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Optimized treatment for the phytostabilization of an acid contaminated soil in La Unión-Cartagena (Spain)

Master's thesis

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Declaration

I hereby certify that work presented in this thesis and entitled as "Optimized treatment for the phytostabilization of an acid contaminated soil in La Unión-Cartagena (Spain)", is, to the best of my knowledge, original, and that the material has not been submitted, either in whole or in part, for a degree at this or any other university. The literature and other sources, which I used, are stated list of references, which are attached to this work.

Prague 17th April 2017   **Juan Carlos Galan Robles**

Acknowledgment

Abstract

Contamination due to trace pollutants compounds has become a serious problem in Cartagena, Spain, where mining activities have been carried out during centuries. The dispersion of these contaminants such as Iron, Lead, Cadmium, Nickel, Arsenic, Magnesium and Manganese, caused mainly by surface run-off, has an important environmental impact on the ecosystem and the high level of toxicity might be affecting nearby regions. Few studies proposing a suitable and realistic soil treatment have been done. In this context, to deal with this situation, phytoremediation with *Dittrichia viscosa* was proposed. Due to the toxicity of the soil, this natural site is almost bare of vegetation. Therefore, in order to promote plant growth, biochar and digestate were used as growth promoters and soil amendments. The aim of this work was to find the optimal doses of biochar and digestate to be mixed with the polluted soil in order to obtain the best performance at stabilizing trace compounds in the soil. The experiment compiled 9 sets of different combinations of the substrates following an optimised statistical design. In every set, plants were grown in polluted-mixed soil, harvested, and analysed to estimate the biomass content, enzymatic activity and the concentration of trace compounds by Inductively Coupled Plasma (ICP). Soil pore water was analysed as well. Results have shown that combining 85.4% v/v of digestate and 34.1 % v/v of biochar, it was obtained the best performance at reducing availability of trace elements (TEs) from soil pore water, it was found as well that digestate promotes the decrease of the concentration of TEs in soil pore water and also it affects the enzymatic activity by being a possible factor reducing stress in plants.

Keywords: Phytostabilization, *Dittrichia viscosa*, biochar, digestate, polluted soil.

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1.Introduction and Literature Review

Pollution due to trace elements (TEs) in abandoned mine areas is one of the most challenging environmental problems to be faced nowadays. Soil erosion facilitates the transport of toxic elements like Arsenic (As), Cadmium (Cd), Cupper (Cu), Manganese (Mn), Iron (Fe), Lead (Pb) and Zinc (Zn), which represents an important source of pollution for the surrounding areas (Conesa and Schulin, 2010; García-Lorenzo et al., 2012) with the consequent risk for human health and for the entire ecosystem (Álvarez-Rogel et al., 2004; Navarro et al., 2006). The restoration of these soils is therefore needed to reduce the dissemination of the contaminants.

When this pollution is located on lands used for agricultural or urban development, this subject becomes a serious problem. Some of the consequences originated by abandoned tailings involve the presence of unfavourable conditions for natural vegetation such as low pH (Wang et al.,1998) toxic metal concentrations, low water retention capacity (Norland and Veith, 1995) and low levels of plants nutrients. Moreover, in some of the cases, the tailings are present on steep slopes, which are unstable and prone to erosion. In this framework, decontamination of polluted soil is necessary. Technics in current are based on either extraction of metals, soil washing, thermal desorption among others; however, all these treatments require specific equipment and operators, being, therefore, costly and difficult to apply in large areas (Del Río-Celestino et al., 2006).

Biological treatments seem to be a viable option reducing operation costs and increasing the efficiency. The benefits of the establishment of a vegetation cover on the soil rely on the capacity of plants to reduce contaminant leaching and to limit the dispersion of the pollutants, as plants reduce water and wind erosion of the soils (Martínez-Sánchez, García-Lorenzo et al., 2012). In this way, phytostabilization can be considered as one of the most suitable ways to ameliorate toxicity in this area (Clemente et al., 2012; Pardo et al., 2014) as it is a non-invasive and low-cost environmentally friendly remediation technique. This technology uses plants and soil amendments to remove and/or

decrease TE bioavailability in soil through their immobilisation in soil or accumulation/adsorption in roots or aerial tissues (Kidd et al., 2007).

This work is focused on the remediation of a polluted soil containing TEs, mainly Pb, Cd, Cu, Ni, Zn and As, which is located in La Cartagena-La Unión district, a former mining area located in semiarid south-east Spain and with more than 200 ha of tailings. Since the use of native species for phytostabilization of TE contaminated soils has shown to be a good option as they are well adapted to the local environmental conditions and to the particular characteristics of the soils to be remediated (Pardo et al., 2014), phytostabilization with *Dittrichia viscosa wa*s proposed as a remediation technic in this work.

The objective of this work is to study the usefulness of different combinations amendments for the restoration of a TEs-contaminated soil of the La Unión-Cartagena mining district. The effectiveness of two different organic amendments, a biochar and a digestate obtained from sewage treatment plant, with regard to soil TEs solubility and the TEs accumulation and growth of a native plant species, *D. viscosa,* was evaluated in a pot experiment.

1.1 Contaminated soils by trace elements (TEs)

TEs are natural metallic elements having a high density, frequently found as trace elements in the environment; Pb, Cd, Cu, As, Ni, Zn, Co, Mn, mercury (Hg) and cobalt (Co) are some of them. However, in high concentrations, they may become toxic affecting the ecosystems and their organisms; the toxicity risks due to the pollutant transfer into the food chain, have become a big concern. Metalliferous mining and processing, including the dumping of wastes, is the principal sources of TEs pollution.

In Europe, there are many examples of TEs pollution due to mining activities; in England and Wales, for instance, the estimated area affected by historical mining, smelting and

heavy metals is approximately 4000 km^2 (Thornton, 1980). Another case is found in Greece, the Lavrion urban area is greatly affected by metal contamination from nearby mining activity, with drastic effects on the human population (Kontopulos et al., 1995). Several abandoned mining areas can be found as well in Italy, especially in Sardinia which underwent intense and long-lasting mining activity. The Montevecchio-Ingurtosu mining area in southern Sardinia is characterized by Pb and Zn ores, which were exploited from Roman times up to 1991 and that, may represent an important environmental risk. In Czech Republic, mining activities in Kutna Hora and Příbram during centuries remains a contamination source in the present day (Horak and Hejcman, 2016). Mining activities carried out in southeast Spain have generated high amounts of wastes; these lands are characterized by acidification processes and accumulation of TEs (Romero et al., 2006).

Many soil decontamination technics can be found and applied in order to remediate a polluted soil; however, some factors such as soils properties, contamination parameters or size of the land need to be considered to select an adequate remediation treatment. Soil washing, bioremediation and thermal treatment are some examples of soil decontamination process (Meuser, 2012).

1.2 The contaminated soil from La Unión (Murcia, Spain)

The Cartagena-La Unión Mining District (110 – 0 m, a.s.l.; 37°37′20″N, 0°50′55″ W – 37°40′03″ N, 0°48′12″ W), which covers an area of 50 km^2, is located on the southeast of the Iberian Peninsula. The Sierra of Cartagena-La Unión, also called Sierra Minera, crosses the Mining District from west to east. The semiarid climate of the zone is typically Mediterranean with an annual rainfall around 250–300 mm and concentrated during spring and autumn (Conesa et al., 2011).

The annual average temperature is 18 °C. This zone constituted an important mining nucleus for more than 2500 years, ceasing activity in 1991. The ore deposits of this zone have Fe, Pb and Zn as main metal components. The northern catchment of the Sierra Minera drains towards the Mar Menor lagoon (Figure 1) which is one of the biggest (135

km^2) hypersaline coastal lagoons in the Mediterranean Sea. The lagoon and its coastal salt marshes cover an area of around 15,000 ha. Environmental consequences of mining waste discharge into the lagoon were already reviewed by (Conesa and Jimenez, 2007).
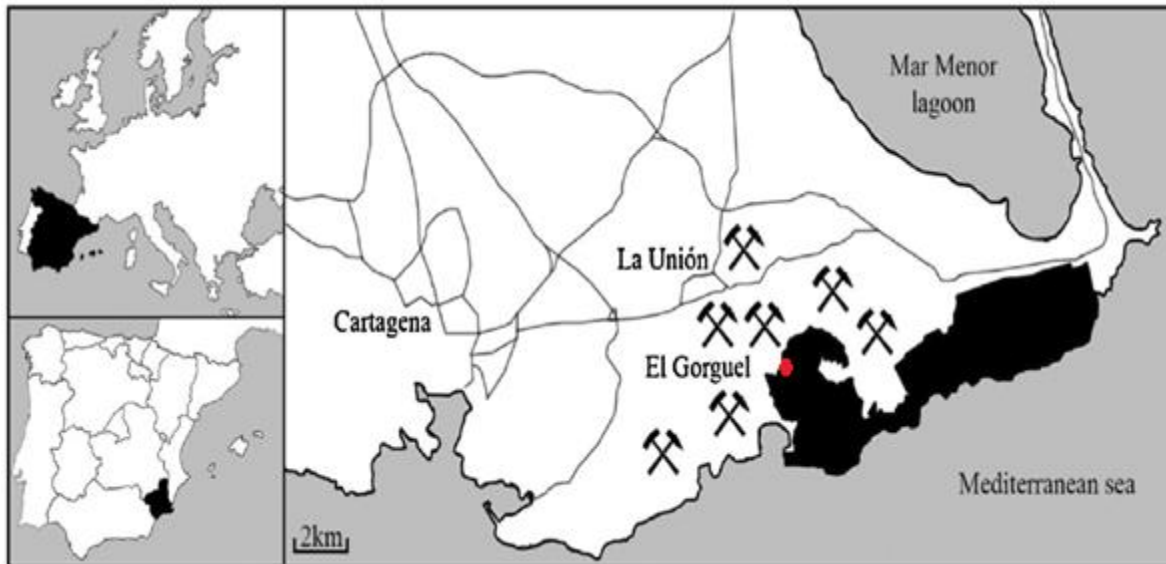
Figure 1: Localisation of the former mining area in Cartagena (Spain) and the affected soils (Martínez-Fernández et al., 2014).

According to Oen (1975) and Ovejero et al. (1976), the Sierra de Cartagena – La Unión or Sierra Minera is one of the largest Zn and Pb ore deposit in the south Europe. One third of the total reserves were extracted in just fifty years (from 1940 to 1990), applying open cast mining and differential flotation processes (Manteca-Martinez et al., 1992). As a result of this massive extraction, a high amount of mining waste was produced in mine tailings. They were spread over the Sierra Minera, constituting a great environmental problem in the immediate mining area and also in the surrounding areas which include the Portman Bay which according to Martinez-Frias (1997) is the most contaminated and a perfect example of ecotoxic pollution of coastal environment by mine tailings (figure 2).

Figure 2: Metal contamination in Portman Bay nowadays.

Several amount of studies have reported that the ecotoxicological risks of mining tailings in "Sierra Minera" are caused mainly by water and wind erosion which promote the transportation of TEs affecting groundwater, soils and natural ecosystems; this fact represent as well a high risk for human health (Navarro et al., 2006; Conesa et al., 2007; Conesa et al., 2011; Gonzáles-Fernández et al., 2011; García-Lorenzoet al., 2012).

According to Gomez-Ros et al. (2013) the removal of mining wastes for disposal in special sites is unrealistic due to financial considerations; phytoremediation is reported as the best method to decontaminate the area. Several studies have been carried out at Sierra Minera, and they show that TEs can be immobilized or stabilized using chemical or phytostabilization technics (Conesa et al., 2006; Navarro et al., 2008; Zanuzzi et al., 2009).

In the region of Murcia (SE Spain), the area of land contaminated with TEs predominantly Cd, Cu, Pb and Zn, by mining activity in the Sierra Minera of La Union-Cartagena (figure 3), and the subsequent dispersal of the contaminants, exceeds 40 km^2 (Gonzáles-Fernández et al., 2011). The establishment of a vegetation cover at the contaminated sites would physically stabilise the soils (phytostabilization) and minimise the erosion and thus the dissemination of the contaminants to neighbouring areas or groundwater (Méndez and Maier, 2008).

Figure 3: Affected area in Sierra Minera (Martínez-Sánchez et al., 2012).

1.3 Phytoremediation

Soil remediation techniques comprise in situ (non excavated soil) and *ex situ* techniques (soils excavated). *Ex situ* remediation requires the soil to be transported to a treatment facility (for example soil washing). The *in situ* method, on the other hand, is a technology which tries to remove or immobilize the metal(loid)s from contaminated soil without performing excavation and transport of contaminants. Experts tend to favour *in situ* techniques, which imply soil disposal as close to the source of contamination as possible. Compared to conventional strategies (*in situ* vitrification, soil incineration,

excavation and landfill, soil washing, soil flushing and solidification), phytoremediation is an aesthetically pleasing, efficient and ecofriendly process in removing contaminants from low to moderate levels. It is also a low cost method which requires less than half of the price of the conventional methods. It also provides an added advantage by not only cleaning polluted soil but by also preventing soil erosion and metal leaching. This method causes less ecological disturbance and its economically aspect makes it a better alternative than *ex situ* technology. This method is further divided into different categories to remove toxic metals from soil and water: phytoextraction, phytostabilization, phytovolatilization, phytodegradation, and rhizofiltration (Brennam and Shelley, 1999)

Phytoremediation is defined as the combined use of plants, soil amendments and agronomic practices to remove, retain environmental pollutants, or to reduce their toxicity (Salt et al., 1995). The term phytoremediation refers to the employment of plants to clean up soils, subsoils or superficial water. In this technique plants are used to reduce the risk of pollution and to avoid transfer of contaminants. Plants may be able to degrade, stabilize, volatilize or uptake numerous types of pollutants such as metals, pesticides or hydrocarbures. Plants also allow preventing soil erosion occasioned by wind, rain and subterranean streams.

