | |
| CEC | $mmol_c$ $kg^{-1}$ | 4.23 ± 0.27 | ISO 23470, 2007 |
| Surface area (BET) | $m^2$ $g^{-1}$ | 135.73 | $N_2$ adsorption isotherm, 77K |
| Porosity | % | 56.69 | Hg porosimetry |
| Mean porus size | nm | 180.6 | Hg porosimetry |

**Table 2.** Dosing of fertiliser (g $pot^{-1}$) and application rates of N, P, K, and biochar (mg $kg^{-1}$ soil) for each fertiliser.

| Fertiliser | g fertiliser pot $^{-1}$ | mg N $kg^{-1}$ | mg P $kg^{-1}$ | mg K $kg^{-1}$ | mg biochar $kg^{-1}$ |
|---|---|---|---|---|---|
| NPK | 4.74 | 66.67 [a] | 171.28 | 108.04 | 0 |
| NPK+B | 4.38 | 66.67 | 160.83 | 99.76 | 1046.23 |
| NP | 2.96 | 66.67 | 110.02 | 0 | 0 |
| NP+B | 2.84 | 66.67 | 106.07 | 0 | 551.62 |
| K | 1.31 | 0 | 0 | 108.04 | 0 |
| K+B | 1.31 | 0 | 0 | 108.04 | 217.52 |

[a] 66.67mg N $kg^{-1}$ = 173.3 kg N $ha^{-1}$

### 3.2.2. Leaching assay

Pots contained a 2 mm mesh gauze at the bottom to allow proper drainage while preventing soil losses. Soil water content was determined gravimetrically before the leachate sampling and used for the estimation of the water to be added to achieve a target leaching volume of 100 ml per pot. Distilled water was used for leaching, and, to simulate natural rainfall, water was slowly added to the pots using perforated bottles. The leachate was collected by placing each pot on a glass tray but raised 1.3 cm to ensure drainage. A total of nine leaching events were carried out. Leachates were collected once per week during the first five leaching events, then, the sampling frequency was extended to two weeks and, finally, to three weeks (see sampling schedule in **Fig. 1**). The collected leachate was then filtered (using Whatman no. 42 filters) under laboratory conditions and stored at -20°C until analysis.

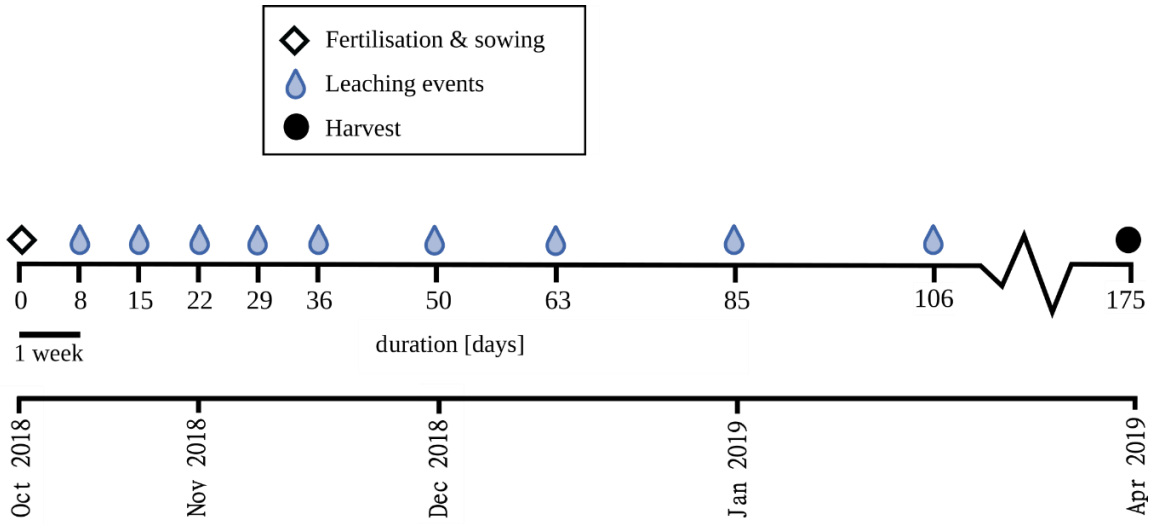


**Fig. 1**. Experimental schedule showing fertilisation and sowing, leaching events, and harvest.

Liquid chromatography was carried out to determine leachate inorganic ions content on a Dionex ICS-1100 ion chromatograph (Dionex, Sunnyvale, USA) using a AS4A-SC Dionex anion column for the quantification of $Cl^-$, $NO_2^-$, $NO_3^-$, $HPO_4^{2-}$ and $SO_4^{2-}$, and a CS12A Dionex cation column for $Na^+$, $NH_4^+$, $K^+$, $Mg^{2+}$, and $Ca^{2+}$ determination. All the ion concentrations were estimated using linear calibration except for $SO_4^{2-}$, $NH_4^+$, $Mg^{2+}$, and $Ca^{2+}$, in which quadratic regression substantially increased the fitting ($R^2$). Detection limit (LOD) estimation was set as three times the standard deviation of five blank values. $NO_2^-$ concentration values were not considered in this study for being almost always below detection limits (i.e., < 0.10 mg $NO_2^-$ $L^{-1}$). Additionally, $HPO_4^{2-}$ was also discarded from analysis since its signal-to-noise ratio in the chromatogram did not exceed the set value of 3. Leachate ionic concentration was quantified both as absolute and cumulative leaching and was expressed as mg per dry weight (kg) of soil, according to the following equation (Eq. (a)):

$$LIC_{soil}\ (mg\ kg^{-1}) = \frac{[LIC_{LC}\ mg\ L^{-1}]\ x\ [water\ added\ for\ leaching + water\ in\ soil\ (L)]}{kg\ soil\ dry\ weight} \qquad (a)$$

Where $LIC_{soil}$ stands for leachate ionic concentration expressed on a soil dry weight basis, and $LIC_{LC}$ for leachate ionic concentration as obtained from liquid chromatography.

### 3.2.3. Plant measurements

Above-ground barley biomass was harvested after plants were fully grown and senescent (after a 6-month growth period) and dried at 60ºC for 48 h. The total number of ears and the number of grains per ear were quantified. Then, straw and grain were weighed separately to determine their biomass. After straw and grain were ground in a ball-mill, nutrient content (N, P, K, Ca, Mg, S, Fe, Mn, and Zn) was determined through near infrared spectrometry (NIRS) by scanning the grounded samples from 1100 to 2500 nm in a NIRSystems 5000 scanning monochromator (FOSS, Hilleröd, Denmark). The calibrations used were developed in a previous study (see the Supplementary Materials in Martos et al., 2020 for more details). P content in straw presented some negative values that were set to 0. Finally, nutrient export was calculated by multiplying each nutrient concentration measurement by its corresponding biomass.

### 3.2.4. Statistical analyses

Since the objective of this research is to compare fertilisers with and without biochar inclusion in its formulation, statistical tests involved comparisons between each organo-mineral fertiliser (without biochar) and its counterpart with biochar (i.e., NPK vs NPK+B; NP vs NP+B, and K vs K+B). Longitudinal data, i.e. variables for which exist a between-subjects factor (biochar inclusion in the fertiliser), and a within-subjects factor (sampling dates) were analysed using two-way mixed ANOVAs, which were computed with the *rstatix* package v0.2.0 (Kassambara, 2019b). Before, Shapiro-Wilk and Levene tests were used to ensure that data had a normal distribution and homogeneous variances, respectively. When these assumptions were not met, the test was run on the $\log_{10}$-transformed variable. The assumption of sphericity was checked using the Mauchly's test, and when violated, the Greenhouse-Geisser correction was applied. Finally, homogeneity of covariances was tested by Box's M. Statistical results of the mixed ANOVA are shown in **Supplementary Table S1** (**S1.1.-S1.48.**). Pairwise comparisons were tested with t-test with Bonferroni adjustment, and the significance level was set at $p < 0.05$. These statistical analyses and results visualisation were performed using R software v. 3.6.1 (R Core Team, 2019) using the packages *ggplot2* (Wickham, 2016) and *ggpubr* v 0.2.3 (Kassambara, 2019a).

For absolute nutrient leaching data the Principal Response Curves (PRC) method (Van den Brink and Ter Braak, 1999), developed for biological community data, was conducted using the CANOCO software version 5.12 (Ter Braak and Šmilauer, 2012). The PRC describe the trajectory over time of the community response (nutrients in this study) in each treatment group, expressed as coefficient of community response, i.e., the canonical coefficient $C_{dt}$, relative to the control (which response is set to 0); whereas the weight ($b_k$) indicates the affinity of the response of each attribute (here ions in leachates) to the overall community response. Positive $b_k$ values indicate attributes whose response pattern follows the PRC, by contrast, negative values indicate attributes whose response pattern is in the opposite fashion to the overall PRC pattern. Near zero $b_k$ values represent weak responses or response patterns unrelated to the PRC. Given the disparity of nutrient concentration ranges, data was standardised to zero-mean and unit-variance prior analysis. Significance of the first axis was checked with a Monte Carlo permutation test while significances at each time point were evaluated by performing redundancy analysis (RDA) on subsets of different sampling dates.

Finally, those parameters analysed at a single sampling date (plant measurements) were assessed by means of Student t-test, Welch's t-test (if homoscedasticity assumption was not met) or the Mann-Whitney-U test (if normality assumption was not met), all of them with Bonferroni correction (*rstatix* package v 0.2.0 (Kassambara, 2019b)). When mixed ANOVA analysis of longitudinal data was not significant, differences at each sampling date were also checked with this approach.

## 3.3. Results

### 3.3.1. Nutrient leaching

The pair of fertilisers NPK vs NPK+B (**Fig. 2a**) was the only one showing significant results on nutrient leaching patterns as measured by the PRC analysis (**Fig. 2**), with 80.18% of the total variance being explained by time and 5.78% by biochar treatment. A significant proportion of the variance (52.21%) was captured by the first canonical axis of the PRC (Monte Carlo permutation test, 499 permutations, $F = 1.8$, $p = 0.018$). The RDA analyses at each sampling time showed contrasted and significant temporal effects associated to biochar inclusion in the formulation. At the first leaching events (day 8 and 15 since experimental onset) the NPK+B leached less nutrients in contrast to the NPK. However, at the third leaching event (day 22) this trend was inverted, and from that moment onwards, NPK+B leached more nutrients than NPK (significantly at days 22, 36, 85 and 106). As shown by $b_k$ values of the PRC, the most responsive nutrients were $N\text{-}NO_3^-$ ($b_k = 1.50$) followed by $K^+$ ($b_k = 1.26$), whereas $NH_4^+$ was the only ion showing an inverse pattern with respect to the PRC ($b_k = -0.53$).

