Phytoremediation of Co-Contaminated Soil using Plant-Bacteria Synergism

by

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DEPARTMENT OF ENVIRONMENTAL SCIENCES

FACULTY OF BIOLOGICAL SCIENCES

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in

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Certificate of Approval

This is to certify that the research work presented in this Thesis, entitled **“Phytoremediation of Co-Contaminated Soil using Plant-Bacteria Synergism”** was conducted by **Zoha Zehra (Reg. No. 02312113003)** under the supervision of **Dr. Sohail Yousaf**. No part of this thesis has been submitted elsewhere for any other degree. This thesis is submitted to the **Department of Environmental Sciences,** in partial fulfilment of the requirements for the degree of **Master of Philosophy** in the field of Environmental Science, Quaid-i-Azam University, Islamabad, Pakistan.

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Abstract

Co-contaminated soils pose a particularly daunting challenge, as they simultaneously bear the burden of multiple pollutants such as heavy metals and petroleum hydrocarbons. Cadmium and total petroleum hydrocarbons co-contamination in agricultural soil is one such example, with unique remediation demands. To address this challenge, a meticulously designed experimental setup was employed, involving soil spiking with the two selected contaminants. Various treatment permutations were applied with *Sorghum bicolor* as the phytoremediator, combined with the bacterial strains *Bacillus cereus* and *Serratia bozhouensis*, and compost as an organic amendment. The contaminant removal efficiency along with the impact on various soil and plant parameters was observed for each treatment. The outcomes of this study were assessed through various statistical analyses, with Principal Component Analysis emerging as a particularly informative tool. The treatment, T4, which incorporated both organic compost and *Serratia bozhouensis* exhibited the highest levels of Cd (61%) and TPH removal (75%), indicating a synergistic effect in enhancing remediation efficiency. T5, involving *Bacillus cereus* and compost, demonstrated similar but slightly lower efficacy. The synergy between compost and bacterial strains demonstrated a promising avenue, merging bioaugmentation and biostimulation. Plant metrics, stress markers, and physiological indicators all experienced positive enhancements through organic amendment application, further accentuated by the inclusion of plant growth-promoting bacteria. T4 exhibited the highest biomass along with the highest levels of photosynthetic pigments. The content of stress markers, MDA and H2O2, and antioxidant enzymes, APX and GPX, were remarkably lower in T4 as well. T5 had similar plant growth characteristics as T5 but at slightly lower levels. Furthermore, T3 showed better plant growth characteristics than both T1 and T2. Additionally, observations highlighted the more dominant influence of Cd contamination on soil and plant parameters compared to TPH contamination. This study offers profound insights into the multifaceted dynamics of co-contaminant remediation, exploring pathways towards sustainable soil restoration techniques.

Keywords: Co-contamination, phytoremediation, cadmium, petroleum hydrocarbons, sorghum

Chapter 1
Introduction

In the pursuit of development and poverty alleviation, developing countries like Pakistan often prioritize goals centered around economic growth and industrialization. However, this approach comes at a cost. As developing countries focus on rapid industrial expansion, environmental concerns are often neglected. Therefore, increasing levels of pollutants such as heavy metals and hydrocarbons become inevitable in these countries. In addition, developing countries have limited resources to address and mitigate environmental contamination. For instance, inadequate waste disposal and treatment technologies, inefficient environmental legislation, and a weak environmental education system contribute to the complexities of addressing environmental issues (Anyanwu et al., 2018; Ding, 2019).

In this study, we looked at an economically viable strategy for the cleanup of pollutants: phytoremediation. Furthermore, since contamination of a single pollutant rarely occurs in isolation, we considered the remediation of co-contamination, specifically that of cadmium (Cd) and total petroleum hydrocarbons (TPH) in agricultural soil (Ye et al., 2017). Additionally, we examined the role of plant-bacteria interactions and organic amendments in enhancing the efficiency of phytoremediation processes. By investigating the applicability of phytoremediation for heavy metal and hydrocarbon removal, we aim to contribute to the development of sustainable and cost-effective soil remediation practices in developing countries.

1.1 Cadmium: Sources and Effects

Cd is a non-essential transition metal that has been classified among the 126 priority pollutants by the US EPA. It has also been declared a probable human carcinogen (Harikumar & Sreedevi, 2023). In natural systems, Cd is released through weathering of rocks containing this element, forest fires, volcanic eruptions, and wastewater. On the other hand, human activities contribute to Cd contamination, primarily through metallurgical works, mining, electroplating, paints, combustion emissions, and the excessive use of fertilizers and pesticides. One notable characteristic of Cd is its high solubility and mobility compared to other metals, making it easily absorbed by plants. Once taken up by plants, Cd is transported and accumulated in the edible parts of these plants (Zulfiqar et al., 2022).

1.1.1 Sources of Cd in Agricultural Soil

The occurrence of Cd in soil, which typically ranges from 0.07 to 1.1 mg/kg, has raised significant concern. While Cd polluted soils can have higher levels, typically between 3 to 10 mg/kg soil, it is important to highlight that agricultural soils have a threshold range of approximately 100 mg/kg (Zulfiqar et al., 2022). One of the primary sources of Cd in agricultural soil stems from the application of phosphate fertilizers in farming practices and the utilization of municipal sewage sludge as a soil amendment (Li et al., 2020).

Phosphate fertilizers are manufactured from phosphate rock which contains varying levels of Cd. This range can be as low as two and as high as 1500 mg Cd/kg P_2O_5. To address the significant impact of phosphorus fertilizers on Cd levels in soil, the European Union enacted Regulation (EU) 2019/1009 to establish restrictions on Cd content in organo-mineral fertilizers. The regulation limits Cd content to a maximum of 60 mg/kg Cd//kg P_2O_5. However, this threshold value may not be deemed effective for control of Cd bioaccumulation. Fertilizers with significantly lower Cd content, not more than 20 mg Cd/kg P_2O_5, prove more effective in limiting the process of Cd bioaccumulation. Since ceasing phosphate fertilizer application is not feasible, adopting remediation strategies like phytoremediation before crop cultivation becomes necessary (Suciu et al., 2022).

1.1.2 Effects of Cd Exposure in Humans, Animals, and Plants

Cd toxicity has significant implications for humans and animals. In humans, the kidneys are particularly susceptible, where Cd deposition leads to observable defects in protein, amino acid, glucose, bicarbonate, and phosphate reabsorption, known as Fanconi syndrome. Approximately 30% of Cd in the body accumulates in the kidney tubule region, causing tubular damage proportional to the amount of Cd unbound to metallothionein. Moreover, individuals with diabetes are more susceptible to renal tubular damage from Cd exposure compared to the general population (Sekar et al., 2022).

Cd also interferes with Vitamin D metabolism in the kidneys, which has adverse effects on bone health. Combined with direct impairment of calcium absorption in the gut and disruption of collagen metabolism, Cd can lead to conditions like osteomalacia and osteoporosis. The most severe example of this is itai-itai disease, a condition observed in Japan, characterized by excruciating pain from osteomalacia, osteoporosis, renal tubular dysfunction, anemia, and calcium malabsorption. The immune system is also affected by Cd-induced impairment. Prenatal exposure to Cd can hinder postnatal T cell production and response to immunization, as well as disrupt thymocyte development (Bernhoft, 2013).

In animal studies, Cd exposure has been linked to male infertility through damage to the blood-testis barrier, reduced germ cell adhesion, decreased sperm count, and subfertility or infertility.

may induce the production of prostaglandin F2alpha, causing vasoconstriction in the cavernosal region and suppressing testosterone synthesis and secretion in males. In females, it can lead to the destruction of the corpus luteum and fetus. However, human epidemiological studies have not strongly supported Cd as a direct cause of male infertility or erectile dysfunction (Alaee et al., 2014).

Cd's toxic effects extend to the central nervous system, leading to oxidative stress, disturbances in membrane function, decreased acetylcholinesterase activity, and depletion of antioxidants. These changes can result in the apoptosis of cortical cells. Clinically, individuals with elevated blood or urine Cd levels may experience decreased attention, memory, and low-frequency hearing. In animal studies, Cd has been shown to impair learning ability, destroy olfactory nerve function, and affect cortical electrical activity (Brance et al., 2020). Finally, Cd is classified as a Class B1 carcinogen by the United States Environmental Protection Agency (Adedapo & Adeoye, 2014).

Cd serves no known physiological function in plants as well. However, it can be absorbed by plants to toxic levels. The predominant form of Cd in the soil is Cd^{2+}. Plant root cells facilitate the entry of Cd^{2+} through a range of micronutrient transporters from the family of zinc-regulated transporter/iron-regulated transporter-like proteins, as well as natural resistance-associated macrophage proteins. Furthermore, the transportation of Cd^{2+} to root cells involves cation channels, including depolarization-activated calcium channels, hyperpolarization-activated calcium channels, and voltage-insensitive cation channels. These mechanisms contribute to the uptake of Cd by plants, potentially leading to elevated toxic concentrations (Asgher et al., 2014).

Cd is phloem-mobile and capable of localizing in any part of the plant. Its toxic effects are manifested through notable anatomical alterations in the roots, stems, and leaves. For example, exposure to Cd results in the closure of abaxial stomata, reduction in stomatal size in leaves, scarification in tracheid walls, narrow xylem vessels, and disorganization in vascular bundles in roots and stems. Other effects include decreased trichome length, density of stomata, and cortex proportion. These structural changes negatively impact plant growth and function by reducing the size of parenchyma in leaves, disrupting the ultrastructure of chloroplasts, altering vascular tissue organization, reducing epidermal tissue thickness, and causing narrow xylem and phloem vessels (Bora & Sarma, 2021).

Cd toxicity is also associated with oxidative damage in plants. Cd, like other heavy metals, triggers the production of reactive oxygen species (ROS), such as hydrogen peroxide (H2O2), and lipid peroxidation, leading to oxidative stress. The excessive ROS production induced by

Cd impairs antioxidant enzymes, such as peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD) (Kapoor et al., 2019). The accumulation of Cd in plants disrupts normal levels of ROS and antioxidants, resulting in oxidative damage to nucleic acids, lipids, proteins, and cellular pigments like chlorophyll and carotenoids. This oxidative stress leads to the accumulation of thiobarbituric acid reactive substances (TBARSs) and malondialdehyde (MDA), causing electrolyte leakage under Cd stress (Younis et al., 2016).

Furthermore, Cd toxicity disrupts the biosynthesis of photosynthetic pigments, inhibits chlorophyll biosynthesis, and impairs photosystems (PSI and PSII). It also hampers the Calvin cycle and inhibits key enzyme activities involved in CO2 fixation. The reduction in carbon metabolism and photosynthesis due to Cd stress adversely affects plant growth, photosynthetic traits, and grain yield in various crops. The severity of Cd-induced yield reduction varies depending on factors such as genotype, Cd concentration, and exposure duration (Abbas et al., 2014).

1.2 Total Petroleum Hydrocarbons: Sources and Effects

The rapid global economic growth has increased the demand for petroleum-based products, leading to detrimental environmental consequences. Petroleum hydrocarbons encompass a diverse range of compounds, including hydrocarbons, polycyclic aromatic hydrocarbons, benzene, toluene, ethylbenzene, xylene, linear alkanes, cyclohexanes, and other organic compounds. The cumulative concentrations of petroleum hydrocarbons in the environment are commonly measured as total petroleum hydrocarbons (TPH), typically spanning carbon numbers from C10 to C40. As a result of petroleum-related activities, soil contamination by TPH has become a significant concern, necessitating effective remediation strategies to mitigate their adverse effects (Grifoni et al., 2020).

