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Biochar-based remediation of water and soils contaminated by organic compounds

Ph.D. Dissertation

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“Il processo di una
scoperta scientifica è un
continuo conflitto di meraviglie”
Albert Einstein.

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Contents

Chapter 1

1 Introduction

1.1 Environmental pollution

The term “environmental pollution” includes the word environmental and pollution. The environment means all that affect and influence the growth and the development of organisms. Pollution is the presence of hazardous material that destroy the delicate balance of the ecosystem. There are different causes that lead to phenomena of pollution, such as gas, that causing air pollution, solids/liquids, that causing water food and land pollution. Therefore the “environmental pollution is an alteration, caused by human or from natural origin, which results in the occurrence of a temporal discomfort or permanent damage on human and/or the environment. In this unbalanced situation, the environment is unable to process the changes or we can say is unable to decompose the non-natural elements known as “anthropogenic pollutants” (Khanka, 2012).

The environment is constantly polluted by a wide range of hazardous chemicals, released from different anthropogenic activities, and having different structures and toxicity levels. The main sources responsible for pollutants in the environment are industrial activities, urban agglomerations, agricultural practices, production of weapons of war, etc. In particular, the development of chemical industries led to the synthesis of a large number of toxic chemical compounds, such as pesticides, solvents, wastewater, polycyclic aromatic hydrocarbons (PAH), chlorophenols (CP), explosives, dyes, etc. (Scelza, 2008). Many countries have introduced law to regulate and mitigate the adverse effect of pollution, in fact the level of pollution need to be controlled if we want to keep the environment safe and healthy. There are three kinds of environmental pollution:

- Air pollution, due to the presence of nitrogen dioxide, sulphur dioxide, carbon monoxide, airborne particle volatile organic compounds (VOCs).
- Water pollution, due to the insecticides, pollutants from livestock operation, (VOCs) food processing and chemical waste.
- Soil pollution, due to hydrocarbons, solvents chlorinated compounds, heavy metal, etc. (Khanka, 2012).

This three environmental media are characterized by migration phenomena of pollutants that are strongly complex and depend on the hydrogeological, meteorological and ecological properties. In addition, the physical and chemical properties of pollutants play the main role in this phenomenon and in particular:

- *solubility* the maximum amount of a substance which, can dissolve in the aqueous phase under specified temperature and pressure conditions;
- *density*, allows to determine that the gaseous substances are heavier than air, or if the polluting substances present in the liquid phase are more or less heavier of the water;
- *partition coefficient solid/liquid*, which expresses the passage of a substance from the liquid phase to the surface of a solid phase, for adsorption, precipitation on the surface of the solid or by diffusion;
- *partition coefficient octanol/water (Kow)*, which is a dimensionless coefficient used as an index of the tendency of an organic compound, to accumulate in fatty tissues of living organisms, and then to be shared between an aqueous phase and an organic phase;
- *vapor pressure*, which provides an indication of the tendency of a substance to move in the atmosphere or in the air of the soil (Haynes, 2014).

Once the pollutants are released into the environment, their spread is associated with:

- runoff on the surface with rainwater;
- direct volatilization into the atmosphere or transport of the dust by wind;

- wash out to the basement or direct percolation into the aquifer.

Typically, the subterranean road is considered the most dangerous both for the temporal persistence and for the large spatial extension (Tedoldi et al., 2016).

1.1.1 Effects of environmental pollution

Environmental pollution can cause effects on human health, ecosystems and the economy, and in particular:

- discharges of contaminants into the soil, groundwater or surface water;
- absorption of contaminants by plants;
- direct contact of humans with contaminated soils;
- inhalation of dust or volatile substances;
- fire or explosion of landfill gas;
- corrosion of underground pipes and other parts of buildings;
- production of secondary hazardous waste;
- conflict with the intended use foreseen for the soil.

The contaminants that are soluble in water can easily seep into the soil and pollute the deeper aquifer where there are reserves of drinking water.

The contamination of surface waters causes the accumulation of contaminants in fish and other organisms and therefore, the entering the food chain, that leading to the bioaccumulation phenomena.

In addition to inhale, volatile substances and soil particles (through the dust) could be possible from contaminated sites. Typical examples of source of volatile substances are former site of petroleum storage, while examples of the particulate dispersion are the landfill of heavy metal waste, the nearby mines and plants of metalworking.

The changes of land use may be the cause of an increase in exposure to pollutants. In the past, many former industrial sites and abandoned landfills were used for other purposes, such as for agriculture, house and school construction

increasing the risk of ingestion or contact of pollutants accumulated over time (EEA, 2012).

1.2 Persistent organic pollutants

Persistent organic pollutants (POPs) are among the most dangerous pollutants released by anthropic activity into the environment every year.

These are highly toxic molecules that can cause a range of negative effects on humans and animals in particular disease, birth defects and death (Carpenter, 2011). Furthermore, their lipoaffinity leads to bioaccumulation phenomena, or to an accumulation in organism in concentrations greater than those found in the environment. Residues were detected in fish, wild animals and in human blood, as well as in food samples (Guerranti et al., 2011; Guerranti and Focardi, 2011). These compounds are highly stable and can last for years or decades before that they are degraded (Puzyn et al., 2011). In addition, they can move on the globe through a process known as the grasshopper effect (Figure 1).

Due to their semi-volatility, these molecules are able to assume, depending on the temperature, the solid (with cold) or gaseous state (with heat) and then evaporate and cover considerable distances in hot weather condition, and condense and precipitate on the coldest point (Gouin et al., 2004).

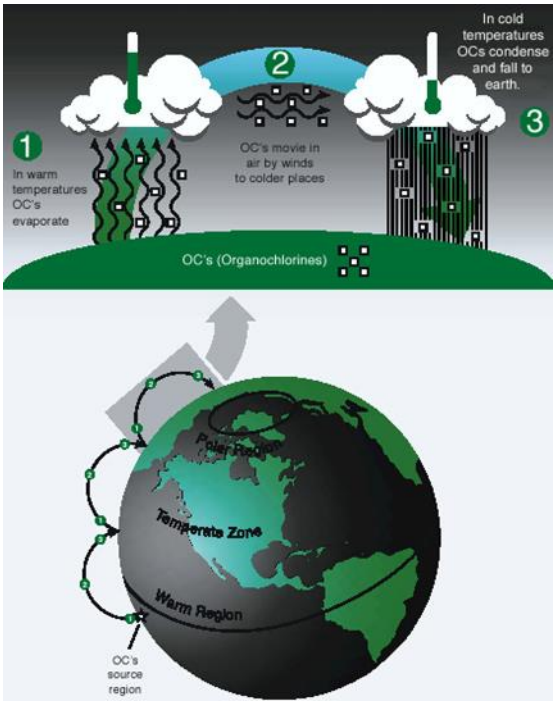

Figure 1. Grasshopper effects.

The international community has been working to eliminate or reduce the POPs, and different organization were involved. In fact, at the Stockholm Convention (2001) 12 POPs were selected based on environmental and toxicological aspect. In last decades based on toxic equivalency factor (TEF) or relative potency (REP), the number of POPs increased to 17. Between the POPs present in the list of Stockholm Convention were considered chlorinated compounds but not PAHs, which are included after two year, based on TEF and REP (Table 1) (Eljarrat and Barceló, 2003).

Table 1. POPs from Eljarrat and Barceló, (2003).

POPs selected on the Stockholm Convention	POPs with an assigned on toxic equivalency factor (TEF) or relative potency (REP)	Emerging POPs
Aldrin		
Chlordane		
DDT		
Dieldrin		
Endrin		
Heptachlor		
Hexachlorobenzene		
Mirex		
Toxaphene		
PCBs	PCBs	
PCCDDs/PCDFs	PCCDDs/PCDFs	
	PCNs	
	PBDEs	PBDEs
	PBDDs/PBDFs	PBDDs/PBDFs
	PBBs	PBBs
	PAHs	

1.3 Polycyclic aromatic hydrocarbon

Polycyclic aromatic hydrocarbons (PAHs) constitute a large class of organic compounds containing only carbon and hydrogen atoms organized in two or more condensed aromatic rings (i.e. PAH unsubstituted and alkyl-substituted derivatives) (Figure 2). In a broader sense the name "polycyclic aromatic compounds" also includes the functional derivatives (e.g. the nitro-PAH) and heterocyclic analogues (e.g. the aza-arenes).

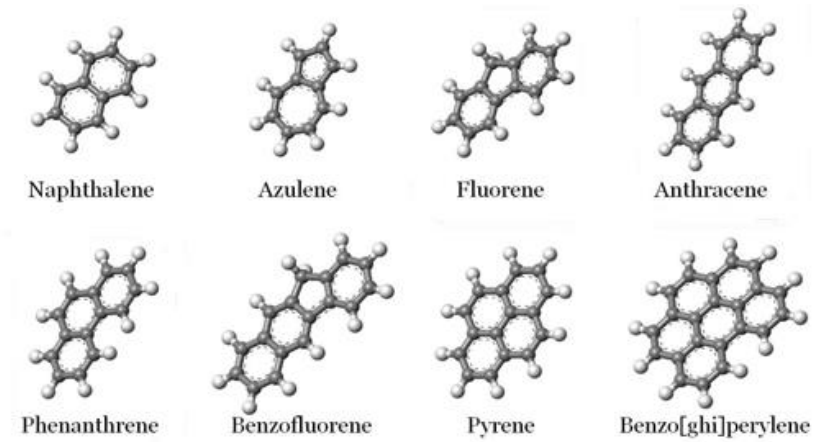

Figure 2. PAHs.

1.3.1 Fate and source of PAHs

PAHs are formed during the incomplete combustion or pyrolysis of organic material, such as coal, wood, petroleum products and wastes, through a complex mechanism of repolymerization, especially in conditions of oxygen deficiency (Figure 3).

The main sources that lead to the release of PAHs are:

- various industrial processes (in particular, aluminium and iron production, steel foundries);
- coal and oil processing;
- electric power generation plants;
- incinerators;
- domestic heating, especially wood and coal;
- emissions from motor vehicles;
- forest fires;
- combustion in agriculture;
- the flame of cooking food;
- tobacco smoke;
- volcanic eruptions, with significant local impact.

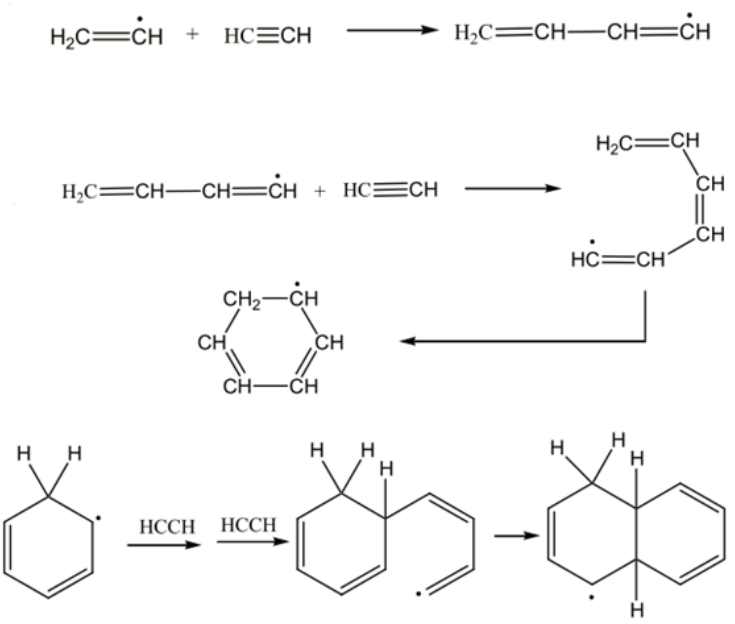

Figure 3. PAH formation mechanism.

Because of these numerous and diffuse sources, PAH are ubiquitous and are found in the environment.

In according to the Italian Agency for Environmental Protection the main emission source for PAHs are fumes of vehicles, associated with the use of oils, diesel fuel lubricant etc. Total global emission of PAHs is estimated to be 520 giga grams per tear (Gg y^{-1}) (Rengarajan et al., 2015).

1.3.2 Physical and chemical properties

PAHs consist of condensed benzene rings and the number of rings determines the chemical, physical, and toxicological characteristics.

They can be divided into low molecular weight compounds with less than four rings (naphthalene, anthracene, phenanthrene etc.) and high molecular weight with four or more rings (benzo(a)anthracene, chrysene, benzo(a)pyrene, etc.). Physical properties of PAHs change with molecular weight and structure. The

vapour pressure decreases by increasing molecular weight therefore low molecular weight compounds at room temperature may be in the vapour state. They are soluble in many organic solvents and they have a lower solubility in water depending on the number of fused benzene rings. In fact, the hydrophobicity increases by increasing of number of rings.

PAHs show, in addition, light sensitivity, heat resistance, conductivity, corrosion resistance, and physiological action. PAHs have very characteristic UV absorbance: each ring structure and isomer have a unique UV absorbance spectrum. This is useful for their identification. Most PAHs are also fluorescent by the emission of a characteristic wavelength of light when they are excited (Kim et al., 2013).

1.3.3 Toxicity

PAHs are also known for the highly adverse effects on humans, in fact, once that PAHs are ingested (or inhaled), they are rapidly absorbed through the gastro-intestinal tract, and distributed in various tissues (especially in the tissue with high fat content), including fetal tissue (Bocca et al., 2003).

Experiments on animals and genotoxicity test *in vitro* and *in vivo* demonstrate that several PAHs are carcinogenic (Table 2). IARC performed the evaluations on the carcinogenicity of PAHs. Because of their toxicity, various legal provisions limit the production and distribution of PAHs in the environment (DFI, 2008):

- the Chemical Risk Reduction ordinance which defines the standard values or the limit for PAHs and benzo(a)pyrene in wood products, treated wood, compost, digestate and oils for the tire production;
- the Foreign Substances ordinance prescribing the tolerance values for PAHs and benzo(a)pyrene in food and drinking water, as well as their limits in food for infants;

- the order against the deterioration of the soil fixing indicative values, for PAHs and benzo(a)pyrene in soil, gardens, vegetable gardens and children's play areas;
- the Water Pollution Control ordinance which regulates the concentration of PAHs in groundwater use as drinking water;
- the Air Pollution Control ordinance that limits the emissions of benzo(a)pyrene.

Table 2. Molecular weight, number of aromatic rings and carcinogenicity of PAH.

PAHs	MW	No aromatic rings	IARC Group
Naphthalene	128	2	2B
Fluorene	166	3	3
Phenanthrene	178	3	3
Anthracene	178	3	3
Fluoranthene	202	4	3
Pyrene	202	4	3
Benzo[a]fluorene	216	4	3
Benzo[a]anthracene	228	4	2A
Chrysene	228	4	3
Benzo[b]fluoranthene	252	5	2B
Benzo[k]fluoranthene	252	5	2B
Benzo[j]fluoranthene	252	5	2B
Benzo[e]pyrene	252	5	3
Benzo[a]pyrene	252	5	1
Perylene	252	5	3
Benzo[ghi]perylene	276	6	3
Indeno[1,2,3-cd]pyrene	276	6	2B
Benzo[b]chrysene	278	6	3
Dibenzo[a,j]anthracene	278	6	-
Dibenzo[a,h]anthracene	278	6	2A
Dibenzo[a,c]anthracene	278	6	-

Meaning of IARC groups: 1, carcinogenic to humans; 2A, probably carcinogenic to humans; 2B, possibly carcinogenic to humans; 3, not classifiable carcinogenic to humans (Bruschweiler et al., 2012).

1.4 Chlorophenols

Chlorophenols (CPs) are organic compounds deriving from phenols (1-hydroxybenzene) by substitution in the ring with one or more chlorine atoms. There are nineteen congeners ranging from monochlorophenol to pentachlorophenol (PCP) (Figure 4).

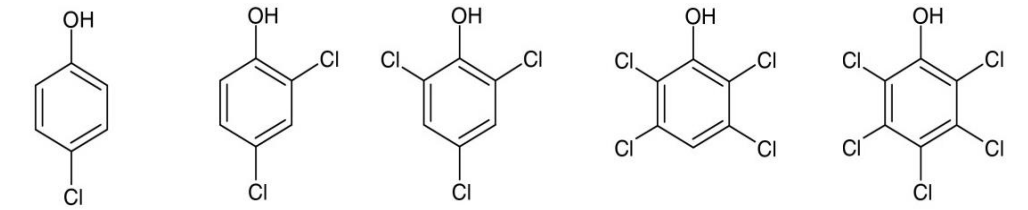

Figure 4. Clorophenols.

CPs, in particular trichlorophenols, tetrachloro-phenol and PCP, are available as salts of sodium or potassium. CPs have a very low solubility in water that decrease by increasing the number of bonded Cl. All CPs are solids at room temperature except 2-CP, which is liquid (Olaniran, and Igbinosa, 2011). CPs may form from several materials (soot, carbon, charred and incomplete combusted materials). CPs can be formed also by PAHs combining catalytic chlorination and oxidative breakdown (Peng et al., 2016).

CPs have an irritating effect on the eyes and the respiratory tract. They can be absorbed through the respiratory tract, the gastro-intestinal tract and the skin. Large doses may cause convulsions, respiratory failure and even lead to death. High concentration of CPs may cause also can develop the symptoms mentioned above including mutagenicity (Greene and Pohanish, 2005).

1.4.1 Formation and fate of chlorophenols in the environment

CPs were used to eradicate microorganisms and as wood preservative as well as the operation of bleaching process. Pentachlorophenol (PCP) was widely used as insecticide but became banned after the Stockholm Convention, for its high toxicity, persistency and low biodegradability (Peng et al., 2016). Therefore, the presence of chlorinated organic compounds in nature is generally attributable to human activities (Annachhatre and Gheewala, 1996). Chlorophenolic compounds are recalcitrant to biodegradation and persistent in the environment. Because of the lipophilicity they can move through the cell membrane and bio-accumulate in aquatic organisms (Pedroza et al., 2007). They are considered harmful for human health due to their potential carcinogenic and mutagenic activity. In natural ecosystems CPs are subjected to a series of physical, chemical and biological process such as sorption, volatilization, degradation, and leaching are the primary processes governing their fate and transport. However, these natural processes can occur with different efficiencies and sometimes may be so slow to make the pollutants persist for years. Organic matter and clay content in soil, sediment and water are important environmental parameters influencing these processes, because CPs may be adsorbed on this matrix (Olaniran, and Igbinosa, 2011).

1.5 Industrial waste

In the last years, the rapid growth of industries such as fertilizer, metal plating, oil industry mining and textile industries have increased the discharge of waste into the environment, particularly in developing countries. Direct disposal of wastewater from various sources is the major cause of the environmental pollution, in particular water bodies. The protection of the water resources has become one of the crucial environmental issues of this century. Water bodies constitute majority of earth crust, but less than 3% are available for human use

due to high salinity of the others. In addition, this water bodies are under continuous contamination by effluents of wastewater hospital, municipal sewage systems, industries, run-off water from agricultural land and others, thus constituting a great threat to the health and safety of both human and environment (Ganiyu et al., 2015; Gude, 2016; Ahmed and Ahmaruzzaman, 2016).

Pollutants reach water in two main ways:

- direct way, when pollutants are discharged directly into waterways without any purification treatment;
- indirect way, when pollutants reach aquifers through other environmental media (air and ground).

The possible causes of water pollution are:

- industrial pollution: when the pollutants are discharged daily in large quantities by industry causing damage to the aquatic ecosystem;
- urban pollution, due to wastewater discharged from homes, offices and other structures. The urban waste, because of their composition (rich in detergents or slurry of organic nature), determine environmental changes such as changes in pH, reduction of oxygen and the transparency of water, increase of concentration of nutrient (from the degradation of substances organic), and contribute to the proliferation, in the coastal area of bacteria and other pathogens;
- agricultural pollution, which comes from the use of fertilizers, pesticides and slurry spread by farms;
- natural pollution, caused by weather and seasonal events;
- thermal pollution due to the excessive use of water to cool the systems of industries, especially in thermal power plants (Lgs. D. 152/2006).

1.5.1 Olive oil wastewater

The olive oil extraction generates large amounts of waste, which can have a great impact on the water and rural environment because of their high toxicity. Several studies have shown negative effects of these wastes on soil and aquatic ecosystems (Dermeche et al., 2013; Keren et al., 2017; Rusan et al., 2016). Therefore, there is a need to manage olive oil waste through technologies that minimize their impact on the environment and make possible the use of this resource.

The olive oil extraction systems provide for different processes: washing of the olives, milling, and extraction, which is the key step of the whole procedure. The amount and the chemical and physical properties of waste products depend on the method used for extraction. There are two main oil extraction methods:

- the pressure method;
- the centrifuge method.

The traditional method is based on crushing the olives into a paste, malaxing this latter, spreading it on fiber disks, which are stacked on top of each other and then placed into a press.

The centrifuge method is the most used technique in the last decades. The paste is pumped into an industrial decanter where the phases are separated using centrifugation and water can be added to facilitate the extraction process. This step can involve a three-phase decanter or a two-phase decanter. In three-phase decanting, system pushes the solids, the water and oil out whereas in two-phase decanting system produces only two fractions: a solid fraction (pomace olive damp or wet past), and a liquid fraction (olive oil).

Nowadays in Mediterranean area, where the three-phase systems are widely adopted, the olive oil plants produces between 7 and 30 million m^3 of olive mill wastewater (OMW) per year (Zbakh and El Abbassi, 2012; Justino et al., 2012). The two-phase method, able to reduce wastewater of 75%, has been launched on

the market with the labelling of "ecological" or "two-phase", because of the absence of wastewater and the reduction of the water consumption, one of the main problems related to the production of olive oil.

OMW (Figure 5) come up as a liquid of variable color between green-yellow and very dark brown, with a strong smell and a solid matrix in suspension. The organo-mineral fraction make OMW toxic for the environment. Phenolic compounds (up to 0.5 - 24 g l^{-1}), fats and salts constitute this fraction (La Cara et al., 2012).

Figure 5. OMW and olive oil.

The phenolic fraction is responsible for the toxicity for microbial communities and for the high value of chemical oxygen demand (COD), which slows down biological mineralization (Barbera et al., 2013). All these factors make difficult to discharge OMW to surface water and to sewers.

Polyphenols, which are absent in drupes, originate thanks to the action of phenoloxidase contained in the fruits and activated during the pressing procedure (Zullo et al., 2014). Also glucosidic enzymes, present in the fruit and activated in the pressing step, catalyse oxidative process on phenolic glucosides of the

fruit. Oleuropein, for example, a bitter glucoside, is present in the olive leaves and drupes, but not generally in OMW. Its degradation products i.e. elenolic acid and hydroxytyrosol can be found instead in OMW. Great interest arises about these high add-value compounds mainly used by chemical and pharmaceutical industry (Mudimu et al., 2012).

1.6 Environmental remediation

Environmental pollution represents a great concern for humans and other living organisms. Several organic and inorganic chemicals have been released into the environment because of anthropogenic activities. The rapid industrialization of agriculture, the expansion of the chemical industry, but mainly the recent resort to cheap energy resources have produced several polluting compounds.

The increase of contaminated sites due to the continuous pollution of the environment has led to possible negative effects on environmental health. Therefore, decontamination and remediation of polluted sites has become an important task, for example recovering soil health and fertility, detoxifying ground water, and reutilizing wastewater (in countries with water deficiencies) (Gianfreda, et al., 2006).

Several research activity turns to find effective, eco-friendly and possibly low-cost tools to mitigate the pollution and restore polluted environments. The remediation of polluted sites becomes difficult because of the nature of the contaminant sources and the presence of contaminant mixtures (organic and inorganic compounds simultaneously) (Thavamani et al., 2012). Moreover, if the contaminated site is soil, interactions of organic and inorganic pollutants with soil colloids, through sorption/desorption mechanisms, may affect the movement of pollutants and hence their availability for plant or microorganisms.

The remediation or restoration of water and soil compartment is possible with physical/chemical approaches or biological strategies. Physical/chemical

methodologies are very expensive and efficient traditional approaches but they do not imply a final safe result for the environment. Biological strategies involving living organisms (i.e. microorganisms, plants and plant-microorganisms associations) are environmental-friendly and, in many case, they may modify the structure and toxicological properties of the contaminants into innocuous products.

1.6.1 Methodologies of environmental remediation

The methodologies of environmental remediation have the aim to remove or reduce pollutants. The selection of remediation technologies is based on the site properties, i.e. soil and aquifer characteristics.

We cannot exclude the natural attenuation that is a process occurring without human action and in all the environmental sites, which leads to a reduction of contaminant concentration (hydrodynamic dispersion, sorption and volatilization) or the mass of contaminants (biodegradation). Sometimes, natural attenuation processes are not enough to achieve the remediation objectives in a reasonable time that is the reason why the remediation measures or the application of enhancers of biological activity should be combined.

In the last decades, the remediation of environmental sites, differently contaminated by organic and inorganic pollutants, was based on physical and chemical methodologies. They include *in situ* and *ex situ* technologies specific for soil or groundwater (Figure 6).