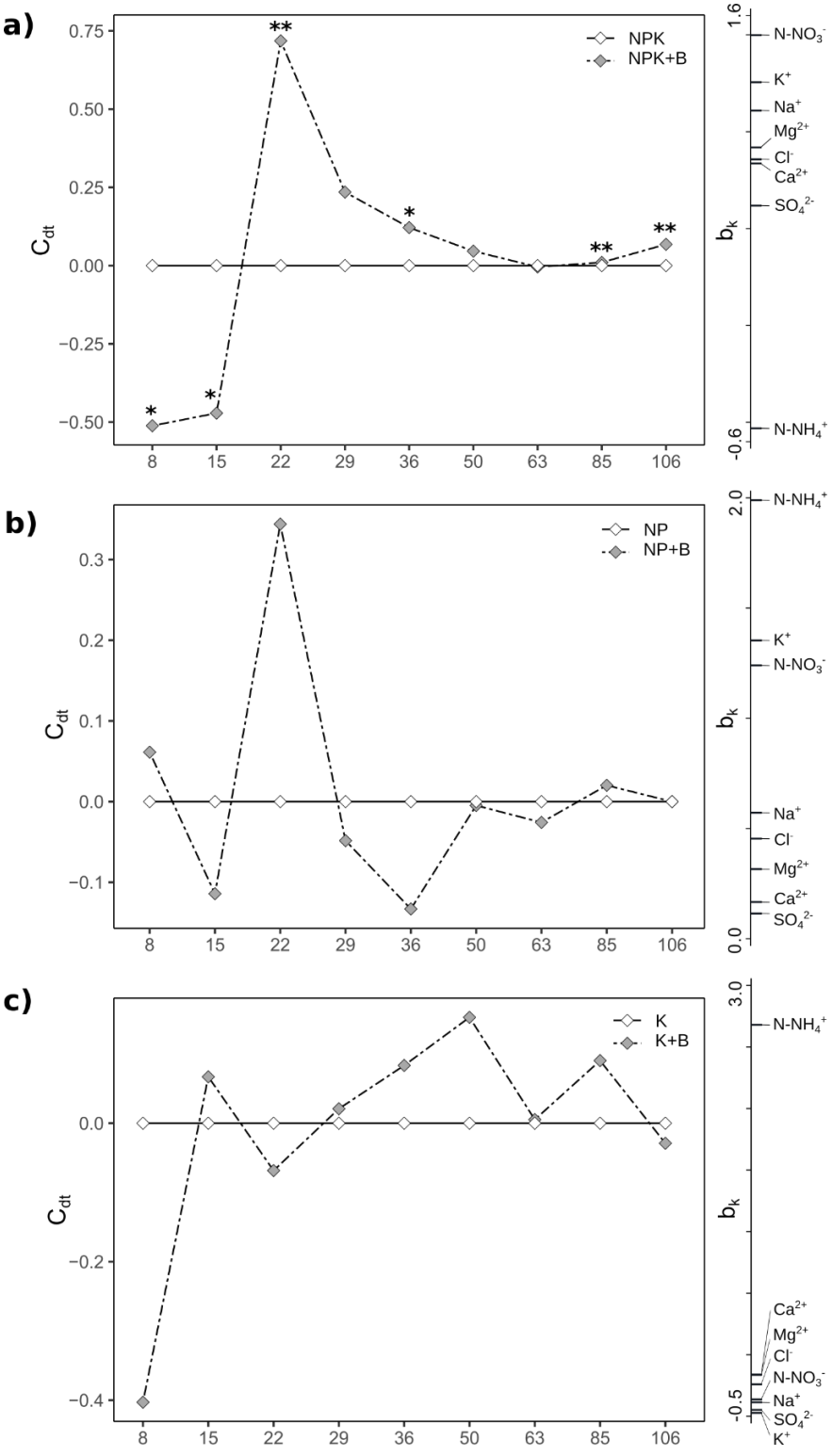


**Fig. 2**. PRC diagram (left) and weight ($b_k$) (right), for the first principal component of nutrient leaching in the fertiliser pairs NPK vs NPK+B (a); NP vs NP+B (b); and K vs K+B (c) showing shifts caused by biochar inclusion in fertiliser formulation in comparison to the conventional (without biochar) fertiliser along nine sampling dates. *,** indicate significant differences at p values comprised between 0.05 and 0.01, and between 0.01 and 0.001, respectively.

Regarding the absolute leaching of each ion plotted separately (**Supplementary Figs. S2 and S3**), the results of mixed ANOVA showed significant interactions between biochar treatment and time for $N\text{-}NO_3^-$, $N\text{-}NH_4^+$, $Na^+$, $K^+$, and $Ca^{2+}$ (**Supplementary Tables S1.1-4** and **S6**) in the NPK vs NPK+B fertiliser pair. In addition, $Mg^{2+}$, $Cl^-$ and $SO_4^{2-}$ also showed significant effects at some sampling dates for the same pair of fertiliser (**Supplementary Figs. S2 and S3**). These ions followed the same trend explained in the PRC analysis, i.e., more leaching in the NPK fertiliser at the first weeks and the inverse trend in the subsequent weeks, except in the case of ammonium, whose leaching was slightly higher (0.5 mg $kg^{-1}$) in the NPK+B compared to NPK at the first sampling time. Concerning the NP and K fertiliser pairs, only sparse significant results were found and thus there was no clear pattern on nutrient leaching as mediated by biochar.

The results of mixed ANOVA evidenced time effects always being significant (**Supplementary Tables S1.1-24**), i.e., there was a generalised plunge in all ions for every fertiliser pair at about the fifth leaching event. Concerning the nitrogen forms, it should be noted that, $N\text{-}NO_3^-$ content was about two orders of magnitude larger than that of $N\text{-}NH_4^+$ at the first samplings, while from the fifth leaching event ionic content was similar (and lower) for the two ions (**Supplementary Figs. S2 and S3**).

Finally, in the matter of cumulative nutrient leaching, whereas sparse significant differences were found at the first sampling dates, there were no significant differences on the final cumulative amount of nutrients leached associated to biochar inclusion in fertiliser formulation (**Supplementary Figs. S4 and S5**).

### 3.3.2. Barley growth parameters

Barley straw weight was significantly enhanced in the NPK+B fertiliser (23.43 % increase) in contrast with NPK according to Welch's t-test, $t$ (5.68) = -2.61, p = 0.04. However, the higher straw biomass in the NPK+B fertiliser did not concur with significantly higher grain yield on this same treatment, although it was marginally improved (Welch's t-test, $t$ (6.0) = -2.25, p = 0.065) (**Fig. 3a** and **b**). Ear number and grain number per ear were not significantly different within any pair of fertilisers (**Fig. 3c** and **d**).

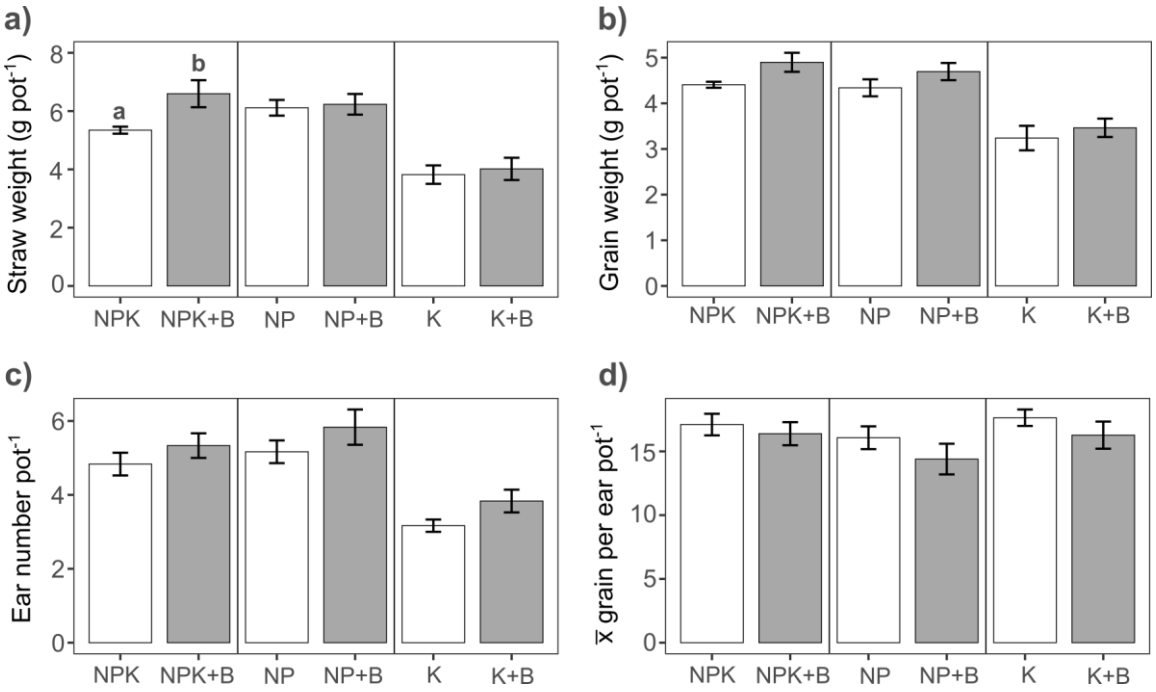


**Fig. 3**. Mean (± SE) (n = 6) straw weight (g pot$^{-1}$) (a); grain weight (g pot$^{-1}$) (b); ear number per pot (c); and grain number per ear averaged per pot. Different letters indicate statistically significant differences within a fertiliser pair (NPK vs NPK+B, NP vs NP+B, and K vs K+B).

### 3.3.3. Barley nutrient content and export

Grain nutrient content was unaffected by any of the treatments within any fertiliser pair (**Table 3**). However, the NP+B fertiliser did increase Ca (t-test, $t$ (10) = -2.59, p = 0.03) and Mn export in grain (Mann-Whitney test, $U$ = 5, z = -2.04, p = 0.04) compared to the NP fertiliser (**Table 4**). Concerning straw nutrient content and export, some beneficial effects were found for the biochar-based fertilisers. Namely, K content (Welch's t-test, $t$ (5.33) = -2.53, p = 0.049) and export (Welch's t-test, $t$ (5.43) = -3.11, p = 0.02), together with S export (Welch's t-test, $t$ (5.69) = -4.39, p = 0.01) were higher at the NPK+B in contrast with the NPK fertiliser (**Tables 3** and **4**). In addition, Mn content (t-test, $t$ (10) = -2.53, p = 0.03) and both Ca content (**Table 3**) (Mann-Whitney test, $U$ = 4, z = -2.17, p = 0.03) and export (**Table 4**) (Mann-Whitney test, $U$ = 5, z = -2.04, p = 0.04) were improved at the K+B compared to the K fertiliser.

