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The use of biochar for soil fertility improvement and increase of crop production

Dissertation thesis

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DECLARATION

I, Nikola Teutscherova, declare that the content presented in this thesis entitled The use of biochar gor soil fertility improvementand increase of crop production, submitted as a partial fulfillment of the requirements for the Ph.D.at Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague, is my own work, unless listed in references or acknowledgements sections.

Furthermore, I declare that no part of this work is being submitted for any other degree to this or any other university.

Prague, August, 2018

Nikola Teutscherova

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LIST OF ABBREVIATIONS

AOA	ammonia-oxidizing archaea
AOB	ammonia-oxidizing bacteria
C	carbon
C_{ox}	potassium dichromate oxidizable carbon
C1	compost at the end of bio-oxidative phase
C2	mature compost
CEC	cation exchange capacity
EC	electric conductivity
EEAs	extracellular enzymes activities
GLM	general linear model
GMM	general mixed model
INT	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride
MBC	microbial biomass carbon
MBN	microbial biomass nitrogen
N	nitrogen
NN	net nitrification
NNM	net nitrogen mineralization
P	phosphorus
PCA	principal components analysis
PE	priming effect
qPCR	real-time polymerase chain reaction
SBR	soil basal respiration
SIR	substrate-induced respiration
SOM	soil organic matter
SW	south-west
TIN	total inorganic nitrogen
TC	total carbon
TN	total nitrogen
TOC	total organic carbon
WHC	water holding capacity
WSA	water-stable aggregates
WSC	water-soluble carbon
WSN	water-soluble nitrogen

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ABSTRACT

Biochar, a carbon-rich material obtained by pyrolysis of organic matter, has been receiving wide scientific and public attention due to its positive effect on soil properties and plant growth. Furthermore, owing to its recalcitrance it is considered to be one of the most promising means of carbon sequestration in agricultural soils. Nevertheless, despite the rapidly increasing number of studies focusing on the influence of biochar on soil fertility, there are still many uncertainties and obtained results are often ambiguous. The aim of the present thesis was detailed evaluation of the effects of biochar produced from holm oak pruning waste on soil nutrient transformations in two contrasting Mediterranean soils. Selected soils were (i) acid degraded Acrisol collected from SW Spain characterized by low pH and high Al availability, and (ii) alkaline Calcisol from central Spain which is commonly used for intensive agriculture. Biochar application to Acrisol caused pH raise which was linked to changes in microbial biomass and activity and was reflected in the enhanced nitrogen (N) mineralization and leaching of N from the soil. While mineralization of nitrogen was stimulated immediately after biochar application, nitrification seemed to be limited by other factors in the studied soil. Thus, the temporal decoupling of ammonification and nitrification caused losses of nitrogen as ammonium rather than in nitrate form. Nevertheless, this effect was rather short-term and no difference between control and biochar amended soil was found in the later stages of soil-biochar incubation, probably as a result of exhaustion of easily decomposable compounds. In alkaline Calcisol, the effect of biochar was much less pronounced, which highlights the importance of liming capacity of biochar. Nevertheless, no negative impact of biochar was detected and soil organic carbon content was increased, suggesting the utilization of biochar as a mean of carbon sequestration. In both soils, the stability of water stable aggregates was increased by biochar in the aerobic incubation, but was reduced in the column leaching experiment, which highlights the lack of our understanding of the biochar effects on soil properties, which is necessary for large-scale biochar implementation in agriculture.

Key words: Acrisol, biochar, Calcisol, extracellular enzymes, nitrificaftion, nitrogen leaching, nitrogen mineralization

SOUHRN

Biouhel, materiál bohatý na uhlík získaný pyrolýzou organické hmoty, získal širokou vědeckou a veřejnou pozornost kvůli pozitivnímu vlivu na vlastnosti půdy a růst rostlin. Dále je vzhledem k jeho vysoké stabilitě považován za jeden z nejslibnějších způsobů sekvestrace uhlíku v zemědělských půdách. Přesto, navzdory rychle rostoucímu počtu studií zaměřených na vliv biouhlu na úrodnost půdy, stále existuje mnoho nejistot a získané výsledky jsou často nejednoznačné. Cílem této práce bylo podrobně vyhodnotit účinky biouhlu vyrobeného z dubu cesmínového na transformaci živin půdy ve dvou kontrastních středomořských půdách. Vybrané půdy byly: (i) degradovaný Acrisol z jihozápadního Španělska, jež je charakterizován nízkým pH a vysokou dostupností Al, a (ii) zásaditý Calcisol z centrálního Španělska, který se běžně používá k intenzivnímu zemědělství. Aplikace biouhlu do Acrisolu vedla ke zvýšení půdního pH, jež bylo spojeno se změnami mikrobiální biomasy a aktivity a odrazilo se ve zvýšené mineralizaci dusíku (N) a vyluhování N z půdy. Zatímco mineralizace dusíku byla stimulována bezprostředně po aplikaci biouhlu, nitrifikace byla ve studované půdě omezena jinými faktory. Časové oddělení amonifikace a nitrifikace tedy způsobilo ztráty dusíku v amoniakální formě spíše než ve formě dusičnanů. Nicméně tento účinek byl spíše krátkodobý a v pozdějších fázích inkubace nebyl zjištěn žádný rozdíl mezi kontrolní půdou a půdou s přídavkem biouhlu, pravděpodobně v důsledku vyčerpání snadno rozložitelných organických látek. V alkalickém Calcisolu byl účinek biouhlu mnohem méně výrazný, což poukazuje na důležitost změny půdního pH po aplikaci biouhlu na mikrobiální aktivitu půdy. Nebyl však zjištěn žádný negativní vliv biouhlu a obsah půdního organického uhlíku se zvýšil, což naznačuje využití biocharů jako prostředku sekvestrace uhlíku. V obou půdách byla aplikací biohlu zvýšena stabilita půdních agregátů při aerobní inkubaci, ale snížena v půdních sloupcích s častým promýváním, jež poukazuje na nedostatky v našich znalostech vlivu biouhlu na půdní vlastnosti, jež jsou nezbytné pro zavedení velkoplošného užívání biouhlu v zemědělství.

Klíčová slova: Acrisol, biouhel, Calcisol, extracelulární enzymy, nitrifikace, mineralizace dusíku, vymývání dusíku

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1 INTRODUCTION

Hidden in the Amazonian rainforest, scattered throughout the Amazon basin in rather unfertile tropical soils, dark patches of soils, formed by consistent and planned agricultural practices, were found by Francisco de Orellana in 1542. Unlike the adjacent Ferralsol and Acrisol, which are of considerable paler color and lower productivity, these dark patches have been observed to sustain intensive agriculture and large populations for centuries (Myers et al. 2003a). These soils, known as Amazonian dark Earths or Terra Preta, are the focus of intensive debate during the last years (Myers et al. 2003b) and many researchers intensively seek the way to mimic the formation of Terra Preta. The anthropogenic formation of Terra Preta is now widely accepted and the soil is believed to be the result of long-term accumulation of charcoal, kitchen waste, human feces, ceramics and other artifacts (Meggers 2001; Petersen et al. 2001a) and of the thermal conversion of organic matter into charcoal-like materials during slash-and-char agriculture (Mann 2002). Nevertheless, up to now no attempts have been fully successful and resulted in the formation of soils of such agronomic value as Terra Preta.

Charcoal is considered the main ingredient contributing to the dark color (Kern et al. 2003a) and high porosity (Tryon 1948; Piccolo et al. 1996). Furthermore, biochar prevents leaching of soil nutrients from the root zone due to its high sorptive capacity (Steiner et al. 2008, 2009). During the last years, the scientific literature evaluating the potential of charcoal as soil amendment increased exponentially (Lehmann et al. 2015a). Charcoal produced from organic residues for the purpose of application to soil has been named biochar to be differentiated from commercial charcoal used for energy production. Biochar application to soil has vast influence on innumerable soil properties including the changes induced by its porosity and low bulk density with impact on soil water movement (Tryon 1948; Piccolo et al. 1996; Sika & Hardie 2014), alteration of soil pH due to usually high alkalinity of biochar (Steinbeiss et al. 2009; Zhao et al. 2014; Teutscherova et al. 2017a), addition of base cations originating from the ash content of biochar or influence on soil biological activity (Steinbeiss et al. 2009; Rutigliano et al. 2014; Tian et al. 2016; Teutscherova et al. 2017a, 2017b).

Biochar is produced by pyrolysis of organic matter at high temperature with limited access of oxygen. During this process, the majority carbon (C) is transformed into recalcitrant carbonaceous forms which are less available to soil microorganisms (Wang et al. 2015a). Nevertheless, small part of C available for soil biota can have an enormous impact on soil transformation processes after biochar application (Zimmerman et al. 2011). For instance, the

increase of available C after biochar addition to soil may stimulate the microbial populations and lead to increased decomposition of soil organic matter (SOM), a phenomenon known as priming effect (PE) (Zimmerman et al. 2011). If biochar addition to soil stimulates the oxidation of native or co-applied SOM, the value of biochar to sequester C would be reduced with possible implication for nutrient cycling and soil properties.

The tight coupling of C and nitrogen (N) cycling in soil also indicates the impact of carbonaceous materials on N transformations. Biochar, rich in C of variable recalcitrance and relatively poor in N, has been observed to affect N mineralization (Ameloot et al. 2015; Teutscherova et al. 2017a), nitrification (Ulyett et al. 2014; Teutscherova et al. 2017a), N fixation (Mia et al. 2014), ammonia (NH_3) volatilization (Mandal et al. 2016), N adsorption and leaching (Laird et al. 2010; Teutscherova et al. 2018a) and denitrification (Cayuela et al. 2013). While biochar additions have been reported to lead to N immobilization, also microbial mining for N and linked N mineralization can occur.

In acid soils, where microbial activity is limited by low pH, soil neutralization by biochar application may lead to enhancement of biological activity (Nugroho et al. 2007; Ulyett et al. 2014; Che et al. 2015), SOM mineralization and production of ammonium (NH_4^+) (Teutscherova et al. 2017a), and consequently, increased oxidation of NH_4^+ to nitrate (NO_3^-) which is highly mobile and prone to leaching (Priha & Smolander 1995; Che et al. 2015), which can result in a severe agronomic drawback of biochar, especially in times when plant N uptake is limited. Furthermore, soil type is the key determinant of biochar-induced changes in soil resulting from changes in soil physical and chemical properties or changes in the abundance and/or structure of microbial community.

Despite its recalcitrance in the soil and long residence time of up to thousands of years (Atkinson et al. 2010), the inherent properties of biochar change in time since application (Hale et al. 2011) as a consequence of superficial oxidation and changes in functional groups on biochar surface (Gai et al. 2014). Furthermore, the losses of base cations from the ash content may impact the properties of soil-biochar mixture. Thus, periodic evaluation of biogeochemical processes after biochar addition to soil should be advisable in order to elucidate the dynamic biochar-induced changes in soil.

Therefore, the aim of the present thesis is the evaluation of the effects of holm oak biochar produced at temperature 600 °C on decomposition of SOM (Chapter 5) and soil microbial activity in relation to plant growth (Chapter 10) and on mineralization of soil

organic N and nitrification rates in two contrasting soils differing in texture and soil pH (Chapter 6 and Chapter 7). Furthermore, the changes induced by biochar application to soil are studied in relation to changes in organic C pools and soil pH with a special emphasis on the evolution of biochar-induced changes in time since biochar application (Chapter 9). Furthermore, the leaching losses of other nutrients, mainly base cations, were evaluated in relation with soil aggregates stability (Chapter 8).

2 LITERATURE REVIEW

2.1 Amazonian dark earths

2.1.1 Origin and distribution of Amazonian dark earths

Amazonian Dark Earths (ADE), in Portuguese *Terra Preta de Índio* or *Terra Preta do Índio*, are anthropogenic soils formed by pre-columbian settlers in the Amazonian basin. They were found scattered throughout the Amazon in the close proximity to human settlements by Francisco de Orellana. Although the particular human activities leading to the formation of patches of dark and highly fertile soils remain largely unknown (Meggers 2001; Petersen et al. 2001b; Mann 2002) as well as whether the creation of such soil was intentional or not, it is generally accepted that several actions taking place simultaneously lead to soil enrichment in nutrients and organic matter and the creation of soil type which can be clearly distinguished from the adjacent soils. Unlike the predominant soil types (Acrisols, Lixisols, Ferralsols and Arenosols according to WRB taxonomy) occurring in the Amazon basin, the ADE are known for their persistent fertility, dark color and high productivity, caused by accumulation of debris of human occupation (Kern et al. 2003b), including high concentrations of charcoal, animal and fish bones, pot-shreds, manure, shells, excrements and urine etc. around the former settlements located in the proximity to the river Amazon (Mann 2002). Thus, intensive nutrient depositions and charring of organic materials, such as garden residues, seem to have played a crucial role. The thermal conversion of organic residues in the absence of oxygen was the key process in formation of these nutrient rich soils with high agronomic value until the recent days. Such techniques, termed slash-and-char (Mann 2002), result in less pollution while converting organic material into recalcitrant carbonaceous form similar to charcoal with the capacity to increase soil physical, chemical and biological properties.

Since the discovery and description of ADE in the Amazon basin, soils with similar properties and origin have been found also in Africa where they are, similarly to Amazon basin, distributed around the human settlements in otherwise unfertile soils. Nevertheless, our knowledge of the agro-ecological practices responsible for the formation of “African Dark Earths” remains limited with the implications for sustainable agriculture planning in tropical Africa being largely unexplored.

2.1.2 Properties and fertility of Amazonian dark earths

During hundreds or thousands of years of human-induced soil amelioration practices, these soils underwent a drastic transformation from the visual, physical, chemical and biological point of view. Shortly after the adoption of sustainable farming practices, such as accumulation of organic matter and charred materials, changes in the abundance and activity of soil macrofauna (soil invertebrates larger than two mm) occur with implications for the decomposition and mixing of the materials within the soil profile (Ponge et al. 2006). Changes have been observed particularly in faunal groups considered as "soil engineers", such as earthworms and ants (Fairhead & Leach 2009), which are responsible for soil structure formation including the stabilization of soil aggregates and soil porosity maintenance which is linked to improved water infiltration and aeration (Zangerlé et al. 2011). Consecutively, improved soil properties result in increased biomass production and higher SOM mineralization rate resulting from the action of soil macrofauna and micoorganisms further stimulating the plant growth by the acceleration of the nutrient release. The increase of both the quantity and the quality of SOM (Liang et al. 2006) plays a role in the enhanced CEC and consequent reduction of nutrient leaching (See Glaser and Birk (2012) and the references therein).

Higher productivity of ADE when compared to common Amazonian soils has been well established (Lehmann et al. 2003) and seem to be responsible for the development and maintenance of highly populated areas, which would not be feasible in the absence of highly productive intensive agriculture (Glaser et al. 2004).When compared to the adjacent soils, where the required fallow period between two consecutive growing seasons ranges between eight and ten years, farming practices on ADE can be sustained with six months of fallow period (German 2004), which could be linked to the reduced necessity to deforest extensive rainforest areas.

2.1.3 Model of sustainable agriculture in tropics

Owing to high and persisting fertility, ADE are one of the most valuable examples of sustainable agriculture in the tropics. Therefore, the conditions under which such soils were formed are a critical research topic with implications for soil degradation mitigation strategies worldwide. The discovery and analysis of ADE resulted in many attempts of their recreation, often by application of organic materials and charcoal to the soil (Lehmann et al. 2015b). The exponentially growing interest of soil and environmental scientists led to the formation of the

term biochar, to distinguish charcoal used as a fuel from charcoal materials produced from residual materials for soil properties improvement and C sequestration in the soil.

Techniques similar to those of pre-columbian populations have the potential to not only increase the fertility of soil and thus drastically improve the productivity, but also deal with the problems related to residues management. Recently, many attempts have been made to re-create ADE, without a particular success, probably due to the complexity of factors involved in the formation of these soils with unprecedentedly long-lasting quality and soil health. Nevertheless, charcoal particles have been identified as the key ingredient with a crucial role in nutrient retention and the prevention of nutrient losses. Biochar, a form of charcoal produced from sustainable source of biomass with the purpose of being applied to soil as soil conditioner, is gaining exponentially increasing amount of attention each year due to its positive effect on soil physical (Herath et al. 2013; Lu et al. 2014; Martinsen et al. 2014), chemical (van Zwieten et al. 2010; Akça & Namlı 2015) and biological properties (Christianson et al. 2010; Lehmann et al. 2011a; Huang et al. 2017). In the recent years, biochar has been cited by many authors to be the win-win-win solution (Laird 2008) with positive impact on soil health and the environment while simultaneously contributing to the recycling of organic materials and to the return of the nutrients to the soil.

2.2 The use of biochar in modern agriculture

2.2.1 Biochar production and properties

Biochar is produced from organic materials under high temperature with limited or no access of oxygen. In such way, the majority of the C proceeding from the plant biomass converts into a recalcitrant form with lower suasceptibility to microbial degradation and longer persistence time (Kuzyakov et al. 2009). In general, biochar has higher porosity and lower decomposition rates when compared to its original feedstock material. Nevertheless, both feedstock and the peak pyrolysis temperature determine the final properties with crucial implications for the effect of biochar on soil properties (Novak et al. 2009).

Feedstock material has been observed by several studies to have an impact on biochar surface characteristics such as surface area, surface functional groups or biochar pH. Zhang et al. (2017) found that the feedstock type determined the electric conductivity and the ash, manganese (Mn), potassium (K), iron (Fe), N, P, calcium (Ca), sodium (Na) and magnesium (Mg) contents, while the content of oxygen (O), hydrogen (H), C, volatile matter and fixed matter as well as pH were influenced by biochar production temperature. In other study (Sohi

et al. 2010), feedstock significantly affected the magnitudes of surface area, pores and functional groups, and consequently, sorption characteristics of biochars.

The pyrolysis temperature, especially the peak temperature during the pyrolysis process, is considered the key factor affecting the stability and sorption capacity of many biochars (Uchimiya et al. 2011b). The pyrolysis at lower temperatures generally results in biochar of lower pH and specific area and with high superficial carboxylic and phenolic hydroxyl functional groups and high CEC (Zhao et al. 2017). On the other hand, higher temperatures promote more efficient carbonization with higher biochar production rates and biochar with high C content and low hydrogen (H) and oxygen (O) contents (Zhang et al. 2017).

2.2.2 The effect of biochar on soil properties and plant growth

2.2.2.1 Physical and chemical properties of biochar-amended soils

The application of highly porous material with low bulk density can clearly impact the aeration and drainage characteristics of soil. Indeed, dramatic changes in soil physical properties induced by biochar application have been reported (Tryon 1948; Herath et al. 2013; Mukherjee & Lal 2013; Obia et al. 2016). The discovery of high porosity (Liang et al. 2006) and surface area (Sigmund et al. 2017) of charred materials, together with the potential impact of biochar application on soil aggregation (Ouyang et al. 2013; Soinne et al. 2014) suggested changes in soil water-holding capacity (WHC) and water movement in the soil profile. At field capacity, biochar application to soil has been observed to affect available water content and/or WHC capacity (Chan et al. 2007). The alteration of water retention in soil may have strong implications for nutrient adsorption and transformations, and, consequently, for nutrient losses via leaching. Several studies have reported a significant reduction of leachate volume when biochar was applied (Sika & Hardie 2014; Sorrenti & Toselli 2016; Xu et al. 2016) as a result of increased water retention, improved soil structure and soil aggregation (Yoo et al. 2014). The addition on C contained in biochar has been reported to facilitate the formation of water-stable aggregates (WSA) (Annabi et al. 2011; Lu et al. 2014), which in turn has been observed to positively affect crop production (Amézketa 1999). Furthermore, besides its highly organized structure, biochar usually contains ash on its surface, which can increase the hydraulic conductivity of soil (Chang et al. 1997) or clog soil pores after swelling when in contact with water (Etiégni & Campbell 1991). Nevertheless, this effect is likely temporal and disappears when ash is leached from the soil.

Pyrolysis induces the formation of large amount of surface functional groups which are directly linked to biochar properties such as electric conductivity (EC) and pH (Li et al. 2013) or sorption capacity (Uchimiya et al. 2011a) and together with ash content lead to often observed changes in soil pH. High quantities of ash may also increase the agronomic value of soils of poor quality, especially in degraded acid soils with low concentrations of base cations (Rajkovich et al. 2012). The changes in soil pH after biochar application to soil may also impact the availability of phosphate and NH_4^+ as the release of these nutrients have been observed to be pH-dependent (Zheng et al. 2013) and the release was reduced with increasing soil pH between pH 2 and 7.

Fresh biochar likely contains high amount of substances on its surface which may have short-lived impact on nutrient availability and plant growth. For instance, Deenik et al 2010 reported reduced microbial activity after application of biochar with high amount of volative matter. Similar conclusion was drawn also by (Asai et al. 2009)where grain yield and plant growth was reduced after application of biochar. Such substances can be toxic to plant and soil biota which may be manifected as reduced plant growth shortly after the application of such biochars. On the other hand, the decomposition of volatile matter substances may result in N immobilization linked to reduced nutrient release (Deenik et al. 2010; Zimmerman et al. 2011) which in the long-turn may contribute to improved plant nutrient uptake (Kuzyakov & Xu 2013).

Once in soil, the biochar-induced changes in soil properties and nutrient cycling could be of variable durability (Song et al. 2016) and are affected by biochar decomposition rate and soil properties (Wang et al. 2015a). The evolution of biochar properties in soil seems to be dependent on biochar source (Heitkötter & Marschner 2015) and could be drastically altered during the initial stages of incubation with soil. Still, the most of the studies evaluate the effect of biochar in short-term studies with only one sampling point selected as representative. Such a study design can give an important insight into some soil-biochar mixture properties but does not necessarily need to catch the highly variable changes of others. Many properties of soil-biochar mixture develop in time and a positive effect of biochar can be observed after partial degradation of biochar in soil (Mukherjee & Lal 2014). For instance, lower NH_4^+-N adsorption capacity of washed biochar when compared with non-washed biochar has been detected by Gai et al. (2014) probably due to the removal of ash and some of the functional groups on the biochar surface.

2.2.2.2 Plant performance and crop yields

The number of studies targeting the impact of biochar on plant productivity and crop yields has been increasing exponentially during the last decade including report from variable climatic conditions, soil types and agronomic systems using plant species with many different traits, which makes the quantitative comparison between studies challenging (Biederman & Harpole 2013). As a consequence of high data variability no general conclusion can be drawn when it comes to biochar effect on plant productivity and crop yields. Nevertheless, several meta-analysis (Jeffery et al. 2011; Biederman & Harpole 2013) aimed to detect the significant changes in growth and productivity of crops after biochar application concluding that despite the high variability of data and confounding factors, biochar application results in overall increase of yields or biomass production, as seen by many authors in individual experiments (Chan et al. 2008; Asai et al. 2009; Graber et al. 2010; Hossain et al. 2010).

Results of the quantitative meta-analysis presented by Jeffery et al (2011) showed an overall small, but statistically significant, effect of biochar on crop productivity, with a grand mean increase of 10%. Furthermore, the authors detected the highest increments of growth in acidic and neutral soils (14% and 13 %, respectively), and in soils with a coarse or medium texture (10% and 13%, respectively), and concluded that the main mechanisms behind the beneficial effect of biochar on plant growth may be soil acidity neutralization, an improved water holding capacity of the soil and increased contents of nutrients available for plants. Biederman and Harpole (2013) reported overall increase of aboveground biomass production and crop yields but no effect of biochar on belowground biomass or plant tissue N content. Clearly, interactions between biochar application with climate, soil type and fertilizer application (Tryon 1948; Van Zwieten et al. 2014), soil type and fertilization are complex and our understanding remains limited.

The application of only biochar to soil may have an adverse effect on plant growth probably as a result of the adsorption of mineral nitrogen and dissolved organic C onto the surface of biochar (Ding et al. 2010). To prevent nutrient immobilization, the co-application of biochar and fertilizer could compensate for biochar-induced N limitation for crops and N immobilization (Tian et al. 2016). The application of biochar together with compost was investigated by Liu et al. (2012), who found synergistic effect on soil fertility and plant growth. In limited number of studies, biochar was applied together with easily degradable C source such as glucose (Hamer et al. 2004), wheat straw (Zavalloni et al. 2011), switchgrass or sugar cane residues (Novak et al. 2010) although the results were highly inconsistent.

Nevertheless, information about existence of such a synergistic effect on biochemical properties is still limited, despite of its potential to help in elucidating complicated biochar-induced changes in soil.

2.2.3 Biochar and soil biological activity

2.2.3.1 Biochar and soil macro-invertebrates

The close relationship between soil physical properties and soil macrofauna has been well documented (Lavelle 1997; Lavelle et al. 2001; Velasquez et al. 2007; Rousseau et al. 2013). While soil macrofauna, particularly soil engineers, directly improve soil structure and other soil properties by their movement and activity, the abundance of smaller pores and soil aggregates directly influence the abundance, activity and diversity of soil mesofauna and microorganisms. Although the relation between biochar and soil biota remains largely unexplored when compared to other soil properties, the presence of biochar itself, as well as the changes in soil properties induced by biochar incorporation to soil, has been observed to influence the soil food web (McCormack et al. 2013) and the ecosystem functioning as macrofauna forms a part of both bacterial and fungal energy channel in the soil food web. The relation between biochar and soil biota has been identified to be a research priority (Lehmann et al. 2011b; McCormack et al. 2013), because biochar application and its effect on soil properties may alter ecosystem functioning through the effect on soil macrofauna, which, consequently, can modify the biochar decomposition rates in soil. Despite the general lack of information dealing with biochar and macro-invertebrates (Lehmann et al. 2011b), (Castracani et al. 2015) found a relationship between ants and biochar which the authors attributed to the changes in soil moisture or in soil temperature which tends to be higher in biochar-amended soil.

2.2.3.2 Effect of biochar on the abundance and activity of soil microorganisms

The overall increase of microbial abundance has been reported and reviewed by many authors (Lehmann et al. 2011b; Thies et al. 2015a) and several mechanisms contributing to improved microbial growth have been hypothesized (Table 2-1).

Table 2-1: Possible mechanisms of biochar effect on the abundance of soil microorganisms

Alteration of soil physical properties	formation of soil pores suitable as soil biota refuge	Ezawa et al 2002, Saito and Marumoto 2002, Thies and Rillig 2009 (all in Ding et al 2016)
	improved water holding capacity linked to reduced dessication risk of soil microorganisms	Wardle et al 1999
	sorption of toxic substances	Thies et al. 2015
Alteration of soil chemical properties	changes in soil pH linked with reduced stress caused by soil acidity	Rousk et al 2010; Aciego Pietry and Brookes 2008
	changes in nutrient avaiability	
	increased of labile organic matter on biochar surface	Pietikainen et al 2000
	priming of SOM	Zimmerman et al 2011
Alteration of plant-microbe signaling		Thies et al. 2015

The often observed dramatic changes of soil physical properties will likely have a key impact on the abundance of soil biota. Owing to its porosity and sorption properties, biochar has been suggested to provide a potential habitat for soil biota resulting from the creation of shelters and refuges (Pietikäinen et al. 2000; Warnock et al. 2007) and both soil bacteria and fungi are believed to benefit from the porous structure of biochar when applied to soil (Ezawa et al. 2002; Thies et al. 2015). The creation of a new environment within the pores of biochar may be beneficial to soil microbes which otherwise would not establish in the soil due to their low competitive ability (Ogawa 1994). Furthermore, these pores may be filled by volatile compounds or bio-oils contained on the biochar surface (usually shortly after biochar application to soil) or may become clogged by organic matter or soil minerals (later stages of biochar aging in soil) making them inhabitable for microbes.

Similarly, the high porosity of biochar is often linked to improved WHC of biochar-amended soil. Thus, the biochar pores may accommodate both soil microbes and water,

creating a habitat suitable for microbial growth protecting soil biota from dessication or drying-rewetting cycles which may be detrimental for microbial growth.

The application of biochar to soil impacts several soil properties which are known to be the key drivers of bacterial abundance, activity and diversity in soil (Lehmann et al. 2011b), including soil temperature, moisture and soil pH. While bacteria generally prefer soil with the pH close to neutral, soil fungi seem to be more tolerant and can be dominant in acid and alkaline soils (Thies et al. 2015c). Consecutively, faunal groups feeding on bacteria or fungi may be affected as observed by Mccormack et al. (2013). Many authors have pinpointed the importance of biochar pH on the abundance and reproduction of soil microbial populations (Rousk et al. 2009, 2010) and positive impact of biochar is often observed especially in acidic soils where low pH limits the microbial growth.

The changes of soil properties after biochar application may also influence the activity of intra- and extracellular enzymes catalyzing key steps of soil organic matter (SOM) decomposition facilitating the liberation of nutrients for plant or microbial uptake. Given that enzyme activity is considered a sensitive indicator of early changes in soil degradation and that the effect of biochar on soil enzymes is among priorities in understanding nutrient cycling (Lehmann et al. 2011a; Gul et al. 2015; Khadem & Raiesi 2017) better understanding of both short- and long-term effects of biochar on soil microbes is necessary. Both bacteria and fungi depend on the production of extracellular enzymes in order to degrade large macromolecules in soil and convert them into easily accessible compounds which could be used as a source of energy (Paul 2006).

The potential activity of extracellular enzymes is a function of many factors including enzymes production rate and enzyme decomposition rate as well as the sorption of enzymes or substrate onto soil (or biochar) surface (Gianfreda & Ruggiero 2006) which may result in enzymes protection or substrate unavailability, respectively. In many studies biochar has been noted to promote the activity of soil enzymes (Kumar et al. 2013; Masto et al. 2013; Ouyang et al. 2014; Paz-Ferreiro et al. 2014; Khadem & Raiesi 2017) which can be explained by increased microbial abundance (Khadem & Raiesi 2017c) related to improved soil properties and enhanced extracellular enzyme production and/or to addition of substrates for enzymatic reaction within biochar (Lehmann et al. 2011b; Thies et al. 2015).

2.2.3.3 Biochar impact on microbial community structure

The majority of soil microbes directly depends on the supply of suitable substrate as energy source. Biochar application to soil alters both the quantity and the quality of C compounds and thus affects the activity of the microbes which are able to decompose them (Ogawa 1994, Zackisson et al 1996, Watzinger et al 2014, Maestrini et al 2014). Freshly produced biochar contains wide range of condensed substances created during the pyrolysis. Such substances may be directly available to soil biota or may be toxic to plants (Mcclellan et al. 2007) and microbes (Painter 1998). On the other hand, specific microbial groups may thrive on such substances which can result in their increased activity (Graber et al. 2014).

As biochar decompose in the soil, different C fractions are being released which may be the C source for distinct microbial groups depending on the microbes capacity to decompose them (Lehmann et al. 2011). Higher bacterial diversity has been observed in ADE soils by several authors (Kim et al. 2007; O'Neill et al. 2009; Grossman et al. 2010) and it has been suggested that the increased diversity of soil biota after biochar application should be expected especially in the long-term experiments as a reduction of microbial diversity has been observed in the short-term experiments (Jin 2010; Khodadad et al. 2011). The substances contained in biochar will likely be decomposed and mineralized shortly after biochar application to soil and the microbial community changes will depend on the ability of microbial groups to utilized biochar compounds as substrates: the microbial groups with higher capacity to decompose biochar will likely prevail in the soil shortly after biochar application.

Significant changes in fungi:bacteria ratio can be expected in biochar-amended soils. Fungi may be better adapted to biochar due to their ability to decompose lignin and more recalcitrant organic compounds (Hofrichter 2002), but some bacteria taxons are often more adaptive to changes in the environement (Lehmann et al. 2011b; Hu et al. 2014) as increased bacterial diversity was observed in a short-term (three months) experiment of Hu et al. (2014). In conclusion, the impact of biochar on the relative abundance of microbial taxons will likely be affected by soil type, biochar properties and the structure of microbial community present in the soil. Changes in microbial community composition and structure may clearly impact the decomposition rate of native SOM as well as the decomposition of biochar itself.

2.2.3.4 Biochar and the mineralization of soil organic matter

The small part of C contained in biochar which remains labile and accessible to microorganisms (Wang et al. 2015a) has been observed to participate in chemical and biochemical reactions and to influence the C mineralization in soil (Zimmerman et al. 2011) with potential implication for SOM turnover in biochar-amended soils. The stimulation of native SOM caused by biochar has been reported to be short- to medium-term and depends on feedstock material and pyrolysis temperature (Zimmerman et al. 2011) and consequently on microbial biomass changes caused by biochar application to soil (Thiessen et al. 2013). Both positive (Zimmerman et al. 2011) and negative (Dempster et al. 2011) PE of biochar have been observed but the mechanisms and causes remain unclear. Biochar-induced stimulation or reduction of CO_2-C losses seems to be attributed to different composition of biochar depending on biochar labile C content.

Despite its recalcitrance, application Hamer et al. (2004) after glucose application. Furthermore, a number of studies have reported that adding biochar to soils may affect soil physical and chemical properties (Atkinson et al. 2010), which have further effect on nutrient turnover and transformation (Atkinson et al. 2010) and can influence soil microorganisms simultaneously. When biochar enters the soil system, it can trigger mechanisms to increase SOC mineralization directly by providing easily accessible C source leading to increased microbial activity or by causing the mining for N, or indirectly by removing obstacles in microbial activity (Whitman et al. 2015). Alternatively, by changing the microbial preferences (Novak et al. 2010) or decreasing labile SOC availability via sorption (among other mechanisms) biochar application can lead to overall decrease of SOC mineralization.

2.2.3.5 Nitrogen transformations in biochar-amended soils

Biochar application to soil induces significant changes in soil biogeochemical processes through many different mechanisms which remain largely unknown. There is an increasing evidence suggesting that biochar impacts a number of soil N reactions (Clough et al. 2013) and the effect is dependent on biochar feedstock and production conditions, in addition to soil properties and environmental conditions. Despite the large scale of potential modes of biochar's effects on N cycling (Clough et al. 2013; Liu et al. 2018), the interactions of these mechanisms and the interplay between soil-microorganisms-biochar system remain poorly understood. Biochar has been observed to affect N fixation (Mia et al. 2014), N mineralization or immobilization (Ameloot et al. 2015), to increase or decrease nitrification

(Ulyett et al. 2014), denitrification (Cayuela et al. 2013), or ammonia volatilization (Mandal et al. 2016).

A stimulation of N mineralization has been observed as a consequence of labile C addition or a result of soil pH neutralization linked to the stimulation of microbial activity and abundance. Similarly, the improvement of soil physical or chemical properties after biochar application can lead to acceleration of SOM mineralization and ammonification in cases when microbial activity was limited by inadequate soil conditions. Consequently, separation of particular modes of action of biochar on N cycling is challenging due to high amount of confounding factors after biochar application to soil. For instance, up to now, the separation of the inherent properties of biochar from the effect of modification of soil pH has not been clearly addressed (Teutscherova et al. 2017a)

Biochar-induced changes in N cycling differ substantially from potential lime-induced changes, as biochar may sorb NO_3^-, NH_3, NH_4^+ and organic-N (Bai et al. 2015) as well as inhibitory compounds such as phenolics that could otherwise inhibit nitrification (DeLuca et al. 2006). In addition, indirect mechanisms associated with soil microbial composition changes can be generated by the specific properties of biochar and thereby have strong implications for soil microbial N processing. Nitrification rates were accelerated by addition of biochar in two acidic arable soils (Zhao et al. 2014). He et al. (2016) also reported an increased nitrification activity in biochar-amended acid oxisol when exogenous NH_4^+ was added, suggesting that nitrification, after successive biochar applications, was not limited by nitrifier activity as was observed in unamended soils. However, Wang et al. (2015) found that peanut shell biochar actually reduced nitrification in an acid soil due to the decreased NH_4^+-N content available to nitrifiers and reduced the abundance of AOB. These inconsistent biochar effects suggest that more attention to nitrification in biochar-amended agricultural soils has to be paid, especially in acidified soils derived from high N-fertilizer inputs.

2.2.4 Concerns and future directions of biochar adoption

Despite the exponentially increasing number of studies focusing on biochar impact on soil properties and nutrient cycling, we are still far away from being able to draw a conclusion due to high variability of results and many confounding factors. Clearly, any soil amendment with simultaneous impact on soil physical, chemical and biological properties need to be evaluated in deep before large-scale implementation and long-term experiments are required to avoid possible negative impacts on the ecosystems (Mukherjee & Lal 2014). The results

obtained from ADE have proved to be a useful tool in biochar investigation. Nevertheless, ADE differ considerably from biochar-amended soils used in the majority of the recent experiments.

2.2.4.1 Negative impacts of biochar on soil and plants

The changes produced by biochar application to soil are often cited to be beneficial to plants due to increased soil pH (in acid soil where soil acidity may be limiting plant and microbial growth) and nutrient availability. Nevertheless, changes of soil pH can also reduce the availability of specific soil nutrients available at lower pH values and plant growth may be diminished (Xu et al. 2012). Furthermore, high CEC biochar may adsorb large amounts of nutrient on their surface and reduce the availability of these nutrients to plant roots (DeLuca et al. 2006; Mukherjee et al. 2011). High temperature biochars have usually higher pH and ash contant when compared to low temperature biochars. Drastic changes of soil pH may be detrimental to some beneficial soil biota as demonstrated for arbuscular mycorrhizal fungi (Gaur & Adholeya 2000; Warnock et al. 2007; Birk et al. 2009) and for earthworms (Topoliantz & Ponge 2005). Furthermore, high salt content and heavy metals or other contaminants contained in biochar may have direct negative impact of soil biota.

The increased sorption capacity of biochar is generally believed to be one of the key mechanisms of reduced nutrient losses in biochar amended soils. Nevertheless, biochar can at least in the short-term lead to increased mineralization of SOM (Zimmerman et al. 2011) which can be linked to accelerated liberation of nutrients into soil solution and increased risk of cumulative leaching losses and reduction of nutrient pool in soil.

3 OBJECTIVES AND HYPOTHESIS

The common objective of the present thesis was to evaluate in detail the impact of biochar on nutrient cycling in Mediterranean soils with a special emphasis on C and N cycling and soil biological activity. The particular aims were following:

3.1 Quantification of biochar effect on soil organic matter mineralization

Depending on soil type, biochar feedstock material, pyrolysis temperature and climatic conditions, biochar may either increase (positive priming effect) or decrease (negative priming effect) the mineralization of SOM. Positive priming effect can be caused for example by increased microbial activity which decomposes SOM to cover its nutrient requirements (N-mining) while reduced SOM mineralization can be caused by sorption of labile C forms making them unavailable for soil microbes or by offering an alternative source of energy for soil microbes (Whitman et al. 2015). Furthermore, the biochar-soil interactions can be influenced by co-application of organic matter with the stability of such organic matter playing a crucial role. Therefore, the specific aims were following:

i) **The determination of possible synergistic effect between compost and biochar**

It was hypothesized that biochar and compost co-application will synergically influence soil microbial activity.

ii) **The evaluation of the influence of compost maturity (and stability) on compost-biochar-soil interactions**

I hypothesized that the compost maturity stage will determine its interaction with biochar and soil organic matter due to the differences in labile organic matter content.

iii) **To determine whether biochar can reduce positive priming effect caused by labile organic matter application**

As hard-wood biochar has been observed to decreased SOM mineralization probably due to labile C adsorption, biochar co-application with unstable compost will reduce the negative impact of such compost application to soil by adsorbing soluble C compounds.

3.2 The effect of biochar on N mineralization and nitrification

i) **To quantify the impact of biochar on ammonification**

Biochar, being stable, porous, C-rich, alkaline material, will likely influence soil microbial activity. I hypothesized that biochar will stimulate the mineralization of organic nitrogen due to changes in soil properties.

ii) To test the relation between ammonification and nitrification in biochar-amended soils

Stimulation of ammonification in soil can lead to increased nitrification, which could have negative environmental consequences. On the other hand, biochar may contain substances directly inhibiting the oxidation of ammonium. I hypothesize that biochar will enhance soil nitrification as a result of the improvement of soil properties and the increment of the supply of ammonium for nitrifiers.

3.3 Potential leaching losses of mineral N form biochar-amended soils

i) To determine if the biochar impacts leaching of ammonium and nitrate

The potential leaching losses of N are a function of mineral nitrogen production (mineralization of organic nitrogen), nitrogen consumption by soil microbes and nitrogen retention in the soil. I hypothesized that the changes in nitrogen mineralization and nitrification will alter the amount of N leached from the soil.

ii) Sorption of ammonium and nitrate

Biochar can affect both cation and anion exchange capacity of the soil and both ammonium and nitrate sorption improvement have been reported. I hypothesize that the changes in ammonium and nitrate production will be compensated by the improvement of N retention in soil and overall leaching losses will be reduced.

3.4 Biochar effect on soil microbial activity

i) To detect the factors affecting soil respiration in biochar-amended soils

I hypothesized that biochar will enhance microbial biomass and activity in both soils as a result of pH neutralization in acrisol and due to C input in calcisol.

ii) To quantify the effect of biochar on soil enzymes activity

Increased microbial biomass and activity are often linked to the activity of soil extracellular enzymes which are released by soil microbes to degrade organic macromolecules. Nevertheless, the activity of soil extracellular enzymes is a function of enzyme production and degradation rate. I hypothesized that biochar will increase the potential enzymatic activity in both soils as a result of enhanced release of enzymes and potentially improved protection of enzymes towards degradation.

3.5 Biochar and soil aggregation

To test whether biochar improves soil aggregation and to analyze under what conditions. I hypothesized that increased biological activity will lead to enhanced formation of WSA under optimal aerobic conditions. Additionally, I test if biochar-induced changes in soil properties affect soil aggregation under intermittently high-moisture conditions. I hypothesized that the changes in soil pH, in the amount of base cations and CEC may hinder the positive effect of biochar on soil aggregation under sub-optimal conditions.

3.6 Biochar effect on plant growth

It was hypothesized that biochar will improve th plant growth in both soil types as a consequence of improved soil physical and/or chemical properties and of the accelerated SOM turnover and nutrient release.

4 MATERIALS AND METHODS

4.1 Biochar production and properties

Biochar used in the present theses was produced from holm oak (*Quercus ilex* L.) pruning waste at 600°C in oxygen-restricted environment in a batch system and crushed to pass 2-mm sieve. Biochar pH and electric conductivity were measured after one hour shaking with deionized water (1:10 w/v). Total C (TC) and total N (TN) were analyzed by automatic analyzer LECO Instrument TruSpec CN (LECO Corporation, St. Joseph, MI, USA), dichromate oxidizable C (C_{ox}) by dichromate oxidation (Walkley & Black 1934) and carbonate content by calcimeter. The content of WSC and WSN were determined in water extract (1:10 w/v). The modified ammonium acetate compulsory displacement method (Gaskin et al. 2008) was used for CEC analysis. Ash content was determined by biochar combustion at 750°C for 6 h in open crucibles. Soil and biochar properties are listed in Table 4-1.

4.2 The properties of used soils

Acid sandy Acrisol (FAO), characterized as clay-skeletal, kaolinitic, acid Palexerult according to Soil Taxonomy, was collected from degraded ecosystem of Cañamero's raña formation in SW Spain. This soil is characterized by low pH, low content of exchangeable bases, low available phosphorus (P) content and exchange complex dominated by aluminum (Al) (Espejo, 1987; Table 4-1). Soil in the area is highly degraded as a consequence of long-term continuous tillage which resulted in loss of SOC and soil acidification. The climax vegetation of the area is cork-oak forest which was replaced by holm-oaks, olive groves and agricultural land resulting in soil degradation, loss of SOC and low pH. Soil contained 80.1% of sand, 6.1% of silt and 13.8% of clay and soil profile can be seen in Fig. 4-1.

Second selected soil is Haplic Calcisol (FAO), corresponding to Typic Calcixerept according to Soil taxonomy, obtained from "La Chimenea" Field Station near Aranjuez (Madrid, Spain). This soil is characterized by high pH, high carbonate content and loamy texture (Table 4-1) and is recently being used for intensive agriculture. Fig. 4-1 displays the profile of selected Calcisol.

Table 4-1: Selected soil and biochar properties

Soil properties	Acrisol	Calcisol	Biochar properties	
pH	5.65	8.00	pH	10.2
Electric conductivity (µS cm^{-1})	49.7	570	Electric conductivity (µS cm^{-1})	940
CEC ($cmol_c$ kg^{-1})	2.73	8.84	TC (%)	68.2
TOC (g kg^{-1})	25.8	9.55	TN (%)	0.67
Carbonate content (%$CaCO_3$)	n.p.	21.9	C_{ox} (%)	4.70
TN (g kg^{-1})	1.28	0.90	Ash content (%)	3.49
WSC (mg kg^{-1})	78.3	29.1	Carbonates content (%$CaCO_3$)	11.9
WSN (mg kg^{-1})	19.0	49.2	WSC (mg kg^{-1})	149
Field moisture capacity (%)	16.9	18.3	WSN (mg kg^{-1})	93.4
Sand (%)	80.1	29.0	CEC ($cmol_c$ kg^{-1})	35.1
Silt (%)	6.10	42.0	NH_4-N sorption (mg NH_4-N g^{-1})	2.22
Clay (%)	13.8	29.0	NO_3-N sorption (mg NO_3-N g^{-1})	n.s.
Exchangeable Ca^{2+} ($cmol_c$ kg^{-1})	1.44	24.6	Exchangeable Ca^{2+} ($cmol_c$ kg^{-1})	4.96
Exchangeable Mg^{2+} ($cmol_c$ kg^{-1})	0.21	2.48	Exchangeable Mg^{2+} ($cmol_c$ kg^{-1})	2.93
Exchangeable K^+ ($cmol_c$ kg^{-1})	0.47	0.42	Exchangeable K^+ ($cmol_c$ kg^{-1})	4.41
Exchangeable Na^+ ($cmol_c$ kg^{-1})	0.05	1.65	Exchangeable Na^+ ($cmol_c$ kg^{-1})	1.02

CEC, cation exchange capacity; TOC, total organic carbon; TN, total nitrogen; TC, total carbon; C_{ox}, dichromate oxidizable organic C; WSC, water soluble carbon; WSN, water soluble nitrogen; n.p., not present, n.s., not significant.

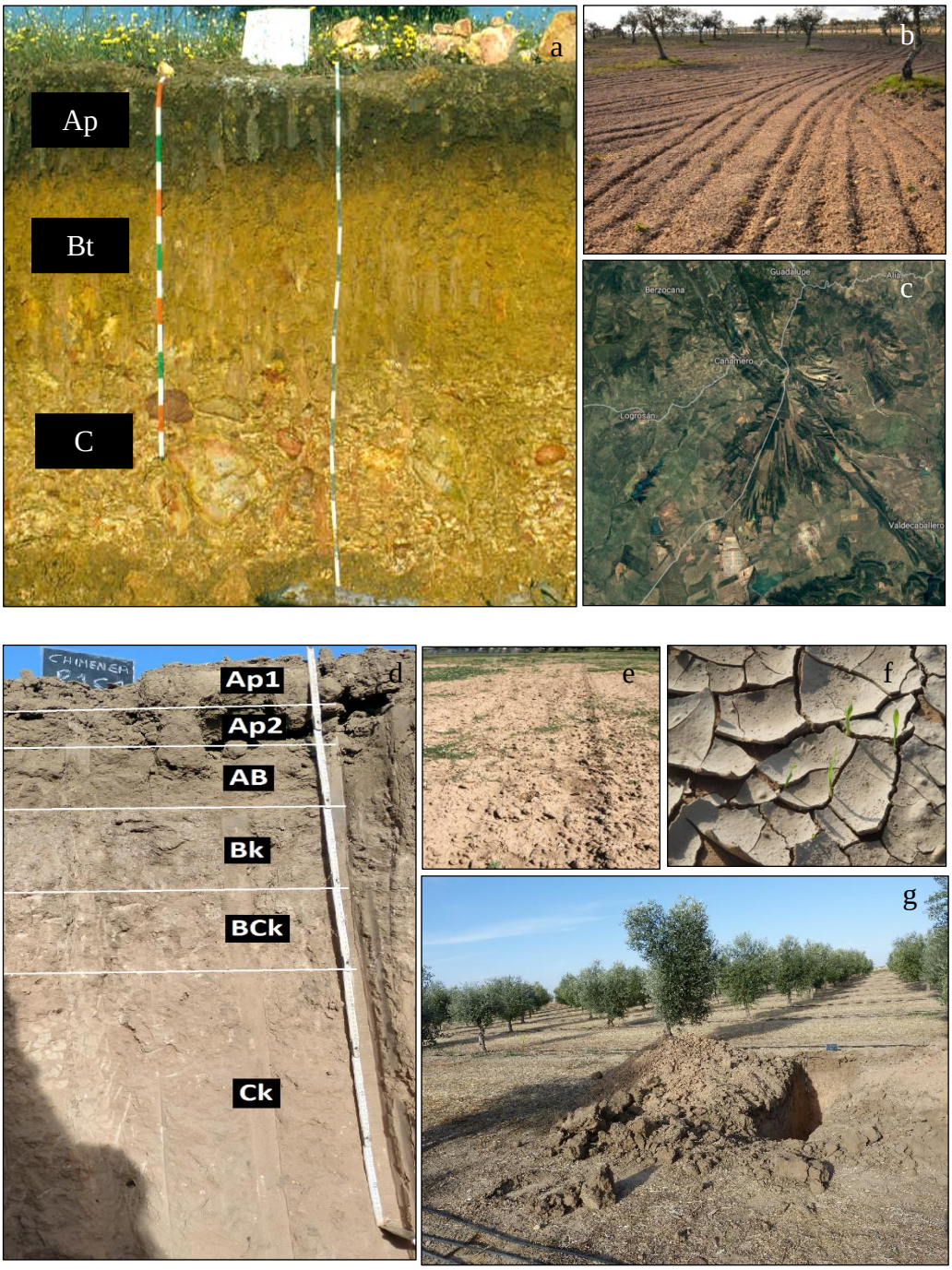

Fig. 4-1: Soil profile of selected Acrisol (a), excessive tillage of Acrisol in the area of Cañamero's raña (b), Cañamero's raña formation (c), soil profile of selected Calcisol (d), superficial crust of Calcisol (e, f) and intensive olive production on Calcisol (g).

4.3 Soil collection and analytical methods

Samples were collected from the top-soil layer (0-10 cm soil depth) and immediately transported to the laboratory, homogenized and sieved at field-moist state within three days. Part of the composite sample was air-dried and sieved to 2 mm for laboratory analysis. Soil pH and electric conductivity were determined in soil: deionized water (1:2.5 w/v) after one hour of shaking. Ammonium acetate (1M, pH 7) method was used for cation exchange capacity (CEC) determination. Soil organic carbon content was measured by dichromate oxidation (Walkley & Black 1934) after carbonates reaction with HCl (Calcisol). The contents of water-soluble C (WSC) and water soluble N (WSN) were determined by extraction with deionized water (1:10 w/v), followed by analysis with automatic analyzer for C and N content, respectively. Field moisture capacity was quantified by pressure plate extractors at -0.33 kPa (Soil Moisture Equipment Corp., Santa Barbara, CA), as described by Dirksen (1999).

4.4 The organization of the experiments

Six independent experiments were established to target the particular steps of nutrient transformations in soil. The overview of the experiments can be seen in Table 4-2.

Table 4-2: The overview of experiments included in the thesis

Chapter number	Chapter name	Experimental design	Reference
5	Biochar and compost synergism	Incubation study	Teutscherova et al. 2017b
6	Biochar and lime effect on nitrogen cycling	Incubation study	Teutscherova et al. 2017a
7	Biochar and mineral nitrogen leaching	Column leaching study	Teutscherova et al. 2018a
8	Base cation leaching and soil structure	Column leaching study	
9	Dynamics and microbial and enzymatic activity	Pot experiment	Teutscherova et al. 2018b
10	Plant growth in biochar-amended soils	Pot experiment	

4.4.1 Soil incubation studies

Chapter 5 and chapter 6 were set-up as laboratory incubations under controlled ambient temperature (25°C) and moisture content (60% of WHC). Fresh soil was sieved to pass 2-mm sieve and in both cases and moist soil in an amount equivalent to 100g dry soil was place in 0.5l plastic incubation jars.

4.4.2 Column leaching study

Column leaching was performed in experiments included in chapter 7 and chapter 8. For column preparation, sieving to 5 mm was selected as the most suitable due to the high content of rock fragments in Acrisol (51%) and to ensure adequate aeration and drainage. Both soils were amended with 1% (B1) and 2% (B2) of biochar (26 Mt ha^{-1} and 52 Mt ha^{-1}, respectively) including soil controls (B0) without biochar addition. Eight replicates were prepared for each soil and biochar application rate, four of them were left without fertilization (B0, B1 and B2 treatments) and four were fertilized with NPK at application rate of 36 kg NH_4^+-N ha^{-1}, 72 kg P ha^{-1} and 72 K kg^{-1} ha^{-1} (B0-F, B1-F and B2-F treatments) in the beginning of each leaching cycle. The amount of applied N was seven mg in each fertilization event per leaching column, resulting in 14 mg of total mineral N applied to each fertilized column.

Soil and biochar mixtures were packed in PVC columns (5 cm diameter and 30 cm height) to a bulk density of approximately 1.3 g cm^{-3}, which corresponds to the bulk density of studied soils. Bulk density was adjusted in control soils and same pressure was used to compact biochar-treated soil. All columns were fitted with fiber mesh and funnel on the bottom and a 5 cm layer of gravel and acid-washed sand was placed inside each column to prevent soil losses. Control columns (without biochar amendment) received 500 g of soil and columns amended with biochar were filled with the amount of mixture equivalent to 500 g of soil and the corresponding amount of biochar (505 g and 510 g for B1and B2, respectively).

4.4.3 Pot experiments

Chapter 9 and chapter 10 were established as pot experiments in greenhouse. Saoil was sieved to 5 mm due to the high content of rock fragments in Acrisol (51%) and in order to ensure adequate aeration during the incubation experiment. Same treatments were used as in column leaching experiments (B0, B1, B2, B0-F, B1-F, B2-F) with the same fertilizer application rate in fertilized treatments. Twenty-four replicates were prepared for each soil and biochar treatment, 12 were not fertilized (B0, B1 and B2 treatments) and 12 were

fertilized with NPK at application rate of 36 kg NH_4^+-N ha^{-1}, 72 kg P ha^{-1} and 72 K kg^{-1} ha^{-1} (B0-F, B1-F and B2-F treatments), which is the fertilization rate used in the study area as reported previously (Gómez-Paccard et al. 2013; Vazquez et al. 2017).

Plastic pots (ten cm in diameter; ten cm height) were filled with 500 g of control soil per pot or biochar-amended soil at 505 and 510 g per pot for B1 and B2, respectively. Four pots were destructively sampled after three weeks, six weeks and 12 weeks of incubation for laboratory analysis. All pots were placed in completely randomized block design in a controlled greenhouse (12 hours of light per day, temperature around 25°C) and watered to 60% of WHC. Moisture content was adjusted gravimetrically every one or two days.

Same treatments were used to study plant growth in additional pots maintained under identical environmental conditions.

