Comparative Phytoremediation of Heavy Metal Contaminated Sterilized and Unsterilized Soil with *Brassica juncea* and Bioaugmentation

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Certificate of Approval

This is to certify that the research work presented in this thesis, titled **"Comparative Phytoremediation of Heavy Metal Contaminated Sterilized and Unsterilized Soil with *Brassica juncea* and Bioaugmentation''** was conducted by **Haider Ali** (Registration No**.: 02312111004**), under the supervision of **Dr. Sohail Yousaf.** No part of this thesis has been submitted else for any other degree. This thesis is submitted to the Department of Environmental Sciences in the partial fulfillment of the requirements for the degree of **Master of Philosophy** in the field of Environmental Science, Quaid-i-Azam University, Islamabad.

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HAIDER ALI

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Abstract

A significant environmental issue is the contamination of the soil and environment by toxic heavy metals and their metabolites. Heavy metals top the list of environmental toxins in terms of their ability to contaminate agricultural soil and water. According to our hypothesis, bacteria are responsible for the increased accumulation of heavy metals by plants. These bacteria can mobilize physiologically inaccessible heavy-metal components, modify root exudation, and stimulate plant growth. Rhizosphere microorganisms may extensively mobilize heavy metals, enhancing their bioavailability. This study concentrated on the remediation of Cd and Cu contaminated soil with *Brassica juncea.* Phytoextraction capabilities of *Brassica juncea* were compared in sterilized soil with inoculant strains and in unsterilized soil with indigenous bacteria. Pot experiment was conducted with heavy metal contaminated soil from an industrial site. The results revealed that treatment (T7) with sterilized soil, *Brassica juncea and Bacillus cereus* exhibited maximum extraction of Cu (87.7%). While (T8) with sterilized soil, *Brassica juncea, Serratia marcescens and* compost amendments exhibited maximum extraction of Cd (57.4%). Maximum root and shoot weight (13.53 g and 1.89 g) was observed in T7. Chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids were also high in T7. Biochemical stress indicators revealed significantly lower levels of APX, GPX, MDA and H_2O_2 in T7. Bacterial colonization was high in sterilized soil as compared to unsterilized soil. Maximum colony forming units (2.60×10^7) were noted in T7. We conclude that sterilized soil with inoculated strains showed better performance as compared to unsterilized soil with indigenous bacteria. It is recommended that integrated approach of bio-augmentation and phytoextraction has a great potential for heavy metal cleanup.

Key Words: Phytoremediation, Bioaugmentation, Brassica, Cadmium, Copper, Compost.

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List of Abbreviations

AC	Abiotic control
APX	Ascorbate peroxidase
B+P	Bioaugmentation + phytoremediation
CAT	Catalase
Car	Carotenoid
CFUs	Colony forming units
Chl a	Chlorophyll a
Chl b	Chlorophyll b
EC	Electrical conductivity
EDTA	Ethylenediamine tetraacetic acid
FW	Fresh weight
GPX	Guaiacol peroxidase
MDA	Malondialdehyde
NPs	Nanoparticles
OM	Organic matter
OOC	Oxidizable organic carbon
PC	Plant control
ROS	Reactive oxygen species
SOD	Superoxide dismutase
T chl	Total chlorophyll
TDS	Total dissolved solids
TOC	Total organic carbon
UV	Ultraviolet

Chapter 1

Introduction

Pollution problems at the local, national, and international level are part of our daily life. The way in which the ecosystems of our planet have been degrading is worrisome, and the superficial layer of the earth's crust is no exception. Industrial activity has caused one of the most serious problems in terms of soil contamination, where the spill of hydrocarbons derived from petroleum occupies one of the first places (Sodango et al. 2018).

Worldwide because of several centuries of mining activity, the basic chemical, petrochemical, and oil refining industries have produced large amounts of hazardous waste that is difficult to quantify. It is known that in 1999, according to figures published by INEGI-INE (2000), the contaminated sites, even in the most conservative estimates, amounted to several thousand places and these were equivalent to 25,967 km 2 of degraded soil surface (Yi, Liang et al. 2017).

In 1995, the mine entered the Environmental Audit program and as a result, natural soil contaminated with hydrocarbons was detected, close to 800 tons, which was confined in a warehouse. The foregoing due to the maintenance of machinery and equipment within the company. To solve this problem, the company sought advice to establish a remediation technology that was simple, flexible, low-cost, and that would allow it to reach acceptable levels in current regulations (Asami 1984).

There are numerous remediation technologies for contaminated soils, and they can be grouped into 3 types: a) biological (bioremediation, bio stimulation, phytoremediation, bio tillage, etc.), where the metabolic activities of certain organisms allow the degradation, transformation or removal of contaminants to innocuous metabolic products; b) physicochemical (electro remediation, washing, solidification/stabilization, etc.), here, the physical and chemical properties of the contaminants are taken advantage of to destroy, separate or contain the contamination; and c) thermal (incineration, vitrification, thermal desorption, etc.), in which heat is used to promote volatilization, burn, decompose or immobilize pollutants in a soil (Wang, Shi et al. 2007).

In addition, it has ventured into the development of emerging and innovative technologies such as phytoremediation, electro remediation and electro

bioremediation, where although it is true today the information is limited, the research developed supports its use and is gaining momentum.

Undoubtedly, there are many alternatives reported as successful, but to select the appropriate remediation technology, the following must be taken into consideration: a) site characteristics, b) type of contaminant, concentration and physicochemical characteristics, c) physicochemical properties and type of soil to treat and d) cost (Liu, Bai et al. 2019).

On the other hand, residual sludge or biosolids are the by-product resulting from the biological treatment of domestic water, which when there is no management plan for them, cause an impact on the environment and the health of the population. Therefore, they are considered as hazardous waste. However, these residual sludges, when they do not contain toxic substances, can be composted, and used to improve the quality of the soil and stimulate the microbial population to promote the degradation of organic pollutants, since they are rich in organic matter, macro and micronutrients. Furthermore, sewage sludge contains a high microbial diversity, much greater than that of any fertile soil.

Since microorganisms are the primary agents of degradation of organic contaminants in the soil, one premise is that by increasing the microbial density in a contaminated soil, the degradation of organic contaminants such as hydrocarbons can also be accelerated (Tyler 1974).

According to the above, the most widely used process is bioremediation and the variable to be controlled is the bio stimulation of native soil microorganisms through the addition of nutrients. This assertion is since the entry of large amounts of carbon (hydrocarbons) disturbs the natural balance of nutrients in the system, causing a rapid decrease in others, such as nitrogen and phosphorus, thus reducing or stopping the bacterial growth rate (Hong-gui et al. 2012).

As already mentioned, sewage sludge contains high concentrations of inorganic nitrogen, phosphorous, and organic matter, making it ideal for stimulating soil microbial activity. Residual sludge can be used as an alternative source of macro and micronutrients and by stimulating microbial activity, greater degradation of hydrocarbons present in the soil will be achieved, if the concentration of pathogens, heavy metals and toxic organic compounds is low. This practice is beneficial for the environment, giving a use to what has commonly been handled as waste (Nwachukwu, Feng et al. 2010).

Bioremediation is a mineralization process, which is also known as composting. Said process is used to stabilize the residual sludge and from which humus is obtained as a product, which acts as an improver of the physical characteristics of a soil. What results from the combination of soil with hydrocarbon and residual sludge is a soil with improved physical characteristics and without the contaminant, suitable for use in any agricultural activity.

Based on said background and to solve the problem of contaminated soil in the mine, the objective of this research was to evaluate the aerobic bioremediation process as a treatment system, considering for this the use of residual sludge as an alternative source of nutrients.

1.1. Effects of Heavy Metals on Soil Health

The soil is disturbed because of mining activities. One of the biogeochemical anomalies that are generated at the time of extraction is the increase in the number of microelements in the soil, converting them to levels of macroelements which negatively affect the biota and soil quality; these affect the number, diversity, and activity of soil organisms, inhibiting the decomposition of soil organic matter. (Huamain, Chunrong et al. 1999) comments that tailings are toxic to living organisms and are inhibitors of ecological factors affecting plant growth. The soils that remain after a mining operation contain all kinds of residual materials, sterile debris, among others, which represents serious problems for the development of vegetation (Awasthi, Nagar et al. 2022).

Soil characteristics play an important role in reducing or increasing the toxicity of metals in the soil. (Ito and Iimura 1976) comment that the distribution of heavy metals in soil profiles, as well as their availability, is controlled by parameters such as intrinsic metal properties and soil characteristics.

Metals tend to accumulate on the soil surface, making them accessible for crop roots to consume. Plants grown in contaminated soils generally take up more trace elements and the concentration of these in plant tissues is often directly related to their abundance in soils, and especially in the moist solution (Ghosh and Singh 2005) mention that excessive concentrations of metals in the soil could impact the quality of food, the safety of crop production and the health of the environment, since they move through the food chain via the consumption of plants by animals and these in turn by humans (Jiang, Adebayo et al. 2019).

Metals accumulated on the soil surface are slowly reduced through leaching, consumption by plants, erosion, and deflation. The objective of the study was to evaluate the concentrations of Lead (Pb), Zinc (Zn), Cadmium (Cd) and Arsenic (As) in different depths of soil affected by tailings dams (Liu, Bai et al. 2019).

Heavy metal contamination in agricultural soils can create a serious problem for human health, since many edible plant species can absorb large amounts of potentially toxic metals from the soil (Chu 2018). The ingestion of metals, through the consumption of contaminated food, can cause malformations, neuronal dysfunctions and even death.

Although heavy metals such as cadmium, lead, nickel, cobalt, copper and zinc are considered potentially toxic, for plants, animals and even for humans it is It is true that other metals, such as potassium, magnesium, iron and manganese, are necessary for the nutrition of plants and agricultural crops in general (Li, Zhou et al. 2019).

It is important to evaluate the metal content, both in soils and in crops, since soil composition is one of the factors that influence the transfer of trace elements within the soil-plant chain as part of the biochemical cycle. Additionally, knowing the metal content makes it possible to demonstrate that the nutrient content is adequate for the crop, and that potentially polluting heavy metals are below the permissible limits, according to national and international environmental regulations.

1.2. Cadmium

Cadmium (Cd) is one of the most toxic heavy metals. Its high mobility and bio accumulative power differentiate it from the rest of its group and motivate the interest of scientists to know its effects and interaction with plants. In the present work, a bibliographic review of the main mechanisms of entry and transport of Cd in plants and its toxic effects on them was carried out. Also, topics such as the defense mechanisms of plants against Cd stress and existing strategies to reduce its toxicity are addressed. Within the different crops, the tomato is of special interest, because it is the most widespread vegetable in the world and has shown to be a plant tolerant to Cd and with potential for its accumulation (Jabeen, Ahmad et al. 2009).

Cadmium is a highly toxic transition metal at very low exposure levels and has acute and chronic effects on the health of plants, animals, humans and all living things in general. Because of industrial and anthropogenic activities, it is estimated that 30,000 tons of Cd are released into the environment each year. Therefore, in different parts of

the planet and in our country, Cd levels have been detected in water, soil and plants that exceed the permissible limits established for different uses (Ali, Khan et al. 2013).

Cadmium is not naturally degradable, so once released into the environment, it will remain in circulation. This property together with its high mobility, bio accumulative power and toxicity at very low concentrations make it one of the most important heavy metals. In the 1960s, environmental contamination with this metal became evident when in Japan more than 100 people died from a disease named Itai-Itai, which was caused by high concentrations of Cd in the Jinzu River, in rice (4.2 mgL^{-1}) and consequently in the human body. These facts motivated the interest of soil and plant science in knowing and controlling the effects that the metal produced in different crops (McIntyre 2003).

This metal is recognized as one of the most toxic and inhibitory of the physiological processes of plants. Studies on various crops have shown that it reduces growth, photosynthetic activity, transpiration and chlorophyll content. Also, it causes chlorosis, oxidative stress, nutritional imbalances and modifies the activity of enzymes, involved in the metabolism of organic acids and in the Krebs cycle 13 – 16 (Sodango, Li et al. 2018). In general, the affectations caused in some physiological processes can be so marked that the plants are not able to evade them and manifest themselves in other processes. Cd toxicity can lead to the death of the plant, and this depends, among other factors, on the exposure time, the metal content and the specific adaptations they develop (Ullah, Heng et al. 2015).

The specific adaptations of plants to Cd stress are based on two main mechanisms; some prevent or regulate its entry and transport and others tolerate certain Cd contents, through its detoxification, through chelation in intracellular organelles. Based on these tolerance mechanisms, several research groups have proposed different strategies to reduce the effects of Cd in plants (Srivastava, Sarkar et al. 2017). Most of the strategies include making modifications in nutrition management. But other practices have also shown favorable results, such as inoculation with beneficial bacteria grafting on resistant rootstocks, adding different growth regulators, and applying soil amendments (Pulford and Watson 2003).

Knowing the interaction of Cd with plants, as well as the search for alternatives to minimize its effects, has caught the interest of the scientific community, due to the accelerated growth of contamination with this metal and its high toxicity. The

objective of this study is to make an updated review of the results of research related to these aspects (Abdu, Abdullahi et al. 2017).

Within the different crops, the tomato is of special interest, because it is the most widespread vegetable in the world and with the highest economic value. It has been used not only as food, but also as a model plant in dissimilar investigations. The tomato plant has many interesting characteristics, such as fleshy fruit, a sympodial shoot, and compound leaves, that other model plants (e.g., rice and Arabidopsis) do not have. Furthermore, some of its varieties have shown to be a Cd-tolerant plant, with potential for its accumulation (He, Shentu et al. 2015).

Absorption and transport of cadmium in plants

Cd enters the plant mainly in the form of Cd, since its chelated ions are generally not available for uptake by the roots. The epidermal cell layer is the first tissue for ion uptake and within it, the root hairs are the most active area for absorbing ions from the soil and it is the structure that facilitates the absorption of Cd. Following three different pathways of Cd entry into the root have been proposed:

First route: in the plasmatic membrane of the epidermal cells of the root, CO_2 (aq) is dissociated in H^+ and HCO^{3-}, through the respiration of the plant. The H^+ is exchanged with the Cd^{2+} of the soil and the metal is adsorbed on the surface of the epidermal cells of the root. This adsorption process is fast and does not require energy and is the stage preceding the subsequent absorption of Cd^{2+} in the epidermis through the apoplast pathway (Mahar, Wang et al. 2016).

Second pathway: Cd is a non-essential element and, therefore, it is assumed that plants do not have specific entry mechanisms for it. It enters plant cells through the essential metal transporters Fe^{2+}, Zn^{2+} and Ca^{2+}, such as the IRT1 and LCT1 proteins. After combining with carrier proteins, Cd enters the epidermal layer of the root, via the symplast pathway.

Third pathway: to increase the availability of ions in the rhizosphere soil, plant roots secrete low molecular mass compounds, such as mugineic acids (MA), which form complexes with Cd^{2+}. Thus, Cd^{2+} enters the root epidermal layer via YSL-like proteins in the form of chelates (Abdu, Abdullahi et al. 2017).

The movement of Cd from the root to the stem is controlled through three processes: the sequestration of metals within the root cells; the transport towards the stele and the release of the metal to the xylem. Retention is the product of apoplastic barriers and

chelation in vacuoles. Phytochelatins and other thiols have been shown to be the main chelators in Cd sequestration in the root. Another of the proposed mechanisms of Cd retention in roots is through the impregnation of suberin in the cell wall during exodermis and endodermis maturation, which affects plasticity and restricts its movement to the stele (Yi, Liang et al. 2017).

The transfer and remobilization of Cd from the xylem to the phloem is another of the crucial processes in the transport of this ion. Other authors identified high concentrations of phytochelatins, glutathione, and Cd in the phloem sap of *Brassica Napus* and suggested that the phloem is also a conduit for the transport of the Cd-phytochelatin and Cd-glutathione complexes (Hong-gui, Teng-feng et al. 2012).

1.2.1. Cd Stress Tolerance Mechanisms

Specific adaptations of plants to Cd stress are based on two main strategies; some prevent or regulate its entry and transport and others tolerate certain Cd contents, through its detoxification, through chelation in intracellular organelles. Other tolerance mechanisms are an increase in the antioxidant defense system, cellular homeostasis, an increase in the endogenous production of plant growth regulators, and the modification of metabolism to repair the damaged cell structure (Ali, Mfarrej et al. 2022).

Plants prevent the entry of Cd by immobilizing it in the cell wall of the roots through links with extracellular exudates, such as polygalacturonate acids, and this limits its transport to the aerial part. Other plants have developed tolerance to stress, accumulating metals in the leaves, in the form of stable, non-toxic metal complexes, with different chelators: organic acids, amino acids, ferritins, phytochelatins and metallothionein (Zea, Souza et al. 2022). Studies have shown that vacuoles are the site of accumulation of heavy metals including Zn and Cd.

Within the different chelators in plants, phytochelatins have shown a greater capacity to form complexes with Cd, hence they have been analyzed in several tolerance studies. Plants that overexpress the enzyme phytochelatin synthase showed greater tolerance to Cd (Hussain, Ashraf et al. 2021).

It has also been shown that the exposure of plants to Cd results in an increase in the assimilation of sulfate and in the activity of enzymes involved in the biosynthesis of GSH, starting substrate in the synthesis of phytochelatins. Two cell lines of tomato

plants tolerant to Cd have been identified, and their tolerance capacity depends on the potentiality of the cells to synthesize phytochelatins, and form complexes with Cd.

However, other evidence indicates that the increased production of phytochelatins is not responsible for the elevated tolerance to Cd in some plants, since both sensitive and tolerant populations produce equivalent amounts of phytochelatins when exposed to equal concentrations of Cd. In addition to phytochelatins, other amino acids and vitamins have also shown alterations against Cd, an increase in the contents of a-tocopherol, asparagine, tyrosine and proline was observed in different tomato cultivars exposed to stress by this metal (Zhao, Lin et al. 2021).

1.2.2. Strategies to Mitigate Cadmium Stress

Due to the damage caused by Cd toxicity in plants and the risk caused by its accumulation in them, several research groups have proposed different strategies to reduce its effects. Most of the strategies include making modifications in nutrition management. But other practices have also shown favorable results, such as inoculation with beneficial bacteria, grafting on resistant rootstocks, adding different growth regulators and applying soil amendments (Zhao, Guan et al. 2021). Many authors suggest optimization in nutrient management as a useful strategy to attenuate Cd toxicity; a review on the subject was carried out by a group of authors in 2012.

