Biochar Effects on Soil Quality as Evaluated by Physical, Chemical, and Biological Parameters

A Dissertation

Presented By:

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Words of thanks

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Contents

List of abbreviations

AEC anion exchange capacity
AIC Akaike information criterion
Al aluminum
ANOVA analysis of variance
As arsenic
ASTM American Society for Testing Materials
β standardized coefficient
BD bulk density
C carbon
Ca calcium
Cd cadmium
CEC cation exchange capacity
$CHCl_3$ chloroform
Cl chlorine
cm centimeter
CO_2 carbon dioxide
Cr chromium
Cu copper
CV coefficient of variation
d day
dm dry matter
DNA deoxyribonucleic acid
DOC dissolved organic carbon
dS deci-Siemens
DW dry weight
EC electrical conductivity
Fe iron
FTIR Fourier-transformed infrared spectroscopy
g gram
g standard gravity
GHG greenhouse gas
h hour
H hydrogen
H:C hydrogen:carbon ratio
ha hectare
H_2PO_4 phosphoric acid
H_2SO_4 sulfuric acid
HPO_4^{2-} hydrogen phosphate
HSD honestly significant difference
ICP-MS inductively coupled plasma mass-spectrometry
IBI International Biochar Initiative
ISO International Standards Organization
K potassium
$K_2Cr_2O_7^{2-}$ potassium dichromate
K_2SO_4 potassium sulphate

Kpa kilopascals
KW Kruskal-Wallis
l liter
LOI loss on ignition
LOI-375 loss on ignition at 375°C
LOI-550 loss on ignition at 550°C
LOI-1100 loss on ignition at 1100°C
m meter
MB microbial biomass
MBC microbial biomass carbon
Mg magnesium
min minute
ml milliliter
MNS mineral, nitrogen, and sulfur
mo. month
MW Mann-Whitney
N nitrogen
Na sodium
NaOH potassium hydroxide
NH_4^+ ammonium
NO_2^- nitrite
NO_3^- nitrate
O:C oxygen:carbon ratio
OC organic carbon
ODW oven dry weight
OECD
p statistical significance
P phosphorous
PAH polycyclic aromatic hyrdocarbons
Pb lead
pH hydrogen ion concentration
r Pearson's product-moment correlation coefficient
R^2 coefficient of determination
RODI reverse osmosis deionized
s second
S sulphur
SD standard deviation
SE standard error
SEM scanning electron microscopy
Si silicon
SIR substrate-induced respiration
SO_4^{2-} sulphate
SOC soil organic carbon
SOM soil organic matter
t metric ton
USDA US Department of Agriculture
VIF variance inflation factor
VM volatile matter

W Mann-Whitney statistic
WHC water-holding capacity
Zn zinc

SI prefixes

μ micro ($x10^{-6}$)
m milli ($x10^{-3}$)
c centi ($x10^{-2}$)
d deci (10^{-1})
k kilo ($x10^{3}$)

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General introduction

1. What is biochar?

Biochar is thermally-decomposed biomass deliberately applied to soil to improve its properties (Lehmann and Joseph 2009a). By nomenclature, biochar is highly related to charcoal, but the latter is fuel and not a soil amendment. While production methods for biochar and charcoal may be similar, the first encompasses technologically-advanced processes designed to produce forms of clean, renewable energy production (thermic energy, combustible gasses, and biooils) (Bridgwater 2006) and biochar (**Figure 1**) as a coproduct.

Figure 1: Pine chips and the corresponding biochar produced by slow pyrolysis.

Biochar production methods include various thermal conversion processes such as dry pyrolysis (including slow, fast), gasification, and hydrothermal (Brown 2009; Libra et al. 2011). Common to all biochar production methods is the total or near-total deprivation of oxygen during thermal conversion, preventing total combustion of carbon-rich biomass (Demirbaş and Arin 2002; Brown 2009). Hydrothermal char (not studied in this thesis) preserves a large proportion of the

feedstock as char (50-80%) and as a technology is well-equipped to deal with agricultural and urban liquid wastes (Libra et al 2011). Slow pyrolysis is the production method most adequate for producing charcoal or biochar as a product, conserving carbon contents to the greatest extent among biochar dry production methods (35%) (Brown 2009). Heating is at low to moderate temperature (around 450 to 650 °C; Sohi et al. 2009), without oxygen, generally from minutes to hours, operating at atmospheric pressure, in continuous reactors such as drum pyrolyzers, rotary kilns or screw pyrolizers (Brown et al. 2009) with higher energy efficiency than traditional kilns (Bruun et al. 2012). Fast pyrolysis is optimized for the production of combustible bio-oils (75%), with low char (12%) and gas (13%) production (Brown 2009). Heating is at moderate temperature (generally around 450 °C; Sohi et al 2009), without oxygen, for milliseconds or seconds, under atmospheric pressure, and consists of a very rapid transfer of heat, typically to crushed/grounded biomass particles blown through hot gases in a confined oxygen-free chamber. Finally, gasification is optimized to produce syngas (including CO, CO_2, H_2, CH_4, and N_2; Bridgwater 2006) for energy generation (85%), with low char output (10%) (Brown 2009). Heating is at high temperatures (>800 °C; Sohi et al. 2009), with a limited supply of oxygen, a moderate residence time of 10-20 seconds (Brown 2009), sometimes at high pressures of 15-50 bars (Bridgwater 2006).

These different thermal conversion processes produce very distinct chemical (Enders et al. 2012; Amonette and Joseph 2009) and physical (Downie et al. 2009) biochar properties. Pyrolysis temperature is the most significant process parameter (Sohi et al. 2010). Maximum temperature and heating rate are the most important factors determining nutrient retention from feedstock to char (Kookana et al. 2011). Most N- and S-based compounds volatilize above 200 and 375 °C, respectively, and since many biochars are produced around or above 450-550 °C, these tend to be depleted in N

and S, with the exception of those produced from feedstocks containing large amounts of N (e.g. sewage sludge) (Kookana et al. 2011). Also, H:C and O:C ratios, used as an estimate of aromaticity (the degree to which aromatic rings, six atoms of linked C, are connected in two or three dimensions), are lower with increasing temperature, hence indicating a greater degree of aromaticity and stability, as has been confirmed by NMR data (Kookana et al. 2011; Krull et al. 2009). Biochar (as well as charcoal) is therefore well defined by its high concentration of C, largely as aromatic rings, having lost most of the other biologically-abundant elements H and O (Antal and Grønli 2003; Lehmann and Joseph 2009a). With increasing temperature the stacking of these rings becomes more ordered, from a largely amorphous mass to increasingly conjugated sheets, whereas the greatest degree of orderedness and stacking represents what is called graphite (Lehmann and Joseph 2009a). Aromaticity confers biochar its high stability in soil since these structures are not easy degraded by microorganisms (Liang et al. 2008), and by the same token higher temperatures lend to higher stability (Luo et al. 2011; Zimmerman 2010). Specific surface area, key for explaining most soil-biochar interactions, also tends to increase with pyrolysis temperature until a maximum and decreases thereafter, though again this is partly dependent on feedstock (Kookana et al. 2011). Whereas maximum specific surface area of wood chars has been seen at around 700-800 °C (Brown et al. 2006), it has been speculated that fine-pore structures are destroyed above this threshold (Chun et al. 2004). Cation exchange capacity (CEC), a key property of soil fertility signifying the potential for adsorption of crucial plant nutrients, is also dependent on temperature to some degree, generally decreasing with increase with temperature partly due to the loss of carboxylic biochar surface functional groups (Enders et al. 2012). Likewise, pH tends to increase with temperature, as well as ash content (Sohi et al. 2010; Enders et al. 2012), and these intrinsically related characteristics both

contribute to biochar's liming value in soils, which is a property of agricultural importance.

The variety of feedstocks that can be used for biochar production is immense, including agricultural, industrial, and urban wastes or residues (Lehmann and Joseph 2009b). The chemical changes that occur during pyrolysis described above may be accompanied by a relatively high preservation of the structure of the original feedstock, as it is the case of some wood chars, retaining the porous cell structure of the original feedstock (Downie et al. 2009), as observed in scanning electron microscopy (SEM) images of some of the materials tested in this thesis, seen in **Figure 2.**

2. Historical precedents

Charcoal production has been an indigenous technology worldwide for the storage of energy, possibly the first synthetic material produced by man (Antal and Grønli 2003). Also, the soil application of charcoal or biomass charring for fertility management has existed traditionally in agriculture in many parts of the world (Sohi et al. 2010). This has included, for instance, the reported use of biochar in traditional agriculture in Britain (Young 1804), the Iberian Peninsula (Miret 2004; Olarieta et al. 2011). In Catalonia, biochar was produced in-field, generally from wood, which was covered by a soil layer after being lit (Miret 2004), a practice known as the so-called 'boïcs/boïgues' or 'formiguers', the last literally meaning 'ant nests'. In this practice, mimicking a small scale charcoal-kiln, relatively small amounts of forest vegetation are placed in the agricultural soil and covered with soil (*formiguer*), and then lit, in a practice intended to 'disinfect' soil (**Figure 3**), but also thereafter incorporating char and ashes, although in small quantities (Olarieta et al. 2011). A similar procedure has been described in India and Bhutan (Mishra and Ramakrishnan 1983a,b; Kerkhoff 2006).

Figure 2: SEM images of fresh (unincubated) biochars used in Chapters 1-3: (A) slow pyrolysis poplar wood, (B) fast pyrolysis poplar wood, (C) slow pyrolysis sewage sludge, (D) gasification pine wood, (E) slow pyrolysis pine wood, (F) fast pyrolysis pine wood.

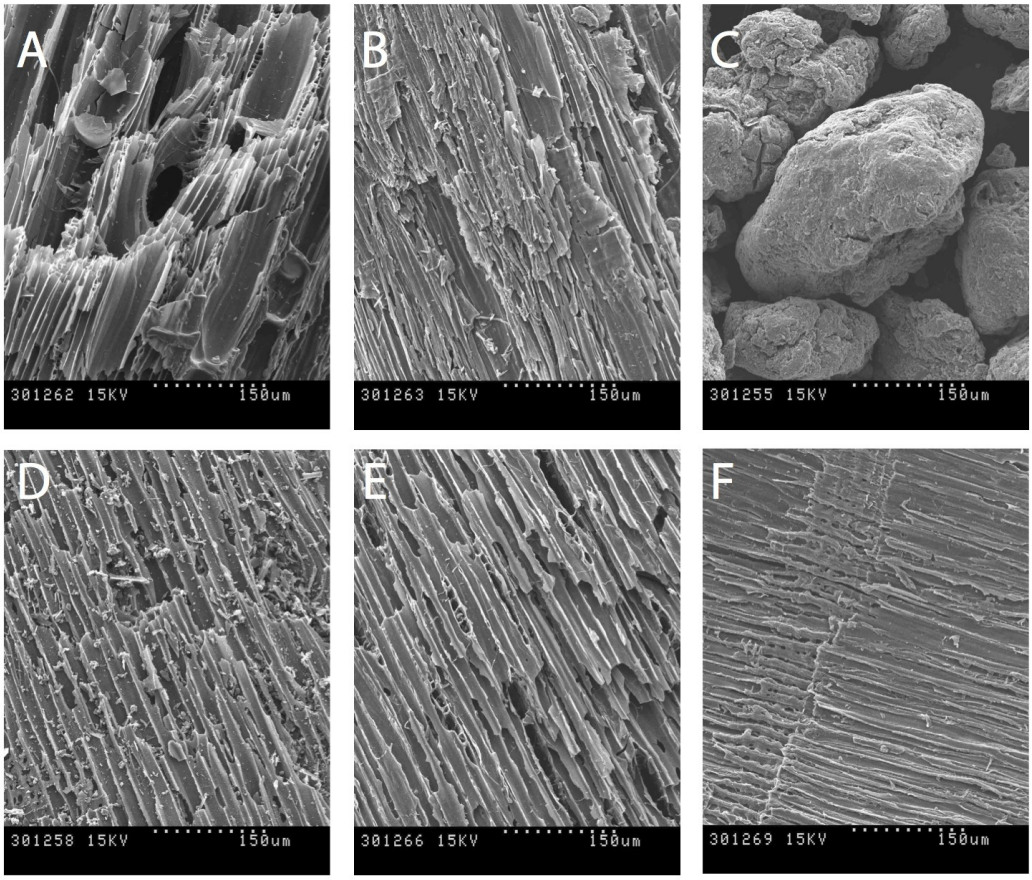
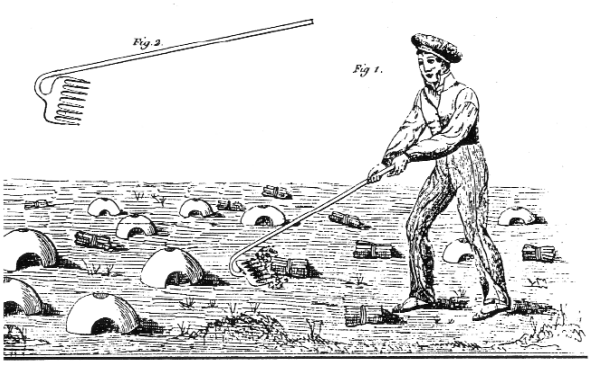


Figure 3. Depiction of the Catalonian practice of '*formiguers'*, reproduced from Miret 2004 (originally from Lasteyrie 1827).



In fact, the field of biochar has garnered widespread interest in part due to studies carried out on Amazonian Dark Earths, or '*Terra Preta do Indio'*, a formation of high-fertility anthrosols in the Amazon Basin, whose dark color and high fertility (compared to surrounding soils) was discovered to be due to high concentrations millennia-old charcoal-derived substances (Glaser et al. 2001; Lehmann 2003). The high temporal stability of such 'black carbon' in the soil makes it unlike any other organic soil amendment. Similar soils have also been described in West Africa (Fairhead and Leach 2009) and recently in Borneo (Sheil et al. 2012).

3. Potential role of biochar in the improvement of modern agriculture

3.1 Current challenges

In contemporary times, agriculture is an enormous driver of land use change (Meyer and Turner 1992; Tilman et al. 2001; Foley et al. 2005). Intensification of agriculture in the last 50 years can be credited for large yield increases, but as a consequence has become a major contributor to greenhouse gas emissions (Vitousek et al. 1997; Vergé et al. 2007), biodiversity loss (Sala et al. 2000), degradation of land and freshwater (Vitousek et al. 1997; Carpenter et al. 1998; Foley et al. 2011), and is a major force driving the environment beyond "planetary boundaries" (Rockström et al. 2009). Population and consumption will increase in the next decades, presenting a mounting challenge to agricultural productivity and sustainability (Vergé et al. 2007), and some believe that biochar may provide a win-win scenario for these (Laird 2008). Increasing soil carbon stocks reduces concentrations atmospheric CO_2 while simultaneously improving soil properties (Steiner et al. 2007; Lal 2009). Increasing agricultural nutrient and water use efficiency and reducing intensity reduces nutrient runoff, pesticide pollution, and greenhouse gas

emissions (Carpenter et al. 1998; Robertson et al. 2000; Tilman et al. 2001; Tilman et al. 2002; Vergé et al. 2007). In recent years, biochar research has grown immensely for its potential role in strategies such as these, and biochar's rapid expansion in soil, agricultural, and bioenergy research areas demonstrates its cross-platform relationship to some of the most severe contemporary global threats. However, as a young scientific field of study, little is proven about biochar's long-term compatibility with modern agriculture.

3.2 Potential environmental benefits of biochar's application to soil

Biochar's high stability in soil has generated much interest as a potential carbon-sequestration mechanism to reduce atmospheric concentrations of CO_2 (Steiner 2007; Laird 2008; Fowles 2007; Mathews 2008), a greenhouse gas (GHG) of much concern. Additionally, some research indicates that biochar may be able to reduce terrestrial production other GHGs such as N_2O (Spokas et al. 2009; Cayuela et al. 2010; Clough and Condron 2010; Taghizadeh-Toosi et al. 2011; Kammann et al. 2012), though biochar weathering may reduce this effect, and much is yet left to elucidate on the topic (Spokas 2013; Cayuela et al. 2013).

Crop productivity may also be improved by biochar use in agriculture, either by providing soil nutrients directly, or indirectly, for its ability to retain nutrients in the soil and reduce leaching losses (Glaser 2001; Ventura et al. 2013), potentially resulting in increased nutrient uptake by plants and higher productivity (Chan and Xu 2009). The surface chemistry of biochar allows the retention of cations, hence reducing nutrient losses when applied to soil, a capacity which increases with biochar aging and surface weathering (Cheng et al. 2008; Cheng and Lehmann 2009). Biochar's natural porosity due to feedstock, as well as any additional porosity created in production,

confers to biochar a high capacity for water retention, but may also aid in clayey soils prone to water-logging, and additionally confers a high specific surface area, comparable to or greater than clay (Downie et al. 2009), therefore maximizing nutrient retention (Liang et al. 2006). Furthermore, porosity has been suggested to act as microbial habitat and refuge from predators, contributing to a more favorable environment for soil biota as whole (Thies and Rillig 2009; Lehmann et al. 2011), whose support of plant growth is crucial for agricultural sustainability (Verhoef 2004).

Also, various biomass electricity generation systems have biochar as a coproduct (Gaunt and Lehmann 2008). So-called "biochar systems" are imagined to integrate the transfer and management of energy and carbon beginning with a crop, the utilization of residues or the biomass product for energy generation, and the return of a portion of the harvested carbon to the soil in the form of biochar, thereby providing additional agricultural and environmental functions (Lehmann and Joseph 2009b; Hammond et al. 2011).

Biochar production can also be an alternative approach to deal with various waste streams, including papermill waste (Van Zwieten et al. 2009), greenwaste (Chan et al. 2007), animal manure (Cao et al. 2011), and sewage sludge (Hossain et al. 2010; Hossain et al. 2011; Méndez et al. 2012). As some biochars have high adsorbance characteristics similar to activated carbons, already used in land remediation, biochars can be useful for remediation of contaminants such as pesticides (Cao et al. 2011) or heavy metals (Uchimiya et al. 2011).

3.3 Knowledge gaps and unintended consequences

While the potential for biochar to improve agricultural efficiencies and intervene in environmental problems is apparently large, there is still major uncertainty about its potential negative impacts on soil quality (Jones et al. 2012). The numerous products potentially

considered as biochars, as a result the wide variety of feedstocks and pyrolysis procedures available, as well as the diversity of soils and management practices, are the sources of these uncertainties, together with the relative scarcity (though quickly growing number) of studies in this new research area. The diminishment of any given soil-mediated service or function for a prescribed application may be considered a reduction in soil quality (Karlen et al. 1997). The large-scale implementation of biochar to address the problems discussed above may provoke unexpected, or unintended, consequences (Kookana et al. 2011), such as a reduced herbicide efficiency (Kookana 2010; Kookana 2013) or herbicide biodegradation (Jones et al. 2011), plant growth inhibition due to provocation of nutrient limitations (Deenik et al. 2010), 'priming' native soils' organic matter leading to its loss (Wardle et al. 2008; Zimmerman et al. 2011), and potential ecotoxicological (Kookana et al. 2011) or ecological (McCormack et al. 2013) effects on soil organisms and soil functions.

4. Justification and structure of the thesis

Biochar's potential effects on soil quality is the topic of this thesis, with an emphasis on biological methods for its evaluation. To date, information on biochar's effects on soil biology is quite limited (Thies and Rillig 2009; Lehmann 2011). Plants are the soil biota best represented in the literature. Meta-analyses have shown that effects on primary productivity have ranged widely from negative to highly positive, though the grand mean of reported studies represents a net increase in crop productivity (Jeffery et al. 2011; Biederman et al. 2012). However, systematic biological testing of biochar effects has been lacking (Lehmann et al. 2011), with most trials normally involving one or two biochar products or test species, and the need of longer studies to deal with biochar aging effects has been expressed (Jeffery

et al. 2011). Information on soil fauna is particularly lacking, especially effects on their abundance and function, and these are considered existing high research priorities (Lehmann et al. 2011).

To address this current lack of systematic testing of biochar's effects on soil biota, and in anticipation of unintended effects, a substantial portion of this thesis is based on ecotoxicological approaches of evaluation. The field of ecotoxicology is equipped to detect important changes in environmental health at multiple levels of biological complexity and organization, specifically the suborganism, organism, population, community, or ecosystem (Moriarty 1999; Newman and Clements 2008). Therefore, laboratory bioassay studies were undertaken, which are highly reductionist and function at the level of the individual, thereby allowing the detection of risks which may be acute or chronic, thereby affecting fitness. Secondly, complementary studies were undertaken in the field for a broader evaluation of effects in a more realistic setting.

4.1 Laboratory-scale experiments

Very little information is available on biochar's effects on soil biota, particularly invertebrates, and ecotoxicological characterization of biochars is scarce in the field. However, its necessity is increasingly recognized, and some limited testing with plants has been proposed as a component of biochar materials characterization (IBI 2013; Busch et al. 2011). In this thesis, the potential toxicity of six biochar materials was tested on plants and invertebrates using organism-level laboratory bioassays. As the work involved materials proceeding from different feedstocks and production methods, the main objective was determine what (tested) biochar materials may cause inhibitory or stimulatory effects on model soil biota, at what application rates, and based on the diverse physico-chemical nature of the products, to what might any responses be attributed. This work corresponds to **Chapter 1** (‘*Short-term unintended effects of biochars on plant growth in an*

alkaline soil') and **Chapter 2** ('*Biochars provoke diverse soil mesofauna responses in laboratory bioassays*'). Also, leading from **Chapter 2**, an alternative, population-level endpoint was developed in **Chapter 3** ('*Soil Collembolan population structure altered by biochar material and application rate in bioassays*').

4.2 Field-scale experiments

Field effects of biochar application were investigated in a mesocosm experiment initiated at the IRTA Torre Marimón research station (Caldes de Montbui, Barcelona, Spain), using a gasification biochar in a simulated agricultural system typical to the region. Namely, this was a spring barley (*Hordeum vulvare* L.) variety with three biochar addition rates (0, 12 and 50 t ha^{-1}) combined with fertilization by dried pig slurry. The aim was to evaluate the effect of biochar on soil quality, by means of physical, chemical and biological properties and indicators, in a model Mediterranean agricultural system, so the success of an agricultural crop in response to biochar was evaluated, as well other soil quality parameters within these compartments. This study comprises of **Chapter 4.1** ('*Pine gasification biochar addition to mesocosms under barley cultivation. I. Effects on soil physical properties*'), **Chapter 4.2** ('*II. Effects on soil chemical properties*) and **Chapter 4.3** ('*III. Effects on soil biological properties*).

5. Additional considerations

While the use of biological methods for evaluation of biochar effects on soil quality has been the emphasis of the thesis at each of these two scales of ecological relevance (laboratory, field), as already mentioned some parameters within the chemical and physical compartments were also considered. Also, it is worth noting that all the fieldwork was carried out in Mediterranean conditions and with

alkaline, calcareous soils. Studies under these conditions are very limited within the current available biochar literature.

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Chapter 1 Short-term unintended effects of biochars on plant growth in an alkaline soil

Abstract

Biochar has demonstrably improved crop yields in weathered and acidic soils, but studies in alkaline soils are particularly lacking. Alkaline soils are characteristic of arid and semiarid climates, so organic amendments which increase soil organic matter content and retain humidity are naturally of interest in these conditions. In this study with biochars obtained from different pyrolysis technologies, we found that some fresh biochars' chemical properties negatively impacted short-term plant growth in an alkaline (pH > 8.2) test soil.

Three biochar production methods, slow pyrolysis, fast pyrolysis, and gasification, were used to obtain biochar from pine wood, poplar and sewage sludge. Chemical characterization of six fresh biochars and biochar-soil mixtures were carried out in conjunction with lettuce and ryegrass growth tests to attempt to relate biochar chemical properties to effects on plant biomass. A sewage sludge slow pyrolysis char was generally stimulatory at the addition rates tested, as was a slow pyrolysis pine wood char at a intermediate concentration, while gasification and fast-pyrolysis pine and poplar wood chars were strongly inhibitory, with reductions in both above- and below-ground biomass at realistic application rates of 5-19 t ha^{-1}. Thus, it was seen that pyrolysis type had a stronger influence on plant responses than feedstock.

To explain inhibition of plant growth, potential effects of biochar components including metals and phytotoxic compounds were considered, but with available data these were seen to be much less probable than effects due to nutrient availability. Statistical association of plant responses with biochar composition and biochar amended soil

chemical properties led to the assessment that plant responses were most related to volatile matter content and total P content, whose availability was regulated by biochar pH and Ca content.

1. Introduction

Biochar is thermally decomposed biomass whose use is destined for application to soils (Granatstein et al. 2009; Sohi et al. 2009; Verheijen et al. 2009). Though very similar to charcoal, a combustible obtained with a traditional technology for storing energy, biochar is defined as pyrolyzed biomass applied to soils to improve crop productivity, enhance soil properties, and increase carbon storage in the soil due to its highly recalcitrant carbon content (Lehmann and Joseph 2009). This practice leads to changes in soil physical (Asai et al. 2009; Oguntunde et al. 2008), chemical (DeLuca et al. 2009), and biological (Lehmann et al. 2011) properties. In tropical systems improvements in crop productivity have often been cited (Verheijen et al. 2009), whereas in many cases this is likely due to improvements in soil pH or alleviation of Al toxicity in highly weathered soils (Blackwell et al. 2009). However, not all biochars are created equal as the saying might go, and different materials may cause different crop responses under the same environmental conditions (Deal et al. 2012; Gaskin et al. 2010), thus provoking the question of what biochar properties might lead to observed differences? There is an immense diversity of biochar materials, with important differences in elemental composition (Brewer et al. 2009), ash content (Deal et al. 2012), ion retention and release (Silber et al. 2010), and recalcitrance to biotic decay (Bruun et al. 2008), among others. These differences have important impacts on soil processes, and also given the need for standardization of agricultural products, they require a classification system (Joseph et al. 2009). Recent efforts have included the proposal of appropriate

laboratory testing guidelines for biochar characterization (IBI 2013), including a germination test. Whereas much information is already available regarding elemental and chemical transformations associated with distinct pyrolytic production methods, these have not been adequately tied to biological responses.

There are a number of reasons why bioassay screening of biochar is critical if biochar is to be applied on large scale for agricultural purposes or atmospheric CO_2 mitigation as a carbon sink. Firstly, despite many studies promoting biochar application to soil as a soil amendment, mainly centered on agronomical benefits, little attention has been paid to potential unintended effects (Kookana et al. 2011), such as the ecotoxicological risks of its application to soils. Since biochar is produced from biomass, including polluted organic wastes, pollutant content such as heavy metals in biochars could be significant and present potential environmental risks when applied to soils, as has been cautioned for biochars produced from animal manures and sewage sludge (Kookana et al. 2011). Also, organic pollutants may include polycyclic aromatic hydrocarbons (PAHs) and dioxins (Garcia Perez 2008; Schimmelpfennig and Glaser 2012).

The field of soil ecotoxicology has long relied on the use of plant bioassays to assess “safe” levels of potential toxins (Paton et al. 2005), which have been codified in test protocols (e.g. OECD 208); we would argue that the screening tools should be utilized when contaminants or unintended effects due to biochar application are suspected. Secondly, bioassays with higher plants measure a direct impact on a critical endpoint of this technology, the primary productivity of crops, which may be impacted by biochar´s demonstrated alterations of soil nutrient availability and soil processes (Clough and Condron 2010; Jones et al. 2011; Zimmerman et al. 2011; Bruun et al. 2012). Finally, standardization of biological characterization may allow future screening and materials characterization by biological methods previous to field implementation

without the need of specialized equipment, which could be critical for the technology's adoption in underdeveloped areas of the world.

Biological assessment is not always integrated into the studies undertaking the chemical and physical characterization of biochar, therefore making it difficult to predict outcomes of measured properties on plants or model organisms. Also, recommendations or guidelines for "appropriate" application rates are lacking within the field (Hossain et al. 2010; Gaskin et al. 2010) and bioassays might have a role for this purpose. Furthermore, what properties may be altered by fresh chars and cause effects on plants in the short term is still under debate.

In this study we addressed the influence of biochar composition on soil-biochar mixture chemical properties and on plant above-ground and below-ground biomass. Specifically, the objective was to associate biochar composition and the changes exerted on soil chemical properties with measured responses of above and below-ground biomass to elucidate possible causes of effects on plant productivity.

2. Methods

2.1 Soil

The test soil corresponded to the 20 cm topsoil of a *Fluventic Haploxerept* agricultural soil, harvested from the Torre Marimón experimental farm site in Caldes de Montbui, NE Spain, uncultured and free of agrochemicals for at least the previous 7 years. Main soil properties are shown in **Table 1**.

Table 1: Test soil main properties.

Parameter	Measurement
Sand (0.05-2mm)	59.6%
Coarse silt (0.02-0.05mm)	12.5%
Fine silt (0.002-0.02 mm)	10.5%

Clay (<0.002 mm)	17.0%
pH (1:2.5 H20)	8.2
Electrical conductivity (25°C)	0.21 dS m^{-1}
Carbonates	6%
Organic matter (Walkley-Black)	1.60%
N (Kjeldahl)	0.08%
P (Olsen)	27 mg kg^{-1}
Ca (ammonium acetate)	5557 mg kg^{-1}
Mg (ammonium acetate)	233 mg kg^{-1}
K (ammonium acetate)	159 mg kg^{-1}
Na (ammonium acetate)	62 mg kg^{-1}
Cd (ICP-MS)	<0.5 mg kg^{-1}
Cu (ICP-MS)	17 mg kg^{-1}
Ni (ICP-MS)	7 mg kg^{-1}
Pb (ICP-MS)	25 mg kg^{-1}
Zn (ICP-MS)	65 mg kg^{-1}
Hg (ICP-MS)	<40 μg kg^{-1}
Cr (ICP-MS)	10 mg kg^{-1}

2.2. Biochars characterization

Biochars proceeded from three feedstocks and three pyrolysis methods (**Table 2**). Elemental analyses were carried out on fresh biochar materials. Analyses for C and H were carried out using a Flash 2000 C.E. elemental analyzer (Thermo Fisher Scientific), N by a Flash EA 1112 elemental analyzer (Thermo Fisher Scientific), and S by ICO-OES spectrometry using a Varian 725-ES Radial ICP Optical Emission Spectrometer (Varian Inc.). Total O was determined by subtraction according to ASTM D1762-84 as follows:

O (%w/w) = 100 - ash (%w/w) – C (%w/w) – N (%w/w) – H (%w/w)

Elemental ratios were calculated using the molar concentrations of the elements concerned, each calculated by dividing the total weight of the element by its molecular weight (Van Krevelen 1961). Metals were analyzed by acid digestion of 0.5 g of sample in Baker Instra-Analyzed HCl - HNO_3 3:1 in a high-pressure teflon reactor heated at 200°C in an Ethos Plus microwave (Milestone Srl.). The digested sample was filtered and the residue washed with HNO_3 0.5M to 100

ml. Determination of Ca, Mg, Na, K, P and Fe were carried out using a Polyscan 61E ICP-MS (Thermo Jarrell-Ash Corp.). Remaining element quantification was carried out using a 7500ce ICP-MS (Agilent Technologies Inc.).

The proximate analysis of biochar products after sample heating at different temperatures was also performed, namely sequential loss on ignition (LOI), volatile matter (VM) and ash content. LOI is a simple method initially developed for estimating the content of organic matter and carbonate minerals in sediments and sedimentary rocks (Dean 1974). It is a gross measure since some losses via oxidation or dehydratation of other minerals may bias the results (Santisteban et al. 2004), although such biasing will usually be limited in biochars, whose composition is mainly organic. LOI was conducted on ground samples in triplicate in a muffle furnace, first at 375° C for 18h, without acid pre-treatment (used for removal of soot and graphitic black carbon) to evaluate biochar organic content except the soot fraction (Gustafsson et al. 1997, Poot et al. 2009); at 550 °C for 5 h to remove soot (Gustafsson et al. 1997), hence representing the complete oxidation of the organic carbon fraction in biochar; and finally at 1100 °C for 5 h, which should mainly remove carbonates (Santisteban et al. 2004). VM was determined in triplicate following ASTM D1762-84 by heating ground samples in a covered (oxygen-limited) crucible at 950 °C for 6 minutes and determining weight loss, which reflects the uncharred organic content in chars (Deenik et al. 2010). Ash content was also evaluated in triplicate following the same ASTM protocol by heating ground samples in an uncovered crucible at 750 °C for 6 h and determining weight loss, representing the mineral content in chars.

Table 2: Biochars studied and identification codes.

Material	Description
CL	*Populus nigra* (Poplar) wood chip biochar produced in a slow pyrolysis reactor 500 – 550 °C
CR	*Populus nigra* wood chip biochar produced in a fast pyrolysis reactor at 430 – 510 °C
FL	Wastewater sludge biochar produced produced in a slow pyrolysis reactor 500 – 550 °C
PG	*Pinus pinaster + P. radiata* wood chip biochar produced in a gasification reactor at 600 – 900 °C
PL	*Pinus pinaster + P. radiata* wood chip biochar produced in a slow pyrolysis reactor 500 – 550 °C
PR	*Pinus pinaster + P. radiata* wood chip biochar produced in a fast pyrolysis reactor at 440 – 480 °C

The characterization of the main chemical bonds that are related to functional groups present in the biochars was done by Fourier-transform infrared spectroscopy analysis (FTIR) of dry (105 °C), ground biochar samples passed through a 100 μm sieve. Spectrum were registered in triplicate at standard infrared resolution (4 cm^{-1}) in the mid-infrared range of 600-4000 cm^{-1} using a Bruker Tensor 27 spectrophotometer working in attenuated total reflectance (ATR) mode with diamond reflection.

2.3. Biochar extractable components

Labile carbon content of the biochars was assessed using a hot water extraction following Rovira and Vallejo (2007), thereafter referred as hot water-extractable carbon (C_{hw}). Briefly, oven-dry ground biochar samples of 0.5 g were extracted with 20 ml of deionized water in sealed Pyrex tubes heated in aluminum blocks at 105° C for one hour after which the extract was filtered with Whatman 42 filter paper and stored at -20 °C. Later, extracts were evaluated for dissolved organic carbon (DOC) using the acid dichromate method described in Brookes and Joergensen (2006) with the modification that DOC extract and acid volumes were halved.

Water soluble components of biochar were also assessed in two successive washings. The first was carried out by submitting 1:10 (g / ml deionized water) biochar slurries to 24 h vertical agitation at 60 rev min^{-1}. Then, slurries were vacuum filtered with Whatman 42 filter paper, pH and electrical conductivity (EC) were measured immediately, and extracts were stored afterwards at -20 °C. The filtered residue was dried overnight at 105 °C, weighed, and the process was repeated again.

A 5:50 ml dilution of frozen extracts was prepared, from which one homogenized 5 ml sample was taken for ion analysis by wet chromatography. Ion chromatography analysis of Na^+, K^+, Mg^{2+}, Ca^{2+}, NH_4^+ was carried out with a CS12A Dionex cation column on a Dionex

ICS-1100 Ion Chromatograph (Dionex, Sunnyvale, USA), and for Cl^-, NO_2^- NO_3^-, HPO_4^{2-} and SO_4^{2-} with a AS4A-SC Dionex anion column on a Dionex DX-100 Ion Chromatograph (Dionex, Sunnyvale, USA).

2.4 Soil-biochar mixtures characterization

The six biochar materials were tested in a wide range of concentrations of 0.4, 0.9, 2.1, 4.9, 11.3 and 26%, in soil (as dry matter) representing a range of theoretical application rates from 10.6 t ha^{-1} to 26% / 676 t ha^{-1}, with the intention that unrealistically high rates would aid the establishment of recommend maximum application rates and force any effects. Maximum water-holding capacity (WHC) at each biochar concentration was determined by water-saturating 50 g samples for 2 h followed by draining for 24 h at room temperature, and measuring moisture as weight loss after drying at 105 °C overnight. EC and pH were determined in triplicate from aqueous extracts. Briefly, 15 g of soil mixture and 75 ml of deionized water (1:5) were vertically agitated in 150 ml polyethylene cups for 2 h at 60 rev min^{-1}. The extract was subsequently centrifuged and the supernatant was filtered through Whatman 42 filter paper. pH and EC were immediately measured, and the extracts stored at -20 °C. Later, a portion of the extracts for pH and EC determinations above were pooled and a 5:50 ml dilution was prepared, from which a homogenized 5 ml sample was taken for ion chromatography analysis to determine water-soluble concentrations of major cations and anions as described in the previous section.

2.5 Plant growth tests

The growth test was conducted following guidelines of OECD 208 using *Lactuca sativa* and *Lolium perenne* as test species. Chars were mixed dry with test soil at the concentrations described above before being transferred to plastic 300 ml germination vessels equipped with

bottom wicks. Four replicates were prepared per test species and concentration, including control. Ten seeds were placed on the surface of soil-biochar mixtures and lightly covered with soil. After, vessels were placed in a germination chamber equipped with bottom-irrigation system with deionized water. Growth tests were conducted within the germination chamber set at 16:8 light:dark cycle at 300 $\mu E/m^2/s$, and constant 22°C and 70% humidity. Seedlings were thinned to the three most 3 vigorous plants after 4 days for *L. sativa* and 7 days for *L. perenne*. Replicates were arbitrarily rearranged within the chamber weekly. After 14 days the test ended and all plants were carefully removed, their roots washed, rinsed with deionized water, dried at 70 °C for 24 h, and above-ground and below-ground parts separated and weighed to obtain dry weight (DW), pooling the three plants in each replicate.

2.6 Statistical analysis

2.6.1 Biochars and mixtures properties

All statistical analyses were carried out in R. Analysis of variance (ANOVA) followed by Tukey´s HSD post-hoc significance tests were used to determine effect of feedstock and pyrolysis method on measured biochar properties. Linear regressions were fit to improve the understanding of effect of biochar concentration on concentrations of water-soluble ions of each mixture. Examination of biochar chemical properties revealed important differences in soluble ion content between materials, with implications for correct model construction and interpretation. NO_2^- was excluded from the statistical assessment since it was undetectable in most of the samples in accordance with its low residence time in the soil, as it is quickly converted to NO_3^- under aerobic conditions.

2.6.2 Plant tests

In the assessment of differences in above-ground and below-ground biomass within materials, non-parametric tests were used since homogeneity of variance was violated according to Levene's test. Kruskall-Wallis test was used for global differences testing, and Mann-Whitney post-hoc tests were used to identify significant differences with respect to control at specific biochar concentrations.

Dose-response models and effective concentrations (EC_x) were calculated using the *drc* package for R (Ritz and Streibig 2005). EC_x of x=10-20 (i.e., concentrations leading 10-20% reduction in measured endpoint), equivalent to the no observed effect concentration or NOEC, are commonly established as acceptable limits for potential contaminants (Isnard et al. 2001; Arnold and Cotsifas 2008), which can be also taken as maximum biochar application rates. Therefore, the EC_{10} was chosen as the more conservative baseline for materials comparison. Two model types were considered, Brain-Cousens (BC) models including a hormesis parameter, and log-logistic (LL); that which minimized residual standard error was chosen as the best model. EC_{10} values were thereafter compared with elemental and chemical properties of the fresh biochars using non-parametric Spearman correlations.

2.6.3 Association of biochar and soil-biochar mixture properties with plant biomass

The effects of measured soil chemical properties on aboveground biomass and belowground biomass (hereafter AGB and BGB, respectively) and root:shoot of lettuce and ryegrass were assessed using multiple linear regression models (MLM) using the *lm* function in R. Plant responses were standardized with respect to the control for each of the material-concentrations. Following the objectives of the study, the effect of the following chemical parameters was evaluated on each of the four endpoints: pH, EC measured as $\mu S\ cm^{-1}$, and water-extractable mg kg^{-1} concentrations of Ca^{2+}, K^{+}, Mg^{2+}, Na^{+}, NH_4^{+}, Cl^{-}, NO_3^{-}

, HPO_4^{2-} and SO_4^{2-} (NO_2^- not included for reasons described above). Concentration was included in the model to account for its association with ion concentration. Following elimination of collinear variables with variance inflation factor >10, the best model was selected as that which minimized the Akaike Information Criteria (AIC) using the *dredge* function in the *MuMIn* package. Standardized coefficients, β_i, were calculated as for each predictor x_i so as to allow comparison between predictors having different units of measurement; the standardized coefficient is calculated by multiplying the unstandardized predictor coefficient by the ratio of the standard deviations for the predictor and dependent variables. Constant error variance was checked visually using standardized residuals. Outliers were identified using the *outlierTest* function of the *car* package, following Bonferroni p-values for Studentized residuals and removed if corrected p was <0.05, and the Shapiro-Wilk test was used to check normality of model residuals which were taken to be normal if p>0.05.

3. Results

3.1 Biochar and soil mixtures characterization

Biochar elemental composition is shown in **Table 3**. Slow-pyrolysis wood chars had the highest C content (81-86%), whereas that of fast-pyrolysis and gasification were similar (71-73%), and the sewage sludge char was the lowest (22%). The sewage sludge char had the highest N content (2.3%), followed by the poplar chars (0.35-0.48%), and pine wood chars the lowest (0.12-0.19%). P content was highest in the sewage sludge char (5.12%), followed by the slow-pyrolysis chars (0.20-0.35%) and lowest in the fast and gasification chars (0.05-0.08%). Thus, comparing wood chars, it appears that pyrolysis method had the strongest influence on C:P ratios (P content highest in low temperature-pyrolysis materials), whereas feedstock determined C:N ratios (N content more similar within feedstocks).

Proximate analyses (LOI, VM, and ash content) and C_{hw} results are shown in **Table 4.** Ash content was significantly affected by both feedstock and pyrolysis method (two-way ANOVA $p<0.001$ for both factors). As expected, the sewage sludge char had high ash and low VM. Comparing materials of the same feedstock, fast pyrolysis and gasification materials had higher ash content than slow pyrolysis materials. Within the wood materials the gasification char had highest ash content (19%), whereas the other pine wood materials had ash content of ~2.5% and those of poplar ~4.5%. VM was significantly affected by pyrolysis method ($p<0.001$ in two-way ANOVA) but not by feedstock ($p=0.065$). Comparing pyrolysis methods fast pyrolysis materials had the highest VM; fast and slow pyrolysis materials had the greatest VM difference (estimated difference in means 14%, $p<0.001$), followed by fast and gasification (12%, $p<0.001$), and least differences in VM were between slow and gasification (2%, $p=0.040$).

FTIR spectra are shown in **Figure 1**. Within the wood feedstocks, spectra were more similar between pyrolysis methods than feedstock, whereas the gasification material (PG) was most markedly distinguished from the slow (CL, PL) and fast pyrolysis (CR, PR) materials by its lack of absorption bands related to functional groups. Overall, the greatest absorption (displacement on the y-axis) was in the order of gasification > slow pyrolysis > fast pyrolysis, particularly <2000 cm^{-1}, indicating increasing degrees of carbonization, aromaticity, and condensation of C (dehydrogenation, rearrangement and polymerization of aromatic rings).

Table 3: Elemental content of the biochar materials with error expressed as SD where applicable. C:N and C:P are mass ratios, whereas H:C and O:C are atomic ratios.

	CL	CR	FL	PG	PL	PR
N (%)	0.48 ± NA	0.35 ± NA	2.26 ± NA	0.12 ± NA	0.12 ± NA	0.19 ± NA
C (%)	81.07 ± 0.11	73.11 ± 0.08	22.34 ± 0.02	71.03 ± 0.16	86.26 ± 0.16	71.76 ± 0.04
H (%)	2.07 ± 0.02	3.27 ± 0.02	1.20 ± 0.02	0.53 ± 0.02	1.97 ± 0.02	3.40 ± 0.04
S (%)	0.04 ± NA	0.02 ± NA	1.01 ± NA	0.08 ± NA	0.02 ± NA	0.02 ± NA
O (%)	12.32	18.78	4.96	8.75	9.08	22.00
P (%)	0.20	0.06	5.12	0.08	0.35	0.05
C:P	405	1219	4	888	246	1435
C:N	169	209	10	592	719	378
H:C	0.31	0.54	0.45	0.09	0.27	0.57
O:C	0.11	0.19	0.17	0.09	0.08	0.23
Ca (ppm)	9573 ± 232	12184 ± 86	89107 ± 4341	92343 ± 3654	3769 ± 138	8273 ± 24
Mg (ppm)	1313 ± 28	1594 ± 38	11827 ± 571	2591 ± 58	980 ± 36	1420 ± 9
Na (ppm)	956 ± 75	1029 ± 18	3843 ± 165	778 ± 3	329 ± 48	476 ± 9
K (ppm)	6570 ± 229	9207 ± 381	9092 ± 598	8249 ± 291	3484 ± 121	6404 ± 220
P (ppm)	1956 ± 222	565± 10	51192 ± 3273	796 ± 113	3505 ± 91	476 ± 69
Fe (ppm)	1966 ± 126	1784 ± 46	42649 ± 2930	1527 ± 85	1213 ± 18	1577 ± 93
Zn (ppm)	130 ± 14.4	540 ± 7	3074 ± 156	823 ± 121	70 ± 24	181 ± 7
Cr (ppm)	213.2 ± 36	40.4 ± 1.4	384.9 ± 50	26.2 ± 2.2	83 ± 21	26.2 ± 0.3
Cu (ppm)	109 ± 27	29 ± 2.2	766.7 ± 87	219 ± 3.0	27 ± 3.1	12.6 ± 0.3

Ni (ppm)	253 ± 22.2	22.6 ± 0.7	249.2 ± 28.2	10.4 ± 2.4	97 ± 0.1	24.5 ± 0.3
As (ppm)	-	-	12.4 ± 1.0	-	-	-
Cd (ppm)	-	-	2.2 ± 0.26	1.2 ± 0.01	-	-
Hg (ppm)	-	-	-	-	-	-
Pb (ppm)	74.7 ± 14.4	62.1 ± 0.9	277 ± 34.4	9.1 ± 0.4	15.7 ± 0.5	10.1 ± 0.1

Results of the fresh biochar washing experiment in the first (L1) and second leachate (L2) are shown in **Table 5**. Washing reduced alkalinity in all materials. The most alkaline materials PG and FL also showed the largest reductions in pH measurements following washing (0.89-1.69 pH points), reductions in fast pyrolysis material alkalinity were intermediate (0.55-0.74), and slow pyrolysis materials showed little change (0.10-0.12). PG and FL liberated the most salts; EC measurements were significantly correlated with Na^+ (Spearman´s ρ=0.90, $p<0.001$). Washing of agronomically important nutrients in respect to their elemental concentrations are shown in **Table 6**. Extractable N as NO_3^--N and NH_4^+-N was low in all cases. K^+, on the other hand was readily extracted in wood biochars, especially in slow pyrolysis materials, but not in FL. Phosphorous extractability as HPO_4^{2-} was relatively high in the slow pyrolysis materials, whereas much lower amounts were measured for the fast pyrolysis materials, and was below detection limits for PG and FL. Soluble ion concentration in the soil-biochar mixtures followed the patterns of the fresh chars per material tested, however a few exceptions are noted: increasing biochar concentration generally decreased concentrations of NO_3^-, and increased NH_4^+, as seen in simple regressions in **Table 7**. Also, Ca^{2+} did not increase in all mixtures. Finally, HPO_4^{2-} was only detected in CL and PL mixtures.

