Interactions between different types of biochar and soil microbial activity:

the effects on the dynamics of labile organic matter and the behaviour of some pesticides

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Ph. D. Thesis

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Summary

Resumen

SUMMARY

Biochar, the particular charcoal obtained through the process of pyrolysis of biomass, has been proposed as a mean of carbon sequestration through soil. It has been also considered useful to improve some relevant properties of the soil fertility and to reduce adverse effects of pollutants.

But the use of biochar must ensure, in addition to its conservation, that it does not generate adverse effects on living organisms, on the soil ecosistemic functions, or on the adjacent natural systems.

This thesis attempts to contribute to knowledge in this regard, by studying:

- The biochar's ability to protect the most labile soil organic matter;
- The hypothetic adverse effects caused by biochar once incorporated into the soil, depending on the type of pyrolysis process and doses applied ; and
- The modulation of the toxic side effects of some pesticides when incorporated into soils that have received a provision of biochar.

To achieve these goals three types of biochars produced from the same starting biomass (pine wood chips) were used to amend soil: biochar produced by slow pyrolysis (PL), biochar obtained from fast pyrolysis (PR) and biochar obtained through the gasification of biomass (PG).

The addition of biochar results in changes of the structure and biology of the soil, then in positive or negative modification of its physical and biological balance.

Considering that microbial community plays a major regulatory role in the soil, conditioning the most important biotic reactions, microbial biomass and soil respiration were used as indicators of the effects produced by the addition of biochar into the soil.

Sorption models were used to assess the ability of biochar to adsorb and protect the most labile soil organic matter. A fully factorial experiment was designed to check the effects of three single factors (biochar, nutrients and glucose addition) and their relationship with the whole SOM mineralization.

The results of this work demonstrated that each kind of biochar established different interactions with the soil.

The ecotoxicological approach suggested that the fast pyrolysis biochar (PR) is the less recommendable if used in doses higher than the calculated ED50.

No evidence of protective interactions with labile organic compounds as glucose could be demonstrated by our results. Although glucose was effectively sorbed in the biochar-amended soil, sorption did not act as a long-term protective mechanism against mineralization.

The biochar produced by slow pyrolysis and gasification, PL and PG, were the safest from the point of view of the response of the microbial biomass. In fact, the increase in the C use efficiency of the microbial biomass may have implications for soil C sequestration and it seems that these types of biochar may positively influence soil organic C preservation. In addition, these types of biochar do not cause detrimental effects when added to the soil, even in highest doses, resulting very resistant to degradation along time.

On the base of this result, PL was chosen to assess if the application of three pesticides has detectable adverse effects on soil microbial activity, and to evaluate if the addition of biochar modifies the toxicity or adverse effects of these chemicals in soil.

Modulation of the effects produced by these chemicals has been proven.

These results remark how different biochars have different interactions with soil, depending on their intrinsic physical-chemical characteristics. Also this thesis emphasizes the importance of future work focused on formulating guidelines to biochar applications to soil.

RESUMEN

El biochar, el carbón especial obtenido a través del proceso de pirólisis de la biomasa, se ha propuesto como un medio de captura de carbono a través del suelo. También se ha considerado útil para mejorar algunas de las propiedades pertinentes del suelo y para reducir los efectos adversos de los contaminantes.

Pero el uso de biochar debe garantizar, además de su conservación, que no genere efectos adversos en los organismos vivos, en las funciones ecosistémicas del suelo, o sobre los sistemas naturales adyacentes.

Esta tesis pretende contribuir al conocimiento en este sentido, mediante el estudio de:

- La capacidad del biochar para proteger la materia orgánica más lábil del suelo;
- Los efectos adversos causados por el biochar una vez incorporado en el suelo, en función del tipo de proceso de pirólisis y las dosis aplicadas; y
- La modulación de los efectos secundarios tóxicos de algunos pesticidas cuando se incorporan a los suelos que han recibido un aporte de biochar

Para alcanzar estos objetivos, se utilizaron tres tipos de biochar producidos a partir de la misma biomasa (astillas de madera de pino) como enmienda del suelo: biochar producido por pirólisis lenta (PL), el obtenido por pirólisis rápida (PR) y el obtenido a través de la gasificación de la biomasa (PG).

La adición de biochar genera cambios en la estructura y la biología del suelo, sea por la modificación positiva o negativa de su equilibrio físico y biológico.

Considerando que la comunidad microbiana juega un importante papel regulador en el suelo, condicionando las reacciones bióticas más importantes, la biomasa microbiana y su actividad respiratoria se utilizaron como indicadores de los efectos producidos por la adición de biochar.

Se han usado modelos de adsorción que se han utilizado para evaluar la capacidad del biochar para adsorber y proteger la materia orgánica más lábil del suelo. Se diseñó un experimento factorial completo para comprobar los efectos de tres factores individuales (biochar, nutrientes y adición de glucosa) y su relación con la mineralización de la materia orgánica del suelo.

Los resultados de este trabajo indicaron que cada tipo de biocarbón estableció diferentes interacciones con el suelo.

El ensayo ecotoxicológico sugirió que el biochar de pirólisis rápida (PR) es el menos recomendado si se utiliza en dosis superiores a la ED50 calculada.

No se obtuvo evidencia de interacciones de protección de materia orgánica lábil, como la glucosa, por nuestros resultados. Aunque la glucosa fue absorbida con eficacia en el suelo tratado con biochar, la absorción no actuó como un mecanismo de protección a largo plazo contra la mineralización.

El biochar producido por la pirólisis lenta o por gasificación, PL y PG, resultaron los más recomendables desde el punto de vista de la respuesta de la biomasa microbiana.

De hecho, el aumento de la eficiencia del uso de C por la biomasa microbiana puede tener implicaciones para el secuestro del C orgánico del suelo y parece que estos tipos de biochar pueden influir positivamente en la preservación de este C. Además, estos tipos de biochar no causaron efectos perjudiciales cuando se añadieron al suelo, incluso en las dosis más altas, resultando muy resistentes a la degradación a lo largo del tiempo.

Sobre la base de estos resultados, PL fue el biochar elegido para evaluar si la aplicación de tres plaguicidas podía tener efectos adversos detectables sobre la actividad microbiana del suelo, y para evaluar si la adición de biochar modifica la toxicidad o los efectos adversos de estas sustancias químicas en el suelo.

La modulación de los efectos producidos por estas sustancias químicas ha quedado demostrada.

Estos resultados remarcan cómo diferentes biochars ejercen diferentes interacciones con el suelo, en función de sus características físico-químicas intrínsecas.

En esta tesis se hace hincapié en la importancia de futuros trabajos centrados en la formulación de directrices para la aplicación de biochar en el suelo.

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Para recordar la importancia de la investigación ecológica, escribo esta frase que siempre ha representado la filosofía de mi vida:

"Only after the last tree has been cut down,
only after the last river has been poisoned,
only after the last fish has been caught,
only then will you find that money cannot be eaten."

Cree Indian prophecy

CONTENTS

ABBREVIATIONS

ANOVA→ analysis of variance

B → biochar

bC → befor Christ

C → carbon

CEC → Cation exchange capacity

d → days

E.C. → Electrical conductivity

G → glucose

h → hours

I → insecticide

OECD → Organisation for Economic Co-operation and Development

P0 → pine splinters

PG → biochar obteined from pine splinter through gassification

PL → biochar obtained from pine splinter through slow pyrolysis

PR → biochar obtained from pine splinter through fast pyrolysis

PAHs → polycyclic aromatic hydrocarbons

qCO_2 → microbial metabolic quotient

SOM → soil organic matter

WHC → water holding capacity

SIR → substrate-induced respiration

Chapter 1

1. INTRODUCTION AND RESEARCH OBJECTIVES

1.1 Climate change: one of the current most important environmental problems

Anthropogenic climate change is one of the most important issues that humanity will face in the coming years. Nowadays, it is possible to observe the first consequences, long been hypothesized and modelled, as the increasing of the temperature of the oceans (Roemmich, 1992), the change of thermohaline circulation (Bryden et al., 2005; Roether et al., 1996), the melting of glaciers (Curran et al., 2003), to name only a few. It is almost commonly accepted the role that increasing greenhouse gases (such as CO2, CH4 and N2O) has on climate change. One of the main reasons for the increasing atmospheric concentration of greenhouse gases is the use of fossil fuels, a practice that can be reduced only through efforts at the international level. In the context of climate mitigation strategies, one of the possible and viable alternatives to fossil fuels is the use of renewable energy. The discovery of Terra Preta (black soil) in the Amazon rainforest, suggest that incomplete charring of biomass could be an important way to produce bioenergy, biofuel and/or biogas giving the possibility to store CO2 in the soil at the same time (a strategy named carbon-negative).

1.2 The discovery of Terra Preta

The “black earth of indios” (Terra Preta do indios in Portuguese) is the significant name of a particularly soil that arouse the attention of the entire global scientific community. It is believed that Terra Preta is the result of the pedological modification of a prior soil as a result of the activities of indigenous cultures. The large amount of pottery and objects of human origin found in these lands clearly reveals its anthropogenic origin (Costa et al., 2004). The discovery of this particular soil happened in the Brazilian Amazon where, in fact, were locates numerous sites characterized by a soil whose features are absolutely different from adjacent land, in spite of mineralogy and texture are the same (Zech et al., 1990).

In contrast to the strongly altered typical soils of the Amazon rainforest (especially Ferralsol and Acrisol), which are red coloured, very unproductive because in the abundance of kaolinite, aluminium and with acid pH, the very fertile

soils called *Terra Preta* do Indios have a black colour, an alkaline pH and host endemic microorganisms (O'Neill, 2006; figure 1.1).

Figure 1.1 A nutrient-poor oxisol (left); an oxisol transformed into fertile terra preta (right). (www.treepower.org).

Terra Preta is characterized by a high content of carbonaceous material (charcoal), over 70 times more than the surrounding soils and up to a depth of 40-80 cm, produced by incomplete combustion of plants parts (probably the remains of fires to cook food) that were voluntarily introduced into the soil by local people (slash and char strategy) over thousands of years (Erikson et al., 2003; Falcão et al., 2003; Glaser et al., 2004). According to Glaser et al., 2001, the carbonaceous fraction owes its chemical and microbiological stability to its complex aromatic polycyclic chemical structure that is able to persist in the environment for centuries. During this period, its aromatic structure is slowly oxidized in surface producing carboxylic groups, and this increases the capacity of the carbonaceous particles to retain the nutrients. Probably the pre-Columbian civilization, that inhabited the Amazon between 2500 and 500 bC, is responsible for this "black fortune" which characterizes relatively large areas of the Amazon basin and other Sud-américian regions (Glaser et al., 2001; Lehmann, 2003). Similar soils have also been described in West Africa (Fairhead and Leach, 2009) and in Borneo (Sheil et al., 2012).

The technique of "slash and char", currently used in Amazonia, has been practiced in various parts of the world as a mean of fertilizing the soil for agricultural purposes.

In Italy, the *carbonaia* o *pojat* (figure 1.2) was a technique widely used in the past to transform the wood, preferably beech, but also spruce, larch, chestnut, holm-oak, oak and pines in charcoal.

Figure 1.2 On the left a museum reproduction of a Carbonaia o Pojat showing the inner part (www.isentieridelmoro.it); on the right a photo of a real Carbonaia o Pojat in function (www.tuscanyiloveyou.com).

Carbonaia is an ancient technique to control the amount of oxygen in the process of combustion of the wood, so as to avoid, on the one hand, the fire goes out and, secondly, that the fire takes effect and burns the woodpile.

The carbonization process could take up to 5 or 6 days. Usually in the Carbonaia from 30 to 40 tons of wood were piled up that produces 6 to 8 tons of coal. The production of charcoal has been an important economic activity for several local realities of Italy in past centuries until the 50s and 60s of the last one (Mantovani, 2006).

1.3 From the past to the future: Biochar

The observations of the ancient agricultural management practices of slash and char that created Terra Preta, inspirited the supplementation of soil with the named biochar, an innovative method designed to sequester carbon (C), on a global scale, while concurrently improving soil functions (Verheijern et al., 2009) and avoiding

short- and long-term detrimental effects to the wider environment as well as human and animal health (Verheijern at al., 2009).

Biochar is a fine-grained and porous substance, similar to charcoal, intended to be added to soil to improve its fertility (figure 3). It is the solid fraction of a pyrolysis process, a thermochemical decomposition of organic materials obtained by the application of heat in absence or limited supply of an oxidizing agent, normally oxygen (Sohi et al., 2009), used to produce renewable energy. On the other side, some of the by-products of this process can be condensed into "bio-oil," a liquid that can be upgraded to fuels including biodiesel, and synthesis gas (syngas). A portion of the non-condensable fraction is burnt to heat the pyrolysis chamber, and the rest can provide heat or fuel to an electric generator (Tenenbaum, 2009).

Figure 1.3 Image showing the appearance of Biochar, similar to charcoal. (www.ambienteambienti.com).

The term biochar was originally associated with a specific type of production, known as "slow pyrolysis". In this type of pyrolysis process, oxygen is absent, heating rates are relatively slow, and peak temperatures relatively low. However, the term biochar has since been extended to products of short duration pyrolysis at higher temperatures known as "fast pyrolysis" and to new techniques such as microwave (Soshi at al., 2009; Shiung Lam and Chase, 2012) and hydrothermal conversion (Brown, 2009; Libra et al., 2011) that actually are the best way to product char by agricultural and urban liquid wastes (Libra et al., 2011). A charred material is

also formed during “gasification” of biomass, which involves thermal conversion at very high temperature (800°C) and in the partial presence of oxygen. This process is designed to maximise the production of synthesis gas (syngas). Materials produced by gasification differ from biochar obtained by the above mentioned technologies in physico-chemical properties, such as particle pore size and heating value (Prins et al., 2006) and have industrial applications, such as production of chemicals (methanol, ammonia, urea) rather than agricultural applications (Saran Soshi at al., 2009). Biochar can be produced by a large variety of feedstocks, including papermill waste (Van Zwieten et al., 2009), greenwaste (Chan et al., 2007), animal manure (Cao et al., 2011), and sewage sludge (Hossain et al., 2010; Hossain et al., 2011; Méndez et al., 2012; Lehmann and Joseph, 2009b).

1.4 Technologies of production and physical-chemical characteristics of biochar

The physical-chemical characteristics of biochar depend not only on the feedstock (biomass), but also on the methods of carbonization and all the operations included pre- and post-treatment of biomass and biochar. These processes mainly influence the degree of alteration of the original structures of biomass, through microstructural rearrangements, friction during the process and formation of fractures (Amonette and Joseph, 2009; Enders et al., 2012; Downie et al., 2009). Pyrolysis maximum temperature and heating rate are the parameters which mostly affect physical-chemical changes that occur in matter and the nutrient retention from feedstock to char (Kookana et al., 2011). Table 1.1 summarizes the pyrolysis processes described before.

Table 1.1 Most important parameters and products (in % of wt) of different carbonization processes.

PARAMETERS	PROCESSES				
	Fast pyrolysis	**Slow Pyrolysis**	**Microwave pyrolysis**	**Hydrothermal conversion**	**Gasification**
Temperature	~450-500°C	~400-500°C	frequencies of: 915 MHz (λ= ~33 cm) and 2.45 GHz (λ = ~12 cm)	~180–250°C	~800°C
Vapour residence time	~1-2 s	h-week	no vapour residence time	no vapour residence time, ~1–12 h processing time	10-20 s
Biochar production	~12%	~35%	~7%	~50-80%	~10%
Bio-oil production	~75%	~30%	~85%	~5-20%	~5%
Syngas production	~13%	~35%	~8%	~2-5%	~85%
References	Fierro, 2011; Bridgwater and Peacocke, 2000	Şensös, 2003	Soshi et al., 2009; Shiung Lam, 2012	Brown 2009; Libra et al., 2011	Balat, 2009; Catherine, 2011

Most biochars contain very few N and S because they volatize above 200 and 375ºC respectively; an exception are those containing large amounts of N (Sohi et al., 2010), such as a biochar from sewage sludge studied in this thesis. Cation exchange capacity (CEC) generally decreases with increasing pyrolysis temperature partly due to the loss of carboxylic biochar surface functional groups (Enders et al., 2012) while pH tends to increase with temperature, as well as ash content (Sohi et al., 2010; Enders et al., 2012).