The mechanisms and efficiency of phytoremediation depend on the type of contaminant, bioavailability and soil properties (Cunningham and Ow, 1996). Phytostabilization and phytoextraction are two common phytoremediation techniques for treating metal-contaminated soils, (Susarla et al., 2002; Wong, 2003).

1.3.1 Phytoextraction

It is also called phytoaccumulation, it refers to the uptake and translocation of metal contaminants in the soil by plant roots into the above ground portions of the plants (shoots, leaves). The figure 4 shows the principle of phytoextraction. Metals such as Ni,

Zn and Cu are the best candidates for removal by phytoextraction because it has been shown that they are preferred by a majority of plants that uptake and absorb unusually large amounts of metals.

There are several advantages of phytoextraction. Its cost is fairly inexpensive compared to conventional methods. Another benefit is that the contaminant is permanently removed from the soil.
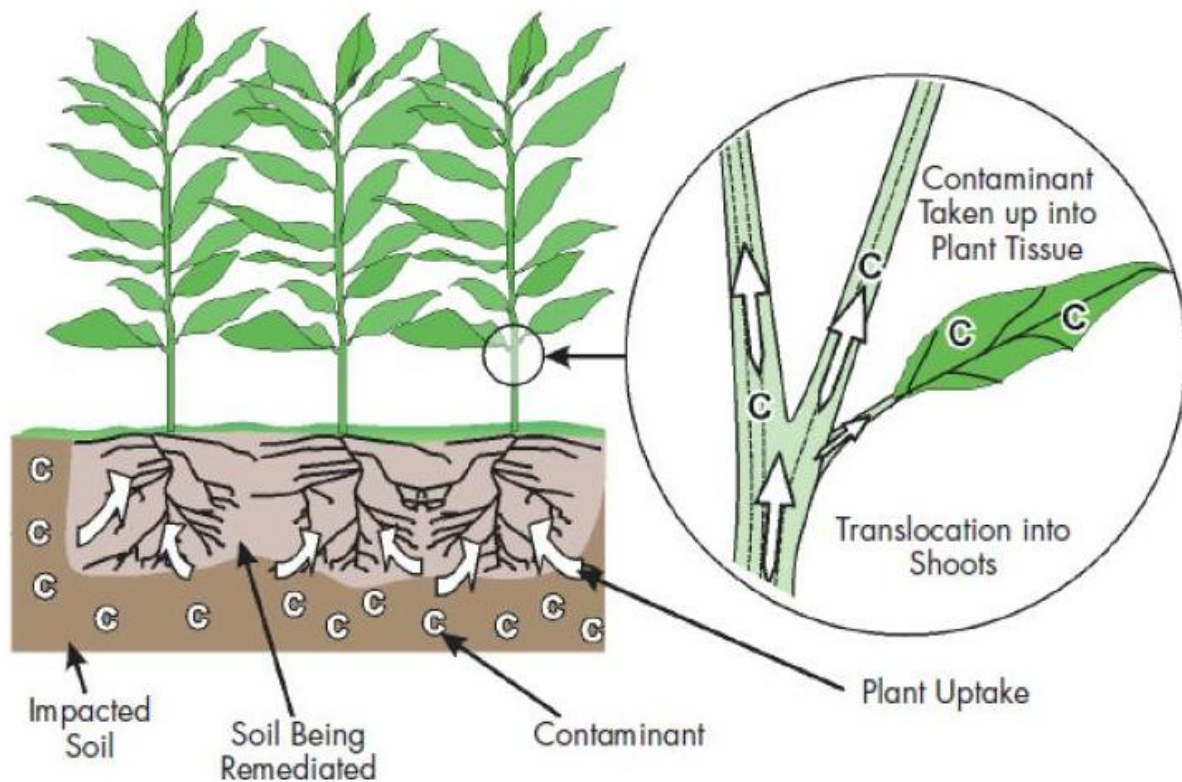
Figure 4: Principle of phytoextraction (BiologyOnline, 2017).

1.3.2 Phytostabilization

This is also referred to as in-place inactivation. It is primarily used for the remediation of soil, sediment, and sludge (USEPA, 2000). It is the use of certain plant species that are able to immobilize contaminants in the soil or ground water through absorption and accumulation by roots, adsorption onto roots or precipitation within the root zone of

plants (rhizosphere; Raskin and Ensley, 2000), the principle of phytostabilization is shown in the figure 5. This process reduces the mobility of the contaminant and prevents migration to the groundwater and it reduces the bioavailability of metal into the food chain. This technique can also be used to reestablish vegetation where natural vegetation cannot survive due to high metals concentrations in surface soils or physical disturbances to surface materials.
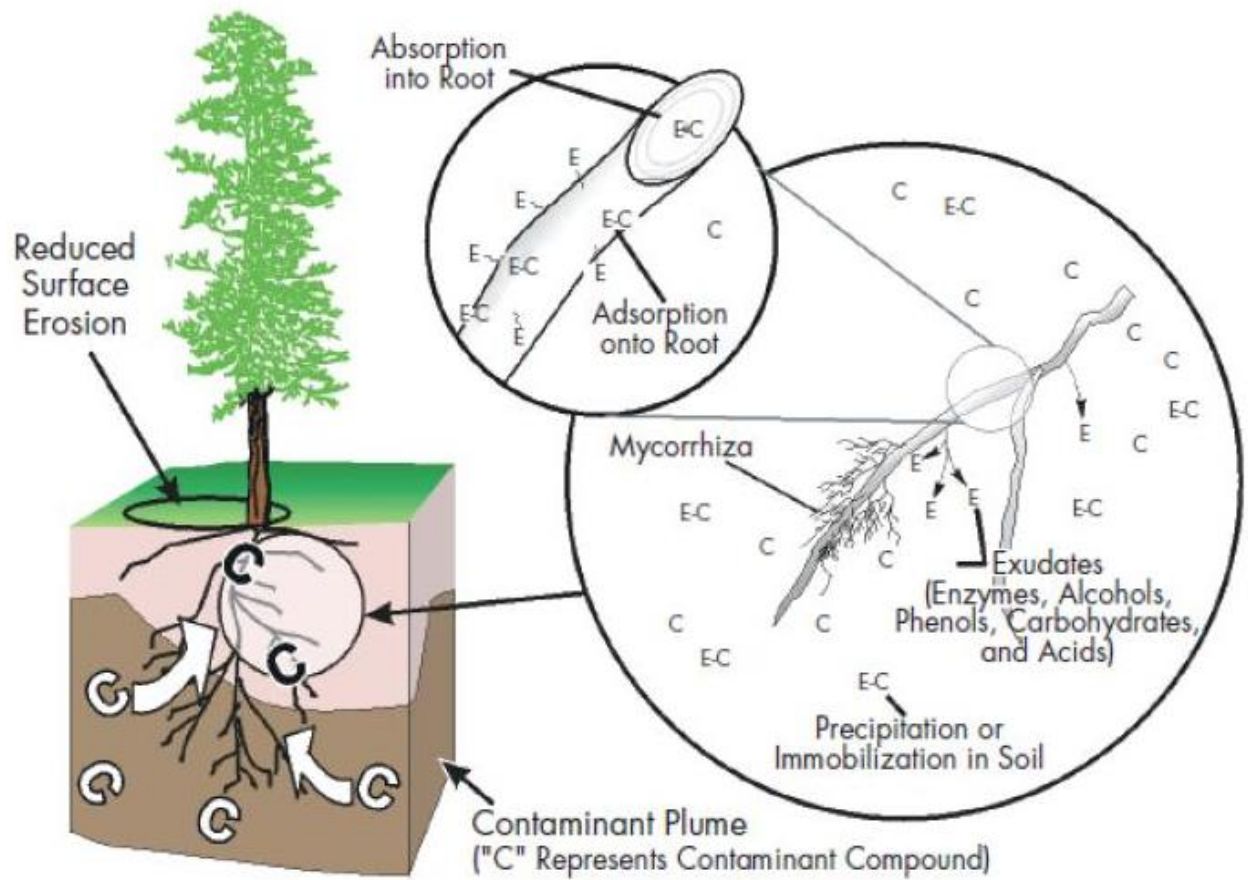
Figure 5: Phytostabilization principle (BiologyOnline, 2017).

Metal-tolerant species are used to restore vegetation at contaminated sites, thereby decreasing the potential migration of pollutants through wind erosion, the transport of exposed surface soils and the leaching of soil contamination to ground water.

Phytostabilization takes advantage of the changes that the presence of the plant induces in soil chemistry and environment. These changes in soil chemistry may induce adsorption of contaminants (Etim, 2012) onto the plant roots or soil, or they might promote metal precipitation onto the plant root.

Some of the advantages associated with this technology are that the disposal of hazardous material is not required and it is very effective when rapid immobilization is needed to preserve ground and surface waters (Zhang et al., 2009). However, this clean-up technology has several major disadvantages including contaminant remaining in the soil, application of extensive fertilization or soil amendments, mandatory monitoring is required, and the stabilization of the contaminants may be primarily due to the soil amendments.

1.4 Soil amendments

Soil organic amendments are often necessary for the improvement of soil properties before the establishment of the vegetation (Tordoff et al., 2000; Clemente et al., 2012). These materials not only provide essential nutrients and organic matter and improve soil structure, but also modify the solubility and phytotoxicity of TEs (Bernal et al., 2007).

1.4.1 Biochar as soil amendment

Biochar is a new term which refers to a product whose fine and porous structure is similar to all appearances to the charcoal produced from the transformation of different types of biomass by pyrolysis, which consists on the thermal decomposition of the organic matter under limited oxygen. Some international organizations like the International Biochar Initiative (IBI) and Lehmann (2009) claim that biochar refers particularity to the pyrolysed material when its destination is no other that as an organic amendment of soils, discarding the use of biochar as a fuel. In this way, its biological origin is outlined and differs from the traditional charcoal destined to fuel.

Numerous publications about possible effects of the application of biochar on the edaphic ecosystem can be found in the literature. Nevertheless, the level of knowledge is still quite recent and the results often vary and depend on the type of study (Sohi et al., 2009).

Some of the effects of using biochar as an amendment of soil are that the carbon can be storage for hundreds of years due to its stability, increasing then, the plant growth and the carbon sequestration in the soil (Lehmann, 2006).

The stability of biochar depends on the proportions of its compounds; the composition is determined by the pyrolysis conditions that are used to produce biochar and by the characteristics of the material used for the production of biochar.

Moreover, some studies have shown that the availability of nutrients for the plants increases due to the cationic exchange capacity that biochar gives to the soil; the biological process is stimulated as well and soil structure and water storage capacity are improved (Glaser et al., 2000). Also, these studies point out the capacity of this material to reduce the leaching and the surface runoff, to increase the pH and to absorb the pesticides and heavy metals (IBI, 2017).

Furthermore, irrigation costs could be reduced due to the increase in the water capacity retention, in this sense (Glaser et al., 2002) found in soils in Terra Preta, a water retention capacity greater in 18% in comparison to neighbouring sites.

According to Sohi et al., (2009), biochar increases the productivity and quality mainly in acid and low nutrients content soils. In addition to this, Lehmann (2006) found out an increment in the plant uptake of P, K, Ca, Zn and Cu. A higher microbial activity has been observed in soils enriched with biochar (Steiner et al., 2008); this, due to the microporous structure.

Nowadays, biochar commercialisation is growing due to its feasible applications. The figure 6 shows a commercial biochar.

Figure 6: Commercial biochar.

1.4.2 Digestate as soil amendment

Digestate is defined as the digested effluent of the biogas production process after extraction of biogas through anaerobic digestion. Its quality and its composition depend on the characteristics of the livestock used. Digestate is a valuable crop fertilizer because it represents an important source of organic carbon and also because of its high content of macro and micronutrients, recycling the digestate as a fertilizer is the most sustainable utilization of it, however in order to be used as a fertilizer, it is desirable that it is free of pathogens or any chemical or physical impurities and pollutants; this can be achieved by controlling the feedstock that is used in the anaerobic digestion or by post treating the digestate (Wellinger et al., 2013).

When compared to untreated waste, digestate presents several advantages such as greater microbial stability and hygiene and a higher amount of nitrogen (N) present as ammonium; land spreading of digestate can, then, lead to benefits if integrated into good agricultural practices, by controlling the nitrogen (N) application rate and heavy metal load, and by securing digestate hygiene (Holm-Nielsen et al., 2009).

A positive effect of digestate in agriculture (mainly for cereal production) has been confirmed by Northern European countries such as Denmark, Sweden, Scotland and Germany (Moller and Stinner, 2009); however, for this work, the digestate was used as a complement of a phytoremediation treatment.

