Universidade de Aveiro Departamento de Ambiente e Ordenamento
2011

MARIJA PRODANA

ECOTOXICOLOGY OF BIOCHAR-BOUND PAHs IN RUNOFF FROM AMENDED SOILS

Universidade de Aveiro
2011

Departamento de Ambiente e Ordenamento

MARIJA PRODANA

ECOTOXICOLOGY OF BIOCHAR-BOUND PAHs IN RUNOFF FROM AMENDED SOILS

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre no contexto do Mestrado ERASMUS MUNDUS JEMES (Joint European Master in Environmental Studies), realizada sob a orientação científica da Doutora Ana Catarina Bastos do Departamento de Biologia e CESAM e do Doutor Nelson Abrantes do Departamento de Ambiente e Ordenamento do Território e CESAM da Universidade de Aveiro .

Financial support of the European Commission through the Erasmus Mundus Program, for the Masters scholarship

O júri

Presidente / President	Prof. Dr. Ana Isabel Couto Neto da Silva Miranda Professora Associada com Agregação / Associate Professor with Aggregation Department of Environment and Planning & CESAM, University of Aveiro
Vogal – Arguente / Examinor	Prof. Dr. Maria Claudia Gonçalves Cunha Pascoal Professora Auxiliar / Assistant Professor Department of Biology (CBMA), University of Minho
Vogal – Co-orientador / Co-supervisor	Dr. Nelson José de Cabaços Abrantes Investigador de Pós-Doutoramento / Post-Doctoral Research Fellow Department of Environment and Planning & CESAM University of Aveiro
Vogal – Orientador / Supervisor	Dr. Ana Catarina Gomes Marcelo Bastos Investigadora de Pós-Doutoramento / Post-Doctoral Research Fellow Department of Biology & CESAM, University of Aveiro

Agradecimentos / Acknowledgements

Firstly, I would like to express my sincerest gratitude to my thesis supervisor Dr. Ana Catarina Bastos and to Dr. Nelson Abrantes, who have supported me throughout my work with their knowledge and patience, while encouraged me and helped me greatly in understanding and writing the dissertation.. I am very grateful to Dr. Frank Verheijen who contributed to improving this work as a scientific advisor. I thank to my co-supervisor Dr Wolfgang Ahlf from Hamburg University of Technology (TUHH) for his professional help.

The work was mainly carried out in the Laboratory of Ecotoxicology and a small part in the Laboratory for Microbiology, both in the Department of Biology, University of Aveiro. I want to thank Professor Amadeu Soares and Professor Fernando Gonçalves for the opportunity to be integrated in their groups and for the laboratory facilities and conditions offered, to perform the experimental part of the work. I also thank Dr. Susana Loureiro for her professional and technical advice. I am especially grateful to Abel Ferreira for his help in the laboratory.

Further, I would like to acknowledge Dr. Xavier Domenes (University of Barcelona) and Dr. Gerardo Ojeda (University of Coimbra) for the information provided and also delivery and elemental analysis of the biochar used in this work.

I would also like to acknowledge JEMES Consortium and the European Commission for awarding me with the scholarship and for giving me the opportunity to study in the context of the Joint European Masters in Environmental Science programme.

Finally, I warmly thank my family, Vladimir, Sanja and all other friends for their support.

Keywords biochar, runoff, polycyclic aromatic hydrocarbons, bioassays, extracts

Abstract While the appeal of biochar application to soils continues growing, so does the concern about the possibility for surface and groundwater contamination, due to biochar-bound contaminants, such as polycyclic aromatic hydrocarbons (PAHs). Up to now, insufficient information exists on to what extent that PAH fraction can become bioavailable in the soil solution over time and which are the associated potential ecotoxicological implications, as a result of processes such as runoff.

This pilot study aimed to evaluate the ecotoxicological effects of biochar-bound PAHs in runoff from soils amended with biochar, having been identified as a gap in current knowledge. Combining soil wetting-drying cycles with PAH water-extraction, a good first approach was obtained for evaluating their potential occurrence in soil solution, while taking into consideration natural soil processes and soil-biochar interactions. LUFA 2.2 soil alone and containing biochar (at usual field rates, 4% ww^{-1}) was subjected to 0, 6 or 12 (sampling times ST-0, ST-1 or ST-2 respectively) consecutive wetting-drying cycles, after which the corresponding test elutriates were extracted. Alongside PAH quantification, a battery of standard aquatic bioassays were used with representative test organisms (*Vibrio fischeri*, *Pseudokirchneriella subcapitata* and *Daphnia magna*), for a robust ecotoxicological evaluation of the biochar-soil (BS) aqueous extracts, while LUFA soil elutriates were used as control (SS).

Compared to the control (SS) and to elutriates of biochar alone (B), BS extracts showed the highest total PAH contents, suggesting that a relevant PAHs fraction in biochar-amended soil may be easily water-extractable, perhaps due to interactions between biochar and soil components. Yet, the number of soil wet-dry cycles on aqueous total PAH concentrations was often not significant, suggesting that natural soil wetting-drying events might have little influence on increased PAH bioavailability in pore water, on the short term.

BS extracts induced toxicity in all tested species, although its extent was species-specific and varied with the number of wet-dry cycles. For example, the highest sensitivity was observed in the acute assay with *D. magna* exposed to BS extract for ST-0, while *P. subcapitata* and *V. fischeri* were most sensitive when exposed to BS, ST-1. Nevertheless, sub-lethal effects were also observed for *P. subcapitata* and *V. fischeri*, when exposed to the control (SS) extracts. Although the levels of individual PAHs in all samples (BS, SS and B) were below the acutely toxic concentrations reported in the literature, it cannot be excluded the combined effects of the multiple PAHs in the test elutriates when explaining these results. Furthermore, although individual PAH concentrations were below that to produce acute effects, chronic effects can occur, and therefore, long-term exposure to these elutriates and using additional non-target species, various biochars and soil properties are necessary for a full evaluation of the bioavailability and ecotoxicity of biochar-bound PAH contaminants in runoff from treated soils.

Table of contents

List of figures

List of tables

List of abbreviations

ANOVA	analysis of variance
ASTM	American Society for Testing and Materials
B	biochar
BS	biochar-enriched soil
BSPT	basic solid phase test
BT	basic test
C	carbon
EC	effective concentration
HOC	hydrophobic organic compound
LOEC	lowest observed effect concentration
mBSPT	modified basic soild phase test

NOEC	no observed effect concentration
NOM	natural organic matter
OECD	Organization for Economic Co-operation and Development
PAH	polycyclic aromatic hydrocarbon
SFE	supercritical fluid extraction
SOM	soil organic matter
SS	standard soil
ST	sampling time
TU	toxic units
USEPA	United States Environmental Protection Agency

Chapter I

General Introduction, Research Aims and Relevance

1.1 Biochar: the material and the concept

1.1.1. Definitions and concepts

Biochar is a subject of current debate. It is commonly defined as the product of thermal decomposition (300-1000ºC) of organic matter (biomass feedstock), under limited oxygen conditions, also known as pyrolysis (IBI, 2011). As a material, biochar is charcoal, since charcoal is the term generally used for 'charred organic matter'. However, from both soil science and environmental quality perspectives, a distinction needs to be made between the two, as recommended by Verheijen et al. (2010). It is the view of these authors that this distinction should be based on their function. While charcoal is mostly used as a fuel (e.g. heating), biochar is meant for application to soils and thus, caution needs to be taken for preventing any deleterious impact on the quality of soil and ground and surface waters (Verheijen et al., 2010).

Charcoal is a product of incomplete combustion of organic material (mostly wood and vegetation), from events such as wildfires, and is therefore naturally present in soils around the world (Preston and Schmidt, 2006). Large deposits of charcoal in soils were specifically found in the Amazon region, commonly known as 'Terra Preta' (or Dark Earths). 'Terra Preta' are highly fertile soils, but this charcoal does not have a natural origin, rather it was concentrated there alongside a mixture of other residues, including animal and fish bones, animal shells and pieces of pottery (Sohi et al., 2009). However, it is the link between the presence of charcoal and their fertility that is the base for today's concept of biochar. Biochar application to soils is thus expected to improve soil properties, processes and functions (Lehmann et al. 2006; Jeffery et al., 2011) and in that way, help to meet current agricultural challenges, including food security (Collison et al., 2009). On a different scale, biochar is also being suggested as a means of sequestering carbon (C) in soils, particularly when used in combination with other strategies for a balanced way to combate climate change (Verheijen et al., 2010). The main reason behind biochar's capability to be a C sink in soils is its environmental recalcitrance, with long mean residence times in soil estimated (for wood biochar) to be in the range of hundreds to thousands of years, compared to that of natural organic matter (NOM) or other common organic soil amendments, which are rapidly mineralised to carbon dioxide (CO_2). The debate on 'biochar' is being further extended to other domains (e.g. remediation of environmental pollutants, renewable energies, waste management), where it is also expected to provide a solution to some

of the current problems (e.g. Collison et al., 2009; Leach et al., 2010; Verheijen et al., 2010), although these areas are beyond this project's scope.

1.1.2. Main biochar characteristics and implications

Physically and chemically, biochar's composition is very heterogeneous (Sohi et al., 2009). Only a brief description of the main properties that are relevant to this work will be discussed here. Carbon, volatile matter, minerals (ash) and moisture are its major constituents. Their relative proportion in biochar is dependent on the combination of the type of feedstock (e.g. ratio of hemicelluloses, cellulose and lignin, mineral matter and water content) and the pyrolysis conditions (mainly temperature) used, since together, they determine the type and degree of the physical and chemical reactions which the biomass goes through during biochar production (Antal and Gronli, 2003; Demirbas, 2004).

The total C content in biochar is generally high and it can be up to 90% (weight) in biochars from woody feedstocks, such as the one used in this study. This C component of biochar is in the form of compacted crystalline graphene sheets, with aromatic rings at the surface, which is responsible for its dense aromatic structure and therefore, its highly recalcitrant nature (Antal and Gronli, 2003; Demirbas, 2004). Compounds such as hydrogen (H), oxygen (O), nitrogen (N), phosphorus (P) and sulphur (S) that were part of the feedstock, are retained in the biochar product within these aromatic rings as functional groups (Bourke et al., 2007). The development of the different functional groups (hydroxyl, nitro, amino etc.) during pyrolysis , results in a very heterogeneous and reactive surface, with neighbouring areas, where properties can alternate between oxidizing and reducing, acidic and basic, hydrophilic and hydrophobic (Amonette and Joseph, 2009). Biochar's surface is not only very reactive, but also very large. The loss of labile microelements (in the form of volatiles) during the pyrolysis of the biomass feedstock leaves an extensive porous network in the biochar product and this is revealed in a large surface area (e.g. Demirbas, 2004).

Together, it is the high porosity and chemically reactive surface of biochar that allows it to interact with the different soil components (Amonette and Joseph, 2009). Interactions of biochar with soil organic matter (SOM), clay minerals and microorganisms (Verheijen et al., 2010) influence soil properties (e.g. improve soil structure; raise of soil pH in acidic soils; Brodowski et al., 2006; Hammes and Schmidt, 2009), processes (e.g. favours soil aggregation and improves

water and nutrient retention; Brodowski et al., 2006; Hammes and Schmidt, 2009) and functions (e.g. enhances crop productivity; Jeffery et al., 2011). In the same way, it can adsorb any contaminants that might be present in soil, and in that way, influencing their mobility and fate, such as that find for various herbicides in contaminated soil (Hiller et al., 2007).

Pyrolysis conditions and feedstock characteristics are both important parameters in controlling the physical and chemical characteristics of the resulting biochar, including particle size, pore-size distribution, exact chemical composition, surface chemistry and type and concentration of contaminants (Antal and Gronli, 2003; Demirbas, 2004) . Different types of biomass can be used as feedstock for producing biochar, among which crop residues (e.g. wheat straw, maize residue, switchgrass), wood (e.g. pine, oak, willow), nut shells and grain husks (e.g. Lua et al., 2004; Martinez et al., 2006; Amonette and Joseph, 2009; Gonzaléz et al., 2009). For example, woody feedstocks such as that used for producing the test biochar used in this study, contain high proportion of lignin, cellulose and hemicellulose and a low amount of mineral matter (Brown et al., 2009). Consequently, the resulting biochar is a stable compound, with coarse and resistant structure, high carbon content (up to 80%) and low concentration of minerals and trace elements (generally <1%) (Demirbas, 2004; Winsley, 2007). In relation to contaminants, studies suggest that wood biochar is generally regarded as low in hydrophobic contaminants, compared to biochars from other source materials, including agricultural wastes (Fernandes and Brooks, 2003).

1.2 Occurrence and implications of contaminants in biochar

1.2.1. Occurrence of contaminants in biochar

Once the soil is loaded with biochar, complex interactions occur between biochar and soil components and differentiation between the various fractions for its removal is then technically very difficult. This is why the presence of polycyclic aromatic hydrocarbons (PAHs) and metals in biochars from a range of feedstocks and pyrolysis conditions, has raised concerns regarding potential adverse effects on environmental quality.

In biochar, contaminants such as these, may come from the biomass itself or be formed during pyrolysis. Various feedstocks have been suggested for producing biochar, such as

biowastes (animal manure, sewage sludge, etc) and composts, although these materials generally contain high amounts of organic pollutants and metals, which may be retained in the biochar. For example, metallic contaminants are more likely to occur as feedstock components (as reviewed by Verheijen et. al., 2010). Sewage sludge-derived biochar contained increased levels of copper (Cu), zinc (Zn), nickel (Ni) and chromium (Cr) (e.g. Bridle and Prichard, 2004), while biochar from poultry litter contained lower metal concentrations when compared to biochars from peanut hull and pine chips (Gaskin et. al, 2008).

Regarding PAHs, more information is available for those emitted as products of the combustion process, than for the PAH fraction that is retained as contaminants in the solid charred residue. The type and concentration of PAHs that are formed during pyrolysis and the level at which they accumulate in the biochar depend both on their type and concentration in the biomass feedstock, combined with pyrolysis temperature (Pakdel and Roy, 1991). The pyrolysis temperature for which PAHs are more likely to be formed are >700ºC, although they have also been found to be produced at temperatures between 350-600ºC (Garcia-Perez, 2008).

Jonker et al. (2005) found that charcoal-associated PAHs are very strongly adsorbed onto their charcoal carrier through physical entrapment, that is, within micro and nanopores, called 'occlusion sites'. According to Fernandes and Brooks (2003), PAH concentrations in pea straw and eucalypt wood biochar (at 450ºC, 1h) were found to be lower (<0,2 μg g^{-1} and <0.07 μg g^{-1} respectively), compared to that found in diesel soot, urban dust and chimney soot (concentrations >8 μg g^{-1}). Brown et al. (2006) has also reported that PAH concentrations in several chars produced at temperatures >500ºC, ranged between 3-16 μg g^{-1} (depending on peak treatment temperature), compared to 28 μg g^{-1} in char from prescribed burn in pine forest. In contrast, a study looking at twelve biochars from a variety of biomass sources and producers, provided evidence that PAH levels in biochar can be comparable (or even lower) than those in some urban soils (Jones, 2008). Overall, quantification of PAHs and other contaminants which come from biochar, as well as their interactions with soil components and the consequences of these interactions over time, are all important issues to be addressed in order to effectively assess the risk of application of biochar into soils.

1.2.2. Aspects of environmental behaviour of PAHs

PAHs are ubiquitous in the environment and generally occur as a mixture of compounds (Hoffman et al., 2002). This group of aromatic hydrocarbons is characterized by the presence of two or more C rings, often including an alkyl group bound to one or more carbon atoms (Figure 1). All PAHs generally have a range of common chemical properties, including high melting and boiling points, low vapour pressure and low solubility in water, although differences in specific chemical behaviour, as well as toxicity effects and mechanisms depend largely on the molecular weight, which is related to the number of rings (Hoffman et al., 2002) .

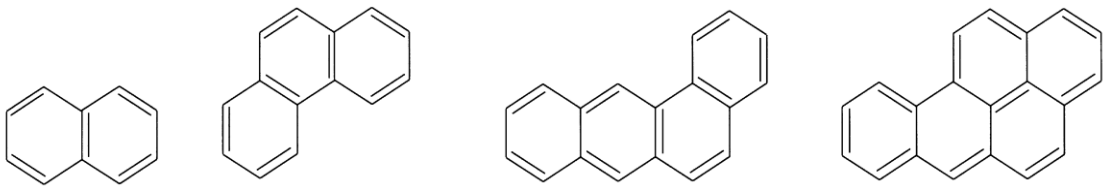


Figure 1. Examples of PAHs evidencing aromatic structure.

According to the European Environment Agency (EEA), persistent organic pollutants (POPs), such as PAHs, are chemical substances that are directly toxic to biota (mostly at the sublethal level; Hoffman et al., 2002) and of relatively high recalcitrance in the environment. Due to their potential to bio-accumulate and/or bio-magnify through the food chain, they pose a risk to human and animal health, as well as to the environment. Their lipophilic character enables PAHs to accumulate in adipose tissue and penetrate the cell membrane where co-metabolites produced during biotransformation can react with DNA, RNA and proteins (Tuvikene, 1995). In humans, PAHs are also considered possible carcinogenic and suppressors of the immunological system and human development, even at low concentrations (EEA, 1999).