1.2.1 Sources of TPH in Agricultural Soil

The United States Environmental Protection Agency has classified several petroleum hydrocarbons as priority pollutants for environmental remediation (EPA, 2015). According to the European Environment Agency (EEA), petroleum hydrocarbons account for approximately 50-60% of the primary contaminants impacting soil. The sources of petroleum hydrocarbon soil contamination are primarily associated with various activities within the petroleum industry, including oil exploration and transportation. Contamination arises from leaks occurring in storage tanks, as well as during the production of refined petroleum products. Accidental oil spills resulting from pipeline accidents also contribute significantly to this form of contamination. Furthermore, in many developing countries, the unauthorized collection of petroleum from oil pipelines is a prevalent and illicit practice. This unlawful activity contributes to petroleum hydrocarbon pollution in soil (Ibeto et al., 2023; Grifoni et al., 2020). The contamination of agricultural soil by petroleum hydrocarbons is exacerbated by the presence of brownfields. Brownfields refer to abandoned, underused, and polarized industrial sites that contain high concentrations of toxic pollutants. In Pakistan, many crude oil-contaminated brownfields have remained unproductive for decades due to their unsuitability for agriculture or commercial use. Particularly in the province of Punjab, these brownfields are frequently located in close proximity to suburban or rural agricultural settings, posing a severe threat to public health. The proximity of these contaminated sites to agricultural areas increases the risk of petroleum hydrocarbon migration into the soil, thereby affecting crop quality and food safety (Rehman et al., 2019).

1.2.2 Effects of TPH on Agricultural Productivity and the Food Chain

Exposure to TPH has been associated with significant risks, including potential long-term damage to the central nervous system. Furthermore, numerous compounds associated with petrogenic contamination possess carcinogenic properties, posing additional health concerns (Sarma et al., 2023). The uptake of petroleum hydrocarbons in plants has significant implications for the food chain and the overall ecosystem. There are two pathways through which petroleum hydrocarbons enter plants: soil-to-plant and air-to-plant. Soil-to-plant uptake involves root absorption and subsequent translocation, while air-to-plant uptake occurs through atmospheric deposition and contact with stomata (Marchal et al., 2014).

The ability of TPHs to be taken up by plants is influenced by various factors, including their physicochemical properties, such as the coefficient of partition octanol-water (Kow), environmental conditions, and plant species. Hydrophilic compounds with a log Kow value of less than four are more likely to be absorbed by the roots and translocated to other plant tissues. In contrast, highly hydrophobic, non-ionized compounds with a log Kow value greater than four are less likely to be incorporated into the inner roots and translocated within the plant. Additionally, plant roots can take up non-ionizable, polar, and highly water-soluble compounds, such as pyrene (with a log Kow value of 4.88), and translocate them to the shoots (Shi et al., 2017).

Plants cultivated in soils contaminated with petroleum hydrocarbons exhibit various abnormalities in growth and metabolism. These effects include reduced chlorophyll content, starch metabolizing enzymes, and antioxidant activity, along with a significant decrease in overall productivity and plant yield (Haider et al., 2021). For instance, one study showed that contamination of soil with 1% w/w crude oil led to a 6.4% reduction in plant height and a

21.9% decrease in plant biomass in maize (Adieze et al., 2012). In another study, petroleum hydrocarbon soil contamination reduced root biomass by 8.0%, leading to a 12.0% decline in shoot biomass yield in perennial ryegrass (Afegbua & Batty, 2018).

1.3 The Challenges of Co-Contamination of Heavy Metals and TPH in Agricultural Soil

The natural attenuation process, performed by indigenous microorganisms, serves as an initial step in the removal of TPH from the environment. However, the presence of co-contaminants, including heavy metals such as lead (Pb), Cd (Cd), copper (Cu), zinc (Zn), and others, poses a significant challenge to the degradation of TPH. These heavy metals directly interact with the enzymes involved in TPH breakdown, causing complications in the degradation process. While essential metals like zinc (Zn) play a crucial role in microbial life, non-essential metals such as lead (Pb), Cd (Cd), and copper (Cu) are toxic to microorganisms (Su et al., 2023).

Some studies show that as the concentration of heavy metals increases, the effectiveness of microbial biodegradation of petroleum hydrocarbons tends to decline. Oriomah (2015) observed that *Achromobacter xylosoxidans* exhibited tolerance to both Cu (II) and waste oil individually. However, when both contaminants coexisted, the ability of *A. xylosoxidans* to degrade waste oil significantly decreased as the concentration of Cu (II) increased. Baltrons (2018) demonstrated that the inhibition of microbial degradation of 3-4-ring PAHs increased with heavy metal concentration.

Heavy metals can hinder microbial petroleum hydrocarbon biodegradation by damaging cells and reducing their viability. Moreover, heavy metals can impact the functional properties of biologically active substances such as enzymes and proteins. Liu (2015) discovered that exposure to Pb (II) altered the spatial conformation of *Bacillus malacitensis* catechol 2,3-dioxygenase, blocking the catalytic active site, inducing reactive oxygen species (ROS), and damaging the enzyme protein. Despite the importance of understanding the impact of heavy metals on petroleum-contaminated sites, earlier research has often overlooked their influence, focusing primarily on hydrocarbon removal without considering the potential interference of heavy metals. Therefore, further investigation is necessary to elucidate the process of petroleum hydrocarbon removal by soil microorganisms under heavy metal stress.

1.4 Phytoremediation of Heavy Metal and TPH Co-Contamination

Phytoremediation is a natural approach that utilizes plants and their associated soil microbes to mitigate the concentrations and harmful effects of contaminants in the environment (Greipsson, 2011). This method is applicable for addressing a wide range of pollutants, including heavy metals, radionuclides, and organic pollutants such as polynuclear aromatic hydrocarbons, polychlorinated biphenyls, and pesticides. One of the significant advantages of phytoremediation is that plants can effectively manage contaminants without negatively impacting the topsoil, thereby preserving its functionality and fertility. In fact, plants can even enhance soil fertility by introducing organic matter into the system. The term "phytoremediation" combines the Greek word "phyto" meaning plant and the Latin word "remedium" meaning to correct or remove an evil. Green plants possess remarkable capabilities to absorb pollutants from the environment and facilitate their detoxification through various mechanisms (Ali et al., 2013).

Phytoremediation offers an aesthetically pleasing and socially accepted solution for addressing environmental contamination, particularly in large field sites where other remediation methods are not feasible in terms of cost and practicality. Compared to alternative remediation options, phytoremediation is characterized by low installation and maintenance costs, making it a cost-effective choice (Van Aken, 2009). In fact, the cost of phytoremediation can be as low as 5% of alternative clean-up methods, making it an attractive option from a financial standpoint. Additionally, the establishment of vegetation on polluted soils serves as a natural safeguard against erosion and the leaching of metals. Phytoremediation can serve multiple purposes: (1) risk containment through phytostabilization, (2) phytoextraction of valuable metals such as Ni, Tl, and Au, (3) rhizospheric degradation of organic pollutants like petroleum hydrocarbons, and (4) sustainable land management, where phytoextraction gradually improves soil quality for subsequent cultivation of high-value crops. Moreover, fast-growing and high-biomass plants like hold the potential for both phytoremediation and energy production, offering a dual benefit (Ali et al., 2013).

Sorghum, a versatile crop, plays an important role in the agricultural landscape of Pakistan. It serves as both food and energy crop. With its adaptability to diverse agro-climatic conditions, sorghum holds significant value in sustaining food security and potentially meeting the growing energy demands of the country. Some studies have demonstrated the potential of sorghum for the phytoextraction of Cd (Wu et al., 2023; Chen et al; 2019). Other studies have shown its promise for use in the removal of TPH from soil (Ma et al., 2023; Koohkan et al., 2023). Therefore, assessing whether this plant is effective in the phytoremediation of TPH and Cd co-contamination is worth further research.

1.5 Enhancing Phytoremediation of Co-Contamination with Plant-Bacteria Synergism

Phytoremediation has emerged as an environmentally and economically favorable approach for remediating contaminated soils. However, a significant challenge in employing this technique is the sensitivity of many plant species to many different contaminants, especially when they occur simultaneously, such as TPH and heavy metal-contaminated soil. To overcome this limitation, a combination of bioaugmentation and phytoremediation, known as microbe-assisted phytoremediation, has been proposed as a solution. By incorporating microorganisms into the phytoremediation process, plant growth, and survival rates can be improved (Girolkar et al., 2021).

Microorganisms play a crucial role in degrading or transforming contaminants through various metabolic processes. While microorganisms are less effective in bioremediating heavy metals compared to organic contaminants, rhizospheric bacteria have been found to contribute to metal uptake in plant tissues, and organic compounds released by plant roots act as signaling agents for plant-bacteria interactions in contaminant removal (Xiang et al., 2022).

Plants can promote the degradation of hydrocarbons through immobilization and removal, as well as by enhancing microbial degradation. Plants can promote the degradation of hydrocarbons through immobilization and removal, as well as by enhancing microbial degradation. Plants provide specific carbon sources to associated bacteria, stimulating their degradation of TPHs. In addition, these bacteria can enhance the solubility of inorganic phosphates and degrade organic compounds containing phosphates, which are preferentially absorbed by plants (Timofeeva et al., 2022). Some plants release root exudates that facilitate the growth of requisite bacteria in hydrocarbon-contaminated soil, thereby increasing the bioavailability of TPHs for enhanced biodegradation (Khan et al., 2013). Therefore, the efficiency of phytoremediation of metal and TPH co-contamination not only depends on the selection of appropriate plant species but also on adequate heavy-metal resistant and hydrocarbon-degrading microbial species.

In a pot experiment involving *Ocimum gratissimum* cultivated in soil containing elevated levels of the heavy metal zinc and petroleum hydrocarbons, Choden et al. (2020) demonstrated that the most effective approach for addressing co-contamination was the introduction of *Pseudomonas putida* inoculation. The incorporation of *P. putida* into vegetated soil led to an elevated removal rate of total petroleum hydrocarbons (TPHs), indicating a favorable interaction between the plant and the introduced inoculant. Although metals differ from organic pollutants in terms of degradation, in situations involving soils contaminated by both organic and inorganic pollutants, microbes can facilitate the breakdown of organic compounds and improve plant growth characteristics to enhance plant tolerance to contaminant-induced toxicity.

Similarly, Jampasri et al. (2019) showed that the effectiveness of the plant, *C. odorata*, and the bacteria, *M. luteus*, as candidates for utilization in bacteria-assisted phytoremediation of soils co-contaminated with lead and fuel oil. Furthermore, the authors note that in the co-contamination setting, the symbiotic relationship between plants and bacteria plays a pivotal role. The primary mechanism driving hydrocarbon remediation appears to be biodegradation facilitated by microorganisms present in the plant's rhizosphere, a process known as rhizodegradation. The interplay between plants and their associated bacteria leads to a mutually beneficial interaction. Plants provide specialized carbon sources like carbohydrates, organic acids, and amino acids to the bacteria. These compounds act as stimuli, prompting the bacteria to engage in the degradation of Total Petroleum Hydrocarbons (TPHs). Furthermore, a notable observation was the plant roots' ability to enhance the tolerance of soil microorganisms to petroleum hydrocarbons, as documented by Jampasri et al (2019).

