Biogeochemical and microbiological processes involved in the rhizospheric area of *Salicaceae* grown on an amended technosol polluted by inorganic toxic elements: a phytostabilization study.

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Abstract

The pollution of soils by metal(loid)s, resulting from anthropogenic activities, is an important issue and subject of research nowadays. Therefore, to remediate such sites, an environment friendly technique has developed over the last decades, called phytoremediation. The phytoremediation is the use of plants and their associated microbiota to remediate *in situ* contaminated area. Among plants that can be used in phytoremediation, *Salicaceae* showed good potential, due to their rapid and high biomass production associated to their tolerance to metal(loid)s. However, contaminated soils are often poor (extreme pH, low nutrient contents), which associated to their elevated metal(loid) concentrations hinder plant growth. That is why amendments must be applied. Three amendment types gathered attention over the last decades, compost, biochar and iron-based amendments. Compost can increase soil nutrient availability and immobilize metal(loid)s. Biochar can ameliorate soil conditions and sorb metal(loid)s. Both amendments showed good potential to improve soil properties, immobilize cations and thus ameliorate plant growth. However, they showed no or negative effects on anions like As. On the contrary, iron-based amendments, such as iron grit and redmud, have an affinity for As and can thus immobilize it. Finally, another parameter that can affect phytoremediation efficiency is the microbial community. Microorganisms are very sensible to the growing conditions and the measure of their diversity and activity can inform on the recovery of the soil during the phytoremediation process. Microorganisms can also affect phytoremediation efficiency: (i) through their effect on metal(loid)s, some bacterial strains showed the capacity to accumulated metal(loid)s or detoxify them and (ii) by increasing plant growth, through the secretion of plant growth promoting factors (*i.e.* hormone and siderophore production, phosphate solubilization).

In this context, the aims of this PhD work were first to evaluate the effect of applying to a former mine technosol highly contaminated by As and Pb different amendments, alone or combined, on (i) soil physico-chemical properties, (ii) *Salicaceae* plant growth and metal(loid) accumulation, (iii) *Salicaceae* physiology and biochemistry and (iv) soil bacterial community. The second objective was to assess the effect of inoculating an endogenous bacterium, using two inoculation methods, on (i) soil physico-chemical properties, (ii) soil bacterial community and (iii) *Salix viminalis* growth and metal(loid) accumulation.

To answer these objectives, a total of seven mesocosm experiments were performed. In general, these different experiments demonstrated that the addition of amendment improved the soil conditions and thus plant growth in most of the cases. *Salicaceae* plants accumulated As and Pb mainly in their roots. Moreover, the application of amendments had an effect on the root physiology of *Salicaceae* plants. Especially, the root proteome profiles of *Salix viminalis* were affected differently by the addition of biochar, compost and/or iron grit; whereas *Salix triandra* roots showed high levels of stress markers on the unamended technosol, which were reduced following the application of redmud and biochar. In addition, the inoculation of an endogenous *Bacillus* strain also improved soil conditions and *Salix viminalis* growth, especially when the bacterium was previously sorbed on biochar surface. Finally, the study of the bacterial community showed an increase of the bacterial activity in the presence of amendments and following bacterial inoculation, in addition to a modification of the bacterial community composition.

To conclude, this PhD work demonstrated that amendment application can improve soil conditions and plant growth; however these effects depend on the amendment(s) applied. This work was also one of the first showing that amendments can affect the plants at a biochemical level and comparing two bacterial inoculation methods, demonstrating the better effect of a biochar carrier inoculation.

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General introduction.

Soil pollution greatly increased with the development of anthropogenic activities such as mining, industries, fertilization… and has become one of the eight threats to soil (Panagos *et al.* 2013). Indeed, more than ten million of contaminated sites have been numbered worldwide, with more than three million in Europe (Khalid *et al.* 2016). Among the different pollutants found in soil, *i.e.* pesticides, polycyclic aromatic hydrocarbons, persistent organic pollutants…, metal(loid)s are the most important ones (Rodríguez-Eugenio *et al.* 2018). Furthermore, contrary to organic pollutants, metal(loid)s cannot be degraded and thus accumulate in soils. They are threatening for the human health as most of them are carcinogenic. Additionally, soil pollution is of great concern for the environment. Indeed, contaminated soils often lack of a vegetation cover, inducing soil wind erosion and water leaching problems that can transport the contamination elsewhere, to a non-contaminated area for instance. Therefore, contaminated soils are in urgent need for remediation.

For that purpose, diverse methods can and have been used for the remediation of contaminated soils. For decades, physical and chemical remediation methods were used, such as surface capping, encapsulation, solidification/stabilization, soil washing… (Liu *et al.* 2018). Their preferential use was related to their fast application. However, such methods are expensive, due to the handling of important soil volumes and often not applicable on large areas. They can also induce a secondary pollution, notably with the use of chemicals to wash the soil or immobilizing agents, and can render the soil unsuitable for (micro)organisms and vegetation. That is why, for several decades now, another remediation technique, a biological method using plants associated to microorganisms and called phytoremediation, has been developed. This technique has been defined by Ghosh and Singh as “the use of green plants and their associated microbiota for *in-situ* treatment of contaminated soil” (Ghosh and Singh 2005). Its principle consists to establish a plant cover that can accumulate metal(loid)s but also reduce the risk of wind erosion and water leaching, thus protecting the surrounding environment. It relies on the combination of amendment application, to improve soil conditions, plant growth and microorganisms, which will be detailed at the end of this introduction. Finally, phytoremediation is divided for metal(loid)s into two main techniques, phytoextraction or phytostabilization. In phytoextraction, (hyper)accumulator plant species are used, they will uptake metal(loid)s from the soil and translocate them into their aboveground tissues (Ali *et al.* 2013). It constitutes a real decontamination method, contrary to phytostabilization. However, the phytoextraction process poses the problem of the treatment of the contaminated biomass, which needs to be harvested and disposed properly to prevent the return of the contaminant into the soil at leaf fall. For instance, the biomass could be burned, with a special system to collect the contaminated smoke (filtering system), in order to prevent atmosphere contamination. Moreover, the contaminated biomass could be pyrolyzed to produce biochar, which could be applied to the soil, after the verification of its metal(loid) contents. In the phytostabilization process, plant establishment, which is sometimes associated with the addition of amendments into the polluted area, aims at decreasing the mobility of metal(loid)s into the soil, while leaving pollutants strongly linked to the soil particles and to the rhizosphere (root zone) by adsorption, absorption and/or precipitation processes (Barceló and Poschenrieder 2003). Phytostabilization is often preferred nowadays to phytoextraction as it is suited for large areas highly contaminated and does not require post-harvest treatments. Moreover, the complete extraction of the pollutants will take decades to centuries and is often not economically interesting, especially for metal(loid)s that do not have an economic value. On the contrary, the aerial biomass produced during phytostabilization can be recovered, as pollutant translocation is reduced, and used for biomass and energy production. Such process is called phytomanagement (Kidd *et al.* 2015).

Since contaminated soils are often not suited for direct establishment of plants due to their extreme pH, low contents in organic and inorganic matter and their elevated levels of metal(loid)s, amendments are required, which will improve soil physico-chemical properties, supply nutrients for plant growth and immobilize metal(loid)s (Galende *et al.* 2014). Amendments are numerous and can be both organic and inorganic. Among the different possible amendments, three types of amendment gathered attention in the last decades and will be detailed here.

Biochar, the solid product obtained from the pyrolysis of biomass (Wiszniewska *et al.* 2016), is mainly characterized by an alkaline pH, an elevated surface area, a high cation exchange capacity and by a surface rich in functional groups (Singh *et al.* 2010, Paz-Ferreiro *et al.* 2014). These properties make biochar a good soil conditioner to reduce soil acidity, increase nutrient availability and immobilize metal(loid)s (Rees *et al.* 2015, Egene *et al.* 2018, Meng *et al.* 2018). Such improvements allowed or increased plant growth (Uzoma *et al.* 2011, Trakal *et al.* 2017, Huang *et al.* 2018). However, biochar properties and thus effects on soil and plant parameters depend on the feedstock used and the pyrolysis conditions to obtain it (Barrow 2012) and can also be improved by biochar functionalization (Tan *et al.* 2017). Functionalization is performed chemically (acidic or basic solutions, salts) or physically (steam, micro-wave), before or after the pyrolysis, to modify the biochar surface and thus improve its sorption capacity.

Another amendment, also much used in agriculture, is compost, the product of microbial degradation of organic wastes (Huang *et al.* 2016a). Compost is rich in humus substances, microorganisms and inorganic components (Huang *et al.* 2016a). It is mainly used to bring nutrients and organic matter to the soil but it can also immobilize metal(loid)s (Walker *et al.* 2004, Alvarenga *et al.* 2014) and thus ameliorate plant growth (Fischer and Glaser 2012, Caporale *et al.* 2013). However, compost is highly degradable and needs to be applied repeatedly. Moreover, depending on the organic matter sources used to produce compost, this compost can contain pollutants such as plastics and microplastics (Ng e*t al.* 2018). For example, Brinton *et al.* (2018) tested several domestic waste, composed of plastic-coated paper, to produce compost and observed that theses plastic-based materials were not biodegradable and thus found microplastic fragments in the compost.

However, although both biochar and compost were shown efficient towards metallic cations such as Pb, they tend to be inefficient or even have negative effects on the mobility of metallic anions such as As

(Hartey *et al.* 2009, Trakal *et al.* 2017). In this goal, another type of amendment has been used, iron-based amendments. Indeed, arsenic is known to have an affinity towards iron (Miretzky and Cirelli 2010). For instance, iron grit amendment showed good potential to immobilize arsenic but also other metal(loid)s and improve plant growth (Michálková *et al.* 2017, Qiao *et al.* 2018, Vítková *et al.* 2018). Similarly, redmud, another iron-based amendment and obtained from the solid waste residue of the alumina production process (Bhatnagar *et al.* 2011), is highly alkaline and possess a large surface area and many Fe and Al oxides that can bind arsenic but also other metal(loid)s (Bertocchi *et al.* 2006, Hua *et al.* 2017). It showed good results to improve soil properties and plant growth (Gray *et al.* 2006, Gautam and Agrawal 2017).

Finally, amendments can be applied singularly or they can be combined. Indeed, a combined application can have better effects than single amendment application as the amendments act differently and can thus have complementary beneficial effects, one amendment can also counteract the negative effect of the other (Beesley *et al.* 2013, Yun *et al.* 2017).

The application of amendments will ameliorate soil conditions and thus allow a better plant development. However, it is important to choose the right plant species:

- A species that accumulates elevated concentrations of metal(loid)s, especially in the aerial parts, for phytoextraction.
- A species producing an important biomass, poorly translocating metal(loid)s to upper parts and developing a dense and deep root system, for phytostabilization.

For phytostabilization processes, as described in the literature, *Salicaceae* are a good option due to their fast growth, deep rooting and high biomass production (Greger and Landberg 1999, Marmiroli *et al.* 2011). Moreover, they are tolerant to high levels of metal(loid)s and can accumulate substantial amounts of them, especially in the roots (Kuzovkina *et al.* 2004, Ruttens *et al.* 2011, Bart *et al.* 2016). Finally, they can be planted in (very) short rotation coppice in order to produce biomass and energy, which will add an economical value to the phytomanagement process that usually is a long-time process.

One of the most important soil compartment for the phytomanagement success corresponds to the rhizosphere, *i.e.* the zone located and corresponding to the root-soil interface in which microorganisms are influenced by the root system and *vice versa* (Martin *et al.* 2014, Seshadri *et al.* 2015). As stated in the definition, rhizosphere is composed of the soil, the associated microorganism and plant roots located nearly.

Plant root activity is influenced by the environment. Therefore, a change in the environmental conditions, by the addition of amendment(s) for instance, can induce a modification of the root activity. For example, in response to a changing environment, roots can modify the expression of some proteins, activating some biological pathways and repressing others (Li *et al.* 2009, Durand *et al.* 2012). Another modification can occur in the root antioxidant system. Indeed, metal(loid) stress induces the overproduction of reactive oxygen species (ROS), which have damaging effects on the cell such as lipid peroxidation. Plants respond to this oxidative stress induced by metal(loid)s through the activation of detoxifying processes such as enzymatic activities (superoxide dismutase, peroxidase, catalase) and non-enzymatic processes (glutathione, carotenoids, flavonoids, phenolic compounds). These antioxidative systems allow to keep the balance between the production and the scavenging of ROS. Finally, plant roots can exudate a multitude of compounds, such as organic acids, sugars and amino acids, into the environment that will affect metal(loid) bioavailability (Dong *et al.* 2007, Meier *et al.* 2012, Huang *et al.* 2016b) but also can serve as carbon sources for microorganisms (Dakora and Phillips 2002).

The other constituent of the rhizosphere is microorganisms, particularly bacteria, and although they cannot degrade metal(loid)s, bacteria possess several resistance mechanisms to metal(loid)s such as external and internal sequestration or detoxification (Yin *et al.* 2019), making them an important parameter to consider in phytomanagement. Moreover, their presence, activity and diversity, which can be assessed through different techniques (measure of the soil enzyme activities, measure of the community level physiological profiling, 16S gene sequencing), is an indication of the recovery of a soil following a phytomanagement process. Finally, bacteria can also improve plant growth, not only by reducing metal(loid) bioavailability, but also by their plant growth promoting properties, *i.e.* siderophore production, phosphate solubilization, indole acetic acid production and aminocyclopropane-1-carboxylate deaminase activity (Marques *et al.* 2013). Thus, bacteria could be inoculated to the soil, especially in addition to amendment application that will increase their survival and activity, in order to improve soil conditions and plant growth, increasing phytomanagement success (Sheng and Xia 2006, Brunetti *et al*. 2012).

This PhD work aimed at answering two main objectives.

The first objective (**objective 1**) was to evaluate the effect of applying different amendments, alone or combined, on different components of the continuum soil-plant, *i.e.* the soil, the plants and the microorganisms. This objective was divided in four sub-objectives:

- **Objective 1a**: evaluation of the effect of amendment application on soil physico-chemical properties.
- **Objective 1b**: evaluation of the effect of amendment application on several *Salicaceae* species growth and metal(loid) accumulation.
- **Objective 1c**: evaluation of the effect of amendment application on *Salix viminalis* and *Salix triandra* physiology and biochemistry.
- **Objective 1d**: evaluation of the effect of amendment application on the soil bacterial community.

The second objective (**objective 2**) was to assess the effect of inoculating an endogenous bacterium, using two inoculation methods, on the soil physico-chemical properties, soil bacterial community and *Salix viminalis* growth and metal(loid) accumulation.

The objectives are synthesized in Figure 1.

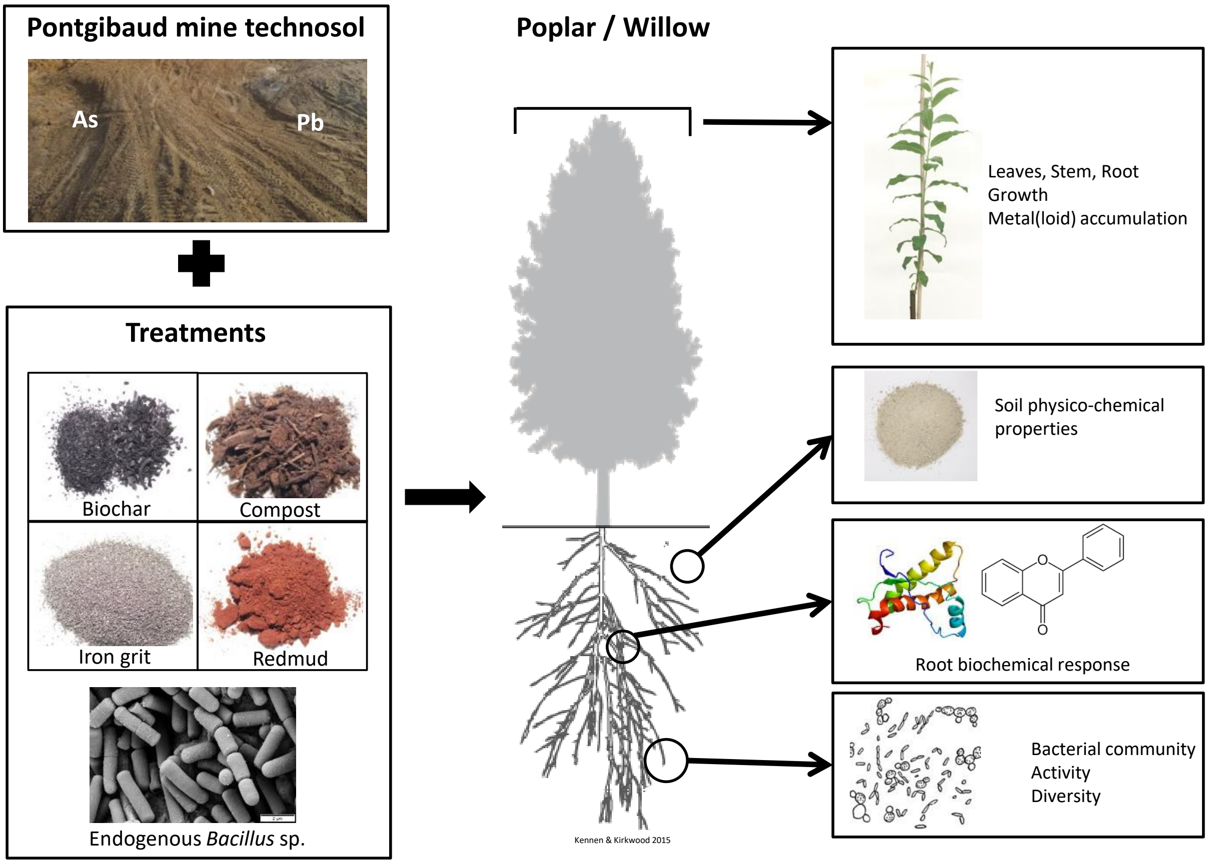

Figure 1 Synthesis of the objectives of the PhD work

To answer these objectives, different mesocosm experiments were set-up and will be detailed in this manuscript.

The first chapter will give a state of the art on the soil pollution and the different remediation techniques, with an accentuation on the phytomanagement and its different components (amendments, plants, rhizosphere, and bacteria).

The second chapter (objectives 1a and 1b) will detail five mesocosm experiments evaluating the effect of amendment application on the soil physico-chemical properties and *Salicaceae* growth and accumulation pattern.

The third chapter (objective 1c) will describe the effect of amendments on plant at the physiological level by the assessment of the root proteome profile, the root exudation pattern and the oxidative stress response.

The fourth chapter (objective 1d) will describe the bacterial response, in terms of diversity and activity, to amendment additions.

The fifth chapter (objective 2) will give the results of the inoculation of the soil in a mesocosm experiment.

Finally, the manuscript will finish by a general conclusion of this work and its perspectives.

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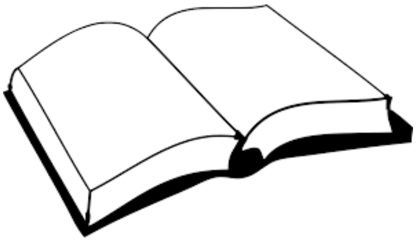
Chapter 1. Bibliographic synthesis: State of the art.



I. Soil pollution.

A. Definition of soil.

Soil is the result of the weathering of Earth crust, at the top layer, a process called pedogenesis and described in Figure 2. It is composed of three phases, solid, liquid and gaseous (Kabata-Pendias 2011), with various components such as mineral particles, organic matter, water, air and living organisms. Soil has a very important place in the ecosystem as it functions as the interface between the earth, the air and the water, and is also the host of most of the biosphere. Moreover, soil provides many ecosystemic services, *i.e.* production of biomass, storage of water and nutrients, pool of biodiversity as it is a habitat for a lot of species, support for human activities… (Payà Pèrez and Rodríguez Eugenio 2018). Furthermore, soil is a non-renewable resource. Thus, its healthy state is of primary importance and as soil is a geochemical sink for any contamination, this good state is in jeopardy, which can also induce a contamination of water streams through leaching.

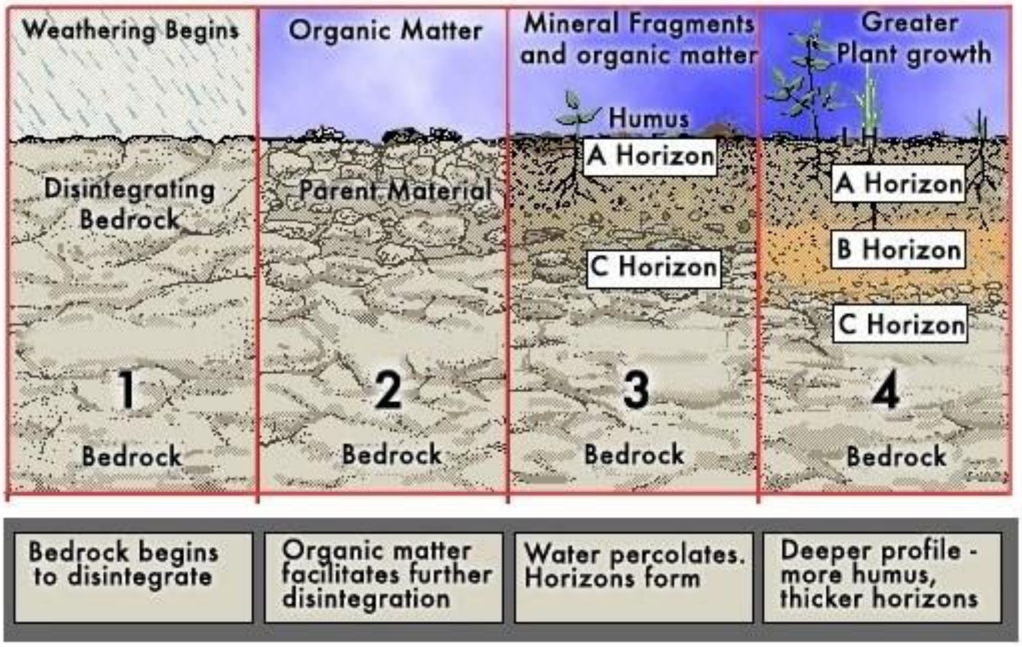


Figure 2 The formation of soil through weathering of the parental rock (source: quora.com).

B. What is soil pollution?

As stated above, the good state of soil is vital; however the past and present human activities led to the degradation of the soil. Soil degradation, defined as the process which lowers the capacity of the soil to produce its services, is recognized as a serious and widespread problem (Oldeman 1992). Soil degradation is caused by eight major threats: erosion, organic matter decline, contamination, salinization, compaction, soil biodiversity loss, landslide and flooding (Payà Pèrez and Rodríguez Eugenio 2018). Among them, soil contamination is the third most important one in Europe (Rodríguez-Eugenio *et al.* 2018).

A soil is considered contaminated when its chemical state deviates from the normal composition (Kabata-Pendias 2011), which causes a loss of its function(s) (Stolte *et al.* 2016). However, a contamination on a site is not necessarily threatening to humans (Rodríguez-Eugenio *et al.* 2018). Oppositely, when such abnormal levels of contaminants are or may become detrimental to human health, it is called pollution (Rodríguez-Eugenio *et al.* 2018).

C. Sources of pollution.

There are two distinct sources of pollution: natural and anthropogenic.

The natural source of pollution comes mainly from the weathering of the earth crust and geological breakdown of the parent rock materials. Other natural sources of pollution are volcanic eruption, continental dust and erosion (Gong *et al.* 2018, Gupta *et al.* 2019, Saxena *et al.* 2019). Such sources usually give low pollution levels.

On the contrary, anthropogenic sources, which have drastically increased with the industrial era, led to high pollution levels. For instance, in Europe in 2004, the total emissions of lead (Pb) and cadmium (Cd) were 5,580 tons and 181 tons, respectively (Van Ginneken *et al.* 2007). Anthropogenic sources are divided in seven categories of activities: waste disposal, industrial and commercial activities, military activity, storage, transport and spills on land, nuclear operations and others (Panagos *et al.* 2013). Figure 3 shows the proportions of those different sources in Europe in 2011 (from Van Liedekerke *et al.* 2014), more than 70 % of the pollution in Europe comes from waste disposal and treatment as well as industrial and commercial activities.

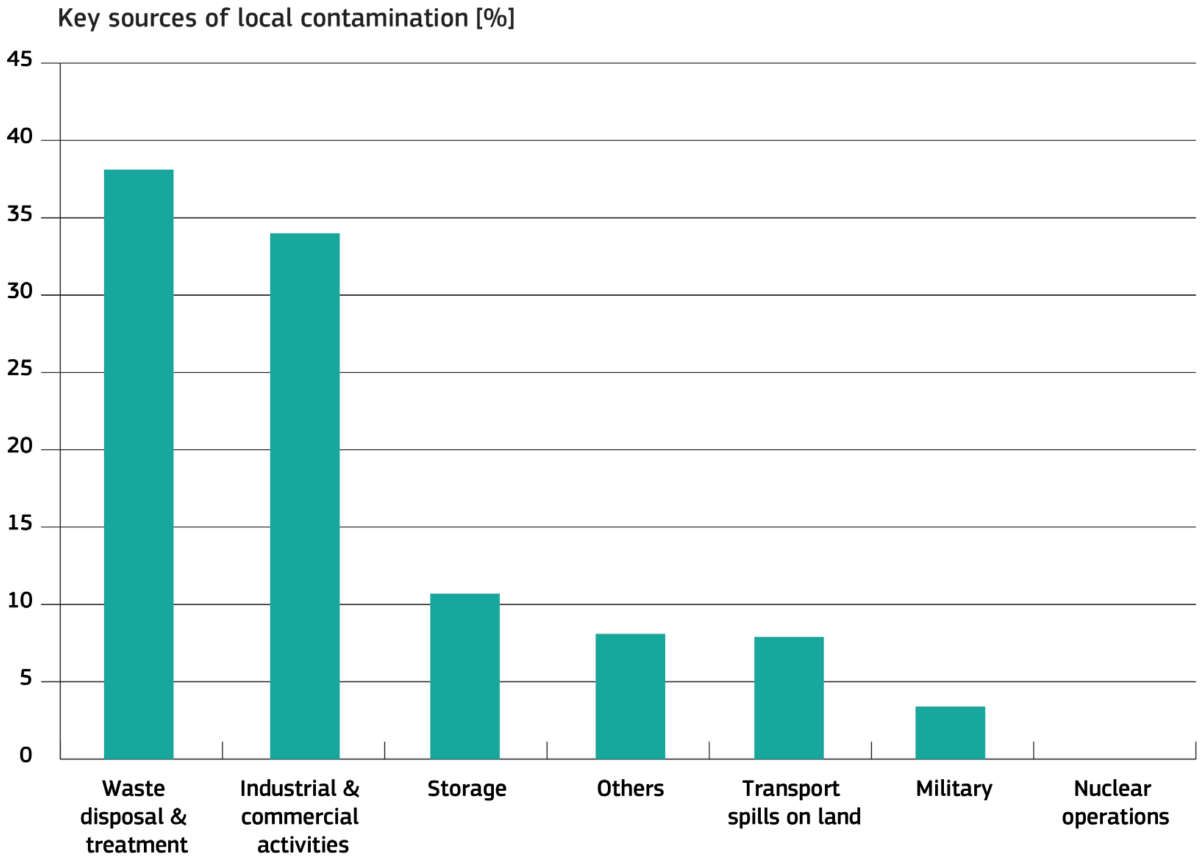


Figure 3 Proportion of the pollution sources in Europe (source: Van Liedekerke et al. *2014).*

D. State of pollution.

Soil pollution is recognized as one of the major issues worldwide. Indeed, more than ten million of contaminated sites have been identified (Khalid *et al.* 2016). Among those, 2.5 million are located in Europe, even though it represents only 7 % of the emerged land, which gives on average 3.6 sites per km² or 5.7 sites per 10,000 habitants (Van Liedekerke *et al.* 2014, Payà Pèrez and Rodríguez Eugenio 2018). Furthermore, more than 11.7 million sites are potentially contaminated (Stolte *et al.* 2016) and, as shown in Figure 4 (from Van Ginneken *et al.* 2007), this number is even underestimated as data are not or poorly available in some part of Europe. Two countries will be detailed, France and Italy.

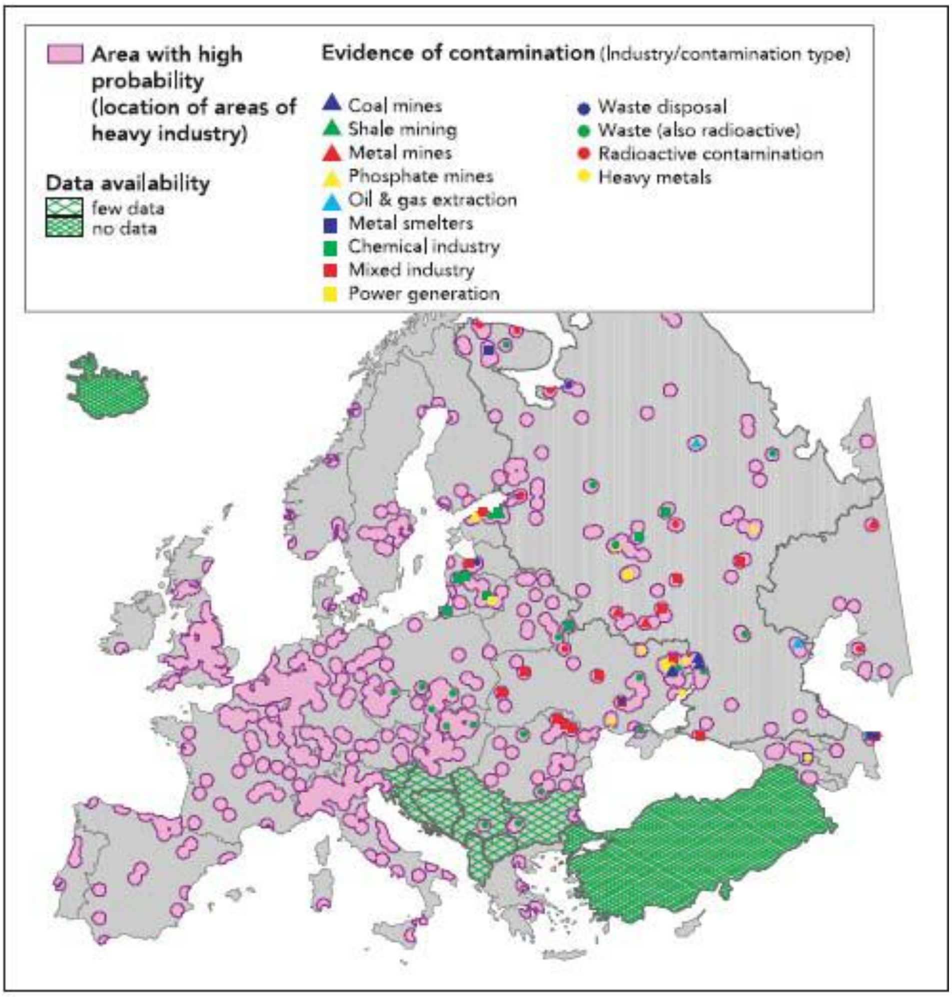


Figure 4 Map showing the contaminated areas in Europe, with their sources (source: Van Ginneken et al. *2007).*

France counts 7,065 polluted sites (BASOL database, June 2019), with almost three quarters coming from industrial and commercial activities (Table 1) (Van Liedekerke *et al.* 2014); indeed, France has an important mining past, notably in the North region with charcoal and iron mines.

Table 1 Repartition of the pollution sources for France and Italy in 2011 (adapted from Van Liedekerke et al. *2014); ND = no data.*

	Waste disposal and treatment		Ind & Commercial activities	Military	Storage	Transport spill on land	Nuclear operations	Others
	Municipal waste	Industrial waste						
France	5%	7%	73%	ND	13%	1%	ND	1%
Italy	20%	20%	52%	1%	5%	2%	0%	0%

Similarly, the first source of pollution in Italy is industrial and commercial activities, accounting for half of it, while the second one is waste disposal and treatment (40 %) (Table 1) (Van Liedekerke *et al.* 2014). Italy counts 12,482 contaminated sites, with 58 severely contaminated (ISPRA database, 2018).

Maps showing the location of the polluted sites in France and Italy are shown in Figure 5.

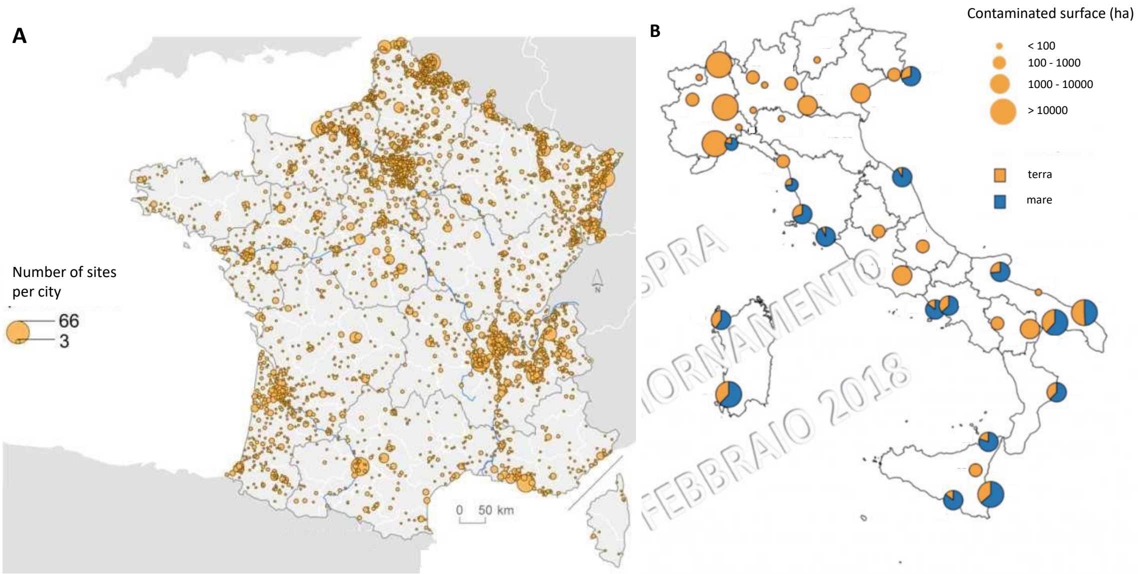


Figure 5 Maps showing the localizations of the polluted sites in France (A) (source: BASOL database, 2018, reported as number of contaminated sites per city) and Italy (B) (source: ISPRA database, 2018, reported as contaminated surface (soil or water) in ha).

E. Metal(loid) pollutants.

Both organic and inorganic pollutants are found in soils, such as nitrogen and phosphorus, polycyclic aromatic hydrocarbons, persistent organic pollutants, radionuclides, emerging pollutants, pathogenic microorganisms… (Rodríguez-Eugenio *et al.* 2018). But the most important ones are metals and metalloids (thereafter referred as metal(loid)s). Indeed, they are found in more than 50 % of the polluted soils worldwide (Khalid *et al.* 2016), while they represent 35 % of the contaminants in the European soils (Figure 6) (Van Liedekerke *et al.* 2014). Metal(loid) pollution is of great concern, as they cannot be degraded and thus persist and accumulate in soils.

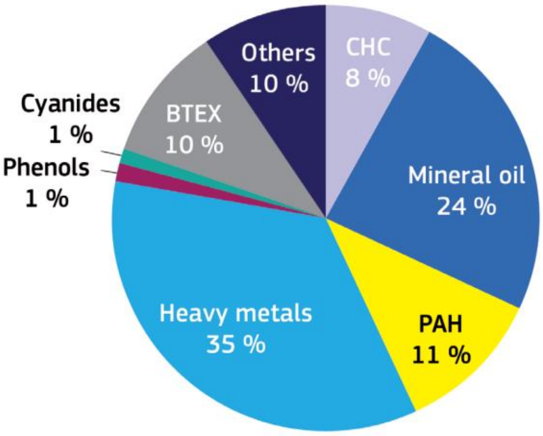


Figure 6 Most frequent pollutants in European soils (source: Van Liedekerke et al. *2014).*

Two types of metal(loid)s are distinguished: the essential ones and the non-essential ones (Figure 7). Essential metal(loid)s are, among others, iron, copper, zinc, manganese, nickel. They are known to participate in cellular functions such as redox reactions and are also part of several enzymes. They are often called micronutrients (Nagajyoti *et al.* 2010, Ashraf *et al.* 2019). On the contrary, other metal(loid)s, such as arsenic (As), Cd, and Pb, have no known function (Ashraf *et al.* 2019). They are toxic even at low concentrations, whereas essential metal(loid)s can become a problem at high concentrations (toxicity) or very low concentrations (deficiencies).

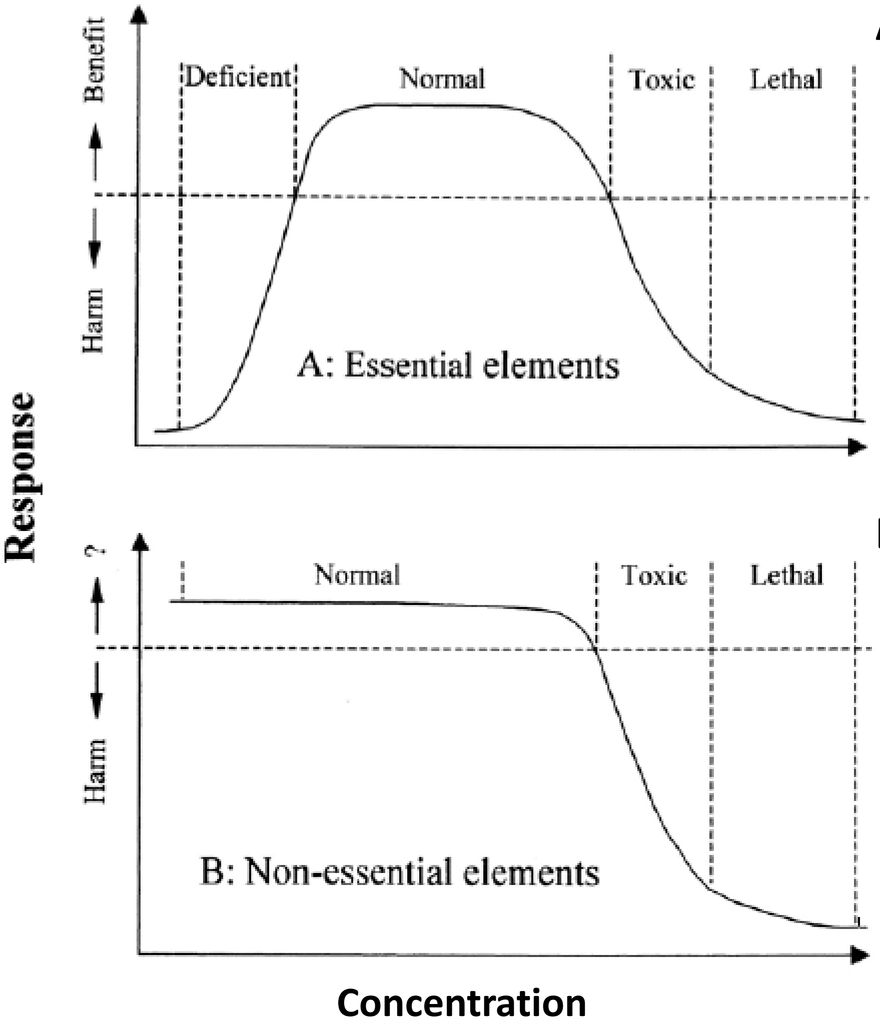


Figure 7 Concentration - response relationships for essential elements (A) and non-essential elements (B). Essential elements can cause deficiencies at too low concentrations and harmful effects at too high concentrations; non-essential elements are generally harmful at low concentrations (modified from Adriano 2001).

There are different ways people can be exposed to metal(loid)s: directly from inhalation of soil dust, drinking contaminated water and soil consumption (such as when children play on soil), or indirectly through the consumption of plants or animals that consumed contaminated materials (Figure 8) (Rodríguez-Eugenio *et al.* 2018, Saxena *et al.* 2019).

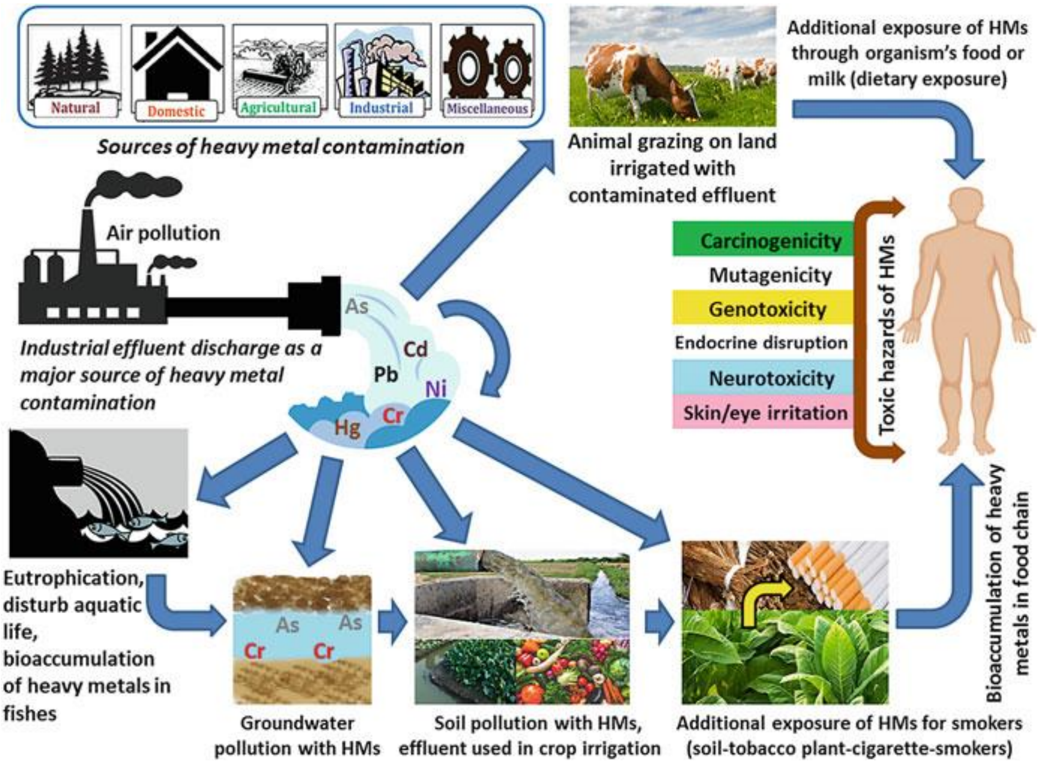


Figure 8 Pathway of human exposure to metal(loid)s (source: Saxena et al. *2019). Sources of metal(loid) contamination are multiple (industries, agriculture, domestic…); these metal(loid)s can contaminate groundwaters and soil on which plants, especially crops, will grow; following, by consuming these crops, animals will be contaminated and* in fine *humans leading to toxic effects on human beings (neurotoxicity, carcinogeninity…).*

However, the detrimental effects caused by metal(loid)s on plants and human health depend mainly on their bioavailability. Indeed, a soil can present very high levels of pollution but this pollution can be poorly available, because pollutants are bound to its residual fraction for instance, and thus present low risk; whereas a soil having low total metal(loid) concentrations with a high bioavailability will represent a higher risk. Peijnenburg and Jager (2003) defined bioavailability as the fraction of pollutant that, within a given time span, is available or will be made available for its uptake by plants and other organisms. Bioavailability depends on the physical, chemical and biological properties of the soil (Rodríguez-Eugenio *et al.* 2018). Therefore, more than the total metal(loid) concentrations in soils, it is important to determine the metal(loid) bioavailable concentrations. Such fraction can be assessed by simple extractions using salt solutions, such as calcium chloride ($CaCl_2$), ammonium nitrate (NH_4NO_3) or acetate (NH_4OAc) which simulate metal(loid) uptake by plants (Gupta *et al.* 1996, Wang *et al.* 2004). In addition, bio-indicator plants, such as *Phaseolus vulgaris*, can be used, as their growth in mesocosm is related to soil toxicity (Meers *et al.* 2007).

Among metal(loid)s, the most occurring ones are As and Pb, accounting, in France for instance, for 4.37 % and 6.09 % of the soil pollution, respectively (BASOL database, June 2019). Moreover, they are the two contaminants studied in this thesis. Thus, a brief overview of their detrimental effects is going to be made.

Arsenic is the most abundant metalloid in nature and the 20th most abundant element on Earth (Jaishankar *et al.* 2014, Ashraf *et al.* 2019). It is one of the major environmental problems around the world due to its negative effects on microbial activities but also on plant and human health (Hettick *et al.* 2015, Rodríguez-Eugenio *et al.* 2018). Indeed, As is a chemical analog of phosphate and thus can be transported by the same carriers (Nagajyoti *et al.* 2010, Farooq *et al.* 2016). Once in plants, As disrupts chloroplast structure and photosynthesis process, as well as the redox homeostasis (Mustafa and Komatsu 2016). Moreover, As has been classified as a carcinogen by the International Agency for Research on Cancer (Hughes 2002). It can also cause other health issues such as chronic weakness, skin lesions, nervous system disorders and the “blackfoot” disease (Bissen and Frimmel 2003, Hettick *et al.* 2015). Moreover, as stated above, arsenic toxicity will depend on its mobile and bioavailable fraction. In general, the available fraction of As decreased with the increasing in silt content as well as free iron content in the soil; however it increases with the rise in soil pH and organic matter content (Huang *et al.* 2006).

Similarly, Pb has no known biological function. It has adverse effects on plant growth and morphology. For instance, Påhlsson (1989) observed that with increasing Pb supply, plants presented smaller and chlorotic leaves, a stunted growth as well as photosynthesis and transpiration rate decreases. Furthermore, as arsenic, Pb is carcinogenic (Jaishankar *et al.* 2014) and it can destroy the plasma membrane, disturb electron transfer reactions and cause biochemical imbalances in diverse organs (Kumar and Aery 2016, Rodríguez-Eugenio *et al.* 2018). Similarly to arsenic, Pb toxicity depends on its bioavailability, which is low at slightly acid to neutral pH (pH 5.5 to 7.5) (Sharma and Dubey 2005). Moreover, soil generally has a high sorption capacity towards Pb, through the precipitation of Pb as carbonates, its fixation to the organic matter and its sorption to hydroxides (Zimdahl and Skogerboe 1977). Other soil parameters will affect Pb bioavailability, such as the redox potential, the cation exchange capacity, the soil mineralogy… (Pourrut *et al.* 2011).

Due to the high levels of pollution, covering an important area, and its negative effects on plant and human health, the necessity to remediate such area is recognized worldwide, socially and politically (Van Ginneken *et al.* 2007).

II. Soil remediation techniques.

A. Definition.

The term remediation, when applied to contaminated soils, refers to the lowering of the harmful effects of pollutants, with the aim to reduce people exposure as well as the recovery of environment functions (Payà Pèrez and Rodríguez Eugenio 2018). It can be divided in two main categories: (1) *in situ* treatments, which consist in the treatment on-site and leave soil structure intact, and (2) *ex situ* techniques, which involve soil excavation and treatment off-site (Payà Pèrez and Rodríguez Eugenio 2018, Rodríguez-Eugenio *et al.* 2018). Finally, remediation processes include physical, chemical and biological processes, which will be detailed in this section (Rodríguez-Eugenio *et al.* 2018, Ashraf *et al.* 2019).

B. Physical methods.

There are four main physical remediation methods: soil replacement, surface capping, encapsulation and thermal desorption (Table 2).

Soil replacement consists on removing the contaminated soil from the site and replacing it by non-contaminated soil. The contaminated soil can be completely or partly replaced. It allows to dilute metal(loid) contents and improve soil potential. However, such approach is costly and usually only applicable on small scales (Khalid *et al.* 2016, Gong *et al.* 2018, Nejad *et al.* 2018).

The remediation by surface capping does not involve the removal of the contaminated soil, but rather the installation of a cover, generally made of geotextile covered with garden soil and sown by plant seeds, on the surface, which will support the vegetation and diminish water leaching. It is less expensive than soil replacement and is a good choice for highly contaminated soils. However, as soil replacement, it is mainly applicable on small areas (Hamby 1996, Mulligan *et al.* 2001, Liu *et al.* 2018).

Similarly, soil encapsulation uses physical barrier systems, but not only on the surface, because barriers are composed of a low permeable cap, an underground barrier and barrier floors. In this way, the contaminated soil is immobilized, preventing the contamination of the surrounding (Khalid *et al.* 2016, Liu *et al.* 2018). Barriers are generally made of synthetic textile or clay layers with low permeability, which prevents surface water infiltration and thus leaching, while the underground barriers prevent horizontal migration of the pollutants.

Finally, thermal desorption is performed by heating the soil. It uses for such approach steam, microwave or infrared radiations. It is applied *in situ* but requires the collection of volatilized metal(loid)s (Gong *et al.* 2018).

Table 2 Definitions of the different physical and chemical remediation techniques (from Hamby 1996, Mulligan et al. *2001, Khalid* et al. *2016, Gong* et al. *2018, Liu* et al. *2018).*

Technique	**Definition**
Soil replacement	Removal of the contaminated soil and replacement by non-contaminated soil.
Surface capping	Installation of a cover on the surface, which will support the vegetation and diminish water leaching
Soil encapsulation	Physical barrier systems which prevents surface water infiltration, leaching and horizontal migration of the pollutants
Thermal desorption	Heating of the soil and collection of the volatilized metal(loid)s
Soil washing	Excavation of the soil, mixing with various reagents or extractants, which removes contaminants and return to the site
Solidification	Encapsulation of the contaminated soil by mixing it with binding agents
Stabilization	Application of chemical reagents to render metal(loid)s less mobile and less toxic
Vitrification	Heating of the soil, between 1,600 and 2,000 °C, which transforms it into glass like solid of a smaller volume
Electrokinetic	Application of a low intensity current and recovery of metal(loid)s accumulated at the two electrodes
Oxidation/neutralization/ reduction	Utilization of solutions to detoxify, precipitate or solubilize metal(loid)s

C. Chemical methods.

Chemical remediation is divided into five techniques: soil washing, solidification/stabilization, vitrification, electrokinetic and oxidation/neutralization/reduction (Table 2).

Soil washing is performed *ex situ*. The soil is excavated and mixed with various reagents or extractants, which removes contaminants. Then, the clean soil is returned to the site. This technique is highly effective and rapid. However, it renders the soil inappropriate for future vegetation (Gong *et al.* 2018, Liu *et al.* 2018).

Similarly, solidification and stabilization are performed *ex situ*. Solidification encapsulates the contaminated soil by mixing it with binding agents, while stabilization uses chemical reagents to render metal(loid)s less mobile and less toxic (Liu *et al.* 2018, Nejad *et al.* 2018, Ashraf *et al.* 2019).

Vitrification is a thermal *ex situ* technique. A high temperature, between 1,600 and 2,000 °C, is applied which incinerates organic and mineral matters, thus transforming the contaminated soil into glass like solid, which has a smaller volume. However, such method is destructive and the material can no longer support vegetation (Hamby 1996, Liu *et al.* 2018, Nejad *et al.* 2018).

Electrokinetic method applies a low intensity current between a cathode and an anode, and a buffer solution is added to maintain the pH. Under such electric current, metal(loid)s migrate towards the anode for negatively charged ions and the cathode for positively charged elements. Following, metal(loid)s that accumulate at the two electrodes can be recovered and treated (Mulligan *et al.* 2001, Gong *et al.* 2018, Liu *et al.* 2018).

Finally, oxidation/neutralization/reduction processes use solutions to detoxify, precipitate or solubilize metal(loid)s. In oxidation, solutions such as potassium permanganate, hydrogen peroxide, hydrochlorite or chlorine gas, are used, while reduction utilizes alkali solutions and neutralization aims at adjusting pH (Hamby 1996, Mulligan *et al.* 2001).

Both physical and chemical methods have been intensively used in the past. However, due to their high cost and negative effects on soil, such "conventional" techniques are less used nowadays, which led to the development of biological methods, such as phytomanagement.

D. Phytomanagement, a biological remediation method.

Contaminated soils can be remediated by plants and their associated microbiota in order to remove, uptake or render harmless contaminants (Ghosh and Singh 2005, Cristaldi *et al.* 2017). Such process has been used for the past 300 years; however the term "phytoremediation" referring to it has been first introduced in 1983 (Ghosh and Singh 2005). Compared to the high cost of conventional techniques, phytoremediation is much less expensive, around 60,000 to 100,000 $ per ha (Tack and Meers 2010), which is on average 1.5 to 5 times cheaper than conventional remediation processes (Gomez *et al.* 2019). Moreover, such process can be optimized by manipulating the soil-plant system in order to recover metal(loid)s and produce biomass for energy, adding economic benefits. Such method is called phytomanagement (Evangelou *et al.* 2015, Kidd *et al.* 2015, Tack and Egene 2019).

In this process, vegetation is established on barren contaminated soils, which in addition to the uptake of contaminants, reduce wind erosion and water leaching, lowering the risk of contamination spreading.

Phytomanagement has many advantages. It is a low cost technique, since it does not require excavation of the soil and demands little labor, as it relies on solar energy-driven technology (Khalid *et al.* 2016, Burges *et al.* 2018, Nejad *et al.* 2018). Moreover, it is aesthetically pleasing and ecofriendly. Indeed, it does not disrupt the ecosystem and leave the site in a good state. It also does not damage the landscape and increases the biodiversity by creating new habitats (Cristaldi *et al.* 2017, Burges *et al.* 2018, Gomez *et al.* 2019). Finally, it is suitable for large contaminated areas.

However, phytomanagement has some limits. The most important one being duration. Indeed, to remediate soils, phytomanagement requires several plant growing periods (Nejad *et al.* 2018). Moreover, it is often limited to root depth. The success depends also on soil and climatic conditions. Finally, phytomanagement process can introduce non-native species into the environment.

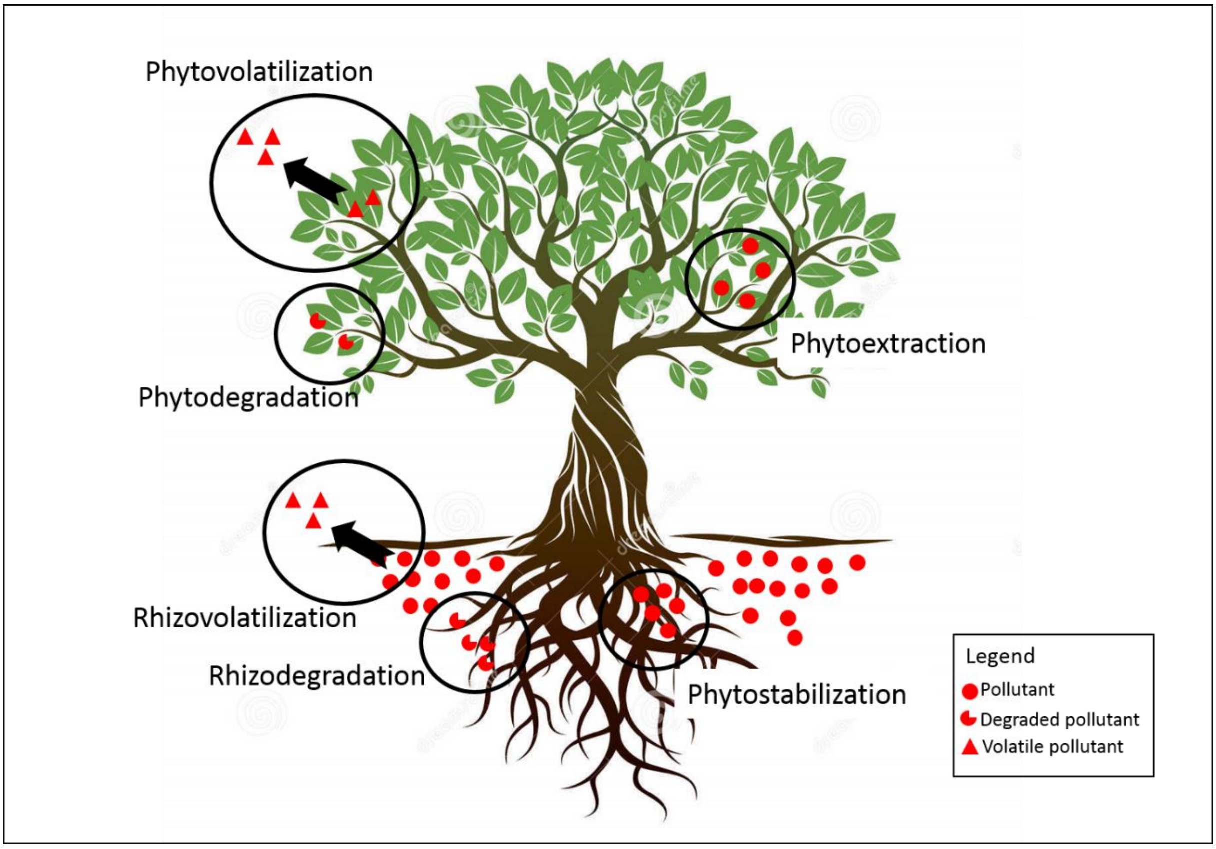


Figure 9 The different techniques of phytomanagement: rhizo- and phyto-degradation (breakdown of organic pollutants by plants and microorganisms), rhizo- and phyto-volatilization (uptake of pollutants from the soil, transformed in volatile forms and release into the atmosphere), phytoextraction (uptake of pollutants from the soil by the roots and translocation to upper parts) and phytostabilization (immobilization of the pollutants by adsorption or absorption by the roots).

There are different techniques in phytomanagement, depending on the goals. They are presented in the Figure 9 and will be detailed, with a focus on the two main techniques, phytoextraction and phytostabilization.

Phyto/Rhizodegradation

This technique is mainly suited for organic contaminants. Plants, and their associated microorganisms, breakdown contaminants through metabolic processes into smaller molecules. It can happen in any plant parts as well as in microorganisms, whose activity (of degradation) can be enhanced by plant presence, and plant root secretion (Mirck *et al.* 2005, Wenzel 2008).

Phyto/rhizovolatilization

In this technique, pollutants are taken up from the soil, converted into volatile forms and released into the atmosphere. It is mainly performed for organic pollutants as well as some metal(loid)s such as mercury (Ali *et al.* 2013, Khalid *et al.* 2016). However, the volatilized pollutant can contaminate the air and also be returned to the soil through rain.

Phytoextraction

It is one of the main biological techniques. Phytoextraction involves the "real removal" of the contamination. Indeed, plant roots take up contaminants from the soil, which are then translocated and accumulated into aboveground parts. Usually, aboveground concentrations are higher than root concentrations (Gomes *et al.* 2016, Khalid *et al.* 2016). Following, aboveground tissues are collected and metal(loid)s, especially the ones of economic interest such as gold and nickel, are recovered. It is called phytomining. Thus, with several growing-harvesting cycles, contamination is removed until a level meeting environment regulation (Liu *et al.* 2018). However, such technique is usually a good option when only one or few elements are concerned, as plants possess the ability to accumulate a limited number of elements. Moreover, it is best suited for low to medium contamination levels, present at the root surface (Ghosh and Singh 2005). Finally, it requires extra work to harvest the contaminated biomass, even when accumulated contaminants present no interest, to prevent it from going back to the soil at leaf falls or through the consumption of this contaminated biomass by animals, which would add the risk of entry of the pollution into the food chain.

Phytostabilization

This is the second most current methods. Contrary to phytoextraction, phytostabilization is not a real decontamination process, as pollutants are not removed from the soil but rather immobilized, to prevent their migration to non-contaminated surrounding areas through soil erosion and water leaching (Khalid *et al.* 2016, Ashraf *et al.* 2019). It is a good option with highly contaminated areas, where phytoextraction would take too much time. Phytostabilization relies on the establishment of a plant cover that will stabilize the soil pollutants. The pollutants will be immobilized into or onto the roots and translocation to upper parts is reduced. Immobilization can be done through root adsorption, root absorption, exudate complex/precipitation (Liu *et al.* 2018). Phytostabilization has the advantage of not requiring biomass harvest and treatment to prevent contamination return. Moreover, as metal(loid) concentrations are low in aboveground parts, the recovered biomass can be used for energy production or other industrial processes. It also prevents the entry of contamination into the food chain (Tack and Meers 2010).

One of the major parameters for the success of phytomanagement is the choice of the plant species. Indeed, to be used in phytomanagement, ideally a plant must have the following characteristics: (1) tolerance to the high metal(loid) concentrations found in the soil, (2) ability to grow on agronomically poor soils, (3) fast growth, (4) high biomass production, (5) deep and wide root system and (6) ability to accumulate metal(loid)s in its different parts, particularly in the roots (Guerra *et al.* 2011, Gerhardt *et al.* 2017).

Based on their metal(loid) accumulation pattern, plants are grouped in three categories (Figure 10):

- Metal(loid) excluders: they accumulate metal(loid)s in their roots but restrict their transport to the aboveground tissues (Alkorta *et al.* 2004, Ali *et al.* 2013). Such species are best suited for phytostabilization.
- Metal(loid) indicators: they accumulate metal(loid)s in their aerial part to levels that reflect the substrate concentrations (Alkorta *et al.* 2004, Ali *et al.* 2013).
- Metal(loid) (hyper)accumulators: they uptake metal(loid)s and translocate them in their aboveground parts. Aerial concentrations are higher than soil levels (Alkorta *et al.* 2004, Ali *et al.* 2013). Hyperaccumulators are best suited for phytoextraction. More than 400 species have been described as hyperaccumulators, most of them being nickel hyperaccumulators (Barceló and Poschenrieder 2003).

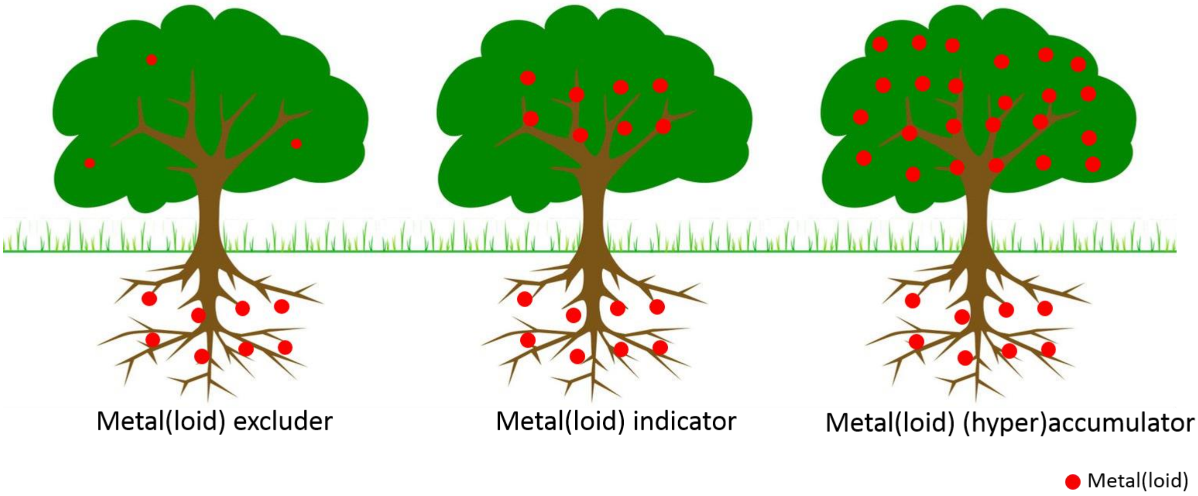


Figure 10 The different categories of plant depending on their capacity to accumulate metal(loid)s: metal(loid) excluders restrict metal(loid)s to the roots with a reduced translocation to upper parts, metal(loid) indicators present metal(loid) concentrations in above ground tissues similar to soil concentrations and metal(loid) (hyper)accumulators concentrate metal(loid)s in their upper tissues, at concentrations higher than the soil concentrations.

However, even though the plant species selected can tolerate such high contamination levels and the climatic conditions, they can still have difficulties to establish due to the low fertility of the contaminated soils. Indeed, in addition to high metal(loid) levels, contaminated soils often present extreme pH conditions, low organic matter and nutrient contents, that hinder plant growth. Therefore, the use of amendments is often recommended in phytomanagement.

III. Amendment applications in phytomanagement processes.

As stated above, contaminated soils are often inappropriate for plant establishment and growth. Indeed, contaminated soils usually present an acidic pH with low nutrient contents and availabilities, as well as a low organic matter content, demonstrating their low fertility. Such conditions, together with the high metal(loid) concentrations, hinder plant growth. Thus as a possible response, amendments must be applied.

Application of amendments to contaminated soils serves multiple purposes:

- It can supply nutrients for plant growth and microorganism activity;
- It can improve soil physico-chemical properties, such as reducing soil acidity; and
- It can immobilize metal(loid)s.

All of this ameliorates soil conditions and thus authorizes plant growth.

Different amendments can be applied, either organic or inorganic (Rizwan *et al.* 2017). The choice of the amendment to apply will depend on the soil type and metal(loid)s present. Many amendments can and have been used in metal(loid) remediation studies, such as peat, sludge, ashes, manure, lime, slurry, fly ash, phosphates, organic matter, biosolids, fertilizers, humic substances, green waste… (Dimitriou *et al.* 2006, Padmavahiamma and Li 2010, Vandecasteele *et al.* 2010, Houben *et al.* 2012, Galende *et al.* 2014, Mahar *et al.* 2015, Tang *et al.* 2015, Fresno *et al.* 2016, Moreno-Jiménez *et al.* 2016, Rehman *et al.* 2016, Zhang *et al.* 2016a, Karer *et al.* 2017, Kiran *et al.* 2017, Zeng *et al.* 2017, Egene *et al.* 2018, Fresno *et al.* 2018, Pukalchik *et al.* 2018, Rocco *et al.* 2018, Kumpiene *et al.* 2019). However, three types of amendments have gathered interests to use in phytomanagement over the last decades and will be detailed here: (1) compost amendments are mainly used to provide nutrients and organic matter, (2) biochars have a high sorption capacity, especially towards cations, and a liming effect, and (3) iron based amendments have affinities towards metal(loid)s, especially arsenic.

A. Compost.

One of the most negative properties of contaminated soils is their poor fertility; thus nutrients need to be applied. Compost is often used in this purpose in agriculture. It is a stabilized and sanitized product that comes from the microbial degradation of organic wastes (Diacono and Montemurro 2010, Huang *et al.* 2016a).

Compost is usually rich in humic substances (high molecular weight substances that are the end point of the soil organic degradation), microorganisms as well as plant nutrients and essential trace elements (Fisher and Glaser 2012, Huang *et al.* 2016a). Indeed, many studies used compost as an amendment and showed that composts had a neutral to alkaline pH, a high electrical conductivity, elevated contents in organic matter and nutrients, characteristics that vary between composts. The properties of different composts used previously, with the references, are listed in Table 3.

Table 3 Physico-chemical properties of different compost used in soil remediation (available concentration, EC = electrical conductivity, OM= organic matter, TOC = total organic carbon, empty cases signify that no data was available).*

Feedstock	**pH**	**EC (μS.cm^{-1})**	**OM content (%)**	**TOC content (mg.kg^{-1})**	**Elemental composition (mg.kg^{-1})**						**Moisture content (%)**	**Reference**
					N	**P**	**K**	**Ca**	**Mg**	**Na**		
Mixed municipal solid waste	8.2	5,690	37.20		18,000	6,100						Alvarenga *et al.* (2014)
Green waste	7.9	2,470	39		10,000	900						Alvarenga *et al.* (2014)
Municipal solid waste	8.7	1,300		232,000	14.8	83 *						Brunetti *et al.* (2012)
Urban organic waste + plant trimmings + tobacco + aromatic plant residues	7.25			321,900		18,730	16,910					Caporale *et al.* (2013)
Grasses + stubles + plant leaves	8.2	4,900	55.2		11,000	2,500	9,800	25,000	2,700			Celik *et al.* (2004)
Green waste	8.11	700	25.20		6,200	2,800	7,400	2,200		1,100		Farrell and Jones (2010)
Green waste + catering waste	7.8	1,500	44.20		12,000	5,000	9,900	4,100		2,000		Farrell and Jones (2010)
Green waste + catering waste + paper waste	8.12	1,400	39.80		13,700	5,900	10,800	7,800		2,500		Farrell and Jones (2010)
Municipal solid waste	7.28	3,600	42.20		9,700	900	10,100	51,800		8,300		Farrell and Jones (2010)
Peat	5.51	700	93.70		10,500	500	5,000	11,100		900		Farrell and Jones (2010)
Urban waste	7.6			274,000	19,000	9,000					29.5	Giusquiani *et al.* (1995)

Table 3. end

Feedstock	**pH**	**EC (μS.cm^{-1})**	**OM content (%)**	**TOC content (mg.kg^{-1})**	**Elemental composition (mg.kg^{-1})**						**Moisture content (%)**	**Reference**
					N	**P**	**K**	**Ca**	**Mg**	**Na**		
Mixed compost	6.09	6,020		26,400		66,000	33,000					Kobayashi *et al.* (2008)
Cow manure	9.82	8,090		24,400								Kobayashi *et al.* (2008)
Alperujo	8.1		29.10			25,400	23,000	138,000	14,800	0.17	14.90	Madejón *et al.* (2014)
Biosolid	7.09		22.60			34,300	8,200	125,000	12,300	0.10	15.60	Madejón *et al.* (2014)
Household	6.7			231,000	37,000	11,000	14,000	32,000	3,900	1,000		Mokolobate and Haynes (2002)
Cattle manure		16,300	63			10,900					49	Ramos (2017)
Sewage sludge	6.8	3,000		100,000	15,000	17,900	12,100	110,700	9,020			Rossini-Oliva *et al.* (2017)
Sewage sludge + olive waste	7	4,200		160,000	26,000	13,600	10,000	146,300	11,600			Rossini-Oliva *et al.* (2017)
Municipal solid waste + bark chippings	6.18				14,000	11,800	5,600	30,100	6,800			Touceda-González *et al.* (2017)
Mean	7.50	4,276	44.65	171,213	14,436	13,512	12,558	57,008	8,731	1,975	27.25	
Min	5.51	700	22.60	24,400	14.8	83*	5,000	4,100	3,900	0.1	14.90	
Max	9.82	16,300	93.70	321,900	26,000	66,000	23,000	146,300	14,800	8,300	49	

Such properties make compost a good soil conditioner in phytomanagement.

Indeed, compost application improves soil physico-chemical properties. Many studies showed that compost amendment increased pH, electrical conductivity (EC), water holding capacity (WHC). For instance, in 2006, Ruttens *et al.* applied a commercial compost to a metal(loid) contaminated soil and found that pH increased, mostly due to the alkalinity of the compost. Later, Brown and Cotton (2011) observed that compost increased soil nutrient content, soil organic content and WHC. Finally, Liu *et al.* (2012) observed a soil pH increase following the application of a compost made of 50 % green waste, 35 % chopped wood and 15 % soil with woody debris.

Moreover, compost being rich in microorganisms, it has beneficial effects on the soil microbial diversity and activity, as observed by Brown and Cotton (2011) and in the reviews of Diacono and Montemurro (2010) and Hargreaves *et al.* (2008).

Furthermore, compost humic substances can complex with metal(loid)s, thus immobilizing them (Huang *et al.* 2016a). Indeed, Walker *et al.* (2004) observed that Pb availability was decreased by compost addition, due to an immobilization of Pb by humified organic matter (OM). Similarly, Ruttens *et al.* (2006) showed that metal(loid) extractable fractions decreased, whereas Alvarenga *et al.* (2014) found that compost decreased Cu bioavailability. However, due to its OM content that can interact with some metal(loid)s such as arsenic, compost has been shown to mobilize arsenic (Hartley *et al.* 2009, Clemente *et al.* 2010).

Finally, all those beneficial effects on soil induce an improvement of plant growth: *Phaseolus vulgaris* (Caporale *et al.* 2013), maize (Rehman *et al.* 2016) and *Agrostis tenuis* (Alvarenga *et al.* 2014), all showed an increase in biomass and other growth parameters such as photosynthesis.

An overview of compost effects on soil and plant is shown Table 4.

Table 4 Overview of compost effects on soil and plants (CEC= cation exchange capacity, WHC = water holding capacity, SOM = soil organic matter, EC = electrical conductivity, OC = organic carbon, ↗ = increase, ↘ = decrease, empty cases signify that data was not available).

Metal(loid)s	**Compost(s)**	**Plant(s)**	**Effects**	**Reference**
As, Cu, Pb, Zn	Mixed municipal solid waste Green waste	*Agrostis tenuis*	↘ soil acidity ↗ organic matter ↘ bioavailable [Cu] ↗ bioavailable [As] ↗ soil enzyme activities ↗ plant biomass	Alvarenga *et al.* (2014)
Cr, Cu, Pb, Zn	Municipal solid waste	*Brassica alba* *Brassica carinata* *Brassica nigra*	↗ plant dry weight	Brunetti *et al.* (2012)
As	Urban organic waste + plant trimmings + tobacco and aromatic plant residues	*Phaseolus vulgaris*	↗ plant biomass ↗ leaf chlorophyll content ↗ P content in plants ↘ [As] in plant tissues	Caporale *et al.* (2013)
As, Pb	Greenwaste		↗ soil pH ↗ soil WHC ↗ soil total [Pb], [As] ↗ As mobility	Clemente *et al.* (2010)
	Grasses + stubles + plant leaves		↗ SOM ↘ soil bulk density ↗ soil WHC	Celik *et al.* (2004)
Cu	Vermicompost		↗ soil OC content ↗ soil pH ↘ available [Cu] ↗ available [K], [P], [Mg] ↘ Al and Zn mobility ↗ Ca, Mg, K, Fe mobility	Ferreira *et al.* (2018)
	Urban waste		↗ soil OC content ↗ soil CEC ↗ soil enzyme activities	Giusquiani *et al.* (1995)
	Green waste + chopped wood + woody debris		↗ nitrogen content ↗ available [P], [Na] ↗ soil CEC ↗ soil water content	Liu *et al.* (2012)

Table 4. end

Metal(loid)s	**Compost(s)**	**Plant(s)**	**Effects**	**Reference**
None	Household	*Zea mays*	↗ exchangeable [Ca], [Mg] ↗ organic [P] ↗ soil pH ↗ plant yield	Mokolobate and Haynes (2002)
	Farmyard manure Vine pruning wastes Sewage sludge		↗ soil OC content ↗ soil nitrogen content ↗ soil basal respiration ↗ soil enzyme activities	Nicolás *et al.* (2012)
Ni	Commercial	*Zea mays*	↗ plant height ↗ root length ↗ plant dry weight ↗ leaf chlorophyll content ↗ plant transpiration rate ↗ stomatal conductance ↗ plant [Cu], [Mn], [Zn], [Fe]	Rehman *et al.* (2016)
As, Cd, Cu, Pb	Sewage sludge Sewage sludge + olive waste	Ryegrass Ahipa Tomato	↗ soil pH ↗ soil OC content ↗ soil enzyme activities ↗ soil microbial activities ↗ plant biomass ↗ leaf chlorophyll and carotenoid contents	Rossini-Oliva *et al.* (2017)
Zn, Cd, Cu	Organic domestic waste + garden debris	*Phaseolus vulgaris*	↗ soil pH ↘ extractable [Zn], [Cd], [Cu] ↘ phytotoxicity	Ruttens *et al.* (2006)
		Natural colonization	↗ leaf area ↘ plant [Zn], [Cd], [Cu]	
Cu, Pb, Zn	Olive leaves + solid fraction of olive mill wastewater	*Chenopodium album*	↗ soil EC ↘ tissue [Pb], [Zn]	Walker *et al.* (2004)
Pb, Cd		Oilseed rape	↗ plant dry weight ↘ shoot [Cd], [Pb]	Wu *et al.* (2011)

Compost has many beneficial effects on soil and plant. Indeed, as seen in Table 4, compost application can reduce soil acidity, metal(loid) bioavailability and provide nutrients which will improve soil microbial activity but also plant growth parameters (dry weight production, pigment contents, nutrient uptake). However it is highly degradable and thus needs to be re-applied regularly. Therefore, a more stable product can be used, *i.e.* biochar.

B. Biochar.

Biochar has gathered attention over the last decades as amendment in phytomanagement processes, and especially phytostabilization, due to its positive effects on soil conditions, plant growth and its sorption capacity. Moreover, biochar has the advantage of being stable compared to compost, with a half-life of several thousand years (Zama *et al.* 2018).

Biochar is defined as the solid product of pyrolysis of biomass (Paz-Ferreiro *et al.* 2014, Wisziewska *et al.* 2016). Pyrolysis process, which produces bio-fuel and biochar (Figure 11), consists of the thermochemical transformation of biomass in the absence of oxygen, or under limited oxygenated conditions (Hagemann *et al.* 2018, Tack and Egene 2019), with a temperature usually ranging from 200 to 1,000 °C (Yu *et al.* 2019). Biochar has the advantages of being sustainably and easy to produce from all kinds of biomass feedstocks, even waste materials (Qian *et al.* 2015). Moreover, biochar permits the sequestration of carbon and thus reduces greenhouse gas emissions (Barrow 2012).

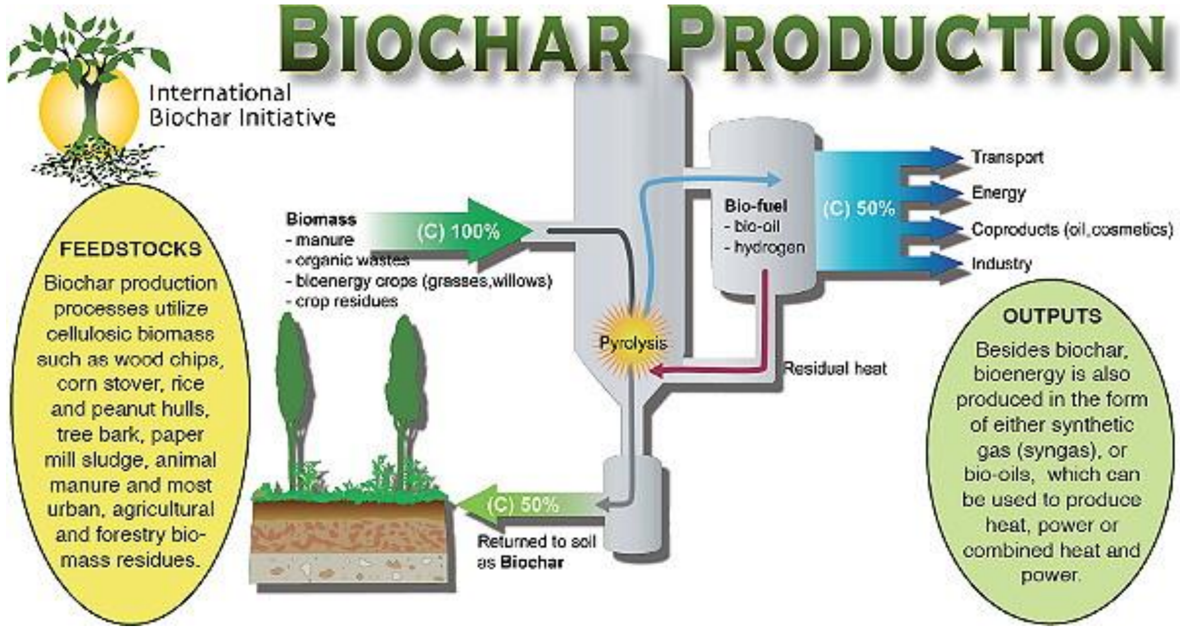


Figure 11 Scheme of the pyrolysis process (source: International Biochar Initiative). The biomass enters the system and is pyrolyzed in absence of oxygen. From this process, two products are formed: (i) bio-fuel, composed of bio-oil and hydrogen, which can serve in the energy and the transport industries, and (ii) biochar, which can be used as soil amendment.

Biochar is characterized by a porous and carbonaceous structure (Paz-Ferreiro *et al.* 2014). Biochar is usually defined by its physico-chemical properties, such as pH, electrical conductivity, cation exchange capacity, ash content, surface area as well as C, H, N and O contents (Table 5).

As shown in Table 5 and the boxplot representations (Figure S1), biochar has a high surface area. Previous studies observed surface areas from 20 cm².g^{-1}, for a biochar obtained from the pyrolysis at 300 °C of eucalyptus wood (Lu *et al.* 2018), to 1,331 m². g^{-1}, for a wheat straw biochar pyrolyzed between 350 and 550 °C (Liu *et al.* 2016a), with an average value of 130 m².g^{-1}. Surface area is highly variable depending on feedstock and pyrolysis temperature. For instance, Jindo *et al.* (2014) characterized biochars made at different temperatures from four biomass materials and observed surface areas from 5.60 to 545.43 m².g^{-1}, whereas Lee *et al.* (2013) found surface areas from 13.6 to 316 m².g^{-1} in biochars made from biogases, cocopeat, paddy straw, palm kernel shell and umbrella tree pyrolyzed at 500 °C. Such elevated surface area is important for metal(loid) complexion (Paz-Ferreiro *et al.* 2014).

As stated above, biochar is mainly composed of carbon, between 38 and 80 % on average (Yuan *et al.* 2018), as well as hydrogen, oxygen and nitrogen (Tan *et al.* 2017). However, some researches observed values outside of this range. For instance, the studies presented in Table 4 revealed that carbon content was 58.22 % on average, with a minimum measured at 3.61 % (cow manure biochar at 500 °C, Uzoma *et al.* 2011) and a maximum at 94.56 % (sawdust of white spruce biochar at 700 °C, Kwak *et al.* 2019). Hydrogen content was found between 0.26 % and 12.2 %, for a manure biochar pyrolyzed at 700 °C (Kwak *et al.* 2019) and a biochar from municipal solid waste pyrolyzed at 400 °C (Jin *et al.* 2014), respectively, the average value was calculated at 2.94 % (Figure S1, Table 5). Nitrogen and oxygen contents showed higher ranges of values, from 0 % to 31.5 % for nitrogen and from 0 % to 66.09 % for oxygen, with averages at 1.39 % and 17.88 % respectively (Figure S1, Table 5). Biochar also contains mineral elements such as silicate, phosphorus (P), calcium (Ca), aluminum (Al), potassium (K), copper (Cu), iron (Fe), zinc (Zn), manganese (Mn) in varying concentrations (Yuan *et al.* 2018, Yu *et al.* 2019).

Biochar is also characterized by a neutral to alkaline pH. Indeed, the pyrolysis process decomposes organic substances, which induces biochar alkalinity (Huang *et al.* 2017). For instance, Singh *et al.* (2010) found pH from 6.93 to 10.26 after pyrolyzing five feedstocks at different temperatures. By analyzing all the pH data from Table 4, average biochar pH was found at 8.9, with a lowest value at acidic pH (pH 3.1) for a wildfire biochar (Zhang *et al.* 2016b) and the highest value at an alkaline pH (pH 12.4) for a chicken manure biochar pyrolyzed at 700 °C (Hass *et al.* 2012).

Moreover, biochars from Table 4 presented electrical conductivity values ranging from 60 µS.cm^{-1} (sawdust biochar at 650 °C, Higashikawa *et al.* 2016) to 91,900 µS.cm^{-1} (wood chips biochar at 500 °C, Sadaf *et al.* 2017), with an average at 4,062 µS.cm^{-1}.

Biochar is also characterized by its ash content, 29.81 % on average (Table 5), ranging from 0.40 % (sawdust of white spruce pyrolyzed at 300 °C, Kwak *et al.* 2019) and 91.25 % (rice husk biochar at 500 °C, Manolikaki *et al.* 2016).

Finally, biochar has a high cation exchange capacity, between 1.43 cmol.kg^{-1} (sugarcane straw biochar at 700 °C, Puga *et al.* 2015) and 304 cmol.kg^{-1} (corn straw biochar at 500 °C, Yuan *et al.* 2011), and its surface carries many functional groups (Wiszniewska *et al.* 2016, Ding *et al.* 2017, Zama *et al.* 2018), which have a major role in metal(loid) sorption.

All of these properties induce positive effects on soil and plant when biochar is applied to soil (Figure 12) (Table 6).

Table 5 Main physico-chemical properties of biochar used in previous studies (EC = electrical conductivity, CEC = cation exchange capacity; empty cases signify that the information was not provided).