Thermal decomposition of organic material (including combustion and pyrolysis) is considered the most common source for PAH emissions, arising either from natural (e.g. volcanoes and wildfires) or anthropogenic processes (e.g. open fires, wood burning from cooking and heating, oil seeps) (EEA, 1999). However, PAHs can generally be found within long distances

from the source and can occur in the environment as gases, or attached to solid adsorbent particles (USEPA, 2002).

It has been suggested that free PAHs in soil are rapidly biodegraded by native bacteria and fungi (Cornelissen et al., 1998). Studies have found that one of the main mechanisms for PAH biodegradation involves the activity of lignin-degrading extracellular enzymes, such as those produced by wood-degrading fungi (Gadd, 2001). These powerful enzymes have low substrate specificity and can co-metabolize various environmental contaminants, which chemical structure is similar to lignin's. The advantage is that these extracellular enzymes are able to metabolize the contaminant at any concentration even if they are not bioavailable or soluble (Cajthaml et al, 2002; Mansur et al., 2003; Pointing, 2003, Veignie, 2004). However, it is unlikely that PAHs occur as free molecules in soils. The most important processes determining PAHs bioavailability and transport in soils and into water resources are probably sorption and desorption. In soils, PAHs are usually found adsorbed to natural organic matter (NOM) and black carbon (including charcoal) particulates and incorporated within soil aggregates. That may partially explain why the lowest concentration of PAHs was found in arable soils, comparing to forest soils and grassland respectively (Cornelissen et al., 2005).

Comparatively to sorption onto NOM, adsorption onto biochar's surface is thought to be a quicker and stronger sorptive process, partly due to biochar's large and reactive surface area, but also due to the PAH's planar molecular structure (Cornelissen et al., 2004, 2005). Consequently, their desorption rates from the charcoal (desorption rate constants of up to 10^{-7} to 10^{-6} h^{-1}) in natural environments is very slow (Jonker et al., 2005). It has been suggested that desorption from the charcoal carrier would take, at least, several decades to occur, dependent on soil and environmental conditions (Jonker et al., 2005). Nevertheless, these authors did not consider biochar interactions in soil which can speed up the desorption process (see sub-section 1.2.3).

1.2.3. Biochar ageing in soil

At present, the largest challenge for biochar researchers is to understand the degree and all the implications of the interactions that biochar establishes in soils with the various soil elements over a period of time, and how these interactions are influenced by natural soil

conditions and processes. As explained before, such interactions are due to the extensive and highly reactive surface of biochar and are generally referred to in this study as 'biochar ageing'. A large gap of current knowledge in this matter relates to the potential effects of biochar ageing in soil on thedesorption of contaminants from biochar, thus increasing their bioavailability, mobility and ecotoxicological implications (Verheijen et al., 2010).

Although it is generally regarded as a recalcitrant compound, biochar is not inert, even if the full range of mechanisms involved in its alteration of properties and finally degradation in soil are not yet fully understood. Binding and adsorption of NOM, clay minerals and microrganisms onto biochar's surface can result in alteration of the charcoal's surface properties, including surface chemistry (Glaser et al., 2002; Hammes and Schmidt, 2009). Consequences of these alterations have been poorly researched so far (Verheijen et al., 2010). It has been suggested, for example, that these processes may be important for biochar oxidation. Oxidation is thought to destabilize the aromatic structure, by removal of electrons with formation of carbonyl, carboxyl and phenolic groups, leaving it more predisposed to biotic (and enzymatic) breakdown (Cheng et al., 2006) and/or more susceptible to further interactions with other soil elements (e.g. Cheng et al., 2006; Chen et al., 2007).

Uchimiya et al. (2010) underlined the importance of biochar ageing in respect to contaminants when assessing the long-term effects of soil amendment with biochar. For example, sorption of NOM (particularly humic and fulvic acids) and of specific metals (e.g. Cu^{2+}; Chen et al., 2007) to charcoal, were found to influence adsorption and desorption of hydrophobic organic compounds to and from its surface, as shown by Pignatello et al.(2006). In fact, the presence in soil of organic compounds with higher molecular sizes have shown to reduce adsorption to charcoals of other compounds with lower molecular weights (e.g. Sander and Pignatello, 2005; Wang et al., 2006). Humic and fulvic acids in NOM can decrease the sorption capacity of organic contaminants to benzene by competition for adsorption sites at the charcoal surface (Pignatello et al., 2006) but the same might be true for other PAHs. For that reason, Verheijen et al. (2010) underlined the importance of analyzing the sorptive properties of biochar but within amended soils, specifically natural soils, which realistically contain a mixture of different compounds. The mechanism of competitive sorption/desorption can influence leaching and thus bioavailability of contaminants, as well as increase their mobility as they are transported from biochar-amended soils into aquatic systems. So far, the data available on this issue mostly come from short-term experiments, where just one or a limited number of potential

competitive chemical species were present, and where the influence of other environmental factors on biochar ageing, were not taken into consideration.

Besides competitive sorption in soil, another way that contaminants (including PAHs) in biochar could reach aquatic systems is by weathering of biochar itself into smaller particles (e.g., through photochemical and microbial breakdown; Goldberg, 1985), that can then be easily transported from soil by wind or water erosion, due to being a light material, as a consequence of the high porosity (Wilcke, 2000; Hammes and Schmidt, 2009). Biochar weathering can thus help disperse any bound/adsorbed contaminants, although little information is available regarding which environmental factors are behind the mobility of biochar through the soil profile and eventually reaching water resources and sediments. What is known is that this is a process which happens overtime and it is likely to be highly dependent on soil and environmental conditions (Cheng et al., 2006; Verheijen et. al, 2010). One can suggest that certain environmental and soil conditions and processes, but also management and land use, can have an impact on biochar weathering. For example, Cheng et al. (2008) studied the influence of temperature on the stability of charcoal in soil and higher rates of breakdown were related to higher mean annual temperatures. In relation to traditional tillage, mechanical mixing of biochar with soil can accelerate the breakdown of biochar to smaller particles and contribute to destruction of soil aggregates, leading to faster biochar loss from soil (Sohi et al., 2009).

1.3 Bioassays

Within the main objectives of the European Water Framework Directive (Directive 2000/60/EC of the European Parliament and of the Council) is the increased focus on protection of the aquatic environment by applying measures for reduction of discharge, emissions and losses of priority hazardous substances. The Directive 2008/105/EC amended the previous suggesting environmental quality standards (EQS) for a range of substances and other pollutants. EQS are listed within the Directive with the recommended concentration limits for certain chemicals expressed in $\mu g\ l^{-1}$ (Directive 2008/105/EC of the European Parliament and of the Council). In addition to this, the European Community requires certain toxicity tests within environmental risk assessment approaches. For the risk assessment of chemicals in the environment, one of the tools usually used involves acute and chronic ecotoxicological tests, which can be performed with species from different trophic levels, following standard protocols

available. Acute toxicity tests are designed with the purpose of establishing concentration-response relationships for survival, while chronic tests enable evaluating the sub-lethal effects of chemicals (e.g. growth, reproduction). It is important to choose species that are sensitive to the effects of the toxic substances, while they also play a representative role in the ecosystem (Hoffman et al., 2002).

Vibrio fischeri is a marine bacterium often used in bioassays in order to evaluate toxicity of solutions of pure chemical substances, or contaminated water or soil samples, in a screening step of ecotoxicological evaluations, in alternative to more elaborate and time consuming tests with aquatic species (Parvez et al., 2006). The test is based on the reduction of luminescence in the bacteria after exposure to toxicants/toxic matrices, reflecting their toxicity (Guzella, 1998). It does not require preparing and maintaining cultures of the bacteria; instead, lyophilized bacteria are generally used, after activation in a saline water suspension. Although the sensitivity of the test has been discussed (Qureshi et al., 1998), its effectiveness was proved for testing the acute toxicity of several chemicals (Van der Grinten et al., 2010). Bacteria have a very important function in aquatic ecosystems as decomposers of organic material (Wang et al., 2009). For this reason *V. fischeri* is very often included in toxicity tests on soil extracts. *Pseudokirchneriella subcapitata* (previously known as *Selenastrum capricornutum*) is a green freshwater microalgae, well known as an indicator of water pollution (Labra et al., 2007). As primary producers (photoautotrophs), green algae are at the bottom of the food web and represent an important food source for herbivorous, as well as primary consumers, such as daphnids. Besides being sensitive to toxic chemicals, this species is also easy to handle, does not require special laboratory facilities, has a relatively short life cycle and for these reasons represents a useful and robust tool in ecotoxicology and in risk assessment of environmental contaminants (Mayer et al., 1997).

Daphnia magna is a planktonic crustacean that reproduces both sexually and asexually. Asexual reproduction, known as parthenogenesis (Alonso, 1996), occurs continuously in conditions of availability of food supply having as a consequence, low genetic variability. The effects of genetic variability on the toxic response are increased in this case, which makes the tests reproducible and the results comparable. The females can survive up to two months in laboratory conditions. This species does not require special laboratory facilities and it is used both for acute and chronic toxicity tests due to its short lifecycle (Terra et al., 2003). *D. magn*a has an important role in the trophic chain in lakes and ponds, very often being the dominant

zooplanktonic species and representing an important food source for planktivorous fish (Alonso, 1996), and therefore plays an essential role in the energy transfer from primary producers to higher levels of the food web. Moreover, among the zooplankton species, individuals from the genus *Daphnia* have a relatively higher sensitivity to toxicant stress, which explains their extensive use in ecotoxicology to evaluate general or more specific toxicity scenarios (Hanazato, 2001).

1.4 Research Aim and Objectives

The main aim of this pilot study was to evaluate the ecotoxicological potential of biochar-bound PAH contaminants in runoff, from soils amended with biochar.

In order to achieve this aim, specific objectives were defined:

i) to develop and optimise a methodology for investigating the potential of biochar-bound PAHs to become available in runoff from biochar-treated soils, based on soil wetting-drying cycles coupled to water-extraction, as an approximation to what would occur in soil systems;

ii) to perform an ecotoxicological evaluation of this PAH fraction in extracts of soil mixed with biochar, based on standard bioassays and test organisms that are representative of different trophic/functional levels.

1.5 Relevance and Applicability of Results

Having the research aim in mind, the results obtained in this pilot study are therefore expected to fill in a gap in the current scientific knowledge on the ecotoxicology of biochar-bound PAH contaminants in runoff from treated soils and may serve as basis for future studies on this matter. Similarly, these results are expected to contribute for standardisation of methodologies for evaluating the full ecotoxicological potential of biochar in soils, and thus may have direct use for regulatory and legislative purposes. This is important, since standardisation of biochar materials and test methodologies have recently been identified as an urgent need, considering the increasing intention of applying biochar to soils, whether it is for improving crop production or as a tool for combating climate change (IBI, 2011).

1.6 Thesis Structure

This thesis is divided into three Chapters as described below, with Chapter II having been structured as a scientific paper.

- Chapter I: General Introduction, Research Aims and Relevance;
- Chapter II: Ecotoxicology of biochar-bound PAHs in runoff from amended soil
- Chapter III: Concluding Remarks and Recommendations for Future Research.

1.7 References

Alonso, M., 1996. *Crustacea*, *Branchiopoda*. En: *Fauna Ibérica*, vol. 7. Ramos,M.A. et al. (Eds.). Museo Nacional de Ciencias Naturales. CSIC. Madrid. 486 pp.

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.

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.

Bridle, T. R., Pritchard, D., 2004. Energy and nutrient recovery from sewage sludge via pyrolysis. Water Science Technology 50: 169-175.

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.

Cajthaml, T. M. Möder P. Kacer V. Šašek P. Popp. 2002. Study of fungal degradation products of polycyclic aromatic hydrocarbons using gas chromatography with ion trap mass spectrometry detection. Journal of Chromatography A 974: 213-22.

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.

Chen, J., Zhu, D., Sun, C., 2007. Effect of heavy metals on the sorption of hydrophobic organic compounds to wood charcoal. Environmental Science and Technology 41: 2536-2541.

Cheng, C-H, Lehmann, J., Thies, J., Burton, S. D., Engelhard, M. H., 2006. Oxidation of black carbon by biotic and abiotic processes. Organic Geochemistry 37: 1477-1488.

Cheng, C-H, Lehmann, J., Thies, J., Burton, S. D., 2008. Stability of black carbon in soils across a climatic gradient. Journal of Geophysical Research 13: 1-10.

Collison, M., Collison, L., Sakrabani, R., Tofield, B. and Wallage, Z. (2009). Biochar and Carbon Sequestration: A Regional Perspective; A report prepared for East of England Development Agency, Norwich, UK: Low Carbon Innovation Centre, University of East Anglia, April 2009, www.uea.ac.uk/lcic/Biochar [accessed 8 November 2011].

Cornelissen, G., Rigterink, H., Ferdinandy, M. M. A., Van Noort, P. C. M., 1998. Rapidly Desorbing Fractions of PAHs in Contaminated Sediments as a Predictor of the Extent of Bioremediation. Environmental Science and Technology 32: 966-970.

Cornelissen, G., Haftka, J., Parsons, J., Gustafsson, Ö., 2005. Sorption to Black Carbon of Organic Compounds with Varying Polarity and Planarity. Environmental Science and Technology 39: 3688-3694.

Cornelissen, G., Gustafsson, Ö., Bucheli, T. D., Jonker, M. T. O., Koelmans, A A., van Noort, P. C. M., 2005. Extensive sorption of organic compounds to black carbon, coal and kerogen in sediments and soils: mechanisms and consequences for distribution, bioaccumulation and biodegradation. Environmental Science and Technology 39: 6881-6895.

Cornelissen, G., Gustafsson, Ö., 2004. Sorption of phenanthrene to environmental black carbon in sediment with and without organic matter and native sorbates. Environmental Science and Technology 38: 148-155.

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.

Directive 2008/105/EC of the European Parliament and of the Council. Of 16 December 2008. On environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 84/513/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Prliament and of the Coucil.Available: http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:348:0084:0097:EN:PDF [accessed 15 June 2011]

European Environment Agency 1999. Criteria for EUROAIRNET: The EEA Air Quality Monitoring and Information Network. Technical Report No.12. Available: http://www.eea.europa.eu/publications/TEC12 [accessed 1 December 2010]

EU Water Framework Directive: Directive 2000/60/EC of the European Parliament and of the Council.Available: http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32000L0060:EN:NOT [accessed 15 June 2011]

Fernandes, M.B. and Brooks, P., 2003. Characterization of carbonaceous combustion residues: II. Nonpolar organic compounds. Chemosphere 53(5): 447-458.

Gadd, G.M., 2001. Fungi in Bioremediation. British Mycological Society. Cambridge University Press.

Garcia-Perez, M., 2008. The formation of polyaromatic hydrocarbons and dioxins during pyrolysis. In: Washington State University.

Gaskin, J.W., Steiner, C., Harris, K., Das, K.C. and Bibens, B., 2008. Effect of low-temperature pyrolysis conditions on biochar for agricultural use. Transactions of the ASABE 51(6): 2061-2069.

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

Goldberg, E. D., 1985. Black carbon in the Environment: properties and distribution. Wiley, NY.

González, J. F., Román, S., Encinar, J. M., Martinéz, G., 2009. Pyrolysis of various biomass residues and char utilization for the production of activated carbons. Journal of Analytical and Applied Pyrolysis 85: 134-141.

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

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.

Hiller, E., Fargasova, A., Zemanova, L., Bartal, M., 2007. Influence of wheat ash on the MCPA imobilization in agricultural soils. Bulletin of Environmental Contamination and Toxicology 78: 345-348

Hoffman, D.J., Rattner, B.A., Burton Jr., G.A., Cairns Jr., J., 2002. Handbook of Ecotoxicology, second ed. CRC Press, New York.

IBI, International Biochar Initiative, 2011. Product Definition and Standard: Preliminary Draft for Review Only. [accessed 20 May, 2011]

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.

Jones, D. M., 2008. Polycyclic aromatic hydrocarbons (PAHs) in biochars and related materials. Biochar: sustainability and security in a changing climate. Proceedings 2nd International Biochar Iniative Conference 2008, Newcastle, UK.

Jonker, M. T. O., Hawthorne, S. B., Koelmans, A. A., 2005. Extremely Slowly Desorbing Polycyclic Aromatic Hydrocarbons from Soot and Soot-like Materials: Evidence by Supercritical Fluid Extraction. Environmental Science and Technology 39: 7889-7895.

Labra, M., Bernasconi, M., Grassi, F., Mattia, D.F., Sgorbati, S., Airoldi, R., Citterio, S., 2007. Toxic and genotoxic effects of potassium dichromate in *Pseudokirchneriella subcapitata*detected by microscopy and AFLP marker analysis, Aquatic Botany. 86: 229-335.

Lehmann, J., Gaunt, J. and Rondon, M., 2006. Bio-char sequestration in terrestrial ecosystems - A review. Mitigation and Adaptation Strategies for Global Change 11: 403-427.

Lua, A. C., Yang, T., and Guo, J., 2004. Effects of pyrolysis conditions on the properties of activated carbons prepared from pistachio-nut shells. Journal of Analytical and Applied Pyrolysis 72: 279-287.