In another study, soil was spiked with diesel fuel, as well as copper (Cu), lead (Pb), and cadmium (Cd). The plant *Festuca arundinacea* was cultivated in the co-contaminated soil environment, with the aim of evaluating the phytoremediation capabilities. Among the trio of heavy metals studied, cadmium (Cd) demonstrated the most efficient uptake by tall fescue and TPH was degraded significantly as well. Importantly, the study highlighted that the dominant genera among the bacterial community within the soil exhibited the capability to biodegrade petroleum hydrocarbons and endure heavy-metal exposure. Certain bacterial communities found in the studied environment included *Paludibaculum*, *Marinobacter*, *Sphingomonas*, *Acidobacterium*, and *Rugosibacter*. These bacterial genera were particularly prominent and played significant roles in the remediation of the heavy metal and TPH co-contamination (Lee et al., 2023). The study further confirms the importance of plant-bacteria synergism for co-contaminated soil.

In another study, researchers isolated a plant growth–promoting (PGP) rhizobacterium, identified as *Novosphingobium* sp., from the rhizosphere of *Festuca arundinacea*. This bacterium exhibited traits associated with promoting plant growth, such as the production of indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, and the generation of siderophores. It also displayed the ability to thrive on growth media containing substantial concentrations of heavy metals (Cu or Pb). To assess the impact of bacterial inoculation, the researchers carried out experiments using soil contaminated with heavy metals (Cu and Pb) and diesel fuel. The soil was then planted with tall fescue, a plant known for its ability to accumulate pollutants. When compared to co-contaminated soil without bacterial inoculation, bacteria-inoculated soil exhibited significantly elevated concentrations of bioavailable Cu, increased tall fescue biomass, and enhanced root growth. The data analysis revealed positive correlations between bacterial inoculation and Cu bioavailability, Cu accumulation in the plant, and diesel removal efficiency (S. Y. Lee et al., 2022).

Collectively, these studies provide valuable insights into the potential of harnessing plant-microbe interactions to combat co-contamination challenges effectively. The findings underscore the necessity of considering both microbial and plant components when designing strategies for remediating environments with multiple pollutant sources.

1.6 Benefits of Organic Amendments for Phytoremediation

Composting is a valuable technique for remediating heavy metal-contaminated soil by stabilizing organic wastes and promoting the formation of mineral ions, humic substances, and beneficial microbes. These components contribute to the immobilization of heavy metals through reactions such as adsorption, complexation, precipitation, and redox reactions. By effectively binding heavy metals, composting reduces their mobility and mitigates their ecological and environmental risks in agricultural soils (Huang et al., 2016).

In addition to its remediation benefits, compost serves as an organic fertilizer, improving soil structure, fertility, and microbial activity. This enhances nutrient cycling and availability, leading to increased crop biomass and improved plant growth. With its low cost, practicality, and environmental friendliness, composting offers a sustainable approach for repairing heavy metal-contaminated agricultural soil. It minimizes reliance on chemical inputs and promotes soil health, making it an attractive option for farmers seeking to restore the quality of their soils and optimize crop production (Huang et al., 2016).

Compost is also useful for TPH-contaminated soil. One of the mechanisms through which compost aids TPH degradation is the formation of metal-humic complexes, which alters the availability of TPHs in the soil. Moreover, composting increases the soil organic matter (SOM) content, leading to decreased TPH bioavailability. Additionally, the cation exchange capacity of humic substances present in compost is comparable to that of clay minerals in the soil, facilitating the retention of essential nutrients. This dual action of compost—enhancing TPH degradation and improving soil fertility—makes it a promising and environmentally friendly strategy for TPH remediation in contaminated soils (Hoang et al., 2021).

In one study, biostimulation using poultry dung, phytoremediation with stubborn grass, *Sporobolus pyramidalis*, and a combined biostimulation and phytoremediation treatment was applied for soil co-contamination with TPH and the heavy metal, Ni. The study's outcomes prominently revealed that the combined application of biostimulation using poultry dung compost and phytoremediation using stubborn grass, *Sporobolus pyramidalis*, yielded the most promising and effective results for co-contaminated soil (Agarry et al., 2013b).

The synergistic effect observed was due to the fact that the introduction of compost enriched the soil with organic matter and nutrients, fueling the growth and activity of hydrocarbon-degrading microorganisms. Concurrently, the plant's root exudates created a conducive rhizosphere environment that attracted and supported hydrocarbon-degrading bacteria, facilitating their interaction with contaminants. The presence of nickel in the soil appeared to stimulate heavy-metal resistant and biodegrading microbial communities, potentially enhancing hydrocarbon degradation. The collaboration between these two methods fostered synergistic interactions, where the enhanced microbial activity from biostimulation combined with the plant's capacity to promote microbial diversity in the rhizosphere created a dynamic remediation environment. This integrated approach effectively addressed co-contamination by leveraging multiple mechanisms, resulting in the observed efficient breakdown and removal of both petroleum hydrocarbons and heavy metals (Agarry et al., 2013b).

In a similar study, compost was introduced to the soil for biostimulation, while maize, known for its phytoremediation capabilities, was cultivated for the phytoextraction approach. It was observed that biostimulation of microbial communities through compost application played a crucial role in enhancing the degradation of total petroleum hydrocarbons (TPH) in the co-contaminated soil (Lee et al., 2023).

In another study, researchers conducted a lab-scale experiment involving the addition of three different types of organic waste amendments (tea leaves, soycake, and potato skin) at a rate of 5% (w/w). These biowaste amendments were introduced to enhance the accumulation of both zinc (Zn) in the plant, *Dracaena reflexa*, and diesel fuel in co-contaminated soil. The biowaste amendments played a dual role in the observed outcome. Firstly, they provided additional organic matter and nutrients to the soil, creating an enriched environment that stimulated the growth and activity of microbial communities responsible for diesel fuel degradation, along with promoting plant growth. Secondly, the presence of these organic amendments improved soil structure and increased the availability of Zn to the plants, thereby enhancing the plant's metal accumulation capability (Agamuthu, 2014).

1.7 Problem Statement

This study addresses the challenging problem of co-contamination in soil, which presents a more complex scenario than single contaminant remediation efforts. By focusing on the simultaneous presence of Cd and total petroleum hydrocarbons (TPH) in agricultural soil, this research explores the potential of phytoremediation as an economically viable strategy for the cleanup of these co-contaminants. Additionally, the study investigates the role of plant-bacteria synergism and organic amendments in enhancing the efficiency of phytoremediation processes. Through this investigation, valuable insights can be gained into the development of sustainable and cost-effective soil remediation practices, particularly in developing countries.

1.8 Research Objectives

This research study has the following objectives:

1. Assessing the effectiveness of phytoremediation with *Sorghum bicolor* in removing Cd and total petroleum hydrocarbons (TPH) from co-contaminated agricultural soil.
2. Investigating the role of plant-bacteria interactions in enhancing phytoremediation efficiency for co-contaminated soil.
3. Evaluate the potential of organic amendments to improve phytoremediation performance in co-contaminated soil.

1.9 Significance of the Study

A large volume of research literature exists about phytoremediation; however, most studies focus on single-contaminant scenarios. In reality, soil contamination often involves multiple pollutants from various sources. For instance, an agricultural field may become contaminated with petroleum hydrocarbons from an oil refinery nearby or a pipeline leakage. However, the same soil can also already have different contaminants present. For example, it may be heavily contaminated with Cd from the extensive use of phosphate fertilizers.

As we have demonstrated earlier, the dynamics of phytoremediation in the presence of co-contaminants are different than in the presence of single contamination. There is a need to select plant and microbial species that are adequate for the phytoremediation of multiple contaminants instead of just one. Studies that demonstrate the effectiveness of amendments for co-contaminants are needed as well. There are few studies that take these considerations into account, and our study fills this gap in phytoremediation literature.

In addition, the plant species we selected in this study, *Sorghum bicolor*, has shown promise for the phytoremediation of Cd and TPH individually. However, studies combining these contaminants in one experiment have not been reported so far. The bacterial strains used in this study have not been reported in studies on the phytoremediation of Cd and TPH co-contamination as well. Therefore, through this study, we aim to provide insight into the plant and microbial species along with amendment choices for the phytoremediation of co-contamination.

Chapter 2

Materials and Methods

This study examines the application of phytoremediation for the removal of Cd and TPH co-contamination in agricultural soil. To enhance the phytoremediation process, two heavy metal and TPH tolerant bacterial strains are used. Compost derived from animal manure and plant waste is also used in combination with plant and bacteria under different treatments.

2.1 Selection of Plant for Phytoremediation

For this study, the plants selected for their potential application in phytoremediation were alfalfa (*Medicago sativa*) and sorghum (*Sorghum bicolor*). Alfalfa was selected because some studies demonstrated its high phytoextraction capacity for Cd. In a pot experiment, Zhang (2019) showed that alfalfa can accumulate up to 356.46 mg/kg Cd in shoots. In another pot experiment, Liu (2017) showed that alfalfa accumulated about 330 mg/kg Cd in roots and 65 mg/kg in shoots. Studies also show that alfalfa has considerable promise in TPH removal (Agnello et al., 2016; Gouda et al., 2016; Lee et al., 2023).

However, in our experiment the presence of Cd and TPH in soil retarded the growth of alfalfa to a great extent and no meaningful results could be produced. Therefore, we proceeded with the experiment using sorghum as the plant species. Sorghum seeds were obtained from sourced from National Agriculture Research Center (NARC), Islamabad.

2.2 Selection of Bacterial Strains

Two pre-isolated bacterial strains were used in this experiment: *Bacillus cereus* (NCCP 2265) and *Serratia bozhouensis* (NCCP 2263). Both strains had been isolated from multi-contaminated sites and, in our laboratory tests, showed tolerance to the Cd and TPH levels used in the experiment. All strains were sourced from Environmental Microbiology and Bioremediation Lab, Quaid-i-Azam University, Islamabad.

2.3 Selection of Organic Amendment

The organic amendment selected for this experiment was animal manure and plant matter compost from NARC. In some studies, compost derived from these materials has shown promise for enhancement of phytoremediation (Sarathchandra et al., 2023; Lu et al., 2023).

2.4 Spiking of Fresh Agricultural Soil

About 15 kg of soil was collected from an agricultural site near Quaid-i-Azam University campus. The soil was spiked with 150 mg/kg of Cd using Cd chloride monohydrate. The dry spiking process was carried out by grinding dry soil through 2 mm sieve and grinding metal chemicals into the fine powder in the agate mortar. Metal compounds were slowly and progressively mixed with ground dry soil (as 10–20 times the mass of metal compounds) and the mixture continued to expand to cover all the soil. Finally, the spiked soil mixture was homogenized through diagonal flipping soil on the plastic sheet for 3–5 times (Chen et al., 2019).

In addition, the soil was also spiked with 2% of diesel oil. The Cd-spiked soil was spread on a plastic sheet, sprayed with diesel oil, and mixed. The soil was then filled in pots with each pot containing around 400g soil. The triplicate pots for the first biotic control was filled with fresh soil.

2.5 Experimental Design

In a pot experiment, sorghum was planted in artificially spiked agricultural soil. Initially, the soil was spiked with 150 mg/kg Cd and 2% TPH. Combinations of plant, bacteria, and compost were applied as illustrated in the following table. The experiment also consisted of one abiotic control (without the plant) and two biotic controls (plant with fresh soil and spiked soil, respectively). Each treatment/control was in triplicate.

Abbreviations:

FS	Fresh Soil
SS	Spiked Soil
B1, B2	Bacterial Strain 1,2
C	Compost

Pot Experiment:

Control 1	SS
Control 2	FS+P
Control 3	SS+P
Treatment 1	SS+P+B1
Treatment 2	SS+P+B2
Treatment 3	SS+P+B1+B2
Treatment 4	SS+P+C

Treatment 5	SS+P+C+B1
Treatment 6	SS+P+C+B2
Treatment 7	SS+P+C+B1+B2

Table 1. Experimental Design

Before the experiment, the physicochemical parameters of the soil were analyzed. These included soil texture, nitrate, phosphate, and organic matter contents. The pH, electrical conductivity, and total dissolved solids in the soil were also recorded.