The temperature range at which these phenomena occur depends on the type of biomass. In biochar production it is useful pay attention in changes in the elemental composition of C, H, O and N and relationships associated with them. In particular, the molar relationship between H/C and O/C are used for the measurement of the degree of aromaticity (Baldock and Smernik, 2002; Braadbaart et al., 2004; Hammes et al., 2006). In general, the H/C ratios and O/C in the biochar produced

experimentally decrease with increasing temperature (Shindo, 1991; Baldock and Smernick, 2002), and increase with decreasing the residence time (Almerndros et al., 2003). Using X-ray diffraction is possible to see how the structure of biochar is generally amorphous, but with some crystalline structures inside formed by highly conjugated aromatic components. The crystalline areas can be viewed as stacks of sheets of aromatic compounds (graphene) cross-linked randomly (Lehmann and Joseph, 2009a). Such as graphite, these structures are good conductors despite their small size (Carmona and Delhaes, 1978). The other non-conductive parts that complement the biochar structure are aliphatic and aromatic organic compounds of complex chemical composition (Antal and Grønli, 2003; Lehmann and Joseph, 2009a), including volatile compounds and inorganic components (ash) (Emmerich et al., 1987). This structure is completed by the voids present in the pores (macro, meso and micro pores) and fracture morphologies and cells cavities of biomass origin (figure 4).

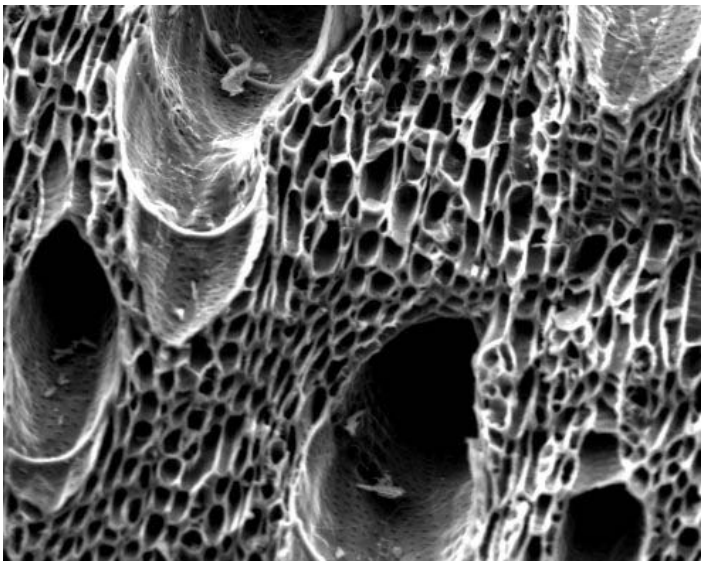

Figure 1.4 Scanning electron microscopy (SEM) of the structure of biochar derived from wood with a slow pyrolysis. (www.PermaCultureScience.org).

The porous structure of biochar because of its high surface area (from ten to a few hundred square meters per gram), their ability to adsorb organic matter, nutrients and gas represent an ideal habitat for host colonization, growth and reproduction of bacteria, actinomycetes and arbuscolar mycorrhizal fungi. Microbes would be protected from their natural predators (Saito and Muramoto, 2002; Warnock et al.,

2007), and those that are less competitive in the soil, may find benefit from a more protected position (Ogawa, 1994). The biggest contribution to total surface area of biochar comes from micropores. It has been demonstrated that high temperatures and high retention times tend to increase their number (Zhang et al., 2004; Kookana et al., 2011).

1.5 Advantages and disadvantages of using biochar

The biochar application in agriculture has been documented from the past until today. It increases nutrient uptake by plants, and as consequence their productivity (Chan and Xu, 2009), for its ability to retain nutrients in the soil and reduce leaching losses (Glaser, 2001; Ventura et al., 2013).

Among the initiatives to mitigate the effects of climate change, has been proposed to increase the use of renewable energy and biofuels to reduce dependence on fossil fuels and CO2 emissions. In the best case, these actions only will help prevent further emissions of CO2 into the atmosphere, which are called carbon-neutral strategies (Lehmann, 2007a). The biochar is very rich in carbon associated with polyaromatic structures and has been proposed as a mean of C sequestration through the soil (carbon-negative methodologies) (Lehmann, 2007a; Fowles, 2007; Steiner, 2007; Laird, 2008). Other uses are for the production of renewable energy and bio-chemicals that could replace petroleum. Biochar con also reduce the emission of CO2 and N2O (Spokas et al., 2009; Cayuela et al., 2010; Cayuela et al., 2013; Spokas, 2013).

It has been documented that the presence of biochar in the soil can improve some of its properties such as structural stability, water holding capacity, cation exchange capacity, nutrient retention, etc. (Lehmann, 2007a). But these effects clearly positive, depend on the residence time of biochar in soil (Preston and Schmidt, 2006; Fowles, 2007; Lehmann, 2007b). On the other hand, biochar has a big adsorbent capacity (Braida et al., 2003; Zhu and Pignatello, 2005; Koelmans et al., 2006; Brändli et al., 2008, Downie et al,. 2009) due to its specific surface area and porosity that has been related in previous work, which can reduce adverse effects of pollutants in soil and can contribute to blocking contaminants (Chen et al., 2005; Koelmans et al., 2006; Qiu et al., 2008). Furthermore, biochar can be used to restore soil contaminated by pesticides (Cao et al., 2011) or heavy metals (Uchimiya et al.,

2011). Also provides a microbial habitat for many group of soil microorganisms (Thies and Rillig, 2009; Lehmann et al., 2011).

The big difference between biochar and a normal charcoal is the particular chemical property that permits the cation retention, a property that increase with biochar ageing and surface weathering (Cheng et al., 2008; Cheng and Lehmann, 2009).

But the potential effects of the use of biochar in soil are still quite unknown and research in this area is relatively new. As every new discovery, it puts the emphasis on the positive qualities but many other aspects such as reducing pesticide efficiency (Kookana, 2010; Kookana, 2013) or biodegradation (Jones et al., 2011), effects on soil organic matter leading to its loss (Wardle et al., 2008; Zimmerman et al., 2011), potential ecotoxic effects (Kookana et al., 2011) or ecological consequence on soil organisms and soil functions still must be investigated (McCormack et al., 2013; Jones et al., 2012).

The carbon sequestration capacity of soils should be estimated not only from the point of view of quantity, but also by the quality in relation to the functions performed by the soil and its organic matter (Almendros, 2008). The use of biochar as a mean of carbon sequestration must ensure, in addition to their conservation into the soil, no adverse effects on organisms living in them, on soil characteristics functions, or adjacent natural systems. Another environmental risk that should be considered in relation to the use of biochar is the possible presence of potentially toxic substances or elements (e.g. PAHs, PCDD / F) generated during the pyrolysis process or already present in the raw materials that are pyrolyzed (Shinogi et al., 2003).

Even if the International Biochar Initiative (IBI, 2013) published a provisional guideline to characterize the biomass feedstock and resulting biochar many secondary effect of the use of biochar must be examined. For example: it is fundamental to consider the possible use of biochar in function of the easiness of production, costs, and transport. Producing and using biochar within the same country may be an excellent carbon negative system, but if we add the transport that requires a huge consumption of fuel, it would probably be just business. Nowadays, the use of biochar is an optimistic assumption, only marginally economically viable,

given the absence of a biochar market and the limited number of production scale biomass pyrolysis plants (USDA, 2013).

1.6 The main goal of the thesis and their organisation in chapters

In view of what discussed above, this thesis proposes to contribute to assess the possibilities of using biochar to improve soil fertility and to sequester C, studying some interactions with organic compounds, its stability over time, and the ecotoxicological effects in the environment especially in presence of pesticides. All these arguments are discussed and assayed in three chapters.

Chapter 2: *"Can biochar protect labile organic matter against mineralization in the soil?"*

The aims of this experiment were to:

-Measure the mineralization of labile organic matter in a soil treated or not with biochar, on which also will test the effect of adding or not a nutrient solution.

-Quantify the amount of labile organic matter that can be adsorbed in the same soil amended or not with biochar.

This work should allow elucidate if biochar has some protective capacity of organic compounds and what extent this property is explained by the absorbent characteristics of the biochar.

Chapter 3: *"Impact of different types of biochar on soil microbial activity: a dose-response study"*

The aim of this chapter was to study the microbial biomass, through fumigation-extraction and substrate-induced respiration methods, in order to assess the potential ecotoxicological effects of adding biochar to the soil. This assessment involves the incorporation of different types of biochar to the same soil in an exponential dose gradient, as to observe whether there is a dose from which to warn the inhibitory effects. The analysis of different curves of dose / response to biochar samples obtained by different pyrolysis processes should provide information on what is the most secure technology from the point of view of ecotoxicological risk of application of biochar to soil.

Chapter 4: *"Would the addition of biochar modulate the adverse effects of some pesticides on soil microorganisms?"*

This chapter evaluated if the application of three current pesticides, an insecticide, a fungicide and an herbicide, have detectable adverse effects on soil microbial activity, and how the presence of biochar modifies the toxicity or adverse effects of these chemicals in soil.

In other words, the objective of this study is to provide information on whether the presence of biochar in a soil can, due its adsorbent capacity, help to mitigate the adverse side effects of common pesticides may have on soil microorganisms.

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Chapter 2

2. CAN BIOCHAR PROTECT LABILE ORGANIC MATTER AGAINST MINERALIZATION IN THE SOIL?

2.1 Introduction

Biochar is a carbon-rich product generated from biomass through pyrolysis (Lehmann et al., 2006: Lehmann, 2007a). The chemical and physical characteristics of biochar depends not only on the original characteristics of the feedstock but also on the conditions of the pyrolysis process, essentially temperature (Lehmann, 2007b; Chen et al., 2008) and time (Encinar et al., 1997) of charring. Its chemical structure generally combines different aromatic carbon structures and can be considered as a transitional form between the carbohydrate-based biomass and the graphitic C (Ogawa et al., 2006), which can also appear as microcrystalline structures (Joseph et al., 2009). These chemical structures, usually named black carbon, are supplemented by voids in the pores (macro-, meso- and micro-pores) derived from cellular fractures of plant cells (Fukuyama et al, 2001). Biochar usually presents a large surface area (Downie et al., 2009) which, together with its structure and chemical properties, gives a great sorption capacity that has been largely demonstrated for a wide range of organic compounds (Cornelissen et al., 2005; Zhang et al., 2006; Chen et al., 2008; Cao et al., 2009).

The role of biochar on soil fertility has been extensively studied during the last decade (Woolf et al., 2010). In the tropical humid soils, the main effects of the biochar on soil fertility seems to be related to the preservation of nutrient losses by leaching (Glaser et al., 2002; Madari et al., 2003; Steiner et al., 2007; 2008). In arid or semiarid climates, such as in a large portion of the Mediterranean area, the risk of nutrients leaching is only high when rainfall exceeds the evaporative demand (Milroy et al., 2008) or in irrigated land. Although worthy of being considered, the effect of biochar on preventing the leaching of nutrients might not be its most significant effect in arid lands. Mediterranean soils are generally poor in organic matter (Rovira & Vallejo, 2003; Zdruli et al., 2004; Larchevêque et al., 2006; Rovira & Vallejo, 2007). In these soils, the major effect of adding biochar could be most likely related to the increase in the total amount of soil organic matter (SOM) due to its intrinsic recalcitrance (Lehmann et al., 2009; Nguyen & Lehmann, 2009) and to a suggested reduction of the mineralization rate of the native SOM (Liang et al., 2009; Thies &

Rillig, 2009; Marchetti et al., 2012). The way on which biochar can reduce the mineralization of the SOM can be probably related to its sorbent properties, which could restrict the microbial access to essential nutrients, therefore limiting its activity, but also to an increase in the amount of SOM physically occluded and chemically adsorbed, and then protected. It has also been suggested that the high C/N ratio of the biochar can cause a significant N immobilization (Yin Chan & Zu, 2009) that can reduce the mineralization of the native SOM as well. Nevertheless, some authors have suggested a priming effect of black carbon that could enhance the mineralization of added glucose (Hamer et al., 2004) or forest humus (Wardle et al., 2008), thus maintaining unclear the effect of the biochar on the mineralization of the native SOM (Luo et al., 2011). Concerning the soil chemical fertility, Lehmann et al (2009) remind that the addition of nutrients limits the decomposition and could increase the mineralization of the native organic matter, as demonstrated by Hobbie (2000). Nevertheless, Brodowski et al. (2007) did not notice any effects of commercial fertilizers on the degradation of biochar in German arable soils.

Despite the diversity in the results collected by the literature, it seems that in most cases the addition of biochar tends to limit the mineralization of SOM, while the addition of nutrients accelerates. This gives a combination of factors which could be probably used to regulate the decomposition of labile organic matter contained in manure or organic amendments applied to agricultural soils, and that could help to stabilize SOM and thus sequestering C into soils.

the aim of this work was (i) to assess the effects of the addition of biochar and nutrients on the SOM mineralization in an artificial soil amended with glucose, and (ii) to measure the amount of labile organic matter (glucose) which can be sorbed, then partially protected in the same soil, amended or not with biochar.

2.2 Material and methods

2.2.1 Experimental design

A fully factorial experiment was designed to check the effects of three single factors (biochar, nutrients and glucose) and their interactions on the whole SOM mineralization. A description of the different combinations of factors that have been tested is shown in table 2.1.

Table 2.1 Different treatments tested in the soil incubation experiment. The amounts of C added as biochar, glucose and the inoculum have been computed from the application rate and by the concentration of C in the biochar (805.7 g kg^{-1}), glucose (400 g kg^{-1}) and the soil inoculum (207.8 µgC ml^{-1}). The soil additions were coded as: B+/- (biochar), N+/- (nutrients), G+/- (glucose).

	Biochar (50 g kg^{-1})	Nutrient solution (40 ml kg^{-1})	Glucose (4.2 g kg^{-1})	Inoculum (25 ml kg^{-1})
C added	40.29 g kg^{-1}	-	1.68 g kg^{-1}	5.2 mg kg^{-1}
Code				
B- N- G-	-	-	-	+
B- N- G+	-	-	+	+
B- N+ G-	-	+	-	+
B- N+ G+	-	+	+	+
B+ N- G-	+	-	-	+
B+ N- G+	+	-	+	+
B+ N+ G-	+	+	-	+
B+ N+ G+	+	+	+	+

2.2.2 *Model soil and amendments*

The experimental soil was derived from the artificial soil proposed by the OECD for testing of chemicals (OECD, 1984), but Sphagnum peat was not used to avoid the presence of organic matter. Therefore, the artificial soil was composed by 38.5% coarse sand (0.2 to 1 mm), 38.5% fine sand (0.05 to 0.2 mm), 22% kaolinite clay and 1% $CaCO_3$.

Biochar was obtained by fast pyrolysis (Ikerlan Energy, Alava's Technology Park, Basque Country, Spain) from a mixture of *Pinus pinaster* Aiton (from the Landes, France) and *Pinus radiata* D. Don (from the Basque Country) woodchips previously air-dried to lower the water content to 10 % and ground to sawdust. The pyrolysis temperature was 440-480ºC, and the residence time of the biomass at this temperature was about 2 seconds. The total C concentration of the biochar was 805.7 g kg^{-1} (489.3 g kg^{-1} in the woodchips), and the total N was 1.9 g kg^{-1} (1.5 g kg^{-1} in the woodchips), which gives C/N ratios of 424 and 326 for the biochar and the sawdust respectively. Biochar was added to the soil at a dose of 50 g kg^{-1}, which

means a surface application rate of about 60 Mg ha^{-1} (considering 50% fine earth, 1200 kg m^{-3} bulk density and a depth of 20 cm).

The nutrient solution was prepared following Cheng et al. (2006). It contained 4mM NH_4NO_3, 4mM $CaCl_2$, 2mM KH_2PO_4, 1mM K_2SO_4, 1mM $MgSO_4$, 25µM $MnSO_4$, 2µM $ZnSO_4$, 0.5µM $CuSO_4$ and 0.5µM Na_2MoO_4, and was applied at the dose recommended by the authors (40 ml kg^{-1}) and mixed with the amount of water required to achieve a soil moisture of 0.5 of their water holding capacity (WHC). Glucose was added at a dose of 4.2 g kg^{-1}. This dose was found to gives the maximum potential respiration rate in a previous test (24 h) with the artificial soil amended with nutrients but without biochar.

All the incubated samples received a microbial inoculum to ensure the existence of a sufficient amount of microorganisms that could use the provided C sources. As the organic C of the soil inoculum was 207.8 µgC ml^{-1} and was added to the samples at a dose of 25 ml kg^{-1}, this means that 5.2 mgC kg^{-1} was added to all samples.

2.2.3 Soil incubation and CO_2 measurement

Soil samples (n=3) were incubated in the dark at 21ºC and at 0.5 WHC during 619 days. WHC was equivalent to 0.27±0.07 and 0.34±0.06g g^{-1} for the B- and the B+ samples, respectively. Prior to the preparation of the incubation, samples were inoculated and preincubated to ensure biological activity was not limited by a small amount of microbial biomass. The inoculum was obtained by extracting 70g of the A horizon (0-15 cm depth) of a *Typic Calcixerept* with 0.3L of sterile physiological serum (NaCl 9 g l^{-1}, 0.15 M). Soil suspension was shacked during 1h, and then liquid inoculum was obtained by centrifugation and filtration through glass wool to eliminate lightweight SOM particles. Inoculum was then spiked to the soil at a dose of 25 ml kg^{-1}.