Subsequent research on other crops and other elements continued to promote adequate nutrition to mitigate Cd stress. Among the different nutrients, P, K, S, Fe, and Zn showed significant favorable effects (Zhao, Guan et al. 2021). The application of P in wheat plants increased the biomass of the shoots, the area of the leaves, the content of photosynthetic pigments and, in turn, favored the assimilation of other nutrients, such as K, Ca, Mg and Mn. It also increased the activity of antioxidant enzymes and decreased the content of Cd and H_2O_2 in the shoots (Singh and Steinnes 2020).

On the other hand, the addition of K reduced the uptake and translocation of Cd in sunflower plants and inhibited the increase in membrane permeability caused by stress. However, in this study no effects were observed in the biomass per organ, nor in the content of photosynthetic pigments, even though in another investigation it was suggested that K participates in the formation of photosynthetic pigments and prevents the decomposition of chlorophylls (Zhou, Ma et al. 2021).

Similarly, KCl supplementation in rice plants grown with high concentrations of CdCl2 increased their growth and decreased the activity of the enzyme NADPH

oxidase. However, other results showed that K deficiency protects rice plants from further oxidative stress caused by Cd, since it increases the activities of antioxidant enzymes (superoxide dismutase, ascorbate peroxidase, glutathione reductase and catalase) (Liu, Bai et al. 2019).

Unlike K sufficiency, its deficiency does not inhibit the entry of Cd into the plant. The results showed that both K sufficiency and deficiency have positive effects in mitigating Cd stress, but with different consequences each. Similarly, to K, the deficiency of Mg, Ca and N allows prior activation of the antioxidant defense but does not prevent the uptake of Cd by the roots (Sodango, Li et al. 2018).

In the case of S, some authors suggest that it is involved in the biosynthesis of heavy metal detoxifying agents. In mustard (*Brassica juncea*) the application of 30 µM and 300 µM of S lessened the effect on the chlorophyll content and increased the activities of the antioxidant enzymes, ascorbatoperoxidase, glutathione reductase and catalase (Yi, Liang et al. 2017).

1.3. Copper (Cu)- Bioavailability and Toxicity

Due to their non-degradable nature, heavy metals are called persistent compounds, that is, they can remain for a long time in the soil. Depending on physicochemical conditions such as soil texture, aeration, pH, water availability, Cu+2 and other metals can accumulate or become bioavailable (soluble and capable of passing into living cells) (Kumar, Pandita et al. 2021). When they become bioavailable, they can be toxic to living organisms including microorganisms, although many of these can activate tolerance mechanisms (Sayara, Sarrà et al. 2010).

The response of plants to heavy metal toxicity involves structural, biochemical and physiological changes that depend on the type and concentration of the elements and the exposure time (Liu, Bai et al. 2019). The most visible symptoms due to phytotoxicity in the plant are related to reduced growth, especially of the root, chlorosis and necrosis in the leaves and, later, symptoms of senescence and abscission (Sodango, Li et al. 2018).

Fruit trees and vines that grow in soils with a high concentration of Cu generally present roots with shorter and thicker apex, due to changes in cell division and in the arrangement of tissues, as well as lower root density that is reflected in an absorption reduced nutrients and, therefore, lower biomass production (Yi, Liang et al. 2017). At

the foliar level, plants can show symptoms that can be confused with deficiencies of other nutrients such as Fe or Zn.

The effects of Cu toxicity on the soil microbiota are related to the decrease in respiration, microbial biomass, and phylogenetic diversity, among others:

Inhibition of microbial biomass: Since the 1990s, when the use of sewage sludge in agriculture became more common, numerous investigations such as those carried out by (Huang, Deng et al. 2018). (Huamain, Chunrong et al. 1999) demonstrated the effect of heavy metals, especially Cu^{+2} applied as a Bordeaux mixture, in the reduction of microbial biomass in relation to the increase in Cu^{+2}.

The presence of high concentrations of Cu^{+2} (1000 mg/kf Cu total) reduces the microbial biomass expressed as microbial C/organic C. Likewise, the production of mycelium in fungi (Bui, Do-Hong et al. 2016). However, this effect is not universal. For example, in one experiment, the addition of Cu to a rice soil, up to 1600 ppm, increased the size of the fungal population, proportional to the concentration of Cu added, but decreased fungal diversity by 40%, determining dominance. from genera such as Aspergillus, Penicillium and Fusarium.

Other tests have indicated that the population of fungi such as Penicillium can increase in the presence of Cu^{+2}, with the inhibition of the evolution of CO_2 in the soil (microbial respiration), observing a positive correlation coefficient between the concentration of Cu and the number of colonies of fungi in the soil. In addition, it was determined that Cu-tolerant fungi, especially the Penicillium genus, are dominant in Cu-contaminated soils (Bosse, Rosen et al. 2014).

Inhibition of respiration in soil: In general, microbial respiration in the soil is negatively affected by high concentrations of Cu^{+2} ions. Several studies have shown a drastic decrease in the aerobic respiratory rate with EC50 values (concentration for a 50% reduction in respiration) of 187 mg kg^{-1} Cu-EDTA/L of soil. The addition of Cu to the soil directly affects the cell membrane of microorganisms or complexes with organic matter making it less available for energy production and therefore favors the death of microorganisms (Hong-gui, Teng-feng et al. 2012).

Inhibition of the degradation of organic matter: The presence of high concentrations of Cu in the soil inhibits the metabolic function of microorganisms. Recent publications indicate that in addition to inhibiting microbial respiration, high concentrations of Cu have a special effect on Gamma proteobacteria and Actinobacteria, groups with genera related to the degradation of organic matter, and

the accumulation of C and N in soils, probably due to the lower rates of degradation. This process leads to a loss of soil fertility and changes in the balance between CO_2 release and long-term C storage (Krishna and Govil 2004).

Genetic selection of microorganisms resistant to Cu: The long-term coexistence of soil microorganisms with high concentrations of Cu ends up selecting the microbial communities with the highest phylogenetic relationship and restricted functional diversity, such as *Acidobacteria*, *Betaproteobacteria* and *Nitrospira*, losing the possibility of participating in various functions in the soil (Maderova, Watson et al. 2011).

Alteration of the N cycle: Different studies in soils (with and without plants) using concentrations between 1 and 100 mg/kg CuO -NP (Cu oxide in nanoparticles) in wheat have shown that the accumulation of Cu in the soil causes effects negative on the populations of denitrifying microorganisms, despite the fact that this microbial group generally presents greater diversity, functional variability and niche width (facultative anaerobes and substrate diversity) that makes it more resistant to abiotic stress, compared to nitrifying microorganisms (Nwachukwu, Feng et al. 2010). The effects of Cu are related to the inhibition of the synthesis of proteins involved in the metabolism of N, the transfer of electrons and the production of transporters.

Additionally, soil experiments have shown a reduction in N_2 fixation, as a product of the accumulation of heavy metals, even present in organic fertilizers of animal origin or sewage sludge (Wang, Shi et al. 2007). Azotobacter is one of the free-fixing genera most sensitive to Cu^{+2} concentration in soil.

1.3.1. Cu Toxicity Symptoms in Maize

Inhibition of enzymatic activity: Numerous studies have shown that high concentrations of Cu inhibit enzymatic activities such as β glucosidase, phosphatase and urease, enzymes associated with the degradation of organic matter, as well as dehydrogenase, an indicator of microbial respiration. The enzymatic activity of urease and nitrate reductase can be affected at different levels of Cu^{++}, but it depends on the texture, pH, available C, among other factors. Likewise, oxidative enzymes such as phenol and peroxidase can be produced by bacteria and fungi to mitigate the toxic effects of Cu. When Cu becomes toxic, reactive oxygen radicals are favored and the activity of oxidative enzymes increases.

1.4. Soil Contamination Detection

Soil is an environmental component that, due to its origin, formation and evolution, cannot be isolated from the environment that surrounds it, representing, in most terrestrial ecosystems, the physical-chemical environment in which life develops. It is fragile, difficult, and long to recover, and of limited extension. Therefore, its inappropriate use can contribute to the degradation of this non-renewable natural resource in the short term.

From all the above, it can be deduced that the traditional concept of soil degradation as loss or reduction of productive potential is currently insufficient, since there are other forms of degradation that, although they are not oriented towards production, reduce environmental quality and, therefore, therefore, the sustainability of the systems. An alternative consists of considering as degradation any change in the properties of the soil that causes a reduction in the functions that it can perform. One can speak of different types of degradation (physical, chemical and/or biological), depending on whether an alteration of said soil properties occurs (Bastida, Jehmlich et al. 2016).

The soil is in equilibrium between processes that act in opposite directions of formation and degradation, but this can break causing accelerated degradation. It performs a wide variety of ecosystem functions, which allows it to play a critical role in issues such as maintaining air quality, storing water and nutrients for plants and microorganisms, and purifying pollutants through physical, chemical, and environmental processes.

Soil contamination occurs when it receives elements or substances in concentrations that exceed its natural self-purification capacity, causing an alteration in its normal functioning.

Contaminated soil is defined as soil whose physical, chemical, or biological characteristics have been negatively altered by the presence of components in concentrations that pose a risk to human health in accordance with the provisions of current legislation in Royal Decree 9/2005, of January 14, 2005, and Law 22/2011 of July 28 on Waste and Contaminated Soils, which establishes potentially polluting activities and the criteria for declaring contaminated soils.

To determine a soil as contaminated, the generic reference levels (NGR) are also considered, which refer to the concentration of a polluting substance in the soil that can generate the highest level of admissible risk to human health and ecosystems. The

NGR is, therefore, an alert level that indicates that there may be an unacceptable ecotoxicological risk that, in any case, must be assessed.

1.4.1. Sources of Contamination by Heavy Metals

Different sources of soil contamination by heavy metals can be distinguished depending on their origin. Sometimes, the very nature of the original material and its alteration are responsible for the contamination; in this case, it is called endogenous contamination. Other times the polluting contributions are external, frequently as a result of anthropogenic activities, called exogenous contamination. Pollution of natural origin is significantly less important than that of anthropogenic origin.

The main sources of exogenous contamination come from indirect contributions through the air, which over time are deposited by sack or wet on the ground, with mining and energy production being the industrial activities that contribute the most to this type of pollution, along with the incineration of plastics, organic waste, and fossil fuels.

Other causes that contribute to soil contamination by direct input are the inappropriate use of mineral fertilizers and phytosanitary products, the dumping of waste generated (manure, slurry, solid urban waste, sludge from wastewater treatment plants), and the use of water of inadequate quality for agricultural use. Industrial discharges or the establishment of landfills where different types of waste accumulate are also other important sources of contamination by heavy metals (Galdames, Mendoza et al. 2017).

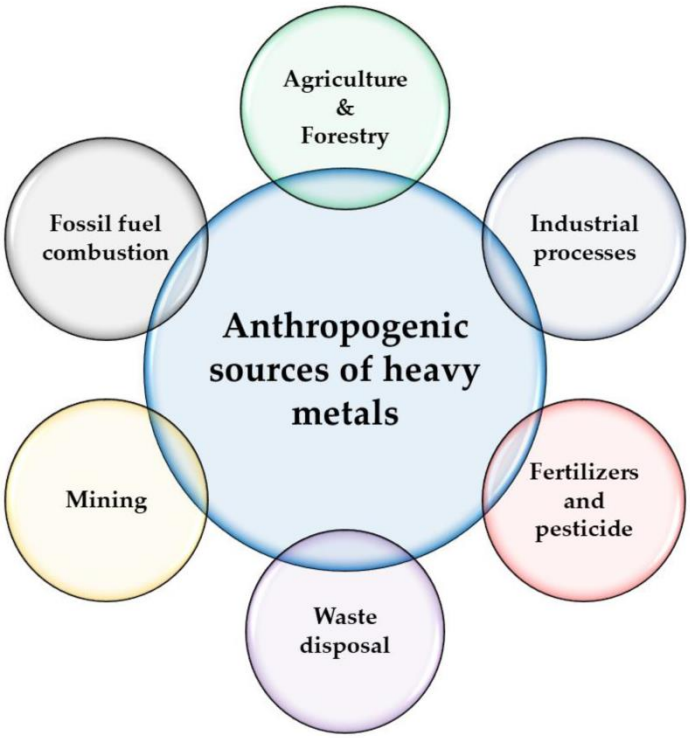

Figure 1.1: Sources of Heavy metals from Anthropogenic activities

(Alengebawy, Abdelkhalek et al. 2021)

1.4.2. Dynamics of Pollutants in the Soil

The soil has become a receptor medium for a multitude of potentially polluting substances. Its condition of interface between the biosphere (terrestrial biomass, marine biomass and man), the lithosphere (crust, soil and sediments), the hydrosphere (fresh water and sea water) and the atmosphere makes it a "transit station" for pollutants, in which they can remain retained for long periods of time (which increases the possibility that they may be degraded and lose their polluting nature) or be so mobile that they are incorporated into other media and, from there, into food webs with the consequent problems that this would entail.

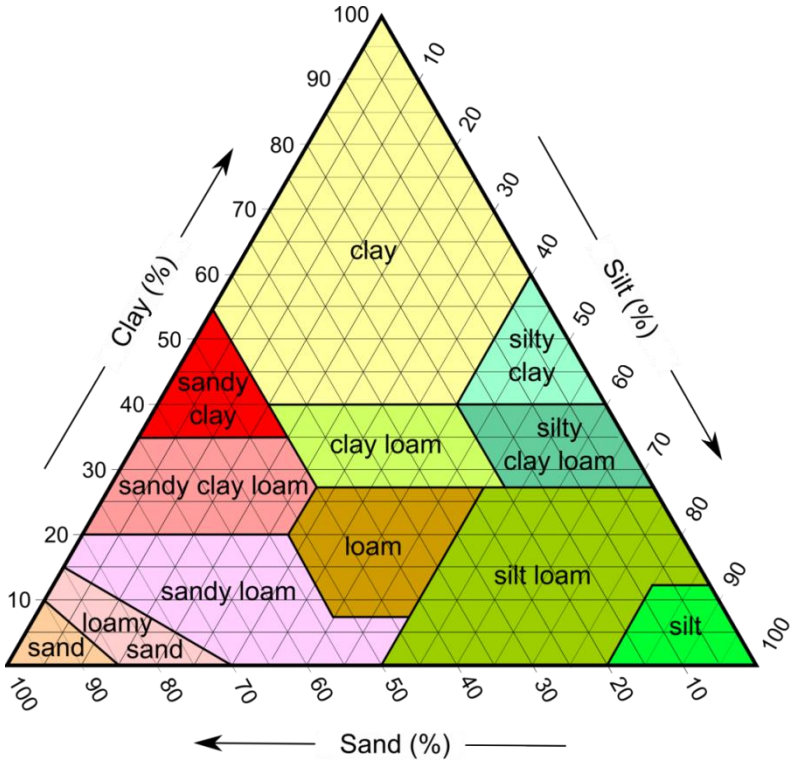

Figure 1.2: Properties of soil with different percentages of clay, silt, and sand (https://thinkingcountry.com/2016/11/30/soil-texture-sand-silt-and-clay/)

1.4.3. Speciation of Pollutants in the Soil

Pollutants dissolve quickly in rivers or in the air. However, in soils they tend to accumulate. For this reason, the soil acts as a sink for most pollutants, including heavy metals.

The toxicity of a polluting agent will not only depend on itself but also on the characteristics of the environment where it is found, so that the sensitivity of soils to the aggression that takes place by polluting agents will be very different depending on of a series of edaphic characteristics.

1.4.4. Forms of Retention and Availability of Metals in the Soil

Heavy metals can occur in the soil in different forms: 1) Soluble in the soil solution. 2) As exchangeable ions of the colloids that make up the exchange complex. 3) Forming complexes with organic matter. 4) Adsorbed on Fe, Mn and Al oxides and hydroxides, sulfides, and phosphates. 5) As constituents of the secondary minerals of the soil.

Factors Affecting the Availability of Metals

In order to know the behavior of heavy metals in soils, the following factors must be considered:

1.5. Soil properties

Such as pH, texture, oxidation-reduction conditions, organic matter content, cation exchange capacity and the presence of other elements.

1.5.1. pH

It is the main factor controlling the availability of metals for plants. Most of the metals tend to be more available at acidic pH, since a decrease in pH improves both the solubility of the metals and their uptake by plant roots. In some cases, it usually happens that an increase in soil pH does not necessarily cause a decrease in the availability of metals, as occurs with As, Mo, Se and Cr. Therefore, pH is an important parameter to define mobility. of the cation, because in moderately alkaline pH media precipitation occurs as hydroxides. However, in very alkaline media these hydroxides can go back into solution as hydroxy complexes.

1.5.2. Texture

Fine-textured soils probably come from secondary minerals that are easily disturbed and are generally the main source of heavy metals. Coarse-textured soils have primary minerals such as quartz, with low heavy metal content.

1.5.3. Oxidation-reduction conditions

Many metals form relatively insoluble sulfides under strongly reducing conditions. These include Cd, Zn, Ni, Co, Cu and Pb. Other metals like Fe and Mn can become more soluble under these conditions.

1.5.4. Organic material

Soil organic matter has a high affinity for certain metals (Co, Cu, Mo, Ni, Pb and Zn), reacting with them and influencing their availability. The availability of metals is generally associated with the formation of metal complexes with humic substances and other high molecular weight compounds. The metals, once they form these complexes, can more easily migrate to the deep layers or remain in the soil solution as soluble organic complexes.

1.5.5. Cation Exchange Capacity (CEC)

The CEC is a function of the clay and organic matter content of the soil, it also controls the availability of metals. In general, an increase in the CEC produces an increase in the time in which these metals are available to plants since the capacity of the soil to fix metals increases.

1.5.6. Presence of other elements

Some metals influence the availability of others (e.g., Cd/Zn). Zn concentration can influence Cd uptake by plants because both elements have a similar ionic structure.

1.5.7. Properties of metals

Such as the ionic potential of the same, the electronegativity, the hydration conditions and the valence of the metals in question.

1.6. Behavior of Metals in Soil-Plant System

The soil-plant system is considered an open system, which is subject to inputs, such as pollutants, fertilizers, and pesticides, and to losses, through leaching, erosion, or volatilization. The incorporation of heavy metals by plants occurs mainly from the soil, through the roots, and is influenced by several factors, including the type of soil, temperature, pH, aeration, redox and fertilization conditions, the plant species, the moment of development and the root system, among others (Sayara and Sánchez 2020). Apart from uptake that takes place via the roots, plants can also take in significant amounts of some elements through foliar uptake. Once the metal ions have been absorbed, they can move throughout the plant. This movement depends on the type of metal, the organ of the plant and its age. In general, the proportion in which the elements are mobilized inside the plants decreases according to the following order: Cd>B>Zn>Cu>Pb. Heavy metals incorporated into the soil can follow four different pathways:

1. Be retained in the soil solution or fixed by adsorption, complexation and/or precipitation.
2. Being absorbed by plants and joining food chains.
3. Pass to the atmosphere by volatilization.
4. Move to surface or groundwater.