Biochar mixture concentration effects on pH, EC, and WHC are seen in **Table 8**. Increases in pH were PG > FL > CR, and decreases were PL > PR > CL. EC increases were highest in PG and FL, though statistically significant increases within realistic application rates were only seen in the latter. As expected, WHC increased in all mixtures.

Table 4: Proximate analyses of loss on ignition (LOI), volatile matter (VM), and ash contents of fresh biochar samples, expressed as percentage of each freaction with respect to total weight, in addition to hot water-extractable C (C_{hw}), all ± SE of three replicates.

	Proximate analyses					C_{hw}
Material	LOI 375°C (%)	LOI 375-550°C (%)	LOI 550-1100°C (%)	VM (950° C 6 min)	Ash content (%)	μg C g^{-1}
CL	95.10 ± 0.01	0.67 ± 0.05	0.67 ± 0.06	14.75 ± 0.46	4.06 ± 0.09	654 ± 55
CR	93.19 ± 0.34	0.45 ± 0.03	0.90 ± 0.14	27.06 ± 0.31	4.49 ± 0.15	3285 ± 42
FL	12.46 ± 0.36	14.39 ± 0.34	3.97 ± 0.01	14.29 ± 0.34	69.24 ± 0.07	1277 ± 42
PG	76.15 ± 0.16	2.22 ± 0.37	6.1 ± 0.46	15.68 ± 0.31	19.57 ± 0.35	613 ± 42
PL	96.75 ± 0.01	0.18 ± 0.00	0.18 ± 0.01	10.67 ± 0.20	2.57 ± 0.09	933 ± 72
PR	94.96 ± 0.13	0.56 ± 0.00	0.37 ± 0.04	28.07 ± 0.22	2.65 ± 0.09	2684 ± 41

Figure 1.1: FTIR spectra of pine feedstock biochars. PG=gasification, PL=slow, PR=fast.

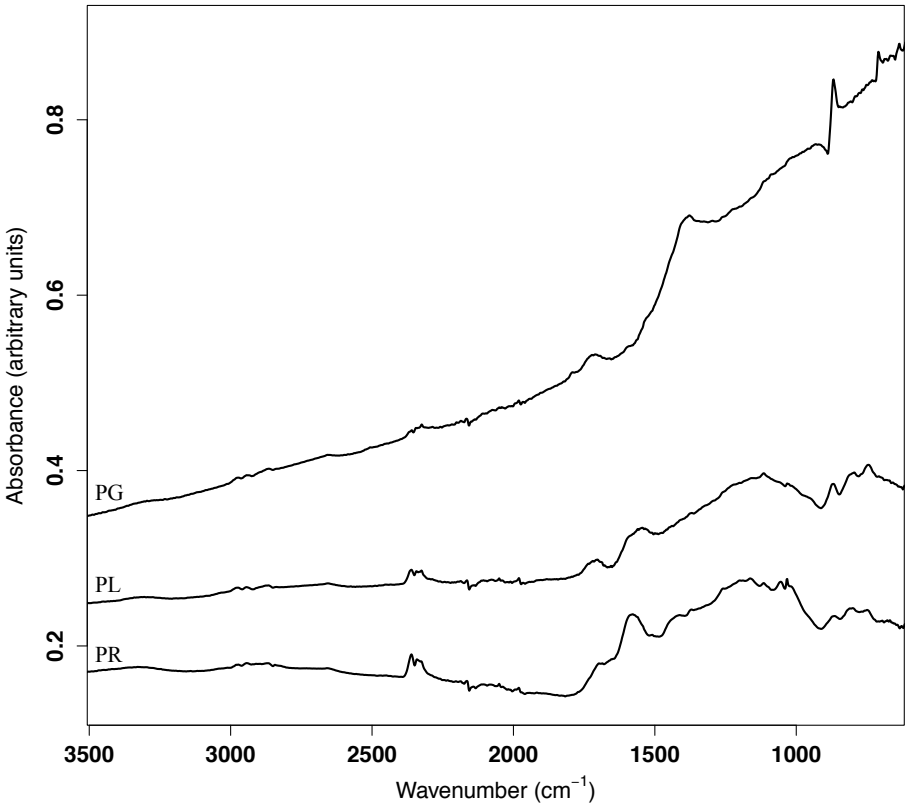


Figure 1.2: FTIR spectra of poplar feedstock biochars. CL=slow, CR=fast.

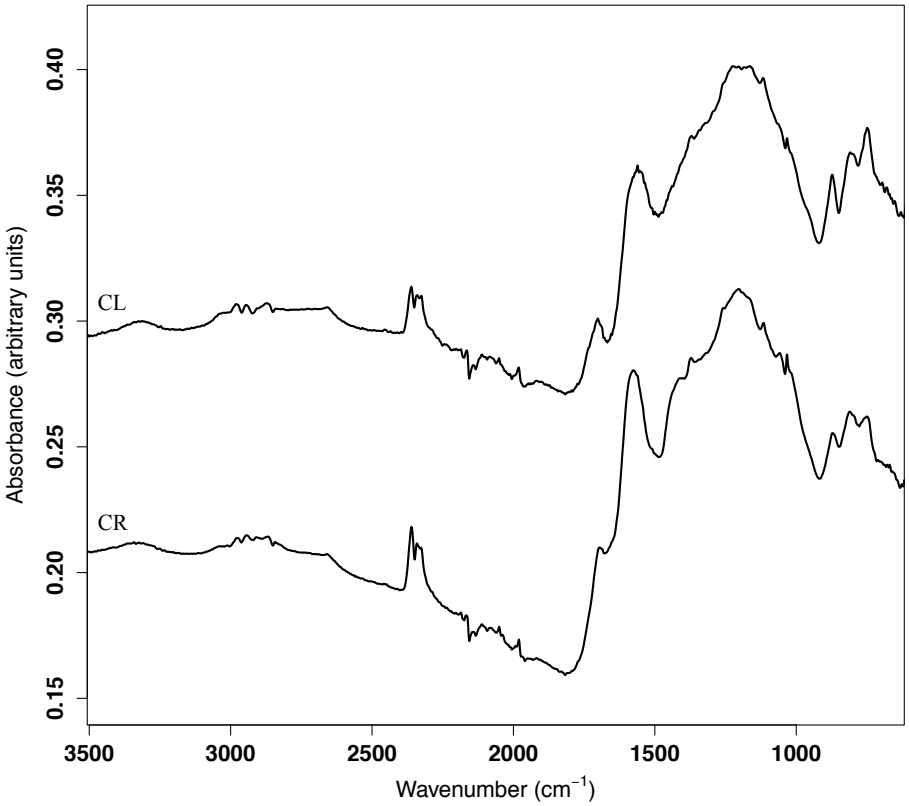


Figure 1.3: FTIR spectra of slow pyrolysis sewage sludge feedstock biochar

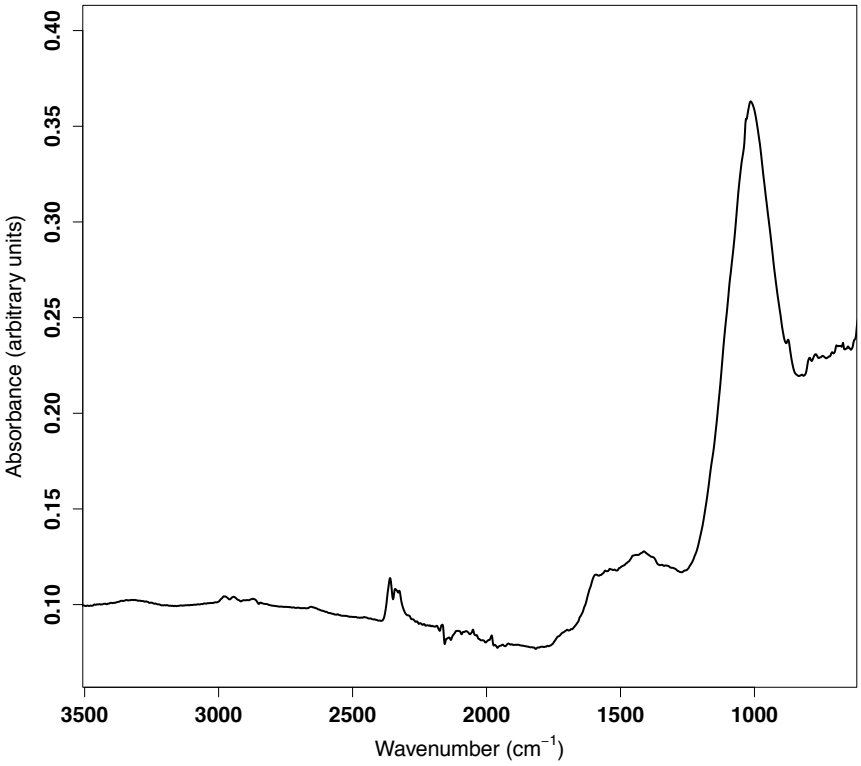


Table 5: Leachate characterization of the first (L1) and second (L2) washing of biochars. Ionic concentrations are expressed as recovered mass in mg kg^{-1} of biochar. `b.d.l.´ indicates concentrations below detection limit. For material codes refer to **Table 2**.

Sample	pH	µS cm^{-1}	Na^+ (mg kg^{-1})	NH_4^+ (mg kg^{-1})	K^+ (mg kg^{-1})	Mg^+ (mg kg^{-1})	Ca^{2+} (mg kg^{-1})	Cl^- (mg kg^{-1})	NO_2^- (mg kg^{-1})	NO_3^- (mg kg^{-1})	HPO_4^{2-} (mg kg^{-1})	SO_4^{2-} (mg kg^{-1})
CL-L1	8.21	784	184.2	105.8	1388.7	49.6	136.8	240.0	b.d.l.	17.4	1674.8	546.0
CL-L2	8.09	638	125.3	71.6	1250.7	40.1	96.9	142.2	4.9	18.7	1391.1	248.4
CR-L1	8.14	610	147.5	31.6	1153.8	50.2	192.8	174.6	b.d.l.	14.5	147.8	84.4
CR-L2	7.59	655	139.9	10.3	1269.7	46.3	161.0	142.4	b.d.l.	10.3	311.1	77.5
FL-L1	8.7	1624	186.9	28.5	115.7	136.5	2093.9	3008.6	b.d.l.	b.d.l.	b.d.l.	1282.2
FL-L2	7.83	967	179.8	28.0	106.6	304.5	1191.2	1052.3	b.d.l.	0.8	b.d.l.	2333.0
PG-L1	11.42	1888	152.8	b.d.l.	2435.7	b.d.l.	79.1	833.5	b.d.l.	27.8	b.d.l.	798.4
PG-L2	9.73	570	70.6	14.6	982.4	154.9	123.9	242.7	b.d.l.	17.8	b.d.l.	287.3
PL-L1	7.29	565	116.2	98.5	952.0	51.0	165.9	210.4	b.d.l.	20.9	3618.2	263.1
PL-L2	7.39	253	34.3	43.8	479.9	22.4	105.7	118.8	b.d.l.	16.7	1044.2	74.2
PR-L1	8.04	639	139.2	35.2	1026.6	74.3	269.7	427.2	b.d.l.	16.6	157.2	131.1
PR-L2	7.3	360	43.3	86.0	465.0	50.2	281.3	201.8	b.d.l.	7.7	65.3	58.1

Table 6: Recovery of agronomically important nutrients in biochar washing experiment repetitions 1 (L1) and 2 (L2). Expressed as percentage of total elemental concentrations of N, K, and P. Material codes in **Table 2**.

	% washing of total elemental concentrations							
	NH_4^+-N		NO_3^--N		K^+		HPO_4^{2-}-P	
Material	L1	L2	L1	L2	L1	L2	L1	L2
CL	2.06	1.40	0.08	0.09	21.14	19.04	27.63	22.95
CR	0.85	0.27	0.09	0.06	12.53	13.79	8.44	17.77
FL	0.12	0.12	0.00	0.00	1.28	1.18	0.00	0.00
PG	0.00	1.17	0.53	0.34	29.53	11.90	0.00	0.00
PL	7.70	3.42	0.40	0.32	27.32	13.78	33.31	9.61
PR	1.72	4.22	0.20	0.10	16.04	7.26	10.65	4.42

Table 7: Linear regressions fit to ion concentration (mg kg^{-1}) in a series of increasing concentrations of biochar (for codes refer to **Table 2**) mixtures (df=7), as determined by wet ion chromotography. Columns are the linear equation (eq.), R^2, and the difference between minimum and maxiumum values (diff.). '^' indicates that the fit has little explanatory power (R^2 < 0.70). NA indicates that concentrations were below detection threshold in all or only 1 concentration. † indicates that the parameter was below detection threshold in all but two concentrations (CL-HPO_4^{2-}, PR-NO_2^-).

	Ca^{2+}			Cl^-			K^+			Mg^+			NH_4^+		
Material	eq.	R^2	diff.	eq.	R^2	diff.	eq.	R^2	diff.	eq.	R^2	diff.	eq.	R^2	diff.
CL	-0.73x + 243	0.53 ^	75	0.73x + 29	0.49^	62	7.95x + 24	0.99	474	0.28x + 25	0.91	16	0.17x + 25	0.18^	22
CR	2.88x + 216	0.91	181	1.07x + 30	0.92	71	4.99x + 31	0.99	297	0.47x + 18	0.96	29	0.38x + 18	0.84	23
FL	21.11x + 223	0.98	121 3	36.11x + 74	0.98	208 9	0.74x + 33	0.81	44	-0.25x + 28	0.39 ^	30	0.28x + 24	0.22^	36
PG	-1.91x + 263	0.56 ^	145	9.05x + 56	0.91	509	20.30x + 8	0.99	117 9	-0.31x + 26	0.66 ^	21	0.70x + 14	0.27^	42
PL	-0.25x + 237	0.18 ^	41	-0.76x + 79	0.65^	51	3.02x + 42	0.95	197	0.14x + 29	0.5^	13	0.60x + 14	0.82	44
PR	3.11x + 251	0.95	189	1.98x + 30	0.98	126	2.64x + 37	0.97	164	0.61x + 24	0.99	37	0.73x + 7.7	0.75	52

	NO_2^-			NO_3^-			Na^+			HPO_4^{2-}			SO_4^{2-}		
Material	eq.	R^2	diff.	eq.	R^2	diff.	eq.	R^2	diff.	eq.	R^2	diff.	eq.	R^2	diff.
CL	NA	NA	NA	-0.17x + 84	0.22^	25	1.03x + 18	0.96	60	0.83x + 116	1†	29	4.02x + 50	0.94	23 4
CR	NA	NA	NA	-0.46x +	0.71	29	0.93x +	0.97	58	NA	NA	NA	0.96x +	0.34^	99

				80			19						35		
				-0.51x +			1.2x +						3.04x +		18
FL	NA	NA	NA	80	0.67^	31	17	0.95	69	NA	NA	NA	51	0.94	7
				-0.20x +			1.98x +						11.28x +		64
PG	NA	NA	NA	25	0.52^	17	19	0.95	113	NA	NA	NA	52	0.93	3
				-0.60x +			-0.10x	0.16		12.78x -		76			12
PL	NA	NA	NA	86	0.80	42	+3 2	^	16	10.65	0.95	1	1.9x + 52	0.96	0
				-0.84x +			0.38x +						0.87x +		
PR	0.71x+3	1†	1	100	0.71	56	16	0.96	24	NA	NA	NA	45	0.69^	71

Table 8: Water holding capacity (WHC), pH, and electrical conductivity (EC) of the soil-biochar mixtures in increasing concentrations. Material codes in left-hand column, refer to **Table 2**. Within a material, boldface value indicates statistically-signficant difference with respect to control as evaluated by a Mann-Whitney U test.

Concentration (%)		0	0.4	0.9	2.1	4.9	11.3	26
CL	WHC (%)	44	41	**49**	**54**	**65**	**102**	**162**
	pH	8.70	8.61	8.64	**8.59**	**8.55**	**8.50**	8.65
	EC (μS cm^{-1})	136	133	132	**132**	155	**190**	**284**
CR	WHC (%)	46	**50**	**50**	**59**	**71**	**95**	**217**
	pH	8.45	**8.54**	**8.53**	**8.54**	**8.58**	**8.54**	**8.78**
	EC (μS cm^{-1})	142	114	114	117	127	171	199
FL	WHC (%)	37	**40**	**39**	**41**	**42**	**45**	**49**
	pH	8.61	8.71	**8.75**	**8.77**	**9.03**	**9.39**	**9.85**
	EC (μS cm^{-1})	115	**127**	**132**	**146**	**185**	**275**	**557**
PG	WHC (%)	41	47	52	**55**	**59**	**83**	**128**
	pH	8.59	**8.62**	**8.94**	**8.86**	**9.46**	**9.72**	**10.12**
	EC (μS cm^{-1})	126	115	124	117	107	**206**	**402**
PL	WHC (%)	44	**46**	**47**	**52**	**60**	**76**	**106**
	pH	8.45	8.42	**8.40**	**8.37**	**8.30**	**8.00**	**7.62**
	EC (μS cm^{-1})	142	142	144	144	164	184	156
PR	WHC (%)	44	**47**	**48**	**51**	**59**	**80**	**115**
	pH	8.65	8.63	8.52	8.60	**8.47**	**8.46**	**8.33**
	EC (μS cm^{-1})	128	123	128	126	150	**170**	**230**

3.2 Plant growth tests

3.3.1 Biochar dosage effects

Biochars had diverse effects on above-ground (AGB) and below-ground biomass (BGB) endpoints (**Figures 2** and **3,** respectively). For lettuce, Kruskal-Wallis tests detected significant effects of concentration on both AGB and BGB for all biochars (data not shown). For ryegrass all biochars had effects on AGB except PL, and all on BGB except PL and FL. However, Mann-Whitney post-hoc tests for ryegrass revealed significant differences on BGB for FL concentration 11.3%. Overall correlations between AGB and BGB were very strong and highly significant for both ryegrass (ρ=0.94, $p<0.001$) and lettuce (ρ=0.95, $p<0.001$). Overall, plant growth inhibition, either measured as ABG and BGB were generally observed for fast-pyrolysis wood biochars and gasification char, while stimulation was observed in the sludge biochar and one concentration of a slow pyrolysis char.

Figure 2: Plant responses for above-ground biomass of *Lactuca sativa* (lettuce), and *Lolium perenne* (ryegrass). Y-axis unit is deviation of concentration mean with respect to control, and x-axis indicates the biochar concentration. Statistical differences with respect to control as evaluated by Mann-Whitney U test are indicated with *.

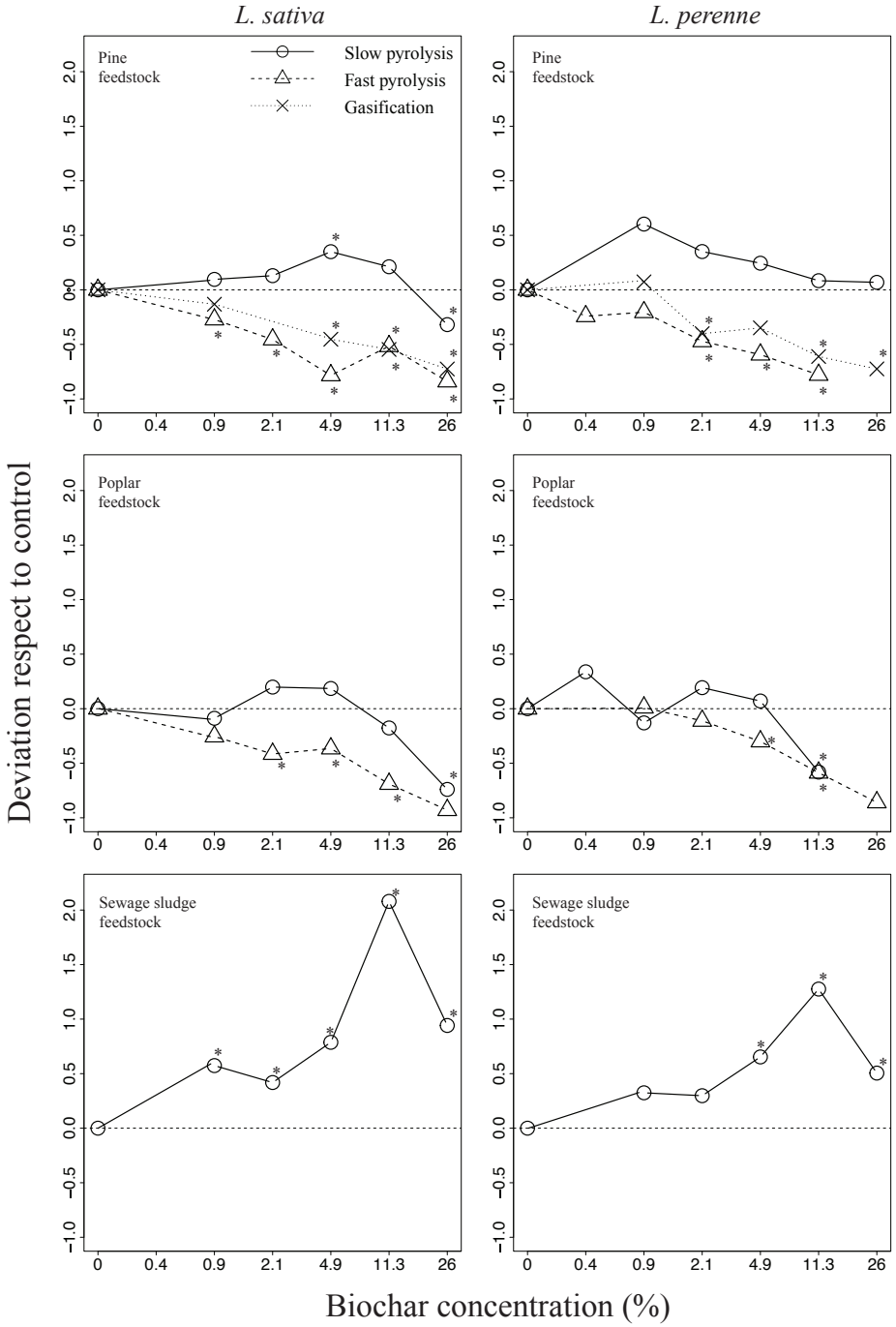
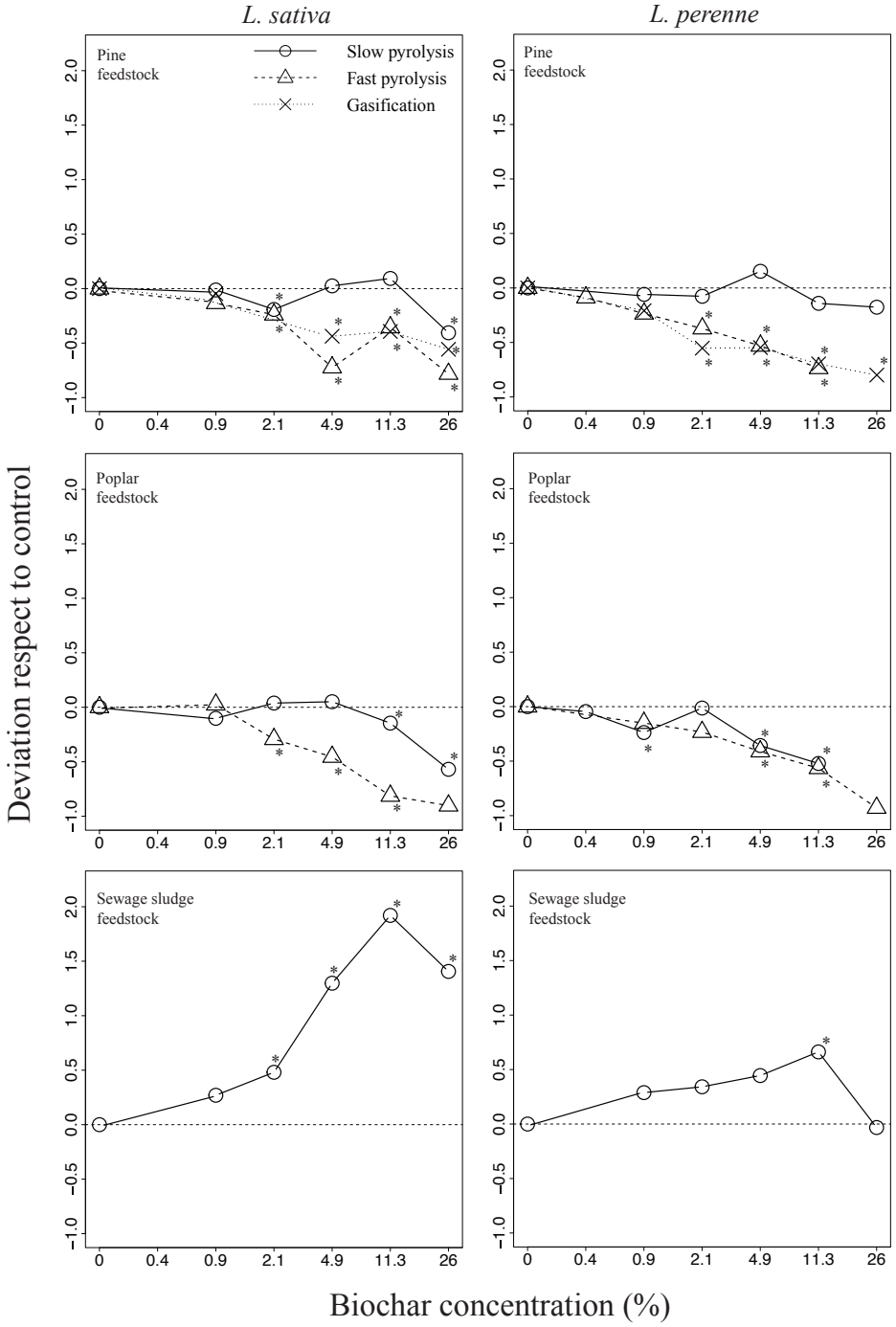


Figure 3: Plant responses for below-ground biomass of *Lactuca sativa* (lettuce), and *Lolium perenne* (ryegrass). Y-axis unit is deviation of concentration mean with respect to control, and x-axis indicates the biochar concentration. Statistical differences with respect to control as evaluated by Mann-Whitney U test are indicated with *.



3.3.2 Dose-response models

EC_{10} values for each of the materials in relation to AGB and BGB are shown in **Table 9**. BC models were chosen over LL in many cases since they were better able to fit stimulatory effects. EC_{10} show that CR, PG and PR were the most inhibitory materials for all endpoints, whereas EC_{10} for CL, PL and FL corresponded to unrealistically high application rates, causing model non-convergence in some cases, in which case these endpoints EC_{10} were reported as over the maximum concentration tested (>26%). Considering ryegrass EC_{10}, the order of most-to-least inhibitory did not change between AGB and BGB, though it is seen that roots were more impacted with the exception of PR, in which AGB was slightly more inhibited. For lettuce, AGB inhibition was PR > CR > PG, and BGB inhibition was PG > PR > CR. In contrast to ryegrass, lettuce AGB was overall more inhibited than BGB.

3.3.3 Plant endpoint models

Each regression model for both plants' BGB and root:shoot endpoints initially met the conditions of model adequacy, whereas for AGB one outlier were identified and removed for each species. Chemical explanatory variables included in the model for each species and endpoint, expressed as standardized coefficients, are shown in **Table 10**. Biochar concentration was significantly associated with negative effects on AGB and BGB but not for root:shoot endpoints. pH was significantly associated with increased lettuce AGB and BGB. Ca^{2+}, NH_4^+, NO_3^-, and HPO_4^{2-} were associated with positive effects. For root:shoot, the models selected only Na^+ (neg. for ryegrass, pos. for lettuce), and had very low explanatory power. Specific ionic concentrations and their implications for characterization of biochar materials will be discussed further in the following sections.

Table 9: Fit dose response models and EC_{10} (CI lower, CI upper) of each species-material combination for aboveground biomass. LL.3 indicates fit of a 3-parameter log-logisitic model, and BC.4 indicates fit of a 4-parameter Brain-Cousens model. Using EC_{10} as criteria, within species results are ordered from most to least harmful. Finally, EC_{10} concentration values have been transformed to t ha^{-1} assuming application to 20 cm depth and bulk density of 1.3 g cm^{-3}. Material codes in **Table 2**.

Test organism	Biochar	Curve fit	EC_{10} (%)	EC_{10} (t ha^{-1})
L. perenne	PR	LL.3	0.21 (-0.12,0.53)	6.0
Above-ground	PG	BC.4	1.61 (0.03,3.18)	18.7
	CR	LL.3	1.97 (-0.15-4-10)	51.2
	CL	LL.3	6.64 (-5.43,18.73)	172.6
	FL	NA	>26	>676
	PL	NA	>26	>676
L. sativa	PR	LL.3	0.20 (0.01,0.38)	5.2
Above-ground	CR	LL.3	0.32 (-0.41,1.06)	8.3
	PG	LL.3	0.45 (-0.12,1.02)	11.7
	CL	BC.4	10.87 (6.38,15.35)	282.6
	PL	BC.4	19.176 (12.95,25.39)	498.7
	FL	NA	>26	>676
L. perenne	PR	LL.3	0.33 (0.06,0.59)	8.6
Below-ground	PG	BC.4	0.45 (-0.13,1.03)	11.7
	CR	LL.3	0.94 (-0.50,2.39)	24.4
	CL	LL.3	2.77 (-1.68,7.22)	72.0
	FL	BC.4	25.29 (13.70,36.89)	657.5
	PL	NA	>26	>676
L. sativa	PG	LL.3	0.35 (-0.26,0.96)	9.1
Below-ground	PR	LL.3	0.91 (0.11,1.72)	23.7
	CR	LL.3	1.06 (0.10,2.02)	27.6
	CL	LL.3	10.35 (3.29,17.41)	269.1
	PL	BC.4	23.96 (-4.61,52.54)	623.0
	FL	NA	>26	>676

Table 10: Multiple linear model results for two plant species endpoints of above-ground (AG) and below-ground (BG) biomass and root:shoot ratio. Values shown for each chemical parameter are the standardized coefficient β with statistical significance at CI=0.95 indicated as 0.05-0.01 (*), 0.009-0.001 (**), and < 0.001 (***). Also provided are the model test statistic, degrees of freedom (df), and significance and corrected R^2. Material intercepts not shown.

	L. sativa			*L. perenne*		
Parameter	AGB	BGB	Root:shoot	AGB	BGB	Root:shoot
Ca^{2+}	0.43 **	0.44 **		0.66 ***	0.43 **	
Mg^{2+}	0.13	0.28		-0.13		
NH_4^+	0.36 *	0.24		0.52 ***	0.47 **	
NO_3^-	0.34	0.36 *			0.25	
Na^+			0.72 **			-0.42 *
HPO_4^{2-}	0.63 ***	0.51 **		0.79 ***	0.73 ***	
pH	0.79 **	1.00 ***	-0.40	0.09	0.27	0.13
Biochar concentration	-1.05 ***	-0.87 ***		-1.16 ***	-1.15 ***	
df	27	28	33	28	28	33
F	7.55	7.83	4.48	10.84	11.18	2.38
p	<0.001	<0.001	0.019	<0.001	<0.001	0.108
R^2	0.57	0.58	0.17	0.63	0.64	0.07

4. Discussion

4.1. Feedstock and pyrolysis effects on biochar composition

As mentioned above, LOI-375 mainly reflects the organic matter fraction of biochars as well as soot (Gustafsson et al. 1997; Poot et al. 2009). Organic matter was the main fraction of most wood chars (93-97%), with the exception of PG (76%) and FL (12%). LOI-550, representing the remaining organic fraction (soot) (Gustafsson et al. 1997), was very low in most biochars (0.2-0.7%) with the exception of PG (2%) and FL (14%), although in the last case losses of minerals other than carbonates may have also occurred (Santisteban et al. 2004). Finally, LOI-1100, mainly reflecting carbonate content, was very low in most biochars (0.2-0.9%) with the exception of FL (4%) and PG (6%). Accordingly, these two were the only chars that showed effervescence when treated with hydrochloric acid. The high positive correlation between LOI-1100 and char Mg and Ca contents (ρ=0.94 both cases) suggest again that Mg and Ca carbonates likely explain the larger losses of these materials.

VM is a useful measure of uncharred content in biochars, and is to some extent related to the stability of the material, mainly corresponding to aliphatic components (Zimmerman et al. 2010) or to less carbonized substances. In our study VM content was associated with pyrolysis method, with higher values in the fast pyrolysis chars, in agreement with Bruun et al. (2012). VM also showed high positive correlation with total organic matter content in chars, i.e. LOI-375 (ρ=0.89, p=0.03), as well as with C:P (ρ=0.94, p=0.01), suggesting that these properties are also conditioned by the pyrolysis method.

As expected, ash content was positively correlated with Ca, Mg, Na, and K content, as well as LOI-1100 (correlations not shown) since these are the main elements in the inorganic fraction of chars.

Biochar functional group composition is important for biochar classification (Joseph et al. 2009), determining char surface sorption (Amonette and Joseph, 2009), including nutrients (Chan and Xu 2009), and also determining important properties such as CEC and pH (Joseph et al. 2009). All biochars showed very limited content of aliphatic groups, whose presence is usually strongest at longer wavelengths (C-H stretching 2800-3000 cm^{-1}, O-H stretching at 3300 cm^{-1}) in FTIR spectra (**Figure 1**). As these groups are easily lost during pyrolysis, they were comparatively most reduced in the high-temperature gasification char (PG). In the range of 1030-1400 cm^{-1}, small peaks of aliphatic methyl and ether/alcohol C-O- and C-O bonds were also evident, though the importance of these was obscured by the strong absorption by aromatic C=C (1570 cm^{-1} and 1415 cm^{-1}) and C=O (1700 cm^{-1}). Overall, the greatest diversity of functional groups is seen in the fast-pyrolysis materials, which were also the least aromatic, evidenced by the lower overall degree of absorption (displacement on the y-axis). On the other extreme, the high temperature gasification char had very reduced abundance of functional groups. Finally, the low absorption of FL spectra demonstrates its limited organic content as reinforced by Si-O signal (1015 -1050 cm^{-1}), though some aromatic C=C bonding was evident at 1410 cm^{-1}. These interpretations are consistent with expectations that increased biochar temperature and residence time results in decreased H and O content of pyrolytic materials (Enders et al. 2012; Brewer et al. 2009), indicative of increasing degree of carbonization, aromaticity, and carbon condensation, as also evidenced by the H:C and O:C ratios in **Table 3,** which follow the order of fast pyrolysis > slow pyrolysis > gasification.

4.2. Feedstock and pyrolysis effects on biochar and soil-biochar mixture chemistry

Biochars had varying effects on soil-biochar mixture pH (**Table 8**). Biochar-mediated pH changes may be related to dissolution or addition of alkaline metals (Kookana et al. 2011; Lehmann et al. 2011), but also due to biochar surface properties such as functional group composition (as mentioned above) which may include carboxyls (strong acids), phenols and carbonyls (weaker acids), and pyrones (basic) (Amonette and Joseph 2009; Joseph et al. 2009). Washing of FL (Table 5) reduced the pH by nearly one point, probably due to the reduction in soluble Ca^{2+} in the second leachate, whereas very similar findings have been reported elsewhere attributed to leaching of base cations (Yao et al. 2010). Also, the elevated pH of PG may have been related to high soluble K^{+} contents. As already mentioned, in soil-biochar mixtures FL, PG, and CR increased pH, whereas CL, PR, and particularly PL, lowered pH. Biochar addition has been often been associated with liming effect in acid soils (Chan and Xu 2009), but our results indicate that some biochar materials may lead to pH decreases in an alkaline soil. These decreases in pH can be explained by adsoption of alkaline cations by those types of biochar that are expected to have more CEC. The lack of studies documenting reductions of pH in alkaline soils following biochar addition has been mentioned elsewhere (DeLuca et al. 2009).

The ionic concentrations reported in **Tables 5** and **7** provide plant-available, water-soluble fractions of nutrients, whereas biochar studies have typically reported the elemental content of chars and nutrients released from char are rarely reported (Chan and Xu 2009). The low N (soluble as well as elemental) in the chars was expected since N is the most sensitive of the macronutrients to heating and most N compounds volatilize above 200 °C (DeLuca et al. 2009). As seen in **Table 3**, FL had the highest amount of N (2.26%), and poplar materials had higher N than the pine materials (0.35-0.48% compared to 0.12-0.19%, respectively), though pine N was more soluble than that of poplar (**Table 5**). In the soil-biochar mixtures, low

concentrations of N-species in the FL extracts (in spite of the high N content of the respective feedstock) indicates that the N contained in FL was organically bound or in otherwise insoluble forms, as has been found by other authors (Bridle and Pritchard 2004). Despite these considerations, N-species resulted significant in the plant response models (**Table 10**), although it must be noted that the quantities of soluble N reported here do not represent all plant-available fractions. With regards to potassium, its limited contribution to the models indicate that their concentrations are not limiting plant growth (Table 10). Fresh FL had very little soluble K (2.4% recovered following L1 and L2), though the elemental concentration was similar to that of the other materials (**Table 3**). Recovered K from wood biochars following L1 and L2 was between 23% (PR) and 41% (PG) (**Table 5**). Overall, washings of the wood biochars show that K was highly available in these materials. During low-temperature pyrolysis (<500°C) K takes plant-available forms, and is considered particularly available (Amonette and Joseph 2009). Accordingly, in the soil biochar mixtures, extracted K^+ increased with concentration in all wood biochar mixtures, but not in FL. Regarding other important alkali metals, Ca^{2+} increased in FL, CR, and PR, and Na^+ increased with concentration in all cases, PG having the highest Na^+ loads, reaching 92 mg kg^{-1} at 26% concentration.

Soluble P was generally scarce in soil-biochar mixtures, only detectable in CL and PL mixtures. P extractability in the washing experiment was relatively high in the slow pyrolysis materials, and undetectable in PG and FL. Since biochar often strongly affects pH, and the availability of P is largely pH-dependent, the possibility that biochar may modify P availability has been previously recognized (DeLuca et al. 2009). Higher solution pH values and higher Ca:P ratios increase the precipitation of phosphate to non-soluble forms (Song et al. 2002). When calcium is abundant (Ca:P of 3.33) it can precipitate with phosphate as quickly as 10 minutes in a pH 8.00 solution (Song et al.

2002). The high alkalinity of the soil of this study (Carreira and Lajtha 1997), as well as the high Ca content and/or the high alkalinization capacity of leachates in FL and PG might explain the low soluble P release from these chars due to phosphate retrogradation. Our results suggest that both biochar liming capacity and Ca release may have been responsible for the low availability of P in PG and FL, and magnified by the fact of being tested in an alkaline soil. PG presented high elemental Ca:P ratio of 117, and though Ca washability was not high, pH was very elevated in leachates (9-11). Regarding FL, it presented a low Ca:P ratio (1.74), but extremely high concentrations of washable Ca. Phosphate precipitation has been described by previous studies with pyrolyzed sewage sludge, though importantly it was also found that P availability increased with incubation time (Bridle and Pritchard 2004; Yao et. al 2010). Yao and colleagues (2010) demonstrated that solubility of P in pyrolyzed sewage sludge was increased by humic acids functioning as chelating agents. Precipitation reactions may also explain why soluble P was maximum in slow pyrolysis chars (CL, PL), which had lower Ca:P ratios (1.07 - 4.8), low washable Ca content, and similar degree of alkalinization of the leachates. Finally, the fast pyrolysis chars, with intermediate Ca:P ratios (17-21), and intermediate Ca washability, presented intermediate P soluble content. The variation in soluble P with biochar addition therefore might have an important role on plant performance, since this, together with N, is the main nutrient limiting primary productivity in ecosystems (Elser et al. 2007), and in fact in our study these nutrients were associated with higher plant biomass (**Table 10**).

4.3. Relating biochar properties and their effect on soil chemistry to effects on plants

Fast-pyrolysis materials (CR, PR) along with the gasification material (PG) had the greatest negative effects on biomass (**Table 9**). The AGB EC_{10} of these materials also fall within realistic potential

application rates of 6-51 t ha^{-1} for ryegrass, and 5-12 t ha^{-1} for lettuce. The other materials, CL, PL, and FL, were not harmful in any realistic range of application rates, CL having the lowest AGB EC_{10} of these at 173 t ha^{-1}, however it is noted that these conclusions are only valid in the context of the test species and test soil utilized. Considering the wood feedstock materials, pyrolysis method had a stronger influence on plant responses than feedstock which is in accordance with the findings above, indicating that for proper biochar characterization detailed information on the pyrolysis procedure should be provided.

In order to identify the key factors explaining plant growth inhibition responses, EC_{10} values were correlated (data not shown) with measured biochar characterization parameters described above. P content in biochars resulted strongly significantly and positively correlated with EC_{10} for both test species (*L. sativa* p=0.016, ρ=0.94; *L. perenne* p=0.007, ρ=0.93) (**Figure 4**). Similarly, C:P was strongly negatively correlated with EC_{10} (*L. sativa* p=0.016, ρ=-0.94; *L. perenne* p=0.007, ρ=-0.93), but the removal of FL resulted in strong though non-significant correlations for both species (p=0.08, ρ=-0.9). These correlations reflect previously discussed results, whereas growth stimulation was observed in sewage sludge biochar with the highest P content, and inhibition was observed in fast pyrolysis biochars with the lowest P content. We reiterate that P content must be distinguished from its availability (soluble P), which we assume was chemically regulated by the alkalinity or calcium richness of the soil and the biochar.

A strong relationship was also found between VM content and plant performance, with significant negative correlations of VM with the EC_{10} of both species (*L. sativa* p=0.03, ρ=-0.89; *L. perenne* p=0.007, ρ=-0.93) (**Figure 5**), but when FL was excluded the relationship was no longer significant (data not shown). VM has been proposed as a critical factor leading to reductions in plant productivity due to its

Figure 4: Fresh biochar P contents are plotted against corresponding EC_{10} values for each test species. EC_{10} values, calculated by dose-response models fit to above-ground growth responses in bioassays (**Table 9**), represent the estimated soil-biochar mixture concentration (%) at which growth is inhibited by 10%, lower EC_{10} values therefore indicating more inhibition. Material codes (see **Table 2**) are printed below the plotted symbols for lettuce, and above for ryegrass.