**Table 3.** Nutrient content in grain and straw of harvested barley for the six organo-mineral fertilisers. Reported values are mean ± standard errors (n = 6). Different letters indicate statistically significant differences within a fertiliser pair (NPK vs NPK+B, NP vs NP+B, and K vs K+B).

| | **NPK** | **NPK+B** | **NP** | **NP+B** | **K** | **K+B** |
|---|---|---|---|---|---|---|
| ***Grain*** | | | | | | |
| N (g $kg^{-1}$) | 8.68 ± 0.2 | 8.84 ± 0.2 | 9.00 ± 0.2 | 8.97 ± 0.3 | 9.23 ± 0.1 | 9.28 ± 0.2 |
| P (g $kg^{-1}$) | 6.18 ± 0.1 | 6.08 ± 0.1 | 6.25 ± 0.1 | 6.13 ± 0.3 | 6.56 ± 0.1 | 6.45 ± 0.1 |
| K (g $kg^{-1}$) | 5.60 ± 0.1 | 5.53 ± 0.3 | 5.87 ± 0.2 | 5.84 ± 0.2 | 6.00 ± 0.1 | 5.77 ± 0.1 |
| S (g $kg^{-1}$) | 0.40 ± 0.0 | 0.38 ± 0.0 | 0.43 ± 0.0 | 0.43 ± 0.1 | 0.47 ± 0.0 | 0.38 ± 0.0 |
| Ca (g $kg^{-1}$) | 1.34 ± 0.0 | 1.30 ± 0.1 | 1.32 ± 0.1 | 1.39 ± 0.0 | 1.20 ± 0.1 | 1.17 ± 0.0 |
| Mg (g $kg^{-1}$) | 1.26 ± 0.0 | 1.25 ± 0.0 | 1.30 ± 0.0 | 1.27 ± 0.0 | 1.32 ± 0.0 | 1.30 ± 0.0 |
| Mn (mg $kg^{-1}$) | 1.41 ± 0.0 | 1.28 ± 0.1 | 1.37 ± 0.0 | 1.38 ± 0.0 | 1.51 ± 0.1 | 1.51 ± 0.0 |
| Zn (mg $kg^{-1}$) | 4.84 ± 0.0 | 4.94 ± 0.1 | 4.95 ± 0.1 | 4.90 ± 0.1 | 4.86 ± 0.0 | 4.81 ± 0.0 |
| ***Straw*** | | | | | | |
| N (g $kg^{-1}$) | 1.38 ± 0.1 | 2.22 ± 0.5 | 2.42 ± 0.5 | 2.65 ± 0.4 | 1.19 ± 0.4 | 1.18 ± 0.3 |
| P (g $kg^{-1}$) | 0.00 ± 0.0 | 1.27 ± 0.6 | 0.00 ± 0.0 | 1.07 ± 0.7 | 1.14 ± 0.5 | 0.00 ± 0.0 |
| K (g $kg^{-1}$) | 5.52 ± 0.5 **a** | 12.54 ± 2.7 **b** | 6.05 ± 0.6 | 11.33 ± 3.4 | 14.04 ± 2.9 | 7.25 ± 0.5 |
| S (g $kg^{-1}$) | 2.10 ± 0.0 | 2.47 ± 0.2 | 2.02 ± 0.0 | 2.39 ± 0.2 | 2.49 ± 0.2 | 2.19 ± 0.1 |
| Ca (g $kg^{-1}$) | 11.32 ± 0.2 | 7.35 ± 1.7 | 10.66 ± 0.2 | 8.66 ± 1.6 | 6.85 ± 1.7 **a** | 11.04 ± 0.2 **b** |
| Mg (g $kg^{-1}$) | 2.07 ± 0.1 | 1.84 ± 0.2 | 2.12 ± 0.1 | 2.10 ± 0.1 | 1.69 ± 0.1 | 1.94 ± 0.1 |
| Mn (mg $kg^{-1}$) | 3.43 ± 0.1 | 3.07 ± 0.2 | 3.26 ± 0.1 | 3.35 ± 0.1 | 2.91 ± 0.1 **a** | 3.36 ± 0.1 **b** |
| Zn (mg $kg^{-1}$) | 3.12 ± 0.1 | 3.22 ± 0.1 | 3.30 ± 0.1 | 3.32 ± 0.1 | 2.85 ± 0.1 | 2.82 ± 0.1 |

**Table 4.** Nutrient export (nutrient content*biomass) in grain and straw of harvested barley for the six organo-mineral fertilisers. Reported values are mean ± standard errors (n = 6). Different letters indicate statistically significant differences within a fertiliser pair (NPK vs NPK+B, NP vs NP+B, and K vs K+B).

| | **NPK** | **NPK+B** | **NP** | **NP+B** | **K** | **K+B** |
|---|---|---|---|---|---|---|
| ***Grain*** | | | | | | |
| N (g) | 3.82 ± 0.1 | 4.34 ± 0.2 | 3.90 ± 0.2 | 4.20 ± 0.2 | 2.98 ± 0.2 | 3.22 ± 0.2 |
| P (g) | 2.72 ± 0.1 | 2.97 ± 0.1 | 2.70 ± 0.1 | 2.86 ± 0.1 | 2.11 ± 0.1 | 2.23 ± 0.1 |
| K (g) | 2.47 ± 0.0 | 2.70 ± 0.2 | 2.54 ± 0.1 | 2.74 ± 0.1 | 1.93 ± 0.1 | 2.00 ± 0.2 |
| S (g) | 0.18 ± 0.0 | 0.19 ± 0.0 | 0.18 ± 0.0 | 0.20 ± 0.0 | 0.15 ± 0.0 | 0.13 ± 0.0 |
| Ca (g) | 0.59 ± 0.0 | 0.64 ± 0.1 | 0.57 ± 0.0 **a** | 0.65 ± 0.0 **b** | 0.39 ± 0.0 | 0.40 ± 0.0 |
| Mg (g) | 0.55 ± 0.0 | 0.61 ± 0.0 | 0.56 ± 0.0 | 0.59 ± 0.0 | 0.42 ± 0.0 | 0.45 ± 0.0 |
| Mn (mg) | 0.62 ± 0.0 | 0.62 ± 0.0 | 0.59 ± 0.0 **a** | 0.64 ± 0.0 **b** | 0.48 ± 0.0 | 0.52 ± 0.0 |
| Zn (mg) | 2.13 ± 0.0 | 2.42 ± 0.1 | 2.14 ± 0.1 | 2.30 ± 0.1 | 1.57 ± 0.1 | 1.67 ± 0.1 |
| ***Straw*** | | | | | | |
| N (g) | 0.74 ± 0.1 | 1.55 ± 0.5 | 1.51 ± 0.3 | 1.68 ± 0.3 | 0.46 ± 0.2 | 0.51 ± 0.2 |
| P (g) | 0.00 ± 0.0 | 0.76 ± 0.3 | 0.00 ± 0.0 | 0.69 ± 0.4 | 0.41 ± 0.2 | 0.00 ± 0.0 |
| K (g) | 2.97 ± 0.3 **a** | 8.00 ± 1.6 **b** | 3.75 ± 0.4 | 7.15 ± 2.3 | 5.21 ± 1.0 | 3.00 ± 0.5 |
| S (g) | 1.12 ± 0.0 **a** | 1.61 ± 0.1 **b** | 1.24 ± 0.1 | 1.49 ± 0.2 | 0.94 ± 0.1 | 0.89 ± 0.1 |
| Ca (g) | 6.04 ± 0.1 | 5.01 ± 1.3 | 6.52 ± 0.4 | 5.35 ± 1.0 | 2.72 ± 0.8 **a** | 4.43 ± 0.4 **b** |
| Mg (g) | 1.11 ± 0.1 | 1.24 ± 0.2 | 1.30 ± 0.1 | 1.31 ± 0.1 | 0.65 ± 0.1 | 0.79 ± 0.1 |
| Mn (mg) | 1.83 ± 0.1 | 2.04 ± 0.2 | 2.00 ± 0.1 | 2.09 ± 0.1 | 1.12 ± 0.1 | 1.35 ± 0.1 |
| Zn (mg) | 1.67 ± 0.1 | 2.14 ± 0.2 | 2.02 ± 0.1 | 2.07 ± 0.1 | 1.10 ± 0.1 | 1.14 ± 0.1 |

## 3.4. Discussion

### 3.4.1. Biochar addition at the NPK+B formulation delayed most of the nutrients release

The fact that nutrient release was only delayed in the NPK+B fertiliser and not in the other biochar-based fertilisers plausibly discards any biochar direct nutrient retention mechanisms such as chemisorption, physisorption or water-pore retention. Namely, despite the higher rate of biochar applied in the NPK+B formulation in relation to NP+B and K+B, we would have expected some kind of effect (albeit weaker) also in the other two biochar fertilisers. Although biochar could also have altered leaching patterns by shifting pH (Laird and Rogovska, 2015), it seems unlikely in the current study, where both the soil ($pH_{1:2.5}$ 8.2; Marks et al., 2016) and biochar ($pH_{1:20}$ 8.5) had an alkaline pH, and this same soil has been proved to resist biochar-induced pH shifts (Marks et al., 2016).

By contrast, as both biochar and macronutrients in NPK+B could influence microbial potential to grow and store nutrients, microbial nutrient immobilisation could be behind the observed temporary nutrient retention. Namely, microbial biomass can grow and store nutrients thanks to C and nutrient provision, but when microbial cells are devoid of these resources, the nutrients immobilised in the microbial biomass are returned to the soil phase via decomposition of dead cells, which can in turn contribute to the pool of available nutrients for plants (Anderson and Domsch, 1980). In particular, low-temperature biochars (< 500 ºC), thus akin to our biochar produced at 400-550 ºC, are more likely to induce net nutrient immobilisation, because they contain higher concentrations of bioavailable C and residual bio-oils (DeLuca et al., 2015). Furthermore, the timing of nutrient release in the NPK+B fertiliser, i.e., retaining nutrients the first two weeks and releasing them from the third week onwards, may support the microbial immobilisation mechanism. Specifically, and although microbial turnover time can vary greatly (normally in the range of days to months; Schmidt et al., 2007), similar values of microbial turnover have been found in cultivated soils (Cheng, 2009).

Additionally, the microbial immobilisation mechanism could also explain the nutrient release delay found for multiple ions ($N\text{-}NO_3^-$, $K^+$, $Na^+$, $Ca^{2+}$, $Mg^{2+}$, $Cl^-$, and $SO_4^{2-}$) since although microbial immobilisation mainly provokes N and P retention (Malik et al., 2013) other nutrients such as K, S, and Ca are also substantially stored in microbial biomass (Anderson and Domsch,

1980; Brookes; 2001; Yamashita et al., 2014). In accordance with this, PRC results of the NPK+B vs NPK pair of fertilisers pointed to major effects on N-$NO_3^-$ and $K^+$ retention, both plausibly as a result of their high relative abundance in microbial biomass or by their direct addition with fertiliser application.