Feedstock	**Temperature of pyrolysis (°C)**	**pH**	**EC (μS.cm^{-1})**	**CEC (cmol.kg^{-1})**	**Ash (%)**	**Specific suface area (m^2.g^{-1})**	**C (%)**	**H (%)**	**N (%)**	**O (%)**	**Reference**
Algae	500	10.24	10,700		59.75		24.62	1.33	3.22	11.44	Yang *et al.* (2018)
Apple tree branch	400	7.02				11.9					Jindo *et al.* (2014)
Apple tree branch	500	9.64				58.6					Jindo *et al.* (2014)
Apple tree branch	600	10.04				208.69					Jindo *et al.* (2014)
Apple tree branch	700	10.03				418.66					Jindo *et al.* (2014)
Apple tree branch	800	10.02				545.43					Jindo *et al.* (2014)
Baby corn peel	450	9.63	860	1.79	22.63				2.71		Rafael *et al.* (2019)
Bagasse	300	7.3				5.2	69.5	4.2	0.9	24.36	Sun *et al.* (2014)
Bagasse	450	7.5				13.6	78.6	3.52	0.92	15.46	Sun *et al.* (2014)
Bagasse	600	7.3				32.9	63.23		0.37		Chen *et al.* (2010)
Bagasse	600	7.5				388.3	76.45	2.93	0.79	18.33	Sun *et al.* (2014)
Bamboo	300	7.9				1.3	66.2	4.7	0.4	27.72	Sun *et al.* (2014)
Bamboo	300	6.7				1.3	66.2	4.7	0.4	27.7	Yao *et al.* (2012)
Bamboo	450	8.5				10.2	76.89	3.55	0.23	18.11	Sun *et al.* (2014)
Bamboo	450	5.2				18.2	76.9	3.6	0.2	18.1	Yao *et al.* (2012)
Bamboo	600	9.2				375.5	80.89	2.43	0.15	14.87	Sun *et al.* (2014)
Bamboo	600	7.9				470.4	80.9	2.4	0.2	14.9	Yao *et al.* (2012)
Bamboo	600	7.9				470.4	80.89	2.43	0.15	16.54	Zhou *et al.* (2013)
Bamboo	700	9.77				42.82	83.3	2.67	0.24	13.71	Ni *et al.* (2018)
Bamboo	750	9.5		15	11.9	907.4	86	1.49	0.45		Lu *et al.* (2017)
Beetle-killed lodge pin	300-550	9.4		15.8	9.39	176	87.2		0.38	1.42	Kelly *et al.* (2014)
Biogas residue	700				58.18	123.93	29.47	0.96	1.28	59.49	Xia *et al.* (2016)
Biosolid	600	7.2				2.2	15.14		1.46		Chen *et al.* (2010)

Table 5. continued

Feedstock	Temperature of pyrolysis (°C)	pH	EC (μS.cm^{-1})	CEC (cmol.kg^{-1})	Ash (%)	Specific suface area (m^2.g^{-1})	C (%)	H (%)	N (%)	O (%)	Reference
Blady grass	450	7.1	978				64		1.24		Zhang *et al.* (2016b)
Brazilian pepperwood	300	6.6				81.1	59.3	5.2	0.3	34.1	Yao *et al.* (2012)
Brazilian pepperwood	450	7.3				0.7	75.6	3.6	0.3	17.2	Yao *et al.* (2012)
Brazilian pepperwood	600	9.1				234.7	77	2.2	0.1	17.7	Yao *et al.* (2012)
Broiler litter	350					59	45.6	4	4.5		Uchimiya *et al.* (2010)
Broiler litter	700					94	46	1.42	2.82		Uchimiya *et al.* (2010)
Canola straw	300	7.68			8.1	2.8	63.25	5.02	1.58	12.64	Kwak *et al.* (2019)
Canola straw	300	6.48		199	10.7		61.6		0.19		Yuan *et al.* (2011)
Canola straw	500	9.43			15	4.8	75.01	2.59	1.63	3.11	Kwak *et al.* (2019)
Canola straw	500	9.39		210	18.4		63.4		0.04		Yuan *et al.* (2011)
Canola straw	700	10.93			15.9	4.2	78.77	1.71	1.37	14.28	Kwak *et al.* (2019)
Canola straw	700	10.76		179	28.55		54.9		0.04		Yuan *et al.* (2011)
Cauliflower leaves	200-600	9.84	1,310								Mary *et al.* (2016)
Chicken manure	350	9.9				2.4	42		3.8	24	Hass *et al.* (2012)
Chicken manure	350	8.21	4,377	32	38.21		38.11	3.4	2.59	55.91	Higashikawa *et al.* (2016)
Chicken manure	500	9.1				6.52	7	0.5	0.71	0.47	Huang *et al.* (2018)
Chicken manure	550	8.8		238		7.27	51.7	2.26	2.1		Park *et al.* (2011)
Chicken manure	650	9.96	4,013	10.56	48.76		32.56	0.91	1.46	65.08	Higashikawa *et al.* (2016)
Chicken manure	700	12.4				10	39		2.3	19	Hass *et al.* (2012)
Coconut	300	7.41		72.86	3.76	4.495					Wu *et al.* (2016)
Coconut	500	10.26		81.17	4.89	6.844					Wu *et al.* (2016)
Coconut	700	10.53		70	6.65	540.63					Wu *et al.* (2016)

Coffee ground	530	9.65		5.56		23.5	68.81		4.3		Lima *et al.* (2018)
Coffee husk	530	10.31		22.64		244	67.11		2.05		Lima *et al.* (2018)

Table 5. continued

Feedstock	**Temperature of pyrolysis (°C)**	**pH**	**EC ($\mu S.cm^{-1}$)**	**CEC ($cmol.kg^{-1}$)**	**Ash (%)**	**Specific suface area ($m^2.g^{-1}$)**	**C (%)**	**H (%)**	**N (%)**	**O (%)**	**Reference**
Coniferous wood chip	700	9.5		10.2							Hailegnaw *et al.* (2019)
Corn stover	850	10	800	24	45.9	176	48		0.41		Sandhu *et al.* (2017)
Corn straw	300	8.25				11.66	48.12	3.94	1.5	42.26	Ni *et al.* (2018)
Corn straw	300	9.37		183	30.15		53.6		1.44		Yuan *et al.* (2011)
Corn straw	500	10.77		304	50.7		41.9		0.9		Yuan *et al.* (2011)
Corn straw	600	10.39			10.17	60.97	85.26	1.75	0.8	5.16	Yu *et al.* (2015)
Corn straw	700	11.32		210	73.3		24.5		0.78		Yuan *et al.* (2011)
Cow manure	500	9.2		4.84			3.61		0.15		Uzoma *et al.* (2011)
Cymbopogon flexuosus	450					3.449	53	3.6	1		Jain *et al.* (2014)
Dairy manure	350	9.2	538		24.2	1.64					Cantrell *et al.* (2012)
Dairy manure	700	9.9	702		39.5	186.5					Cantrell *et al.* (2012)
Date palm	300	8.32	3,260		14.42		57.99	4.08	0.54	20.82	Al-Wabel *et al.* (2017)
Date palm	500	9.59	3,500		19.68		72.3	2.11	0.42	4.5	Al-Wabel *et al.* (2017)
Date palm	700	11.5	3,660		21.05		73.42	1.14	0.35	3.19	Al-Wabel *et al.* (2017)
Empty fruit bunch		9.4			13.9	1.89	48	3.8	1.3	30	Samsuri *et al.* (2013)
Eucalyptus	400	7.47				10.35	77.8	5.38	0.41	18.3	Sun *et al.* (2013)
Eucalyptus	600	10.4			1.74	334.56	81.03		1.07		Lu *et al.* (2014a) Lu *et al.* (2015)
Eucalyptus saligna	500	9.5	170	27.21			80.2		0.12		Namgay *et al.* (2010)
Eucalyptus wood	300					0.028	76		1.1		Lu *et al.* (2018)
Eucalyptus wood	500					0.039	63		0.87		Lu *et al.* (2018)
Farmyard manure	500	7.6	30,540				54.2		5.8		Sadaf *et al.* (2017)

Fraxinus excelsior + Fagus sylvatica + Quercus robur	870	11.06	17,400	26.1		34.2	16.7		0.24		Reed *et al.* (2017)
Gliricidia sepium	900	10.1			70	714	50	1	0.5	44	Herath *et al.* (2014)

Table 5. continued

Feedstock	Temperature of pyrolysis (°C)	pH	EC (μS.cm^{-1})	CEC (cmol.kg^{-1})	Ash (%)	Specific suface area (m^2.g^{-1})	C (%)	H (%)	N (%)	O (%)	Reference
Grape husk	600	9.98		18.7		77					Trakal *et al.* (2014)
Grape pomace	300	10.8	1,058		25.67						Manolikaki *et al.* (2016)
Grape pomace	500	11	528		48.39						Manolikaki *et al.* (2016)
Grape stalk	600	10		40.2			70.2	1.7	1.45	12.5	Mitchell *et al.* (2018)
Greenwaste	450	9.4		24			36		0.18		Chan *et al.* (2008)
Greenwaste	450	9.6	720			271	59.7		0.51		Zhang *et al.* (2016b)
Greenwaste	550	7.7		250		6.87	77.5	2.62	0.26		Park *et al.* (2011)
Hardwood	650-700	9.6	443	33.8	18.8		72		1.02		Bakshi *et al.* (2014)
Hardwood		7.6			13.9		71.5		0.72		Laird *et al.* (2010)
Hardwood + Softwood	450	9.46		4.6			68		0.18		Rees *et al.* (2016)
Hibiscus cannabinus	500	7.5				117.61	75.1	2.9	0.39	0.21	Huang *et al.* (2018)
Hickory	300	7.1				< 0.1	69.13	4.85	0.39	24.36	Sun *et al.* (2014)
Hickory	450	7.9				12.9	83.62	3.24	0.17	11.46	Sun *et al.* (2014)
Hickory	600	8.4				401	81.81	2.17	0.73	14.03	Sun *et al.* (2014)
Hickory wood	600	8.4				401	81.81	2.16	0.73	15.3	Zhou *et al.* (2013)
Holm oak wood	650	9.6		17.4	14.7		51.4	1.42	0.45		Van Poucke *et al.* (2018)
Jarrah	750	10.8	315			206	86		0.23		Zhang *et al.* (2016b)
Kitchen waste	500	6.3	29,690				49.5		25.1		Sadaf *et al.* (2017)
Loblollypine	600					209.6	85.68	2.13	0.33	11.19	Wang *et al.* (2015a)
Macadamia nutshell	465	10.29	170			202.49	74.72		0.66		Xu *et al.* (2018)

Mallee	750	10.2	1,373			233	69		0.43		Zhang *et al.* (2016b)
Mango tree branches	450	9.26	250	4.5	52.77				0.8		Rafael *et al.* (2019)
Manure	300	7.91			69.4	0.8	19.61	1.66	1.46	7.89	Kwak *et al.* (2019)
Manure	500	9.39			80	8.4	13.6	0.61	0.84	5	Kwak *et al.* (2019)

Table 5. continued

Feedstock	**Temperature of pyrolysis (°C)**	**pH**	**EC ($\mu S.cm^{-1}$)**	**CEC ($cmol.kg^{-1}$)**	**Ash (%)**	**Specific suface area ($m^2.g^{-1}$)**	**C (%)**	**H (%)**	**N (%)**	**O (%)**	**Reference**
Manure	700	9.77			85.3	3	12.86	0.26	0.5	1.03	Kwak *et al.* (2019)
Miscanthus	700	10			18.5		76.7	1.4	0.3	3	Novak *et al.* (2018)
Miscanthus giganteus	550-600	10.1			34.93	864.2	60.8		0.4		Schimmelpfennig *et al.* (2014)
Miscanthus straw	600	10.24		29.47					0.31		Houben *et al.* (2013a)
Miscanthus straw	600	10.24					53.5		0.31		Houben *et al.* (2013b)
Municipal solid waste	400	8			6.1	20.7	48.6	12.2	1.3	31.7	Jin *et al.* (2014)
Municipal solid waste	500	8.5			9.2	29.1	59.5	9.1	1.4	20.8	Jin *et al.* (2014)
Municipal solid waste	600	9			6.2	29.8	70.1	8.4	1.3	13.7	Jin *et al.* (2014)
Nut shield	600	8.63		8.44		465					Trakal *et al.* (2016)
Oak tree	400	6.43				5.6					Jindo *et al.* (2014)
Oak tree	500	8.1				103.17					Jindo *et al.* (2014)
Oak tree	600	8.85				288.58					Jindo *et al.* (2014)
Oak tree	700	9.54				335.61					Jindo *et al.* (2014)
Oak tree	800	9.68				398.15					Jindo *et al.* (2014)
Olive mill waste	400-450	9	310		7.5		76.92	3.51	0.9		Hmid *et al.* (2015)
Olive tree prunings	300	10	204		31.27						Manolikaki *et al.* (2016)
Olive tree prunings	450	9.34	2,430	36.6		265	75.52				Brennan *et al.* (2014)
Olive tree prunings	450	9.45	1,360	29.6			64.22		1.21		Olmo *et al.* (2014)

Olive tree prunings	500	11	204		43.77						Manolikaki *et al.* (2016)
Orange peel	200-600	9.43	231								Mary *et al.* (2016)
Orchard pruning	500	9.7	7,500	21.3		410	77.81	4.53	0.91		Amendola *et al.* (2017), Baronti *et al.* (2014)
Palm bark	400	7.1				2.46	68.9	5.38	0.88	20.8	Sun *et al.* (2013)

Table 5. continued

Feedstock	**Temperature of pyrolysis (°C)**	**pH**	**EC (μS.cm^{-1})**	**CEC (cmol.kg^{-1})**	**Ash (%)**	**Specific suface area (m^2.g^{-1})**	**C (%)**	**H (%)**	**N (%)**	**O (%)**	**Reference**
Paved-feedlot manure	350	9.1	713		28.7	1.34					Cantrell *et al.* (2012)
Paved-feedlot manure	700	10.3	1,140		44	145.2					Cantrell *et al.* (2012)
Pea pod	200-600	8.84	589								Mary *et al.* (2016)
Peanut hull	300	7.8				0.8	73.9	3.9	1.6	19.1	Yao *et al.* (2012)
Peanut hull	450	8.2				21.8	81.5	2.9	1	13	Yao *et al.* (2012)
Peanut hull	600	8				27.1	86.4	1.4	0.9	10	Yao *et al.* (2012)
Peanut hull	600	6.9				27.1	86.39	1.36	0.94	11.26	Zhou *et al.* (2013)
Peanut shell	450	8.7	495			112	35.1		0.73		Zhang *et al.* (2016b)
Peanut shell	500	9.68	6,570			4.25	52.5	3.26	1.58	11.8	Khan *et al.* (2015)
Peanut shell	550	10.1			6.65		67.4		1.3		Xu *et al.* (2015)
Peanut straw	300	8.6		229	20.1		53.7		2.6		Yuan *et al.* (2011)
Peanut straw	350	8.88		81	40.9	2.1	41		2.46		Qian *et al.* (2013)
Peanut straw	500	10.86		230	32.5		48.5		1.51		Yuan *et al.* (2011)
Peanut straw	700	11.15		254	38.5		47		1.51		Yuan *et al.* (2011)
Pine	750	8.6	321			322	84.2		0.15		Zhang *et al.* (2016b)
Pine cone	500				2.13	6.6	67.88	3.89	0.55	22.07	Van Vinh *et al.* (2015)
Pine woodchip	450	7.52	256	12.6		288	83.71				Brennan *et al.* (2014)
Pinewood	850	9.3	120	9	39.7	233	55		0.33		Sandhu *et al.* (2017)

Poultry litter	300					1.1	20		2		Lu *et al.* (2018)
Poultry litter	400	10.02			74.95	7.418	16.77		1.37		Lu *et al.* (2014a), Lu *et al.* (2015)
Poultry litter	500					1.2	14		0.93		Lu *et al.* (2018)
Poultry litter manure	350	8.7	1,405		30.7	3.93					Cantrell *et al.* (2012)
Poultry litter manure	700	10.3	2,217		46.2	50.9					Cantrell *et al.* (2012)
Poultry manure	420	10.3		12.3		18.7	48.9	0.8	3.4		Marchand *et al.* (2016)
Poultry manure	500	6.6	16,920				53		31.5		Sadaf *et al.* (2017)

Table 5. continued

Feedstock	**Temperature of pyrolysis (°C)**	**pH**	**EC (µS.cm^{-1})**	**CEC (cmol.kg^{-1})**	**Ash (%)**	**Specific suface area (m^{2}.g^{-1})**	**C (%)**	**H (%)**	**N (%)**	**O (%)**	**Reference**
Residues of anaerobic digestion	400	8.83				7.6	63.5	5.28	0.94	18.1	Sun *et al.* (2013)
Rice hull	500	10.2	820	50.4			20.5		0.26		Kim *et al.* (2015)
Rice husk	300	7.5	265		63.54						Manolikaki *et al.* (2016)
Rice husk	350	8.44	227	13.89	40.44		32.79	1.09	0.04	66.09	Higashikawa *et al.* (2016)
Rice husk	400	6.84				193.7					Jindo *et al.* (2014)
Rice husk	450	7.56	160	3.86	6.57				0.6		Rafael *et al.* (2019)
Rice husk	500	8.99				262					Jindo *et al.* (2014)
Rice husk	500	7.6	252		91.25						Manolikaki *et al.* (2016)
Rice husk	600	9.41				243					Jindo *et al.* (2014)
Rice husk	600	8.7		17.57			18.72				Masulili *et al.* (2010)
Rice husk	650	8.72	283	16.47	41.97		49.48	1.47	0.02	49.04	Higashikawa *et al.* (2016)
Rice husk	700	9.52				256					Jindo *et al.* (2014)
Rice husk	800	9.62				295.57					Jindo *et al.* (2014)
Rice husk		8.5			27.2	25.16	45	2.3	0.17	17	Samsuri *et al.* (2013)

Rice husk + Cotton seed shell	400	10.64	1,020	12.5	12.6						Akhtar *et al.* (2014)
Rice straw	300					4.49	50.1	4.65	0.58	30	Wu *et al.* (2018)
Rice straw	350	7.69		152	40.3	19.3	42.5		1.65		Qian *et al.* (2013)
Rice straw	400	10.41	8,890		28	2.01	54.98	2.24	1.5	13.28	Meng *et al.* (2018)
Rice straw	500	10.05	1,037			2.21	47.4	3.13	1.65	14.7	Khan *et al.* (2015)
Rice straw	500	10		45	42.7	36.7	50.8	1.72	1.66		Lu *et al.* (2017)
Rice straw		10			22.5	2.51	42.3		1.5		Abbas *et al.* (2018)
Rice straw	400	8.62				46.6					Jindo *et al.* (2014)
Rice straw	500	9.82				59.91					Jindo *et al.* (2014)

Table 5. continued

Feedstock	**Temperature of pyrolysis (°C)**	**pH**	**EC (µS.cm^{-1})**	**CEC (cmol.kg^{-1})**	**Ash (%)**	**Specific suface area (m^2.g^{-1})**	**C (%)**	**H (%)**	**N (%)**	**O (%)**	**Reference**
Rice straw	600	10.19				129					Jindo *et al.* (2014)
Rice straw	700	10.39				149					Jindo *et al.* (2014)
Rice straw	800	10.47				256.96					Jindo *et al.* (2014)
Rice straw + Swine manure (1:1)	400	10.04	3,880		41	3.42	42.62	2.13	1.77	12.49	Meng *et al.* (2018)
Rice straw + Swine manure (1:3)	400	9.85	2,800		45	2.35	40.12	2.07	1.95	10.86	Meng *et al.* (2018)
Rice straw + Swine manure (3:1)	400	10.13	5,560		32	2.3	48.57	2.6	1.83	15	Meng *et al.* (2018)
Salicornia bigelovii		8.6			41.1	1.72	43.4	3.1	0.7	11	Al Marzooqi and Youssef (2017)
Sawdust	350	7.59	74	20.67	1.24		71.63	3.94	0.1	24.35	Higashikawa *et al.* (2016)
Sawdust	650	7.48	60	91.17	1.19		84.6	2.84	0.22	12.35	Higashikawa *et al.* (2016)
Sawdust of white spruce	300	5.7			0.4	0.8	57.33	6	0.04	36.2	Kwak *et al.* (2019)
Sawdust of white spruce	500	5.42			1.3	100	85.07	2.96	0.11	13.34	Kwak *et al.* (2019)

Sawdust of white spruce	700	7.2			1.5	302	94.56	1.74	0.22	1.87	Kwak *et al.* (2019)
Sewage sludge	300	5.32	4,120		52.8		25.6	2.55	3.32	8.33	Hossain *et al.* (2011)
Sewage sludge	400	4.87	4,150		63.3		20.2	1.28	2.4	4.61	Hossain *et al.* (2011)
Sewage sludge	500	7.27	4,700		68.2		20.3	0.88	2.13	0.65	Hossain *et al.* (2011)
Sewage sludge	500	7.1				14.1	19.3	1.3	2.75	0.35	Huang *et al.* (2018)
Sewage sludge	500	7.43	6,690			4.07	20.6	2.01	2.83	14.2	Khan *et al.* (2015)
Sewage sludge	500	9.54	554	2.36	67.5	32.24					Méndez *et al.* (2012)
Sewage sludge	700	12	2,500		72.5		20.4	0.51	1.2	0	Hossain *et al.* (2011)
Sorghum	500	7.43	5,950		29.36		46.73	2.98	0	12.99	Yang *et al.* (2018)
Sorghum	600	9.62	5,920		45.1		47.45	2.31	0	9.85	Yang *et al.* (2018)

Table 5. continued

Feedstock	**Temperature of pyrolysis (°C)**	**pH**	**EC (μS.cm^{-1})**	**CEC (cmol.kg^{-1})**	**Ash (%)**	**Specific suface area (m^2.g^{-1})**	**C (%)**	**H (%)**	**N (%)**	**O (%)**	**Reference**
Soybean stover	300	7.3	3,800		10.4	5.6	68.8	4.3	1.8	25	Vithanage *et al.* (2017)
Soybean straw	300	7.66		279	11.15		57.6		1.27		Yuan *et al.* (2011)
Soybean straw	350	9.02		98	23.3	1	54.1		3.62		Qian *et al.* (2013)
Soybean straw	500	10.06	5,120			2.63	65.6	3.17	1.72	15.6	Khan *et al.* (2015)
Soybean straw	500	10.92		216	17.85		62.6		0.37		Yuan *et al.* (2011)
Soybean straw	700	11.1		222	23.7		57.9		0.1		Yuan *et al.* (2011)
Straw	525	9.7	5,180	14.85	28.1	12.26					Kloss *et al.* (2014)
Sugar cane straw	700	10.2		1.43	13.4	5	68.8	2.2	0.9		Puga *et al.* (2015)
Sugarcane bagasse	300	7.2				5.2	69.5	4.2	0.9	24.5	Yao *et al.* (2012)
Sugarcane bagasse	350	5.9	614				70.8		0.34		Zhang *et al.* (2016b)
Sugarcane bagasse	450	7.9				15.3	78.6	3.5	0.9	15.5	Yao *et al.* (2012)
Sugarcane bagasse	600	7.9				4.2	76.5	2.9	0.8	18.3	Yao *et al.* (2012)
Sugarcane bagasse	600	7.5				557.4	76.45	2.93	0.79	19.81	Zhou *et al.* (2013)

Sugarcane straw	350	8.67	1,213	28	24.22		60.13	2.44	1.66	35.78	Higashikawa *et al.* (2016)
Sugarcane straw	650	9.17	1,903	16.88	13.32		69.37	2.45	1.5	26.69	Higashikawa *et al.* (2016)
Swine manure	400	9.45	1330		47	3.46	36.34	1.95	2.08	12.63	Meng *et al.* (2018)
Swine solid manure	350	8.4	216		32.5	0.92					Cantrell *et al.* (2012)
Swine solid manure	700	9.5	194		52.9	4.11					Cantrell *et al.* (2012)
Switchgrass	850	10.8	550	19	45.8	188	49.5		0.45		Sandhu *et al.* (2017)
Timber	750	9.6	474			382	84.7		0.23		Zhang *et al.* (2016b)
Tobacco stem	450	10.51		32.45	25.46	368.9	59.75	3.26	2.01		Zhang *et al.* (2019)
Turkey litter manure	350	8	651		34.8	2.6					Cantrell *et al.* (2012)
Turkey litter manure	700	9.9	981		49.9	66.7					Cantrell *et al.* (2012)
Vineyard pruning	400	8.3	1,480	12.35	4.3	1.69					Kloss *et al.* (2014)

Table 5. end

Feedstock	**Temperature of pyrolysis (°C)**	**pH**	**EC ($\mu S.cm^{-1}$)**	**CEC ($cmol.kg^{-1}$)**	**Ash (%)**	**Specific suface area ($m^2.g^{-1}$)**	**C (%)**	**H (%)**	**N (%)**	**O (%)**	**Reference**
Vineyard pruning	525	8.8	1,080	7.88	7.7	4.85					Kloss *et al.* (2014)
Wastewater sludge	550	8.2	1,900		35				0.23		Hossain *et al.* (2010)
Wheat straw	300	9.66			13.1	1.9	65.43	4.38	0.9	16.23	Kwak *et al.* (2019)
Wheat straw	350-550	7.23		17.3		1331	69.2	3.4	1.53	11.8	Liu *et al.* (2016a)
Wheat straw	500	10.15			16.8	3.3	71.4	2.55	0.84	8.43	Kwak *et al.* (2019)
Wheat straw	700	10.34			18	5.8	75.14	1.46	0.74	4.65	Kwak *et al.* (2019)
Wildfire	350–500	3.1	110			109	69.7		0.22		Zhang *et al.* (2016b)
Willow	350	8.6			5.6	6.2	73.9	3.5	0.8		Gregory *et al.* (2015)
Willow	550	8.3	710	11.2			47.5		0.38		Agegnehu *et al.* (2015)
Willow	550	8.6			4.1	59.8	77.4	3.3	0.7		Gregory *et al.* (2015)
Willow	700				7.72	11.4	69.94	2.08	1.13	19.13	Kołtowski *et al.* (2017)
Wood chips	500	6.1	91,900				50.9		2.2		Sadaf *et al.* (2017)
Woodchips	525	8.9	1,580	9.3	15.2	26.41					Kloss *et al.* (2014)

Mean	8.9	4,062	67.42	29.81	130.18	58.22	2.94	1.39	17.88
Min	3.1	60	1.43	0.40	0.028	3.61	0.26	0	0
Max	12.4	91,900	304	91.25	1,331	94.56	12.2	31.5	66.09

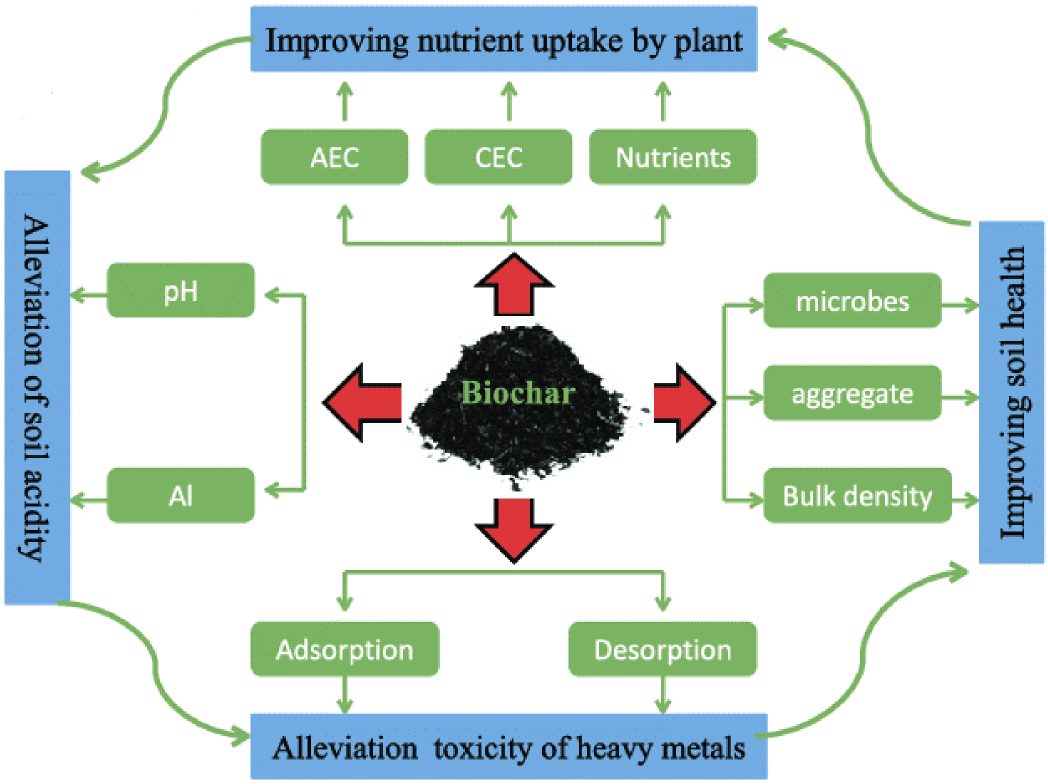


Figure 12 Scheme of the different effects of biochar application to soil (source: Shaaban et al. 2018); AEC =anion exchange capacity, CEC = cation exchange capacity.

Indeed, biochar has a high sorption capacity towards metal(loid)s, especially positively charged elements. For instance, Doumer *et al.* (2016) found that biochars made from sugarcane bagasse, eucalyptus forest residues, castor meal, green pericarp of coconut and water hyacinth were capable to sorb more than 95 % of Cd, Cu, Pb, Zn in solutions containing these metals in a range of 0.025 to 5 mmol.L^{-1}. Similarly, biochars made from olive mill solid waste were effective in removing more than 80 % of Pb from a solution of 50 ppm Pb (Abdelhadi *et al.* 2017). Biochar can sorb metal(loid)s through several mechanisms: surface electrostatic attraction, precipitation with minerals such as P, surface complexation with functional groups, which can be assessed by Fourier-transform infrared spectroscopy (FTIR) (Inyang *et al.* 2012, Ding *et al.* 2014, Wang *et al.* 2015b).

In addition to its sorption capacity, biochar can decrease metal(loid) bioavailability and mobility when added to the soil through metal(loid) sorption but also by affecting soil properties, especially pH, which is one of the most important parameters affecting metal(loid) behavior. Hmid *et al.* (2015) applied until 15 % of olive mill biochar to a Zn smelter, which immobilized metal(loid)s, related to biochar-induced pH increase that rise the overall negative charges of the soil. Similarly, Meng *et al.* (2018) observed that biochar (rice straw and swine manure mixture) decreased $CaCl_2$-extractable metals when applied at 1 % and 3 % to a Pb-Zn abandoned mine. They also attributed this to an improvement of soil pH, which favors metal(loid) precipitation (Houben *et al.* 2013b). Biochar also decreased $CaCl_2$-extractable Pb concentrations due to pH and SO_4^{2-} concentration increases (Li *et al.* 2018a). Finally, holm oak wood biochar decreased Zn and Cd soil pore water (SPW) concentrations (Egene *et al.* 2018). However grape stalk biochar decreased SPW Zn concentrations but increased the ones of As, Cu and Cr (Trakal *et al.* 2017). Similarly, Ahmad *et al.* (2014) observed a mobilization of As and Pb with biochar application.

Biochar amendment also affects other soil physico-chemical properties, such as pH, EC, WHC and nutrient contents (Table 5). Indeed, most biochars are alkaline, which induce a liming effect, especially in acidic soils (Rees *et al.* 2015, Xu *et al.* 2017, Lima *et al.* 2018). Such liming effect was attributed to: (i) biochar alkalinity, (ii) biochar high surface area, (iii) biochar soluble carbonates and (iv) biochar base cations (Hmid *et al.* 2015, Xu *et al.* 2017, Meng *et al.* 2018). Additionally, due to its ash and soluble salts contents, biochar can increase soil EC (Nigussie *et al.* 2012, Hmid *et al.* 2015, Meng *et al.* 2018). Moreover, as observed in Agegnehu *et al.* (2017) and Laghari *et al.* (2015), biochar can increase soil WHC. Indeed, biochar has a porous structure that can retain water, but biochar also reduces soil bulk density and the total porosity of the soil, by increasing the number of large pores, and also increases the soil aggregation, which increases the water retention capacity of the soil (Hardie *et al.* 2014, Obia *et al.* 2016). Biochar is also characterized by a high CEC, which can lead to soil CEC increase (Tan *et al.* 2017, Xu *et al.* 2017). Finally, biochar can increase soil nutrient content and availability. Indeed, Nigussie *et al.* (2012) observed an increase in total nitrogen (N) content following maize stalk biochar amendment, while Laghari *et al.* (2015) showed that pine sawdust biochar increased total N and K contents in two desert soils. De Tender *et al.* (2016) measured an increase in plant-available concentrations of P, K, Ca and magnesium (Mg) with holm oak wood biochar application and Hass *et al.* (2012) found that chicken manure biochar rose extractable concentrations of both micro and macro nutrients.

Finally, biochar was shown to improve soil microbiological aspects (Molnár *et al.* 2016). Indeed, biochar amendment increased microbial community activity and diversity due to its effect on soil properties but also its role as microbial habitat.

Finally, all of these beneficial effects on the soil properties induced an improvement of plant growth parameters (Table 6). Ryegrass yield was increased following grape stalk biochar application to two agricultural soils (Trakal *et al.* 2017). Similarly, biochar obtained from a mix of rice husk and cotton shell increased tomato fruit yield, water use efficiency, stomatal density and pore aperture (Akhtar *et al.* 2014). Pak choi yield and biomass were also found to increase with the application of sugarcane bagasse biochar to a metal smelter (Nie *et al.* 2018). Finally, all the biochars applied to a Pb/Zn tailing increased *Cassica alata* dry weight, height and root length (Huang *et al.* 2018).

Table 6 gives a panorama of the diverse effects of biochar on soil and plants.

Table 6 Biochar effects on soil and plants parameters (EC = electrical conductivity, WHC = water holding capacity, SPW = soil pore water, CEC = cation exchange capacity, SOC = soil organic carbon, SOM = soil organic matter ↘ = decrease, ↗ = increase, empty cases signify that the data was not available).

Biochar feedstock	**Temperature of pyrolysis (°C)**	**Pollutant(s)**	**Plant**	**Effect**	**Reference**
Baby corn peel	450		Cowpea	↗ soil pH ↗ available [P] ↗ soil CEC ↗ plant growth ↗ nutrient uptake	Rafael *et al.* (2019)
Bagasse	600		Sugarcane	↗ soil [N], [C] ↗ plant growth	Chen *et al.* (2010)
Bamboo	700	Pb		↘ Pb bioaccessibility	Ni *et al.* (2018)
Bamboo	750	Cd, Cu, Pb, Zn		↘ $CaCl_2$ extractable [Cd], [Cu], [Pb], [Zn] ↘ plant available [Cu], [Pb], [Zn]	Lu *et al.* (2017)
Beetle-killed lodgepole pin	300-550	Al, As, Cd, Cu, Fe, Pb, S, Zn		↗ soil pH ↗ soil moisture content ↗ pH leachate ↗ [Cd], [Zn] leachate ↗ [Ca], [Mg], [K] leachate	Kelly *et al.* (2014)
Biosolid	600		Sugarcane	↗ soil [N], [C] ↗ plant growth	Chen *et al.* (2010)
Chicken manure	350, 700			↗ soil pH ↗ extractable micro and macronutrient concentrations ↗ extractable [Cu], [Zn] ↘ extractable [Al], [Ca], [Ni], [Pb]	Hass *et al.* (2012)

Table 6. continued

Biochar feedstock	Temperature of pyrolysis (°C)	Pollutant(s)	Plant	Effect	Reference
Chicken manure	500	As, Cd, Cu, Pb, Zn	*Cassia alata*	↗ soil pH ↗ extractable [As], [Cd], [Zn] ↗ shoot and root biomass ↗ shoot height and root length ↘ shoot [As], [Cd], [Pb], [Zn] ↗ root [As], [Cd], [Pb], [Zn]	Huang *et al.* (2018)
Chicken manure	550		Indian mustard	↗ soil pH ↗ soil respiration ↗ soil microbial activity ↗ soil dehydrogenase activity ↘ NH_4NO_3 extractable [Cd], [Pb] ↗ NH_4NO_3 extractable [Cu] ↘ SPW [Cd], [Pb] ↗ SPW [Cu] ↗ plant dry weight ↘ shoot [Cd], [Pb] ↘ root [Cd], [Cu], [Pb]	Park *et al.* (2011)
Coffee ground	530		*Zea mays*	↗ soil WHC ↗ available [P] ↗ plant dry weight	Lima *et al.* (2018)

Table 6. continued

Biochar feedstock	Temperature of pyrolysis (°C)	Pollutant(s)	Plant	Effect	Reference
Coffee husk	530		*Zea mays*	↗ soil WHC ↗ soil pH ↗ exchangeable [K] ↗ available [P] ↗ plant dry weight	Lima *et al.* (2018)
Corn straw	300	Pb		↗ Gram negative bacteria biomass ↗ soil microbial diversity ↘ Pb bioaccessibility	Ni *et al.* (2018)
Corn straw	600	As	Rice	↗ plant growth ↘ plant [As]	Yu *et al.* (2017)
Cow manure	500		Maize	↗ soil pH ↗ soil C, N contents ↗ exchangeable [K], [Ca], [Mg] ↗ soil CEC ↗ plant growth ↗ plant N, P, K, Mg uptake	Uzoma *et al.* (2011)
Date palm	300	Cd, Cu, Pb, Zn, Mn, Fe		↘ soil pH ↗ soil EC ↗ SOC content ↗ soil microbial biomass and activity ↘ available [Cd], [Cu], [Pb], [Zn]	Al-Wabel *et al.* (2017)
Date palm	500	Cd, Cu, Pb, Zn, Mn, Fe		↗ soil pH ↗ soil EC ↗ SOC content	Al-Wabel *et al.* (2017)

Table 6. continued

Biochar feedstock	Temperature of pyrolysis (°C)	Pollutant(s)	Plant	Effect	Reference
Date palm	700	Cd, Cu, Pb, Zn, Mn, Fe		↗ soil pH ↗ soil EC ↗ SOC content ↘ soil microbial biomass carbon	Al-Wabel *et al.* (2017)
Eucalyptus	600	Cd	Amaranth	↗ soil pH ↗ soil C, N contents	Lu *et al.* (2014a)
Eucalyptus	600	Cd	Amaranth	↗ soil basal respiration ↗ soil metabolic quotient ↗ soil enzyme activity	Lu *et al.* (2015)
Eucalyptus saligna	500	As, Cd, Cu, Pb, Zn	Maize	↗ soil pH ↗ soil EC ↘ extractable [Pb] ↗ extractable [As], [Cd], [Zn] ↘ shoot [As], [Cd], [Cu], [Pb], [Zn]	Namgay *et al.* (2010)
Fraxinus excelsior + *Fagus sylvatica* + *Quercus robur*	870		Italian ryegrass	↗ soil pH ↗ available [P], [K] ↗ soil C content ↗ SOM content	Reed *et al.* (2017)
Gliricidia sepium	900		Tomato	↗ soil pH ↗ soil EC ↗ SOC content ↗ soil CEC ↗ available [K], [Ca], [Mg] ↗ plant biomass ↘ plant [Ni], [Cr], [Mn]	Herath *et al.* (2014)

Table 6. continued

Biochar feedstock	Temperature of pyrolysis (°C)	Pollutant(s)	Plant	Effect	Reference
Grape stalk	600	As, Cu, Cr		↘ $CaCl_2$ extractable [As], [Cu], [Cr]	Mitchell *et al.* (2018)
Greenwaste	450		Radish	↗ soil pH ↗ SOC content ↗ exchangeable [Na], [K], [Ca] ↗ extractable [P] ↘ exchangeable [Al] ↗ field capacity ↗ plant [P], [K], [Ca]	Chan *et al.* (2008)
Green waste	550		Indian mustard	↗ soil pH ↗ soil respiration ↗ soil dehydrogenase activity ↘ NH_4NO_3 extractable [Cd], [Cu], [Pb] ↘ SPW [Cd], [Pb] ↗ SPW [Cu] ↗ plant dry weight ↘ shoot [Cd], [Pb]	Park *et al.* (2011)
Hibiscus cannabinus	500	As, Cd, Cu, Pb, Zn	*Cassia alata*	↗ soil pH ↗ extractable [As], [Cd], [Zn] ↗ shoot and root biomass ↗ shoot height and root length ↘ shoot [As], [Cd], [Pb], [Zn] ↗ root [As], [Cd], [Pb], [Zn]	Huang *et al.* (2018)
Hardwood	650-700	Cu		↗ Cu immobilization ↗ soil pH ↗ leachate [Ca], [K], [Na] ↘ water soluble [P]	Bakshi *et al.* (2014)

Table 6. continued

Biochar feedstock	Temperature of pyrolysis (°C)	Pollutant(s)	Plant	Effect	Reference
Hardwood				↗ soil C, N content ↗ soil WHC ↗ soil pH ↗ soil CEC ↗ available [K], [Ca], [Mn], [P] ↘ bulk density	Laird *et al.* (2010)
Hornbeam	400	Cd, Cu, Pb	White willow	↗ soil pH ↗ soil N content ↗ soil organic carbon content ↘ available [Cd], [Cu], [Pb] ↗ available [K], [P] ↗ plant height ↗ plant dry biomass ↗ leaf gas exchange ↘ plant [Cd], [Cu], [Pb]	Mokarram-Kashtiban *et al.* (2019)
Macadamia nutshell	465	Cd, Pb		↗ soil pH ↘ bioavailable [Cd], [Pb] ↗ soil microbial respiration ↗ soil microbial biomass carbon	Xu *et al.* (2018)
Maize stalk	500	Cr	Lettuce	↗ soil pH ↗ soil EC ↗ soil organic carbon content ↗ soil N content ↗ available [P] ↗ soil CEC ↗ nutrient uptake ↘ shoot [Cr]	Nigussie *et al.* (2012)

Table 6. continued

Biochar feedstock	**Temperature of pyrolysis (°C)**	**Pollutant(s)**	**Plant**	**Effect**	**Reference**
Mango tree branches	450		Cowpea	↗ soil pH ↗ available [P] ↗ soil CEC ↗ plant growth ↗ nutrient uptake	Rafael *et al.* (2019)
Miscanthus straw	600	Cd, Pb, Zn	Rapeseed	↗ soil pH ↗ soil CEC ↗ available nutrients ↘ total [Pb], [Zn] ↗ plant biomass ↘ shoot [Cd], [Pb], [Zn]	Houben *et al.* (2013a)
Miscanthus straw	600	Cd, Pb, Zn	Ryegrass	↗ soil pH ↘ $CaCl_2$ extractable [Cd], [Pb], [Zn] ↘ shoot [Cd], [Pb], [Zn]	Houben *et al.* (2013b)
Olive mill waste	400-450	Cd, Pb, Zn	*Phaseolus vulgaris*	↗ soil pH ↗ soil EC ↘ extractable [Cd], [Pb], [Zn] ↗ leaf and root dry weight ↘ leaf and root [Cd], [Pb], [Zn]	Hmid *et al.* (2015)
Olive tree prunings	450			↗ soil EC ↗ soil N content ↗ available [P], [K], [Mg] ↗ extractable [Cu], [Zn]	Olmo *et al.* (2014)

Table 6. continued

Biochar feedstock	**Temperature of pyrolysis (°C)**	**Pollutant(s)**	**Plant**	**Effect**	**Reference**
Olive tree prunings	450	As, Cu, Zn, Mn	Maize	↗ root length and surface area ↗ leaf surface area ↗ root and shoot biomass ↘ shoot [Mn], [Zn] ↘ extractable [Cu], [Zn] ↗ extractable [As] ↗ As leaching	Brennan *et al.* (2014)
Orchard prune residue	500	As	Tomato	↗ SPW [As] ↗ soil pH ↘ plant dry weight ↘ plant organ [As]	Beesley *et al.* (2013)
Peanut shell	550		Peanut	↗ soil EC ↗ soil N content ↗ plant biomass ↗ peanut yield	Xu *et al.* (2015)
Pine woodchip	450	As, Cu, Zn, Mn	Maize	↗ root length and surface area ↗ leaf surface area ↘ extractable [Cu], [Zn]	Brennan *et al.* (2014)
Poultry litter	300	Cd, Cu, Pb, Zn	Rice	↗ soil pH ↘ leachate [Pb] ↘ plant [Cu], [Pb], [Zn]	Lu *et al.* (2018)
Poultry litter	400	Cd	Amaranth	↗ soil pH ↘ extractable [Cd] ↗ soil N content ↗ plant biomass	Lu *et al.* (2014a)

Table 6. continued

Biochar feedstock	Temperature of pyrolysis (°C)	Pollutant(s)	Plant	Effect	Reference
Poultry litter	400	Cd	Amaranth	↗ soil basal respiration ↗ soil microbial biomass ↗ soil enzyme activity	Lu *et al.* (2015)
Poultry litter	500	Cd, Cu, Pb, Zn	Rice	↗ soil pH ↘ leachate [Pb] ↗ plant growth ↘ plant [Cu], [Pb], [Zn]	Lu *et al.* (2018)
Poultry manure	420	Cd, Cu, Pb, Zn	Rapeseed	↗ SPW [As], [Cd], [Fe], [Mo], [Ni], [Pb], [Zn] ↗ SPW [K], [Mg] ↘ SPW [Ca], [P] ↘ seed yield	Marchand *et al.* (2016)
Prune residues from orchard	500	Pb, Zn		↗ soil pH ↗ soil [Mn], [K], [P] ↗ soil CEC ↘ bioavailable [Cd], [Pb], [Zn] ↘ Al, Cd mobility ↗ Cu leachability	Fellet *et al.* (2011)
Rice hull	500	Cd, Cu, Pb, Zn	Lettuce	↗ soil pH ↘ phytoavailable [Cd], [Cu], [Pb], [Zn] ↘ plant [Cd], [Pb], [Zn]	Kim *et al.* (2015)
Rice husk	450 °C		Cowpea	↗ soil pH ↗ available [P] ↗ soil CEC ↗ plant growth ↗ nutrient uptake	Rafael *et al.* (2019)

Table 6. continued

Biochar feedstock	**Temperature of pyrolysis (°C)**	**Pollutant(s)**	**Plant**	**Effect**	**Reference**
Rice husk	600		Rice	↘ bulk density ↗ soil pH ↗ soil CEC ↘ exchangeable [Al], [Fe] ↗ plant growth	Masulili *et al.* (2010)
Rice husk + Cotton seed shell	400		Tomato	↗ soil water content ↗ fruit yield ↗ chlorophyll content index ↗ gas exchange ↗ water use efficiency ↗ stomatal density and pore aperture	Akhtar *et al.* (2014)
Rice straw		Cd	Wheat	↗ soil pH ↗ soil EC ↗ plant growth ↘ plant [Cd] ↗ chlorophyll content ↗ gas exchange	Abbas *et al.* (2018)
Rice straw		Cd, Cu, Pb		↗ soil CEC ↗ Cd, Cu, Pb immobilization	Jiang *et al.* (2012)
Rice straw	450	As, Cd, Pb		↘ bioavailable [Cd], [Pb] ↗ bioavailable [As]	Yin *et al.* (2016)
Rice straw	500	Cd, Cu, Pb, Zn		↘ $CaCl_2$ extractable [Cd], [Cu], [Pb], [Zn] ↘ plant available [Cu], [Pb], [Zn]	Lu *et al.* (2017)

Table 6. continued

Biochar feedstock	Temperature of pyrolysis (°C)	Pollutant(s)	Plant	Effect	Reference
Rice straw	500	Cd, Pb	Rice	↗ soil pH ↗ soil C, N contents ↗ SOM content ↗ soil EC ↘ extractable [Cd] ↘ root, shoot and leaf [Pb]	Li *et al.* (2018a)
Salicornia bigelovii				↗ soil pH ↗ soil EC ↗ SOC content ↗ soil microbial biomass carbon ↗ soil enzyme activity	Al Marzooqi and Youssef (2017)
Sewage sludge	500			↗ soil respiration ↗ SOC content ↗ soil pH ↗ soil EC	Méndez *et al.* (2012)
Sewage sludge	500	As, Cd, Cu, Pb, Zn	*Cassia alata*	↗ soil pH ↗ extractable [As], [Cd], [Zn] ↗ shoot and root biomass ↗ shoot height and root length ↘ shoot [As], [Cd], [Pb], [Zn] ↗ root [As], [Cd], [Pb], [Zn]	Huang *et al.* (2018)
Soybean stover	300	Pb, Cu, Sb		↗ available [Ca], [K], [Mg] ↘ exchangeable [Cu], [Pb] ↗ dissolved organic carbon content ↘ extractable [Cu], [Pb] ↗ extractable [Sb]	Vithanage *et al.* (2017)

Table 6. continued

Biochar feedstock	**Temperature of pyrolysis (°C)**	**Pollutant(s)**	**Plant**	**Effect**	**Reference**
Sugarcane bagasse	450	Cd, Cu, Pb	Pak choi	↗ plant biomass ↘ exchangeable [Cd] ↘ labile [Cu] ↗ residual [Pb] ↘ plant [Cd], [Cu], [Pb] ↗ soil enzyme activity ↗ microbial population and activity	Nie *et al.* (2018)
Sugarcane straw	700	Cd, Pb, Zn	Jack bean *Mucuna aterrima*	↗ soil [P] ↘ available [Cd], [Pb], [Zn] ↘ SPW [Cd], [Pb], [Zn] ↗ plant dry weight ↗ shoot [K] ↘ plant [Cd], [Pb], [Zn]	Puga *et al.* (2015)
Tobacco stem	450	Cr, Cr, Pb	Tobacco	↗ soil pH ↗ leaf biomass and yield ↘ plant [Cr], [Cu], [Pb]	Zhang *et al.* (2019)
Wastewater sludge	550	As, Cd, Cr, Cu, Ni, Pb, Se, Zn	Tomato	↗ soil pH ↗ soil EC ↗ soil total N content ↗ extractable [P] ↗ soil CEC ↗ plant growth	Hossain *et al.* (2010)
Water Hyacinth	450	As, Cd, Pb		↘ bioavailable [Cd], [Pb] ↗ bioavailable [As] ↘ leachate [Cd] ↗ leachate pH ↗ leachate [Pb]	Yin *et al.* (2016)

Table 6. end

Biochar feedstock	**Temperature of pyrolysis (°C)**	**Pollutant(s)**	**Plant**	**Effect**	**Reference**
Wheat straw	350-550	Cd, Cu, Pb, Zn	Italian ryegrass	↘ $CaCl_2$ extractable [Cd], [Cu], [Pb], [Zn] ↗ soil pH ↘ shoot [Cd], [Cu], [Pb], [Zn] ↗ shoot biomass ↘ urease soil activity ↗ catalase, phosphatase soil activity ↗ soil microbial diversity	Liu *et al.* (2016a)
Wheat straw	350-550	Cd, Pb	Rice	↗ SOC content ↗ soil pH ↗ available [P], [K] ↘ available [Cd], [Pb] ↗ rice yield ↘ grain [Cd], root [Pb] ↘ Cd, Pb uptake	Bian *et al.* (2014)
Wheat straw	350-550	Cd	Wheat	↗ soil pH ↗ SOC content ↘ extractable [Cd] ↘ Cd uptake	Cui *et al.* (2012)
Willow	350, 550	As		↘ water soluble [As] ↗ soil dehydrogenase activity	Gregory *et al.* (2015)
Willow	550		Peanut	↗ soil organic carbon content ↗ soil nitrogen content ↗ available [P], [K], [Mg], [Al] ↗ plant yield ↗ chlorophyll content	Agegnehu *et al.* (2015)
Willow	700	Cd, Zn, Pb	*Lepidium sativum*	↗ root growth	Kołtowski *et al.* (2017)

However, as shown in Tables 5 and 6, all of these beneficial effects are dependent on soil type but also on biochar properties. Indeed, as it can be seen in Table 5, each biochar is different and biochar properties depend on feedstock, pyrolysis conditions (Table 5) and particle sizes (Barrow 2012). As examples, Chen *et al.* (2011) measured a pH slightly acidic for a hardwood biochar, while the pH of the corn straw biochar was alkaline (pH 9.54). Biochars made from oak wood, pine wood, oak bark and pine bark presented differing surface areas of 2.04, 2.73, 25.4 and 1.88 $m^2.g^{-1}$, respectively (Mohan *et al.* 2007). Similarly, Arán *et al.* (2016) showed that biochar surface area was influenced by feedstock, especially its structural and morphological structures. The authors also showed that biochars made from different feedstocks presented different sorption efficiency. Moreover, Wang *et al.* (2015c) observed that all of their studied biochars had a high Pb sorption capacity; however the pine biochar was the most efficient. Similarly, Khan *et al.* (2015) found that between biochars made from soybean, rice straw, peanut shell and sewage sludge, the peanut shell biochar was the most effective to increase soil pH and decrease metal(loid) bioaccumulation in *Brassica rapa*. Lu *et al.* (2014b) studied biochars obtained from rice straw and bamboo, and harboring two particle sizes (fine/coarse). Rice straw biochars presented a higher pH and alkalinity as well as a lower organic C and surface area than the bamboo biochars. They were thus more effective at increasing soil pH. Moreover, all biochars increased dry weight of *Sedum plumbizincola*, except for the coarse bamboo biochar. Finally, Zhang *et al.* (2013) showed that with decreasing particle size, biochar immobilization increased.

All of these studies showed that the selection of a biochar presenting the appropriate characteristics is important. Indeed, the overview of biochar effects on soil and plant given in Table 5 shows that in some cases, biochar can have negative effects. Moreover, biochar properties can be modified and improved by modifying biochar surface. This process is called biochar functionalization or activation. There are two main types of activation: the physical activation (magnetization, steam activation, gas activation) and the chemical activation (amino modification, methanol modification, combination with mineral sorbent, acid and base treatments) (Figure 14) (Rajapashka *et al.* 2016, Tan *et al.* 2017). The physical steam activation will produce a biochar with a high porosity and will increase biochar surface area, micropore surface area and micropore volume; the gas activation increases biochar surface area and pore volume (Tan *et al.* 2017). The chemical activation uses different reagents. For instance, acid chemical activation improves biochar surface area and porosity and can introduce more functional groups on the biochar surface. Another chemical activation can be performed using microwave (Tan *et al.* 2017). These functionalizations, or activations, improve biochar surface and can create more sites available for metal(loid)s, thus increasing their affinity for metal(loid)s (Table 6).

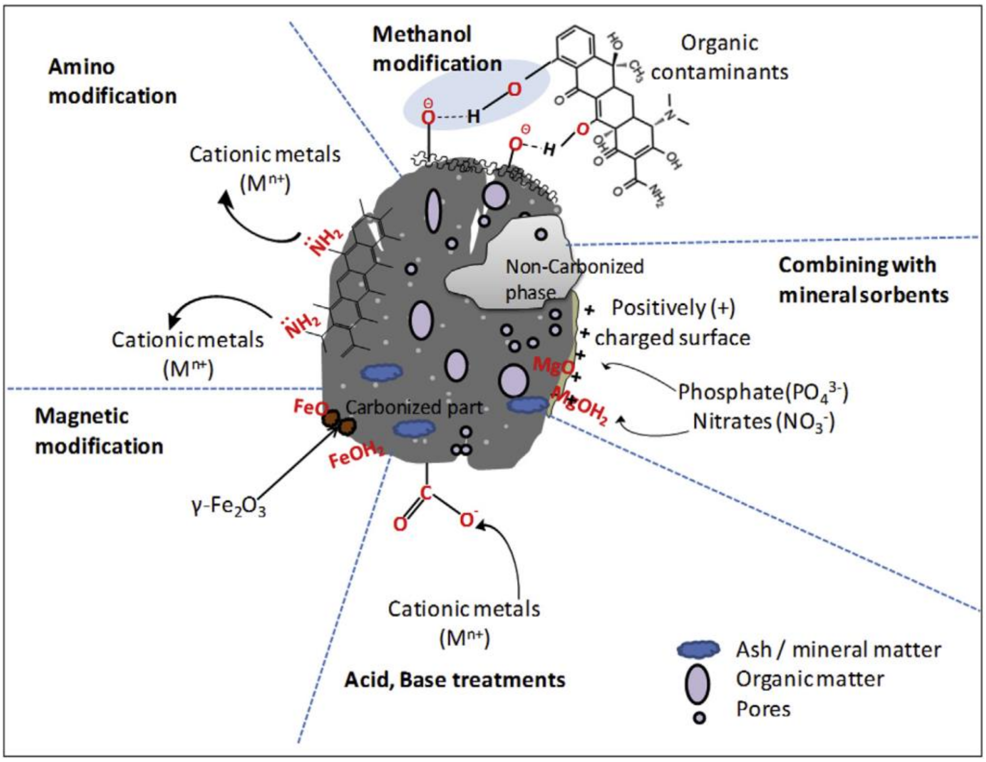


Figure 13 Biochar modifications (source: Rajapashka et al. *2016). Biochar can be modified through chemical modification (acid/base treatment, modification of functional groups, organic solvents, surfactant modification, coating), physical modification (steam modification, gas purging), impregnation with mineral oxides and magnetic modification*.

Indeed, Ding *et al.* (2016) showed that an alkali-modified biochar adsorbed metal(loid)s five times more efficiently than its pristine (non-modified) biochar, due to its much higher surface area and CEC but also to the presence of more oxygen containing functional groups on this modified biochar. Furthermore, the amelioration of the sorption capacity of modified-biochar can also be attributed to the presence of minerals and precipitates on its surface, for instance the presence of Fe or Co having an affinity for metal(loid)s (Rajapaksha *et al.* 2016). For instance, biochar functionalization can be very efficient for arsenic, for which biochar is not so efficient. Al functionalization increased As sorption capacity of crop straw biochar (Qian *et al.* 2013). Similarly, to increase As sorption, Fe functionalization was shown effective in many studies (Payne and Abdel-Fattah 2005, Samsuri *et al.* 2013).

Table 7 Biochar functionalization: modification of biochar properties and effects on soil and plant (empty cases signify that no data was available, CEC=cation exchange capacity, EC = electrical conductivity, ↗= increase, ↘ = decrease).

Feedstock	**Temperature of pyrolysis (°C)**	**Modification type (pre/post pyrolysis)**	**Properties modified**	**Effects**	**Reference**
Bamboo	550	$KMnO_4$ (post-pyrolysis)	↘ surface area		Li *et al.* (2014)
Bamboo	550	HNO_3 (post-pyrolysis)	↘ surface area ↗ N content		Li *et al.* (2014)
Bamboo	550	NaOH (post-pyrolysis)	↘ surface area ↘ N content		Li *et al.* (2014)
Bamboo	550	Heat (post-pyrolysis)	↗ surface area ↗ C content ↘ H content		Li *et al.* (2014)
Bamboo	600	Chitosan (post-pyrolysis)	↗ pH ↘ surface area ↗ N, H, O contents ↘ C content	↗ Cd, Cu, Pb removal	Zhou *et al.* (2013)
Biogas residue	700	$ZnCl_2$ (pre-pyrolysis)	↗ C, N contents ↘ O content ↗ ash content ↗ surface area	↗ As adsorption	Xia *et al.* (2016)
Broiler litter	350	Steam activation (post-pyrolysis)	↗ surface area ↘ C, H, N contents		Uchimiya *et al.* (2010)
Broiler litter	700	Steam activation (post-pyrolysis)	↗ surface area ↘ C, N contents		Uchimiya *et al.* (2010)
Canola straw	300	Steam activation (post-pyrolysis)	↗ pH ↗ C, N, O contents ↗ surface area	↗ Pb adsorption capacity	Kwak *et al.* (2019)
Canola straw	500	Steam activation (post-pyrolysis)	↗ O content ↘ ash content	↗ Pb adsorption capacity	Kwak *et al.* (2019)

Table 7. continued

Feedstock	Temperature of pyrolysis (°C)	Modification type (pre/post pyrolysis)	Properties modified	Effects	Reference
Canola straw	700	Steam activation (post-pyrolysis)	↘ H, O contents ↘ ash content ↗ surface area	↗ Pb adsorption capacity	Kwak *et al.* (2019)
Chicken manure	350, 700	Steam activation (post-pyrolysis)	↗ pH ↗ surface area	↗ available nutrient concentrations ↘ extractable [Al], [Cd], [Ni], [Pb] ↗ extractable [Cu], [Zn]	Hass *et al.* (2012)
Coconut	300	H_2O_2 (post-pyrolysis)	↘ pH ↘ ash content ↗ CEC ↗ surface area		Wu *et al.* (2016)
Coconut	300	NH_3 (post-pyrolysis)	↗ pH ↘ ash content ↗ CEC ↗ surface area	↗ Pb sorption	Wu *et al.* (2016)
Coconut	300	HNO_3 (post-pyrolysis)	↘ pH ↘ ash content ↗ CEC	↗ Pb sorption	Wu *et al.* (2016)
Coconut	500	H_2O_2 (post-pyrolysis)	↘ pH ↘ ash content ↗ CEC ↗ surface area	↘ Pb sorption	Wu *et al.* (2016)
Coconut	500	NH_3 (post-pyrolysis)	↘ pH ↘ ash content ↗ surface area	↘ Pb sorption	Wu *et al.* (2016)

↗ CEC

Table 7. continued

Feedstock	Temperature of pyrolysis (°C)	Modification type (pre/post pyrolysis)	Properties modified	Effects	Reference
Coconut	500	HNO_3 (post-pyrolysis)	↘ pH ↘ ash content ↗ surface area ↗ CEC	↘ Pb sorption	Wu *et al.* (2016)
Coconut	700	H_2O_2 (post-pyrolysis)	↘ pH ↘ ash content ↗ CEC	↘ Pb sorption	Wu *et al.* (2016)
Coconut	700	NH_3 (post-pyrolysis)	↘ pH ↘ ash content ↗ surface area	↘ Pb sorption	Wu *et al.* (2016)
Coconut	700	HNO_3 (post-pyrolysis)	↘ pH ↘ ash content ↗ CEC ↘ surface area	↘ Pb sorption	Wu *et al.* (2016)
Corn straw	600	Fe (pre-pyrolysis)	↘ C content ↗ H, O contents ↗ ash content ↗ surface area	↗ As(V) removal	He *et al.* (2018)
Corn straw	600	$KMnO_4$ (post-pyrolysis)	↗ pH ↗ ash content ↘ C, H, N contents ↗ O content	↗ As sorption ↗ soil pH	Yu *et al.* (2015)

Table 7. continued

Feedstock	**Temperature of pyrolysis (°C)**	**Modification type (pre/post pyrolysis)**	**Properties modified**	**Effects**	**Reference**
Corn straw	600	$KMnO_4$ (post-pyrolysis)		↗ rice growth ↘ rice [As]	Yu *et al.* (2017)
Empty fruit bunch		Fe (post-pyrolysis)		↗ As (III) and As (V) sorption	Samsuri *et al.* (2013)
Eucalyptus wood	300	Magnetization (pre-pyrolysis)	↘ C, N contents ↘ surface area		Lu *et al.* (2018)
Eucalyptus wood	500	Magnetization (pre-pyrolysis)	↘ C, N contents ↘ surface area		Lu *et al.* (2018)
Eucalyptus saligna wood	400, 500	Steam activation (post-pyrolysis)	↗ pH ↗ EC ↗ ash content		Singh *et al.* (2010)
Grape stalk	600	Magnetization (post-pyrolysis)	↗ surface area ↘ pH	↗ Cd sorption	Trakal *et al.* (2016)
Grape husk	600	Magnetization (post-pyrolysis)	↗ surface area ↗ CEC	↗ Cd sorption	Trakal *et al.* (2016)
Hickory	600	Alkali modification (post-pyrolysis)	↗ H, O contents ↗ surface area ↗ CEC	↗ Cd, Cu, Pb, Zn sorption capacity	Ding *et al.* (2016)
Hickory wood	600	Chitosan (post-pyrolysis)	↘ surface area ↗ N, H, O contents ↘ C content	↗ Cd, Cu, Pb removal	Zhou *et al.* (2013)
Loblollypine	600	Mn oxide (pre-pyrolysis)	↘ C, H contents ↗ O content ↗ surface area	↗ As (V) and Pb (II) sorption	Wang *et al.* (2015a)
Loblollypine	600	Birnessite (post-pyrolysis)	↘ C, H, N contents	↗ As (V) and Pb (II) sorption	Wang *et al.* (2015a)

			↗ O content ↘ surface area		

Table 7. continued

Feedstock	Temperature of pyrolysis (°C)	Modification type (pre/post pyrolysis)	Properties modified	Effects	Reference
Loblollypine	600	Magnetization (pre-pyrolysis)	↘ C, H, N contents ↗ O content	↗ As sorption	Wang *et al.* (2015d)
Manure	300	Steam activation (post-pyrolysis)	↗ pH ↘ C, O contents ↗ ash content ↗ surface area	↗ Pb adsorption capacity	Kwak *et al.* (2019)
Manure	500	Steam activation (post-pyrolysis)	↗ O content ↗ surface area		Kwak *et al.* (2019)
Manure	700	Steam activation (post-pyrolysis)	↗ O content ↗ surface area	↗ Pb adsorption capacity	Kwak *et al.* (2019)
Miscanthus x giganteus	350	H_2O_2 activation (post-pyrolysis)	↗ surface area ↘ pH ↗ O content	↗ Cu removal	Cibati *et al.* (2017)
Miscanthus x giganteus	600	H_2O_2 activation (post-pyrolysis)	↗ surface area ↘ pH		Cibati *et al.* (2017)
Municipal solid waste	500	KOH (post-pyrolysis)	↗ ash content ↗ C content ↘ H, N contents ↗ surface area	↗ As(V) adsorption	Jin *et al.* (2014)
Nut shield	600	Magnetization (post-pyrolysis)	↘ surface area ↗ pH ↗ CEC	↗ Cd, Pb sorption	Trakal *et al.* (2016)
Peanut hull	600	Chitosan (post-pyrolysis)	↗ pH ↘ surface area	↗ Cd, Cu, Pb removal	Zhou *et al.* (2013)

↗ N, H, O contents
↘ C content

Table 7. continued

Feedstock	Temperature of pyrolysis (°C)	Modification type (pre/post pyrolysis)	Properties modified	Effects	Reference
Pine cone	500	Zinc coating (post-pyrolysis)	↗ surface area ↗ C content ↘ H content	↗ As (III) adsorption	Van Vinh *et al.* (2015)
Plum stone	600	Magnetization (post-pyrolysis)	↘ surface area ↗ pH ↗ CEC	↗ Cd , Pb sorption	Trakal *et al.* (2016)
Poultry litter	300	Magnetization (post-pyrolysis)	↘ C, N contents ↘ surface area		Lu *et al.* (2018)
Poultry litter	500	Magnetization (post-pyrolysis)	↘ C, N contents ↘ surface area		Lu *et al.* (2018)
Rice husk		Fe (post-pyrolysis)		↗ As (III) and As (V) sorption	Samsuri *et al.* (2013)
Rice straw	300	FeOS (post-pyrolysis)	↘ C, H contents ↗ N, O contents	↘ extractable [As]	Wu *et al.* (2018)
Rice straw	300	$FeCl_3$ (post-pyrolysis)	↘ surface area ↘ C, H contents ↗ N content	↘ extractable [As]	Wu *et al.* (2018)
Rice straw	300	$Fe^{(0)}$ (post-pyrolysis)	↘ surface area ↘ C, H, O contents ↗ N content	↘ extractable [As]	Wu *et al.* (2018)
Sawdust of white spruce	300	Steam activation (post-pyrolysis)	↗ C, N contents ↘ O content ↗ surface area	↗ Pb adsorption capacity	Kwak *et al.* (2019)

Table 7. continued

Feedstock	Temperature of pyrolysis (°C)	Modification type (pre/post pyrolysis)	Properties modified	Effects	Reference
Sawdust of white spruce	500	Steam activation (post-pyrolysis)	↗ C, N contents ↘ O content ↗ surface area ↘ ash content		Kwak *et al.* (2019)
Sawdust of white spruce	700	Steam activation (post-pyrolysis)	↗ pH ↘ C, H contents ↗ N, O contents ↗ ash content ↗ surface area	↗ Pb adsorption capacity	Kwak *et al.* (2019)
Sugarcane bagasse	600	Chitosan (post-pyrolysis)	↗ pH ↘ surface area ↗ N, H, O contents ↘ C content	↗ Cd, Cu, Pb removal	Zhou *et al.* (2013)
Wheat straw	300	Steam activation (post-pyrolysis)	↗ surface area	↗ Pb adsorption capacity	Kwak *et al.* (2019)
Wheat straw	500	Steam activation (post-pyrolysis)	↗ C, N contents ↗ surface area ↘ O content	↗ Pb adsorption capacity	Kwak *et al.* (2019)
Wheat straw	600	Magnetization (post-pyrolysis)	↘ surface area ↘ pH ↗ CEC	↗ Cd sorption	Trakal *et al.* (2016)
Wheat straw	700	Steam activation (post-pyrolysis)	↘ H, O contents ↗ surface area	↗ Pb adsorption capacity	Kwak *et al.* (2019)
Willow	700	Steam activation (post-pyrolysis)	↘ C, H, N contents ↗ surface area	↘ soil phytotoxicity ↗ *Lepidium sativum* root growth	Kołtowski *et al.* (2017)

			↗ ash content		

Table 7. end

Feedstock	Temperature of pyrolysis (°C)	Modification type (pre/post pyrolysis)	Properties modified	Effects	Reference
Willow	700	Microwave activation (post-pyrolysis)	↗ C content ↘ H, N, O contents ↗ surface area ↗ ash content	↘ soil phytotoxicity ↗ *Lepidium sativum* root growth	Kołtowski *et al.* (2017)

However, such modifications have mostly been studied in batch tests but poorly in soil. Moreover, modifications can also be negative or inefficient regarding the targeted metal(loid), especially after its incorporation in soil. Furthermore, biochar modification has an extra cost, therefore other amendments can be used, especially regarding As. In this purpose, iron-based amendments showed good results.

C. Iron-based amendments.

As shown in Tables 4 and 6, biochar and compost can have beneficial effects but they can also mobilize some metal(loid)s, such as As, Cu and Zn (Clemente *et al.* 2010, Hass *et al.* 2012, Alvarenga *et al.* 2014, Brennan *et al.* 2014, Kelly *et al.* 2014, Wang *et al.* 2015a). However, here, only the case of arsenic will be discussed.

Iron based amendments showed good potential in immobilizing arsenic, due to the affinity between As and Fe. Indeed, As(V) binded strongly to Fe(III) oxide minerals in the study of Miretzky and Cirelli (2010). Different iron sources can be used, *i.e.* iron oxides, iron sulfates, zero valent iron (ZVI) and iron rich industrial by-products. For instance, Fe sulfate amendment reduced As accumulation in lettuce (Warren and Alloway 2003). In addition to their positive effects on arsenic, iron-based amendments are also efficient on cationic metal elements such as Pb.

Two iron-based amendments will be detailed in this section: iron grit, a zero valent iron product, and redmud, an industrial by-product. An overview of their properties and effects on the soil and the plants is given Tables 8 and 9, respectively.

Iron grit

Iron grit is made of Fe^0, which can adsorb, surface precipitate and/or co-precipitate As when the iron corrodes after being applied to the soil (Mak *et al.* 2009). In another study, Kumpiene *et al.* (2006) demonstrated that ZVI amendment decreased SPW As concentration, As bioaccumulation and increased plant biomass. Later on, Qiao *et al.* (2018) showed that ZVI decreased As bioavailability by increasing the quantity of As bound to amorphous Fe-oxide. Indeed, ZVI is very reactive and can be oxidized and transformed into amorphous iron oxyhydroxides, which creates adsorption sites for As. Similarly, applying 1 % ZVI decreased As solubility by adsorption on Fe oxyhydroxides; it also improved sunflower and ryegrass biomass productions and decreased plant As uptake (Vítkovà *et al.* 2018).

Redmud

Redmud is a solid waste formed during the bauxite ore process to produce alumina (Bhatnagar *et al.* 2011). Each year, 90 to 120 million of tons of redmud are produced (Liu *et al.* 2011, Hua *et al.* 2017). Redmud is characterized by its highly alkaline pH, generally around 10-13 (Bhatnagar *et al.* 2011, Zhou *et al.* 2017). Its composition is variable but redmud is mainly composed of silica, Al, Fe, Ca and some other minor elements, such as Na, Ni, Cu, Pb, Zn … (Liu *et al.* 2011). Although it is classified as a hazardous waste, redmud has been used as an amendment in As and other metal(loid) contaminated soil stabilization studies. For instance, redmud application increased pH and decreased extractable metal(loid) concentrations (Lee *et al.* 2011, Gautam and Agrawal 2017). Similarly, redmud was efficient in the immobilization of As and other metal(loid)s, due to its large surface area and its high amount in Fe and Al oxides (Bertocchi *et al.* 2006). Moreover, Castaldi *et al.* (2009) showed that 4 % redmud decreased metal(loid) uptake in peat and wheat and increased their growth. Finally, Lee *et al.* (2014) found that applying 2 % redmud to a Pb/Zn mine increased soil pH and decreased $CaCl_2$-extractable metal(loid) concentrations. They showed that redmud could immobilize metal(loid)s through three mechanisms: (i) adsorption to highly accessible sites on the surface, (ii) precipitation with Al, Fe and Mn oxides and (iii) formation of minerals and diffusion across mineral surfaces. However, redmud application was also shown to have some negative effects on plants, such as chlorosis and nutrient-deficiency symptoms (Lombi *et al.* 2002). Therefore, it could be a good solution to add redmud in addition to another amendment.

Table 8 Overview of iron-based physico-chemical properties (ampty cases signify that no data was available, ZVI = zero-valent iron, nZVI = nano size zero-valent iron).

Amendment	**pH**	**Cation exchange capacity (cmol.kg^{-1})**	**Surface area (m^2.g^{-1})**	**Elemental composition (mg.kg^{-1})**							**Reference**
				Cd	**Cu**	**Pb**	**Zn**	**As**	**Al**	**Fe**	
Redmud	11.32			2.12	6.83	78.21	60.61				Lee *et al.* (2014)
Redmud	12.55		16.48								Liang *et al.* (2012)
Redmud	10.73	45.24	44.13		15	48	131				Nejad and Jung (2017)
Redmud	11.32			2.1		78.2	60.6	62.32			Lee *et al.* (2011)
Redmud	11.1		19.5						96,500	303,500	Garau *et al.* (2011)
Redmud	11.5	10.7				13.4	5.2				Garau *et al.* (2007)
Redmud	10.9			0.27	55.7	41.2	10.7	4.68		90,200	Clemente *et al.* (2019)
Hematite			8.4							995	Mamindy-Pajany *et al.* (2013)
Fe-rich water treatment residual	7.78		35						19.30	245.1	Garau *et al.* (2014)
Fe-rich water treatment residual	7.15	75.02		0.24	51.48	25.69	235.33			134.2	Manzano *et al.* (2016)
ZVI			1.8							962.8	Mak *et al.* (2009)
nZVI	7.53		25								Michalkova *et al.* (2017)
Geothithe					0	3.04	0	0.23			Hartley and Lepp (2008)
Goethite		129	32								Molla *et al.* (2017)
Iron grit					291.33	1.15	0	18.40			Hartley and Lepp (2008)

Table 9 Overview of the effects of iron-based amendments on soil and plants parameters (EC = electrical conductivity, SPW = soil pore water, ↗ = increase, ↘ = decrease).

Amendment	**Pollutant(s)**	**Effects**	**Reference**
Redmud	Cd, Cu, Pb, Zn	↗ soil pH ↗ soil EC ↘ $CaCl_2$ extractable [Cd], [Cu], [Pb], [Zn] ↘ SPW [Cd], [Cu], [Pb], [Zn]	Lee *et al.* (2014)
Redmud	Cd, Pb, Zn	↗ soil pH ↘ Cd, Zn, Pb leachability ↘ phyto-available [Cd], [Zn], [Pb]	Liang *et al.* (2012)
Redmud	Ni, Zn	↗ soil pH ↘ Ni, Zn leachability	Friesl *et al.* (2003)
Redmud	As, Cd, Cu, Pb, Zn	↗ soil pH ↘ As, Cd, Pb, Zn mobility ↗ Cu mobility	Feigl *et al.* (2012)
Redmud	As, Cd, Pb, Zn	↗ soil pH ↗ soil EC ↗ soil microbial activity ↘ lettuce [As], [Cd], [Pb], [Zn]	Lee *et al.* (2011)
Redmud	As	↗ soil pH ↗ number of As resistant bacteria ↗ soil enzyme activity	Garau *et al.* (2011)
Redmud	Cd, Pb, Zn	↗ soil enzyme activity ↘ Cd, Pb, Zn extractability	Garau *et al.* (2007)
Redmud	Cd, Pb, Zn	↗ pea and wheat biomass ↘ plant [Cd], [Pb], [Zn]	Castaldi *et al.* (2009)

Table 9. continued

Amendment	Pollutant(s)	Effects	Reference
Redmud (added with pig slurry)	As, Pb, Zn	↗ soil pH ↗ extractable [As], [Mn] ↘ extractable [Al], [Cu], [Pb], [Zn] ↘ SPW [Al], [Pb], [Zn] ↗ *Silybum marianum* biomass ↗ *Peptatherum miliaceum* biomass	Clemente *et al.* (2019)
Redmud	Cd,Pb, Zn	↗ soil pH, EC ↘ water extractable [Cd], [Zn] ↗ water extractable [Pb] ↘ Pb bioaccessibility ↗ soil enzyme activity ↗ lettuce biomass ↘ lettuce [Cd], [Zn]	Lee *et al.* (2009)
Redmud	As	↗ *Panax notuginseng* biomass ↘ plant [As]	Yan *et al.* (2013)
Iron grit	As, Cu, Cr	↘ As, Cr solubility ↘ soil pH	Kumpiene *et al.* (2009)
$FeSO_4$	As, Cu	↘ SPW [As], [Cu] ↘ extractable [As], [Cu]	Fresno *et al.* (2017)
$FeSO_4$	As	↘ soil pH ↘ lettuce yield ↘ lettuce [As]	Warren and Alloway (2003)
Zero-valent iron	As, Cd, Pb, Zn	↗ soil pH ↘ $CaCl_2$ extractable [Cd], [Pb], [Zn] ↗ soil enzyme activity ↘ sunflower [Zn]	Michálková *et al.* (2017)

Table 9. end

Amendment	**Pollutant(s)**	**Effects**	**Reference**
Zero-valent iron	As, Cu	↘ dissolved [As], [Cu] ↗ soil pseudo-total [Fe]	Tiberg *et al.* (2016)
Zero-valent iron	As	↗ *Panax notuginseng* biomass ↘ plant [As]	Yan *et al.* (2013)
Nano Zero-valent iron	As, Cd, Pb, Zn	↘ dissolved organic carbon content ↘ As solubility ↗ sunflower root and shoot biomass ↘ sunflower [As], [Cd], [Pb], [Zn]	Vítkovà *et al.* (2018)

D. Combination of amendments.

This section showed that many amendments have beneficial effects on soil and plants, but they also have limits and negative effects. Therefore, a good solution could be to apply different amendments in combination, in order to have complementary effects and better results.

For instance, Agegnehu *et al.* (2016) applied biochar and compost alone or combined to an agricultural soil and observed that in all cases, amendments had positive effects on most of the maize growth parameters and also increased soil organic carbon and soil exchangeable Ca, Mg, Na and CEC. Similarly, Beesley *et al.* (2014) used a single and combined application of biochar and compost to a mine soil (As, Cd, Zn) and observed an increase of soil pH and SPW phosphate concentration as well as an increase of *Lolium perenne* germination, but only in the case of biochar and biochar+compost amendment. In a previous study, Beesley *et al.* (2010) also used, on a soil contaminated by As, Cd, Cu and Zn, biochar and compost amendments, alone or combined; however, results were different. They observed that soil pH increased in all cases, but the highest rise was observed for the combination. Amendments also increased total organic carbon, water soluble carbon and dissolved organic carbon contents and decreased extractable Cd concentrations. However, all the amendment treatments increased water extractable As and Cu concentrations. Furthermore, shoot emergence also increased; however, the combination showed lower benefits than the single amendments. Padmavathiamma and Li (2010) showed that a combined application of diverse amendments (lime, phosphate, compost) to a Cu, Mn, Pb and Zn contaminated soil lowered Pb and Mn plant concentrations more importantly than their single application. Another study also showed that when applied together, biochar and compost interact. Indeed, biochar physico-chemical properties and functional groups can be modified with the humus oxidation and the microorganisms of the compost. Moreover, biochar improves compost humification and quality (Liang *et al.* 2017). The combined application of biochar and compost increased watermelon yield compared to alone application (Yun *et al.* 2017), whereas Ohsowski *et al.* (2018) showed that biochar, compost and arbuscular mycorrhizal fungi had a synergistic effect in increasing grass biomass on post-mine sandpits. Fresno *et al.* studied the application to a former smalting factory (As and Cu) of $FeSO_4$ with different amendments in several studies (Fresno *et al.* 2016, 2017, 2018) and showed either positive, negative or no-effects. For instance, soil pH increased in all cases except the combination of Fe+biochar, whereas As mobility and leaching decreased in all treatments. However, in most of the cases, no effect on plant growth was observed, except for the increase with Fe+compost and the decrease with Fe+papermill sludge and Fe+biochar. Jones *et al.* (2016) observed that biochar and compost combination decreased Cu leaching more importantly than biochar alone and also increased sunflower height better than their single application. Biochar and zero-valent iron were used alone or combined in the study of Li *et al.* (2018b), which showed that biochar increased *Brassica rapa* biomass whereas the combined amendment decreased water soluble As more importantly than the single amendment. The three studies of Oustriere *et al.* (2016, 2017a, 2017b) applied biochar, compost and/or iron grit and showed that combining biochar with iron grit increased soil pH more importantly than biochar alone. In addition, the increase in SPW Cu concentration was lowest with iron grit while bean shoot yield decreased with the biochar – iron grit combination (Oustriere *et al.* 2016). However, biochar and biochar + iron grit amendments had no effect on plant growth (Oustriere *et al.* 2017a, b). Finally, two studies showed that although biochar and compost had beneficial effects on soil and plant, their combination had no synergistic or additional effects, and even showed some antagonistic effects on plant growth and physiological performance (Seehausen *et al.* 2017, Trupiano *et al.* 2017). Finally, biochar could also be introduced directly during the composting process. Such composted biochar showed beneficial effect on plant growth (Schulz *et al.* 2013), combined to a reduction of the ammonia emission and nitrogen loss of the composting process (Malinska *et al.* 2014). Moreover, Zeng *et al.* (2015) evaluated the effect of applying compost, biochar, biochar and compost combined, composted-biochar and biochar-composting to a contaminated soil. They observed that all the treatments reduced the bioavailable fractions of metal(loid)s and their concentrations in soil pore water, but the most effective were composted-biochar and biochar-composting. Finally, such process could “aged” the biochar faster, and thus modify its properties. This could ameliorate its effects on the soil and the plant, notably for biochar that have a negative primary effect when applied to the soil.

All of these studies showed that amendment combinations can have better effects than the single application of amendments, but it also demonstrated that amendment combination was in some cases, not better or worse than alone amendments.

To conclude, amendments are usually required on metal(loid) contaminated sites in order to improve soil conditions and thus allow plant growth. Furthermore, in addition to choose the right amendment(s) to use, select the appropriate species is an important parameter for the phytomanagement success.

IV. Plants used in phytomanagement.

As stated previously, phytomanagement of polluted soils relies on the combination of an efficient amendment to allow plant growth and the choice of a plant species harboring the right properties, depending on the technique used. Indeed, in the case of phytoextraction, plants should tolerate high levels of pollutants, have a rapid growth and produce an important biomass and a deep root system, accumulate metal(loid)s at high concentrations especially in the above ground tissues (Alkorta *et al.* 2004, Ernst 2005). Plants used in phytostabilization should present an extensive root system, grow rapidly and limit the translocation of metal(loid)s towards harvestable parts (Vamerali *et al.* 2010, Gomes *et al.* 2016).

A. Herbaceous species.

Many studies evaluated diverse plant species for their potential in phytoremediation. For instance, Fellet *et al.* (2007) observed that *Glycine m*ax and *Sorghum bicolor* accumulated more arsenic and metals than *Zea mays* and *Helianthus annuus*. Foucault *et al.* (2013) found that borage, white mustard and phacelia grew well on different polluted sites and that borage and white mustard improved soil conditions. Lehmann and Rebele, in 2004, found that *Calamagrostis epigejos* was tolerant to Cd whereas *Elymus repens* was tolerant to Zn. Finally, *Sorghum bicolor* and *Helianthus annuus* showed good phytoremediation potential towards As, Cd, Co, Cu and Zn (Marchiol *et al.* 2007) whereas *Helianthus tuberosus* was suitable for the phytoextraction of Mn, Zn, Cd and Ni (Willscher *et al.* 2016). Moreover, a lot of herbaceous species have been characterized as hyperaccumulator species. For instance, *Noccaceae caerulescens* was found to be able to hyperaccumulate Zn and Cd whereas *Pteris vitata* can hyperaccumulate As (Burges *et al.* 2018). Similarly, many species of the *Brassicaceae* family are known for their hyperaccumulation capacity (Chibuike and Obiora 2014).

However, even though these species showed good metal(loid) accumulation potential, they produce a low biomass compared to tree species and are often annual plant species. Moreover, their root system is often small and do not explore a deep soil surface. Therefore, when used in phytoextraction, only the soil surface can be de-contaminated and it will take a long time due to the low biomass produced and thus the low phytoextraction potential. Similarly, when used in phytostabilization, the reduced root system will only immobilize metal(loid)s at the surface. Thus, higher plants with a more important biomass production potential are a better solution.

B. Woody species.

Contrary to annual herbaceous species, trees are a good option due to their perennial property and long life as well as their important biomass and deep and wide root system, which allow the exploration of an important soil area (Gomez *et al.* 2019). Moreover, trees have essential ecological roles. They intervene for instance in biogeochemical cycles, environmental health, climate regulation, soil conservation and habitat protection (Gomez *et al.* 2019).

Several tree species have been studied and showed a good tolerance towards metal(loid)s and accumulation properties. For instance, Alagić *et al.* (2013) demonstrated that *Tillia sp.* and *Betula sp.* had a low As uptake but that they both developed defense mechanisms towards arsenic. Evangelou *et al.* (2013) observed that birch was suitable on soil presenting elevated Zn and Cd concentrations, based on its metal accumulation and dry weight production whereas oak was the species presenting no growth reduction and no toxicity symptoms but generally produced low biomass. Hartley *et al.* (2011) observed an improvement of the soil conditions following *Alnus cordata* and *Alnus glutinosa* growth. Finally, the study of Hermle *et al.* in 2006 revealed that *Picea abies* was highly tolerant to metal(loid) stress and showed no growth reduction and an important metal(loid) accumulation. However, these species are less studied than another tree family, *Salicaceae*.

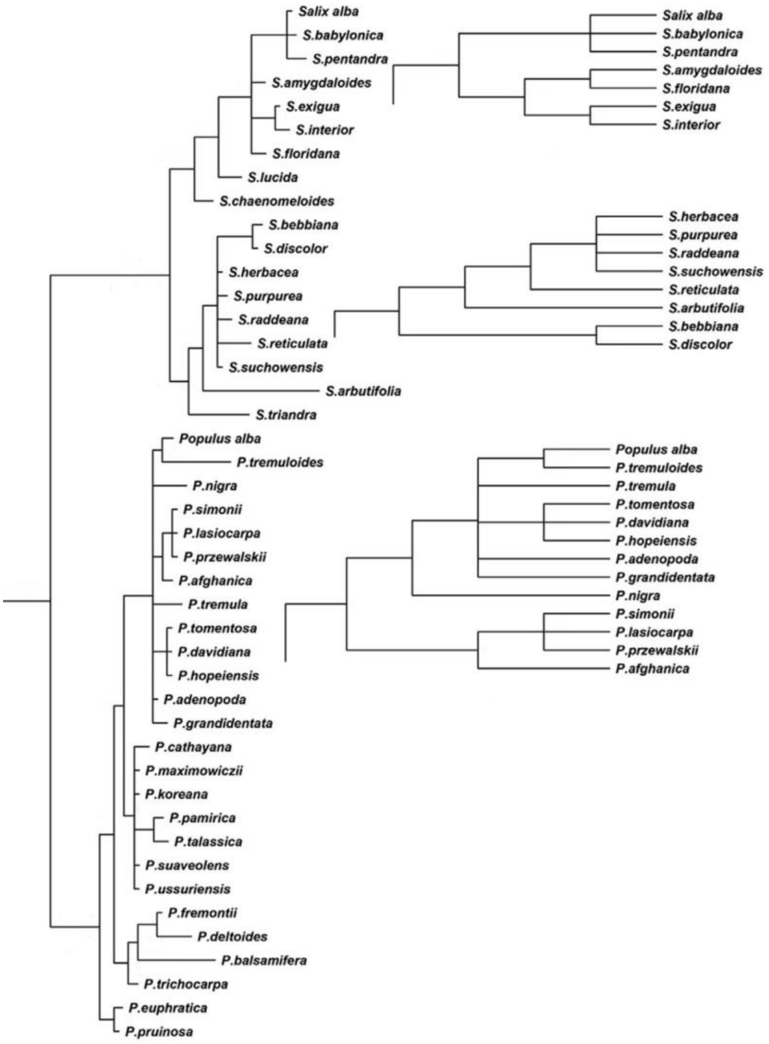


Figure 14 Phylogeny of Salicaceae *family, showing the main species of Salix and Populus (modified from Liu* et al. *2016b).*

Salicaceae are divided into two genera, the genus *Salix*, composed of 450 species, and the genus *Populus*, composed of 30 species divided into six major sections. An overview of the main species of *Salicaceae*, with their phylogenetic relation, is given Figure 15. They are widely distributed in the Northern hemisphere, are pioneer species and have the ability to colonize poorly fertile sites, making them a good option in degraded contaminated soils (Kuzovkina and Quigley 2005). However, their choice as phytoremediators relies more on other properties: they produce an important biomass, from 10 to 30 $t.ha^{-1}.year^{-1}$ on average on non-contaminated soils (Guerra *et al.* 2011, Marmiroli *et al.* 2011, Courchesne *et al.* 2017), present a fast growth associated to a high transpiration rate, a deep and wide root system and they are also tolerant to diverse stresses (Marmiroli *et al.* 2011). Indeed, they showed the ability to grow in hydropony solutions containing until 100 µM Cu, 0.5 M Pb or 1 mM Zn (Borghi *et al.* 2007, Chen *et al.* 2013) and to develop on soils presenting pseudo-total metal(loid) concentrations of several thousands milligrams per kilograms (Sebastiani *et al.* 2004, Laidlaw *et al.* 2012, Kácalková *et al.* 2014, Bart *et al.* 2016).

Many previous studies showed the potential of *Salicaceae* species to tolerate metal(loid) stress, in hydropony experiments for instance, and their efficiency in the remediation of metal(loid) contaminated soils.

For instance, Kuzovkina *et al.* (2004) performed a hydropony experiment to evaluate the ability of five willow species, *Salix discolor*, *Salix eriocephala*, *Salix exigua*, *Salix nigra* and *Salix lucida*, to accumulate Cu and Cd. They found a 100 % survival rate for all species and that willows were Cd accumulators but Cu excluders. An Asian willow species was used to treat an As-contaminated water. Results showed that this species presented a translocation factor, *i.e* the ratio between the aboveground concentrations to the root concentrations, below 1, showing its suitability in arsenic phytostabilization (Chen *et al.* 2014b). Indeed, the establishment of such species would prevent the erosion, leaching and runoff of the pollutants. Moreover, plant growth will also convert the pollutant into a less bioavailable form (Chen *et al.* 2014b).

In a pot experiment, *Salix viminalis* and *Salix fragilis* plants accumulated high concentrations of Cu, Cr, Ni and Pb in their roots (Vandecasteele *et al.* 2005). Additionally, diverse willow and poplar clones were tested on two soils collected on a former Zn and Pb smelter and all were capable of growing on both moderately and highly polluted soils, showing their tolerance to these elements (Dos Santos Utmazian and Wenzel 2007). Two willow species, *Salix viminalis* and *Salix purpurea*, were able to develop a root system and produce an aboveground biomass on a former gold mine, and strongly accumulated arsenic (Bart *et al.* 2016).

In 2003, Vervaeke *et al.* published the results of their field experiment on dredged sediment disposal sites using *Salix viminalis*. They showed that *Salix viminalis* establishment made an effective green capping and helped in the metal(loid) stabilization and the prevention of their leaching, as water infiltration was reduced. The study of Unterbrunner *et al.* (2007) showed that *Salix caprea*, *Salix purpurea* and *Salix tremula* plants, collected at four European sites contaminated by Cd and Zn, possessed a large accumulation potential. Similarly, on the Metaleurop contaminated site, soil, leaves and stems were sampled to evaluate the remediation efficiency. On this site, willow and poplar plants accumulated in their leaves and stems between 200 and 950 mg.kg^{-1} Zn, 13 to 44 mg.kg^{-1} Cd in their leaves and 9 to 15 mg.kg^{-1} Cd in their stems. Finally, Pb concentrations were about two to four times higher in the stems than the leaves, with stem concentrations between 50 and 100 mg.kg^{-1} (Migeon *et al.* 2009). In the same way, shortly after the cessation of the disposal activity at four dredged sediment landfills and four infrastructure spoil landfills, a vegetation composed of willows was observed (Vandecasteele *et al.* 2009). Moreover, Hartley *et al.*, in 2011, showed that willows were shoot accumulators of Zn and Cd and root accumulators of Cu, Cr, Ni and Pb. Such behavior could be due to the uptake mechanism of the plants. For instance, Trakal *et al.* (2015) showed that transportation of metal(loid)s towards upper parts was dependent on the metal(loid): Ag, B, Li, Mo and Se being easily transported; Mn, Ni, Cd and Zn being moderately mobile whereas Co, Cu, Cr, Pb, Hg and Fe were strongly bind to the roots, particularly the cell wall. Moreover, Trakal *et al.* (2013) observed that the accumulation of Cd and Zn in willow was higher that the concentrations predicted by their model based on water flow transport, showing that these elements were not only transported with water and that the plant, with their exudates, could affect the bioavailability of the metal(l(oid)s. Four willow clones and three poplar clones rooted well on a former maize field contaminated by Cd, Cu, Pb and Zn. Also, among those clones, willow plants produced a higher shoot biomass than poplars. Finally, Zn concentrations were higher in leaves than shoots (Ruttens *et al.* 2011). It was also observed that willows and poplars performed a normal growth on a former waste incineration plant contaminated by Cu, Cd and Zn. Plants did not present any visual symptoms of toxicity and Cd and Zn were found in higher amounts in willow leaves (Kácalková *et al.* 2014). Finally, on a field experiment, Nandillon (2019) showed in a field experiment that four *Salix* species (*S. purpurea*, *S. alba*, *S. triandra* and *S. viminalis*) were able to grow on an As and Pb contaminated soil amended with biochar, compost and/or iron sulfate and that among those species, *Salix triandra* produced the most important biomass, associated to the lowest metal(loid) translocation towards the upper parts.