The choice of the more suitable technology needs a wise knowledge of all parameters characterizing sites and contaminants. Besides remediation strategy, in some cases, when the contamination levels are high and sites can be only confined, safety measures could be necessary (vitrification, reactive permeable barriers). Traditional methodologies, especially *ex-situ* ones, requiring transport of polluted matrices to specific plants and the use of expensive equipment.

Biological methodologies are a valid alternative approach with reduced environmental and economic impact. They are e*x situ* and *in situ* strategies that consist in the use of living organisms such as plants and microorganisms able to degrade contaminants or transform them in less hazardous form or biomolecules such as enzymes.

Some biological technologies (Figure 6) are:

- *Bioventing* is an in situ remediation technology that used airflow (or oxygen) or adding also nutrients if necessary, in the unsaturated zone, to enhance the development of indigenous microorganisms to biodegrade organic constituents adsorbed to soils.
- *Biosparging* is an in situ remediation technology that injects air (or oxygen) and nutrients (if needed) into the saturated zone to increase the biological activity of the indigenous microorganisms.
- *Biopiles* are used to reduce concentrations of petroleum constituents in excavated soils by stimulating aerobic microbial activity through the aeration and/or addition of minerals, nutrients, and water.
- *Phytoremediation* consists in the direct use of plants and the associated microorganisms to stabilize or reduce contamination (organic and inorganic contaminants) in soils, sludge, sediments, surface water, or groundwater.
- *Mycoremediation* is a biotechnology based on the use of fungi for cleaning up contaminated soils. Principally white-rot fungi are very effective in degrading a wide range of organic molecules as they release extra-cellular lignin-modifying enzymes (lignin-peroxidase, manganese peroxidase, and laccase). Carbon sources such as sawdust, straw and corncob can be added to polluted sites to improve the degradation processes.
- *Composting* is a treatment where polluted materials (generally with organic compounds) are mixed in piles together with a solid organic substance readily degradable such as straw, wood chips, etc.

- *Land farming* involves spreading of excavated contaminated soils in a thin layer on the ground surface and stimulating aerobic microbial activity through aeration and/or the addition of nutrients and water.
- *Bioreactors* consist in container where biological degradation of contaminants in soil and water occur by existing and/or added microorganisms is isolated and controlled.
- *Microbial filters* are packed-bed bioreactors, where microorganisms are allowed to grow in order to degrade volatile compounds once adsorbed on solid supports (activated carbon, biochar, soil, peat, etc.).

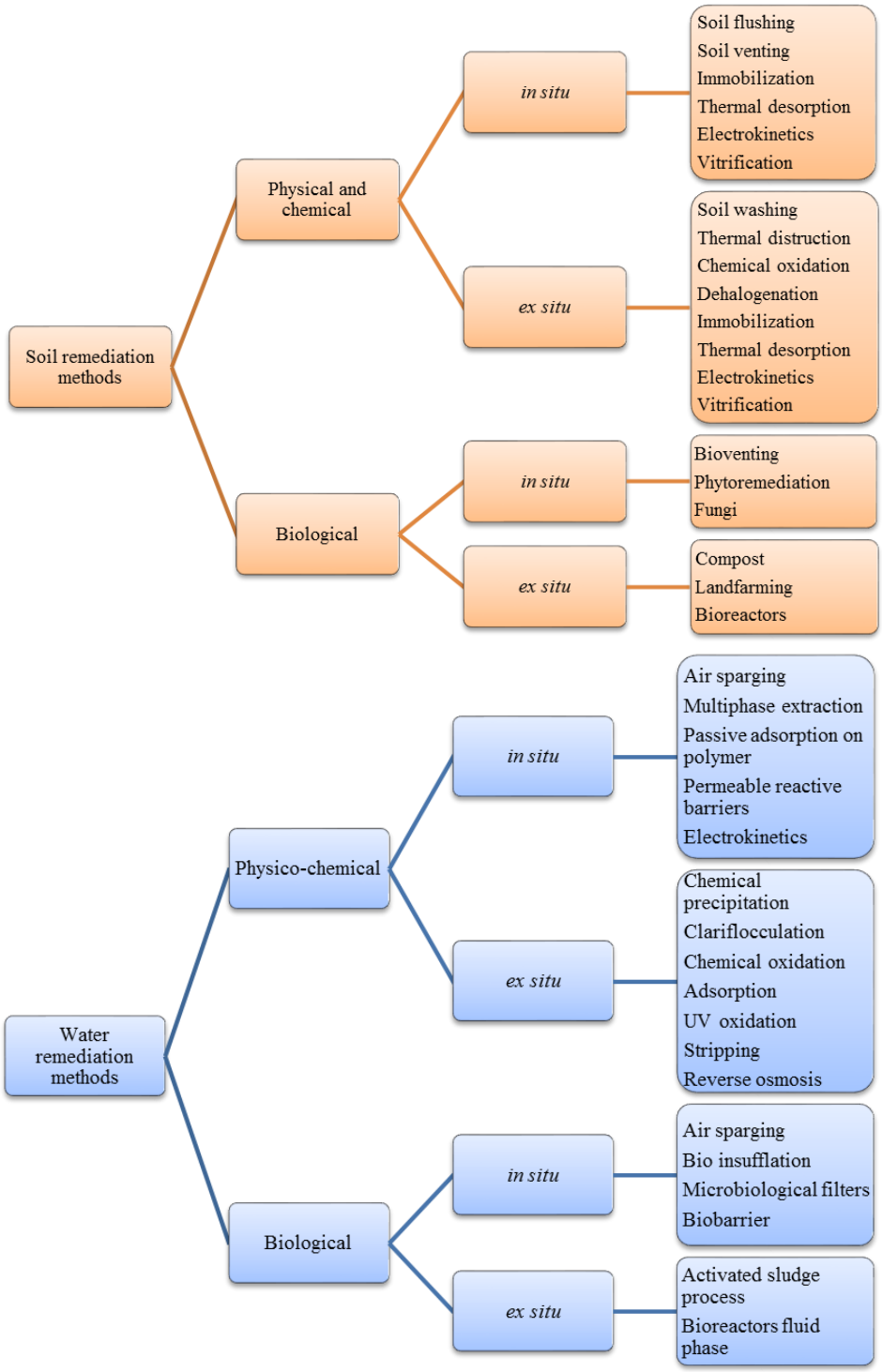

Figure 6. Soils and water remediation methodologies.

1.7 Biochar

1.7.1 Origins

"Biochar is the carbon-rich product obtained when biomass, such as wood, manure or leaves, is heated in a closed container with little or no available air. In more technical terms, biochar is produced by so-called thermal decomposition of organic material under limited supply of oxygen (O_2), and at relatively low temperatures (<700°C)" (Lehmann and Joseph, 2009). Its origin is linked to the ancient populations of the Amazon, and is locally known as Terra Preta de Indio (Ahmad et al., 2014) (Figure 7).

Figure 7. Profile of Terra Preta.

In some areas of the Amazon, during the explorations by European settlers, the soil in this area seemed to have high fertility. This was linked to the presence of

dark soil, with very different characteristics compared to the soils typical for Amazon region (Acrisol and Ferrasol), red in colour because they are rich in kaolinite, acid pH and rich in aluminium. Terra Preta, in fact, has black colour, an alkaline pH, with high percentage of endemic microorganisms (O'Neill et al., 2006). Richness in nutrients, high and stable organic matter levels, and cation exchange capacity are the reasons of the great fertility of these soils.

There are different hypotheses about the formation of Terra Preta. It could be from volcanic eruptions or the former lakes, or it could be due to the Indios' population, which buried vegetable residues through incomplete combustion (residues from cooking, forest and crops) (Erikson et al., 2003; Falcão et al., 2003; Glaser et al., 2004; Zech et al., 1990). These practices lead to an increase of carbonaceous material content of black carbon, about 70 times more than the surrounding soils and to a depth of 40-80 cm (Lehmann et al., 2003).

Several researchers studied the effects of adding coal to the soil to identify the factors and interactions that contributed to the fertility of Terra Preta.

Steiner (2006) compares the results of the two different land management practices, i.e. the slash and char as an alternative to "slash and burn". The "slash and burn", widely used in tropical areas leads to the loss of soil fertility. The production and the burying of vegetable coal (biochar) is a key factor for soil fertility and sustainability with the reduction of CO_2 emissions (Kuhlbusch et al., 1996; Steiner, 2006). In fact, thanks to the high recalcitrance of its aromatic structure, biochar represents a sink to immobilize carbon (more than 50%) (Lehmann et al., 2002), (Figure 8). This aromatic structure is slowly oxidized by producing carboxyl groups, responsible for the increase of the capacity of the carbonaceous particles to retain nutrients (Glaser et al., 2001).

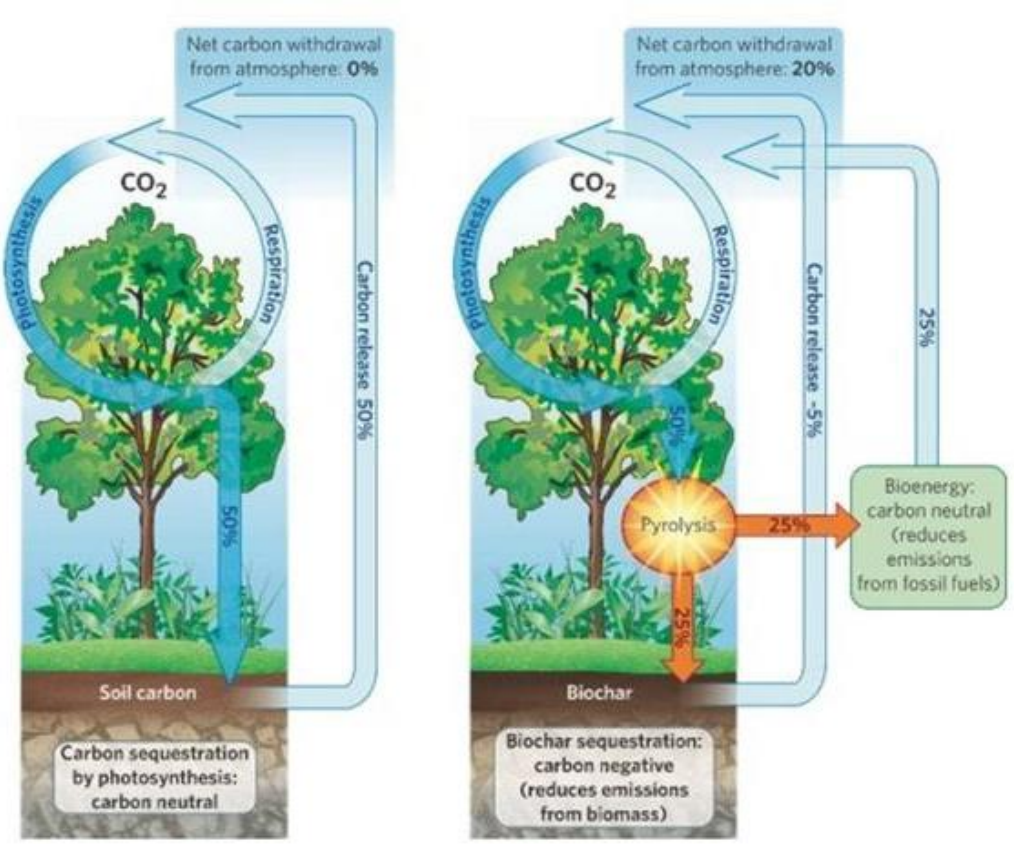

Figure 8. Natural carbon cycle and carbon sequestration in biochar.

1.7.2 Production of biochar

Biochar is the product of the pyrolysis of vegetable biomasses and is similar to coal. In fact, it could be produced in charcoal kiln, in rudimentary furnace, or in those made of steel, or with new technology providing special reactors that allow the recovery of the different products of pyrolysis (solid, liquid and gaseous fractions). While these last two fractions are used for energy purposes, the solid part is produced both for agronomic purposes (improve soil properties) and for environmental management (carbon sequestration) (Lehmann and Joseph, 2009).

Pyrolysis is a thermochemical process of organic materials decomposition, similar to the combustion, but occurring in the absence of an oxidizing agent (oxygen) (Verheijen et al., 2010).

The pyrolysis, through cracking and polycondensation reactions, leads to the transformation of organic materials in three different components:

- *biochar*, the solid part;
- *bio-oil*, which is the liquid fraction, containing organic substances such as alcohols, ketones and hydrocarbons;
- *syngas*, the gaseous fraction formed by hydrogen, carbon monoxide, carbon dioxide and light hydrocarbons (IEA, 2007).

The process temperature and the residence time of the material influence the pyrolysis process that can be fast, intermediate and slow.

The fast pyrolysis (Figure 9), which has a shorter duration than 2 seconds, is used for the production of bio-oil (about 75%).

The intermediate and slow process (Figure 10), lasting from few minutes to several hours, or even days, are preferred for the production of biochar (25-35%) (Mohan et al., 2006; Brown et al., 2009; Ahmad et al., 2014).

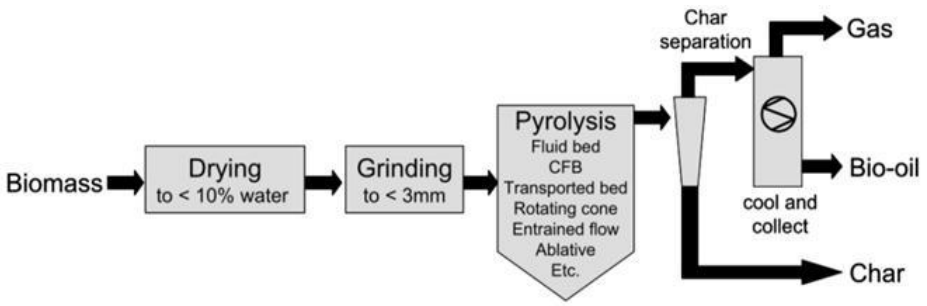

Figure 9. Fast pyrolysis (Bridgewater, 2006).

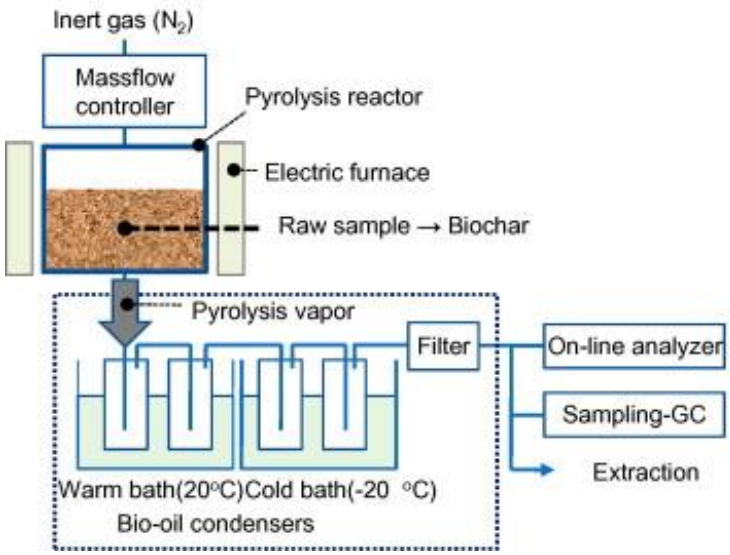

Figure 10. Slow pyrolysis (Lee et al., 2013).

The gasification differs from conventional pyrolysis processes because the biomass is converted at high temperatures (> 700 °C) into carbon monoxide and hydrogen, under controlled level of oxygen and/or steam. The resulting gas mixture is synthetic gas or syngas (Mohan et al., 2006) (Table 3).

Table 3. Pyrolysis process and percentage of the product (modified from Ahmad et al., 2014).

Process	**Temperature (°C)**	**Residence time**	**Product**		
			bio-oil (%)	*biochar (%)*	*syngas (%)*
Fast pyrolysis	300-1000	< 2 s	75	12	13
Intermediate pyrolysis	≈ 500	10-20 s	50	25	25
Slow pyrolysis	100-1000	5-30 min	30	35	35
Gasification	> 800	10-20 s	5	10	85

1.7.3 Physical and chemical properties of biochar

1.7.3.1 Physical properties

The structural characteristics of biochar depend not only on organic matter, but also on the pyrolysis system, including all the operations of pre- and post-treatment. During the thermochemical conversion, in fact, there is a loss of mass (volatile organic compounds) with the formation of fractures and microstructural rearrangements.

Lua et al. (2004) evaluated the importance of various pyrolysis conditions by determining the standard deviation and the variation coefficients of different physical parameters (surface area, production and the surface area of micropores), concluding that the main role can be attributed mainly to the pyrolysis temperature. Up to 120 °C begins the thermal decomposition, hemicellulose is degraded in the range of 200-380 °C, cellulose between 250-380 °C, and lignin between 180 and 900 °C (Slopiecka et al., 2011). Therefore, the ratio of these components (biomass) will affect the degree of reactivity and the variability level during the thermal processes.

Another structural feature to consider is the presence of fractures. The biochar is typically characterized by macrofractures that may depend on both the starting biomass and thermal processes (Byrne and Nagle, 1997). For example, biochar produced from wood is more fractured due to the different speed of the material degradation (faster outside and slowly inside). The structure is more or less similar to the graphite, but there are various orders of rearrangement (Emmerich and Luengo, 1996). The surface of biochar from wood consists of a set of faces and edges (Boemh, 2002) (Figure 11). This type of crystallite structure of various size is called turbostratic (Lehmann and Joseph, 2009).

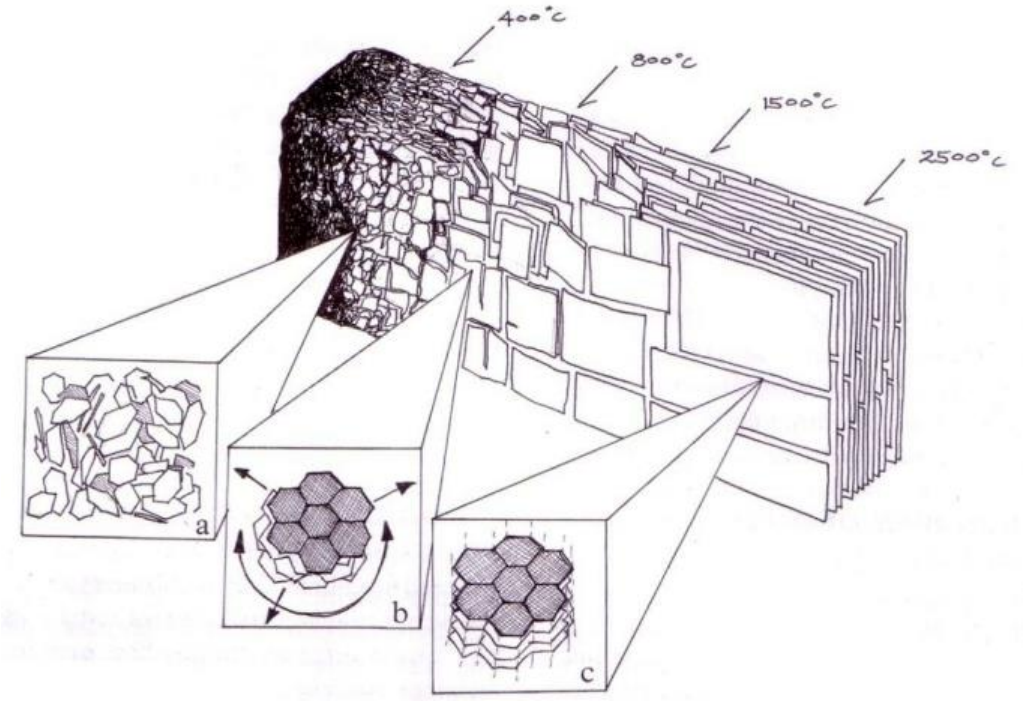

Figure 11. Structure of biochar at different temperature (Joseph and Lehmann, 2009).

1.7.3.2 Chemical properties

Biochar is produced from a wide range of biomass derived from different plants; that lead to a large variability in terms of chemical composition. This wide heterogeneity is due to the thermal process that produce, according to the temperature, changes in the chemistry of the biochar surface leading to hydrophilic, hydrophobic, acidic or basic property.

On the surface of biochar there are of different functional groups with hydrogen, nitrogen, oxygen, phosphorus and sulphur atoms responsible for the heterogeneity in the surface chemistry because of their different electronegativity (Brennan et al., 2001).

Elizalde-Gonzalez et al. (2007) demonstrated how the relative concentration of the various functional groups depends on the composition of the starting

biomass, the final temperature of reaction, the composition of the gas that surrounds the particles, the heating rate and any post-treatment.

The presence of various functional groups on the surface of biochar influences sorption properties, in according to the charge that these groups give to the surface and to the presence of π electrons.

1.7.4 Agricultural and environmental applications of biochar

To understand better the real contribution that biochar could exercise on improving the soil properties, it will be necessary to know interactions and changes occurring in soil system.

An important factor to consider for biochar application is the residence time that depends on its resistance to biotic and abiotic degradation (oxidation). In order to increase this properties there are isotope techniques in which biochar is enriched with. The residence time of biochar is estimated about hundreds or thousands years and depends on environmental conditions and the characteristics of biochar itself (Brewer, 2012).

1.7.4.1 Improving agronomic properties

The application of biochar in soils is not a new idea. The first observations about the positive effects of carbon storage in soil derive from ancient farm management practices carried out in the Amazon region known as Terra Preta. These soils are characterized by high levels of fertility without external input of fertilizers, suggesting that the application of biochar could be economically sustainable and advantageous.

Terra Preta soil in fact has high levels of organic matter and nutrients such as nitrogen, phosphorus, potassium and calcium. These features are attributed to their high content of char, and that is why the interest on biochar is growing up over the years.

In addition, because of its shape biochar gives structure to the soil improving the mechanical properties of soils.

The cation exchange capacity increases due to oxidation of the biochar surface, heaving a high O/C ratio (Brodowski et al., 2005). As organic matter, biochar is able to retain exchangeable cations, thanks also to its high porosity. The water holding capacity of the soil increases with the increase of its organic carbon. Glaser et al. (2002) have observed an increase about 18% of water holding capacity in soils containing biochar, that showed, in addition, a significant increase in seed germination, plant growth and crop yield. Biochar affected also microbial populations by increasing their activity (Verheijen et al., 2010; Lehmann et al., 2011). All these changes in the physical and chemical properties of the soil amended with biochar determine changes also in the soil ecosystem. There are new relationships among roots, bacteria and fungi thanks to the increased availability of nutrients and high porosity, which create safety habitats where soil bacteria and fungal hyphae can grow (Yamato et al., 2006; Warnock et al., 2007) (Figure 12).

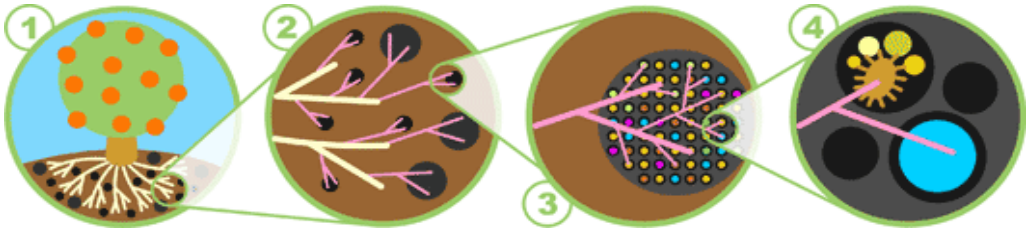

Figure 12. Biochar can be a habitat for microorganisms and nutrients storage.

All of these characteristics, therefore, make biochar a useful tool for environmental management (Lehmann and Joseph, 2009).

Biochar application in environmental management target to four main objective (Figure 13):

- improvement of soil characteristics;

- waste management;
- climate change mitigation;
- energy production.

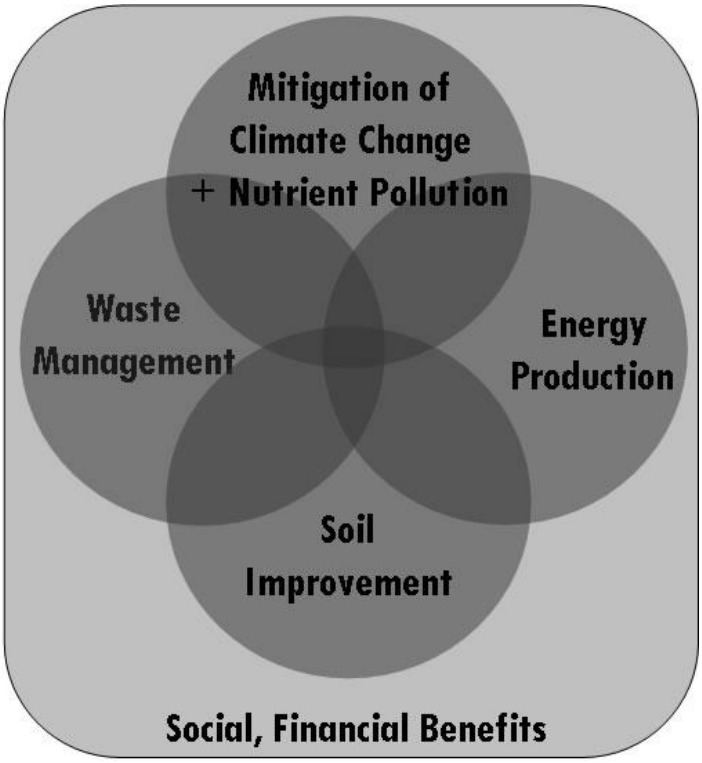

Figure 13. Benefits of biochar application (Lehmann e Joseph, 2009).

