Rehabilitation of Total Petroleum Hydrocarbons and Heavy Metals Co-contaminated Soil by Bioaugmentation, Co-planting and Organic Amendments

By

Hafiz Abdul Malik

Reg No. 02312011010

Department of Environmental Sciences

Faculty of Biological Sciences

Quaid-i-Azam University Islamabad

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Submitted in partial fulfillment of the requirements.

For the degree of Master of Philosophy in Environmental Sciences

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_____

Dr. Sohail Yousef

Department of Environmental Sciences,

Quaid-I-Azam University, Islamabad.

Submitted through:

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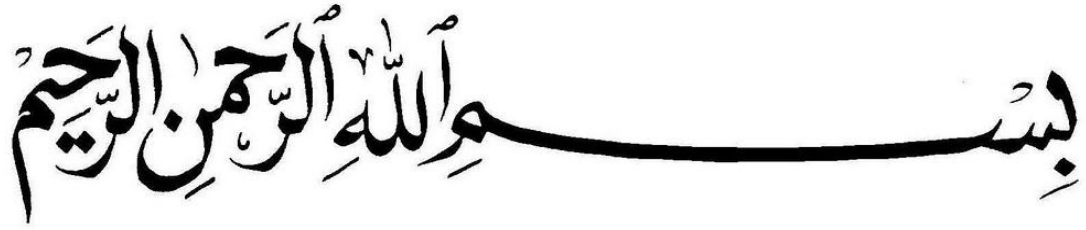
Dr. Sohail Yousaf

Chairman

Department of Environmental Sciences,

Quaid-I-Azam University, Islamabad.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Hafiz Abdul Malik

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List of Abbreviations

AOPs	Advance oxidative processes
APX	Ascorbate peroxidase
As	Arsenic
Car	Carotenoids
CAT	Catalase
Chl a	Chlorophyll a
Chl b	Chlorophyll b
CNS	Central nervous system
DCM	Dichloromethane
DMA	Dimethylarsine
DNA	Deoxyribonucleic acid
EC	Electrical conductivity
EDTA	Ethylenediamine tetra-acetic acid
EM	Electromagnetic
EPA	Environmental protection agency
FW	Fresh weight
GPX	Guaicol peroxidase
MDA	Malondialdehyde
OM	Organic matter
OOC	Oxidizable organic carbon
PAHs	Polyaromatic hydrocarbons
PC	Plant control
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PGPR	Plant growth promoting rhizobacteria

Abstract

Soil is the receptor of all the organic and inorganic pollutants in the environment. Different anthropogenic activities lead to soil contamination with variety of different contaminants which then can be taken up by plants and enter the food chain posing many health hazards. The phenomenon of co-contamination is very common as there are very less chances of presence of single pollutant in contaminated area. Sometimes a bioremediation strategy applied for one pollutant causes the mobility or availability of the pollutants residingnext to it, which again contaminate the matrix. Current bioremediation and phytoremediation technologies are more focused into the method which can deal the multiple pollutants at the same time. In this study, co-contamination of heavy metals and total petroleum hydrocarbons (TPHs) is targeted by co-planting of *Zea mays L.* and *Ricinus Communis* in phase-I and *Lolium perenne* and *Ricinus Communis* in Phase-II, which will degrade TPHs in soil and phytoextract metal in plants. Pot experiment was conducted on co-contaminated soil with TPH, and HMs along selected bacterial strains. Compost was also used as organic amendment in some treatments. Treatments with *Bacillus safensis* strain inoculum and co-plantation and compost showed highest TPHs removal. Cd, uptake was 46.47 mg/kg, Pb 54 mg/kg and Zn 95.77 mg/kg. Highest plant biomass (3.97g) was observed in treatment. Chlorophyll a, b and carotenoids content improved plant health in C.B + M + *Bacillus safensis* + Compost treatments. MDA, H_2O_2, Apx etc. were high in treatment and significant reduction was observed in inoculated treatments. CFU was highest in the treatments with *Bacillus safensis* strain inoculum and co-plantation and compost. Results of current study indicated that phyto assisted remediation by co-plantation, bacterial inoculation and organic amendment not only helps to degrade the TPHs but also aids plant in removal of HMs.

Key words: Co-contamination, heavy metals, co-cropping, phytoremediation, bacteria, compost

Chapter 1
Introduction

Increasing heavy metal pollution is causing serious environmental problems and therefore a highly deserved environmental problem to be addressed. “Heavy metals” the elements that possess a high density value in comparison with water are termed as heavy metals. They may have a density range between 4-6 gram cubic centimeters. By the increased in density of metals they seemed to be more toxic in nature, they includes metalloids also such as arsenic that cause toxicity even at low levels of exposure. After industrial revolution, heavy metals came to expose with the environment in such a manner that was never before. In current scenario the toxicity by heavy metals is causing not only serious health problems but major threats to environment by challenging the natural environment in soil-plant-microbe interaction, disruption in natural physiochemical functioning of living organisms, interfering with food chains, and posing serious threats to animals, plants and humans directly. The situation is going adverse day by day due to expanding use of these toxic metals in the fields of agriculture (as pesticides, herbicides, fungicides) in domestic, industrial use in paints and varnishes, construction and technological industry. There are various sources of heavy metals and reported one are as geogenic, industrial, agricultural, pharmaceutical, domestic effluents, and atmospheric sources. It has been seen that in point source areas such as mines, foundries, refineries and smelters, and other metal-based manufacturing operations, heavy metals are more evident.

Industrial revolution results in pollution level which is above self-cleaning capacity/self-rehabilitation of the environment. “Silent spring”, a book by Rachael Carson in1960s, triggered the environmental movement. It was more than the study and effects of the pesticides. It presented the idea that if humans poisoned nature, nature as revenge would poison humans. Technological innovations were made without diligence to the environment and irrevocably destroy the natural system. Thus, it is the need of time to take an account for our actions and put forward the efforts for the restoration and remediation of the ecosystem.

The main pollution problem faced by developed and developing countries is the disposal of waste from various activities, particularly agricultural practices, such as crop residues, farm animal feed, as well as household and municipal waste. Industries also contribute to

polluting land and soil through the release of toxins and various chemical products that are eventually deposited on the ground, whether in the atmosphere. The increase in population and the thirst for more resources lead to the exploitation of natural resources through rapid urbanization, further industrialization and the conversion of natural land into agricultural fields. These features add more and more contaminants to the environment that will eventually reach the land. This pollution is way more difficult to deal with since contaminants in soil take more longer time to degrade or disappear and as a result a heap of garbage and other solid waste comes into existence and a good amount of land is occupied by the pile of solid waste and can increase in volume day by day and definitely have bad effects on human health as well, and leachate from this waste can seep down the soil, reaching the aquifer.

Soil contamination by different (organic & inorganic) pollutants cause worldwide concerns and intentional or unintentional introduction of these chemicals pose serious hazards to human health and environment. Soil behaves as the final acceptor of organic and inorganic pollutants released into environment. Other than soil, water and air may contain both natural and anthropogenic pollutants with a wide range of compositions and concentrations. Release of solid, liquid, and gaseous waste containing lethal pollutants like heavy metals, hydrocarbons, and/or organic solvents into environmental matrices (water, air and soil) causedetrimental effects to flora, fauna, and environment (Cristaldi et al. 2017).

1.2. Co-contamination of soil

In nature, total single contamination is rare, and combination of organic and inorganic pollutants is commonly found, which have become key environmental and health concern globally. These pollutants can have antagonistic or synergetic effects on each other. Remediation efficiency of co-contaminated soil would change due to mutualistic or antagonistic effects of pollutants on remediation process. Co-occurrence of mixed pollutants can also affect the bioremediation potential of plants and their related microorganisms. For example, degradation of organic pollutants in the process of bioremediation mainly depends upon the microorganism but due to heavy metals present in soil plant growth patterns got inhibited partially or permanently (Montenegro et al. 2017). The co-occurrence of heavy metals and total petroleum hydrocarbons (TPHs) are

frequently found and largely evaluated in different anthropogenic industries like mining and metallurgy industry, manufactured gas plant sites, and even in the sediments of natural water bodies.

Soil contamination with total petroleum hydrocarbons TPHs is a serious environmental problem posing threat to humans and environment thus requires considerable public attention. The recalcitrant nature of these contaminants lead to reduced or slow degradation and their persistence is the key issue (Hussain et al. 2018). There is a dire need to establish efficient, eco-friendly and cost-effective remediation technique for reclamation of soil, water and sediments. Aim of sustainable remediation is to bring the concentration of pollutant to a level well below regulatory toxic limit such that it is no more harmful for people. Various physiochemical methods such as excavation, incineration, landfilling and storage are available for removal of TPHs but associated cost and working complexities are problem thus they are difficult to execute (Gong 2012). They can also result in secondary contamination. Intensive chemical treatments can add to the problems of contamination. They are disruptive to the environment. Biological methods on other hand are preferred as suitable alternative due to their natural and eco-friendly approach. These are low cost and energy involved processes. The most commonly opted method by industries for TPHs treatment is land piling followed by bioremediation sometimes assisted with the plants. Biodegradation is a naturally occurring process. Though this process is sustainable but it takes quite a lot of time as it takes months to years to complete. Another limitation of bioremediation is the possible toxicity of pollutant to the bacterial strain. Biodegradation mostly treats low levels of organic contamination as it is difficult to apply it for remediation of highly contaminated sites (Jorfi et al. 2013).

1.3. Heavy metals

These include metallic elements with comparatively high density, and deleterious health consequences even at low concentration (Duruibe et al. 2007). Heavy metal, refers to the group of metals and metalloids having atomic density more than 4 g cm^{-3}, or having density 5 times higher than water (Laghlimi et al. 2015). According to this definition, 53 out of 92 naturally elements are heavy metals (Javed et al. 2019). Few of these minerals

are biologically significant for plants, and these included Copper (Cu), Manganese (Mn), Iron (Fe), Cobalt (Co), and Zinc (Zn). These heavy metals are acknowledged as micro-nutrients and are essentially needed in small quantities to carry out various biochemical functions in plants, animals, and humans. However other metals have no precise role in normal biological functions, such as mercury (Hg), cadmium (Cd) and lead (Pb), termed as non-essential heavy metals (Javed et al. 2019). Although these metals are found naturally in lithosphere, but the incremented problem has arisen due to the drainage of metal loaded industrial discharges intowater bodies, especially freshwater (Tripathi et al. 2014). Their presence in the environment can also cause soil pollution, deterioration of soil structure and physical and chemical properties, destruction of ecological landscapes and decline in biodiversity (Bello et al. 2018). Amplified concentrations of these heavy metals to xenobiotic levels in the soil pose serious threats to all living organisms. For example, copper high concentration cause harm to the brain, kidneys, and intestinal irritation, while arsenic can lead to cancer (Sarma 2011). Anthropogenic activities are one of the key sources of heavy metals in the environment, and these included mining, smelting, filtering, fertilizer and pesticides production and use, and industrialization (Nagajyoti et al. 2010). Most of these toxic heavy metals (e.g. Cd, Pb, and Hg) or trace metals in excess quantities (e.g. Cu, and Zn) have harmful effects on plants, causing unevenness of nutrient ions, DNA breakdown and abduct formation, inhibition of photosynthesis, and the risk of bioaccumulation in the food chain. This eventually led to serious problems for humans (Nagajyoti et al. 2010). Unlike other organic materials, heavy metals do not degrade through chemical or biological processes, yet they can only change their form (Shafi, 2005). Eliminating heavy metals completely is therefore very difficult compared to other organic pollutants, but they can be tackling via various remediationtechniques.

1.3.2. Toxicity of heavy metals

The heavy metals toxicity depends upon the two basic facts (1) Concentration of heavy metals available to cells; thus, no substance is always toxic in nature. The toxicity depends upon the dose-response data of specific substance and organism effecting from that substance. (2) Some metals and substances are critical to the breakdown of cells at low concentration but highly toxic when taken in high amounts; referred as

micronutrients. Micronutrients are important for some key processes happening in body like biosynthesis, growth, for carbohydrates, proteins, and lipids. Some heavy metals (non-essential) have a stimulating effect when they are applied in minor/low concentrations. (Khoei et al. 2018).

Plant growth is considered as major indicator to study the effect of stressor on growth rate inhibition as plant reaction. Root is the very first plant system which came across to the toxic ions. Other common stress responses of plants include leaf discoloration, reduced stomatal opening, damage to cell structure, and water balance issues. Photosynthesis is a majorparameter to examine heavy metals responses in plants.

Table 1 Heavy metal sources

Heavy Metals	**Sources**	**Reference**
As	Semiconductors, petroleum refining, wood preservatives, animal feed additives, coal power plants, herbicides, volcanoes, mining, and smelting	(Nriagu, 1994; Walsh et al., 1979
Cu	Electroplating industry, smelting, and refining, mining, bio solids	Liu et al., 2005
Cd	Gelogenic sources (Baize, 1997), anthropogenic activities, metal smelting and refining, fossil fuel burning, application of phosphate fertilizers, sewage sludge	Nriagu and Pacyna, 1988, Alloway, 1995; Kabata-Pendias, 2001
Cr	Electroplating industry, sludge, solid waste, tanneries	Knox et al., 1999
Pb	Mining and smelting of metalliferous ores, burning of leaded gasoline, municipal sewage, industrial wastes enriched in Pb, paints	Gisbert et al., 2003; Seaward Richardson, 1990
Hg	Volcano eruptions, forest fire, emissions from industries producing caustic soda, coal, peat and wood burning	Lindqvist, 1991
Se	Coal mining, oil refining, combustion of fossil fuels, glass manufacturing industry, chemical synthesis (e.g., varnish, pigment formulation)	Seaward Richardson, 1990
Ni	Volcanic eruptions, land fill, forest fire, bubble bursting and gas exchange in ocean, weathering of soils and geological materials	Knox et al., 1999
Zn	Electroplating industry, smelting, and refining, mining, bio solids	Liu et al., 2005

1.3.3. Pathways of heavy metals access

To cause any effect in living organisms heavy metals need to come in contact with the living organisms, this might happen in three possible ways/routes.

1. Through atmosphere: Organisms can be exposed to heavy metals by respiration of naturalor anthropogenic emissions. It is estimated that these substances are released in the environment in thousand tons annually, and this number is increasing day by day due to increased industrialization and population. These heavy metals entering organisms can be volatile (e.g. Hg) or particulate in nature. The major health damages caused are liver and kidney diseases, cancer, visual and neurological diseases, negative effects on immune systems, allergies, abortions, and anemia.

2. Through Water: The second pathway for heavy metals to access organisms is through water whether used for drinking purpose or for using that water for cooking or irrigation purposes. Globally about one third people of the world have little or no access to safe drinking water for their daily use i.e., drinking, cooking, sanitation and for personal hygiene which causes different diseases especially in children and infants.

3. Through food: The third pathway of heavy metals to enter humans or living organisms is through the food with high content of heavy metals accumulated. One of the major routesis through plants, as plants grown in soil can be loaded with heavy metals or water irrigated to them could have heavy metals in it and plants can hyperaccumulate them which then pollute food crops and animal forage. Then from plants it can reach through higher tropic levels to humans. The extent of a human access to metal from plant depends upon the metal's form in plant i.e. how heavy metal is bounded to the soil, and the soil phase with which it is bounded and chemical form of heavy metal. Pollutants can be present in soil as particulates, liquids absorbed in soil pores, absorbed ions, adsorbed ions, and liquid films.

1.3.4. Bioavailability and bioaccumulation of heavy metals

The term bioavailability refers to the accessibility of heavy metals, or mobile form of metals which can expose to organisms. If we consider the ecotoxicological definition of bioavailability it means "the amount of chemical present in environment and available for biological activities, for example uptake by an organism or plant".

Metals essential for metabolism fall in three categories: first, the deficiency range, where natural (biological) activities (growth, metabolism) can be enhanced by increasing the dose of metal, second, the buffering range, where biological functions are optimal, and third, the toxicity range, where increase in amount of metal can inhibit the metabolism and even can belethal to humans. The metals concentration needed by organisms depends upon its chemical nature, sensitivity of organism receiving the dose, and nature of the environmental medium concerned i.e. land or water systems.

1.3.5. Bioavailability of metals in soil-plant system

Heavy metal uptake and its bioaccumulation is of great importance because its future impacts the flora and fauna. There are three major classes of plants in terms of metal intake;

1. Excluders: These are the plants which are not sensitive to heavy metals over a high concentration range. They can easily limit the metal translocation in their shoots but have a large amount of metals in their roots. These include the members of grass family for example suntan grass, brome grass and others.

2. Indicators: These are the plants which can accumulate the metals in their above ground parts and metal levels in their shoots and stems etc. reflect the amount of metals in soil. But in case of continuous accumulation of heavy metals these plants can die-off. These plants render the biological and ecological functions in that and can be used as best representatives of pollution. Examples of indicator plants include grains and cereal crops, such as corn, wheat, soybean, oats etc.

3. Accumulators: These are the ones who store the heavy metals in their above ground parts far more than in the soil or in the non-accumulating species growing nearby. These plantshave a great potential to store the metals in their shoots and concentrate them for a longer period of time. Due to this property these plants are widely used in the Phytoremediation. Determination of a plant whether its hyperaccumulator or excluder is done on the basis ofstrict criteria. A plant is considered as hyperaccumulator when (1). Shoot/root quotient > 1, (2). Extraction coefficient > 1, (3). Heavy metals concentration level is 10-50 times higher than in normal plants (Mganga et al. 2011).

1.3.3. Problems associated with heavy metals exposure

Heavy metals exposure cause many problems to flora and fauna as well as environment. As with increasing levels of these pollutants at an alarming rate, there is increased risk of toxicity in animals, plants and human, when consumed above the recommended threshold limit, for acute and chronic exposure (Ashraf et al. 2019). Around 10 million people worldwide suffer from health problems, due to the soil heavy metal contamination, according to a report by the US Environmental Protection Agency (EPA 2016). Many heavy metals can have a direct impact on physiological and biochemical pathways on micro and macro organisms. Heavy metals usually accumulate in the upper layer of soil because of the ability of large organic horizons to bind these molecules (Ashraf et al. 2019), as a result they are easily taken up by plants and thus the adsorption process of essential nutrients (Cu^{2+}, Fe^{2+}, Mn^{2+} and Zn^{2+}) is adversely effected, which may lead to abnormal functioning of the plant, because these nonessential/toxic components occupied the sites for required element and would disrupt the natural mechanism (Khan Anwarzeb et al. 2016)23. Roots is the main entry source of heavy metals from the contaminated site inside the plants and when taken by humans, they will result in various diseases e.g. respiratory disorders, Kidney damage, heart and brain related issues (Ashraf et al. 2019). There are three different mechanisms of heavy metal toxicity in biological molecules by production of ROS, replacing basic metal ions, blockage of functional groups (Küpper and Andresen, 2016). Metal contamination in soil can also lead to change in soil native microbial community, leading to a change in biochemical properties.