Periodical measurements of C mineralized to CO_2 were carried out with 1M NaOH traps (Anderson, 1982) along the 619-days incubation to allow the mineralization of both active and slow organic matter pools. The length of the incubation periods between measurements was shorter at the beginning and was progressively enlarged as respiration decreased. Cumulative CO_2-C results were

fitted to a first order kinetic model shown in equation 1 (Paul et al., 2006; Ribeiro et al., 2009):

$$CO_2 - C_t = Org - C_0 \cdot (1 - e^{(-k \cdot t)}) \qquad [1]$$

Where CO_2-C_t is the cumulative respired C at time t, Org-C_0 is the total amount of organic C at the beginning of the incubation, and *k* is the mineralization rate of the organic matter along the incubation. As *t* is expressed as days, the units of *k* are d^{-1}, and the mean residence time (MRT) of the soil organic matter, which is the inverse of *k*, is computed as days. Respiration rate (mg CO_2-C kg^{-1} d^{-1}) was computed for each period of incubation as the amount of CO_2-C produced during the time period divided by the length of the period and the dry weight of the samples.

The effects of biochar, nutrients and glucose on cumulative CO_2-C production and MRT of organic matter were analyzed by ANOVA. Firstly, a two-way ANOVA (Biochar, Nutrients) of the cumulative CO_2-C released by the samples not amended with glucose was used to assess the effect of the addition of the biochar and its interaction with nutrients. Secondly, a two-way ANOVA (Glucose, Nutrients) of the cumulative CO_2-C released by the samples not amended with biochar was used to assess the effect of the addition of glucose and its interaction with nutrients. Finally, a three-way ANOVA (Biochar, Glucose, Nutrients) of the cumulative CO_2-C released by all the samples was used to assess the joint effects of the different C sources on C mineralization.

2.2.4 *Glucose sorption assay*

These measurements were only made with the samples B-N-G- and B+N-G- of the same soil described above, without microbial inoculum. Soil samples (1g) with or without biochar (50 g kg^{-1}) were imbibed with 10 ml of glucose solutions at different concentrations (0.5 to 100 mg l^{-1}) during 8h at 4ºC in polypropylene centrifuge tubes with regular agitation (twice per hour). The amounts of glucose added to the soil ranged from 0.004 to 0.909 g kg^{-1}. The time of imbibition was determined in a previous test by the time needed to reach a constant amount of glucose sorbed. After soaking, soil suspensions were centrifuged and the supernatant containing the non sorbed glucose filtered. The imbibition solutions and the extracts were stored overnight at 3ºC until the next day, when they were analyzed.

Glucose was analyzed by the colorimetric method of Dubois et al. (1956). Although this is not a glucose-specific method, due to the composition of the artificial soil, it can be assumed that there were no significant amounts of other sugars in the solutions. The amount of sorbed glucose, S (µg g^{-1}) was calculated as the difference between the total amount added and the amount of glucose in the extract. Two different models were selected to assess the equilibrium isotherms of glucose sorption: the model of Freundlich (Kano et al., 2000) and the Langmuir (Langmuir, 1918; Kano et al., 2000).

According Freundlich, sorption isotherms can be fitted to the equation 2, where S is the amount of glucose sorbed in soil at equilibrium (µg g^{-1}), C_e is the amount of glucose in the supernatant (µg ml^{-1}) and K and n are constants of the model that explains how favorable is the sorption process (n) and the sorption capacity of the solid matrix (K), also called as the sorption affinity.

$$S = K \cdot C_e^n \qquad [2]$$

According Langmuir, the sorption isotherms can be fitted to the equation 3, where in addition to the S and C_e terms described above, ST and C are constants of the model that explain the rate of sorption (ST) and the sorption capacity (C).

$$S = \frac{ST \cdot C_e}{C + C_e} \qquad [3]$$

Having analyzed the values of C_e and computed the amounts of sorbed glucose (S), the most probable values of the terms *K* and *n* of the Freundlich model, and of the ST and C terms of the Langmuir model, were fitted with the statistical software SAS. The confidence intervals of the fitted parameters were used compare the sorption characteristics of the synthetic soil amended or not with biochar and, therefore, to assess the effects of the addition of biochar onto the glucose sorption.

2.3 Results

2.3.1 Effects of biochar, nutrients and labile organic matter addition on soil respiration

As expected, the lower CO_2-C production (Figure 2.1) was found in the samples which received only the soil inoculum (5.2 mgC kg^{-1}) as a sole C source. The B-N-

G- and B-N+G- treatments produced 192.5±13.0 and 246.7±47.9 mg CO2-C kg, respectively, and the addition of nutrients was not significant (P=0.3173, Table 2.2).

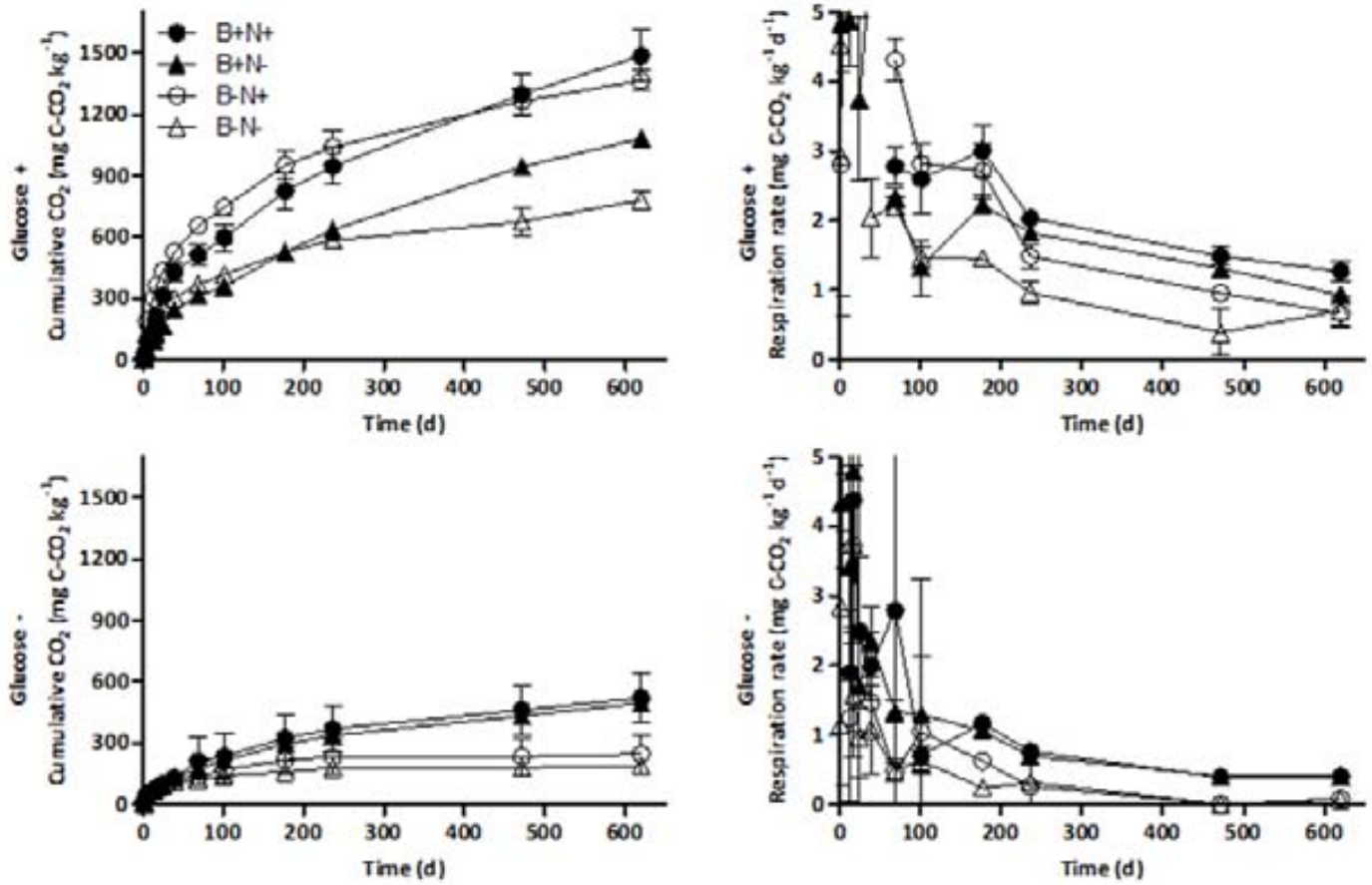

Figure 2.1 Cumulative C -CO_2 production (left) a nd r espiration r ates (right) o f s amples tr eated with (above) o r without glucose (below) d uring t he whole experimental period. The Y axis o f the graphs o n the r ight hav e been truncated to a maximum value of 5 mg CO_2-C kg^{-1} d^{-1} to clearly display the changes of respiration rates along the incubation. Thus, higher values of the first days are not plotted.

The addition of biochar as a C source increased significantly the cumulative CO_2-C release ($P<0.0001$) of the treatments not amended with glucose (495.9±9.0 and 522.2±59.5 mg CO_2-C kg^{-1} for the B+N-G- and B+N+G-, respectively), but no significant effect of the addition of nutrients was found between these samples ($P=0.323$). If this C release is taken as the sum of the CO_2-C provided by the inoculum and the CO_2-C provided by the biochar, the difference between the CO_2-C production of the samples B+N-G- and the samples B-N-G- could be used as an estimation of the basal C mineralization of the biochar. In the same way, the difference between the samples B+N+G- and B-N+G- gives an estimation of the increased C-mineralization of the biochar when nutrients were added. These estimations gave C mineralization estimates ranging from 276 and 303 mg CO_2-C kg^{-1} when biochar was incubated with or without nutrients, which means in both cases a C loss of 0.4% in a year.

The addition of glucose without biochar caused a noticeable increase of the amount of CO_2-C released ($P<0.0001$) which ranged from 781.95±21.75 and 1365.12±24.48 mg CO_2-C kg^{-1} depending on the addition of nutrients (values of samples B-N-G+ and B-N+G+, respectively, $P<0.0001$). The addition of nutrients caused a major increase in CO_2-C release of the glucose amended samples than in unamended samples ($P<0.0001$). Applying the same correction for the CO_2-C produced by the mineralization of the inoculum, these values gave cumulative CO_2-C amounts ranging from 589 to 1118 mg CO_2-C kg^{-1}, which implies C losses in a year of 21% without nutrients and 39% when nutrients were added.

The production of CO_2-C in samples amended with both biochar and glucose ranged from 1082.63±11.60 to 1487.21±62.85 mg CO_2-C kg^{-1} (B+N-G+ and B+N+G+ samples, in that order). CO_2-C released increased mainly by the addition of glucose ($P<0.0001$) and nutrients ($P<0.0001$), but also by the addition of biochar ($P<0.0001$). As seen before, the addition of nutrients caused a major increase in soil respiration when glucose was added ($P<0.0001$), but not when biochar was added ($P=0.0634$). Subtracting the C released by the inoculum, cumulative CO_2-C production ranging from 890 to 1232 mg CO_2-C kg^{-1} can be computed for the samples treated with biochar and glucose, with or without nutrients (B+N-G+ and B+N+G+, respectively). Therefore, C losses ranging from 1.3 to 1.8% of the total C

(biochar + glucose) could be estimated respectively in samples B-N-G+ and B+N+G+.

When nutrients were not added to the soil, the CO_2-C lost from the B+G+N- samples (corrected to 890 mg CO_2-C kg^{-1}) was virtually equal to the sum of the CO_2-C produced by the mineralization of the biochar (B+G-N-, 303 mg CO_2-C kg^{-1}) and by the mineralization of glucose (B-G+N-, 589 mg CO_2-C kg^{-1}). In the samples enriched with nutrients, the CO_2-C produced by the B+G+N+ samples (corrected to 1232 mg CO_2-C kg^{-1}) was only slightly lower than the sum of the CO_2-C produced by the mineralization of the biochar (B+G-N+, 276 mg CO_2-C kg^{-1}) and by the mineralization of glucose (B-G+N-, 1118 mg CO_2-C kg^{-1}).

Concerning the pattern of CO_2-C release of samples amended with biochar and glucose, biochar reduced the release of CO_2 during the first stage of incubation, but from day 400 in the samples with nutrients and from day 200 in the samples without nutrients, the total production of CO_2-C increased in samples treated with biochar. On the contrary, when the samples were not enriched with glucose, the addition of biochar always gave the highest cumulative CO_2 production.

The mineralization rates of the organic matter (right graphs on figure 2) were always higher at the beginning of the incubation, but a fast stabilization was seen in all cases. In fact, the mineralization rate dropped at the end of the incubation to values ranging from 0.67±0.20 to 1.28±0.15 mg CO_2-C kg^{-1} d^{-1} for the glucose-enriched samples, and to values ranging from 0.08±0.06 to 0.41±0.08 mg CO_2-C kg^{-1} d^{-1} for the samples not enriched (small graphs on the figure 1). From the day 200-250, the addition of biochar increased the respiration rates in all samples enriched with glucose. In the samples not enriched with glucose, the addition of biochar increased the respiration rate almost throughout all the incubation period.

Table 2.2. Results of the ANOVAs of the cumulative CO_2-C released by the samples along the 619-d of incubation.

Samples analyzed (C sources)	***Source of variation / Factors***	**Cum. CO_2-C F-Value**	**Cum. CO_2-C P-Value**
B- G- (inoculum)	Nutrients	1.190	0.3173
B+/- G- (biochar, inoculum)	Biochar	55.020	<0.0001
	Nutrients	1.063	0.3228
	Biochar x Nutrients	0.127	0.7277
B- G+/- (glucose, inoculum)	Glucose	824.820	<0.0001
	Nutrients	114.860	<0.0001
	Glucose x Nutrients	79.146	<0.0001
B+/-G+/- (glucose, biochar, inoculum)	Biochar	89.205	<0.0001
	Glucose	944.773	<0.0001
	Nutrients	101.465	<0.0001
	Biochar x Glucose	2.165	0.1541
	Biochar x Nutrients	3.788	0.0634
	Glucose x Nutrients	73.195	<0.0001
	Biochar x Glucose x Nutrients	2.021	0.1680

The application of the first order kinetic model of the organic matter mineralization allows computing a potential mean residence time (MRT) of the organic matter for all the measured treatments (Figure 2.2). MRT of organic matter were notably increased by the addition of biochar ($F=110.7$, $P<0.0001$), and decreased by the addition of glucose ($F=18.9$, $P=0.0005$), but no overall effects of the addition of the nutrients were observed ($F=1.4$, $P=0.2497$). However, significant interactions of nutrients x biochar ($F=5.5$, $P=0.0324$) and nutrients x glucose ($F=4.9$, $P=0.0417$) were found, indicating that the addition of nutrients reduced the MRT particularly in biochar or glucose treated samples. MRT of biochar-amended samples ranged from 158±31 days for the treatment B+N+G- to 265±21 days for the treatment B+N-G+. Concerning the samples without biochar, the MRT of the organic matter ranged from 34±5 days for the treatment B-N-G- to 86±8 days for the treatment B-N+G+.

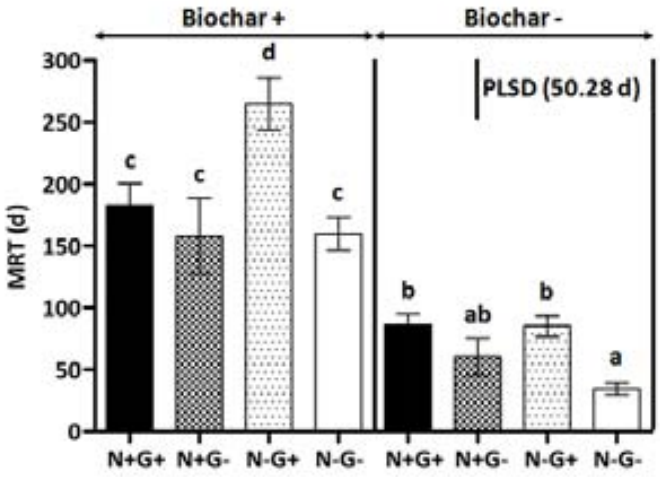

Figure 2.2. Mean residence times (MRT) of the whole soil organic matter on combined biochar, nutrients and glucose treatments (see table 1 for codenames). Error bars correspond to standard deviation. Different letters indicate significant differences at P =0.05 in the ANOVA test.

2.3.2 *Glucose sorption on soil amended or not with biochar*

The values of the parameters of the Freunlinch and the Langmuir models and the evaluation of their goodness of fit are given in Table 2.3. Although there is a high variability in the results, all models could be fitted, but Freundlich isotherm gave the highest values of R2 and slightly lower error.