1.5 Selection of suitable plant species

The selection of phytoremediation techniques depends on site, size, location, history, soil characteristics, type and physical state of contaminants, degree of pollution, desired final land use, technical and financial means available, and environmental, legal, and social issues (Vangronsveld and Cunningham, 1998).

Selection of the suitable plant species is crucial for the success of any phytoremediation project. Plants having the ability to fix nitrogen, being tolerant to TEs, having low nutrient needs, and being able to grow fast, are recommended to be used in phytoremediation.

In principle, the best plants for the purpose are those that can absorb high amounts of the contaminants and have economic value. Species that are able to accumulate large amounts of TEs that are toxic for other species are known as hyperaccumulators and their use in phytoextraction is considerable interesting (Rai, 2008).

Hyperaccumulation of TEs ions is a phenomenon exhibited by less than 0.2% of angiosperms (Baker et al., 2000). These hyperaccumulator plants are considered endemic to metalliferous substrates, and are characterized by their tolerance and sequestration of large quantities of TEs in their shoots or leaves at concentrations that would be toxic to normal plants.

Fast growth is ideal and part of the reason for selection of early succession species which typically exhibit such a pattern. Plants that exhibit tolerance for metal contaminated soils may then be used for soil stabilization instead phytoextraction (Schat and Verkleij, 1998).

Plant-based strategies for the large scale remediation of abandoned mining areas require prolonged periods of time and the use of plant species tolerant to soil pollution and to the environmental conditions of the area to be restored. Consequently, the plants used may remain in the soil for enough time to show their ability for expansion, reproduction, competition with nearby plants and colonisation of the surrounding areas. This is of vital importance, especially for soils close to areas of faunistic and floristic interest, where the use of exogenous species may alter the natural habitat. This is the case of the mining area of La Unión-Cartagena, which surrounds the Natural Park of "Calblanque, Monte de las Cenizas y Peña del Águila" that has one of the most important heritages of the Iberian Peninsula in terms of plant and animal species (Dirección General de Patrimonio Natural y Biodiversidad, 2008). The use of native species for the restoration of this type of soils is of special interest in order to maintain the ecosystem equilibrium. Additionally, native plants are adapted to specific climate and soil conditions, providing the basis for natural ecological succession (Méndez et al., 2007) with a greater chance of survival, growth and reproduction in environmental stress conditions than plants introduced from other environments (Adriano, 2001).

1.5.1 *Dittrichia viscosa* L. in contaminated soils

Dittrichia viscosa (figure 7) is a common weed 1 – 1.2m tall (*Asteraceae* family), colonizing abandoned fields and roads thanks to its wind spreadable seed, and easily regrows after mowing. Moreover this specie is appreciated by pollinators for its flowering and abundant production of pollen, while it is refused by herbivores because its resinous content. It is resistant and perennial (Barbafieri et al., 2011).

Figure 7: *Dittrichia viscosa* (Alberti, 2017)

Many plants species have been studied to restore polluted soils due to mining tailings. However, *D. viscosa* has been reported as a good candidate for applying phytoremediation in semiarid soils (Barbafieri et al., 2011; Martínez-Sánchez et al., 2011). Some characteristics of *D. viscosa* such as high tolerance and metal accumulation have been confirmed by (Fernández et al., 2013).

According to Adriano (2001), the best plants for phytoremediation purposes are native plants because of their greater chance of survival, growth and reproduction in environmental stress conditions than plants introduced from other environments. *D. viscosa* is abundant in the Sierra Minera, which gives it the characteristic of native plant (Martínez-Sánchez et al., 2011).

Dittrichia viscosa is usually grown in semiarid area because its low nutritional needs (Martínez-Sánchez et al., 2012); it is also able to grow in wet and saline areas.

Some reports have shown that this specie has grown under high Cd pollution in Asturias (Spain) and has been found in many other metal polluted sites of the Mediterranean area, where it accumulates As, Cd, Pb and Zn (Fernández et al., 2011).

Dittrichia viscosa has been described in other similar studies as a widespread species; it was defined as a good biomonitor for metal pollution, especially for Pb and Zn (Swaileh et al., 2004). It has been reported that in mining areas from Spain it accumulated an enormous amount of Pb (Melendo et al., 2002). This plant has also been commonly found in soil with high As content, showing an interesting As accumulation in shoots (Gisbert et al., 2008). The same has been observed with Zn, Pb and Cu.

Dittrichia viscosa has shown to be suitable for phytostabilization and revegetation, Pérez-Sirvent et al. (2012) and Martínez-Fernández et al. (2014) for instance, claim that the plant transfers the antimony (Sb) and the As to the roots but not to the aerial parts.

For the characteristic mentioned above, *Dittrichia viscosa* has proved to be a good candidate for phytoremediation procedure in la Sierra Minera.

Classification of *Dittrichia viscosa* (USDA, 2017):

- Kingdom: Plants

- Subkindom: Tracheobionta (Vascular plants)

- Division: Spermatophyta (Seed plants)

- Class: Magnoliopsida (Dicotyledons)

- Subclass: Asteridae

- Order: Asterales

- Family: Asteraceae (Aster Family)

- Genus: *Dittrichia Greuter (Dittrichia)*

- Species: *Dittrichia viscosa (L.) Greuter* (False yellow head)

1.6 Bioavailability and soil pore water

There are several definitions of bioavailability, they depend on the context, but in a general idea bioavailability can be defined as the representation of the fraction of a chemical accessible to an organism for absorption, it can defined as well as the rate at which a substance is absorbed into a living system, or as a measure of the potential to cause a toxic effect (National Academies Press, 2003).

Bioavailability processes are important in order to evaluate exposures of ecological receptors to persistent compounds. When referring to bioavailability in soil remediation, from some studies (Tye, et al., 2002; Weng et al., 2002), it has emerged that total soil metal content alone is not a good indicator of bioavailability; therefore, it is not a very useful tool to determine potential risks from soil contamination (Tack and Verloo, 1995). In fact, since plants uptake most of the nutrients from the soil pore water; it is often assumed that the dissolved potentially toxic elements are readily bioavailable to organisms (Vig et al., 2003). It is clear that the concentration and speciation of metals in the pore water might provide more useful information on metal bioavailability and toxicity than total soil concentration (Percival, 2003; Prokop et al., 2003).

As it has already said, to assess the environmental bioavailability, mobility and geochemical cycling of TEs in soil, analyses of soil pore water composition are frequently more useful than those from soil extracts. Soil pore water analysis can be carried out to model the nature, direction, extent, and rate of chemical reactions. The figure 8 represents the soil pore water in a soil sample.
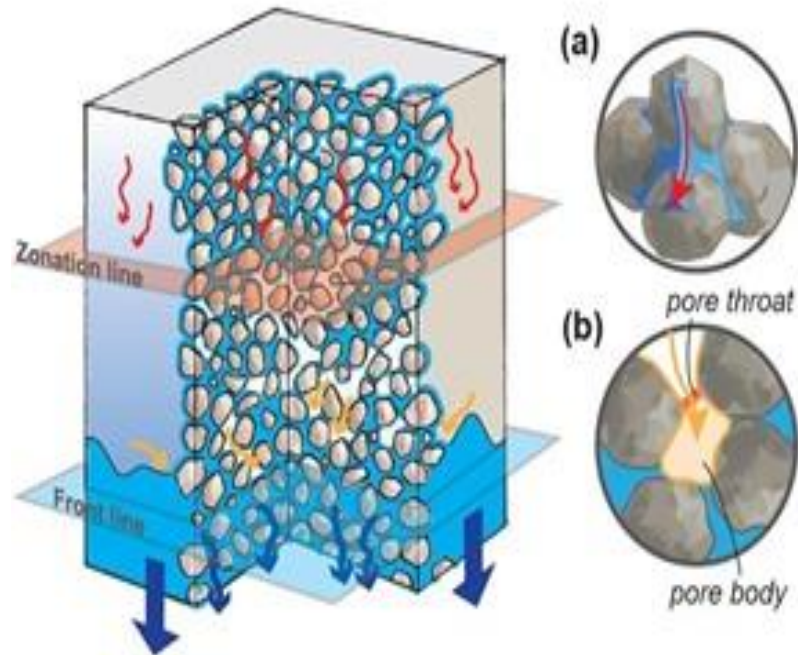
Figure 8: Conceptual image of flow transitions and soil pore water drainage mechanisms behind a drainage front invading a porous medium.

Many sampling methods can be used to obtain and analysed soil pore water without altering it, one of the most used one is called Rhizon Soil Moisture Sampling. Rhizon samplers are devices that can be used in the laboratory or in the field (Cabrera, 1998); a syringe, a vacuum tube or a pump are needed to apply suction to withdraw pore water; it is one of the latest developments in terms of tension samplers.

Using this device, a soil pore water sample is obtained by inserting the sampler into a wet soil, and applying suction. For the soil pore water sampling, the procedure described by Tye et al. (2003) is normally followed.

1.7 Enzymatic activity as an indicator of plant stress

Based on the literature, it is known that some TEs are involved in key metabolic events such as respiration, photosynthesis, and fixation and assimilation of some major elements like nitrogen and sulfur. Considerable evidence was given by Kabata-Pendias and Pendias (2001), pointing that tolerant plants may also be stimulated in their growth by important amounts of metal, which reveals a physiological need for an excess of a particular metal by a single plant genotype or species. The great flexibility of enzymatic systems and variability among plant species are involved in the evolution of metal tolerance of which several points are still not clear.

However, some other studies (Soudek et al, 2014) reveal that TEs can cause a blockage of essential functional groups in biomolecules, inducing oxidative stress. Moreover, the presences of metals, provokes the disruption of the electron transport chains or the induction of lipid peroxidation (Fernández et al., 2013).

In order to detoxify the excess of reactive oxygen, plants are able to produce antioxidant enzymatic systems involving superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GOPX) and glutathione reductase (GR) (Smeets et al., 2008).

The SOD is the first line of defence against reactive oxygen species (ROS), and it is essential for the removal of oxygen radicals (Takahashi and Asada, 1983). The decomposition of O_2 is always followed by H_2O_2 production, which rapidly diffuses across the membrane being toxic as it acts as an oxidant and as a reductant at the same time (Foyer et al., 1997). H_2O_2 can be scavenged in the cell either by CAT or APX.

CAT is a universally present oxidoreductase that decomposes H_2O_2 to water and O_2. It is a crucial enzyme for the removal of toxic peroxides. H_2O_2 can also be reduced to water and oxygen by APX in the ascorbate-glutathione cycle, which plays an important role in detoxification of ROS through successive oxidation and reduction reactions involving ascorbate and glutathione (Kuzniak and Ekladowska, 1999). In this cycle, APX plays an important role in removing H_2O_2 and GR can provide a substrate for APX through a catalysing reaction (Shi et al., 2005).

Trace metals of the transition metal group are known to activate enzymes or to be incorporated into metalloenzymes as electron transfer systems (Cu, Fe, Mn, and Zn) and also to catalyse valence changes in the substrate (Cu, Co, Fe, and Mo). Some TEs (Al, Cu, Co, Mo, Mn, and Zn) seem to be involved in protection mechanisms of plant varieties (Marutian, 1972); this has been confirmed with studies showing a positive correlation between the activity of some enzymes and the levels of TEs in plant tissues.

Enzymatic activity is then an indicator of plant stress due to the action of TEs.

2. Objectives

General objective:

The aim of this work was to identify the optimal doses of biochar and digestate to be combined with the extremely polluted soil in order to obtain the most useful combination for the stabilization of the TEs in polluted soils.

Particular objectives:

- To find out the influence of Biochar and Digestate on the concentrations of pollutants in soil pore water and in the plant.
- To find out the influence of Biochar and Digestate on the enzymatic activity in the plant.
- To make mathematical models to calculate the concentration of pollutants and pH in soil pore water according to the doses of biochar and digestate used.

3. Materials and methods

In this chapter, the materials required to develop the project were characterised: soil, Biochar and Digestate. Then, the experimental design was explained, and finally the analytical methods used to treat the data are pointed out.

3.1 Soil characterisation

The selected soil was collected from an abandoned mining-pond, with no edaphic development, situated in El Descargador 37°37'03.9" N, 0°51' 49.9" W (red dot in figure 1; semi-arid climate), within the former mining area of La Unión-Cartagena (Murcia, Spain). The soil was air-dried, sieved through a 2-mm sieve and homogenised for its characterisation (Table 1). The area from where the soil was collected is almost bare of vegetation, with high TEs concentrations and low nutrient levels in the soils.

Table 1: Soil characterisation

TEs	Concentration (mg kg^{-1})
Al	841
As	0.29
Ba	0.394
Ca	15213.2
Cd	15.94
Co	1.11
Cu	31.89
Fe	23.27
K	3.64
Mg	3704.83
Mn	809.69
Na	43.25
Ni	2.91
Pb	108.48
S	19590.7
Se	0.765
Si	93.91
Sr	13.38
Zn	3537.5

3.2 Biochar characterization

Previous to the elaboration of the biochar, the material was homogenised, air dried and analysed by Trakal et al. (2014) to determine the bulk density, moisture, ash content and material composition. After that, the material was pyrolysed in a muffle at 600°C under 16.7 mL min^{-1} nitrogen flow at a retention time of 30 min. The biochar was analysed to determine the yield of biochar from waste material, specific surface, volume of micropores, pH and composition the characterisation of this material is given by Trakal et al. (2014) and it is shown in the table 2. The biochar was made from grape stalks (by-product originated from the wine production).

