Universidade de Aveiro Departamento de Biologia
2018

MARIJA PRODANA

FERRAMENTAS INTEGRATIVAS PARA AVALIAÇÃO DOS EFEITOS DO BIOCHAR NA BIOTA DO SOLO

INTEGRATIVE TOOLS TO ASSESS EFFECTS OF BIOCHAR TO SOIL BIOTA

Universidade de Aveiro Departamento de Biologia
2018

MARIJA PRODANA

FERRAMENTAS INTEGRATIVAS PARA AVALIAÇÃO DOS EFEITOS DO BIOCHAR NA BIOTA DO SOLO

INTEGRATIVE TOOLS TO ASSESS EFFECTS OF BIOCHAR TO SOIL BIOTA

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Susana Patrícia Mendes Loureiro, professora auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro e do Centro de Estudos do Ambiente e do Mar e co-orientação do Doutora Ana Catarina Bastos, investigadora em pós-doutoramento do Departamento de Biologia da Universidade de Aveiro e do Centro de Estudos do Ambiente e do Mar.

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio através de uma bolsa de doutoramento atribuída a Marija Prodana (SFRH / BD / 89891 / 2012).

o júri

presidente

Doutor Amadeu Mortágua Velho da Maia Soares
Professor Catedrático da Universidade de Aveiro

Doutora Cláudia Saramago de Carvalho Marques dos Santos Cordovil
Professora Auxiliar da Universidade de Lisboa

Doutor Tiago Manuel Ferreira Natal da Luz
Investigador em Pós-doutoramento da Universidade de Coimbra

Doutora María Nazaret González Alcaraz
Investigadora da Universidade de Aveiro

Doutora Susana Patrícia Mendes Loureiro
Professora Auxiliar com Agregação da Universidade de Aveiro (Orientadora)

agradecimentos/ acknowledgements

I would like to express my sincerest gratitude to my mentors Dr. Susana Loureiro and Dr. Ana Catarina Bastos, who have supported me throughout this work with their knowledge. Thank you for sharing with me your expertise and enthusiasm for work, but also for great understanding when difficulties and obstacles were on the way! Many thanks to my cosupervisor Dr. Frank Verheijen for his contribution in the development of this work, and useful discussions we had along the way.

I want to thank to professor Amadeu Soares for the given opportunity to be integrated in the AplEE group and for the laboratory facilities and conditions offered to perform the experimental part of the work. I would also like to acknowledge the collaboration within the work on vineyards with Dr. Rui Morgado and Diogo Cardoso, and the work on biomarkers and fluorimetry with Dr. Carlos Gravato and Carlos Silva.

Great thanks to Abel Ferreira for his enormous help and patience in the laboratory during all these years! Further, I would also like to acknowledge the contribution of Dr. João Serôdio, Silja Frankenbach and Dr. Glória Pinto with providing analytical instruments and sharing the laboratory facilities, and to Dr. Joana Barata and Dr. Anabela Pereira from CESAM who assisted with DOC analysis.

Huge thank you to all my lab colleagues. Special thanks to Fátima, Cátia V., Carlos P, Carlos S, Diogo, Rui, Giuliana, Violeta, Joana, Carolina, Liliana, Andreia, Patrícia, Rafael, António, Gonçalo, Rita S., Sandra, Maria P, Cátia S., Diana, Sara P., Sara C., Catarina M., Zahra, Pearl, Hugo M., Althiéris, Rita B., Natália, Maria J. It was a great pleasure to have you all around!

Many thanks to Flávio, my 'biochar school friend', for all the laughter and friendship!

Because of my PhD work that brought me back to Aveiro, I have got the amazing friends – Luísa and Smriti. Thank you, girls, for being there for me so many times!

I warmly thank my brother, my parents and Tiago for their love and care!

palavras-chave ecotoxicidade, biochar, biochar-composto, biota do solo, microcosmos, modelos de ecossistemas terrestres de pequena escala

resumo

A aplicação de biochar no solo como aditivo agrícola, bem como fonte de carbono, é um foco de crescente interesse, apesar de vários fatores subjacentes determinarem o seu comportamento, toxicidade e destino no solo, apesar de pouco compreendidos. O principal objetivo deste estudo foi avaliar de forma integrada o potencial ecotoxicológico de aplicações representativas de um biochar produzido de raspas e resíduos de madeira no solo, combinando respostas de vários organismos edáficos e parâmetros estruturais e funcionais, em escalas espaciais e temporais relevantes. Para isso, os objetivos específicos foram definidos e abordados em quatro capítulos experimentais. Os efeitos sobre a biota do solo deste biochar e de uma mistura de biochar com compostagem (biochar-composto) em vinhas com fins comerciais no centro de Portugal foram monitorizados em bioensaios de laboratório. O biochar e o biochar-composto foram testados através da avaliação da sobrevivência e reprodução do colêmbolo *Folsomia candida* e do consumo de alimento e biomassa do isópode terrestre *Porcellionides pruinosus*. O solo imediatamente modificado com a adição do biochar e biochar-composto não induziu mudanças significativas no desempenho dos organismos, enquanto a aptidão dos organismos foi reduzida quando expostos ao esse solo envelhecido em campo e ao solo retificado, que foi submetido a vários fatores climáticos e pesticidas convencionais. Os resultados sugerem que a biodisponibilidade de compostos potencialmente tóxicos, como pesticidas, pode não diminuir em termos temporais pela presença de biochar e biochar-composto em vinhas que recebem este tipo de produtos fitofarmacêuticos convencionais. Posteriormente, a toxicidade inerente do biochar foi avaliada na biota, tendo em conta a influência do tamanho das partículas e taxas de aplicação, onde o delineamento experimental foi baseado num ensaio preliminar de comportamento (evitamento) no lumbricídeo *Eisenia andrei*. A experiência principal foi conduzida durante 28 dias em microcosmos de estufas onde foram avaliadas a sobrevivência, perda de peso e distribuição vertical de E. andrei e o consumo de “bait-lamina”, combinando a avaliação da toxicidade dos lixiviados com o objetivo de determinar a inibição de luminescência da bactéria *Vibrio fischeri* e a imobilização do cladócero *Daphnia magna*. Além disso, foi realizada uma experiência de alimentação em laboratório para abordar a alteração de peso e a possível ligação com metabólitos de hidrocarbonetos poliaromáticos (HPAs) nos tecidos dos lumbricídeos. Os resultados mostraram que partículas pequenas (< 0.5 mm) de biochar de madeira podem causar toxicidade sub-letal no biota do solo, sugerindo que há uma relação com o comportamento (evitamento), ao nível individual (alterações de peso, metabólitos tipo naftaleno em tecido de lumbricídeos) e parâmetros funcionais (consumo de “bait-lamina”). Em seguida, explorou-se a interação entre invertebrados de solo de diferentes grupos funcionais, os lumbricídeos (*E. andrei*) e os isópodes (*P. pruinosus*), e a sua relação com a atividade enzimática do solo, em solo biologicamente alterado, juntamente com os principais mecanismos de respostas dos lumbricídeos. Este último foi avaliado com biomarcadores de efeito. A resposta microbiana mostrou ser dependente do tempo de amostragem, da presença de invertebrados e da enzima em causa. A reprodução de *E. andrei* não foi afetada pela exposição ao biochar de madeira. Os biomarcadores responderam como ferramentas de alerta precoce, mostrando um aumento na peroxidação lipídica e diminuição da alocação de energia celular em lumbricídeos expostas. Finalmente, testes de complexidade mais elevada foram conduzidos em modelos de ecossistemas terrestres de pequena escala em 42 dias, avaliando os efeitos de biochar, biochar-composto e fertilizante inorgânico (NPK) e as suas combinações, na sobrevivência e perda de peso de *E. andrei*, consumo de “bait-lamina”, assim como a componente morfológica e de produção da planta *Brassica rapa* (de ciclo de vida rápido), bem como a inibição do crescimento da macrófita aquática *Lemna minor* exposta aos respetivos lixiviados. Os resultados revelaram poucos ou nenhuns efeitos nos lumbricídeos e pequenas estimulações nos parâmetros de produção nas plantas, nomeadamente no tratamento de biochar-composto combinado com fertilizante mineral. O crescimento de *L. minor* foi um dos parâmetros sensível. O estudo indicou que a possibilidade de estímulo de lixiviação de nutrientes pode não ser excluída, o que pode representar um risco para os sistemas aquáticos.

Assim sendo, os resultados demonstram que as respostas biológicas ao biochar de resíduos de madeira variaram de efeitos subletais a neutros e / ou de estímulo, dependendo do organismo e parâmetro do teste, do tratamento com biochar e da taxa de aplicação. Além disso, é de destacar que, para uma compreensão abrangente dos efeitos de biochar na biota e nos mecanismos associados, é fundamental avaliar várias espécies e parâmetros indicadores, que incluam diferentes vias de exposição e níveis de organização biológica e interações, sob cenários de exposição representativos.

keywords ecotoxicity, biochar, biochar-compost, soil biota, microcosms, small scale terrestrial ecosystem models

abstract

Biochar application to soil as an agricultural amendment, as well as a carbon sink, is a focus of increasing interest, despite the underlying factors determining its behaviour, toxicity and fate in soil remaining poorly understood. The main aim of this study was to integratively evaluate the ecotoxicological potential of a wood chip biochar in soil at representative application rates, through combining the responses of multiple soil organisms, and structural and functional parameters, at relevant spatial and temporal scales. To achieve this, the specific objectives were defined and addressed within four experimental chapters. The effects on soil biota of biochar alone and a biochar-compost mixture from a commercial vineyard in Central Portugal, were monitored with laboratory bioassays. Both fresh and field-aged biochar and biochar-compost were tested by evaluating the endpoints survival and reproduction of the collembolan *Folsomia candida* and food consumption and biomass change of terrestrial isopod *Porcellionides pruinosus*. Freshly-amended soil did not induce significant changes on organisms' performance, while the organisms' fitness was reduced when exposed to the field-aged soil and amended-soil, which was subjected to various climatic factors and conventional pesticides. The results suggested that bioavailability of potentially toxic compounds, like pesticides, might not decrease over time by the presence of biochar and biochar-compost in vineyards that receive conventional plant protection products. Subsequently, research was conducted on the potential inherent toxicity of biochar on biota, as influenced by particle size and application rates, where the experimental design was based on a preliminary earthworm (*Eisenia andrei*) avoidance behaviour assay. The main experiment was conducted over 28 days in greenhouse microcosms in which survival, weight losses and vertical distribution of *E. andrei* and bait-lamina consumption were assessed, and combined the evaluation of leachates toxicity looking into endpoints luminescence inhibition of bacterium *Vibrio fischeri* and immobilisation of the cladoceran *Daphnia magna*. In addition, a laboratory feeding experiment was performed to address the weight change and the possible link with polyaromatic hydrocarbons (PAH)-type metabolites in the earthworms' tissues. The results showed that smaller particles (<0.5 mm) of woodchip biochar might pose sub-lethal toxicity to soil biota, suggesting that there is a connection in behavioural (avoidance), individual (weight changes, naphthalene-type metabolites in earthworms' tissue) and functional (bait-lamina consumption) endpoints. Next, the link was explored between the interaction of soil invertebrates from different functional groups, such as earthworms (*E. andrei*) and isopods (*P. pruinosus*), and activity of soil microbial enzymes in biochar-amended soil, alongside the main mechanisms of earthworm' responses. The latter was investigated with biomarkers of effect. Microbial response was sampling time-, invertebrate presence-, and enzyme-dependent. Reproduction of *E. andrei* was not affected by the exposure to the woodchip biochar. Biomarkers responded as early warning tools, by showing an increase in lipid peroxidation and cellular energy allocation decrease in exposed earthworms. At last, higher tier testing was conducted in indoor small-scale terrestrial ecosystem models over 42 days, by assessing the effects of biochar, biochar-compost and inorganic fertilizer (NPK) and their combinations, on the earthworm *E. andrei* survival and weight loss, bait-lamina consumption and a morphological and production traits of rapid cycling plant *Brassica rapa*, as well as of their leachates on growth inhibition of aquatic macrophyte *Lemna minor*. The results revealed low-to-no effect on earthworms, and slight stimulations in production parameters in plants, namely in the treatment of combined biochar-compost with mineral fertilizer. *L. minor* growth was a sensitive endpoint. The study indicated that possibility of nutrients leaching stimulation might not be excluded, which could pose a hazard to aquatic systems.

Together, the results demonstrate that biological responses to woodchip biochar varied from sub-lethal to neutral and/or stimulatory, depending on the test organism and endpoint, biochar treatment and application rate. Further, they highlight that for a comprehensive understanding of biochar effects on biota and associated mechanisms, it is paramount to evaluate various indicator species and endpoints, that include different exposure routes and levels of biological organisation and interactions, under representative exposure scenarios.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AChE	acetylcholine-esterase
ANOVA	analysis of variance
CAT	catalase
CEA	cellular energy allocation
DHA	dehydrogenase
DBC	dissolved black carbon
DOC	dissolved organic carbon
EBC	European Biochar Certificate
EC	effective concentration
ETS	electron transport system
GST	glutathione-S-transferases
IBI	International Biochar Initiative
ISO	International Organization for Standardization
LC	lethal concentration
LOEC	lowest observed effect concentration
LPO	lipid peroxidation
Nap	naphthalene
NOEC	no observed effect concentration
OECD	Organization for Economic Co-operation and Development
PAHs	Polycyclic aromatic hydrocarbons
PEC	predicted environmental concentration
Phe	Phenanthrene

PMS	postmitochondrial supernatant
PTE	potentially toxic element
RQ	risk quotient
SOM	soil organic matter
WHC	water holding capacity

Chapter 1

General Introduction

General introduction

1.1. Biochar – definition, properties and role as environmental management tool

According to the International Biochar Initiative, biochar is defined as a *"solid material obtained from the carbonization thermochemical conversion of biomass in an oxygen-limited environment*. *In more technical terms, biochar is produced by thermal decomposition of organic material (biomass such as wood, manure or leaves) under limited supply of oxygen (O_2), and at relatively low temperatures (<700°C)*" (IBI, 2017).

Biochar can be defined as charcoal for application to soil, and what makes it different from charcoal is actually the concept and application (Verheijen et al., 2009). Charcoal is formed during the incomplete combustion of organic material, namely wood, and in nature it can be found, for instance, after wildfires (Preston and Schmidt, 2006). The motivation behind the use of biochar for soils has roots in the knowledge about "Terra Preta do Indio" (Portuguese "black earth"), the Amazonian fertile soils, characterized by neutral to high pH, with a high proportion of soil organic matter (SOM), and high water holding capacity. These anthropogenic soils contain mixtures of animal bones, broken pieces of pottery, shells and other organic residues, including charcoal, deposited there by the indigenous people, and which together contribute to the fertility of these soils (Glaser et al., 2001). For the maximum benefit to society and the environment, biochar should be perceived in a systematic approach, to target five main objectives: soil improvement, waste management, climate change mitigation, pollution control and energy production (Lehmann and Joseph, 2015).

Biochar started receiving more attention in 2010 with the work of Woolf and co-authors who calculated that a globally implemented biochar system had a potential of 12 % reduction in anthropogenic CO_2-C_e emissions (Woolf et al., 2010). The concept of sustainable biochar application presented in **Figure 1.1.** highlights the high overall potential of biochar as one of the major outputs of pyrolysis and with potential application as soil amendment (Woolf et al., 2010). Although, it is important to note that Woolf and co-authors were addressing sustainable biochar application in the context of technical potential of biochar to mitigate climate change, with 'sustainable' referring to the offsets in emissions vs. C sequestration.

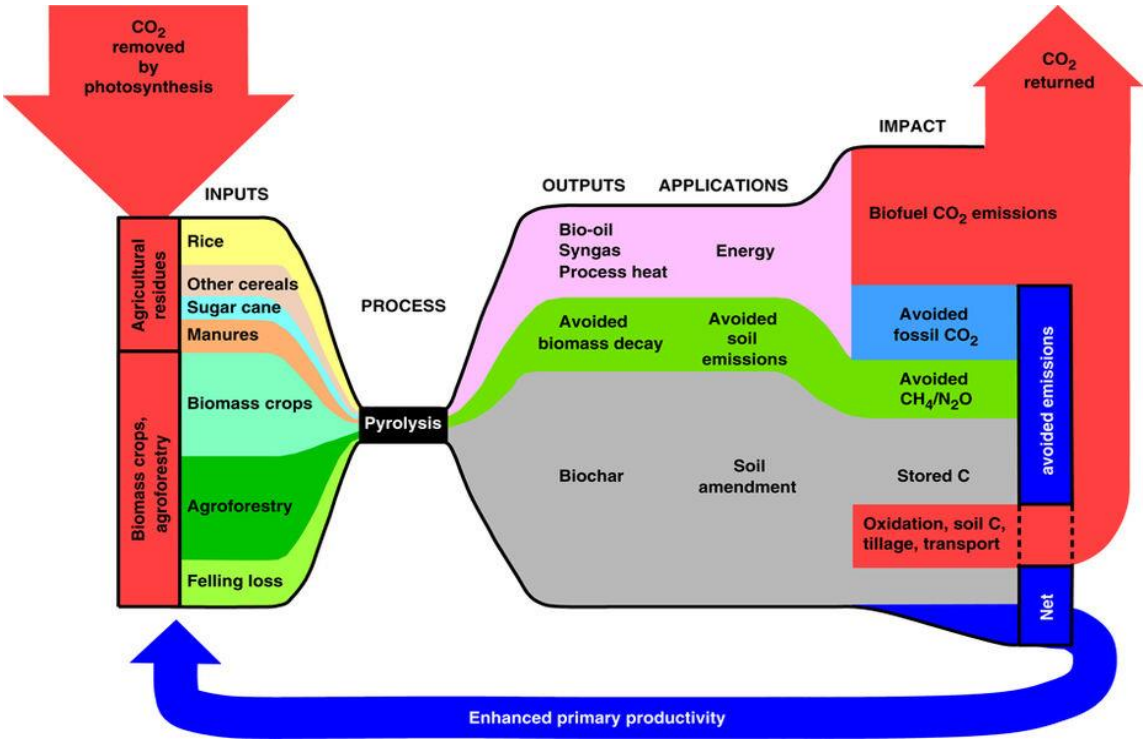

Figure 1.1. Schematic presentation: concept of sustainable use of biochar. From 'Sustainable biochar to mitigate global climate change', by Woolf et al. (2010).

The concept of sustainable use of biochar has expanded towards maximizing environmental benefits while avoiding the negatives. In relation to this, biochar application as a soil amendment and a carbon sink became the focus of increasing interest in recent years, as the Food and Agriculture COST Action TD1107 ('Biochar as option for sustainable resource management') fostered rapid developments in biochar production and research. Tammeorg and co-authors (2017) emphasised the future aspects in biochar research that are essential for the sustainable policy development, by splitting them in five broad research areas discussed within thematic groups, as follows: (1) soil biodiversity and ecotoxicology; (2) soil organic matter (SOM); greenhouse gas (GHG) emissions; (3) soil physical properties; (4) nutrient cycles and crop production; and (5) soil remediation. The key research priorities are identified based on the required level of scientific understanding (**Figure 1.2.**).

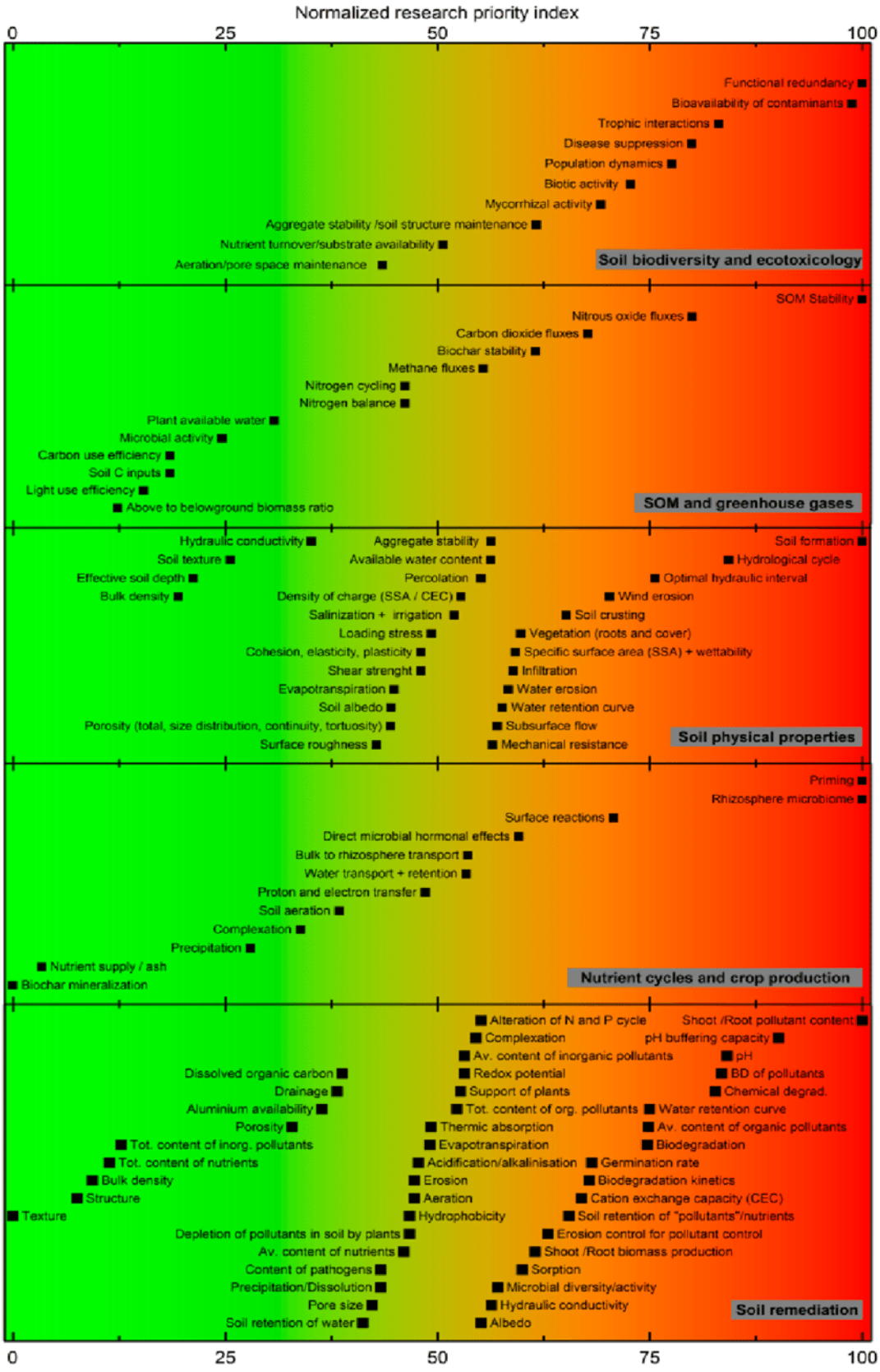
Normalized research priority index
0
25
50
75
100
Functional redundancy
Bioavailability of contaminants
Trophic interactions
Disease suppression
Population dynamics
Biotic activity
Mycorrhizal activity
Aggregate stability /soil structure maintenance
Nutrient turnover/substrate availability
Aeration/pore space maintenance
Soil biodiversity and ecotoxicology
SOM Stability
Nitrous oxide fluxes
Carbon dioxide fluxes
Biochar stability
Methane fluxes
Nitrogen cycling
Nitrogen balance
Plant available water
Microbial activity
Carbon use efficiency
Soil C inputs
Light use efficiency
Above to belowground biomass ratio
SOM and greenhouse gases
Hydraulic conductivity
Aggregate stability
Soil formation
Soil texture
Available water content
Hydrological cycle
Effective soil depth
Percolation
Optimal hydraulic interval
Bulk density
Density of charge (SSA / CEC)
Wind erosion
Salinization + irrigation
Soil crusting
Loading stress
Vegetation (roots and cover)
Cohesion, elasticity, plasticity
Specific surface area (SSA) + wettability
Shear strenght
Infiltration
Evapotranspiration
Water erosion
Soil albedo
Water retention curve
Porosity (total, size distribution, continuity, tortuosity)
Subsurface flow
Surface roughness
Mechanical resistance
Soil physical properties
Priming
Rhizosphere microbiome
Surface reactions
Direct microbial hormonal effects
Bulk to rhizosphere transport
Water transport + retention
Proton and electron transfer
Soil aeration
Complexation
Precipitation
Nutrient supply / ash
Biochar mineralization
Nutrient cycles and crop production
Alteration of N and P cycle
Shoot /Root pollutant content
Complexation
pH buffering capacity
Av. content of inorganic pollutants
pH
Dissolved organic carbon
Redox potential
BD of pollutants
Drainage
Support of plants
Chemical degrad.
Aluminium availability
Tot. content of org. pollutants
Water retention curve
Porosity
Thermic absorption
Av. content of organic pollutants
Tot. content of inorg. pollutants
Evapotranspiration
Biodegradation
Tot. content of nutrients
Acidification/alkalinisation
Germination rate
Bulk density
Erosion
Biodegradation kinetics
Structure
Aeration
Cation exchange capacity (CEC)
Texture
Hydrophobicity
Soil retention of "pollutants"/nutrients
Depletion of pollutants in soil by plants
Erosion control for pollutant control
Av. content of nutrients
Shoot /Root biomass production
Content of pathogens
Sorption
Precipitation/Dissolution
Microbial diversity/activity
Pore size
Hydraulic conductivity
Soil retention of water
Albedo
Soil remediation
0
25
50
75
100

Figure 1.2. Normalized research priority of the indicators as identified by the thematic groups. Higher values of RPI (and red background) refer to higher priorities. *Abbreviations:* Av. = Available; BD = Biodegradability; Org. = organic; Tot. = Total. From 'Biochars in soils: towards the required level of scientific understanding', by Tammeorg et al. (2017).

One of the main characteristics of biochar is its heterogeneity. Processing conditions, mainly temperature and properties of the biomass used as feedstock, determine together the physico-chemical composition of any biochar (Demirbas, 2004; Zhao et al., 2013). However, the properties that are common to all biochars are aromatic structure and high carbon content, as well as neutral to basic pH (Sohi et al., 2009; Verheijen et al., 2009). The relative contribution (w/w) of the major constituents of biochar can be summarized as follows: 50-90 % of C, up to 40 % of volatiles, up to 15 % of moisture and, ideally 0.5-5 % of mineral matter (ashes) (Antal and Gronli, 2003; Brown, 2009; Verheijen et al., 2009). Total nitrogen (N), potassium (K) and phosphorous (P) can be found in different biochars at broad ranges (1.8-56.4 g/kg N, 1-58 g/kg K, and 2.7-480 g/kg P), as reported by Chan and Xu (2009). Due to the process of thermochemical conversion of biomass, biochar contains a significantly higher aromatic carbon proportion than the source feedstock, which is the main cause of biochar recalcitrance, i.e. chemical resistance and reduced susceptibility to microbial degradation (Baldock and Smernik, 2002; Zimmerman, 2010).

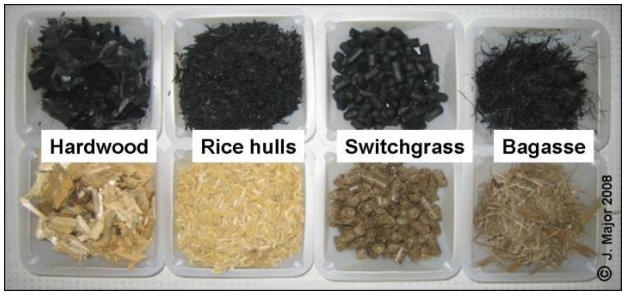

Figure 1.3. Various feedstocks (lower) used to produce biochar (upper). Copyright 2008 by J. Major. Retrieved from *http://www.biochar-international.org.*

During processing, the aromatic rings retain hydrogen, nitrogen, sulphur, oxygen from the feedstock as functional groups (Bourke et al., 2007), such as hydroxyl, amino, carboxylic acids

and esters. The presence of these groups makes the surface of biochar highly reactive, with specific properties ranging from hydrophilic or hydrophobic, oxidizing or reducing, in adjacent areas of the biochar surface (Amonette and Joseph, 2009). The pyrolysis conditions also have a major role in forming biochar's porous structure, as obtained from loss of mineral and small organic molecules, and its large internal surface area, obtained as the volatile compounds that evaporate during the treatment leave the spaces or pores on the biochar surface (Demirbas, 2004). Particle size is another physical property of biochar, which is primarily determined by that of the feedstock (Verheijen et al., 2009), as is captured in **Figure 1.3.** where biochars produced from various feedstocks are presented.

As the properties of biochars are dependent on processing conditions and biomass characteritics, the prospective of biochar application to soil is in a thorough understanding of these properties, which will allow for matching the soil needs with the adequate biochar (Enders et al., 2012; Abiven et al., 2014). Biochar properties will determine the way each biochar acts in soil (e.g. interactions with biota, interactions with soil mineral and organic matter such as aggregate formation/dispersal, translocation of biochar in soil profile), which in turn determines its affinity for adsorption/desorption of contaminants and its bioavailability (Malev et al., 2015; Conti et al., 2016). For instance, ecotoxicological characterization of gasification char and fast pyrolysis wood biochar already demonstrated the adverse effects to soil biota, e.g. phytotoxicity due to volatile matter presence and therefore limited nutrient availability (Marks et al., 2014), while neutral to positive effects were observed in the case of slow pyrolysis corn stover biochar (Domene et al., 2014) and slow pyrolysis wood biochar (Marks et al., 2014). Wood biochars are in general characterized by relatively low levels of volatiles when subjected to slow pyrolysis, followed by degassing procedure, the process which assures that volatiles do not accumulate on the surface of the biochar (Verheijen et al., 2009).

1.2. Biochar as a soil amendment

Biochar application to soil can be motivated from a carbon sequestration perspective, and from a perspective of improving agronomic function of soil. The latter considers the use of biochar as input source of nutrients (e.g. slow release fertilizer), or it can be used as a soil conditioner, by means of improving soil properties and processes linked to the agronomic function. The highest potential in the first case, is for biochars originated from manures (source of N, P, K),

food waste (N, K), biosolids (N), paper mill waste (K), cereals like barley or wheat (P, K), while in the other case, potentially all biochars could be used, since it relies on its generally high specific surface area (Ippolito et al., 2015).

As mentioned in the previous section, the surface of biochar is characterized by high porosity and chemical reactivity. That allows biochar to interact with other components in soil, such as organic matter (SOM), clay minerals and microorganisms. In this way, the properties of amended soil, like structure or pH change, and consequently the processes in soil, like increased water holding capacity (WHC) and nutrient retention, and/or aggregation, might be favored (Brodowski et al., 2006; Hammes and Schmidt, 2009). High cation exchange capacity (CEC) of biochar is responsible for nutrient retention potential and buffering against soil acidification in biochar-amended soil (reviewed by Verheijen et al., 2009). It can go up to 40 cmolc/g (Lehmann et al., 2007), and Glaser and co-authors reported that the aged biochar can be characterized by higher level of CEC (Glaser et al., 2001). Soil bulk density normally decreases upon biochar application to soil (Busscher et al., 2011; Mankasingh et al., 2011), which, combined with improved aggregate stability, can favour aeration and root propagation. It is known that pH levels of enriched soil may increase because of the liming effect of biochar (Singh et al., 2017; Verheijen et al., 2009), which is of high importance for correcting the pH of acidic soils in some regions (Masulili et al., 2010; Molnar et al., 2016; Jeffery et al., 2017).

All such changes that biochar can trigger regarding soil properties and processes, are often seen as a means to increase the agronomic production capacity of soil and combat food scarcity challenges in the future. Therefore, much reasearch effort is directed to looking into biochar effects on crop yields. A recently published review based on meta-analysis suggests that biochar potential for crop yield improvement is limitted to the low-nutrient acidic soils, like those in tropical regions (Jeffery et al., 2017). Tropical soils can benefit from biochar fertilization and liming effects, while regions with temperate climate may take the advantage of reduced pH correction costs, when biochar is used as a liming agent, or of other environmental benefits, such as greenhouse gases emissions reduction. On the other hand, a meta analysis on effects of biochar on trees by Thomas and Gale (2015) underlined that the scale of the effects on the trees is generally greater than on the agricultural crops, but also that the angiosperms might be less affected by biochar application than conifers (Thomas and Gale, 2015). Biochar effects will depend on the combined soil and environmental conditions but also on the agricultural management practices. For instance, combination of climate and management may affect the

result, as the water retention benefits are more pronounced in drier soils and conditions. This can be seen in the two studies in vineyards, one located in Mediterranean and the other in temperate climate. Due to improvement in soil water retention, biochar showed positive effects on the productivity of non-irrigated vines in Italy (Genesio et al, 2015), while in Swiss vineyards there were no observed significant economic benefits when studying the health of the vines and grape quality (Schmidt et al, 2014). Most current biochar applications are combined with other soil amendments, such as compost (to enhance nutrient retention further) and/or inorganic (NPK) fertilizers. However, neither applications of biochar to soil as a source of nutrients is straightforward. For example, if biochar is to be used as additional source of N, one should bear in mind that the total N concentration in biochar may not be representive of the N available in soil after biochar application, due to the recalcitrant nature of biochar, with N being mostly present in heterocyclic form, i.e. being tightly bound between C atoms in an aromatic structure (Verheijen et al., 2009). Yet, more research is still needed towards optimization of the application rates of biochar and of the concentration ratio between biochar and different amendments in the case of combined applications (Schultz et al., 2012). In the work of Jeffery et al. (2015) on the future steps in biochar research and use in practise, the authors point out the need to indentify 'trade-offs' between the possible benefits that biochar can bring, like in the case of fungal desease supression in tomatoes on one side (Elad et al., 2010), and reduction of efficacy of pesticides on the other when biochar is used for remediation purposes (Graber et al., 2012). Another trade-off, of main relevance in the present work, is related to potential toxicity seen as bioavailability of biochar-bound contaminants.

1.3. Effect-based approach in quality assessment of biochar and biochar-amended soils

Soil ecosystem functions and services are defined with interconnected physical (climatic factors, soil porosity, aggregates, etc.), chemical (transformation and decomposition of organic residues), biological (microbial and faunal functions) and human factors (e.g. agricultural activities). Soil biota, with its role in soil organic matter fragmentation, decomposition and redistribution, soil porosity and hydrology regulation, structure maintenance and soil aggregates stabilization, have the key role in maintaining soil health and functioning. Depending on the type of pressure or stress to which the soil ecosystem is exposed (e.g.

environmental, pollution, joint stressors), different species can be used as bioindicators of soil quality changes (Orgiazzi, et al., 2016).

Once biochar is applied to soil, due to its recalcitrant nature and highly reactive surface, it will establish different types of interactions with soil biota. Direct effects of biochar can be caused by changes in soil nutrient status, input of water extractable (bioavailable) metals or organic compounds, or by combined environmental and chemical stressors. Biochar might also affect biota indirectly, due to its contribution to changes in pH, CEC, soil hydrology and sorption of soil contaminants i.e. when biochar is used in remediation of contaminated soils, the effects of biochar addition in many cases are directed towards reduction of toxicity. The types of effects, however, are also dependent on specific biochar properties and application rates used, and benefits can be offset at higher application rates of biochar (Bielska et al., 2018).

Analogous to the possibility for black carbon mobilization from soils to aquatic systems, one can hypothesise a similar scenario in the case of field-scale biochar application (Jaffe et al., 2013). In this case, aquatic species can be affected directly, through changes in pH, dissolved organic carbon (DOC), bioavailable potential contaminants (or their mixtures), or through the combination of stressors. It has been demonstrated higher concentration of water-extractable metals and PAHs in soil-biochar mixtures than in biochar alone, probably due to competition for reactive sites by SOM that can result in increased desorption of potentially toxic elements (PTE) (Bastos et al., 2014). Alternatively, aquatic ecosystems could be indirectly affected through increased dissolved organic matter (DOM) occurrence in runoff or leachates from biochar amended soils (Lindh et al., 2015).

Like in the case of soil quality assessment and contaminated soil screening, the evaluation of the ecotoxicological risk of biochar-amended soils can be done through complementing the analytical approach, (physicochemical characterization) with effect based approaches, (ecotoxicological characterization). Biochar analytical characterization methods were developed quickly and resulted in two international voluntary biochar quality standards, the European Biochar Certificate (EBC, 2012) and the International Biochar Initiative (IBI, 2015). Moreover, in the case of screening biochar-amended soils or leachates, one can compare the concentrations of PTE in these fractions with the established benchmark levels in soil quality frameworks, directives and/or regulations, such as the Canadian soil quality standard (CCME, 1999), Finnish guideline (MEF, 2007), or European Water Framework Directive for the aqueous

component (EU WFD, 2000). So far, only the IBI biochar standard recommends the use of plant germination assays for biochar quality assessment (IBI, 2015).

Biological methods have advantages as they represent direct toxicity assessment, provide the information on bioavailable fraction of contaminants in soil or in aqueous solutions as well as in mixtures (Loureiro et al., 2005a; 2006a), and account for the interaction effects with soil and between co-existing chemicals (Santos et al., 2011; Morgado et al., 2016). Moreover, they are characterized as substantial tools in risk communication through indication of the presence or absence of the components and functions which constitute a healthy ecosystem (Spurgeon et al., 2009). Many of the available standardized and well-established guidelines (OECD, ISO) can be applied to biochar ecotoxicological assessment, measuring a range of responses, from a molecular genetic level, up to those assessing the ecological function (Spurgeon et al., 2009; van Gestel and van Brummelen, 1996).

Ecotoxicity of biochar as a heterogeneous matrix has started to be addressed only recently (Bastos et al., 2014; Bielska et al., 2018; Conti et al., 2016; Domene et al., 2014; Malev et al., 2015; Marks et al., 2014). Nevertheless, the underlying factors determining behaviour, toxicity and fate of biochar in soil remain poorly understood. Widespread implementation of biochar systems should rely on robust risk assessment to ensure sustainability before policy can be developed adequately. Compromised biological communities can lead to significant shifts in element cycles (Grossman et al., 2010), plant-pathogen interactions and crop growth (Warnock et al., 2007). It is thus, timely and vital to achieve an integrative ecotoxicological assessment of biochar in soils, for a range of physical (e.g. particle size distribution) and chemical (e.g. pH, contents of mineral and organic compounds, including metals and PAHs) properties at recommended applications rates and at different scales (Tammeorg et al., 2017). There is a knowledge gap in understanding the interactions that biochar establishes in soils with the various soil elements over a certain period of time, and how these interactions are influenced by natural soil conditions and processes. The effects of alterations that biochar can go through, the so called “biochar ageing” in soil, on the desorption of contaminants from biochar, and the risks of increasing their bioavailability, mobility and ecotoxicological implications are a challenge for biochar researchers (Hilber et al., 2017).

1.4 Study organisms

Folsomia candida is a collembolan species, also known by the colloquial name of springtail. It is one of the most frequently used soil model organisms in ecotoxicology, parthenogenetic, and already included in the standardized ecotoxicological guidelines (ISO, 1999, OECD, 2009). It is widely distributed in soil and has the role of micro-decomposer in the soil food web (Fountain and Hopkin, 2005, Tully et al., 2006). Through their feeding as fungivores, these organisms have an important contribution in maintaining soil microbial biomass abundance and activity (Kaneda and Kaneko, 2002; Fountain and Hopkin, 2005). The uptake of chemicals in collembolans occurs when in contact with soil pore water, mainly through a ventral tube (Fountain and Hopkin, 2005). It is indeed a very common model organism in terrestrial ecotoxicology of contaminants (e.g. Cardoso et al., 2015) or mixture of stressors (e.g. Cardoso et al., 2014), in ecotoxicological characterization of biochar amended agricultural soil (Domene et al., 2014; Marks et al., 2014; Conti et al., 2017), and/or in ecotoxicological assessment of biochar remediated contaminated soils (e.g. Bielska et al., 2018).

Porcellionides pruinosus, the terrestrial arthropod from the order Isopoda, is a cosmopolitan species, known by the colloquial name of woodlouse. Through litter decomposing, these organisms contribute to microbial activity and nutrient cycling in soil (Orgiazzi et al, 2016). *P. pruinosus* is a model organism in ecotoxicology due to its known sensitivity to pesticides (Loureiro et al., 2006b). They are mostly exposed to environmental contaminants though the uropodes or via the cuticle. Nevertheless, standardized guidelines for using isopods to assess toxicity of environmental contaminants are yet to be developed (van Gestel, 2012). Loureiro and co-workers suggested an avoidance bioassay as a screening tool, as well as food consumption and biomass change bioassays to assess soil quality and contamination (Loureiro et al., 2005; Loureiro et al., 2006). Recently, *P. pruinosus* is used as a model species in assessment of the effects of nanoparticles (Tourinho et al., 2013), or combination of chemical and/or chemical and physical stressors (Tourinho et al., 2015 and Morgado et al., 2016, respectively)

Eisenia andrei is an earthworm from the family *Annelida*. Earthworms have an important role in soil processes, such as organic matter decomposition and redistribution, and bioturbation and structure maintenance. *E. andrei* is simple to maintain in laboratory cultures and due to its sensitivity to environmental contaminants, this epigeic earthworm species is, along with collembolans, a frequently studied soil model organism in ecotoxicology. Together with *E.*

fetida, it is included in standardized guidelines for soil quality assessment, including acute and chronic endpoints within ISO and/or OECD. *Eisenia andrei/fetida* are shown to be sensitive in responses to biochar in soil, in the various approaches such as avoidance behaviour (Li et al., 2011; Amaro et al., 2016), bioaccumulation of PAHs (Malev et al., 2015), biomarkers of effects (Li et al., 2011).

Rapid cycling *Brassica rapa* is also very practical for use in ecotoxicology and terrestrial microcosms and mesocosms experiments as the full cycle lasts relatively short - 36 days (Lima et al., 2011; Santos et al., 2011). Recently, it was also used in the context of higher-tier assessment of biochar amended soil (Amaro et al., 2016).

Daphnia magna is a planktonic crustacean species, with the ability to reproduce both asexually and sexually. However, parthenogenesis (Allonso, 1996) occurs in conditions of higher food availability. This means that low genetic variability that is created due to asexual reproduction will induce a less variable response to the toxic compounds. Individuals from this genus are characterized by increased sensitivity to stress, and this is why they are equally used to test general, as well as specific scenarios in ecotoxicology (Hanazato, 2001). *D. magn*a is very often the dominant zooplankton in ponds and lakes and food for fish (Ebert, 2005). *D. magna* is easy to maintain and, due to its short lifecycle, it is used frequently in acute as well as chronic toxicity bioassays (Terra et al., 2003). Besides a very common use of D. magna in ecotoxicology, it is yet not much used in biochar studies, although it has shown to be sensitive to biochar-amended soil (Bastos et al., 2014).

Vibrio fischeri is a marine bacterium. It is used frequently for evaluation of toxicity of solutions of chemicals or water, wastewater or contaminated soil, in ecotoxicological evaluations, as an alternative to more time-consuming assays with other aquatic species (Parvez et al., 2006). Bacteria are decomposers of organic material in aquatic ecosystems, and therefore have an important role in the trophic chain (Wang et al., 2009). For these reasons is *V. fischeri* very often included in ecotoxicological evaluation of soil elutriates or soil aqueous extracts (e.g. Loureiro et al., 2005a; Bastos et al., 2014).

Lemna minor is a fresh water macrophyte from the duckweed family (*Lemnaceae*) and, since it is only absent from some tropical and polar regions, it can be classified as a cosmopolitan species (Cronk and Fennessy, 2009). *L. minor* is characterized by asexual reproduction, which starts with asexual propagules, subsequently branching of shoots and developing fronds

(Lemon et al., 2001 and the references therein). Besides being a common ecotoxicological model organism, it is also used to study the behaviour of invasive aquatic plants due to its fast growth and as it is easy to cultivate and handle under laboratory conditions (Palacci et al., 2016).

1.5. Study aim, approach and objectives

This study aims at providing an integrative ecotoxicological evaluation of a wood chip biochar in soil, added at typical application rates, alone or in combination with traditional soil amendments (e.g. vegetable compost, mineral fertilizer), through combining the responses of soil organisms and key processes.

In order to achieve higher ecological relevance, the study approach considers:

- *Biological scale*: individual (e.g. evaluating endpoints on biochemical level) to population (e.g. reproduction) and community level (through evaluation of functional parameters such as feeding or changes in the activity of soil enzymes).
- *Spatial scale*: starts by using single species toxicity tests in the laboratory, and continues to multispecies microcosms tests, up to the higher-tier tests in small-scale terrestrial ecosystem models (STEMs).
- *Temporal scale*: time series (e.g. up to 18 months in the field, and/or sampling events over a 56-days experiment).
- *Environmental scale:* considers testing of both terrestrial component of amended soil and the aquatic component through assessing toxicity of the amended-soil leachates.

The species selected for the study are used as model organisms in ecotoxicology, and the bioassays are standardized and/or well established. Besides the contribution to soil health through affecting dynamics of nutrients and organic matter, the selected organisms have different exposure routes to contaminants present in soil and therefore they respond with variable sensitivity to environmental stressors. In the case of the aquatic species, they were chosen as representatives of different trophic levels in the aquatic ecosystem.

The ecotoxicological evaluation is complemented with physicochemical characterization of biochar and soil, and of the respective leachates. This integrative way of addressing potential

toxicity of biochar can provide datasets for development present initiatives to establish frameworks for biochar risk assessment. In addition, it may contribute to product standardisation in relation to specific potentially bioavailable contaminants or to other properties that might contribute to the increased risk of biochar application to soil.

All experiments were performed with natural agricultural soil, sampled from field sites in Portugal. The choice to use natural soil in the ecotoxicological evaluation of biochar is because the bioavailability of toxic substances can significantly change based on soil properties (Amorim et al., 2005, Leitao et al., 2014). Bearing that in mind and with the purpose to increase the environmental representativeness of the performed work, we opted for a natural soil that is a representative soil type of Central Portugal. Moreover, the choice of woodchip biochar is, as explained in section 1.1., related to its properties by means of low levels of contaminants and less heterogenous characteristics, making it more relevant in a real field application, which altogether increases the reproducibility of the current work.

The following main specific objectives were identified:

a) To quantify the exposure and effects on representative soil biota through standard and/or widely established soil ecotoxicological tests, using soil invertebrates with different physiological features and complementary ecological roles *(Chapter 2).*

b) To evaluate the effects of biochar particle size distribution on soil biota, and on soil water retention function (*Chapter 3).*

c) To evaluate the link between the interaction of soil invertebrates from different functional groups and activity of soil enzymes in biochar amended soil, and study the potential mechanism of earthworms' response to biochar-soil using the biomarkers approach *(Chapter 4).*

d) Higher tier testing in a laboratory terrestrial microcosms study over 42 days: to assess the effects of biochar, biochar-compost and inorganic fertilizer (NPK) and their combinations, on earthworms, bait-lamina consumption and a rapid cycle plant, as well as of their leachates on a common duckweed, the aquatic macrophyte *(Chapter 5)*.

1.6. Framework

This thesis is organized into six chapters. After a theoretical introduction to the study and presentation of study aims in the first chapter, the second, third, fourth and fifth chapters constitute the experimental sections, each presented as an independent scientific paper. In the sixth chapter, a general discussion and conclusions regarding the main findings of the thesis are provided.

In the current chapter (**Chapter 1)** introduces a definition, properties and role of biochar as environmental management tool in general, and as a soil amendment in particular. It further presents the arguments for the use of effect-based approaches in the quality assessment of biochar and biochar-amended soils, identifying the knowledge gaps regarding the biochar effects on biota. It also includes a section that characterizes the model organisms used in the experiments. The chapter ends with the overall study aims, objectives alongside the study approach, including the flowchart of the thesis methodology (Figure 1.4.).

Chapter 2 presents a case study of biochar and biochar-compost in a field experiment in the Bairrada region of Portugal. Biomonitoring of biochar and biochar-compost amended soil using bioassays with the invertebrates *F. candida* and *P. pruinosus* was performed in the laboratory, in order to assess the potential ecotoxicological effects in freshly amended soils and 18 months after the application.

Chapter 3 addresses the effects of biochar particle size distribution on biota. Preliminary laboratory avoidance bioassays with *E. andrei* and a follow-up greenhouse experiment with *E. andrei* and bait-lamina were performed in order to evaluate the effects of three biochar particle sizes at two application rates. Toxicity of leachates from the greenhouse experiment was assessed with the *D. magna* acute toxicity bioassay and *V. fischeri* luminescence inhibition assay. A laboratory feeding experiment with the same treatments was conducted in order to evaluate the body mass change as a sublethal endpoint and to quantify PAHs in the earthworms tissue using a fixed fluorescence method.

Chapter 4 aims to identify potential interactions between isopods (*P. pruinosus*) and earthworms (*E. andrei*) in biochar-amended soil in two-species microcosms. Soil (unamended) and amended soil were sampled over time (56 days) in order to evaluate the activity of soil enzymes as soil quality indicators. The reproduction output of *E. andrei* was asssessed. Further, oxidative stress and metabolic biomarkers were analysed in the adult earthworms specimens in order to assess the possible response mechanism to biochar amendment.

Chapter 5 evaluates the impact of biochar, biochar-compost, mineral fertilizer and the combination of these amendments, on *B. rapa*, earthworm *E. andrei* and bait-lamina consumption. The experiment was performed in small-scale terrestrial ecosystem models (STEMs), previously developed as a higher-tier approach for evaluating pesticide toxicity . Effects of the corresponding leachates from the amended soil on growth of the water macrophyte *L. minor* were also evaluated.

In **Chaper 6** an overall discussion of the main study results and observations is provided, along with study limitations, major conclusions and future directions.