When a contaminant is incorporated into the soil, a series of physical, chemical or biological processes are triggered that condition the effects that it can cause not only on the soil system but also on the rest of the environmental compartments and on the trophic chain. In order to assess the environmental impact of contamination in the soil-plant system, the characteristics of the pollutant, the receiving environment and its environment must be known, as well as the models that govern the behavior of the pollutant and its transfer to plants. Once the contaminant is incorporated, it can be

influenced by processes such as transformation, retention and transport (Barker and Bryson 2002).

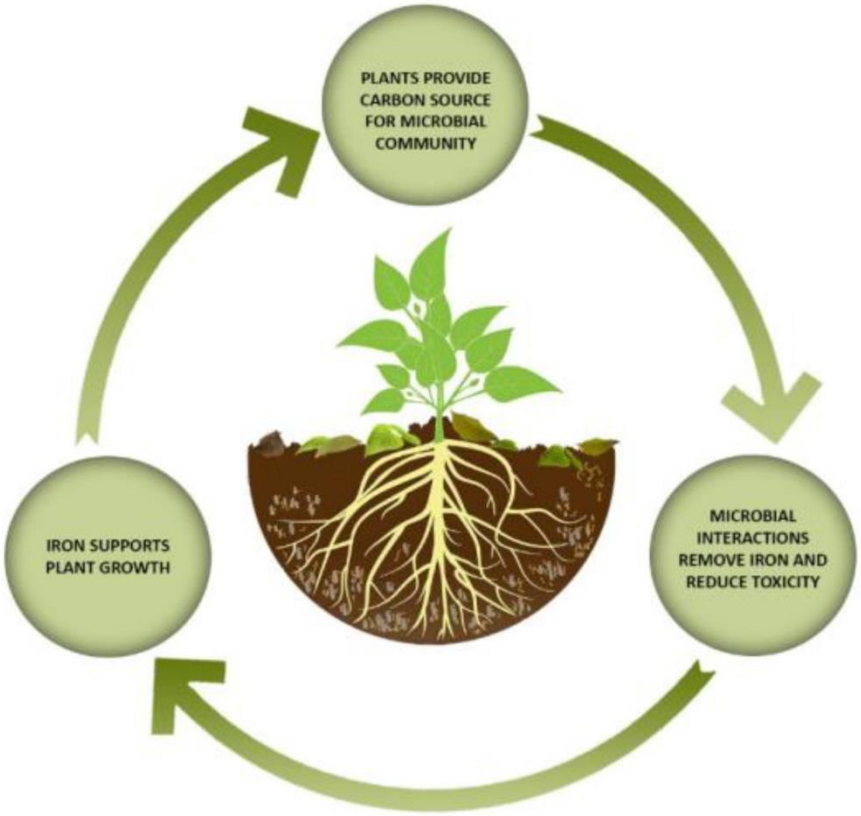

Figure 1.3: Behavior of Metals in plant-soil system.

(Alengebawy, Abdelkhalek et al. 2021)

1.7. Contamination Threshold in Edaphology

The contributions of waste of industrial, urban or agricultural origin can be optimized considering that the soil can act as a filter and reactor through physical-chemical and biological processes. However, when considering the soil as a waste receptor, it must be recognized that its acceptance capacity is not unlimited. So, the usual agricultural practices such as the agricultural use of compost and sewage sludge, have determined that in different countries such as the Netherlands and certain official organizations, they put in place legislation based on maximum reference values for heavy metal content that could be reached in soils, so that above these thresholds it can be considered that there is contamination.

After its implementation, it became clear that standards based on thresholds or limits, regardless of the type of soil, are not generally effective. This is because the mobility or bioavailability of soil elements depends on their characteristics, such as pH or organic matter, as well as climatic conditions. Therefore, the need to consider the type

of receiving soil or some of its characteristics is currently accepted to establish reference thresholds in relation to contamination by substances such as heavy metals or other compounds of an inorganic and organic nature (Kästner and Miltner 2016).
Consequently, more recently, the need to know the background values that are independent of agricultural practices has been established, so they must be measured in natural soils.

1.7.1. Unfavorable Effects of Pollution

Pollution can be defined as the contribution of an element or a chemical compound from outside the place, which causes an increase with respect to the initial concentration, which produces unfavorable effects, both due to its deactivating action, and if they cause an excessive increase in activity.
Pollutants generally cause negative effects on the environment that can act directly or indirectly on the soil system. Some of the effects of pollution are described below.

1.7.2. Direct effects on soil

Inhibition of their enzymatic activity due to the destruction of their self-purification power by normal biological regeneration processes, as the acceptance capacity of the soil has been exceeded. The biogeochemical cycle and the biofilter function are affected. Qualitative and quantitative decrease in the normal growth of populations of microorganisms and soil fauna, or alteration of their diversity, which increases the fragility of the system. Consequently, decreasing in crop yields and changing the composition of the products, with a risk to the health of consumers, when certain elements enter the food chain.

1.7.3. Indirect effects on soils

Following are the indirect effects of pollution on soil health:

- Contamination of surface and groundwater by transfer processes. Concentrations higher than those considered acceptable are reached.
- Variation of the availability of elements in the long term in the soils, because of changes in their physical-chemical properties.
- Reduction of soil fertility, by reducing its flora and fauna.
- Modification of the soil structure due to the loss of its fertility.

Contamination generally causes a disturbance of the soil which translates into a loss of quality and suitability for use or makes it unusable, unless it is subjected to prior

treatment. The soil can contain a wide variety of chemical elements, so it can be difficult to establish from what moment an element ceases to be beneficial or non-toxic to the soil and becomes a pollutant. Likewise, it is also difficult to specify when a soil that is undergoing a recovery process is no longer contaminated.

In nature, there are practically no soils that are totally "free" from anthropogenic contributions, since even forest soils far from industrial activity receive elements and compounds transported by atmospheric circulation over long distances, even in minute amounts.

1.7.4. Origin and Consequences of Soil Contamination by Metallic Elements

Contamination by heavy metals causes a particular problem in soils, due to its impossibility of biodegradation and the fact that many metallic elements can accumulate in the soil in forms that are not very bioavailable. A heavy metal is defined as any metallic element whose specific weight is greater than 5 g/cm3 or its atomic number is greater than 20. All metals exist naturally, but in minimum concentrations that do not cause adverse effects. Thus, heavy metals in the soil can be of geogenic or anthropogenic origin. The geogenic ones come from the alteration of the parent material by chemical, physical or biological processes during edaphogenesis, and they are the ones that pass from the parent material to the soil.

On the contrary, anthropogenic are those that have their origin in discharges related to anthropogenic activities such as the metallurgical industry, the combustion of certain fossil fuels, or certain agricultural activities, among others. They constitute the largest contribution in contaminated soils and can become toxic depending on their concentration and bioavailability, causing a wide variety of lethal or sub- lethal adverse effects in living beings.

This double origin of metals in soils implies the need, in contamination assessment work, to discriminate between the portion naturally present in the soil and the portion originating from polluting activities. Thus, background levels are defined as the concentration of a substance, systematically present in the natural environment that has not been influenced by localized human activities.

In general, it can be stated that when the real concentrations of a metal in the soil exceed the background levels, and this causes damage to the normal functioning of the soil, for example, causing mortality or dysfunctions in living organisms, the soil can be considered contaminated. From a legal point of view, the declaration of

contaminated soil automatically generates the need to undertake soil remediation work.

The bioavailability and mobility of an element depends on the characteristics of the soil where it is found, as well as the chemical form in which they are found. However, the mobility of metals in the soil is usually low, being accumulated in the first centimeters of the soil. The main edaphic attributes that act on the bioavailability of metallic elements are:

- The pH, since metals tend to be more available in an acid soil as they are less strongly absorbed (except As, Mo, Se, Cr).
- Its cation exchange capacity (CEC), clayey soils retain more metals when adsorbed.
- Organic matter (OM) that, in addition to generating cation exchange capacity, reacts with metals forming organometallic complexes, and can be so strongly adsorbed that they remain stabilized like Cu or form stable chelates like Pb.
- Redox conditions, the metal can be oxidized or reduced.
- Presence of carbonates that guarantee a high pH, which makes the metals precipitate.
- Salinity, if it increases, mobilization can be increased.

1.8. Decontamination treatments

Remediation techniques can be physical, chemical or biological and are often used in sequential combination, called a process train, to achieve the most economical and efficient recovery.

1.8.1. Physical remediation

1. Soil replacement: We replace contaminated soil with another that is not. Thus, we dilute the concentration of heavy metals in the soil, increasing its functionality.
2. Soil isolation: It is achieved by placing a layer of impermeable material such as clay under the contaminated region of the soil. It is very useful to avoid contamination of groundwater.
3. Vitrification: In vitrification an electric current is passed through the soil by vertically inserting electrodes into the contaminated area. This

technique is applicable to large volumes of soil and temperature is a key factor.

4. Electrokinetic remediation: Separates metals by electrophoresis, electrical filtration or electro-migration thus reducing contamination.

1.8.2. Chemical remediation

1. Immobilization techniques: They decrease the mobility of the metal and its bioavailability in the soil by adding immobilizing agents to the soil, resulting in reactions of complex formation, precipitation and adsorption that causes the redistribution of metals to solid particles, limiting their transport and bioavailability. It is carried out with the contribution of organic and inorganic amendments. The most common being those that include cement, clay, zeolites, phosphates, minerals and microbes.

2. Encapsulation: The contaminated soil is mixed with other products such as concrete, lime or asphalt, making it immobile and avoiding contamination of the surrounding materials. In addition, it prevents the leaching of organic materials.

3. Soil washing: Reagents and extractants are used that can leach heavy metals from the soil. The solution and the extraction are mixed for a certain time and through precipitation, ion exchange, chelation or adsorption the metals are transferred to the liquid phase and subsequently separated by leaching. Synthetic chelating agents such as EDTA, organic acids, humic substances, surfactants and cyclodextrin can be used.

1.8.3. Biological Remediation or Bioremediation

Born from the use of microorganisms or plants to detoxify or remove contaminants from the soil, bioremediation is profitable, economical, non-invasive, and provides a permanent solution.

1. Phytoremediation: It is the mechanism by which plants can immobilize, degrade or remove metals.

2. Phytovolatilization: The plant absorbs metals from the soil and converts them into less toxic vapors that are later released into the atmosphere through the process of transpiration. These metals are assimilated into volatile organic compounds (VOCs) that are released into the atmosphere as biomolecules.

3. Phytostabilization: Plants are used to reduce the availability and mobility of heavy metals in the soil, this technique does not reduce the concentration of metals, but rather limits this movement and can do so in several ways: Reducing leaching, reducing soil erosion stabilizing it with the roots and decreasing runoff.
4. Phytoextraction: This method involves cleaning heavy metals from the soil through absorption by plants. It is based on the ability of plant roots to absorb, transfer and concentrate heavy metals to the aerial parts of the plant, resulting in a decrease in the contaminated mass. During the process the heavy metals are transferred from the soil to plant biomass which is very easy to treat compared to the soil and this guarantees a permanent removal of the metals, although not all plant species can be used.
5. Phytoremediation assisted by microbes: Refers to the use of microorganisms to induce the absorption, precipitation, oxidation and reduction of heavy metals in the soil.

1.9. Bioremediation

Due to human activity, today there are different types of pollution that aggravate the health of the planet. The productive sector is responsible for this, but thanks to bioremediation this evil can be reversed.

Bioremediation is one of the many biotechnological applications that can be used today in order to reduce contamination. It is undoubtedly some help to the environment, and it is a strategy that presents numerous advantages to face today's challenges.

1.9.1. Bioremediation as a decontamination tool

Bioremediation is a biotechnological process that uses microorganisms, fungi, plants, enzymes, yeasts, and bacteria to remedy a biological problem, such as the contamination of natural spaces such as forests, the ocean, soil, etc.

The catabolic capacities of living beings are used to attack different polluting agents. Some of these are: polycyclic aromatic hydrocarbons, oil, pesticides, chlorophenols, heavy metals, dyes, sulfates, etc. Thus, a large part of the environmental space is recovered without resorting to invasive practices.

This practice involves oxidation-reduction reactions to reduce contamination. In addition, it can be used in two ways: bio stimulation and bioaugmentation. In the first, limiting nutrients are added to support or stimulate native microorganisms existing in the environment that can carry out bioremediation. On the other hand, the second consists of the addition of living cells that can accelerate the degradation of certain pollutants. As a biological process, it presents a series of advantages such as:

- It does not produce significant waste for the environment.
- It requires little energy for its application.
- It is cheaper than other decontamination techniques.
- It can work as a complement to other techniques, or sequentially to them.
- It is not invasive or harmful to the environment.
- Results in simple operations and low requirements

1.9.2. Types of Contamination that Bioremediation can Treat

In general, bioremediation can treat almost all types of pollution that are caused by human activity. Among the most common are oil spills and substances derived from it in natural spaces. There is also the degradation of the fields due to the use of fertilizers, chemical pesticides, and intensive livestock. Undoubtedly, today there are multiple natural disasters to which we are exposed. However, bioremediation is presented as an ecological alternative to reduce the negative impact of human practice. Below is described how bioremediation can help treat three types of contaminants: hydrocarbons, heavy metals, and dyes.

1.9.2.1. Bioremediation of hydrocarbons

This is applied to reduce or eliminate pollution produced by oil, oil, diesel and grease spills in terrestrial and aquatic ecosystems. These events can reduce or inhibit the development of flora and fauna in the contaminated sector, having a serious environmental impact. Acting urgently before the contamination of ecosystems is essential.

Therefore, bio stimulation and bioaugmentation strategies to improve and stimulate microbial development are important to apply. The use of biosolids as a source of macro and micronutrients can favor the stimulation of native microorganisms and, at the same time, degrade hydrocarbons through the oxidation-reduction reaction.

1.9.2.2. Bioremediation of heavy metals

These pollutants affect the quality of water and soil, causing damage to the environment and human health. As a result, the efficient extraction of heavy metals thanks to bioremediation is essential today. This biotechnological process can be applied by three methods: biosorption, metal precipitation and bioleaching, which are very economical for the treatment of wastewater and contaminated soils.

1.9.2.3. Bioremediation of contamination by dyes

The textile, leather tanning, stationery, and food industries, among others, use coloring techniques that are highly polluting for the environment and even carcinogenic. Dissolved dyes in water interfere with sunlight penetration (interfering with photosynthesis) and inhibit the growth of aquatic flora and fauna. To avoid this problem, microorganisms are introduced into the polluted water to absorb and degrade the dyes.

1.10. Phytoremediation

Contamination by heavy metals causes a particular problem for the soil due to its impossibility of biodegradation. Many of the decontamination techniques, although they reduce their concentration, cause significant soil disturbance. Phytoextraction is a technique that makes it possible to reconcile the extraction of metals with the improvement of soil quality. It consists of the use of plants that extract metals from the soil and store them in their harvestable parts, which facilitates their collection.

A recently widely used species is Indian mustard (*Brassica juncea*), due to its ability to simultaneously accumulate numerous metals and its adaptability to diverse soil conditions. A study was carried out to evaluate the capacity of B. juncea in a Lead, Nickel and Copper phytoremediation process. In an artificially contaminated limestone soil, it was planted and cultivated for 45 days. Plants took up appreciable amounts of all three metals, although concentration factors were always less than 1 in contaminated soils. Its development was also very scarce, with such a reduced production that it leads to the conclusion that this species is not suitable for this type of practice on this soil and in the concentrations studied.

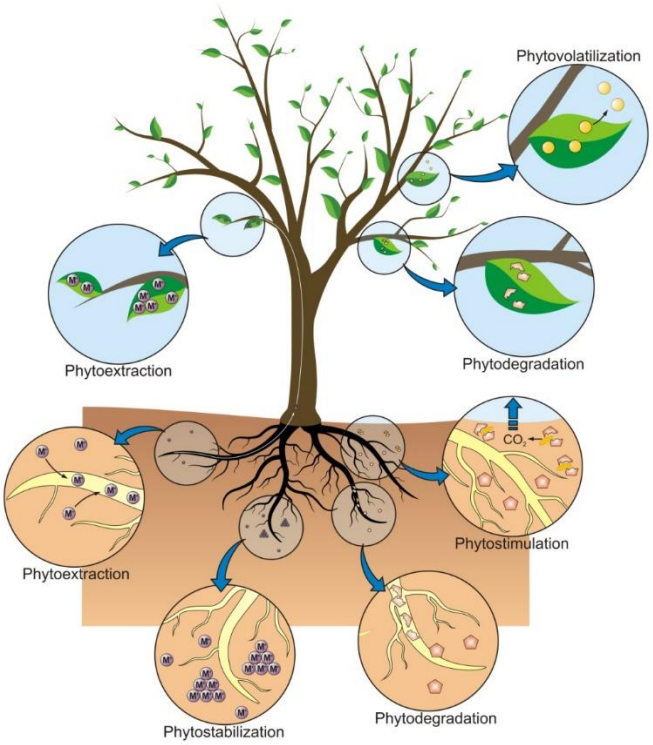

Figure 1.4 shows different types of phytoremediation

(Favas, Pratas et al. 2014)

1.10.1. Phytoremediation and *Brassica juncea* efficacy

At present there are numerous methods to treat soils contaminated by metals. However, most of the physical or chemical methods are very aggressive and, although they reduce the concentration of metals, they cause a significant alteration to the physical, chemical and biological attributes of the soil.

In soils such as those of La Hoya de Huesca, with carbonate contents that are frequently not less than 30 or 40%, decarbonation prior to acid washing of metals, for example, implies the destruction of a third of the soil, if not more, and the alteration of all the attributes that the soil inherits from these components.

In this context, phytoextraction techniques can be a viable way to reconcile the progressive extraction of heavy metals and the improvement of their quality. Phytoextraction consists of the use of plants that extract metals from the soil and store them in harvestable parts, in such a way that, with minimal soil disturbance, the goal of decontamination can be gradually achieved.

The entire heavy metal phytoextraction mechanism has five basic aspects: (1) mobilization of heavy metals in the soil, (2) absorption of metal ions by plant roots, (3) translocation of accumulated metals from the roots to aerial tissues, (4)

sequestration of metal ions in the plant, and (5) tolerance to metals. Metal tolerance is a key prerequisite for metal accumulation and therefore phytoremediation.