Some examples of studies of *Salicaceae* in metal(loid) contaminated conditions, *i.e* hydropony, pot and field experiments, are given Table 10.

Table 10 Examples of use of Salicaceae *species in metal(loid) contamination studies.*

Pollutant(s)	***Salicaceae* species**	**Experiment type**	**Observations**	**Reference**
Pb	*Salix integra*	Aeropony	Tolerance, transport and accumulation of Pb	Wang *et al.* (2014)
Cu, Zn	*Populus deltoides* *P. x canadensis*	Hydropony	Cu accumulated mainly in leaves and stems Zn accumulated mainly in roots	Benyó *et al.* (2016)
Cu	*P.x euramericana*	Hydropony	Reduction of plant biomass Accumulation in roots Tolerance to high Cu levels	Borghi *et al.* (2007)
Cd, Ni, Pb	*Salix alba* (clone 68/53/1) *Salix alba* (clone 106/54/0) *Salix matsudana* (clone SM 4041) *Salix nigra* (clone 0408)	Hydropony	Accumulation in roots Clone 0408 best suited for Cd and Ni phytoextraction	Borišev *et al.* (2009)
Pb, Zn	*Populus beijingensis* *Populus cathayana*	Hydropony	*Populus beijingensis* more tolerant Slight Pb toxicity Accumulation in roots *Populus beijingensis* more effective for phytoremediation	Chen *et al.* (2013)
As	*Salix jiangsuensis* J172 *Salix matsudana* *Salix integra* Yizhibi *Salix integra* Weishanhu *Salix mongolica*	Hydropony	*S. integra* Yizhibi highest root As concentration	Chen *et al.* (2014b)
Cu, Cd	*Salix discolor* *Salix eriocephala* *Salix exigua* *Salix nigra* *Salix lucida*	Hydropony	100 % survival Cd accumulation Cu exclusion	Kuzovkina *et al.* (2004)
Cd, Cu, Fe, Pb, Zn	*Popolus alba* AL22 *Populus nigra* N12	Pot	$[\mathrm{Cu, Fe, Pb}]_{\mathrm{root}} > [\mathrm{Cu, Fe, Pb}]_{\mathrm{leaf,\ stem}}$ $[\mathrm{Zn, Cd}]_{\mathrm{leaf}} > [\mathrm{Zn, Cd}]_{\mathrm{stem,\ root}}$	Baldantoni *et al.* (2014)

Table 10. continued

Pollutant(s)	***Salicaceae* species**	**Experiment type**	**Observations**	**Reference**
As, Pb, Sb	*Salix viminalis* *Salix pupurea*	Pot	Ability to develop a root system Strong accumulation of As	Bart *et al.* (2016)
Cd, Pb, Zn	*Salix smithiana*	Pot	Efficient phytoextraction of Cd and Zn	Puschenreiter *et al.* (2013)
Cd, Cu, Cr, Zn	*Populus deltoides x maximowiczii* *P. x euramericana*	Pot	Increased growth Increased accumulation	Sebastiani *et al.* (2004)
Cu, Cr, Ni, Pb	*Salix viminalis* *Salix fragilis*	Pot	High accumulation in the roots	Vandecasteele *et al.* (2005)
As, Cd, Cu, Ni, Pb, Zn	*Salix purpurea Fish Creek* *S. x dasyclados SV1* *S. miyabeana SX67*	Field	Fish Creek efficient for Cu, Ni, Zn extraction SV1 efficient for As, Cd, Pb extraction	Courchesne *et al.* (2017)
Zn, Cd, Pb, As, Cu, Co, Hg, Al	*Populus deltoides Dvina* *Populus deltoides Lena* *Populus x canadensis Neva* *Salix matsudana S76-005* *Salix matsudana S76-008* *Salix alba clone SI64-017*	Field	Good phytoextraction and phytostabilization	De Paolis *et al.* (2011)
Cd, Cu, Zn	*Salix x smithiana* *Salix rubens* *Populus nigra x maximowiczii* *Populus nigra*	Field	Normal growth No symptom of toxicity Highest removal of Cd and Zn by *Salix smithiana*	Kácalková *et al.* (2014)
As, Cr, Cu, Hg, Pb	*Salix x caprea* *Salix x calodendron* *Salix x calodendron* *Salix chilensis* *Salix x reichardtii* *Salix matsudana* *Salix viminalis*	Field	Good growth Reduced extraction	Laidlaw *et al.* (2012)

Table 10. end

Pollutant(s)	*Salicaceae* species	Experiment type	Observation	Reference
Cd, Zn, Pb	*P. deltoides x Populus nigra* *Populus tremulaxPopulus alba* *P. tremula x Populus tremuloides* *P. trichocarpa x P. deltoides*	Field	High accumulation properties	Migeon *et al.* (2009)
Cu, Pb, Zn	*Populus deltoides x Populus nigra* *Salix matsudana*	Field	Species suitable for Cu, Pb, Zn phytomanagement	Nissim *et al.* (2018)
As, Pb	*Salix purpurea* *Salix alba* *Salix triandra* *Salix viminalis*	Field	Good growth of all species Highest growth for *Salix triandra*	Nandillon (2019)

All these studies demonstrated that both willows and poplars were tolerant to elevated metal(loid) concentrations. They also perform good growth on contaminated sites and accumulated substantial amounts of metal(loid)s, making them a good option in water and soil remediation. Furthermore, their growth on contaminated soils in short rotation coppice can add an economical benefit to the phytomanagement process. For instance, in its PhD, Romain Nandillon (Nandillon 2019) showed that the biomass production of *Salix triandra*, after two years of growth on an amended contaminated technosol and under field conditions, was close to the one observed in short rotation coppice on uncontaminated soils. Moreover, poplars and willows can also have an effect on metal(loid)s at the rhizosphere level, through their root activity. Indeed, root exudates can modify metal(loid) bioavailability and the microbiota composition (Gomez *et al.* 2019).

V. The rhizosphere.

A. Definition.

The rhizosphere has been first described in 1904 (Seshadri *et al.* 2015). It is the zone of the soil where plant roots, microorganisms and soil interact and influence each other; it is localized 2 mm radially from the root surface (Martin *et al.* 2014). It is an important zone where many physical, chemical and biological processes occur (Seshadri *et al.* 2015). This zone is important in terms of root growth, exudate production as well as microbial community composition and activities (Bertin *et al.* 2003) and its good state is crucial for the success of the phytomanagement process (Wenzel 2008). Rhizosphere is composed of two main components: the microorganisms, which will be discussed in the next section, and roots, which will be detailed in this section.

B. The roots and metal(loid) stress.

Plant roots are very important organs. In addition to being the anchor of plants in the soil and assuring plant nutrition, they can also modify the soil conditions and alter the microbial community composition and activity. The main mechanisms by which plant roots influence the rhizosphere is exudation and association with bacteria and fungi. In return, plant root activity is also driven by the soil conditions (Seshadri *et al.* 2015). Therefore, the study of the root activity is crucial in phytomanagement. To do so, different analyses can be performed. The compounds exuded by the roots, called root exudates in general, can be evaluated in terms of composition and quantity. In addition, the proteomic profile can be evaluated to have a picture of the protein produced under a specific condition. Finally, roots can be examined at the biochemical level, with the analysis of the oxidative stress markers.

1) Root exudates.

Plant rhizodeposits, which come mainly from photosynthesis (Dong *et al.* 2007) can be grouped into five general classes: exudates, secretion, plant mucilage, mucigel and root lysates (Kidd *et al.* 2009). This part will focus only on root exudates. Their amounts and composition vary between plant species, cultivar, age and stress factors (Bertin *et al.* 2003).

Root exudates can be released into the soil through diverse mechanisms (Figure 16) (Bertin *et al.* 2003):

- Diffusion, using a passive process against a gradient;
- Ion channel;
- Vesicle transport.

The process used will depend on the compound exuded. Indeed, root exudates are composed of low molecular weight organics, such as sugars, amino acids, carboxylic acids and phenolic compounds, which are released through diffusion; carboxylates that are exported using ion channels and high molecular weight compounds, such as proteins and mucilage, that use vesicle transport (Bais *et al.* 2006, Bertin *et al.* 2003).

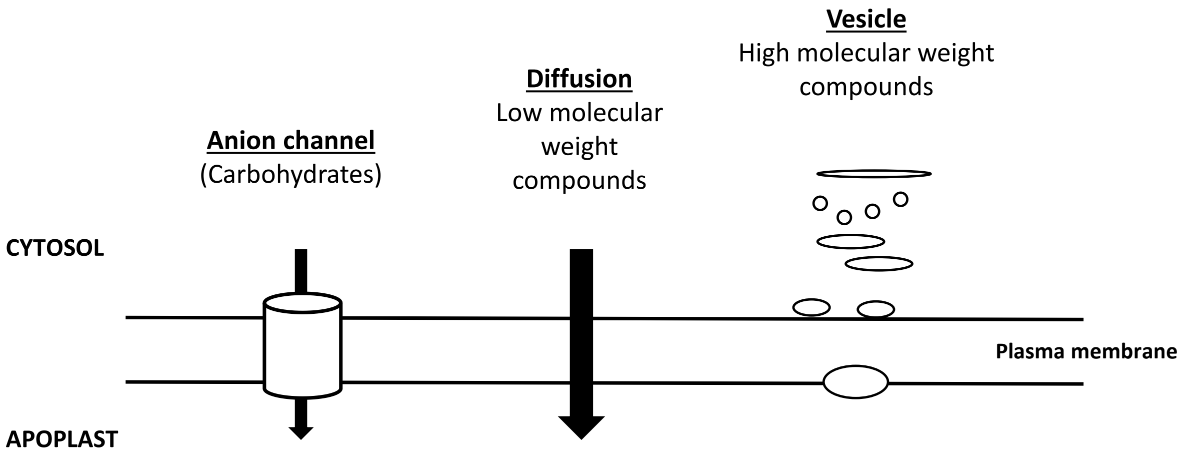


Figure 15 Mechanisms of root exudation (source: Bertin et al. *2003). Molecules produced by plants can be released into the rhizosphere through three main mechanisms: diffusion for low molecular weight compounds, anion channel transport for carbohydrates and vesicle transpot for high molecular weight compounds.*

As said before, root exudates have an important role in the rhizosphere: they can serve as a carbon source for microbial growth (Dakora and Philips 2002) and can also alter metal(loid) bioavailability by modifying soil conditions, especially soil pH and redox potential, but also by precipitating metal(loid)s, though the release of metal(loid) chelators (Dong *et al.* 2007, Gomez *et al.* 2019). This last function is mainly performed by organic acids exuded by roots (Dong *et al.* 2007). Indeed, organic acids are chelating substances, which can form complexes with metal(loid)s (Rosas *et al.* 2007, Huang *et al.* 2016b). For instance, low molecular weight organic acids (LMWOA) can modify the rhizosphere conditions and can chelate Pb in the rhizosphere, reducing its phytotoxicity (Shahid *et al.* 2012). Similarly, citric acid showed a strong affinity to form stable complexes with metal(loid)s (Schwab *et al.* 2008, Meier *et al.* 2012). Finally, the concentration of succinic, tartaric and malic acids and the amount of organic acids in general increased with increasing Cu dose as a detoxification mechanism (Huang *et al.* 2016b).

These results show the importance of organic acids exudation in metal(loid) immobilization. Therefore, their collection and study can give information regarding the mechanism used by plants to tolerate metal(loid) stress.

2) Oxidative stress.

The production of reactive oxygen species (ROS) is a common feature in plants when they are exposed to metal(loid)s (Xu *et al.* 2019). ROS are free radicals and act as the second messenger in a variety of cellular processes. However, whether they will act as messenger or damaging molecules depends on the equilibrium between ROS production and ROS scavenging (Sharma *et al.* 2012). Indeed, ROS are continually produced, even under normal conditions, but are over expressed under stress. Plants possess diverse scavenging mechanisms to maintain ROS at a normal level, but these mechanisms can be overlapped, causing oxidative damage such as lipid peroxidation. For instance, lipid peroxidation increased with Pb stress (Verma and Dubey 2003). To counteract the oxidative stress caused by metal(loid) exposure, plants use both non enzymatic (*i.e.* glutathione, ascorbic acid, carotenoids, flavonoids, phenolic compounds) and enzymatic systems (*i.e.* superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPOD) and glutathione-S-transferase (GST)) (Sharma *et al.* 2012, Ishtiyaq *et al.* 2018).

Glutathione is the most important non enzymatic intracellular defense (Ahmad *et al.* 2009, Ishtiyaq *et al.* 2018). Similarly, ascorbic acid is a powerful antioxidant that can scavenge ROS (Ahmad *et al.* 2009) and is one of the first line of non-enzymatic defense (Ishtiyaq *et al.* 2018). Carotenoids have three major functions in plants: they absorb the light and transfer it to the chloroplasts, they intervene in the assembly of the photosystem I but more importantly they protect the photosynthesis apparatus by quenching free radicals (Ahmad *et al.* 2009). Flavonoids are a large family of plant secondary metabolites (Sytar *et al.* 2013). They are strong antioxidants that assist in the reduction of ROS (Salam *et al.* 2019), acting against a variety of compounds. For instance, they are ideal scavengers of H_2O_2 due to their reductive potential (Ahmad *et al.* 2009). Similarly, phenolics also assist in ROS reduction (Salam *et al.* 2019). Phenolics are plant carbon-based secondary metabolites, which protect plants from oxidative stress (Esteban *et al.* 2008). They tend to be accumulated under stress (Sakihama *et al.* 2002).

Among the enzymatic system, SOD is a metalloenzyme and four SODs are described in plants based on the metallic ions attached to their active site: Cu/Zn-SOD found in the cytosol and the chloroplast, Mn-SOD localized in the mitochondria and the peroxisomes, Fe-SOD and Ni-SOD. SODs are over expressed to counteract the negative effects of the oxidative stress (Alscher *et al.* 2002). Indeed, they can induce the deprotonation of O_2^- to H_2O_2 and O_2, reducing the metal(loid)-induced oxidative stress (Salam *et al.* 2019). It has a central role in defense against oxidative stress as it is one of the most effective intracellular enzyme (Sharma *et al.* 2012, Ishtiyaq *et al.* 2018). Following the action of SOD that produces H_2O_2, CAT, a universally present oxidoreductase, intervenes and converts H_2O_2 into O_2 and H_2O (Verma and Dubey 2003, Salam *et al.* 2019). Similarly, GPOD can eliminate H_2O_2 excess and preferentially oxidizes aromatic election donors (Sharma *et al.* 2012, Ishtiyaq *et al.* 2018). Finally, GST can serve as intracellular detoxification mechanism of radicals (Reddy *et al.* 2005) and is part of the last group of enzymes involved in plant defense against ROS (Smolinska and Szczodrowska 2016).

All of these non-enzymatic and enzymatic systems were shown to increase under metal(loid) stress. For instance, Salam *et al.* (2019) found that antioxidative enzymes increased in activity with high concentrations of Cu, Zn and Ni. They also showed that such higher antioxidation could increase the ability of plants to scavenge free radicals.

Therefore, study the expression of both enzymatic and non-enzymatic antioxidative systems can inform on the oxidative stress level plants are under. Moreover, it can provide information on which system is more induced under a specific stress.

3) Root proteomic.

Proteomic research is a high throughput analytical technique (Ahsan *et al.* 2009). This technology rapidly developed and has been much applied to analyze the different levels of synthesis of proteins in response to diverse stresses (Komatsu and Ahsan 2009). Indeed, proteins of diverse functions were reported to be related to stress response, such as response to metal(loid)s, heat, drought, salinity… (Bonhomme *et al.* 2009, Li *et al.* 2009, Durand *et al.* 2012, Rodziewicz *et al.* 2014, Liu *et al.* 2017). Under stress conditions, specific pathways are either repressed or activated, which is shown by the down- and over-synthesis of proteins (Abreu *et al.* 2013, Rodziewicz *et al.* 2014).

For instance, using hydroponic growth and proteomic analysis, Li *et al.* (2009) build a model of the response of *Elsholtzia splendens* to copper stress: *E. splendens*, under Cu stress, modified the abundance of proteins involved in redox homeostasis, energy metabolism, cell wall metabolism, cytoskeleton rearrangement and cell defense. Each protein class have a role: redox homeostasis acts as a signal, cell defense act to bind Cu to the cell walls, sequester Cu into vacuole and reduce Cu mobility for instance. In their review, Ahsan *et al.* (2009) showed that in response to metal(loid) stress, the generation of ROS induced an up regulation of proteins involved in the antioxidative defense mechanisms, signaling molecules and chaperones (Figure 17).

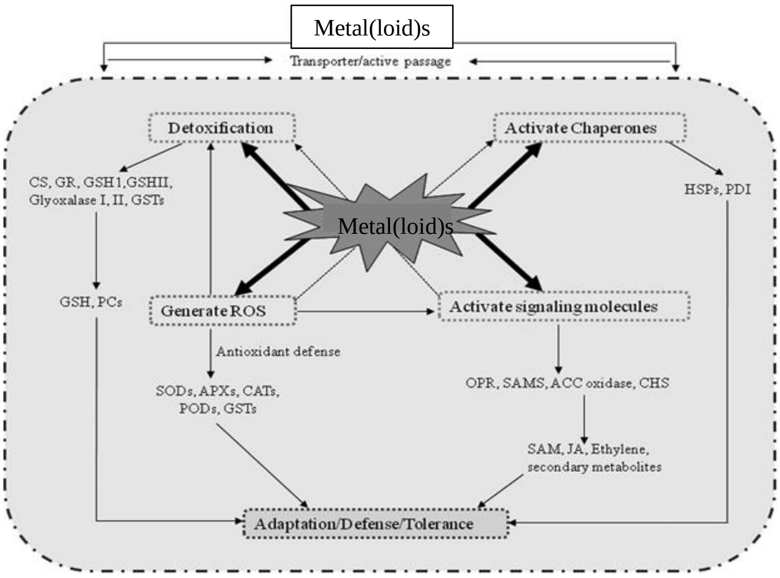


Figure 16 General scheme of plant reponse to metal(loid) stress. Metal(loid) entry in cell induces the generation of reactive oxygen species (ROS), which activates detoxification mechanisms, signaling molecules and chaperones, involved in plant tolerance to stress (source: Ahsan et al. *2009).*

Similarly, Liu *et al.* (2017) showed that in response to As, poplars up-regulated their carbohydrate and energy metabolisms, showing that to maintain their survival under such stress, poplars consumed a lot of energy. Gutierrez-Carbonell *et al.* (2013) also demonstrated that the metabolic pathways most affected by Zn were energy related followed by carbohydrate metabolism and oxidative stress. Finally, Sharmin *et al.* (2012) found 36 proteins differentially represented in roots treated with Cu, the majority being related to ion transporters, energy and nitrogen metabolism.

Therefore, as the root exudates, the analysis of the proteome, *i.e.* the complete set of proteins expressed at a given time and under specific conditions (Cvjetko *et al.* 2014), can provide information about which plant functions are affected by metal(loid)s but also on how plants react to metal(loid) stress by modifying the synthesis of their proteins to tolerate such stress.

VI. Soil microorganisms.

The soil, and especially the rhizosphere, constitutes a habitat for many microorganisms (Thavamani *et al.* 2017), such as bacteria, fungi, actinomycetes, protozoa and algae. Bacteria are the most important and the most abundant ones, with 10^8 to 10^9 cells per gram of soil on average (Yu *et al.* 2019). They can live freely in the rhizosphere or in the cellular spaces of the root tissues (rhizobacteria) (Ahmad *et al.* 2018).

The measure of the activity and composition of the soil bacterial community is an indicator of soil health. This can be assessed through diverse techniques, *i.e.* the measure of the soil enzyme activities, the evaluation of the community level physiological profiles (CLPP) and soil DNA extraction followed by next generation sequencing (NGS).

Indeed, soil enzymes constitute the biochemical status of the soil. They are an early and sensitive indicator of the ecological restoration status of the soil (Alvarenga *et al.* 2014), as they are highly sensitive to the changes of the soil conditions (Mierzwa-Hesztek *et al.* 2016). Furthermore, the majority of the enzymes present in a soil are believed to be mainly of microbial origin. Thus, the evaluation of their activities can provide information about the modification of the microbial community (Al-Marzooqi and Youssef 2017) following biotic or abiotic stress. Moreover, it is generally believed that a soil with a high activity of diverse enzymes is of good quality (Mierzwa-Hersztek *et al.* 2016).

Additionally, CLPP can provide information on the activity of the cultivable fraction of the bacterial community. CLPP is usually based on the ability of the microorganisms to oxidize different carbon substrates (Gomez *et al.* 2006, Lombi *et al.* 2002). It aims to inform about the functional diversity of the community (Lombi *et al.* 2002). To assess CLPP, bacterial community is extracted from the soil and inoculated into Biolog EcoPlates^{TM} (Figure 18).

Finally, in addition to the microbial activity, the composition of the microbial community can be assessed through NGS, which identifies the microbial community based on operational taxonomic units (OTUs). Such technique could be used to give the pattern of the microbial community in response to diverse environmental conditions (Bucci *et al.* 2015).

Such evaluation can inform on the quality of the soil, following contamination but also following amendment application. Indeed, as discussed previously, amendments can modify the soil physical-properties and thus influence the bacterial community, as microorganisms are very sensitive to soil conditions. However, such influence can be negative or positive for the microorganisms. For instance, Latini *et al.* (2020) tested two different biochars and observed that woody biochar induced a decrease in bacterial richness, probably due to a nutrient deficiency, while the wheat straw biochar maintain the diversity stable.

A1 **Water**	**A2** **β-Methyl-D-Glucoside**	**A3** **D-Galactonic Acid γ-Lactone**	**A4** **L-Arginine**	**A5** **Water**	**A6** **β-Methyl-D-Glucoside**	**A7** **D-Galactonic Acid γ-Lactone**	**A8** **L-Arginine**	**A9** **Water**	**A10** **β-Methyl-D-Glucoside**	**A11** **D-Galactonic Acid γ-Lactone**	**A12** **L-Arginine**
B1 **Pyruvic Acid Methyl Ester**	**B2** **D-Xylose**	**B3** **D-Galacturonic Acid**	**B4** **L-Asparagine**	**B5** **Pyruvic Acid Methyl Ester**	**B6** **D-Xylose**	**B7** **D-Galacturonic Acid**	**B8** **L-Asparagine**	**B9** **Pyruvic Acid Methyl Ester**	**B10** **D-Xylose**	**B11** **D-Galacturonic Acid**	**B12** **L-Asparagine**
C1 **Tween 40**	**C2** **i-Erythritol**	**C3** **2-Hydroxy Benzoic Acid**	**C4** **L-Phenylalanine**	**C5** **Tween 40**	**C6** **i-Erythritol**	**C7** **2-Hydroxy Benzoic Acid**	**C8** **L-Phenylalanine**	**C9** **Tween 40**	**C10** **i-Erythritol**	**C11** **2-Hydroxy Benzoic Acid**	**C12** **L-Phenylalanine**
D1 **Tween 80**	**D2** **D-Mannitol**	**D3** **4-Hydroxy Benzoic Acid**	**D4** **L-Serine**	**D5** **Tween 80**	**D6** **D-Mannitol**	**D7** **4-Hydroxy Benzoic Acid**	**D8** **L-Serine**	**D9** **Tween 80**	**D10** **D-Mannitol**	**D11** **4-Hydroxy Benzoic Acid**	**D12** **L-Serine**
E1 **α-Cyclodextrin**	**E2** **N-Acetyl-D-Glucosamine**	**E3** **γ-Amino Butyric Acid**	**E4** **L-Threonine**	**E5** **α-Cyclodextrin**	**E6** **N-Acetyl-D-Glucosamine**	**E7** **γ-Amino Butyric Acid**	**E8** **L-Threonine**	**E9** **α-Cyclodextrin**	**E10** **N-Acetyl-D-Glucosamine**	**E11** **γ-Amino Butyric Acid**	**E12** **L-Threonine**
F1 **Glycogen**	**F2** **D-Glucosaminic Acid**	**F3** **Itaconic Acid**	**F4** **Glycyl-L-Glutamic Acid**	**F5** **Glycogen**	**F6** **D-Glucosaminic Acid**	**F7** **Itaconic Acid**	**F8** **Glycyl-L-Glutamic Acid**	**F9** **Glycogen**	**F10** **D-Glucosaminic Acid**	**F11** **Itaconic Acid**	**F12** **Glycyl-L-Glutamic Acid**
G1 **D-Cellobiose**	**G2** **Glucose-1-Phosphate**	**G3** **α-Keto Butyric Acid**	**G4** **Phenylethylamine**	**G5** **D-Cellobiose**	**G6** **Glucose-1-Phosphate**	**G7** **α-Keto Butyric Acid**	**G8** **Phenylethylamine**	**G9** **D-Cellobiose**	**G10** **Glucose-1-Phosphate**	**G11** **α-Keto Butyric Acid**	**G12** **Phenylethylamine**
H1 **α-D-Lactose**	**H2** **D,L-α-Glycerol Phosphate**	**H3** **D-Malic Acid**	**H4** **Putrescine**	**H5** **α-D-Lactose**	**H6** **D,L-α-Glycerol Phosphate**	**H7** **D-Malic Acid**	**H8** **Putrescine**	**H9** **α-D-Lactose**	**H10** **D,L-α-Glycerol Phosphate**	**H11** **D-Malic Acid**	**H12** **Putrescine**

Figure 17 Design of the Biolog EcoPlates^{TM}. These microplates are composed of 96 wells, each one containing an organic carbon substrate associated to tetrazolium dye indicator, except for three wells that contain only water and constitute the controls. In total, 31 carbon sources are repeated in triplicates.

Moreover, in addition to the bacterial activity and composition that provide information on the microbiological state of the soil, microorganisms are also essential in the metal(loid) remediation processes (Ojuerderie and Babalola 2017). Indeed, even though metal(loid)s cannot be degraded, microorganisms, and especially bacteria, can affect metal(loid) behavior. Bacteria possess several tolerance mechanisms towards metal(loid)s (Figure 19), which have been well described in Etesami (2018) and Yin *et al.* (2019):

- Extracellular sequestration: metal(loid)s are accumulated in different structures; *i.e.* extracellular polymeric substances (EPS), glutathione, bio-surfactants.
- Intracellular sequestration: metal(loid)s can also enter bacterial cells and are trapped into the cytoplasm to protect sensitive cellular components.
- Active export of metallic ions: after their entry into the bacteria, metal(loid)s can also be exported from the cell; this process involves ion transports.
- Enzyme detoxification: finally, once they are inside the bacterial cell, metal(loid)s can also be transformed into a less harmful form.

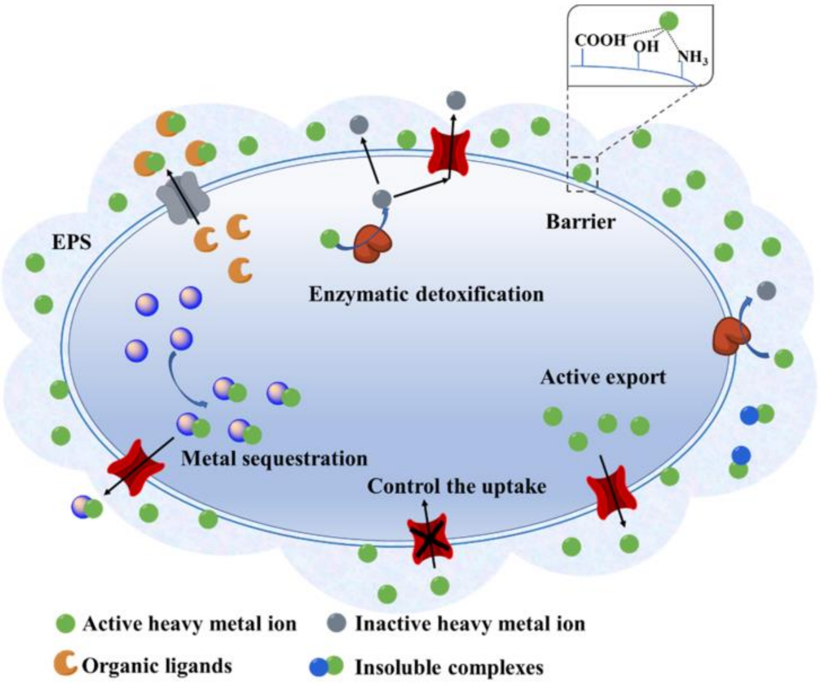


Figure 18 Tolerance of bacteria towards metal(loid)s (source: Yin et al. *2019). To survive metal(loid) stress, bacteria can sequester metal(loid)s in extracellular strucutres (extracellular polymeric substances (EPS), glutathione, bio-surfactants), sequester metal(loid)s in intracellular structures (cytoplasm), export metal(loid)s using ion transport and detoxify them using enzymes that will transform metal(loid)s into a less harmfull form.*

All of these mechanisms confer to few bacteria a contrasted and sometimes an elevated tolerance to metal(loid)s (Table 11). For instance, *Bacillus weihenstephanensis* tolerated Ni up to 1,500 mg.L^{-1}, Cu up to 500 mg.L^{-1} and Zn up to 700 mg.L^{-1} (Rajkumar *et al.* 2008). Similarly, a *Bacillus* strain and a *Pseudomonas* sp. isolated from a Cd and Pb contaminated soil showed a multi-tolerance towards Cd, Cu, Pb, Ni and Zn at levels of 50 mg.L^{-1}, 20 mg.L^{-1}, 100 mg.L^{-1}, 10 mg.L^{-1} and 150 mg.L^{-1}, respectively (He *et al.* 2009).

In addition to this tolerance, some bacterial strains possess plant growth promoting (PGP) properties, *i.e.* indole acetic acid (IAA) production, siderophore production, phosphate solubilization capacity and aminocylcopropane-1-carboxylate (ACC) deaminase ability (Table 11).

Table 11 Characterisitcs of several bacterial strains used in previous studies (IAA = indole acetic acid, ACC = aminocyclopropane-1-carboxylate, empty cases signify that no data was available).

Bacteria species	Pollutants	Characterization	Reference
Bacillus sp.		ACC deaminase activity IAA production Resistance to Ni (500 mg.L^{-1})	Akhtar *et al.* (2018)
Bacillus sp.	Cd, Pb	Resistance to Cd (50 mg.L^{-1}), Cu (20 mg.L^{-1}), Pb (100 mg.L^{-1}), Ni (10 mg.L^{-1}), Zn (150 mg.$^{L-1}$) Siderophore production ACC deaminase activity	He *et al.* (2009)
Bacillus subtilis SJ101	Ni	IAA production P - solubilization	Zaidi *et al.* (2006)
Bacillus thuringiensis	As, Cu, Cd, Ni, Pb, Zn	IAA production (0.8 µg.L-1) Siderophore (8 mm) P - solubilization Metal(loid) tolerance (As = 800 mg.kg^{-1}, Pb = 1,400 mg.kg^{-1}, Cd = 400 mg.kg^{-1}, Zn = 2,600 mg.kg^{-1}, Ni = 50 mg.kg^{-1}, Cu = 50 mg.kg^{-1})	Babu *et al.* (2013)
Bacillus weihenstephanensis		Metal tolerance (Ni = 1,500 mg L^{-1}), Cu = 500 mg L^{-1}, Zn = 700 mg L^{-1}) P - solubilization IAA production	Rajkumar *et al.* (2008)
Microbacterium sp.		IAA production Siderophore production ACC deaminase activity	Sheng *et al.* (2008)
Pseudomonas aeruginosa	Zn	IAA production Siderophore production P - solubilization	Islam *et al.* (2014)
Pseudomonas sp.	Cd, Pb	Resistance to Cd (50 mg.L^{-1}), Cu (20 mg.L^{-1}), Pb (100 mg.L^{-1}), Ni (10 mg.L^{-1}), Zn (150 mg.L^{-1}) Siderophore production ACC deaminase activity	He *et al.* (2009)

Table 11. end

Bacteria species	Pollutants	Characterization	Reference
Rhanella sp.	Cd, Pb, Zn	IAA production ACC deaminase activity P - solubilization	He *et al.* (2013)
Stenotrophomonas sp.		ACC deaminase activity IAA production Resistance to Ni (300 mg.L^{-1})	Akhtar *et al.* (2018)

IAA is a phytohormone involved in cell elongation, cell division and root initiation (Vessey 2003, Babu *et al.* 2013). It also participates in other processes such as seed germination and lateral root formation (Santoyo *et al.* 2019). Siderophore is a small compound having a high affinity for iron (Etesami 2018). It can chelate iron and also other metal(loid)s (Munir and Faisal 2016). Finally, ACC deaminase can reduce the formation of ethylene in response to stress, which normally leads to senescence, chlorosis and abscission (Babu *et al.* 2013, Santoyo *et al.* 2019). All of these properties, when expressed by bacteria, can improve plant growth, which is important in phytomanagement.

Finally, to improve plant growth and phytomanagement ability, bacteria (after isolation or by making consortium) can be inoculated, either to the soil, by liquid inoculation or using a carrier, or to the plants by fixing the bacteria to the seeds or plant roots. Such process is called bioaugmentation or microbial assisted phytoremediation (Tack and Meers 2010, Khalid *et al.* 2016). In this technique, inoculated bacteria will reduce metal(loid) toxicity and improve the growing conditions, leading to a better plant growth. Indeed, previous studies evaluated the effect of bacterial inoculation on soil, polluted or not, and plant (Table 11). For instance, Yu *et al.* (2012) showed that inoculating a rock phosphate with *Streptomyces pactum* increased *Sorghum bicolor* height and dry weight, attributing such effect to a better absorption of nutrients and bacterial IAA production. In the same way, the inoculation of *Pseudomonas* BA-8, *Bacillus* OSU-142 and *Bacillus* M3 increased strawberry yield and growth, due to the bacterial production of auxin and cytokinine, N_2 fixation, P solubilization and microbial production of antimicrobial substances (Esitken *et al.* 2010). The same two *Bacillus* strains increased raspberry yield (Orhan *et al.* 2006). The authors attributed this beneficial effect to N_2 fixation, P solubilization but also to an increase of plant nutrient availability. Indeed, organic acids produced by bacteria decreased soil pH, which increased Ca, Fe, Mn and P availability. Other strains of the genus *Bacillus,* which is a genus often found in contaminated soils, showed good results. In the study of Brunetti *et al.* (2012), *Bacillus licheniformis* inoculation to a Cr, Cu, Pb and Zn contaminated soil increased *Brassica alba*, *Brassica nigra* and *Brassica carinata* dry matter production. In another study, *Bacillus licheniformis* inoculation into a hydropony solution supplemented by Ni also showed positive effects on rice germination and rice chlorophyll and nutrient contents (Jamil *et al.* 2014). On a As, Cd, Cu, Ni, Pb and Zn contaminated mine soil, *Bacillus thuringiensis* inoculation protected *Alnus firma* from metal(loid) stress and increased its root length, shoot height, dry biomass production and chlorophyll contents (Babu *et al.* 2013).

Table 12 Effects of bacterial inoculation observed in previous studies (↘ = decrease of the parameter, ↗ = increase of the parameter, empty cases signify that no data was available).

Bacteria species	**Pollutant(s)**	**Plant species**	**Effects**	**Reference**
Bacillus licheniformis		*Pinus pinea*	↗ aerial surface ↗ aerial length	Probanza *et al.* (2002)
Bacillus licheniformis	Ni	Rice	↗ germination ↘ Ni uptake ↗ plant [Na], [Ca], [K] ↗ chlorophyll content ↗ carotenoid content	Jamil *et al.* (2014)
Bacillus M3		Raspberry	↗ yield ↗ [Fe], [Mn] in leaves ↗ N, P, Ca plant uptake	Orhan *et al.* (2006)
Bacillus OSU142		Raspberry	↗ N, P, Ca plant uptake	Orhan *et al.* (2006)
Bacillus PSB10	Cr	*Cicer arietinum*	↗ root and shoot length ↗ nodule number and dry weight ↗ chlorophyll content	Wani and Khan (2010)
Bacillus pumilus		*Pinus pinea*	↗ aerial surface ↗ aerial length	Probanza *et al.* (2002)
Bacillus sp.		*Phaseolus vulgaris*	↗ growth and yield ↗ bacterial population	Saxena *et al.* (2013)
Bacillus sp.	Ni	*Raphanus sativus*	↗ radish dry biomass ↗shoot/root growth ↗ chlorophyll content ↗ Ni uptake	Akthar *et al.* (2018)
Bacillus sp.	Cd, Pb	*Lycopersicon esculentum*	↗ available [Cd], [Pb] ↗ root length ↗ shoot and root dry weight ↗ root [Pb]	He *et al.* (2009)

Table 12. continued

Bacteria species	Pollutant(s)	Plant species	Effects	Reference
Bacillus subtilis		Tomato	↗ radical system ↗ root length and dry weight	Mena-Violante and Olalde-Portugal (2007)
Bacillus subtilis SJ101	Ni	*Brassica juncea*	↗ plant Ni accumulation ↗ plant biomass	Zaidi *et al.* (2006)
Bacillus thuringiensis	As, Cu, Cd, Ni, Pb, Zn	*Alnus firma*	↗ root length ↗ shoot height ↗ dry biomass ↗ chlorophyll content ↗ nodules ↗ pollutant accumulation	Babu *et al.* (2013)
Bacillus weihenstephanensis	Cu, Ni, Zn	*Helianthus annuus*	↗ fresh and dry weights ↗ Cu, Zn accumulation ↘ Ni accumulation	Rajkumar *et al.* (2008)
Chryseobacterium humi	Cd, Zn	*Helianthus annuus*	↘ shoot [Zn] ↘ root [Cd], [Zn]	Marques *et al.* (2013)
Microbacterium sp.	Pb	*Brassica napus*	↗ water-soluble Pb ↗ root length ↗ root and shoot dry weight ↗ shoot [Pb]	Sheng *et al.* (2008)
Pseudomonas aeruginosa	Zn	*Triticum aestivum*	↗ plant growth ↘ oxidative stress	Islam *et al.* (2014)
Pseudomonas fluorescens	Pb	*Brassica napus*	↗ water-soluble Pb ↗ root length ↗ root and shoot dry weight ↗ shoot [Pb]	Sheng *et al.* (2008)

Table 12. end

Bacteria species	Pollutant(s)	Plant species	Effects	Reference
Pseudomonas sp.	Cd, Pb	*Lycopersicon esculentum*	↗ available [Cd], [Pb] ↗ root length ↗ shoot and root dry weight ↗ root [Pb] ↗ shoot [Cd]	He *et al.* (2009)
Rhanella sp.	Cd, Pb, Zn	*Brassica napus*	↗ water-soluble [Cd], [Pb], [Zn] ↘ pH ↗ chlorophyll content ↗ plant height ↗ root length ↗ root and shoot dry weight ↗ Cd, Pb, Zn uptake	He *et al.* (2013)
Sphingomonas SaMR12	Cd	*Sedum alfredii*	↗ shoot and root biomass ↗ root hair length ↗ root branching improved root morphology ↗ oxalic and succinic acid secretion ↘ tartaric acid secretion ↘ oxidative stress ↗ shoot [Cd]	Chen *et al.* (2014a)
Stenotrophomonas sp.	Ni	*Raphanus sativus*	↗ radish dry biomass ↗shoot/root growth ↗ chlorophyll content ↗ Ni uptake	Akthar *et al.* (2018)

These studies used a single bacterial strain inoculation. However, bacterial strains can also be inoculated in combination, which can be beneficial or detrimental. For instance, Orhan *et al.* (2006) inoculated two *Bacillus* strains, OSU-142 and M3, alone or combined and found that all inoculations increased raspberry uptake of Ca, Na and P while only M3 and OSU-142 + M3 treatments increased raspberry yield and leaf Fe and Mn concentrations. Two other *Bacillus* strains, *B. licheniformis* and *B. pumilus*, were inoculated singularly or combined and only their single application increased *Pinus pinea* aerial surface and aerial length, whereas their combined inoculation had no effect (Probanza *et al.* 2002). Finally, the single inoculation of *Bacillus subtilis* and *Azospirillum brasilense* improved *Lycopersicum esculentum* growth, while their co-inoculation induced a lower plant height, nodule number and total biomass compared to the single inoculation.

Another type of microorganism showed good potential in phytomanagement. Indeed, fungi are tolerant to metal(loid)s and can associate with plant roots to form mycorhizae and improve plant growth. For instance, Achal *et al.* (2011) showed that *Gloeophyllum sepiarium* was capable of remediating highly contaminated Cr(VI) soil, by bio-transforming Cr(VI) to Cr(III) and increasing soil nutrient content. Moreover, *Trichoderma virens* PDR-28 showed some plant growth promoting traits (ACC deaminase activity, phosphate solubilization ability and siderophore production), was able to remove metal(loid)s from solution and increased maize growth and metal(loid) accumulation when inoculated to a As, Cd, Cu, Pb and Zn contaminated soil (Babu *et al.* 2014). Finally, *Pseudomonas koreensis* AGB-1 isolated from a mine soil was able to grow well in presence of elevated concentrations of Zn (3,000 mg.L^{-1}), Cd (2,700 mg.L^{-1}), As (2,700 mg.L^{-1}), Pb (1,800 mg.L^{-1}) or Cu (500 mg.L^{-1}). It was also able to produce IAA and biosurfactants, solubilize P and had a high ACC deaminase activity, which increased *Miscanthus sinensis* growth and its accumulation of metal(loid)s (Babu *et al.* 2015).

To conclude on this part, bacteria activity and community composition is an important parameter to evaluate soil recovery in the process of phytomanagement. Additionally, bacteria can be inoculated to soil to improve phytomanagement success. However, it is important to choose the appropriate bacterial strain(s), as some studies showed no or negative effects.

VII. Presentation of the site.

This PhD work was located on two main structures, the University of Molise in Italy and the University of Orleans in France. Due to the difficulty in the access of contaminated sites, especially in Italy, a French site was studied.

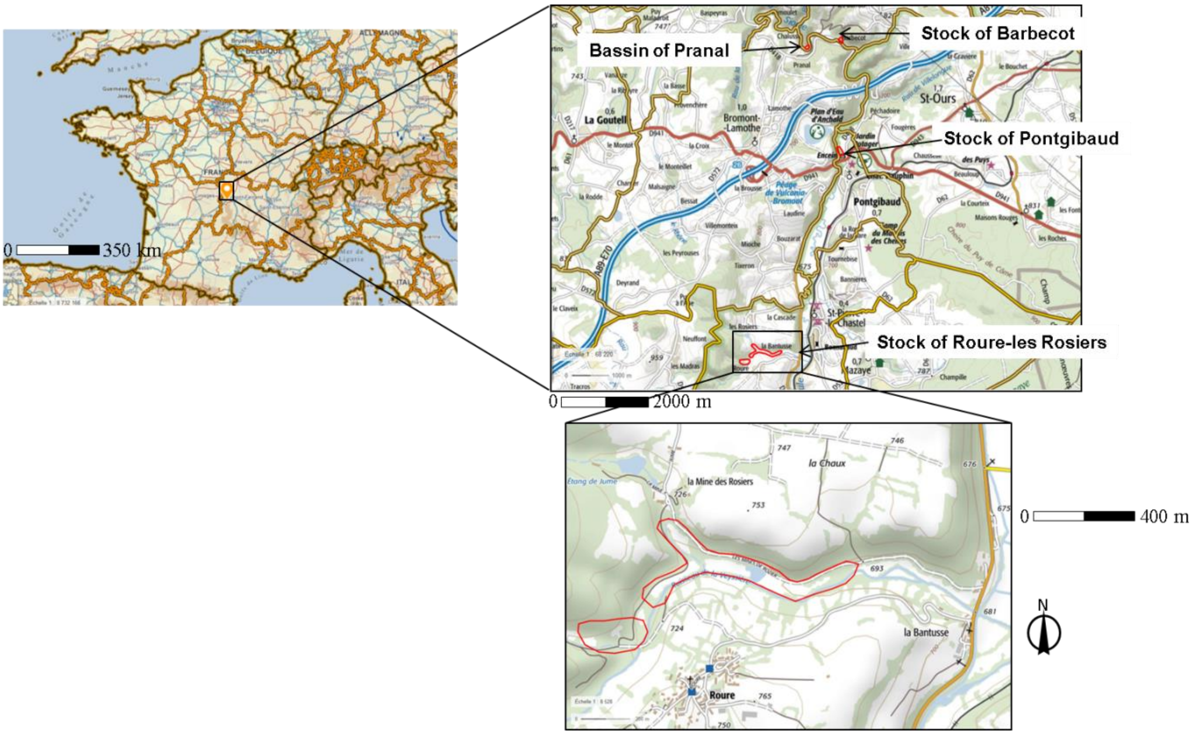


Figure 19 Localization of the studied site (modified from Nandillon 2019)

This contaminated site was part of the Pontgibaud mine district, in the Massif Central (Figure 19). This silver-lead extraction mine was active during the Gallo-roman period as well as during the nineteenth century (Nandillon 2019, Cottard 2010). This site was divided into four locations: Pontgibaud, Pranal, Barbecot and Roure-les-Rosiers. This study focused on the last one, corresponding to 84,700 m^3 of wastes highly contaminated with As and Pb in average concentrations of 539 mg.kg^{-1} and 11,453 mg.kg^{-1}, respectively, with a sandy texture. The physico-chemical properties of the soil are given Table 13.

Table 13 Physico-chemical properties of Pontgibaud technosol (from Lebrun et al. *2017 and Van Poucke* et al. *2019)*

Properties	Value	Unit
pH	4.60	
Organic matter	2.60	%
Sand	93	%
Silt	6.5	%
Clay	0.4	%
Cation exchange capacity	2.34	cmol.kg^{-1}

The site poses a serious threat to the environment and the public heath, as in addition to the elevated metal(loid) concentrations, the soil presents also a reduced fertility, *i.e.* acidic pH, low nutrient content and availability (Nandillon 2019). Furthermore, such conditions prevent plant growth, as illustrated by the picture in Figure 20, and thus the site also suffers from soil erosion that can spread the contamination to the surrounding environment.

Figure 20 Picture of the studied mine in January 2015 (picture taken by Domenico Morabito).

A phytoextraction process could not be suitable for such site, as contamination is elevated and concerns a huge amount of waste deposited deeply. Consequently a phytostabilization remediation was chosen, associated to amendments and/or bacterial inoculation to reduce metal(loid) toxicity, improve soil conditions and thus ameliorate plant growth.

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Supplemantary material

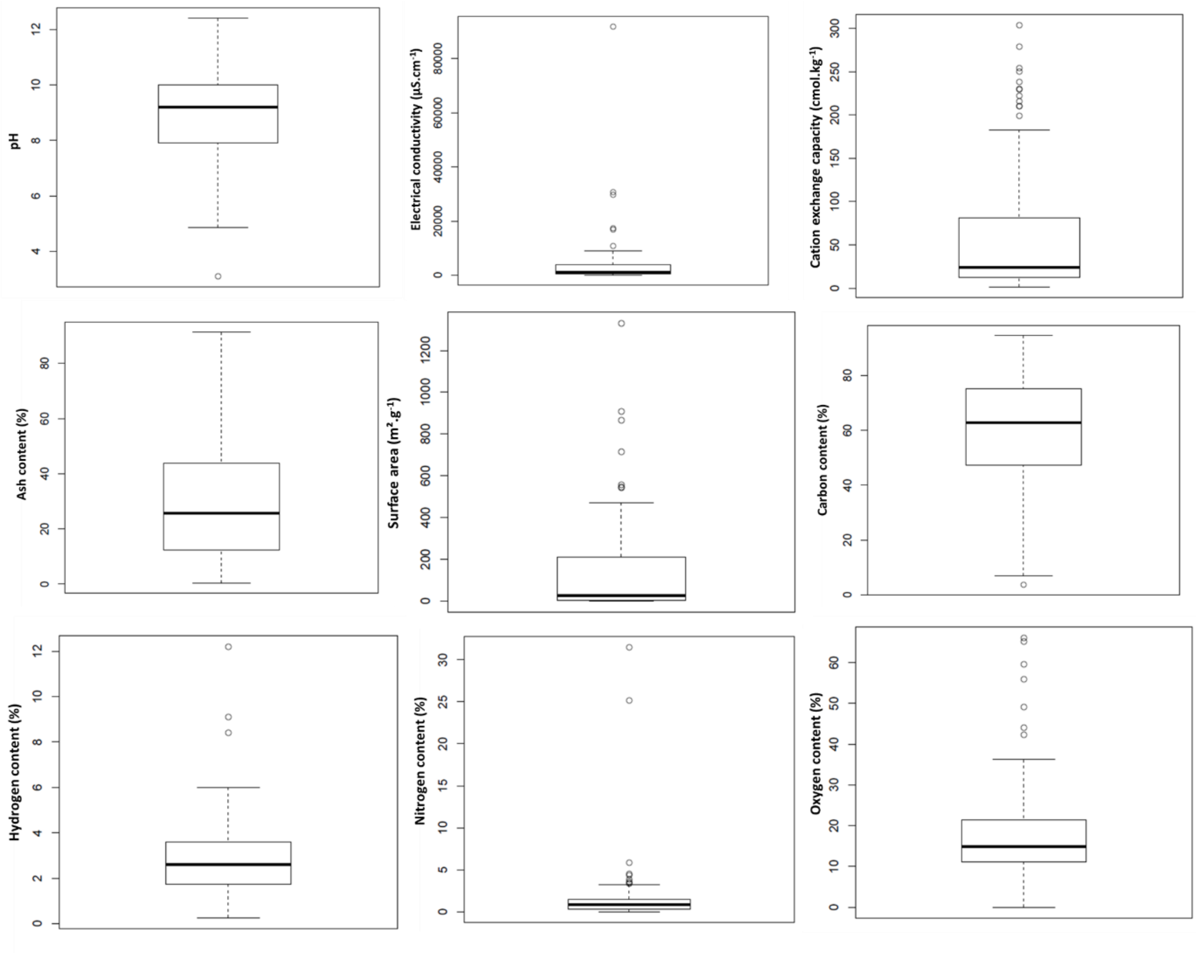
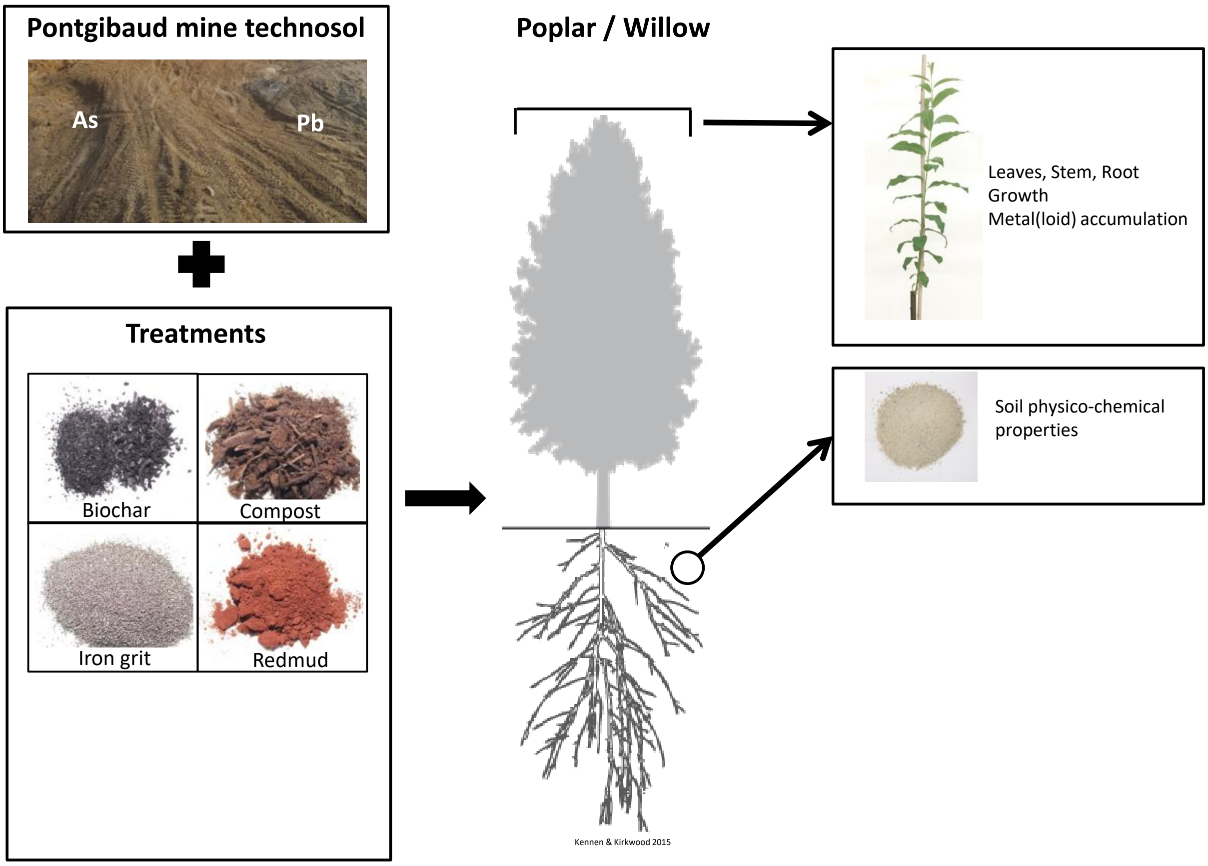


Figure S1 Boxplots of the different properties of biochars (extracted from Table 5).

Chapter 2. Amendment effects on soil physico-chemical properties, plant growth and metal(loid) accumulation.



This chapter will show the results obtained from five different experiments, answering the objectives 1a and 1b of this PhD thesis, which evaluated the effects of adding different amendments, biochar, compost, iron grit and redmud, alone or combined, to the mining technosol of Pontgibaud (France) on the soil physico-chemical properties and the growth and metal(loid) accumulation abilities of three *Salicaceae* species, *Salix viminalis*, *Populus euramericana* Dorskamp and *Salix dasyclados*. This chapter will be divided in five parts, each one corresponding to a mesocosm experiment.

The first part aimed at evaluating the effect of applying four biochars (La Carbonerie, Crissey, France) obtained from a same hardwood biomass but differing by their particle sizes (inf. to 0.1 mm, 0.2-0.4 mm, 0.5-1 mm, 1-2.5 mm) on the soil physico-chemical properties, *Salix viminalis* growth and metal(loid) distribution in plants in order to determine which particle size biochar has the best effect. This work was published in the *Journal of Soils and Sediments* (Lebrun *et al*. 2018).

The second part assessed biochar feedstock (pinewood *vs* lightwood) and particle size (inf. to 0.1 mm *vs* 0.2-0.4 mm) effects on the soil physico-chemical properties and the growth and phytostabilization capacities of two *Salicaceae* species, *Salix viminalis* and *Populus euramericana* Dorskamp. The aim of this study was to find which feedstock and which particle size induced the best effect and between the two plant species, which one grew better and showed the lowest translocation towards upper parts. This study was published in the journal *Chemosphere* (Lebrun *et al*. 2018, 194, 316-326).

The third part studied the effects of functionalizing a biochar by impregnation with iron on the As sorption capacity, and its possible effect on Pb sorption, in a batch experiment and the effect of amending the mine soil with such modified biochar. This study focused on the four biochars used in the first study. The biochars were first characterized for their sorption capacity and one of them was modified to improve arsenic sorption capacity in batch and arsenic immobilization in soil. This work was published in the journal *Environmental Science and Pollution Research* (Lebrun *et al*. 2018, 25, 33678-33690).

In the fourth part, amendment combination was used. This experiment aimed at studying the effect of amending the Pontgibaud mine soil with biochar, compost and iron grit, applied alone or combined, on the soil physico-chemical properties and *Salix viminalis* phytostabilization capacities. The goal was to test if an amendment combination showed better results than a single amendment application. This work was published in the journal *Chemosphere* (Lebrun *et al*. 2019, 222, 810-822).

Finally, the last experiment was performed at the Ecochem laboratory in Ghent University, during an exchange program. It evaluated different biochars (hardwood, coconut) (La Carbonerie, Jacobi Carbons), one activated carbon (coconut, steam activation) (Jacobi Carbons) and three redmuds for their ability to sorb Pb and reduce its availability. The study also assessed the effects of adding a hardwood biochar, an activated carbon and two commercial redmuds (un-treated and neutralized) (Alteo Environnement) to the Pontgibaud mine soil, chosen based on the first experimental part, on the soil physico-chemical properties and *Salix dasyclados* growth and metal(loid) accumulation. This work has been submitted to the journal *Land degradation and Development* (LDD-19-0882).

Part A. Eco-restoration of a mine technosol according to biochar particle size and dose application: study of soil physico-chemical properties and phytostabilization capacities of *Salix viminalis.*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Eco-restoration of a mine technosol according to biochar particle size and dose application: study of soil physico-chemical properties and phytostabilization capacities of *Salix viminalis*

Manhattan Lebrun, Florie Miard, Romain Nandillon, Nour Hattab-Hambli, Gabriella S. Scippa, Sylvain Bourgerie & Domenico Morabito

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Abstract

Purpose

Anthropic activities induce severe metal(loid)s contamination of many sites, which is a threat to the environment and to public health. Indeed metal(loid)s cannot be degraded, and thus accumulate in soils. Furthermore, they can contaminate surrounding ecosystems through run-off or wind erosion. This study aims to evaluate the phytostabilization capacity of *Salix viminalis* to remediate As and Pb highly contaminated mine site, in a biochar-assisted phytoremediation context and to assess biochar particle size and dose application effects.

Materials and methods

To achieve this, mesocosm experiments were conducted using the contaminated technosol and four different size fraction of one biochar as amendment, at two application rates (2 and 5%). Non-rooted cuttings of *Salix viminalis* were planted in the different mixtures. In order to characterize the mixtures, soil pore waters were sampled at the beginning and at the end of the experiment and analyzed for pH, electrical conductivity, and metal(loid) concentrations. After 46 days of *Salix* growth, roots, stems, and leaves were harvested and weighed, and As and Pb concentrations and distributions were measured.

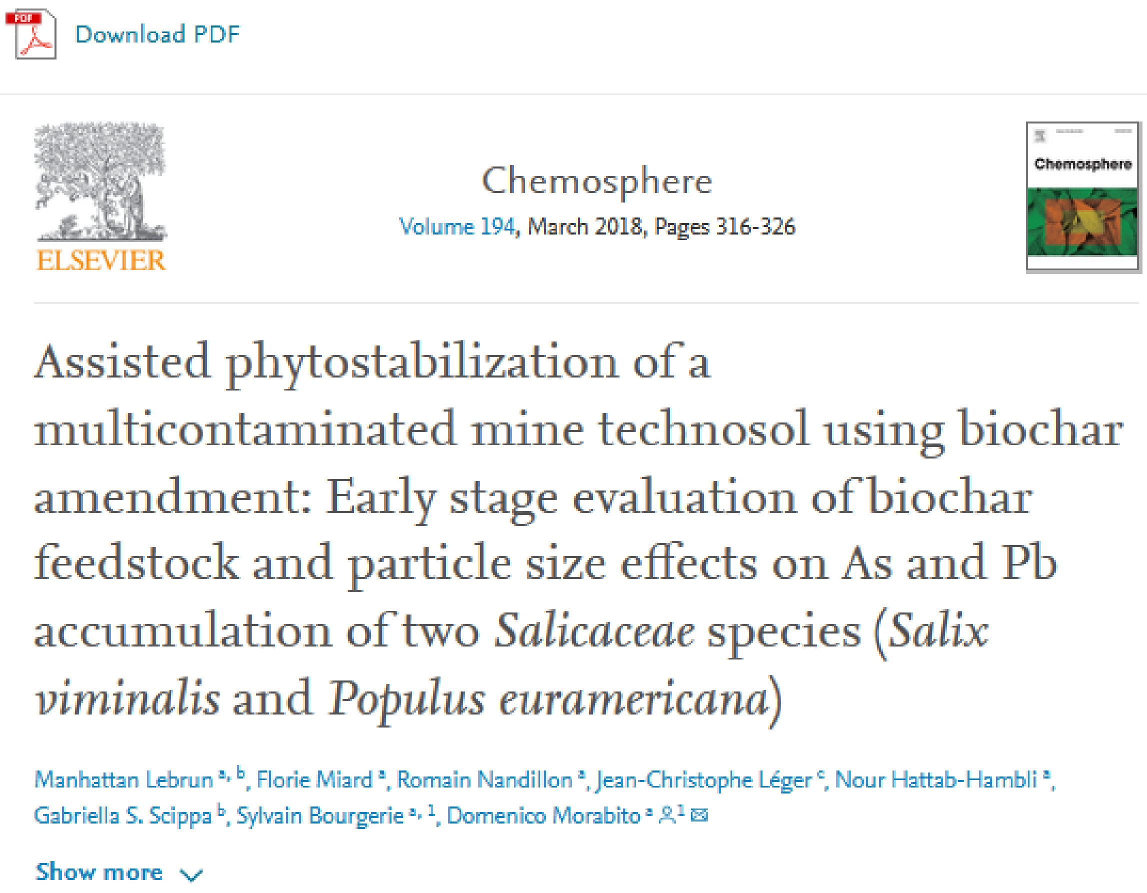
Results and discussion

Soil fertility improved (acidity decrease, electrical conductivity increase) following biochar addition, whatever the particle size, and the Pb concentration in soil pore water decreased. *Salix viminalis* did not grow on the non-amended contaminated soil while the biochar amendment permitted its growth, with a better growth with the finest biochars. The metal(loid)s accumulated preferentially in roots.

Conclusions

Fine biochar particles allowed *S. viminalis* growth on the contaminated soil, allowing this species to be used for technosol phytostabilization.

Part B. Assisted phytostabilization of a multicontaminated mine technosol using biochar amendment: early stage evaluation of biochar feedstock and particle size effects on As and Pb accumulation of two *Salicaceae* species (*Salix viminalis* and *Populus euramericana*).



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Assisted phytostabilization of a multicontaminated mine technosol using biochar amendment: Early stage evaluation of biochar feedstock and particle size effects on As and Pb accumulation of two *Salicaceae* species (*Salix viminalis* and *Populus euramericana*)

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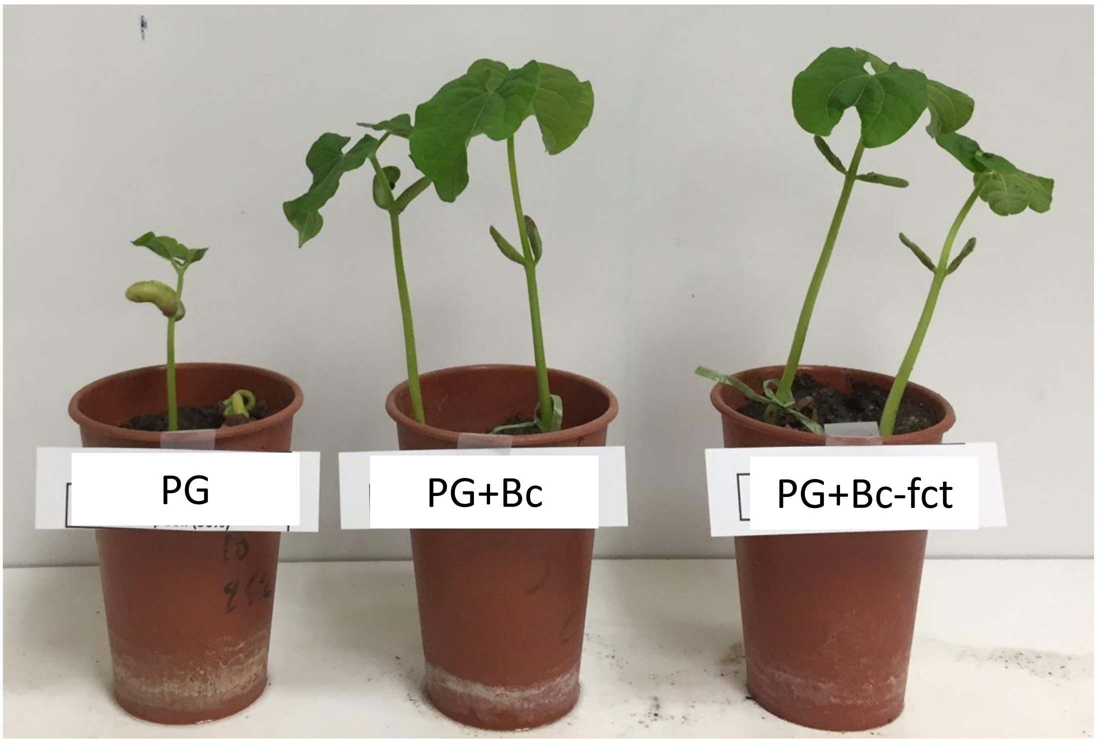
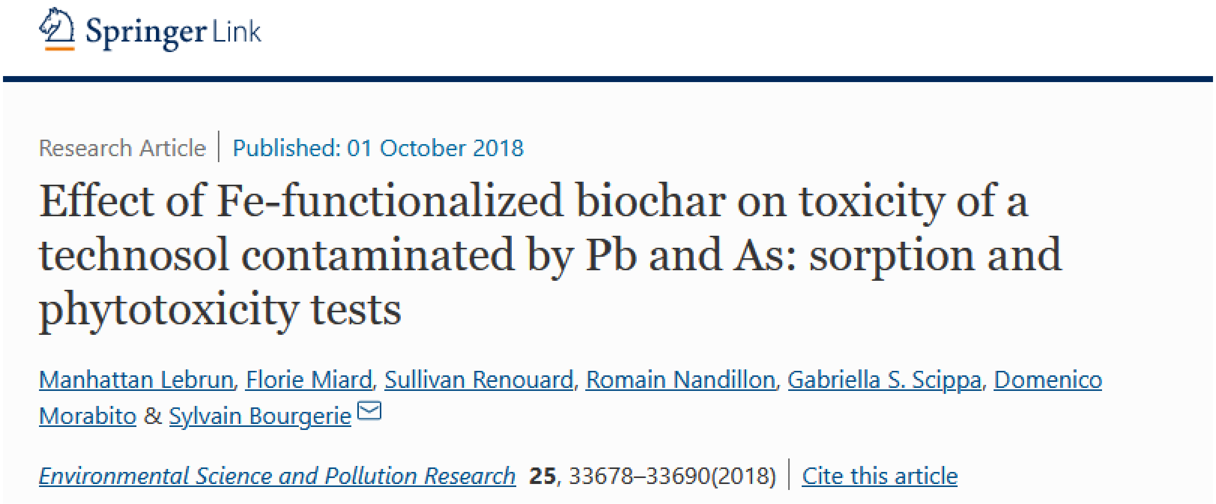
Abstract

Soil contamination by metal(loid)s is one of the most important environmental problem. It leads to lossof environment biodiversity and soil functions and can have harmful effects on human health. Therefore,contaminated soils could be remediated, using phytoremediation. Indeed, plant growth will improve soilconditions while accumulating metal(loid)s and modifying their mobility. However, due to the poorfertility and high metal(loid)s levels of these soils, amendments, like biochar, has to be applied. Thisstudy was performed on a former mine technosol contaminated by As and Pb and aimed to study (i) theeffect of biochar on soil physico-chemical properties and plant phytostabilization potential (ii) biocharfeedstock and particle size effects. In this goal, a mesocosm experiment was set up using four differentbiochars, obtained from two feedstocks (lightwood and pinewood) and harboring two particle sizes (inf.0.1 mm and 0.2e0.4 mm) and twoSalicaceaespecies. Soil and soil pore water physico-chemical prop-erties as well as plant growth and metal(loid)s distribution were assessed. The results showed thatbiochar was efficient in improving soil physico-chemical properties and reducing Pb soil pore waterconcentrations. This amelioration allowed plant growth and increased dry weight production of bothspecies. Regarding metal(loid)s distribution, willow and poplar showed an As and Pb accumulation inroots and low translocation towards edible parts, i.e stems and leaves, which shows a phytostabilizationpotential. Finally, the 2 biochar parameters, feedstock and particle size, only affected soil and soil porewater physico-chemical properties while having no effect on plant growth.

Part C. Effect of Fe-functionalized biochar on toxicity of a technosol contaminated by Pb and As: sorption and phytotoxicity tests.





Research Article | Published: 01 October 2018

Effect of Fe-functionalized biochar on toxicity of a technosol contaminated by Pb and As: sorption and phytotoxicity tests

Manhattan Lebrun, Florie Miard, Sullivan Renouard, Romain Nandillon, Gabriella S. Scippa, Domenico Morabito & Sylvain Bourgerie

Environmental Science and Pollution Research **25**, 33678–33690(2018) | Cite this article

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Abstract

Biochar, produced by the pyrolysis of biomass under low oxygen conditions, has gathered attention in the last few years due to its capability to reduce metal(loid)s bioavailability and mobility in soils, as well as its beneficial effects on soil fertility. Indeed, biochar amendment to polluted soil induced usually an increase of pH, water holding capacity, and nutrient contents, associated with a decrease of metal(loid)s concentrations in soil pore water, through sorption. However, biochar has been shown efficient in sorbing cation pollutants, like Pb, but present a low sorption capacity towards anions like As. This contrasted behavior poses a problem, as most polluted soils are multi-contaminated, with both cation and anion pollutants. One of the solutions to overcome such problem is to functionalize biochar, by modifying its surface. However, most studies actually focused on functionalization effect on metal(loid)s sorption towards batch experiments, and only a few dealt with modified biochar incorporation to the soil. Therefore, this study aimed (i) to assess the sorption capacity of hardwood biochars, harboring different particle sizes, towards Pb and As; (ii) to evaluate the effect of a Fe-functionalization on Pb and As sorption; and (iii) to validate the results, in a phytotoxicity test using *Phaseolus vulgaris* as bioindicator plant. The batch experiments showed that all four biochars were able to efficiently sorb Pb, the fine biochars showing higher sorption values than the coarse biochars. As sorption was very low. Fe-coating increased As sorption value, while having no effect on Pb sorption. However, when incorporated in the soil, Fe-coated biochar did not improve soil physico-chemical properties compared to the pristine biochar; especially, it did not reduce As soil pore water concentrations. Finally, bean plant did not show differences in terms of biomass production between the two biochars incorporated into polluted soil, demonstrating that Fe-functionalization did not improve biochar capacity to decrease soil toxicity.

Part D. Biochar effect associated with compost and iron to promote Pb and As soil stabilization and *Salix viminalis* L. growth.

Chemosphere
Volume 222, May 2019, Pages 810-822

Biochar effect associated with compost and iron to promote Pb and As soil stabilization and *Salix viminalis* L. growth

Manhattan Lebrun [a, b], Florie Miard [a], Romain Nandillon [a, c, d, e], Gabriella S. Scippa [b], Sylvain Bourgerie [a], Domenico Morabito [a]

Show more

Accepted: 30 January 2019

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Abstract

Soil pollution by metal(loid)s is one of the most significant problems in Europe. To remediate and potentially rehabilitate these contaminated sites, phytoremediation procedures are being put into place, often using amendments to help offset the extreme conditions of such soils. The aim of this study was to define the best amendment to use on the field. This was done by studying how the addition of three different amendments (biochar, compost and iron grit), alone or in combination, could affect: (i) soil physico-chemical properties, (ii) Salix viminalis growth, and (iii) metal(loid) stabilization. A 69 day-mesocosm study was thus set up using a former mine technosol, the three amendments applied alone or combined, and S. viminalis cuttings. The results showed that biochar and/or compost improved the soil fertility and the soil pore water characteristics, with reductions of acidity, metal(loid) mobility and toxicity, while iron grit amendment presented negative effects on such parameters. Such ameliorations allowed better plant growth and higher biomass production. In addition, stress indicators (leaf pigment content and root guaiacol peroxidase activity) showed a reduction in plant stress following biochar and/or compost application. Finally, among the different treatments, the use of compost or a biochar-compost combination showed better results in terms of improvement of soil conditions, increase in plant growth and reduced translocation of metal(loid)s towards upper parts, making these two treatments a valuable option for a field trial.

Supplementary material

Additionally to the results presented in the published paper, other parameters were measured.

On all the substrates samples, collected at T0 and T69 in non-vegetated and vegetated pots, the total C, H, N and S contents were determined, using a Flash 2000 (Thermo).

Results showed that at the beginning of the experiment, Pontgibaud technosol presented a low total carbon content (0.14 %) (Table S4). This C content was increased in all treatments compared to P, except in PI condition which presented a similar C level (Table S4). The highest C content increase was observed in PBCI treatment (4.77 %), followed by PBI and PB, which presented similar C contents of 4.15 % and 3.76 %, respectively. Finally, the combination biochar-compost led to a higher C rise (3.40 %) than the addition of compost alone (1.25 %). At the end of the experiment time course (T69), the same trends in carbon content variations were observed in both vegetated and non-vegetated conditions. As found at T0, the addition of iron grit alone (PI treatment) did not induce significant change in C content. Indeed, C contents in P were 0.17 % and 0.11 % whereas in PI they were 0.20 % and 0.14 %, in non-vegetated and vegetated conditions, respectively. However, all the other treatments increased soil C contents, with a higher rise observed in PBC (5.07 %) for the non-vegetated condition and in PBCI (4.50 %) in the vegetated condition, while the lowest improvement of soil C content was found in PC substrates in both non-vegetated (1.32 %) and vegetated (0.92 %) conditions. Regarding the evolution of soil C content with time, no change was observed in P and PBI treatments, while in PB, PI and PBC treatments, soil C content increased after 67 days in non-vegetated pots, until 4.29 %, 0.20 % and 5.07 %, respectively compared to 3.76 %, 0.12 % and 3.40 % observed at T0, respectively. Oppositely, in PBCI, a decrease in soil C content was observed after 69 days in the non-vegetated condition, until 4.17 % compared to the 4.77 % found at T0. However, in three of these four treatments, PB, PI and PBCI, a decrease in soil C content was observed in vegetated pots compared to their respective un-vegetated condition, until levels similar to those observed at T0; which shows a plant effect. Finally, in PC treatment, soil C content decreased after 69 days of *S. viminalis* growth.

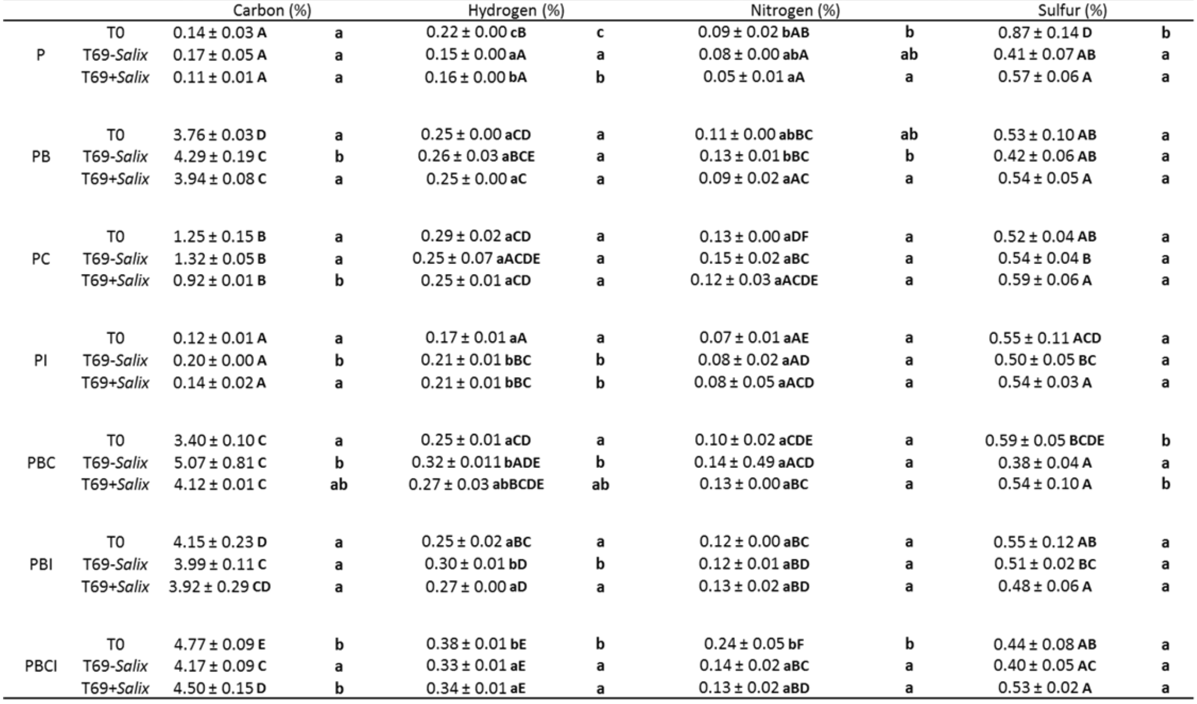
As for carbon, non-amended Pontgibaud technosol presented at the beginning of the experiment (T0) a low hydrogen content of 0.22 % (Table S4), which decreased following iron grit application alone (0.17 %) (Table S4), while biochar-iron grit addition had no effect. On the contrary, the other amendment treatments led to a significant increase in soil H content: biochar, compost and their combination induced a similar increase (0.25 %, 0.29 % and 0.25 % respectively) whereas the application of the three amendments together resulted in the highest soil H content rise (0.38 %). At the end of the experiment, in the non-vegetated condition, soil H content was 0.15 % in P, 0.25 % in PC and 0.32 % in PBC substrates, with no significant difference among the three treatments. The addition of biochar increased soil H content until 0.26 % and the addition of compost together with biochar did not induce a higher rise of soil H content compared to PB. A similar soil H content rise than the one observed with biochar was found after iron grit addition to P (0.21 %). Finally, higher improvements of soil H content were observed in PBI (0.30 %) and PBCI (0.33 %) treatments, with PBCI presenting a significantly higher soil H content than PBI. Regarding the vegetated pots, soil H content was 0.16 % in P substrate and increased with all treatments. The soil H content rises declined in the order PBCI (0.34 %), PBI (0.27 %), PBC (0.27%), PB (0.25 %), PC (0.25 %) and PI (0.21 %). Soil H content did not show change with time in PB and PC treatments. On the contrary, P treatment presented a decrease in soil H content after 69 days compared to the level observed at T0, with a higher drop in non-vegetated pots compared to the one with *S. viminalis*. The soil H content in PI substrate increased to the same level after 69 days in both vegetated and non-vegetated conditions, compared to T0 levels, whereas in PBCI, it decreased to the same level. Finally, in PBC and PBI treatments, a soil H content increase was observed but it was only significant in the non-vegetated condition.

Soil nitrogen content was 0.09 % in P at T0 (Table S4) and the addition of biochar and iron, alone or in combination, had no effect (Table S4). The three other amendments increased soil N content in the order PBC (0.10 %), PC (0.13 %) and PBCI (0.24 %). After 69 days without plant growth, P substrate presented a soil N content of 0.08 %, which was not affected by the application of iron or the biochar-compost combination. The four other treatments improved the soil N contents, with no significant difference among them (0.13 % for PB, 0.15 % for PC, 0.12 % for PBI and 0.14 % for PBCI). In the vegetated condition, only the amendments combinations, biochar-compost, biochar-iron and biochar-compost-iron, increased soil N content, with values of 0.13 % in all cases, compared to P (0.05 %). In four out of the seven treatments (PC, PI, PBC and PBI), soil N content did not show significant variations with time. On the contrary, plant growth decreased soil N content in P and PB substrates, while a decrease of soil N content was observed in PBCI treatment after 69 days, with no difference between the non-vegetated and the vegetated conditions.

At T0, P technosol had a sulfur content of 0.87 % and concentration was not affected by the addition of iron and biochar-iron to P. The four remaining treatments led to a decrease in soil S levels, until values between 0.44 % and 0.55 %, with no significant difference among treatments. After 69 days, soil S contents in P were 0.41 % and 0.57 % for the non-vegetated and vegetated conditions, respectively. In both conditions, the different amendment treatments had no significant effect on the soil S content (Table S4). Finally, only two treatments showed variations in terms of soil S content with time: in P, a decrease of soil S content was observed, with no significant difference between the non-vegetated (0.41 %) and the vegetated (0.57 %) conditions; whereas in the case of PBC treatment, soil S content decrease was only significant in the non-vegetated samples (0.38 %).

The increases in terms of soil C, H and N contents observed with the different amendments were in accordance with previous studies. In 2012, Jones *et al*. observed an increase in soil total carbon content from 2.27 % to 2.78 % three years after the application to a sandy soil of 50 t.ha^{-1} of wood biochar (*Fraxinus excelsior*, *Fagus sylvatica* and *Quercus robur*). Moreover, in 2015, Agegnehu *et al*. showed that the application of biochar and/or compost increased the total N content of the soil. Such improvements can be explained by the amount of C, H and N found in the biochar and the compost used as amendment (Nigussie *et al*. 2012). On the contrary, the non- or negative- effect of the amendments on soil S content can be explained by the low S content of the biochar and the compost. Moreover, the higher soil C, H and N content improvements usually observed with biochar compared to compost can be attributed to the fact that the C, H and N elements are more stable in biochar than in compost (Laird *et al*. 2010). Regarding the evolution of the soil CHNS contents during the experiment time course, although the increase in the non-vegetated condition compared to T0 was not expected and thus difficult to explain, the decrease observed in some treatments can be attributed to a leaching of CHNS. Moreover, plant growth can affect CHNS soil contents, resulting in either a decrease or an increase, through nutrient uptake or release of root exudates.

Table S4. Total soil carbon, hydrogen, nitrogen and sulfur content (%) determined at the beginning of the experiment (T0) and at the end of the experiment in both non-vegetated (T69-*Salix*) and vegetated (T69+*Salix*) conditions on Pontgibaud technosol (P) alone and amended with 5% biochar (B), 5% compost (C) or 1.5% iron grit (I), alone or combined. Minuscule letters indicate significant difference with time for each treatment and capital letters indicate dignificant difference between treatment for each time ($p < 0.05$) (n= 3 ± SE).



		Carbon (%)		Hydrogen (%)		Nitrogen (%)		Sulfur (%)	
	T0	0.14 ± 0.03 **A**	**a**	0.22 ± 0.00 **cB**	**c**	0.09 ± 0.02 **bAB**	**b**	0.87 ± 0.14 **D**	**b**
P	T69-*Salix*	0.17 ± 0.05 **A**	**a**	0.15 ± 0.00 **aA**	**a**	0.08 ± 0.00 **abA**	**ab**	0.41 ± 0.07 **AB**	**a**
	T69+*Salix*	0.11 ± 0.01 **A**	**a**	0.16 ± 0.00 **bA**	**b**	0.05 ± 0.01 **aA**	**a**	0.57 ± 0.06 **A**	**a**
	T0	3.76 ± 0.03 **D**	**a**	0.25 ± 0.00 **aCD**	**a**	0.11 ± 0.00 **abBC**	**ab**	0.53 ± 0.10 **AB**	**a**
PB	T69-*Salix*	4.29 ± 0.19 **C**	**b**	0.26 ± 0.03 **aBCE**	**a**	0.13 ± 0.01 **bBC**	**b**	0.42 ± 0.06 **AB**	**a**
	T69+*Salix*	3.94 ± 0.08 **C**	**a**	0.25 ± 0.00 **aC**	**a**	0.09 ± 0.02 **aAC**	**a**	0.54 ± 0.05 **A**	**a**
	T0	1.25 ± 0.15 **B**	**a**	0.29 ± 0.02 **aCD**	**a**	0.13 ± 0.00 **aDF**	**a**	0.52 ± 0.04 **AB**	**a**
PC	T69-*Salix*	1.32 ± 0.05 **B**	**a**	0.25 ± 0.07 **aACDE**	**a**	0.15 ± 0.02 **aBC**	**a**	0.54 ± 0.04 **B**	**a**
	T69+*Salix*	0.92 ± 0.01 **B**	**b**	0.25 ± 0.01 **aCD**	**a**	0.12 ± 0.03 **aACDE**	**a**	0.59 ± 0.06 **A**	**a**
	T0	0.12 ± 0.01 **A**	**a**	0.17 ± 0.01 **aA**	**a**	0.07 ± 0.01 **aAE**	**a**	0.55 ± 0.11 **ACD**	**a**
PI	T69-*Salix*	0.20 ± 0.00 **A**	**b**	0.21 ± 0.01 **bBC**	**b**	0.08 ± 0.02 **aAD**	**a**	0.50 ± 0.05 **BC**	**a**
	T69+*Salix*	0.14 ± 0.02 **A**	**a**	0.21 ± 0.01 **bBC**	**b**	0.08 ± 0.05 **aACD**	**a**	0.54 ± 0.03 **A**	**a**
	T0	3.40 ± 0.10 **C**	**a**	0.25 ± 0.01 **aCD**	**a**	0.10 ± 0.02 **aCDE**	**a**	0.59 ± 0.05 **BCDE**	**b**
PBC	T69-*Salix*	5.07 ± 0.81 **C**	**b**	0.32 ± 0.011 **bADE**	**b**	0.14 ± 0.49 **aACD**	**a**	0.38 ± 0.04 **A**	**a**
	T69+*Salix*	4.12 ± 0.01 **C**	**ab**	0.27 ± 0.03 **abBCDE**	**ab**	0.13 ± 0.00 **aBC**	**a**	0.54 ± 0.10 **A**	**b**
	T0	4.15 ± 0.23 **D**	**a**	0.25 ± 0.02 **aBC**	**a**	0.12 ± 0.00 **aBC**	**a**	0.55 ± 0.12 **AB**	**a**
PBI	T69-*Salix*	3.99 ± 0.11 **C**	**a**	0.30 ± 0.01 **bD**	**b**	0.12 ± 0.01 **aBD**	**a**	0.51 ± 0.02 **BC**	**a**
	T69+*Salix*	3.92 ± 0.29 **CD**	**a**	0.27 ± 0.00 **aD**	**a**	0.13 ± 0.02 **aBD**	**a**	0.48 ± 0.06 **A**	**a**
	T0	4.77 ± 0.09 **E**	**b**	0.38 ± 0.01 **bE**	**b**	0.24 ± 0.05 **bF**	**b**	0.44 ± 0.08 **AB**	**a**
PBCI	T69-*Salix*	4.17 ± 0.09 **C**	**a**	0.33 ± 0.01 **aE**	**a**	0.14 ± 0.02 **aBC**	**a**	0.40 ± 0.05 **AC**	**a**
	T69+*Salix*	4.50 ± 0.15 **D**	**b**	0.34 ± 0.01 **aE**	**a**	0.13 ± 0.02 **aBD**	**a**	0.53 ± 0.02 **A**	**a**

Finally, a column leaching test was set up: 20 g (dried) of each substrate was placed in a syringe (n = 5) and distilled water was added to field capacity. Following, 20 mL of distilled water was passed through the soil after 0, 30, 56 and 232 days (L0, L30, L56 and L232). Water after leaching was recovered and analyzed for pH, EC and As, Pb and Fe concentrations. Such test was performed to evaluate amendment effect on a longer time period but also assess the composition of the water that can leached from the soil under field condition after a rain event (Doherty *et al.* 2017).

On these leachates, pH and metal(loid) (As, Fe and Pb) concentrations were measured.

At L0, pH of P0% leachate was acidic (pH 3.96) (Table S5) and all amendments increased leachate pH, the least efficient being iron amendment (+ 0.5 pH unit) and the most efficient biochar + compost amendment (+ 4.3 pH units). After 30 days, leachate pH of P0% and PI were not different; all the other treatments showed higher pH values compared to P0%, the most efficient one being again biochar + compost. After 56 days, the same pattern as L0 was observed: all amendments increased leachate pH compared to P0%, the least and the most efficient were iron and biochar + compost, respectively. Finally, after 232 days, all substrates showed acidic to nearly neutral pH and all amendments increased pH compared to P0%. The highest rise was observed in PBC (+ 2.3 pH units). The results of the leachate pH reflected what was observed in the SPW (Table 3). The alkalization induced by biochar and compost can thus be explained by the alkalinity of these two amendments (Houben *et al*. 2013, Montiel-Rozas *et al*. 2015) while the increase following iron application can be related to iron grit corrosion (Qiao *et al*. 2018).

Finally, a trend towards acidification was observed with time, probably due to the high acidity of Pontgibaud technosol that released acidic compounds.

Leachate As concentration was 0.07 mg.L^{-1} in P0% at L0 and amendment applications decreased by seven-fold As concentration, except the three treatments containing compost. Indeed, the combination biochar + compost + iron grit had no effect, whereas compost alone and, to a lesser extent, biochar + compost increase As leaching (Table S5). Similarly, at L30, leachate As concentration was decreased with biochar, iron grit, biochar + iron grit but also biochar + compost + iron grit, whereas biochar + compost application had no effect and compost amendment alone greatly increased leaching of As. The same pattern was observed at L56 while at L232, no difference was observed between treatments. Similarly, Doherty *et al.* (2017) observed the capacity of iron-based amendments to reduce metal(loid) leaching. Contrary to what was observed in the SPW, As concentration was not below detection limit in most of the cases. However, similarly to SPW, compost application increased As concentration, which could be due to the dissolved organic compounds of compost that can interact with As as well as the pH increase which can mobilize As (Beesley *et al*. 2013).