Mansur, M. M. E. Arias J. L. Copa-Patino M. Flärdh and A. E. González. 2003. Thewhite-rot fungus *Pleurotus ostreatus* secretes laccase isozymes withdifferent substrate specificities. *Mycologia* 95, no. 6: 1013-20.

Martínez, M. L., Torres, M. M., Guzmán, C. A., Maestri, D. M., 2006. Preparation and characteristics of activated carbon from olive stones and walnut shells. Industrial crops and products 23: 23-28.

Mayer, P., Cuhel, R., Nyholm, N., 1997. A simple in vitro fluorescence method for biomassmeasurements in algal growth inhibition tests, Water Research 31: 2525-2531.

Pakdel, H., and Roy, C., 1991. Hydrocarbon Content of Liquid Products of Tar from Pyrolysis and Gasification. Energy & Fuels: 427-436.

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.

Pignatello, J. J., Kwon, S., and Lu, Y., 2006. Effect of Natural Organic Substances on the Surface and Adsorptive properties of Environmental Black Carbon (Char): Attenuation of Surface Activity by Humic and Fulvic Acids. Environmental Science and Technolology. 40: 7757-7763.

Pointing, S., 2001. Feasibility of bioremediation by white rot fungi: Applied Microbiology and Biotechnology. 57: 20-33.

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.

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.

Rondon, M.A., Lehmann, J., Ramírez, J. and Hurtado, M., 2007. Biological nitrogen fixation by common beans (Phaseolus vulgaris L.) increases with bio-char additions. Biology and Fertility of Soils. 43 (6): 699-708.

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.

Sander, M., and Pignatello, J. J., 2005. Characterisation of charcoal adsorption sites for aromatic compounds: Insights drawn from single and bi-solute competitive experiments. Environmental Science and Technology 39: 1606-1615.

Stein, J.R., 1973. Handbook of Phycological Methods: Culture Methods and Growth Measurements. University Press, London, UK.

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.

Tuvikene, A., 1995. Responses of fish to polycyclic aromatic hydrocarbons (PAHs**).** Annales Zoologici Fennici. 32: 295-309.

Uchimiya, M., Lima I. M., Klasson K. T., Wartelle L. H., 2010. Contaminant immobilization and nutrient release by biochar soil amendment: Roles of natural organic matter. Chemosphere. 80: 935-940.

U.S. Environmental Protection Agency, 2002. Atmospheric Concentrations of Greenhouse Gases Available: http://cfpub.epa.gov/eroe/index.cfm?fuseaction=detail.viewPDF&ch=46&lShowInd=0&subtop=342&lv=list.listByChapter&r=209837. [accessed February 2010]

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.

Veignie, E. Rafin, C., Woisel, P., Cazier, F., 2004. Preliminary evidence of the role of hydrogen peroxide in the degradation of benzo[a]pyrene by a non-white rotfungus *Fusarium solani*. Environmental Pollution. 129: 1-4.

Verheijen, F.G.A., Jeffery, S., Bastos, A.C., van der Velde, M., and Diafas, I. (2010). 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.

Wang, X., Sato, T., and Xing, B., 2006. Competitive sorption of pyrene on wood chars. Environmental Science and Technology 40: 3267-3272.

Wang, F., Leung, A. O. W., Wu, S. C., Yang, M. S., Wong, M. H., 2009. Chemical and ecotoxicological analyses of sediments and elutriates of contaminated rivers due to e-waste recycling activities using a diverse battery of bioassays. Environmental Pollution 157: 2082–2090.

Wilcke, W., 2000. Polycyclic aromatic hydrocarbons (PAHs) in soil - A review. Journal of Plant Nutrition and Soil Science 163 (3): 229-248.

Winsley P., 2007. Biochar and Bionenergy Production for Climate Change. New Zealand Science Review 64 (1): 1-10.

Wolf, D., 2008. Biochar as a Soil Amendment: A Review of the Environmental Implications. Swansea University, School of the Environment and Society. Unpublished document.

Chapter II

Ecotoxicology of Biochar-bound PAHs in Runoff from Amended Soils

2.1. Introduction

While the interest in biochar application to soils continues to grow, so does the concern regarding the possibility for soil contamination, associated to bound contaminants (Verheijen et al., 2010). The presence and concentration of biochar-bound contaminants, such as polycyclic aromatic hydrocarbons (PAHs), is determined by the feedstock type and pyrolysis conditions used (e.g. temperature; Antal and Gronli, 2003; Demirbas, 2004) but little is still known on the implications of this fraction. It is fundamental to ensure that the quality of soil, surface and ground water are not threatened, as a result of adding biochar to soils (Collison et al., 2009). PAHs have their main source in processes of incomplete combustion of organic matter, including pyrolysis (EEA, 1999). These are ubiquitous compounds, which have received considerable attention in recent decades, for being directly toxic to biota and environmentally recalcitrant (EEA, 1999). Yet, more is known on the PAH fraction that is emitted as volatiles during pyrolysis, than on the PAHs that are retained in the biochar product.

Adsorption of PAHs in charcoals is known to be strong (e.g. Chen and Yuan, 2011), both because of the char's large and reactive surface area, but also because of the planarity of the PAH molecule (Cornelissen et al., 2005). Their desorption rates to water are therefore considered very slow (desorption rate constants of 10^{-7} to 10^{-1} h^{-1}) and evidence suggests that this process could take several decades to occur in soils (Jonker et al., 2005). Although this appears to imply a reduced environmental and ecotoxicological risk for bound PAHs through leaching from biochar-amended soil, through processes such as runoff, very little data exists to confirm it. In fact, those authors (Jonker et al., 2005) did not count with the influence of natural soil processeswet-dry cycles, which might trigger and accelerate the release of PAHs from biochar, increasing their bioavailability. This is important because it means that perhaps aquatic systems and sediments are in danger of becoming important sinks for contaminants that may be transported from treated soils over time, through runoff. In this thesis, 'runoff' is used as a general term for excess water, both that flows along the surface without infiltration (surface flow), and the water that infiltrates and moves through the soil into groundwater (underground runoff).

One natural soil process that might have important consequences for enhancing soil-biochar interactions is wetting/drying events. Wetting and drying are known to enhance microbial activity and soil natural organic matter (NOM) decomposition and nutrient cycling,

including increasing the bioavailability of contaminants that might already be present in soil bount to and within soil aggregates (e.g. van Gestel et al., 1993; Denef et al., 2001a, b; Mikha et al., 2005; Jablonowski et al., 2011). One way in which wet-dry cycles can trigger the release of SOM and leaching of soil contaminants, is by influencing soil physical properties, such as aggregation. Rapid intake of water during wetting results in swelling followed by aggregate disruption, hereby contributing to release aggregate-associated compounds (Dennef et al., 2001; Mikha et al., 2005). One can then hypothesise that when biochar is present in soil, it is likely that drying and rewetting of the biochar-soil mixture, will not only have an important impact on releasing NOM to the soil solution , but also on enhancing the interactions between biochar and all soil components (NOM, clay minerals and biological matter; e.g. Brodowski et al., 2005). In the context of this work, such interactions are overall referred to as 'biochar ageing'. Up to now, the extent and implications of biochar ageing on enhancing leaching, and hence biovailability and potential ecotoxicity of the PAH contaminant fraction has been scarcely studied.

In soils and sediments, the large humic, fulvic (Pignatello et al., 2006) and lipidic (Salloum et al., 2002) molecules, but also some metalic species (e.g. Cu^{2+}; Chen et al., 2007), have been found to alter charcoal's adsorption affinity/capacity (Kwon and Pignatello, 2005) for pyrene (Hockaday, 2006) dichlorobenzene and naphthalene (Chen et al., 2007), by mechanisms of pore blockage (Kwon and Pignatello, 2005; Pignatello et al., 2006) and/or by their capacity to compete (e.g. Cornelissen and Gustafsson, 2005) and remove the organic compound from the sorption sites on the charcoal's surface (Hockaday, 2006). It is possible that interactions like these occurring in biochar-amended soil, over time, can increase desorption of PAHs from the biochar, which then become bioavailable to be transported to water courses through runoff, where it may induce toxicity to edaphic organisms.

In this study, it is assumed that PAH toxicity is related to enhanced PAH bioavailability in the soil solution and consequently an increased possibility for transport to aquatic systems through runoff. In this context, ecotoxicological tests using aqueous soil extracts (or elutriates) are useful for studying their potential effects on aquatic organisms, as demonstrated in previous studies when exposure to environmental contaminants is via water (in this case soil solution) (Hund-Rinke et al., 2002; Loureiro et al., 2005, Lors et al., 2011).

This pilot study aimed at evaluating the potential ecotoxicity of biochar-bound PAH contaminants in runoff from biochar-amended soils. This was based on combining soil wet-dry cycles (that simulate a natural soil process, which can enhance soil-biochar interactions) and

water-extraction of biochar-bound PAHs (to simulate what would naturally occur during processes such as runoff, where water flowing through the soil carries the released PAHs). Analytical methods that are commonly used for quantifying contaminants in soil extracts do not provide on their own any information on their bioavailability and are recommended to be applied together with ecotoxicological tests on soil elutriates (Loureiro et al., 2005). For that reason,a battery of standard aquatic bioassays were used alongside PAH quantification, with test organisms (*Vibrio fisheri, Pseudokirchneriella subcapitata* and *Daphnia magna*) that are representative of different trophic/functional levels, for a more complete ecotoxicological assessment of this biochar contaminant fraction.

2.2. Materials and methodology

2.2.1. Soil and biochar characteristics

LUFA 2.2 was the soil used throughout this study, as it is a natural (agricultural) loamy sand soil, which is widely used in soil ecotoxicology and environmental risk assessment. Relevant background information and preliminary soil treatments were provided in the form of a description sheet from the manufacturer (LUFA Speyer). After collection, (0-20 cm depth), the soil was sieved (<2 mm) and air-dried, in accordance to the corresponding ISO guidelines for soil collection, handling and storage (ISO 10381-6: 1993E). Its main physico-chemical characteristics are summarized as follows: pH (0.01 M, $CaCl_2$): 5.5 ± 0.1; soil organic C (%): 1.93 ± 0.2; cation exchange capacity (meq 100^{-1} g^{-1}): 10.0 ± 0.8; sand (%): 81.3 ± 2.3; silt (%): 12.1 ± 1.3; clay (%): 6.60 ± 1.3; water holding capacity (g $100^{-1}g^{-1}$): 45.2 ± 5.0; density (g ml^{-1}): 1.13 ± 0.045.

The selected biochar is product of fixed-bed gasification of pine wood (particle size range < 50 µm; temperature of approximately 800ºC; residence time of 75 min, Xavier Domenes, personal communication, 2011), collected in a ceramic filter at the bottom of the gasification unit.. C (86.92%), N (0.16%) and S (0.22%) in biochar were estimated by elemental analysis and Dumas inductively coupled plasma-optical emission spectrometry (ICP-OES), respectively. Dry matter (95.80%) was estimated by gravimetry and moisture content (4.20%) was calculated by difference. Biochar's particle size distribution (mm) was performed by serial sieving and

expressed as % weight: > 5.0mm, 0.08%; 5.0 - 2.0mm, 1.06%; 2.0 - 0.5mm, 41.52%; 0.5 - 0.25mm, 11.17%; 0.25 - 0.1mm, 7.40%; 0.1 - 0.05mm, 28.09%; < 0.05mm, 10.72%.

2.2.2. Preliminary procedures

2.2.2.1. Volumetric water content curves

Lufa soil was stored (4ºC, in the dark) until use (ISO 10381-6: 1993E). In order to establish the relationship between volumetric water content (θ) and the volume of added water (ml) θ, water content curves were developed for LUFA 2.2 (alone) and for the mixture soil + biochar (4% w w^{-1}) (Annexe 1) by calculating gravimetric water content (W) and then converting it to θ. W was determined by weight loss of 10 g for each treatment (in triplicate), oven-dried to constant weight at 50ºC for 24 h. The selection of a lower oven temperature for the calculation of W , as opposed to the standard 105ºC, is explained by the fact that 50ºC is the temperature of interest for subsequent steps, namely, for the wet-dry cycles , in order to prevent thermal PAH degradation and/or transformation (Pakpahan et al., 2009). Subsequently, the conversion of W to θ was done using Equation 1:

$$\theta = W \rho s / \rho w \qquad \text{(Equation 1)}$$

where θ is the volumetric water content (ml ml^{-1}), W is the gravimetric soil water content, ρs is the soil bulk density (1.13 g ml^{-1} was used, as it is characteristic of a loamy sand texture) and ρw is the density of pure water (1 g ml^{-1}). It was important to develop an individual curve for each treatment, to ensure reproducibility and comparison of results during the wet-dry cycles, because due to its large surface area, biochar addition to soil increases soil water retention.

2.2.2.2. Initial drying experiment

Before starting the wet-dry cycle, a preliminary slow drying experiment was performed, in order to determine the time required to oven-dry (at 50°C) 100 g of the soil-biochar mixture (4% ww^{-1}) from saturation (four times field capacity) to a θ of ≈ 0.8 ml ml^{-1}, corresponding to the wilting point for most natural soils with a loamy sand texture. With no added water, the θ of LUFA alone was already very near this value and thus it was assumed to be that for simplification. The remaining procedure was done, based on the corresponding volumetric water

content curves and using a bulk soil density of 1.13 g ml^{-1}. The drying time was estimated to be approximately 24 h.

2.2.2.3. Test species and culturing conditions

New-born females of *D. magna* were maintained within 800 ml glass bottles with ASTM (American Society for Testing and Materials) hard water (ASTM, 1980) and a seaweed extract (organic additive prepared from *Ascophilum nodosum*; Baird et al., 1989), at 20ºC±1 and a photoperiod of 16h:8h (light/dark). The daphnids were fed every two days with the microalgae *P. subcapitata* at a rate of $3.0x10^5$ cells ml^{-1} $daphnia^{-1}$. The organisms were transferred to fresh culture medium every two days.

Unialgal cultures of *P. subcapitata* were prepared in 250 ml erlenmeyers containing Hoods Hole MBL medium (Stein, 1973) and kept under stirring (orbital shaker, 150 rpm) at 20ºC±1 and a photoperiod of 16h:8h (light/dark). The organisms were transferred to fresh culture medium weekly.

The marine bacteria *V. fischeri* was used in the form of freeze-dried reagent, following reconstitution according to the standard MICROTOX® protocols (Microbics Inc. Protocols).

2.2.3. Soil wet-dry cycles

Considering the objective of this study, consecutive drying and fast-rewetting of the soil-biochar mixture, was expected enhance soil-biochar interactions. Drying and wetting events are referred to, throughout this study, as wet-dry cycles and the method was adapted from Jablonowski et al. (2011). For each sampling time, 0 (ST-0), 1 (ST-1) and 2 (ST-2), a series of wet-dry cycles of 0, 6 and 12 (respectively) were performed. The reference to the various treatments is shown in Table 1.

Each cycle started with fast rewetting of the soil treatments. De-ionised water (100 ml) was added to 100 g (oven-dry basis) of soil alone and containing biochar (4% ww^{-1}) within 250 ml glass-erlenmeyers, to raise (in excess) the volumetric water content of the mixture, from the initial 0.08 (ml ml^{-1}) to over-saturation (>0.42 ml ml^{-1}), based on the characteristic water content curves previously developed. The choice of adding water in excess, was to ensure enough volume of extract for the ecotoxicological assays, yet this water availability interval is representative to

natural water content range after heavy rainfall. Three replicates of each treatment were prepared for three different treatments: LUFA 2.2 soil (SS) used as the control, LUFA soil with biochar (BS) and biochar alone (B). Following quick wetting and stirring to homogenise, soil treatments were stored at 4ºC for 24 h to equilibrate, allowing the water to be in equilibrium throughout the soil volume.

Table 1. The reference to the various sampling times. Each sampling is conducted after corresponding number of cycles.

Sampling time	Number of wet-dry cycles
sampling time 0 (ST-0)	0 cycles *
sampling time 1 (ST-1)	6 cycles
sampling time 2 (ST-2)	12 cycles

*0 cycles means that the initial sampling (ST-0) is conducted after only wetting, equilibration, agitation and resting steps of the standards soil sample (SS), biochar enriched standard soil sample (BS) and biochar alone sample (B); the step which is missing in the case of ST-0 is oven drying at 50ºC.

2.2.4. Preparation of soil extracts

Water-extraction of biochar-bound PAHs was used to assess their potential to become water-extractable and thus be easily transported into water systems during processes such as runoff. The method used was adapted from Jablonowski et al. (2011). Following rewetting and equilibration, slow agitation of the samples was performed using a bench top orbital shaker (150 rpm, 24 h) at constant temperature (≈18-20ºC), under artificial lighting. During agitation, which intended to increase surface contact between the soil-biochar particles and the water, the erlenmeyers were wrapped in foil to avoid photodegradation or photomodification of PAHs (e.g. Fasnacht and Blough, 2002). Samples were then allowed to settle overnight at 4ºC, in the dark. Decantation to collect the supernatant was performed through suction, while the biochar-soil residue was weighted (wet weight) before being put in the oven to dry for the next cycle, according to the treatments. Centrifugation of the supernatant (4000 rpm, 15 min), was done to clear out organic matter in suspension. For the removal of the fine biochar particles still in suspension, vacuum filtration was performed using the Buckner devise, with glass microfiber

filters (Whatmann GFC Ø 47 mm, 1.2 µm porosity). The filtrate was then stored at 4ºC in glass containers (while not in use). The ecotoxicological assessment of extracts was always performed with fresh extract, with no more than a storage time of 1 week at 4ºC.