1.3.4. Environmental occurrence, industrial production and use

Lead is a bluish-grey metal that occurs naturally in small amounts in the earth's crust. Despite the fact that lead is naturally present in our environment, many anthropogenic activities such as mining, fossil fuel combustion, and various manufacturing operations emit a significant quantity of lead into the environment. This metal has a wide range of agricultural, residential, and industrial uses. Lead is now mostly utilized in metal products, lead-acid batteries, ammunition, and X-ray shielding devices. In 2004, almost 1.52 million metric tons of lead were projected to have been consumed in the United States for various uses. The manufacturing of lead-acid batteries accounted for 83 percent of the total, with the remaining amount covering all other processes and products.

In recent years, there has been a significant decrease in the usage of lead in industry. Its use in ceramic items, pipes, paints, and caulking has decreased substantially. Despite the fact that the reduction is ongoing, it has been reported that 25% of 16.4 million US houses have considerable amounts of lead-contaminated paint that has deteriorated 10. Even if the dwellings are cleansed, lead-contaminated dirt and dust can recontamination them 23, resulting in an increase in lead concentrations in the blood of children who like playing outside 22. Currently, Lead is widely available in the form of dust and chips that can be found on paints and interior surfaces. According to studies, children exposed to decaying lead paint have lead concentrations as high as 20 g/dL or even higher in some circumstances.

1.3.5. Potential for human exposure

Inhalation of lead-containing dust particles or aerosols, as well as ingestion of lead-contaminated food or water, are the main sources of lead exposure. Drinking water containing lead accounts for 35 to 50 percent of lead absorption in adults, and this rate is more than 50 percent in children. Multiple factors, such as physiological status and age, influence lead absorption. In the human body, lead is absorbed mostly through the kidneys, followed by the liver, and finally soft tissues such as the brain and heart. The largest body fraction, however, is represented by lead, which is found in the skeleton. The neurological system is the most vulnerable to lead toxicity. Poor focus, memory loss, headaches, dullness, and irritability are some of the signs of lead toxicity. These signs and symptoms indicate that lead is affecting the body's central nervous system. Multiple initiatives to eliminate lead in fuel, as well as bans on leaded paints, drink and food cans, and plumbing systems, have resulted in a major reduction in lead exposure since the 1970s. Multiple federal programmers aimed at eliminating lead in food cans and plaits, as well as promoting lead poisoning screening programs for young people and children, have been implemented by local health governments and state-level authorities. Although progress has been made in this area, lead poisoning remains a severe health concern in modern times. It is a toxin that has adverse effects on the liver, hematological system, reproductive system, kidneys, CNS, and endocrine system, among other organs.

Lead poisoning is caused by decaying house paints, lead-based items in the workplace, and lead-based ceramic containers, all of which leach into food and drink, as well as lead used in hobbies, cosmetics, and medicines. The National Health and Nutrition Examination Surveys (NHANES) have undertaken a number of studies in which blood lead levels in the US population were examined, as well as the amount of exposure based on race, age, degree of urbanization, income, and gender. These surveys have shown a clear drop in lead in blood since the 1970s, but they have also discovered that a huge proportion of youngsters had elevated levels of lead in their blood (> 10g/dL). As a result, lead poisoning is currently one of the most serious risks to children and other people in the United States and around the world .When it comes to pregnant women, lead exposure is a major concern since lead ingested by the mother is passed on to the developing baby. Human research supports animal findings 38, and has linked prenatal lead exposure to lower birth weight 38, as well as delivery problems and neurological impairments in the newborn.

1.3.6. Molecular mechanisms of toxicity and carcinogenicity

Lead has been shown to have detrimental effects in both children and adults in a number of studies. According to studies, there is a link between children's lower IQ and blood lead poisoning. Lower IQ, reduced neurobehavioral development, speech and language impairments, poor attention span, obsessive behaviors, anti-social behavior, growth retardation, and decreased hearing are all linked to this poisoning. Adults, on the other hand, incur reproductive consequences such as reduced sperm count in men and miscarriages in women. Kidney damage, GI disorders, and brain damage are all caused by acute lead exposure. Chronic exposure has also been shown to have a deleterious impact on the central nervous system, kidneys, blood, vitamin D metabolism, and blood pressure.Biochemical mechanisms, such as lead's capacity to disrupt basic calcium functions and interact with proteins, are a primary mechanism that aids lead in demonstrating its harmful effects. Lead is incorporated into the skeleton in the form of calcium. It's also related to biological molecules, causing their functions to change in a variety of ways. It binds to the sulfhydryl and amide groups of enzymes, causing them to

modify their structure and reduce their activity. Lead competes for binding sites with metallic cations, altering critical cation transport and inhibiting enzyme activity.

Lead poisoning induces cellular damage due to the formation of reactive oxygen species (ROS), according to many studies. Furthermore, according to Jiun and Hseien, the levels of malondialdehyde (MDA) in blood were found to be significantly associated with the concentration of lead in exposed employees' blood. In other studies, antioxidant enzyme activity, such as superoxide dismutase (SOD) and glutathione peroxidase, was found to be considerably higher in the erythrocytes of lead-exposed workers than in non-exposed workers. In a series of recent studies in our lab, induction of cell death and oxidative stress, transcriptional activation of stress genes 2, DNA damage, externalization of phosphatidylserine, and activation of caspase-3 were all discovered to be involved in lead-induced toxicity and apoptosis in human cancer cells.

According to a large number of studies, lead functions by interfering with calcium-dependent mechanisms involved in neuronal signaling and intracellular signal transduction. Intracellular calcium cycling is disrupted by lead, making organelle reserves such as the endoplasmic reticulum and mitochondria less releasable. Calcium-dependent activities in glutamatergic neurons are suppressed by lead, including the calcium-dependent release of many neurotransmitters and receptor-coupled ionospheres. In other situations, lead appears to promote calcium-dependent processes such protein kinase C and calmodulin.

In experimental studies, lead has been proven to induce kidney cancer in rats and mice , and the International Agency for Research on Cancer (IARC) has classed it as a potential human carcinogen . Gene mutations and sister chromatid swaps , as well as morphological abnormalities in cultured rodent cells [203] and enhanced anchorage independence in diploid human fibroblasts , have all been linked to lead exposure. According to in vitro and in vivo studies, lead compounds cause genetic harm through a number of indirect mechanisms, including suppression of DNA synthesis and repair, oxidative damage, and interactions with DNA-binding proteins and tumour suppressor proteins. According to Roy and colleagues, lead acetate caused mutagenicity in the E. coli gpt locus transfected to V79 cells at a dangerous level. They also discovered that

lethal doses of lead acetate and lead nitrate transfected into V79 cells resulted in DNA breaks in the E. coli gpt gene . In another study, Wise and his colleagues discovered no evidence of direct genotoxic or DNA-damaging effects of lead, save for lead chromate. They suspect that hexavalent chromate, not lead, is to blame for the genotoxicity .

1.4. Prospects

According to a comprehensive review of published evidence, heavy metals such as arsenic, cadmium, chromium, lead, and mercury are found naturally. Anthropogenic activities, on the other hand, have a significant part in pollution of the environment. These metals are systemic poisons that have been associated in humans to heart disease, developmental abnormalities, neurologic and neurobehavioral disorders, diabetes, hearing loss, hematologic and immunologic disorders, and a variety of cancers. The most prevalent modes of exposure are ingestion, inhalation, and skin contact. The severity of negative health effects vary based on the type of heavy metal used, its chemical form, period, and dose. In metal toxic kinetics and toxic dynamics, speciation is crucial, because it is affected by valence state, particle size, solubility, biotransformation, and chemical form, among other factors. Several studies have linked toxic metals exposure to long-term health issues in people. Although the acute and chronic effects of some metals are well documented, less is known about the health consequences of hazardous mixtures. These harmful ions may interfere with the metabolism of nutritionally important metals such as iron, calcium, copper, and zinc, according to recent research. Regrettably, research on the combined toxicity of heavy metals is lacking. Long-term exposure to heavy metals can have severe additive, antagonistic, or synergistic effects.

According to a recent assessment of a number of individual studies that addressed metals interactions, co-exposure to metal/metalloid mixes of arsenic, lead, and cadmium had more severe effects at both relatively high and low dose levels in a biomarker-specific manner at both relatively high and low dose levels. These effects were found to be influenced by dose, exposure time, and genetic factors. Furthermore, co-exposure to cadmium and inorganic arsenic in humans resulted in more severe kidney damage than either element alone. In many metal-contaminated areas, chronic low-dose exposure to many elements represents a substantial public health danger. To detect health concerns

and control chemical combinations, the molecular foundation of heavy metal interactions must be known. As a result, further research is needed to better understand the molecular mechanisms of dangerous metal combinations in humans, as well as the public health implications.

1.5. Total petroleum hydrocarbons and its impacts

Petroleum is one of the dominant energy source to meet the worldwide demand of energy (Peng et al. 2009)3. Despite the fact petroleum hydrocarbons are used as major energy sources, they can have deleterious impacts on earth's ecosystem. They may contaminate the environment; at oil drilling sites (Maddela et al. 2015), or through accidental spills during exploration, manufacturing, refining, storage and transportation of petroleum and its products; or by leaking of storage tanks and pipelines; or by improper disposal of the industrial sludge and leads to progressive deterioration of quality of the environment (Yen et al. 2011). Petroleum refinery effluent contains either floating or emulsified oil which requires appropriate treatment or separation before disposal. Safe disposal of oily sludge is a problem for refineries. If not remediated, industries have to face financial losses in terms of fines by environmental protection associations and they cannot apply for labels like Ecofriendly product, ISO 14001 and other NEQs. Total petroleum hydrocarbons (TPHs) describe the addition of all the hydrocarbons of various molecular weights that are contained in crude oil or any product produced from it like gasoline, creosote or diesel (Hussain et al. 2018). Crude oil is a naturally occurring flammable liquid found in geological formations formed millions of years ago from fossilized organic material (Hunt, et al. 2018). It is composed of complex aliphatic, aromatic, asphalting and resin hydrocarbons (Liu Jianv et al. 2018). It is commonly refined into various types of fuels or products via fractional distillation. Both unprocessed as well as processed hydrocarbons are termed as petroleum. Composition of TPHs varies depending upon the source of crude oil and refining practice used to produce the product (Hunt et al. 2019). Generally saturated hydrocarbons (composed of normal alkanes, up-to 40 carbons and cyclic alkanes) comprise the pre-dominant fraction of crude oil followed by aromatics. Remaining are asphaltene and resin portion of crude oil. Asphaltenes are very high molecular weight hydrocarbons (Logeshwaran et al. 2018). Petroleum

contamination in soil is a serious concern globally. Due to their toxicity, mutagenicity, carcinogenicity and recalcitrance they pose severe environmental problems. They get bind with soil and sediment particles due to hydrophobic behavior thus they get accumulated and their bioavailability gets very much reduced (Khan et al. 2017). Weathering of TPHs enhances the sorption of contaminant into solid matrix thus aging plays a significant role in contaminant bioavailability, biodegradation and potential toxicity. These contaminants affect quality of the soil by changing the physical, chemical and biological properties of the soil (Masakorala et al. 2014) and produces infertile and nutrient deprived soils (Varjani and Upasani 2019). Due to their presence in soil pores and binding with the soil particles; availability of water, oxygen and nutrients decreases thus producing extreme harsh surroundings for plants and microorganisms (Shahzad et al. 2016). Their presence in soil adversely affect growth and development of plant by; altering or inhibiting seed germination rate, causing oxidative damage to photosynthetic pigments and slowing down nutrient uptake and absorption (Peng et al. 2009). Their contamination produces extensive damage to biodiversity of flora and fauna. Researchers have investigated toxic effects of TPHs contamination on survival of earthworms, bacteria and plants and found that no earthworm can survive in soil contaminated with 3% or more TPHs whereas 100% inhibition of bacteria was observed at 1% TPHs contaminated soil. Germination inhibition of plants specifically wheat and maize was observed at 3% TPHs contaminated soils (Lim et al. 2016).

Usability of the land also gets affected as weathered petroleum residuals may bind for years. They can also be the source of groundwater contamination. Petroleum residuals in soil act as continuous source of groundwater contamination. In some cases TPHs may float on the surface of the water table known as light non aqueous phase liquids (LNAPLS) which is due to their buoyancy and results in plume of hydrocarbons in groundwater (Logeshwaran et al. 2018). Marine oil spills produce devastating impacts on shorelines as well as seas. Marine life is severely affected due to oil spills in water bodies. This also reduces the aesthetic appeal thus can have an economic impact on tourism (Dave and Ghaly 2011). Human health effects from environmental exposure to TPHs may vary depending upon type and quantity. Smaller hydrocarbons like benzene, toluene and Xylene may affect the CNS. Other hydrocarbons can cause breathing

problems; blood and liver abnormalities; and some of them affect skin, lungs, bladder, kidneys, liver, spleen and stomach (Hunt et al. 2019). However, for assessment of health effects due to TPHs exposure requires much more detailed information than what is provided by single TPHs value. Petroleum aromatic hydrocarbons PAHs are one type of TPHs contributed to environment both naturally and through anthropogenic activities. They are known for carcinogenic and mutagenic activities. Out of all the petroleum products, diesel oil was found to be more toxic due to increased levels of PAHs contained in diesel oil (Wante and Leung 2018). Petroleum derived synthetic organic compounds are highly resilient to biodegradation.

1.6. Techniques used for the treatment of co-contaminated soil

Soil contaminated with multiple pollutants is a worldwide problem for food safety, human health and for environment itself which is also increasing day by day. It is estimated that globally almost >5 million sites casing 20 million hector lands are polluted with heavy metals and other multiple pollutants. Over the years various in site and ex-situ techniques have been used to remediate the contaminated soils. To minimize potential adverse effects of TPHs' and metals exposure, on humans and ecological health, remediation of petroleum contamination is necessary. The cleanup of co-contaminated soils should be a priority as it poses risk to groundwater and soil fertility. Remediation is basically an approach to manage contaminated site by preventing, reducing, mitigating and monitoring contaminant to overcome its consequences to human health and environment. Applied methods for TPHs remediation ranges from physical, chemical and biological methods and in recent decades much of the attention have been given for advancement of these. Sustainable method for remediation of soil is required which is cost effective and environmentally friendly i.e., it not only lowers the level of existing pollution problem, but also low or no secondary pollution is produced. Balancing cost and gain are very challenging as environmental costs are usually not considered or ignored. Generally, gain through remediation is native (cleaner soil), but environmental cost is most frequently regional or global (particle or other air emissions, affecting biodiversity.

1.6.1 Bioremediation

Bioremediation means use of biological entities, such as bacteria, fungi, algae or plants, that play role in; degradation, detoxification, stabilization, immobilization or transformation of toxic contaminants into a harmless state or to the permissible limits given by monitoring authorities to cure environmental problems such as contaminated groundwater or soil. Bioremediation is a naturally occurring process and eco compatible means of reclaiming polluted land. Bioremediation was recognized as the first commercial application in 1972. Since 1972 bioremediation has advanced greatly as a method of cleaning up spills of gasoline, diesel, heavy metals and other easily degraded petroleum products (National Research Council). Bioremediation for restoration of contaminated site can be categorized into; in-situ treatment or ex-situ treatment, based on the site of application. In-situ treatment involves on spot treatment, without being excavated and transported whereas in cases where soil cannot be treated in-situ due to regulatory reasons, unavailability of land for treatment or due to risk of groundwater or air contamination, ex-situ treatment is used which contains excavation of polluted soil and its transportation to a suitable place for treatment.

1.6.2. Phytoremediation

Phytoremediation is a type of bioremediation process. It is an environmentally friendly solar energy driven; cost effective; in-situ technique, which involves use of specific plants which along with their associated microbial community; remove, transform, stabilize or assimilate toxic chemicals present in soils, sludges, sediments, groundwater and surface water (Peng et al. 2009). Phytoremediation has been proposed as “green biotechnology” which can effectively degrade organic pollutants including petroleum hydrocarbons thus mitigating the undesirable effects of petroleum contamination (Escalante-Espinosa et al. 2005). It involves low input wherein sowing plants may be the only investment (Liu Rui et al. 2012).

Phytoremediation is successfully used for treatment of pollutants from soil, sediments, shallow aquifers and brown fields (Moubasher et al. 2015). Plants perform multiple mechanisms influencing fate of metal and TPHs in soil. These include; phytoaccumulation, phyto-volatilization, phyto-degradation, phytostimulation,

rhizodegradation and endophytic. Degradation. A key element for successful phytoremediation of TPHs contaminated soil is the use of plants that can tolerate high levels of contaminant in combination with beneficial plant related rhizospheric and endophytic microorganisms (Fatima et al. 2018). Oxidative coupling reactions take place during rhizosphere degradation of hydrocarbons as follows:

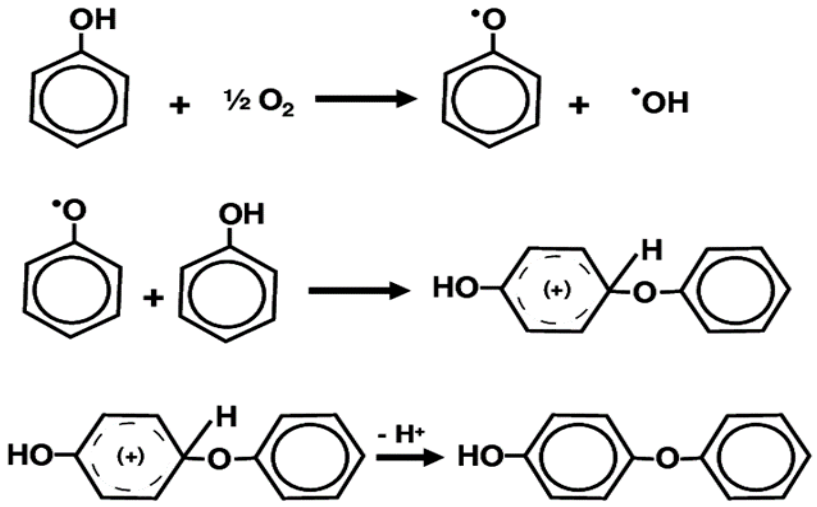

Figure 1 Oxidative coupling reaction

Rhizospheric microbial degradation is considered as the primary mechanism controlling the phytoremediation process. The rhizosphere of plant is very active zone compared to bulk soil. In this zone, microbial activities, soil enzyme activities, nutrient exchange and degradation is very dynamic. Generally intensive microbial activity is observed in this zone due to occurrence of high amount of available carbon as root exudates. The influence of roots on soil microbial population start immediately after seed germination and increases as the plant grows. This mechanism is often termed as phyto-stimulation term rhizodegradation and phyto-stimulation are often used interchangeably (Daryabeigi Zand and Hoveidi 2016). It involves release of different organic compounds like sugars, organic acids, amino acids, hormones and vitamins in rhizosphere region through the roots of plants which increases the diversity, density and activity of specific microorganism which in turn facilitate rhizo- degradation or can be plant growth promoting microorganism by controlling nutrient availability and uptake thus enhance

efficiency of Phytoremediation (Liu Hong et al. 2009). Root exudates can be utilized as electron donors or as carbon substrate to support metabolismthus stimulating degradation of hydrocarbons. Plant roots are considered as significant parameter to improve soil aeration by increasing the porosity and decreasing soil moisture. Plants promote the dynamic environment for aerobic microorganisms. Oxen microorganisms produce di-oxygenase and monooxygenase enzymes that induce transformation and mineralization of TPHs (McIntosh et al. 2017).