Table 2: Biochar characterization

Variable		Value
Yield (%)		30.6
Bulk density (g cm^{-3})		0.16
Specific surface (m^2 g^{-1})		72
Volume of micropores (mm^3 g^{-1})		30
pH		10
Element composition w (%)	Ash content in dry sample	16.1
	Carbon	70.2
	Oxygen	12.5
	Hydrogen	1.70
	Nitrogen	1.45

3.3 Digestate characterization

For this experiment digestate from a Biogas station (BPS Žitín) was used, characterisation is given in table 3.

Table 3: Digestate characterization

Variable		Value
Content of NH_4^+ and N	NH_4^+ (mgkg^{-1})	3758
	Total N (%)	0.91
Dry matter (%)		37.7
pH		9.19
EC (mScm-1)		2.965
Macronutrients (mg kg^{-1})	P	8526
	K	25321
	Ca	18075
	Mg	4215
	S	3654
Micronutrients (mg kg^{-1})	Fe	1753
	Mn	234
	B	12.6
	Mo	1.13
TEs (mg kg^{-1})	Zn	133
	Pb	3.53
	As	2.69
	Cd	0.17
	Cr	5.04
	Cu	31.1

3.4 Experimental design: Central Composite Design

A central composite design (CCD) is the most commonly used response surface designed experiment for building a second order model for the response variable without needing to use a complete three-level factorial experiment. CCD is a factorial or fractional factorial design with center points, augmented with a group of axial points that

let us estimate curvature. CCD models a response variable with curvature by adding center and axial points to a previously-done factorial design (Minitab, 2017).

In our experiment, an incubation experiment was carried out in 30 pots of 1 l volume following a CCD in a glasshouse. For this experiment, a CCD of 13 runs (sets 9 to 13 having the same concentration) in triplicate, was designed using MINITAB software version 16.1.1., for dose of digestate and biochar. The software helped to optimize the design of the combinations of both independent variables (digestate from 0 to 100% v/v and biochar from 0 to 40% v/v) in order to detect a significant response in the dependent variables (concentrations of nutrients in the plant tissues and soil pore water). The 13 combinations are shown in the table 4.

Table 4: Central Composite Design with 13 runs

Run	(% v/v)	
	Digestate	Biochar
1	14.6	5.9
2	85.4	5.9
3	14.6	34.1
4	85.4	34.1
5	0	20
6	100	20
7	50	0
8	50	40
9	50	20
10	50	20
11	50	20
12	50	20
13	50	20

In the figure 9 it is observed the 30 pots already containing the mixture of soil, biochar and digestate according to the experimental design (Central Composite Design). The rhizon-samplers (for soil pore water sampling) were allocated before transplanting the plants into the pots.

Figure 9: Preparation of the pots according to the CCD

3.5 Plant growth

Dittrichia viscosa (L.) Greuter (syn. *Inula viscosa* (L.) (Asteraceae) was selected due to its capacity to grow in soil polluted by mining activities (Martínez-Fernández et al., 2014). Firstly the plants were grown on perlite substrate (figure 10) in order to assure suitable conditions for growth; once reaching the sufficient size, they were transplanted (figure 9) into the pots containing the mixture of soil, digestate and biochar specified by the CCD. Six individuals of *Dittrichia viscosa* were allocated in each pot (figure 11, 12 and 13). Plants need to be harvested to the first sign of toxicity.

Figure 10: Plants grown in perlite substrate

Figure 11: Plants being transplanted

Figure 12: Plants transplanted into the pots

Figure 13: Plant sample after transplanting

3.6 Soil pore water analysis

Soil pore water sampling was done when plants were harvested. The soil pore water samplings was made using rhizon-samplers (Rhizosphere Research Products, The Netherlands), and it was analysed using inductively coupled plasma-optical emission spectroscopy (ICP-OES) in order to estimate the concentration of TEs in soil pore water. pH was also analysed by a pH meter.

3.7 Plant analysis

Biomass:

The plants (shoot and roots separately) were weighed just after harvesting (fresh weight), and then they were lyophilised to constant weight to conserve the organic components. After lyophilisation they were weighed again for dry weight determination.

Trace elements content:

The nutrients and total TEs were extracted from the freeze-dried plant material by acid digestion (HNO_3/H_2O_2) at 210°C and determined by inductively-coupled plasma-optical emission spectrometry (ICP-OES). For each set of samples, a reference plant material was also digested.

Enzymatic activity:

Plant tissues were homogenised with mortar and pestle in cold 0.1 M Tris/HCl buffer (supplemented by 5mM EDTA. 1% PVP K30. 5mM DTE. 1% Nonidet P40) at pH 7.8 (10 mL of extraction buffer per 1 g FW). The homogenate was centrifuged at 20 000 rpm at 4 °C for 30 min. Sample was filtered with Miracloth Filter and supernatant volume was measured. For the first precipitation, ammonium sulphate (40%) was added and solution was agitated for 30 min. The suspension was centrifuged at 20 000 rpm at 4 °C for 30

min again. Sample was filtered through Miracloth Filter (Calbiochem) and supernatant volume was again measured. For the second precipitation, ammonium sulphate (80%) was added and solution was agitated for 30 min. The suspension was then centrifuged at 20 000 rpm at 4 °C for 30 min. The pellet was resuspended with 2.5 mL 25 mMTris/HCl buffer (pH 7.8)filled into the columns PD 10.eluted with 3.5 mL of 25 mMTris/HCl buffer (pH 7.8) and freezed and stored at -80 °C.

Antioxidative enzyme assays:

All enzyme assays were performed in a microplate reader TECAN Infinite N200.

- Peroxidase (POX) activity was detected on the base of colour reaction with guaiacol. The reaction mixture used contained guaiacol (3.4 mM. 0.6 mL). Tris/HCl buffer (50 mM. pH = 6.0. 27 mL) and H_2O_2 (9 mM. 0.6 mL). The supernatant (0.01 mL) was added to 0.19 mL of reaction mixture into a whole in a microplate and POX activity was measured at 436 nm (ε = 26.6 mM^{-1} cm^{-1}) (Drotar et al., 1985).

- Glutathion-S-transferase (GST) activity was detected on the base of reaction with CDNB. The reaction mixture used contained CDNB (0.06 mM. 1 mL).Tris/HCl buffer (100 mM. pH = 6.4. 23.7 mL) and GSH (0.12 mM. 0.5 mL). The supernatant (0.04 mL) was added to 0.15 mL of reaction mixture into a whole in a microplate and GST activity was measured at 340 nm (ε = 9.6 mM^{-1} cm^{-1}) (Lyubenova and Schroder, 2011).

- Catalase (CAT) activity was detected on the base of hydrogen peroxide disappearing. The reaction mixture used contained phosphate buffer (100 mM KH_2PO_4. pH = 7.0. 30 mL) and H_2O_2 (200 mM. 12 mL). The supernatant (0.01 mL) was added to 0.14 mL of reaction mixture into a whole in a microplate and CAT activity was measured at 240 nm (ε = 0.036 mM^{-1} cm^{-1}) (Verma and Dubey, 2003).

- Ascorbate peroxidase (APX) activity was detected on the base of a decrease of absorbance of ascorbate. The reaction mixture contained sodium ascorbate (60 mM, 0.01 mL).Tris/HCl buffer (50 mM, pH = 6.0. 27 mL) and H_2O_2 (3%, 0.041 mL). The supernatant (0.02 mL) was added to 0.18 mL of reaction mixture into a whole in a microplate and APX activity was measured at 290 nm (ε = 2.8 mM^{-1} cm^{-1}) (Vanacker et al., 1998).

The enzyme activities were expressed in µkat mg^{-1} protein. The protein extraction followed a procedure by Brandford (1976) using serum albumin as a standard. All reported values for enzyme activities were the means of determinations of three separate extracts for each sampling point. Each extract was prepared from both parts (root and leaf) of the plant.

3.8 Data analysis tools

In order to analyse the data, two statistical software were used: SPSS and Minitab 16.1. The results obtained from concentration of TEs in soil pore water were reintroduced into MINITAB to determine the statistically significant effects of the digestate and biochar on the plant and soil response using the CCD. The quadratic models were only created for the nutrients and TEs which showed a R^2_{Adj} higher that 95%, using their deduced response surface plots to graphically illustrate the significant relationship between the experimental variables and the responses. The equations then were created using the significant terms of the polynomial function (y) expressed in quadratic terms:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j + \varepsilon$$

Where x_1, x_2, x_k are the independent variables, which have an influence on the response y; β_0, β_i (i = 1, 2, …, k), β_{ij} and β_{ij} (i = 1, 2, …, k; j = 1, 2, …, k) are the intercept, linear, quadratic, and interaction constant coefficients, respectively, and ε is either a random

error or allows for the description of uncertainties between the predicted and measured values.

Concerning enzymatic activity in plants, biomass and concentration of TEs in plants mean values were calculated from three replicates and then they were organized to be analysed by the CCD. However, due to no significance difference when using the CCD, analysis of variances (ANOVA), using Student t-test for P=0.05, was used to estimate statistically significant differences among the sets.

The differences among treatments were tested by one-way ANOVA with Turkey HSD multiple comparison test. Significance level P = 0.05 was used for both analyses.

4. Results

Results were divided into two sections:

I. Soil pore water analysis, which includes TEs content analysis pH and electrical conductivity (EC).

II. Plant analysis which includes biomass, TEs content and enzymatic activity.

All the analyses were made after plant harvesting, which took place when they showed their first sight of toxicity. In our experiment, this happened after six days of having transplanted the plants into the pots. Signs of toxicity are represented in figure 14.

Figure 14: Toxicity in leaves after 6 days of growing in the treated soils.

4.1 Soil pore water

Table 5 sums up the data of TEs concentrations, EC and pH values for soil pore water for each set. The values were expressed as the average of the data in triplicate that were obtained for each variable.

Table 5: Concentration of TEs in soil pore water

Amendment		Lowest		Low		High			Highest		
Digestate (A)	(% v/v)	0		14.6		85.3			100		
Biochar (B)		0		5.9		34.1			40		
Run		Actual level of variables (% v/v)			mS m^{-1}	Response (as mean of triplicate)						
						mg L^{-1}					
		Digestate	Biochar	pH	EC	Cd	K	Mn	Ni	Pb	Zn
1		14.6	5.9	3.74	9.40	6.41	247.7	293.4	1.13	1.49	792.2
2		85.4	5.9	4.69	6.83	1.56	356	142.7	0.31	0.19	291.4
3		14.6	34.1	5.89	14.42	0.70	2175	94.22	0.12	<0.01	68.31
4		85.4	34.1	5.85	12.15	0.09	1774	8.90	0.01	<0.01	7.62
5		0	20	4.58	12.14	5.08	1153	286.4	1.00	0.77	649.4
6		100	20	5.77	9.85	0.36	952	48.2	0.10	<0.01	58.7
7		50	0	3.82	8.58	4.79	82.22	239.6	0.84	1.27	639.2
8		50	40	6.36	14.97	0.12	2196	8.83	0.01	<0.01	9.97
9		50	20	5.35	11.42	1.66	1082	181.1	0.35	<0.01	248.1
10		50	20	5.37	10.94	1.48	968	163.2	0.31	<0.01	222
11		50	20	5.34	11.60	1.68	1092	183.6	0.35	<0.01	251.9
12		50	20	5.34	11.99	1.78	1148	195.4	0.38	<0.01	267.2
13		50	20	5.31	12.06	1.81	1169	194.9	0.37	<0.01	270.2
			R^2_{adj}	97.59	93.32	97.83	98.54	96.44	95.85	99.11	96.11

The results showed that just the pH and concentrations of Cd, Mn, Ni, Pb, Zn and potassium (K) can be expressed as functions of digestate and biochar (being the coefficient of correlation greater than 95%). EC, having a R^2_{adj} of 93.32 was included in the table as well.

Concerning the concentration of Al, Co, Cu, Mg, calcium (Ca),magnesium (Mg), sodium (Na), selenium (Se) and strontium (Sr) the results were not statistically significant;

meaning that the concentration of these nutrients cannot be expressed as a function of biochar nor digestate; they may rely then, on other variables. More experiments need to be carried out to determine the factors influencing these elements.

All the variables that were statistically significant can be expressed with mathematical models. Equations were designed using the CCD with the statistical software MINITAB 16.1 in order to express the concentration of TEs, pH and EC as a function of digestate and biochar doses. The table 6 synthetizes the mathematical models for each variable:

Table 6: Mathematical models for pH, EC and TEs in soil pore water

Digestate dose	A	Mathematic models						
Biochar dose	B							
Variable	Unities	Constant	A	B	AB	A^2	B^2	R^2_{adj}
pH		2.66187	0.02752	0.11539	-0.00049		-0.00075	97.59
EC	mS m^{-1}	8.63072		0.18106				93.32
Cd	mg L^{-1}	9.79919	-0.11894	-0.28523	0.00212		0.00034	97.83
K				61.922	-0.255			98.54
Mn		347.478	-1.808				-0.162	96.44
Ni		1.70986	-0.02088	-0.04223	0.00036	0.00006		95.85
Pb		2.57872	-0.03122	-0.11644	0.00060	0.00009	0.00112	99.11
Zn		1179.69	-12.44	-32.57	0.22			96.13

The aim of the mathematical models was to understand how digestate and biochar influence the behaviour of pH, EC and TEs concentration in soil pore water. It is observed, for instance, that EC relies only on the amount of biochar added; pH and concentration of K are also much more influenced by the biochar than the digestate. Concerning Cd, Mn, Pb, Ni and Zn concentrations both, digestate and biochar, play an important role. These equations allow us to estimate the concentration of TEs, the pH and the EC in soil pore water when we do know the digestate and biochar doses.