5 BIOCHAR AND COMPOST SYNERGISM

Adopted from: Teutscherova N, Vazquez E, Santana D, Navas M, Masaguer A, Benito M. 2017. Influence of pruning waste compost maturity and biochar on carbon dynamics in acid soil: Incubation study. European journal of soil biology **78**: 66-74.

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Nikola Teutscherova contributed by establishing the experimental design, analytical and statistical analysis and manuscript preparation including required revisions

ABSTRACT

Compost is the most common organic fertilizer supplying nutrients and organic carbon to soil as well as improving soil physical, chemical and biochemical properties. On the contrary, biochar application to soil usually does not add nutrients, but can have effect on nutrient transformations and microbial community and also alleviates soil acidity. Although these two products of organic residues recycling have different function in soil, their co-application could result in synergistic effect on soil biochemical properties. Therefore, the aim of present study was to determine how the application of biochar and compost in two stages of maturity (one month old after bio-oxidative phase; and final mature compost), applied alone or together, affects soil pH, water soluble carbon and nitrogen contents, carbon and nitrogen mineralization, microbial biomass and enzymes activities in acid soil in a short-term (60 days) incubation study. Additionally, same treatments were tested in a ryegrass growth assay. Application of all organic materials increased soil pH, which probably resulted in microbial community changes and overall decrease of microbial biomass carbon. Soil respiration was increased after application of immature compost (903 μg CO_2-C g^{-1}) or its mixture with biochar (823μg CO_2-C g^{-1}), but we did not observe significant increase in respiration after biochar application respect to control (402 μg CO_2-C g^{-1}). Biochar decreased β-glucosaminidase activity and increased the activity of dehydrogenase. The higher values in β-glucosidase and dehydrogenase activities, as well as soil respiration, when immature compost and biochar were applied together, showed the synergism between these materials. Ryegrass growth was stimulated by all organic amendments, but combined application of immature compost and biochar resulted in growth increment lower than only biochar or only compost application. Adequate stabilization of pruning waste compost avoided priming of SOM induced by biochar co-application.

Key words: carbon mineralization; microbial biomass carbon; priming effect; soil respiration

5.1 Introduction

Sustainable organic residues management is a key step in nutrients recycling and is essential in order to maintain soil fertility. To date, the most common form of organic wastes recycling is composting which consists of decomposition of organic matter (OM) by the action of thermophilic and mesophilic microorganisms. When pruning waste is used, the final product, pruning waste compost, is relatively cheap and suitable for soil application (Benito et al. 2003). However, care needs to be taken in determining the compost maturity, which during composting process undergoes four stages: (i) initial stage (no decomposition), (ii) the thermophilic phase (high temperatures, rapid degradation), (iii) the end of bio-oxidative phase (drop of temperature), and (iv) maturation phase (stabilization). Final compost is stabilized, humified and pathogen-free product which continues mineralization in slower rate liberating nutrients after its application to soil. Depending on composting facility and actual demand, also compost at the end of bio-oxidative stage can be applied to soil, because according to some parameters at this point it could be considered mature enough to be applied to soil (Benito et al. 2003). In some cases, when immature compost is used, its high content of water soluble carbon (WSC) can lead to stimulation of microbial activity followed by an increased carbon dioxide (CO_2) fluxes, a higher soil organic matter (SOM) decomposition that it is referred to as priming effect (PE), and a nitrogen (N) immobilization (Benito et al. 2005).

As an alternative to aerobic composting, organic residues can be processed anaerobically via fermentation or biogas digestion, or via pyrolysis by heating the material to high temperature in oxygen limited environment, which leads to carbonization of organic matter and production of biochar. During pyrolysis, the major part of CO_2 trapped by plants to form biomass is converted into recalcitrant form of C with hundreds to thousands of years of stability (Atkinson et al. 2010), leaving only small part labile and accessible to microorganisms (Wang et al. 2015a). This small labile part has been observed to participate in chemical and biochemical reactions and to influence C mineralization in soil (Zimmerman et al. 2011). The stimulation of native SOM caused by biochar has been reported to be short- to medium-term and depends on feedstock material and pyrolysis temperature (Zimmerman et al. 2011) and consequently on microbial biomass changes caused by biochar application to soil (Thiessen et al. 2013). Both positive (Zimmerman et al. 2011) and negative (Dempster et al. 2011) PE of biochar have been observed but the mechanisms and causes remain unclear. Biochar-induced stimulation or reduction of CO_2-C losses seems to be attributed to different

composition of biochar depending on biochar labile C content. Hard-wood biochar was observed to cause the highest long-term decrease in soil organic carbon (SOC) mineralization (Zimmerman et al. 2011), possibly for its great sorption capacity that could protect labile C from microorganisms. Despite its recalcitrance, application of labile substrate could lead to increased biochar C mineralization, as it was found by Hamer et al. (2004) after glucose application. Furthermore, a number of studies have reported that adding biochar to soils may affect soil physical and chemical properties (Atkinson et al. 2010), which have further effect on nutrient turnover and transformation (Atkinson et al. 2010) and can influence soil microorganisms simultaneously. When biochar enters the soil system, it can trigger mechanisms to increase SOC mineralization directly by providing easily accessible C source leading to increased microbial activity or by causing the mining for N, or indirectly by removing obstacles in microbial activity (Whitman et al. 2015). Alternatively, by changing the microbial preferences (Novak et al. 2010) or decreasing labile SOC availability via sorption (among other mechanisms) biochar application can lead to overall decrease of SOC mineralization.

On the other hand, it should be taken into account that application of only biochar to soil may have an adverse effect on plant growth probably as a result of the adsorption of mineral nitrogen and dissolved organic C onto the surface of biochar (Ding et al. 2010b). To prevent nutrient immobilization, the co-application of biochar and fertilizer could compensate for biochar-induced N limitation for crops and N immobilization (Tian et al. 2016). The application of biochar together with compost was investigated by Liu et al. (2012), who found synergistic effect on soil fertility and plant growth. In limited amount of studies, biochar was applied together with easily degradable C source such as glucose (Hamer et al. 2004), wheat straw (Zavalloni et al. 2011), switchgrass or sugar cane residues (Novak et al. 2010) with highly inconsistent results. Nevertheless, information about existence of such a synergistic effect on biochemical properties is still limited, despite of its potential to help in elucidating complicated biochar-induced changes in soil.

The main aim of this study is to test whether hard-wood biochar, previously cited to have the greatest long term potential to decrease SOC mineralization (Zimmerman et al. 2011), interacts with composts of two stages of maturation when applied together to the soil. In continuation, if this possible interaction reflects in a decreased priming of SOC and previously documented N mineralization (Benito et al. 2005) when pruning waste compost rich in labile C is applied to soil. Based on the information gap in this possible synergistic

functioning of both products, we set a hypothesis, that different organic amendments will have different effect on microbial biomass and activity in the soil, and that this effect would be related to increased soil pH of these acid soils. To better understand changes in soil respiration, four soil enzymes participating in OM decomposition and nutrient cycling were selected and measured at the end of the 60-days incubation. Simultaneously, ryegrass assay was setup to determined possible detrimental or synergistic effects of application of biochar and both composts.

5.2 Materials and methods

5.2.1 Soil and organic materials

This experiment was performed using acid Acrisol, biochar and compost in two stages of maturity. The production conditions and properties of biochar can be found in Table 4-1 (Materials and methods section) as well as the location and properties of the used Acrisol. Compost was produced from pruning waste, leaves and grass clippings (60-70% of the waste volume woody material, 30-40% green waste) in the composting facility “Migas Calientes” in Madrid. The mixture of waste was composted in trapezoidal windrow piles (2.5 m high, 5 m wide and 30 m long). Forced aeration was used during the first 30 days (bio-oxidative phase) followed by maturation period, during which the piles were turned periodically to maintain adequate O_2 levels. For the present study we used compost (< 4 mm) in two stages of maturation, one-month old taken at the end of bio-oxidative phase (C1) and six months old compost (C2). Both C1 and C2 were relatively rich in C, low in N and with high pH values. The properties C1 and C2 are listed in Table 5-1 as well as the relevant properties of used biochar and Acrisol.

Table 5-1: Selected properties of biochar, composts and soil

	Biochar (Bc)	Compost 1 (C1)	Compost 2 (C2)
pH	10.2	8.2	9.2
EC ($\mu S\ cm^{-1}$)	940	515	869
TC (%)	68.2	46.23	34.59
TN (%)	0.67	1.43	2.01
C:N ratio	101.8	31.7	17.0
Cox (%)	4.7	45.3	34.1
carbonates content (% $CaCO_3$)	11.89	-	-
WSC ($mg\ kg^{-1}$)	148.9	4729	4229
WSN ($mg\ kg^{-1}$)	93.44	1213	1009
CEC ($cmol_c\ kg^{-1}$)	35.09	155.51	201.49
exchangeable Ca^{2+} ($cmol_c\ kg^{-1}$)	4.96	11.40	26.31
exchangeable Mg^{2+} ($cmol_c\ kg^{-1}$)	2.93	3.60	3.71
exchangeable K^{+} ($cmol_c\ kg^{-1}$)	4.41	4.11	8.86

EC, electric conductivity; TC, total carbon; TN, total nitrogen; Cox, potassium dichromate-oxidizable carbon; WSC, water-soluble carbon; WSN, water-soluble nitrogen; CEC, cation exchange capacity

5.2.2 Incubation procedure and soil respiration

Soil sieved to 2 mm was amended with Bc, C1, C2, the mixture of C1 with Bc and C2 with biochar (S-Bc, S-C1, S-C2, S-C1-Bc and S-C2-Bc, respectively), in order to increase the SOC content by one per cent, from 2.58 to 3.58% total organic carbon (TOC) (equivalent to application of 24 t C ha^{-1}) in all cases. Application rates of all organic materials were calculated according to their C content. In case of mixtures, each component provided 50% of applied C. For incubation, equivalent to 100g dry weight soil with addition of organic amendment containing one gram of organic C, were placed in airtight plastic jars (0.5L) for aerobic incubation with four replicates. All treatments and control were moistened until the 60% of their water holding capacity and incubated for 60 days at 25 °C in dark. Water content was regularly checked gravimetrically and adjusted with deionized water. Carbon mineralization was measured as CO_2-C loss using alkaline trap during the 60 days of incubation. The emitted CO_2 was trapped in 10ml of NaOH which was titrated with HCl on days 1, 2, 6, 9, 16, 23, 30, 51 and 60 after carbonate precipitation with $BaCl_2$ (Iannotti et al. 1993).

5.2.3 Priming effect

Additionally, jars containing 50 g of each compost (C1 and C2), biochar (Bc), and 50 g of their mixtures, where 50% of C were derived from biochar and 50% from C1 (C1-Bc) or C2 (C2-Bc), were prepared in another set of jars (n=4) and maintained in the same conditions as amended soil treatments in completely randomized design. The evolution of CO_2 was monitored using the same alkaline traps as amended soil on the same days.

The difference between the amount of C-CO_2 released during incubation of amended soil (measured CO_2 production) and the sum of C-CO_2 mineralized in control soil and C-CO_2 released from organic material incubation (expected CO_2 production) is referred to as priming effect (PE), similarly to Gómez-Muñoz et al.(2016) , according to equation (1).

PE = Measured CO_2 production – Expected CO_2 production (1)

5.2.4 Analytical methods

At the end of incubation period, soils from all jars were analysed for selected chemical properties, microbial biomass C and the activity of soil enzymes. The C and N contents were determined by automatic analyzer LECO Instrument TruSpec CN (LECO Corporation, St. Joseph, MI, USA). For pH and EC measurement, water extracts (1:2.5 w/v) were prepared. Ammonium content was extracted with 2M KCl (1:10 w/v) after shaking for two hours and determined colorimetrically using the salicylate method as the variation of Berthelot-Phenate method (Bower & Holm-Hansen 1980). In water extract (1:10 w/v), WSC and WSN were analyzed by automatic analyzer and NO_3^- content determined as a result of nitrification of salicylic acid colorimetrically (Robarge et al. 2008).

Microbial biomass C was quantified using substrate induced respiration method as a respiration of 20g moist soil samples after glucose-talcum powder mixture (1:3 ratio) addition during four hours incubation followed by conversion of emitted CO_2 to microbial biomass C according to Anderson and Domsch (Anderson & Domsch 1978). Dehydrogenase activity was determined using 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) as a substrate using method of Trevors et al. (1982) modified by García et al. (1993). For β-glucosidase activity method of Hoffmann and Dedeken (1965) modified by Strobl and Traunmueller (1996) was selected using β-glucoso-saligenin (salicin) as substrate. The β-glucosaminidase activity was determined according to method proposed by Parham & Deng (2000) with p-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate. For the activity of urease, method proposed by Kandeler & Gerber (1988) and modified by Kandeler et al. (1999) was selected and the activity was determined as NH_4^+ produced during incubation. For possible adsorbtion effect of biochar, series of incubations were set up. However, no significant adsorption effect was found. All laboratory analysis were replicated at least twice.

5.2.5 Carbon and nitrogen mineralization

Data regarding changes in the CO_2 evolution rate during incubation were fitted to a kinetic function by a non-lineal least squares technique (Marquardt-Levenberg algorithm)

using the SigmaPlot 2013 program and the values of Snedecor F test and residual mean square (RMS) were calculated to determine the best fit.

Kinetic function selected was first-order kinetic model (2)

$$C = C_o (1\text{-}exp^{-kt}), \quad (2)$$

where C is the amount of mineralized C (%TOC) at time *t* (day $^{-1}$), C_o is the potentially mineralizable C (% TOC) and k is the mineralization rate constant (day $^{-1}$). As same amount of C was applied in all treatments, the original C content was 3.58%.

Nitrogen mineralization and net nitrification rates were determined using the equations (3) and (4) according to Hart et al. (1994).

$$\text{N mineralization } (\mu g\ N\ g^{-1}\ d^{-1}) = [(NH_4^+{}_{final} + NO_3^-{}_{final}) - (NH_4^+{}_{initial} + NO_3^-{}_{initial})] / \text{days of incubation} \quad (3)$$

$$\text{Net nitrification } (\mu g\ NO_3^-\text{-}N\ g^{-1}\ d^{-1}) = (NO_3^-{}_{final} - NO_3^-{}_{initial}) / \text{days of incubation} \quad (4)$$

5.2.6 Plant growth assay

Pot experiment was carried out in a greenhouse to evaluate the effect of the same treatments used in incubation study on ryegrass (*Lolium perenne* L.) growth. A randomised block design was used, with four replications for each treatment. Three grams of ryegrass seeds was sown into each pot (300 ml capacity). The pots were settled at room temperature (22 ºC) and watered daily, after 60 days the ryegrass biomass was cut and dried at 60 °C until constant weight

5.2.7 Statistical analysis

The statistical analyses were performed using SPSS 19.0 program. After testing of assumptions, analysis of variance (ANOVA) was performed followed by Tukey's post-hoc test. For priming effect, paired t-test was used to compare the measured CO_2 production and the expected CO_2 production. Results marked as significantly different are different at $p<0.05$ unless specified in text. For correlation between variables Pearson's correlation coefficients were calculated. All reported values are means of four replicates.

5.3 Results

5.3.1 Carbon mineralization

Biochar application to soil did not increase CO_2-C release (Fig.5-1) respect to soil alone. The mineralization of C was stimulated by both composts with the highest values of S-C1 and S-C1-Bc where the cumulative CO_2-C production doubled compared to control with no significant difference between S-C1 and S-C1-Bc. The respiration of S-C2 was 1.6 times higher compared to control (710 μg CO_2-C g^{-1} with respect to 441 μg CO_2-C g^{-1}) while S-C2-Bc only 1.3 times higher (Fig. 5-1). After one day of incubation, there was no difference between treatments in respiration rates. Significant increase of CO_2-C production from S-C1 was observed on second day of incubation where S-C1 respiration rate was 2.5 times higher than control soil while other treatments were not different from control ($p<0.05$). From the sixth day, there was no difference found between S-C1 and S-C1-Bc. The respiration of S-C2 was slightly higher than S-Bc and generally higher than S-C2-Bc treatment. The mineralization of C was positively correlated with WSC (r= 0.8283; $p \leq 0.0001$) which was also the highest in S-C1 and S-C1-Bc and lowest in S-Bc and S (Table 5-2, Table 5-3).

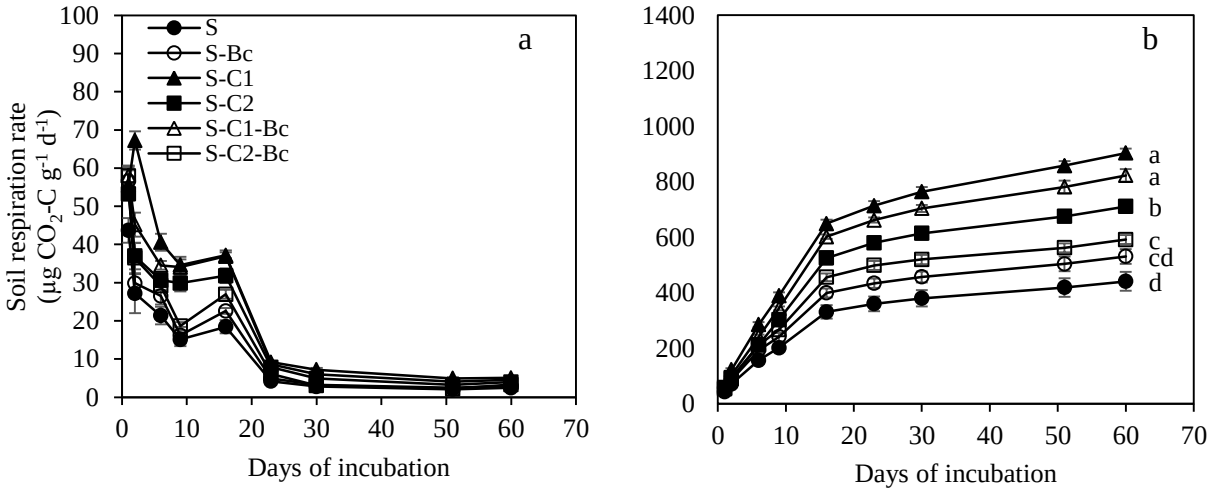

Fig. 5-1: Respiration rate (a) and cumulative CO_2-C production (b) of soil amended with organic carbon (10 mg C kg^{-1} soil) in form of biochar (S-Bc), immature compost (S-C1), mature compost (S-C2), mixture of immature compost and biochar (S-C1-Bc) and mixture of mature compost with biochar (S-C2-Bc) (Means±SE). Different letters at the end of cumulative CO_2-C production curves indicate significant differences in total amount of mineralized C ($p<0.05$).

The amount of CO_2-C evolved from all incubated treatments fitted well to first-order kinetic model with the fit being significant at $p<0.01$ (Table 5-2). Potentially mineralizable C pool (C_o) varied between treatments being the lowest in S-Bc and S-C2-Bc treatments (1.49 and 1.68% of total organic C of amended soil, respectively). There was found no difference in the mineralization rates between organic amendments used.

Table 5-2: Parameter values of the equations describing CO_2 evolution rate

	C_o (%TOC)			k(day^{-1})			RMS	F
S-Bc	1.49	(0.04)	c	0.0735	(0.0059)	a	0.0030	702.26 (p<0.01)
S-C1	2.62	(0.06)	a	0.0657	(0.0045)	a	0.0058	1,097.7 (p<0.01)
S-C2	2.04	(0.06)	b	0.0660	(0.0051)	a	0.0051	874.35 (p<0.01)
S-C1-Bc	2.37	(0.07)	a	0.0645	(0.0052)	a	0.0052	833.00 (p<0.01)
S-C2-Bc	1.68	(0.04)	c	0.0752	(0.0057)	a	0.0057	804.53 (p<0.01)

Different letters within each column indicate difference ($p<0.05$; one-way ANOVA). (Means±SE, n=4). S, control soil; S-Bc, soil amended with biochar; S-C1, soil amended with compost 1; S-C2, soil amended with compost 2; S-C1-Bc, soil amended with the mixture of compost 1 and biochar; S-C2-Bc, soil amended with the mixture of compost 2 and biochar. RMS residual mean square.

5.3.2 Priming effect

Priming effect was observed only in S-C1 and S-C1-Bc where, in both cases, the C-CO_2 released from amended soil resulted 25% higher ($p<0.05$) than additive amount of C-CO_2 evolved from soil and from organic material (Fig. 5-2). In both S-C1 and S-C1-Bc the differences between expected and observed CO_2-C production became significant on day 16 of the incubation. During the first two days, the observed CO_2-C production was slightly lower than the expected respiration, although these differences were not significant. While the amount of extra CO_2-C respired (primed C) from S-C1 continued to increase until the end of the incubation, the amount of primed C was stabilized in case of S-C1-Bc on day 16 (Fig. 5-2). Primed C from the amended soil was well correlated ($r=-0.8584$; $p\leq0.0001$) with total amount of mineralized N.

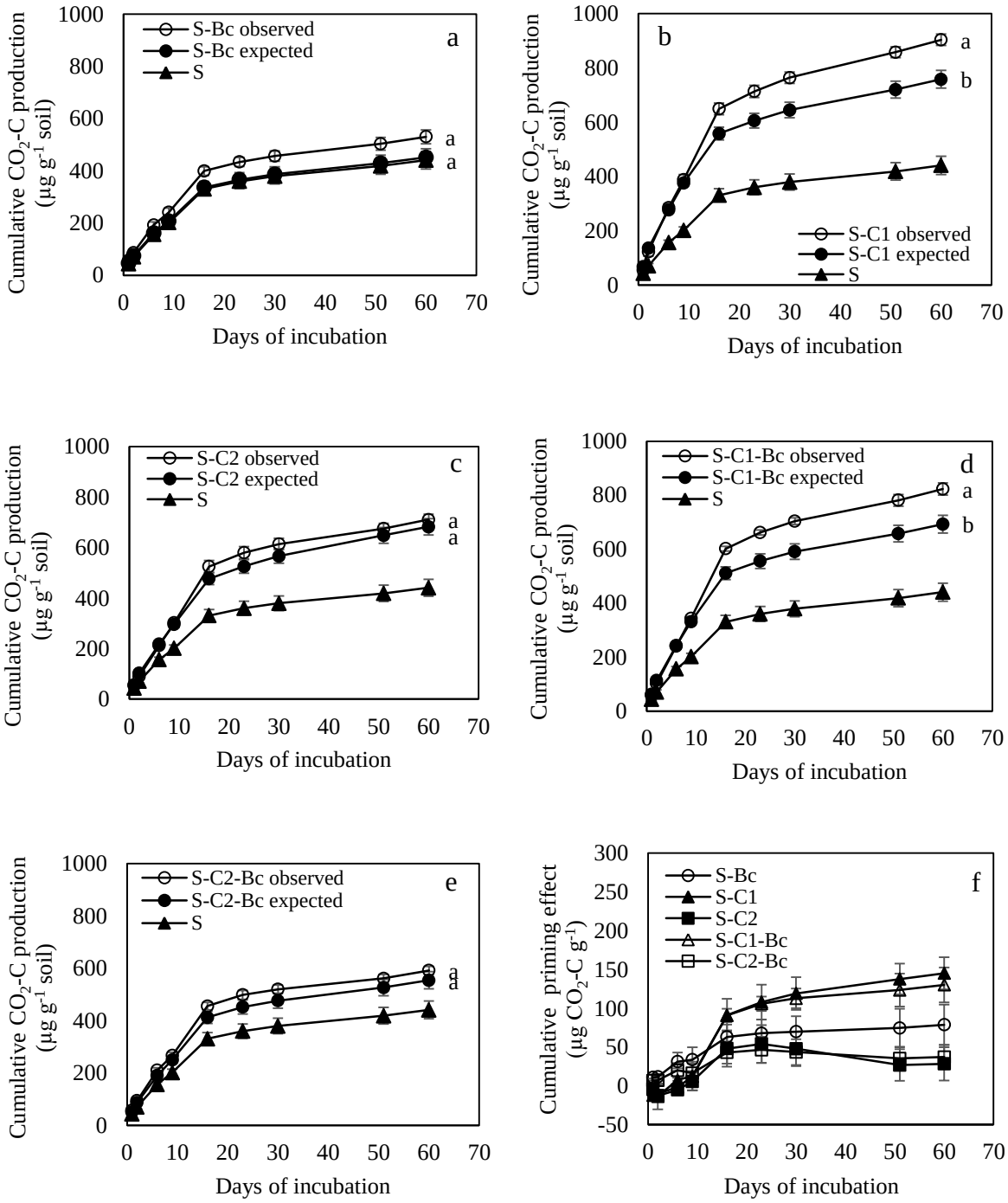

Fig. 5-2: Expected and observed cumulative CO_2-C production from S-Bc (a), S-C1 (b), S-C2 (c), S-C1-Bc (d), and S-C2-Bc treatments (e), and cumulative priming effect calculated as a difference between expected and observed cumulative CO_2-C production (f) (Means±SE). Different letters at the end of cumulative CO_2-C production curves indicate significant differences between expected and observed CO_2-C production ($p<0.05$). S control soil; S-Bc soil amended with biochar; S-C1 soil amended with compost 1; S-C2 soil amended with compost 2; S-C1-Bc soil amended with the mixture of compost 1 and biochar; S-C2-Bh soil amended with the mixture of compost 2 and biochar

5.3.3 Soil chemical properties

After 60 days of incubation, soil pH was increased by all organic materials with S-Bc resulting in the highest pH of 6.17 at the end of incubation (Table 5-3). The application of C1 caused the lowest increment of pH from all amendments and was increased by 0.49 unit compared to control. The EC remained unaffected (data not shown). The C:N ratio of soil was relatively low (14.6) with no difference ($p<0.05$) after C2 application. The application of Bc, C1-Bc, C2-Bc and C1 all increased the ratio to 18.6, 17.1, 16.7 and 15.6, respectively. The content of WSN was the lowest in both S-C1 and S-C1-Bc and without difference in S-Bc, S-C2 and S-C2-Bc compared to control. The ratio of WSC:WSN was higher only in S-C1 and less also in S-C1-Bc. Ammonium content at the end of incubation was negligible. The content of NO_3^--N was lowest in S-C1 and highest in S-C2 in order S-C1<S-C1-Bc<S-Bc=S-C2-Bc<S<S-C2.

Table 5-3: Soil chemical properties and nutrient content at the end of the incubation

	TN ($g\ kg^{-1}$)	C:N	pH_{H2O}	WSC ($\mu g\ g^{-1}$)	WSN ($\mu g\ g^{-1}$)	WSC:WSN	NO_3^--N ($\mu g\ g^{-1}$)
S	1.71 (0.01)b	14.6 (0.02)de	5.25 (0.08)c	510 (5.95)cd	78.1 (4.13)a	6.78 (0.37)c	54.02 (1.21)b
S-Bc	1.75 (0.02)b	18.6 (0.02)a	6.17 (0.02)a	482 (8.94)d	67.2 (1.87)a	7.19 (0.32)c	30.84 (0.19)c
S-C1	2.01 (0.01)ab	15.6 (0.19)cd	5.74 (0.03)b	664 (1.02)a	46.56 (1.64)c	14.3 (0.50)a	5.92 (0.17)e
S-C2	2.57 (0.1)a	13.9 (0.62)e	5.91 (0.08)ab	573 (8.11)bc	75.6 (2.29)a	7.59 (0.15)c	58.52 (1.18)a
S-C1-Bc	1.97 (0.02)ab	17.1 (0.25)b	5.99 (0.04)ab	606 8.97)ab	52.7 (0.52) b	11.5 (0.26)b	15.42 (0.58)d
S-C2-Bc	1.97 (0.03)b	16.7 (0.16)bc	5.95 (0.04)ab	551 (7.62)bc	72.9 (1.79)a	7.56 (0.09)c	30.96 (0.55)c

Different letters within each column indicate difference ($p<0.05$). (Means±SE, n=4). S control soil; S-Bc soil amended with biochar; S-C1 soil amended with compost 1; S-C2 soil amended with compost 2; S-C1-Bc soil amended with the mixture of compost 1 and biochar; S-C2-Bc soil amended with the mixture of compost 2 and biochar. TN total nitrogen; WSC water soluble carbon; WSN water soluble N

5.3.4 Microbial biomass and enzymatic activity

All organic amendments resulted in decreased MBC compared to control (Fig. 5-3) and increased dehydrogenase activity, which were both well correlated with soil pH ($r=-0.740$ and $r=0.765$, respectively, both at $p\leq0.001$). The S-C1-Bc resulted in values 2.7 times higher than non-amended soil, followed by S-Bc, S-C1 and S-C2-Bc which all doubled the dehydrogenase activity (Fig. 5-3). The β-glucosidase activity was only increased in S-C1-Bc and without differences between other treatments or compared to control (Fig. 5-3). Urease was increased in both S-C1 and S-C1-Bc four- and two-fold, respectively, and was negatively correlated with WSN ($r=-0.688$; $p<0.001$).

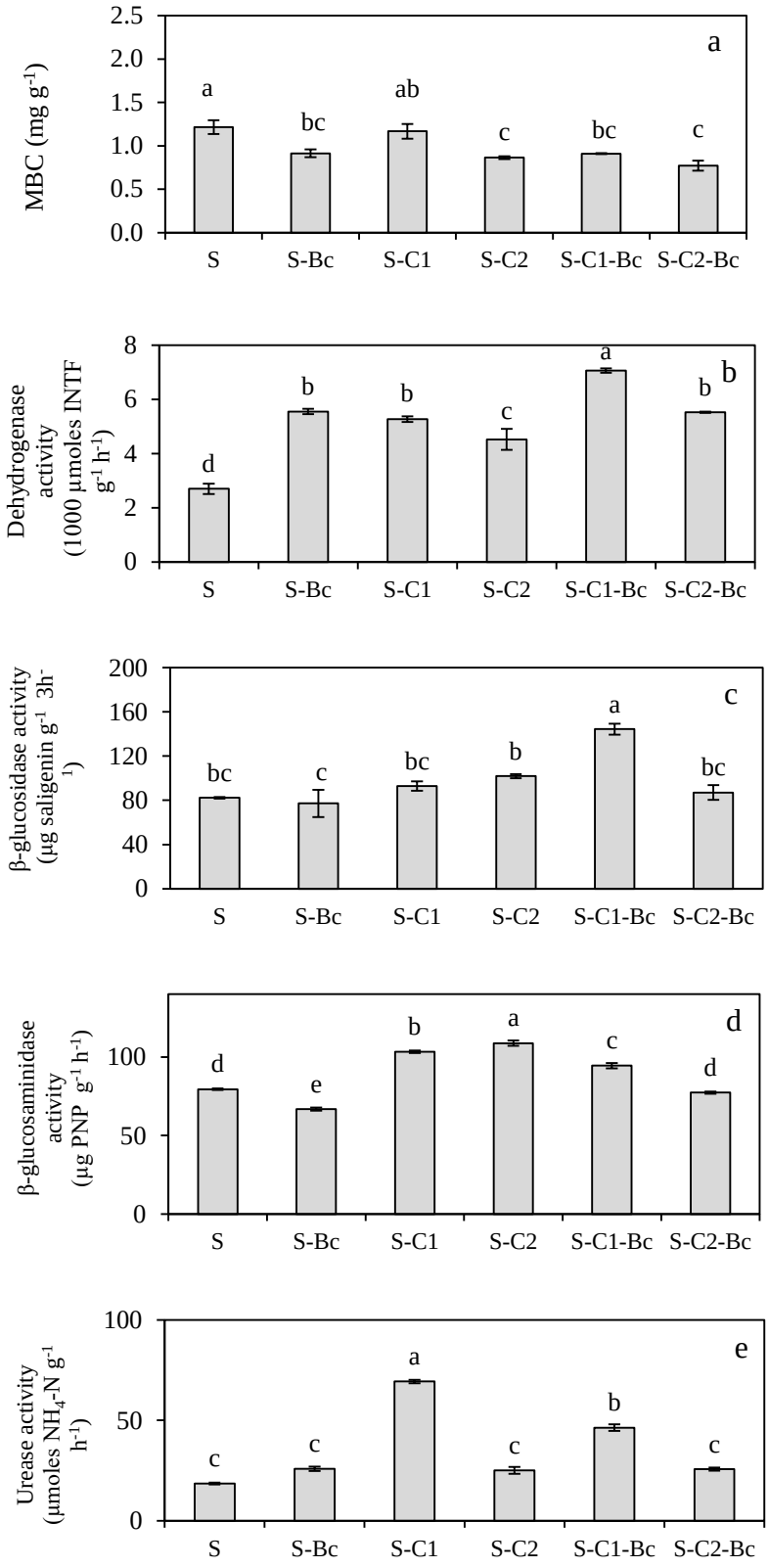

Fig. 5-3: The effect of different organic amendments on (a) microbial biomass carbon, (b) dehydrogenase activity; (c) β-glucosidase activity; (d) β-glucosaminidase activity and (e) urease activity (Means±SE). Different letters indicate significant difference at $p<0.05$. S control soil; S-Bc soil amended with biochar; S-C1 soil amended with compost 1; S-C2 soil amended with compost 2; S-C1-Bc soil amended with the mixture of compost 1 and biochar; S-C2-Bc soil amended with the mixture of compost 2 and biochar.

5.3.5 Nitrogen mineralization and net nitrification

Nitrogen mineralization rates of control soil and of S-C2 were 0.63 µg N g-1 d-1 and 0.58 µg N g-1 d-1, respectively, with no difference between S and S-C2 (Fig.5-4). Biochar application decreased the mineralization rate by 60% reaching down to only 0.25 µg N g-1 d-1 ($p<0.05$). When composts were applied together with biochar, the observed mineralization rates for S-C1-Bc and S-C2-Bc treatments were 0.03 and 0.26 µg N g-1 d-1, respectively. Nitrogen immobilization occurred in case of application of immature compost resulting in negative values of -0.08 µg N g-1 d-1 in S-C1 treatments. Net nitrification rate revealed similar patterns as N mineralization. However, S-C2 treatment resulted in net nitrification rate higher than control ($p<0.05$). Net nitrification rate of all treatments followed the order S-C2>S>S-Bc=S-C2-Bc>S-C1-Bc>S-C1. In case of S-C1, similarly to N mineralization, also net nitrification reached negative values as a result of N immobilization.

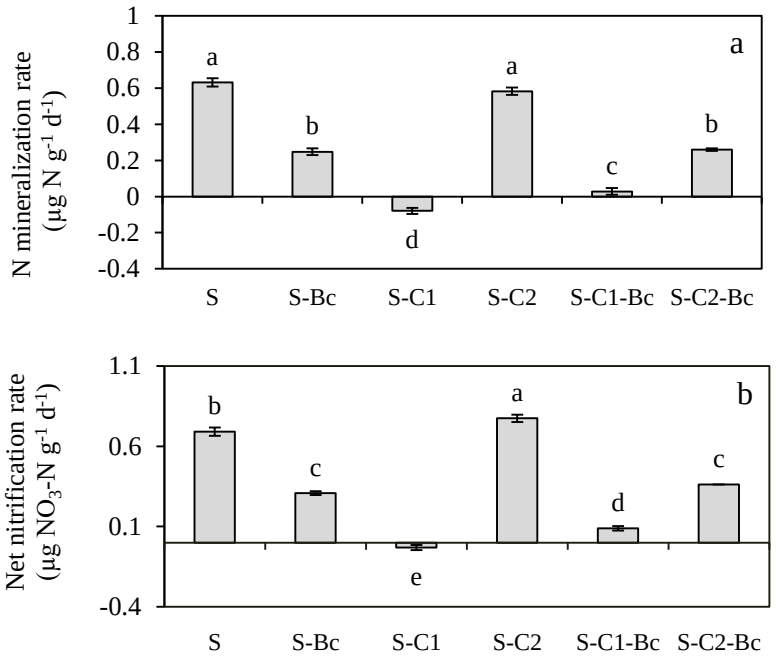

Fig.5-4: Effect of different organic amendments on (a) nitrogen mineralization rate and (b) net nitrification rate at the end of 60-days incubation. (Means±SE). Different letters indicate significant difference at $p<0.05$. S control soil; SBc soil amended with biochar; S-C1 soil amended with compost 1; S-C2 soil amended with compost 2; S-C1-Bc soil amended with the mixture of compost 1 and biochar; S-C2-Bc soil amended with the mixture of compost 2 and biochar.

5.3.6 Plant growth assay

Ryegrass biomass production was increased by all treatments compared to control and was positively correlated with soil pH (r=0.723; p<0.0001). The highest biomass production was observed in S-C2 and S-Bc treatments with no significant difference between both materials (p<0.05). The S-C2-Bc treatment was not different from S-Bc (Fig.5-5). In case of immature compost, the S-C1-Bc treatment was significantly lower than both S-Bc and S-C1.

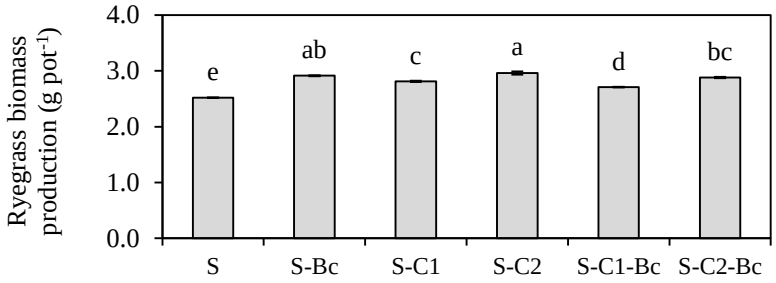

Fig.5-5: Effect of different organic amendments on ryegrass biomass production. (Means±SE). Different letters indicate significant difference at p<0.05. S control soil; SBc soil amended with biochar; S-C1 soil amended with compost 1; S-C2 soil amended with compost 2; S-C1-Bc soil amended with the mixture of compost 1 and biochar; S-C2-Bc soil amended with the mixture of compost 2 and biochar.

5.4 Discussion

The additive approach for PE evaluation used in this study could lead to overestimation of SOM priming by biochar, due to the fact that fresh biochar does not naturally possess microbial population for its mineralization and when it is incubated without soil it usually shows low CO_2 release. When it is applied to soil, the small part of labile C can be used by soil biota. Furthermore, biochar application to acid soil may lead to release of inorganic C contained in biochar (if present) also resulting in increase of CO_2 evolution (Jones et al. 2011). Nevertheless, as there was no significant difference found between the respiration of control soil and soil amended with biochar, the overestimation of PE is rather unlikely. On the other hand, considering both biochar-C and inorganic C release possibility, the lack of difference between observed and expected CO_2 release from S-Bc could be the result of no PE or negative PE caused by biochar application to soil, which could, however, be detected only using isotopic techniques.

Our results indicate that the application of compost or biochar to the soil induced short-term effects on soil properties. The short-term stimulation of microbial activity could be a result of C source addition, of pH increase resulting in stimulated bacterial community (Rousk

et al. 2009) or both. As the process of pyrolysis converted the large proportion of C into recalcitrant form unavailable to microorganisms (Wang et al. 2015a), the soil respiration was only affected by compost application and compost-biochar mixtures. After two months, we observed that WSC content and soil respiration of S-Bc remained both without difference compared to control. Similar findings were reported by Zavalloni et al. (2011) who also did not observe any increase of respiration after application of biochar made from coppiced woodland. In a similar way, Gómez-Muñoz et al. (2016) observed positive correlation of WSC with the magnitude of priming effect, which was also caused only by the application of pruning waste but not by biochar incorporation to soil. However, Zavalloni et al. (2011) found synergistic functioning of biochar and wheat straw on formation of soluble C compounds which they explained by easier decomposition and/or desorption of compounds on the biochar surface in presence of crop residues. Nevertheless, no strong priming effect of biochar and straw co-application was detected. In our experiment, S-C1 showed the highest amount of soluble organic carbon with no difference (p<0.05) between S-C1 and S-C1-Bc. These same treatments also showed the highest respiration and positive PE. In our case, the synergistic effect of immature compost and biochar on increased WSC content reflected in the stimulation of soil respiration and priming effect, while in the experiment of Zavalloni et al. (2011), the application of straw increased MBC. This increase of microbial MBC could immobilize part of the C, which could be the cause of discrepancies between their and our results, correspondingly to findings of Steiner et al. (2004) who concluded that biochar application together with readily decomposable OM did not stimulate respiration, but rather immobilize OM as a result of increased MBC. On the other hand, Castaldi et al. (2011) found no effect of biochar application on MBC and short-term increase of soil respiration, possibly due to small part of biochar C available for microorganisms. Wardle et al. (2008) reported that application of humus-biochar mixture to boreal forest soil resulted in increased C losses which they attributed rather to stimulation of humus decomposition than by decomposition of biochar. However, mineralization of applied labile organic matter, SOC and biochar seems to be rather interactive (Hamer et al. 2004). In case of mature compost, soil respiration was increased in S-C2 and S-C2-Bc, however S-C2-Bc was not different from simple mean between S and S-C2, and there was no PE observed, suggesting no interaction between biochar and stable OM in form of mature compost.

As previously commented, high dose of C applied to soil could lead to microbial community shift resulting in overall decrease in MBC. Moreover, the negative correlation of

MBC with soil pH ($r = -0,740$; $p<0.001$) could also affect the community structure. Generally, soil acidity alleviation after biochar application to soil could stimulate microbial biomass (Lehmann et al. 2011a) and cause significant shifts in microbial community structure (Anderson et al. 2011). To date, the majority of studies shows increase of soil biota after biochar application (Lehmann et al. 2011a) and few reports no effect of biochar (Rutigliano et al. 2014; Elzobair et al. 2016). In our study, changes in composition of microbial communities could have been caused by a turnover of bacteria or a trade-off between fungi and bacteria as a result of pH rise. Rousk et al. (2009) further stated high correlation between the decrease in fungal growth and the increase of bacteria alongside the pH raise, suggesting relationship between the growth of the two microbial groups rather than simple unrelated but opposite response to soil pH. They further revealed the negative effect of bacteria on fungal growth by manipulating the bacterial contribution to respiration (Rousk et al. 2008). Furthermore, fungal biomass has higher C content compared to bacterial biomass (Ekenler & Tabatabai 2003), thus, the decrease of soil fungi community and increase in soil bacteria can lead to overall decline of MBC after pH increase.

Helpful tool in understanding the processes leading to PE could be the activity of soil enzymes, reflecting the decomposition processes taking place in soil. As Hamer et al. (2004) suggested, important mechanisms causing biochar mineralization could be co-metabolism. When immature C1 was applied together with Bc, we observed synergistic effect on β-glucosidase activity and dehydrogenase activity which were both higher in S-C1-Bc compared to either S-Bc or S-C1. Dehydrogenases in soil have one of the most important roles for their function in microbial oxidation of organic matter and by some they are considered to be direct indicator of overall microbial activity. In the present study, the activity was stimulated by all organic amendments and was well correlated with soil pH ($r = -0.765$; $p<0.01$), which is in agreement with data obtained by Quilchano & Marañón (2002) who also observed strong correlation of dehydrogenases with soil pH in acid soil rich in organic matter. However, available data in literature about the relation between soil pH and the activity of these enzymes are still ambiguous. One of the factors influencing dehydrogenase activity in soil is SOM content and its quality (Fontaine et al. 2003). In our case, we obtained positive correlation with soil C:N ratio ($r=0.668$, $p<0.001$) and with soil respiration ($r=0.598$, $p<0.01$), resulting in S-C1-Bc with the highest dehydrogenases activity.

The β-glucosidase is the predominant glycosidase enzyme in soil and its function is the hydrolysis of the glucoside bonds in soils which is why it is usually well correlated with

organic C content (Eivazi & Tabatabai 1988). The application of organic material to soil involves the incorporation of large amounts of carbohydrates available for microorganism which further increases the β-glucosidase activity in the soil (Bastida et al. 2008). Similarly to our results, after application of biochar to soil, Tian et al. (2016) reported no significant effect on the activity of this enzyme.

Urease activity was only affected by the application of C1, where the activity was increased in response to the application of high amount of fresh organic matter, similarly to findings of Torres et al. (2015) after the application of compost. The strong negative correlation between WSN and urease suggests the repressive effect of WSN on the activity of this enzyme (Nannipieri et al. 1980). The high urease activity after C1 application could be directly linked to microbial population within C1 which was applied to soil along with C1, as the mixed application of C1 and Bc resulted in roughly half of the value.

The activity of β-glucosaminidase shows an opposite response to the application of biochar and both composts. There are several factors affecting this enzyme: (i) β-glucosaminidase activity is increased by the presence of easily mineralizable C and N (Ekenler & Tabatabai 2002); and (ii) β-glucosaminidase is correlated with the presence of fungi populations (Acosta-Martinez et al. 2004). In our case, the decrease in β-glucosaminidase activity after biochar application could be possibly explained by the increase in the pH which may have induced a decline of fungi population in favour of bacteria. This increase in the pH was similar after the compost amendments, where, however, the large amount of organic C contained in both materials could explain the overall increase in β-glucosaminidase activity.

Based on this synergistic effect of Bc and C1 on soil respiration, dehydrogenases and β-glucosidase activity and keeping in mind that both S-C1 and S-C1-Bc caused PE, we assume that in case of S-C1, the PE could be more apparent resulting from accelerated C turnover as response to high amount of energy added in form of easily degradable C. On the other hand, in S-C1-Bc treatment a shift towards the real PE may have been initiated resulting in increased production of SOM degrading enzymes.

The mineralization of N was the highest in control soil and in soil amended with C2 which had similar C:N ratio as the soil. Although degraded, soil collected from “raña” have still relatively high content of organic matter, which can decompose under favourable conditions. Application of fresh compost with high C:N ratio resulted in N immobilization

similarly to Benito et al. (2005), who also used pruning waste compost, suggesting that the large amount of C in C1 was not stabilized and was readily used by microorganisms. Biochar application to soil did not increase soil respiration, however, decreased to less than half the N mineralization occurring in control soil possibly due to adsorption by biochar. The final soil contained practically no NH_4^+ which suggests rich nitrifying community of the soil and the limitation of the ammonification rather than nitrification. Also in the study of DeLuca et al. (2006), soils with active nitrifier population showed no effect of biochar on soil nitrification. Despite no difference in respiration between S-C1 and S-C1-Bc, the final nitrate content was higher in S-C1-Bc compared to S-C1, possible also as a result of priming effect and more decomposition of SOM in S-C1-Bc.

Soil acidity alleviation was probably one of the main reasons of increased plant biomass production after application of all organic amendments. The co-application of immature compost and biochar resulted to be the least efficient in improving plant growth as ryegrass biomass production was significantly lower in S-C1-Bc treatment respect to S-C1 and S-Bc.

5.5 Conclusions

In conclusion, we observed a drop in MBC after application of both composts and biochar probably as a result of rapid pH raise. Biochar was not observed to cause any priming effect and its solely application to soil did not influence soil respiration. Synergism was found between C1 and biochar resulting in increased C mineralization and the activity of dehydrogenases and β-glucosidase enzymes. Although biochar could have adsorbed part of WSC from the soil as we expected, this was more than compensated by increased soil respiration and stimulation of soil enzymes. This synergistic effect could be caused by active microbial population of immature compost which could benefit from the improved habitat after biochar application or by priming of biochar after addition of WSC-rich material.

5.6 References

Acosta-Martínez V, Zobeck TM, Allen V. 2004. Soil Microbial, Chemical and Physical Properties in Continuous Cotton and Integrated Crop–Livestock Systems. Soil Science Society of America Journal **68**:1875. Soil Science Society.

Anderson CR, Condron LM, Clough TJ, Fiers M, Stewart A, Hill RA, Sherlock RR. 2011. Biochar induced soil microbial community change: Implications for biogeochemical cycling of carbon, nitrogen and phosphorus. Pedobiologia **54**:309–320.

Anderson JPE, Domsch KH. 1978. A physiological method for the quantitative measurement of microbial biomass in soils. Soil Biology and Biochemistry **10**:215–221.

Atkinson CJ, Fitzgerald JD, Hipps NA. 2010. Potential mechanisms for achieving agricultural

benefits from biochar application to temperate soils: A review. Plant and Soil **337**:1–18.

Bastida F, Kandeler E, Moreno JL, Ros M, García C, Hernández T. 2008. Application of fresh and composted organic wastes modifies structure, size and activity of soil microbial community under semiarid climate. Applied Soil Ecology **40**:318–329.

Benito M, Masaguer A, Moliner A, Arrigo N, Palma RM. 2003. Chemical and microbiological parameters for the characterisation of the stability and maturity of pruning waste compost. Biology and Fertility of Soils **37**:184–189.

Benito M, Masaguer A, Moliner A, Arrigo N, Palma RM, Effron D. 2005. Evaluation of maturity and stability of pruning waste compost and their effect on carbon and nitrogen mineralization in soil. Soil Science **170**:360–370.

Bower CE, Holm-Hansen T. 1980. A Salicylate–Hypochlorite Method for Determining Ammonia in Seawater. Canadian Journal of Fisheries and Aquatic Sciences **37**:794–798.

Castaldi S, Riondino M, Baronti S, Esposito FR, Marzaioli R, Rutigliano FA, Vaccari FP, Miglietta F. 2011. Impact of biochar application to a Mediterranean wheat crop on soil microbial activity and greenhouse gas fluxes. Chemosphere **85**:1464–1471.

DeLuca TH, MacKenzie MD, Gundale MJ, Holben WE. 2006. Wildfire-Produced Charcoal Directly Influences Nitrogen Cycling in Ponderosa Pine Forests. Soil Science Society of America Journal **70**:448.

Dempster DN, Gleeson DB, Solaiman ZM, Jones DL, Murphy D V. 2011. Decreased soil microbial biomass and nitrogen mineralisation with Eucalyptus biochar addition to a coarse textured soil. Plant and Soil **354**:311–324.

Ding Y, Liu YX, Wu WX, Shi DZ, Yang M, Zhong ZK. 2010. Evaluation of biochar effects on nitrogen retention and leaching in multi-layered soil columns. Water, Air, and Soil Pollution **213**:47–55.

Eivazi F, Tabatabai MA. 1988. Glucosidases and galactosidases in soils. Soil Biology and Biochemistry **20**:601–606.

Ekenler M, Tabatabai MA. 2002. β-Glucosaminidase activity of soils: effect of cropping systems and its relationship to nitrogen mineralization. Biology and Fertility of Soils **36**:367–376.

Ekenler M, Tabatabai MA. 2003. Effects of liming and tillage systems on microbial biomass and glycosidases in soils. Biology and Fertility of Soils **39**:51–61.

Elzobair KA, Stromberger ME, Ippolito JA, Lentz RD. 2016. Contrasting effects of biochar versus manure on soil microbial communities and enzyme activities in an Aridisol. Chemosphere **142**:145–52.

Espejo R. 1987. The soils and ages of the "raña" surfaces related to the Villuercas and Altamira mountain ranges (Western Spain). CATENA **14**:399–418.

Fontaine S, Mariotti A, Abbadie L. 2003. The priming effect of organic matter: a question of microbial competition? Soil Biology and Biochemistry **35**:837–843.

García C, Hernández T, Costa F, Ceccanti B, Ganni A. 1993. Hydrolases in the organic matter fractions of sewage sludge: Changes with composting. Bioresource Technology **45**:47–52.

Gómez-Muñoz B, Valero-Valenzuela JD, Hinojosa MB, García-Ruiz R. 2016. Management of tree pruning residues to improve soil organic carbon in olive groves. European Journal

of Soil Biology **74**:104–113.

Hamer U, Marschner B, Brodowski S, Amelung W. 2004. Interactive priming of black carbon and glucose mineralisation. Organic Geochemistry **35**:823–830.

Hart SC, Stark JM, Davidson EA, Firestone MK. 1994. Nitrogen mineralisation, immobilisation and nitrification. Pages 985–1013 MEthods of Soil Analysis: Microbiological and Biochemical Properties, Part 2, 3rd edition. ASA and SSSA, Madison.

Hoffmann GG, Dedeken M. 1965. Eine Methode zur colorimetrischen Bestimmung der β-Glucosidase-Aktivität in Böden. Zeitschrift für Pflanzenernährung, Düngung, Bodenkunde **108**:193–198.

Iannotti DA, Pang T, Toth BL, Elwell DL, Keener HM, Hoitink HAJ. 1993. A Quantitative Respirometric Method for Monitoring Compost Stability. Compost Science & Utilization **1**:52–65.

Jones DL, Murphy D V., Khalid M, Ahmad W, Edwards-Jones G, DeLuca TH. 2011. Short-term biochar-induced increase in soil CO2 release is both biotically and abiotically mediated. Soil Biology and Biochemistry **43**:1723–1731.

Kandeler E, Gerber H. 1988. Short-term assay of soil urease activity using colorimetric determination of ammonium. Biology and Fertility of Soils **6**:68-72.

Kandeler E, Stemmer M, Klimanek E-M. 1999. Response of soil microbial biomass, urease and xylanase within particle size fractions to long-term soil management. Soil Biology and Biochemistry **31**:261–273.

Lehmann J, Rillig MC, Thies J, Masiello CA, Hockaday WC, Crowley D. 2011. Biochar effects on soil biota - A review. Soil Biology and Biochemistry **43**:1812–1836.

Liu J, Schulz H, Brandl S, Miehtke H, Huwe B, Glaser B. 2012. Short-term effect of biochar and compost on soil fertility and water status of a Dystric Cambisol in NE Germany under field conditions. Journal of Plant Nutrition and Soil Science **175**:698–707.

Nannipieri P, Ceccanti B, Cervelli S, Matarese E. 1980. Extraction of Phosphatase, Urease, Proteases, Organic Carbon, and Nitrogen from Soil1. Soil Science Society of America Journal **44**:1011.

Novak JM, Busscher WJ, Watts DW, Laird DA, Ahmedna MA, Niandou MAS. 2010. Short-term CO2 mineralization after additions of biochar and switchgrass to a Typic Kandiudult. Geoderma **154**:281–288.

Parham JA, Deng SP. 2000. Detection, quantification and characterization of??-glucosaminidase activity in soil. Soil Biology and Biochemistry **32**:1183–1190.

Quilchano C, Marañón T. 2002. Dehydrogenase activity in Mediterranean forest soils. Biology and Fertility of Soils **35**:102–107.

Robarge WP, Edwards A, Johnson B. 2008. Water and waste water analysis for nitrate via nitration of salicylic acid 1. Communications in Soil Science and Plant Analysis **14**:1207–1215.

Rousk J, Brookes PC, Bååth E. 2009. Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. Applied and environmental microbiology **75**:1589–96.

Rousk J, Demoling LA, Bahr A, BÃ¥Ã¥th E. 2008. Examining the fungal and bacterial niche

overlap using selective inhibitors in soil. FEMS Microbiology Ecology **63**:350–358.

Rutigliano FA, Romano M, Marzaioli R, Baglivo I, Baronti S, Miglietta F, Castaldi S. 2014. Effect of biochar addition on soil microbial community in a wheat crop. European Journal of Soil Biology **60**:9–15.