Plants carry heavy metals from the soil solution to their roots. After entering the roots, heavy metal ions can be stored in the roots or they are transferred to the shoots mainly through the xylem vessels, to the stems, pits or fruits, where they are deposited mainly in vacuoles. Phytoextraction efficiency depends on many factors, such as the bioavailability of metals in the soil, soil properties, heavy metal speciation, and plant species.

Plants suitable for phytoextraction should ideally have the following characteristics: (1) high growth rate, (2) production of more biomass above ground than below ground, (3) widely distributed and highly branched root system, (4) larger accumulation of heavy metals in plant than in soil, (5) translocation of accumulated heavy metals from roots to shoots, (6) tolerance to the toxic effects of target heavy metals, (7) good adaptation to environmental and climatic conditions prevailing, (8) resistance to pathogens and pests, (9) easy cultivation and harvest, and (10) repulsion to herbivores to avoid contamination of the food chain.

Thus, the phytoextraction potential of a plant species is mainly determined by two factors: the concentration of metals in the shoots and the biomass produced by the plant. However, hyperaccumulation and hyper tolerance are more important in phytoremediation than high biomass production. Hyperaccumulator plants have the potential to accumulate heavy metals in their shoots to levels that are toxic to other plants, thanks to having evolved effective detoxification mechanisms.

The term hyperaccumulator was first coined to describe plants with Ni concentrations greater than 1000 mg/kg dry weight. Currently, hyperaccumulator plants are considered those that can accumulate in their aerial biomass more than 100mg of Cd or Se, more than 1000mg/kg of Pb, Ni, Co or Cu, more than 10000mg/kg of Mn or Zn. Hyperaccumulator plants are characterized by their portion of roots in relation to plant mass, and more intense transpiration and slow growth. Therefore, its effective use for decontamination is limited. To enhance phytoremediation, chelating agents such as ethylenediaminetetraacetic acid (EDTA) or diethylenetriaminepentaacetic acid (DTPA) can be used.

The use of hyperaccumulators will produce a low volume, metal-rich biomass that is inexpensive and easy to handle in case of metal recovery and safe disposal. On the other hand, the use of non-accumulators will produce a biomass of great volume but

poorer in metals, which will be uneconomical to process for metal recovery and expensive to dispose of safely.

Indian mustard (*Brassica juncea* L.) is part of the *Brassicaceae* family, which is characterized by being able to accumulate large amounts of metals in roots and shoots. It can reach 1 m in height and its roots reach a depth of 90-120 cm, it has a cylindrical and branched glaucous stem, it has lower leaves with long petioles and upper leaves with or without petiole. Its flowers are bisexual, bright yellow with 6 stamens. It can tolerate high rainfall and a pH of 4.3 to 8.3.

It produces high biomass even in soils that contain metals as contaminants, it grows easily in various locations and climates. It is especially effective with Pb, which is concentrated in the roots and greatly restricts its translocation to shoots. Phytoremediation has some limitations:

- The scarcity of usable plant species and that can sometimes affect local biodiversity.
- The low growth and biomass of most of these species slowing down the process.
- The excessive time required for soil recovery.
- The difficulty of mobilizing the fraction of metal ions most strongly bound to the substrate, limiting its bioavailability.
- It is applicable to sites with low or moderate levels of metal contamination because the growth of these plants is not possible in highly contaminated soils. In addition, there is a risk of contamination of the food chain.

Although phytoextraction is a widely studied and documented technique, both in decontamination works and in Phyto mining activities, its use in strongly carbonated soils such as the predominant soils in Aragon and with not excessively high levels of contamination has not been sufficiently studied.

Industrial areas often suffer from soil contamination due to the discharge of heavy metals, such as Cadmium (Cd) and Copper (Cu), which can pose significant environmental risks. Bioremediation, a promising approach for soil remediation, involves the use of microorganisms and plants to degrade or immobilize pollutants. In this study, the aim was to evaluate the potential of two bacterial strains, *Bacillus cereus and Serratia marcescens*, in combination with *Brassica juncea*, a commonly

used phytoremediation plant, for the remediation of Cd and Cu-contaminated soil. The soil was taken from industrial Area I-9/2. It was naturally contaminated with Cd and Cu. The cadmium metal was 5 ppm while copper metal (Cu) was 300 ppm. Cadmium was raised to 100 ppm after spiking.

Bacillus cereus is known for its ability to enhance the degradation of various organic contaminants and heavy metals. Studies have reported its successful application in the bioremediation of soils contaminated with heavy metals, including Cd and Cu (Dixit, Malaviya et al. 2015). Similarly, *Serratia marcescens* has shown promising results in the degradation of organic pollutants and has the potential to enhance metal mobilization and bioavailability in contaminated environments.

Furthermore, the use of *Brassica juncea* in phytoremediation has gained considerable attention due to its ability to accumulate heavy metals in its tissues. This plant possesses unique characteristics, including a high biomass production rate, deep-rooting system, and metal hyperaccumulation properties. *Brassica juncea* has been widely studied for its effectiveness in the phytoremediation of Cd and Cu-contaminated soils (Chigbo, Batty et al. 2013). It can accumulate these metals in its tissues, thereby reducing their concentration in the soil.

Based on the literature and previous research, an experiment to compare the performance of unsterilized soil, containing naturally occurring microorganisms, with sterilized soil inoculated with *Bacillus cereus* and *Serratia marcescens* was designed. The aim was to assess the potential of these bacterial strains, along with *Brassica juncea*, in remediating Cd and Cu-contaminated soil.

By analyzing the results obtained from this study, insights into the effectiveness of the inoculated bacterial strains and the role of *Brassica juncea* in phytoremediation have been obtained. This research contributes to the field of environmental microbiology and bioremediation and provides valuable information for the development of sustainable strategies for soil remediation in industrial areas.

1.11. Problem Statement

A well-known problem associated to the existence of heavy metals, is that they tend to remain for larger period of time in soil than other forms of pollutants like organic pollutants. It is factually impossible to degrade those through the means of biochemical degradation. However, it is very much possible to change their nature of bioavailability thus reducing their toxic effects. There are many problems associated

to the presence of heavy metals is soil. On one hand where it might affect agricultural outputs and the quality of groundwater, on the other hand it can adversely affect the human health too. Moreover, the presence of HMs directly affect the degradation process related to other pollutants.

1.12. Objectives

- Comparison of phytoremediation potential of *Brassica juncea* in unsterilized heavy metal contaminated soil having indigenous bacteria and sterilized heavy metal contaminated soil with inoculation of two bacterial strains i.e., *Serratia marcescens* and *Bacillus cereus.*
- To investigate the effect of compost amendment on heavy metal uptake, plant growth, and bacterial colonization in sterilized and unsterilized soil.

Chapter 2

Materials and Methods

2.1. Collection and Processing of Soil

Heavy metal contaminated soil was obtained from the industrial area in Sector I-9/2, Islamabad. The soil was air dried and sieved using 2mm sieve to remove debris and obtain homogenized soil (pH 7 ± 0.1). One half of the soil was then autoclaved at 121 °C and 15 psi pressure. The soil was prepared for heavy metal analysis on an atomic absorption spectrometer. Two heavy metals i.e., Cd (100 mg/kg) and Cu (300 mg/kg) were found above the permissible limit.

Figure 2.1: Dried Sieved Soil Prior to Autoclaving

2.2. Bacterial Strains

Two pre-isolated heavy metal resistant bacterial strains i.e., *Serratia marcescens* (accession number LC763411) and *Bacillus cereus* (accession number LC763407) were used for inoculation. All strains were sourced from Environmental Microbiology and Bioremediation Lab, Quaid-i-Azam University, Islamabad.

2.3. Plant Materials

Brassica juncea a known hyperaccumulators of heavy metals, was selected for this experiment. Seeds were sourced from National Agriculture Research Center (NARC), Islamabad. Healthy seeds were surface sterilized with 70% ethanol, rinsed and washed with distilled water.

2.4. Experiment Design

Pot experiment was conducted in a greenhouse of the botanical garden (QAU, Islamabad). Pots with the dimensions (15×7×7 cm) were filled with 400 g pot^{-1} of soil. In experimental pots, 20 seeds *Brassica juncea* were sown in each pot and housed in greenhouse for three months (from December 2022 to March 2023 with prevailing seasonal growth conditions). The greenhouse conditions of 16 hours of light and 8 hours of darkness, at 30-33 ºC, and soil moisture in the pots at around 60% of their water-holding capacity, were maintained throughout the experiment.

Abiotic control treatments were also used containing sterilized and unsterilized soil to check effect of environmental conditions. Each treatment had three replicates. The pots were closely monitored for seed germination. Upon germination, the seedlings were thinned to five in number. To prevent contamination from leaching, each pot was set on a saucer. For the arrangement of the pots in the greenhouse, complete randomized block design (CRBD) was used.

C1 = Unsterilized contaminated soil

C2 = Sterilized contaminated soil

C3 = Unsterilized contaminated soil + compost

C4 = Sterilized contaminated soil + compost

T1 = Unsterilized contaminated soil + *Brassica juncea*

T2 = Sterilized contaminated soil + *Brassica juncea*

T3 = Unsterilized contaminated soil + compost + *Brassica juncea*

T4 = Sterilized contaminated soil +compost + *Brassica juncea*

T5 = Sterilized contaminated soil + *Brassica juncea* + *Serratia marcescens*

T6 = Sterilized contaminated soil + *Brassica juncea* + *Serratia marcescens* + Compost

T7 = Sterilized contaminated soil + *Brassica juncea* + *Bacillus cereus*

T8 = Sterilized contaminated soil + *Brassica juncea* + *Bacillus cereus* + Compost

T9 = Sterilized contaminated Soil + Brassica juncea + Serratia marcescens + Bacillus cereus

T10 = Sterilized contaminated Soil + *Brassica juncea* + *Serratia marcescens* + *Bacillus cereus* + Compost

Figure 2.2: Sowing of *Brassica juncea* seeds in the pots.

Figure2.3: Pots at Day 1 after sowing seeds

2.5. Inoculum preparation

The sterilized soil was inoculated with two selected bacterial strains. Nutrient broth was prepared for both strains. Single colonies were picked with a loop and then dipped into the broth and the flasks were placed on a shaker at 30 ºC for 24 hours. At 30 ºC, bacterial suspensions were nurtured in nutrient broth before being centrifuged and resuspended in 0.9% (w/v) NaCl. After seedlings stage the inoculation of 15ml bacterial suspension were applied at each pot of bacterial treatment containing 1.2 $*10^8$ bacterial cells/ml. As a control, 0.9% NaCl was used to treat spiking soil in place

of the inoculum suspension (Ren et al., 2019). After 60 days experimental plants were harvested for further analysis.

2.6. Analytical Procedures

Soil samples prior to experimentation, and after harvesting were used to examine physicochemical characteristics, heavy metals contents and nutrient analyses (phosphorous, nitrates, and organic matter). A subsample of sieved soil (2 mm) Soil pH, TDS and EC was measured by the EUTECH instrument pc 510. 10g of soil was taken using top balance machine in a glass beaker. Then 50ml of deionized water was poured into it for making (1:5 w/v) soil-water suspension. The suspension was mixed using orbital shaker and allowed to stand for 30 min. Using standard buffer solution, the pH meter was first calibrated at 6.86 and room temperature was also adjusted. The electrode was carefully rinsed with distilled water and with the use of tissue paper, drops of water were cleaned from the tip of electrode. The probe was put in the sample solution for at least 1 min and the reading was noted. The same procedure was applied for determination of total dissolved solids and electric conductivity.

2.6.1. Heavy Metal Analysis of Collected Samples

For elemental evaluation of soil samples, they were oven dried in a single day at 80ºC. After drying, samples were crushed manually and sieved by using 0.59 mm ASTM sieve to obtain homogeneous soil sample and used for further evaluation. For this test, aqua regia (containing 1:3 ratio of HNO_3 and HCL) was made. After preparing the aqua regia, 1 g of the sample was added in 15 ml aqua regia and boiled till the volume reduces to 3 to 5 ml. Then on the next day 5 ml of perchloric acid ($HClO_4$) was added into the leftover and boiled again till the volume of 3 to 5 ml was left. The leftover was cooled down and filtered using the Whatman filter paper (Number 42). Deionized water was used to raise the volume up to 15 ml. A blank sample was also analyzed in the same way but without the soil sample addition to remove any error during the procedure. A spectrophotometer for atomic absorption was used to analyze each sample in triplicate (Charles, 1991).

2.6.2. Determination of Nitrates

Soil nitrates were quantified by the chromotropic acid method (Estefan et al., 2013). In a nutshell, 1 g of sieved and dried soil was combined with 5 ml of 0.02 N $CuSO_4.5H_2O$ and shaken for 15 min at 100 rpm on an orbital shaker. Following

mixing, each sample was filtered using Whatman No. 42 filter paper, and 3 ml of the resulting filtrate was combined with 1 ml of 0.1% chromotropic acid before being placed in an ice bath. After this 6 ml of sulfuric acid (concentrated) was added in the solution and swirled. To prevent excessive heat formation, prepared mixture was left on shaking to cool down at room temperature. After 45 min yellow color was formed, to which absorbance of the mixture were taken on 430 nm, using spectrophotometer. A blank control was also prepared, containing all ingredients except soil, further standards of NO_3, using KNO_3 dissolved in 0.02 N $CuSO_4.5H_2O$, were also prepared. The concentration of NO_3 in ppm was quantified using values derived from the calibration curve.

2.6.3. Determination of Extractable Phosphorous

The standard Olsen sodium bicarbonate procedure was used to determine the amount of extractable phosphorus in soil samples (Estefan et al., 2013). For 30 minutes, samples were shaken at 150 rpm using an orbital shaker, then filtered through filter paper (Whatman no. 40). 3-5 drops of 0.25% nitrophenol indicator were added in filtrate (5ml) and mixed with 5N H_2SO_4 drop wise until the solution changes from yellow to colorless. After acidification the volume of the acidified solution was increased to 20 ml by using distilled water and 4 ml of ascorbic acid solution. A blank control of all ingredients except soil was made, and a phosphate standard of 1 to 5 ppm was made. After 10 minutes, the Rayleigh spectrophotometer UV9200 / VIS7220G was used to measure the absorbance of the blank, standard, and sample at 882 nm. The amount of extractable P in mg/kg was calculated using the values derived from calibration curve

2.6.4. Total Organic Carbon, Oxidizable Organic Carbon, and Organic Matter Analysis

The Walkley Black technique was used to calculate the organic matter of the soil (Nelson and Sommers 1982). Take around 0.5 g of the dry soil and place it in a 500 ml beaker. By using pipette took 5 ml solution of potassium dichromate (1N) and added approximately 10 ml of H_2SO4, after adding mixed the suspension by stirring. After leaving it for 30 minutes 100 ml of distilled water was added and after that 5ml of concentrated H_3PO_4 and let the mixture to cool. After adding almost 15 drops of the diphenylamine indicator in the beaker placed it on the magnetic stirrer. After that by applying the method of titration, titrated this solution with solution of ferrous

ammonium sulfate (0.5 M) and noted when color changes from violet to green. Blanks with no soil were made and analyze in the same way.

2.6.5. Determination of Soil Texture

The texture of soil was assessed by hydrometer method. In this method 40 g of soil was taken in the glass beaker and mixed with 60 ml of dispersion solution of sodium hexa meta phosphate. After covering the beaker with watch glass, it was left overnight. Then carefully transferred this mixture into the soil stirring cup on next day and filled the cup to three quarters with water. The suspension was kept on shaking overnight.

Figure 2.4: Hydrometer to check soil texture.

On next day the suspension was transferred to 1 liter cylinder or hydrometer jar and 1 liter volume was made using water. Then sand, silt and clay content were assessed by using hydrometer (ASTM 152H GILSON Comp Inc., USA) in the suspension (Strickland et al. 1988).

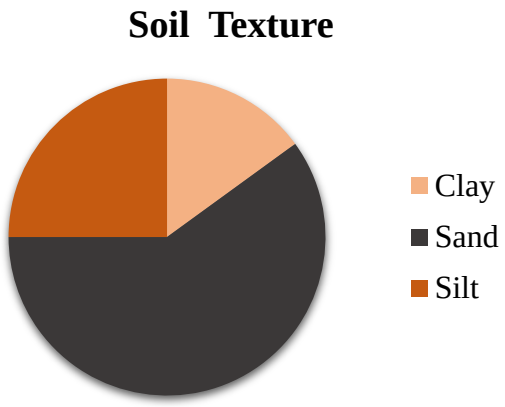

Figure 2.5: Constituents' Proportion in Soil

The same procedure was applied for blank but without soil. The texture of the soil was sandy loam. The soil had 10% clay, 65% sand and 25% silt.

2.7. Plant Analysis

2.7.1. Morphological Parameters

Harvested plants were subjected to physiological analysis. The final plant growth period was set at 60 days to investigate the effects of plant density on heavy metal uptake. The root and shott length were recorded. Gravimetric readings recorded on an electric weighing balance were used to measure the fresh and dried weight of the root, shoot, and were expressed in g plant 1. Samples were dried in an oven at 70°C until their dry weight remained constant. A representative number of fresh leaves were also preserved at -20 °C for biochemical and enzymatic analysis.

2.7.2. Heavy Metal Quantification in Plants

Heavy metals concentration in plants was quantified through the method of wet oxidation (Estefan et al., 2013). It involves the digestion of plants by mixture of acids (HNO_3 and $HClO_4$). 1g of the plant sample was grinded and soaked with concentrated HNO_3. Samples were left for pre digestion for 6 to 8 hours. After that 10 ml of acid mixture (HNO_3 and $HClO_4$ in 9:4) were added and placed on the hot plate at the temperature of about 120 to 180 °C. When white fumes started to appear and white content was left then samples were placed at room temperature to cool down. After that samples were filtered using Whatman No. 42 filter paper. By adding distilled water, the capacity was increased to 15 ml. Blank sample was also prepared in the

same way without plant content. Heavy metals were quantified using the atomic absorption spectrophotometer.