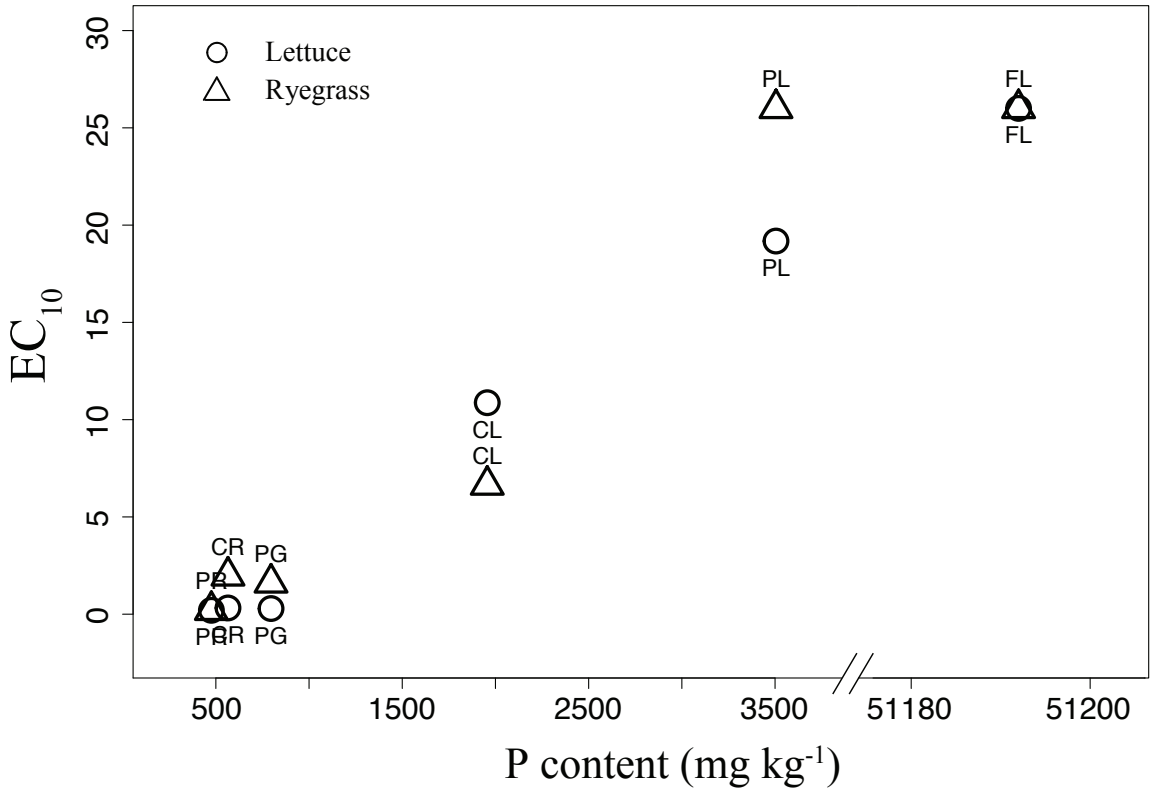
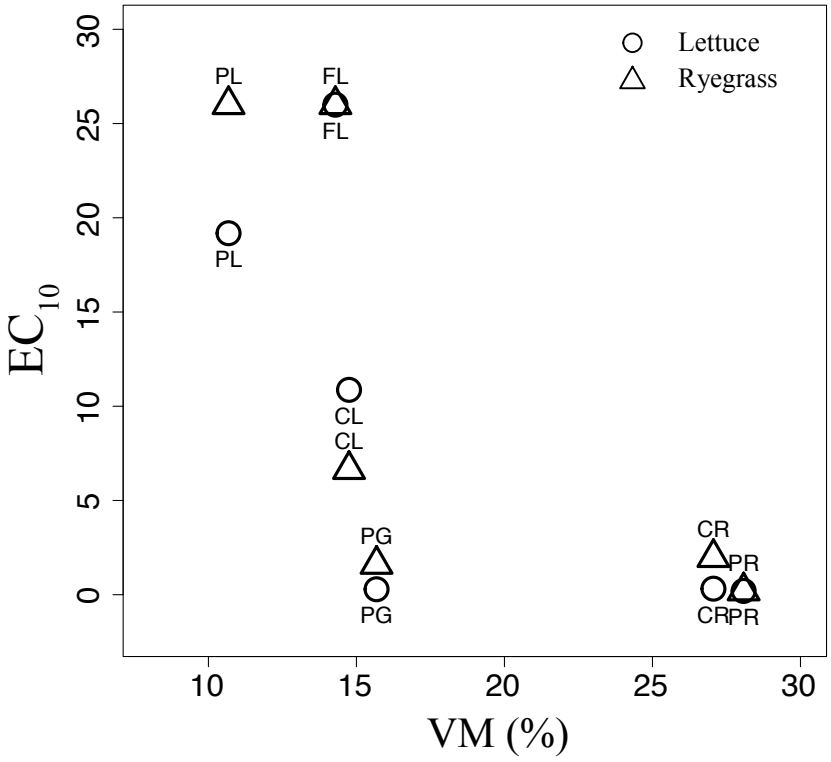


Table 5: Fresh biochar volatile matter (VM) contents are plotted against corresponding EC_{10} values for each test species. EC_{10} values, calculated by dose-response models fit to above-ground growth responses in bioassays (**Table 9**), represent the estimated soil-biochar mixture concentration (%) at which growth is inhibited by 10%, lower EC_{10} values therefore indicating more inhibition. Material codes (see **Table 2**) are printed below the plotted symbols for lettuce, and above for ryegrass.



influence on N-immobilization in weathered soils (Deenik et al. 2010, Bruun et al. 2012). Higher VM indicates higher labile carbon content and microbial biomass, resulting in significant N immobilization (Bruun et al. 2012). Alternatively, the possibility of phytotoxic compounds in chars with high VM has also been suggested (Villar et al. 1998; Van Zwieten et al. 2009; Deenik et al. 2010). Although IR absorption at some wavelengths were obscured by strong aromatic peaks, thus limiting identification of potential differences, strong effects due to phytotoxic pyrolytic condensates seems unlikely due to the close similarity of slow (stimulating or non-inhibitory) and fast pyrolysis (strongly inhibitory) FTIR spectrograms (**Figure 1**). Regarding potentially hazardous heavy metals such as Cu, Cr, and Zn, concentrations in soil-char mixtures were compared to the values limiting applications of sewage sludge for alkaline soils (pH≥7) set in the Working Draft for the use of sewage sludge in agriculture (European Commission 2000). FL slightly exceeded the Zn limit (200 mg/kg dm) at 11.3%, exceeded the Cr limit (100 mg kg^{-1} dm) at 26%, and the Cu limit (100 mg kg^{-1} dm) at 26%. PG exceeded the Zn limit at 26%. As such, limits were only reached at highly unrealistic application rates. Additionally, bioavailability of heavy metals should be low both due to the alkaline pH of the soil (Uchimiya et al 2011a) and the high metal sorption capacity of biochars (Uchimiya et al 2011b).

Somewhat surprisingly, C_{hw} had no relationship with either VM or EC_{10}, despite the fact that VM and C_{hw} are proposed as alternative methods for evaluating the most labile fractions of biochars (Joseph et al. 2009). PG, a strongly inhibitory material in the context of this study, had VM and C_{hw} content similar to the non-inhibitory materials CL and PL, indicating that inhibition by this material was probably not due to microbial nutrient immobilization. The low P availability in mixtures with this char, as discussed in the previous section, might be an alternative explanation to the high inhibition observed with this material.

Finally, Cr concentration was also positively significantly correlated with EC_{10} (data not shown) due to the fact that FL, CL, and PL had the highest Cr concentrations, though this seems to be an artifact since it the opposite of what would be expected and the concentrations are too low to be of real importance or concern.

In the multiple- and general linear models developed for this study, chemical properties explaining AGB and BGB responses were very similar, though somewhat distinguished by magnitude of effect of macronutrients N and P. These nutrients were positively associated with growth, supporting the evidence that nutrient limitation caused the plant growth inhibition, whereas increasing biochar concentration had a negative effect, reflecting the general inhibition showed by most chars in this study. It is noted that the models considered here are only representative of the conditions of the study, and the strength and direction of the predictor coefficients are conditioned by the factors included in the model. Their direct extension to other materials sets and species would thus be inappropriate without cross-validation.

All this indicates that growth inhibition observed in our study is not related to phytotoxic effects, but rather due to reduced nutrient availability, either by nutrient competition with microorganisms (i.e., VM content) or P content.

5. Conclusions

Plant growth tests with six biochars in an alkaline soil showed that pyrolysis method had a strong influence on chemical properties affecting plant responses. Gasification and fast-pyrolysis chars were strongly inhibitory at realistic application rates, while slow-pyrolysis chars generally did not affect plant growth. Inhibition was not related to phytotoxic substances in chars such as aliphatic compounds or heavy metals, but rather due to short-term effects on nutrient plant availability, limited by both VM content which increases competition with microorganisms, and P content and/or P availability, the latter of

which was in turn likely related to phosphate precipitation to non-available forms due to biochar chemistry and the initial P content. These mechanisms can explain the stimulatory effect of sewage sludge char (low VM and high P content, despite the low P washability in fresh char which likely increased with time), the stimulation or non-effect of slow pyrolysis wood chars (low VM and intermediate P content which was highly available), and the inhibition reported for fast pyrolysis wood chars (with high VM and low P content and availability) and especially for the wood gasification char (low VM but low elemental P and no soluble P).

These results may aid practitioners to avoid any short-term unintended effects of biochars on plant growth that may occur with the application of fresh biochar in an alkaline soil, and also suggest the potential fertilization and acidification value of slow pyrolysis wood biochars under such conditions.

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Chapter 2 Biochars provoke diverse soil mesofauna responses in laboratory bioassays

Abstract

Biochar application to soil has the potential to improve soil fertility under certain conditions. However, potential ecological effects remain largely unexplored and poorly understood, particularly those on soil biota. Six biochars were tested on two soil-dwelling invertebrates in short-term bioassays to determine effects on survival and reproduction. A pine wood gasification char increased collembolan adult mortality at high concentrations. Wood slow and fast pyrolysis biochars had a strong stimulatory effect on collemboan reproduction, but no stong effect on enchytraeids. A sewage sludge char was slightly stimulatory for both organisms, and a pine gasification char was inhibitory in both cases. Inhibitory effects were associated with biochars with high carbonate and Ca content and pH. Unexpectedly, stimulatory effects were not associated with microbial biomass, neither did this parameter change with increasing biochar concentration. In light of high stimulation of the collemboan, potential explanations such as microbial community shifts or gut symbiont use of biochar are suggested.

1. Introduction

Biochar is a carbon-rich coproduct of pyrolyzation of biomass destined as an agricultural amendment (Lehmann and Joseph 2009). Biochar addition to agricultural soils can improve crop growth (Biederman and Harpole 2012; Jeffery et al. 2011), reduce mobility of contaminants (Cao et al. 2011; Song et al. 2012), and has been proposed as a means to sequester carbon as a utilizable by-product of bioenergy generation (Laird 2008; Mathews 2008). However, the

effects of biochar use on soil ecology, especially in the long term, are not well understood, with most available information relating to bacteria and fungi (Thies and Rillig 2009), while a few studies have been published for invertebrates such as earthworms (Noguera et al. 2010; Van Zwieten et al. 2009; Li et al. 2011; Noguera et al. 2012) and collembolans (Salem et al. 2013). Overall, effects on eukaryotic soil biota have yet to be systematically studied, representing an important gap of knowledge within the field (Lehmann et al. 2011).

Soil invertebrates influence physical, chemical, and biological processes (Lavelle et al. 2006), in the last case mainly through the regulation of microbial communities which in turn influence plant-available nutrients (Wardle et al. 1998), while others such as earthworms act as ecosystem engineers (Lavelle et al. 2006). Thus, given recent impetus for large-scale deployment of biochar, it is not surprising that the need to understand the effect of biochar on the soil biota has been urged forward (McCormack et al. 2013).

Soil invertebrates are commonly used to evaluate soil quality (Coleman et al. 2004) and ecotoxicity (van Straalen 2004), and emerging soil quality assessments have been based on mesofauna responses because of observations of their sensitivity to unfavorable conditions and toxins, and also due to observed correlations between invertebrate community structure and soil quality parameters (Bone et al. 2010; van Straalen 1998; Verhoef 2004).

Addition of biochar to soils has the potential to impart direct or indirect effects on soil invertebrates. Direct effects are those which affect individuals' performance through release of salts, heavy metals or organic chemicals, and can be mediated through multiple exposure routes, which are oral, dermal, or through gas exchange (Eijsackers 1993). Indirect effects, on the other hand, are those mediated through changes in the environment (Didden and Rombke 2001), e.g. pH, humidity, aeration, or other factors related to the ecological tolerance ranges of the exposed species.

Concerning potential direct effects leading from soil amendment with biochar, substances released are potentially diverse since biochar feedstocks may proceed from a great variety of agricultural as well as urban organic wastes, including manures (e.g. Wang et al. 2012), crop residues such as corn stover (Lee et al. 2010), forestry residues (which may have different chemical properties depending on their physiological function, i.e. leaves, bark, e.g. Wu et al. 2011), sewage sludge (e.g. Méndez et al. 2012, Hossain et al. 2010), or papermill waste (Van Zwieten et al. 2009), among others. As an example, direct effects might be expected with sewage sludge biochar, having high heavy metal content and electrical conductivity (Hossain et al. 2010; Paz-Ferreiro et al. 2012). Also, polycyclic aromatic hydrocarbons (PAHs) can be generated during pyrolysis, pyrolysis process being more influential than feedstock in this case (Brown et al. 2006; Kloss et al. 2012). Regarding indirect effects of biochar, it is known that biochar induces changes in soil physicochemical properties, such as pH (Jeffrey et al. 2011), salinity (Hossain et al. 2010), water retention (Verheijen et al. 2009), porosity, and aeration (Downie et al. 2009) just to name a few, all of which might alter the suitability of the operative environment for invertebrates or the microorganism communities they rely on (McCormack et al. 2013). Biochar has normally been found to increase soil microbial biomass (Biederman et al. 2012), in temperate (Kolb et al. 2009; Bruun et al. 2012) as well as tropical soils (Liang et al. 2010). Increases in decomposer abundance have the potential to precipitate an increase in microbial grazer populations through a stimulatory trophic effect, which is one of the experimental questions of this study.

In our study we tested the effect of increasing concentrations of biochars on soil invertebrate endpoints using standard bioassay methods, endpoints being survival and reproductive response of the soil organisms *Folsomia candida* (Isotomidae: Collembola) and the enchytraeid *Enchytreus crypticus* (Enchytraeidae:Oligochaeta),

whereas survival reflects direct effects, effects on these organisms' reproduction is an individual-level response useful for either studying stress due to habitat suitability variation (Hund-Rinke et al. 2003), pollution (Maltby 1999), or variation in food availability (Domene et al. 2007). Biochars tested in the study came from different feedstocks and pyrolysis methods, so different responses following material identity were expected. Considering the above, the aims here were to (1) determine which biochar materials and potential application rates may cause inhibitory or positive effects on the survival and reproductive response of the test organisms as related to biochar-mediated changes to soil chemical properties, and (2) test for a potential trophic effect by associating microbial abundance with any stimulatory reproductive responses.

2. Materials and Methods

2. 1 Characterization of biochars

The six biochars tested in the study proceeded from mixed pine wood chips (*Pinus radiata* and *P. pinaster*), poplar (*Populus nigra*), and a thermally dried sewage sludge from the Prat del Llobregat WWTP (Barcelona, Spain), produced by fast pyrolysis, slow pyrolysis, and gasification processes (**Table 1**). Soil aqueous extracts were prepared by adding water to biochar in the proportion of 1:10 (g biochar:ml deionized water) and vertically agitating for 24 h at 60 rev min^{-1}. Then, the suspensions were vacuum filtered with Whatman 42 filter paper, and pH and electrical conductivity (EC) were measured immediately. Biochar elemental composition was analyzed, with determinations of C and H carried out using a Flash 2000 C.E. elemental analyzer (Thermo Fisher Scientific), N by a Flash EA 1112 elemental analyzer (Thermo Fisher Scientific), and S by ICO-OES spectrometry using a Varian 725-ES Radial ICP Optical Emission Spectrometer (Varian Inc.). Total O was determined by subtraction according to ASTM D1762-84 as follows:

O (%w/w) = 100 - ash (%w/w) – C (%w/w) – N (%w/w) – H (%w/w) ,

whereas ash content was determined as explained below. O:C and H:C ratios were calculated using the molar concentrations of the elements concerned, each calculated by dividing the total weight of the element by its molecular weight (Van Krevelen 1961).

Loss on ignition (LOI) was evaluated on ground samples in triplicate in a muffle furnace, first at 375 °C for 18 h without acid pre-treatment (used for removal of soot and graphitic black carbon) to evaluate biochar organic content except the soot fraction (Gustafsson et al. 1997, Poot et al. 2009); at 550 °C for 5 h to remove soot (Gustafsson et al. 1997), hence representing the complete oxidation of the organic carbon fraction in biochar; and finally at 1100 °C for 5 h, which should mainly remove carbonates (Santisteban et al. 2004). Volatile matter (VM) was determined in triplicate by proximate analysis following ASTM D1762-84 by heating ground samples in a covered crucible at 950 °C for 6 min and determining weight loss, and ash content was also evaluated in triplicate following the same ASTM protocol by heating ground samples in an uncovered crucible at 750 °C for 6 h and determining weight loss, representing the mineral content in chars.

Labile carbon content of the biochars was evaluated in triplicate using a hot water extraction following Rovira and Vallejo (2007), hereafter referred to as hot

Table 1: Materials tested in the study and identification codes.

Material	Description
CL	*Populus nigra* (Poplar) wood chip biochar produced in a slow pyrolysis reactor 500 – 550 °C
CR	*Populus nigra* wood chip biochar produced in a fast pyrolysis reactor at 430 – 510 °C
FL	Wastewater sludge biochar produced produced in a slow pyrolysis reactor 500 – 550 °C
PG	*Pinus pinaster + P. radiata* wood chip biochar produced in a gasification reactor at 600 – 900 °C
PL	*Pinus pinaster + P. radiata* wood chip biochar produced in a slow pyrolysis reactor 500 – 550 °C
PR	*Pinus pinaster + P. radiata* wood chip biochar produced in a fast pyrolysis reactor at 440 – 480 °C

water extracted carbon (C_{hw}). Briefly, oven-dry ground biochar samples of 0.5 g were extracted with 20 ml of deionized water in sealed Pyrex tubes digested in aluminum blocks at 105 °C for one hour after which the extract was filtered with Whatman 42 filter paper and stored at -20 °C. Later, extracts were evaluated for dissolved organic carbon (DOC) using the acid dichromate method described in Brookes and Joergensen (2006); briefly, this involves oxidation with 2:1 H_2SO_4:H_3PO_4 mixture and 0.4 M $K_2Cr_2O_7$ at 150 °C for 30 min followed by back-titration with 40 mM $(NH_4)Fe(SO_4)2 \cdot 6H_20$, with the modification that extract and wet chemical volumes were halved. The surface chemistry of the biochars was assessed by Fourier-transform infrared spectroscopy (FTIR) performed on dry (105 °C), ground, biochar samples passed through a 100 μm sieve. Spectrum were registered in triplicate at standard infrared resolution (4 cm^{-1}) in the mid-infrared range of 600-4000 cm^{-1} using a Bruker Tensor 27 spectrophotometer working in attenuated total reflectance (ATR) mode with diamond reflection.

2.2 Characterization of soil-biochar mixtures

The test soil was collected in an olive grove in La Granadella (Lleida, Spain), and corresponded to the topsoil of a Hypercalcic Calcisol, whose main properties are reported in **Table 2**. Heavy metal content in the soil was low, which excluded possibility of any excessive concentration therein. Soil was dried and sieved to 2 mm before being defaunated by dual freezing (-20 °C) and thawing (20 °C) cycles of four days. Soil-biochar mixtures were prepared at 0 (control), 0.5, 1.3, 3.2, 8, 20, and 50%. WHC of each material-concentration was determined gravimetrically by saturating 50 g, draining for 24 h at room temperature and weighing, drying overnight at 105 °C and weighing, and calculating the proportion of drained water content to ODW. pH and conductivity were determined in triplicate from 1:5 (w/v) aqueous abstracts: 15 g soil mixture and 75 ml deionized water were

vertically agitated in 150 ml polyethylene cups for 2 h at 60 rev min^{-1}, the extract was subsequently centrifuged, the supernatant was filtered through Whatman 42 filter paper, pH and conductivity were immediately measured, and the extracts stored at -20 °C. Ion chromatography was used to determine water-soluble concentrations of major cations and anions derived from biochar-soil mixtures. 10 ml of the extracts described above were pooled and then a

Table 2: Main properties of the test soil.

Locality	La Granadella
Soil classification	Hypercalcic Calcisol
Soil use	Olive grove
WHC (%)	49.8
pH (soil:water 1:5)	8.2
Sand (%)	27.8
Silt (%)	48.5
Clay (%)	23.69
Organic matter (%)	1.7
Organic C (%)	0.99
Total N (%)	0.11
C/N	9
CEC (cmol kg^{-1})	14.2
Cd (mg kg^{-1})	0.2
Cu (mg kg^{-1})	26
Cr (mg kg^{-1})	19
Ni (mg kg^{-1})	28
Pb (mg kg^{-1})	10
Zn (mg kg^{-1})	42

5:50 ml dilution was prepared, from which a homogenized 5 ml sample was taken for analysis. Ion chromatography analysis of Na^+, K^+, Mg^{2+}, Ca^{2+}, NH_4^+ was carried out with a CS12A Dionex cation column on a Dionex ICS-1100 ion chromatograph (Dionex, Sunnyvale, USA), and for Cl^-, NO_2^- NO_3^-, HPO_4^{2-} and SO_4^{2-} with a AS4A-SC Dionex anion column on a Dionex DX-100 ion chromatograph (Dionex, Sunnyvale, USA).

Soil microbial biomass was assessed in triplicate for each material-concentration by incubating 60 g moistened to 40% WHC under the same conditions as the bioassays (described later), but destructively sampled at half the bioassay duration (14 d) in order to better relate this parameter with test mid-point conditions influencing reproduction. Microbial biomass was analyzed by the fumigation extraction method (Brookes and Joergensen 2006): two portions of moist soil from the same replicate (10 g FW each) were weighed and noted as fumigated and non-fumigated samples. In the first (non-fumigated), dissolved soil carbon (DOC) was immediately extracted with 40 ml 0.5 M K_2SO_4 by vertical agitation at 60 rev min^{-1} for 2 h, centrifuged, filtered in Whatman no. 42, and stored at -20 °C. The second was fumigated for 24 h at 25° C with ethanol-free $CHCl_3$ and then DOC was extracted as described above. DOC was determined by wet oxidation with potassium dichromate followed by titration with Mohr's salt. Soil microbial biomass carbon (C_{mic}) was calculated as the difference between DOC in fumigated soil and that of non-fumigated soil.

Sorption of DOC on biochar is expected to cause MBC underestimation (Jin, 2010) requiring the correction of the C_{mic} values obtained. Therefore, DOC sorption for each material-concentration was quantified using a portion of soil-biochar mixture from the previous 14 d incubation reserved for this purpose. A solution of DOC stock was prepared by stirring 400 g soil taken from a forest with 1000 ml deionized water for 2 h, centrifuging at 13,800 *g* for 5 min, recovering the supernatants, and DOC in was assessed per methods described above. Then, for each soil-biochar mixture concentration, and in

duplicate, 6 g (FW) were placed in a 40 ml centrifuge tube, and 23 ml of 0.5 M K_2SO_4 were added together with 1 ml containing a known DOC concentration (0, 70 or 100 μg C/ml) obtained by dilution of the stock solution. The centrifuge tubes were vertically agitated at 60 rev min^{-1} for 2 h and afterwards centrifuged for 5 min at 13,800 *g*. The supernatant was filtered through Whatman 42 filter paper and the samples were stored at -20 °C until analysis. DOC concentrations in supernatants were determined following the methods described above. Then, at each added DOC concentration and replicate, measured DOC (M) was evaluated by the method previously described, and the expected DOC (E) was calculated as the sum of the initial DOC content of sample (that without DOC addition) and the added DOC. Correction factors (CF) were then calculated as CF = M / E. The CF values of the two replicates were averaged to obtain a final CF for each material-concentration. Corrected microbial biomass carbon was then calculated as $C_{mic\text{-}c} = C_{mic} / CF$. Adsorption-corrected microbial biomass (MB_c) was then calculated as $MB = C_{mic\text{-}c} / 0.38$, according to Vance et al. (1987).

2.3 Mesofauna bioassays

All mesofauna tests were conducted at 21 ± 1 ºC, and followed a 16:8 h light:dark cycle at 12 ± 10μE m^{-2} s^{-1} luminosity to ensure that organisms would not avoid the substrate. Five replicates of 30 g were prepared for each material-concentration adjusted to 40% WHC, placed in 50 ml polyethylene test vessels with sealable tops. Test vessels were aerated and arbitrarily rearranged 2 times weekly for the duration of each test (indicated below).

F. candida is a parthenogenetic soil springtail of world-wide distribution, commonly used in ecological testing (Fountain and Hopkin 2005). In accordance with ISO Guideline 11267 (1999), cultures were synchronized to obtain 10-12 day old springtails. 10 synchronized individuals were separated from cultures and placed in test vessels using a pump-powered pooter. Appx. 2 mg of baker's yeast was

provided as food at the beginning of the test and at 14 d. At 28 d, vessels were flooded with water to float organisms to the surface, the water was tinted with a dark dye to improve visibility of the white Collembola, and photos were taken of the floating organisms using a high-resolution digital camera. Later, adults and juveniles were counted with ImageJ v1.43 (National Institutes of Health, Bethesda, MD, USA).

E. crypticus is commonly known as the white potworm. Soil aqueous phase animals such as enchytraeids have advantages for ecological testing due to their ease of culture and exposure to all three soil phases (Didden and Rombke 2001). Several methodological modifications have been made to the ISO Guideline 16387 (2003). First, a shorter test duration (28 d vs. 42 d when *E. albidus* is used) following Kuperman (2006), and secondly, adults were kept in the substrate during the entire test period instead of being removed and counted after 14 d. Five replicates were prepared for each concentration, each consisting of a container filled with 20 g of wet soil-biochar mixture. Ten *E. crypticus* adults (clitellated individuals) were individually separated from juveniles in a Petri dish with water and placed in test vessels using jeweler's tweezers. Following previous experience ground rolled oats were provided at the beginning of the test and after 14 d, which is half the amount specified by Kuperman (2006), in which food was provided each week. At 28 d all vessels were filled with 70% alcohol solution to 1 cm above substrate level to fix the worms, and 10 drops of Bengal red (1% solution in alcohol) were added for staining. Vessels were resealed, gently agitated, and set aside for a minimum of 12 h before counting to allow adequate time for the stain to set. To count surviving adults and juveniles, all vessel contents were cleaned of fine particles using a 0.2 mm sieve and tap water, after which remaining contents were transferred to quadrated petri dishes placed on a light box to ease counting.

2.4 Statistical analyses

Statistical analyses were performed with R 2.15.2 (R Development Core Team 2012). Global significance tests of biochar effects on reproduction were performed using Kruskal-Wallis tests; this treatment is based on rank-sum methods and is therefore recommended by standardized ecotoxicology protocols for nonparametric data (OECD 2004). If significant differences existed between the concentrations, post-hoc tests were carried out comparing biochar concentrations with the control using the Mann-Whitney U method.

The effect of biochar characteristics on test species performance was assessed as follows: mean fauna performance for each biochar was calculated as the percent of the total averaged within-material reproduction with respect to the control (within-material). Thus, values >100 corresponded to a material's global stimulation, while values <100 indicated global inhibition. Material-averaged performance was then correlated with biochar characteristics using Pearson correlations.

For soil-biochar mixtures, the effects of microbial biomass and measured chemical properties (pH, EC, and ions) on mesofauna reproduction were assessed using multiple linear regression using the *lm* function in R. Fauna percent-of-control reproduction within each material-concentration were taken as response variables, and soil chemical and biological measurements as explanatory. NO_2^- was only detected in FL-50; for this reason, it was excluded from the models. Biochar concentration was also included in the model to account for its association with ion concentration. A global model was constructed, and the variables having a variable inflation factor (VIF) >10 were removed beginning with the largest VIF, recalculating VIFs, and repeating the process until all variables had VIF<10. Next, the best model was selected as that which minimized the Akaike Information Criteria (AIC) using the *dredge* function of the package *MuMIn* of R. Standardized coefficients, β_i, were calculated for each predictor x_i so as to allow comparison between predictors having different units of

measurement. Constant error variance was checked visually using standardized residuals.

3. Results

3.1 Feedstock and pyrolysis procedure effects on biochar composition and soil chemical and biological properties

Biochar elemental composition is shown in **Table 3**. Slow-pyrolysis wood chars had the highest C content (81-86%), whereas that of fast-pyrolysis and gasification was similar (71-73%), and the sewage sludge char was the lowest (22%). The sewage sludge char had the highest N content (2.3%), followed by the poplar chars (0.35-0.48%), and pine wood chars the lowest (0.12-0.19%). P content was highest in the sewage sludge char (5.12%), followed by the slow-pyrolysis chars (0.20-0.35%) and lowest in the fast and gasification chars (0.05-0.08%). Thus, comparing wood chars, it appears that pyrolysis method had the strongest influence on C:P ratios (P content highest in low temperature-pyrolysis materials), whereas feedstock determined C:N ratios (N content more similar within feedstocks).

Biochar LOI, VM and C_{hw} results are shown in **Table 4**. For LOI, fast and slow pyrolysis materials had very similar LOI-375 (93-96%), LOI-550 (0.18-0.67%), and LOI-1100 (0.18-0.90%). FL and PG and, on the other hand, were distinguished by their lower (especially FL) LOI-375 (12% and 76%, respectively). Also, FL had a very high LOI-550 (14%), representing soot. Finally, both FL and PG had relatively high LOI-1100 (4% and 6%, respectively), indicating carbonate content. For C_{hw}, Analysis of variance showed significant differences between feedstocks ($p=0.02$) as well as pyrolysis methods ($p<0.001$). Comparing feedstocks, the slow pyrolysis pine char had significantly less C_{hw} than the sewage sludge char ($p=0.03$). Comparing pyrolysis

methods, fast-pyrolysis wood materials had more C_{hw} than the gasification or slow-pyrolysis materials (Tukey HSD $p<0.001$ for both contrasts). As for VM, comparing wood materials VM was not significantly affected by feedstock ($p>0.05$ in all Tukey HSD contrasts), but comparing pyrolysis methods fast-pyrolysis materials had the highest VM (27-28%); fast and slow pyrolysis materials had the greatest VM difference ($p<0.001$), followed by fast and gasification ($p<0.001$), and least differences in VM were between slow and gasification ($p=0.040$).

FTIR spectrum of each biochar and their detailed interpretation are found in **Appendix 1**. As expected, spectra of sewage sludge and wood biochars were highly dissimilar owing to their different origins. Within the wood feedstocks, spectra were more similar in pyrolysis method than feedstock, whereas the gasification material (PG) was most markedly distinguished from the slow (CL, PL) and fast-pyrolysis (CR, PR) materials. Overall, the greatest absorption (displacement on the y-axis) was in the order of gasification > slow pyrolysis > fast pyrolysis, particularly <2000 cm^{-1}, indicating increasing carbonization, aromaticity, and condensation of C.

Linear regressions of bioassay mixture concentration (biochar %) with pH, EC, and ionic concentrations are shown in **Table 5**. Though trends were varied between materials, a few general statements can be made; increasing biochar concentration increased soluble K^+ (except CR), Na^+ (except PL), and EC (except CL). Also, materials where concentration significantly increased NH_4^+ showed a concomitant decrease in NO_3^- (in fact, NO_3^- decreased with concentration in all materials except

Table 3: Elemental content of the biochar materials (column names, refer to **Table 1**) with error expressed as SD where applicable. C:N and C:P are mass ratios, whereas H:C and O:C are atomic ratios.

	CL	CR	FL	PG	PL	PR
N (%)	0.48 ± NA	0.35 ± NA	2.26 ± NA	0.12 ± NA	0.12 ± NA	0.19 ± NA
C (%)	81.07 ± 0.11	73.11 ± 0.08	22.34 ± 0.02	71.03 ± 0.16	86.26 ± 0.16	71.76 ± 0.04
H (%)	2.07 ± 0.02	3.27 ± 0.02	1.20 ± 0.02	0.53 ± 0.02	1.97 ± 0.02	3.40 ± 0.04
S (%)	0.04 ± NA	0.02 ± NA	1.01 ± NA	0.08 ± NA	0.02 ± NA	0.02 ± NA
O (%)	12.32	18.78	4.96	8.75	9.08	22.00
P (%)	0.20	0.06	5.12	0.08	0.35	0.05
C:P	405	1219	4	888	246	1435
C:N	169	209	10	592	719	378
H:C	0.31	0.54	0.45	0.09	0.27	0.57
O:C	0.11	0.19	0.17	0.09	0.08	0.23
Ca (ppm)	9573 ± 232	12184 ± 86	89107 ± 4341	92343 ± 3654	3769 ± 138	8273 ± 24
Mg (ppm)	1313 ± 28	1594 ± 38	11827 ± 571	2591 ± 58	980 ± 36	1420 ± 9
Na (ppm)	956 ± 75	1029 ± 18	3843 ± 165	778 ± 3	329 ± 48	476 ± 9
K (ppm)	6570 ± 229	9207 ± 381	9092 ± 598	8249 ± 291	3484 ± 121	6404 ± 220
P (ppm)	1956 ± 222	565± 10	51192 ± 3273	796 ± 113	3505 ± 91	476 ± 69
Fe (ppm)	1966 ± 126	1784 ± 46	42649 ± 2930	1527 ± 85	1213 ± 18	1577 ± 93
Zn (ppm)	130 ± 14.4	540 ± 7	3074 ± 156	823 ± 121	70 ± 24	181 ± 7
Cr (ppm)	213.2 ± 36	40.4 ± 1.4	384.9 ± 50	26.2 ± 2.2	83 ± 21	26.2 ± 0.3
Cu (ppm)	109 ± 27	29 ± 2.2	766.7 ± 87	219 ± 3.0	27 ± 3.1	12.6 ± 0.3

Ni (ppm)	253 ± 22.2	22.6 ± 0.7	249.2 ± 28.2	10.4 ± 2.4	97 ± 0.1	24.5 ± 0.3
As (ppm)	-	-	12.4 ± 1.0	-	-	-
Cd (ppm)	-	-	2.2 ± 0.26	1.2 ± 0.01	-	-
Hg (ppm)	-	-	-	-	-	-
Pb (ppm)	74.7 ± 14.4	62.1 ± 0.9	277 ± 34.4	9.1 ± 0.4	15.7 ± 0.5	10.1 ± 0.1

Table 4: Proximate analyses of loss on ignition (LOI), volatile matter (VM), and ash contents of fresh biochar samples, expressed as percentage of each freaction with respect to total weight, in addition to hot water-extractable C (C_{hw}), all ± SE of three replicates. Material codes in **Table 1**.

	Proximate analyses					C_{hw}
Material	LOI 375°C (%)	LOI 375-550°C (%)	LOI 550-1100°C (%)	VM (950° C 6 min)	Ash content (%)	μg C g^{-1}
CL	95.10 ± 0.01	0.67 ± 0.05	0.67 ± 0.06	14.75 ± 0.46	4.06 ± 0.09	654 ± 55
CR	93.19 ± 0.34	0.45 ± 0.03	0.90 ± 0.14	27.06 ± 0.31	4.49 ± 0.15	3285 ± 42
FL	12.46 ± 0.36	14.39 ± 0.34	3.97 ± 0.01	14.29 ± 0.34	69.24 ± 0.07	1277 ± 42
PG	76.15 ± 0.16	2.22 ± 0.37	6.1 ± 0.46	15.68 ± 0.31	19.57 ± 0.35	613 ± 42
PL	96.75 ± 0.01	0.18 ± 0.00	0.18 ± 0.01	10.67 ± 0.20	2.57 ± 0.09	933 ± 72
PR	94.96 ± 0.13	0.56 ± 0.00	0.37 ± 0.04	28.07 ± 0.22	2.65 ± 0.09	2684 ± 41

Table 5: Linear regressions fit to ion concentration (mg kg^{-1}) in a series of increasing concentration of biochar mixtures as determined by wet ion chromotography. Columns are the linear equation (eq), R^2, and the difference between minimum and maxiumum values (diff.). NA indicates below detection threshold in all concentrations or present in only one concentration. † indicates that the parameter was below detection threshold in all but two concentrations. Significance codes: <0.05, '*', <0.01, '**', <0.001, '***'. Material codes in **Table 1**.

	Ca^{2+}			Cl^-			K^+			Mg^+		
Material	eq	R^2	diff.	eq	R^2	diff.	eq	R^2	diff.	eq	R^2	diff.
CL	412 + -4.33x*	0.66	302	200 + -0.47x	-0.18	237	182 + 10.41x***	0.94	557	55 + -0.69x*	0.59	47
CR	401 + -4.96x*	0.61	308	105 + 0.23x	0.01	24	208 + 7.03x	0.30	484	57 + -0.81x*	0.60	48
FL	432 + 27.01x***	0.96	1341	154 + 45.30x***	0.96	2238	76 + 3.88x***	0.98	207	67 + 9.73x***	0.98	479
PG	378 + -1.93x***	0.95	103	73 + 1.72x***	0.95	83	92 + 6.01x***	0.94	293	61 + -0.24x*	0.66	14
PL	400 + 1.27x	0.45	86	72 + -0.12x	-0.17	51	86 + 6.59x***	0.93	357	58 + 0.21x	0.28	18
PR	366 + -4.87x***	0.92	256	82 + 5.10x***	0.90	252	113 + 12.13x***	0.90	583	64 + -0.75x	0.38	59

	NH_4^+			NO_2^-			NO_3^-			Na^+		
Material	eq	R^2	diff.	eq	R^2	diff.	eq	R^2	diff.	eq	R^2	diff.
CL	16 + 1.85x**	0.82	88	NA	NA	NA	523 + -6.22x*	0.69	395	13.7 + 1.80x***	0.95	90
CR	47 + -0.12x	-0.15	34	NA	NA	NA	487 + -11.06x**	0.75	567	15.23 + 1.46x***	0.93	73
FL	4.19 + 0.82x*	0.66	40	NA	NA	NA	307 + -4.99x*	0.67	337	15.62 + 4.64x***	0.98	232
PG	42 + 0.27x	-0.03	30	NA	NA	NA	323 + -6.27x*	0.79	315	11 + 0.50x***	0.95	27
PL	32 + 0.82x	0.31	57	NA	NA	NA	223 + -2.95x	0.29	255	13 + 0.26x	0.29	18
PR	42 + 0.17x*	0.50	12	NA	NA	NA	251 + -5.45x*	0.67	339	14 + 1.36x**	0.86	66

	HPO_4^{2-}			SO_4^{2-}			pH			EC ($\mu S\ cm^{-1}$)		
Material	eq	R^2	diff.	eq	R^2	diff.	eq	R^2	diff.	eq	R^2	diff.
CL	-18 + 6.19x***	0.96†	306	110 + 6.01x***	0.93	305	8.12 + 0.004x	0.39	0.29	319 + 3.57x	-0.02	464
CR	NA	NA	NA	360 + -4.84x	-0.11	854	7.99 + 0.009x**	0.75	0.52	243 + 3.80x**	0.82	213
FL	NA	NA	NA	228 + 50.10x***	0.95	2501	7.89 + -0.002x	0.16	0.24	261 + 26.64x***	0.96	1348
PG	NA	NA	NA	79 + 0.21x	0.03	21	8.15 + 0.002x	0.37	0.11	219 + 1.41x*	0.73	72
PL	-25 + 8.12x***	0.96†	403	124 + 0.69x	0.13	70	7.92 + -0.002x*	0.54	0.14	195 + 3.03x***	0.90	159
PR	NA	NA	NA	115 + 5.76x**	0.86	312	8.06 + 0.02x***	0.95	0.99	235 + 3.77x**	0.74	189

PL). Soluble Ca^{+} decreased with concentration in most wood material mixtures (except PL) and strongly increased with concentration in FL. Also, HPO_4^{2-} was only detected in the slow-pyrolysis wood materials. pH increased in the fast-pyrolysis materials, and decreased in PL. FL strongly increased concentrations of the greatest number of ions, whereas PL affected the fewest, increasing only HPO_4^{2-} and K^{+}.

Microbial biomass results are shown in **Figure 1**. No differences were found between control sets (Kruskal-Wallis p=0.413). Against expectations, no clear trend was seen with increasing biochar concentration within any material. However, significant differences in MB were found in the FL dataset (Kruskal-Wallis p=0.016) though without any consistent trend.

3.2 Soil fauna reproduction tests

In *F. candida*, the coefficient of variation (CV=(SD/$\bar{x}$)*100) for juvenile reproduction was <30%, adult mortality did not exceed 20%, and the mean number of juveniles was 100 in control replicates, fulfilling the validity criteria of the ISO guideline. However, in PL and PR validity criteria were not met for number of counted adults at the end of the test, with means of 7 and 7.8 respectively, whereas 8 is established as the minimum. However, as reproduction CV within controls for PL and PR was well within accepted limits (15 and 3% respectively), the results from these biochars were accepted, and was likely due to our inability to refloat all adults. For *E. crypticus*, in CR a mean of 7 adults was found at the end of the test.

Adult survival was not affected within any material for either organism, with the exception of *F. candida* PG, where adult numbers were significantly lower than the control at 3.2, 20, and 50% biochar.

Reproduction of the two species for each material-concentration is shown in **Figure 2**. For *F. candida* reproduction, there were significant effects of biochar concentration in all materials with the exception of FL. With the exception of PG, significant stimulatory

effects on collembolan reproduction were found for all materials concentrations >0% except at CL-1.3, FL-3.2, and PR-3.2. In PG, inhibitory effects were found at concentrations 20% and 50%. For *E. crypticus,* reproduction (as evaluated by the global significance test) was only significant for FL, PG and PR, though in post-hoc testing inhibitory effects were also found for CL

Figure 1: DOC adsorption-corrected microbial biomass (MB_c) of the six biochar materials with increasing concentration following two-week incubation with the test soil. Error bars represent SE. Material codes in **Table 1**.

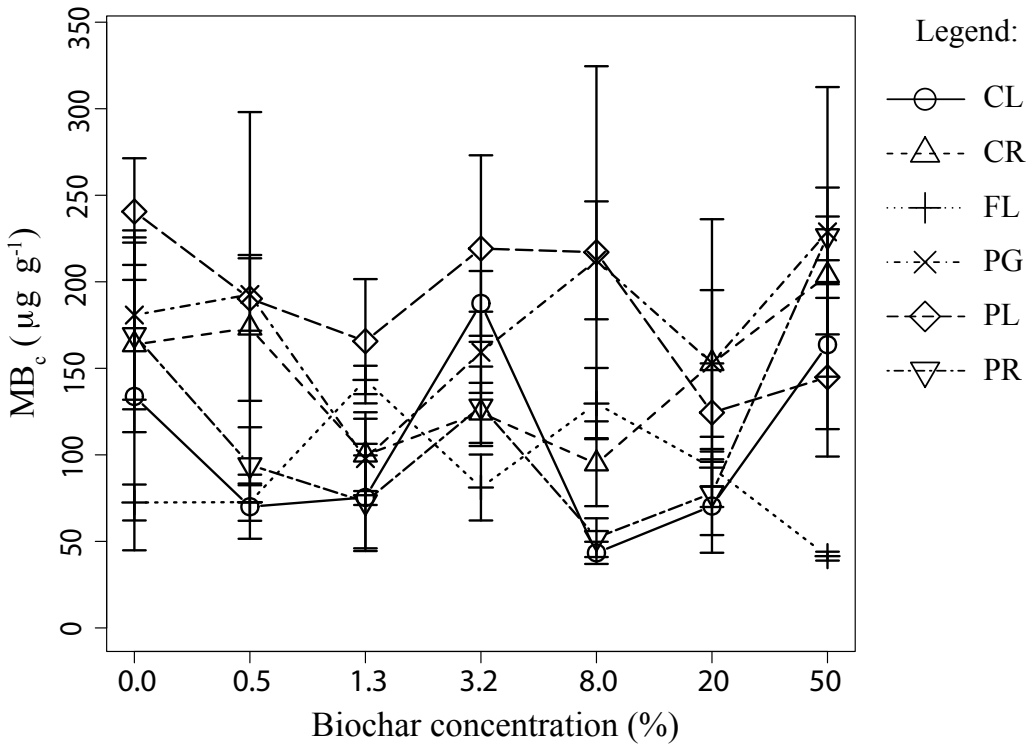
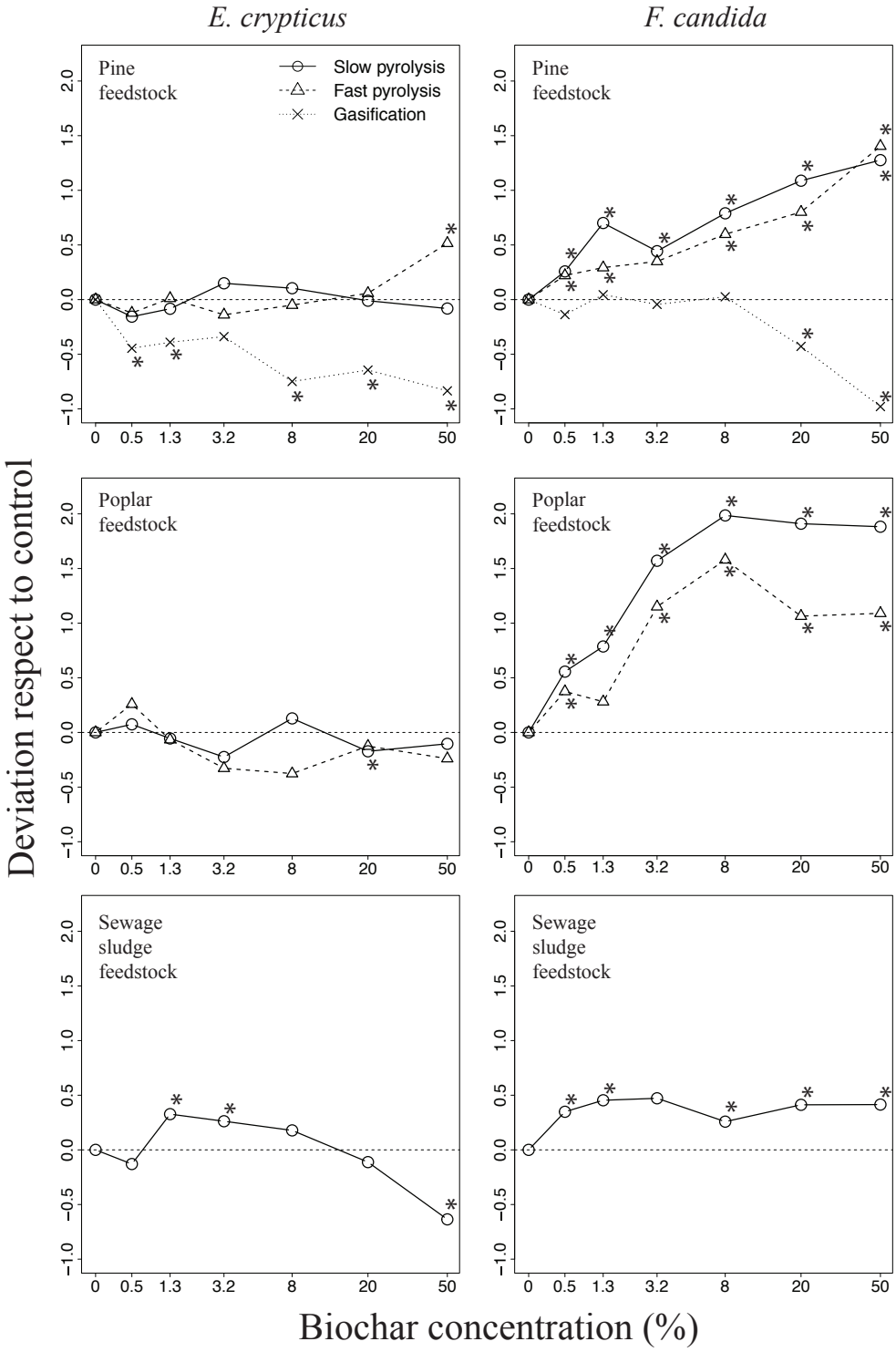


Figure 2: Mesofauna reproduction in each biochar tested.



20%. Effects were stimulatory in FL at concentrations 1.3% and 3.2%, and negative at 50%. For PG, the effects were inhibitory for all concentrations >0% with the exception of 3.2%. Finally, PR 50% was stimulatory.

3.3 Fresh biochar composition effects on species performance

Mean reproductive performance of the test species for each biochar is shown in **Table 6**. Pearson correlations revealed a significant negative relationship between biochar LOI-1100 and mean *F. candida* performance (r=-0.83, p=0.042). LOI-1100 was itself strongly correlated with biochar Ca content (r=0.96, p=0.002), pH (r=0.92, p=0.009), and EC (r=0.98, p<0.001). For *E. crypticus* performance the relationships were similar, as there was a negative relationship with biochar pH (r=-0.92, p=0.008), whereas pH was itself highly correlated with LOI-1100 (see above), Ca (r=0.80, p=0.058) and EC (r=0.86, p=0.028).