1.7.5 Use of biochar for environmental remediation

One of the most important properties of biochar consists of the ability to adsorb and retain persistent pollutants, especially those with a planar structure such as polycyclic aromatic hydrocarbons (PAH), but also other forms of pollutants, organic and inorganic, including heavy metals, protecting the environment and organisms by the accumulation phenomenon (Koelmans, 2005). Biochar is emerging just as a tool to optimize the reduction of bioavailability of contaminants in the environment by making benefits to soil fertility and mitigating climate change (Sohi, 2012). Environmental remediation, then, was

recently recognized as one of the areas where the biochar could be successfully applied (Ahmad et al., 2014).

Unlike activated carbon, biochar is not activated or treated (Cao and Harris, 2010). Furthermore, biochar contains a fraction not carbonized, which could interact with soil contaminants and water. In particular, the functional groups containing oxygen bonds (carboxylic, hydroxyl and phenolic) on the surface of the biochar could retain the contaminants (Uchimiya et al., 2011).

1.7.5.1 Adsorption of organic contaminant

Biochar, thanks to its characteristic, (high surface area and microporosity) can be used to remove organic contaminant from soil and water (Lou et al., 2011; Rhodes et al., 2008; Yang et al., 2010; Yu et al., 2009; Zhang et al., 2013) (Figure 14).

Biochars produced at temperatures of 400 °C are the most efficient in the adsorption of organic contaminants due to their high surface area and micropores (Uchimiya et al., 2010; Yang et al., 2010; Ahmad et al., 2012). Chen et al. (2008) assumed that the mechanism of organic contaminant adsorption on biochar derived from pine needles at low pyrolysis temperatures (100-300 °C) take place on non-carbonized fraction. The adsorption in biochar obtained at higher temperatures (400-700 °C), takes place on the porous carbonized fractions. The polarity and aromaticity of the surface, in fact, are important features of biochar, because they affect the adsorption of the organic contaminants in the aqueous system (Chen et al., 2008). In general, at higher temperatures (500 °C) the surface of the biochar becomes less polar and more aromatic because of the loss of functional groups containing hydrogen bonds (H) and oxygen bonds (O-), that could influence the adsorption of organic contaminants (Ahmad et al., 2014).

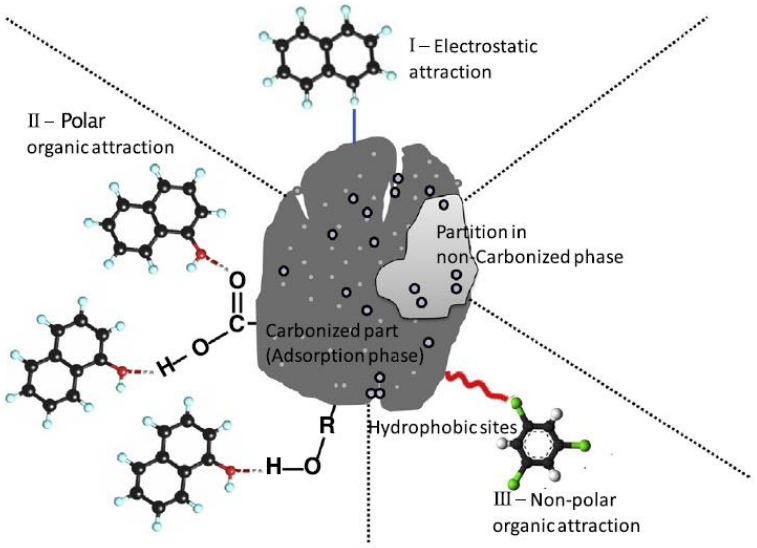

Figure 14. Possible interaction between biochar and organic contaminants (from Ahmad et al., 2014).

Electrostatic repulsions or attractions between biochar and organic contaminants represent another mechanism to adsorb pollutants. Biochar surfaces normally are negative charged: this makes easy the adsorption of positive charged organic compounds. However, an electrostatic repulsion between negative charged organic compounds and biochar could promote adsorption with hydrogen bonds (Ahmad et al., 2014).

1.7.5.2 Adsorption of inorganic contaminant

The inorganic contaminants in the environment (metals) derive from anthropogenic sources (Zhang et al., 2013) (Figure 15).

Mohan et al. (2011) have shown that biochar derived from oak by fast pyrolysis can adsorb Cr (VI). The adsorption is due to the bulge of the pre-existing pores in the dry biochar that increase the internal surface area (Mohan et al., 2011).

The biochar can affect differently metal mobility in soils. Beesley et al. (2010) applied biochar made from hardwood in soils contaminated with different

elements (As, Cu, Cd and Zn). After biochar application a change in ion mobility occurred: copper and arsenic were mobile, while cadmium and zinc were not mobile. The leaching of the copper and arsenic was due to the increase of the soil pH induced by biochar application. The pH increase, in turn, led to the reduction of cadmium and zinc solubility (Beesley et al., 2010). Therefore, biochar, increasing soil pH could affect the metal adsorption.

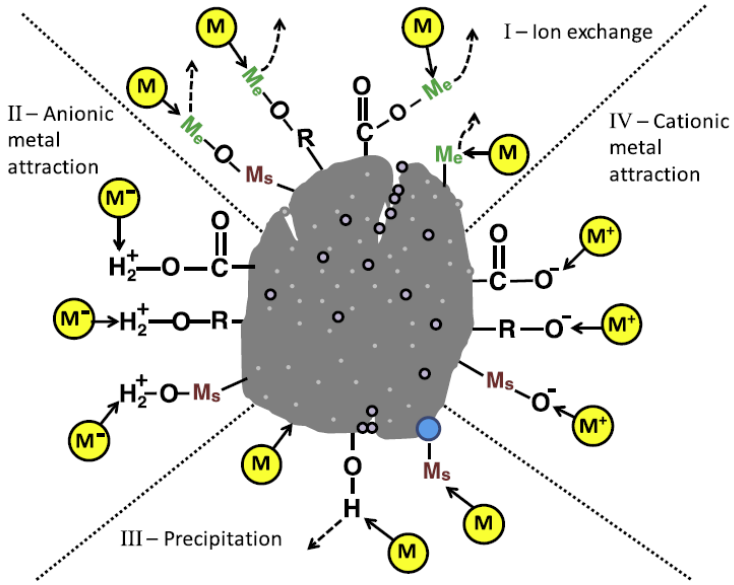

Figure 15. Possible interaction between biochar and inorganic contaminants (from Ahmad et al., 2014).

1.8 The aim of the thesis

Since environmental pollution is currently one of the main issues, irreversible situations may occur and seriously threaten the survival not only of human but also of any form of life on Earth. Several remediation techniques could be necessary for environmental protection or safeguard, the recovery of environmental compartments, and the mitigation of the effect of industrial, agricultural and urban waste on the environment.

Biochar is well known for agronomic benefits due to the enhancement of liming effects, water holding capacity, soil structure, cation exchange capacity, soil microbial activities and finally the plant growth. However, biochar amendment of degraded soil has also been shown to enhance sorption phenomena for hydrophobic organic pollutants and reduce their desorbing fraction and bioaccessibility.

Therefore, the aim of this work was:

- to evaluate the capacity of biochar, from different biomass, to remediate water and soil contaminated by organic compounds;
- to assess the efficiency of biochar-based treatments of agro-industrial wastewater, i.e. olive oil mill wastewater, and the impact of the disposal of biochar + remediated wastewater mixtures, as soil amendment.

Moreover, as the biochar supply induces an increase of black carbon content in soil (Lehmann et al., 2003) it would be interesting to follow the fate of black carbon and its metabolites in soil deriving from oxidative degradation (Haumaier 2010). Therefore, a method through ion-exchange chromatography system was set up to evaluate black carbon metabolites in soil.

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Chapter 2

2 Biochar based remediation of water and soil contaminated by phenantrene and pentachlorophenol

2.1 Introduction

According to Lehmann and Joseph (2009), biochar can be defined as a carbonaceous material deliberately applied to soils in order to enhance fertility. This carbonaceous system is obtained from thermal decomposition (i.e. pyrolysis) of biomasses under oxygen starved conditions and at temperatures ranging from 350 °C up to >1000 °C. However, the aforementioned biochar definition is considered weak by the European Biochar Certificate (EBC) because it is only oriented towards agronomic biochar applications (Conte et al., 2015). As an example, Lehmann and Joseph's definition does not consider the sustainability of the methodology used for biochar achievement, does not account for any possible biochar application apart of the agronomical one, does not include the very important greenhouse gas reduction property, nor accounts for the nature of the biomasses used for biochar production. The biochar defined by Lehmann and Joseph (2009) can be obtained by any kind of contaminant-free biomass feedstock, regardless of the sustainability of its procurement. Just as an example, many plant biomass species take a long time to grow. If not controlled by sustainability standards, their use for biochar production may pose serious problems for biodiversity protection, wildlife habitats, soil protection, and water production, thereby limiting the eco-sustainability of biochar applications. For these reason, EBC proposed a new definition of biochar that is: "biochar is a heterogeneous substance rich in aromatic carbon and minerals. It is produced by pyrolysis of sustainably obtained biomass under controlled conditions with clean technology and it is used for any purpose that does not involve its rapid mineralization to CO_2 and preserves its capacity to become eventually a soil amendment" (Conte et al., 2015).

Due to its nature, biochar is not only used as a soil amendment to increase soil fertility and to mitigate climate change by sequestering C from atmosphere to soils, but it can also be applied for energy production, thereby ensuring future source for green energy (Lehmann and Joseph, 2009; Lehmann et al., 2011; Conte et al., 2015). In addition, its particular porous structure and high specific surface area make it a very effective sorbent for organic and inorganic contaminants in soil and water (Ahmad et al., 2014; Caporale et al., 2014), thus lowering their bioavailability and toxicity to living organisms.

Biochar is composed by different organic and inorganic fractions that can react with contaminants (Ahmad et al., 2014; Beesley et al., 2011) through different mechanisms such as partitioning, adsorption, and electrostatic interactions (Ahmad et al., 2014).

Ahmad et al. (2014) reviewed several studies on biochar application in remediating soil and water contaminated by organic and inorganic pollutants. For example, biochars from different organic materials showed great capacity to immobilize some pesticides in soils thus reducing their bioavailability and plant uptake (Cao et al., 2011; Jones et al., 2011; Yu et al., 2009). Tong et al. (2014) demonstrated that biochar from rape-straw enhanced pentachlorophenol dechlorination by stimulating soil microorganism growth. Biochar produced from pine needles can be used to adsorb aromatic compounds from water samples (Chen et al., 2008). Kong et al. (2011) found that biochar from soybean stalk was very efficient in removing phenanthrene and mercury from contaminated water.

The interactions between biochar and contaminants change with pyrolysis temperature and nature of the parent material (Jindo et al., 2014; Mukome et al., 2013). Pyrolysis temperature strongly affects the partitioning of contaminants into non-carbonized and carbonized biochar fractions as it influences surface area and micropores development (Ahmad et al., 2014). Also the starting

material can significantly act on biochar adsorbent properties (Jindo et al., 2014; Mukome et al., 2013). Crop residues, forestry waste, animal manure, food processing waste, paper mill waste, municipal solid waste, and sewage sludge are reported to be the most commonly used waste biomasses for biochar production (Ahmad et al., 2014). Conversion of these materials to biochar is an economical and eco-friendly tool for environmental remediation because it allows recycling of existing resources (Conte et al., 2015).

Several studies reported the contemporary use of biochar and other organic amendments such as compost. Beesley et al. (2010) investigated the capability of biochar and green-waste compost in reducing the mobility of As and some PAHs in soil. Both of them showed a great potential, although biochar was more efficient. A synergistic effect occurs when biochar and compost were mixed to reduce Cu and Pb concentrations in soil (Karamia et al., 2011). This combination biochar-compost also results in an increased soil organic matter and fertility (Schulz and Glaser, 2012; Kamman et al., 2015). Furthermore, use of compost can contrast the reduced bioavailability of contaminants caused by biochar as compost can stimulate pollutant degradation (Kästner and Mahro, 1996).

The majority of scientific investigations is focused on water remediation, and just few studies are available on biochar application into contaminated soils (Ahmad et al., 2014).

In this work we hypothesize that biochar, due to its porosity and surface area, could adsorb organic compounds from contaminated water and soil. Though biochar can have good efficiency in the contaminant removal, biochar dust could remain after the treatment. This side effect could be beneficial for soil quality, but conversely it could represent an additional concern in the treatment of contaminated water. To avoid this side effect a focused experiment should test a system in which biochar is confined without getting water dirty with own dust.

Therefore, the aim of the present study was to evaluate the efficiency of poplar and conifer biochars in water and soil remediation. The attention was focused on two different organic contaminants, phenanthrene and pentachlorophenol, as representative of polycyclic aromatic hydrocarbons (PAHs) and chlorophenols (CPs), respectively. In addition, a specific experiment using biochar confined in dialysis tube for remediation of water bodies was performed. Studies of adsorption, sequestration and reduction of bioavailability of these contaminants in soil were carried out in a long-term experiment. A combination of biochar and compost was also tested to verify a synergistic effect on pollutant removal.

2.2 Material and Methods

2.2.1 Chemicals

Reagent-grade phenathrene (Phe) (>99% purity, m.w.: 178.23, H_2O solubility: 1.6 mg l^{-1}) and pentachlorophenol (PCP) (>99% purity, m.w.: 266.34, H_2O solubility: 20 mg l^{-1}) were purchased from Sigma Aldrich (Germany). HPLC-grade solvents and all other chemicals were supplied by Carlo Erba Reagents.

2.2.2 Physical-chemical properties of biochar

Biochar was produced from poplar (*Populus* spp. L.) (BP) and conifer wood chips (BC). Conifer wood chips were obtained from different species (*Larix decidua*, *Pinus sylvestris* L., *Pinus nigra* A., *Abies alba* M., *Picea excelsa* L.). Biochars were provided by the Department of Agricultural and Forestry Science of the University of Palermo (Italy). The details of the gasification process as well as biochar properties (pH, elemental composition, content of ash and metals) have been already reported in De Pasquale et al. (2012). The Brunauer–Emmett–Teller (BET) analysis was carried out on biochar, previously powdered (0.5 mm), to determine surface area and pores size and volume. Infrared spectra were recorded on Perkin Elmer FT-IR spectrometer using the Diffuse

Reflectance Infrared Fourier Transformed (DRIFT) analysis to highlight structural differences between biochars, in the transmittance mode by mixing with a mortar 5 mg of biochar with 95 mg of KBr. The DRIFT infrared spectra were recorded in 400-4000 cm^{-1} range, collecting 32 scans, with 4 cm^{-1} of resolution, and correcting the background noise (Novak et al., 2010). The spectra were acquired and processed using Spectrum 10 software.

2.2.3 Physical-chemical properties of soil and compost

Fresh soil, without no history of organic contamination, was collected from the citrus orchard located at the Department of Agriculture of University of Naples Federico II in Portici (Naples, Italy) at 0-20 cm of depth, air-dried and <2 mm sieved. The main physical-chemical soil properties were performed in triplicate following the standard techniques (Sparks, 1996) (Table 1). According to the USDA the soil was classified as a loamy sand soil (clay 27 ± 5, sand 879 ± 42, lime 93 ± 47 g kg^{-1}).

Table 1. Physical and chemical properties of soil.

Properties	Value
Sand (g kg^{-1})	879 ± 42
Lime (g kg^{-1})	93 ± 47
Clay (g kg^{-1})	27 ± 5
pH (in H_2O)	7.90 ± 0.06
EC (dS m^{-1})	0.099 ± 0.003
Limestone (g kg^{-1})	6.2 ± 0.4
CEC (cmol(+) kg^{-1})	15 ± 1
TOC (g kg^{-1})	12 ± 1
O.M. %	2 ± 0.1
Total N (g kg^{-1})	1.22 ± 0.02
C/N	10 ± 0.7
P_2O_5 (mg kg^{-1})	46 ± 1

A compost from olive pomace (Comp), characterized in details by Altieri and Esposito (2008), was used. It consisted of 72% olive pomace, 14% wheat straw and 14% sawdust.

2.2.4 Adsorption of Phe or PCP on biochars in liquid medium

Experiments to assess the capacity of BP or BC to adsorb and retain phenathrene (Phe) or pentachlorophenol (PCP) from liquid matrix were performed. Glass tubes (10 ml) were used to incubate 40 mg of each biochar, from BP or BC, with 1 ml of contaminant solutions (Phe or PCP). Suitable amounts of Phe or PCP stock solution (50 mg l^{-1}) in acetone were used to obtain different contaminant concentration (5, 7.5, 10, 12.5, and 15 mg l^{-1}). After 1, 3 and 7-d incubation, solutions were collected, filtered (0.45 µm, Phenomenex), centrifuged at 3000 rpm for 15 min, concentrated by evaporation under vacuum (LABOROTA 4000, Heidolph), suspended in 1 ml methanol and analysed by high-performance liquid chromatography (HPLC) to evaluate the residual concentration of contaminants. After centrifugation solid phase (biochar) was dried overnight at 25 °C and extracted by using organic solvents suitable for each contaminant, as described by Scelza et al. (2007, 2008).

2.2.5 Adsorption of Phe or PCP on biochar confined in dialysis tubes

Biochar was confined in porous membrane. In particular, 500 mg of biochar from poplar (BP) or conifer (BC) were put in 9-cm dialysis tubes (Spectra/Por, Millipore, Ø 34 mm, cut-off 3500 MWCO) together with 5 ml water/methanol (50:50, v:v) solution. Tubes were submerged in 250-ml glass bottles containing 100 ml of 15 mg l^{-1} Phe or PCP solutions in water/methanol (50:50, v:v). Each tube was prepared in triplicate and control tubes without biochar were also considered. All bottles were kept under shaking (100 rpm) over time. Aliquots of solution in which the dialysis tubes were submerged were daily collected and

analysed by HPLC to determine the residual Phe or PCP concentration. When contaminants concentration reached values < 1 mg l^{-1} or the value was stable for 2-3 days the tubes with biochar were transferred in a Phe or PCP fresh solution prepared as described above. This process was repeated for 4 cycles, after which the mixture confined in the dialysis tubes (contaminant solution + biochar) was centrifuged (15 min at 3000 *g*) and was performed contaminant extraction.

2.2.6 Kinetic models

Very often, remediation treatments of contaminated water do not allow direct addition of biochar because it could release, in turn, dusty residues in water bodies. Hence, after evaluating the biochar efficiency of Phe or PCP sorption directly in contaminated water, biochar was confined into dialysis tube immersed in contaminated water. In this way, the contaminant molecules may enter the dialysis tube due to a suitable cut off of selected membrane, and then be adsorbed on biochar surface. This procedure prevented that biochar came out through the same dialysis tube membrane and it can be easily removed after sorption process (Figure 1).

Kinetic studies were performed to study the Phe and PCP sorption process and the chemical reaction on biochars. Various kinetic models were applied to investigate which of these equations were fitting the mechanism of adsorption. The mathematical model was evaluated on R^2, in order to determine the best fit kinetic model (Ahmad et al., 2013).

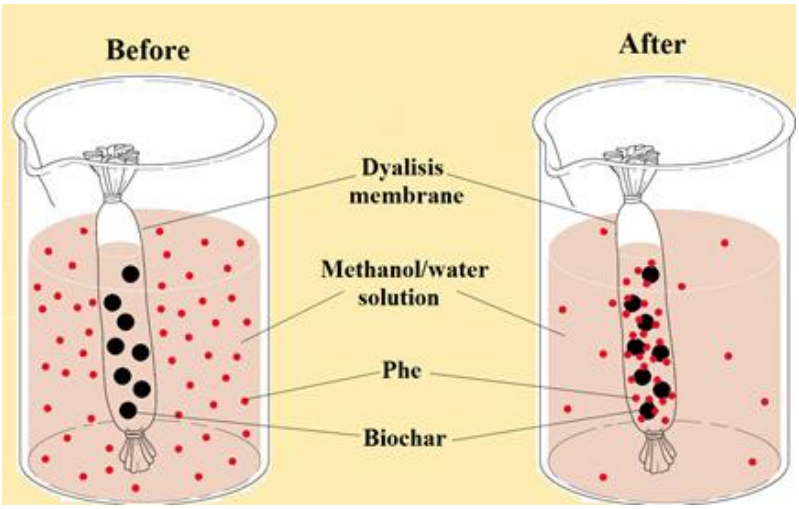

Figure 1. Biochar confined in dialysis tube.

The Phe adsorption kinetic on biochars was described by the following equation:

$$C_t = C_0 \cdot e^{-kt} \quad (1)$$

$$1/C_t = kt + 1/C_0 \quad (2)$$

where Ct is the mean concentration of Phe as a function of time in days (mg l^{-1}), C_0 is the initial Phe concentration (mg l^{-1}), k is the rate constant (d^{-1}), and t is time (d).

PCP adsorption kinetic on biochars was described by the following equation:

$$C_t = kt + C_0 \quad (3)$$

where the parameters are the same of the previous equations (1 and 2).

2.2.7 Treatment of Phe or PCP contaminated soil with biochar

Soil samples were rewet to 30% of water holding capacity before spiking procedure according to Scelza et al. (2007). Suitable amounts of Phe or PCP stock solutions (15 g l^{-1} in acetone) were added to soils to have 150 mg kg^{-1} as final concentration.

Contaminated soils were incubated at 25 °C for 21 days in 1l glass jars. After this time, samples were split in 5-g aliquots to which biochars and compost were

added. In particular, poplar and conifer biochars (BP and BC, respectively) were added to contaminated soils (Sc) at two different rates, BP1 or BC1 = 2.5 mg g^{-1} of soil and BP2 or BC2 = 5 mg g^{-1} of soil. Compost from olive pomace (Comp) was also added, singularly or together with biochars, in an amount equal to 2.2 mg g^{-1} of soil. Triplicates were performed for each sample. Samples were placed in the dark in a climatic chamber set at 25 °C and after 10 and 30 incubation days contaminant (Phe or PCP) extraction and germination tests were carried out on these samples. The experimental design is schematically represented in Table 2.

Table 2. Adsorption of the contaminants in solid matrix: experimental design.

Samples	Soil	BP	BC	Compost
	g		(mg g^{-1})	
Sc[a]	5	-	-	-
Sc + Comp[b]	5	-	-	2.2
Sc + BP1[c]	5	2.5	-	-
Sc + BP1 + Comp	5	2.5	-	2.2
Sc + BP2[d]	5	5	-	-
Sc + BP2 + Comp	5	5	-	2.2
Sc + BC1[e]	5	-	2.5	-
Sc + BC1 + Comp	5	-	2.5	2.2
Sc + BC2[f]	5	-	5	-
Sc + BC2 + Comp	5	-	5	2.2

[a]Sc = contaminated soil with phenanthrene or pentachlorophenol; [b]Comp = compost from olive pomace 2.2 mg g^{-1} of soil; [c]BP1 = poplar biochar 2.5 mg g^{-1} of soil; [d]BP2 = poplar biochar 5 mg g^{-1} of soil; [e]BC1 = conifer biochar 2.5 mg g^{-1} of soil; [f]BC2 = conifer biochar 5 mg g^{-1} of soil.

2.2.8 Phe extraction from solid matrices

Extractable Phe from biochar and soil samples was evaluated according to Scelza et al. (2007). Contaminated soil samples (0.6 g of dry weight at 25 °C) were extracted with ethanol (12 ml) and then with an ethanol/*n*-hexane (75:25, v:v) mixture (12 ml). Extracts were combined, concentrated by evaporation under

vacuum (LABOROTA 4000, Heidolph), and then dissolved in 2 ml of methanol before HPLC analysis.

A Soxhlet extraction procedure was also applied to soil samples to evaluate the adsorbed Phe on biochar and/or compost. As described by Saifuddin e Chua (2003), 3 g of soils were extracted with an acetone/*n*-esane (1:1; v:v) mixture for 24 h. Extract was concentrated by evaporation under vacuum (LABOROTA 4000, Heidolph), and then dissolved in 5 ml of methanol before HPLC analysis. For HPLC analysis a Phenomenex C-18 RP column (250mm × 4.6mm × 4µm) and a diode-array detector (Agilent® Serie 1100) were used. Methanol and water (86:14, v:v) were the mobile phase and the flow rate was 1.0 ml min^{-1}. The retention time for Phe was about 6 min. Detection was carried out at 254 nm.

2.2.9 PCP extraction from solid matrices

The extraction of PCP from soil and biochar (0.6 g of dry weight at 25 °C) was performed using a water-ethanol (50:50, v:v) mixture (12 ml) as described by Scelza et al. (2008). The concentrate was dissolved in 2 ml of methyl alcohol for HPLC analysis. Instrument and column were described above. Methyl alcohol and buffered water (1% acetic acid) were used as mobile phase (90:10, v:v) and the flow rate was set on 1.0 ml min^{-1}. Detection was carried out at 220 nm.