The behaviour of each one of these variables was independently explained and commented. Surface plots relating the different variables with digestate and biochar doses were built to provide better understanding of mathematical models.

4.1.1 pH
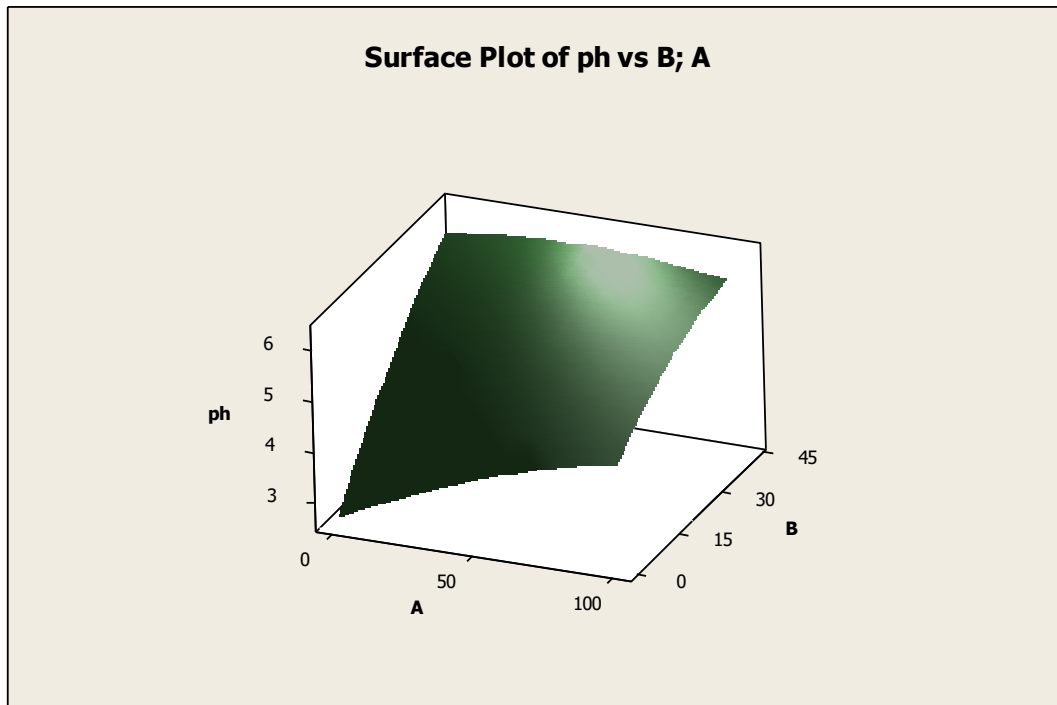
Figure 15: Surface plot of pH vs digestate (A) and biochar (B) doses (% v/v).

The pH is represented by the Y axe; the digestate and the biochar are represented by the X and Z axes respectively. In this graph it is observed that both digestate and biochar increased the pH in the soil pore water; however, the biochar, whose pH is initially basic, influences the most. According to this plot, the biochar can increase the pH up to 6, whereas the digestate increases it up to 4. A neutral pH (close to 7) is desired to promote plant growing.

4.1.2 Electrical conductivity (EC)
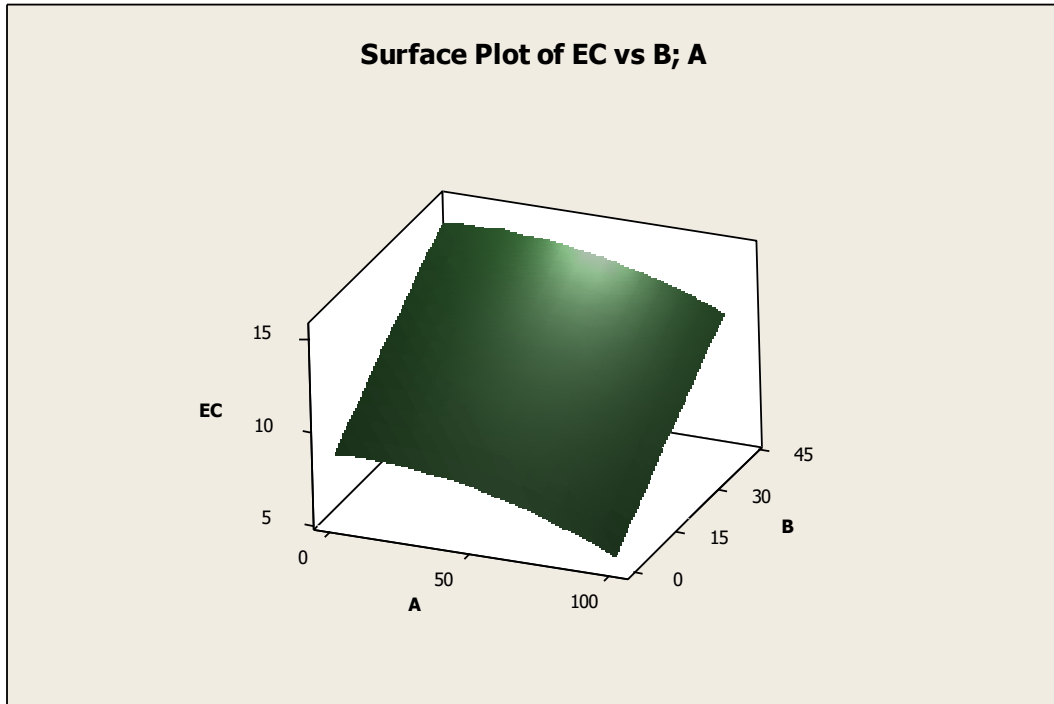
Figure 16: Surface plot of EC (mS m^{-1}) vs digestate (A) and biochar (B) doses (%v/v).

It is observed that the behaviour of the EC is significantly influenced by the biochar; this can be confirmed by the equation in table 6. It was found that there was a proportional relation between the amount of biochar and EC.

4.1.3 Trace elements concentration

Surface plots were used as well to explain concentration of TEs, in all the plots, the concentration of the element in mg L^{-1} is represented by the Y axe, and the digestate (A) and the biochar (B) doses are represented by the X and Z axes respectively.

Cadmium (Cd)
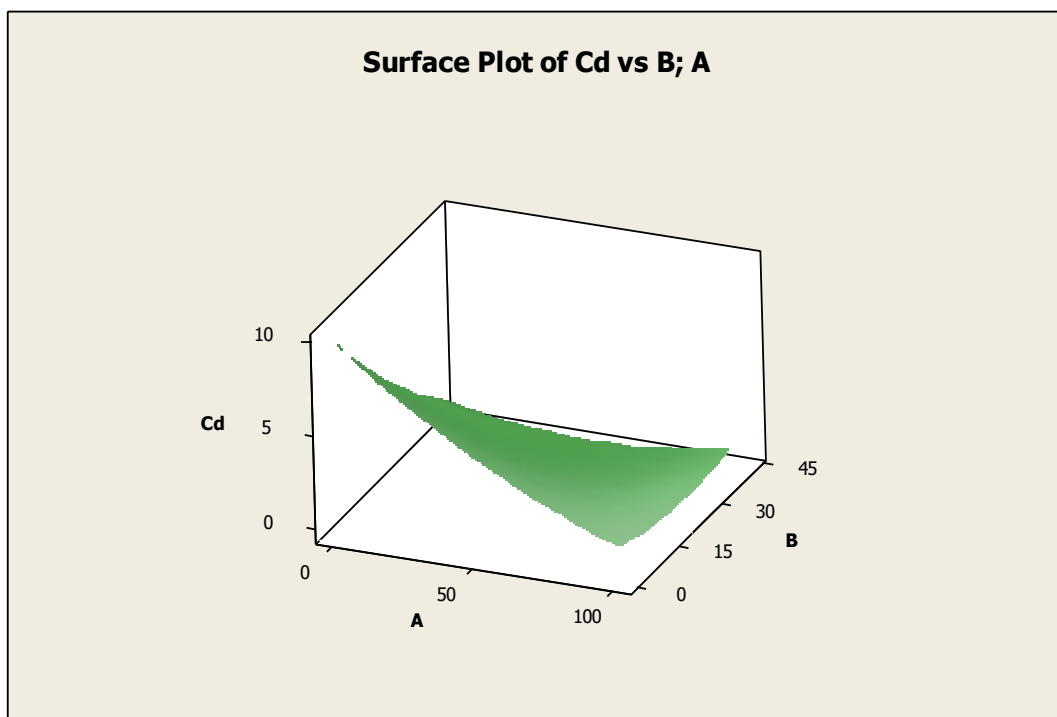
Figure 17: Surface plot of Cd concentration in mg L^{-1} vs digestate (A) and biochar (B) doses (%v/v).

Cd concentration was influenced by the presence of digestate; however, according to the equation, biochar, is also significant. In the figure 17, it is observed that there was a negative correlation between the Cd concentration and the amount of digestate, meaning that the presence of digestate decreases the concentration of Cadmium in soil

pore water. Concerning biochar, it is observed from the equation that it favoured the decrease of Cd.

Lead (Pb)

Figure 18: Surface plot of Pb concentration in mg L^{-1} vs digestate (A) and biochar (B) doses (%v/v).

In figure 18, it is shown a significant indirect proportional association between the concentration of Pb and the amount of both digestate and biochar, this relation is verified as well by the obtained coefficients for Pb equation. It means that both amendments promote the decrease of Pb concentration of in soil pore water.

Manganese (Mn)
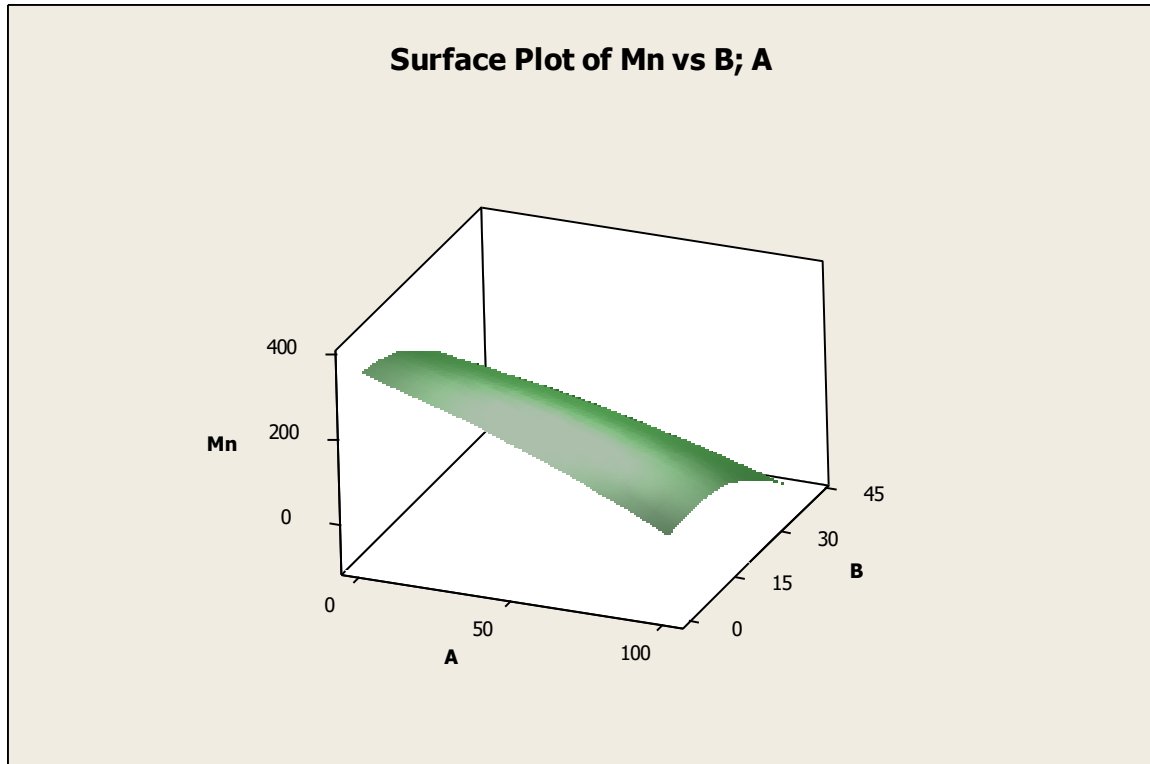
Figure 19: Surface plot of Mn concentration mg L^{-1} vs digestate (A) and biochar (B) doses (%v/v).