Similarly to pH, leachate As concentrations tended to decrease with time which could demonstrate that Pontgibaud technosol did not re-supply the arsenic lost through leaching.

At L0, leachate Fe concentration was below detection limit in all treatments except PI (Table S5). Similarly at L30, leachate Fe concentrations were below detection or low in all treatments except PI, in which a great increase in Fe leaching was observed. At L56, Fe concentration was below detection limit in P0%, as well as PB, but it increased in several cases: all the treatments containing iron grit, with the highest rise observed with its application alone but also with the addition of compost. Finally, at L232, as for arsenic, no different was found between treatments. Generally, results matched SPW data: an increase in PI, due to the supply of Fe by iron grit amendment, which was lower in combination with biochar and compost, probably due to their sorption capacity and pH increase (Park *et al.* 2011). Finally, Fe leaching was also observed to decrease in PI, probably due to a lower supply of Fe by iron grit with time, after its complete oxidation.

Table S5. Leachate pH and metal(loid) (As, Fe and Pb) concentrations (mg.L^{-1}) determined after passing 20 mL of solution, after 0, 30, 56 and 232 days (L0, L30, L56, L232) into columns filled with the different substrates (Pontgibaud technosol (P) alone and amended with 5% biochar (B), 5% compost (C) or 1.5% iron grit (I), alone or combined). Minuscule letters indicate significant difference with time for each treatment and capital letters indicate significant difference between treatment for each time ($p < 0.05$) (n= 5 ± SE).

	L0		L30		L56		L232	
Leachate pH								
P0%	3.96 ± 0.04 **c**	**A**	3.79 ±± 0.04 **b**	**A**	3.51 ± 0.05 **a**	**A**	3.64 ± 0.02 **a**	**A**
PB	7.92 ± 0.05 **d**	**E**	6.95 ± 0.07 **c**	**C**	6.51 ± 0.09 **b**	**DE**	6.11 ± 0.06 **a**	**D**
PC	6.82 ± 0.29 **b**	**C**	6.70 ± 0.46 **b**	**CD**	6.96 ± 0.39 **b**	**EF**	4.93 ± 0.51 **a**	**BCD**
PI	4.47 ± 0.08 **b**	**B**	3.90 ± 0.08 **a**	**A**	3.93 ± 0.08 **a**	**B**	4.55 ± 0.05 **b**	**B**
PBC	8.30 ± 0.02 **c**	**F**	7.47 ± 0.14 **b**	**D**	7.30 ± 0.19 **b**	**F**	5.95 ± 0.39 **a**	**CD**
PBI	7.71 ± 0.02 **b**	**D**	5.20 ± 0.16 **a**	**B**	5.13 ± 0.14 **a**	**C**	5.51 ± 0.04 **a**	**C**
PBCI	7.93 ± 0.11 **c**	**DE**	6.55 ± 0.36 **b**	**C**	5.92 ± 0.27 **ab**	**D**	5.50 ± 0.34 **a**	**BC**
Leachate [As] (mg.L^{-1})								
P0%	0.07 ± 0.01 **c**	**B**	0.05 ± 0.00 **b**	**D**	0.04 ± 0.00 **b**	**C**	0.00 ± 0.00 **a**	**A**
PB	0.01 ± 0.00 **b**	**A**	0.00 ± 0.00 **ab**	**A**	0.01 ± 0.00 **b**	**A**	0.00 ± 0.00 **a**	**A**
PC	0.67 ± 0.07 **b**	**D**	0.64 ± 0.07 **b**	**E**	0.32 ± 0.08 **a**	**D**	0.16 ± 0.08 **a**	**A**
PI	0.01 ± 0.00 **b**	**A**	0.02 ± 0.00 **bc**	**BC**	0.03 ± 0.00 **c**	**B**	0.00 ± 0.00 **a**	**A**
PBC	0.32 ± 0.03 **c**	**C**	0.16 ± 0.04 **b**	**CD**	0.10 ± 0.02 **b**	**C**	0.01 ± 0.00 **a**	**A**
PBI	0.01 ± 0.00 **ab**	**A**	0.01 ± 0.00 **ab**	**AB**	0.02 ± 0.00 **b**	**AB**	0.00 ± 0.00 **a**	**A**
PBCI	0.07 ± 0.02 **a**	**B**	0.02 ± 0.00 **a**	**B**	0.02 ± 0.00 **a**	**A**	0.00 ± 0.00 **a**	**A**
Leachate [Fe] (mg.L^{-1})								
P0%	0.00 ± 0.00 **a**	**A**	0.02 ± 0.02 **a**	**AB**	0.00 ± 0.00 **a**	**A**	0.00 ± 0.00 **a**	**A**
PB	0.00 ± 0.00 **a**	**A**	0.00 ± 0.00 **a**	**A**	0.00 ± 0.00 **a**	**A**	0.00 ± 0.00 **a**	**A**
PC	0.00 ± 0.00 **a**	**A**	0.74 ± 0.36 **b**	**B**	0.89 ± 0.27 **b**	**B**	2.42 ± 1.36 **ab**	**A**
PI	3.11 ± 0.03 **b**	**B**	70.62 ± 25.90 **c**	**C**	69.49 ± 12.26 **c**	**D**	0.03 ± 0.03 **a**	**A**
PBC	0.00 ± 0.00 **a**	**A**	0.00 ± 0.00 **a**	**A**	0.03 ± 0.03 **a**	**AC**	0.77 ± 0.77 **a**	**A**
PBI	0.00 ± 0.00 **a**	**A**	0.92 ± 0.92 **a**	**AB**	13.73 ± 10.15 **a**	**B**	0.11 ± 0.11 **a**	**A**
PBCI	0.00 ± 0.00 **a**	**A**	0.25 ± 0.25 **a**	**AB**	8.82 ± 6.74 **a**	**BC**	0.82 ± 0.74 **a**	**A**
Leachate [Pb] (mg.L^{-1})								
P0%	8.50 ± 0.28 **a**	**E**	9.70 ± 0.13 **ab**	**D**	9.57 ± 0.37 **ab**	**D**	10.01 ± 0.42 **b**	**C**
PB	0.34 ± 0.03 **a**	**C**	0.14 ± 0.04 **a**	**A**	0.25 ± 0.09 **a**	**B**	0.63 ± 0.15 **a**	**A**
PC	0.33 ± 0.11 **a**	**C**	0.29 ± 0.04 **a**	**B**	0.11 ± 0.04 **a**	**AB**	2.49 ± 0.69 **a**	**AB**
PI	6.48 ± 0.40 **b**	**D**	2.66 ± 0.24 **a**	**C**	2.72 ± 0.26 **a**	**C**	20.12 ± 1.09 **c**	**D**
PBC	0.02 ± 0.01 **a**	**B**	0.07 ± 0.04 **a**	**A**	0.02 ± 0.02 **a**	**AB**	1.56 ± 0.72 **b**	**AB**
PBI	0.02 ± 0.02 **a**	**AB**	2.20 ± 0.10 **a**	**C**	1.93 ± 0.24 **a**	**C**	1.95 ± 0.29 **a**	**B**
PBCI	0.00 ± 0.00 **a**	**A**	0.54 ± 0.29 **ab**	**AB**	0.24 ± 0.12 **ab**	**AB**	1.46 ± 0.60 **b**	**AB**

A high concentration of Pb leached from P0% at L0 but all amendment treatments decreased Pb leaching, the least efficient was iron grit (24 %) and the most efficient was PBCI treatment, in which Pb was below detection limit (Table S5). Similarly, the elevated Pb leaching (9.70 mg.L^{-1}) in P0% was reduced by the amendments, between 73% (PI) and 99% (PBC), at L30. The same decrease (between 72% and 99%) was observed at L56 whereas at L232, a 2 fold increase in Pb leaching occurred in PI compared to P0% while the other treatments decreased Pb leaching. Such reduction in Pb leaching was previously observed by Gul *et al.* (2019) and can be attributed to the sorption capacity of the different amendments (Park *et al*. 2011, Jiang *et al*. 2012, Huang *et al*. 2016, Houben *et al*. 2012).

Finally, contrary to what was observed for As and Fe, Pb leaching either did not evolve or increased with time, probably due to a supply of Pb by the soil highly contaminated by Pb.

These leaching tests demonstrated that amendment application decreased acid leaching, although such effect was reduced with time. Amendments also permitted to reduce metal(loid) leaching, especially the combination containing the three amendments. Moreover, As and Fe leaching reduced with time while Pb tended to increase, demonstrating that with time, amendments are less efficient to immobilize Pb supplied by the soil, probably due to the fact that they reached their maximum sorption capacity.

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Part E. Effects of carbon-based materials and redmuds on metal(loid) immobilization and *Salix dasyclados* growth on a former mine technosol contaminated by As and Pb.

Effects of carbon-based materials and redmuds on metal(loid) immobilization and *Salix dasyclados* growth on a former mine technosol contaminated by As and Pb

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Abstract

Metal(loid) soil contamination is a widespread issue and its remediation a priority. One remediation technique suited is phytoremediation, *i.e.,* the use of plants to stabilize/extract metal(loid)s and prevent contamination spreading. *Salicaceae* are fast growing trees that can tolerate and accumulate metal(loid)s, showing their potential for phytoremediation. However, amendments need to be applied to allow plant growth because of poor soil fertility. Biochar, the solid product of pyrolysis, can ameliorate soil properties and reduce metal(loid) bioavailability but showed low affinity towards arsenic. Redmud, a waste product, can immobilize arsenic and other metal(loid)s and improve soil conditions. To evaluate the potential of those two amendment types to immobilize metal(loid)s, a pot experiment was conducted using several carbon-based materials (biochar and activated carbon) and redmuds. Beforehand, a characterization and a sorption test towards Pb were performed to select two carbon-based materials and two redmuds with the best results. A former mining soil was amended with those products and *Salix dasyclados* cuttings were planted. This experiment revealed that only biochar and one redmud improved growing conditions and *Salix dasyclados* growth. Moreover, redmud yielded similar effects than biochar at a lower application rate, and is also cheaper than biochar. It can therefore be concluded that the application of redmud, associated to *Salix dasyclados*, could be an option for the phytoremediation of an As and Pb contaminated soil. However these results need to be evaluated on a bigger scale to assess the gain brought by the combination of redmud and *Salix dasyclados* on the contaminated area.

Keywords

Activated carbon; Biochar; Metal(loid)s; Phytoremediation; Redmud; *Salix dasyclados*

Highlights

Carbon-based materials and redmuds were evaluated for metal(loid) immobilization.

Biochar and neutralized redmud immobilized Pb and improved soil fertility.

Biochar and neutralized redmud application increased *Salix dasyclados* growth.

1. Introduction

Soils are fundamental in the ecosystem and provide many services such as biomass production and support for human activities (agriculture, construction…). Thus, their good state is crucial but human activities induced a huge contamination of the soils worldwide. For instance, in Europe, it was estimated that around 2.5 million sites are contaminated, which gives five to seven sites per 10,000 habitants (Paya Pérez and Rodriguez Eugenio 2018). Such high amount of contaminated sites comes mainly from waste disposal, industrial and commercial activities, storage, transport and spills on land and nuclear operations (Panagos *et al.* 2018). There is a multitude of pollutants in soils but the most important ones are metal(loid)s. In addition to their abundance, soil contamination by metal(loid)s is of great concern as metal(loid)s cannot be degraded and thus accumulate in soils for decades. Moreover, this contamination has negative effects on both the environment, *i.e.* decrease of biodiversity, de-vegetation, and human health, such as the induction of cancer and other diseases. For instance, arsenic (As) is a metalloid without biological function and of great environmental concern due to its toxicity and abundance. Lead (Pb) is a widespread toxic metal which has no known biological function (Peralta-Videa *et al.* 2009). Both elements are classified as carcinogenic. Therefore, the remediation of areas contaminated by those elements is needed.

One remediation method that gathered attention over the last decades is phytoremediation, which involves the manipulation of the soil-plant system with the goal to reduce the environmental risks posed by metal(loid)s (Robinson *et al.* 2009, Tack and Meers 2010). Phytoremediation includes diverse approaches including phytoextraction, phytostabilization, phytovolatilization… and aims at establishing a vegetation cover. The plants will take up contaminants and store them in their tissues. This plant cover will also prevent contaminant spreading by wind erosion and leaching. However, the success of phytoremediation relies on the selection of the right plant species as well as on the application of an appropriate amendment, as contaminated soils are often poor from an agronomic point of view.

Among possible plants to use in phytoremediation, *Salicaceae* are fast growing trees with a high biomass production and a deep and wide root system (Marmiroli *et al.* 2011). They also have a resistance to metal(loid)s. Numerous studies demonstrated their ability to grow on contaminated soils and to accumulate metal(loid)s, especially in their roots. For instance, Zárubová *et al.* (2015) showed that diverse *Salix* clones were capable of growing on a soil moderately contaminated by Cd, Pb and Zn. In the study of Bart *et al.* (2016), *Salix viminalis* and *Salix purpurea* were able to develop a root system and to produce an aboveground biomass on a former gold mine contaminated by As, Pb and Sb. Finally, Unterbrunner *et al.* (2007), Migeon *et al.* (2009) and Lebrun *et al.* (2017) showed the ability of *Salix* species to accumulate metal(loid)s when grown on former mining areas. Moreover, *Salicaceae* can be planted in short rotation coppice system to produce biomass for energy, adding an economic value to the remediation.

The other component of the phytoremediation success is the amendment application. Contaminated soils often present a poor fertility (extreme pH, low nutrient content and availability, low organic matter content). The combination with elevated contamination levels makes it difficult for the plant to germinate. One possibility is to apply fertilizer that can increase nutrient content and availability. However fertilization does not reduce metal(loid) stress and generally needs to be re-applied. Thus, amendments have been studied as potential ameliorators of the growing conditions. Two amendments gathered attention recently and showed good results, biochar and redmud (Agegnehu *et al.* 2017, Hua *et al.* 2017). Biochar is the solid product of pyrolysis of biomass (Wisniewska *et al.* 2016), characterized by a porous and carbonaceous structure (Paz-Ferreiro *et al.* 2014). Its properties, such as alkaline nature, high surface area, high content in carbon, hydrogen, nitrogen and oxygen, elevated water holding capacity (WHC) (Paz-Ferreiro *et al.* 2014, Wisziewska *et al.* 2016), make biochar a good product to ameliorate soil properties and reduce metal(loid) bioavailability. Biochar application was shown to increase soil pH, WHC and nutrient contents (Hmid *et al.* 2015, Rees *et al.* 2015). It can also sorb metal(loid)s, reducing their mobility and bioavailability (Hmid *et al.* 2015, Meng *et al.* 2018). Biochar can also be activated to improve its performances; the product is thus called activated carbon (Tan *et al.* 2017). However, previous studies also showed that biochar was not efficient regarding negatively charged metal(loid)s, such as arsenic, and even induced negative effects, *i.e.* increase of mobility (Beesley *et al.* 2010, Huang *et al.* 2018). On the contrary, Bertocchi *et al.* (2006) showed that redmud was able to immobilize arsenic and other metal(loid)s. Redmud is a solid waste residue obtained from the production of alumina or charcoal mines (Bhatnagar *et al.* 2011). It is highly alkaline and contains elevated concentrations of aluminum, iron, calcium, silicon and titanium (Bhatnagar *et al.* 2011, Liu *et al.* 2011). Previous studies found that the application of redmud to contaminated soils improved soil condition and plant growth (Castaldi *et al.* 2009, Lee *et al.* 2011). However, the effects of those two amendment types are highly dependent on the amendment properties, soil conditions and metal(loid) type (Lebrun *et al.* 2018a).

Although those two amendments have been much studied, few analyzed simultaneously several biochars and several redmuds, as well as their combined application. Therefore, the aims of this study were first to characterize a former mining soil and six amendments, three carbon-based materials and three redmuds. Based on this characterization, two carbon-based materials and two redmuds were selected to perform a pot experiment. The aims of this pot experiment were to assess the effect of applying a mineral fertilization, carbon-based material and/or redmud on: (i) the soil physico-chemical properties, (ii) metal(loid) immobilization and (iii) *Salix dasyclados* growth and metal(loid) accumulation pattern.

2. Materials and Methods

2.1 Studied site

The study focused on a former silver-lead extraction mine site of the Pontgibaud district (Auvergne-Rhône-Alpes, France). Mining activities lasted until the end of the nineteenth century and resulted in the production of large amounts of wastes contaminated mainly by arsenic and lead (Cottard 2010). Previous studies showed that this soil was very acidic, with low organic matter content (Lebrun *et al.* 2018a, 2019). Soil samples were collected in one of the settling ponds (between 0 and 20 cm of depth) in the area called Roure-les-Rosiers.

2.2 Amendments

Two types of amendments were used: carbon-based materials and redmuds. Three carbon-based materials were tested: a commercial hardwood biochar, provided by La Carbonerie (Crissey, France) (Lebrun *et al.* 2019), a coconut-based biochar provided by Jacobi Carbons (Paris, France) and a coconut-based steam activated carbon also provided by Jacobi Carbons. These carbon-based materials will subsequently be referred to as biochar 1 (Bc1), biochar 2 (Bc2) and activated carbon (AC), respectively. Similarly, three redmuds were employed: a commercial redmud provided by Alteo Environnement (Gardanne, France), the same redmud neutralized by gypsum (Hurel *et al.* 2017), also provided by Alteo Environnement, and a redmud directly collected from a charcoal mine located in Alès (France). Those three products will be referred to as redmud 1 (RM1), redmud 2 (RM2) and redmud 3 (RM3).

2.3 Characterization of the soil and the amendments

The soil was analyzed for its carbonate content, as well as for its organic and available phosphorus and total nitrogen, using methods described in Van Ranst *et al.* (1999). Total carbonate content was determined by adding an excess amount of acid to the soil, which will be neutralized by the bases of the soil; the excess acid was then titrated with sodium hydroxide to determine $CaCO_3$ equivalent content. Organic phosphorus was determined by calcination of the soil sample and extraction of the mineralized phosphorus by sulfuric acid. Available phosphorus was determined by the Olsen's method, using sodium bicarbonate, activated charcoal for P solubilization and molybdenum blue for P quantification. Total nitrogen was determined by the Kjedahl method, using a mixture of sulfuric acid and salicylic acid to mineralize the nitrogen of the soil, followed by a steam distillation and a titration with acid.

Additionally, both the soil and the amendments were analyzed for cation exchange capacity, using ammonium acetate, and metal(loid) concentrations after *aqua-regia* extractions and ICP-OES (Varian Vista MPX, Varian Palo Alto, California, USA) measurements, as described in Van Ranst *et al.* (1999). Furthermore, the pH of the 1:7 mixture of distilled H_2O with the amendments was analyzed after four hours agitation.

Additionally, a sorption test was performed using lead as $Pb(NO_3)_2$. Only Pb was used as it is the main contaminant in Pontgibaud. For this test, a series of Pb solutions were prepared at increasing concentrations of 1, 5, 10, 15, 20 and 25 mM. Afterwards, 10 mL of these solutions were mixed with 0.1 g of amendment, and shaken for 24 h. Suspensions were then filtered and pH was measured. Solutions were acidified with HNO_3 and analyzed for Pb concentration by ICP-OES. Sorbed Pb quantity was calculated as g of Pb sorbed per kg of amendment material. In addition, sorption coefficients K_d were calculated using the formula $K_d = Q_e/C_e$ (Wang *et al.* 2015), with Q_e the quantity of Pb sorbed per kg of amendment and C_e the concentrations in the tubes after the sorption process.

2.4 Calcium chloride extractions

Calcium chloride (0.01 M $CaCl_2$) extractions (1:5 soil:extractant ratio) were performed on the contaminated soil alone and on the soil mixed with each biochar at 2 % (w/w) and/or each redmud at 1 % (w/w), as described in Van Ranst *et al.* (1999) and Meers *et al.* (2007a).

2.5 Pot experiment

Based on the tests described above, seven conditions were selected for testing in a pot experiment: non amended Pontgibaud technosol (PG), PG + fertilization, PG + 2 % Bc1, PG + 1 % RM2, PG + 2 % Bc1 + 1 % RM2, PG + 1 % RM1 and PG + 2 % AC. For the first five treatments, ten pots of 2 kg were prepared and seven were vegetated with a non-rooted cutting of *Salix dasyclados* (cv. Loden), whereas for the last two treatments, three and four pots were prepared respectively, with one left un-vegetated. All amendments were added on a weight basis. The fertilization treatment involved adding 100 mL of nutrient solution containing NH_4NO_3 (5 $g.L^{-1}$), $Ca(H_2PO_4)_2{\cdot}H_2O$ (2.5 $g.L^{-1}$), K_2SO_4 (2.5 $g.L^{-1}$) and $MgSO_4{\cdot}7H_2O$ (2.5 $g.L^{-1}$).

The prepared substrates were watered at 80 % of the WHC (Lebrun *et al.* 2019) and left equilibrated for two weeks before planting the cuttings. Mixtures were maintained at 80 % WHC throughout the experiment by weighting and watering every two days, using distilled water.

Soil pore waters (SPWs) were sampled after the equilibration period, using soil moisture samplers (Rhizon®) (Cattani *et al.* 2006). On those samples, pH and metal(loid) concentrations were measured by ICP-OES after sample acidification.

At the middle of the experiment (41 days after mixture preparation), 50-100 grams of wet soil were sampled from each non-vegetated pot and $CaCl_2$ extractions were performed as previously described. Additionally, soil was also sampled at the end of the growing period (*i.e.* 69 days after mixture preparation) to determine available macro-element concentrations (Ca, K, Mg, Na, P, S) by ICP-OES after ammonium acetate + EDTA (pH 4.65) extraction (1:5 soil:extractant ratio) (Van Ranst *et al.* 1999, Meers *et al.* 2007b).

After 55 days of growth, leaf pigments were measured on five mature leaves per stem using a Yara N-Tester® (Ortuzar-Iragorri *et al.* 2005). It was not possible to measure pigments for the plants grown on the fertilized condition, due to the state of the leaves. Following, plants were harvested. Stem length and total number of leaves were determined. All the leaves of each plant were photographed to measure total leaf area using ImageJ software. The three organs formed during growth period (leaves, stems, roots) were dried for four days at 60 °C and dry weight (DW) was determined. Finally, the plant material was acid digested in microwave: (i) 0.2 g of plant material was mixed with 10 mL pico-pure HNO_3 in tubes and left at room temperature for 30 min; (ii) tubes were incubated in a sonicator for 30 min at 50 °C; (iii) tubes were heated in a microwave (210 °C, 15 hold, 800 psi, 900-1050 W) (Microwave mars 6). After the digestion, samples were recovered and diluted to 50 mL using MilliQ water and analyzed for metal(loid) concentration by ICP-OES.

2.6 Statistical analysis

The data were analyzed using R software version 3.5.1 (R Development Core Team, 2009). The means of the seven treatments were compared using one way Anova test followed by a post-hoc Tukey test for two-by-two comparison. Difference was considered significant at $p < 0.05$.

3. Results

3.1 Soil properties

The studied soil had a low fertility, with low contents in organic phosphorus, available phosphorus and nitrogen (Table 1). The soil also had a low carbonate content and a low CEC (Table 1). Finally, As and Pb concentrations were highly above permissible limits (Ashraf *et al.* 2019).

Table 1: Properties of Pontgibaud technosol (n = 3 ± SE)

	Value	Limits[a]	Unit
Organic Phosphorus	465 ± 17		mg.kg^{-1}
Available Phosphorus	6 ± 1		mg.kg^{-1}
Total Nitrogen	75 ± 3		mg.kg^{-1}
Total Carbonates	0.7 ± 0.4		% $CaCO_3$
Cation Exchange Capacity	2.3 ± 0.1		cmol.kg^{-1}
[Al]	2,010 ± 187		mg.kg^{-1}
[As]	**1,501 ± 326**	20	mg.kg^{-1}
[Cd]	0.26 ± 0.00	3	mg.kg^{-1}
[Cr]	5 ± 0	150	mg.kg^{-1}
[Cu]	52 ± 5	140	mg.kg^{-1}
[Fe]	6,518 ± 1639	50,000	mg.kg^{-1}
[Mn]	7 ± 0	80	mg.kg^{-1}
[Ni]	<DL	50	mg.kg^{-1}
[Pb]	**19,228 ± 1531**	300	mg.kg^{-1}
[Zn]	284 ± 20	300	mg.kg^{-1}

[a]Maximum permissible limit in soil (from Ashraf et al. 2019); bold values are above these limits
< DL = below detection limit (10 mg.kg^{-1} Ni)

3.2 Amendment characteristics

All tested amendments had an alkaline pH, between 8.00 and 12.26 depending on the type and the feedstock (Table 2).

Biochars Bc1 and Bc2 presented a CEC (1.5 and 2.4 cmol.kg^{-1}, respectively) similar to the one measured for PG soil (2.3 cmol.kg^{-1}) (Table 1, Table 2). Amendment AC had a higher CEC (13.1 cmol.kg^{-1}) than the soil and the two biochars (Table 1, Table 2). All redmuds had a higher CEC than the soil. The higher value was observed for the non-processed redmud (RM3).

The two types of materials, carbon-based materials and redmuds, differed in their elemental composition. Redmuds tended to have higher metal(loid) concentrations than biochars (Table 2). Only biochar 1 had an elevated Fe concentration. In addition, As, Cd, Ni and Pb contents were below detection limits (As = 90 mg.kg^{-1}, Cd = 1.25 mg.kg^{-1}, Ni = 10 mg.kg^{-1}, Pb = 25 mg.kg^{-1}) in all three biochars, while Cr was below detection limits in AC. Redmuds were also characterized by high levels of Fe.

Table 2: Properties of the amendments (n = 3 ± SE)

	Biochar 1	Biochar 2	Activated carbon	Redmud 1	Redmud 2	Redmud 3
pH	8.98 ± 0.02	10.03 ± 0.02	10.70 ± 0.00	12.26 ± 0.04	8.58 ± 0.43	8.00 ± 0.05
CEC (cmol.kg^{-1})	1.5 ± 0.3	2.4 ± 0.6	13.1 ± 2.6	4.7 ± 0.8	6.0 ± 1.0	26.0 ± 1.3
[Al] (mg.kg^{-1})	705 ± 7	50 ± 0	66 ± 6	45,706 ± 1,641	32,315 ± 3,328	12,229 ± 932
[As] (mg.kg^{-1})	<DL	<DL	<DL	<DL	<DL	**620 ± 35**
[Cd] (mg.kg^{-1})	<DL	<DL	<DL	**22 ± 1**	**15 ± 2**	**34 ± 2**
[Cr] (mg.kg^{-1})	5 ± 0	2 ± 0	<DL	**1,453 ± 52**	**925 ± 86**	1 ± 0
[Cu] (mg.kg^{-1})	10 ± 0	11 ± 8	6 ± 0	26 ± 0	28 ± 2	<DL
[Fe] (mg.kg^{-1})	4,456 ± 304	66 ± 2	70 ± 8	286,081 ± 10,158	199,555 ± 19,958	281,097 ± 19,278
[Mn] (mg.kg^{-1})	241 ± 3	4 ± 0	6 ± 1	187 ± 3	246 ± 18	1,747 ± 162
[Ni] (mg.kg^{-1})	<DL	<DL	<DL	<DL	32 ± 4	**71 ± 4**
[Pb] (mg.kg^{-1})	<DL	<DL	<DL	66 ± 1	71 ± 5	26 ± 0
[Zn] (mg.kg^{-1})	16 ± 1	12 ± 7	3 ± 0	50 ± 1	53 ± 3	**15,860 ± 681**

Bold values = above French upper critical limit for organic amendments (Oustriere et al. 2017)

CEC = cation exchange capacity

< DL = below detection limit (90 mg.kg^{-1} As, 1.25 mg.kg^{-1} Cd, 1 mg.kg^{-1} Cr, 2 mg.kg^{-1} Cu, 10 mg.kg^{-1} Ni, 25 mg.kg^{-1} Pb)

Based on this first characterization, redmud 3 presented too high metal(loid) concentrations to be applied to the soil. Consequently RM3 was not used in the following mesocosm experiment.

3.3 Sorption test

Redmud materials had a higher sorption capacity than the biochars (Figure 1). Amendment RM3 presented higher values of Pb sorbed than the other two redmuds at the higher Pb concentrations. Among biochars, the lowest sorption capacity was found for Bc2, while its steam activation (AC) increased its sorption capacity.

Finally, as shown in Table S1 and Figure 1, *K*$_d$ values of redmuds were higher than the one of biochars and decreased with increasing Pb concentrations.

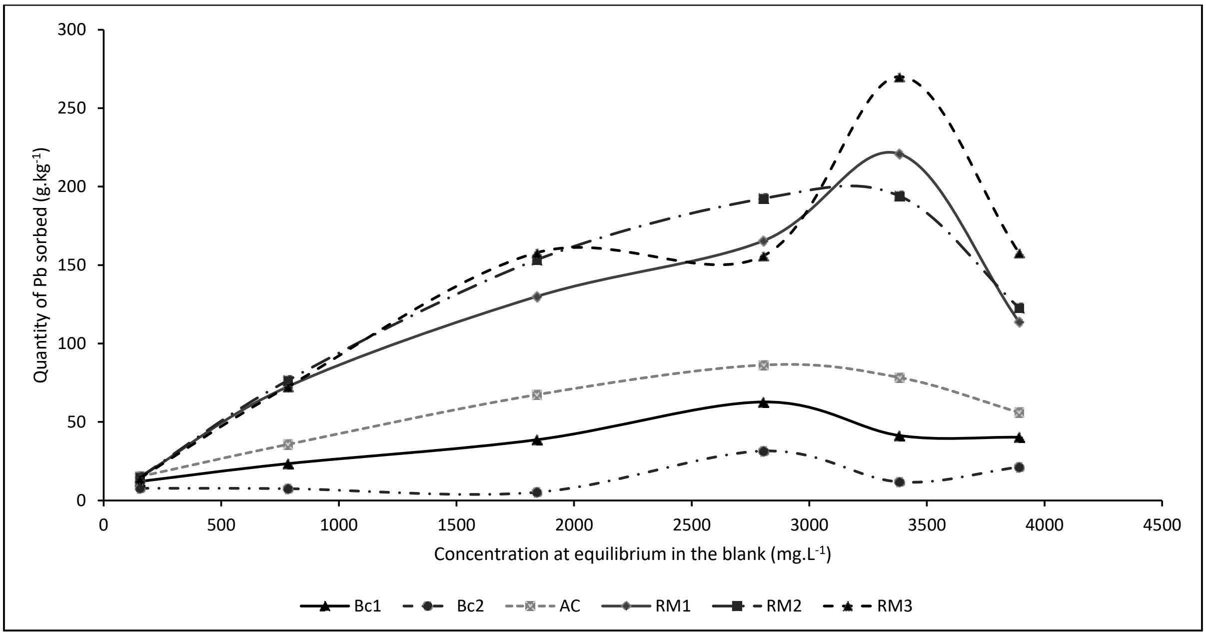

Figure 1: Variation in Pb sorbed quantity (g.kg^{-1}) on biochars or redmuds compared to the concentration in the blank (mg.L^{-1}). Bc1 = hardwood biochar, Bc2 = coconut biochar, AC = activated coconut carbon, RM1 = commercial redmud, RM2 = neutralized commercial redmud, RM3 = redmud from charcoal mine.

3.4 $CaCl_2$ extraction test

Because Pb is the main contaminant in Pontgibaud, its concentration in the extract was measured. Biochar and activated carbon (2 %) alone was not reducing $CaCl_2$-extractable Pb concentration. All redmud treatments showed a decrease in $CaCl_2$ extractable Pb concentration (Table S2), the most effective being RM2.

Some amendments presented high levels of Cd and Zn, above regulation limits (Table 2), thus those two elements were also analyzed. Concentrations of $CaCl_2$ extractable Cd were low in all treatments and not affected by almost all amendments. Only the treatment Bc1+RM3 increased $CaCl_2$ extracted Cd (Table

S2), although it was not significant. Finally, $CaCl_2$ extractable Zn concentration was highly increased in all RM3 treatments but the association of RM3 with the different carbon-based materials decreased $CaCl_2$ extracted Zn compared to RM3 alone.

3.5 SPW physico-chemical properties

SPW was sampled at the beginning of the experiment. Pontgibaud soil had a very acidic pH (pH 3.74) (Table 3). The fertilization treatment further decreased pH, by 0.7 units. On the contrary, applying biochar, activated carbon and redmuds, alone or combined, increased SPW pH (Table 3). The addition of Bc1 and RM2 increased pH until neutrality. The other two amendments, RM1 and AC, induced a lower pH rise than the first two.

Concentration in As in SPW was below detection limit in all conditions except when fertilization was applied (Table 3). The application of a mineral fertilization greatly increased SPW As concentration, until 2.5 $mg.L^{-1}$.

Oppositely to As, Pb concentration in SPW of PG was high, almost 15 $mg.L^{-1}$ (Table 3) and adding fertilizer had no effect. However, the other treatments highly decreased the Pb in the SPW by more than 70 %. Biochar and redmud similarly decreased Pb concentration in SPW, and the combination of Bc1 and RM2 did not show better results than their single application.

Table 3: Soil pore water pH and arsenic and lead concentrations ($mg.L^{-1}$) measured at the beginning of the experiment in Pontgibaud (PG) fertilized (fert) or amended with hardwood biochar (Bc1), coconut activated carbon (AC) and/or two commercial redmuds (RM1 and RM2). Letters indicate difference between the treatments assessed by one-way Anova and post-hoc Tukey tests (n = 3-7 ± SE) ($p < 0.05$).

	pH	[As] ($mg.L^{-1}$)	[Pb] ($mg.L^{-1}$)
PG	3.74 ± 0.03 e	< DL	14.8 ± 1.8 a
PG+fert	3.05 ± 0.03 f	2.5 ± 2.1	13.0 ± 9.0 a
PG+Bc1	5.83 ± 0.03 b	< DL	5.0 ± 0.2 b
PG+RM2	5.92 ± 0.03 b	< DL	2.2 ± 0.3 b
PG+Bc1+RM2	7.06 ± 0.04 a	< DL	1.1 ± 0.2 b
PG+RM1	5.57 ± 0.12 c	< DL	3.5 ± 0.3 b
PG+AC	5.23 ± 0.08 d	< DL	4.7 ± 0.2 b

< DL = below detection limit (0.9 $mg.L^{-1}$)

3.6 Soil extractions at mid term

Concentrations of $CaCl_2$ extractable metal(loid)s were the highest for Pb, the principal contaminant of Pontgibaud (Table 4). All the treatments decreased $CaCl_2$ extractable Pb concentration, except AC. The most efficient treatment was the combination Bc1+RM2.

Table 4: $CaCl_2$-extractable Al, Cd, Cr, Cu, Fe, Mn, Pb and Zn concentrations (mg.kg^{-1}) and solution pH after 0.01 M $CaCl_2$ extraction on soils sampled 41 days after mixture preparation in Pontgibaud (PG) fertilized (fert) or amended with hardwood biochar (Bc1), coconut activated carbon (AC) and/or two commercial redmuds (RM1 and RM2). Letters indicate difference between the treatments assessed by one-way Anova and post-hoc Tukey tests (n = 3 ± SE) ($p < 0.05$).

	Solution pH	[Al] (mg.kg^{-1})	[Cd] (mg.kg^{-1})	[Cu] (mg.kg^{-1})	[Fe] (mg.kg^{-1})	[Mn] (mg.kg^{-1})	[Pb] (mg.kg^{-1})	[Zn] (mg.kg^{-1})
PG	3.36 ± 0.01	52.7 ± 1.0 a	0.13 ± 0.01 b	1.10 ± 0.04 a	0.15 ± 0.02	1.18 ± 0.08 b	442 ± 14 a	12.5 ± 0.9 b
PG+fert	3.33 ± 0.03	47.3 ± 2.2 b	0.11 ± 0.01 c	1.09 ± 0.03 a	< DL	0.85 ± 0.11 c	235 ± 17 c	10.4 ± 0.7 c
PG+Bc1	3.77 ± 0.04	1.9 ± 0.2 ef	0.13 ± 0.00 b	0.32 ± 0.01 d	< DL	1.63 ± 0.04 a	311 ± 2 b	7.5 ± 0.1 d
PG+RM2	3.80 ± 0.06	2.7 ± 0.6 de	0.08 ± 0.00 d	0.26 ± 0.03 d	< DL	0.73 ± 0.02 cd	199 ± 19 c	3.4 ± 0.2 e
PG+Bc1+RM2	3.86 ± 0.18	1.4 ± 0.1 f	0.06 ± 0.00 e	0.22 ± 0.00 d	< DL	0.28 ± 0.01 e	99 ± 17 d	1.7 ± 0.2 e
PG+RM1	3.59 ± 0.12	15.6 ± 2.4 d	0.11 ± 0.00 c	0.46 ± 0.04 c	< DL	0.55 ± 0.02 d	323 ± 20 b	9.3 ± 0.1 c
PG+AC	3.50 ± 0.02	39.2 ± 0.2 c	0.17 ± 0.00 a	0.65 ± 0.01 b	< DL	0.64 ± 0.01 cd	422 ± 7 a	14.5 ± 0.1 a

< DL = below detection limit (0.125 mg.kg^{-1} Fe)

Table 5: Ammonium acetate + EDTA-extractable Ca, K, Mg, Na, P, S concentrations (mg.kg^{-1}) in soils sampled at harvest time in Pontgibaud (PG) fertilized (fert) or amended with hardwood biochar (Bc1), coconut activated carbon (AC) and/or two commercial redmuds (RM1 and RM2). Letters indicate difference between the treatments assessed by one-way Anova and post-hoc Tukey tests (n = 3 ± SE) ($p < 0.05$).

	[Ca] (mg.kg^{-1})	[K] (mg.kg^{-1})	[Mg] (mg.kg^{-1})	[Na] (mg.kg^{-1})	[P] (mg.kg^{-1})	[S] (mg.kg^{-1})
PG	34 ± 2 e	28 ± 2 c	7.7 ± 0.2 c	6 ± 1 c	1.0 ± 0.1 b	847 ± 39 a
PG+fert	44 ± 4 ce	81 ± 4 b	21.0 ± 1.2 a	5 ± 1 c	9.0 ± 2.1 a	1,138 ± 322 a
PG+Bc1	321 ± 31 bc	53 ± 7 c	8.1 ± 1.0 c	5 ± 1 c	1.4 ± 0.3 b	1,371 ± 254 a
PG+RM2	304 ± 28 b	33 ± 3 c	12.2 ± 1.4 b	173 ± 15 a	3.4 ± 1.1 b	1,432 ± 501 a
PG+Bc1+RM2	488 ± 41 a	45 ± 8 c	8.6 ± 1.3 bc	123 ± 15 b	4.2 ± 1.2 b	973 ± 417 a
PG+RM1	150 ± 19 d	35 ± 3 c	7.3 ± 0.5 c	105 ± 17 b	2.5 ± 1.0 b	1,362 ± 280 a
PG+AC	39 ± 0 e	133 ± 7 a	10.8 ± 0.2 bc	12 ± 1 c	2.6 ± 0.0 b	1,966 ± 66 a

The Al concentration was the highest in PG and decreased in all other treatments, with the highest drop again observed in the Bc1+RM2 treatment. The $CaCl_2$ extractable Cd concentration was low on PG soil but still decreased with the application of the different treatments, except with AC. Similarly, the Cu concentration was low on PG soil and the application of biochar and/or redmud slightly decreased $CaCl_2$ extracted Cu while the fertilization had no effect. Iron was only detectable in $CaCl_2$ extraction of PG. Concentration in Mn was lower in the treated conditions compared to PG, except Bc1 which increased $CaCl_2$ extracted Mn. Finally, Zn concentrations were decreased by the fertilization and the application of amendments, except for the increase observed with AC.

$CaCl_2$ extractable metal(loid) concentrations highlighted the beneficial effects of applying biochar and/or redmud. It also showed that steam activation, performed to improve biochar effects, was not beneficial in this case, although it showed higher Pb sorption capacity than its non-activated biochar in the sorption test.

3.7 Soil extraction at harvest time

Concentration in Ca of PG soil was 33.5 mg.kg^{-1}. All treatments, except fertilization and AC, increased Ca concentration, with the most efficient being the combination Bc1+RM2 (Table 5). On the contrary, only fertilization and AC application increased K concentrations. Concentration in Mg was only increased with fertilization and to a lesser extent RM2. Only the application of redmud rose Na concentration. Finally, P concentration was only improved with fertilization whereas S concentration did not evolve.

Taken together, data showed that soil fertility was not recovered, at least not entirely, with the application of a mineral fertilizer, activated carbon, biochar and/or redmud.

3.8 Plant growth

On PG soil, plants showed a low average stem length (5 cm). Only the application of RM2, alone or combined to Bc1, significantly increased stem length, more than three times (Table 6). On PG soil, plants produced 24 leaves on average and none of the treatments had an effect. Leaf surface area was increased with Bc1 and/or RM2 addition, by 5 fold on average (Table 6). Leaf pigments could not be measured on plants grown on the fertilized condition, but it was possible for the other conditions. Leaf pigments decreased with RM2, more importantly when it was combined with Bc1, which can be due to a dilution effect, as leaves were larger in such conditions. Finally, organ dry weight (DW) was low on PG (Figure 2). Fertilization did not improve DW production, as well as RM1 and AC. On the contrary, adding Bc1 and/or RM2 greatly increased leaf, stem and root DW (Figure 2).

Fertilization as well as RM1 and AC amendments did not improve *Salix dasyclados* growth. On the contrary, adding Bc1 and/or RM2 greatly improved *Salix* growth, in terms of stem length, leaf area and DW production. However, no difference was observed between Bc1 and RM2, even though biochar was added at a higher rate, and there was no additional effect when they were applied together.

Table 6: Stem length (cm), number of leaves, leaf pigments and total leaf surface area (cm²) of *Salix viminalis* plants grown on Pontgibaud (PG) fertilized (fert) or amended with hardwood biochar (Bc1), coconut activated carbon (AC) and/or two commercial redmuds (RM1 and RM2). Letters indicate difference between the treatments assessed by one-way Anova and post-hoc Tukey tests (n = 2-7 ± SE) ($p < 0.05$).

	Stem length (cm)	Number of leaves	Leaf pigments	Total leaf surface area (cm²)
PG	5 ± 1 b	24 ± 6 abc	500 ± 12 a	68 ± 18 b
PG+fert	5 ± 1 b	15 ± 3 bc	ND	33 ± 5 b
PG+Bc1	12 ± 2 ab	34 ± 5 ab	457 ± 10 ab	313 ± 32 ac
PG+RM2	17 ± 3 a	38 ± 4 a	437 ± 10 b	371 ± 36 a
PG+Bc1+RM2	19 ± 3 a	35 ± 5 ab	391 ± 10 c	353 ± 55 ac
PG+RM1	9 ± 4 ab	26 ± 17 abc	497 ± 33 ab	138 ± 112 bc
PG+AC	5 ± 2 ab	7 ± 5 c	529 ± 18 ab	44 ± 7 b

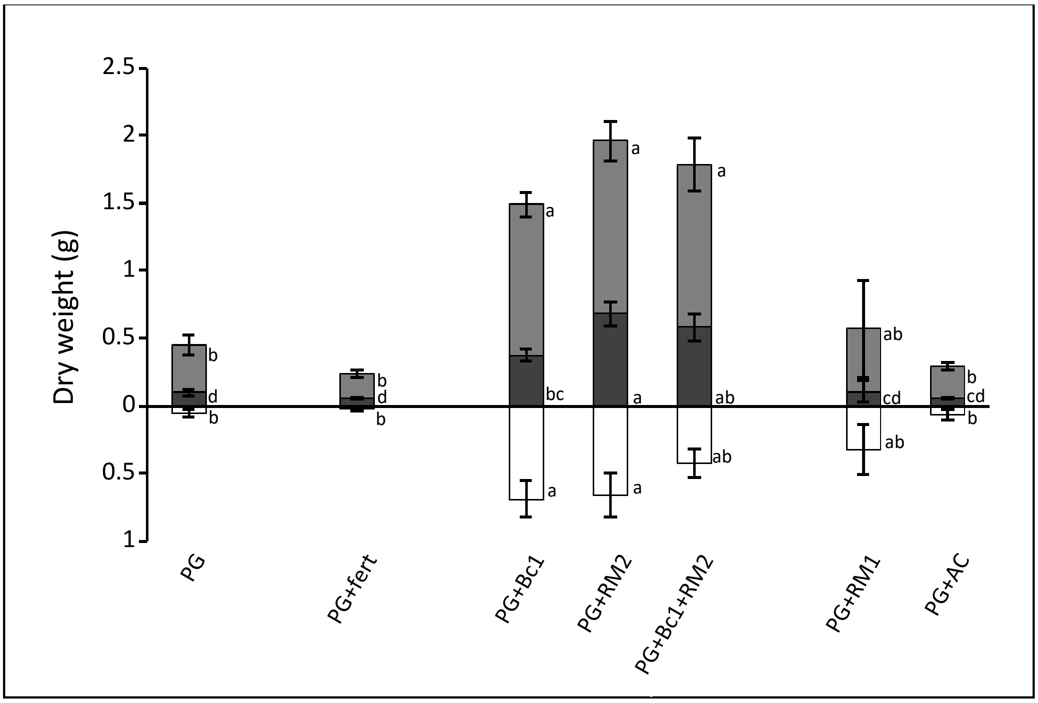

Figure 2: Leaf (light grey), stem (dark grey) and root (white) dry weight (g) of *Salix viminalis* plants grown on Pontgibaud (PG) fertilized (fert) or amended with hardwood biochar (Bc1), coconut activated carbon (AC) and/or two commercial redmuds (RM1 and RM2). Letters indicate difference between the treatments assessed by one-way Anova and post-hoc Tukey tests (n = 2-7 ± SE) ($p < 0.05$).

3.9 As and Pb concentrations in plants

Both elements were mainly accumulated in or on the roots (Figure 3). Concentrations in As in the aerial parts (leaf and stem) were increased with fertilization and RM1 amendment, but those rises were not significant (Figure 3a). Amendment application to Pontgibaud did not significantly affect As concentrations in *Salix* aerial parts. Similarly, root As concentrations were not affected, expect for the increase observed following fertilization. Concentrations in Pb in the leaves were slightly increased with Bc1 and RM2 applied alone, while concentrations in the stems rose with Bc1, RM2 and RM1 (Figure 3b). Finally, root Pb concentrations were decreased in all conditions compared to PG, except with the addition of RM1 and AC.

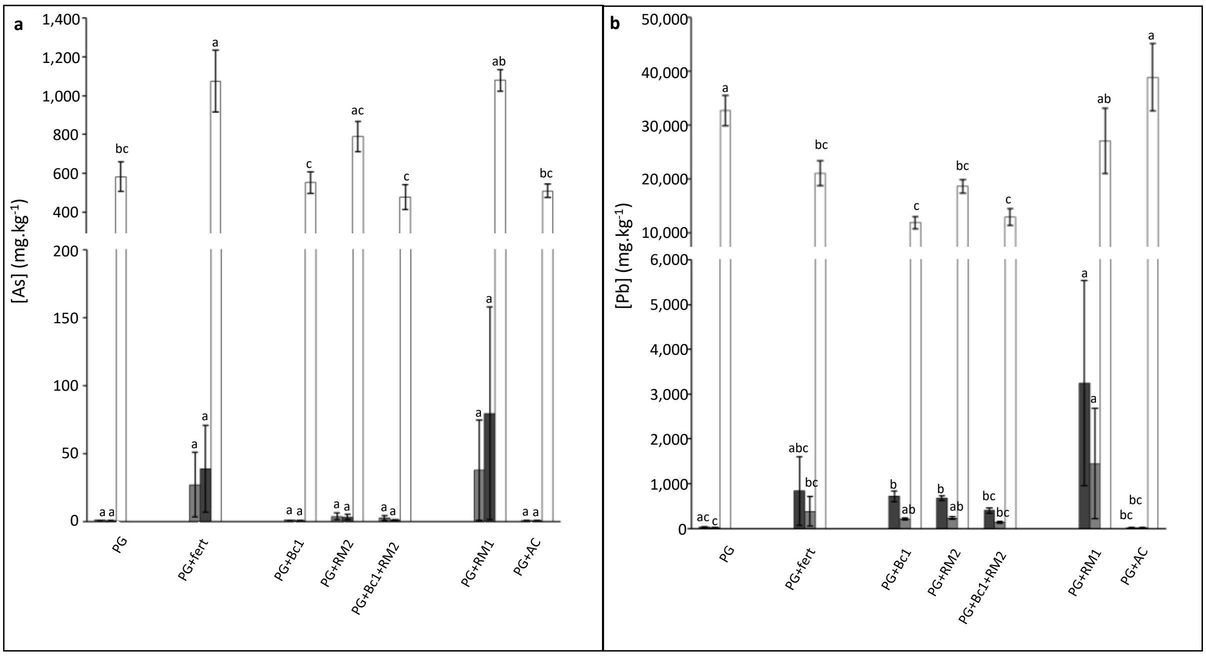

Figure 3: Leaf (light grey), stem (dark grey) and root (white) arsenic (a) and lead (b) concentrations (mg.kg^{-1}) of *Salix viminalis* plants grown on Pontgibaud (PG) fertilized (fert) or amended with hardwood biochar (Bc1), coconut activated carbon (AC) and/or two commercial redmuds (RM1 and RM2). Letters indicate difference between the treatments assessed by one-way Anova and post-hoc Tukey tests (n = 2-7 ± SE) ($p < 0.05$).

4. Discussion

4.1 Soil properties

Soil available phosphorus concentration required for plants is set between 10 and 20 mg.kg^{-1} (Rodríguez-Vila *et al.* 2017). The studied soil presented content under this range, showing its low fertility. Such a low fertility is generally observed in contaminated mining soils (Salam *et al.* 2019).

4.2 Amendment characteristics

Carbon based materials have been found to have a neutral to alkaline pH, dependent on feedstock. For instance, Fidel *et al.* (2017) measured pH between 6.4 and 10.7 in different biochars. Sabir *et al.* (2013) determined the pH of an activated carbon to be 10.6. Such alkalinity has been related to the inorganic minerals and ash formed during pyrolysis and carbonization (Chen *et al.* 2019). Many studies measured an alkaline pH when analyzing redmud (Friesl *et al.* 2003, Garau *et al.* 2007). Redmud alkalinity can be explained by the origin of this material. Redmud comes from the alkali leaching of the alumina bauxite residue (Zhou *et al.* 2017). Such alkaline pH of the amendments indicates a potential liming effect upon their incorporation to soil, which could reduce PG soil acidity (Kloss *et al.* 2012).

The CEC is the total capacity of a material to hold exchangeable cations (Suliman *et al.* 2016, Dai *et al.* 2017). Cation exchange capacity values were in accordance with previous studies (Garau *et al.* 2007, Kloss *et al.* 2012). The higher value observed for AC showed its higher potential for retention of cations. Biochar generally have low metal(loid) concentrations. However, Cantrell *et al.* (2012) observed an elevated Fe concentration in biochar, similar to the founding for the hardwood biochar. Redmud commonly presents Al as it comes from aluminum mine. In addition, As and Cr are common components of redmud (Hua *et al.* 2017). Argyraki *et al.* (2017) showed that redmud was rich in Fe, sequestered in goethite and hematite. Finally, metal(loid) concentrations in all biochars were below the limits stated for organic amendments of 18 $mg.kg^{-1}$ As, 3 $mg.kg^{-1}$ Cd, 300 $mg.kg^{-1}$ Cr, 120 $mg.kg^{-1}$ Cu, 60 $mg.kg^{-1}$ Ni, 180 $mg.kg^{-1}$ Pb and 600 $mg.kg^{-1}$ Zn (Oustriere *et al.* 2017). On the contrary, redmuds presented values above those limits for As (RM3), Cd (all redmuds), Cr (RM1, RM2), Ni and Zn (RM3), which can be an issue for their use as amendment.

4.3 Sorption test

Both carbon-based and redmud materials are known for their sorption capacity towards Pb (Liu *et al.* 2011, Kołodyńska *et al.* 2017).

Activated carbon had a higher sorption capacity than the two biochars. Steam activation can increase the surface area, which is an important parameter in metal(loid) sorption (Kwak *et al.* 2019). The higher CEC of the activated carbon compared to the two biochars could also explain its higher sorption capacity. Amendment AC was also characterized by a higher CEC compared to RM1 and RM2 but did not show higher sorption capacity. It could mean that other parameters than CEC can affect sorption capacity, such as surface area and surface functional groups (Lebrun *et al.* 2018b).

K_d values showed that on these materials, Pb sorption was not linear (Wang *et al.* 2015).

4.4 $CaCl_2$ extraction test

Calcium chloride extraction provides information about the available fraction of metal(loid)s in the soil (Meers *et al.* 2007a). Both biochar and redmud have immobilizing properties towards metal(loid)s (Yin *et al.* 2016, Zhou *et al.* 2017). However, each one will have different immobilization efficiencies.

The decrease in extractable Pb observed with the amendments can be related to the pH of the solution, as stated by the correlation coefficient ($R^2 = -0.77$, $p < 0.001$). At pH > 6, Pb can form hydroxide precipitates (Oustriere *et al.* 2017). The amendment RM3 increased the content in extractable Zn and Cd, which could be explained by the high level of these two elements in RM3. When RM3 was combined with a carbon-based material, the immobilization of Zn by this material could have explained the lower increase observed in this case (Dai *et al.* 2018).

4.5 SPW physico-chemical properties

Previous studies showed the acidic nature of the mine soil from Pontgibaud (Lebrun *et al.* 2018a, 2019). Tang *et al.* (2015) also observed a soil acidification following superphosphate fertilizer application, consistent with the results of this study with the fertilization treatment.

Both amendment types, biochar and redmud, due to their alkalinity, are known to increase soil pH, which is an important parameter in metal(loid) immobilization. Pavel *et al.* (2014) applied redmud at 1 % and observed a rise of pH by 2 units, which was also found in this study. Garau *et al.* (2014) found that redmud application increased soil pH due to its alkalinity and high Na content. Biochar is formed through pyrolysis process, which creates alkaline oxides and carbonates, responsible for the alkalinity of biochar. Those oxides and carbonates are released into the soil after biochar application, then react with H^+ and thus increase pH (Abewa *et al.* 2014). Here, pH increase was similar between biochar and redmud but redmud was added at a lower rate. Therefore, redmud was more effective in increasing pH. Similar results were observed by Friesl *et al.* (2003).

Arsenic concentrations in soil pore water increased with fertilization. This is consistent with the studies of Fitz and Wenzel (2002) and Moreno-Jimenez *et al.* (2016). Such an increase in arsenic mobility was surprising as the pH of the SPW decreased and was acidic. This is usually a condition in which As is immobilized (Beesley *et al.* 2010). However, the fertilizer composition can explain such an observation. The fertilizer was composed of phosphorus, a chemical analog of As that competes for the same sorption sites. Phosphorus addition to the soil displaces As from its sorption sites on soil, releasing As to the soil solution (Fitz and Wenzel 2002, Cao *et al.* 2003).

On the contrary, Pb concentrations in soil pore water decreased with amendment addition. Redmud was shown to be efficient to immobilize metallic elements in several studies (Gray *et al.* 2006, Liang *et al.* 2012). Such positive effect can be attributed to the high content of Fe and Al oxides, which can complex metals (Gray *et al.* 2006, Liang *et al.* 2012). Similarly, biochar can interact with metal(loid)s through electrostatic interaction, metal-ligand complex and sorption by the interaction with aromatic π electronic system (Oustriere *et al.* 2017). Moreover, the pH increase induced by the application of redmud and biochar can have contributed to the observed Pb immobilization. With the increase in soil pH, the negative charges of the soil increase which favors the sorption of positively charged metal(loid) onto soil matrix and amendment surfaces (Gray *et al.* 2006, Dai *et al.* 2018).

4.6 Soil extractions at mid term

Extractable Pb was decreased with the amendments, which was consistent with the study of Sabir *et al.* (2013). In general, biochar amendment was shown to decrease $CaCl_2$ extractable Pb concentration (Lebrun *et al.* 2019, Nandillon *et al.* 2019). Immobilization of Pb can be attributed to the pH increase induced by biochar application (Li *et al.* 2016), as observed in the SPW data and with the pH of the solution after extraction. However, AC did not affect $CaCl_2$ extracted Pb, even though its application increased SPW pH and extraction pH. The decrease in $CaCl_2$ extractable Pb concentration can also be explained by the formation of Pb-carbonate species and the interaction between Pb and biochar functional groups, which are less abundant in activated carbon (Dai *et al.* 2018). The increase in soil pH can also explain the decrease in Pb bioavailability induced by redmud amendment. Garau *et al.* (2007) found that redmud decreased Pb bioavailability due to the sorption of Pb on organic matter and Fe and Al oxides present at the redmud surface, which is favored by the increase in soil pH.

Aluminum toxicity is one of the key factors limiting root growth in acid soil. However it can be sorbed on biochar surface and can precipitate under alkaline conditions (Dai *et al.* 2017), explaining its reduction in bioavailability due to the amendments.

The observed decreases in $CaCl_2$ extracted metals (Cd, Cu, Fe, Mn and Zn) could be related to the sorption capacity of the amendments, which differed depending on the amendment and element types, but could also be explained by the pH rise induced by amendment applications (Lebrun *et al.* 2019).

4.7 Soil extraction at harvest time

Ammonium acetate extractions inform on the exchangeable fractions of the elements and thus on the nutritious status of the different treatments. Concentrations measured for the contaminated soil of PG were below the one observed by Salam *et al.* (2019). These authors stated that the contaminated soil studied did not satisfy nutrient requirements.

The improvement of K, Mg and P concentrations with fertilization can be directly related to the composition of the fertilizer solution. Biochar can improve soil fertility by increasing nutrient availability (Dai *et al.* 2017). But Yuan *et al.* (2018) showed that plant-based biochars are better soil conditioner rather than fertilizer due to their low leachable nutrient contents. This could explain the reduced effect of Bc1 (hardwood based biochar).

The higher concentrations in Ca and Na observed with redmud amendment can be due to its elevated content in those two elements. Indeed, the disposal of the redmud and incomplete washing lead to high content in sodium hydroxide, sodium carbonate and sodium aluminate (Hua *et al.* 2017). Feigl *et al.* (2012) also stated that redmud was mainly composed of Fe_2O_3 and Al_2O_3 but also contained, in smaller amounts, SiO_2, CaO and Na_2O.

4.8 Plant growth

Although the application of a fertilizing solution increased the available concentration of three important nutrients (K, Mg, P), fertilization did not improve plant growth. Therefore, it can be hypothesized that the reduced growth observed here on PG soil, but also in general, is not mainly due to a nutrient deficiency but is more related to (i) its elevated metal(loid) concentrations and (ii) its acidity which mobilizes metal(loid)s, especially Pb in this case. The amelioration of plant growth with Bc1 and RM2 can be explained by an increase in soil pH, which reduced metal(loid) bioavailability and mobility, as shown by the soil pore waters and $CaCl_2$ extractions (Castaldi *et al.* 2009, Rafael *et al.* 2019). Moreover, other ameliorations of the soil conditions could have also contributed, such as increases in CEC, WHC, alteration of the microbial community and activity (Hmid *et al.* 2015, Rafael *et al.* 2019). However, the results found here emphasized the fact that biochar and redmud effects are highly dependent on the biochar/redmud type as only harwood biochar and neutralized redmud had beneficial effects. Previous studies observed positive outcomes with the use of redmud or biochar (Castaldi *et al.* 2009, Sabir *et al.* 2013, Rafael *et al.* 2019). Negative effects were also observed. For instance, Ohsowski *et al.* (2018)

found that *Andropogon gerardi* biomass production was reduced by biochar amendment compared to the non-amended treatment. In the present case, the non-effect of the activated carbon and the non-neutralized redmud on plant growth, even though they reduced Pb mobility, could be explained by their effects on other soil parameters. Indeed, they both increased soil pH but values were still around pH 5.5. At such soil pH, aluminum is known to cause a reduced biomass production (Pescod 1992). Moreover, in both treatments, extractable Al was higher than in the other amended conditions. Therefore, the reduced growth on conditions amended with activated carbon and non-neutralized redmud could be explained by a combination of low pH and Al toxicity compared to the other amended treatments.

4.9 As and Pb concentrations in plants

Previous studies on *Salicaceae* already observed elevated concentrations in metal(loid)s in the roots (Meers *et al.* 2005, Lebrun *et al.* 2019), due to their direct contact with the soil. Such elevated amounts of metal(loid)s could be either absorbed in the roots and/or adsorbed on the root surface.

The higher As concentration in the roots observed with the fertilization can be related to the elevated As concentrations in SPW but also the high concentration of available phosphorus found in this condition, as arsenic and phosphorus are chemical analog (Cao *et al.* 2003). However, such higher root uptake did not induce a more important translocation towards upper parts. It could be due to an immobilization of As into the roots but also on the root surface, as the measure did not differentiate the elements adsorbed from the ones absorbed. On the contrary, the decreased Pb concentration in the roots can be related to the efficient Pb immobilization by biochar and redmud (Hmid *et al.* 2015, Castaldi *et al.* 2019). However, such amendments could have impacted plant physiology, leading to a higher translocation of Pb to the above ground parts, especially in stems.

5. Conclusions

Several carbon-based materials and redmuds were tested for their ability to immobilize metal(loid)s, especially As and Pb, in batch and mesocosm experiments. Batch tests showed that redmuds were more effective than carbon-based materials in decreasing soluble Pb and $CaCl_2$ extractable metal(loid) concentrations. Following, a pot experiment was performed using a former mine technosol, contaminated with As and Pb, fertilized or amended with two carbon-based materials (one biochar and one activated carbon) and two redmuds, alone or combined. Results showed that fertilization had no beneficial effects, due to its acidification and metal(loid) mobilization effects. Moreover, among the different amendments, only the hardwood-based biochar and the neutralized redmud showed positive effects on both soil and plants, reducing its acidity and metal(loid) mobility together with increasing plant growth. The activation of biochar products aims at increasing the benefits biochar have on soil and plants. Activated carbon (AC) showed a more effective Pb sorption in batch tests and a higher pH than biochar. However, AC did not have beneficial effects once incorporated in the soil. No difference was observed between beneficial biochar (Bc1) and redmud (RM2) and their combination did not lead to better results. Redmud was applied to a lower rate and is also cheaper, being a waste product. Thus, compared to biochar, redmud could be more appropriate in order to reduce acidity and metal(loid) mobility and increase plant growth. However, these beneficial effects need to be assessed on a bigger scale to measure the gain in terms of biomass production and vegetation cover and thus evaluate if the cost of applying such material, which requires a neutralization before its application, provides a sufficient benefit for an efficient phytoremediation. Moreover, a longer incubation is required to evaluate the long term effect of applying such industrial waste.

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Supplementary material

Table S1: Sorption coefficient of the six amendments towards Pb, after 24 h in contact of increasing Pb concentrations. Bc1 = hardwood biochar, Bc2 = coconut biochar, AC = activated coconut carbon, RM1 = commercial redmud, RM2 = neutralized commercial redmud, RM3 = redmud from charcoal mine.

Concentration Equilibrium	Bc1	Bc2	AC	RM1	RM2	RM3
1mM	0.367	0.101	4.836	11.393	7.448	2.793
5mM	0.044	0.011	0.087	9.471	11.332	9.018
10mM	0.027	0.003	0.056	0.263	0.470	0.575
15mM	0.029	0.013	0.044	0.156	0.225	0.132
20mM	0.014	0.004	0.030	0.176	0.138	0.391
25mM	0.012	0.006	0.017	0.041	0.046	0.070

Table S2: CaCl$_2$-extractable Pb, Cd and Zn concentrations (mg.kg^{-1}) and solution pH after 0.01 M CaCl$_2$ extraction on Pontgibaud soil (PG) amended with the different amendments (Bc1 = hardwood biochar, Bc2 = coconut char, AC = activated coconut char, RM1 = commercial redmud, RM2 = modified commercial redmud, RM3 = redmud from charcoal mine field) (n=3).

Significant difference from PG is presented by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$). Letters in brackets indicate difference between the treatments being significantly different compared to PG.

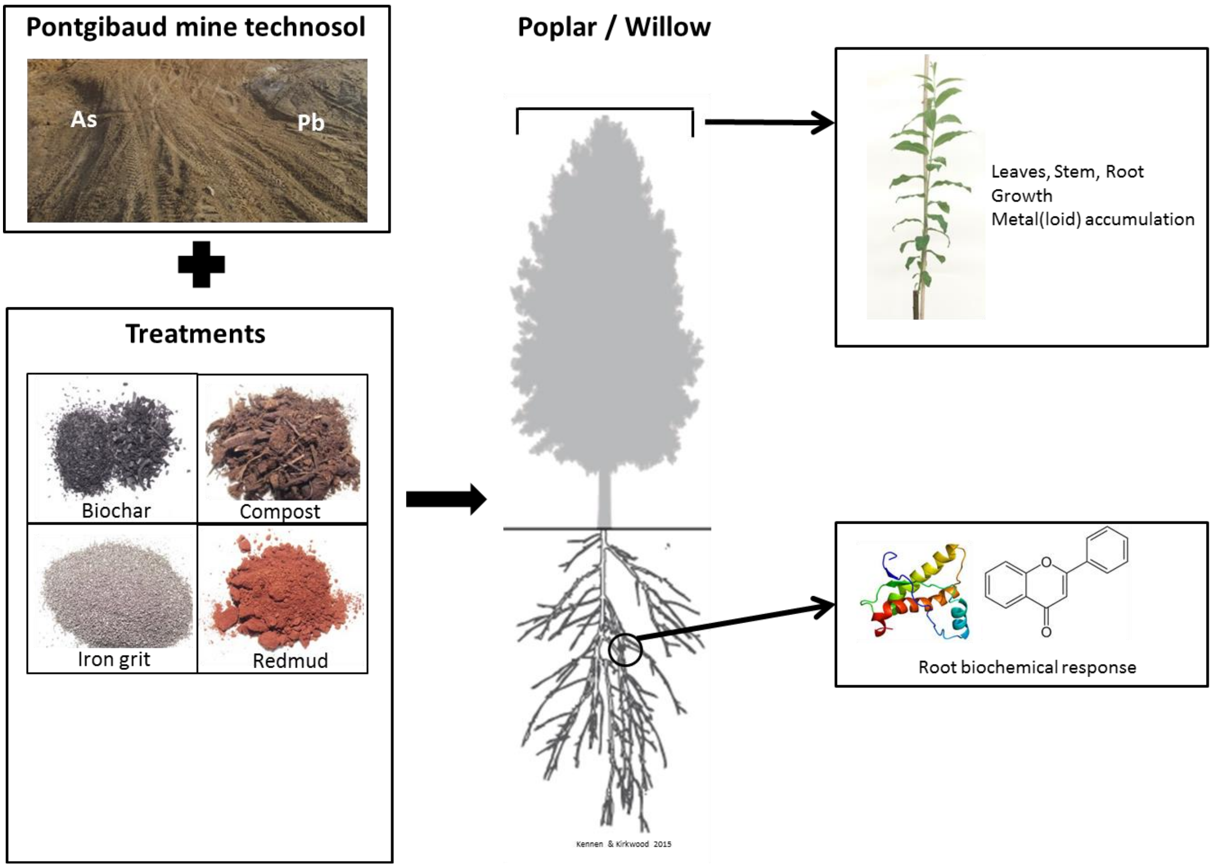
Treatment	pH solution	CaCl$_2$ [Pb] (mg.kg^{-1})	CaCl$_2$ [Cd] (mg.kg^{-1})	CaCl$_2$ [Zn] (mg.kg^{-1})
PG	3.23 ± 0.03	247 ± 2	0.12 ± 0.00	15.5 ± 0.5
PG+Bc1	3.50 ± 0.03	212 ± 6	0.13 ± 0.02	15.2 ± 0.9
PG+Bc1+RM1	3.63 ± 0.04	93 ± 12 *** (cd)	0.13 ± 0.01	12.2 ± 0.9
PG+Bc1+RM2	3.67 ± 0.01	73 ± 2 *** (d)	0.12 ± 0.00	12.7 ± 0.4
PG+Bc1+RM3	3.60 ± 0.07	118 ± 16 *** (ac)	0.17 ± 0.01 *	23.4 ± 1.5 *** (bc)
PG+Bc2	3.39 ± 0.04	266 ± 3	0.16 ± 0.01	17.9 ± 0.1
PG+Bc2+RM1	3.72 ± 0.06	124 ± 9 *** (bc)	0.12 ± 0.00	12.1 ± 0.4
PG+Bc2+RM2	3.70 ± 0.05	80 ± 3 *** (d)	0.14 ± 0.01	13.7 ± 0.3
PG+Bc2+RM3	3.62 ± 0.06	133 ± 3 *** (ac)	0.16 ± 0.00	26.3 ± 0.5 *** (c)
PG+AC	3.54 ± 0.06	236 ± 1	0.13 ± 0.01	16.9 ± 0.2
PG+AC+RM1	3.72 ± 0.07	99 ± 5 *** (cd)	0.08 ± 0.00	9.8 ± 0.7 ** (d)
PG+AC+RM2	3.67 ± 0.07	74 ± 1 *** (d)	0.09 ± 0.01	11.6 ± 0.3
PG+AC+RM3	3.68 ± 0.09	106 ± 5 *** (bcd)	0.10 ± 0.01	21.5 ± 1.0 *** (b)
PG+RM1	3.70 ± 0.05	110 ± 6 *** (bcd)	0.07 ± 0.01	10.6 ± 0.1 *(d)
PG+RM2	3.64 ± 0.05	79 ± 2 *** (bcd)	0.11 ± 0.00	13.3 ± 0.2
PG+RM3	3.57 ± 0.08	137 ± 13 *** (ab)	0.14 ± 0.01	29.3 ± 1.8 *** (a)

This chapter focused on comparing diverse amendments to improve Pontgibaud soil conditions and plant growth and the metal(loid) accumulation capacities of three *Salicaceae* species. The first part showed that biochar amendment improved soil properties and plant growth. Moreover, the fine biochars showed better results than the coarser ones. In the second experiment, lightwood and pinewood biochars also ameliorated soil characteristics and *Salix viminalis* and *Populus euramericana* Dorskamp growth. In general, lightwood biochars were more efficient than pinewood biochars in ameliorating soil conditions. The sorption tests performed in the third part revealed the high sorption efficiency towards Pb of the four hardwood biochars used in the first part, with more than 60 % Pb sorbed, depending on the particle size. On the contrary, As was poorly sorbed (< 4 %). Furthermore, the functionalization of one biochar with Fe increased As sorption capacity (av. 50 %) without affecting Pb sorption. However, when this modified biochar was applied to the soil, it did not show better results than the non-modified biochar. Therefore, it was decided to use iron grit as amendment, combined with biochar and compost, in a fourth experiment. The results of this experiment showed that biochar and/or compost ameliorated soil physico-chemical properties and *Salix viminalis* growth. However, adding iron grit, alone or combined to biochar, had no positive effects and even had for some parameters a negative effect. Additionally, leaching test mimicked results of the soil pore water parameters, but it also revealed that with time, amendments were less efficient, as shown by the slight leachate acidification and increase in Pb leachate after 232 days. Following, another iron-based amendment was studied, redmud. The characterization of the soil in the fifth part showed that the mine soil presented a low fertility whereas the characterization of three biochars and three redmuds showed that all were alkaline with an elevated Pb sorption capacity, redmuds being more efficient compared to biochars. Based on these evaluations, two biochars and two redmuds were applied to the soil and a fertilization treatment was also performed, to test whether the deficiency in plant growth was mainly due to soil fertility shortage. This mesocosm experiment revealed that fertilization had no beneficial effects on plant growth, demonstrating that the no vegetation observed on the site was more due to soil acidity and elevated metal(loid) contamination rather than nutrient deficiency. Additionally, among the other amendment treatments, only one biochar and one redmud had beneficial effects on soil and plants, with no difference between the two. Moreover, their combined application had no additional effects. Finally, as redmud was applied at a lower rate than biochar, the neutralized redmud, although it is considered as an industrial waste, seemed more appropriate.

In conclusion, this chapter showed the beneficial effect of amendments on soil and plants, especially biochar and redmud, for their alkalinity and sorption capacity, and compost, for its nutritious state. Furthermore, all three *Salicaceae* species showed good growth on most of the amended substrates with a preferential As and Pb accumulation in the roots. Finally, the effects depended on plant species, amendments and metal(loid)s. Indeed, in the first part, amending with HW biochar 0.5-1 mm showed no translocation of As and Pb towards *Salix viminalis* upper parts, while HW biochar < 0.1 mm showed a low translocation. In the second part, *Populus* plants performed better growth than *Salix*, however *Salix* plants had a more important root system. Translocation also differed depending on the metal(loid)s and plant species: *Salix* showed the lower As translocation while *Populus* had the lowest Pb translocation. Finally, *Salix dasyclados* had a very poor As and Pb translocations on the non-amended Pontgibaud technosol, while amendments tended to increase translocation, except fertilization and activated carbon.

However, this chapter only focused on short-time mesocosm experiments, but amendment effects need to be evaluated over a longer period of time, as leaching test over 232 days revealed that beneficial amendment effects reduced with time. Furthermore, in this chapter, only the soil physico-chemical parameters as well as plant growth and accumulation were evaluated. But amendments can also affect soil microbial community and the biochemical status of the plant. These two elements will be detailed in the next chapters.

Chapter 3. Amendment effects on plant physiology/biochemistry.



This chapter 3 (objective 1c) will focus on the physiological and biochemical responses of roots of *Salix* plant grown on the Pontgibaud contaminated soil differentially amended. It will be divided in three parts.

The first part is a continuation of the work described in chapter 2, part D (pages 197 to 221). This part related the effect of biochar, compost and iron grit amendments, alone or combined, on the soil physico-chemical properties and *Salix viminalis* growth. The results showed that compost, associated or not with biochar, was a good amendment to improve soil condition and *Salix* growth. In this chapter, the study will focus on the root proteome profiles of the *Salix viminalis* plants grown on six out of seven treatments. Indeed, root growth was too low on the iron alone treatment to allow protein extraction. Treatment analyzed were thus: (i) non-amended Pontgibaud, (ii) Pontgibaud amended with 5 % biochar, (iii) Pontgibaud amended with 5 % compost, (iv) Pontgibaud amended with 5 % biochar and 5 % compost, (v) Pontgibaud amended with 5 % biochar and 1.5 % iron grit, and (vi) Pontgibaud amended with 5 % biochar, 5 % compost and 1.5 % iron grit. This work aimed at evaluating the effect of biochar, compost and/or iron grit amendments on plant growth and metal(loid) accumulation indexes and on root proteome profiles. This work has been accepted for publication in the journal *Chemosphere* (Lebrun *et al*., in press).

The second part is also a continuation of the work described in chapter 2, part D (pages 197 to 221) and will describe the organic acids exudation pattern of *Salix viminalis* plants grown on the seven treatments: (i) non-amended Pontgibaud, (ii) Pontgibaud amended with 5 % biochar, (iii) Pontgibaud amended with 5 % compost, (iv) Pontgibaud amended with 1.5 % iron grit (v) Pontgibaud amended with 5 % biochar and 5 % compost, (vi) Pontgibaud amended with 5 % biochar and 1.5 % iron grit, and (vii) Pontgibaud amended with 5 % biochar, 5 % compost and 1.5 % iron grit. This study evaluated the effect of amendment application on *Salix viminalis* organic acids root exudation. This work has been submitted to the journal *Soil Science and Plant Nutrition* (SSPN-19-275-F).

The third part will describe a new mesocosm experiment, focusing on the oxidative stress markers of the roots of *Salix triandra* plant. In this new experiment, amendment combinations were tested. The aims of this study were to evaluate the effect of combining different biochars to one redmud on the soil physico-chemical properties, *Salix triandra* growth, metal(loid) accumulation in plant organs and oxidative stress level of the roots. This work has been submitted to the *Journal of Plant Physiology* (JPLPH-D-19-00712).

Part A. Effect of biochar, compost and/or iron grit application on a contaminated former mine technosol: changes in *Salix viminalis* growth, root proteome profiles and metal(loid) accumulation indexes.

Amending an As/Pb contaminated soil with biochar, compost and iron grit: effect on *Salix viminalis* growth, root proteome profiles and metal(loid) accumulation indexes

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Keywords

Amendment; arsenic; lead; heavy metals; phytoremediation; phytostabilization.

Highlights

Compost and biochar+compost amendments show better improvements to *Salix* growth

Compost and biochar+compost show best decrease in metal(loid) mobility and toxicity

For compost and biochar+compost, plants elicit proper defense/signaling response

Iron grit is harmful for plant growth (rise in soil acidity and metal(loid) toxicity)

Biochar+iron enhances plant oxidative stress and protein degradation dysfunction

Abstract

There is currently a large amount of research being done into the phytoremediation of polluted soils. Plant installation in contaminated soils may require the application of soil amendments, such as biochar, compost and/or iron grit, which can improve the soil conditions and reduce the metal(loid) phytoavailability and mobility. The beneficial effects of these amendments on soil properties, plant growth, and metal(loid) accumulation ability have already been described, although their effect on the physiological response of plants has been poorly studied. This study aimed to assess the effect of these amendments on plant growth and metal(loid) accumulation, as well as elucidating associated molecular mechanisms. Therefore, a pot experiment was performed on *Salix viminalis* cuttings using a former mine technosol amended with biochar, compost and iron grit. The results showed that the application of amendments improved plant growth by three fold, except for the biochar plus iron combination. It also revealed that metal(loid)s were not effectively translocated from the roots to the shoots (translocation factors <1), and that the bioaccumulation peaked in the roots, and increased in the presence of iron-based amendments. Corresponding proteomic profiles revealed 34 protein spots differentially represented and suggested that plants counteracted metal(loid)-induced oxidative stress after the addition of biochar and/or compost by eliciting proper defense and signaling pathways, and by redirecting the metabolic fluxes towards primary and secondary metabolism. However, they did highlight the occurrence of oxidative stress markers when the biochar plus iron amendment was applied, which could be both the cause and result of protein degradation impairment.

1. Introduction

The reclamation of disturbed areas aims to return sites to their original state (Thavami et *al.* 2017). For polluted soils, this is a particularly important research field as it is a major issue worldwide, and one of the eight main threats to soils (Panagos *et al.* 2013). Phytoremediation uses the ability of plants to accumulate the pollutants from soil as they grow, and thus either remove them from the environment or reduce their harmful effects (Vamerali *et al.* 2010). Plants can also secrete molecules (root exudates) that can alter the behavior of pollutants. Moreover, the plant cover will reduce the risk of wind erosion and contaminated water leaching.

However, the success of the phytoremediation process depends on the establishment of plants, which can be difficult on extremely contaminated and poorly fertile soils. Different amendments can be applied in order to decrease mobility of metal(loid)s or to contribute to the recovery of valuable elements (nitrogen, phosphorus, and other nutrients) important for plant growth. Among diverse soil amendments, the use of biochar and compost have been the subject of increasing attention.

Biochar is a solid product obtained by the pyrolysis of diverse biomasses (Wiszniewska *et al.* 2016), and is characterized by a porous structure, a high cation exchange capacity, an alkaline pH, and a large surface area which can bind metal(loid)s (Beesley *et al.* 2011, Paz-Ferreiro *et al.* 2014). Compost is a stabilized product of the composting process, which is rich in organic matter and nutrients (Diacono and Montemurro 2011). Iron-based amendments, such as iron grit, can also be employed, especially with anionic element pollution, because Fe possesses a high affinity for As. Indeed, iron-based amendments can decrease As mobility and bioavailability through adsorption or surface precipitation mechanisms (Komárek *et al.* 2013).

All three amendments improved soil fertility when applied to contaminated soils, by reducing soil acidity and pollutant bioavailability and mobility, and increasing the soil water holding capacity, organic matter and nutrient contents, as well as the microbial biomass and activity (Hargreaves *et al.* 2008, Oustriere *et al.* 2016, Michàlkovà *et al.* 2017). Such improvements lead to better plant growth (Chen *et al.* 2010, Caporale *et al.* 2013, Yan *et al.* 2013). However, although our previous studies evaluated the effects of adding amendments, the plant's responses at physiological and molecular levels have been poorly studied. In our previous study, we also proved that biochar, compost and iron grit application, alone or in combination, led to considerable variations in soil physico-chemical properties, plant growth and pollutant accumulation abilities, without investigating the molecular mechanisms behind these different responses (Lebrun *et al.* 2019). In detail, results showed that biochar and/or compost improved the soil fertility and the soil pore water characteristics, by reducing acidity, metal(loid) mobility and toxicity. Such improvements allowed better plant growth and higher biomass production, and showed a reduction in some stress indicators. However, biochar+iron and biochar+compost+iron decreased Pb and As, but had a negative effect on plant growth and oxidative stress response, probably due to the application of a high dose of Fe.

Proteomic-based approaches are currently used to evaluate the effect of several different stresses on the plant protein profiles, including metal stress. However, such studies were performed in a hydroponic system using mono-element solutions, such as arsenic (Ahsan *et al.* 2008) or cadmium (Alvarez *et al.* 2009, Lomaglio *et al.* 2015). Studies in real polluted soil conditions are scarce and to the best of our knowledge, there is no study analyzing the soil amendment effect on the plant proteome.

In the present work, the aims of this study were to assess the effect of biochar, compost and/or iron grit addition to a metal(loid) contaminated former mine technosol on *Salix viminalis* performance and tolerance, describing the changes in plant growth, metal(loid) accumulation indexes and the alteration of root proteome profiles.

Results obtained offer new insights into molecular mechanisms regulating the plant growth-stress response to metal(loid) contamination, understanding the complex tolerance and removal potential of *Salix* for modern phytotechnologies.

2. Materials and Methods

2.1 Experiment design

The soil was sampled from a former silver-lead extraction mine, located in Pontgibaud (Auvergne-Rhone-Alpes, France). This mining district was very active, and one of the most important French mines up until the nineteenth century. The area is highly contaminated with lead (11,453.63 ± 0.18 mg kg^{-1}) and arsenic (539.06 ± 0.01 mg kg^{-1}) due to the intense mining activity. The soil physic-chemical properties are given in Lebrun *et al.* (2018ab, 2019). Briefly, the soil was moderately acidic (pH 3.74 ± 0.04); it was characterized by low electrical conductivity (EC 222 ± 12 µS.cm^{-1}) and CHNS contents (C 0.14 ± 0.03%, H 0.22 ± 0.00%, N 0.09 ± 0.02% and S 0.87 ± 0.14%).

Three amendments were applied to this soil: i) a commercial biochar derived from hardwood biomass (La Carbonerie, Crissey), applied at 5% (w/w); ii) a commercial compost derived from manure and vegetated biomass, also applied at 5%; iii) an industrial iron grit, applied at 1.5%. The amendment doses were set up based on some previous studies (Lebrun *et al.* 2018a, Feng *et al.* 2016, Codling and Dao 2007). The biochar and compost characteristics were described in Lebrun *et al.* (2019).

These amendments were applied alone as well as in combination, giving seven treatments: non-amended Pontgibaud (P), Pontgibaud amended with biochar (PB), Pontgibaud amended with compost (PC), Pontgibaud amended with iron grit (PI), Pontgibaud amended with biochar and compost (PBC), Pontgibaud amended with biochar and iron grit (PBI) and Pontgibaud amended with biochar, compost and iron grit (PBCI). In our previous work, the effect of iron grit alone was also tested on P, but this treatment was not considered here, due to the low growth observed, especially regarding the root system. *Salix viminalis* non-rooted cuttings were grown for 69 days. At the end of the growing period, as stated in our previous paper (Lebrun *et al.* 2019), plants were harvested and subjected to different pre-processing procedures. Out of the 14 plants per treatment, three were randomly chosen for proteomic analysis, and the roots of these plants were lyophilized until further analysis. Five plants were used for calculating dry weight and metal(loid) concentrations.

2.2 Plant growth and metal(loid) accumulation

Oven-dried root, leaf and stem samples of each treatment were used to determine plant growth and metal(loid) accumulation/translocation indexes.

For each treatment, total biomass was reported as g of plant dry weight (DW). Relative plant and organ (root, leaf and stem) DW of the diverse amended samples (PB, PC, PBC, PBI and PBCI) in respect to P sample was calculated by using the equation reported below:

$$Relative\ organ/plant\ dry\ weight = \frac{\text{organ/plant dry weight (treatment)}}{\text{organ/plant dry weight (Pontgibaud)}}\ x\ 100$$

To assess the As, Pb and Fe accumulation and translocation capacity of *Salix viminalis*, the translocation (TF) and bioaccumulation (BAF) factors were calculated. The TF, indicating metal(loid)s translocation from root to shoot, was calculated by dividing the metal(loid) concentrations in the shoot by the metal(loid) concentrations in the root; TF > 1 indicates that the translocation of the metal(loid) was effectively made from the root to the shoot (Fayiga and Ma 2006). The BAF was calculated by dividing the metal(loid) concentrations of each organ by the metal(loid) concentrations in soil. Samples showing BAF > 1 and < 1 were further categorized as accumulator and excluder, respectively (Cluis 2004).

Soil and plant metal(loid) content analyses were fully described in Lebrun *et al.* (2019). Metal(loid) concentration was expressed in mg kg^{-1}.

2.3 Statistical analysis

Data was analyzed using R software version 3.1.2 (R Development Core Team 2009). Normality and homoscedasticity of the data were analyzed using the Shapiro test and the Bartlett test respectively. Means were then compared two at a time using the Student test for normal data and Wilcox test for non-parametric data. Difference was considered significant when $p < 0.05$.

2.4 Root protein profiles

2.4.1 Protein extraction and separation

Total proteins were extracted from 0.5 g of fresh lyophilized root tissue using the phenol extraction protocol, as described in De Zio *et al.* (2016). Protein quantity in the extracts was measured following the Bradford method, using bovine serum albumin as standard.

For isoelectric focusing (IEF) analysis, immobilized pH gradient (IPG) strips (17 cm; pH 3–10 non-linear; Bio-Rad) were rehydrated overnight with 300 mL of rehydration buffer [6 M urea, 2 % (w/v) CHAPS, 0.5 % (v/v) Triton X-100, 20 mM dithiothreitol (DTT) and 1 % (w/v) carrier ampholytes pH 3–10] and 700 mg of total proteins. IEF was performed in a PROTEAN IEF Cell (Bio-Rad) with the following program: (1) 250 V for 90 min in linear mode; (2) 500 V for 90 min in linear mode; (3) 1000 V for 180 min in linear mode; and (4) 8000 V in rapid mode until 56 kVh was reached. After IEF, the IPG strips were equilibrated in 10 mL of equilibration buffer [50 mM Tris–HCl, pH 8.8, 6 M urea, 30 % (w/v) glycerol, 2 % (w/v) SDS] supplemented with 1 % (w/v) DTT for 20 min. The IPG strips were then treated with 10 mL of equilibration buffer containing 2.5 % (w/v) iodoacetamide for 20 min. The latter two treatments allowed protein reduction and alkylation, respectively.

Proteins were separated by using 12 % polyacrylamide gel (17 cm x 24 cm x 1 mm) electrophoresis (SDS–PAGE); in detail, analysis was performed in a PROTEAN (Bio-Rad) vertical apparatus containing 25 mM Tris–HCl, pH 8.3, 1.92 M glycine, and 1 % (w/v) SDS as running buffer. A constant voltage of 70 V was applied for 16 h, until the dye front reached the bottom of the gel. Three replicates were run for each sample. Finally, separated proteins were fixed by treating gels with 40 % (v/v) ethanol, 10 % (v/v) acetic acid for 1 h, and then stained with Coomassie Brilliant Blue G-250 (Bio-Rad) in order to visualize the proteins. Gels were scanned using a GS- 800 calibrated densitometer (Bio-Rad); corresponding digital images were recorded and analyzed using PDQuest software (Bio-Rad). Finally, statistical analysis was conducted by applying a Student test ($P < 0.05$). A 2-fold change (< 0.5 and > 2) of normalized spot densities was considered indicative of differential expression between samples.

2.4.2 In-gel spot digestion, mass spectrometry analysis and protein identification

2-DE spots of interest were excised from the gels, triturated, S-alkylated with iodoacetamide, and hydrolyzed with trypsin (Shevchenko *et al.* 2006). Peptide mixtures were desalted/concentrated with μZip-TipC18 devices (Millipore, USA) and analysed using a nanoLC-ESI-Q-Orbitrap-MS/MS platform, consisting of a Q ExactivePlus Orbitrap mass spectrometer with a Nanospray Flex ion source (Thermo Fisher Scientific, USA) connected to an UltiMate 3000 RSLC nano-liquid chromatographer (Dionex, USA). Protein digests were done on a 15 cm length × 75 μm inner diameter column packed with Acclaim PepMap RSLC C18 resin (Thermo Fisher Scientific, USA). Mobile phases were 0.1% v/v formic acid in water (eluent A) and 0.1% v/v formic acid in acetonitrile/water 4/1 v/v (eluent B), running at a total flow rate of 300 nL/min. A linear gradient started 20 min after sample loading; eluent B ramped from 3% to 40% v/v over 40 min, and from 40% to 80% v/v over 5 min. The mass spectrometer operated in data-dependent scan mode, allowing the acquisition of all MS spectra in the positive ionization mode within a scan range of *m/z* 375-1500. Up to 8 of the most intense ions in MS were selected for collision

ion fragmentation (CID). A nominal resolving power at 70,000 full width at half-maximum (FWHM), an automatic gain control (AGC) target of 3 × 10^6 ions, and a maximum ion injection time (IT) of 80 ms were set to generate precursor spectra. MS/MS fragmentation spectra were obtained by applying a normalized collision energy of 28%, a nominal resolving power of 17,500 FWHM, an AGC target of 5 × 10^4 ions, a maximum IT of 110 ms, and an isolation window of *m/z* 1.2. In order to prevent repeated fragmentation of the most abundant ions, a dynamic exclusion of 30 s was applied. Precursor ions which were singly charged or with more than six charges were excluded from the fragmentation.