The retention time for PCP was about 5 min.

2.2.10 Germination test

Germination tests were performed on Phe- and PCP-contaminated soils amended or not with biochars and/or compost after 10 and 30 days of incubation (APAT, 2004). *Lepidium sativum* L. seeds were placed on Petri dishes (10 × 90 mm) for 72 h at 25 ± 2°C in the dark, in a climatic chamber. Control tests were carried out with uncontaminated soils (S) and contaminated soils without biochars addition (Sc). A primary root >1 mm was considered as the end germination

point. Experiments were performed in four replicates. The relative germination RG=100 (Gs/Gc), the relative length RL=100 (Ls/Lc), and the germination index GI=100 (Gs/Gc) (Ls/Lc) were calculated for each treatment. Gs and Gc are the numbers of seeds germinated in the sample and control, respectively, and Ls and Lc are the length of root in the sample and control, respectively.

2.2.11 Statistical analysis

Two-way ANOVA (incubation time, treatment) was used to examine the results from water-remediation experiment. A three-way ANOVA analysis (incubation time, type of biochar, amount of added biochar) was performed on data from soil-remediation experiment. The significant differences between means at $P < 0.05$ were assessed according to Duncan test. All statistical analysis were performed by SPSS (21.0 version).

2.3 Results and discussion

2.3.1 Biochar characterization

Both biochars were produced by gasification process and are characterized by alkaline pH, as described in details in De Pasquale et al. (2012). The high metal content (K^+, Ca^{2+}, Cu^{2+} and Mn^{2+}) was responsible for the alkaline reaction of both the biochars when suspended in water (De Pasquale et al, 2012). In fact, the process of metal exchange from biochar surface with H^+ from water appeared to be predominant on a possible acid reaction of metals (De Pasquale et al 2012). In addition, the presence of inorganic salts in the parent biomass could lead to the formation of complexes in which metal ions substitute H atoms of aromatic groups (Amonette e Joseph, 2009).

BET analysis showed a higher specific surface area for BC (114.67 $m^2 g^{-1}$) than for BP (76.88 $m^2 g^{-1}$). Moreover, no differences were observed between the pore

sizes of the two biochars, although pore volume appeared larger in BC than in BP. As a consequence, specific surface areas were different (Table 3).

Table 3. BET analysis of biochars.

Biochar	Origin biomass	Specific surface area	Pore volume	Pore size
		$m^2\ g^{-1}$	$cm^3\ g^{-1}$	Å
BP	poplar	76.88	0.046	24.08
BC	conifer	114.67	0.067	23.23

As already reported in literature, biochar properties are strongly influenced by biomasses and by pirolysis process (Mukome et al., 2013; Cimò et al., 2014; Conte et al., 2014; Jindo et al., 2014).

Figure 2 shows no differences between the FT-IR spectra of the two biochars. Both of them showed C–H (3050 cm^{-1}), C═C and C═O stretching (1707 cm^{-1}), aromatic C═C and C—H deformation modes of alkenes (1591−1455 cm^{-1}), and C═O stretching (1080 cm^{-1}) characteristic of ketones, ethers, phenols, and chain anhydride. There are also absorption bands due to aromatic C—H out-of-plane vibrations (three peaks at 876, 820, and 760 cm^{-1}) (Lee et al., 2010; Li et al., 2017). This result is in according with CPMAS ^{13}C NMR spectra reported in De Pasquale et al. (2012). In fact, the authors found aromatic groups and few oxygenated functional groups, in both biochars due to the partial oxidative condition during the gasification process.

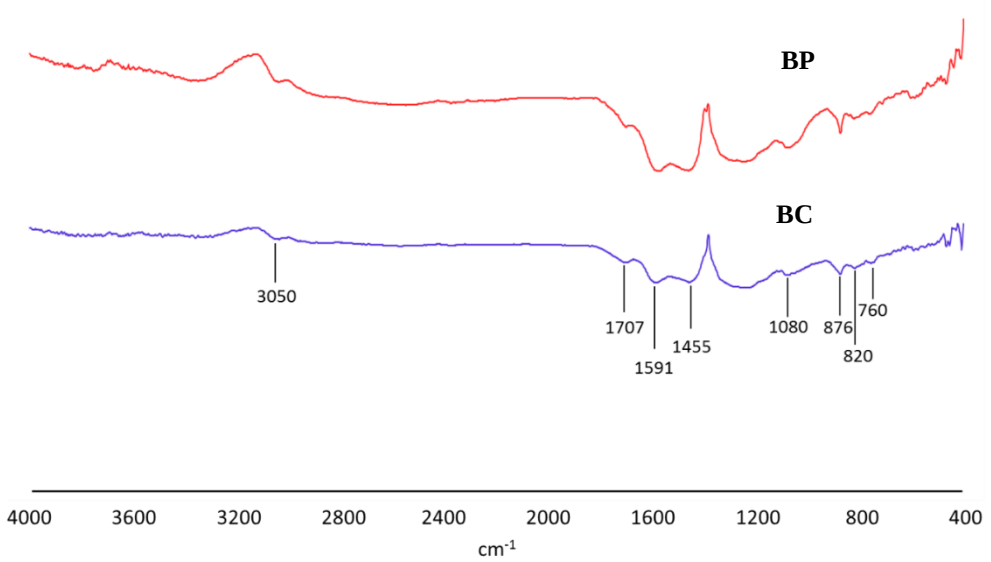

Figure 2. FT-IR spectra of BP and BC.

2.3.2 Phe removal from contaminated water

As reported in Table 2, Phe initially added was completely adsorbed by both biochars right after 1 day of incubation. Therefore, both biochars showed a good adsorbing capacity as also reported in James et al. (2005), Kong et al. (2011), and Sun et al. (2011).

Only a small amount of Phe adsorbed on biochars was extracted by organic solvents (Table 2). BC showed the major efficiency to retain the contaminant by increasing Phe concentration. The extractable Phe ranged from 17.6 ± 0.3% in 1BC-Phe sample with 5.0 mg l^{-1} of Phe initial concentration to 14.5 ± 0.3% in 5BC-Phe with 15.0 mg l^{-1} of Phe after 1 day of incubation, whereas the extractable Phe ranged from 23.6 ± 0.5% to 15.9 ± 0.2% in the samples amended with BP.

The extractable amount of Phe reduced with increasing incubation time. After 7-incubation time, extractable Phe ranged from 6.1 ± 0.3% to 8.4 ± 0.2% in samples amended with BP and from 2.1 ± 0.2% to 4.1 ± 0.3% in samples with

BC, thus confirming the better BC efficiency as compared to BP, due to BC larger surface area.

As reported in literature these results confirmed that biochar is a good sorbent for PAHs. Valili et al. (2013) showed that the pyrolysis of raw malt spent rootlets increased the surface area and the number of pore, which contributed positively to the sorption of Phe. Zielińska and Oleszczuk, (2015) showed high values for Phe and pyrene by the sewage-sludge-derived biochar. Furthermore, the process of Phe and pyrene adsorption on biochar was a physical interaction depending on surface area and porosity of this material. Pore size and organic contaminant size were important factors that influenced the adsorption process. The authors found that the adsorption of Phe was higher than pyrene, because Phe was sorbed in micro- and meso-pores, while pyrene was sorbed only in macro-pores. Physical interactions are not the only mechanism of the Phe adsorption. In fact, Li et al. (2014) and Sun et al. (2011) confirmed that the presence of aromatic groups could favour hydrophobic interaction with organic pollutants highlighting that hydrophobic and π–π interactions were the principal mechanism of pyrene adsorption. In particular there are three possible mechanisms of interaction between biochar and PAH. According to Anyika et al. (2015) the principal and strongest adsorption process was π–π interaction between the benzene rings of PAH and the biochar rings.

2.3.3 PCP removal from contaminated water

PCP showed different response as it interacted with the two biochars. In fact, after 1d-incubation, the adsorbed PCP ranged from 89.7 ± 0.6% to 94.1 ± 0.3% in samples amended with BP and from 89.8 ± 0.4% to 94.4 ± 0.5% in samples with BC, thus indicating a not complete adsorption as observed for Phe (Table 4).

Complete adsorption (100%) of PCP initially added was registered after 3d, regardless of biochar nature.

The PCP amount extracted from each sample was lower than that of Phe retrieved from analogous samples: only 8.8 ± 0.1% of PCP was extracted from 1BP-PCP versus 23.6 ± 0.5% extracted from 1BP-Phe after 1-d incubation time. In addition, no extraction was obtained from 1BC-PCP after 1-d incubation time. The extraction percentage continuously decreased in all samples treated with BC. After 3-d incubation no PCP desorption was achieved in samples with 5.0 μg ml^{-1} whereas the extractable PCP changed from 6.4 ± 0.1% to 11.6 ± 0.1% and from 3.5 ± 0.1% to 5.7 ± 0.1% in BP and BC samples, respectively, by increasing initial PCP concentration (Table 4). After 7-d incubation, extractable PCP showed only little changes ranging from 2.7 ± 0.1% to 3.0 ± 0.1% in samples amended with BP and no extraction was possible from BC amended samples, thus confirming the best capability of BC to adsorb the contaminant.

PCP showed different characteristics and solubility as compared to Phe. In fact, PCP is an acid (pKa 4.75) and its adsorption on soil or other matrix depends on pH. Larger PCP adsorption is usually observed as pH lowers (Lafrance et al., 1994; Lee et al., 1990; Peng et al., 2016). Adsorption at low pH values is due to the hydrophobic interactions following PCP protonation. As pH increases also PCP solubility enhances, thereby leading to a decrease of adsorption on biochar surface. The pH of PCP solution used for this experiment was 5.5 ± 0.1, thus indicating a possible complete and irreversible adsorption of PCP into biochar pores. In addition, hydrophobic and π–π interactions are valuable mechanisms for PCP adsorption on biochar produced at pyrolysis temperature > 500 °C (Peng et al. 2016).

Table 4. Adsorbed and extractable Phe and PCP in/from BP and BC biochars after 1, 3 and 7d-incubation in liquid matrix.

Samples	Added (μg)	Incubation time 1 d Adsorbed	1 d Extractable	3 d Adsorbed	3 d Extractable	7 d Adsorbed	7 d Extractable
		---------- % ----------					
Phe							
1 BP-Phe	5.0	100.0 ± 0.1	23.6 ± 0.5	100.0 ± 0.2	14.8 ± 0.5	100.0 ± 0.2	6.8 ± 0.3
2 BP-Phe	7.5	100.0 ± 0.2	17.1 ± 0.2	100.0 ± 0.2	11.5 ± 0.5	100.0 ± 0.1	6.1 ± 0.3
3 BP-Phe	10.0	100.0 ± 0.2	18.2 ± 0.2	100.0 ± 0.1	9.8 ± 0.6	100.0 ± 0.2	6.4 ± 0.3
4 BP-Phe	12.5	100.0 ± 0.1	16.8 ± 0.1	100.0 ± 0.3	10.7 ± 0.4	100.0 ± 0.1	7.4 ± 0.2
5 BP-Phe	15.0	100.0 ± 0.3	15.9 ± 0.2	100.0 ± 0.1	10.0 ± 0.4	100.0 ± 0.2	8.4 ± 0.2
1 BC-Phe	5.0	100.0 ± 0.1	17.6 ± 0.3	100.0 ± 0.2	6.0 ± 0.1	100.0 ± 0.2	3.6 ± 0.2
2 BC-Phe	7.5	100.0 ± 0.3	13.6 ± 0.3	100.0 ± 0.1	5.6 ± 0.1	100.0 ± 0.1	2.7 ± 0.2
3 BC-Phe	10.0	100.0 ± 0.1	14.8 ± 0.4	100.0 ± 0.2	6.2 ± 0.1	100.0 ± 0.1	2.1 ± 0.2
4 BC-Phe	12.5	100.0 ± 0.2	14.2 ± 0.3	100.0 ± 0.2	5.4 ± 0.2	100.0 ± 0.3	3.5 ± 0.2
5 BC-Phe	15.0	100.0 ± 0.1	14.5 ± 0.3	100.0 ± 0.1	5.7 ± 0.2	100.0 ± 0.1	4.1 ± 0.3
PCP							
1 BP-PCP	5.0	94.0 ± 0.1	8.8 ± 0.1	100.0 ± 0.1	0.0 ± 0.1	100.0 ± 0.2	0.0 ± 0.1
2 BP-PCP	7.5	94.1 ± 0.3	10.1 ± 0.2	100.0 ± 0.2	6.4 ± 0.1	100.0 ± 0.1	2.7 ± 0.1
3 BP-PCP	10.0	92.2 ± 0.4	11.0 ± 0.6	100.0 ± 0.3	9.6 ± 0.1	100.0 ± 0.2	3.0 ± 0.1
4 BP-PCP	12.5	90.8 ± 0.5	10.7 ± 0.1	100.0 ± 0.1	11.5 ± 0.2	100.0 ± 0.3	3.0 ± 0.1
5 BP-PCP	15.0	89.7 ± 0.6	12.1 ± 0.1	100.0 ± 0.2	11.6 ± 0.1	100.0 ± 0.1	2.9 ± 0.1
1 BC-PCP	5.0	94.4 ± 0.5	0.0 ± 0.1	100.0 ± 0.2	0.0 ± 0.1	100.0 ± 0.1	0.0 ± 0.1
2 BC-PCP	7.5	93.3 ± 0.7	2.9 ± 0.1	100.0 ± 0.2	3.5 ± 0.1	100.0 ± 0.2	0.0 ± 0.2
3 BC-PCP	10.0	91.2 ± 0.8	6.2 ± 0.1	100.0 ± 0.1	3.4 ± 0.1	100.0 ± 0.3	0.0 ± 0.2
4 BC-PCP	12.5	92.3 ± 0.5	8.3 ± 0.2	100.0 ± 0.1	4.0 ± 0.1	100.0 ± 0.1	0.0 ± 0.2
5 BC-PCP	15.0	89.8 ± 0.4	11.7 ± 0.1	100.0 ± 0.1	5.7 ± 0.1	100.0 ± 0.3	0.0 ± 0.2

2.3.4 Biochar confined in dialysis tubes: Phe or PCP adsorption kinetics and efficiency

As reported in Figure 3a, Phe concentration decreased over time faster with BC in the first cycle, thereby reaching amounts of < 1 mg l^{-1} after 7 days. Conversely, 10 days were necessary to reach the same results by using BP.

During the first cycle, the fast sorption rates of Phe was well fitted with the first-order kinetic model for both BP and BC with R^2 value of 0.9471 and 0.9648, respectively. The calculated rate constant was higher for BC (0.33 d^{-1}) than for BP (0.29 d^{-1}). Therefore, in this first cycle the adsorption of Phe molecules was affected by the available surface of biochar in dialysis tubes and the Phe molecules arranged in a surface layer entering also in pores. This adsorption was faster in BC because of its higher surface area and pore volume (Table 3). Smernik (2009) reported that these latter biochar characteristics accelerate adsorption and favour interactions between contaminant and adsorbent.

The second cycle ended after 11 days when Phe concentration in each system remained unchanged for more than three days (2.19 ± 0.43 mg l^{-1} and 3.01 ± 0.02 mg l^{-1} in in BC and BP, respectively). The efficiency clearly decreased over time for both biochars, probably due to a partial saturation of the carbonaceous material by contaminant molecules (Figure 3a). At the end of the third and fourth cycles, Phe concentration reached values around 7 and 8 mg l^{-1} with BC and BP, respectively. This slow adsorption rate of Phe in the second and in the further cycles was well fitted with second order kinetic model (Table 5) for both biochars. The rate constant (k) decreased from the second to the fourth cycle, indicating that a reduction of physical interaction between Phe and biochar occurred after the second cycle. Phe molecules arriving in the last phases found occupied or blocked biochar sorption sites due to Phe molecules already placed in the first stage. Cornelissen et al. (2004) concluded that the surface properties of biochar may be changed due to surface coverage pore blockage and surface

oxidation. Therefore, Phe molecules could interact with Phe molecules already arranged in the first layer through hydrophobic and π–π interactions as demonstrate by second order kinetics (Li et al. (2014).

At the end of the last cycle, biochar inside the dialysis tube was recovered and Phe was extracted. Extractable Phe from BP and BC was very low, 0.96% ± 0.02 and 1.49% ± 0.04, respectively. In order to force the extraction process, Soxhlet method was also adopted. A larger amount of contaminant (corresponding to 9.79 ± 0.72% and 10.89 ± 0.85% with BP and BC, respectively) was extracted. This result remained very low as compared to the total Phe added and immobilized (6 mg deriving from 1.5 mg per cycle), thus indicating a strong immobilization of Phe into the structure of both biochars.

The response of PCP during the remediation process was completely different compared to Phe (Figure 3b). PCP adsorption was well fitted with a zero-order kinetic model with both biochars and for all of four cycles (Table 5). PCP concentration of solution where dialysis tubes were immersed reached values < 1 mg l^{-1} after 24-d incubation during the first cycle. This is almost a double time if compared to the Phe experiment. No significant difference due to biochar nature was found: the rate constant was 0.58 d^{-1} with BC and 0.55 d^{-1} with BP (Table 2). PCP concentration reached a value < 1 mg l^{-1} after 26 days during the second cycle and after 30 days during the third and the fourth cycle. The rate constant remained unchanged in each cycle (Table 5), contrarily to what observed in Phe adsorption cycles. In PCP experiment, the cycles lasted longer, but the biochars efficiency remained unchanged over time, because biochars continued to adsorb PCP in solution. The zero-order kinetic model explains that the adsorption process did not depend on the PCP concentration. At the end of the last cycle, the PCP extracted from biochar recovered from the dialysis tubes was very low, 2.15% ± 0.06 and 1.79% ± 0.03, in BP and BC, respectively. The

efficiency was due to the biochar properties deriving, in particular, from the pyrolysis temperature.

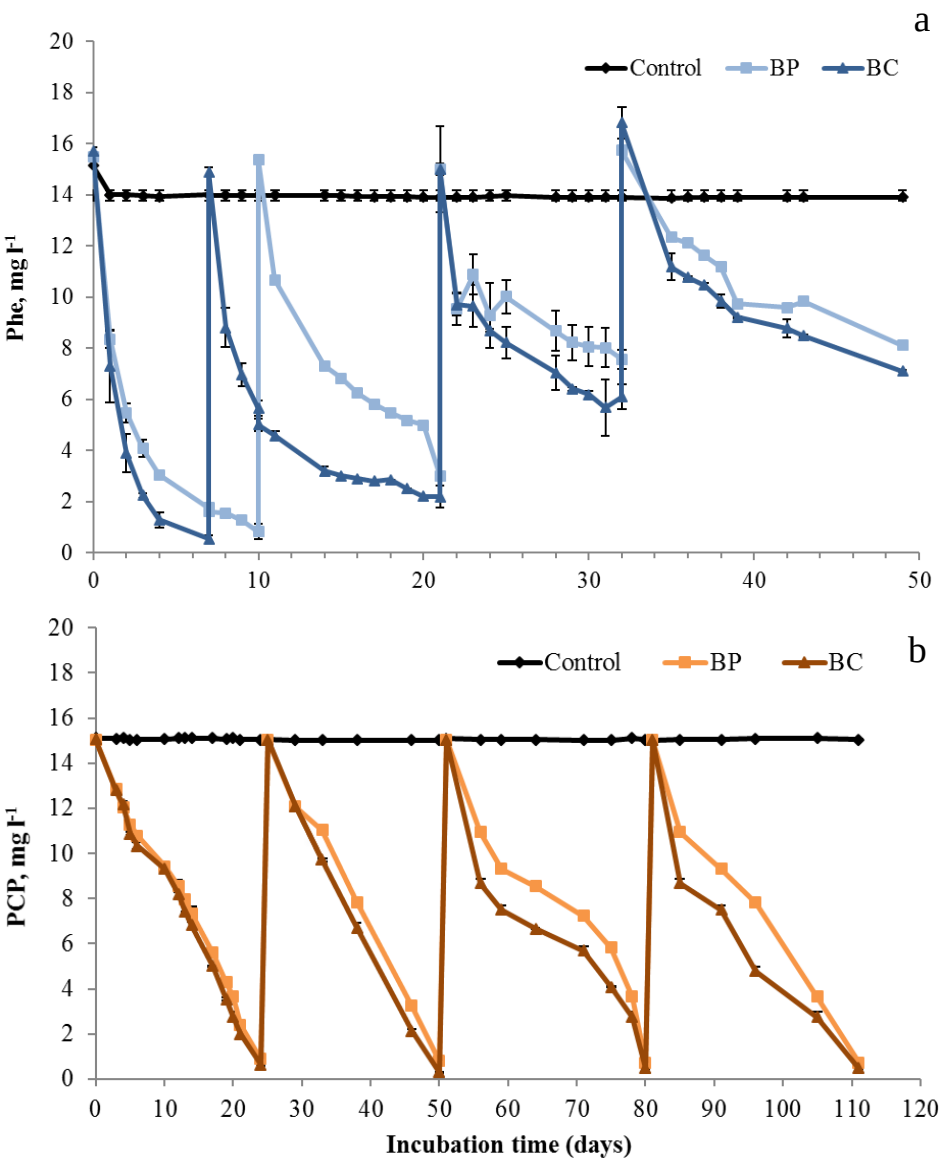

Figure 3. Variation of a) Phe and b) PCP concentration during the different remediation cycles of contaminated water.

As reported by Devi and Saorha, (2015) the PCP adsorption on biochar also increases by increasing the pyrolysis temperature. In fact, a pyrolysis

temperature > 400 °C led to the decrease of the biochar polarity and to the increase of its aromaticity. Moreover, these authors explained the higher value of the PCP adsorption in biochar produced at 700 °C not only with hydrophobic interactions but also by considering the pore filling mechanism (Devi and Saorha, 2015).

The longest time of the PCP adsorption as compared to Phe, could be attributed to the solution pH, since it is well known that PCP adsorption increases at lower pH by reaching a maximum at around pH 3. In our experiment, pH was 5.5 ± 0.1 thereby leading to an increase of the time of adsorption (Peng et al., 2016).

Table 5. Adsorption kinetics of remediation cycles of Phe/PCP contaminated waters.

	BP		BC		
Cycle	k (d^{-1})	R^2	k (d^{-1})	R^2	Reaction rate
Phe					
I	0.29	0.9471	0.33	0.9648	First order
II	0.02	0.9902	0.03	0.9992	Second order
III	0.005	0.7961	0.01	0.9344	Second order
IV	0.004	0.9308	0.005	0.9320	Second order
PCP					
I	0.55	0.9887	0.58	0.9895	Zero order
II	0.56	0.9948	0.59	0.9971	Zero order
III	0.39	0.9199	0.38	0.8751	Zero order
IV	0.44	0.9768	0.42	0.8944	Zero order

2.3.5 Biochar-based remediation of Phe or PCP contaminated soil

The remediation efficiency of BP and BC was tested also with contaminated soils. Either Phe or PCP contaminated soil was treated with different amounts of BP and BP and amended with compost.

After 21 days-incubation, the extractable Phe decreased to 11.9 ± 0.6 mg kg^{-1} corresponding to 7.90 % of the initial concentration (150 mg kg^{-1}) before

biochar-based treatment (Figure 4a). This value remained unchanged after 10 days in the presence of both biochars with the exception of the sample amended with compost (Sc+Comp). After 30 days, a further reduction of extractable Phe was observed in this latter sample as evidenced also in soil samples treated with BC at both rates (BC1 and BC2) (Figure 4a).

After 21 day-incubation the extractable PCP decreased to 132 ± 3.3 mg kg^{-1} equal to 88.8 % of the initial concentration (150 mg kg^{-1}). Also in this case, the treatment with either BP or BC did not affect the amount of the extractable PCP after incubation of 10 days (Figure 4b).

After 30 days the treatment with both biochars led to a significant reduction ($p<0.05$) of the extractable PCP. It was approximately 75% of the initial value. The organic amendment realized with compost did not changed the amount of extracted PCP in all samples treated or not treated with biochar whatever was biochar concentration. As the biochar concentration used in this experiment resulted not able to retain strongly PCP molecules, another experimental test was carried out by using larger biochar concentrations, i.e. 20 and 50 mg g^{-1} (Figure 5). The improved experimental conditions led to a marked reduction of the contaminant extraction after 30 day of incubation time (41 ± 2 and 29 ± 1% with BP and 33 ± 1 and 24 ± 1% with BC at 20 and 50 mg g^{-1}, respectively).