The pot experiment was conducted in a greenhouse on the Quaid-i-Azam university campus. Pots with the dimensions (15×7×7 cm) were filled with 400 g soil. 15 sorghum seeds were sown in each pot and allowed to grow for 30 days in the months of May and June 2023. The average temperature in the day was around 35 degrees Celsius and around 22 degrees Celsius at night. Each pot was set in a saucer to avoid contaminant leaching. For the arrangement of the pots in the greenhouse, complete randomized block design (CRBD) was used.

2.7 Preparation and Administration of Bacterial Inoculum

A pure culture of the selected bacterial strains was obtained and inoculated into the sterile nutrient broth. The culture was then incubated at 30 degrees Celsius for 24 hours in a shaking incubator. The growth of the bacterial culture was monitored by measuring its optical density. When the culture reached an optical density of one, it was used to inoculate the designated experimental pots (Madariaga-Navarrete et al., 2017). About 30 ml of the culture was applied to each designated three days after germination. Another round of inoculum preparation and administration was conducted a week later.

2.8 Physicochemical Characterization of Soil

The following physiochemical parameters were investigated in this experiment.

2.8.1 Soil Texture

The soil texture was analyzed through the hydrometer method (Estefan et al., 2013). 40 g of air-dried soil was weighed into a 500 ml beaker. 60 ml of dispersing solution was added (40 g sodium hexametaphosphate and 10 g sodium carbonate in 1 liter distilled water) and kept overnight. The next day, the solution was stirred with a magnetic stirrer for 3 minutes and transferred to a 1-liter measuring cylinder.

Determination of Blank

A blank solution was also prepared by adding 60 ml of the dispersing solution in a 1-liter measuring cylinder and increasing the volume up to 1 liter by adding distilled water. The hydrometer was inserted, and the reading was recorded. The temperature was also measured using a thermometer.

Determination of Silt Plus Clay

To determine the hydrometer reading for silt and clay, the soil suspension was stirred for one minute, and the hydrometer was immersed immediately after the withdrawal of the stirring rod. The reading was taken at 40 seconds. The temperature was noted as well.

Determination of Clay

The soil suspension was mixed with a stirring rod and then kept for 6 hours. The reading was taken subsequently, and the temperature was noted as well.

Determination of Sand

Finally, the soil suspension was filtered through a 50-µm sieve. The filtrate was dried in an oven at 105 degrees Celsius overnight in the pre-weighed beaker. After drying, the beaker was weighed, and the weight of the sand was noted.

Calculating quantities of silt, sand, and clay

The following formulas were used to determine quantities of silt, sand, and clay:

1. [Silt + Clay] (% w/w) = (Rsc - Rb) × 100/Oven - Dry soil (g)
2. Clay (% w/w) = (Re- Rb) X-100/Oven - Dry soil (g)
3. Silt (% w/w) = [Silt + Clay (% w/w)]- [Clay (%w/w)]
4. Sand (% w/w) = Sand weight (g) x 100/Oven - Dry soil (g)
5. Sand weight (g) = [Beaker + Sand (g)]- [Beaker (g)]

Where Rsc is the reading (silt+clay), Rb is the reading for blank, Rc is the reading for clay.

2.8.2 Determination of Soil pH, Electrical Conductivity, and Total Dissolved Solids.

The pH of the spiked soil was determined by mixing 50 g of soil with 50 ml of distilled water in a 250 ml beaker. The suspension was mixed at ten-minute intervals for 30 minutes and kept for an hour in the mechanical shaker. Next, buffers of 4 and 7 pH were prepared and used to calibrate the pH meter. After calibration, the reading of the suspension was noted (Estefan et al., 2013).

Standard potassium chloride and sodium chloride solutions were prepared to measure electrical conductivity. Readings for the standards were noted and compared with readings provided by the protocol. Subsequently, the readings for electrical conductivity and total dissolved solids were noted (Estefan et al., 2013).

2.8.3 Determination of Soil Organic Matter and Total Organic Carbon

Five grams of soil were weighed and placed in ceramic crucibles. The total weight of the crucibles containing soil samples was measured and recorded. The crucibles were then placed in a muffle furnace for 2 hours at 800 degrees Celsius. The crucibles were allowed to cool overnight and weighed again. This weight was subtracted from the original weighed recorded to determine the amount of organic matter (Ameen et al., 2016). Total organic carbon was calculated through the following formula:

Organic matter (%) = Total organic carbon (%) x 1.72

2.8.4 Determination of Nitrates in Soil

To quantify nitrates in the soil, a standardized procedure was followed (Estefan et al., 2013). Five grams of air-dried soil were taken in a conical flask, and 50 mL of 0.02 N $CuSO_4$.5H2O solution was added. After a 15-minute period in the mechanical shaker, the mixture was filtered through a Whatman filter paper. Then, 1 mL of 0.1% chromotropic acid solution was added, followed by the addition of 6 mL concentrated H_2SO_4. A blank solution and standards ranging from 0.5-3.5 mg/L were prepared. The absorbance of each solution was measured at 430 nm using a UV spectrophotometer. The concentration of nitrates in the soil was determined by constructing a calibration curve using the absorbance values of the standards against their known concentrations.

2.8.5 Determination of Extractable Phosphorus

The extractable phosphorus was quantified in soil using the standard Olsen sodium bicarbonate procedure (Estefan et al., 2013). Soil samples were shaken at 150 rpm on an orbital shaker for 30 minutes, followed by filtration through Whatman no. 40 filter paper. To the resulting filtrate (5 ml), 3-5 drops of 0.25% nitrophenol indicator were added and mixed with 5N H_2SO_4 dropwise until the solution turned colorless from yellow. After acidification, the volume of the acidified solution was adjusted to 20 ml with distilled water, and 4 ml of ascorbic acid solution was added. A blank control was prepared without soil, and a phosphate standard ranging from 1 to 5 ppm was also prepared. After 10 minutes, the absorbance of the blank, standard, and sample was measured at 882 nm using a spectrophotometer. The amount of extractable P in mg/kg was calculated using the values derived from calibration curve.

2.9 Quantification of Cd in Soil

To quantify Cd in soil samples, the following procedure was followed. The soil samples were oven-dried at 80ºC for a single day. Once dried, the samples were manually crushed and sieved through a 0.59 mm ASTM sieve to obtain a homogeneous soil sample for further evaluation. Aqua regia, which consists of a 1:3 ratio of HNO_3 and HCl, was prepared. In a test tube, 1 g of the soil sample was added to 15 ml of aqua regia and boiled until the volume reduced to 3 to 5 ml. The following day, 5 ml of perchloric acid ($HClO_4$) was added to the remaining solution and boiled until the volume reduced to 3 to 5 ml. After cooling down, the solution was filtered using Whatman filter paper (Number 42). The volume was raised to 15 ml using deionized water. A blank sample was also prepared without the soil sample to account for any procedural errors. Each sample, along with the blank, was analyzed in triplicate using a spectrophotometer for atomic absorption (Hseu et al., 2002).

2.10 Quantification of TPH in Soil

To quantify the total petroleum hydrocarbons (TPH) in soil samples, a series of steps were followed (Villalobos et al., 2008). First, the soil samples were sieved to obtain a particle size of 2 mm using ASTM Sieve 10, followed by a finer sieving using ASTM Sieve 200 with a particle size of 75 µm. The sieved samples were then dried at 105°C for 12 hours. After drying, the soil samples were mechanically homogenized, and 10 grams of samples were accurately weighed and placed in dried round flasks. To facilitate extraction, 10 grams of anhydrous Na2SO4 were added to create a free-flowing powder.

Extraction of the TPH was carried out using n-hexane as the solvent in an ultrasound bath. The optimal conditions for extraction efficiency were determined through investigations. After extraction, the resulting extracts were filtered through a funnel packed with treated cotton. This filtration process was done to remove impurities. Additional hexane was used to wash down the funnel, resulting in a final liquid extract of 60 ml for analysis.

The extracted hexane was evaporated by keeping the flasks uncovered overnight. Finally, the residue was weighed using an analytical balance, and the weight was designated as the total petroleum hydrocarbons (TPH) present in the soil sample.

2.11 Plant Analysis Post-Harvest

2.11.1 Morphological Parameters

To assess the physiological characteristics of the harvested plants, we measured the root and shoot length, as well as the fresh and dried weight of the root and shoot. The samples were dried in an oven at 70°C until a constant dry weight was achieved. Additionally, a representative number of fresh leaves were preserved at -20°C for further biochemical and enzymatic analysis.

2.11.2 Heavy Metal Quantification in Plants

To determine the concentration of heavy metals in the plants, we utilized the wet oxidation method. The plant samples were digested using a mixture of acids (HNO_3 and $HClO_4$). After the digestion process, the samples were filtered, and the volume was adjusted to 15 ml with distilled water. A blank sample was prepared without the addition of plant material. The concentration of heavy metals was measured using an atomic absorption spectrophotometer (Shirisha et al., 2014).

2.11.3 Chlorophyll A, Total Chlorophyll, Chlorophyll B, and Carotenoid Contents Assay:

To evaluate the levels of chlorophyll and carotenoids in the fresh leaf samples, a 2 ml extract was prepared using 80% acetone solution. The extract was centrifuged, and the supernatant was collected. Absorbance values were obtained at specific wavelengths, and the equations from Lichtenthaler (1987) were used to calculate the amounts of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids.

2.11.4 Determination of Lipid Peroxidation

The analysis of lipid peroxidation was conducted following the method described by Venkatachalam et al. (2017). Fresh leaf samples were macerated in cold 5% TCA, and the resulting supernatant was mixed with a solution of TBA. After heating and centrifugation, absorbance was measured at specific wavelengths to determine the level of lipid peroxidation.

2.11.5 Hydrogen Peroxide Production

The content of hydrogen peroxide was quantified using the method reported by Yusuf et al. (2011). Fresh leaf samples were mashed in an extraction buffer, and the resulting supernatant was collected. A reaction mixture was prepared, and the absorbance at 410 nm was measured to determine the H_2O_2 content.

2.11.6 Determination of Antioxidant Enzymes Activity

Enzyme activity was measured using the method described by Venkatachalam et al. (2017). Leaf samples were macerated, and the resulting supernatant was collected. The enzymatic activities of catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX) were assessed using specific reaction mixtures. The absorbance at specific wavelengths was measured to determine the enzyme activity.

The concentrations of enzyme units were calculated using Beer's law. The values obtained from the absorbance measurements were multiplied by the appropriate factors to express the enzyme activity per gram of fresh weight.

2.12 Statistical Analysis

In this study, descriptive statistics were used to summarize the key variables. A one-way analysis of variance (ANOVA) was conducted to establish a significant difference among control/treatment groups with respect to contaminant removal and soil/plant parameters. Each control/treatment group was plotted against contaminant removal and measured parameters to observe patterns and trends. Subsequently, contaminant removal percentages were calculated and correlated with soil/plant parameters through Pearson correlation. After observing emerging patterns in correlation coefficients, regression and ANOVA tests were applied to understand contaminant removal and associated variation in soil/plant parameters for the treatment/control groups. Finally, a principal component analysis (PCA) was used to explain the trends emerging across the experimental groups and related variations in soil/plant parameters.

Chapter 3

Results

In this study, we investigated the effectiveness of our phytoremediation strategy for the removal of Cd and petroleum hydrocarbons in agricultural soil. The experimental design encompassed a range of treatments, which included various combinations of the selected plant, compost, and bacterial strains. To assess the outcomes, soil parameters, plant characteristics, Cd and TPH contents, as well as removal percentages, were measured.