3.4 Soil fauna performance modeling

Models explaining species performance based on mixture chemical properties are shown in **Table 7**. Microbial biomass was not a selected parameter. Overall, the models explained little variation of fauna reproduction for either the *F. candida* dataset (R^2=0.44) or that of *E. crypticus* (R^2=0.19).

4. Discussion

4.1 Biochar characteristics

4.1.1 Biochar labile carbon

Labile carbon content of biochars is associated with its degree of resistance to degradation by microbial action, and hot-water extraction

has been considered an appropriate estimation of soil organic matter available to microorganisms in the short term (Pereira et al. 2011; Rovira et al. 2007; Joseph et al. 2009). VM is another estimate of the labile fraction, of interest in biochar studies due to its potential influence on nutrient availability (Lehmann et al. 2011; Deenik et al. 2010). Fast pyrolysis materials were shown to have substantial VM and labile C contents (see

Table 6: Mean reproductive performance of the two test species for each biochar, calculated as the mean of reproduction in all mixtures >0% with respect to the control. For material abbreviations see **Table 1**.

Material	*F. candida*	*E. crypticus*
CL	244.9	94.1
CR	192.3	85.4
FL	139.3	98.1
PG	74.7	43.3
PL	175.9	98.7
PR	161.1	104.5

Table 7: Multiple linear model associating measured parameters with reproduction of *Enchytraeus crypticus* and *Folsomia candida*. Values shown for each parameter are the standardized coefficient β, with statistical significance at CI=0.95 indicated as non-significant (NS), 0.05-0.01 (*), 0.009-0.001 (**), and < 0.001 (***). Also provided are the model test statistic, degrees of freedom (df), model significance and corrected R^2. Material intercepts not shown.

Parameter	*E. crypticus*	*F. candida*
HPO_4^{2-}	0.34	0.41 **
K^+		0.86 ***
NH_4^+	-0.22	
SO_4^{2-}		-0.61 *
EC		0.92 **
pH	0.58 **	
Concentration	-0.45 *	-0.90 ***
df	36	35
stat	F=3.3	F=7.2
p	0.021	<0.001
R^2	0.19	0.44

results above). If these labile contents have an effect of increasing microbial biomass, then material identity as well increasing concentration should influence this parameter. This was tested statistically by a two-way ANOVA with microbial biomass as the dependent variable and concentration and material as predictors; in this test, concentration resulted non-signficant ($p=0.40$), and material significant ($p=0.004$). However, post-hoc tests contrasting materials did not follow expectations, with significant differences only between PL and CL, and PL and FL, whereas significant differences with the fast-pyrolysis materials would instead be expected. For instance, Bruun et al. (2012) found that a fast-pyrolysis char supported more microbial biomass than a slow-pyrolysis char. In summary, we found no evidence to support the hypothesis that labile C increased microbial biomass, therefore potentially stimulating reproduction of the two saprophagous test speices.

4.1.2 FTIR

FTIR spectra are indicative of biochar surface chemistry, including surface-adhering aromatic and aliphatic pyrolysis condensates, as well as the degree of material aromaticity, which provides information on its value as a substrate. All biochars showed very limited content of aliphatic groups, whose presence is usually strongest at longer wavelengths (C-H stretching 2800-3000 cm^{-1}, O-H stretching at 3300 cm^{-1}). As these groups are easily lost during pyrolysis, they were most reduced in the high-temperature gasification char (PG). In the range of 1030-1400 cm^{-1}, small peaks of aliphatic methyl and ether/alcohol C-O- and C-O bonds were also evident, though the importance of these was obscured by the strong absorption by aromatic C=C and C=O. Aliphatic water-soluble functional groups found on the surface of chars such as acids, alcohols, aldehydes, ketones and sugars may serve as substrate for microbes, but pyrolysis conditions may also produce bactericidal or fungicidal compounds such as cresols, xylenes, and

formaldehydes (Thies and Rillig 2009). Fast-pyrolysis materials were also the least aromatic, evidenced by the lower overall degree of absorption (displacement on the y-axis). The aromaticity of black carbon increases its stability against microbial attack (Glaser et al. 2001), and conversion of aliphatic C to aromatic C during pyrolysis is accompanied by a reduction in C mineralization rates (Chan and Xu 2009). Thus, the FTIR spectra would also suggest that the fast pyrolysis materials should be the higher-quality substrate for microbial growth. These qualitative observations are coherent with the measurements of labile C described above, though as already mentioned, the results generally do not support any effect of material on microbial abundance, either positive or negative.

4.2 Mesofauna responses

Stimulation of reproduction was strong and significant for *F. candida* in all biochars except PG, where strong inhibition of reproduction occurred. In the case of *E. crypticus*, effects were similar for PG, while in FL a parabolic-type response occurred with initial stimulation and inhibition at high concentrations, but no consistent response was seen for other materials. Firstly, these results point to the importance of biochar screening with multiple species. These mesofauna are generally considered saprophagous, with distinct physiology, exposure routes, ecological niches, and life histories (Bardgett, 2005; Jänsch et al. 2005), therefore it is expected that responses may differ. In both cases, inhibitory effects were only seen with PG and high concentrations of FL.

In both *F. candida* and *E. crypticus*, mean performance was negatively associated with high carbonate content (LOI-1100) and pH, respectively, hence indicating that alkalization limited reproduction. PG and FL were the materials with the highest carbonate and calcium contents and pH. PG reduced mean reproduction for both organisms. For FL, *F. candida* mean reproduction was only slightly stimulated,

whereas for *E. crypticus*, mean reproduction was similar to other materials.

Contradicting the results of the correlations described above, in the multiple linear models based on mixtures, pH showed a positive effect on enchytraied reproduction, and was not included in the collembolan performance model. However, these models explained very little variability of the response, hence they are of low reliability.

Elsewhere, *F. candida* has been found to be a pH-sensitive species (see below), but the reported thresholds at which acute (mortality) and chronic (reproduction) effects are seen are varied and somewhat discordant within the literature. One study using an artificial soil found that reproduction and adult survival (to a lesser extent) were significantly negatively impacted by pH>7 (Greenslade and Vaughan 2003). Crouau et al. (1999), testing the effect of pH on reproduction and adult survival in an artificial soil nearly identical to that of Greenslade and Vaughan (2003), found that increases in pH up to 6.9 did not seem to influence adult mortality, while the rate of reproduction declined steadily with the rise in pH. However, two other studies using a wide variety of natural test soils concluded that soil pH was not a significant factor, having tested soils up to pH 7.4 (Amorim et al. 2005) and pH 8.4 (Domene et al. 2011).

pH-mediated effects on *E. crypticus* reproduction are also possible, since the pH of the test soil was already ~8.0, exceeding the optimal pH range for this species (Jänsch et al. 2005). However, it is considered that among Oligochaete the pH tolerance range for reproduction of this species is relatively broad (4.2-7.7; Chelinho et al. 2011), though high pH (e.g. > 6.7; Chelinho et al. 2011) has been shown to provoke a behavioral avoidance response.

Regarding the increased reproduction observed with some chars, the lack of evidence for a relationship between biochar concentration and microbial biomass went against our expectations. However, the methodology utilized may not represent additional changes in the

microbial community, such as shifts in microbial/fungal dominance due to biochar-mediated pH changes, whereas increased pH is expected to increase fungal dominance (McCormack et al. 2013; Thies and Rillig 2009). If such a shift did occur, it might explain differing responses since the animals have different feeding habits. Many uncertainties still exist as to the feeding preferences of enchytraeids, though there is evidence to indicate their preference for decaying organic matter (i.e. saprovores) (Briones and Ineson 2002). On the other hand, most collembolans are fungal grazers, and *F. candida* is no exception (Fountain and Hopkin 2005), though it is also known to feed on bacteria and nematodes (Bakonyi 1989; Lee and Widden 1996).

An alternative explanation for stimulation may arise from mutualistic relationships with gut microbiota capable of using organic compounds in biochar. The composition of gut symbionts in insects is now recognized to be often highly diverse and abundant (Dillon and Dillon 2004). Investigations on these communities in *F. candida* demonstrated the presence of symbiotic bacteria isolated from the gut were of using aromatic compounds (Thimm et al. 1998). Also, Borkott and Insam (1990) demonstrated the presence of symbiotic chitinolytic microorganisms in the gut and faeces of this species. A closely related species, *Folsomia fimateria* has been shown to complete its entire lifecycle consuming only a hydrochar (Salem et al. 2013), and we have also readily observed ingestion of biochar particles by *F. candida,* evidenced by coloring of the digestive tract due to its translucent nature (personal observation of the authors), so the use of some biochar compounds by symbiotic bacterial gut communities is plausible, imparting a nutritional advantage with increasing concentrations of the majority of the materials tested. Less information is available to discuss any similar mechanism for *E. crypticus*, though mutualistic bacteria within the gut have also been described (Krištůfek et al. 1999).

5. Conclusions

Possible effects of different biochars on reproduction of two soil invertebrates was investigated in relation to fresh biochar characteristics, and chemical properties and microbial abundance of soil-biochar mixtures. Performance of the two species was analogous for both the sewage sludge and pine gasification char, which were slightly stimulatory and inhibitory, respectively. For the slow and fast-pyrolysis wood materials, *F. candida* reproduction was strongly stimulated, whereas generally no effects were seen in the case of *E. crypticus*. Our results suggest alkalization-related increases in mortality and inhibition of reproduction. As for stimulation, we hypothesized that increased microbial abundance would explain stimulation of fauna performance, but this showed no clear trend with biochar concentration, providing no evidence for a trophic effect. Other potential mechanisms, such as microbial community shifts or the use of biochar compounds by symbiotic gut bacteria may explain the increased performance of the collembolans, whose reproduction in some treatments was two times that of the control, whereas for enchytraeids no strong stimulation was observed.

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Chapter 3 Soil Collembolan population structure altered by biochar material and application rate in bioassays

Abstract

Ecotoxicological bioassays with bioindicator organisms have been proposed as a component of biochar materials screening before field application. However, toxicity is not to be expected with most biochars, and stimulation of bioindicator endpoints might also occur given that biochar is known to enhance various soil properties. Therefore, in order to improve materials characterization using biological methods, following standardized reproduction assays with the soil collembolan *Folsomia candida* we evaluated additional non-lethal endpoints by measuring somatic length of juveniles. Somatic length measurement was carried out on sample populations raised in soil-biochar mixtures with 6 different biochars, obtained from varied feedstock materials (pine, poplar, and sewage sludge) by slow, fast, and gasification pyrolysis processes, tested in increasing application rates in an agricultural soil. Multi-Gaussian models were used to identify juvenile size classes, and estimated biomass and proportional representation for each size-class cohort were calculated for each. Biomass, shown to be a sensitive endpoint, was stimulated in many cases, up to a factor of two above the controls. Biomass was also highly sensitive to noxious effects of some biochars and was seen to be a more sensitive endpoint than juvenile number. Juvenile population structure, interpreted as the number and proportional representation of size classes, was altered by biochar addition, with increasing number of size classes under stimulatory conditions and reduced number of size classes in less favorable conditions. Therefore, the population structure endpoint may be

useful for the characterization of biological effects of diverse agricultural amendments.

1. Introduction

Biochar is a pyrolyzed biomass product very similar to charcoal whose use is destined for application as a soil amendment as opposed to storing energy (Lehmann and Joseph 2009). While much research has centered on improvement of agriculture, particularly in tropical zones, little attention has been paid to potential unintended effects (Kookana et al. 2011), including the ecotoxicological risks of the application of particular biochars to soils. Most biochars produced from forestry and agricultural residues are not expected to contain pollutants, such as corn stover, but also from materials from which noxious effects can be expected, such as sewage sludges with high heavy metal content. Additionally, biochar characteristics differ greatly in their physical and chemical properties due to not only feedstock, but also the pyrolysis method used in their production (e.g. Wu et al. 2011).

Given the large potential diversity of products owing to the multitude of factors influencing biochar's physiochemical properties, characterization efforts must be undertaken for wider adoption within agriculture (Joseph et al. 2009). Recent efforts in this regard have been the development of product testing guidelines, which include the ecotoxicological screening of biochars potentially containing hazardous substances (IBI 2013). Drawing from the field of soil ecotoxicology, some studies have included standard bioassay methods (Van Zwieten et al. 2010). Bioassays are cost effective because they do not require specialized equipment and thus are easily adoptable, and the use of test organisms has higher ecological relevance than chemical or in-vitro methods. Therefore, our objective has been the biological characterization

of biochar materials using the collembolan *Folsomia candida*. *F. candida* (Collembola), a parthenogenetic soil springtail of world-wide distribution, is considered as a "standard" soil arthropod (Fountain and Hopkin 2005), whose use is common in soil ecotoxicology screening protocols. Some biochars might present toxic effects, with measurable changes in parameters such as survival, growth and reproduction; however, not all biochar products and application rates are noxious, and since Collembola are microorganism grazers, positive effects on reproduction or growth might be expected due to the known enhancement of microbial communities associated with biochar application to the soil (Kolb et al. 2009; Bruun et al. 2012). Therefore, a bioassay that is sensitive to direct or indirect effects on non-lethal endpoints may give additional information about biochar´s potential influence on soil quality before field application. Here we evaluate, in a short-term assay, the effects of different biochars on the somatic length of juveniles and recruitment to juvenile size-classes as indicators of potential impacts of biochar on soil quality.

Changes in *F. candida*'s somatic length (Crouau and Moïa 2006; Domene et al. 2007; Bur et al. 2012) or mass (Crommentuijn et al. 1997; Smit et al. 2004) in response to contaminants have often been used as endpoints in ecotoxicological studies. The use of sub-lethal, measurable responses of this organism has also been extended to serve as a bioindicator approach for the assessment of soil quality when no specific harmful contaminant is suspected; Kaneda and Kaneko (2002) tested contrasting effects between "high-quality" forest soil versus pure sand substrate on *F. candida* body length, demonstrating a relationship between soil biological and chemical parameters and organism response. Domene et al. (2011) compared bioassay endpoints of survival and reproduction for *F. candida* using 19 Mediterranean soils and found that reproduction was positively affected by moisture content, coarser texture, and decreasing nitrogen. Nelson and colleagues (2011) compared

the effects of greatly differing soils on reproduction and growth of different aged *F. candida* juveniles in the context of organic potato cropping, and found that somatic growth was found to be the most sensitive parameter to changes in soil quality. Additionally, they observed that reproduction may serve as complementary information when growth is not sensitive, whereas survival is not an effective factor in soil quality monitoring.

Studies such as those described above measured changes in lengths of individuals over time; less common is the measurement of population structure (e.g. by using the same measurements such as length or mass), which is possible in collembolans due to their successive molts along the life cycle, which results in a measureable discontinuous increase in size with moulting events that can be used to estimate changes in the population structure. Population-level parameters are of great value due to their higher ecological significance with respect to individual-level tests (Kammenga and Laskowski 2000; Stark and Banks 2003). One example of population-level information was by Crouau and Moïa (2006), who developed histograms of *F. candida* juvenile length in a population exposed to four xenobiotics, though these results were not analyzed quantitatively. More quantitative was an approach used by Son et al. (2009) in the parameterization of a "baseline" population based on measurement of head capsule widths of the collembolan *Paronychiurus kimi* (Lee). This baseline population was then compared to populations exposed to increasing cadmium concentrations in order to assess the effects of this metal on population structure, whereas individuals measured in the toxicity assay were assigned to head capsule size classes previously established in the baseline population. Block (1982) also assigned sampled field populations of Collembola to pre-determined size classes. However, other authors have commented that the analysis of cohort (class) structure should give a more biologically realistic

description of demography than the initial arbitrary definition of size classes (Hertzberg et al. 2000); these previously mentioned authors used statistical methods to estimate separate size class parameters from histogram distributions of samples of field populations of arctic Collembola.

Here, we aim to assess short-term changes in juvenile population structure due to exposure to different biochars at increasing concentrations. Our approach is based on quantitative tools which allow the differentiation of somatic length classes of *F. candida* juveniles following the 1-month survival/reproduction standardized test. This provides additional sub-lethal and population-level endpoints, whereas this test is normally limited to the level of the individual. Specifically, the research questions posed are 1) if somatic length and separation of size-classes are sensitive to biochar material and application rate, and 2) if they can be used to interpret biological effects of biochar products and thus their influence on soil quality.

2. Methods

F. candida bioassays were carried out using mixtures of biochar with an agricultural soil as detailed later in this section. The soil consisted of the topsoil of an olive field in La Granadella, Lleida, NE Spain, whose main properties are reported in Domene et al. (2011). Six biochars from different feedstocks and pyrolysis processes **(Table 1)** were tested in 6 concentrations in an increasing geometric progression of 0 (control), 0.5, 1.3, 3.2, 8, 20 and 50% (dw/dw), thus representing potential application rates spanning two orders of magnitude.

In accordance with ISO Guideline 11267 (1999), *F. candida* cultures were synchronized to obtain 10-12 day-old springtails, which were individually separated from cultures using a pump-powered pooter and

placed in test vessels at ten animals per replicate. Five replicates were prepared for each concentration, each consisting of a container filled with 30 g of soil-biochar mixture at 40% of the maximum water holding capacity. Test vessels were 100 ml polyethylene containers with sealable tops, and were aerated and randomly rearranged 2 times weekly. All tests were conducted at 21 ± 1 ºC, and followed a 16/8 hour light/dark cycle at 12 ± 10 μE m^{-2} s^{-1} luminosity to ensure that organisms would not avoid the substrate. Approximately 2 mg of brewer´s yeast were provided for food at the beginning of the test and after 14 d. After 28 d, replicate contents were carefully transferred to a 1000 ml glass beaker (10.5 cm diameter) by multiple iterations of flooding and decanting. A dark dye was added to improve visibility of the white collembolans, and a final gentle stirring was done to assure that all individuals floated to the surface. Photographs of the floating organisms were taken using a high-resolution digital camera. Subsequently, counting and measuring were performed manually on a computer using the ImageJ software version 1.43 (National Institutes of Health, Bethesda, MD, USA). Total number of juveniles in each replicate was counted. For somatic length measurement, defined as the end of the posterior abdominal segment to the anterior margin of the head (Crouau and Moïa 2006), the scale was set using the known diameter of the beaker (10.5 cm) and carried out using existing tools in ImageJ. Measurement of individuals proceeded in a quadrant of the photograph as specified by a random number generator. Measurement of individuals began in the extreme outwards corner of the selected quadrant and proceeded in a progressive up-and down fashion and to the left or right in a viewing region of 140 mm^2 at 20X magnification until 50 juveniles were measured. This resulted in a total of 50 × 5 = 250 juvenile measurements for each material-concentration, a total of 10,500 measurements.

Table 1: Biochar materials tested in the study and identification codes.

Material	Description
CL	*Populus nigra* (Poplar) wood chip biochar produced in a slow pyrolysis reactor 500 – 550 °C
CR	*Populus nigra* wood chip biochar produced in a fast pyrolysis reactor at 430 – 510 °C
FL	Wastewater sludge biochar produced produced in a slow pyrolysis reactor 500 – 550 °C
PG	*Pinus pinaster + P. radiata* wood chip biochar produced in a gasification reactor at 600 – 900 °C
PL	*Pinus pinaster + P. radiata* wood chip biochar produced in a slow pyrolysis reactor 500 – 550 °C
PR	*Pinus pinaster + P. radiata* wood chip biochar produced in a fast pyrolysis reactor at 440 – 480 °C

To quantify the differences in size classes of juveniles, a multi-Gaussian fitting approach was used, following the assumption that size distribution within each class can be approximately defined by a normal distribution. This analysis was carried out in R. For each material-concentration, histograms of length measurements were first created with bin size and number calculated following the criteria set up by Sturges (1926), and these graphs were assessed visually to identify the number of modes (size classes) that were present. In all cases we tried to follow the principle of parsimony, keeping low the number of identified modes. Next, the final number of bins was thereafter defined as the Sturges bin number multiplied by the number of identified modes. In other words, if a population was uni-modal, then no modification to the Sturges bin number was made, but if two modes were present it was multiplied by a factor of two. If model convergence in a given population was not successful, the number of modes was increased until convergence was achieved. The observed abscissa x_{jil} for class j of concentration i of material l were defined as the midpoint of the interval of each bin. The Gaussian function to be fit to observed abscissa was defined as,

$$f(x) = \frac{k}{\sigma\sqrt{2\pi}} e^{\frac{-(x-\mu)^2}{2\sigma^2}}$$

whereas x is the observed abscissa (length), where k is the area enclosed by the corresponding Gaussian function, σ is the standard deviation of the set of x_{jil}, and μ is the mean of x_{jil}. Fitting was done by nonlinear least-squared regression techniques (function "nls" in R) and approximate starting estimates were provided for the parameters described above. Using the fitted parameters, the area a_{jil} of each curve was transformed into the proportion p of the population represented by each size class as $p_{jil} = a_{jil} / \text{sum}(a_{ji1}...a_{jin})$. The number of individuals n_{jil} of each class was

calculated as $n_{jil} = p_{jil} * n_{il}$, whereas n_{il} is the mean number of individuals for the material-concentration population (mean of 5 replicates). Thereafter, the total biomass per each size class expressed as mg dry weight of individuals b_{jil} was calculated following Fountain and Hopkin (2001) as *b=0.8317*e^(m*1.8626), m* being the fitted abscissa parameter for the class. The equation provided by these last authors was considered appropriate since it was constructed using *F. candida* specimens of the range 0.1-2.2 mm length, which includes the full range of somatic lengths measured in the present study.

3. Results

3.1 Validity

Modality of the controls was between 1 and 3 (**Table A.1**). CL, CR, PL, and PR were bi-modal, whereas PR had 1 mode and PG had 3. In the PG control, a population class corresponding to 0.99 mm was fit (not present in other control populations) representing 31% of the sample population. This was likely due to slight differences in the animals introduced in this test (e.g. due to slight age differences in the 2-day hatching window during synchronization or nutritional state), which was also reflected in the higher mean number of juveniles (**Table 3**). However, a Kruskal-Wallis test of difference in control group juvenile number between materials resulted non-significant (Chi-squared=24.7, df=25, p=0.47), supporting the comparability of all tests. PR control was uni-modal due to the fact that a bi-modal population fit was unsuccessful (non-convergence); this is reflected in the high standard residual error for this fit (**Table A.1**), suggesting the existence of an additional mode(s). Considering overall mode number and location in the controls, similar results were found by Crouau and Moïa (2006), who found three *F. candida* juvenile size groups present in controls after 35 d at ~0.4, ~0.5,

and ~ 1.0 mm, although these results were not analyzed quantitatively and thus exact location or group proportions of total animal number are unknown. A notable difference between the present study and that of Crouau and Moïa is the smaller size of the second-smallest size class (~0.5 compared to 0.74-0.94 mm in the present study). Finally, it was seen that trends in size class recruitment and animal size were consistent within materials, demonstrating that effects were not due to random differences in control animals.

3.2 Multi-Gaussian fits

3.2.1 Goodness of fit

Modality for all material-concentration ranged between 1 and 4; uni-modal populations had 2 cases, bi-modal 29 cases, three modes 10 cases, and 4 modes 1 case (**Table A.1**). The methodology was able to minimize to a large degree the residual sum of squares. It is seen that lower goodness of fit was typically caused by peaks and troughs of adjacent histogram bars within the same size class (e.g. FL 8, PG 0.5, PG 3.2, PL 20) (**Figure B.1)**, possibly indicating small margins between the size of animals of distinct instars (see Discussion below for details on its methodological implications). Calculating the area of each fit yields a total population number estimated by the fit, the "observed" animals, which optimally should be equal to the total number of measured animals for that treatment, or "expected". The differences from the expected animal number are given in **Table 2**, having a median of 1.89, mean of 4.98, and SD of 7.96. The fits with differences higher than 1 SD from the median (more conservative than the average) were FL 0.5, FL 20, PG 3.2, PL 0, PR 0, PR 0.5, and PR 1.3. Firstly, these treatments with high population number error do not correspond to those with lower goodness of fit described above, indicating a different source of estimated

population number error. Visual inspection of these treatments (**Figure B.1**) shows that these fits did not include points on the right side of the graphs, and thus these instances of high population number error are an indication of small populations of larger animals which were not fit with additional Gaussian curves. Therefore, taken into consideration with the experiment validity discussed above, it is seen that some populations of larger animals did likely exist in a number of cases (including the controls PL and PR), though model adjustment was not possible using this particular methodology due to the small number of points, impeding model convergence.

3.2.2 Parameters

The numerical fits are provided in **Table C.1**. In general, the fitted parameters *k*, *m*, and *s* were highly significant. Of the 282 parameters fitted, 16 were not significant at the 0.05 level of confidence. Based on the values of *m* (size class midpoint), it was seen that the location of abscissa defining each of the size classes was not stable with increases in

Table 2: The difference between actual measured (expected, *e*) and modeled animal count as a result of the multi-Gaussian fits (observed, *o*). Animal number *o* was calculated as the sum of area of the multi-Gaussian curves. The difference *e* - *o* indicates whether the fits over- (negative values) or underestimated (positive values) the population. See **Table 1** for material abbreviations.

Treatment	*e* - *o*	Treatment	*e* - *o*
CL 0	-0.79	PG 0	5.62
CL 0.5	3.07	PG 0.5	0.57
CL 1.3	-0.16	PG 1.3	-0.25
CL 3.2	0.28	PG 3.2	11.51
CL 8	3.61	PG 8	0.69
CL 20	-0.7	PG 20	-0.96
CL 50	3.22	PG 50	0.55
CR 0	3.67	PL 0	13.72
CR 0.5	-1.37	PL 0.5	8.77
CR 1.3	-1.78	PL 1.3	7.46
CR 3.2	2.01	PL 3.2	-1.27
CR 8	1.09	PL 8	2.25

biochar concentration, likely indicating that treatments affected the size of the animals, which will be discussed in the later sections of this communication. Considering all fitted *m* irrespective of material or concentration shows that three generalized size classes dominated in terms of frequency, those at ~0.45, ~0.75, and ~0.9 mm.

3.3 Juvenile number and biomass endpoints

Biochar treatments had a strong effect on *F. candida* juvenile number and estimated biomass for each material (**Table 3**). Significant increases in juvenile number were found for CL, CR, PL, and PR, and in PG juvenile number was significantly reduced in concentrations 20% and 50%. FL caused initial stimulation up to 8% concentration for both endpoints, after which juvenile number remained relatively stable and biomass decreased. Juvenile number and biomass can be compared for their relevance to the objective of detecting biological effects of biochar

application. Statistically significant ($p<0.05$) linear relationships were found between these two endpoints for all materials with the exception of FL (**Figure 1**). In this case, whereas FL juvenile number remained relatively unchanged at biochar concentrations >3.2%, biomass was progressively reduced with each increase in concentration.

3.4. Juvenile population structure

Somatic length class recruitment was altered due to biochar treatment. First, biochar concentration changed the modality of all material groups with the exception of PL, which had two modes in all concentrations tested. It is seen that the change in number of classes was in most cases associated with the appearance and/or disappearance of the largest identified size class. The exception was PR, for which the changes in modality are associated with the recruitment of intermediate classes corresponding to 0.7 and 0.59 mm at concentrations 0.5% and 1.3%.

Population structure as each class' proportion of total biomass for each treatment is shown in **Figure 2**. Within materials it is possible to trace effects on each size class based on *m* in conjunction with visual inspection (**Figure B.1**; **Table C.1**). Structure changed with concentration within materials, and effects were dissimilar between materials. CL and CR had notable proportional increases in biomass allocated to the smallest size class with increasing biochar concentration. In CL, FL, and PR, higher structural complexity (more classes) emerged at low to intermediate concentrations. In PG, structural complexity was quickly reduced with increasing concentration. Finally, in PL, structural complexity remained relatively even, but the size of animals corresponding to the larger group progressively decreased.

Regarding the size of individuals in each size-class (**Figure 2**), the treatments with greater population structural complexity (FL and CL) had

wider overall size range and smaller sized animals in the intermediate size class. This is particularly evident in FL, with the promotion of size classes corresponding to 1.0 and 0.96 mm at concentrations 3.2 and 8%, respectively. In the case of PG, it has already been noted that the control population presented a more complex initial structure compared to other materials; this structural complexity present at low concentrations was thereafter reduced, as well as the size of animals pertaining to the largest class.

Table 3: Estimated *F. candida* biomass of each identified size class for each material-concentration (ordered based on smallest to largest animal sizes), total biomass (sum of classes 1-4), normalized biomass *B* as the ratio of the estimated biomass with respect to the control, mean juvenile number ± SE of the 5 replicates, and normalized juvenile number *N* as the ratio of the mean juvenile number with respect to the control. For mean juvenile number, replicates with significant differences with respect to the control following Mann-Whitney contrasts at CI=0.95 are indicated with *. See **Table 1** for material abbreviations.

Treatment	Class 1 biomass (mg odw replicate^{-1})	Class 2 biomass (mg odw replicate^{-1})	Class 3 biomass (mg odw replicate^{-1})	Class 4 biomass (mg odw replicate^{-1})	Total biomass (mg odw replicate^{-1})	Normalized total biomass (*B*)	Mean juvenile number	Normalized juvenile number (*N*)
CL 0	31	669	-	-	700	1.00	167 ± 21	1.00
CL 0.5	174	553	282	-	1009	1.44	260 ± 45*	1.56
CL 1.3	289	337	411	-	1037	1.48	298 ± 25*	1.79
CL 3.2	535	135	640	-	1309	1.87	429 ± 43*	2.57
CL 8	718	752	-	-	1470	2.10	498 ± 15*	2.99
CL 20	800	521	-	-	1321	1.89	485 ± 42*	2.91
CL 50	662	733	-	-	1395	1.99	481 ± 38*	2.88
CR 0	41	56	531	-	628	1.00	162 ± 13	1.00
CR 0.5	77	755	-	-	832	1.33	223 ± 14*	1.37
CR 1.3	163	602	-	-	766	1.22	208 ± 25	1.28
CR 3.2	370	733	-	-	1103	1.76	349 ± 40*	2.15
CR 8	407	769	-	-	1176	1.87	418 ± 36*	2.58

CR 20	342	637	-	-	979	1.56	335 ± 43*	2.06
CR 50	435	555	-	-	991	1.58	339 ± 47*	2.09
FL 0	69	564	-	-	634	1.00	188 ± 19	1.00
FL 0.5	130	642	-	-	772	1.22	254 ± 11*	1.35
FL 1.3	160	697	-	-	857	1.35	274 ± 14*	1.45
FL 3.2	141	555	194	-	890	1.41	277 ± 48	1.47
FL 8	89	445	237	-	771	1.22	237 ± 14*	1.26
FL 20	224	518	-	-	742	1.17	266 ± 29*	1.41
FL 50	113	487	-	-	600	0.95	266 ± 13*	1.41
PG 0	135	574	327	-	1037	1.00	304 ± 40	1.00
PG 0.5	105	582	281	-	968	0.93	263 ± 40	0.86
PG 1.3	221	499	249	-	969	0.93	318 ± 34	1.04
PG 3.2	175	636	-	-	811	0.78	291 ± 11	0.96
PG 8	133	698	-	-	831	0.80	312 ± 24	1.03
PG 20	118	345	-	-	462	0.45	174 ± 18*	0.57
PG 50	14	-	-	-	14	0.01	6 ± 2*	0.02
PL 0	23	740	-	-	764	1.00	164 ± 2	1.00
PL 0.5	69	834	-	-	903	1.18	207 ± 27*	1.26
PL 1.3	230	807	-	-	1037	1.36	280 ± 20*	1.70
PL 3.2	101	794	-	-	895	1.17	237 ± 18*	1.44
PL 8	197	864	-	-	1060	1.39	294 ± 24*	1.79

PL 20	144	953	-	-	1097	1.44	343 ± 18*	2.09
PL 50	239	931	-	-	1169	1.53	374 ± 19*	2.28
PR 0	0	711	-	-	711	1.00	156 ± 11	1.00
PR 0.5	61	112	563	-	736	1.04	190 ± 24*	1.22
PR 1.3	42	81	31	629	782	1.10	201 ± 22*	1.29
PR 3.2	150	623	-	-	773	1.09	210 ± 52	1.35
PR 8	98	841	-	-	938	1.32	249 ± 23*	1.60
PR 20	308	673	-	-	981	1.38	281 ± 12*	1.80
PR 50	255	866	-	-	1121	1.58	375 ± 32*	2.40

Figure 1: Linear regressions of estimated biomass and average juvenile number (mean of 5 replicates) for each material. Regression results were as follows (material, coefficient ± SE, significance): CL, 0.47 ± 0.04, <0.001. CR, 0.47 ± 0.04, <0.001. FL, 0.15 ± 0.10, 0.21. PG, 0.29 ± 0.04, <0.001. PL, 0.51 ± 0.05, <0.001. PR, 0.46 ± 0.04, <0.001. An ANCOVA to evaluate influence of material on slope of the relationship resulted significant (df=5, F=5.6, p<0.001). Subsequently, a multiple linear model was used to test for differences of slopes between materials. Using CL as the baseline, FL and PG materials had significant interactions with biomass in predicting juvenile number (p=0.005 and p<0.001, respectively), though there was no significant interaction between these. It is noted that the FL linear model was non-significant. See **Table 1** for material abbreviations.

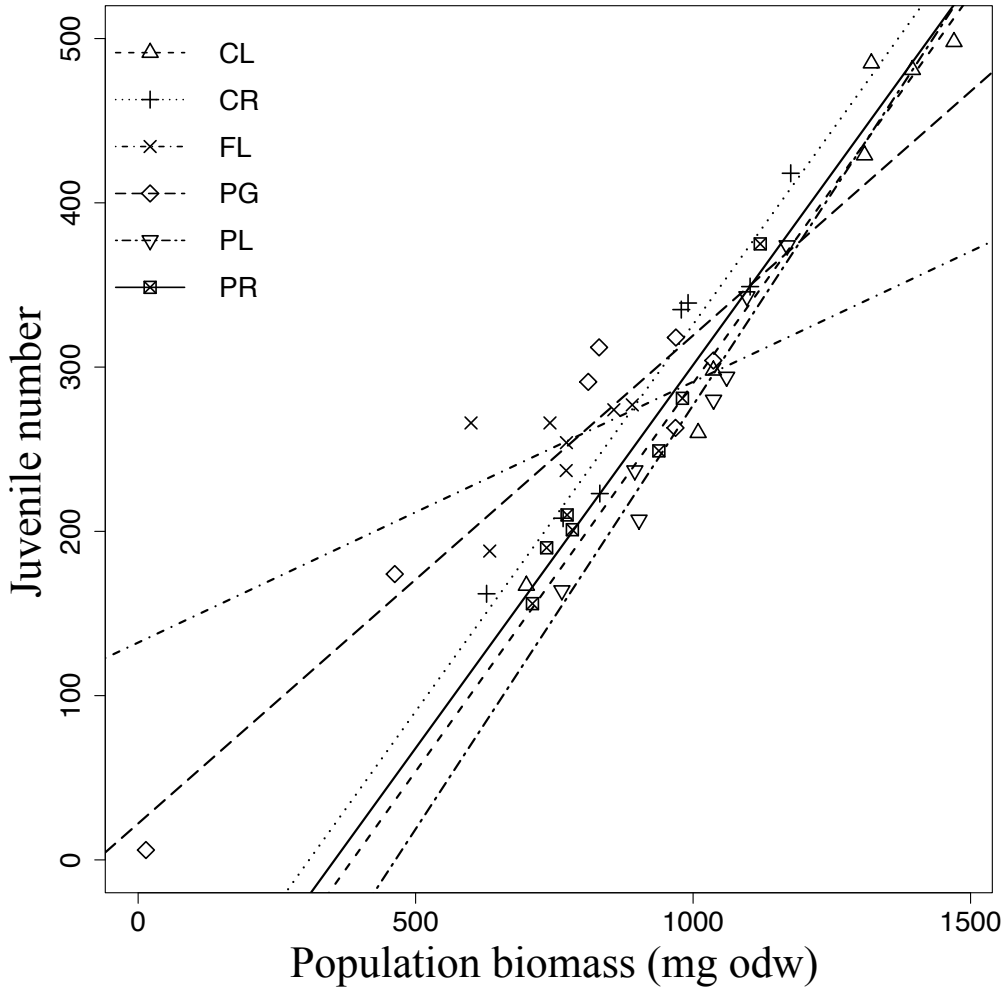
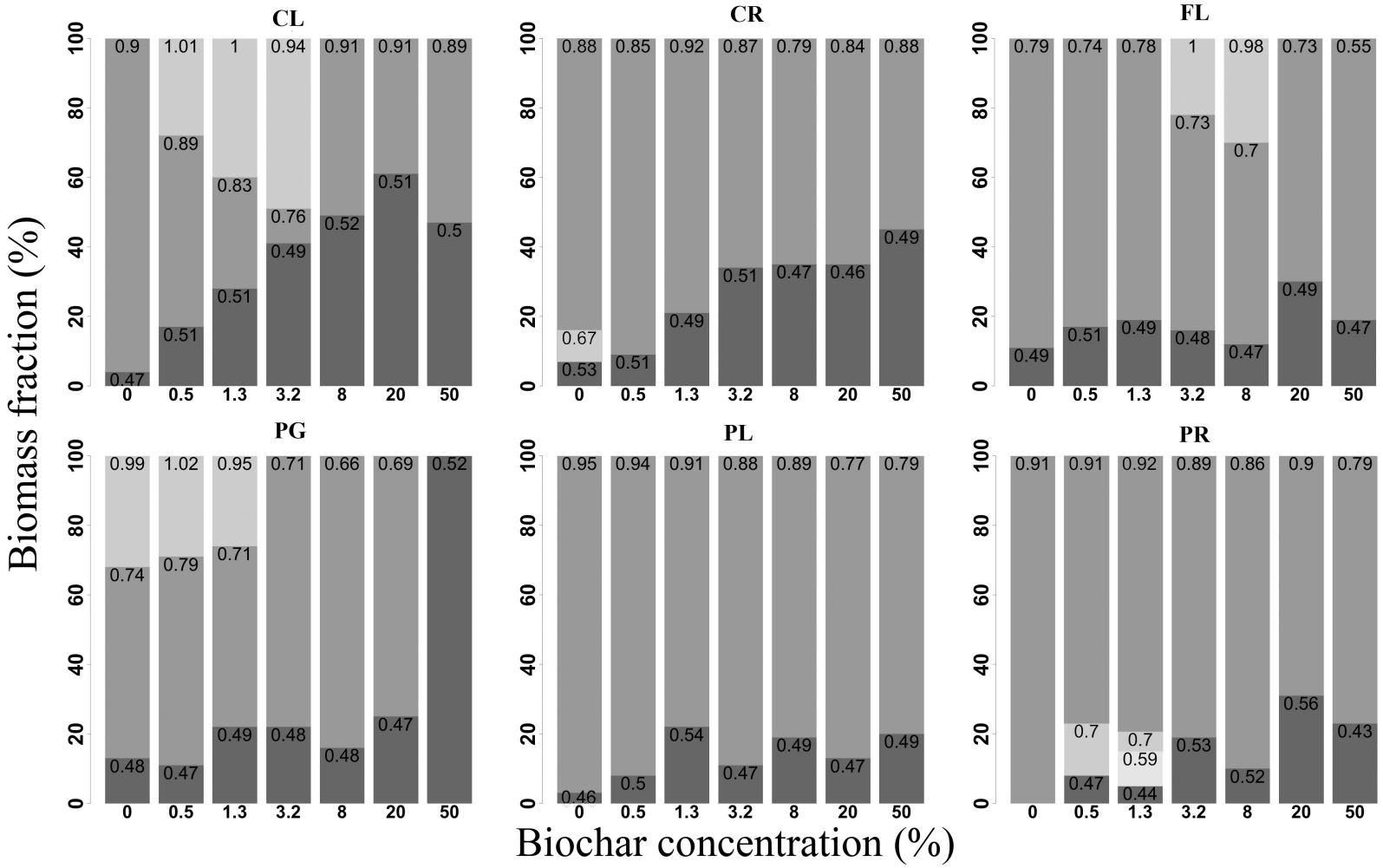


Figure 2: Class recruitment based on estimated biomass of each class. Different colored bars represent different size classes present in each material concentration (x-axis) and their proportional representation (y-axis). Numbers within each class bar define the fit class midpoint (animal length). See Table 1 for material abbreviations.


CL
CR
FL
PG
PL
PR
Biomass fraction (%)
Biochar concentration (%)
0
0.5
1.3
3.2
8
20
50

4. Discussion

4.1 Comparison of juvenile number and biomass endpoints

Following animal measurement, the calculation of biomass is straightforward for *F. candida*, providing an alternative endpoint beyond the standard juvenile count. When comparing these endpoints, it was seen that their relationship depended on the effect. When regressing these two endpoints the materials with strong stimulation (CL, CR, PL, PR) had similar linear regression coefficients (**Figure 1**); the material with noxious effects (PG) had a significantly different slope from the stimulatory materials; and finally, the material with clear non-linear effects on biomass (FL) did not conform to a linear model. As shown in the case of FL, the biomass endpoint was necessary for detecting effects to which juvenile number was not sensitive since FL biomass was reduced in the highest concentrations, which was not clearly reflected in the juvenile count.

Normalizing with respect to control the endpoints of total population juvenile number (hereafter *N*) and total biomass (hereafter *B*) allows comparisons between materials (**Table 3**). Considering the maximum values of *N* or *B*, the order of effect from most “beneficial” to least (harmful), the ranking does not change: CL, CR, PR, PL, FL, and PG. Maximum and minimum effects on *N* and *B* occurred at the same concentrations, with the exception of PG for which *B* was more sensitive to noxious effects. Fast and slow pyrolysis wood materials of the same feedstock had the same maximum effect concentration for both *N* and *B*, indicating that for these endpoints feedstock was more important (having more influence) than pyrolysis method.

4.2 Population structure endpoint

4.2.1 Methodological considerations

The multi-Guassian method used allowed precise and reliable determination of size classes; this was evidenced both visually by the excellent agreement between observed distributions and their corresponding fitted curves, and statistically by the high statistical significance of the fit parameters. Furthermore, this methodology was more capable of detecting changes to size class recruitment and abscissa than other methods encountered in the literature due to the fact that the modeling exercise is carried out individually for each treatment. Perhaps the study most similar to the present of any encountered in the literature is that of Hertzberg and colleagues (2000), whereas size class mixtures were separated and parameters were estimated quantitatively, one crucial difference however being their use of Bayesian methods. As commented by these authors, statistical separation of groups was impeded by confidence interval overlap of cohorts in the mixture of (assumed) normal distributions, in contrast to the present study in which groups have been separated with high confidence using classical (frequentist) methods.

Fit values for abscissa (somatic length) of the same class were not stable between concentrations of the same material (**Figure 2**). This can be expected due to natural variation or measurement errors, but it is also likely that this was caused by effects of treatment on juvenile development, as will be discussed in the following sections.

Importantly, it is assumed that this methodology does not allow the identification of true *F. candida* instars. Various authors have discussed the difficulty of identifying each instar of Collembola (Peterson 1971; Son et al. 2009), as an adult *F. candida* may pass through as many as 45 moults in its lifetime (Snider 1973). Collembola are ametabolous and therefore postembryonic development is dominated by discontinuous

increase in size until sexual maturity by numerous and successive moulting events, even beyond sexual maturity. For instance, Green (1964) found that after the 10th instar, size and form of *Folsomia candida* var *distincta* changed very little. For these reasons, we therefore must assume that more instars are present beyond the 1-4 groups detected using these methods.

All considered, given the comparability of tests (3.1), the goodness of the fits (3.2.1), and the emerging patterns witnessed in each case (3.4), we therefore conclude that the observed changes in population structure are real and not due to random effect. As such, with regards to experimental question 1, we can conclude that somatic length and population structure of the test organism were sensitive to treatments including the effects of both biochar concentration and material identity.

4.2.2 Use of population structure to interpret effects on soil quality

Population-level parameters are of interest in ecotoxicology as a best approximation to possible field effects of contaminants; however, extrapolation to potential effects on field populations is more precise if addressed by a multi-generational study, allowing for environmental adaptation which can become evident phenotypically (Niewiarowski 2001). For instance, Moe et al. (2001) studied cadmium in different larval stages to determine how this affected sublethal responses and delayed stages. Nonetheless, the results presented in our study show that different biochars have distinct effects on population class size structure, which has been associated with effects at the population level (Crommetuijn et al. 1995). Also, neonate fitness has been also shown to be important in determining future population parameters (Hammers-

Wirtz and Ratte 2000). Furthermore, we expect that due to differing physicochemical properties, biochars incorporated into the soil matrix will cause indirect effects on organisms with potential to modify mortality, birth or migration rates by mechanisms such as the release or immobilization of toxic agents (Cao et al. 2011; Xu et al. 2012), the suitability or not of the physicochemical environment created with biochar (Van Zwieten et al. 2009), increased or decreased food availability via changes in bacterial (Pietikainen et al. 2000; Steiner et al. 2008) or fungal (Warnock et al. 2007) abundances, or changes in root biomass (Makoto et al. 2010; Solaiman et al. 2010).

A number of biological explanations may be posited for the observed changes in population structure due to treatment, such as (i) conditions affecting the survival and fertility and/or oviposition of the adults (Stam et al. 1996), (ii) advancement or delay in time to hatching (e.g. due to the fact that abiotic factors as well as toxins are known to change life-history traits of collembolans by acting on egg development and size; see Ellers and Driessen 2010, Smit et al. 2004, respectively), (iii) accelerated development of instars related to food quality (Booth and Anderson 1979; Stam et al. 1996), or delayed development due to toxicity (van Straalen et al. 1989; Crommentuijin et al. 1997; Smit et al. 2004) or unsuitability of environmental conditions (iv), density dependent factors, such that increased population number and competition reduces the available nutrients per individual (Noël et al. 2006), or any combination of these.