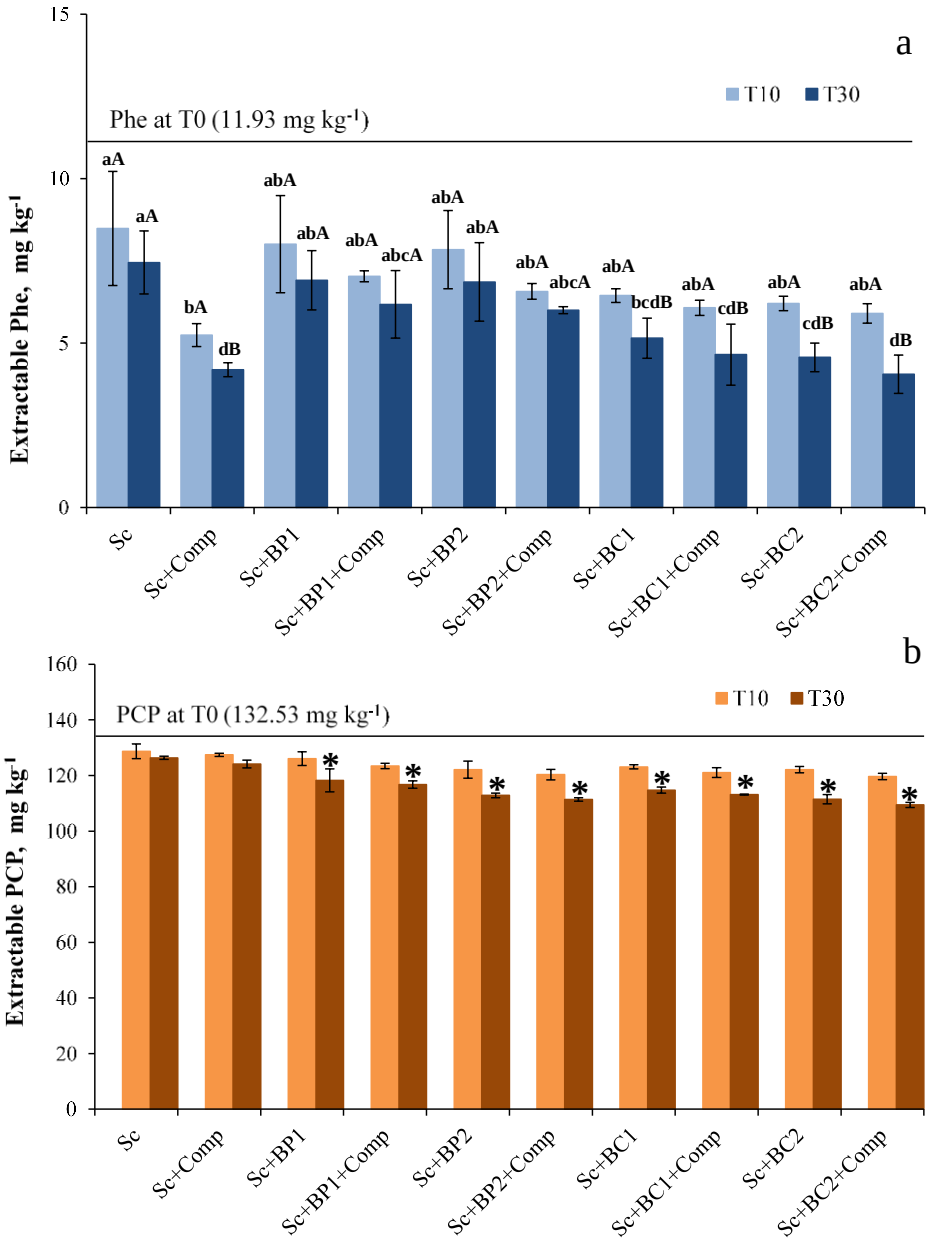

Figure 4. Extractable a) Phe and b) PCP from soil amended with biochar and/or compost. Sc = contaminated soil with 150 mg kg^{-1} phenanthrene or pentachlorophenol; Comp = compost from olive pomace 2.2 mg g^{-1} of soil; BP1 = poplar biochar 2.5 mg g^{-1} of soil; BP2 = poplar biochar 5 mg g^{-1} of soil; BC1 = conifer biochar 2.5 mg g^{-1} of soil; BC2 = conifer biochar 5 mg g^{-1} of soil. Capital letters indicate significant differences between two incubation time; lower case letters indicate differences between the treatments ($P < 0.05$); * indicates statistically different values ($P < 0.05$) from the control.

The reduction of the extractable contaminant, very marked in Phe experiment, could be first explained by natural attenuation occurring in soils because of additive phenomena such as chemical and microbial degradation, adsorption/immobilization/sequestration of contaminant molecules, etc. (Scelza et al., 2007; Ouvrard et al., 2013; Pernot et al., 2014; Dagois et al., 2016; Kuppusamy et al., 2016). The nature of contaminant and soil and, above all, soil organic matter content affect the fate of contaminant molecules through the "ageing" process (Scelza et al., 2010). PCP in contaminated soil showed the same behavior of Phe contaminated soil but after 21 days there was only a reduction of 17.47 ± 3.3 mg kg^{-1} (Figure 4b) due to a natural attenuation process for the intrinsic soil properties (Hansen et al., 2004 Scelza et al., 2008).

The ability to boost the process begun with the natural attenuation was shown just by BC with Phe after 30 days and by both biochars with PCP (Figure 4). The percentage of extracted PCP (29 ± 1% and 24 ± 1% for BP and BC respectively) obtained with both biochar after 30 days was reached only by increasing ten-fold the biochar amount (Figure 5).

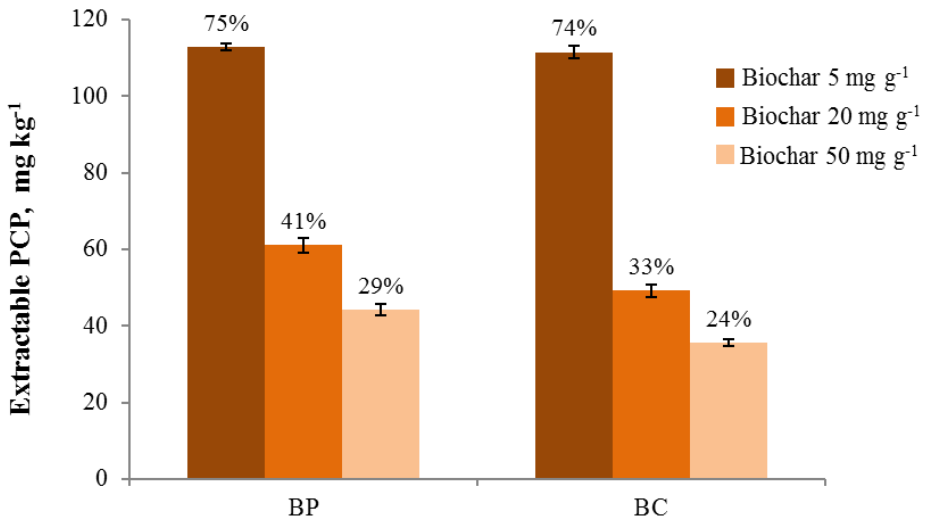

Figure 5. Extractable PCP from soil amended with increasing amount of BP or BC after 30 days.

Firstly, the chemical properties of Phe, a polycyclic aromatic hydrocarbon, having lower solubility and a smaller molecular weight than PCP, favored hydrophobic interactions with biochar surface and the entrapment in the biochar porosity. In addition, the physical properties of BC, such as specific surface area and, in particular, the pore volume, affected positively the retention of Phe molecules in its pores. In fact, BC had a specific surface area of 114.67 m^2 g^{-1} against that of BP (76.88 m^2 g^{-1}) and a pore volume of 0.067 cm^3 g^{-1} against 0.047 cm^3 g^{-1} of BP. This difference in pore volume measured by BET analysis (Table 1) was consistent with data obtained by De Pasquale et al. (2012) through FFC NMR analysis. PCP molecules have a larger molecular weight that can hinder their entrance in biochar porosity or just in part of biochar pores. In fact, a satisfying amount of retained PCP was achieved only by using 20 and 50 mg g^{-1} (Figure 5). Also in this case hydrophobic interactions as well as π–π interactions can be claimed to explain PCP retention in biochar structure (Devi and Saorha, 2015; Peng et al., 2016).

2.3.6 Germination tests on remediated or treated soil

Soils contaminated by PAHs and PCs could produce negative effects on seed germination as result of their phytotoxic activity (Scelza et al 2010; Lou et al. 2011; Khan et al. 2014).

Germination tests with *Lepidium sativum L.* seeds performed on Phe- and PCP-contaminated soil amended with BP or BC and/or compost showed contrasting results. Phe contaminated soil did not affected the seed germination (RG=100%) even when treated with biochar and/or compost (data not shown). By contrast, PCP contaminated soil completely inhibited seed germination regardless of biochar nature and concentration. Not even compost addition contrasted this negative effect (data not shown). The negative response of PCP contaminated soil disappeared when larger biochar concentration, i.e. 20 and 50 mg g^{-1} were

used. As reported in Table 6, the soil treatment with larger biochar doses led the RG values (96%) and the root growth (RL) to values very close or even greater than those of control soil (S), except for Sc+BP 50. This behavior could be explained by considering contaminant immobilization on biochar surface by reducing toxic effect (Beesley et al., 2010; Gomez-Eyles et al., 2011; Lehmann et al., 2011), and the increased availability of nutrients deriving from biochar, thereby leading to a stimulating effect on root growth (Lehman and Joseph, 2009; Kamman et al., 2015). Any inhibitory effect of BP and BC on cress germination can be excluded by control samples (Table 6). These results also accord to a previous paper by Visioli et al. (2016).

The lack of Phe inhibition on cress seed germination could be explained by claiming the Phe low bioavailability in our experimental systems due to the strong sorption affinity to soil organic matter (Scelza et al., 2010). Lipińska et al. (2015) found 10% and 22.5% inhibition of seed germination and root growth, respectively, only at 4000 mg kg^{-1} of Phe soil contamination.

Table 6. Relative germination (RG), relative length (RL) and germination index (GI) of PCP-contaminated soils (Sc) amended with 20 and 50 mg g^{-1} BP or BC.

Sample	RG	RL	GI
		%	
S	100 a	100 a	100 a
Sc	0 c	0 d	0 d
BP	100 a	100 a	100 a
BC	100 a	100 a	100 a
Sc + BP 20	96 b	110 a	108 a
Sc + BP 50	96 b	92 c	89 c
Sc + BC 20	96 b	110 a	105 a
Sc + BC 50	96 b	113 a	109 a

Furthermore, structural and physiologic properties of cress seeds could also contribute to understand the process. Cress seed, during first germination step,

produces mucilage gel in the envelope alongside its outer layer. This hydrophilic gel consisting of cellulose, uronic acid and polysaccharides allows water to penetrate and, in the same time, makes unlikely the transfer of hydrophobic Phe molecules (solubility in water, 1.6 mg l^{-1}) (Behrouzian et al., 2014). Conversely, the complete inhibition of cress seed germination by the untreated PCP contaminated soil could be explained by the greater amount of extractable PCP (132 ± 3.3 mg kg^{-1}) as compared to the amount of extractable Phe from Phe contaminated soil (11.9 ± 0.6 mg kg^{-1}) and by its solubility (20 mg l^{-1}) approximately twelve fold higher than Phe. The treatment with BP and BC did not improve the germination index if not at higher doses (20 and 50 mg g^{-1}) when the biochar surface area available for adsorption was much larger. In fact, a reduction of extractable PCP occurred simultaneously with an increment of the germination index. A reduction of PCP contamination rate could determine a limited inhibition of seed germination as demonstrated by Lou et al. (2011) with 50 mg kg^{-1} PCP concentration and wheat seeds. The seed germination already took place in soil amended with 5 mg g^{-1} rice straw biochar, amount resulted inefficient in our experimental conditions.

2.4 Conclusions

Overall, the results showed that biochar is a matrix suitable for soil and water remediation as both environmental compartments are contaminated by organic pollutants. In contaminated water, both biochar characteristics and contaminant natures played an important role in the sorption kinetics and the efficiency of contaminant removal. However, the methodology based on confining biochar in dialysis tube resulted in an interesting and easy way to remediate water bodies.

In a contaminated soil, the treatment with a biochar from conifer biomasses induced a better response with both Phe and PCP, thereby highlighting a direct relationship with the biochar amount used in soil treatment. The biochar

amendment, therefore, reduced successfully the extractability of contaminants and as consequence their bioavailability leading to the disappearance of phytotoxicity phenomena.

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Chapter 3

3 Effect of olive mill wastewater treated with biochar on soil fertility

3.1 Introduction

The olive mill wastewater (OMW) derive from three-phase systems of the olive oil industry and their large production amount is a serious problem for different areas of the Mediterranean, due to high toxicity and the short time of production (October-February). OMW are generally scattered on soil at controlled rate in according to specific environmental law to avoid possible toxicity phenomena for crops and microbial biomass, and superficial and groundwater contamination for leaching. However, the cautious use of OMW as organic fertilizer for agricultural soils can represent a valid disposal method with a great advantage for soil fertility (Bonari, 2007).

The toxicity of OMW for plant and soil microbial community is due to the high organic matter content and the presence of monomeric phenolic compounds, fatty acid salts, and recalcitrant compounds (lignins and tannins) (Justino et al., 2012). OMW are characterized by high chemical oxygen demand (COD, approximately 15,000-135,000 mg l^{-1}) and biological oxygen demand (BOD, approximately 37,000-318,000 mg l^{-1}) (Kilic and Solmaz, 2013). The OMW contain high amount of polyphenols (until to 10 g l^{-1}) being responsible for phytotoxicity (Capasso et al., 1991; Aliotta et al., 2000) and antibacterial activity (Capasso et al., 1995; Borja et al., 1998) and nevertheless representing high add-value products for industries, related to pharmaceuticals, cosmetics, and medical and in the food storage (Bertin et al., 2011).

The disposal of these huge volumes of OMW needs pre-treatments to make the waste less toxic (Paraskeva and Diamadopoulos 2006). Biological process are more suitable and less expensive for the treatment of OMW. However, the presence of some inhibitors or toxic compounds, such as polyphenols and lipids,

make the OMW not susceptible to direct biological treatments (Justino et al., 2012). Cassano et al. (2011) have shown that ultrafiltration, with different types of membranes, can be useful in the treatment of these wastes. In particular, they observed polyethersulfone membranes were more efficient in the removal of phenolic compounds compared to the membranes of regenerated cellulose. El-Abbassi et al. (2012) have tested successfully distillation in direct contact (DCMD - direct contact membrane distillation) with membranes of polytetrafluoroethylene. However, all these processes are more expensive due to membrane obstruction. Thus, appropriate pre-treatment steps are necessary to decrease membrane fouling and increase filtration efficiency (Cassano et al., 2011) and as consequence operational costs (Kilic and Solmaz, 2013).

Biochar is a carbonaceous material product by thermal decomposition (pyrolysis) of vegetable biomass under oxygen limited conditions (Lehmann et al., 2011). Unlike activated carbon, biochar requires less energy and cost, and no activation processes for production (Cao and Harris, 2010). Biochar is able to sorb organic contaminants thanks to its high surface area and microporosity (Yu et al., 2009; Yang et al., 2010; Lou et al., 2011) and, therefore, environmental remediation represents one of the areas where biochar can be successfully applied (Ahmad et al., 2014). Biochar produced at temperatures higher than 400 °C is the most effective in adsorption of organic contaminants due to their high surface area and the increased size of micropores (Uchimiya et al., 2010; Yang et al., 2010; Ahmad et al., 2012). Liu et al. (2010) found that the temperature of pyrolysis influenced the adsorption because the pyrolysis temperature was a key factor affecting surface areas and adsorption capacities. In particular, catechol sorption capacity increased with biochar pyrolysis temperature (Mohan et al., 2014). Biochar produced from oak, pine and grass showed increasing catechol adsorption by increasing the pyrolysis temperature (from 250 to 650 °C) (Mohan et al., 2014). The authors demonstrated that the different biomasses showed

different degree of catechol adsorption (pine < oak< grass), related also to the dimension of biochar particle (coarse < fine). In fact, the origin biomass could also affect its ability in removing specific contaminants: for example, biochar from soybean stalk was very efficient in removing phenanthrene and mercury from contaminated water (Kong et al. 2011).

If biochar is able to adsorb phenols and other more complex contaminants in simplified contaminated systems (Kasozi et al., 2010; Yang et al., 2010; Lou et al., 2011), it could be efficient also in a complex system like olive mill wastewater in which phenols are the principal cause of toxicity. The process of phenol removal from OMW should lead to a reduction of toxicity of this waste. As consequence, the disposal of biochar-treated OMW on agricultural soils could represent a beneficial organic amendment that improves physical, chemical and biochemical soil properties also because biochar added to OMW ameliorates itself soil fertility. A positive effect on soil properties would allow increasing the rate of OMW disposal on soil favouring the re-use of a waste produced in big volumes and in a limited period of time.

Therefore, the aim of this work was to evaluate the ability of biochar produced from poplar and conifer wood to remove phenol compounds from OMW thus reducing toxicity and the effect of biochar-treated OMW on the chemical and biochemical properties of an agricultural soil when they are used as organic fertilizer.

3.2 Materials and Methods

3.2.1 Biochar characteristic

Biochar was produced from poplar (BP) and conifer (BC) wood and characterized (pH, elemental composition, content of ash and metals) by De Pasquale et al. (2012). Furthermore, the BET (Brunauer–Emmett–Teller) analysis showed that the surface area of BP and BC were 76.88 $m^2 g^{-1}$ and 114.67

m^2 g^{-1}, respectively. The FT-IR analysis (Chapter 2) demonstrated poplar and conifer biochar did not differ in terms of functional groups.

3.2.2 Soil

The soil used in this experiment was collected in Portici in South of Italy (40°49'11" N, 14°20'28" E) from the 10-30 cm soil layer, sieved at 2 mm, and immediately used in the lab-scale experiment. Suitable amount of soil was air-dried at room temperature and analyses to measure pH, electrical conductivity (EC), C, N, P, and soil texture in according to Sparks et al. (1996) were performed. Another suitable amount was saved at 4 °C for biochemical analyses.

3.2.3 Olive mill wastewater

OMW were collected from three phase olive mill extraction plant located in San Prisco (Caserta) in Southern Italy. They were stored at -18 °C until use.

3.2.4 Characterization of OMW

The pH and EC (μS cm^{-1}) of OMW samples were measured using a pH-meter (Hanna Instruments, Hi 9017 electrode CW711) and conductivity meter (Hanna Instruments, Hi 8733), respectively. Total phosphorous, COD and BOD_5 were measured following the APAT methods (2003).

The extraction of the phenolic compounds was performed in according to Brenes et al. (1995). Briefly, in 50 ml tube 20 ml of distilled water was added to 20 ml of OMW and 5 mg of sodium metabisulfite (400 mg l^{-1}) to prevent phenol oxidation. Syringic acid (1 ml of 200 mg l^{-1}) was added like internal standard. In order to remove the lipidic fraction the solution was washed three time with 15 ml of *n*-hexane. All solutions was collected in separatory funnel and each sample was washed five time with 20 ml of ethyl acetate. The extract was concentrated

by evaporation under vacuum (LABOROTA 4000, Heidolph) and dissolved in 2 ml of methanol.

Total phenolic compounds of ethyl acetate extracts were measured by using the Folin-Ciocalteu reagent (Sigma, Italy). A suitable amount of Milli-Q water (830 µl) and Folin-Ciocalteau reagent (50 µl) was added to 20 µl of sample. After 3 minutes 100 µl of 6% NaOH were added and the absorbance at 725 nm was measured after 1 h of incubation. The phenol content was calculated by calibration curve obtained with catechol. All analyses were carried out in triplicate.

3.2.5 HPLC analysis

The HPLC analysis of phenolic extracts was performed with an Agilent® 1100 instrument equipped with a pump and a diode-array detector. A Phenomenex 250×4.6 mm C-18 column with 4 µm particle size and a Phenomenex C-18 (4.6 × 30 mm) guard column were used. Detection was carried out at 279 nm. Elution gradients at a flow of 0.5 mL min^{-1} with a mobile phase composed of water acidified with *o*-phosphoric acid (solvent A) and acetonitrile-water (70:30 v/v) (solvent B) was adopted. The elution program for the OMW extract was as follows: isocratic elution with 85% A for 5 min; gradient to 50% A in 35 min; to 100% B in 10 min; to 85% A in 10 min; isocratic elution 85% A for 5 min. The phenolic compounds were identified by comparison with standards. Coinjection with different concentrations of the related standards and the direct standard elution were used to ensure the identity and concentration of the compounds.

3.2.6 Biochar based treatment of OMW

OMW has been left to decant and already after 1 day OMW showed a sediment. BP and BC (5 and 10%) was added to 40 ml of decanted or non-decanted OMW

and after 20 and 60 days phenolic content was evaluated and the germination test was performed to assess their phytotoxicity.

3.2.7 Catechol adsorption

Experiments to assess the capacity of BP to adsorb phenols from OMW were performed simplifying the system. Biochar interacted with only one phenol, chatechol (Sigma, Italy). Glass tubes (10 ml) were used to incubate 5% and 10% BP, with 1 ml of catechol solution at three different concentration (0.5, 1, and 1.5 mg ml^{-1}). After 0.5 h, 24 h and 7 days of incubation, the mixtures were centrifuged at 14000 rpm for 15 min and filtered (0.45 µm, Phenomenex). Filtrates were analysed by high-performance liquid chromatography (HPLC) (Kasozi et al., 2010).

3.2.8 Soil amendment with biochar treated OMW

As the treatment with poplar biochar induced a larger reduction of phenolic compounds in comparison with conifer biochar, OMW treated with BP (for 60 days) were spread on soil in lab scale experiment (Figure 1) following a detailed experimental design (Table 1).

Figure 1. PVC tube used in the lab-scale experiment.

Table 1. Experimental design of lab-scale treatment.

Sample	Composition
S	Control soil
BP5	Soil + 5% BP
BP10	Soil + 10% BP
OMW	Soil + OMW
OMWd	Soil + OMW decanted for 24h
OMW+BP5	Soil + OMW treated with 5% biochar
OMW+BP10	Soil + OMW treated with 10% biochar
OMWd+BP5	Soil + OMWdec treated with 5% biochar
OMWd+BP10	Soil + OMWdec treated with 10% biochar

Soil was placed in PVC tubes (30 cm x 5 cm) closed in the bottom with non-tissue disk. Soil moisture was maintained at 30% of the WHC by adding water during the experiment. The OMW disposal occurred at 80 $m^3 ha^{-1} year^{-1}$ dose in according with the law specification. After 30 and 90 days, chemical and biochemical analysis and seed germination test of amended soil were carried out.

3.2.9 Chemical and biochemical analysis of soil

Soils amended with treated OMW and related control samples were characterized chemically by following methodologies described in Par. 4.2.2.

As regards biochemical properties, microbial biomass C was measured as soon as possible from the end of the incubation by fumigation-extraction method (Vance et al. 1987). Enzyme activities were determined within 15–20 days from the collection of the samples. β-glucosidase (GLU) was determined as described Eivazi and Tabatabai (1988), and phosphatase (PHO) was determined in according to Tabatabai and Bremner (1969). Urease (UR) activity was measured according to Kandeler and Gerber (1988) whereas dehydrogenase (DH) activity in according to Alef e Nannipieri (1995). The activity of the *o*-diphenol oxidase (DPO) was determined using a mixture of catechol and proline as the substrate

(Perucci et al. 2000).The fluorescein diacetate hydrolase (FDAH) activity was assessed as described by Green et al. (2006). Triplicates were analysed for each activity assay.

3.2.10 Phytotoxicity of soil amended with biochar treated OMW

The germination test was performed as described by APAT (2004) in presence of OMW treated with biochar (BP or BC) and soil amended with treated OMW. Briefly, *Lepidium sativum* L. seeds were placed in 10x90 mm Petri dishes (10 seeds per dish), equipped with 5 ml of OMW or 10 g of soil and incubated for 72 h at 25 ± 2 °C in the dark in a climatic chamber. Control test was performed with distilled water (experiment with OMW) or untreated soil (experiment with soil). A primary root >1 mm was considered as the end germination point. Experiments were performed in four replicates. The relative germination RG=100 (Gs/Gc), the relative length RL=100 (Ls/Lc), and the germination index GI=100 (Gs/Gc) (Ls/Lc) were calculated for each treatment. Gs and Gc are the numbers of seeds germinated in the sample and control, respectively, and Ls and Lc are the length of root in the sample and control, respectively.

3.2.11 Statistical analysis

Two-way ANOVA (incubation time, treatment) was used to examine the results. The significant differences between means at $P < 0.05$ were assessed according to Duncan test. All statistical analysis was performed by SPSS (21.0 version).

3.3 Results

3.3.1 Properties of OMW

Analyses of OMW showed an acidic pH (4.39 ± 0.01) and a high conductivity of 6.55 dS m^{-1} ± 0.02 (Table 3).

Table 3. Chemical properties of OMW.

Chemical properties	Value
pH	4.39 ± 0.01
EC (µS cm^{-1})	3553.3 ± 15.3
Total P (mg l^{-1})	177.50 ± 9.03
Dry matter (%)	3.6 ± 0.2
Humidity (%)	96.43 ± 0.23
Chlorides (mg l^{-1})	177.50 ± 9.03
COD (mg l^{-1})	76227.5 ± 3626.1
BOD (mg l^{-1})	1583 ± 144
Total phenols (mg ml-1)	1.35 ± 0.02

The values of COD and BOD were consistent with literature data and also those of total phenols placing however in intermediate levels of the range reported in literature (El Abbassi et al., 2017; Ochando-Pulido et al., 2017; Table 3). Most of phenols were gallic acid, caffeic acid, catechol, syringic acid, protocatechuic acid, 2-hydroxybenzoic acid and of 2,6-hydroxybenzoic acid (Figure 2).