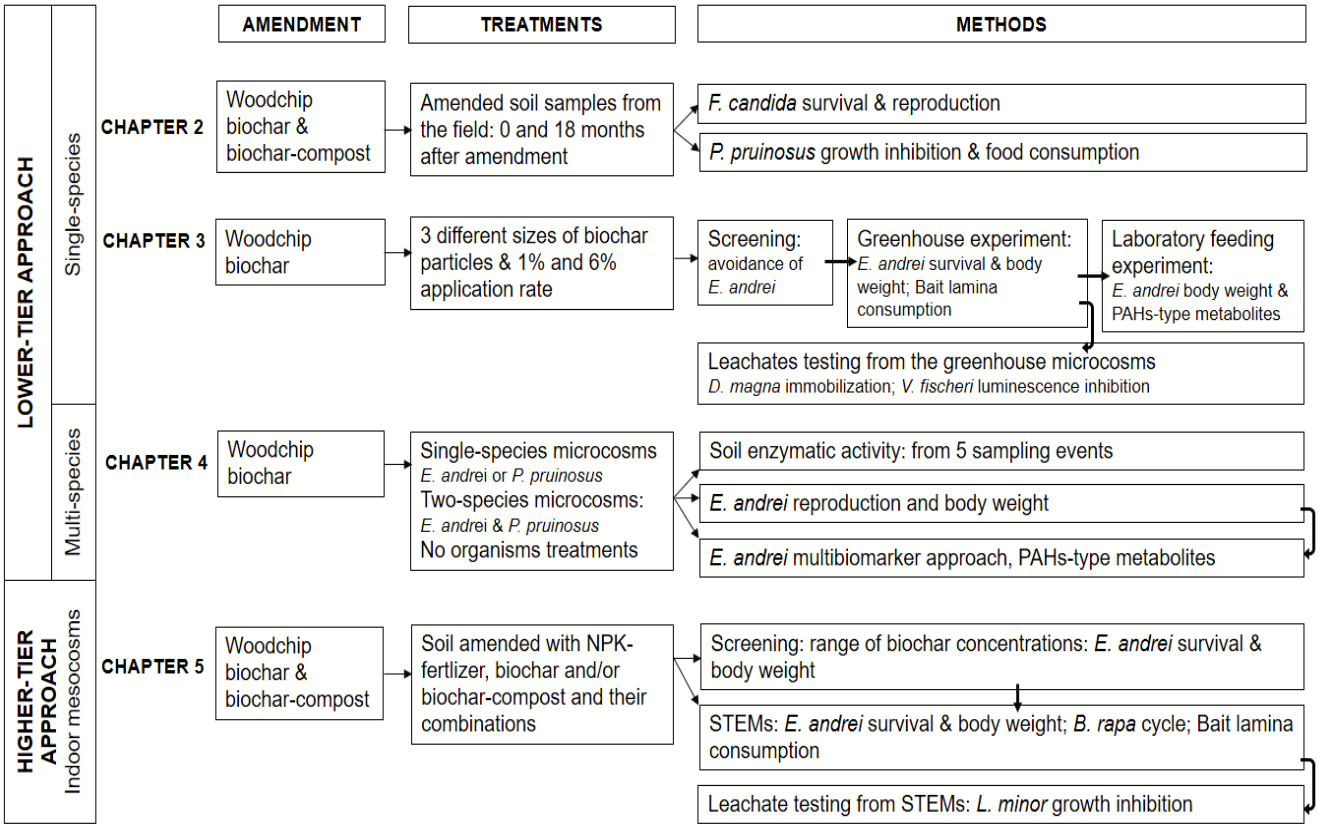

Figure 1.4. The scheme of the experimental approaches used in Chapters 2 to 5 to investigate effects of biochar on biota.

1.7. References

Abiven S., Schmidt M. W. I. and Lehmann, J. ,2014. Biochar bydesign Nat. Geosci 7 326–7.

Alonso M. 1996. Crustacea, Branchiopoda. Fauna Iberica. 7. Madrid: Consejo Superior de Investigaciones Cientificas.

Amaro A, Bastos AC, Santos MJG, Verheijen FGA, Soares AMVM, Loureiro S (2016) Ecotoxicological assessment of a biochar-based organic N-fertilizer in small-scale terrestrial ecosystem models (STEMs). Appl Soil Ecol 108:361–370

Amonette, J.E., Jospeh, S., 2009. Characteristics of Biochar: Microchemical Properties. In: J. Lehmann, Joseph, S. (Editor), Biochar for Environmental Management Science and Technology. Earthscan, London.

Amorim, M.J.D.B., Römbke, J., Schallnass, H.-J., Soares, A.M.V.M., 2005. Effect of soil properties and aging on the toxicity of copper for Enchytraeus albidus, Enchytraeus luxuriosus, and Folsomia candida. Environ. Toxicol. Chem. 24, 1875–1885. doi:10.1897/04-505R.1

Antal Jr, M.J. and Grönli, M., 2003. The art, science, and technology of charcoal production. Industrial and Engineering Chemistry Research 42(8): 1619-1640.

Baird, D.J., Soares, A.M.V.M., Girling, A., Barber, I., Bradley, M.C., Calow, P., 1989. The long-term maintenance of *Daphnia magna* Straus for use in ecotoxicity tests: problems and prospects. In: Lokke, H., Tyle, H., Bro-Rasmussen F, editors. Proceedings of the First European Conference on Ecotoxicology. Denmark: Lyngby, pp. 144–8.

Baird, D.J., Barata, C., 1998. Variability in the response of Daphnia clones to toxic substances: are safety margins being compromised? Archives of Toxicology Supplement 20: 399-406.

Baldock, J. A., Smernik, R. J., 2002. Chemical composition and bioavailability of thermally altered Pinus resinosa (red pine) wood. Organic Geochemistry 33: 1093-1109.

Bastos, A.C., Prodana, M., Abrantes, N., Keizer, J.J., Soares, A.M.V.M., Loureiro, S., 2014. Potential risk of biochar-amended soil to aquatic systems: an evaluation based on aquatic bioassays. Ecotoxicology 23, 1784–1793.

Beesley, L., Marmiroli, M., 2011. The immobilisation and retention of soluble arsenic, cadmium and zinc by biochar. Environmental Pollution 159: 474-480.

Bielská, L., Škulcová, L., Neuwirthová, N., Cornelissen, G., Hale, S.E., 2018. Sorption, bioavailability and ecotoxic effects of hydrophobic organic compounds in biochar amended soils. Sci. Total Environ. 624, 78–86.

Brodowski, S., Amelung, W., Haumaier, L., Abetz, C., Zech, W., 2005. Morphological and chemical properties of black carbon in physical soil fractions as revealed by scanning electron microscopy and energy-dispersive X-ray spectroscopy. Geoderma 128: 116-129.

Brown, R.A., Kercher, A.K., Nguyen, T.H., Nagle, D.C., Ball, W.P., 2006. Production and characterization of syntheticwood chars for use as surrogates for natural sorbents. Organic Geochemistry 37, 321–333.

Canadian Council of Ministers of the Environment (CCME), 1999. Canadian Soil Quality Guidelines for the Protection of Environmental and Human Health.

Cardoso, D.F.N., Bastos, A.C., Soares, A.M.V.M., Loureiro, S., 2014. Short-term exposure to carbaryl and UV radiation increases the reproduction output of the collembolan Folsomia candida. J. Soils Sediments 14, 1559–1567. doi:10.1007/s11368-014-0892-6

Cardoso, D.F. N., Santos, M.J.G., Soares, A.M.V.M., Loureiro, S., 2015. Molluscicide baits impair the life traits of Folsomia candida (Collembola): Possible hazard to the population level and soil function. Chemosphere 132, 1–7.

Chan, K. Y., Xu, Z., 2009. Biochar: Nutrient Properties and Their Enhancement. In: Biochar for Environmental Management: Science and Technology (Eds. Lehmann, J. & Joseph, S.), Earthscan.

Conti, F.D., Visioli, G., Malcevschi, A., Menta, C., 2017. Safety assessment of gasification biochars using Folsomia candida (Collembola) ecotoxicological bioassays. Environ. Sci. Pollut. Res. 2015, 1–12.

Cronk, J.K. and Fennessy, M.S. 2009. In: Encyclopedia of Inland Waters, *Editor-in-Chief Gene E. Likens,* First Edition, Elsevier.

Demirbas, A., 2004. Effects of temperature and particle size on bio-char yield from pyrolysis of agricultural residues. Journal of Analytical and Applied Pyrolysis 72(2): 243-248.

Domene, X., Mattana, S., Hanley, K., Enders, A., Lehmann, J., 2014. Medium-term effects of corn biochar addition on soil biota activities and functions in a temperate soil cropped to corn. Soil Biol. Biochem. 72, 152–162.

Elad Y, David DR, Harel YM, Borenshtein M, Kalifa HB, Silber A, Graber ER, 2010. Induction of systemic resistance in plants by biochar, a soil-applied carbon sequestering agent. Phytopathology, 100, 913–921.

Enders, A., Hanley, K., Whitman, T., Joseph, S., Lehmann, J., 2012. Characterization of biochars to evaluate recalcitrance and agronomic performance. Bioresour. Technol. 114, 644–653.

European Biochar Certificate (EBC), 2012. Guidelines for a Sustainable Production of Biochar. European Biochar Foundation (EBC), Arbaz, Switzerland. http://www.europeanbiochar.org/en/download. Version 6.3E of 14th August 2017

Fountain, M.T., Hopkin, S.P., 2005. *FOLSOMIA CANDIDA* (COLLEMBOLA): A "Standard" Soil Arthropod. Annu. Rev. Entomol. 50, 201–222.

Genesio, L., Miglietta, F., Baronti, S., Vaccari, F.P., 2015. Biochar increases vineyard productivity without affecting grape quality: Results from a four years field experiment in Tuscany. Agric. Ecosyst. Environ. 201, 20–25.

Glaser, B., Haumaier, L., Guggenberger, G., Zech, W., 2001. The "Terra Preta" phenomenon: A model for sustainable agriculture in the humid tropics. Naturwissenschaften 88, 37–41. doi:10.1007/s001140000193

Graber ER, Tsechansky L, Gerstl Z, Lew B, 2012. High surface area biochar negatively impacts herbicide efficacy. Plant and Soil, 353, 95–106.

Grossman, J.M., O'Neill, B.E., Tsai, S.M., Liang, B., Neves, E., Lehmann, J., Thies, J.E., 2010. Amazonian anthrosols support similar microbial communities that differ distinctly from those extant in adjacent, unmodified soils of the same mineralogy.Microbial Ecology 60, 192-205.

Hammes, K. and Schmidt, M.W.I. ,2009. Changes of biochar in soil. In Lehmann J. and Joseph, S. (Eds.) Biochar for environmental management, Science and Technology. London, Earthscan.

Hanazato, T.,2001. Pesticide effects on freshwater zooplankton: an ecological perspective. Environmental Pollution. 112:1–10.

Hilber, I., Bastos, A.C., Loureiro, S., Soja, G., Marsz, A., Cornelissen, G., Bucheli, T.D., 2017. The different faces of biochar: contamination risk versus remediation tool. J. Environ. Eng. Landsc. Manag. 6897, 1–19.

International Biochar Initiative (IBI), 2015. Standardized Product Definition and Product Testing Guidelines for Biochar 7 That Is Used in Soil. http://www.biochar-international.org/sites/default/files/IBI_Biochar_Standards_V2.1_Final.pdf

IBI [WWW Document], 2017. URL http://www.biochar-international.org (accessed 10.9.17).

Ippolito, J.A., Spokas, K.A., Novak, J.M., Lentz, R.D., Cantrell, K.B., 2015. Biochar elemental composition and factors influencing nutrient retention. In: Lehmann, J., Joseph, S. (Eds.). Biochar for Environmental Management, Science, Technology and Implementation. Routledge, London and New York.

Jaffe R, Ding Y, Niggemann J, Vahatalo AV, Stubbins A, Spencer RGM, Campbell J, Dittmar T. 2013 Global charcoal mobilization from soils via dissolution and riverine transport to the oceans. Science 340, 345–347.

Jeffery S., Verheijen F.G.A., van der Velde M., Bastos A.C. 2011. A quantitative review of the effects of biochar application to soils on crop productivity using meta-analysis. Agriculture, Ecosystems and the Environment. 144, 1, 175-187.

Jeffery, S., Bezemer, T.M., Cornelissen, G., Kuyper, T.W., Lehmann, J., Mommer, L., Sohi, S.P., van de Voorde, T.F.J., Wardle, D.A., van Groenigen, J.W., 2015. The way forward in biochar research: targeting trade-offs between the potential wins. GCB Bioenergy 7, 1–13.

Jeffery, S., Abalos, D., Prodana, M., Bastos, A.C., van Groenigen, J.W., Hungate, B.A., Verheijen, F., 2017. Biochar boosts tropical but not temperate crop yields. Environ. Res. Lett. 12, 53001.

Lehmann, J., Gaunt, J. & Rondon, M. 2006. Bio-char Sequestration in Terrestrial Ecosystems – A Review. *Mitigation and Adaptation Strategies for Global Change,* 11**,** 395-419.

Lehmann, J., 2007. A handful of Carbon. Nature 447: 143-144

Lehmann, J., Rillig, M. C., Thies, J., Masiello, C. A., Hockaday, W. C. & Crowley, D. 2011. Biochar effects on soil biota – A review. *Soil Biology and Biochemistry,* 43**,** 1812-1836.

Lehmann, J., Joseph, S., 2015. Biochar for environmental management: an introduction. In: Lehmann, J., Joseph, S. (Eds.). Biochar for Environmental Management, Science, Technology and Implementation. Routledge, London and New York.

Leitão, S., Cerejeira, M.J., Van den Brink, P.J., Sousa, J.P., 2014. Effects of azoxystrobin, chlorothalonil, and ethoprophos on the reproduction of three terrestrial invertebrates using a natural Mediterranean soil. Appl. Soil Ecol. 76, 124–131. doi:10.1016/j.apsoil.2013.12.013

Lemon G.D., Posluszny, U., Husband, B.C., 2001. Potential and realized rates of vegetative reproduction in Spirodela polyrhiza, Lemna minor, and Wolffia borealis. Aquat. Bot. 70, 79–87. doi:10.1016/S0304-3770(00)00131-5

Li, D., Hockaday, W. C., Masiello, C. A., Alvarez, P. J. J. 2011. Earthworm avoidance of biochar can be mitigated by wetting. Soil Biol. Biochem. 43(8), 1732–1737.

Lindh, M. V., Riemann, L., Baltar, F., Romero-Oliva, C., Salomon, P.S., Granéli, E., Pinhassi, J., 2015. Consequences of increased temperature and acidification on bacterioplankton community composition during a mesocosm spring bloom in the Baltic Sea. Environ. Microbiol. Rep. 5, 252–262.

Loureiro S., Ferreira A.L.G, Soares A.M.V.M, Nogueira A.J.A, 2005a. Evaluation of the Toxicity of Two Soils from Jales Mine (Portugal) Using Aquatic Bioassays. Chemosphere. 61, 168-177.

Loureiro, S., Soares, A. M. & Nogueira, A. J. 2005b. Terrestrial avoidance behaviour tests as screening tool to assess soil contamination. *Environmental Pollution,* 138**,** 121-131.

Loureiro S., Santos S., Pinto G., Costa A., Monteiro M., Nogueira A.J.A., Soares A.M.V.M., 2006a. Toxicity Assessment of Two Soils from Jales Mine (Portugal) Using Plants: Growth and Biochemical Parameters. Arch. Environ. Contam. Toxicol.. 50, 182–190.

Loureiro, S., Sampaio, A., Brandao, A., Nogueira, A. J. & Soares, A. M. 2006b. Feeding behaviour of the terrestrial isopod< i> Porcellionides pruinosus</i> Brandt, 1833 (Crustacea, Isopoda) in response to changes in food quality and contamination. *Science of the total environment,* 369**,** 119-128.

Malev, O., Contin, M., Licen, S., Barbieri, P., De Nobili, M., 2016. Bioaccumulation of polycyclic aromatic hydrocarbons and survival of earthworms (Eisenia andrei) exposed to biochar amended soils. Environ. Sci. Pollut. Res. 23, 3491–3502.

Marks, E.A.N., Mattana, S., Alcañiz, J.M., Domene, X., 2014. Biochars provoke diverse soil mesofauna reproductive responses in laboratory bioassays. Eur. J. Soil Biol. 60, 104–111.

Masulili, A., Utomo, W.H., 2010. Rice Husk Biochar for Rice Based Cropping System in Acid Soil 1. The Characteristics of Rice Husk Biochar and Its Influence on the Properties of Acid Sulfate Soils and Rice Growth in West Kalimantan, Indonesia. J. Agric. Sci. 2, P39.

Ministry of the Environment Finland (MEF), 2007. Government Decree on the Assessment of Soil Contamination and Remediation Needs 214/2007, March 1 2007.

Molnár, M., Vaszita, E., Farkas, É., Ujaczki, É., Fekete-Kertész, I., Tolner, M., Klebercz, O., Kirchkeszner, C., Gruiz, K., Uzinger, N., Feigl, V., 2016. Acidic sandy soil improvement with biochar — A microcosm study. Sci. Total Environ. 563, 855–865.

Morgado, R.G., Gomes, P.A.D., Ferreira, N.G.C., Cardoso, D.N., Santos, M.J.G., Soares, A.M.V.M., Loureiro, S., 2016. Toxicity interaction between chlorpyrifos, mancozeb and soil moisture to the terrestrial isopod Porcellionides pruinosus. Chemosphere 144, 1845–1853.

Orgiazzi, A., Bardgett, R.D., Barrios, E., Behan-Pelletier, V., Briones, M.J.I., Chotte, J-L., De Deyn, G.B., Eggleton, P., Fierer, N., Fraser, T., H., K., Jeffery, S., Johnson, N.C., Jones, A., Kandeler, E., Kaneko, N., L., P., Lemanceau, P., Miko, L., Montanarella, L., Moreira, F.M.S., R., K.S., Scheu, S., Singh, B.K., Six, J., van der Putten, W.H., Wall, D.H. (Eds.), 2016. Global Soil Biodiversity Atlas. Publications Office of the European Union, Luxemburg, p. 176. doi:10.2788/2613.

Preston, C. M., and Schmidt, M. W. I., 2006. Black (pyrogenic) carbon in boreal forests: a synthesis of current knowledge and uncertainties. Biogeosciences Discussions 3:211-271.

Parvez, S., Venkataraman, C., Mukherji, S., 2006. A review on advantagesluminescence inhibition test (*Vibrio fischeri*) for acute toxicity prediction

ofchemicals. Environmental International. 32: 265-268.

Qureshi, A.A., Bulich, A.A., Isenberg, D.L., 1998. Microtox toxicity test systems— where they stand today. In Wells PG, Lee K, Blaise C, eds, Microscale Testing in Aquatic Toxicology: Advances, Techniques, and Practice. CRC, New York, NY, USA, pp. 185–199.

Schmidt, H.-P., Kammann, C., Niggli, C., Evangelou, M.W.H., Mackie, K.A., Abiven, S., 2014. Biochar and biochar-compost as soil amendments to a vineyard soil: Influences on plant growth, nutrient uptake, plant health and grape quality. Agric. Ecosyst. Environ. 191, 117–123.

Schulz, H., Glaser, B. Effects of biochar compared to organic and inorganic fertilizers on soilquality and plant growth in a greenhouse experiment. J. Plant Nutr. Soil Sci. 2012, 175, 410–422

Sohi, S., Loez-Capel, E., Krull, E., Bol, R., 2009. Biocharclimate change and soil: A review to guide future research. CSIRO Land and Water Science Report 05/09.

Spurgeon, D., Svedsen, C., Hankard, K. P., 2009. Biological methods for assessing potentially contaminated soils. In: Thompson, C, Wadhia, K. and Loibner, A. (Eds.). Environmental Toxicity Testing, Blackwell Publishing.

Tammeorg, P., Bastos, A.C., Jeffery, S., Rees, F., Kern, J., Graber, E.R., Ventura, M., Kibblewhite, M., Amaro, A., Budai, A., Cordovil, C.M. d. S., Domene, X., Gardi, C., Gascó, G., Horák, J., Kammann, C., Kondrlova, E., Laird, D., Loureiro, S., Martins, M.A.S., Panzacchi, P., Prasad, M., Prodana, M., Puga, A.P., Ruysschaert, G., Sas-Paszt, L., Silva, F.C., Teixeira, W.G., Tonon, G., Delle Vedove, G., Zavalloni, C., Glaser, B., Verheijen, F.G.A., 2017. Biochars in soils: towards the required level of scientific understanding. J. Environ. Eng. Landsc. Manag. 25, 192–207. doi:10.3846/16486897.2016.1239582

Terra, N.R., Feiden, I.R., 2003. Reproduction and Survival of *Daphnia magna Straus*, 1820 (Crustacea: Cladocera) under different hardness conditions. Acta Limnologica Brasiliensia. 15 (2): 51-55.

Thomas, S.C., Gale, N., 2015. Biochar and forest restoration: a review and meta-analysis of tree growth responses. New For. 46, 931–946.

Tourinho, P.S., Waalewijn-kool, P.L., Zantkuijl, I., Jurkschat, K., Svendsen, C., Soares, A.M.V.M., Loureiro, S., Gestel, C.A.M. Van, 2015. CeO 2 nanoparticles induce no changes in phenanthrene toxicity to the soil organisms Porcellionides pruinosus and Folsomia candida.

Ecotoxicol. Environ. Saf. 113, 201–206

Tully, T., D'haese, C. A., Richard, M. & Ferriere, R. 2006. Two major evolutionary lineages revealed by molecular phylogeny in the parthenogenetic collembola species Folsomia candida. *Pedobiologia,* 50**,** 95-104.

Van der Grinten, E., Pikkemaat, M.G., Van den Brandhof, E., Stroomberg, G. J., Kraak, M.H.S., 2010. Comparing the sensitivity of algal, cyanobacterial and bacterial bioassays to different groups of antibiotics. Chemosphe 80: 1-6.

Van Gestel, C. A. 2012. Soil ecotoxicology: state of the art and future directions. *ZooKeys***,** 275.

Van Gestel, C.A.M. and Van Brummelen, T.C., 1996. Incorporation of the biomarker concept in ecotoxicology calls for a redefinition of terms. Ecotoxicology, 5: 217.

Verheijen, F., Jeffery, S., Bastos, A.C., van der Velde, M. and Diafas, I., 2009. Biochar application to soils - A critical scientific review of effects on soil properties, processes and functions 166.

Warnock, D.D., Lehmann, J., Kuyper, T.W., Rillig, M.C., 2007. Mycorrhizal responses to biochar in soil–concepts and mechanisms. Plant Soil 300, 9–20.

Woolf, D., Amonette, J. E., Street-Perrott, F. A., Lehmann, J., Joseph, S. 2010. Sustainable biochar to mitigate global climate change. Nat. Commun. 1, 56.

Zhao, L., Cao, X., Mašek, O., Zimmerman, A., 2013. Heterogeneity of biochar properties as a function of feedstock sources and production temperatures. J. Hazard. Mater. 256–257, 1–9.

Chapter 2

Biomonitoring tools for biochar and biochar-compost amended soil under viticulture: looking at exposure and effects

Submitted

Biomonitoring tools for biochar and biochar-compost amended soil under viticulture: looking at exposure and effects

2.1. Abstract

Benefits that biochar can bring to unirrigated vineyards are related mainly to soil chemistry, soil structure and water retention improvements. Little is still known about effects of biochar on soil biotic processes and on organisms that mediate them. For a sustainable use of biochar in agriculture, alone or in combination with other soil amendments, there is a need for better understanding of soil-biochar-biota interactions, particularly in the long term. Here we applied an ecotoxicological monitoring programme to evaluate the effects of field plot-scale biochar and biochar-compost mixture into vineyards soil. Standard and well described laboratory bioassays were used, assessing the survival and reproduction of *Folsomia candida* and food consumption and biomass change of *Porcellionides pruinosus.* The present study examined the effects of biochar and biochar-compost enriched soil treatments in a commercial vineyard subjected to conventional pesticide management practices. We considered two sampling times: i) immediately after initial application of fresh biochar and biochar-compost; and ii) 18 months after the application of the amendments. Based on the time of application and the application rates of pesticides relative to the second sampling event, a theoretical exposure was estimated alongside with risk quotients. The estimated risk quotient was elevated for certain active ingredients in the mixture, namely the fungicides cyprodinil, propiconazole, copper oxychloride and copper sulfate, respectively. This corroborates the overall decrease in organisms' performance observed for the second sampling time. The ecotoxicological response to the tested biochar and biochar-compost enriched soil was species specific, time-dependent, and to some extent, treatment-dependent. The most sensitive endpoint obtained in the study was the collembolan reproduction output. Freshly-amended soil did not induce significant changes on organisms' performance. However, the organisms' fitness was significantly reduced when exposed to the soil and amended-soil from the second sampling event which was subjected to various climatic factors and conventional pesticides. Regarding food consumption of *P. pruinosus*, and adults' survival and juveniles' number of *F. candida* the effects were more pronounced in the 40 t/ha biochar and biochar-compost amended treatments than in 4 t/ha treatment. Results of the study show that bioavailability of potentially toxic elements might not

be prevented over time by the presence of biochar and biochar-compost in commercial vineyards that receive conventional plant protection products.

Key words biochar, biochar-compost, vineyards, biomonitoring, soil invertebrates, mixture exposure

2.2. Introduction

The capacity of biochar to improve soil chemistry, soil structure, water retention, and possibly, plant disease suppression (Tammeorg et al., 2017), is leading to increased interest on its application in vineyards. Benefits from biochar application in grape yield and quality in European vineyards has been investigated, in both temperate (Schmidt et al., 2014) and Mediterranean climates (Baronti et al., 2014; Genesio et al., 2015; Maienza et al., 2017). Nonetheless, the impact of biochar application to soil as a complex ecosystem remains far from being understood. The available studies are very broad in terms of effect size, highlighting the need for testing representative combinations of soil and biochar characteristics (Sakrabani et al., 2017; Verheijen et al., 2014; Verheijen et al., 2017). Biochar effects on soil biota have previously been shown to be linked to feedstock type, pyrolysis temperature (Domene et al., 2015), species and exposure conditions (Amaro et al., 2016). Reproduction stimulation in the collembolan *Folsomia candida* has been reported in soil amended with corn stover biochar at 2 % w/w, although it was accompanied by growth inhibition of the earthworm *Aporrectodea caliginosa* (Hale et al., 2013). Woody feedstock biochars produced by slow and fast pyrolysis have also been observed to stimulate *F. candida's* reproduction, with no effects observed on the enchytraeid *Enchytraeus crypticus* (Marks et al., 2014). Specific mechanisms leading to the stimulation of collembolan reproduction have not been identified. However, enhanced microbial biomass, shifts in community structure or stimulation of symbiotic gut bacteria have been proposed as potential reasons (Marks et al., 2014). No medium-term negative impacts were reported for biological activity of soils cropped with corn in temperate regions (measured as microbial and faunal feeding activity), neither 3 years after amendment with corn biochar at 3, 12 and 30 t/ha, or at an average annual application rate of 1 t/ha (Domene et al., 2014).

On the other hand, earthworm weight loss and mortality were observed from exposure to pine chip and poultry litter biochar applied to artificial soil at 22.5, 45.0, 67.5 and 90.0 Mg/ha (Liesch et al., 2010). Moreover, gasification char increased collembolan and enchytraeid mortality at

concentrations that are relevant to agricultural biochar applications, possibly as the result of liming (Marks et al., 2014). Other reasons for adverse effects of biochar were bioavailability of potentially toxic elements individually or as a mixture (Bastos et al., 2014; Oleszczuk et al., 2013; Smith et al., 2013;), and/or bioaccumulation of these compounds in the organism (Malev et al., 2016).

Vineyards are a potential beneficiary of biochar application due to the lack of irrigation, particularly in Central Portugal. This is the first long-term trial involving biochar application to vineyards in Portugal. This on-going trial includes monitoring of a wide range of soil physical, chemical and biological properties, conducted by an interdisciplinary team of researchers. One of its components includes biomonitoring changes in soil quality and function. The impact of biochar on organisms that are representative of vineyard soils has not yet been explored, especially long-term. The investigation of biochar's potential risks to soil invertebrates that participate in primary soil processes (e.g. organic matter break-down, regulation of microbial abundance and activity) over time, is the basis for ensuring sustainable soil management practices (Nair et al., 2017; Verheijen et al., 2012).

Hence, the present study aimed at assessing the effects on soil organisms of plot-scale biochar and biochar-compost application in a vineyard. For that, two cosmopolitan invertebrate species (*Folsomia candida* and *Porcellionides pruinosus)* were used, due to their sensitivity to changes in soil conditions (e.g. moisture, metals, pesticides). These frequently studied model organisms in ecotoxicology differ by the route of exposure to chemicals in the environment, which is an important criterion for experimental design in ecotoxicology used to assure the ecological relevance of experimental results (Lock and Janssen, 2003; Tourinho et al., 2015). In the case of collembolans, intake of chemicals occurs in contact with soil pore water, mainly through a ventral tube (Fountain and Hopkin, 2005). For terrestrial isopods, contaminants may become available through litter consumption and/or while ingesting soil particles (Zimmer, 2002). The approach used in the present study included treatments of: 1) biochar and a biochar-compost, and 2) sampling of freshly amended soil and 18 months after application. The field site is a part of commercial vineyards, managed with conventional plant protection products (PPPs). Therefore, the theoretical exposure and potential risk were estimated using site specific data for the pesticides applied during two growth seasons and available toxicity data from the literature.

2.3. Materials and Methods

2.3.1. Field site and soil properties

The study field site is located in Anadia (40°26'22.71"N 8°26'20.60"W), part of the Bairrada region (Central Portugal), and belongs to the Estação Vitivinícola da Bairrada - Regional Ministry of Agriculture (Direção Regional de Agricultura e Pescas do Centro-DRAPC). The soil is a Cambisol with a sandy loam texture (sand 69 %, silt 16 %, clay 14 %), topsoil pH of 6.4, WHC_{max} of 38.3 % (maximum water holding capacity; 105°C), soil organic carbon content of 1.21 %, and bulk density of 1.45 g/cm^3. The field had established vines of the Sauvignon Blanc variety, which were un-irrigated and received conventional crop management. Available on-site meteorological data are presented in supplementary **Table S2.1.** Conventional plant protection products (PPPs) applied during two growth seasons, in 2013 and 2014, are shown in supplementary **Table S2.2**.

2.3.2. Characterization and incorporation of biochar and biochar-compost

The biochar and the biochar-based amendment (mixture of biochar and vegetal compost, with 4 % biochar, w/w) were acquired from Swiss Biochar gmbh (Switzerland). The biochar was produced by slow pyrolysis (620°C) of residues from wood chip production. The main physical and chemical properties of the biochar and the biochar-compost mixture can be found in **Table 2.1.**

Table 2.1. Summary of the main physical and chemical characteristics of the selected biochar from mixed wood residues (alone), the biochar-compost mixture containing biochar at 4% (w/w) and the compost (dry weight). Abbreviations: WHCmax stands for maximum water holding capacity, EC for electrical conductivity, and n.d. for 'not determined'.

	Biochar	**Biochar-compost mix**	**Compost**
pH	10.1 (1:5, H_2O)	7.5 (1:5, H_2O)	7.8 (1:20, H_2O)
WHC_{max} (105°C) (%)	73.2	n.d.	n.d.
Bulk density (g/cm^3)	0.55	n.d.	n.d.
EC (µS/cm)	3 000	1 240	1 370
Salts (g/kg)	8.40	11.13	n.d.
Organic carbon (%)	75.0	22.5	12.9
Organic matter (%)	n.d.	38.7	n.d.
H (%)	47	n.d.	n.d.

Ash (550°C) (%)	18.6	5.4	n.d.
N (%)[1]	1.8	4.8	10.6[6]
H:C (molar ratio)	0.07	18.4	n.d.
O:C (molar ratio)	0.04	n.d.	n.d.
P (mg/kg)[2]	1 300	2 400	6 400[7]
K (mg/kg)	10 400	8 400	11 000
S (mg/kg)	372	190	14 200
Ca (mg/kg)	42 200	59 150	103 000
Mg (mg/kg)	2 980	5 400	12 900
B (mg/kg)	39	n.d.	n.d.
Na (mg/kg)	744	930	1 000
Metals (mg/kg)[3]			
Fe	2 420	19 000	n.d.
Hg	<0.07	0.25	n.d.
Ni	17	20.63	n.d.
Pb	<2	14.91	n.d.
Cr	27	21	n.d.
Cu	16	28.93	n.d.
Zn	70	101.16	n.d.
Cd	<0.2	0.21	n.d.
PAHs (mg/kg)[4]			
Naphtalene	0.48	n.d.	n.d.
Acenaphthylene	<0.1	n.d.	n.d.
Acenaphthene	<0.1	n.d.	n.d.
Fluorene	<0.1	n.d.	n.d.
Phenanthrene	<0.1	n.d.	n.d.
Anthracene	<0.1	n.d.	n.d.
Fluoranthene	<0.1	n.d.	n.d.
Pyrene	<0.1	n.d.	n.d.
Benz-[a]-anthracene	<0.1	n.d.	n.d.
Chrysene	<0.1	n.d.	n.d.
Benzo[b]fluoranthene	<0.1	n.d.	n.d.
Benzo[k]fluoranthene	<0.1	n.d.	n.d.
Benzo[a]pyrene	<0.1	n.d.	n.d.
Indeno[1,2,3-cd]-pyrene	<0.1	n.d.	n.d.
Dibenz-[a,h] anthracene	<0.1	n.d.	n.d.
Benzo[ghi]perylene	<0.1	n.d.	n.d.

Σ_{16}PAHs (mg/kg)	0.48	n.d.	n.d.
Σ_7 ind. PCBs (mg/kg)[5]	<0.002	n.d.	n.d.

[1]N, H, C in biochar and biochar-compost were determined with CHN analyzer (DIN/ISO 51732). [2]P, K, S, Ca, Mg, B, Na, Si were determined by ICP-MS (DIN/ISO 17294-2) after melting digestion (DIN 51729). [3]Metals in biochar and biochar-compost were determined by ICP-MS (DIN/ISO 17294-2) after microwave digestion (DIN 22022-1). [4]PAHs in biochar were determined by SPME coupled to GC/MS (DIN EN 15527), where individual PAH values were below or equal to the limit of detection (0.1 mg/kg). [5]The 7 indicator PCBs in biochar (incl. BG) were determined by HRGC/HRMS. [6]N Kjeldahl in g/kg. [7]P as PO_2, K as K_2O, Mg as MgO, Ca as CaO, Na as Na_2O, S as SO_3.

The amendments were incorporated into the topsoil (15 cm depth) of 6 m^2 field plots and with three replicate plots (per treatment) in a random block design. The study treatments were: reference plot B-0 (un-amended soil, 0 t/ha of amendment); biochar-enriched soil B-4 and B-40 (4 t/ha and 40 t/ha, respectively); and soil amended with biochar-compost mixture BC-40 (40 t/ha).

2.3.3. Soil sampling

The first soil sampling (0-15 cm topsoil layer) for bioassays and chemical analysis was conducted at the end of March 2013, at the time when the amendments were applied to the vineyards. This will be referred as the first sampling time or ST1 further in the text, and includes the reference (un-amended) soil and amended soils. The addition of biochar and biochar-compost to the soil samples was performed in the laboratory with the objective to run bioassays and chemical analysis with freshly amended soil. The second sampling event occurred on the 13th October 2014 when composite samples of the un-amended soil, biochar- and biochar-compost-enriched soil were collected from the field plots (18 months after the first application of soil amendments to the plots). Approximately 6 kg of soil were sampled from each replicate plot. These samples were mixed and homogenized in the laboratory, and both reference soil and each treatment were used as composite sample. This sampling event will be further referred as the second sampling time or ST2 and includes the un-amended soil and the amended soil after two growing seasons. B-0, B-4, B-40 and BC-40 were tested as composite samples both in bioassays and in chemical analyses. These sampling times were selected as suitable for the biomonitoring in view of the study aim, while also avoiding disturbance of amended plots, which would compromise subsequent samplings of the long-term study and on-site probe readings.

2.3.4. Chemical analyses of the reference soil and the amended soil samples

Total metal and nutrient contents in un-amended and amended soil samples from both sampling series were analysed in an external laboratory by inductively coupled plasma mass spectrometry (ICP-MS) screening after an aqua regia digestion (DIN EN ISO 17294, 2003), except for sulfur which was determined from the leachate (DIN EN ISO 12457-4, 2002) by inductively coupled plasma optical emission spectrometry (ICP-AES) (DIN EN ISO 11885, 2007). Soil pH was determined in soil-water solution (1:5 v/v) following the ISO standard protocol for soil quality (ISO 10390, 1994).

2.3.5. Predicted exposure and risk assessment

Background content of pesticides' residues in the soil collected during the first sampling event was not part of the risk assessment exercise in the present study because the period before 2013 did not involve the application of any biochar/biochar-compost amendments in this field site. Thus, it was assumed that a potential impact of the initial residual fraction would be negligible and that possible bioavailability/non-availability of potentially toxic elements (PTEs) or any effects observed in ST1 can be attributed to the freshly introduced amendments.

The growing seasons in 2013 and 2014 differed by a long dry summer in 2013 and rainfall events during the summer in 2014. This resulted in an early grape harvest during August 2013 and no need for the planned insecticide treatment. In 2014, however, the insecticide thiamethoxan was applied, as well as an extra grey mold treatment with cyprodinil in late August. Consequently, the grapes were harvested during September. All plots were equally treated, including the reference plot (un-amended soil, 0 t/ha of biochar/biochar-compost). Supplementary information **Table S2.3** depicts all the active ingredients of PPPs applied to the field, their application rates and the main properties together with toxicity levels reported in the literature, the data accessed through Pesticide Properties DataBase (PPDB, 2017). Estimation of predicted environmental concentrations (PECs), derived predicted no effect concentrations (PNECs) and their ratio expressed as risk quotient (RQ) were calculated for the second sampling event (13th October 2014). Two criteria were applied for including a pesticide's active ingredient in the PEC calculation: (1) presence in the soil at the sampling day according to the soil degradation period (DT_{50}) of an active ingredient in the field, and (2) active ingredients of the pesticides that were sprayed within the last 100 days prior to the sampling (i.e. those applied

starting from the beginning of July 2014, and on). A simple model was applied, following the recommendations of Forum for the Coordination of Pesticide Rate Models and their Use (FOCUS, 1997). First, an initial PEC_{soil} (mg/kg) was calculated for each active ingredient:

$$PEC_{soil} = \frac{A \times (1 - f_{int})}{100\ x\ d\ x\ bd} \qquad (Eq.\ 1)$$

where A (g/ha) is an application rate, f_{int} (%) is a fraction intercepted by crop canopy (for large plants, 50 %), d (cm) is a depth of a soil layer (used depth is 15 cm due to the sampling in the same depth of the layer), bd (g/cm^3) is a bulk density of soil (used 1.40 g/cm^3 as mean number of the values measured for all the treatments in the second sampling; please see results section 3.1.). The next step was to calculate actual concentrations in soil $PEC_{soil,act}$ for the day of sampling:

$$PEC_{soil,act} = PEC_{soil} * e^{-kt} \qquad (Eq.\ 2)$$

where PEC_{soil} is an initial predicted environmental concentration of an active ingredient (from Eq. 1), k (days^{-1}) is dissipation rate constant ($k=ln2/DT_{50}$), and t (days) is time between the last application date of a specific pesticide and the day of sampling, 13th October 2014.

Regarding ecotoxicity, the data available from the literature and/or PPDB were used for each active ingredient, namely the NOECs (no observed effect concentrations) for soil invertebrate reproduction as a chronic endpoint. The lowest reported NOEC for each compound was selected by comparing the values found in the literature or in the databases. Predicted no effect concentration (PNEC) is further estimated using a safety factor (SF) as a measure of data uncertainty, following the guideline of European Chemical Agency for assessment factors for derivation of PNECs in terrestrial environment (ECHA, 2008). SF of 100 was used which means that the lowest NOEC was divided by 100. Supplementary **Table S2.4**. contains the toxicity data and safety factors applied for PNEC estimation for every active ingredient. The Risk quotient (RQ) was assessed as a ratio between $PEC_{soil,act}$ and $PNEC$:

$$RQ = \frac{PECsoil,act}{PNEC} \qquad (Eq.\ 3)$$

Risk quotient (RQ) expressed according to the *Eq. 3* is frequently used in risk characterization of industrial chemicals, biocides, various pharmaceuticals, etc. RQ ≥ 1 considers that the ecological risk is likely to occur, while RQ ≤ 1 indicates low likelihood that a substance could pose an ecological risk (Backhaus and Faust, 2012).

2.3.6. Organisms and bioassays

Isopod food consumption and biomass change after 14 days was evaluated following the procedure described by Loureiro et al. (2006). Specimens of *P. pruinosus* were maintained in a laboratory culture at 22±2 °C and 16/8 h of light/dark. Soil used for the culture boxes was a commercially available potting soil, adjusted to 40 % to 60 % WHC. Isopods in the culture were fed *ad libitum* with alder leaves. Bioassays were performed with animals ranging from 15 mg to 25 mg of weight, excluding pregnant females and moulting individuals. Plastic test recipients (6.5 cm diameter) were filled with 50 g of soil/amended-soil. Experiments were conducted with 10 replicates and 1 individual in each per treatment. All isopods were fed with alder leave disks. The weight measurements of every individual and the leave disks were taken at the beginning and at the end of the two weeks-experiment. Changes in isopods biomass and consumption ratio were calculated with the formulas as presented with Eq. 4 and Eq. 5, respectively, according to Loureiro et al. (2006):

$$B_{\Delta} = \frac{W_{ii} - W_{if}}{W_{ii}} \times 100 \qquad \textit{(Eq. 4)}$$

$$C_r = \frac{W_{li} - W_{lf}}{W_{ii}} \qquad \textit{(Eq.5)}$$

where, in Eq. 4, B_{Δ} is the % of change in biomass, W_{ii} (mg fresh weight) is the isopod initial weight, and W_{if} (mg fresh weight) the isopod final weight. In the Eq. 5, C_r (mg food/mg isopod) stands for a consumption ratio, W_{li} (mg dry weight) is the leaf disk initial weight, W_{lf} (mg dry weight) the leaf disk final weight, and W_{ii} (mg fresh weight) is the isopod initial weight.

Collembolan adult survival and reproduction assay (OECD 232, 2009) was performed with collembolans (10-12 days old) from synchronised laboratory cultures maintained in the dark, at 20±2°C, and fed weekly with dry yeast granules. The bioassay was performed at 20±2°C, and 16/8 h of light/dark, for 28 days. Glass test recipients (20 mL of volume) contained 30 g of soil/amended soil and 10 *Folsomia candida*. Tests were performed with five replicates per treatment, for non-amended and amended soil treatments. WHC of the non-amended soil and of the treatments was maintained in the range between 40 % and 60 %. Soil moisture and amount of food in the test recipients were monitored weekly and corrected if needed. After 4 weeks, the soil and animals were transferred to glass crystallizers and filled with water.

Collembolans were photographed at the water surface and the number of adults and juveniles was counted using the SigmaScan Pro5 software.

2.4. Statistical analyses

Sub-lethal data were checked for normality and homogeneity of variance with Shapiro-Wilk test ($p>0.05$) and Leven's test ($p>0.05$), respectively. The endpoints from both bioassays were tested with two-way ANOVA looking into effects of two factors, "sampling time" and "treatment", and their interaction, followed by Dunnett's post hoc test when significant differences were found. Estimates of effect size (R^2) were calculated by dividing the sum of squares for factor 'sampling time' and/or 'treatment' and for their interaction by total sum of squares (Hullet and Levine, 2003). Statistical analysis was performed with sofware package SigmaPlot 12.5.

2.5. Results

2.5.1. Chemical analysis

Results from the analysis of selected metals and nutrients in un-amended soil and amended soil samples from ST1 (with fresh amendments applied) and ST2 (18 months after biochar and biochar-compost application) are provided in **Table 2.2**, together with the pH values. Soil pH was higher in the treatments than in the reference soils, for both sampling events. No large fluctuations between the two sampling events were observed in nutrient and metal contents, or within the sampling times when looking into treatments relative to the un-amended soil. Measured bulk density (bd) of ST2 treatments were: bd(B-0)=1.45 g/cm^3, bd(B-4)=1.42 g/cm^3, bd(B-40)=1.38 g/ cm^3 and bd(BC-40)=1.37 g/ cm^3.

Table 2.2. Contents of metals and nutrients in sandy loam soil (mg/kg dry weight): un-amended soil (B-0), soil amended with biochar at 4 and 40 t/ha (B-4 and B-40) and with a biochar-compost mixture at 40 t/ha (BC-40) for sampling 1 and sampling 2.

	Sampling time 1				**Sampling time 2**			
	B-0	**B-4**	**B-40**	**BC-40**	**B-0**	**B-4**	**B-40**	**BC-40**
pH (H_2O)	6.4	6.5	6.8	6.7	5.9	6.0	6.3	6.9
P Olsen (mg/kg)	270	280	300	280	260	260	260	240
K (mg/kg)	1 000	1 200	940	910	720	1 000	1 100	750
S (mg/ml)	0.1	0.2	0.1	2.9	0.2	0.1	0.1	0.1
Mg (mg/kg)	550	600	600	600	530	580	530	510
Ca (mg/kg)	590	700	910	1 100	3 500	750	850	660
Na (mg/kg)	110	61	23	38	23	43	42	21
Al (mg/kg)	7 300	7 300	6 500	6 200	5 100	6 900	6 700	5 400
As (mg/kg)	6	6	5	6	5	6	5	5
Ba (mg/kg)	35	34	26	27	25	32	34	25
Be (mg/kg)	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Pb (mg/kg)	12	10	9	9	7	9	9	8
B (mg/kg)	<5	<5	<5	<5	<5	<5	<5	<5
Cd (mg/kg)	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Cr (mg/kg)	9	10	9	9	8	10	9	8
Fe (mg/kg)	6 400	7 000	6 700	7 000	6 000	6 900	6 100	6 000
Cu (mg/kg)	60	64	67	63	57	66	62	64
Li (mg/kg)	<10	<10	<10	<10	<10	<10	<10	<10
Mn (mg/kg)	150	170	170	160	150	150	150	180
Hg (mg/kg)	0.54	0.58	0.35	0.23	0.32	0.56	0.72	0.22
Mo (mg/kg)	<5	<5	<5	<5	<5	<5	<5	<5
Ni (mg/kg)	6	6	6	6	5	6	5	5
Se (mg/kg)	<10	<10	<10	<10	<10	<10	<10	<10
Sr (mg/kg)	<5	<5	<5	7.2	8.6	<5	<5	<5
Ti (mg/kg)	80	84	69	98	62	80	72	72
V (mg/kg)	12	13	10	11	10	13	12	10
Zn (mg/kg)	38	28	27	28	23	26	26	26

2.5.2. Predicted exposure and risk characterization

Earthworms are generally highlighted as more sensitive to fungicides, compared to collembolans and enchytraeids (mandipropamid, azoxystrobin). Collembolans are more sensitive to the insecticide thiamethoxam. This is demonstrated by the low chronic toxicity values (NOECs) in supplementary **Table S2.4.**

The exposure parameters (PECs), predicted actual environmental concentrations in soil ($PEC_{soil,act}$), derived predicted no effect concentrations (PNECs) and risk quotients (RQs) are shown in **Table 2.3.** Overall, potentially highest risk to non-target invertebrates pose as follows: cyprodinil, propiconazole, copper oxychloride and copper sulfate. Among these, the first three were applied to vineyards twice during the period relevant for the study.

Table 2.3. Risk characterization of active ingredients in the pesticide mixture: predicted no effect concentrations (PNEC, in mg/kg) derived, predicted actual concentrations for the day of sampling ($PEC_{soil,\ act}$, in mg/kg) and the corresponding risk quotients (RQ).

Application date	Action[1]	Active ingredient	PNEC	$PEC_{soil,act}$	RQ[2]
16/04/2014	F	azoxystrobin	0.200	0.013	0.067
06/05/2014	F	propiconazole	0.008	0.022	2.587
16/05/2014	F	propiconazole	0.008	0.022	2.673
07/07/2014	F, B	Cu oxychloride	0.089	0.144	1.609
07/07/2014	F	mandipropamid	0.160	0.000	0.001
07/07/2014	F	proquinazid	0.509	0.001	0.002
07/07/2014	F	cyprodinil	0.011	0.016	1.388
07/07/2014	F	fludioxonil	0.013	0.002	0.132
07/07/2014	I	thiamethoxam	0.010	0.002	0.167
22/08/2014	F	cyprodinil	0.011	0.043	3.783
03/07/2013	F, B	Cu oxychloride	0.089	0.140	1.569
17/07/2013	F, B	Cu sulphate	0.150	0.196	1.304

[1]F-fungicide, B-bactericide I-insecticide
[2]RQ ≥ 1 - ecological risk is likely to occur; RQ ≤ 1 - low likelihood for an ecological risk

2.5.3. Bioassays

Biomass changes and food consumption ratios of *P. pruinosus* at ST1 and ST2 are presented in **Figure 2.1**. For both ST1 and ST2 the isopods' growth was stimulated in the amended soils

compared to un-amended soil. Growth response followed the general pattern B-4>BC-40>B-40>B-0. On the contrary, alder leaves consumption was the lowest in B-4 treatment during ST1. At ST2, for B-4 and B-40 lower consumption ratios were obtained. Integrating the sampling times and the treatments, both factors significantly affected isopod body mass fluctuations and equally contributed to the total variation, with 12 % of the effect size (two-way ANOVA, p=0.008 for sampling time and p=0.021 for treatment; **Table 2.4.**). However, the interaction effect (sampling time*treatment) was not significant (two-way ANOVA, p=0.771). The food consumption ratio was significantly lower in the ST2, while treatment as a factor, or the factors' interaction did not significantly affect this parameter (two-way ANOVA, p=0.014, p=0.243 and p=0.416 respectively). Factor 'sampling time' explained only 10.8 % of the total variation in the food consumption ratio. For the treatment B-40 the difference was statistically significant between sampling times (Dunnett's method, p=0.018).

I. II.

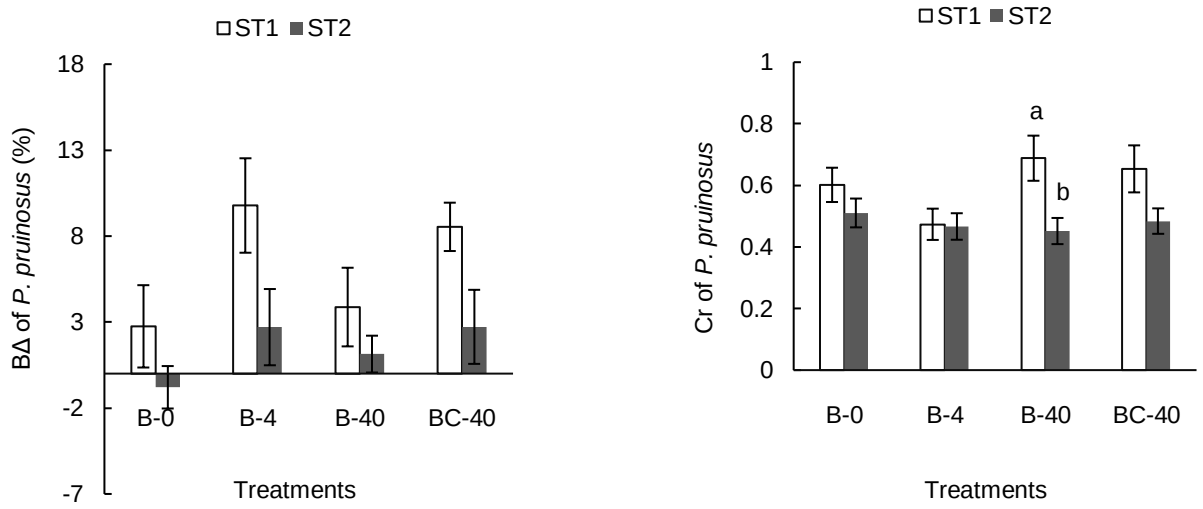

Figure 2.1. (I.) Biomass change (B_{Δ}, in %) and (II.) food consumption ratio (C_r, in mg food/mg isopod) of the isopod *Porcellionides pruinosus* exposed for 14 days to the reference (un-amended) soil (0 t/ha biochar, B-0), biochar amended soil (at 4 t/ha B-4, and 40 t/ha, B-40) and biochar-compost amended soil (at 40 t/ha, BC-40), from sampling time 1 (ST1) and sampling time 2 (ST2). Vertical bars represent standard errors of the means. Lower case letters (a, b) indicate significant differences between sampling times within a treatment (Two-Way ANOVA; Dunnett's method, *p<0.05*).