For protein identification, the MASCOT software (v. 2.2.06, Matrix Science, UK) was used to compare raw data from nanoLC-ESI-Q-Orbitrap-MS/MS analyses to the SwissProtKB sequence database (08/2018 release; 558,362 total entries), selecting *Viridiplantae* as the taxonomy. Database searches were carried out allowing carbamidomethylation of cysteines as fixed protein modification and oxidation of methionines as variable modification, a mass tolerance value of 20 ppm for parent ions and of 0.05 Da for MS/MS fragment ions, trypsin as proteolytic enzyme, and a maximum of 2 missed cleavages. All other parameters were kept as default. At least two sequenced peptides with an individual peptide expectation value of less than 0.001, which corresponds to a confidence level for peptide attribution greater than 99.9%, determined the identification of protein candidates. Decoy databases were used to calculate the false discovery rate, which was less than 1% of all identifications. In all cases, the fragmentation assignment was verified manually for all peptide spectra matches prior assigning the protein candidates.

3. Results

3.1 Plant biomass allocation

Biomass allocation was assessed in terms of total plant dry weight (DW) and as relative plant / organ-specific DW (Figure 1 and Table 1). In detail, taking into account the total plant biomass production, the values were higher in PB, PC, PBC and PBCI compared to P and PBI. No significant different was observed between PB, PC, PBC and PBCI (Figure 1A). Relative plant DW confirmed that biomass improved in amended samples in the order PB> PC> PBC>PBCI compared to P, and remained unchanged in PBI (Figure 1B)

The relative organ (leaf, stem and root) DW calculation showed that, compared to P, the DW of the three organs were higher in all amended conditions except PBI (Table 1).

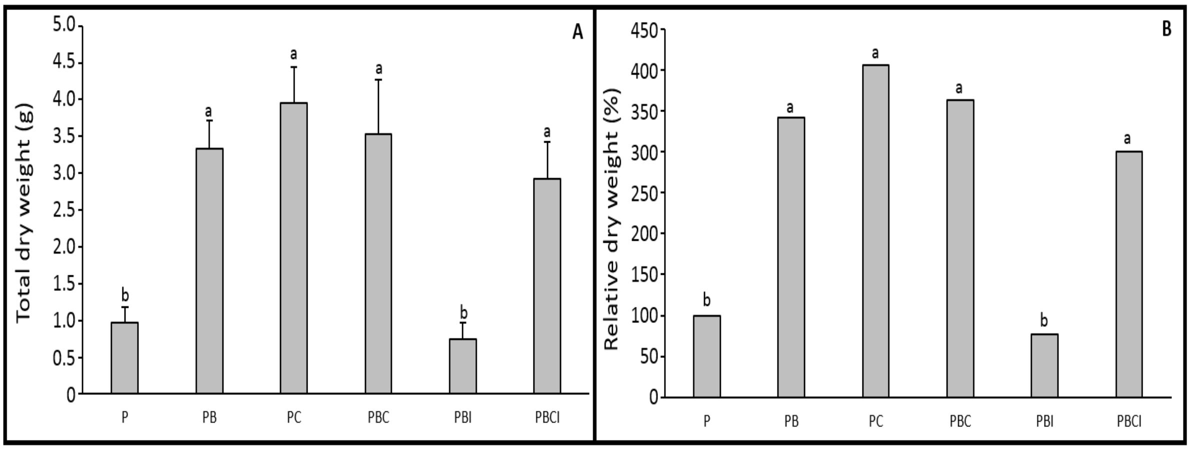

Figure 1: Total and relative plant biomass. A) total plant biomass showing the g of dry weight (DW) and B) relative plant biomass (%) expressing the percentage of DW allocated in *Salix viminalis* plant grown on ameded soils (PB, PC, PBC, PBI and PBCI) compared to DW allocated in unamended soil (P). P = non amended Pontgibaud technosol, PB = Pontgibaud amended with 5% biochar, PC = Pontgibaud amended with 5% compost, PBC = Pontgibaud amended with 5% biochar and 5% compost, PBI = Pontgibaud amended with 5% biochar and 1.5% iron grit, PBCI = Pontgibaud amended with 5% biochar, 5% compost and 1.5% iron grit. Letters indicate significant differences ($p < 0.05$) (n = 5-8).

Table 1: Relative organ-specific biomass production (%). Relative organ (leaves, stem and root) specific biomass production expressing the percentage of organ dry weight (DW) of *Salix viminalis* plants grown on amended soils (PB, PC, PBC, PBI and PBCI) compared to the organ DW of those grown on P. P = non amended Pontgibaud technosol, PB = Pontgibaud amended with 5% biochar, PC = Pontgibaud amended with 5% compost, PBC = Pontgibaud amended with 5% biochar and 5% compost, PBI = Pontgibaud amended with 5% biochar and 1.5% iron grit, PBCI = Pontgibaud amended with 5% biochar, 5% compost and 1.5% iron grit. Letters indicate significant differences ($p < 0.05$) (n = 5-8).

	Leaves	Stems	Roots
P	100 **c**	100 **b**	100 **b**
PB	259 **ab**	463 **a**	282 **a**
PC	326 **a**	658 **a**	446 **a**
PBC	297 **ab**	599 **a**	431 **a**
PBI	81 **cd**	101 **b**	71 **b**
PBCI	248 **b**	454 **a**	445 **a**

3.2 Metal(loid) accumulation

The TFs, calculated for each metal(loid), were < 1 in all plants, with the highest value being for Fe, followed by Pb and As (Table 2). However, in amended plants, compared to P plants, the TF_{As} only increased in PBCI, the TF_{Fe} decreased in PBCI and PB, with the greatest decrease observed in PBCI, while the TF_{Pb} was not affected by the application of amendments.

Calculations for the BAF showed that As, Fe and Pb were mainly accumulated in *Salix* roots (Table 3). In detail, BAF_{As} and BAF_{Fe} values were highest in the roots, followed by the leaves, and lowest in the stems, whereas for BAF_{Pb} the order was roots > stem > leaves. All values were < 1, except in the case of PBI and PBCI roots, where BAF_{Fe} values were about 1.8 and 1.6, respectively. For the leaves and stem, the BAF_{As}, BAF_{Fe} and BAF_{Pb} showed no difference between the amended conditions and P, except for the decrease in BAF_{Fe} for PB and PBCI compared to P in the two organs. Additionally, the leaf and stem BAF_{Fe} presented higher values for PBI than PB, PC and PBCI, and leaf BAF_{As} was higher in PC and PBCI compared to PB. Conversely, for the roots, the highest BAF values were observed in plants grown on iron amended substrates: BAF_{As} was higher in PBI than PB, and BAF_{Fe} values were the highest in PBI and PBCI, while the BCF_{Pb} was higher in PBI compared to all treatments, except PBCI.

Table 2: Translocation factors (TF). As, Fe and Pb translocation factors measured in *Salix viminalis* plants grown on the different soil substrates (P, PB, PC, PBC, PBI and PBCI). P = non amended Pontgibaud technosol, PB = Pontgibaud amended with 5% biochar, PC = Pontgibaud amended with 5% compost, PBC = Pontgibaud amended with 5% biochar and 5% compost, PBI = Pontgibaud amended with 5% biochar and 1.5% iron grit, PBCI = Pontgibaud amended with 5% biochar, 5% compost and 1.5% iron grit. Letters indicate significant differences ($p < 0.05$) ($n = 5$).

	As	Fe	Pb
P	0.0009 ± 0.0005 ^{bc}	0.0169 ± 0.0029 ^{a}	0.0056 ± 0.0020 ^{a}
PB	0.0000 ± 0.0000 ^{c}	0.0091 ± 0.0013 ^{b}	0.0042 ± 0.0010 ^{a}
PC	0.0017 ± 0.0010 ^{ab}	0.0105 ± 0.0026 ^{ab}	0.0075 ± 0.0031 ^{a}
PBC	0.0022 ± 0.0024 ^{ac}	0.0201 ± 0.0084 ^{ab}	0.0121 ± 0.0058 ^{a}
PBI	0.0056 ± 0.0039 ^{ac}	0.0115 ± 0.0027 ^{ab}	0.0106 ± 0.0046 ^{a}
PBCI	0.0039 ± 0.0012 ^{a}	0.0045 ± 0.0008 ^{c}	0.0051 ± 0.0009 ^{a}

Table 3: Bioaccumulation factor (BAF). As, Fe and Pb bioaccumulation factors measured in leaves, stem, roots of *Salix viminalis* plants grown on the different soil substrates (P, PB, PC, PBC, PBI and PBCI). P = non amended Pontgibaud technosol, PB = Pontgibaud amended with 5% biochar, PC = Pontgibaud amended with 5% compost, PBC = Pontgibaud amended with 5% biochar and 5% compost, PBI = Pontgibaud amended with 5% biochar and 1.5% iron grit, PBCI = Pontgibaud amended with 5% biochar, 5% compost and 1.5% iron grit. Letters indicate significant differences ($p < 0.05$) ($n = 5$).

	Leaves	**Stem**	**Roots**
Arsenic			
P	0.0003 ± 0.0002 **ab**	0.0011 ± 0.0011 **a**	0.7579 ± 0.1062 **ab**
PB	0.0000 ± 0.0000 **a**	0.0000 ± 0.0000 **a**	0.6455 ± 0.0551 **a**
PC	0.0020 ± 0.0011 **b**	0.0003 ± 0.0002 **a**	0.8170 ± 0.0878 **ab**
PBC	0.0017 ± 0.0019 **ab**	0.0000 ± 0.0000 **a**	0.7475 ± 0.1073 **ab**
PBI	0.0052 ± 0.0053 **ab**	0.0071 ± 0.0036 **a**	0.9945 ± 0.0900 **b**
PBCI	0.0036 ± 0.0018 **b**	0.0021 ± 0.0014 **a**	0.7937 ± 0.0557 **ab**
Iron			
P	0.0139 ± 0.0020 **ac**	0.0052 ± 0.0023 **abc**	0.7697 ± 0.1441 **a**
PB	0.0061 ± 0.0007 **b**	0.0027 ± 0.0018 **ac**	0.5502 ± 0.0394 **a**
PC	0.0094 ± 0.0023 **ab**	0.0029 ± 0.0018 **ac**	0.6992 ± 0.0765 **a**
PBC	0.0144 ± 0.0029 **acd**	0.0106 ± 0.0064 **ab**	0.7894 ± 0.1849 **a**
PBI	0.0184 ± 0.0031 **c**	0.0188 ± 0.0077 **b**	1.7979 ± 0.3209 **b**
PBCI	0.0090 ± 0.0018 **bd**	0.0021 ± 0.0006 **c**	1.5554 ± 0.1774 **b**
Lead			
P	0.0024 ± 0.0005 **ab**	0.0033 ± 0.0015 **a**	0.5493 ± 0.0666 **ad**
PB	0.0012 ± 0.0006 **a**	0.0030 ± 0.0005 **a**	0.4387 ± 0.0154 **ab**
PC	0.0025 ± 0.0013 **ab**	0.0025 ± 0.0011 **a**	0.3603 ± 0.0440 **b**
PBC	0.0039 ± 0.0015 **ab**	0.0050 ± 0.0016 **a**	0.4222 ± 0.0638 **ab**
PBI	0.0081 ± 0.0051 **b**	0.0136 ± 0.0064 **a**	0.8293 ± 0.0976 **c**
PBCI	0.0034 ± 0.0010 **ab**	0.0030 ± 0.0008 **a**	0.6430 ± 0.0565 **cd**

3.3 Proteomic profiles

Root proteome maps for *Salix viminalis* plants grown on P, PB, PC, PBC, PBI and PBCI contained an average of 338 well-resolved spots, varying in Mr between around 15 and 140 kDa (Figure A.1). These maps were highly reproducible, most spots detected in 2-DE gels showed analogous positions and intensities. Computer-assisted comparison of 2-DE maps revealed 34 statistically ($p < 0.05$) and quantitatively (2-fold change) differentially represented protein spots (Figure 2). These spots were subjected to trypsinolysis and nanoLC-ESI-Q-Orbitrap-MS/MS analysis, which allowed the identification of a total of 33 protein spots. Only one spot, the number 11, was not identified. The list of all protein spots, together with their information and representation profiles are shown in Table 4. Based on Bevan's classification (Bevan *et al.* 1998), the identified protein spots were grouped into seven different functional classes: energy was the most represented group (21.21 %), followed by protein destination and storage (18.18 %), secondary metabolism (15.15 %), disease/defense (12.13 %), intracellular traffic (12.12 %), metabolism (9.09 %), signaling (9.09 %) and protein synthesis (3.03 %).

Figure 2: Master gel. Map shows the 34 proteins differentially expressed between all samples (P, PB, PC, PBC, PBI and PBCI). Arrow indicated the position of each proteins spots and relative spot ID reported in Table 4.

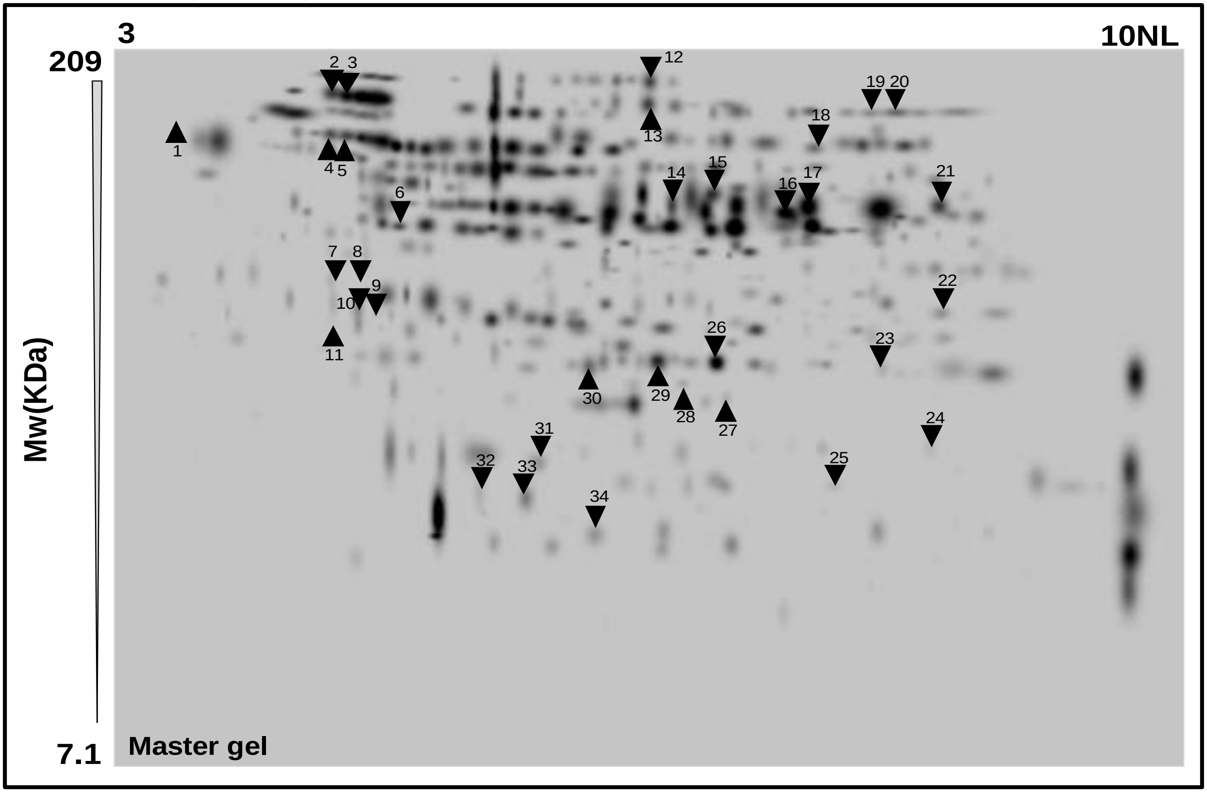

Table 4: Differentially expressed protein spots between *Salix* root grown on different substrates. The list includes: functional classification, protein description, spot number on the reference gel (see Figure 2), Uniprot code, theoretical protein Mr and pI values, experimental protein Mr values, sequence coverage (%), Mascot Score, Organism and protein representation levels. Level of expression: high (red), low (green), very low (grey), absent (yellow). Letters indicate significant difference (p<0.05; n = 3). P = non-amended Pontgibaud, PB = Pontgibaud amended with biochar, PC = Pontgibaud amended with compost, PBC = Pontgibaud amended with biochar and compost, PBI = Pontgibaud amended with biochar and iron, PBCI = Pontgibaud amended with biochar, compost and iron.

Functional classification	Description	Spot ID	UniProt Code	pI / Mr (kDa) th	Mr (kDa) obs	Peptides	Unique peptides	Seq coverage (%)	Mascot Score	Organism	P	PB	PC	PBC	PBI	PBCI
Metabolism	Fructokinase	6	SCRK_SOLTU	5.47 / 33.972	39.28	27	7	25.7	555	*Solanum tuberosum*	a	a	a	a	b	a
	Glycine dehydrogenase	12	GCSP_PEA	7.17 / 115.411	114.67	15	9	11.6	555	*Pisum sativum*	a	ab	b	ab	c	b
	Serine hydroxymethyltransferase 4	18	GLYC4_ARATH	6.8 / 52.141	57.01	29	9	21.9	575	*Arabidopsis thaliana*	a	a	a	a	b	a
Energy	ATP synthase subunit beta	1	ATPBM_HEVBR	5.95 / 60.335	67.96	11	6	14.4	415	*Hevea brasiliensis*	a	a	b	bc	a	c
	Fructose-bisphosphate aldolase	17	ALF_MAIZE	7.52 / 39.036	42.54	31	13	20	922	*Zea mays*	a	ac	b	bc	d	bc
	Fructose-bisphosphate aldolase	21	ALF_MAIZE	7.52 / 39.036	42.64	33	12	20	821	*Zea mays*	a	ab	b	b	c	b
	Glyceraldehyde-3-phosphate dehydrogenase 2	14	G3PC2_ORYSJ	6.34 / 36.921	42.76	18	12	32.3	826	*Oryza sativa*	a	b	b	b	b	b
	Glyceraldehyde-3-phosphate dehydrogenase 2	16	G3PC2_ORYSJ	6.34 / 36.921	41.57	62	14	37.4	964	*Oryza sativa*	ab	ab	ab	a	b	a
	Glyceraldehyde-3-phosphate dehydrogenase 2	29	G3PC2_ORYSJ	6.34 / 36.921	28.27	27	11	32.3	756	*Oryza sativa*	a	a	a	a	b	b
	RuBISCO	27	RBL_ABIFI	6.57 / 49.258	26.38	2	2	4.7	97	*Abies firma*	a	a	a	b	b	b
Secondary metabolism	Caffeoyl-CoA O-methyltransferase	8	CAMT_POPTM	5.3 / 28.020	32.68	12	6	33.2	338	*Populus tremuloides*	a	ab	ab	b	c	c
	Caffeoyl-CoA O-methyltransferase	9	CAMT_POPTM	5.3 / 28.020	30.92	5	4	30	218	*Populus tremuloides*	a	b	ab	b	ab	ab
	Caffeoyl-CoA O-methyltransferase	10	CAMT_POPTM	5.3 / 28.020	31.2	3	3	20.2	165	*Populus tremuloides*	a	ab	b	b	b	b
	Chalcone synthase	15	CHS1_SOYBN	6.21 / 42.888	44.73	14	7	21.9	481	*Glycine max*	a	a	a	a	b	b
	Phenylalanine ammonia-lyase	13	PALY_POPTR	5.8 / 78.497	75.43	28	14	25.6	1037	*Populus trichocarpa*	a	a	ab	ab	b	b
Disease/Defense	Heat shock 70 kDa protein 3	2	HSP7C_ARATH	4.97 / 71.559	84.79	85	25	35.7	1741	*Arabidopsis thaliana*	ab	ab	ab	b	ab	a
	Heat shock 70 kDa protein 3	3	HSP7C_ARATH	4.97 / 71.559	80	91	24	35.6	1677	*Arabidopsis thaliana*	a	b	b	ab	b	b
	NAD(P)H dehydrogenase	30	FQR1_ARATH	5.96 / 21.782	28	37	3	19.1	210	*Arabidopsis thaliana*	a	ab	a	a	b	c
	Superoxide dismutase	33	SODC_PAUKA	5.6 / 15.316	21.9	6	3	21.7	185	*Paulownia kawakamii*	a	bc	b	bc	ac	bc
Protein synthesis	Eukaryotic translation initiation factor	31	IF5A2_MEDSA	5.41 / 17.502	23.42	26	8	45.3	598	*Medicago sativa*	a	b	a	ab	c	a
Protein destination and storage	26S proteasome regulatory subunit	4	PS6AB_ARATH	3.54 / 47.236	55.79	20	13	33.8	754	*Arabidopsis thaliana*	a	ab	b	ab	a	b
	26S proteasome regulatory subunit	5	PS6AB_ARATH	3.54 / 47.236	54.56	88	24	51.3	1736	*Arabidopsis thaliana*	a	b	b	b	a	b
	Peptidyl-prolyl cis-trans isomerase	24	CYPH_CATRO	8.36 / 18.501	24.06	7	2	15.1	154	*Catharanthus roseus*	ab	abc	a	ab	c	b
	Peptidyl-prolyl cis-trans isomerase	32	FK152_ARATH	5.27 / 17.761	22.18	4	4	22.1	225	*Arabidopsis thaliana*	a	bc	b	ab	ac	b
	Peptidyl-prolyl cis-trans isomerase	34	FK152_ARATH	5.27 / 17.761	20.46	3	3	22.1	170	*Arabidopsis thaliana*	ab	ab	a	ab	b	c
	Ubiquitin-conjugating enzyme E2 variant 1D	25	UEV1D_ARATH	6.2 / 16.693	22.43	4	2	20.5	155	*Arabidopsis thaliana*	a	b	b	b	c	d
Signaling	14-3-3-like protein	7	1433_LILLO	4.79 / 29.349	32.94	8	6	22.8	447	*Lilium longiflorum*	a	ab	b	ab	c	c
	GTP-binding protein	23	SAR1B_ARATH	6.52 / 22.029	27.92	19	9	48.7	606	*Arabidopsis thaliana*	a	a	a	a	b	a
	GTP-binding protein	28	SAR1B_ARATH	6.52 / 22.029	27.12	5	3	22.3	220	*Arabidopsis thaliana*	abc	ab	ab	a	bc	c
Intracellular traffic	Dynamin-related protein 5A	19	SDL5A_SOYBN	8.04 / 68.628	71.36	3	2	4.6	109	*Glycine max*	a	b	b	b	c	c
	Dynamin-related protein 5A	20	SDL5A_SOYBN	8.04 / 68.628	71.62	5	4	7.9	230	*Glycine max*	a	ab	bc	c	d	d
	V-type proton ATPase subunit E	22	VATE_CITLI	7.14 / 26.498	30.91	18	11	27.4	561	*Citrus limon*	ab	a	a	a	b	b
	Ras-related protein	26	RAB1B_ARATH	6.52 / 23.389	28.21	3	2	11.4	132	*Arabidopsis thaliana*	a	a	a	a	b	c

In the “Energy” functional class, we grouped seven protein spots: an ATP-synthase subunit β (ATPsyn; spot 1), two fructose-bisphosphate aldolases (FBA; spots 17 and 21), three glyceraldehyde-3-phosphate dehydrogenases 2 (GAPDH; spots 14, 16 and 29) and a RuBiSCO large subunit (rbLc; spot 27) (Table 4). In detail, the ATP-synthase subunit β (spot 1) was completely absent in PB and PBI, as well as in P, and over-represented in the other three treatments (PC, PBC and PCBI), with a higher expression in PC compared to PBCI. The two FBAs (spots 17 and 21) were under-represented in PC, PBC, PBI and PBCI, with the minimum value being in PBI. One GADPH isoform (spot 14) was under-represented in all amended conditions, while the other two isoforms only in PBI and PBCI (spot 29), and in PBI (spot 16). Finally, the rbLc (spot 27) was down-represented in three treatments at similar levels: PBC, PBI and PBCI.

In the “Protein destination and storage” functional class, we grouped six protein spots: two 26S proteasome regulatory subunits (spots 4 and 5), three peptidyl-prolyl cis-trans isomerases (PPI; spots 24, 32 and 34), and one ubiquitin-conjugating enzyme E2 variant 1D (spot 25) (Table 4). Among the two 26S proteasome isoforms, the spot 4 decreased equally in PC and PBCI, whereas the spot 5 decreased equally in PB, PC, PBC and PBCI. Among the PPI isoforms, one isoform (spot 24) was under-represented in all treatments, except in PBI, the isoform 32 increased in PB, PC and PBCI, while the isoform 34 was strongly down-represented only in PBCI. Finally, the ubiquitin-conjugating enzyme E2 variant 1D (spot 25) showed a lower representation in PB, PC and PBC, and even less represented in PBCI, while was completely absent in PBI.

In the functional class “Secondary metabolism” five spots were grouped: three caffeoyl-CoA-O-methyltransferases (CCoAOT; spots 8, 9 and 10), a chalcone synthase (CHS; spot 15) and a phenylalanine ammonia-lyase (PAL; spot 13) (Table 4). In detail, one CCoAMT isoform (spot 10) was over-represented in all treatments except in PB, the isoform 9 in PB and PBC, while the isoform 8 was over-represented in PBC and completely absent in PBI and PBCI. The CHS (spot 15) and PAL (spot 13) were completely absent in PBI and PBCI.

We grouped four protein spots in the “Disease/defense” functional class: two heat shock 70 kDa protein 3 (HSP; spots 2 and 3), one superoxide dismutase (SD; spot 33) and one NAD(P)H dehydrogenase (spot 30) (Table 4). One HSP isoform (spot 2) did not show any difference in expression in amended conditions compared to P, in which it was highly represented. Its expression level was lower only in PBC compared to PBCI. The other isoform (spot 3) showed low representation in all treatments compared to P, except in PBC. The SD (spot 33) had low representation in P and PBI but was over-represented in the other conditions. The NAD(P)H dehydrogenase (spot 30) was under-represented in PBI and PBCI, reaching the minimum in PBCI.

We grouped four protein spots in the “Intracellular traffic” functional class: a Ras-related protein (spot 26), a V-type proton ATPase subunit E (spot 22), and two dynamin-related proteins 5A (DRPs; spots 19 and 20) (Table 4). The Ras-related protein (spot 26) decreased in PBI and PBCI compared to the other treatments, with a lower expression in PBI compared to PBCI. The V-type proton ATPase subunit E (spot 22) was under-represented in PBI and PBCI compared to PB, PC and PBC, although no difference in expression was observed compared to P. The two DRPs 5A (spots 19 and 20) were completely absent in PBI and PBCI. Furthermore, compared to P, spot 19 was under-represented in the other three treatments (PB, PC and PBC) whereas spot 20 was under-represented only in PC and PBC, remaining unchanged in PB.

Three protein spots were grouped in the "Metabolism" functional class: a fructokinase (FRK2; spot 6), a glycine dehydrogenase (GDH; spot 12) and a serine hydroxymethyltransferease 4 (SHMT; spot 18) (Table 3). In detail, the FRK2 (spot 6) was unchanged in all treatments, except in PBI, where it was absent. The GDH (spot 12) was reduced in PC and PBCI, and absent in PBI; the SHMT (spot 18) was under-represented only in PBI.

In the functional class "Signaling", we grouped two GTP-binding proteins (spots 23 and 28) and a 14-3-3 like protein (spot 7) (Table 4). The two GTP-binding protein isoforms showed opposite behaviors: spot 28 was abundant in all treatments and showed no difference with P, however, it was under-represented in PBCI compared to PB, PC and PBC, and also in PBI compared to PBC; the spot 23 was under-represented in all conditions except in PBI, in which its expression level increased. The 14-3-3 like protein (spot 7) was under-represented in PC and completely absent in PBI and PBCI.

In the functional class of "Protein synthesis", we found only one protein spot, the eukaryotic translation initiation factor (spot 31), down-represented in PB and absent in PBI (Table 3).

4. Discussion

In a previous work (Lebrun *et al.* 2019), compost, biochar and iron grit amendments were tested, alone and in combination, on an As and Pb contaminated soil, in order to verify their effects on metal(loid) mobility/availability and *Salix viminalis* growth. Results showed that biochar and/or compost improved the soil fertility and the soil pore water characteristics, with reductions in acidity, metal(loid) mobility and toxicity. The SPW_{Pb} content decreased in biochar and/or compost amended conditions (PC -90 %, PB -91 % and PBC -97 %) while SPW_{As} content decreased in biochar alone but increased, 4.7-fold and 18-fold, in compost and/or biochar treatments, respectively. However, biochar and/or compost amendment did not affect leaf and stem As and Pb accumulation and induced a decrease in root (-25 %). Such improvements allowed better plant growth and higher biomass production showing a reduction in plant stress (leaf pigment content and root guaiacol peroxidase activity). Conversely, biochar+iron and biochar+compost+iron had a negligible effect on pH acidity and decreased Pb (PBI -77 % and PBCI -92 %) while they had no effect on As but, probably due to a high dose of Fe, negative effects on plant growth and stress indicators were observed.

In the present work, we found biochar and compost amendments, alone or in combination, led to an improvement in plant biomass production, as shown by the relative organ and total plant biomass values in PC, PB and PBC. Also, iron addition in combination with compost and biochar (PBCI) seemed to have a positive effect on plant growth and biomass allocation, whereas the biochar+iron (PBI) amendment was harmful for *Salix* growth.

A possible explanation is that biochar and/or compost could enhance available nutrients for plants and, consequently, biomass accumulation, ameliorating soil physic-chemical characteristics. Indeed, Lebrun *et al.* (2019), according to Liu *et al.* (2012) and Akhtar *et al.* (2014), showed that biochar and compost amendments, alone and in combination, improved water holding capacity (WHC), organic matter (OM) content and soil pore water (SPW) pH of Pontgibaud technosol; the combination of iron, biochar and compost (PBCI) raised SPW pH, WHC and OM content while a negligible effect was shown for a biochar and iron combination (PBI).

This is related to biochar alkalinity, porous structure, hydrophobicity and high surface area (Molnár *et al.* 2016, Polzella *et al.* 2019), as well as to its organic fraction content (Janus *et al.* 2015), and the capacity of compost to improve soil structure by directly increasing organic matter and the micro and macro-porosity (Celik *et al.* 2004; Trupiano *et al.* 2017). The combined application of compost and biochar had, also, a positive synergistic effect on plant growth enhancing soil physic-chemical and biological properties, nutrient contents and water-holding capacity (Fischer and Glaser 2012, Liu *et al.* 2012; Shulz and Glaser 2012). Conversely, iron grit is known for being unable to retain water and add organic material to soil (Tiberg *et al.* 2016, Vítková *et al.* 2018).

Considering the above mentioned evidences, the differences in plant biomass accumulation can be explained by the specific or cumulative effect that amendments had on soil characteristics. However, the chemical behavior of amendments with metal(loid)s need to be also considered. Indeed, it is widely reported that each amendment has a specific capacity to interact with metal(loid)s, modifying their availability and mobility and, thus, their bio-accessibility and toxicity (Galende *et al.* 2014; Lebrun *et al.* 2018ab).

Overall, results showed that compost and biochar, alone or in combination, reduced soil metal(loid) availability whereas had a negligible effect on their mobility (TFs < 1) or accumulation in the tree different organs (leaf, shoot and root), although they were mainly accumulated in roots. Contrarily, the iron grit amendment enhanced Fe and Pb root accumulation (BAF ≥ 1).

It is reported that biochar precipitates As and Pb through its structural phosphates and carbonates (Zheng *et al.* 2013, Lomaglio *et al.* 2016, Lu *et al.* 2017), or they could sorb onto its surface (Park *et al.* 2011) through interactions with oxygen functional groups (Jiang *et al.* 2012). Concerning compost, Huang *et al.* (2016a) showed that it potentially had a biosorbent role towards As and Pb, due to humic substances which contain many organic functional groups (Lebrun *et al.* 2018b). Also, when iron grit is incorporated into the soil it is oxidized (Fe0), and has the capacity to sorb metals, in particular Pb (Houben *et al.* 2012). However, this reaction is accompanied by the reduction of nitrate into ammonium, which results in a net release of H^+ (Haynes 1990) or an irreversible sorption of soil nutrients, such as phosphate (Hanauer *et al.* 2011, Huang *et al.* 2016b), that could be responsible for the growth reduction in iron-based treated *Salix* plants.

The restriction of all metal(loid)s in the root apparatus is considered as the tolerance mechanism to protect the plant photosynthetic tissues; we hypothesized an involvement of Casparian strip of the endodermis as a barrier to avoid metal(loid)s translocation toward aerial part (Chen *et al.* 2013).

However, in iron-treated plants (PBI and PBCI), the increase of metal(loid) roots accumulation could be controlled by biochar and/or iron ability to induce change in the chemistry of root exudates able to directly or indirectly affect metal(loid)s behavior (Kidd *et al.* 2009, Akhter *et al.* 2015, Fresno *et al.* 2018).

Some suggestions of how and by which mechanisms the different amendments influence root *Salix* growth and defense toward metal stress came from our proteomic investigations.

Data seems to indicate that enzymes mainly involved in carbon metabolism such as fructose bisphosphate aldolases (FBAs), RuBiSCO large subunit (rbcL) and glyceraldehyde-3-phosphate dehydrogenases (GAPDHs) were strongly under-represented in plants grown on iron grit amended substrates, especially in combination with biochar alone. Iron grit supply seemed to affect ATP production as well, as indicated by the complete absence of ATP synthase β subunit. Conversely, the functionality of carbon metabolism-related enzymes and the ATP synthesis seemed to be guaranteed in compost and biochar+compost amendments, as also observed by Li *et al.* (2009).

FBA is a key metabolic enzyme involved in glycolysis and gluconeogenesis in the cytoplasm, while in the plastids, it catalyzes the condensation of fructose-1,6-biphosphate in the Calvin cycle. FBAs may also play important roles in sugar, ABA, stress signaling in plants (Uematsu *et al.* 2012). Plant GAPDHs are abundant and ubiquitous enzymes playing essential metabolic roles in glycolysis and photosynthetic carbon assimilation. It has been widely demonstrated that in *Arabidopsis* roots subjected to Cd stress, GAPDH (after its relocalization in the nucleus) could have alternative functions in oxidative stress signaling or protection in plants, mainly linked to its susceptibility to oxidative post-translational modifications (Vescovi *et al.* 2013).

Salix plants to create possible growth-defense tradeoffs could promote some functions/pathways and neglecting others.

Modulation of enzymes involved in the phenyl propanoid pathway frequently occurs under metal(loid) stress in order to use their carbon skeletons dynamically for growth or defense (Kieffer *et al.* 2008, Rodríguez-Celma *et al.* 2010).

In metal(loid) stressed *Salix* roots, secondary metabolism was differentially modulated by amendments. The expression of key enzymes such as chalcone synthase (CHS), phenylalanine ammonia-lyase (PAL), caffeoyl CoA 3-O-methyltransferase isoforms (CCoAOMTs) were up-regulated mostly in plants grown on compost and biochar+compost, and almost absent in plants grown on iron treatments (PBI and PBCI).

The PAL is a key enzyme that catalyzes the first reaction of the phenylpropanoid pathway, leading to the synthesis of *p*-coumaroyl CoA. The *p*-coumaroyl CoA is an important intermediate in the metabolic routes of flavonoids or phenylpropanoid compounds *sensu stricto*, and is the common substrate of two specific enzymes: (1) CHS, which catalyzes the formation of the flavonoid skeleton, and (2) hydroxycinnamoyl transferase, which leads to the biosynthesis of the two major lignin building units (guaiacyl and syringyl units). The CCoAOMT catalyzes an important step in the lignin biosynthesis pathway, synthesizing feruloyl CoA from caffeoyl CoA (Humphreys and Chapple 2002).

Both flavonoids and lignin are important in counteracting metal(loid) stress due to their ability to neutralize ROS by donating electrons to hydrogen atoms (Labra *et al.* 2006, Sarry *et al.* 2006, Kieffer *et al.* 2008, Rodríguez-Celma *et al.* 2010).

Besides the well-studied role of flavonoids as effective antioxidants (Michalak 2006), they are also considered natural regulators of auxin gradients (by inhibiting polar auxin transport), local auxin concentrations (inhibiting peroxidase-mediated IAA oxidation), and thus are potentially involved in the so-called "stress-induced morphogenic responses" (Agati *et al.* 2012). Also, the CCoAOMT regulated plant growth; Sobhanian *et al.* (2010) showed that the down regulation of CCoAOMT was associated with a reduced plant growth.

We can hypothesize that for *Salix* roots, compost and biochar amendments (alone or in combination), redirected the phenylpropanoid flux towards enhancing the defense system and morphogenic responses, whereas iron seemed to have a negative effect on this pathway, leading to a decrease in flavonoids or phenylpropanoid compounds. This iron negative effect seems to be accentuated in presence of biochar only, as showed in our previous work by the reduction of flavonoids content essentially in PBI (Lebrun *et al.* 2019).

The activation of ROS scavenging is another common way to protect the cells from the adverse effects of metal(loid)s, this defense system involves different enzymes, such as NAD(P)H dehydrogenase FQR1, Cu-Zn superoxide dismutase (SD), glycine decarboxylase (GDC) and serine hydroxymethyltransferase (SHMT), that here were over-represented in all amended conditions, except in PBI. Conversely, molecular chaperones, such as peptidyl-prolyl cis-trans isomerases (PPIs) and heat shock proteins (HSP), involved in protein stabilization, proper folding, assembly, and translocation (Immel *et al*., 2012, Štefanic *et al*., 2018), were under-represented in all amended plants, especially in those grown on PBC and PBCI substrates.

The NAD(P)H-dependent quinone dehydrogenase is a flavin mononucleotide-binding enzyme functioning as a protective agent in oxidative stress response and detoxifying reactions (Berczi and Moller 2000, Laskowski *et al.* 2002). In particular, it can reduce quinones to the hydroquinone state, which prevents the interaction of the semiquinone with O_2 and thus disables production of toxic superoxide. Proteomic studies highlighted a role of NAD(P)H-dependent quinone dehydrogenase to counteract Cd (Sarry *et al.* 2006, Lee *et al.* 2010) and As (Pandey *et al.* 2012) stress.

The SD constitutes the first line of plant defense, converting superoxide to molecular oxygen and H_2O_2 (Alvarez *et al.* 2009, Chen *et al.* 2012). Three different SDs are present in plants: the Mn SD (mitochondrial), the Fe SD (chloroplastic), and the Cu-Zn SD, which is both cytosolic and chloroplastic (Babu *et al.* 2003). Previous studies showed a down-regulation of Cu-Zn SD with Cd, due to the reduced availability of Cu and Zn under metallic stress (Kieffer *et al.* 2008) or the inhibition of the respiratory system in the case of metal(loid) excess (Demirevska-Kepova *et al.* 2004, Alvarez *et al.* 2009).

Glycine and serine levels, through glycine decarboxylase complex (GDC) and serine hydroxymethyltransferase (SHMT) enzymes, respectively, represent the metabolic signals regulating the expression of genes encoding photorespiratory metabolism enzymes (Timm *et al.* 2013), involved in stress responses for preventing ROS accumulation. Indeed, photorespiratory reactions help to minimize ROS production either directly (using ATP, NAD(P)H and reduced ferredoxin), or indirectly (*e.g.* via alternative oxidase and providing an internal CO_2 pool), by dissipating excess reducing equivalents and energy. Some of the most important reactions in photorespiration lead to the biosynthesis of L-Ser through the glycolate pathway. In these reactions, one glycine molecule is decarboxylated and deaminated by the GDC, with the formation of CO_2 and NH_3 and the concomitant reduction of NAD^+ to NADH (Oliver 1994). The remaining methylene carbon of glycine is transferred to tetrahydrofolate (THF) to form methylene-THF, which reacts with a second glycine to form L-Ser in a reaction catalyzed by SHMT. Glycine and serine catabolism also supplies C1 units for the biosynthesis of primary (*e.g.* nucleic acids and proteins) and secondary metabolites (Cossins and Chen 1997).

Thus, overall the results seem to indicate that oxidative stress and correlated protein misfolding and aggregation could be reduced in compost and/or biochar amended conditions, whereas plants grown on PBI seemed to be under more metal(loid)-induced oxidative stress conditions. Some confirmations came from our previous results (Lebrun *et al.* 2019) showing that guaiacol peroxidase (GPOD), a biomarker for metal(loid)-induced stress in plants, was decreased in all amended samples (with the lowest value in PC, PBC and PBCI), except in biochar+iron (PBI). This decrease in GPOD activity in roots indicated a lower metal(loid) stress, especially in compost amended plants, maybe related to the capacity of compost to induce the immobilization of metal(loid)s and decrease plant metal(loid) content, consequently reducing the metal(loid)-induced stress.

Specific signaling networks and trafficking also seem to be activated or regulated differently by the diverse amendments. In more detail, in *Salix* roots, factors such as Fructokinase (FRK), 14-3-3 like protein, Ras related protein (Ras), GTP-binding proteins, a V-type proton ATPase subunit E and Dynamin related proteins (DRPs) seems to be effective in all amended conditions, except in PBI. Additionally, a dysregulation of the ubiquitin-proteasome pathway was hypothesized in all amended conditions, especially in plants grown on iron grit substrates where the ubiquitin-conjugating enzyme was completely absent.

FRK is a fructose-phosphorylating enzyme playing a central role in the regulation of plant sugar metabolism and sensing (Gupta and Kaur 2005). Soluble sugars can act as primary messengers, like phytohormones, and regulate signals that control the expression of different genes involved in plant growth, metabolism and defense. This ensures optimal synthesis and use of carbon and energy resources (Rosa *et al.* 2009). FRK is generally up-regulated under stress to ensure the re-establishment of the plant homeostasis (Kieffer *et al.* 2008).

The 14-3-3 proteins act as hubs of a cellular web encompassing different signaling pathways, transducing and integrating diverse hormone signals in the regulation of physiological processes. On the whole, evidence indicates that 14-3-3 proteins are versatile regulators of IAA action, intervening both downstream, as final transducers of IAA growth-promoting signaling, and upstream, controlling the formation of IAA gradients (Keicher *et al.* 2017).

The Rab GTPases protein, belonging to a Ras superfamily, and DRPs, which are large multidomain GTPases, could play crucial roles in vesicle formation and trafficking, especially at vacuolar level (Nielsen *et al.* 2008, Fujimoto and Tsutsumi 2014), where metal(loid)s are internalized by vacuolar proton-ATPase (Lee *et al.* 2010) and/or damaged proteins are subjected to degradation.

The ubiquitin-proteasome pathway (UPP) is the primary cytosolic proteolytic machinery for the selective degradation of damaged proteins. A fully functional UPP is required for cells to cope with oxidative stress, so UPP activity is modulated by cellular redox status. In the canonical UPP, proteins are first tagged by multiple ubiquitin molecules and then degraded by the 26S proteasome. However, in non-canonical UPP, proteins can be degraded by the 26S or the 20S proteasome without being ubiquitinated. Thus, the proteasome is responsible for a selective degradation, while ubiquitination is involved in the degradation of some forms of oxidized proteins (Shang and Taylor 2011).

In the iron grit amendment, the signaling/regulatory pathways that transmit the external stimuli into an intracellular response in order to boost the defense responses (*e.g.* through the sequestration of metal(loid)s into the vacuole or the intensification of antioxidative mechanisms), seemed to be strongly affected, especially in PBI, where protein synthesis and degradation machine dysfunction was also affected. Although a UPP dysregulation involved plants grown on biochar and/or compost treatments, these treatments also seemed to better preserve the protein synthesis and recycling, compared to PBI.

5. Conclusion

In summary, out of all the different amendments tested, the compost or biochar+compost combination showed better results in terms of *Salix viminalis* growth improvement, amelioration of soil characteristics and fertility, and decreasing metal(loid) mobility and toxicity. Conversely, the iron grit amendment negatively affected plant growth, as it increased soil acidity and thus metal(loid)s phytoavailability, accumulation/toxicity in root system, compromising the sorption of soil nutrients.

The analysis of proteome profiles showed that plants grown on compost and biochar+compost amended contaminated soil and used carbon skeletons dynamically from the primary to secondary metabolism to counteract metal(loid) oxidative stress, and induce stress morphogenic responses. Furthermore, in stress conditions, we observed an activation of defense mechanisms and signaling/regulatory pathways involved in the sequestration of metal(loid)s into vacuole and antioxidative mechanisms. All these processes were affected by the iron grit amendment, especially when in combination with only biochar. In PBI the complete proteasome dysregulation may be related to an accumulation of damaged proteins which most likely leads to disorders in plant growth.

In conclusion, *Salix viminalis* plants alongside a compost or biochar/compost mixture could be used as an effective assisted-phytostabilization strategy to manage As and Pb contaminated soil. However, further long-term field experiments should be planned to monitor whether: i) stabilizing conditions are maintained over time *in loco;* ii) prolonged exposure to metal(loid) toxic effects may prevent long-term plant growth; iii) further amendments are necessary over time to preserve the immobilization effectiveness of contaminants. Additionally, gene expression studies and hormonal profiling (De Zio *et al.* 2019) should be carried out to better decipher, in a network-type fashion, the key factors and mechanisms related to the complex plant-substrate interaction. This information will be useful to scientists/managers involved in smart selection and innovation of remediation strategies.

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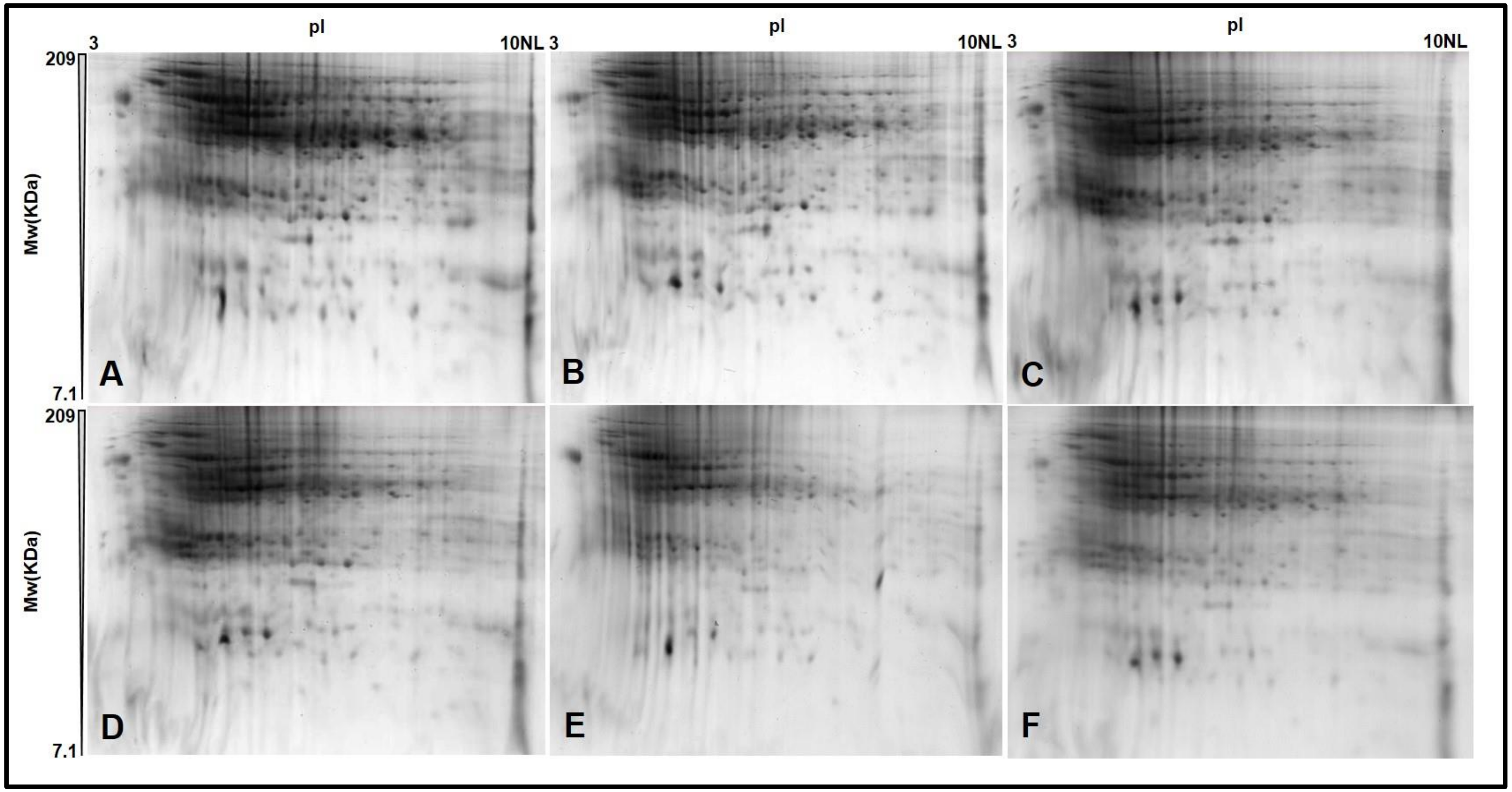
Figure A.1. Two-dimensional proteome maps of *Salix viminalis* roots in control and mechanical stress conditions. IEF was performed with non-linear pH 3-10 IPG strips, followed by SDS-PAGE on 12% polyacrylamide gels. Gels were stained with Coomassie Brillant Blue and images were analyzed using the PDQuest software. Panels show maps of P (A), PB (B), PC (C), PBC (D), PBI (E) and PBCI (F). P= *Salix viminalis* plants grown on non-amended Pontgibaud technosol, PB = Pontgibaud amended with 5 % biochar, PC = Pontgibaud amended with 5 % compost, PBC = Pontgibaud amended with 5 % biochar and 5 % compost, PBI = Pontgibaud amended with 5 % biochar and 1.5 % iron grit, PBCI = Pontgibaud amended with 5 % biochar, 5 % compost and 1.5 % iron grit.



Part B. Effect of biochar, compost and/or iron grit amendments on the organic acid excretion by *Salix viminalis* roots grown on an As and Pb contaminated mining technosol.

Effect of biochar, compost and/or iron grit amendments on the organic acid excretion by *Salix viminalis* roots grown on an As and Pb contaminated mining technosol

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Abstract

Salicaceae are much used in phytoremediation process due to their tolerance and ability to accumulate metal(loid)s. However, the poor fertility of contaminated soils often requires the application of amendments to improve the growth conditions. In this goal, biochar, compost and iron based amendments (such as iron grit) showed good potential and their single and combined effects have been demonstrated in a previous study. Moreover, phytoremediation success also depends on the plant activity such as root exudates. Indeed, organic acids secreted by roots in the environment can have an important role in metal(loid) phytoremediation by modifying metal(loid) bioavailability and increase plant metal(loid) tolerance. Therefore, a mesocosm study was set up to evaluate the effect of amendment applications to a former mining technosol on the organic acid root exudation profiles of *Salix viminalis* plants. The results showed that the five organic acids measured (citric, fumaric, malic, succinic and tartaric acids) were only little affected by the amendments, although their exudation tended to increase in the amended conditions, related to a higher growth and leaf surface. Moreover, the study showed that citric and malic acids were the most represented in almost all conditions, which could reflect their role in As and Pb toxicity tolerance as well as in Fe deficiency tolerance.

Keywords
Amendments; Metal(loid)s; Organic acids; Root exudates; *Salix viminalis*

1. Introduction

Soil pollution has become a major issue of the current time. Indeed, more than ten million sites are contaminated worldwide (Khalid *et al.* 2016). Such important pollution, of which more than 50% is with metal(loid)s (Khalid *et al.* 2016), poses a great risk to the environment and human health. Contaminated soils usually present a low diversity with a poor fertility and thus often lack of vegetation, increasing the risk of contamination by leaching and erosion. Furthermore, when vegetation is present, plants often suffer from oxidative stress. Moreover, with the consumption of contaminated plants by animals, metal(loid)s can enter the food chain and cause great problems. Indeed, many metal(loid)s, such as arsenic and lead, are classified as carcinogenic (Singh *et al.* 2015). Therefore, the remediation of such contaminated areas, is the object of many researches.

One possibility is to use plants which will take up pollutants and store them in their root and/or aerial parts. In addition, the implementation of a plant cover will reduce the risk of erosion and water leaching. Such technique is called phytoremediation (Thavamani *et al.* 2017). *Salicaceae* showed good potential due to their fast and important growth as well as their tolerance and accumulation capacity towards metal(loid)s (Bart *et al.* 2016, Lebrun *et al.* 2017).

However, due to usually a poor fertility of contaminated soils, amendments must be applied. For instance, biochar, a carbon rich product obtained from the pyrolysis of biomass, has been often used to improve soil conditions (Paz-Ferreiro *et al.* 2014). Indeed, due to its alkaline nature, large surface area and high cation exchange capacity (Wisniewska *et al.* 2016), it can reduce soil acidity and metal(loid) availability. Furthermore, biochar can also increase nutrient content and availability (Trakal *et al.* 2017, Lima *et al.* 2018). Another amendment often studied is compost. Indeed, in addition to being rich in nutrients and organic matter, its humic substances possess sorption capacity towards metal(loid)s (Walker *et al.* 2004, Brown and Cotton 2011). Finally, iron based amendments, such as iron grit, showed good potential to reduce anions mobility, especially arsenic (Tiberg *et al.* 2016), which can be increased with biochar and compost addition.

As a biological process, phytoremediation success relies on plant development and activity. More particularly, root exudates can have an important role. Root exudates come from plant metabolism, especially photosynthesis. Indeed, about half of the products obtained by the photosynthesis are transported to the roots, and among this, 12 to 40 % are released to the rhizosphere as exudates (Dong *et al.* 2007). Root exudates can be divided in two categories: low molecular weight compounds and high molecular weight compounds (Bais *et al.* 2006). The composition and amount of the different compounds in the root exudates is greatly influenced by the growing environment: presence or absence of particular minerals or toxic metal(loid)s, nutrient content, soil moisture, soil texture (Badri and Vivanco 2009, Haichar *et al.* 2014). In return, root exudates can also affect phytoremediation by altering pollutant bioavailability as well as affecting the composition of plant associated microbiota (Gomez *et al.* 2019). Among the root exudates, organic acids are an important factor as they can increase plant tolerance to high metal(loid) concentrations (Goliński *et al.* 2015). Indeed, they possess metal(loid) chelation properties that can either increase or decrease metal(loid) accumulation in plants (Bais *et al.* 2006, Seshadri *et al.* 2015). Thus, organic acids study is important to assess plant ability to remediate metal(loid) contaminated soils. However, most of the works have been performed with mono-element medium in hydroponic conditions or with artificially contaminated soils, especially Cd and to a lesser extent Al, Ni and Zn (Yang *et al.* 1997, Saber *et al.* 1999, Agrawal *et al.* 2012, Hawrylak-Novak *et al.* 2015, Javed *et al.* 2017). But to the best of our knowledge, no work evaluated the effect, in real soil conditions, of amendment application on root exudation profiles.

Therefore, the objectives of this study were to evaluate the effect of biochar, compost and iron grit single or combined application on the root organic acid exudation profiles of *Salix viminalis* plants grown on such amended As and Pb contaminated mine technosol.

2. Material and Methods

2.1 Studied site and experimental design

This study focused on a former silver-lead extraction mine site, located in the Pontgibaud mine district (Puy-de-Dome, Auvergne-Rhone-Alpes, France), whose activity stopped at the end of the nineteenth century. Due to an intense activity, the Pontgibaud district was one of the most important mine district in the XIXth century, the area is now highly contaminated with elevated concentrations of lead (Pb) and arsenic (As) (Lebrun *et al.* 2019).

The experimental design as well as the first results of the study have been fully described in a previous paper (Lebrun *et al.* 2019) but a brief description of the experiment will be reminded here. The Pontgibaud technosol (P) was mixed or not with three different amendments: a hardwood biochar added at 5% (B), a commercial compost added at 5% (C) and an industrial iron grit added at 1.5% (I), giving seven treatments in total (P0%, PB, PC, PI, PBC, PBI and PBCI). After equilibration of the mixtures, one non-rooted cutting of *Salix viminalis* was added per pot and plants were grown for 69 days.

2.2 Collection of root exudates

After 69 days of growth on the different substrates, plants were harvested and subjected to different treatments. Three plants of each substrate were used for the analysis of the root exudates. To do so, the plants were removed from the pot and the roots were carefully washed in distilled water until no soil particles were observed on the roots. Following, the roots were immerged in milliQ water. The volume of milliQ water was adjusted depending on the root system of each plant in order to cover all the roots. After four hours, at room temperature, the plants were removed and the solution were filtered to remove any particle left and put at -20 °C. The frozen solutions were then lyophilized and the dry powder was recovered, weighted and solubilized in acetone, at different volumes (between 0.5 and 2 mL) depending on powder quantity (from 0.4 to 10 mg/ml). These extracts were kept at -20 °C until further analysis.

2.3 Analysis

Based on previous studies performed on willow (Drzewiecka *et al.* 2014; Goliński *et al.* 2015) and especially on *Salix viminalis* (Mleczek *et al.* 2018), five organic acids were analyzed: citric acid, fumaric acid, malic acid, succinic acid and tartaric acid.

The samples were analyzed for organic acids by ultra-high-performance liquid chromatography (UHPLC) (Destandau *et al.* 2005) using an Ultimate 3000 RSLC system (Dionex, Germering, Germany) consisting of a binary pump, an online vacuum degasser, an autosampler and a column compartment. Separation of compounds was achieved on a Phenomenex Luna Omega PS C18 column 1.6 µm, 100 mm x 2.1 mm, kept at 25°C. Mobile phase A was water containing 0.1% formic acid; mobile phase B was acetonitrile containing 0.1% formic acid. The flow was 0.5 mL/min, and the gradient profile was 0 at 50% B in 5 min, return in 0.1 min to initial conditions. The injection volume of both the standard solutions and the samples was 20 µL. Equilibration time between two injections was 2 minutes.

UHPLC was coupled with mass spectrometry detection. It was performed on a TSQ Endura triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) instrument equipped with an electrospray ionization ion source (H-ESI). Capillary voltage was -2.5 kV in negative mode; the vaporizer temperature was at 500°C; ion transfer tube temperature at 380°C. Gas flow is in arbitrary unit, sheath gas was fixed at 27, Aux gas at 9 and sweep gas at 0. MS acquisitions were done in full scan mode from *m/z* 80 to *m/z* 250. Data processing was done using Xcalibur version 3.0 SP2.

2.4 Statistical analysis

The data were analyzed on R software version 3.5.1 (R Core Development Team). For each organic acid, a one-way ANOVA was performed followed by a post-hoc Tukey test. Difference was considered significant at $p < 0.05$.

3. Results and Discussion

Five organic acids were measured in the root exudates: one tricarboxylic acid, citric acid, and four dicarboxylic acid, fumaric, malic, succinic and tartaric acids. Among those five organic acids, citric and malic acids were the most abundant in all conditions except PI, which did not present those two acids but presented elevated contents of succinic and to a lesser extent tartaric acid (Figure 1). Zeng *et al.* (2008) also observed that oxalic and malic acid contents were higher than the four others (lactic, acetic, citric, succinic acids) in the root exudates of rice plants exposed to chromium. In the same way, Haoliang *et al.* (2007) identified nine organic acids in the root exudates of *K. candel* plants exposed to cadmium and found that acetic, lactic, malic and citric acids were the most abundant. Such higher contents of these two organic acids can be explained by their high ability to complex with metal(loid)s (Seshadri *et al.* 2015).

3.1 Citric acid

The citric acid ($C_6H_8O_7$) has a function in microbial mineralization, phosphorus mobilization, aluminum detoxification, as well as in the mobilization of several elements (Fe, Cu, Mn, Ca, Mg, Zn and Ni) (Martin *et al.* 2014).

After 69 days of growth, the content of malic acid in *Salix viminalis* root exudates was higher in the biochar and compost amended substrates, compared to P0%, although this increase was significant only for their combined application (PBC) (Figure 1A). Moreover, its content was below detection limit in PI and PBI. Such findings are in contradiction with previous studies showing a higher citric acid content in root exudates after treatment with metal(loid)s. For instance, the treatment of *Solanum nigrum* by increasing levels of chromium rose root exudation of citric acid (UdDin *et al.* 2015). Similarly, Javed *et al.* (2017) showed that citric acid exudation increased with increasing cadmium concentration treatment. But our previous study showed that metal(loid) stress was reduced with biochar and/or compost amendments (Lebrun *et al.* 2019). However, in this same study, soil pore water (SPW) As concentration were observed to increase in PB and PBC conditions, which could explain the increase in citric acid exudation observed here, as found with increasing Cd and Cr medium concentration by previous researchers. Moreover, such more important exudation of citric acid in response to higher SPW As concentration could have led to the chelation of As to render it less available (Pinto *et al.* 2008), as root As concentrations were observed reduced in those two conditions even with higher concentrations in SPW (Lebrun *et al.* 2019). In addition, SPW Pb concentrations were decreased in the vegetated pots of the conditions amended with biochar and compost, compared to P0% (Lebrun *et al.* 2019), which could be attributed to the uptake of Pb by the plant but also to a chelation of Pb by citric acid, since root and leaf Pb concentrations were also decreased with biochar and compost amendments (Lebrun *et al.* 2019). Indeed, Duarte *et al.* (2007) observed that with increasing concentrations of citric acid, Ni uptake was decreased due to the formation of a complex that prevented its uptake, but also citric acid was observed to decrease Cd translocation to the upper parts. Finally, another parameter that could have contributed to the higher exudation of citric acid in the biochar-compost amended treatment is the leaf area that was higher in this condition compared to P0% and as root exudates come from photosynthesis metabolism, a higher leaf surface area means more photosynthetic surface and thus potentially more photosynthesis products.

Citric acid was not detected in PI and PBI, which could be explained by a low leaf surface area (Lebrun *et al.* 2019) but also by the high levels of Fe measured in SPWs. Indeed, citrate is a potential complexing reagent of Fe in soil but can also induce the dissolution of non-available ferric oxyhydroxide to the soil solution (Jones 1998), but since here SPW Fe levels were already high, a dissolution was not necessary.

3.2 Fumaric acid

Fumaric acid ($C_4H_4O_4$) was not detected in P0%, PI and PBI while low concentrations were detected in the biochar and/or compost amended treatments, although such higher fumaric acid exudation was not significantly different from P0% (Figure 1B). Moreover, fumaric acid presented the lowest level of the five organic acids measured. Depending on the treatment, its levels were nine to 150 times lower than the citric acid, three to 190 times lower than the malic acid, two to four times less than the succinic acid and two to 55 times lower than the tartaric acid. Such low exudation could mean that fumaric acid had no major role in the metal(loid) detoxification and tolerance.

3.3 Malic acid

Similarly to citric acid, malic acid ($C_4H_6O_5$) has several functions such as microbial mineralization, phosphorus mobilization, Al detoxification and Fe, Cu, Mn, Ca and Mg mobilization (Martin *et al.* 2014).

Although there was no significant difference between P0% and the amended conditions, malic acid content in *Salix* root exudates tended to be higher in PBC. In addition, it was below detection limit in PI (Figure 1C). Such observation is not consistent with the finding of UdDin *et al.* (2015) who measured increasing malic acid exudation with increasing Cr concentrations. However, the tendency of malic acid exudate content to increase in PBC could be related to the increase in SPW As concentration, similarly to citric acid. Moreover, plant growth was better with biochar + compost amendment, which could be linked to the higher malic acid production. Indeed, Hawrylak-Nowak *et al.* (2015) observed that sunflower growth indicators were higher after the application of exogenous malic acid. Furthermore, plants grown on PBC accumulated lower concentrations of metal(loid)s, which is in contradiction with previous studies. For instance, in Hawrylak-Nowak *et al.* (2015), the application of exogenous malic acid increased root Cd concentrations in sunflower while in Yang *et al.* (1997), the higher Ni root concentration was related to higher malic acid concentration in the roots. Finally, the non-detection of malic acid in PI could be due to the high Fe concentrations found in SPW and plants (Jones 1998), similarly to citric acid.

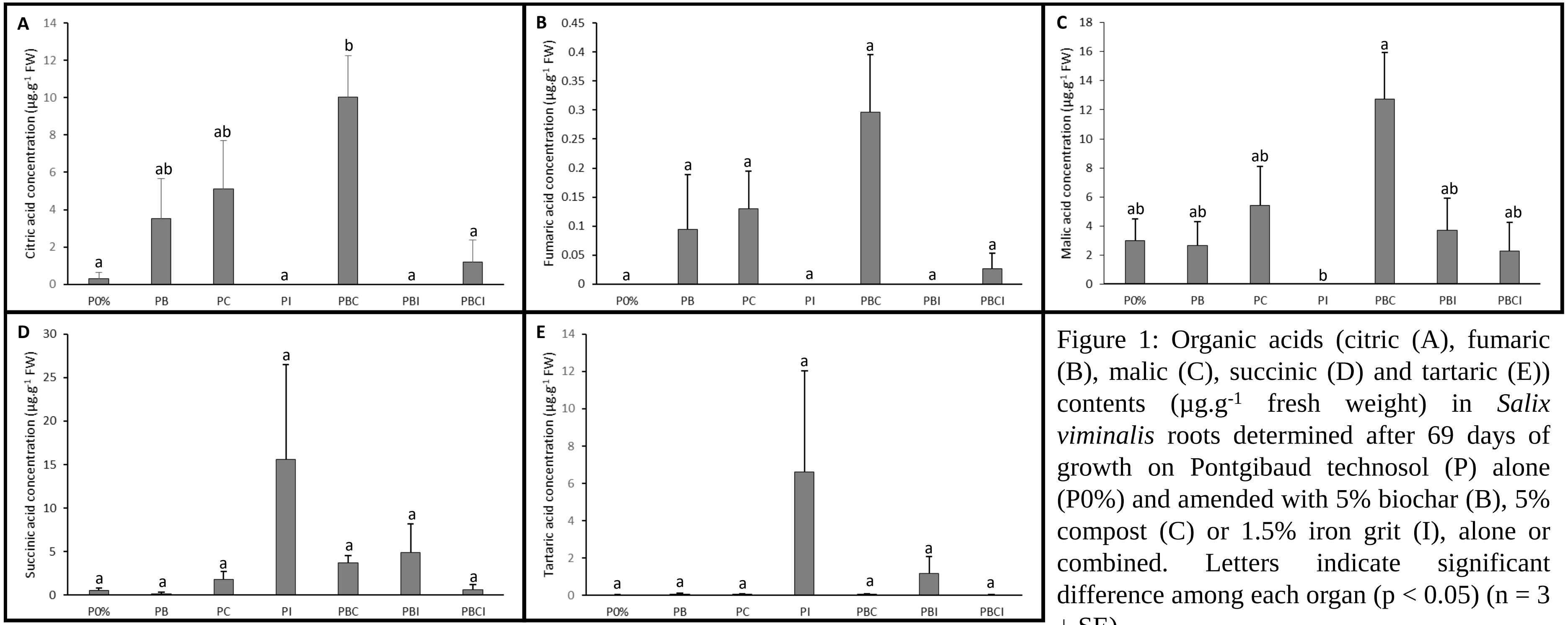

Figure 1: Organic acids (citric (A), fumaric (B), malic (C), succinic (D) and tartaric (E)) contents (µg.g^{-1} fresh weight) in *Salix viminalis* roots determined after 69 days of growth on Pontgibaud technosol (P) alone (P0%) and amended with 5% biochar (B), 5% compost (C) or 1.5% iron grit (I), alone or combined. Letters indicate significant difference among each organ ($p < 0.05$) ($n = 3 \pm SE$).

3.4 Succinic acid

Succinic acid ($C_4H_6O_4$) has a role in microbial mineralization, phosphorus mobilization and micronutrient mobilization (Martin *et al.* 2014).

No significant variation was observed in the succinic acid concentrations (Figure 1D). However its content tended to be higher in PI, which could be due to Fe toxicity (Javed *et al.* 2017). Moreover, in all conditions, even P0%, succinic acid contents in roots exudates were low, which could indicate that in this case, succinic acid played little role in As and Pb detoxification and tolerance.

3.5 Tartaric acid

Tartaric acid ($C_4H_6O_6$) is known to be involved in phosphorus mobilization and micronutrient mobilization (Martin *et al.* 2014).

Similarly to succinic acid, no significant difference in tartaric acid amounts between the seven treatments was observed. However, concentrations were very low in P0%, as well as in PB, PC, PBC and PBCI, while it was higher in PI and PBI (Figure 1E), which could be also related to Fe toxicity. Indeed, Chen *et al.* (2014) showed that tartaric acid may play a role in plant tolerance to Cd toxicity whereas Shahid *et al.* (2012) observed that organic acids were released at higher levels under higher concentrations of Pb and Fe. Finally, as succinic acid, tartaric acid contents were generally low, except in PI and PBI, which could signify that it played little role in As and Pb tolerance, while it had a more important role in Fe tolerance.

3.6 General discussion

When taken together, the results showed a general increase in organic acid exudation in the amended conditions presenting a reduced metal(loid) stress, in contradiction with previous studies. For instance, Javed *et al.* (2013) showed that metal stress increased concentrations of organic anions in the rhizosphere. However, such improvement could be related to a better growth and leaf surface area observed in most of these amended conditions, as shown in our previous study (Lebrun *et al.* 2019). Indeed, a higher leaf surface means a more important photosynthetic surface and thus more products synthesized.

Furthermore, malic and citric acids were the most important organic acids measured in all conditions, except PI and PBI, and they tended to increase with biochar and compost amended conditions, in which plant growth was better and metal(loid) accumulation was reduced. Therefore, these two organic acids seem to have a role in As and Pb tolerance in *Salix viminalis* plants and they could protect the plants by forming complex with the metal(loid)s (Javed *et al.* 2017). Especially, they tended to increase in biochar and/or compost amended conditions, in which SPW As concentrations showed a trend to increase. Furthermore, their increase could be related to a Fe deficiency (Sun *et al.* 2006), which has been sorbed by biochar and compost. Such explanation was attested by their no detection in PI and PBI treatments and the low SPW Fe concentrations measured in those treatments, even lower than in P0%, as well as the lower Fe availability measured by $CaCl_2$- and NH_4NO_3-extractions (Lebrun *et al.* 2019).

4. Conclusion

This study is the first evaluation of the effect of three different amendments on the root organic acid exudation profiles of *Salix viminalis* plants grown on a contaminated soil. The results of this study showed low but significant effects of biochar, compost and iron grit amendments, even though a trend was observed, separating the organic acids in two groups: malic and citric acids, and to a lesser extent fumaric acid, seemed to have an important role in As and Pb tolerance and maybe in the control of Fe deficiency, while succinic and tartaric acids seemed to have a role in Fe tolerance in *Salix viminalis* pants.

However, further research is needed, such as analyzing the contents of the organic acids inside the diverse *Salix viminalis* organs to evaluate if the amendments impacted metal(loid) compartmentation by modifying organic acid contents in plants. Moreover, even though organic acids were little affected by amendments, other root exudate compounds could have been more impacted, such as sugars or amino acids.

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Part C. Effect of biochar and redmud amendment combinations on *Salix triandra* growth and oxidative stress response.

Effect of biochar and redmud amendment combinations on *Salix triandra* growth, metal(loid) accumulation and oxidative stress response

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Abstract

Remediation of metal(loid) polluted soils is an important area of research nowadays. In particular, one remediation technique is much studied, phytomanagement. Phytomanagement combines amendment application and plant growth in order to reduce the risk posed by contaminants. *Salicaceae* plants showed tolerance towards metal(loid)s and the ability to accumulate high amounts of metal(loid)s in their tissue. Amendments are often applied to counterbalance the reduced soil fertility and high metal(loid) concentrations. Two amendments gathered attention over the last decades, biochar (product of biomass pyrolysis), which can be activated for better effects, and redmud (by-product of alumina production). Those two amendments showed ability to improve soil conditions and thus plant growth, although few studied their combined application. Moreover, no study evaluated the response of *Salicaceae* plants to metal(loid) stress and amendment application at the biochemical level in a real soil condition. Therefore, a mesocosm study was set up to evaluate the effect of amending a mine soil with redmud combined to diverse biochars on the soil properties and *Salix triandra* growth, metal(loid) accumulation and stress marker levels. Results showed that all amendment combinations improved the soil fertility, reduced metal(loid) mobility and thus ameliorated *Salix triandra* growth, which accumulated metal(loid)s mainly in its roots. Moreover, among the different amendment combinations, *Salix triandra* plants still suffered from oxidative stress when grown on PG soil amended with redmud and chemically activated carbon. Finally, one treatment showed stress markers similar or lower than the control, the combination of redmud with steam activated carbon. In conclusion, this treatment seemed a good solution in a phytomanagement strategy using *Salix triandra*.

Keywords
Activated carbon; biochar; metal(loid)s; *Salix triandra*; oxidative stress markers

1. Introduction

Soil is a geochemical sink for contamination (Kabata-Pendias 2011) and thus, the development of anthropogenic activities, *i.e.* mining, industry, fertilizer and pesticide uses in agriculture, transport… (Vamerali *et al.* 2010, Panagos *et al.* 2013), led to an important contamination of the soils worldwide (Panagos *et al.* 2013). Particularly, metal(loid)s, encountered in more than 50 % of the contaminated soils (Khalid *et al.* 2016), are of great concern, due to their non-degradability compared to organic pollutants, but also due to their negative effects on the environment and human health. Therefore, the necessity to remediate such contaminated soils has become a priority (Van Ginneken *et al.* 2007).

Among possible remediation techniques, phytomanagement gained attention these last decades over the conventional physical and chemical methods. The goal of phytomanagement is to reduce metal(loid) mobility and thus the risk posed by such pollutants (Domínguez *et al.* 2008). To accomplish this, phytomanagement involves the manipulation of the soil-plant system and combines plant establishment and amendment application (Tack and Meers 2010). Plant establishment will reduce wind erosion and water leaching risk, thus diminishing spreading of contamination. Moreover, plants will take up contaminants and store them in their roots (phytostabilization) and their aerial parts (phytoextraction).

As described in the literature, *Salicaceae* species showed a good potential for phytostabilization, often chosen in the case of elevated and deep contamination, in terms of metal(loid) tolerance (Kuzovkina *et al.* 2004, Ruttens *et al.* 2011), growth potential on contaminated soil (Vervaeke *et al.* 2003, Bart *et al.* 2016, Lebrun *et al.* 2018) and metal(loid) accumulation (Migeon *et al.* 2009, Hartley *et al.* 2011, Lebrun *et al.* 2019).

However, contaminated soils are often characterized by a poor agronomic value (extreme pH, low organic matter and nutrient contents, high metal(loid) concentrations); therefore, amendments often must be applied. Among amendments, biochar has received particular attention in recent years. Biochar is obtained through the pyrolysis of biomass, mostly of vegetal and manure origins, under low oxygen conditions (Wiszniewska *et al.* 2016). It is characterized by an alkaline pH, a high surface area, a porous structure, a high cation exchange capacity and the presence of many oxygen containing functional groups at its surface (Singh *et al.* 2010, Cantrell *et al.* 2012, Paz-Ferreiro *et al.* 2014). All these properties make biochar a good amendment for metal(loid) contaminated soils that will increase pH, nutrient content and availability, but also and more importantly reduce metal(loid) bioavailability through its sorption capacity (Trakal *et al.* 2017, Lima *et al.* 2018, Meng *et al.* 2018). Such improvements of the soil conditions lead to a better plant growth, demonstrated in many previous studies for diverse plant species: ryegrass (Trakal *et al.* 2017), maize (Uzoma *et al.* 2011), tomato (Akthar *et al.* 2014), and willow (Lebrun *et al.* 2017, 2018, 2019). Moreover, biochar can also undergo “activation”, *i.e.* a modification of its surface using steam or chemical activations, to further increase its beneficial effects on soil and plants. Such post-activation product is called “activated carbon”. However, biochar showed good potential mainly for cation metal(loid) contaminated soils but it was revealed inefficient or even negative for anions like arsenic (Beesley *et al.* 2010, 2014). On the contrary, redmud, a by-product of alumina production (Hua *et al.* 2017), is rich in iron and aluminum oxides and hydroxides that can interact with arsenic and other metal(loid)s (Bertocchi *et al.* 2006). Redmud is also characterized by a very alkaline pH and a highly corrosive property (Liu *et al.* 2011). Redmud application to soil can thus increase soil pH and immobilize metal(loid)s (Lee *et al.* 2011, Gautam and Agrawal 2017), improving consequently plant growth (Gray *et al.* 2006, Castaldi *et al.* 2009, Gautam and Agrawal 2017,).

Furthermore, in addition to hinder plant growth, metal(loid)s also induce an oxidative stress in plants, through the overproduction of reactive oxygen species (ROS) (Ishtiyaq *et al.* 2018). In response to such elevated ROS content, plants can activate their antioxidant system, composed of both enzyme and non-enzyme elements. For instance, flavonoids and phenolic compounds generally increase under stress as they participate in the scavenging of ROS (Sakihama *et al.* 2002, Jaskulak *et al.* 2018). Phenolic compounds can also chelate metal(loid)s (Dresler *et al.* 2017). Finally, enzymes such as superoxide dismutase and peroxidase can scavenge ROS and thus decrease oxidative damage (Wang *et al.* 2008). The assessment of these different stress markers can thus give an indication of the stress level the plant is under.

Although both biochar and redmud have been much studied for their effects on soil properties and plant growth, few studies assessed the effect of their combined application on such parameters. Furthermore, to the best of our knowledge, no studies evaluated the effect of biochar and redmud amendment on *Salix* oxidative stress. Therefore, the goals of this study were to evaluate the effects of amending a former mine technosol contaminated by As and Pb with redmud associated to diverse biochars on: (i) the soil physico-chemical properties, (ii) *Salix triandra* growth and metal(loid) accumulation and (iii) *Salix triandra* oxidative stress status.

2. Materials and Methods

2.1 Studied site and amendments

This experiment focused on a mine technosol, resulting from the silver-lead extraction on the Pontgibaud mine district (Région Auvergne-Rhone-Alpes, France). All extraction activities stopped in the middle of the nineteenth century but the intense activity generated an important amount of wastes highly contaminated by arsenic (539 mg.kg^{-1}) and lead (11,453 mg.kg^{-1}) (Cottard 2010). Soil was sampled at one of the four parts of the Pontgibaud mine district: Roure-les-Rosiers.

Five amendments were used in this study: a bamboo based biochar (BA) (Jacobi Carbons), a biochar obtained from bark and sapwood of oak (BS2) (La Carbonerie), a wood activated carbon (steam activation) (EK5) (Jacobi Carbons), a wood activated carbon (chemical activation) (L27) (Jacobi Carbons) and a commercial redmud modified to be less alkaline (R) (Alteo Environment). The amendments were characterized for their pH, electrical conductivity, redox potential, as described in Lebrun *et al.* (2019) using a multimeter (Mettler-Toledo, Serveur Excellence) and results are presented Table 1.

2.2 Substrates preparation

In total, six substrates were prepared. The first one was a non contaminated "control" (Ctr) prepared by mixing garden soil with perlite (ratio 4:1); the second treatment was the non-amended Pontgibaud technosol (PG); the third substrate was PG amended with 1 % R and 2 % BA (RBA); the fourth one was PG amended with 1 % R and 2 % BS2 (RBS2); the fifth treatment was composed of PG amended with 1 % R and 2 % EK5 (REK5) and the sixth one was PG amended with 1 % R and 2 % L27 (RL27). All amendments were added on a w/w basis. Four replicates were prepared for each treatment.

2.3 Plant growth and physiological analysis

After the mixture preparation, one non-rooted cutting of *Salix triandra* was placed in each pot. After buds broke, one stem was left to develop and plants were grown for 41 days (16 h of light / 8 h of darkness, 25 °C / 21 °C with a photon flux of approximately 800 $mol.m^{-2}.s^{-1}$). At the end of the growing period, plants were harvested and subjected to diverse treatments. Leaves were removed, numbered and scanned in order to determine total leaf area of each plant using Image J software. In addition, the average leaf surface was calculated. Stem lengths were measured. Roots were washed twice with tap water and once with distilled water. One root ramification was sampled for each plant, immediately frozen and stored at -80 °C until further analysis (part of the material was lyophilized). The other part of the root as well as leaves and stems were dried at 60 °C for 72 h to determine dry weight (DW). Finally, the dried biomass was subjected to acid digestion and ICP-AES analysis (Inductively Coupled Plasma Atomic Emission Spectroscopy; ULTIMA 2, HORIBA, Labcompare, San Francisco, USA) to measure As and Pb concentrations, as described in Bart *et al.* (2016).

2.4 Soil pore water (SPW) sampling and analysis

SPWs were sampled at the end of the growing period, just before plant harvest, in each pot using soil moisture samplers (Rhizon®) (model MOM, Rhizosphere Research Produces, Wageningen, The Netherlands) as described in Lebrun *et al.* (2017). SPW samples were used directly to measure pH, electrical conductivity (EC) and redox potential (Mettler-Toledo, Serveur Excellence). They were then acidified with a few drops of concentrated nitric acid (65%) and analyzed for As and Pb concentrations, using ICP-AES.

2.5 Plant non-enzymatic oxidative stress markers

The lyophilized material was used to determine oxidative stress markers. First, total anthocyanin and phenolic contents, free radical scavenging and chelating capacity were measured, using the following protocols.

Extraction procedure. Root extracts were obtained by grinding 3 to 30 mg of lyophilized material in 1 mL of 50 % ethanol (v/v) (HPLC grade, Thermo) followed by an ultra-sonication (60 min, 50 °C, 400 W, 45 kHz) (ultrasonic bath USC1200TH, Prolabo). Extracts were then centrifuged 10 min at maximum speed (14 000 x g) and supernatant was recovered. The rest of the root biomass was lyophilized for further analysis.

Quantification of total phenolic content (TPC). TPC was determined by the Folin-Ciocalteu method. The reagent was prepared by mixing 25 mL of Na_2CO_3 (4 %), 250 µL $CuSO_4$ (2 %) and 250 µL tartrate sodium potassium (2 %). 190 µL of this reagent were mixed with 10 µL of root extract and absorbance at 735 nm was measured after 10 min at room temperature. Gallic acid (Sigma) was used for standard calibration curve and TPC was expressed as milligrams gallic acid equivalent per gram.

Quantification of total anthocyanin content. Total anthocyanin content was determined by the aluminum chloride colorimetric method described in Lopez-Contreras *et al.* (2015).

Determination of free radical scavenging capacity. Free radical scavenging capacity was evaluated by the DPPH method, assessing the scavenging capacity through hydrogen atom transfer, and the CUPRAC method, evaluating the scavenging capacity through electron transfer. The DPPH method was described by Lopez-Contreras *et al.* (2015). Briefly, 10 µL of root extract were mixed with 190 µL of DPPH solution (60 mM prepared in EtOH) and the absorbance at 630 nm was read after 10 min incubation at room temperature. For the CUPRAC method, a solution containing Cu(II) (10 mM), neocuproine (7.5 mM) and ammonium acetate buffer (1 M, pH 7) was prepared by adding each component at the same volume. Following, 190 µL of this solution were mixed with 10 µL of root extract and absorbance at 450 nm was read after 10 min incubation at room temperature.

Chelation capacity. The chelation capacity of the root extracts was determined by the method of Dinis *et al.* (1994) using ferrous ions. For this, a solution containing $FeCl_3$ and ferrozine was prepared and 190 µL of this solution were mixed with 10 µL of root extract. After 10 min incubation at room temperature, absorbance at 490 nm was measured. Chelation capacity was calculated as $[(A_0 – A_s)/A_s] \times 100$, where A_0 was the absorbance of the control and A_s the absorbance of the extract.

2.6 *Salix triandra* root salicinoid contents

Salicinoids were quantified by HPLC using HPLC-grade solvents (Sigma Aldrich). Lyophilized material (100 mg) from each sample was homogenized in 500 µL of 75% (v/v) aqueous ethanol using ultra-turrax (T25, Ika) set at 8,000 rpm for 30 seconds and then sonoextracted during 60 minutes with the help of an ultrasonic bath USC1200TH (Prolabo) set at an operating frequency of 45 kHz and an extraction temperature of 50°C. The characteristics of the US bath were: inner dimension of 300 × 240 × 200 mm, electrical power of 400W (*i.e.* acoustic power of $1W.cm^{-2}$), maximal heating power of 400W, variable frequencies, equipped with a digital timer, a frequency and a temperature controller. Following extraction, the extract was centrifuged during 15 min at 3,000 rpm and the supernatant was filtered (0.45 µm; Merck Millipore) before HPLC analysis. HPLC separation was performed on a Zorbax SB C18-column (Agilent Technology) at 35 °C with a Varian a HPLC system (Agilent Technology) composed of Varian Prostar 230 pump Meta chem Degasit, Varian Prostar 410 autosampler and Varian Prostar 335 Photodiode Array Detector (PAD) and driven by Galaxie version 1.9.3.2 software (Agilent Technology, Les Ulis, France). Separation was performed using the binary gradient of methanol and water (with 2% tetrahydrofuran; 20 µL/min) as described by Rubert-Nason *et al.* (2014). Detection of compounds for quantification was realized DAD (set at 274 nm). Quantification was done based on retention time compared to authentic standards (Sigma Aldrich). Examination of each sample was realized three times.

2.7 *Salix triandra* root cell wall analysis

The lyophilized root biomass was subjected to Fourrier-Transformed Infra-Red analysis using a Nicolet iS10 (Thermo Scientific) (Plateforme des Techniques Analytiques, ICOA, France) in order to assess qualitatively and semi-quantitatively the cell wall components.

After experimental analysis, data were normalized using the band at 1670 cm^{-1} (Hano *et al.* 2006). Next, bands characteristics were measured. For the lignin, two bands were used, the one at 1328 cm^{-1} corresponds to the stretching of the bonds of the syringyl groups present on the aromatic nuclei and is characteristic of the subunit S of the lignin. The band at 1234 cm^{-1} corresponds to the stretching of the bonds of the guaiacyles groups present on the aromatic nuclei and is characteristic of the subunit G of the lignin (Bykov 2008). Similarly, two bands were used for the cellulose, the band at 897 cm^{-1} corresponds to the stretching of the bonds C-O-C, characteristic of the presence of amorphous cellulose. The band at 1375 cm^{-1} corresponds to the stretching of the bonds C-H and to the vibration of the bonds COO, characteristic of the presence of crystalline cellulose. The ratio of these two bands indicates the crystallinity of the cellulose (Kavkler *et al.* 2011). Two bands were used for the hemicellulose: the band at 1078 cm^{-1} corresponds to the xyloglucanes and the band at 1089 cm^{-1} corresponds to the xylanes (Scheller and Uluskov 2010). Finally, the band at 1610 cm^{-1} was used for the pectin (Wróbel-Kwiathowska *et al.* 2009).

2.8 Antioxidant enzyme activities in *Salix triandra* roots

Extraction procedure. 0.1 g of fresh root biomass was mixed with 1 mL of phosphate buffer (50 mM, pH 7) containing 1 % polyvinylpyrrolidone (PVP) and 140 mM β-mercapto-ethanol, and crushed in a frozen mortar. The solution was then centrifuged (14 000 x g, 10 min) and the supernatant recovered and stored at -20 °C until further analysis.

Glutathione-S-transferase (GST). GST activity was measured following the protocol of Mauch and Dudler (1993). Briefly, 30 µL of root extract were mixed in a microplate with 1 µmol CNDB, 1 µmol reduced GSH and 100 µmol buffer. Absorbance was read at 340 nm. Activity was calculated using $\varepsilon_{340} = 9.6\ mM^{-1}.cm^{-1}$.

Peroxidase (POD). POD activity was measured following the protocol described in Khan *et al.* (2019). Twenty µL of root extract were mixed with 40 µL buffer, 100 µL dH_2O, 20 µL guaiacol (100 mM) and 20 µL H_2O_2 (10 vol.). Absorbance was measured at 470 nm and activity calculated using $\varepsilon_{470} = 26.6\ mM^{-1}.cm^{-1}$.

Superoxide dismutase (SOD). Similarly, SOD activity was measured based on the protocol of Khan *et al.* (2019). Thirty µL of extract were mixed with 39 µL buffer, 10 µL EDTA (1 mM), 1 µL riboflavin (0.02 mM), 10 µL methionine (130 mM) and 20 µL NBT (0.75 mM). Absorbance was read at 660 nm and SOD activity was calculated using $\varepsilon_{660} = 43.6\ mM^{-1}.cm^{-1}$.

Protein quantification. In order to normalize enzymatic activity values, protein content was quantified using the Bradford method, using BSA as standard.

2.9 Statistical analysis

Data were analyzed using R software version 3.5.1 (R Development Core Team, 2009). After evaluation of the homogeneity (Shapiro test) and homoscedasticity (Bartlett/Levene tests) of the data, means were compared using Anova (parametric data) or Kruskal (non-parametric data) test, followed by a Tukey post-hoc test. Difference was considered significant when $p < 0.05$.