On the other hand, the low recovery in leachates of added P in both NPK+B and NPK, is not surprising as phosphate is immobile in most soils because of precipitation reactions and adsorption to mineral surfaces (Wild, 1988). In our experiment, the negligible P release is mainly attributed to P precipitation with Ca due to the high Ca content and high soil pH (Hopkins and Ellsworth, 2005) found in the soil used for this experiment. Regarding the $Cl^-$ leaching delay, it is less straightforward to understand because its retention in microbial biomass is not expected (Kanwar et al., 1997). As a possible explanation, nitrification of the ammoniacal N provided in fertilisers is known to cause acidification and therefore to displace basic cations from the cation exchange complex (Bouman, 1995; Poss and Saragoni, 1992), but this process can also affect anion availability and leaching. Specifically, when cations like $Ca^{2+}$ or $Mg^{2+}$ are displaced by $H^+$ resulting from nitrification, anions (as $Cl^-$) could be concurrently released since they can be directly bounded to these polyvalent cations by bridging or be present in the diffuse double layer around the cation exchange complex. Then, if microbial immobilisation was promoted in the NPK+B fertiliser, less $NH_4^+$ would have been available for nitrification as it would be stored in microbial biomass and, as a result, acidification and leaching of ions (including $Cl^-$) could have been prevented to some extent at this treatment. It is worth mentioning that if this process occurred, it could imply complex interactions between microbial immobilisation, nitrification and nutrient leaching, making difficult to discern why $NH_4^+$ was the only ion whose release was not delayed by the NPK+B formulation, with its leaching indeed being higher at the NPK+B than in NPK at the first sampling date. However, the low quantities found for soluble $NH_4^+$ even at the first sampling date, especially in comparison with $NO_3^-$, make us disregard $NH_4^+$ release as an important process for understanding nutrient dynamics.

Regarding the causative mechanisms of the potential microbial nutrient immobilisation, different drivers could have played a role. Since microorganisms in agricultural soils are usually C limited (Schimel, 1986), biochar application rate (the amount of labile C provided) could be the only limiting factor. On the other hand, microbes could have been both C and NPK co-limited, specifically, the combination of commensurate C supply (here labile C from biochar) with mineral fertilisation (NPK instead of reduced nutrient combinations) could fulfil the

microbial stoichiometric requirements (Ashraf et al., 2020) and thus allow for microbial growth and retention of nutrients within their biomass. Provided that the NPK+B fertiliser not only supplied a combination of labile C from biochar and the three macronutrients but also represented the highest biochar rate of application among all biochar-based fertilisers (**Table 2**) it remains unresolved which underlying mechanism could have acted.

### 3.4.2. All biochar-based fertilisers enhanced barley nutrient status and NPK+B increased straw biomass

As expected given the more gradual nutrient release achieved in the NPK+B fertiliser, thus potentially improving plant uptake, this fertiliser was the only one significantly enhancing barley straw biomass, although grain biomass was only marginally improved. However, it should be noted that the high number of leaching events performed in this experiment, which led to a soluble nutrient shortage at about the fifth sampling, could have diluted the possible NPK+B beneficial effects on grain biomass.

Regarding barley nutrient content and export, all biochar-based fertilisers exerted some beneficial effects compared to the corresponding non-biochar formulations. The majority of effects became apparent in straw nutrient and export, i.e., the NPK+B fertiliser enhanced K content and export, and S export in contrast with NPK, while K+B improved Ca content and export and Mn content compared to the K fertiliser. On the other hand, the NP+B fertiliser provoked effects on grain export, i.e., enhanced Ca and Mn export in comparison with the NP fertiliser. Nutrient content and export improvement caused by the NPK+B fertiliser might result from its more gradual nutrient release pattern, by directly providing nutrients, i.e., biochar acting as a source of nutrients itself (Chan and Xu, 2009), or a combination of these two mechanisms. On the other hand, regarding the NP+B and K+B fertilisers, since nutrient leaching patterns were not found to improve in relation to its conventional counterpart, the beneficial effects on plant nutrient status would mostly fit the direct nutrient provision hypothesis. In support of this, the studied biochar has been proven to release K in water extracts (unpublished results). In addition K, Ca, and S are nutrients commonly reported to be released after biochar application (Laird et al., 2010b; Lehmann et al., 2003; Marks et al., 2016). Finally, although less commonly reported, biochar could also have released Mn (Elmer and Pignatello, 2011), alternatively, soil Mn solubilisation due to redox properties of biochar has also been

reported (Graber et al., 2014), but only at pH levels below 8, and therefore this mechanism is not likely in our alkaline system.

Our results could be in line with those of Güereña et al. (2012), who reported not significant biochar effects on grain yield whereas N leaching was reduced in biochar treatments, this result being potentially attributable to a three-fold increase in microbial biomass. However, if the microbial immobilisation mechanism is confirmed, caution must be laid on fertiliser development and application, since this positive effect might be soil-specific and highly dependent on the native microbial activity, biochar dosing and biochar composition. For instance, no microbial immobilisation might be observed in soils with already high SOC contents and microbial biomass (Subedi et al., 2016; Yanardağ et al., 2017). On the other hand, excessive microbial immobilisation on larger time frames could cause competition for nutrients between plants and soil microorganisms (Anderson and Domsch, 1980).

Despite these potential constraints, biochar-based fertilisers are of high interest for being able to provide immediate benefits for soil fertility by supplying and, more importantly, by retaining nutrients. Additionally, biochar-based fertilisers can also provide multiple environmental benefits widely proposed in the literature, such as a higher C sequestration (Shackley et al., 2013), greenhouse gas mitigation (Kammann et al., 2017), or water retention (Omondi et al., 2016). Further research is needed to avoid unintended effects and to maximise the number of services which biochar can deliver as a fertiliser ingredient.

## 3.5. Conclusions

The inclusion of biochar in fertiliser formulation did not alter the final cumulative amount of nutrients leached, nevertheless, there were distinct temporal patterns of nutrient release in the NPK+B fertiliser. While most fertilisers contributed to a large flush of available nutrients upon application, the NPK+B biochar-based fertiliser caused a delayed and more sustained release of nutrients throughout the growing season. Microbial nutrient immobilisation could be behind this response since the effects were only found in the biochar fertiliser which simultaneously supplied biochar-C and the three macronutrients (reduced nutrient combinations could have limited microbial growth) and/or because NPK+B was also the biochar-based fertiliser with a highest proportion of biochar, and thus, of labile C. Although there were no significant differences on grain production, straw biomass was indeed increased in the NPK+B treatment,

potentially indicating that this fertiliser might aid in the synchronisation of nutrient availability and crop requirements. Furthermore, nutrient content and export were enhanced in all biochar-based fertilisers, likely as a result of biochar acting as a direct nutrient source. To conclude, the slow nutrient release of NPK+B might facilitate the gradual provision of nutrients for plants and holds promise for the development of new generation biochar-based fertilisers.

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*Supplementary Material Chapter 3*

**Table S1.** Summary of the results of two-way mixed ANOVAs on different variables, with treatment (control fertiliser without biochar vs biochar-based fertiliser) as between-subjects factor, and time (sampling dates) as within-subjects factor. Degrees of freedom (df) are shown as: (degrees of freedom numerator, degrees of freedom denominator); the effect size is reported as generalised eta squared ($\eta_G^2$), and significant p-values ($p < .05$) are marked in bold.

**Table S1.1.** $N\text{-}NO_3^-$ absolute leaching (NPK vs NPK+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.04 | 0.840 | 0.00 |
| time | (2.07, 20.74) | 73.77 | **< .001** | 0.87 |
| treatment x time | (2.07, 20.74) | 6.30 | **0.007** | 0.37 |

**Table S1.2.** $N\text{-}NH_4^+$ absolute leaching (NPK vs NPK+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.04 | 0.847 | 0.00 |
| time | (2.24, 22.39) | 17.26 | **< .001** | 0.61 |
| treatment x time | (2.24, 22.39) | 6.20 | **0.006** | 0.36 |

**Table S1.3.** $Na^+$ absolute leaching (NPK vs NPK+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 1.79 | 0.211 | 0.03 |
| time | (3.06, 30.56) | 99.96 | **< .001** | 0.89 |
| treatment x time | (3.06, 30.56) | 4.92 | **0.006** | 0.29 |

**Table S1.4.** $K^+$ absolute leaching (NPK vs NPK+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.42 | 0.532 | 0.01 |
| time | (8, 80) | 126.11 | **< .001** | 0.91 |
| treatment x time | (8, 80) | 4.04 | **< .001** | 0.24 |

**Table S1.5.** $Mg^{2+}$ absolute leaching (NPK vs NPK+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.48 | 0.503 | 0.00 |
| time | (2.05, 20.55) | 110.57 | **< .001** | 0.91 |
| treatment x time | (2.05, 20.55) | 3.41 | 0.052 | 0.24 |

**Table S1.6.** $Ca^{2+}$ absolute leaching (NPK vs NPK+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.18 | 0.681 | 0.00 |
| time | (3.44, 34.38) | 360.53 | **< .001** | 0.97 |
| treatment x time | (3.44, 34.38) | 6.19 | **0.001** | 0.36 |

**Table S1.7.** $Cl^-$ absolute leaching (NPK vs NPK+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 2.75 | 0.128 | 0.03 |
| time | (1.83, 18.28) | 87.96 | **< .001** | 0.89 |
| treatment x time | (1.83, 18.28) | 2.69 | 0.099 | 0.20 |

**Table S1.8.** $SO_4^{2-}$ absolute leaching (NPK vs NPK+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.48 | 0.505 | 0.00 |
| time | (2.26, 22.6) | 126.93 | **< .001** | 0.92 |
| treatment x time | (2.26, 22.6) | 2.82 | 0.075 | 0.21 |

**Table S1.9.** $N\text{-}NO_3^-$ absolute leaching (NP vs NP+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.17 | 0.685 | 0.00 |
| time | (2.08, 20.76) | 41.78 | **< .001** | 0.80 |
| treatment x time | (2.08, 20.76) | 0.50 | 0.623 | 0.04 |

**Table S1.10.** $N\text{-}NH_4^+$ absolute leaching (NP vs NP+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.20 | 0.661 | 0.00 |
| time | (2.75, 27.54) | 21.43 | **< .001** | 0.66 |
| treatment x time | (2.75, 27.54) | 0.67 | 0.568 | 0.06 |

**Table S1.11.** $Na^+$ absolute leaching (NP vs NP+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.05 | 0.823 | 0.00 |
| time | (2.31, 23.07) | 70.38 | **< .001** | 0.86 |
| treatment x time | (2.31, 23.07) | 0.26 | 0.807 | 0.02 |