Extracts were prepared for the study treatments: soil with biochar (BS) and LUFA 2.2 soil (SS) used as the control. Besides the study treatments, extracts of biochar-alone (B) were also prepared for assessing the individual contribution of biochar for the total PAH content in soil-biochar extracts, although no ecotoxicological tests will be performed with biochar-alone extracts. Extracts of the three treatments were characterised in an independent laboratory in relation to PAH and metal content. PAH analysis was restricted to the sixteen PAHs that the United States Environmental Protection Agency (USEPA) identified as priority contaminants. They were the following: naphthalene (NAP), acenaphthylene (ACY), acenaphthene (ACE), fluorene (FLU), phenanthrene (PHE), anthracene (ANT), fluoranthene (FLT), pyrene (PYR), benz(a)anthracene (BaA), chrysene (CHR), benzo(a)pyrene (BaP), benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), indeno(1,2,3-cd)pyrene (IND), dibenz(a,h)anthracene (DBA) and benzo(g,h,i)perylene (BGP). The PAH concentrations of the several extracts were analyzed using solid phase micro-extraction (SPME) in a 100 µm poly-dimethysiloxane (PDMS) that was used as the absorbing material. After the extraction, the fibre was desorbed in a gas chromatograph (GC) (Varian CP-3800), with a split/splitless injector. The GC was coupled to a mass spectrometer Ion Trap Saturn 2200 (GC-MS) for the identification of the PAHs. The analytical procedure was validated by doping the sample with standards of the 16 PAHs. The recovery rates of the individual PAHs ranged from 80% to 117%. The detection limits (DL) were between 0.75 and 1.94 ng l^{-1}. The result for each sample corresponds to the average of two independent replicates.

Similarly, SS and BS extracts were analysed for their metal content, in respect to cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), lead (Pb), arsenic (As) and mercury (Hg) by inductively coupled plasma-atomic emission spectroscopy (ICP-AES)(Annexe 2). The metal analysis was performed exclusively for ensuring that any observed toxicity was not caused by metals that are often also present in biochar, alongside PAHs. The pH of the extracts was measured at all sampling times for the study treatments, using a bench-top pH metre.

2.2.5. Ecotoxicological assays

2.2.5.1. *Daphnia sp. acute immobilization assay*

The acute immobilization test with the cladoceran *D. magna* followed the OECD's standard methodology (OECD, 2004). The test used 5 neonates from clone K6 (third- to fifth-brood, <24h; (Baird and Barata, 1998)) per treatment (including negative controls). For each sampling time (ST-0, ST-1, ST-2) tests were conducted for standard soil extracts (SS) and for biochar-soil extracts (BS). Extracts of the biochar-soil treatment were used as test media in the experiments, while extracts of standard LUFA 2.2 soil were used as test media for (positive) control tests. 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 in order to obtain 6.25, 12.5, 25% and 50%, while 100% represents pure extracts without addition of the medium. Four replicates per each test concentration were applied as well as for the controls (containing ASTM only). Following an exposure time of 48h (during which the organisms were not fed), at 20±1ºC and a photoperiod of 16h:8h (light/dark), the number of immobilised/dead organisms was recorded. Physico-chemical 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 in any of them prior to the test.

2.2.5.2. P. subcapitata growth inhibition assay

The test followed the corresponding OECD guideline (OECD, 2006), with adaptations by Geis et al. (2000), who used 24 multi-well plates, instead of 100 ml erlenmeyers. The algae were exposed to a series of extract test solutions 6.25, 12.5, 25, 50, 100% (the last one represents pure sample/extract) for 72h at 20±1°C and a photoperiod of 16h:8h (light/dark), re-suspending the test wells with the algae cells twice a day. No pH adjustments of the samples were made. The test concentration of 100% extract was supplied with proportional amount of nutrients, in order to ensure unrestricted growth (OECD, 2006). The negative control sample contained only MBL medium and the organisms. Three replicates were used per each concentration, as well as six negative controls, as recommended in the OECD guideline (2006). For each sampling time (ST-0, ST-1, ST-2) separate tests were conducted for standard soil extracts (SS) and for biochar-soil extracts. Extracts of the biochar-soil treatment were used as test media in the experiments, while

extracts of standard LUFA 2.2 soil were used as test media for (positive) control tests Algae's growth rate (expressed per day) was determined through microscopic (Olympus CKX41) cell count in a Neubauer chamber.

2.2.5.3. V. fischeri luminescence inhibition test

MICROTOX® test was used to assess inhibition of bioluminescence in the marine bacterium *V. fischeri*. Two different approaches were taken: a Basic Test (BT) and a Basic Solid Phase Test (BSPT), in which the bacteria were exposed to the treatment extracts or the corresponding solid matrices, respectively. For the BT, different extract dilutions of the biochar-soil mixture and the control (LUFA soil) were pipette 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 15 min after transferring of the bacteria into the extract vials, the toxicity response was evaluated and a 50% reduction of luminescence was calculated using Microtox Data Collection and Reduction software (Microbics). Results were reported as % of extracts. For the BSPT, 7 g of the biochar-soil mixture and the control (LUFA soil) were suspended in 35 ml of the solid phase test diluent solution (Azur Environmental, Carlsbad, CA, US) and stirred on a magnetic stirrer for 10 minutes. A series of dilutions and the blank (100% diluent) were prepared into glass cuvettes. The bacterium was added (10 µl and following 30 min of incubation, reduction of luminescence was measured using Microbics (model 500) toxicity analyser. Results were reported as concentration of dry soil-biochar or control soil.

2.2.6. Statistical analysis

For *D. magna* immobilisation, EC_{50} values (extract concentration for which a 50% mobility reduction was observed) and corresponding 95% confidence interval (CI) were estimated using Probit regression analysis. The statistical software used was IBM SPSS.19.

For the MICROTOX® test, EC_{50} (corresponding to a 50% luminescence reduction in *V. fischeri*) was obtained through the Microtox Data Collection and Reduction software (Microbics). When appropriate, sample toxicity data were converted to Toxic Units (TU) for ease of

interpretation and comparison between tests, using the inverse of the X (where X = LC_{50} or EC_{50}) expressed as %: TU = [1/X] x 100 (Loureiro et al., 2005).

For *P. subcapitata* growth tests, and considering that growth is a continuous parameter, EC_{50}, EC_{20} and EC_{10} together with CI were calculated by nonlinear regression using the STATISTICA software. LOEC and NOEC were calculated using the IBM SPSS.19 software by one-way ANOVA followed by the Dunnett multiple comparisons in order to test a significant difference between the control and the test dilutions (Zar, 1999).

2.2.7. Flowchart of the experimental steps

The flowchart in Figure 2 show the various experimental steps involved in this study.

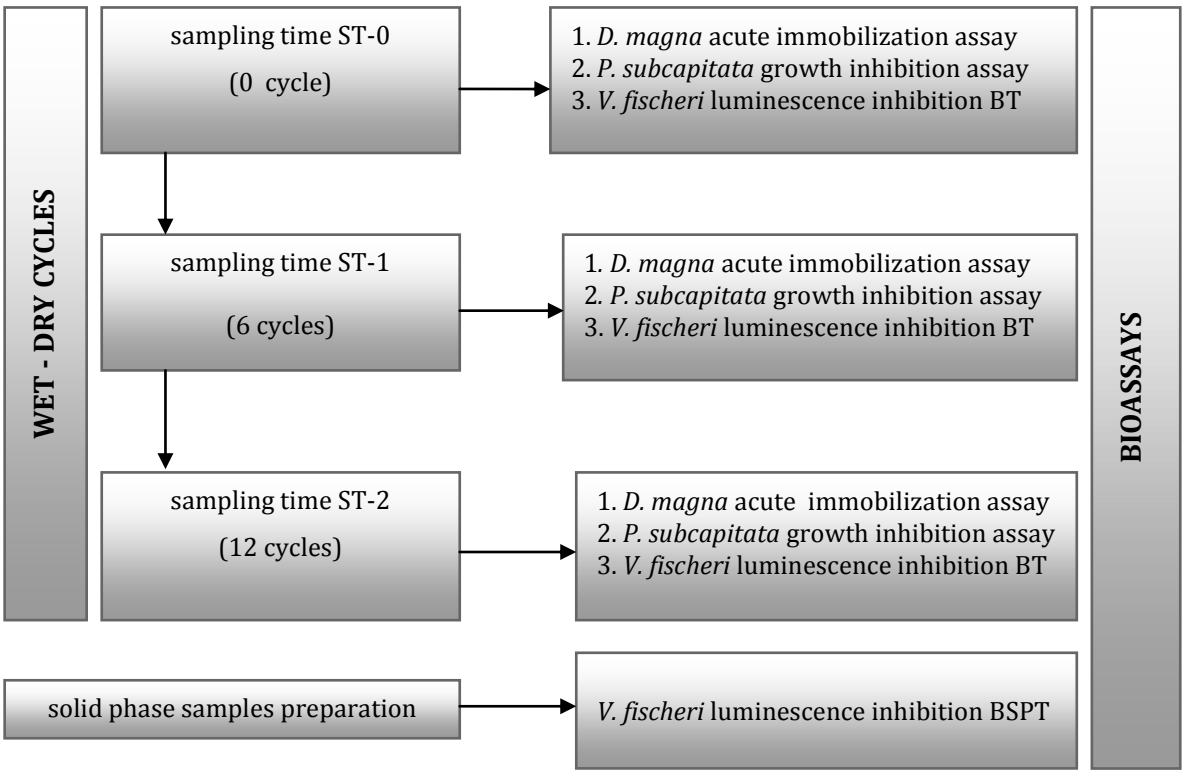


Figure 2. Flowchart of the experimental steps involved in the project. BT refers to MICROTOX® 81.9% Basic Test and BSPT to MICROTOX® Basic Solid Phase Test.

2.3. Results

2.3.1. Chemical analyses

The content of selected PAHs (in respect to individual and total concentrations) in the treatment extracts of standard soil (SS), biochar-soil mixture (BS), as well as on the additional biochar (B) alone treatment, are shown in Table 2. When compared to SS and B extracts, extracts of BS generally had higher total PAH contents (ΣPAHs), independently of the number of soil wet - dry cycles. While the ΣPAHs determined in SS decreased slightly with the increasing number of cycles (i.e. from 72.10 ng l^{-1} in ST-0 to 68.31 ng l^{-1} in ST-2), the opposite trend was observed for ΣPAHs in BS (ranging from 106.42 ng l^{-1} in ST-0 to 112.05 ng l^{-1} in ST-2). Nevertheless, in both later treatments, differences between ΣPAHs as influenced by the number of cycles were often not significant. In contrast, the ΣPAHs in biochar extract samples (B), showed higher fluctuations in relation to number of cycles.

The composition pattern of PAHs by ring size revealed that 2 and 3 ring PAHs, corresponding to lower molecular weights, were clearly dominant (57-86%) independently of the treatment, such as for NAP (10-56%) and PHE (14-25%). In contrast, high molecular weight PAHs (5 or 6 rings) had generally lower contributions to the total amount (5-25%) (Table 2). The metal content for the same extracts is, as previously mentioned, in Annex 2.

Table 2. Results of the chemical analysis of the extracts on the occurrence of certain PAHs.

PAHs (ng l^{-1})	Rings	Standard soil extract (SS)			Biochar-soil extract (BS)			Biochar extract (B)		
		ST-0 (0cycle)	ST-1 (6cycles)	ST-2 (12cycles)	ST-0 (0cycle)	ST-1 (6cycles)	ST-2 (12cycles)	ST-0 (0cycle)	ST-1 (6cycles)	ST-2 (12cycles)
NAP	2	27.46±3.59	32.49±4.04	35.80±5.28	19.64±3.1	43.13±4.06	37.25±6.51	9.43±1.02	41.61±3	29.71±1.11
ACY	3	4.13±0.3	<LD	<LD	6.43±0.14	10.63±0.5	8.14±0.84	10.16±1.02	7.33±0.3	5.88±0.04
ACE	3	5.48±0.1	<LD	5.49±0.53	3.87±0.5	<LD	<LD	3.82±0.44	5.11±0.44	<LD
FLU	3	<LD	0.99±0.03	<LD	1.18±0.09	<LD	<LD	1.95±0.01	1.17±0.02	<LD
PHE	3	10.99±0.23	14.73±0.83	11.27±1.45	23.3±2.32	21.76±2.47	23.21±3.67	23.33±1.76	16.89±1.58	13.71±2.03
ANT	3	5.67±0.42	2.58±0.11	2.4±0.19	6.7±0.4	6.51±0.49	7.07±1.07	6.35±0.25	4.96±0.17	3.84±0.45
FLT	4	6.58±0.94	6.63±0.18	3.58±0.02	8.91±0.72	7.35±0.58	10.01±1.52	6.71±1.09	7.47±0.54	6.15±0.8
PYR	4	1.97±0.04	3.36±0.03	2.03±0.33	7.49±1.49	6.02±0.45	10.08±1.0	8.87±1.15	6.66±0.59	5.40±0.19
CHR	4	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
BaA	4	<LD	<LD	<LD	3.61±0.33	<LD	2.32±0.33	<LD	<LD	<LD
BbF	5	<LD	<LD	1.16±0.16	<LD	<LD	<LD	4.98±0.7	<LD	<LD
BkF	5	4.84±0.61	4.4±0.51	2.08±0.15	9.1±0.3	4.95±1.1	3.95±0.14	7.25±1.12	8.1±0.35	5.22±5.01
BaP	5	<LD	<LD	<LD	5.29±0.87	1.57±0.31	2.62	<LD	5.53±0.98	<LD
DBA	5	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
BGP	6	2.33±0.41	3.19±1.17	<LD	6.18±0.88	4.21±0.86	3.99±0.96	7.25±0.74	4.98±1.01	5.27±0.76
IND	6	2.65±0.13	3±0.09	<LD	4.73±0.88	5.57±0.64	3±0.47	3.4±0.07	<LD	<LD
∑PAHs		**72.1**	**71.36**	**63.81**	**106.42**	**111.69**	**112.05**	**93.49**	**109.83**	**75.18**

Compound abbreviations: naphthalene (NAP), acenaphthylene (ACY), acenaphthene (ACE), fluorene (FLU), phenanthrene (PHE), anthracene (ANT), fluoranthene (FLT), pyrene (PYR), chrysene (CHR), benz(a)anthracene (BaA), benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), benzo(a)pyrene (BaP), dibenz(a,h)anthracene (DBA) and benzo(g,h,i)perylene (BGP), indeno(1,2,3-cd)pyrene (IND). Standard deviations are shown as ± SD. LD stands for limit of detection.

In the current study, pH of the extracts of the study treatments was measured after each sampling time and the values are listed in Table 3.

Table 3. pH values of the extracts.

Sample	Sampling time (number of cycles)	pH
SS	ST- 0 (0 cycle)	5.32
	ST-1 (6 cycles)	6.98
	ST-2 (12 cycles)	7.48
BS	ST-0 (0 cycle)	7.63
	ST- 1 (6 cycles)	7.68
	ST- 2 (12 cycles)	7.5

2.3.2. Toxicity tests

All ecotoxicological tests fulfilled the validity requirements established in their corresponding guidelines.

2.3.2.1. Daphnia sp. acute immobilisation assay

D. magna acute test has shown that the extracts obtained from standard soil (SS), which were used as control, were not toxic to *D. magna* (Figure 3 and Table 4). Although 20% of immobilization was recorded in the 100% concentration of the ST-1 extract, the response observed was also generally independent of the number of wet -dry cycles.

In contrast, toxicity from exposure to biochar-soil extracts (BS) has shown to be dependent on the number of cycles (Table 4). The biochar-soil extract corresponding to ST-0, showed to be highly toxic to *D. magna,* with an EC_{50} of 2.95% (CI: 0.03-7.43%), and further decreasing for increasing number of wet-dry cycles. For ST-1, biochar-soil extract induced toxic effects, which were observed only at the highest concentrations, being of approximately 40% at maximum concentration (100%). Finally, the extract obtained from ST-2 had no observed deleterious effects on *D. magna*.

Table 4. EC values (% dilution) and respective 95%-confidence intervals calculated for *D. magna* exposed to the standard soil extract (SS) and to the biochar-soil extract (BS) at each sampling time (ST-0, ST-1, ST-2). ND stands for not determined due to the low toxicity.