Plant microbe association plays important role in making PHCs more available for biodegradation. Along with the exudates plant releases various enzymes that stimulate biochemical activities in the surrounding soil to support bioremediation. It is reported that plant biomass significantly reduces due to presence of petroleum hydrocarbons (Spiares et al. 2016). High level of TPHs inhibits the plant growth. TPHs are phytotoxic and hydrophobic innature (Cai et al. 2010). Saturated hydrocarbons have high degradation rates because they are not as much of toxic to microorganisms in rhizosphere and be responsible for carbon source for microbial metabolism (Liu Jianv et al. 2018). It has been reported that plants grown in TPHs contaminated soils enhance prevalence of endophytes that have genes encoded for production of enzymes for hydrocarbon degradation (Yousaf S et al. 2010b). For TPHs phytoremediation, those plants are preferred which have; ability to tolerate high contaminant concentration with rapid growth; extensive root system and large root surface area (Yousaf Sohail et al. 2010). There are many plants and grass species reported for their abilities to tolerate and efficiently remove TPHs from Soil. These include maize (Shahzad et al. 2016), ryegrass (Hussain et al. 2018), birds foot trefoil (Yousafet al. 2010), alfalfa (Zand et al. 2016), sorghum (Iraji et al. 2016), Bermuda grass (Basumatary and Bordoloi 2016), suitable for TPHs removal.

Table 2 Plants reported for remediation.

Reported plants	Contaminant concentration	Growth time	Season
S. alfredii **Stone crop**	(6.38 mg kg−1 DW)	60 days	**S**pring
Fava bean broad bean	slightly to moderately	4 m	Oct-Nov
S.alfredii, **ryegrass, Castor**	Moderately to high	90 days	Mar-Apr
Phragmites australis **(common reed)**	40 mg/kg	75 days	Spring-Summer
Maize	4.5 mg/kg	60 days	Spring-Autumn
Alfalfa Lucerne		90	Oct-Nov
Festuca L **Fescue Fire phoenix**	169 mg/kg PAH	150 days	Spring
Melia azedarach **Bakain, Dhrek**	60 mg/kg pyrene	60 days	Mar-Apr
Fava bean ***S.alfredii***	136 mg/kg		

Problem statement

After the advent of industrialization, co contamination emerged as a serious threat to environment because of its high toxicity and ability to persist in the environment. Especially the soils Co-contaminated with heavy metals and Total Petroleum Hydrocarbons is needed to be rehabilitated by using eco-friendly remediation techniques.

Objectives

1. To achieve bioremediation total petroleum hydrocarbon and heavy metal contaminated soil with co-plantation.
2. To evaluate the effect of co-plantation of *Zea mays L*, *Ricinus communis*, with bacterial inoculation on TPHs degradation and heavy metal uptake.
3. To evaluate the effect of compost amendment on TPHs degradation, heavy metal uptake and bacterial colonization.
4. To evaluate the effect of co-plantation of *Lolium perenne*, *Ricinus communis*, with bacterial inoculation and compost amendment on TPHs degradation and heavy metal uptake.

Chapter 2
Materials and Methods

2.1. Collection of contaminated soil

The contaminated soil was collected from Austria State: Lower Austria District: Gänserndorf Municipality: Drösing Collected soil was air dried and air-dried soil was sieved to remove the debris. The Drösing petroleum refinery was operated from 1899 to 1937. There were among others Kerosene, wound gasoline, light, medium and heavy gasoline as well as petroleum produced. Acidic Highly viscous mineral oil hydrocarbons were not processed further in the production process and ended up as waste in an acid tar pit that lasted until the end of production 2,000 m^3. Mineral oil contamination has occurred on a large part of the company premises detected. The expansion of the underground areas heavily contaminated with mineral oil can be caused by approx. 55,000 m^2 and approx. 100,000 m^3, of which around 30,000 m^3 in the groundwater fluctuation range, be estimated. The spread of pollutants in the groundwater is currently low. It is also in the future with no significant pollutant emissions in the groundwater runoff are to be expected. The considerable contaminated area poses a significant threat to the environment. It is classified in Priority class 3 proposed.

2.2. Inoculation of selected strains

After the germination started, two bacterial strains (BACILLUS CEREUS 25 and BACILLUS SAFENSIS 28) already reported as TPH stress tolerant were used to assist the plant-microbe interaction. Then 15ml tube was added in each plant pot. An equal volume of sterile water was used in the un-inoculated control pot (Ren et al., 2019).

2.3. Plant material

Seeds of *Lolium perenne*,*Ricinus communis* and *Zea mays* L were collected from the National Agricultural Research Center (NARC), Islamabad. Healthy seeds were washed several times with autoclaved distilled water before planting.

2.4. Germination trials (pre-experiment)

Co-contaminated soil tested for seed germination prior to the final experiment as follows:

Table 3 Seed germination trial with co-contaminated soil

Dated	Trial Plant	Soil Status	Status
02-06-21	Ryegrass Type 1	Co-contaminated	Not Germinated
02-06-21	Castor bean Type 1	Co-contaminated	Not Germinated
09-06-21	Ryegrass Type 2	Co-contaminated	Not Germinated
09-06-21	Ryegrass Type 1	Fresh	Germinated
09-06-21	Ryegrass Type 2	Fresh	Germinated
09-06-21	Castor bean Type 2	Co-contaminated	Not Germinated
09-06-21	Castor bean Type 2	Fresh	Germinated
09-06-21	Castor bean Type 1	Fresh	Germinated
17-06-21	Mari Gold	Co-contaminated	Not Germinated
17-06-21	Sorghum	Co-contaminated	Not Germinated
17-06-21	Maize	Co-contaminated	Germinated
30-06-21	Maize OPV 1-2	10% Compost	Germinated
30-06-21	Mari Gold	10% Compost	Germinated
30-06-21	Castor bean	10% Compost	Germinated
30-06-21	Ryegrass	10% Compost	Germinated

2.5. Experiment design Phase 1.

Pot experiment was designed for bioremediation of co-contaminated (heavy metals + TPHs) soil by co plantation of hyper-accumulated *Zea mays L.* with heavy metal resistant and PAH degrading *Ricinus Communis*. The experiment will be carried out in a green house. The treatments will be applied as follows:

Contaminated Soil = C.S — Control

Fresh Soil + Maize = F. S+M — Treatment

Fresh Soil+ Castor bean = F. S+C.B — Treatment

Fresh Soil+ Maize+ Castor bean = F. S+Maize+C.B — Treatment

Treatment 1	C.S + M	C.S + C. B	C.S + M + C.B
Treatment 2	C.S + M + C	C.S + CB + C	C.S + M + C.B + C
Treatment 3	C.S + M + *Bacillus cereus*	C.S + C.B + *Bacillus cereus*	C.S + M + C.B +*Bacillus cereus*
Treatment 4	C.S + M +*Bacillus safensis*	C.S + C.B + *Bacillus safensis*	C.S + M+ C.B + *Bacillus safensis*
Treatment 5	C.S + M + *Bacillus cereus* + C	C.S + C.B + *Bacillus cereus* + C	C.S + M + C.B + *Bacillus cereus* + C
Treatment 6	C.S + M + *Bacillus safensis* + C	C.S + C.B + *Bacillus safensis* + C	C.S + M + C.B + *Bacillus safensis* + C
Total Ts. = 19*3 = 57	Soil Weight = 19.7kg	Per pot wt= 300g	Total wt= 300g*57 = 17.1kg
Compost Ts=9*3=27	Compost = 10%	Per pot wt= 30g	Total wt=30*27=810g

Martials Used:

- Seeds
- Bacteria
- Pots
- Green House
- Distilled Water
- Ethanol
- Hoagland Solution

Pot experiment was conducted in greenhouse of botanical garden, Quaid I Azam University, Islamabad. 300 g soil was added in each pot. Eight seeds of castor bean and fifteen seeds of maize were added as per treatment desighn.Inoculation was applied after 15 days of experiment. Watering. Predefined greenhouse conditions 16 h light: 8 h dark, at 30-33 ºC, and constant moisture levels (after every two days, 20 ml of water) were maintained throughout the growth period. Two abiotic control treatments were also used, one containing fresh soil with plant while second pot containing only spiked soil to check effect of environmental conditions. Each treatment had three replicates. Each of the pots were placed on the saucers to avoid contaminant leaching. Complete randomized block design (CRBD) was followed for pots placement in the greenhouse. Light and dark conditions. Harvesting was done after 78 days. Soil and plant samples were stored for further analysis.

2.6. Experiment Design Phase 2.

Pot Experiment was designed for bioremediation of co-contaminated (heavy metals + TPHs) soil by co plantation of hyper-accumulated Ryegrass with heavy metal resistant and PAH degrading Castor bean. The experiment will be carried out in a green house. The treatments will be applied as follows:

Contaminated Soil = C.S — Control

Fresh Soil + Lolium perenne = F.S+M

Fresh Soil+Castor bean = F.S+C.B

Treatment

Fresh Soil+Ryegrass+Castor bean = F.S+R.G+C.B

Abiotic Control

Control: F.S + C.B

Control: F.S + R.G

Control: F.S + C.B + R.G

T1= C.B + C

T2= C.B + C + *Bacillus cereus*

T3= C.B + C + *Bacillus safensis*

T4= R.G + C

T5= R.G + C + *Bacillus cereus*

T6= R.G + C + *Bacillus safensis*

T7= C.B + R.G + C

T8= C.B + R.G + C + *Bacillus cereus*

T9= C.B + R.G + C + *Bacillus safensis*

Pot experiment was conducted in greenhouse of botanical garden, Quaid I Azam University, Islamabad. 300 g soil was added in each pot. Eight seeds of castor bean and fifteen seeds of rye grass was added as per treatment desighn.Inoculation was applied after 15 days of experiment. Watering. Predefined greenhouse conditions 16 h light: 8 h dark, at 30-33 ºC, and constant moisture levels (after every two days, 20 ml of water) were maintained throughout the growth period. Two abiotic control treatments were also used, one containing fresh soil with plant while second pot containing only spiked soil to check effect of environmental conditions. Each treatment had three replicates. Each of the pots were placed on the saucers to avoid contaminant leaching. Complete randomized block design (CRBD) was followed for pots placement in the greenhouse. Light and dark conditions. Harvesting was done after 78 days. Soil and plant samples were stored for further analysis.

2.7. Soil analysis

2.7.1. Soil physicochemical status and nutrients

Soil samples were taken from each treatment before and after the addition and during the collection treatment. They were used for a large number of studies, including the physical and chemical state of the soil and nutrients (including soil electrical conductivity (EC), pH, extractable phosphorus, available nitrates, total organic carbon, oxidizable organic carbon and organic matter), soil enzymatic status (including catalase) and bacterial strain

survival rate to the corresponding program. The composition of the soil was also determined. Determination of the additional content of heavy metals in each soil sample was done. The brief details of all the analyses are presented in this section.

2.7.2. Soil texture determination

In a beaker, combine 40 g of dry soil with 60 ml of sodium hexa-meta-phosphate dispersion (4:1; $(NaPO_3)_{13}$: Na_2CO_3). Watch glass is used to cover the beaker and let it sit overnight. On next day quantitatively transfer the contents of the beaker the next day. In a glass of mixed soil, fill the glass with approximately three-quarters of water. Shake the suspension overnight. Quantitatively transfer the suspension to a calibrated 1 liter cylinder (hydrometer tank) and dilute to volume with water. Do the same solution for the blank, but without soil. The hydrometer method is used to evaluate the soil texture; then the hydrometer (ASTM 152H GILSON Comp Inc., USA) suspension is used to calculate the sand, silt and clay content (%) (Strickland et al. 1988).

2.7.3. Soil pH, EC, TDS quantification

The pH, EC, TDS of soil was measured by using Cyber scan PC 510 by Eutech instrument. One gram of soil was taken in 50 ml beaker and mixed in 5 ml of distilled water for pH, EC and TDS as done by Khan et al. (2019b), while 1 g soil mixed in 10 ml of distilled water, for ORP (Upadhyay et al., 2019). The mixture was allowed to stand without agitation for 30 minutes. The respective electrode of pH, EC, TDS was immersed in solution prepared for each sample, and readings were taken.

2.7.4. Soil extractable phosphorus quantification

Extractable phosphorous in soil samples were quantified by using the standard Olsen's sodium bicarbonate method, as provided by (Estefan et al., 2013). For quantification of 0.5 g of air-dried soil was in 10 ml 0.5 M $NaHCO_3$ solution and was place on orbital shaker at 150 rpm for 30 min, and then filtered using filter paper (Whatman No. 40). Filtrate (5 ml) was added with a 3-5 drops of 0.25% p-nitro phenol indicator, prepared with distilled water, and mixed, drop by drop, with 5 N H_2SO_4, till the solution become colorless from yellow. After acidification, the volume of the acidified solution was raised to 20 ml, using distilled water, and 4 ml of ascorbic acid solution. A blank control was also prepared, containing all ingredient except soil, and standards of 1 to 5 ppm

phosphate were also prepared. The absorbance of blank, standards, and samples was taken after 10 minutes at 882 nm, using UV-9200/VIS-7220G, and Rayleigh spectrophotometer. The concentration of extractable P in ppm was quantified using following formula;

$$P\ (in\ ppm) = Value\ derived\ from\ calabration\ curve\ using\ Absorbance \times \frac{V \times V_2}{W_t \times V_1}$$

Where, V= total volume of the extract (ml), $W_{t.}$ = Weight of air-dry soil (g), V_1= Volume of soil extract used for measurement (ml), and V_2= Volume of flask used for measurement (ml).

2.7.5. Soil nitrates quantification

Soil nitrates was quantified by the chromo trophic acid method (Estefan et al., 2013). Briefly, 1 g of sieved air-dried soil was mixed with 5 ml 0.02 N $CuSO_4.5H_2O$ and was placed on an orbital shaker for 15 min at 100 rpm. After mixing, each sample was filtered using filter paper (Whatman No. 42), and 3 ml of filtrate was mixed with 1 ml 0.1% chromo trophic acid and left on ice bath. After this the solution is added with 6 ml of sulphuric acid (concentrated) and were swirled. To prevent excessive heat formation, prepared mixture was left with shaking to cool down at room temperature. After 45 min yellow color was formed, to which absorbance of the solution was taken on 430 nm, using spectrophotometer. A blank control was also prepared, containing all ingredient except soil, further standards of NO_3, using KNO_3 dissolved in 0.02 N $CuSO_4.5H_2O$, were also prepared as suggested in the standard protocol. The concentration of NO_3 in ppm was quantified using following formula;

$$NO_3(in\ ppm) = Value\ derived\ from\ calabration\ curve\ using\ Absorbance \times \frac{V \times V_2}{W_t \times V_1}$$

Where, V= total volume of the extract (ml), $W_{t.}$ = Weight of air-dry soil (g), V_1= Volume of soil extract used for measurement (ml), and V_2= Volume of flask used for measurement (ml).

2.7.6. Oxidizable organic carbon, total organic carbon, and organic matter

The Walkley-Black method was used for the quantification of OOC, TOC, and OM in the soil (Estefan et al., 2013). Briefly, 0.5 g of sieved air-dried soil was mixed with 5 ml of 1 N potassium dichromate, and the resulted solution was mixed with 10 mL concentrated H_2SO_4, and left undisturbed for 30 min. After 30 min, 100 ml of distilled water was added to the solution and then mixed with 5 ml H_3PO_4 (concentrated). The resulted solution was mixed with 15 drops of diphenylamine and was titrated with 0.5 M ferrous ammonium sulfate solution. Change in color was noted carefully from dark black to violet blue, upon titration, and the addition of 0.5 M ferrous ammonium sulfate solution was halted when the color change reached green color. A blank containing all ingredient except soil was also prepared. The quantity of OOC, TOC, and OM was quantified using following equations;

$$OOC\ (\%) = \frac{(V_B - V_S) \times 0.3 \times 10}{W_t \times V_B}$$

$$TOC\ (\%) = 1.334\ \times OOC\ (\%)$$

$$OM\ (\%) = 1.724\ \times OOC\ (\%)$$

Where, V_B= Volume of ferrous ammonium sulfate solution required for the titration of the blank (ml), V_S= Volume of ferrous ammonium sulfate solution required for the titration of the sample (ml), W_t= Weight of air dried soil (g), and $0.3 = 3 \times 10^{-3} \times 100$, where 3 is the equivalent weight of C.