**Table S1.12.** $K^+$ absolute leaching (NP vs NP+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.00 | 0.956 | 0.00 |
| time | (2.46, 24.57) | 57.16 | **< .001** | 0.83 |
| treatment x time | (2.46, 24.57) | 0.75 | 0.508 | 0.06 |

**Table S1.13.** $Mg^{2+}$ absolute leaching (NP vs NP+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.16 | 0.702 | 0.00 |
| time | (1.67, 16.68) | 52.54 | **< .001** | 0.83 |
| treatment x time | (1.67, 16.68) | 0.09 | 0.881 | 0.01 |

**Table S1.14.** $Ca^{2+}$ absolute leaching (NP vs NP+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.08 | 0.784 | 0.00 |
| time | (1.28, 12.84) | 41.34 | **< .001** | 0.79 |
| treatment x time | (1.28, 12.84) | 0.03 | 0.920 | 0.00 |

**Table S1.15.** $Cl^-$ absolute leaching (NP vs NP+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.95 | 0.353 | 0.01 |
| time | (2.85, 28.49) | 92.79 | **< .001** | 0.89 |
| treatment x time | (2.85, 28.49) | 0.83 | 0.481 | 0.07 |

**Table S1.16.** $SO_4^{2-}$ absolute leaching (NP vs NP+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.00 | 0.948 | 0.00 |
| time | (1.65, 16.48) | 88.47 | **< .001** | 0.89 |
| treatment x time | (1.65, 16.48) | 0.43 | 0.620 | 0.04 |

**Table S1.17.** $N\text{-}NO_3^-$ absolute leaching (K vs K+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.37 | 0.558 | 0.01 |
| time | (8, 80) | 216.83 | **< .001** | 0.95 |
| treatment x time | (8, 80) | 0.76 | 0.637 | 0.06 |

**Table S1.18.** $N\text{-}NH_4^+$ absolute leaching (K vs K+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.09 | 0.773 | 0.00 |
| time | (2.85, 28.54) | 21.84 | **< .001** | 0.67 |
| treatment x time | (2.85, 28.54) | 1.47 | 0.245 | 0.12 |

**Table S1.19.** $Na^+$ absolute leaching (K vs K+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.68 | 0.429 | 0.01 |
| time | (2.2, 22.04) | 46.95 | **< .001** | 0.81 |
| treatment x time | (2.2, 22.04) | 0.20 | 0.839 | 0.02 |

**Table S1.20.** $K^+$ absolute leaching (K vs K+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.32 | 0.585 | 0.01 |
| time | (2.3, 23) | 31.13 | **< .001** | 0.71 |
| treatment x time | (2.3, 23) | 0.29 | 0.781 | 0.02 |

**Table S1.21.** $Mg^{2+}$ absolute leaching (K vs K+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.00 | 0.983 | 0.00 |
| time | (1.64, 16.44) | 42.35 | **< .001** | 0.79 |
| treatment x time | (1.64, 16.44) | 0.02 | 0.967 | 0.02 |

**Table S1.22.** $Ca^{2+}$ absolute leaching (K vs K+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.07 | 0.796 | 0.00 |
| time | (1.27, 12.69) | 47.24 | **< .001** | 0.81 |
| treatment x time | (1.27, 12.69) | 0.09 | 0.824 | 0.01 |

**Table S1.23.** $Cl^-$ absolute leaching (K vs K+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.03 | 0.870 | 0.00 |
| time | (1.87, 18.68) | 28.56 | **< .001** | 0.73 |
| treatment x time | (1.87, 18.68) | 0.07 | 0.926 | 0.01 |

**Table S1.24.** $SO_4^{2-}$ absolute leaching (K vs K+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.02 | 0.887 | 0.00 |
| time | (2.49, 24.9) | 174.20 | **< .001** | 0.93 |
| treatment x time | (2.49, 24.9) | 0.10 | 0.942 | 0.01 |

**Table S1.25.** $N\text{-}NO_3^-$ cumulative leaching (NPK vs NPK+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.13 | 0.725 | 0.01 |
| time | (1.16, 11.6) | 101.03 | **< .001** | 0.60 |
| treatment x time | (1.16, 11.6) | 5.40 | **0.035** | 0.08 |

**Table S1.26.** N-$NH_4^+$ cumulative leaching (NPK vs NPK+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.79 | 0.394 | 0.06 |
| time | (1.75, 17.49) | 529.49 | **< .001** | 0.88 |
| treatment x time | (1.75, 17.49) | 2.03 | 0.165 | 0.03 |

**Table S1.27.** $Na^+$ cumulative leaching (NPK vs NPK+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.48 | 0.506 | 0.04 |
| time | (1.39, 13.88) | 618.10 | **< .001** | 0.89 |
| treatment x time | (1.39, 13.88) | 5.42 | **0.027** | 0.07 |

**Table S1.28.** $K^+$ cumulative leaching (NPK vs NPK+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.04 | 0.845 | 0.00 |
| time | (1.25, 12.49) | 276.34 | **< .001** | 0.89 |
| treatment x time | (1.25, 12.49) | 2.06 | 0.176 | 0.06 |

**Table S1.29.** $Mg^{2+}$ cumulative leaching (NPK vs NPK+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 1.10 | 0.320 | 0.09 |
| time | (1.32, 13.18) | 316.93 | **< .001** | 0.71 |
| treatment x time | (1.32, 13.18) | 2.71 | 0.117 | 0.02 |

**Table S1.30.** $Ca^{2+}$ cumulative leaching (NPK vs NPK+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 2.25 | 0.165 | 0.18 |
| time | (1.5, 15) | 362.74 | **< .001** | 0.55 |
| treatment x time | (1.5, 15) | 3.27 | 0.077 | 0.01 |

**Table S1.31.** $Cl^-$ cumulative leaching (NPK vs NPK+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.00 | 0.969 | 0.00 |
| time | (1.12, 11.21) | 72.56 | **< .001** | 0.70 |
| treatment x time | (1.12, 11.21) | 2.74 | 0.124 | 0.08 |

**Table S1.32.** $SO_4^{2-}$ cumulative leaching (NPK vs NPK+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.00 | 0.954 | 0.00 |
| time | (1.17, 11.66) | 229.72 | **< .001** | 0.87 |
| treatment x time | (1.17, 11.66) | 1.39 | 0.269 | 0.04 |

**Table S1.33.** $N\text{-}NO_3^-$ cumulative leaching (NP vs NP+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.06 | 0.813 | 0.01 |
| time | (1.56, 15.61) | 157.95 | **< .001** | 0.61 |
| treatment x time | (1.56, 15.61) | 0.99 | 0.373 | 0.01 |

**Table S1.34.** $N\text{-}NH_4^+$ cumulative leaching (NP vs NP+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.27 | 0.616 | 0.02 |
| time | (2.42, 24.23) | 486.08 | **< .001** | 0.87 |
| treatment x time | (2.42, 24.23) | 0.22 | 0.846 | 0.00 |

**Table S1.35.** $Na^+$ cumulative leaching (NP vs NP+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.17 | 0.691 | 0.02 |
| time | (2.13, 21.33) | 923.31 | **< .001** | 0.90 |
| treatment x time | (2.13, 21.33) | 0.52 | 0.615 | 0.00 |

**Table S1.36.** $K^+$ cumulative leaching (NP vs NP+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.00 | 0.988 | 0.00 |
| time | (1.44, 14.36) | 406.77 | **< .001** | 0.91 |
| treatment x time | (1.44, 14.36) | 0.32 | 0.662 | 0.01 |

**Table S1.37.** $Mg^{2+}$ cumulative leaching (NP vs NP+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.11 | 0.743 | 0.01 |
| time | (1.62, 16.23) | 446.93 | **< .001** | 0.58 |
| treatment x time | (1.62, 16.23) | 0.25 | 0.740 | 0.00 |

**Table S1.38.** $Ca^{2+}$ cumulative leaching (NP vs NP+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.06 | 0.813 | 0.01 |
| time | (1.53, 15.32) | 425.49 | **< .001** | 0.46 |
| treatment x time | (1.53, 15.32) | 0.14 | 0.812 | 0.00 |

**Table S1.39.** $Cl^-$ cumulative leaching (NP vs NP+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 1.25 | 0.290 | 0.08 |
| time | (1.33, 13.3) | 58.05 | **< .001** | 0.61 |
| treatment x time | (1.33, 13.3) | 1.47 | 0.257 | 0.04 |

**Table S1.40.** $SO_4^{2-}$ cumulative leaching (NP vs NP+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.11 | 0.746 | 0.01 |
| time | (2.19, 21.85) | 459.53 | **< .001** | 0.77 |
| treatment x time | (2.19, 21.85) | 0.85 | 0.452 | 0.01 |

**Table S1.41.** $N\text{-}NO_3^-$ cumulative leaching (K vs K+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.02 | 0.885 | 0.00 |
| time | (1.3, 13.04) | 67.02 | **< .001** | 0.35 |
| treatment x time | (1.3, 13.04) | 0.06 | 0.875 | 0.00 |

**Table S1.42.** $N\text{-}NH_4^+$ cumulative leaching (K vs K+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.66 | 0.434 | 0.06 |
| time | (2.15, 21.5) | 792.21 | **< .001** | 0.90 |
| treatment x time | (2.15, 21.5) | 1.42 | 0.265 | 0.02 |

**Table S1.43.** $Na^+$ cumulative leaching (K vs K+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.69 | 0.425 | 0.06 |
| time | (1.64, 16.42) | 698.25 | **< .001** | 0.88 |
| treatment x time | (1.64, 16.42) | 0.33 | 0.681 | 0.00 |

**Table S1.44.** $K^+$ cumulative leaching (K vs K+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.16 | 0.700 | 0.01 |
| time | (1.25, 12.51) | 241.11 | **< .001** | 0.80 |
| treatment x time | (1.25, 12.51) | 0.53 | 0.520 | 0.01 |

**Table S1.45.** $Mg^{2+}$ cumulative leaching (K vs K+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.00 | 0.966 | 0.00 |
| time | (1.18, 11.79) | 138.83 | **< .001** | 0.41 |
| treatment x time | (1.18, 11.79) | 0.02 | 0.915 | 0.00 |

**Table S1.46.** $Ca^{2+}$ cumulative leaching (K vs K+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.17 | 0.690 | 0.02 |
| time | (1.19, 11.95) | 151.64 | **< .001** | 0.34 |
| treatment x time | (1.19, 11.95) | 0.30 | 0.634 | 0.00 |

**Table S1.47.** $Cl^-$ cumulative leaching (K vs K+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.02 | 0.882 | 0.00 |
| time | (1.47, 14.68) | 61.01 | **< .001** | 0.38 |
| treatment x time | (1.47, 14.68) | 0.13 | 0.812 | 0.00 |