2.7.3. Chlorophyll A, Total Chlorophyll, Chlorophyll B, and Carotenoid Contents Assay

According to the Arnon (1949) methodology, 40 mg of fresh leaf samples were briefly immersed to generate a homogenous leaf extract in around 2 ml of 80% acetone solution (v/v). This extract was then used to assess the amount of chlorophyll and carotenoids in the sample. For five minutes, the extract was centrifuged at 5000 g. A fresh, clean falcon tube was used to properly store the supernatant. The pellets were vortexed for 1 minute at 5000 g with 1 cc of 80% (v/v) acetone in water. The previously harvested supernatant and the newly obtained supernatant were mixed for analysis. After obtaining absorbance (A) values at wavelengths of 663, 645, and 470 nm, the equations from Lichtenthaler (1987) were used to calculate photosynthetic pigments such as chlorophyll a, chlorophyll b, the total chlorophyll, and carotenoids.

2.7.4. Determination of Lipid Per-Oxidation

Method of Venkatachalam et al., 2017 was adopted for the analysis. In this procedure, 0.1 g of fresh leaf samples were obtained and macerated in cold 1 ml of TCA (5%) until they became homogeneous before being centrifuged at 10,000 g for 10 min. Then, in a 1:1 ratio, TBA solution (0.67%) was added to the supernatant, and the combination was heated for almost 30 minutes at 95 °C. The mixture was heated, then chilled for about a minute before being centrifuged at 10,000 g for ten minutes. The absorbance was measured using a UV spectrophotometer at wavelengths of 450 nm, 532 nm, and 600 nm. Malondialdehyde g-1 of fresh weight was used to measure lipid peroxidation.

2.7.5. Hydrogen Peroxide Production

Yusuf et al., 2011 reported the method of H_2O_2 and this method was adopted to quantify the content of hydrogen peroxide. In this, 0.1 g of fresh leaf was mashed in 1 ml of pH 7.4 extraction buffer that also contained 50 mM potassium phosphate buffer and 0.5 mM EDTA (PPB). After that, this mixture was centrifuged for 15 minutes at 10,000 rpm. For the purpose of further estimating the H_2O_2 content, the supernatant was subsequently collected and taken as a leaf extract. To create the reaction mixture for measuring the amount of H_2O_2, 40 l of leaf extract, 1 ml of PPB with a pH of 6.5 (0.05 mM), and 352.8 l of 1% $Ti(SO_4)_2$ produced in 20% H_2SO_4 were all combined.

The mixture was then centrifuged for roughly 15 minutes at 6000 g. The absorbance at 410 nm was measured using a UV spectrophotometer as part of an analysis of the supernatant to determine the strength of the yellow colour that was forming. By using the molar extinction coefficient (ε) of 0.28 M^{-1} cm^{-1}, H_2O_2 was expressed as M H_2O_2 contents g^{-1} of fresh weight.

2.7.6. Determination of Antioxidant Enzymes Activity

The following section introduces a method to quantify enzyme activity. The Venkatachalam et al. method was used to create leaf extract (2017). In a nutshell, leaf samples (0.1 g fresh samples) were macerated in 1 ml of pre-chilled extraction buffer (pH 7.4) containing roughly 50 mM potassium phosphate (PPB) and 0.5 mM EDTA, and then centrifuged at 10,000 g at 4 °C for 15 minutes. In order to measure the enzymatic activity of the homogenized sample, the obtained supernatant from the homogenized sample was collected, employed as a leaf extract, and kept at 4°C. For all the enzyme activities in the sample, the values were given in units of g^{-1} of FW.

Assay for Catalyze (CAT) Activity

CAT behaviour was assessed by determining the rate of H_2O_2 evaporation using the method (Maehly, 1954). The reaction mixture included 50 µl of diluted enzyme extract and 2.5 ml of 50 mM phosphate buffer pH 7.4, as well as 0.1 ml of 1% H_2O_2 and 0.1 ml of H_2O_2. The drop in absorbance coincided with the decrease in H_2O_2 at 240 nm (ε = 39.4 mM^{-1} cm^{-1}). The values are given in units g^{-1} of the fresh weight of the sample.

Assay for Ascorbate Peroxidase (APX) Activity

Using a modified version of Chen and Asada's (1989) procedure, ascorbate peroxidase activity (APX) was measured. For this, a reaction mixture made by mixing 50µL of leaf extract with 1mL of reaction buffer made of 500µM ascorbate, 100µM EDTA, 1.54mM H_2O_2, and 50mM PPB, having pH at 7.0 was used to observe the absorbance at 240nm. To compute the APX activity ε of 2.8 mM^{-1} cm^{-1} was used.

Assay for Guaiacol Peroxidase (GPX)

The method of Upadhyay et al. (2019) to quantify the activity of guaiacol peroxidase (GPX) was applied. The reaction mixture was prepared by mixing 20µl of leaf extract with 2.5mL reaction buffer made by 50mM PPB at pH 6.1, 1mL 1% Guaiacol and

1mL 1% H_2O_2. A420 was examined after 1 minute to determine the changes. The activity was calculated, using ε equal to 26.6 mM^{-1} cm^{-1}.

Calculation for APX, CAT, and GPX

The concentrations of enzyme unit were calculated by using Beer's law, which is

C (Units ml^{1}) = A / ε.L

Where, C= concentration, A= Absorbance, ε= Molar extinction coefficient, and L= Length of cuvette (1 cm)

Then for each expressing the values for gram of fresh weight C is multiplied with DF:

$$C \text{ (Units } g^{-1}) = (C) \times (W/1000) \times B$$

Where, C = Concentration derived from Beer's Law, V= ul of enzyme extract used for assay, and W= plant sample per ml of extraction buffer (0.1 g per ml of extraction buffer).

2.8. Statistical analysis

Before any analysis, the data were checked for normality using the Shapiro-Wilk Test. To compare various means, a one-way analysis of variance was performed on all treatments, followed by Duncan's multiple range post hoc test. All data was gathered in triplicate, and a p value of 0.05 was considered statistically significant. SPSS 20 was used for all statistical work.

Chapter 3

Results

3.1. Results

3.1.1. Physico-chemical Properties

The table 3.1 presents the results of the analysis of various physico-chemical properties for different treatments applied to unsterilized soil (US) and sterilized soil (SS) samples. The treatments are labeled as T1, T2,…..T10, representing different combinations of unsterilized soil, sterilized soil, and the addition of bacteria (*Serratia marcescens*, *Bacillus cereus*) with plant (*Brassica juncea*). The pH values indicate the acidity or alkalinity of the soil. The observed pH values range from 7.01 to 8.03, suggesting slightly alkaline soil conditions.

EC (Electrical Conductivity) of Control C4 (SS + Compost) had the highest EC value of 1916 uS/cm, indicating high salt content while Treatment T7 (SS + *Brassica juncea* + *Bacillus cereus)* had the lowest EC value of 185 uS/cm. Moreover, TDS (Total Dissolved Solids) of Abiotic Control C2 (Sterilized Soil) had the highest value of 858 mg/l while Treatment T7 (SS + *Brassica juncea* + *Bacillus cereus)* had the lowest TDS value of 124 mg/l.

NO_3 (Nitrate concentration) was found highest for the Treatment T7 (SS + *Brassica juncea* + *Bacillus cereus)* with the value of 11.5 mg/kg followed by T9 (SS + *Brassica juncea* + *Serratia marcescens* + *Bacillus cereus)* having value of 10.1 mg/kg and T8 (SS + *Brassica juncea* + *Bacillus cereus* + Compost) having value of 9.9 mg/kg. While it was lowest for the treatment C3 (Unsterilized Soil + Compost) with a value of 5.1 mg/kg. PO_4 (Phosphate concentration) of Treatment T7 (SS + *Brassica juncea* + *Bacillus cereus)* had the highest value of 205 mg/kg however, Control 3 and 4 having compost along with unsterilized soil and sterilized soil respectively, had the lowest PO_4 value of 15.6 mg/kg. OOC (Organic Carbon Content) of Treatment T7 (SS + *Brassica juncea* + *Bacillus cereus)* had the highest OOC value of 2.53% and control C2 (Sterilized Soil) had the lowest OOC value of 0.56%.

TOC (Total Organic Carbon) of Treatment T7 (SS + Plant + *Bacillus cereus)* had the highest TOC value of 3.38% while Control C2 (Sterilized Soil) had the lowest TOC value of 0.75%. OM (Organic Matter) was highest for Treatment T7 (SS + Plant + *Bacillus cereus)* with a value of 4.36% while Abiotic Control C2 (Sterilized Soil) had the lowest OM value of 0.97%. Overall, the results indicate variations in pH, EC,

nitrate and phosphate concentrations, and organic carbon content among the different treatments.

Table 3.1: Physico-chemical properties of soil

Description	Treatments	pH	EC uS/cm	TDS mg/l	NO_3 $mgkg^{-1}$	PO_4 $mgkg^{-1}$	OOC	TOC	OM
Initial Readings (Before Plantation)		7.1	1500	865	4.8	22.5	0.82	1.08	1.41
Unsterilized Soil	**C1**	7.53 ± 0.57^{ab}	1132 ± 5^{d}	742 ± 10^{b}	5.3 ± 0.6^{ef}	19.1 ± 17.5^{g}	0.83 ± 0.16^{f}	1.10 ± 0.22^{f}	1.42 ± 0.28^{f}
Sterilized Soil	**C2**	7.51 ± 0.59^{ab}	1288 ± 9^{c}	858 ± 12^{a}	4.3 ± 0.4^{gh}	26.1 ± 17.5^{g}	0.56 ± 0.10^{f}	0.75 ± 0.13^{f}	0.97 ± 0.17^{f}
US + Compost	**C3**	7.58 ± 0.52^{ab}	1645 ± 14^{b}	414 ± 32^{ef}	4.1 ± 0.3^{h}	15.6 ± 14.0^{g}	0.73 ± 0.17^{f}	0.97 ± 0.22^{f}	1.25 ± 0.28^{f}
SS + Compost	**C4**	7.01 ± 0.27^{b}	1916 ± 14^{a}	520 ± 28^{d}	4.9 ± 0.4^{fg}	15.6 ± 7.0^{g}	0.83 ± 0.12^{f}	1.10 ± 0.16^{f}	1.42 ± 0.21^{f}
Unsterilized Soil+ *Brassica juncea*	**T1**	7.82 ± 0.37^{a}	647 ± 14^{f}	431 ± 14^{e}	5.3 ± 0.3^{ef}	26.1 ± 10.5^{g}	1.43 ± 0.27^{e}	1.91 ± 0.36^{e}	2.47 ± 0.46^{e}
Sterilized Soil + *Brassica juncea*	**T2**	8.00 ± 0.27^{a}	839 ± 15^{e}	553 ± 18^{c}	5.1 ± 0.6^{ef}	19.1 ± 10.5^{g}	1.33 ± 0.26^{e}	1.78 ± 0.35^{e}	2.29 ± 0.46^{e}
US + *Brassica juncea* + Compost	**T3**	7.85 ± 0.27^{a}	436 ± 21^{h}	293 ± 13^{g}	6.3 ± 0.4^{d}	68.2 ± 10.5^{ef}	1.69 ± 0.26^{de}	2.26 ± 0.34^{de}	2.92 ± 0.44^{de}
SS + *Brassica juncea* + Compost	**T4**	7.84 ± 0.27^{a}	601 ± 17^{g}	399 ± 13^{f}	5.8 ± 0.4^{de}	57.7 ± 14.0^{f}	1.50 ± 0.26^{e}	2.00 ± 0.34^{e}	2.58 ± 0.44^{e}
SS + *Brassica juncea* + *Serratia marcescens*	**T5**	7.95 ± 0.24^{a}	261 ± 11^{j}	169 ± 14^{i}	9.3 ± 0.4^{c}	106.8 ± 21.1^{cd}	2.12 ± 0.17^{bc}	2.83 ± 0.23^{bc}	3.66 ± 0.29^{bc}
SS + *Brassica juncea* + *Serratia marcescens* + Compost	**T6**	7.86 ± 0.35^{a}	283 ± 16^{j}	188 ± 13^{i}	6.0 ± 0.4^{d}	92.8 ± 21.1^{de}	1.99 ± 0.21^{bcd}	2.66 ± 0.29^{bcd}	3.43 ± 0.37^{bcd}
SS + *Brassica juncea* + *Bacillus cereus*	**T7**	8.03 ± 0.25^{a}	185 ± 13^{k}	124 ± 13^{j}	11.5 ± 0.3^{a}	205 ± 21.1^{a}	2.53 ± 0.12^{a}	3.38 ± 0.15^{a}	4.36 ± 0.20^{a}
SS + *Brassica juncea* + *Bacillus cereus* + Compost	**T8**	7.89 ± 0.20^{a}	199 ± 17^{k}	127 ± 13^{j}	9.9 ± 0.3^{b}	133.1 ± 12.3^{bc}	2.26 ± 0.23^{ab}	3.01 ± 0.31^{b}	3.89 ± 0.40^{ab}
SS + *Brassica juncea* + *Serratia marcescens* + *Bacillus cereus*	**T9**	7.92 ± 0.27^{a}	190 ± 12^{k}	125 ± 11^{j}	10.1 ± 0.3^{b}	134.9 ± 21.1^{b}	2.34 ± 0.18^{ab}	3.13 ± 0.25^{b}	4.04 ± 0.32^{ab}
SS + *Brassica juncea* + *Serratia marcescens* + *Bacillus cereus* + Compost	**T10**	7.82 ± 0.35^{a}	328 ± 14^{i}	219 ± 17^{h}	5.6 ± 0.4^{de}	85.7 ± 14.0^{de}	1.88 ± 0.22^{cd}	2.51 ± 0.29^{cd}	3.24 ± 0.37^{cd}

Treatments, C= Control, OOC= Oxidizable Organic Carbon, TOC= Total Organic Carbon, OM= Organic Matter, NO_3 = Nitrates, PO_4 = Extractable Phosphorous, EC= Electrical Conductivity, SS= spiked soil, Data are shown as means (n =3 ± SD). Significantly the highest mean was "a" column wise followed by later alphabets for lower means. Similar small letters in column are non-significant.

3.1.2. Physiological Parameters of *Brassica juncea*

The physiological parameters including shoot length, root length, fresh weight and dry weight of roots and shoots were calculated after 80 days. The treatment T7 (SS + *Brassica juncea* + *Bacillus cereus*) exhibits the highest shoot length (54.67 cm), shoot fresh weight (13.53 g), and shoot dry weight (0.62 g). It suggests that the combination of *Brassica juncea* and the bacterial strain *Bacillus cereus* has a positive impact on shoot growth and development. This combination promotes the elongation and biomass of shoots.

Treatment T7 also showed the highest values for root length (22.7 cm), root fresh weight (1.89 g), and root dry weight (1.33 g). It implies that the inclusion of both *Brassica juncea* and *Bacillus cereus* enhanced root growth and biomass production. The presence of these components in the soil likely promotes root elongation and increases nutrient uptake and overall root system development.

The lowest values for plant growth parameters in both roots and shoots are observed in T1 and T2 where no additional treatment is applied (Table 3.2).

Table 3.2: Impact of different treatments on the physiological parameters of *Brassica juncea*.

Description	Treatments	Shoots			Roots		
		Length (cm)	Fresh wt. (g)	Dry wt. (g)	Length (cm)	Fresh wt. (g)	Dry wt. (g)
Unsterilized Soil+ *Brassica juncea*	T1	35.67 ± 3.51 ^{d}	3.70 ± 0.46 ^{e}	0.24 ± 0.05 ^{d}	7.0 ± 1.2 ^{de}	0.38 ± 0.18 ^{d}	0.14 ± 0.01 ^{b}
Sterilized Soil + *Brassica juncea*	T2	19.67 ± 4.04 ^{e}	3.60 ± 0.62 ^{e}	0.15 ± 0.03 ^{e}	5.9 ± 1.4 ^{e}	0.37 ± 0.09 ^{d}	0.09 ± 0.02 ^{b}
US + *Brassica juncea* + Compost	T3	44.00 ± 6.24 ^{bcd}	5.30 ± 0.80 ^{d}	0.29 ± 0.06 ^{cd}	8.4 ± 1.6 ^{de}	0.76 ± 0.14 ^{bc}	0.16 ± 0.03 ^{b}
SS + *Brassica juncea* + Compost	T4	40.00 ± 3.00 ^{cd}	5.23 ± 0.85 ^{d}	0.28 ± 0.03 ^{d}	7.9 ± 1.9 ^{de}	0.73 ± 0.08 ^{c}	0.15 ± 0.03 ^{b}
SS + *Brassica juncea* + *Serratia marcescens*	T5	48.33 ± 4.51 ^{abc}	9.77 ± 1.34 ^{b}	0.38 ± 0.04 ^{b}	12.0 ± 5.5 ^{bcd}	0.84 ± 0.11 ^{bc}	0.20 ± 0.04 ^{b}
SS + *Brassica juncea* + *Serratia marcescens* + Compost	T6	46.17 ± 6.29 ^{abc}	7.13 ± 0.97 ^{c}	0.37 ± 0.06 ^{bc}	11.0 ± 2.7 ^{bcde}	0.84 ± 0.11 ^{bc}	0.19 ± 0.03 ^{b}
SS + *Brassica juncea* + *Bacillus cereus*	T7	54.67 ± 7.09 ^{a}	13.53 ± 0.65 ^{a}	0.62 ± 0.07 ^{a}	22.7 ± 3.6 ^{a}	1.89 ± 0.51 ^{a}	1.33 ± 0.55 ^{a}
SS + *Brassica juncea* + *Bacillus cereus* + Compost	T8	50.67 ± 3.06 ^{ab}	12.37 ± 1.11 ^{a}	0.39 ± 0.05 ^{b}	14.4 ± 3.4 ^{bc}	1.01 ± 0.12 ^{bc}	0.22 ± 0.03 ^{b}
SS + *Brassica juncea* + *Serratia marcescens* + *Bacillus cereus*	T9	51.67 ± 4.04 ^{ab}	12.53 ± 0.75 ^{a}	0.56 ± 0.06 ^{a}	15.0 ± 4.3 ^{b}	1.13 ± 0.10 ^{b}	0.35 ± 0.04 ^{b}
SS + *Brassica juncea* + *Serratia marcescens* + *Bacillus cereus* + Compost	T10	44.67 ± 3.51 ^{bc}	5.73 ± 0.80 ^{cd}	0.32 ± 0.04 ^{bcd}	9.1 ± 2.8 ^{cde}	0.76 ± 0.14 ^{bc}	0.18 ± 0.01 ^{b}

Treatments, C= Control, FS + P= Fresh soil + Plant, AC= Abiotic control, P= Phytoremediation, P+B= Phytoremediation + Bioaugmentation, SS= spiked soil, the data are shown as means (n =3 ± SD). Significantly the highest mean was "a" column wise followed by later alphabets for lower means. Similar small letters in column are non-significant.