2.7.6 Total organic carbon, oxidizable organic carbon, and organic matter

The Walkley-Black method was used to quantify OOC, TOC, and OM in soil (Estefan et al., 2013). In brief, 0.5 g of sieved air-dried soil was mixed with 5 mL of 1 N potassium dichromate, and the resulting solution was mixed with 10 mL of concentrated H2SO4 and allowed to stand for 30 minutes. After 30 minutes, 100 ml of distilled water was added to the solution, followed by 5 ml of H3PO4 (concentrated). The resulting solution was titrated with 0.5 M ferrous ammonium sulphate solution after being combined with 15 drops of diphenylamine. During titration, the colour changed from dark black to violet blue, and the addition of 0.5 M ferrous ammonium sulphate solution was stopped when

the colour changed to green. A blank with all ingredients except dirt was also made. The following formulae were used to calculate the amount of OOC, TOC, and OM;

$$OOC\ (\%) = \frac{(V_B - V_S) \times 0.3 \times 10}{W_t \times V_B}$$

$$TOC\ (\%) = 1.334\ \times OOC\ (\%)$$

$$OM\ (\%) = 1.724\ \times OOC\ (\%)$$

Where VB is the volume of ferrous ammonium sulphate solution necessary for the blank titration (ml), VS is the volume of ferrous ammonium sulphate solution required for the sample titration (ml), Wt is the weight of air dried soil (g), and 0.3 is the equivalent weight of C.

2.8. Soil microbial count

Materials and Procedures 2.7.6 Total organic carbon, oxidizable organic carbon, and organic matter the soil for all treatments including bacterial inoculum is tested for bacterial colony forming units (CFU) and the survival of aggregated bacterial strains that can survive heavy metals. Plate counting was used to collect bacterial isolates. The soil suspension was prepared with a 0.9N NaCl saline solution (10 grammes of soil in 90 ml of normal saline) and serially diluted by combining 9 ml of 0.1 percent (w/v) sterile saline solution with 1 ml of the preceding diluent.

For each procedure, 100 l of the diluent from 10-1 to 10-4 was spread on a nutrient agar plate containing 400 ppm of lead, then the plate was incubated at 30°C for 24 hours and the number of colonies on the plate was counted (Liu et al., 2020).

2.9. TPH quantification

TPHs were detected directly utilizing a modified Quenchers approach as well as gas chromatography along with mass spectrometry detection (GC-MS). To properly measure the remaining TPHs, 10 g homogenized dried material was combined with 3 ml distilled water for one minute 50 ml glass tube, and then 3 ml of dichloromethane were added and was again mixed for 1 minute. The salt mixture was added in a 1:4:1:0.5 g ratio to a

solution containing MgSO4, Nacl disodium hydrogen citrate trispdium citrate hydrate and sesquihydrate, and also the sample was agitated for 1 minute again. The resultant samples were centrifuged for 8 minutes at 4000 rpm. After centrifugation, the supernatant was then collected in a clean centrifuge tube for cleaning. A salt combination was added to the supernatant once again. All samples were extracted & filtered using a 45 m nylon disc filter, yielding 300 L, which was placed inside a glass vial with 700 L DCM and used for GC-MS analysis.

Individual alkanes were detected and also quantified with the use of a Shimadzu QP2020 mass selective detector and a gas chromatograph equipped with the mass spectrometer detector (GC-MS). A 30 m long SH-Rxi-5Sil MS capillary column with a 0.5 mm diameter and fused silica capillary with a 0.25 m film thickness was employed. The temperature of oven was set as 50 degrees Celsius for one minute, then continued to increase at a rate of 25 degrees Celsius per minute to 120 degrees Celsius, then to 160 degrees Celsius at 10 degrees Celsius per minute, 240 degrees Celsius at 6 degrees Celsius per minute, and finally to 315 degrees Celsius at 2 degrees Celsius per minute for ten minutes. The carrier gas was high quality helium at 1.2 mL/min. The sample volume was 1L using split less mode at 300 °C. The selective mass detector was a quadrupole (Agilent Technologies Model 5975) with an electronic impact ionization mechanism at 70 eV and 230 °C. To identify chemicals, pure standards were employed, and mass spectra were examined using the NIST5.0/EPA/NIH (version 2.0 d) library. Standard curves for quantification were obtained by generating a solution containing a mixture of several standard alkane compounds (Sigma, UST122) in doses of 0.5, 1, 5, 10, 25, and 50 mg kg-1. The concentrations of the each standard chemical were estimated using just a linear regression equation (R2 from 0.991 to 0.998). TPH was estimated by summing the 27 distinct hydrocarbons identified in the extract and using dry soil (at 105°C) as a reference. 2.10 Plant analysis 2.10.1 Physiological growth evaluation Physiological analysis was performed on harvested plants. Plant factors evaluated were shoot and root length (RL and SL, respectively, and expressed in cm). The lengths were measured using a centimeter scale, from base to tip (for roots, from plant base to tip of the tap root, and for shoots, from the plant base to the apical buds of the plants), Total plant leaves were physically counted and expressed LN (n, plant-1) in specific treatment. Image J was used

to calculate the leaf area (LA, cm2). In brief, a snaps of all leaves per plant were captured onto white paper using a 3*3 cm red box scale as a reference. Shoot and root lengths were measured with a ruler and expressed in centimeters. The dried and fresh weights of the root, leaf and shoot were calculated using gravimetric readings acquired on an electric weighing balance and expressed in g plant-1. Fresh leaves were also stored at -80 °C in representative amounts for enzymatic and biochemical analyses. Fresh weight was taken right after harvest, whereas dried weight was oven dried at 60 °C till the constant weight was achieved. All plant physiological parameters were recorded using the standard method for plant physiological analysis developed by Khan et al (2019).

2.10.2 Measuring the biochemical and stress injury parameters of the plants

2.10.2.1 Contents of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid

The Arnon (1949) technique was used to prepare the extract required for the measurement of chlorophyll and carotenoid levels. In brief, 40 mg of fresh leaf sample was macerated in 2 ml of 80 percent aqueous acetone (v/v) to obtain a homogeneous leaf extract. The resulting macerated suspension was centrifuged for 5 minutes at 5000 g. Supernatant was transferred to a new falcon tube, and the pellet was vortexed with 1 ml of 80 percent aqueous acetone (v/v) before centrifugation at 5000 g for 5 minutes. The resulting supernatant was combined with previously collected supernatant and analyzed. Lichtenthaler (1987) developed formulae for calculating photosynthetic pigments such as chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid concentrations (abbreviated as Chl a, Chl b, T Chl, and carotenoid content). After measuring absorbance (A) at wavelengths of 663, 645, and 470 nm, the calculations were performed using the following equations:

2.10.2.2 Lipid peroxidation quantification

Malondialdehyde (MDA) levels were determined in samples used to quantify lipid peroxidation. The Venkatachalam et al. approach was used for the analysis (2017). In general, 0.1 g fresh leaf sample was dissolved to create a homogenized solution in 1 ml pre-chilled 5 percent TCA (w/w) in an ice bath. Homogenized samples were centrifuged for 10 minutes at 10,000 g, and the supernatant was mixed with a 1:1 ratio of TBA

solution (0.67 percent). The solution then heated for 30 minutes at 95 °C before being placed for 1 minute in an ice bath. The cold liquid was then centrifuged for 10 minutes at 10,000 g. After computing the values using the following formulae, the absorbance of prepared samples were analyzed at 450, 532, and 600 nm, and the total lipid peroxidation was provided in M of malondialdehyde g-1 of FW.

Where Vt equals 0.001 L and W equals 0.1 g.

2.10.2.3 Production of hydrogen peroxide

The methodology employed by Khan et al. (2019b), with adjustments in the leaf extract preparation methodological scheme of Venkatachalam et al., was used to quantify the generation of ROS, specifically hydrogen peroxide content (2017). In brief, a leaf fresh sample was macerated in a pre-chilled 1 ml extraction buffer (pH 7.4) containing 50 mM potassium phosphate buffer (PPB) and 0.5 mM EDTA and centrifuged for 15 minutes at 10,000 g at 4 °C. The homogenized sample's supernatant was then collected and used as a leaf extract in the quantification of H2O2 content, as well as to prevent deterioration of the prepared sample, which was kept at 4 °C.

The homogenized sample's supernatant was collected and employed as a leaves extract in the determination of H2O2 concentration, as well as to prevent deterioration of the produced sample, which was maintained at 4 °C. A reaction mixture for H2O2 contents was made by combining 40 l of leaf extract, 1 ml of 0.05 mM PPB (pH 6.5), and 352.8 l of 1 percent Ti(SO4)2 produced in 20 percent H2SO4 (v/v), then centrifuging for 15 minutes at 6000 g. The supernatant was collected to determine the intensity of a yellow color, which was evaluated by measuring absorbance at 410 nm. The value of H2O2 content was obtained as M H2O2 contents g-1 of FW using Beer-Lambert law and a molar extinction coefficient of 0.28 M-1 cm-1.

Where A = sample absorbance at selected wavelength, = molar extinction coefficient of desired substance,

b = sample route length (which was 1 cm in the instance of cuvette),

c = compound concentration in solution

2.10.3 Enzymatic activation quantification

The approach used to quantify enzyme activity is described further down in this section. The leaf extract was generated using the method described by Venkatachalam et al. (2017), as was the H2O2 activity. In brief, a fresh leaf sample i-e 0.1 g was macerated in an already chilled 1 ml extraction buffer of pH 7.4 containing 50 mM potassium phosphate buffer (PPB) and 0.5 mM EDTA and centrifuged for 15 minutes at 10,000 g at 4 °C. The homogenized sample's supernatant was collected and employed as a leaf extract in the quantification of enzyme activities, as well as to prevent deterioration of the produced sample, which was maintained at 4 °C. For all enzyme activities, values are provided in Units g-1 of FW of sample, except for GST, which is expressed in M min-1 g-1 of FW of plant. 2.10.3.1 Activity of ascorbate peroxidase the modified Chen and Asada procedure was used to measure ascorbate peroxidase activity (APX) (1989). The ascorbate oxidation was monitored by measuring the reduction of A at 240 nm in a reaction mixture made up of 1 ml reaction buffer (500 M ascorbate, 100 M EDTA, 1.54 mM H2O2, and 50 mM PPB, pH 7.0) and also 50 microliter of leaf extract. A value of 2.8 mM1 cm1 was used to calculate the APX activity.

2.10.3.2 Catalase activity

Catalase (CAT) activity was evaluated using the Mealy and Chance (1954) procedure. After one minute, the H2O2 decrease was quantified by monitoring A240. 2.5 ml of reaction buffer (50 mM PPB, pH 7.4), 100 l 1 percent H2O2, and 50 l leaf extract comprised the reaction mixture (which was also diluted in some cases to preserve observations inside the analytical linear range). Catalase activity was determined to be 39.4 mM1 cm1.

2.10.3.3. Activity of guaiacol peroxidase

Activity of catalase was evaluated by ε of 39.4 mM^{-1} cm^{-1}. The method of Upadhyay et al., (2019) was used for the quantification of GPX activity. The mixture for reaction was obtained by mixing 20 µl of leaf extract with 2.5 ml reaction buffer (50 mM PPB at pH 6.1)1 ml of 1% H guaiacol, and 1 ml of 1% H2O2. The change in A_{420} was examined for 1 min. By using ε of 26.6 mM^{-1} cm^{-1} GPX activity was calculated.

Heavy metal analysis of collected samples

For elemental evaluation of soil samples, they were oven dried in a single day at 80 ºC. 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. All samples were processed in triplicates and were analyzed using atomic absorption spectrophotometer (Charles, 1991).

Statistical analysis

Data obtained were analyzed by using the Shapiro-Wilk Test for checking the normality before any analysis. One way analysis of variance was applied on of all treatments, followed by Duncan's multiple range post hoc test, to compare multiple means. All the data was collected in triplicate, the p value of 0.05 was considered significant. All statistical work were done using SPSS 20.

Chapter 3

Results and Discussion

Phase 1 Results

3.1. Plant biomass

Following eighteen treatments were added to co-contaminated soil in which treatment 18 C.S+Maize+Castorbean+Bacteria2+Comp possess the maximum plant biomass among all eighteen treatments.

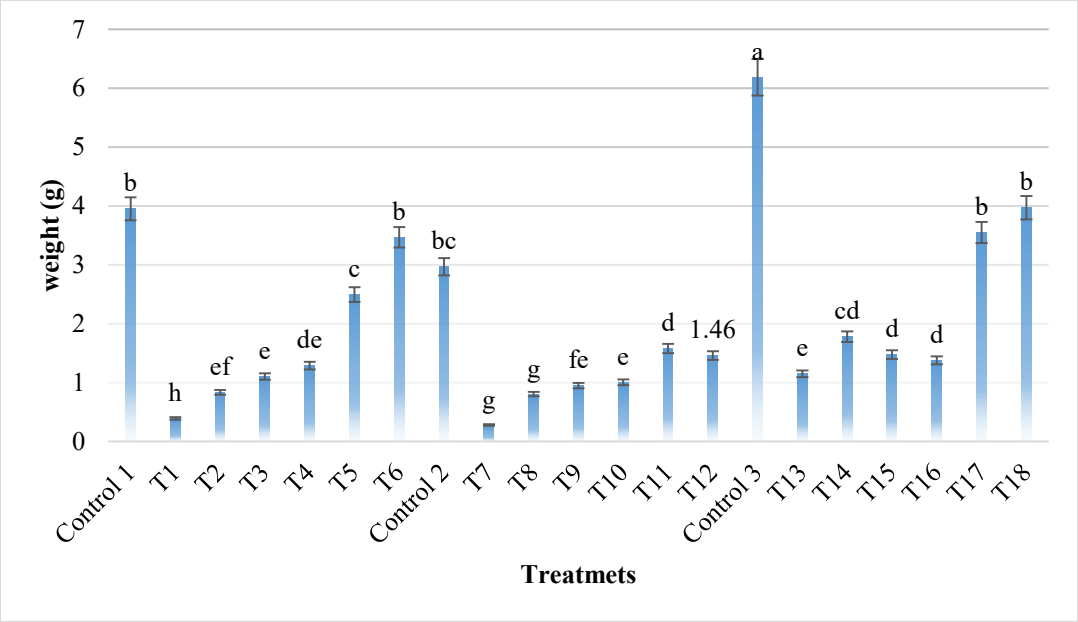

Figure 2 Total biomass in different treatments

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later Alphabets for lower means. Similar small letters in are non-significant.

Above treatments shows biomass of plants having three controls with fresh soil and eighteen treatments with diversity of organic amendment and bacterial strains in C.S (co-contaminated soil).

3.3. Plant enzymes parameters.

Plant stress enzymes were analyzed to evaluate the toxicological damage and stress to plants in co-contaminated soil.

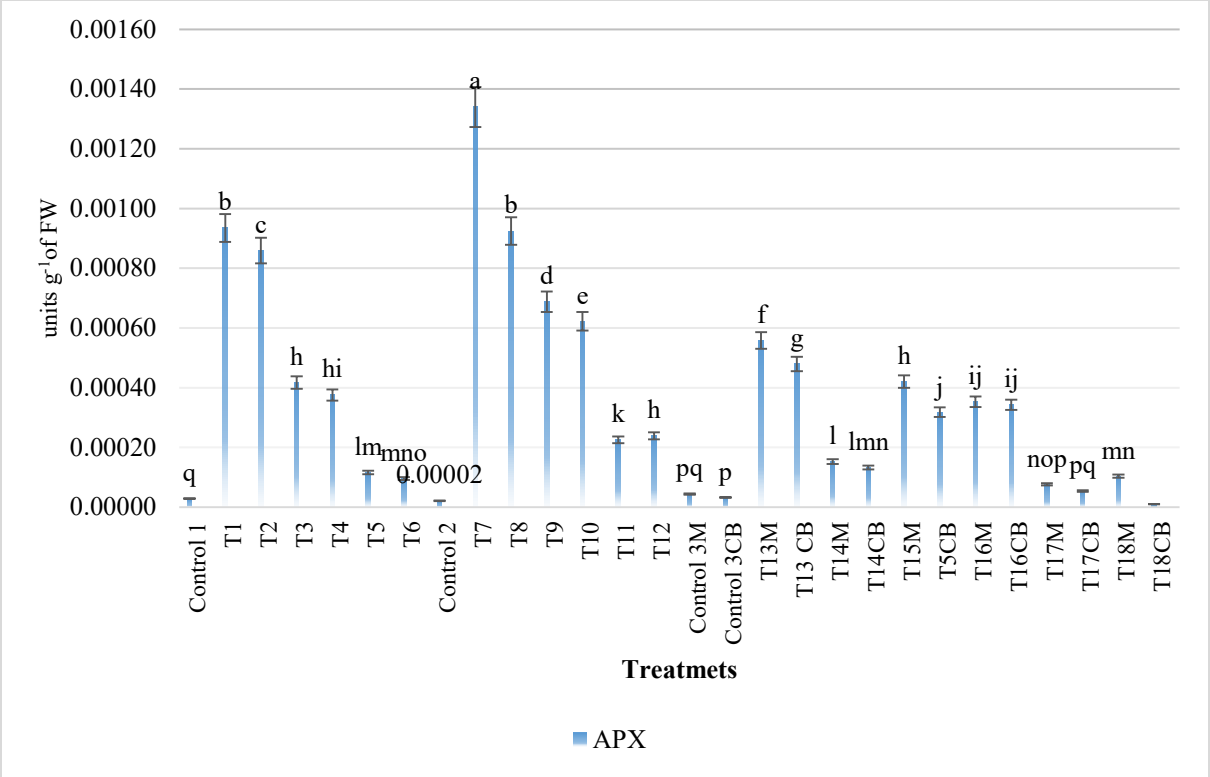

Figure 3 The enzymatic profile (APX) of *Zea mays* L. and *Ricinus communis* with reference to different applied treatment* Values are expressed in units $g^{-1}$of FW of plant leaf sample.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later Alphabets for lower means. Similar small letters in are non-significant.