**Table S1.48.** $SO_4^{2-}$ cumulative leaching (K vs K+B)

| Source | df | F | p | $\eta_G^2$ |
|---|---|---|---|---|
| treatment | (1, 10) | 0.24 | 0.637 | 0.02 |
| time | (2.17, 21.74) | 480.96 | **< .001** | 0.84 |
| treatment x time | (2.17, 21.74) | 0.06 | 0.953 | 0.00 |

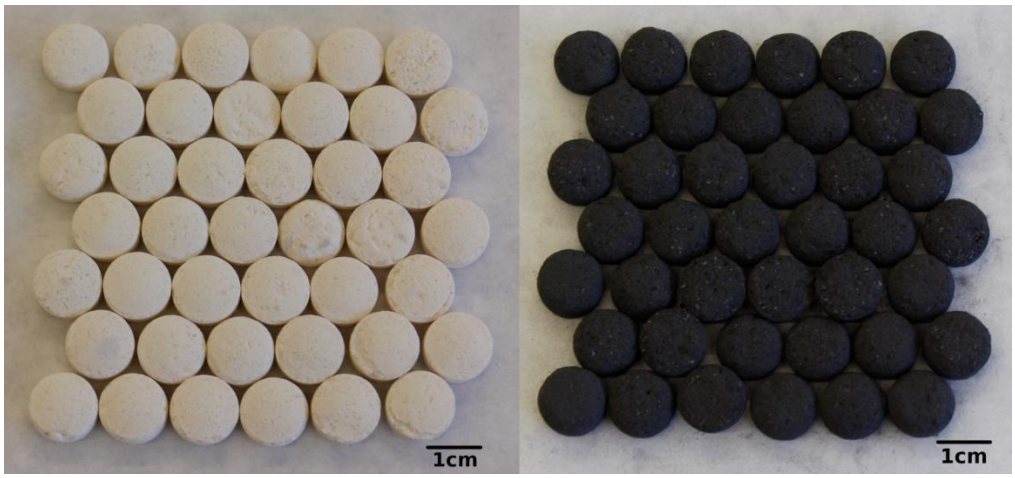


**Fig. S1.** Fertiliser tablets without (left) and with biochar (right).

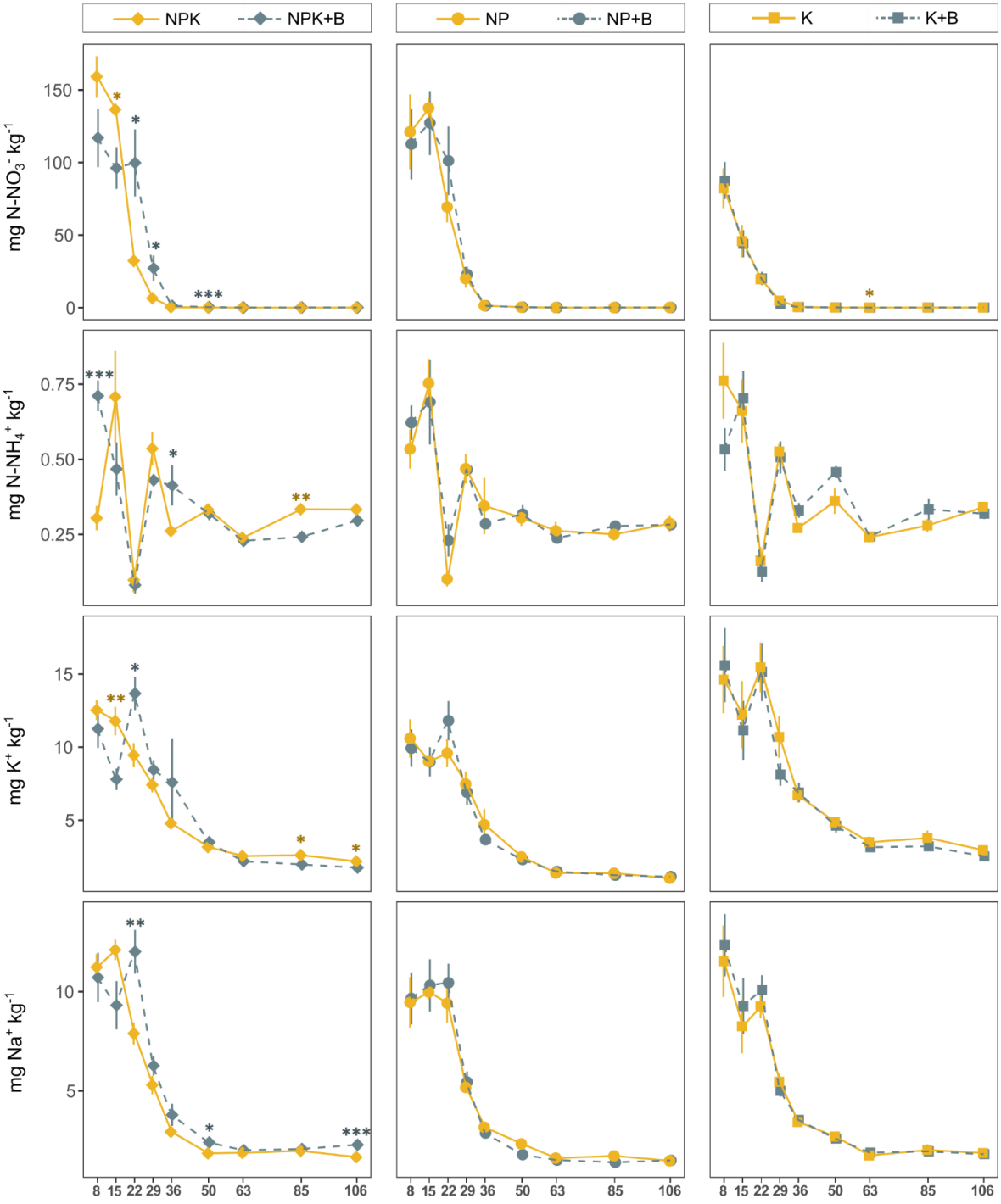


**Fig. S2.** Absolute leaching of $N\text{-}NO_3^-$, $N\text{-}NH_4^+$, $K^+$, and $Na^+$ (mg $kg^{-1}$ dry soil) for the three pairs of organo-mineral fertilisers (NPK vs NPK+B, NP vs NP+B, and K vs K+B) along nine leaching events. *,**,*** indicate significant differences at p values comprised between 0.05 and 0.01, between 0.01 and 0.001, and <0.001, respectively. The colour of the asterisk matches the colour of the treatment with higher leached amount.

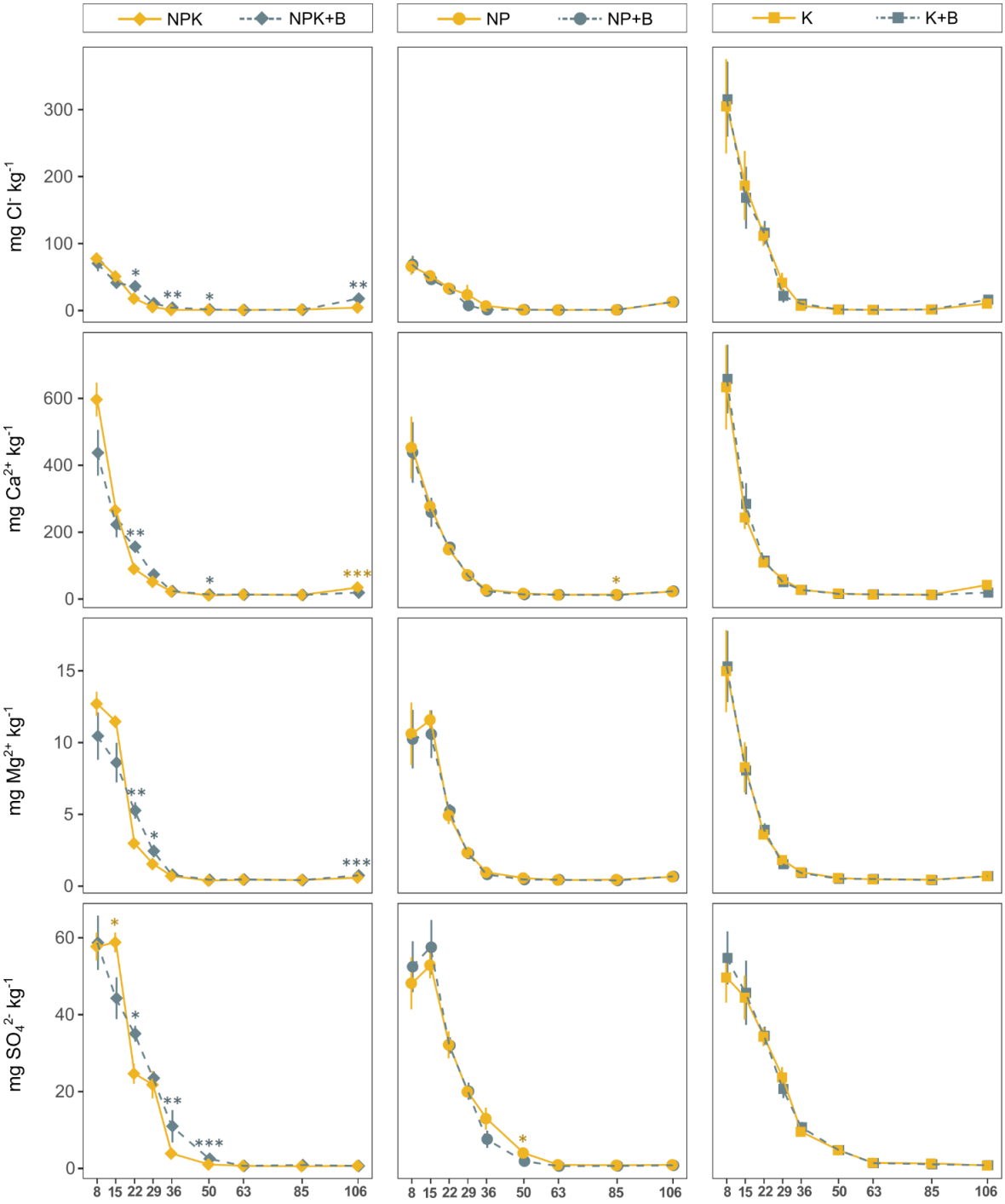


**Fig. S3.** Absolute leaching of $Cl^-$, $Ca^{2+}$, $Mg^{2+}$, and $SO_4^{2-}$ (mg $kg^{-1}$ dry soil) for the three pairs of organo-mineral fertilisers (NPK vs NPK+B, NP vs NP+B, and K vs K+B) along nine leaching events. *,**,*** indicate significant differences at p values comprised between 0.05 and 0.01, between 0.01 and 0.001, and <0.001, respectively. The colour of the asterisk matches the colour of the treatment with higher leached amount.