3.1.3. Chlorophyll A, Chlorophyll B, Total Chlorophyll, and Carotenoid Content

Chlorophyll a, chlorophyll b, total chlorophyll and carotenoids were assessed for each treatment after plant harvesting. The Chla, Chlb and total chlorophyll showed significant variation. Total Chlorophyll content as well as carotenoids content was highest for T7 (0.24 mg/g plant FW, and 28.77 mg/g plant FW) and lowest for T2 (0.058 mg/g plant FW, and 3.09 mg/g plant FW), T3 (0.052 mg/g plant FW, and 4.61 mg/g plant FW) and T4 (0.052 mg/g plant FW, and 4.68 mg/g plant FW) which is in accordance with the physiological parameters results discussed in Table 7 where T7 treatment showed high values for above and below ground biomass while the values of above and below ground biomass (shoot parameters) were not significantly high in T2. This explains poor growth has led to lower chlorophyll and carotenoids content. The Chlorophyll a, chlorophyll b, total chlorophyll content is presented in the figure 3.1 while carotenoid content is represented in figure 3.2.

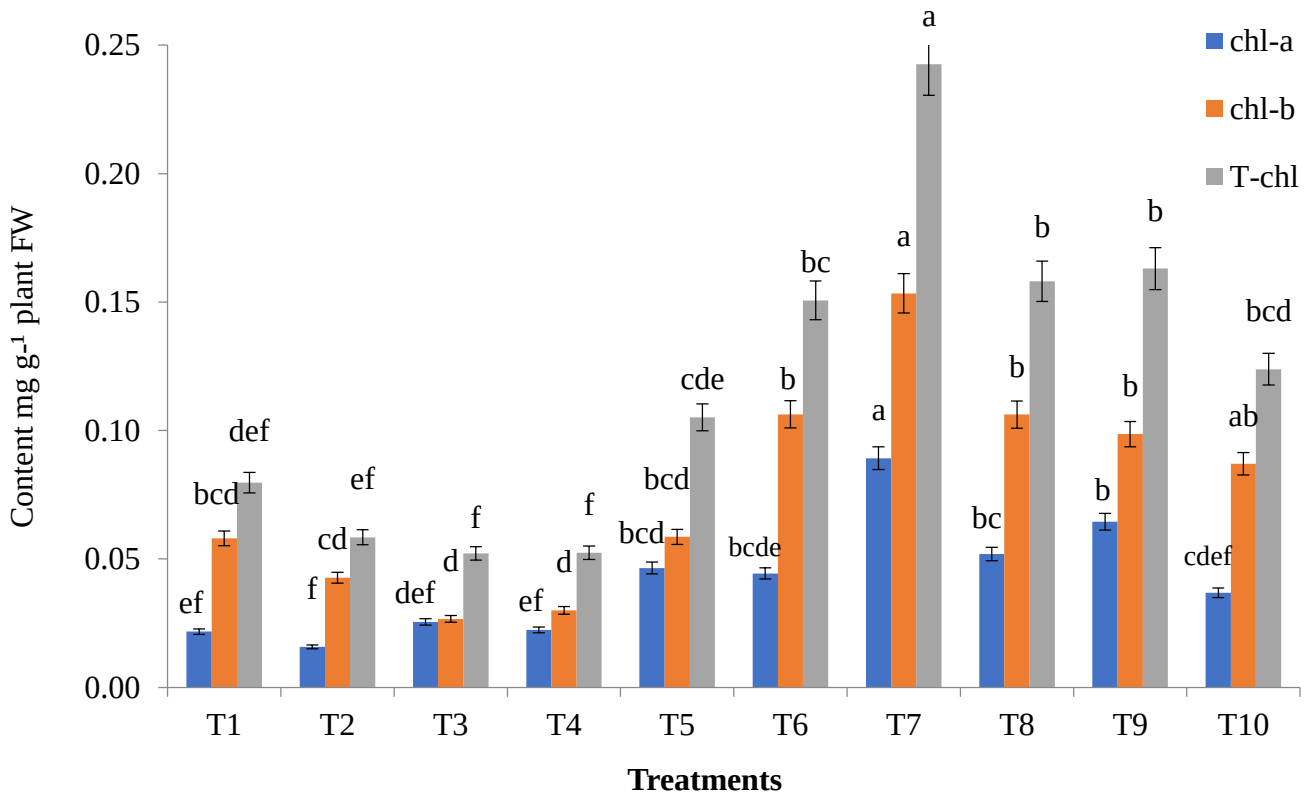

Figure 3.1: Chlorophyll a, Chlorophyll b and total Chlorophyll in plants.

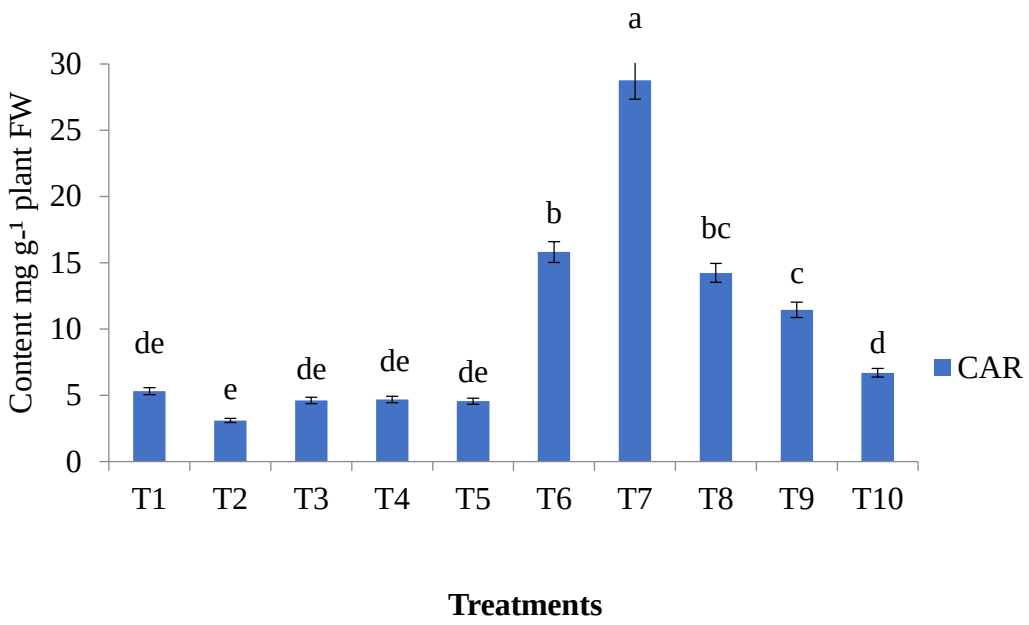

Figure 3.2: Carotenoid levels in different treatments.

3.1.4. Effects on enzymatic activities of *Brassica juncea*

Enzyme activities such as APX, GPX, MDA, H_2O_2, and CAT in *Brassica juncea* on exposure to the heavy metal stress are presented in figure 3.3, 3.4, 3.5, 3.6 and 3.7, respectively. The results showed that, treatments T2 with *Brassica juncea* in sterilized soil exhibit higher values for antioxidant enzyme activity (APX and GPX), indicating higher heavy metal stress. However, they also show higher levels of lipid peroxidation (MDA) and the presence of reactive oxygen species (H_2O_2). It also suggests that high level of heavy metal stress contributed to higher stress and so higher antioxidants. Catalase is also highest for T2 and lowest for T7 (SS+ *Brassica juncea* + *Bacillus cereus)* as represented in figure 3.7, which indicates inclusion of bacteria has significantly reduced the number of antioxidants and stress.

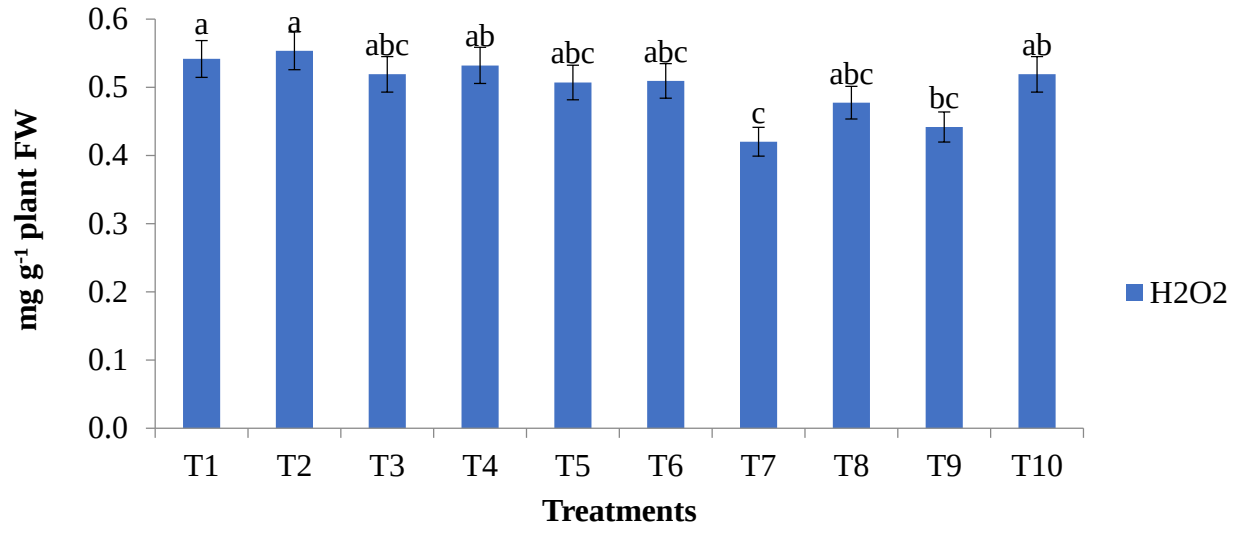

Figure 3.3: Stress injury due to HMs exposure to Brassica *juncea*.

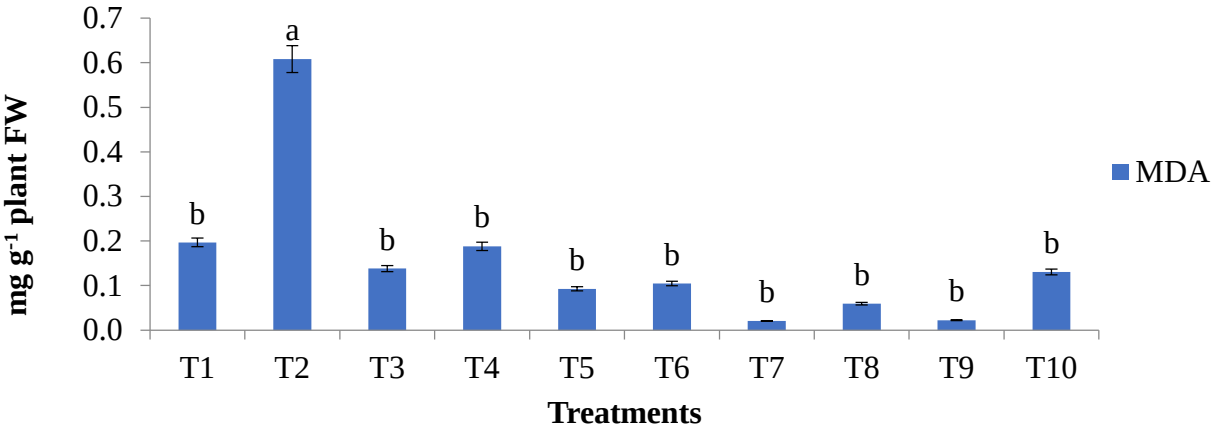

Figure 3.4: Stress injury due to HMs exposure to Brassica *juncea*.

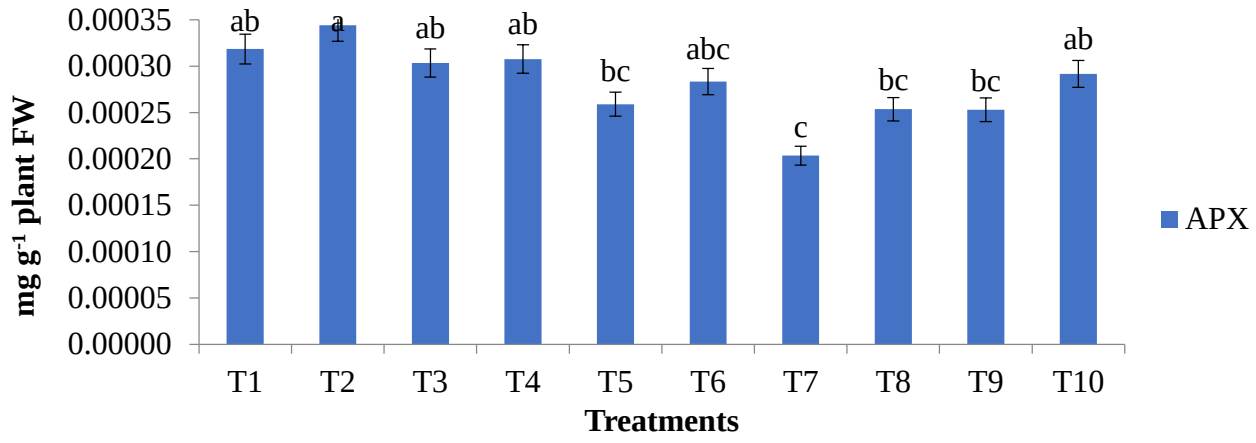

Figure 3.5: The enzymatic profile (APX) of *Brassica juncea* with different treatments.

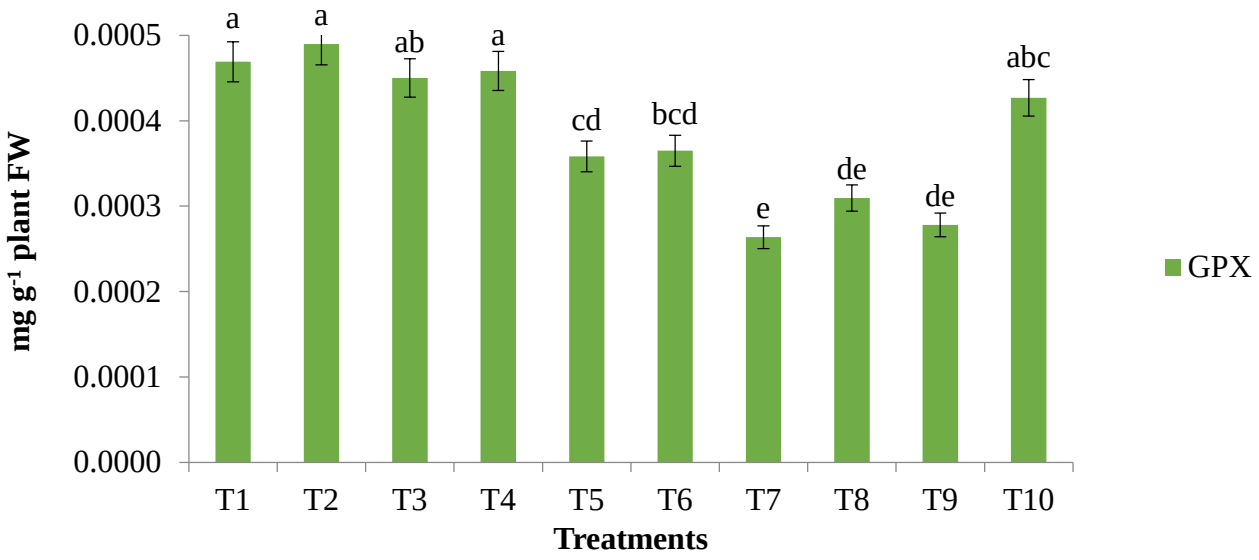

Figure 3.6: The enzymatic profile (GPX) of Brassica *juncea* with different treatments.

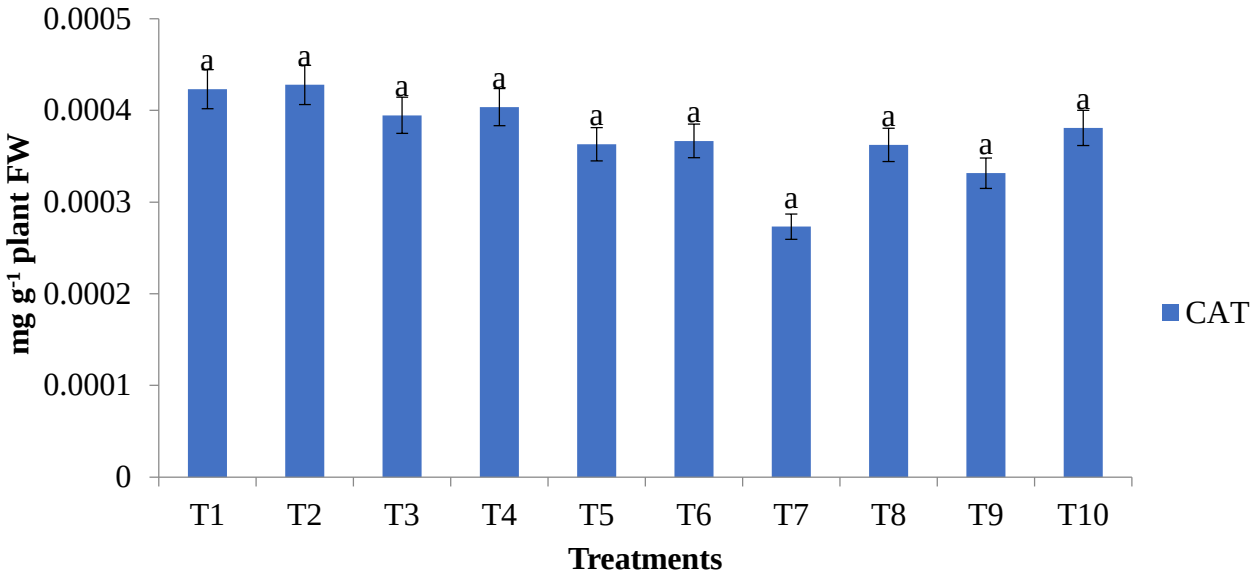

Figure 3.7: The enzymatic profile (CAT) of Brassica *juncea* with different treatments.

3.1.5. Cadmium content in soil, roots, and shoots

The concentrations of Cadmium metal in soil after harvesting were quantified. All the treatments showed significant differences in cadmium contents in soil. The results showed that inoculation of bacterial strains enhanced Cd uptake by plant.