3.1 An Overview of Soil Parameters

The analysis of soil parameters serves as a fundamental exploration in understanding the environmental context of the phytoremediation experiment. This section presents a comprehensive assessment of key soil attributes, including electrical conductivity, total dissolved solids, pH levels, nitrate and phosphate concentrations, organic matter content, and total organic carbon. The initial values for soil parameters in fresh soil are given in the table below.

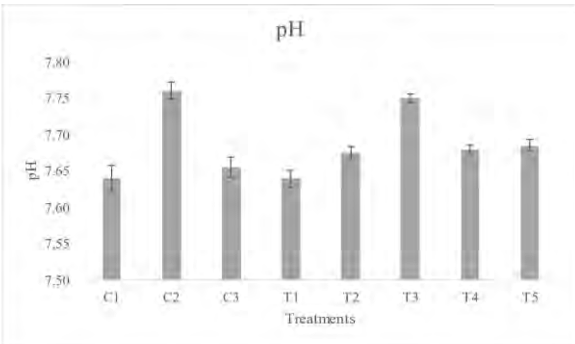
Soil Parameters	**Mean Values**
pH	7.84
EC uS/cm	315
TDS mg/kg	201.92
Nitrates mg/kg	41.42
Phosphates mg/kg	8.22
Organic Matter %	10.2
Total Organic Carbon %	5.90
Cd Conc. mg/kg	0.15

TPH Conc. %	-

Table 3.1. Average values of soil parameters in fresh soil

The average pH values in each of the treatments and controls ranged from a minimum of 7.64 (T1) to a maximum of 7.76 (C2). The pH values among the treatments have a low variance, however, a one-way ANOVA (analysis of variance) with all the replicates showed a significant difference among the treatment/control groups ($p<0.05$).

For electrical conductivity (EC) and total dissolved solids (TDS), the recorded average values spanned a range across treatments. EC values varied from 197.50 µS/cm (C2) to 396.00 µS/cm (C1), while TDS values ranged from 119.39 mg/kg (C2) to 240 mg/kg (C1). Notably, groups T4, and T5 share similar EC and TDS values indicating common conductivity traits among these treatment groups. Moreover, the experimental groups, T1, T2, and T3 share similar EC and TDS values. One-way ANOVA for both showed significant differences among treatment/control groups.



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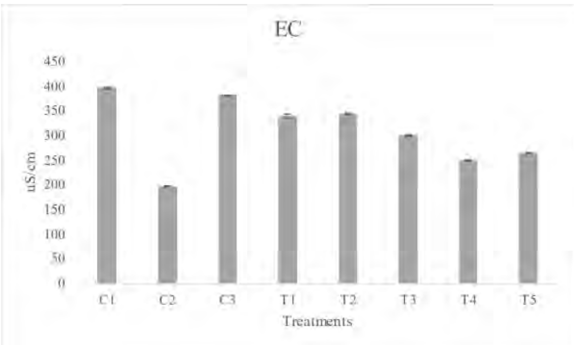

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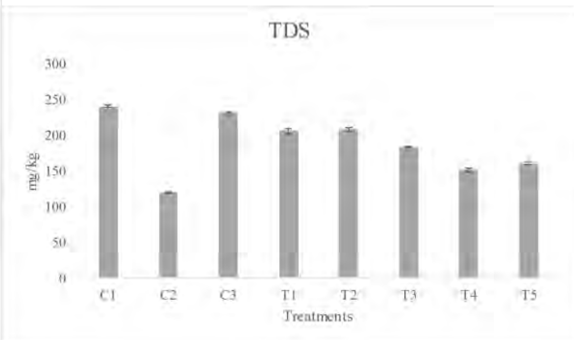
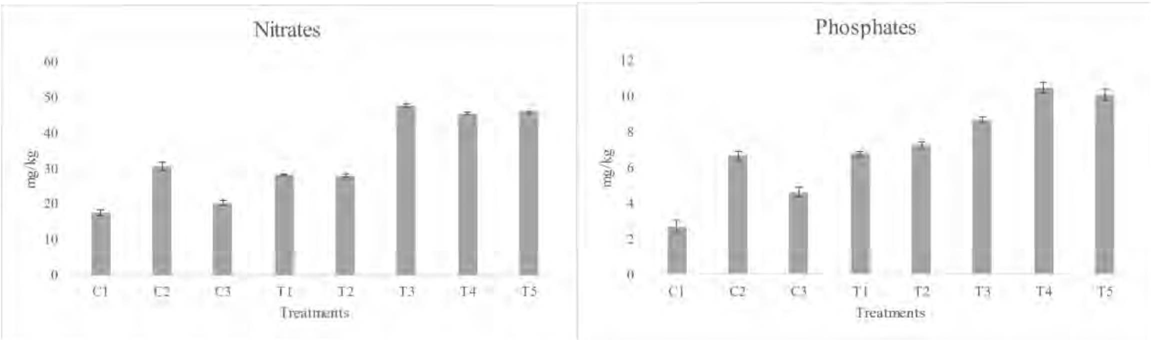


c.

Figure 3.1 Average values for pH (a), EC (b), and TDS (c) in soil

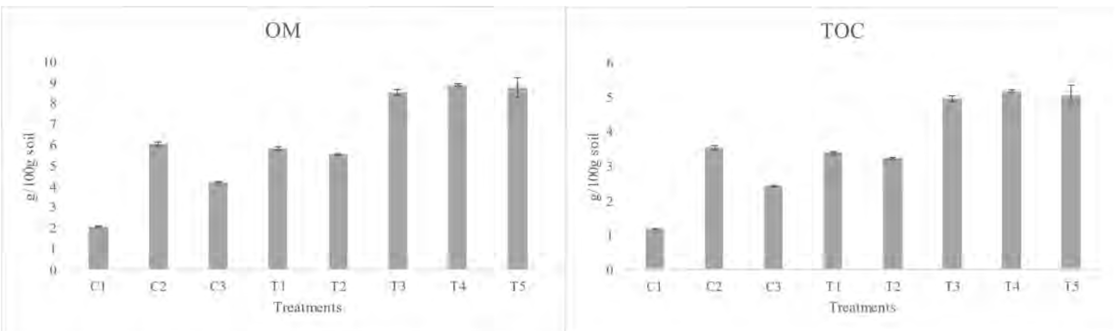
Similarly, average values for nitrates ranged from 17.39 mg/kg (C1) to 47.70 mg/kg (T3), while phosphates ranged from 2.65 mg/kg (C1) to 10.45 mg/kg (T4). However, while nitrate levels exhibited high variance, phosphate levels had low variance among the treatment/control groups. Nevertheless, a one-way ANOVA showed significant differences across the experimental groups. Nitrate levels for treatment groups, T1, T2, and T3 appear to be similar. In the case of phosphates, T3 appears to have greater levels than previous experimental groups, but T4 and T5 both have higher levels than T3.



a. b.

Figure 3.2 Average values for nitrates (a) and phosphates (b) in soil

Furthermore, the average organic matter content ranged from 2.05% (C1) to 8.89% (T4), while total organic carbon content ranged from 1.19% (C1) to 5.17% (T4). Both parameters exhibit low variance among treatment and control groups. However, a one-way ANOVA shows significant differences among each experimental group. The last three experimental groups, T3, T4, and T5 have higher contents for organic matter and total organic carbon than other groups.



a. b.

Figure 3.3 Organic matter (a) and total organic carbon (b) in Soil

In addition to the above parameters, the soil texture was also analyzed. With the greatest fraction of silt, followed by sand and then clay, the soil texture was found to be silt loam.

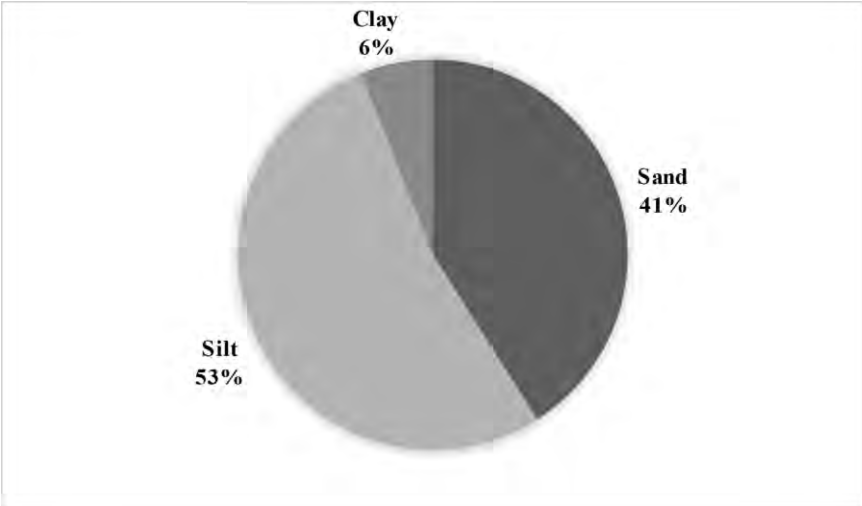
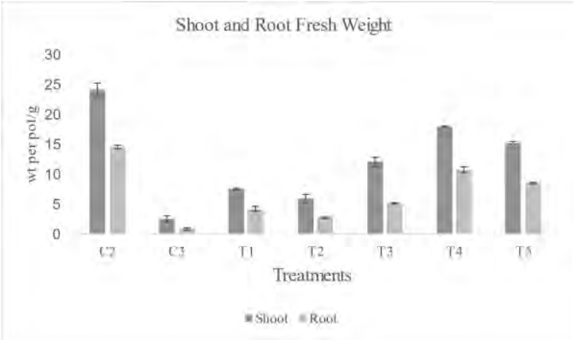


Figure 3.4 Soil texture fractions for silt, sand, and clay

3.2 An Overview of Plant Parameters

A comprehensive assessment of various plant parameters was conducted to reveal the intricate responses of plant-soil interactions to the introduction of Cd and total petroleum hydrocarbons (TPH). The parameters examined included shoot, root length, and fresh and dry weight. The fresh leaf samples were also examined for chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, malondialdehyde (MDA), hydrogen peroxide (H2O2) contents, along with key stress enzymes—catalase (CAT), ascorbate peroxidase (APX), and glutathione peroxidase (GPX).

The average measurements of the shoot and root length and their corresponding weights for each experimental group reveal a certain across the controls and treatments. C2 has the highest values for all these measurements, while C1 appears to have the lowest values, and therefore, the lowest biomass. Looking at treatment groups, we see that T4 and T5 appear to have the largest and second-largest biomass, respectively. Treatment groups T1, T2, and T3 seem to have lower biomass, with T3 having higher values than T1 and T2. A one-way ANOVA for all these parameters shows a significant difference between all experimental groups.



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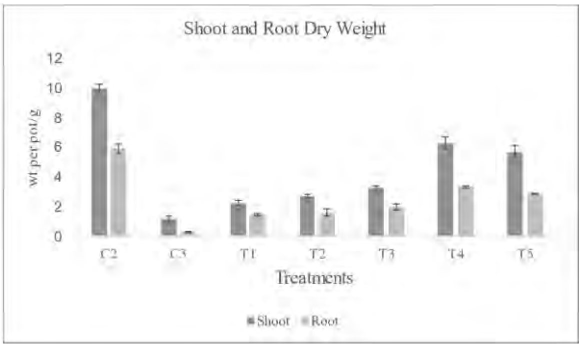
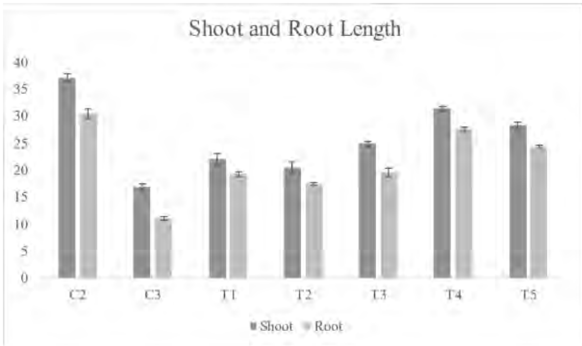


b.



c.