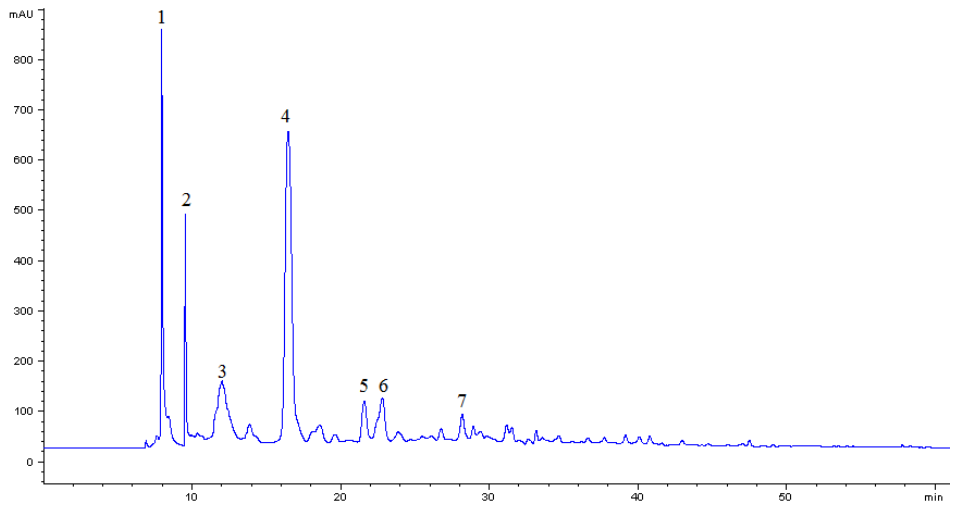

Figure 2. Chromatogram of OMW at 279 nm. Numbers correspond to 1) gallic acid; 2) 2,6-hydroxybenzoic acid; 3) protocatechuic acid; 4) caffeic acid; 5) 2-hydroxybenzoic acid; 6) catechol; 7) syringic acid.

3.3.2 Remediation of OMW

The treatments of OMW with BP and BC determined no reduction of phenolic content after 20 days. In fact, the total phenols of the treated OMW were approximately 1.30 mg ml^{-1}, value very close to that of the control sample (1.35 mg ml^{-1}) except for decanted OMW (OMWd) treated with 5 and 10% BP where the value decreased to 1.20 and 1.0 mg ml^{-1}, respectively (Figure 3a).

After 60 days the value of the control samples OMW remained unchanged whereas that of the control OMWd fell down to 1.08 mg ml^{-1} (Figure 3b).

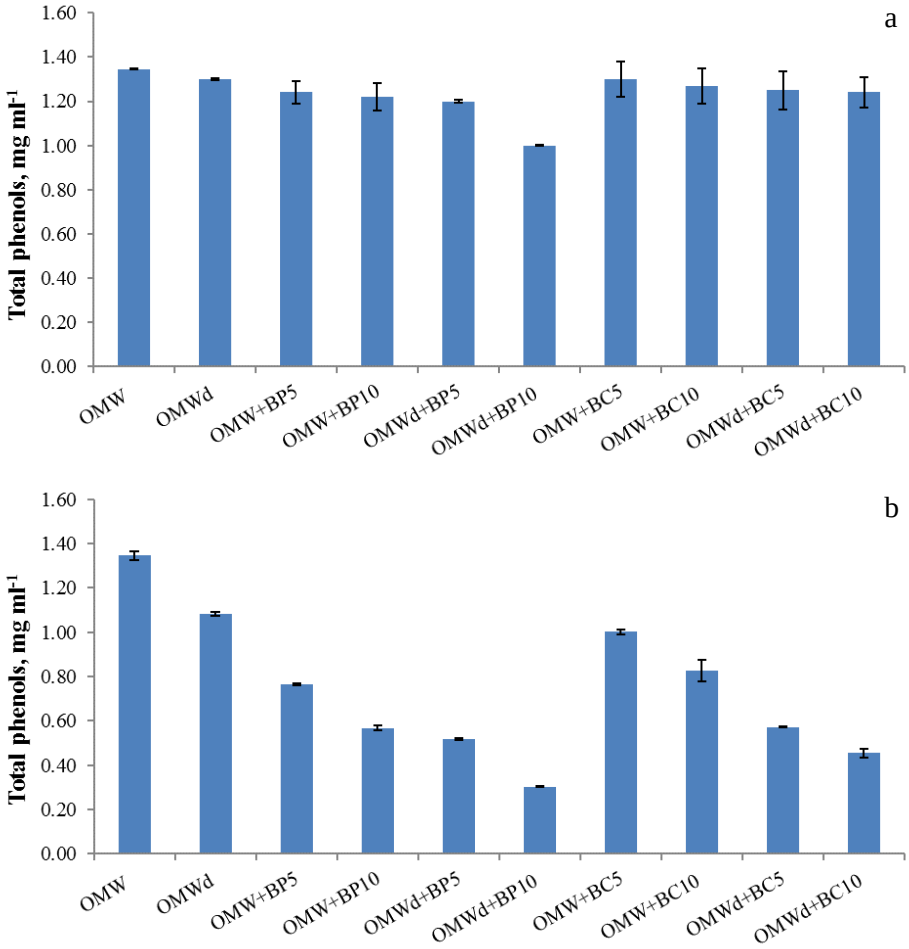

Figure 3. Total phenols in OMW differently treated with BP and BC after a) 20 day and b) 60 days (bars represent standard errors).

All OMW treated with biochar (BP and BC) showed a more marked reduction of total phenols, although BP resulted more efficient in taking phenols off. OMWd treated with 5% and 10% BC showed 52 and 42% of total phenols relieved in control OMWd, respectively. Conversely, the treatment based on 5 and 10% BP led to a more marked reduction of total phenols, i.e. 43 and 28%, respectively. The positive effect of OMW decantation arose from all results.

In order to exclude the phenol polimerization in OMW due to the possible oxidative polimerization promoted by biochar addition, catechol adsorption experiment on BP was carried out. No catechol polymerization occurred within 7 d, but the catechol adsorption was rather detected and the process depended on catechol concentration, biochar concentration and incubation time. In fact, in the sample Cat 0.5+BP5 0.4 mg ml^{-1} of catechol (80% of the amount initially added) was adsorbed after 30 min, whereas doubling BP concentration (Cat 0.5+BP10) catechol was completely adsorbed. By increasing the catechol concentration up to 1 and 1.5 mg ml^{-1}, the complete catechol adsorption occurred after 24 h and 7 days, respectively (Figure 4). The catechol concentration reflected the concentration of total phenols in OMW. In accordance with Kasozi et al. (2010) catechol adsorption was achieved.

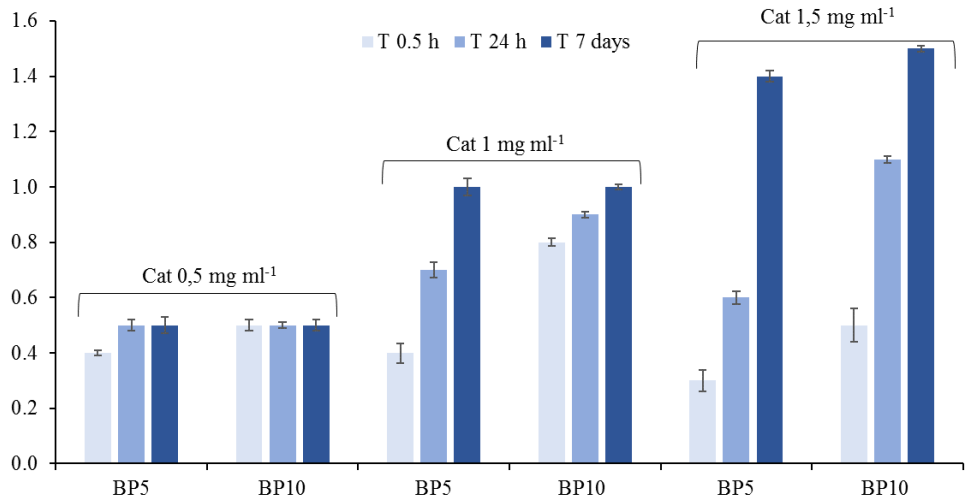

Figure 4. Catechol adsorption on 5% and 10% BP.

The germination test of *Lepidium sativum* seeds carried out in presence of treated or not treated OMW confirmed the relationship between total phenols and phytotoxicity (Table 4). In fact, the treatment with 10% BP allowed annulling completely the inhibition observed in OMW and OMWd and only partially (RG 80%) with BP 5%. Unfortunately, the response of the root growth (RL) to the presence of OMW treated with biochar was not in according to RG. In fact, the RL value remained rather small (<40%) also with BP based treatment of OMW, which was able to take the RG back to 80 and 100% (5% and 10% BP, respectively). Thus, the resulting GI values remained very small to indicate seed suffering occurred in the root elongation stage rather than in seed germination despite the OMW remediation treatment. The phenolic content in OMW, still present also after the biochar treatment, could be still responsible of toxicity effects, even if weaker.

Considering the interesting phenol reduction in OMW and RG values in germination test obtained with BP, experiments continued by using exclusively BP based remediated OMW for their disposal in soil.

Table 4. Phytotoxycity test of biochar-treated OMW with *Lepidium sativum* L. seeds.

Sample	RG	RL	GI
	---------- % ----------		
OMW	0 a	0 a	0 a
OMWd	0 a	0 a	0 a
OMW+BP5	0 a	0 a	0 a
OMW+BP10	60 b	35 d	20 e
OMWd+BP5	80 c	33 d	30 f
OMWd+BP10	100 b	40 d	40 d
OMW+BC5	0 a	0 a	0 a
OMW+BC10	0 a	0 a	0 a
OMWd+BC5	0 a	0 a	0 a
OMWd+BC10	80 c	35 d	40 d

3.3.3 Response of soil chemical properties to biochar-treated OMW

The soil used to test the effect of the disposal of OMW treated with BP was a sandy soil (Table 2) having an alkaline reaction (pH 7.9) and a average content of organic carbon (12.13 g kg^{-1}), total nitrogen (1.22 g kg^{-1}), and available phosphate (46.09 g kg^{-1}). The value of EC 0.099 dS m^{-1} indicated a typology of non-saline soil.

Changes of chemical properties of soil amended with BP treated soil did not occur in terms of pH and EC: no significant differences were observed, after of 30 and 90 days from OMW disposal (data not shown).

Organic carbon (OC) content showed significant differences only between incubation time (30 and 90 days) (Figure 5). In fact, the OC increased significantly in all samples after 30 days of incubation time but, after 90 days, the OC returned to the initial values (approximately 12.00 g kg^{-1}) or still smaller as occurred in the sample OMWd+BP10 (10.94 g kg^{-1}) (Figure 5).

Table 2. Physical and chemical properties of soil.

Properties	Value
Sand (g kg^{-1})	879 ± 42
Lime (g kg^{-1})	93 ± 47
Clay (g kg^{-1})	27 ± 5
pH (in H_2O)	7.90 ± 0.06
EC (dS m^{-1})	0.099 ± 0.003
Limestone (g kg^{-1})	6.2 ± 0.4
CEC (cmol(+) kg^{-1})	15 ± 1
TOC (g kg^{-1})	12 ± 1
O.M. %	2 ± 0.1
Total N (g kg^{-1})	1.22 ± 0.02
C/N	10 ± 0.7
P_2O_5 (mg kg^{-1})	46 ± 1

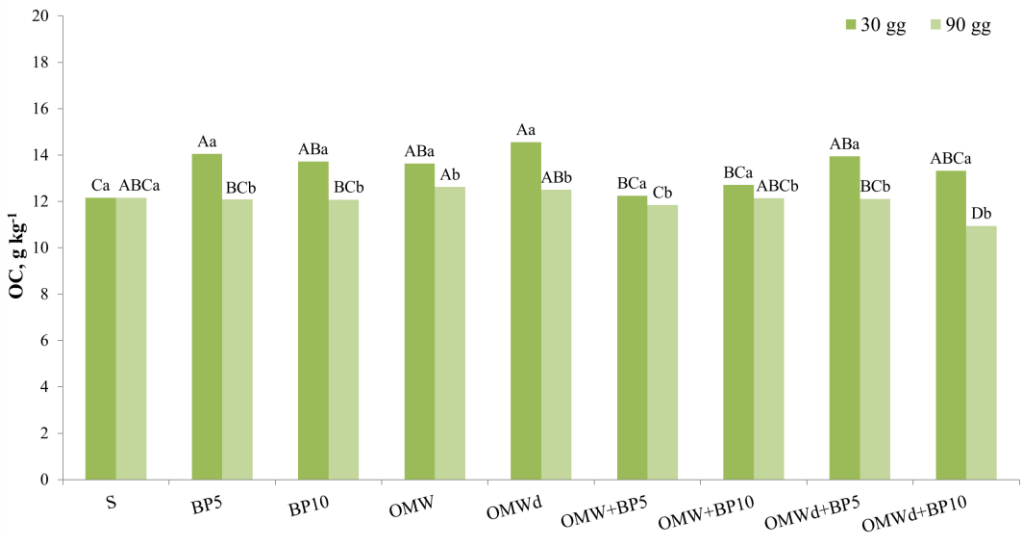

Figure 5.Organic carbon of soil amended with biochar-treated OMW. Capital letters indicate significant differences between the treatments; lower case letters indicate differences between two incubation times ($P < 0.05$).

Nitrogen content changed slightly only after 90 days of incubation time in amended soil samples that showed significantly increased values respect the control (Figure 6).

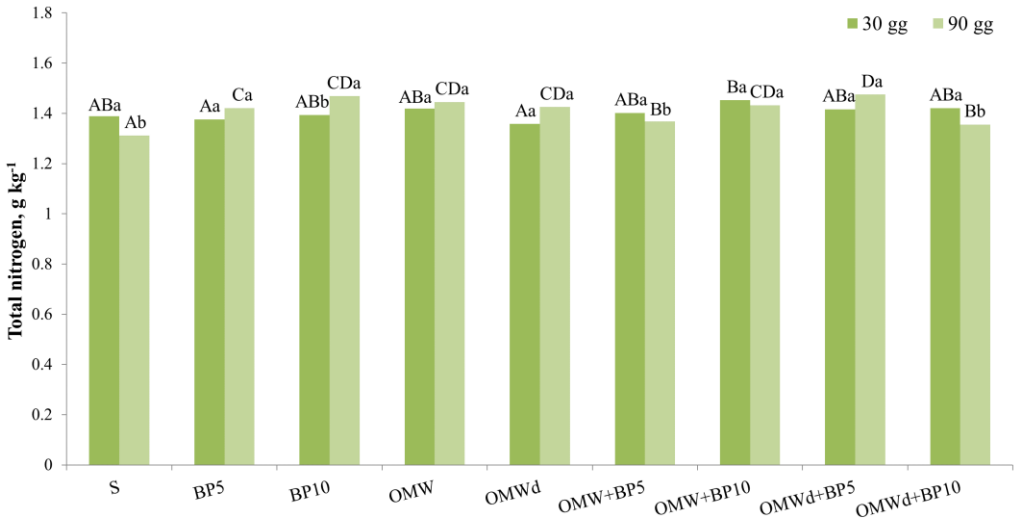

Figure 6. Total nitrogen of soil amended with biochar-treated OMW. Capital letters indicate significant differences between the treatments; lower case letters indicate differences between two incubation times ($P < 0.05$).

An increase of phosphorous content of 32% and 28%, instead, was observed in BP10 and OMWd+BP10 respectively, after 30 days of incubation (Figure 7). The value of phosphorous decreased after 90 days in all soil samples where the disposal of treated OMW occurred.

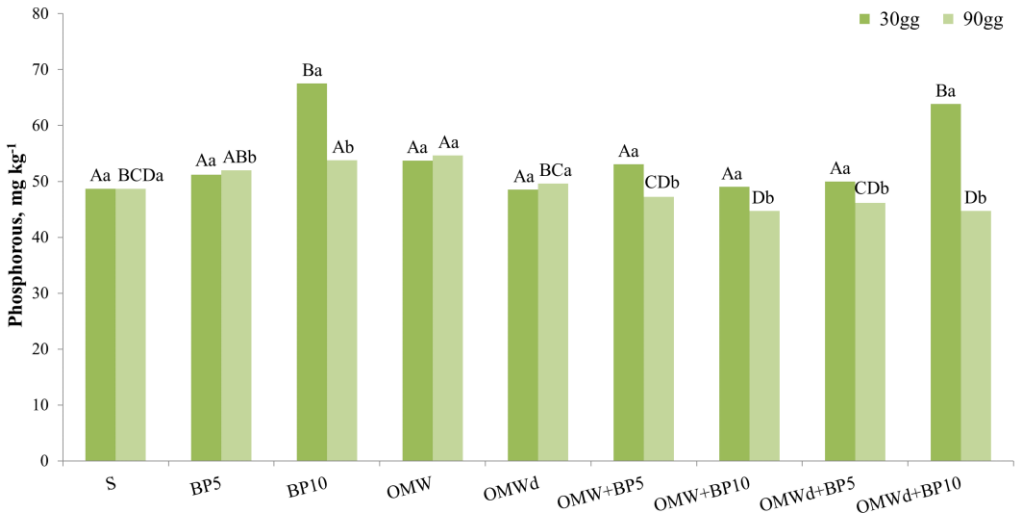

Figure 7. Phosphorous in soils differently amended with biochar-treated OMW. Capital letters indicate significant differences between the treatments; lower case letters indicate differences between two incubation times ($P < 0.05$).

3.3.4 Response of soil biochemical properties to biochar-treated OMW

All treatments with OMW containing biochar stimulated the development of the biomass microbial C biomass (MB-C) that represents the fraction of organic carbon due to soil microorganisms (Figure 8). After 30 days the value of MB-C was higher than 150 mg kg^{-1}, except for the sample BP10 (97.02 mg kg^{-1}) against the control value of 59.96 mg kg^{-1}. After 90 days, the MB-C was still higher in most of samples and different response to the different treatments was observed. The value of MB-C in OMW+BP5 and OMW+BP10 samples increased at 257.08 and 310.49 mg kg^{-1}, respectively. MB-C increased also in the control samples BP10, OMWd, and, in particular, OMW showing the biggest value of 412.27 mg kg^{-1}.

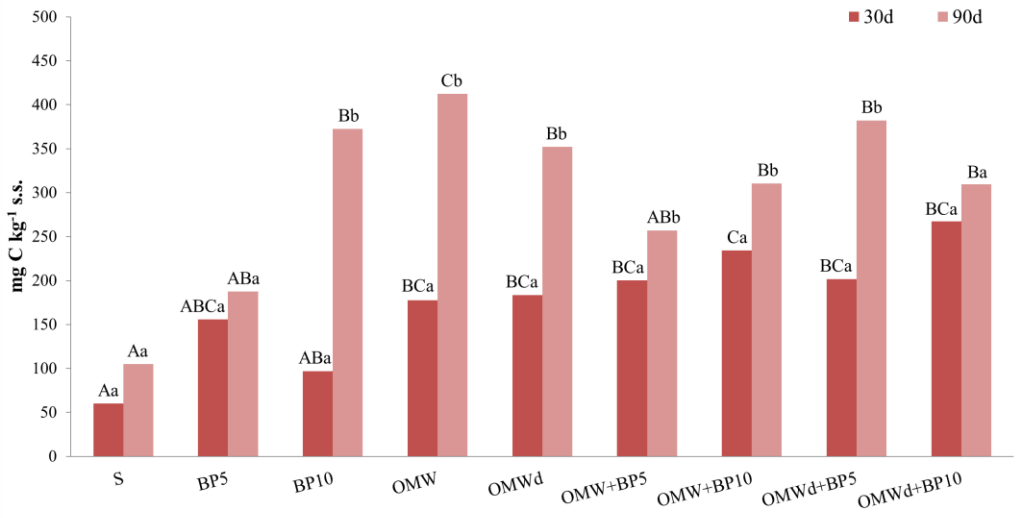

Figure 8. Microbial C biomass of soil amended with biochar-treated OMW. Capital letters indicate significant differences between the treatments; lower case letters indicate differences between two incubation times ($P < 0.05$).

Soil enzymatic activities, measured as bioindicators of soil fertility, showed a diverse response to OMW treatments. DH significantly increased in soil after 30 days from the amendment with OMW (i.e., OMW and OMWd) treated/untreated with BP10 (Figure 9). While, after 90 days in the same samples treated with BP10 (OMW+BP10 and OMWd+BP10) the activity return to the value registered in the control sample (S). However, the DH was stable in the time in the samples OMW and OMWd treated with 5% of biochar.

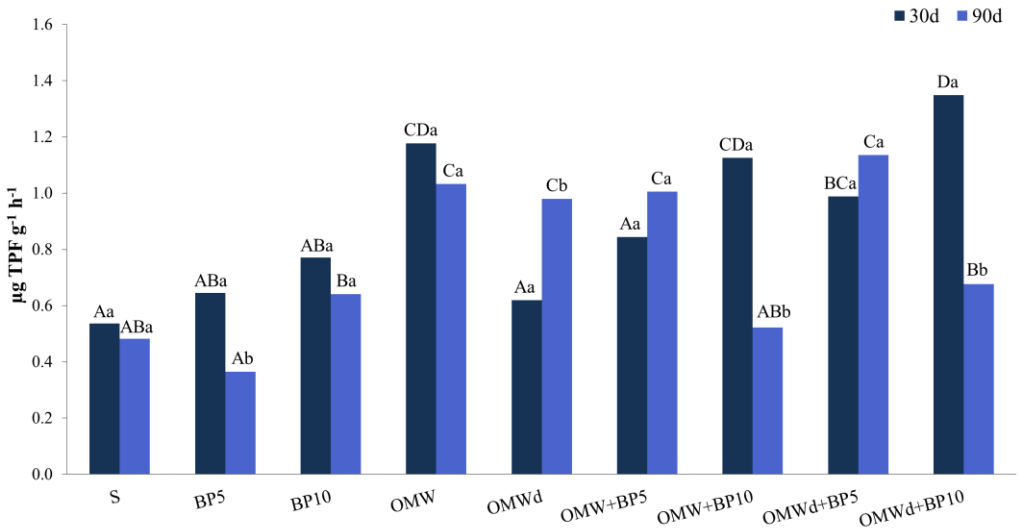

Figure 9. Dehydrogenase activity of soil amended with biochar-treated OMW. Capital letters indicate significant differences between the treatments; lower case letters indicate differences between two incubation times ($P < 0.05$).

The activity of FDAH showed a significant increase after 30 days from the application of OMW treated with biochar (Figure 10). The sample OMW+BP5 reached the highest value of the activity (42.97 mg g^{-1} h^{-1}), three times higher than the control sample (S). The level of FDAH, after 90 days, returned close to the control values in all samples.

The activity of GLU showed, after 30 days, a significant increase only in the samples treated with OMW+BP5 and OMW+BP10, as well as in OMWd control where there was the highest level of this activity (0.486 µmol pNP g^{-1} h^{-1}) (Figure 11). After 90 days, the GLU activity was reduced significantly both in soil control sample (S) and in all of the other samples, with the exception of OMW+BP5, OMW+BP10, OMWd+BP5 and OMWd+BP10 sample. This suggests that in these samples the organic substance has been stabilized ensuring, the availability of a substrate over time.

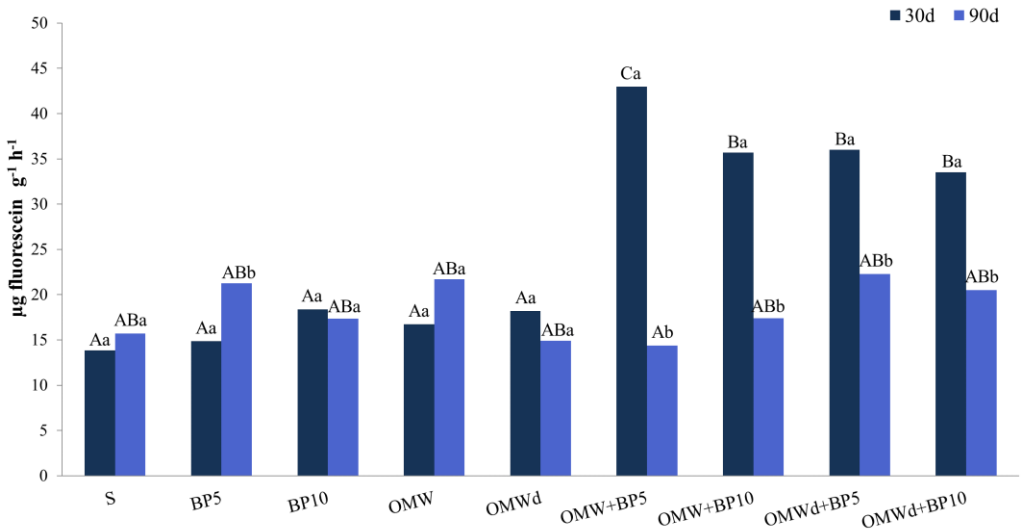

Figure 10. Fluorescein diacetate hydrolase activity of soil amended with biochar-treated OMW. Capital letters indicate significant differences between the treatments; lower case letters indicate differences between two incubation times ($P < 0.05$).