Table 2.3. Parameters of the Freundlich and Langmuir adsorption isotherms for glucose in a model soil amended or not with biochar. The coefficients of determination, R^2, have been computed as the ratio SSR/SST, being SSR the sum of squares of the regression, and SST the total sum of squares. The proportions of the error of the model (SSE, %) have been computed as the ratio SSE/SST, being SSE the error sum of squares.

	Freundlich				***Langmuir***			
	n	***K***	***R^2***	***SSE (%)***	***ST***	***C***	***R^2***	***SSE (%)***
Biochar amended soil	0.17 ±0.07	46.85 ±10.56	0.82	0.18	91.06 ±14.53	2.10 ±1.76	0.76	0.24
Control	0.04 ±0.09	21.23 ±5.80	0.53	0.47	25.73 ±4.40	0.31 ±0.49	0.55	0.45

The adsorption of glucose to control soil was significantly lower (Figure 2.3) than the adsorption to the soil amended with biochar. The maximum amount of glucose sorbed in the control soil was found to be 42.92±14.40 µg g^{-1} when the concentration in solution was 10.71±1.44 µg ml^{-1}, and did not increased when glucose concentration rose until 96.60±1.49 µg ml^{-1}. In contrast, similar concentrations of glucose in biochar-amended soil solution gave amounts of sorbed glucose ranging from 54.37±15.28 and 74.64±20.72 µg g^{-1} respectively. In the biochar-amended soil, increasing the concentration of glucose in soil solution increased the sorption to a maximum amount of sorbed glucose of 132.79±5.40 µg.g^{1}. The Freundlich's isotherm constants which describe the affinity of a compound to the sorbent (*n* and *K*) were clearly higher in the biochar amended soil (0.17±0.07 and 46.85±10.56 in that order) than in the control soil (0.04±0.09 and 21.23±5.80 respectively).

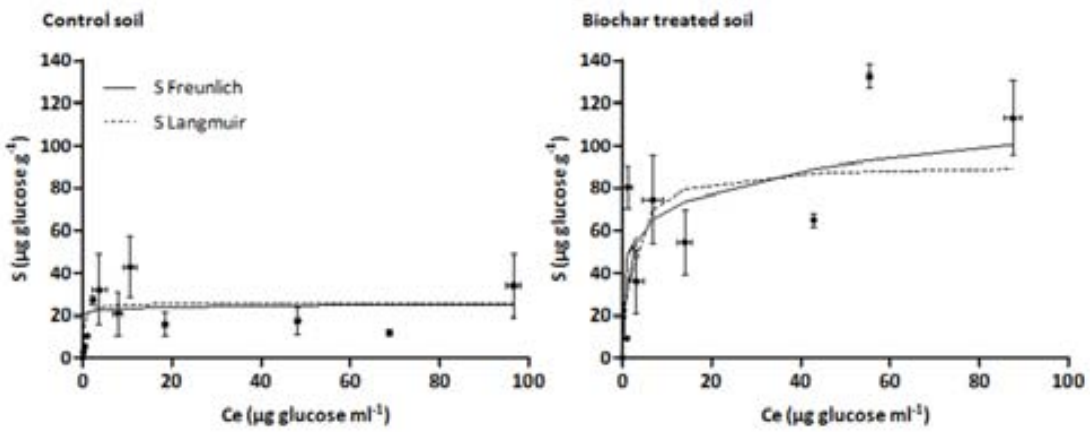

Figure 2. 3. Equilibrium adsorption isotherms of glucose for biochar treated soil (right) and control soil (left) fitted to the Freundlich equation (solid line) and Langmuir equation (broken line). The error bars indicate standard deviations. Ce corresponds to the concentration of glucose in the soil solution, and S to the concentration of glucose sorbed into soil.

2.4 Discussion

In the control soil not amended with glucose, no other organic C sources than the inoculum was added (5.2 mgC kg^{-1}). Even assuming that the water of the inoculum was saturated with CO_2 (ca 1.45 g l^{-1} at 25ºC), which implies an additional CO_2-C source of ca 10 mg C kg^{-1}, the amounts of CO_2-C released (with an average of 220 mg CO_2-C kg^{-1}) were much higher than the total C provided by the inoculum. This suggests that, at least in these samples, the CO_2-C released came mainly from the microbial decarbonation. It must be kept in mind that $CaCO_3$ was added to the artificial soil to ensure a soil pH similar for all treatments at a rate of 10 g kg^{-1}, which provided an extra C source of 1200 mgC kg^{-1}.

Although biochar is not all considered as inherently inert, their mineralization rates have been frequently considered much slower than other organic sources (Thies & Rillig, 2009). Nevertheless, it is also accepted that the fresh nature of the recently charred biomass could cause relatively high initial mineralization rates when applied to the soil (Lehmann et al., 2009). For instance, Nguyen and Lehmann (2009) found C losses ranging from 8.08 and 21.21% when oak wood and corn residues charred at 350ºC where incubated during one year in an unsaturated soil. Kuzyakov et al (2008) found C losses under 0.5% for biochar produced from *L. perenne* litter charred during 13h at 400ºC. In our experiment, the C loss of the biochar when incubated without glucose has been estimated as a 0.4% in a year. The measured C loss might

be due to the mineralization of a large variety of organic compounds. Steiner et al (2008b) and Thies and Rillig (2009) pointed that bio-oils, pyroligneous acids, alcohols, aldehydes, ketones and sugars can be present in biochars as surface-adhering pyrolysis condensates, which can be easily mineralized by soil microorganisms. Before that, Knicker (2007) suggested that the common model of biochar as a graphite-like material formed mainly by highly condensed polyaromatic clusters might be over-simplified, and that this type of structures might be combined with highly oxidizable heat-altered biopolymers that facilitate both microbial attack and dissolution. In our case, the relative high temperature of pyrolysis (between 440 and 480ºC) suggests a highly transformed biochar, but the low time of charring probably allows the presence of both partially charred domains and low molecular weight condensates adhered to biochar surfaces.

The computation of the MRT of biochar in soil gave values that suggest the persistence of biochar in soil should be much lower than reported by several authors, which is usually between 600 and 9000 years (Liang et al., 2008; 2009; Lehmann et al., 2009). Kuzyakov et al. (2008) suggested that decomposition of biochar in field conditions with temperate climate should be approximately 10 times slower than in the optimal settings obtained in laboratory incubation. Assuming this, MRT ranging from 1500 to 2000 days could be estimated from our results. These are much higher than those reported in previous works, and can be explained by the surface oxidation that occurs rapidly in fresh biochars leading to an overestimation of C mineralization when measured in incubation experiments (Lehmann et al., 2009)

The mineralization of glucose without the addition of biochar caused an estimated yearly C loss ranging from 20 to 40% of the added C, which can be considered low regarding the high degradability of this compound (van Hees et al., 2005). However, the amount of C evolved to CO_2 might be a poor predictor of the microbial use of glucose, as a large fraction of the C could be transformed to other microbial metabolites or biomass and therefore remain in the soil system (Dilly, 2004; van Hees et al., 2005; Strickland et al., 2010). In addition, the strong dependency of the CO_2-C release on the addition of nutrients, which was not seen when biochar was the sole C source, suggests that microbial activity could be nutrient-limited in our measurements.

Kuzyakov et al., (2008) suggested that the addition of glucose caused a cometabolic mineralization of black carbon, although only during a short time ranging from 1 week to 1 month. Previously, Hamer et al., (2004) also described a cometabolic decomposition of biochar and glucose after 26 and 34 days of the glucose addition. Nevertheless, other authors proposed that black carbon helps to protect the native SOM from decay (Glaser et al., 2002b; Liang et al., 2009). Our measurements of the cumulative CO_2-C production over the whole incubation time when the artificial soil received biochar and glucose could be explained by the CO_2-C release by the two C sources separately, and a cometabolic decay of the two C sources could not be demonstrated by our results.

On the other side, our results demonstrated that a significant amount of glucose can be sorbed on the biochar-amended soil, therefore suggesting the existence of physico-chemical mechanisms of glucose protection. Nevertheless, it has been reported that even if glucose can be effectively sorbed on soil surfaces, its fate in soil is mostly determined by the microbial uptake (Fischer et al., 2010). Therefore, it can be suggested that the amount of glucose sorbed onto biochar surfaces is only a transient pool formed immediately after its addition into the soil, and that desorption occurs as its concentration in soil solution decreases along time due to its consumption by soil microorganisms. This hypothesis would be confirmed in future works using labile organic substrates with different adsorption affinity to biochar or by isotopic analysis (Keith et al., 2011).

In field conditions, when the mineralization of SOM takes place slower than in optimal laboratory conditions, it is feasible that the sorption of glucose occurs for a larger time span, thus contributing to the preservation of labile organic matter occluded in microsites of biochar for medium to large time periods. However, this has not been possible to confirm in the present work.

2.5 Conclusions

The mineralization of pine-wood biochar obtained by fast pyrolysis was relatively slow and accounted for approximately a C loss of 0.4% in a year. The mineralization of glucose was faster and dependent on the addition of nutrients. The mineralization of organic matter in the soil treated with both biochar and glucose could be explained as the sum of the mineralization of the two C sources separately.

Therefore, no evidence of protective interactions could be demonstrated by our results. Although glucose was effectively sorbed in the biochar-amended soil, sorption did not act as a long-term protective mechanism against mineralization in this artificial soil.

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Chapter 3

3. IMPACT OF DIFFERENT TYPES OF BIOCHAR ON SOIL MICROBIAL ACTIVITY: A DOSE-RESPONSE STUDY

3.1 Introduction

In recent years the scientific world focuses great attention on the possibility of using biochar as soil amendment with the aim to store C which contributes to reduce the release of atmospheric greenhouse gases from soil (Sohi et al., 2010). In effect, the long persistence of biochar in soil (Lehmann and Joseph, 2009) can help to mitigate climate change as the soil potential to be a sink for atmospheric CO_2 increase (Vaccari et al., 2011).

The use of biochar, as soil amendment, has several advantages: it increases the retention of nutrients in the soil, giving rise a slow-release source of minerals, and can constitute a refuge for microorganisms. In a fact, it has been hypothesised that biochar, due to its porous nature, can provide a microbial refuge and, as consequence, favouring microbial growth (Peitikainen et al., 2000; Lehman et al., 2011, Glaser and Birk 2012; Watzinger et al., 2013; Ferrel e al., 2013). Several researches demonstrated that biochar can improve soil fertility (Lehmann et al., 2006; Steiner et al., 2007; Lehmann and Joseph, 2009). In this regard, some studies shown positive findings of biochar application to soil such as significantly increase of plant production through improvement of mycorrhizae associations (Nisho and Okano, 1991; Ishii and Kadoya, 1994; Warnock et al., 2007), or a liming effect, caused by the increase of soil pH (Hoshinel, 2001; Yamato et al., 2006; Rondon et al., 2007; Van Zweiten et al., 2007). Nevertheless, presently, biochar research is in its initial phase and it requires more investigation to enrich the current knowledge and to focus further research relating to biochar effects on soils (Verheijen et al., 2009).

Pyrolysis conditions and feedstock characteristics largely control the physical-chemical properties of the resulting biochar (e.g. elemental composition, particle and pore size distribution), which in turn determine the suitability for a given application (Downie et al., 2009). In view of the numerous feedstocks from which biochar could be produced, specific studies are still needed to be able to define guidelines of biochar quality and applicability in order to answers to all doubts of scientific

community. Recently, the International Biochar Initiative (IBI, 2013) published a provisional guideline to characterize the biomass feedstock and resulting biochar intended to be applied to soils.

Considering the important role of the soil microbial biomass in nutrient recycling, one of the purposes of the research should be to monitor the response of the microorganisms after the addition of different types of biochar. The microbial biomass represents the main living part of the soil and it is considered a good indicator of soil quality (Jenkinson and Ladd, 1981; Powlson, 1994; Stockdale and Brookes, 2006). This chapter, therefore, would provide elements for the assessment of the ecotoxicology aspects of biochar through the study of microbial activity of a treated soil. Whit this objective, a soil was amended, in an exponential gradient of dose, with biochar obtained from pine wood splinters submitted to three different technologies of production.

Therefore, the purpose was to observe if exist any dose from which inhibitory effects are noticed. The study of dose-response curves, using soil microbial activity as an endpoint, allows establishing the "safe" dose range, checking if agronomic applications are within this range.

Focused in this direction, the aim of this work is to establish what kind of pyrolysis process is the more suitable to obtain biochar for soil application, and to propose a safe dose range using different soil microbial parameters as indicators.

3.2 Materials and methods

This research would provide information about the ecotoxicological risk of biochar incorporation to the soil through the use of biological indicators and standardized procedures as the dose-response protocol according to OECD-217 (OECD, 2000).

3.2.1 *Soil characterization*

The soil used comes from an uncontaminated agricultural field; it was taken from a stack containing a mixture of Ap and Bw horizons of a *Fluventic Haploxerept* (SSS, 2010), located at the experimental farm of Torre Marimón (Catalonia, NE Spain). The soil has a sandy-loam texture, a basic pH according its high lime content,

a relatively low organic matter and nitrogen content, and was moderately rich in phosphorus (table 3.1). The soil stock was air dried and sieved to 2 mm previously to its use in greenhouse experiments.

Table 3.1 Main characteristics of the reference soil.

Parameter	Units	Value
Clay (<0.002 mm)	g kg^{-1}	174
Fine silt (0.002-0.02 mm)	g kg^{-1}	125
Coarse silt (0.02 - 0.05 mm)	g kg^{-1}	105
Sand (0.05 - 2 mm)	g kg^{-1}	596
pH (H_2O) 1:2,5 w:v	---	8.3
E.C.25ºC(1:5 w:v)	dS m^{-1}	0.21
Organic matter	g kg^{-1}	16
$CaCO_3$ equiv.	g kg^{-1}	60
N (Kjeldahl)	g kg^{-1}	0.8
P (Olsen)	mg kg^{-1}	27
K (NH_4Ac extract)	mg kg^{-1}	159
Ca (NH_4Ac extract)	mg kg^{-1}	5557
Mg (NH_4Ac extract)	mg kg^{-1}	233
Na (NH_4Ac)	mg kg^{-1}	62
Cd (acid digestion)	mg kg^{-1}	<0.5
Cu (acid digestion)	mg kg^{-1}	17
Ni (acid digestion)	mg kg^{-1}	7
Pb (acid digestion)	mg kg^{-1}	25
Zn (acid digestion)	mg kg^{-1}	65
Cr (acid digestion)	mg kg^{-1}	10
Hg (acid digestion)	mg kg^{-1}	<40

3.2.2. *Characterization of biochars*

The three biochars tested in this study were made from mixed pine splinter (Pinus radiata and Pinus pinaster) produced by fast pyrolysis, slow pyrolysis, and gasification processes. Details of pine splinters and corresponding biochars are described in table 3.2.

Biochar obtained by gasification (PG) was produced in an industrial plant of the *Centro de Investigación en Gasificación de Biomasa – Guascor* in Júndiz (Álava, Spain). Gasification is a process similar to other pyrolysis types, with two main differences: first, biomass is exposed to significantly higher temperatures (typically between 800 and 1300°C); secondly, it is not carried out in complete anoxic conditions since oxygen is supplied in controlled quantities. The main end products are gases, a small quantity of charcoal (biochar) and ashes (table 2).

Biochar obtained by fast pyrolysis (PR) was produced in the plant from IKERLAN company (Mondragón, Guipuzcoa, Spain), which uses moderate pyrolysis temperatures (450ºC). In this case, biomass is rapidly heated by the progressive introduction of small quantities of finely particulate biomass. In this procedure, gas and condensates are produced almost instantaneously, obtaining around 65% of liquid oil and 15% of charcoal (biochar), (table 2).

Biochar obtained by slow pyrolysis (PL) was supplied by the *Grupo de Ingeniería Química y Ambiental del Instituto de Medio Ambiente, Recursos Naturales y Biodiversidad* of the *Universidad de León* (León, Spain). In the slow pyrolysis, a process similar to that carried out in traditional charcoal production, a fixed amount of biomass is heated slowly within a chamber in a process extended in time at temperatures below 400ºC. At the end, 40% of the initial biomass becomes charcoal (biochar), (table 2).

Table 3.2 Main pyrolysis process attributes and analytical parameters of t biochars used in this study: PR (biochar from fast pyrolysis), PL (biochar from slow pyrolysis) and PG (biochar from gasification). Elemental concentrations are mean values. Ash content is expressed as percentage with respect to total weight. The number in parentheses indicate the replicates standard error.