This surface plot shows how both (digestate and biochar) influence the concentration of manganese. There is an indirect proportional relation between the concentration of Mn and the digestate. It means that the concentration of Mn is lower when the amount of digestate is higher. Concerning the biochar, it is observed that the concentration of Mn reached the highest point in a middle point of the biochar axe, it indicates a quadratic relation between Mn concentration and biochar, this relation is confirmed by the mathematic model for Mn concentration where factor B^2 is significant.

Nickel (Ni)
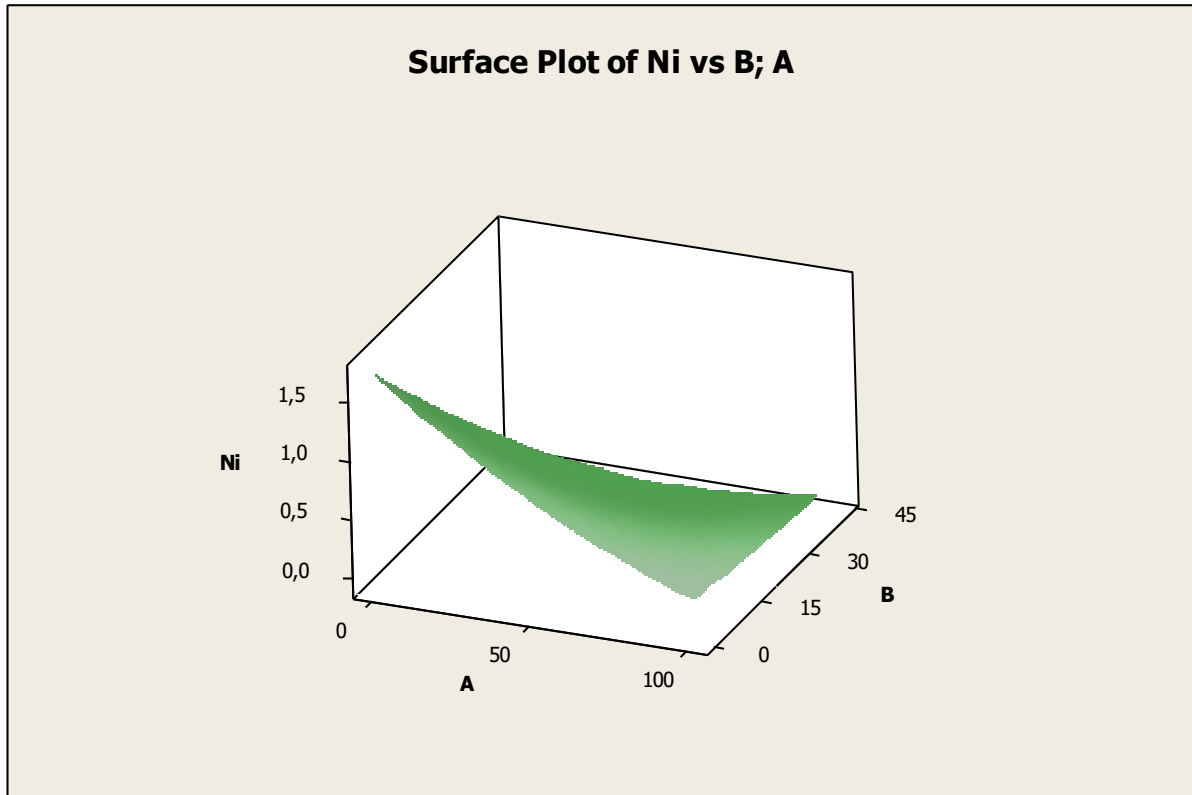
Figure 20: Surface plot of Ni concentration in mg L^{-1} vs digestate (A) and biochar (B) doses (%v/v).

This plot confirmed that the digestate influences the behaviour of the Ni concentration more than the biochar, The Ni concentration remains almost the same whether the amount of Biochar is. It is observed as well that the presence of digestate decreases the concentration of Nickel in soil pore water.

Zinc (Zn)
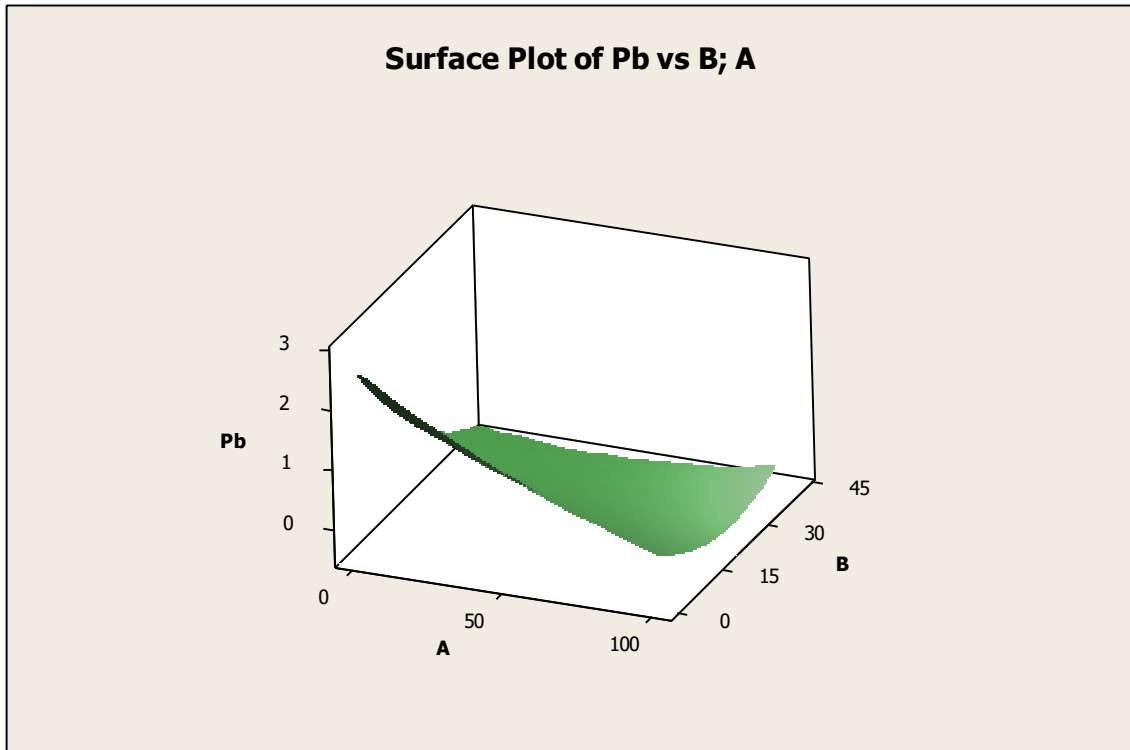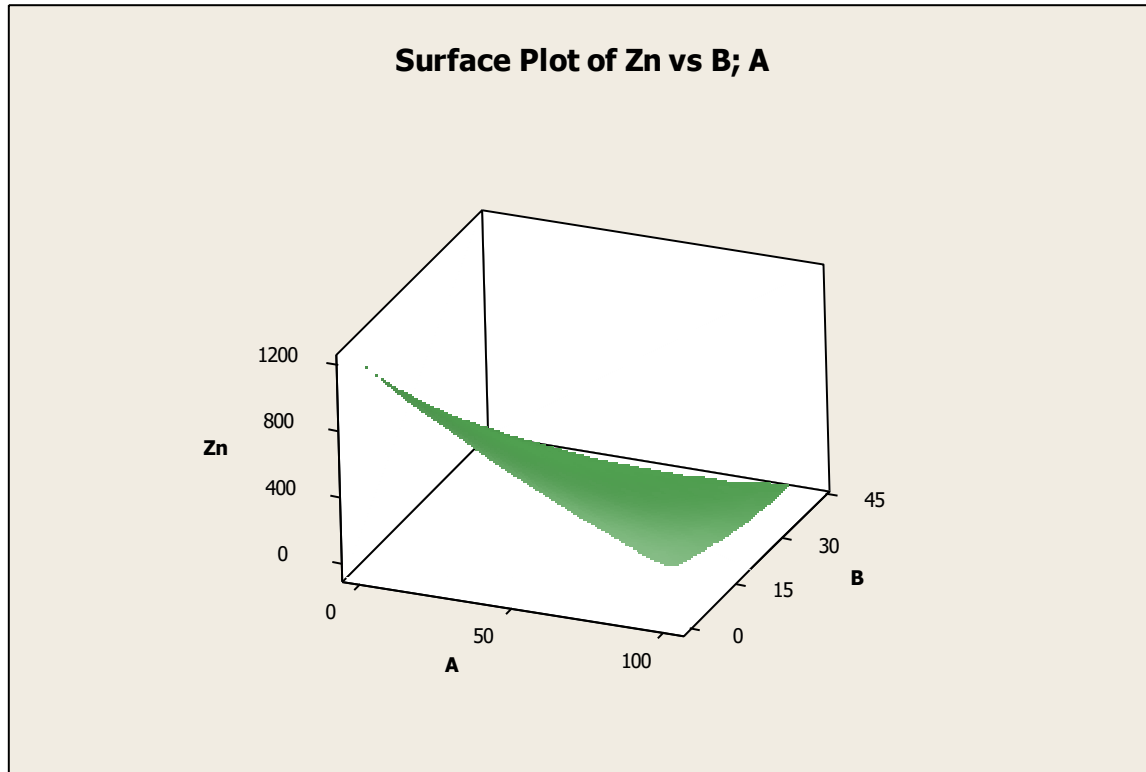

Figure 21: Surface plot of Zn concentration in mg L^{-1} vs digestate (A) and biochar (B) doses (%v/v).

Figure 21 shows an indirect proportional relation between the concentration of Zn and the amount of digestate. It means that digestate influences by decreasing the concentration of Zn in soil pore water. This response is supported by the mathematical model for Zn, where it is observed that both digestate and biochar have a significant influence in the concentration of Zn in soil pore water.

Potassium (K)
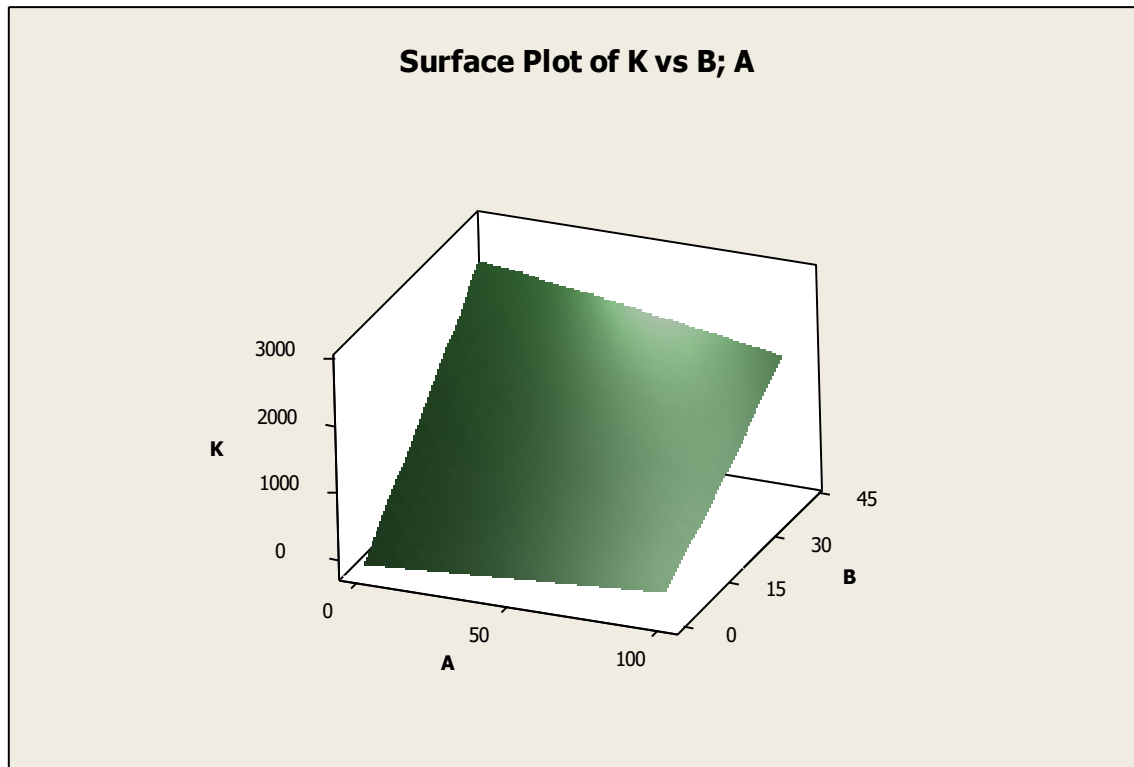
Figure 22: Surface plot of K concentration in mg L^{-1} vs digestate (A) and biochar (B) doses (%v/v).

Respecting K concentration, no significance differences were found for digestate, meaning that the digestate does not influence the concentration of K in soil pore water. This could be observed in the figure 22 where it is noted that the K concentration barely changed in relation to the digestate doses. On the other hand, the biochar is playing the main role showing a significant positive correlation between the concentration of K and the amount of biochar. So, the higher biochar a dose is, the higher the concentration of K will be.