Sample	Effect concentration	ST-0 (0 cycle)	ST-1 (6 cycles)	ST-2 (12 cycles)
SS	EC_{50}	ND	ND	ND
	EC_{20}	ND	ND	ND
	EC_{10}	ND	ND	ND
BS	EC_{50}	2.95 (0.03-7.43)	ND	ND
	EC_{20}	0.45 (0-2.28)	ND	ND
	EC_{10}	0.17 (0-1.26)	ND	ND

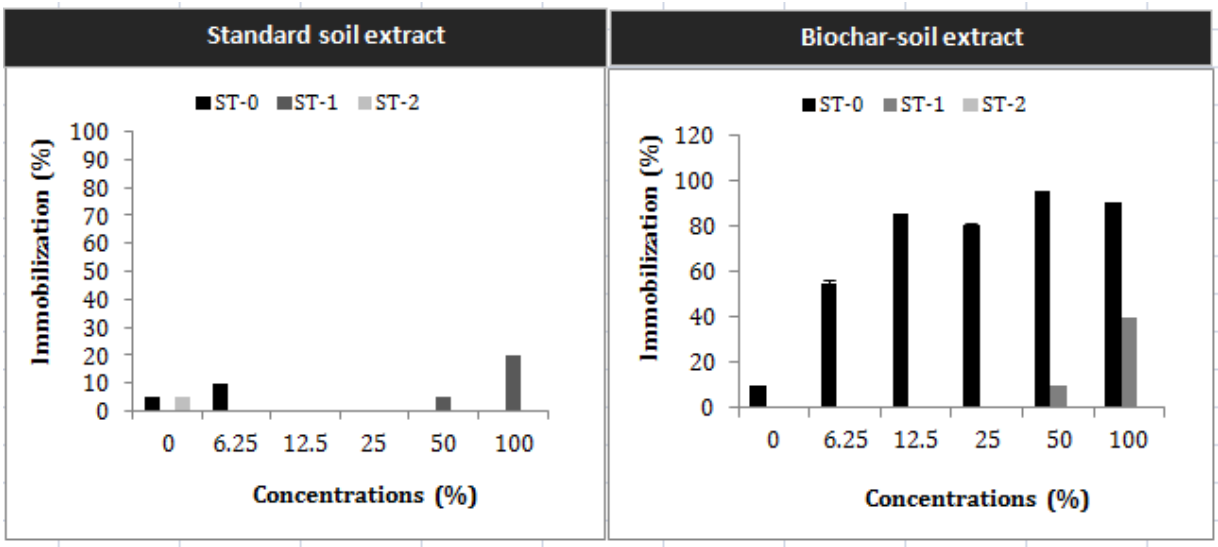


Figure 3. Percentage of immobilization of *D.magna* when exposed to a range of dilutions of the extracts SS (standard soil) and BS (biochar-soil) for each sampling time (ST-0: 0 cycle, ST-1: 6 cycles, ST-2: 12 cycles). Error bars above the charts indicate standard deviations.

2.3.2.2 P. subcapitata growth inhibition assay

Regarding the extracts obtained from the standard soil (SS), different responses were observed depending on the number of wet-dry cycles applied to the samples (Figure 4 and Table 5). An analogous dose-response curve was obtained for ST-0 and ST-2, which was characterized by low inhibitory effects on the microalgae growth. Significant toxic effects were only observed in the maximum concentration, allowing the estimation of the LOEC and the NOEC, which corresponds to 100% and 50%, respectively (Table 5 and 6). Compared with ST-0 and ST-2, extracts from the ST-1 were markedly more toxic to the microalgae, which is pointed out by the LOEC and NOEC values (25 and 12.5%, respectively). Even low, stimulatory effects on *P. subcapitata* growth rate were recorded for low concentrations (6.25 and 12.5%) of extracts of ST-1.

In the case of soil-biochar extract (BS), and as already reported, the toxicity was dependent on the number of wet-dry cycles (Tables 5 and 6 and Figure 4). ST-0 extracts induced , in general, very low toxicity to *P. subcapitata.* However, though mild toxicity, significant deleterious effects ($p<0.05$) where found on *P. subcapitata* growth rate (GR) when exposed to the maximum concentration (100%), which corresponds to a LOEC of 100%, and a NOEC of 50% (Table 5). By comparing with ST-0, *P. subcapitata* was more sensitive to ST-1 and ST-2 extracts, showing an EC_{50} of 85.23% (C.I.: 72.23-98.23) and 90.74% (85.89-95.87), respectively (Table 5). Regarding the dose-response curve obtained for ST-1, it is clear a similar trend to the ST-1 from the SS, with a slight stimulus in the lowest experimental concentration (6.25%). In terms of EC_{20} and EC_{10}, the low values obtained for ST-1 [30.03% (22.15-37.91%) and 16.30% (9.86-22.74%)], respectively, pointed out its higher toxicity compared to ST-2 for intermediate concentrations. Although there was a clear difference between the three extracts obtained (ST-0, ST-1 and ST-2) for BS, the LOEC and NOEC values determined were the same in all. Since the estimation of this toxicological values are depend on the concentration range tested, they should be carefully considered. Although the highest toxicity associated to the biochar-soil extract (BS) compared with the standard soil extract (SS) it is noticeable, in a general way, a similar dose-response curve when comparing each pair of sampling times between SS and BS.

Table 5. Toxicity parameters [effect concentrations EC_{50}, EC_{20} and EC_{10} values (%) with 95% confidence intervals (in brackets), lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC)] for *P. subcapitata* exposed to each sampling time (ST-0, ST-1, ST-2) of standard soil extracts(SS) and biochar-soil extracts(BS). ND stands for not determined due to the low toxicity.

Sample	Toxicity parameters	ST-0 (0 cycle)	ST-1 (6 cycles)	ST-2 (12 cycles)
SS	EC_{50}	ND	ND	ND
	EC_{20}	ND	47.75 (38.08-57.42)	ND
	EC_{10}	ND	30.47 (20.83-40.12)	ND
	LOEC	100	25	100
	NOEC	50	12.5	50
BS	EC_{50}	ND	85.23 (72.23-98.23)	90.47 (85.89-95.87)
	EC_{20}	ND	30.03 (22.15-37.91)	54.25 (48.66-59.85)
	EC_{10}	ND	16.3 (9.86-22.47)	40.15 (34.09-42.6)
	LOEC	100	100	100
	NOEC	50	50	50

ND-not determined

Table 6. One-way ANOVA outcome summary for the growth rate (day^{-1}) of *P. subcapitata*.

Sample	statistical output	ST-0 (0 cycle)	ST-1 (6 cycles)	ST-2 (12 cycles)
SS	df	5	5	5
	MS	0.026	0.436	0.0461
	F	10.655	96.37	53.541
	P	<0.001	<0.001	<0.001
BS	df	5		
	MS	0.0207		
	F	18.432		
	H		19.147	16.688
	P	<0.001	0.002	0.005

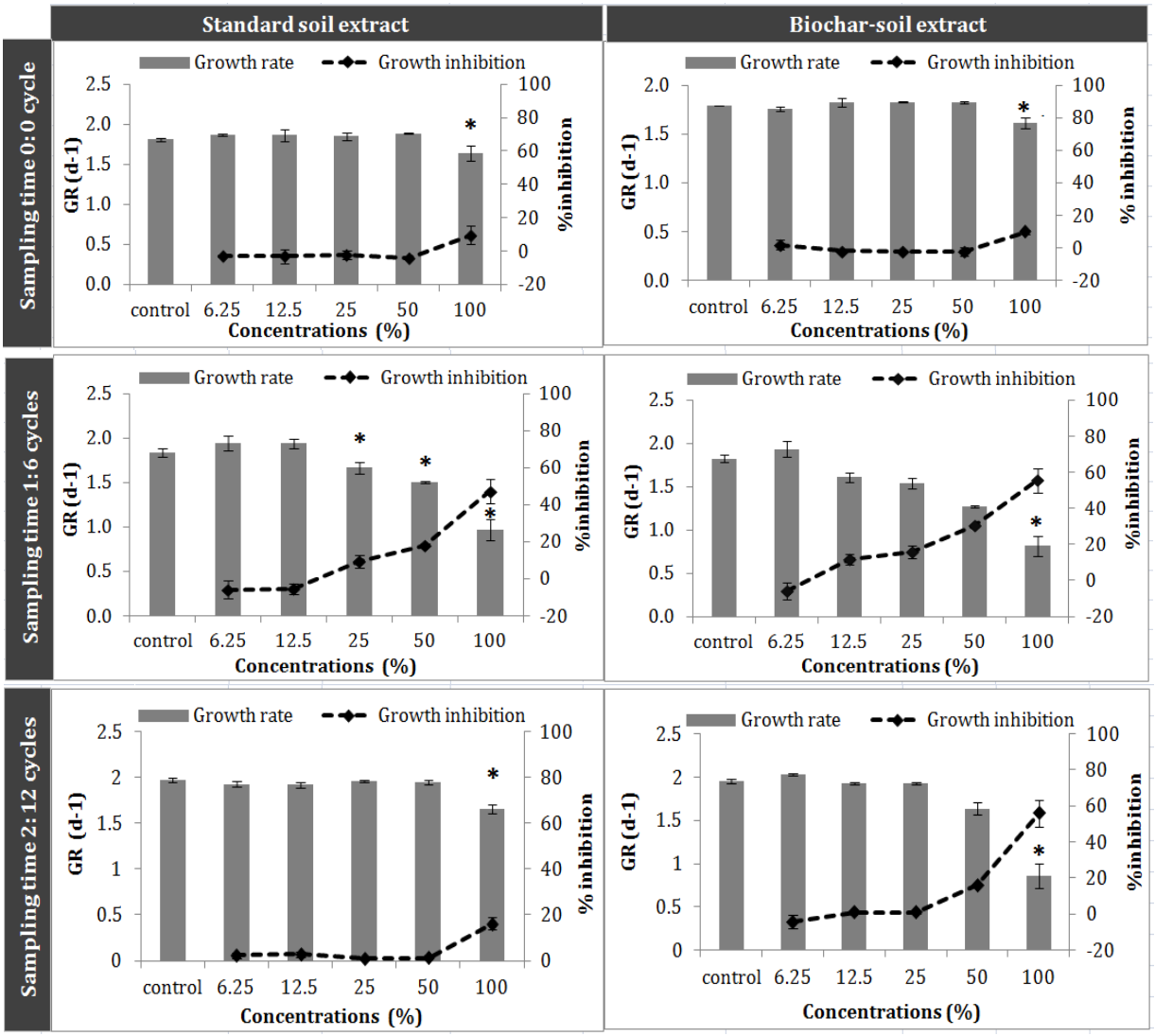


Figure 4. Growth rate (expressed per day) and growth inhibition (expressed in percentage) of *P. subcapitata* when exposed to each sampling time (ST-0, ST-1, ST-2) of standard soil extracts (SS) and biochar-soil extracts (BS). Error bars represent standard deviations. Asterisk refers to a significant difference from the control ($p<0.05$).

2.3.2.3. V. fischeri luminescence inhibition test

In this test, two approaches were applied. Both MICROTOX® 81.9% Basic Test (BT) and MICROTOX® Basic Solid Phase Test (BSPT) were conducted. Firstly, MICROTOX® 81.9% BTs were conducted in order to observe the toxicity of the extracts towards the bacteria. The EC

estimated values are presented in Tables 7 and 8. In each extract, for the three sampling times, the effect on bacterial luminescence was slightly higher after 5 minutes than after 15 minutes of exposure (Figure 5).

Table 7. MICROTOX® 81.9% Basic Test. EC_{50} (%) values of bacterium *V.fischeri* exposed to each sampling time for standard soil (SS) and biochar-soil (BS) extracts. The values in brackets refer to the 95% confidence limits.

Sample		ST-0 (0 cycle)	ST-1 (6 cycles)	ST-2 (12 cycles)
SS	EC_{50}(5 min)	ND	25.25 (22.5-28.4)	ND*
	EC_{50}(15 min)	ND	39.59 (26.41-67.5)	ND*
BS	EC_{50}(5 min)	ND	10.59 (8.08-14.26)	42.42 (17.47-103)
	EC_{50}(15 min)	ND	14.18 (9.94-20.58)	47.46

ND-not determined
*hormesis detected

It is notorious that the toxicity was dependent on the number of wet-dry cycles. Regarding the control (SS), low toxicity was observed and a marked fluctuation characterized the dose response curve obtained in the samples obtained after 0 cycle (ST-0). Comparatively, ST-1 was highly toxic to *V. fischeri* [EC_{50}= 25.25% (22.5-28.4%) for 5 minutes and 39.59% (26.41-67.5%) for 15 minutes]. Although toxic to *V. fischeri*, the ST-2 showed a lower toxicity response compared with the former one [EC_{50}= 42.42% (17.47-103%) for 5 minutes and 47.46% (-) for 15 minutes]. Stimulatory effects where observed in general for all tests in the lowest dilutions tested.

By comparing the extract obtained from the biochar-soil mixture (BS) with the control (SS), a higher toxicity was detected in BS for the several sampling times. Likewise SS, BS showed a similar trend of toxicity along the sampling times, with the highest inhibitory effect in the ST-1 [EC_{50}= 10.59% (8.08-14.26) for 5 minutes and 14.18 (9.94-20.58) for 15 minutes].

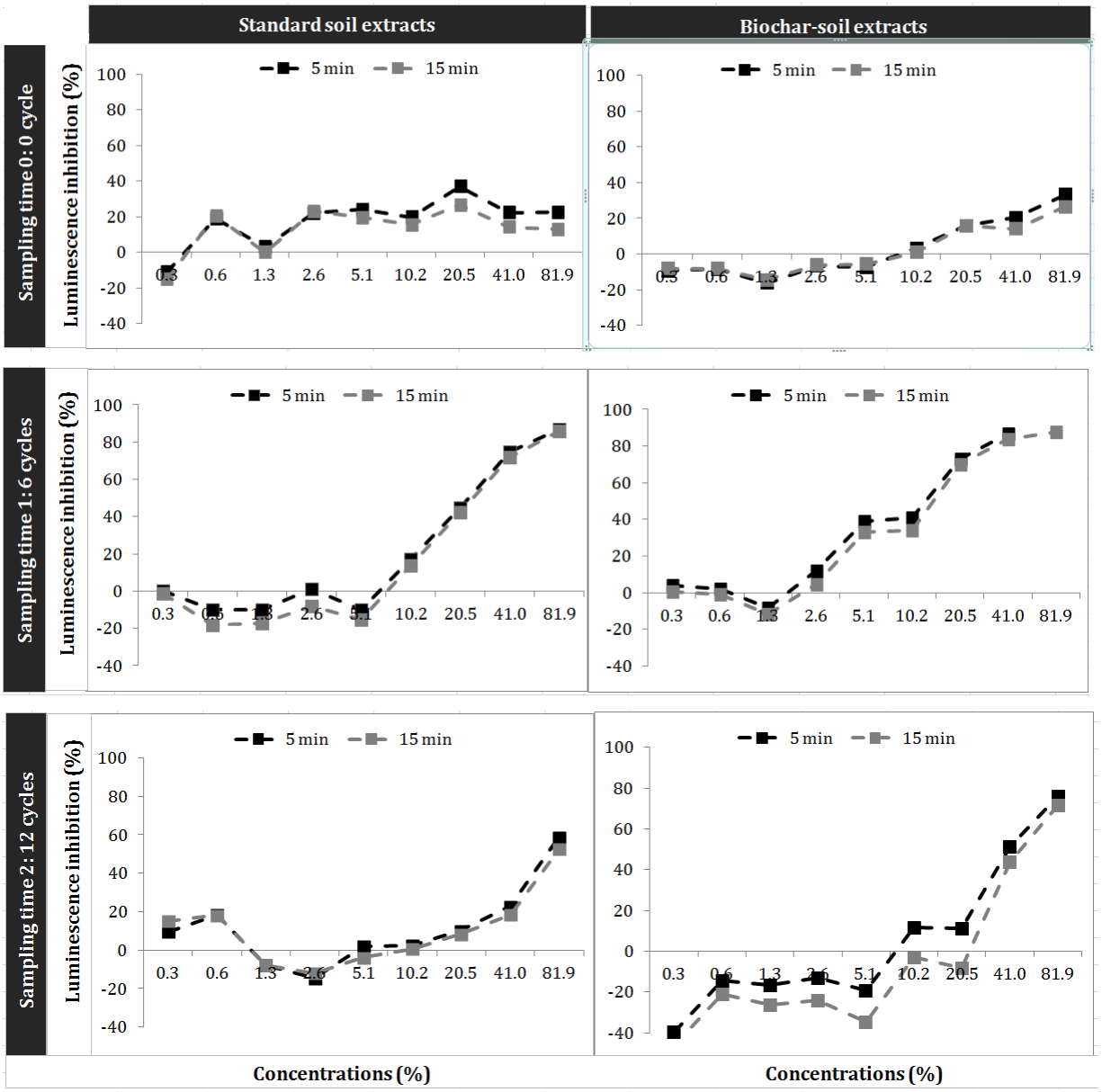


Figure 5. MICROTOX® 81,9% Basic Test (BT). Luminescence inhibition of *V. fischeri* (expressed in percentage) exposed to the standard soil extracts-SS and standard soil enriched with biochar extracts - BS. Each graph represents the values measured 5 minutes and 15 minutes after the exposure for each sampling time (ST-0, ST-1, ST-2).