Table 2.4. Two-way ANOVA testing output for the factors 'sampling time' and 'treatment' and their interaction effect (sampling time x treatment) on biomass change and food consumption of *P. pruinosus* and on adults' survival and reproduction of *F. candida*. Asterisks refer to the levels of statistical significance *p<0.05, **p<0.01 and ***p<0.001.

	DF	SS	MS	F	P	R^2
Porcellionides pruinosus						
Biomass change						
Sampling time	2	437.0	218.5	5.217	0.008**	0.120
Treatment	3	435.9	145.3	3.469	0.021*	0.120
Sampling time x treatment	6	137.4	22.90	0.547	0.771	0.038
Food consumption						
Sampling time	2	0.289	0.144	4.575	0.014*	0.108
Treatment	3	0.135	0.045	1.427	0.243	0.051
Sampling time x treatment	6	0.194	0.032	1.026	0.416	0.073
Folsomia candida						
Adults survival						
Sampling time	1	1.600	1.600	4.414	0.044*	0.101
Treatment	3	2.100	0.700	1.931	0.144	0.132
Sampling time x treatment	3	0.600	0.200	0.552	0.651	0.038
Reproduction						
Sampling time	1	32 262	32 262	110.1	<0.001***	0.688
Treatment	3	3 631.8	1 211	4.130	0.014*	0.077
Sampling time x treatment	3	1 639.8	546.6	1.865	0.155	0.035

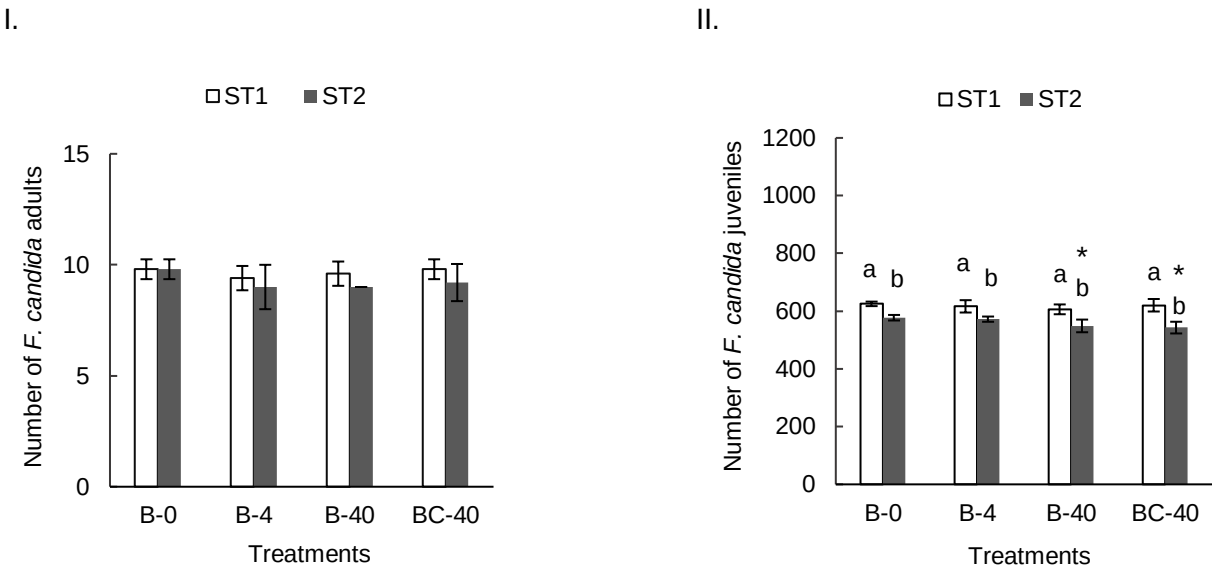

Figure 2.2. (I.) Survival (number of adults) and (II.) reproduction (number of juveniles) of *Folsomia candida* exposed for 28 days to the reference (un-amended) soil (0 t/ha biochar, B-0), biochar amended soil (at 4 t/ha, B-4, and 40 t/ha, B-40) and biochar-compost amended soil (at 40 t/ha, BC-40), from sampling time 1 (ST1) and sampling time 2 (ST2). Vertical bars represent standard errors of the means. Lower case letters (a, b) indicate significant differences between sampling times within a treatment, and asterisk (*) indicates significant differences between treatments and un-amended soil within a sampling time (Two-Way ANOVA; Dunnett's method, $p<0.05$).

F. candida bioassays fulfilled the validity criteria of the guideline related to the reference (un-amended) soil used in the bioassays. These criteria propose that the non-contaminated soil has adult mortality below 20 %, the juveniles number above 100 and the coefficient of variation regarding number of juveniles lower than 30 % (OECD 232, 2009). **Figure 2.2** depicts the obtained survival and reproduction outcomes in *F. candida* bioassays for ST1 and ST2. Table 4 further summarises the two-factorial ANOVA testing of *F. candida* bioassays data, for the factors 'sampling time', 'treatment' and their interaction effect. A significant difference in the number of *F. candida* adults for the factor 'sampling time' was derived ($p=0.044$), which was not the case for the factor 'treatment' or for the interaction effect ($p=0.144$, $p=0.651$ respectively). Factor 'sampling time', however, only explained 10.1 % of the total variability in the number of adults. Regarding the number of juveniles, a statistically significant effects of sampling time ($p<0.001$) and of treatment ($p=0.014$) were observed, but their interaction was not significant ($p=0.155$). Concerning the effect size, 68.8 % of the variation, can be explained by the sampling time factor, and only 7.7 % by the treatment factor. Further on, post hoc tests

revealed specific differences. In terms of treatments, the differences were found between un-amended soil B-0 and both B-40 and BC-40 (Dunnett's method, p=0.011 and p=0.042, respectively). Significant differences between sampling times were obtained for B-0, B-4, B-40 and BC-40 (Dunnett's method, p<0.001). Within ST2, in both B-40 and BC-40, average number of juveniles was lower than in B-0 by 5 % and 6 %, respectively. This decrease was marked as statistically significant when compared to the corresponding unamended soil B-0 (Dunnett's method, p=0.035 and p=0.009 respectively).

2.6. Discussion

2.6.1. Chemical analysis

Besides the existing national quality standard for biochar in some countries (e.g. Germany, Austria, Switzerland, Italy, United Kingdom), two international biochar standardization documents are available as voluntary standards: the European Biochar Certificate (EBC) and the International Biochar Initiative (IBI) (Meyer et al., 2017). Chemical composition of the biochar used in the present study shows that the sum content of PAHs (Σ_{16}PAHs=0.48 mg/kg) is below EBC and IBI threshold concentrations (<4 mg/kg in EBC 2016, <12 mg/kg in IBI, 2015). The Σ_{16}PAHs is comparable to the woodchip biochars produced at 550-620°C (Hilber et al., 2012). Metals and PCBs are also beneath the benchmark concentrations (EBC, 2012; IBI, 2015). Due to low levels of the contaminants it has been classified as "premium grade" biochar according to EBC (EBC, 2012).

To contextualise the metal concentrations in our soil samples at the two sampling events, the measured values were compared with the available soil quality guidelines. Copper was the only metal with concentrations comparable to the ones in the guidelines (57-67 mg/kg). The Canadian soil quality standard sets the concentration of 63 mg/kg as a value for soil quality guideline for environmental health of agricultural soils (CCME, 1999), while the Finnish guideline sets it to 100 mg/kg, where 150 mg/kg and 200 mg/kg represent lower and higher levels of ecological risk respectively (MEF, 2007).

In terms of physicochemical properties, pH levels increased in the amended soil due to the liming effect of biochar on soils (Singh et al., 2017; Verheijen et al., 2009). Upon biochar application, a decrease in soil bulk density may be expected (Busscher et al., 2011; Mankasingh et al., 2011). Despite this, the bulk density values measured in the amended soil

in this study were not substantially different, either within treatments, or in comparison to the un-amended soil.

2.6.2. *P. pruinosus* bioassays

In the bioassays with *P. pruinosus* the reference (un-amended) soil (B-0) exhibited the consumption ratios and biomass changes comparable to those of LUFA 2.2, a frequently used natural soil in ecotoxicology (Morgado et al., 2016; Tourinho et al., 2015).

The two endpoints obtained, changes in biomass and consumption ratios of alder leaves, did not disclose the same pattern. This is especially important because it indicates that alder leaves might not have been the only food source utilised during the bioassays, but that isopods also consumed soil/amended soil particles at different proportions depending on the treatment. The peak increase of biomass in treatment B-4 of ST1 might be due to additional readily available nutrients from fresh biochar (Ippolito et al., 2015). Alternatively, an additional organic matter input from biochar-compost in BC-40 could contribute to a certain extent to the increase in the growth rate. Indeed, this is not a surprising behaviour for terrestrial isopods, as they can use more than one food source to supply their body with necessary nutrients. The study with the desert isopod *Hemilepistus reaumuri* showed that this species preferably feeds on a mixed diet, including detritus, herbaceous material, microbiota and soil particles (Shachak et al., 1976).

To our knowledge, this is the first study so far that addresses the effects of biochar and biochar-compost amendments to *P. pruinosus*. Mechanisms behind isopods' behaviour in biochar and biochar-compost amended soils are not known, and particularly, the palatability degree of these amendments to terrestrial isopods, and how their nutrition is affected over time. Although, it has been shown that some other soil invertebrates, like the endogeic earthworm *Pontoscolex corethrurus,* consume biochar particles possibly due to their gut stimuli by microbiota from the biochar surface (Topoliantz and Ponge, 2003; Topoliantz and Ponge, 2005). Considerably more information is available on the impact of biochar to the earthworm species of the *Eisenia* genus. Belonging to the epigeic group of earthworms, they are involved in litter decomposition (Coleman and Wall, 2014; Domene, 2016), just like terrestrial isopods (Zimmer et al., 2005). Van Zwieten et al. (2010) observed the preference of *Eisenia fetida* for ferrosol type of soil amended with paper mill residues biochar, but not for calcarosol type of soil. Unlike the growth stimulation that we observed for *P. pruinosus* in the amended soil, the weight changes reported

for *Eisenia sp.* ranged from neutral (Amaro et al., 2016; Liesch et al., 2010), to negative (Gomez-Eyles et al., 2011; Li et al., 2011; Liesch et al., 2010). The latter was caused by the feeding inhibition in the presence of biochar, though it has been highlighted the dependence of responses on the biochar and soil properties and their combinations (reviewed by Weyers and Spokas, 2011).

In our study, the isopods performance was strongly dependent on the sampling time. This difference based on sampling times coincides with contrasting management. The soil sampled in 2013 did not contain any additional pesticides and was amended with fresh biochar and biochar-compost, while the samples brought from the field in 2014 underwent 18-months of weathering alongside conventional pesticides application (see section 2.5). It is known that *P. pruinosus* can sense chemical compounds, whether they are present alone, or as mixtures (Loureiro et al., 2009). Decline in feeding performance of *P. pruinosus* in multiple stress conditions has also been reported (Morgado et al., 2016). Furthermore, the presence of several pesticides and/or their residues might have affected the isopods either indirectly, by altering the rate and quality of the leaf litter colonisation by microbiota during the 2-weeks bioassay (Zimmer et al., 2003), or directly by affecting their fitness. Although it is known that terrestrial isopods are sensitive animals when exposed to several pesticides, e.g. dimethoate (Ferreira et al., 2015), glyphosate (Santos et al., 2010), no information is available regarding the active ingredients of the organic pesticides identified in ST2. As mentioned previously, copper concentration was maintained within the values advised by soil guidelines. Moreover, this metal is an essential nutrient for isopods and the constituent of their respiratory pigment hemocyanin, stored in a form of copper granules in the hepatopancreas (Zimmer, 2002). Therefore, it is not expected that copper might have induced this decrease in isopods fitness, unless exposure is considered as a mixture, where no information is available on the interaction of Cu and the other pesticides.

While obviously being stimulated with fresh biochar and biochar-compost, a significant decline in their fitness from ST1 to ST2 raises the concern regarding the adverse effects of the vineyards soil on the terrestrial isopods under the conventional pesticide management. Yet, more research is needed to understand how biochar amendments alter these effects.

2.6.3. *F. candida* bioassays

The adequacy of the reference soil (B-0) used in the current study is evident as the number of *F. candida* juveniles obtained in the un-amended soil (B-0) is comparable to those reported for LUFA 2.2 soil (Cardoso et al., 2015; Tourinho et al., 2015).

Like in the case of *P. pruinosus, F. candida* bioassays' outcome is characterised with a high dependence on sampling time. While the freshly-amended soil did not cause any significant effects on collembolan fitness, the number of adults and number of juveniles were significantly reduced when organisms were exposed to the treatments from ST2. Reproduction of *F. candida* is the most sensitive endpoint observed in the study and the negative impact on collembolans' reproduction was somewhat more pronounced in BC-40 then in B-40 treatments. Albeit the significance, one should bear in mind that the scale of the negative responses to the treatments within ST2 was not large (please see section 3.3.). More prominent effects of biochar on collembolans reproduction, that are reported in the literature so far, were related to the higher application rates (Bielska et al., 2018), or to the initial, biochar-contained, toxic compounds (Domene et al., 2015; Marks et al., 2014). In the study of Domene et al. (2015) collembolans avoidance of biochar-soil was related to microbial biomass decrease, while their reproduction was either stimulated or inhibited, depending on the feedstock used and the processing conditions. Bielska et al. (2018) reported reduction in *F. candida* reproduction rates by 27 % for rice husk biochar, and 38 % for wood biochar, both added at concentration of 10 %. Nonetheless, at the concentrations of 5 % and 1 % of both biochars, that are comparable to the application rates of 40 and 4 t/ha in our study, reduction in number of juveniles was not observed (Bielska et al., 2018).

The investigations on the use of biochar in highly contaminated soils (metals) demonstrated different biochar affinities for various metals and dependence of the metals mobility on the soil pH, dissolved and total organic carbon (Beesley et al., 2010; Uchimiya and Bannon, 2013). In our study, despite the low detected concentrations of PTEs (e.g. copper, lead, mercury, arsenic), a direct toxicity due to higher bioavailability of PTEs, present in the soil pore water as a mixture, may be the factor causing the slight decline in the number of adults and juveniles in the amended treatments of ST2. *F. candida* is more sensitive to the metals spiked soil than to the contaminated food (Fountain and Hopkin, 2001). However, there is also evidence that collembolans are capable to palatalize charred materials (Salem et al., 2013) contributing to the hypothesis that they could be directly affected by ingestion of biochar particles. It is also

possible that available residues of fungicides caused a decrease in microbial biomass in the amended soil. Although being fed with yeast during the assay, collembolans as fungivore organisms could, to some extent, suffer from a decrease in microbial biomass while they were exposed to the treatments from ST2.

The sensitivity of *F. candida* response observed in the current study demonstrates the adequacy of using this species when addressing the mechanisms of biochar effects on soil organisms and particularly the quality of biochar-amended soil over time.

2.6.4. Risk assessment and role of biochar-amendments in ecotoxicological response

Even though most of the fungicides generally used in vineyards are applied foliarly, a simulation study demonstrated that many of them (e.g. cyprodinil, fludioxonil, mandipropamid, etc.) can still be found in rainwater collected from canopy wash-off at concentrations that are far higher than benchmark levels reported in European drinking water quality regulations (Perez-Rodriguez et al., 2017). In our study, individual active ingredient risk quotients higher than 1 indicate an elevated ecological risk for the soil ecosystem. Bioassays, using an effect-based approach, supported this theoretical estimation, while accounting for bioavailability of the entire mixture.

The lower biochar application rate (B-4), and biochar-compost (BC-40), when applied fresh, had stimulatory effects on isopods and low-to-no effect on collembolans. On the contrary, samples collected 18 months after application induced a decrease in bioassays performance. This result in the context of the PPPs applied during the seasons 2013 and 2014 in the vineyards indicate that the effects were mainly caused by the potential exposure to these pesticides. Further, this raises the question of sorption/desorption capacity of biochar and weathering or ageing effects on such processes. Yang et al. (2005) analysed the residual concentration of herbicide diuron in a four-weeks pot experiment and found that it was higher in the amended soil, but less bioavailable to microbiological degradation, thus increasing the survival and biomass of barnyard grass. Affinity of biochar to absorb and desorb the herbicides atrazine and diuron was studied by Martin et al. (2012) with the objective to compare fresh and 32-months-aged biochar application. The authors found that the sorption capacity of paper mill biochar decreased by 47 % for atrazine, while a 68 % decrease was observed for diuron in the aged poultry litter biochar. The quality of amended soil changes under combined anthropogenic

and environmental pressures, thereby precluding accurate previsions on how long a biochar can have a soil conditioning effect (Ippolito et al., 2015), and when/if biochar can become a possible source of PTEs (Hilber et al., 2017). To fill in this knowledge gap, further long-term laboratory and field studies under different environmental scenarios are required.

2.7. Conclusions

Biochar and biochar-compost amendments in vineyard soil induced a positive or neutral improvement on isopods and collembolans fitness. Upon pesticide applications, negative effects were observed for both organisms, with an overall decrease for collembola reproduction and isopod consumption or body mass, irrelevant of the presence/absence of the amendment. A theoretical exposure estimation and risk assessment approach, based on the available site-specific, experimental and literature data, was in agreement with the outcome of the laboratory bioassays. The study findings outline the need to carefully consider biochar application to agricultural soils as a conditioner when conventional pesticides are applied, as it may have different behaviours regarding different chemical compounds. In addition, multiple application of pesticides in vineyards should be considered in further studies bearing in mind the deleterious effects observed after their application. Case-by-case assessment is necessary for a safe use of biochar and biochar-compost as soil amendments. The sensitivity demonstrated by *P. pruinosus* and *F. candida* makes these bioassays promising tools to assess the quality of biochar and biochar-compost amended soils. Revisions of the actual biochar quality standards to include invertebrate bioassays as part of ecotoxicological biomonitoring programmes are needed to assure sustainable biochar application.

2.8. References

Amaro A, Bastos AC, Santos MJG, Verheijen FGA, Soares AMVM, Loureiro S (2016) Ecotoxicological assessment of a biochar-based organic N-fertilizer in small-scale terrestrial ecosystem models (STEMs). Appl Soil Ecol 108:361–370

Backhaus, T., Faust, M., 2012. Predictive Environmental Risk Assessment of Chemical Mixtures: A Conceptual Framework. Environmental Science and Technology 46 (5):2564-2573.

Baronti, S., Vaccari, F.P., Miglietta, F., Calzolari, C., Lugato, E., Orlandini, S., Pini, R., Zulian, C., Genesio, L., 2014. Impact of biochar application on plant water relations in Vitis vinifera (L.). Eur. J. Agron. 53, 38–44.

Bastos, A.C., Prodana, M., Abrantes, N., Keizer, J.J., Soares, A.M.V.M., Loureiro, S., 2014. Potential risk of biochar-amended soil to aquatic systems: an evaluation based on aquatic bioassays. Ecotoxicology 23, 1784–1793.

Beesley, L., Moreno-jiménez, E., Gomez-eyles, J.L., 2010. Effects of biochar and greenwaste compost amendments on mobility, bioavailability and toxicity of inorganic and organic contaminants in a multi-element polluted soil. Environ. Pollut. 158, 2282–2287.

Bielská, L., Škulcová, L., Neuwirthová, N., Cornelissen, G., Hale, S.E., 2018. Sorption, bioavailability and ecotoxic effects of hydrophobic organic compounds in biochar amended soils. Sci. Total Environ. 624, 78–86.

Busscher, W.J., Novak, J.M., Ahmedna, M., 2011. Physical Effects of Organic Matter Amendment of a Southeastern US Coastal Loamy Sand. Soil Sci. 176(12):661-667.

Canadian Council of Ministers of the Environment (CCME), 1999. Canadian Soil Quality Guidelines for the Protection of Environmental and Human Health.

Cardoso, D.N., Santos, M.J.G., Soares, A.M.V.M., Loureiro, S., 2015. Molluscicide baits impair the life traits of Folsomia candida (Collembola): Possible hazard to the population level and soil function. Chemosphere 132, 1–7.

Coleman, D.C., Wall, D.H., 2014. Soil fauna: occurrence, biodiversity, and roles in ecosystem function. In: Paul, E.A. (Ed.), Soil Microbiology, Ecology, and Biochemistry, fourth ed. Elsevier Publishers.

DIN/EN/ISO 12457-4, 2002. Characterization of waste - Leaching; Compliance test for

leaching of granular waste materials and sludges - Part 4: One stage batch test at a liquid to solid ratio of 10 l/kg for materials with particle size below 10 mm (without or with limited size reduction). German version EN 12457- 4. International Organization for Standardization.

DIN/EN/ISO 17294, 2003. Water quality — Application of inductively coupled plasma mass spectrometry (ICP-MS). International Organization for Standardization. German version EN 17294.

DIN/EN/ISO 11885, 2007. Water quality -- Determination of selected elements by inductively coupled plasma optical emission spectrometry (ICP-OES). International Organization for Standardization. German version EN 11885.

DIN 15527, 2008.Characterization of waste - determination of polycyclic aromatic hydrocarbons (PAH) in waste using gas chromatography mass spectrometry (GC/MS). Deutsches Institut für Normung e. V.

DIN 51729, 2011. Testing of solid fuels - Determination of chemical composition of fuel ash. Deutsches Institut für Normung e. V.

DIN 22022-1, 2014. Solid fuels - determination of contents of trace elements - part 1: general rules, sampling and sample preparation - preparation of samples for the analyses (dissolution method). Deutsches Institut für Normung e. V.

DIN 51732, 2014. Testing of solid mineral fuels - determination of total carbon, hydrogen and nitrogen - instrumental methods. Deutsches Institut für Normung e. V.

Domene, X., Mattana, S., Hanley, K., Enders, A., Lehmann, J., 2014. Medium-term effects of corn biochar addition on soil biota activities and functions in a temperate soil cropped to corn. Soil Biol. Biochem. 72, 152–162.

Domene, X., Enders, A., Hanley, K., Lehmann, J., 2015. Ecotoxicological characterization of biochars: Role of feedstock and pyrolysis temperature. Sci. Total Environ. 512, 552–561.

Domene, X., 2016. A critical analysis of meso- and macrofauna effects following biochar supplementation. In: Komang Ralebitso, T. and Caroline Orr, C. (Eds.). Biochar Application – Essencial Soil Microbial Ecology. Elsevier Publishers.

European Biochar Certificate (EBC), 2012. Guidelines for a Sustainable Production of

Biochar. European Biochar Foundation (EBC), Arbaz, Switzerland. http://www.europeanbiochar.org/en/download. Version 6.3E of 14th August 2017.

European Chemicals Agency (ECHA), 2008. Characterization of dose [concentration]-response for environment. Guidance on Information Requirements and Chemical Safety Assessment.

Ferreira, N.G.C., Morgado, R., Santos, M.J.G., Soares, A.M.V.M., Loureiro, S., 2015. Biomarkers and energy reserves in the isopod Porcellionides pruinosus: The effects of long-term exposure to dimethoate. Sci. Total Environ. 502, 91–102.

FOCUS, 1997. Soil persistence models and EU registration. The final report of the work of the Soil Modelling Work group of FOCUS (FOrum for the Co-ordination of pesticide rate models and their Use). European Commission Document 7617/VI/96.

Fountain, M.T., Hopkin, S.P., 2001. Continuous monitoring of *Folsomia candida* (Insecta: Collembola) in a metal exposure test. Ecotoxicol. Environ. Saf. 48, 275–286.

Fountain, M.T., Hopkin, S.P., 2005. *FOLSOMIA CANDIDA* (COLLEMBOLA): A "Standard" Soil Arthropod. Annu. Rev. Entomol. 50, 201–222.

Genesio, L., Miglietta, F., Baronti, S., Vaccari, F.P., 2015. Biochar increases vineyard productivity without affecting grape quality: Results from a four years field experiment in Tuscany. Agric. Ecosyst. Environ. 201, 20–25.

Gomez-Eyles, J. L., Sizmur, T. Collins, C. D., Hodson, M. E., 2011. Effects of biochar and the earthworm Eisenia fetida on the bioavailability of polycyclic aromatic hydrocarbons and potentially toxic elements. Environ. Pollut. 159(2), 616–622.

Hale, S.E., Jensen, J., Jakob, L., Oleszczuk, P., Hartnik, T., Henriksen, T., Okkenhaug, G., Martinsen, V., Cornelissen, G., 2013. Short-term effect of the soil amendments activated carbon, biochar, and ferric oxyhydroxide on bacteria and invertebrates. Environ. Sci. Technol. 47, 8674–83.

Hilber, I., Blum, F., Leifeld, J., Schmidt, H.-P., Bucheli, T.D., 2012. Quantitative determination of PAHs in biochar: a prerequisite to ensure its quality and safe application. J. Agric. Food Chem. 60, 3042–50.

Hilber, I., Bastos, A.C., Loureiro, S., Soja, G., Marsz, A., Cornelissen, G., Bucheli, T.D.,

2017. The different faces of biochar: contamination risk versus remediation tool. J. Environ. Eng. Landsc. Manag. 6897, 1–19.

Hullett, C.R., Levine, T.R., 2003. The Overestimation of Effect Sizes from F Values in Meta-Analysis: The Cause and a Solution. Commun. Monogr. 70, 52–67.

International Biochar Initiative (IBI), 2015. Standardized Product Definition and Product Testing Guidelines for Biochar 7 That Is Used in Soil. http://www.biochar-international.org/sites/default/files/IBI_Biochar_Standards_V2.1_Final.pdf

ISO 10390, 1994. Soil quality – Determination of pH. International Organization for Standardization, Geneva, Switzerland.

Ippolito, J.A., Spokas, K.A., Novak, J.M., Lentz, R.D., Cantrell, K.B., 2015. Biochar elemental composition and factors influencing nutrient retention. In: Lehmann, J., Joseph, S. (Eds.). Biochar for Environmental Management, Science, Technology and Implementation. Routledge, London and New York.

Li, D., Hockaday, W. C., Masiello, C. A., Alvarez, P. J. J. 2011. Earthworm avoidance of biochar can be mitigated by wetting. Soil Biol. Biochem. 43(8), 1732–1737.

Liesch, A.M., Weyers, S.L., Gaskin, J.W., Das, K.C., 2010. Impact of Two Different Biochars on Earthworm Growth and Survival. Ann. Environ. Sci. 4, 1–9.

Lock, K., Janssen, C.R., 2003. Effect of new soil metal immobilizing agents on metal toxicity to terrestrial invertebrates. Environ. Pollut. 121, 123–127.

Loureiro, S., Sampaio, A., Brandão, A., Nogueira, A.J.A., Soares, A.M.V.M., 2006. Feeding behaviour of the terrestrial isopod Porcellionides pruinosus Brandt , 1833 (Crustacea , Isopoda) in response to changes in food quality and contamination. Sci. Total Environ. 369, 119–128.

Loureiro, S., Amorim, M.J.B., Campos, B., Rodrigues, S.M.G., Soares, A.M.V.M., 2009. Assessing joint toxicity of chemicals in Enchytraeus albidus (Enchytraeidae) and Porcellionides pruinosus (Isopoda) using avoidance behaviour as an endpoint. Environ. Pollut. 157, 625–636.

Maienza, A., Baronti, S., Cincinelli, A., Martellini, T., Grisolia, A., Miglietta, F., Renella, G., Stazi, S.R., Vaccari, F.P., Genesio, L., 2017. Biochar improves the fertility of a Mediterranean vineyard without toxic impact on the microbial community. Agron. Sustain. Dev.

37(5), 47.

Malev, O., Contin, M., Licen, S., Barbieri, P., De Nobili, M., 2016. Bioaccumulation of polycyclic aromatic hydrocarbons and survival of earthworms (Eisenia andrei) exposed to biochar amended soils. Environ. Sci. Pollut. Res. 23, 3491–3502.

Mankasingh, U., Choi, P.-C., Ragnarsdottir, V., 2011. Biochar application in a tropical, agricultural region: A plot scale study in Tamil Nadu, India. Appl. Geochemistry 26, S218–S221.

Marks, E.A.N., Mattana, S., Alcañiz, J.M., Domene, X., 2014. Biochars provoke diverse soil mesofauna reproductive responses in laboratory bioassays. Eur. J. Soil Biol. 60, 104–111.

Martin, S.M., Kookana, R.S., Zwieten, L. Van, Krull, E., 2012. Marked changes in herbicide sorption – desorption upon ageing of biochars in soil. J. Hazard. Mater. 232, 70–78.

Meyer, S., Genesio, L., Vogel, I., Schmidt, H.-P., Soja, G., Someus, E., Shackley, S., Verheijen, F.G.A., Glaser, B., 2017. Biochar standardization and legislation harmonization IN legislation. J. Environ. Eng. Landsc. Manag. 6897.

Ministry of the Environment Finland (MEF), 2007. Government Decree on the Assessment of Soil Contamination and Remediation Needs 214/2007, March 1 2007.

Morgado, R.G., Gomes, P.A.D., Ferreira, N.G.C., Cardoso, D.N., Santos, M.J.G., Soares, A.M.V.M., Loureiro, S., 2016. Toxicity interaction between chlorpyrifos, mancozeb and soil moisture to the terrestrial isopod Porcellionides pruinosus. Chemosphere 144, 1845–1853.

Nair, V.D., Nair, P.K.R., Dari, B., Freitas, A.M., Chatterjee, N., Pinheiro, F.M., 2017. Biochar in the Agroecosystem–Climate-Change–Sustainability Nexus. Front. Plant Sci. 8.

OECD 232, 2009. Collembolan Reproduction Test in Soil. Organisation for Economic Co-operation and Development. OECD Publishing.

Oleszczuk, P., Jośko, I., Kuśmierz, M., 2013. Biochar properties regarding to contaminants content and ecotoxicological assessment. J. Hazard. Mater. 260, 375–382.

Pérez-Rodríguez, P., Soto-Gómez, D., Paradelo, M., López-Periago, J.E., 2017. Concentration levels of new-generation fungicides in throughfall released by foliar wash-off from vineyards. J. Environ. Manage. 203, 467–475.

Pesticide Properties DataBase (PPDB), 2017**.** University of Hertfordshire, UK. Accessed on 15/10/2016 http://sitem.herts.ac.uk/aeru/ppdb/en/atoz.htm

Sakrabani, R., Kern, J., Mankasingh, U., Zavalloni, C., Zanchettin, G., Bastos, A.C., Tammeorg, P., Jeffery, S., Glaser, B., Verheijen, F.G.A., 2017. Representativeness of European biochar research: part II – pot and laboratory studies. J. Environ. Eng. Landsc. Manag. 25, 152–159.

Salem, M., Kohler, J., Rillig, M.C., 2013. Palatability of carbonized materials to Collembola. Appl. Soil Ecol. 64, 63–69.

Santos, M.J.G., Soares, A.M.V.M., Loureiro, S., 2010. Joint effects of three plant protection products to the terrestrial isopod Porcellionides pruinosus and the collembolan Folsomia candida. Chemosphere 80, 1021–1030.

Schmidt, H.-P., Kammann, C., Niggli, C., Evangelou, M.W.H., Mackie, K.A., Abiven, S., 2014. Biochar and biochar-compost as soil amendments to a vineyard soil: Influences on plant growth, nutrient uptake, plant health and grape quality. Agric. Ecosyst. Environ. 191, 117–123.

Shachak, M., Chapman, E.A., Steinberger, Y., 1976. Feeding, Energy Flow and Soil Turnover in the Desert Isopod, Hemilepistus reaumuri. Oecologia 69, 57–69.

Singh, B., Dolk, M. M., Shen, Q., Camps-Arbestain, M., 2017. 3 Biochar pH, electrical conductivity and liming potential. Biochar: A Guide to Analytical Methods, 23.

Smith, C.R., Buzan, E.M., Lee, J.W., 2013. Potential Impact of Biochar Water-ExtracTable Substances on Environmental Sustainability. ACS Sustain. Chem. Eng. 1, 118–126.

Tammeorg, P., Bastos, A.C., Jeffery, S., Rees, F., Kern, J., Graber, E.R., Ventura, M., Kibblewhite, M., Amaro, A., Budai, A., Cordovil, C.M. d. S., Domene, X., Gardi, C., Gascó, G., Horák, J., Kammann, C., Kondrlova, E., Laird, D., Loureiro, S., Martins, M.A.S., Panzacchi, P., Prasad, M., Prodana, M., Puga, A.P., Ruysschaert, G., Sas-Paszt, L., Silva, F.C., Teixeira, W.G., Tonon, G., Delle Vedove, G., Zavalloni, C., Glaser, B., Verheijen, F.G.A., 2017. Biochars in soils: towards the required level of scientific understanding. J. Environ. Eng. Landsc. Manag. 25, 192–207.

Topoliantz, S., Ponge, J.-F., 2003. Burrowing activity of the geophagous earthworm Pontoscolex corethrurus (Oligochaeta: Glossoscolecidae) in the presence of charcoal. Appl. Soil Ecol. 23, 267–271.

Topoliantz, S., Ponge, J.-F., 2005. Charcoal consumption and casting activity by

Pontoscolex corethrurus (Glossoscolecidae). Appl. Soil Ecol. 28, 217–224.

Tourinho, P.S., Waalewijn-kool, P.L., Zantkuijl, I., Jurkschat, K., Svendsen, C., Soares, A.M.V.M., Loureiro, S., Gestel, C.A.M. Van, 2015. CeO 2 nanoparticles induce no changes in phenanthrene toxicity to the soil organisms Porcellionides pruinosus and Folsomia candida. Ecotoxicol. Environ. Saf. 113, 201–206.

Uchimiya, M., Bannon, D.I., 2013. Solubility of Lead and Copper in Biochar-Amended Small Arms Range Soils: Influence of Soil Organic Carbon and pH. J. Agric. Food Chem. 61, 7679–7688.

Van Zwieten, L. Van, Kimber, S., Morris, S., 2010. Effects of biochar from slow pyrolysis of papermill waste on agronomic performance and soil fertility. Plant Soil 327, 235–246.

Verheijen, F.G.A., Jeffery, S., Bastos, A.C., van der Velde, M., and Diafas, I., 2009. Biochar Application to Soils - A Critical Scientific Review of Effects on Soil Properties, Processes and Functions. EUR 24099 EN, Office for the Official Publications of the European Communities, Luxembourg, 149pp.

Verheijen, F. G. A., Montanarella, L., Bastos, A. C., 2012 Sustainability, certification, and regulation of biochar Pesqui. Agropec. Bras. 47 649–53.

Verheijen, F.G.A., Graber, E.R., Ameloot, N., Bastos, A.C., Sohi, S., Knicker, H., 2014. Biochars in soils: New insights and emerging research needs. Eur. J. Soil Sci. 65, 22–27.

Verheijen, F. G. A. , Mankasingh, U., Penizek, V., Panzacchi, P., Glaser, B., Jeffery, S., Bastos, A. C. Tammeorg, P., Kern, J., Zavalloni, C., Zanchettin, G., Sakrabani, R. , 2017. Representativeness of European biochar research: part I–field experiments. Journal of Environmental Engineering and Landscape Management, 25(2), 140-151.

Weyers, S.L., Spokas, K.A., 2011. Impact of Biochar on Earthworm Populations: A Review. Appl. Environ. Soil Sci. 2011, 1–12.

Yang, Y., Sheng, G., Huang, M., 2006. Bioavailability of diuron in soil containing wheat-straw-derived char. Sci. Total Environ. 354, 170–178.

Zimmer, M., 2002. Nutrition in terrestrial isopods (Isopoda : Oniscidea): an evolutionary-ecological approach. Biol. Rev. 455–493.

Zimmer, M., Kautz, G., Topp, W., 2003. Leaf litter-colonizing microbiota: supplementary food source or indicator of food quality for Porcellio scaber (Isopoda : Oniscidea) Eur. J. Soil Biol. 39, 209–216.

Zimmer, M., Kautz, G. and Topp, W., 2005. Do woodlice and earthworms interact synergistically in leaf litter decomposition? Funct. Ecol. 19, 7-16.

2.9. Supplementary Information

Table S2.1. Summary of meteorological data in the field site at Estação Vitivinícola da Bairrada (Bairrada, Portugal) between October 2012 and October 2014.

	Min. T (ºC)	Max. T (ºC)	Mean T (ºC)	Rel. humidity (%)	Precipitation (mm)
October 2012-March 2013	5.35	15.6	11.3	83.9	360
April 2013-September 2013	14.5	29.7	23.4	67.4	10.0
October 2013-March 2014	6.55	19.1	13.5	81.1	280
April 2014-October 2014	12.9	25.0	20.0	76.2	89.4

Table S2.2. Plant protection products applied at the vineyards in Estação Vitivinícola da Bairrada (Bairrada, Portugal) during 2013 and 2014.

Date of treatment	**PPPs and action[1]**	**Active ingredient**	**Application rate of active ingredient kg/ha (as stated by Estação Vitivinícola Bairrada)**	**Application rate of active ingredient kg/ha (as recommended by PPPs producers)[2]**
2013				
17/4/2013	Quadris Max (F)	azoxystrobin	0.112	
7/5/2013	Pergado F (F)	folpet and mandipropamid	1.2 of folpet and 0.15 of mandipropamid	
	Topaze (F)	propiconazole	0.152	
20/5/2013	Ridomil Gold (F)	mefenoxam	1.68	
	Topaze (F)	propiconazole	0.152	
5/6/2013	Pergado F (F)	folpet and mandipropamid	1.2 of folpet and 0.15 of mandipropamid	
	Talendo (F)	proquinazid	0.04	
3/7/2013	Pergado C (F, B)	Cu oxychloride 13.95 % and mandipropamid 2.5 %		0.607 of Cu oxychloride and 0.112 of mandipropamid
	Talendo (F)	proquinazid	0.04	
17/7/2013	Bordeaux mixture (F)	Cu sulfate		1
	Cosan WDG (F)	sulfur		2.4
2014				
25/3/2014	Quadris Max (F)	azoxystrobin	0.112	
7/4/2014	Quadris Max (F)	azoxystrobin	0.112	
9/4/2014	Folar Max (H)	glyphosate and oxifluorfen		0.8 0.12
16/4/2014	Quadris Max (F)	azoxystrobin	0.112	

6/5/2014	Pergado F (F)	folpet (40 %) and mandipropamid (5 %)	1.2 of folpet and 0.15 of mandipropamid	
	Topaze (F)	propiconazole	0.152	
16/5/2014	Ridomil Gold (F)	mefenoxam	1.68	
	Topaze (F)	propiconazole	0.152	
26/5/2014	Ridomil Gold (F)	mefenoxam	1.68	
	Dynali (F)	difenoconazol (6 %) and cyflufenamid (3 %)		0.039 of difenoconazole and 0.0195 of cyflufenamid
20/6/2014	Pergado F (F)	folpet and mandipropamid	1.2 of folpet and 0.15 of mandipropamid	
	Talendo (F)	proquinazid	0.04	
7/7/2014	Pergado C (F, B)	Cu oxychloride 13.95 % and mandipropamid 25 %		0.607 of Cu oxychloride and 0.112 of mandipropamid
	Talendo (F)	proquinazid	0.04	
	Switch (F)	Cyprodinil (37.5 %) and (25 %) fludioxonil		0.298 of cyprodinil 0.198 of fludioxonil
	Actara (I)	thiamethoxam	0.04	
	Stimufol K - fertilizer	n.a.	n.a.	n.a.
22/8/2014	Chorus (F)	cyprodinil		0.4

[1]Action: F-fungicide, B-bactericide, H-herbicide, I-insecticide, A-acaricide, n.a. – not applicable.

[2]Where the amount of pesticide is not stated, the producers' recommended dose is applied in the PEC calculation

Table S2.3. Active ingredients of the PPPs with their main physicochemical properties and ecotoxicity data available in the Pesticide Properties DataBase (PPDB).

Active ingredient	Action	Molecular mass (g/ mol)	Bulk density (g/ml)	Vapour pressure at 25° C (mPa)	Log Kow[1]	Soil degradation DT_{50} soil – field (days)	Earthworms acute 14 days LC_{50} (mg/kg)	Collembola
Azoxystrobin	F	403.4	1.34	$1.10x10^{-07}$	2.5	180.7	283	-
Glyphosate	H	169.1	1.71	0.013	-3.2	23.79	>5 600	-
Oxyfluorfen	H	361.7	1.53	0.026	4.86	73	>1 000	*F. candida* NOEC reproduction 1.25 mg/kg
Folpet	F	296.56		$2.10x10^{-0.2}$	3.02	3	>500	-
Mandipropamid	F	411.9	-	$9.40x10^{-0.4}$	3.2	13.6	>500	-
Propiconazole	F	342.22	1.32	0.056	3.72	214	686	-
Mefenoxam	F	279.33	1.2	0.75	1.75	113	>1 000	-
Difenoconazol	F	406.26	1.37	$3.33x10^{-0.5}$	4.36	85	>610	-
Cyflufenamid	F	412.36	1.35	0.0354	4.7	25.3	>500	-
Proquinazid	F	372.2	1.57	0.09	5.5	30.5	>1 000	-
Cu oxychloride	F, B	427.14	-	0.00001	0.44	10 000[2]	>489.6	-
Cyprodinil	F	225.29	1.21	$5.10x10^{-0.1}$	$1.00x10^{04}$	45	192	-
Fludioxonil	F	248.19	1.54	$3.90x10^{-0.4}$	4.12	20.5	≥1 000	-
Thiamethoxam	I	291.71	1.57	$6.60x10^{-0.6}$	-0.13	39	>1 000	-
Cu sulphate	F, B	461.3	2.29	$3.40x10^{-10}$	0.44	1 600[2]	>155	-
Sulfur	F, A	32.06	2.36	0.098	0.23	-	>2 000	-

[1]Octanol-water partition coefficient at pH 7, 20°C.

[2]As 'copper does not degrade' (Paranjape et al., 2015; PPDB, 2017) and the DT50 values (PPDB, 2017) are used for PECs calculation for the purpose of estimation of the fate of these compounds, as it was already done for vineyard soil (Vaj et al., 2014).

Table S2.4. Chronic toxicity of the active ingredients (a.i.) reported in the literature for soil invertebrates and predicted no effect concentrations (PNECs) derived out of lowest no observed effect (NOEC) using safety factor of 100. The underlined NOECs were used in PNECs calculation.

Active ingredient	Chronic effects (reproduction) (mg a.i. kg−1 dw soil)	PNEC derived with safety factor of 100
azoxystrobin	*E. fetida* NOEC=20 (EFSA, 2010; PPDB) *E. andrei* NOEC<50, EC50=42 (Leitao et al., 2014) *E. crypticus* EC50=99.2 (Leitao et al., 2014) *F. candida* EC50=92 (Leitao et al., 2014)	0.200
Cu oxychloride	*E. fetida* NOEC<8.92 (Helling et al., 2000) *E. fetida* NOEC<15 (EFSA, 2008; PPDB)	0.089
Cu sulphate	*E. fetida* NOEC<15 (EFSA, 2008; PPDB)	0.150
cyprodinil	Earthworms NOEC=1.13 (EFSA, 2005)	0.011
fludioxonil	Earthworms NOEC=1.3 (EFSA, 2007) Earthworms NOEC=20 (PPDB)	0.013
mandipropamid	*Eisenia sp.* NOEC≥16 (EFSA, 2012) *F. candida* NOEC≥20 (EFSA, 2012)	0.160
propiconazole	Earthworms LOEC=0.833 (PPDB)	0.008
proquinazid	Earthworms LOEC=50.9 (EFSA, 2009; PPDB)	0.509
thiamethoxam	*F. candida* NOEC=1 (Alves et al., 2013) Eearthworms NOEC=5.4 (PPDB) *F. candida* NOEC=12.27 (Seres et al., 2016)	0.010

2.9.1. Supplementary References

Alves, P.R.L., Cardoso, E.J.B.N., Martines, A.M., Sousa, J.P., Pasini, A., 2014. Seed dressing pesticides on springtails in two ecotoxicological laboratory tests. Ecotoxicol. Environ. Saf. 105, 65–71. doi:10.1016/j.ecoenv.2014.04.010

European Food Safety Authority. Conclusion on the peer review of the pesticide risk assessment of the active substance azoxystrobin. EFSA Journal, 2010. 8(4):15421542. [110 pp.]. doi:10.2903/j.efsa.2010.1542. Available online: www.efsa.europa.eu

European Food Safety Authority. Conclusion on the peer review of the pesticide risk assessment of the active substance copper (I), copper (II) variants namely copper

hydroxide, copper oxychloride, tribasic copper sulfate, copper (I) oxide, Bordeaux mixture. EFSA Journal 2008; 187, 1-101. Available online: www.efsa.europa.eu

European Food Safety Authority. Conclusion on the peer review of the pesticide risk assessment of the active substance cyprodinil. EFSA Scientific Report, 2005. 51, 1-78. Available online: www.efsa.europa.eu

European Food Safety Authority. Conclusion on the peer review of the pesticide risk assessment of the active substance fludioxonil. EFSA Scientific Report, 2007. 110, 1-85. Available online: www.efsa.europa.eu

European Food Safety Authority. Conclusion on the peer review of the pesticide risk assessment of the active substance mandipropamid. EFSA Journal, 2012. 10(11): 2935. [76pp.] doi:10.2903/j.efsa.2012.2935. Available online: www.efsa.europa.eu/efsajournal

European Food Safety Authority, Arena M, Auteri D, Barmaz S, Bellisai G, Brancato A, Brocca D, Bura L, Byers H, Chiusolo A, Court Marques D, Crivellente F, De Lentdecker C, De Maglie M, Egsmose M, Erdos Z, Fait G, Ferreira L, Goumenou M, Greco L, Ippolito A, Istace F, Jarrah S, Kardassi D, Leuschner R, Lythgo C, Magrans JO, Medina P, Miron I, Molnar T, Nougadere A, Padovani L, Parra Morte JM, Pedersen R, Reich H, Sacchi A, Santos M, Serafimova R, Sharp R, Stanek A, Streissl F, Sturma J, Szentes C, Tarazona J, Terron A, Theobald A, Vagenende B, Verani A and Villamar-Bouza L. Conclusion on the peer review of the pesticide risk assessment of the active substance propiconazole. EFSA Journal, 2017. 15(7):4887, 28 pp. https://doi.org/10.2903/j.efsa.2017.4887

European Food Safety Authority. Conclusion on the peer review of the pesticide risk assessment of the active substance proquinazid on request from the European Commission. EFSA Journal, 2009. 7(10):1350. [132 pp.]. doi:10.2903/j.efsa.2009.1350. Available online: www.efsa.europa.eu

European Food Safety Authority. Conclusion on the peer review of the pesticide risk assessment for bees for the active substance thiamethoxam considering all uses other than seed treatments and granules. EFSA Journal, 2015. 13(8):4212, 70 pp. doi:10.2903/j.efsa.2015.4212. Available online: www.efsa.europa.eu/efsajournal

Helling, B., Reinecke, S.A., Reinecke, A.J., 2000. Effects of the fungicide copper oxychloride on the growth and reproduction of Eisenia fetida (Oligochaeta). Ecotoxicol. Environ. Saf. 46, 108–116. doi:10.1006/eesa.1999.1880

Paranjape, K., Gowariker, V., Krishnamurthy, V.N., Gowariker, S., 2015. The Pesticide Encyclopedia. CAB International.

PPDB. Pesticide Properties DataBase, 2017. http://sitem.herts.ac.uk/aeru/ppdb/en/atoz.htm

Seres, A., Hracs, K., Gyursco, G., Sarospataki, M., Szakalas, J., Nagy, P.I., 2016. Laboratory studies on the effects of a neonicotinoid-containing seed treatment product on non-target soil animals 3, 7–14. doi:10.18380/SZIE. COLUM.2016.3.2.7.

Vaj, C., Van Gestel, A. M. C., Vighi, M., 2014. Year-round behaviour of soil microarthropod communities under plant protection product application. Ecotoxicology 23, 898–913. doi:10.1007/s10646-014-1232-8.

Chapter 3

Influence of biochar particle size distribution on biota responses

Influence of biochar particle size distribution on biota responses

3.1. Abstract

Despite the increasing interest for biochar as a soil amendment, a knowledge gap remains on its impacts on non-target soil species that have a leading role in both structure and function of soils. The present study tested the hypothesis that biochar particle size and application rate can play a role in the toxicity to biota. Pine woodchip biochar was incorporated in a clean soil at three particle size classes: small (<0.5 mm), medium (1-2 mm), and large (<4 mm), and at two concentrations: 1 % and 6 % w/w, giving a total of six treatments. Soil without biochar was used as a negative control. A first screening to study the most adequate soil-biochar equilibration period was carried out by using an avoidance behaviour of the earthworm *Eisenia andrei* in laboratory-controlled conditions (48h). Moving towards a more ecologically representative approach, a 28-days microcosm experiment was conducted in a greenhouse and survival, vertical distribution and weight changes of *E. andrei*, and feeding activity with the bait-lamina method were recorded. After 28 days, soil leachates from the microcosms were collected to assess their effects on *Daphnia magna* immobilisation and *Vibrio fischeri* bioluminescence. Feeding experiments with *E. andrei* were also performed to address changes in body mass and to conduct a screening of PAHs/PAH-type metabolites in earthworms' tissue. The 6 % <0.5 mm treatment induced significant avoidance behaviour of earthworms in the laboratory bioassays when incubated for 96h. Significant reduction in bait-lamina consumption in microcosms was also observed in 6 % <0.5 mm treatment. Moreover, particle size as a factor was statistically significant considering the loss of weight in the feeding experiment and Naphthalene-type metabolites detected in the earthworms' tissue. Elevated concentrations when exposed to <0.5 mm biochar particles were observed, both at 1 % and 6 % application rates. Aquatic bioassays with leachates resulted in the absence of toxicity to *D. magna* and *V. fischeri*. Overall results suggest that particles <0.5 mm of pine woodchip biochar can induce sub-lethal effects to soil biota.

Key words: biochar, earthworms, soil microcosms, bait-laminas, leachates, PAHs

3.2. Introduction

Biochar is a product obtained in the process of thermo-chemical conversion of biomass under low-to-no-oxygen conditions (IBI, 2015), which physicochemical characteristics are determined by the type of biomass and the processing conditions (Demirbas, 2004; Verheijen et al., 2009). Bearing in mind the growing intentions for the employment of biochar as a soil amendment and a carbon sink, a robust hazard assessment is necessary to ensure sustainability before large-scale implementation can be considered in policy development (Verheijen et al., 2012; Meyer et al., 2017).