	P0	PG	PR	PL
Parameter	---			
Production plant	---	Guascor, Júndiz, Spain	Ikerland IK4, Álava, Spain	Irene-University of León, Spain
Process type	---	Gassification	Fast Pyrolysis	Slow Pyrolysis
Process temperature (ºC)	---	600-900	440-480	500-550
Process time	---	75 min.	<2sec	15 min
pH (H_2 0,1:10)	5.2	11.42 (0.02)	8.04 (0.04)	7.29 (0.02)
EC (mS m^{-1})	42	189 (0.57)	64 (0.13)	57 (1.06)
C (g kg^{-1})	475	710	718	863
N (g kg^{-1})	2.0	1.2	1.9	1.2
H (g kg^{-11})	59.3	5.3	34.0	19.7
S (g kg^{-1})	<2	0.8	0.2	0.2
O (g kg^{-1})	455	88	220	91
P (g kg^{-1})	0.2	0.8	0.5	3.5
Ca (g kg^{-1})	7.1	92.3	8.3	3.8
Mg (g kg^{-1})	0.2	2.6	1.4	1.0
Na (g kg^{-1})	0.05	0.8	0.5	0.3
K (g kg^{-1})	7.0	8.2	64	3.5
Fe (g kg^{-1})	0.4	1.5	1.6	1.2
Zn (mg kg^{-1})	8.1	823	181	70
Cr (mg kg^{-1})	0.1	26	26	83
Cu (mg kg^{-1})	0.1	219	13	27
Ni (mg kg^{-1})	0.5	10	25	97
As (mg kg^{-1})	<0.1	<2.0	<2.0	<2.0
Cd (mg kg^{-1})	0.08	1.2	<0.5	<0.5
Hg (mg kg^{-1})	<0.1	<1.0	<1.0	<1.0
Pb (mg kg^{-1})	<0.1	9	10	16
Ash content (%)	2.0	19.6 (0.35)	2.7 (0.09)	2.6 (0.09)

Slow pyrolysis wood chars had the highest C content (86%), whereas that of fast pyrolysis and gasification was similar (71%). N content ranged between 0.12 and 0.19%. P content was highest in slow-pyrolysis char (35%) and lowest in the fast and gasification chars (0.05%). It appears that pyrolysis method had the strongest influence on C: P ratios (P content highest in low temperature-pyrolysis materials), whereas feedstock determined C: N ratios (N content more similar within feedstocks). Biochars in general were slightly alkaline, with the exception of PG, which was strongly alkaline. Ash content was highest in PG (19,6%). As concern heavy metals PG appears particularly reach in Zn and Cu (823 and 219 mg kg-1

respectively), while PL displayed the highest content in Cr and Ni (83 and 97 mg kg^{-1} respectively).

Table 3.3 shows the biochar loss on ignition (LOI), volatile matter (VM) and hot water extracted carbon (C_{hw}) comparative data obtained from Marks et al., 2014. Table 3.3. Proximate analyses of loss on ignition (LOI), volatile matter (VM), and ash contents of fresh biochar samples, expressed as percentage of each fraction with respect to total weight, in addition to hot water-extractable C (C hw). SE of three replicates is indicated in parentheses. Data from Marks et al., 2014).

	LOI 375 °C (%)	LOI 375-550 °C (%)	LOI 550e 1100 °C (%)	VM (%)	C_{hw} mg C kg^{-1}
PG	76.2 (0.16)	2.22 (0.37)	6.10 (0.46)	15.8 (0.31)	613 (42)
PL	96.8 (0.01)	0.18 (0.00)	0.18 (0.01)	10.7 (0.20)	933 (72)
PR	95.0 (0.13)	0.56 (0.00)	0.37 (0.04)	28.1 (0.22)	2684 (41)

Fast and slow pyrolysis biochars had very similar LOI-375 (95-96%), LOI-550 (0.18-0.56%), and LOI-1100 (0.18-0.37%), while PG was distinguished by its lower LOI-375 (76%). PG had also relatively high LOI-1100 (6%), indicating carbonate content. Regarding hot water-soluble C (C_{hw}), fast-pyrolysis wood materials had more C_{hw} than the gasification or slow-pyrolysis materials. In the same sense, PR has high volatile matter content than the other biochars (table 3).

3.2.3. *Soil-biochar mixtures*

Pine splinters and derived biochar have been crushed to pass a 2mm sieve and then mixed with the soil to obtain four groups of treatments named with the same acronym:

P0 (soil + splinters)

PL (soil + biochar from slow pyrolysis)

PR (soil + biochar from fast pyrolysis)

PG (soil + biochar from gasification)

Each type of biochar has been added at 8 doses, respectively: D1, 5.00; D2, 9.65; D3, 18.64; D4, 35.98; D5, 69.47; D6, 134.13; D7, 258.97 and D8, 500.00 g kg-1. D0

represent the control. All treatments were done by triplicate. Soil incubations were performed at 21ºC, in a dark chamber, at 40% of the soil water holding capacity.

3.2.4. *Microbial assays*

For each dose tested, the microbial biomass carbon (MBC), through fumigation and extraction method (Vance et al., 1987), and substrate-induced respiration (SIR) were measured (Anderson and Domsch, 1978) following the 217 OECD protocol (OECD, 2000). Soil samples were incubated during a period of 28 days, and microbial biomass were measured the first and the last day of incubation. The SIR was performed during the same period and the quantification of respiratory activity was carried out by measuring the O_2 consumption after 12 hours of incubation in gauge bottles, Velp Scientifica (García-Orenes et al., 2010). All data were expressed as a percentage respect to D0 (untreated soil).

3.2.5. *Electrical conductivity and pH of soil extracts*

10 g of soil mixture and 50 ml of deionised water (1:5 w:v) were vertically shaken in 150 ml polyethylene cups for 2 h at 60 rev min^{-1}. The extract was subsequently centrifuged and the supernatant was filtered through Whatman 42 filter paper. The pH and EC were immediately measured.

3.2.6 *Effective Concentration 50% (EC50)*

Half maximal effective concentration, EC50, was calculated for SIR at 28 day of incubation. A polynomial regression analysis was used to predict the values assumed by a SIR variable from the knowledge of the logarithmic basis of the doses of biochar added.

3.2.7 *Statistical analyses*

Statview software was used to carry out the following statistical analysis:

-Analysis of variance (ANOVA one way), followed by Fisher´s HSD exact test, was used to determine the effects of soil treatment with feedstock and biochars to MBC and SIR.

-Exponential models [Y=a*e (b*X)] was chosen to describe the curves of dose-response effect. Factors a) and b) are, respectively, the amplitude and the slope of the curve and X represents the logarithm of the dose. The slope of the curve was used to describe the rate of microbial parameter measured within the biochar dose used.

-Polynomial regression (Y=a-b*X) was used to assess the EC50.

3.3 Results

3.3.1 *Effect of biochars over soil pH and electrical conductivity*

Figure 3.1 shows the trend of the pH in the different treatments as a function of the dose of pine splinters or their derived biochar added. The addition of pine feedstock (P0) generated a decrease of pH to 7.8 for D8, while EC showed a quite contrary behaviour. Indeed the EC, decreases until D 3 with a value of 63.3 ($dS.m^{-1}$), remained constant for D4, D5, D6 and increased significantly for D7 and D8 with values of 153.1 and 194.4 ($dS.m^{-1}$), respectively.

Soil amended with biochar of fast pyrolysis (PR) shown a slight increase pH for highest doses, with a maximum of 8.4 corresponding to D8. EC for highest doses reach a maximum value of 177.2 ($dS.m^{-1}$), corresponding to D8.

For that concern the addition of biochar of slow pyrolysis (PL), it caused a very slight increase of pH with a maximum value of 8.5 corresponding to D6. The values of EC are quite constant.

Regarding the addition of biochar of gasification (PG) we notice an increase of pH with the doses until a value of 8.9 corresponding to D8. EC shows an exponential increase with a maximum value of 475.6 $dS.m^{-1}$ corresponding to D8.

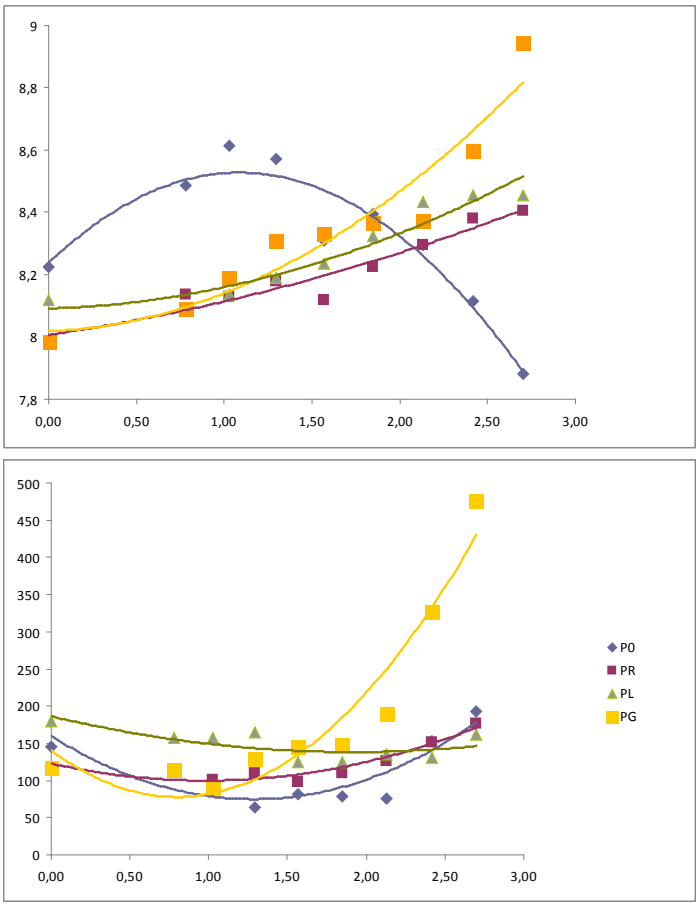

Figure 3.1. pH (top) and EC in $dS.m^{-1}$ (below) of soil extracts as a function of the dose of pine splinters and their derived biochars added to the soil.

3 3.2 *Effects of biochar type and dose on soil microbial biomass and activity*

Table 3.4 illustrates the values of microbial activity (SIR) and microbial biomass (MBC) as a function of the dose of pine splinters (P0) and respective biochars obtained by slow pyrolysis (PL), fast pyrolysis (PR) or gasification (PG).

Table 3.4. Parameters of the model [Y=a*e(b*X)] used to describe substrate induced respiration(SIR) and microbial biomass (MBC) as a function of the dose of (P0), (PL), (PR) or (PG); X were computed as log(dose+1). The values of P were < 0.0001 for all analysis performed.

SIR ($mg\ O_2\ kg^{-1}d^{1}$)	Incubation time (d)	a	b	R^2
P0	0.5	64.2±6.7***	0.5±0.0***	0.97
	28	57.8±6.5***	0.4±0.0***	0.96
PG	0.5	116.3±9.8***	0.1±0.0***	0.96
	28	97.2±9.8***	-0.1±0.1***	0.94
PR	0.5	103.2±9.2***	0.4±0.0***	0.97
	28	36.8±8.3***	0.7±0.1***	0.92
PL	0.5	117.0±5.7***	-0.1±0.0***	0.98
	28	119.3±5.1***	-0.1±0.0*	0.99
Microbial Biomass-C ($\mu gC.g^{-1}$)				
P0	0.5	47.4±15.9*	0.1±0.1***	0.88
	28	23.9±13.4ns	0.9±0.2*	0.77
PG	0.5	75.5±18.5*	0.5±0.1***	0.90
	28	74.5±7.3***	0.3±0.0***	0.97
PR	0.5	80.1±18.4***	0.2±0.1 ns	0.88
	28	116.2±13.9***	0.1±0.1 ns	0.94
PL	0.5	83.9±22.7*	0.3±0.1*	0.84
	28	139.4±31.8***	0.6±0.1***	0.93

****p*<0.0001
* *p* <0.05
ns not significant

The pine feedstock treatment (P0) showed a positive SIR curve denoting an exponential enhancement of respiration rate at 12h of incubation that persist at 28th day with a mean values of 260.9 and 584.1 mg O_2 $kg^{-1}d^{-1}$ corresponding to D1 and D8, respectively (table 4).

As concerning the amendment with biochar of gasification (PG), it caused an enhancement of respiration after 12h of incubation with a maximum mean value of 415.5mg O_2 $kg^{-1}d^{1}$ corresponding to D5 (table 3). After 28 days, respiration rate showed a decreasing trend presenting a mean minimum value of 72.3mg O_2 $kg^{-1}d^{-1}$correspondig to D8 (table 3.4).

The biochar obtained by fast pyrolysis (PR) produced a general enhancement of respiration rate at first time of incubation. This tendency is maintained after 28 days with means values of 93.2 mg O_2 $kg^{-1}d^{-1}$for D1 and 450.7mg O_2 $kg^{-1}d^{-1}$ for D8, respectively (table 3.4).

Regarding soil treated with biochar of slow pyrolysis (PL), a downward trend of respiration has been noted during the first 12h of incubation. This tendency keeps almost unchanged at 28 days with minimum mean values of 308.9mg O_2 $kg^{-1}d^{-1}$ corresponding to D8 (table 3.4).

For that concerning MBC, biomass curve of pine feedstock treatment (P0), denoted a positive tendency at 12h of incubation. At day 28 a more pronounced exponential enhancement of microbial biomass has been noted with mean values of 6.5µgC.$g^{-1}$for D1 and 40.2µgC.$g^{-1}$for D8 (table 3.4).

As concern biochar of gasification (PG), was possible to observe a increase in MBC at 12h that linger at 28 days with means values of 61.2 and 124.3 µgC.g^{-1} for D1 and D8 respectively (table 3.4).

Regarding the biochar obtained by fast pyrolysis (PR), the dose-response curve of MBC denoted a no significant growth trend (table 3.4).

Biochar obtained from slow pyrolysis (PL) shown an enhancement trend of MBC particularly pronounced at day 28, presenting a minimum mean value of 18.7 µgC.g^{-1} corresponding to D2 and a maximum of 83.3 µgC.g^{-1} to D8 (table 3.4).

3.3.3 *Comparisons between treatments*.

Figures 3.2 permits an easy view of the effects of soil amendments with different types of biochar, at different doses, on soil induced respiration and microbial biomass, trough the representation of the regression trend of these microbiological parameters.

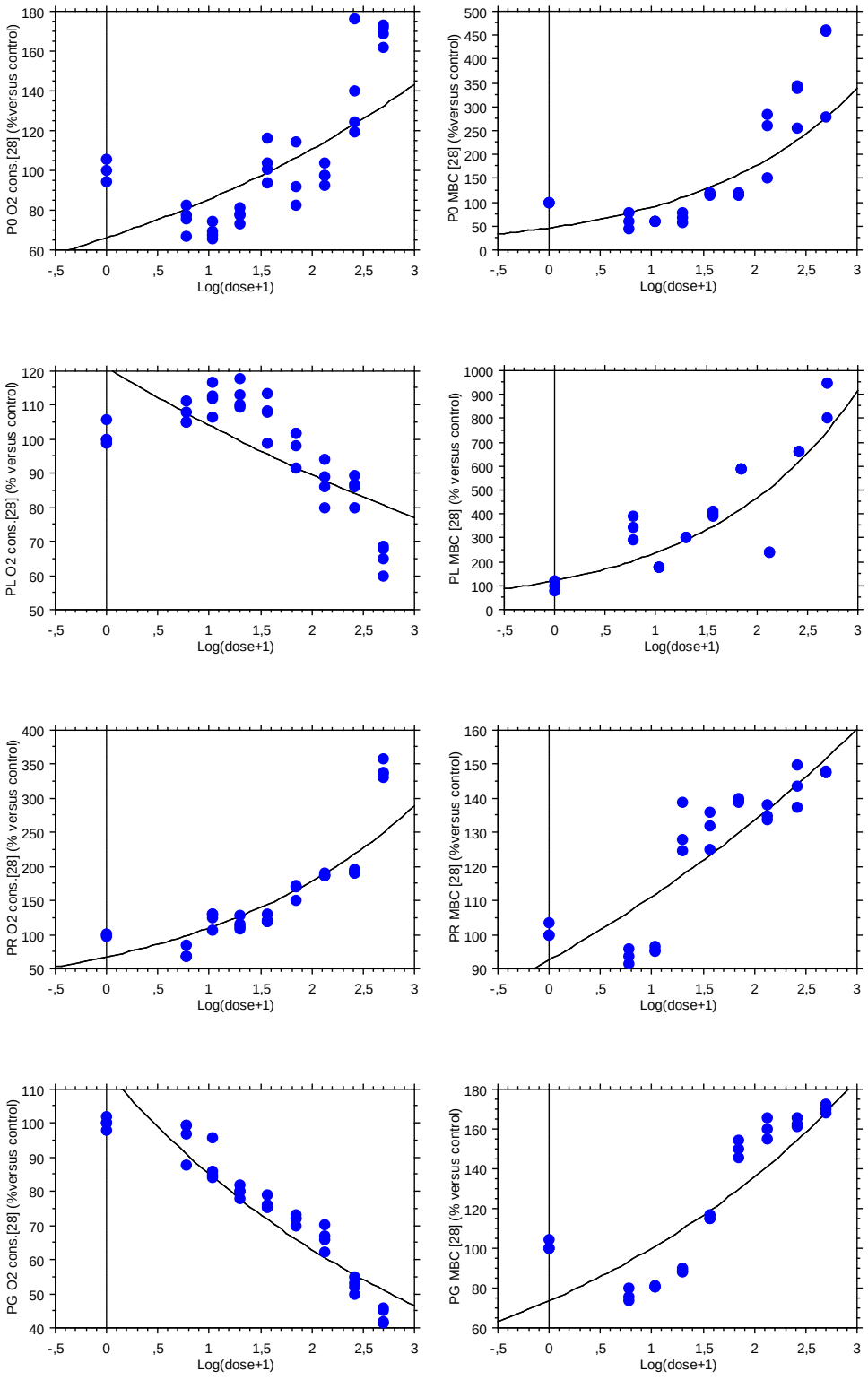

Figure 3.2 Substrate induced respiration (SIR) and microbial biomass carbon (MBC) of the pine feedstock (P0), PL, PR and PG in % versus control as a function of the dose applied, 28 days after the addition.