4.1.4 Comparison of TEs in soil pore water for the different combinations of biochar and digestate

In order to better compare the concentrations of TEs in soil pore water among the 13 sets (combination of biochar and digestate doses) bar charts were built to show whether which set had the best performance at removing TEs from soil pore water. It is important to remark that sets from 9 to 13 are represented only by the set 9, this reminding that sets 9 – 13 had the same substrate doses.

The figure 23 and 24 shows the concentration of Mn and Zn, respectively, for each set.
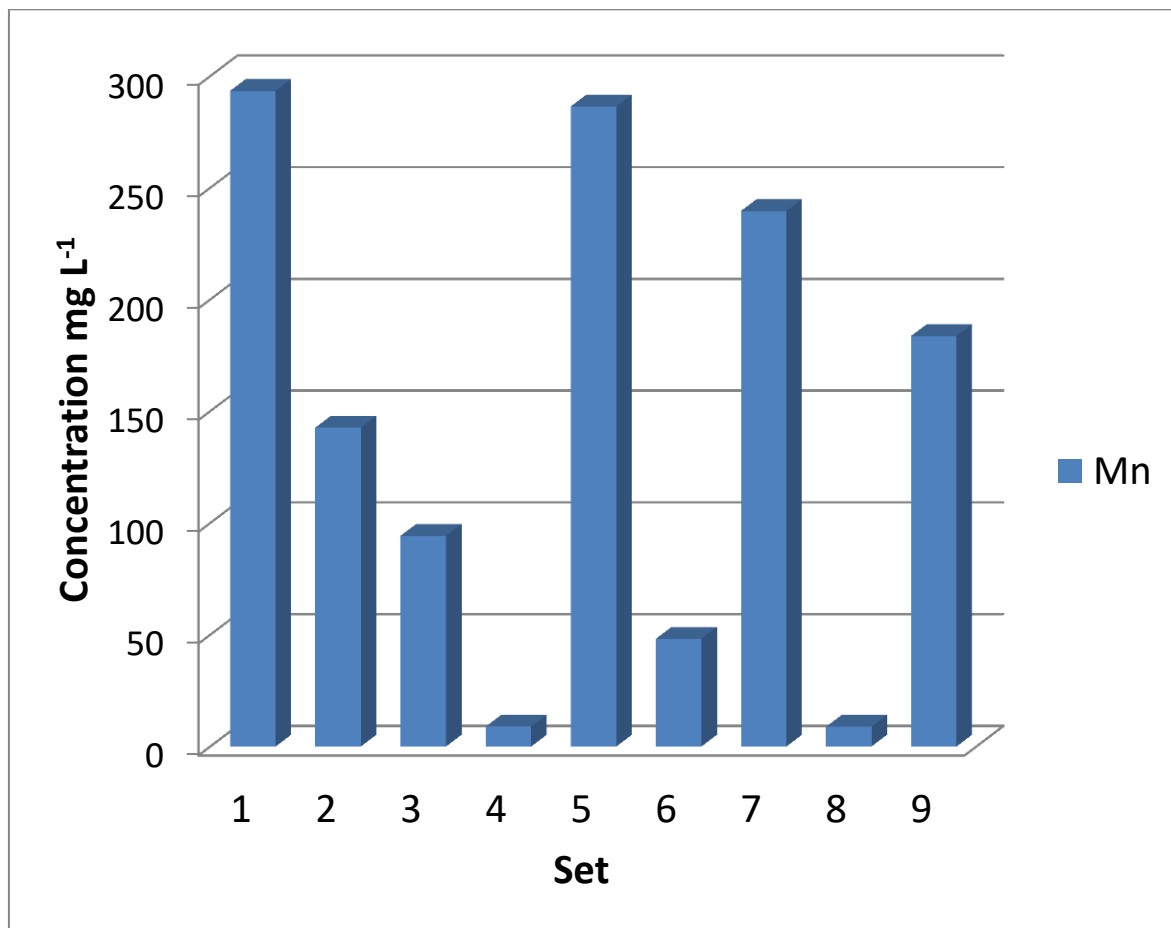
Figure 23: Bar chart: Concentration of Mn in soil pore water.
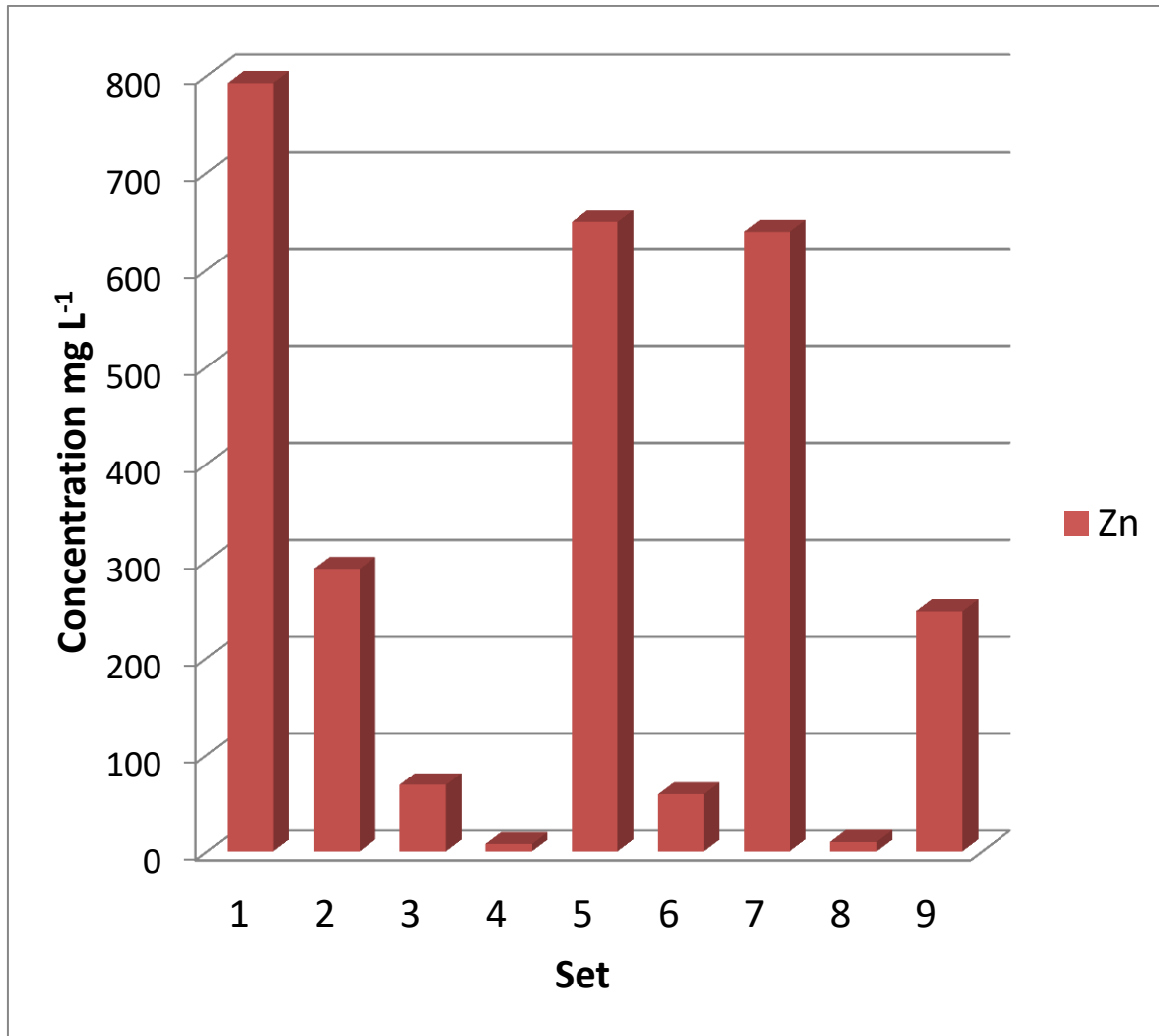
Figure 24: Bar chart: Concentration of Zn in soil pore water

As shown in the figures, there were important differences when comparing the concentration of soil pore water among the different sets. Set number 1, 5, 7 and 9 showed the worst performance at removing Mn and Zn from soil pore water. On the other hand, it is observed that set number 4 (85.4 v/v of digestate and 34.1 v/v of biochar) and set number 8 (50v/v in digestate and 40 v/v in biochar) present the lowest concentration of pollutants in soil pore water meaning that, for Mn and Zn, with these combinations, it is obtained the best performance at removing Ni and Mn from soil pore water.

The figure 25 shows the concentration of Cd, Ni and Pb for every set.
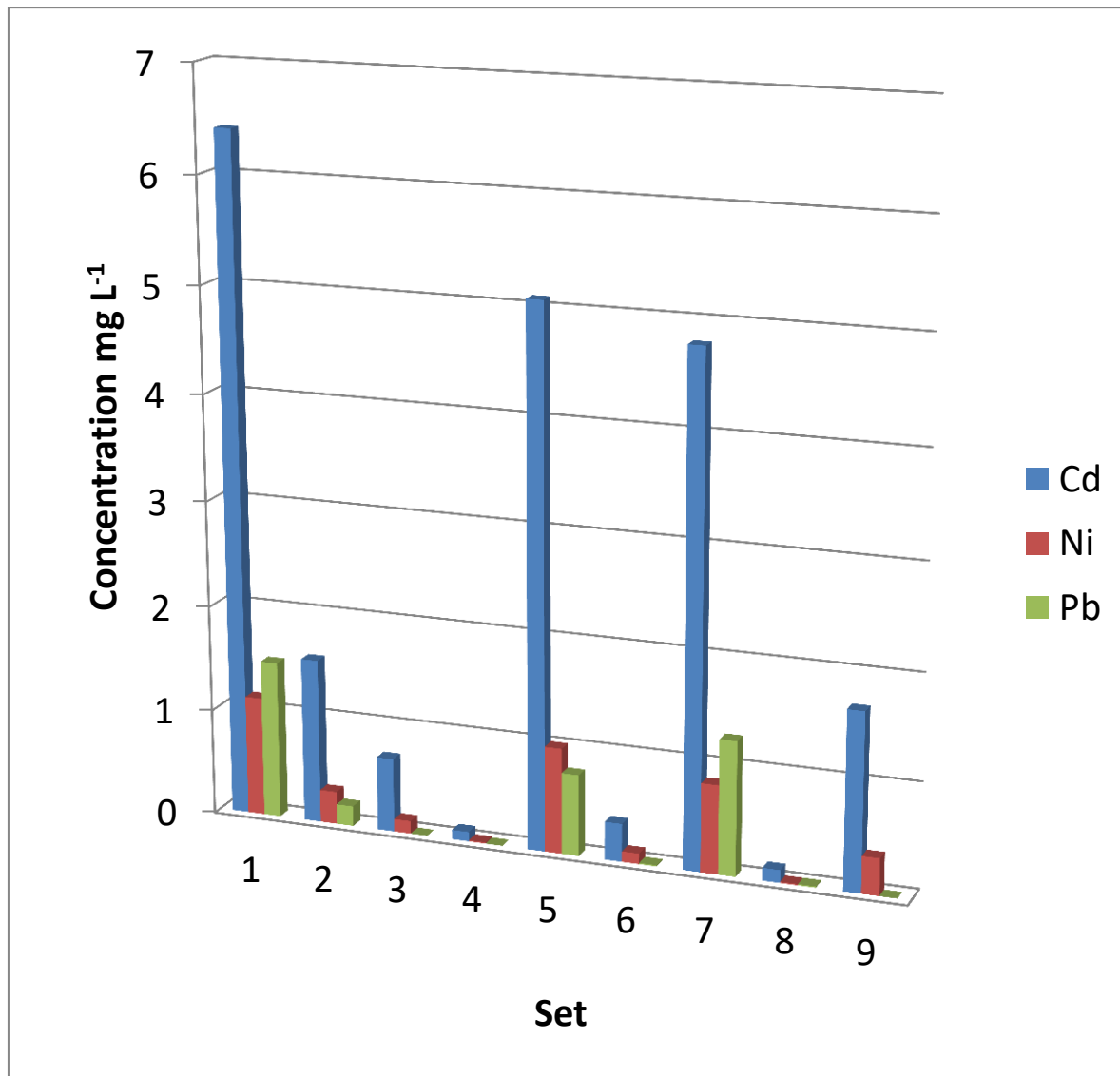
Figure 25: Bar chart: Concentration of Cd, Ni and Pb in soil pore water

It was interesting to notice that the pattern shown in the figures 23 and 24 was repeated in figure 25, meaning that the removal of Cd, Ni and Pb was lower for the sets number 1, 5, 7 and 9; whereas for sets 4 and 8 it was much more noticed. When comparing just the set 4 and 8 it was observed that the lowest values for TEs are represented by set 4. From the figures 23 to 25, it is concluded that the set 4, corresponding to 85.4v/v and 34.1v/v digestate and biochar respectively, shows the lowest values in concentration of TEs and the best performance at removing TEs from soil pore water.

4.2 Plants

For plant analysis, the data could not be represented as functions of the biochar and digestate doses due to the low R^2 (<95) that was observed for the models. However, as stated in the methodology, the differences among treatments were tested by one-way ANOVA with Turkey HSD multiple comparison test. The results were divided in biomass, TEs concentrations and enzymatic activities.