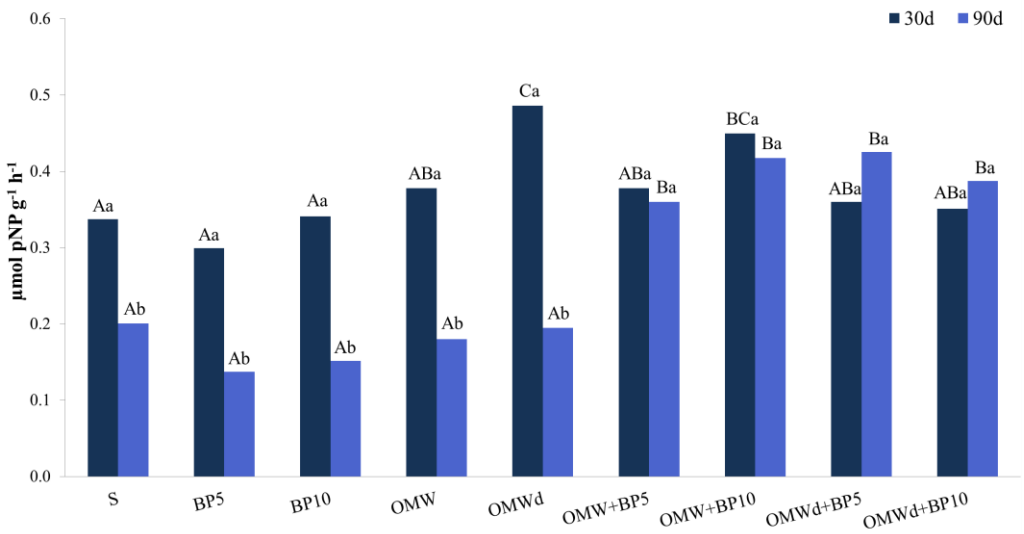

Figure 11. β-glucosidase activity of soil amended with biochar-treated OMW. Capital letters indicate significant differences between the treatments; lower case letters indicate differences between two incubation times ($P < 0.05$).

The application of OMW treated with biochar resulted in an increase of the phosphatase activity (PHO) after 30 days, except for OMWd+BP5 samples and OMWd+BP10 (Figure 12). In these samples, the value of the activity remained similar to the control sample (S). After 90 days the PHO decreased in all soil samples, but not in those amended with biochar-treated OMW, indicating that the greater availability of inorganic phosphorus in these samples also in the long period.

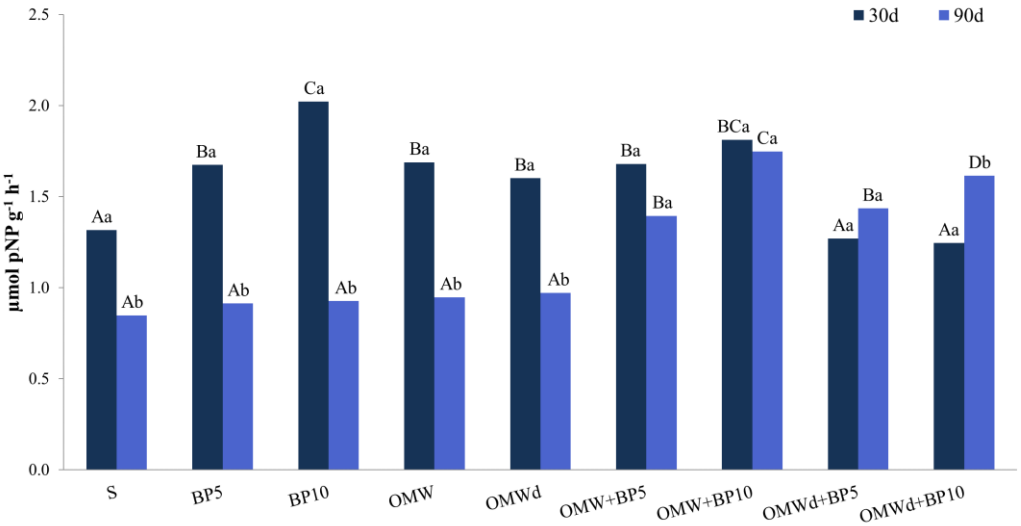

Figure 12. Phosphatase activity of soil amended with biochar-treated OMW. Capital letters indicate significant differences between the treatments; lower case letters indicate differences between two incubation times ($P < 0.05$).

The activity of DPO significantly decreased in the OMW, OMWd and OMW+BP5 samples compared to the control sample after 30 days; while the activity significantly increased in OMW+BP10, OMWd+BP5 and OMWd+BP10 samples (Figure 13). After 90 days, all samples showed a significant decline maintaining the same trend observed after 30 days.

The urease activity (UR) (Figure 14) in soils amended with OMW treated with biochar was not influenced significantly by the treatment. After 30 days, there

was a decrease of the activity in the samples OMW+BP10 and OMWd+BP5. After 90 days, no significant differences were observed between the different treatments.

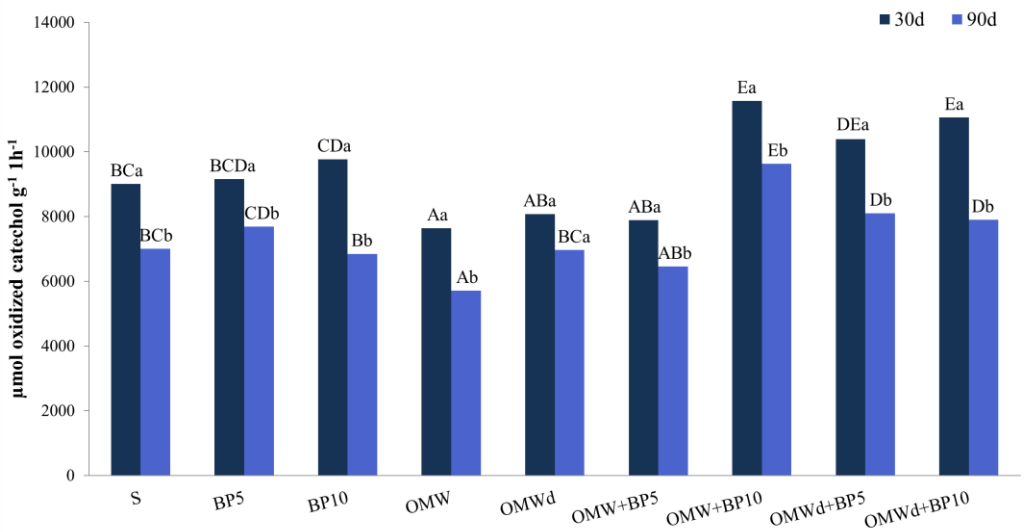

Figure 13. Ortho-diphenoloxidase activity of soil amended with biochar-treated OMW. Capital letters indicate significant differences between the treatments; lower case letters indicate differences between two incubation times ($P < 0.05$).

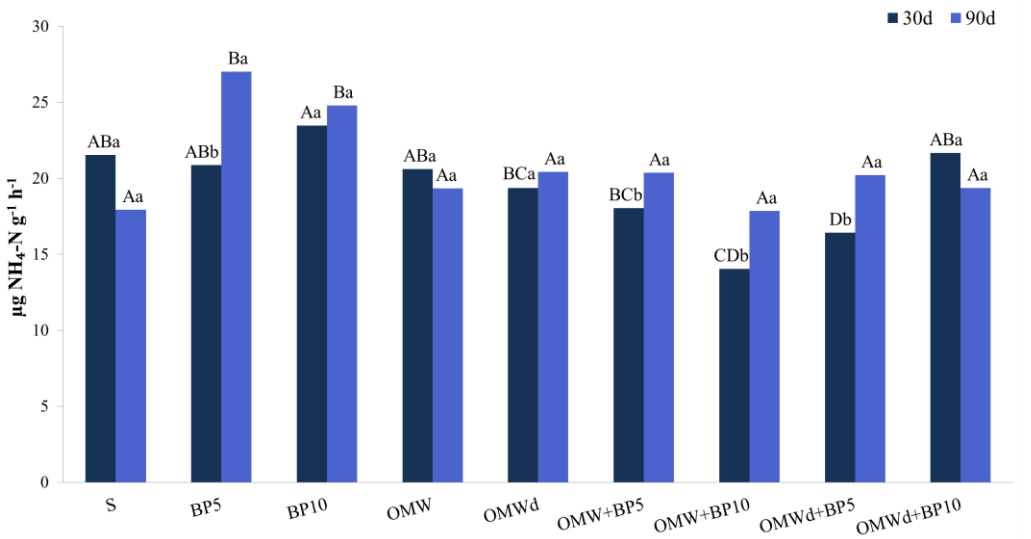

Figure 14. Urease activity of soil amended with biochar-treated OMW. Capital letters indicate significant differences between the treatments; lower case letters indicate differences between two incubation times ($P < 0.05$).

3.3.5 Phytotoxicity of soil amended with biochar treated OMW

In addition to chemical and biochemical analysis of the soil, the phytotoxicity of soil amended with biochar-treated OMW was evaluated through the germination test with *Lepidium sativum* L. seeds. Soil incubated with BP-treated OMW for 30 and 90 days was tested.

As reported in Table 5, after 30 days from the amendment, the value of relative germination in presence of biochar-treated OMWwas close to the control sample (S), with the exception of OMW+BP5, which showed a reduced germination to 80%. The germination tests performed at 90 days of incubation (Table 6) showed a further increase of the relative germination in soil amended with biochar-treated OMW. The values were similar to the control (100%) except for OMWd+BP5 and OMWd+BP10 (90%). In these latter, a reduction of the relative length and a consequent reduction of the germination index were observed.

Table 5. Phytotoxycity test of soil after 30 days.from the amendment with biochar-treated OMW.

Sample	RG	RL	GI
	----------	% ----------	
S	100 a	100 a	100 a
BP5	90 b	100 a	100 a
BP10	100 a	100 a	100 a
OMW	90 b	100 a	100 a
OMWd	90 b	100 a	100 a
OMW+BP5	80 c	100 a	80 c
OMW+BP10	90 b	100 a	100 a
OMWd+BP5	90 b	100 a	100 a
OMWd+BP10	100 a	100 a	100 a

Table 6. Phytotoxycity test of soil after 90 days.from the amendment with biochar-treated OMW.

Sample	RG	RL	GI
	---------- % ----------		
S	100 a	100 a	100 a
BP5	100 a	100 a	100 a
BP10	90 b	100 a	90 b
OMW	100 a	100 a	100 a
OMWd	100 a	100 a	100 a
OMW+BP5	100 a	100 a	100 a
OMW+BP10	100 a	90 a	90 b
OMWd+BP5	90 b	90 a	90 b
OMWd+BP10	90 b	80 c	75 c

3.4 Discussion

The use of biochar could be an alternative to the several remediation methods suitable to reduce the toxicity of OMW. As advantages, biochar is a low cost material, useful to improve soil fertility because able to sorb nutrients for plant and microorganisms (Lehman and Joseph, 2009; Kammann et al, 2015). Exploiting the same property biochar is also able to retain numerous organic pollutant drastically reducing their environmental impact (Lehman and Joseph, 2009; Lou et al., 2011; Mohan et al., 2014).

In OMW management, the more important purpose is to fall down the phenolic content that depends on olive variety, olive maturity at harvesting, conservation in post-harvesting and extraction process (Ramos-Cormenzana et al., 1997)

In our study the treatment of OMW with biochar whatever was the origin biomass (poplar or conifer) has significantly reduced the phenolic content. The initial concentration of phenolic compounds in OMW was within the common range for this waste (1.35 mg ml^{-1}). Unfortunately, the treatment showed greater efficiency only in long incubation time since 20 day-incubation time of biochar in OMW led to no changes from the value of control sample. After 60 days, instead, different responses to different treatments with BP and BC took shape:

BP determined a higher reduction of phenolic compounds compared to the BC treatment. The reason of this different effect was not consistent with different functional groups since FT-IR analysis did not highlight any different pick in the spectra of two biochars (Figure 2 in Chapter 2). The reason was not even consistent with biochar physical characteristics such as surface area and pore volume because BP had less large surface area and smaller pore volume as reported by De Pasquale et al., 2012). However, BP was characterized by a bigger number of small pore therefore by a higher porosity (De Pasquale et al. 2012), a physical property that could affect the phenol adsorption (Kasozi et al., 2010).

The phenol removal from OMW grew up by doubling the biochar concentration. The further reduction was from 56% to 37% of residual phenols for OMW and from 48% to 28% of residual phenols for OMWd in BP-treated samples against 10 percentage points (from 71% to 61% of residual phenols for OMW and from 53% to 42% of residual phenols for OMWd) in BC-treated samples. This behaviour confirmed the better efficiency of BP in OMW remediation (Figure 3b).

In the previous study the adsorption of phenantherene occurred more efficiently with BC than BP, explaining this behaviour with the more easy access of big phenantrene molecules in the BC structure having higher surface area and larger pore dimension (Chapter 2). Kasozi et al. (2010) found that catechol adsorption increased by increasing the pyrolysis temperature and the adsorption process took place in the micropores and nanopores. In our study the pyrolysis temperature was the same for the production of both biochars (1200 °C), but anyway the different porosity has been observed possibly resulting from the origin biomass (Jindo et al., 2014).

The microbial and plant toxicity of OMW is the main concern as confirmed by germination test of OMW of this study. Results confirmed the relationship

between phenol content and phytotoxicity. The larger removal of phenols obtained with BP determined a greater reduction of phenol bioavailability for cress seeds, although the negative impact on root growth remained. The RL values remained very far from 100% (pure water) to indicate that the residual phenols or intermediate products of phenol transformation could however affect root elongation especially in the first phase of growth. After all young roots can explore also little pores where phenols are adsorbed and undergo the negative effect on own growth.

At the light of this finding, successive experiments to remediate OMW to spread on soil in lab-scale were carried out only with poplar biochar (BP) at two different rate (5 and 10%). This experiment had the purpose to understand if biochar that already if a material suitable for improvement of soil fertility (Lehman and Joseph, 2009; Lehman et al., 2011) may be used to detoxify OMW before their disposal in soil.

In Italy, a specific law regulates the management of OMW and limites to 80 t ha^{-1} per year the disposal of this waste according to physical soil properties to avoid leaching phenomena. A mitigated phytotoxic potential of OMW after biochar-based treatment could be a very interesting result to prevent in any case toxicity effect occurring in the first phase after the disposal. Once in soil, OMW undergo degradation as organic matter in a complex system as soil: in particular, phenolic compounds could be substrates of oxidative polymerization thus lacking its toxic potential or leading to more toxic intermediate products.

The advantage of the use of biochar in detoxifying OMW is to make possible the disposal of the entire mixture OMW+biochar, without a previous separation of biochar. In fact, biochar is not toxic and harmful for soil health, rather it is beneficial and good for improving soil fertility (Lehman et al., 2011). This could make easier and less expensive the application of this proposed methodology of OMW remediation.

Once soil was amended with OMW treated with BP at different rate, amended soil samples showed OC significantly enhanced after 30 days. Both biochar and OMW positively affected values as demonstrated by BP5, BP10, OMW and OMWd samples having higher values than control sample. While it is obvious the OMW contribute to OC, the biochar contribute could be explained analysing the biochar composition. In the pyrolysed biomass recalcitrant material prevails but also a more labile fraction remains. This latter, indeed, can be taken into account by analytical method of OC (Lehman and Joseph, 2009). The beneficial effect of biochar was observed on soil phosphorous and in particular, in the samples BP10 and OMWd+BP10 in which the phosphorous content significantly increased. Biochar is able to retain on its surface nutrients and to behave as a nutrient source (Lehman and Joseph, 2009; Kammann et al., 2015). The MB-C that is an index of the active and sporulated microbial biomass showed an enhancement over time (after both 30 and 90 days). This response was due above all to the labile organic carbon from OMW but also to the improved soil physical properties and nutrients derived by the BP arrival in soil samples (Moreno et al. 2013; Lehman and Joseph, 2009; Lehman et al., 2011; Lehmann et al., 2015). MB-C significantly increased already after 3 h from spreading (Moreno et al., 2013).

The increment of MB-C was positively correlated ($p<0.05$) with the activity of DH and FDAH after 30 and 90 days of incubation time, in according to numerous studies on the effect of organic amendments on soil biochemical properties (Scotti et al., 2015). The labile organic fraction from OMW stimulated the activity of enzymes involved in C, N and P cycles such as GLU, FDAH and PHO. Conversely, UR activity was not affected by the OMW amendment, in according with Moreno et al. (2013). In fact, ammonium content of OMW could control this activity (Piotrowska et al., 2006). DPO activity, reflecting the phenol turnover in soil, was inhibited in samples having higher phenol content (OMW,

OMWd), conversely, DPO activity was enhanced in soil samples amended with OMW treated with biochar having lower phenol content (Figure 13; Tsiknia et al., 2014).

The biochar-based treatment of OMW did not affect phytotoxycity of this waste. Cress seeds were able to germinate also in presence of soil amended with no treated OMW or differently treated in according with Magdich et al. (2012) who demonstrate that only continue spreading of OMW can lead to the decrease of germination index after six years.

3.5 Conclusions

Overall, biochar can be used in the treatment of OMW, in order to reduce the phenolic compounds. The disposal of biochar-trested OMW showed a synergistic effect for soil properties. Temporary and permanent changes in chemical and biochemical soil properties were observed, showing that soil has an intrinsic buffer capacity to resist to possible perturbation. Further investigations need to verify if greater amount, also with repeated disposals of biochar-treated OMW could further improve soil properties. Field trial may be helpful for better understand the real potential of biochar-based remediation of OMW.

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Chapter 4

4 Determination of black carbon metabolites by ion-exchange chromatographic separation and ultraviolet spectroscopic detection of benzenepolycarboxylic acids

4.1 Introduction

Black carbon is formed by incomplete combustion of organic matter and fossil fuels and occurs along a continuum from slightly charred material over char, charcoal to soot and graphite (Goldberg, 1985). Being a ubiquitous material circulating around the surface of the earth, black carbon can be found e.g. in the air, soils, sediments and water (Goldberg, 1985). That is why, over the last decades, the interest on origin and fate of black carbon in the environment was increasing due to its potential prominent role in the global carbon cycle (Glaser et al., 1998; Schmidt and Noack, 2000; Glaser and Amelung, 2003; Haumaier, 2010; Rodionov et al., 2010). Furthermore, it can be used as indicator of fire history. In fact, black carbon may represent up to 45% of total organic carbon in agricultural soil, which is often burned (Czimezik snd Masiello, 2007) and black carbon can give information about physical, chemical and biological properties of charcoal in soil (Glaser et al., 1998). However, it has been shown that polycondensed aromatic structures identical to black carbon can be produced biologically in significant amounts in soils (Glaser and Knorr 2008). Nevertheless, there are various methods for the quantification and characterization of black carbon in soils including optical techniques, thermal and chemical degradation and spectroscopic methods (Knicker, 2007; Schmidt et al., 2001). Among these methods, benzenepolycarboxylic acids (BPCA) are widely used as molecular marker for black carbon (Glaser et al. 1998). This method is based on the principle that degradation of charcoal with hot

concentrated nitric acid at elevated pressure leads to a conversion of the polycondensed aromatic structures into BPCA.

The procedure requires a tedious sample clean-up and derivatization prior to gas chromatographic (GC) separation and flame ionization detection, which is time- and cost-consuming. That is why within the last years, the BPCA method was improved and simplified several times. For example, Dittmar (2008) used liquid chromatography (LC) for BPCA analysis in marine dissolved organic matter. The main advantage of using LC instead of GC is that sample processing is less, and derivatization is not required. These characteristics make the method suitable for routine analysis and reduce the risks of artefacts production, because the BPCA are not produced during the procedure. Schneider et al. (2011) who compared GC and LC for the determination of black carbon outlined the advantages of LC. Thereby, the authors stated that by LC, a more robust quantification of BPCA can be obtained due to a minimum level of sample preparation. Wiedemeier et al. (2013) confirmed the findings of Schneider et al. (2011), in fact the sample analysed by LC showed a smaller variation (5%), in contrast to the 16-23% of GC analysis. The authors concluded that black carbon analysis using LC is useful for small sample amounts and showed higher reproducibility of the results compared to the often used GC method.

In our study, we will further optimize ion-exchange chromatography (IEC) in order to separate the BPCA based on the findings of Yarnes et al. (2011). They used IEC for the separation of BPCA because it is particularly well suited for the separation of water soluble ionic compounds without the use of organic solvents. In fact, there are several applications for the separation of ionic organic compounds, including carbohydrates (Abave et al., 2011), amino acids (Rinne et al., 2012) and amino sugars (Dippold et al., 2014). The quality of the separation was affected by the presence of other organic anions, thus the cleaning process must be improved to remove polyvalent cations. High concentrations of

polyvalent cations omnipresent in soil samples, could affect the retention time of BPCA through local electrostatic interaction.

Haumaier (2010) demonstrated that free BPCA can also be found in soils under natural conditions. The author tested different types of soils from different countries and hypothesized that black carbon metabolites are as ubiquitous as black carbon in soil. Therefore, free BPCA in soil can be used as black carbon metabolites and their proper analysis can contribute to understand the fate of black carbon in the environment. The aim of this study is to establish an improved analysis method for black carbon metabolites measurements using IEC. The common procedure proposed by Haumaier (2010), requires three days of extraction and this study aimed at the reduction of the extraction time, sample clean-up procedure and BPCA quantification. For this purpose, we used pure quartz sand and real soil samples spiked with BPCA and subjected them to optimization of extraction time, sample clean-up and IEC separation and UV detection. Furthermore, on the same soil samples, we evaluated black carbon in accordance with Glaser at al. (1998) with modifications of Browdowski et al. (2005) to understand better if black carbon metabolites are directly linked or correlated with black carbon content in soil.

4.2 Material and Methods

4.2.1 Chemicals

BPCA were used as a molecular marker of black carbon. Pure standard compounds (Sigma-Aldrich) including hemimellitic acid (benezene tricarboxylic acid, B3CA, 1,2,3-B3CA), trimellitic acid (1,2,4-B3CA) and trimesic acid (1,3,5-B3CA); benzene tetracarboxlic acid (B4CA), 1,2,4,5-B4CA (pyromellitic acid); benzenepentacarboxylic acid (B5CA); and benzenehexacarboxylic acid (B6CA; mellitic acid) and phthalic acid, were used as standards. The mobile phase for IEC was produced from 50-52% w/w Sodium

hydroxide solution (Sigma-Aldrich) was used, as it contained lower carbonate content compared to preparation from solid sodium hydroxide.

4.2.2 Soil Samples

Soil samples used in this experiment were selected to cover a wide range of soil properties. Samples of sandy soil were taken from Wendland (Germany) and silty soil from Etzdorf (Germany). Samples of clayey soil were taken from Santarem (Brasil). Basic characteristics of the soils are listed in Table 1. The investigated soils cover a wide range of sand (30 - 750 g kg^{-1}), silt (20 - 630 g kg^{-1}), and clay contents (150 - 960 g kg^{-1}) as well as different pH (3.8 - 7.2) and TOC concentrations (4 - 65 g kg^{-1}).

Table 1. Basic properties of soil samples used for method optimization.

Soil type	Country	Location	Texture	Sand (%)	Silt (%)	Clay (%)	pH (KCl)	TOC (g kg^{-1})
Cambisol	Germany	Wendland	Sandy	89.2	7.7	3.1	7.2	4.3
Chernozem	Germany	Etzdorf	Silty clay loam	6.0	63.0	31.0	5.7	22.0
Ferralsol	Brasil	Santarem	Heavy clay	15.0	15.2	69.7	3.8	14.0
Anthrosol	Brasil	Santarem	Heavy clay	2.9	1.6	95.6	6.0	65.2

4.2.3 Extraction of benzenepolycarboxylic acdis

Prior to the extraction process, the soil samples were spiked with 100 µg phthalic acid solution (1 mg ml^{-1}) as internal standard. Furthermore, the samples were spiked with 100 µl of BPCA solution (1 mg ml^{-1}) to calculate the recovery of the individual BPCA in quartz sand and soil samples.

The extraction of BPCA was performed according to Haumaier (2010). In brief, around 5 g of air-dried soil samples were extracted with 25 ml of 500 mM NaOH on a horizontal shaker for 24 h and centrifuged at 4000 g per 15 min. The extract was acidified with 4 mL of 4 M HCl and centrifuged at 4000 g per 15 min. The

same sample was extracted for 48 h and 72 h in the same way and at the end of the extraction process, all extracts were purified in the same way.

4.2.4 Purification with cation exchange column

For the analysis of BPCA, it is necessary to remove all polyvalent cations (Glaser et al., 1998). A cation exchange column (Dowex 50 W X 8, 200-400 mesh, Sigma Aldrich, St. Louis, MO, USA) was used in accordance with Glaser et al. (1998), including some modifications. The resin was filled in a glass column (350 mm long and with an inner diameter of 26 mm) up to a level of 100 mm corresponding to a volume of 53 cm^3. The extracts (30 ml) were eluted through the column. After the sample was completely eluted, the resin was washed with 100 mL of water into 250 ml conical flask. The eluate was freeze-dried to remove the water. The dried sample was re-dissolved in 2 ml of 100 mM NaOH, filtered (< 0.2 µm) and analysed by IEC.