Fig 3.5 Shoot and root fresh weight (a), dry weight (b), and length (c)

Similarly, the control group C2 exhibits the highest average levels of photosynthetic pigments, which include chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids, while C3 has the lowest contents. Among the treatment groups, T4 and T5 have the highest and second-highest photosynthetic pigments, respectively. Furthermore, the treatment group T3 has higher levels of photosynthetic pigments than both T1 and T2. A one-way ANOVA for all these parameters shows a significant difference among experimental groups.

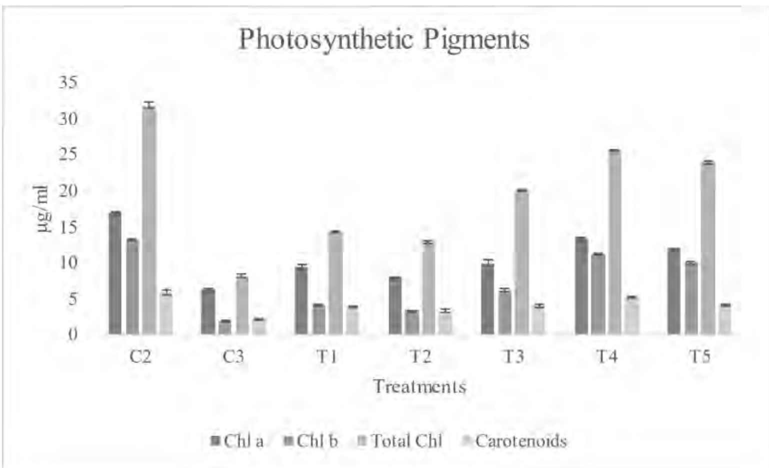
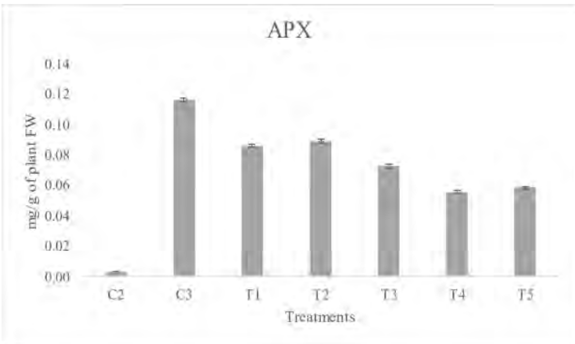


Fig 3.6 Photosynthetic Pigments

Furthermore, similar trends can be observed in the stress marker contents in plants—malondialdehyde (MDA) and hydrogen peroxidation (H2O2), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX). The stress markers seem to have greater levels in the control group C3. They are dramatically lower in the control group C2. The treatment groups, T4 and T5, appear to have lower levels than other treatments as well. However, a different trend can be observed in catalase (CAT) levels. In contrast with the other two antioxidant enzymes, CAT appears to have a higher concentration in treatment groups T3, T4, and T5 and a lower concentration in T1, T2, and C3 groups. However, a one-way ANOVA for all five stress markers shows significant differences among the experimental groups.



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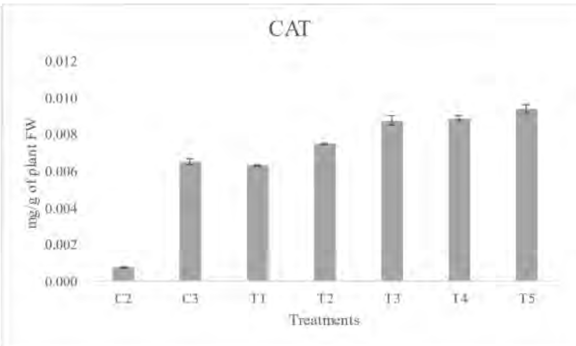

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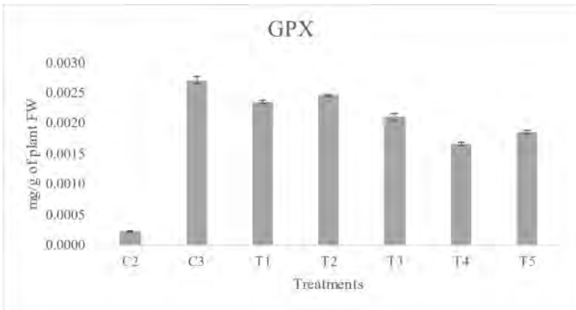
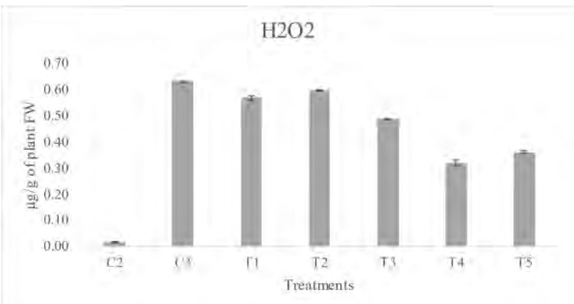
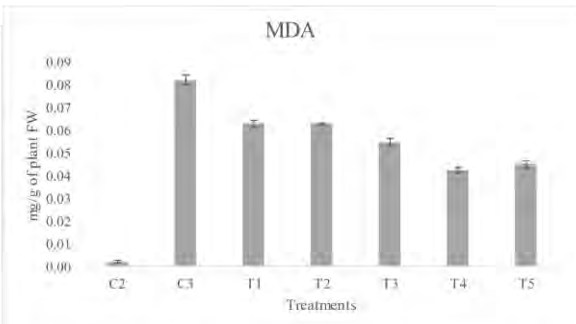


c.

Figure 3.7 Contents of antioxidant enzymes, APX (a), CAT (b), GPX (c) in leaf samples





a. b.

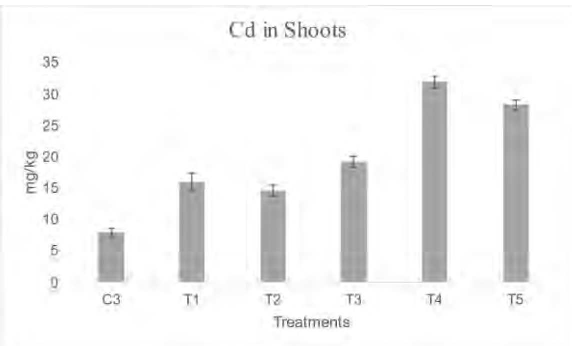
Fig 3.8 Contents of stress markers, H2O2 (a) and MDA (b) in leaf samples

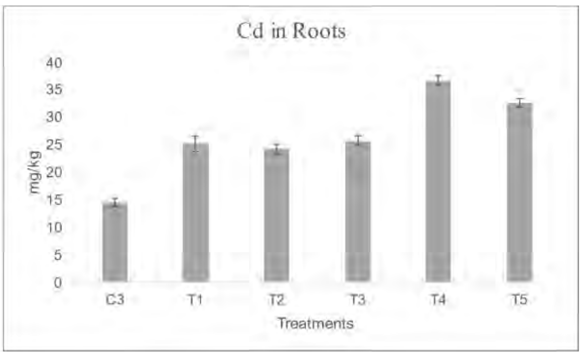
3.3 Remediation of Cd

In this section, we analyze the performance of our treatments at Cd removal from soil and compare their efficacy against control groups. Control group C1 showed a conspicuously high level of Cd in the soil, which is expected as there was no plant in it. Control group C2 (fresh soil with plant) revealed no detectable Cd in either shoots or roots, however, the soil showed an average Cd content of 1.5 mg/kg. In contrast, Control Group C3 (spiked soil with plant) showed discernible Cd concentrations in both shoot and root tissues, quantified at 7.9 mg/kg and 16.14 mg/kg, respectively. The Cd in soil reached about 88.7 mg/kg in this group, resulting in a total Cd level of 112.7 mg/kg.

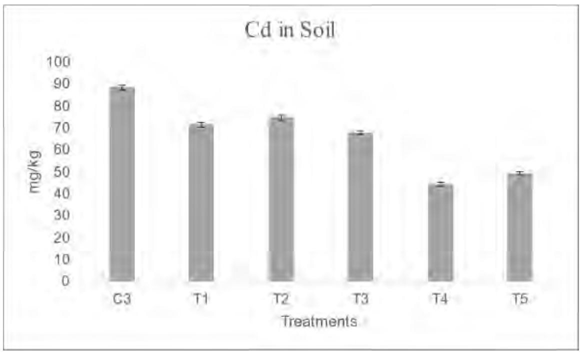
Treatment group T1 contained average Cd concentrations of 13.0 mg/kg in shoots and 23.75 mg/kg in roots. Correspondingly, the soil showed a Cd content of 72.2 mg/kg, contributing to an overall Cd level of 109.0 mg/kg. T2 demonstrated a similar pattern of Cd accumulation. Cd concentrations measured 14.6 mg/kg in shoots and 24.35 mg/kg in roots. The soil's Cd content for this group was 73.3 mg/kg, resulting in a total Cd level of 112.3 mg/kg.

The group T3 displayed a higher Cd content in both shoots (19.2 mg/kg) and roots (25.80 mg/kg) compared to T1 and T2. The soil Cd content was 66.9 mg/kg, resulting in a total Cd level of 111.8 mg/kg. Notably, T4 exhibited the highest Cd content in both shoots (32.0 mg/kg) and roots (36.67 mg/kg). Similarly, T5 demonstrated substantial Cd concentrations in shoots (29.3 mg/kg) and roots (33.74 mg/kg). The soil Cd content for T5 was 48.2 mg/kg, resulting in a total Cd level of 111.3 mg/kg. Finally, the one-way ANOVA test was conducted and it showed significant differences among treatment groups.





a. b.



c.

Figure 3.9 Cd content in shoots (a), plant roots (b), and soil (c)

The percentage of Cd removal in each control/treatment group can be calculated by subtracting the amount of Cd accumulated by the plant (both shoots and roots) from the total detected Cd level (in shoots, roots, and soil) and obtaining a percentage. The standard error was also calculated accordingly. This can help us analyze Cd removal only in the context of phytoremediation and exclude Cd loss through other processes, such as strong adsorption to soil particles and leaching after irrigation. The highest removal of Cd was achieved by T4 (61%) followed by T5 (56.7%). About 40% removal was achieved by T3. T1 and T2 showed 34.7% and 33.8% removal from soil, respectively. The lowest removal percentage was observed in C3 (21.3%).

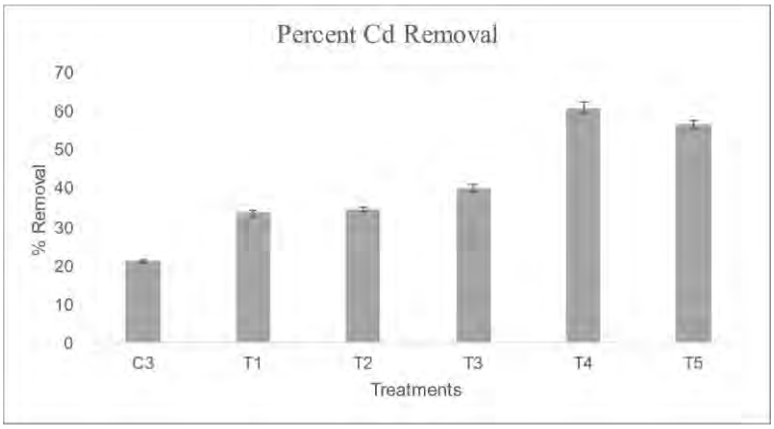


Figure 3.10 Percent Cd removal from soil

After observing substantial amounts of Cd removal from the soil, we can examine the translocation factor (TF) and accumulation coefficient (AC) in the plant. TF is a measure used to assess the ability of a plant to transfer a specific element, in this case, Cd from its roots to other parts of the plant, typically the shoot or aerial parts. The formula for calculating the translocation factor is:

TF = (Concentration in Shoots) / (Concentration in Roots)

AC measures the ability of a plant to accumulate a specific element, in this case, Cd, in its tissues. It's calculated as the ratio of the concentration of the element in the plant tissue (roots) to the concentration of the element in the external substrate, in this case, soil.