The incidence of higher population structural complexity may therefore indicate that additional resources were available, thereby accelerating juvenile development and the promotion of larger size classes (e.g. FL), increase in the number of classes (e.g. PR), or both (e.g. CL). As previously mentioned, it is known that biochars can stimulate microorganism abundance (see Lehmann et al. 2011), and this may be a

potential source of the “positive” effects on population structure which were found at low to intermediate concentrations, although such measurements were not undertaken in this study. For instance, in CL the biomass of the largest size class of 0.94-1.01 mm increased in biochar concentrations 0.5 to 3.2 from 282 to 410 and 639 mg; why this group was thereafter not present is likely due to less favorable conditions at extremely high biochar concentrations also evidenced by the reduced estimated biomass at biochar concentrations 20% and 50%. Accordingly, we interpret that in less favorable conditions larger classes were reduced (e.g. PG, CR). In PG, the biomass of the class of 0.95-1.02 mm present at biochar concentrations 0% to 1.3% was progressively reduced from 327 to 280 and 249 mg and thereafter absent at biochar concentrations >1.3%; concordantly, the number of classes was reduced from three in the control to only one (corresponding to the smallest class) at the highest concentration. Progressive reductions in the proportion of larger size classes until elimination of all but the smallest size classes was reported in the Cd toxicity experiment carried out by Crouau and Moïa (2006), agreeing with the pattern found for PG in this study.

Therefore, with regards to experimental question 2, our results suggest that the observed changes in number and (biomass) proportion of size classes are biologically meaningful, indicating acceleration or delay in the development of the test organism, which we interpret as (respectively) stimulatory and toxic effects at the population level, and this may serve as additional information for the characterization of agricultural amendments. The methodology proved effective since it succeeded in detecting differences in small increments of concentration as well as between materials. Also, it exposed effects which the biomass and/or juvenile number endpoints did not reflect, such as the limitation of stimulatory effects to low-intermediate concentrations (i.e. CL), and

reductions of structural complexity when juvenile number was unaffected (i.e. PG).

Overall, changes to population structure were diverse, offering no clear patters between feedstocks and pyrolysis methods; for this reason a ranking based on structural complexity is not offered, but three principal response types might be generalized as those with no effect (PL), positive effects at intermediate and low concentrations (CL, FL PR), and negative effects (CR, PG). Further study is required to determine whether these effects are transitory or may lead to persistent changes under more realistic conditions.

Population-level parameters such as the size structure endpoint used in our study are of interest in ecotoxicology as a best approximation to possible field effects of contaminants; however, extrapolation to potential effects on field populations is more precise if addressed by a multi-generational study, allowing for environmental adaptation which can become evident phenotypically (Niewiarowski 2001), which would then permit the measurement of population growth rate of each of the identified size classes. For instance, Moe et al. (2001) studied cadmium in different larval stages to determine how this affected sublethal responses and delayed stages. Nonetheless, the results presented in our study show that different biochars have distinct effects on population class size structure, which has been associated with effects at the population level (Crommetuijn et al. 1995). Also, neonate fitness has been also shown as important in determining future population parameters (Hammers-Wirtz and Ratte 2000). Furthermore, we expect that due to differing physicochemical properties, biochars incorporated into the soil matrix will cause indirect effects on organisms with potential to modify mortality, birth or migration rates by mechanisms such as the release or immobilization of toxic agents (Cao et al. 2011; Xu et al. 2012), the suitability or not of the physicochemical environment created with biochar

(Van Zwieten et al. 2009), increased or decreased food availability via changes in bacterial (Pietikainen et al. 2000; Steiner et al. 2008) or fungal (Warnock et al. 2007) abundances, or changes in root biomass (Makoto et al. 2010; Solaiman et al. 2010).

4.3 Endpoint comparison

The results show that the evaluation of biological effects of biochar depends on the endpoint considered (Table 3). Whereas *N* and *B* were usually highly correlated, the population structure showed different trends, which can be expected from foundational theory of ecotoxicology and population ecology, such that any given individual-level endpoint cannot fully represent effects on populations (Newman and Clements 2008). For juvenile number and biomass, effects were either stimulatory for all concentrations tested (CL, CR, PL, and PR), with strong negative effects (PG), or non-linear (FL). A different but complementary perspective is provided with population-level endpoint. Firstly, stimulation occurred only at intermediate and low concentrations (CL, FL, PR). Also, stimulation of numbers and total biomass did not always result in increased juvenile development and thus higher structural complexity (PL, CR). Finally, in one case, stimulation of juvenile number and biomass contrasted with reduction of the population structure (CR).

5. Conclusions

Two bioindicator endpoints, juvenile biomass and size class structure, were developed using a multi-Gaussian fitting approach as an extension of the standard Collembolan reproduction test with the objective of testing biological effects of different agricultural amendments. Biomass largely followed juvenile number, but it was shown to be a more sensitive endpoint under noxious conditions. Increases in number of size classes,

accompanied by greater relative allocation of biomass to individuals of larger somatic length, were interpreted as stimulatory conditions, likely signifying upward promotion of smaller groups. Reduction in the number of classes and proportional representation of larger classes were associated with inhibitory effects, possibly signifying delayed development of the juveniles. Overall, since the endpoints were sensitive to each of the experimental factors under both stimulatory and noxious conditions, they can be considered as appropriate for the evaluation of diverse agricultural amendments such as biochar, since feedstocks and pyrolysis production methods are known to yield materials that differ greatly in their physicochemical properties.

Using a model bioindicator organism, the present study supports the view that biochar application will lead to measurable short-term changes in soil quality. However, further study is necessary to investigate specifically which material physical and chemical properties provoke observed changes in the endpoints. Also, since the goal of bioindicator methods is the detection of effects relevant to populations, future experiments could implement a more ecologically relevant experimental set-up (e.g. microcosms) in order to examine if the effects on the population structure hold under more realistic conditions and whether they are persistent with successive generations.

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Chapter 3 supplementary information

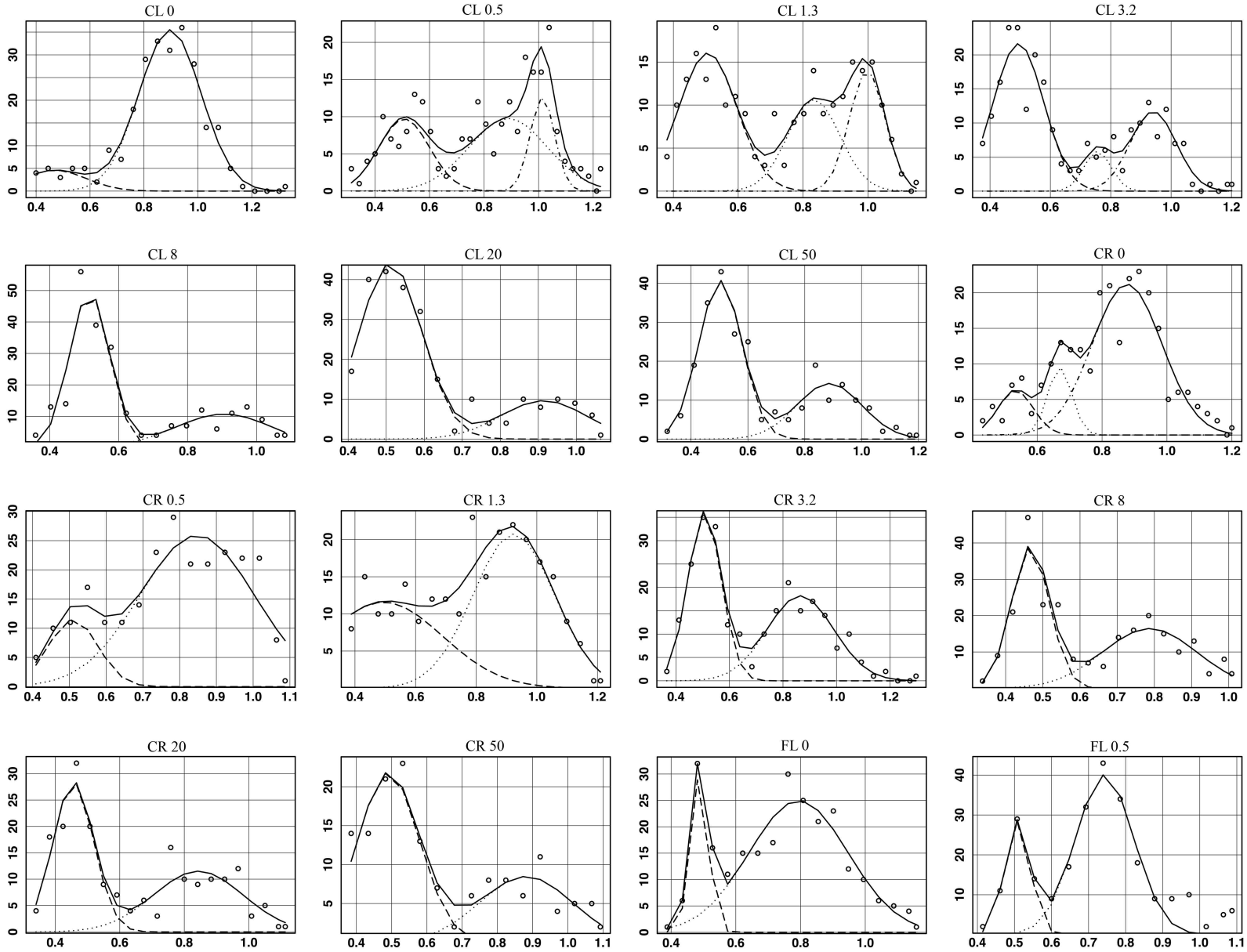
- Table A.1
- Figure B.1
- Table C.1

Table A.1: Multi-Gaussian fits of *F. candida* size classes. Fits are ordered by material and concentration. Provided are the residual standard error of the model, the total sum of squares, residual sum of squares, the degrees of freedom, and the number of fit modes (size-classes). See **Table 1** for material abbreviations.

Treatment	Res. Std. Error	Total SS	Res. SS	1 - (res. SS / tot. SS)	df	Modes
CL 0	2.55	3107	104	0.97	16	2
CL 0.5	3.034	888	221	0.75	24	3
CL 1.3	2.288	640	94	0.85	18	3
CL 3.2	2.82	1285	167	0.87	21	3
CL 8	5.718	3424	392	0.89	12	2
CL 20	3.834	3020	147	0.95	10	2
CL 50	3.469	2703	168	0.94	14	2
CR 0	3.106	1293	174	0.87	18	3
CR 0.5	4.714	912	222	0.76	10	2
CR 1.3	2.953	741	122	0.84	14	2
CR 3.2	2.564	2171	105	0.95	16	2
CR 8	4.966	1912	296	0.85	12	2
CR 20	3.402	1172	162	0.86	14	2
CR 50	2.74	587	75	0.87	10	2
FL 0	2.896	1522	101	0.93	12	2
FL 0.5	4.653	2306	216	0.91	10	2
FL 1.3	4.672	1810	262	0.86	12	2
FL 3.2	3.213	1470	248	0.83	24	3
FL 8	3.716	1472	331	0.78	24	3
FL 20	4.485	2046	201	0.9	10	2
FL 50	2.78	1600	154	0.9	20	2
PG 0	2.252	1211	106	0.91	21	3
PG 0.5	3.078	500	170	0.66	18	3
PG 1.3	2.251	1356	76	0.94	15	3
PG 3.2	4.886	1752	239	0.86	10	2
PG 8	3.023	1344	91	0.93	10	2
PG 20	2.656	2688	70	0.97	10	2
PG 50	0.4668	796	1.3	1	6	1
PL 0	3.421	4010	234	0.94	20	2
PL 0.5	3.693	1629	218	0.87	16	2
PL 1.3	4.467	1207	279	0.77	14	2
PL 3.2	4.762	1237	272	0.78	12	2
PL 8	4.141	1385	274	0.8	16	2
PL 20	3.518	706	173	0.75	14	2

PL 50	3.837	1441	206	0.86	14	2
PR 0	7.477	1928	308	0.84	7	1
PR 0.5	2.281	1456	109	0.93	21	3
PR 1.3	2.548	709	208	0.71	32	4
PR 3.2	3.032	1885	129	0.93	14	2
PR 8	3.059	845	150	0.82	16	2
PR 20	3.214	1348	124	0.91	12	2
PR 50	4.537	1511	288	0.81	14	2

Figure B.1: Graphical fits of the multi-Gaussian models defining *F. candida* size classes. Figures are labeled following the treatments of material (CL, CR, FL, PG, PL, PR) and concentration % (0, 0.5, 1.3, 3.2, 8, 20, 50). See **Table 1** for material abbreviations.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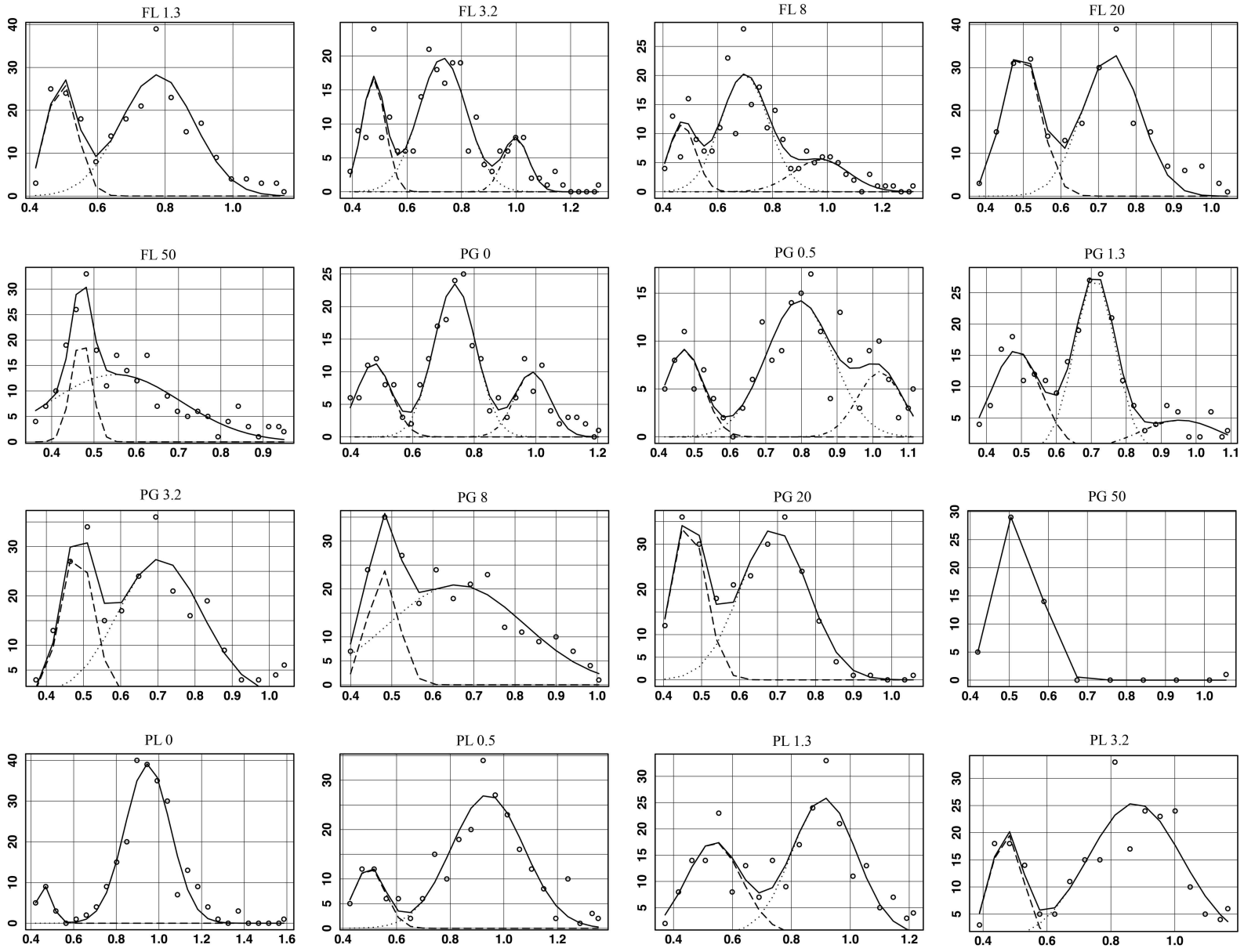
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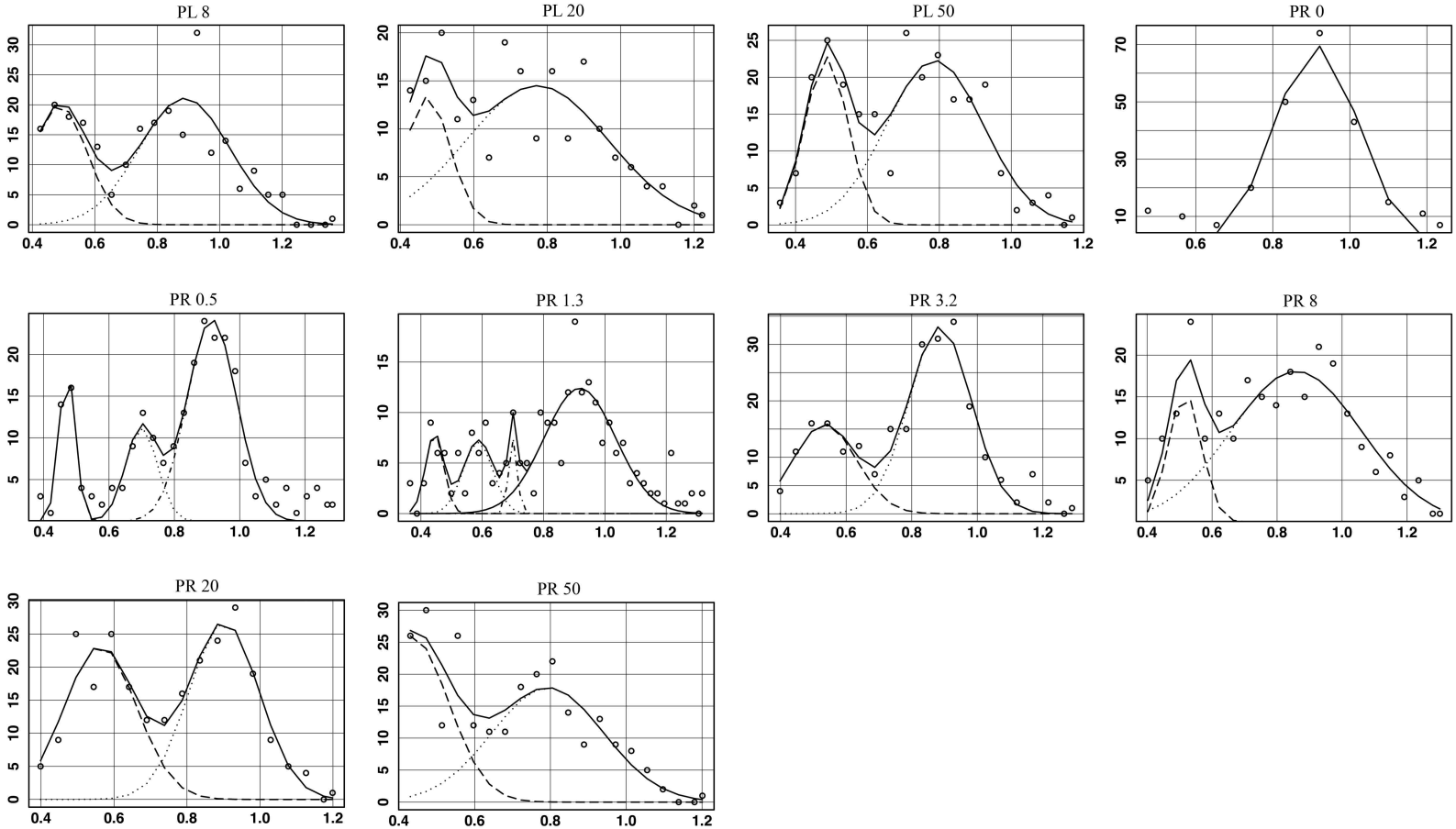
PL 3.2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PL 20
PL 50
PR 0
PR 0.5
PR 1.3
PR 3.2
PR 8
PR 20
PR 50

Table C.1: Numerical fits of the multi-Gaussian models defining *F. candida* size classes. The first two columns *mat* and *conc* define the treatments of material (see **Table 1** for material abbreviations) and concentration % (0, 0.5, 1.3, 3.2, 8, 20, 50), respectively. Three parameters *k*, *m*, and *s* were estimated for each fit, where *k* is the area enclosed by the corresponding Gaussian function, *m* is the midpoint abscissa for the curve, and *s* is the standard deviation of the set of lengths used to fit the curve. Between 1 and 4 curves were fit for each population; accordingly, the parameters for each curve are labeled *k1*, *s1*, etc. Provided for each of these parameters are the estimate (*est*), the error (*err*), the test statistic (*t*), and the resulting p-value of the significance test at CI=0.95 (*p*).

mat	conc	k1_est	k1_err	k1_t	k1_p	m1_est	m1_err	m1_t	m1_p
CL	0	1.341	0.970	1.382	0.186	0.469	0.077	6.084	0.000
CL	0.5	2.256	0.559	4.036	0.000	0.507	0.023	21.881	0.000
CL	1.3	3.597	0.364	9.891	0.000	0.506	0.009	56.041	0.000
CL	3.2	4.526	0.371	12.204	0.000	0.494	0.007	67.842	0.000
CL	8	7.177	0.738	9.723	0.000	0.515	0.007	79.140	0.000
CL	20	9.176	0.644	14.251	0.000	0.511	0.006	82.796	0.000
CL	50	7.747	0.512	15.143	0.000	0.503	0.006	88.808	0.000
CR	0	0.847	0.472	1.793	0.090	0.534	0.033	16.415	0.000
CR	0.5	1.944	1.011	1.923	0.083	0.510	0.029	17.607	0.000
CR	1.3	5.642	4.462	1.265	0.227	0.494	0.064	7.715	0.000
CR	3.2	5.605	0.347	16.166	0.000	0.507	0.004	119.876	0.000
CR	8	4.961	0.614	8.075	0.000	0.467	0.007	70.559	0.000
CR	20	4.460	0.451	9.880	0.000	0.456	0.007	65.955	0.000
CR	50	4.778	0.571	8.362	0.000	0.491	0.010	47.865	0.000
FL	0	2.068	0.296	6.978	0.000	0.488	0.005	103.796	0.000
FL	0.5	2.471	0.441	5.605	0.000	0.508	0.007	67.990	0.000
FL	1.3	3.051	0.564	5.410	0.000	0.494	0.009	55.669	0.000
FL	3.2	1.785	0.290	6.159	0.000	0.477	0.008	62.792	0.000
FL	8	1.418	0.515	2.756	0.011	0.470	0.016	28.885	0.000
FL	20	4.308	0.549	7.846	0.000	0.494	0.007	69.749	0.000
FL	50	1.238	0.244	5.065	0.000	0.470	0.004	131.909	0.000
PG	0	1.601	0.236	6.772	0.000	0.475	0.009	52.596	0.000
PG	0.5	1.207	0.346	3.485	0.003	0.471	0.016	29.280	0.000
PG	1.3	2.786	0.328	8.491	0.000	0.485	0.008	57.564	0.000
PG	3.2	3.237	0.874	3.704	0.004	0.483	0.009	54.680	0.000
PG	8	2.188	0.741	2.953	0.014	0.479	0.005	87.027	0.000
PG	20	3.962	0.361	10.978	0.000	0.465	0.004	115.942	0.000
PG	50	4.113	0.073	56.070	0.000	0.521	0.001	416.061	0.000
PL	0	0.839	0.333	2.516	0.021	0.462	0.017	27.131	0.000
PL	0.5	1.810	0.480	3.770	0.002	0.498	0.017	29.133	0.000
PL	1.3	4.107	0.790	5.202	0.000	0.537	0.019	27.547	0.000
PL	3.2	2.546	0.584	4.362	0.001	0.473	0.013	36.649	0.000
PL	8	4.389	1.198	3.664	0.002	0.488	0.021	23.148	0.000
PL	20	2.055	1.190	1.727	0.106	0.474	0.019	25.150	0.000
PL	50	3.419	0.681	5.019	0.000	0.486	0.011	45.002	0.000
PR	0	19.057	1.698	11.223	0.000	0.914	0.011	81.233	0.000
PR	0.5	1.133	0.149	7.601	0.000	0.472	0.004	131.158	0.000
PR	1.3	0.584	0.182	3.202	0.003	0.445	0.010	44.313	0.000
PR	3.2	3.835	0.599	6.400	0.000	0.535	0.016	34.425	0.000
PR	8	1.971	0.573	3.442	0.003	0.516	0.010	50.337	0.000
PR	20	5.747	0.670	8.582	0.000	0.563	0.012	46.444	0.000
PR	50	6.346	4.781	1.327	0.206	0.433	0.060	7.230	0.000

mat	conc	s1_est	s1_err	s1_t	s1_p	k2_est	k2_err	k2_t	k2_p
CL	0	0.116	0.100	1.169	0.260	10.252	0.521	19.683	0.000
CL	0.5	0.093	0.023	3.985	0.001	3.507	0.899	3.901	0.001
CL	1.3	0.089	0.011	7.774	0.000	2.155	0.908	2.374	0.029
CL	3.2	0.083	0.009	9.635	0.000	0.662	0.405	1.635	0.117
CL	8	0.058	0.007	8.784	0.000	3.816	1.238	3.083	0.009
CL	20	0.083	0.007	11.434	0.000	2.706	0.700	3.863	0.003
CL	50	0.076	0.006	13.022	0.000	4.107	0.620	6.628	0.000
CR	0	0.054	0.035	1.525	0.145	0.886	0.562	1.576	0.132
CR	0.5	0.067	0.032	2.109	0.061	10.074	1.339	7.523	0.000
CR	1.3	0.194	0.166	1.172	0.261	6.996	2.575	2.716	0.017
CR	3.2	0.062	0.004	14.359	0.000	5.675	0.476	11.920	0.000
CR	8	0.051	0.007	7.571	0.000	5.216	0.920	5.669	0.000
CR	20	0.063	0.007	8.749	0.000	4.014	0.643	6.243	0.000
CR	50	0.087	0.012	7.025	0.000	2.792	0.613	4.551	0.001
FL	0	0.028	0.004	7.481	0.000	9.548	0.618	15.448	0.000
FL	0.5	0.035	0.006	5.531	0.000	7.893	0.668	11.820	0.000
FL	1.3	0.045	0.009	4.933	0.000	7.749	0.821	9.441	0.000
FL	3.2	0.043	0.008	5.457	0.000	4.358	0.426	10.237	0.000
FL	8	0.049	0.019	2.634	0.015	4.398	1.025	4.291	0.000
FL	20	0.050	0.007	6.813	0.000	6.355	0.665	9.563	0.000
FL	50	0.024	0.004	5.724	0.000	5.072	0.582	8.711	0.000
PG	0	0.057	0.010	5.454	0.000	3.965	0.250	15.841	0.000
PG	0.5	0.053	0.019	2.795	0.012	3.307	0.636	5.203	0.000
PG	1.3	0.070	0.010	6.741	0.000	3.974	0.549	7.237	0.000
PG	3.2	0.043	0.010	4.414	0.001	7.751	1.158	6.693	0.000
PG	8	0.036	0.008	4.508	0.001	8.667	1.319	6.573	0.000
PG	20	0.044	0.004	10.247	0.000	7.440	0.461	16.149	0.000
PG	50	0.054	0.001	48.141	0.000	NA	NA	NA	NA
PL	0	0.036	0.016	2.243	0.036	10.418	0.560	18.589	0.000
PL	0.5	0.058	0.019	3.096	0.007	9.175	0.675	13.599	0.000
PL	1.3	0.093	0.022	4.326	0.001	7.020	0.820	8.563	0.000
PL	3.2	0.051	0.013	3.833	0.002	9.383	0.951	9.870	0.000
PL	8	0.088	0.028	3.184	0.006	7.643	0.969	7.885	0.000
PL	20	0.061	0.028	2.223	0.043	7.046	1.462	4.819	0.000
PL	50	0.060	0.011	5.429	0.000	7.554	0.892	8.470	0.000
PR	0	0.109	0.011	9.747	0.000	NA	NA	NA	NA
PR	0.5	0.025	0.004	6.071	0.000	1.370	0.256	5.349	0.000
PR	1.3	0.030	0.010	2.897	0.007	0.863	0.255	3.386	0.002
PR	3.2	0.096	0.019	5.113	0.000	7.896	0.551	14.328	0.000
PR	8	0.051	0.012	4.161	0.001	9.071	0.925	9.812	0.000
PR	20	0.099	0.014	7.055	0.000	6.493	0.638	10.182	0.000
PR	50	0.097	0.062	1.582	0.136	6.595	1.740	3.790	0.002

mat	conc	m2_est	m2_err	m2_t	m2_p	s2_est	s2_err	s2_t	s2_p
CL	0	0.897	0.006	138.607	0.000	0.115	0.007	16.586	0.000
CL	0.5	0.889	0.050	17.805	0.000	0.144	0.035	4.109	0.000
CL	1.3	0.833	0.034	24.748	0.000	0.081	0.033	2.478	0.023
CL	3.2	0.757	0.026	29.538	0.000	0.045	0.026	1.755	0.094
CL	8	0.906	0.048	18.956	0.000	0.142	0.060	2.375	0.035
CL	20	0.914	0.031	29.428	0.000	0.112	0.037	3.032	0.013
CL	50	0.887	0.020	45.319	0.000	0.115	0.021	5.545	0.000
CR	0	0.670	0.018	37.723	0.000	0.037	0.019	1.960	0.066
CR	0.5	0.848	0.021	40.200	0.000	0.155	0.026	5.907	0.000
CR	1.3	0.924	0.039	23.595	0.000	0.135	0.025	5.284	0.000
CR	3.2	0.865	0.012	73.723	0.000	0.124	0.013	9.850	0.000
CR	8	0.787	0.024	32.250	0.000	0.126	0.028	4.538	0.001
CR	20	0.844	0.025	33.932	0.000	0.139	0.027	5.107	0.000
CR	50	0.881	0.031	28.543	0.000	0.131	0.036	3.606	0.005
FL	0	0.794	0.011	71.739	0.000	0.153	0.012	12.657	0.000
FL	0.5	0.743	0.008	98.347	0.000	0.079	0.008	9.953	0.000
FL	1.3	0.778	0.013	60.075	0.000	0.109	0.014	7.738	0.000
FL	3.2	0.731	0.009	78.821	0.000	0.088	0.011	8.151	0.000
FL	8	0.698	0.016	42.806	0.000	0.087	0.022	4.036	0.000
FL	20	0.735	0.009	82.102	0.000	0.076	0.010	7.890	0.000
FL	50	0.551	0.020	27.633	0.000	0.153	0.018	8.473	0.000
PG	0	0.737	0.005	157.641	0.000	0.067	0.005	12.932	0.000
PG	0.5	0.794	0.019	41.691	0.000	0.093	0.021	4.500	0.000
PG	1.3	0.710	0.006	128.842	0.000	0.058	0.006	9.068	0.000
PG	3.2	0.706	0.019	37.832	0.000	0.112	0.020	5.511	0.000
PG	8	0.657	0.029	22.507	0.00	0.166	0.025	6.619	0.00

					0				0
				113.51	0.00			13.10	0.00
PG	20	0.691	0.006	9	0	0.089	0.007	6	0
PG	50	NA	NA	NA	NA	NA	NA	NA	NA
				143.57	0.00			16.09	0.00
PL	0	0.947	0.007	5	0	0.106	0.007	8	0
					0.00			11.61	0.00
PL	0.5	0.940	0.011	82.332	0	0.135	0.012	5	0
					0.00				0.00
PL	1.3	0.911	0.014	64.494	0	0.108	0.015	7.112	0
					0.00				0.00
PL	3.2	0.876	0.017	51.993	0	0.147	0.018	8.213	0
					0.00				0.00
PL	8	0.888	0.020	43.752	0	0.145	0.022	6.434	0
					0.00				0.00
PL	20	0.773	0.046	16.935	0	0.194	0.045	4.319	1
					0.00				0.00
PL	50	0.788	0.018	44.516	0	0.135	0.020	6.897	0
PR	0	NA	NA	NA	NA	NA	NA	NA	NA
					0.00				0.00
PR	0.5	0.699	0.010	71.267	0	0.049	0.010	4.889	0
					0.00				0.01
PR	1.3	0.586	0.014	40.851	0	0.049	0.018	2.685	1
				120.09	0.00			12.03	0.00
PR	3.2	0.887	0.007	7	0	0.095	0.008	6	0
					0.00				0.00
PR	8	0.859	0.022	38.221	0	0.200	0.025	7.987	0
					0.00				0.00
PR	20	0.902	0.010	86.084	0	0.097	0.011	8.695	0
					0.00				0.00
PR	50	0.793	0.044	18.018	0	0.147	0.041	3.592	3

mat	conc	k3_est	k3_err	k3_t	k3_p	m3_est	m3_err	m3_t	m3_p
CL	0	NA	NA	NA	NA	NA	NA	NA	NA
CL	0.5	1.410	0.672	2.099	0.047	1.014	0.011	92.727	0.000
CL	1.3	1.929	0.806	2.392	0.028	0.998	0.018	55.959	0.000
CL	3.2	2.232	0.449	4.972	0.000	0.941	0.017	55.313	0.000
CL	8	NA	NA	NA	NA	NA	NA	NA	NA
CL	20	NA	NA	NA	NA	NA	NA	NA	NA
CL	50	NA	NA	NA	NA	NA	NA	NA	NA
CR	0	5.697	0.567	10.044	0.000	0.877	0.012	74.022	0.000
CR	0.5	NA	NA	NA	NA	NA	NA	NA	NA
CR	1.3	NA	NA	NA	NA	NA	NA	NA	NA
CR	3.2	NA	NA	NA	NA	NA	NA	NA	NA
CR	8	NA	NA	NA	NA	NA	NA	NA	NA
CR	20	NA	NA	NA	NA	NA	NA	NA	NA
CR	50	NA	NA	NA	NA	NA	NA	NA	NA
FL	0	NA	NA	NA	NA	NA	NA	NA	NA

FL	0.5	NA	NA	NA	NA	NA	NA	NA	NA
FL	1.3	NA	NA	NA	NA	NA	NA	NA	NA
FL	3.2	0.925	0.296	3.129	0.005	0.999	0.017	59.666	0.000
FL	8	1.391	0.883	1.575	0.128	0.976	0.070	13.860	0.000
FL	20	NA	NA	NA	NA	NA	NA	NA	NA
FL	50	NA	NA	NA	NA	NA	NA	NA	NA
PG	0	1.416	0.221	6.395	0.000	0.988	0.010	98.259	0.000
PG	0.5	1.057	0.573	1.844	0.082	1.020	0.031	32.991	0.000
PG	1.3	1.344	0.666	2.017	0.062	0.955	0.048	19.880	0.000
PG	3.2	NA	NA	NA	NA	NA	NA	NA	NA
PG	8	NA	NA	NA	NA	NA	NA	NA	NA
PG	20	NA	NA	NA	NA	NA	NA	NA	NA
PG	50	NA	NA	NA	NA	NA	NA	NA	NA
PL	0	NA	NA	NA	NA	NA	NA	NA	NA
PL	0.5	NA	NA	NA	NA	NA	NA	NA	NA
PL	1.3	NA	NA	NA	NA	NA	NA	NA	NA
PL	3.2	NA	NA	NA	NA	NA	NA	NA	NA
PL	8	NA	NA	NA	NA	NA	NA	NA	NA
PL	20	NA	NA	NA	NA	NA	NA	NA	NA
PL	50	NA	NA	NA	NA	NA	NA	NA	NA
PR	0	NA	NA	NA	NA	NA	NA	NA	NA
PR	0.5	4.599	0.306	15.032	0.000	0.915	0.006	163.703	0.000
PR	1.3	0.265	0.136	1.948	0.060	0.700	0.008	86.858	0.000
PR	3.2	NA	NA	NA	NA	NA	NA	NA	NA
PR	8	NA	NA	NA	NA	NA	NA	NA	NA
PR	20	NA	NA	NA	NA	NA	NA	NA	NA
PR	50	NA	NA	NA	NA	NA	NA	NA	NA

mat	conc	s3_est	s3_err	s3_t	s3_p	k4_est	k4_err	k4_t	k4_p
CL	0	NA	NA	NA	NA	NA	NA	NA	NA
CL	0.5	0.045	0.014	3.170	0.004	NA	NA	NA	NA
CL	1.3	0.055	0.013	4.283	0.000	NA	NA	NA	NA
CL	3.2	0.076	0.018	4.176	0.000	NA	NA	NA	NA
CL	8	NA	NA	NA	NA	NA	NA	NA	NA
CL	20	NA	NA	NA	NA	NA	NA	NA	NA
CL	50	NA	NA	NA	NA	NA	NA	NA	NA
CR	0	0.107	0.013	8.234	0.000	NA	NA	NA	NA
CR	0.5	NA	NA	NA	NA	NA	NA	NA	NA
CR	1.3	NA	NA	NA	NA	NA	NA	NA	NA
CR	3.2	NA	NA	NA	NA	NA	NA	NA	NA
CR	8	NA	NA	NA	NA	NA	NA	NA	NA
CR	20	NA	NA	NA	NA	NA	NA	NA	NA
CR	50	NA	NA	NA	NA	NA	NA	NA	NA
FL	0	NA	NA	NA	NA	NA	NA	NA	NA
FL	0.5	NA	NA	NA	NA	NA	NA	NA	NA
FL	1.3	NA	NA	NA	NA	NA	NA	NA	NA
FL	3.2	0.047	0.017	2.747	0.011	NA	NA	NA	NA
FL	8	0.100	0.067	1.503	0.146	NA	NA	NA	NA
FL	20	NA	NA	NA	NA	NA	NA	NA	NA
FL	50	NA	NA	NA	NA	NA	NA	NA	NA
PG	0	0.057	0.010	5.425	0.000	NA	NA	NA	NA
PG	0.5	0.062	0.030	2.040	0.056	NA	NA	NA	NA
PG	1.3	0.115	0.071	1.609	0.128	NA	NA	NA	NA
PG	3.2	NA	NA	NA	NA	NA	NA	NA	NA
PG	8	NA	NA	NA	NA	NA	NA	NA	NA
PG	20	NA	NA	NA	NA	NA	NA	NA	NA
PG	50	NA	NA	NA	NA	NA	NA	NA	NA
PL	0	NA	NA	NA	NA	NA	NA	NA	NA
PL	0.5	NA	NA	NA	NA	NA	NA	NA	NA
PL	1.3	NA	NA	NA	NA	NA	NA	NA	NA
PL	3.2	NA	NA	NA	NA	NA	NA	NA	NA
PL	8	NA	NA	NA	NA	NA	NA	NA	NA
PL	20	NA	NA	NA	NA	NA	NA	NA	NA
PL	50	NA	NA	NA	NA	NA	NA	NA	NA
PR	0	NA	NA	NA	NA	NA	NA	NA	NA
PR	0.5	0.076	0.006	12.282	0.000	NA	NA	NA	NA
PR	1.3	0.014	0.006	2.443	0.020	3.614	0.331	10.933	0.000
PR	3.2	NA	NA	NA	NA	NA	NA	NA	NA
PR	8	NA	NA	NA	NA	NA	NA	NA	NA
PR	20	NA	NA	NA	NA	NA	NA	NA	NA
PR	50	NA	NA	NA	NA	NA	NA	NA	NA

mat	conc	m4_est	m4_err	m4_t	m4_p	s4_est	s4_err	s4_t	s4_p
CL	0	NA	NA	NA	NA	NA	NA	NA	NA
CL	0.5	NA	NA	NA	NA	NA	NA	NA	NA
CL	1.3	NA	NA	NA	NA	NA	NA	NA	NA
CL	3.2	NA	NA	NA	NA	NA	NA	NA	NA
CL	8	NA	NA	NA	NA	NA	NA	NA	NA
CL	20	NA	NA	NA	NA	NA	NA	NA	NA
CL	50	NA	NA	NA	NA	NA	NA	NA	NA
CR	0	NA	NA	NA	NA	NA	NA	NA	NA
CR	0.5	NA	NA	NA	NA	NA	NA	NA	NA
CR	1.3	NA	NA	NA	NA	NA	NA	NA	NA
CR	3.2	NA	NA	NA	NA	NA	NA	NA	NA
CR	8	NA	NA	NA	NA	NA	NA	NA	NA
CR	20	NA	NA	NA	NA	NA	NA	NA	NA
CR	50	NA	NA	NA	NA	NA	NA	NA	NA
FL	0	NA	NA	NA	NA	NA	NA	NA	NA
FL	0.5	NA	NA	NA	NA	NA	NA	NA	NA
FL	1.3	NA	NA	NA	NA	NA	NA	NA	NA
FL	3.2	NA	NA	NA	NA	NA	NA	NA	NA
FL	8	NA	NA	NA	NA	NA	NA	NA	NA
FL	20	NA	NA	NA	NA	NA	NA	NA	NA
FL	50	NA	NA	NA	NA	NA	NA	NA	NA
PG	0	NA	NA	NA	NA	NA	NA	NA	NA
PG	0.5	NA	NA	NA	NA	NA	NA	NA	NA
PG	1.3	NA	NA	NA	NA	NA	NA	NA	NA
PG	3.2	NA	NA	NA	NA	NA	NA	NA	NA
PG	8	NA	NA	NA	NA	NA	NA	NA	NA
PG	20	NA	NA	NA	NA	NA	NA	NA	NA
PG	50	NA	NA	NA	NA	NA	NA	NA	NA
PL	0	NA	NA	NA	NA	NA	NA	NA	NA
PL	0.5	NA	NA	NA	NA	NA	NA	NA	NA
PL	1.3	NA	NA	NA	NA	NA	NA	NA	NA
PL	3.2	NA	NA	NA	NA	NA	NA	NA	NA
PL	8	NA	NA	NA	NA	NA	NA	NA	NA
PL	20	NA	NA	NA	NA	NA	NA	NA	NA
PL	50	NA	NA	NA	NA	NA	NA	NA	NA
PR	0	NA	NA	NA	NA	NA	NA	NA	NA
PR	0.5	NA	NA	NA	NA	NA	NA	NA	NA
PR	1.3	0.918	0.012	77.325	0.000	0.116	0.013	8.926	0.000
PR	3.2	NA	NA	NA	NA	NA	NA	NA	NA
PR	8	NA	NA	NA	NA	NA	NA	NA	NA
PR	20	NA	NA	NA	NA	NA	NA	NA	NA
PR	50	NA	NA	NA	NA	NA	NA	NA	NA

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Chapter 4.1 Pine gasification biochar addition to mesocosms under barley cultivation. I. Effects on soil physical properties

Abstract

Biochar, when used as a soil amendment, is expected to influence basic soil physical properties, and may be beneficial in agricultural systems due to predicted decreases in bulk density, increases in water retention, among other properties. However, limited information is available on actual changes in the field. Therefore, we evaluated important soil physical properties up to 18 months following application of a pine chip gasification biochar at application rates of 12 and 50 t ha^{-1} in a model agronomic system of barley cultivation under Mediterranean field conditions. As expected, biochar increased soil water holding capacity and field moisture retention, with the potential to reduce hydric stress of the barley crop. Also, bulk density decreased linearly with increasing biochar application rate, in our case corresponding to a decrease of 1.4 kg m^{-3} / t biochar / ha. A decrease in penetration resistance was also linearly related to application rate. Also, we investigated the effects of biochar on aggregate size distribution and the distribution of stable aggregates at 18 months. Biochar treatments increased the proportion of larger aggregates, associated with a reduction in the proportion of small macroaggregates 250-500 μm. Finally, biochar increased the proportion of stable aggregates >500 μm, but did not effect the proportion of stable aggregates in the 50-250 μm and 250-500 μm fractions. Considering these results, we conclude that biochar improved soil physical attributes of the system studied.

1. Introduction

Knowledge on biochar's effects on the soil physical properties is still incomplete (Gaunt and Cowie 2009; Sohi et al. 2010; Mukherjee and Lal 2013), despite their importance for agricultural sustainability, including plant water availability (Horn et al. 1994), nutrient release (Lindquist et al. 1997), and erosion prevention (Liu et al. 2012a), among others.

Some specific changes in soil physical characteristics due to biochar addition have been documented, although most inferences are based on biochar's general physico-chemical characteristics, and the fact that surprisingly little information has been collected on basic physical parameters has been noted elsewhere (Sohi et al. 2010). Many physical properties are expected to be affected by biochar addition, including structure, porosity and consistency, as well as water retention and aeration through an altered bulk surface area, pore-size distribution, particle density, and particle packing as aggregates (Downie et al. 2009; Mukherjee and Lal 2013). Improvement in soil physical properties can potentially allow higher yields due to an enhanced penetration depth and availability of air and water within the root zone (Downie et al. 2009). In turn, biochar's persistence in soil might benefit from such physical improvements, due to the poor accessibility of biochar when physically enveloped by soil particles, which could help to enhance the recalcitrant nature of biochar (Brodowski et al. 2006, Nguyen et al. 2008), but also due to its mobility into deeper soil profile layers (Mukherjee and Lal 2013).

Regarding biochar's potential effect on water retention, organic amendments generally increase water holding capacity of soil (Zebarth et al. 1999; Liebig et al. 1999; Major et al. 2009). Increases in field capacity due to biochar application are expected and have been documented (Chan et al. 2007; Laird et al. 2010; Novak et al. 2012) as well as increases in soil moisture (Jones et al. 2012; Liu et al. 2012b), whereas the micro- and mesoporosity of biochars have been

suggested as the main explanation for increased soil water retention (Downie et al. 2009, Mukherjee and Lal 2013, Liu et al. 2012b).

Biochar's bulk density is lower than its feedstocks (Downie et al. 2009) or mineral soils (Major et al. 2009) due to its high porosity, and this explains reductions in bulk density and increased porosity found under charcoal kilns (Oguntunde et al. 2008) and reduced bulk density in agricultural settings (Zhang et al. 2012a; Zhang et al. 2012b).