The dose response curves obtained from the Basic Solid Phase Test (BSPT) for the two samples are presented in Figure 6. In MICROTOX® BSPT, both samples had toxic effects on bacteria with higher EC50 of standard soil solid sample (SS) [EC_{50}=50450 mg l^{-1}(26830-94880) for standard soil sample (SS), and EC_{50}=65960 mg l^{-1}(33520-125900) for biochar amended standard soil (BS)]. SS was more toxic than BS in medium test concentrations while less toxic at lower test concentrations. As it can be seen on the figure, at the highest concentrations one can notice very similar response of bacteria between SS and BS samples.

Table 8. MICROTOX® Basic Solid Phase Test (BSPT). EC_{50} (mg l^{-1})values of bacterium *V. fischeri* exposed to two solid samples (standard soil SS and standard soil amended with biochar BS). The values in brackets refer to the 95% confidence limits.

MICROTOX® BSPT	EC_{50}
standard soil	50450 (26830-94880)
standard soil enriched with biochar	65960 (33520-125900)

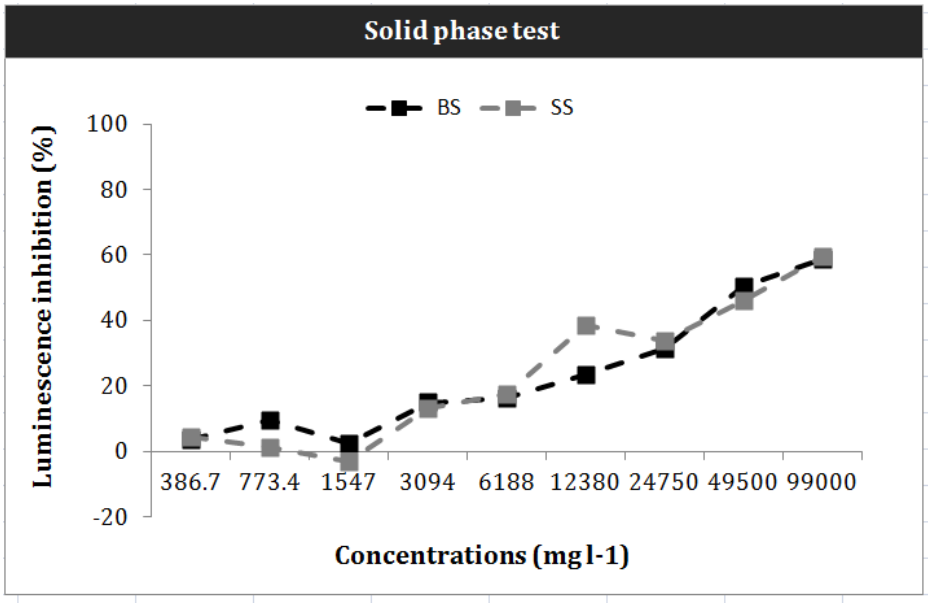


Figure 6. MICROTOX® Basic Solid Phase Test (BSPT). Luminescence inhibition (expressed in percentage) of *V. fischeri* exposed to two solid samples: standard soil (SS) and standard soil

amended with biochar (BS). Graph represents the values measured 30 minutes after the exposure for the two solid samples.

2.4. Discussion

The main aim of this pilot study was to evaluate the potential ecotoxicological implications of biochar-bound PAH contaminants in runoff from soils containing biochar, having been identified as a gap in current knowledge. By combining soil wetting-drying cycles with PAH water-extraction, a good first approach was obtained for evaluating their potential occurrence in the soil solution, while taking into consideration two aspects of environmental relevance, such as natural soil processes and soil-biochar interactions. A battery of standard aquatic bioassays were used (*Vibrio fisheri, Pseudokirchneriella supcapitata and Daphnia magn*a), in order to provide a global picture of the potential ecotoxicity of soil-biochar extracts in relation to PAHs, at various trophic/functional levels.

The approach of combining wet-dry cycles with water-extraction was based on a study by Jablonowski et al. (2011), which has shown that repeated soil drying and rewetting coupled to water-extraction was effective in enhancing aqueous extraction of a range of aged hydrophobic pesticides from soil (e.g. ethidimuron, methabenthiazuron, anilazine and atrazine). Although using a milder water-extraction (weaker agitation at 150 rpm for 24h, as opposed to agitation followed by centrifugation at 4000 rpm in the later study) and considering that charcoal is a stronger adsorbing matrix to hydrophobic compounds than soil (Gustafsson et al., 1997; Chiou and Kile, 1998; Jonker et al., 2005), the chemical analysis of the treatment extracts in the present study has shown that relevant amounts of priority PAHs according to UEPA, were available in the aqueous fraction of standard soil (SS), biochar-soil mixture (BS) and biochar alone (B). This demonstrates that it is possible for these PAH contaminants to become available in pore water and thus be transported through runoff into aquatic systems. It could be hypothesised that the agitation step used within each cycle, and which intended to increase surface contact between soil-biochar particles and the water, might have contributed to enhance PAH water-extraction and may not be representative to what would happen in a biochar-enriched field during runoff. However, it could be argued that a mild agitation can be an approximation to the physical forces of the raindrops falling in soil surface. Interestingly, total PAH concentration in LUFA 2.2 soil and biochar-soil elutriates was only lightly influenced by the number of wet-dry cycles that the soil

was subject to, since differences between cycles were often not significant or did not follow any particular pattern. This might suggest that the natural process of soil drying and rewetting may not be enough on its own to enhance desorption of PAHs from biochar increasing their bioavailability in soil solution in amended soil, at least on the short term. However, this needs to be interpreted carefully, particularly because of the short study duration, which is not representative for natural soil processes. Also, the method used for preparation of the extracts (mostly the drying step at 50ºC) could have induced loss and/or transformation of the more labile PAH compounds. In the context of this work, there are two ways by which soil-biochar interactions over time could result in increased PAHs bioavailability in runoff from soils to which biochar was applied. The other way, is indirectly, as changes in biochar's physical properties as a result of those interactions, including breakdown of large biochar particles into smaller ones over a longer-time period, which could increase PAH bioavailability and even transport across larger distances. Once released into soil, evidence exists that PAHs can be transported through and from soils into ground and surface water systems, together with dissolved organic matter or black carbon particles (Wilcke, 2000).In comparison to the amount of PAHs that were extracted from biochar-amended soil (BS), that extracted from biochar-alone (B) was generally significantly lower, but also seemed to be more strongly influenced by wetting and drying the biochar residue. This suggests that it is easier to water-extract PAHs from biochar-amended soil than from biochar-alone after being treated similarly. Also, total concentrations of PAHs in biochar-soil elutriates did not equal the sum between PAHs the control soil and PAHs in biochar-alone extracts. For example, for ST-0, total PAH concentrations were 72.1 ng l-1, 93.49 ng l-1, 106.42 ng l-1 for elutriates of LUFA, biochar-alone, biochar-soil, respectively), while after the wet-dry treatments the resulted total content of PAHs in BS extracts was in average approximately 60% higher than in SS, and 20% higher than in B extracts. This distribution pattern of total PAHs might be evidence that soil-biochar interactions over time may result in increased PAHs bioavailability in runoff from soils to which biochar has been applied. It suggests that interactions between biochar and soil components may after all explain an increased desorption of PAHs from biochar when this is added to soil. This would corroborate other studies in the literature (Kwon and Pignatello, 2005; Hockaday, 2006), which have shown increased desorption of hydrophobic compounds from charcoals due to interactions between biochar and soil components, such as competition or pore blockage by NOM's humic, fulvic and lipidic fractions.

Total PAH contents of standard LUFA 2.2 soil were higher than expected for priority PAHs. The PAH content of standard soil LUFA 2.2 found in the literature is 0.2 μg g^{-1} (Frische, 2003). It was also observed that low molecular weight PAHs (2-3 rings), particularly NAP and PHE, were clearly dominant in all extract samples, in contrast with the low contribution of high molecular weight PAHs (5-6 rings). It is well known that physical and chemical characteristics of PAHs vary with molecular weight. For instance, PAH vaporization and solubility decreases with increasing molecular weight, whereas the mobility and potential for bioaccumulation of these compounds increases (Hoffman et al, 2002). As a result, PAHs differ in their fate and distribution in the environment, as well as on their effects on biota (Tuvikene, 1995). For example, in water, the toxicity of individual PAHs to both plants and animals increases as molecular weight increases up to 4 rings (FLT, PYR). For PAHs with more than 4 rings, a rapid decline in solubility reduces PAH concentrations to sublethal levels, regardless of their intrinsic toxicity (Tuvikene, 1995). Nevertheless, sub-lethal effects can be depicted from exposure to these very low concentrations of high molecular weight PAHs (Hoffman et al., 2002).

Environmental Quality Standards (EQS) for PAHs set the sum of the concentrations of indeno(1,2,3,cd)pyrene (IND) and benzo(ghi)perylene (BGP) of 2 ng l-1, while at concentrations above this value, such compounds are considered a threat to inland surface waters (WFD, 2000). In this study, extracts from both standard soil and standard soil enriched with biochar have shown levels of these two PAHs that were significantly higher than the corresponding EQS . For the remaining PAHs, and according to the toxicological benchmarks proposed by Suter and Tsao (1996), none were found at concentrations above the benchmarks in extracts from biochar-treated soil. There are no benchmarks for IND and BGP in the same literature. Perhaps results in this study could have direct use in legislative matters, such as help setting threshold levels for individual and total PAH contents in biochar-amended soil, above which, toxicity to aquatic organisms, as a result of runoff can be observed. In fact, this would be an important aspect to consider, when attempting to fully evaluate the potential ecotoxicity of biochar to soil, surface and ground waters. The majority of studies on the occurrence and environmental impacts of PAHs are focused on those produced during forest fires and that are then found in runoff from burned soils. These studies, generally involve solvent extraction-based methodologies, such as dichloromethane (DCM)–acetone (1+1). Vila-Escale et al. (2007) quantified PAH levels in a Mediterranean creek after runoff from a burned area. The highest levels of total PAHs (386.26 ng

l^{-1}) were detected 12 days after the wildfire in both dissolved and particulate phases (Vila-Escale et al., 2007). In another study, focusing on environmental effects of forest fires and within one month of the fire, the total PAH concentrations in a nearby stream ranged between 2 and 336 ng l^{-1}, depending on the sampling location (Olivella et al., 2005). Campos et al. (2011) underline that due to PAHs's persistency in aquatic environment some particular PAHs were present in higher concentrations immediately after the fire, while other PAHs were present in higher concentrations eleven months after the wildfire. The total concentrations of PAHs in biochar-soil extracts in our study were in average approximately 108 ng l^{-1} (already taking into account soil wetting and drying events), meaning that they are in a range comparable (maybe even lower) to those in runoff from forest fires. This appears to suggest that in respect to soil and water contamination, the threat of adding biochar to soils might not be higher than that caused by forest fires. Nevertheless and once again, this needs to be interpreted very carefully, not only because different extraction methods were used but also since these concentration ranges will vary largely with the type of biochar used (particularly type of biomass and pyrolysis temperature) and maybe also on soil conditions, as demonstrated in the literature. In fact, it is well known that the type and concentration of PAHs that are formed during pyrolysis and the extent to which they accumulate in the biochar depend on their type and concentration in the biomass feedstock, combined with pyrolysis conditions used, mostly temperature (Pakdel and Roy, 1991).

Alongside PAH quantification, a battery of standard aquatic bioassays were used with representative test organisms (*Vibrio fischeri*, *Pseudokirchneriella subcapitata* and *Daphnia magna*), for a robust ecotoxicological evaluation of the PAH fraction that is actually bioavailable in biochar-soil (BS) aqueous extracts. Extracts of soil amended with biochar (BS) caused lethal or sublethal effects in all tested species. Notwithstanding, the influence of the number of wet-dry cycles on the toxicity of the extracts was found to be species-specific. Among the short-term tests performed, the highest sensitivity in the current study was expressed in the acute bioassay with the aquatic invertebrate *D. magna* for BS, ST-0 (TU= 33.90%). Specific toxicity patterns could be established in terms of the sensitivity response on different sampling times for the algae *P. subcapitata* and the bacteria *V. fischeri.* In both bioassays, the samples from ST-1 (6 cycles) showed to have the highest observed toxicity (TU=1.17% and 9.44%, respectively), though for *V. fischeri*, these samples induced toxicity at lower concentrations.

In bioassays with standard soil extracts (SS), sub-lethal effects were also observed for *P. subcapitata* and *V. fischeri*, but not so expressive as when exposed to BS (TU=ND and 3.96%, respectively). A similar pattern was found between SS and BS for these species, with the highest observed effects on ST-1. In contrast, *D. magna* was not acutely affected by SS exposure.

Short-term tests with aquatic invertebrates exposed to PAHs showed LC_{50}s in the range of 0.1 to 5.6 mg l^{-1}, with adults and juveniles exhibiting higher tolerance than eggs or larvae (reviewed by Hoffman et al., 2002). Regarding bacteria and algae, available literature reports that individual PAH compounds, mostly 2- and 3-ring, at high concentrations (0.2 to 10 mg l^{-1}) can impair cell division and photosynthesis of algae and cell division of bacteria; as ultimate effect they can also cause death. The same PAHs at low concentrations (5 to 100 µg l^{-1}) can inhibit or stimulate growth and cell division in aquatic bacteria and algae (Eisler, 2000). Notwithstanding, all these toxicity values are highly above the levels of individual PAHs measured in all samples (BS, SS and B), even if we consider the total PAH_S measured in this study. However, it is important to keep in mind that the extracts obtained in this study contained multiple PAHs, and therefore we cannot exclude the occurrence of additional or synergistic effects, which can explain the toxicity observed. Moreover, although concentrations of individual PAHs in the extracts are much lower than concentrations that are acutely toxic to aquatic organisms, chronic effects (e.g reproduction) can be produced, and for this reason, studies which include long-term exposure tests are required.

Despite the attempt to compare toxicity values in the literature with those obtained in this study, this task is made difficult by species-specific differences in relation to PAH-metabolism and by the fact that the majority of literature is focused in individual compounds and not in complex natural soil samples characterised by multiple PAHs. Nevertheless, so far, toxicological and ecotoxicologial studies have mainly focused on PAHs that are heavily carcinogenic and mutagenic (such as those produced at pyrolysis temperatures >700ºC), whereas those considered less toxic (e.g. produced <500ºC), have generally been less researched (Hoffman et al., 2002).

In the study on effects of wildfires on aquatic species the toxicity was observed in the assays with *P. Subcapitata* and *V. fischeri,* while in chronic tests with *D. magna* the effect were not statistically significant (Campos et al., 2011). Lors et al. (2011) analysed the toxicity of PAHs contaminated industrial soils and compared the sensitivity of batteries of solid and liquid bioassays. They found a significant correlation between the toxic endpoints and 3-rings PAHs in

both type of bioassays. Due to their chemical characteristics 3-rings PAHs are able to easily cross the cell membrane, and therefore are not dependent on whether they are ingested from soil or directly absorbed from aquatic environment (Lors et al., 2011: Leaner and Mason, 2002; Van de Wiele et al., 2004). Considering this, it is important to highlight the expressive concentrations of phenanthrene (PHE; 3-ring PAH) measured in this study, which certainly have played a role in the toxicity observed.

Moreover, Becker et al. (2002) underline the importance of formation of photoproducts and their potential toxic effects on the organisms. These authors found that UVB treatment was less effective when phenantrene (PHE) was in a mixture with sediment particles due to possible inhibition of photoproducts formation because of the presence of particles. In this study the toxicity is increased under UVB treatmen only in the absence of sediment. It has been shown that phenanthrequinone, as the first photoproduct of PHE, has higher solubility than phenanthrene and consequently higher bioavailability (McConkey et al, 1997). Having in mind that in this study algae and daphnids are subjected to the photoperiod of 16:8 hours, it is possible that to a certain extent, toxicity may be derived from the photoproducts formed during incubation.

In *P. subcapitata* bioassays with soil-biochar extracts (BS) decline in toxicity was observed in ST-2 (12 cycles) and very low toxicity in ST-0 (0 cycles). This toxicity trend, when compared to daphnids, might be attributed to the darker observed colour of the sample BS, ST-1 which was the most toxic for algae. It has been demonstrated that there is a correlation in higher fluorescence and absorbance values in water after wildfires, due to contribution of PAHs. The authors suggested that these effects on light transmission and absorption could affect the primary producers in aquatic ecosystems (Vila Escale et al., 2007).

Since the pH values of the samples are in the range of optimal demands for pH needed for the test species, with exception for SS, ST-0, detrimental effects of the treatment extracts on the organisms are not explained based on pH changes of the media.

According to the results of the current study, MICROTOX®BSPT (solid phase test) showed a different response of the bacteria to the samples, when compared to the MICROTOX®BT (basic test). The reason for this difference might be the use of the special solid phase diluent (Azur Environmental, Carlsbad, CA, USA), which is more effective in dissolving the PAH fraction of the samples, considering the low solubility of PAHs in water. Harkey and Young (1999) demonstrated the lower availability of PAHs from contaminated soils when using saline solutions (physiologically based procedure), when compared to SFE (supercritical fluid

extraction) approach, being a more vigorous method based on physical extraction. Nevertheless a chemical analysis of the solid samples would be needed, in order to better explain the difference in toxicity of the SS and BS solid samples. MICROTOX®BT is a more relevant approach in the context of this study, because the experimental design is based on the assumption that PAH toxicity is through the water route, i.e., its availability in soil solution is related to the possibility for transport through runoff into aquatic systems.