Moreover, a principal component analysis was performed on the plant parameters using the software PAST (Hammer *et al.* 2001).

3. Results

3.1 Amendment characteristics

All amendments presented an alkaline pH, except for L27 (chemically activated carbon) that was very acid (Table 1). Similarly, all the amendments were characterized by a high electrical conductivity, except for one. Amendment BS2 had an EC of 162 $\mu S.cm^{-1}$, while the other amendments were characterized by an EC between 1,004 $\mu S.cm^{-1}$ (redmud) and 2,629 $\mu S.cm^{-1}$ (bamboo biochar) (Table 1). Finally, except for the amendment EK5 that had a negative redox potential (-7 mV) (Table 1), all the other amendments had a positive redox potential, between 83 mV (bamboo biochar) and 525 mV (chemically activated carbon) (Table 1).

Table 1: Amendment physico-chemical properties (pH, electrical conductivity (EC) (µS.cm^{-1}), redox potential (mV)). R = neutralized redmud, BA = bamboo biochar, BS2 = bark-sap biochar, EK5 = steam activated carbon and L27 = chemical activated carbon. (n = 3)

	pH	EC (µS.cm^{-1})	Redox potential (mV)
R	8.6 ± 0.4	1004 ± 12	237 ± 8
BA	10.6 ± 0.1	2629 ± 175	83 ± 15
BS2	8.1 ± 0	162 ± 2	250 ± 1
EK5	12.6 ± 0.1	1637 ± 4	-7 ± 5
L27	1.9 ± 0.1	2680 ± 2	525± 1

3.2 SPW physico-chemical properties

SPWs were sampled at the end of the experiment and analyzed for pH, EC, redox potential and As and Pb concentrations.

In the non-contaminated control soil, pH was neutral at 7.1 (Table 2), while the contaminated PG soil was acidic at pH 4.5. Moreover, all amendments increased SPW pH at a similar level than the control.

Similarly, EC of the control soil was 1,087 µS.cm^{-1} and PG soil had a twice lower EC (536 µS.cm^{-1}) (Table 2) which was increased by all amendments, to levels three times higher than the control and six times higher than PG, on average.

On the contrary, redox potential was lower in control (326 mV) than PG (441 mV) and decreased with amendment application compared to PG (Table 2).

SPW As concentration was low in all substrates and amended conditions did not differ from control and PG (Table 2).

Finally, SPW Pb concentration was high on PG (13.73 mg.L^{-1}) and decreased with all amendments, from 82 % to 96 % (Table 2).

Table 2: Soil pore water physico-chemical properties (pH, electrical conductivity (EC) (µS.cm^{-1}), redox potential (mV), As and Pb concentrations (mg.L^{-1})) determined after 41 days of *Salix triandra* growth on the different substrates. Ctr = control (garden soil), PG = non-amended Pontgibaud, RBA = PG + redmud + bamboo biochar, RBS2 = PG + redmud + bark-sap biochar, REK5 = PG + redmud + steam activated carbon and RL27 = PG + redmud + chemical activated carbon. Letters indicate significant difference ($p < 0.05$) (n = 4).

	pH	EC (µS.cm^{-1})	Redox potential (mV)	[As] (mg.L^{-1})	[Pb] (mg.L^{-1})
Ctr	7.1 ± 0.1 **ab**	1087 ± 96 **b**	326 ± 6 **bc**	0.14 ± 0.01 **ab**	0.15 ± 0.02 **c**
PG	4.5 ± 0.2 **c**	536 ± 29 **c**	441 ± 10 **a**	0.12 ± 0.00 **ab**	13.73 ± 0.83 **a**
RBA	7.1 ± 0.2 **ab**	3785 ± 386 **a**	329 ± 5 **bc**	0.12 ± 0.01 **ab**	1.50 ± 0.20 **bc**
RBS2	6.7 ± 0.0 **a**	3746 ± 452 **a**	347 ± 2 **b**	0.11 ± 0.00 **b**	1.79 ± 0.12 **b**
REK5	7.4 ± 0.1 **b**	3359 ± 235 **a**	315 ± 3 **c**	0.11 ± 0.00 **b**	2.43 ± 0.25 **b**
RL27	6.7 ± 0.2 **a**	2675 ± 334 **a**	344 ± 7 **b**	0.16 ± 0.02 **a**	1.08 ± 0.12 **bc**

3.3 *Salix triandra* growth parameters

Stem height was low on the non-amended soil PG, corresponding to 7.9 cm and for all amended conditions *Salix triandra* presented a higher stem height, between 25.1 and 30.8 cm (Table 3). No difference was observed between amended treatments; however on REK5 and RL27, plant stem height was not significantly different than on control.

On the control substrate, plants produced 63 leaves on average, much more than on PG (13 leaves). Compared to PG, only RBS2 treatment increased plant leaf number (Table 3).

Similarly, leaf area was seven times lower on PG compared to control. Amendment application increased leaf area compared to PG, to levels still lower than the control (Table 3). When looking at the average leaf area, a different trend was observed. Average leaf area was lower on PG (2.43 cm^2) compared to the control (3.84 cm^2) (Table 3). However, only the treatment REK5 had a significant higher average leaf area compared to PG, which was similar to the control (Table 3).

Table 3. *Salix triandra* total leaf area (cm^2), stem height (cm), number of leaves and average leaf area (cm^2.leaf^{-1}) after 41 days of growth on the different substrates. Ctr = control (garden soil), PG = non-amended Pontgibaud, RBA = PG + redmud + bamboo biochar, RBS2 = PG + redmud + bark-sap biochar, REK5 = PG + redmud + steam activated carbon and RL27 = PG + redmud + chemical activated carbon. Letters indicate significant difference ($p < 0.05$) (n = 4).

	Total leaf area (cm^2)	Stem height (cm)	Number of leaves	Average leaf area (cm^2.leaf^{-1})
Ctr	238.49 ± 6.92 **a**	40.3 ± 4.3 **a**	63 ± 4 **a**	3.84 ± 0.34 **a**
PG	34.55 ± 11.62 **c**	7.9 ± 2.0 **c**	13 ± 3 **c**	2.43 ± 0.46 **b**
RBA	89.14 ± 7.64 **b**	25.8 ± 2.2 **b**	24 ± 1 **bc**	3.72 ± 0.27 **ab**
RBS2	100.79 ± 15.72 **b**	25.1 ± 4.4 **b**	27 ± 5 **b**	3.78 ± 0.05 **ab**
REK5	117.99 ± 21.92 **b**	30.8 ± 4.2 **ab**	25 ± 2 **bc**	4.73 ± 0.56 **a**
RL27	91.86 ± 4.76 **b**	28.9 ± 3.1 **ab**	24 ± 1 **bc**	3.79 ± 0.19 **ab**

Finally, DW production was low on PG, with 17 mg roots, 195 mg leaves and 49 mg stem, which was 98 %, 87 % and 95 % lower than the DW produced on the control, respectively (Figure 1). All amendments similarly increased organ DW by three fold for leaves, seven fold for stem and 15 fold for roots. However, DW production was still 30 to 50 % lower than the control (Figure 1).

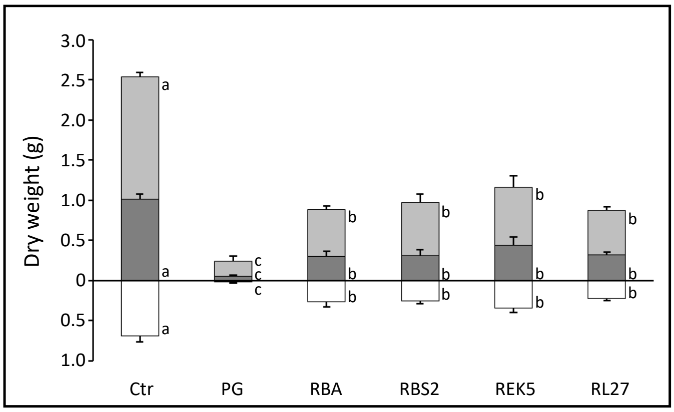

Figure 1: Leaf (light grey), stem (dark grey) and root (white) dry weight (g) of *Salix triandra* plant grown for 41 days on the different substrates. Ctr = control (garden soil), PG = non-amended Pontgibaud, RBA = PG + redmud + bamboo biochar, RBS2 = PG + redmud + bark-sap biochar, REK5 = PG + redmud + steam activated carbon and RL27 = PG + redmud + chemical activated carbon. Letters indicate significant difference ($p < 0.05$) (n = 4).

3.4 *Salix triandra* metal(loid) accumulation

Salix triandra plants accumulated elevated As and Pb concentrations on PG (Figure 2), with higher concentration in the roots compared to the aerial tissues.

Regarding As, all amendments decreased organ As concentrations, except RL27 in leaves and roots. Moreover, As aerial concentration was similar to the control in all amended conditions. Regarding Pb, only root concentrations were decreased by amendments.

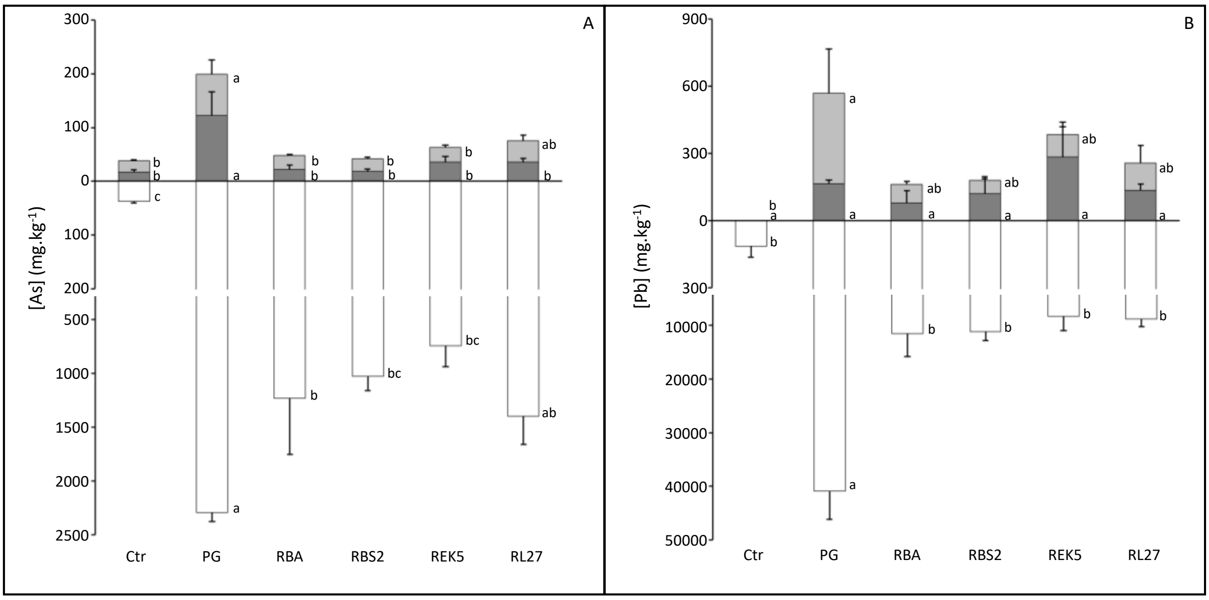

Figure 2: Leaf (light grey), stem (dark grey) and root (white) As (A) and Pb (B) concentrations (mg.kg^{-1}) of *Salix triandra* plant grown for 41 days on the different substrates. Ctr = control (garden soil), PG = non-amended Pontgibaud, RBA = PG + redmud + bamboo biochar, RBS2 = PG + redmud + bark-sap biochar, REK5 = PG + redmud + steam activated carbon and RL27 = PG + redmud + chemical activated carbon. Letters indicate significant difference ($p < 0.05$) (n = 4).

3.5 *Salix triandra* root stress markers

Total anthocyanin content was low on PG and only the condition RBA had a significant higher anthocyanin level compared to PG (Figure 3).

On the contrary, total phenolic content was high on PG compared to the control and a lower content was observed with all amendments, until level similar to the control (Figure 3).

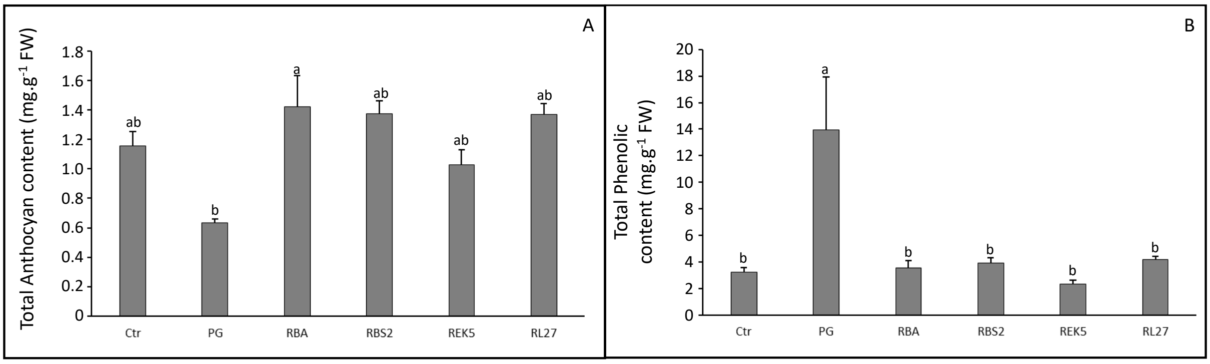

Figure 3: Root total anthocyan (A) and phenolic contents (B) (mg.g^{-1} FW) of *Salix triandra* plant grown for 41 days on the different substrates. Ctr = control (garden soil), PG = non-amended Pontgibaud, RBA = PG + redmud + bamboo biochar, RBS2 = PG + redmud + bark-sap biochar, REK5 = PG + redmud + steam activated carbon and RL27 = PG + redmud + chemical activated carbon. Letters indicate significant difference ($p < 0.05$) (n = 4).

Antioxidant activity, determined by the CUPRAC and DPPH tests, was high on PG compared to the control and the addition of the amendments lowered antioxidant activity measured at the end of the experiment, compared to PG. The antioxidant activity by electron transfer was lower than PG but at a same level than the control in all cases (Figure 4A) whereas the antioxidant activity by hydrogen atom transfer was similar than control level for all the amended conditions except RL27 treatment that presented an antioxidant activity higher than control (Figure 4B). Finally, the antioxidant activity by electron transfer was higher than by hydrogen atom transfer (Figure 4).

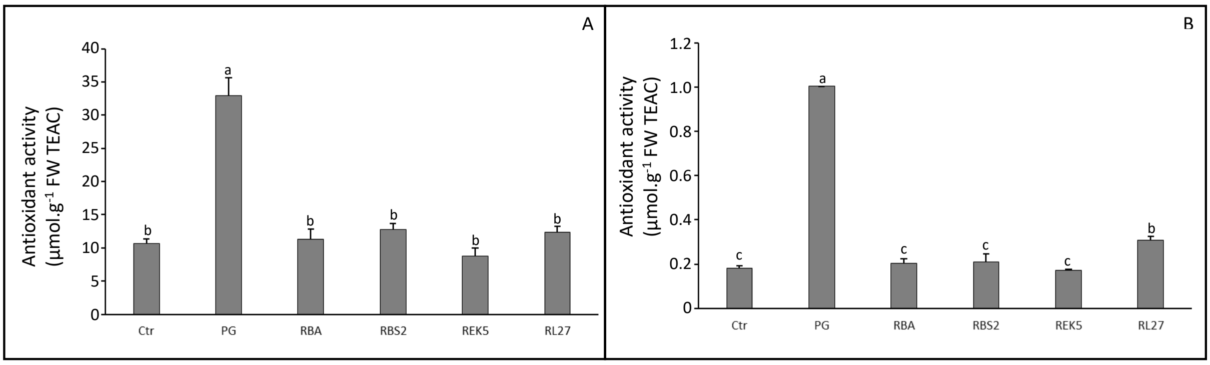

Figure 4: Root antioxidant activity (µmol.g^{-1} FW TEAC) in terms of electron transfer (CUPRAC test) (A) and hydrogen atom transfer (DPPH test) (B) of *Salix triandra* plant grown for 41 days on the different substrates. Ctr = control (garden soil), PG = non-amended Pontgibaud, RBA = PG + redmud + bamboo biochar, RBS2 = PG + redmud + bark-sap biochar, REK5 = PG + redmud + steam activated carbon and RL27 = PG + redmud + chemical activated carbon. Letters indicate significant difference ($p < 0.05$) (n = 4).

Additionally, root chelation capacity was assessed and revealed that plants grown on control and PG soils had a similar chelation capacity and only RL27 treatment presented a lower chelation capacity compared to PG (Figure 5).

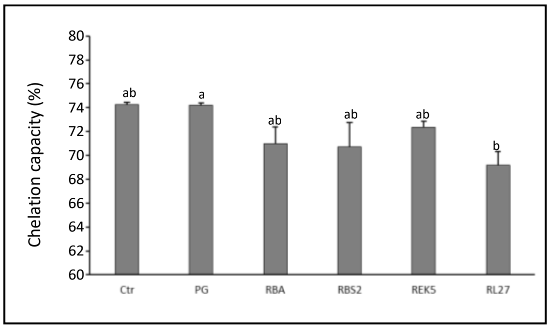

Figure 5: Root chelation capacity (%) of *Salix triandra* plant grown for 41 days on the different substrates. Ctr = control (garden soil), PG = non-amended Pontgibaud, RBA = PG + redmud + bamboo biochar, RBS2 = PG + redmud + bark-sap biochar, REK5 = PG + redmud + steam activated carbon and RL27 = PG + redmud + chemical activated carbon. Letters indicate significant difference ($p < 0.05$) ($n = 4$).

Finally, three enzyme activities were evaluated: glutathione-S-transferase (GST), peroxidase (POD) and superoxide dismutase (SOD). GST activity did not differentiate between PG and the amended conditions, only RL27 condition presented a higher GST activity than the control condition. A similar pattern was observed for POD, whereas SOD activity did not show any different between treatments (Figure 6).

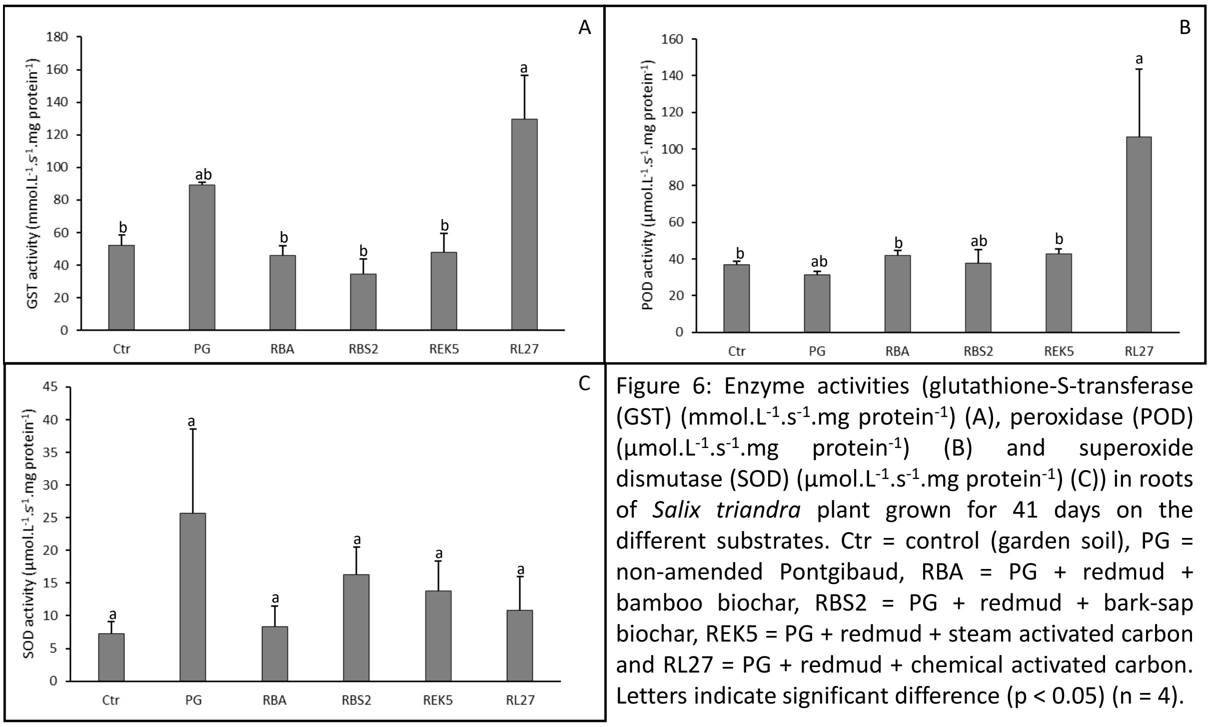

Figure 6: Enzyme activities (glutathione-S-transferase (GST) ($mmol.L^{-1}.s^{-1}.mg\ protein^{-1}$) (A), peroxidase (POD) ($\mu mol.L^{-1}.s^{-1}.mg\ protein^{-1}$) (B) and superoxide dismutase (SOD) ($\mu mol.L^{-1}.s^{-1}.mg\ protein^{-1}$) (C)) in roots of *Salix triandra* plant grown for 41 days on the different substrates. Ctr = control (garden soil), PG = non-amended Pontgibaud, RBA = PG + redmud + bamboo biochar, RBS2 = PG + redmud + bark-sap biochar, REK5 = PG + redmud + steam activated carbon and RL27 = PG + redmud + chemical activated carbon. Letters indicate significant difference ($p < 0.05$) ($n = 4$).

3.6 *Salix triandra* root salicinoid contents

Root extracts were analyzed to measure their contents in salicinoids. The HPLC analysis revealed seven molecules: arbutin, salicin, salicinoside, salicortin, 2’-O-acetylsalicortin, tremuloidin and tremulacin

(Figure S1). All these salicinoids presented a similar trend: a higher content in PG compared to the control, and a lower content with all amendments compared to PG (Figure 7). In more detail, arbutin content was similar in RBA, RBS2 and RL27, whereas its content in REK5 was lower and similar to the control (Figure 7A). Salicin content was the lowest on REK5 while RBS2 and RL27 treatments presented higher contents than REK5 (Figure 7B). Similarly, salidroside content was the lowest in REK5, followed by the control, RBA, RBS2, RL27 and PG (Figure 7C). Salicartin and 2'-O-acetulsalicortin contents followed the same variations than arbutin (Figures 7D and 7E). Finally, tremuloidin and tremulacin contents presented similar variations: lowest contents in control and REK5, followed by RBA, then RBS2 and RL27 and finally PG (Figures 7F and 7G).

Globally, control and REK5 conditions presented the lowest salicinoid contents and PG the highest, whereas RBA, RBS2 and RL27 presented similar intermediary contents. Finally, salicinoids were found in different quantities, in the decreasing order: salicin, salidroside, salicortin, 2'-O-acetylsalicortin, arbutin, tremulodin and tremulacin.

3.7 *Salix triandra* root cell wall content

The total lignin content of the cell wall tended to be higher when plants were grown on the contaminated substrates compared to the control, although it was only significant in the case of RBS2 and REK5 treatments (Table 4). In addition, when considering the two lignin types, only the content in lignin S significantly increased in the treatments PG, RBA and RBS2 compared to the control; whereas the content in lignin subunit G only increased in the condition REK5 compared to the control (Table 4). Finally, the ratio lignin G/lignin S did not show variation compared to the control; however this ratio was higher in the REK5 treatment compared to RBA (Table 4).

The two cellulose forms, amorphous and crystalline, were not affected by the different treatments compared to the control (Table 4). However, the content in crystalline cellulose was higher in REK5 compared to RBA. Finally, the crystallinity of the cellulose did not show variation between the treatments (Table 4).

Similarly to the cellulose content, the content in hemicellulose was not affected by the treatments compared to the control; however the contents in xyloglucanes (XylG) and xylanes (XylA) were again superior in REK5 compared to RBA (Table 4). Finally the ratio XylG/XylA was not affected by the treatments (Table 4).

Similarly to the hemicellulose, the content in pectin did not show differences among the treatments (Table 4).

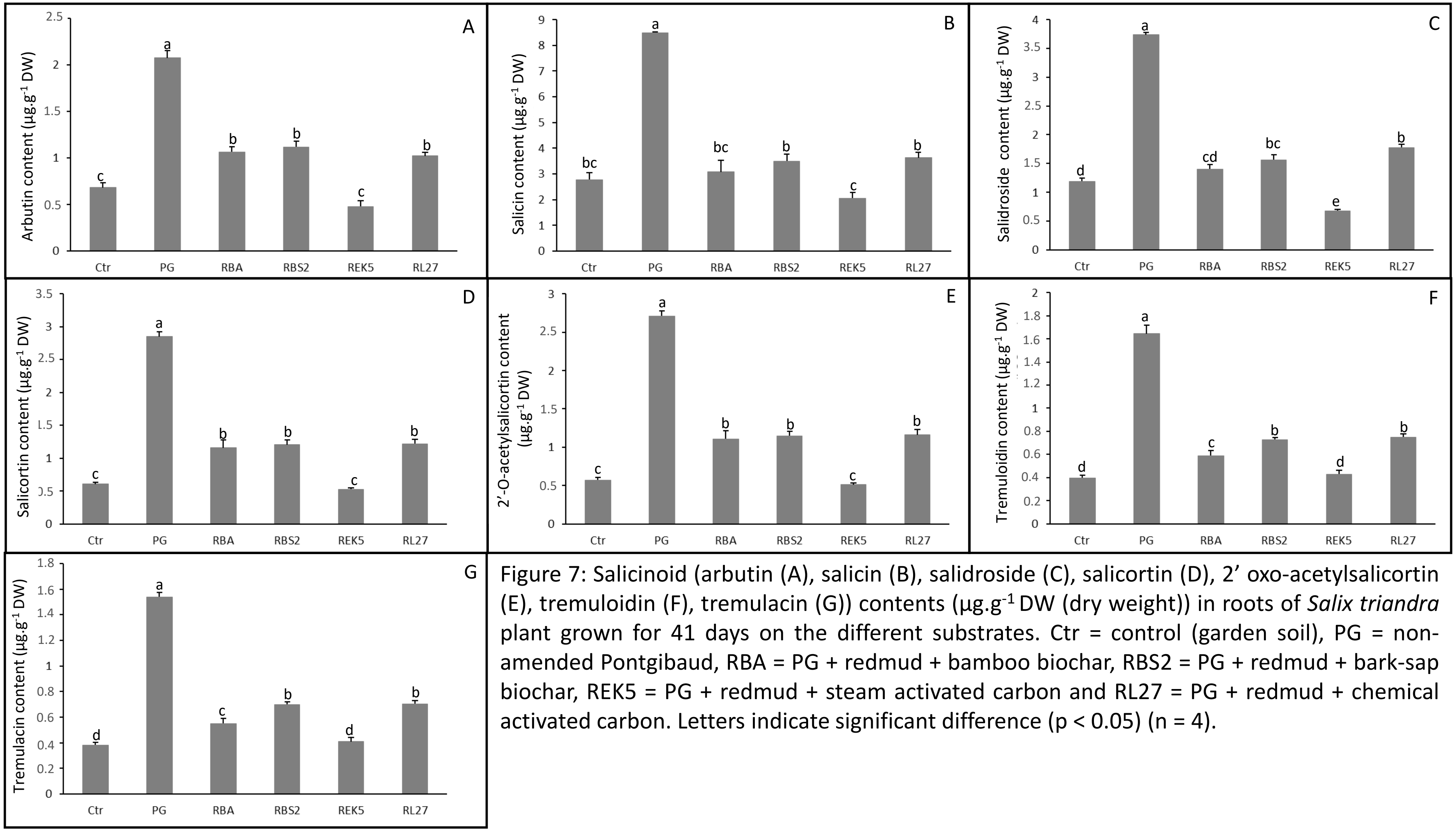

Figure 7: Salicinoid (arbutin (A), salicin (B), salidroside (C), salicortin (D), 2' oxo-acetylsalicortin (E), tremuloidin (F), tremulacin (G)) contents (µg.g^{-1} DW (dry weight)) in roots of *Salix triandra* plant grown for 41 days on the different substrates. Ctr = control (garden soil), PG = non-amended Pontgibaud, RBA = PG + redmud + bamboo biochar, RBS2 = PG + redmud + bark-sap biochar, REK5 = PG + redmud + steam activated carbon and RL27 = PG + redmud + chemical activated carbon. Letters indicate significant difference ($p < 0.05$) ($n = 4$).

Table 4. FTIR analysis of cell wall components (normalized transmittance values) of *Salix triandra* roots after 41 days of growth on the different substrates. Ctr = control (garden soil), PG = non-amended Pontgibaud, RBA = PG + redmud + bamboo biochar, RBS2 = PG + redmud + bark-sap biochar, REK5 = PG + redmud + steam activated carbon and RL27 = PG + redmud + chemical activated carbon. Data were acuired by Fourrier Transformed Infra Red method and results were normalized using the band at 1670 cm^{-1}. XylG = xyloglucanes; Xyl1 = xylanes. Letters indicate significant difference ($p < 0.05$) ($n = 4$).

	Lignin				Cellulose			Hemicellulose			
	Total (1328, cm^{-1} 1234 cm^{-1})	Sub-unit S (1328 cm^{-1})	Sub-unit G (1234 cm^{-1})	Ratio G/S	Amorphous form (897 cm^{-1})	Crystallin form (1375 cm^{-1})	Index of cristallinity	XylG (1078 cm^{-1})	XylA (1089 cm^{-1})	Ratio XylG/XylA	Pectin (1610 cm^{-1})
Ctr	0.970 ± 0.005 **b**	0.979 ± 0.003 **b**	0.960 ± 0.007 **b**	0.981 ± 0.004 **ab**	0.972 ± 0.007 **a**	0.872 ± 0.026 **ab**	89.70 ± 2.07 **a**	0.885 ± 0.023 **ab**	0.953 ± 0.009 **ab**	92.83 ± 1.50 **a**	0.999 ± 0.001 **a**
PG	0.996 ± 0.003 **ab**	1.006 ± 0.004 **a**	0.986 ± 0.001 **ab**	0.980 ± 0.003 **ab**	0.986 ± 0.005 **a**	0.857 ± 0.017 **ab**	86.90 ± 1.35 **a**	0.873 ± 0.016 **ab**	0.951 ± 0.011 **ab**	91.86 ± 0.54 **a**	0.998 ± 0.007 **a**
RBA	0.991 ± 0.008 **ab**	1.001 ± 0.007 **a**	0.981 ± 0.009 **ab**	0.980 ± 0.003 **b**	0.967 ± 0.005 **a**	0.830 ± 0.025 **b**	85.80 ± 2.46 **a**	0.843 ± 0.024 **b**	0.950 ± 0.006 **b**	88.73 ± 2.04 **a**	0.990 ± 0.010 **a**
RBS2	0.994 ± 0.007 **a**	1.002 ± 0.006 **a**	0.987 ± 0.009 **ab**	0.985 ± 0.004 **ab**	0.974 ± 0.011 **a**	0.861 ± 0.031 **ab**	88.35 ± 2.52 **a**	0.872 ± 0.028 **ab**	0.959 ± 0.011 **ab**	90.82 ± 2.05 **a**	0.991 ± 0.001 **a**
REK5	0.991 ± 0.002 **a**	0.995 ± 0.002 **ab**	0.988 ± 0.003 **a**	0.993 ± 0.002 **a**	0.994 ± 0.003 **a**	0.931 ± 0.016 **a**	93.71 ± 1.30 **a**	0.936 ± 0.015 **a**	0.986 ± 0.005 **a**	94.97 ± 1.09 **a**	1.005 ± 0.001 **a**
RL27	0.981 ± 0.002 **ab**	0.989 ± 0.002 **ab**	0.973 ± 0.003 **ab**	0.984 ± 0.002 **ab**	0.981 ± 0.006 **a**	0.862 ± 0.014 **ab**	87.86 ± 1.36 **a**	0.875 ± 0.013 **ab**	0.962 ± 0.007 **ab**	90.87 ± 0.79 **a**	1.002 ± 0.003 **a**

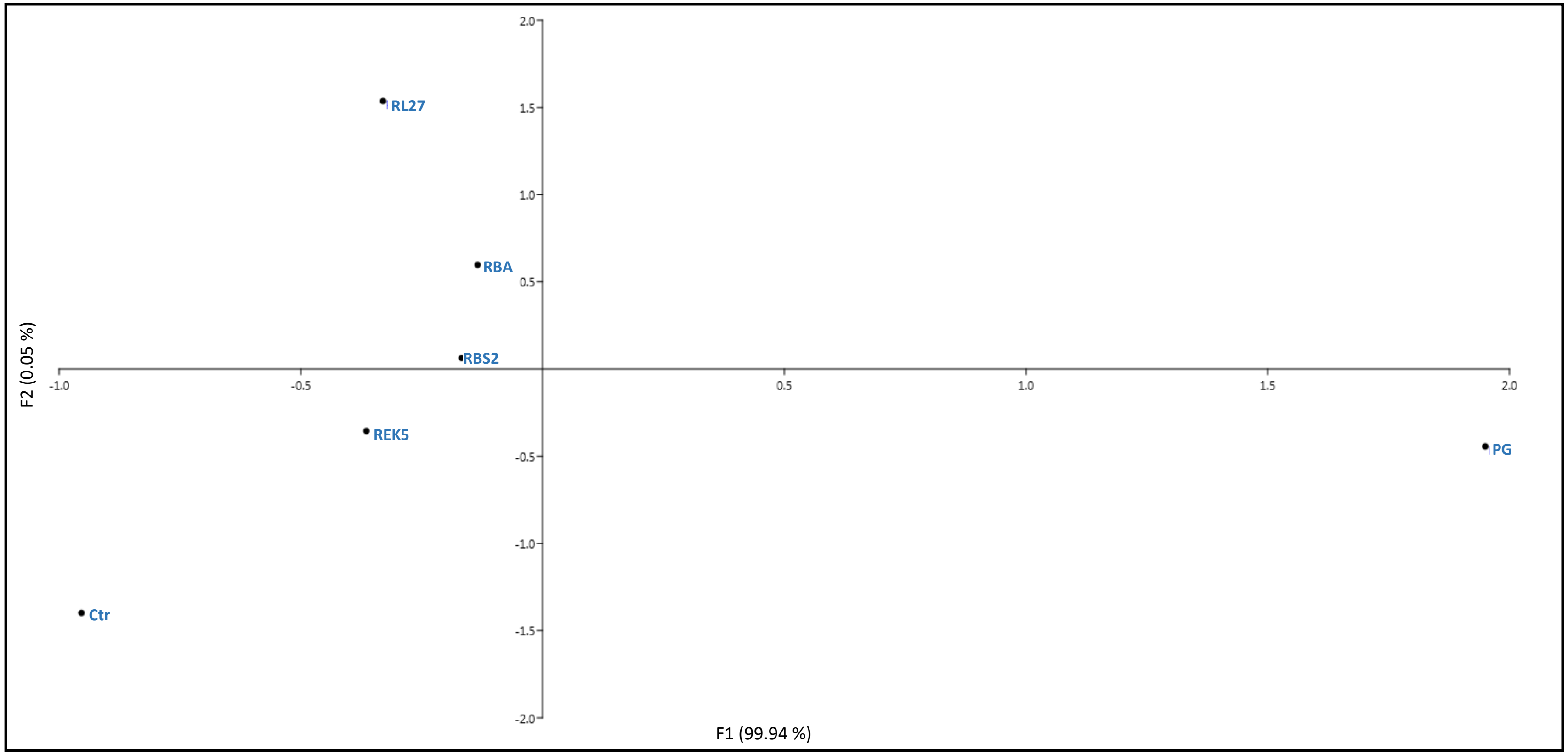

Figure 8: Principal component analysis of the parameters measured in *Salix triandra* plant grown for 41 days on the different substrates. Ctr = control (garden soil), PG = non-amended Pontgibaud, RBA = PG + redmud + bamboo biochar, RBS2 = PG + redmud + bark-sap biochar, REK5 = PG + redmud + steam activated carbon and RL27 = PG + redmud + chemical activated carbon.

3.8 Correlation analysis of the plant parameters

Principal component analysis was applied on plant parameters in order to discriminate the treatments. The resulting biplot showed that 99.94 % of the variability was explained by F1 axis whereas F2 axis only explained 0.05 % of the variability (Figure 8). Moreover, the biplot representation showed that three groups could be formed along the F1 axis, which was mainly constrained by Pb root concentrations, and to a lesser extent As root concentrations and Pb leaf concentrations (Figure S2A): PG and control treatments were located at the two extremities, whereas the third group was composed of the amended conditions, located more closely to the control condition. From the second axis (F2), constrained by root As concentrations and to a lesser extent leaf surface area, number of leaves, Pb concentrations in root and stem, GST and POD activities (Figure S2B), three groups could be made among the amended treatments: REK was located below the axis, RL27 at the top, whereas RBA and RBS2 conditions could be grouped together and were located between the other two treatments.

4. Discussion

4.1 SPW physico-chemical properties

Soil pH is an important parameter to assess as it affects many processes in soil and especially metal(loid) behavior but also nutrient availability. Previous studies showed the potential of biochar and redmud to increase pH of an acidic soil (Lebrun *et al.* 2017, 2019, Nandillon *et al.* 2019a, Zhou *et al.* 2017), mostly explained by their alkalinity (Moore *et al.* 2017, Zhou *et al.* 2017, Dai *et al.* 2018). Indeed, except amendment L27 that was very acidic (pH 1.2) (Table 1), all amendments used were alkaline between pH 8.1 and pH 12.6 (Table 1). Moreover, even though L27 was very acidic, its application together with redmud still led to a SPW pH increase, which was similar to the treatment combining redmud (pH 8.6) and BS2 (pH 8.1). Therefore, redmud seems to be efficient to increase soil pH and counteract L27 acidity.

Similarly, SPW EC was increased with all amendments, which is consistent with previous studies and could be related to the high EC of the amendment used (Table 1) (Lee *et al.* 2009, 2014, Garau *et al.* 2014, Lebrun *et al.* 2019). However, SPW EC values of the amended substrates were, in most cases, higher than amendment EC values. Thus the increase in SPW EC can be explained by the dissolution of soil and amendments organic matter and other soluble salts into SPW which happened during the entire experiment time course through the interaction between the soil and the amendment (Lebrun *et al.* 2017, Nandillon *et al.* 2019b).

Contrary to pH and EC, SPW redox potential (Eh) decreased following amendments, which can be related to the low redox potential of most amendments, especially EK5. Moreover, soil Eh is known to behave oppositely to pH, as demonstrated by the highly significant negative correlation between SPW pH and Eh ($R^2 = - 0.99$, $p < 0.001$) (Rinklebe *et al.* 2016).

In previous studies, biochar amendment applied alone to metal(loid)s polluted soils showed generally negative effects on SPW As concentration, *i.e.* increase of As mobility, although some studies also showed that biochar had no effect or had a positive effect, *i.e.* decrease in SPW As concentration (Beesley *et al.* 2010, 2014, Lebrun *et al.* 2017, Nandillon *et al*. 2019a). Altundoğan *et al.* (2000) and Garau *et al.* (2011) showed that redmud had an affinity toward As and thus can immobilize it. Therefore, the non-effect of amendment application on SPW As concentration observed here could be due to a compensation of the negative effect of biochar by the positive effect of redmud, leading to a neutralized effect, or to a non-effect of the two amendments. Moreover, As is known to be mobilized with increasing pH, which could have happened here. However, such mobilized As could have been directly sorbed by redmud. Finally, even though SPW As concentrations were not modified, the application of amendment could have modified As speciation, rendering it less toxic.

Finally, SPW Pb concentrations were shown greatly decreased by amendments. Indeed, both biochar and redmud can sorb positively charged elements due to their compositions. Biochar surface is negatively charged which allows electrostatic attraction with positively charged ions (Ahmad *et al.* 2016) whereas redmud contains many iron and aluminum oxides than can sequester metal(loid)s (Zhou *et al.* 2017). However, here the association redmud + biochar did not lead to a better Pb immobilization than the one observed in previous studies with biochar and iron grit (Lebrun *et al.* 2018, 2019). Therefore, other than the sorption on amendment surface, Pb immobilization can be explained by the pH increase induced by amendment, explanation supported by the highly significant negative correlation between SPW pH and SPW Pb concentration ($R^2 = -0.91$, $p < 0.001$). Indeed, the soil pH increase promotes the sorption of metal(loid)s on soil colloids as well as the formation of metal(loid) carbonates and hydroxide precipitates, leading to their immobilization and thus decrease concentration in SPW (Ahmad *et al.* 2016, Zhou *et al.* 2017, Dai *et al.* 2018).

4.2 Salix triandra growth

Compared to the control condition, *Salix triandra* growth parameters were highly decreased on PG, which is one of the negative effects of metal(loid)s (Chaoui *et al.* 1997, Ali *et al.* 2006, Fernández *et al.* 2013). Additionally, *Salix triandra* growth could have been impaired by the low fertility of the soil, *i.e.* low nutrient availability, low organic matter content and acidic pH, as shown in previous studies (Lebrun *et al.* 2017, 2018, 2019). Compared to another study, *Salix triandra* presented a higher impairment of leaf DW than *Salix alba* (80 %), *Salix viminalis* (70 %) and *Salix purpurea* (68 %) but a similar decrease in stem and root DW (Lebrun *et al.* 2017).

Amendment application to PG soil increased all *Salix triandra* growth parameters except leaf number, which could be directly related to the amelioration of the soil conditions. Indeed, many studies observed an improvement of plant growth with the amelioration of soil conditions induced by amendment application (Agegnehu *et al.* 2016, Fresno *et al.* 2017, Mehmood *et al.* 2018, Clemente *et al.* 2019, Zhang *et al.* 2019).

4.3 *Salix triandra* As and Pb accumulation

Arsenic and lead plant contents were decreased in the amended conditions compared to PG, which could be related to the soil immobilization observed following amendment application or a modification of their speciation, which reduced their uptake. Moreover, a lower concentration could also be due to a dilution effect, as organ DWs were higher in the amended treatments.

Finally, As and Pb were mainly accumulated in the roots with a low translocation towards upper parts, which is often observed in *Salix* plants (Bart *et al.* 2016, Lebrun *et al.* 2017, 2019) and underlines the "trap" function of the roots to protect photosynthetic organs (Drzewiecka *et al.* 2012) but also the ability of roots to not only absorb metal(loid)s but also adsorb them on their surface.

4.4 *Salix triandra* biochemical profiles at the end of the experiment

Exposure to elevated concentrations of metal(loid)s is known to induce an oxidative stress through the overproduction of ROS (Demirevska-Kepova *et al.* 2004, Ahmad *et al.* 2009, Kováčik *et al.* 2009). In response to such oxidative stress, plants activate their antioxidative system composed of non-enzymatic and enzymatic elements.

Total phenolic compounds (TPC) content is a highly sensitive stress marker that generally increases in response to stress (Jaskulak *et al.* 2018). Indeed, plants submitted to oxidative stress promote phenolic production that participates in the scavenging of ROS (Kováčik and Klejdus 2008). The high level of TPC in plants grown on non-amended PG soil reflected the important oxidative stress encountered by these plants. On the contrary, TPCs in plants grown on amended PG were low and similar to the control, revealing that plants did not suffer from oxidative stress when grown on amended PG. Comforting this fact, antioxidant activity was increased in PG compared to control, showing that an important free radical scavenging activity occurred in roots of *Salix triandra* grown on PG (Ali *et al.* 2006). Moreover, by comparing the two tests used to assess antioxidant activity, CUPRAC and DPPH, it can be seen that the scavenging activity occurred mainly through electron transfer even in non-stressed conditions. Furthermore, even though amendments decreased oxidative stress, antioxidant activity through hydrogen atom transfer was higher in RL27 compared to the other amended treatments, showing a slightly higher oxidative stress in this condition, which can be related to the lower chelation capacity of the roots in such condition.

Moreover, in response to stress, plants can also activate enzymes that will detoxify free radicals (Bai *et al.* 2009). Enzyme activities differed depending on treatment and enzyme type. However, in general, PG plants showed an elevated GST and SOD activities whereas RL27 plants presented high activities of GST and POD. The other amended treatments showed similar activities to the control. Elevated enzyme activity is a marker of enhanced ROS production (Goswani and Das 2016) and related scavenging. Indeed, GST, POD and POD have a role in metal(loid) and ROS detoxification (Tamás *et al.* 2008). SOD is a metalloprotein catalyzing the dismutation of superoxide to H_2O_2 (Goswani and Das 2016) and its elevated level might protect plants from oxidative damage (Wang *et al.* 2008, Gao *et al.*

2010). Similarly, POD is one of the principal enzymes involved in the elimination of ROS (Goswani and Das 2016). These first results showed that on Pontgibaud soil, plants greatly suffered from oxidative stress. This stress was suppressed by the addition of amendment combinations, except for one treatment (RL27) that only reduced oxidative stress but in which oxidative stress was still higher than the control condition.

Salicinoid contents were also greatly increased when grown on the contaminated PG soil. Such observation was commonly observed when plants were under stress. Indeed, although their study did not focus on the response to metal(loid) stress, previous studies showed that *Salicaceae* species increased the synthesis of secondary metabolites, such as salicin, arbutin and other phenolic glycosides, when exposed to water, Ag and herbivory stresses (Boeckler *et al.* 2011, Cheynier *et al.* 2013, Popović *et al.* 2016, Zhang *et al.* 2018). Such observations underlined the importance of salicinoid compounds in the defense towards the high metal(loid) (As and Pb) concentrations encountered on Pontgibaud. Moreover, such elevated salicinoid contents were reduced when amendments were added, especially for one treatment (REK5) that presented similar or lower levels of salicinoids than in control. These results again testified that adding amendments reduced the stress plants were under, which could be related to the reduced acidity and metal(loid) mobility induced by amendments.

Finally, the content of the cell wall of *Salix triandra* root did not show great modification in response to metal(loid) stress and amendment application. The most important response was an increase in lignin, especially in the case of the amended conditions. Lignin has the effect to enhance the rigidity of the cell wall and is an important barrier against plant stress. Indeed, the stress induced by biotic or abiotic factors to the plant is often accompanied by an increase in ROS content but also in lignin content (Liu *et al.* 2018). Lignin contains an elevated number of functional groups that can bind metal(loid)s and thus prevent their entry in the cytoplasm and thus their translocation towards upper parts (Liu *et al.* 2018). Therefore, the increase in lignin observed under the contaminated treatments can be a direct response of the presence of metal(loid)s in the soil and their entry into the roots.

5. Conclusion

A mesocosm study was set up in order to evaluate the effect of diverse amendment combinations on soil properties and *Salix triandra* growth, metal(loid) accumulation and oxidative stress level, and thus their potential in phytomanagement.

The results showed that on Pontgibaud soil, plants greatly suffered from stress, as shown by their reduced growth and high stress markers. The addition of redmud combined with different carbon-based materials, biochar and activated carbon, improved soil conditions and immobilized Pb. Such ameliorations led to a better plant growth. In general, plant growth and metal(loid) accumulation patterns did not discriminate amended conditions. However, biochemical analysis of the root material showed that among the diverse treatments, plants grown on RL27 amendment still presented high enzymatic and non-enzymatic antioxidative compounds. Moreover, salicinoid contents were the lowest with REK5 amendments. Finally, when taking all of the plant parameters together, REK5 seems to be the best amendment, showing clearly a better growth than on PG and stress marker levels similar or lower than on control.

In conclusion, in a phytomanagement strategy, the combination of neutralized redmud associated to stream activated carbon could be applied on Pontgibaud soil in order to improve soil conditions and thus ameliorate *Salix triandra* growth and reduce its oxidative stress.

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Supplementary material

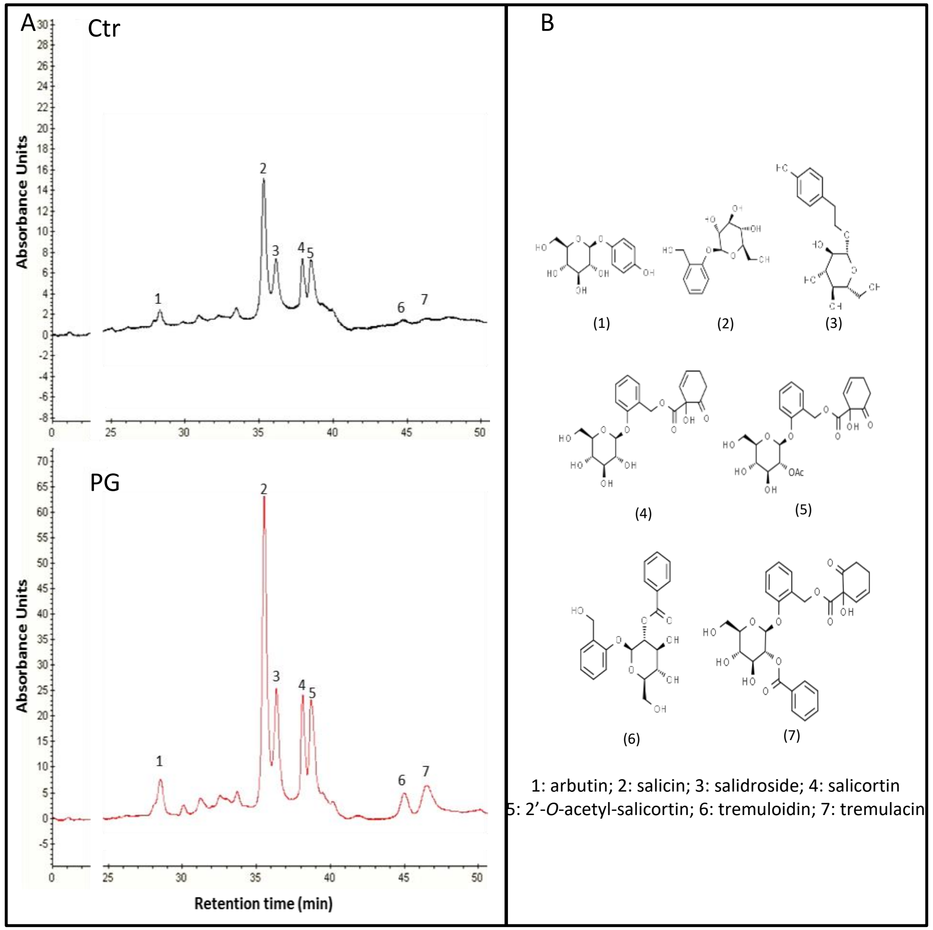


Figure S1: HPLC chromatograms (A) of two conditions and salicinoid structures (B). Ctr = control (garden soil), PG = non-amended Pontgibaud. Numbers on the chromatograms correspond to the number of the molecule structures.

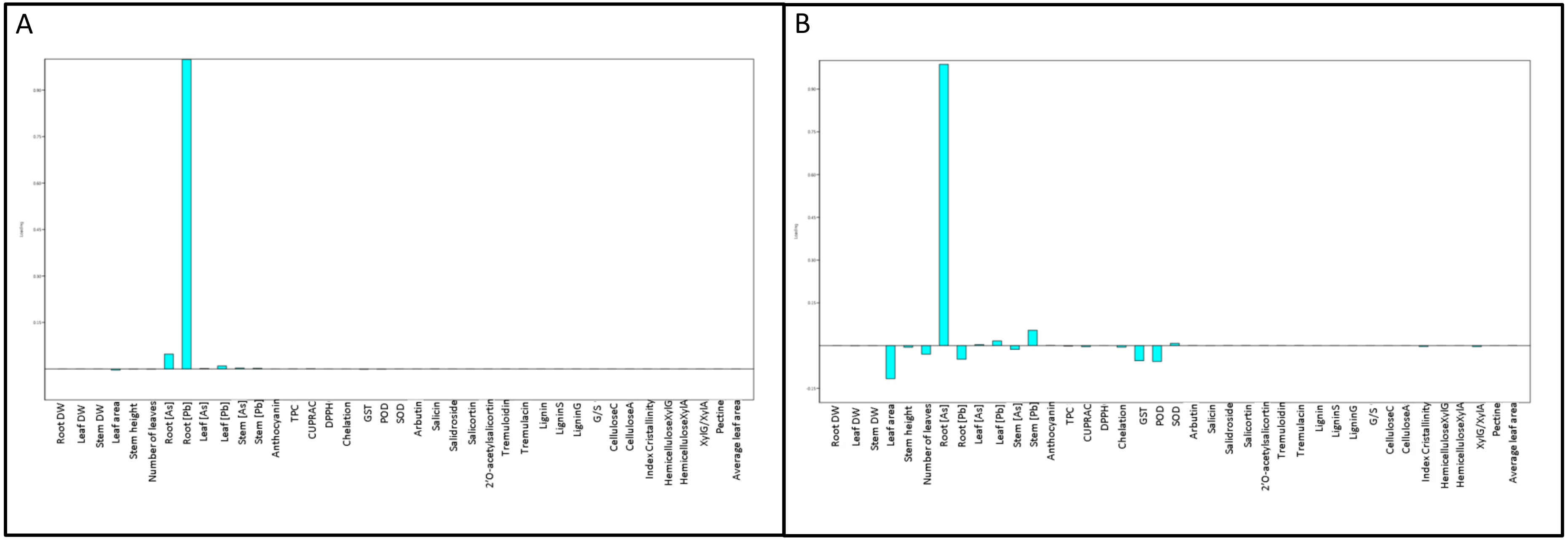
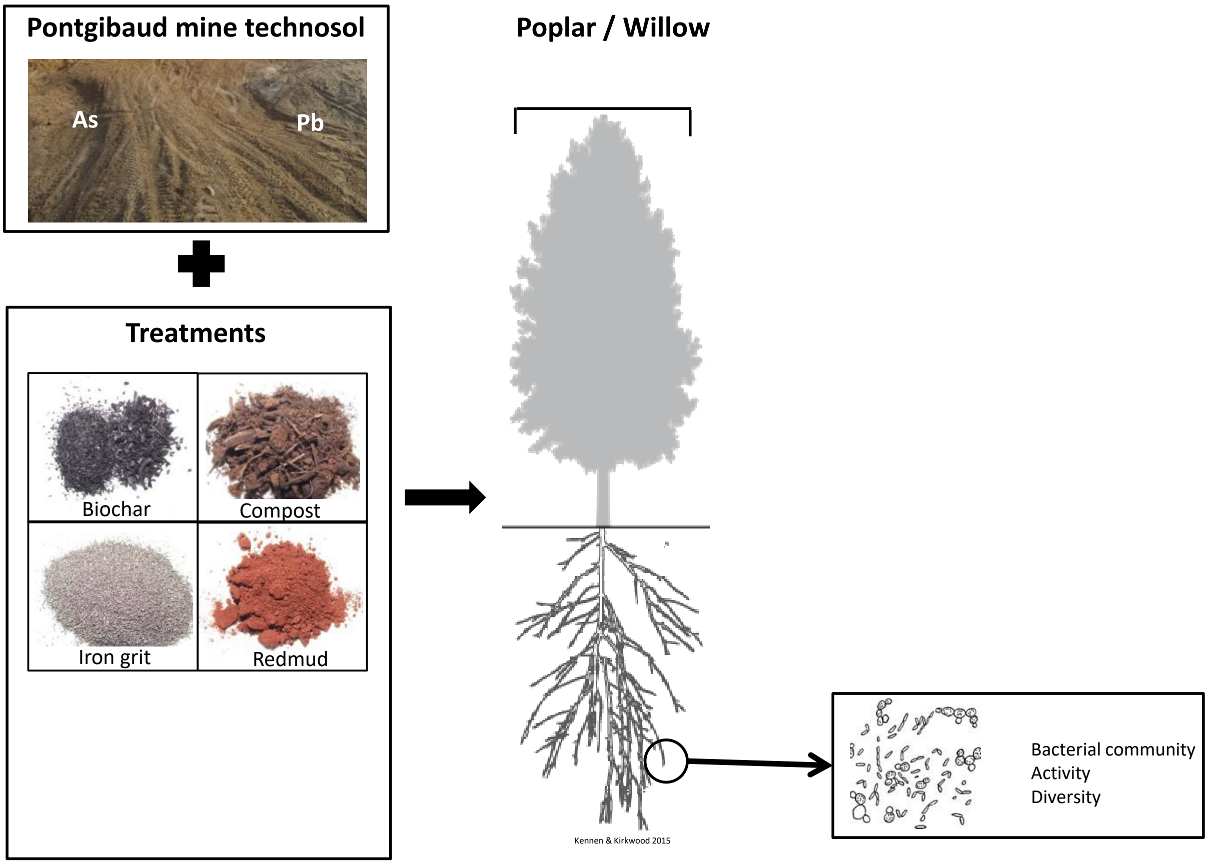


Figure S2: Loading scores of the first two axis (F1 (A), F2 (B)) of the principal component analysis of the parameters measured in *Salix triandra* plant grown for 41 days on the different substrates.

This chapter gave an overview of the physiological and biochemical responses of plants to metal(loid) stress and amendment application, at the root level. The three studies showed that, in response to amendments, *Salix viminalis* plants modified their root proteome profile, especially by redirecting the metabolic fluxes towards the primary and secondary metabolisms in response to biochar and compost. Moreover, plant roots still suffered from oxidative stress after biochar + iron amendments. However, the same amendments had a small effect on organic acids root exudation, although a trend was observed for the plant presenting a reduced metal(loid) stress. Indeed, with biochar and/or compost, organic acids exudation tended to increase. Finally, the third study showed that when grown on Pontgibaud, *Salix triandra* plants suffered from oxidative stress, which was repressed with amendment application, except for one condition, using an acidic activated carbon, which still showed oxidative stress, although reduced compared to the non-amended Pontgibaud.

Chapter 4. Amendment effects on the soil bacterial diversity and activity.



This fourth chapter (objective 1d) will focus on the soil bacterial community, in terms of activity and diversity. It will be divided in two parts and will be a continuation of two works presented in the chapter 2.

The first part is a continuation of the experiment described in the part D of the chapter 2 (pages 197 to 221), evaluating the effect of biochar, compost and/or iron amendment on the soil physico-chemical properties and *Salix viminalis* growth and metal(loid) accumulation capacity. The previous study showed that compost, alone or in combination with biochar, was effective in ameliorating soil condition and *Salix viminalis* growth. The study presented in this chapter aimed at evaluating the effect of biochar, compost and/or iron grit amendments on the soil bacterial community activity and diversity. This work has been written for a future submission to the *Journal of Soils and Sediments*.

The second part is a continuation of the experiment of part E (pages 225 to 251) demonstrating the efficiency of diverse amendments (fertilizer, biochar, activated carbon, redmud) to stabilize metal(loid)s, sustain *Salix dasyclados* growth and affect plant metal(loid) accumulation. The study showed that neutralized redmud was a good amendment to improve soil condition, immobilize Pb and ameliorate *Salix dasyclados* growth. In this chapter, the study aimed at assessing the effects of amending Pontgibaud mine soil with a fertilizer, two biochars and/or two redmuds on the soil bacterial community. This work has been written for a future submission.

These two parts of the study used the same methods, *i.e* enzyme activity measurements, Biolog EcoPlates^{TM} and DNA extraction followed by 16S sequencing, which will allow a comparison of the results at the end of this chapter, regarding the effect of contrasted amendments on the soil bacterial community.

Part A. Effect of amendment application and *Salix viminalis* growth on the soil bacterial community composition and activity of a former mining technosol.

Effect of amendment application and *Salix viminalis* growth on the soil bacterial community composition and activity of a former mining Technosol

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Abstract

Purpose. Amendment application in phytoremediation to improve soil condition can help in establishing a plant cover. Biochar, compost and iron grit showed good results on soil and plants. However, their combined application was less studied, especially regarding the bacterial community, an important parameter in soil remediation. This study aimed to assess the effect of the amendment treatments on: (i) the soil enzymatic activities, (ii) the bacterial community level physiological profiles and (iii) the bacterial community composition.

Methods. A mesocosm experiment was conducted using a former mine Technosol highly contaminated by As and Pb and amended with 5 % biochar, 5 % compost and 1.5 % iron, either alone or combined. After 69 days of *Salix viminalis* growth, soil samples were collected in both non-vegetated (bulk) and vegetated (rhizosphere) conditions. Soils were analyzed for soil enzyme activities (acid phosphatase, alkaline phosphatase and fluorescein diacetate hydrolysis), community level physiological profiles, using Biolog EcoPlates^{TM} and bacterial community composition through 16S rRNA sequencing.

Results. At the end of the experiment, soil enzyme activities were higher in the amended conditions compared to the mine soil. Moreover, amendment application increased the capacity of the bacterial community to use diverse carbon sources. The carbon sources already used by the bacterial community of the mine soil were also more importantly used in the amended conditions. Next-generation sequencing revealed the presence of phyla commonly retrieved in soil ecosystems (*Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, *Gemmatimonadetes*, and *Firmicutes*) but the different amendments, and compost application specifically, induced a shift in bacterial and archaeal communities. As compost is an amendment rich in microorganisms, its application to soil introduced new microorganisms, which can explain the more important effect of compost amendment.

Conclusion. All of the observed effects were more important when compost was added, either alone or combined, which is consistent with its high content in microorganisms. However, plant growth had little effect. Moreover, these results were in correlation with the results observed on *Salix viminalis* plants in a previous study.

Keywords

Amendments; Bacteria; Biolog EcoPlates^{TM}; Soil enzyme activities; Metal(loid)s; Next Generation Sequencing

1. Introduction

Assisted phytostabilization of metal(loid) polluted soils associates amendment application with the establishment of a plant cover, to diminish the toxic effects of the pollutants (Cristaldi *et al.* 2017). The application of diverse amendments, such as biochar, compost and iron grit, to highly contaminated and poorly fertile soils will improve the soil physico-chemical properties (reduction of acidity, supply of organic matter and nutrients) (Adriano *et al.* 2004, Lebrun *et al.* 2018b) and reduce metal(loid) stress (reduction of metal(loid) exchangeable, extractable fractions and soil pore water concentrations) (Lebrun *et al.* 2018a, b). Such ameliorations will allow a better plant growth. Plant cover success is an important part of the phytostabilization process because plants will accumulate metal(loid)s in their roots and their root activities will modify the rhizosphere environment conditions impacting metal(loid) behavior. Moreover, the plant cover can stabilize the soil and consequently limits wind erosion and water leaching (Barceló and Poschenrieder 2003).

In addition to the amendments and plants, another soil biotope plays an important role for phytostabilization success: bacteria. Indeed, even though metal(loid)s cannot be degraded, bacteria can control the transformation of metal(loid)s by various mechanisms, such as oxidation, reduction, (de)methylation, complex formation and biosorption (Adriano *et al.* 2004) and influence the solubility and speciation of metal(loid)s (Wenzel 2008). Finally, the bacterial diversity is hypothesized to play a crucial role in the stability of the ecosystem productivity, functions and resilience towards stress (Wu *et al.* 2019). In return, the bacterial community is also influenced by the environment conditions.

Both bacterial community composition and activities are important parameters to assess in a remediation approach. This can be done by diverse techniques, for instance the measurement of the soil enzymatic activities, community level physiological profiles (CLPP) and next-generation sequencing (NGS).

Soil enzymatic activities are important indicators of the soil fertility as well as the changes taking place in the soil environment (Mierzwa-Hersztek *et al.* 2016). Indeed, such activities reflect the status of the whole bacterial community and represent one of the most reactive components of the soil (Epelde *et al.* 2008). Moreover, they are highly sensitive to excess concentrations of soluble metal(loid)s and have been recommended as biochemical indicators for the evolution of the soil quality during the remediation process (Touceda-González *et al.* 2017). It is generally believed that high soil enzymatic activities imply a good quality of the soil (Mierzwa-Hersztek *et al.* 2016). However, contrary to CLPP, the enzyme tests in soil are associated with both living and dead bacterial cells (Al Marzooqi and Youssef 2017).

Community level physiological profiles are based on the ability of bacteria to oxidize different carbon substrates (Gomez *et al.* 2006). CLPP can be assessed through the use of Biolog Ecoplates™. They consist of plates of 96 wells containing 31 different carbon sources and a blank, all in triplicates. When the carbon source is used by the cultivable portion of the bacterial community, the tetrazolium violet present in each well is reduced and a purple color develops (Epelde *et al.* 2008, Cesarano *et al.* 2017). Such color development can be assessed spectrophotometrically. Ecoplates are a simple and rapid method to observe the biological response of the bacterial community (Epelde *et al.* 2008, Cesarano *et al.* 2017).

Finally, the bacterial community composition can also be revealed through the sequencing of the 16S rRNA gene. The high-throughput sequencing approach identifies the bacterial communities based on OTUs (Operational Taxonomic Units). Contrary to the two previous techniques, which inform about the activity of the bacterial community, this technique gives a pattern of the bacterial community arrangement in terms of Domain, *Phylum*, Class, Order, Family, *Genus* and Species (when possible).

This study was a continuation of a previous work (Lebrun *et al.* 2019) evaluating, in mesocosm, the effects of amendment application to a mining soil on the soil physico-chemical properties and *Salix viminalis* growth and metal(loid) accumulation pattern.

Additionally, soil samples were collected at the end of the experiment and analyzed at the microbiological level. The aims of the present study were thus to evaluate the effect of biochar, compost and/or iron grit amendment, as well as *S. viminalis* growth, on (i) soil enzymatic activities, (ii) CLPP, (iii) bacterial community composition. The use of diverse techniques to assess the bacterial community activity and structure allowed having the most complete picture of the bacterial community and how it is shaped by amendments and/or plant growth.

2. Materials and Methods

2.1 Experimental design

The study focused on a former silver-lead mine extraction site, located in Pontgibaud (Auvergne-Rhône-Alpes, France). The mining activity led to high contamination with As (539 $mg.kg^{-1}$) and Pb (11,454 $mg.kg^{-1}$) (Lebrun *et al.* 2017).

This Technosol was amended with three different amendments, a commercial hardwood biochar (5 %, w/w), a commercial compost (5 %, w/w) and an industrial iron grit (1.5 %, w/w). These amendments were applied alone or combined, giving seven treatments in total: unamended Pontgibaud (P), Pontgibaud + biochar (PB), Pontgibaud + compost (PC), Pontgibaud + iron grit (PI), Pontgibaud + biochar + compost (PBC), Pontgibaud + biochar + iron grit (PBI), Pontgibaud + biochar + compost + iron grit (PBCI). One non-rooted cutting of *Salix viminalis* was placed in 14 pots and five were left un-vegetated. The experiment lasted for 69 days, under greenhouse conditions: temperature 22 ± 2 °C, light intensity 800 $\mu mol.m^{-2}.s^{-1}$, and photoperiod 16 h.

2.2 Soil sampling

After 69 days, soil samples were collected in each pot, both vegetated (rhizosphere) and un-vegetated (bulk). For the vegetated condition, rhizosphere soil was collected by shaking the roots inside a sterile plastic bag. For each treatment and condition (bulk/rhizosphere), a composite sample was realized, by mixing the samples of all the replicates of each treatment. The composite samples were used to perform the following analysis.

2.3 Soil enzymatic activities

Three soil enzymatic activities were measured: acid phosphatase, alkaline phosphatase and fluorescein diacetate (FDA) hydrolysis.

Acid and phosphatases were determined using PNPP (*p* nitrophenyl phosphate disodium) substrate: 2 g of air dry soil were mixed with 2 mL of buffer (sodium acetate 0.1 M, pH 5 for acid phosphatase; Tris-HCl 0.1 M, pH 8 for alkaline phosphatase) and shaken overnight (150 rpm, ambient temperature). Solutions were centrifuged and supernatants collected. Following, the reactions were realized in a microplate: 100 µL of extract were mixed with 100 µL of PNPP 5 mM or buffer (for control) and the plate was incubated one hour at 25 °C. The reaction was stopped by adding 100 µL NaOH 0.1 M and absorbance was read at 410 nm (against blank reactant). Calculation was done using ε (PNPP) = 19,500 $L.mol^{-1}.cm^{-1}$.

For the FDA hydrolysis, 0.1 g of air dry soil was mixed with 5 mL of potassium phosphate buffer (60 mM, pH 7.6) and 50 µL FDA (50 mM in acetone), except in control tubes. The tubes were shaken for three hours (150 rpm, 37 °C). Solutions were centrifuged, and then 200 µL of supernatant were put in the well microplate and absorbance was measured at 490 nm. Calculation was done using ε (FDA) = 8,000 $L.mol^{-1}.cm^{-1}$.

2.4 Biolog Ecoplates™

Fresh soil (2.5 g) was mixed with 10 mL of sterile NaCl 0.9 % and vortexed 3 min. Solutions were centrifuged and 600 µL of supernatant was mixed with 17.4 mL of sterile NaCl 0.9 %. Following, 150 µL of the bacterial extract were put in each well of the Ecoplate. One plate was prepared by treatment. Plates were incubated at 25 °C and absorbances at 590 nm were measured each day for two weeks.

2.5 Next generation sequencing

Soil DNA was extracted using PowerSoil^{TM} DNA Kit (MO BIO Laboratories, Inc), following the manufacturer's instructions. NGS sequencing protocol was performed at INRA Transfert (Narbonne, France): extracted DNA were amplified by PCR using primers 515F (5'-GTGYCAGCMGCCGCGGTA-3') and 909R (5'-CCCGYCAATTCMTTTRAGT-3') that target the variable regions V4-V5 of the 16S ribosomal RNA gene of the prokaryotes. The sequencing was performed by MiSeq Illumina and the identification on the base of the taxonomy on Greengenes.

2.6 Statistical analyses

Data were analyzed using R software version 3.1.2 (R Development Core Team, 2009). Firstly, the global treatment (seven treatments) and plant (bulk/rhizosphere) effects were assessed using the following procedure: normality and homoscedasticity of the data were evaluated using Shapiro and Bartlett tests, respectively; following treatment effect was measured using an Anova or a Kruskal test while plant effect was determined using a Student or a Wilcox test, for normal and non-normal data, respectively. Following, to have a deeper analysis, treatments were compared two by two using Student test for normal data and Wilcox test for non-normal data. Moreover, bulk and rhizosphere conditions were compared within each treatment using the same protocol.

Finally, to evaluate the possible link between environmental parameters (described in Lebrun *et al.* 2019) and the bacterial community structure, data sets were subjected to a canonical correspondence analysis (CCA) using the vegan package in the R software.

3. Results

3.1 Soil enzymatic activities

Three soil enzymatic activities were measured at the end of the experiment for the different modalities, for both non-vegetated (bulk) and vegetated (rhizosphere) soils: acid phosphatase, alkaline phosphatase and hydrolytic activity (against FDA).

Global treatment effect was highly significant ($p < 0.001$) for the three activities, whereas plant effect was only very significant ($p < 0.01$) for alkaline phosphatase, while having no effect on the other two activities (Table 1).

Table 1: General treatment and plant effects, determined on the three soil enzymatic activities (acid phosphatase, alkaline phosphatase and hydrolytic (FDA) activities) as well as on the parameters measured with the Biolog Ecoplate test (average well color development (AWCD), Shannon-Weaver index H’, eveness E, and richness). This parameters were measured at the end of the experiment on both bulk and rhizospheric soils. Level of significance: * (p < 0.05), ** (p < 0.01) and *** (p < 0.001), ns (non-significant).

	Treatment effect	Plant effect
Soil enzymatic activities		
Acid phosphatase	***	ns
Alkaline phoshatase	***	**
FDA	***	ns
Biolog Ecoplate		ns
AWCD	***	ns
H'	**	ns
E	**	ns
Richness	***	ns
%AWCD-carbohydrates	***	ns
%AWCD-amino acids	*	ns
%AWCD-carboxylic acids	**	ns
%AWCD-polymers	ns	ns
%AWCD-amines	ns	ns
%AWCD-phenolic compounds	ns	ns

Acid phosphatase activity was shown to increase following the addition of biochar, compost and iron grit alone in the bulk soil while in the rhizosphere it decreased with biochar-compost treatment and increased in the three treatments containing iron grit (Figure 1A). Alkaline phosphatase activity increased with all amendments except for biochar alone in bulk while it increased with all amendments except for biochar and biochar-iron treatments in the rhizosphere (Figure 1B).

Plant growth had different effects on enzymatic activities depending on the enzyme and treatment: acid phosphatase activity was higher in the rhizosphere compared to the bulk for PI and PBI modalities, whereas it was lower for PC and PBC. *Salix viminalis* plant growth increased alkaline phosphatase activity on P, PB and PC treatments.

The overall hydrolytic activity was assessed by the hydrolysis of FDA. Compared to the non-amended Technosol, this activity greatly increased in the three compost treatments as well as with iron alone in the bulk soil. In the rhizosphere, it decreased following biochar application and increased in the three compost-amended substrates. It has to be noted that the level of response of the FDA hydrolytic activity was higher in the bulk compared to the rhizosphere compartment (Figure. 1C). Finally, plant growth decreased the hydrolytic activity of the soil in three cases, PB, PC and PBC.

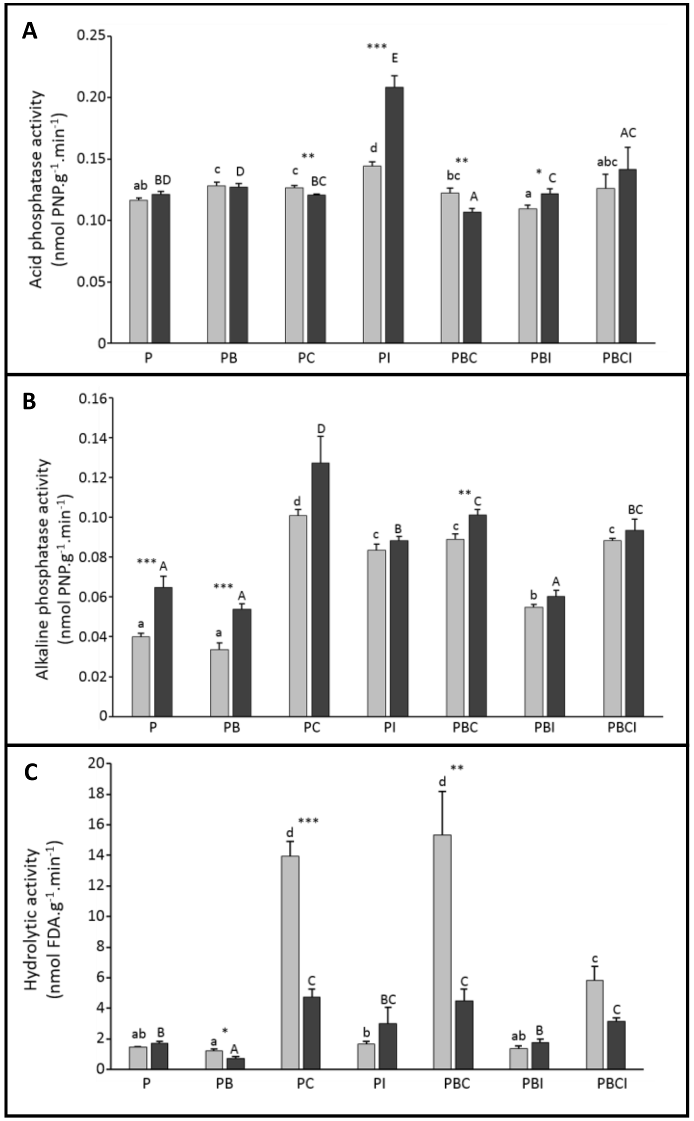

Figure 1: Soil enzymatic activities (acid phosphatase (A) (nmol PNP.g^{-1}.min^{-1}); alkaline phosphatase (B) (nmol PNP.g^{-1}.min^{-1}) and hydrolytic (C) (nmol FDA.g^{-1}.min^{-1}) measured on Pontgibaud (P) technosol amended or not with biochar (B), compost (C) or iron grit (I), alone or combined, after 69 days of Salix viminalis growth on bulk (☐) and rhizosphere (■) soils. Minuscule letters indicate significant difference ($p < 0.05$) between the treatment in the bulk condition while capital letters indicate significant difference in the rhizosphere condition. Significant difference between bulk and rhizosphere condition indicated by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

Taken together, these data showed that in general, soil amendment increased the soil enzymatic activities and a lower effect of plant development on soil enzyme activities.

3.2 Bacterial activity (Biolog Ecoplates^{TM})

In addition to the soil enzymatic activities, Biolog Ecoplate^{TM} tests were performed to assess the bacterial activity toward various substrates. The values of absorbance at 590 nm were used to calculate several indices: the average well color development (AWCD), the Shannon-Weaver diversity index (H'), the Eveness (E) and the richness.

Table 2: Biolog Ecoplate data (AWCD = average well colour development, H' = Shannon-Weaver index, E = eveness, Richness = number of positive well (OD_{590} > 0.25)) measured at the end of the experiment (T69) on the bulk and rhizosphere soil of Pontgibaud (P) amended or not with biochar (B), compost (C) or iron grit (I), alone or combined. Minuscule letters indicate significant difference ($p < 0.05$) between the treatment in the bulk condition while capital letters indicate significant difference in the rhizosphere condition. Significant difference between bulk and rhizosphere condition indicated by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

Treatment		AWCD	H'	E	Richness
P	Bulk	0.09 ± 0.02 **a**	2.4 ± 0.1 **a**	0.69 ± 0.04 **a**	3 ± 1 **a**
	Rhizosphere	0.17 ± 0.02 **AB** *	2.5 ± 0.1 **B**	0.72 ± 0.02 **B**	7 ± 1 **BC** **
PB	Bulk	0.12 ± 0.02 **a**	2.4 ± 0.1 **a**	0.69 ± 0.01 **a**	4 ± 1 **ab**
	Rhizosphere	0.12 ± 0.01 **A**	2.5 ± 0.0 **B**	0.72 ± 0.00 **B**	5 ± 1 **AB**
PC	Bulk	0.55 ± 0.02 **d**	2.9 ± 0.1 **b**	0.85 ± 0.02 **b**	17 ± 1 **d**
	Rhizosphere	0.39 ± 0.00 **C** **	2.5 ± 0.0 **B** *	0.73 ± 0.01 **B** *	11 ± 0 **D** *
PI	Bulk	0.22 ± 0.01 **b**	2.5 ± 0.1 **a**	0.73 ± 0.03 **a**	8 ± 1 **bc**
	Rhizosphere	0.17 ± 0.02 **AB**	2.4 ± 0.1 **AB**	0.69 ± 0.04 **AB**	8 ± 1 **ABCD**
PBC	Bulk	0.34 ± 0.02 **c**	2.6 ± 0.1 **ab**	0.76 ± 0.03 **ab**	11 ± 1 **b**
	Rhizosphere	0.31 ± 0.05 **ABC**	2.4 ± 0.1 **AB**	0.69 ± 0.01 **AB**	9 ± 0 **C**
PBI	Bulk	0.16 ± 0.00 **a**	2.3 ± 0.0 **a**	0.67 ± 0.00 **a**	6 ± 0 **bc**
	Rhizosphere	0.19 ± 0.02 **B**	2.2 ± 0.0 **A** **	0.63 ± 0.00 **A** **	6 ± 1 **AB**
PBCI	Bulk	0.14 ± 0.03 **ab**	2.4 ± 0.1 **a**	0.69 ± 0.02 **a**	4 ± 0 **ac**
	Rhizosphere	0.10 ± 0.01 **A**	2.4 ± 0.0 **AB**	0.71 ± 0.03 **AB**	4 ± 1 **A**

Treatment had a highly significant (for AWCD and richness) and very significant (for H' and E) effect on the parameters measured, while plant had no significant effect for all the parameters (Table 1).

In the non-vegetated condition (bulk), AWCD values increased in PC, PI and PBC treatments compared to P (Table 2), with the highest increase observed in PC (6.1-fold). In the vegetated condition (rhizosphere), AWCD only increased in the compost treatment (PC). Plant growth only affected AWCD values on P and PC substrates, in which it increased and decreased AWCD values, respectively.

Shannon-Weaver index (H') and Eveness (E) developed similar trends. In the bulk compartment, they only increased in PC compared to P (Table 2), while in the rhizosphere zone, they decreased with the addition of biochar-iron (Table 2). Moreover, plant development led to a decrease in H' and E on PC and PBI treatments.

Richness index represents the number of wells having an absorbance above 0.25; this reflects the number of utilized carbon substrates. Richness increased in all the amended-treatments, except PB and PBCI compared to the non amended Pontgibaud soil, in the bulk condition. The highest increase (5.7-fold) was observed in PC and the lowest (2-fold) in PBI (Table 2). For the rhizosphere compartment, it only increased following single compost amendment (Table 2). Again, plant growth had little and contradictory effects on the richness: it doubled richness value on P whereas it decreased it on PC substrates.

Furthermore, for a deeper analysis, the different carbon substrates were grouped in six categories (carbohydrates, amino acids, carboxylic acids, polymers, amines and phenolic compounds) and the percentages of utilization of the different categories were calculated based on AWCD. The statistical analysis revealed a highly significant treatment effect for the carbohydrates, a very significant one for the carboxylic acids and a significant one for the amino acids; whereas treatment had no effect on the three other substrates and plant development did not show any significant effect for the all six categories (Table 1). Moreover, the results showed that carbohydrates were the most used substrates (Figure 2) while amines and phenolic compounds were the least. Those two last substrate categories and carboxylic acids did not show change in relative utilization following amendments in both bulk and rhizosphere compartments. Overall, few changes in percentage of utilization were observed in the amended treatments compared to P. In the bulk condition, amino acids use increased in PC while polymers increased in PB and decreased in PBI. In the rhizosphere, only carbohydrates utilization was decreased in PBC. Even though the statistical analysis showed few significant changes in categories utilization, it can be seen from Figure 2 that, contrary to the other treatments, PBC presented a more diverse profile, with the six categories having similar utilization percentage, more or less, in both bulk and rhizosphere.

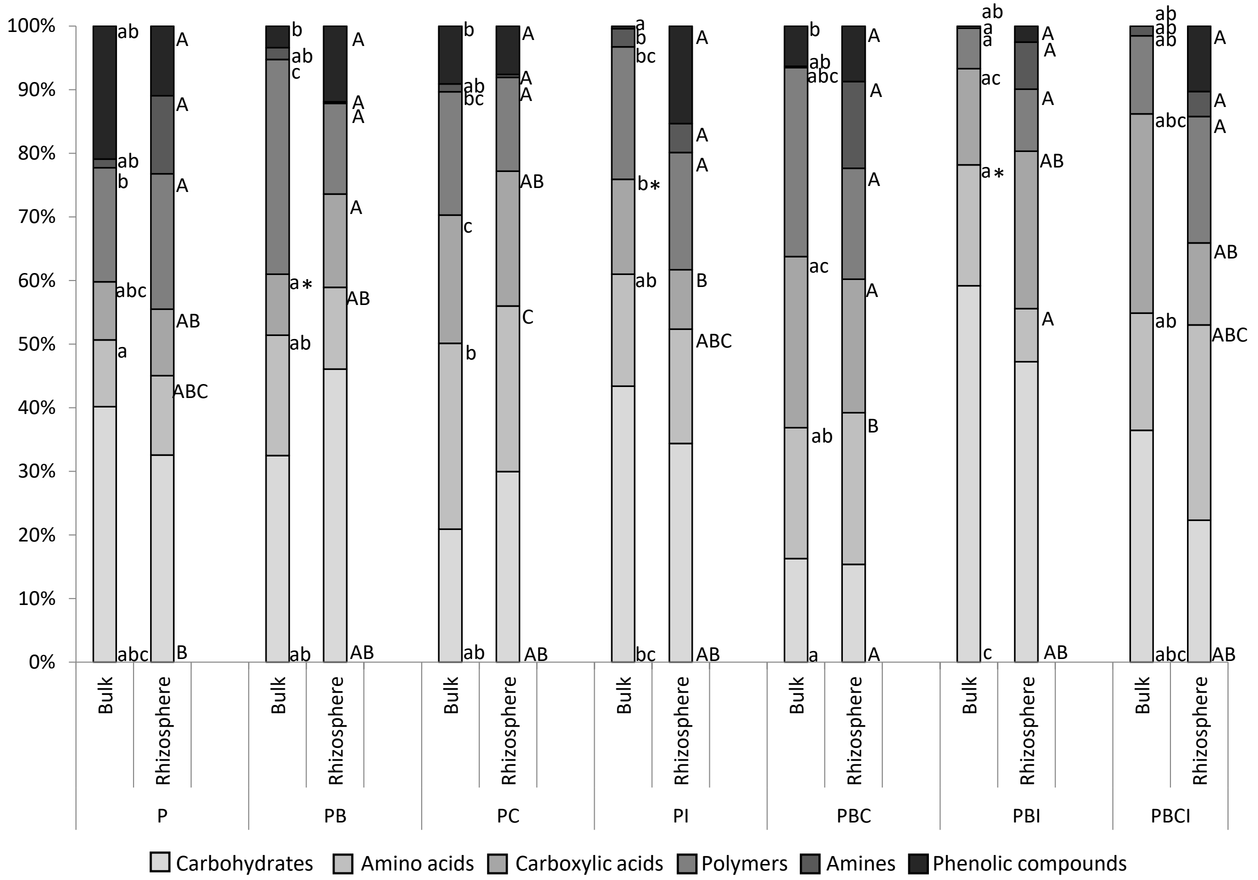

Figure 2: Pourcentage of utilization of the substrates categories (carbohydrates, amino acids, carboxylic acids, polymers, amines, phenolic compounds) determined by the Biolog Ecoplates measured at the end of the experiment (T69) on the bulk and rhizosphere soil of Pontgibaud (P) amended or not with biochar (B), compost (C) or iron grit (I), alone or combined. Minuscule letters indicate significant difference between the three treatments for the bulk condition while capital letters indicate significant difference between the three treatments for the rhizosphere condition ($p < 0.05$) ($n = 5$). Significant difference between the bulk and rhizosphere conditions is indicated by: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3.3 Bacterial community analysis

The characterization of the bacterial communities by using NGS showed a dominance of the bacterial phyla *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, *Gemmatimonadetes*, and *Firmicutes* (Figure S1). *Cyanobacteria* also represented an important component of the studied communities, being retrieved at relatively high abundances in some samples, such as P rhizospheric soil (11.26 %). After the addition of biochar and iron grit, alone or in combination, an increase of relative abundance of *Proteobacteria* was observed compared to the soils not amended or with compost. Moreover, a decrease in *Acidobacteria* was shown in all the treatments. *Bacteroidetes* relative abundance increased significantly in biochar and compost amended soils whereas *Actinobacteria* increased in biochar and iron grit treated samples (alone and combined). In addition, DNA sequences from microorganisms belonging to the domain of *Archaea* were retrieved in soil samples. They were found at relatively high abundances in P soils (bulk and rhizosphere soils; ≈16.61 % and ≈12.40 %, respectively) and after the amendments addition with compost (PC), biochar + compost (PBC), and biochar + compost + iron grit (PBCI) with representatives of the phyla *Crenarchaeota* and *Euryarchaeota.*

At class taxonomic level, *Betaproteobacteria*, *Saprospirae*, *Cytophagia*, *Anaerolineae*, *Acidobacteriia* and *Thaumarchaeota* represented the dominant groups in the sample set (Figure S2). *Betaproteobacteria* was the most abundant class in PB bulk soil, PI and PBI bulk and rhizosphere soils, and PBCI non-vegetated soil. *Saprospirae* dominated PB and PC rhizospheric soils and *Cytophagia* ranked first in PC bulk and PBC rhizosphere soils. *Anaerolineae* was the main class retrieved in samples PBC bulk and PBCI rhizosphere whereas *Acidobacteriia* and *Thaumarchaeota* represented the main groups in P rhizosphere and P bulk soils, respectively.

Given the complexity of the bacterial community structure and the number of treatments planned in the experimental design, a statistical approach was used to analyse NGS data and link it to the environment data measured in the same treatments and described in Lebrun *et al.* (2019). The Canonical Correspondence Analysis (CCA) analysis explained 33.96 % of the variability (CCA1 = 19.22 %; CCA2 = 14.74 %) (Fig. S3) and the resulting biplot showed a clear separation of the bacterial communities. It also showed that the bacterial communities were shaped by several key factors such as pH, electrical conductivity (EC), soil organic matter (SOM), *Salix* root dry weight (DW), As, Pb and Fe contents. At least three different clusters can be observed: the first one comprising communities from bulk and rhizospheric soil samples (P bulk and P rhizosphere) positively correlated with Pb, the second including the PBI and PI samples mainly influenced by iron content and a third group with the remaining samples positively correlated with pH, EC and SOM, and to a lower extent with As and root DW. Finally, the CCA analysis did not show a clear separation between the "bulk" samples and the "rhizosphere" samples, meaning that plant development did not seem to have a high influence on the soil bacterial community structure.