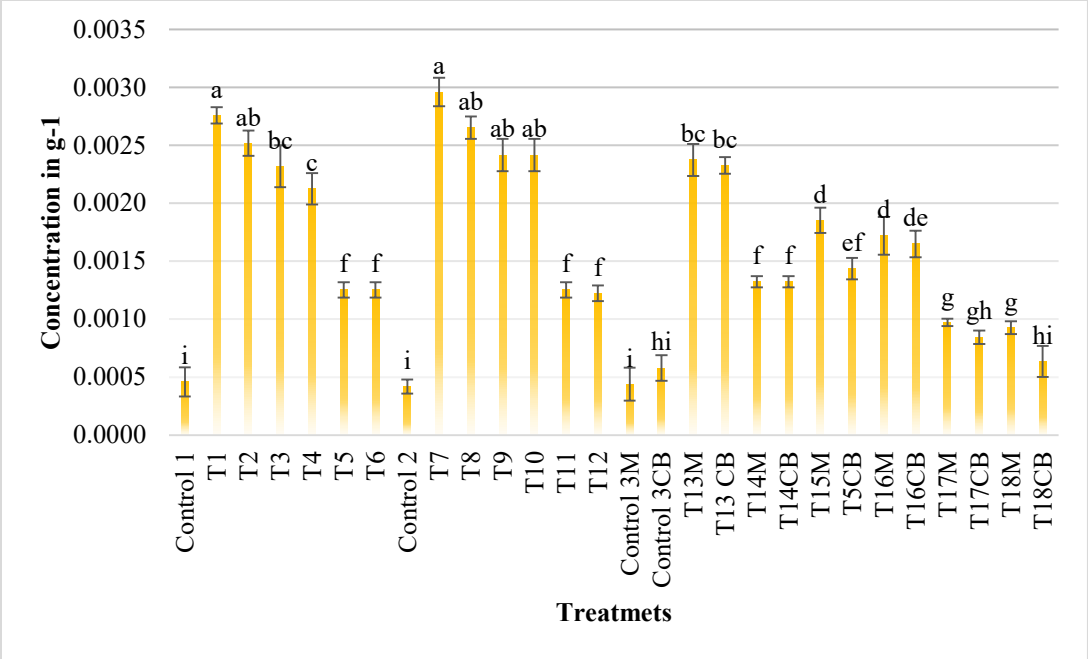

Figure 4. The enzymatic profile (GPX) of *Zea mays* L. and *Ricinus communis* with reference to different applied treatment*Values are expressed in Units $g^{-1}$of FW of plant leaf sample.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later Alphabets for lower means. Similar small letters in are non-significant.

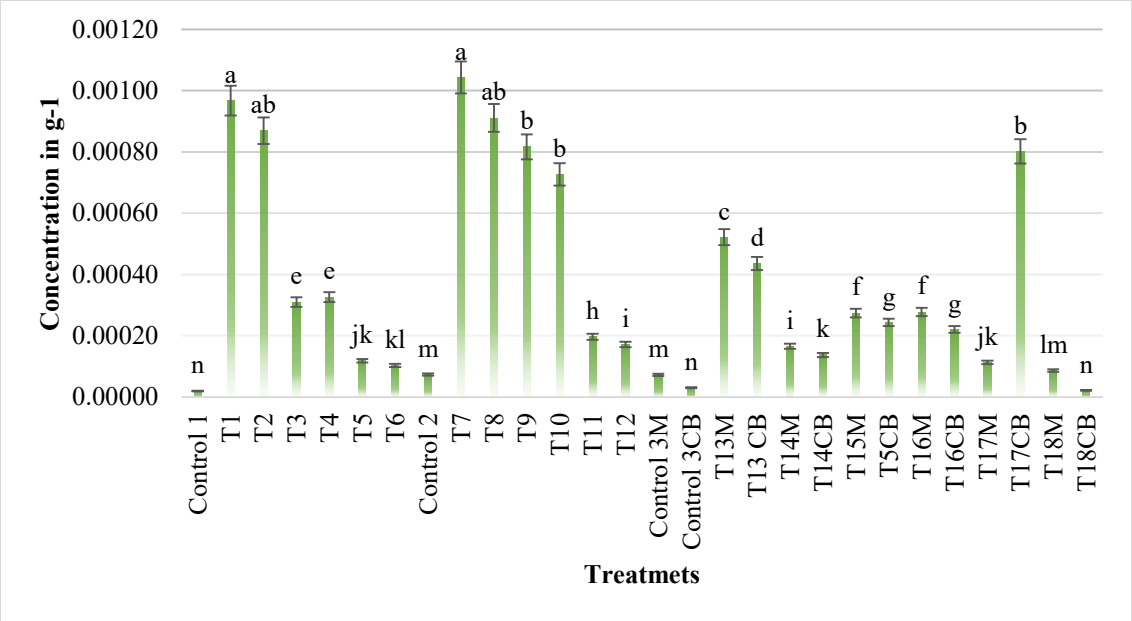

Figure 5 The enzymatic profile (GPX) of *Zea mays* L. and *Ricinus communis* with reference to different applied treatment*Values are expressed in Units $g^{-1}$of FW of plant leaf sample.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later Alphabets for lower means. Similar small letters in are non-significant.

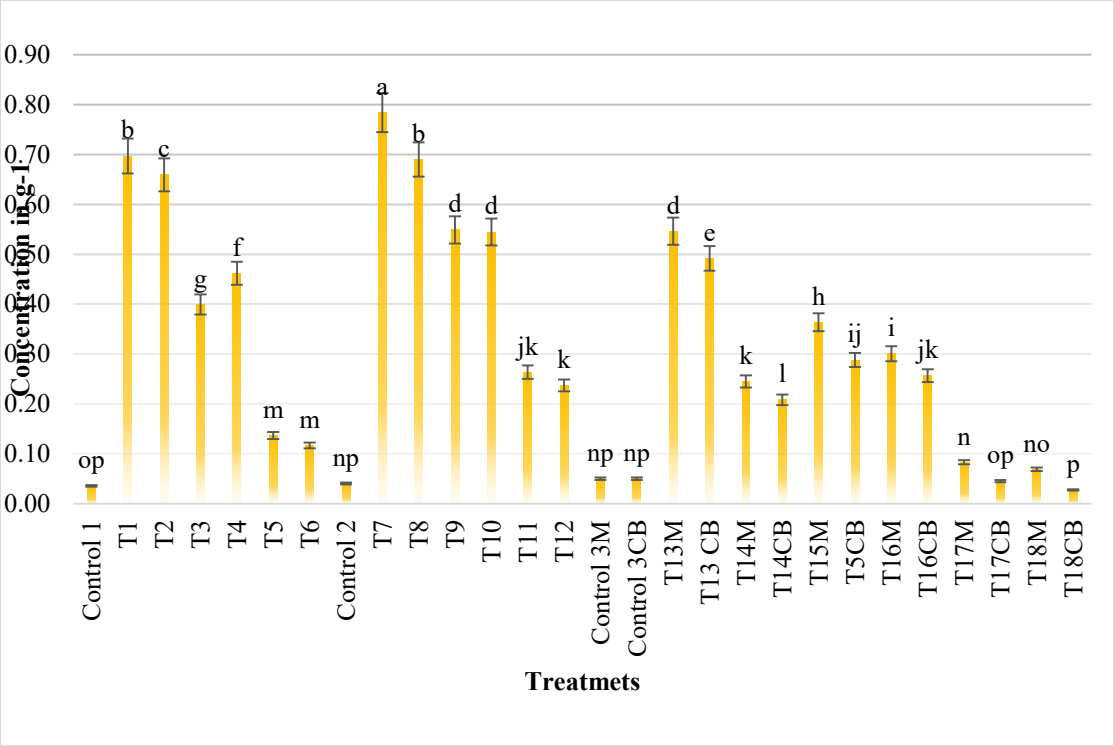

Figure 6 Enzymatic profile (GPX) of *Zea mays* L. and *Ricinus communis* with reference to different applied treatment*Values are expressed in Units g-1of FW of plant leaf sample.

Data represented in means (n=3±SD). Significantly highest mean was “a” followed by later Alphabets for lower means. Similar small letters in are non-significant.

3.4. Soil Physicochemical Parameters.

Table 4 Physicochemical parameters of phase 1

Treatment	Description	pH	EC	OOC	TOC	OM
			(µScm-¹)	%	%	%
A.C	Abiotic Control	8.54±0.09^{ab}	1.48±0.29^{a}	0.15±0.05^{h}	0.20±0.07^{h}	0.36±0.12^{h}
Control 1	Fresh Soil+Castor bean	7.63±0.12^{bc}	0.36±0.03^{d}	0.59±0.03^{g}	0.79±0.03^{g}	1.37±0.06^{g}
T1	C.S + Castor bean	8.62±0.1^{a}	0.43±0.11^{cd}	0.98±0.05^{de}	1.31±0.07^{de}	2.26±0.12^{de}
T2	C.S+ Castor bean +Compost	8.86±0.06^{a}	0.51±0.07^{bc}	1.03±0.1^{cd}	1.38±0.14^{cd}	2.38±0.24^{cd}
T3	C.S+ Castor bean +Bacteria1	8.82±0.13^{a}	0.40±0.05^{cd}	1.03±0.1^{cd}	1.38±0.14^{cd}	2.38±0.24^{cd}
T4	C.S+ Castor bean +Bacteria2	8.76±0.06^{a}	1.16±0.13^{ab}	0.88±0.16^{ef}	1.17±0.21^{ef}	2.02±0.36^{ef}
T5	C.S+Castorbean+Bacteria1+Compost	8.72±0.12^{a}	0.70±0.05^{b}	1.29±0.16^{a}	1.72±0.21^{a}	2.97±0.36^{a}
T6	C.S+Castorbean+Bacteria2+Compost	8.76±0.19^{a}	0.71±0.03^{b}	1.10±0.23^{bc}	1.52±0.31^{bc}	2.68±0.54^{bc}
Control 2	Fresh Soil+Maize	7.08±0.09^{c}	0.47±0.11^{cd}	0.72±0.1^{ef}	0.99±0.14^{ef}	1.67±0.24^{ef}
T7	C.S + Mazie	8.71±0.04^{a}	0.55±0.02^{c}	0.83±0.01^{fg}	1.11±0.01^{fg}	1.91±0.01^{fg}
T8	C.S+Maize+Compost	8.96±0.16^{a}	0.18±0.06^{ef}	0.88±0.16^{ef}	1.17±0.21^{ef}	2.02±0.36^{ef}
T9	C.S+Maize+Bacteria1	8.72±0.01^{a}	0.99±0.17^{ab}	0.88±0.16^{ef}	1.17±0.21^{ef}	2.02±0.36^{ef}
T10	C.S+Maize+Bacteria2	8.77±0.06^{a}	0.94±0.03^{b}	0.78±0.16^{fg}	1.03±0.21^{fg}	1.78±0.36^{fg}
T11	C.S+Maize+Bacteria1+Compost	8.88±0.19^{a}	0.65±0.04^{bc}	1.03±0.1^{cd}	1.38±0.14^{cd}	2.38±0.24^{bc}
T12	C.S+Maize+Bacteria2+Compost	8.86±0.04^{a}	0.30±0.04^{de}	1.03±0.21^{cd}	1.38±0.28^{cd}	2.38±0.48^{bc}
Control 3	Fresh Soil+Maize+Castor bean	7.20±0.04^{bc}	0.52±0.07^{bc}	0.62±0.1^{g}	0.82±0.14^{g}	1.43±0.24^{g}
T13	C.S+Maize+ Castor bean	8.63±0.06^{bc}	0.38±0.01^{bc}	1.04±0.05^{bc}	1.38±0.06^{bc}	2.38±0.11^{bc}
T14	C.S+Maize+Castorbean+Compost	8.83±0.01^{a}	0.29±0.11^{f}	1.00±0.15^{bc}	1.42±0.2^{bc}	2.44±0.35^{bc}
T15	C.S+Maize+Castorbean+Bact1	8.67±0.04^{ab}	0.46±0.11^{cd}	1.04±0.09^{cd}	1.38±0.13^{cd}	2.39±0.22^{cd}
T16	C.S+Maize+Castorbean+Bacteria2	8.86±0.13^{a}	0.47±0.07^{cd}	1.00±0.26^{cd}	1.33±0.35^{cd}	2.31±0.6^{cs}
T17	C.S+Maize+Castorbean+Bacteria1+Comp	8.59±0.04^{ab}	0.44±0.06^{d}	1.24±0.13^{ab}	1.65±0.17^{ab}	2.85±0.3^{an}
T18	C.S+Maize+Castorbean+Bacteria2+Comp	8.55±0.01^{a}	0.73±0.05^{b}	1.29±0.05^{a}	1.73±0.07^{a}	2.97±0.12^{a}

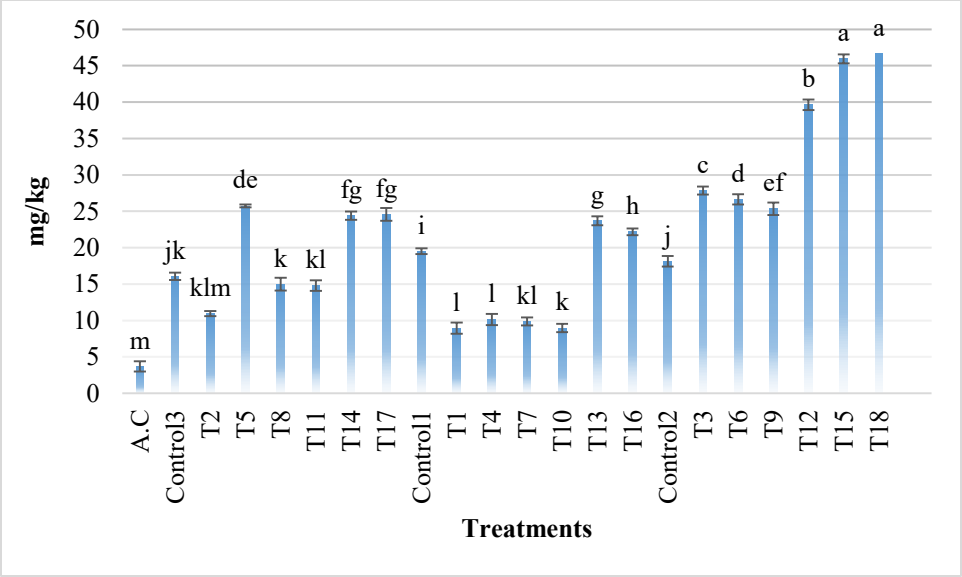

Figure 7 Extractable phosphorus content in soil depicting different treatments profile.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later alphabets for lower means. Similar small letters in are non-significant.

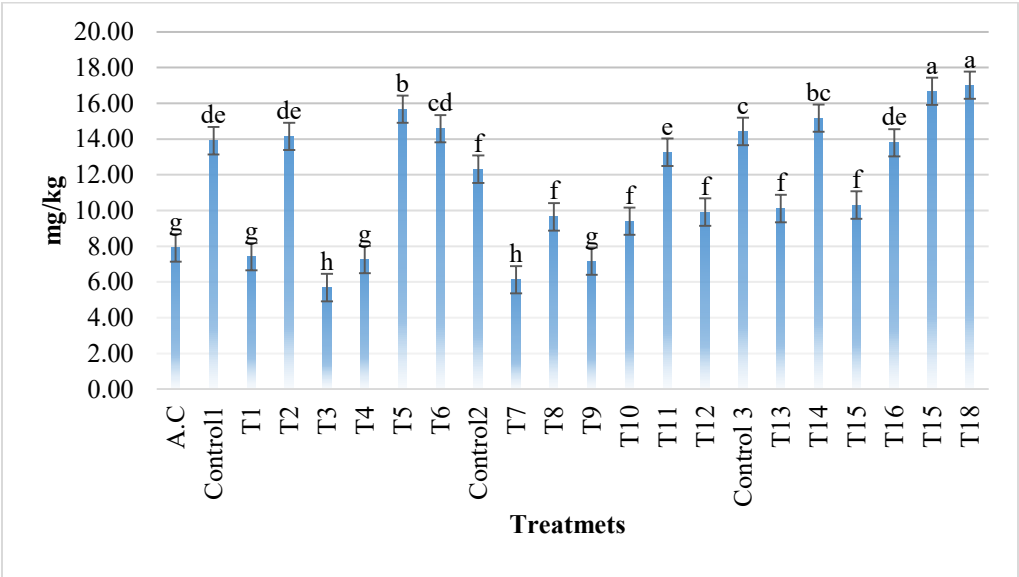

Figure 8 Nitrate content in soil depicting different treatments profile.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later Alphabets for lower means. Similar small letters in are non-significant.

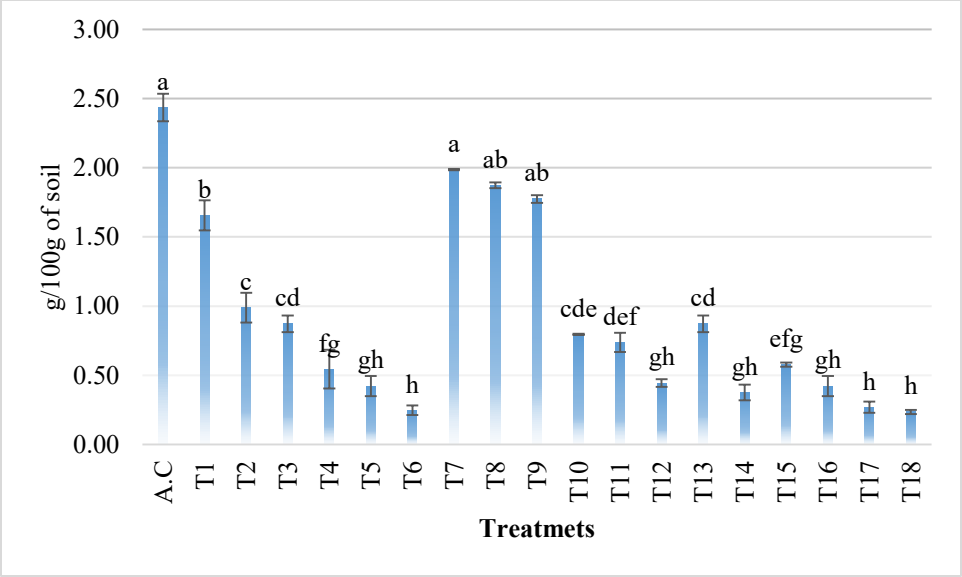

Figure 9 Residual total petroleum hydrocarbon content in soil

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later alphabets for lower means. Similar small letters in are non-significant.