AC = (Concentration in Plant Tissue) / (Concentration in External Substrate)

The table below provides values for the TF and AC for Cd in the selected plant, sorghum, and how it varies as different treatments are applied. The highest TF and AC levels are observed in treatment groups T4 and T5. The control group, C3, has the lowest values for TF and AC, as expected. The groups T1 and T2 have higher levels than C3 but lower levels than T3.

Table 3.1 Translocation factors and accumulation coefficients for Cd in sorghum across experimental groups

Treatment/ Control	TF (shoot/root)	AC (root/soil)
C1	0	0
C2	0	0
C3	0.49	0.18
T1	0.60	0.33
T2	0.55	0.33
T3	0.74	0.39
T4	0.87	0.84
T5	0.87	0.70

3.4 Remediation of TPH

The quantification of TPH removal was calculated by subtracting the remaining TPH after treatment from the initial spiked TPH concentration to determine the reduction. The percentage removal was then derived by dividing the reduction by the initial TPH concentration and multiplying by 100. The standard error was also calculated accordingly. In addition, a one-way ANOVA showed that there was a significant difference among experimental groups in terms of TPH reduction.

Control group C1, devoid of plant, demonstrated a TPH remediation of 16.00%. This value can be attributed to natural attenuation. Control group C2, with no contaminant introduction, displayed no TPH removal.

Control group C3 exhibited a remediation of 21.34%. Treatment groups T1 and T2 showcased substantial removals of 54.00% and 51.50% respectively, indicating effective TPH reduction. Treatment Group T3 achieved a moderate remediation of 24.75%. The most significant TPH remediation was evident in Treatment Groups T4 and T5, displaying removals of 75.06% and 72.00% respectively.

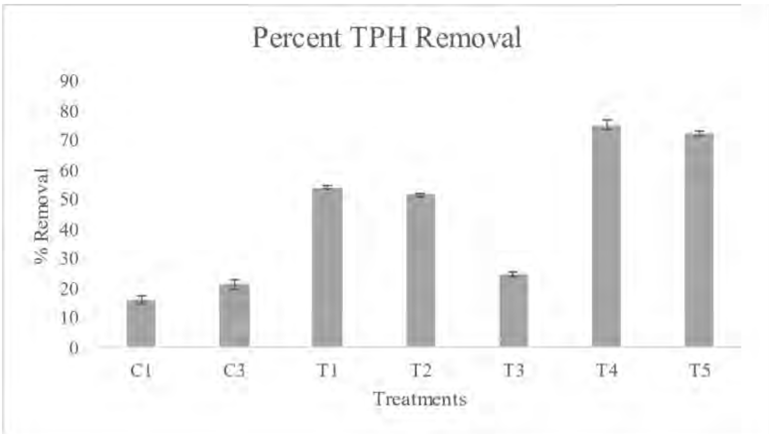


Fig 3.11 Average TPH removal from soil

3.5 Linking Cd, TPH Reduction, and Soil/Plant Parameters

In this experiment, we can notice certain trends across different experimental groups for various parameters, although not consistently across all of them. For many parameters, control group C3 stands out at one end, while treatment group T4 stands out at the opposite end. T1 and T2 show similar trends, T3 is somewhere in the middle, and T4 and T5 have similar trends as well. In many cases, T1 performs better than T2 and T4 performs better than T5. This trend is evident in the removal percentages of Cd and TPH in soil, as well.

Given these patterns, it's valuable to use a method like Pearson correlation to examine which specific soil and plant parameters strongly relate to the effectiveness of Cd and TPH reduction. We can then conduct a regression analysis to check whether linear or closely linear relationships exist between parameters and the removal efficacy of Cd and TPH.

Furthermore, the Pearson correlation provides insights into the correlations existing between various parameters themselves. Such insights can help us identify redundant parameters and facilitate a refined analysis. In the figure below, a Pearson correlation heatmap shows the associations between soil parameters and the percentages of Cd and TPH reduction.

It’s important to note that the analysis excludes the first two controls, (C2 and C2, which were spiked soil without plant and fresh soil with plant, respectively). This exclusion ensured that our focus remained on treatment and control groups where active phytoremediation processes were at play. By omitting these control groups, we can concentrate on assessing the impact of the applied treatments and better understand their effects on contaminant reduction and related soil/plant parameters.

3.5.1 Analysing Correlations Between Soil/Plant Parameters and Contaminant Removal

Pearson correlation is a statistical measure that quantifies the strength and direction of a linear relationship between two variables. It ranges from -1 to 1:

- A correlation coefficient of 1 indicates a perfect positive linear relationship.
- A correlation coefficient of -1 indicates a perfect negative linear relationship.
- A correlation coefficient of 0 means there's no linear relationship between the variables.

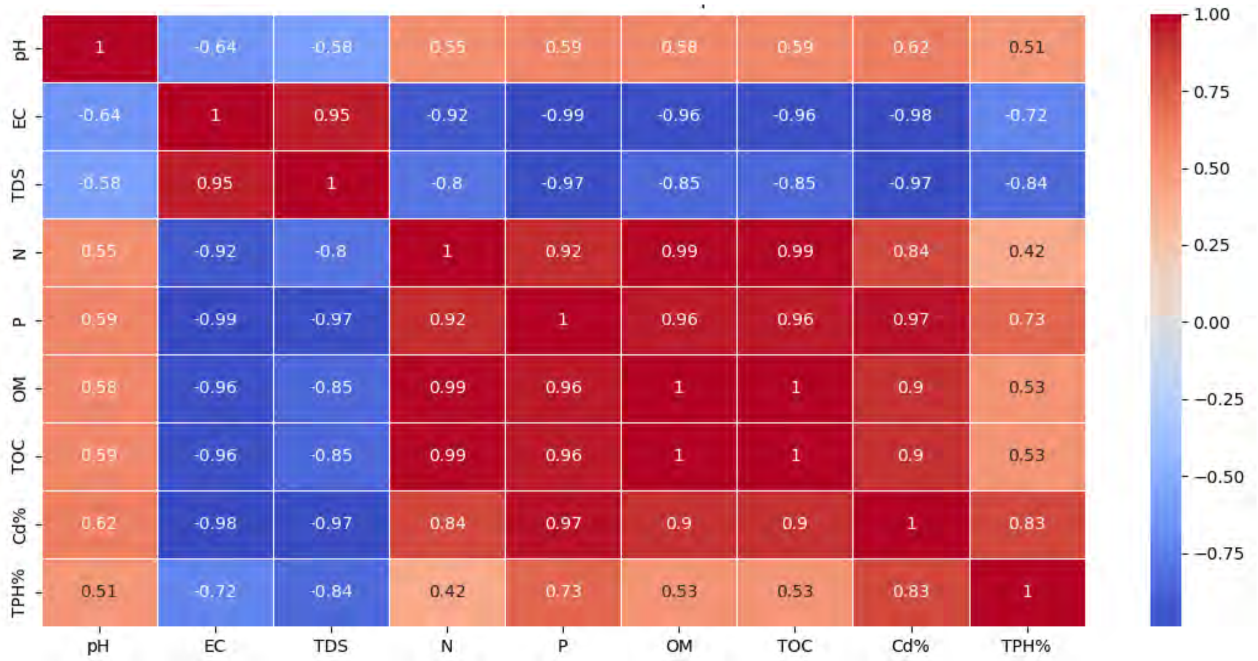


Figure 3.12 Heatmap showing correlations between soil parameters and the reduction of Cd and TPH in soil

In the context of Figure 3.10, the dark red color is used to represent a strong positive correlation of 1. The dark blue color represents a strong negative correlation of -1. As the correlation moves away from these extremes (closer to 0), the colors become lighter. This corresponds to weaker correlations, indicating that while there might still be a relationship between the parameters, it's not as strong or straightforward.

Therefore, the soil pH does not appear to have a very strong correlation with either Cd or TPH removal. However, this does not mean that a relationship may be absent. It just means that a linear relationship may not exist. Next, we can see that EC and TDS have a high negative correlation with both Cd and TPH reduction. Hence, the result indicates that experimental groups with low levels of EC and TDS have high percentage removals of both Cd and TPH. With a high correlation, we can explore these parameters further to check if they have a valid linear correlation. However, EC and TDS have a very high correlation with each other in addition to having a high correlation with Cd and TPH reduction. To mitigate redundancy, we can select one of these parameters for further analysis.
Next, we see that nitrates have a high positive correlation with Cd removal but a lower Pearson coefficient value for TPH removal. In contrast, phosphates have a high positive correlation with both Cd and TPH reduction, although the coefficient is higher in the case of Cd reduction.

This result, therefore, necessitates further analysis. OM and TOC both have a high positive correlation with Cd removal. However, they appear to have a weaker correlation with TPH removal. In addition, both parameters are strongly correlated to each other while showing similar results for Cd and TPH removal. Therefore, we can select one of these parameters for further analysis.

The relationships between plant parameters and Cd or TPH removal can be examined through Pearson correlation as well. The following heatmap shows these relationships.

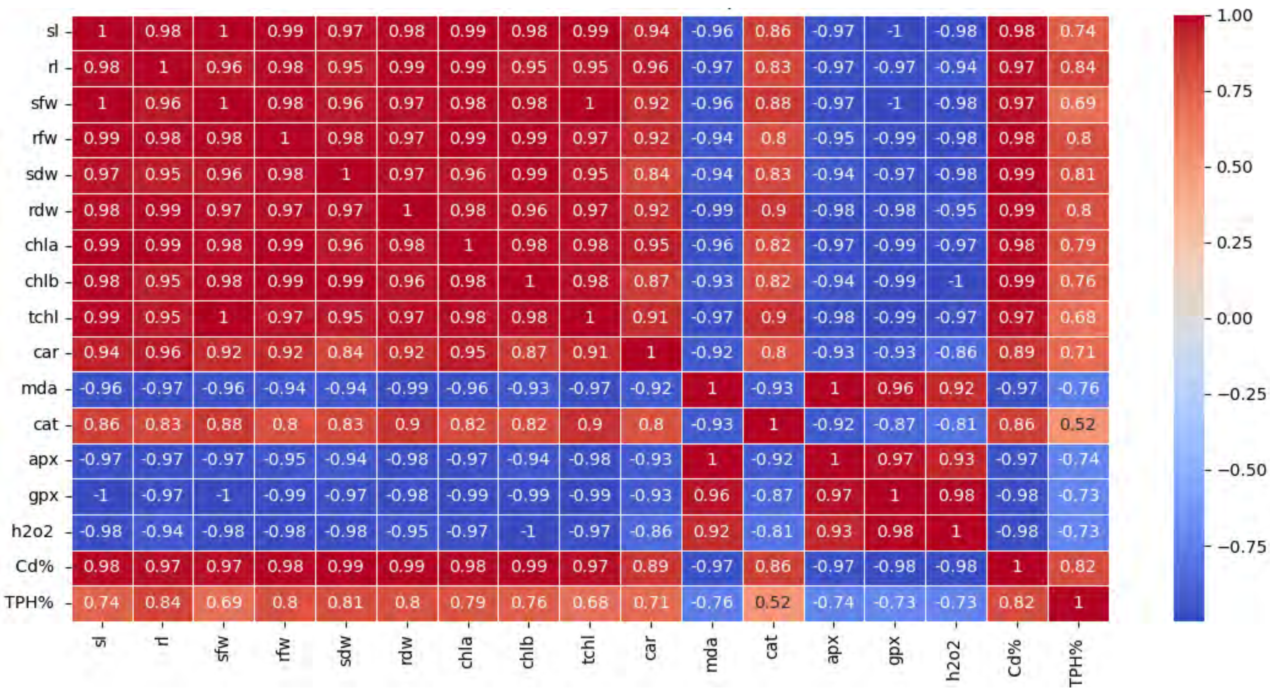


Figure 3.13 Heatmap showing correlations between plant parameters and the reduction of Cd and TPH in soil

The above figure shows that the plant's physical parameters such as shoot or root length and corresponding fresh and dry weights are all highly positively correlated with Cd removal. There is a positive correlation with TPH reduction as well for these parameters, but the coefficient values are lower than in the case of Cd removal. In addition, since these physical parameters are highly correlated with each other and show similar results for Cd and TPH removal, we can proceed with the analysis with only one physical parameter for shoots and roots, respectively. This will help us mitigate redundancy in subsequent analyses.