Steiner C, Teixeira WG, Lehmann J, Zech W. 2004. Microbial Response to Charcoal Amendments of Highly Weathered Soils and Amazonian Dark Earths in Central Amazonia — Preliminary Results. Pages 195–212 in B. Glaser and W. I. Woods, editors. Amazonian Dark Earths: Explorations in Space and Time. Springer Berlin Heidelberg, Berlin, Heidelberg.

Strobl W, Traunmueller M. 1996. β-Glucosidase activity. Pages 198–200 in F. Schinner, R. Öhlinger, E. Kandeler, and R. Margesin, editors. Methods in Soil Biology. Springer Berlin Heidelberg, Berlin, Heidelberg.

Thiessen S, Gleixner G, Wutzler T, Reichstein M. 2013. Both priming and temperature sensitivity of soil organic matter decomposition depend on microbial biomass – An incubation study. Soil Biology and Biochemistry **57**:739–748.

Tian J, Wang J, Dippold M, Gao Y, Blagodatskaya E, Kuzyakov Y. 2016. Biochar affects soil organic matter cycling and microbial functions but does not alter microbial community structure in a paddy soil. Science of the Total Environment **556**:89–97.

Torres IF, Bastida F, Hernández T, García C. 2015. The effects of fresh and stabilized pruning wastes on the biomass, structure and activity of the soil microbial community in a semiarid climate. Applied Soil Ecology **89**:1–9.

Trevors JT, Mayfield CI, Inniss WE. 1982. Measurement of Electron Transport System (ETS) activity in soil. Microbial Ecology **8**:163–168.

Wang J, Xiong Z, Kuzyakov Y. 2015. Biochar stability in soil: meta-analysis of decomposition and priming effects. GCB Bioenergy **8**:512-523.

Wardle DA, Nilsson MC, Zackrisson O. 2008. Fire-derived charcoal causes loss of forest humus. Science **320**:629.

Whitman T, Singh BP, Zimmerman AR. 2015. Priming effects in biochar-amended soils: Implications of biochar-soil organic matter interactions for carbon storage. Pages 455–488 in J. Lehmann and S. Joseph, editors. Biochar for Environmental Management: Science, Technology and Implementation, 2nd edition. Routledge.

Zavalloni C, Alberti G, Biasiol S, Vedove GD, Fornasier F, Liu J, Peressotti A. 2011. Microbial mineralization of biochar and wheat straw mixture in soil: A short-term study. Applied Soil Ecology **50**:45–51.

Zimmerman AR, Gao B, Ahn M-Y. 2011. Positive and negative carbon mineralization priming effects among a variety of biochar-amended soils. Soil Biology and Biochemistry **43**:1169–1179.

6 BIOCHAR AND LIME EFFECT ON NITROGEN CYCLING

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ABSTRACT

Ca-amendments are recommended for soil fertility enhancement in acid soils. Biochar (Bc) can be used as an alternative for the same purpose. Biochar additions have been reported to alter microbial communities in soils and biogeochemical processes including nitrogen (N) cycling. In a microcosm experiment we investigated the interactive effects of soil pH, the type of soil amendment (lime or biochar) and the NH_4^+ supply on net N mineralization and nitrification in a degraded acid soil, and on the abundance of ammonia-oxidizing bacteria (AOB) and archaea (AOA). Soil was incubated under native pH and $CaCO_3$ or biochar manipulated pH to reach pH 6.2 and 6.8 in the presence or absence of added ammonium for 70 days. Our results showed that Bc had longer-lasting effect on soil pH than $CaCO_3$, suggesting that Bc could be a preferable liming agent. Increased pH stimulated microbial activity and led to increased N mineralization, which was higher when $CaCO_3$ was applied. Although pH increase and NH_4^+-N addition had no immediate effect on nitrification, they synergically enhanced nitrification at the end of the experiment. The *amoA* gene of AOA consistently outnumbered that of AOB, whereas only AOB *amoA* gene abundance number was significantly correlated with nitrification and their abundance followed similar trend as NO_3^--N during the incubation. In acid soils where AOB could play a significant role in nitrification biochar could result in more pronounced changes in N cycle than lime application which could be of especially high interest in intensively managed soils with high N inputs.

Key words: archaea; bacteria; liming; net nitrification; qPCR

6.1 Introduction

In degraded and resource-limited cropping systems, the combined application of mineral fertilizers and organic inputs is a recommended practice for soil fertility and crop productivity enhancement (Partey et al. 2013). This strategy is pertinent to the old Ultisols in the Cañamero's raña formation in SW of the Iberian Peninsula, Spain, where base extraction by harvest, SOM loss and soil fertilization has accelerated soil acidification and Al solubilisation (Goméz-Paccard et al. 2013). Restoration of these degraded soils, where the main constraints for crop production are Al toxicity and Ca^{2+} deficiency, would require lime or other Ca-amendment to raise soil pH and alleviate Al toxicity. Amendment with organic soil amendments, such as biochar (Bc), can increase soil pH as well as decrease nutrient loss through enhanced cation adsorption, improved organic matter content and water retention (Joseph et al. 2010).

Most of the N fertilizer used in agriculture is in the form of ammonium (NH_4^+) or NH_4^+-based compounds (Wang et al. 2015), which can be rapidly converted into nitrate (NO_3^-) via nitrification. Fertilizer N not recovered by crops is vulnerable to losses via NO_3^- leaching or nitrous oxide (N_2O) emissions, both with significant negative environmental impact. Soil pH and the NH_4^+ supply are key factors affecting nitrification (Homyak et al. 2014; Hanan et al. 2016) which can interact to magnify their effect on nitrification (Hanan et al. 2016). The increase of pH alone does not stimulate nitrification in soils in which both pH and ammonia availability are limiting, but combining N fertilization with liming has been observed to increase nitrification rates (Priha & Smolander 1995; Che et al. 2015). Although lime or biochar have been reported to increase net N mineralization and nitrification in soil (Nugroho et al. 2007; Ulyett et al. 2014; Che et al. 2015), the mechanism of lime- and biochar-mediated changes in nitrification remains unclear as does the effect on the abundance of ammonia oxidizers in degraded acid soils.

Both ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) possess the *amoA* gene, which encodes a subunit of ammonia monooxygenase, the key enzyme of aerobic ammonia oxidation, which is the first step of nitrification. Quantification of *amoA* gene copies showed that both groups are distributed in a wide range of ecosystems (Yao et al. 2011b). However, there is still debate about the relative contribution of each group and the factors influencing their activity in soils (Rudisill et al. 2016). Nevertheless, recent studies revealed that AOA may be particularly active when ammonia concentrations in soil are low, soil pH is low, or oxygen is limiting (Chen et al. 2011; Stewart et al. 2012; Xu et al.

2012; Qin et al. 2013) whereas AOB may be active at higher ammonia concentrations (Di et al. 2010; Wang et al. 2016), possibly due to metabolic advantage of AOA over AOB (Wang et al. 2014) at low substrate concentrations.

Biochar application to soil induces significant changes in soil biogeochemical processes through many different mechanisms which remain largely unknown. There is increasing evidence suggesting that Bc impacts a number of soil N reactions (Cough et al. 2013) and the effect is dependent on biochar feedstock and production conditions, in addition to soil properties and environmental conditions. Biochar-induced changes in N cycling differ substantially from potential lime-induced changes, as biochar may sorb NO_3^-, NH_3, NH_4^+ and organic-N (Bai et al. 2015) as well as inhibitory compounds such as phenolics that could otherwise inhibit nitrification (DeLuca et al. 2006). In addition, indirect mechanisms associated with soil microbial composition changes can be generated by the specific properties of biochar and thereby have strong implications for soil microbial N processing (Anderson et al. 2011). Nitrification rates were accelerated by addition of Bc in two acidic arable soils (Zhao et al. 2014). He et al. (2016) also reported an increased nitrification activity in biochar-amended acid oxisols when exogenous NH_4^+ was added, suggesting that nitrification, after successive biochar applications, was not limited by nitrifier activity as was observed in unamended soils. However, Wang et al. (2015) found that peanut shell Bc actually reduced nitrification in an acid soil due to the decreased NH_4^+-N content available to nitrifiers and reduced the abundance of AOB. These inconsistent biochar effects suggest that more attention to nitrification in biochar-amended agricultural soils has to be paid, especially in acidified soils derived from high N-fertilizer inputs.

The present study aims to compare the effect which two potential liming agents, the typically used lime and holm oak biochar, have on soil N cycling processes. Therefore, we investigated the interaction of soil pH, soil amendment (biochar and lime) and NH_4^+ supply on net N mineralization, nitrification and abundance of nitrifying organisms. We hypothesized that (1) regardless the liming agent used, increases in soil pH stimulate net mineralization and nitrification and that this effect is amplified after NH_4^+ addition and that (2) the response of AOA and AOB to changes in soil pH differs in biochar- and lime-amended soil as both materials show different chemical and physical characteristics that can affect nitrifying organisms. Because the lime amendment application is a common practice in restoration of acid soils and biochar is being used more and more frequently, the importance of the present study lies in the fact that we compared both liming agents to be able to make a

recommendation of a suitable C-rich alternative to lime in degraded acid soils as the Cañamero´s raña soil.

6.2 Materials and methods

6.2.1 Soil and biochar characterization

For the present study, acid degraded Acrisol was used. The properties of soil and biochar can be seen in Table 4-1 (Materials and methods chapter).

6.2.2 Incubation experiment

Aerobic incubation experiments were conducted with the following treatments (4 replicates each): (i) control (C, no amendment); (ii) soil amended with biochar in order to raise the pH to 6.2 (B6.2) corresponding to the application rate of 24.8 Mg ha^{-1} (iii) soil amended with biochar to increase pH to 6.8 (B6.8) equivalent to application rate of 46 Mg ha^{-1} (iv) soil amended with $CaCO_3$ for pH increase to 6.2 (L6.2) and soil with $CaCO_3$ addition to raise pH to 6.8 (L6.8) (Table 6-1). For each treatment, 100 g of soil were placed in eight jars, four were fertilized with diammonium phosphate (DAP) at rate of 200 mg $NH4^+$-N kg^{-1} and the rest four were not fertilized. Samples were incubated for 70 days at 25 ºC in dark. The moisture content (60% of the water holding capacity) was checked gravimetrically every two to three days and adjusted by adding deionized water.

Table 6-1: Biochar and lime ($CaCO_3$) application rates and pH values of amendmed soil

Treatment	Biochar (Mt ha^{-1})	$CaCO_3$ (kg ha^{-1})	pH
C	-	-	5.3
B6.2	24.8	-	6.2
B6.8	46.0	-	6.8
L6.2	-	458	6.2
L6.8	-	847	6.8

C – control; B6.2 – biochar application for pH increase to 6.2; B6.8 – biochar application for pH increase to 6.8; L6.2 – lime application to pH increase to 6.2; L6.8 – lime application for pH increase to 6.8

Soil was sampled on days 3, 7, 11, 21, 31, 45 and 70 for soil pH, NH_4^+-N and NO_3^--N content determination. On days 11 and 70, microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN), total archaea, total bacteria, archaeal ammonia oxidizers (AOA) and bacterial ammonia oxidizers (AOB) abundances were determined.

6.2.3 Analytical methods

Soil pH was measured after one hour shaking in water (1:2.5; soil:water). Inorganic N was extracted with 2M KCl (1:10) and the concentration of exchangeable NH_4^+-N and NO_3^--N were determined colorimetrically using sodium salicylate method (Forster JC 1995) and sulphanilamide and N-(1-naphthyl) ethylendiamine dihydrochloride method (Miranda et al. 2001), respectively.

Microbial biomass C and N were determined using the fumigation-extraction method (Vance et al. 1987) by fumigating 15 g of soil with ethanol-free chloroform followed by 0.5 M K_2SO_4 extraction (1:4). The concentration of organic C was determined colorimetrically by measuring Cr^{3+} produced by reduction of Cr^{6+} (578nm) after microwave digestion (Speedwave four, Berghof, Eningen, Germany) at 135°C for 30 minutes. Microbial biomass N content was determined by Kjeldahl digestion of extracts followed by steam distillation (Bremner & Mulvaney 1982). Microbial biomass C and N were calculated as the difference between the C and N content in fumigated and non-fumigated samples, divided by 0.38 (Joergensen 1996) and 0.54 (Brookes et al. 1985), respectively.

6.2.4 DNA extraction

Soil DNA was extracted from 0.25 g (total humid weight) of soil samples (days 11 and 70 after incubation) using the Power Soil DNA Isolation Kit (MoBIO Laboratories, Carlsbad, CA, USA), according to the manufacturer's instructions. DNA concentration and purity were determined by 260/280 nm and 260/230 nm measurements using a Nanodrop spectrophotometer (DeNovix, Wilmington, DE, USA).

6.2.5 Real-time PCR quantification

Quantitative PCR (qPCR) was performed to assess the abundance of the following genes: 16S rRNA gene for total bacteria, 16S rRNA gene for total archaea and *amoA* gene for AOA and AOB. The qPCR was performed in 20-µL reaction mixtures containing the following components: 10 µL of SYBR GreenER™ qPCR SuperMix (Invitrogen, NJ, USA), 0.5 µM of each primer (Table 6-2) and 4 µL of diluted DNA extracts.

The optimal dilution of DNA extracts was tested to compensate any reaction inhibition by humic acids co-extracted during DNA isolation (data not shown). All qPCR assays were run on an Applied Biosystems (Applied Biosystems, NJ, USA) ABI 7300 sequence detection system starting with the initial denaturation step at 95 ºC for 10 min, followed by amplification cycles specific for each target gene (Table 6-2). A melting curve

analysis was performed after each assay to ensure that only the products of the desired melting temperature were generated from the SYBR Green qPCR. The R^2 values for the standard curves were 0.99 or higher in all runs. The standard curves for quantifying gene copy numbers were determined by cloning the PCR products in a plasmid using the procedures reported by Okano et al. (2004). The population sizes of total bacteria, total archaea, AOB, and AOA were estimated as the normalized copies per gram of dry soil.

Table 6-2: Target genes, used primers and qPCR conditions

Target gene	Primers / Sequence (5´-3´) (reference)	qPCR
Bacteria 16S rRNA	341 F/ CCT ACG GGA GGC AGC AG 534 R/ ATT ACC GCG GCT GCT GGC A (Muyzer et al. 1995; Muyzer et al. 1996; López-Gutiérrez et al. 2004)	95ºC 10 min; 40 cycles: 95ºC 15 s, 60ºC 30 s, 72ºC 30 s, 80ºC 30 s,
Archaea 16S rRNA	Arc 771F/ ACGGTGAGGGATGAAAGCT Arc 957R/ CGGCGTTGACTCCAATTG (Ochsenreiter et al. 2003)	95ºC 10 min; 40 cycles: 95ºC 15 s, 55ºC 30 s, 72ºC 30 s, 80ºC 30
AOB-*amoA*	*amoA* A189F/ GGH GAC TGG GAY TTC TGG *amoA* 2R'/ CCT CKG SAA AGC CTT CTT C (Holmes et al. 1995; Okano et al. 2004)	95ºC 10 min; 40 cycles: 95ºC 30 s, 55ºC 30 s, 72ºC 30 s
AOA-*amoA*	*amo*Ars03F/ GCAGGWGACTAYATYTTCTAC amoArs03R/ GCATAATAKGTBCGMGTKCC (See note below)	95ºC 10 min; 40 cycles: 95ºC 15 s, 54ºC 30 s, 72ºC 30 s

A primer set designed to amplify the archaeal *amoA* gene (AOA) *Arch_amoA*_F 5'-AATGGTCTGGSTTAGAMG-3' (De La Torre et al. 2008) and *Arch-amoA*-R 5'-GCGGCCATCCATCTGTATGT-3' (Francis et al. 2005) (PCR conditions - 95ºC 10 min; 35 cycles: 95ºC 30 s, 58ºC 30 s, 72ºC 1 min, 72ºC 5 min) was used to amplify DNA extracted from local soil sample (Davis, California, USA) and the PCR product was cloned into pCR2.1-TOPO vector (Invitrogen, California). A clone with sequence identified as *Candidatus Nitrososphaera gargensis amoA* (BLASTX against refseq_protein database 2/4/2013 – 96% identity, 0 gaps) was selected as qPCR standard. To take advantage of newer AOA sequences in the database than those used to design older AOA qPCR primers, degenerate primers *amo*Ars03 F 5'-GCAGGWGACTAYATYTTCTAC-3' and *amo*Ars03 R 5'-GCATAATAKGTBCGMGTKCC-3' were designed based on alignment of 358 AOA sequences identified through BLAST searches using known AOA genes (2/13/13). The *amo*Ars03 F and R primers were tested by cloning and sequencing of PCR product amplified from DNA extracted from local soils (Davis, California, USA). All ten clones analyzed matched to archaeal *amoA* sequences. Primers were further tested against the cloned *Candidatus Nitrososphaera gargensis amoA* standard (see Table S-2 for PCR conditions) and shown to give satisfactory results (single peak in qPCR melt curve analysis, 92% efficiency, R2 = 0.995).

6.2.6 Statistical analysis

The effects of amendment type (Amend), pH (pH) and fertilizer application (Fert) on the concentration of exchangeable NH_4^+-N and NO_3^--N during the incubation were evaluated by the repeated measures three-way ANOVA for all sampling points using the software SPSS 19 (IBM SPSS, Inc., Chicago, USA). For this analysis, the control treatments were excluded to simplify the data set to only two amendment types (Biochar or $CaCO_3$) and two pH levels

(6.2 and 6.8). The used model was full factorial design for the between-subjects factors excluding the analysis of control treatments. The effects of the treatments on the MBC, MBN, pH, the gene copies (16S rRNA for total bacteria, 16S rRNA for total archaea, *amoA*-AOA and *amoA*-AOB) were analysed using the general linear model (GLM) for each sampling date separately using three-way ANOVA (amendment type, pH and fertilizer application as fixed factors) excluding the control treatment. Bivariate correlations were determined using all data by the Pearson's correlation coefficient.

6.3 Results

6.3.1 Amendment effect on soil pH, MBC and MBN

The pH was affected by the type of amendment used for both sampling dates (Table 6-3), with higher values in biochar-amended soil than limed treatments of the equivalent initial pH. Fertilization also affected pH; the application of ammonium-based fertilizer increased soil pH early in the experiment (day 11) but it had decreased by the end of the incubation (day 70). Differences between fertilized and unfertilized samples were greater in lime than in biochar-amended soil, as can be seen in the interaction between amendment and fertilizer (Table 6-3). An interactive effect of initial pH and amendment type on pH was also detected on day 70; while pH in biochar-amended soils (B6.8) increased with time (from 6.5 to 6.67), in the equivalent lime treatments (L6.8) pH decreased from 6.44 on day 11 to 6.20 on day 70. The MBC and MBN were highly variable across the examined samples (Table 6-3) with no significant differences in MBC. Significantly higher MBN values were found in lime- than biochar-amended soils, regardless of sampling date, while fertilization had no effect on MBN. On day 11, treatments adjusted to higher pH (6.8) showed higher MBN compared to treatments adjusted to pH of 6.2. Significant interactions between pH and fertilizer were found on both sampling dates (Table 6-3); on day 11 MBN was increased by fertilization at the higher pH, but on day 70 MBN was lower in fertilized than unfertilized samples at pH of 6.8.

Table 6-3: Microbial biomass C, microbial biomass N and pH

Treatment	MBC (mg kg^{-1})		MBN (mg kg^{-1})		pH	
	day 11	day 70	day 11	day 70	day 11	day 70
Without DAP application						
C	154 (±16.0)	120 (±12.6)	16.6 (±3.27)	23.7 (±8.05)	4.81 (±0.05)	4.79 (±0.00)
B6.2	127 (±4.04)	124 (±19.1)	14.3 (±1.20)	13.8 (±0.78)	5.90 (±0.06)	6.14 (±0.02)
B6.8	88.7 (±8.03)	133 (±26.5)	16.5 (±1.04)	38.4 (±6.22)	6.50 (±0.03)	6.67 (±0.03)
L6.2	84.4 (±7.39)	159 (±14.9)	32.6 (±4.81)	37.3 (±4.28)	5.89 (±0.05)	5.91 (±0.03)
L6.8	110 (±27.3)	150 (±9.73)	32.6 (±5.26)	28.0 (±2.66)	6.44 (±0.07)	6.20 (±0.03)
With DAP application						
C-F	257 (±32.7)	109 (±12.02)	43.5 (±3.51)	28.3 (±7.45)	5.32 (±0.03)	4.79 (±0.02)
B6.2-F	98.7 (±10.6)	135 (±16.04)	12.5 (±2.02)	20.0 (±3.53)	6.10 (±0.01)	5.73 (±0.07)
B6.8-F	119 (±17.8)	165 (±21.13)	39.4 (±3.61)	29.9 (±2.17)	6.66 (±0.07)	6.19 (±0.07)
L6.2-F	118 (±13.6)	174 (±21.7)	22.2 (±4.29)	43.4 (±6.45)	6.07 (±0.03)	5.36 (±0.02)
L6.8-F	156 (±21.4)	170 (±9.94)	34.6 (±5.26)	27.9 (±2.66)	6.43 (±0.05)	5.58 (±0.02)
Effects						
Amend	n.s.	n.s.	**	***	*	***
Initial pH	n.s.	n.s.	**	n.s.	***	***
Fert	n.s.	n.s.	n.s.	n.s.	**	***
Amed x initial pH	n.s.	n.s.	n.s.	**	n.s.	***
Amend x Fert	n.s.	n.s.	*	n.s.	n.s.	*
Initial pH x Fert	n.s.	n.s.	***	**	n.s.	n.s.
Amend x initial pH x Fert	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

DAP, Diammonium phosphate ($(NH_4)_2HPO_4$); C, control; B6.2, biochar application for pH increase to 6.2; B6.8, biochar application for pH increase to 6.8; L6.2, lime application to pH increase to 6.2; L6.8, lime application for pH increase to 6.8. n=4. Mean (±SE).
* $p<0.05$; ** $p<0.01$; *** $p<0.001$; n.s. not significant

6.3.2 Amendment effect on NH_4^+-N and NO_3^--N dynamics during incubation

Without additional NH_4^+-N supply, the concentrations of exchangeable NH_4^+-N decreased during the first three weeks in C, B6.2 and L6.2 (Fig.6-1) and remained at a low level (below 2 mg NH_4^+-N kg^{-1} soil) during the entire incubation. In contrast, in B6.8 and L6.8 the exchangeable NH_4^+-N concentration increased to a maximum (32 mg NH_4^+-N kg^{-1} soil) on day 31 and then sharply decreased (Fig. 6-1) with significant differences between L6.8 and the rest of the samples on day 70. As would be expected, application of NH_4^+-based fertilizer increased the ammonium content during the incubation period (Fig. 6-1). With the exception of the C treatment, a similar temporal pattern during the incubation was found for exchangeable NH_4^+-N accumulation in all treatments with fertilizer added, showing a slight increase the first week followed by a rapid drop (Fig. 6-1). B6.8 showed the lowest exchangeable NH_4^+-N content at the end of 70-day incubation. The exchangeable NH_4^+-N content was significantly affected by the type of amendment (Table 6-4). Values were significantly higher in L than Bc treatments. An interaction was found between pH and amendment type, with higher values in Bc-treated soil when the initial pH was 6.2, and higher values in L treatments when initial pH was the highest (6.8). On the other hand, the interaction between initial pH and fertilization showed that differences between fertilized and unfertilized samples were higher for the lowest than highest initial pH. The effects and interactions are listed in Table 6-4.

Table 6-4: Effects of amendment type (Amend), initial pH and fertilizer application (Fert) on exchangeable NH_4^+-N, and NO_3^--N

	exchangeable NH_4^+-N	NO_3^--N
	($mg\ g^{-1}$ soil)	
Amend	***	***
Initial pH	n.s.	n.s.
Fert	***	***
Amend x Initial pH	***	n.s.
Amend x Fert	n.s.	n.s.
Initial pH x Fert	***	**
Amend x initial pH x Fert	n.s.	n.s.

* $p<0.05$; ** $p<0.01$; *** $p<0.001$; n.s. not significant

In the initial stage of incubation, the unfertilized amended soils showed decreased nitrification with respect to control (Fig. 6-1). Nevertheless, after 21 days, nitrification for L6.2 was higher than control and remained lower than control until day 45 for the rest of the samples. At the end of the incubation period the highest NO_3^--N content was found in B6.8 and L.6.8 treatments, while the lowest were found in C and B6.2. The NO_3^--N values for

fertilized treatment increased during incubation, regardless of the type of amendment (Fig. 6-1) and fertilized were significantly higher ($p<0.001$, Table 6-4) than unfertilized treatments. The NO_3^--N values were higher in L than in Bc samples (Table 6-4). There was an interaction between initial pH and fertilizer at an initial pH of 6.2, with unfertilized samples showing a higher NO_3^--N content than fertilized treatments. In contrast, at pH 6.8, NO_3^--N content was higher in fertilized soils. The accumulation of NO_3^--N was correlated with the abundance of AOB ($r = 0.380$ $p<0.01$).

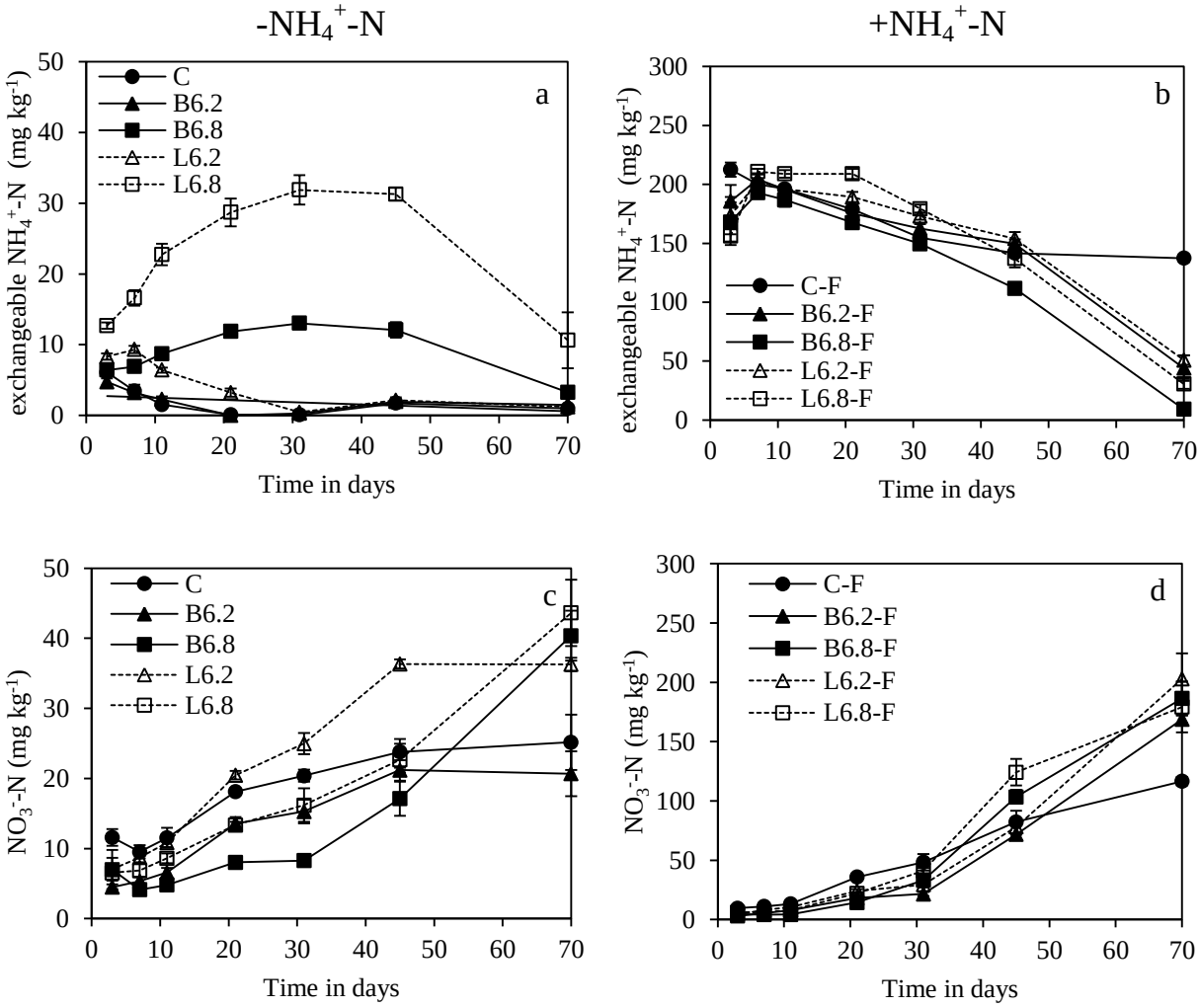

Fig.6-1: The content of exchangeable NH_4^+-N (a, b) and NO_3^--N (c, d) of treatments without fertilizer application (a, c,) and of fertilized treatments (b, d). Bars represent standard errors, n=4; C, control; B6.2, biochar application for pH increase to 6.2; B6.8, biochar application for pH increase to 6.8; L6.2, lime application to pH increase to 6.2; L6.8, lime application for pH increase to 6.8. C-F, control without amendment and with fertilizer application; B6.2-F, biochar application for pH increase to 6.2 and with fertilizer application; B6.8-F, biochar application for pH increase to 6.8 and with fertilizer application; L6.2-F, lime application to pH increase to 6.2 and with fertilizer application; L6.8-F, lime application for pH increase to 6.8 and with fertilizer application.

The Net Nitrification (NN) rate was more than four times higher on day 70 than at day 11 (Fig. 6-2), and this increase was most pronounced in fertilized treatments. On day 11, the NN was significantly higher in the lime-treated soil and also at the lower pH (6.2) (Table 6-4). The NN was positively correlated with AOB (r=0.339) and slightly negatively correlated with soil pH (r=-0.231).

The Net N Mineralization (NNM) rate followed a different pattern. On day 11 NNM was almost 3.5 times higher than on day 70 (Fig. 6-2). The effect of the different treatments was more marked on day 11, when NNM was significantly higher in the lime treatments and at 6.8 pH (Table 6-4). In addition, the application of fertilizer significantly increased the NNM. The interaction between amendment type and fertilizer showed that the highest rate in the fertilized lime treatments, while lowest in the unfertilized biochar treatments. No effect of fertilizer application on NNM was found on day 70, but the interaction between pH and fertilizer revealed that the fertilized treatments at higher pH were the only negative rates. The NNM rate was correlated with soil pH ($r = 0.371$ $p<0.01$).

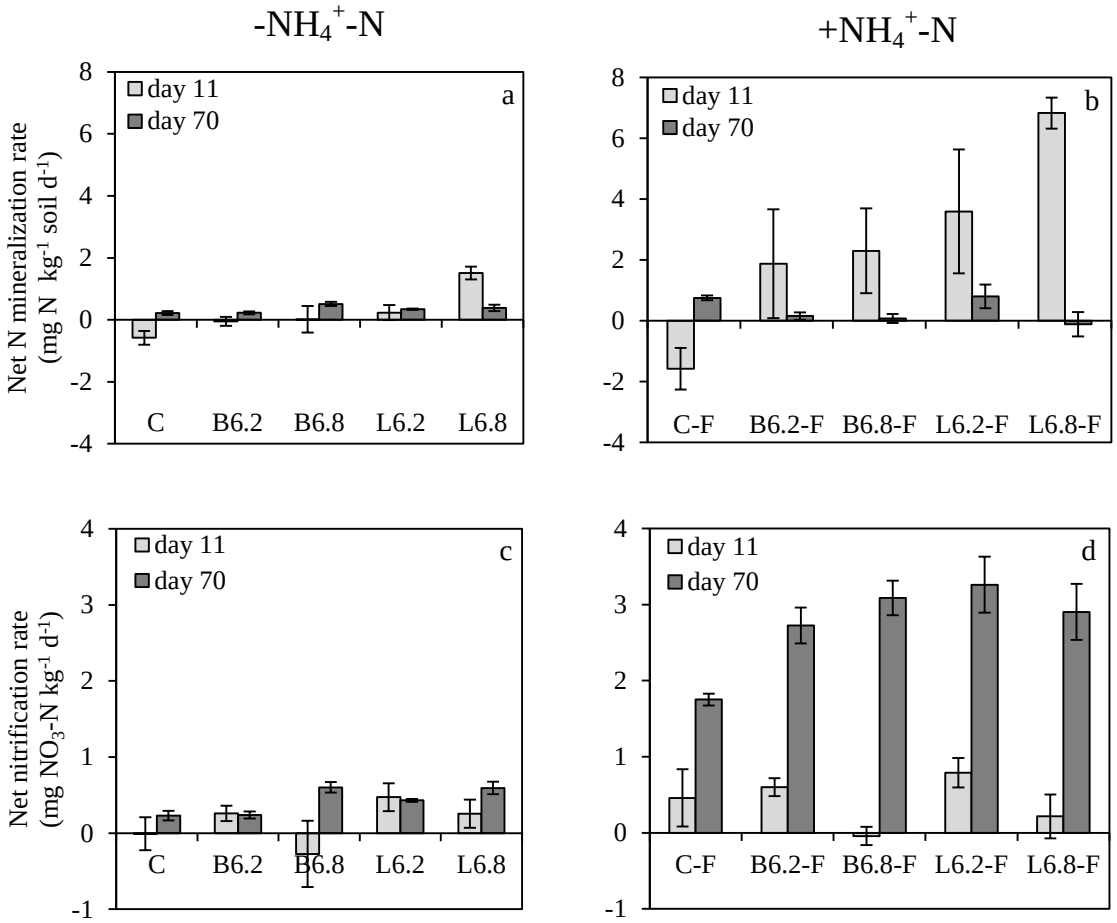

Fig.6-2: Net N mineralization rate (a, b) and net nitrification rate (c, d) of treatments without fertilizer application (a, c) and of fertilized treatments (b, d) from the beggining of the experiment until day 11 (day 11) and from the period between day 11 and day 70 (day 70). Bars resperesent standard errors, n=4; C, control; B6.2, biochar application for pH increase to 6.2; B6.8, biochar application for pH increase to 6.8; L6.2, lime application to pH increase to 6.2; L6.8, lime application for pH increase to 6.8. C-F, control without amendment and with fertilizer application; B6.2-F, biochar application for pH increase to 6.2 and with fertilizer application; B6.8-F, biochar application for pH increase to 6.8 and with fertilizer application; L6.2-F, lime application to pH increase to 6.2 and with fertilizer application; L6.8-F, lime application for pH increase to 6.8 and with fertilizer application

6.3.3 Amendment effect on the abundance of AOA, AOB, total archaea and total bacteria

The abundance of AOA and AOB were estimated by quantifying their respective *amoA* gene copy number. The abundance of AOA *amoA* was clearly affected by the amendment application, regardless the type of amendment (Fig. 6-3). This effect was higher in unfertilized samples than in those fertilized. AOA numbers ranged from 3.81×10^6 to 55.70×10^6 per gram of dry soil and from 3.61×10^6 to 18.40×10^6 per gram of dry soil in unfertilized and fertilized samples, respectively. Neither amendment type nor initial pH

significantly affected AOA abundance in any of the sampling dates (Table 6-4); meanwhile fertilization effect showed that unfertilized had higher values than fertilized treatments on day 70. On day 11, two significant three-way interactions ($p<0.01$) were found, showing an opposite response to NH_4^+-N application in lime and biochar treatments and depending on the initial pH level.

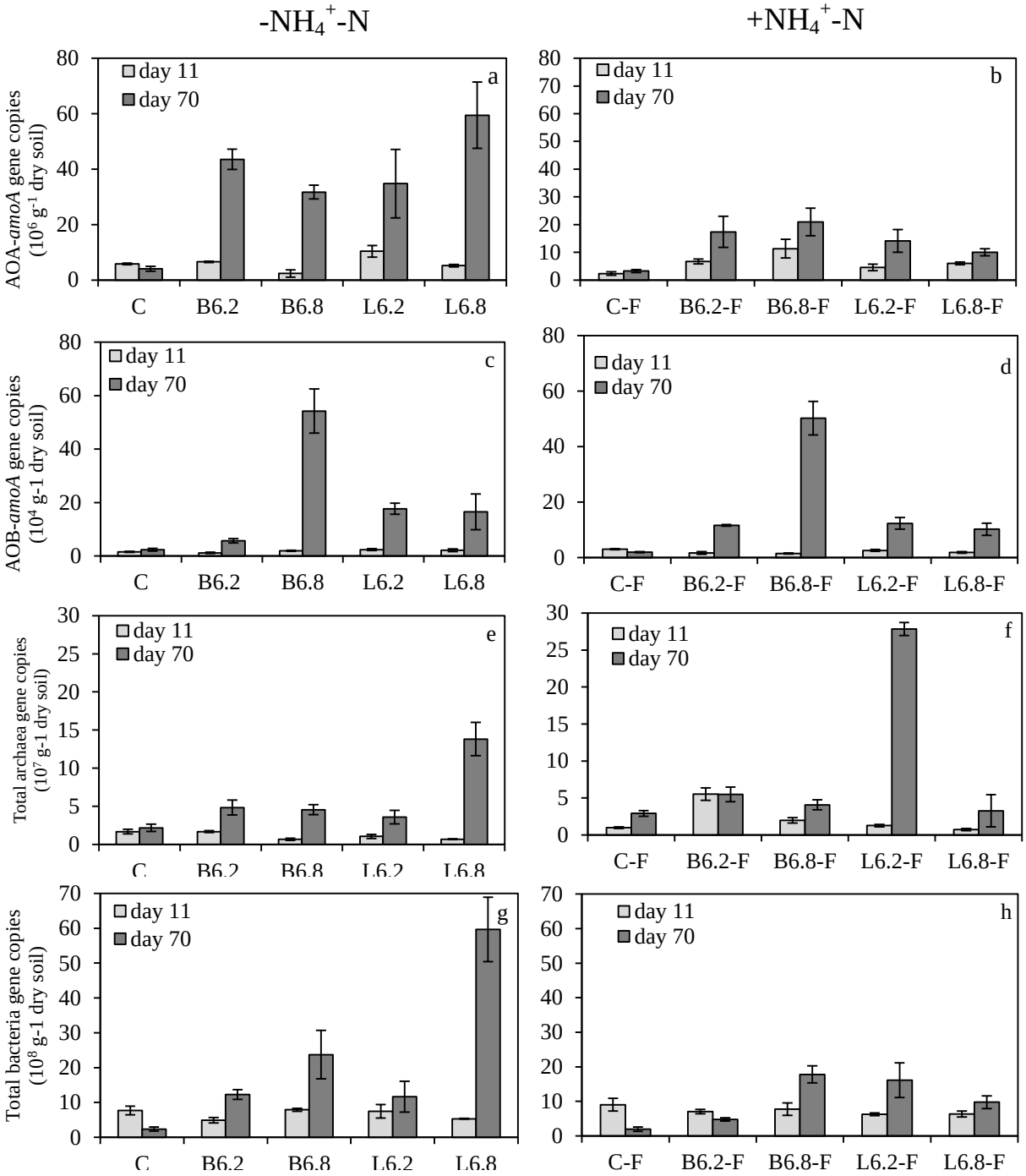

Fig.6-3: Gene copies of AOA-amoA (a, b), AOB-amoA (c, d), total archaea (e, f) and total bacteria (g, h) of treatments without fertilizer application (a, c, e, g) and of fertilized treatments (b, d, f, h). Bars represent standard errors, n=4; C, control; B6.2, biochar application for pH increase to 6.2; B6.8, biochar application for pH increase to 6.8; L6.2, lime application to pH increase to 6.2; L6.8, lime application for pH increase to 6.8; C-F, control without amendment and with fertilizer application; B6.2-F, biochar application for pH increase to 6.2 and with fertilizer application; B6.8-F, biochar application for pH increase to 6.8 and with fertilizer application; L6.2-F, lime application to pH increase to 6.2 and with fertilizer application; L6.8-F, lime application for pH increase to 6.8 and with fertilizer application.

Table 6-4: Effects of amendment type (Amend), pH and fertilizer application (Fert) on AOA-*amoA* gene copies, AOB-*amoA* gene copies, total archaea (16S rRNA gene copies) , total bacteria (16S rRNA gene copies), net N mineralization rate and net nitrification rate after 11 and 70 days of incubation

	AOA-*amoA*		AOB-*amoA*		Archaea total		Bacteria total		NNM		NN	
	day		day		day		day		day		day	
	11	70	11	70	11	70	11	70	11	70	11	70
Amend	n.s.	n.s.	**	***	***	***	n.s.	*	**	n.s.	*	n.s.
Initial pH	n.s.	n.s.	n.s.	***	***	*	n.s.	***	*	n.s.	**	n.s.
Fert	n.s.	***	n.s.	n.s.	***	n.s.	n.s.	***	***	n.s.	n.s.	***
Amed x Initial pH	n.s.	n.s.	n.s.	***	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Amend x Fert	**	n.s.	n.s.	n.s.	***	n.s.	n.s.	*	*	n.s.	n.s.	n.s.
Initial pH x Fert	**	n.s.	n.s.	n.s.	*	***	n.s.	**	n.s.	*	n.s.	n.s.
Amend x Initial pH x Fert	n.s.	*	n.s.	n.s.	*	***	n.s.	***	n.s.	n.s.	n.s.	n.s.

NNM, net nitrogen mineralization rate; NN, net nitrification rate
* p<0.05; ** p<0.01; *** p<0.001; n.s. not significant

The abundance of AOB was affected by amendment type (Fig. 6-3). Samples ranged from 9.44×10^4 to 52.30×10^4 per gram of dry soil and from 1.90×10^4 to 49.00×10^4 per gram of dry soil in unfertilized and fertilized samples, respectively. In this case, the amendment type showed an effect on AOB, being higher for L samples at day 11 and on the contrary, higher for biochar samples at day 70. The initial pH only affected AOB abundance on day 70, being higher when pH was 6.8 against 6.2. The interaction between type of amendment and pH showed that if biochar was used, AOB abundance in B6.8 treatment was higher than in B6.2 (Table 6-4). The AOA and AOB abundances were positively correlated ($r = 0.438$ $p<0.01$) and both AOA and AOB correlated positively with soil pH ($r = 0.278$ $p<0.05$ and $r = 0.293$ $p<0.05$, respectively). Fertilization did not affect AOB abundance.

The archaea 16S rRNA gene copies revealed that amendment has an effect in gene abundance (Table 6-4). Values ranged from 6.26×10^6 to 1.34×10^8 per gram dry and from 6.65×10^6 to 2.51×10^8 per gram dry in unfertilized and fertilized samples, respectively (Fig. 6-3). Values were higher in biochar treatments than in lime treatments for day 11 and, conversely, higher in lime than in biochar for day 70. The initial pH also had a significant effect on total archaea with highest numbers at the lowest pH ($p<0.05$). This effect was greater for biochar treatment than for lime treatment and in fertilized samples compared to unfertilized samples. A triple interaction was also found (Table 6-4), showing that for pH=6.2 and fertilized samples, the archaea 16S rRNA gene copies were higher for lime samples. In contrast, the archaea 16S rRNA gene copies were higher for lime samples, in pH=6.8 and unfertilized samples. Bacterial 16S rRNA gene copies values ranged from 2.07×10^8 to 5.71

$\times 10^9$ per gram dry soil and from 1.73×10^8 to 2.10×10^9 per gram dry soil in unfertilized and fertilized samples, respectively (Fig. 6-3). They did not show any difference at day 11, however, significant differences were found between different type of amendment, pH or fertilization (Table 6-4) at day 70. Bacterial 16S rRNA gene copies were higher in lime samples *vs* biochar samples, for pH=6.8 *vs* pH=6.2 and in unfertilized samples *vs* fertilized. Interaction between type of amendment and fertilization revealed that for unfertilized samples, total bacteria were higher in lime treatment than in biochar treatment. Bacterial 16S rRNA gene copies correlated positively with soil pH ($r = 0.240$ $p<0.05$).

6.4 Discussion

Both pH and liming amendment type affected N transformation in acid raña soil, but both effects were short-lived and were detected only in the beginning of the incubation study and no differences in net N mineralization and net nitrification were found after 70 days. Although the number of studies focusing on the effect of biochar on N transformation processes in soil is rapidly increasing, the effect of biochar on soil pH has been difficult to separate from the inherent role of biochar in N cycling. In this study, the use of lime in parallel with biochar application in order to reach the same final soil pH permitted us to evaluate the effects of both materials regardless the soil pH. Our results revealed that in this acid degraded soil AOB abundance, and not AOA abundance, was affected by the amendment type being enhanced by biochar application during the whole incubation period while net N transformation rates differed in lime- and biochar treated only in the very beginning of the experiment. In a similar way, Zhang et al. (2017) concluded that AOA abundance was not affected by the ameliorant used in an acid soil, however, AOB abundance was higher when wheat biochar was used against lime in combination with organic fertilizer. These authors pointed out in their study the importance of soil pH as a factor affecting the abundance of AOB.

6.4.1 The role of pH and NH_4^+-N supply in nitrogen cycling

The higher NNM rate and NH_4^+-N accumulation in treatments with initial pH adjustment to 6.8 were the result of ammonification stimulation in early stage of incubation when additional NH_4^+-N was not supplied. This confirms the importance of soil pH on microbial activity in the Cañamero's raña soil, as also observed in previous field study in the same area (León et al. (2017) where a sugar beet foam amendment enhanced activities of the

enzymes involved in N cycle, due to the prevention of their inhibition by acidic soil pH. Moreover, the application of fertilizer also stimulated the NNM in the short-term, which is a well described phenomenon (Jenkonson et al. 1985). However, the combination of the highest soil pH and the fertilization resulted in negative NNM rates on day 70, demonstrating that the effects of these factors on NNM were short-lived possibly due to depletion of the easily decomposable organic N substrate.

The stimulation of NN by NH_4^+-N amendment in acid soils has been observed in several studies (Ross & Hales 2003; Che et al. 2015), where low substrate concentrations were proposed to be the main factor limiting nitrification (Homyak et al. 2014). We found no effect of NH_4^+-N application on NN in the first 11 days of the incubation and a decrease of NN in high-pH treatments, suggesting that supplementation of NH_4^+-N and increase of soil pH did not overcome the limitations of nitrification in this soil in the short-term, the initial populations of nitrifiers were probably responsible for these limitations. Furthermore, if we consider the acidity and low fertility of this soil and the preference of AOA for acid soils with low ammonia concentration (Stopnišek et al. 2010; Hatzenpinchler 2012), we could expect that rapid increase of soil pH could have led to decrease of activity of AOA, or at least part of AOA community, which are more sensitive to ammonia inhibition (Prosser & Nicol 2012). In the same line, the relatively much lower abundance of AOB could not compensate for the decrease in ammonia oxidizing activity in this short period of time (11 days).

On the other hand, by the end of incubation, NN was higher only with NH_4^+-N addition, suggesting that at least three factors were limiting soil nitrification, namely insufficient NH_4^+-N supply, low soil pH and low activity of nitrifiers. The interactive effect of pH and NH_4^+-N application on NO_3^--N content suggests that the substrate can promote nitrification more in soils which already have activate nitrifiers to some extent (Yao et al. 2011). Hanan et al. (2016) concluded that only when the NH_4^+-N was at high enough concentrations, was nitrification in a Mediterranean soil in chaparral affected by the pH. We detected much lower NO_3^--N in the fertilized treatments with high pH, which also confirms the multiplication effect of pH and substrate addition in ecosystems where both of these factors are limiting nitrification, similar to the conditions at the end of the incubation period. Furthermore, both AOA and AOB abundance increased between day 11 and 70 in treatments with elevated soil pH, suggesting that both ammonia oxidizing groups could be affected positively by pH increase in acid soils. Nevertheless, despite being outnumbered by their counterpart AOA, the correlation of AOB with net nitrification and with NO_3^--N content

suggest that probably AOB were largely responsible for nitrification in these acid soils. Indeed, others have observed that higher AOA abundance does not necessarily mean that they are the primary nitrifiers in a soil (Nicol et al. 2008; Tian et al. 2014).

Xu et al. (2012) pointed out the importance of N inputs on abundance and activity of AOB and AOA in soils. Application of fertilizer did not influence the abundance of AOB in our study, similar to what was observed by Phillips et al. (2000), Hallin et al. (2009) and Wessén et al. (2010). In these studies, the growth of bacterial ammonia oxidizers was reported to be limited more by pH-related factors than by insufficiency of substrate.

6.4.2 The response of nitrogen cycling to $CaCO_3$ or biochar application

Despite the fact that the pH was initially the same, the type of amendment had a significant effect on NNM and NN in the beginning of the incubation. The lower concentration of NH_4^+-N in B6.8 than L6.8 could have resulted from higher rates of NNM in L6.8 than B6.8, or due to NH_4^+-N adsorption onto biochar due to the presence of negatively charged functional groups onto biochar surface (Novak et al. 2010; Bai et al. 2015) or a physical entrapment of NH_4^+-N in biochar pore structures (Saleh et al. 2012). Alternatively, the labile fraction of biochar C may have stimulated microbial growth immobilizing N, which, however, was neither confirmed by MBN nor by the abundance of total archaea and bacteria, which were all higher or same in $CaCO_3$-amended treatments. Furthermore, if C was not a limiting factor for microbial growth in this soil, the application of biochar containing some labile C and N could partly cover the need for N resulting in decreased need of SOM mineralization (negative priming effect).

One interpretation of the higher number of AOB *amoA* gene copies in the B6.8 sample in both fertilized and unfertilized treatments suggests that biochar could create a better habitat for AOB. Numerous reports have shown that AOB are favored in nutrient-rich environments with high organic C content (He et al. 2016). The B6.8 treatment had the highest input of biochar and, consequently, a highest organic C input. The higher abundance of AOB in biochar treatments after 70 days of incubation indicates that biochar application increased the community size of bacterial ammonia oxidizers.

6.5 Conclusions

The main finding of the present study is that increase of soil pH and NH_4^+-N addition did not result in stimulated nitrification in early stages of incubation, suggesting that the low activity of nitrifiers could have been the limiting factor at the beginning of the experiment.

Furthermore, we observed that both AOA and AOB *amoA* gene copies increased in number when soil pH was increased, suggesting that pH increment can have a positive effect on both ammonia oxidizing groups in acid soils. Nevertheless, only AOB positively correlated with net nitrification and their abundance followed similar trend as NO_3^--N during the incubation. The abundance of archaeal *amoA* gene decreased after fertilizer application but was not affected by the amendment type while AOB seemed to be limited more by the pH-related factors than by N supply. Biochar seems to be a suitable alternative to lime and no increased nitrification rates after biochar application were detected when compared to lime at a comparable soil pH. Nevertheless, the correlation of soil nitrification with AOB abundance rather than AOA along with the AOB preference for biochar-treated soil could raise the concern that biochar could have stronger effect on nitrification in long-term in soil where nitrification could be AOB-driven. It must be noted that our findings were obtained under specific microcosm conditions and although they are valid as a first approach, including quantification of transcripts would be necessary to clarify the importance of AOB in the studied soil.

6.6 References

Anderson CR., Condron LM, Clough TJ, Fiers M, Stewart A, Hill, RA, Sherlock RR. 2011. Biochar induced soil microbial community change: Implications for biogeochemical cycling carbon, nitrogen and phosphorus. Pedobiologia **54**:309-320.

Bai SH, Reverchon F, Xu CY, Xu Z, Blumfield TJ, Zhao H, Van Zwieten L, Wallace HM. 2015. Wood biochar increases nitrogen retention in field settings mainly through abiotic processes. Soil Biology and Biochemistry **90**:232–240.

Bremner JM, Mulvaney CS. 1982. Nitrogen-Total. Pages 595–624in A. L. Page, H. Miller, R, and K. D.R., editors.Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties (Agronomy series n 9) ASA. SSSA Madison, Wisconsin.

Brookes PC, Landman A, Pruden G, Jenkinson DS. 1985. Chloroform fumigation and the release of soil nitrogen: A rapid direct extraction method to measure microbial biomass nitrogen in soil. Soil Biology and Biochemistry **17**:837–842.

Clough T, Condron L, Kammann C, Müller C. 2013. A Review of Biochar and Soil Nitrogen Dynamics. Agronomy **3**:275–293.

DeLuca TH, MacKenzie MD, Gundale MJ, Holben WE. 2006. Wildfire-Produced Charcoal Directly Influences Nitrogen Cycling in Ponderosa Pine Forests. Soil Science Society of America Journal **70**:448.

Di HJ, Cameron KC, Shen J-P, Winefield CS, O'Callaghan M, Bowatte S, He J-Z. 2010. Ammonia-oxidizing bacteria and archaea grow under contrasting soil nitrogen conditions. FEMS microbiology ecology **72**:386–94.

Espejo R. 1987. The soils and ages of the “raña” surfaces related to the Villuercas and Altamira mountain ranges (Western Spain). CATENA **14**:399–418.

Forster JC. 1995. Soil Nitrogen. Pages 79–87in N. P. Alef K, editor.Methods in Applied Soil Microbiology and Biochemistry.

Gómez-Paccard C, Mariscal-Sancho I, León P, Benito M, González P, Ordóñez R, Espejo R, Hontoria C. 2013. Ca-amendment and tillage: Medium term synergies for improving key soil properties of acid soils. Soil and Tillage Research **134**: 195-206.

Hallin S, Jones CM, Schloter M, Philippot L. 2009. Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment. Isme Journal **3**:597–605.

Hanan EJ, Schimel JP, Dowdy K, D’Antonio CM. 2016. Effects of substrate supply, pH, and char on net nitrogen mineralization and nitrification along a wildfire-structured age gradient in chaparral. Soil Biology and Biochemistry 95:87–99.

Hatzenpichler R. 2012. Diversity, physiology, and niche differentiation of ammonia-oxidizing archaea. Applied and environmental microbiology **78**:7501–10. American Society for Microbiology.

He L, Liu Y, Zhao J, Bi Y, Zhao X, Wang S, Xing G. 2016. Comparison of straw-biochar-mediated changes in nitrification and ammonia oxidizers in agricultural oxisols and cambosols. Biology and Fertility of Soils **52**:137–149.

Homyak PM, Sickman JO, Miller AE, Melack JM, Meixner T, Schimel JP. 2014. Assessing Nitrogen-Saturation in a Seasonally Dry Chaparral Watershed: Limitations of Traditional Indicators of N-Saturation. Ecosystems **17**:1286–1305.

Che J, Zhao XQ, Zhou X, Jia ZJ, Shen RF. 2015. High pH-enhanced soil nitrification was associated with ammonia-oxidizing bacteria rather than archaea in acidic soils. Applied Soil Ecology **85**:21–29.

Chen X, Zhang L-M, Shen J-P, Wei W-X, He J-Z. 2011. Abundance and community structure of ammonia-oxidizing archaea and bacteria in an acid paddy soil. Biology and Fertility of Soils **47**:323–331.

Jenkinson DS, Fox RH, Rayner JH. 1985. Interactions between fertilizer nitrogen and soil nitrogen—the so called “priming”effect. Journal of Soil Science **36**:425–444.

Joergensen RG. 1996. The fumigation-extraction method to estimate soil microbial biomass: Calibration of the kEC value. Soil Biology and Biochemistry **28**:25–31.