4. Discussion

4.1 Soil enzymatic activities

Phosphatase enzymes are involved in the mineralization of organic phosphate and they develop a rapid response to soil management (Yang *et al.* 2016, Al Marzooqi and Youssef 2017). Similarly to what was observed here, compost has been shown to increase phosphatase activities. For instance, municipal solid waste compost applied on the tailings of a copper mine increased acid and alkaline phosphomonoesterase activities (Touceda-González *et al.* 2017). Ros *et al.* (2006) applied on the field different kinds of compost (urban organic waste, green waste, cattle manure and sewage sludge) and all such composts increased phosphatase activity (evaluated at pH 6) compared to the control plot. Similarly, biochar application to soil induced an increase in alkaline phosphatase activity in several studies (Oleszczuk *et al.* 2014, Al Marzooqi and Youssef 2017). However, it was surprising to observe in this study an increase in acid phosphatase activity in the biochar treated substrate, as an increase in SPW pH was observed in this case (Lebrun *et al.* 2019). But both increase and decrease in acid phosphatase activity were observed after biochar application. Indeed, Liu *et al*. (2017) and Pukalchik *et al.* (2018) showed a decrease activity of the acid phosphatase whereas Oleszczuk *et al.* (2014) observed a rise of the same activity following biochar amendment. Moreover, Yang *et al.* (2016) evaluated the effect of two biochars and dose application and found that applying 5 % bamboo biochar decreased acid phosphatase activity while 1 % fine rice straw biochar increased it. These results showed that other factors, in addition to soil pH, clearly influenced phosphatase activities. For instance, the decrease in metal(loid) toxicity effect as well as the improvement of soil fertility by the addition of organic matter could have also contributed to the improvement of both acid and alkaline phosphatase activities. Indeed, Zeng *et al.* (2007) applied increasing Pb concentrations to two different soils and observed that Pb had a stimulatory effect on acid phosphatase activity at low concentration; but when added at high concentrations (above 500 mg.kg^{-1}), it induced inhibitory effects due to the suppression of the bacterial growth as well as the direct antagonist effect that exists between enzymes and metal(loid)s. Moreover, they also observed that the soil presenting the higher organic matter content also had the higher enzyme activities since this soil supplied more carbon source for the microorganisms. Such explanations are corroborated with the soil physico-chemical properties (Lebrun *et al.* 2019), which showed that amendments, and particularly biochar and compost, decreased metal(loid) extractable fractions and soil pore water concentrations, especially for Pb, together with an increase of the soil organic matter contents.

The FDA activity is related to the overall microbial activity (Pukalchik *et al.* 2018). Indeed, FDA can be hydrolyzed by several different enzymes, which makes the FDA hydrolysis activity an index of the overall enzyme activity (Lee *et al.* 2008). Similarly to the present study, Pardo *et al.* (2014) observed an increase in FDA hydrolysis after compost amendment, indicating a stimulation of the soil microbial community, while Yun *et al.* (2017) observed a rise in FDA hydrolysis when both biochar and compost were added and no effect of their single application.

Overall, amendment application increased soil enzyme activities, indicating an increase of the bacterial activity and/or biomass (Karaca *et al.* 2010, Khadem and Raies 2017). Such improvement can be explained by an amelioration of the soil physico-chemical properties (Khadem and Raies 2017, Touceda-González *et al.* 2017) (increase in pH, EC, water holding capacity), a supply of organic matter by the amendments used (Nie *et al.* 2018) and a decrease of metal(loid) stress (Khan *et al.* 2007). Indeed, the measure of the soil physico-chemical properties (Lebrun *et al.* 2019) showed that amendment application increased soil pore water pH as well as soil water holding capacity and organic matter content, while decreasing metal(loid) availability ($CaCl_2$- and NH_4NO_3-extractable fractions) and mobility (soil pore water concentrations), which could have reduce the negative pressure put on microorganisms and thus improve their growth and activity.

Finally, although plants are known to increase enzymatic activity, through the gradual formation of the rhizosphere microbiota and the release of enzymes (Nannipieri *et al.* 2007, 2011), both increase and decrease were observed in this case, which could be due to different root exudate release depending on the treatments, which could affect differently the microbial community composition and activity. However, such hypothesis needs to be verified by the analysis of the root exudates, in addition to the organic acids. Moreover, the plant cover can induce a loss of carbon due to mineralization, which leads to decrease enzymes activities (Pascual *et al.* 2000).

4.2 Bacterial activity (Biolog Ecoplates^{TM})

The AWCD is an important index of the microbial function diversity, which represents the ability of the soil microorganisms to use different carbon sources (Zhu *et al.* 2017). The increase in AWCD values observed in this study have been previously observed following compost and biochar amendments (Pardo *et al.* 2014, Liao *et al.* 2016) and reflects the increase in bacterial abundance and the stimulation of the soil microbial community (Pardo *et al.* 2014, Chen *et al.* 2016). Indeed, amendments can provide a habitat to the soil microorganisms and stimulate their activities (Liao *et al.* 2016). They can also provide nutrients (Pérez-Piqueres *et al.* 2006) and reduce stress pressure (Garau *et al.* 2017), such as metal(loid) toxicity. Indeed, Kenarova *et al.* (2014) found that AWCD value was negatively correlated with As, Cu, Pb and U contents, demonstrating the adverse effect that pollution can exercise on the microbial activity. Similarly, the soil data (Lebrun *et al.* 2019) showed that amendment applications reduced $CaCl_2$- and NH_4NO_3-extractable As and Pb as well as soil pore water Pb concentrations. Additionally, soil pore water toxicity decreased at the end of the experiment in the amended substrates, testifying of a reduction of the stress, which could have allow an improvement of the microbial community growth and activity. Moreover, the higher increase observed in PC treatment can be related to the elevated nutrient levels of the compost but also to the addition of microorganisms from the compost itself.

The functional diversity H', or Shannon-Weaver index, can be defined as the number of distinct functions carried out by a microbial community (Boshoff *et al.* 2014). The increase induced by compost in the bulk condition can be explained by the organic matter incorporation (Pardo *et al.* 2014) and the lowering of metal(loid) availability, rendering them less toxic to both plants and microorganisms (Hmid *et al.* 2015), as shown by the soil measurements (Lebrun *et al.* 2019). However, the non- or negative effect of biochar seemed surprising as these data showed that biochar application improved soil conditions. However, such result has been also found by Zhu *et al.* (2017) and could be attributed to the possible release of toxic compounds by biochar, which could affect the activity of some bacteria. Moreover, in their study, authors also revealed the negative relation between Shannon-Weaver H' index and soil pH. High pH could also contribute to the non- or negative effect of biochar, as PB showed higher SPW pH than PC but lower than PBC. However, contrary to the biochar-compost combination, biochar alone or combined to iron could have not provided enough nutrient to sustain microbial diversity and activity (Lebrun *et al.* 2019).

The improvement of substrate utilization (richness) can be attributed to a supply of organic matter (Pardo *et al.* 2014), especially with compost, as well as a reduction of metal(loid) toxicity (as shown in Lebrun *et al.* 2019) that rendered the substrates more suitable for microbial growth and activity (Hmid *et al.* 2015).

The analysis by functional substrate categories showed that biochar and compost amendment shifted the bacterial community structure, with a more diverse C source utilization. This can be attributed to the direct addition of microorganisms with compost and a better survival of them when a carbon rich material (biochar) is also present in the soil.

4.3 Bacterial community analysis

An understanding of the temporal and spatial structures, functions, interactions, and population dynamics of bacterial communities is critical for many aspects of life, including scientific discovery, biotechnological development, sustainable agriculture, energy security, environmental protection, and human health (Bucci *et al.* 2017). Accordingly, several methods (cultivation-dependent and molecular approaches) have been employed to reveal bacterial community composition and responses to environmental changes in several and various environments and in different contexts (Bucci *et al.* 2011, 2014, 2015 a,b, Crescenzo *et al.* 2017, Di Luccia *et al.* 2018, Petrella *et al.* 2018, Pietrangelo *et al.* 2018).

Microorganisms living in soil are abundant, highly diverse and represent the key players of many soil functions such as biogeochemical cycling, plant productivity or climate regulation. Thus, they are essential for the integrity of terrestrial ecosystems (Griffiths and Philippot 2013). In the process of soil remediation, their activities affect the growth and remediation efficiencies of plants (Liu *et al.* 2018).

The dominant phyla observed in this study are often found in the heterogeneous and complex soil systems (Janssen 2006, Schulz *et al.* 2013, Wolińska *et al.* 2017). *Cyanobacteria* phylum comprises photosynthetic organisms which can easily survive on bare minimum requirement of light, carbon dioxide and water (Woese 1987, Castenholz 2001). They are phototrophic, fulfill their own nitrogen requirement by nitrogen-fixation, produce some bioactive compounds which promote the crop growth, protect them from pathogens, improve the soil nutrient status (Singh *et al.* 2016) and have the ability to degrade various toxic compounds and to detoxify metals (Cohen 2006, Singh *et al.* 2016). *Proteobacteria* are heterotrophic microorganisms, generally found in higher abundance in nutrient and carbon rich substrates (Liu *et al.* 2019). Their abundance was shown to both increase or decrease with Cr and Cd, respectively (Liu *et al.* 2019), showing that microorganisms respond differently depending on the contaminants. This could explain the difference in abundance between treatments: *Proteobacteria* could have be promoted by the decrease in As and Pb availabilities induced by biochar and iron, while being restrained by the increase in As availability in compost amended soils (Lebrun *et al.* 2019). *Acidobacteria* are involved in several biogeochemical cycles and are able to decompose and use natural polymers (Huang *et al.* 2018). *Actinobacteria* have a role in carbon cycle and as *Proteobacteria,* they are dominant in carbon rich environment (Xu *et al.* 2017), which could explain their higher abundance in biochar-soils, biochar being a carbon-rich product. They were shown to have a crucial role in the decomposition of organic materials (Wu *et al.* 2019). Finally, *Archae* play important roles in C and N cycling (de Araújo *et al.* 2018). Their different abundances in the treatments revealed that the addition of the compost as amendment, alone or in combination, determined shifts in archaeal community composition promoting the growth of members of *Euryarchaeota* and excluding *Crenarchaeota*.

Finally the CCA analysis of the sequencing data combined with the environment data showed that on P, the bacterial community was mostly affected by Pb contamination, which is consistent with the fact that Pb is the main contaminant of Pontgibaud Technosol. Similarly, the high influence of Fe concentration on the shape of the bacterial community of PI and PBI was in accordance with the addition of Fe with the iron grit amendment, which led to important increases in soil pore water Fe concentrations as well as in available Fe (Lebrun *et al.* 2019). Finally, in the other treatments, containing biochar and/or compost, bacterial community structure seemed more affected by As concentrations, root DW, pH, EC and SOM, parameters that tended to increase in the same treatments (Lebrun *et al.* 2019). Such influence of the soil parameters on the soil bacterial community structure has been previously studied at different taxonomic levels (Guo *et al.* 2017, Tipayno *et al.* 2018). For instance, Cesarano *et al.* (2017) showed that *Actinobacteria* abundance was positively correlated with EC and negatively with pH, whereas *Acidobacteria* abundance was negatively correlated with EC. Tipayno *et al.* (2017) also showed that soil chemical variables such as pH and EC but also metal(loid) concentrations were correlated with the relative abundances of bacterial phyla and genera. Moreover, Liu *et al.* (2019) observed that Cr presence in soil promoted the growth of *Actinobacteria* and *Proteobacteria* while restraining the growth of *Firmicutes*, whereas Cd promoted *Firmicutes* and *Actinobacteria* while restraining *Proteobacteria* growth. Therefore, the molecular analysis of the bacterial community revealed that amendment applications, but modifying the soil properties, induced shifts in the community, promoting some phyla/classes and restraining others. The reduce effect of the plant over soil properties in shaping the microbial community was also observed by Xu *et al.* (2014) after biochar addition.

5. Conclusion

An assisted phytostabilization experiment was set up using a former mine Technosol (Pontgibaud) mainly contaminated by As and Pb amended with biochar, compost and/or iron grit. Previous data (Lebrun *et al.* 2019) showed the effects of the diverse amendment treatments on the soil and soil pore water physico-chemical properties as well as on *Salix viminalis* growth, metal(loid) accumulation and stress indicators. The present paper aimed at evaluating the effect of these amendments, in both bulk and rhizosphere soils, on the bacterial community composition and activity. The study revealed that the amendments led to an increase in the soil enzymatic activities (acid and alkaline phosphatases and FDA hydrolysis). Their application also increased the capacity of the bacterial community to use diverse carbon sources. These two parameters were especially increased when compost was applied. Next-generation sequencing revealed the presence of phyla commonly retrieved in soil ecosystems such as *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, *Gemmatimonadetes*, and *Firmicutes*. Nevertheless, in general the different amendment treatments and compost application specifically induced a shift in the bacterial and archaeal communities. Such effect could be the result of the addition of microorganisms present in the compost itself.

In conclusion, compost, applied alone or combined with biochar and iron grit, was the amendment showing the better soil microbial bacterial activity increases, confirming previous results (Lebrun *et al.* 2019).

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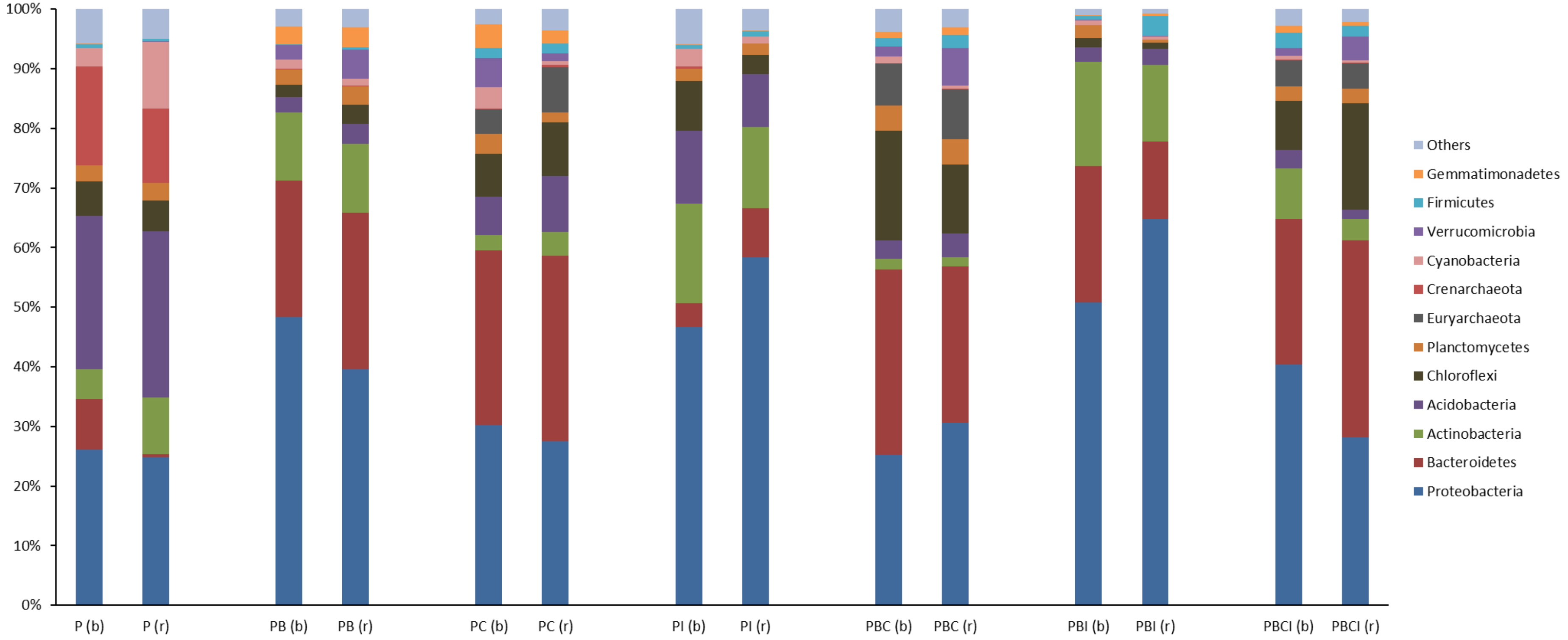

Figure S1: Phylum level microbial community composition in the bulk (b) and the rhizosphere (r) compartment of Pontgibaud (P) amended or not with biochar (B), compost (C) or iron grit (I), alone or combined. Phyla with relative abundance < 1% are grouped in the category “Others”.

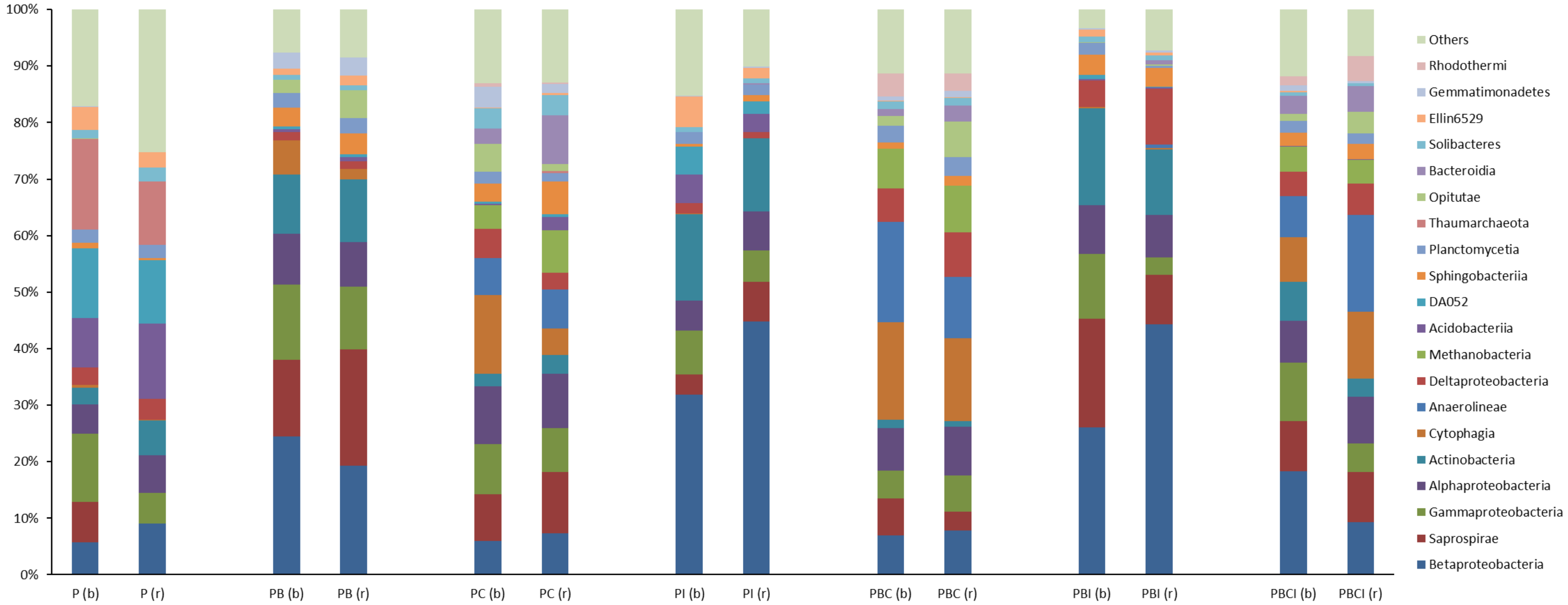

Figure S2: Class level microbial community composition in the bulk (b) and the rhizosphere (r) compartment of Pontgibaud (P) amended or not with biochar (B), compost (C) or iron grit (I), alone or combined. Classes with relative abundance < 1% are grouped in the category “Others”.

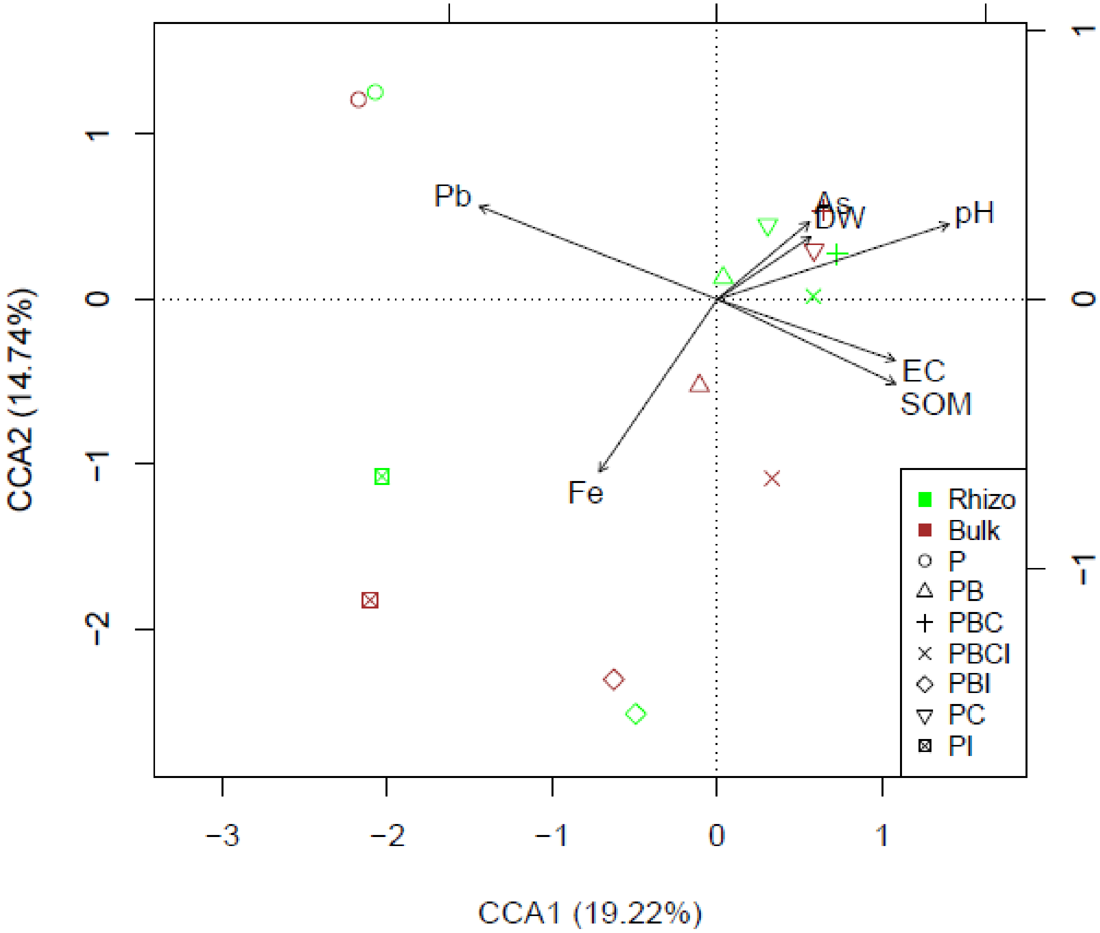

Figure S3: Canonical-correlation analysis (CCA) based on bacterial 16S rRNA genes retrieved from the bulk (Bulk) and the rhizosphere (Rhizo) compartment of Pontgibaud (P) amended or not with biochar (B), compost (C) or iron grit (I), alone or combined.

Part B. Effect of fertilization, carbon-based material and redmud amendments on the bacterial diversity and activity of a metal(loid) contaminated mining soil.

Effect of fertilization, carbon-based material and redmud amendments on the bacterial diversity and activity of a metal(loid) contaminated mining soil

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Abstract

Amendment application is a common practice in the phytomanagement of polluted soils. Their effects on the soil physico-chemical properties, metal(loid) immobilization and plant growth have been largely demonstrated. However, the addition of amendments may also impact the soil bacterial community, in terms of activity and composition. Such evaluation of the effects of single or combined amendment addition on the bacterial community has been scarce. The aims of this study were to assess the effect of two amendments types, two carbon-based amendments, a biochar and an activated carbon, and two different redmuds, a bauxaline and a neutralized redmud, applied alone or combined, and a fertilizer applied alone to a former mine technosol polluted by lead and arsenic on the bacterial community using three methods: the measure of four enzyme activities, Biolog EcoPlates™ and DNA extraction followed by 16S sequencing. This study, which was a continuation of a previous one (Lebrun *et al.*, in prep, pages 225 to 251) showing the effects of the same amendments on the soil physico-chemical properties and plant growth, revealed that all amendments affected the bacterial community differently. The neutralized redmud increased the overall hydrolytic activity of the soil while the other amendments had different effects on the other enzyme activities. The activity of the cultivable bacterial community, assessed with the Biolog EcoPlates™, was increased with the two redmuds tested and the addition of activated carbon. Finally, all treatments induced a shift in the bacterial community structure, with the treatments containing redmuds and/or biochars clustering together, whereas the treatments with fertilization and activated carbon were more distant from the previous treatments.

Keywords

Amendments; Bacterial community; Biolog EcoPlates™; Enzyme activities; Next Generation Sequencing

1. Introduction

The bacterial community is an important parameter to take into account during the phytomanagement process of metal(loid) polluted soils. Indeed, the soil hosts many diverse microorganisms, such as bacteria, fungi, actinomycetes, protozoa and alga; bacteria being the most important (Yu *et al.* 2019). Bacteria are negatively affected by metal(loid)s and are sensitive to soil conditions, such as pH, organic matter content and moisture content. Moreover, although metal(loid)s are not degradable, bacteria can affect metal(loid) mobility and availability (Violante *et al.* 2010), either directly through different mechanisms, *i.e* extracellular and intracellular sequestrations, or detoxification (Yin *et al.* 2019), or indirectly by modifying soil conditions. Moreover, bacteria can improve plant growth, through plant growth promoting properties (*e.g.* siderophore and indole acetic acid production, phosphate solubilization) and their effects on nutrient cycles. This will indirectly ameliorate phytomanagement efficiency. The evaluation of the bacterial community, in terms of activity and composition, can be done through different methods, such as the measure of the soil enzyme activities, Biolog EcoPlates^{TM} and soil DNA extraction followed by next generation sequencing (NGS). It will give an indication regarding the recovery of the soil functions following a phytomanagement process.

Bacterial enzymes are the major components of the biological soil processes (Baldrian 2009) and are considered good bio-indicators, correlated to natural and anthropogenic disturbances of soils (Karaca *et al.* 2010). For instance, metal(loid)s can decrease enzyme activities through their interaction with the enzyme-substrate complex as well as the denaturation of the enzyme or the interaction with the active protein groups (Pan and Yu 2011). Similarly, amendments, which are often used in phytomanagement, can also influence soil enzyme activities, generally in a positive way. Indeed, they can stimulate the intracellular and extracellular enzymes but also interact with metal(loid)s through functional groups, thus improving soil conditions and indirectly increasing enzyme activities (Karaca *et al.* 2010, Lu *et al.* 2015, Al-Wabel *et al.* 2017).

In order to evaluate the potential activity of the bacterial activity, Biolog technique is often used. It allows for an evaluation of the community level physiological profiles (CLPP). It is a fast and reliable way to differentiate bacterial communities based on their potential functional diversity (Garau *et al.* 2011).

These two methods, enzyme activities and Biolog measurements, provide information regarding the activity and functionality of the soil bacterial community. But it is also interesting to investigate how taxonomically the soil bacterial community is organized. This can be done by extracting the soil DNA and by setting up a NGS analysis, which provides the list and the proportion of the *phyla*, orders, families and *genera* present in the soil bacterial community. By means of this method, shifts in the bacterial community, by the enhancement and/or repression of specific taxonomic groups, can be evaluated.

In a phytomanagement approach, amendments such as carbon-based materials and redmud have been mainly studied for their beneficial effects on soil physico-chemical properties, metal(loid) immobilization and plant growth (Marks *et al.* 2014, Gautam and Agrawal 2017, Lebrun *et al.* 2017, Trakal *et al.* 2017, Zhou *et al.* 2017, Nie *et al.* 2018). Carbon-based materials are composed of two main types: biochar, the product of pyrolysis of biomass under low oxygen condition (Paz-Ferreiro *et al.* 2014) and activated carbon, which corresponds to wood biomass pyrolyzed and that was subjected to a further activation in order to improve its properties, particularly for the treatment of contaminated water but also to improve its effects on soil and plants (Tan *et al.* 2017). However, studies on their combined application are scarce, especially on the effects on the bacterial community.

Therefore, the aims of this study were to evaluate the effects of diverse amendments, alone or combined, on (i) the soil enzyme activities, (ii) the community level physiological profiles and (iii) the soil bacterial community composition. For this purpose, a mesocosm experiment was set-up using two carbon-based materials, biochar and activated carbon, and two redmuds, bauxaline and neutralized redmud, applied alone or combined, and finally a fertilization treatment applied alone. These amendments were selected based on a previous characterization and a sorption test; the effect of these different treatments on the soil physico-chemical properties and plant growth (*Salix dasyclados*) were reported in a previous paper, presented in the Chapter 1 of this PhD manuscript (Lebrun *et al.* in prep, pages 225 to 251). This paper will focus on the soil bacterial community and, together with the previous paper, will give a complete picture of the amendment effects on the physico-chemical and biological status of a polluted soil to be restored by phytomanagement.

2. Materials and Methods

2.1 Experimental design

This study was a continuation of a mesocosm experiment detailed in another paper (Lebrun *et al.* in prep, pages 225 to 251) but a brief description will be given below.

A former silver-lead extraction mine was studied. This mine, active until the nineteenth century, was part of the Pontgibaud mine district (Auvergne-Rhône-Alpes, France) and its intensive extraction activity led to the implementation of a polluted technosol mainly contaminated by Pb (11,000 mg.kg^{-1} on average) and As (500 mg.kg^{-1} on average) (Lebrun *et al.* 2017).

Amendments were chosen and applied to this technosol according to their capability to immobilize metal(loid)s. Moreover, a fertilizer was also applied, as it can also be used to improve soil fertility and plant growth. Seven treatments were applied (the same codes than the previous study will be used for easier reading): a non-amended Pontgibaud technosol (PG), PG fertilized (PG+fert), PG amended with 2 % hardwood biochar (PG+Bc1), PG amended with 1 % neutralized redmud (PG+RM2), PG amended with 2 % hardwood biochar and 1 % neutralized redmud (PG+Bc1+RM2), PG amended with 1 % redmud (PG+RM1) and PG amended with 2 % coconut activated carbon (PG+AC). These substrates were vegetated with *Salix dasyclados*, with one to three pots left un-vegetated.

After 69 days of maturation and regular weathering at 80 % field capacity, soil was sampled in each non-vegetated pot to assess amendment effects after a period of maturation. The same quantity of all the samples of the same treatment were pooled together and homogenized to form a composite sample, which was air dried.

2.2 Measure of soil enzyme activities

The activity of four enzymes, *i.e.* alkaline phosphatase, acid phosphatase, hydrolysis of fluorescein diacetate (FDA) and β-glucosidase, was measured in each soil. All substrates used were purchased from Sigma (Lyon, France).

Alkaline and acid phosphatase activities were determined after extraction with the appropriate buffer (sodium acetate, 0.1 M, pH 5 for acid phosphatase; Tris-HCl, 0.1 M, pH 8 for alkaline phosphatase) at room temperature. Next, the enzyme extract was mixed with 5 mM PNPP (4-nitrophenyl phosphate disodium salt hexahydrate) and incubated three hours at 25 °C; the reaction was stopped by adding 0.1 M NaOH and the absorbance at 410 nm was measured. The activity was calculated using the molar extinction coefficient ε^{PNPP} = 19 500 $L.mol^{-1}.cm^{-1}$ and expressed as $mU.g^{-1}$ soil (1 mU = 1 $\mu g.min^{-1}$).

The hydrolysis of FDA was performed using potassium phosphate buffer (60 mM, pH 7.6), FDA (50 mM prepared in acetone) and incubation for 3 h at 37 °C and 105 rpm on a stirring platform. The absorbance at 490 nm was measured (Shnürer and Rosswall 1982). The activity was calculated using ε^{FDA} = 8 000 $L.mol^{-1}.cm^{-1}$ and expressed as $mU.g^{-1}$ soil.

Finally, the β-glucosidase test was realized with citrate phosphate buffer (0.15 M, pH 4-5) and 10 mM PNPG (4-nitrophenyl β D glucopyranoside) for the extraction. Next, enzyme activity was determined by mixing the enzyme extract with 2 % Na_2CO_3 and measure of the absorbance at 410 nm (Li *et al.* 2009). The activity was calculated using ε^{PNPG} = 18 400 $L.mol^{-1}.cm^{-1}$ and expressed as $mU.g^{-1}$ soil.

2.3 Biolog EcoPlates^{TM}

Two grams of soil were mixed with 10 mL of sterile NaCl (0.9 % w/w). The mixture was vortexed for three min followed by a centrifugation (5 min, 1500xg). Six hundreds µL of the supernatant were again mixed with 17.4 mL sterile NaCl (0.9 %). Finally, each well of the plate was filled with 150 µL of the bacterial extract. Plates, one by treatment, were incubated at 25 °C and absorbance was measured at 590 nm each day for one week.

Absorbance data at 96 h were used to calculate several parameters:

- AWCD (average well color development) = mean of absorbance
- Shannon-Weaver index $H' = -\Sigma\, p_i * \ln p_i$

  with $p_i = Abs_i / \Sigma Abs$ and i representing the substrate
- Evenness $E = H' / \ln (31)$
- Richness = number of wells having $Abs_{590} > 0.25$.

2.4 Soil DNA extraction and 16S sequencing

Soil DNA was extracted using E.Z.N.A® Soil DNA Kit (Omega Bio-Tek) following the manufacturer's instructions. Following, soil DNAs were sent to INRA Transfert (Narbonne, France) for next generation sequencing: first, an amplification by PCR using primers 515F (5'-GTGYCAGCHGCCGCGGTA-3') and 909R (5'-CCCGYCAATTCMTTTRAGT-3') targeting the V4-V5 variable region of the 16S rRNA; second, sequencing by Illumina MiSeq; and finally identification on the base of the taxonomy using Greengenes.

Three extractions were performed per treatments and each DNA extract was sequenced.

2.5 Statistical analyses

Data were analyzed using R software version 3.5.1 (R Development Core Team, 2009). After verification of data normality (Shapiro test) and homoscedasticity (Bartlett and Fligner test), treatments were compared using Anova test (parametric data) or Kruskal test (non-parametric data) followed by a post-hoc Tukey test.

Difference was considered significant at $p < 0.05$.

Finally, the NGS data were analyzed using a Principal Component Analysis performed on the software PAST (Hammer *et al.* 2001).

3. Results

3.1 Soil enzyme activities

Four enzyme activities were measured: FDA hydrolysis, β-glucosidase, alkaline phosphatase and acid phosphatase.

On PG, FDA hydrolytic activity was 4 mU per gram of soil. This activity only increased with the addition of RM2 (neutralized redmud) alone into Pontgibaud soil, which doubled the FDA activity; the other treatments had no effect (Figure 1A).

β-glucosidase activity was less than 0.1 $mU.g^{-1}$ soil on PG and it was decreased when the biochar Bc1 and the activated carbon AC were applied alone. The other treatments had no effect (Figure 1B).

Regarding the two phosphatases, acid phosphatase activity was three times higher than alkaline phosphatase activity, on average (Figures 1C and 1D). On PG, alkaline phosphatase activity was less than 0.06 $mU.g^{-1}$ soil and was only increased in two conditions: fertilization and Bc1+RM2 amendment (Figure 1C). Acid phosphatase activity was less than 0.2 $mU.g^{-1}$ soil on PG and was not affected by amendments addition. The only differences in activity were observed with the fertilization treatment that presented a higher acid phosphatase activity than PG+RM2 and PG+Bc1+RM2 (Figure 1D).

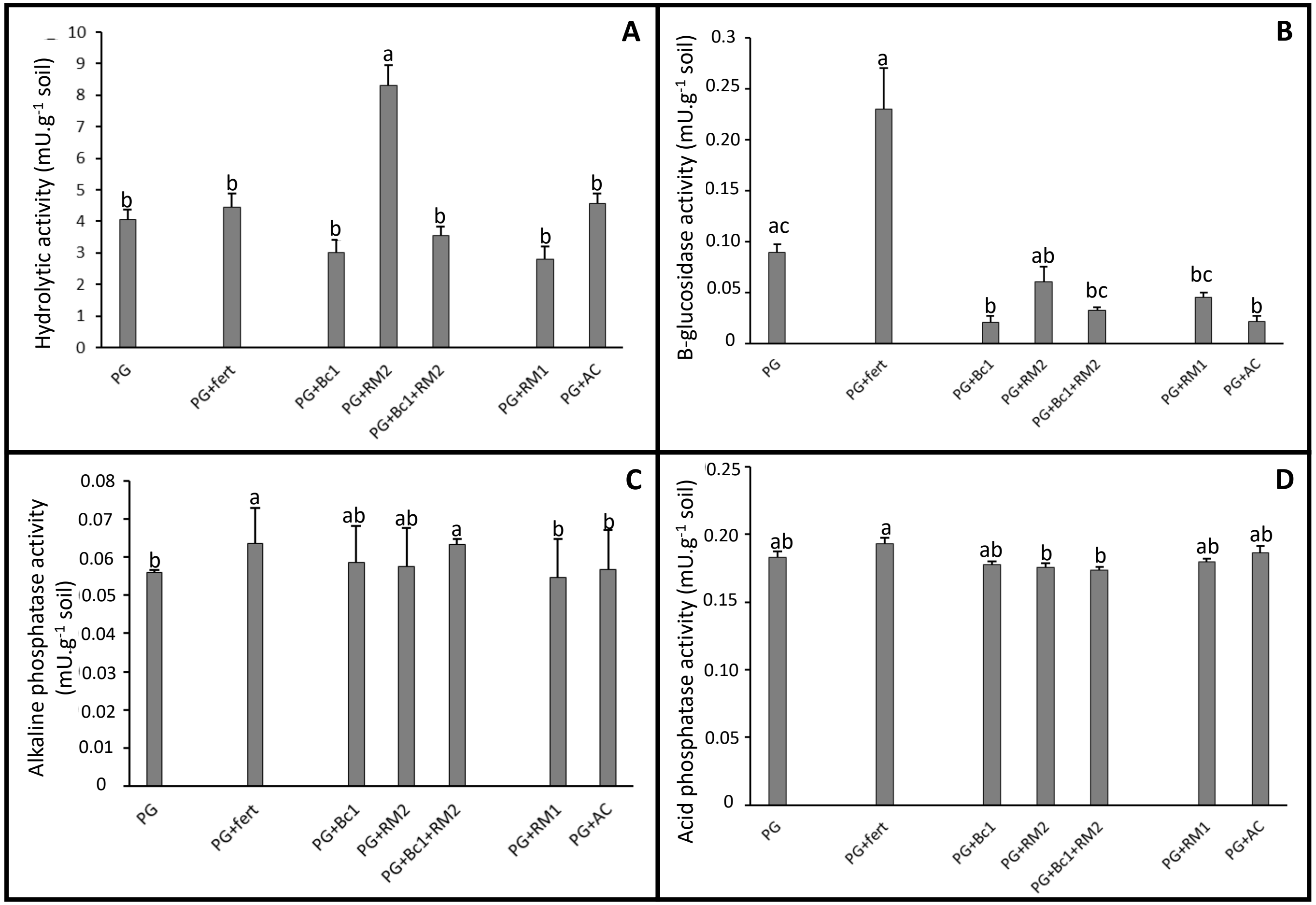

Figure 1: Soil enzymatic activities (hydrolytic (A), β glucosidase (B), alkaline phosphatase (C) and acid phosphatase (D)) (mU. g^{-1} soil) measured after 69 days of maturation in the different treatments. PG = Pontgibaud; PG + fert = PG fertilized; PG + Bc1 = PG amended with 2 % hardwood biochar; PG + RM2 = PG amended with 1 % neutralized redmud; PG + Bc1 + RM2 = PG amended with 2 % hardwood biochar and 1 % neutralized redmud; PG + RM1 = PG amended with 1 % redmud; PG + AC = PG amended with 2 % steam coconut-based activated carbon. Letters indicate significant difference ($p < 0.05$) (n=9).

3.2 Biolog EcoPlates^{TM}

Using absorbance values, diverse indices were calculated.

The AWCD represents the index of the global activity of the bacterial community. It was low on PG (0.054) and increased in all amended conditions except fertilization and Bc1 amendment (Table 1). The highest increase was observed with RM1 and AC. In addition, the application of the fertilizer decreased AWCD value, even though it was not significant.

Table 1: Biolog Ecoplate^{TM} data (AWCD = average well color development, H' = Shannon-Weaver index, E = eveness, Richness = number of positive well ($abs_{590} > 0.25$)) measured after 69 days of maturation in the different treatments. PG = Pontgibaud; PG + fert = PG fertilized; PG + Bc1 = PG amended with 2 % hardwood biochar; PG + RM2 = PG amended with 1 % neutralized redmud; PG + Bc1 + RM2 = PG amended with 2 % hardwood biochar and 1 % neutralized redmud; PG + RM1 = PG amended with 1 % redmud; PG + AC = PG amended with 2 % steam coconut-based activated carbon. Letters indicate significant difference ($p < 0.05$) (n=3).

	AWCD	H'	E	Richness
PG	0.054 ± 0.009 cd	2.3 ± 0.2 a	0.68 ± 0.04 a	1 ± 0 d
PG+fert	0.014 ± 0.002 d	0.7 ± 0.4 b	0.21 ± 0.11 b	1 ± 0 d
PG+Bc1	0.099 ± 0.008 c	2.3 ± 0.0 a	0.68 ± 0.01 a	5 ± 0 c
PG+RM2	0.181 ± 0.008 ab	2.4 ± 0.0 a	0.70 ± 0.00 a	8 ± 1 bc
PG+Bc1+RM2	0.149 ± 0.002 b	2.0 ± 0.1 a	0.59 ± 0.02 a	5 ± 0 c
PG+RM1	0.211 ± 0.018 a	2.5 ± 0.1 a	0.74 ± 0.03 a	12 ± 1 a
PG+AC	0.228 ± 0.015 a	2.7 ± 0.0 a	0.77 ± 0.01 a	10 ± 1 ab

On the contrary, Shannon-Weaver H' and Evenness E values were not affected by the application of the carbon-based materials and/or redmuds; whereas they decreased in the case of fertilizer addition (Table 1).

Finally, richness, representing the number of positive wells, was low on PG and PG+fert, substrates that only presented one positive well on average. All the other treatments increased richness in the order PG+Bc1 – PG+Bc1+RM2 < PG+RM2 < PG+AC < PG+RM1 (Table 1).

Moreover, when looking more closely at the substrates "positively" used in each substrates, it is possible to conclude that none of the soil bacterial community used amines (phenylethylamine and putrescine) and phenolic compounds (2-hydroxy benzoic acid and 4-hydroxy benzoic acid), whereas they all used carbohydrates (β-methyl-D-glucoside, D-galactonic-acid-γ-lactone, D-xylose, i-erythritol, D-mannitol, N-acetyl-D-glucosamine, D-cellobiose, glucose-1-phosphate, α-D-lactose, D-L-α-glycerol-phosphate). Furthermore, all amended conditions, except PG+fert, used other substrate categories in addition to carbohydrates. In detail, in addition to carbohydrates, the microbial communities of PG+Bc1 and

PG+Bc1+RM2 used carboxylic acids (pyruvic-acid-methyl-ester, D-galacturonic acid, γ-hydroxybutyric acid, D-glucosaminic acid, itaconic acid, α-ketobutyric acid, D-malic acid), PG+RM2, PG+RM1 and PG+AC used carboxylic acids, polymers (tween 40, tween 80, αcyclodextrin, glycogen) and amino acids (L-arginine, L-asparagine, L-phenylalanine, L-serine, L-threonine, glycyl-L-glutamic acid).

3.3 Bacterial community structure

The data set of the 16S rRNA sequences showed that the bacterial communities were composed of between 47 and 417 OTUs per sample (Table 2). Treatments presented different richnesses, in terms of number of OTUs. In detail, the treatment PG+fert presented the lowest OTU number, four times less than on PG, while the highest number of OTUs was found in the treatment PG+Bc1+RM2. However, compared to the non-amended Pontgibaud treatment, only the combined application of biochar and redmud increased OTU number (Table 2). The soil bacterial communities presented a similar diversity (Shannon index at OTU level), between 3.59 and 4.32 (Table 2), except for two treatments, fertilization and activated carbon, whose application decreased Shannon index compared to PG, to 2.66 and 2.85, respectively (Table 2).

Table 2: Sequencing data characteristics of soils sampled after 69 days of maturation in the different treatments. PG = Pontgibaud; PG + fert = PG fertilized; PG + Bc1 = PG amended with 2 % hardwood biochar; PG + RM2 = PG amended with 1 % neutralized redmud; PG + Bc1 + RM2 = PG amended with 2 % hardwood biochar and 1 % neutralized redmud; PG + RM1 = PG amended with 1 % redmud; PG + AC = PG amended with 2 % steam coconut-based activated carbon. Letters indicate significant difference ($p < 0.05$) (n=3). OTU = Operational Taxonomic Unit

	Sequencing results			Shannon diversity indices	
	OTUs	Reads	Phyla	OTU level	Phylum level
PG	199 ± 65 bce	51261 ± 5963 a	13 ± 2 bc	4.11 ± 0.22 a	2.06 ± 0.09 a
PG+fert	47 ± 14 e	44518 ± 3339 a	8 ± 3 c	2.66 ± 0.07 b	1.44 ± 0.20 b
PG+Bc1	277 ± 36 b	44203 ± 2631 a	18 ± 1 a	4.30 ± 0.10 a	1.83 ± 0.03 ab
PG+RM2	301 ± 14 abc	43962 ± 2510 a	17 ± 0 ab	4.32 ± 0.01 a	1.84 ± 0.02 ab
PG+Bc1+RM2	417 ± 39 a	45230 ± 2270 a	18 ± 1 a	4.06 ± 0.02 a	1.65 ± 0.02 ab
PG+RM1	118 ± 42 cd	39640 ± 5001 a	10 ± 0 c	3.59 ± 0.06 ab	1.74 ± 0.10 ab
PG+AC	76 ± 30 de	54515 ± 4093 a	11 ± 0 c	2.85 ± 0.61 b	1.48 ± 0.24 b

The bacterial communities were also evaluated at the *phylum* level. They were composed of 8 to 18 *phyla* and only the application of Bc1, alone or combined with RM2, significantly increased *phylum* number. Similarly to what was observed at the OTU level, Shannon diversity index at the *phylum* level was lower in the fertilization and activated carbon treatments compared to PG (Table 2). The relative abundance of the ten most represented *phyla* was calculated and presented in Figure 2. The most represented *phylum* was *Proteobacteria*, followed by *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, *Planctomycetes*, *Acidobacteria*, *Chloroflexi*, *WPS-2* and *Gemmatimonadetes* (Figure 2). Compared to the relative abundance observed in PG soil, *Proteobacteria* abundance increased in the treatment with activated carbon. The addition of fertilizer or amendments did not affect *Actinobacteria* relative abundance compared to PG. However, the relative abundance of *Actinobacteria* was higher in PG+RM2 compared to PG+AC. *Firmicutes* relative abundance on PG was 4.5 %, and this abundance greatly increased only with the fertilization treatment, by five-fold. On the contrary, *Planctomycetes* relative abundance was 9.3 % on PG and highly decreased in PG+fert (< 0.1 %). *Acidobacteria* abundance was more affected by the different treatments. The relative abundance of *Acidobacteria* was 9.5 % on PG and was not affected by the addition of Bc1 alone, whereas it decreased in all the other treatments. The most important decrease was observed in the case of PG+fert (< 1 %), followed by PG+AC (1.4 %), PG+Bc1+RM2 (2.2 %), PG+RM1 (4.5 %) and PG+RM2 (5 %). Similarly, *Chloroflexi* relative abundance was the highest on PG soil (11.5 %) and decreased with all treatments. The lowest abundances were found in PG+Bc1+RM2 (1.1 %) and PG+fert (1.5 %). On the contrary, the relative abundance of *Gemmatimonadetes* was the lowest on PG (< 1 %) and only increased when Bc1 and/or RM2 were applied. The highest increase was observed with the combined application of biochar and redmud. The relative abundance of the three last *phyla*, *Bacteroidetes*, *Cyanobacteria* and *WPS-2*, did not differ among treatments.

Finally, to visualize the overall community at the *phylum* level, a PCA analysis was performed. In this multi-dimensional representation, the closer samples present a closer bacterial composition. The biplot representation (Figure 3) resulting from this PCA analysis explained around 72 % (component 1 + component 2) of the variability. The first component explained around 45 % of the variability and separated the samples according to the *phyla Proteobacteria, Firmicutes, Cyanobacteria, WPS-2* and *Bacteroidetes* (Figure S1); whereas the second component explained around 27 % of the variability and separated the samples according to *phyla Firmicutes*, *Bacteroidetes*and to a lesser extent *Planctomycetes, Chloroflexi* and *Actinobacteria* (Figure S2). Finally, it can be seen from the biplot representation that treatments containing redmud and/or biochar clustered together at the center of the biplot (green circle), and were closer to PG soil than the last two treatments, fertilizer and activated carbon, that showed a bacterial community at the *phylum* level more different from PG, showing that these two treatments greatly affected bacterial community structure.

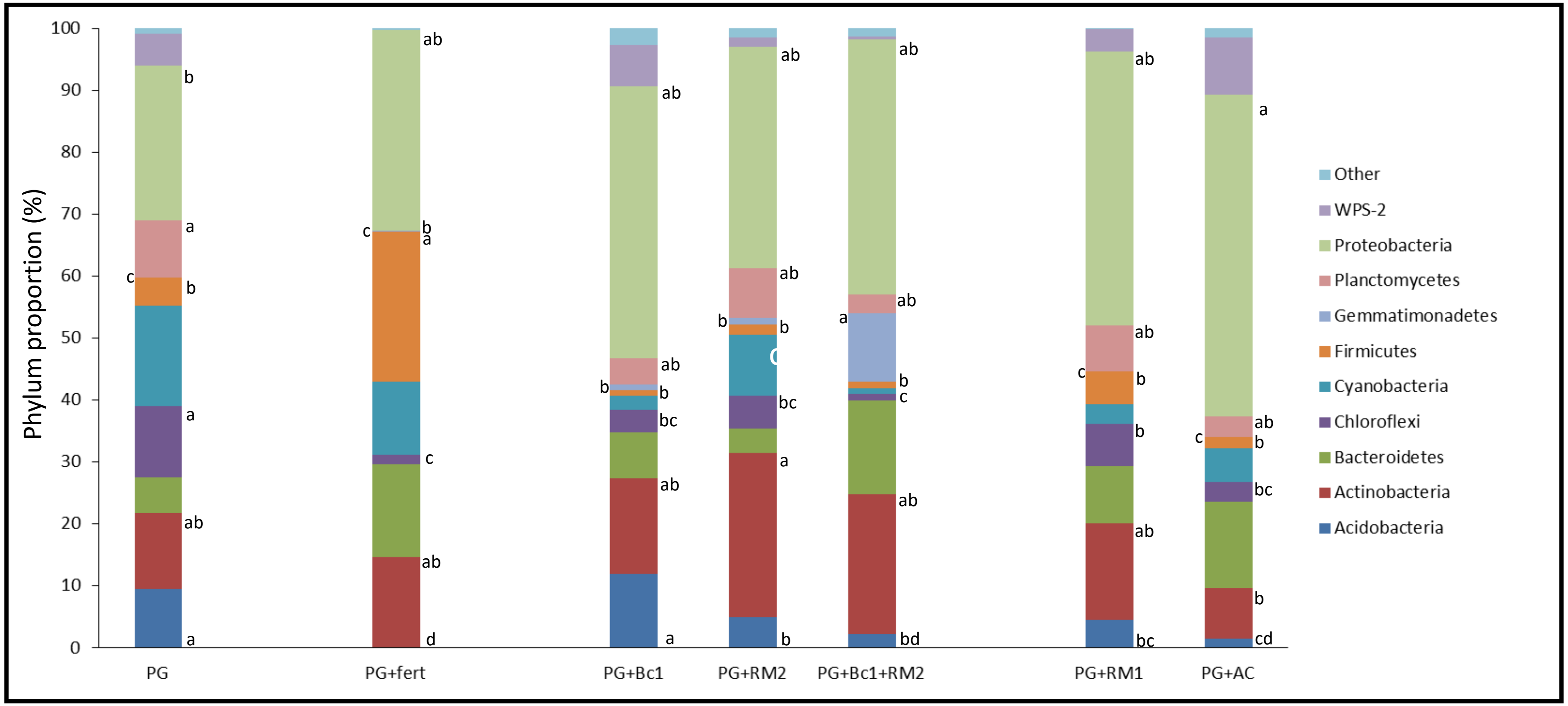

Figure 2: Proportion of the 10 most represented bacterial *phyla* ($p > 1$ %) in the soils sampled after 69 days of maturation in the different treatments. PG = Pontgibaud; PG + fert = PG fertilized; PG + Bc1 = PG amended with 2 % hardwood biochar; PG + RM2 = PG amended with 1 % neutralized redmud; PG + Bc1 + RM2 = PG amended with 2 % hardwood biochar and 1 % neutralized redmud; PG + RM1 = PG amended with 1 % redmud; PG + AC = PG amended with 2 % steam coconut-based activated carbon. Phyla with $p < 1$ % were grouped as "Other". Letters indicate significant different between treatments, only for the phyla differing among treatments ($p < 0.05$) (n=3). No letters are indicated for *Bacteroidetes*, *Cyanobacteria* and *WPS-2* due to the non significant treatment effect.

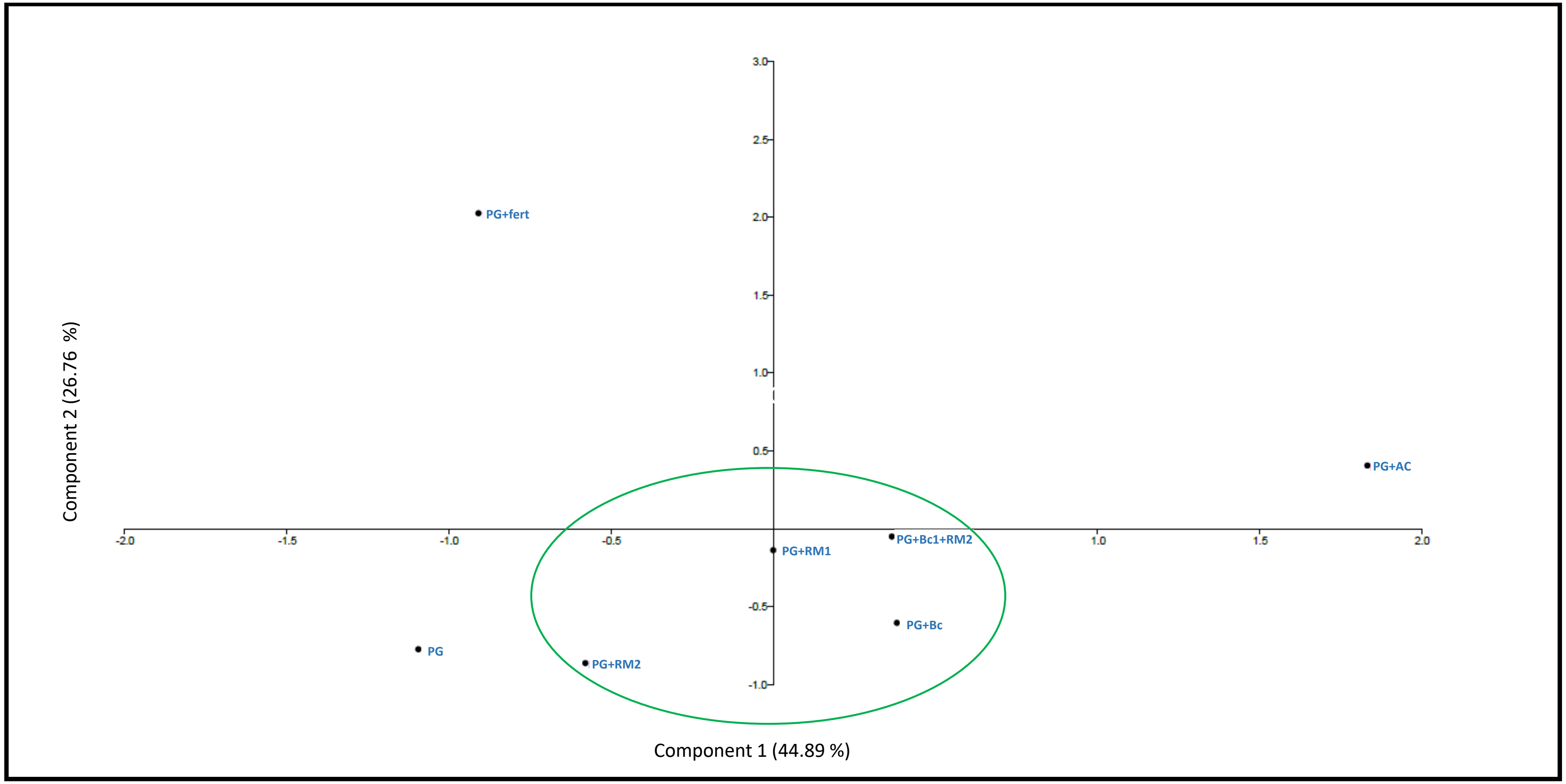

Figure 3: Principal Component Analysis of the bacterial community at the phylum level of the soils sampled after 69 days of maturation in the different treatments. PG = Pontgibaud; PG + fert = PG fertilized; PG + Bc1 = PG amended with 2 % hardwood biochar; PG + RM2 = PG amended with 1 % neutralized redmud; PG + Bc1 + RM2 = PG amended with 2 % hardwood biochar and 1 % neutralized redmud; PG + RM1 = PG amended with 1 % redmud; PG + AC = PG amended with 2 % steam coconut-based activated carbon. The green circle represents the cluster of the amendments containing redmud and/or biochar.

4. Discussion

4.1 Soil enzyme activities

Soil enzyme activities were assessed to have the activity status of the whole bacterial community in the different substrates. For this, four enzyme activities were measured: FDA hydrolysis, β-glucosidase, alkaline phosphatase and acid phosphatase. However, from such measurements at an end-point of 69 days, no clear trend was observed. Indeed, the effect of amendment depended on the treatment applied and the enzyme type.

The FDA hydrolysis was only increased with RM2 application alone. This enzyme activity reflects the activity of several enzymes, such as lipases, esterases and proteases (Araujo *et al.* 2015) and was considered to be directly proportional to the bacterial population by Adam and Duncan (2001). Since metal(loid)s have a negative impact on several enzymes (Yun *et al.* 2017), the increase in FDA hydrolysis activities with RM2 can indicate a reduced metal(loid) toxicity in this treatment, attested by the improvement of the soil conditions (pH increase, soil pore water Pb concentration decrease, Pb availability decrease and nutrient availability increase) (Lebrun *et al.*, in prep, pages 225 to 251). However, other treatments also showed an improvement of their conditions, at similar levels than RM2 in some cases, but did not show an improvement of the global microbial activity assessed by FDA hydrolysis. Furthermore, the treatment PG+RM2 did not show a rise of the other enzyme activities measured. It could signify that although global microbial activity was increased, some specific enzymes, which were not measured here, could have been negatively affected by the different treatments. It could also explain the non-increase in FDA hydrolysis in the other treatments, in which some enzymes could have been stimulated while other were repressed, leading to a neutral effect on the global activity.

β-glucosidase is an enzyme involved in the carbon cycle in the soil (Baldrian 2009, Liao *et al.* 2016). Only the single application of biochar to PG affected β-glucosidase, leading to a decrease of its activity. Previous studies showed contrasting effects of biochar on β-glucosidase. Indeed, Chen *et al.* (2016), Jain *et al.* (2016) and Zheng *et al.* (2016) observed a negative influence of biochar on β-glucosidase activity, while Liao *et al*. (2016) observed a positive effect. Since β-glucosidase enzyme contributes to the degradation of cellulose and consequently the release of glucose used by microorganisms for their growth (Jain *et al.* 2016, Zheng *et al.* 2016), the observed decrease in β-glucosidase activity could mean that in these treatments, bacteria did not need to degrade cellulose for energy source or that cellulose was not in a form available for its degradation, by being sorbed on biochar for instance (Jain *et al*. 2016, Xu *et al*. 2017).

Phosphatase enzymes have an essential role in the mineralization of organic phosphorus (Garcia-Gil *et al.* 2000). Amendments did not greatly affect acid and alkaline phosphatase activities, which could be related to the non-effect of amendments on available phosphorus content, except for fertilization which increased available P content and alkaline phosphatase activity (Lebrun *et al.*, in prep, pages 225 to 251). This result was surprising, as phosphatase activity is generally increased when phosphate availability is low (Garcia-Gil *et al.* 2000, Ros *et al.* 2006) and fertilization decreased soil pH to a level below the optimal pH of alkaline phosphatase (Sardans and Penuelas 2005). Moreover, the combination Bc1+RM2 also increased alkaline phosphatase activity. This increase in activity occurring in those two treatments could be related to an alteration of the phosphorus cycle and/or of the bacterial community. Finally, acid phosphatase activity was higher than the alkaline phosphatase activity, related to soil pH. Indeed, the optimal pH for acid phosphatase is 6.5 while the one for alkaline phosphatase is 11 (Sardans and Penuelas 2005).

Overall, the evaluation of soil enzyme activities showed that amending PG with RM2 improved the global activity of the bacterial community. On the soil physico-chemical properties and plant growth, the amendment RM2 similarly improved soil pH, reduced metal(loid) mobility and availability and greatly increased plant growth. On the contrary, fertilization, RM1 and AC treatments had less strong effects on the soil properties and did not improve plant growth. However, amendment Bc1 showed similar effect on the soil and plant than RM2 but did not ameliorate the overall bacterial activity. This could be related to lower nutrient availability with Bc1 compared to RM2. The measurement of the enzyme activities also revealed that amendments had different effects depending on the enzymes.

4.2 Biolog EcoPlates^{TM}

Contrary to enzyme activities, the Biolog EcoPlates^{TM} method measures the metabolic potential of the cultivable fraction of the bacterial community only (Epelde *et al.* 2008).

AWCD represents the ability of the soil bacteria to use diverse carbon sources (Zhu *et al.* 2017). The increase in AWCD values in four treatments compared to PG (PG+RM2, PG+Bc1+RM2, PG+RM1, PG+AC) showed that in such conditions, the bacterial community had a greater ability to use carbon sources, related to a stimulation of the microbial community by such treatments (Chen *et al.* 2016, Tian *et al.* 2016). However, these treatments did not affect the metabolic diversity of the bacterial community, as revealed by the similar H' value compared to PG (Tian *et al.* 2016). Moreover, fertilization decreased the metabolic diversity of the bacterial community.

Similarly, all the treatments containing biochar and/or redmud presented a higher richness than PG, which was consistent with other studies (Garau *et al.* 2007, 2011, Liao *et al.* 2016). However, in this case, the amendments had a similar effect.

Such beneficial effects of amendments on AWCD and richness values can be explained by the benefits of these amendments on the soil parameters, particularly the decrease in Pb availability and mobility, which was similar in the different amended treatments. Indeed, metal(loid)s are known to have a negative impact on the microbial community functionality (Garau *et al.* 2011). Therefore, the reduced pressure of metal(loid)s on microorganisms induced by amendments led to a higher utilization of carbon substrates and the use of more substrates, such as carboxylic acids, polymers and amino acids, showing a recovery of certain metabolic capacities.

4.3 Bacterial community composition

The analysis of the 16S rRNA sequences showed that biochar amendment increased the number of *phyla* present in the soil, which could be attributed to the recalcitrant carbon of the biochar, which can be assimilated by soil microorganisms (Ameloot *et al.* 2013, Kolton *et al.* 2017). Moreover, the highly porous structure of biochar can act as a protective microhabitat for bacteria, helping in their proliferation (Chintala *et al.* 2016).

Moreover, the dominant *phyla* found in the soil samples were consistent with other studies that observed, in soils contaminated by metal(loid)s, a dominance of bacteria belonging to the *phyla Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Gemmatomonadetes*, *Chloroflexi*, *Bacteroidetes* and *Firmicutes* (Herrmann *et al.* 2019, Liu *et al.* 2019, Lopez *et al.* 2019, Thouin *et al.* 2019, Wang *et al.* 2019, Wu *et al.* 2019). *Proteobacteria* are heterotrophic bacteria known to be able to decompose soluble sugars into monosaccharides (Liu *et al.* 2019). They are found in higher abundance under rich nutrient conditions and high content in carbon sources (Li *et al.* 2019), which could explain the higher abundance in *Proteobacteria* observed in the activated carbon amended condition. *Firmicutes* are copiotrophs and r-strategists (Mackie *et al.* 2015). They can degrade cellulose (Liu *et al.* 2019) and are generally found in soils rich in available carbon (Cesarano *et al.* 2017), which could explain the non-effect of carbon-based amendments, presenting recalcitrant carbon. *Firmicutes* abundance increased with the fertilizer application. This could be attributed to the nutrients added, which favors *Firmicutes* growth (Xu *et al.* 2017). *Acidobacteria* are oligotrophs (Cesarano *et al.* 2017) and usually dominate under low pH (Herrmann *et al.* 2019, Wu *et al.* 2019). This could explain their lower abundance under redmud and activated carbon treatments. However, their abundance also decreased with fertilization, when pH decreased (Lebrun *et al.*, in prep, pages 225 to 251). This decrease could be due to unfavorable conditions, induced by high metal(loid) concentrations in soil pore water and available fractions of the soil, especially arsenic, which increased in soil pore water (Lebrun *et al.*, in prep, pages 225 to 251).

Overall, a shift in the bacterial community composition at the *phylum* level was observed, with four groups formed (PG, activated carbon amendment, fertilization, biochar/redmud amendments). Such shifts in the bacterial community structure could have been induced by the influence of the different treatments on the soil physico-chemical properties. Indeed, the previous paper describing the influence of the same amendments on the soil physico-chemical properties (Lebrun *et al.*, in prep, pages 225 to 251) demonstrated that the diverse treatments had an influence on soil pore water pH and Pb concentrations and on soil extractable metal(loid) fractions. Specifically, Bc1 and RM2 treatments, which clustered together, showed a higher influence on the soil properties (increase in pH and nutrient availability) and metal(loid) immobilization (decrease in Pb concentration in soil pore water and available fraction) (Lebrun *et al.* in prep, pages 225 to 251) than the other two amendments.

5. Conclusion

Following the evaluation of the effect of fertilization as well as activated carbon, biochar and/or redmud amendments on the soil physico-chemical properties and *Salix dasyclados* growth and metal(loid) accumulation pattern, an analysis of the soil bacterial community was performed on the same soils. This study provided a picture at the ending point of 69 days of the effects of these amendments on the soil bacterial communities. This study revealed that soil enzyme activities were influenced by amendments. However, the effects varied depending on the amendment(s) applied and the enzyme considered. On the contrary, the Biolog EcoPlates^{TM} method showed that redmud and activated carbon amendments increased the bacterial activity of the soil. Finally, the NGS method revealed common *phyla*, frequently observed in contaminated soils. Moreover, amendments affected the bacterial community, by increasing or decreasing the relative abundance of certain *phyla*.

Finally, the present study did not match the results observed in a previous paper (Lebrun *et al*., in prep, pages 225 to 251). Indeed, the previous paper revealed that RM2 could be a better amendment due to its beneficial effect on the soil physico-chemical properties and plant growth. However, in the present study, all amendments affected the bacterial community differently and a single amendment could not be selected as the best: RM2 amendment was the only one improving the general hydrolytic activity of the soil but the other amendments also affected the other soil enzyme activities; both redmuds as well as the activated carbons increased the activity of the cultivable bacterial community whereas all amendments induced modification in the structure of the bacterial community. Such results amplify the need to evaluate the effect of amendment application not only on the plant but also on the microbial community in order to evaluate the efficiency of a phytoremediation technique on a metal(loid) contaminated soil. Indeed, to confirm that phytoremediation was successful, several criteria have to be met: (i) improvement of the soil condition, (ii) immobilization of metal(loid)s, (iii) amelioration of plant growth, and (iv) recovery of the microbial activity.

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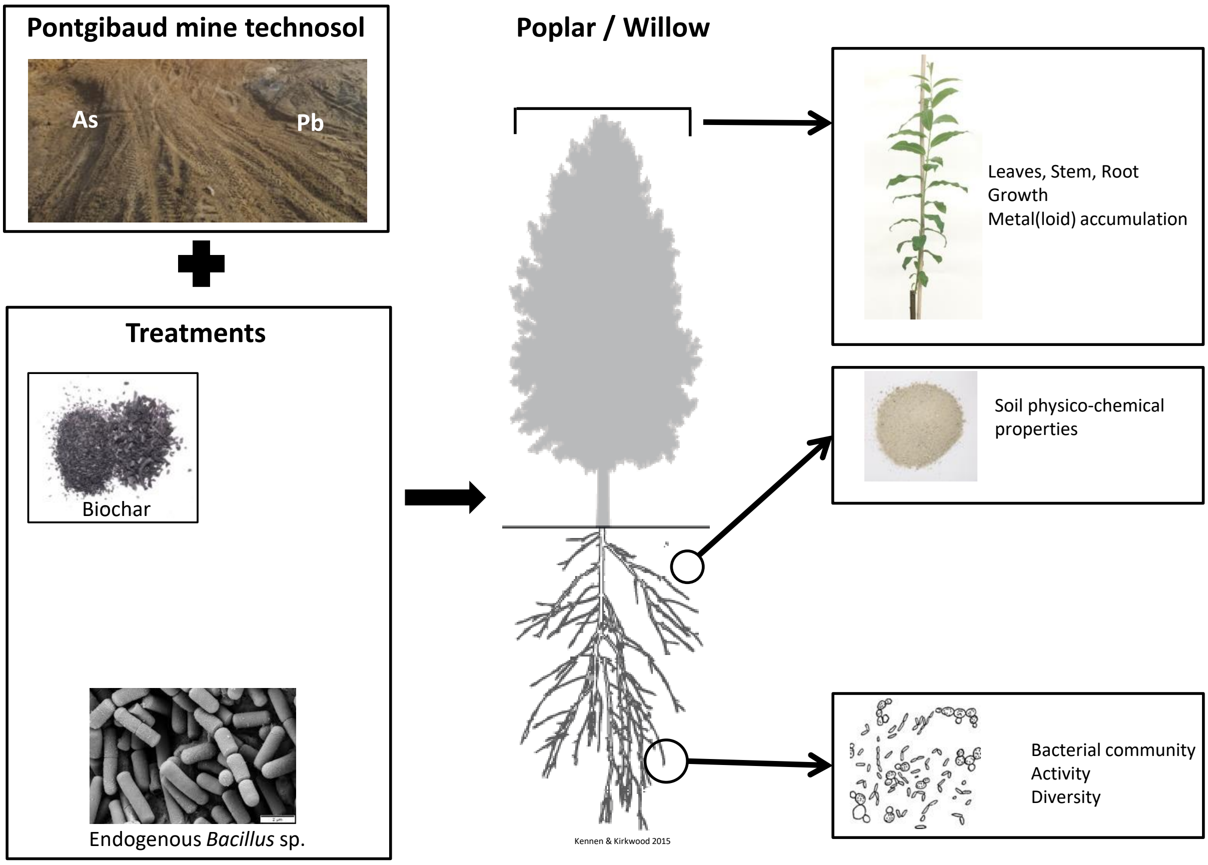
This chapter reported the effects of different amendments on the soil bacterial community.

The two studies showed similar levels of bacterial activity for Pontgibaud and that amendment application tended to improve the soil bacterial activity, visible by the increase in soil enzyme activities and Biolog EcoPlate^{TM} parameters, which can be related to the reduced pressure of metal(loid)s on the soil bacterial community. Moreover, among the different amendments, *i.e.* biochar, compost, fertilizer, iron grit and redmud, compost was the one inducing the best improvement of the bacterial activity, consistent with its elevated nutrient and microorganism contents.

Finally, amending the mining soil induced a shift in the bacterial community, assessed by the 16S sequencing.

Chapter 5. Effect of bacterial inoculation on soil properties, *Salix* growth, metal(loid) immobilization and microbial community.



This last chapter will present the results of a new mesocosm experiment focusing on bacterial inoculation.

Indeed, as stated in the chapter 1, microorganisms, and especially bacteria, are very important in phytomanagement. Indeed, they can affect soil conditions, metal(loid) behavior but also ameliorate plant growth through their plant growth promoting properties. They can thus be inoculated to the soil to improve phytomanagement success.

The study reported in this chapter used a *Bacillus* bacterial strain, previously isolated from Pontgibaud technosol, which was inoculated to a biochar amended Pontgibaud substrate. Two inoculation methods were used: a “direct” liquid inoculation and a “biochar carrier” inoculation.

The first goal of this experiment was to evaluate the effect of the bacterial inoculation on the soil physico-chemical properties, the soil bacterial community and *Salix viminalis* growth and As and Pb accumulation pattern. The second goal of this study was to assess which inoculation method showed better results.

This experiment was submitted to the *Journal Applied Soil Ecology* (APSOIL_2019_1065).

Endogenous *Bacillus* strain inoculation to improve *Salix viminalis* growth and soil bacterial activity for the remediation of an As and Pb contaminated technosol

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Abstract

Phytomanagement manipulates the soil-plant system to lower the risk posed by contaminated soils. In this process, amendments such as biochar can be applied in order to improve the fertility of poor contaminated soils and thus ameliorate plant growth. Moreover, phytomanagement success can also be increased by inoculating bacterial strains which will affect metal(loid) mobility and availability. In addition, many bacteria possess plant growth promoting properties, which improve plant development. Numerous *Bacillus* strains have been isolated from contaminated soils and inoculated, showing positive results on soil properties and plant growth. Furthermore, the association biochar-*Bacillus* strains showed better results than the inoculation alone, as biochar can ameliorate soil properties and can also serve as habitat for microorganisms. Therefore, a mesocosm study was set-up using a mining technosol amended with biochar and inoculated with an endogenous *Bacillus* strain, to evaluate the effect of inoculation on the soil and plant parameters as well as on the metal(loid) immobilization. Moreover, a comparison between two inoculation methods, *i.e.* direct inoculation or inoculation using biochar as a carrier, was done. The results showed that the *Bacillus* strain inoculation decreased soil pore water pH and increased electrical conductivity, plant leaf number and chlorophyll content. Moreover, biomass production increase was higher with the direct inoculation than the carrier inoculation. Although *Bacillus* strain inoculation did not have a great effect on metal(loid) accumulation in plants, its direct inoculation tended to increase Pb concentrations in the aboveground parts. Finally, the microbial activity was stimulated in the inoculated conditions and a higher improvement was observed when biochar was used as a carrier. The *Bacillus* strain inoculation also induced a shift in the microbial community composition, which was more important with the carrier inoculation method.

In conclusion, this study showed an improvement of the plant growth and microbial activity when a *Bacillus* strain was inoculated to a former mining soil with better results recorded when a carrier was used.

Highlights

Two methods were compared to inoculate a metal(loid)s contaminated technosol by a *Bacillus* strain
Biochar was used as bacterial carrier or direct inoculation was assessed
Bacillus strain have an effect on metal(loid)s accumulation in *Salix*
Overall soil microbial activity was stimulated after inoculation
Bacillus inoculation induced a shift in the microbial community composition

Keywords
Bacillus sp.; Biochar; Carrier inoculation; Metal(loid)s; Mine soil; *Salix*

1. Introduction

Phytomanagement can be defined as the use of vegetation and soil amendments in the goal of reducing the environmental risk that is posed by contaminated soils (Domínguez *et al.* 2008). In this process, the soil-plant system is manipulated in order to affect the metal(loid) fluxes in the environment. Such handling aims to remediate contaminated soils, recover metal(loid)s of interest or increase micronutrient concentrations in crops (Tack and Egene 2019).

As a soil amendment in phytomanagement, biochar can be necessary to sustain plant growth on poor mining soils, and has thus been much studied. Biochar is a porous and carbon-rich product that is produced by the pyrolysis of organic materials (Paz-Ferreiro *et al.* 2014). Each biochar shows diverse properties depending on the feedstock, the particle size and the pyrolysis conditions. However, most biochars present an alkaline pH, a high cation exchange capacity, an important water holding capacity, a large surface area as well as the ability to sorb metal(loid)s on their surface (Jiang *et al.* 2012, Angin 2013, Wiszniewska *et al.* 2016). Its application to soil, either contaminated or not, was shown to improve the soil physico-chemical properties, such as pH, nutrient availability, organic matter content…, and to ameliorate the crop development and biomass production (Tang *et al.* 2013, Janus *et al.* 2015).

Another component of the soil system that influences greatly phytoremediation success is the microbiota. Microorganisms are ubiquitous in the environment, notably in the soil. Indeed, soils host a complex web of organisms, among which bacteria are the most abundant (Yu *et al.* 2019). Microorganisms were shown essential in soil remediation (Ojuederie and Babalola 2017). They can control the transfer of metal(loid)s by several mechanisms, such as oxidation, reduction, (de)methylation, complex formation, biosorption (Mishra *et al.* 2017) and also sequester them (Adriano *et al.* 2004). Moreover, many microorganisms, and particularly bacteria, show plant growth promoting (PGP) properties, such as hormone (indole-3-acetic acid (IAA)) production, siderophore secretion, 1-aminocycloropane-1-caroxylate deaminase activity and phosphate solubilization ability. In addition to these direct effects, they can indirectly facilitate plant growth by preventing or reducing the harmful effects of pathogens (Nadeem *et al.* 2014). Therefore, one way to improve plant development and phytomanagement success could be to inoculate the contaminated soil with microorganisms. Inoculation can be done using either exogenous or endogenous strains. However, the inoculation of an endogenous strain isolated from the soil could show better results, as this strain is already adapted to the environmental conditions.

Several studies isolated and identified *Bacillus* strains in contaminated soils, and showed that they were tolerant to high metal(loid) concentrations (Zahoor and Rehman 2009, Çolak *et al.* 2011, Brunetti *et al.* 2012, Besaury *et al.* 2013). Moreover, studies demonstrated that *Bacillus* spp. were able to produce IAA and siderophores as well as to solubilize phosphate, leading to plant growth promotion (Hrynkiewicz *et al.* 2012, Shin *et al.* 2012, Babu *et al.* 2013). Thus, many commercially available biofertilizers are composed of *Bacillus* strains.

Moreover, as shown by Liao *et al.* (2016), biochar, due to its surface properties and amelioration of the soil fertility, can increase the microbial biomass. It acts as a habitat for the microorganisms (Gul *et al.* 2015), improving microbial activity and growth (Gul *et al.* 2015, Zhu *et al.* 2017). Additionally, the association soil-biochar-*Bacillus* strain has been shown the best for almost all plant parameters (Saxena *et al.* 2013).

The inoculation method can have a great influence on the bacterial implementation success and effects on plant and soil: microorganisms can be inoculated directly to the soil by liquid inocula or they can be fixed on a carrier, such as biochar, and then applied to the soil (Zhang *et al.* 2017). Hale *et al.* (2014, 2015) studies showed that the bacterial population was ten times greater when biochar was used as a carrier compared to the liquid inoculation. However, such beneficial effects are dependent on biochar type and bacterial strain.

Therefore, the objectives of the study were to evaluate and compare the effect of direct and carrier endogenous *Bacillus* strain inoculations on (i) the soil physico-chemical properties, (ii) *Salix viminalis* growth parameters and (iii) As and Pb immobilization.

2. Materials and Methods

2.1 Study site

A former silver-lead extraction mine site was used in the present study. This mine was part of the Pontgibaud mining district, which had an important activity during the nineteenth century. Such high extraction activity led to large amounts of wastes, highly contaminated with arsenic and lead (Lebrun *et al.* 2018a, b).

2.2 Biochar

Based on a previous study (Lebrun *et al.* 2018a), a hardwood derived biochar was used as amendment. This biochar was provided by La Carbonerie (Crissey, France) and was produced by the slow pyrolysis at 500 °C of oak, charm and beech biomasses. Following pyrolysis, biochar was sieved to obtain a particle size between 0.2 and 0.4 mm. The biochar characteristics were given in Lebrun *et al.* (2019).

2.3 Bacterial strain

To isolate a bacterial strain tolerant to As and Pb, 1 g of Pontgibaud soil was homogenized in nutrient broth (NB) and incubated at 28 °C for 2 h with shaking. Then, soil suspension was serially diluted and plated on nutrient agar (NA) medium. The plates were incubated at 28 °C for 24 h.

The best candidate for the experiment was chosen after a preliminary bacterial isolates screening based on phenotypic traits and tolerance to different As and Pb concentrations. Then, the 16S rRNA gene was amplified and sequenced through Bact16S protocol at BMR Genomics (Padova, Italy). The isolated bacterial strain was identified as *Bacillus* sp.

A characterization of the strain was realized in terms of IAA and siderophore productions, and phosphate solubilization. The production of IAA was assessed using LB medium (5 $g.L^{-1}$ yeast extract, 10 $g.L^{-1}$ NaCl and 10 $g.L^{-1}$ peptone) supplemented with L-tryptophan, as described in Rajkumar *et al.* (2008). Incubation was done at 30 °C and under shaking (150 rpm) for 96 h. Siderophore production was evaluated by the appearance of an orange color when the strain was cultivated in blue chrome azurol S medium (Shin *et al.* 2012), after 6 h incubation at 30 °C and under 150 rpm shaking. Finally, the ability to solubilize phosphate was determined after bacterial growth on a Pikovskaya's agar medium, supplemented with non-soluble tricalcium phosphate (Babu *et al.* 2013).

Moreover, a growth test was performed in LB media supplemented by As, as a standard solution, at 0.5 $mg.L^{-1}$ or Pb, as two different salts, $PbNO_3$ and $PbCl_2$, at 50 $mg.L^{-1}$. The concentrations used were chosen to be above the concentrations usually measured in Pontgibaud SPW (Lebrun *et al.* 2017, 2018a, b, 2019). Bacterial growth was performed at 30 °C under shaking (250 rpm) and monitored every two hours for eight hours, by measuring the optical density (OD) at 595 nm. Finally, under the same growth conditions, different Pb (0, 1, 5, 10, 20, 50, 100, 200 $mg.L^{-1}$) and As (0, 0.1, 0.2, 0.5, 1, 2, 5, 10 $mg.L^{-1}$) concentrations were tested, using As and Pb standard solutions added to LB media, in order to determined EC50 of both As and Pb towards *Bacillus* sp. after six hours, by measuring the OD at 595 nm. EC50 was calculated by: (i) determining the survival percentage for each concentration compared to the control; (ii) plotting the survival percentage against the concentration, and (ii) determining the equation of the linear curve in the decreasing phase of the curve. The following equation was used: $EC50 = (50 - b)/a$, with a and b being constant of the linear curve equation.

2.4 Treatments

Based on previous studies showing almost no growth on non-amended Pontgibaud and improvement of it by biochar amendment (Lebrun *et al.* 2017; 2018a), the inoculation of the soil by the endogenous bacterial strain was realized on Pontgibaud amended with 5% biochar, using two inoculation methods. Therefore, three treatments were tested:

i. Pontgibaud amended with 5 % biochar and non-inoculated (PB);
ii. PB inoculated with the *Bacillus* strain directly in solution (PB + *Bacillus* direct). The bacterial culture was grown in LB medium at 30 °C for six hours under shaking. Following, the bacterial suspension was centrifuged (10 min; 5500 x g) and washed to recover the bacterial biomass, which was finally resuspended in sterile water (to adjust OD to 10 units) before its inoculation to PB;
iii. PB inoculated with the *Bacillus* strain using biochar as a carrier (PB + *Bacillus* carrier). The bacterial culture was grown in LB medium containing 10% (w/v) biochar (as described in Zhang *et al.* 2017) for six hours under shaking at 30 °C. Following, the solution was filtered and the biochar air dried before its mixing with Pontgibaud soil at 5 % (w/w).

2.5 Soil pore water (SPW) sampling and analysis

SPWs were sampled in all pots using Rhizon® (model MOM, Rhizosphere Research Product, Wageningen, The Netherlands) at two times during the experiment time course: before plant introduction (T0) and at the end, before harvest (T43). These SPWs were analyzed for pH and electrical conductivity (EC), using respectively a pHmeter (FE20/EL20, Mettler-Toledo AG2007) and a conductimeter (SOLEA, Tacussel electronique, Lyon, France); as well as for As and Pb concentrations using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) (ULTIMA 2, HORIBA, Labcompare, San Francisco, USA), after acidification.

2.6 Plant material and analysis

Salix viminalis was chosen as the remediator plant for its high biomass production, dense root system and metal(loid) tolerance (Bart *et al.* 2016, Lebrun *et al.* 2017). For each condition, one rooted cutting was placed in five pots, while three pots were let un-vegetated. Plants were grown for 43 days under greenhouse conditions: temperature 22 ± 2 °C, light intensity 800 $\mu mol.m^{-2}.s^{-1}$, photoperiod 16 h.

After 43 days, leaf chlorophyll content was measured using DUALEX mobile sensor clip (DUALEX SCIENTIFIC^{+TM} FORCE A) (Cerovic *et al.* 2012) on the last three leaves having completed their development. In addition, the number of leaves per plant as well as stem length were determined. Following, new formed organs (leaves, stems, roots) were harvested and dried for three days at 60 °C to determine organ dry weight (DW). As and Pb concentrations in the different plant organs were determined by ICP-AES after acid digestion. Finally, metal(loid) mineralomasses were calculated by multiplying the DW by the concentration.

2.7 Microbial soil analysis

Soils of the different treatments were sampled at the end of the experiment. For each treatment, a sample of similar volume was taken in all of the replicates, five for the vegetated pots and three for the non-vegetated pots. Following a composite sample was made by mixing the samples of the same treatment and compartment (bulk/rhizosphere) together to obtain an homogeneous sample for each condition (Quoreshi *et al.* 2019, Stacey *et al.* 2019). These samples were used to analyze the bacterial community diversity and activity, using Biolog EcoPlates^{TM} and next generation sequencing (NGS), respectively.

For the Biolog EcoPlates^{TM}, bacterial suspensions were obtained by vortexing 2.5 g of fresh soil with 10 mL of sterile NaCl (0.9 %) for 3 minutes. After centrifugation, 600 µL of the supernatant were added in 17.4 mL of sterile NaCl (0.9 %), and 150 µL of the bacterial solution were put in each well of the plate. One plate was prepared by condition. Plates were inoculated at 25 °C and absorbance was measured at 590 nm every 24 h for two weeks.

Moreover, total soil DNA was extracted, based on the manufacturer's instructions, using a PowerSoil^{TM} DNA kit (MO BIO Laboratories, Inc.). The sequencing of the extracts was realized by INRA Transfert (Narbonne, France) using the following procedure: the amplicons were amplified by PCR using primers targeting the regions V4-V5 of the 16S RNAr (primers 515F and 909R), and sequenced by MiSeq Illumina. Following, the computer pipeline Mothur (version 1.36.1) was used to analyze the sequences. First, the barcodes, primers and sequences presenting homopolymers of more than 8 bp as well as the chimers were removed. Second, the sequences presenting 100% homology between each other were grouped in OTU (Operational Taxonomic Unit). Finally, OTUs were identified based on taxonomy using Greengenes.

2.8 Statistical analyses

R software version 3.1.2 (R Development Core Team, 2009) was used to analyze the data. The normality and homoscedasticity of the data were evaluated using respectively the Shapiro and the Bartlett tests. Based on the results of these tests, means were compared using either Anova test or Kruskal test, for normal and non-normal data respectively; followed by a post-hoc test (TukeyHSD or Pairwise Wilcox, respectively).

In addition, on the SPW data, the "time" effect was evaluated using the same procedure whereas on the soil data, a "plant" effect was assessed by comparing the means of the bulk and rhizosphere conditions using a Student test for normal data and a Wilcox test for non-normal data.

Finally, a canonical correlation analysis was performed on the NGS data.

3. Results and Discussion

3.1 Characterization of the bacterial strain

The endogenous bacterial strain, isolated from Pontgibaud technosol and identified as *Bacillus* sp. was characterized for three PGP properties: phosphate solubilization, siderophore production and IAA production. Such PGP properties help to promote plant growth, facilitating resource acquisition and modulating phytohormones levels (direct promotion) and/or also decreasing the inhibitory effects of pathogens (indirect promotion) (Yu *et al.* 2019). In detail, the solubilization of phosphate by bacteria supplies phosphorus to plants (Ojuederie and Babalola 2017). Siderophores, metal binding molecules having low molecular mass (Ojuederie and Babalola 2017), can chelate toxic metals reducing their availability. IAA can enhance the elongation as well as the surface area of the roots, to make nutrients more available (Ojuederie and Babalola 2017).

Results showed that endogenous Pontgibaud *Bacillus* strain was able to solubilize phosphate (Pikovskaya medium test; Table 1) whereas it was not possible to detect siderophore production (chrome azurol S medium test; Table 1). On the other hand, the strain was able to produce IAA using L-tryptophan as a source (Table 1).

Such results indicated that this bacterium harbors some PGP properties and thus is possibly capable to increase plant growth and protect the plant against metal stress (Marques *et al.* 2013) when inoculated in soil.

Furthermore, the growth test in presence of one concentration of As or Pb showed that the *Bacillus* strain was able to grow in presence of 0.5 mg.L^{-1} As and 50 mg.L^{-1} without its growth being affected (Figure S1), as shown by the growth curve that were similar in the presence of the contaminants than in the control. Such results showed the resistance of *Bacillus* sp. towards As and Pb. Finally, the test performed with increasing concentrations of As and Pb determined the EC50 values to 1.95 mg.L^{-1} for As and 14.48 mg.L^{-1} for Pb.