In order to address the relevance of MICROTOX® tests in assessing toxicity of the soil extracts in this study, more experimental work needs to be done. The turbidity and colour of samples might mask the bacterial luminescence and provide the information on false inhibition (higher toxicity) (Campisi et al., 2005). Though the BS extracts induced higher toxic effects, it is not clear whether a certain bacterial adhesion or optical interference (Guzzella, 1998) might be responsible for unexpectedly high toxicity of standard soil extract and if these factors contributed to the toxicity of BS extracts.

2.5. Conclusions

This pilot study has shown that it is possible to mildly water-extract a relevant PAH fraction from biochar, when it is added to soil at common application rates, and that the amount extracted is relatively independent of the number of soil wet-dry cycles applied to the biochar-soil mixture. This suggests that, in the short-term, increased PAH bioavailability in runoff from biochar treated soils is influenced by soil-biochar interactions, whereas relatively independent of natural soil wetting-drying events. . Using a battery of standard aquatic ecotoxicological procedures using representative species (e.g. green algae, daphnids and bioluminescent bacteria) of different functional levels, it has been demonstrated thatthese PAHs in biochar-soil aqueous extracts can induce toxicity to aquatic organisms, although the extent and pattern of the responses were species-specific and also influenced by the number of wet-dry cycles.. The highest sensitivity to extracts was observed in the case of acute assay with *D. magna* when subjected to the extract obtained from BS, ST-0. *P. Subcapitata* and *V. fischeri* were most sensitive when exposed to BS, ST-1 (corresponding to 6 wet-dry cycles). In the context of this pilot study, the method employed coupled to the selected aquatic bioassays was useful and adequate for evaluating the ecotoxicological effects of biochar-bound PAH contaminants in runoff from

enriched soils. Yet, longer term exposure to the test elutriates are necessary using these, as well as other non-target species, alongside various biochars and soil properties.

In *D. magna* acute test BS, ST-0, showed to be highly toxic, and further trend of decreasing effects with the number in wet- dry cycles increasing was observed. Inhibition of growth of *P. subcapitata* was observed in both SS and BS extracts and was dependent on the number of wet-dry cycles. The highest sensitivity of *P. subcapitata* was to BS, ST-1. BS, ST-2 extract though inhibiting the algae growth, in the medium test concentrations, the effect was lower than in the case of BS, ST-1. When *V. fischeri* was exposed to the extracts (MICROTOX®BT), the toxicity observed was dependent on the number of wet-dry cycles. SS and BS extracts showed a similar trend of toxicity through the sampling times, with higher toxicity of BS. The bacterial assay showed the highest sensitivity in the case of BS, ST-1 extract likewise algae. In MICROTOX®BSPT the standard soil was more toxic than the sample of biochar-enriched soil.

Having in mind that in this study algae and daphnids are subjected to the photoperiod of 16:8 hours, it is possible that a certain toxicity is derived from the photoproducts formed during incubation. Generally, the higher observed toxicity in the BS extracts may be explained with the higher content of total PAHs in the extracts and possible synergistic effects of these contaminants due to the fact they here were present in the mixture. Microalgae were affected with the colour of the samples which is likely to occur in runoff after the wildfires. To a certain extent the bacterial luminescence was inhibited due to high turbidity of the samples. In order to fully address the effects of the extracts on *V. fischeri* further adjusting the samples' turbidity need to be done as preliminary procedure.

2.6. References

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.

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.

Ankley, G. T., Schubauer-berigan, M. K., Dierkes, J. R., 1991. Predicting the toxicity of bulk sediments to aquatic organisms with aqueous test fractions: pore water vs. extract. Environmental Toxicology and Chemistry 10: 1359-1366.

Antunes, S. C., Pereira, J. L., Cachada, A., Duarte, A. C., Gonçalves, F., Sousa, J. P., Pereira R., 2010. Structural effects of the bioavailable fraction of pesticides in soil: Suitability of extract testing. Journal of Hazardous Materials 184: 215–225.

ASTM, American Society for Testing and Materials, 1998. Standard Practice for conducting toxicity tests with fish, microinvertebrates and amphibians. E729-90. In: Annual Book for ASTM Standards (American Society for Testing Materials). Philadelphia. 271-296.

ASTM, American Society for Testing and Materials, 1980. Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians. Report E 729-80. American Society for Testing and Materials, Philadelphia, USA.

Ayodele, A., Oguntunde, P., Joseph, A., de Souza Dias Junior, M., 2009. Numerical analysis of the impact of charcoal production on soil hydrological behavior, runoff response and erosion susceptibility. Revista Brasileira de Ciência do Solo. 33:137-145.

Baird, D.J., Barber, I., Calow, P., 1990. Clonal variation in general responses of Daphnia magna Straus to toxic stress. 1. Chronic life-history effects. Functal Ecology. 4: 399-407.

Becker, A. M., S. Heise and W. Ahlf (2002). Effects of phenanthrene on Lemna minor in a sediment-water system and the impacts of UVB. Ecotoxicology 11(5): 343-348.

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

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.

Bucheli, T., Gustafsson, Ö., 2001. Ubiquitous observations of enhanced solid affinities for aromatic organochlorines in field situations: are in situ dissolved exposures overestimated by existing portioning models? Environmental Toxicology and Chemistry 20: 1450-1456.

Bucheli, T., Gustafsson, Ö., 2003. Soot sorption of non-ortho and ortho substituted PCBs. Chemosphere 53: 515-522.

Campisi, T., Abbondanzi, F., Casado-Martinez, C., DelValls, T.A.,Guerra, R., Iacondini, A., 2005. Effect of sediment turbidity and color on light output measurement for Microtoxs Basic Solid-Phase Test. Chemosphere. 60: 9–15.

Campos, I. , Abrantes, N.,Vidal, T., Gonçalves, F., Keizer, J.J., 2011. Off-site enironmental impacts of wildfires: evaluation of the toxicity of runoff from burnt area on freshvater aquatic species. Poster presentation. Erosfire II Project.

CCME, Canadian Council of Ministers of the Environment, 2008. Canadian Soil QualityGuidelines for Carcinogenic and Other Polycyclic Aromatic Hydrocarbons .Environmental and Human Health Effects. Scientific Supporting Document. 218 pp.

Cetin, E., Moghtaderi, B., Gupta, R., Wall, T. F., 2004. Influence of pyrolysis conditions on the structure and gasification reactivity of biomass chars. Fuel 83: 2139-2150.

Chen, J., Zhu, D., Sun, C., 2007. Effect of heavy metals on the sorption of hydrophobic organic compounds to wood charcoal. Environmental Science and Technology 41: 2536-2541.

Chen, B., Yuan, M., 2011. Enhanced sorption of polycyclic aromatic hydrocarbons by soil amended with biochar. Journal of Soils and Sediments 11: 62–71.

Cheng , X.K. , Kan, A.T. , Tomsom, M.B., 2004. Naphthalene adsorption and desorption from aqueous C-60 fullerene. Journal of Chemical and Engeneering Data 49:675–83.

Cheng, C-H, Lehmann, J., Thies, J., Burton, S. D., Engelhard, M. H., 2006. Oxidation of black carbon by biotic and abiotic processes. Organic Geochemistry 37: 1477-1488.

Chiou, C. T., Kile, D. E., 1998. Deviations from sorption linearity on soils of polar and nonpolar organic compounds at low relative concentrations. Environmental Science and Technology 32: 338-343.

Collison, M., Collison, L., Sakrabani, R., Tofield, B. and Wallage, Z., 2009. Biochar and Carbon Sequestration: A Regional Perspective; A report prepared for East of England Development Agency, Norwich, UK: Low Carbon Innovation Centre, University of East Anglia, April 2009. Available: www.uea.ac.uk/lcic/Biochar [accessed 8 November 2011].

Cornelissen, G., Gustafsson, Ö., 2005. Importance of unburned coal carbon, black carbon, and amorphous organic carbon to phenanthrene sorption in sediments. Environmental Science and Technology 39: 764-769.

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.

Denef, K., Six, J., Bossuyt, H., Frey, S.D., Elliott, E.T., Merckx, R., Paustian, K., 2001a. Influence of dry–wet cycles on the interrelationship between aggregate, particulate organic matter, and microbial community dynamics. Soil Biology & Biochemistry 33, 1599–1611.

Denef, K., Six, J., Paustian, K., Merckx, R., 2001b. Importance of macroaggregate dynamics in controlling soil carbon stabilization: short term effects of physical disturbance induced by dry–wet cycles. Soil Biology & Biochemistry 33, 2145–2153.

Directive 2008/105/EC of the European Parliament and of the Council. Of 16 December 2008. On environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 84/513/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Prliament and of the Coucil.Available: http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:348:0084:0097:EN:PDF [accessed 15 June 2011]

Eisler, R., Polycyclic aromatic hydrocarbons, in *Handbook of Chemical Risk Assessment*, Vol. 2, Lewis Publishers, Boca Raton, FL, 2000, Chap. 25.

European Environment Agency 1999. Criteria for EUROAIRNET: The EEA Air Quality Monitoring and Information Network. Technical Report No. 12. Available: http://www.eea.europa.eu/publications/TEC12 [accessed 1 December 2010]

EU Water Framework Directive: Directive 2000/60/EC of the European Parliament and of the Council.Availbale: http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32000L0060:EN:NOT [accessed 15 June 2011]

Fasnacht, M.P., Blough N.V., 2002. Aqueous photodegradation of polycyclic aromatic hydrocarbons. Environ Sci Technol. 15; 36 (20), 4364-9.

Fernandes, M.B. and Brooks, P., 2003. Characterization of carbonaceous combustion residues: II. Nonpolar organic compounds. Chemosphere 53(5): 447-458.

Frische, T., 2003. Ecotoxicologica evaluation of in situ bioremediation of soilf contaminated by the explosive 2,4,6-trinitrotoluene (TNT). Environmental Pollution 120: 103-113.

Geis, S. W., Fleming, K. L., Korthals, E. T., Searle, G., Reynolds, L., & Karner, D. A. (2000). Modifications to the algal growth inhibition test for use as a regulatory assay. Environmental toxicology and chemistry, 19(1), 36-41.

Goldberg, E. D., 1985. Black carbon in the Environment: properties and distribution. Wiley, NY

Guzzella, L., 1998. Comparison of test procedures for sediment toxicity evaluation with *Vibrio fischeri* bacteria. Chemosphere 37: 2895-2909.

Gustafsson, Ö., Haghseta, F., Chan, C., Macfarlane, J., Gschwend, P., 1997. Quantification of the dilute sedimentary soot phase: implications for PAH speciation and bioavailability. Environmental Science and Technology 31: 203-209.

Harkey, G.A., Young, T.M., 2000. Effect of soil contaminant extraction method in determining toxicity using the Microtox assay. Environ. Toxicol. Chem. 19, 276–282.

Hiller, E., Fargasova, A., Zemanova, L., Bartal, M., 2007. Influence of wheat ash on the MCPA imobilization in agricultural soils. Bulletin of Environmental Contamination and Toxicology 78: 345-348

Hockaday, W. C., 2006. The organic geochemistry of charcoal black carbon in the soils of the University of Michigan Biological Station. Doctoral Thesis, Ohio State University, US.

Hoffman, D.J., Rattner, B.A., Burton Jr., G.A., Cairns Jr., J., 2002. Handbook of Ecotoxicology, second ed. CRC Press, New York.

Hund-Rinke, K.H. , Kordel, W., Hennecke, D., Eisentraeger, A., Heiden, S., 2002. Bioassays for the Ecotoxicological and Genotoxicological Assessment of contaminated soils (Results of a Roun Robin Test): Part I: Assessment of a possible groundwater contamination: Ecotoxicological and genotoxicological tests with aqueous soil extracts. Journal of Soils and Sediments 2, 43-50.

IBI, International Biochar Initiative, 2011. Product Definition and Standard: Preliminary Draft for Review Only. [accessed 20 May, 2011]

ISO 7027: 1999. Water Quality-Determination of Turbidity. ISO-Intenational Organization for Standardization, Geneva.

ISO 10381-6: 1993E. Soil quality -- Sampling -- Part 6: Guidance on the collection, handling and storage of soil under aerobic conditions for the assessment of microbiological processes, biomass and diversity in the laboratory**.** ISO-Intenational Organization for Standardization, Geneva.

Jablonowski, N. D., Linden, A., Koppchen, S., Goebbels, D., Thiele, B., Hofmann, D., Burauel, P., 2011. Influence of dry-wet cycles on the water-extracability of aged ^{14}C-pesticide residues in soils. Paper presented at the General Assembley European Geoscience Union. April, 2011. http://meetings.copernicus.org/egu2011/ [accessed May, 2011]

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.

Jonker, M. T. O., Hawthorne, S. B., Koelmans, A. A., 2005. Extremely Slowly Desorbing Polycyclic Aromatic Hydrocarbons from Soot and Soot-like Materials: Evidence by Supercritical Fluid Extraction. Environmental Science and Technology 39: 7889-7895.

Kilduff, J. E., Wigton, A., 1999. Sorption of TCE by humic-preloaded activated carbon: Incorporating size-exclusion and pore blockage phenomena in a competitive adsorption model. Environmental Science and Technology 33: 250-256.

Kwon, S. and Pignatello, J. J., 2005. Effect of Natural Organic Substances on the Surface and Adsorptive Properties of Environmental Black Carbon (Char): Pseudo Pore Blockage by Model Lipid Components and Its Implications for N2-Probed Surface Properties of Natural Sorbents. Environmental Science and Technology 39: 7932-7939.

Leach, M., Fairhead, J., Fraser, J. and Lehner, E. (2010). Biocharred Pathways to Sustainability? Triple Wins, Livelihoods and the Politics of Technological Promise, STEPS Working Paper 41, Brighton: STEPS Centre.

Leaner, J.J., Mason, R.P., 2002. Methylmercury accumulation and fluxes across the intestine of channel catfish, Ictalurus punctatus. Comparative Biochemistry and Physiology, Part C, Toxicology and Pharmacology 132, 247e259.

Lehmann, J., Gaunt, J. and Rondon, M., 2006. Bio-char sequestration in terrestrial ecosystems - A review. Mitigation and Adaptation Strategies for Global Change 11: 403-427.

Liang, B., Lehmann, J., Solomon, D., Kinyangi, J., Grossman, J., O'Neill, B., Skjemstad, J.O., Thies, J., Luizão, F.J., Petersen, J., and Neves, E.G., 2006. Black carbon increases cation exchange capacity in soils. Soil Science Society of America Journal 70(5): 1719-1730.

Lors, C., Ponge, J.F., Martínez Aldaya, M., Damidot, D., 2011. Comparison of solid and liquid phase bioassays using ecoscores to assess contaminated soils. Environmental Pollution 159, 2974-2981.

Loureiro, S., Ferreira, A.L.J., Soares, A.M.V.M., Nogueira, A.J.A., 2005. Evaluation of the toxicity of two soils from Jales Mine (Portugal) using aquatic bioassays. Chemosphere 61, 168–177.

McConkey,B.J., Duxbury,C.L.,Dixon,D.G., Greenberg,B.M. ,1997. Toxicity of a PAH photooxidationproduct to the bacteria *Photobacterium phosphoreum* and the duckweed *Lemna gibba:* effects of phenanthrene and its primary photoproduct, phenanthrenequinone. Environmental Toxicology and Chemistry 16: 892-899

Microbics Corporation, 1992. Microtox_ Manual. AToxicity Testing Handbook. Carlsbad, CA, USA.

Mikhaa, M.M,, Riceb, C.W., Millikenc, G.A., 2005. Carbon and nitrogen mineralization as affected by drying and wetting cycles. Soil Biology & Biochemistry 37, 339–347

Moore, M.N., 2006. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? Environment International 32(8): 967-976.

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

OECD, 2006. Freshwater Algae and Cyanobacteria, Growth Inhibition Test nº 201. OECD-Organisation for Economic Cooperation and Development, Paris.

Olivella, M. A., Ribalta, T. G., de Febrer, A. R., Mollet, J. M., de las Heras, F. X. C., 2006. Distribution of polycyclic aromatic hydrocarbons in riverine waters after Mediterranean forest fires. Science of the Total Environment 355: 156– 166.

Parvez, S., Venkataraman, C., Mukherji, S., 2006. A review on advantages luminescence inhibition test (*Vibrio fischeri*) for acute toxicity prediction of chemicals. Environment International 32: 265-268.

Pignatello, J. J., Kwon, S., and Lu, Y., 2006. Effect of Natural Organic Substances on the Surface and Adsorptive properties of Environmental Black Carbon (Char): Attenuation of Surface Activity by Humic and Fulvic Acids. Environmental Science and Technolology 40: 7757-7763.

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.

Salloum, M. J., Chefetz, B., Hatcher, P. G., 2002. Phenanthrene sorption by alliphatic-rich natural organic matter. Environmental Science and Technology 36: 1953-1958.