While the feedstock (P0) and PR had a tendency to increase substrate induced respiration (SIR) with the dose, slow pyrolysis (PL) and gasification (PG) biochar shown the opposite trend. However, at smaller doses, in soil amended with PO and PR an inhibition of SIR is reported respect to non-treated soil.

Regarding MCB all treatments shown the same tendency to increase with the dose, more pronounced in PL.

3.3.4 *Dose-response slope factor of substrate induced respiration and microbial biomass.*

The slope of microbial biomass and activity identifies the lean of the relationship between dose and response during time. This in turn allows deriving the power of the effect of the treatment on the studied parameters. Positive rate indicate an increase in parameter considered; while negative rate indicates a decrease of the same over biochar doses respect to control soil. Figure 3.3 allow comparing and summarising the effects of adding the three types of biochar to the soil through the analysis of the slope factor of SIR, and MCB, in a dose-response curve, at 12h and after 28 days of incubation.

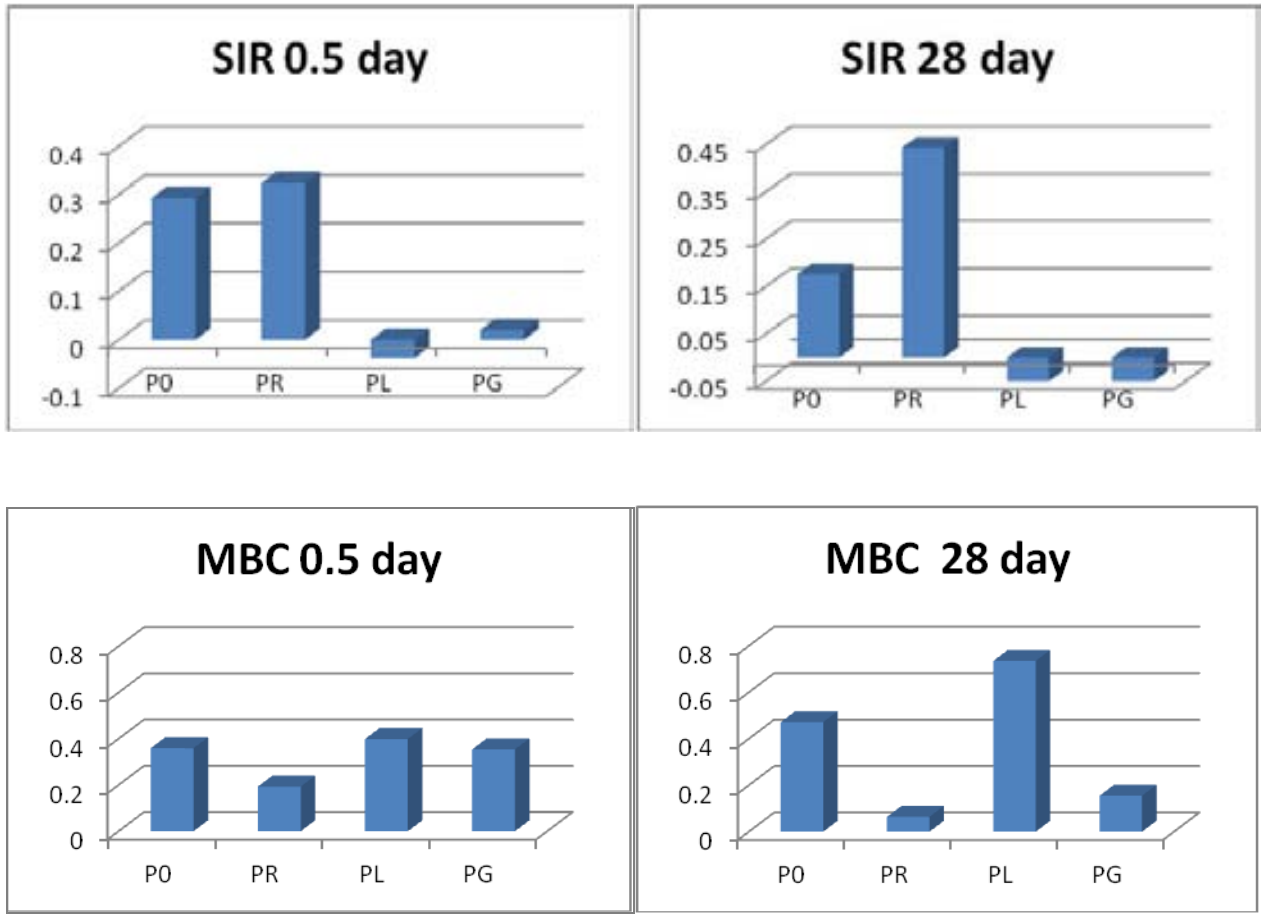

Figure 3.3 Slope of SIR (graphics at the top) and MCB (graphics below) in the dose-response curves corresponding to the pine feedstock and respective biochars (see codes in methods section) at 0.5 and 28 days of incubation.

As regards the rate of SIR, soil treated with PL shown a negative tendency both at 12h and 28 days after incubation. Samples amended with PR shown a positive trend at 12h that increase at day 28. Soil treated with P0 show a positive tendency at 12h that slight decreases after 28 days. PG showed a positive lean at 12h that become negative after 28 days of incubation.

Regarding MBC a positive trend was noticed in all combinations of soil and biochar tested, both at 12h and after 28 days. This tendency was much steeper in samples treated with PL, while decreased for samples treated PG and PR.

3.3.5 *EC50*

Only samples treated with biochar coming from fast pyrolysis and pine feedstock showed a negative effect on soil microbial biomass. The half maximal effective concentration, EC50, calculated at day 28 of incubation were 1.42 (p<0.0001) and 2.60 g.Kg^{-1}(p<0.001) for PR and P0 respectively.

3.4 Discussion

The chemical characterization of the biochars used in this study corroborates the fact that biochar properties are highly variable depending on pyrolysis conditions (Marks et al., 2014).While more investigation exist about the study of biochar effect over soil microbial activity (Saito and Muramoto, 2002; Warnock et al, 2007) and structure (Amonette and Joseph 2009; Enders et al. 2012; Downie et al. 2009), to date, few studies have analyzed the effect of biochars from a same feedstock obtained at different pyrolysis conditions.

Soil microbial activity greatly depends of suitable microhabitats and available nutrients furnished by the soil components (Madsen, 1996). In this experiment the adding biochar or pine splinters, could modify these soil characteristics and enhance microbial activity. This in turn generate changes in soil biomass populations thereby providing an early sign of soil improvement or an early warning of soil degradation (Powlson et al., 1987; Kennedy and Papendick, 1995; Pankhurst et al., 1995; Giller et al., 1998). Also the pH and the electrical conductivity (EC) of soil are factors directly related to the solubilisation of the mineral elements, and then to their availability which could influence microbial activity.

Typically, an increase in microbial biomass is considered beneficial and an improvement to the soil system, while a decline is considered detrimental, but this level of interpretation is too simple and not sufficient in the case of microbial activity. Considering this, was decide to use the method of substrate-induced respiration (SIR) proposed by Anderson and Domsch, (1978) to understand better the behaviour of microbial biomass in different condition. The method consists in the measurement of microbial respiration after amending soil samples with an excess of a readily nutrient source, usually glucose, to trigger microbial activity. SIR is therefore a function of size of active microbial biomass (Anderson and Domsch, 1978) and also energy requirement for soil microorganism. Higher values are considered positive were correlated with higher biomass content and decreased values can indicate an inhibition effect due to physical o chemical condition (Anderson and Domsch, 1978; Visser and Parkinson, 1989). On the other hand, in presence of lows values of microbial biomass with high values of SIR could indicate higher maintenance energy caused by some stress (Visser and Parkinson, 1989). Moreover, generally, a lack of correlation between activity and size of microbial biomass may indicate shifts in the structure and physiology of the microbial community (Dilly and Munch1998).

Soil microbial activity stimulation or inhibition is related to substrate quality or recalcitrance. Lability of the carbon of biochar used in this research was evaluated indirectly in a previous work on the basis of C_{hw} and VM (Marks et al., 2014). As reported in table 3.3, PR biochar is quite different to the others (higher VM and C_{hw}), suggesting that effects on microbial biomass and activity could be diverse, mainly at high doses. Labile carbon content of biochars is associated with its degree of ease of degradation by microbial action, and hot-water extraction has been considered an appropriate estimation of soil organic matter available to microorganisms in the short term (Santisteban et al., 2004; Joseph et al., 2009; Calvelo et al., 2011). The VM parameter has been also suggested as an estimate of the labile fraction, due to its potential influence on plant nutrient limitations associated with their immobilization in microbial biomass (Deenik et al., 2010; Lehmann et al., 2011).

3.4.1 *Biochar treatments and dose effects*

The addition of pine splinters to the soil has an effect obviously different of the respective biochars due to the different chemical composition (Schmidt and Noack, 2000). A clear example of this statement is given in Figure 2 which compares the microbial parameters used in this work between treatments. It is possible to note an inhibitory effect in lower doses, then SIR and biomass increases in higher doses probably due to the variation of pH and EC caused by the resinous wood (figure 1). Furthermore, this type of material is easily mineralizable compared to biochar, as indicated by the values of volatile matter (VM) and water extracted carbon (C_{hw}) reported in table 3. It is possible that, at the beginning, the bacterial and fungal communities used the labile substrate experimentally added; subsequently, changes of pH generated conditions suitable for fungi able to degrade more resistant carbonyl structures as cellulose, lignin and humus (Thies and Grossman, 2006; Paul, 2007). The pH is one of the environmental factors that most influence the abundance, activity and diversity of microbial populations (Wardle, 2002).

The trend of SIR in PG amended soil changed over time becoming negative after 28day of incubation (figure 3). This effect is more evident for the higher doses and could be explained considering physical-chemical properties of biochar produced by gasification (Lua et al., 2004). During gasification process the majority of non-carbon atoms are removed. The carbon concentration passes from a 40-50% to more than 90% after carbonization (Antal and Grønli, 2003). The same was also observed for naturally occurring black carbon (Cornelissen et al., 2005). These conditions could influence the retention of the soil native organic matter increasing the efficiency of microbial biomass due to a greater availability of energy resources (Odum, 1969), as indicates by the result of this experiment. The reduction in the microbial CO_2 production at highest doses of PG, may be due to an increase of the stability of the microbial community over time.

Results of samples treated with PL prove that the microbial biomass become more efficient in the use of carbon if compared with other treatments (Figures 2 and 3). In fact, the reduction of respiration parallel to an increase of size of microbial biomass indicates a better use of C resources by soil microorganisms (Insam and Haselwandter, 1989). This could be partially explained considering the particular structure and the physical chemical characters of this biochar (PL). The slow pyrolysis is characterized by relatively low temperatures and long residence times

that result in completely pyrolysed biomass, containing none or very small fractions of labile organic matter (table 3). For this reason, the derived biochar produced has a greater resistance to mineralization by soil microorganisms. Moreover, it could be an ideal habitat for soil microbial biomass due to its high porosity. The macropores are reported to be an ideal habitat for soil microbes, due to the size of the microbes themselves and their colonies (Lal 2006). In these conditions, soil microbial biomass reaches a favourable habitat that increase biomass with the dose of biochar added.

A very different behaviour was observed for soil treated with biochar of fast pyrolysis (PR). In fact, both biomass and respiration slightly decrease at lower doses although showing a positive tendency (figure 3) due to the particularly pronounced increase in the higher doses. This trend is maintained at day 28 although with a lower rate (figure 3). Microbial degradation of organic matter of PR could be strongly controlled by the relative high amount of labile C present in this type of biochar. Depending on the conditions of fast pyrolysis, the resulting biochar may contain a certain fractions of labile carbohydrates. This aspect was found to be correlated to the short-term degradation rates of the PR-biochars when applied to soil (Yanik et al., 2007). Therefore, the addition of PR biochar to soil was shown to stimulate microbial growth compared to the PL biochar or the control soil. Greater microbial pools have mostly been explained by the availability of easily decomposable fractions of the added biochars (Kolb et al., 2009; Kuzyakov et al., 2009; Novak et al., 2010; Steiner et al., 2008a).

It is interesting to note that many types of biochars generate an increase of EC with increasing doses due to its ash content; only samples treated with P0 and PL keeps values almost constant. If EC reaches high values, inhibitory effects of microbial activity can appear. The increase of pH as biochar dose raises can be explained by the basic cations (mainly Ca, K and Mg) contained in the ashes that accompanied the char.

4.2 *Analysis of EC50*

The EC50 represent the median effective concentration that produces a specific effect, other than death, on about 50% of the population. In this study was decided to calculate this important parameter using substrate induced respiration (SIR) as indicator because it represents an important parameter of mineralization capability.

On the base of this experiment it is recommended not to add pine splinters in doses >1.42 g kg^{-1}. Application of pine splinters to soil generates an unstable situation due to the proportion of degradable organic substances present in this type of substrate (Table 2 and 3) and to changes on soil pH induced with increasing dose applied. As had been reported in previous works, these pH changes may influence the microbial biomass, the rate of respiration (Anderson and Domsch, 1993), and the structure of microbial communities (Pennanen et al., 1998). Also the availability and the imbalance between nitrogen (N), phosphorus (P) and carbon (C) can thus feedback on dynamics of soil microbial biomass (Wang et al. 2010; Brookes 2001). As regard, biochar of fast pyrolysis, the result of EC50 suggests an evident effect that could be detectable from a dose < 5.0 g kg^{-1} (D1) in this experiment. One explanation is that PR could be easily degraded by microorganisms and causes a deficiency of selectivity for resources, and therefore, a reduced balance of the microbial community (Anderson and Domsch, 1993). The PR biochar begins to be no recommendable at doses higher than 2.6 g kg^{-1}.

3.5 Conclusion

As expected, all types of biochar result more resistant to microbial degradation if compared with the feedstock. Considering the importance of the maintenance of the equilibrium of microbial biomass in the soil, the results of this study suggests that biochar from fast pyrolysis is the less recommendable if used in doses higher than the calculated ED50. Biochar proceeding from slow pyrolysis and gasification, as tested in this experiment, represent the safest ones from the point of view of the response of microbial biomass. In fact, the better efficiency of C use by microbial biomass may have implications for soil C sequestration and seems that these types of biochar may positively influence soil organic C retention. As result indicates, PL and PG do not cause detrimental effects on microbial community when added to the soil, even in high doses, and were very resistant to degradation along time.

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Chapter 4

4. WOULD THE ADDITION OF BIOCHAR MODULATE ADVERSE EFFECTS OF SOME PESTICIDES ON SOIL MICROORGANISMS?

4.1 Introduction

In the last decades, the huge population growth and the increased demand for agricultural products, particularly cereals, led to the use of pesticides in order to maintain high production rates, mainly for monocultures. Many pesticides are known to develop a series of secondary effects that may adversely interfere with soil microorganisms (Perucci et al., 2000; Busse et al., 2001), influencing their biodiversity or activity and therefore, affecting biological soil functions that could reduce soil quality. Modern agricultural production is characterized in most countries by an intensive use of pesticides that could remain in the soils, in some cases for a long time. Some pesticides, such as chlorinated derivatives, are very toxic and persistent compounds in the environment; for this reason most of them have been banned decades ago, but they can still be found in the environment (Goncalves and Alpendurada, 2005; Hildebrandt et al., 2009; Jiang et al., 2009; Yang et al.2010). Last generation of pesticides tends to be more specific for target organisms and less persistent, but secondary effects cannot be excluded (Heinz et al., 2013). One practical problem is that an important fraction of pesticides applied to crops goes directly to soil surface without interacting with target species, but affecting other organisms or being adsorbed to soil particles.