Particle size distribution is a physical characteristic of biochar mostly dependent on the feedstock used (Chia et al., 2015). Together with biochar particles' shape and porosity, these factors affect the hydrology of biochar-enriched soils (Liu et al., 2017). Nonetheless, due to the heterogeneity of biochar as a product and complexity of the interactions within biochar-soil matrices, conflicting results were revealed in different studies focused on the effect of biochar particle size distribution on the sorption of contaminants, its PAH content and the effects on organisms. Zheng et al. (2017) concluded that smaller biochar particles are responsible for immobilizing Cd, Pb and Zn in contaminated soil, but that was not the case of As. In another study, biochar particle <2 mm had higher sorption capacity for simazine than that of larger particles (>2 mm) when applied at the rate of 100 t/ha, while also reducing the pesticide' mineralization and leaching from soil (Jones et al., 2011). The increase of total PAHs concentrations at lower particle size ranges have been reported (Hilber et al., 2012; Li et al., 2016) due to changes in the surface area-to-volume and/or mass ratio (Hilber et al., 2012).

Biochar particle size- and concentration-dependent effects on microorganisms have been demonstrated (Chen et al., 2017; Liang et al., 2016). Up to date, studies of biochar particle size effects on soil invertebrates and their interactions are lacking. There is evidence that two collembolan species, *Coecobrya tenebricosa* and *Folsomia fimetaria*, ingested hydrochar independently on the particle size ranges tested, while the fitness of those fed only on the hydrochar was slightly decreased (Salem et al., 2013). An epigeic earthworm species *Eisenia fetida* ingested fine biochar particles in the range of 50 µm (Sevin et al., 2017), while a geophagous earthworm *Pontoscolex corethrurus* probably benefited from biochar (<2 mm) due to changes in their gut pH, rather than using it as a direct source of nutrients (Topolianz and Ponge, 2005; reviewed by Weyers and Spokas, 2011).

The aim of the present study was to infer the contribution of particle size and application rate of biochar to the toxicity in soil biota. For that, the earthworm species *Eisenia andrei* was chosen as a model organism in the experiments with unamended and biochar-amended soil, while the bioluminescent bacterial species *Vibrio fischeri* and the cladoceran species *Daphnia magna* were used in the experiments with aqueous leachates of the unamended and amended soil. Behavioural, functional and individual endpoints were evaluated over the series of experiments in the following order: preliminary earthworms' avoidance bioassays, greenhouse microcosms experiment with earthworms and bait-laminas, leachates toxicity assessment with daphnids and bacteria, and an earthworm feeding experiment in the laboratory. To our knowledge, this is the first study in the biochar literature addressing inherent toxicity of biochar particles to biota while combining terrestrial and aquatic approaches for a robust ecotoxicological assessment. A slow pyrolysis woodchip biochar was chosen with levels of trace metals, PAHs and other potentially toxic elements lower than the benchmark concentrations proposed by biochar quality standards (EBC, 2012; IBI, 2015). We, therefore, expected different sub-lethal responses to biochar-amended soil relative to the unamended control soil, as well as to the particle sizes and concentrations of biochar applied.

3.3. Materials and methods

3.3.1. Test soil and biochar

The soil used in the study is a natural agricultural topsoil (0-15 cm), sampled in October 2014 from a pristine field in agricultural area located in the Mondego valley (Central Portugal), with no history of contamination or inputs of pesticides and inorganic fertilizers in the last 4 years (Lemos et al., 2010; Santos et al., 2011). It is a loamy sand of the following characteristics: sand 86.6 %, silt 7.6 %, clay 5.8 %, pH (H_2O) of 6.9, soil organic matter 1.88 % and maximum water holding capacity of 32 %.

Biochar, obtained from the Swiss Biochar gmbh, Switzerland, was produced by slow pyrolysis (620°C highest treatment temperature, 20 min) of a mixture of wood chip residues. Particle size distribution (w/w) was as follows: 4% (<0.1 mm), 25% (0.1-0.5 mm), 34% (0.5-2 mm), 37% (>2 mm), with an average of 29.5 µm and pH (H2O) of 9.1. Total 16 PAHs (US EPA) concentration was 0.48 mg/kg, where naphthalene alone was 0.48 mg/kg, and the rest of the PAHs were

below the detection limit (<0.1 mg/kg). Sum of the 7 indicator PCBs (dioxins) was <0.002 mg/kg.

Table 3.1. summarizes the main physical and chemical characteristics of the biochar.

Table 3.1. Summary of the main physical and chemical characteristics (dry weight) of the woodchip residue biochar. Abbreviations stand for maximum water holding capacity (WHC_{max}) and electrical conductivity (EC).

	Biochar
pH (1:5, H_2O)	10.1
WHC_{max} (105°C) (%)	73.2
Bulk density (g/cm^3)	0.55
EC (µS/cm)	3,000
Salts (g/kg)	8.40
Organic carbon (%)	75.0
H (%)	0.47
Ash (550°C) (%)	18.6
N (%)	1.8
H:C (molar ratio)	0.07
O:C (molar ratio)	0.04
P (mg/kg)	1,300
K (mg/kg)	10,400
S (mg/kg)	372
Ca (mg/kg)	42,200
Mg (mg/kg)	2,980
B (mg/kg)	39
Na (mg/kg)	744
Metals (mg/kg)[1]	
Fe	2,420
Hg	<0.07
Ni	17
Pb	<2
Cr	27
Cu	16
Zn	70
Cd	<0.2

PAHs (mg/kg)[2]	
Naphtalene	0.48
Acenaphthylen	<0.1
Acenaphthen	<0.1
Fluoren	<0.1
Phenanthren	<0.1
Anthracen	<0.1
Fluoranthen	<0.1
Pyren	<0.1
Benz-[a]-anthracen	<0.1
Chrysen	<0.1
Benzo[b]fluoranthene	<0.1
Benzo[k]fluoranthene	<0.1
Benzo[a]pyren	<0.1
Indeno[1,2,3,-cd]-pyren	<0.1
Dibenz-[a,h]-anthracen	<0.1
Benzo[ghi]perylen	<0.1
ΣPAHs (mg/kg)	0.48
Σ_7 ind. PCBs (mg/kg)[3]	<0.002

[1]Metals were determined by microwave digestion (DIN/ISO 17294-2)
[2]PAHs were determined by SPME coupled to GC/MS (DIN EN 15527), where individual PAH values were below or equal to the limit of detection (0.1 mg/kg);
[3]The 7 indicator PCBs (incl. BG) were determined by HRGC/HRMS

3.3.2. Treatments

After the soil was brought to the laboratory it was air dried and sieved to < 4 mm. Biochar was first air-dried for 96 h at 20±1°C in a dark, and then mechanically crushed and sieved to the following particle sizes: <0.5 mm (referred as S – small further in the text), 1-2 mm (referred as M – medium further in the text) and <4 mm (referred as L – large further in the text). Biochar was applied to soil at concentrations of 1 % (w/w) and 6 % (w/w). Water holding capacity (WHC) was determined by loss of weight, for the unamended soil and for each of the amended treatments. Unamended soil (0 % biochar) was used as a negative control.

3.3.3. Chemical analysis

Total contents of selected metals and trace elements in the soil and biochar-amended treatments were analysed in an external laboratory by inductively coupled plasma mass

spectrometry (ICP-MS) screening after an aqua regia digestion (DIN EN ISO 17294-2). The pH was measured in water at 1:5 soil-water ratio following the ISO standard protocol for pH determination in soil (ISO 10390, 1994).

3.3.4. Experimental design

Screening test: earthworms' avoidance bioassay

The earthworms *Eisenia andrei* (Bouché, 1972) were obtained from established laboratory cultures maintained at 20±1ºC with a photoperiod of 16:8 hours (light:dark). Cultures are kept in opaque 24 l plastic containers, with a mixture of soil potting mix and peat, at pH between 6 and 7, adjusted with CaCO3, and at 70 % of its water holding capacity (WHC). The earthworms were fed with horse manure previously frozen to kill fly eggs, if present. It was gradually thawed afterwards and used weekly as a food source, by covering the surface of container with a 3-4 cm layer. Adult individuals were three months old, with developed clitellum and in a range of 300-600 mg of body weight.

The biochar treatments with the soil moisture adjusted to 60 % were incubated with soil for 96 hours and for 14 days. The unamended soil was treated in the same way. A screening avoidance test with earthworms was conducted in the laboratory following the standardized ISO avoidance protocol (ISO/DIS 17512-1, 2005). Each test vessel A was divided by a removal plastic barrier in the middle before applying the unamended soil and the test treatments in a uniform way. Each vessel side contained around 350 g of unamended soil in one half, and the same amount of the amended soil in the other half. As a test validation, the same procedure was carried out with unamended soil in both halves of the vessel. Five replicates were used for the treatments, and for the unamended soil. Ten adult earthworms, each of them at least 3 months old, were positioned in the middle, on the border of the soil and the amended-soil. After 48 h under a 20±1ºC and a 16:8 hours photoperiod (light:dark), the number of earthworms in each half of the vessel was recorded. This bioassay was carried out in order to get an insight on possible influence of soil-biochar structural equilibration to earthworms' avoidance/preference behaviour and thus, study the most adequate equilibration period to be used for the follow-up experiments. After 48 h of exposure, the number of earthworms in each half of the vessel was recorded. Mean avoidance per treatment (A, in %) was calculated according to the *Equation 1* (ISO/DIS 17512-1, 2005):

$$A = \frac{(C-T)*100}{N} \qquad \textit{(Eq. 1)}$$

where C is the number of earthworms in unamended soil, T represents the number of earthworms in amended soil, and N is the total number of 10 earthworms used per replicate.

These results served in decision making in terms of treatments' choices and biochar-soil equilibrium duration, both for the greenhouse and for the laboratory feeding experiments, before introducing the earthworms and bait-laminas, or only earthworms in case of the feeding experiment.

Greenhouse microcosms experiment

Greenhouse soil microcosms were conducted with six replicates per treatment, including unamended soil, in a fully randomized design. Three particle size classes were used: small (< 0.5 mm), medium (1-2 mm), large (<4 mm), and two concentrations of the biochar: 1 % and 6 % w/w, giving six treatments. Unamended soil was used as a negative control. Microcosms consisted of PVC tubes covered with a nylon mesh at the bottom and over the top to prevent the earthworms from escaping (**Figure 3.1.**). The block of 42 microcosms was protected with polystyrene panels on the lateral sides to avoid uneven heating of the PVC tubes and, therefore, to prevent uneven water evaporation. The microcosm configuration is presented in **Figure 3.1**. Each contained around 1.6 kg of soil/amended soil in total. The unamended soil (800 g) was at the bottom half of the column, and the same amount of biochar-amended soil in the top layer (treatments), both layers at 60 % of the maximum water holding capacity (WHC). Three bait-laminas and five adult earthworms were introduced in each column, in this order. The amended soil layer was added over an unamended layer to simulate topsoil biochar application, a common way of applying biochar to arable soils (Verheijen et al., 2010). During the experiment, moisture content was maintained by weighing each pot and adding the corresponding amount of water that was lost by evaporation. At each weighing step, every third day, a new randomisation of the microcosms was performed. Feeding activity was assessed using the ISO bait-lamina consumption assay (ISO TC 190/SC 4 N, 2012). Bait-laminas were filled with the mixture of L-cellulose, oat bran and activated charcoal made in proportion 70:27:3 (Kratz, 1998; Santos et al., 2011). The mean values (± standard deviations) for humidity and

temperature during the experiment in the greenhouse were 65.6±22.9 % and 17.6±8.2 °C. The experiment lasted for 28 days, including the biochar-soil equilibrium period of 96 h.

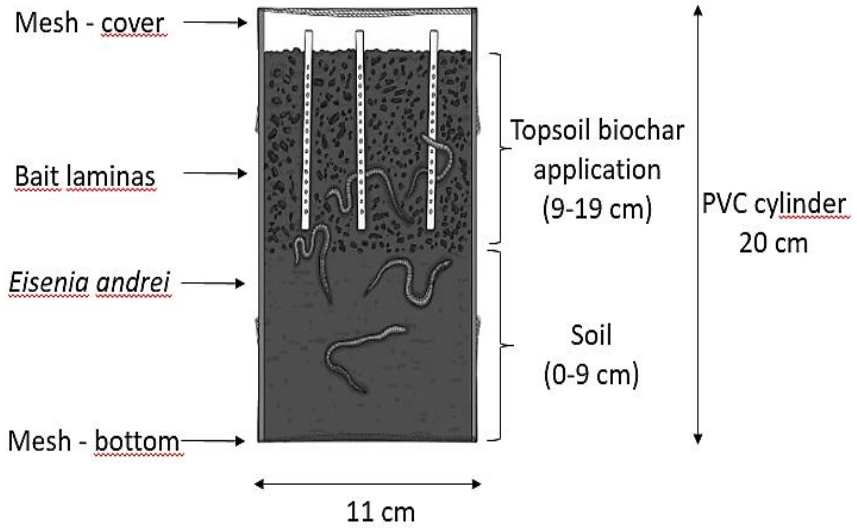

Figure 3.1. Schematic diagram of a microcosm used in the greenhouse experiment.

At the end of the experiment the presence of the animals in each layer was recorded. The endpoints observed were survival and location of the earthworms, body weight changes, and number of empty apertures in bait-laminas. Earthworms biomass per microcosm was recorded by pooling five earthworms and expressing the mean weight per earthworm. Loss of body weight (LW, expressed in grams) was calculated according to the *Equation 2* (Lima et al., 2011):

$$\mathrm{LW} = \frac{\mathrm{wi}-\mathrm{wf}}{\mathrm{wi}} \qquad \textit{(Eq. 2)}$$

where wi represents the initial mean weight of pooled earthworms per microcosm, and wf is the final mean weight of polled earthworms per microcosm, recorded at the end of the greenhouse experiment.

Leaching procedure and aquatic bioassays

The OECD guideline for leaching the soil columns was adapted in order to be used for water percolation through the disturbed soil cores, after removing organisms and bait-laminas from the greenhouse microcosms (OECD 312, 2004). The schematic diagram of the leaching procedure applied is depicted in **Figure 3.2**. The volume of the microcosm as an approximate to a cylinder (11 cm diameter and 20 cm high) was 1,900.66 cm3. According to the OECD 312:2004 protocol, proportionally to the amount of rainwater that is recommended for the cylinder of 4 cm diameter and 30 cm high, we applied 1,265 ml of deionized water. The step of adding 0.1 M CaCl2 to water for creating artificial rainwater was omitted because we intended to use the leachates for both chemical analysis and aquatic bioassays. Therefore, a possible interference if 0.1 M CaCl2 in the bioassays' results and chemical analysis was excluded. The amount of water used represents a simulation of an extremely high rainfall event of approximately 200 mm over 48 hours at 18-25°C (OECD 312, 2004). By applying this volume of deionized water, we assured that enough leachate was produced for the planned ecotoxicity bioassays as well as for the chemical analysis. The collected samples were centrifuged at 3,000 rpm for 20 minutes and stored up to one week at 4°C prior to use for ecotoxicological and chemical analysis. Aquatic bioassays were conducted with freshly produced samples, not older than one week.

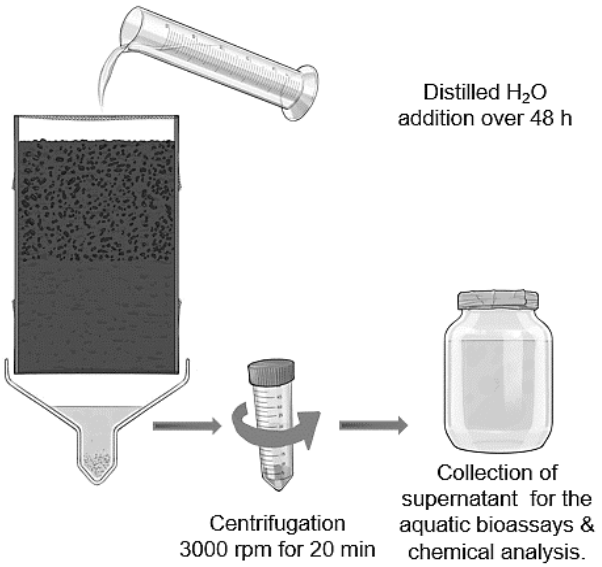

Figure 3.2. Scheme of the leaching procedure: water percolation and leachate collection from a disturbed soil core of a microcosm, after the greenhouse experiment.

The *Daphnia magna* immobilisation assay was conducted following the OECD standard methodology (OECD 202, 2004). The cultures of *D. magna* from clone K6 were maintained in a controlled laboratory conditions, with photoperiod of 16/8 hours of light/dark, at 20±1°C. Neonates for the acute bioassay were obtained from a synchronized culture. Five neonates (third- to fifth-brood, <24h) were used per treatment (including negative controls). The leachates of the biochar-soil treatments were used as test media. The leachates of unamended soil were used as test soil control. ASTM (American Society for Testing and Materials) solution (ASTM, 1998) was used both as eluent and negative control. All extracts were diluted with the ASTM solution giving 12.5 %, 25 %,50 % and 75 % concentration range, while 100% presents pure leachates without addition of the medium. Four replicates per each test concentration as well as for the controls (containing ASTM only) were applied. Following an exposure time of 48h (during which the organisms were not fed), at 20±1ºC and at photoperiod of 16h:8h (light/dark), the number of immobilized/dead organisms was recorded. Physicochemical parameters, such as pH and oxygen were measured for all extract treatments at the beginning and at the end of the assay. No adjustments were made prior to the test.

The Microtox® Basic Test 81.9 was applied, where bacteria *Vibrio fischeri* were exposed to serial series of dilutions of the leachates. Leachates of the biochar-soil treatments and the unamendedsoil were pipetted into glass cuvettes and the salinity was adjusted with MOAS (Microtox Osmotic Adjusting Solution, Azur Environmental, Carlsbad, CA, US), as recommended by the manufacturer (Microbics Corporation, 1992). Five and fifteen minutes after transferring the bacteria into the extract vials, changes in bioluminescence were assessed.

Laboratory earthworms' feeding experiment and screening of PAH-type metabolites

The *Eisenia andrei* feeding experiment was performed with six replicates per un—amended or biochar-amended soil treatment. Each replicate consisted of one adult earthworm in an opaque pot filled with 100 g of amended or unamended soil. Animals were weighed prior to the exposure, first while selecting them, and then after 24 h of gut purging in the dark. Organisms were not fed during the 14 days of the exposure, in constant conditions of 20±1ºC and 16 h/8 h (light/dark) photoperiod. In the absence of mortality, the test endpoint was body mass of

individual earthworms – assessed after gut depuration. Loss of earthworms' body weight (LW, expressed in grams) was calculated using *Equation 2* for each replicate (individual) and then expressed as a mean loss of weight, where wi represents the initial weight of an individual after 24 h of gut depuration, and wf represents the weight after 14 days of the exposure and 24 h of gut depuration. Subsequently, all the individuals were kept frozen at -20 ºC before the fixed fluorescence analyses.

PAH-type metabolites were analysed with fixed fluorescence. The method was adapted from the protocol developed for fish bile samples (Aas et al., 1998; 2000a, 2000b). Prior to analysis every specimen was defrost and individually homogenized on ice by sonication (for 2x30 s, using 250 Sonifier, Branson Ultrasonics) in 3,000 µL of K-phosphate buffer (0.1 M, pH 7.4). Samples were then mixed with 50 % methanol (50 µL sample and 4950 µL methanol), vortexed, and sonicated for 1 min at 25°C. Aliquots of 300 µL were transferred to multi-well plates for the readings. Four blanks per plate containing the K-phosphate buffer (50 µL of 0.1 M, pH 7.4) and 50 % methanol (4,950 µL) were employed for calibration. Each sample was pipetted in four wells of the multi-well plate, giving four technical replicates. The concentrations of PAH-type metabolites were expressed in ng/mg of earthworm body weight, relative to the standard calibration curves with known concentrations of naphthalene (Nap), phenantrene (Phe), pyrene (Pyr) and benzo[a]pyrene (BaP). Fluorescence was determined in a spectrofluorometer (Hitachi F-7000) using several excitation/emission wavelength pairs: 290 nm/335 nm for Nap, 341 nm/383 nm for Pyr, 256 nm/380 nm for Phe, and 380 nm/430 nm for Bap (Gravato and Santos, 2003). For quality assurance, limits of detection (LOD) and limits of quantification (LOQ) were calculated for each of these metabolites from a calibration curve at low concentrations, as described in Shrivastava and Gupta (2011). The results were interpreted based on the obtained values defining LOD as a minimum detectable concentration of an analyte in a sample under the given test conditions. LOQ considers a minimum determined concentration of an analyte in a sample under the given test conditions, that can be claimed with an acceptable level of precision and accuracy (Shrivastava and Gupta, 2011).

3.4. Statistical analysis

To evaluate biochar amendment effects on the behaviour of the earthworms, a one-tailed Fischer test was conducted at a level of significance of $\alpha<0.05$, with Graph Pad Software. All

the observed endpoints were expressed as percentage of the unamended control soil and used in the factorial ANOVA. A three-way ANOVA was used to analyse the effects of factors 'incubation time', 'particle size', 'application rate' of biochar and their interaction on earthworms' avoidance behaviour. Two-way ANOVA was applied for testing significance of the factors biochar 'particle size', 'application rate' and their interaction effect on the endpoints obtained in the greenhouse experiment and the feeding experiment. To interpret the main effects when significant, a Tukey post hoc test was used. Normality of data was checked with Shapiro-Wilk test ($p>0.05$) and homogeneity of variance with Leven's test ($p>0.05$). Where data distribution was not normal, residual values were checked for normality (Keough and Quinn, 2006). In case that the assumption of equality of variances was not fulfilled (Leven's test, $p<0.05$), the two-way ANOVA was still considered to be robust enough as the ratios between the largest group variance and the smallest group variance were lower than three (Jaccard, 1998). Estimates of effect size (R^2) were obtained by dividing the sum of squares for a factor and/or the interaction of factors by total sum of squares (Hullet and Levine, 2003). The statistical analyses were performed with SigmaPlot 12.5 statistical package.

3.5. Results

3.5.1. Chemical analysis

Concentrations of the selected analysed metals and trace elements in the unamended soil (0 %), in the amended soil used in the experiments (1 % S, 6 % S, 1 % M, 6 % M, 1 % L, 6 % L), and in the respective leachates are presented in **Table 3.2.**

Table 3.2. pH values and contents of selected trace metals and nutrients measured in unamended soil (0 %), biochar-amended soil (1 % S, 6 % S, 1% M, 6 % M, 1% L, 6 % L), and in the respective leachates.

Solid samples (concentration of elements in mg/kg)							
	1 % S	**6 % S**	**1 % M**	**6 % M**	**1 % L**	**6 % L**	**0 % unamended soil**
pH (H_2O, 1:5)	7.52	8.07	7.49	7.92	7.29	7.81	6.90
As	10	9	10	9.8	11	10	12
Sb	< 1	<1	< 1	< 1	< 1	< 1	< 1
Be	1.5	1	1.4	1.2	1.5	1.2	1.4
Pb	64	55	67	71	74	72	68
Bo	3	5	3	19	4	9	2
Cd	0.2	<0.2	< 0.2	< 0.2	< 0.2	0.2	< 0.2
Ca	2 900	3 100	4 200	4 400	3 700	4 000	3 200
Cr	12	11	12	11	12	12	12
Fe	15 000	14 000	15 000	18 000	15 000	18 000	15 000
K	3 300	2 500	3 500	3 000	3 500	3 300	2 900
Co	5	5	6	5	6	5	22
Cu	17	13	15	14	14	16	15
Li	60	61	60	52	64	55	62
Mg	2 800	2 100	3 000	3 600	3 000	3 600	2 900
Mo	< 2	<2	< 2	< 2	< 2	< 2	< 2
Ni	8	7	9	8	9	9	8
Se	8	<10	6	<1	5	<1	4
Ag	< 5	< 5	< 5	< 5	< 5	5	< 5
Sr	10	13	13	12	12	12	9
Tl	0.4	<2	0.4	0.4	0.4	0.4	0.4
Ti	440	480	430	480	450	520	460
V	15	15	16	23	15	26	15
Zn	100	90	100	86	110	96	110
Sn	< 10	<10	< 10	< 10	< 10	< 10	< 10
Leachates (concentration of elements in mg/ml)							
pH	8.11	8.14	8.14	8.16	8.17	8.22	8.16
As	0.007	0.009	0.007	0.008	0.007	0.009	0.007
Sb	0.032	0.045	0.050	0.037	0.029	0.036	0.017
Be	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Pb	0.001	0.002	< 0.001	0.001	0.001	< 0.001	0.001
Bo	0.06	0.06	0.05	0.05	0.05	0.06	0.05
Cd	< 0.0002	< 0.0002	< 0.0002	< 0.0002	< 0.0002	< 0.0002	< 0.0002
Ca	92	86	96	77	100	120	96
Cr	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Fe	0.040	0.031	0.020	0.020	0.023	0.026	0.021
K	23	35	21	31	21	40	18
Co	0.0003	0.0003	0.0003	0.0002	0.0003	0.0003	0.0003
Cu	0.011	0.012	0.011	0.010	0.012	0.015	0.012
Li	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
Mg	12	11	11	8.8	12	14	12
Mo	0.002	0.004	0.002	0.003	0.002	0.006	0.002
Na	29	21	19	18	20	23	20
Ni	0.002	0.002	0.002	0.001	0.002	0.002	0.002
Se	0.003	0.002	0.001	< 0.001	0.004	0.002	< 0.001
Ag	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Sr	0.17	0.12	0.14	0.11	0.14	0.17	0.13
Tl	< 0.0002	< 0.0002	< 0.0002	< 0.0002	< 0.0002	< 0.0002	< 0.0002
Ti	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
V	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002
Zn	0.036	0.026	0.013	0.016	0.016	0.006	0.031
Sn	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

3.5.2. Earthworms' avoidance bioassay

The avoidance behaviour test fulfilled the validity criteria of the ISO guideline (ISO/DIS 17512-1, 2005), with the homogeneous distribution of earthworms in the control pots. When comparing the earthworms' distribution in the treatments to the expected distribution, a statistically significant difference was observed for the treatment 6% S 96 h (Fischer exact test, $P<0.05$, **Figure 3.3.**). This bioassay served as a preliminary approach, conducted with the aim to study possible differences in avoidance behaviour caused by particle size, application rate and/or incubation time of biochar-amended soil. No significant differences were observed for any of the factors, nor for their interaction, as presented in Table S1 of Supplementary information (SI) file (three-way ANOVA, $p>0.05$; **Table S3.1**. in SI). Therefore, an incubation period of 96 h was used in the follow-up experiments.

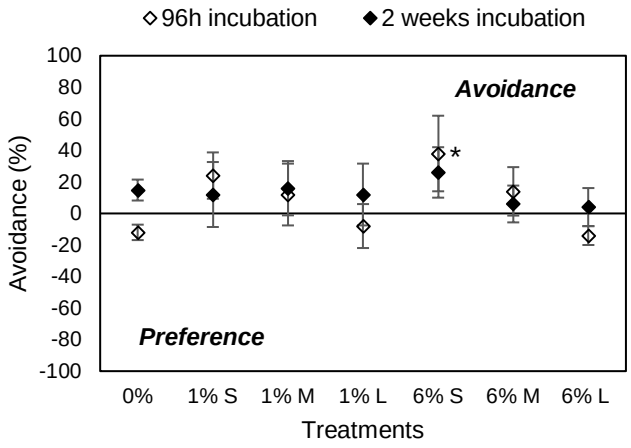

Figure 3.3. *Eisenia andrei* avoidance response to the unamended and biochar-amended soil treatments, after the soil-biochar mixture was allowed to equilibrate for 96 hours or 2 weeks prior to the bioassays. Error bars represent standard errors of the mean. Asterisk (*) refers to significant avoidance response (Fischer exact test, $p<0.05$).

3.5.3. Greenhouse experiment – earthworms' survival, vertical distribution, weight changes, and bait-lamina consumption

Survival, vertical distribution and weight change of *E. andrei* in the treatments are presented in **Figure 3.4.I.** The values of the treatments are expressed as the percentage of the following mean observed values (+/- standard error of the mean) in the unamended soil: 4.8±0.4 individuals for the survival, 2.3±1.0 individuals for the presence in the amended soil, and 0.12±0.30 g for the loss of weight. Loss of weight is also presented in the Table S4 of SI. After the 28-days greenhouse microcosms experiment, there was 13 % mortality of earthworms in the treatments 1 % M and 1 % L. This level of mortality in the 28-days experiment can be considered as relatively low and acceptable, bearing in mind that even the validity criteria of the *E. andrei* survival bioassay (ISO/DIS 17512-1, 2005) accepts up to 10 % mortality in controls. Further, there was no evidence of earthworm vertical avoidance behaviour towards the unamended bottom soil layer. It is notable that exposure to all the treatments caused an increase in weight loss of the earthworms, relative to those exposed to the unamended soil (**Figure 3.4.I.**). However, there was no significant effect observed for any of the factors – particle size or application rate, nor for their interaction (two-way ANOVA, $p>0.05$; **Table S3.2**. in SI).

Bait-lamina consumption in the microcosms is shown in Figure **3.4.II.** The data are expressed as percentage of unamended soil, in which the mean number of empty apertures was 9.83±1.01 (+/- standard error of the mean). The application rate and particle size as well as their interaction had a significant impact on bait-lamina consumption (two-way ANOVA, $p<0.05$, **Table S3.2.**). Particle size is the factor explaining 24 %, application rate 20 %, and the interaction of the factors 14 % of the total variability. With regard to the interaction, by looking at the least square means in two-way ANOVA output for each group of application rate versus particle size, the lowest mean bait-lamina consumption is associated to small particles (S) at 6 % application rate.

I. **II.**

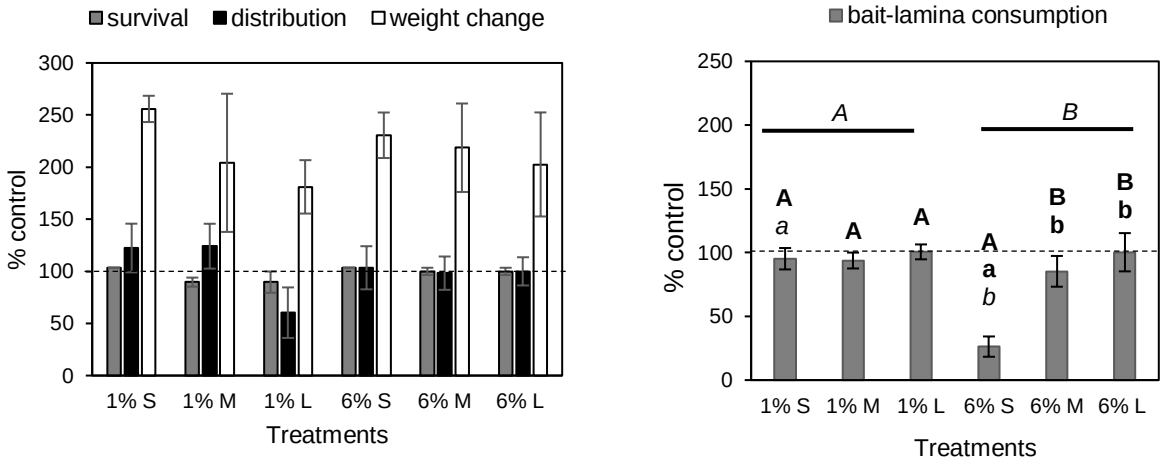

Figure 3.4. (I.) *Eisenia andrei* survival, weight loss, and distribution of the recovered earthworms from the amended soil (topsoil layer 9—10 cm of a microcosm), and **(II.)** bait-lamina consumption obtained in the 28-day greenhouse microcosms experiment. All the results are presented as percentage to the unamended control. Error bars represent standard errors of the mean. Different italic upper case letters (*A, B*) indicate significant differences for factor application rate (Tukey test, $p<0.05$). Different bold upper case letters (**A, B**) indicate significant differences for factor particle size (Tukey test, $p<0.05$). Different bold lower case letters (**a, b**) indicate significant differences between particle sizes within 6 % application rate of biochar (Tukey test, $p<0.05$). Different italic lower case letters (*a, b*) indicate significant differences between application rates within small particle sizes of biochar (Tukey test, $p<0.05$).

3.5.4. Aquatic bioassays

When *D. magna* juveniles were exposed to the leachates of biochar-amended soil, no toxicity was observed for any of the treatments (data not shown). None of the leachates induced any negative effects to *V. fischeri* either (data not shown).

3.5.5. Earthworms feeding experiment and PAH quantification in tissue

When the earthworms were fed on biochar-amended soil their body weight was generally lower than in the unamended control. Loss of weight in the treatments was presented as percentage of the unamended soil in which the mean loss was 0.07±0.02 g (+/- standard error of the mean). The most pronounced loss of body mass was in 1 % S and 6 % S treatments (**Figure 3.5.I.**; **Table S3.4** in SI). **Table S3.3.** in SI depicts factorial ANOVA output for this endpoint. Particle size had a significant impact on the weight of the earthworms (two-way ANOVA, $p<0.05$), with the absence of statistical significance for the application rate and for the factors' interaction effect (two-way ANOVA, $p>0.05$). The factor particle size had the largest contribution to the total variability of 17 %.

PAHs metabolites' screening in the earthworms' tissue resulted in detectable levels of Nap-type metabolites ranging from 54.65 µg/ml to 93.71 µg/ml, with limit of detection (LOD) of 2.83 µg/ml and limit of quantification (LOQ) of 9.46 µg/ml. For Phe and its metabolites the calculated LOD and LOQ were LOD= 1.60 µg/ml, LOQ=5.33 µg/ml, while the screened levels in the animals were in the range of 1.80 µg/ml to 4.70 µg/ml, suggesting lower reliability of the data. Pyrene- and Benzo[alpha]pyrene-type were not detected in the samples. Naphthalene-type metabolites observed in the tissue of the earthworms are presented as percentage to the unamended control soil in Figure 3.5.II. The mean concentration in the control was 161.3±12.6 ng/mg of body mass (+/- standard error of the mean). Table S3.4. in SI file depicts the concentrations of Nap-type metabolites measured in the earthworms' tissue. The particle size had a statistically significant effect (two-way ANOVA, $p<0.05$) and explained 23 % of the total variation in the Nap-type metabolites presence. The effect was not significant for the application rate or the interaction of the two factors (two-way ANOVA, $p >0.05$, Table S3.3.).

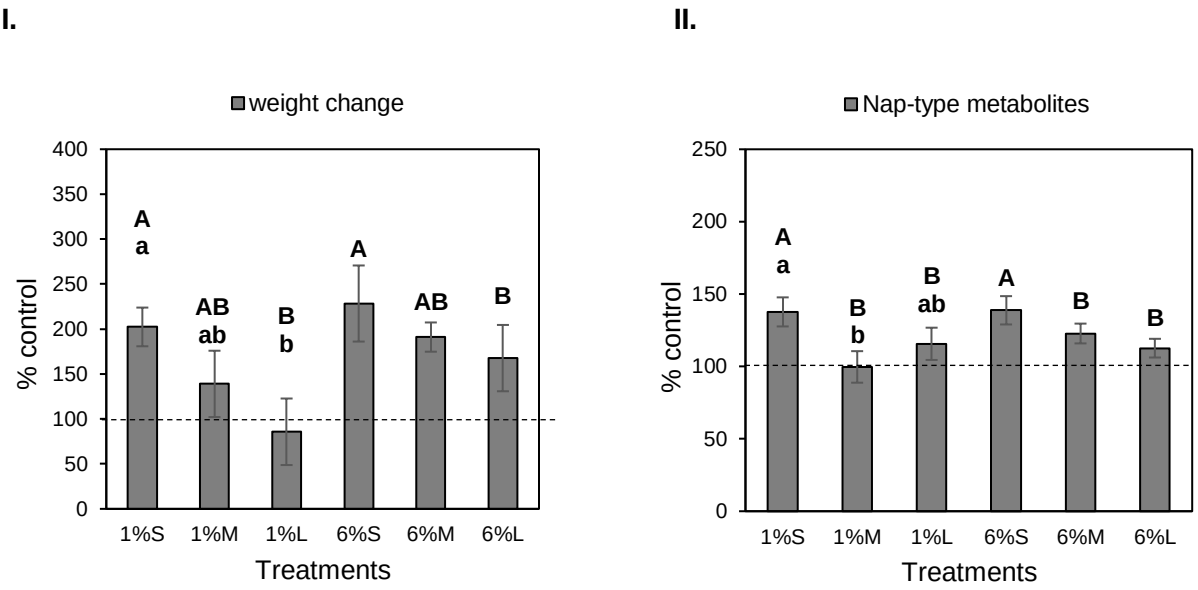

Figure 3.5. (I.) *Eisenia andrei* weight change and **(II.)** Naphthalene-type metabolites detected in tissue of *Eisenia andrei* after the feeding experiment. All the results are presented as percentage to the unamended control. Error bars represent standard errors of the mean. Different bold upper case letters (**A, B**) indicate significant differences for factor particle size (Tukey test, $p<0.05$). Different bold lower case letters (**a, b**) indicate significant differences between particle sizes within 1 % application rate of biochar (Tukey test, $p<0.05$).

3.6. Discussion

3.6.1. Chemical analysis

The concentrations of metals and PCBs in the biochar were below the benchmark concentrations recommended by two voluntary international biochar quality standards, i.e. the European Biochar Certificate (EBC, 2012) and the International Biochar Initiative (IBI, 2015). The sum content of PAHs (Σ16PAHs=0.48 mg/kg) was below the threshold concentrations defined in both guidelines (<4 mg/kg in EBC, and <12 mg/kg in IBI). Σ16PAHs is comparable to the other woodchip biochars produced at 550-620°C (Hilber et al., 2012). Due to low levels of the contaminants, this biochar has been classified as “premium” grade biochar (EBC, 2012).

The levels of selected trace metals detected in the treatments are comparable to the values in the unamended soil. Due to the composition of the biochar used in the study, it is not surprising that the concentrations of trace elements in the amended soil and the respective leachates were not elevated. This was confirmed when the concentrations were compared to available

soil quality standards (CCME, 1999). The concentrations of the same elements in leachates did not exceed the benchmarks defined for ground water quality (EU WFD, 2000).

3.6.2. Effects on earthworms and bait-lamina consumption: *E. andrei* screening test and greenhouse microcosms

From the initial screening trial, the avoidance of biochar-amended soil at the concentration of 6% w/w is comparable to the reported for 50 % *E. andrei* individuals at biochar rate of 122 t/ha, in OECD artificial soil (Malev et al., 2015). In a study with the geophagous earthworm *Aporrectodea caliginosa*, no avoidance was observed at 30 t/ha biochar after 48 h, but significant avoidance occurred after 14 days. The authors explained this referring to the decrease in the soil water potential (Tammeorg et al., 2014).

Extrapolating the outcome of laboratory screening bioassays, such as the avoidance behaviour, to higher tier approaches is not straightforward, as it was already demonstrated for biochar-enriched soils. Tammeorg et al. (2014) conducted a 4.5-months field trial in Finland and showed that biochar amendment did not significantly change the density or the biomass of the earthworms. In a 28-day study with small scale terrestrial ecosystem models (STEMs) in laboratory conditions, there was no observed changes in the body mass, but *E. andrei* avoided the soil amended with biochar-N-fertilizer at 25 t/ha (Amaro et al., 2016). This perceived avoidance behaviour was manifested as vertical movement towards the unamended bottom soil layer in the columns of the STEMs, using an experimental set-up comparable to that used here. In contrast, when using standardized laboratory avoidance test, avoidance behaviour was significant at a higher biochar concentration (50 t/ha; Amaro et al., 2016). Our study showed no difference in earthworms' vertical distribution, contrary to the laboratory avoidance behaviour observed previously.

Regarding bait-lamina consumption, a reduction in biochar-amended soil was observed. This method has been included so far in investigating the effects of two biochars contrasting in physicochemical properties. One was a corn stover biochar produced with slow pyrolysis at the temperature of 600°C (Domene et al., 2014), and the other was a gasification pine wood char, produced at the temperature range of 600°C to 900°C (Marks et al., 2016). The corn stover biochar did not have a negative impact on collembolans' and enchytraeids' reproduction in a field study during a summer and autumn season. Neither the seasonality, nor different corn

biochar rates, had a substantial impact on the feeding activity. However, besides the increased microbial abundance, a slightly stimulatory effect of the corn biochar on fauna consumption was observed (Domene et al., 2014). On the contrary, a negative effect of gasification char was reported regarding the reproduction of collembolans and enchytraeids (Marks et al., 2014), as well as considering the bait-lamina consumption assessed in a field study, particularly after one year and two years since the initial application (Marks et al., 2016). This charred material was characterized with very high levels of total sum of 16 USEPA PAHs (321 mg/kg), and relatively low levels of metals (apart from Cd) (Marks et al., 2016). These studies, together with the current, demonstrate the adequacy of using bait-lamina test method over various experimental designs to investigate biochar-soil-biota interactions.

In the present study, the results obtained from the preliminary laboratory bioassay and from the greenhouse experiment were in concordance for certain endpoints, namely in terms of significant effects for 6 % S treatment on the behavioural (avoidance/preference), and functional (bait-lamina consumption) endpoints observed. However, although in the microcosms the lower observed mean body mass in the treatments 1 % S and 6 % S respectively, might suggest a possible role of smaller particles in sub-lethal toxicity, this needs to be considered with caution as the result could not be confirmed statistically or supported with the estimated effects sizes for the factors. On the other hand, bait-laminas were immersed in the amended-soil layer, therefore reflecting the feeding activity in the biochar-amended topsoil only.

3.6.3. Leachates and aquatic bioassays

The results of the aquatic bioassays corroborated with the presented chemical composition of the leachates. Nonetheless, the fact that leachates from the biochar-amended soil did not cause any adverse effects to *D. magna* and *V. fischeri* should also be interpreted carefully. This outcome represents a first screening approach without addressing a chronic toxicity of the leachates, e.g. effects on *D. magna* reproduction. Previously reported toxic effects to daphnids in miscanthus biochar aqueous extract were correlated with high total concentration of PAHs in biochar (Oleszczuk et al., 2013). The highest luminescence inhibition of *V. fischeri* was observed with the miscanthus biochar. Also, the same study found that those biochars with low concentrations of PAHs posed toxicity too, raising questions as to the possible role of biochar

PAHs in explaining its ecotoxicological effects (Oleszczuk et al., 2013). A study by Bastos et al. (2014) addressed toxicity of biochar-amended natural LUFA soil elutriates with a battery of standardized aquatic bioassays, resulting in *V. fischeri* being the most sensitive species tested (EC_{20}= 20.5 % for soil-biochar, and EC_{20}= 8.73 % for elutriate of biochar alone) and in immobilisation of daphnids at the higher elutriate concentrations (EC_{20}=79.3 % for soil-biochar, and no toxicity for elutriate of biochar alone). While the soil texture was similar to the one reported in this study, the biochar contained potential contaminants at higher concentrations, e.g. total 16 USEPA PAHs of 0.712 mg/kg (Bastos et al., 2014). The dilution caused with the quantity of water applied in the leaching procedure in our experiment could have led to an underestimation of toxicity to some extent. Comparison of toxic responses in aquatic bioassays over various studies is limited, due to the small number of studies in the literature and to differences in methodologies to produce the aqueous extracts (Smith et al., 2013; Oleszczuk et al., 2013; Bastos et al., 2014). In the current study, the leachates were produced from biochar-amended natural soil. Moreover, the biochar was applied at typical rates for use in agriculture and representative of a typical topsoil incorporation strategy. These factors together make this ecotoxicological evaluation more environmentally relevant. Thus, the approach taken in the study highlights the importance of direct toxicity assessment (Gruiz et al., 2016), analogous to those conducted for contaminated soils (Loureiro et al., 2005), as well as the necessity for development of the standardized methodologies for biochar-soil-aqueous extraction.

3.6.4. Earthworms feeding and PAH-type metabolites

Fixed fluorescence for determination of PAH-metabolites in soil organisms is not a rarely employed method. Phe-type metabolites were screened with fixed fluorescence in bioaccumulation study with *E. albidus*, demonstrating the adequacy of the method for soil organisms (Amorim et al., 2011). The uptake and elimination (14 days plus 14 days) of Phe were tracked in the soil initially spiked with 8 mg/kg Phe (dry soil) (Amorim et al., 2011). The lowest concentrations (in the early uptake and late elimination phases) were in the range of 10-15 mg/kg fresh weight of *E. albidus* (Amorim et al., 2011). This is comparable to the highest concentration of phenanthrene in the present study, i.e. 9.91 mg/kg for the 1 % S treatment (data not shown), expressed per body weight of the animals. This, added to the low reliability of the measured levels in the earthworms' tissue according to LOD and LOQ criteria applied,

suggests little contribution of Phe-type metabolites to the overall sub-lethal effects in the present study.

Malev et al. (2016) demonstrated that bioavailability of PAHs from biochar-amended soil is not necessarily dependent on initial concentrations of PAHs, but rather on the soil texture and capacity of biochar to retain PAHs trough surface interactions and adsorption into micropores. They showed that accumulation of biochar-originated PAHs in the body of *E. andrei* is possible, while testing the effects of two different biochars applied to uncontaminated soils of different textures. Lower concentrations of PAHs were accumulated in clay-loam soil, than in the sandy soil, particularly the higher molecular weight PAHs. Biochar that contained 2.3 mg/kg total PAHs was produced at lower temperature than the one containing 6.8 mg/kg total PAHs. The lower biochar production temperature resulted in a less charred structure, which contributed to the higher bioavailability of PAHs to the earthworms, according to the authors (Malev et al., 2016). The soil used in our experiments is of a similar texture as the sandy soil from the study of Malev et al. (2016), but the biochar contained only 0.48 mg/kg total PAHs. Nevertheless, in our study the loss of weight and increased levels of Nap-type metabolites for the earthworms exposed to the treatments with <0.5 mm confirm our hypothesis that there is a link between potential toxicity and particle-size of biochar. Gomez-Eyles et al. (2011) studied a remediation potential of biochar by applying 10 % (dry weight) of biochar (total mean levels PAHs of 1.21 mg/kg) to a contaminated soil (with total mean PAHs of 773 mg/kg). They observed significant losses of *E. fetida* weight in the biochar-amended soil, relative to the contaminated soil without the amendment, on the 28th and 56th day since the exposure. The decrease in weight can be partly justified by the relatively small amount of soil provided during that experiment (200 g per replicate for 10 individuals). As a positive effect, high molecular weight PAHs were reduced in the earthworms' tissues in the presence of biochar. On the contrary, significantly higher concentrations of 2-ring PAHs were recorded, both after 28 and after 56 days (Gomez-Eyles et al., 2011), which is in a line with our study. This together is supported by the fact that Nap is among the dominant PAH compounds in biochars (Bucheli et al., 2015; Hilber et al., 2017b). Besides, as a low molecular weight PAH it is characterized with higher bioaccessibility, or in other words a higher 'readily desorbed fraction' (Hilber et al., 2017b). Biochar particles smaller than 1 mm can improve hydrological properties of coarse-textured soil in comparison to the 1-2 mm fraction (Ibrahim et al., 2016). In the present study, the small particles <0.5 mm may have affected the soil hydrology, thereby potentially increasing the intake of readily available Nap

fraction by the earthworms, the process described for contaminated soil by Qi and Chen (2010). Further research is, however, necessary to investigate this in biochar amended soils.

The fixed-fluorescence-screened levels of PAH-type metabolites were expected considering the low initial levels of these compounds in the biochar, with Nap being the only one within the detectable levels by the GC/MS. Yet, biota responses to the biochar-amended soil should be perceived as responses to the mixture of potentially toxic compounds (even those at low concentrations and not detectable). The consistency in the response, such as the small particle size impact on the earthworm weight losses and on the increase in Nap-type metabolites, are important findings in the present study. They demonstrate that under certain factors, or combination of factors, there is a likelihood for the transfer of toxic elements from biochar through the food chain or for the occurrence of secondary effects (e.g. the earthworms might become a lower quality food for their predators). Generally, the study results sustain already raised concerns by other authors regarding the need for biochar ecotoxicological risk assessment using representative methodologies, coupled to their chemical and physical characterization (Gomez-Eyles et al., 2011; Domene et al., 2014; Bastos et al., 2014; Hilber et al 2017; Bielska et al., 2018).

While involving aforementioned limitations, the results of fixed fluorescence indicate the necessity for more research on the applicability of biochar-originated PAHs screening in soil organisms with this method. This technique represents an important asset for this kind of studies as it is cost-effective, with rapid manipulation and processing of large number of samples. In addition to this method, the demonstrated sensitivity of the bait-lamina test in responses to biochar-enriched soil makes it equally suitable tool for field and laboratory assessments.

3.7. Conclusions

The current study employed the integrative approach in studying the effects of biochar to biota, taking into consideration the spatial scale (from standardized laboratory conditions to greenhouse), biological scale (from assessing individual endpoints to functional) and environmental scale (by testing both soil and aquatic phase). The applied methods are expected to contribute further in the evaluation and understanding of biota responses to biochar-amended soil.

Together, the outcomes of the conducted experiments suggest that smaller particles (<0.5 mm) of slow pyrolysis woodchip biochar may pose sub-lethal toxicity to soil biota, even at lower application rates. There is a close link between behavioural (avoidance), individual (weight changes, Nap-type metabolites in earthworms' tissue) and functional (bait-lamina consumption) endpoints obtained. The results suggest that earthworms may respond to small particles by two mechanisms. The first one is an indirect mechanism – using the strategy of avoiding and/or not eating. The evidence for this is the earthworms' avoidance observed in 48-hours bioassay, the lower bait-lamina consumption in 28-days microcosms experiment, and the reduced body mass observed in the laboratory feeding experiment. The second one is a direct mechanism – through ingesting biochar particles and/or skin sorption of biochar's inherent contaminants. This is supported primarily by the detected Nap-type metabolites, which were available at lower and higher biochar rates. Certain toxicity of single compounds and/or mixture of compounds possibly contributed to the loss of weight in the feeding experiment, involving different metabolic reactions and/or changes in the energy homeostasis in the organisms. This, however, should be further investigated in the context of impacts on different biomarkers of exposure and biomarkers of effects.

3.8. References

Aas, E., Beyer, J., Goksøyr, A., 2000a. Fixed wavelength fluorescence (FF) of bile as a monitoring tool for polyaromatic hydrocarbon exposure in fish: an evaluation of compound specificity, inner filter effect and signal interpretation. Biomarkers 5, 9–23.