Joseph SD et al. 2010. An investigation into the reactions of biochar in soil. Australian Journal of Soil Research **48**:501–515.

León P, Espejo R, Gómez-paccard C, Hontoria C, Mariscal I, Renella G, Benito M. 2017. No tillage and sugar beet foam amendment enhanced microbial activity of degraded acidic soils in South West Spain. Applied Soil Ecology **109**:69–74.

Miranda KM, Espey MG, Wink DA. 2001. A Rapid, Simple Spectrophotometric Method for Simultaneous Detection of Nitrate and Nitrite. Nitric Oxide **5**:62–71.

Nicol GW, Leininger S, Schleper C, Prosser JI. 2008. The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. Environmental Microbiology **10**:2966–2978.

Novak JM, Busscher WJ, Watts DW, Laird DA, Ahmedna MA, Niandou MAS. 2010. Short-term CO2 mineralization after additions of biochar and switchgrass to a Typic Kandiudult. Geoderma **154**:281–288.

Nugroho RA, Röling WFM, Laverman AM, Verhoef HA. 2007. Low nitrification rates in acid Scots pine forest soils are due to pH-related factors. Microbial ecology **53**:89–97.

Okano Y, Hristova KR, Leutenegger CM, Jackson LE, Denison RF, Gebreyesus B, Lebauer D, Scow KM. 2004. Application of Real-Time PCR To Study Effects of Ammonium on Population Size of Ammonia-Oxidizing Bacteria in Soil. Applied and Environmental Microbiology **70**: 1008–1016.

Partey ST, Preziosi RF, Robson GD. 2013. Maize Residue Interaction with High Quality Organic Materials: Effects on Decomposition and Nutrient Release Dynamics. Agricultural Research **2**:58–67.

Phillips CJ, Harris D, Dollhopf SL, Gross KL, Prosser JI, Paul EA. 2000. Effects of agronomic treatments on structure and function of ammonia-oxidizing communities. Applied and environmental microbiology **66**:5410–8.

Priha O, Smolander A. 1995. Nitrification, denitrification and microbial biomass N in soil from two N-fertilized and limed Norway spruce forests. Soil Biology and Biochemistry **27**:305–310.

Prosser JI, Nicol GW. 2012. Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. Trends in Microbiology **20**:523–531.

Qin HL, Yuan HZ, Zhang H, Zhu YJ, Yin C, Tan Z, Wu JS, Wei WX. 2013. Ammonia-oxidizing archaea are more important than ammonia-oxidizing bacteria in nitrification and NO_3^--N loss in acidic soil of sloped land. Biology and Fertility of Soils **49**:767–776.

Ross DS, Hales HC. 2003. Sampling-Induced Increases in Net Nitrification in the Brush Brook (Vermont) Watershed. Soil Science Society of America Journal **67**: 318-326.

Rudisill MA, Turco RF, Hoagland LA. 2016. Fertility practices and rhizosphere effects alter ammonia oxidizer community structure and potential nitrification activity in pepper production soils. Applied Soil Ecology **99**:70–77.

Saleh ME, Mahmoud AH, Rashad M. 2012. Peanut biochar as a stable adsorbent for removing NH4-N from wastewater: A preliminary study. Advances in Environmental Biology **6**:2170–2176.

Stewart FJ, Ulloa O, DeLong EF. 2012. Microbial metatranscriptomics in a permanent marine oxygen minimum zone. Environmental microbiology **14:**23–40.

Stopnišek N, Gubry-Rangin C, Höfferle Š, Nicol GW, Mandič-Mulec I, Prosser JI. 2010. Thaumarchaeal ammonia oxidation in an acidic forest peat soil is not influenced by ammonium amendment. Applied and Environmental Microbiology **76**:7626–7634.

Tian XF, Hu HW, Ding Q, Song MH, Xu XL, Zheng Y, Guo LD. 2014. Influence of nitrogen

fertilization on soil ammonia oxidizer and denitrifier abundance, microbial biomass, and enzyme activities in an alpine meadow. Biology and Fertility of Soils **50**:703–713.

Ulyett J, Sakrabani R, Kibblewhite M, Hann M. 2014. Impact of biochar addition on water retention, nitrification and carbon dioxide evolution from two sandy loam soils. European Journal of Soil Science **65**:96–104.

Vance ED, Brookes PC, Jenkinson DS. 1987. An extraction method for measuring soil microbial biomass C. Soil Biology and Biochemistry **19**:703–707.

Wang J, Zhang L, Lu Q, Raza W, Huang Q, Shen Q. 2014. Ammonia oxidizer abundance in paddy soil profile with different fertilizer regimes. Applied Soil Ecology **84**:38–44.

Wang Q, Zhang LM, Shen JP, Du S, Han LL, He JZ. 2016. Nitrogen fertiliser-induced changes in N2O emissions are attributed more to ammonia-oxidising bacteria rather than archaea as revealed using 1-octyne and acetylene inhibitors in two arable soils. Biology and Fertility of Soils **52**:1163–1171.

Wang Z, Zong H, Zheng H, Liu G, Chen L, Xing B. 2015. Reduced nitrification and abundance of ammonia-oxidizing bacteria in acidic soil amended with biochar. Chemosphere **138**:576–583.

Wessén E, Nyberg K, Jansson JK, Hallin S. 2010. Responses of bacterial and archaeal ammonia oxidizers to soil organic and fertilizer amendments under long-term management. Applied Soil Ecology **45**:193–200.

Xu Y gang, Yu W tai, Ma Q, Zhou H. 2012. Responses of bacterial and archaeal ammonia oxidisers of an acidic luvisols soil to different nitrogen fertilization rates after 9 years. Biology and Fertility of Soils **48**:827–837.

Yao H, Campbell CD, Qiao X. 2011a. Soil pH controls nitrification and carbon substrate utilization more than urea or charcoal in some highly acidic soils. Biology and Fertility of Soils **47**:515–522.

Yao H, Gao Y, Nicol GW, Campbell CD, Prosser JI, Zhang L, Han W, Singh BK. 2011b. Links between ammonia oxidizer community structure, abundance, and nitrification potential in acidic soils. Applied and environmental microbiology **77**:4618–25.

Zhao X, Wang S, Xing G. 2014. Nitrification, acidification, and nitrogen leaching from subtropical cropland soils as affected by rice straw-based biochar: Laboratory incubation and column leaching studies. Journal of Soils and Sediments **14**:471–482.

7 BIOCHAR AND MINERAL NITROGEN LEACHING

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ABSTRACT

Biochar is a carbon-rich porous material intensively studied for its agronomic benefits, such as decrease of greenhouse gases emission and nutrient losses via leaching, increased crop production and improved soil physical and chemical properties. We investigated the effect of holm oak biochar produced at 600°C on mineral nitrogen (N) leaching from two contrasting soils (Acrisol and Calcisol). Biochar was applied at three rates (0 %, 1 % and 2 % w/w) with (B0-F, B1-F and B2-F, respectively) and without (B0, B1 and B2, respectively) ammonium-based fertilizer. Soil columns were leached with deionized water and mineral N in leachate was monitored during ten weeks after each fertilization. Sorption behavior of biochar-amended soils was assessed in batch experiments before and after leaching. Biochar increased ammonium (NH_4^+-N) sorption in sandy Acrisol but had no effect on nitrate (NO_3^- -N) sorption. Furthermore, sorption properties of soil decreased by up to 25% during the study. In Acrisol, biochar affected NH_4^+-N leaching, which was increased by both doses of biochar without fertilization, but decreased by the lower biochar application rate when fertilizer was added. The leaching of NO_3^--N was not affected by biochar in Acrisol. The ability of Calcisol to adsorb NH_4^+-N was high and was not further increased by biochar, which corresponds to no NH_4^+-N leaching from Calcisol regardless the biochar application rate. Moreover, biochar had no effect on NO_3^--N leaching from Calcisol. Our results demonstrate that biochar effect on leaching of inorganic N forms is inconsistent, evolves in time and is highly dependent on soil properties. Alleviation of soil acidity by biochar application to Acrisol resulted in short-term stimulation organic N mineralization, which resulted in enhanced amount of NH_4^+-N being leached. Furthermore, the fact that biochar lost its effect on N leaching already after the second fertilizer application could cast a doubt on the efficiency of biochar application to soil in order to increase N retention and decrease N leaching.

Key words: Acrisol; adsorption; ammonium leaching; biochar; Calcisol; nitrate leaching

7.1 Introduction

Biochar, a carbon-rich material obtained by pyrolysis of organic matter, has been a focus of wide attention due to its potential to mitigate climate change via carbon (C) sequestration (Lehmann et al. 2006) and reduction of greenhouse gas emissions (Cayuela et al. 2013; Case et al. 2015). There is an exponentially growing number of studies focusing on the agronomic benefits of biochar including positive effects on soil properties (Mukherjee et al. 2014) and crop growth (Jeffery et al. 2011; Wang et al. 2012). Although biochar can contain significant amount of nutrients, from the practical or economical point of view it is usually not suitable for annual application to the soil. Instead, its potential lies in the fact that it can serve as a slow-release fertilizer liberating the nutrients continuously and thus, preventing the losses caused by the excess of available and mobile nutrients when there is no crop demand. Despite the large scale of potential modes of biochar's effects on nitrogen (N) cycling (Clough et al. 2013), the interactions of these mechanisms and the interplay between soil-microorganisms-biochar system remain poorly understood. Biochar has been observed to affect N fixation (Mia et al. 2014), N mineralization or immobilization (Ameloot et al. 2015), to increase or decrease nitrification (Ulyett et al. 2014), denitrification (Cayuela et al. 2013), or ammonia volatilization (Mandal et al. 2016).

Soil degradation, contamination of ground water and the high cost of fertilizers all lead to an urgent need of mechanisms to improve nutrient retention in soil and to prevent nutrient losses. Due to its porous structure and surface charge, biochar is a promising tool for N leaching mitigation (Laird et al. 2010). Clough et al. (2013) reviewed the effects of biochar on N dynamics and summarized the potential mechanisms of reduced N leaching to (i) adsorption onto biochar surface, (ii) anion or cation exchange reactions and (iii) immobilization as a result of the addition of a labile C contained in biochar. Increasing number of studies demonstrate that biochar could decrease N leaching due to its large surface area and surface charge (Ding et al. 2010; Yao et al. 2012). Indeed, its cation exchange capacity (CEC) is most likely the reason for ammonium (NH_4^+-N) retention by biochar (Dempster et al. 2011). Nevertheless, the sorption properties of biochar depend on feedstock and pyrolysis temperature. For example, Yao et al. (2012) reported that biochars produced at temperatures of 600°C or higher displayed the highest nitrate (NO_3^--N) adsorption. Furthermore, also soil properties will affect the final sorption behavior of the soil-biochar mixture (Streubel et al. 2011).

During the pyrolysis, the major part of the biomass C is transformed and becomes recalcitrant, leaving only a minor part available to microorganisms (Wang et al. 2015a). This small C pool can lead to a short-term stimulation of microbial processes, N immobilization (Zavalloni et al. 2011) or N-mining resulting in increased soil organic matter (SOM) mineralization. Indeed, increased (Castaldi et al. 2011), decreased (Dempster et al. 2011) and unaffected (Castaldi et al. 2011; Streubel et al. 2011) net N mineralization have been reported. Although both biotic and abiotic degradation of biochar have been demonstrated to have an effect on biochar surface properties and sorption capacity (Hale et al. 2011; Liu et al. 2013), most studies focus on sorption behavior of fresh biochar and fewer studies deal with aged or degraded biochar (Liu et al. 2013; Gronwald et al. 2015). Furthermore, the stage of oxidation of biochar could affect N transformation processes. For instance, nitrification rates were observed to increase after charcoal application (Berglund et al. 2004; DeLuca et al. 2006) but also decreased when fresh biochar was used (Zheng et al. 2012).

Growing number of studies aims to clarify the mechanism involved in biochar-induced reduction of leaching of highly mobile NO_3^--N from soil profile (Kanthle et al. 2016). The application of biochar to soil may affect the fate of applied fertilizer by various mechanisms, such as direct sorption of NO_3^--N (Mukherjee et al. 2014) which retains NO_3^--N in soil for a longer period of time increasing the opportunities of NO_3^--N uptake by plants or soil microorganisms; or by adsorption of NH_4^+-N which can prevent nitrification (Liang et al. 2006). Biochar sorption could limit NH_4^+-N assimilation by soil microorganisms or plants or its use as energy source of ammonia oxidizers, processes that need NH_4^+-N in solution (Thies et al. 2015).

Apart from biochar feedstock and production conditions, also soil type determines the properties of soil-biochar mixtures and the final agronomic or environmental impact of biochar application to soil (Streubel et al. 2011). Soil N transformations are likely to be affected distinctly in different soil types (Clough et al. 2013). While biochar porosity and its positive effect on soil aggregation (Herath et al. 2013) could be of higher interest in poorly drained soils, biochar sorption potential and high CEC could be a promising mean to lower nutrient losses in sandy (Sika & Hardie 2014) or kaolinitic soils (Laird et al. 2010). Furthermore, the native SOM content has been observed to affect the role of biochar in NO_3^--N leaching mitigation (Kanthle et al. 2016). Besides soil C and N content, the recalcitrance of biochar and of the soil N and C pools are believed to determine the mineralization of bioavailable forms of N contained in biochar (Clough et al. 2013). Therefore, we selected

two soils with contrasting properties: degraded acid sandy Acrisol rich in organic matter, and alkaline C-depleted Calcisol with loamy texture, in order to study the effect of holm oak biochar on inorganic N leaching. In particular, we focused on the sorption properties of biochar and biochar-soil mixtures as well as on their effects on net N mineralization and net nitrification. Furthermore, two leaching cycles were performed in order to detect the effect of fresh biochar when applied together with mineral fertilizer (first leaching cycle) and the effect of biochar already 'aged' in the soil when fertilizer is applied (second leaching cycle). We hypothesized that biochar produced at temperature of 600°C will decrease both NH_4^+-N and NO_3^--N leaching from both soil types, but that the sorption effect of biochar will be more pronounced in sandy Acrisol. Furthermore, the pH change in acid Acrisol can stimulate both N mineralization and nitrification and thus increase NO_3^--N losses or diminish the effect of biochar caused by its potential to adsorb NO_3^--N.

7.2 Materials and methods

7.2.1 Soil and biochar

Both Acrisol and Calcisol were used for the present study. The properties of both soils and applied biochar can be seen in Table 4-1 (Materials and methods chapter).

7.2.2 Experimental design and column preparation

For column preparation, sieving to 5 mm was selected as the most suitable due to the high content of rock fragments in Acrisol (51%) and to ensure adequate aeration and drainage. Both soils were amended with 1% (B1) and 2% (B2) of biochar (26 Mt ha^{-1} and 52 Mt ha^{-1}, respectively) including soil controls (B0) without biochar addition. Eight replicates were prepared for each soil and biochar application rate, four of them were left without fertilization (B0, B1 and B2 treatments) and four were fertilized with NPK at application rate of 36 kg NH_4^+-N ha^{-1}, 72 kg P ha^{-1} and 72 K kg^{-1} ha^{-1} (B0-F, B1-F and B2-F treatments) in the beginning of each leaching cycle. The amount of applied N was seven mg in each fertilization event per leaching column, resulting in 14 mg of total mineral N applied to each fertilized column.

Soil and biochar mixtures were packed in PVC columns (5 cm diameter and 30 cm height) to a bulk density of approximately 1.3 g cm^{-3}, which corresponds to the bulk density of studied soils. Bulk density was adjusted in control soils and same pressure was used to compact biochar-treated soil. All columns were fitted with fiber mesh and funnel on the bottom and a 5 cm layer of gravel and acid-washed sand was placed inside each column to

prevent soil losses. Control columns (without biochar amendment) received 500 g of soil and columns amended with biochar were filled with the amount of mixture equivalent to 500 g of soil and the corresponding amount of biochar (505 g and 510 g for B1and B2, respectively).

7.2.3 Leaching experiment

All columns were wetted with deionized water (pH 5.9, EC 0.09 μS cm^{-1}) to 40% of their water-holding capacity (WHC, determined by pressure plate extractor) and pre-incubated for one week in dark. After one week, four columns of each treatment were wetted with additional water to 60% of their WHC and four were fertilized with the same amount of water with dissolved fertilizer. No leaching occurred during the preparatory phase. The PVC columns were placed in a randomized design in a custom-made wooden rack.

Leaching events started one week after fertilizer (or water) application. Columns were leached with 100 ml of deionized water for ten consecutive weeks (leaching events) and the leachate was collected during 24 hours after each leaching event. After ten weeks, columns were left for four weeks, which were followed by application of the same dose of fertilizer (only fertilized treatments) or water to bring the columns again to 60% of their WHC. One week after fertilization or watering, columns were subjected to the first leaching event of the second leaching cycle. At the end of the second leaching cycle (ten weeks), soil from all columns was fresh sieved to <2 mm for determination of NH_4^+-N content, NO_3^--N content, pH, TOC, TN, potentially mineralizable nitrogen (PMN), net nitrogen mineralization (NNM), net nitrification (NN), NH_4^+-N sorption and NO_3^--N sorption.

7.2.4 Analytical methods

Collected leachate was quantified gravimetrically and analyzed for NH_4^+-N and NO_3^--N content colorimetrically using the salicylate Berthelot-Phenate method with variations (Bower & Holm-Hansen 1980) and the salicylic acid nitrification method (Robage et al. 2008), respectively.

Soil pH and electric conductivity were determined in the same way as described for the initial soil analysis. The PMN was determined by 7-day anaerobic incubation followed by NH_4^+-N determination and distraction of initial NH_4^+-N content (Waring & Bremner 1964). For NNM and NN estimation, 10 g of soil (60% WHC) were incubated under aerobic conditions at 25°C for two weeks. NNM and NN rates were calculated as the difference between final and initial total inorganic nitrogen (TIN) content (NH_4^+-N and NO_3^--N) and NO_3^--N content, respectively, divided by days of incubation (Hart et al. 1994). Inorganic N

was extracted with 2M KCl (1:10) and NH_4^+-N and NO_3^--N contents were determined colorimetrically (UV-1203, Shimadzu, Kyoto, Japan) using the sodium salicylate method (Foster 1995) and the sulphanilamide and N-(1-naphthyl)ethylendiamine dihydrochloride method (Miranda et al. 2001), respectively.

The sorption ability of biochar to NH_4^+-N and NO_3^--N was evaluated in batch sorption experiments, using 1 g of soil or 0.1 g of biochar as sorbents (Gao et al. 2015). Sorbents were placed in centrifuge tubes with 30 ml of aqueous solution containing NH_4^+-N (50 mg l^{-1}) or NO_3^--N (50 mg l^{-1}). Tubes were placed on horizontal shaker (120 rpm) for 24 hours at 25°C. Subsequently, tubes were centrifuged and filtered, and NH_4^+-N and NO_3^--N concentrations were determined colorimetrically as described above.

7.2.5 Statistical analysis

The effect of biochar application on the initial soil-biochar mixtures before leaching experiment (pH, sorption properties and PMN) was evaluated using one-way ANOVA separately for each soil type followed by post-hoc LSD test ($p<0.05$). The effect of soil type, biochar application and fertilization in leaching losses were analyzed by a repeated measures three-way ANOVA separately for the first and the second leaching cycle, considering the ten leaching events of each cycle as a within subject factor. The used model was full factorial design for the between-subjects factors and a post-hoc LSD test was used to evaluate the differences between the three doses of biochar ($p<0.05$). The within-subject factors are presented in the Table S-1 of supplementary material. In addition, the cumulative leaching losses at the end of both cycles and the final soil properties were analyzed using a full factorial General Linear Model (GLM) with soil type, biochar doses and fertilizer application as fixed factors and a post-hoc LSD to test the differences between the three biochar application rates ($p<0.05$). In all the analyses where the NH_4^+-N leaching was tested, only the Acrisol results were analyzed because no NH_4^+-N losses were detected in Calcisol. These variables were analyzed only considering biochar doses and fertilizer application as fixed factors in their respective models. Similarly, the relative amount of NO_3^--N losses respect to total amount of leached inorganic N was only calculated and statically analyzed in Acrisol. All the analyses were performed using the SPSS 22 (IBM SPSS, Inc., Chicago, USA) software.

7.3 Results

7.3.1 Soil properties and leachate volume

There was no difference between the leachate volume of control and biochar-amended soil (data not shown). The majority of soil properties data variability could be attributed to soil type (Table 2) explaining 96%, 64%, 96%, 82% and 93% of soil pH, TOC, TN, PMN and NH_4^+-N sorption data variability, respectively. Biochar effect on soil properties was of minor importance and explained less than 2% of pH data variability at the end of the leaching experiment (Table 7-1). Nevertheless, biochar application rate of 1% and 2% to acid Acrisol (original pH values 5.65) increased significantly ($p<0.05$) the pH of soil-biochar mixture (prior the initiation of the leaching) to 6.56 and 7.08, respectively (Fig. 7-1). This effect remained obvious at the end of the leaching experiment (Table 7-1), when the pH of biochar-amended soil remained higher than the pH of B0.

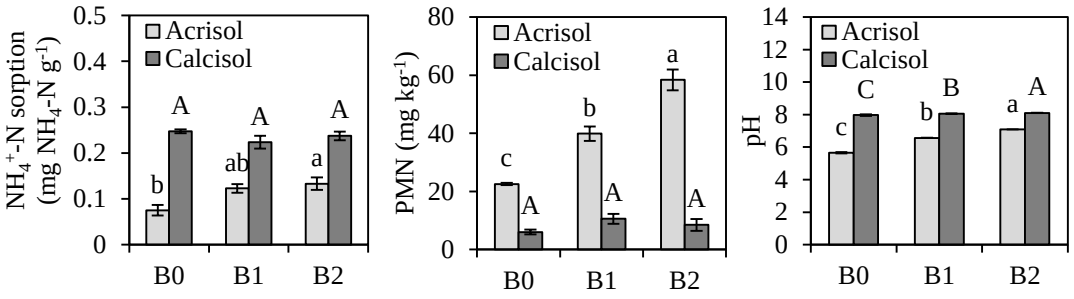

Fig. 7-1: NH_4^+-N sorption, potentially mineralizable nitrogen (PMN) and pH of Acrisol and Calcisol amended with biochar before leaching experiment. Different letters within the same soil type indicate significant difference (one-way ANOVA, $p<0.05$). B0 and B0-F, 0 % biochar with and without fertilization, respectively; B1 and B1-F, 1 % biochar with and without fertilization, respectively; B2 and B2-F, 2 % biochar with and without fertilization, respectively.

At the end of the leaching experiment, both TOC and TN were significantly higher ($p<0.001$) in biochar-amended treatments in both soil types although the effect size was larger in TOC than in TN ($\eta^2=0.341$ and $\eta^2=0.027$, respectively) and no effect of fertilization was detected (Table 7-1). Biochar application rate explained 32% of 2 M KCl-extractable NH_4^+-N data variability ($p<0.05$) and the NH_4^+-N content decreased ($p<0.001$) with increasing biochar application rate in Acrisol (Table 7-1). The amount of NO_3^--N remained without difference between fertilized and non-fertilized treatments and between control and biochar-amended soil and only 52.9% of data variability could be explained by the studied factors (Table 7-1).

Similarly, the NNM and NN rates remained largely unexplained with the majority of data variability attributed to unknown factors (75.2%, 80.8% and 47.1%, respectively).

Table 7-1: The effects of soil type (S), biochar application (B) and fertilization (F) on soil chemical properties at the end of the leaching experiment

	pH (H_2O)	TOC	TN	PMN[a]	NH_4^+-N	NO_3^--N	NNM[b]	NN[c]	NH_4^+ sor[d]	NO_3^- sor[e]
		(g kg^{-1})		(mg kg^{-1})						
Acrisol										
B0	3.88	22.80-	1.40	14.02	7.93	10.71	0.42	0.19	0.05	n.d.
B1	4.51	30.79	1.46	19.69	5.12	10.32	0.27	0.15	0.07	n.d.
B2	5.06	39.40	1.54	15.49	1.38	12.78	0.21	0.12	0.09	n.d.
B0-F	3.74	23.01	1.40	9.04	2.59	12.29	0.31	0.13	0.06	n.d.
B1-F	4.35	31.52	1.48	16.91	2.48	15.11	0.14	0.04	0.09	n.d.
B2-F	5.03	40.01	1.54	13.01	1.95	14.12	0.00	0.00	0.10	n.d.
Calcisol										
B0	8.17	9.55	0.87	1.85	0.00	6.43	0.00	0.00	0.21	n.d.
B1	8.19	15.50	0.86	2.07	0.00	3.95	0.37	0.32	0.23	n.d.
B2	8.14	21.72	0.92	0.72	0.00	14.60	0.28	0.25	0.24	n.d.
B0-F	8.17	8.42	0.79	2.84	0.00	4.92	0.02	0.00	0.22	n.d.
B1-F	8.19	15.79	0.86	1.39	0.00	9.33	0.27	0.21	0.22	n.d.
B2-F	8.09	20.30	0.95	0.75	0.00	7.01	0.00	0.00	0.22	n.d.
Effects (η^2 and p-value)										
S	0.964^{***}	0.637^{***}	0.963^{***}	0.824^{***}	-	0.231^{***}	$0.006^{n.s.}$	$0.001^{n.s.}$	0.927^{***}	-
B	0.016^{***}	0.341^{***}	0.027^{***}	0.035^{***}	0.317^{***}	0.086^{*}	$0.067^{n.s.}$	$0.045^{n.s.}$	0.024^{***}	-
F	0.000^{***}	$0.000^{n.s.}$	$0.000^{n.s}$	0.014^{**}	0.225^{***}	$0.004^{n.s.}$	$0.018^{n.s.}$	$0.015^{n.s.}$	$0.000^{n.s.}$	-
SxB	0.019^{***}	0.010^{***}	$0.001^{n.s.}$	0.042^{***}	-	$0.024^{n.s.}$	$0.024^{n.s.}$	$0.013^{n.s.}$	0.007^{*}	-
SxF	0.000^{***}	$0.001^{n.s.}$	$0.000^{n.s.}$	0.014^{*}	-	$0.035^{n.s.}$	$0.002^{n.s.}$	$0.002^{n.s.}$	0.003^{*}	-
BxF	$0.000^{n.s.}$	$0.001^{n.s.}$	0.002^{*}	$0.001^{n.s.}$	0.320^{***}	0.112^{*}	$0.077^{n.s.}$	$0.080^{n.s.}$	$0.000^{n.s.}$	-
SxBxF	0.000^{**}	$0.000^{n.s.}$	$0.001^{n.s.}$	$0.003^{n.s.}$	-	$0.037^{n.s.}$	$0.055^{n.s.}$	$0.036^{n.s.}$	$0.003^{n.s.}$	-
Error	0.000	0.010	0.006	0.068	0.139	0.471	0.752	0.808	0.035	

[a] Potentially mineralizable nitrogen (mg N kg^{-1})
[b] Net nitrogen mineralization (mg total inorganic nitrogen kg^{-1} d^{-1})
[c] Net nitrification (mg NO_3^--N kg^{-1} d^{-1})
[d] NH_4^+-N sorption (mg NH_4^+-N g^{-1})
[e] NO_3^--N sorption (mg NO_3-N g^{-1})
* indicates significant effect at p<0.05; ** p<0.01; *** p<0.001, n.s. not significant
n.d. not detected

7.3.2 Nitrogen leaching

Ammonium was detected only in the leachate from Acrisol, where it was significantly increased by both biochar and fertilizer application (Fig. 7-2, Table 7-2) and the biochar effect was more important than the fertilizer effect in the first leaching cycle (η^2=0.799 and η^2=0.066, respectively). Nevertheless, while fertilization explained the same part of variability in both leaching cycles (6.6%), the importance of biochar decreased and only 18.1% of NH_4^+-N leaching data variability could be explained by biochar application (Table 7-2) while 47.2% remained unexplained by studied factors. Total amount of leached NH_4^+-N during both cycles was not satisfactorily explained by studied factors with only 14.6% of data

variability explained by soil type, biochar application, fertilization and their interactions (Table 7-2).

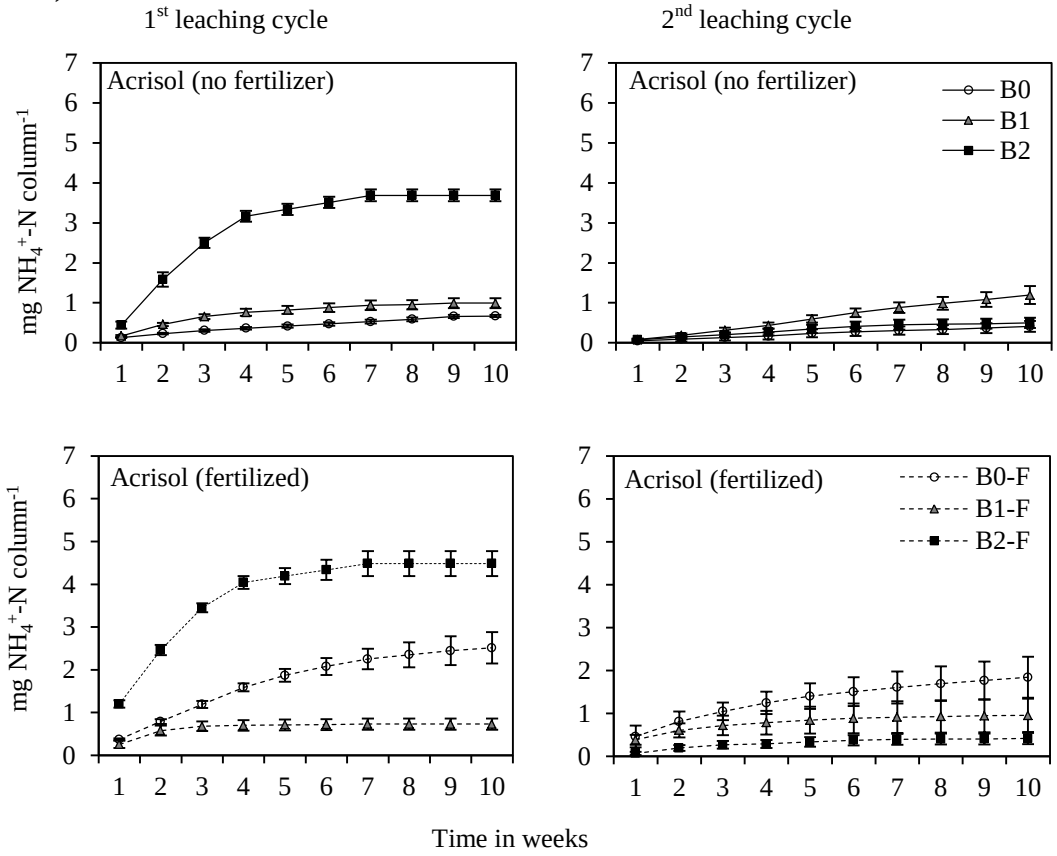

Fig. 7-2: Cumulative NH_4^+-N losses leached during the first and the second leaching cycle from unfertilized and fertilized Acrisol. Means±SE (n=4). B0 and B0-F, 0 % biochar with and without fertilization, respectively; B1 and B1-F, 1 % biochar with and without fertilization, respectively; B2 and B2-F, 2 % biochar with and without fertilization, respectively.

Table 7-2: The results of repeated measures ANOVA for the between-subject effects of soil type (S), biochar application (B), fertilization (F) and interactive effects on leaching of ammonium (NH_4^+), nitrate (NO_3^-) and total inorganic nitrogen (TIN) from Calcisol and Acrisol

	NH_4^+-N				NO_3^--N				TIN			
	1st leaching cycle		2nd leaching cycle		1st leaching cycle		2nd leaching cycle		1st leaching cycle		2nd leaching cycle	
	Sig.	η^2	Sig.	η^2	Sig.	η^2	Sig.	η^2	Sig.	η^2	Sig.	η^2
S	-	-	-	-	***	0.236	***	0.293	***	0.046	*	0.020
B	***	0.799	n.s.	0.181	**	0.037	n.s.	0.005	***	0.259	*	0.025
F	***	0.066	n.s.	0.066	***	0.562	***	0.530	***	0.477	***	0.703
SxB	-	-	-	-	**	0.043	**	0.021	***	0.086	n.s.	0.014
SxF	-	-	-	-	*	0.013	***	0.081	***	0.038	**	0.044
BxF	***	0.077	*	0.281	n.s.	0.006	n.s.	0.000	n.s.	0.004	*	0.027
SxBxF	-	-	-	-	n.s.	0.008	n.s.	0.000	n.s.	0.012	*	0.035
Error	-	0.058	-	0.472	-	0.095	-	0.070	-	0.077	-	0.133

* indicates significant effect at p<0.05; ** p<0.01; *** p<0.001; n.s. not significant
η^2 eta squared

The leaching of NO_3^--N was strongly increased by fertilization, with 56.2 and 53.0% of data variability explained by fertilization in the first and the second leaching cycle, respectively. Soil type also influenced the amount of leached NO_3^--N and the losses were significantly higher in Calcisol when compared to Acrisol (Fig. 7-3, Table 7-3). The fertilization increased more the leaching of NO_3^--N from Acrisol (by 67%) when compared to Calcisol where it was increased only by 34%. The biochar effect was low and only the highest biochar dose increased significantly the losses respect to the control. After the second fertilization, NO_3^--N leaching remained significantly higher in Calcisol when compared to Acrisol (Table 7-1) but no significant effect of biochar was detected. The interaction between soil type and biochar application revealed that the highest amount of NO_3^--N was leached from Calcisol in B1 treatment while in Acrisol the highest NO_3^--N leaching was found in B2. The increase in NO_3^--N leaching caused by fertilization was higher in Acrisol (Table 7-2).

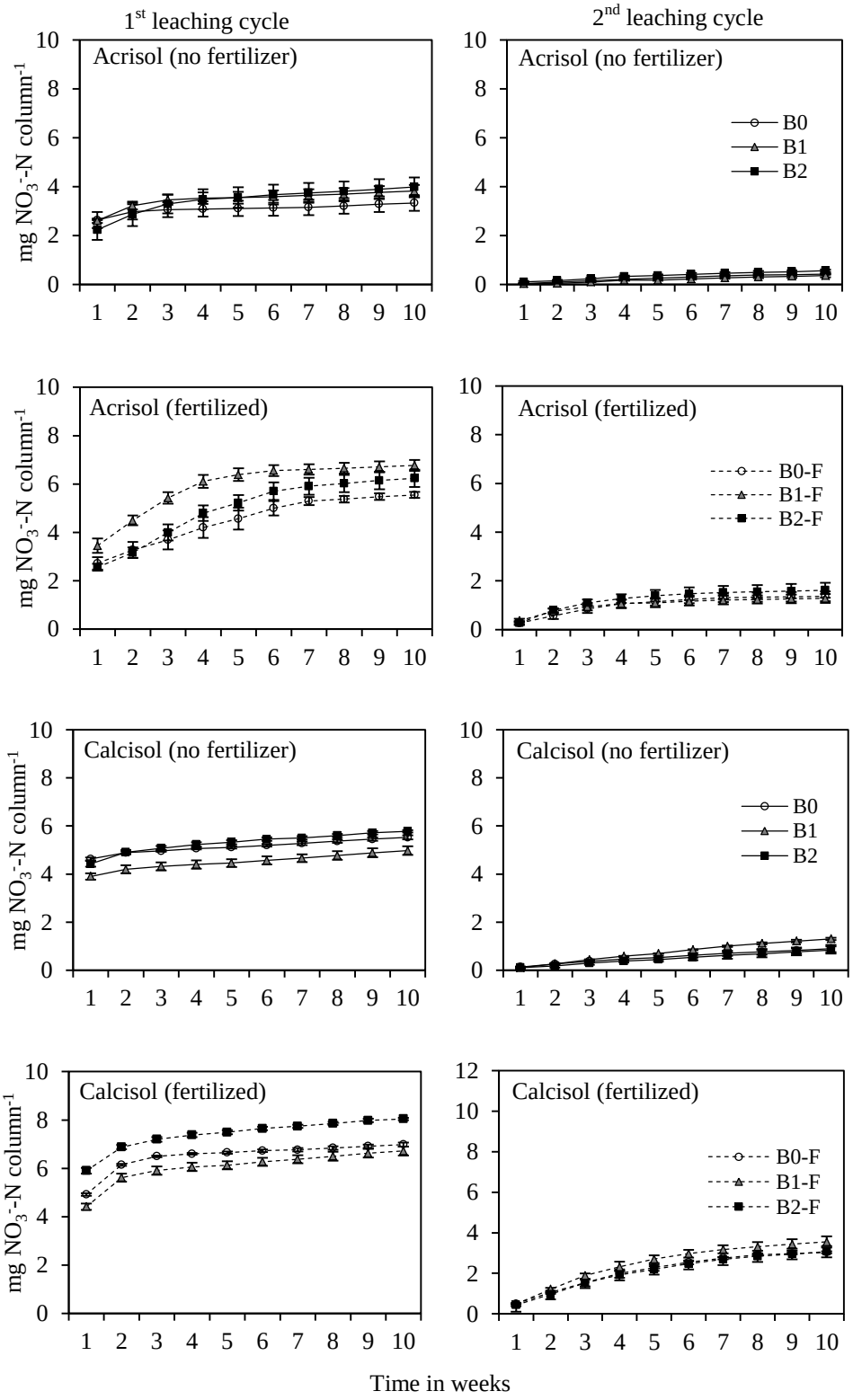

Fig. 7-3: Cumulative NO_3^--N losses leached during the first and the second leaching cycle from unfertilized and fertilized Acrisol and Calcisol. Means±SE (n=4). B0 and B0-F, 0 % biochar with and without fertilization, respectively; B1 and B1-F, 1 % biochar with and without fertilization, respectively; B2 and B2-F, 2 % biochar with and without fertilization, respectively.

Furthermore, the amount of TIN (NH_4^+-N + NO_3^--N) could be explained mainly by fertilization (η^2=0.477) and biochar application (η^2=0.259) in the first leaching cycle, but only by fertilization (η^2=0.703) in the second cycle (Table 7-2). After the first leaching cycle, the total amount of leached TIN was higher in Acrisol when compared to Calcisol and was significantly increased only by the higher biochar application rate (Fig. 7-4, Table 7-2). Nevertheless, the effect of fertilization was higher in Acrisol (6.66 mg TIN column^{-1} in non-fertilized treatments respect to 11.24 mg TIN column^{-1} leached from columns receiving fertilization) while in Calcisol the TIN leaching increased from 6.44 mg column^{-1} to 10.47 mg TIN column^{-1} (Table 7-4). The TIN leaching during the second leaching cycle was higher in Calcisol respect to Acrisol (Fig. 7-4, Table 7-2) and the highest losses were detected in B1 treatment. However, the effect size of biochar application in the first and the second cycle was low (η^2=0.259 and η^2=0.025, respectively).

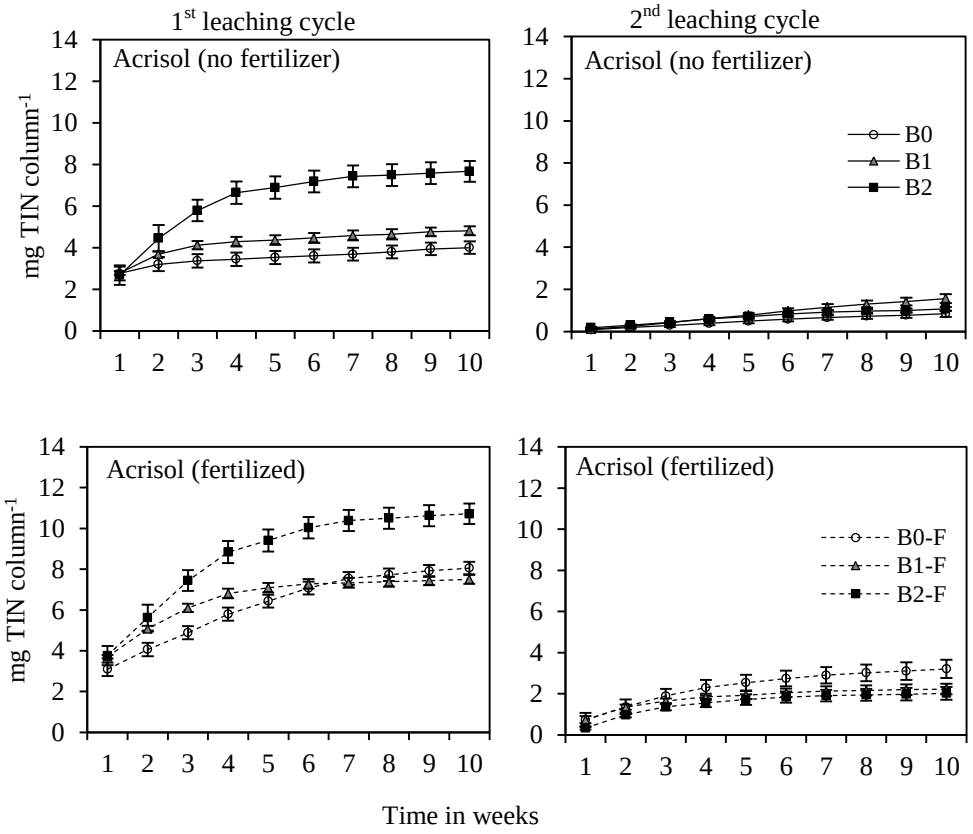

Fig. 7-4: Cumulative total inorganic nitrogen (TIN = NH_4-N+NO_3-N) leached during the first and the second leaching cycle from unfertilized and fertilized Acrisol and Calcisol. Means ± SE (n=4). B0 and B0-F, 0 % biochar with and without fertilization, respectively; B1 and B1-F, 1 % biochar with and without fertilization, respectively; B2 and B2-F, 2 % biochar with and without fertilization, respectively.

The combined cumulative leaching of TIN during both leaching cycles was strongly affected by soil type (η^2=0.738, p<0.001) and was higher in Acrisol when compared to Calcisol. The effect of biochar was significant (p<0.05) only in B2 (Table 7-3). In Acrisol, the difference between fertilized and non-fertilized treatments was decreasing with increasing biochar application rate (Table 7-3): while in B0 the fertilization increased TIN leaching by 6.42 mg column^{-1}, in B1 and B2 treatments the differences were only 3.35 and 4.00 mg column^{-1}, respectively. Furthermore, in the case of Acrisol, the relative amount of leached NO_3^--N respect to TIN decreased with biochar application rate but when fertilizer was applied, the highest ratio was found in B1 during the first leaching cycle and in B2 in the second leaching cycle (Table 7-3).

Table 7-3: The results of general linear model (GLM) for the effects of soil type (S), biochar application (B) and fertilization (F) on total leaching losses of ammonium (NH_4^+), nitrate (NO_3^-) and total inorganic nitrogen during both leaching cycle, and the relative amount of nitrate losses during both leaching cycles respect to total amount of leached inorganic nitrogen in Acrisol. No detectable amount of NH_4^+-N was found in the leachate of Calcisol

	Total leaching losses			Recovered N[1]	NO_3^--N / TIN	
	NH_4^+-N	NO_3^--N	TIN		1st cycle	2nd cycle
	(mg column^{-1})			(%)		
Acrisol						
B0	1.08	3.77	4.85	137.19	0.83	0.58
B1	2.19	4.19	6.38	137.17	0.79	0.25
B2	4.19	4.56	8.74	154.68	0.52	0.55
B0-F	4.35	6.92	11.3	76.93	0.69	0.45
B1-F	1.68	8.05	9.73	76.41	0.90	0.61
B2-F	4.90	7.85	12.7	87.36	0.58	0.80
Calcisol						
B0	0.00	6.43	6.43	33.64	-	-
B1	0.00	6.28	6.28	35.85	-	-
B2	0.00	6.62	6.62	49.11	-	-
B0-F	0.00	10.0	10.0	29.25	-	-
B1-F	0.00	10.3	10.3	46.16	-	-
B2-F	0.00	11.1	11.1	34.45	-	-
Effects (η2 and p value)						
S	-	0.294^{***}	0.010^{*}	0.643^{***}	-	-
B	0.081^{***}	0.017^{**}	0.097^{***}	0.034^{***}	0.775^{***}	0.196^{*}
F	0.023^{**}	0.619^{***}	0.738^{***}	0.155^{***}	$0.002^{n.s.}$	0.117^{*}
SxB	-	$0.004^{n.s.}$	0.038^{***}	0.005^{*}	-	-
SxF	-	$0.004^{n.s.}$	$0.003^{n.s.}$	0.121^{***}	-	-
BxF	0.042^{***}	$0.003^{n.s.}$	$0.012^{n.s.}$	0.010^{**}	0.141***	0.213^{*}
SxBxF	-	$0.002^{n.s.}$	$0.025^{n.s.}$	$0.003^{n.s.}$	-	-
Error	0.854	0.057	0.077	0.029	0.082	0.474

[1] The TIN recovered in leachate and final soil as a percentage of initial TIN content (in soil and applied fertilizer)

* indicates significant effect at p<0.05; ** p<0.01; *** p<0.001, n.s. not significant

The total amount of recovered N (in leachate and final soil) was higher in Acrisol than in Calcisol, increased by biochar dose (B0<B1<B2; p<0.05) and decreased with fertilization

(Table 7-3). In the case of Acrisol, the N recovered was 37% higher than the initial amount of mineral N contained in B0 and B1 and 54% higher in B2 treatment (Table 7-3). In fertilized soil, 76, 77 and 86% of initial inorganic N and applied N was recovered in B0-F, B1-F and B2-F, respectively.

7.3.3 Sorption

Prior to leaching experiment, the NH_4^+-N sorption capacity of Acrisol was enhanced by biochar application (Fig. 7-1) while the sorption of NO_3^--N was not affected (data not shown). Sorption properties of Calcisol were not affected by biochar amendment. Sorption capacity of all treatments (NH_4^+-N adsorption) decreased during the leaching experiment (Table 7-1).

7.4 Discussion

Several studies have reported a significant reduction of leachate volume when biochar was applied (Sika & Hardie 2014; Sorrenti & Toselli 2016; Xu et al. 2016) as a result of increased water retention, improved soil structure and soil aggregation (Yoo et al. 2014). Although Xu et al. (2016) observed differences in leached volume even at the lowest application rate (2% biochar), no differences were found in leachate volume even at the higher application rate (2% biochar) used in our study. Thus, in agreement with Sorrenti & Toselli (2016), the effect of biochar on the amount of leachate is rather inconsistent. Furthermore, the volume of water applied in our experiment was relatively high compared to watering applied by Xu et al. (2016). Thus, the changes in the amount of leached N forms could not be related to the reduced water movement.

7.4.1 The production and leaching of NH_4^+-N

The leaching of NH_4^+-N was strongly affected by soil type, with no detectable amount of NH_4^+-N leached from Calcisol which indicates that the inherent soil properties were the key factors controlling NH_4^+-N leaching from the soils during this experiment. Nevertheless, biochar had strong effect on NH_4^+-N leaching during the first leaching cycle from Acrisol as it explained 80% of data variability.

The leaching of NH_4^+-N is the function of sorption, NH_4^+-N production and NH_4^+-N consumption, and other potential losses. Over 92% of variability in NH_4^+-N sorption was attributed to soil type with biochar being of lower importance. Nevertheless, significant differences in NH_4^+-N sorption between biochar application rates were observed in Acrisol which may have impacted the NH_4^+ leaching. The pH of Calcisol rich in $CaCO_3$ was not affected by biochar application similarly to findings of previous studies (Lentz & Ippolito

2012; Kumari et al. 2014) probably due to a high buffering potential of $CaCO_3$. Calcisol showed higher adsorption of NH_4^+-N which was not affected by biochar application and no NH_4^+-N leaching occurred. No effect of biochar on CEC in calcareous soils has been observed by van Zwieten et al. (2010) who argued that CEC of the soil was similar to that of biochar resulting in no difference after biochar application. Furthermore, a higher content of clay materials could have led to a competition between clay and biochar surface for the adsorption and reduce the biochar effect, confirming that the sorption properties of biochar depends on the soil type (Gronwald et al. 2015).

In sandy soil, biochar application increased soil capacity to retain NH_4^+-N both in the beginning and at the end of the experiment, but this capacity decreased significantly in time. Gronwald et al. (2015) found out that after field application, biochar lost more than half of its adsorption capacity in only seven months. Acrisol soil pH after the application of both doses of biochar ranged from 6.5 to 7, which according to Kizito et al. (2015) is the optimum range for NH_4^+-N retention by biochar. Decreased NH_4^+-N removal from solution at low pH observed by Kizito et al. (2015) was suggested by the authors to be a result of high protonation of functional groups on the biochar surface and changes of the charge. Additionally, changes in N leaching may be partly attributed to ash content of biochar, which could have increased the hydraulic conductivity (Chang et al. 1997) or clog soil pores after swelling when in contact with water (Etiégni & Campbell 1991). This effect would likely be temporal and disappear when ash is leached from the soil. Nevertheless, the reduction of sorption capacity of Acrisol was detected also in B0 suggesting that at least a part of the reduced sorption could be due to changes in soil properties, such as decreased amount of SOM or leaching of other captions and consequent reduction of soil pH. Furthermore, using deionized water could have caused severe changes in soil properties including the pore water chemistry and could result in disruption of soil aggregates, which in turn could have impact on sorption properties. The disaggregation of soil particles could increase the mineralization of SOM by uncovering the organic matter protected within the stable aggregates, which may at least partly explain the relatively low difference between N leaching from fertilized and non-fertilized treatments. Ultimately, lower NH_4^+-N adsorption capacity of washed biochar when compared with non-washed biochar has been detected (Gai et al. 2014) probably due to the removal of ash and some of the functional groups on the biochar surface.

Biochar-induced increase in sorption capacity can decrease the vertical movement of NH_4^+-N (Ding et al. 2010) and thus lower the risk of NH_4^+-N losses via leaching in soils with

low CEC. Nevertheless, in the present study, we observed that biochar application to soil increased significantly the NH_4^+-N content in the leachate, which suggests that biochar-induced NH_4^+-N production exceeded the capacity of NH_4^+-N retention by soil-biochar mixture.

Besides its neutralizing effects and sorption in Acrisol, biochar application may induce changes in soil properties and thus indirectly affect the N transformations. As SOM decomposition is a complex of enzyme-mediated processes, it is reasonable to expect a higher microbial activity in biochar amended soil if soil physical and chemical properties were improved. The alleviation of soil acidity was observed to reduce the pH-related inhibition of extracellular enzymes involved in N cycle (León et al. 2017; Vazquez et al. 2017), with a potential impact on SOM decomposition and ammonification. Furthermore, lower biochar application rate could have weaker effect on SOM mineralization while slightly increasing the sorption capacity of the soil, resulting in decreased NH_4^+-N losses from B1. When larger amount of biochar was applied, the content of mineralized NH_4^+-N was probably too high to be retained by adsorption. Although considered relatively recalcitrant, biochar can contain labile C fractions which can have short-term effect on soil biota and affect nutrient dynamics. Such a stimulation of microbial activity by labile C input may lead to priming of SOM and result in N immobilization as observed by Zavalloni et al. (2011) or SOM mining for N (Whitman et al. 2015). Nevertheless, despite this short-term SOM stimulation, at the end of the incubation period all biochar-amended treatments displayed TOC contents much higher than control soil which demonstrates the high residence time of biochar in soil. The amount of PMN significantly increased after biochar application prior to leaching experiment and this increase was proportional to biochar application rate. Nevertheless, at the end of the second leaching cycle, the PMN was the highest in the B1 when compared to B0 or B2, which further confirms the enhanced mineralization of N during the incubation in B2 treatments and the relative exhaustion of easily mineralizable N pool.

The leaching of NH_4^+-N was strongly affected by the time and the highest losses were observed in the very beginning of the first leaching cycle (Fig. 2; Table S1), and after 4-5 weeks a clear reduction in NH_4^+-N losses (the curve shifts into horizontal trend, especially in case of B2 and B2-F) could indicate temporal priming of native SOM induced by biochar amendment. It is in agreement with Nguyen et al. (2017) who recommended biochar application at least one month prior to planting of the crops/trees to prevent possible adverse effect of mineral N reduction on plant growth. The PMN resulted to be a relatively reliable

predictor of potential NH_4^+-N losses from acid sandy Acrisol as it followed similar trend and was the highest in B2 in the beginning of the experiment but in B1 at the end of the second leaching cycle, which could indicate rapid SOM mineralization during the first leaching cycle in B2 and slower mineralization in B1. Rapid raise of soil pH and consequent reduction of soil acidity resulted in accelerated depletion of easily mineralization SOM (and PMN). In an incubation study using the same soil and biochar (Teutscherova et al. 2017a) the biochar application led to immediate stimulation of SOM mineralization and temporal accumulation of NH_4^+-N which supports the results of the present study. Nevertheless, this effect was rather short-term and disappeared before the end of the incubation study (70 days). Furthermore, in all unfertilized treatments of Acrisol, the amount of recovered N was higher than the initial amount of mineral N present in the soil, which clearly indicates the decomposition of SOM. Nevertheless, we only measured inorganic N forms and possible leaching of organic N forms was not addressed. It is possible that increased NH_4^+-N leaching from biochar-amended soil is related to retention of dissolved organic matter which could be degraded by soil microbes while releasing NH_4^+-N. The difference in total leached TIN between B0-F and B0 accounted for 46% of applied N, while in B1 and B2 treatments it was only 24 and 29%, respectively, suggesting that increased N mineralization could be at least partly caused by N contained in biochar which could have a priming effect on mineralization of SOM. Therefore, when fertilizer was applied, no significant effect of biochar was observed.

7.4.2 Nitrification and leaching of NO_3^--N

Fertilization and, to a lesser extent, soil type resulted to be the most important factors affecting the NO_3^--N leaching during both leaching cycles, explaining together 79.8% and 82.3% in the first and the second leaching cycle, respectively. The lack of the biochar effect in NH_4^+-N oxidation to NO_3^--N could be caused by the insufficient time of the experiment given the relatively slow growth of nitrifiers.

The increase of SOM ammonification may lead to enhanced nitrification in case there are no other factors limiting the substrate oxidation (Hanan et al. 2016). In the present study, we observed increased leaching of NH_4^+-N but no differences in NO_3^--N between biochar application rates, which suggests that there were other factors limiting the oxidation of NH_4^+ to NO_3^- in B2 treatment, such as insufficient activity or abundance of nitrifiers as previously described by Teutscherova et al. (2017). This is further confirmed by high unexplained variability for NNM and NN (75.2% and 80.8%, respectively) and low effect of biochar on NO_3^--N leaching (less than 5%). Thus, additional factors, such as the activity and diversity of

the community of soil nitrifiers should be included in the future studies in order to increase the accuracy of the prediction of biochar effect on N leaching. Furthermore, the alleviation of soil acidity in Acrisol by biochar and co-application of NH_4^+-N could lead to increased net nitrification at lower biochar application rate (B1), but at higher biochar dose (B2), nitrification could be affected by volatile compounds contained in biochar as pointed out also by other studies (Spoas et al. 2010; Wang et al. 2011). This is also supported by the relative amount of NO_3^--N released during the first leaching cycle, which was the highest in B1-F treatment where 90% of TIN was leached in the form of NO_3^--N. No difference between B2 and B2-F treatment could be explained by increased nitrification in the presence of mineral fertilizer, increased losses via volatilization as NH_3, microbial immobilization or slightly lower mineralization when compared to non-fertilized soil.