Soil contamination may be due to a wide range of organic and inorganic compounds that interact with soil components. Agrochemicals tend to be sorbed to soil organic matter or clay minerals; for this reason, organic amendments can be useful to block pesticides avoiding losses by leaching. Recently, the use of biochar in contaminated soils has been proposed as a potential, inexpensive, and natural tool in mitigating/remediating contaminated soils (Uchenna and Kirk, 2013). Biochar is a very recalcitrant product, rich in carbon, obtained from the pyrolysis of different types of biomass (i.e. charcoal) that is intended to be applied to soil to enhance soil fertility. Its chemical structure and composition ranges from partly charred to highly condensed forms of organic carbon (Schmidt et al., 2000). Considering the high sorption capacity of biochar, due to its greater surface area, high microporosity and

chemical functional groups (Accardi-Dey and Gschwend, 2003; Chun *et al.*, 2004; Yu *et al.*, 2006), it has been suggested that it could influence the mobility, extractability and/or bioavailability of organic contaminants in soil (Sundelin et al., 2004; Cornelissen et al., 2005; Rhodes et al., 2008a; Cao et al., 2011). Further, biochar has been shown to aid in stabilizing and restoring soil organic matter levels (Amonette et al., 2003). Several authors have evaluated the adsorbent characteristics of biochar and the possibility to use it as a way to block the toxic effects of certain substances or pollutants in the soil (Bornemann et al., 2007, Chen et al., 2008, Chen, B., Yuan, M., 2011). Thus, biochar can result useful in remediation of pesticide contaminated soil because of its capacity of interaction with some functional groups of these agrochemicals (Lou et al., 2011, Yu et al., 2009). Moreover, biochar makes the xenobiotics present in soils and sediments less available to organisms and hinder their off-site transport into receiving environments (Burgess et al., 2009).

Despite the increasing interest in biochar application to soil for carbon sequestration to abate climate change (Lehmann et al., 2006), currently less information exists in literature if biochar amendment to soil can reduce the uptake of pesticides or their residues (Kookana, 2010). Such a practice, if found effective, could contribute to the remediation of contaminated agricultural and urban soils polluted by pesticides.

Nowadays, 220.000 tons of agrochemicals, basically fungicides, herbicides, insecticides and growth regulators, were released into the European environment along 2012 (Pesticide Action Network, 2012). It must be hypothesized that if a pesticide can reach the soil surface, it could be partially immobilized by a biochar present in soil, reducing its mobility/activity, and the possible adverse effects on soil microbiota.

Therefore, our main objectives were (i) to assess if the application of three pesticides based on imidacloprid, methyl thiophanate and glyphosate have detectable adverse effects on soil microbial activity, and (ii) to evaluate if the addition of biochar modifies the toxicity or adverse effects of these chemicals in soil.

4.2 Material and methods

The surface Ap horizon (0-30 cm) of an agricultural soil (*Fluventic Haploxerept*, SSS 2010) from the experimental farm of Torre Marimon (Catalonia, NE Spain) was selected to be amended in the greenhouse with 0, 1.9 and 11.5 g $kg^{-1}$of biochar, which roughly correspond to agronomic contributions of 0, 5 and 30 Mg ha^{-1}. These three different dosages of biochar-amended soil received realistic amounts of currently used pesticides.

The soil displayed a sandy loam texture (Table 4.1), a relatively low content of organic matter, an alkaline pH caused by its noticeable amount of carbonates, low levels of N and K, a moderate concentration of P and a high amount of Ca. The concentrations of heavy metals in this soil were low. The soil was sieved to 2 mm and brought to 40% of its water holding capacity (WHC) before biochar addition or pesticide treatments.

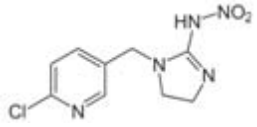
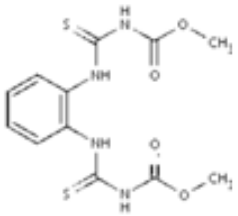
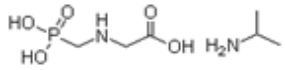
The biochar was made of pine wood biomass by slow pyrolysis and supplied by the "Grupo de Ingeniería Química y Ambiental del Instituto de Medio Ambiente, Recursos Naturales y Biodiversidad" of the Universidad de León (León, Spain). Pine chips were charred during approximately 15 minutes at a pyrolysis temperature ranging from 500ºC to 550ºC. The obtained biochar had a concentration of 862 g kg^{-1} of total C (elemental C).

Table 4.1. Main characteristics of the unamended soil used in this work.

Parameter	Units	Value
Clay (< 0.002 mm)	g kg^{-1}	174
Fine silt (0.002-0.02 mm)	g kg^{-1}	125
Coarse silt (0.02 – 0.05 mm)	g kg^{-1}	105
Sand (0.05 – 2 mm)	g kg^{-1}	596
pH (H_2O) 1:2,5 w:v	---	8.3
$E.C._{25ºC}$(1:5 w:v)	dS m^{-1}	0.21
Organic matter (dichromate	g kg^{-1}	16.0
$CaCO_3$ equiv.	g kg^{-1}	60.0
N (Kjeldahl)	g kg^{-1}	0.8
P (Olsen)	mg kg^{-1}	27.0
K (NH_4Ac extract)	mg kg^{-1}	159
Ca (NH_4Ac extract)	mg kg^{-1}	5557
Mg (NH_4Ac extract)	mg kg^{-1}	233
Na (NH_4Ac extract)	mg kg^{-1}	62
Cd (acid digestion)	mg kg^{-1}	<0.5
Cu (acid digestion)	mg kg^{-1}	17
Ni (acid digestion)	mg kg^{-1}	7
Pb (acid digestion)	mg kg^{-1}	25
Zn (acid digestion)	mg kg^{-1}	65
Cr (acid digestion)	mg kg^{-1}	10
Hg (acid digestion)	µg kg^{-1}	<40

The pesticides were selected from the list established for use in Europe according to the Directive 91/414/EEC. The application rates of the three pesticides have been chosen from the doses suggested by the manufacturers (Table 4.2) assuming that all the product could be distributed and concentrated in the first millimetres of the soil surface, thus considering that the amount of treated soil was 2600 kg ha^{-1}. The three pesticides have been incorporated into the soil at doses 50% higher than those recommended by the manufacturer. A distilled water solution/emulsion of the pesticides was applied by irrigating the surface of the soil placed in 5-cm depth trays, in order to reach the desired concentration of these agrochemicals. Then the soil was mixed to assure the homogeneous distribution of the products. Three replicates of each treatment were separately prepared then, treated soils were transferred to polyethylene containers analyzed and stored at 21ºC and 50% WHC for a period of 28 days under dark conditions.

Table 4.2 Recommended and provided doses of the three selected pesticides on the experimental biochar-amended soil.

	Insecticide	**Fungicide**	**Herbicide**
Commercial name	Confidor, Bayer	Pelt, Bayer	Lograd, Massó
Active principle	Imidacloprid	Thiophanate methyl	Glyphosate, Mono Isopropylamine salt solution
Recommended dose	0.65 l ha^{-1} [potatoe] (0.25 ml kg^{-1})	1.7 l ha^{-1} [cereal] (0.65 ml kg^{-1})	4.5 l ha^{-1} [general use] (1.73 ml kg^{-1})
Provided dose	0.38 ml kg^{-1}	0.98 ml kg^{-1}	2.60 ml kg^{-1}
Molecular structure	 $C_9H_{10}ClN_5O_2$	 $C_{12}H_{14}N_4O_4S_2$	 $C_6H_{17}N_2O_5P$
CAS number	138261-41-3	23564-05-8	38641-94-0
Octanol:water partition coefficient (log K_{ow})	0.57	1.4	-3.2
Water solubility	0.61 g/l at 20°C	24.6 mg/l at 25°C	12 g/l at 25°C
Reported half-life in soil	40-124 d (Singh D.K.2012)	< 60 d (European Commission 2005)	2-197 d (European Commission 2001)

Experimental combinations between soil, biochar and selected pesticides are shown in Table 4.3.

Table 4.3 Experimental design to evaluate the possible modulation effect of the biochar towards the three pesticides added to the soil.

Biochar dose in soil (g kg^{-1})	Insecticide (Confidor) (ml kg^{-1})	Fungicide (Pelt) (ml kg^{-1})	Herbicide (Logrado) (ml kg^{-1})	Code
0	0	0	0	B- I- F- H-
1.9	0	0	0	B1 I- F- H-
11.5	0	0	0	B2 I- F- H-
0	0.38	0	0	B- I+ F- H-
1.9	0.38	0	0	B1 I+ F- H-
11.5	0.38	0	0	B2 I+ F- H-
0	0	0.98	0	B- I- F+ H-
1.9	0	0.98	0	B1 I- F+ H-
11.5	0	0.98	0	B2 I- F+ H-
0	0	0	2.60	B- I- F- H+
1.9	0	0	2.60	B1 I- F- H+
11.5	0	0	2.60	B2 I- F- H+

Substrate-induced respiration (Anderson and Domsch, 1978) was measured after 6 and 12 hours, and after 28 days of the addition of the pesticides. Oxygen consumption (García-Orenes et al., 2010) was measured for 12 consecutive hours after 2.5 g glucose kg^{-1} were added as a microbial activator. Microbial biomass C was (under)-estimated from the difference between the amount of C in 0.5M K_2SO_4 extracts of $CHCl_3$ fumigated soil and the extractable C in non-fumigated samples (Vance et al., 1987), and no k_{eC} was applied. These measurements were made at 12 h and 28 d after the addition of the pesticides.

Specific respiration was computed as the amount of O_2 consumed per unit of microbial C. The specific respiration at 6 hours was calculated from the ratio between O_2 consumption measured at 6 hours and the measure of microbial biomass at 12 hours.

4.2.1 *Statistics*

Statistical analyses were carried out in Statview software. The effects of the addition of biochar on the microbiological properties have been tested by a one way ANOVA, comparing data of three doses of biochar treated soil (three levels) with control soil (soil not treated with pesticides or biochar). The effects of the addition of the selected pesticides in the soil, amended or not with biochar, have been analysed

for each agrochemical by two-way ANOVA [biochar (3 doses) and pesticide (yes / no)].

4.3 Results

4.3.1. *Biochar effects*

Figures 4.1 to 4.3 show the effect of biochar addition on the O_2 consumption, microbial biomass-C and the specific respiration when agrochemicals have been added.

The addition of biochar does not increase the O_2 consumption neither after 6 hours (F = 1.673, P = 0.2548), 12 hours (F = 2.467, P = 0.1545) or at 28 days (F = 2.332, P = 0.1781) of its addition. By contrast, the higher dose of biochar cause remarkable effects on the microbial biomass, which increases after 12 hours of the addition (F = 65.418, P <0.0001) and decreased 28 days later (F = 60,331, P <0.0001). Therefore, biochar causes a small reduction of the specific respiration activity at 6 and 12 hours (F = 12.600, P = 0.0048, F = 52.209, P <0.0001, respectively), but a significant increase at 28 days (F = 45.711, P = 0.002).

4.3.2. *Insecticide effects*

As showed in figure 4.1, at 6 hours of incubation, O_2 consumption was not affected by adding the insecticide Imidacloprid (F = 2.854, P = 0.1118), with no significant interaction with the presence of biochar (F = 2.275, P = 0.1371). By contrast, a moderate inhibitory effect of insecticide appears at 12 hours, and was greater at 28 days (F=11.127, P=0.0042 y F=75.003, P=< 0.0001, respectively).

The addition of insecticide also caused, 12 h after its addition, a significant increase of the microbial biomass (F = 39.311, P <0.0001), but only in soils not treated with biochar, or amended with its lower dose (F = 23.180, P <0.0001). After 28 days of incubation, the insecticide caused a decrease in the microbial biomass in soil not treated with biochar, while generated an increase in that amended (F= 49.819, P= <0.0001) especially for the lower dose of biochar (F= 145.170, P= <0.0001).

Specific respiration calculated after 6 hours of incubation was very low in samples treated with insecticide ($F = 41.958$, $P =< 0.0001$) and the control ones, with a significant interaction with biochar ($F = 5.086$, $P = 0.0195$). The inhibitory effect of the insecticide persisted at 12 h ($F=111.514$ $P<0.0001$). After 28 d of incubation the effect of insecticide shows a moderate increase of specific respiration for samples not treated with biochar and a big decrease for the others ($F=84.453$, $P=< 0.0001$) with a clear interaction with biochar ($F= 145.170$, $P= <0.0001$).

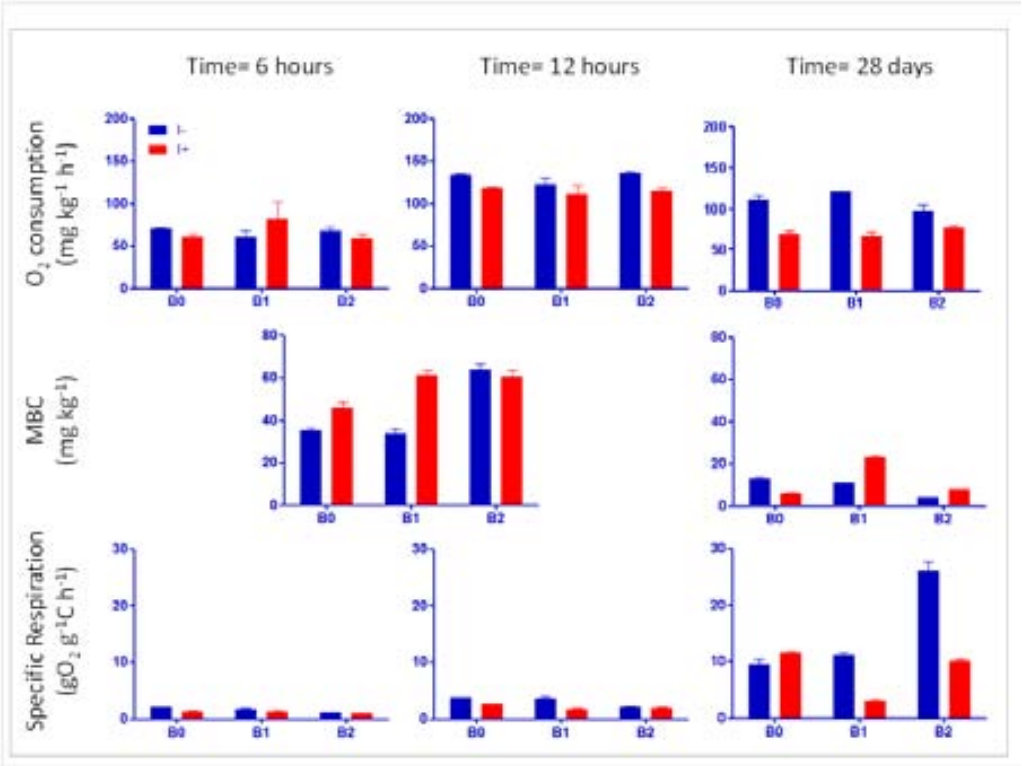


Figure 4.1 Insecticide effects (treatments I+, I-/ F- H-). Average values of O_2 consumption (upper row), microbial biomass-C (middle row) and specific consumption of O_2 (bottom row) along the time since the insecticide was added: 6 hours (left column), 12 hour (middle column) and 28 days (right column). Within the graphs, blue bars correspond to the soil without insecticide, while red bars indicate the results of the treated ones. B0: soil without biochar; B1 and B2: soil amended with biochar (1.9 and 11.5 g kg^{-1}, respectively).

3.3. *Fungicide effects*

Figure 4.2 illustrates the effects of the addition of the fungicide Thiophanate. It caused a slight increase in soil O_2 consumption at 6 hours of incubation ($F = 5.468$, $P = 0.0360$), most visible at 12 hours ($F = 18.257$, $P = 0.0009$) and at 28 days ($F = 8.206$, $P = 0.0118$), not depending on biochar dose ($F= 0.684$, $P= 0.5218$; $F=1.823$, $P= 0.2005$; $F= 2.892$, $P = 0.0866$; respectively). Microbial biomass, practically was not affected by the incorporation of fungicide at 12 hours after its addition ($F =$

2.099., P = 0.1656), and presented a sharp increase at 28 days after the treatment (F = 273.177, P <0.0001).

Regarding the specific respiration, no effects were observed along the first 6 or 12 hours of incubation, but a strong decrease was found after 28 days (F = 138.549, P <0.0001). This decrease was dependent on the dose of biochar (F = 45.995, P = <0.0001), being greater as highest biochar dose was added.