4.2.1 Biomass

In order to determine the effect of digestate and biochar in biomass an ANOVA was carried out. When compared biomass in terms of biochar no different groups were detected. On the other hand, significant different groups were observed in terms of digestate doses. Table 7 shows the analysis in terms of the digestate for 0, 50 and 100 v/v. Biomass content is expressed in fresh (FW) and dry (DW) weight for every 3 plants.

Table 7: ANOVA for Biomass in relation to Digestate content

	Digestate doses	Weight
FW (g)	0	3.713 a
	50	5.762 b
	100	6.653 b
	Post-Hoc	Tukey HSD
DW (g kg^{-1}) of fresh weight	0	0.552b
	50	0.881a
	100	0.891a
	Post-Hoc	Tukey HSD

Table 7 shows the two different groups (a,b) that were obtained by Post-Hoc analysis for the digestate doses when biochar reminds constant (20 v/v). In our experiment the biomass was directly affected by the amount of digestate: in absence of digestate, the

biomass in both cases (fresh and dry) was always lower than in presence of it. The digestate might be playing an important role promoting plant growth.

4.2.2 Trace elements concentration

The content of every nutrient in the plant was analysed by ANOVAs from the results. None of the TEs can be expressed as a function of digestate and biochar doses. When analysing the data, just the iron (Fe) showed statistically significant differences in terms of biochar doses; furthermore it is observed that phosphorus uptake is also affected by the biochar doses. The results are shown in table 8.

Concerning the analysis of Al, Ba, Ca, Cu, Mg, Mn, Sr and Zn, the results obtained were not statistically significant and no differences between groups were observed.

Table 8: ANOVA for Fe and P concentrations in relation to Biochar content (digestate=50)

TEs	Biochar	Means of concentrations $(g\ Kg^{-1})$
Fe	0	0.243 b
	40	0.340 a
	20	0.342 a
	Post-Hoc analysis	Tukey HSD
P	40	5.452 b
	20	5.546 b
	34.14	5.557 b
	5.86	5.740 b
	0	6.964 a
	Post-Hoc analysis	Tukey HSD

According to the ANOVA, two groups (a,b) can be distinguished when comparing the concentration of Fe in plants and the biochar doses, when biochar is different to zero (a) the concentration of Fe in the plant is higher than when biochar is equal to zero (b). Biochar might represent then a source of Fe.

When comparing the concentration of P in plants according to the amount of biochar, two groups can be significantly distinguished as well. In absence of biochar the highest content of P is observed, whereas in presence of biochar, P concentration is significantly lower.

4.2.3 Enzymatic activity (EA)

When analysing the activities it was observed that none of them can be expressed as a function of digestate and biochar doses with the CCD due to the lower (>95%) correlation coefficient. Nevertheless, when analysing the data *via* ANOVA significant differences were found, just the results being significant different are shown.

It was observed that the enzymatic activity did not rely on the biochar dose; any statistically different group was evidenced when comparing the enzymatic activity in terms of the biochar. On the other hand, according to the obtained results, the enzymatic activity was influenced mainly by the digestate. Table 9 summarizes the results for enzymatic activities in the plant with level of significance equal to 5 (P = 0.05).

Table 9: Enzymatic activity in plats tissues

Enzyme		Digestate doses	Activity means ($\mu katmg^{-1}$)
Ascorbate-Peroxidase APX		0	5.209 a
		50	2.973 b
		14.64	2.114 bc
		85.35	1.739 c
		100	1.364 c
		Post-Hoc	Turkey HSD
Catalase CAT		0	0.626 a
		50	0.389 ab
		100	0.292 abc
		14.64	0.227 bc
		85.35	0.103 c
		Post-Hoc	Turkey HSD
Peroxidase		0	52.18 a
		50	21.84 b
		14.64	16.94 b
		100	13.39 b
		85.35	12.36 b
		Post-Hoc	Turkey HSD
Peroxidase-Guaiacol (GOPX)		0	2.266 a
		50	0.772 b
		14.64	0.64 bc
		100	0.566 bc
		85.355	0.353 c
		Post-Hoc	Turkey HSD
Glutathione - S transferase (GST)	Fluorodifen	0	0.421 a
		50	0.0792 b
		14.64	0.0631 b
		85.35	0.0511 b
		100	0.0425 b
		Post-Hoc	Turkey HSD
	pNBoC	0	16.03 a
		50	8.866 b
		14.64	6.536 b
		85.35	5.145 b
		100	4.943 b
		Post-Hoc	Turkey HSD

According to the statistical analysis for APX, the results showed that there are significant differences between its means, there being higher values (5.209a), intermediates values and lower values (1.739 and 1.364c). The same pattern was observed for CAT and GOPX: three statistically different groups can be distinguished when comparing their activity with the digestate doses. It is remarkable that the highest enzymatic activity is observed in absence of digestate.

Concerning peroxidase, the results exhibited that means were significantly different showing higher (52.18a) and lower (remaining) values. For GST (fluorodifen and pNBoC), there were also two groups that were statistically distinguished when comparing the means. When the digestate dose was equal to zero, the highest enzymatic activity was observed; likewise it was noted that the lowest activity occurred in the samples with maximal doses of digestate.

From these results, a common pattern could be noticed in all the cases for EA: in absence of digestate, the activities present the highest value; then, considering the EAs as an indicator of plant stress it was confirmed that the digestate reduces the enzymatic activity and therefore it may be an important factor decreasing plant stress.

5. Discussion

5.1 Soil pore water

In our experiment it was found that biochar was significantly influencing the behaviour of EC in soil pore water. The increase of EC could be attributed to the mineral salts that are provided by the biochar. According to our findings, pH pore water also raised when biochar doses increased, meaning that both variables (EC and pH) presented a similar behaviour, both being influenced by biochar. Wong et al. (1998) explained that EC is pH related due to under acidic conditions the tailings matrix will dissolve more salts.

Concerning TE composition it was observed that Cd concentration decreased in soil pore water when the digestate dose increased. This behaviour was also noted by Beesley et al. (2010) who applied hardwood-derived biochar and green waste compost to a multi-element polluted soil and observed that Cd was immobilized. Cd in soil pore water is relatively insoluble when the pH is increased (Ross, 1994), so with the application of soil amendments the pH increases and the solubility of Cd may be reduced and so the concentration in soil pore water.

For Pb concentration, it was found that both biochar and digestate play a significant role, both amendments promoted the decrease of Pb concentration in soil pore water.
Respecting Mn concentration, we found out that it tended to decrease when the amount of digestate was higher. Martínez-Fernández et al. (2014) observed that Mn concentration in soil pore water decreased when using compost treatments in soil.

According to our experiment, and just like Cd, Zn concentration in soil pore water showed an indirect relation in terms of our amendments, meaning that both of them decreased the concentration of Zn. This behaviour was also observed by Beesley et al. (2010) who noticed Zn immobilization in soil after application of hardwood-derived biochar to a multi-element contaminated; on the other hand, Perez-Esteban (2013)

found that an organic (manure) amendment also reduced Zn bioavailability; Clemente and Bernal (2006) found that Zn was immobilized by humic acids from organic amendments as well and finally, according to Ross (1994) the application of soil amendments increases the pH and then the solubility of Zn may be reduced.

All these TEs results is soil pore water, coincided with Alvarenga (2008) who also found a decrease in bioavailability in Pb, Zn and Cd when using organic material as amendment. Gonzáles et al. (2012) found as well that treatments with compost significantly reduced the concentrations of Cd, Cu, and Pb in lixiviates and pore water.

Concentration of K in soil pore water was found to be positively affected by the biochar, meaning the our biochar might be a source of K. Lehmann (2006), Rodríguez-Vila et al. (2016) and Sohi et al., (2009) also noted an increase of K bioavailability when using biochar as amendment.

The general objective of this work was to find out the optimal doses of biochar and digestate to be combined with the polluted soil, from the figures 23 to 25, it is concluded that the set 4, corresponding to 85.4v/v and 34.1v/v digestate and biochar respectively, shows the lowest values in concentration of TEs. So, it is concluded that among all the combinations carried out in the framework of this experiment, set 4 is retained as the best one reducing TEs (Cd, Pb, Mn, Ni and Zn) in soil pore water. Meaning that bioavailability of these TEs can be reduced when using these doses.

Several studies (Bernal et al., 1998; Carrasco et al., 2009; Opatokun et al., 2017)) confirmed that soil amendments favours phytoremediation; however few of them have specified the optimal doses of substrate to be used in order to obtain the best performance in phytostabilization.

5.2 Plant analysis

It was found in our research that plant biomass increased when the amount of digestate increased as well. From the literature it has been also verified that organic amendments such as digestate play the main role in plat growth, being the principal source of carbon (Kabas et al., 2012). Martínez-Fernández et al. (2014) also found that biomass is favoured by organic amendments such as digestate.

For TEs content in plant tissues the analysis with the CCD for Al, Ba, Ca, Cu, Mg, Mn, Sr and Zn showed that they were not statistically significant and therefore they could not be expressed in terms of digestate nor biochar doses. Also no significant differences between groups were observed by ANOVA.

Only Fe and P concentration showed a significant response in terms of biochar dose. It was noted that the presence of biochar promoted the uptake of Fe by the plant. Kappler et al. (2014) found that biochar may affect mineral forms of Fe by acting as an electron shuttle in redox-mediated reactions, Fe may be released and available to be uptaken by the plats roots This pattern was also observed by Graber et al. (2014) who noted how redox catalytic activity associated to biochar solubilized Fe from soil.

In absence of biochar the highest content of P was observed, whereas in presence of biochar, P concentration is significantly lower. It means that, even when biochar can suppose a source of P for the plants, its uptake and accumulation was affected. According to Cui et al. (2014) the addition of biochar alter P availability probably by modifying the P sorption and precipitation processes; in acid soils, for example, biochar liming effect leads to P sorption decreases and P availability increases (Farrell et al., 2014); the impact of biochar on P availability relies on complex nutrient–biochar–soil interactions because it is affected by soil pH, changes of Fe- and Al-oxides, and biochar composition and properties such as surface area, cation exchange capacity and liming capacity. Therefore the concentration of P in plants is highly related to the biochar (Shen et al., 2016).

When analysing EA, a common pattern could be noticed in all the cases for all the enzymes: in absence of digestate, the activities presented the highest value; then,

considering the EAs as an indicator of plant stress, it was confirmed that the digestate reduces the enzymatic activity and therefore it may be an important factor decreasing plant stress.

According to the literature TEs in high concentrations cause oxidative stress in plants, inducing oxygen radical production. In order to combat the reactive oxygen, the defence system of plants produces the enzymes. Gratao et al. (2005) and Pandey et al. (2009) listed several studies showing that Cd, Zn and Ni are some of the principal TEs inducing plant stress.

For example, it has been reported by Araving and Prasad (2003) that APX activity raises considerably in presence of Cd, Zn and Ni. Regarding GOPX activity, it was reported an increase in the activity in several plant species due to Cd presence (Cho and Sohn, 2004). GTS activity is considerably raised also by Cd (Moons, 2003) and Ni (Becher et al., 2004) induced stress. Concerning CAT activity, plants species exposed to Ni have exhibited greater activities than with other TEs (Gratao et al., 2005).

As it was found in our experiment, the digestate is playing an important and significant role at reducing the bioavailability of several TEs including Cd, Ni and Zn. Likewise, the digestate was also found to decrease the enzymatic activity and the stress in *Dittrichia viscosa*. These two behaviours can be directly related meaning that when the TEs are less bioavailable, the plant experiences less stress and so its enzymatic activity decreases. It can be conclude the stress in *Ditriccia viscosa* is considerable reduced because the digestate influences the behaviour of TEs by reducing their bioavailability. However, it needs to be considered that the TE stress varies considerably depending on plant species and metal exposure (Gratao et al. 2005).

6. Conclusions and perspectives

The results of this study brought by the Central Composite Design (CCD) demonstrated that combining 85.4% v/v of digestate and 34.1 % v/v of biochar, it is obtained the best performance at removing Pb, Cd, Zn and Mn from soil pore water; meaning that the bioavailability of these pollutants potentially transferred by erosion to farther sites can be significantly reduced.

Concerning the influence of our two substrates it is observed that digestate decreases the concentration of Zn, Pb, Ni, Mn and Cd in soil pore water, even when the biochar had more potential effect on the pH and Electrical conductivity.

Respecting plant analysis, it was proved that biochar promotes the uptake of Fe by the plant. On the other hand, it was noted that in absence of biochar, plants uptake more P even when biochar supposes a source of P for the plants. It was demonstrated in this experiment as well that the digestate increase the biomass, confirming that this amendment, in effect, promotes plant growth.

It was observed that the enzymatic activity is much higher in absence of digestate, meaning that the digestate helps to dissipate the stress provoked by TEs in plants. Considering that the enzymatic activity can be used as an indicator of plant stress induced by TEs, the digestate might be promoting the reduction of stress in plants.

Another remarkable fact is that *Dittrichia viscosa* was able to regrow in the pots after harvesting, which undoubtedly is good indicator of tolerance and adaptability of this specie. In the framework of this research more experiments should be contemplated in order to complement and extend this work. Experiments analysing the soil pore water and the plant over the time would give a better understanding about the influence of our substrates. Field research should be carried out as well to verify the results under real conditions.

7.References

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