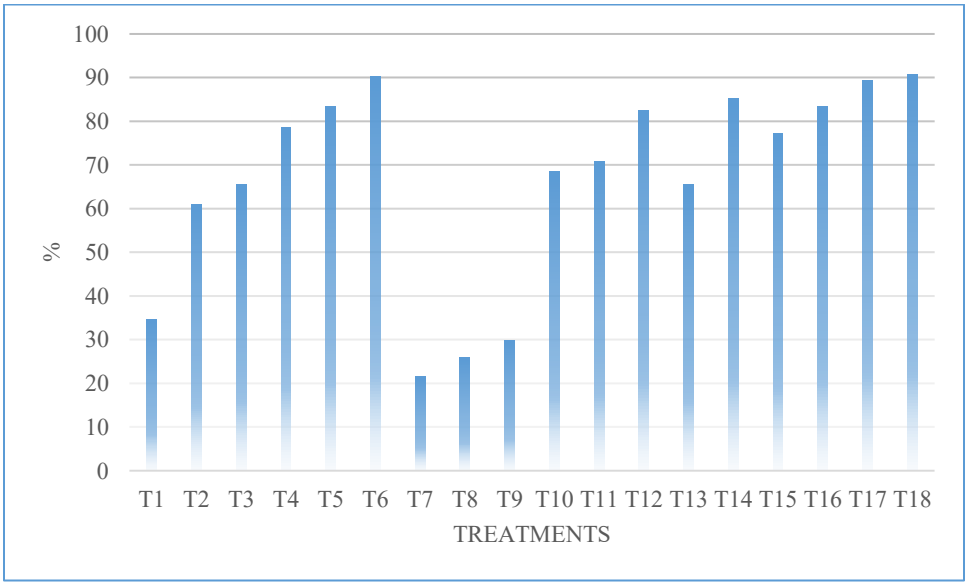

Figure 10 Removal percentage of TPH phase 1.

Heavy Metals Content in plants.

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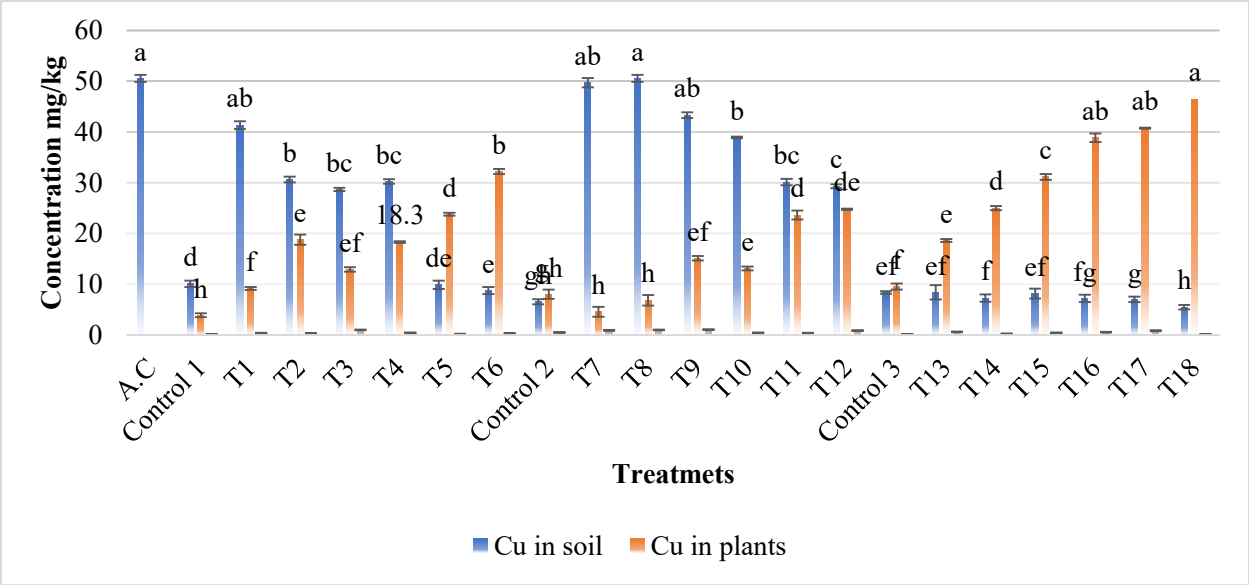

Figure 11 Cu content in soil and p lants in diverse setup of treatments.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later alphabets for lower means. Similar small letters in are non-significant.

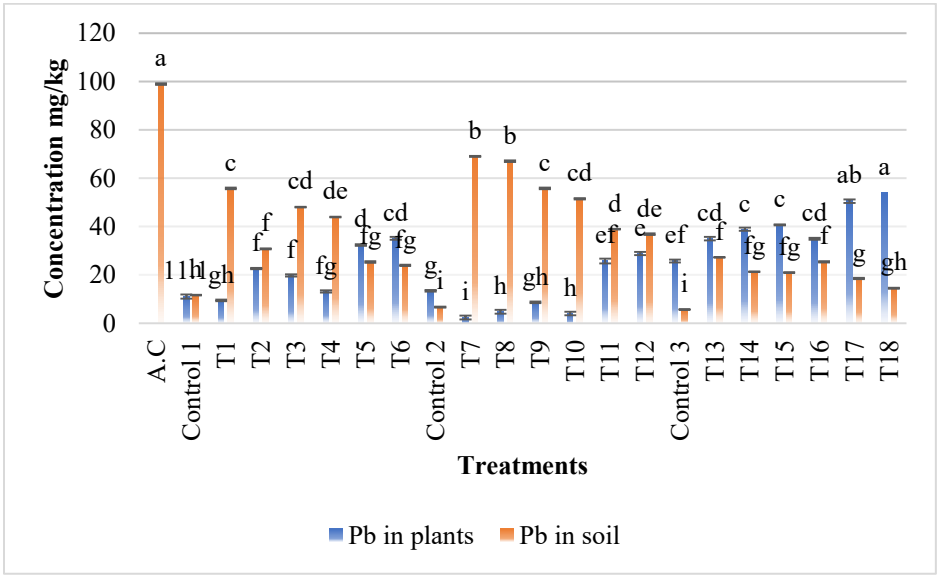

Figure 12 Pb content in soil and plants in diverse setup of treatments.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later

alphabets for lower means. Similar small letters in are non-significant.

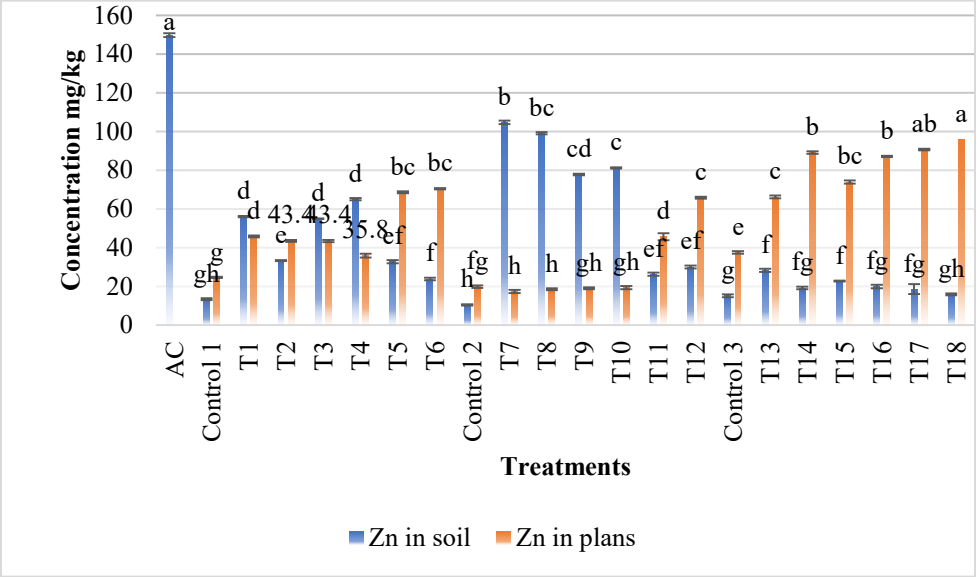

Figure 13 Zn content in soil and plants in diverse setup of treatments.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later Alphabets for lower means. Similar small letters in are non-significant.

Soil bacterial count for different treatments

Table 5 Bacterial Count for different treatments.

Description	Treatment	CFU (Cells $g^{-1}$of soil)
C.S+Castor Bean+Bacteria1	T8	$1.28*10\text{^}4^{ef}$
C.S+Castor Bean+Bacteria2	T11	$1.32*10\text{^}4^{d}$
C.S+Castorbean+Bacteria1+Compost	T14	$1.67*10\text{^}5^{a}$
C.S+Castorbean+Bacteria2+Compost	T17	$1.18*10\text{^}5^{b}$
C.S+Maize+Bacteria1	T7	$9.52*10\text{^}3^{fg}$
C.S+Maize+Bacteria2	T10	$1.08*10\text{^}4^{de}$
C.S+Maize+Bacteria1+Compost	T13	$1.63*10\text{^}4^{d}$
C.S+Maize+Bacteria2+Compost	T16	$1.46*10\text{^}4^{d}$
C.S+Maize+Castorbean+Bact1	T9	$1.29*10\text{^}5^{bc}$
C.S+Maize+Castorbean+Bacteria2	T12	$1.25*10\text{^}5^{ab}$

C.S+Maize+Castorbean+Bacteria1+Comp	T15	$1.47*10\text{^}5^{b}$
C.S+Maize+Castorbean+Bacteria2+Comp	T18	$1.67*10\text{^}5^{a}$

Phase 2 Results

3.5. Plant biomass

Following nine treatments were added to co-contaminated soil in which treatment 9 C.S+Ryegrass+Castorbean+Bacteria2+Comp possess the maximum plant biomass among all nine treatments.

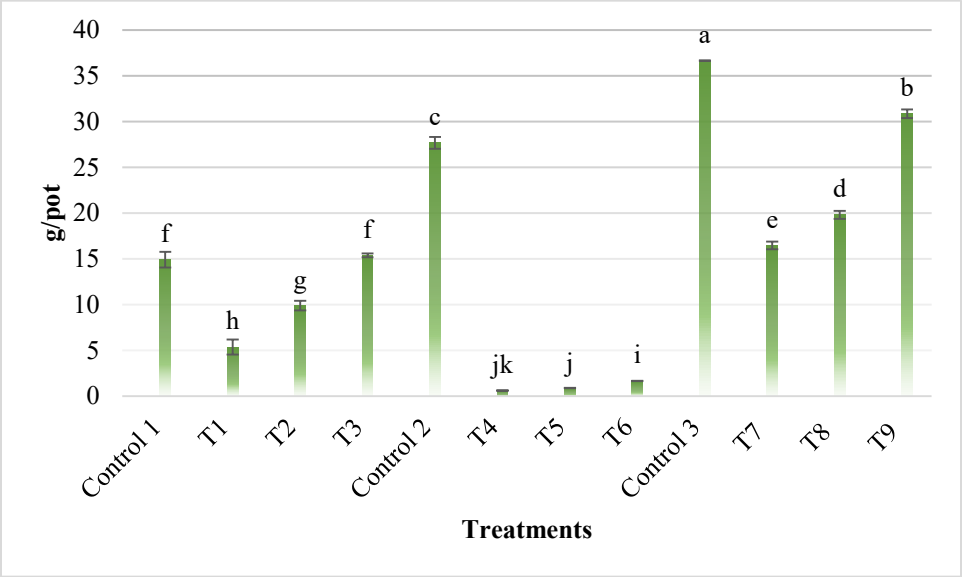

Figure 14 Total plant biomass content in different setting of treatments.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later alphabets for lower means. Similar small letters in are non-significant**.**

Total chlorophyll and carotenoids

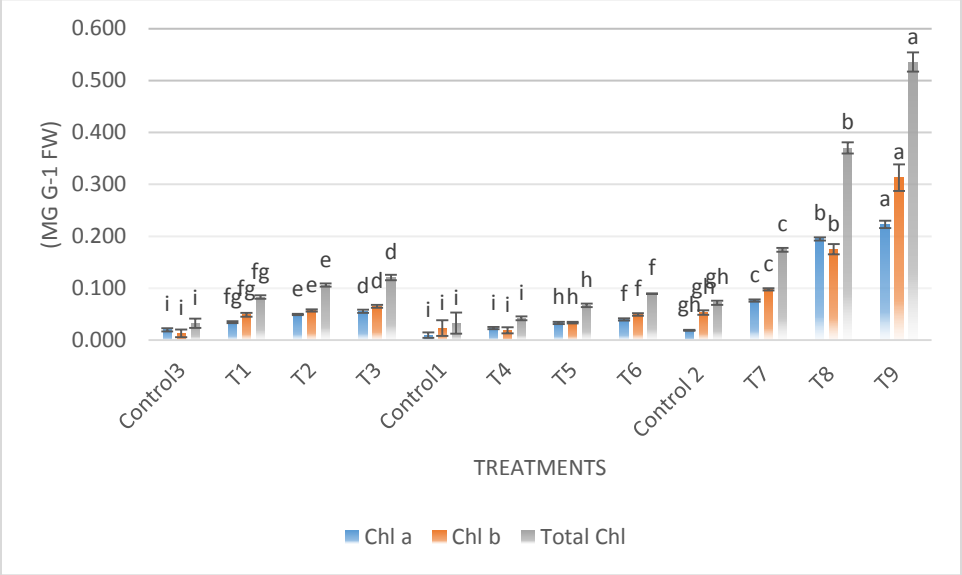

Figure 15 Chlorophyll a, Chlorophyll b, Total Chlorophyll content in different setting of treatments.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later alphabets for lower means. Similar small letters in are non-significant**.**

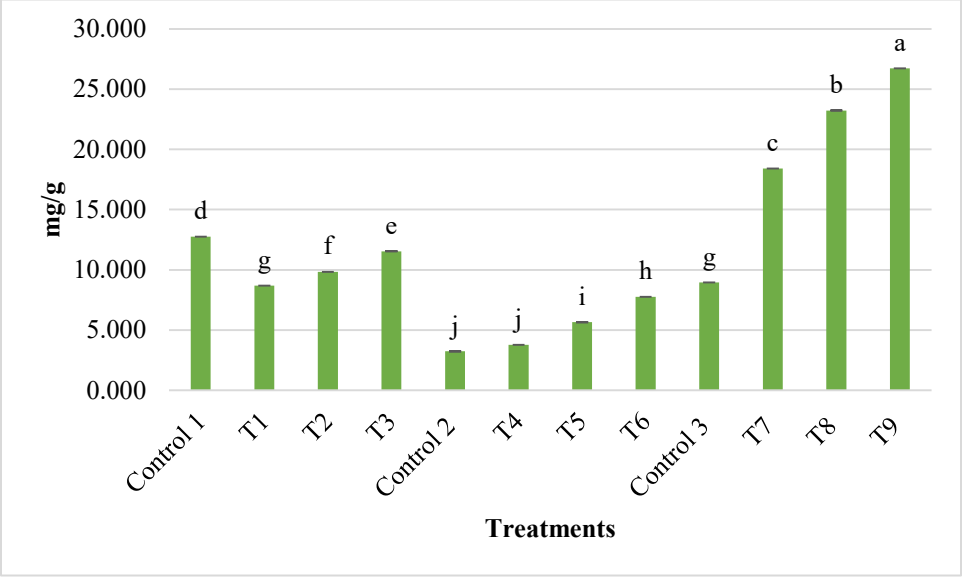

Figure 16 Carotenoids content in different setting of treatments.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later alphabets for lower means. Similar small letters in are non-significant**.**

Enzymatic profile of plants (Phase 2)

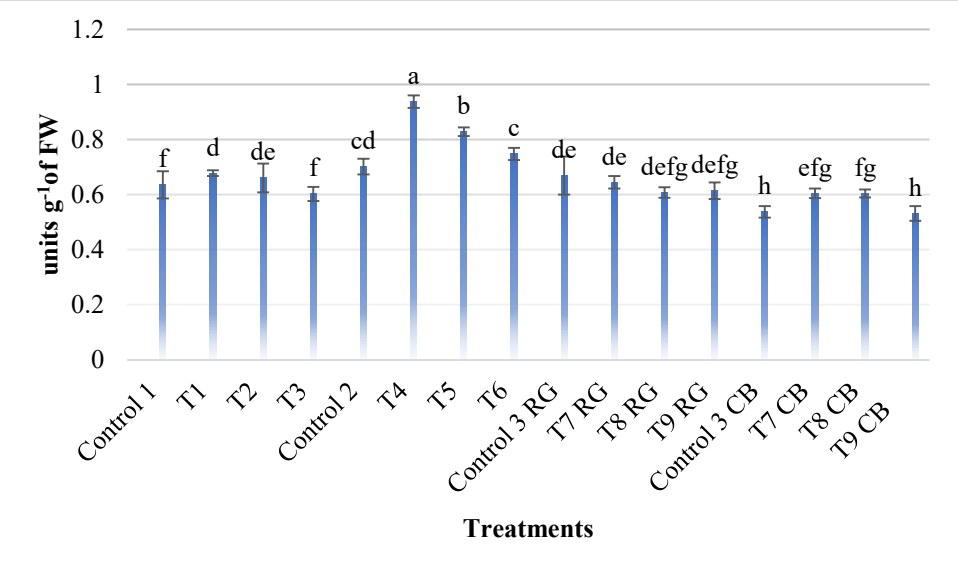

Figure 17 The enzymatic profile (SOD) of *Lolium perenne* and *Ricinus communis* with reference to different applied treatment*Values are expressed in units $g^{-1}$of FW of plant leaf sample.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later alphabets for lower means. Similar small letters in are non-significant.

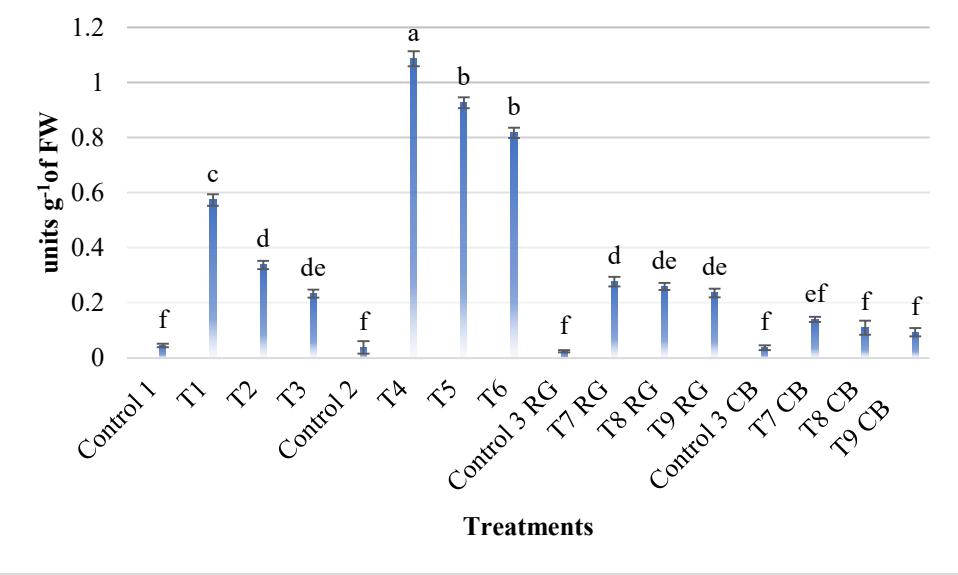

Figure 18 The enzymatic profile (MDA) of *Lolium prenne* and *Ricinus communis* with reference to different Applied treatment*Values are expressed in units $g^{-1}$of FW of plant leaf sample.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later alphabets for lower means. Similar small letters in are non-significant.