Maximum concentration of Cd in soil (98 mg/kg) was noted in Abiotic Control having unsterilized soil (C1) treatment followed by C2, C3, C4. The minimum concentration of Cd in soil (42.6 mg/kg) was observed in T8 treatment where bacterial strain (*Bacillus cereus)* as well as compost were applied in combination with *Brassica juncea* (Fig. 3.8).

On the other hand, maximum Cd in roots (50.3 mg/kg) and shoots (3.8 mg/kg) was present in T8 where compost and bacteria were applied. The lowest level of Cd in soil of T8 suggests that the bacterial inoculation facilitated the uptake of cadmium from soil into roots and then to shoots (Fig. 3.9).

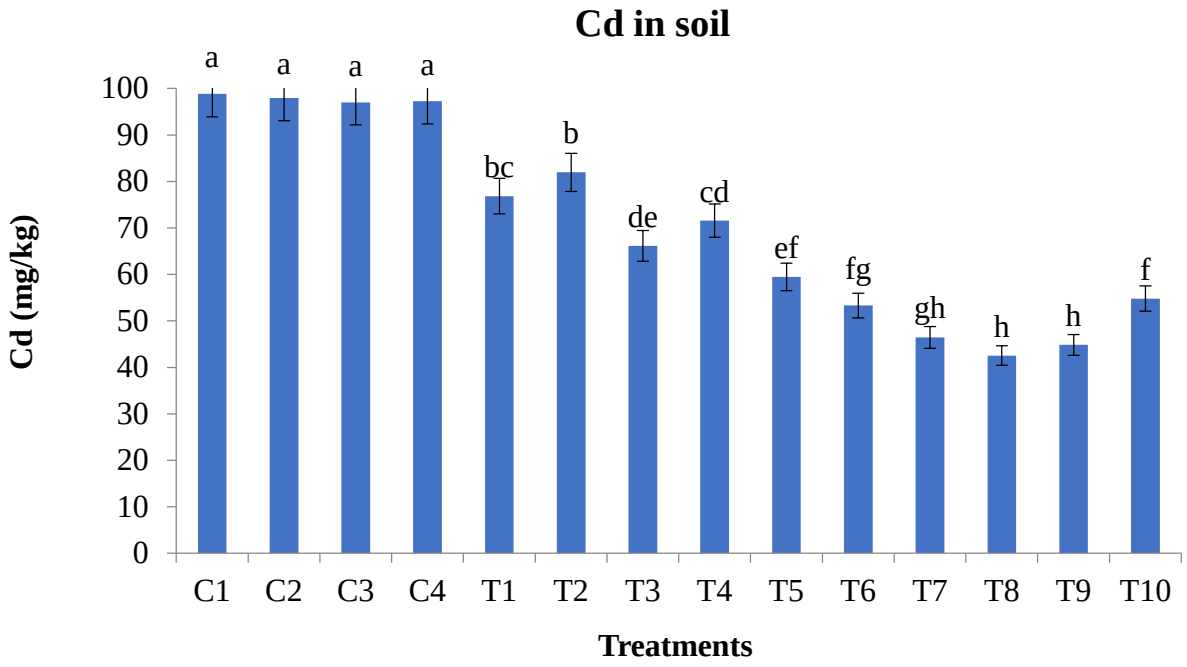

Figure 3.8: Cadmium content in soil after harvesting of plants.

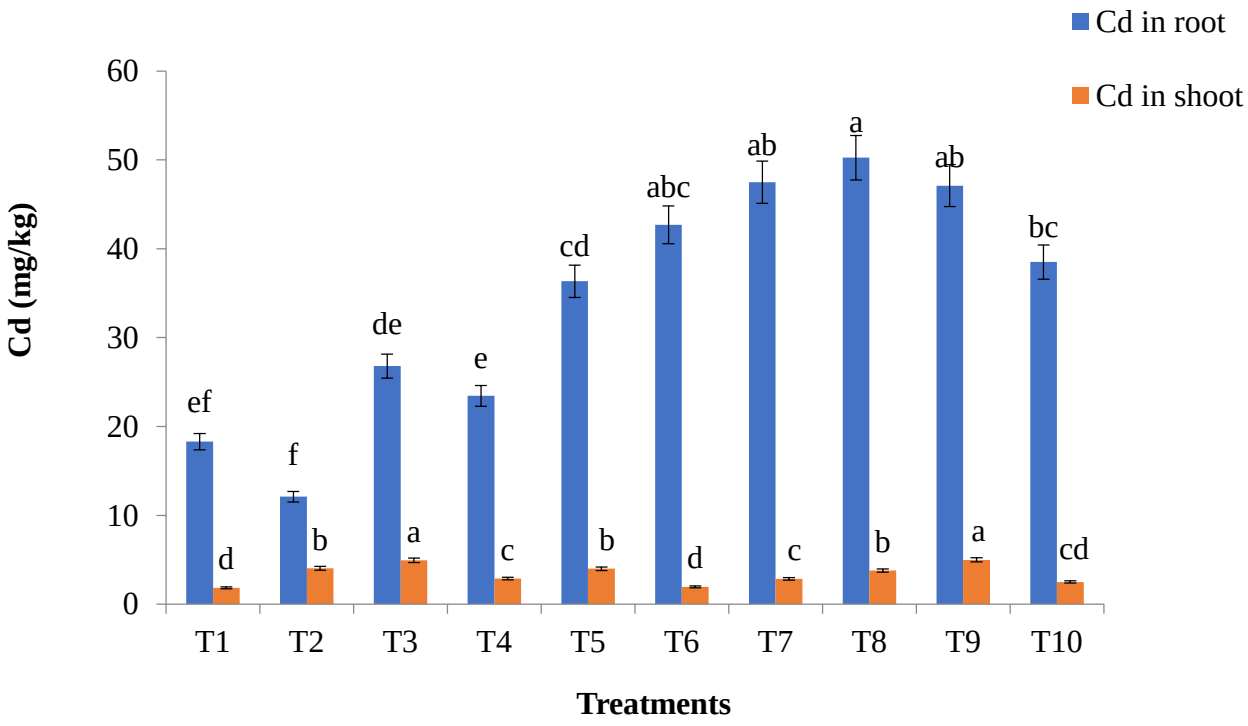

Figure 3.9: Cadmium content in roots and shoots after harvesting of plants.

3.1.6. Copper content in soil, roots, and shoots

Copper showed maximum value (296.5 mg/kg) in unsterilized soil (C1) control followed by C3 (292 mg/kg), C2 (290 mg/kg), and C4 (284 mg/kg). Lowest copper 36.9 mg/kg) was observed in soil of T7 (SS+ Brassica + *Bacillus cereus*) (Fig. 3.10). However, in shoots and roots the copper concentration was highest in T7 (57.5 mg/kg and 202.6 mg/kg) which explains the lowest in soil in T7 as much of the copper has been taken up by roots and shoots of the plant in T7 treatment (Fig. 3.11). Moreover, the lowest copper concentration in roots and shoots is observed in T2 (111.3 mg/kg and 18.6 mg/kg) with sterilized soil and *Brassica juncea*.

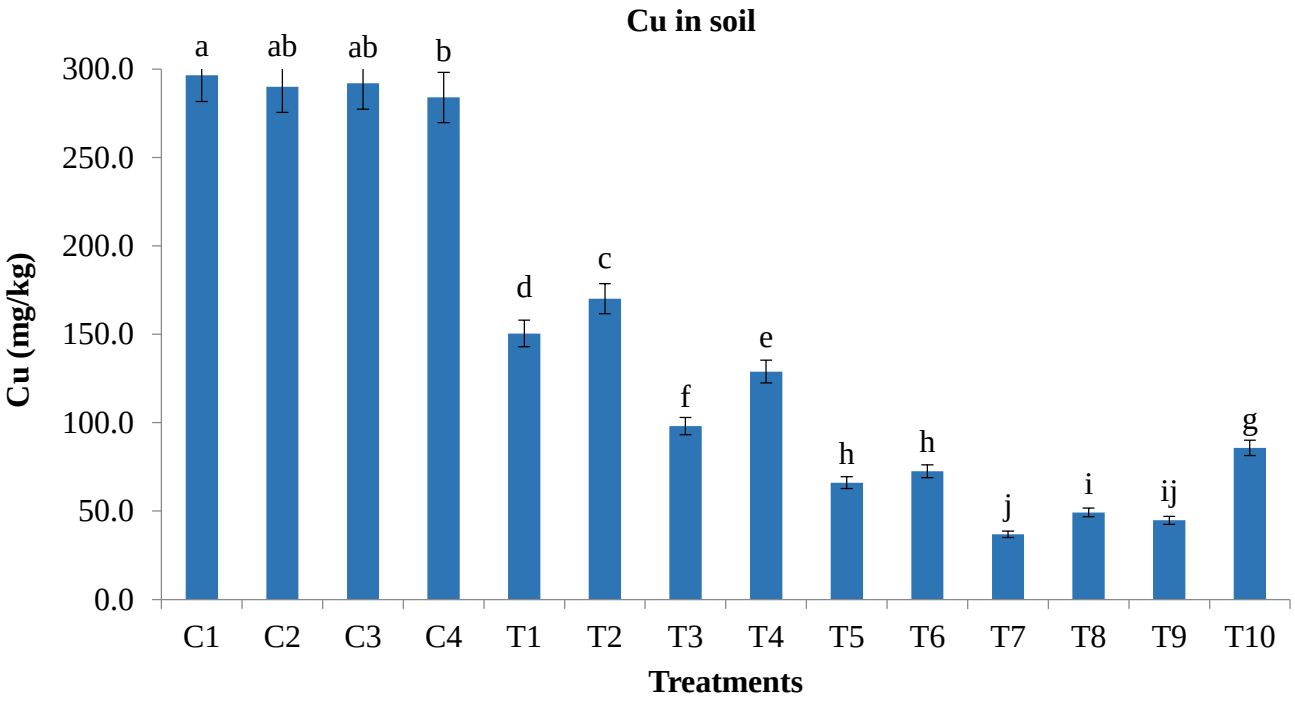

Figure3.10: Copper content in soil after harvesting of plants.

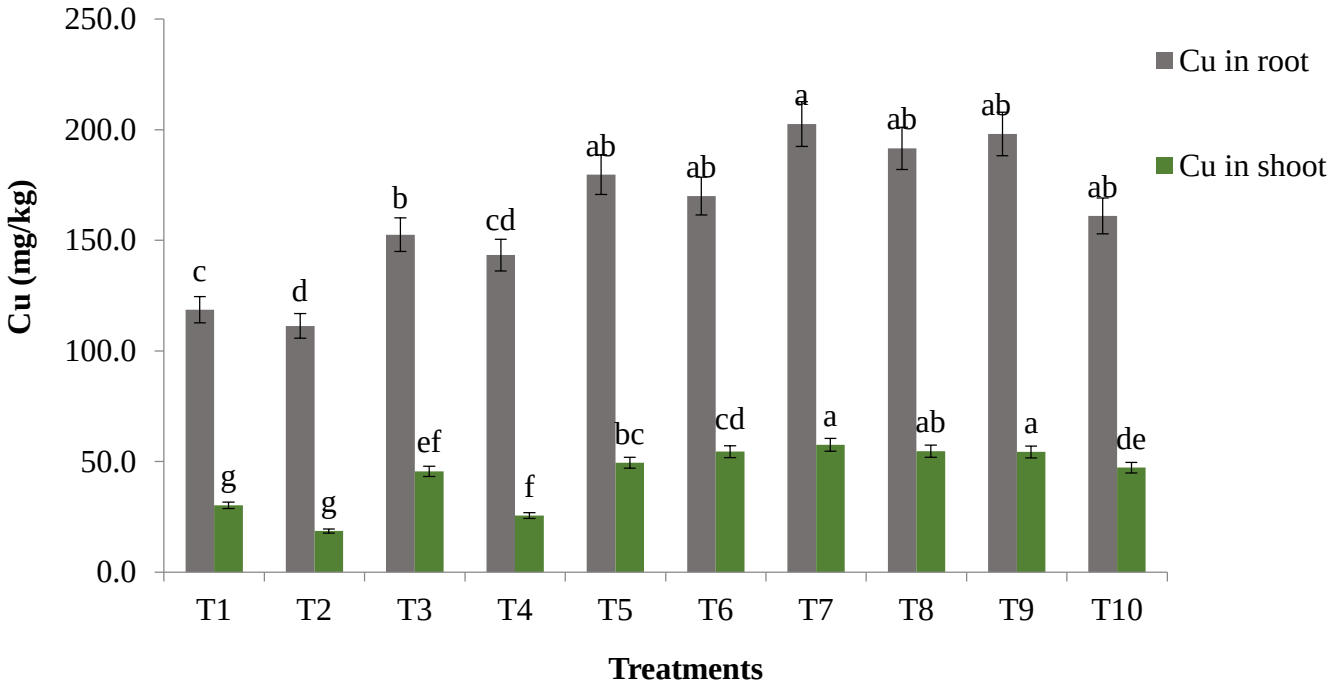

Figure 3.11: Copper content in roots and shoots after harvesting of plants.

3.1.7. Accumulation coefficient and Translocation factor for Cadmium and Copper Metals

A high translocation factor indicates that the plant species in that treatment has a greater ability to transport metal from the roots to the shoots. It suggests that a significant proportion of the metal taken up by the roots is being translocated and accumulated in the above-ground parts of the plant. In other words, the plant is more efficient in moving metal from the roots to the shoots.

Conversely, a low translocation factor suggests that the plant species in that treatment has a relatively lower ability to transport metal from the roots to the shoots. It implies that a smaller proportion of the metal taken up by the roots is being translocated and accumulated in the aboveground parts of the plant. In this case, the plant is less efficient in moving metal from the roots to the shoots.

Therefore, in case of cadmium metal, treatment 2 exhibited the highest translocation factor, indicating a higher degree of Cadmium transportation from the roots to the

shoots, while treatment 6 showed the lowest translocation factor, indicating a lower degree of Cadmium transportation from the roots to the shoots (Table 8).

Table 3.3: The average concentration, the accumulation coefficient, and the translocation factor of cadmium, in the roots and shoots of *Brassica juncea*.

CADMIUM							
	Treatments	**Concentration (mg kg^{1})**			**Accumulation Coefficient (AC)**		**Translocation Element Factor (TF)**
		Roots	**Shoots**	**Soil**	**Root/Soil**	**Shoot/Soil**	**Shoot/Root**
Unsterilized Soil	**C1**	0.00	0.00	98.85	0.00	0.00	0.00
Sterilized Soil	**C2**	0.00	0.00	97.95	0.00	0.00	0.00
US + Compost	**C3**	0.00	0.00	97.00	0.00	0.00	0.00
SS + Compost	**C4**	0.00	0.00	97.25	0.00	0.00	0.00
Unsterilized Soil+ *Brassica juncea*	**T1**	18.30	1.85	76.85	0.24	0.02	0.10
Sterilized Soil + *Brassica juncea*	**T2**	12.10	4.05	81.95	0.15	0.05	0.33
US + *Brassica juncea* + Compost	**T3**	26.80	4.95	66.15	0.41	0.07	0.18
SS + *Brassica juncea* + Compost	**T4**	23.45	2.90	71.60	0.33	0.04	0.12
SS + *Brassica juncea* + *Serratia marcescens*	**T5**	36.35	4.00	59.45	0.61	0.07	0.11
SS + *Brassica juncea* + *Serratia marcescens* + Compost	**T6**	42.70	1.95	53.30	0.80	0.04	0.05
SS + *Brassica juncea* + *Bacillus cereus*	**T7**	47.50	2.85	46.45	1.02	0.06	0.06
SS + *Brassica juncea* + *Bacillus cereus* + Compost	**T8**	50.25	3.80	42.55	1.18	0.09	0.08
SS + *Brassica juncea* + *Serratia marcescens* + *Bacillus cereus*	**T9**	47.10	5.00	44.85	1.05	0.11	0.11
SS + *Brassica juncea* + *Serratia marcescens* + *Bacillus cereus* + Compost	**T10**	38.50	2.50	54.80	0.70	0.05	0.06

The AC shows the accumulation coefficient that was calculated to assess how differently Cu and Cd are absorbed by *Brassica juncea*. The data in the tables 3.3 and 3.4 represents, that the accumulation of cadmium, and copper is generally higher in the roots compared to the shoots. The concentration values for both metals in the roots are consistently higher than the concentration values in the shoots across the different treatments in *Brassica juncea*.

Table 3.4 represents the average concentration of copper in the roots and shoots of *Brassica juncea* as well as the accumulation coefficient and the translocation factor. In case of copper, Treatment 7 has the highest accumulation coefficient of 5.49660787 while Treatment 2 has the lowest accumulation coefficient of 0.109053498. On the other hand, Treatment 6 has the highest translocation factor of 0.320294118 (Table 3.4).

The differences observed in the accumulation coefficient and translocation factor among the treatments suggest variations in the plants' abilities to take up and distribute metals, possibly due to different genetic characteristics, physiological responses, or environmental conditions.

Table 3.4: The average concentration, the accumulation coefficient and the translocation factor of copper, in the roots and shoots of *Brassica juncea*.

COPPER							
	Treatments	**Concentration (mg kg^{1})**			**Accumulation Coefficient (AC)**		**Translocation Element Factor (TF)**
		Roots	**Shoots**	**Soil**	**Root/Soil**	**Shoot/Soil**	**Shoot/Root**
Unsterilized Soil	**C1**	0.00	0.00	296.50	0.00	0.00	0.00
Sterilized Soil	**C2**	0.00	0.00	290.00	0.00	0.00	0.00
US + Compost	**C3**	0.00	0.00	292.00	0.00	0.00	0.00
SS + Compost	**C4**	0.00	0.00	284.00	0.00	0.00	0.00
Unsterilized Soil+ *Brassica juncea*	**T1**	118.55	30.20	150.50	0.79	0.20	0.25
Sterilized Soil + *Brassica juncea*	**T2**	111.30	18.55	170.10	0.65	0.11	0.17
US + *Brassica juncea* + Compost	**T3**	152.50	45.55	98.15	1.55	0.46	0.30
SS + *Brassica juncea* + Compost	**T4**	143.30	25.55	128.90	1.11	0.20	0.18
SS + *Brassica juncea* + *Serratia marcescens*	**T5**	179.65	49.45	66.10	2.72	0.75	0.28
SS + *Brassica juncea* + *Serratia marcescens* + Compost	**T6**	170.00	54.45	72.60	2.34	0.75	0.32
SS + *Brassica juncea* + *Bacillus cereus*	**T7**	202.55	57.50	36.85	5.50	1.56	0.28
SS + *Brassica juncea* + *Bacillus cereus* + Compost	**T8**	191.50	54.60	49.25	3.89	1.11	0.29
SS + *Brassica juncea* + *Serratia marcescens* + *Bacillus cereus*	**T9**	198.05	54.30	44.75	4.43	1.21	0.27
SS + *Brassica juncea* + *Serratia marcescens* + *Bacillus cereus* + Compost	**T10**	161.00	47.20	85.75	1.88	0.55	0.29

3.1.8. Soil Bacterial Count

Two different heavy metal resistant bacterial strains *Serratia marcescens* and *Bacillus cereus were* used for inoculation in this experiment. Table 3.5 shows the results of bacterial colonies survived in each treatment. The treatment T7 (SS + *Brassica juncea* + *Serratia marcescens* + *Bacillus cereus* + Compost) showed highest number of microbial colonies in soil (2.60×10^7) followed by T8 (2.99×10^6) and T9 (1.80×10^6). Treatment 10 showed the lowest bacterial count (2.42×10^4) with both bacteria and *Brassica juncea* plant which shows the growth of bacteria reduced due to competition of resources. The findings indicated that plants have a favorable impact on bacterial development in soil.