Although biochar did not affect the total amount of leached NO_3^--N in Calcisol, leaching was increased in the B2 during the first leaching cycle, while it was increased by B1 during the second cycle. As this soil type is relatively low in TOC, short-term immobilization after biochar application could occur (Bruun et al. 2012) and explain the reduction of the NO_3^--N in the B1 unfertilized treatment during the first leaching cycle and the subsequent higher leaching during the second cycle. Short-term immobilization could create a reserve pool of organic N temporarily stored in the microbial biomass, which could be prone to leaching after its re-mobilization (Zheng et al. 2013a). Additionally, higher N mineralization after biochar addition to Calcisol leading to higher N losses by lixiviation would probably be rather low because the initial PMN was not affected by biochar addition and at the end of the experiment was only slightly decreased in B2. The reason might be the low native SOM of the Calcisol and the initial alkaline pH of the Calcisol which eliminate the liming effect of biochar and thus the microbial biomass stimulation and consequent N mineralization (Ameloot et al. 2015).

7.5 Conclusions

This study reports that the effect of biochar on soil properties and nutrient leaching is highly dependent on soil properties. Furthermore, we demonstrated decreased sorption capacity of soil and soil-biochar mixture which dropped during the leaching experiment by more than 25%. This reduction of sorption behavior along with the pH decline could lower the potential benefits given by biochar application to acid soils. Moreover, based on our medium-term results, biochar application to Calcisol resulted to be an efficient mean of C sequestration without any detected negative impact on N cycle. Biochar application resulted

efficient to increase soil TOC and TN and to alleviate soil acidity of Acrisol but its effect on leaching of mineral N was rather inconsistent. In acid Acrisol with relatively high content of SOM, alkaline biochar application resulted in alleviation of soil acidity and enhancement of SOM mineralization followed by lixiviation of NH_4^+-N without significant effect on NO_3^--N leaching. Nevertheless, this microbial-stimulating effect decreased in time and when fertilizer was applied 15 weeks after biochar amendment, no effect of biochar was observed, possibly due to exhaustion of potentially mineralizable N pool. The limited effect of biochar suggests that biochar did not affect nitrification in this soil as it was probably limited by other factors besides soil pH and NH_4^+-N supply and thus, other parameters should be included in future studies, such as the activity and diversity of soil nitrifiers, to better understand the biochar-induced changes and their impact on mineral N leaching. This temporal decoupling of ammonification and nitrification deserves more attention in future investigation due to its possible implications in N cycling in agricultural soils. On the other hand, in Calcisol, pH was not limiting factor and biochar application did not affect net N transformations or N leaching. As pH and NH_4^+-N supply are considered the main factors driving nitrification activity in soil, the better understanding of both direct and indirect effects of biochar in soil is essential in order to lower environmental drawbacks of agronomic practices while increasing economic feasibility.

7.6 References

Ameloot N, Sleutel S, Das KC, Kanagaratnam J, de Neve S. 2015. Biochar amendment to soils with contrasting organic matter level: Effects on N mineralization and biological soil properties. GCB Bioenergy **7**:135–144.

Berglund LM, DeLuca TH, Zackrisson O. 2004. Activated carbon amendments to soil alters nitrification rates in Scots pine forests. Soil Biology and Biochemistry **36**:2067–2073.

Bower CE, Holm-Hansen T. 1980. A Salicylate–Hypochlorite Method for Determining Ammonia in Seawater. Canadian Journal of Fisheries and Aquatic Sciences **37**:794–798.

Bruun EW, Ambus P, Egsgaard H, Hauggaard-Nielsen H. 2012. Effects of slow and fast pyrolysis biochar on soil C and N turnover dynamics. Soil Biology and Biochemistry **46**:73–79.

Case SDC, McNamara NP, Reay DS, Stott AW, Grant HK, Whitaker J. 2015. Biochar suppresses N2O emissions while maintaining N availability in a sandy loam soil. Soil Biology and Biochemistry **81**:178–185.

Castaldi S, Riondino M, Baronti S, Esposito FR, Marzaioli R, Rutigliano FA, Vaccari FP, Miglietta F. 2011. Impact of biochar application to a Mediterranean wheat crop on soil microbial activity and greenhouse gas fluxes. Chemosphere **85**:1464–1471.

Cayuela ML, Sanchez-Monedero MA, Roig A, Hanley K, Enders A, Lehmann J. 2013. Biochar and denitrification in soils: when, how much and why does biochar reduce N_2O emissions? Scientific reports **3**:1732.

Clough T, Condron L, Kammann C, Müller C. 2013. A Review of Biochar and Soil Nitrogen Dynamics. Agronomy **3**:275–293.

DeLuca TH, MacKenzie MD, Gundale MJ, Holben WE. 2006. Wildfire-Produced Charcoal Directly Influences Nitrogen Cycling in Ponderosa Pine Forests. Soil Science Society of America Journal **70**:448.

Dempster DN, Gleeson DB, Solaiman ZM, Jones DL, Murphy D V. 2011. Decreased soil microbial biomass and nitrogen mineralisation with Eucalyptus biochar addition to a coarse textured soil. Plant and Soil **354**:311–324.

Ding Y, Liu Y-X, Wu W-X, Shi D-Z, Yang M, Zhong Z-K. 2010. Evaluation of Biochar Effects on Nitrogen Retention and Leaching in Multi-Layered Soil Columns. Water, Air, & Soil Pollution **213**:47–55.

Dirksen C. 1999. Soil physics measurement. Catena Verlag.

Espejo R. 1987. The soils and ages of the "raña" surfaces related to the Villuercas and Altamira mountain ranges (Western Spain). CATENA **14**:399–418. 2016.

Etiégni L, Campbell AG. 1991. Physical and chemical characteristics of wood ash. Bioresource Technology **37**:173–178.

Forster JC. 1995. Soil Nitrogen. Pages 79–87 in N. P. Alef K, editor.Methods in Applied Soil Microbiology and Biochemistry.

Gai X, Wang H, Liu J, Zhai L, Liu S, Ren T, Liu H. 2014. Effects of feedstock and pyrolysis temperature on biochar adsorption of ammonium and nitrate. PLoS ONE **9**:1–19.

Gao F, Xue Y, Deng P, Cheng X, Yang K. 2015. Removal of aqueous ammonium by biochars derived from agricultural residuals at different pyrolysis temperatures. Chemical Speciation & Bioavailability **27**:92–97.

Gaskin JW, Steiner C, Harris K, Das KC, Bibens B. 2008. Effect of Low-Temperature Pyrolysis Conditions on Biochar for Agricultural Use. Transactions of the ASABE **51**:2061–2069.

Gronwald M, Don A, Tiemeyer B, Helfrich M. 2015. Effects of fresh and aged chars from pyrolysis and hydrothermal carbonization on nutrient sorption in agricultural soils **1**:475–489.

Hale S, Hanley K, Lehmann J, Zimmerman A, Cornelissen G. 2011. Effects of Chemical, Biological, and Physical Aging As Well As Soil Addition on the Sorption of Pyrene to Activated Carbon and Biochar. Environmental Science & Technology **45**:10445–10453.

Hanan EJ, Schimel JP, Dowdy K, D'Antonio CM. 2016. Effects of substrate supply, pH, and char on net nitrogen mineralization and nitrification along a wildfire-structured age gradient in chaparral. Soil Biology and Biochemistry **95**:87–99.

Hart SC, Stark JM, Davidson EA, Firestone MK. 1994. Nitrogen mineralisation, immobilisation and nitrification. Pages 985–1013MEthods of Soil Analysis: Microbiological and Biochemical Properties, Part 2, 3rd edition. ASA and SSSA, Madison.

Herath HMSK, Camps-Arbestain M, Hedley M. 2013. Effect of biochar on soil physical properties in two contrasting soils: An Alfisol and an Andisol. Geoderma **209**:188–197.

Chang AC, Lund LJ, Page AL, Warneke JE. 1997. Physical Properties of Fly Ash-Amended Soils. Journal of Environmental Quality **6**:267–270.

Jeffery S, Verheijen FGA, van der Velde M, Bastos AC. 2011. A quantitative review of the effects of biochar application to soils on crop productivity using meta-analysis. Agriculture, Ecosystems & Environment**144**: 175-187.

Kanthle AK, Lenka NK, Lenka S, Tedia K. 2016. Biochar impact on nitrate leaching as influenced by native soil organic carbon in an Inceptisol of central India. Soil and Tillage Research **157**:65–72.

Kizito S, Wu S, Kirui WK, Lei M, Lu Q, Bah H, Dong R. 2015. Evaluation of slow pyrolyzed wood and rice husks biochar for adsorption of ammonium nitrogen from piggery manure anaerobic digestate slurry. Science of the Total Environment **505**: 102-112.

Kumari KGID, Moldrup P, Paradelo M, Elsgaard L, Hauggaard-Nielsen H, de Jonge LW. 2014. Effects of Biochar on Air and Water Permeability and Colloid and Phosphorus Leaching in Soils from a Natural Calcium Carbonate Gradient. Journal of Environment Quality **43**:647.

Laird D, Fleming P, Wang B, Horton R, Karlen D. 2010. Biochar impact on nutrient leaching from a Midwestern agricultural soil. Geoderma **158**:436–442.

Lehmann J, Gaunt J, Rondon M. 2006. Bio-char sequestration in terrestrial ecosystems - A review. Mitigation and adaptation strategies for global change **11**: 403-427.

Lentz RD, Ippolito JA. 2012. Biochar and Manure Affect Calcareous Soil and Corn Silage Nutrient Concentrations and Uptake. Journal of Environment Quality **41**:1033.

León P, Espejo R, Gómez-Paccard C, Hontoria C, Mariscal I, Renella G, Benito M. 2017. No tillage and sugar beet foam amendment enhanced microbial activity of degraded acidic soils in South West Spain. Applied Soil Ecology **109**:69–74.

Liang B et al. 2006. Black Carbon Increases Cation Exchange Capacity in Soils. Soil Science Society of America Journal **70**:1719.

Liu Z, Demisie W, Zhang M. 2013. Simulated degradation of biochar and its potential environmental implications. Environmental Pollution **179**:146–152.

Mandal S, Thangarajan R, Bolan NS, Sarkar B, Khan N, Ok YS, Naidu R. 2016. Biochar-induced concomitant decrease in ammonia volatilization and increase in nitrogen use efficiency by wheat. Chemosphere **142**:120–127.

Mia S, van Groenigen JW, van de Voorde TFJ, Oram NJ, Bezemer TM, Mommer L, Jeffery S. 2014. Biochar application rate affects biological nitrogen fixation in red clover

conditional on potassium availability. Agriculture, Ecosystems and Environment **191**:83–91.

Miranda KM, Espey MG, Wink DA. 2001. A Rapid, Simple Spectrophotometric Method for Simultaneous Detection of Nitrate and Nitrite. Nitric Oxide **5**:62–71.

Mukherjee A, Lal R, Zimmerman AR. 2014. Effects of biochar and other amendments on the physical properties and greenhouse gas emissions of an artificially degraded soil. Science of the Total Environment **487**:26–36.

Nguyen TTN, Xu CY, Tahmasbian I, Che R, Xu Z, Zhou X, Wallace HM, Bai SH. 2017. Effects of biochar on soil available inorganic nitrogen: A review and meta-analysis.

Robarge WP, Edwards A, Johnson B. 2008. Water and waste water analysis for nitrate via nitration of salicylic acid 1. Communications in Soil Science and Plant Analysis **14**:1207–1215.

Sika MP, Hardie AG. 2014. Effect of pine wood biochar on ammonium nitrate leaching and availability in a South African sandy soil. European Journal of Soil Science **65**:113–119.

Sorrenti G, Toselli M. 2016. Soil leaching as affected by the amendment with biochar and compost. Agriculture, Ecosystems and Environment **226:**56–64.

Spokas KA, Baker JM, Reicosky DC. 2010. Ethylene: potential key for biochar amendment impacts. Plant and Soil **333**:443–452.

Streubel JD, Collins HP, Garcia-Perez M, Tarara J, Granatstein D, Kruger CE. 2011. Influence of Contrasting Biochar Types on Five Soils at Increasing Rates of Application. Soil Science Society of America Journal **75**:1402.

Teutscherova N, Vazquez E, Masaguer A, Navas M, Scow KM, Schmidt R, Benito M. 2017. Comparison of lime- and biochar-mediated pH changes in nitrification and ammonia oxidizers in degraded acid soil. Biology and Fertility of Soils **53**:811–821.

Thies JE, Rillig MC, Graber ER. 2015. Biochar effects on the abundance, activity and diversity of the soil biota. Pages 327–389 in J. Lehmann and S. Joseph, editors.Biochar for environmental management: science, technology and implementation 2nd edition.

Ulyett J, Sakrabani R, Kibblewhite M, Hann M. 2014. Impact of biochar addition on water retention, nitrification and carbon dioxide evolution from two sandy loam soils. European Journal of Soil Science **65**:96–104.

van Zwieten L, Kimber S, Morris S, Chan KY, Downie A, Rust J, Joseph S, Cowie A. 2010. Effects of biochar from slow pyrolysis of papermill waste on agronomic performance and soil fertility. Plant and Soil **327**:235–246.

Vazquez E, Teutscherova N, Almorox J, Navas M, Espejo R, Benito M. 2017. Seasonal variation of microbial activity as affected by tillage practice and sugar beet foam amendment under Mediterranean climate. Applied Soil Ecology **117**–118:70–80.

Walkley A, Black IA. 1934. An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. Soil Science **37**:29–38.

Wang J, Pan X, Liu Y, Zhang X, Xiong Z. 2012. Effects of biochar amendment in two soils on greenhouse gas emissions and crop production. Plant and Soil **360**:287–298.

Wang J, Xiong Z, Kuzyakov Y. 2015. Biochar stability in soil: meta-analysis of decomposition and priming effects. GCB Bioenergy **8**: 512-523.

Wang J, Zhang M, Xiong Z, Liu P, Pan G. 2011. Effects of biochar addition on N2O and CO2 emissions from two paddy soils. Biology and Fertility of Soils **47**:887–896.

Waring SA, Bremner JM. 1964. Ammonium Production in Soil under Waterlogged Conditions as an Index of Nitrogen Availability. Nature **201**:951–952.

Whitman T, Singh BP, Zimmerman AR. 2015. Priming effects in biochar-amended soils: Implications of biochar-soil organic matter interactions for carbon storage. Pages 455–488in J. Lehmann and S. Joseph, editors.Biochar for Environmental Management: Science, Technology and Implementation, 2nd edition. Routledge.

Xu N, Tan G, Wang H, Gai X. 2016. Effect of biochar additions to soil on nitrogen leaching, microbial biomass and bacterial community structure. European Journal of Soil Biology **74**:1–8.

Yao Y, Gao B, Zhang M, Inyang M, Zimmerman AR. 2012. Effect of biochar amendment on sorption and leaching of nitrate, ammonium, and phosphate in a sandy soil. Chemosphere **89**:1467–1471.

Yoo G, Kim H, Chen J, Kim Y. 2014. Effects of Biochar Addition on Nitrogen Leaching and Soil Structure following Fertilizer Application to Rice Paddy Soil. Soil Science Society of America Journal **78**:852.

Zavalloni C, Alberti G, Biasiol S, Vedove GD, Fornasier F, Liu J, Peressotti A. 2011. Microbial mineralization of biochar and wheat straw mixture in soil: A short-term study. Applied Soil Ecology **50**:45-51.

Zheng H, Wang Z, Deng X, Herbert S, Xing B. 2013. Impacts of adding biochar on nitrogen retention and bioavailability in agricultural soil. Geoderma **206**:32–39.

Zheng J, Stewart CE, Cotrufo MF. 2012. Biochar and nitrogen fertilizer alters soil nitrogen dynamics and greenhouse gas fluxes from two temperate soils. J Environ Qual **41**:1361–1370.

8 BASE CATIONS LEACHING AND SOIL STRUCTURE

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Nikola Teutscherova contributed by establishing the experimental design, analytical and statistical analysis and manuscript preparation

ABSTRACT

The use of biochar for the improvement of cation exchange capacity (CEC) and consequent prevention of nutrient losses is now widely accepted. Furthermore, biochar can contain large amount of ash and cations on its surface which can improve the nutritional status of the soil. The effect of biochar application on the base cation leaching and the stability of macroaggregates were evaluated in two contrasting soils: Acrisol and Calcisol. The pH and EC of leachate was increased by biochar application in both soil types, indicating that biochar aported more nutrients than the amount that could be retaind by soil-biochar mixtures. All studied base cations (Ca, Mg, K, and Na) were also more abundant in the leachate of biochar-amended soils when compared to control. Nevertheless, at the end of the leaching experiment (15 weeks), the content of all nutrients remained higher in Acrisol with biochar respect to unmended Acrisol as well as the CEC. On the other hand, in alkaline Calcisol, biochar had no effect on the CEC or Ca content, slightly reduced the content of Mg and increased the content of K in the exchange complex. In both soils, the stability of soil aggregates was reduced by biochar application, which could be attributed to (i) enhanced mineralization of soil organic matter, (ii) the changes in base cations composition towards higher K/CEC ratio which caused aggregated disruption; or to (iii) higher sensitivity of biochar-amended soils to aggregates disruption under intermittent flooding caused by frequent and intensive leaching events. These results highlight the gaps in our understanding of biochar impact on soil structure which can have crucial implications for soil erodibility or restoration of degraded lands.

Key words: Acrisol; aggregates; base cations; Calcisol; leaching

8.1 Introduction

The positive impact of biochar on soil physical properties has been widely accepted, with reduced bulk density and increased porosity being among the most commonly observed effects. Many authors have observed enhanced formation of water-stable aggregates (WSA) after biochar application, which is often linked to increased microbial activity (Demisie et al. 2014; Teutscherova et al. 2018b) and aggregate stabilization by polysaccharides from microbial metabolism and to adsorption of organic matter onto biochar surface (Glaser et al. 2002). Similarly, liming of acid soils (Blomquist et al. 2018) or application of organic matter (Sun et al. 2017) usually reflect in enhanced stabilization of soil aggregates in soils where high acidity or low substrate availability limit the microbial activity, respectively. Thus, owing to its alkalinity and high carbon (C) content, biochar application to soil is not only a powerful mean to sequester organic C in soil (Amonette et al. 2007), but could be also a promising strategy in soil structure improvement and related environmental functions, such as erosion control or nutrient losses.

According to biochar production temperature and feedstock material, biochar may differ considerably in the content of ashes and available nutrients (Rehrah et al. 2014), especially potassium (K), which could be very high in fresh biochars. Furthermore, the ash contained in biochar is readily soluble (Etiégni & Campbell 1991) and can be lost from the soil, taken up by plants or microbes or clog pores and thus impact soil physical properties. High application of monobasic ions can lead to displacement of cations increasing the saturation of exchange complex by monobasic ions and reducing amount of calcium (Ca) and magnesium (Mg) on the exchange sites. Both losses of Ca and Mg and application of large amount of K have been observed to be detrimental for soil aggregation (Heil & Sposito 1993; Auerswald et al. 1996). Although the effect of biochar-contained ash and cations on soil properties is likely to be temporal and disappear once ash is leached out of the soil, the potential impact on soil aggregates disruption have not been addressed.

The enhanced microbial activity in biochar-amended soil may have crucial impact on microbial metabolism in recently flooded or intermittently flooded soils (Fig. 8-5). Shortly after flooding soil microbes use up oxygen from the soil to maintain aerobic respiration. Therefore, the increased microbial activity in biochar-amended soil may directly influence the speed of oxygen utilization and the timing of the switch to anaerobic metabolism. On the other hand, great porosity of biochar may increase soil aeration and thus reduce the anaerobic conditions (Liu et al. 2017). Furthermore, the direct effect of biochar on soil pH, CEC and the

concentration of cations may play a key role in soil aggregation or the disruption of soil aggregates. In addition, the soil aggregation response to liming depends on the period of time since the amendment (Haynes and Naidu 1998): in the short-term, the raise of soil pH can promote the dispersion of clay coloids, while the increase of calcium (Ca) and hydroxy-Al polymers formers by the precipitation of exchangeable Al can enhance the particle aggregation acting as a cement agent. Finally, in the long- term, the expected enhance in the crop productivity can increase the content of soil organic matter and therefore, the soil aggregation.

The majority of the studies focusing on biochar impact on soil structure and WSA aggregates formation have been done under controlled laboratory conditions under adequate moisture content for microbial growth and activity (Jien & Wang 2013; Lu et al. 2014; Soinne et al. 2014; Teutscherova et al. 2017), which, however, does not necessarily need to reflect the situation in the field, where soil moisture fluctuates. Furthermore, ponding conditions or intermittent flooding have been demonstrated to have a crucial impact on soil aggregate formation or disruption (De-Campos et al. 2009) due to changes in chemical conditions, such as redox state of the soil (Fig. 8-5). Reducing conditions can affect key soil properties, such as the increased cation exchange capacity (CEC) and the availability of ions in the soil and soil solution (Ponnamperuma 1972; De-Campost et al. 2009) or the increase of soil pH, which in turn both influence soil structure. Favre et al. (2004, 2002) and Kirk et al. (2003) observed increased instability of soil aggregates resulting from increased CEC while Suarez et al. (1984) detected increased clay dispersion and reduction of soil aggregation with increasing soil pH. Clearly, many factors determine the stabilization/disruption of soil aggregates, especially in the intermittently flooded soils.

Despite the rapidly increasing number of studies demonstrating the possible benefits of biochar to improve soil structure and soil aggregation, our understanding remains limited, probably due to high number of biochar-induced changes in soil which all potentially influence biological activity and soil structure, being challenging to disentangle one from the other. The aim of the present study is to evaluate how biochar application to two contrasting soils affects the content of WSA in a column leaching experiment published by Teutscherova et al. (2018a), where high watering rates likely ensured intermittently reducing conditions which could have caused an increase of soil aggregates instability. We hypothesize that aggregate disruption will be reduced in acid Acrisol due to increased soil pH, CEC and

potassium content after biochar application, and will be unaffected in Calcisol which has both pH and CEC comparable to the pH and CEC of the used holm oak biochar.

8.2 Materials and methods

8.2.1 Soil and biochar characterization

Both Acrisol and Calcisol were used for the present experient. The properties of both soil types and biochar can be found in Table 4-1 (Materials and methods chapter).

8.2.2 Experimental design and column preparation

Columns were prepared as described in chapter 4 (Materials and methods) and in chapter 7 (Biochar and mineral nitrogen leaching). Same columns were used as in chapter 7, but only unfertilized treatments were included.

8.2.3 Leaching experiment

Leaching of the columns is described in Chapter 7.

8.2.4 Analytical methods

At the end of the experiment, soil pH and electric conductivity were determined in soil:water suspension (1:2.5). The content of exchangeable base cations (Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}) was quantified by atomic absorption spectroscopy (AAnalyst 400, PerkinElmer, Wellesley, MA) in Mehlich III extraction solution (Mehlich 1984). Soil available P was extracted by Bray 1 method in Acrisol and by Olsen method in Calcisol and analyzed colorimetrically (Murphy & Riley 1962). Water-stable aggregates (WSA) were determined by wet-sieving of air-dried 1–2 mm aggregates through a 250 mm sieve (Kemper & Rosenau 1986) and calculated as described in Chapter 5. The aggregates were expressed as percentage of WAS as well as in grams of WSA per kg of soil. Soil acidity and Al content (Acrisol) were determined using the method of Yuan (1959). The effective cation exchange capacity (ECEC) of the soil at the end of the experiment was calculated as the sum of exchangeable H and Al and exchangeable base cations (Pansu and Gautheyrou, 2006). The amount of leached cations was measured with atomic absorption spectroscopy.

8.2.5 Statistical analysis

The effect of soil type and biochar application in leaching losses were analyzed by a repeated measures two-way ANOVA separately for the first and the second leaching cycle,

considering the ten leaching events of each cycle as a within subject factor. The used model was full factorial design for the between-subjects factors and a post-hoc LSD test was used to evaluate the differences between the three doses of biochar ($p<0.05$). In addition, the cumulative leaching losses at the end of both cycles and the final soil properties were analyzed using a full factorial General Linear Model (GLM) with soil type and biochar doses as fixed factors and a post-hoc LSD to test the differences between the three biochar application rates ($p<0.05$). In all the analyses where the Al, H and acidity were tested, only the Acrisol results were analyzed because these parameters were not detected in Calcisol. These variables were analyzed only considering biochar doses as fixed factor in their respective models. In addition, multiple stepwise regressions were used to analyze the main drivers of soil aggregation at each soil. The stepping criteria employed for the entry and removal were based on the significance level of the F-value and were set at 0.05. All the analyses were performed using the SPSS 22 (IBM SPSS, Inc., Chicago, USA) software.

8.3 Results

8.3.1 Leachate and final soil pH and EC

Both pH and EC of leachate were higher in Calcisol than in Acrisol during both leaching cycles (Fig.8-1, Table 8-1, Table 8-2). In addition, pH and EC was increased by the application of both biochar application rates (Fig.8-1, Table 8-1, Table 8-2). Nevertheless, the effect of biochar was stronger in Acrisol when compared to Calcisol.

At the end of the leaching experiment, soil pH was significantly higher in Calcisol. The pH of Acrisol without biochar amendment dropped by almost 1.8 units during both leaching cycles while the pH of Calcisol increased by 0.1 pH unit (Table 8-3). The pH of biochar-amended Acrisol was significantly higher than control soil at the end of the experiment. Nevertheless, the pH of Calcisol was comparable between biochar treatments and EC was only slightly higher in B2 when compared to B0 ($p<0.05$) (Table 8-3).

Table 8-1: The results of repeated measures ANOVA for the between-subject effects of soil type (S), biochar application (B) and interactive effects on leachate pH and EC and leaching of calcium (Ca), magnesium (Mg), potassium (K) and sodium (Na) from Acrisol and Calcisol.

	pH		EC		Ca		Mg		K		Na
	1st cycle	2nd cycle	1st cycle	2nd cycle	1st cycle	2nd cycle	1st cycle	2nd cycle	1st cycle	2nd cycle	1st cycle
Effects $\eta^{2\ p=value}$											
S	32.90***	47.68***	94.68***	60.01***	96.94***	66.06***	91.93***	52.92***	37.22***	44.48***	66.63***
B	36.52***	28.18***	5.05***	25.66***	2.17***	25.40***	6.78***	26.11***	58.33***	49.64***	14.63***
S x B	28.80***	11.85**	0.06n.s.	1.14n.s.	0.10n.s.	0.96n.s.	0.77***	1.75n.s.	1.60*	0.17n.s.	2.43n.s.
Error	1.78	12.29	0.22	13.19	0.79	7.58	0.52	19.22	2.85	5.72	16.31

* indicates significant effect at p<0.05; ** p<0.01; *** p<0.001; n.s. not significant
η^2 eta squared

Table 8-2: The results of repeated measures ANOVA for the between-subject effects of soil type (S), biochar application (B) and interactive effects on leachate pH and EC and leaching of calcium (Ca), magnesium (Mg) and potassium (K) from Acrisol and Calcisol.

	Total Ca	Total Mg	Total K
Effects $\eta^{2\ p=value}$			
soil	94.80***	89.43***	40.77***
Biochar	4.15***	8.94***	55.75***
SxB	0.06n.s.	0.38n.s.	0.87n.s.
Error	0.99	1.24	2.60

* indicates significant effect at p<0.05; ** p<0.01; *** p<0.001; n.s. not significant
η^2 eta squared

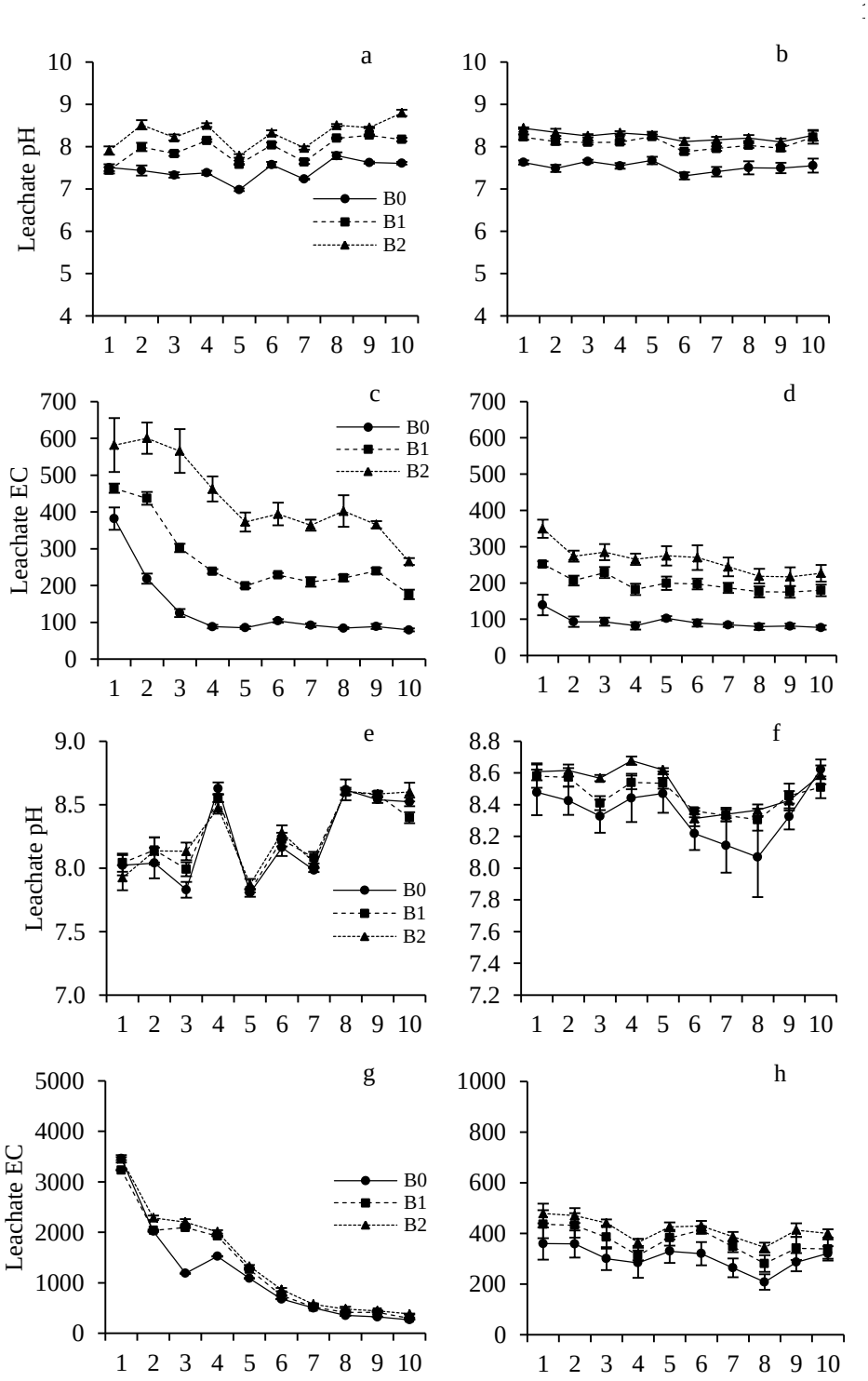

Fig. 8-1: The pH (a, b, e, f) and EC (c, d, g, h) of leachate of Acrisol (a, b, c, d) and Calcisol (e, f, g, h) during the first (a, c, e, g) and the second (b, d, f, h) leaching cycle.

8.3.2 Cumulative leaching losses

The leaching of base cations (Ca, Mg, K) was higher in Calcisol during both leaching cycles (Fig. 8-2, Fig. 8-3, Table 8-1) and was increased by both biochar application rates in

both soils. Similarly, the Na in the leachate was significantly higher in Calcisol than in Acrisol and significantly higher in B2 respect to BO during the first leaching cycle, while in the second cycle, the Na losses were below the detection limit in both soils (Fig. 8-2, Fig. 8-3, Table 8-1). With exception of Mg and K leachated during the first leaching cycle, the interaction of soil type and biochar was not statistically significant neither in the the second leaching cycle, nor in the total cation leaching after both cycles (Fig. 8-2, Fig. 8-3, Table 8-1)

During the first leaching cycle, the leaching of Ca, Mg and Na leaching was more affected by soil type (η^2 94.94, 91.93 and 66.63 %, respectively) when compared to the effect of biochar (η^2 2.17, 6.78 and 14.63%, respectively). Nevertheless, leaching of K was influenced mainly by biochar application rate, which explained 59.33 % of variability when compared to soil type explaining 37.22%. During the second leaching cycle, the differences between the η^2 of soil type and biochar were comparable for Ca, Mg and K leaching although the effect of soil type on Ca and Mg remained higher than the effect of biochar.

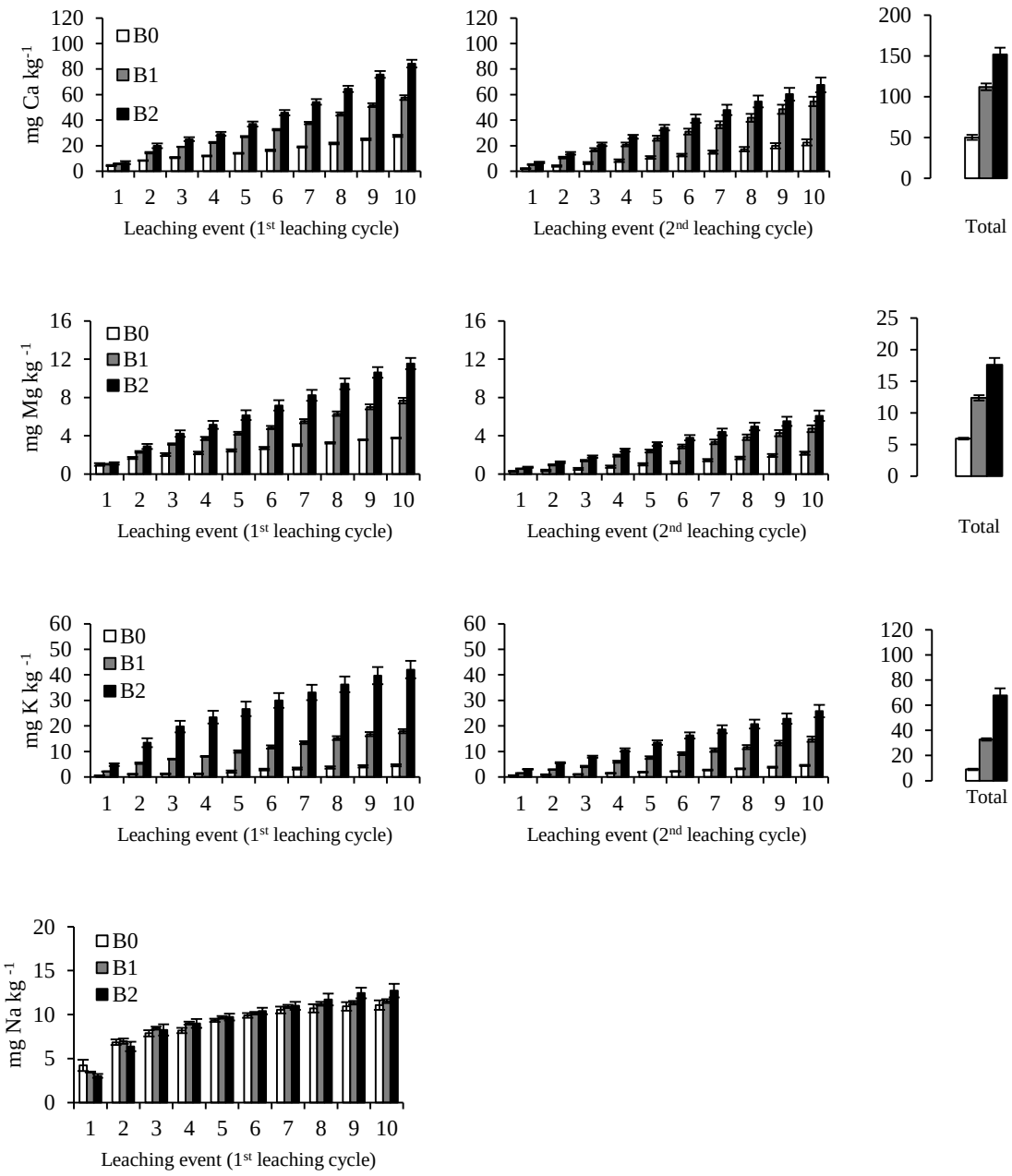

Fig.8-2: Cumulative leaching losses of Ca, Mg, K and Na from Acrisol

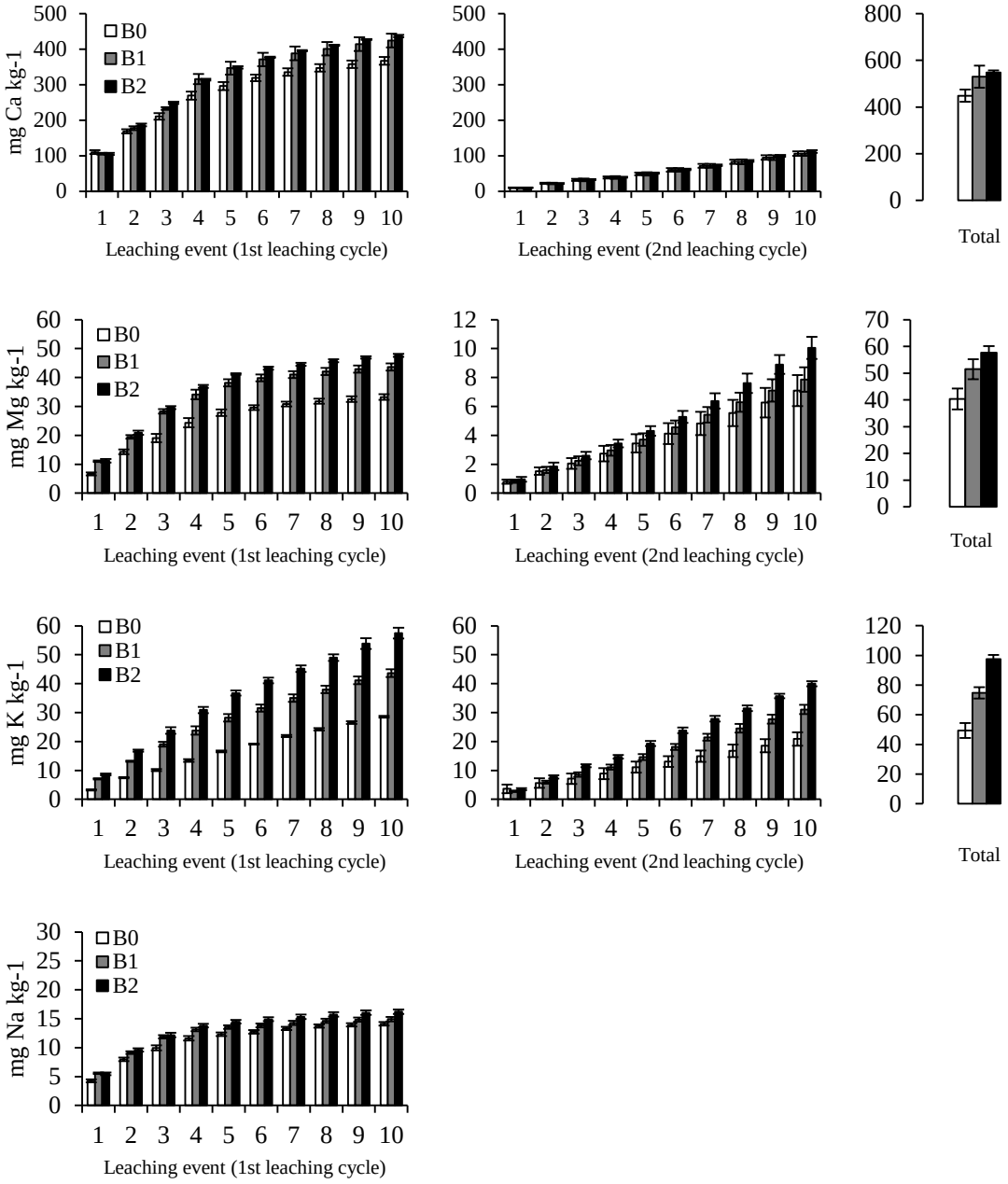

Fig. 8-3: Leaching of Ca, Mg, K and Na during two leaching cycles from acid Calcisol

8.3.3 Final soil properties

The content of Ca, Mg and K was higher in Calcisol when compared to Acrisol with soil type explaining 89.8%, 96.6 and 88.8% of data variability, respectively (Table 8-3). The application of both doses of biochar increased significantly the final soil content of Ca and K, while Mg was significantly higher only in B2 when compared to B0 (Table 8-3). The content of Mg was increased by biochar in Acrisol but decreased in Calcisol (Table 8-3). The Na content in the soil at the end of the leaching experiment was below the detection limit.

Biochar reduced the Al content in Acrisol but had no impact on soil acidity (Table 8-3). The ECEC was significantly higher in Calcisol than in Acrisol, and was increased by biochar application in Acrisol. The relation between K and ECEC (K/ECEC) was significantly higher in Acrisol than in Calcisol and was increased by both biochar application rates (Table 8-3).

The available P content at the end of the leaching experiment was significantly higher in Calcisol than in Acrisol (Table 8-3) and the effect of biochar application varied between soil types: in Acrisol, P content was increased by both biochar applications, while in Calcisol, P content was lowered by B1 and unaffected by B2, when compared to B0.

Table 8-3: The soil properties at the end of the leaching experiment and the effects of soil type (S), biochar application rate (B) and their interactions obtained by GLM

	pH	P	Ca	Mg	K	Al	H	ECEC	K/(Ca+Mg)	K/ECEC
		mg kg^{-1}	$cmol_c$ kg^{-1}						%	
Acrisol										
B0	3.88	29.32	0.96	0.11	0.05	1.11	0.47	2.71	0.050	1.98
B1	4.51	38.47	2.56	0.18	0.11	0.01	0.56	3.42	0.041	3.34
B2	5.06	50.58	3.82	0.22	0.14	0.08	0.20	4.45	0.035	3.20
Calcisol										
B0	8.17	80.86	24.44	1.34	0.49	-	-	26.27	0.019	1.86
B1	8.19	63.45	24.55	1.27	0.57	-	-	26.39	0.022	2.16
B2	8.14	77.81	24.12	1.29	0.62	-	-	26.03	0.024	2.38
Effects										
S	93.9 ***	75.6 ***	89.8 ***	96.6 ***	88.8 ***	-	-	97.9 ***	84.5 ***	30.2 ***
B	2.94 ***	9.71 ***	4.98 ***	1.41 ***	7.66 ***	98.9 ***	43.2 n.s.	0.95 ***	0.35 n.s.	49.2 ***
SxB	3.17 ***	12.3 ***	5.13 ***	1.89 ***	3.00 ***	-	-	1.03 ***	11.8 ***	10.7 **
Error	0.00	2.39	0.05	0.10	0.53	1.13	56.8	0.17	3.34	9.92

Table 8-4: The factors explaining the stability of soil aggreagates in both soil types detected in stepwise regression analysis

Acrisol	3.032	-0.744 K/ECEC	-0.333 SIR		R^2=.670	p<0.01
Calcisol	5.757	-0.457 SOM	-2.54 P	+7.107 Mg	R^2=0.834	p<0.01

K/ECEC, the ration of potassion and cation exchange capacity; SIR, substrate-induced respiration; SOM, soil organic matter; P, available phosphorus; Mg, magnesium
Exluded variables were Ca, K, K/(Ca+Mg), pH, BR, TC, NNM, NN, ECEC
SOM values taken from Teutscherova et al. (2018a)

8.3.4 Water-stable aggregates

The stability of soil aggregates was affected by soil type with higher amount of WSA found in Acrisol when compared to Calcisol. In both soil types, the content of WSA was reduced by biochar application (Fig. 8-3, Table 8-5) and the stability was related to K/CEC and SIR (data not shown here) in Acrisol and to SOM, P and Mg in Calcisol according to the stepwise regression analysis (Table 8-4).

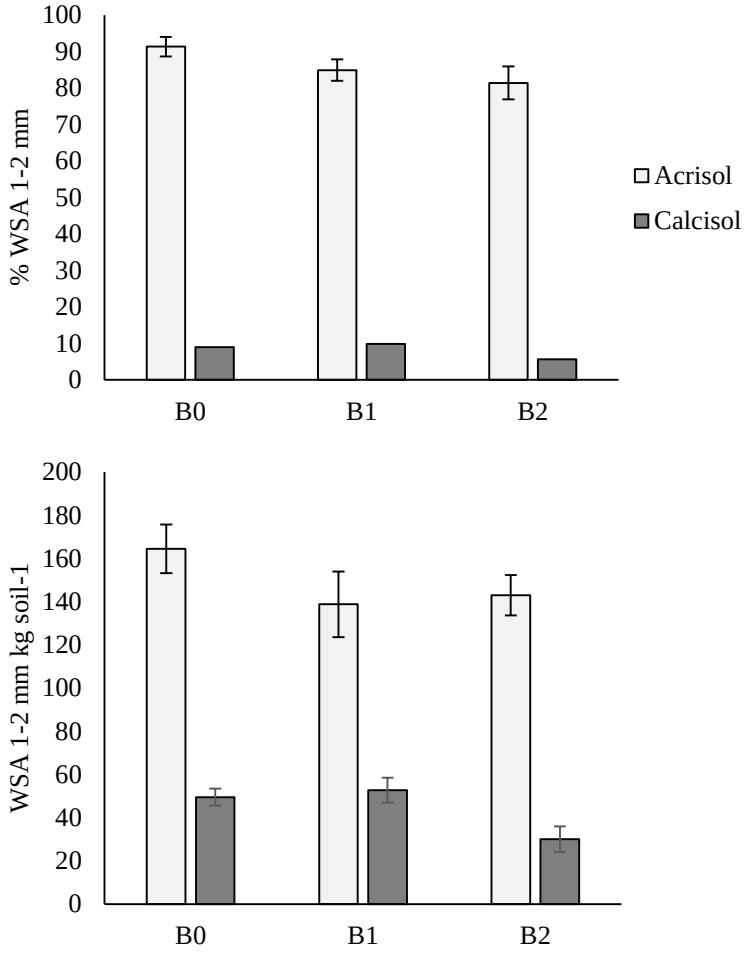

Fig. 8-3: The content of water-stable aggregates (a) and the stability of soil aggregates (b).

Table 8-5: The effect of soil type (S) and biochar application rate (B) on the percentage of water stable aggregates (WSA)

Effects $\eta 2$ p-value	$WSA_{1\text{-}2mm}$ %	$WSA_{1\text{-}2mm}$ g kg^{-1} soil
S	95.23 ***	84.66 ***
B	1.54 **	4.67 **
SxB	0.97 *	3.68 *
Error	2.26	6.99

8.4 Discussion

The incorporation of biochar into the soil induces wide range of changes in soil physical and chemical properties, increases SOM content and fertilizer-use efficiency while often improving plant growth (Chan et al. 2007; Deenik et al. 2010; van Zwieten e al. 2010) and enhancing soil microbial activity (Castaldi et al. 2011; Lehmann et al. 2011; Alburquerque et al. 2013; Khadem & Raiesi 2017). The application of the same biochar to the same soil types resulted in enhanced stability of soil macroaggregates (1-2 mm fraction) in the study of Teutscherova et al. (2018b) in a greenhouse experiment under controlled moisture content maintained at 60% of soil WHC, suggesting that biochar enhanced microbial activity of the soils which contributed to the stabilization of aggregates. Despite the same soil types, biochar and biochar application rate, the stability of soil aggregates was reduced by biochar application in the present study possibly due to (i) addition and leaching of base cations which could result in changes in the concentration of monobasic and dibasic ions, (ii) changes in moisture content of the soil resulting from frequent flooding of soil and leaching, or (iii) changes in SOM status after biochar application.

8.4.1 Base cations dynamics

Although biochar has been suggested by many authors to act as a sponge to adsorb nutrients from the soil solution and thus prevent their losses by leaching, according to the feedstock material and production temperature, biochar can contain variable quantity of cations in the ash fractions, which are readily soluble (Etiégni & Campbell 1991). Such an addition of ash material to soil can be observed as increase of EC after biochar application to soil. The EC of the leachate from both soils was increased after biochar application, especially during the first leaching cycle, but maintained higher in biochar-amended columns until the end of the experiment. The application of the cations of biochar to soil caused the higher leaching of Ca, Mg, K and Na in the biochar-amended treatments of both soils, similarly to the results obtained by Laird et al (2010b) and Cheng et al (2017). The absence of significant effect of the interaction between soil type and biochar in the total cumulative leaching after both cycles supports the hypothesis that excess of cations leachated from the biochar-treatments respect to the control was caused by the incorporation of mobile cations with biochar addition.

At the end of the leaching experiment, the pH of Acrisol was strongly lowered in relation with the initial soil while in Calcisol it remained comparable to the initial value. However, despite of the higher leaching of cations from the biochar-amended treatments in

Acrisol, the soil pH was significantly higher in both doses of biochar treatments. This result, in accordance with the data presented in Laird et al., (2010a) and Laird et al., (2010b), support previous studies where the capacity of holm oak biochar as liming agent of acid soils was tested (Teutscherova et al. 2017) and highlight its durability over time even under strong leaching conditions. In addition, the presence of exchangeable Al was reduced by both biochar doses despite of the acidic pH of B1 and B2. For example, in a field experiment performed in a plot adjacent to the soil collection site of the present study (Gomez-Paccard et al. 2013), the exchangeable Al of the soil was much higher with higher soil pH levels than the measured in B1 and B2. Despite of the different methods used to quantify the exchangeable Al used in both studies, the alleviation of Al toxicity observed in our biochar treatments could be related to the demonstrated ability of biochar to adsorpt Al by mean of surface complexation (Quian et al. 2013). Such impact of biochar on soil pH neutralilzation and Al toxicity allevation are of high importance as the excessive use of N fertilizers linked with the base cation extraction by crops have promoted the soil acidification and the increase of Al phytotoxicity of extensive agricultural regions (Guo et al. 2010). Biochar have been proposed as an alternative to traditional liming materials due to the lack or the high costs in some regions of lime or dolomite (Yuan and Xu 2011). The present study supports the liming capacity of biochar even under a strong leaching regime.

Similarly, despite of the higher Ca, Mg and K leaching in the biochar treatments respect to control, higher contents of available Ca, Mg and K were found in biochar-amended Acrisol when compared to control. The increase of cation availability with increasing biochar application rate can support the hypothesis that the higher cation content in the biochar treatments after leaching was caused by the application of these nutrients with biochar. However, the effect of biochar application on final cation content in Calcisol was more erratic, with absence of effect in Ca, a trend of lower Mg content with biochar application and a trend of higher availability of K. These differences between both soils in the effect of biochar on the final cation content was linked to a contrasting effect of biochar on ECEC: while in Acrisol the lower initial ECEC was increased by increasing biochar application rate, in Calcisol, whose ECEC was several times higher than in Acrisol, biochar application did not changed the ECEC. Therefore, we observed that the capacity of biochar to increase the soil nutrient availability and ECEC depended on the soil type. This finding could be contradictory with the most of the published studies evaluating the effect of biochar in ECEC and base cation content, because the effect of biochar is often tested in incubation or greenhouse

experiments with acid soils where there are no leaching losses of nutrients (El-Naggar et al. 2015; Inal et al. 2015; Yuan and Xu 2011; Yue et al. 2017). However, biochar application did not affect the amount of available base cations or ECEC in alkaline soil under field experiment where nutrient leaching likely occurred (Lentz and Ippolito 2012). The amount of base cations applied by biochar is relatively lower (to the cations contained in the soil) in the alkaline soils respect to the acid soil which dilutes the effect of cation supply by biochar. In addition, the absence of significant changes in the soil pH in alkaline soils does not change the nutrients availability. Finally, we highlight the increase in the K/ECEC caused by biochar appliaciton in both soils. These changes in the relation between monovalent and bivalent base cantions in soil could affect the aggregate stability and soil structure as is discussed in the section 8.4.2

The biochar application increased the P availability in Acrisol while Calcisol was unaffected. Despite of the amount of P released from biochar, the effect of biochar application in soil P availability depends on the changes in the sorption and desorption of P which are related to soil acidity (Xu et al., 2014). The raise of soil pH caused by biochar application to Acrisol could be related to an increase of the amount of desorbed P (Xu et al. 2014) or an increase in the organic P mineralization (DeLuca et al. 2009). The absence of biochar effect on P availability in Calcisol could be caused by the high amount of bivalent cations provided by biochar which could increase the P sorttion and precipitation in form of Ca and Mg phosphates (Chintala et al., 2014; Xu et al., 2014)

8.4.2 Changes in soil aggregation under intensive leaching conditions

The formation of soil aggregates is a result of flocculation and cementation processes. While flocculation is affected mainly by pH, EC and the Na content, the cementation depends on the amount and quality of binding agents, such as Ca carbonate, Mg carbonate, gypsum, sesquioxides, clay particles and SOM (Tisdall & Oades 1982). The bridging effect of Ca^{2+} between clay and SOM is of particularly high importance and plays a key role in soil aggregate formation. Therefore, biochar containing high amounts of Ca^{2+} may be more efficient in soil structure re-building, especially when compared to biochars with higher quantities of monobasic (K^+, Na^+ ions). Nevertheless, biochar often contains large amount of K^+, which has been known to cause aggregate disruption (Auerswald et al. 1996), due to low charge-to-size ion ratio. In addition, biochar application have been found to increase the soil aggregation by mean of an stimulation of soil microbial activity and SOM mineralization

which increases the synthesis of polysaccharides, a bonding agent responsible of soil aggregation (Demisie et al., 2014)

Althought an increase in WSA was expected according to literature (Demisie et al., 2014; Obia et al., 2016; Ouyang et al., 2013), our results showed that biochar application decreased the WSA in both soils. In addition, this result is the opposite that the obtained in a previuos study where the same biochar-soil mixtures were tested in a greenhouse pot experiment (Teutscherova et al., 2018b). This contradictory result could be caused by the different experimental setup, as in the present study, the soil-biochar mixture had a strong leaching regime which may affect the relation between bivalent and monovalent cations. Indeed, the regression analysis revealed that the saturation of exchange complex with K played significant role soil stability as increased ratio of K/ECEC was responsible for reduced flocculation in Acrisol. In the case of Calcisol, the Mg content in final soil was identified as a driver of WSA. The increase of Mg leaching in Calcisol promoted by biochar could have caused the decrease of WSA as Mg is a bivalent cation which acts as a bonding agent.

In addition, the longer term of the present study and the absence of organic matter inputs could lead to a depletion of the SOM pool in the biochar amended treatments which may decrease the soil aggregation stability. This could be supported by the stepwise regression in Calcisol where the WSA was negatively related with SOM and in Acrisol where WSA was negatively affected by SIR.