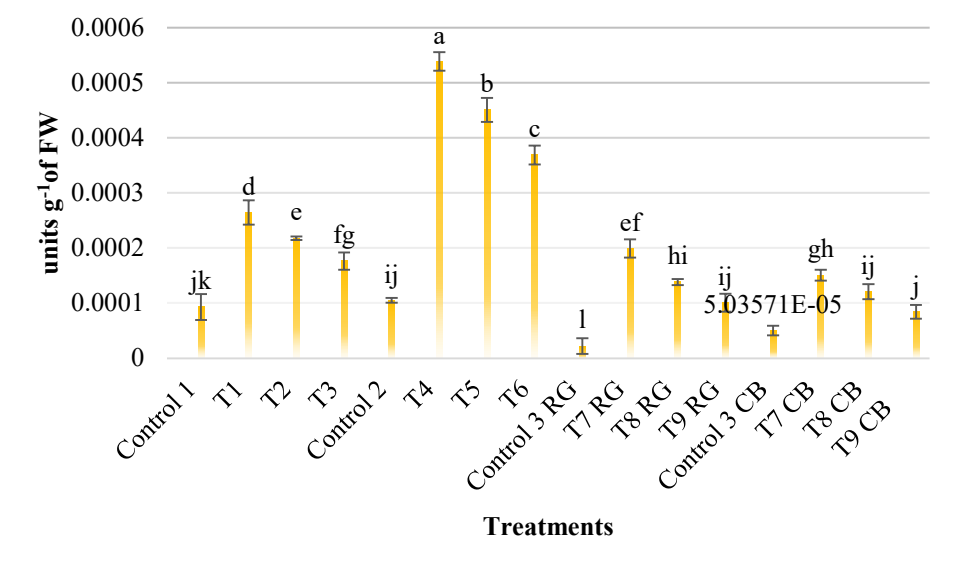

Figure 19 The enzymatic profile (GPX) of *Lolium perenne* and *Ricinus communis* with reference to different applied treatment*Values are expressed in units $g^{-1}$of FW of plant leaf sample.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later alphabets for lower means. Similar small letters in are non-significant.

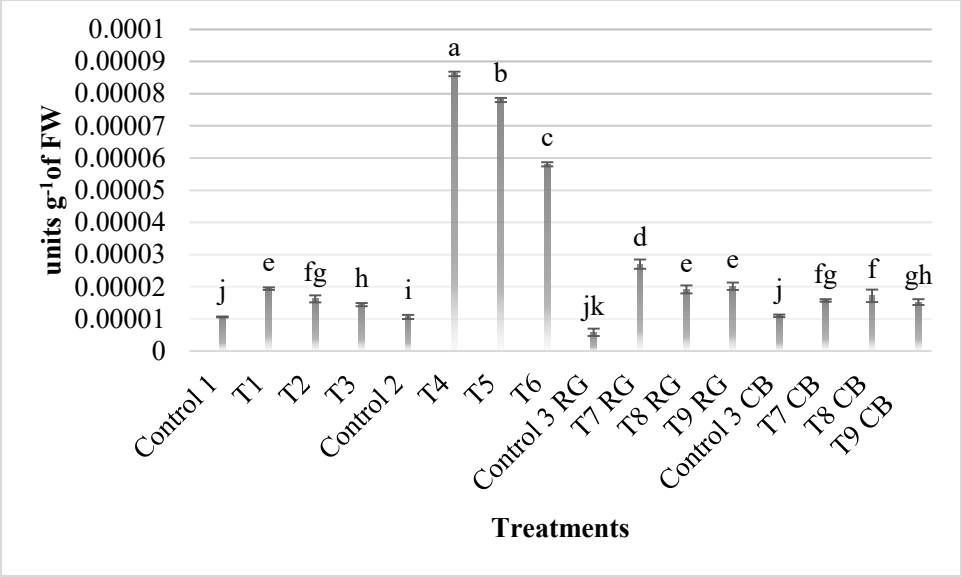

Figure 20 The enzymatic profile (APX) of *Lolium prenne* and *Ricinus communis* with reference to different applied treatment*Values are expressed in units $g^{-1}$of FW of plant leaf sample.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later alphabets for lower means. Similar small letters in are non-significant.

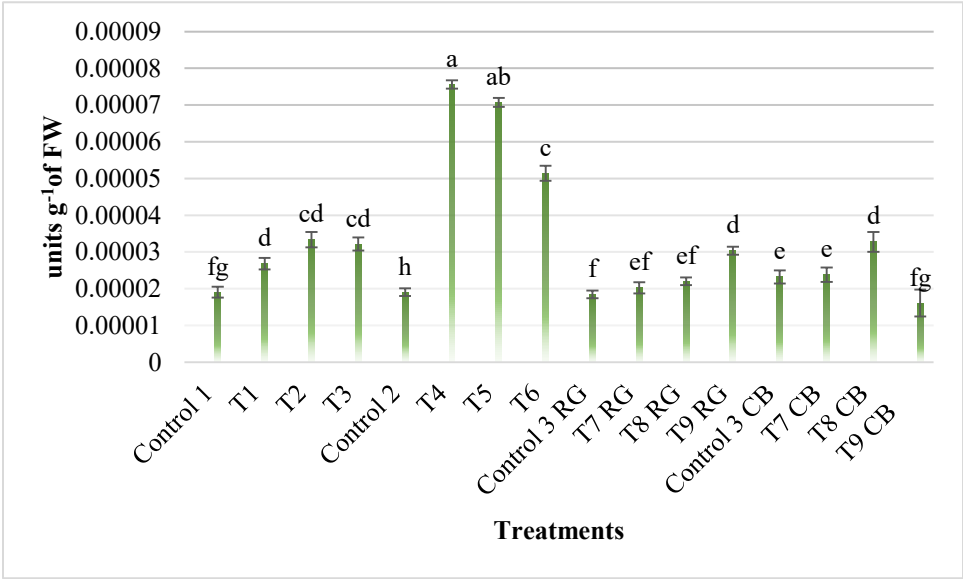

Figure 21 The enzymatic profile (CAT) of *Lolium perenne* and *Ricinus communis* with reference to different applied treatment*Values are expressed in units $g^{-1}$of FW of plant leaf sample.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later alphabets for lower means. Similar small letters in are non-significant.

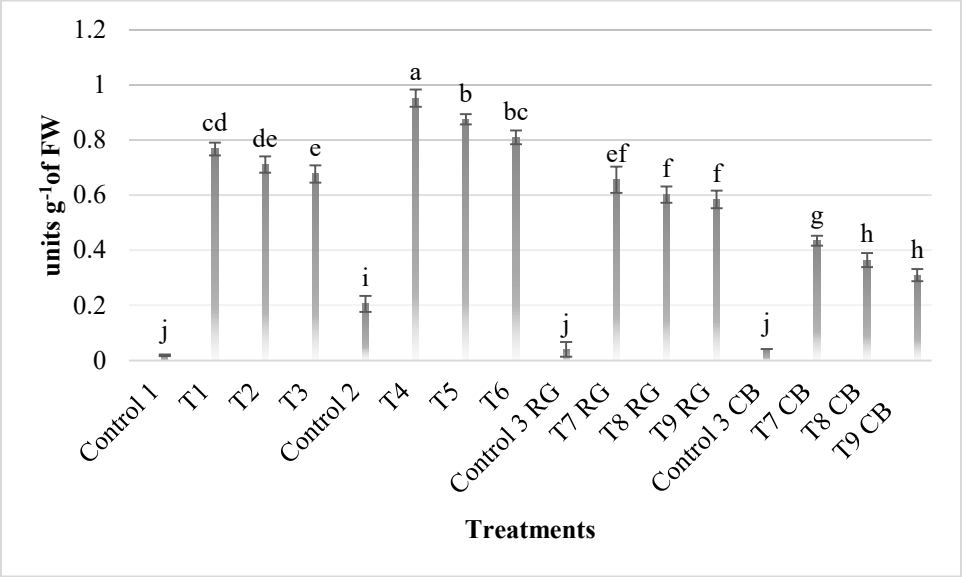

Figure 22 Enzymatic profile (H_2O_2) of *Lolium perenne* and *Ricinus communis* with reference to different Applied treatment*Values are expressed in units $g^{-1}$of FW of plant leaf sample.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later Alphabets for lower means. Similar small letters in are non-significant.

Physicochemical Parameter phase 2

Table 6 Physicochemical parameters phase 2

Description	**Treatments**	**TDS** mg/kg	**EC** (µScm-¹)
Contaminated Soil	CS	566.00	988.67
Fresh Soil+Castor Bean	Control 1	191.00	348.33
C.S + Castor Bean + Compost	T1	998.33	298.33
C.S+Castor Bean +Compost+Bacteria1	T2	711.00	246.00
C.S+Castor Bean+Compost+Bacteria 2	T3	777.67	257.33
Fresh Soil+Rye Grass	Control 2	215.00	422.00
C.S+Rye Grass+Compost	T4	771.33	506.33
C.S+Rye Grass+Compost+Bacteria1	T5	878.33	446.00
C.S+Rye Grass+Compost+Bacteria 2	T6	812.33	380.67
Fresh Soil+Rye Grass+Castor Bean	Control 3	215.00	421.67
C.S+Castor Bean+Rye Grass+Compost	T7	803.00	250.33
C.S+Castor Bean+Rye Grass+Compost+Bacteria1	T8	798.00	291.00
C.S+Castor Bean+Rye Grass+Compost+Bacteria2	T9	811.33	280.67

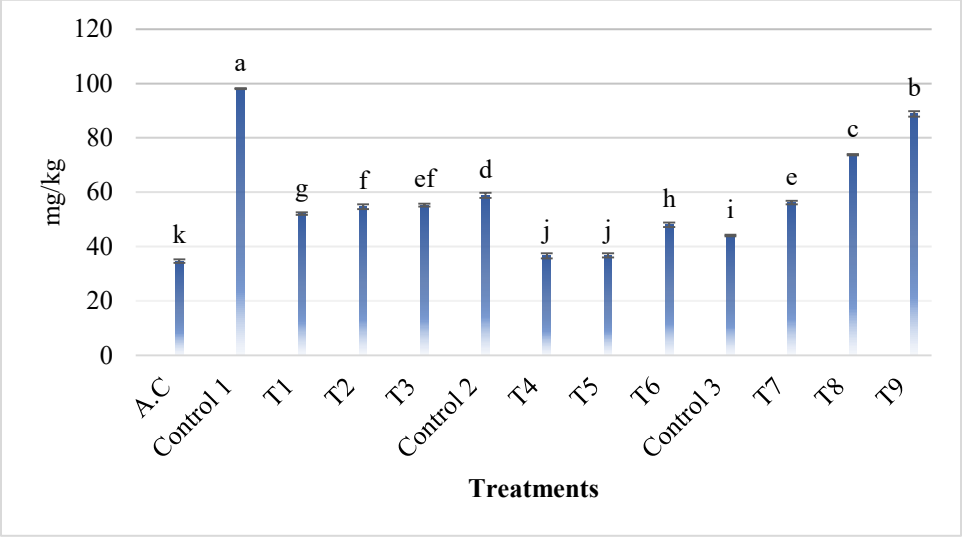

Figure 23 Nitrate content in soil with different treatments.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later Alphabets for lower means. Similar small letters in are non-significant.

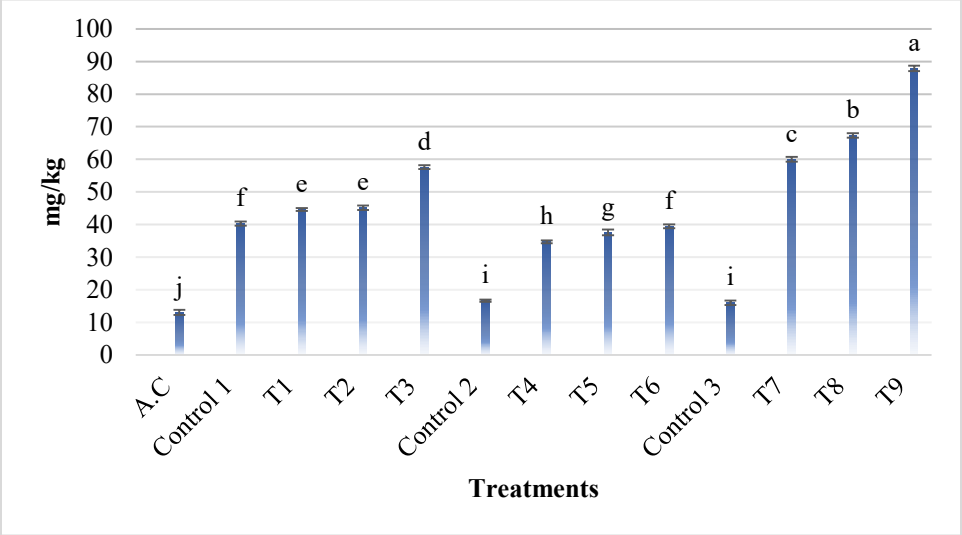

Figure 24 Extractable phosphorous content in soil with different treatments.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later Alphabets for lower means. Similar small letters in are non-significant.

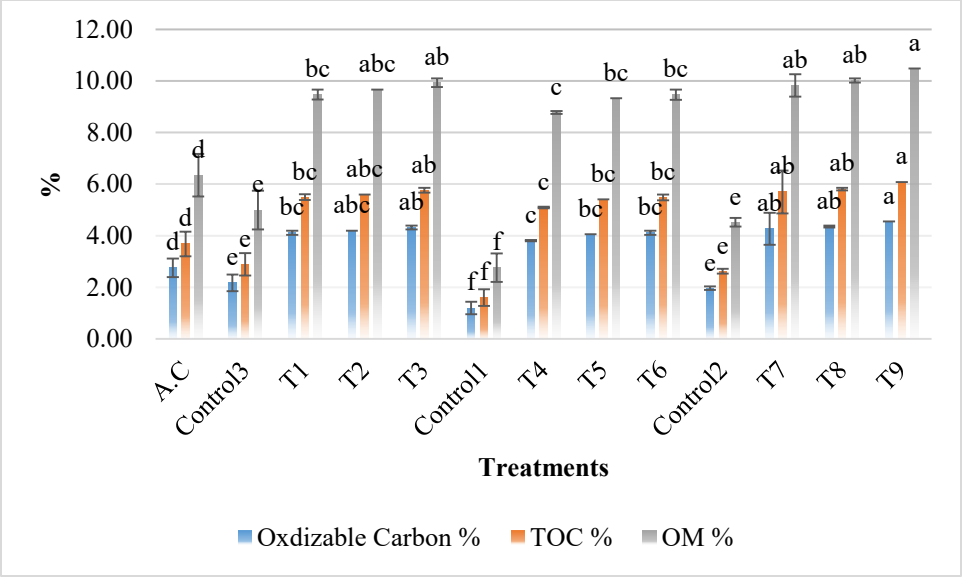

Figure 25 Oxidizable Carbon, total organic carbon, organic matter content in soil with different treatments.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later alphabets for lower means. Similar small letters in are non-significant.

3.8. Heavy metals content in soil and plants.

Heavy metal accumulation was analyzed in the roots and shoots of plants to rate the overall accumulation of heavy metals among various treatments applied to co-contaminated soil.

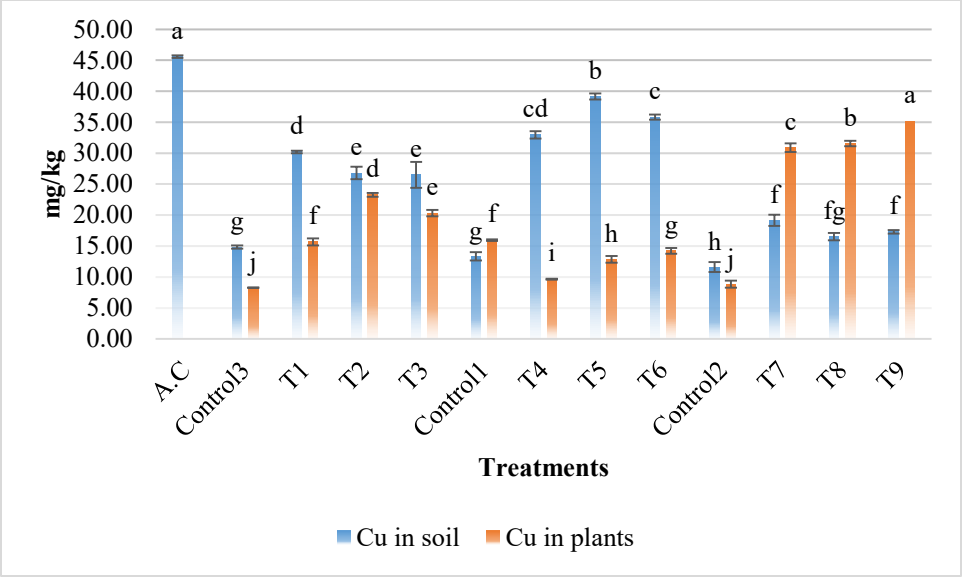

Figure 26 Cu content in soil and plants in diverse setup of treatments.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later alphabets for lower means. Similar small letters in are non-significant.