Sohi, S1.Loez-Capel, E2., Krull, E3., Bol, R4., 2009. Biochar's roles in soil and climatechange: A review of research needs. CSIRO Land and Water Science Report 05/09, 64 pp

Suter II, G.W., Tsao, C.L., 1996. Toxicological Benchmarks forScreening Potential Contminants of Concern for Effects on Aquatic Biota: 1996 Revision. US department of Energy. Oak Ridge.

Tuvikene, A., 1995. Responses of fish to polycyclic aromatic hydrocarbons (PAHs). Annales Zoologici Fennici 32: 295-309.

Pakpahan, E.N., Isa, M.H., Kutty, S.M.N., Malakhamad, K.., 2009. Effect of temperature on the formation and degradation of polycyclic aromatic hydrocarbons. International Conference of Emerging Technologies in Environmental Science and Engineering, 26-28 October, Aligarh, India, 2009.

Uchimiya, M., Lima I. M., Klasson K. T., Wartelle L. H., 2010. Contaminant immobilization and nutrient release by biochar soil amendment: Roles of natural organic matter. Chemosphere 80: 935-940.

U.S. Environmental Protection Agency, 2002. Atmospheric Concentrations of Greenhouse Gases http://cfpub.epa.gov/eroe/index.cfm?fuseaction=detail.viewPDF&ch=46&lShowInd=0&subtop=342&lv=list.listByChapter&r=209837. [accessed February 2010]

Van de Wiele, T.R., Verstraete, W., Siciliano, S.D., 2004. Polycyclic aromatic hydrocarbon release from a soil matrix in the in vitro gastrointestinal tract. Journal of Environmental Quality 33, 1343e1353

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.

Verheijen, F.G.A., Jeffery, S., Bastos, A.C., van der Velde, M., and Diafas, I. (2010). 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.

Vila-Escalé, M., Vegas-Vilarrúbia, T., Prat, N., 2007. Release of polycyclic aromatic compounds into a Mediterranean creek (Catalonia, NE Spain) after a forest fire. Water Research 41: 2171 – 2179.

Wilcke, W., 2000. Polycyclic aromatic hydrocarbons (PAHs) in soil - A review. Journal of Plant Nutrition and Soil Science 163(3): 229-248.Wolf, D., 2008.

Biochar as a Soil Amendment: A Review of the Environmental Implications. SwanseaUniversity, School of the Environment and Society. Unpublished document.

Xavier Domenes, 2011. Personal communication.

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

Zitko, V., 2000. Marine pollution. In: Wangersky, P. (Ed.). The Handbook of Environmental Chemistry, Springer Verlag, Heidelberg, vol. 5, Part D, 75-109

Chapter III

Concluding Remarks, On-going Research and Recommendations for Future Studies

3.1. Concluding remarks

Biochar research is just beginning to bloom and much information is still required, such as on the environmental consequences in relation to possible leaching of biochar contaminants, over time. The literature often provides incomplete or contradictory results on this matter, as reviewed by Verheijen et al. (2010). The reason for this can be, at some extent, the limited amount of long-term studies carried out with biochar in natural systems, and partly due to the lack of standardised methods for simulating biochar-soil interactions over time and long-term environmental monitoring for biochar in soil (Verheijen et al., 2010). In this pilot study, by considering the influence of natural soil processes and soil-biochar interactions on PAH bioavailability in pore water, and integrating the responses of different species (e.g. green algae, daphnids and bioluminescent bacteria) that are representative of different trophic and functional levels, a broad picture was obtained on the potential ecotoxicity of biochar-bound PAH contaminants in runoff from treated soils. Outcomes from this study are thus expected to fill in gaps in current knowledge on this subject, and perhaps help identify future research directions on contaminant bioavailability and ecotoxicology of biochar-bound contaminants. In this context, the use of a standard natural soil such as LUFA 2.2 will make easier to compare methodologies and results to other studies in the literature, while contributing for standardisation of methodologies in respect to a full ecotoxicological evaluation of biochar in soils.

This is important, since standardisation of biochar materials and test methodologies (including soil and application site characteristics) have recently been identified as an urgent need, in view of the increasing intention of widespread application of biochar to soils, (Verheijen, et al., 2010; IBI, 2011). At the European scale, this type of research is expected to have direct use for regulatory and legislative purposes, contribute to the processes of adjusting existing methodologies and help developing new ones in ecotoxicological risk assessment, under the European Soil Directive (2006). In Canada, authorities went further in regulatory measures for soil quality, establishing The Canadian Soil Quality Guidelines for Carcinogenic and Other PAHs (2008), where combustion-derived PAHs are identified as the second source (after petroleum) of antropogenic PAH contamination of soils, groundwater and surface waters. Guidelines on soil quality consider two approaches (environmental and human health soil quality guidelines), both being developed for four important land uses (agricultural, residental, industrial and commercial; CCME, 2008).

So far, the majority of studies on the occurrence and environmental impacts of PAHs in runoff, come from combustion processes (generally forest fires) and involve methodologies based on solvent extraction. It is likely that PAH extractions using solvents result in overestimating PAH concentrations in runoff samples and this provides little realistic information on their bioavailable fraction in soil water. In the present study, water-based extraction was proposed, aiming for a more robust representation of the PAHs actual potential for desorbing from the biochar-soil carrier into water, and induce toxicity to aquatic organisms, when subject to runoff. However, differences in PAH extraction methods make results from this study less comparable to those found in the literature. Also, dealing with natural samples of high heterogeneity means that it is difficult to know for sure, whether the toxicity observed was due to the PAHs alone, or due to synergistic or antagonistic effects between PAHs and other soil or biochar components, including metals.

One way of addressing the effects of co-existing contaminants in biochar on the observed toxicity is perhaps including different biochars. For example, the biochar used in this study was derived from a widely available and commercially relevant feedstock (pine) and was used at common field application rates (80 ton ha-1). Wood biochar is generally regarded as low in hydrophobic contaminants, compared to biochars from other source materials, including agricultural wastes (Preston and Schmidt, 2006). Nevertheless, the test biochar was produced through gasification at temperatures (~800°C) above those generally recommended for biochar production, which is expected to favour accumulation of PAHs in the final charred residue. Biochars produced at temperatures ranging between 300-500º°C are perhaps more representative of those expected to be applied to soil at larger scales and this decrease may be sufficient to reduce the potential risk for soil contamination from biochar-bound PAHs (Garcia-Perez, 2008).

3.2. On-going Research and Recommendations for Future Studies

Work is still in progress to include short-term characterisation of the ecotoxicological effects of elutriates of biochar alone on *V. fischeri*, *P. subcapitata* and *D. magna*, Looking at the responses of the various tests organisms to elutriates of biochar alone, will complement and help to better understand the influence of soil-biochar interactions (mostly in respect to organic

matter) on short-term bioavailability and toxicity of PAHs in runoff. Preliminary results of on-going work can be found in Annexe 3.

Although results of the current pilot study may provide sound ground basis in this context, a great deal of research of this kind, involving various biochars will be essential for a comprehensive evaluation of PAH bioavailability and ecotoxicology in runoff from soils amended with biochar. It is recommended that biochars made from representative feedstocks and pyrolysis conditions are used in long-term ecotoxicological tests. Long-term exposure to biochar-soil elutriates using a wider range of non-target organisms, soil types (i.e., texture and amount and type of natural organic matter) and biochar characteristics (e.g., particle and pore sizes) are also strongly recommended as a direction for further research, in order to comprehensively examine both acute and chronic toxicity of biochar-bound PAH contaminants in runoff from soils amended with biochar.

In order to further evaluate the applicability and usefulness of BSPT, one group of authors suggests modifications in the BSPT (mBSPT) by additional light readings after re-suspension of the bacteria in contact with the samples. They suggest the application and subsequent comparison of both approaches (Campisi et al., 2005).

In better addressing the extracts' effects on bacterial luminescence the samples of equal turbidity of e.g. 50 FNU (formazine nephelometric units; ISO 7027, 1999) might give more comparable results and thus more reliable information on the toxicity (Hund-Rinke at al., 2002) .

In terms of toxicity assessment of contaminated sediments with a mixture of different pollutants Liβ and Ahlf (1996) underline the importance of applying extract, pore water as well as whole soil and sediment testing. Analogous to this recommendation, the importance of toxicity assessment of the soil amended with biochar by solid phase bioassays and using a range of representative soil organisms should not be neglected. So far, tests with earthworms have shown that in soil contaminated with PAHs, biochar was effective in reducing the total and cyclodextrine extractable PAHs, as well as the PAHs concentrations in *Eisenia fetida,* but decrease in the earthworms' weight was observed (Jose et al., 2010), demonstrating the uncertainties that still need to be addressed.

3.3. References

Campisi, T., Abbondanzi, F., Casado-Martinez, C., DelValls, T.A.,Guerra, R., Iacondini, A., 2005. Effect of sediment turbidity and color on light output measurement for Microtoxs Basic Solid-Phase Test. Chemosphere. 60: 9–15.

CCME, Canadian Council of Ministers of the Environment, 2008. Canadian Soil QualityGuidelines for Carcinogenic and Other Polycyclic Aromatic Hydrocarbons .Environmental and Human Health Effects. Scientific Supporting Document. 218 pp.

EU Proposal for a Directive of the European Parliament and of the Council: establishing a framework for the protection of soil and amending Directive 2004/35/EC. Brussels, 2006. Available: http://ec.europa.eu/environment/soil/pdf/com_2006_0232_en.pdf [accessed 30 September 2011]

Hund-Rinke, K.H. , Kordel, W., Hennecke, D., Eisentraeger, A., Heiden, S., 2002. Bioassays for the Ecotoxicological and Genotoxicological Assessment of contaminated soils (Results of a Roun Robin Test): Part I: Assessment of a possible groundwater contamination: Ecotoxicological and genotoxicological tests with aqueous soil extracts. Journal of Soils and Sediments 2, 43-50.

IBI, International Biochar Initiative, 2011. Product Definition and Standard: Preliminary Draft for Review Only. [accessed 20 May, 2011]

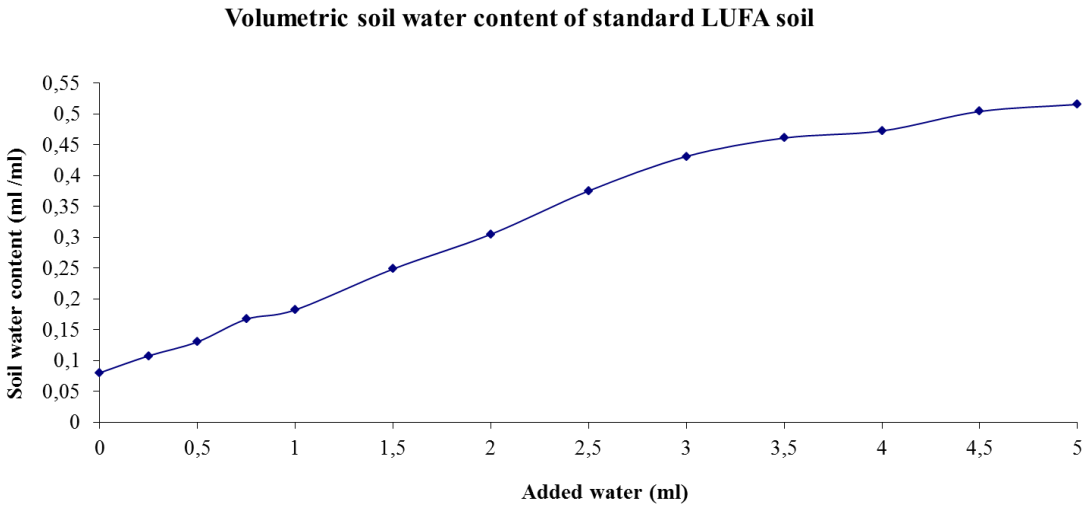
Liß, W., Ahlf, W., 1997. Evidence from Whole-sediment, porewater, and elutriate testing in toxicity assessment of contaminate sediments. Ecotoxicology and Environmental Safety. 36: 140-147.

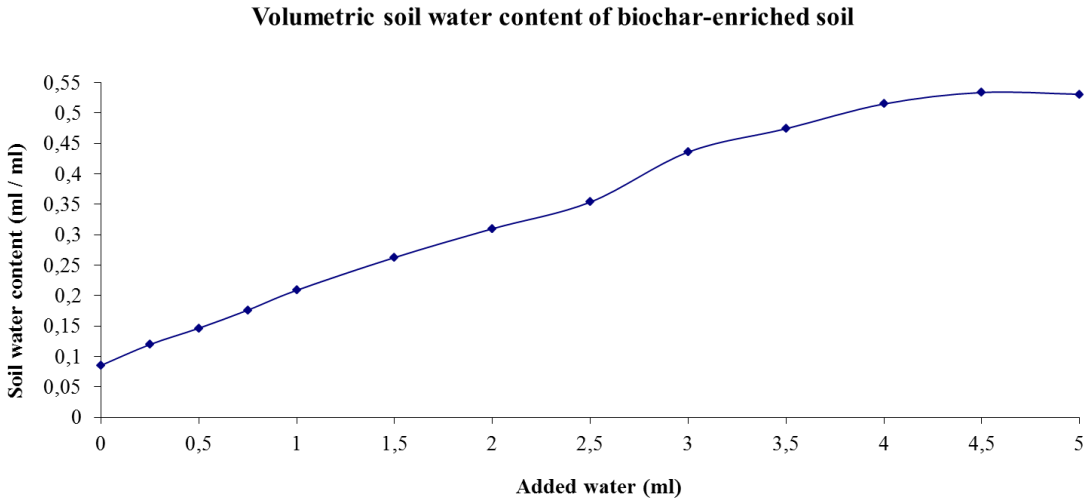
Preston, C. M., and Schmidt, M. W. I., 2006. Black (pyrogenic) carbon in boreal forests: a synthesis of current knowledge and uncertainties. Biogeosciences Discussions. 3: 211-271.

Verheijen, F.G.A., Jeffery, S., Bastos, A.C., van der Velde, M., and Diafas, I. (2010). 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.

Annexe 1


Volumetric soil water content of standard LUFA soil
Soil water content (ml /ml)
0,55
0,5
0,45
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
0
0,5
1
1,5
2
2,5
3
3,5
4
4,5
5
Added water (ml)


Volumetric soil water content of biochar-enriched soil
Soil water content (ml / ml)
0,55
0,5
0,45
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
0
0,5
1
1,5
2
2,5
3
3,5
4
4,5
5
Added water (ml)

Annexe 2

The results of the chemical analysis of the extracts on the occurrence of certain metals.

heavy metals (µg l-1)	Standard soil extract (SS)			Biochar-soil extract			Biochar extract		
	ST-0 (0 cycle)	ST-1 (6 cycles)	ST-2 (12 cycles)	ST-0 (0 cycle)	ST-1 (6 cycles)	ST-2 (12 cycles)	ST-0 (0 cycle)	ST-1 (6 cycles)	ST-2 (12 cycles)
Cr	1.60	4.00	3.30	1.70	4.50	3.80	3.30	1.20	<1
Mn	117.00	846.00	643.00	67.00	1000.00	744.00	43.00	27.00	75.00
Co	0.49	2.10	1.30	0.54	1.70	4.60	0.31	0.14	0.43
Ni	5.90	8.90	10.00	5.30	8.30	6.90	7.20	2.50	6.70
Cu	6.50	10.00	8.30	7.80	1.50	10.00	1.70	<1	<1
Zn	44.00	15.00	18.00	12.00	15.00	19.00	12.00	76.00	14.00
As	<1	4.50	4.00	2.60	6.40	5.80	2.20	<1	<1
Cd	0.27	0.15	<0,1	<0,1	0.15	0.11	<0,1	<0,1	<0,1
Pb	0.93	2.30	1.30	0.32	1.10	0.83	0.47	0.13	0.16
Hg	0.18	0.22	0.13	0.08	0.17	0.14	0.09	0.13	<0.05

Annexe 3

Measured pH values of biochar only (B) extract for ST-0 (0cycles), ST-1 (6 cycles) and ST-2 (12 cycles):

Sample	Sampling time (number of cycles)	pH
B	ST- 0 (0 cycle)	10.05
	ST-1 (6 cycles)	9.29
	ST-2 (12 cycles)	8.26

Daphnia magna acute immobilization assay

The three assays fulfilled the validity criteria according to the respective guideline (OECD, 2004). Biochar extracts (B) for ST-0 (0 cycles), ST-1(6 cycles) and ST-2 (12 cycles) did not induce toxic effect on juveniles of *Daphnia magna*.

V. fischeri luminescence inhibition test

MICROTOX® 81.9% Basic Test (BT). EC_{50} (%) values of bacterium *V.fischeri* exposed to each sampling time for biochar only (B) extracts. The values in brackets refer to the 95% confidence limits:

Sample		ST-0 (0 cycle)	ST-1 (6 cycles)	ST-2 (12 cycles)
B	EC_{50}(5 min)	33.82 (31.13-36.73	ND	ND
	EC_{50}(15 min)	34.85 (27.86-43.60)	ND	ND

ND-not determined

MICROTOX® Basic Solid PhaseTest (BSPT). EC_{50} (mg l^{-1})value of bacterium *V. fischeri* exposed to solid sample of biochar. The values in brackets refer to the 95% confidence limits: EC_{50}=11880 (892.7-158100)