4.2.5 Ion-exchange chromatographic separation

Measurement of BPCA were performed using a Dionex ICS 5000 (Thermo Fisher, Bremen, Germany) IEC equipped with variable wavelength detector. The chromatography was performed with a Dionex IonPac AS11-HC column (2 x 250 mm) equipped with an AG11-HC guard column, in accordance with Yarnes et al. (2011). Please note that our system was equipped with polyether ether ketone (PEEK) tubes throughout the whole system including the flow cell of the variable wavelength detector. Sample injection of 5 µl was carried out by a Dionex AS-AP auto sampler. BPCA standards were injected accordingly. Sodium hydroxide (100 mM, eluent A) and deionized water (eluent B) were used as mobile phase delivered by a Dionex ICS 5000 single pump. The time of the analysis was 50 min and followed this gradient elution: (% of eluent A): 0-1 min 30-35%; 1-5 min 35-40%; 5-20 min 40-80%; 20-30 min 80-100%; 30-35 min

100%; 35-40 min 100-30%; 40-50 min 30%. The flow rate was 350 µl min^{-1}. The UV detection was carried out at 254 nm. Before the analysis of each batch, the system was washed with 200 mM NaOH for 1 h, followed by a washing cycle of 1 h with water to equilibrate the system.

4.2.6 Black carbon analysis

Black carbon was analyzed using BPCA as molecular marker (Glaser et al., 1998; Brodowski et al., 2005). An amount of 0.5 g of ground soil sample was digested with 10 mL of 4 M trifluoroacetic acid (TFA) for 4 h at 105 °C. The dried residue was oxidized with 2 mL of 65% nitric acid for 8 h at 170 °C. The digested solution was diluted to 10 mL. An aliquot of 2 mL was subjected to a cation exchange clean-up procedure, by using a Dowex 50 W X 8, 200-400 mesh. After freeze-drying, the aromatic acids were analyzed as trimethylsilyl (TMS) derivatives by capillary gas chromatography (GC-FID) using a Shimadzu GC-2010 instrument (Shimadzu Ltd., Tokyo, Japan) equipped with a DB5 capillary column (30 m x 0.32 mm x 0.25 mm film thickness) and an Flame ionization detector (FID). Phthalic acid (1 mg ml^{-1} in water) was used as internal standard 1 and added prior to the sample clean-up; 2.2′-biphenyldicarboxylic acid (1 mg ml^{-1} methanol, internal standard 2) was added prior to derivatization. The sum of BPCA after nitric acid oxidation is a relative measure of black carbon and when multiplied with 2.27, a charcoal equivalent can be calculated (Glaser et al., 1998).

4.2.7 Standard preparation

External standards were used for calibration. The external standard solution was measured at six different concentrations (10, 25, 50, 100, 250, 500 µg of BPCA per vial) during the sample analysis and was prepared by dissolving 1 mg of each BPCA in 1 ml of 100 mM NaOH. The corresponding volume (10, 25, 50, 100,

250, 500 µl) was then transferred into a vial and filled up to 1 ml with 100 mM NaOH.

4.3 Results and Discussion

4.3.1 Ion-exchange chromatography separation

We modified the IEC separation of Yarnes et al. (2011), so that the total run time could be reduced from 70 min to 50 min. Before the analysis of quartz sand and soil samples, each BPCA standard was injected in order to identify the compounds through retention time. The chromatograms of BPCA standards (Figure 1a) and Chernozem sample (Figure 1b) showed that 50 min was enough to elute all of the BPCA. For both, the soil sample and the standard, a good efficiency and selectivity of the separation could be demonstrated. However, the chromatogram of the soil sample showed some background during analysis but nevertheless the BPCA could be baseline-separated and detected. The noisy background in the chromatogram of the soil sample was probably due to the interferences with clay minerals and metal compounds. This is the reason, why a cleaning step with 200 mM NaOH is required after ten samples.

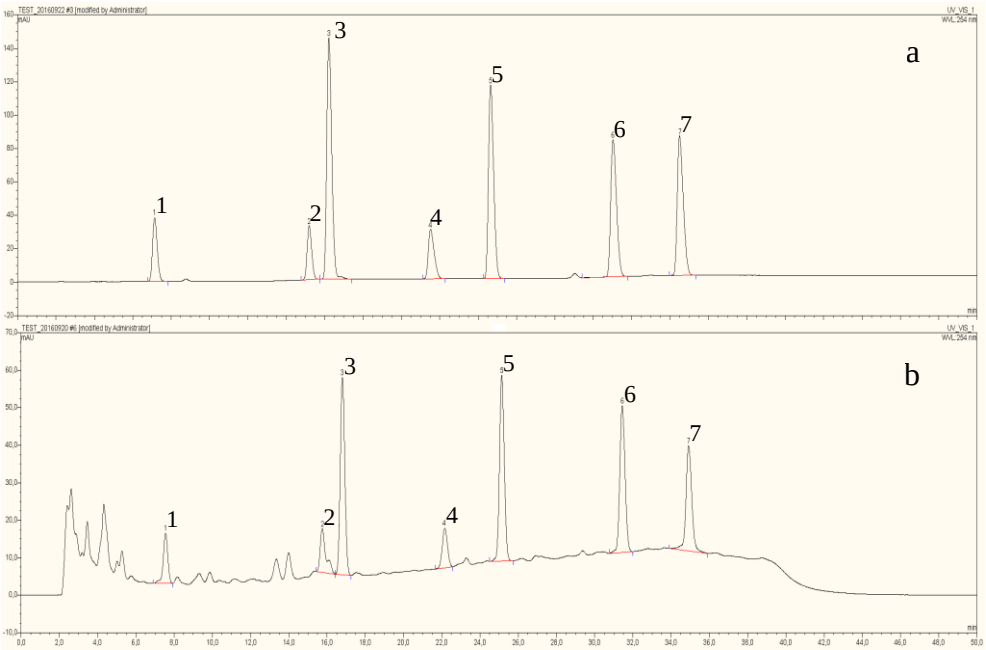

Figure 1. Ion-exchange chromatography of a benzenepolycarboxylic acids standard (a) and a Chernozem sample (b). Numbers correspond to 1) Phthalic acid (internal standard); 2) hemimellitic acid; 3) trimellitic acid; 4) trimesic acid; 5) pyromellitic acid; 6) benzene pentacarboxylic acid; 7) mellitic acid.

4.3.2 Optimization of black carbon metabolite extraction: purification

To extract black carbon metabolites, we used a sample of quartz sand spiked it with 100 µl BPCA solution (1 mg ml^{-1}) and followed the procedure of Haumaier (2010). We aimed at optimizing this proposed method in order to reduce the extraction time and analyse black carbon metabolites through IEC to avoid the derivatization procedures that require time and the use of solvents. We followed the procedure of Haumaier (2010) but used a different resin (Dowex 50 W X 8, 200-400 mesh) as it was suggested by Glaser et al. (1998). With 53 cm^3 of resin, the salts that could interfere during the subsequent analysis were removed. Other methods for removing salt suggested by Haumaier (2010) with 50 cm^3 of cation exchange resin (H^+ form) and by Glaser et al. (1998) with 4.70 cm^3 Dowex resin were not successful.

4.3.3 Recovery of black carbon metabolite from spiked quartz sand sample

In order to optimize the extraction time, quartz sand was spiked with black carbon metabolites and extracted for 24, 48 and 72 h. Thereby we found that within 48 hours all metabolites could be extracted (Figure 2a), whereas an extraction time of 3 days (72 hours) as suggested by Haumaier (2010) did not show an improvement of extraction efficiency.

The overall recovery of the added BPCA to quartz sand was between 54% and 74% (Table 2). Quartz sand is an inert material that should not interfere with black carbon metabolite extraction that is why the main losses probably occurred during the purification process. The procedure was repeated with soil samples in order to clarify if soil samples need longer to extract black carbon metabolites compared to quartz sand.

Table 2. Recovery (%) of spiked benzenepolycarboxylic acids (100 µg) in quartz sand and soil samples.

Sample	Hemimellitic acid	Trimellitic acid	Trimesic acid	Pyromellitic acid	B5CA	Mellitic acid
			%			
Quartz sand	61	54	59	64	71	74
Cambisol	77	75	68	76	66	62
Chernozem	71	67	70	65	67	83
Ferralsol	98	74	108	106	107	108
Anthrosol	107	65	99	54	68	113

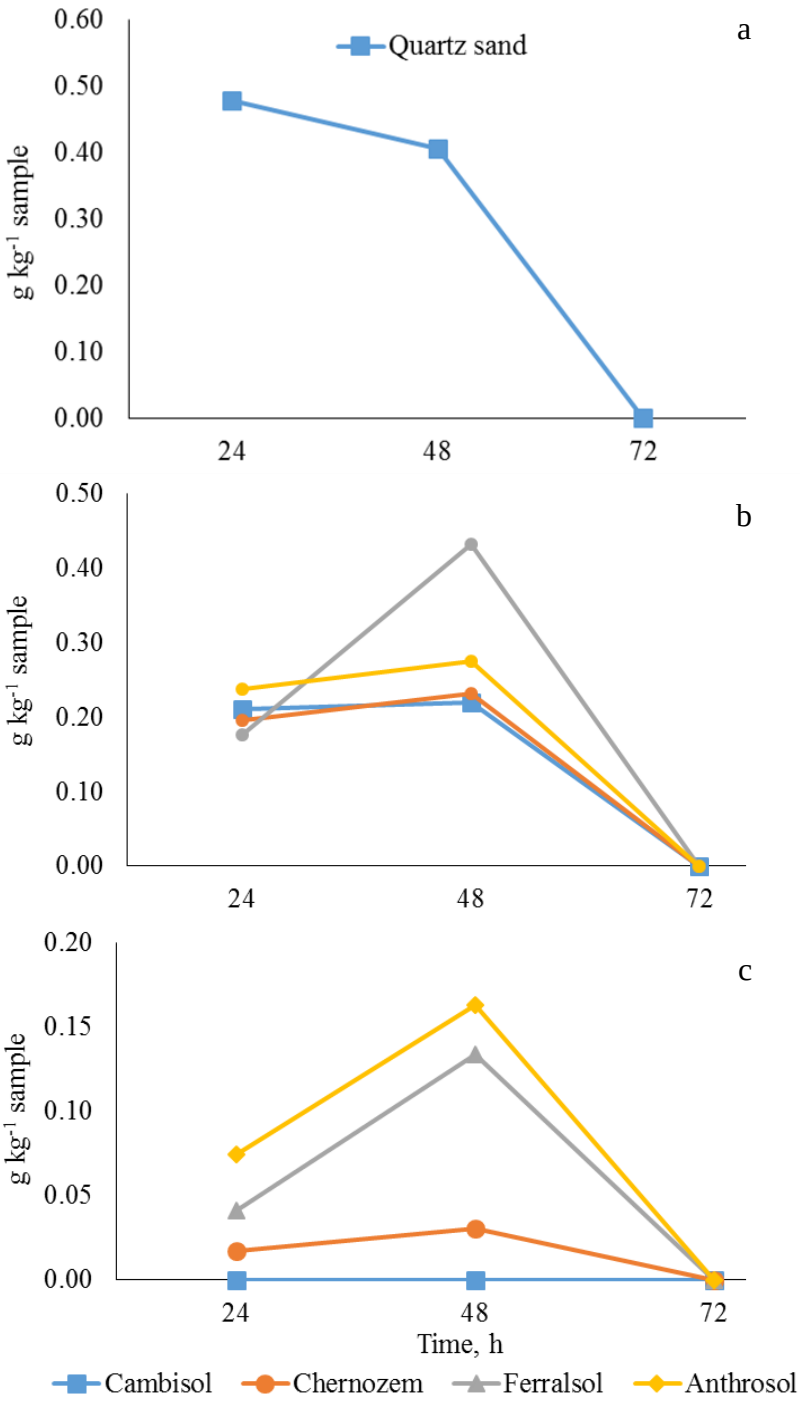

Figure 2. Extraction time (24, 48, 72 h) of black carbon metabolites in spiked (100 µg individual benzenepolycarboxylic acids) (a) quartz sand, (b) soil samples, and (c) un-spiked soil samples**.**

4.3.4 Recovery of black carbon metabolites from spiked and un-spiked soil samples

After the extraction of the black carbon metabolites from quartz sand, the optimized method was used with different soil samples covering a wide range of physical and chemical properties (Table 1). Similar to the procedure used for quartz sand samples, we used soil samples spiked with 100 µl of BPCA solution (1 mg ml^{-1}) to verify the complete extraction of these compounds in a complex matrix like soil.

The optimum extraction time for soil samples was 48 hours (Figure 2b) which is in agreement with the optimum extraction time in quartz sand. The recovery of spiked BPCA in soil samples was 81.4 ± 18.6% which was as good as or even better than the previous extraction with quartz sand (Table 2). The high variability in the BPCA recovery compared to quartz sand might be explained by different matrix effects of the soil samples, where different soil properties such as texture, pH and TOC may affect the recovery.

All spiked soils showed similar black carbon metabolites concentrations ranging between 0.4 and 0.6 g kg^{-1} (Figure 3a), which is not surprising as all soils received the same amount of spiked BPCA. For improving the understanding of black carbon metabolite in soil, the extraction of these compounds from the same soil samples without an additional spiking of BPCAs was performed. Testing the different extraction times of un-spiked soil samples showed that in the Cambisol sample no black carbon metabolites could be detected during the applied 72 h. For the other samples, including a Chernozem, Ferralsol and Anthrosol, the extractable amount of black carbon metabolites increased from 24 h to 48 h, especially in Ferralsol and Anthrosol samples. Unlike for spiked quartz sand and soil samples (Figure 2c) no black carbon metabolite could be extracted after 72 h.

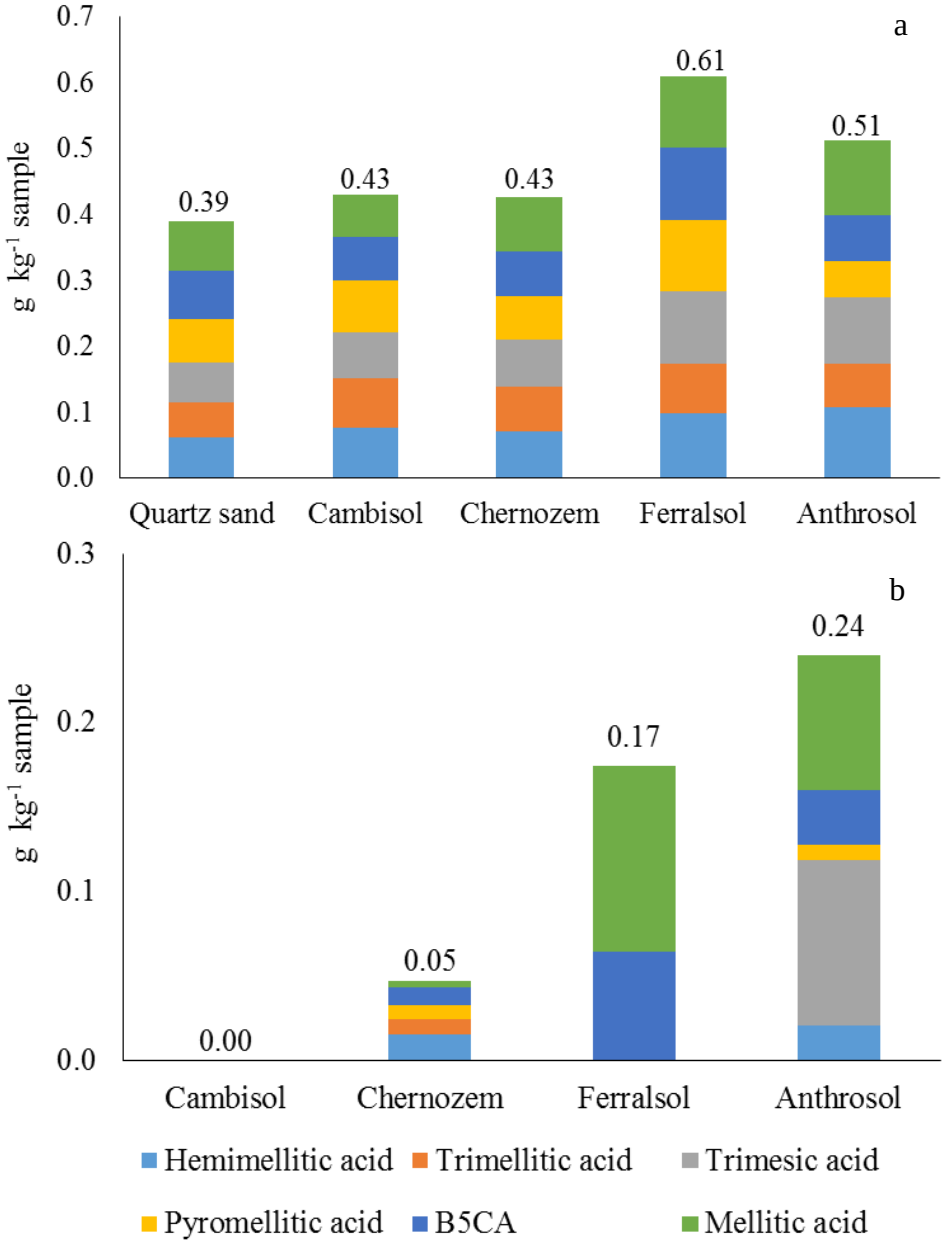

Figure 3. Sum of individual black carbon metabolites in (a) spiked quartz sand and soil samples and (b) un-spiked soil samples.

4.3.5 Black carbon metabolites in natural soils

Black carbon metabolites in the soils under study varied between 0 g kg^{-1} and 0.24 g kg^{-1} and increased in the order Cambisol < Chernozem < Ferralsol < Anthrosol (Figure 3b). It is interesting to note that temperate soils (Cambisol and Chernozem) obviously exhibited lower amounts of black carbon metabolites than tropical soils (Ferralsol and Anthrosol).

With respect to individual black carbon metabolites, temperate soils are dominated by lower condensed aromatic moieties (B3CA and B4CA), while the BPCA pattern of tropical soils is dominated by higher condensed aromatic moieties (B5CA and B6CA) (Table 3). Obviously, the amount and distribution of black carbon metabolites in the soil is more dependent on climatic conditions and / or soil physical and/or chemical properties rather than on black carbon concentration (Table 1).

Table 3. Relative amount of black carbon metabolites present in un-spiked soil samples.

Sample	Hemimellitic acid	Trimellitic acid	Trimesic acid	Pyromellitic acid	B5CA	Mellitic acid
			%			
Cambisol	0	0	0	0	0	0
Chernozem	33	19	0	16	23	8
Ferralsol	0	0	0	0	37	63
Anthrosol	9	0	41	4	14	33

The negligible black carbon metabolites concentrations of the Cambisol was probably due to specific soil characteristics compared to other soil samples under investigation, including an higher pH (7.2), lower TOC (4.3 g kg^{-1}), and the texture (sandy soil). Soil pH is an important factor for the mobility of black carbon metabolites. In fact, alkaline pH increases the mobility of black carbon metabolites and supports an eluviation of these compounds in deeper soil layers (Haumaier, 2010). However, soil samples under investigation do not show this high level of pH and the maximum of 7.2 was found in Cambisol samples.

Nevertheless, the increased pH compared to other soils under study might explain the missing extractable black carbon metabolites due to increased translocation into deeper soil layers, which were not sampled.

The black carbon metabolites content in the Chernozem sample is lower compared to Ferralsol and Anthrosol which supports our observation that black carbon metabolites are not directly linked with the black carbon content and mainly depend on the physical and chemical characteristics of the soil and / or climatic conditions. In addition, individual BPCA were more or less evenly distributed among total black carbon metabolites (Table 3). Chernozems are known for their high black carbon concentrations and stable soil organic matter (Rodionov et al., 2010). They are assumed being of natural origin although some of them are anthropogenically over-printed (Kleber et al., 2003), which certainly alters soil organic matter stability, and thus, occurrence of black carbon metabolites. Furthermore, Ding et al. (2015) proposed that the enrichment of hemimellitic, trimellitc (33%) and pyromellitic acid (16%) as shown for the Chernozem sample in this study (Table 3) derived from anthropogenic sources.

The Ferralsols under study (Table 3) show an enrichment in B5CA (37%) and mellitic acid (63%) which represents a minor contribution from anthropogenic sources (Ding et al., 2015). The same was suggested by Brodowski et al. (2005), showing that the black pigment of *Aspergillus niger*, a saprothrophic soil fungi was dominated by mellitic acid. Also Glaser and Knorr (2008) showed by an isotopic labelling experiment, that up to 9% of black carbon was produced biologically in tropical soils.

In the Anthrosol samples, hemimellitic acid and trimesic acid summed up to 50% of the total amount of black carbon metabolites, whereas B5CA and mellitic acid contributed 47% to the total amount of black carbon metabolites. In this case, it was not possible to distinguish between a natural and anthropogenic source of

these compounds because both classes (B3CA and B4CA) and (B5CA and B6CA) had an equal percentage.

Physical and chemical properties (Table 1) also influence soil biological properties such as microbial biomass and composition and enzyme activity, which are able to degrade aromatic structures such as laccases and manganese peroxidase (Haumaier, 2010). The optimum of these enzymes is in acid range which might explain the higher occurrence of black carbon metabolites in Chernozem, Ferralsol and Anthrosol compared to the Cambisol in our study. In addition, microbial degradation can be considered as another factor of black carbon metabolites loss. However, microorganisms are able to degrade black carbon metabolites only when they grow in their presence (Haumaier, 2010). In conclusion, these results show clearly that further analyses of additional soil samples of different regions are needed to study the fate of black carbon metabolites in soil. As this study shows especially, soil physical, chemical, and biological properties as well as climatic conditions have to be considered when interpreting black carbon metabolites.

4.3.6 Black carbon metabolites related to black carbon concentrations

It can be assumed that the amount of black carbon metabolites is a function of black carbon content of soils that means the higher the black carbon amount of a soil, the higher should also be the amount of black carbon metabolites found in this soil. In our case, the contribution of black carbon metabolites to total black carbon contents ranged from 3.3% of the Anthrosol and Chernozem to 37.8% of the Ferralsol (Figure 4). Therefore, there seems to be no direct link between black carbon concentration of a certain soil and its black carbon metabolite concentration. Given the wide range of physical and chemical properties of the soils in our study, it can be assumed that physical and/or chemical soil properties determine black carbon metabolism more than the occurrence of black carbon.

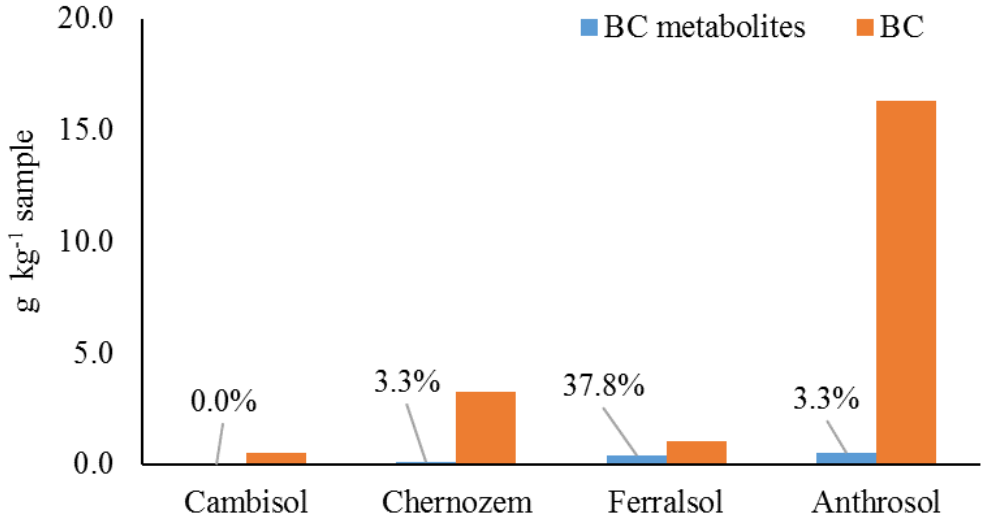

Figure 4. Comparison of black carbon metabolites and total black carbon in soils samples.

4.3.7 Conclusions

We optimized a method of soil black carbon metabolites extraction and measurement originally presented by Haumaier (2010) with respect to extraction time, sample clean-up and analysis. We showed that extraction duration can be reduced to 48 h and that after thorough salt removal by IEC, free BPCA can be separated by IEC and detected by absorbance at 254 nm without further sample preparation. Recovery of spiked BPCA was 81 ± 19% in soil samples, which covered a wide range of texture, pH, and TOC. Black carbon metabolites are ubiquitous in soil like black carbon itself. However, black carbon concentration in soil seems to be a less suitable predictor for black carbon degradation. Rather, physical, chemical and probably also biological soil properties could control black carbon metabolism in soil.

4.3.8 References

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