Biochar directly decreases bulk density due to its high porosity, but may also actuate indirectly if enhancing soil aggregate formation. According to the aggregate hierarchy concept (Tisdall and Oades 1982), free primary particles (sand, silt, and clay) and silt-sized aggregates are bound together into stable microaggregates kept together by persistent binding agents (such as humified organic matter and polyvalent metal cation complexes), oxides, and highly disordered aluminosilicates, later bound together as macroaggregates (>250 μm) by temporary and transient binding agents (such as fungal hyphae/plant roots and microbial- and plant-derived polysaccharides, respectively). According to Oades (1984), these temporary macroaggregates are held together by roots and hyphae, and once they disappear, macroaggregates can break into new fragments, some of them constituting new microaggregates. Summarizing, aggregation is mediated by inorganic binding agents, soil biota (microorganisms, plant roots and fauna) and environmental factors, such drying/wetting cycles, fire, as well as soil management (Six et al. 2004), and biochar might influence soil aggregation and aggregate stability due to its interaction with any of the factors mentioned above. Effects on aggregation are expected to be linked to biochar's surface charge characteristics, which develop gradually by weathering and are affected by soil pH (Major et al. 2009; Cheng et al. 2006), but may also be determined by interactions with soil organic matter and microorganisms (Warnock et al. 2007). Physical and chemical interactions of biochar with soil particles will be driven by the biochars'

surface chemistry, and expected to be faster than biologically-mediated interactions, which are in turn modulated by other factors such as moisture content, organic matter or nutrient source, biochar's surface area and porosity (Mukherjee and Lal 2013). Hence, a rapid aggregate stabilization by physicochemical processes can be expected after the addition of biochar, although the presence of a labile carbon fraction in fresh biochars that can be consumed by microorganisms may subsequently enhance the biological component of soil aggregation (Mukherjee and Lal 2013). However, few biochar studies have investigated nanoscale biochar-mineral interactions (Mukherjee and Lal 2013). Recently, Lin et al. (2012) demonstrated that soil mineral phase appeared directly attached to the organic phase of aged biochars' surface within the first year of incubation. The changes in biochar's surface with aging was identified as the explanation, with mineral particles attached to biochar through surface carboxylic and phenolic functional groups (via multivalent cations and oxides) and the organic matter adsorbed to the biochars' surface (mainly via calcium cations).

Finally, penetrability is also expected to improve due to its association with aggregation and bulk density, and was shown to decrease in laboratory soil columns with pecan biochar amendment (Busscher et al. 2010).

Here, we investigated the effects of biochar addition on soil water holding capacity, field moisture, bulk density, penetration resistance, and aggregation in a field mesocosm study simulating barley cultivation under Mediterranean climatic conditions.

2. Methods

Field mesocosms were constructed at the IRTA Torre Marimón experimental station (Caldes de Montbui, Barcelona, Spain), where mean annual precipitation is 616 mm $year^{-1}$, MAT = 14.7 °C, and

potential annual evapotranspiration (Thornthwaite) is 787 mm (Josa-March and Hereter-Quintana 2000). Mesocosms were established in March 2011 in a flat, previously cultivated agricultural area, where no pesticides or fertilizers had been applied in at least the past 7 years. A grid was prepared for 18 plots of 1 m^2 with six replicates for each of three treatments consisting of the addition of 0, 12, and 50 t ha^{-1} biochar (hereafter designated as B0, B12, and B50, respectively), assigned in a complete random block. The biochar applied was produced by gasification (600-900 ºC) of mixed pine chips (*Pinus pinaster* and *P. radiata*). Each mesocosm was constructed by excavating the upper 20 cm, delimiting horizontally on each side with four 30 cm-height steel plates protruding to 10 cm above ground level to exclude exterior contamination or losses of any matter. Excavated soil was thoroughly mixed with biochar to achieve a homogeneous mixture before refilling the excavated area. The same procedure was followed for control plots. In late winter 2011 and 2012, barley (*Hordeum vulgare* L.) was seeded at a density of 250 seeds m^{-2} in each plot.

Soil textural analyses were undertaken for each plot to account for any unknown differences. Cores of 4 x 20 cm were taken in each plot for laboratory texture analyses following the pipette method (Gee and Bauder 1986) without the iron oxide and carbonate removal steps, mechanically dispersed in sodium hexametaphosphate, and particle separation by sedimentation, calibrated for international classification which defines clay (<2 μm), silt (2-20 μm), fine sand (20-200 μm) and coarse sand (200-2000 μm).

Water holding capacity (WHC) of each of the 18 plots was determined in duplicate using samples collected by 4 x 20 cm cylinders at two points within each plot two months following mesocosm construction, using the small core method (Cassel and Nielsen 1986). WHC was determined gravimetrically by saturation of 50 g sieved (< 2 mm) samples for 2 h followed by draining for 24 h at room

temperature, weighing the 24-hour field capacity, and overnight drying at 105 °C. To validate the previous results in the field, soil moisture was assessed at five moments from Autumn 2011 to Autumn 2012, and evaluated in the laboratory gravimetrically (drying at 105 °C until constant weight) on 5 mm-sieved samples. Bulk density was assessed in intact 193 cm^3 cores, taken from each plot in triplicate after 9 and 18 months following biochar addition, and evaluated in the laboratory following Method 3B6a (Soil Survey Staff 2004).

Samples for soil dry-aggregate and wet-aggregate (stable) distributions were taken at 18 months by two 5 x 20 cm cores in each plot, and were assessed based on the general recommendations in Kemper and Rosenau (1986). The two cores were combined, air-dried, and additional physical alteration was avoided to the highest degree possible. For analyses, representative samples were taken by coning the whole sample in a bin, flattening, and scooping from opposite quadrants. Dry aggregate size distribution was evaluated by sieving using 250 g placed in a column of six 200 mm-diameter sieves with mesh sizes 5000, 2000, 1000, 500, 250, and 50 μm, and sieved on a Haver ELM 200 sieving machine for 5 minutes at an intensity of 5 on a scale of 1 to 10. These timing and intensity parameters were chosen as they resulted in a reasonably equitable distribution of aggregate mass between the sieves without excessive abrasion.

Stable aggregate size distribution was assessed by simulating rainfall using a water dispersion accessory for the sieving apparatus, though no vibration was applied. Sieves of 500, 250, and 50 μm were chosen following the high fraction of biochar particles at these diameters, as seen in **Table 1**. Pressure of the input hose was set at 150 Kpa to allow proper functioning of the water dispersing accessory, corresponding to a discharge of 1.86 L $minute^{-1}$, while exposure time was determined following the

Table 1: Particle size distribution of the pine gasification char (n=2, SE not available)

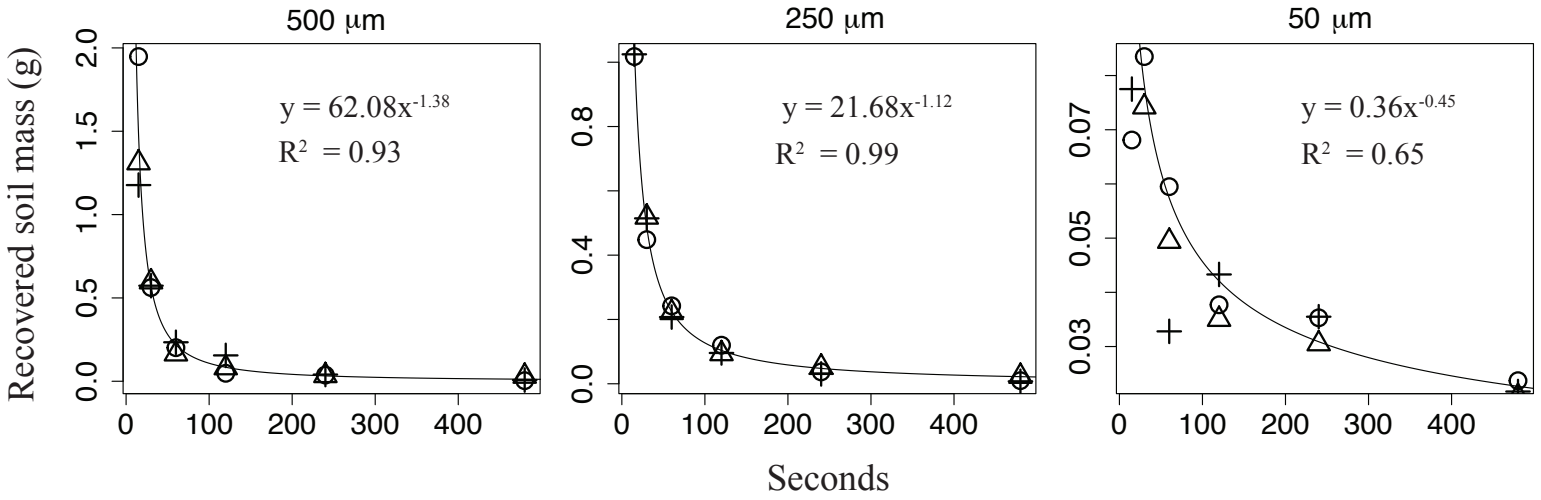
Particle size diameter (μm)	Fraction (% ODW)
> 5000	0.08
2000 - 5000	1.06
500 - 2000	41.52
250 - 500	11.17
100 - 250	7.40
50 - 100	28.09
< 50	10.72

construction of disaggregation curves. To construct the curves, samples were taken from one plot of each treatment, selected for their spatial proximity and textural similarities. For each sieve and treatment, 60 g sample was taken per the methodology described above and placed in the simulator, water was applied at the prescribed pressure, and 50 ml samples were taken at the water output hose at 15, 30, 60, 120, 240, and 480 seconds. Water was evaporated at 105 °C, and disaggregated material was determined gravimetrically in each container whose weight had been noted beforehand. Inspection of the curves showed that within this time period tested, 60 seconds resulted in 53-84% disaggregation (**Figure 1**), and was therefore chosen to reflect the majority of aggregate slaking without excessive water treatment. Hence, stable aggregate size distribution was evaluated as the variation in stable aggregate size distribution after 60 seconds of simulated rainfall exposure, subsequent drying of the sieves at 105 °C until constant weight, and weighing remaining mass of each. Initial soil weight (60 g) was corrected for hygroscopic water by drying 50 g of soil from each sample at 105 °C overnight. Stable aggregate proportions between 50-250 and 250-500 μm were calculated as the fraction retained on each sieve minus the fraction considered unstable in sieves of smaller diameter. Mean weight diameter (MWD) was

calculated for dry-sieved aggregates and stable aggregates as described in Hillel (1998).

Penetration resistance was evaluated by a Eijkelkamp Pocket Penetrometer 06.03 with plug diameter 6.4 mm, 9 months after the experiment start. Evaluation was carried out at 10 points in a 90 cm transect, measurements taken every 10 cm.

Figure 1: Mass loss curves of soil subjected to simulated rainfall in 500, 250, and 50 μm sieves, whereas soil was recovered at 15, 30, 60, 120, 240, and 480 seconds. "o"=0 t ha $^{-1}$ biochar (control), "Δ"= 12 t ha $^{-1}$, and "+"= 50 t ha $^{-1}$.



To test the effect of biochar treatments, linear models using the *lm* function in R were applied, and non-significant terms were dropped using F-tests until only significant parameters remained. For the field moisture measurements, a two-way repeated measures ANOVA was applied, nested by plot using the *aov* function in R. Pairwise tests of significant differences between biochar treatments were carried out with Mann-Whitney U-tests.

3. Results

Though some slight differences in relative abundance of particle sizes were evident on the plot level (**Table A.1**), the treatments had been previously assigned randomly in the experimental area to eliminate any localized textural biases. Accordingly, no differences were found in the defined soil particle size classes between treatments (as evaluated by non-parametric Kruskal-Wallis tests; data not shown).

WHC was 38.8 ± 0.86, 39.2 ± 0.91, and 40.0 ± 0.97% for B0, B12 and B50 respectively. When evaluated in a linear model with application rate as the only independent variable, WHC was not affected by biochar addition ($p=0.33$, $R^2 = 0.00$). However, when clay, silt and sand content in were also included as explanatory variables, the model was highly improved ($p<0.001$, $R^2 = 0.74$), and biochar addition rate was significant, as shown in **Table 2**. As indicated by standardized coefficients β, it is seen that the magnitude of the effects of biochar application rate and coarse sand and silt materials were similar, with application rate and silt increasing water-holding capacity, and coarse sand materials reducing water-holding capacity, thereby following expectations. In agreement, field moisture increased due to biochar application as indicated by a two-way repeated measures ANOVA (**Table 2**). Also, sampling date and the interaction of treatment and date were significant (**Table 3**). As shown in **Figure 2**, during wet periods of the year soil moisture was always in the order of B50 > B12 > B0, though these differences were only statistically

significant at the final Autumn sampling date (B0 vs. B50 p=0.016, W=3; B12 vs. B50 p=0.037, W=5).

Bulk density means ± SE for B0, B12, and B50 were 1.21 ± 0.03, 1.18 ± 0.02, and 1.14 ± 0.02 g cm $^{-3}$ at 6 months and 1.00 ± 0.03, 0.97 ± 0.03, and 0.93 ± 0.02 g cm $^{-1}$ at 18 months, respectively. Linear models for both dates were constructed including soil textural components. Biochar application rate linearly reduced bulk density at both dates, shown in **Table 4**. Additionally, as seen in **Table 4**, at 6 mo. clay content influenced decreased bulk density, and at 12 mo. silt content influenced decreased bulk density.

Dry-sieved aggregate distribution and the corresponding MWD at each biochar application rate are shown in **Table 5**. Both biochar addition rates reduced the proportion of aggregates between 250-500 μm compared to unamended plots (p=0.055, W=30 in both cases), coupled to the corresponding increase, though not significant, in the proportion of size-classes over 500 μm. No significant differences in dry MWD due to biochar treatment were detected (**Table 5**), though that of B50 was slightly higher. In the evaluation of stable aggregate size distribution, no differences were found in the 50-250 μm or 250-500 μm fractions, nor in the MWD (**Table 6**). However, a greater proportion of water-stable aggregates were retained on the 500 μm sieve in the biochar treatments with respect to the control (**Table 6**; p=0.055, W=6 in both cases).

Penetration resistance means for B0, B12, and B50 were 2.11 ± 0.09, 2.09 ± 0.10, and 1.67 ± 0.08 kg cm^{-2}. Here, textural parameters were not selected. As was the case with bulk density, biochar application rate linearly reduced penetration resistance (**Table 5**).

Table 2: Multiple linear model factors significantly affecting water holding capacity following backward selection, including standardized coefficients (β). Model parameters were the following: df=14, R^2=0.74, F=17.21, p<0.001.

Model parameter	Estimate	Std. error	β	*t*	p
intercept	42.416	2.588	NA	16.385	<0.001
biochar application rate	0.031	0.012	0.315	2.513	0.025
coarse sand (200-2000 μm)	-0.265	0.061	-0.574	-4.286	<0.001
silt (2-20 μm)	0.229	0.069	0.452	3.326	0.005

Table 3: Two-way repeated measures ANOVA of field humidity with biochar treatment, date, and interaction as factors. Residual df=78, SS=96.5.

Model parameter	df	SS	F	p
treatment	1	10.8	8.7	0.004
date	4	1824.6	368.6	<0.001
treatment*date	4	18.7	3.8	0.007

Figure 2: Soil field moisture evaluated over a year (sampling dates shown on x-axis).

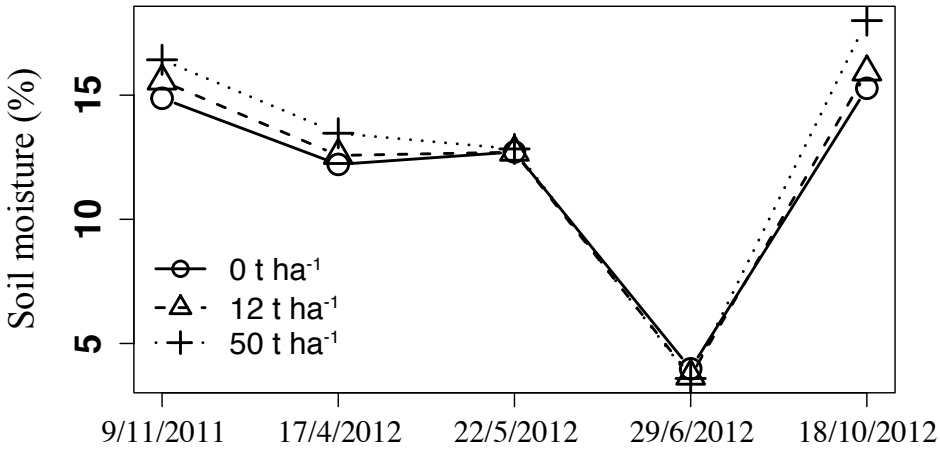


Table 4: Linear models explaining effect of biochar application on bulk density, expressed as g cm^{-3}, and penetration resistance, expressed as kg cm^{-2}. df=16 in both cases, bulk density 6-month R^2 was 0.18, 18-month R^2 was 0.17, and penetration resistance R^2 was 0.45.

Field parameter	Model parameters	Est.	Std. error	*t*	p
Bulk density 6-month	Intercept	1.4	0.08	17.4	<0.001
	t ha^{-1} biochar	-0.001 (β = -0.44)	<0.01	-2.3	0.034
	% clay (<2 μm)	-0.008 (β = -0.48)	<0.01	-2.6	0.021
Bulk density 18-month	Intercept	1.14	0.06	20.4	<0.001
	t ha^{-1} biochar	-0.001 (β = -0.50)	<0.01	-2.7	0.017
	% silt (2-20 μm)	-0.008 (β = -0.51)	<0.01	-2.7	0.015
Penetration resistance 9-month	Intercept	2.15	0.071	30	<0.001
	t ha^{-1} biochar	-0.0093 (β = -0.70)	0.0024	-3.9	0.001

Table 5: Mean values of dry-sieved aggregate diameter classes and MWD ± SE for each treatment (n=6 for each). Within each aggregate diameter class, same letters represent no significant difference between treatments ($p > 0.05$).

Biochar application rate (t ha $^{-1}$)	>5000 μm (%)	2000-5000 μm (%)	1000-2000 μm (%)	500-1000 μm (%)	250-500 μm (%)	50-250 μm (%)	MWDd (mm)
0	34.04 ± 4.83 a	21.84 ± 2.73 a	16.73 ± 1.38 a	13.35 ± 1.66 a	7.46 ± 1.66 a	5.37 ± 1.25 a	1.15 ± 0.10 a
12	39.72 ± 8.02 a	22.96 ± 2.48 a	15.75 ± 2.71 a	10.70 ± 2.32 a	5.37 ± 1.58 b	4.31 ± 0.95 a	1.15 ± 0.13 a
50	35.00 ± 8.45 a	23.87 ± 1.84 a	17.34 ± 3.47 a	12.17 ± 3.23 a	5.70 ± 1.21 b	4.32 ± 0.69 a	1.21 ± 0.10 a

Table 6: Stable aggregate masses within indicated diameter classes and MWD ± SE for each treatment (n=6 for each). Within each diameter class same letters represent no significant difference between treatments ($p > 0.05$).

Biochar application rate (t ha $^{-1}$)	>500 μm (%)	250-500 μm (%)	50-250 μm (%)	<50 μm	MWD (mm)
0	31.34 ± 1.66 a	29.10 ± 3.42 a	47.20 ± 3.60 a	10.36 ± 2.17 a	0.182 ± 0.07 a
12	38.78 ± 5.29 b	25.51 ± 3.53 a	48.51 ± 3.95 a	9.79 ± 1.49 a	0.171 ± 0.10 a
50	36.10 ± 7.45 b	28.60 ± 5.50 a	45.61 ± 4.93 a	9.86 ± 1.80 a	0.178 ± 0.16 a

4. Discussion

4.1 Biochar effects on soil water

As expected, biochar increased WHC linearly with application rate, which has already been reported in laboratory settings (Chan et al. 2007; Laird et al. 2010). Here, we found a positive effect of biochar application rate on WHC, partly counteracting the negative effect of coarse sand in this soil, so this may be a useful strategy in sandy soils, a benefit which has been suggested elsewhere (Atkinson et al. 2010). Accordingly, a meta-analysis found that biochar addition to coarse- and medium-textured soils improved crop yields, indicating that increases in WHC may be one main mechanism for yield improvement (Jeffery et al. 2011). In our study, consistent with our expectations and laboratory-evaluated WHC results, field soil moisture sampled during the campaign was slightly higher in biochar–amended soils. This effect has the potential to improve crop growth where water is limiting, especially during critical periods of the growing season (Laird et al. 2010). In Australia, improvements in wheat tiller survival, head formation, kernel weight, and therefore grain yield were attributed to biochar's effect of reducing drought stress (Blackwell et al. 2010). However, greater water retention does not necessarily mean more plant-available water (Tolk 2003), but the few studies available indicate that plant water availability might also be improved by biochar addition (Solaiman et al 2009; Liu et al. 2012b; Pereira et al. 2012a).

4.2 Biochar effects on bulk density

Also as expected, biochar decreased bulk density at both sampling times, whereas the differences in measurements between the Autumn 2011 and Autumn 2012 sampling dates are likely due to previous climatic conditions (compaction due to rainfall and settling of the tilled soil). However, if we calculate the difference between the highest (B0) and lowest bulk densities (B50) and divide by applied biochar (t ha^{-1}),

the magnitude of effect of biochar on bulk densities did not change between dates, resulting in reductions of 1.4 kg m^{-3} / t biochar / ha. Using the same calculation, a maize cultivation with a wheat-straw biochar in a calcareous loamy soil reported reductions of 0.7 kg m^{-3} / t biochar / ha (Zhang et al. 2012b, and with the same biochar applied to rice paddy decreases were 2.5 and 1.4 kg m^{-3} / t biochar / ha for different years (Zhang et al. 2012a). However, a different field study in a temperate soil reported no changes to bulk density at biochar applications of 25 and 50 t ha^{-1} (Jones et al. 2012). Biochar´s effect of decreasing bulk density has also been found in a tropical soil at high field application rates (116 t ha^{-1}) (Major et al. 2010) and in the laboratory (Laird et al. 2010). With scarce studies reporting decreased bulk densities under field conditions, our study provides additional evidence for this effect in a well-structured, temperate soil. This is an agriculturally-important parameter, since higher bulk densities are associated with restricted water infiltration and restricted root growth (Brady and Weil 1996).

4.3 Biochar effects on aggregates

With regards to the dry aggregate distribution, biochar reduced the 250-500 μm size class, though the fate of these materials (i.e. as larger or smaller aggregates, silt, or clay) in the biochar treatments was not identified with certainty. However, based on the aggregate size distribution in **Table 4**, proportions of some macroaggregates (>250 μm) appear to have been enhanced by B12 and B50, and the average MWD of B50 was greater, though these were not statistically significant. Macroaggregate formation is associated with biological activities, namely fungal hyphae and roots and microbial- and plant-derived polysaccharides (Tisdall and Oades 1982); here, the influence of these processes was not validated directly, and there were no statistically-significant differences in microbial biomass between biochar treatments (**Chapter 4.3**). Greater dry-sieved MWD has been

related to soil organic carbon content (Yang and Wander 1998; Álvaro-Fuentes et al. 2008) as well as shifts to larger-sized aggregates in response to additions of organic matter (Hurisso et al. 2013). The contribution of the biggest biochar particles to these size-classes should be minimal, since less than 50% of biochar particles were >250 μm representing ~1% total weight in B50. Similarly, no significant variation in the stable aggregate distribution or the corresponding MWD with biochar treatments was found, but we did find a larger water-stable mass fraction >500 μm due to biochar treatment. Therefore, the trend of increasing aggregate size observed for dry-sieved aggregates was also observed for water-stable aggregates.

The importance of organic matter in aggregation is well established (Six et al. 2004), but the role that biochar may play in this process is still largely unknown, and is limited by the lack of a fundamental understanding of biochar's interactions in soil over the long term (Mukherjee and Lal 2013), which obfuscates the timescale for potential aggregation effects. In the soil science literature, various components of aggregation dynamics in agricultural systems have been identified on the monthly (De Gryze et al. 2005), year (Álvaro-Fuentes et al. 2008), or decadal (Jastrow 1996) timescales (Abiven et al. 2009). In the short term, application of fresh biochar having organic aliphatic functional groups (associated with biochar volatile matter; Mukherjee et al. 2011; Rutherford et al. 2008) may, for example, lead to initial aggregation but which may later become susceptible to destruction (Mukherjee and Lal 2013). In the longer term, a recent 2-year field study observed an increase in macroaggregates due to a biochar-compost treatment in the first year, but these were disrupted in the second year, freeing microaggregates and silt and clay particles (Pereira et al. 2012b). In the same study a biochar-only treatment did not have any effect. Though biochar may have short to medium-term aggregation or disaggregation effects such as these, one would expect that in the long term aggregation effects of biochar would increase

due to changing surface chemistry with aging (Lin et al. 2012). Here, aggregation was evaluated at one point in time, 18 months after biochar application, so it is not known to what temporal phase of aggregation the effects discussed above may pertain to. All considered, longer-term studies will be necessary in the future, since other longer-term processes such as particle fragmentation, freeze-thaw cycles, rain and wind, penetration by plant roots and hyphae, and bioturbation all make biochar more accessible to further chemical and biological processes (Hammes and Schmidt 2009).

4.4 Biochar effects on soil penetrability

Biochar linearly decreased penetration resistance with application rate. As previously mentioned, decreases in penetration resistance with biochar application has also been found in the laboratory (Busscher et al. 2010), but to our knowledge not in the field. Furthermore, this was evaluated in the winter before tilling and seeding for the next crop cycle, and should therefore correspond to maximum soil compaction. Soil penetrability is associated with enhanced root growth and water infiltration, and is related to biochar's effect of increasing porosity, both directly by it's low particle density, and indirectly by improving soil aggregation (decreased bulk density described above).

5. Conclusions

Soil physical parameters were evaluated between 6 and 18 months in mesocosms simulating barley cultivation with two biochar application rates of 12 t ha^{-1} and 50 t ha^{-}. Biochar improved all measured soil physical parameters, including higher soil water retention, decreased bulk density and penetration resistance, and increased proportions of the larger dry and water-stable macroaggregate fractions considered. All these parameters have the potential to improve crop growth, especially in water-limited environments such as the Mediterranean,

and the results represent a contribution to biochar's effects on physical properties in the field, which are currently scarce.

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Chapter 4.1 supplementary information

- Table A.1

Table A.1: Fine earth fraction soil particle diameters of each mesocosm, with particle size classes defined following the system of the International Society of Soil Science.

Plot	Biochar treatment (t ha^{-1} biochar)	Coarse sand (200-2000 μm) %	Fine sand (20-200 μm) %	Silt (2-20 μm) %	Clay (<2 μm) %
1	12	24.59	28.96	17.05	29.40
2	50	27.40	25.52	18.39	28.69
3	0	24.78	29.53	16.82	28.87
4	12	26.26	27.13	17.63	28.98
5	0	26.07	32.23	15.60	26.09
6	12	30.09	29.34	14.34	26.23
7	0	31.18	30.75	12.81	25.26
8	50	32.50	32.85	11.30	23.35
9	12	37.91	26.78	12.79	22.52
10	50	36.94	26.90	12.67	23.49
11	0	36.15	24.32	14.41	25.12
12	0	34.63	23.29	28.33	13.76
13	12	30.75	26.70	17.86	24.69
14	50	26.40	29.12	20.23	24.25
15	50	26.52	26.70	20.51	26.27
16	12	23.63	28.36	20.13	27.88
17	0	24.49	26.51	22.50	26.50
18	50	25.53	26.56	21.14	26.77

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Chapter 4.2 Pine gasification biochar addition to mesocosms under barley cultivation. II. Effects on soil chemical properties

Abstract

The effects of a pine gasification biochar on soil chemical properties was investigated in field mesocosms simulating barley cultivation. Soil pH, electrical conductivity, soil cation exchange capacity (CEC), soluble ions, and soil organic carbon (SOC) were evaluated 6-18 months following biochar application. Mesocosm pH and EC were unchanged by biochar, so it appears that field weathering quickly leached the biochar and reduced any transitory effects. CEC, evaluated at 18 months, was also unaffected by biochar, though it is recognized that this may increase with time due to progressive field weathering and oxidation of the biochar surface. Soil concentrations of K^+ and Cl^- were both increased by biochar addition, and may be due to either direct biochar inputs or indirect effects such as reduced leaching. Biochar strongly decreased soil NO_3^- at one sampling date, and potential mechanisms are discussed, though whose identification was outside the scope of the study. Finally, biochar addition increased labile SOC in an additive fashion, there being no evidence that biochar indirectly influenced additions C inputs or losses over the growing season.

1. Introduction

The positive effects of some biochars on crop yields have been associated with an improved nutrient status. This can be due to the direct provision of nutrients of fresh chars, as well as the indirect effects such as enhanced fertilizer use efficiency or liming, or increased moisture (Steiner et al. 2008; Chan and Xu 2009). The direct fertilizing effect of biochars

should be moderate and transitory following the addition of fresh biochars (DeLuca et al. 2009), related to the presence of leachable nutrients such as N, P and K, whose concentrations depend on feedstock and production conditions (Gaskin et al. 2008; Chan and Xu 2009; Amonette and Joseph 2009; Enders et al. 2012). In previous experience with a gasification biochar produced in the same gasification plant and from the same feedstock as used in this study (**Chapter 1**), we observed that a significant portion of its inorganic nutrients were easily washed with water, which is also likely to occur under field conditions. The maximum temperature and heating rate are the key factors that explain nutrient content; maximum temperature is important in relation to each nutrient's volatilization temperature, and heating rate determines the intensity of this loss by volatization (Kookana et al. 2011). For example, most N- and S-based compounds volatilize above 200 and 375 ºC respectively, while K and P volatilize between 700 and 800 ºC (DeLuca et al. 2009). For this reason, wood biochars produced at 450-550 ºC tend to be depleted in N and S, while other chars produced from feedstocks containing large amounts of these nutrients, such as sewage sludge, that can retain up to 50% of their original N and S contents (Kookana et al. 2011).

Apart from direct nutrient release, it has been suggested that biochar can indirectly improve fertilizer use efficiency, potentially increasing plant nutrient uptake and thereby enhancing yields (Chan and Xu 2009), although this has mainly been observed in tropical soils, and less is known about this effect in temperate soils (Laird et al. 2010). The main mechanism proposed for this increased efficiency has been nutrient retention in biochar's cation exchange complex (Lehmann et al. 2003), but also pH increases which improve nutrient bioavailability and reduce Al toxicity in acid soils (Blackwell et al. 2009). Biochar may also provoke beneficial microbial community shifts such as enhancement of mycorrhizal interactions (DeLuca et al. 2009). However, it has also been suggested

that biochar addition might indirectly decrease nutrient availability, for instance immobilization of N in microbial biomass following addition of biochars with high C:N ratios (as we suspect may have occurred in pot trials in **Chapter 1**), excessive sorption of ammonium or sequestration of soil nutrient-containing solution into fine pores (Sohi et al. 2010), or excessive liming, reducing the plant bioavailability of nutrients such as P (another suggested mechanisms for negative effects on plant growth in **Chapter 1**).

All these uncertainties underscore the need for a better understanding of how biochar can affect soil chemical properties and thereby consequences for crop yields. There is a need for medium to long-term data from varied scenarios which reflect different biochar types, soil properties, and climatic conditions. In our study, we assessed the impact of increasing application rates of a gasification biochar on chemical properties of a Mediterranean alkaline soil under barley cultivation and with pig slurry fertilization a year after its application.

2. Methods

2.1 Mesocosm construction

Field mesocosms with treatments of 0, 12, and 50 t ha^{-1} biochar (hereafter B0, B12, and B50) were prepared in March 2011 as described in **Chapter 4.1**. A barley crop (*Hordeum vulgare* L.) was planted at late winter in 2011 and 2012, seeded at a density of 250 seeds m^{-2} in each plot. To test biochar's potential for improving fertilization efficiency, it was decided that the fertilization regime would be reduced to half the recommended dosage of N (100 / 2 = 50 kg N ha^{-1} $year^{-1}$), applied at a rate corresponding to the hydrolyzable fraction of a thermally-dried pig slurry whose properties are shown in **Table 1**, and whose heavy metal concentrations were within acceptable limits. Fertilization occurred at two

times during the growing season: at seeding, and approximately 1.5 months following seeding date. As such, ¼ of the recommended dose of N (25 kg ha^{-1}) was applied at each of these moments.

Table 1: Content of the dried pig slurry fertilizer. Contents expressed based on fresh (*f.w.*) or dry (*d.w.*) masses are indicated accordingly.

Parameter	Units	Value
dry matter	g kg^{-1} (*f.w.*)	865
WHC	% (*f.w.*)	55.9
pH	water, 1:5 (w/v)	6.4
electrical conductivity	dS m^{-1}, 25ºC	64.65
organic matter	g kg^{-1} (*d.w.*)	612
stable organic matter	%	36.6
N	g kg^{-1} (*d.w.*)	62.5
non-hydrolyzable N	g kg^{-1} (*d.w.*)	10.9

2.2 Biochar characterization

Pine (*Pinus pinaster + P. radiata*) chip gasification biochar had been obtained from a pilot gasification facility in Basque Country, Spain. pH and electrical conductivity (EC) of the fresh biochar were evaluated in triplicate in 1:20 (g:ml) slurries subjected to vertical agitation at 60 rev min^{-1} for 1.5 h (Rajkovich et al. 2012). Loss on ignition (LOI) was evaluated on ground samples in triplicate in a muffle furnace, first at 375 °C for 18h, without acid pre-treatment (used for removal of soot and graphitic black carbon) to

hydrolyzable N	g kg^{-1} (*d.w.*)	51.6
NH_4^+-N	g kg^{-1} (*f.w.*)	52.9
P	g kg^{-1} (*d.w.*)	20.4
K	g kg^{-1} (*d.w.*)	55
Cd	mg kg^{-1} (*d.w.*)	<0.7
Cr	mg kg^{-1} (*d.w.*)	15
Cu	mg kg^{-1} (*d.w.*)	780
Hg	mg kg^{-1} (*d.w.*)	0.12
Ni	mg kg^{-1} (*d.w.*)	29
Pb	mg kg^{-1} (*d.w.*)	<20
Zn	mg kg^{-1} (*d.w.*)	2060

evaluate biochar organic content except the soot fraction (Gustafsson et al. 1997, Poot et al. 2009); at 550°C for 5 h to remove soot (Gustafsson et al. 1997), hence representing the complete oxidation of the organic carbon fraction in biochar; and finally at 1100 °C for 5 h, which should mainly remove carbonates (Santisteban et al. 2004). Volatile matter (VM) was determined in triplicate by proximate analysis following ASTM D1762-84 by heating ground samples in a covered crucible at 950 °C for 6 min and determining weight loss, and ash content was evaluated in triplicate following the ASTM protocol by heating ground samples in an uncovered crucible at 750 °C for 6 h and determining weight loss.

Surface chemistry was assessed by Fourier-transform infrared spectroscopy (FTIR) was performed on dry (105 °C), ground biochar

samples passed through a 100 μm sieve. Spectrum were registered in triplicate at standard infrared resolution (4 cm^{-1}) in the mid-infrared range of 600-4000 cm^{-1} using a Bruker Tensor 27 spectrophotometer working in attenuated total reflectance (ATR) mode with diamond reflection.

2.3 Soil analyses

Five soil samplings were conducted between Autumn 2011 and Autumn 2012, representing the period of 6 to 18 months after the application of the biochar. Samples were taken at 5 points by 2.5 cm diameter auger to 20 cm depth and combined in a plastic bag. In the laboratory, within 24 h each bulk sample was sieved to 5 mm and humidity was determined gravimetrically by drying at 105 °C overnight. Once the humidity of each plot was determined, the remaining sample was weighed and adjusted to 45% WHC (previously determined for each mesocosm) with deionized water. Samples were thereafter stabilized for 7-10 days at 22 °C before chemical analyses.

Cation exchange capacity (CEC) was assessed in triplicate using samples from the Autumn 2012 sampling by the ammonium acetate extraction method. A 1 cm layer of fiberglass was placed in a plastic syringe, followed by a 1 cm layer of washed quartz sand. Then 15 g of wet soil sample of were added, followed by additional sand and fiberglass layers. The sample was soaked for 15 min in deionized water supplied to the syringe using medical tubing, and then drained off. Next, the sample was soaked with 1 N ammonium acetate for 24 h, following which the sample was washed drop by drop with 100 ml of the same ammonium acetate extractant for a minimum of 2 h to saturate cation exchange sites with ammonium, followed by washing with 80% ethanol to clear any excess ammonium acetate. The sample was then washed drop by drop with 100 ml of 1 M $BaCl_2$ for a minimum of 2 h to displace the exchangeable ammonium, and the extract was collected in 150 ml polypropylene vessels containing 1 ml of H_2SO_4 to

avoid ammonia volatilization. Finally, ammonium in the extracts was quantified using a Crison NH_4^+ selective electrode, and this measurement was used to calculate the CEC.

Additional chemical analyses were carried out on stabilized soils of each sampling date. Extracts for determination of pH, EC, and ions were with 15 g ODW soil and 60 mL deionized water (1:5) subjected to vertical agitation at 60 rev min^{-1} for 2 h, followed by centrifuging and filtering with Whatman 42 filter paper. pH and EC were determined immediately using a Crison GLP 21 pH meter and a Crison Basic 30 conductometer, and the extract was frozen at -20 °C for determination of ion concentrations at a later date. Ion chromatography was used to determine water-soluble concentrations of major cations and anions. A 5:50 mL dilution was prepared from the extracts used for pH and EC, and a 5 mL sample was taken for analysis. Ion chromatography analysis of Na^+, K^+, Mg^{2+}, Ca^{2+}, and NH_4^+ was carried out with a CS12A Dionex cation column on a Dionex ICS-1100 ion chromatograph (Dionex, Sunnyvale, USA), and for Cl^-, NO_2^- NO_3^-, HPO_4^{2-} and SO_4^{2-} with a AS4A-SC Dionex anion column on a Dionex DX-100 ion chromatograph (Dionex, Sunnyvale, USA).

Soil total organic C (SOC) was evaluated at the beginning and end of the growing season. Since the plots were maintained weed-free, we assumed that these should represent seasonal moments of lowest and highest soil organic matter, respectively. SOC was estimated by performing the Walkley-Black method in duplicate for each plot and date by digesting 100 μg oven-dry ground samples with 15 mL 0.2 N chromic acid at 150°C for 10 min and back-titrating remaining potassium dichromate with ferrous ammonium sulfate.

2.4 Statistical analyses

For each chemical parameter, a two-way repeated measures ANOVA was performed, using plot identity as subject, and including as factors

biochar *treatment*, *date*, and *treatment*date*. If either factor *treatment* or *treatment*date* were found to be significant, pairwise tests of significant differences between treatments were carried out with Mann-Whitney U-tests over the complete time series.

3. Results

The biochar's pH, EC, proximate analyses, ash, and elemental (C, N, S) content are shown in **Table 2**. Surface chemistry (FTIR spectrum) is shown in **Figure 1**. Notable is the overall lower abundance and diversity of functional groups and the higher absorbance (situation on the y-axis) with respect to slow and fast pine biochars (**Appendix 1**), which can be attributed to high condensation of aromatic structures (Mochidzuki et al. 2003). The peak at 870 cm^{-1} has previously been assigned to biochar carbonates (Kloss et al. 2012). See **Appendix 1** for a detailed interpretation of peaks. In agreement, LOI-1100 also indicated some carbonate contents (~1%).

Table 2: Chemical properties and selected elemental content of the pine gasification biochar used in the study.

Parameter	Value
pH	11.4 ± 0.02
EC ($\mu s\ cm^{-1}$ 25°C)	644 ± 3.8
VM (%)	8.0 ± 0.28
LOI-375 (%)	88.83 ± 0.06
LOI-550 (%)	0.73 ± 0.03
LOI-1100 (%)	1.08 ± 0.06
Ash (%)	9.49 ± 0.05
C (%)	88.41 ± NA
N (%)	0.30 ± NA
S (%)	0.06 ± NA

Soil mesocosms' pH and EC at each sampling of the study period are shown in **Figure 2**. As indicated in **Table 3**, there were no treatment effects on either of these parameters. CEC of B0, B12, and B50 were 63.0 ± 1.2, 66.1 ± 1.8, and 65.1 ± 2.1 cmol kg^{-1} (mean of 6 replicates ± SE), respectively. There was no significant difference in CEC due to biochar treatment (Kruskal-Wallis chi-squared=1.55, p=0.45). Biochar application affected the availability of K^+, Cl^-, and NO_3^- (**Figure 3**; **Table 3**). For concentrations of available K^+ and Cl^-, the effect of biochar application was additive, increasing with biochar addition rate. Additionally, status of Cl^- and NO_3^- concentrations over the year depended on biochar treatment and sampling date (*date*treatment*), whereas during the growing season Cl^- was particularly enhanced by biochar, and NO_3^- was particularly reduced. For NO_3^- this occurred at the end of the growing season where concentration in B50 was lower than that in both B0 (p=0.01) and B12 (p=0.003), mean water-soluble concentrations being 51.2 ± 7.8, 44.0 ± 3.8, and 23.6 ± 2.6 mg $kg^{-1}$for B0, B12 and B50 respectively.

Walkley-Black SOC was increased by biochar addition at both sampling dates (**Figure 4**; ANOVA p<0.001 for *treatment*, **Table 4**), with means of 0.77 ± 0.06, 0.96 ± 0.07, and 1.6 ± 0.10 % at the spring sampling date, and 0.93 ± 0.08, 1.20 ± 0.07, and 1.91 ± 0.12 % for B0, B12, and B50 (respectively) at the summer sampling date (**Figure 4**). SOC was higher in all treatments at the end of the growing season (ANOVA p=0.002 for *date*, **Table 4**). There was no significant interaction in the ANOVA between *date* and *treatment* (**Table 4**).

Figure 1: FTIR spectra of the biochar used in the study.

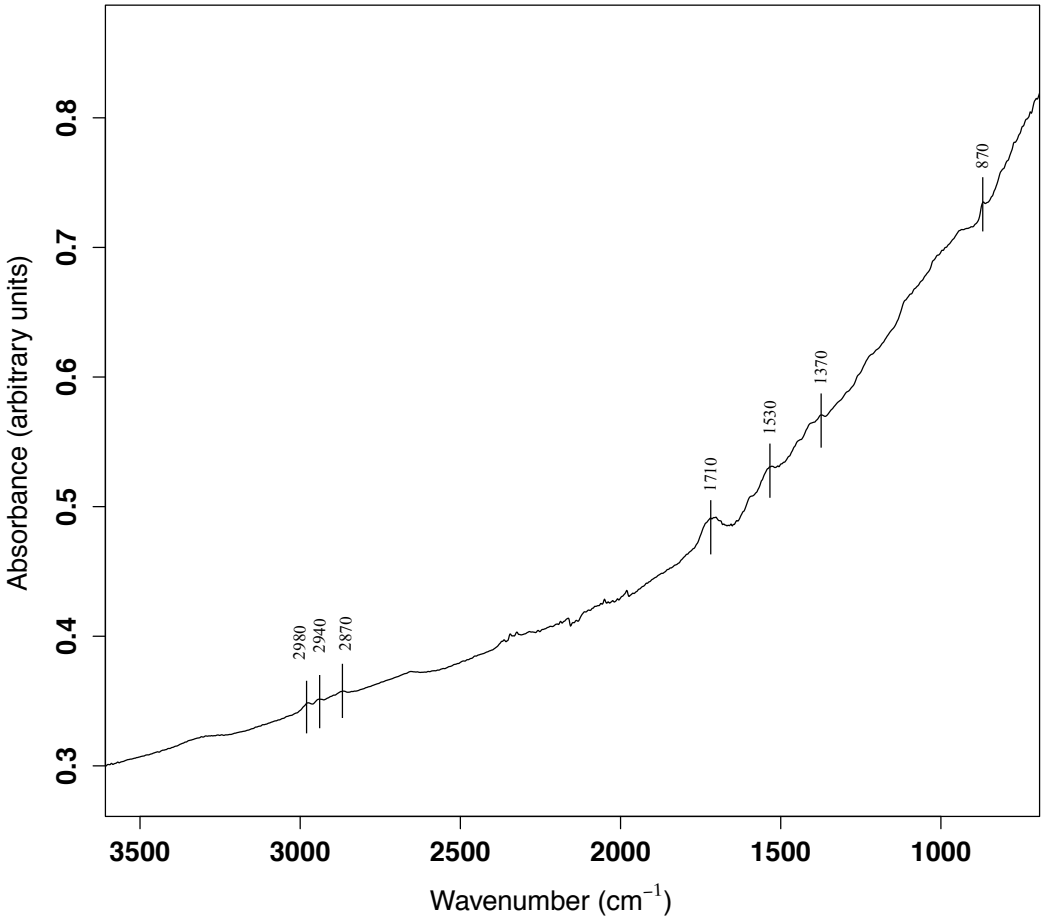

Absorbance (arbitrary units)
0.3
0.4
0.5
0.6
0.7
0.8
3500
3000
2500
2000
1500
1000
Wavenumber (cm^{-1})
2980
2940
2870
1710
1530
1370
870

Figure 2: pH and electrical conductivity (EC) measured in the mesocosm treatments of 0, 12, and 50 t ha^{-1} biochar during the study period, x-axis indicating sampling date, and error bars indicating SE.

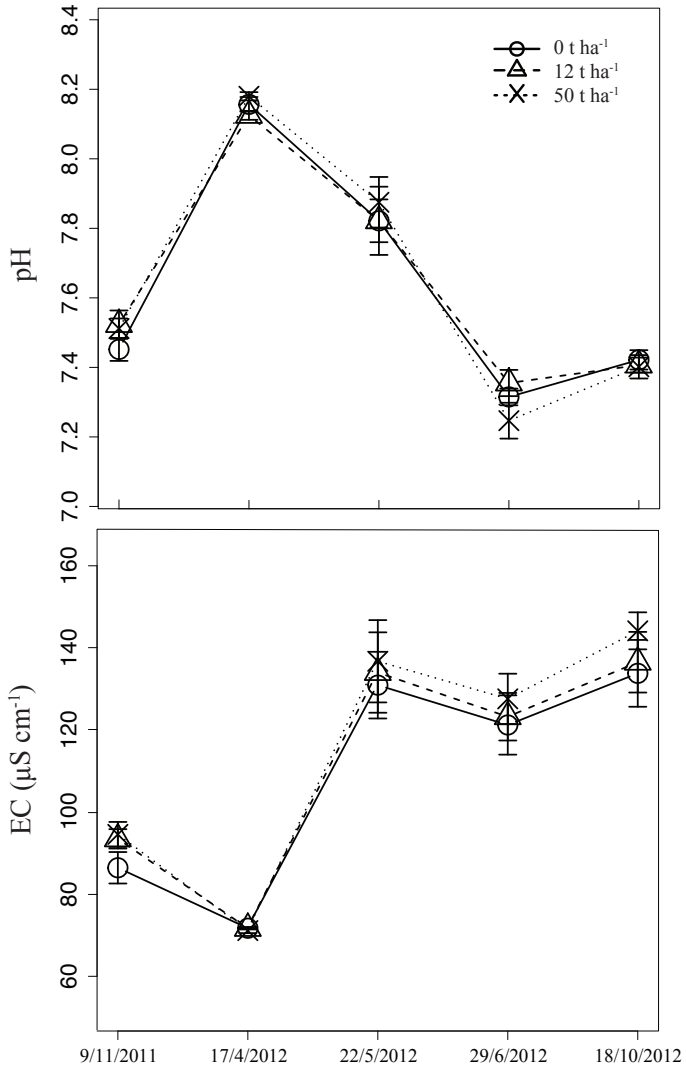


Figure 3: Soil concentrations of ions affected by biochar treatments evaluated at each sampling date (x-axis), error bars indicating SE.