Despite the serious lack of knowledge of biochar-soil interactions under flooded or intermittently flooded conditions, it could be speculated that biochar-amended soil will act distinctly when comparison to control soil under anaerobic conditions. Immediately after the watering of soil columns (100ml of water per 500g of soil weekly), soil likely became saturated with water at least for a short period of time, when soil microorganisms depended on the O_2 stock. Clearly, soils with higher microbial activity may use up O_2 faster when compared with low-activity soils, which accelerate the transition to anaerobic conditions in the soil. Consequently, under such conditions, biochar-induced changes including pH raise, increase of CEC or changes in base saturation, could be of high importance in flocculation/disruption of soil particles (De-Campos et al. 2009). On the other hand, biochar-amended soils usually display higher porosity and improved aeration, which may delay reducing conditions. In such case, the formation of soil aggregates could be increased by biochar application due to large quantities of aggregate binding agents which are of microbial

origin. Short-term changes in microbial activity during the initial stages (Teutscherova et al. 2018b) could determine the biochar impact on soil aggregates.

In the case of CEC, fresh biochar generally present lower CEC than aged biochar because the biochar surface is oxidized under environmental conditions which increases the CEC of biochar (Cheng et al., 2008). Feng et al (2018) demonstrated that under anaerobic conditions, the CEC capacity of biochar was lower in comparison with the same biochar under aerobic conditions and related this phenomenon with the biochar surface oxidation under aerobic conditions. At this point, the periodical anaerobic conditions of soil columns could have reduced the biochar surface oxidation in comparison with biochar under aerobic conditions and reduce the CEC of soil biochar mixture proposed by Laird et al. (2010a) to explain the reduction of the effective CEC of soil-biochar mixture of the bottom part of their soil columns. This reduction of the soil biochar mixture CEC in relation with soil under aerobic conditions could have promoted the leaching of cations which finally caused the unexpected decrease in soil aggregation. This hypothesis cannot be determined with the available experimental setup and dataset, but highlight the need of an evaluation of the suitability of the use of soil columns to evaluate the feasibility of biochar to reduce the cation leaching. In addition, the biochar capacity to improve soil aggregation must be evaluated for these soils which are affected by periodic flooding conditions.

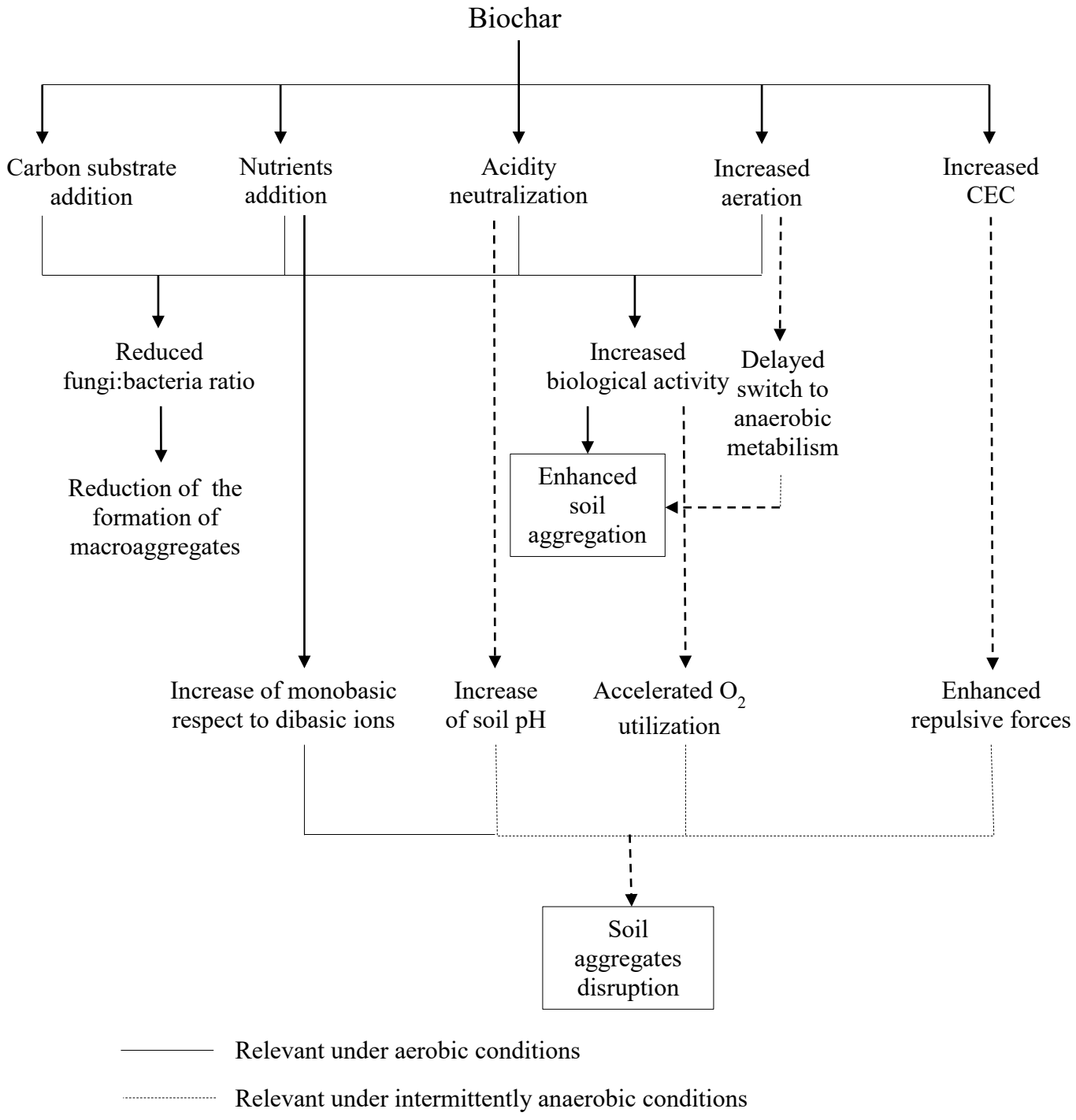

Fig. 8-5: Possible mechanisms explaining reduction of WSA content in soil

8.4.3 Changes in SOM status

While the majority of the studies, including the study of Teutscherova et al. (2018b) focus on biochar effect on soil microbial activity and soil aggregation under controlled conditions, much less attention is being paid to the impact of biochar on soil aggregation under other, sub-optimal, conditions. The biochar-induced changes of soil properties generally result in enhanced microbial activity which can influence the decomposition of native soil organic matter (SOM), a phenomenon termed priming effect. Positive priming effect, increased mineralization of SOM, could be caused directly by the input of labile organic carbon (C) contained in biochar (Wang et al. 2016) or indirectly by removing obstacles limiting microbial growth (Whitman et al. 2015), which could be reduction of soil acidity or addition of soil nutrients necessary for microbial metabolism. On the other hand, biochar can reduce SOM mineralization (Dempster et al. 2011), a negative priming effect, and the interactive effect of SOM-biochar depends on biochar feedstock material, production temperature as well as soil properties (Zimmerman et al. 2011). Clearly, any change in soil SOM status can have crucial implications in other soil properties, including soil structure and the formation of WSA. Given that the stabilization of soil WSA is a function of clay content and the quantity and quality of SOM, increased (or decreased) mineralization of SOM may impact soil structure. Soil macroaggregates (1-2 mm) are considered to be most responsive aggregate fraction to changes in land-use, and could be therefore used as indicators of soil changes induced by the alteration of soil management. If biochar application to soil enhances biological activity, the increased SOM mineralization could be reflected in reduction of soil macroaggregates in soil. On the other hand, the co-application of biochar with labile organic matter can increase the biochar-contained C (Hamer et al. 2004), which may reduce the C sequestration potential. Similarly, if biochar application to soil removes obstacles of microbial activity (e.g. soil pH modification, nutrient addition), SOM accumulated in soil may be mineralized rapidly which can enhance biochar mineralization and hinder biochar's positive effect on C storage and soil aggregation. The impact of biochar application on N mineralization and leaching under this experimental set-up has been published previously (Teutscherova et al. 2018a) and increased N losses from Acrisol were observed which the authors attributed to enhanced mineralization of N in Acrisol resulting from the amelioration of soil chemical properties (acidity and Al toxicity), which could hinder the positive effect of biochar on C sequestration potential in soils.

8.5 Conclusions

The impact of biochar application to two contrasting soils subjected to frequent and intensive leaching was evaluated in a column leaching study for 15 weeks. Biochar increased the pH, EC and base cations contents in the leachate, which indicates that biochar-contained cations exceeded the sorptive capacity of biochar linked with the negative functional groups on biochar surface. Despite the positive effect of the same biochar on has been observed by Teutscherova et al. (2018b) in a greenhouse experiment with constant moisture content (60% WHC), in this study we found less WSA aggregates in biochar-amended treatments in both soil types. In acid degraded Acrisol, this reduction of WSA content seemed to be attributed to the increase of K in the exchange complex and by to microbial biomass as K/CEC ratio and SIR together explained 67% of WSA data variability. In alkaline Calcisol, the reduction of WSA was related negatively to SOM and P content and positively to Mg concentration. Although biochar is a promising mean to sequester large quantities of organic C in soil and thus help to mitigate climate change, its impact on soil properties remains still poorly understood. As climate change is predicted to bring unregularly distributed precipitations, the biochar-soil interactions need to be evaluated under wide range of environmental conditions, including intermittent flooding and drying, in order to predict biochar impact on soil erodibility, nutrient cycling and, ultimately, plant growth.

8.6 References

Alburquerque JA, Salazar P, Barr??n V, Torrent J, Del Campillo MDC, Gallardo A, Villar R. 2013. Enhanced wheat yield by biochar addition under different mineral fertilization levels. Agronomy for Sustainable Development **33**:475–484.

Amonette JE, Lehmann J, Joseph S. 2007. Terrestrial Carbon Sequestration with Biochar: A Preliminary Assessment of its Global Potential. Proceedings of the American Geophysical Union, Fall Meeting.

Auerswald K, Kainz M, Angermüller S, Steindl H. 1996. Influence of exchangeable potassium on soil erodibility. Soil Use and Management **12**:117–121.

Blomquist J, Simonsson M, Etana A, Berglund K. 2018. Structure liming enhances aggregate stability and gives varying crop responses on clayey soils. Acta Agriculturae Scandinavica, Section B — Soil & Plant Science **68**:311–322.

Castaldi S, Riondino M, Baronti S, Esposito FR, Marzaioli R, Rutigliano FA, Vaccari FP, Miglietta F. 2011. Impact of biochar application to a Mediterranean wheat crop on soil microbial activity and greenhouse gas fluxes. Chemosphere **85**:1464–1471.

Chan KY, Van Zwieten L, Meszaros I, Downie A, Joseph S. 2007. Agronomic values of greenwaste biochar as a soil amendment. Australian Journal of Soil Research **45**:629–634.

Cheng CH, Lehmann J, Engelhard MH. 2008. Natural oxidation of black carbon in soils: changes in molecular form and surface charge along a climosequence. Geochimica et Cosmochimica Acta 72: 1598-1610.

Chintala R, Schumacher TE, Mcdonald LM, Clay DE, Malo DD, Papiernik SK, Clay SA, Julson JL. 2014. Phosphorus sorption and availability from biochars and soil/biochar mixtures. Clean Soil Air Water **42**: 626e634.

De-Campos AB, Mamedov AI, Huang C. 2009. Short-Term Reducing Conditions Decrease Soil Aggregation. Soil Science Society of America Journal **73**:550.

Deenik JL, McClellan T, Uehara G, Antal MJ, Campbell S. 2010. Charcoal Volatile Matter Content Influences Plant Growth and Soil Nitrogen Transformations. Soil Science Society of America Journal **74**:1259.

Demisie W, Liu Z, Zhang M. 2014. Effect of biochar on carbon fractions and enzyme activity of red soil. Catena **121**:214–221.

Dempster DN, Gleeson DB, Solaiman ZM, Jones DL, Murphy D V. 2011. Decreased soil microbial biomass and nitrogen mineralisation with Eucalyptus biochar addition to a coarse textured soil. Plant and Soil **354**:311–324.

El-Naggar AH, Usman ARA, Al-Omran A, Ok YS, Ahmad M, Al-Wabel MI. 2015. Carbon mineralization and nutrient availability in calcareous sandy soils amended with woody waste biochar. Chemosphere **138**: 67–73.

Etiégni L, Campbell AG. 1991. Physical and chemical characteristics of wood ash. Bioresource Technology **37**:173–178.

Favre F, Jaunet AM, Pernes M, Badraoui M, Boivin P, Tessier D. 2004. Changes in clay organization due to structural iron reduction in a flooded vertisol. Clay Minerals **39**:123–134.

Favre F, Tessier D, Abdelmoula M, Génin JM, Gates WP, Boivin P. 2002. Iron reduction and changes in cation exchange capacity in intermittently waterlogged soil. European Journal of Soil Science **53**:175–183.

Feng M, Zhang W, Wu X, Jia Y, Jiang C, Wei H, Qiu R, Tsang DC. 2018. Continuous leaching modifies the surface properties and metal (loid) sorption of sludge-derived biochar. Science of The Total Environment **625**: 731-737.

Gaskin JW, Steiner C, Harris K, Das KC, Bibens B. 2008. Effect of Low-Temperature Pyrolysis Conditions on Biochar for Agricultural Use. Transactions of the ASABE **51**:2061–2069.

Glaser B, Lehmann J, Zech W. 2002. Ameliorating physical and chemical properties of highly weathered soils in the tropics with charcoal - A review. Biology and Fertility of Soils **35:**219–230.

Gómez-Paccard C, Mariscal-Sancho I, León P, Benito M, González P, Ordóñez R, Espejo R, Hontoria C. 2013. Ca-amendment and tillage: Medium term synergies for improving key soil properties of acid soils. Soil and Tillage Research **134**: 195-206.

Guo JH, Liu XJ, Zhang Y, Shen JL, Han WX, Zhang WF, Christie P, Goulding KW, Vitousek PM, Zhang FS. 2010. Significant acidification in major Chinese croplands. Science, 1182570.

Hamer U, Marschner B, Brodowski S, Amelung W. 2004. Interactive priming of black carbon and glucose mineralisation. Organic Geochemistry **35**:823–830.

Haynes RJ, Naidu R. 1998. Influence of lime, fertilizer and manure applications on soil organic matter content and soil physical conditions: a review. Nutrient cycling in agroecosystems **51**: 123-137.

Heil D, Sposito G. 1993. Organic Matter Role in Illitic Soil Colloids Flocculation: I. Counter Ions and pH. Soil Science Society of America Journal **57**:1241.

Inal, A., Gunes, A., Sahin, O. Z. G. E., Taskin, M. B., & Kaya, E. C. (2015). Impacts of biochar and processed poultry manure, applied to a calcareous soil, on the growth of bean and maize. *Soil Use and Management*, *31*(1), 106-113.

Jien SH, Wang CS. 2013. Effects of biochar on soil properties and erosion potential in a highly weathered soil. Catena **110**:225–233.

Kemper WD, Rosenau RC. 1986. Aggregate Stability and Size Distribution. Methods of soil analysis. Part 1. Physical and mineralogical methods **9**:425–442.

Khadem A, Raiesi F. 2017. Influence of biochar on potential enzyme activities in two calcareous soils of contrasting texture. Geoderma **308**:149–158.

Kirk GJD, Solivas JL, Alberto MC. 2003. Effects of flooding and redox conditions on solute diffusion in soil. European Journal of Soil Science **54**:617–624.

Laird D, Fleming P, Davis DD, Horton, R, Wang B, Karlen DL. 2010a. Impact of biochar amendments on the quality of a typical Midwestern agricultural soil. Geoderma **158**: 443-449.

Laird D, Fleming P, Wang B, Horton R, Karlen D. 2010b. Biochar impact on nutrient leaching from a Midwestern agricultural soil. Geoderma **158**: 436-442.

Lehmann J, Rillig MC, Thies J, Masiello CA, Hockaday WC, Crowley D. 2011. Biochar effects on soil biota - A review. Soil Biology and Biochemistry **43**:1812–1836.

Lentz RD, Ippolito JA. 2012. Biochar and manure affect calcareous soil and corn silage nutrient concentrations and uptake. Journal of Environmental Quality **41**:1033–1043.

Liu Z, Dugan B, Masiello CA, Gonnermann HM. 2017. Biochar particle size, shape, and porosity act together to influence soil water properties. PLoS ONE **12**.

Lu SG, Sun FF, Zong YT. 2014. Effect of rice husk biochar and coal fly ash on some physical properties of expansive clayey soil (Vertisol). Catena **114**:37–44.

Mehlich A. 1984. Mehlich 3 soil test extractant: A modification of Mehlich 2 extractant. Communications in Soil Science and Plant Analysis **15**:1409–1416.

Murphy J, Riley J. 1962. A modified single solution method for the determinatio of phosphate in natural waters. Analytical Chemistry ACTA **27**:31 – 36.

Obia A, Mulder J, Martinsen V, Cornelissen G, Børresen T. 2016. In situ effects of biochar on aggregation, water retention and porosity in light-textured tropical soils.

Soil and Tillage Research **155**: 35–44.

Ouyang L, Wang F, Tang J, Yu L, Zhang R. 2013. Effects of biochar amendment on soil aggregates and hydraulic properties. Journal of Soil Science and Plant Nutrition **13**: 991–1002.

Pansu M, Gautheyrou J. 2006. Handbook of soil analysismineralogical, organic and inorganic methods. Springer-Verlag, Heidelberg

Ponnamperuma FN. 1972. The Chemistry of Submerged Soils. Advances in Agronomy **24**:29–96.

Qian L, Chen B, Hu D. 2013. Effective alleviation of aluminum phytotoxicity by manure-derived biochar. Environmental science & technology **47**: 2737-2745.

Rehrah D, Reddy MR, Novak JM, Bansode RR, Schimmel KA, Yu J, Watts DW, Ahmedna M. 2014. Production and characterization of biochars from agricultural by-products for use in soil

Soinne H, Hovi J, Tammeorg P, Turtola E. 2014. Effect of biochar on phosphorus sorption and clay soil aggregate stability. Geoderma **219**-220:162–167.

Suarez DL, Rhoades JD, Lavado R, Grieve CM. 1984. Effect of pH on saturated hydraulic conductivity and soil dispersion. Soil Sci. Soc. Am. J. **48**:50–55.

Sun D, Li K, Bi Q, Zhu J, Zhang Q, Jin C, Lu L, Lin X. 2017. Effects of organic amendment on soil aggregation and microbial community composition during drying-rewetting alternation. Science of The Total Environment **574:**735–743.

Teutscherova N, Houška J, Navas M, Masaguer A, Benito M, Vazquez E. 2018a. Leaching of ammonium and nitrate from Acrisol and Calcisol amended with holm oak biochar: A column study. Geoderma **323**:136–145.

Teutscherova N, Lojka B, Houška J, Masaguer A, Benito M, Vazquez E. 2018b. Application of holm oak biochar alters dynamics of enzymatic and microbial activity in two contrasting Mediterranean soils. European Journal of Soil Biology **88**:15–26.

Teutscherova N, Vazquez E, Santana D, Navas M, Masaguer A, Benito M. 2017. Influence of pruning waste compost maturity and biochar on carbon dynamics in acid soil: Incubation study. European Journal of Soil Biology **78**:66–74.

Tisdall JM, Oades JM. 1982. Organic matter and water-stable aggregates in soils. Journal of Soil Science **33**:141–163.

van Zwieten L, Kimber S, Morris S, Chan KY, Downie A, Rust J, Joseph S, Cowie A. 2010. Effects of biochar from slow pyrolysis of papermill waste on agronomic performance and soil fertility. Plant and Soil **327**:235–246.

Walkley A, Black IA. 1934. An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. Soil Science **37**:29–38.

Wang J, Xiong Z, Kuzyakov Y. 2016. Biochar stability in soil: Meta-analysis of decomposition and priming effects. GCB Bioenergy **8**:512–523.

Whitman T, Singh BP, Zimmerman A, Lehmann J, Joseph S. 2015. Priming effects in biochar-amended soils: implications of biochar-soil organic matter interactions for carbon storage. Pages 455–488 Biochar for Environmental Management - Science, Technology and Implementation, 2nd edition.

Xu G, Sun J, Shao H, Chang SX. 2014. Biochar had effects on phosphorus sorption and desorption in three soils with differing acidity. Ecological Engineering **62**:54–60.

Yuan TL. 1959. Determination of exchangeable hydrogen in soils by titration method. Soil Science **88**:164-167.

Yue Y, Cui L, Lin Q, Li G, Zhao X. 2017. Efficiency of sewage sludge biochar in improving urban soil properties and promoting grass growth. Chemosphere **173**:551–556.

Yuan JH, Xu RK. 2011. The amelioration effects of low temperature biochar generated from nine crop residues on an acidic Ultisol. Soil Use and Management **27**:110-115.

Zimmerman AR, Gao B, Ahn M-Y. 2011. Positive and negative carbon mineralization priming effects among a variety of biochar-amended soils. Soil Biology and Biochemistry **43**:1169–1179.

9 DYNAMICS OF MICROBIAL AND ENZYMATIC ACTIVITY

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ABSTRACT

The application of biochar in Mediterranean region has been considered a promising mean to enhance the soil quality and health. However, the results of previous studies are inconsistent and the effects of biochar on biological activity seem to depend on the initial soil properties. To elucidate this relation, the evolution of microbial and enzymatic activity in two contrasting soils was evaluated in the course of 12 weeks of greenhouse incubation after biochar application and fertilization with nitrogen, phosphorus and potassium. Biochar enhanced the activity of soil biota in an acid Acrisol as a result of soil pH neutralization which was observed as an increase of microbial biomass carbon (MBC) and soil basal respiration (SBR) during the initial six weeks of the incubation. On the other hand, in an alkaline Calcisol, the effect of biochar application on SBR and MBC was short-term (three weeks) and seemed to be related to the nitrogen availability. Dehydrogenase and urease activity in Acrisol were enhanced by biochar application while the activity of all the other enzymes decreased or remained unaffected by biochar application. Biochar application enhanced the aggregates stability in both soils. In summary, general decrease of the enzymatic activities and the inconsistency of the soil biological properties, specifically MBC and SBR, highlight the need of long-term investigation and periodic sampling to target the dynamic changes induced by biochar application. Nevertheless, the improved aggregation of both soils could indicate biochar as a useful mean to combat soil degradation in Mediterranean areas.

Key words: Acrisol; Calcisol; microbial biomass; soil aggregation; soil enzymes

9.1 Introduction

Biochar is a carbonaceous material obtained by pyrolysis of organic matter, differing considerably from its original feedstock. The pyrolysis process transforms the major part of organic carbon (C) into stable form which is generally recalcitrant towards biotic and abiotic oxidation and which is believed to persist in soil for centuries or even up to thousands of years (Atkinson et la. 2010). Although only minor part of original C content remains labile and available for the use by soil biota (Smith et al. 2010), addition of biochar to soil may alter soil biogeochemical processes as a result of organic C addition and the unique properties of biochar. Pyrolysis induces the formation of large amount of surface functional groups which are directly linked to biochar properties such as electric conductivity (EC) and pH (Li et al. 2013b) or sorption capacity (Uchimiya et al. 2011) and together with ash content lead to often observed changes in soil pH. These biochar properties are affected by both feedstock material (Sohi et al. 2010) and pyrolysis temperature with more alkaline biochars with higher sorption capacity formed at higher temperatures (Uchimiya et al. 2011). These characteristics have high importance in degraded soils where acidity can limit microbial activity and plant growth, such as many Mediterranean soils where inappropriate agricultural practices caused drastic reduction of soil C stocks and soil acidification. It has been suggested that changes in soil pH and salinity could be the key drivers of microbial changes in biochar-amended soils (Masto et al. 2013; Khadem & Raiesi 2017a).

Biochar can sequester C in soil, while simultaneously making use of organic wastes (Sohi et al. 2010) and potentially increasing soil quality. Once in soil, biochar has been observed to induce changes in nutrient cycling which could be attributed to the addition of C substrate (Cross & Sohi 2011) or nutrients, or to transformation of chemical or physical properties. Aside from these variable interactions in soils, the duration of change (Song et al. 2016) also depends on the rate of decomposition and the initial soil properties (Wang et al. 2015a) and biochar feedstock (Heitkötter & Marschner 2015). The inherit variability within soils and biochar amendments calls for long-term studies with multiple sampling points to capture complex dynamics.

The interaction of biochar an soil will also impact microbial abundance and activity of intra- and extracellular enzymes (Hale et al. 2011). These are critical for key steps of soil organic matter (SOM) decomposition, facilitating the liberation of nutrients for plant or microbial uptake. Given that enzyme activity is considered a sensitive indicator of soil health, the effect of biochar on soil enzymes is a key to understanding changes to short and long-term

impacts on microbial nutrient cycling (Gul et al 2015; Khadem & Raiesi 2017a). Incorporation of biochar into the soil may have several direct or indirect impacts on soil biota, including alteration of abiotic factors including soil pH and increased availability or altered quality of substrate as a source of energy (Thies et al. 2015).

Despite their key role in soil nutrient cycling and increasing body of literature, the effect of biochar on the activity of soil enzymes remains largely unclear. For example, dehydrogenase is an intracellular enzyme and its activity in soil is linked to respiratory processes, often tightly correlated with organic C availability. Nevertheless, strong enhancing effect of soil pH have also been detected in degraded acid soils (León et al. 2017; Teutscherova et al. 2017; Vazquez et al. 2017), suggesting that biochar could affect dehydrogenase activity either by labile C input or through soil pH neutralization. Similarly, the activity of β-glucosidase, the enzyme catalyzing the final step of cellulose degradation releasing glucose, has been observed to be increased, unaffected or decreased by biochar application (Lammirato et al. 2011; Yoo & Kang 2012; Ventura et al. 2014) as well as the activity of other hydrolases involved in SOM transformation and nutrient cycling, such β-glucosaminidase (Yoo & Kang 2012), phosphatase (Yoo & Kang 2012; Ventura et al. 2014) or urease (Teutscherova et al. 2017). Soil organic C content is directly linked to the formation and stabilization of soil aggregates which are the key element in soil structure rebuilding and erosion prevention. The increase of soil C leads to stimulation of biological activity in soil including the activity of soil enzymes, which in turn result in enhanced formation of soil macroaggregates and their stabilization (Demisie et al. 2014).

Extensive areas in Mediterranean regions are characterized by severely degraded soils resulting from an unsustainable soil management causing a reduction of SOM and often related soil acidification (Goméz-Paccard et al. 2013), such as for example large areas of SW Spain covered with degraded Acrisols (FAO). Climax vegetation these areas is a cork oak (*Quercus suber* L.) forest, which has been largely substituted by holm oak (*Quercus ilex* L.), olive groves, pastures or agricultural lands. Holm oak agroforestry systems (*Dehesa*) are of high economic and traditional importance due to Iberian pigs, which are largely or exclusively fed on acorns. However, the value of utilization of holm oak pruning residues declined drastically in the past decades and their conversion to biochar could simultaneously solve the residues problems as well as improve degraded soil properties and increase their productivity. Under Mediterranean climate, often severely C-poor Calcisols occupy the largest area of all World Reference Base (WRB) groups (85 million ha) (Verheye & de la Rosa 2005). These

soils are often poor in nutrients and SOM and have high soil pH. There is limited amount of information about the effect of biochar application on Calcisols, despite their large extension areas under Mediterranean climate (Song et al. 2016; Elzonair et al. 2016).

The aim of this study is the evaluation of enzymatic and microbial activity dynamics in two contrasting soils (an alkaline Calcisol with low organic C content and an acid degraded Acrisol) after biochar application and fertilization in a greenhouse experiment with a special emphasis on development of biochar-induced changes over twelve weeks since application. We hypothesized that the biochar will increase microbial biomass and microbial activity, related to labile C inputs in the Calcisol and related to pH neutralization in the Acrisol. Furthermore, biochar will enhance stabilization of soil aggregates in both soils as a result of stimulated microbial activity and that biochar induced changes will decrease over time.

9.2 Materials and methods

9.2.1 Soil and biochar characterization

Both Acrisol and Calcisol were used for the present study. The properties of both soils and applied biochar can be found in chapter 4 (Materials and methods section).

9.2.2 Experimental design

Soil was sieved to 5 mm due to the high content of rock fragments in Acrisol (51%) and in order to ensure adequate aeration during the incubation experiment. Both soils were amended with 1% (B1) and 2 % (B2) of biochar by weight (26 Mg ha^{-1} and 52 Mg ha^{-1}, respectively) including also soil controls (B0) without biochar addition. Twenty-four replicates were prepared for each soil and biochar treatment, 12 were not fertilized (B0, B1 and B2 treatments) and 12 were fertilized with NPK at application rate of 36 kg NH_4^+-N ha^{-1}, 72 kg P ha^{-1} and 72 K kg^{-1} ha^{-1} (B0-F, B1-F and B2-F treatments), which is the fertilization rate used in the study area as reported previously (Gómez-Paccard et al. 2013; Vazquez et al. 2017).

Plastic pots (ten cm in diameter; ten cm height) were filled with 500 g of control soil per pot or biochar-amended soil at 505 and 510 g per pot for B1 and B2, respectively. Four pots were destructively sampled after three weeks, six weeks and 12 weeks of incubation for laboratory analysis. All pots were placed in completely randomized block design in a controlled greenhouse (12 hours of light per day, temperature around 25°C) and watered to 60% of WHC. Moisture content was adjusted gravimetrically every one or two days.

9.2.3 Analytical methods

At determined sampling points, soil from the pots was sieved in moist state immediately after sampling. Part of the soil was kept at 4 °C for microbial analysis and inorganic N pools and the other part was air-dried for chemical properties determination. In the air-dried soil, soil pH and EC measurement, water extracts (1:2.5 w/v) were prepared. Water-stable aggregates (WSA) were determined by wet-sieving of air-dried 1–2 mm aggregates through a 250 mm sieve (Kemper & ROsenau 1986). The percentage of $WSA_{1–2\ mm}$, was calculated as the weight of stable aggregates divided by the sum of stable and unstable aggregates.

Inorganic nitrogen (N) was extracted from fresh soil samples with 2M KCl (1:10) and NH_4^+-N and NO_3^--N contents were determined colorimetrically using the same methods as in Teutscherova et al. (2017).

Microbial biomass C (MBC) was quantified using the fumigation-extraction method by fumigating 15 g of fresh soil with ethanol-free chloroform followed by 0.5 M K_2SO_4 extration (1:4) (Vance et al. 1987). The concentration of organic C was determined colorimetrically by measuring Cr^{3+} produced by reduction of Cr^{6+} (578nm) after microwave digestion (Speedwave four, Berghof, Eningen, Germany) at 135°C for 30 minutes. The content of MBC was then calculated as the difference between the C content in fumigated and non-fumigated samples, divided by 0.38 following the recommendation of Joergensen (1996) for the C analysis by dichromate consumption. Soil basal respiration (SBR) was determined by aerobic incubation of 20 g of moist soil (60 % field capacity) for three days in air-tight jars with alkaline trap followed by titration of NaOH with HCl after carbonate precipitation with $BaCl_2$. Substrate-induced respiration (SIR) as a mean to estimate the active microbial biomass was determined in a similar way by incubation of 20 g field moist soil sample with talco:glucose mixture (3:1) for four hours at 25 °C (Anderson & Domsch 1978). The extractable organic C (EOC) was extracted with 0.5M K_2SO_4 and determined as in MBC.

Dehydrogenase activity was determined using 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) as a substrate using method modified by García et al. (1993). For β-glucosidase activity method modified by Strobl and Traunmueller (1996) was selected using β-glucoso-saligenin (salicin) as substrate. The β-glucosaminidase activity was determined according to method proposed by Parham and Deng (2000) with p-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate. Acid (Acrisol) and alkaline (Calcisol)

phosphomonoesterase activity was determined using the method of Tabatabai and Bremner (1969). For the activity of urease, method modified by Kandeler et al. (1999) was selected and the activity was determined as NH_4^+-N produced during the incubation. A series of biochar amended samples analysis were performed to account for possible adsorption of the substrate or final product of colorimetric reactions. Nevertheless, no negative impact of biochar on the methods was detected.

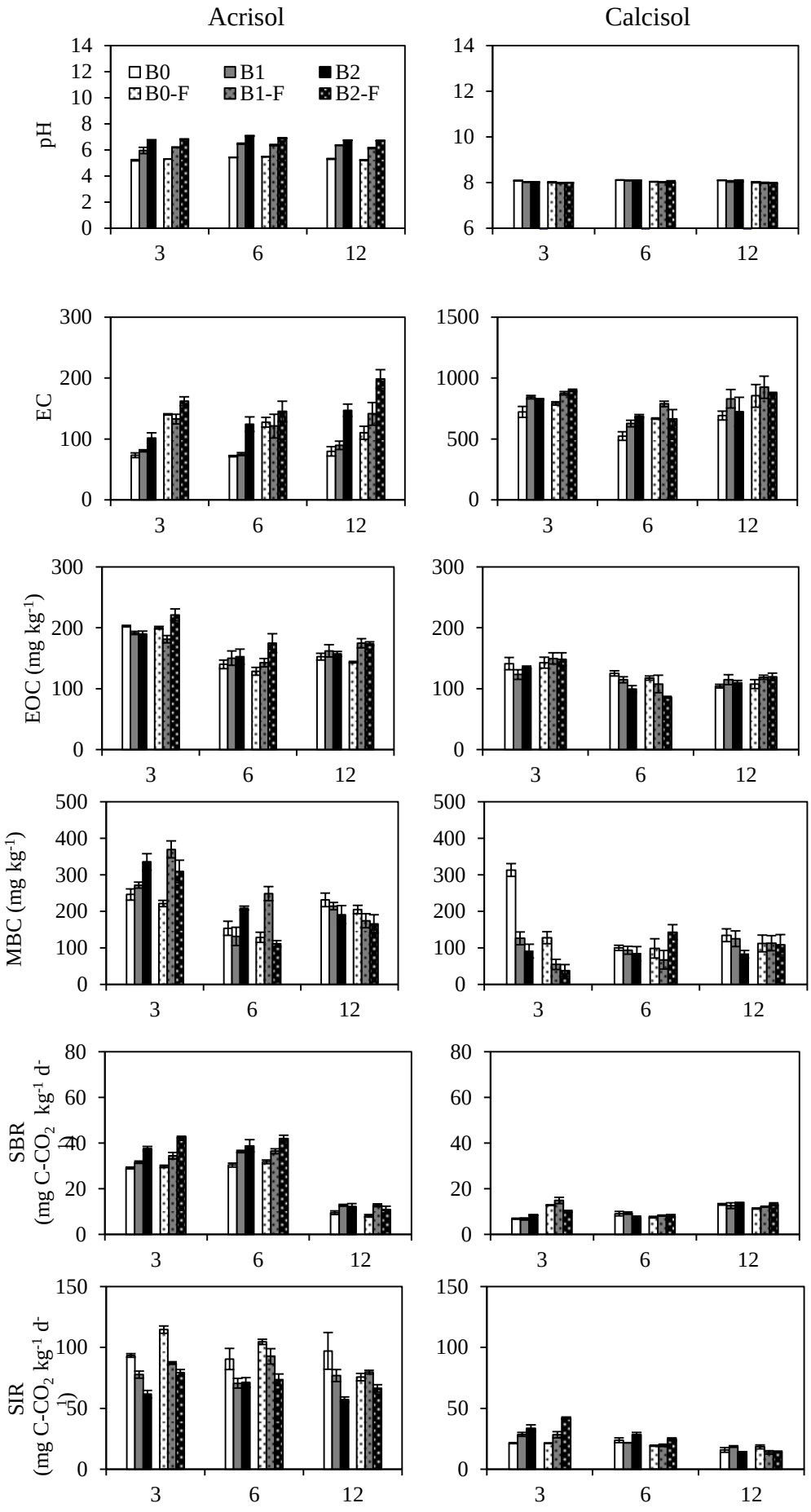
9.2.4 Statistical analysis

Data were analyzed statically using SPSS 22.0 program (IBM SPSS, Inc., Chicago, USA) using a full factorial Linear Mixed Model for each soil type separately, using biochar application rate, fertilizer application and sampling date as fixed factors and block as random factor followed by post-hoc LSD test ($p<0.05$) between the different biochar treatments and sampling dates. For each soil, principal components analyses (PCA) were applied and the two first components (PC1 and PC2) were extracted through Varimax orthogonal rotation. Pearson correlation tests were performed between the soil properties and scores of PC1 and PC2. Additionally, the treatments were plotted in the orthogonal space defined by PC1 and PC2. For identification of the main drivers of the SBR, separate stepwise regressions were applied for each of the sampling dates. The stepping criteria employed for the entry and removal were based on the significance level of the F-value and were set at 0.05. In addition, multiple stepwise regressions were used to analyze the main drivers of soil aggregation using the mean values from all three sampling dates.

9.3 Results

9.3.1 Effects on properties of Acrisol

The application of holm oak biochar raised the soil pH of acid Acrisol ($p<0.001$) (Fig. 9-1, Table 9-1) and the increase was directly proportional to the application rate. The addition of fertilizer did not affect the soil pH (Fig.9-1, Table 9-1) but pH decreased in the fertilized pots over time. Similarly, the application of the B2 biochar treatment increased the EC of the Acrisol significantly over the control (Fig. 1, $p<0.05$). The highest EC was observed after 12 weeks ($p<0.05$) due to great increase in B1 and B2 respect to the previous weeks.


Acrisol
Calcisol
B0
B1
B2
B0-F
B1-F
B2-F
pH
EC
EOC (mg kg-1)
MBC (mg kg-1)
SBR (mg C-CO2 kg-1 d-1)
SIR (mg C-CO2 kg-1 d-1)
3
6
12

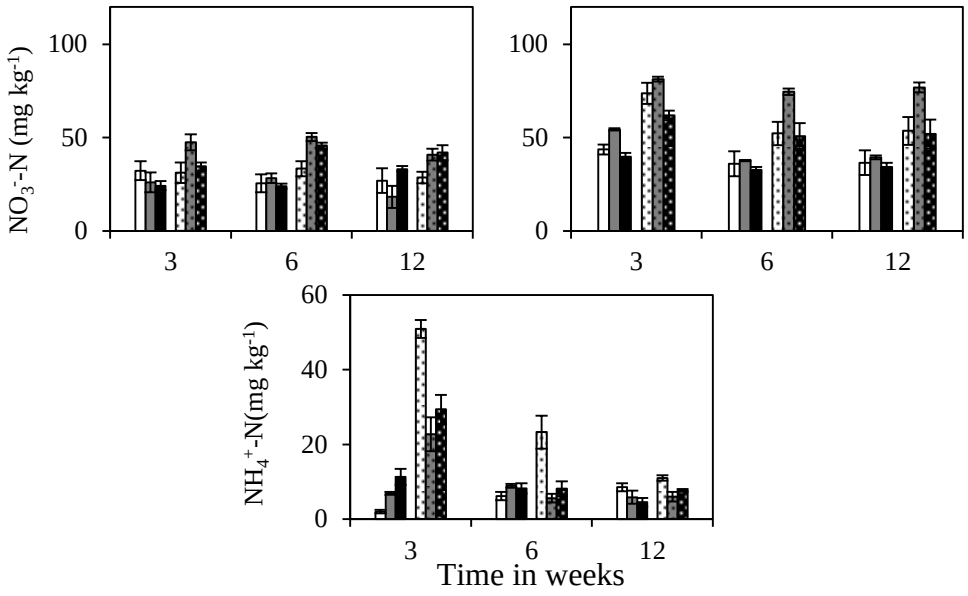


Fig.9-1: Soil pH, electric conductivity, extractable organic carbon, soil basal respiration, substrate-induced respiration, microbial biomass carbon, nitrate content and ammonium content evolution during the incubation. Bars indicate standard error of the mean (n=4).

Table 9-1: Effect of time (T), biochar application rate (B), fertilization (F) and their interactions on soil properties

	pH	EC	EOC	NH_4^+-N	NO_3^--N	MBC	SBR	SIR
						F value (*p* value)		
Acrisol								
T	26.69 (***)	4.335 (*)	82.54 (***)	68.82 (***)	0.853 (n.s.)	78.78 (***)	1208 (***)	6.514 (**)
B	876.9 (***)	34.71(**)	9.283 (***)	21.76 (***)	3.266 (*)	6.250 (**)	89.38 (***)	43.16 (***)
F	0.293 (n.s.)	98.14 (***)	2.102 (n.s.)	136.2 (***)	48.09 (***)	9.406 (n.s.)	6.170(*)	12.25 (**)
TxB	2.089 (n.s.)	3.715 (*)	4.481 (**)	2.034 (n.s.)	2.600 (*)	7.394 (***)	12.58 (***)	1.060 (n.s.)
TxF	6.591 (**)	1.422 (n.s.)	0.306 (n.s.)	69.96 (***)	1.417 (n.s.)	2.397 (n.s.)	5.728 (**)	5.965 (**)
BxF	0.363 (n.s.)	0.191(n.s.)	8.454 (**)	35.28 (***)	9.055 (***)	14.15 (***)	1.603 (n.s.)	0.704 (n.s.)
TxBxF	1.773 (n.s.)	1.128 (n.s.)	1.036 (n.s.)	9.583 (***)	0.434 (n.s.)	5.486 (**)	1.127 (n.s.)	3.373 (*)
Calcisol								
T	9.009 (***)	19.10 (***)	39.66 (***)	n.d.	15.45 (***)	3.084 (n.s.)	85.86 (***)	134.6 (***)
B	5.314 (**)	6.404 (**)	1.655 (n.s.)	n.d.	24.06 (***)	15.87 (***)	1.333 (n.s.)	31.22 (***)
F	57.78 (***)	15.12 (***)	1.150 (n.s.)	n.d.	171.2 (***)	12.45 (**)	18.89 (***)	0.556 (n.s.)
TxB	0.554 (n.s.)	0.404 (n.s.)	4.830 (**)	n.d.	0.351 (n.s.)	12.49 (***)	3.253 (*)	24.60 (***)
TxF	1.435 (n.s.)	0.832 (n.s.)	4.585 (*)	n.d.	0.196 (n.s.)	15.82 (**)	51.49 (***)	7.157 (**)
BxF	0.543 (n.s.)	0.460 (n.s.)	0.618 (n.s.)	n.d.	5.797 (**)	6.634 (**)	3.424 (*)	2.965 (n.s.)
TxBxF	0.483 (n.s.)	0.895 (n.s.)	0.696 (n.s.)	n.d.	1.486 (n.s.)	1.904(n..s.)	7.661 (***)	3.605(*)

EOC, extractable organic C; MBC, microbial biomass C; SBR, soil basal respiration; SIR, substrate induced respiration

***, **, * correspond to $p<0.001$, $p<0.01$ and $p<0.05$, respectively; n.s., not significant; n.d., not detectable

The EOC content of Acrisol was increased by the higher application rate of biochar (Fig.9-1, Table 9-1). The EOC fluctuated in time with the maximum average of all treatments after three weeks and the minimum after six weeks ($p<0.05$). In addition, the effect of biochar application rate was dependent on time (Table 9-1), and after three weeks, the lowest EOC was found in B1 treatment, while after six and 12 weeks the lowest values were detected in control. Although fertilizer did not affect the EOC, we observed that the mean value of all three samplings of fertilized B2 treatment was more than a 14% higher than the unfertilized B2.

The mineral N in Acrisol was increased by the application of fertilizer (Fig. 9-1). In the case of NH_4^+-N, the content decreased with time and the lowest amount was detected after 12 weeks ($p<0.05$). Both biochar treatments decreased significantly the NH_4^+-N content respect to B0. The increase of NO_3^- content due to fertilization was more evident after six and 12 weeks.

9.3.2 Effects on properties of Calcisol

Contrary to acid Acrisol, both the application of biochar and fertilization decreased significantly the soil pH of the alkaline Calcisol (Fig. 9-1) without differences between the B1 and B2 treatment.

The EOC of Calcisol decreased significantly with time (Fig. 9-1, Table 9-1). Although the biochar effect was not significant, we observed an evolution of the biochar-induced effect on EOC, which was 10.5% and 8.3% higher in B1 and B2, respectively, when compared to control at the end of the end of the experiment. The positive effect of fertilization on EOC was observed only during the first three weeks of the experiment.

No detectable amount of NH_4^+-N was extracted from Calcisol and all mineral N was extracted in the form of NO_3^--N. The content of NO_3^--N was increased by the fertilizer application (Fig. 9-1) and decreased in time between the first and the second sampling and then was increased between the second and the third sampling (Table 9-1). Biochar application affected the NO_3^--N content, although only B1 treatment had NO_3^--N content significantly higher than B0 ($p<0.05$).

9.3.3 Micorbial and enzymatic activity in Acrisol

The SBR of Acrisol (Fig. 9-1) decreased over time with the rates more than three times lower after 12 weeks when compared to values determined after three or six weeks. The

SBR was increased ($p<0.05$) by biochar application and fertilization (Fig. 9-1) and the magnitude of both effects decreased in time (Table 9-1). Similarly, both biochar treatments increased the MBC (Fig. 9-1, Table 9-1) during the first six weeks of incubation, although no significant differences were found between B1 and B2. The effect of biochar depended on time: the MBC content was increased by biochar three weeks after biochar application but decreased at the end of the incubation (12 weeks). Although the fertilizer application did not affect the MBC, fertilizer application increased the MBC in B1 treatment but decreased MBC in both B0 and B2 (Table 9-1). The SIR decreased with time and with biochar application (Fig. 9-1, Table 9-1) but increased with fertilizer application in the beginning of the experiment.

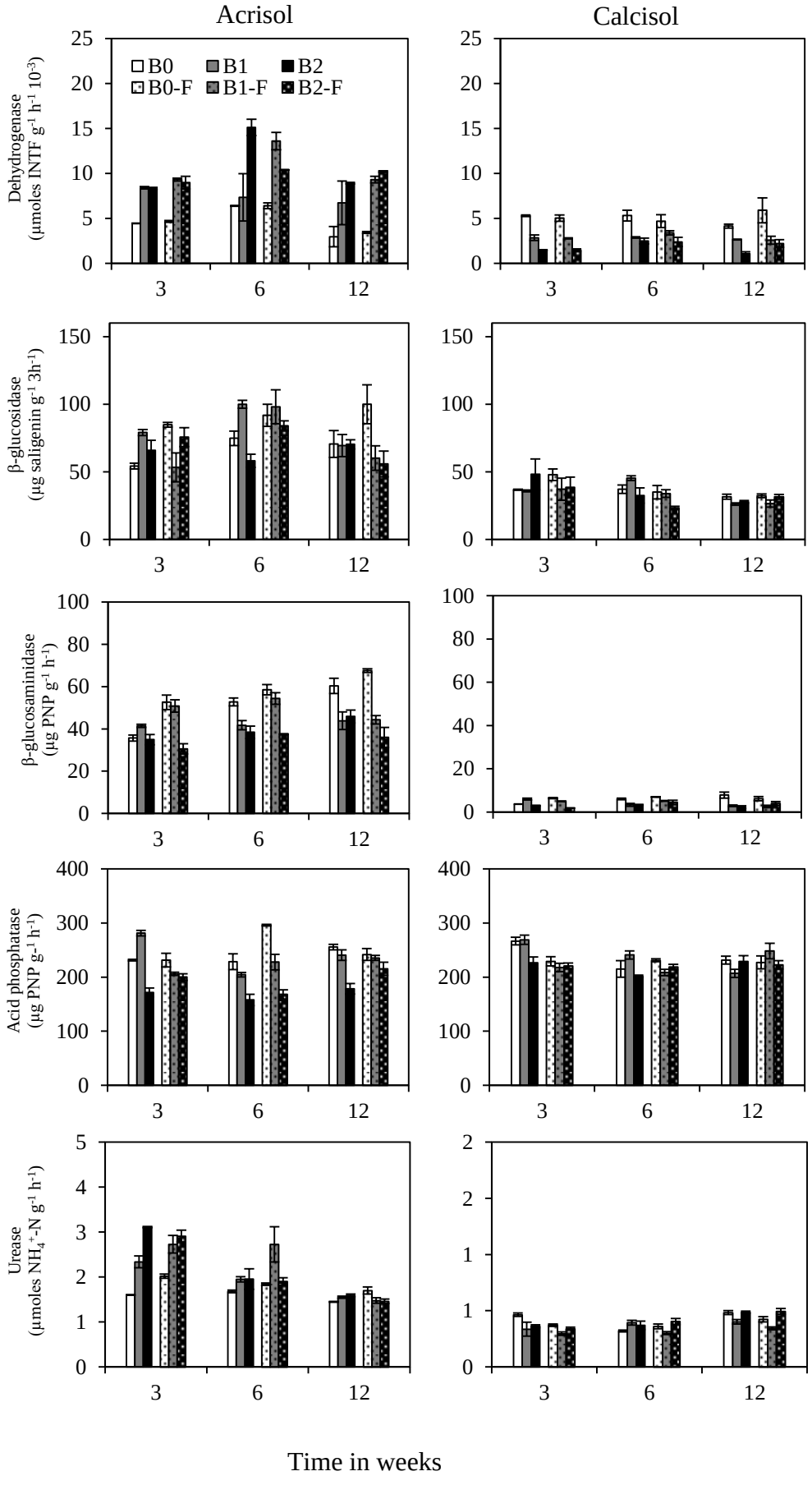


Fig.9-2: The enzymatic activity in Acrisol and Calcisol during the incubation. Bars indicate standard error of the mean (n=4).

Application of holm oak biochar increased the dehydrogenase activity of the acid Acrisol (Fig. 9-2, Table 9-2) and the increase was directly proportional to biochar application rate ($p<0.05$). With the exception of urease, which was enhanced by biochar application (Fig. 9-2), the other three measured extracellular enzymes decreased in biochar-amended treatments. The effect was more evident in β-glucosaminidase and phosphatase, where the decline was significantly higher in B2 than B1 ($p<0.05$). The fertilization of Acrisol enhanced significantly the activity of β-glucosaminidase, phosphatase and urease (Fig. 9-2). The combination of biochar and fertilization revealed significant interaction in all determined EAs (Table 9-2). The activity of β-glucosidase, β-glucosaminidase and urease followed a similar pattern and the positive effect of fertilization on enzymes activities was reduced with increasing biochar application rates.

Table 9-2: Effect of time (T), biochar application rate (B), fertilization (F) and their interactions on soil enzymes activities

	Dehydrogenase	β-glucosidase	β-glucosaminidase	Phosphatase	Urease
	F value (*p* value)				
Acrisol					
T	71.32 (***)	7.080 (**)	16.49 (***)	4.932 (*)	82.99 (***)
B	229.6 (***)	3.278 (*)	62.35 (***)	117.7 (***)	24.12 (***)
F	0.490 (n.s.)	3.378 (n.s.)	10.61 (**)	4.809 (*)	8.733 (**)
TxB	2.961 (*)	4.196 (**)	8.323 (***)	9.982 (***)	17.98 (***)
TxF	1.793 (n.s.)	0.927 (n.s.)	3.798 (*)	15.16 (***)	2.005 (n.s.)
BxF	9.849 (***)	8.878 (***)	13.40 (***)	13.96 (***)	6.818 (**)
TxBxF	13.28 (***)	2.083 (n.s.)	1.433 (n.s.)	11.70 (***)	2.360 (n.s.)
Calcisol					
T	1.752 (n.s.)	9.451 (***)	1.987 (n.s.)	7.102 (**)	23.05 (***)
B	87.00 (***)	0.816 (n.s.)	41.41(***)	4.273 (*)	13.97 (***)
F	1.499 (n.s.)	0.634 (n.s.)	2.982 (n.s.)	2.909 (n.s.)	8.079 (**)
TxB	0.948 (n.s.)	2.366 (n.s.)	8.773 (***)	0.873 (n.s.)	5.773 (**)
TxF	2.843 (n.s.)	1.900 (n.s.)	2.213 (n.s.)	9.207 (***)	1.467 (n.s.)
BxF	0.095 (n.s.)	1.382 (n.s.)	0.263 (n.s.)	1.230 (n.s.)	3.404 (*)
TxBxF	1.574 (n.s.)	0.952 (n.s.)	5.598 (**)	6.115 (***)	2.114 (n.s.)

***, **, * correspond to $p<0.001$, $p<0.01$ and $p<0.05$, respectively; n.s., not significant

9.3.4 Microbial and enzymatic activity in Calcisol

The fertilization of Calcisol had a positive effect on SBR (Fig. 9-1) only at the three week time point. In contrast to Acrisol, biochar application to Calcisol decreased the MBC

but without significant differences between the biochar application rates (Fig. 9-1). Initially, fertilization reduced MBC, evident after three weeks. The differences between fertilized and control pots were gradually decreasing with the increasing biochar application rate. In Calcisol, SIR decreased with time (Fig. 9-1, Table 9-1) and was increased by biochar application. The highest values were found in B2 treatment ($p<0.05$) with the differences between biochar application rates decreasing in time. Fertilization did not affect the mean SIR values over the whole experimental period but significantly higher values were found in fertilized treatments in the beginning of the experiment.

In Calcisol, only β-glucosidase was unaffected by biochar application and all other measured EAs decreased with biochar application (Fig. 9-2, Table 9-2). In addition, the activity of dehydrogenase, β-glucosaminidase and phosphatase reached the lowest activity with the highest biochar application rate ($p<0.05$). However, different interactions with the time were found and the highest differences in β-glucosaminidase between biochar application rates were found after 12 weeks, while the highest differences in urease activity were detected already after three weeks of incubation.

The effect of fertilization on EAs in Calcisol was lower than in Acrisol. A reduction of phosphatase activity by 13.4% was observed after three weeks of incubation, but this effect disappeared in the subsequent sampling points. The fertilization decreased the urease activity over the whole sampling period in B0 and B1 treatments by 7 and 18%, respectively, but had no effect in case of B2.

9.3.5 Soil aggregation

The stability of soil macroaggregates (1-2 mm fraction) was significantly improved by both biochar application rates in Acrisol and by B2 treatment in Calcisol (Table 9-3). While in Acrisol both biochar application rates enhanced the percentage of WSA to the same extend (by 11.3% and 12.9% in B1 and B2 treatment, respectively), in Calcisol the stability was doubled by B2 treatment. Significant interaction between biochar and fertilization was found in Acrisol and biochar increased the aggregate stability only in unfertilized treatments meanwhile there was no difference between biochar-amended soil and control in fertilized pots.

Table 9-3: The percentage of WSA_{1-2mm} in Acrisol and Calcisol as affected by biochar (B) and fertilization (F)

	Acrisol	Calcisol
B0	80.37	9.13
B1	89.47	7.53
B2	90.72	20.53
B0-F	89.12	9.90
B1-F	89.02	10.59
B2-F	91.65	24.80
Effects		
F value (*p* value)		
B	25.149 (***)	153.831 (***)
F	16.343 (***)	14.109 (***)
BxF	14.160 (***)	2.023 (n.s.)