Aas, E., Baussant, T., L. Balk, B. Liewenborg, O.K. Andersen, 2000b. PAH metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil exposure: a laboratory experiment with Atlantic cod Aquat. Toxicol., 51, pp. 241-258

Aas, E., Beyer, J., Goksøyr, A., 1988. PAH in fish bile detected by fixed wavelength fluorescence Mar. Environ. Res., 46, pp. 225-228

Amaro, A., Bastos, A.C., Santos, M.J.G., Verheijen, F.G.A., Soares, A.M.V.M., Loureiro, S., 2016. Ecotoxicological assessment of a biochar-based organic N-fertilizer in small-scale terrestrial ecosystem models (STEMs). Appl. Soil Ecol., 108:361–370

Amorim, M.J.B., Oliveira, E., Teixeira, A.S., Gravato, C.S., Loureiro, S., Guilhermino, L.C., Van Gestel, C.A.M., Soares, A.M.V.M., 2011. Toxicity and bioaccumulation of phenanthrene in Enchytraeus albidus (Oligochaeta: Enchytraeidae). Environ. Toxicol. Chem. 30.

Bastos, A.C., Prodana, M., Abrantes, N., Keizer, J.J., Soares, A.M.V.M., Loureiro, S., 2014. Potential risk of biochar-amended soil to aquatic systems: an evaluation based on aquatic bioassays. Ecotoxicology 23, 1784–1793.

Bielská, L., Škulcová, L., Neuwirthová, N., Cornelissen, G., Hale, S.E., 2018. Sorption, bioavailability and ecotoxic effects of hydrophobic organic compounds in biochar amended soils. Sci. Total Environ. 624, 78–86.

Bucheli, T.D., Hilber, I., Schmidt, H., 2015. Polycyclic aromatic hydrocarbons and polychlorinated aromatic compounds in biochar. In: Lehmann, J., Joseph, S. (Eds.), Biochar for Environmental Management: Science, Technology and Implementation. Earthscan, London, UK, pp 595-624.

CCME, 1999. Canadian Council of Ministers of the Environment, 1999. Canadian Soil Quality Guidelines for the Protection of Environmental and Human Health.

Chen, J., Li, S., Liang, C., Xu, Q., Li, Y., Qin, H., Fuhrmann, J.J., 2017. Response of microbial community structure and function to short-term biochar amendment in an intensively managed bamboo (Phyllostachys praecox) plantation soil: Effect of particle size and addition rate. Sci. Total Environ. 574, 24–33.

Chia, H.C., Downie, A., Munroe, P., 2015. Characteristics of biochar-physical and structural properties, in: Lehmann, J., Joseph, S. (Eds.), Biochar for Environmental Management, Science, Technology and Implementation. Routledge, London and New York.

Demirbas, A., 2004. Effects of temperature and particle size on bio-char yield from pyrolysis of agricultural residues. Journal of Analytical and Applied Pyrolysis 72(2): 243-248.

DIN/EN/ISO 12457-4, 2002. Characterization of waste - Leaching; Compliance test for leaching of granular waste materials and sludges - Part 4: One stage batch test at a liquid to solid ratio of 10 l/kg for materials with particle size below 10 mm (without or with limited size reduction). German version EN 12457- 4.

DIN/EN/ISO 17294, 2003. Water quality — Application of inductively coupled plasma mass spectrometry (ICP-MS). International Organization for Standardization. German version EN 17294.

DIN/EN/ISO 11885, 2007. Water quality -- Determination of selected elements by inductively coupled plasma optical emission spectrometry (ICP-OES). International Organization for Standardization. German version EN 11885.

Dissanayake, A., Galloway, T.S., 2004. Evaluation of fixed wavelength fluorescence and synchronous fluorescence spectrophotometry as a biomonitoring tool of environmental contamination. Mar. Environ. Res. 58, 281–285.

Dissanayake, A., Bamber, S.D., 2010. Monitoring PAH contamination in the field (South west Iberian Peninsula): Biomonitoring using fluorescence spectrophotometry and physiological assessments in the shore crab Carcinus maenas (L.) (Crustacea: Decapoda). Mar. Environ. Res. 70, 65–72.

Domene, X., Mattana, S., Hanley, K., Enders, A., Lehmann, J., 2014. Medium-term effects of corn biochar addition on soil biota activities and functions in a temperate soil cropped to corn. Soil Biology and Biochemistry 72, 152-162.

European Biochar Certificate (EBC), 2012. Guidelines for a Sustainable Production of Biochar. European Biochar Foundation (EBC), Arbaz, Switzerland. http://www.europeanbiochar.org/en/download. Version 6.3E of 14th August 2017. DOI: 10.13140/RG.2.1.4658.7043

EU Water Framework Directive: Directive 2000/60/EC of the European Parliament and oftheCouncil.Availbale:http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32000L0060:EN:NOT [accessed 15 June 2017]

Gomez-Eyles, J.L., Sizmur, T., Collins, C.D., Hodson, M.E., 2011. Effects of biochar and the earthworm Eisenia fetida on the bioavailability of polycyclic aromatic hydrocarbons and potentially toxic elements. Environ. Pollut. 159, 616–622.

Gravato, C., Santos, M., 2003. Genotoxicity biomarkers' association with B(a)P biotransformation in Dicentrarchus labrax L. Ecotoxicology and Environmental Safety 55, 352e358.

Hilber, I., Blum, F., Leifeld, J., Schmidt, H.-P., Bucheli, T.D., 2012. Quantitative determination of PAHs in biochar: a prerequisite to ensure its quality and safe application. J. Agric. Food Chem. 60, 3042–50.

Hilber, I., Schmidt, H.P., Bucheli, T.D., 2017a. Sampling, storage and preparation of biochar for laboratory analysis. In: Singh, B., Camps-Arbestain, M., Lehmann, J. (Eds.), Biochar: a Guide to Analytical Methods. CSIRO Publishing.

Hilber, I., Mayer, P., Gouliarmou, V., Hale, S.E., Cornelissen, G., Schmidt, H.P., Bucheli, T.D., 2017b. Bioavailability and bioaccessibility of polycyclic aromatic hydrocarbons from (post-pyrolytically treated) biochars. Chemosphere 174, 700–707.

Hilber, I., Bastos, A.C., Loureiro, S., Soja, G., Marsz, A., Cornelissen, G., Bucheli, T.D., 2017c. The different faces of biochar: contamination risk versus remediation tool. J. Environ. Eng. Landsc. Manag. 6897, 1–19.

International Biochar Initiative (IBI), 2015. Standardized Product Definition and Product Testing Guidelines for Biochar 7 That Is Used in Soil. http://www.biochar-international.org/sites/default/files/IBI_Biochar_Standards_V2.1_Final.pdf

Ibrahim, A., Usman, A.R.A., Al-Wabel, M.I., Nadeem, M., Ok, Y.S., Al-Omran, A., 2017. Effects of conocarpus biochar on hydraulic properties of calcareous sandy soil: influence of particle size and application depth. Arch. Agron. Soil Sci. 63, 185–197.

ISO 10390 Soil quality – Determination of pH, 1994.

ISO TC 190/SC 4 N, 2012 Method for testing effects of soil contaminants on the feeding activity of soil dwelling organisms — Bait-lamina test

ISO, 2005. Avoidance test for testing the quality of soils and the toxicity of chemicals – Part 1: Test with earthworms (Eisenia andrei).

ISO/DIS 15799, 2001 Soil quality — Guidance on the ecotoxicological characterization of soil and soil materials.

Jaafar, N.M., Clode, P.L., Abbott, L.K., 2015. Soil Microbial Responses to Biochars Varying in Particle Size, Surface and Pore Properties. Pedosphere 25, 770–780.

Jaccard, J., 1998. Interaction Effects in Factorial Analysis of Variance. SAGE Publications, Inc.

Jones, D.L., Edwards-Jones, G., Murphy, D. V., 2011. Biochar mediated alterations in herbicide breakdown and leaching in soil. Soil Biol. Biochem. 43, 804–813.

Keough, M.J and Quinn, G.P. Experimental design and data analysis for biologist, Cambridge, 2006.

Kratz, W., 1998. The bait-lamina test. General aspects, applications and perspec- tives. Environ. Sci. Pollut. Res. 5 (2), 94–96.

Lemos, M.F.L., Van Gestel, C.A.M., Soares, A.M.V.M., 2010. Developmental toxicity of endocrine disrupters bisphenol A and vinclozolin in a terrestrial isopod. Arch. Environ. Contam. Toxicol. 59, 274–281.

Li, Y., Liao, Y., He, Y., Xia, K., Qiao, S., Zhang, Q., 2016. Polycyclic Aromatic Hydrocarbons Concentration in Straw Biochar with different Particle Size. Procedia Environ. Sci. 31, 91–97.

Lima, M.P.R., Soares, A.M.V.M. & Loureiro, S., 2011. Combined effects of soil moisture and carbaryl to earthworms and plants: Simulation of flood and drought scenarios. Environmental Pollution, 159(7), pp.1844–1851.

Liu, Z., Dugan, B., Masiello, C.A., Gonnermann, H.M., 2017. Biochar particle size, shape, and porosity act together to influence soil water properties. PLoS ONE 12,6.

Malev, O., Contin, M., Licen, S., Barbieri, P., De Nobili, M., 2016. Bioaccumulation of polycyclic aromatic hydrocarbons and survival of earthworms (Eisenia andrei) exposed to biochar amended soils. Environ. Sci. Pollut. Res. 23, 3491–3502. doi:10.1007/s11356-015-5568-2

Marks, E.A.N., Mattana, S., Alcañiz, J.M., Domene, X., 2014. Biochars provoke diverse soil mesofauna reproductive responses in laboratory bioassays. Eur. J. Soil Biol. 60, 104–111.

Marks, E.A.N., Mattana, S., Alcañiz, J.M., Pérez-Herrero, E., Domene, X., 2016. Gasifier biochar effects on nutrient availability, organic matter mineralization, and soil fauna activity in a multi-year Mediterranean trial. Agric. Ecosyst. Environ. 215, 30–39.

Meyer, S., Genesio, L., Vogel, I., Schmidt, H. P., Soja, G., Someus, E. & Glaser, B., 2017. Biochar standardization and legislation harmonization. Journal of Environmental Engineering and Landscape Management, 25(2), 175-191.

Microbics Corporation, 1992. Microtox Manual. A Toxicity Testing Handbook. Carlsbad, CA, USA.

OECD, 2004. Daphnia sp., Acute Immobilisation Test nº 202. OECD- Organisation for Economic Cooperation and Development, Paris.

OECD, 2004. Leching in soil comlumns, Test nº 312. OECD- Organisation for Economic Cooperation and Development, Paris.

Oleszczuk, P., Jośko, I., Kuśmierz, M., 2013. Biochar properties regarding to contaminants content and ecotoxicological assessment. J. Hazard. Mater. 260, 375–382.

Qi, Y., Chen, W., 2010. Comparison of earthworm bioaccumulation between readily desorbable and desorption-resistant naphthalene: Implications for biouptake routes. Environ. Sci. Technol. 44, 323–328.

Salem, M., Kohler, J., Rillig, M.C., 2013. Palatability of carbonized materials to Collembola. Appl. Soil Ecol. 64, 63–69.

Santos, M. J., Morgado, R., Ferreira, N. G., Soares, A. M. & Loureiro, S., 2011. Evaluation of the joint effect of glyphosate and dimethoate using a small-scale terrestrial ecosystem. Ecotoxicol Environ Saf, 74, 1994-2001.

Servin, A.D., Castillo-Michel, H., Hernandez-Viezcas, J.A., De Nolf, W., De La Torre-Roche, R., Pagano, L., Pignatello, J., Uchimiya, M., Gardea-Torresdey, J., White, J.C., 2018. Bioaccumulation of CeO 2 Nanoparticles by Earthworms in Biochar-Amended Soil: A Synchrotron Microspectroscopy Study. J. Agric. Food Chem.

Shrivastava, A., Gupta, V.B., 2011. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. Chronicles of Young Scientists 2 (1): 21-25.

Silva, C., Oliveira, C., Gravato, C., Almeida, J.R., 2013. Behaviour and biomarkers as tools to assess the acute toxicity of benzo(a)pyrene in the common prawn Palaemon serratus. Mar. Environ. Res. 90, 39–46.

Smith, C.R., Buzan, E.M., Lee, J.W., 2013. Potential Impact of Biochar Water-Extractable Substances on Environmental Sustainability. ACS Sustain. Chem. Eng. 1, 118–126.

Statistical Package for the Social Sciences. 2015. IBM SPSS 23.0 for Windows. New York, USA.

Topoliantz, S. and Ponge, J. F., 2003. Burrowing activity of the geophagous earthworm Pontoscolex corethrurus (Oligochaeta: Glossoscolecidae) in the presence of charcoal, App. Soil Ecol., 23(3), 267–271.

Topoliantz, S. and Ponge, J. F., 2005. Charcoal consumption and casting activity by Pontoscolex corethrurus (Glossoscolecidae), App. Soil Ecol., vol. 28(3), 217–224,

Van Gestel, C.A.M., Kruidenier, M., Berg, M.P., 2003. Suitability of wheat straw decomposition, cotton strip degradation and bait-lamina feeding tests to determine soil invertebrate activity. Biol. Fert. Soils 37, 115-123

Verheijen, F.G.A., Jeffery, S., Bastos, A.C., van der Velde, M., and Diafas, I. (2009). Biochar Application to Soils - A Critical Scientific Review of Effects on Soil Properties, Processes and Functions. EUR 24099 EN, Office for the Official Publications of the European Communities, Luxembourg, 149pp. 11.

Verheijen, F. G., Montanarella, L., Bastos, A. C., 2012. Sustainability, certification, and regulation of biochar. Pesquisa Agropecuária Brasileira, 47(5), 649-653.

Wang, F., Ji, R., Jiang, Z., Chen, W., 2014. Species-dependent effects of biochar amendment on bioaccumulation of atrazine in earthworms. Environ. Pollut. 186, 241-247.

Weyers, S.L., Spokas, K.A., 2011. Impact of Biochar on Earthworm Populations: A Review. Appl. Environ. Soil Sci. 2011, 1–12.

Wu, S., Wu, E., Qiu, L., Zhong, W., Chen, J., 2011. Effects of phenanthrene on the mortality, growth, and anti-oxidant system of earthworms (Eisenia fetida) under laboratory conditions. Chemosphere 83, 429–434.

Zar, J.H., 1996. Biostatistical Analysis. Prentice-Hall International, Inc., New Jersey.

Zheng, R., Li, C., Sun, G., Xie, Z., Chen, J., Wu, J., Wang, Q., 2017. The influence of particle size and feedstock of biochar on the accumulation of Cd, Zn, Pb, and As by Brassica chinensis L. Environ. Sci. Pollut. Res. 24, 22340–22352.

3.9. Supplementary Information

Table S3.1. Three-way ANOVA output table for *Eisenia andrei* avoidance bioassay.

	SS	DF	MS	F	P	R^2
Avoidance						
incubation time	41.667	1	41.667	0.030	0.862	0.0005
application rate	15.000	1	15.000	0.011	0.917	0.0002
particle size	7 023.3	2	3 511.6	2.568	0.087	0.0825
incubation time * application rate	81.667	1	81.667	0.060	0.808	0.0010
incubation time * particle size	2 503.3	2	1 251.6	0.915	0.407	0.0294
application rate * particle size	1 290.0	2	645.00	0.472	0.627	0.0152
incubation time * application rate * particle size	103.33	2	51.667	0.038	0.963	0.0012

Table S3.2. Two-way ANOVA output table for *Eisenia andrei* survival, vertical distribution (as % of individuals present in amended soil of the top layer in a microcosm), weight change (as loss of weight) and bait-lamina consumption (as % of empty apertures) in a greenhouse experiment. Asterisks refer to the levels of statistical significance *P<0.05, **P<0.01 and ***P<0.001.

	SS	DF	MS	F	P	R^2
Survival						
application rate	428.062	1	428.062	2.903	0.099	0.0756
particle size	594.530	2	297.265	2.016	0.151	0.1051
application rate * particle size	214.031	2	107.015	0.726	0.492	0.0378
Vertical distribution						
application rate	26.754	1	26.754	0.011	0.918	0.0003
particle size	8156.96	2	4078.478	1.656	0.208	0.0908
application rate * particle size	7776.46	2	3888.228	1.579	0.223	0.0865
Loss of weight						
application rate	115.022	1	115.022	0.012	0.915	0.0004
particle size	16153.1	2	8067.54	0.809	0.455	0.0505
application rate * particle size	3821.17	2	1910.58	0.191	0.827	0.0119
Bait-lamina consumption						
application rate	6034.74	1	6034.74	10.45	0.003**	0.1440
particle size	10094.9	2	10094.9	8.737	0.001**	0.2410
application rate * particle size	8433.56	2	8433.56	7.299	0.003**	0.2013

Table S3.3. Two-way ANOVA output table for *Eisenia andrei* weight change and naphthalene-type metabolites screened in the *Eisenia andrei* tissue obtained in the laboratory feeding experiment. Asterisks refer to the levels of statistical significance *P<0.05, **P<0.01 and ***P<0.001.

	SS	DF	MS	F	P	R^2
Loss of weight						
application rate	25614.5	1	25614.5	3.876	0.058	0.0925
particle size	47465.9	2	23732.9	3.591	0.040*	0.1714
application rate * particle size	4697.55	2	2348.77	0.355	0.704	0.0170
Naphthalene-type metabolites						
application rate	450.131	1	450.131	0.853	0.363	0.0198
particle size	5275.28	2	2637.64	4.999	0.013*	0.2320
application rate * particle size	1179.70	2	589.851	1.118	0.340	0.0519

Table S3.4. *Eisenia andrei* weight change (presented as mean loss of weight +/- standard error of the mean, in g) observed in greenhouse microcosms experiment and in laboratory feeding experiment, and Naphthalene-type metabolites (presented as mean concentration in ng/mg of body mass +/- standard error of the mean) screened in the tissue.

	0 %	1 % S	6 % S	1 % M	6 % M	1 % L	6 % L
Greenhouse microcosms	0.12±0.30	0.31±0.15	0.28±0.27	0.25±0.80	0.27±0.50	0.22±0.30	0.25±0.60
Feeding experiment	0.07±0.02	0.15±0.16	0.17±0.30	0.10±0.27	0.14±0.12	0.06±0.27	0.12±0.27
Naphthalene-type metabolites	161.3±12.6	222.1±16.2	223.9±15.8	160.8±17.6	198.1±28.0	186.6±18.0	181.7±10.4

Chapter 4

Interspecies interaction in soil enriched with biochar: effects on microbial enzymatic activity and biomarkers in earthworms

Interspecies interaction in soil enriched with biochar: effects on microbial enzymatic activity and biomarkers in earthworms

4.1. Abstract

Albeit the increasing number of studies on the effects of biochar on soil enzymatic activity, up to date, none of them has linked microbial enzymatic activities with the activity of representative soil invertebrates. Here we are addressing the knowledge gap, by exploring enzymatic activity in soil enriched with 1.5 % (w/w) woodchip biochar, as influenced by presence of representative invertebrate species, the terrestrial isopod *Porcellionides pruinosus* and the earthworm *Eisenia andrei*. The earthworm reproduction was also assessed, alongside with biomarkers of effect in order to get an insight on the mechanism behind the effects of this dynamic matrix to biota. Overall microbial response was enzyme-specific, characterized as sampling time-, and invertebrate-dependent. Reproduction of *E. andrei* was not affected by the exposure to the woodchip biochar. Biomarkers responded as early warning tools, by showing an increase in lipid peroxidation and cellular energy allocation decrease in exposed earthworms. The multibiomarker approach applied in the current study provides a useful base for case-to case assessment of biochar impact on biota.

Key words: soil, biochar, invertebrates, soil enzymes, interaction, biomarkers of effect

4.2. Introduction

Besides prospective benefits that biochar can provide in numerous environmental management applications, many unknowns remain to be addressed, like those regarding the use of adequate feedstock (i.e. biomass quality), matching biochar with type of soil, effects of biochar-contained contaminants, long term effects, etc. (Mukherjee et al., 2014). Xenobiotics such as metals, PAHs or dioxins are found in various biochars, as determined by feedstock and production conditions (Sohi et al., 2009; Verheijen et al., 2010). Biochar physicochemical characterization methods were developing quickly and up to date resulted in two international voluntary biochar quality standards, the European Biochar Certificate (EBC) and the International Biochar Initiative (IBI) (EBC, 2012; IBI, 2015). Nonetheless, in the context of biochar utilization as soil amendment, its potential ecotoxicity has been highlighted in recently published works (e.g. Bastos et al., 2014; Domene et al., 2014; Marks et al., 2014; Malev et al., 2015; Conti et al., 2016; Bielska et al., 2018). There have been reports of increases in microbial abundance and enzymatic activities (Jin et al., 2003), as well as associated shifts in community composition, as response to biochar application (Grossman et al., 2010). On the other side, decreases in mycorrhizal fungi biomass have also been found (Liang et al., 2010). It has been suggested that pulse increases in nutrient availability and/or sorption of growth-inhibiting compounds by char, may play important roles in explaining these observations (Lehmann et al., 2011). Mechanisms of biochar effects to biota still need thorough understanding in terms of bioavailability of biochar-bound contaminants, sub-lethal effects, species interactions and functional redundancy, soil organic matter priming, among others (Tammeorg et al., 2017)

Although there is an increasing number of studies on effects of biochar in soil enzymatic activity, up to date, the information is lacking on the link between microbial enzymatic activities and activity of representative invertebrates (Paz-Ferreiro et al., 2014). Soil invertebrates are promoters and indicators of soil ecosystem services due to their contribution in nutrients cycling, primary production (e.g. through interactions with plants), soil formation and structure, etc. (reviewed by Lavelle et al., 2006). Through litter fragmentation, they stimulate microbial activity, resulting in increased mineralisation and humification of organic matter (Lavelle et al., 2006), and/or in stabilization of soil organic matter (e.g. within earthworms' casts, Bertrand et al., 2015). It has been demonstrated that the earthworm *Lumbricus rubellus* and the isopod *Porcellio scaber* can behave synergistically in litter decomposition in the presence of high quality litter (Zimmer et al., 2005). Soil enzymes are indicators of organic matter decomposition

in soil and nutrient cycling, reflecting both microbial and physicochemical characteristics of soil (Sinsabaugh et al., 2008).

Therefore, the aim of the study was to assess how woodchip biochar application to soil changes microbial activity in the presence or absence of key decomposer invertebrate species, looking at a key functional level. The current work used the terrestrial isopod *Porcellionides pruinosus,* a representative litter macrodecomposer (Loureiro et al., 2005; 2006), and the earthworm *Eisenia andrei,* known by its role in redistributing organic material and contributing to maintenance of soil structure and stability of aggregates (Lavelle et al., 1997). In addition, after assessing the potential effects on earthworm reproduction (inferring on results at the population level), other different organisational levels were explored to infer on mechanisms of toxicity of the applied biochar– at the biochemical level (oxidative stress), at the level of organism (energy related parameters in earthworms). Relating biomarker responses to toxicity is a widely used approach in terrestrial ecotoxicology (e.g. Santos et al., 2010; Novais and Amorim, 2013; Ferreira et al., 2016; Morgado et al., 2013). Due to their sensitivity biomarkers can serve as early warning signs of stress, and as an approach that can offer a mechanistic understanding behind an induced toxicity (van Gestel, 2012). Woodchip slow pyrolysis biochar was chosen for the study, characterised according to the EBC product quality guideline with the levels of potentially toxic elements bellow the benchmarks (EBC, 2012). The activity of soil enzymes in unamended soil and in 1.5 % (w/w) biochar amended soil was measured in five sampling events during a 56-days laboratory microcosm experiment.

The null hypotheses to be tested in the current work are: (i) soil microbial enzymatic responses are maintained in the presence of woodchip biochar and representative soil invertebrates *E. andrei* and *P. pruinosus* and (ii) exposure to woodchip biochar amended soil alone and/or the presence of *P. pruinosus* maintain the reproduction output and metabolic responses of *E. andrei* stable.

4.3. Materials and methods

4.3.1. Soil and biochar

The soil used in this experiment is a natural agricultural topsoil (10 cm), sampled in August 2015 from an agricultural area located in the Mondego valley (Central Portugal), with no history of contamination or inputs of pesticides and fertilizers in the last 6 years. It is a sandy loam of the following characteristics: sand 69.2 %, silt 18.8 %, clay 12.0 %, pH (H_2O) of 7.6, soil organic matter 2.9 % and maximum water holding capacity of 49%. The physicochemical characteristics of the soil and biochar are presented in **Table 4.1.**

Table 4.1. Physicochemical characteristics of the soil and woodchip biochar used in the study.

	Soil	**Biochar**
texture class	sandy loam	n.a.
sand (%)	69.2	n.a.
silt (%)	18.8	n.a.
clay (%)	12	n.a.
WHC_{max} (%)	49	73.2
Bulk density (g/cm^3)	n.a.	0.55
EC (µS/cm)	n.a.	3 000
Ash (550°C) (%)	n.a.	18.6
Organic C (%)	n.a.	75
Organic matter (%)	2.9.	n.a.
pH (H_2O)	7.6	10.1
pH (KCl)	7.4	n.a.
Salts (g/kg)	n.a.	8.4
$CaCO_3$ (g/kg)	89	n.a.
H (%)	n.a.	47
H:C (molar ratio)	n.a.	0.07
O:C (molar ratio)	n.a.	0.04
N total (g/kg)	1.98	n.a.
N (%)	n.a.	1.8
P_2O_5 (mg/kg)	805	n.a.
K_2O (mg/kg)	250	n.a.
Al (mg/kg)[1]	17 000	n.a.
Sb (mg/kg)	<5	n.a.
As (mg/kg)	18	n.a.
Ba (mg/kg)	110	n.a.
Be (mg/kg)	1.8	n.a.
Pb (mg/kg)	210	<2
B (mg/kg)	13	39

Cd (mg/kg)	<0.5	<0.2
Ca (mg/kg)	25 000	42 200
Cr (mg/kg)	17	27
Hg (mg/kg)	n.a.	n.a.
Fe (mg/kg)	23 000	2 420
K (mg/kg)	3 200	10 400
Cu (mg/kg)	82	16
Li (mg/kg)	70	n.a.
Mg (mg/kg)	5 000	2 980
Mn (mg/kg)	1 100	n.a.
Mo (mg/kg)	<5	n.a.
Na (mg/kg)	120	744
Ni (mg/kg)	17	17
P (mg/kg)	1 500	1 300
S (mg/kg)	n.a.	372
Se (mg/kg)	<10	n.a.
Sr (mg/kg)	90	n.a.
Tl (mg/kg)	<2	n.a.
Ti (mg/kg)	600	n.a.
V (mg/kg)	23	n.a.
Zn (mg/kg)	200	70
Sn (mg/kg)	15	n.a.
ΣPAHs (mg/kg)[2]	n.a.	0.48
Σ7 ind. PCBs (mg/kg)[3]	n.a.	<0.002

[1]Metals were determined by microwave digestion (DIN/ISO 17294-2).
[2]PAHs were determined by SPME (solid-phase microextraction) coupled to gas chromatography/mass spectrometry GC/MS (DIN EN 15527), where individual PAH values were below or equal to the limit of detection (0.1 mg/kg).
[3]The 7 indicator PCBs were determined by HRGC/HRMS (high resolution gass chromatography and mass sectrometry)

Biochar was acquired from Swiss Biochar gmbh (Switzerland). The biochar was produced from slow pyrolysis (620°C) of wood chip production residues. It is characterized with the following particle size distribution (w/w): 4% (<0.1 mm), 25% (0.1-0.5 mm), 34% (0.5-2 mm), 37% (>2 mm), with an average of 29.5 µm and pH (H_2O) of 10.1.

4.3.2. Soil invertebrates

The earthworm *Eisenia andrei* (Bouché 1972) and the isopod *Porcelionides pruinosus* (Brandt 1883) were obtained from established laboratory cultures maintained at 20±1ºC (earthworms) and 22±1ºC (isopods), with a photoperiod of 16:8 (light:dark). Earthworms were kept in opaque 24 L plastic containers, with a mixture of soil potting mix and peat, at pH between 6 and 7, and at 70% of its water holding capacity (WHC). Earthworms were fed weekly with horse manure previously frozen and gradually thawed. Adult earthworm individuals were three months old,

with developed clitella and with a body weight ranging 300-600 mg. Isopod cultures were maintained in soil moistened to approximately 40-50% of its WHC, where animals were fed with alder leaves (*Alnus glutinosa*) *ad libitum* (Morgado et al., 2013). Only adult isopods with antenna were selected (15-25 mg fresh weight) to ensure suitable perception of chemical stimuli via antennae (Takeda, 1980) and pregnant females were excluded from the experiment.

4.3.3. Experimental treatments and set-up

The experimental design included several soil treatments kept for 56 days: S (soil), Sm (soil-manure), SB (soil-biochar), SBm (soil-biochar-manure), Smi (soil-manure-isopods), Sme (soil-manure-earthworms), Smie (soil-manure-isopods-earthworms), SBmi (soil-manure-isopods), SBme (soil-manure-earthworms), SBmie (soil-manure-isopods-earthworms). All treatments consisted of four replicates (four microcosms) and the biochar application in soil corresponded to 1.5 % (w/w) of biochar. Soil used in the experiments was previously sieved (<2 mm). Every microcosm contained 400 g of soil/biochar-amended soil, adjusted to 60 % of maximum water holding capacity (WHC). During the experiment the moisture was checked daily and adjusted gravimetrically by spraying with distilled water, when needed.

The experimental treatments consisted of soil and/or biochar-amended soil and for those with organisms, manure was provided as food. The single species treatments included four isopods, or six earthworms each. The combined species treatments included together four isopods and six earthworms. Dried (at 70°C in the oven) and sieved (<2 mm) horse manure used also for the culture maintenance in the laboratory was supplied weekly as a source of food. Food (2 g) was provided weekly in the first four weeks, and always after previous sampling for soil enzymatic activity assays. It was also added to those treatments without organisms. This amount of food was previously defined in a small reproduction trial experiment (data not shown). This trial ensured no negative effects on the earthworm reproduction, but also to see if the food supplied was sufficient when isopods were present. For the soil enzymatic activities assays, soil only, soil with manure, soil with biochar, and soil with manure and biochar were added as reference microcosms to serve for determination of enzymatic activities without impact of the isopods and earthworms. This ensured the quality and interpretation of results, but also to serve as comparison of levels reported for biochar-amended soil in other studies.

The microcosms were incubated at 21±1°C and with photoperiod of 16:8 h of light:dark. The experiment duration (eight weeks) ensured the production of *E. andrei* cocoons in the first four weeks plus four more weeks for the cocoons to hatch and to obtain juveniles. In the middle of the experiment, after four weeks, the number of cocoons was reported, as well as the adults' body weight in order to compare the initial adults' weight. Both isopods and earthworms were removed from the pots at the end of the fourth week. Earthworms were frozen in a liquid nitrogen and stored at -80°C for the biochemical and PAHs analysis. Isopod mortality was randomly detected and in order to maintain the community for the enzymatic assessment, the dead individuals were replaced with the ones from the same culture, at any point of the experiment. The mortality recorded was lower than 10 %, yet remained as the study limitation due to inability to assess the weight changes and biochemical parameters in isopods.

Sampling for enzymatic activity assays was conducted after the first (sampling time 1), second, third, fourth, and eighth week of the experiment (sampling time 2 to sampling time 5). Sampling was done by carefully taking two small corers of of the topsoil in each microcosm. The dimensions of corer were 4-5 cm (height), and 1.5 cm (diameter). At the end of the experiment (sampling time 5 for the enzymatic assays) *E. andrei* juveniles were counted by manual sorting.

4.3.4. Soil enzymatic activity methods

Activities of dehydrogenase (EC 1.1.1.49) and ß-glucosidase (EC 3.2.1.21) were performed according to the protocols of Tabatabai (1994), and as described in Dick et al. (1996). For dehydrogenase soil solution was suspended in a triphenyl-tetrazoliumchloride solution (TTC). The samples were incubated at 37°C for 24 h. The product triphenylphormasan (TPF) was extracted with pure methanol (analytical grade) and the absorbance was measured at 546 nm using microplate reader. Activity of ß-glucosidase was performed with incubation of soil in buffered p-nitrophenyl- ß-D-glucoside solution at pH=6, for 1 h at 37°C. Production of p-nitrophenol resulted in a change of color which was measured at 405 nm with a microplate reader. Urease (EC 3.5.1.5) was determined according to the protocol of Kendeler and Gerber (1988), by suspending the samples of soil and biochar amended soil in borate buffer at pH=10, and in solution of urea, following 2h of incubation at 37°C. The absorbance was measured at 690 nm using microplate reader MultiSkan Spectrum (Thermo Fisher Scientific). NH_4^+ release was expressed as mg N/kg soil/2 h.

4.3.5. Biomarkers in *Eisenia andrei*

Specimen were individually homogenized on ice by sonication (for 30s, 250 Sonifier, Branson Ultrasonics) in 3000 µL of K-phosphate buffer (0.1 M, pH 7.4). Aliquots were taken for the analysis of lipid (300 µL), sugar, and protein content (300mL) and electron transport system (ETS) activity (300 µL),100 µL for PAHs, 200 µL was used for the determination of lipid peroxidation. Remaining homogenate of around 1900 µL was centrifuged for 20min at 10 000 g and at 4°C. Postmitochondrial supernatant (PMS) was afterwards split into 5 microtubes. The PMS samples were kept at –80°C for further analysis: 100 µL for catalase (CAT), 100 µL for proteins, 300 µL for glutathione-S-transferases (GST), and 300 µL for acetylcholine-esterase (AChE).

All protocols for homogenisation, biomarkers analysis, energy reserves, cellular energy allocation (CEA) were followed according to Ferreira et al. (2010), with adaptations for the earthworm tissue where stated. AChE activity was conducted according Ellman's method (Ellman et al., 1961), and adapted to microplate reader according Guilhermino et al. (1966). Absorbance increase at 412 nm was read for the substrate acetylthiocholine. GST was performed following Habig et al. (1974), and CAT according to the protocol of Clairborne (1985). LPO was performed according to Bird and Draper (1984) and Ohkawa et al. (1979) and adapted to microplate (Ferreira et al., 2010). Protein concentration was obtained from a 50-mL PMS aliquot as described in Bradford's method (Bradford, 1976). This methodology was adapted from BioRad's Bradford microassay for 96-well plate, using bovine g-globulin as a standard.

PAH-type metabolites were quantified with fixed fluorescence analysis. This method was developed for fish bile samples, showing to be a good proxi for PAHs as a biomarker of exposure (Aas et al. 1998; 2000a, 2000b). Prior to analysis every specimen was defrost and individually homogenized on ice by sonication (for 2*30 s, 250 Sonifier, Branson Ultrasonics) in 3000 µL of K-phosphate buffer (0.1 M, pH 7.4).

The PAH-type metabolites are expressed in ng/mg of earthworm body weight, relative to the standard calibration curves with known concentrations of naphthalene (Nap), phenantrene (Phe), pyrene (Pyr) and benzo[a]pyrene (BaP). Homogenized samples were mixed with 50 % methanol (50 µL sample and 4950 µL), vortexed, and sonicated for 1 min at 25°C. 300 µL of each sample were transferred to multi-well plates for the readings. Fluorescence was determined in a spectrofluorometer (Hitachi F-7000) in excitation-emission wavelength pairs:

290 nm-335 nm for Nap, 341 nm-383 nm for Pyr, 256 nm-380 nm for Phe, and 380 nm-430 nm for Bap (Gravato and Santos, 2003). For quality assurance, limits of detection (LOD) and limits of quantification (LOQ) were calculated for each of these metabolites from a calibration curve at low concentrations, as described in Shrivastava and Gupta (2011). The results were interpreted based on the obtained values defining LOD as a minimum detectable concentration of an analyte in a sample under the given test conditions. LOQ considers a minimum determined concentration of an analyte in a sample under the given test conditions, that can be claimed with an acceptable level of precision and accuracy (Shrivastava and Gupta, 2011).

Available energy (carbohydrates, lipids, proteins) and energy consumption (activity of ETS) were determined according to method of De Coen and Janssen (1997), with adaptation for microplates (Ferreira et al., 2010; Rodrigues et al., 2015). Further on, the methodology used is described in Ferreira al. (2016). The energy consumed (Ec) value was transformed into caloric values using the specific oxyenthalpic equivalent to average of lipid, protein, and carbohydrate mixture of 480 kJ/mol O2. Calculated values were expressed by the organisms' fresh weight.

The available energy (Ea) was calculated as the sum of the total lipid, carbohydrate, and protein fraction, calculating first the difference as mg per organism and converting into caloric values using enthalpy of combustion: 39.5 kJ/g lipid, 17.5 kJ/g glycogen and 24 kJ/g protein. At last, the CEA was calculated as the ratio between Ea and Ec (CEA=Ea/Ec).

Spectrophotometric readings were all conducted in the Microplate reader MultiSkan Spectrum (Thermo Fisher Scientific).

4.4. Statistical analysis

A Principal Component Analysis (PCA) was carried out to explore the whole matrix of data consisting of: activities of three soil enzymes recorded over five sampling occasions in 10 experimental treatments. This exploratory approach was used to investigate the relationship between the experimental treatments and soil enzymatic activities. Standardization of the enzymatic activity was applied in order to obtain the same weight of each enzyme, i.e. to be used in one ordination plot (ter Braak and Smilauer, 2002). CANOCO 4.5 software for Windows was used for PCA.

Permutational multivariate analysis of variance (PERMANOVA) was also carried out based on the whole matrix of data, to investigate potential differences in the global enzymatic activity based on the experimental design, namely on the factors 'treatment' and 'sampling time', and the factors' interaction. Like in the case of PCA, variables were previously standardized. PERMANOVA was performed with R 3.4.4 software, Vegan package.

At last, the treatments without organisms and the treatments with organisms were separately analysed by factorial analysis of variance (ANOVA). Data were examined for normality and homoscedasticity with Shapiro-Wilk and Leven's tests, respectively. In the case of the treatments without organisms, 'treatment' was used as a first two-levels factor (with biochar/without biochar) and manure as a second two-levels factor (with manure/without manure). For the treatments with organisms, 'biochar' was used as a first two-levels factor (no biochar/biochar) and 'invertebrates' as a second factor consisting of three-levels (earthworms alone/isopods alone/combined earthworms and isopods). Earthworms' reproduction, changes in body weight and all biomarkers of effect and exposure (PAHs-type metabolites) measured were also assessed by factorial analysis of variance (ANOVA), with 'biochar as a first two-levels factor (no biochar/biochar) and 'invertebrates as a second two-levels factor (earthworms alone/earthworms with isopods). When statistical significance was detected with ANOVA, Tukey post hoc method was applied to test for specific differences. ANOVA was conducted with statistical software Sigma Plot 12.5.

4.5. Results

4.5.1. Soil enzymatic activity

Figure S4.1. in the Supplementary Information (SI) file is integrating the soil enzymatic activities observed for soil enzymes over five sampling times. pH values of soil and biochar amended soil at the start of the experiment were 7.7 (S), 7.9 (Sm), 8.1 (SB) and 8.0 (SBm), as measured in H_2O, in a proportion 1:5 v/v of soil/amended soil and deionised water. **Table S.4.1** is presenting pH values of the treatments at the end of experiment. The pH of all treatments ranged between 7.9 and 8.3.

The PCA performed separated the effects of different treatments and the sampling times on soil enzymatic activity. **Figure 4.1. i**s presenting the first two ordination axes that explained 89.7 % of the total variability. Principle component 1 (PC1) explained major variability (53.8 %)

in the enzymatic activity, which is closely related to sampling time. ß-glucosidase and urease were strongly positively related with PC1, which is separating sampling time 3, as an overall peak in the activities of these two enzymes, from the other sampling times with lower activities. Principle component 2 (PC2) explained 35.9 % variability, with dehydrogenase being strongly positively related to it. PC2 provides a sampling time dependency gradient of dehydrogenase activity, separating sampling time 1 with an increased activity, from sampling time 2 which is characterized by the lowest overall activity. No clear separation was achieved in the case of other sampling times.

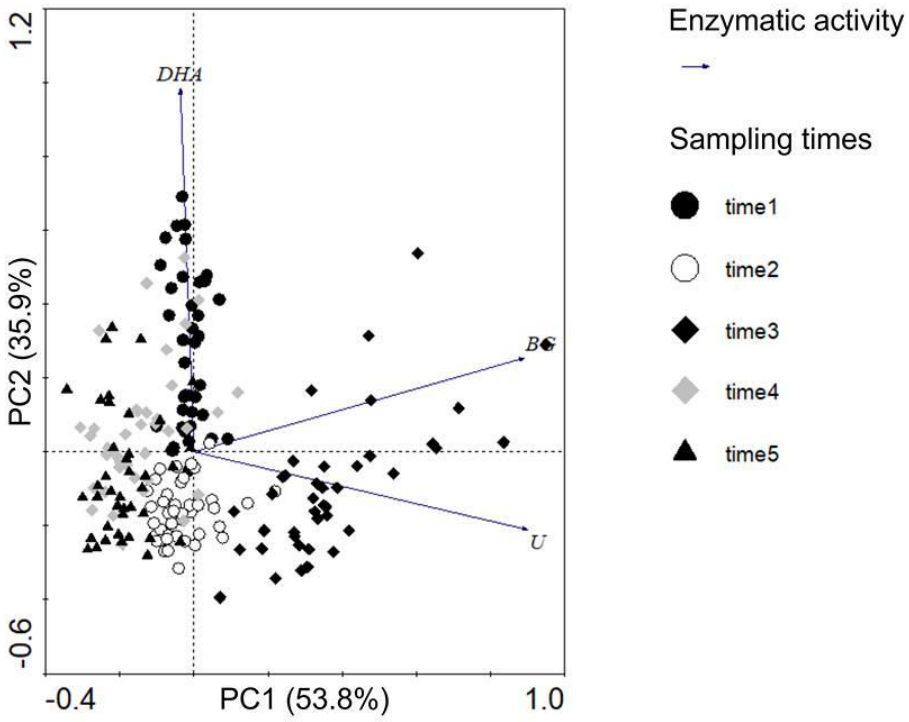

Figure 4.1. Enzymatic activity response diagram from the principal component analysis (PCA) of the experimental treatments in five sampling times (time 1 to time 5) used as samples data. Endpoints datasets of soil/amended soil treatments used as response variable (DHA-dehydrogenase, BG-ß-glucosidase and U-urease). Data sets were obtained from the experimental treatmens (*S* soil; *Sm* soil-manure; *SB* soil-biochar; *SBm* soil-biochar-manure; *Smi* soil-manure-isopods; *Sme* soil-manure-earthworms; *Smie* soil-manure-isopods-earthworms, *SBmi* soil-manure-isopods; *SBme* soil-manure-earthworms; *SBmie* soil-manure-isopods-earthworms).

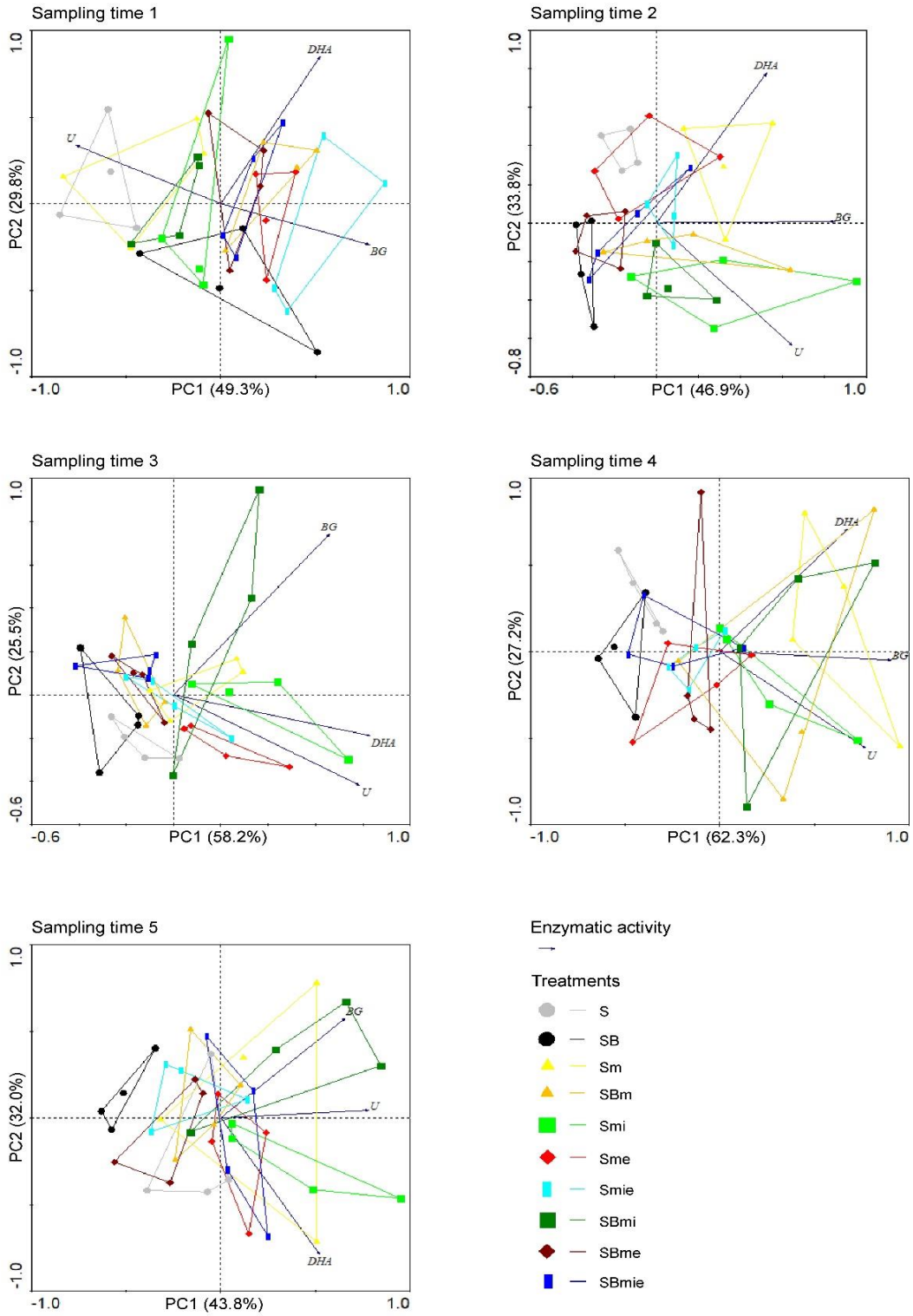
Sampling time 1
PC2 (29.8%)
PC1 (49.3%)
DHA
U
BG
Sampling time 2
PC2 (33.8%)
PC1 (46.9%)
DHA
BG
U
Sampling time 3
PC2 (25.5%)
PC1 (58.2%)
BG
DHA
U
Sampling time 4
PC2 (27.2%)
PC1 (62.3%)
DHA
BG
U
Sampling time 5
PC2 (32.0%)
PC1 (43.8%)
BG
U
DHA
Enzymatic activity
Treatments
S
SB
Sm
SBm
Smi
Sme
Smie
SBmi
SBme
SBmie

Figure 4.2. Enzymatic activity response diagram from the principal component analysis (PCA) of the experimental treatments for each time of sampling (from Sampling time 1 to 5). Endpoints datasets of soil/amended soil treatments used as species data (DHA-dehydrogenase, BG-ß-glucosidase and U-urease). Data sets were obtained from the samples - experimental treatmens (*S* soil; *Sm* soil-manure; *SB* soil-biochar; *SBm* soil-biochar-manure; *Smi* soil-manure-isopods; *Sme* soil-manure-earthworms; *Smie* soil-manure-isopods-earthworms, *SBmi* soil-manure-isopods; *SBme* soil-manure-earthworms; *SBmie* soil-manure-isopods-earthworms).

Figure 4.2. is depicting PCA of experimental treatments as a trend in enzymatic activities for each of the five sampling times separately. In the first sampling (ST1) PC1 explained 49.3 % variability in the soil enzymes, with ß-glucosidase being positively related to it, and urease negatively. PC2 explained 29.8 % variability, with dehydrogenase strongly positively related to it. Treatments are separated along the first axis, showing that differences are mainly due to the activity of ß-glucosidase and urease. In the second sampling (ST2) PC1 explained 46.9 % variability, and PC2 variability of 33.8 %. The trend of the enzymatic activities through the treatments can be seen as overall low activities of ß-glucosidase and dehydrogenase, particularly in the treatment SB (for both enzymes), and in SBme (for ß-glucosidase). In the third sampling (ST3) PC1 explained 58.2 % variability, while PC2 explained 25.5 % variability. It is separating high ß-glucosidase activity (particularly in SBmi) and high dehydrogenase and urease activities in Smi and Sme from the low enzymatic activities recorded in SB and S. In the fourth sampling time (ST4) 62.3 % variation is explained by PC1, clearly separating lower S and SB activities from higher ones in Sm and SBmi. PC2 explained 27.2 % of the variation. In the fifth sampling time (ST5) PC1 explained 43.8 % of variation, and PC2 32.0 %. The separation of PC1 between mainly S, SBme and SB, on negative side of the axis from the rest of the treatments on the positive side is due to the low activities of the enzymes in these treatments.

The PERMANOVA depicted the significant differences between the sampling times ($F = 0.080$, $p= 0.001$), and between treatments ($F=0.076$, $p=0.008$), which supports the presented ordination provided with PCA. However, significant interaction effects for the two factors was not detected by the PERMANOVA ($F= 0.022$, $p=0.077$).