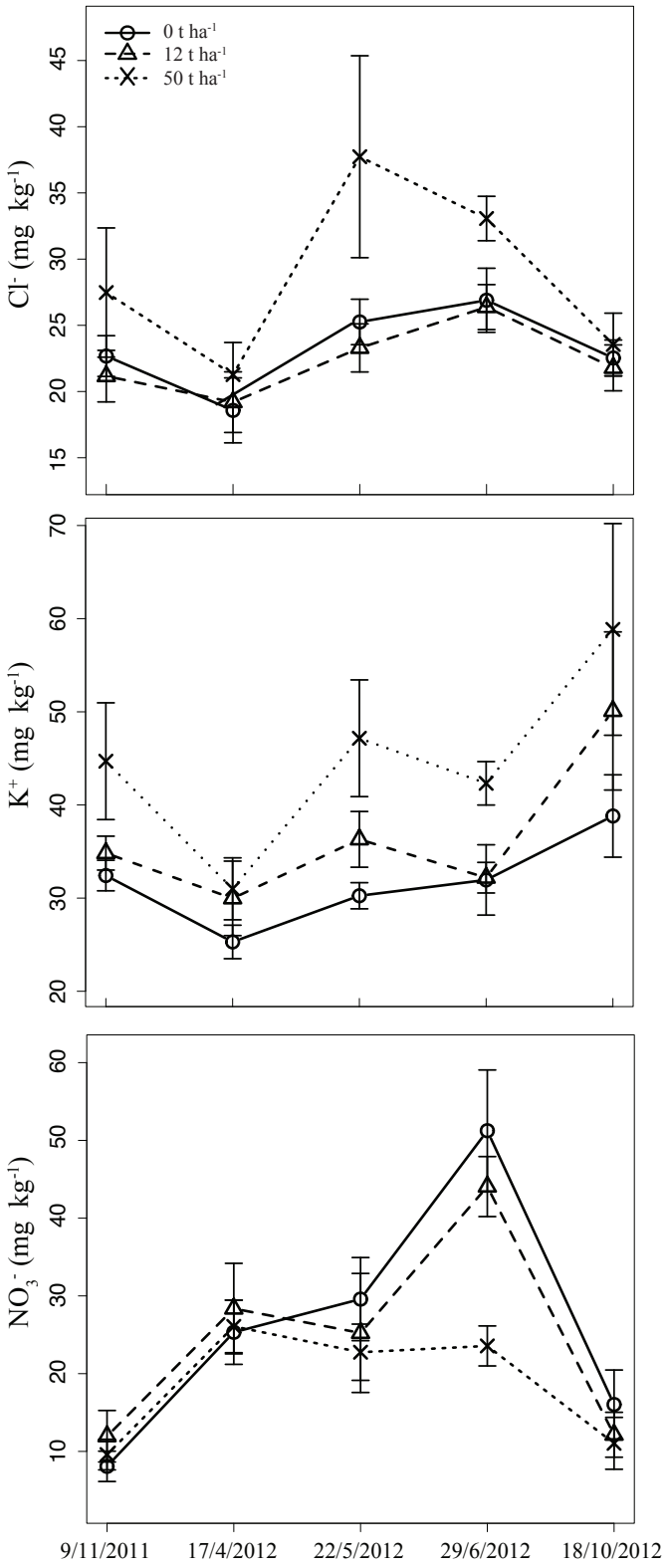


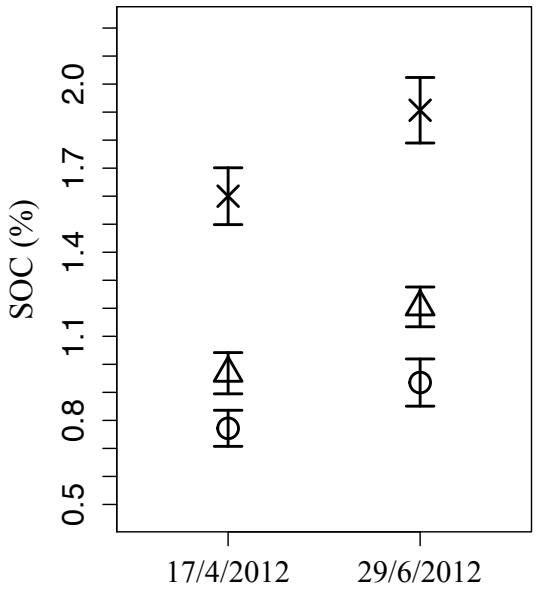
Table 3: P-values of two-way repeated measures ANOVAs of effect of biochar treatment, sampling date, and their interaction on soil ionic concentrations, pH, and electrical conductivity (EC). Sampling dates are the same as indicated in **Figure 2**.

Model variable	Ca	Na	Mg	Cl^-	NH_4^+	NO_3^-	NO_2^-	HPO_4^{2-}	K^+	SO_4^{2-}	pH	EC
Treatment	0.237	0.780	0.943	0.008	0.187	0.201	0.844	0.731	0.056	0.654	0.847	0.571
Date	<0.001	<0.01	<0.01	<0.01	<0.001	<0.001	0.116	<0.001	<0.01	<0.001	<0.01	<0.01
Treatment*date	0.474	0.698	0.092	0.014	0.704	0.008	0.822	0.345	0.652	0.330	0.430	0.915

Table 4: Two-way repeated measures ANOVA of field soil organic carbon with biochar treatment, date, and interaction as factors. Sampling dates indicated in **Figure 4**. Residual df=30, SS=1.26.

Model variable	df	SS	F	p
Treatment	1	1.53	36.9	<0.001
Date	1	0.50	12.0	0.002
Treatment*date	1	0.03	0.6	0.418

Figure 4: Walkely-Black soil organic carbon (SOC) at two sampling dates, error bars indicating SE. Point designation is as follows: "o" = control, "Δ" = 12 t ha^{-1} biochar, and "x" = 50 t ha^{-1} biochar.



4. Discussion

Studies on effects of gasification chars on soil processes are still scarce (Brewer et al. 2009). Gasification chars are distinguished from other biochar production methods by their high production temperature (>750 °C) and intermediate residence time of 10-20 seconds, whereas slow pyrolysis usually lasts hours, and fast pyrolysis milliseconds (Bridgwater 2006; Brown 2009); high temperatures produce high surface areas (Brown 2009), promote the condensation of C, and increase the proportion of fixed C (Keiluweit et al. 2010). As a matter of precision, due to controlled O_2 input to allow partial combustion within the reactor to further raise temperatures driving biomass devolatilization to maximize gas production, gasification is not formally considered a pyrolysis process (Brown 2009; Lugato et al. 2013).

4.1 Biochar characterization

Ash content was relatively high for a wood biochar (9.5%). EC was not expected to have any effect on soil salinity (664 $\mu S\ cm^{-1}$). VM was relatively low within biochars (8%) in accordance with the biochar's high production temperature. LOI-375 was moderate for a wood biochar (88%), representing most organic contents, whereas LOI-550 (0.73%) and LOI-1100 (1%) indicate soot and carbonate contents, respectively. Carbonates were also detected in this biochar as effervescence following treatment with HCl, and the FTIR peak near 870 cm^{-1} (**Figure 1**) may be attributable to carbonates (Kloss et al. 2012). The availability of these carbonates outside of the organic matrix helps explain the biochar's high pH.

4.2 Effects on soil pH and salinity

The pH of the soil and biochar are both important, as they have both been shown to affect productivity outcomes (Biederman et al. 2012). The high pH is probably due to the biochar's carbonate contents which were ~1%, as well as the relatively high ash content (for a wood biochar) which can be ascribed to a partial combustion as part of the gasification process. Also, the relatively high pH of wood gasification chars (see PG in **Chapter 1**) can be attributed in part to a temperature-driven reduction of surface acidic functional group composition (-C-H or -OH bonds, namely carboxylic, phenolic, or amine) (**Appendix 1**; Mochidzuki et al. 2003; Conklin 2005; Keiulweit et al. 2010; Mukherjee et al. 2011), but is also related to ash content (see PG in **Chapter 1**), which increases with pyrolysis temperatures (Enders et al. 2012; Lehmann et al. 2011).

Despite high fresh biochar pH, during the campaign, representing the period of 6-12 months following biochar application, we detected no effect of biochar addition on soil pH. Since biochar surface carbonates are readily dissolved (Joseph et al. 2009), this likely explains why we found no biochar-mediated increment in soil pH during the study period, whereas the biochar had already been weathered 6 months. As seen in **Chapter 1**, a very similar gasification char was easily leached, and both pH and EC were substantially reduced by washing. Therefore, we interpret that any previous transitory effects on soil pH and EC rapidly subsided with field incubation and leaching of the mesocosms.

Some information is available on biochar effects on pH in other field studies. A two-year field study with a pine chip char did not find any effect on soil pH in the first year, but caused a decrease in the second (Gaskin et al. 2010). Elsewhere, soil pH increases have normally been found: a one-year study in a calcareous soil with maize cropping and a slow-pyrolysis wheat straw biochar documented an increase in soil pH at 40 t ha^{-1} (but not at 20 t ha^{-1}), but this effect was less clear with mineral fertilizer (Zhang et al. 2012a); a two-year study in rice paddy with the same biochar also

documented an increment in pH at 10 t ha^{-1} (Zhang et al. 2012b); finally, a sustained 3-year pH increase was found in a study with a 50 t ha^{-1} application of biochar of mixed woody residue feedstock in a temperate soil (Jones et al. 2012). It is not clear to what extent such differences may be explained by biochar feedstocks or pyrolysis methods. For instance, in the study by Gaskin et al. (2010) above, an increase in pH was found for a peanut hull char under the same conditions, but evidently not for the pine chip char. Also, in our previous work, we found that slow pyrolysis and gasification chars of the same pine feedstock provoked opposite pH changes in a pot trial, decreasing with the slow pyrolysis char and increasing with the gasification char (**Chapter 1**).

With regards to soil EC, the biochar's salinity indicates that soluble salts were present, but the total amount added to soil is probably not enough for significant salinity increases to be observed, and rapid field leaching might also explain why no persistent effects were seen. Less bibliographic information than for pH is available on biochar's effects on soil EC in the field, but the previously mentioned study of Jones et al. (2012) did not find any effects on this parameter, either.

4.3 Soil cation exchange capacity (CEC)

Although high production temperatures, such as those used to produce the gasification char of our study, have been linked to maximum biochar CEC values in biochars (Lehmann et al. 2007), the particularities of gasification technology, strictly not a pyrolysis process, may explain the lack of effects of this gasification char on soil CEC. In a previous study of our group, a similar gasification pine biochar presented lower CEC than the corresponding slow and fast pyrolysis pine biochars (Pérez-Herrero, personal communication). Similarly, Lee et al. (2010) reported that a cornstover gasification char presented lower CEC than the corresponding fast pyrolysis char. However, it is known that CEC can increase with biochar

aging in soil (Hammes and Schmidt 2009), so long-term positive effects on CEC cannot be discarded for this char or model agro-ecosystem.

4.4 Effects on soil soluble nutrient concentrations

4.4.1 Potential direct effects

We observed that biochar application resulted in sustained increased concentrations of soluble K^+ and Cl^-. Other studies have reported dominant quantities of water-extractable Cl^- in charcoal and charcoal ash (Yanai et al. 2007), and increases in available K^+ after biochar addition have been frequently reported in field studies (Chan and Xu 2009). Gasification chars are expected to be depleted in K due to the high temperatures achieved in their production (600-900 °C) (Kookana et al. 2011); however, comparing a similar gasification char with slow and fast pyrolysis materials of the same feedstock (**Chapter 1**), K was higher in the gasification char, which goes against expectations, and in water-washing experiments it also had higher water-soluble concentrations of both K and Cl than the other materials.

4.4.2. Potential indirect effects

Nutrients provided with fertilization may have been retained to a higher extent in biochar-amended soils (fertilizer nutrients seen in **Table 1,** Cl not available; these values reflect total contents, not necessarily ionic forms). Nutrient retention is most likely to occur via increases in cation or anion-exchange capacities. However, we did not find significant increases in CEC with biochar treatments, therefore any such increased retention of K^+ should be limited. Likewise, as to explain the sustained high Cl^- concentrations, anion retention capacity of chars is low in alkaline pH (Cheng et al. 2008). Microorganisms may also assimilate Cl (Öberg and Sandén 2005), and it thereby may accumulate.

Interpretation of our result of biochar's effect decreasing NO_3^- is limited by the available data. Biochar is likely to interfere in nitrification and denitrification (Clough and Condron 2010), and numerous recent studies documenting biochar's effect on denitrification (Van Zwieten et al. 2009; Clough et al. 2010; Bruun et al. 2011; Singh et al. 2010), or reduced leaching of N-species (Dempster et al. 2012a; Knowles et al. 2011; Ding et al. 2010; Ventura et al. 2013) demonstrate current interest in this topic. In fact, the effects of biochar on nitrogen retention in soils is one of the most debated topics in the field, since both positive and negative effects on nitrification have been described in the literature. In forest soils increased NO_3^- concentrations have been explained by several biological mechanisms (Lehmann et al. 2011) including direct stimulation of nitrifiers due to alkalinization (Atkinson et al. 2010). In agricultural and grassland soils, nitrification has been unaffected or decreased (DeLuca et al. 2009).

In our study, the decrease in NO_3^- concentrations at some sampling dates may be explained by several mechanisms, although we cannot validate any of them with the available data: (i) a reduction in NH_4^+ availability due to increased CEC with biochar addition (Lehmann et al. 2003, Major et al. 2009), but as already mentioned, biochar affected neither (water-extractable) NH_4^+ nor CEC; (ii) increased N assimilation and immobilization by microorganisms due to stimulation by biochar VM (Deenik et al. 2010); (iii) an increased leaching of NO_3^- due to the improved hydraulic conductivity of soils amended with biochar (Downie et al. 2009), although this is unlikely since Cl^-, which is equally leachable, remained higher in biochar-amended plots during the study; (iv) increased N uptake by plants (Clough et al. 2013); (v) microbial-mediated processes, most notably denitrification or possible inhibition of nitrification (DeLuca et al. 2009; Clough and Condron 2010; Dempster et al. 2012b).

P, a critical plant nutrient, is released from fresh biochars (DeLuca et al. 2009), though its quantity and availability depend on feedstock and

production method, which may therefore determine plant responses (**Chapter 1**). Here, HPO_4^{2-} was not affected by treatment, which is consistent with previous knowledge that a similar gasification char had little elemental P (0.08%) and no water-soluble P (**Chapter 1**) which we supposed was due to alkaline conditions and high Ca contents. The alkaline soil should not favor high P availability, whereas in alkaline (pH >8.0) media P is easily complexed in carbonates (Song et al. 2002), and in Mediterranean, calcareous soils, an increase in soluble Ca^+ to the system can increase P sorption (Carreira and Lajtha 1997). One laboratory study found that additions of a wheat residue biochar up to 1% increased water-soluble P, but rates of 2% and 4% decreased soluble P in certain soils, which the authors ascribed to the possible mechanisms of (i) binding and adsorption to soil P binding sites; (ii) binding to positively charged metal complexes formed on biochar surfaces; and (iii) flocculation of colloidal P through Ca, Mg and K ions added with ash in the biochar (Parvage et al. 2013). While direct biochar sorption of HPO_4^{2-}-P on positively-charged surface sites may also be a possibility, as previously mentioned we do not expect a high anion adsorption capacity of the biochar, and a study of biochar effects on P sorption in temperate soils showed that a softwood biochar either had no effect or negatively affected P sorption (Hovi et al. 2013).

4.3 Organic carbon

SOC was increased by biochar treatment at both sampling dates. Based on elemental C (**Table 2**), biochar additions in B12 and B50 corresponded to an estimated increase of soil C by 0.4% and 1.76% respectively. This calculation includes inorganic carbon as carbonates, but carbonate content, as evaluated by LOI-1100, was very small compared to total C (1% and 88%, respectively). Adding these calculated quantities of C to actual SOC measured in the control mesocosms (B0) results in values which are less

than actual SOC measured in B12 and B50. This is not surprising, as it is known that acid dichromate methods do not fully reflect all black carbon (Manning et al. 2009), rather reflecting labile C (Calvelo Pereira et al. 2011; these authors, however, discuss the possibility that aromatic structures may interfere in the acid dichromate wet oxidation, resulting in overestimation of the labile fraction). Another possible explanation may be that a portion of the biochar had mineralized. Unpublished data from our laboratory have shown that 65% of the C of a similar biochar was oxidizable by acid dichromate, and therefore potentially mineralizable. However, *a priori* we do not expect that such biotically-mediated oxidation would be important due to the high aromaticity of the biochar shown by FTIR, and as demonstrated elsewhere (Baldock and Smernik 2002).

Considering the above, direct contributions of biochar to SOC are clear. However, as with nutrients, biochar may also provoke indirect changes in soil organic carbon inputs or losses. For instance, any enhanced (or reduced) plant growth will affect SOC inputs via root turnover and root exudates (Kuzyakov and Domanski 2000). Also, biochar is known to absorb DOC, so the retention of soluble C-containing compounds in the soil may be increased; Laird et al. (2010) demonstrated that biochar is able to retain DOC released by simultaneous addition of manures. Finally, native organic carbon in the soil might be also influenced by positive or negative priming effects (Zimmerman et al. 2011).

By choosing two sampling dates representing moments just previous to and following the growing season, we postulated that any indirect effect on the soil C pool, e.g. by increasing/decreasing barley root growth, protecting organic matter, priming native SOC, etc., would be best detected during this interval. However, despite the above considerations, such an effect (*date*treatment* term in the statistical model) was not supported (*date* and *treatment* were only additive effects), therefore indicating that biochar

application rate directly contributed to SOC levels, but did not indirectly change organic carbon inputs or losses (**Table 3**).

All considered, organic carbon content is a crucial property of soils, linked to increased soil quality through improvements in water retention, nutrient reserve capacity, higher efficiency of added inputs, and improved soil structure, among others, and depleting SOC can even exacerbate increasing atmospheric CO_2 levels associated with climate change due to reduced ability to sequester atmospheric CO_2 (Lal 2011).

5. Conclusions

A wood gasification biochar did not change soil pH, EC, or CEC from 6-18 months following application. Therefore, we conclude that biochar field weathering quickly leached the biochar and reduced any transitory effects on pH and EC, but additional future weathering and oxidation of the biochar surface, potentially increasing CEC, cannot be discounted. Higher concentrations of soil available K was sustained for up to 18 months in biochar treatments, showing that biochar application may be particularly useful in K-deficient soils, and though unconfirmed this effect was likely due to direct biochar addition. Biochar also had the effect of decreasing soil NO_3^-, the mechanisms for which may be due to a number of proposed mechanisms, but whose identification was outside the scope of the study and deserves further attention in the future. Finally, direct increases in labile SOC were observed with biochar application, with no detectable indirect effects on the SOC pool over one growing season.

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Chapter 4.3 Pine gasification biochar addition to mesocosms under barley cultivation. III. Effects on soil biological properties

Abstract

Soil biota regulate key soil processes, including decomposition of organic matter and nutrient availability for higher plants. Biochar, when applied as an agricultural soil amendment, has the potential to affect soil biota though many potential alterations of the soil environment. These impacts and interactions may be either negative or positive, depending on the biological property or biota considered. Despite the high importance of ecosystem services provided by soil biota, to date little is known about how biochar affects their activities or abundances in the field. For this reason, we investigated biochar's impact on soil biological quality indicators following additions of a pine gasification biochar at application rates of 12 and 50 t ha^{-1} in a field mesocosm study under Mediterranean conditions with barley cultivation. Measured parameters included microorganism activity, abundance, and mineralization, soil fauna activity, and barley seedling emergence, plant height, flag leaf length, and primary productivity. Total soil microbial abundance was enhanced by biochar addition, whereas activities were generally unaffected, though there was limited evidence indicating increased C-use efficiency. Whereas mineralization of NH_4^+ and C were largely unaffected, NO_3^- mineralization rate was reduced by biochar. Soil fauna, unaffected by the 12 t ha^{-1} treatment, were inhibited by the 50 t ha^{-1} treatment. Finally, the barley crop seedling emergence was marginally increased at 50 t ha^{-1} biochar. Also, plant height was marginally increased at 12 t ha^{-1} biochar. However, at 50 t ha^{-1}, plant height and flag leaf length were reduced. While neither application rate significantly affected barley

primary productivity with respect to the control, that in the 12 t ha^{-1} application rate was significantly greater than that in the 50 t ha^{-1} application rate. These results go to show that while this pine gasification biochar may improve some soil biological parameters at moderate application rates, high application rates can negatively impact soil quality in a temperate, alkaline soil.

1. Introduction

Biochar addition can enhance soil health, but may also pose a direct risk to soil organisms and their functions (Lehmann et al. 2011). In turn, soil biota may influence biochar stability in soil, since part of its mineralization is directly carried out by microorganisms (Zimmerman 2010) but indirect mineralization is also expected to be facilitated by soil animals, although no studies on this topic are available besides one reporting biochar ingestion by earthworms (Topoliantz and Ponge 2005).

Risks might arise from the release of toxic compounds in biochars such as pyrolysis oils (Gell et al. 2011), PAHs (Hale et al. 2012) or heavy metals (Chan and Xu 2009), but also through short- or long-term changes in the soil physicochemical environment as affected by biochar richness in labile carbon, EC, pH, porosity/aeration, water, and nutrient retention (Thies and Rillig 2009). The long-term sustainability of biochar systems hinges on the continued provision key ecosystem services provided by soil biota, together with suitable soil physical and chemical parameters, ultimately impacting crop growth (Verhoef 2004). In turn, biochar can indirectly affect soil communities through effects on primary production (Biederman and Harpole 2012), driving detritus production (Cebrian 1999), which is crucial for the support of the soil food web (Moore et al. 2004).

Soil organisms are key for processes such as organic matter decomposition and nutrient recycling (Verhoef 2004; Coleman et al. 2004) and play a role in soil aggregation promotion (Shipitalo and Protz 1989),

hence potentially inducing important above-ground effects in terms of plant growth. Abundance and activity of the soil microbial community has been used to describe impairment (or enhancement) of soil functions associated with various human impacts or contaminants (Lagomarsino et al. 2011; Brookes 1995). Soil invertebrates participate in the fragmentation of organic matter and regulate microbial communities, so practices effecting diversity and activity of mesofauna such as collembolans, mites, and enchytraeids are also of concern (Verhoef 2004; van Straalen 2004; Bardgett 2005). This is why studies dealing with invertebrates are currently a top priority in biochar research (Lehmann et al. 2011).

Biochar is expected to encourage microbial populations, as it constitutes a favorable site for microorganisms, possibly due to higher concentrations of adsorbed compounds that can be used by them (Pietikäinen et al. 2000), but also because of the presence of pores that could serve as refuge sites for bacteria and fungi, since pore size excludes predators like protozoa and nematodes (Thies and Rillig 2009; Warnock et al. 2007), though targeted experimentation is needed (Lehmann et al. 2011). It has been also suggested increases in microbial abundances with biochar addition could partly be explained by a reduced leaching of microorganisms sorbed to biochar surfaces (Pietikäinen et al. 2000).

Regarding potential impacts on soil fauna, biochar may have positive effects though changes in soil porosity, pH (in acid soils), moisture retention and soil temperature, as well as nutrient retention (McCormack et al. 2013). However, negative effects may also occur with the enhanced retention of toxic substances and the release of pollutants already mentioned.

Concerning crop yield effects, recent meta-analysis studies from extensive biochar research reviews have shown that, on average, biochar additions to agricultural soils improve crop yields (Jeffery et al. 2011; Biederman and Harpole 2012), although not directly linked to biochar

addition rate, and with varied results at higher addition rates (Biederman and Harpole 2012). Jeffery et al. (2011) indicated that the positive effects on yield are higher in acidic and neutral pH soils and those with coarser texture. Many of the published studies have been carried out in tropical soils, acidic and poor in nutrients because of their abundance in low activity clays, possibly biasing generalizations about biochar's positive effects (Blackwell et al. 2009). As an example, in the previously mentioned meta-analysis Jeffery et al. (2011) indicated that soil pH was greater than 6.0 in only 14% of studies surveyed, but when considering only these the response in productivity was nonetheless significantly positive. Studies in moderately alkaline soils (e.g. pH >8.0; Brady and Weil 1996) such as that in our study are even scarcer.

In this chapter we assess the impact of additions of a pine gasification biochar to mesocosms under barley cultivation in an alkaline soil. Effects on soil biological quality were evaluated by several ecologically relevant biological endpoints. As the endpoints considered represent key biologically-mediated soil functions, this chapter complements physical and chemical parameters presented in **Chapters 4.1** and **4.2**. Microbiological endpoints included microbial abundance (microbial biomass), activity (basal respiration), C-use efficiency (metabolic quotient), and C and N mineralization rates. Fauna activity was assessed by the bait lamina test, while barley crop fitness was evaluated by emerged seedling number, plant height, flag leaf length, and end-of-season primary productivity. Finally, to explain crop responses, we discuss pertinent changes in soil quality of the model agricultural system described in **Chapters 4.1-4.3**.

2. Methods

2.1. Mesocosm setup, maintenance and sampling

Biochar production and mesocosm construction are described in

Chapter 4.1. Main soil properties are described in Chapter 4.1, and biochar chemistry is described in Chapter 4.2. In late Winter 2012 barley (*Hordeum vulgare* L.) was seeded at a density of 250 seeds m^{-2}, and was fertilized with dried pig slurry (Chapter 4.2) at an application rate corresponding to half the recommended dose of nitrogen (50 kg hydrolyzable N ha^{-1}) divided into two applications, one at the seeding date, and the second approximately 8 weeks following seeding.

Soil biological samplings were conducted at four moments in 2012, three in the growing season, at approximately 1, 2, and 3 months from the seeding date, and one in the following Autumn. In each mesocosm samples were taken at 5 points by 2.5 cm diameter auger to 20 cm depth and combined in a plastic bag. Between samplings the auger was sterilized with 70% alcohol. In the laboratory, within 24 h each bulk sample was sieved to 5 mm and moisture was determined gravimetrically by drying at 105 °C overnight. Once the moisture of each plot was determined, the remaining sample was weighed and adjusted to 45% WHC (previously determined for each mesocosm) with deionized water. Samples were thereafter stabilized for 7-10 days at 22 °C before biological and chemical analyses.

2.2. Microbial endpoints

Following stabilization, soil biological analyses were carried out as follows: soil basal respiration (BR) was evaluated with gas traps following Pell et al. (2004). Briefly, ~30 g stabilized soil was placed in 150 ml polypropylene vessels placed in 1 l hermetic glass jars with a vessel of 2 ml freshly prepared 0.1 M NaOH solution, and incubated in darkness at 22 °C for 24 h. Following incubation, 4 ml 0.05 M $BaCl_2$ was immediately added to the gas trap vials to precipitate the absorbed CO_2 as barium carbonate and the remaining sodium hydroxide was titrated against 0.05 M HCl using 3-4 drops phenolphtalein solution as indicator. The same sample was then used to estimate microbial biomass by the fumigation-extraction method

following Brookes and Joergensen (2006) as follows: two portions of the moist soil (~15 g ODW) were weighed and noted as fumigated and non-fumigated samples. In the first (non-fumigated), dissolved organic carbon (DOC) was immediately extracted with 40 ml 0.5 M K_2SO_4 by vertical agitation at 60 rev min^{-1} for 2 h, centrifuged, filtered (Whatman no. 42), and stored at -20 °C. The second was fumigated for 24 h at 22 °C with ethanol-free $CHCl_3$ and then DOC was extracted as described above. Extracted organic DOC of both fumigated and unfumigated samples was evaluated by oxidation with 2:1 H_2SO_4:H_3PO_4 mixture and 0.4 M $K_2Cr_2O_7$ at 150 °C for 30 min followed by back-titration with 40 mM $(NH_4)Fe(SO_4)2 \cdot 6H_20$, with the modification that extract and wet chemical volumes were halved. Microbial biomass (MB) was then calculated as MB = C_{mic} / 0.38, where C_{mic} is the difference between organic C extracted from fumigated soil and from non-fumigated soil, and 0.38 is the conversion factor from C_{mic} into microbial biomass. As biochar may increase adsorption of organic C, leading to underestimation of microbial biomass, adsorption correction factors (CF) for each mesocosm plot were determined largely following Jin (2010): first, a DOC stock solution was prepared by stirring 400 g of a forest topsoil with 1000 mL deionized water, centrifuging at 13,800 *g* for 8 min, and recovering the supernatants. Next, four replicates of moist soil corresponding to 6 g ODW were placed in 40 mL-capacity centrifuge tubes and 24 mL of K_2SO_4 containing the suitable amount of stock solution to provide, in duplicate, 0, 400 or 550 μg C g^{-1}. The centrifuge tubes were vertically agitated at 60 rev min^{-1} for 2 h and afterwards centrifuged for 8 min at 13,800 *g*. The supernatant was filtered through Whatman 42 filter paper and the samples were stored at -20 °C until analysis. DOC concentrations in supernatants were determined following the same method described above. Expected DOC (E) was calculated as the sum of DOC content of sample and DOC added, and measured DOC (M) as that evaluated. CF were then calculated as CF = M /

E. These CF, corresponding to two replicates for each of the two DOC concentrations tested were then averaged (n=4) to obtain a final CF for each mesocosm. Corrected microbial carbon and biomass estimates were then calculated as $C_{mic\text{-}c} = C_{mic} / CF$, and $MB_c = C_{mic\text{-}c} / CF$. Finally, the metabolic quotient (qCO_2) was calculated as $qCO_2 = (\mu g\ CO_2\ g\ soil^{-1}\ hour^{-1} / \mu g\ C_{mic\text{-}c}\ g\ soil^{-1}) * 1000$ (Anderson and Domsch 1990). Statistical analyses for differences in BR, MB_c, and qCO_2 due to biochar treatment and sampling date were carried out by two-way repeated measures ANOVA followed by non-parametric Mann-Whitney contrasts at each sampling date.

Using soil samples collected in the mesocosms 6 mo. and 12 mo. after biochar addition, C and N mineralization tests were carried out for each of the 18 plots. Four 3 cm cores were taken to 20 cm depth and combined. Sample humidity was measured using gravimetric methods, and water was subsequently added to achieve moistures of 45% of the WHC. After stabilization for 7 d, a 15 g (ODW) sample was taken to evaluate existing NO_3^- and NH_4^+ concentrations using selective electrodes. Water-extractable NO_3^- and NH_4^+ were extracted 1:5 (g ODW:ml RODI H_2O) by 2 h vertical agitation at 60 rev min^{-1}, and subsequently divided into two subsamples which were adjusted for ionic strength, and NO_3^- and NH_4^+ concentrations were evaluated using selective electrodes.

A low C:N ratio substrate (*Medicago sativa,* C:N=1:12-1:15) was mixed into appx. 400 g ODW sample from each mesocosm at 5 g kg^{-1}, placed in plastic incubation vessels with lids and incubated at 22 °C. Vessels were aerated two times weekly, and after 14 d water loss was evaluated gravimetrically by drying 2 g from each sample at 105 °C and adjusting accordingly. Nutrient mineralization and transformation was were subsequently evaluated beginning day 0, and reoccurring at days 7, 14, and 28, following standardized methods (OECD 216; OECD 217). At each sampling date, C mineralization as CO_2 evolution was evaluated using ~30 g ODW soil from each incubated sample per methods described above. This

sample was subsequently divided into two portions, and NO_3^- and NH_4^+ were extracted and quantified as described above.

For each mesocosm, N mineralization/transformation rates were calculated between the four sampling dates (0-7 d, 7-14 d, 14-28 d) as the amount of nutrient extracted minus that measured the previous date (in the case of day 0, initial concentrations), divided by days elapsed. This allows standardization accounting for any initial differences in the nutrient status of each sample. Also, a global rate was calculated for each plot by linearly regressing measured nutrient concentrations with days elapsed, and the slope of the regression was taken as the global mineralization rate.

2.3. Fauna and crop endpoints

Feeding activity of mesofauna was evaluated using the bait lamina method (Filzek et al. 2004) at 8 mo. (Autumn) and 14 mo. (Spring) following experiment start. In this test, each bait lamina is equipped with 16 holes spaced 0.5 cm apart to 8 cm, the holes are filled with a substrate of wheat flour 25% + activated carbon 5% + cellulose 70%, and the laminae are inserted into the soil so that the first hole is at 0.5 cm depth. Nine bait laminae were arranged within each plot in a 3 x 3 grid with 10 cm spacing. Total feeding activity for 0.5-8 cm depth was calculated as the percentage of total punctured holes for each plot, and feeding activity was also calculated at depth intervals of 0.5-3 cm, 3.5-5.5 cm, and 6-8 cm as the proportion of holes punctured within those intervals. In Autumn, bait lamina were exposed for 35 days due to very low feeding activity, while in Spring bait laminae were exposed for 12 days, at which point approximately half of the lamina holes were punctured (bait eaten) (checked weekly in extra laminae in each plot).

Barley emerged seedling number, and later plant height and flag leaf length, were evaluated at approximately 1 and 2 mo. following the seeding date, respectively. During the 2012 growing season, these sampling dates

corresponded to the late seedling growth stage / beginning of the tillering stage, and the opening of the flag leaf vein (stage 4.7) (Zadocks et al. 1974). Emerged seedling number was evaluated as a complete census of emerged tillers within each mesocosm. Plant height was considered as the length from the base of the plant to the bottom of the flag leaf inflection, and length of the flag leaf inflection was evaluated on the same plant; for these measurements, the 15 most-central plants within each mesocosm were used to avoid any edge effect. Above-ground barley biomass (primary productivity) of each plot was harvested at approx. 3 mo. following seeding, dried at 70 °C 48 h, and weighed.

Analysis of effects due to biochar treatment in feeding rates and barley parameters were by Mann-Whitney pairwise contrasts.

3. Results

3.1 Microbial responses

BR, MB_c, and qCO_2 results over the year are shown in **Figure 1**. Two-way repeated-measures ANOVA results are shown in **Table 1**. For BR, the ANOVA detected significant changes due to date, but none due to treatment; however, at the first sampling date BR of B12 was significantly lower than the control (p=0.034, W=31). MB_c was significantly increased by biochar treatment and changed with date, as indicted by the ANOVA. As seen in **Figure 1**, MB_c means in biochar-amended plots were higher at most dates, though these did not result significant in contrasts. Finally, as evaluated by ANOVA qCO_2 changed with date but was not affected by treatment, though at the first sampling date qCO2 of B50 was lower than the control (p=0.028, W=27).

Concentrations of NH_4^+, NO_3^-, and evolved CO_2 measured in mineralization tests carried out in samples from 6 and 12 mo.-incubated soils are shown in **Figure 2**, and mineralization rates are shown in **Table 2**.

As seen in **Figure 2**, the amount of measured NH_4^+ at 0 and 7 d at 6 mo. was higher than at 12 mo. (t-test $p<0.001$), possibly due to the different statuses of the soil (Spring vs. Autumn sampling). Global NH_4^+ mineralization rate at both 6 and 12 mo. was negative, with no significant difference due to biochar (**Table 2**). However, at 12 mo. significant differences due to treatment were detected over 0-7 d, where NH_4^+ mineralization was higher in B50 compared to B0, but then decreased faster compared to the control over 7-14 d, and finally over 14-28 d mineralization continued to decrease faster in B50 compared to B12.

At 6 mo. the global NO_3^- mineralization rate was decreased by biochar (**Table 2**), but not at 12 mo. At 6 mo., between 14-28 d NO_3^- mineralization was significantly reduced in

Figure 1: Field microbiological parameters assessed over four sampling dates (x-axis) in the study.

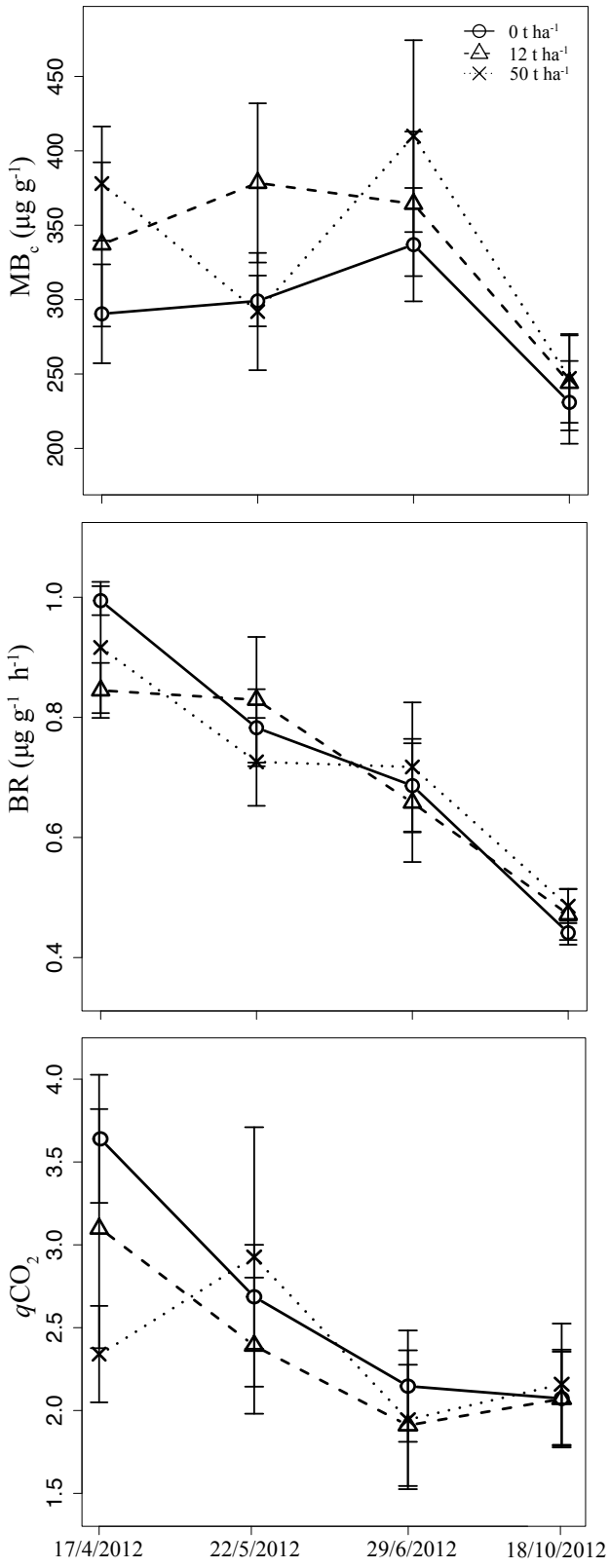


Table 1: Two-way repeated measures ANOVA table for soil microorganism parameters measured in the study.

Parameter	Model parameters	df	SS	F	p
Basal respiration (BR)	Treatment	1	0.07	2.6	0.113
	Date	3	1.94	21.1	<0.001
	Treatment*date	3	0.03	0.3	0.784
	Error	62	1.9		
Microbial biomass (MB_c)	Treatment	1	88455	9.6	0.003
	Date	3	161418	5.8	0.001
	Treatment*date	3	24791	0.9	0.447
	Error	60	551331		
Metabolic coefficient (qCO_2)	Treatment	1	2.3	2.1	0.154
	Date	3	12.8	3.9	0.013
	Treatment*date	3	4.2	1.3	0.290
	Error	60	65.8		

Figure 2: Concentrations of nutrients measured over 0-28 days (x-axis) in laboratory nutrient mineralization and transformation tests at the two sampling dates.

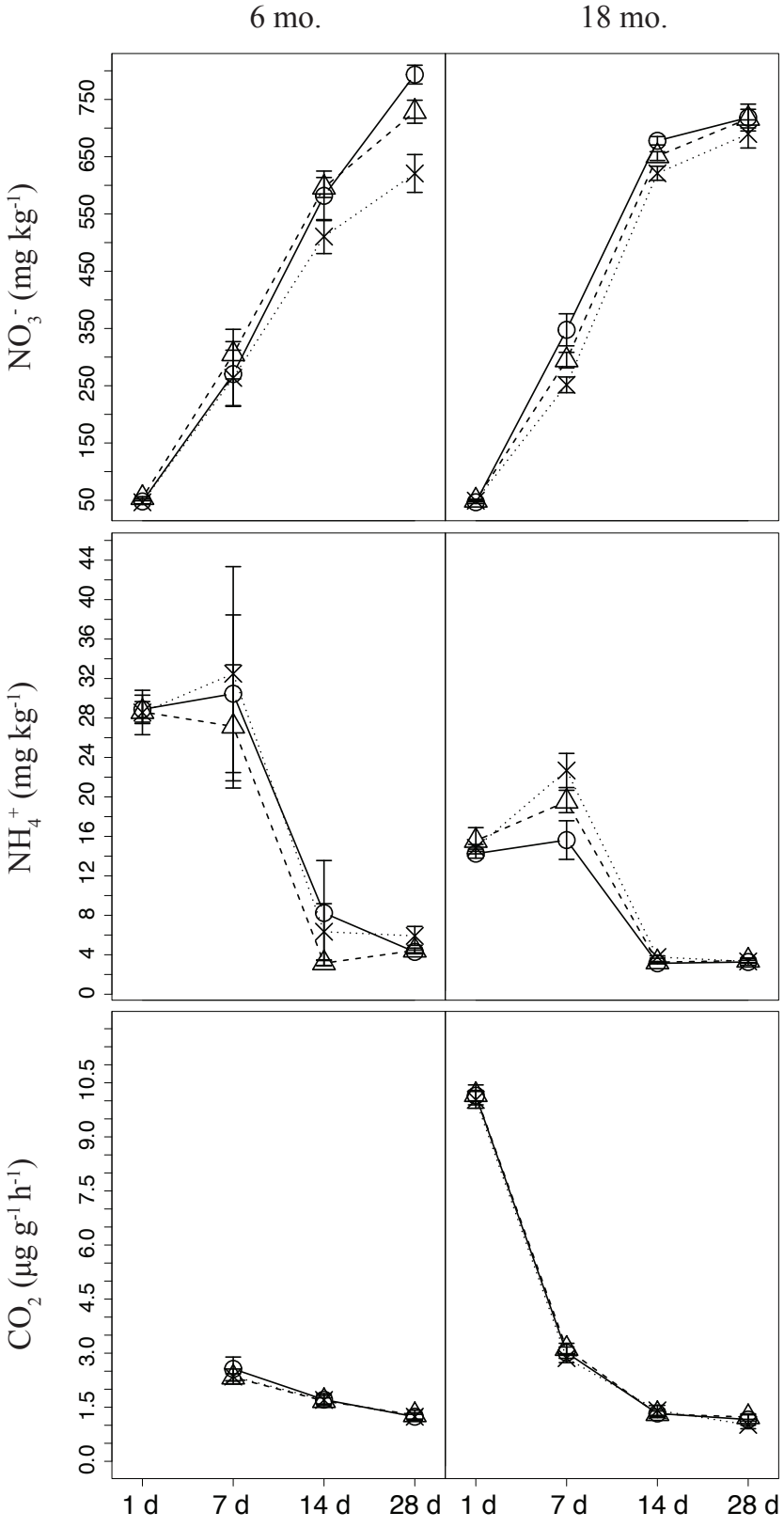

6 mo.
18 mo.
NO_3^- (mg kg^{-1})
NH_4^+ (mg kg^{-1})
CO_2 (µg g^{-1} h^{-1})
50
150
250
350
450
550
650
750
0
4
8
12
16
20
24
28
32
36
40
44
0.0
1.5
3.0
4.5
6.0
7.5
9.0
10.5
1 d
7 d
14 d
28 d

Table 2: Nutrient mineralization and transformation rates over the periods indicated. Within a row for each nutrient, different letters indicate a statistically significant difference.

		Water-extractable NH_4^+ (mg kg^{-1} day^{-1})			Water-extractable NO_3^- (mg kg^{-1} day^{-1})			CO_2 evolution (μg g^{-1} h^{-1} day^{-1})		
Sampling date		0 t ha^{-1}	12 t ha^{-1}	50 t ha^{-1}	0 t ha^{-1}	12 t ha^{-1}	50 t ha^{-1}	0 t ha^{-1}	12 t ha^{-1}	50 t ha^{-1}
6 months	0-7 days	0.226 a	-0.211 a	0.561 a	31.8 a	35.9 a	31.0 a	-	-	-
	7-14 days	-3.175 a	-3.430 a	-3.736 a	44.5 a	41.6 a	35.2 a	-	-	-
	14-28 days	-0.282 a	0.092 a	-0.030 a	15.1 a	9.4 ab	7.9 b	-	-	-
	0-28 days	-1.007 a	-0.974 a	-0.971 a	26.8 a	23.9 b	20.3 c	-0.06 a	-0.05 a	-0.05 a
12 months	0-7 days	0.199 a	0.573 ab	1.130 b	41.9 a	34.1 b	28.2 c	-	-	-
	7-14 days	-1.800 a	-2.346 ab	-2.723 b	46.0 a	49.6 a	51.4 a	-	-	-
	14-28 days	0.010 ab	0.012 a	-0.032 b	2.8 a	4.6 a	4.8 a	-	-	-
	0-28 days	-0.469 a	-0.549 a	-0.569 a	23.0 a	23.3 a	22.8 a	-0.27 a	-0.27 a	-0.27 a

B50 with respect to B0 (**Table 2**), and at 12 mo. over 0-7 d NO_3^- mineralization rate was decreased by biochar (**Table 2**).

For C mineralization, the 0 d point is missing for 6 mo. Globally, no differences were detected in mineralization rates at 6 or 12 mo. (**Table 2**).