*** p<0.001; n.s. not significant

9.3.6 Correlations among variables

In the case of Acrisol, the two extracted components in PCA accounted for a 47.18% of the variance, while in Calcisol, only 40.22% of the variance was explained (Fig. 9-3, Table 9-4). In both soils, soil pH, dehydrogenase activity correlated with PC1 scores, while urease and EOC were correlated with PC2 scores, showing similar dependencies across soil type (Table 9-4). Urease and EOC correlated with PC2 scores positively in Acrisol, but in Calcisol, urease correlated negatively and EOC positively with PC2 scores. In addition, SBR and MBC in Acrisol correlated with the same axis as urease and EOC suggesting a positive relationship. Nevertheless, in Calcisol both SBR and MBC correlated with the same axis as pH and dehydrogenase activity. Finally, the SBR seemed to be N-driven loading in the same axis as ammonium in Acrisol and nitrate in Calcisol.

Table 9-4: Pearson correlation coefficients between soil properties and scores of PC1 and PC2 of PCA

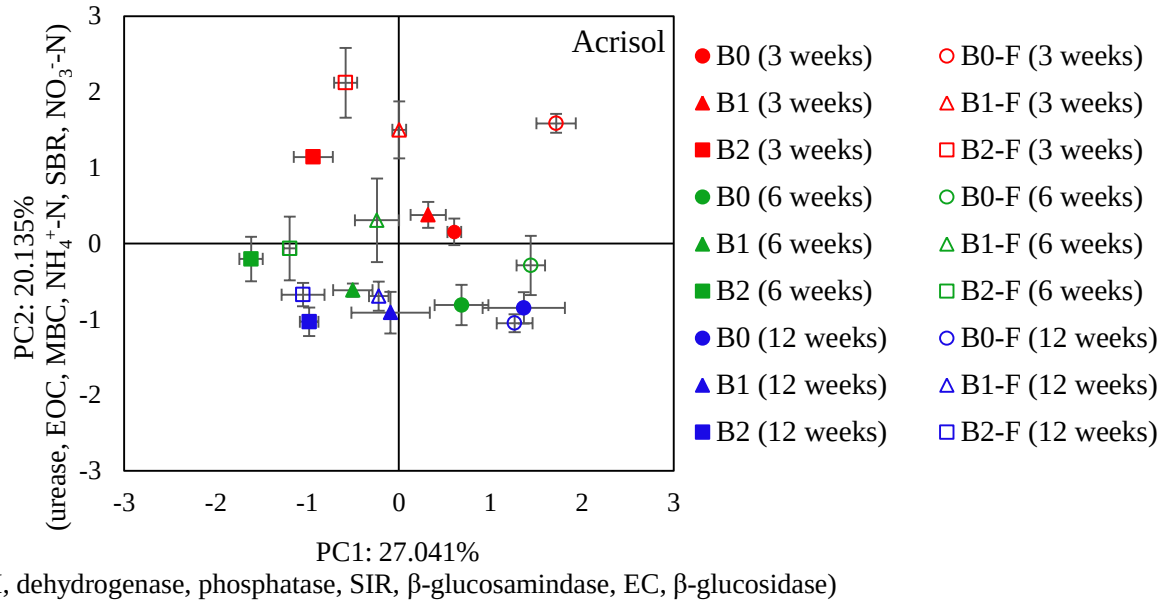
Acrisol			Calcisol		
Soil parameter	PC1	PC2	Soil parameter	PC1	PC2
	27.045 %	20.135 %		22.391 %	17.827 %
pH	-0.93**	0.13	pH	0.80**	-0.24*
Dehydrogenase	-0.79**	0.06	EC	-0.80**	0.15
Phosphatase	0.75**	-0.16	Dehydrogenase	0.59**	0.13
SIR	0.73**	0.27*	MBC	0.51**	-0.14
β-glucosaminidase	0.68**	-0.31	SBR	-0.50**	-0.39**
EC	-0.31**	0.26*	NO_3^--N	-0.50**	0.43**
β-glucosidase	0.29*	-0.09	β-glucosaminidase	0.44**	0.11
Urease	-0.20	0.75**	Phosphatase	0.20	0.20
EOC	-0.12	0.69**	Urease	0.03	-0.70**
MBC	-0.02	0.65**	SIR	-0.07	0.64**
NH_4^+-N	0.39**	0.66**	EOC	-0.15	0.63**
SBR	-0.27*	0.64**	β-glucosidase	0.12	0.58**
NO_3^--N	-0.15	0.19			

SIR, substrate-induced respiration; EOC, extractable organic carbon; MBC, microbial biomass carbon; SBR, soil basal respiration

* Significant correlation coefficients at a significant levels of 0.05.

** Significant correlation coefficients at a significant levels of 0.01.

In addition, the treatments were plotted in the orthogonal space defined by PC1 and PC2 (Fig. 9-3). In both soils we can observe that the treatments of the first sampling were plotted upwards the PC2 while the treatments in the second and third samplings were accumulated around the middle of the PC2 in Acrisol. In case of Calcisol, both samplings were distributed downwards the PC2 with the third sampling points reaching negative values. The differences between the fertilized and unfertilized treatments were observed: in Acrisol the fertilized treatments were generally plotted above the controls along the y (PC2) axis. However, in Calcisol the most of the fertilized treatments were located more to the left side of the PC1 axis than the unfertilized treatments. Finally, a clear pattern of distribution of the treatments according to the biochar application rate was found in Acrisol with the B0 in the positive side of the PC1, B1 in the middle and B2 in the negative side of the PC1.



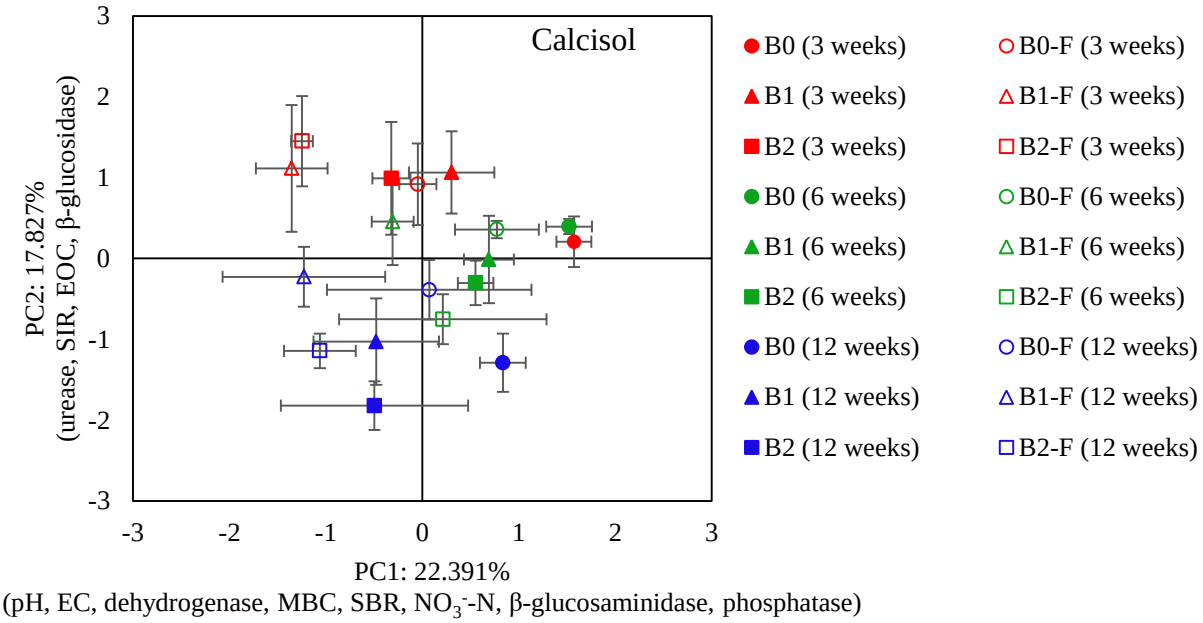


Fig. 9-3: PCA loading plots of the pots based on soil pH, electric conductivitiy (EC), extractable organic carbon (EOC), NH_4^+-N, NO_3^--N, microbial biomass carbon (MBC), soil basal respiration (SBR), substrate-induced respiration (SIR), dehydrogenase activity, β-glucosidase activity, β-glucosaminidase activity, acid phosphomonoesterase activity and urease activity. In Acrisol, the highest PC1 scores in Acrisol had soil pH (-0.927) and dehydrogenase activity (-0.790) and the highest PC2 scores were found for urease activity (0.746) and EOC (0.694). In the Calcisol, soil pH (0.804) and EC (-0.802) scored the highest in PC1 and urease activity (-0.702) in the PC2. Scores of all PC1 and PC2 variables can be found in Table S1 (Supplementary material). B0, B1 and B2 indicate the 0%, 1% and 2% biochar application rates without fertilization (closed symbols), respectively. B0-F, B1-F and B2-F indicate the 0%, 1% and 2% biochar application rates with addition fertilizer (open symbols), respectively. Red, green and blue colors symbolize the samples collected three weeks, six weeks and twelve weeks since the establishment of the experiment, respectively.

9.3.7 Main drivers of soil respiration and aggregate stability

From the measured variables, the stepwise regression revealed the soil pH and EOC as two of the main controls of SBR in Acrisol in the two first samplings (Table 9-5). Together with the EC after three weeks and with urease and nitrate content after six weeks both regressions reached a high R^2 (=0.87). However, after 12 weeks the variation was explained by only 39% with dehydrogenase as the only selected factor (Table 9-5). In the case of Calcisol, after three weeks 87% of SBR variability was explained by nitrate content, phosphatase and SIR. In the final sampling, the SBR was explained by urease activity only.

Table 9-5: Stepwise regression analysis for identification of soil parameters controlling soil basal respiration

	Constant	pH	EOC	EC	Ure	NO_3^-	DHase	PHase	SIR	R^2	p-value[1]
Acrisol											
3 weeks	-18.1	5.75	0.07	0.03						0.87	***
6 weeks	-1.55	4.83	0.56		-3.12	0.15				0.87	***
12 weeks	7.01						0.53			0.39	***
Calcisol											
3 weeks	25.5					0.11		-0.08	-0.10	0.87	***
6 weeks											n.s.
12 weeks	7.29				12.3					0.35	**

EOC, extractable organic C; Ure, urease activity; DHase, dehydrogenase activity; PHase, phosphatase activity; SIR, substrate-induced respiration
Discarded parameters (p>0.05) were: MBC, NH_4^+-N, Gls, Glm. NH_4^+-N was not included in Calcisol
** p<0.01; *** p<0.001; n.s. not significant
[1] p-value of the model

For water-stable macroaggregates, the stepwise regression analyses showed that soil pH, NH_4^+-N and phosphatase activity in the case of Acrisol, and SIR in the case of Calcisol were the main drivers of the formation (Table 9-6).

Table 9-6: Stepwise regression analysis for identification of soil parameters controlling WSA

	Constant	pH	NH_4^+-N	PHase	SIR	R^2	p-value [1]
Acrisol	20.858	8.116	0.261	0.065		0.79	***
Calcisol	-25.687				1.742	0.60	***

PHase, phosphatase activity; SIR, substrate-induced respiration
Discarded parameters (p>0.05) were: MBC, SBR, EOC, DHase, Gls, Glm. Urease, NO_3^--N, EC.
NH_4^+-N was not included in Calcisol. All the parameters introduced in the stepwise regression were the average of the three samplings
*** p<0.001
[1] p-value of the model

9.4 Discussion

The present study evaluated the evolution of effects of holm oak biochar application on microbial biomass, soil respiration and soil extracellular enzymes activity during 12 weeks of

greenhouse incubation. Three destructive sampling points in the course of the study permitted us to evaluate not only the interactive effects of biochar application and fertilization but also the evolution of these changes in time. Our results demonstrate that the effect of biochar application to soil depends both on the soil type and the time that has elapsed since the treatment application. Therefore, our initial hypothesis of biochar-induced enhancement of microbial biomass and activity was only confirmed in Acrisol. Although MBC and SBR was increased in Acrisol after the biochar application, in Calcisol the MBC was slightly decreased and the SBR unaffected by biochar application. In addition, the microbial changes in Acrisol seemed to be related to the liming capacity of biochar in acid soils as hypothesized.

9.4.1 Soil pH, basal respiration and microbial biomass in Acrisol

In acid soils, biochar tends to increase pH likely as a result of high ash content and due to the presence of alkaline functional groups on its surface (Li et al. 2013). Besides the high alkalinity of biochar itself, the pH of soil-biochar mixtures tends to raise as a result of protons removal from soil solution and their binding onto negatively charged groups on biochar surface (Brewer & Brown 2012). We observed immediate increase of pH in acid Acrisol with biochar application, which is in line with other studies (Chintala et al. 2014).

Previous research shows pH as a key mechanism behind the biochar-induced changes in soil microbial communities and no effect is found when soil pH was not affected by biochar application (Meynet et al. 2012). Such a neutralization effect could be of especially high interest in degraded acid soils where low pH and high Al toxicity decelerate microbial activity and nutrient turnover in soil. In agreement with previous studies (Teutscherova et al. 2017a; Teutscherova et al. 2017b), pH of Acrisol used in our study was significantly increased by both biochar application rates. Using the soil from the same study area (Cañamero's raña formation from SW Spain), Teutscherova et al. (2017) showed decreased SIR after application of biochar and compost with an increase in pH. The authors suggested that such a drop of microbial biomass could be a result of rapid pH raise resulting in microbial community structure changes. Nevertheless, the MBC obtained by fumigation-extraction method revealed the opposite trend and seemed to be increased by biochar in the early stages of the present experiment, suggesting that rapid raise of soil pH or decline of Al toxicity may have stimulated SOM mineralization and microbial growth in the short-term, which is also in agreement with increased soil respiration peaking after three and six weeks of the incubation. The content of MBC and soil respiration has been observed to be stimulated after biochar application in previous studies (Demisie et al. 2014; Khadem & Raiesi 2017) but this effect

seemed to be dependent on biochar application rate (Xu et al. 2016). In our study, the significant role of biochar application in the SBR was supported by the regression analyses, which found soil pH and EOC as two of the main drivers of SBR with strong correlation between SBR with pH and EOC which decreased in time. The decrease of the differences in SBR caused by biochar and the lower MBC in the biochar pots after 12 weeks suggests that the effects of biochar on C mineralization decrease in time, which can be caused by a depletion of the labile C fraction of biochar (Smith et al. 2010) or decelerated mineralization of SOM (Zimmerman et al. 2011). Increased SOM mineralization in the beginning of the experiment could be also seen in the accumulation of NH_4^+-N after three and six weeks. The application of N fertilizer may have initiated microbial growth which could have led to accumulation of mineral N in soil owing to the absence of plants which could take up the ammonium or nitrate from the soil solution. Furthermore, the amount of NH_4^+-N after three and six weeks of incubation clearly exceeds the application rate, which directly indicates the initial SOM mineralization.

It has been observed that biochar can increase the MBC without enhancing the C utilization capacity of the soil biota (Jiang et al. 2017). The alleviation of soil acidity by biochar application to degraded Acrisol resulted in enhanced MBC and soil respiration but declined SIR, which could suggest that the part of microbial biomass stimulated by the presence of biochar did not use glucose as their primary energy source and were rather specialized on other C-rich compounds potentially contained in biochar. It has been generally accepted that biochar could be utilized as a substrate for specific microbial groups which could become dominant in biochar-amended soils (Zheng et al. 2016). Furthermore, the active microbial biomass would likely be the part affected by rapid changes in soil properties. If biochar application to soil initiated changes in the community structure, it could lead to temporal decline of active microbial biomass (Dempster et al. 2011).

9.4.2 Effect of biochar on microbial biomass and activity in Calcisol

In the Calcisol, with pH values similar to those of biochar, microbial activity is less likely to be affected by pH change, although short-term increase of pH in calcareous soil has been reported. We observed significant effect on soil pH of Calcisol but the decrease was only around 0.1 pH units. This decrease, more evident in the fertilized pots, can be related to stimulated nitrification of the applied fertilizer (Song et al. 2016) which is also supported by the results of PCA which loaded in the same axis. This can explain the distribution along the PC1 with most of the fertilized treatments on the negative side of the PC1 axis and the

unfertilized in the positive side. It can be concluded that microbial activity in the studied Calcisol was limited by insufficient supply of nutrients, most apparent in the relation between SBR and NO_3^--N content in the beginning of the experiment This hypothesis can be supported by the stepwise regression analyses which showed that the nitrate content after three weeks and the urease in the last sampling were playing a significant role in the soil microbial activity. Therefore, the changes in SBR related to the N fertilization in the first sampling and the microbial requirements to mineralize N in the last sampling. This agree with the general trend of net N immobilization between the first and second sampling and the net mineralization between the second and the third sampling. This important role of N in the microbial activity in Calcisol can be observed in the positive effect of fertilizer in SIR only shortly after fertilization and the general decrease of SIR in time. Similar pattern is also obvious from the PCA loadings where the treatments shifted downward along the PC2 axis in time. The enhanced SIR caused by biochar have been demonstrated in several studies (Kolb et al. 2009; Khadem & Raiesi 2017) but the absence of differences in the last samplings could be related to the mentioned insufficient supply of N.

Nevertheless, at the three week time point, biochar application reduced significantly the MBC in Calcisol both with and without fertilization. The initial decrease of MBC could be caused by a temporal shift in the soil microbial population that increased with biochar addition in another calcareous soil (Ippolito et al. 2014). The increase of the relative abundance of bacteria could imply a lower allocation of C in MBC at the same respiration status (Jones et al. 2012) causing this slight decrease in the MBC. However, after the initial effect of biochar application, the differences disappeared with the aging soil-biochar mixture and the consumption of most labile C, similar to the results found in other long term experiments (Ventura et al. 2014; Elzobair et al. 2016).

9.4.3 Soil enzymes activities

Soil enzymes are highly sensitive to changes in soil properties and many factors have been suggested to affect their activity in biochar-amended soils (Bailey et al. 2011; Masto et al. 2013; Demisie et al. 2014). Both biotic and abiotic factors seem to affect the half-life of extracellular enzymes in soil (Burns et al. 2013) and the pool of enzymes in the soil is determined by the rates of enzymes production and degradation. Furthermore, owing to its porosity and large surface area biochar has been suggested to trap either enzyme or its substrate in biochar's pores, which may impede the enzymatic reaction and lead to an overall drop of potential enzymatic activity in soil.

The dehydrogenase is an intracellular enzyme which is only present in active organisms. Therefore, it correlates well with the microbial activity and soil respiration (Garcia et al. 1997). Our results corroborate this relationship between dehydrogenase activity and soil basal respiration at the end of the experiment according to the stepwise regression. The dehydrogenase activity in Acrisol was strongly affected by soil pH as both correlated with PC1. Several other studies have detected the positive response of dehydrogenase to the application of liming agents in acids soils to alleviate the Al toxicity (León et al. 2017; Teutscherova et al. 2017; Vazquez et al. 2017). The increase of dehydrogenase, considered as a general indicator to evaluate the recovery of degraded soil (Garcia et al. 1997), confirms the potential role of biochar as liming material to enhance the microbial activity in degraded acid soils.

In the Acrisol, biochar has a positive impact on MBC and dehydrogenase activity but decreased β-glucosidase, β-glucosaminidase, and phosphatase activities. We could attribute the reduction of the activity of β-glucosidase activity to an adsorption ability of biochar as also found by some authors (Lammirato et al. 2011). The decoupling of dehydrogenase and β-glucosidase activity in biochar-amended soil was also found by Chen et al. (2013) who proposed a potential improvement of resource utilization due to co-location of resources and microorganism on the biochar surface. The possible adsorption of substrates or enzymes on biochar surface could also be translated into observed decline in the activity of β-glucosaminidase and phosphatase after biochar addition. Alternatively, this reduction in activity could be attributed to pH increase (Tabatabai 2000), which is in agreement with the results found by Teutscherova et al. (2017) and with the PCA, where the correlation between the PC1 scores and pH was negative but positive with phosphatase and β-glucosaminidase. The increase of urease activity by the application of biochar is in agreement with several studies reporting stimulation of the activity of enzymes involved in N-cycling after the application of biochar (Demisie et al. 2014; Wang et al. 2015). The application of C by biochar could enhance the relative microbial N requirements to equilibrate the microbial requirements stoichiometry. The PCA analyses support this hypothesis as urease and EOC correlated with the PC2 scores. Furthermore, the loading plot showed that this relation was more significant after three weeks and was stimulated by the fertilizer application.

The decrease of dehydrogenase and MBC in Calcisol can be related to the similar drop of the activity of the extracellular enzymes after biochar application, further confirming the relationship between dehydrogenase and microbial activity. Similar negative effect on

extracellular enzymes caused by biochar application in other alkaline soils were found by Foster et al. (2016) and Wang et al. (2015) who also observed a higher decrease in enzymes activities at higher application rate of maize straw biochar produced at 450°C. Several mechanism can explain this decrease of the extracellular enzymes activity: (i) the decrease of the MBC which reflects in the reduction of the enzyme production and release, (ii) the sorption or blocking of either the enzymes or substrates (Bailey et al. 2011), or (iii) the absence of the liming capacity of biochar in an alkaline soil (Thies et al. 2015). The absence of effect of biochar application on β-glucosidase activity, an enzyme involved in the decomposition of glucoside bonds, could be related to one of the mentioned mechanism or even to the absence of significant differences in the EOC after biochar application to the Calcisol. This last hypothesis is further supported by the results of the PCA where the activity of β-glucosidase and EOC were positively correlated with the PC2 scores.

Our results did not confirm our initial hypothesis of a general increase of the enzymatic activity in both soils after biochar application resulting from the pH raise in Acrisol and C input in Calcisol. On the contrary, with the exception of dehydrogenase and urease in Acrisol, the activity of studied enzymes was reduced or remained unaffected by biochar application. These findings suggest that other mechanism, such as adsorption of substrates or enzymes onto biochar surface, could be affecting the enzymatic activity in our studied soils.

9.4.4 The stability of soil macroaggregates

An increase in the stability of soil aggregates was observed in both soils after the application of biochar and fertilization regardless of their differences in soil texture, initial SOM content an initial amount of water stable aggregates. Several studies have found positive effects of biochar on soil aggregation (Ouyang et al. 2013; Demisie et al. 2014; Obia et al. 2016). The enhanced macroaggregate formation can be attributed to biochar surface characteristics, which can retain soil particles and thus directly bind materials as the SOM (Glaser et al. 2002). In the Acrisol, this increase in WSA could be related to increased microbial activity and augmented synthesis of polysaccharides (Demisie et al. 2014). The studied variables revealed in stepwise regression that NH_4^+-N was one of the significant factors of soil aggregation and can be explained by the positive effect of fertilization on the extracellular enzymes (Table S3 in Supplementary material). The fact that aggregate stabilization was affected by biochar only in unfertilized treatments indicates that biochar could at least partially cover the nutritional requirements of soil biota. The neutralization of soil pH was found to be the main driver of the water-stability of aggregates in Acrisol, which

could be considered a direct impact of biochar application due to: (i) the formation of complexes of soil minerals, SOM and biochar particles, (ii) the increase of the hydrophobicity of the aggregates in the presence of biochar (Glaser et al. 2002), or (iii) the increased content of cations originating from ash fraction of biochar which can act as a cement agent between soils particles (Haynes & Naidu 1998). In the case of Calcisol, the stepwise regression showed a clear influence of the microbial activity in the water-stability of aggregates as SIR was found as the main driver of the soil aggregation. Our initial hypothesis was supported only in the Calcisol, in which microbial activity correlated with soil aggregate stabilization.

9.5 Conclusions

The adoption of inappropriate agricultural practices resulted in serious degradation of many Mediterranean soils due to the depletion of C stocks and/or acidification. The application of biochar combat soil degradation and ameliorated some properties of an alkaline C-depleted Calcisol and an acid degraded Acrisol. Our study revealed a high influence of soil type on the results, as contrasts between the Acrisol and Calcisol existed in several measured responses (*e.g.*, pH, SIR, MBC, dehydrogenase, urease and β-glucosidase). According to our results, the effect of biochar was more significant in the Acrisol because of the liming capacity of biochar, which enhanced some of the studied microbial parameters. However, in the alkaline Calcisol the application of biochar reduced the microbial biomass and activity (MBC, SBR, and all the measured enzymes with the exception of β-glucosidase which was not affected) which appeared to be limited by the insufficient supply of N. In both soils, the application of biochar increased the water-stability of soil aggregates. The three consecutive samplings in the course of the 12 weeks revealed that the differences in SBR caused by biochar fluctuate in time and in general are related to the increase of labile C fraction, neutralization of soil pH and dehydrogenase activity in Acrisol and to mineral N content and urease activity in Calcisol. The general decrease of the enzymatic activities highlights the need of long-term investigation and the mechanistic understanding and careful laboratory work. Nevertheless, the improved aggregation of both contrasting soils could indicate biochar as a useful mean to combat soil degradation of soils with poor structure or on erosion-prone sites.

9.6 References

Anderson JPE, Domsch KH. 1978. A physiological method for the quantitative measurement of microbial biomass in soils. Soil Biology and Biochemistry **10:**215–221.

Atkinson CJ, Fitzgerald JD, Hipps NA. 2010. Potential mechanisms for achieving agricultural benefits from biochar application to temperate soils: A review. Plant and Soil **337**:1–18.

Bailey VL, Fansler SJ, Smith JL, Bolton H. 2011. Reconciling apparent variability in effects of biochar amendment on soil enzyme activities by assay optimization. Soil Biology and Biochemistry **43**:296–301.

Brewer CE, Brown RC. 2012. Biochar. Pages 357–384 Comprehensive Renewable Energy.

Burns RG, DeForest JL, Marxsen J, Sinsabaugh RL, Stromberger ME, Wallenstein MD, Weintraub MN, Zoppini A. 2013. Soil enzymes in a changing environment: Current knowledge and future directions. Soil Biology and Biochemistry **58**: 216-234.

Cross A, Sohi SP. 2011. The priming potential of biochar products in relation to labile carbon contents and soil organic matter status. Soil Biology and Biochemistry **43**:2127–2134.

Demisie W, Liu Z, Zhang M. 2014. Effect of biochar on carbon fractions and enzyme activity of red soil. Catena **121**:214–221.

Dempster DN, Gleeson DB, Solaiman ZM, Jones DL, Murphy D V. 2011. Decreased soil microbial biomass and nitrogen mineralisation with Eucalyptus biochar addition to a coarse textured soil. Plant and Soil **354**:311–324.

Elzobair KA, Stromberger ME, Ippolito JA, Lentz RD. 2016. Contrasting effects of biochar versus manure on soil microbial communities and enzyme activities in an Aridisol. Chemosphere **142**:145–52.

Foster EJ, Hansen N, Wallenstein M, Cotrufo MF. 2016. Biochar and manure amendments impact soil nutrients and microbial enzymatic activities in a semi-arid irrigated maize cropping system. Agriculture, Ecosystems and Environment **233**:404–414.

Garcia C, Hernandez T, Costa F. 1997. Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. Communications in Soil Science and Plant Analysis **28**:123–134.

García C, Hernández T, Costa F, Ceccanti B, Ganni A. 1993. Hydrolases in the organic matter fractions of sewage sludge: Changes with composting. Bioresource Technology **45**:47–52.

Gaskin JW, Steiner C, Harris K, Das KC, Bibens B. 2008. Effect of Low-Temperature Pyrolysis Conditions on Biochar for Agricultural Use. Transactions of the ASABE **51**:2061–2069.

Glaser B, Lehmann J, Zech W. 2002. Ameliorating physical and chemical properties of highly weathered soils in the tropics with charcoal - A review. Biology and Fertility of Soils **35**:219–230.

Gómez-Paccard C, Mariscal-Sancho I, León P, Benito M, González P, Ordóñez R, Espejo R, Hontoria C. 2013. Ca-amendment and tillage: Medium term synergies for improving key soil properties of acid soils. Soil and Tillage Research **134**: 195-206.

Gul S, Whalen JK, Thomas BW, Sachdeva V, Deng H. 2015. Physico-chemical properties and microbial responses in biochar-amended soils: Mechanisms and future directions.

Hale S, Hanley K, Lehmann J, Zimmerman A, Cornelissen G. 2011. Effects of Chemical, Biological, and Physical Aging As Well As Soil Addition on the Sorption of Pyrene to Activated Carbon and Biochar. Environmental Science & Technology **45:**10445–10453.

Haynes RJ, Naidu R. 1998. Influence of lime, fertilizer and manure applications on soil organic matter content and soil physical conditions: A review. Nutr **51**:123–137.

Heitkötter J, Marschner B. 2015. Interactive effects of biochar ageing in soils related to feedstock, pyrolysis temperature, and historic charcoal production. Geoderma 245–**246**:56–64.

Chen J et al. 2013. Biochar soil amendment increased bacterial but decreased fungal gene abundance with shifts in community structure in a slightly acid rice paddy from Southwest China. Applied Soil Ecology **71**: 34-44.

Chintala R et al. 2014. Molecular characterization of biochars and their influence on microbiological properties of soil. Journal of Hazardous Materials **279**:244–256.

Ippolito J a., Stromberger ME, Lentz RD, Dungan RS. 2014. Hardwood Biochar Influences Calcareous Soil Physicochemical and Microbiological Status. Journal of Environment Quality **43**:681.

Jiang L lin, Han G ming, Lan Y, Liu S nan, Gao J ping, Yang X, Meng J, Chen W fu. 2017. Corn cob biochar increases soil culturable bacterial abundance without enhancing their capacities in utilizing carbon sources in Biolog Eco-plates. Journal of Integrative Agriculture **16**:713–724.

Joergensen RG. 1996. The fumigation-extraction method to estimate soil microbial biomass: Calibration of the kEC value. Soil Biology and Biochemistry 28:25–31.

Jones DL, Rousk J, Edwards-Jones G, DeLuca TH, Murphy D V. 2012. Biochar-mediated changes in soil quality and plant growth in a three year field trial. Soil Biology and Biochemistry **45**:113–124.

Kandeler E, Stemmer M, Klimanek E-M. 1999. Response of soil microbial biomass, urease and xylanase within particle size fractions to long-term soil management. Soil Biology and Biochemistry **31**:261–273.

Kemper WD, Rosenau RC. 1986. Aggregate Stability and Size Distribution. Methods of soil analysis. Part 1. Physical and mineralogical methods **9**:425–442.

Khadem A, Raiesi F. 2017a. Influence of biochar on potential enzyme activities in two calcareous soils of contrasting texture. Geoderma **308:**149–158.

Khadem A, Raiesi F. 2017b. Responses of microbial performance and community to corn biochar in calcareous sandy and clayey soils. Applied Soil Ecology **114**:16–27..

Kolb SE, Fermanich KJ, Dornbush ME. 2009. Effect of Charcoal Quantity on Microbial Biomass and Activity in Temperate Soils. Soil Science Society of America Journal **73**:1173.

Lammirato C, Miltner A, Kaestner M. 2011. Effects of wood char and activated carbon on the hydrolysis of cellobiose by ??-glucosidase from Aspergillus niger. Soil Biology and Biochemistry **43**:1936–1942.

León P, Espejo R, Gómez-Paccard C, Hontoria C, Mariscal I, Renella G, Benito M. 2017. No tillage and sugar beet foam amendment enhanced microbial activity of degraded acidic soils in South West Spain. Applied Soil Ecology **109**:69–74.

Li X, Shen Q, Zhang D, Mei X, Ran W, Xu Y, Yu G. 2013. Functional Groups Determine Biochar Properties (pH and EC) as Studied by Two-Dimensional 13C NMR Correlation Spectroscopy. PLoS ONE 8:e65949.

Masto RE, Kumar S, Rout TK, Sarkar P, George J, Ram LC. 2013. Biochar from water hyacinth (Eichornia crassipes) and its impact on soil biological activity. Catena **111**:64–71.

Meynet P, Hale SE, Davenport RJ, Cornelissen G, Breedveld GD, Werner D. 2012. Effect of activated carbon amendment on bacterial community structure and functions in a PAH impacted urban soil. Environ Sci Technol **46**:5057–5066.

Obia A, Mulder J, Martinsen V, Cornelissen G, Børresen T. 2016. In situ effects of biochar on aggregation, water retention and porosity in light-textured tropical soils. Soil and Tillage Research **155**:35–44.

Ouyang L, Wang F, Tang J, Yu L, Zhang R. 2013. Effects of biochar amendment on soil aggregates and hydraulic properties. Journal of soil science and plant nutrition **13**:991–1002.

Parham JA, Deng SP. 2000. Detection, quantification and characterization of β-glucosaminidase activity in soil. Soil Biology and Biochemistry **32**:1183–1190.

Smith JL, Collins HP, Bailey VL. 2010. The effect of young biochar on soil respiration. Soil Biology and Biochemistry **42**:2345–2347.

Sohi SP, Krull E, Lopez-Capel E, Bol R. 2010. A review of biochar and its use and function in soil. Page Advances in Agronomy, 1st edition. Elsevier Inc.

Song D, Xi X, Huang S, Liang G, Sun J, Zhou W, Wang X. 2016. Short-Term Responses of Soil Respiration and C-Cycle Enzyme Activities to Additions of Biochar and Urea in a Calcareous Soil. PloS one 11:e0161694.

Strobl W, Traunmueller M. 1996. β-Glucosidase activity. Pages 198–200in F. Schinner, R. Öhlinger, E. Kandeler, and R. Margesin, editors.Methods in Soil Biology. Springer Berlin Heidelberg, Berlin, Heidelberg.

Tabatabai MA, Bremner JM. 1969. Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biology and Biochemistry **1**:301–307.

Tabatabai VAMA. 2000. Enzyme activities in a limed agricultural soil:85–91.

Teutscherova N, Vazquez E, Masaguer A, Navas M, Scow KM, Schmidt R, Benito M. 2017a. Comparison of lime- and biochar-mediated pH changes in nitrification and ammonia oxidizers in degraded acid soil. Biology and Fertility of Soils **53**:811–821.

Teutscherova N, Vazquez E, Santana D, Navas M, Masaguer A, Benito M. 2017b. Influence of pruning waste compost maturity and biochar on carbon dynamics in acid soil: Incubation study. European Journal of Soil Biology **78**:66–74.

Thies JE, Rillig MC, Graber ER. 2015. Biochar effects on the abundance , activity and diversity of the soil biota. Pages 327–389 in J. Lehmann and S. Joseph, editors.Biochar for environmental management: science, technology and implementation 2nd edition.

Thomas GW. 1982. Exchangeable cations. Methods of soil analysis. Part 2. Chemical and microbiological properties **9**:159–165.

Uchimiya M, Chang S, Klasson KT. 2011a. Screening biochars for heavy metal retention in soil: Role of oxygen functional groups. Journal of Hazardous Materials **190**:432–441.

Uchimiya M, Wartelle LH, Klasson KT, Fortier CA, Lima IM. 2011b. Influence of pyrolysis temperature on biochar property and function as a heavy metal sorbent in soil. Journal of Agricultural and Food Chemistry **59**:2501–2510.

Vance ED, Brookes PC, Jenkinson DS. 1987. An extraction method for measuring soil microbial biomass C. Soil Biology and Biochemistry **19:**703–707.

Vazquez E, Teutscherova N, Almorox J, Navas M, Espejo R, Benito M. 2017. Seasonal variation of microbial activity as affected by tillage practice and sugar beet foam amendment under Mediterranean climate. Applied Soil Ecology **117–118**:70–80.

Ventura M, Zhang C, Baldi E, Fornasier F, Sorrenti G, Panzacchi P, Tonon G. 2014. Effect of biochar addition on soil respiration partitioning and root dynamics in an apple orchard. European Journal of Soil Science **65**:186–195.

Verheye W, de la Rosa D. 2005. Mediterranean soils. Encyclopedia of Life Support Systems: 1–26.

Wang J, Xiong Z, Kuzyakov Y. 2015a. Biochar stability in soil: meta-analysis of decomposition and priming effects. GCB Bioenergy **8**:512-523.

Wang X, Song D, Liang G, Zhang Q, Ai C, Zhou W. 2015b. Maize biochar addition rate influences soil enzyme activity and microbial community composition in a fluvo-aquic soil. Applied Soil Ecology **96:**265–272.

Xu N, Tan G, Wang H, Gai X. 2016. Effect of biochar additions to soil on nitrogen leaching, microbial biomass and bacterial community structure. European Journal of Soil Biology:1–8.

Yoo G, Kang H. 2012. Effects of Biochar Addition on Greenhouse Gas Emissions and Microbial Responses in a Short-Term Laboratory Experiment. Journal of Environment Quality **41**:1193.

Zheng J, Chen J, Pan G, Liu X, Zhang X, Li L, Bian R, Cheng K, Jinwei Z. 2016. Biochar decreased microbial metabolic quotient and shifted community composition four years after a single incorporation in a slightly acid rice paddy from southwest China. Science of the Total Environment **571**:206–217.

Zimmerman AR, Gao B, Ahn M-Y. 2011. Positive and negative carbon mineralization priming effects among a variety of biochar-amended soils. Soil Biology and Biochemistry **43**:1169–1179.

10 PLANT GROWTH IN BIOCHAR-AMENDED SOILS

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Nikola Teutscherova contributed by establishing the experimental design, analytical and statistical analysis and manuscript preparation

10.1 Introduction

Based on the high and long-lasting productivity of *Terra preta* soils, the use of pyrolyzed organic matter, biochar, for soil fertility improvement and crop production stimulation has been in the focus of wide public and scientific attention. Until now, many studies have demonstrated the improvement of plant growth and enhanced yield of agricultural crops (Graber et al. 2010; Uzoma et al. 2011; Alburquerque et al. 2013; Carter et al. 2013; Lai et al. 2013; Thomas et al. 2013) after biochar application. Nevertheless, the effect of biochar on plants cannot be generalized as it depends on many interrelated factors, including biochar type (variable according to pyrolysis temperature and feedstock material) soil type, climate and management practices (Jeffery et al. 2011; Biederman & Harpole 2013).

Although some biochar may at least partly substitute organic fertilizers (Glaser et al. 2015) due to their elevated nutrient contents, the capacity of biochar to improve plant growth generally lies in the improvement of soil physical (Novak et al. 2012) and/or chemical properties (van Zwieten et al. 2010; Oleszczuk et al. 2014). Liming effect of biochar has been identified to be the key mechanism behind the biochar positive effect on plant growth in the acidic soils (Jeffery et al. 2011).

The degraded soils of Cañamero's raña have been acidified due to the loss of soil organic matter (SOM) as a consequence of excessive tillage (Mariscal et al. 2007) and related release of organically bound aluminum (Al) from the organo-mineral complexes. During the recent years, the attempts to restore large areas of raña included the adoption of no-tillage agriculture in order to elevate the SOM content (Goméz-Paccard et al. 2013; Vazquez et al. 2017), and the application of liming materials, such as sugar foam residues in combination with red gypsum (Goméz-Paccard et al. 2013; Vazquez et al. 2017) or biochar (Teutscherova et al. 2017a, 2017b). The application holm oak biochar proceeding from pruning waste to the acidic Acrisol from Cañamero's raña resulted to affect the nitrogen (N) cycling (Teutscherova et al. 2017a) influenced the sorption capacity of soil (Teutscherova et al. 2018a) and stimulated the microbial activity (Teutscherova et al. 2018b), which was partly attributed to the pH increase and partly to the inherent biochar properties (Teutscherova et al. 2017a). In the study of Teutscherova et al. (2018a), the authors found enhanced leaching of ammonium after the application of the same biochar and concluded that the neutralization of soil acidity caused stimulation of SOM mineralization. In another study, Teutscherova et al. (2017a) found that the soil pH neutralization did not affect the nitrification immediately, indicating that ammonia oxidation was limited by other factor such as low activity of ammonia oxidizers.

Nevertheless, comparable biochar application rate resulted in stimulation of soil basal respiration and growth of microbial biomass carbon (MBC) but decreased substrate-induced respiration (SIR). Thus, biochar likely affected the C utilization preferences in the studied soil which could affect the availability of other soil nutrients.

When the same biochar was applied to alkaline Calcisol, the effect on microbial biomass and activity was the opposite and total MBC was reduced, SIR increased and soil basal respiration was related mainly to the availability of N (Teutscherova et al. 2018b), probably as a consequence of labile C input within biochar. The microbial immobilization of N after C application was hypothesize to reduce N leaching losses Teutscherova et al. (2018a), which was not observed and biochar did not influence leaching of nitrate from Calcisol.

As plant growth promotion after biochar application is a function of many biochar-induced changes in soil, plant growth could be enhanced even if no effect on nutrient cycling or soil pH is detected, as long as other plant growth limitations are overcome by biochar amendment. In a loamy Calcisol, it could be enhanced aggregation and related increased drainage and aeration (Mukherjee et al. 2014; Alburquerque et al. 2014). As soil aggregation has been observed to be enhanced in both Acrisol and Calcisol under aerobic conditions, plant growth could be stimulated in both soil types: in Acrisol due to the reduction of soil acidity, and in Calcisol due to improved soil physical properties.

Therefore, this chapter focuses on the effect of holm oak biochar on aboveground biomass production of three plant species (lettuce, bean and ryegrass) in a greenhouse experiment. In order to correlate the obtained growth data with previously published results of microbial activity, same treatments were established and the pots were maintained under identical conditions as the greenhouse experiment of Teutscherova et al. (2018b).

10.2 Materials and methods

10.2.1 Soils and biochar characterization

Both acid degraded Acrisol and loamy alkaline Calcisol were used for the present study. The properties of both soil types and used biochar can be seen in table 4-1 (Materials and mehods chapter).

10.2.2 Experimental design

Pot experiment with ryegrass (*Lolium perenne* L.), lettuce (*Lactuca sativa* L.) and common bean (*Phaseolus vulgaris* L.) were established in a controlled greenhouse simultaneously with the experiment described in Chapter 4 using the same treatments. Plants were grown in a greenhouse with controlled conditions (20-30 °C) at the Polytecnical University of Madrid. Studied factors were: (i) soil type (Acrisol or Calcisol); (ii) biochar application rate (0%, 1% or 2%) and fertilization (with or without NPK), all with four repetitions. In total there were 144 pots. All plants were water on regular basis (every 1-2 days) with deionized water.

Lettuce: 10 seeds of *L. sativa* L. were sown in plastic pots (10 cm diameter, 10 cm height) in 500 grams of soil. Lettuces plants were harvested after 12 weeks of growth for determination of the production of aboveground biomass.

Ryegrass: Seven grams of *L.perenne* L. seeds were sown in plastic pots (10 cm diameter, 10 cm height) in 500 grams of soil. The aboveground biomass of ryegrass was cut after three weeks, nine weeks and 15 weeks to determine the cumulative aboveground biomass production.

Bean: Four seeds of *P.vulgaris* L. were sown in pots (15 cm diameter, 20 cm height) in two kg of soil. Bean plants were harvested after 12 weeks of growth for determination of the production of aboveground biomass.

10.2.3 Statistical analysis

The data of total produced biomass of all three species were analyzed in SPSS software using general linear model (GLM) with soil type (S), biochar application rate (B) and fertilization (F) as fixed factors. Additionally, ryegrass biomass production was analyzed with general mixed model (GMM) with time (T) as an additional fixed factor.

10.3 Results and discussion

The growth and aboveground biomass production of the selected plant species responded differently to studied factors. While biochar application explained 16.5% and 26% of data variability of lettuce and ryegrass growth, respectively, no effect of biochar was observed in the growth of leguminous bean plants (Table 10-1; Fig. 10-1, Fig. 10-2, Table 10-2). Similarly, the application of fertilizer explained more than 50% of lettuce growth variability (Table 10-1) and 34.5% of bean biomass variability, but only 8% of bean grown,

where more than 65% of aboveground biomass variability remained unexplained by the studied factors. Despite the contrasting properties of selected soils, soil type had no impact on aboveground biomass production during this experiment.

Plant response to biochar application and fertilization varied between soil types and plant species. While fertilization explained 52% ad 34% of data variability of the growth of lettuce and ryegrass when the aboveground biomass production was enhanced by fertilizer, no effect was observed in case of bean plants. Furthermore, while in Acrisol the application of NPK without biochar had strong effect on lettuce growth, in Calcisol, the growth of lettuce was not improved and fertilizer incrased lettuce growth only in the presence of biochar. These results indicate that in alkaline Calcisol plant growth was restrained by other factors besides nutrient availability and applied mineral fertilizer could be efficiently used by lettuce plants only when there limitations were reduced by the presence of biochar. Synergistic functioning of applied biochar and inorganic fertilizer has been observed by several authors (Shamim et al. 2015) and is believed to be a promising strategy to reduce the risks of possible growth reduction induced by biochar application to soil (Spokas et al. 2011). On the other hand, in the present study, biochar did not reduce plant growth of none of the selected plant species, which indicates that N immobilization resulting from labile C input did not limit plant nutrient uptake or that increased N mineralization (Teutscherova et al. 2017a; 2018a) more than compensated increased microbial growth (Teutscherova et al. 2018b) with a nutrient supply sufficient for both plant roots and soil microbes. In Acrisol, the lettuce growth was explained by EC and EOC, which can suggest that plant growth in this degraded soil was limited by insufficient nutrient supply and the biomass production was incrased by direct addition of nutrients contained in biochar. The crop production on acid Acrisol from SW Spain is limited by high acidity linked with low base cations contents, thus, the base cations contained in the ash fraction of biochar could directly stimulate plant growth. The aboveground biomass production of bean plants was explained only in 8% by fertilization, which indicates that bean N nutrition could be covered by N fixation by symbiotic bacteria. Biochar or biochar-fertilizer interaction did not affect bean growth.

Repeated monitoring of the biomass production of ryegrass revealed significant effect of time, which explained 62% of ryegrass biomass variability (Table 10-2).The second harvest of aboveground biomass was significantly lower when compared to the last harvest, despite the same time between harvests (Fig. 10-2). Nevertheless, both biochar application and fertilization increased plant growth (Table 10-2).

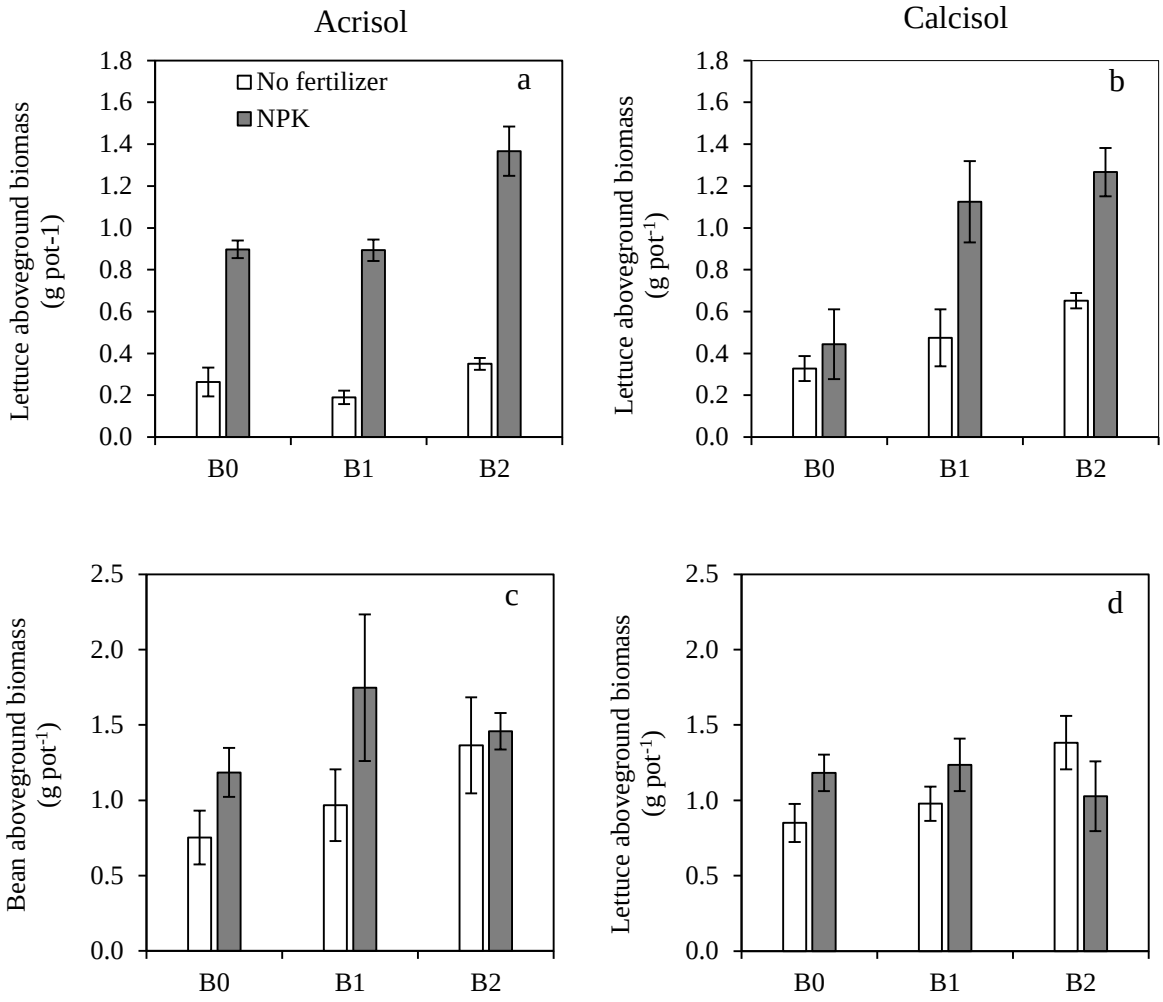

Figure 10-1: Aboveground biomass production of lettuce (*Lactuca sativa* L.) (a, b) and cmmon bean (*Phaseolus vulgaris* L.) (c, d) grown in Acrisol (a, c) and Calcisol (b, d) after three months of pot experiment.

Table10-1: The effects of soil type (S), biochar application rate (B) and fertilization (F) on the cumulative aboveground biomass production during the experiment

	Lettuce	Bean	Ryegrass
	η^2 (p-value)		
S	0.40 (n.s.)	2.89 (n.s.)	1.79 (n.s.)
B	16.47 (***)	9.86 (n.s.)	25.96 (***)
F	52.28 (***)	8.32 (*)	34.62 (***)
S x B	4.78 (*)	0.66 (n.s.)	5.45 (*)
S x F	3.56 (**)	1.99 (n.s.)	0.58 (n.s.)
B x F	4.58 (*)	8.76 (n.s.)	6.79 (*)
S x B x F	1.33 (n.s.)	1.96 (n.s.)	0.02 (n.s.)
Error	16.60	65.56	24.78

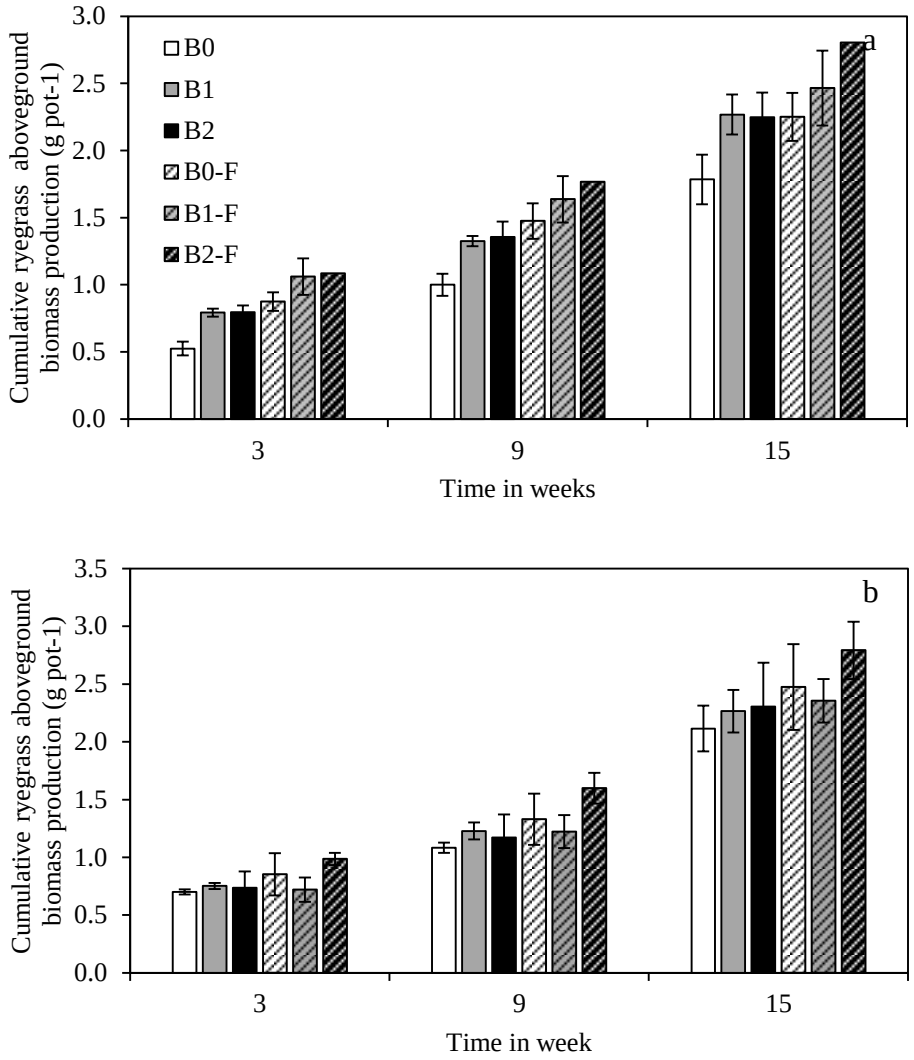

Figure 10-2: Cumulative aboveground biomass production of ryegrass (*Lolium perenne* L.) grown in Acrisol (a) and Calcisol (b) harvested after three, nine and 15 weeks of greenhouse pot experiment.

Stepwise regression analysis identified the key fators affecting the plant growth in Acrisol a MBC, EC, ammonium content, SIR, phosphatase ad EOC (ryegrass), EC and EOC (lettuce) and pH and ammonium content (bean). In Calcisol, the ryegrass was explained by EC, BR, EOC and urease, the lettuce growth by MBC and bean growth by the activity of β-glucosidase.

Table 10-2: The effect of soil type (S), biochar application rate (B), fertilization (F) and time (T) on the aboveground biomass production of ryegrass

	η^2 (p-value)
S	0.31 (n.s.)
B	4.45 (***)
F	5.93 (***)
T	61.97 (***)
S x B	0.93 (*)
S x F	0.10 (n.s.)
S x T	9.11 (***)
B x F	1.16 (**)
B x T	0.29 (n.s.)
F x T	1.97 (***)
S x B x F	0.00 (n.s.)
S x B x T	1.12 (*)
S x F x T	1.24 (**)
B x F x T	0.07 (n.s.)
S x B x F x T	0.97 (*)
Error	10.37

10.4 References

Alburquerque JA, Calero JM, Barrón V, Torrent J, del Campillo MC, Gallardo A, Villar R. 2014. Effects of biochars produced from different feedstocks on soil properties and sunflower growth. Journal of Plant Nutrition and Soil Science **177**:16–25.