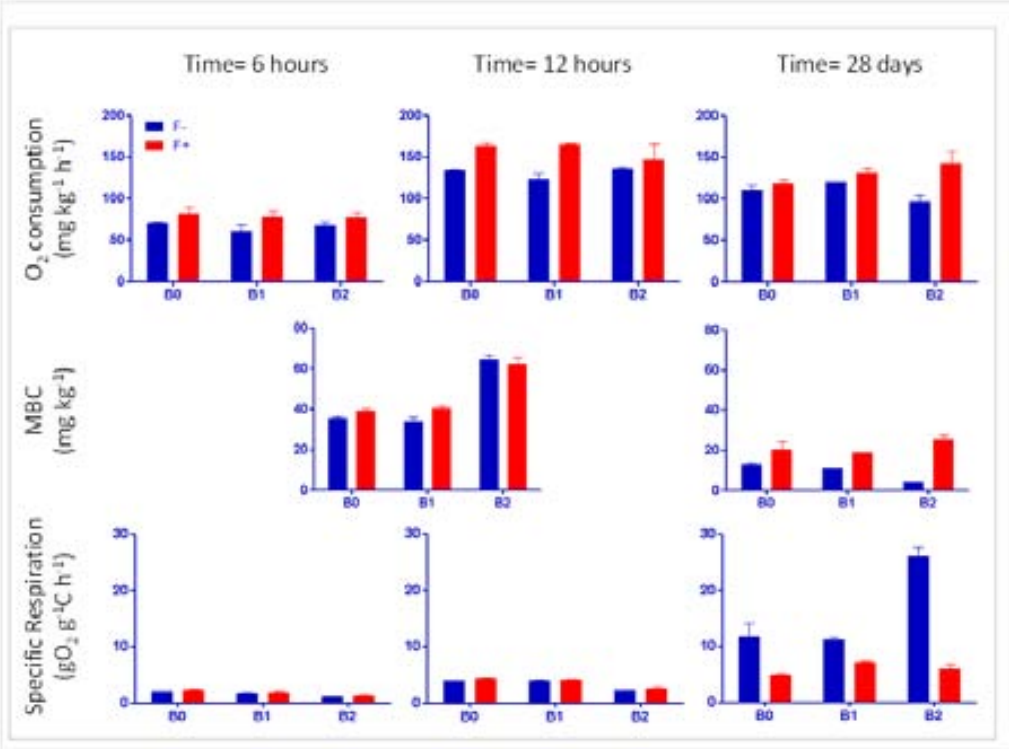


Figure 4.2 Fungicide effects (treatments I- F+/- H-). Average values of O_2 consumption (upper row), microbial biomass-C (middle row) and specific consumption of O_2 (bottom row) along the time since the fungicide was added: 6 hours (left column), 12 hour (middle column) and 28 days (right column). Within the graphs, blue bars correspond to the soil without fungicide, while red bars indicate the results of the treated ones. B0: soil without biochar; B1 and B2: soil amended with biochar (1.9 and 11.5 g kg^{-1}, respectively).

3.4. *Herbicide effects*

The addition of the herbicide Glyphosate (Figure 4.3) did not generate a global effect on O_2 consumption at 6h, 12h and 28d of incubation (F = 0.633, P = 0.4395; F = 0.175, P = 0.6818 and F = 0.707, P = 0.4137, in that order).

Nevertheless, microbial biomass was strongly affected by the addition of the herbicide, which displayed an increasing or decreasing trend depending on the

interaction with the dose of biochar ($F = 7.091$, $P = 0.0054$ and $F = 293.637$, $P < 0.0001$, at 12h and 28d respectively). However, the effects of the herbicide vary remarkably throughout incubation. At 12h, the inhibitory effect was detected in the soil treated with the higher dose of biochar, while larger amounts of microbial C were found in the untreated soil. On the contrary, these effects were the opposed at 28d.

The specific respiration was low at short incubation times (6 and 12 h) but still showed marked effects of the addition of the herbicide. At 6 and 12 hours ($F = 7.456$, $P = 0.0163$ and $F = 25.554$, $P = 0.0002$, respectively) the herbicide reduced the specific respiration of the soil not amended with biochar, had no noticeable effect on the soil amended with the lowest dose, and slightly increased on the soil amended with the highest dose ($F = 22.086$, $P = 0.0003$). After 28 days of incubation, the effect of the herbicide was clearly reflected in an increase of specific respiration of the unamended soil but, concerning the soil treated with biochar, the addition of the herbicide decreased the specific respiration at the B1 and B2 doses of biochar, respectively ($F = 121.643$, $P < 0.0001$).

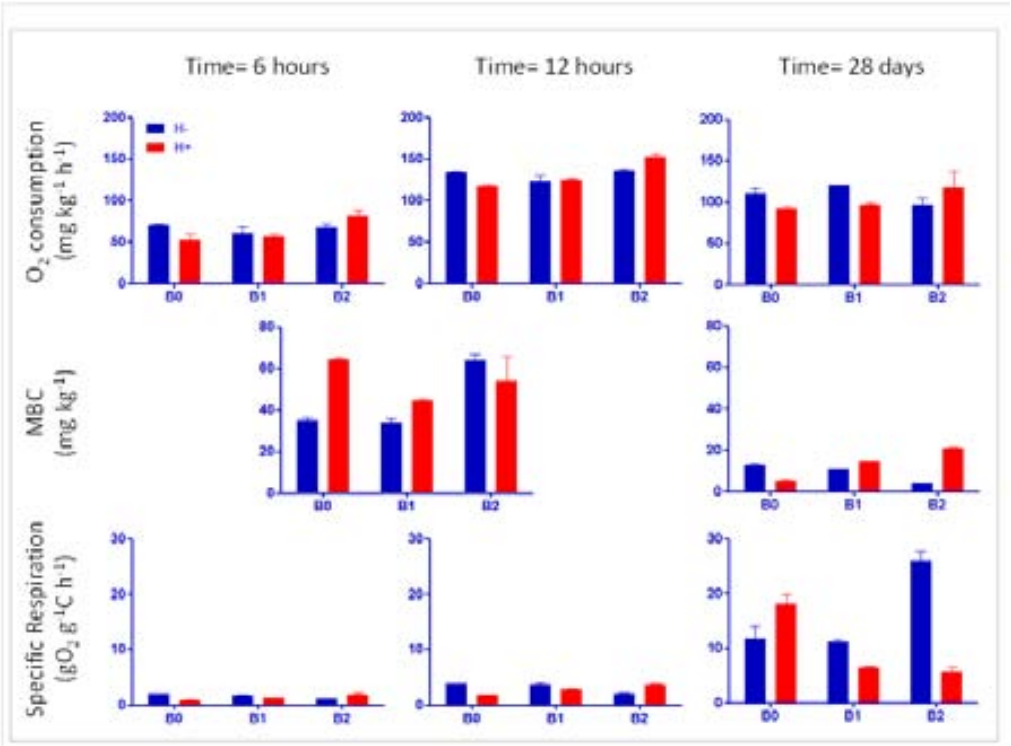


Figure 3. Herbicide effects, treatments I- F- H+/-. Average values of O_2 consumption (upper row), microbial biomass-C (middle row) and specific consumption of O_2 (bottom row) along the time since the herbicide was added: 6 hours (left column), 12 hour (middle column) and 28 days (right column). Within the graphs, blue bars correspond to the soil without herbicide,

while red bars indicate the results of the treated ones. B0: soil without biochar; B1 and B2: soil amended with biochar (1.9 and 11.5 g kg^{-1}, respectively.

4.4 Discussion

4.4.1 *Influence of biochar on microbial indicators*

The addition of biochar represents a C source that may be partially available for soil microorganisms (Hamer et al., 2004; Stariner et al., 2008), although no noticeable changes in soil O2 consumption have been found in the present work after this amendment. The mineralization of biochar has been extensively described as a slow process that mainly affects its most labile fraction (Nguyen & Lehmann, 2009; Novak et al., 2009), so it could not be easily detected by overall respirometric measurements due to the bigger fluxes of gases (CO2 and O2) caused by the mineralization of the native soil organic matter (Kuzyakow et al., 2008), and particularly after the addition of glucose. Nevertheless, the addition of the high dose of biochar caused a notable increase in the amount of microbial biomass-C at short term. Although remarkable improvement of the soil attributes have been described as a consequence of the addition of the biochar (Yamato et al., 2006; Boehm 1994; 2002; Fukuyama et al., 2001), as this change has been produced very quickly, this suggests that some amount of microbial C was colonizing the partially charred biomass, even before its addition to the soil. Indeed, biochar is a porous, nutrient sorbent and C-rich material suitable for the colonization of a large variety of microorganisms (Thies & Rillig, 2009).

The microbial metabolic quotient (qCO_2) has been used as an indicator of the efficiency of the C use by soil microorganisms (Anderson & Domsch, 1990; 1993). Being computed in this paper as the ratio between the O_2 consumption and the microbial C, the specific respiration has probably the same mean that the qCO_2. Therefore, the slightly lower values of specific respiration found after 6 and 12 hours of the addition of the biochar can be explained by (i) a higher C use efficiency of the soil microorganisms after the addition of biochar (Jin, 2010), or that (ii) the microbial biomass provided with the biochar was more efficient metabolizing C substrates than the soil microbial biomass.

The significant reduction of microbial biomass along the incubation period suggests a progressive depletion of the most labile C sources, which probably selects microorganisms able to use more stable C sources (Pietikainen et al., 2000). Labile organic matter depletion by mineralization and/or protection by biochar sorption (Ogawa et al., 2006; Liang et al., 2009; Thies 2009; Marchetti et al. 2012) probably reduce the C use efficiency of soil microorganisms, thus increasing the specific respiration along time.

4.4.2 *Insecticide effects*

The reduction of O_2 consumption 12h after the addition of the insecticide and along the time suggests an adverse effect on the metabolism of the soil microorganisms. Nevertheless, our results did not indicate that the insecticide caused a significant lethal effect on soil microbiota, as the amount of microbial biomass did not changed, or even has increased, immediately after the addition of the insecticide. After 28 days of incubation microbial biomass decreased in soil not treated with biochar while increased in the other.

As a consequence, the specific respiration of these samples was also lower than non-treated ones, suggesting that biochar had a blocking effect of the functional toxicity of the pesticide but this effect is more evident in the low dose of biochar. Several authors have proposed that biochar has the ability to adsorb some pesticides and then partially modulate their adverse effects to soil microbiota (Ennis et. al., 2012; Wang et al., 2010).

4.4.3 *Fungicide effects*

Unlike what observed in the case of insecticide, the enhancement of O_2 consumption 6h and 12h after the addition of the fungicide suggests that the addition of this agrochemical does not produce any adverse effect and can be used as a mineralisable substrate, therefore as an energy source, by soil microorganisms (Frioni, 1999). Moreover, after 28 d of incubation the consumption of O_2 recovered normal values; this may be due to the progressively consumption of the chemical added, as suggested by its known half-life in soil (European Commission, 2005).

Fungicide does not cause lethal effects on soil microbial biomass (probably dominated by bacteria) which remains fairly stable after 6 and 12 hours of

incubation, and increases after 28 days. This growth indicates that the microbial community found the equilibrium in the later period of incubation as suggested by the decrease of the specific respiration rate.

Regarding the modulator effect of biochar on the soil response to the addition of the fungicide, it appears noticeable only at the end of the incubation. At this time, all fungicide-treated soils are showing microbial biomass values greater than their respective controls, but this difference is maximal in the case of soils that received the higher dose of biochar. This is probably due to the capacity of biochar to block labile organic matter (Sohi, 2010).

4.4 *Herbicide effects*

No significant changes in O_2 consumption were observed as consequence of the addition of herbicide during all period of soil incubation, in spite of a slightly reduction in herbicide treatments that were neutralised by biochar at high dose.

On the contrary, an irregular pattern of microbial growth were detected at 12h which increased in soil not amended with biochar (B0), remained stable in B1, while decreased in B2 treated soil. This result suggests that, at short term, herbicide modifies the microbial population, needing more biomass to maintain similar respiration levels. The presence of biochar modulates this effect. After 28 days is possible to observe the opposite situation of microbial pattern suggesting the beneficial effects of biochar to enhance microbial biomass. This result clearly demonstrates the ability of biochar to modulate the effects of this herbicide on soils microbial biomass (Nag et al., 2011). As the immobilisation of the pesticide on biochar surface needs some time to be produced (Hammes and Schmidt, 2009) this effect appeared after 28 days of incubation but not in the early stages.

5 Conclusion

The results of this work suggest that the expected protective effect of biochar against harmful pesticide actions is not detectable in the early stage of incubation but it increase over time. High doses of biochar best perform this task. A modulation due to the presence of biochar of the effects produced by the agrochemicals tested has been proved.

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Chapter 5

5.1 General discusions

The general aim of this thesis was to contribute to understanding some interactions between biochar and soil. To achieve this goal we decided to study the biochar stability/degradability over time as well as its relationship with the microbial activity of the soil, especially in the presence of pesticides.

Our results remark how different biochars acted in different ways depending on their intrinsic physical-chemical characteristics. The design of the experiments of this thesis gave the opportunity to underline how the same feedstock can be transformed in different biochars having different properties depending on the pyrolysis process selected. This leads to different to positive or negative interaction with soil.

An example of unexpected disadvantages came out from an experiment intended to understand the absorption capacity of of labile organic matter. Sorption did not acted as a long-term protective mechanism against the mineralization of the most labile organic fraction in a soil amended with biochar produced by fast pyrolysis (PR). No evidence of long-term protective interactions could be established by our results. But later experiments suggest that this upshot strongly depends on the type of biochar used to perform this task, as demonstrated by a second experiment of this thesis focused in establishing what kind of pyrolysis process is the most suitable to obtain biochar for soil applications. It was possible to reach this conclusion trough the study of dose-response curves, using soil microbial activity as an endpoint. This allowed establishing the "safe" dose, checking if usual agronomic applications were within this range. For instance, PR showed a faster degradation rates when applied to the soil. Considering the importance of the equilibrium of microbial biomass, the results of this study also suggested that biochar produced by fast pyrolysis is the less recommendable if used in doses higher than the calculated ED50. By the contrary, biochars obtained by slow pyrolysis (PL) and gasification (PG) represent the safest biochars concerning the response of the whole microbial biomass.

In view of these results, PL was chosen to evaluate if the addition of biochar could modulate the toxicity of three pesticides once added in a reference soil. This work suggested that biochar proceeding from slow pyrolysis (PL), unlike what we had previously observed with biochar produced by fast pyrolysis (PR), establishes a protective effect of the labile soil organic matter, which in addition increases over

time. The effect of the dose was there relevant, as high doses of biochar were more effective to perform this task. A modulation of the adverse effects produced by the chosen agrochemicals due to the presence of biochar was proved.

In the light of the results of this thesis, it is unavoidable wondering how many types of biochar could be produced. Moreover, and keeping in mind that each kind of biochar showed different interactions with soil, it is clear that research in this area is still in its infancy. This remarks the need to study at different levels and from different approaches to reach enough knowledge about the interactions between different types of biochar and soils.

More in-depth researches are needed before using biochar as a mean of global scale carbon sequestration. These experiments would be a contribution in this direction by giving some starting points for further works focused in establishing a guideline for biochar applications.

5.2 General conclusions

The specific conclusions of the experimental chapters of this thesis are:

With regard to the capacity of fast pyrolysis biochar (PR) to adsorb and protect the labile organic matter the main conclusion is:

The mineralization of pine-wood biochar obtained by fast pyrolysis was relatively slow and accounted for approximately a C loss of 0.4% per year. The mineralization of glucose was faster and dependent on the addition of nutrients. The mineralization of organic matter in the soil treated with both biochar and glucose could be explained as the sum of the mineralization of the two C sources separately. Therefore, no evidence of protective interactions could be demonstrated by our results. Although glucose was effectively sorbed in the biochar-amended soil, sorption did not act as a long-term protective mechanism against mineralization in this artificial soil.

Concerning the response of the microbial biomass to different doses of three types of biochar produced from the same feedstock it is possible to say:

As expected, all types of biochar result more resistant to microbial degradation when compared with the feedstock. Considering the importance of the equilibrium of microbial biomass in the soil, the results of this study suggests that biochar from fast pyrolysis (PR) is the less recommendable if used in doses higher than the calculated ED50. Biochar produced by slow pyrolysis (PL) and gasification (PG) tested in this experiment represent better biochars from the point of view of the response of microbial biomass. In fact, the better efficiency of C use by microbial biomass may have implications for soil C sequestration and seems that these types of biochar may positively influence soil organic C retention. As result indicates, PL and PG do not cause detrimental effects on microbial community when added to the soil, even in high doses, and were very resistant to degradation along time.

In reference to the capacity of the biochar to modulate the adverse effects of pesticides in soil the main conclusion is:

The results of this work suggest that the expected protective effect of biochar against harmful pesticide actions is not detectable in the early stage of incubation (6

to 12h) but it increases over time (28 d). High doses of biochar perform better this task. A modulation due to the presence of biochar of the effects produced by the agrochemicals tested has been proved.

5.3 Comments for future reserchs

This thesis gave information about positive and negative effects of adding biochar to the soil.

The contribution for the research in this direction has been performed studying the response of microbial biomass.

The most curious thing was discover how many types of biochar can be produced depending on pyrolysis process. Each one have a different effect once added to the soil therefore suggesting to:

-investigate more types of biochar before to define a guideline for a global application.

-Study in deep the potential absorption and adsorption of biochar in realation to the possibility to use it as a mean of carbon sequestration in soil and to block the effects of contaminants.