Firstly, for a closer insight on the differences in the treatments without the organisms (S, Sm, SB, SBm) a two-way ANOVA was carried out, for each enzyme and for five sampling times, analysing factors manure (presence/absence of manure) and treatment (presence of biochar

or SB/absence of biochar or S). Interactions of the factors were not statistically different, in any of the sampling times (two-way ANOVA, $p>0.05$). The ß-glucosidase activity in treatments without organisms was statistically significant for the factor treatment in ST1, due to increased activity in biochar amended soil SB and SBm, but post hoc test did not reveal any specific differences (two-way ANOVA, $p<0.05$; Tukey test, $p>0.05$). In ST3 presence of manure increased the activity only within unamended soil S (two-way ANOVA, Tukey test, $p<0.05$), and in ST4 within both S and SB (two-way ANOVA, Tukey test, $p<0.05$). ST2 and ST5 did not result in significant differences in ß-glucosidase activity among the treatments without organisms (two-way ANOVA, $p>0.05$). Regarding urease activity in ST1 presence of biochar significantly reduced the enzyme activity, with or without manure (two-way ANOVA, Tukey test, $p<0.05$). For ST2, ST4 and ST5 effects were driven by the presence of manure, i.e. being significantly stimulated both in Sm and SBm (two-way ANOVA, Tukey test, $p<0.05$). No differences were detected in the urease activity in ST3 (two-way ANOVA, $p>0.05$). Dehydrogenase activity was characterized with manure driven effects in ST1 as stimulation of SBm (two-way ANOVA, Tukey test, $p<0.05$), and in ST4, but no specific differences were detected with post hoc test (two-way ANOVA, $p<0.05$; Tukey test, $p>0.05$). In ST2 the response of dehydrogenase was significantly different for both factors 'manure' and 'biochar' (treatment), generally being reduced in the case of biochar presence (two-way ANOVA, Tukey test, $p<0.05$), but stimulated with the presence of manure both in unamended and biochar-amended soil (two-way ANOVA, Tukey test, $p<0.05$). In ST3 and ST5 statistically significant reduction of activity in the presence of biochar was observed (two-way ANOVA, Tukey test, $p<0.05$), while presence of manure did not have statistically significant impact (two-way ANOVA, Tukey test, $p>0.05$)

Secondly, for the treatments with organisms (Sme, Smi, Smie, SBme, SBmi, SBmie), a two-way ANOVA was carried out on the enzymatic activities and allowed to distinguish whether the factors 'invertebrates' (e-earthworms, i-isopods, ie-earthworms and isopods) or 'biochar' (presence of biochar SBm/absence of biochar Sm), or their interaction had significant effects on these endpoints. It is important to note that manure was present in all the microcosms with organisms as a source of food. Interactions of the factors analysed were not statistically different, in any of the sampling times (two-way ANOVA, $p>0.05$). The activity of ß-glucosidase in ST1 was invertebrate-dependent only within S, and in Smi it was significantly lower than in Sme and Smie (two-way ANOVA, Tukey test, $p<0.05$). The presence of biochar reduced significantly the enzymatic response of of ß-glucosidase in ST2, but post hoc test did not detect

any specific differences (two-way ANOVA, $p<0.05$; Tukey test, $p>0.05$). In the ST3 factor species was statistically significant within SB treatments, namely the presence of isopods (SBi) stimulated ß-glucosidase activity comparing to the treatment with only earthworms (SBe) and in the combined species treatment (SBmie) (two-way ANOVA, $p<0.05$; Tukey test, $p<0.05$). The activity of ß-glucosidase in ST4, even overall decreased relative to the ST3, responded with a similar pattern as factor species was statistically significant within SB treatments, namely presence of isopods (SBi) stimulated ß-glucosidase activity comparing to the treatment with only earthworms (SBe). The response of ß-glucosidase in ST5 was not statistically different for any of the factors, or their interaction (two-way ANOVA, $p>0.05$).

Measurements of urease in ST1 resulted in statistically significant differences in the presence of both invertebrate species, namely within S due to the reduction in activity in the Smie treatment (two-way ANOVA, $p<0.05$; Tukey test, $p<0.05$), while in the presence of biochar stimulation of urease was observed in SBmie (two-way ANOVA, $p<0.05$; Tukey test, $p<0.05$). In ST2, in the presence of isopods in unamended soil (Smi) urease activity was significantly higher than is the treatments with earthworms or combined species treatments (Sme, or Smie) (two-way ANOVA, $p<0.05$; Tukey test, $p<0.05$). In ST3 statistically significant reduction in the presence of biochar was observed within SBme and SBmie (two-way ANOVA, $p<0.05$; Tukey test, $p<0.05$). Statistically significant invertebrate-specific effects were also observed in the ST4 as a stimulation in the presence of isopods over reduction in the presence of two species. However, no specific differences were detected in the post hoc (two-way ANOVA, $p<0.05$; Tukey test, $p>0.05$). In ST5 the response pattern was the same like in ST2, isopods driven, but only within S by means of significantly higher activity in Smi over Sme and Smie (two-way ANOVA, $p<0.05$; Tukey test, $p<0.05$).

The dehydrogenase activity in treatments with organisms was statistically significant only in ST3 for the factor 'biochar', within single species treatments as follows: Smi was significantly higher than SBmi, and Sme than SBme ((two-way ANOVA, $p<0.05$; Tukey test, $p<0.05$).

4.5.2. Earthworms weight changes, reproduction, biomarkers of effectparameters

Weight loss and reproduction of *E. andrei* were not affected by either the presence of biochar or the presence of the isopods (two-way ANOVA, $p>0.05$; **Figure 4.3.**).

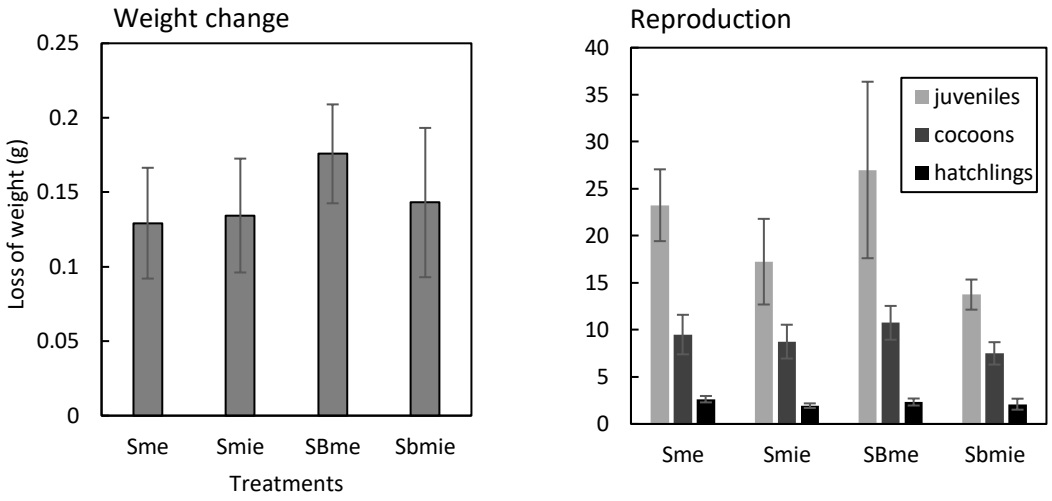

Figure 4.3. *Eisenia andrei* weight loss (g) and reproduction (as number of juveniles, number of cocoons and hatchlings number per cocoon) resulting from the four-week- exposure to soil (Sm) and biochar amended soil (SBm, at 1.5 % w/w), in the absence (e) and presence (ie) of *Porcellionides pruinosus.*

Biomarker responses in *E. andrei* after a four-weeks exposure to soil and biochar amended soil in the microcosms in the presence and/or absence of *P. pruinosus* are presented on **Figure 4.4.** Glutathione s-transferase (GST) activity and acetylcholinesterase (AChE) activity did not reveal significant fluctuations amongst exposed earthworms (two-way ANOVA, $p>0.05$). Catalase (CAT) was significantly reduced for factor treatment (two-way ANOVA $p<0.05$), but no specific differences could be detected with Tukey post hoc test ($p>0.05$). In the presence of biochar lipid peroxidation was higher, both when earthworms were alone (SBme) and with isopods (SBmie) (two-way ANOVA; Tukey test, $p<0.05$).

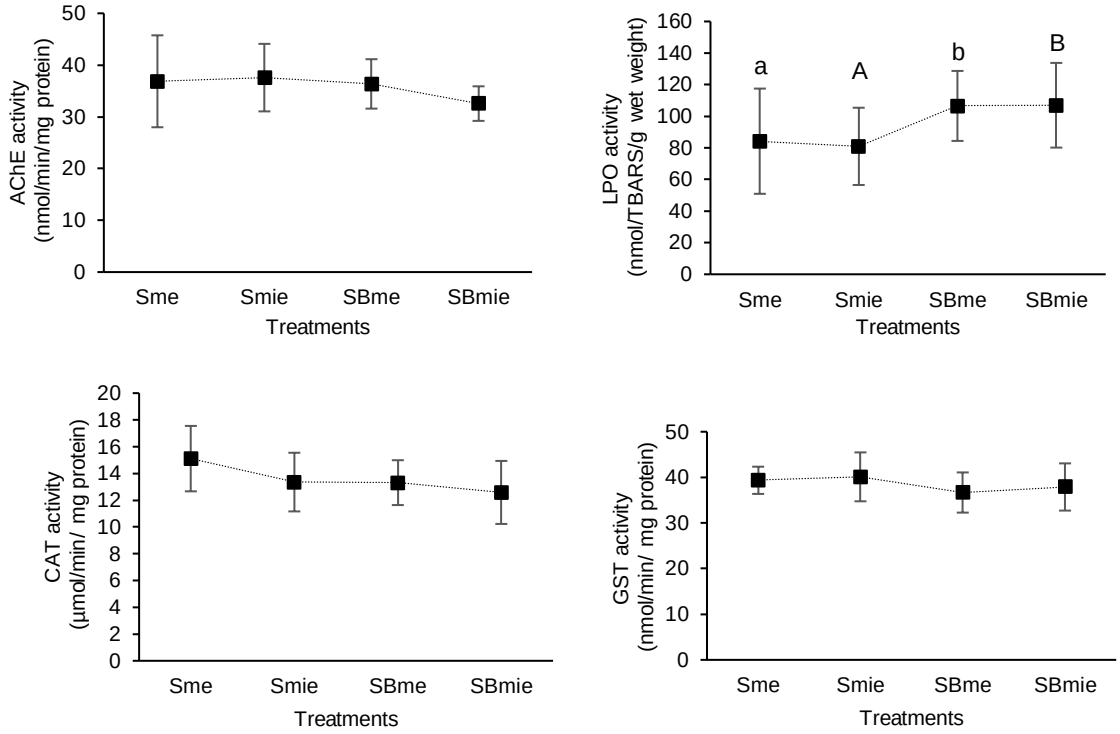

Figure 4.4. *Eisenia andrei* biomarker of effects responses resulting from the four-week-exposure to soil (Sm) and biochar amended soil (SBm, at 1.5 % w/w), in the absence (e) and presence (ie) of *Porcellionides pruinosus*. catalase (CAT); glutathione-S-transferase (GST); lipid peroxidation (LPO); acetylcholinesterase activity (AChE). All values are presented as means with standard deviation. Different lowercase and uppercase letters represent significant comparisons for factor treatment (Sm/SBm) within single species (e) and within two species (ie) microcosms, respectively (two-way ANOVA, Tukey test $p<0.05$).

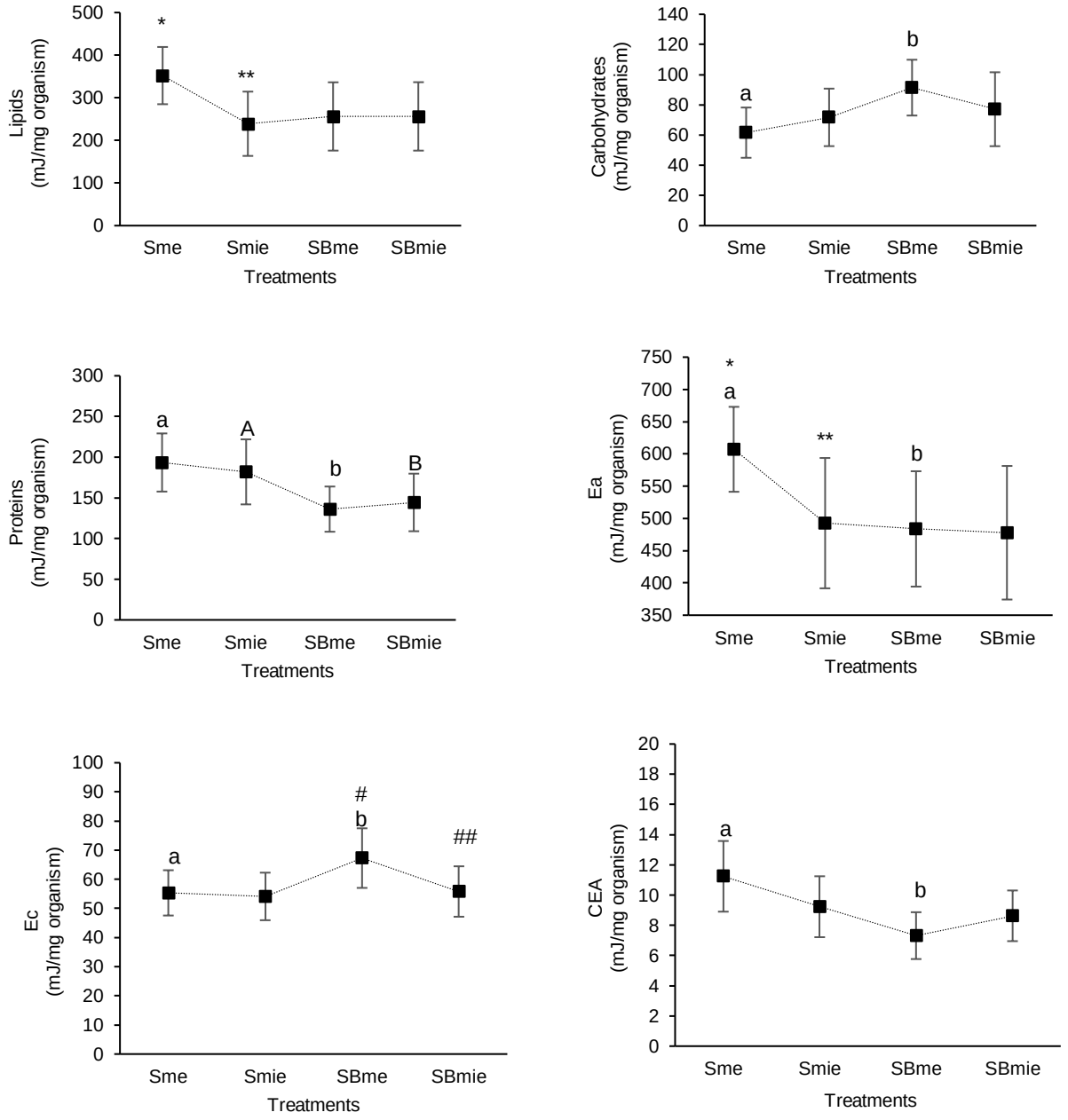

Figure 4.5. *Eisenia andrei* energy related parameters resulting from a four-week-exposure to soil (Sm) and biochar amended soil (SBm, at 1.5 % w/w), in the absence (e) and presence (ie) of *Porcellionides pruinosus*: lipids, carbohydrates and proteins content, and the balance for the energy available (Ea), energy consumed (Ec) and cellular energy allocation (CEA) are presented as means with standard deviations. Different lowercase and uppercase letters represent significant comparisons for factor treatment (Sm/SBm) within single species (e) and within two species (ie) microcosms respectively; * and ** represent significant comparisons for factor species (e/ie) within Sm, while # and ## represent significant comparisons for factor species within SBm (two-way ANOVA, Tukey test $p<0.05$).

Figure 4.5. depicts energy budget of *E. andrei* after 4-weeks of exposure to soil and biochar amended soil in the microcosms in the presence and/or absence of *P. pruinosus*. The lipids' content was significantly lower in the combined invertebrate microcosms (two-way ANOVA, $p<0.05$). Post hoc test revealed statistically significant difference for factor species within S (two-way ANOVA, $p<0.05$; Tukey test, $p<0.05$), with higher lipids observed when earthworms were kept alone with no biochar. Levels of carbohydrates were significantly increased for the factor biochar, within single species microcosms (two-way ANOVA, $p<0.05$; Tukey test, $p<0.05$). Protein contents were significantly reduced in the biochar treatment, both within single and two species microcosms (two-way ANOVA, $p<0.05$; Tukey test, $p<0.05$). In terms of the energy available (Ea), statistically significant reductions were observed in the presence of biochar (within single species micorcosms, two-way ANOVA, Tukey test, $p<0.05$) and the factor invertebrate (within Sm, two-way ANOVA, Tukey test, $p<0.05$; **Figure 4.5.**). Increase in consumed energy (Ec) was statistically significant for the factor invertebrates, by means of higher consumed energy within SB in a single species microcosms (SBme) over two species microcosms (SBmie) (two-way ANOVA, Tukey test, $p<0.05$), and for the factor treatment, by means of higher consumed energy in SBme over Sme (two-way ANOVA, Tukey test, $p<0.05$). Cellular energy allocation (CEA) was significantly lower in the treatment SBme, over Sme, i.e. within single species microcosms treatments (two-way ANOVA, Tukey test, $p<0.05$).

Naphthalene-metabolites were the only PAH-type metabolites quantified in the earthworms tissue, with no statistically significant difference between the treatments (two-way ANOVA, $p>0.05$; **Figure S4.2.** in SI).

4.6. Discussion

4.6.1. Soil and biochar amended soil enzymatic activity

Biochar used in the present study is considered a technically safe biochar, fully characterized according to the EBC guideline (EBC, 2012), to assure for homogeneity in physicochemical properties. Wood residues are very common feedstocks for biochar production, which together makes it a representative biochar. In addition, this biochar was previously tested (data not shown), and had no impact on the survival and body weight of *E. andrei* at the concentration chosen in the present work of 1.5 % w/w.

To infer on the changes this biochar application can induce to soil microbiota, along with the presence of the two invertebrates' species, activities of soil enzymes ß-glucosidase, dehydrogenase and urease were assessed over a 56 days laboratory microcosms' incubation, in five sampling occasions (sampling times). Overall the responses were dependent on the sampling time and treatment. In general, ß-glucosidase and urease peaks in activities occurred after three weeks of incubation (i.e. third sampling). In treatments without invertebrates the activity of these enzymes was higher in the presence of manure, with exception of the responses in the first week. Dehydrogenase peak of activity occurred after the first week, where the input of manure induced an increase in dehydrogenase activity, while presence of biochar reduced and even inhibited dehydrogenase in the treatments without organisms. Biochars produced of lignin-rich feedstocks, like it is the case in our study, are more likely to induce negative priming, a process known as induced changes (positive or negative) in soil organic matter mineralisation (SOM) as consequence of addition of organic substrates (Kuzyakov et al., 2000). Possibly negative priming occurred due to higher proportion of recalcitrant carbon in wood biochar (Yu et al., 2018), while in the treatments with manure, on the contrary, the manure might have caused a positive priming due to readily available, labile carbon. More experiments are needed, however, to relate the effects observed in the current study with SOM priming.

The most pronounced increase in ß-glucosidase treatments with organisms was obtained in the biochar amended soil in the presence of isopods. Isopods could benefit from the presence of biochar in soil while using it as a food source, as it has been reported recently by Madzaric et al. (2017). ß-glucosidase can be a good indicator of changes in soil organic matter content (Bandick and Dick, 1999; Paz-Ferreiro et al., 2014), and was considered a sensitive endpoint in the current study, responding to the different treatments.

Urease is an enzyme involved in the hydrolysis of urea, commonly used to distinguish between soils enriched with crop residues, nitrogen and animal manure (Bandick et al., 1994; Dick et al., 1999). In this work urease was overall characterised by lower fluctuations amongst the treatments. The effect of invertebrate species (namely isopods) is dominant in the urease activity measured. It was increased in the presence of isopods, but only without biochar. There may be a possibility that isopods are affecting or inducing urease by the excretion of ammonia (Loureiro et al., 2006).

Dehydrogenase is an intracellular enzyme, an indicator of overall microbial activity in soil (Dick et al., 1996). The most pronounced outcome observed for dehydrogenase was in ST3, the

substantially higher activity in the absence of biochar in both single species microcosms, indicating possible positive effect of individual species on this enzyme, but disappearance of the positive effect when biochar was present and in the case of the two-species presence. However, there was not a clear response pattern in the last two sampling times (ST4 and ST5). Elzobair et al. (2016) reported contrasting effects of hard wood fast pyrolysis biochar versus diary manure applied in the field, observed as neutral impacts of biochar and higher efficiency of diary manure in enhancing microbial biomass and activity (Elzobair et al., 2016).

The observed effects of woodchip biochar in this study are not in line with results reported by other authors for biochars from various feedstocks. Chicken manure biochar (Park et al., 2011) and sewage sludge biochar (Paz-Ferreiro et al., 2012) stimulated dehydrogenase, while Masto and authors reported that dehydrogenase activity was proportional to biochar application increase (biochar from water hyacinth; Masto et al., 2013). However, the information on the role of invertebrates in modification of these processes in biochar amended soil is scarce. A three months study on the effects of earthworms *Pontoscolex corethrurus* and biochar on soil enzymes (ß-glucosidase, ß-glucosaminidase, arylsulphatase, phosphomonoesterase and urease) has been conducted by Paz-Ferreiro et al. (2014). Increases in enzymatic activities were observed, being more pronounced in high mineral ash biochars (sewage sludge biochars; mineral ash ranging from 64.81 % to 78.53 %), than in low mineral ash biochars (Miscanthus biochar and wood gasification char; mineral ash content of 18.75 % and 29.82 %, respectively), while underlining the higher impact in low fertile soils (acidic pH, low organic matter content) as result of liming effect (pH increase). They reported that biochar and earthworms did not interact in relation to soil enzymes, with the exception of arylsulphatase, and that only for ß-glucosidase activity there was an observed interaction between soil type and presence of earthworms (Paz-Ferreiro et al., 2014). In our study the ash content of biochar was as low as 18.6 %, comparable to that in Miscanthus biochar (Paz-Ferreiro et al., 2014). However, this kind of mechanistic effects regarding possible contribution of biochar ash contents in changes of enzymatic activity of biochar amended soil need to be specifically addressed in the follow-up studies. Additionally, in the current study the alkaline pH in all the treatments was relatively stable, and is less likely that could influence the results.

When microbial activity enzymes are assessed as indicators of soil quality, like for instance, in the case of contaminated soil and recovery evaluation, it is recommended to be used as a complementary approach, i.e. within a battery of assays (Loureiro et al., 2007). Our study

demonstrates that soil enzymes responses were overall specific for each of the enzyme evaluated, time- and invertebrates-dependent, highlighting that they are sensitive tools in biochar amended soil quality assessment and can be also suggested as complementary approach within a soil test battery with invertebrates.

4.6.2. Effects on earthworms

Earthworms body mass loss, number of cocoons or number of juveniles were not affected by the presence of 1.5 % (w/w) biochar, or altered in the presence of other detritivore species, in this case the isopod *P. pruinosus*. Similar results to ours were obtained for much higher concentrations, of up to 20 % of apple woodchip biochar regarding earthworms' reproduction. The same work, reported a weight loss in *E. fetida* (Li et al. 2011), as opposite of our results.

Soil invertebrate have been widely used as model organisms in multibiomarkers approaches to assess the effects of soil contaminants, e.g. *Porcellionides pruinosus* (e.g. Santos et al., 2010; Ferreira et al., 2016), *Eisenia andrei* (e.g. Cataldo et al., 2011; Wu et al., 2012; Nusair et al., 2017). Also, energy related parameters have been previously related to chemical exposures in several studies, using the potworm *Enchytraeus albidus* (Novais et al., 2013; Gomes et al., 2015), or the isopod *Porcellionides pruinosus* (e.g. Ferreira et al., 2016; Morgado et al., 2013). Nevertheless, the works on biomarkers of exposure and effects in soil organisms exposed to biochar-amended soils are very scarce. Only recently fewer studies reported the effects of biochar amendment on biomarkers of exposure *in Eisenia fetida* (Li et al., 2011), and in two other earthworm species *Aporrectodea icterica* and *Aporrectodea longa* (Marchand et al., 2017).

In the current study biochar did not induce changes in the AChE activity (indicator of inhibited neurotransmission), neither in the measured oxidative stress biomarker, GST, nor substantial changes in CAT. An increase in the LPO was observed in the presence of biochar, indicating the occurrence of cellular membrane damage. Nap-type metabolites in the *E. andrei* tissue were not increased compared to those measured in the absence of biochar, therefore indicating low probability that Nap-type metabolites might have any contribution in the observed increase in LPO. A recently published study found that biochar-associated free radicals from rice-straw biochar were responsible for neurotoxic effect in *Caenorhabditis elegans,* while excluding potential adverse effects of biochar-bound compounds on this model organism (Lieke et al.,

2018). Previously we observed that the increase in Nap-type metabolites was related to the exposure to smaller particles of biochar (<0.5 mm), probably due to higher toxicity caused as a consequence of larger surface area and bioavailability of mixture of contaminants (data not published). As for the energy parameters evaluated in this study, changes in energy reserves were statistically significant. Reduction in lipids and proteins led to lower available energy, and with significantly increased energy consumed the CEA ratio consequently decreased. The presented results are not in accordance with the work published by Li and co-authors, who evaluated oxidative stress biomarkers in *E. fetida* exposed to biochar over 14 days, where no lipid peroxidation or anti-oxidative defence were observed (Li et al., 2011). Earthworms in the study of Marchand et al. (2017) exposed to poultry manure biochar (2 %) in a metal contaminated soil showed a reduced GST and increase in lipids and proteins content upon biochar application. However, they did report the reduction of body mass in the presence of biochar (Marchand et al., 2017). This, taken together with the fact that biochar-induced loss of weight in earthworms is commonly observed (Liesch et al., 2010; Gomez-Eyles et al., 2011; Li et al., 2011), should be considered with caution. In the current work, the effects on the energy reserves may indicate that organisms are using this energy to retrieve physiological damages, and return to a homeostatic equilibrium. Here we report the reduction in CEA and occurrence of LPO after 28 days of exposure to 1.5 % wood chip biochar applied to non-contaminated soil which highlights physiological and biochemical changes, while also indicating that more investigation is necessary to infer on these mechanistic effects.

Isopods have been widely used in multibiomarker approaches but their enzymatic activities could not be measured in the present study due to the mortality rate observed in the experiment. Although the mortality rate was low, organisms were replaced every time a dead animal was observed, in order to maintain their ratio and function in soil and to not compromise the soil enzymatic activity approach nor the continuous interaction with earthworms.

4.7. Conclusions

Isopods and earthworms have a significant role in soil processes and understanding their behaviour and possible interactions in biochar amended soil is important for future safe application of biochar. The approach taken to study complex relationships in the scenario soil-biochar-biota, showed that microcosms with combined detritivore species can provide relevant

insights on potential changes in biochar amended soil, while accounting for the interactions. This study is the first one to use the integrative ecotoxicological tools in investigating biota relationships in biochar amended soil, to the best of our knowledge. Woodchip biochar can induce sub-lethal changes in earthworms and reduction in enzymatic activities, while on the other side, it might be beneficial for isopods. More research is suggested to further address these issues. The multibiomarker approach applied in the current study provides a useful insight on the mechanisms behind biochar impact on soil biota.

4.8. References

Aas, E., Beyer, J., Goksøyr, A., 2000a. Fixed wavelength fluorescence (FF) of bile as a monitoring tool for polyaromatic hydrocarbon exposure in fish: an evaluation of compound specificity, inner filter effect and signal interpretation. Biomarkers 5, 9–23.

Aas, E., Baussant, T., L. Balk, B. Liewenborg, O.K. Andersen, 2000b. PAH metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil exposure: a laboratory experiment with Atlantic cod Aquat. Toxicol., 51, pp. 241-258

Aas, E., J. Beyer, A. Goksøyr, 1988. PAH in fish bile detected by fixed wavelength fluorescence Mar. Environ. Res., 46, pp. 225-228

Amelot, N., Sleutel, S., Case D. C. S., Alberti, G., McNamara, N.P., Zavalloni, C., Vervisch, B., delle Vedove, G., De Neve, S., 2014. C mineralization and microbial activity in four biochar field experiments several years after incorporation. Soil Biology and Biochemistry. 195-203.

Baker M, Cerniglia G, Zaman A. 1990. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Anal Biochem 190:360–365.

Bandick A.B., Dick, R.P., 1999. Field management effects on soil enzyme activities. Soil Biol Bioche 31:1471–1479

Bastos, A.C., Prodana, M., Abrantes, N., Keizer, J.J., Soares, A.M.V.M., Loureiro, S., 2014. Potential risk of biochar-amended soil to aquatic systems: an evaluation based on aquatic bioassays. Ecotoxicology 23, 1784–1793.

Bielská, L., Škulcová, L., Neuwirthová, N., Cornelissen, G., Hale, S.E., 2018. Sorption, bioavailability and ecotoxic effects of hydrophobic organic compounds in biochar amended soils. Sci. Total Environ. 624, 78–86.

Biochar. European Biochar Foundation (EBC), Arbaz, Switzerland. http://www.europeanbiochar.org/en/download. Version 6.3E of 14th August 2017

Bird RP, Draper HH. 1984. Comparative studies on different methods of malonaldehyde determination. Methods Enzymol 105:299–305.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Cataldo, J.R., Hidalgo, M.E.L., Neaman, A., Gaete, H.O., 2011. Use of molecular biomarkers in Eisenia foetida to assess copper toxicity in agricultural soils affected by mining activities. J. Soil Sci. Plant Nutr. 11, 57–70.

Clairborne A. 1985. In Greenwald RA, ed, CRC Handbook of Methods in Oxygen Radical Research. CRC, Boca Raton, FL, USA, pp 283–284.

Conti, F.D., Visioli, G., Malcevschi, A., Menta, C., 2017. Safety assessment of gasification biochars using Folsomia candida (Collembola) ecotoxicological bioassays. Environ. Sci. Pollut. Res. 2015, 1–12.

De Coen W, Janssen CR. 1997. The use of biomarkers in Daphnia magna toxicity testing. IV. Cellular Energy Allocation: A new methodology to assess the energy budget of toxicant-stressed Daphnia populations. J Aquat Ecosyst Stress Recov 6:43–55.

Dick, R.P., Breakwell, D.P., Turco, R.E., 1996. Soil Enzyme Activities and Biodiversity Measurements as Integrative Microbiological Indicators. In: Methods for Assessing Soil Quality. SSSA Spec. Publ. 49. SSSA, Madison, Wisconsin, USA.

Domene, X., Mattana, S., Hanley, K., Enders, A., Lehmann, J., 2014. Medium-term effects of corn biochar addition on soil biota activities and functions in a temperate soil cropped to corn. Soil Biol. Biochem. 72, 152–162.

Ellman GL, Courtney KD, Andres V, Featherstone RM. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95.

Elzobair, K.A., Stromberger, M.E., Ippolito, J.A., Lentz, R.D., 2016. Contrasting effects of biochar versus manure on soil microbial communities and enzyme activities in an Aridisol. Chemosphere 142, 145–152.

Ercal, N., Gurer-Orhan, H., Aykin-Burns, N., 2001. Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage. Current Topics in Medicinal Chemistry 1, 529e539.

European Biochar Certificate (EBC), 2012. Guidelines for a Sustainable Production of

Ferreira, N. G. C., Santos, M. J. G., Domingues, I., Calhoa, C. F., Monteiro, M., Amorim, M. J. B., Soares, A. M. V. M. & Loureiro, S. 2010. Basal levels of enzymatic biomarkers and energy reserves in Porcellionides pruinosus. Soil Biol. Biochem*.,* 42**,** 2128-2136.

Ferreira, N.G.C., Morgado, R.G., Amaro, A., Machado, A.L., Soares, A.M.V.M., Loureiro, S., 2016. The effects of temperature, soil moisture and UV radiation on biomarkers and energy reserves of the isopod Porcellionides pruinosus. Appl. Soil Ecol. 107, 224–236.

Gomes, S., Scott-Fordsmand, J., Amorim, M., 2015. Cellular Energy Allocation to Assess the Impact of Nanomaterials on Soil Invertebrates (Enchytraeids): The Effect of Cu and Ag. Int. J. Environ. Res. Public Health 12, 6858–6878. doi:10.3390/ijerph120606858

Gomez-Eyles, J.L., Sizmur, T., Collins, C.D., Hodson, M.E., 2011. Effects of biochar and the earthworm Eisenia fetida on the bioavailability of polycyclic aromatic hydrocarbons and potentially toxic elements. Environ. Pollut. 159, 616–622. doi:10.1016/j.envpol.2010.09.037

Gravato, C., Santos, M., 2003. Genotoxicity biomarkers' association with B(a)P biotransformation in Dicentrarchus labrax L. Ecotoxicol. Environ. Saf. 55, 352e358.

Grossman, J.M., O'Neill, B.E., Tsai, S.M., Liang, B., Neves, E., Lehmann, J., Thies, J.E., 2010. Amazonian anthrosols support similar microbial communities that differdistinctly from those extant in adjacent, unmodified soils of the same mineralogy. .Microb.l Ecol. 60, 192-205.

Guilhermino L, Lopes MC, Carvalho AP, Soares AMVM. 1996.Acetylcholinesterase activity in juveniles of Daphnia magna Straus. Bull Environ Contam Toxicol 57:979–985.

Habig WHH, Pabst MJJ, Jacoby WB, Jakoby WB. 1974. Glutathione S transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249:7130–7139.

International Biochar Initiative (IBI), 2015. Standardized Product Definition and Product Testing Guidelines for Biochar 7 That Is Used in Soil. http://www.biochar-international.org/sites/default/files/IBI_Biochar_Standards_V2.1_Final.pdf

ISO 11268-2, 2012. Soil quality – Effects of pollutants on earthworms – Part 2: Determination of effects on reproduction of Eisenia fetida/Eisenia andrei. International Organization for Standardization, Geneve.

Jin, H., 2010. Characterization of microbial life colonizing biochar and biochar amended soils. PhD Dissertation, Cornell University, Ithaca, NY.

Kendeler E, Gerber H. 1988. Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol Fert Soil. 6:68–72.

Kuzyakov, Y., Friedel, J.K., Stahr, K., 2000. Review of mechanisms and quantification of priming effects. Soil Biol. Biochem. 32, 1485–1498.

Lavelle, P., Bignell, D., Lepage, M., Wolters, V., Roger, P., Ineson, P., Heal, O.W., Dhillion, S., 1997. Soil function in a changing world: the role of invertebrate ecosystem engineers. Eur. J. Soil Biol. 33, 159–193.

Lavelle, P., Decaëns, T., Aubert, M., Barot, S., Blouin, M., Bureau, F., Margerie, P., Mora, P., Rossi, J.P., 2006. Soil invertebrates and ecosystem services. Eur. J. Soil Biol. 42.

Lehmann, J., Rillig, M.C. Thies, J., Masiello, C.A., Hockaday, W.C., Crowley, D., 2011. Biochar effects on soil biota - A review. Soil Biol. Biochem., 1-25.

Li, D., Hockaday, W.C., Masiello, C.A., Alvarez, P.J.J., 2011. Earthworm avoidance of biochar can be mitigated by wetting. Soil Biol. Biochem. 43, 1732–1737.

Liang, B., Lehmann, J., Sohi, S.P., Thies, J.E., O'Neill, B., Trujillo, L., Gaunt, J.,Solomon, D., Grossman, J., Neves, E.G., Luizão, F.J., 2010. Black carbon affects thecycling of non-black carbon in soil. Org. Geochem. 41, 206-213.

Lieke, T., Zhang, X., Steinberg, C.E.W., Pan, B., 2018. Overlooked Risks of Biochars - Persistent Free Radicals trigger Neurotoxicity in *C. elegans*. Environ. Sci. Technol.

Loureiro, S., Nogueira, A.J.A., Soares, A.M.V.M., 2007. A microbial approach in soils from contaminated mine areas: The jales mine (Portugal) case study. Fresen. Environ. Bull. 16, 1648–1654.

Loureiro, S., Sampaio, A., Brandao, A., Nogueira, A. J. & Soares, A. M. 2006. Feeding behaviour of the terrestrial isopod Porcellionides pruinosus, Brandt, 1833 (Crustacea, Isopoda) in response to changes in food quality and contamination. Sci.Total Environ., 369**,** 119-128.

Loureiro, S., Soares, A. M. & Nogueira, A. J. 2005. Terrestrial avoidance behaviour tests as screening tool to assess soil contamination. Environ. Pollut. 138, 121-131.

Madžarić, S., Kos, M., Drobne, D., Hočevar, M., Jemec Kokalj, A., 2018. Integration of behavioral tests and biochemical biomarkers of terrestrial isopod Porcellio scaber (Isopoda, Crustacea) is a promising methodology for testing environmental safety of chars. Environ. Pollut. 234, 804–811.

Malev, O., Contin, M., Licen, S., Barbieri, P., De Nobili, M., 2015. Bioaccumulation of polycyclic aromatic hydrocarbons and survival of earthworms (Eisenia andrei) exposed to biochar amended soils. Environ. Sci. Pollut. Res. 23, 3491–3502.

Marchand, L., Brunel-Muguet, S., Lamy, I., Mench, M., Pelosi, C., 2017. Modulation of trace element bioavailability for two earthworm species after biochar amendment into a contaminated technosol. Ecotoxicology 26, 1378–1391. doi:10.1007/s10646-017-1862-8

Marks, E.A.N., Mattana, S., Alcañiz, J.M., Domene, X., 2014. Biochars provoke diverse soil mesofauna reproductive responses in laboratory bioassays. Eur. J. Soil Biol. 60, 104–111.

Masto, R.E., Kumar, S., Rout, T.K., Sarkar, P., George, J., Ram, L.C., 2013. Biochar from water hyacinth (Eichornia crassipes) and its impact on soil biological activity. Catena 111, 64–71.

Morgado, R., Ferreira, N.G.C., Tourinho, P., Ribeiro, F., Soares, A.M.V.M., Loureiro, S., 2013. Environmental- and growth stage-related differences in the susceptibility of terrestrial isopods to UV radiation. J. Photochem. Photobiol. B 126, 60–71.

Mukherjee A., Lal R., 2014. The biochar dilemma. Soil Research 52, 217-230.

Novais, S.C., Amorim, M.J.B., 2013. Changes in cellular energy allocation in Enchytraeus albidus when exposed to dimethoate, atrazine, and carbendazim. Environ. Toxicol. Chem. 32, 2800–2807.

Nusair, S.D., Abu Zarour, Y.S., 2017. Molecular and cellular response of earthworm Eisenia andrei (Oligochaeta, Lumbricidae) to PCDD/Fs exposure. Environ. Sci. Pollut. Res. 24, 902–910. doi:10.1007/s11356-016-7893-5

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal _ tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.

Park, J.H., Choppala, G.K., Bolan, N.S., Chung, J.W., Chuasavathi, T., 2011. Biochar reduces the bioavailability and phytotoxicity of heavy metals. Plant Soil 348, 439–451. d

Paz-Ferreiro, J. Fu, S. ,Méndez, A., Gascó, G., 2014. Interactive effects of biochar and the earthworm Pontoscolex corethrurus on plant productivity and soil enzyme activities. J Soils Sediments 14:483–494

Paz-Ferreiro, J., Gascó, G., Gutiérrez, B., Méndez, A., 2012. Soil biochemical activities and the geometric mean of enzyme activities after application of sewage sludge and sewage sludge biochar to soil. Biol. Fertil. Soils 48, 511–517.

Rodrigues, A.C.M., Gravato, C., Quintaneiro, C., Golovko, O., Zlabek, V., Barata, C., Soares, A.M.V.M., Pestana, J.L.T., 2015. Life history and biochemical effects of chlorantraniliprole on Chironomus riparius. Sci Total Environ 508:506–513.

Rossel, D., Tarradellas, J., Bitton, G. and Morel, J.-L. (1997) Use of Enzymes in Soil Ecotoxicology: A Case for Dehydrogenase and Hydrolytic Enzymes. In Soil Ecotoxicology. J. Tarradellas, G. Bitton and D. Rossel, D. (Eds.) Boca Raton. 179-206.

Santos, M. J., Morgado, R., Ferreira, A, N. G., Soares, A. M. & Loureiro, S. 2010. Evaluation of the joint effect of glyphosate and dimethoate using a small-scale terrestrial ecosystem. Ecotoxicol. Environ. Saf*,* 74**,** 1994-2001.

Shrivastava, A., Gupta, V.B., 2011. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. Chronicles of Young Scientists 2 (1): 21-25.

Sinsabaugh, R.L., Lauber, C.L., Weintraub, M.N., Ahmed, B., Allison, S.D., Crenshaw, C., Contosta, A.R., Cusack, D., Frey, S., Gallo, M.E., Gartner, T.B., Hobbie, S.E., Holland, K., Keeler, B.L., Powers, J.S., Stursova, M., Takacs-Vesbach, C., Waldrop, M.P., Wallenstein, M.D., Zak, D.R., Zeglin, L.H., 2008. Stoichiometry of soil enzyme activity at global scale. Ecol. Lett. 11, 1252–1264.

Sohi, S. Loez-Capel, E., Krull, E., Bol, R., 2009. Biocharclimate change and soil: A review to guide future research. CSIRO Land and Water Science Report 05/09.

Tabatabai, M.A., 1994.Soil enzymes. In: Weaver RW, Angle JS, Bottomley PS (eds) Methods of soil analysis, part 2. Microbiological and biochemical properties. SSSA Book Series No. 5. Soil Science Society of America, Madison, WI, pp 775–833.

Takeda, N., 1980. The aggregation pheromone of some terrestrial isopod crustaceans. Experientia 36, 1296–1297.

Tammeorg, P., Bastos, A.C., Jeffery, S., Rees, F., Kern, J., Graber, E.R., Ventura, M., Kibblewhite, M., Amaro, A., Budai, A., Cordovil, C.M. d. S., Domene, X., Gardi, C., Gascó, G., Horák, J., Kammann, C., Kondrlova, E., Laird, D., Loureiro, S., Martins, M.A.S., Panzacchi, P., Prasad, M., Prodana, M., Puga, A.P., Ruysschaert, G., Sas-Paszt, L., Silva, F.C., Teixeira, W.G., Tonon, G., Delle Vedove, G., Zavalloni, C., Glaser, B., Verheijen, F.G.A., 2017. Biochars in soils: towards the required level of scientific understanding. J. Environ. Eng. Landsc. Manag. 25, 192–207.

ter Braak, C.J.F., Smilauer, P., 2002. CANOCO reference manual and CanoDraw for Windows user's guide: software for canonical community ordination (version 4.5). Microcomputer power, Itaca.

Tietze F. 1969. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues. Anal Biochem 27:502–522.

van Gestel, C.A.M., 2012. Soil ecotoxicology: State of the art and future directions. Zookeys 176, 275–296.

Verheijen, F.G.A., Jeffery, S., Bastos, A.C., van der Velde, M., and Diafas, I. (2009). Biochar Application to Soils - A Critical Scientific Review of Effects on Soil Properties, Processes and Functions. EUR 24099 EN, Office for the Official Publications of the European Communities, Luxembourg, 149pp.

Wu, S., Zhang, H., Zhao, S., Wang, J., Li, H., Chen, J., 2012. Biomarker responses of earthworms (Eisenia fetida) exposured to phenanthrene and pyrene both singly and combined in microcosms. Chemosphere 87, 285–293.

Yu, Z., Chen, L., Pan, S., Li, Y., Kuzyakov, Y., Xu, J., Brookes, P.C., Luo, Y., 2018. Feedstock determines biochar-induced soil priming effects by stimulating the activity of specific microorganisms. Eur. J. Soil Sci. 69, 521–534.

Zimmer, M., Kautz, G. and Topp, W., 2005. Do woodlice and earthworms interact synergistically in leaf litter decomposition? Funct. Ecol. 19, 7-16.

4.9. Supplementary Information

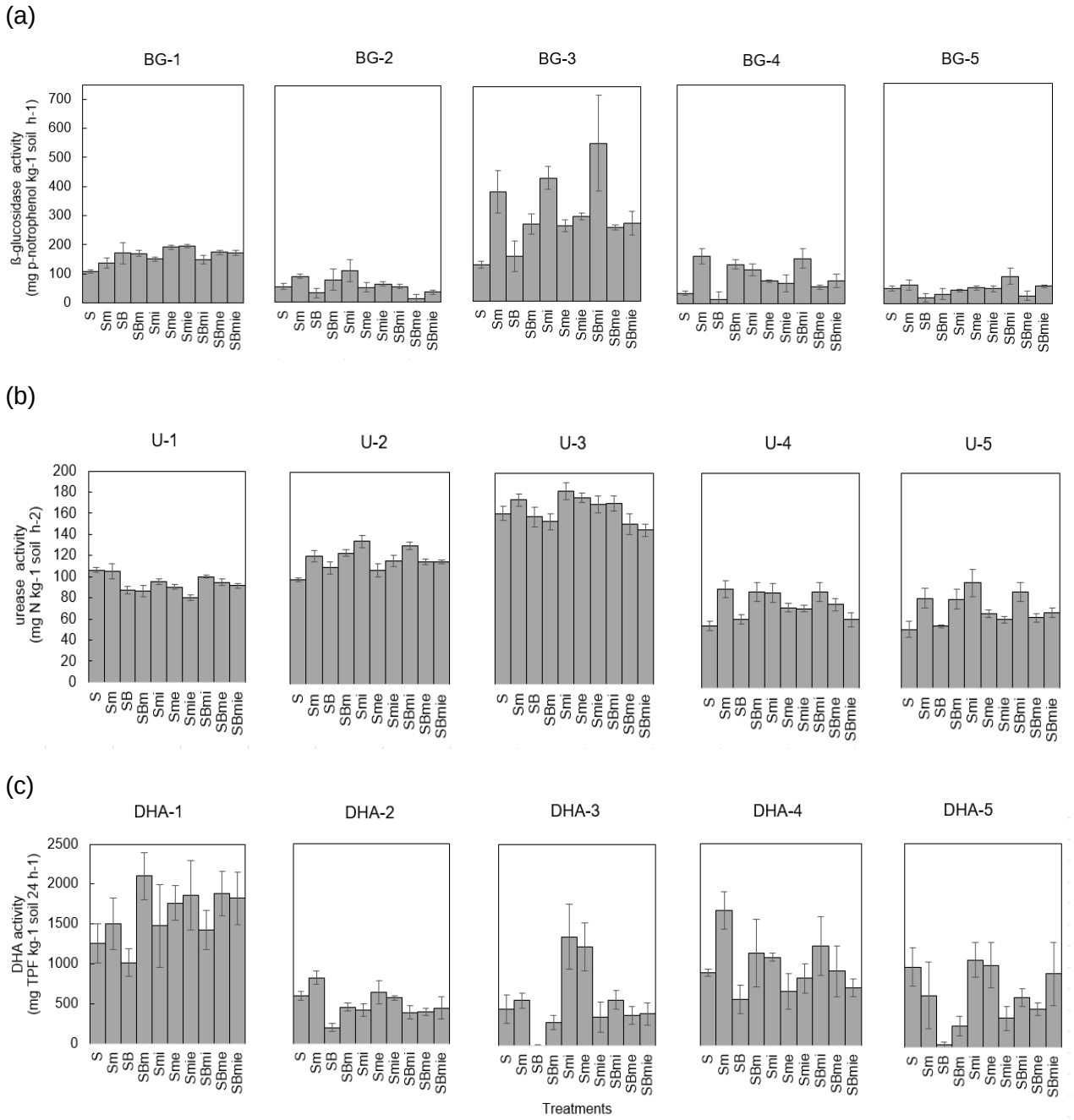

Figure S4.1. Enzymatic activities of (a) ß-glucosidase (BG) expressed as mg p-nitrophenol per kg of soil per hour (b) urease (U) expressed as mg of nitrogen per kg of dry soil per two hours and (c) dehydrogenase (DHA) expressed in mg of triphenyl formazan (TPF) per kg of soil per 24 hours. Numbers from 1-5 stand for the sampling time series: 1 – first sampling (week one of the experiment), 2 – second sampling (week two of the experiment), 3 – third sampling (week three of the experiment), 4 – fourth sampling (week four of the experiment, and 5 – fifth sampling (final sampling in the week eight of the experiment).

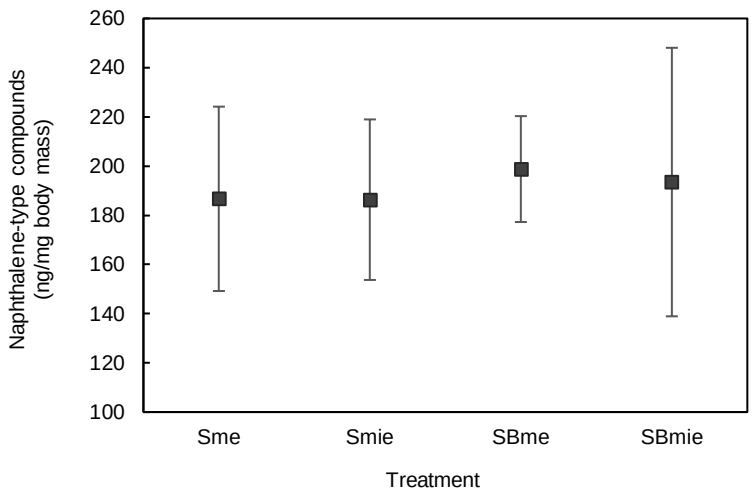

Figure S4.2. Naphthalene-type metabolites detected in tissue of *Eisenia andrei* after four-week-exposure to soil (Sm) and biochar amended soil (SBm, at 1.5 % w/w), in the absence (e) and presence (ie) of *Porcellionides pruinosus*. Values are presented as means with standard deviations.

Table S4.1. Mean pH values (±standard deviations) of the treatments at the end of experiment. *Treatments:* S (soil), Sm (soil-manure), SB (soil-biochar), SBm (soil-biochar-manure), Smi (soil-manure-isopods), Sme (soil-manure-earthworms), Smie (soil-manure-isopods-earthworms), SBmi (soil-manure-isopods), SBme (soil-manure-earthworms), SBmie (soil-manure-isopods-earthworms).

Treatment	S	Sm	SB	SBm	Smi	Sme	Smie	SBmi	SBme	SBmie
pH (H_2O) 1:5	7.9±0.3	8.1±0.2	8.2±0.2	8.2±0.1	8.2±0.2	8.2±0.3	8.2±0.1	8.2±0.2	8.3±0.3	8.3±0.2

Chapter 5

Combined effects of biochar, organic amendments and fertilizer on biota in small-scale terrestrial ecosystem models

Combined effects of biochar, organic amendments and fertilizer on biota in small-scale terrestrial ecosystem models

5.1. Abstract

The present study evaluated the impact of biochar, biochar-compost, NPK-based mineral fertilizer and their combinations on biota, in a natural agricultural soil at application rates that are relevant for agronomic applications. The ecotoxicological assessment was carried out in two phases: (i) assessment of the effects of amended soil on earthworms (*Eisenia andrei*), rapid-cycling plants (*Brassica rapa*) and bait-lamina consumption in small-scale terrestrial ecosystem models (STEMs) using an agricultural soil, and (ii) assessment of potential toxicity of the leachates collected from STEMs on the aquatic macrophyte *Lemna minor*. Additionally, treated soils, soil pore water and leachates were also characterized for selected nutrients and/or dissolved organic carbon, to complement the bioassays. Treatments had low to no-effects on *E. andrei*. There was no observed significant change in water content of *B. rapa*, indicating that the plants were not under hydric stress. In general, plant biomass was slightly stimulated in all the treatments, with the most pronounced effects in those where biochar-compost was applied with mineral fertilizer. Yet, the increase in morphological traits measured was not statistically significant. Amongst the production characteristics of *B. rapa* obtained, the number of seeds and mean number of seeds per pod increased significantly in the treatments of biochar-compost combined with mineral fertilizer and biochar-compost, respectively. Bait-lamina consumption evaluated during the experiment was reduced over time, being the lowest in the treatment of soil with biochar. Leachates assessment indicated a slight stimulation at lower leachates concentrations. The leachates of the soil without any amendment and the amended soil induced an inhibition in *L. minor* growth, when exposed to the pure (non-diluted) leachates. The lowest EC_{20} and EC_{10} were obtained in the leachate of soil amended with biochar-compost. In response to stress, significantly higher dry to fresh weight ratios in *L. minor* were observed, indicating that a possibility of nutrients leaching stimulation might not be excluded, which could pose a hazard to aquatic systems. The sensitivity of the responses observed with different functional groups indicate that STEMs methodology is an adequate higher tier approach for ecotoxicological assessment of biochar and/or biochar-compost and mineral fertilizer applications.