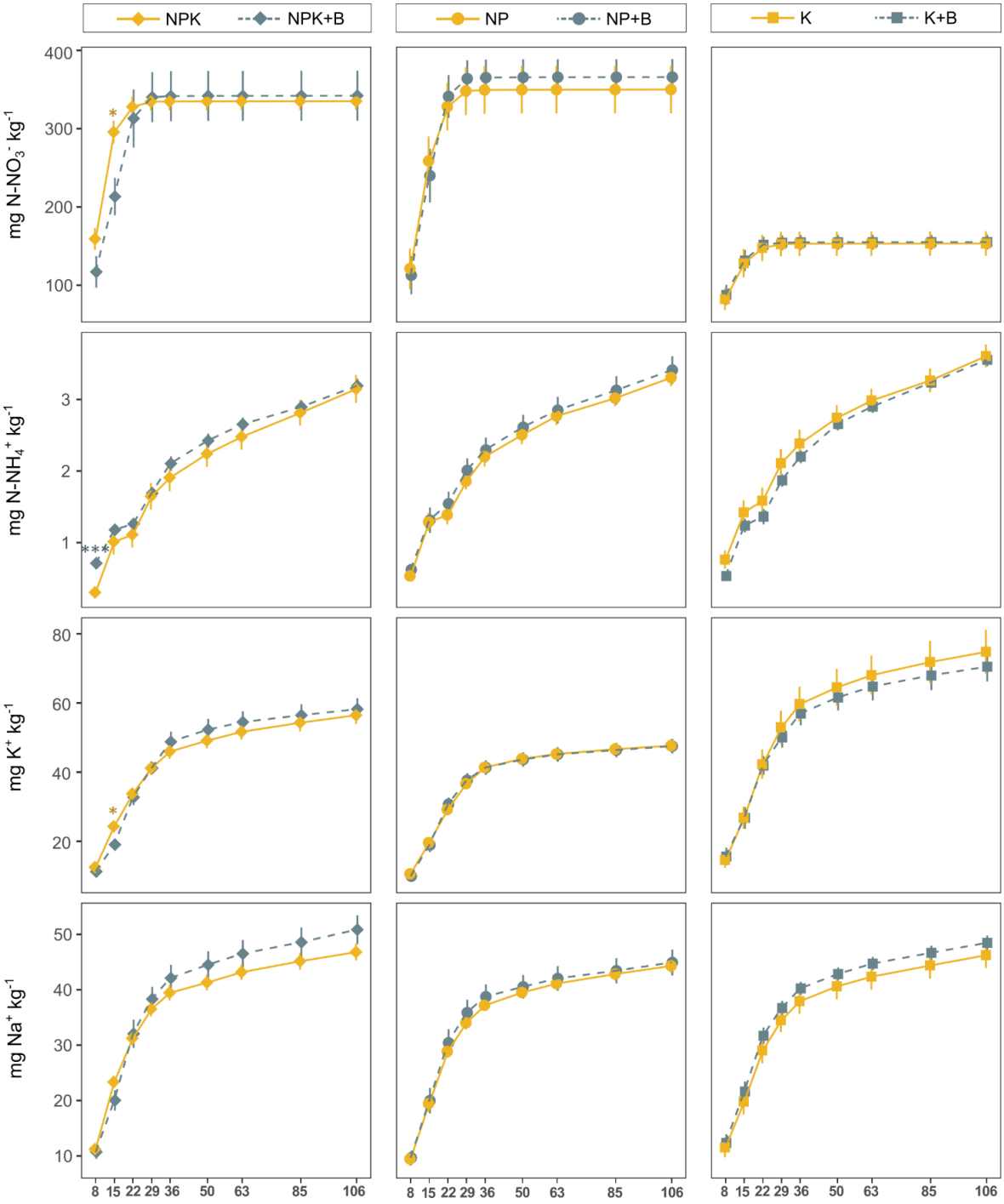


**Fig. S4.** Cumulative leaching of $N\text{-}NO_3^-$, $N\text{-}NH_4^+$, $K^+$, and $Na^+$ (mg $kg^{-1}$ dry soil) for the three pairs of organo-mineral fertilisers (NPK vs NPK+B, NP vs NP+B, and K vs K+B) along nine leaching events. *,**,*** indicate significant differences at p values comprised between 0.05 and 0.01, between 0.01 and 0.001, and <0.001, respectively. The colour of the asterisk matches the colour of the treatment with higher leached amount.

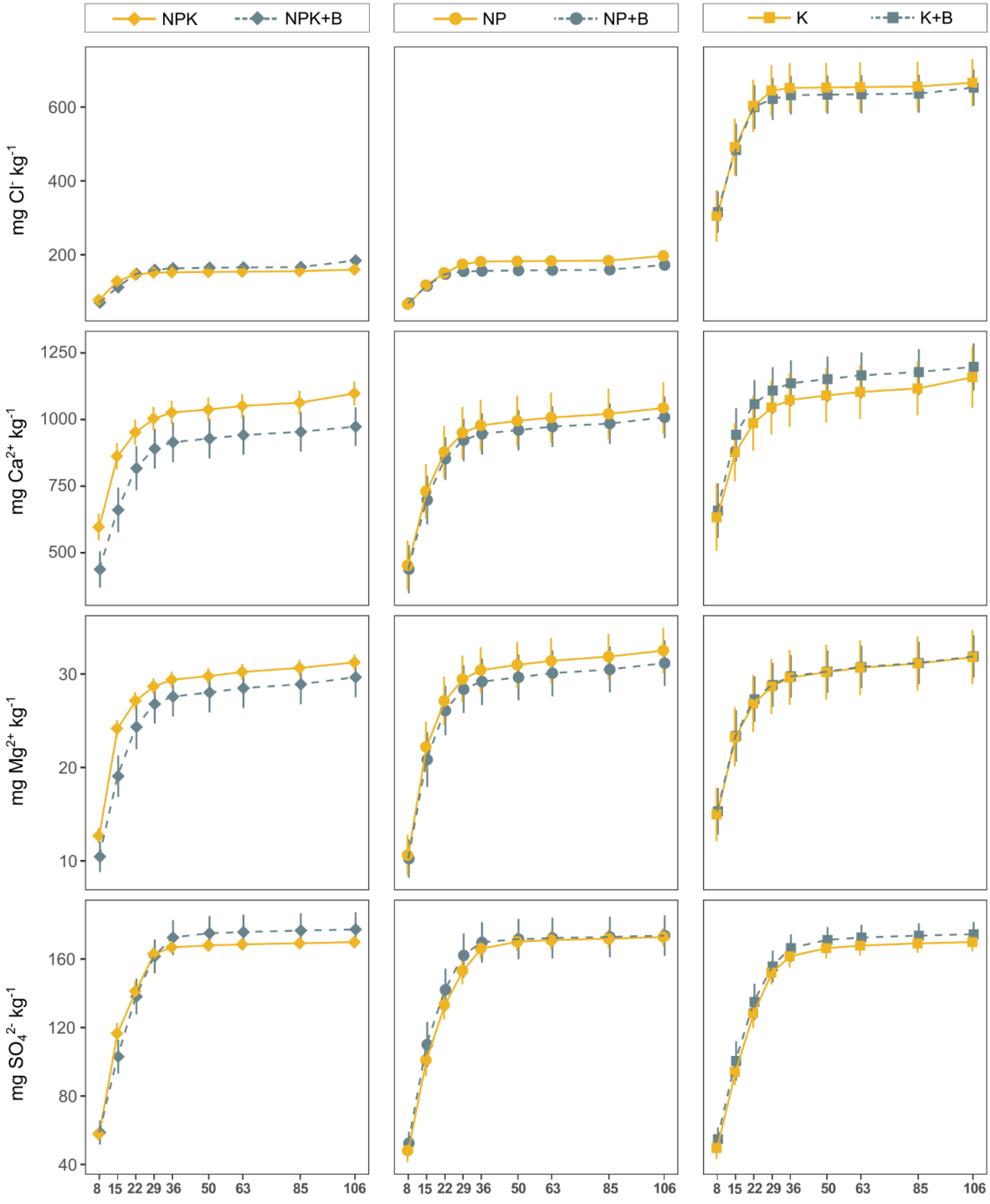


**Fig. S5.** Cumulative leaching of $Cl^-$, $Ca^{2+}$, $Mg^{2+}$, and $SO_4^{2-}$ (mg $kg^{-1}$ dry soil) for the three pairs of organo-mineral fertilisers (NPK vs NPK+B, NP vs NP+B, and K vs K+B) along nine leaching events. There were no significant statistical differences between each pair of fertilisers at each sampling date.

# GENERAL DISCUSSION

## Biochar effects on N cycling as a function of production technology, ageing and application rates

The overarching goal of this thesis was to evaluate how biochar application could improve N cycling in agroecosystems. As discussed in the introduction to this thesis, intensification of agriculture has resulted in enhanced fluxes of reactive N, mainly through $NO_3^-$ leaching and $N_2O$ and $NH_3$ emissions, and biochar soil amendment has the potential to mitigate these three detrimental N losses (Borchard et al., 2019; Hagemann et al., 2016). Nevertheless, our results pointed to main biochar effects on $NO_3^-$ dynamics, while $N_2O$ (Chapter 1 and 2) and $NH_3$ emissions (Chapter 1) were unaffected by biochar addition. Particularly, we demonstrated $NO_3^-$ content alleviation in soil solution in the short term after the application of 12 and 50 t $ha^{-1}$ of a fresh pine gasification biochar (Chapter 1). In addition, when a slow pyrolysis biochar was included as ingredient in the fertiliser investigated in Chapter 3, we detected a $NO_3^-$ leaching delay and a more gradual release pattern. By contrast, the aged pine gasification biochar did not exert significant effects upon $NO_3^-$ (Chapter 1 and 2).

First, we will consider biochar effects on $NO_3^-$ dynamics as explained by its production technology. Two different biochars were examined in the present thesis: a gasification biochar (Chapters 1 and 2) and a slow-pyrolysis biochar (Chapter 3), which were applied as a direct amendment and as a fertiliser ingredient, respectively. Since both biochars had wood-based feedstocks, differences in biochar properties and behaviour in the soil would most likely derive from their production technologies. In this regard, gasification typically yields biochars with a higher degree of aromaticity and aromatic condensation than pyrolysis biochars, as temperatures within the gasifier reactor reach higher values (You et al., 2017). Thus, it is expected that pyrolysis biochars could retain a higher amount of labile C, available to microorganisms. Our results are in agreement with this general trend, namely, the gasification biochar had a much lower H/C ratio (0.14) (thus indicating higher aromaticity; Lopez-Capel et al., 2016) than the slow pyrolysis biochar (0.41). Hence, the $NO_3^-$ leaching delay mediated by the slow pyrolysis biochar-based fertiliser was consistent with a microbial immobilisation mechanism coupled to microbial biomass growth, whereas the reduction in soluble $NO_3^-$ found for the gasification biochar was ascribed to an organo-mineral organic coating (after ruling out the effect of other potential mechanisms proposed in the available literature, also including microbial immobilisation).