3.3 Fauna and crop responses

Fauna activity as evaluated by bait laminae is shown in **Figure 3**. No significant differences in activity due to biochar treatment were detected for the Autumn sampling, either overall (0-8 cm) or at any of the depth intervals. On the other hand, at the Spring sampling B50 showed 7.43 ± 4.2 % overall reduced feeding activity with respect to B0 (Mann-Whitney p=0.012, W=32). Also, at specific depth intervals, at 3.5-5.5 cm activity in B50 was lower than both B0 and B12, and at 6-8 cm activity at B50 was lower than B0.

Emerged seedling averages for B0, B12, and B50 were 263 ± 5.0, 265 ± 3.1, and 279 ± 8.8 seedlings per plot (**Figure 4**). The global test resulted non-significant (Kruskal-Wallis p=0.12, chi-squared=4.16, df=2), but the higher emerged seedling number in B50 compared to B12 was marginally significant (Mann-Whitney p=0.054, W=6).

Barley height averages for B0, B12, and B50 were 38.4 ± 0.8, 40.1 ± 0.8, and 36.6 ± 0.6 cm (**Figure 4**). Global differences were detected (Kruskal-Wallis p<0.001, chi-squared=19.40, df=2), as well as in the pairwise contrasts (B0-B12 Mann-Whitney p=0.055, W=3380; B0-B50 p=0.005, W=5023; B12-B50 p<0.001, W=5519), demonstrating that height was greatest at B12 and lowest in B50.

Flag leaf length (**Figure 4**) averages were 4.1 ± 0.1, 4.0 ± 0.1, and 3.6 ± 0.1 cm, respectively, and again significant differences were found (Kruskal-Wallis p=0.026, chi-squared=7.28, df=2). There was no significant

difference between flag leaf lengths of B0 and B12 (Mann-Whitney p=0.85, W=4113), though B50 was lower than both B0 (p=0.012, W=4918) and B12 (p=0.032, W=4791).

Barley primary productivity means for B0, B12, and B50 were 420 ± 19.7, 461 ± 41.7, and 374 ± 13.2 g m^{-2}, respectively (**Figure 4**). Significantly higher biomass yield was found in B12 compared to B50 (Mann-Whitney W=30, p=0.027), but there were no differences between B0 and B12/B50.

4. Discussion

4.1 Field microbial response to biochar addition

In our study microbial abundance (MB_c) was increased by biochar treatment, which follows with expectations expressed elsewhere (Pietikäinen et al. 2000; Thies and Rillig 2009, Warnock et al. 2007, Lehmann et al. 2011), and agrees with some field studies that have shown increased microbial abundance in biochar-added plots (Lehmann et al. 2011), such as reported by Jin (2010) after 6 months of a cornstover biochar addition to a corn crop, and also by Jones et al. (2012), whom in the second year after biochar application reported that microbial biomass and fungal growth was increased, although this effect was transient as disappeared in the third year.

BR and qCO_2 were unaffected by biochar, with the exception of the first sampling date where lower BR, coupled with overall higher average biomass, caused a lower qCO_2 of biochar-amended soils at this date. This indicates decreased activity (Nannipieri et al. 2003) and enhanced C-use efficiency (Anderson and Domsch 1990; Gomez and Correa 2008), respectively. Others have found reduced respiration rather than increased microbial biomass causing reductions in qCO_2 in laboratory soil-biochar incubations (Paz-Ferreiro et al. 2012; Jin 2010). In contrast, Jones et al. (2012),

reported increases in microbial biomass coupled with increased respiration two years after the application of a wood biochar. qCO_2 is an ecophysiological indicator whose decreases have been interpreted as improved soil quality (Franchini et al. 2007; Jinbo et al. 2007), due to decreased energy spending of the community (Anderson 1994), potentially explained by improvement of the soil environment as a habitat for soil microorganisms, and may be enabled by, for instance, increased soil moisture (**Chapter 4.1**) or increased availability of certain nutrients with biochar (**Chapter 4.2**). However, microbial community shifts might be also an explanation for the enhanced C-use efficiency (Anderson and Domsch 1990), although we lack data to support this statement. For instance, Jin (2010) reported a shift in fungal communities after

Figure 3: Fauna feeding activity as evaluated by bait laminae for biochar treatments (x-axis) at two sampling dates, by depth interval. Within each depth interval and sampling date considered, different letters indicate statistically significant differences.

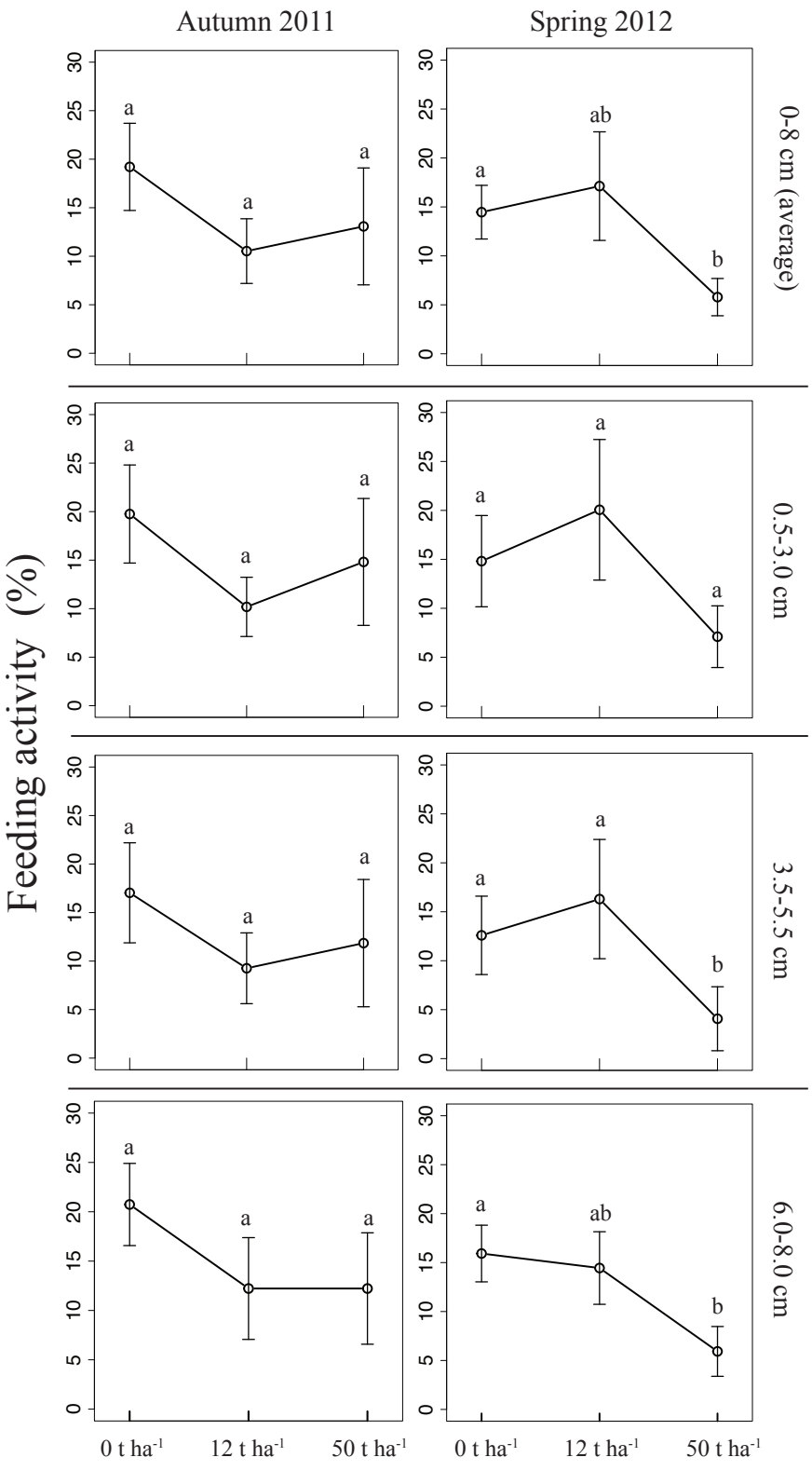
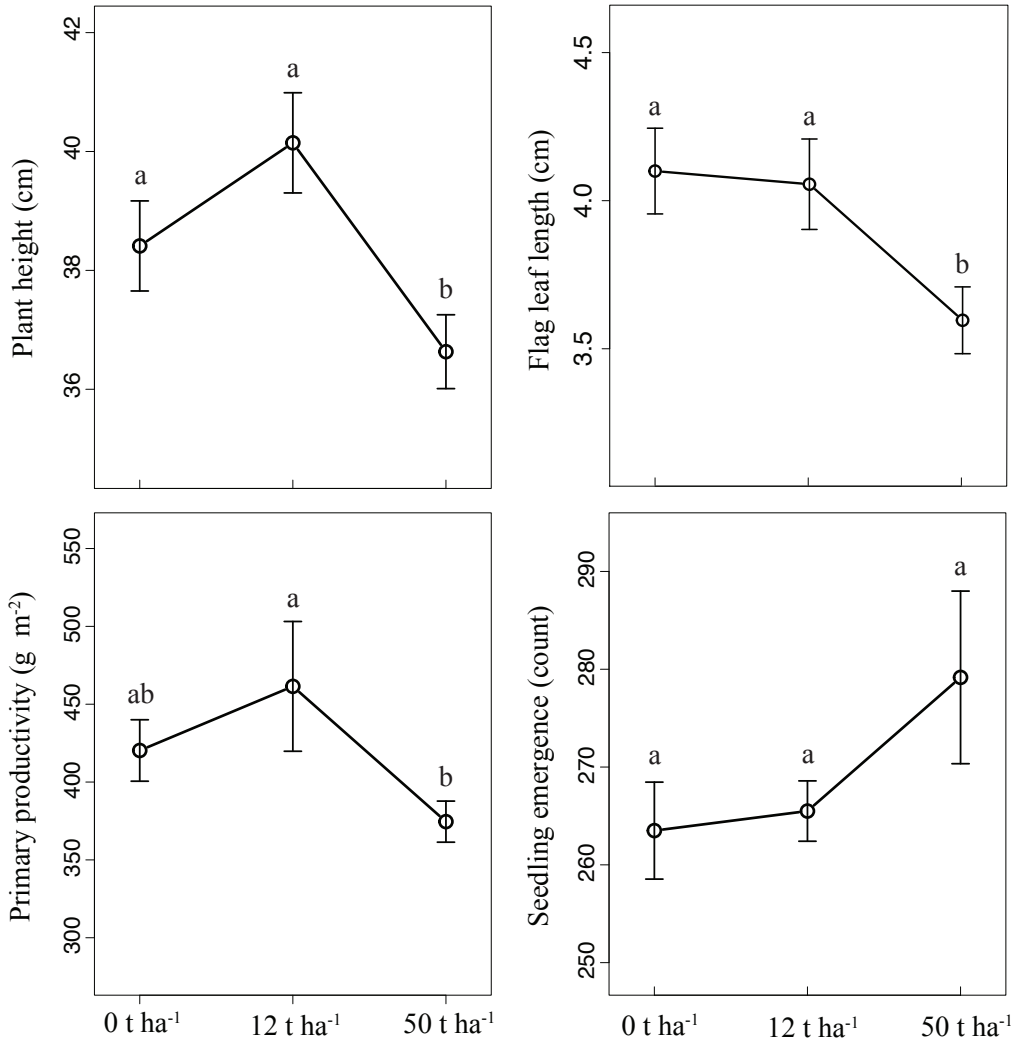

Autumn 2011
Spring 2012
Feeding activity (%)
0-8 cm (average)
0.5-3.0 cm
3.5-5.5 cm
6.0-8.0 cm
0 t ha-1
12 t ha-1
50 t ha-1

Figure 4: Responses of barley crop parameters in each biochar treatment (x-axis). Different letters indicate statistically significant differences.



4 weeks of incubation of a cornstover biochar, suggesting that freshly-added biochar may have favored the proliferation of fungal families more capable of using some biochar fractions. Jin (2010) also reported a shift to increased fungal diversification over that of bacteria in biochar-amended plots which they proposed might have been due to the observed increase in soil pH, although this pH effect is unlikely to occur in our already alkaline soil, and was not detected in **Chapter 4.2**. In a medium-term field study, Jones et al. (2012) found that biochar addition favored bacteria over fungi in the second year, but not in the third, and was thereby considered a transient effect.

4.2 Biochar effects on C and N mineralization

As mentioned above, nutrient mineralization is an indispensable soil function, and is often used to test for potential inhibitory effects of contaminants or pesticides (van Beelen and Doelman 1997; Engelen et al. 1998). C-mineralization as CO_2 decreased along the 28 d incubation period, explaining the overall negative rates (**Table 1**), and can be interpreted as the progressive exhaustion of the more labile fraction of the organic matter added with litter. However, no effect of biochar treatments was observed. A similar study reported decreased 21 d C-mineralization of ^{14}C-labelled ryegrass litter incubated in field soil samples collected from 50 t biochar ha^{-1} plots applied 2-3 years previous (Jones et al. 2012). We assume that in our experiment most of the mineralized C measured as CO_2 originated from the added litter, although emissions from native soil organic matter or biochar itself cannot be excluded, something that we cannot validate due to the use of a non-labeled litter substrate. The general lack of effects of biochar addition rate on C-mineralization in the laboratory is in agreement with generally no effects on BR in the field, though these are not altogether comparable.

Regarding biochar's effects on N-transformation products, several explanations have been provided, some of the most prominent being (i) N retention as ammonium (NH_4^+) in soil (Laird et al. 2010); (ii) denitrification reductions (Rondon et al. 2006; Yanai et al. 2007; Van Zwieten et al. 2009) or increases in denitrification enzyme activity (Jones et al. 2012); (ii) increased NO_3^- leaching due to the higher hydraulic conductivity in biochar-amended soils and its low or lack of anion retention capacity (Cheng et al. 2008; Mukherjee et al. 2011), (iii) increased plant uptake (Clough et al. 2013); (iv) impacts on microbial functional groups important for the N-cycle such as nitrifiers (Thies and Rillig 2009) and N_2 fixers (Ogawa and Okimori 2010); (v) lower concentrations of oxygen in micropores which normally facilitates nitrogenase activity (Thies and Rillig 2009); (vi) increased N immobilization in microbial biomass due to the availability of labile carbon in freshly added biochars (Bruun et al. 2012; Deenik et al. 2010; Novak et al. 2010; Laird et al. 2010), and; (vii) in forest soils, the possible reduced inhibition of nitrifiers by phenolic substances due to their sorption in biochar (Lehmann et al. 2011).

Overall, NO_3^- mineralization rates of field-incubated samples, evaluated in the laboratory, were significantly reduced by biochar (**Table 1**), while overall NH_4^+ mineralization rates were globally unaffected, although increased by biochar during the first days of the incubation period. The initial increased concentrations of NH_4^+ were not reflected later in higher concentrations of NO_3^-; in fact, NO_3^- concentrations remained lower in biochar-amended soils during the whole 28 d test. This result suggests a possible blocking of nitrification and and/or increased losses of NH_4^+ as NH_3, which is plausible in the alkaline test soil (Mills et al. 1974). For instance, others have hypothesized that biochar can lead to higher aeration of soil, and therefore facilitate higher rates of volatilization of N species (Case et al. 2012). Also, it might reflect increased N incorporation into microbial biomass, something we cannot confirm as it was not measured in this test.

This short-term result may partly explain reduced NO_3^- concentrations in the field, as well (**Chapter 4.2**). In both cases, we lack of evidences in favor of any of the mechanisms previously mentioned, but we can at least discount plant uptake or leaching due to the confined nature of the laboratory mineralization tests. Other studies have reported similar effects, (DeLuca et al. 2006, Novak et al. 2010; Dempster et al. 2012a), no effects (Dempster et al. 2012b), or increases with biochar (Gundale and DeLuca 2006; Laird et al. 2010) or activated carbon (Berglund et al. 2004).

4.3 Soil fauna activity

The bait lamina method mainly reflects mesofauna feeding activities, such as that of collembolans and enchytraeids (Helling et al. 1998; Gongalsky et al. 2008), but also of macrofauna taxa such as earthworms (Van Gestel et al. 2003; Förster et al. 2004; Hamel et al. 2007; Gongalsky et al. 2008).

Feeding activity was significantly impaired in B50 in the Spring, but not in Autumn. When assessed at different depths, trends were similar. This finding is in line with our previous study in **Chapter 2**, where we found that a very similar biochar inhibited collembolans and enchytraids at similar or lower application rates (**Chapter 2, 3**). Thus we conclude that at some concentration between 12 and 50 t ha^{-1}, this biochar becomes detrimental to soil mesofauna in the field, and that the effect is not transitory in the short term since it was detected 1 year after biochar application.

As of yet we have not identified with confidence any possible mechanism(s) explaining inhibition of invertebrates in this experiment. It may be related to alkalizing effects such as discussed in **Chapter 2**, notwithstanding that we detected no biochar-induced pH changes in the field (**Chapter 4.2**). pH increases, hypothesized elsewhere to potentially positively affect soil fauna due the amelioration in some soil properties such

as pH (McCormack et al. 2013), was proposed in reference to acidic soils, and may not apply to alkaline soils like the one in our study.

Trophic changes might explain decreased fauna activity, but the decreased feeding activity in B50 was not coupled with decreases in total microbial abundance, if not the contrary, although this could also be consistent with a community shift. As an example, biochar addition has been linked to changes in the bacteria-to-fungi ratio (Jin 2010; Jones et al. 2012), favoring either fungivore or microbivore fauna, with important consequences for the soil mesofauna community directly or indirectly relying on soil microorganisms (Bardgett 2005).

Finally, the release of noxious chemicals such as PAHs or the enhanced retention of NH_4^+, toxic to soil fauna, might be explanation (McCormack et al. 2013), but our data do not support the latter.

Whatever be the underlying mechanism(s), our results demonstrate a reduction in fauna activities, which is of concern within the framework of environmental risk assessment, and may potentially translate to effects on other biologically-mediated processes such as decomposition and nutrient cycling.

4.4 Vegetation responses to biochar addition

Seedling emergence was enhanced by 6% in B50 (only marginally significant), contrasting with the lack of effect on this endpoint in similar field studies, such as that of Jones et al. (2012), who found no effects on maize germination in the same range of biochar addition rates. We can also discard any negative effects due to salinity, which was not affected by biochar addition (**Chapter 4.2**). Although we lack direct evidence, increased seedling emergence at 50 t ha^{-1} biochar could be related to either (i) increased soil moisture or (ii) increased soil temperature, since these are known to importantly influence germination in this species and other grains (Bishop 1944; Ellis and Roberts 1980; Hampson and Simpson 1990). As described in **Chapter 4.1**, field moisture was higher in biochar-amended

soils, so this explanation seems likely. Additionally, recent work by others has shown that biochar has significantly reduced surface albedo in a field trial, causing an increase in soil temperatures, and since this effect was strongest early in the growing season it was suggested that this may positively impact seedling emergence (Genesio et al. 2012).

Barley height was marginally improved by the B12 treatment, but significantly inhibited by the B50 treatment, but these did not translate to statistically-significant effects on primary productivity, since there were no significant differences between treatments and control, though notably, B12 biomass was higher than B50, despite a greater number of emerged seedlings in the latter.

In the previous chapters we have identified changes in soil quality parameters which should benefit the crop, most notably increased field moisture (**Chapter 4.1**), and increased available K^+ (**Chapter 4.2**). At the same time however, increasing biochar addition rate led to potential impediments to crop growth, evidenced in B50, such as reduced availability of NO_3^--N in the field during a crucial moment of growth (**Chapter 4.2**), or potential immobilization of P, as suggested in **Chapter 1**. However, with the available data we cannot determine with certainty which nutrients may have been limiting, which would necessarily require plant uptake and/or soil analyses which take into account all plant-available fractions.

Our results demonstrate the importance of testing biochar at multiple application rates, and more results such as these are needed within the field to inform recommendations for appropriate application rates, which are currently lacking (Steiner 2007, Hossain et al. 2010; Gaskin et al. 2010).

5. Conclusions

A wood gasification biochar added to a Mediterranean alkaline soil at 0, 12 and 50 t ha^{-1} increased microbial abundance. Globally, no effects on microbial activity and C-use efficiency were found, though activity was found to be reduced at one of the samplings, resulting in a significantly increased microbial C-use efficiency at the same moment; these effects were detected at the field scale and at the medium–term (from one year to 18 months following application). In laboratory tests with field-incubated soils, over 0-28 days no changes in the C mineralization or N mineralization as NH_4^+ were observed, but concentrations of NO_3^- were reduced with increasing biochar addition rate.

Biochar did not significantly affect barley biomass. However, at 12 t ha^{-1} biochar, barley plant height was marginally improved, and at 50 t ha^{-1} barley seedling emergence rate was marginally improved, while barley plant height and flag leaf length were inhibited, as well as fauna feeding activity.

With few biochar studies available in Mediterranean environments, more research will be required in the future to improve the scientific basis for biochar applications under these conditions, both of potential agronomical benefits and risks to soil quality.

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General Discussion

Chapters 1-4 have considered the effect of seven different biochar materials on physical (**Chapter 4.1**), chemical (**Chapters 1, 2, 4.2**), and biological (**Chapters 1-3**, **4.3**) soil quality parameters. Biological methods, the emphasis of the thesis, are useful in biochar materials screening and characterization since biological responses can reflect the sum of all intervening changes in the soil environment, whereas biochar impacts physical, chemical, and biological compartments. As previously indicated, systematic biological testing of biochar materials has been lacking, and one of the main objectives of this thesis has been to build basic knowledge in that respect, mainly exposed in **Chapters 1-3**. In addition, little information is available on biochar's effects on soil properties in Mediterranean environments or alkaline soils, so a field study was initiated to investigate medium-term effects under those conditions, considering basic soil physical and chemical parameters as well. Here, the results of these two levels of study, laboratory and field, are summarized, and further lines of research are proposed.

Soil bioassay suitability for the characterization of biochars

What can be concluded about which biochars are "good" or "bad" for soil biota? As can be expected, this manner of framing the topic is overly simplistic, since this not only depends on the nature of biochar itself, but also on application rate, the type of soil used, or any other particularity such as climate and soil management. Bioassays (**Chapters 1-3**) pose short-term, necessarily reductionist and unrealistic conditions to reduce the influence of non-treatment effects, and as with any methodology, there are limitations to their interpretation, as the results are valid only for the system considered. For instance, different or evolving effects may be seen in the field due

to material weathering, as it is well understood that biochar chemistry is decidedly altered with leaching (Chapters 1), incubation, and oxidation, which can occur in relatively short periods (Cheng et al. 2006). Nevertheless, the information garnered from bioassays can be used as a tool to (i) allow the relative comparison of biochars, with the aim to determine the best feedstocks and pyrolysis procedures, (ii) provide an initial basis for regulatory limits to agricultural applications of the product by the derivation of maximum addition rates taking the information from different test organisms and soils, and (ii) suggest the mechanisms underlying the effects observed and can help improve the understanding of potential effects observed in the field. Here, both positive and negative effects on numerous taxa were found, so following the precautionary principle, unintended consequences of biochar application are plausible. The classical individual-level endpoints for plants (germination and growth) and fauna (survival and reproduction), but also a new fauna endpoint at the population–level was assessed, providing a more refined information that allowed a better classification of chars.

Key biochar properties explaining positive and negative reponses in bioassays

With the above in mind, some general conclusions can be made based on the results of Chapters 1-3. **Pyrolysis/production method** has a large influence on biological effects observed. Biochar physicochemical properties are dependent on production method as discussed in the introduction to this thesis, but among other characteristics, this determines degree of carbonization, volatile matter, and pH via ash and carbonate contents; the evidences presented herein have indicated that these are crucial to understanding biological responses in the alkaline soils used in this thesis. Specifically, biochars with high **volatile matter** may be

inhibitory to plant growth in the short term, likely via an *indirect* effect on N availability due to competition with microbial biomass, which has been suggested elsewhere (Deenik et al. 2010). Also, large biochar-mediated increments in soil **pH** or alkalizing effects can be *directly* inhibitory for invertebrates and *indirectly* inhibitory for plants, whereas for plants the mechanism of reduced P availability was proposed.

Despite expectations and evidences that organism responses to biochar will vary depending on their particular physiology, some biochar materials provoked very similar responses in very different taxa; namely, these were the **sewage sludge char,** which was stimulatory, and the gasification pine wood char which was inhibitory. In the case of the sewage sludge char, this is probably due to its high N and P contents, increasing plant nutrient availability, but the mechanism explaining fauna stimulation remained unexplained. The **pine gasification char**, on the other hand, was highly inhibitory in the short term for both plants and animals, likely due to increases in soil pH via alkalizing biochar elements such as carbonate content which directly and indirectly impacted on these taxa, respectively, as already indicated.

Fast-pyrolysis materials, while stimulatory or provoking no response for invertebrates, were inhibitory for plants probably due to high volatile matter contents stimulating microorganisms and thereby immobilizing N in the short term, as mentioned above. Weathering and oxidation of the labile C components of the fast pyrolysis materials is likely to reduce negative effects on plants, though this was not tested in the present work.

Slow pyrolysis materials are the least nocive, and may even provoke stimulatory responses for both plants and animals. We would suggest that this is in part due to the pyrolysis method which effectuates a high degree of carbonization and fixed C due to the long residence times in production, producing low content of volatile matter, carbonates, and ash. Additionally, with particular interest for

alkaline soils, slow pyrolysis chars even had the effect of reducing soil pH under laboratory conditions, an effect which to our knowledge has not been widely documented for a biochar, and this effect may have practical applications.

Joseph et al. (2009) discussed biochar classification methods. Within criteria for categorizing and defining the value of charcoal and other fuels, fixed C, volatile C, and ash content are prominent. While these criteria were developed to establish fuel qualities, Joseph et al. (2009) suggested that they may be suitable for predicting biochar stability in soils as well. Interestingly, though perhaps not surprisingly, we found that these criteria also describe important parameters for biological effects, as well.

Effects on soil quality following the addition of a pine gasification char to an alkaline agricultural soil

Results of measured soil quality parameters in the field study are summarized in **Table 1.** Within the physical compartment, it is clearly seen that biochar addition promoted conditions more favorable to crop growth. This largely followed expectations, as the literature has generally predicted positive effects of biochar on soil physical properties. Also, we found indications that aggregation might have been enhanced in the 1.5-year period following biochar application, although this deserves more detailed and longer-term study.

Chemical effects, on the other hand were varied. Biochar effects on soil pH, widely discussed in the literature, were not detected in this study, likely due to the already high soil pH. Perhaps the most notable positive effect was increased soluble and potentially plant-available K, and the most important negative effect on the crop was decreased field NO_3^- concentrations. Biochar also reduced NO_3^- transformation rates in laboratory mineralization assays. Despite potential negative consequences for crop growth, elsewhere there is interest in taking

advantage of precisely this effect to reduce environmental risks following applications of inorganic fertilizer, namely groundwater pollution by nitrates (Venterea et al. 2013).

In contrast to many parameters in the physical and chemical compartments, some biological effects were non-linear or non-additive with increasing biochar application rate. These included marginally-stimulatory effects on some barley crop parameters at 12 t ha^{-1} and negative effects on others at 50 t ha^{-1}. This was a crucial observation, as it helps define a limit or threshold for appropriate biochar application rates, which are not well described or defined within the literature at this moment. The enhanced barley seedling emergence of the 50 t ha^{-1} treatment, while interesting, did not translate to larger yields within that treatment, as the inhibition of growth was apparently more important. Also, mesofauna activity was reduced at 50 t ha^{-1}, but not at 12 t ha^{-1}, indicating that the high application rate may present risks to the soil ecosystem as whole and their functions. By contrast, microorganism responses were globally unaffected in the assessed perio in terms of activity or efficiency, although a significant enhancement of microbial abundance with biochar. s.

Future research

Some important effects of biochar on soil biota have been identified in this thesis, including both stimulatory and inhibitory responses, and though many mechanisms have been suggested to explain these responses, some supported by ample evidences collected in the elaboration of the thesis, for the most part they remain as hypotheses. Therefore, future lines of research are suggested to elaborate the hypothesized mechanisms.

1. Confirm hypothesis of reduced plant nutrient availability leading to inhibition of plant growth by high volatile matter chars and highly alkalizing chars in alkaline soils

2. Investigate hypothesis of soil fauna inhibition as a direct effect of highly alkalizing chars

3. Identify cause of general strong stimulation of collembolans with biochar addition (except PG)

4. Investigate the impact of biochar-mediated increased K availability and reduced N in alkaline soils

5. Identify the mechanism underlying the reduction in nitrate concentrations in an alkaline soil

6. Investigate the increased microbial abundance as related to shifts in the bacterial and fungal communities

7. Investigate observed inhibition of soil mesofauna in the field

Table 1: Summary of soil quality impacts found in the field study.

Compartment	Soil Quality Impact			
	Positive (+)	Negative (-)	No change (=)	Unclear (?)
Physical	- Increased soil **water holding capacity** - Increased **field moisture** - Decreased **penetration resistance** - Decreased soil **bulk density** - **Aggregation** effects - some evidence that biochar may have increased aggregate size, but deserves more study			
Chemical	- Increased water-soluble **K^+** availability - Increased **soil organic carbon** (**SOC**)	- Water-soluble **NO_3^-** unchanged with the exception of one sampling date, where it was low	- Water-soluble concentrations of **Ca^{2+}, Na^+, NH_4^+, NO_2^-, Mg^+, SO_4^{2-}, HPO_4^{2-}** - **pH** - **Electrical conductivity** (**EC**)	- Increased water-soluble **Cl^-** availability
Biological	- Increased soil **microbial biomass** - Marginally increased **barley height** 12 t ha^{-1} - Marginally increased **seedling emergence** 50 t ha^{-1}	- Barley **primary productivity** in 50 t ha^{-1} reduced with respect to 12 t ha^{-1} - Decreased **barley height** and **flag leaf length** 50 t ha^{-1} - Decreased **NO_3^-** mineralization rate	- Laboratory **C mineralization**	- Lower field **basal respiration**

	- Increased microbial **C-use efficiency**	- Impaired soil **fauna feeding activity**

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Appendix 1 Fourier transform infrared spectroscopy (FTIR) analysis and detailed interpretation of six biochar materials

1. Methods

FTIR spectrum were obtained from milled dry (105 °C) biochar (**Table 1**) samples passed through a 100 μm sieve. Spectrum were registered in triplicate at standard infrared resolution (4 cm^{-1}) in the mid-infrared range of 600-4000 cm^{-1} using a Bruker Tensor 27 spectrophotometer equipped with a Golden Gate single reflection diamond accessory working in attenuated total reflectance (ATR) mode.

Material	Description
CL	*Populus nigra* (Poplar) wood chip biochar produced in a slow pyrolysis reactor 500 – 550 °C
CR	*Populus nigra* wood chip biochar produced in a fast pyrolysis reactor at 430 – 510 °C
FL	Wastewater sludge biochar produced produced in a slow pyrolysis reactor 500 – 550 °C
PG	*Pinus pinaster + P. radiata* wood chip biochar produced in a gasification reactor at 600 – 900 °C
PL	*Pinus pinaster + P. radiata* wood chip biochar produced in a slow pyrolysis reactor 500 – 550 °C
PR	*Pinus pinaster + P. radiata* wood chip biochar produced in a fast pyrolysis reactor at 440 – 480 °C

Table 1: Biochars studied and identification codes.

2. Results

FTIR spectrum of pine, poplar, and sewage sludge biochar samples are shown in **Figures 1A**, **1B**, and **1C**, respectively. As expected, spectra of sewage sludge and wood biochars were highly dissimilar owing to their different origins.

2.1 Sewage sludge char

In the FL FTIR spectrum strongest absorption can be seen at 1015-1050 cm^{-1}, which can be attributed to Si-O- silicate bonds, due to the large, non-metal mineral fraction of this material (>50%). The strong absorption by these silicates obscures other potential adsorption bands in the shorter wavelength end of the spectrum. Absorption at 1410 cm^{-1} can be attributed to aromatic C=C bonds. Moderate absorption was also detected at 1600 cm^{-1}, likely due to C=N or N-H bonds, which can be explained by the high nitrogen content of the sludge feedstock (2.26%). Small absorption peaks in the range of 2800-3000 cm^{-1} can be attributed to aliphatic C-H groups.

2.2 Wood feedstocks

Within the wood feedstocks, spectra were more similar between pyrolysis method than feedstock, whereas the gasification material (PG) was most markedly distinguished from the slow (CL, PL) and fast pyrolysis (CR, PR) materials. Overall, the greatest absorption (displacement on the y-axis) was in the order of gasification > slow pyrolysis > fast pyrolysis, particularly <2000 cm^{-1}, indicating a gradient of carbonization, aromaticity, and condensation of C. This increase of the baseline shift (increased diffusive absorption) is assigned to low-energy electron excitations of condensed aromatic structures (Mochidzuki et al. 2003).

Some H-bonded OH stretching can be seen in the area surrounding 3320 cm^{-1} of the slow and fast pyrolysis materials (Schnitzer and Khan 1978). Common to all wood materials was slight absorption in the range of 2800-3000 cm^{-1} indicating some aliphatic C-H stretching (Schnitzer and Khan 1978). Absorption at 1700 can be attributed to C=0 aromatic carbonyl or carboxyl stretching (Guo et al. 1998; Kloss et al. 2012), which was not significantly influenced by

pyrolysis method, though it was notably strongest in PL. Absorption at 1570 cm^{-1} and 1415 cm^{-1} is likely due to aromatic C=C bonds (Coates 2000; Ozçimen and Ersoy-Meriçboyu 2010; Kloss et al. 2012), whereas the former were most accentuated in the poplar materials, and the latter in the fast pyrolysis materials. Absorption at 1370 cm^{-1} is indicative of aliphatic CH_3 deformation (Guo et al. 1998), and was most pronounced in PG, and while present in the fast and slow pyrolysis materials in these cases it was likely obscured by other absorption bands. Strong peaking in the slow and fast pyrolysis materials around 1200 cm^{-1} may be indicative of C-O phenol stretching (Coates 2000), and small peaks at 1160 cm^{-1} may indicate aromatic CO- stretching (Ozçimen and Ersoy-Meriçboyu 2010), both of which were reduced in PG. Slight absorption was found at 950 cm^{-1} (PG; not identified in Figure) and 1115 cm^{-1} (others) likely due to Si-O-Si silicate oxides (Chia et al. 2012), strongest in the fast-pyrolysis materials due to sand grains used in their production. Absorption at 1030-1050 cm^{-1} is indicative of stretching of aliphatic ether C-O-, primary alcohol C-O, or carbohydrate C-O (Coates 2000; Schnitzer et al. 2007; Chia et al. 2012), which were strongest in fast pyrolysis materials, slight in slow pyrolysis materials, and nearly absent in the gasification char. Finally, peaks in the range of 750-870 cm^{-1} are likely due to aromatic C-H out-of-plane bending (Guo et al. 1998; Coates 2000; Chia et al. 2012), which was strongest in the order of slow-pyrolysis > fast pyrolysis > gasification, whereas the pine feedstocks had more aromatic groups of this type than the poplar feedstocks. Other authors have attributed bands at 1436 cm^{-1} and 875 cm^{-1} in biochars to carbonates (Kloss et al. 2012).

3. Discussion

All biochars showed very limited content of aliphatic groups, whose presence is usually strongest at longer wavelengths (C-H

stretching 2800-3000 cm^{-1}, O-H stretching at 3300 cm^{-1}). As these groups are easily lost during pyrolysis, they were most reduced in the high-temperature gasification char (PG). In the range of 1030-1400 cm^{-1}, small peaks of aliphatic methyl and ether/alcohol C-O- and C-O bonds were also evident, though the importance of these was obscured by the strong absorption by aromatic C=C and C=O.

Overall, the greatest diversity of functional groups is seen in the fast-pyrolysis materials, which were also the least aromatic, evidenced by the lower overall degree of absorption (displacement on the y-axis). On the other extreme, the high temperature gasification method produced very reduced abundance of functional groups, and whose structural form likely resembles matrices of polycyclic aromatic hydrocarbons with peripheral functional groups. Finally, the low absorption of FL spectra demonstrates its limited organic content, though some aromatic C=C bonding was evident.

Figure 1A: FTIR spectra of pine feedstock biochars. PG=gasification, PL=slow, PR=fast.

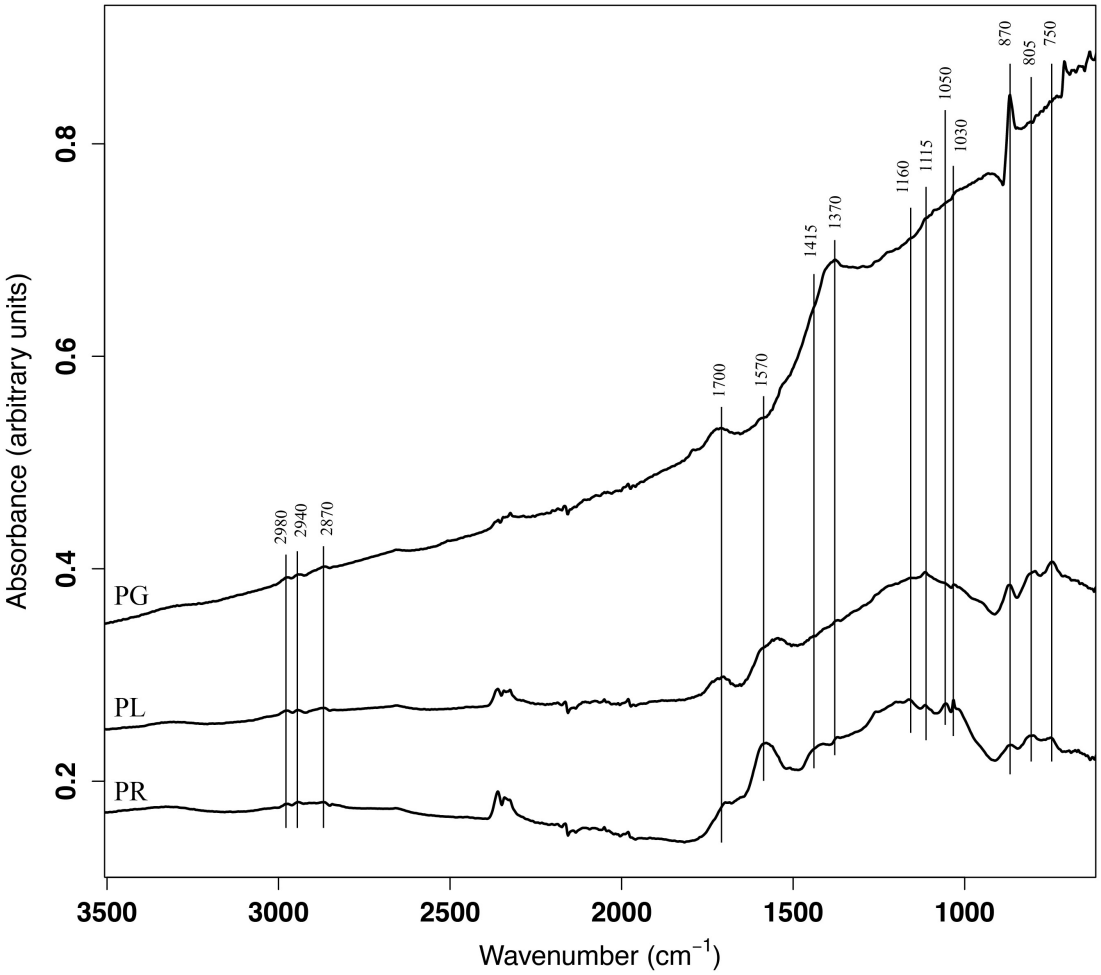


Figure 1B: FTIR spectra of poplar feedstock biochars. CL=slow, CR=fast.

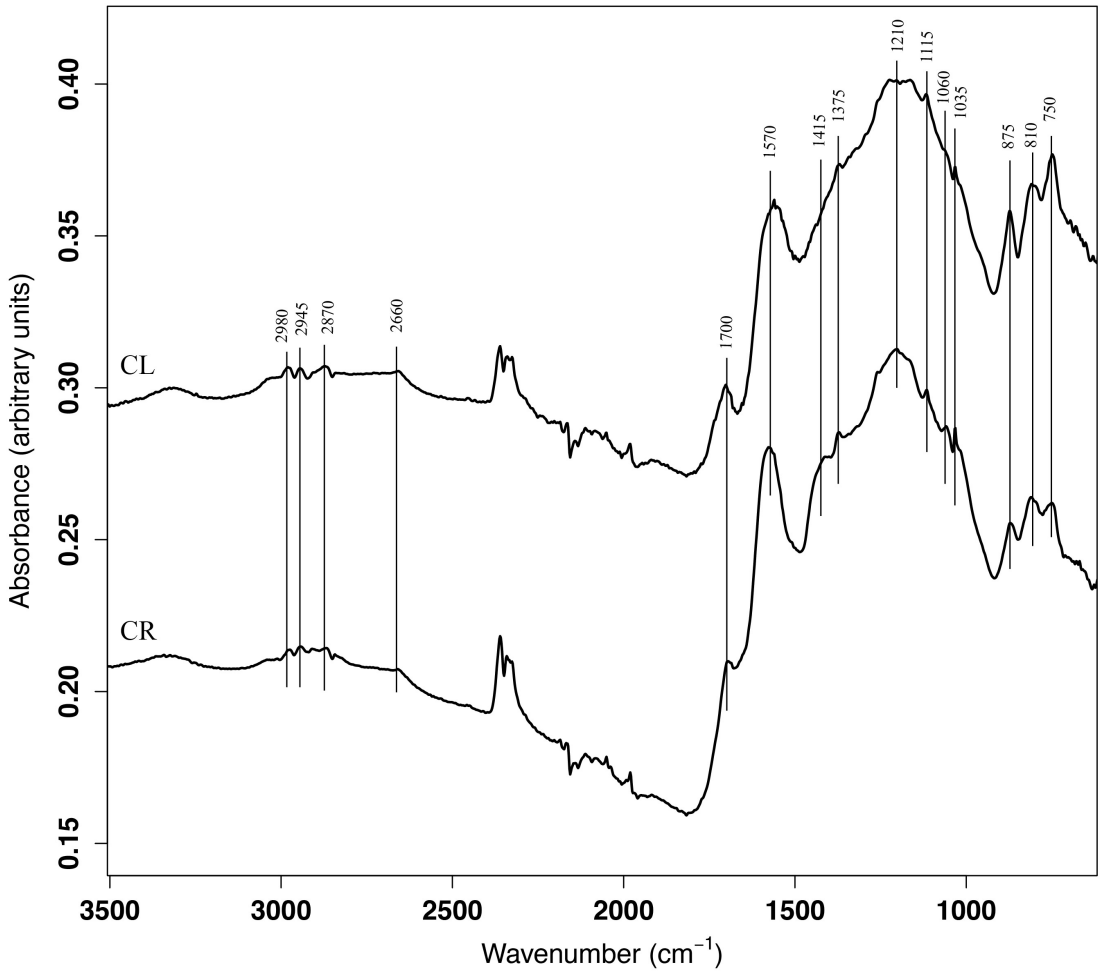
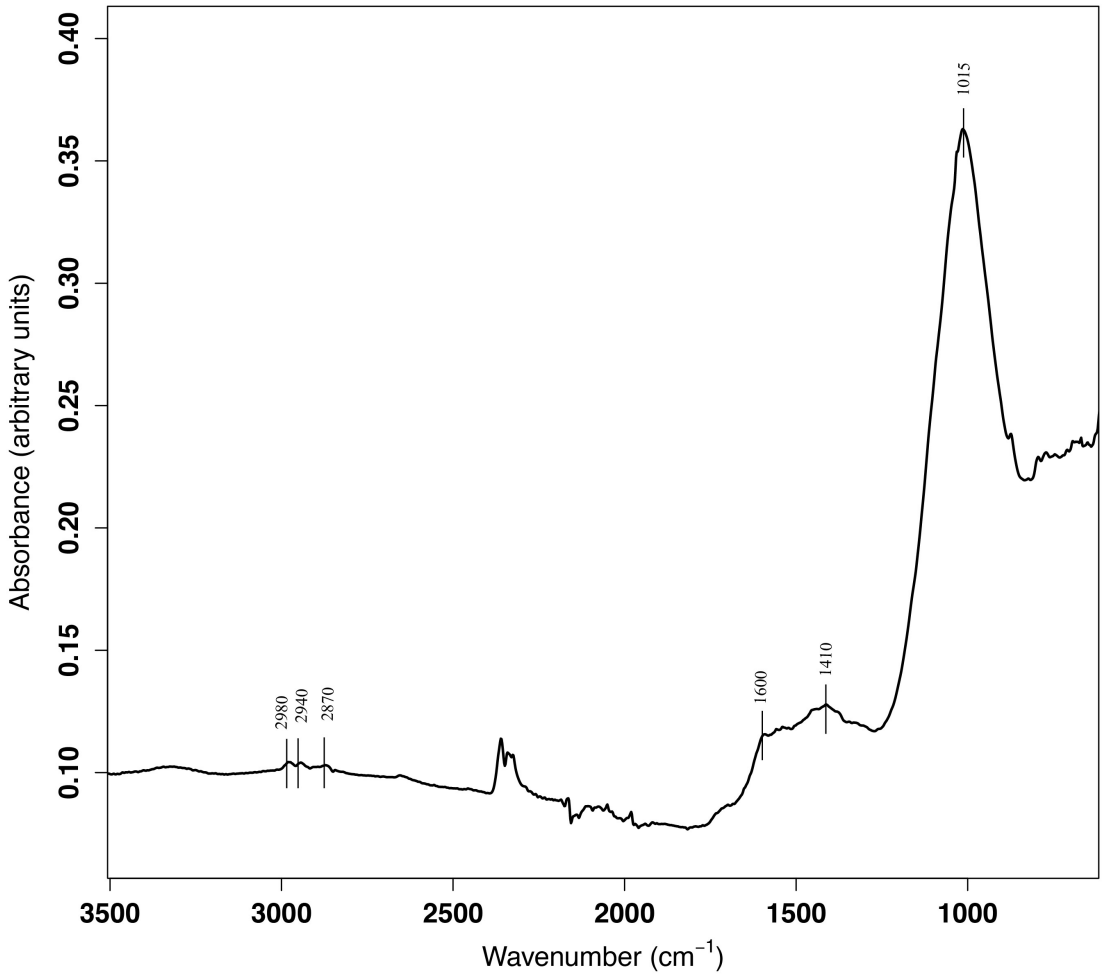


Figure 1C: FTIR spectra of slow pyrolysis sewage sludge feedstock biochar



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