Key words: biochar, biochar-compost, earthworms, rapid-cycling plants, bait-lamina, small scale terrestrial ecosystem models, aquatic macrophyte

5.2. Introduction

In contrast to the relatively high proportion of studies addressing agronomic strengths and weaknesses from biochar application to soil, information is scarce on potential environmental and ecological consequences of biochar utilization in soil alongside other traditional organic amendments and fertilizers. These may include interactions in the system soil-biochar-biota as well as possible negative impact of their combined effect on non-target soil and aquatic organisms.

Investigations of the agronomic benefits of biochar application to soils have been focused on its use as conditioner for improving soil properties and processes (e.g. correction of pH of acidic soils, improve soil aggregation and hydrologic characteristics (Masulili et al., 2010; Molnar et al., 2016; Schulz and Glaser, 2012), or on its use to improve crop yield, alone or in combination with other organic amendments or fertilizers (Ippolito et al., 2015; Jeffery et al., 2017). Another knowledge gap is related to the likelihood of biochar particles and biochar-bound contaminants to reach groundwaters as a consequence of leaching, or surface water bodies through runoff (Jaffe et al., 2013; Bastos et al., 2014a; Buecker et al., 2016). The same problem has been often highlighted regarding fertilizers, which can reach aquatic systems and/or underground water by runoff or leaching.

The ecotoxicological effects of slow pyrolysis wood biochar on soil organisms have been already studied, e.g. on the earthworm *Eisenia fetida* (Li et al., 2011), collembolan *Folsomia candida* (Bielska et al., 2018; Marks et al., 2014), and on the enchytraeid *Enchytraeus crypticus* (Marks et al., 2014). Molnar and co-authors applied a battery of complementary bioassays when studying ecotoxicity of wood biochar when applied to acidic soil, by carrying out single species tests with *Aliivibrio fischeri, Folsomia candida, Sinapis alba* and *Triticum aestivum* (Molnar et al., 2016). Alongside the tests and methodologies already used for biochar assessment, fewer studies addressed the issue by using more ecologically relevant approaches, like accounting for multi-species presence and interactions (Amaro et al., 2016), or from laboratory earthworms' avoidance to 4.5 months field-experiment with wheat in which earthworms' biomass and density was evaluated (Tammeorg et al., 2014).

Bearing in mind the uncertainties in the context of safe and sustainable biochar application, including the lack of long-term, chronic, and ecologically representative studies, an integrated ecotoxicological evaluation of biochar-amended soil in small-scale terrestrial ecosystem models (STEMs), coupled with aqueous leachates testing was planned as a way of bridging the gap between laboratory experiments and natural field conditions. Such setup provides a possibility to look at multiple test species and endpoints simultaneously, as well as to assess both the terrestrial and the aquatic component through leachate collection. STEMs were previously developed by Santos et al. (2011a, 2011b) for the assessment of the effects of pesticide mixtures on soil biota. Recently, the use of STEMs was adapted by Amaro et al. (2016) for assessing biochar effects and potential toxicity to soil organisms, simulating the biochar topsoil incorporation (0-15 cm) practice (Amaro et al., 2016). The epigeic earthworm *Eisenia andrei* was selected as a representative species of soil biota, mediating key soil processes and functions, such as structure maintenance, organic matter redistribution and nutrient cycling (Brown et al., 2000; Edwards, 2004). More specifically, it is known that earthworms are involved in nitrogen mineralization from soil organic matter (Cortez et al., 2000), in which way they can, to different extent, contribute to mediating plant uptake of nitrogen and regulating soil carbon dioxide and nitrous oxide emissions (Lubbers et al., 2011; van Groenigen et al., 2014). Therefore, combining earthworms and plants in mesocosms testing also accounts for possible interactions between organisms (Amaro et al., 2016). The plant species chosen for the experiment is the rapid-cycling turnip (*Brassica rapa)* (Williams, 1989). It is often used as a model organism in cell and molecular biology, plant biochemistry (Williams and Hill, 1986), and more recently in ecotoxicological studies (Lima et al., 2011; Santos et al., 2011). Considering the biochar from wood as a feedstock, it is frequently studied biochar in both agronomic and environmental contexts. The combination of processing conditions (400-600°C) and woody feedstock typically results in low levels of PAHs accumulated in the biochars (e.g. Hale et al. 2012; Kloss et al., 2012; Yargicoglu et al., 2015).

In the present work, an integrated approach was employed to address the ecotoxicological implications of woodchip-waste biochar in STEMs, applied to soil individually, as a mixture with vegetal compost, and combined with mineral (NPK) fertilizer. Specifically, the study aimed at investigating:

i) the effect of the treatments on the performance of edaphic organisms, namely *E. andrei* survival and body weight, bait-lamina consumption, and morphological and production traits of rapid-cycling *B. rapa*, when applied at common or recommended concentrations, and

ii) the ecotoxicological potential of leachates from (i) to the aquatic macrophyte *L. minor.*

Analytical characterization of amended soil, soil pore water, plant tissue, and leachates were also performed thus, providing complementary information.

5.3. Materials and methods

5.3.1. Characterization of soil, biochar, biochar-compost and mineral fertilizer

The physicochemical characteristics of the soil, biochar and biochar-compost are presented in **Table 5.1**. Soil in this experiment is a natural agricultural topsoil (10-15 cm) with sandy loam texture, sampled in August 2015 from an agricultural field located in the Mondego valley (Central Portugal), with no recent history of contamination or inputs of pesticides and inorganic fertilizers (Lemos et al., 2010; Santos et al., 2011). Soil sampled from the field was sieved in the laboratory (< 2 mm) prior to the use in the experiments.

Table 5.1. Summary of the main physicochemical characteristics of the soil, biochar and biochar-compost (4% w/w) used in the study. Abbreviations: WHC_{max} stands for maximum water holding capacity, EC for electrical conductivity, and n.d. for 'not determined'.

	Soil	Biochar	Biochar-compost
texture class	sandy loam	n.d.	n.d.
sand (%)	69.2	n.d.	n.d.
silt (%)	18.8	n.d.	n.d.
clay (%)	12	n.d.	n.d.
WHC_{max} (%)	49	73.2	n.d.
Bulk density (g/cm^3)	n.d.	0.55	n.d.
EC (µS/cm)	n.d.	3 000	1 240
Ash (550°C) (%)	n.d.	18.6	5.4
Organic C (%)	n.d.	75	22.5
Organic matter (%)	2.9.	n.d.	38.7
pH (H_2O)	7.6	10.1	7.2
pH (KCl)	7.4	n.d.	n.d.
Salts (g/kg)	n.d.	8.4	11.1
$CaCO_3$ (g/kg)	89	n.d.	n.d.
H (%)	n.d.	47	n.d.
H:C (molar ratio)	n.d.	0.07	18.4
O:C (molar ratio)	n.d.	0.04	n.d.
N total (g/kg)	1.98	n.d.	n.d.

N (%)	n.d.	1.8	4.8
P_2O_5 (mg/kg)	805	n.d.	n.d.
K_2O (mg/kg)	250	n.d.	n.d.
Al (mg/kg)[1]	17 000	n.d.	n.d.
Sb (mg/kg)	<5	n.d.	n.d.
As (mg/kg)	18	n.d.	n.d.
Ba (mg/kg)	110	n.d.	n.d.
Be (mg/kg)	1.8	n.d.	n.d.
Pb (mg/kg)	210	<2	14.9
B (mg/kg)	13	39	n.d.
Cd (mg/kg)	<0.5	<0.2	0.21
Ca (mg/kg)	25 000	42 200	59 150
Cr (mg/kg)	17	27	21
Hg (mg/kg)	n.d.	n.d.	0.25
Fe (mg/kg)	23 000	2 420	19 000
K (mg/kg)	3 200	10 400	8 400
Cu (mg/kg)	82	16	28.9
Li (mg/kg)	70	n.d.	n.d.
Mg (mg/kg)	5 000	2 980	5 400
Mn (mg/kg)	1 100	n.d.	n.d.
Mo (mg/kg)	<5	n.d.	n.d.
Na (mg/kg)	120	744	930
Ni (mg/kg)	17	17	20.6
P (mg/kg)	1 500	1 300	2 400
S (mg/kg)	n.d.	372	190
Se (mg/kg)	<10	n.d.	n.d.
Sr (mg/kg)	90	n.d.	n.d.
Tl (mg/kg)	<2	n.d.	n.d.
Ti (mg/kg)	600	n.d.	n.d.
V (mg/kg)	23	n.d.	n.d.
Zn (mg/kg)	200	70	101.2
Sn (mg/kg)	15	n.d.	n.d.
ΣPAHs (mg/kg)[2]	n.d.	0.48	n.d.
Σ7 ind. PCBs (mg/kg)[3]	n.d.	<0.002	n.d.

[1]Metals were determined by microwave digestion (DIN/ISO 17294-2).

[2]PAHs were determined by SPME (solid-phase microextraction) coupled to gas chromatography/mass spectrometry GC/MS (DIN EN 15527), where individual PAH values were below or equal to the limit of detection (0.1 mg/kg).

[3]The 7 indicator PCBs were determined by HRGC/HRMS (high resolution gass chromatography and mass sectrometry)

Biochar and biochar-compost were both acquired from Swiss Biochar gmbh (Switzerland). The biomass feedstock was woodchip residues, subjected to the process of slow pyrolysis (highest treatment temperature 620°C). The biochar had the following particle size distribution (w/w): 4% (<0.1 mm), 25% (0.1-0.5 mm), 34% (0.5-2 mm), 37% (>2 mm), with an average particle size of 29.5 µm and pH (H_2O) of 10.1. The biochar-compost was prepared by mixing 4% w/w of the biochar with vegetal compost, at the end of the composting process.

Mineral fertilizer under the commercial name Osmocote was used, consisting of nitrogen (N), phosphorous (P), and potassium (K), in the proportions of 14-13-13.

5.3.2. Study organisms

The earthworms *Eisenia andrei* (Bouché 1972) were obtained from laboratory cultures maintained at 20±1ºC and a photoperiod of 16:8 hours (light:dark). Earthworms were kept in 24 L plastic containers, with a mixture of soil potting mix and peat, at pH 6 to 7, and at 70% of its water holding capacity (WHC). The animals were fed once per week with horse manure previously frozen and gradually thawed as needed. The individuals used in the experiments were three months old, with developed clitella and an average body weight between 300 and 600 mg.

Seeds of rapid cycling *Brassica rapa* were obtained from the commercial supplier Carolina Biological Supply Company (Williams, 1989).

The freshwater macrophyte *L. minor* was maintained in sterile 250 ml Erlenmeyers filled with Steinberg medium (OECD 2006a). The vessels were closed with sterile cotton pads to minimize eventual evaporation and contamination during 8 weeks before the bioassays. The culture medium of *L. minor* was renewed twice per week. The culture was maintained in an incubator chamber, with controlled temperature (20±1°C), photoperiod of 16-:8 hours (light:dark) and light intensity of approximately 6500 lux.

5.3.3. Experimental design

Screening bioassay: *Eisenia andrei* survival and body weight

Firstly, a screening bioassay based on earthworm survival and changes in body weight was performed according to the guideline OECD 207 (OECD, 1984), to infer on the experimental design and biochar concentration in the follow-up STEMs experiment. Umamended soil was used as negative control, and biochar treatments of 1%, 2%, 3%, 5%, 8%, 16%, 26% and 36% (w/w) at 60% WHC were prepared at 3 replicates per treatment (including the unamended soil). Each replicate contained 10 earthworms. The test duration was two weeks (20±1ºC; photoperiod of 16:8 hours, light:dark). The endpoints observed were survival and biomass. The animals were weighted before and after the bioassay, and the pooled weight

was expressed per replicate. Changes in body weight were expressed as loss of weight, by subtracting the animals weight at the end of experiment (final weight) from the weight at the beginning (initial weight), and dividing it by the initial weight (Lima et al., 2011).

STEMs experiment

The experiment was conducted using indoor mesocosms, or small-scale terrestrial ecosystem models (STEMs) in a climate-controlled laboratory chamber, based on the methodology described by Santos et al. (2011a, 2011b) with adaptations by Amaro et al. (2016) for biochar testing. Briefly, each STEM consisted of a PVC cylinder of 20 cm height and a diameter of 11 cm. The cylinders were sealed with a 1 mm thick plastic mesh at the bottom to hold soil. The STEMs were inserted in acclimatized moveable carts (83 cm length; 55 cm width; 55 cm depth), each cart with the capacity for five STEMs. Carts had an automatic control of soil temperature set to 15°C.

The reference, i.e. un-amended soil (S) was used as negative control. The experimental treatments were: soil amended with NPK mineral fertilizer (Sf), soil amended with biochar at 2% (w/w), equivalent to 40 t/ha (SB), soil amended with biochar at 2% (w/w) and NPK mineral fertilizer (SBf), soil amended with biochar-compost at 2% (w/w) (SCB), and soil amended with biochar-compost at 2% (w/w) and NPK mineral fertilizer (SCBf). The experiment was performed with four replicates per treatment, including the un-amended soil. Each mesocosm contained around 1.7 kg of soil/amended soil in total. As the NPK fertilizer was in granular form it was previously ground with an electric mill and dissolved in distilled water in order to be homogenously applied to soil/soil amended with biochar and biochar-compost. Water holding capacity (WHC) was adjusted to 60-65 % of the maximum soil/ amended soil WHC before filling in each column with also homogeneously mixed biochar and biochar-compost, NPK fertilizer and/or their mixtures.

The information on the effect concentration from the abovementioned *E. andrei* screening bioassay was used to select the adequate concentration (i.e. biochar application rate) for the higher tier approach. The selected 2 % w/w is equivalent to maximum of 40 t/ha (in the case of 15 cm layer of biochar/biochar-compost topsoil application and soil bulk density of 1.3 g/cm^3). NPK fertilizer was added at a rate of 50 g/m^2**,** according to the suppliers' recommendation (Osmocote, NPK 14-13-13), which corresponded to 0.43 g per mesocosm,

per replicate. This amount of the mineral fertilizer is equivalent to 0.5 t/ha. The experiment lasted for six weeks. This period is expected to allow the full life cycle of *B. rapa*, which germinates within two days, develops flowers after 13 to 18 days and finishes its life cycle in 36 days under the constant light supply of 24 h (Williams, 1989). The experiment was conducted at 20±5°C and a photoperiod of 16:8 hours (light:dark), which led to slightly slower development.

The first 96 h of the experiment were used for the soil-biochar pre-incubation (namely for pH equilibration). Earthworms (10 adult individuals of *E. andrei* per column, previously weighed), seeds (10 seeds of *B. rapa*) and bait-laminas (three per column) were introduced on the fifth day. Later, while growing, the plants were thinned to seven to eight per replicate. Plants became less fragile and with an adequate size at the third week, where the yield of photosynthesis measurements started, and were repeated in the fourth and fifth week of the experiment. Measurements of the chlorophyll fluorescence were carried out on *B. rapa* leaves with PAM (pulse amplitude modulation system). The equipment consists of computer-operated PAM-Control Unit (Walz) and a WATER-EDF-Universal emitter–detector unit (Gademann Instruments GmbH, Germany). The measurement was applied on the adaxial side of five mature leaves in every mesocosm. Minimal fluorescence (F0) was measured by applying a weak modulated light to leaves which were pre-adapted to darkness for 30 min. F0 is emitted when the reactions centres are open (plants adapted to darkness). Maximal fluorescence (Fm) was measured by applying a 0.7 s saturating pulse, which causes the reaction centres to close. Fv represents a variable fluorescence, a difference between F0 and Fm. The ratio Fv/Fm corresponds to maximum quantum yield and it is a measure of the health state of the plant's photosynthetic apparatus (Krause and Weis, 1991; Govindjee, 2004). Cross pollination was performed in order to obtain the production of pods and seeds (representative of yield endpoints). The procedure was carried out on the 20^{th} and 21^{st} day of the experiment, when the flowers on all the plants were opened. For mimicking natural cross pollination in *B. rapa*, pollination sticks with a handle on one side and a small brush on the opposite side were used, thus resembling the shape and structure of an insect pollinator and to allow the successful attachment and transfer of pollen. Morphological endpoints obtained for *B. rapa* were fresh and dry weight, root and shoot length as root/shoot ratio, and hydric content that represents a difference in fresh and dry weight divided by fresh weight and

expressed as percentage (according to Lima et al., 2011). The production yield traits observed were number of pods and seeds, also expressed as number of seeds per pod.

Additionally, bait-lamina consumption was also assessed over time, applying the baits three times during the experiments, i.e. every 12 days. Bait-lamina test, was primarily created for *in situ* and field measurements, which at the time of the experiment, it was available as a draft ISO/TC 190/SC 4 N (ISO, 2012). Bait-laminas were filled with a mixture of L-cellulose, oat bran and activated charcoal in the proportion of 70:27:3 (Kratz, 1998; Santos et al., 2011) and inserted vertically in the soil mesocosm. In each reading, three bait-lamina sets were used per mesocosm. Each set of bait-laminas was assessed after 12 days, by counting the number of empty apertures on each bait-lamina. Since the consumption rate was relatively high, 12 days allowed for the assessment of eventual differences over time.

The endpoints observed for the earthworms were survival and body mass expressed as loss of weight. Animals were weighed at the beginning of the experiment. At the end of 42 days experiment they were counted and weighed again to account for the loss of body mass. Earthworms biomass per microcosm was recorded by pooling 10 earthworms and expressing the mean weight per earthworm. Loss of body weight was calculated by subtracting the final weight from the initial weight of animal and dividing it by initial weight (Lima et al., 2011).

Soil pore water extraction

Right after the bait-lamina test, plants and earthworms were collected from all soil mesocosm and the measurements recorded, the soil/amended soil samples were collected for soil pore water extraction. The followed procedure was adapted from Tourinho et al. (2013). Sampling was performed by placing 50 g of soil in a Falcon tube. Three tubes per replicate of the amended treatments and un-amended soil were used. The follow-up steps were saturation of the samples with ultrapure water and 48 hours incubation/equilibration at 4ºC in the dark. After that, centrifugation at relative centrifugal force of 2860 g was performed for 90 minutes. The supernatant was collected, approximately 6 ml from each Falcon tube. Samples for dissolved organic carbon (DOC) analysis were taken with a syringe containing a filter of 0.45 µm pore size, to separate the particulate organic carbon fraction (> 0.45 µm) from the dissolved organic carbon fraction (< 0.45 µm). The supernatant was passed through a filter paper of 11 µm pore size (Whatman 1) for nutrient analysis. Samples (< 24 h aged) were

prepared according to the procedure described below and used for DOC quantification (see section 5.5.). The fraction of the samples used for nutrient analysis was stored at 4ºC for one week, except those analysed for potassium, which were acidified with nitric acid and stored at room temperature for no longer than six months prior to the analysis, according to the HACH Sampling and Storage procedure within Method 8049.

Leaching procedure and toxicity assessment of the leachates

The procedure applied was adapted from the OECD guideline for leaching in soil columns (OECD 312, 2004), and used on the disturbed soil cores after 42 days, when bait-laminas, plants and earthworms were removed from them. The volume of the mesocosm (11cm diameter and 20 cm high) was 1900.66 cm^3. According to the OECD 312:2004 protocol, and proportional to the amount of rainwater recommended, we recalculated the amount of water to be applied, in order to simulate the highest average rainfall in the district of Aveiro, Portugal, where the study was conducted, for a more realistic scenario. This estimated volume of water of 600 ml per column/mesocosm is equivalent to 140-150 mm of rainfall (estimation used as characteristic to the period between November and January in Aveiro, Portugal), according to Climate-Data.Org (www.en.climate-data.org). Leaching was performed with ultrapure water for consistency. The water was gradualy applied at the surface of the mesocosm over 48 hours at 21°C. The step of adding 0.1 M $CaCl_2$ to water was skipped since the leachates were intended for DOC and nutrient analysis, as well as for the aquatic bioassay. Centrifugation of the leachate was performed at 3000 rpm for 20 minutes and stored at 4°C prior to use for ecotoxicological and chemical analysis. The storage time was as described for soil pore water samples.

The aquatic component testing of the leachates from STEMs was carried out with fresh samples, not older than one week. The *Lemna minor* growth inhibition assay was performed according to the guideline OECD 207 (2006). The cultures of *L. minor* were incubated for 7 days prior to test in a climatized chamber, under constant light (6500 lux) and temperature of 24±1°C. Due to the large number of leachates samples, concentrations and number of replicates, the bioassay was performed in 6-well plates. Leachate were diluted with the Steinberg medium to achieve dilutions of 12.5%, 25%, 50%, 75% and 100% (pure leachate), in order to allow for calculation of the toxicity endpoints, such as effect concentration (EC),

lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC). The Steinberg medium used for culture maintenance was used as negative control. Three replicates were used for all leachate concentrations, and six replicates for the control (Steinberg medium). The initial total number of fronds per well was 11. Test duration was seven days in the climatized chamber used for incubation (6500 lux, 24±1°C). On the second, fourth and sixth day of the bioassay, 1.5 ml of medium were replenished to the controls and leachates (of corresponding dilutions) to the treatment wells, to compensate for media loss due to evaporation. This volume was calculated during the trial prior to bioassay.

5.3.4. Chemical analysis

At the end of the STEMs experiment, replicates of un-amended and amended soil were pooled into a composite sample for chemical analysis. Soil samples were analysed in an external laboratory for soil organic matter content (SOM), total nitrogen (N), inorganic nitrogen in a nitric N (NO_3^-) and ammoniacal N form (NH_4^+), total calcium carbonate ($CaCO_3$), plant available phosphorous (P_2O_5, analysed with Egner-Riehm method) and potassium (K_2O, Egner-Riehm method).

Nutrients in the dry plant material, soil pore water and leachates were analysed spectrophotometrically due to limited amounts of samples, with a portable HACH spectrophotometer (model DR/2000). The analyses were conducted following the DR2000 Spectrophotometer Procedure Manual (HACH Co. USA DR/2000). Plant tissue extraction was performed by homogenising 0.5 g of dry tissue with 100 ml of deionized water with a pestle and mortar. The homogenate was then filtered through 0.11 µm pore size (Whatman 1) and used for nitrate and phosphate analyses. Nitrate was measured using the modified cadmium reduction method with gentisic acid, with the reading range up to 1.5% of NO_3^- N (Method 8151, HACH Co. USA DR/2000). Phosphate was analysed using the Ascorbic acid method, with the reading range up to 0.4% of PO_4^{3-} P in plant tissue (Method 8179, HACH Co. USA DR/2000).

Available nutrients in soil pore water and leachates were evaluated following the methodology for water, wastewater and seawater (HACH Co. USA DR/2000). Nitrate was measured using the Cadmium reduction method (Method 8039), with the maximum reading range up to 30 mg/L NO_3^- N. Due to the highly concentrated samples, the dilution step of 1:9

(sample:deionized water) was applied to all samples and taken into consideration for the final calculation. Phosphate was measured with the Ascorbic acid method for reactive phosphorus, with the maximum reading range up to 2.5 mg/L PO_4^{3-} (Method 8048). Potassium was measured with the Tetraphenylborate method, with the maximum reading range up to 7 mg/l K.

Dissolved organic carbon fraction in soil pore water and leachates was analysed after filtration (Whatman, 0.45 µm filter pore size) and acidification of the samples to pH 2, using acetic acid. DOC quantification in the samples was performed according to NPOC method (non-purgable) with the TOC/TN analyser Analytic Jena AG.

5.4. Statistical analysis

Data were first analysed for normality and homoscedasticity (with Shapiro-Wilk and Leven's tests, respectively). One-way ANOVA, followed by Dunnett's test was applied to test the differences between un-amended soil and treatments. When the assumption of normality failed and the transformation of data could not correct for normality, a Kruskal-Wallis test or ANOVA on ranks was performed (Zar, 1996), followed by the Dunn's test in case of significant differences. Ecotoxicity parameters, LOECs and NOECs in *E. andrei* survival and *L. minor* growth inhibition assays were thereafter derived. Two-way repeated measures ANOVA was performed to test for the effects of factors 'treatment', 'time' and/or their interaction, for both endpoints yield of photosynthesis and bait-lamina consumption in STEMs. Yield and/or bait-lamina consumption was used as dependent variable, where factor time was considered a random factor, while factor treatment was a fixed factor. Statistical analysis was done with Sigma Plot software. Effective concentrations in *L. minor* growth inhibition assay, EC_{20} and EC_{10}, together with 95 % confidence intervals (CI), were calculated with nonlinear regression using the logistic equations in STATISTICA 10 software.

5.5. Results

5.5.1. Screening bioassay: *Eisenia andrei* survival and body weight

In the preliminary bioassay earthworms' survival was not affected when exposed to the tested biochar concentrations (**Figure S5.1.** in Supplementary material). Statistical significance was

observed when comparing the weight loss of individuals between the amended and un-amended soil (one-way ANOVA; Dunnett's test, $p<0.05$). This allowed estimating *E. andrei* body weight no observed effect concentrations (NOEC) and lowest observed effect concentration (LOEC) as 5% (equivalent to 100 t/ha) and 8% biochar in soil (equivalent to 160 t/ha), respectively.

5.5.2. Chemical analysis

Selected chemical characteristics of the soil samples, soil pore water and leachates, as well as nutrients in plant tissue at the end of the STEMs experiment are presented in **Table 5.2.**

Table 5.2. Chemical characterization of soil samples, soil pore water and leachates and nutrient content in dry *Brassica rapa* tissue after a six week exposure to soil treatments in small scalled terrestrial ecosystems. S-unamended soil, Sf-soil with NPK fertilizer (f), SB-soil with biochar, SBf-soil with biochar and NPK fertilizer(f), SBC -soil amended with biochar-compost, and SBCf-soil with biochar-compost and NPK fertilizer (f).

Soil samples	**S**	**Sf**	**SB**	**SBf**	**SBC**	**SBCf**
pH (H_2O)	7.1	7.2	7.5	7.4	7.5	7.5
SOM (g/kg)[1]	26.4	31.9	30.1	27.9	29.3	28.4
N total (g/kg)	1.72	1.96	1.80	1.87	1.79	1.90
N NH_4 (mg/kg)	2.50	3.13	2.52	2.19	2.06	2.51
N NO_3^- (mg/kg)	60	115	58	83	76	102
P_2O_5 (mg/kg)[2]	527	576	595	498	593	667
K_2O (mg/kg)[2]	182	192	245	235	232	390
Soil pore water						
pH (H_2O)	7.8	7.7	8.1	7.3	7.5	7.4
DOC (mg/L)[3]	26.1	16.2	51.2	16.7	23.6	50.7
N NO_3^- (mg/L)	98.1	103.5	38.7	67.5	76.5	126
PO_4^{3-} (mg/L)	0.84	1.29	1.04	1.65	0.94	1.45
K (mg/mL)	15.7	38.4	24.2	32.0	29.5	41.5
Leachates						
pH (H_2O)	7.3	7.1	7.4	7.1	7.2	7.2
DOC (mg/L)[3]	17.9	27.5	27.2	19.9	31.9	28.5
N NO_3^- (mg/L)	147.1	152.1	98.1	128.7	128.7	145.8
PO_4^{3-} (mg/L)	0.28	0.31	0.15	0.20	0.08	0.18
K (mg/mL)	18.6	13.2	23.4	34.5	33.9	45.9
Plant tissue						
N NO_3^- (mg/kg DM)[4]	4.70	3.80	4.65	3.55	3.70	3.60
P PO_4^{3-} (mg/kg DM)	1.35	1.05	0.90	1.05	1.00	1.20

[1]SOM stands for soil organic matter

[2]plant available phosphorus and pottasium analyzed with the Egner-Riehm method

[3]DOC stands for dissolved organic carbon

[4]DM stands for dry matter

The pH of soil and treated soil was 7.1 and 7.5 respectively, suggesting only a slight increase in the presence of the organic amendments. SOM was varying from 26.4 (the lowest measured in S) to 31.9 g/kg (the highest measured in Sf). Total N concentration was the highest in Sf and SBCf (1.96 g N/kg and 1.90 N g/kg respectively). Un-amended soil was characterized with the lowest total N of 1.72 g/kg. Plant available phosphorus and potassium were present at the highest concentrations in the SBCf treatment, 667 mg P/kg and 390 mg K/kg respectively, while in the case of nitrate this treatment contained 102 mg/kg as the second highest measured concentration after Sf (115 mg/kg). Also, the highest concentration of ammoniacal N (3.13 mg/kg) was measured in Sf (**Table 5.2.**).

As for the soil pore water pH, values were in the range of 7.3 to 8.1, with SB having the highest pH. Somewhat higher DOC levels were measured in SB and SBCf, 51.2 mg/mL and 50.7 mg/l, respectively. These concentrations are approximately double of those in S and SBC, and approximately three-fold larger than those in Sf and SBf (**Table 5.2.**). Nitrate concentrations were in the range of 38.7 to 126 mg/l. Nitrate was present at the highest concentrations in soil pore water of SBCf, Sf and S. Phosphate concentrations in soil pore water, on the other hand, revealed a different pattern, with the treatments with mineral fertilizer (SBf, SBCf and Sf) containing higher levels than those in SB, SBC and S (from 0.84 to 1.65 mg/L). Potassium concentrations in the soil pore water generally expressed a similar pattern to phosphate, with the concentration ranging from 15.7 to 41.5 mg/l (**Table 5.2.**).

The soil and amended soil leachates had comparable pH values, ranging from 7.1 to 7.4. DOC fluctuations were less contrasting than in the case of soil pore water, ranging from 17.9 mg/l in S, up to 31.9 mg/L in SBC. Nitrate levels measured in the leachates were also comparable between treatments, ranging from 98.1 to 152.1 mg/L. However, like for soil pore water samples, there were higher nitrate concentrations in Sf, S, and SBCf, compared to SBC, SBf and SB. Phosphate levels were between 0.08 and 0.31 mg/L, and potassium between 13.2 and 45.9 mg/L. Phosphate concentration was higher in the leachates without the organic amendments (S and Sf). In contrast, SCBf, SBf and SBC had the highest potassium concentrations, compared to the remaining treatments (**Table 5.2.**).

The contents of nutrients, namely nitrates and phosphates in *B. rapa* tissue were in the range of 3.55 to 4.70 mg/kg of dry weight for nitrates, and 1.00 to 1.35 mg/kg dry weight for phosphate (**Table 5.2.**).

5.5.3. STEMs experiment

Eisenia andrei survival and weight change

The endpoints obtained for the earthworms at the end of the STEMs experiment regarding survival and body weight are presented in **Figure 5.1**. Although body weight revealed a decrease in the treatments, no significant differences were observed in relation to the control (Kruskal-Wallis test, $p>0.05$). Mortality of 15% was recorded in the SB treatment, also with the absence of statistical significance (Kruskal-Wallis test, $p>0.05$).

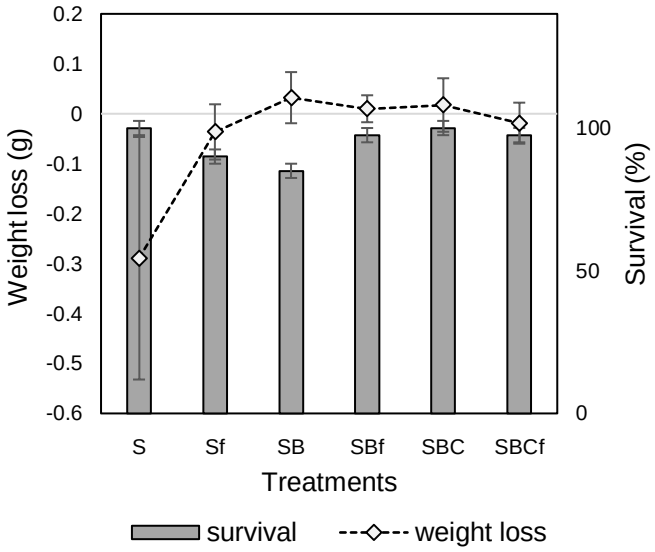

Figure 5.1. *Eisenia andrei* survival (expressed as %) and body weight change (expressed as average pooled loss of weight in g) when exposed un-amended soil and treatments in STEMs. S-unamended soil, Sf-soil with NPK fertilizer (f), SB-soil with biochar, SBf-soil with biochar and NPK fertilizer (f), SBC-soil amended with biochar-compost, and SBCf-soil with biochar-compost and NPK fertilizer (f).

Bait-lamina consumption

The bait-laminas evaluated every 12 days during the STEMs experiment (three bait-lamina sets in total, from the 5th day until 42nd day) resulted in statistically significant response for the factor 'time' (two-way RM ANOVA, $p<0.05$), but not for the factor 'treatment' or their interaction (two-way RM ANOVA, $p>0.05$), as presented in **Table S5.1.** However, the third time (bait-laminas set) was also tested with one-way ANOVA and statistically significant difference in consumption was detected between S and SB (Tukey test, $p<0.05$; **Figure 5.2.**).

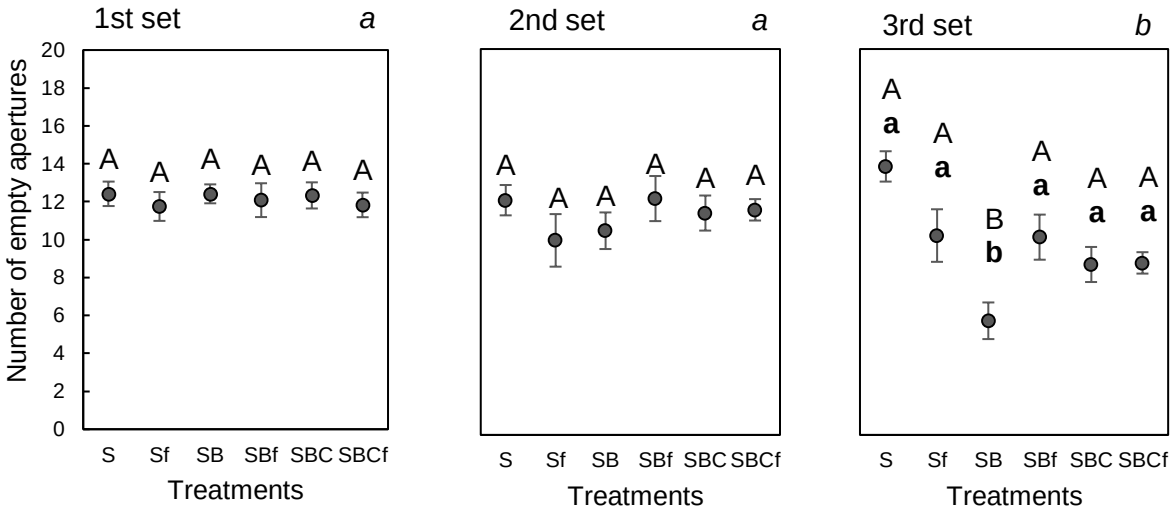

Figure 5.2. Bait-lamina cosumption in STEMs measured over time (expressed as number of empty apertures), on three successive sets of bait-laminas in STEMs. Error bars represent standard errors of the means. Different lower case italic letters (*a*, *b*) indicate significant differences for factor 'time' (i.e. between the three sets of bait-laminas); uppercase letters indicate significant differences for factor 'time' within treatments (two-way ANOVA, $p<0.05$; Tukey test, $p<0.05$); different lower case bold letters (**a, b**) indicate significant differences only within the third set of bait-laminas. (one-way ANOVA; Tukey test, $p<0.05$). S-unamended soil, Sf-soil with NPK fertilizer (f), SB-soil with biochar, SBf-soil with biochar and NPK fertilizer (f), SBC-soil amended with biochar-compost, and SBCf-soil with biochar-compost and NPK fertilizer (f).

Brassica rapa chlorophyll fluorescence, morphological and production traits

Yield of photosynthesis observed in *B. rapa* leaves in the third, fourth and fifth weeks was significantly reduced both in the fourth and fifth week, compared to the initial measurement at the third week (two-way ANOVA, $p<0.05$), as shown in **Table S5.2**. Photosynthetic yield changes in the treatments over time are shown on **Figure 5.3.** There was a reduction in photosynthetic yield in the treatments Sf, SBC and SBCf in week 4, relative to those observed in the initial measurement (Tukey posthoc test, $p<0.05$; **Figure 5.3.**).

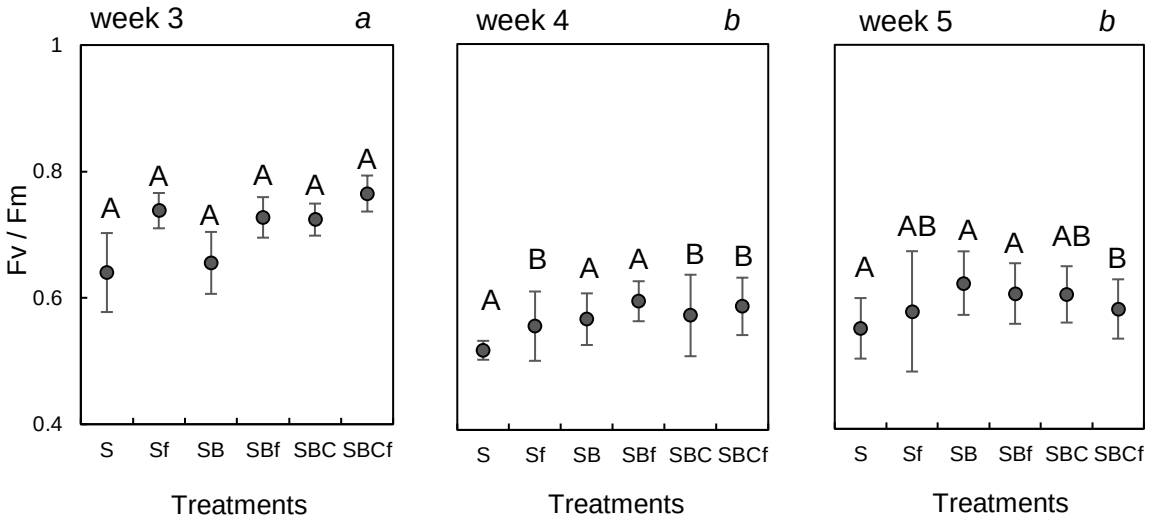

Figure 5.3. *Brassica rapa* maximal quantum yield of PSII (Fv/Fm), as measured in the third, fourth and fifth weeks in STEMs. Error bars represent standard errors of the mean. Lowercase italic letters indicate significant differences in the yield of photosynthesis for the factor 'time' (over a three-week period), and uppercase letters indicate significant differences for factor 'time' within each treatment (Tukey test, $p<0.05$). S-unamended soil, Sf-soil with NPK fertilizer (f), SB-soil with biochar, SBf-soil with biochar and NPK fertilizer (f), SBC-soil amended with biochar-compost, and SBCf-soil with biochar-compost and NPK fertilizer (f).

The mean seedlings emergence in the STEMs was between 87.5% and 100%, with statistically significant differences (Kruskal-Wallis test, $p<0.05$; **Figure 5.4.**). However, a pairwise multiple comparison did not reveal specific differences (Dunn's test, $p>0.05$). The morphological traits, namely the ratio root to shoot length (one-way ANOVA, $p>0.05$), fresh weight (one-way ANOVA, $p>0.05$; Figure 4) and dry weight (Kruskal-Wallis test, $p>0.05$; **Figure 5.4.**) also did not differ between treatments in a statistically significant manner. Hydric content was also similar between the treatments and the control (Kruskal-Wallis test, $p>0.05$; **Figure 5.4.**).

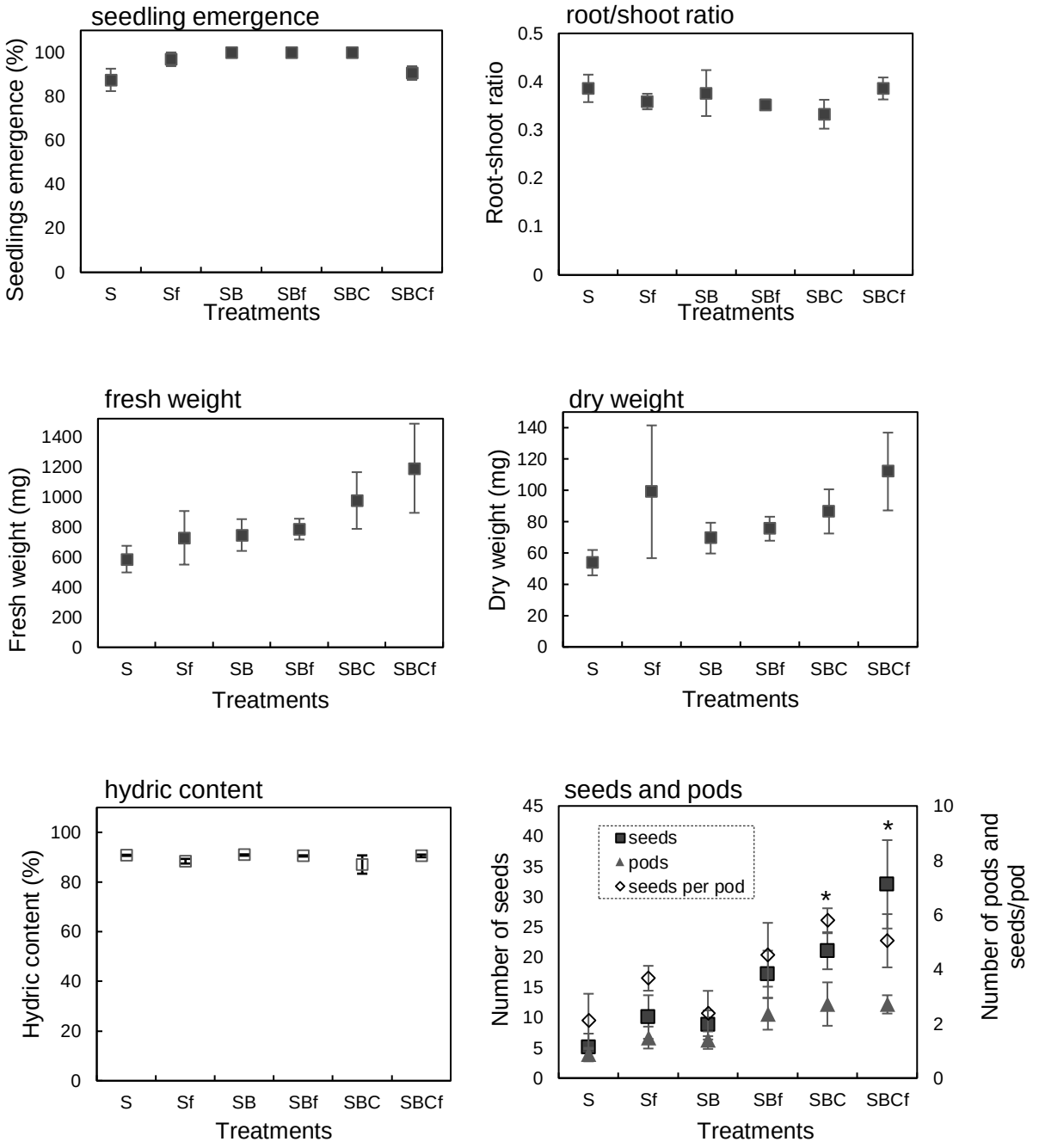

Figure 5.4. Traits of *Brassica rapa* exposed for 6 weeks to soils with different tratments in Small Scale Terrestrial Ecosystems: emergence, morphological traits (fresh and dry weight expressed in mg, root/shoot ratio), hydric content (expressed as %), number of pods, seeds, and seeds per pod. Error bars present standard error of the means. Asterisks (*) refer to significant difference when compared to the control (un-amended soil, S) (Dunnett's test, $p<0.05$). S-unamended soil, Sf-soil with NPK fertilizer (f), SB-soil with biochar, SBf-soil with

biochar and NPK fertilizer (f), SBC-soil amended with biochar-compost, and SBCf-soil with biochar-compost and NPK fertilizer (f).

As for the reproductive yield traits of *B. rapa,* the number of pods observed also did not differ statistically between un-treated and treated soil (one-way ANOVA, $p>0.05$; **Figure 5.4.**). Statistically significant difference in the number of seeds was observed for SBCf when comparing the treatments with that in un-amended soil (one-way ANOVA, $p<0.05$; Dunnetts test, $p <0.05$; Figure 4). Expressing number of seeds per pod, a statistical significance was obtained for the SBC treatment (one-way ANOVA, $p<0.05$; Dunnett's test, $p<0.05$; **Figure 5.4.**).

5.5.4. *Lemna minor* growth inhibition bioassay

In the leachate toxicity assessment, the un-amended soil as well as amended treatments induced growth inhibition in *L. minor*, revealing a dose response pattern (**Figure 5.5.**). Table 3 presents the estimated EC_{20} and EC_{10} values, alongside the LOEC and NOEC, where possible for the several dilutions of the leachates. As presented in Figure 5, a slight, but statistically significant stimulation of growth was observed at the lowest leachate concentration of 12.5% in S, Sf, SBf, and at 25% of the leachate concentration in the case of SBCf (one-way ANOVA; Dunnett's test, $p<0.05$). The lowest EC_{20} and EC_{10} were obtained for SBC (EC_{20}=62.7 %, CI 48.0-77.3; EC_{10}=38.9, CI 21.9-55.9), while the least toxic treatments revealed similar EC values, Sf (EC_{20}=80.6 %, CI 69.8-91.4; EC_{10}=58.6, CI 42.8-74.4) and SB (EC_{20}=78.7 %, CI 68.0 – 89.3; EC_{10}=59.4, CI 44.0-74.8) (**Table 5.3.**). The dry weight to fresh weight ratio (DW/FW) were calculated for *L. minor* (**Figure S5.2.** in Supplementary material). Statistically significant differences were observed due to the increase in DW/FW ratios in the pure leachates (100%) of Sf, SB, SBf, SBC when compared to those in the bioassay controls consisting of Steinberg growth medium (ctrl-0) (one-way ANOVA, $p<0.05$; Dunnett's test, $p<0.05$).

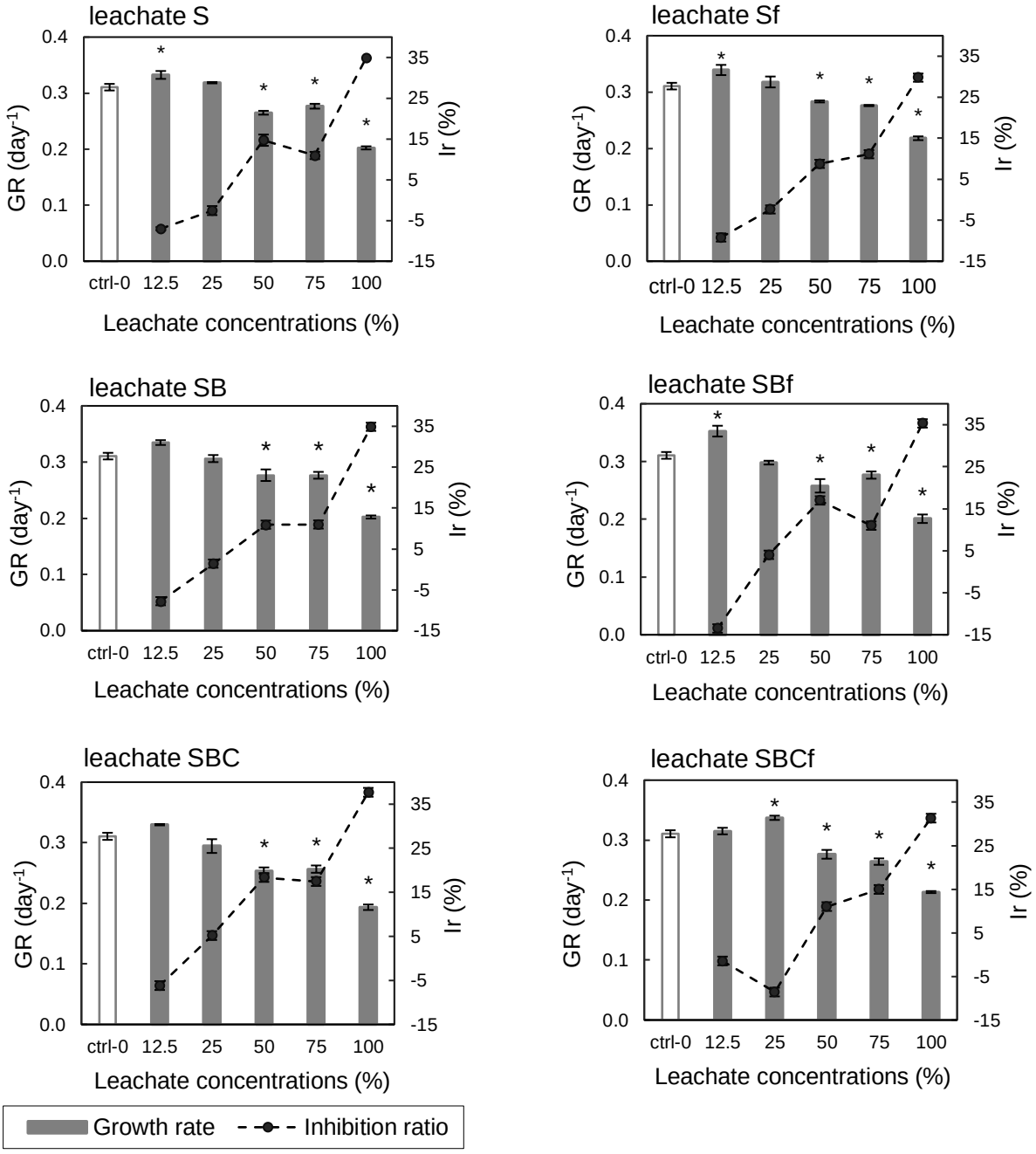

Figure 5.5. *Lemna minor* growth rate (GR; day^{-1}) and inhibition of growth (Ir; %) as a result of a 7day exposure to leachates collected from un-amended soil (S) and amended soil treatments (Sf, SB, SBf, SBC, SBCf) from the STEMs experiment. Leachates were diluted to 12.5%, 25%, 50% and 75% with Steinberg growing medium, which was also used as a test control (ctrl-0). 100% represents non-diluted leachate. Error bars represent standard errors of the mean. Asterisk (*) refers to significant difference when compared to the control that consists of Steinberg medium, ctrl-0 (Dunnett's test, $p<0.05$). S-unamended soil, Sf-soil with

NPK fertilizer (f), SB-soil with biochar, SBf-soil with biochar and NPK fertilizer (f), SBC-soil amended with biochar-compost, and SBCf-soil with biochar-compost and NPK fertilizer (f).

Table 5.3. Effects of leachates on *L. minor* growth rate. EC_{20}, EC_{10}, LOEC, NOEC parameters calculated for un-amended soil leachate (S), and for the treatments leachates (Sf, SB, SBf, SBC, SBCf). Values in brackets refer to 95 % confidence intervals. n.d. stands for not determined. S-unamended soil, Sf-soil with NPK fertilizer (f), SB-soil with biochar, SBf-soil with biochar and NPK fertilizer(f), SBC -soil amended with biochar-compost, and SBCf-soil with biochar-compost and NPK fertilizer (f).