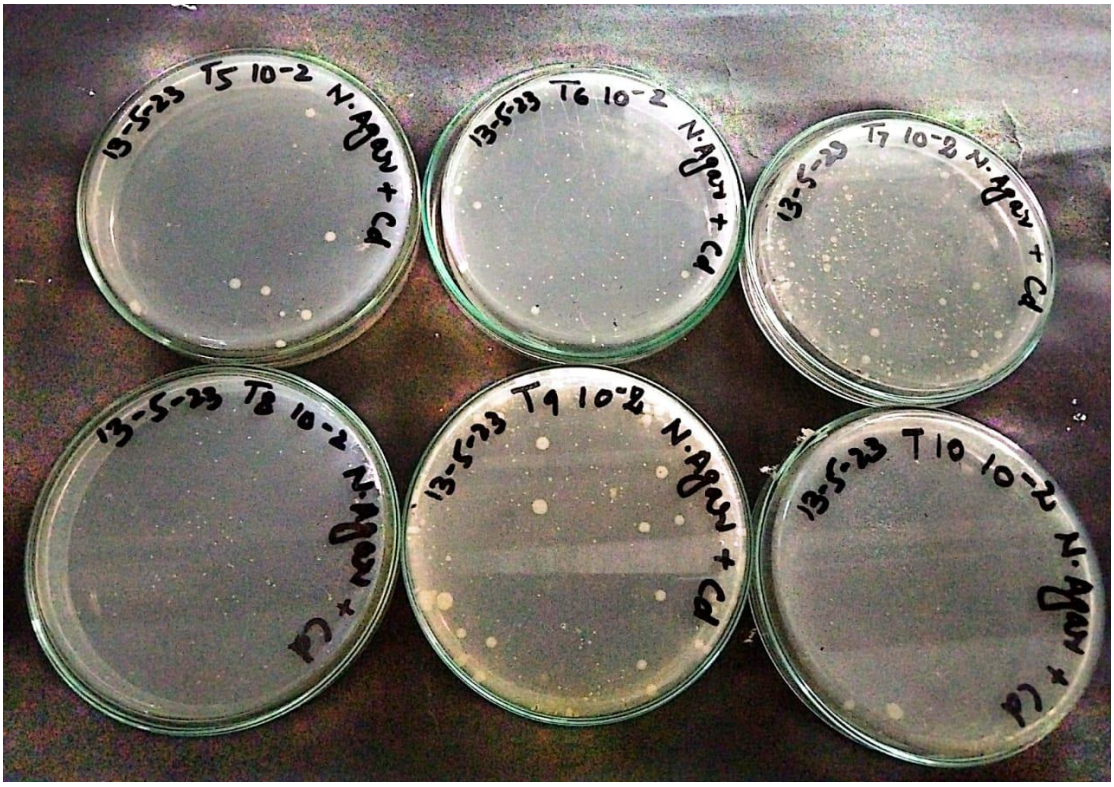

Figure 3.12: Soil Bacteria Count for Different Treatments

Table 3.5: Soil Bacteria Count for Different Treatments

Description	Treatment	CFU (Cells g^{-1} of soil)
C1	Unsterilized Soil	$2.31 \times 10^{2} \pm 1.06 \times 10^{f}$
C3	US + Compost	$1.14 \times 10^{2} \pm 1.25 \times 10^{f}$
T1	US + *Brassica juncea*	$2.55 \times 10^{3} \pm 1.36 \times 10^{2e}$
T3	US + *Brassica juncea* + Compost	$1.89 \times 10^{3} \pm 1.45 \times 10^{2e}$
T5	SS + *Brassica juncea* + *Serratia marcescens*	$4.18 \times 10^{5} \pm 1.32 \times 10^{3c}$
T6	SS + *Brassica juncea* + *Serratia marcescens* + Compost	$2.75 \times 10^{5} \pm 1.20 \times 10^{3c}$
T7	SS + *Brassica juncea* + *Bacillus cereus*	$2.60 \times 10^{7} \pm 1.76 \times 10^{4\,a}$
T8	SS + *Brassica juncea* + *Bacillus cereus* + Compost	$2.99 \times 10^{6} \pm 1.35 \times 10^{4b}$
T9	SS + *Brassica juncea* + *Serratia marcescens* + *Bacillus cereus*	$1.80 \times 10^{6} \pm 2.70 \times 10^{3b}$
T10	SS + *Brassica juncea* + *Serratia marcescens* + *Bacillus cereus* + Compost	$2.42 \times 10^{4} \pm 1.16 \times 10^{2d}$

Chapter 4

4. Discussion

The direction of first experiment in current research was towards the use of integrated methods for heavy metal remediation. This experimental study used *Brassica juncea* to test the efficacy of bioaugmentation and phytoremediation, two independent remediation approaches used to treat industrial soil contaminated with cadmium and copper Along with phytoextraction, the effectiveness of bioaugmentation was also investigated.

In soils polluted with heavy metals, certain plants thrive. High potential exists for the soil to be cleaned up by hyperaccumulator plants (gathered mainly in the root or shoots). Heavy metals are removed from the contaminated soil layer by the plants once they reach the permitted standards level for heavy metals. Using plants with consortia of microbial systems to remove heavy metals is a new technology (Su et al., 2014). In this study, *Brassica jucea* was used with different combinations of *Bacillus cerues* and *Serratia marcescens* for remediation of Cd and Cu with concentrations of 100 mg kg^{-1} and 300 $mgkg^{-1}$ in soil.

Bioaugmentation involves the use of microbial systems, such as specific bacterial strains, to assist in the remediation process. In this study, we used *Bacillus cerues* and *Serratia marcescens*, which are heavy metal-resistant bacterial strains. By adding these microbial strains to the contaminated soil, they aimed to enhance the remediation of Cd and Cu.

Phytoremediation, on the other hand, relies on plants' ability to absorb and accumulate heavy metals from the soil. Certain plants, known as hyperaccumulators, have a higher capacity to accumulate metals. *Brassica juncea* has been found to be effective in hyperaccumulating Cd, and CU from contaminated soils.

In this experiment, a comparison between the bioremediation potential of sterilized soil and unsterilized soil was performed. The effects of soil treatments and plant-microbe interactions on soil physicochemical properties and plant growth has been investigated under several different conditions. In this study, variations in soil pH among different treatments was observed. Similar findings have been reported in

previous research. For example, a study by (Trivedi, Singh et al. 2017) investigated the effects of different soil amendments on soil pH and found that the addition of organic matter and microbial inoculants significantly influenced soil pH levels. This aligns with the current study's observations that the addition of compost and bacterial strains influenced soil pH.

The electrical conductivity (EC) and total dissolved solids (TDS) are indicators of salt content in soil. The study identified differences in EC and TDS values among treatments. This is consistent with previous studies that have shown the influence of soil amendments on salt accumulation. For instance, a study by (Wichern, Islam et al. 2020) investigated the effects of organic amendments on soil salinity and found that the addition of organic materials reduced salt content in the soil. These findings support the current study's observation that compost addition can influence salt content.

The study analyzed nitrate (NO_3) and phosphate (PO_4) concentrations in the soil. Previous research has also highlighted the role of plant-microbe interactions in nutrient availability. For example, a study by (Çakmakçi, Dönmez et al. 2006) investigated the impact of plant growth-promoting bacteria on nutrient availability and found that bacterial inoculation increased the availability of nitrate and phosphate in the soil. This aligns with the current study's findings that the addition of specific bacterial strains influenced nitrate and phosphate concentrations.

The organic carbon content, total organic carbon, and organic matter in the soil were also measured. Previous literature supports the idea that organic matter addition enhances soil organic carbon. For instance, a study by (Whitman, Zhu et al. 2014) investigated the effects of organic amendments on soil organic carbon and found that organic matter addition increased soil organic carbon levels. This supports the current study's observation that the inclusion of compost and *Brassica juncea* enhanced organic carbon content.

In this research, various physiological parameters of *Brassica juncea* were also evaluated. Previous research has extensively documented the positive effects of plant-microbe interactions on plant growth and development. For example, a study by (Wu, Cheung et al. 2006) investigated the growth-promoting effects of rhizobacteria on *Brassica juncea* and reported increased shoot and root growth in inoculated plants. This is in line with the current study's findings that the inclusion of bacterial strains positively influenced shoot and root length, as well as biomass accumulation.

The presented results provide insights into the physicochemical properties and physiological parameters of soil samples treated with various combinations of unsterilized soil, sterilized soil, bacteria (*Serratia marcescens, Bacillus cereus*), and the plant *Brassica juncea*. These findings contribute to understanding the effects of different treatments on soil conditions and the growth and development of *Brassica juncea* plants.

The analysis of physicochemical properties revealed variations among the different treatments. The pH values indicated slightly alkaline soil conditions, ranging from 7.01 to 8.03. Treatment T7 (SS + *Brassica juncea* + *Bacillus cereus)* had the highest pH value (8.03), indicating alkaline conditions, while treatment C4 (Sterilized Soil + *Brassica juncea*) had the lowest pH value (7.01). This suggests that the addition of *Brassica juncea* and specific bacterial strains can influence the soil pH.

The electrical conductivity (EC) and total dissolved solids (TDS) provide insights into the salt content of the soil. Treatment C4 (SS + Compost) exhibited the highest EC value (1916 uS/cm), indicating high salt content, while Treatment T7 (SS + *Brassica juncea* + *Bacillus cereus)* had the lowest EC value (185 uS/cm). Treatment C2 (Sterilized Soil) had the highest TDS value (858 mg/l), while Treatment T7 (SS + *Brassica juncea* + *Bacillus cereus)* had the lowest TDS value (124 mg/l). These results suggest that sterilization of the soil and the addition of bacterial strains can influence the salt content of the soil.

The concentrations of nitrate (NO3) and phosphate (PO4) were measured to assess the nutrient content of the soil. Treatment T7 (SS + *Brassica juncea* + *Bacillus cereus*) showed the highest nitrate concentration (11.5 mg/kg), while Control C3 (US + Compost) had the lowest (4.1 mg/kg). Treatment T7 (SS + *Brassica juncea* + *Bacillus cereus*) also exhibited the highest phosphate concentration (205 mg/kg), while Control C3 and C4 (US + Compost and SS + Compost) had the lowest (15.6 mg/kg). These findings indicate that different treatments can affect the availability of nitrate and phosphate in the soil.

The organic carbon content (OOC), total organic carbon (TOC), and organic matter (OM) provide information about the organic composition of the soil. Treatment T7 (SS + *Brassica juncea* + *Bacillus cereus)* had the highest values for OOC, TOC, and OM (2.53, 3.38 and 4.36 %), while Control C2 (Sterilized Soil) had the lowest values (0.56, 0.75 and 0.97%). This suggests that the inclusion of *Brassica juncea*, *Bacillus cereus,* and compost can enhance the organic carbon content of the soil.

Moreover, the physiological parameters of *Brassica juncea,* including shoot length, root length, shoot fresh weight, shoot dry weight, root fresh weight, and root dry weight, were evaluated to assess the growth and development of the plant under different treatments.

Treatment T7 (SS + *Brassica juncea* + *Bacillus cereus)* exhibited the highest values for shoot length, shoot fresh weight, shoot dry weight, root length, root fresh weight, and root dry weight. These results indicate that the combination of *Brassica juncea* and *Bacillus cereus has* a positive impact on shoot and root growth and biomass accumulation. The presence of these components in the soil likely promotes elongation, nutrient uptake, and overall development of both the shoot and root systems. However, it was observed that the treatments having compost amendments showed relatively low growth as compared to the corresponding treatments having no compost.

The results of the study indicate significant variations in chlorophyll and carotenoid content among the different treatments. Treatment T7, which involved the application of SS+ *Brassica juncea* + *Bacillus cereus,* exhibited the highest levels of total chlorophyll and carotenoids, while treatments T2, T3, and T4 showed the lowest levels. This correlation between plant growth parameters and chlorophyll/carotenoid content suggests that poor growth resulted in lower chlorophyll and carotenoid production.

The enzymatic activities of *Brassica juncea,* including APX, GPX, MDA, H_2O_2, and CAT, were also assessed. Treatment T2, which involved *Brassica juncea* with stressed soil, generally exhibited higher antioxidant enzyme activity (APX and GPX), indicating better antioxidative defense systems. However, T2 also showed higher levels of lipid peroxidation (MDA) and reactive oxygen species (H_2O_2), suggesting increased stress. The inclusion of bacteria in T7 (SS+ *Brassica juncea* + *Bacillus cereus*) resulted in reduced levels of antioxidants and stress, as indicated by lower CAT activity.

The concentrations of cadmium and copper in soil, roots, and shoots were measured after harvesting. The highest cadmium content in soil was observed in treatments involving unsterilized soil, stressed soil, and unsterilized soil + compost. However, in roots and shoots, the highest cadmium concentrations were found in treatments involving the inclusion of bacterial strains (*Bacillus cereus)* and compost. This

suggests that the inoculation of bacterial strains enhanced the uptake of cadmium by the plant, leading to higher concentrations in the roots and shoots.

Similarly, the highest copper content in soil was observed in unsterilized soil treatments, while the highest concentrations in roots and shoots were found in treatments involving the inclusion of *Brassica juncea* and *Bacillus cereus.* This indicates that the bacterial and compost treatments increased the uptake of copper from the soil into the roots and shoots of the plant.

The accumulation coefficient (AC) and translocation factor (TF) were calculated to assess the plants' ability to absorb and transport metals. Treatment 2 exhibited the highest translocation factor for cadmium, indicating a greater ability to transport cadmium from the roots to the shoots. Treatment 6 showed the lowest translocation factor, suggesting a lower degree of cadmium transportation. In the case of copper, treatment 7 had the highest accumulation coefficient, indicating a higher accumulation of copper in the plant, while treatment 2 had the lowest accumulation coefficient. Treatment 6 exhibited the highest translocation factor for copper.

Overall, the study demonstrates that different treatments, including the use of bacterial strains and compost, affect the growth, chlorophyll and carotenoid content, enzymatic activities, and metal accumulation in *Brassica juncea*. The results suggest that the inclusion of bacterial strains and compost can enhance plant growth and improve antioxidative defense systems, but they can also lead to increased metal uptake. The findings provide insights into the physiological responses and metal accumulation patterns in *Brassica juncea* under different treatments, which can contribute to the development of strategies for phytoremediation and metal detoxification.

Furthermore, the treatments with the maximum bacterial count (CFU - Colony Forming Units per gram of soil) are T7 (SS + *Brassica juncea* + *Bacillus cereus)* with a count of $2.60 \times 10^7$and T8 (SS + *Brassica juncea* + *Bacillus cereus* + Compost) with a count of 2.99×10^6. On the other hand, the treatment with the lowest bacterial count is T10 (SS + *Brassica juncea* + *Serratia marcescens* + *Bacillus cereus* + Compost*)* with a count of 2.42×10^4. The variation in bacterial counts among these treatments can be attributed to several factors, including the specific bacterial strains used, the presence of compost, and the interaction between the bacteria and the plant (*Brassica juncea*).

In the treatments with higher bacterial counts (T7 and T8), the separate application of bacterial strains (*Serratia marcescens* and *Bacillus cereus*) seems to have promoted bacterial growth and colonization in the soil.

In contrast, the treatment with the lowest bacterial count (T10) includes the combination of *Serratia marcescens* and *Bacillus cereus with* the addition of compost. The presence of compost could have bound the nutrients necessary for bacterial growth, resulting in a lower bacterial count compared to the other treatments.

Finally, the association of the current study's findings to existing literature helped establish a broader understanding of the effects of soil treatments and plant-microbe interactions on soil properties and plant growth, providing a foundation for future research and practical applications in agriculture and soil management.

Conclusions

Three key components of the global environment—soil, water, and air—are necessary for life to survive. However, contamination is constantly harming these environmental aspects; for example, a rise in heavy metal pollution in soil has become problematic to the environment and food security. To address these issues, a comparative study on the impact of bioaugmentation and phytoextraction alone, and in combination has been conducted, using unsterilized and sterilized soil contaminated with Cd and Cu. To conclude, *Brassica juncea* grown in a sterilized soil inoculated with bacterial strains not only enhanced the growth of roots and shoots of plant, but also performed better bioremediation of cadmium and copper than unsterilized contaminated soil having indigenous microbial colonies. Additionally, this study reveals that in the comparison of bioremediation capabilities, T7 with sterilized soil, *Brassica juncea* and *Bacillus cereus* exhibited maximum extraction of Cu (87.7%). T7 also showed the maximum values of plant growth parameters of *Brassica juncea*. While (T8) with sterilized soil, *Brassica juncea, Serratia marcescens* and Compost amendments exhibited maximum extraction of Cd (57.4%). Thus overall, Cu uptake was better than Cd. Hence, the application of this combination can enhance the phytoremediation by the extraction of heavy metals and other pollutants along with the plant growth promotion in field.

Future Recommendations

Based on the results of this experimental study, we may draw the conclusion that the development of remediation techniques has increased our understanding of the remediation of toxic heavy metals, which have negative impacts on both human health and our ecology. For the treatment of soil contaminated with heavy metals, the bioaugmentation method with Brassica offers tremendous potential. The processes of signaling between plants and rhizospheric bacteria through root exudates and their unique role have been established. In connection to bacterial inoculation, we have not yet identified the precise genes that cause heavy metal accumulation. To optimize heavy metal accumulation processes for the restoration of polluted sites, a deeper understanding of these aspects in particular plant-microbe interactions is needed.

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