Alburquerque JA, Salazar P, Barr??n V, Torrent J, Del Campillo MDC, Gallardo A, Villar R. 2013. Enhanced wheat yield by biochar addition under different mineral fertilization levels. Agronomy for Sustainable Development **33**:475–484.

Biederman LA, Harpole WS. 2013. Biochar and its effects on plant productivity and nutrient cycling: A meta-analysis. GCB Bioenergy **5**:202–214.

Carter S, Shackley S, Sohi S, Suy T, Haefele S. 2013. The Impact of Biochar Application on Soil Properties and Plant Growth of Pot Grown Lettuce (Lactuca sativa) and Cabbage (Brassica chinensis). Agronomy **3**:404–418.

Glaser B, Wiedner K, Seelig S, Schmidt HP, Gerber H. 2015. Biochar organic fertilizers from natural resources as substitute for mineral fertilizers. Agronomy for Sustainable Development **35**:667–678.

Gómez-Paccard C, Mariscal-Sancho I, León P, Benito M, González P, Ordóñez R, Espejo R, Hontoria C. 2013b. Ca-amendment and tillage: Medium term synergies for improving key soil properties of acid soils. Soil and Tillage Research **134**:195–206.

Graber ER, Harel YM, Kolton M, Cytryn E, Silber A, David DR, Tsechansky L, Borenshtein M, Elad Y. 2010. Biochar impact on development and productivity of pepper and tomato grown in fertigated soilless media. Plant and Soil **337**:481–496.

Jeffery S, Verheijen FGA, van der Velde M, Bastos AC. 2011. A quantitative review of the effects of biochar application to soils on crop productivity using meta-analysis. Agriculture, Ecosystems & Environment **144:** 175-187.

Lai WY, Lai CM, Ke GR, Chung RS, Chen CT, Cheng CH, Pai CW, Chen SY, Chen CC. 2013. The effects of woodchip biochar application on crop yield, carbon sequestration and greenhouse gas emissions from soils planted with rice or leaf beet. Journal of the Taiwan Institute of Chemical Engineers **44**:1039–1044.

Mariscal I, Peregrina F, Terefe T, González P, Espejo R. 2007. Evolution of some physical properties related to soil quality in the degraded ecosystems of "raña" formations from SW Spain. The Science of the total environment **378**:130–2.

Mukherjee A, Lal R, Zimmerman AR. 2014. Effects of biochar and other amendments on the physical properties and greenhouse gas emissions of an artificially degraded soil. Science of the Total Environment **487**:26–36.

Novak JM et al. 2012. Biochars impact on soil-moisture storage in an ultisol and two aridisols. Soil Science 177:310–320.

Oleszczuk P, Jo??ko I, Ku??mierz M, Futa B, Wielgosz E, Ligeza S, Pranagal J. 2014. Microbiological, biochemical and ecotoxicological evaluation of soils in the area of biochar production in relation to polycyclic aromatic hydrocarbon content. Geoderma **213**:502–511.

Shamim MIA, Dijkstra FA, Abuyusuf M, Hossain AI. 2015. Synergistic effects of biochar and NPK fertilizer on soybean in an alkaline soil. Pedopshere **25**: 713-719.

Spokas K, Cantrell K, Novak JM, Archer DW, Ippolito JA, Collins HP, Boateng AA, Lima IM, Lamb MC, McAloon AJ, Lentz RD, Nichols KA. 2011. Biochar: A synthesis of its agronomic impact beyond carbon sequestration. Journal of environmental quality **41:**973-989.

Teutscherova N, Houška J, Navas M, Masaguer A, Benito M, Vazquez E. 2018a. Leaching of ammonium and nitrate from Acrisol and Calcisol amended with holm oak biochar: A column study. Geoderma **323**:136–145.

Teutscherova N, Lojka B, Houška J, Masaguer A, Benito M, Vazquez E. 2018b. Application of holm oak biochar alters dynamics of enzymatic and microbial activity in two contrasting Mediterranean soils. European Journal of Soil Biology **88**:15–26.

Teutscherova N, Vazquez E, Masaguer A, Navas M, Scow KM, Schmidt R, Benito M. 2017a. Comparison of lime- and biochar-mediated pH changes in nitrification and ammonia oxidizers in degraded acid soil. Biology and Fertility of Soils **53**:811–821.

Teutscherova N, Vazquez E, Santana D, Navas M, Masaguer A, Benito M. 2017b. Influence of pruning waste compost maturity and biochar on carbon dynamics in acid soil: Incubation study. European Journal of Soil Biology **78**:66–74.

Thomas SC, Frye S, Gale N, Garmon M, Launchbury R, Machado N, Melamed S, Murray J, Petroff A, Winsborough C. 2013. Biochar mitigates negative effects of salt additions on two herbaceous plant species. Journal of Environmental Management **129**:62–68.

Uzoma KC, Inoue M, Andry H, Fujimaki H, Zahoor A, Nishihara E. 2011. Effect of cow manure biochar on maize productivity under sandy soil condition. Soil Use and Management **27**:205–212.

van Zwieten L, Kimber S, Morris S, Chan KY, Downie A, Rust J, Joseph S, Cowie A. 2010. Effects of biochar from slow pyrolysis of papermill waste on agronomic performance and soil fertility. Plant and Soil **327**:235–246.

Vazquez E, Teutscherova N, Almorox J, Navas M, Espejo R, Benito M. 2017. Seasonal variation of microbial activity as affected by tillage practice and sugar beet foam amendment under Mediterranean climate. Applied Soil Ecology **117–118**:70–80.

Table 10-3: The main factors explaining the variability of crop growth in both soils revelease by stepwise regressions analysis.

Acrisol									
Ryegrass	0.076	+0.001MBC	+0.002EC	+0.005NH_4^+	-0.003SIR	+0.001PHase	+0.001EOC	R^2=0.71	p<0.001
Lettuce	-1.681	+0.011EC	0.006EOC					R^2=0.84	p<0.001
Bean	-1.474	+0.407pH	+0.013NH_4^+					R^2=0.33	p<0.05
Calcisol									
Ryegrass	-0.357	+0.001EC	+0.015BR	+0.002EOC	+0.507Urease			R^2=0.46	p<0.001
Lettuce	1.072	-0.003MBC						R^2=0.42	p<0.001
Bean	0.579	+0.013Glc						R^2=0.22	p<0.05

MBC, microbial biomass carbon; EC, electric conductivity; SIR, substrate-induced respiration; PHase, the activity of soil phosphomonoesterasel EOC, extractable organic carbon; BR, basal respiration; Glc, the activity of soil β-glucosidase

11 DISCUSSION AND CONCLUSIONS

The impact of biochar application on soil properties was evaluated in a series of experiments targeting C and N mineralization and their implications for nutrient cycling as well as the impact of biochar on three selected plant species. The impact of biochar on nutrient cycling was studied in two Mediterranean soils with contrasting properties, such as texture, pH and SOM content. Soil type has been identified to play the key role in the determination of biochar impact on nutrient (N in particular) cycling and nutrient leaching losses.

11.1 Biochar effects on nutrients dynamics in Acrisol

Soil degradation and acidification has becoming a serious concern worldwide and especially under Mediterranean climate, a suitable strategy to combat SOM losses and increase crop production is urgently needed. The application of biochar to acid degraded Acrisol from SW Spain (Cañamero's raña) resulted in an immediate increase of SOC content and soil pH which influenced the majority of biological indicators evaluated in this study.

The application of alkaline hard wood biochar produced at high temperature (600 °C) as well as alkaline pruning compost resulted in a reduction of microbial biomass (obtained as SIR) in an incubation study (Chapter 5) which could be explained by the immediate changes in the microbial community structure after rapid pH change. Similar trend was observed also in the greenhouse experiment (Chapter 9) where SIR was reduced but MBC (obtained by fumigation-extraction method) was increased. These seemingly conflicting results indicate that biochar may impact the microbial C utilization capacity and part of the microbial biomass grown after biochar application to soil does not use glucose as its primary energy source.

While the application of biochar to soil did not affect soil basal respiration, the CO_2 emissions were increased when biochar was co-applied with immature compost and this increase was directly correlated with WSC content (Chapter 5). Thus, we could conclud that such synergistic functioning between immature compost with high C:N ratio and high WSC, and biochar could have caused the solubilization of organic compounds and resulted

in positive priming effect on SOM which, however, was not seen in the case of stabilized organic matter in the form of mature pruning waste compost. Soil basal respiration in Acrisol seemed to be determined by the availability of labile C fractions, the activity of dehydrogenase and the neutralization of soil pH (Chapter 9) at least during the first three months after biochar application to soil in a greenhouse experiment (Chapter 9).

Similaly, positive effect of soil neutralization was also observed in the activity of soil dehydrogenase, intracellular enzyme present in active soil microorganisms, which supports the hypothesis that the increased of soil pH will lead to enhanced microbial activity in degraded acid soil.

Soil pH has also been observed to play a key role in N transformation processes in soil (Chapter 6). The improvement of soil properties will result in the alleviation of microbial stress which can be manifested by increased mineralization of SOM and linked potential losses of nutrients (Chapter 7), which could be the case especially in soils with low CEC as observed in the kaolinic Acrisol used in this thesis. The stimulation of microbial activity by pH neutralization together with high content of SOC in Acrisol resulted in rapid mineralization of labile N compounds and leaching of NH_4^+, which, however, seemed to be rather short-lived as biochar had an impact on N leaching only during the initial few weeks of the experiment. After this time, the amount of potentially mineralizable N was likely exhausted.

The separation of the inherent effect of biochar on N cycling from the effect of the changes in soil pH after biochar application has been challenging (Chapter 6). The evaluation of net nitrogen mineralization rates and net nitrification revealed that the increase of pH and application of substrate for soil nitrifiers did not influence the rate of ammonium oxidation and that nitrification rates were limited by other factors besides soil pH and substrate supply. Possibly, soil nitrifiers were inactive in the studied soil and time is required for their activation. Although no changes were observed between lime and biochar application (applied at application rates in order to increase soil pH to a same value) in nitrification, we observed stronger effect of biochar on the growth of AOB, which seemed to be more adaptive to a new environment formed by biochar. Furthermore, AOB abundance was correlated with nitrification rates, which may lead to enhanced nitrification rates in long-term after biochar application when compared to lime.

Overall, the microbial activity of Acrisol was stimulated by biochar amendment and this increment was likely related to pH neutralization and amelioration to soil acidity-related stress and to labile C content either released from biochar itself or resulting from decomposition of organic matter. Nevertheless, while enhanced biological activity lead to improved soil health and related soil structure building, at least short-term negative impacts may be related to increased mineralization of SOM and decoupling of ammonification and nitrification, which

may be manifested by increased NH_4^+ leaching, especially in soil with low CEC. Nevertheless, biochar-related changes in soil properties improved the growth of all three plant species, which indicated that in the presence of plant roots, the rapid liberation of nutrients from organic matter may be taken up by plants. Thus, careful planning and timing of biochar application to soil may overcome the potential short-term negative impacts and environmental drawback related to biochar application to acid soil.

11.2 Biochar effects on nutrients dynamics in Calcisol

The pH of Calcisol was comparable to pH of biochar and therefore, the changes in biological activity were expected to be caused by changes of substrate availability rather than by changes in soil pH. The TOC content was significantly increased by biochar application to Calcisol while limited influence of biochar was detected in N cycling. These results indicate that the application of biochar to C-poor alkaline soils can be a useful tool to combat soil degradation and SOC losses. We observed that soil respiration of Calcisol seemed to be related more to the N content, rather than to C supply (Chapter 9) as soil basal respiration was explained the best by mineral N content and the activity of urease.

Calcisol used in the present thesis had high CEC which was not further increased by the application of biochar. Furthermore, no NH_4^+ was detected in the leachate of Calcisol which could indicate strong NH_4^+ retention or high nitrification rates, which were not affected by biochar application rate as no difference in total leached NO_3^- was observed between studied treatments.

Nevertheless, unlike acid Acrisol where the majority of studied parameters was directly correlated with biochar application rate, the growth of fertilized ryegrass plants was improved at higher biochar application rate and slightly diminished at lower rate, which could be attributed to a short-term immobilization of N which is in line with the regression analysis revealing the importance of N in the microbial activity of biochar-amended Calcisol.

11.3 The implications of presented results

11.3.1 Similarity to Amazonian dark earths

Although biochar is generally believed to be the essential ingredient of Amazonian dark earths, amending soil with only biochar is unlikely to results in soils of such properties as those created by pre-columbian populations. Biochar serves as soil conditioner with may functions being narrowed down to (i) increased SOC content; (ii) increased sorption capacity;

(iii) modification of soil pH and nutrient availability; (iv) improvement of soil physical properties. Clearly, the improvement of soil physical properties usually leads to the improvement of crop growth, especially in soils where the growth is limited by excessive soil compaction, poor aeration or water-logging. Nevertheless, the largest effects of biochar application to soil will likely be observed when biochar is co-applied with fertilizer. After the application of nutrients to soil, biochar serves as a sponge adsorbing nutrients from soil solution and therefore preveing their losses. Similarly, the addition of biochar to soil, usually linked with increased biological activity in soil, can be benefitial especially when organic material is applied to soil along with biochar. In such case, the stimulation of organic matter decomposition leads to faster nutrient turnover and release of nutrients available for plants uptake. In turn, improved plant growth leads to higher organic matter production and further stimulation of the nutrient transformations, similarly as observed in Amazonian dark earths.

Although the application of biochar to soil does not lead to Amazonian dark earths formation, all aspects of biochar-soil-plant-environment interactions should be carefuly evaluated before large scale biochar implementation.

11.3.2 Feasibility of biochar use

Despite its potential benefits on soil properties and productivity, the main economical interest in biochar may lay in the fact that biochar contains stable C which persists in soil for hundreds or up to thousands of years, which has implications for climate changes mitigations strategies. Nevertheless, there are still uncertainties about biochars impact on soil quality in the long-term due to low amount of long-term biochar trials. Furthermore, large-scale biochar adoption is limited by insufficient amount of commercial biochar avaialable to the farmers and linked possible pollution originating from biochar production (Kato et al. 2004). Furthermore, in many areas the land-application of biochar remains restricted.

On the other hand, many areas in both developed and developing countries, face a serious problem with high production of waste products which largely remain without possible utilization. This is also the case of extensive areas in Spain, which are covered with perennial crops (olives, holm oaks, pines) due to harsh environmental conditions which do not allow annual crops to be economically feasible. Prunings and other residues from silvopastoral or tree-based agricultural practices are often burn on the field. Localized recycling of such materials, either composting, conversion to biochar or both, could have a beneficial impact on soil properties and plant growth in the area.

11.3.3 Final remarks

The separation of the study in the specific experiments and the extensive evalution of the effects of biochar on different soil parameters increased the robustness of the obtained results and improved our understanding of biochar-induced changes in biological activity and nutrient cycling in some Mediterranean soils. The obtained results indicate that biochar substantially contributed to the enhancement of SOC content and soil structure formation, regardless of the soil type. Furthermore, the plant growth is enhanced in both soil types, which can be explained by increased SOM mineralization (Acrisol) or to other factors (Calcisol) which could be the improvement of soil aeration or addition of nutrients contained in the biochar.

In conclusion, biochar originating from holm oak pruning waste resulted to be a useful strategy to improve crop production of Mediterranean soils. Nevertheless, the co-application of fertilizers, the timing of biochar application and biochar application rate all need to be carefully evaluated before the implementation of biochar as a soil conditioner in order to avoid possible negative impacts on crop growth or the environment.

12 REFERENCES

Akça MO, Namlı A. 2015. Effects of poultry litter biochar on soil enzyme activities and tomato , pepper and lettuce plants growth **4**:1–8.

Ameloot N, Sleutel S, Das KC, Kanagaratnam J, de Neve S. 2015. Biochar amendment to soils with contrasting organic matter level: Effects on N mineralization and biological soil properties. GCB Bioenergy **7**:135–144.

Amézketa E. 1999. Soil Aggregate Stability: A Review. Journal of Sustainable Agriculture **14**:83–151.

Annabi M, Le Bissonnais Y, Le Villio-Poitrenaud M, Houot S. 2011. Improvement of soil aggregate stability by repeated applications of organic amendments to a cultivated silty loam soil. Agriculture, Ecosystems and Environment **144**:382–389.

Asai H, Samson BK, Stephan HM, Songyikhangsuthor K, Homma K, Kiyono Y, Inoue Y, Shiraiwa T, Horie T. 2009. Biochar amendment techniques for upland rice production in Northern Laos. 1. Soil physical properties, leaf SPAD and grain yield. Field Crops Research **111**:81–84.

Atkinson CJ, Fitzgerald JD, Hipps NA. 2010. Potential mechanisms for achieving agricultural benefits from biochar application to temperate soils: A review. Plant and Soil **337**:1–18.

Bai SH, Reverchon F, Xu CY, Xu Z, Blumfield TJ, Zhao H, Van Zwieten L, Wallace HM. 2015. Wood biochar increases nitrogen retention in field settings mainly through abiotic processes. Soil Biology and Biochemistry **90**:232–240.

Biederman LA, Harpole WS. 2013. Biochar and its effects on plant productivity and nutrient cycling: A meta-analysis. GCB Bioenergy **5**:202–214.

Birk JJ, Steiner C, Teixiera WC, Zech W, Glaser B. 2009. Microbial response to charcoal amendments and fertilization of a highly weathered tropical soil. Pages 309–324 Amazonian Dark Earths: Wim Sombroek's Vision.

Castracani C, Maienza A, Grasso DA, Genesio L, Malcevschi A, Miglietta F, Vaccari FP, Mori A. 2015. Biochar-macrofauna interplay: Searching for new bioindicators. Science of the Total Environment **536**:449–456.

Cayuela ML, Sanchez-Monedero MA, Roig A, Hanley K, Enders A, Lehmann J. 2013. Biochar and denitrification in soils: when, how much and why does biochar reduce N(2)O emissions? Scientific reports **3**:1732.

Chan KY, Van Zwieten L, Meszaros I, Downie A, Joseph S. 2007. Agronomic values of greenwaste biochar as a soil amendment. Australian Journal of Soil Research **45**:629–634.

Chan KY, Van Zwieten L, Meszaros I, Downie A, Joseph S. 2008. Using poultry litter biochars as soil amendments. Australian Journal of Soil Research **46**:437–444.

Chang AC, Lund LJ, Page AL, Warneke JE. 1997. Physical Properties of Fly Ash-Amended Soils. Journal of Environmental Quality **6**:267–270.

Che J, Zhao XQ, Zhou X, Jia ZJ, Shen RF. 2015. High pH-enhanced soil nitrification was associated with ammonia-oxidizing bacteria rather than archaea in acidic soils. Applied

Soil Ecology **85**:21–29.

Christianson L, Hedley M, Camps M, Free H, Saggar S. 2010. Influence of Biochar Amendments on Denitrification. Methods:1–8.

Clough T, Condron L, Kammann C, Müller C. 2013. A Review of Biochar and Soil Nitrogen Dynamics. Agronomy **3**:275–293.

Deenik JL, McClellan T, Uehara G, Antal MJ, Campbell S. 2010. Charcoal Volatile Matter Content Influences Plant Growth and Soil Nitrogen Transformations. Soil Science Society of America Journal **74**:1259.

DeLuca TH, MacKenzie MD, Gundale MJ, Holben WE. 2006. Wildfire-Produced Charcoal Directly Influences Nitrogen Cycling in Ponderosa Pine Forests. Soil Science Society of America Journal **70**:448.

Dempster DN, Gleeson DB, Solaiman ZM, Jones DL, Murphy D V. 2011. Decreased soil microbial biomass and nitrogen mineralisation with Eucalyptus biochar addition to a coarse textured soil. Plant and Soil **354**:311–324.

Ding Y, Liu YX, Wu WX, Shi DZ, Yang M, Zhong ZK. 2010. Evaluation of biochar effects on nitrogen retention and leaching in multi-layered soil columns. Water, Air, and Soil Pollution **213**:47–55.

Dirksen C. 1999. Soil physics measurement. Catena Verlag.

Espejo R. 1987. The soils and ages of the "raña" surfaces related to the Villuercas and Altamira mountain ranges (Western Spain). CATENA **14**:399–418.

Etiégni L, Campbell AG. 1991. Physical and chemical characteristics of wood ash. Bioresource Technology **37**:173–178.

Ezawa T, Yamamoto K, Yoshida S. 2002. Enhancement of the effectiveness of indigenous arbuscular mycorrhizal fungi by inorganic soil amendments. Soil Science and Plant Nutrition **48**:897–900.

Fairhead J, Leach M. 2009. Amazonian dark earths in Africa? Amazonian Dark Earths: Wim Sombroek's Vision:265–278.

Gai X, Wang H, Liu J, Zhai L, Liu S, Ren T, Liu H. 2014. Effects of feedstock and pyrolysis temperature on biochar adsorption of ammonium and nitrate. PLoS ONE **9**:1–19.

Gaskin JW, Steiner C, Harris K, Das KC, Bibens B. 2008. Effect of Low-Temperature Pyrolysis Conditions on Biochar for Agricultural Use. Transactions of the ASABE **51**:2061–2069.

Gaur A, Adholeya A. 2000. Effects of the particle size of soil-less substrates upon AM fungus inoculum production. Mycorrhiza **10**:43–48.

German LA. 2004. Ecological praxis and blackwater ecosystems: A case study from the Brazilian Amazon. Human Ecology **32**:653–683.

Gianfreda L, Ruggiero P. 2006. Enzyme activities in soil. Pages 257–311 in P. Nannipieri and K. Smalla, editors. Nucleic acids and proteins in soil. Soil Biology. Springer Berlin Heidelberg.

Glaser B, Birk JJ. 2012. State of the scientific knowledge on properties and genesis of Anthropogenic Dark Earths in Central Amazonia (terra preta de ??ndio). Geochimica et Cosmochimica Acta **82**:39–51.

Glaser B, Zech W, Woods WI. 2004. History, Current Knowledge and Future Perspectives of Geoecological Research Concerning the Origin of Amazonian Anthropogenic Dark Earths (Terra Preta). Pages 9–17 Amazonian Dark Earths: Explorations in Space and Time. Springer Berlin Heidelberg, Berlin, Heidelberg.

Gómez-Paccard C, Mariscal-Sancho I, León P, Benito M, González P, Ordóñez R, Espejo R, Hontoria C. 2013. Ca-amendment and tillage: Medium term synergies for improving key soil properties of acid soils. Soil and Tillage Research **134**: 195-206.

Graber ER, Frenkel O, Jaiswal AK, Elad Y. 2014. How may biochar influence severity of diseases caused by soilborne pathogens? Carbon management **5**: 169-183.

Graber ER, Harel YM, Kolton M, Cytryn E, Silber A, David DR, Tsechansky L, Borenshtein M, Elad Y. 2010. Biochar impact on development and productivity of pepper and tomato grown in fertigated soilless media. Plant and Soil **337**:481–496.

Grossman JM, O'Neill BE, Tsai SM, Liang B, Neves E, Lehmann J, Thies JE. 2010. Amazonian anthrosols support similar microbial communities that differ distinctly from those extant in adjacent, unmodified soils of the same mineralogy. Microbial Ecology **60**:192–205.

Gul S, Whalen JK, Thomas BW, Sachdeva V, Deng H. 2015. Physico-chemical properties and microbial responses in biochar-amended soils: Mechanisms and future directions. Agriculture, Ecosystems & Environment **206**:46-59.

Hale S, Hanley K, Lehmann J, Zimmerman A, Cornelissen G. 2011. Effects of Chemical, Biological, and Physical Aging As Well As Soil Addition on the Sorption of Pyrene to Activated Carbon and Biochar. Environmental Science & Technology **45**:10445–10453.

Hamer U, Marschner B, Brodowski S, Amelung W. 2004. Interactive priming of black carbon and glucose mineralisation. Organic Geochemistry **35**:823–830.

He L, Liu Y, Zhao J, Bi Y, Zhao X, Wang S, Xing G. 2016. Comparison of straw-biochar-mediated changes in nitrification and ammonia oxidizers in agricultural oxisols and cambosols. Biology and Fertility of Soils **52**:137–149.

Heitkötter J, Marschner B. 2015. Interactive effects of biochar ageing in soils related to feedstock, pyrolysis temperature, and historic charcoal production. Geoderma **245**–**246**:56–64.

Herath HMSK, Camps-Arbestain M, Hedley M. 2013. Effect of biochar on soil physical properties in two contrasting soils: An Alfisol and an Andisol. Geoderma **209**:188–197.

Hina K, Bishop P, Arbestain MC, Calvelo-Pereira R, MacIá-Agulló JA, Hindmarsh J, Hanly JA, MacÍas F, Hedley MJ. 2010. Producing biochars with enhanced surface activity through alkaline pretreatment of feedstocks. Pages 606–617 Australian Journal of Soil Research.

Hofrichter M. 2002. Review: Lignin conversion by manganese peroxidase (MnP).Enzyme and Microbial Technology **30**:454-466.

Hossain MK, Strezov V, Yin Chan K, Nelson PF. 2010. Agronomic properties of wastewater sludge biochar and bioavailability of metals in production of cherry tomato (Lycopersicon esculentum). Chemosphere **78**:1167–1171.

Hu L, Cao L, Zhang R. 2014. Bacterial and fungal taxon changes in soil microbial community composition induced by short-term biochar amendment in red oxidized loam soil. World Journal of Microbiology and Biotechnology **30**:1085–1092.

Huang D, Liu L, Zeng G, Xu P, Huang C, Deng L, Wang R, Wan J. 2017. The effects of rice straw biochar on indigenous microbial community and enzymes activity in heavy metal-contaminated sediment. Chemosphere **174**:545–553.

Jeffery S, Verheijen FGA, van der Velde M, Bastos AC. 2011. A quantitative review of the effects of biochar application to soils on crop productivity using meta-analysis.Agriculture, Ecosystems & Environment **144**:175-187.

Jin H. 2010. August 5. Characterization Of Microbial Life Colonizing Biochar And Biochar-Amended Soils. Available from http://ecommons.cornell.edu/handle/1813/17077 (accessed February 22, 2016).

Kato M, Loomis D, Brooks LM, Gattas GFJ, Gomes L, Carvalho AB, Rego M a V, DeMarini DM. 2004. Urinary biomarkers in charcoal workers exposed to wood smoke in Bahia State, Brazil. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology.

Kern DC, D'aquino G, Rodrigues TE, Frazao FJL, Sombroek W, Myers TP, Neves EG. 2003a. Distribution of Amazonian Dark Earths in the Brazilian Amazon. Pages 51–75 Amazonian Dark Earths. Kluwer Academic Publishers, Dordrecht.

Khadem A, Raiesi F. 2017a. Influence of biochar on potential enzyme activities in two calcareous soils of contrasting texture. Geoderma **308**:149–158.

Khadem A, Raiesi F. 2017b. Responses of microbial performance and community to corn biochar in calcareous sandy and clayey soils. Applied Soil Ecology **114**:16–27.

Khadem A, Raiesi F. 2017c. Influence of biochar on potential enzyme activities in two calcareous soils of contrasting texture. Geoderma **308**:149–158. Elsevier.

Khodadad CLM, Zimmerman AR, Green SJ, Uthandi S, Foster JS. 2011. Taxa-specific changes in soil microbial community composition induced by pyrogenic carbon amendments. Soil Biology and Biochemistry **43**:385–392.

Kim JS, Sparovek G, Longo RM, De Melo WJ, Crowley D. 2007. Bacterial diversity of terra preta and pristine forest soil from the Western Amazon. Soil Biology and Biochemistry **39**:684–690.

Kumar S, Masto RE, Ram LC, Sarkar P, George J, Selvi VA. 2013. Biochar preparation from Parthenium hysterophorus and its potential use in soil application. Ecological Engineering **55**:67–72.

Kuzyakov Y, Subbotina I, Chen H, Bogomolova I, Xu X. 2009. Black carbon decomposition and incorporation into soil microbial biomass estimated by 14C labeling. Soil Biology and Biochemistry **41**:210–219.

Kuzyakov Y, Xu X. 2013. Competition between roots and microorganisms for nitrogen: Mechanisms and ecological relevance.

Laird D, Fleming P, Wang B, Horton R, Karlen D. 2010. Biochar impact on nutrient leaching from a Midwestern agricultural soil. Geoderma **158**:436–442.

Laird DA. 2008. The charcoal vision: A win-win-win scenario for simultaneously producing bioenergy, permanently sequestering carbon, while improving soil and water quality. Agronomy Journal **100**:178–181.

Lavelle P. 1997. Faunal Activities and Soil Processes: Adaptive Strategies That Determine Ecosystem Function. Advances in Ecological Research **27**:93–132.

Lavelle P, Barros E, Blanchart E, Brown G, Desjardins T, Mariani L, Rossi JP. 2001. SOM management in the tropics: Why feeding the soil macrofauna? Nutrient Cycling in Agroecosystems **61**:53–61.

Lehmann J, Joseph S, Earthscan from Routledge. 2015a. Biochar for environmental management : science, technology and implementation. Page (Lehmann J, Joseph S, editors)second edition.

Lehmann J, Kern D, German L, Mccann J, Martins GC, Moreira A. 2003. Soil Fertility and Production Potential. Pages 105–124 Amazonian Dark Earths: Origin Properties Management.

Lehmann J, Rillig MC, Thies J, Masiello CA, Hockaday WC, Crowley D. 2011a. Biochar effects on soil biota - A review. Soil Biology and Biochemistry **43**:1812–1836.

Lehmann J, Rillig MC, Thies J, Masiello CA, Hockaday WC, Crowley D. 2011b. Biochar effects on soil biota – A review. Soil Biology and Biochemistry **43**:1812–1836.

Li X, Shen Q, Zhang D, Mei X, Ran W, Xu Y, Yu G. 2013. Functional Groups Determine Biochar Properties (pH and EC) as Studied by Two-Dimensional 13C NMR Correlation Spectroscopy. PLoS ONE **8**.

Liang B et al. 2006. Black Carbon Increases Cation Exchange Capacity in Soils. Soil Science Society of America Journal **70**:1719.

Liu J, Schulz H, Brandl S, Miehtke H, Huwe B, Glaser B. 2012. Short-term effect of biochar and compost on soil fertility and water status of a Dystric Cambisol in NE Germany under field conditions. Journal of Plant Nutrition and Soil Science **175**:698–707.

Liu Q, Zhang Y, Liu B. 2018. How does biochar influence soil N cycle? A meta-analysis. Plant and Soil **426**:211-225.

Lu SG, Sun FF, Zong YT. 2014. Effect of rice husk biochar and coal fly ash on some physical properties of expansive clayey soil (Vertisol). Catena **114**:37–44.

Mandal S, Thangarajan R, Bolan NS, Sarkar B, Khan N, Ok YS, Naidu R. 2016. Biochar-induced concomitant decrease in ammonia volatilization and increase in nitrogen use efficiency by wheat. Chemosphere **142**:120–127.

Mann CC. 2002. The real dirt on rainforest fertility.

Martinsen V, Mulder J, Shitumbanuma V, Sparrevik M, B??rresen T, Cornelissen G. 2014.

Farmer-led maize biochar trials: Effect on crop yield and soil nutrients under conservation farming. Journal of Plant Nutrition and Soil Science **177**:681–695.

Masto RE, Kumar S, Rout TK, Sarkar P, George J, Ram LC. 2013. Biochar from water hyacinth (Eichornia crassipes) and its impact on soil biological activity. Catena **111**:64–71.

Mcclellan AT, Deenik J, Uehara G, Antal M. 2007. Effects of Flashed Carbonized © Macadamia Nutshell Charcoal on Plant Growth and Soil Chemical Properties. ASA-CSSA-SSA International Annual Meetings, New Orleans, Louisiana:14.

Mccormack SA, Ostle N, Bardgett RD, Hopkins DW, Vanbergen AJ. 2013. Biochar in bioenergy cropping systems: Impacts on soil faunal communities and linked ecosystem processes. GCB Bioenergy **5**:81–95.

Meggers B. 2001. The Continuing Quest for El Dorado : Round Two. Latin American Antiquity **12**:304–325.

Mia S, van Groenigen JW, van de Voorde TFJ, Oram NJ, Bezemer TM, Mommer L, Jeffery S. 2014. Biochar application rate affects biological nitrogen fixation in red clover conditional on potassium availability. Agriculture, Ecosystems and Environment **191**:83–91.

Mukherjee A, Lal R. 2013. Biochar Impacts on Soil Physical Properties and Greenhouse Gas Emissions. Agronomy **3**:313–339.

Mukherjee A, Lal R. 2014. The biochar dilemma. Soil Research **52**:217–230.

Mukherjee A, Zimmerman AR, Harris W. 2011. Surface chemistry variations among a series of laboratory-produced biochars. Geoderma **163**:247–255.

Myers TP, Denevan WM, Winklerprins A, Porro A. 2003a. Historical Perspectives on Amazonian Dark Earths. Pages 15–28 Amazonian Dark Earths. Kluwer Academic Publishers, Dordrecht.

Novak JM et al. 2009. Characterization of designer biochar produced at different temperatures and their effects on a loamy sand. Annals of Environmental Science **3**:195–206.

Novak JM, Busscher WJ, Watts DW, Laird DA, Ahmedna MA, Niandou MAS. 2010. Short-term CO2 mineralization after additions of biochar and switchgrass to a Typic Kandiudult. Geoderma **154**:281–288.

Nugroho RA, Röling WFM, Laverman AM, Verhoef HA. 2007. Low nitrification rates in acid scots pine forest soils are due to pH-related factors. Microbial Ecology **53**:89–97.

O'Neill B, Grossman J, Tsai MT, Gomes JE, Lehmann J, Peterson J, Neves E, Thies JE. 2009. Bacterial community composition in Brazilian Anthrosols and adjacent soils characterized using culturing and molecular identification. Microbial Ecology **58**:23–35.

Obia A, Mulder J, Martinsen V, Cornelissen G, Børresen T. 2016. In situ effects of biochar on aggregation, water retention and porosity in light-textured tropical soils. Soil and Tillage Research **155**:35–44.

Ogawa M. 1994. The symbiosis of people and nature in the tropics. Farming Japan **28**:10–34.

Ouyang L, Tang Q, Yu L, Zhang R. 2014. Effects of amendment of different biochars on soil enzyme activities related to carbon mineralisation. Soil Research **52**:706–716.

Ouyang L, Wang F, Tang J, Yu L, Zhang R. 2013. Effects of biochar amendment on soil aggregates and hydraulic properties. Journal of soil science and plant nutrition **13**:991–1002.

Painter TJ. 1998. Carbohydrate polymers in food preservation: An integrated view of the Maillard reaction with special reference to discoveries of preserved foods in Sphagnum-dominated peat bogs. Carbohydrate Polymers **36**:335–347.

Paul EA. 2006. Soil microbiology, ecology and biochemistry: Third edition. Page Soil Microbiology, Ecology and Biochemistry: Third Edition.

Paz-Ferreiro J, Fu S, Méndez A, Gascó G. 2014. Interactive effects of biochar and the earthworm Pontoscolex corethrurus on plant productivity and soil enzyme activities. Journal of Soils and Sediments **14**:483–494.

Petersen JB, Neves EG, Heckenberger MJ. 2001a. Chapter 3 - "Gift from the past: Terra Preta and prehistoric Amerindian Occupation in Amazonia" [in] Unknown Amazon: culture in nature in ancient Brazil | University of Exeter. Pages 86–107 in C. McEwan, C. Barreto, and E. Neves, editors. Unknown Amazon: culture in nature in ancient Brazil. British museums, London.

Piccolo a., Pietramellara G, Mbagwu JSC. 1996. Effects of coal derived humic substances on water retention and structural stability of Mediterranean soils. Soil Use and Managaement **12**:209–213.

Pietikäinen J, Kiikkilä O, Fritze H. 2000. Charcoal as a habitat for microbes and its effect on the microbial community of the underlying humus. Oikos **89**:231–242.

Ponge JF, Topoliantz S, Ballof S, Rossi JP, Lavelle P, Betsch JM, Gaucher P. 2006. Ingestion of charcoal by the Amazonian earthworm Pontoscolex corethrurus: A potential for tropical soil fertility.

Priha O, Smolander A. 1995. Nitrification, denitrification and microbial biomass N in soil from two N-fertilized and limed Norway spruce forests. Soil Biology and Biochemistry **27**:305–310.

Rajkovich S, Enders A, Hanley K, Hyland C, Zimmerman AR, Lehmann J. 2012. Corn growth and nitrogen nutrition after additions of biochars with varying properties to a temperate soil. Biology and Fertility of Soils **48**:271–284.

Rousk J, Bååth E, Brookes PC, Lauber CL, Lozupone C, Caporaso JG, Knight R, Fierer N. 2010. Soil bacterial and fungal communities across a pH gradient in an arable soil. The ISME journal **4**:1340–51.

Rousk J, Brookes PC, Bååth E. 2009. Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. Applied and environmental microbiology **75**:1589–96.

Rousseau L, Fonte SJ, Téllez O, Van Der Hoek R, Lavelle P. 2013. Soil macrofauna as indicators of soil quality and land use impacts in smallholder agroecosystems of western Nicaragua. Ecological Indicators **27**:71–82.

Rutigliano FA, Romano M, Marzaioli R, Baglivo I, Baronti S, Miglietta F, Castaldi S. 2014. Effect of biochar addition on soil microbial community in a wheat crop. European Journal of Soil Biology **60**:9–15.

Sigmund G, Hüffer T, Hofmann T, Kah M. 2017. Biochar total surface area and total pore volume determined by N2 and CO2 physisorption are strongly influenced by degassing temperature. Science of The Total Environment **580**:770–775.

Sika MP, Hardie AG. 2014. Effect of pine wood biochar on ammonium nitrate leaching and availability in a South African sandy soil. European Journal of Soil Science **65**:113–119.

Sohi SP, Krull E, Lopez-Capel E, Bol R. 2010. A review of biochar and its use and function in soil. Page Advances in Agronomy, 1st edition. Elsevier Inc.

Soinne H, Hovi J, Tammeorg P, Turtola E. 2014. Effect of biochar on phosphorus sorption and clay soil aggregate stability. Geoderma **219**–**220**:162–167.

Song D, Xi X, Huang S, Liang G, Sun J, Zhou W, Wang X. 2016. Short-Term Responses of Soil Respiration and C-Cycle Enzyme Activities to Additions of Biochar and Urea in a Calcareous Soil. PloS one **11**:e0161694.

Sorrenti G, Toselli M. 2016. Soil leaching as affected by the amendment with biochar and compost. Agriculture, Ecosystems and Environment **226**:56–64.

Steinbeiss S, Gleixner G, Antonietti M. 2009. Effect of biochar amendment on soil carbon balance and soil microbial activity. Soil Biology and Biochemistry **41**:1301–1310.

Steiner C, Garcia M, Zech W. 2009. Effects of charcoal as slow release nutrient carrier on N-P-K dynamics and soil microbial population: Pot experiments with ferralsol substrate. Pages 325–338 Amazonian Dark Earths: Wim Sombroek's Vision.

Steiner C, Glaser B, Teixeira WG, Lehmann J, Blum WEH, Zech W. 2008. Nitrogen retention and plant uptake on a highly weathered central Amazonian Ferralsol amended with compost and charcoal. Journal of Plant Nutrition and Soil Science **171**:893–899.

Teutscherova N, Houška J, Navas M, Masaguer A, Benito M, Vazquez E. 2018a. Leaching of ammonium and nitrate from Acrisol and Calcisol amended with holm oak biochar: A column study. Geoderma **323**:136–145.

Teutscherova N, Lojka B, Houška J, Masaguer A, Benito M, Vazquez E. 2018b. Application of holm oak biochar alters dynamics of enzymatic and microbial activity in two contrasting Mediterranean soils. European Journal of Soil Biology **88**:15–26.

Teutscherova N, Vazquez E, Masaguer A, Navas M, Scow KM, Schmidt R, Benito M. 2017a. Comparison of lime- and biochar-mediated pH changes in nitrification and ammonia oxidizers in degraded acid soil. Biology and Fertility of Soils **53**:811–821.

Teutscherova N, Vazquez E, Santana D, Navas M, Masaguer A, Benito M. 2017b. Influence of pruning waste compost maturity and biochar on carbon dynamics in acid soil: Incubation study. European Journal of Soil Biology **78**:66–74.

Thies JE, Rillig MC, Graber ER. 2015. Biochar effects on the abundance , activity and diversity of the soil biota. Pages 327–389 in J. Lehmann and S. Joseph, editors. Biochar for environmental management: science, technology and implementation2nd editio.

Thiessen S, Gleixner G, Wutzler T, Reichstein M. 2013. Both priming and temperature sensitivity of soil organic matter decomposition depend on microbial biomass – An incubation study. Soil Biology and Biochemistry **57**:739–748.

Tian J, Wang J, Dippold M, Gao Y, Blagodatskaya E, Kuzyakov Y. 2016. Biochar affects soil organic matter cycling and microbial functions but does not alter microbial community structure in a paddy soil. Science of the Total Environment **556**:89–97.

Topoliantz S, Ponge JF. 2005. Charcoal consumption and casting activity by Pontoscolex corethrurus (Glossoscolecidae). Applied Soil Ecology **28**:217–224.

Tryon EH. 1948. Effect of charcoal on certain physical, chemical, and biological properties of forest soils. Ecol. Monographs **18**:81–115.

Uchimiya M, Chang S, Klasson KT. 2011a. Screening biochars for heavy metal retention in soil: Role of oxygen functional groups. Journal of Hazardous Materials **190**:432–441.

Uchimiya M, Wartelle LH, Klasson KT, Fortier CA, Lima IM. 2011b. Influence of pyrolysis temperature on biochar property and function as a heavy metal sorbent in soil. Journal of Agricultural and Food Chemistry **59**:2501–2510.

Ulyett J, Sakrabani R, Kibblewhite M, Hann M. 2014. Impact of biochar addition on water retention, nitrification and carbon dioxide evolution from two sandy loam soils. European Journal of Soil Science **65**:96–104.

van Zwieten L, Kimber S, Morris S, Chan KY, Downie A, Rust J, Joseph S, Cowie A. 2010. Effects of biochar from slow pyrolysis of papermill waste on agronomic performance and soil fertility. Plant and Soil **327**:235–246.

Van Zwieten L, Singh BP, Kimber SWL, Murphy D V., Macdonald LM, Rust J, Morris S. 2014. An incubation study investigating the mechanisms that impact N2O flux from soil following biochar application. Agriculture, Ecosystems and Environment **191**:53–62.

Vazquez E, Teutscherova N, Almorox J, Navas M, Espejo R, Benito M. 2017. Seasonal variation of microbial activity as affected by tillage practice and sugar beet foam amendment under Mediterranean climate. Applied Soil Ecology **117**–**118**:70–80.

Velasquez E, Lavelle P, Andrade M. 2007. GISQ, a multifunctional indicator of soil quality. Soil Biology and Biochemistry **39**:3066–3080.

Walkley A, Black IA. 1934. An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. Soil Science **37**:29–38.

Wang J, Xiong Z, Kuzyakov Y. 2015a. Biochar stability in soil: meta-analysis of decomposition and priming effects. GCB Bioenergy **8**:512-523.

Wang Z, Zong H, Zheng H, Liu G, Chen L, Xing B. 2015b. Reduced nitrification and abundance of ammonia-oxidizing bacteria in acidic soil amended with biochar. Chemosphere **138**:576–583.

Warnock DD, Lehmann J, Kuyper TW, Rillig MC. 2007. Mycorrhizal responses to biochar in soil - Concepts and mechanisms. Plant and Soil **300**:9–20.

Whitman T, Singh BP, Zimmerman AR. 2015. Priming effects in biochar-amended soils: Implications of biochar-soil organic matter interactions for carbon storage. Pages 455–488 in J. Lehmann and S. Joseph, editors. Biochar for Environmental Management: Science, Technology and Implementation, 2nd edition. Routledge.

Xu G, Lv Y, Sun J, Shao H, Wei L. 2012. Recent Advances in Biochar Applications in Agricultural Soils: Benefits and Environmental Implications.

Xu N, Tan G, Wang H, Gai X. 2016. Effect of biochar additions to soil on nitrogen leaching, microbial biomass and bacterial community structure. European Journal of Soil Biology **74**:1–8.

Yoo G, Kim H, Chen J, Kim Y. 2014. Effects of Biochar Addition on Nitrogen Leaching and Soil Structure following Fertilizer Application to Rice Paddy Soil. Soil Science Society of America Journal **78**:852.

Zangerlé A, Pando A, Lavelle P. 2011. Do earthworms and roots cooperate to build soil macroaggregates? A microcosm experiment. Geoderma **167**–**168**:303–309.

Zavalloni C, Alberti G, Biasiol S, Vedove GD, Fornasier F, Liu J, Peressotti A. 2011. Microbial mineralization of biochar and wheat straw mixture in soil: A short-term study. Applied Soil Ecology **50**:45–51.

Zhang H, Chen C, Gray EM, Boyd SE. 2017. Effect of feedstock and pyrolysis temperature on properties of biochar governing end use efficacy. Biomass and Bioenergy **105**:136–146.

Zhao SX, Ta N, Wang XD. 2017. Effect of temperature on the structural and physicochemical properties of biochar with apple tree branches as feedstock material. Energies **10**.

Zhao X, Wang S, Xing G. 2014. Nitrification, acidification, and nitrogen leaching from subtropical cropland soils as affected by rice straw-based biochar: Laboratory incubation and column leaching studies. Journal of Soils and Sediments **14**:471–482.

Zheng H, Wang Z, Deng X, Zhao J, Luo Y, Novak J, Herbert S, Xing B. 2013. Characteristics and nutrient values of biochars produced from giant reed at different temperatures. Bioresource Technology **130**:463–471.

Zimmerman AR, Gao B, Ahn M-Y. 2011. Positive and negative carbon mineralization priming effects among a variety of biochar-amended soils. Soil Biology and Biochemistry **43**:1169–1179.

13 APPENDICES

13.1 Curriculum vitae

13.1.1 Education

02/2018 – 08/2018	**International Center for Tropical Agriculture, CIAT** (Palmira, Colombia) Disentangling the relationship between soil properties and biological nitrification inhibition in the tropical signalgrass pastures and the diversity of arbuscular mycorrhizal fungi Visiting researcher (Supervision by Dr. Mirjam Pulleman and Dr. Jacobo Arango)
09/2017– 12/2017	**University of Florence** (Florence, Italy) The impact of soil management practices on greenhouse gases and volatile organic compounds emissions from soil after soil re-wetting Visiting researcher (Supervision by Dr. Giancarlo Renella)
07/2017	**The World Reference Base (WRB) International Soil Classification System Workshop** (Latvia, Estonia)
01/2017– 06/2017	**International Center for Tropical Agriculture**, **CIAT** (Palmira, Colombia) Soil Research Area. Research Project on the effects of pasture and silvopastoral systems on soil ecological processes and indicator of soil health; arbuscular mycorrhizal symbiosis and its potential relation to biological nitrification inhibition in signalgrass pastures Visiting researcher (Supervision by Dr. Mirjam Pulleman)
10/2016 –10/2016	**The World Reference Base (WRB) International Soil Classification System** (Quito, Ecuador) Sociedad Latinoamericana de la Ciencia del Suelo, Instructor: Dr. Peter Schad. 1 Week.
08/2016 – 09/2016	**Karlsruhe Institute of Technology** (Garmisch-Partenkirchen, Germany) Institute of Meteorology and Climate Research, Atmospheric Environmental Research (IMK-IFU). Department 'Bio-Geo-Chemical Processes. The impact of soil management practice on gross nitrogen transformation rates Visiting researcher (Supervision by Dr. Eugenio Diaz-Pines)
03/2015 – 09/2015	**Universidad Politécnica de Madrid** (Spain) Department of Agricultural Production ERASMUS Studies
09/2014 – 03/2015	**Universidad Politécnica de Madrid** (Spain) Department of Agricultural Production

ERASMUS Traineeship

07/2013 – 10/2013 **Karlsruhe Institute of Technology** (Garmisch-Partenkirchen, Germany)
Institute of Meteorology and Climate Research, Atmospheric Environmental Research (IMK-IFU)
Internship

09/2013 **2nd European Biochar Summer School** (Valais, Switzerland)
Ithaca Institute, 1 week

07/2012 – 10/2012 **Nong Lam University** (Ho Chi Minh City, Vietnam)
Soil, water and plant analysis
Internship

09/2010 – 06/2011 **Czech University of Life Sciences Prague** (Czech Republic)
Faculty of Tropical AgriSciences,
The use of locally produced charcoal for soil fertility improvement in Peruvian Amazon
M.Sc. student (graduated with distinction)

06/2011 – 10/2011 **Universidad Nacional de Ucayali** (Peru)
M.Sc. thesis research, soil and plant analysis
Visiting student

09/2010 – 06/2011 **Universidad Politécnica de Valencia** (Spain)
ERASMUS student

09/2007 – 06/2010 **Czech University of Life Sciences Prague** (Czech Republic)
Faculty of Tropical AgriSciences
B.Sc. student (graduated with distinction)

13.1.2 Brief relevant work experience

10/2013 – 05/2014 **Caritas Czech Republic** (Aceh, Indonesia)
Internship in Sustainable Agriculture
Colsulting on agriculture, farmers‘ manuals preparation, workshops for farmers organization, demoplots establishment

01/2012 – 02/2012 **SAELAO Community Project** (Vang Viang, Laos)
Volunteer. Organic farming, teaching English, working in vegetable garden, floating vegetable garden, biogas production

13.1.3 Languages

Czech: native speaker
English: proficient user
Spanish: proficient user
German: intermediate user
Indonesian: intermediate user

13.2 List of publications

Teutscherova N, Lojka B, Houška J, Masaguer A, Benito M, Vazquez E. 2018. Application of holm oak biochar alters dynamics of enzymatic and microbial activity in two contrasting Mediterranean soils. European Journal of Soil Biology **88**:15-26.

Teutscherova N, Houška J, Navas M, Masaguer A, Benito M, Vazquez E. 2018. Leaching of ammonium and nitrate form Acrisol and Calcisol amended with holm oak biochar: A column study. Geoderma **323**: 136-145.

Teutscherova N, Vazquez E, Masaguer A, Navas M, Scow K, Schmidt R, Benito M. 2017. Comparison of lime- and biochar-mediated pH changes in nitrification and ammonia oxidizers in an acid degraded soil. Soil Biology and Fertility **53**: 811-821

Vazquez E, Teutscherova N, Almorox J, Navas M, Espejo R, Benito M. 2017. Seasonal variation of microbial activity as affected by tillage practice and sugar beet foam amendment under Mediterranean climate. Applied Soil Ecology **117-118**: 70-80

Teutscherova N, Vazquez E, Santana D, Navas M, Masaguer A, Benito M. 2017. Influence of pruning waste compost maturity and biochar on carbon dynamics in acid soil: Incubation study. European Journal of Soil Biology **78**:66-74

13.3 Scientific conferences

Teutscherova N, Vazquez E, Arevalo A, Chagueza Y, Diaz E, Benito M, Arango J, Pulleman M. 2017. *Is there a link between biological nitrification inhibition and mycorrhizal symbiosis in Brachiaria grasses?*
Tropentag (Bonn, Germany)

Vazquez E, Teutscherova N, Chagueza Y, Botero C, Benito M, Gutierrez JF, Sotelo M, Arango J, Pulleman M. 2017. *Soil macrofauna as indicator of soil quality in improved (silvo)pastoral systems in the tropics*
Tropentag (Bonn, Germany)

Teutscherova N, Vazquez E, Navas M, Espejo R, Benito M. 2016. *Arbuscular mycorrhizae and plant reproduction patterns under no-tillage and calcium amendment*.
XXI Congreso Latinoamericano y XV Congreso Ecuatoriano de la Ciencia del Suelo (Quito, Ecuador)

Teutscherova N, Vazquez E, Navas M, Espejo R, Benito M. 2016. *No-tillage and sugar beet residues amendment affect carbon mineralization in degraded Palexerult*.
XXI Congreso Latinoamericano y XV Congreso Ecuatoriano de la Ciencia del Suelo (Quito, Ecuador)

Vazquez E, Teutscherova N, Navas M, Espejo R, Benito M. 2016. *Metabolic activity evolution during exceptionally dry year: effect of no-tillage and Ca-amendment*
XXI Congreso Latinoamericano y XV Congreso Ecuatoriano de la Ciencia del Suelo (Quito, Ecuador)

Vazquez E, Teutscherova N, Navas M, Espejo R, Benito M. 2016. *Soil enzyme activities and microbial biomass: long-term effects of no-tillage and Ca-amendment*. **XXI Congreso Latinoamericano y XV Congreso Ecuatoriano de la Ciencia del Suelo** (Quito, Ecuador)

Teutscherova N, García-González, I, Sastre B, Benito M, Almorox J, Bienes R, Espejo R, Hontoria C. 2016. *Arbuscular mycorrhizal fungi response to cover crops in an olive orchard grown in a gypsiferous soil under semiarid climate*
VII Congresso Ibérico de Ciências de Solo (Beja, Portugal)

Teutscherova N, Vazquez E, Fernandez E, Benito M, Masaguer A. 2016. *Evolución de las propiedades del compost mediante la combinación del vermicompostaje y compostaje respecto al solo compostaje de los residuos.*
V Jornadas de la Red Española de Compostaje (Sevilla, Spain)

Vazquez E, Teutscherova N, Fernandez E, Benito M, Masaguer A. 2016. *La combinación del vermicompostaje y compostaje mejora las propiedades agronómicas del producto final respecto al solo compostaje de los resíduos.*
V Jornadas de la Red Española de Compostaje (Sevilla, Spain)

Teutscherova N, Vazquez E, Santana D, Masaguer A, Benito M. 2016. *Carbon mineralization determined by the compost stability stage when applied with biochar to degraded acid soil.*

International Conference of the European Society for Soil Conservation (Cluj-Napoca, Romania)

Vazquez E, Teutscherova N, Espejo R, Benito M. 2016. Comparison of four different land-use systems in degraded area of "raña" in SW Spain.
International Conference of the European Society for Soil Conservation (Cluj-Napoca, Romania)

Teutscherova N, Vazquez E, Santana D, Benito M, Masaguer A. 2015. *Impact of biochar addition on nitrification and CO_2 evolution from acid Palexerult.*
Tropentag (Berlin, Germany)

Teutscherova N, Vazquez E, Masaguer A, Benito M. 2015. *Response of two contrasting soils to biochar amendment: microbial biomass, soil respiration and nitrification*
RENS (Reunion Nacional de Suelos) (Granada, Spain)

Vazquez E, Espejo R, Teutscherova N, Mariscal-Sancho I, Hontoria C, Benito M. 2015. *Siembra directa beneficiosa para la productividad de cultivos exetensivos en suelos de raña tanto en campanas con deficit como con exceso de precipitaciones*.
RENS (Reunion Nacional de Suelos) (Granada, Spain)

Teutscherova N, Lojka, B. 2012. *Amazonian Dark Earths and the Potential of Biochar for their Re-creation*.
Tropentag 2012 (Goettingen, Germany)