Growth rate	S	Sf	SB	SBf	SBC	SBCf
EC_{20}	68.5	80.6	78.7	68.5	62.7	77.1
	(48.1- 88.9)	(69.8 - 91.4)	(68.0 - 89.3)	(48.1 - 89.0)	(48.0 – 77.3)	(66.4 - 88.9)
EC_{10}	44.1	58.6	59.4	44.1	38.9	55.5
	(18.7 - 69.5)	(42.8 - 74.4)	(44.0 - 74.8)	(18.7- 69.5)	(21.9 – 55.9)	(40.3 – 70.7)
NOEC	n.d.	n.d.	25	n.d.	25	n.d.
LOEC	n.d.	n.d.	50	n.d.	50	n.d.

5.6. Discussion

5.6.1. STEMs: Responses of *Eisenia andrei*, bait-lamina consumption and *Brassica rapa*

The levels of pH in the amended soil were higher than in the un-amended soil, as expected due to the alkaline pH of the biochar. This is in accordance with the reported pH in the biochar amended soils (Major et al., 2010; Buecker et al., 2016; Jeffery et al., 2017). Nevertheless, already being alkaline, the pH of the soil increased only up to 0.4 units in the amended treatments, which can be attributed to the high buffering capacity of the soil used in our study (Gonzaga et al., 2018).

Regarding nutrients, the highest concentrations of the analysed compounds in the solid samples, such as total and ammoniacal nitrogen, were present in the treatment Sf, followed by SBCf. Also, SBCf contained high initial input of N and P due to the high levels of this compounds in biochar-compost. Even though SBf sample contained the same amount of NPK alongside with biochar-introduced nutrients, the measured concentration of nitrate was lower in Sf.

Woodchip biochar was chosen in the present study mainly due to its chemical properties, such as the low concentrations of potentially toxic elements, which are within or lower than

the benchmark concentrations proposed by the two international voluntary quality standards: 'European Biochar Certificate' (EBC, 2012), and International Biochar Initiative 'Standardized product definition and product testing guidelines for biochar that is used in soil (IBI, 2015). The fact that there was no mortality at the end of the screening bioassay with *E. andrei,* and that the NOEC and LOEC were obtained for body weight as a sublethal endpoint, indicated the adequacy of the chosen woodchip biochar for the higher tier biochar assessment approach in STEMs. Moreover, the obtained NOEC of 5% biochar, allowed for choosing a lower test concentration, 2% w/w (equivalent to 40 t/ha application rate) in the follow-up experiment.

Thereafter, using the STEM procedures, at the concentration of 2% w/w in STEMs, none of the differences in earthworm survival and weight loss in STEMs were statistically significant. Absence of significant differences in body weight of *E. andrei* has also been reported in soil containing wood-waste biochar in STEMs (Amaro et al., 2016). Significant drop in the bait-lamina consumption occurred in the last, third set of bait-laminas. The lowest observed statistically significant bait-lamina consumption in the SB treatment might be linked with the incidence of earthworms' mortality in this treatment, as the earthworm community decreased. One of the possibilities is also that bait-lamina consumption could be, to some extent, affected by reduction in nitrogen availability in biochar amended soil. That might be a consequence of reduced nitrogen mineralization and microbial biomass carbon, as reported for coarse-textured agricultural soil (Dempster et al., 2012). Interaction processes between plants, earthworms and microbial community over 42 days might have altered the bait-lamina consumption, yet there is a demand for further research to investigate the possible links. Up to date it has been reported that higher wood biochar application rates than the one used in the current study caused more pronounced effects on soil organisms. Wood biochar applied at 10 % w/w caused a reproduction drop in *Folsomia candida* by 38 %, higher than the effect of rice husk that caused 27 % reduction (Bielska et al., 2018). The effects of slow pyrolysis pinewood biochar to edaphic organisms reported by Marks et al. (2014), ranged from stimulation of *Folsomia candida* reproduction, to no effect on the *Enchytraeus crypticus*, at biochar concentration in soil up to 50% w/w (Marks et al., 2014).

Brassica rapa responded differently to the amended soil, demonstrating variation in sensitivity depending on the endpoints. An over-time decrease in the maximum quantum yield (Fv/Fm) coincides with *B. rapa* life cycle stage as in this period plants started developing flower buds,

and afterwards, the last photosynthesis yield reading overlapped with the phase of pods and seeds development. This means that this possible drop in quantum yield was a consequence of allocation of energy from the leaves (Pavlovic et al., 2014; Poorter and Nigel, 2000). It is thus, less probable that this over time decrease can be attributed to some other stress condition arising from the test substrates. Therefore, this may be rather due to the plants' life development stage and the fact that they were subjected to a photoperiod regime instead to the constant light supply (Poorter and Nigel, 2000), as recommended by Williams (1989) and by the seeds supplier for the optimum performance. The nutrient contents measured in dry plant tissue did not reveal any pattern that could be explained as an alteration in the availability or uptake of nutrients. Hydric content was not changed, indicating absence of stress conditions for the plants. Regarding the morphological traits in *B. rapa*, they were characterised with high variability, consequently resulting in the absence of detected statistical significance. However, the increase in biomass was notable. Furthermore, the significant difference in the reproductive traits of *B. rapa*, namely the highest mean seeds number in SBCf, coincide with the overall higher concentrations of nitrates, phosphates and potassium in the solid sample, but also in the corresponding soil pore water extract that is representing plants available nutrient concentrations. The trend of higher soil pore water nutrients levels can be observed in the SBCf primarily due to the measured concentrations of nitrate and potassium, but also of the phosphate.

5.6.2. Leachates from STEMs: Responses of *Lemna minor*

Leachates from the STEMs experiment, as expected according to the initial soil, biochar biochar-compost physicochemical properties, were not highly toxic to *L. minor*, thus resulting in the absence of estimated EC_{50}s. Therefore, for a mechanistic understanding of the toxicity of biochar-based amendments and fertilizer applications, a thorough characterization of leachates, and of the DOC fraction itself would be important for future work. The most pronounced growth inhibition in *L. minor* occurred in the exposure to SBC leachate, while the EC_{20}s and EC_{10}s obtained for the other treatments were not substantially different, particularly in S and SBf. Additionally, the significant increase in dry weight to wet weight ratios is an indication of the stress occurrence. This ratio is shown to be a relevant endpoint, as it can be elevated due to bioaccumulation of contaminants, causing changes in hydric content and, consequently, the inhibition of growth (Radic et al., 2009). Sensitivity of *L. minor* when

exposed to leachates showed that the amendments applied at 2 % w/w or 40 t/ha might cause changes in the water macrophyte growth dynamics. To the best of our knowledge, the present study is the first to report the effects of biochar-based amendment and fertilizer applications on this aquatic macrophyte. Whether it is a stimulation or inhibition of growth, these contrasting outcomes might both trigger a misbalance in an aquatic ecosystem. Possible projection would be that in the case of higher availability of nutrients, an invasive species might start competing for them, as it was shown for *L. minor* and the invasive species *L. minuta*, under a certain combination of environmental factors and nutrients (Paolacci et al., 2016).

In general terms, it is becoming increasingly clear that biochar application to soil for improving soil agronomic properties will soon be in the form of biochar-compost or mineral fertilizer mixtures (Schulz et al., 2012; Glaser and Birk, 2013; Hagemann et al., 2017). Moreover, biochar and biochar-based amendments are also in attention in the context of carbon sequestration. On the other side, a recently reported study has been estimated that dissolved charcoal (i.e. dissolved black carbon, DBC, from forest fires) contributes to the riverine dissolved organic matter (DOC) flux, with around 10% on a global scale. Jaffe and authors argued that there is a link in the processes of DOC and DBC release involving sorption/desorption, hydrophobic interactions, suggesting that biochar-amended sites might become another significant source of DBC (Jaffe et al., 2013). The current work proposes an environmentally relevant approach in studying potential ecotoxicological effects of biochar and biochar-based amendments, or their mixtures, in order to bridge the gap between laboratory and filed studies. Increased environmental relevance in the demonstrated indoor mesocosms experiment was achieved through extended duration of the experiment to six weeks, and through combination of the plant and earthworm species known for their interactions in soil, which together allowed obtaining the endpoints from the individual (earthworms weight) to functional (bait-lamina feeding) and population level (full plant cycle – reproduction traits). Moreover, the experimental design allowed for testing both soil and aquatic component.

5.7. Conclusions

Low to no effects on earthworms and plants indicate that habitat function of soil was not affected with 40 t/ha of biochar and biochar-compost, alone and mixed with mineral fertilizer at recommended doses. Bait-lamina consumption was sensitive in differentiating the unamended from the biochar-amended soil over time, therefore being a useful tool in complementary ecotoxicological evaluation of woodchip biochar. The sensitivity of *L. minor* growth to the tested leachates emphasizes this bioassay as a promising tool in direct assessment of retention function and leaching potential of soils that are receiving additional input of biochar-based amendments and/or their combinations with conventional fertilizers. More advancements are, however, necessary for thorough understanding of these processes. In practical terms, a detailed characterization of the leachates would provide more information about the mechanisms behind the impact of such complex mixtures to the aquatic ecosystem. The evaluation of biochar and biochar-based amendments on case-by-case bases is essential for comprehensive understanding and matching of their properties with those of soil and with the application context.

5.8. References

Amaro, A., Bastos, A.C., Santos, M.J.G., Verheijen, F.G.A., Soares, A.M.V.M., Loureiro, S., 2016. Ecotoxicological assessment of a biochar-based organic N-fertilizer in small-scale terrestrial ecosystem models (STEMs) 108, 361–370.

Bastos, A.C., Prodana, M., Abrantes, N., Keizer, J.J., Soares, A.M.V.M., Loureiro, S., 2014a. Potential risk of biochar-amended soil to aquatic systems: an evaluation based on aquatic bioassays. Ecotoxicology 23, 1784–1793.

Bastos, A.C., Prodana, M., Oliveira, J.M.M., Calhôa, C.F., Santos, M.J.G., Soares, A.M.V.M., Loureiro, S., 2014b. Water-extractable priority contaminants in LUFA 2.2 soil: back to basics, contextualisation and implications for use as natural standard soil. Ecotoxicology 23, 1814–1822.

Bielská, L., Lucia, Š., Neuwirthová, N., Cornelissen, G., Hale, S.E., 2018. Science of the Total Environment Sorption, bioavailability and ecotoxic effects of hydrophobic organic compounds in biochar amended soils 624, 78–86.

Brown, G.G., Edwards, C.A., Brussaard, L., 2004. How earthworms affect plant growth burrowing into mechanisms. In: Edwards, C.A. (Ed.), Earthworm Ecology. CRC Press, Boca Raton, USA, pp. 13–49.

Buecker, J., Kloss, S., Wimmer, B., Rempt, F., Zehetner, F., Soja, G., 2016. Leachate Composition of Temperate Agricultural Soils in Response to Biochar Application. Water. Air. Soil Pollut. 227.

Dempster, D.N., Gleeson, D.B., Solaiman, Z.M., Jones, D.L., Murphy, D. V., 2012. Decreased soil microbial biomass and nitrogen mineralisation with Eucalyptus biochar addition to a coarse textured soil. Plant Soil 354, 311–324.

Eriksen-Hamel, N.S., Whalen, J.K., 2007. Impacts of earthworms on soil nutrients and plant growth in soybean and maize agroecosystems. Agr. Ecosyst. Environ. 120, 442–448.

European Biochar Certificate (EBC), 2012. Guidelines for a Sustainable Production of Biochar. European Biochar Foundation (EBC), Arbaz, Switzerland. http://www.europeanbiochar.org/en/download. Version 6.3E of 14th August 2017. DOI: 10.13140/RG.2.1.4658.7043

Fischer, D. and Glaser, B. (2012). Synergisms between compost and biochar for sustainable soil amelioration. In K. Sunil and A. Bharti (eds), Management of Organic Waste, 167–198. Rijeka: InTech.

Godlewska, P., Schmidt, H.P., Oleszczuk, P., 2017. Biochar for composting improvement and contaminants reduction. A review. Bioresour. Technol. 246, 193–202.

Gonzaga, M.I.S., Mackowiak, C., de Almeida, A.Q., de Carvalho Junior, J.I.T., Andrade, K.R., 2018. Positive and negative effects of biochar from coconut husks, orange bagasse and pine wood chips on maize (Zea mays L.) growth and nutrition. CATENA 162, 414–420.

Govindjee JF (2004) Chlorophyll a Fluorescence: A Bit of Basics and History. In: Papageorgiou GC & Govindjee JF (eds). Chlorophyll a Fluorescence: A Probe of Photosynthesis. pp. 2-42 Kluwer Academic. Dordrecht, Netherlands.

Van Groenigen, J.W., Lubbers, I.M., Vos, H.M.J., Brown, G.G., De Deyn, G., van Groenigen, K.J., 2014. Earthworms increase plant production: a meta-analysis. Nat. Sci. Reports 1–7.

Hagemann, N., Joseph, S., Schmidt, H., Kammann, C.I., Harter, J., Borch, T., Young, R.B., Varga, K., Taherymoosavi, S., Elliott, K.W., McKenna, A., Albu, M., Mayrhofer, C., Obst, M., Conte, P., Dieguez-Alonso, A., Orsetti, S., Subdiaga, E., Behrens, S., Kapple, A., 2017b. Organic coating on biochar explains its nutrient retention and stimulation of soil fertility. Nat. Commun.

Hagemann, N., Kammann, C.I., Schmidt, H., Kappler, A., Behrens, S., 2017a. Nitrate capture and slow release in biochar amended compost and soil. PlosONE. 1–16.

International Biochar Initiative (IBI), 2015. Standardized Product Definition and Product Testing Guidelines for Biochar 7 That Is Used in Soil. http://www.biochar-international.org/sites/default/files/IBI_Biochar_Standards_V2.1_Final.pdf

Ippolito, J.A., Spokas, K.A., Novak, J.M., Lentz, R.D., Cantrell, K.B., 2015. Biochar elemental composition and factors influencing nutrient retention. In: Lehmann, J., Joseph, S. (Eds.). Biochar for Environmental Management, Science, Technology and Implementation. Routledge, London and New York.

ISO 18311, 2016. Soil quality – Method for testing effects of soil contaminants on the feeding activity of soil dwelling organisms – Bait-lamina test. International Organization for Standardization, Geneva, Switzerland.

Jaffe R, Ding Y, Niggemann J, Vahatalo AV, Stubbins A, Spencer RGM, Campbell J, Dittmar T. 2013 Global charcoal mobilization from soils via dissolution and riverine transport to the oceans. Science 340, 345–347.

Jeffery, S., Abalos, D., Prodana, M., Bastos, A.C., van Groenigen, J.W., Hungate, B.A., Verheijen, F., 2017. Biochar boosts tropical but not temperate crop yields. Environ. Res. Lett. 12, 53001.

Kammann, C. I., Glaser, B., Schmidt, H.P., 2016. In: Biochar in European Soils and Agriculture: Science and Practice. Eds Shackley, S., Ruysschaert, G., Zwart, K. & Glaser, B. Routledge, 2016.

Kratz, W., 1998. The bait-lamina test. General aspects, applications and perspectives. Environ. Sci. Pollut. Res. 5 (2), 94–96.

Krause G.H., Weis, E.,1991. Chlorophyll fluorescence and photosynthesis: the basics. Annual Review Plant Physiology. 42: 313-349

Lemos, M.F.L., Van Gestel, C.A.M., Soares, A.M.V.M., 2010. Developmental toxicity of endocrine disrupters bisphenol A and vinclozolin in a terrestrial isopod. Arch. Environ. Contam. Toxicol. 59, 274–281.

Li, D., Hockaday, W.C., Masiello, C.A., Alvarez, P.J.J., 2011. Soil Biology & Biochemistry Earthworm avoidance of biochar can be mitigated by wetting. Soil Biol. Biochem. 43, 1732–1737.

Lima, J.R. de S., de Moraes Silva, W., de Medeiros, E.V., Duda, G.P., Corrêa, M.M., Martins Filho, A.P., Clermont-Dauphin, C., Antonino, A.C.D., Hammecker, C., 2018. Effect of biochar on physicochemical properties of a sandy soil and maize growth in a greenhouse experiment. Geoderma 319, 14–23.

Lima, M.P.R., Soares, A.M.V.M., Loureiro, S., 2011. Combined effects of soil moisture and carbaryl to earthworms and plants: Simulation of flood and drought scenarios. Environmen. Pollut., 159(7), 1844–1851.

Liu, Q., Zhang, Y., Liu, B., Amonette, J.E., Lin, Z., Liu, G., Ambus, P., Xie, Z., 2018. How does biochar influence soil N cycle? A meta-analysis. Plant Soil 1–15. doi:10.1007/s11104-018-3619-4

Lubbers, I.M., Brussaard, L., Otten, W., van Groenigen, J.W., 2011. Earthworm-induced N mineralization in fertilized grassland increases both N 2 O emission and crop-N uptake. Eur. J. Soil Sci. 62, 152–161.

Major, J., Steiner, C., Downie, A., & Lehmann, J., 2009. Biochareffects on nutrient leaching. In J. Lehmann & S. Joseph (Eds.) Biochar for environmental management—science and technology. pp. 271–288. London: Earthscan.

Malev, O., Contin, M., Licen, S., Barbieri, P., De Nobili, M., 2016. Bioaccumulation of polycyclic aromatic hydrocarbons and survival of earthworms (Eisenia andrei) exposed to biochar amended soils. Environ. Sci. Pollut. Res. 23, 3491–3502.

Marks E. A. N., Mattana, S., Alcañiz, M. J., Domene X., 2014. Biochars provoke diverse soil mesofauna reproductive responses in laboratory bioassays. European Journal of Soil Biology 60, 104-111.

Masulili, A., Utomo, W.H., 2010. Rice Husk Biochar for Rice Based Cropping System in Acid Soil 1. The Characteristics of Rice Husk Biochar and Its Influence on the Properties of Acid Sulfate Soils and Rice Growth in West Kalimantan, Indonesia. J. Agric. Sci. 2, P39.

Molnár, M., Vaszita, E., Farkas, É., Ujaczki, É., Fekete-Kertész, I., Tolner, M., Klebercz, O., Kirchkeszner, C., Gruiz, K., Uzinger, N., Feigl, V., 2016. Acidic sandy soil improvement with biochar — A mesocosm study. Sci. Total Environ. 563, 855–865.

OECD 207, 1984. Earthworm Acute Toxicity Tests. OECD – Organization for Economic Cooperation and Development, Paris.

OECD, 2006. Organization for Economic Cooperation and Development. Lemna sp. Growth inhibition test. OECD guidelines for testing of chemicals 221. Paris, France.

Paolacci, S., Harrison, S., Jansen, M.A.K., 2016. A comparative study of the nutrient responses of the invasive duckweed Lemna minuta, and the native, co-generic species Lemna minor. Aquat. Bot. 134, 47–53.

Pavlovic, D., Nikolic, B., Djurovic, S., Waisi, H., Andjelkovic, A., Marisavljevic, D., 2014. Chlorophyll as a measure of plant health: Agroecological aspects. Pestic. i fitomedicina 29, 21–34.

Poorte, H.A., Nagel, O., 2000. The role of biomass allocation in the growth response of plants to different levels of light, CO2, nutrients and water: A quantitative review. Funct. Plant Biol. 595–607.

Qayyum, M.F., Liaquat, F., Rehman, R.A., Gul, M., Zafar, M., Rizwan, M., Zia, M., 2017. Effects of co-composting of farm manure and biochar on plant growth and carbon mineralization in an alkaline soil 26060–26068.

Radic, S., Stipanicev, D., Cvjetko, P., Lovrencic Mikelic, I., Marijanovic Rajcic, M., Sirac, S., Pevalek-Kozlina, B., Pavlica, M., 2010. Ecotoxicological assessment of industrial effluent using duckweed (Lemna minor L .) as a test organism 216–222.

Santos, M. J., Ferreira, V., Soares, A. M. & Loureiro, S., 2011a. Evaluation of the combined effects of dimethoate and spirodiclofen on plants and earthworms in a designed mesocosm experiment. Appl. Soil Ecol., 48, 294-300.

Santos, M. J., Morgado, R., Ferreira, N. G., Soares, A. M., Loureiro, S., 2011b. Evaluation of the joint effect of glyphosate and dimethoate using a small-scale terrestrial ecosystem. Ecotoxicol. Environ. Saf., 74, 1994-2001. 15

Schulz, H.; Glaser, B., 2012. Effects of biochar compared to organic and inorganic fertilizers on soil quality and plant growth in a greenhouse experiment. J. Plant Nutr. Soil Sci. 175, 410–422.

Smebye, A., Alling, V., Vogt, R.D., Gadmar, T.C., Mulder, J., Cornelissen, G., Hale, S.E., 2016. Biochar amendment to soil changes dissolved organic matter content and composition. Chemosphere 142, 100–105.

Tammeorg, P., Parviainen, T., Nuutinen, V., Simojoki, A., Vaara, E., 2014. Effects of biochar on earthworms in arable soil: avoidance test and field trial in boreal loamy sand. Agriculture, Ecosyst. Environ. 191, 150–157.

Troy, S.M., Lawlor, P.G., O'Flynn, C.J., Healy, M.G., 2014. The impact of biochar addition on nutrient leaching and soil properties from tillage soil amended with pig manure. Water. Air. Soil Pollut. 225.

Verheijen, F., Jeffery, S., Bastos, A.C., van der Velde, M. and Diafas, I., 2009. Biochar application to soils - A critical scientific review of effects on soil properties, processes and functions 166.

Williams, P. H. ,1989. Rapid Cycling Brassicas (RCBs) in Hands-on Teaching of Plant Biology. Asociation for Biology Laboratory Education. Available at http://www.ableweb.org/volumes/vol-10/1-williams.pdf

Williams, P. H., and Hill, C., 1986. Rapid-Cycling Populations of Brassicas. Science 232, 1385-1389.

Yargicoglu, E.N., Yamini, B., Reddy, K.R., Spokas, K., 2015. Physical and chemical characterization of waste wood derived biochars. Waste Manag. 36, 256–268.

Zar, J.H., 1996. Biostatistical Analysis. Prentice-Hall International, Inc., New Jersey.

5.9. Supplementary Information

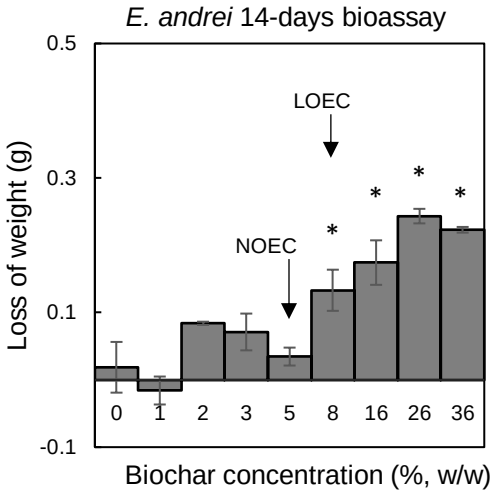

Figure S5.1. Body weight changes of *E. andrei* expressed as average loss of weight (in g) when exposed to a range of biochar concentrations (%, w/w). "0%" concentration refers to un-amended soil (control). Error bars represent standard errors of the means. Asterisk (*) refers to significant differences when compared to un-amended control. NOEC stands for no-observed effect concentration, LOEC for lowest observed effect concentration (Dunnett's test, $p>0.05$).

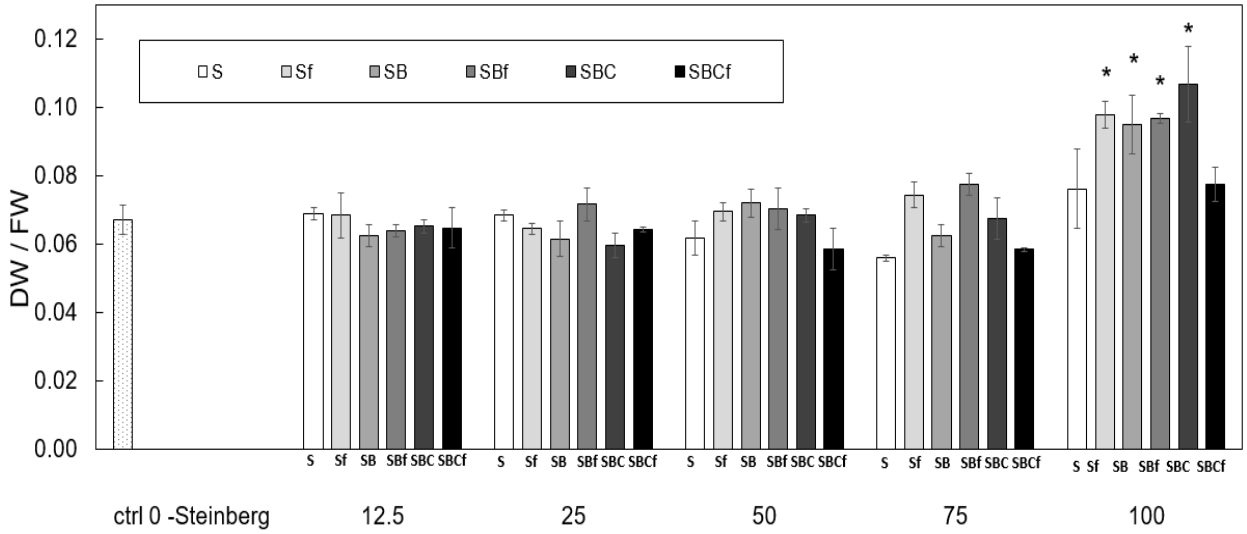

Figure S5.2. *Lemna minor* dry weight:fresh weight ratios (DW/FW) as a result of exposure to the leachates of un-amended soil (S) and amended soil treatments (Sf, SB, SBf, SBC, SBCf) from the STEMs experiment. Leachates were diluted to 12.5 %, 25 %, 50 % and 75% with Steinberg growing medium, which was also used as a control (ctrl-0) during the 7-days exposure. Concentration of 100% represents non-diluted leachate. Error bars represent

standard errors of the mean. Asterisk (*) refers to significant difference when different leachate concentrations are compared to the control that consists of Steinberg medium, ctrl-0 (Dunnett's test, $p<0.05$).

Table S5.1 . Two-way RM ANOVA output table for the effects of treatments on bait-lamina consumption in STEMs. Asterisk (*) indicates statistically significant differences ($p<0.01$).

Source of Variation	DF	SS	MS	F	p
Bait-lamina consumption					
Time (bait-lamina sets)	2	75.06	37.53	7.429	0.002*
Treatment	5	67.09	13.42	2.500	0.069
Time x treatment	10	84.47	8.647	1.712	0.116

Table S5.2. Two-way RM ANOVA output table for the measured yield of photosythesis in *Brassica rapa* plants. Asterisk (*) indicates statistically significant differences ($p<0.001$).

Source of Variation	DF	SS	MS	F	p
Photosynthesis yield					
Treatment	5	0.044	0.009	0.851	0.532
Time (weeks)	2	0.268	0.134	15.68	<0.001*
Treatment x time	10	0.032	0.003	0.369	0.952

Chapter 6

General Discussion and Conclusions

6.1. General discussion

The main outcomes of the work carried out in this doctoral thesis are discussed in the current section, by summarising and integrating the main findings, alongside the study limitations, major conclusions and future research directions. The effects of woodchip biochar on soil biota were explored in different experimental contexts that combine multiple test organisms and structural and functional endpoints, splitting the work into four sections (Chapters 2-5).

6.1.1. Overall methodology

Biochar's increased attention in recent years is evident through the expansion of research in various fields of biochar applications. The main biochar research literature has been summarised by meta-analyses-based quantitative reviews, such as those on biochar effects on crop yield (Jeffery et al., 2011; Jeffery et al., 2017), tree growth responses (Thomas and Gale, 2015), root traits (Xiang et al., 2017), available inorganic nitrogen (Nguyen et al., 2017), decomposition and priming effects (Wang et al., 2016), nitrous oxide emissions (Cayuela et al., 2013), methane emissions (Jeffery et al., 2016), as well as by qualitative reviews on biochar effects on soil biota (Lehmann et al., 2011; Ameloot et al., 2013), particularly earthworms (Weyers and Spokas, 2011). More recent reviews have analysed the required level of scientific understanding for sustainable biochar application (Tammeorg et al., 2016), biochar as a source versus a sink of potentially toxic elements (Hilber et al., 2017), and compost improvement with biochar for agriculture (Godlewska et al., 2017).

Nevertheless, bioavailability and fate of biochar contaminants by means of effects on soil and aquatic biota is not well understood. Biochar-soil interactions depend on the biochar feedstock characteristics, pyrolysis/processing conditions, but also on the properties of the soil to which biochar is applied and overall environmental factors, as discussed in more detail in **Chapter 1**. For example, biochar can contribute to remediation of contaminated soil, in contrast to a possibility of becoming a source of contaminants itself in the course of time (Hilber et al., 2017). Bioavailability of biochar-contained contaminants, sub-lethal effects on edaphic and aquatic organisms, species interactions and functional redundancy, using representative experimental designs are stated as some of the most important gaps to be addressed in biochar research field (Tammeorg et al., 2017).

This work is expected to contribute to the aforementioned knowledge gaps by studying the impact of selected slow pyrolysis woodchip biochar on biota, using an integrative effects based approach and considering: (1) the biological scale (e.g. biochemical responses; behavioural responses, survival, reproduction, etc.); (2) spatial scale (e.g. from standardized bioassays under laboratory conditions, to the assessment in multispecies microcosms, up to higher tier assessment with indoor mesocosms); (3) time scale (in biomonitoring of freshly amended and field-aged amended soils with biochar and biochar-compost); and (4) environmental scale (by testing both terrestrial and aquatic components of biochar amended soil). Each experimental section investigated a set of specific research questions, while including the scaling, in order to obtain data sets that are complementary, thus ecologically relevant. In general, it was designed to start with an evaluation of single species bioassays, using standardized and/or established methodologies (Chapter 2, partially Chapter 3), for ecotoxicological characterization of the biochar substrates, representing a base for further experiments by selecting suitable biochar application rates, moisture adjustments and incubation period of biochar-amended soil. Further, the evaluation was carried out with multispecies test approaches (Chapter 4), within which the experimental designs also allowed for addressing a mechanistic effect of biochar on biota (Chapters 3 and 4). The final experimental section represents a higher-tier approach, as a way of bridging the gap from laboratory to field, to enhance ecological and environmental relevance (Chapter 5).

6.1.2 Summary of results

Chapter 2 presents biochar and biochar-compost effects on survival and reproduction of *Folsomia candida* and food consumption and biomass change of *Porcellionides pruinosus*, using as a case study a commercial vineyard in Central Portugal. Un-irrigated commercial vineyards could benefit from the amendments, mostly due to potential increase in water retention and additional organic matter input. However, the effects of these amendments to soil dwelling organisms are not fully understood, particularly in the long-term. Besides, vineyard soil is exposed to additional pressure due to application of conventional pesticides. In this study we evaluated the effects of fresh and 18 months field-aged biochar and biochar-compost, while complementing the ecotoxicological laboratory bioassays with soil chemical analysis, and the theoretical/predicted exposure and risk assessment of the pesticides applied in the vineyard during the study. The ecotoxicological response to the tested biochar and biochar-compost

enriched vineyard soil was species specific, time-dependent, and to some extent, treatment-dependent. The most sensitive endpoint obtained in the study was collembolan reproduction output. Freshly-amended soil did not induce significant changes on organisms' performance. Isopods were stimulated in the freshly amended soil, and the results indicate possibility that they are using biochar and biochar-compost as a source of food. However, the organisms' fitness was reduced when exposed to the soil and amended-soil from the second sampling event, which was subjected to various climatic factors and conventional pesticides. Estimated risk quotients for some of the pesticides were elevated. The results suggest that the bioavailability of potentially toxic compounds like pesticides, might not be prevented over time by the presence of biochar and biochar-compost in the vineyards that receive conventional plant protection products, as often is suggested as one of biochar capabilities. Our findings can contribute in further understanding of long-term effects of biochar and biochar-compost on representative soil organisms. Specifically, the indications of *P. pruinosus* feeding behaviour in amended soil are in the line with those of Madžarić et al. (2018), the only available study up to date, who showed that terrestrial isopods *P. scaber* feed on biochar.

Chapter 3 addresses the potential inherent toxicity of biochar particles on soil and aquatic biota, as influenced by particle sizes and application rates. Pine woodchip biochar was incorporated in a clean soil at three particle size classes: small (<0.5 mm), medium (1-2 mm), and large (<4 mm), and at two concentrations: 1 % and 6 % (w/w). A first screening to study the most adequate soil-biochar equilibration period was carried out by using avoidance behaviour of *Eisenia andrei*. A follow-up 28-days microcosm experiment was conducted in a greenhouse and survival, vertical distribution and weight changes of *E. andrei*, and fauna feeding activity (bait-lamina) were recorded. Soil leachates from the microcosms were collected at the end of the greenhouse experiment to assess their effects on *Daphnia magna* immobilisation and *Vibrio fischeri* bioluminescence. Feeding experiments with *E. andrei* were also performed to address changes in body mass and to conduct a screening of PAHs/PAH-type metabolites in earthworms' tissue. The 6% <0.5 mm treatment induced significant avoidance behaviour of earthworms in the laboratory bioassays when incubated for 96 h. Pre-incubation of 96h was therefore used in the greenhouse microcosms experiment. The results showed that smaller particles (<0.5 mm) of woodchip biochar might pose sub-lethal toxicity to soil biota suggesting that there is a connection in behavioural (avoidance), individual (weight

changes, Nap-type metabolites in earthworms' tissue) and functional (bait-lamina consumption) endpoints.

Chapter 4 presents the laboratory experiment on the activity assessment of three soil enzymes (dehydrogenase, urease and ß-glucosidase) in the unamended soil and 1.5% biochar-amended soil over five sampling events during 56-days. This was carried out in microcosms consisting of single species treatments (*E. andrei* or *P. pruinosus*), combined species treatments (*E. andrei* and *P. pruinosus*), and in those without organisms. Besides, a multi-biomarker approach was applied to *E. andrei* exposed to unamended soil and biochar amended soil (from 1) in the presence and/or absence of *P. pruinosus*. Enzymatic activities in biochar amended soil showed time-dependency. In the absence of animals, dehydrogenase and ß-glucosidase reduction and even inhibition was observed. In the treatments with animals, the responses of ß-glucosidase were species-dependent with stimulations in the biochar-amended soil in the presence of isopods. Urease activity also showed dominance of species as a factor, namely isopods, but mostly in soil without biochar. Dehydrogenase activity showed significant fluctuations only in the third week of sampling. This response was treatment-driven in the single species microcosms, meaning that it was reduced in biochar-amended soil. However, it is interesting that this pattern was not observed when both species were present. While the body mass and reproduction of *E. andrei* were not affected, toxicity biomarkers in earthworms revealed occurrence of lipid peroxidation and cellular energy allocation in response to biochar.

The final experiment conducted in the study (**Chapter 5)** utilized a higher-tier approach, analogous to those recommended for pesticide risk assessment (Santos et al., 2011), and also for biochar-amended soil testing (Amaro et al., 2016). The study duration was extended to 42 days in order to obtain the full life cycle of plants, with an additional testing of leachates from the soil columns at the end of the 42 days-experiment. Impact of biochar, biochar-compost, NPK-based mineral fertilizer and their combinations on biota, while added to natural agricultural soil at relevant application rates was investigated in indoor mesocosms. The experiments were carried out in two phases. First was the six week-experiment where the effects of soils amendments on *Eisenia andrei*, rapid-cycling *Brassica rapa* and fauna feeding were evaluated in a small-scale terrestrial ecosystem study (STEMs). Second was the potential toxicity study of the amended soil leachates from the STEMs on *Lemna minor*. Applied amendments had low to no-effects on earthworms. In general, the plants' biomass even stimulated in the treatments of biochar-compost with mineral fertilizer, did not respond in a statistically significant manner.

Amongst the production characteristics of *B. rapa*, the number of seeds and mean number of seeds per pod increased significantly in the treatments of biochar-compost combined with mineral fertilizer and biochar-compost, respectively. The aquatic component testing the lowest EC_{20} and EC_{10} were obtained in the leachate of soil with biochar-compost. Significantly increased dry to fresh weight ratios in *L. minor* were observed, even though the intensity of the response was not high. Here a possibility of leaching stimulation (e.g. of nutrients, and/or potentially toxic compounds in mixture) may not be excluded, and consequently a hazard to aquatic systems. Nevertheless, this demands further research. The sensitivity of the responses observed with different functional groups indicate that STEMs methodology is an adequate higher tier approach for ecotoxicological assessment of biochar- based amendments.

6.1.3. Practical outcomes

The direct risk on representative organisms associated with application of the woodchip biochar used in this study was low. Regarding the application rate, the recommendation for this biochar might be <2% (equivalent to around 40 t/ha maximum, in the case of a 15 cm topsoil application and soil bulk density of 1.3 g/cm^3). However, direct risks might also be linked with effects of the woodchip biochar application, in combination with conventional pesticides, in which case neither the biochar or biochar mixed with compost should be used in the arable soils by farmers, as the long-term effects of the mixtures are not at the required level of understanding yet. EBC/IBI certifications are currently primarily based on biochar properties, i.e. without providing a guidance regarding the application rates. Although EBC gives a reference of 40 t/ha in 100 years period in the context of PAHs benchmarks set by this guideline (4 mg/kg dry matter, or 12 mg/kg dry matter), any environmental factors and risks associated to bioavailability were not taken into account up to date (EBC, 2012; IBI, 2016). Considering biochar particle sizes, before any recommendation on the safe biochar application can be issued based on ecotoxicological characterizations of biochars, the currently available biochar quality guidelines, such as EBC (V6.2, last reviewed in 2016) and IBI (V2.1, last reviewed in 2015) should be supplemented with effects-based approaches that address different representative organisms and biochar particle sizes required for the producers, as was already recommended within IBI (2016).

6.2. Limitations of the study

Bearing in mind that 'biochar' includes a diverse and physicochemically heterogenous group of materials, the universality of the inferences drawn from the research carried out in this doctoral thesis need to be confirmed in further research, through a case by case evaluation. Similar rationale should be applied in the case of soil diversity and heterogeneity, e.g. in terms of climate temperate versus tropical.

In Chapter 2 the main limitation was related to the lack of an adequate reference soil with similar characteristics as the treated soil, but exempted from pesticide treatment. The experimental field site was located within a large area under intensive agricultural management and it was not feasible to find similar soil without recent/historic pesticide treatment. This is why the decision was made to sample the vineyards soil immediately before biochar/biochar-compost were applied and before the pesticide application season started, and to apply the fresh amendments to soil in the laboratory. Another reason for this is that the freshly amended plots would be disturbed by extracting large amounts of amended topsoil, which could cause issues regarding the use and reporting the accurate amount of biochar bearing in mind that 18 month sampling time was planned for the ecotoxicological assessment.

The relatively low number of replicates (4 to 6) is the main drawback of the microcosms study in the greenhouse (Chapter 3) and of the mesocosms study in the laboratory (STEMs) (Chapter 5). Reasons for this include the high amount of soil needed for the experiments, limited space in laboratory and reduced number of available carts, in the case of the STEMs study. Consequently, relatively low amounts of dry plant material, soil/amended soil pore water extracts and leachates in the latter experiment were limiting factors for the replication in nutrients measurements of the samples. Increasing the number of replicates could overcome the issue of high variability among the replicates, but also assure the higher available amounts/aliquots of samples for the chemical analysis.

6.3. Main conclusions and directions for future work

The work conducted in this PhD thesis shows that slow pyrolysis woodchip biochar did not induce strong adverse effects on the tested organisms, and the responses varied from sublethal to neutral and/or stimulatory. The responses of representative model organisms were to some extent species-specific, and application rate- and/or treatment- dependent. It is worth

remembering that the biochar used in the study contains relatively low concentrations of the potentially toxic elements (metals, PAHs, PCBs, etc.), as expected for wood biochars produced under highly controlled conditions, including degassing. It is, thus considered of premium quality and therefore, safe according to the EBC quality standards (EBC, 2016). Nevertheless, the observed sublethal effects of the woodchip biochar on organisms reflect bioavailability of the whole matrix as a mixture of potentially toxic compounds. under certain physicochemical characteristics (e.g. particle size), including application rate, exposure route, soil/environmental combinations, and/or biochar ageing processes.

The approach used in this thesis highlights the importance of a case-by-case biochar assessment, by means of avoiding contaminants while taking into consideration the overall context of the specific application, such as environmental conditions and/or site-specific pesticide management practices in arable soils (Chapter 2). Due to sensitivity of *F. candida* reproduction and *P. pruinosus* feeding and body mass obtained in the bioassays, the ecotoxicological evaluation in the laboratory can be recommended as a useful biomonitoring tools for biochar/biochar-compost field application. Care should be taken in the case of intended use of biochar and biochar-based amendments in arable soil that is receiving conventional pesticide treatments. The questions of biochar ageing in soil, sorption/desorption capacity of biochars related to pesticides and other emerging contaminants and to biochar-bound contaminants, as well as the effects of environmental factors on these processes, remain to be addressed in more detail. Long-term field and laboratory studies are generally lacking in the assessment of biochar effect to non-target organisms. Soil invertebrate community studies in biochar-amended field sites as part of soil screening or ecological surveys are still scarce, to the best of our knowledge.

This study shows that for a comprehensive understanding of biochar effects on biota it is paramount to evaluate various endpoints, exposure routes and levels of biological organisation, under representative exposure scenarios. This is well-demonstrated in the experiment in Chapter 3, where the resulting response pattern revealed sub-lethal effects of small biochar particles. The obtained result emphasises the importance of evaluating the bioavailable fraction of biochar-bound contaminants coupled to the assessment of total concentrations in biochar. The reported consistency in responses and in addition to that, using time- (fixed fluorescence, avoidance) and cost-effective techniques (bait-lamina consumption, fixed fluorescence), open the possibility of integration of such bioassays as routine procedures in biochar quality

standards (Chapter 3). There is an urge to include more effect-based approaches using different organisms, to complement the physicochemical analysis in the assessment of biochar within the existing quality standards (EBC, 2012; IBI, 2016), as so far only IBI is recommending the use of germination inhibition bioassay with the reference to OECD (1984) with three plant species (IBI, 2016)

Although the biomarkers showed to be sensitive in evaluating earthworm responses, and generally can offer a large set of information in a short time, their broader practical use within quality guidelines might be limited due to high costs (Chapter 4). Biomarker approaches can be used as an early warning signs and are promising tools in biochar ecotoxicity studies for understanding the mechanism behind the earthworm responses to biochar-amended soil.

The soil/amended soil enzymatic activity results (Chapter 4) highlighted the relevance of considering the species interactions when evaluating the quality of biochar-enriched soil. It offers robust information output not only on the effects of biochar on soil quality, but also on the role of representative soil organisms in modifications of these effects. This is the first study to address isopod and earthworm interactions in biochar-amended soil, and it is a useful base for further research in this field.

In technical terms it is important to mention that this work can also contribute to the practical side of the use of ecotoxicity bioassays in biochar assessment, like complementing the lack of information on the soil-biochar pre-incubation duration (namely for pH equilibration) prior to exposure of animals in the chronic bioassay, for example. Besides the pH measurement, performing avoidance behaviour bioassay with amended soils incubated for different periods of time is recommended, as it is specific for the soil-biochar combination used, as demonstrated in Chapter 3. Also, prior to testing ranges of concentrations/application rates of a biochar following ecotoxicological guidelines, it is necessary to determine water holding capacity (WHC) for each one of them separately due to biochar's potential to retain moisture, when freshly applied to soil. This is normally not the case in ecotoxicological tests of chemicals, for which no significant changes in soil moisture are expected to occur after spiking the soil with the test substance.

Increased ecological relevance within the STEMs study (Chapter 5) was achieved through extended duration of the experiment to six weeks, and through combination of the plant and earthworm species known for their interactions in soil, which together allowed obtaining the

endpoints from the individual (earthworms weight) to functional (bait-lamina feeding) and population level (full plant cycle – reproduction traits), while also testing the aquatic component as leachates from the mesocosms. The approach proposed in the STEMs study serves in bridging the gap between laboratory and filed studies. Possible risks of increased organic carbon, or nutrients, and/or potentially toxic compounds and mixtures in the water bodies remain as a recommendation for additional investigation in the context of biochar-based amendments, being mandatory within the criteria of sustainable biochar application to soil.

Aquatic bioassays are already taking its place in biochar literature, being an important source of information on the leaching potential of the contaminants from biochar, of their bioavailability in soil pore water, but also on the impact of biochar application to aquatic ecosystems. Lack of standardisation in leaching procedure and/or elutriate extractions from biochar-amended soils in general, is a limitation encountered during the work (Chapters 3 and 5 on leachates production and use in aquatic bioassays). Efforts within the scientific community towards development of such procedures would increase the confidence in results comparability between studies (e.g. within ring trials or inter-laboratory tests). Moreover, it would contribute to reproducibility, and consequently to easier integration of aquatic bioassays to biochar assessment quality guidelines. Additionally, chronic exposure assessments are necessary for better understanding of biochar particles' mobility and potential risk to aquatic ecosystems. Besides there are still fewer available aquatic toxicity studies when compared to terrestrial ones in general, a research on effects of biochar or biochar-based amendments on sediment dwelling organisms has not been reported yet. Laboratory simulations to study representative conditions of temperate climate regions, such as taking into account soil freezing and thawing cycles, soil wetting and drying, temperature and conductivity fluctuations in the context of climate changes are also scarce. In order to explore this in the future research, a detailed characterization of the elutriates, leachates and/or aqueous extracts would provide more information about the mechanisms behind the impact of such complex mixtures to the aquatic ecosystem.

6.4. References

Amaro, A., Bastos, A.C., Santos, M.J.G., Verheijen, F.G.A., Soares, A.M.V.M., Loureiro, S., 2016. Ecotoxicological assessment of a biochar-based organic N-fertilizer in small-scale terrestrial ecosystem models (STEMs). Appl. Soil. Ecol. 108, 361–370.

Ameloot, N., Graber, E.R., Verheijen, F.G.A., De Neve, S., 2013. Interactions between biochar stability and soil organisms: Review and research needs. European Journal of Soil Science, 64 (4), pp. 379-390.

Cayuela, M.L., van Zwieten, L., Singh, B.P., Jeffery, S., Roig, A., Sánchez-Monedero, M.A., 2014. Biochar's role in mitigating soil nitrous oxide emissions: A review and meta-analysis. Agric. Ecosyst. Environ. 191, 5–16.

European Biochar Certificate (EBC), 2012. Guidelines for a Sustainable Production of Biochar. European Biochar Foundation (EBC), Arbaz, Switzerland. http://www.europeanbiochar.org/en/download. Version 6.3E of 14th August 2017. DOI: 10.13140/RG.2.1.4658.7043

Godlewska, P., Schmidt, H.P., Oleszczuk, P., 2017. Biochar for composting improvement and contaminants reduction. A review. Bioresour. Technol. 246, 193–202.

Hilber, I., Bastos, A.C., Loureiro, S., Soja, G., Marsz, A., Cornelissen, G., Bucheli, T.D., 2017. The different faces of biochar: contamination risk versus remediation tool. J. Environ. Eng. Landsc. Manag. 6897, 1–19.

International Biochar Initiative (IBI), 2015. Standardized Product Definition and Product Testing Guidelines for Biochar 7 That Is Used in Soil. http://www.biochar-international.org/sites/default/files/IBI_Biochar_Standards_V2.1_Final.pdf

Jeffery S., Verheijen F.G.A., van der Velde M., Bastos A.C. 2011. A quantitative review of the effects of biochar application to soils on crop productivity using meta-analysis. Agriculture, Ecosystems and the Environment. 144, 1, 175-187.

Jeffery, S., Verheijen, F.G.A., Kammann, C., Abalos, D., 2016. Biochar effects on methane emissions from soils: A meta-analysis. Soil Biol. Biochem. 101, 251–258.

Jeffery, S., Abalos, D., Prodana, M., Bastos, A.C., van Groenigen, J.W., Hungate, B.A., Verheijen, F., 2017. Biochar boosts tropical but not temperate crop yields. Environ. Res. Lett. 12, 53001.

Lehmann, J., Rillig, M.C., Thies, J., Masiello, C.A., Hockaday, W.C., Crowley, D., 2011. Biochar effects on soil biota – A review. Soil Biol. Biochem. 43, 1812–1836.

Liang, C., Zhu, X., Fu, S., Méndez, A., Gascó, G., Paz-Ferreiro, J., 2014. Biochar alters the resistance and resilience to drought in a tropical soil. Environ. Res. Lett. 9.

Madžarić, S., Kos, M., Drobne, D., Hočevar, M., Jemec Kokalj, A., 2018. Integration of behavioral tests and biochemical biomarkers of terrestrial isopod Porcellio scaber (Isopoda, Crustacea) is a promising methodology for testing environmental safety of chars. Environ. Pollut. 234, 804–811.

Nguyen, T.T.N., Xu, C.Y., Tahmasbian, I., Che, R., Xu, Z., Zhou, X., Wallace, H.M., Bai, S.H., 2017. Effects of biochar on soil available inorganic nitrogen: A review and meta-analysis. Geoderma 288, 79–96.

Santos, M. J., Morgado, R., Ferreira, N. G., Soares, A. M., Loureiro, S., 2011b. Evaluation of the joint effect of glyphosate and dimethoate using a small-scale terrestrial ecosystem. Ecotoxicol Environ Saf, 74, 1994-2001. 15

Tammeorg, P., Bastos, A.C., Jeffery, S., Rees, F., Kern, J., Graber, E.R., Ventura, M., Kibblewhite, M., Amaro, A., Budai, A., Cordovil, C.M. d. S., Domene, X., Gardi, C., Gascó, G., Horák, J., Kammann, C., Kondrlova, E., Laird, D., Loureiro, S., Martins, M.A.S., Panzacchi, P., Prasad, M., Prodana, M., Puga, A.P., Ruysschaert, G., Sas-Paszt, L., Silva, F.C., Teixeira, W.G., Tonon, G., Delle Vedove, G., Zavalloni, C., Glaser, B., Verheijen, F.G.A., 2017. Biochars in soils: towards the required level of scientific understanding. J. Environ. Eng. Landsc. Manag. 25, 192–207.

Thomas, S.C., Gale, N., 2015. Biochar and forest restoration: a review and meta-analysis of tree growth responses. New For. 46, 931–946.

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

Weyers, S.L., Spokas, K.A., 2011. Impact of Biochar on Earthworm Populations: A Review. Appl. Environ. Soil Sci. 2011, 1–12.

Xiang, Y., Deng, Q., Duan, H., Guo, Y., 2017. Effects of biochar application on root traits: a meta-analysis. GCB Bioenergy 9, 1563–1572.