Another important factor to take into account for understanding biochar-$NO_3^-$ interactions is biochar ageing. One the one hand, some studies have proposed that biochar mainly alters N cycling in the short-term (less than one year), possibly due to the rapid and transitory effects of any labile C introduced by biochar or as a result of biochar pore-blocking, and therefore inactivation with ageing (Borchard et al., 2019; Gao et al., 2019; Mukherjee et al., 2011). On the other hand, other research suggests that biochar is more prone to show effects in the long-term. Specifically, one commonly cited mechanism involves biochar oxidation over time, thus increasing its CEC and, consequently, enabling $NH_4^+$ retention (Cheng et al., 2006; Liang et al., 2006). While Chapter 3 pointed to immediate biochar effects driven by biochar labile C content, results in Chapter 1 are in line with those that suggest that biochar need some time before affecting N cycle. However, the formation of an organo-mineral coating (observed eight months after biochar application) was the potential underlying mechanism for $NO_3^-$ retention, instead of CEC enhancement with biochar ageing. Despite this time-dependent effect, it should be noted that the 6-yr aged biochar was not capable of reducing $NO_3^-$ contents (Chapter 1 and 2). Indeed, in field mesocosms, $NH_4^+$ contents in soil solution were increased at the 50 t $ha^{-1}$ biochar rate, indicating either a pore blockage or changes in N cycling over time (Chapter 2).

Biochar application rate can add further variability on $NO_3^-$ dynamics. In Chapter 1, $NO_3^-$ soil solution reduction did not increase with application rate, indicating that some biochar responses are non-linear and therefore difficult to predict. Finally, it must be considered that even the same biochar-soil-plant-system could elicit different responses. Namely, in the study of Marks et al. (2016), $NO_3^-$ reduction was not accompanied by any other nutrient reduction so it is likely that a specific N-cycling effect acted. On the other hand, in Chapter 1, $NO_3^-$ reduction was accompanied by a chloride, sodium, calcium and magnesium soluble contents decrease, indicating a non-specific N-related mechanism.

## Is gasification biochar application safe at the tested amendment rates?

According to the soil function-based quality assessment conducted in Chapter 2, the most responsive parameters to biochar application were C sequestration and soil food web functioning, the former being increasingly promoted with biochar rate, and the latter impaired at the highest biochar rate.

On the one hand, the results concerning enhanced C sequestration after 6 years of biochar amendment to soil are not surprising, since biochar presents residence times within the centurial to millenial timescale. Specifically, highly aromatic biochars with important stable C contents, such as the studied gasification biochar, are more prone to this response (Schmidt and Noack, 2000; You et al., 2017). This biochar effect could be of crucial importance in Mediterranean areas such as the studied agroecosystem, with typically low SOC contents (Aguilera et al., 2013; Romanyà and Rovira, 2011).

On the other hand, the harmful effects of the highest biochar application on soil food web functioning, i.e., reduced functional diversity of nematodes and collembola, are less straightforward to understand and more research is warranted to elucidate the causative mechanisms. Previous research from our group also recorded negative effects on collembolans as a response of the same gasification biochar in laboratory conditions (although at higher application rates than the ones here investigated) (Marks et al., 2014), which were attributed to an excessive alkalinisation as a subjacent mechanism. Related to this, it is noteworthy that no biochar pH-induced changes were found in our study, indicating that alkaline biochar application in already alkaline soils does not necessarily result in further alkalinisation of soils, as suggested elsewhere (IBI, 2021). As stated by Alburquerque et al. (2015), alkaline soils with calcareous nature could prevent pH shifts due to its high buffering capacity.

Although the highest biochar rate in this study (50 t $ha^{-1}$) sequestered more carbon and presented transient improvements in moisture retention, it also exceeded the biochar loading capacity, i.e., it significantly compromised the soil nematode and collembolan communities. Furthermore, methane net emission was promoted at the highest biochar rate in contrast with the medium biochar rate, which acted as a sink. All things considered, the medium application rates of biochar of the monitored gasification biochar (12 t $ha^{-1}$) seem safer to apply as those detrimental effects were not observed. This suggestion concurs with the findings of a Liu et al. (2018) who conducted a meta-analysis exploring biochar effects on N cycling and concluded that biochar application rate should not exceed 40-80 t $ha^{-1}$, being the optimum 10-40 t $ha^{-1}$.

## Opportunities to improve agroecosystem management: reconciling C and N cycles

Whether high rates of synthetic nitrogen fertiliser cause net losses of SOC and SON is a hotly debated topic (Khan et al., 2007; Mulvaney et al., 2009; Powlson et al., 2010; Reid, 2008). Regardless of these controversies, excessive N inputs without C additions clearly lead to a decoupling of these two highly intertwined elements in natural systems. In a meta-analysis performed by Gardner and Drinkwater (2009), which investigated the fate of N inputs in temperate grain agroecosystems, it was found that management practices focused to improve nutrient use efficiency of inorganic fertilisers (such as appropriate application timing) were less efficient in N recovery (both in crop and soil) than the ones aimed at re-coupling N and C (such as planting cover crops, returning crop residues to the soil, or adding manure).

Nevertheless, C and N re-coupling strategies are not exempt from trade-offs in both N and C cycles. As an illustration, crop residue return has been proved to increase ammonia volatilisation losses at a global scale in comparison with mineral N fertilisers (Xia et al., 2018), and can also cause C loss via positive priming (Liang et al., 2012). In this regard, conversion of crop residues or manure into biochar (as well as from other feedstocks), could be a more effective way of re-coupling C and N cycles, as it offers greater potential for carbon sequestration (Wang et al., 2016) while with lower risks of GHG emissions and positive priming (Bolan et al., 2021). Despite the above-mentioned benefits of biochar, it has the drawback of having little fertiliser value when applied on its own, especially in already fertile soils or alkaline soils where liming it is not necessary. For this reason, complementing C-rich substrates (biochar) with N-rich products (e.g., by composting or by blending biochar with inorganic fertilisers) is a management practice advocated for optimising multiple soil functions at a time (Joseph et al., 2013; Kammann et al., 2016).

Results from the present work indicate that biochar is a promising tool to formulate new-generation fertilisers (Chapter 3). The most plausible underlying mechanism being microbial immobilisation and the subsequent turnover of microbial biomass and nutrients stored within their cells. Thus, this type of biochar application could be ascribed to C-N coupling strategies that intensify internal N cycling (immobilisation) (Gardner and Drinkwater, 2009; Norton and Ouyang, 2019). However, further research is needed in order to not provoke an unintentional excessive nutrient retention in microbial biomass with this type of biochar-based fertiliser. In

another direction, regarding the gasification biochar, if the organo-mineral coating hypothesis suggested in Chapter 1 could be confirmed it should also be of great interest to corroborate if the nutrient-rich water entrapped in pores would be available to plants as suggested by Kammann et al. (2015).

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## General conclusions

### Chapter 1

- Fresh application of a pine gasification biochar provoked a significant decrease in soil $NO_3^-$ contents (69 % at the 12 t $ha^{-1}$ rate and 64% at the 50 t $ha^{-1}$ rate), as well as of other soluble ions, including $Cl^-$, $Ca^{2+}$, $Mg^{2+}$ and $Na^+$. Conversely, no effects were found at the aged biochar scenario.

- We were unable to found empirical evidence of biochar influence in commonly cited explanatory mechanisms for soluble $NO_3^-$ reduction, such as sorption, leaching, ammonia volatilisation, microbial immobilisation, reduced mineralisation, denitrification, plant export, or ecotoxicological effects on biological groups regulating N-cycling.

- Given that the ionic content reduction affected multiple ions and was observed after eight months of biochar application, the formation of an organo-mineral coating, which potentially entrapped water and nutrients into biochar pores, emerged as a plausible underlying mechanism. On the other hand, excessive pore occlusion in the longer term could explain the lack of effects in the aged biochar scenario.

- Although $NO_3^-$ leaching was not reduced in practice, the lower $NO_3^-$ concentrations in soil solution found for the fresh biochar treatments are highly desirable. Namely, further leaching events could indeed wash away the $NO_3^-$ contained in topsoil solution, especially at the post-harvest period, when plant uptake is ceased and N loss risk is the highest.

### Chapter 2

- A generalised SOC loss in all the treatments occurred after 6 years of mesocosm establishment and it was associated to a continued cropping in a field previously under fallow. However, SOC contents were higher in biochar treatments and increased linearly with biochar addition rate. Nevertheless, we found evidence for biochar stimulation of recalcitrant decomposition at the 12 t $ha^{-1}$ treatment, thereby implying a possible reduction of C sequestration potential in a longer-term.

- Effects on N cycling were minor, the only significant result being the concentration decrease of soluble $NH_4^+$ at the 50 t $ha^{-1}$ treatment at the post-harvest period. Nevertheless, the total mineral N concentration at this period did not raise concerns for important N leaching risks.

- Biochar application did not alter $CO_2$-equivalent emissions (including $CO_2$, $N_2O$, and $CH_4$). However, the system shifted from being a $CH_4$ sink at the 12 t $ha^{-1}$ treatment, to a net source at the 50 t $ha^{-1}$ treatment.

- Soil food web functioning was impaired by the highest biochar treatment, indicating a risk to soil processes mediated by soil invertebrates. Concretely, there was a reduction in the omnivore nematode footprint and a concomitant increase of the fungivore and herbivore footprints, indicating a loss of functionality of higher soil food web levels and a higher risk of pest pressure. Furthermore, functional diversity of collembolans was also impaired.

- Although this gasification biochar is expected to be quite stable and therefore of interest for carbon sequestration, its utilisation at high application rates poses serious threats to biologically-mediated soil processes. Despite the soil quality index did not point to a biochar scenario being more favourable than the other, we conclude that the medium biochar rate is safer to apply.

**Chapter 3**

- Biochar inclusion in fertiliser formulation in the NPK+B fertiliser resulted in higher efficiency since nutrient release that was not reduced in quantity but was slowed down. This effect could potentially permit a more gradual nutrient release meeting plant demands and preventing undesirable N losses.

- Microbial immobilisation of nutrients and posterior release upon microbial turnover was postulated as the most likely mechanism. Specifically, labile C supplied with biochar (either by its concurrent addition with NPK or by its own effects) could be behind this initial boosting of microbial populations.

- Straw growth was enhanced at the NPK+B treatment whereas grain biomass was only marginally enhanced. On the other hand, nutrient content and export was improved in all biochar-based fertilisers (NPK+B, NP+B, and K+B), mainly in relation to K, Ca, S, and Mn.

“Soil animals exist.

I like soil animals.

They respire too little.

Ergo, they must CONTROL something!”

``Faunophilic logic'', 1987, Andrén, O.