Table 1: Characterization of the endogenous *Bacillus* strain

Measured parameters	Methods	Results
Phosphate solubilization	Pikovskaya medium	Positive
Siderophore production	Chrome Azurol S medium	Unconclusive
IAA production*	LB + Tryptophane medium	Positive

*IAA: indole-3-acetic acid

3.2 Soil pore water (SPW) physico-chemical properties

At T0, SPW pH in PB treatment was around neutrality (pH 6.15) (Table 2), and was lower when the *Bacillus* strain was inoculated through biochar carrier (PB + *Bacillus* carrier). The same trend was observed in the non-vegetated pots at T43: neutral pH on PB, which decreased with carrier-*Bacillus* inoculation (PB + *Bacillus* carrier). Such pH decrease has already been observed following the inoculation of *Burkolderia* sp J62 (Jiang *et al.* 2008) as well as *Azotobacter chroococcum*, *Bacillus* *megaterium* and *Bacillus mucilaginosus* (Wu *et al.* 2006). This can be attributed to the production of organic acids by the bacteria inoculated (Orhan *et al.* 2006, Ali *et al.* 2017) and to the secretion of protons and amino acid (Wu *et al.* 2006). On the opposite, in the *Salix* vegetated pots at T43, pH was not influenced by the bacterial inoculation. Such no effect was also observed after the single or associated application of a PGPR and a rhizobium (Ju *et al.* 2019). Finally, in all conditions, SPW pH was observed to increase with time, between 1.3 and 1.6 unit, with no plant effect. This can be due to the aging of the biochar in the mixture, which alters biochar surface groups (Murkherjee *et al.* 2014).

Table 2: Soil pore water physico-chemical properties (pH, electrical conductivity (EC) (µS.cm^{-1}), As and Pb concentrations (mg.L^{-1})) determined at the beginning of the experiment (T0) and at the end of the experiment in non-vegetated (T43 - *S. viminalis*) and vegetated (T43 + *S. viminalis*) pots in Pontgibaud contaminated technosol amended with biochar (PB), biochar + *Bacillus* strain in direct inoculation (PB + *Bacillus* direct) or biochar + *Bacillus* strain using biochar as carrier (PB + *Bacillus* carrier). Minuscule letters indicate significant differences between T0, T43 - *S. viminalis* and T43 + *S. viminalis* conditions while capital letters indicate significant difference between the three treatments at each sampling time (n = 3-5) ($p < 0.05$).

		pH	EC (µS.cm^{-1})	[As] (mg.L^{-1})	[Pb] (mg.L^{-1})
PB	T0	6.15 ± 0.03 **aB**	570 ± 43 **aA**	0.005 ± 0.004 **aA**	4.53 ± 2.31 **bA**
	T43 - *S. viminalis*	7.70 ± 0.07 **bB**	1247 ± 74 **bA**	0.052 ± 0.003 **bA**	0.50 ± 0.06 **aA**
	T43 + *S. viminalis*	7.57 ± 0.03 **bA**	1077 ± 131 **bA**	0.051 ± 0.005 **bA**	0.43 ± 0.05 **aA**
PB + *Bacillus* direct	T0	6.24 ± 0.02 **aB**	548 ± 39 **aA**	0.004 ± 0.006 **aA**	3.65 ± 0.48 **bA**
	T43 - *S. viminalis*	7.58 ± 0.03 **bAB**	1458 ± 77 **bA**	0.054 ± 0.001 **bA**	0.47 ± 0.02 **aA**
	T43 + *S. viminalis*	7.64 ± 0.09 **bA**	1878 ± 219 **bA**	0.058 ± 0.005 **bA**	0.55 ± 0.06 **aA**
PB + *Bacillus* carrier	T0	5.97 ± 0.06 **aA**	2591 ± 402 **aB**	0.007 ± 0.003 **aA**	15.81 ± 5.58 **bB**
	T43 - *S. viminalis*	7.46 ± 0.01 **bA**	1407 ± 51 **aA**	0.088 ± 0.023 **bA**	0.58 ± 0.02 **aA**
	T43 + *S. viminalis*	7.56 ± 0.09 **bA**	3924 ± 1614 **aA**	0.046 ± 0.002 **abA**	0.49 ± 0.07 **aA**

PB substrate presented an EC of 570 µS.cm^{-1} in the SPW at T0 (Table 2). EC was increased 4.5 times when the *Bacillus* strain was inoculated on biochar (PB + *Bacillus* carrier), while the direct inoculation had no effect (PB + *Bacillus* direct). *Bacillus* inoculation, with both methods, had no effect on SPW EC at T43. Wu *et al.* (2006) also observed an increase in EC following bacterial inoculation. The authors explained such increase by the effect of the bacterial inoculation on pH. Indeed, with decreasing soil pH, bases are released into the soil solution. Such effect can also explain the EC increase observed at T0. Indeed, at T0, both an EC increase and a pH decrease were observed in PB + carrier *Bacillus* treatment, compared to PB. Finally, with time, increases in EC were observed in PB and PB + *Bacillus* direct conditions, while no change was observed in PB + *Bacillus* carrier.

SPW As concentrations were low in all substrates and at all times, between 0.004 mg.L^{-1} and 0.088 mg.L^{-1} (Table 2). *Bacillus* inoculation, either through biochar carrier or directly, had no effect on SPW As concentration. However, an increase in SPW As concentration was observed in all treatments, between 10 and 15-fold, in T43 compared to T0. Such increase can be attributed to the ability of biochar to mobilize As and can also be related to the pH increase induced by biochar aging (Beesley *et al.* 2013).

SPW Pb concentration in PB was 4.53 mg.L^{-1} (Table 2). The inoculation of the endogenous *Bacillus* strain highly increased SPW Pb concentration at T0, but only when the inoculation was done using biochar as a carrier (PB + *Bacillus* carrier). Previous studies showed an increase in Pb mobility following bacterial inoculation. Jiang *et al.* (2008) observed a rise in water-soluble Pb in *Burkholderia* sp J62 culture. Similarly, the inoculation of Pb-resistant strains in presence of Pb contamination increased SPW Pb concentration (Sheng *et al.* 2008). Finally, He *et al.* (2013) showed that the concentration of water soluble Cd, Pb and Zn increased with the inoculation of the strain JN6. Such increase can be related to the pH decrease observed in this treatment (Jiang *et al.* 2008, He *et al*. 2013). However, after 43 days, no effect of the inoculation was detected in both non-vegetated and vegetated pots (comparison between PB, PB + *Bacillus* strain direct inoculation and PB + carrier-*Bacillus* strain at T43). Finally, a SPW Pb concentration decrease, from 85 % to 97 %, was observed with time, which could be related to biochar aging, as biochar is known to be able to sorb Pb, and to the pH rise observed with time (Lebrun *et al.* 2018a).

3.3 Plant parameters

At the end of the experiment, leaf chlorophyll content was determined using Dualex. Measurements showed that on PB substrate, leaf presented 16.03 µg.cm^{-2} chlorophyll (Table 3). Only when inoculated through biochar carrier (PB + *Bacillus* carrier) the *Bacillus* strain increased leaf chlorophyll content by 1.2-fold. Similarly, Wani and Khan (2010) inoculated an industrial site with *Bacillus* PSB10 and observed a rise in leaf chlorophyll content, while Akhtar *et al.* (2018) spiked a non-contaminated soil with Ni and added *Bacillus* sp. CIK-516 as well as *Stenotrophomonas* sp. CIK-517Y and found higher chlorophyll contents in leaves. Such increase in pigment contents showed a decrease in metal(loid) stress (Ali *et al.* 2017). It can also be explained by a more important photosynthetic leaf area (Jamil *et al.* 2014) as well as an increase in iron availability induced by bacteria (Islam *et al.* 2014).

Table 3: Leaf number, chlorophyll content (µg.cm^{-2}) and stem length (cm) of *Salix viminalis* plant after 43 days of growth on Pontgibaud contaminated technosol amended with biochar (PB), PB + *Bacillus* strain in direct inoculation (PB + *Bacillus* direct) or PB + *Bacillus* strain using biochar as carrier (PB + *Bacillus* carrier). Letters indicate significant differences between the three treatments (n = 5) (p < 0.05).

	Number of leaves	Chlorophyll content (µg.cm^{-2})	Stem length (cm)
PB	13 ± 1 **a**	16.03 ± 0.93 **a**	14.76 ± 2.06 **a**
PB + *Bacillus* direct	18 ± 1 **b**	15.11 ± 0.71 **a**	25.26 ± 1.31 **b**
PB + *Bacillus* carrier	19 ± 1 **b**	19.61 ± 0.94 **b**	21.93 ± 2.34 **b**

The number of leaves as well as stem length showed similar trends: lower values observed on PB (13 leaves.plants^{-1} and 14.76 cm, respectively) (Table 3) compared to the soil inoculated with *Bacillus* strain, in which both parameters were increased by 1.4 and 1.6-fold on average, respectively (Table 3). However, there was no significant difference between the direct inoculation and the carrier inoculation. PB plants produced on average 0.28 g leaves, 0.09 g stem and 0.38 g roots (Figure 1). Leaf DW was increased by both inoculation methods, with no difference between the two methods which showed a 2.2-fold DW production improvement compared to PB. Stem DW was not affected by the *Bacillus* strain inoculation, whereas root DW was shown to increase 2.2 times, but only when the strain was inoculated directly (PB + *Bacillus* direct). Finally, taking into account the whole plant DW production, *Salix viminalis* plants grown on PB + *Bacillus* direct treatment produced the highest DW (1.68 g), followed by the carrier inoculation (PB + *Bacillus* carrier) (1.20 g) and the non-inoculated condition (PB) (0.75 g).

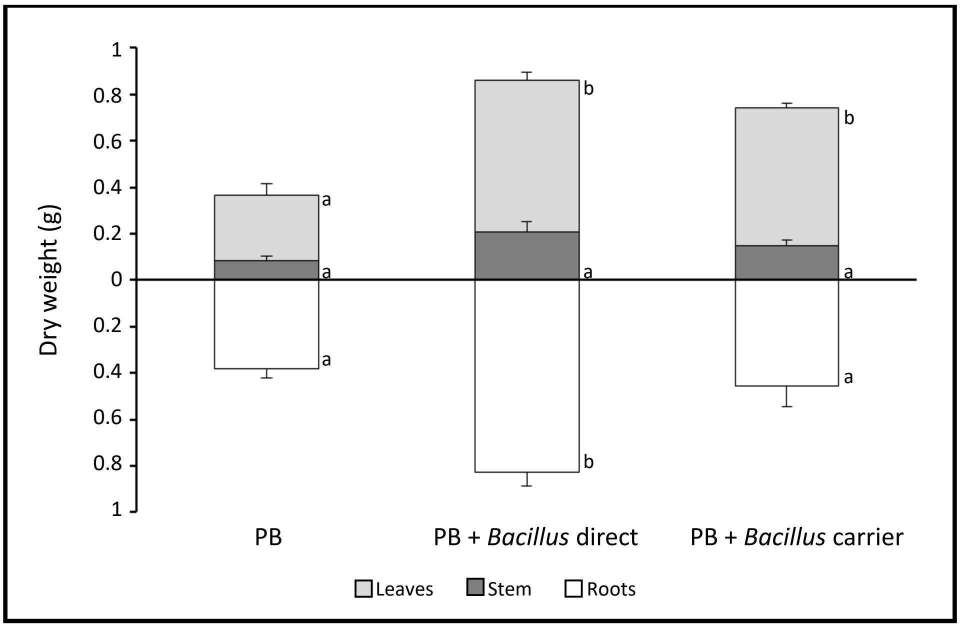

Figure 1: *Salix viminalis* organs dry weight (g) after 43 days of growth on Pontgibaud contaminated technosol amended with biochar (PB), PB + *Bacillus* strain in direct inoculation (PB + *Bacillus* direct) or PB + *Bacillus* strain using biochar as carrier (PB + *Bacillus* carrier). Letters indicate significant difference between the three treatments (p < 0.05) (n = 5).

Such improvements of growth are consistent with previous studies evaluating the effect of inoculation. For instance, Rajkumar and Freitas (2008) observed an increase in mustard shoot length following the inoculation of either *Pseudomonas* sp. Ps29C or *Bacillus megaterium* Bm4C in presence of Ni. Similarly, Sheng and Xia (2006) inoculated a contaminated electroplate factory by a bacteria isolated from the soil and observed a rise in root length. Finally, Yu *et al.* (2012) showed that the inoculation of a phosphate mine by four bacterial strains induced an improvement in walnut plant height, and shoot and root DW. Higher DW production with the *Bacillus* strain inoculation can be explained by: (i) the mitigation of metal(loid) toxicity (Wu *et al.* 2006), (ii) the production of IAA by the bacterial strain (Mena-Violante and Olalde-Portugal 2007, Felici *et al.* 2008, Babu *et al.* 2013), and (iii) higher chlorophyll production (Akhtar *et al.* 2018).

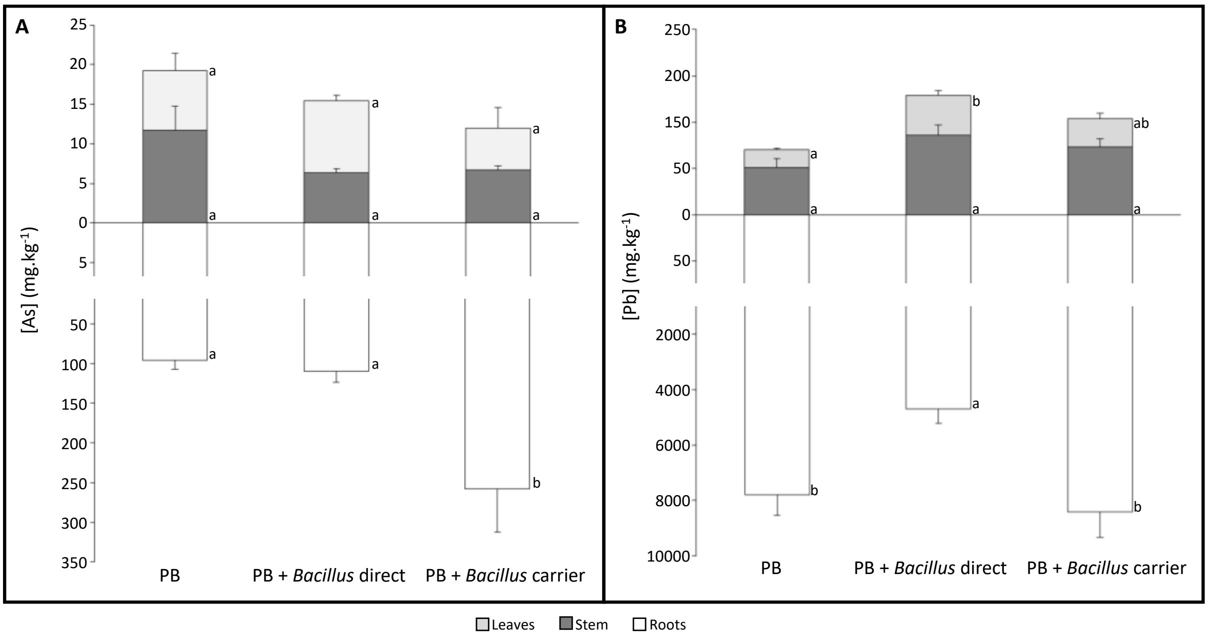

Figure 2: *Salix viminalis* organs arsenic (A) and lead (B) concentrations (mg.kg^{-1}) after 43 days of growth on Pontgibaud contaminated technosol amended with biochar (PB), PB + *Bacillus* strain in direct inoculation (PB + *Bacillus* direct) or PB + *Bacillus* strain using biochar as carrier (PB + *Bacillus* carrier). Letters indicate significant difference between the three treatments ($p < 0.05$) ($n = 5$).

Contrary to the plant growth parameters, As and Pb concentrations were little affected by the *Bacillus* strain inoculation. Indeed, As concentration was only higher in roots of plants grown on PB inoculated using the carrier (PB + *Bacillus* carrier), compared to PB (Figure 2A). Pb concentration was only affected by the direct inoculation (PB + *Bacillus* direct), where it increased in leaves and decreased in roots (Figure 2B).

As and Pb mineralomasses in plant parts were calculated based on DW production and metal(loid) concentrations. As for the concentrations, the *Bacillus* strain inoculation had small effects: As quantities increased following direct inoculation (PB + *Bacillus* direct) in both leaves and roots, whereas Pb quantities in leaves were higher in both inoculation treatments, compared to PB (Figures 3A and 3B).

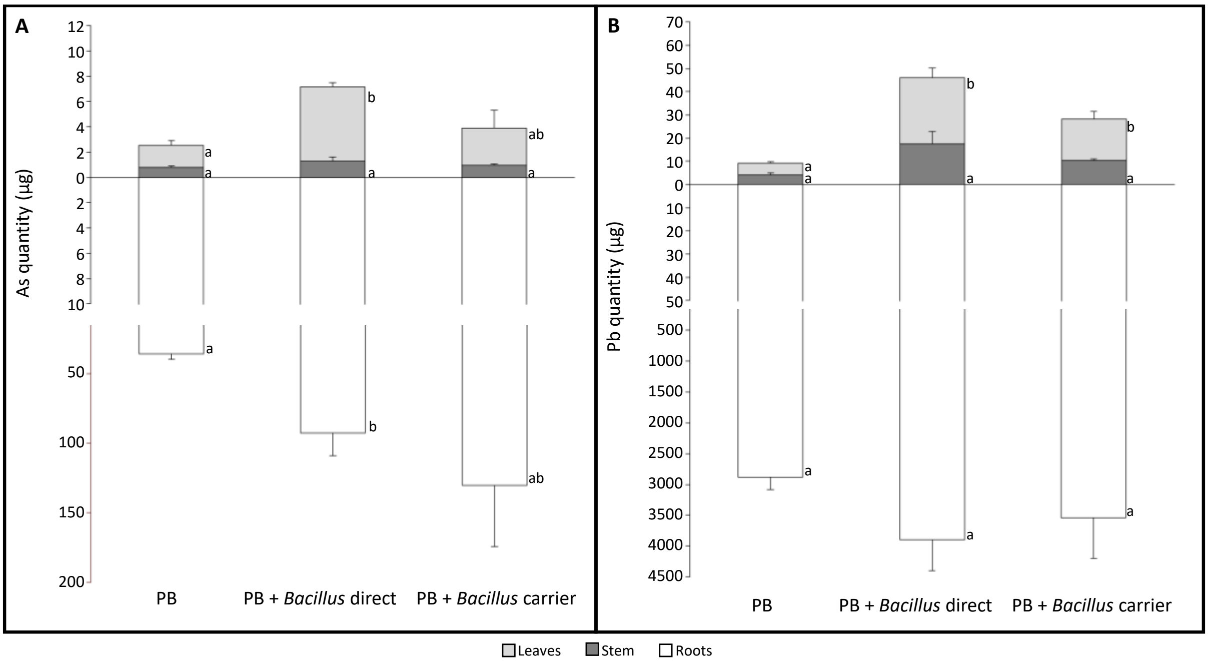

Figure 3: *Salix viminalis* organs arsenic (A) and lead (B) contents (µg) after 43 days of growth on Pontgibaud contaminated technosol amended with biochar (PB), PB + *Bacillus* strain in direct inoculation (PB + *Bacillus* direct) or PB + *Bacillus* strain using biochar as carrier (PB + *Bacillus* carrier). Letters indicate significant difference between the three treatments ($p < 0.05$) ($n = 5$).

Bacterial inoculation was shown to increase metal(loid) accumulation in plants, due to the production of siderophores and organic acids, the induced decrease of pH and the mobilization of metals by phosphates (Babu *et al.* 2013). However, few studies also showed no change in metal(loid) concentrations after soil inoculation. Indeed, Rajkumar and Freitas (2008) did not observe a modification of Ni accumulation in the root and shoot systems following *Pseudomonas* sp. or *Bacillus megaterium* inoculation. Similarly, Jiang *et al.* (2008) studied the response of Indian mustard, tomato and maize to *Burkholderia* sp J62 inoculation. They observed no effect on Pb and Cd shoot concentrations in Indian mustard and tomato. However, shoot Cd concentration in maize decreased, while root Pb and Cd concentrations in maize and Indian mustard increased. Moreover, Lu and Zhang (2014) stated that the influence of inoculation on plant metal accumulation was dependent on the metal type, as well as the interaction between the inoculated strain and the plant species.

3.4 Soil microbial community analysis

An understanding of the temporal and spatial structures, functions, interactions, and population dynamics of microbial communities is critical for many aspects of life such as scientific discovery, biotechnological development, environmental protection, and human health (Bucci *et al.* 2017). Accordingly, several methods have been employed to reveal microbial community composition, function and responses to environmental changes, in several and various environments and in different contexts (Bucci *et al.* 2011, 2014, 2015 a,b, Crescenzo *et al.* 2017, Di Luccia *et al.* 2018, Petrella *et al.* 2018, Pietrangelo *et al.* 2018).

Biology EcoPlates^{TM} were used to evaluate the soil microbial community.

The Average Well Color Development (AWCD), index of the bacterial diversity (Zhu *et al.* 2017), in both bulk and rhizosphere compartments, was higher in the inoculated treatments compared to PB (Table 4). In the bulk condition, there was no significant difference between the two inoculation methods, while in the rhizosphere, the inoculation using biochar as a carrier induced a higher increase than the direct inoculation. Moreover, in all cases, plant development increased the AWCD value compared to the non-vegetated condition.

Table 4: Biolog Ecoplate data (AWCD = average well color development, H' = Shannon-Weaver indice, E = Eveness indice, Richness) determined at the end of the experiment in non-vegetated (Bulk) and vegetated (Rhizosphere) pots in Pontgibaud contaminated technosol amended with biochar (PB), PB + *Bacillus* strain in direct inoculation (PB + *Bacillus* direct) or PB + *Bacillus* strain using biochar as carrier (PB + *Bacillus* carrier). Minuscule letters indicate significant differences between the three treatments for the bulk condition while capital letters indicate significant difference between the three treatments for the rhizosphere condition (n = 3) ($p < 0.05$). Significant difference between the bulk and rhizosphere conditions is indicated by: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

		AWCD	H'	E	Richness
PB	Bulk	0.18 ± 0.02 **a** *	2.5 ± 0.1 **a**	0.73 ± 0.03 **a**	7 ± 1 **a** **
	Rhizosphere	0.30 ± 0.02 **A**	2.7 ± 0.0 **A**	0.80 ± 0.01 **A**	13 ± 1 **A**
PB + *Bacillus* direct	Bulk	0.28 ± 0.01 **b** **	2.8 ± 0.1 **ab** *	0.81 ± 0.02 **ab** *	11 ± 1 **b** **
	Rhizosphere	0.40 ± 0.01 **B**	2.7 ± 0.0 **A**	0.78 ± 0.01 **A**	15 ± 1 **A**
PB + *Bacillus* carrier	Bulk	0.32 ± 0.01 **b** ***	2.9 ± 0.0 **b**	0.85 ± 0.01 **b**	15 ± 1 **c** *
	Rhizosphere	0.69 ± 0.03 **C**	3.0 ± 0.0 **B**	0.89 ± 0.01 **B**	22 ± 1 **B**

Shannon-Weaver H', representing the number of distinct functions carried out by a bacterial community (Boshoff *et al.* 2014), and Eveness E indices were only increased when the *Bacillus* strain was inoculated with biochar as a carrier (PB + *Bacillus* carrier), compared to PB, in both bulk and rhizosphere soil (Table 4). In addition, only in this treatment (PB + *Bacillus* carrier) plants were shown to increase both indices.

Finally, richness, or the number of wells having an absorbance above 0.25, rise was higher with the carrier inoculation (PB + *Bacillus* carrier) than the direct inoculation (PB + *Bacillus* direct), compared to PB, in bulk soil. However, in the rhizosphere soil, richness increased only when biochar was used as a carrier (PB + *Bacillus* carrier) (Table 4). In addition, as for AWCD, plant development induced an increase in the richness value.

All of these data obtained by Biolog EcoPlates^{TM} analysis revealed that the inoculation of PB substrate using an endogenous *Bacillus* strain enhanced the microbial diversity and activity. Moreover, plant development increased AWCD, Shannon-Weaver and Eveness indices and richness, which can be explained by the presence of easily mobilizable root exudates and root surfaces for the microbial colonization (Epelde *et al.* 2009, He *et al.* 2018).

Furthermore, the substrates of the Biolog EcoPlates^{TM} were grouped in six categories (carbohydrates, amino acids, carboxylic acids, polymers, amines and phenolic compounds) and AWCD values of each substrate category was used to determine the percentage of utilization of the difference substrates. The data revealed that carboxylic acids and polymers were the two categories mostly used in bulk, whereas amino and carboxylic acids were the main categories used in the rhizosphere. This can show that the plant development modifies the bacterial activity from polymers-based community to amino acids. Moreover, in the bulk, a higher use of carbohydrates was observed following the *Bacillus* strain inoculation using carrier (PB + *Bacillus* carrier) while polymer use increased with both inoculation methods, compared to PB (Figure 4). The rhizosphere community was more affected by inoculation. Indeed, all categories showed changes in their relative utilization. Inoculation by using the carrier (PB + *Bacillus* carrier) induced an increase in carbohydrates and phenolic acids use, while lower relative use was found for amino and carboxylic acids. Moreover, direct inoculation (PB + *Bacillus* direct) increased the relative utilization of carboxylic acids whereas it decreased the one of polymers and amines (Figure 4). Such results showed that the *Bacillus* strain inoculation shifted the bacterial diversity and activity, and that the inoculation method influenced in which direction the community was affected. Finally, plant development on PB only decreased polymer use. On the carrier inoculation condition, the rhizosphere soil showed a lower carboxylic acid use together with a higher utilization of amines and phenolic compounds compared to the bulk. Plant growth on the PB soil directly inoculated with the *Bacillus* strain induced a decrease in the relative utilization of carbohydrates and polymers (Figure 4). This showed that the plant development had different effects depending on the substrate on which plants grew, which can be explained by a difference in plant exudation.

The analysis of the soil bacterial community by the sequencing of the 16S RNA revealed the presence of the following *phyla*: *Bacteroidetes*, *Proteobacteria*, *Planctomycetes*, *Firmicutes*, *Cyanobacteria*, *Acidobacteria*, *Chloroflexi*, *Actinobacteria*, *Armatimonadetes*, *Verrucomicrobia*, *Gemmatimonadetes* and *Nitrospirae* (Figure 5).

The *phylum Bacteroidetes* includes species which have an important role in the degradation of organic matter and in the carbon cycle (Liu *et al.* 2019). Indeed, these microorganisms have the ability to rapidly exploit the organic matter bioavailable (Cesarano *et al.* 2017). The relative abundance of this *phylum* was lower in the bulk of PB soil inoculated using a carrier (PB + *Bacillus* carrier) compared to PB, while it was higher in the rhizosphere of the same condition. Moreover, plant development decreased

Bacteroidetes proportion in PB and PB directly inoculated, while it increased it in the last treatment (PB + *Bacillus*-carrier).

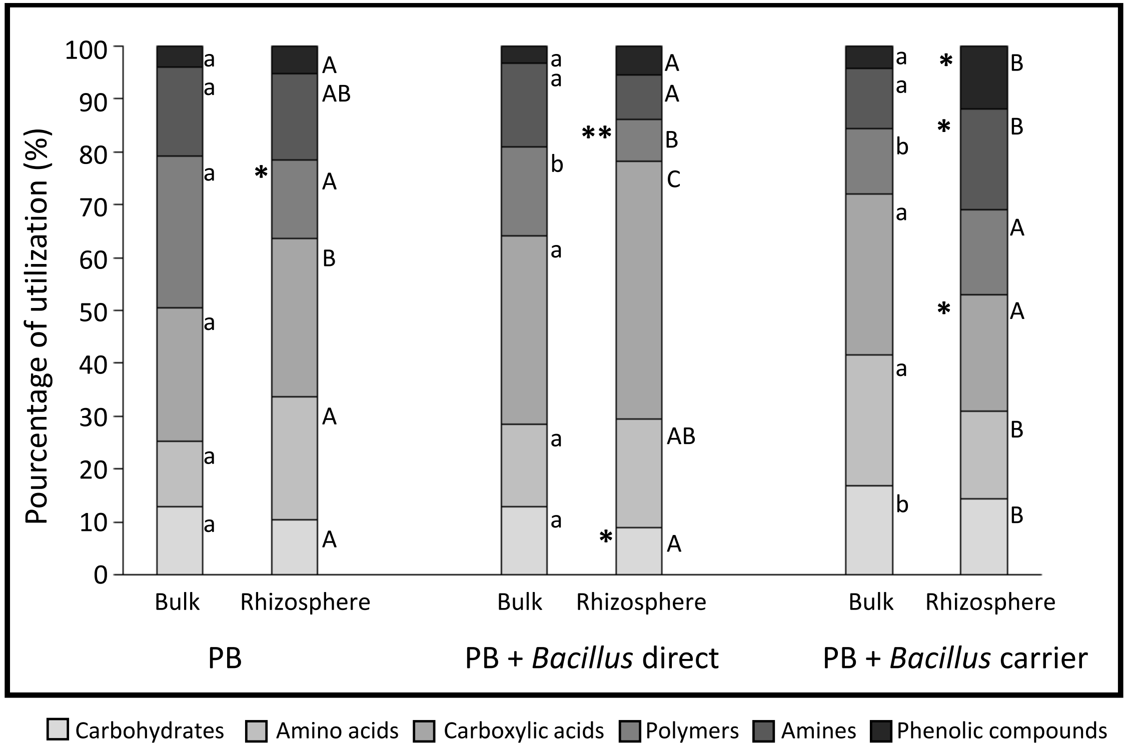

Figure 4: Percentage of utilization of the substrates categories (carbohydrates, amino acids, carboxylic acids, polymers, amines, phenolic compounds) determined by the Biolog Ecoplates at the end of the experiment on the Pontgibaud contaminated technosol amended with biochar (PB), PB + *Bacillus* strain in direct inoculation (PB + *Bacillus* direct) or PB + *Bacillus* strain using biochar as carrier (PB + *Bacillus* carrier) for both non vegetated (Bulk) and vegetated (Rhizosphere) conditions. Minuscule letters indicate significant difference between the three treatments for the bulk condition while capital letters indicate significant difference between the three treatments for the rhizosphere condition ($p < 0.05$) ($n = 5$). Significant difference between the bulk and rhizosphere conditions is indicated by: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

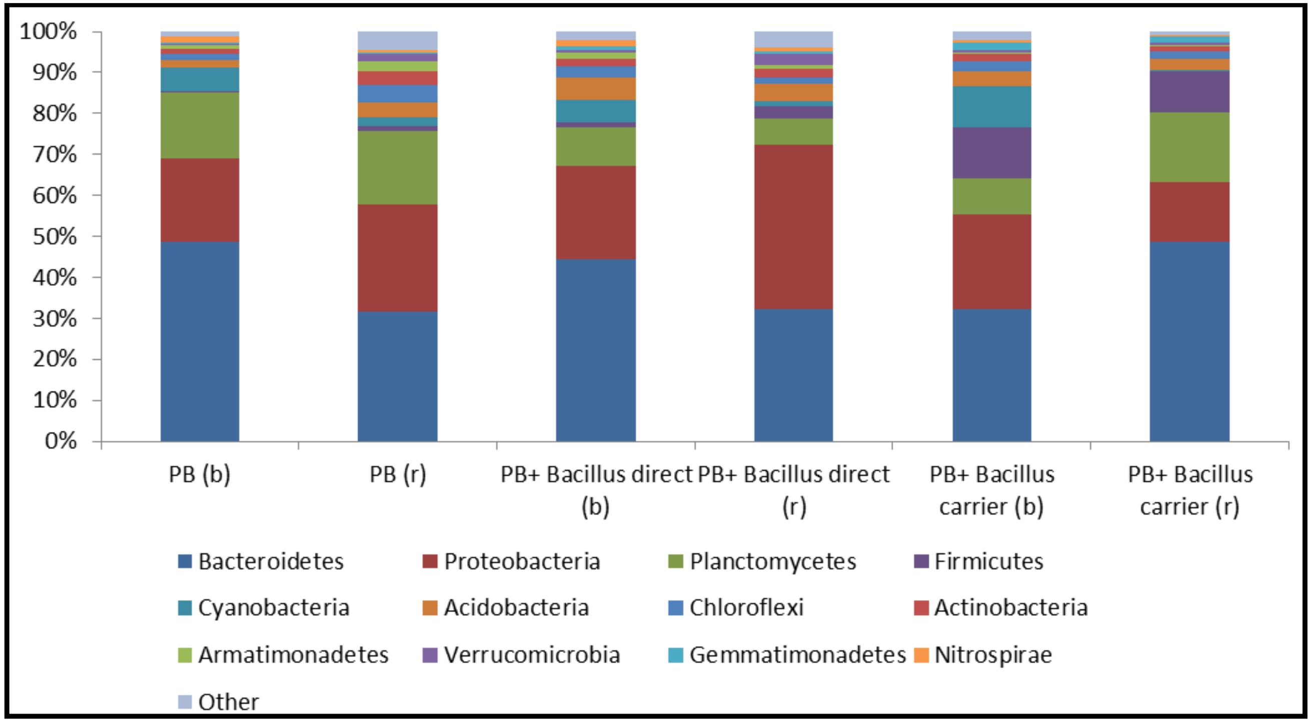

Figure 5: Relative abundance of bacterial taxa at the phylum level, identified using 16S rRNA gene amplicon sequence analysis, in the bulk (b) and the rhizosphere (r) compartment of Pontgibaud contaminated technosol amended with biochar (PB), PB + *Bacillus* strain in direct inoculation (PB + *Bacillus* direct) or PB + *Bacillus* strain using biochar as carrier (PB + *Bacillus* carrier).

Proteobacteria are heterotrophic bacteria that are generally found at higher relative abundance under nutrient rich conditions and with high contents of carbon sources (Li *et al.* 2019). A slight increase in *Proteobacteria* proportion was observed in the inoculated bulk while in the rhizosphere, it was increased with direct *Bacillus* strain inoculation (PB + Bacillus direct) and decreased following inoculation using biochar as a carrier (PB + *Bacillus* carrier). Moreover, the plant "effect" was the opposite of the one observed with the *Bacteroidetes*.

Finally, *Firmicutes* are r-strategists, predominantly present in suitable conditions. They are generally found to increase as the ecological risk posed by metal(loid)s decreases (Xu *et al.* 2017). They have a role in the degradation of cellulose as well as in the anaerobic and methanogenic phases of the organic matter decomposition (Liu *et al.* 2019). Usually, they are highly abundant in soils presenting a high carbon availability. In the present study, *Firmicutes* relative abundances increased with *the Bacillus* strain inoculation in both bulk and rhizosphere compartments. Moreover, plant development had contrasting effects depending on the substrate: *Firmicutes* relative abundance was higher in the rhizosphere of PB and PB directly inoculated (PB + *Bacillus* direct) while it was lower in PB inoculated with a carrier (PB + *Bacillus* carrier), compared to the bulk compartment.

At the class level, *Bacillus* proportion was observed higher in the inoculated substrates compared to PB, in both bulk and rhizosphere conditions (data not shown). Inoculation using biochar as a carrier (PB + *Bacillus* carrier) showed a higher increase than the direct inoculation (PB + *Bacillus* direct), showing that biochar carrier inoculation was more efficient for the establishment of *Bacillus* species. Regarding the difference between the rhizosphere and the bulk, *Bacillus* abundance increased with plant development in PB while a decrease was observed after the carrier-*Bacillus* strain treatment.

Finally, the canonical-correlation analysis of the bacterial community at the OTU (operational taxonomic unit) level showed a clear separation of the bacterial bulk communities from the ones of the rhizosphere, which were mainly driven by root DW. Moreover, the three treatments were separated: the communities in the bulk and the rhizosphere of the direct inoculation treatments (PB + *Bacillus* direct) were closer to the ones of PB compared to the communities of the PB substrates inoculated using a carrier (PB + *Bacillus* carrier). PB bacterial communities were mainly explained by SPW pH, the community in the bulk of PB + *Bacillus* strain carrier substrate (PB + *Bacillus* carrier) was driven by the SPW As and Pb concentrations, and the rhizosphere community of the same treatment by the SPW EC. Furthermore, this representation explained 22.02 % and 21.43 % of the bacterial diversity (Figure 6).

To conclude, the analysis of the bacterial community composition showed that it was shifted by the inoculation of the *Bacillus* strain in the soil (Correa *et al.* 2017). It also revealed that the method of inoculation as well as plant growth influenced the way the bacterial community was shifted.

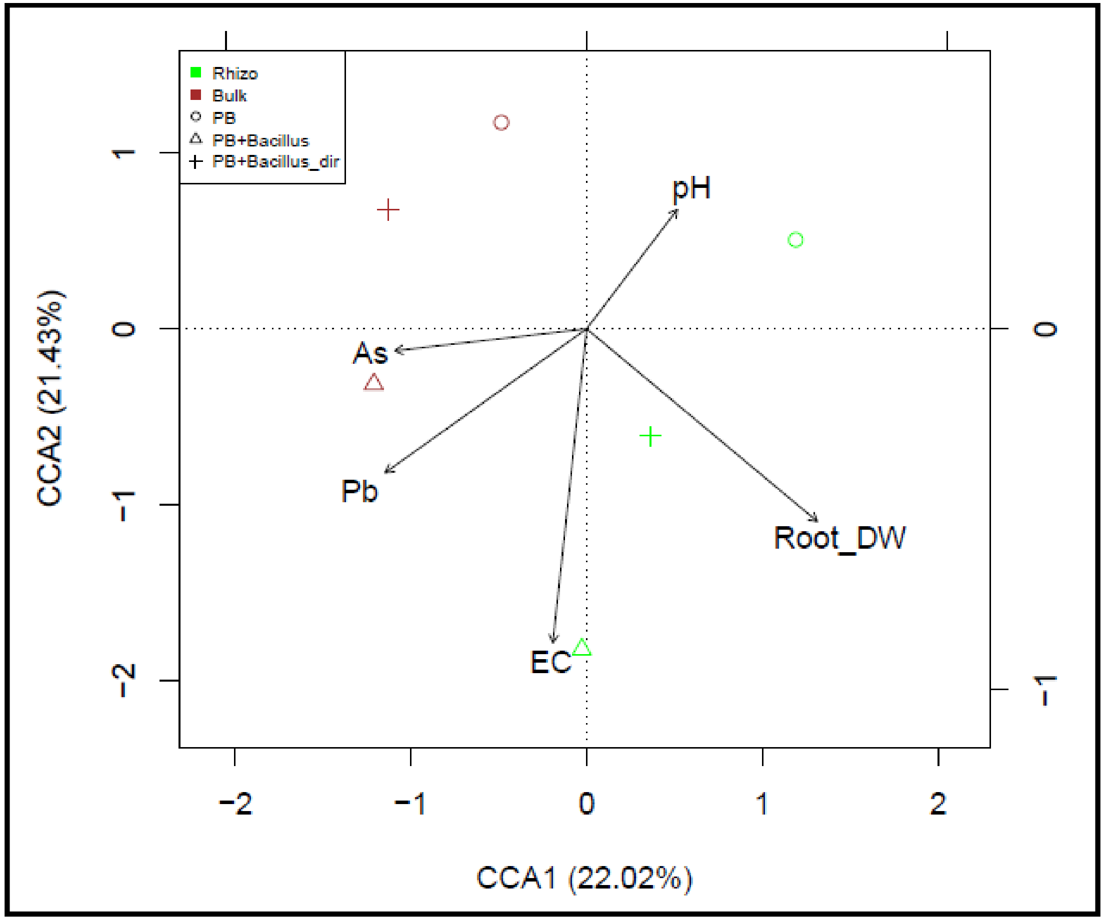

Figure 6: Canonical-correlation analysis (CCA) of bacterial 16S rRNA genes retrieved from the bulk (Bulk, red) and the rhizosphere (Rhizo, green) compartment of Pontgibaud contaminated technosol amended with biochar (PB), PB + *Bacillus* strain in direct inoculation (PB + *Bacillus*_dir) or PB + *Bacillus* strain using biochar as carrier (PB + *Bacillus*). Arrows represent the influence of the soil pore water data (pH, EC = electrical conductivity, As and Pb concentrations) and plant root dry weight (Root_DW).

4. Conclusion

A mesocosm experiment was set-up in order to study the effect of the inoculation of an endogenous *Bacillus* strain, and its inoculation method, on the properties of a former mining soil and the growth and metal(loid) accumulation of *Salix viminalis*. The results showed that the strain inoculation decreased soil pore water pH and increased EC. The use of a carrier also increased SPW Pb concentrations, although it was observed only at the beginning of the experiment. The *Bacillus* sp. inoculation showed beneficial effects on plant growth. Indeed, it increased leaf number and chlorophyll content as well as stem length and dry weight production. However, little effect was observed on metal(loid) accumulation in plants, even though the direct inoculation tended to increase aerial Pb concentration. Finally, the strain inoculation also affected the soil bacterial community, which showed a higher activity with the carrier inoculation compared to the direct inoculation, although both were superior to the non-inoculated condition. Moreover, the bacterial community composition was shifted when the *strain* was inoculated to the soil, more importantly when biochar was used as a carrier. To conclude, the results seem to indicate that the isolated *Bacillus* strain inoculation is beneficial for soil and plant growth amelioration, as well as the bacterial community activity; and that the carrier inoculation showed higher effect than the direct inoculation.

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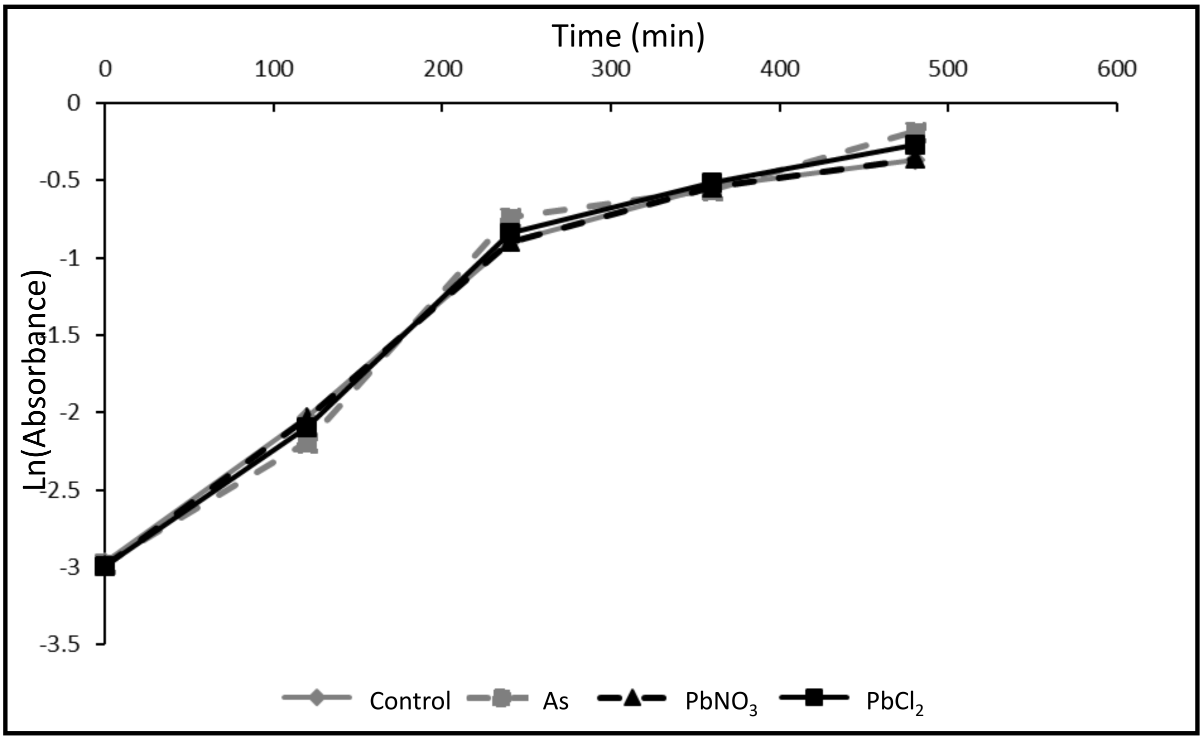

Figure S1: Growth curves of *Bacillus* sp. strain isolated on Pontgibaud, when grown in LB media (control), supplemented with As, $PbNO_3$ and $PbCl_2$.

This chapter focused on the effect of inoculating an endogenous *Bacillus* strain on the soil physico-chemical properties and bacterial community and *Salix viminalis* growth and metal(loid) accumulation pattern. The mesocosm experiment showed that the *Bacillus* strain isolated from Pontgibaud technosol was able to grow in a medium supplemented with elevated As (0.5 mg.L^{-1}) and Pb (50 mg.L^{-1}) concentrations, without significant reduced growth. This bacterial strain also showed some plant growth promoting properties, *i.e.* indole acetic acid production and phosphate solubilization. Moreover, its inoculation to biochar amended Pontgibaud increased Pb mobility, although only at the beginning of the experiment, and increased *Salix viminalis* growth, without greatly affecting metal(loid) accumulation. Furthermore, *Bacillus* inoculation also affected the bacterial community, increasing its activity and inducing a shift in its composition. Finally, inoculation *Bacillus* using biochar as a carrier showed better results than the liquid inoculation.

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General conclusions and perspectives.

The remediation of metal(loid) contaminated soils, using biological techniques, is an important subject of research nowadays. Indeed, with the rise of polluted soils, their harmful effects on the environment and the human health and the cost of physical and chemical remediation techniques, research turned to a more environment friendly method, the phytomanagement, combining plant establishment and amendment application.

This PhD work focused on the (aided-)phytostabilization, a phytomanagement method, of a former mine technosol highly contaminated with As and Pb, using *Salicaceae* species and diverse amendments. To answer the different objectives of this work, seven mesocosm experiments were performed.

The first two objectives (objectives 1a and 1b) were to evaluate the effects of amendment application on the soil physico-chemical properties and *Salicaceae* plant growth and metal(loid) accumulation. These two objectives were answered in the Chapter 2. The five mesocosm experiments presented in this chapter showed that amending Pontgibaud mine technosol improved soil conditions, *i.e.* increased soil pH, decreased Pb availability and mobility, increased soil organic matter and nutrient contents, and thus promoted plant growth. Furthermore, results described in Chapter 2 revealed that biochar effects depended on its particle size and the feedstock used to obtain it, as the fine biochars had a better effect than the coarser ones and lightwood biochars were more efficient than pinewood biochars. Moreover, the functionalization of one biochar showed efficacy to increase its As sorption capacity but only in batch tests. Sorption tests performed on two amendment types also showed that redmud had a higher Pb sorption capacity compared to biochar. Among the different amendments tested, the combination of biochar and compost showed the best improvement of plant growth, mainly due to the sorption capacity of biochar associated with the high nutritive value of compost. However, during the different experiments, some amendments did not induce benefits to soils and plants. Indeed, the addition of the mineral fertilizer, the non-neutralized redmud and the steam activated coconut carbon to Pontgibaud mine technosol did not improve plant growth, although the last two improved soil conditions, whereas iron grit amendment was harmful for plants growth, especially alone or with biochar only. Regarding *Salicaceae* plants, they all showed a poor growth on Pontgibaud mine technosol, especially *Salix dasyclados* did not develop a root system, which was clearly improved with amendments in most cases. Moreover, the different *Salix* and *Populus* species tested accumulated As and Pb mainly in the roots. Additionally, *Populus euramericana* Dorskamp showed a poor Pb translocation, *Salix viminalis* a poor As translocation whereas *Salix dasyclados* had a very poor both As and Pb translocation towards aerial parts on the non-amended Pontgibaud technosol that was increased with amendments.

The third objective (objective 1c) was to assess amendment effects on plant root physiology and biochemistry. This objective was answered in Chapter 3. Indeed we showed that amendment addition to Pontgibaud modified root proteome profiles. Root protein production system shifted towards primary and secondary metabolisms with biochar and compost addition, while oxidative stress markers were over-represented with biochar+iron amendments combination. However, the same amendments had a small effect on organic acid root exudation, although biochar and compost tended to increase organic acid exudation. Finally, the last part of this chapter showed that plants grown on non-amended Pontgibaud suffered from oxidative stress and redmud+biochar/activated carbon amendments repressed this stress, reducing stress markers to control levels, except one condition that only induced a decrease of oxidative stress compared to non-amended Pontgibaud, but still presented higher stress marker levels than the control.

The fourth objective (objective 1d) concerned the bacterial community response to amendment application and was presented in Chapter 4. Results revealed that bacterial metabolic activity was very low on Pontgibaud and amendment application generally improved it. Furthermore the bacterial community composition was also shifted with the addition of amendments. Among amendments tested, compost was the one inducing the best activity improvement and a more important shift in the community, due to its high content in microorganisms and organic matter.

Finally, the last objective (objective 2) was to measure the effect of inoculating an endogenous bacterial strain to a biochar amended Pontgibaud substrate. Chapter 5, relating the results of this mesocosm experiment, showed that the *Bacillus* strain isolated from Pontgibaud was highly tolerant to As and Pb and possessed few plant growth promoting properties. Therefore, its inoculation improved soil conditions and *Salix viminalis* growth. It also increased soil bacterial community and induced a shift in the bacterial community composition. Finally between the two inoculation methods, we demonstrated that the biochar-carrier inoculation showed better results than the direct liquid inoculation.

To conclude, this PhD work showed the beneficial, and in some cases negative, effects of amendment application on the soil, the microorganisms and the plants. It also revealed that plants responded differently depending on the amendments and plant species. Therefore, it is crucial to find the right amendment-plant species combination for an efficient phytostabilization process.

Some complementary researches could be initiated to answer several questions raised during this PhD work.

All these experiments were performed in mesocosm in a short-time period (usually less than three months). It could thus be interesting to perform longer experiments to assure that the effects observed are long-lasting. Moreover, in laboratory experiments, growing conditions, *i.e.* light, temperature, humidity, watering, are optimum. However in the field, plants will be submitted to environmental conditions that can be unfavorable, such as extreme temperatures, drying… They can also be subjected to insect pests and diseases. Thus it is possible that the beneficial outcomes of amendment application are not met on the field. Moreover, in mesocosm experiments, amendments are applied on the all pot height, making a homogeneous substrate. On the contrary, amendments in the field are applied only at the soil surface, on the first 30 cm in general. Therefore, with time and plant growth, roots will probably arrive to a non-amended part of the soil. After this, if the plants are well developed and tolerant enough, they will continue to grow, but the contact with the non-amended soil could also be detrimental to the plants, inducing an important metal(loid) and oxidative stress, possibly leading to plant death. Such response should be evaluated. Additionally, amendments, even biochar, are not inert materials. Thus, they will age in contact with soil but also through the plant development, that can release compounds into the rhizosphere. For instance, after its incorporation into the soil, as demonstrated, biochar is slowly oxidized and carboxylic functional groups are formed. Other biochar parameters are modified, such as CEC and O/C ratio (Baronti *et al.* 2010). Such “aging” of the biochar will affect the soil properties and plant growth but also the microbial community. This will need to be evaluated. Organic amendments, such as compost, will be degraded by microorganisms. Therefore, their effects will be enhanced or repressed, leading to positive or negative effects on the microorganisms and the plants. Finally, with the amelioration of the soil conditions, amendment application can allow the natural development of plants, whose effects on phytoremediation success will need to be evaluated.

Additionally, as one of the aims of phytostabilization is not only to implement a plant cover but also to reduce water leaching and soil erosion, such parameters could be assessed. Indeed, in pot experiment, a device can be placed at the bottom of the pot in order to collect leachate solution. On these solutions, the volume, the concentrations of soil particles but also metal(loid) concentrations can be measured in order to determine if the application of amendments but also the development of plants reduce the volume of water that leaches from the soil and if these leached solutions contain less contaminated soil particles and less mobile metal(loid)s (Doherty *et al.* 2017). Furthermore, on the field, soil wind erosion could be assessed by putting a device collecting air particles to again evaluate the effectiveness of the aided-phytostabilization process.

Furthermore, soil, leachates and soil pore water can be evaluated for their toxicity using for example bioluminescent bacteria or earthworm (Girotti *et al.* 2008). This allows a measurement of the toxicity of the substrates towards (micro)-organisms. It can also allow an optimization of the amendment combination and/or application rate.

Regarding the pollutants, a deeper analysis of their behavior could be performed. For instance, in addition to the measure of their concentrations in the soil and the soil pore water, an evaluation of their speciation is important, particularly arsenic. Indeed, As(III) is more toxic than As(V) (Bissen and Fimmel 2003). Therefore, the ability of amendment application and/or plant development and bacterial community to oxidize As(III) into As(V) is a parameter to take into account when selecting amendment treatments and plant species. Moreover, plants can also have a preference to uptake some metal(loid) species over others (Wang *et al.* 2002).

The repartition of metal(loid)s into the different soil phases can also be assessed through sequential extraction procedures such as the BCR (Community Bureau of Reference) method. This test provides information on which phases are localized metal(loid)s: exchangeable fraction, weak acid soluble fraction, reducible fraction, oxidizable fraction and residual fraction (Baran and Tarnawski 2015). The first fractions are less strongly bound to the soil particles than the last ones and can be thus a potential source of metal(loid) supply to the soil solution and for plant uptake.

Finally, the bioaccessibility of metal(loid)s needs to be evaluated for health reasons. Indeed, as explained in the Chapter 1, humans are exposed to metal(loid)s through diverse pathways, direct and indirect. Therefore, we need to know the risk posed by such possible consumption of metal(loid)s. Such test informs on the fraction of metal(loid)s than can move from one compartment into another until human being. Diverse *in vitro* tests were developed. In particular, the SBET (Simplified Base Extraction Test) is a test that reproduce what happens in the stomach and can thus measure the fraction of metal(loid) processed by the stomach. An evaluation of the effect of amendment application on the bioaccessible fraction can allow selecting amendments that will reduce this bioaccessibility to animals and humans and thus reduce the risk on human health.

Moreover, instead of using one plant species, diverse species combinations could be used to test if the presence of one species favors or represses the development of the other. For instance, Desjardins *et al.* (2018) showed that, on a former petroleum refinery, the presence of *Festuca arundinacea* was beneficial for the growth of *Medicago sativa*, the first one increasing the availability of nitrogen for the second one. Similarly, phytoremediation success was higher with a mixed cultivation compared to the single cultivation in the study of Luo *et al.* (2017). With the mixed cultivation, a more important biomass was produced, transpiration rate was higher and metal(loid) accumulation was increased. On the contrary, Nandillon (2019) observed that the growth of *Trifolium repens* was repressed by the presence of *Salix viminalis*, probably due to a competition for nutrient resource, whereas *Salix viminalis* was not affected by its association with clover. Moreover, the association of an herbaceous species with a wood perennial woody species will allow a better soil plant cover. Indeed, the woody plant will colonize the deep area of the soil, while the herbaceous plant will only colonize the surface of the soil but will cover a higher soil surface than the tree. Therefore, the soil surface will be completely covered and thus soil erosion through wind will be greatly reduced.

Furthermore, the present work focused on woody species that, for some were found on the contaminated site. However cuttings used for mesocosm experiments were of commercial provenance, and not collected on the site. But plants (named endemic species) found on the site could be used for its remediation. Especially, these plants are supposed to have a better tolerance to the metal(loid) pollution of the soil and thus being able to grow better than the plants of the same species coming from other areas. For instance, Lehman and Rebele (2004) grew several population of *Calamagrostis epigejos* on a highly contaminated substrate. They observed that the population coming from a copper smelter performed better that the populations collected on an unpolluted site. Similarly, in 2005, Liu and Xiong found that the plants grew from seeds of *Elsholtzia haichowensis* collected on a copper mine had a higher ability to exclude copper from uptake and translocation than the ones of the uncontaminated site. Such study of endemic *Salix* or annual species clone has been initiated on the Pontgibaud site, with the project "Phytoselect", funding by Région-Centre Val de Loire. For instance, a work using *Agrostis capillaris* seeds demonstrated that populations coming from the Pontgibaud site performed better on this soil, amended or not, than the population coming from commercial seeds.

A deeper analysis at the molecular level could be performed on the plants to better understand their tolerance mechanisms towards metal(loid)s and how amendments modify them. For instance, studying another type of stress, De Zio *et al.* (2016, 2019) assessed the answer of poplar roots to bending stress, using microscopic, proteomic and hormone analyses. They showed that a bending stress induced an increase in xylem thickness and lignin content associated to a low vessel density and the activation of the cambium cell activity. Such modifications were triggered by auxin and used protein factors controlling cell wall deformation, lignification and xylem differentiation. Moreover, the perception of the bending forces, compression and stretching, activated specific signal pathways, which induced structural variation at the tissue level. Similarly, a complete study of the structure of the different tissue at the microscopic level, of the proteome profiles of the roots but also the stems and the leaves as well as the hormone synthesis levels in the plants grown on the contaminated soil and in presence of amendments will give a complete picture of the pathways plants are using to tolerate metal(loid)s and the ones that are modify by amendment application, leading to a better and lower metal(loid) tolerance. For instance, the study of Lomaglio *et al.* (2015) showed that in response to Cd, *Populus nigra* leaves accumulated Cd in the cell wall of guard cells. Moreover, exposure to Cd increased the levels of abscisic acid, gibberellins and ethylene, whereas auxin and cytokinin levels decreased. Finally, the proteomic analysis showed that ROS scavenging proteins and molecular chaperones were activated. However, their study focused on detached leaves exposed to Cd and was not performed on real soil conditions. Theredore, it will be interesting to apply the same molecular and histologic analyses on plants grown in the real Pontgibaud soil condition, amended or not, and compare it to a control non contaminated condition, in the goal to assess the effect of soil pollution on plant physiology as well as the effects amendment application will induce. Finally, in addition of the enzyme activities measured in the plant as well as the stress markers already measured, other parameters could be measured, such as the amino acid and proline concentrations in the plant tissues, as they are involved in the tolerance to metal(loid)s, by acting as ligand (Hossain *et al.* 2012, Eutropio *et al.* 2016).

On the amendment part, only three main types of amendment were tested here but other amendments exist. For instance, phosphate can immobilize Pb through precipitation and adsorption mechanisms (Zeng *et al.* 2017). Cow slurry was also shown effective in reducing the labile pool of Pb and Zn and improved *Festuca rubra* biomass production (Galende *et al.* 2014). The application of chitosan reduced

ROS level in *Vigna radiata* and immobilized Cd in soil (Ramzani *et al.* 2017). Finally, in 2012, Houben *et al.* studied diverse amendments and showed that $CaCO_3$, fly ash, bentonite and bone meal were effective in reducing the leaching and phytoavailability of Cd, Zn and Pb, except for the negative effect of bone meal on Pb leaching.

Moreover, biochar types are infinites. In this work, only woody-based biochars were studied but other feedstocks could be used to produce biochars, such as plant-based feedstock, since plant-based biochars are better fertilizers compared to wood-based biochars, which are better soil conditioner (Yuan *et al.* 2018). The use of such biochar will ameliorate the fertility of contaminated soils and thus plant growth but also the microbial activity. Moreover biochar cost is still high (5 $ per kg on average) (Alhashimi and Aktas 2017) due to the lacking of a real market. To reduce the cost, an *in situ* resource could be used. Indeed, the biomass produced on the contaminated site during the phytoremediation process could be recovered and used to produce biochar, if pollutant translocation towards upper parts is reduced. However, as this biomass will certainly contain metal(loid)s, the behavior of these pollutants during pyrolysis and after biochar application to soil will need to be monitored. In addition, as stated in Chapter 1, adding biochar to the composting process will probably modify the properties of both amendments: more functional groups on the biochar surface will improve its sorption capacity and composting process will be accelerated, by increasing the microbial activity (Wu *et al.* 2017). After composting, composted-biochar could be added to the soil; such amendment will combine the high carbon sequestration and sorption capacity of biochar with the fertilization potential of compost (Schulz *et al.* 2013).

Furthermore, amendments could be better characterized in terms of sorption capacity toward metal(loid)s. Indeed, sorption batch experiments can be performed by varying different parameters, *i.e.* pH, metal(loid) concentrations, and sorbent concentrations. *In fine*, sorption patterns can be fitted to sorption models such Langmuir and Freundlich, to better understand the mechanisms of sorption.

In addition to the sorption tests and to verify the sorption capacity of amendments when added to the soil, amendments could be collected after their incorporation into the soil for microscopic analysis that will study the amendment surfaces, using electronic scanning microscope. This will allow to verify if the immobilization effect of amendments is due to a sorption of the metal(loid)s on the amendments surface or if it is more an indirect effect through the modification of soil conditions, notably the modification of soil pH.

Finally, this work only used three types of modified biochars (Fe-functionalized, acid-activation and steam-activated) but another biochar modification gathered attention recently, *i.e.* magnetization. Such modification uses iron, which can increase As sorption capacity. Moreover, the magnetic property of the biochar allows an easier recovery of the product, which makes its analysis easier. And secondary it can also permit its recycling.

Regarding microorganisms, as the analysis in this work mainly focused on bacteria, the fungal community, the second most important component of the microbial community, could be evaluated, in terms of abundance and activity to have a complete picture of the soil microbial community. Moreover, as stated in Chapter 1, some fungal strains also possess a tolerance towards metal(loid)s and can also act on metal(loid) toxicity by transforming Cr(VI) to Cr(III) for instance. They can also form mycorrhizae in association to plant roots and improve plant growth, by increasing nutrient availability and their plant growth promoting properties.

The microbial community could also be characterized in terms of potential functionality. For instance, Tipayno *et al.* (2018) used the Vikodak software to predict the functional diversity of their soil samples, based on the taxonomic abundance of the samples. Other tools can be used for such functional analysis of the microbial community, such as the Tax4Fun package on R and the software Picrust. Such tools use the 16S data of the NGS, which is matched to a reference genome database to infer metabolic functions using phylogenetic proximity.

Finally, in addition to the *Bacillus* strain isolated from Pontgibaud, other bacterial strains could be isolated and characterized. Indeed, many studies isolated bacterial strains from contaminated soils and found strains belonging to diverse genus. For instance, Durand *et al.* (2018) isolated an Hg-resistant strain identified as *Pseudomonas graminis* and also determined that this strain had plant growth promoting properties. *Bacillus sp*. RJ16 and *Pseudomonas sp*. RJ10 were both isolated from a metal(loid)-polluted soil (He *et al.* 2009). Finally, *Pseudomonas aeruginosa* was found to be resistant to Zn (Islam *et al.* 2014) whereas *Burkholderia sp*. was resistant to several metal(loid)s (Jiang *et al.* 2008). Moreover, Pepi *et al.* (2007) isolated from polluted sediments ten As resistant bacterial strains, four belonging to the *Bacillus* genus, five to *Aeromonas* and one to *Pseudomonas*, and showed that all were good candidates for bioremediation. Similarly, the inoculation of the PGPR strains *Ralstonia eutropha* and *Chrystiobacterium humi* improved the stabilization potential of sunflower plants (Marques *et al.* 2013). Finally, Pinter *et al.* (2017) demonstrated that the inoculation of *Bacillus licheniformis*, *Micrococcus luteus* or *Pseudomonas fluorescens* was beneficial to the growth of grapevine plants and increased their biomass production. Therefore, by taking advantages of the diversity of the soil (217 genus), other resistant strains could be isolated and tested for their tolerance towards As and Pb as well as for their plant growth promoting properties. Finally, the most tolerant strains could be used to inoculate the soil. Moreover, such strains could be used as an alone inoculum or as a consortium of several strains, carefully selected. For instance, Sprocati *et al.* (2012) demonstrated the efficiency of a microbial formula, composed of 12 strains, to promote hydrocarbon degradation. Titah *et al.* (2013) used a consortium including six rhizobacterial strains in combination to a NPK fertilizer and observed that such association alleviated the toxic effects of As on *Ludwigia octovalvis* and thus increased its biomass production.

Moreover, bacterial strain efficiency towards metal(loid) resistance and plant growth promotion can be enhanced through genetic engineering. Such technique consists in the inclusion of a specific gene into the bacteria to modify its machinery. The genetic modification can aim to increase metal(loid) sequestration or metal(loid) detoxification, and/or to induce the production of plant growth metabolites, which will finally improve plant growth.

However, most of these studies on microorganisms will need to be restricted to the laboratory tests, as the European legislation does not allow the inoculation of microorganisms without homologation into the field, especially genetically engineered- microorganisms. Therefore, an evaluation of the already available bio-fertilizers (used for commercial purposes) composed of bacterial strains for their resistance to metal(loid)s and their effects on plant growth and stabilization properties could lead to the selection of one promising fertilizer that could be applied on the field to improve the phytomanagement process.

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List of scientific productions during the PhD program.

I. Published papers.

A. As first author.

I. Effect of biochar amendments on As and Pb mobility and phytoavailability in contaminated mine technosols phytoremediated by *Salix*

Manhattan Lebrun, Carmelo Macri, Florie Miard, Nour Hattab-Hambli, Michael Motelica-Heino, Domenico Morabito and Sylvain Bourgerie

(2017) *Journal of Geochemical Exploration*, 182, 149-156. (IF = 3.47)

II. Eco-restoration of a mine technosol according to biochar particle size and dose application: study of soil physico-chemical properties and phytostabilization capacities of *Salix viminalis*

Manhattan Lebrun, Florie Miard, Romain Nandillon, Nour Hattab-Hambli, Gabriella S. Scippa, Sylvain Bourgerie and Domenico Morabito

(2018) *Journal of soils and sediments*, 18(6), 2188-2202. (IF = 2.67)

III. Assisted phytostabilization of a multicontaminated mine technosol using biochar amendment: Early stage evaluation of biochar feedstock and particle size effects on As and Pb accumulation of two Salicaceae species (*Salix viminalis* and *Populus euramericana*)

Manhattan Lebrun, Florie Miard, Romain Nandillon, Jean Christophe Léger, Nour Hattab-Hambli, Gabriella S. Scippa, Sylvain Bourgerie and Domenico Morabito

(2018) *Chemosphere*, 194, 316-326. (IF = 5.11)

IV. Assisted phytoremediation of a multi-contaminated industrial soil using biochar and garden soil amendments associated with *Salix alba* or *Salix viminalis*: abilities to stabilize As, Pb, and Cu

Manhattan Lebrun, Florie Miard, Nour Hattab-Hambli, Sylvain Bourgerie and Domenico Morabito

(2018). *Water, Air, & Soil Pollution*, 229(5), 163. (IF = 1.77)

V. Effect of Fe-functionalized biochar on toxicity of a technosol contaminated by Pb and As: sorption and phytotoxicity tests

Manhattan Lebrun, Florie Miard, Sullivan Renouard, Romain Nandillon, Gabriella S. Scippa, Domenico Morabito and Sylvain Bourgerie

(2018). *Environmental Science and Pollution Research*, 25(33), 33678-33690. (IF = 2.91)

VI. Biochar effect associated with compost and iron to promote Pb and As soil stabilization and *Salix viminalis* L growth.

Manhattan Lebrun, Florie Miard, Romain Nandillon, Gabriella S. Scippa, Sylvain Bourgerie and Domenico Morabito
(2019) *Chemosphere*, 222, 810-822. (IF = 5.11)

The contributions of Manhattan Lebrun on these papers were:

- Design and set up of the experiment;
- Analysis of the amendments, soils and plants;
- Statistical analysis of the data;
- Redaction of the manuscript.

VII. Amending an As/Pb contaminated soil with biochar, compost and iron grit: effect on *Salix viminalis* growth, root proteome profiles and metal(loid) accumulation indexes.

Manhattan Lebrun, Elena De Zio, Florie Miard, Gabriella S. Scippa, Giovanni Renzone, Andrea Scaloni, Sylvain Bourgerie, Domenico Morabito and Dalila Trupiano
Chemosphere, in press (IF = 5.11) (accepted on November, 17th 2019)
The contributions of Manhattan Lebrun in this paper were:

- Protein extraction and 2 DE gels and analysis;
- Redaction and revision of the manuscript.

VIII. Effect of different tissue biochar amendments on As and Pb stabilization and phytoavailability in a contaminated mine technosol

Manhattan Lebrun, Florie Miard, Nour Hattab-Hambli, Gabriella S Scippa, Sylvain Bourgerie and Domenico Morabito
Science of the Total Environment, in press (IF = 5.589) (accepted on November, 18th 2019)
The contributions of Manhattan Lebrun in this paper were:

- Statistical analysis;
- Redaction of the manuscript.

B. As contributing author

I. Cd, Pb, and Zn mobility and (bio) availability in contaminated soils from a former smelting site amended with biochar

Tonia Lomaglio, Nour Hattab-Hambli, Florie Miard, Manhattan Lebrun, Romain Nandillon, Dalila Trupiano, Gabriella S. Scippa, Arnaud Gauthier, Michael Motelica-Heino, Sylvain Bourgerie, and Domenico Morabito
(2018) *Environmental Science and Pollution Research*, 25(26), 25744-25756. (IF = 2.91)

II. Capability of amendments (biochar, compost and garden soil) added to a mining technosol contaminated by Pb and As to allow poplar seed (*Populus nigra* L.) germination.

Romain Nandillon, Manhattan Lebrun, Florie Miard, Marie Gaillard, Stéphane Sabatier, Marc Villar, Sylvain Bourgerie, and Domenico Morabito

(2019) *Environmental Monitoring and Assessment*, 191(7), 465. (IF = 1.96)

III. Potential use of biochar, compost and iron grit associated with *Trifolium repens* to stabilize Pb and As on a multi-contaminated technosol

Romain Nandillon, Oumaima Lahwegue, Florie Miard, Manhattan Lebrun, Marie Gaillard, Stéphane Sabatier, Fabienne Battaglia-Brunet, Domenico Morabito and Sylvain Bourgerie

(2019) *Ecotoxicology and environmental safety*, 182, 109432. (IF = 4.53)

IV. Effect of biochar and amendments on Pb and As phytotoxicity and phytoavailability in a Technosol

Romain Nandillon, Florie Miard, Manhattan Lebrun, Marie Gaillard, Stéphane Sabatier, Fabienne Battaglia-Brunet, Sylvain Bourgerie, and Domenico Morabito

(2019) *CLEAN–Soil, Air, Water*, 47(3), 1800220. (IF = 1.51)

The contributions of Manhattan Lebrun on these papers were:

- Help in the set-up of the experiment and for the collect and analysis of soils and plants.

V. Contrasted tolerance of *Agrostis capillaris* metallicolous and non-metallicolous ecotypes in the context of a mining technosol amended by biochar, compost and iron sulphate.

Romain Nandillon, Manhattan Lebrun, Florie Miard, Marie Gaillard, Stéphane Sabatier, Domenico Morabito and Sylvain Bourgerie

Environmental Geochemistry and Health, in press (IF = 3.23) (accepted on October, 9th 2019)

The contribution of Manhattan Lebrun in this paper was:

- Help in the set-up of the experiment and for the collect and analysis of soils and plants;
- Help in the redaction of the manuscript;
- Revision of the manuscript

II. Papers in preparation.

A. As first author.

I. Effect of biochar and redmud on metal(loid)s immobilization and *Salix dasyclados* growth on a former mine technosol contaminated by As and Pb.

Manhattan Lebrun, Reinhart Van Poucke, Florie Miard, Gabriella S. Scippa, Sylvain Bourgerie, Domenico Morabito and Filip Tack

Submitted to *Land Degradation and Development* (LDD-19-0882)

The contributions of Manhattan Lebrun in this paper were:

- Design and set-up of the experiment;
- Amendments, soil and plant collect and analysis;
- Statistical analysis;
- Redaction of the manuscript.

II. Effect of biochar, compost and/or iron grit amendments on the organic acid excretion by *Salix viminalis* roots grown on an As and Pb contaminated mining technosol.

Manhattan Lebrun, Laëtitia Fougere, Florie Miard, Gabriella S. Scippa, Domenico Morabito, Emilie Destandau and Sylvain Bourgerie

Submitted to *Soil Science and Plant Nutrition* (SSPN-19-275-F)

The contributions of Manhattan Lebrun in this paper were:

- Collect and analysis of the samples;
- Statistical analysis;
- Redaction of the manuscript.

III. Effect of biochar and redmud amendment combinations on *Salix triandra* growth and oxidative stress response.

Manhattan Lebrun, Florie Miard, Gabriella S. Scippa, Christophe Hano, Domenico Morabito and Sylvain Bourgerie

Submitted to *Journal of Plant Physiology* (JPLPH-D-19-00712)

The contributions of Manhattan Lebrun in this paper were:

- Collect and analysis of the samples;
- Statistical analysis;
- Redaction of the manuscript.

IV. Effect of amendment application and *Salix viminalis* growth on the soil microbial community composition and activity of a former mining technosol.

Manhattan Lebrun, Florie Miard, Bucci Antonio, Romain Nandillon, Gino Naclerio, Gabriella S. Scippa, Domenico Morabito and Sylvain Bourgerie

In preparation (*Journal of Soil and Sediments*)

The contributions of Manhattan Lebrun in this paper were:

- Collect and analysis of the samples;
- Statistical analysis;

- Redaction of the manuscript.

V. Effect of biochar and redmud amendments on soil bacterial diversity and activity.

Manhattan Lebrun, Florie Miard, Reinhart Van Poucke, Filip M. G. Tack, Gabriella S. Scippa, Domenico Morabito and Sylvain Bourgerie

In preparation

The contributions of Manhattan Lebrun in this paper were:

- Collect and analysis of the samples;
- Statistical analysis;
- Redaction of the manuscript.

VI. Endogenous *Bacillus* strain inoculation to improve *Salix viminalis* growth and soil bacterial activity for the remediation of an As and Pb contaminated technosol.

Manhattan Lebrun, Florie Miard, Bucci Antonio, Dalila Trupiano, Romain Nandillon, Gino Naclerio, Gabriella S. Scippa, Domenico Morabito and Sylvain Bourgerie

Submitted to *Journal of Applied Soil Ecology* (APSOIL_2019_1065)

The contributions of Manhattan Lebrun in this paper were:

- Design and set-up of the experiment;
- Soil and plant collect and analysis;
- Statistical analysis;
- Redaction of the manuscript.

VII. Effect of biochar, iron sulfate and chicken dung application on the phytotoxicity of a former tin mine.

Manhattan Lebrun, Florie Miard, Gabriella S Scippa, Sylvain Bourgerie and Domenico Morabito

In preparation

The contributions of Manhattan Lebrun in this paper were:

- Statistical analysis;
- Redaction of the manuscript.

VIII. Effect of biochar, redmud and manure amendments associated to metallicolous *Agrostis capillaris* on As and Pb stabilization of a former mine technosol.

Manhattan Lebrun, Romain Nandillon, Florie Miard, Domenico Morabito and Sylvain Bourgerie

Submitted to *Environmental Geochemistry and Health* (EGAH-19-00881)

The contributions of Manhattan Lebrun in this paper were:

- Help in the harvest of the plant and collect of the soil;
- Statistical analysis;

- Redaction of the manuscript.

IX. Influence of particle size and concentration of biochar on Pb and As availability in contaminated mining soil and effect on poplar growth.

Manhattan Lebrun, Florie Miard, Romain Nandillon, Nour Hattab-Hambli, Jean Christophe Léger, Gabriella S. Scippa, Domenico Morabito and Sylvain Bourgerie

In preparation (*International Journal of Phytoremediation*)

The contributions of Manhattan Lebrun in this paper were:

- Help in the harvest of the plant and collect of the soil;
- Statistical analysis;
- Redaction of the manuscript.

X. Biochar rate application effect to improve soil fertility and *Linum usitatissimum* growth on an arsenic and lead contaminated technosol.

Manhattan Lebrun, Florie Miard, Domenico Morabito and Sylvain Bourgerie

In preparation

The contributions of Manhattan Lebrun in this paper were:

- Supervision of the student and help during the experiment set and samples analysis;
- Statistical analysis;
- Redaction of the manuscript.

XI. Variations in growth, metal uptake and metabolic responses of 5 *Linum usitatissimum* cultivars under multicontaminated technosol.

Manhattan Lebrun, Florie Miard, Samantha Drouet,, Eric Laine, Domenico Morabito, Christophe Hano and Sylvain Bourgerie

In preparation

The contributions of Manhattan Lebrun in this paper were:

- Design and set-up of the experiment;
- Soil and plant collect and analysis;
- Statistical analysis;
- Redaction of the manuscript.

XII. Preliminary characterization of a post-industrial soil for long-term remediation by phytomanagement: mesocosm study of its phytotoxicity before field application

Nour Hattab-Hambli, Manhattan Lebrun, Florie Miard, Lydie Le Forestier, Sylvain Bourgerie and Domenico Morabito

Submitted to *International Journal of Environmental Research* (IJER-D-19-00883R1) (in revision)

The contributions of Manhattan Lebrun (co first author) in this paper were:

- Statistical analysis;
- Redaction of the manuscript;
- Revision of the manuscript.

B. As contributing author

I. Assisted phytoremediation of a former mine contaminated soil using amendments: evaluation of biochar and iron sulphate effects on As soil immobilization and accumulation in three *Salicaceae* species (*Populus x euramericana* clone I-45/51, *Salix purpurea* and *Salix viminalis*) in a field mesocosm experiment.

Melissa Simiele, Manhattan Lebrun, Florie Miard, Olivier Forestier, Philippe Poupart, Gabriella S. Scippa, Sylvain Bourgerie and Domenico Morabito

Submitted to *Science of the Total Environment* (STOTEN-S-19-23182)

The contributions of Manhattan Lebrun in this paper were:

- Help in the harvest of the plant and collect of the soil;
- Help in the statistical analysis;
- Correction of the manuscript.
- Redaction of the manuscript.

II. The effect of bioaugmentation and biochar-stimulation on As and Pb contaminated soil and on plant growth.

Melissa Simiele, Antonio Bucci, Manhattan Lebrun, Gabriella S. Scippa, Domenico Morabito, Sylvain Bourgerie, Gino Naclerio, Claudio Caprari and Dalila Trupiano

In preparation

The contributions of Manhattan Lebrun in this paper were:

- Plant and soil pore water analysis;
- Correction of the manuscript.

III. Application of biochar to stabilize Cd, Pb and Zn in contaminated acid sandy soils.

Reinhart Van Poucke, Caleb Egene, Simon Allaert, Manhattan Lebrun, Sylvain Bourgerie, Domenico Morabito, Yong Sik Ok, Frederik Ronsse, Erik Meers and Filip Tack

Submitted to *Environmental Geochemistry and Health* (in revision) (EGAH-D-19-00111)

The contributions of Manhattan Lebrun in this paper were:

- Soil characterization;
- Measurements of some biochar properties;
- Revision of the manuscript.

IV. Assessment of compost and three different biochar feedstocks associated with Ailanthus altissima (Miller) Swingle on Pb and As stabilization in a post-mining technosol.

Ibrahim Alidou Arzika, Florie Miard, Romain Nandillon, Manhattan Lebrun, Gulriz Bayçu, Sylvain Bourgerie and Domenico Morabito

Submitted to *Pedosphere* (pedos201907480)

The contributions of Manhattan Lebrun in this paper were:

- Help in the harvest of the plant and collect of the soil;
- Help in the statistical analysis;
- Correction of the manuscript.

V. Effect of biochar and compost as amendments on lead and arsenic contaminated soils vegetated by *Robinia pseudoacacia* L. (black locust).

Ibrahim Alidou Arzika, Manhattan Lebrun, Florie Miard, Romain Nandillon, Ghaya Hmida, Gulriz Bayçu, Sylvain Bourgerie and Domenico Morabito

In preparation

The contributions of Manhattan Lebrun in this paper were:

- Help in the harvest of the plant and collect of the soil;
- Help in the statistical analysis.

III. Presentations in scientific conferences

A. As presenting author

Oral Presentations

I. Effects of biochar and garden soil as amendments on the physicochemical characteristics of contaminated soils and on the growth and the potential use of 6 *Salix* species for phytoremediation.

Tonia Lomaglio, Manhattan Lebrun, Nour Hattab, Florie Miard, Francis Cottard, Marie Gaillard, Stéphane Sabatier, Michael Motelica-Heino, Sylvain Bourgerie and Domenico Morabito

Eurosoil 2016 (Istanbul, Turkey)

II. Effect of lightwood and pinewood biochar amendments on the growth and assisted phytostabilizing capacities of *Salix viminalis*.

Manhattan Lebrun, Florie Miard, Romain Nandillon, Jean-Christophe Léger, Gabriella S. Scippa, Domenico Morabito and Sylvain Bourgerie

14th International Phytotechnologies Conference 2017 (Montréal, Canada)

III. Biochar Amendment Associated to Compost and/or Iron in Order to Improve Lead and Arsenic Soil Stabilization and *Salix Viminalis* Growth.
Manhattan Lebrun, Florie Miard, Romain Nandillon, Dalila Trupiano, Elena De Zio, Gabriella S. Scippa, Sylvain Bourgerie and Domenico Morabito
International Conference on Environmental Pollution and Remediation 2018 (Madrid, Spain)
BEST PAPER AWARD

IV. Quel est le potentiel du lin en phytoremédiation assistée ? Etude exploratoire de l'effet d'un amendement au biochar sur les capacités phytoremédiatrices de 5 cultivars de lin placés sur un ancien site minier présentant une pollution polymétallique.
Manhattan Lebrun, Florie Miard, Samantha Drouet, Sullivan Renouard, Christophe Hano, Eric Laine, Domenico Morabito and Sylvain Bourgerie
French Flax Research Network Meeting 2017 (Amiens, France)

V. Does biochar associated to compost and iron allow a better Pb and As soil stabilization and tree growth?
Manhattan Lebrun, Florie Miard, Romain Nandillon, Dalila Truppiano, Elena DeZio, Gabriella S. Scippa, Sylvain Bourgerie and Domenico Morabito
GRS-DIBT 2018 (Isernia, Italy)

VI. Effect of hardwood biochar and endogen *Bacillus* inoculation on *Salix viminalis* growth and pollutant immobilization of an As and Pb contaminated former mine technosol.
Manhattan Lebrun, Florie Miard, Romain Nandillon, Dalila Trupiano, Antonio Bucci, Gabriella S. Scippa, Sylvain Bourgerie and Domenico Morabito
Soils of Urban, Industrial, Traffic, Mining and Military Areas 10 (Seoul, Korea)

Poster presentations

I. Effects of a biochar amendment to improve the physico-chemical characteristics of a former mine extraction soil contaminated mainly by Pb and As and to enhance the growth of three willow species.
Manhattan Lebrun, Carmelo Macri, Nour Hattab-Hambli, Florie Miard, Michael Motelica-Heino, Sylvain Bourgerie and Domenico Morabito
Biotechnocentre 2016 (Seillac, France)

II. Biochar functionalization to improve As sorption capacity and utilization for mine technosol technosol stabilization: phytotoxicity test using *P. vulgaris.*
Manhattan Lebrun, Romain Nandillon, Florie Miard, Ibrahim Alidou-Arzika, Nour Hattab-Hambli, Sylvain Bourgerie, Domenico Morabito and Gabriella S. Scippa
GRS-DIBT 2017 (Isernia, Italy)

III. Effet de trois amendements (biochar, compost, grenaille de fer), seuls ou combinés, sur l'activité et la diversité microbienne d'un ancien sol minier pollué à l'arsenic et au plomb.
Manhattan Lebrun, Florie Miard, Gabriella S. Scippa, Domenico Morabito and Sylvain Bourgerie
IXème colloque de l'AFEM (Bussang, France)

B. As contributing author

Oral Presentations

I. Organic amendments effects on the physicochemical characteristics of a contaminated soil and on the growth of three willow species in a phytoremediation goal.
Manhattan Lebrun, Carmelo Macri, Nour Hattab, Florie Miard, Michael Motelica-Heino, Tonia Lomaglio, Gabriella S. Scippa, Sylvain Bourgerie and Domenico Morabito
3rd Asia Pacific Biochar Conference (Korea)

II. Effects of different biochars as amendments on the physicochemical characteristics of a contaminated soil and on the growth of *S. viminalis* in a phytoremediation end.
Manhattan Lebrun, Carmelo Macri, Nour Hattab, Florie Miard, Michael Motelica-Heino, Jean-Christophe Leger, Sylvain Bourgerie and Domenico Morabito
3rd Asia Pacific Biochar Conference (Korea)

III. Effect of organic amendments to improve the physicochemical characteristics of a soil and to enhance the growth of three willow species.
Manhattan Lebrun, Carmelo Macri, Nour Hattab, Florie Miard, Luigi Minuto, Michael Motelica-Heino, Sylvain Bourgerie and Domenico Morabito
Internaltional Conference of Heavy Metals in the Environment 2016 (Ghent, Belgium)

IV. Use of biochar obtained from wood feedstock to reduce lead contamination in mining soil, effect on *Ailanthus altissima* growth and lead plant accumulation.

Domenico Morabito, Ibrahim Alidou-Arzika, Manhattan Lebrun, Florie Miard, Romain Nandillon, Oumaima Lahwegue, Jean-Christophe Léger, Sylvain Bourgerie and Gulriz Bayçu
Ecology 2017 (Kayseri, Turkey)

V. Effect of wood biochar associated to compost on the phytotoxicity of a mining technosol mainly contaminated by Pb and As.
Romain Nandillon, Manhattan Lebrun, Florie Miard, Marie Gaillard, Stéphane Sabatier, Sylvain Bourgerie and Domenico Morabito
14th International Phytotechnologies Conference 2017 (Montréal, Canada)

VI. Biochar an efficient tool to decrease Pb and As in metal(loid)s contaminated soils and to allow assisted phytoremediation of multicontaminated technosols using tree species.
Manhattan Lebrun, Romain Nandillon, Nour Hattab-Hambli, Florie Miard, Gabriella S. Scippa, Sylvain Bourgerie and Domenico Morabito
Global Symposium on soil Pollution 2018 (Rome, Italy)

VII. Phytotoxicity Test to Assess Biochar Associated To Others Amendments Effect on Pb and As from Mining Technosol.
Romain Nandillon, Manhattan Lebrun, Florie Miard, Marie Gaillard, Stéphane Sabatier, Sylvain Bourgerie, Fabienne Battaglia-Brunet and Domenico Morabito
International Conference on Environmental Pollution and Remediation 2018 (Madrid, Spain)

VIII. Biochar Obtained from Different Wood Trunk Layers Allow to Stabilize Pb and As in a Mining Technosol.
Simon Chevolleau, Florent Beaumont, Florie Miard, Manhattan Lebrun, Romain Nandillon, Pascale Gautret, Jean-Christophe Léger, Sylvain Bourgerie and Domenico Morabito
International Conference on Environmental Pollution and Remediation 2018 (Madrid, Spain)

IX. Poplar Seeds Capabilities to Germinate on a Metal(loid)s Contaminated Mining Technosol Differently Amended.
Florie Miard, Romain Nandillon, Manhattan Lebrun, Marie Gaillard, Stéphane Sabatier, Sylvain Bourgerie and Domenico Morabito
International Conference on Environmental Pollution and Remediation 2018 (Madrid, Spain)

X. Capabilities of Fe-Functionalized Biochar to Decrease Soil Pb and As Phytodisponibility.
Manhattan Lebrun, Florie Miard, Sullivan Renouard, Romain Nandillon, Gabriella S. Scippa, Domenico Morabito and Sylvain Bourgerie

International Conference on Environmental Pollution and Remediation 2018 (Madrid, Spain)

XI. Effect of biochar and amendments on *Ailanthus altissima* capacities to remediate a Pb and As contaminated mining soil.

Ibrahim Alidou Arzika, Manhattan Lebrun, Florie Miard, Romain Nandillon, Jean-Christophe Léger, M. Kryvtsova, Gulriz Baycu, Sylvain Bourgerie and Domenico Morabito

Ecolgie conference 2018 (Kastamonu, Turkey).

XII. Biochar: an effective amendment to reduce soil pollution and for the implementation of phytomanagement strategies.

Manhattan Lebrun, Romain Nandillon, Nour Hattab Hambli, Simon Chevolleau, Justine Garraud, Solène Tuffigo, Florie Miard, Melissa Simiele, Sylvain Bourgerie and Domenico Morabito

The 3rd International Conference on Bioresources, Energy, Environment, and Materials Technology 2019 (Hong-Kong)

XIII. Immobilization of heavy metal in contaminated mine technosols using biochar: a phytomanagement strategy.

Manhattan Lebrun, Romain Nandillon, Nour Hattab-Hambli, Simon Chevolleau, Florie Miard, Melissa Simiele ,Gabriella S. Scippa, Sylvain Bourgerie and Domenico Morabito

Biochar II (Calabria, Italy)

Poster presentations

I. Effect of a biochar amendment to improve the physicochemical characteristics of a former mine extraction soil contaminated mainly by Pb and As and to enhance the growth of three willow species.

Sylvain Bourgerie, Carmelo Macri, Manhattan Lebrun, Nour Hattab, Florie Miard, Michael Motelica-Heino and Domenico Morabito

International Conference on Heavy Metals in the Environment 2016 (Ghent, Belgium)

II. Immobilization of heavy metal in contaminated mine technosols using biochar: a phytomanagement strategy.

Manhattan Lebrun, Romain Nandillon, Nour Hattab-Hambli, Simon Chevolleau, Florie Miard, Melissa Simiele ,Gabriella S. Scippa, Sylvain Bourgerie and Domenico Morabito

Biochar II (Calabria, Italy)

III. Phytomanagement d’un technosol minier : Etude des effets d’amendements et d’un couvert végétal sur la biodisponibilité de l’As et du Pb.

Romain Nandillon, Manhattan Lebrun, Florie Miard, Marie Gaillard, Stéphane Sabatier, Louis De Lary De Latour, Fabienne Battaglia-Brunet, Domenico Morabito Et Sylvain Bourgerie

Quatrièmes Rencontres nationales de la Recherche sur les sites et sols pollués (Paris, France)

IV. The effect of bioaugmentation and biochar-stimulation on metal(loid)s contaminated soil and plant growth

Melissa Simiele, Antonio Bucci, Manhattan Lebrun, Gabriella S. Scippa, Sylvain Bourgerie, Domenico Morabito, Gino Naclerio and Dalila Trupiano

4th Edition of global Conference on Plant Science and Molecular Biology