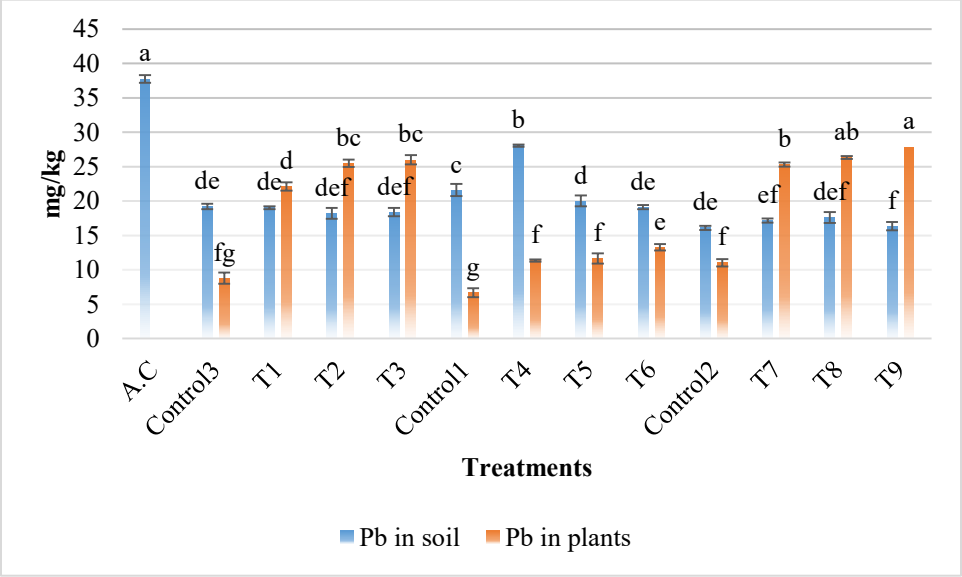

Figure 27 Pb content in soil and plants in diverse setup of treatments.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later alphabets for lower means. Similar small letters in are non-significant

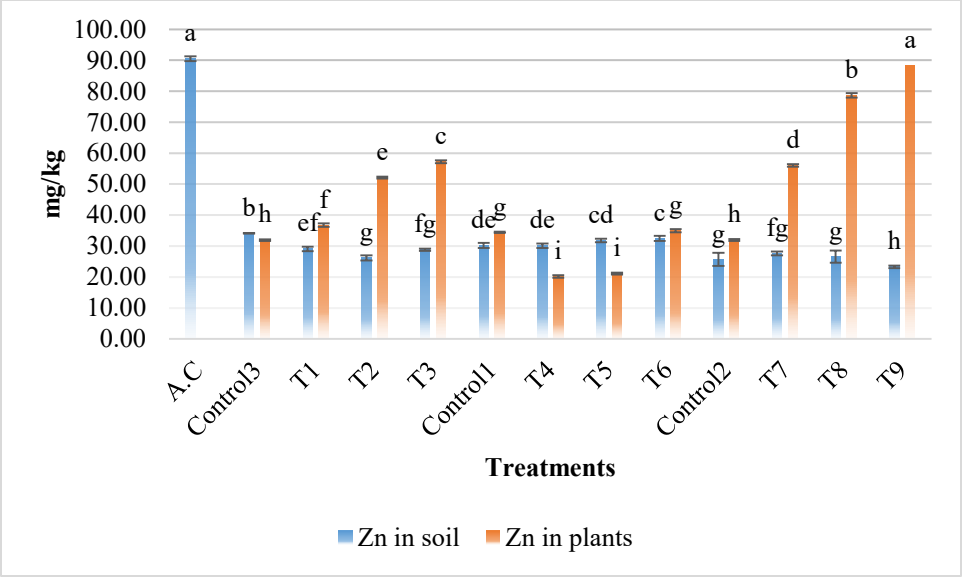

Figure 28 Zn content in soil and plants in diverse setup of treatments.

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later Alphabets for lower means. Similar small letters in are non-significant.

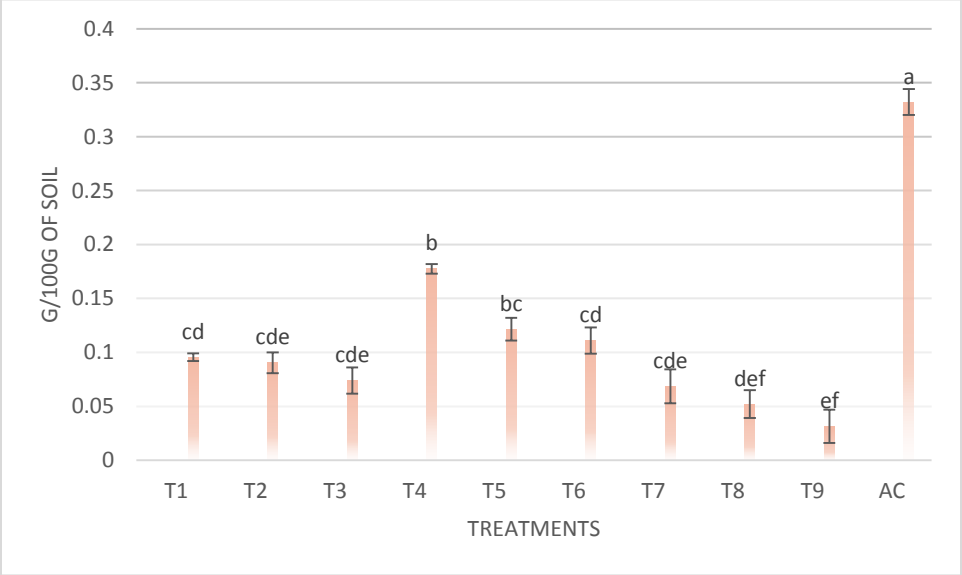

Figure 29 Residual total petroleum hydrocarbon content in soil

Data represented in means (n=3±SD). Significantly highest mean was "a" followed by later Alphabets for lower means. Similar small letters in are non-significant.

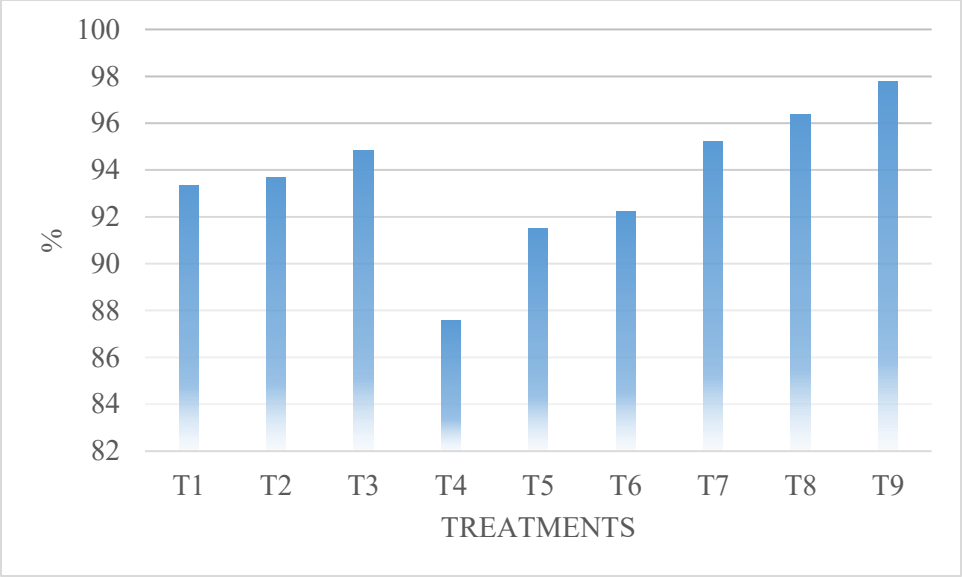

Figure 30 Removal percentage of TPH phase 2

Soil bacterial count for different treatments

Table 5 Phase 2 Bacterial count

Description	**Treatments**	**CFU (Cells g^{-1} of soil)**
C.S+Castor Bean +Compost+Bacteria 1	T2	$1.09*10\text{^}4^{c}$
C.S+Castor Bean+Compost+Bacteria 2	T3	$1.71*10\text{^}4^{b}$
C.S+Rye Grass+Compost+Bacteria 1	T5	$1.35*10\text{^}4^{bc}$
C.S+Rye Grass+Compost+Bacteria 2	T6	$9.00*10\text{^}3^{de}$
C.S+Castor Bean+Rye Grass+Compost+Bacteria 1	T8	$1.22*10\text{^}5^{ab}$
C.S+Castor Bean+Rye Grass+Compost+Bacteria2	T9	$1.59*10\text{^}5^{a}$

Discussion

Nowadays, many sites in the world are largely contaminated with multiple type of pollutants, where bioremediation phenomenon is need to be handled carefully as it is seen that sometimes the remediation of single contaminant triggers the mobility or availability of pollutants residing next to that contaminant. So, it is need of the time to design such bioremediation strategies which can target multiple pollutants at the same time. One such strategy is used of phytoremediation along with bio augmentation and co-plantation where a suitable microbe- plant combination is used to degrade the polluted matrix. Different treatments were arranged in this study with two types of plant combinations i.e. one combination is *Zea mays L.*with *Ricinus Communis* and other combination is *Lolium perenne* with *Ricinus Communis* .

Contamination with TPHs and HMs alters the physiochemical status of the soil which in turn affects the soil microbial and enzymatic activities. Soil pH is considered to be a key soil parameter as it influences many processes and activities in the soil. In this study pH of the non-contaminated i.e. fresh soil and contaminated soil i.e. TPHs and HMs co-contaminated soil was observed almost the same (very minute fluctuations) which indicates that TPHs contamination does not alter the soil pH. Moreover, no significant difference was observed after all the treatments were applied. The soil pH in all initial and final treatments was found almost neutral. Masakorala et al., (2014) reported significant pH increase in TPHs contaminated soils.

Soil electrical conductivity (EC) and total dissolved solids (TDS) are indicators of soil health, mainly indicates the salinity of soil which refers to the quantification of soil salts present in the soil sample. The optimum level of EC for healthy soils is 0-2 mS cm-1 i.e. salts in soil is in adequate amount for healthy plant growth. Generally higher EC and TDS were observed for TPHs contaminated soils compared to non-contaminated soils. In this study, similar trend was obtained with highest EC and TDS observed for Aged and Spiked soil contaminated with TPHs (Table 3.1). According to (Sanchez et al. 2019) it was reported that due to addition of TPHs and HMs in the soil, the soil EC was increased

because the charged species were added into it. It was also found that the EC and TDS of the fresh soil was less than the EC and TDS of the contaminated soil.

Nutrient status of the contaminated soil indicates the degradation efficiency. Nitrogen and phosphorus are considered as primary limiting nutrients (Sarkar et al. 2005). In this study, nitrates content in the soil were observed interestingly diverse setting for all the treatments whereas concentration of extractable phosphorus alters in the applied treatments.

Soil organic matter OM varies in composition and is representative of remains of roots, plant material and soil organisms in various stages of decay. It is considered as a nutrient reservoir in the soil thus improves soil fertility. It also enhances soil porosity and aeration, improves water holding capacity and reduces nutrient leaching (Hussain et al., 2018). Total organic carbon TOC is the carbon stored in organic matter. Usually oxidizable organic carbon OOC content in the soil is measured and converted into TOC and OM using a constant factor. The highest value of OOC, TOC and OM were observed for C.S+Maize+Castorbean+ *Bacillus safensis* +Compost (Fig 3.4.3) Phase 1and C.S+Ricinus Communis +Lolium perenne+Compost+*Bacillus safensis* (Fig 3.7.2) Phase 2 which is an indicator for enhanced plant growth and high microbial activity. Least value of OOC, TOC and OM were observed for treatments followed single plant or bacterium inoculant by this might be due to absence of plant because plant is essential for maintaining the optimal levels of organic matter.

Petroleum hydrocarbons in combination with heavy metals are considered among most phytotoxic contaminants and several studies reported that TPHs and HMs persistivity in soil would negatively impact plant growth. Hussain et al., (2018) reported that suppressed plant growth was observed for TPHs contaminated soils due to limited availability of nutrients however use of organic amendments not only enhance soil fertility but also improves soil porosity and aeration thus promoting plant growth. HMs-TPHs contamination produces nutrient deprived, infertile soils. Toxicity of low molecular weight hydrocarbons and hydrophobic behavior reduce the ability of plants to uptake

water and nutrients and are considered to be primary factors that inhibit plant growth (Yousaf et al., 2011). Hydrocarbons may form thin film coating around root cells thus reducing or preventing absorption of water and nutrients and gaseous exchange. They may damage cell membranes if they successfully penetrate into plant tissues and adversely impact metabolic activities. The germination response of seeds may be inhibited due to hydrocarbon coating around the seeds which act as a physical barrier and prevents entry of water and oxygen. Hydrocarbons may enter the seeds where they directly kill the embryo due to acute toxicity or alter the metabolic activities (Serrano et al., 2009). Different plant species behave differently in HMs-TPHs stress. Some exhibit best performance in regard to tolerance while others under unfavorable conditions showed reduced growth (Yousaf et al., 2010).

Environmental stresses whether biotic or abiotic generates oxidative stress due to enhanced production of ROS i.e. overflow of ROS that exceeds defense mechanism. This oxidative stress can cause peroxidation of lipids, protein oxidation, damage to nucleic acid, enzyme inhibition and activation of programmed cell death (Martí et al., 2009). TPHs contamination induces oxidative stress on plants. From results, it can be seen that TPHs contamination negatively influenced SOD (Fig 3.6.1) MDA (Figure 3.6.2) while negatively to photosynthetic pigments (Figure 3.5.2) and resulted in increased activities by APX and CAT while reduced activity by GPX (Figure 3.6.3).

Plant growth is generally assessed by its biomass or growth parameters which are root length, fresh weight, dried weight, shoot length, fresh and dried weight. This study showed the maximum plant growth for fresh Fresh Soil+Maize+Castor Bean (Fig 3.2.1). Both root and shoot length and fresh weights were higher in this treatment followed by co-contaminated soil Castor bean + Maize *Bacillus safensis*+Compost where inoculum and both plant combined to cope with pollutant stress and plant exhibits improvement in growth.

Chlorophyll is a vital chemical substance found in autotrophic organisms which plays key role in carrying photosynthesis – life sustaining process. Any stress or nutrient injury can adversely affect chlorophyll thus reducing its content. Measurement of chlorophyll content represent the state of organism and conditions in which it is present thus

chlorophyll is considered to be biomarker of environmental stress (Rastogi et al. 2017). High chlorophyll levels indicate high availability of nutrients (mostly N and P), whereas low levels indicate stressed environment (Hussain et al., 2018). Carotenoids are also plant pigments which facilitates plant by absorbing light from across the color spectrum. The highest chlorophyll level was noted for FS+ Castor bean which is indicator of high nutrient availability and lowest was observed for plant grown in co-contaminated soil which indicated poor nutrient status due to TPHs and HMs stress.

Lipid peroxidation is a process where ROS attack lipids especially PUFAS (polyunsaturated fatty acids); structural component of cell membranes and damage cellular integrity. Lipid peroxidation is comprised of three steps i.e. initiation, propagation and termination and produces variety of oxidation products. Among many different aldehydes which are produced as secondary products; malondialdehyde MDA, is one of them that has been used as convenient biomarker for lipid peroxidation due to oxidative stress. Hydrogen peroxide is a type of ROS produced inside the plants act either as damaging or signaling molecule depending upon the delicate balance between its formation and scavenging (Niu and Liao, 2016). Plant control (FS+ Maize) exhibit lowest levels of MDA and H2O2 content among all treatments which indicates stress free environment. Highest level of MDA was observed for contaminated soil+ Maize and Contaminated soil+ Castor bean.

Production of antioxidant enzymes is counteract mechanism of plants in response to oxidative stress and is considered as a first line of defense by plants upon stress exposure (Khan et al., 2019). Antioxidants are crucial for our existence otherwise ROS would end eaten up all the cells. Antioxidants are those molecules which can safely donate their electron to free radical specie to stop chain reaction. They can be enzymatic as well as non-enzymatic. Ascorbate peroxidase APX, guaicol peroxidase GPX and catalase CAT are categorized into enzymatic antioxidants. Usually an organelle possesses more than one ROS scavengers (Caverzan et al., 2012). Upon stress exposure fluctuations in levels of these enzymes were observed. Shahzad et al., (2016) reported increase in plant antioxidant enzyme production when exposed to hydrocarbons.

Catalase and guaicol peroxidase are defense mechanism against H2O2 which is considered as one of the major ROS in the cells. They decompose H2O2 into water and oxygen. Ascorbate peroxidase also scavenges H2O2 with much higher affinity than CAT. CAT and APX are highly specific for H2O2. CAT have very fast turnover rate i.e. 1 molecule of CAT catalyzes 40 million molecules of H2O2 per second but they have much lower affinity for H2O2 than APX and peroxides (Mhamdi et al., 2010).

In this study antioxidant enzyme assays were carried out for ascorbate peroxidase, catalase, guaiacol peroxidase, and higher levels of APX, CAT, and GPX were observed in CS soil+ Maize and CS+Castor bean treatment (Figure 3.3.) that indicates high levels of stress induced by TPHs. Up regulation of these enzymes reduces direct cytotoxicity caused by ROS production (Liu et al., 2009). Significant reduction in level of these enzymes was observed in treatments where microbial strain and both plants are used along with the compost for remediation. It can be inferred that combination of biological and organic amendments methods for TPHs removal is an effective remediation strategy. Production of GPX in Ricinus Communis exhibited completely different trend in response to stress. It was observed highest in control where no TPHs were present and least for TPHs contaminated soil. GPX might be downregulated or damaged due to cytotoxins. Liu et al., (2009) reported that at high stress levels, antioxidant enzymes can be damaged or down regulated

Chapter 4

Conclusions

Advent of industrialization led to the more and more release of toxic contaminants in the environment which causes the pollution of all matrices (soil, air, and water). These pollutants reside with each other in soil and can be taken up by plants through which they enter into the food chain. Remediation of these contaminated sites is quite tricky and needs proper strategic planning. This study concludes that plant-microbe combination applied for remediation of co-contaminated sites with organic (TPHs) and inorganic (heavy metals) pollutants can not only degrade the petroleum hydrocarbons but also immobilize the heavy metals too and make them unavailable to flora and fauna thus remediating the soil. This study proves that unique co-plant combination can be the best remediation strategy for soil contaminated with multiple pollutants. Co-planted mediated bioremediation also helps soil to rejuvenate its fertility by adding nutrients and enzymes to it. However, there is still need to explore other aspects of co-plant combination with bacteria and efficacy of this combination can also be tested against persistent organic pollutants in fields.

Future recommendations

Conclusively, based on this experimental study, the advances of remediation techniques have improved our knowledge about remediation of toxic heavy metals which has detrimental effects on our ecosystem as well on human health. This study was conducted for phytoremediation of Zn, Cd, Pb and TPH co-contaminated soil by using co-plantation of castor bean ryegrass and maize with compost addition and bacterial strains in two phases. This study paved a path to find out more sustainable and ecofriendly ways against co-contamination of soil that co-plantation of different plant combinations with organic amendments and bacteria could be more impactful remediation technique. Further studies can also be made to evaluate the potential of co-plantation with fungus to remediate co-contaminated soi

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