Next, we see that chlorophyll a, b, and total chlorophyll contents have high positive correlations with Cd removal. They also have positive correlations with TPH removal but to a lesser extent. The three parameters have strong correlations with each other and similar results for Cd and TPH, therefore, we can select one of these parameters for further analysis. Similar to the chlorophyll content, the carotenoid content also has a high positive correlation with Cd removal, and to a lesser extent, with TPH removal.

Lastly, we can see that the stress markers, MDA and H2O2, and the antioxidant enzymes, APX and GPX have a high negative correlation with Cd removal, and to a lesser extent, TPH removal. The antioxidant enzyme, CAT, however, shows a different trend than the other four stress indicators. To explain this exception and examine the relationships between stress indicators and contaminant removal, further analyses are necessary. Therefore, even though the other four stress markers show strong correlations with each other, we will still proceed with them for the next step.

3. 5. 2 Assessing Linear Relationships Between Soil/Plant Parameters and Contaminant Removal

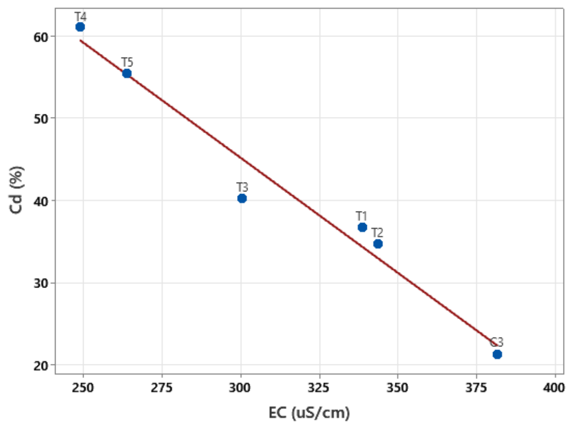
After conducting the Pearson correlation analysis, we observed that certain parameters demonstrated a notable positive or negative correlation with contaminant removal, specifically with Cd. However, while Pearson's correlation provides insight into these relationships, it does not address how treatment/control groups are linked to soil/plant parameters, nor does it indicate the statistical significance of the results.

Therefore, after identifying parameters showing a notable correlation with Cd and TPH removal, subjected these parameters to ANOVA and linear regression tests. The linear regression test incorporated data labels that specify treatment/control groups. This approach helped us uncover how these groups are associated with soil/plant parameters.

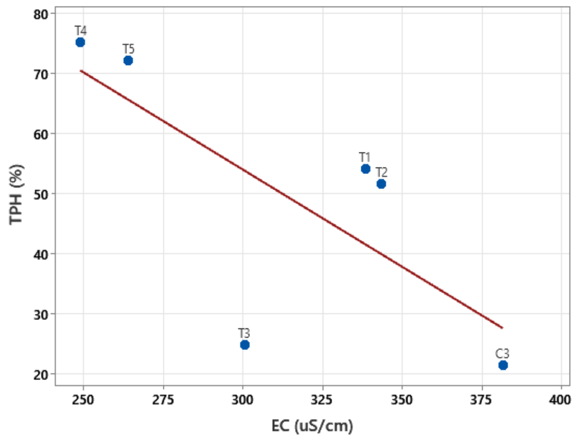
Before proceeding with ANOVA and linear regression, we assessed the data for normality using the Ryan-Joiner test, ensuring that the assumptions for these analyses were met and that the results were reliable. The first two control groups, C1 and C2, are excluded from these analyses as well.

The ANOVA results for Cd removal against pH indicated that the observed relationship wasn't statistically significant, as the p-value was relatively high (>0.05). Similarly, ANOVA findings for TPH removal against pH indicated no significant relationship as well. This suggested that the variation in Cd and TPH removal explained by pH variation might have occurred by chance.

In contrast, the ANOVA analysis exploring the relationship between Cd removal and EC revealed a statistically significant correlation ($p < 0.05$). The regression analysis further confirmed a substantial linear connection between Cd removal and EC, emphasizing the significance of EC in explaining variations in Cd removal. However, the ANOVA results for TPH removal against EC indicated that the relationship between these two parameters wasn't statistically significant. For a comparison, linear regression plots for both relationships are shown below.



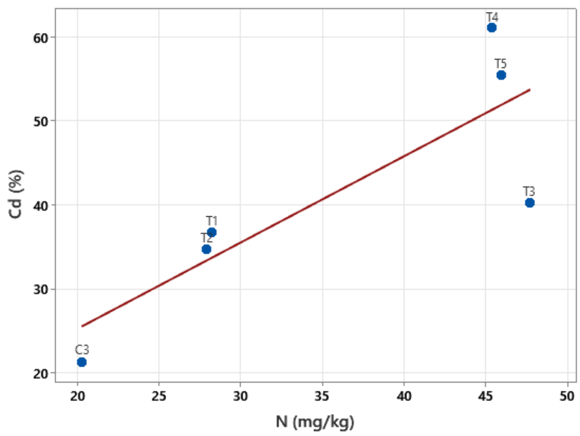
a.



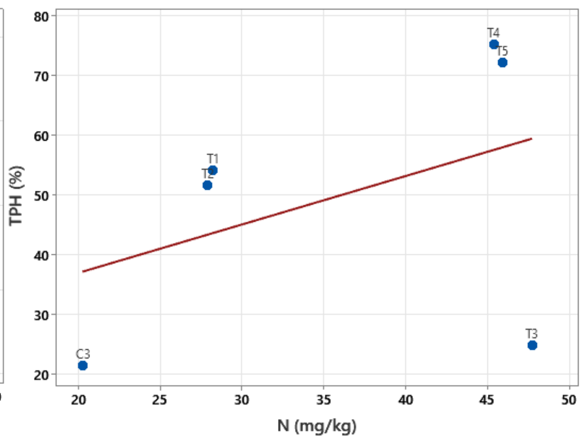
b.

Fig 3.14 Regression analysis of Cd (a) and TPH (b) removal with EC

Next, the ANOVA test was applied for Cd removal against nitrates and it revealed statistical significance, as indicated by the calculated p-value (>0.05). This suggests a meaningful relationship between Cd removal and nitrates, but the linear regression test suggests that this relationship may be non-linear. In contrast, the observed relationship between TPH removal and nitrate levels lacked statistical significance.



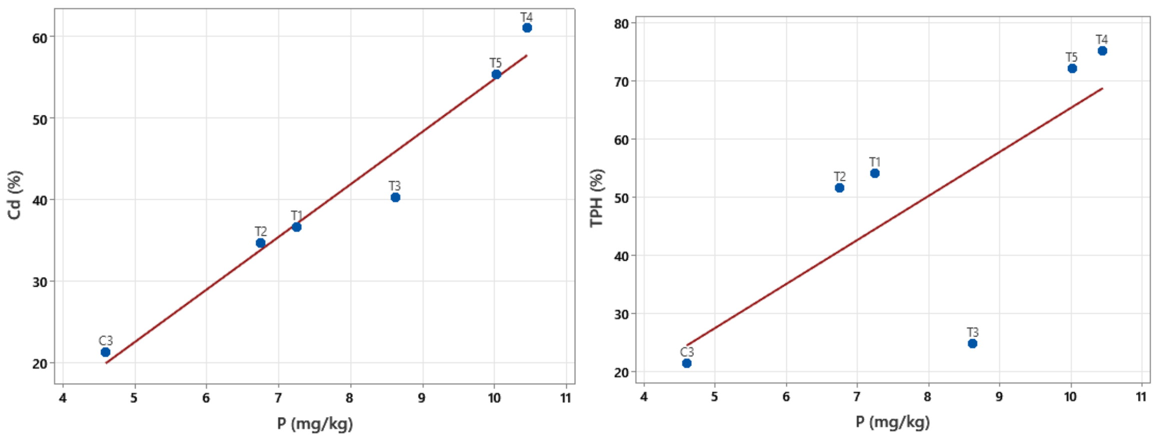
a.



b.

Fig 3.15 Regression analysis of Cd (a) and TPH (b) removal with nitrates

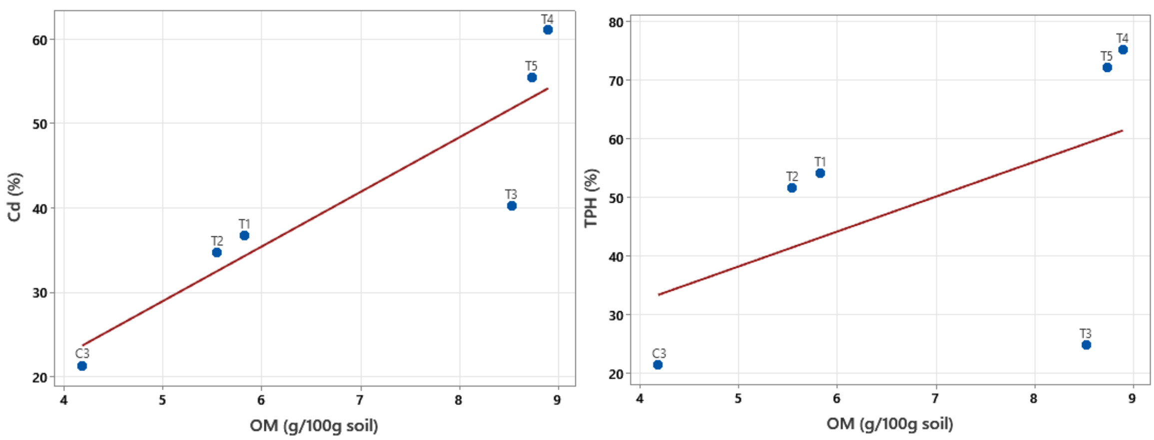
Phosphate levels were observed to have a statistically significant and linear relationship with Cd removal. In the case of TPH, however, a p-value of 0.099 indicates that the result is close to, but not less than, the conventional significance level of 0.05. This suggests that while the observed relationship may show some indication of significance, it falls just short of being statistically significant at the 0.05 significance level.



a. b.

Fig 3.16 Regression analysis of Cd (a) and TPH (b) removal with phosphates

In the same vein, the relationship between Cd removal and soil organic matter content was calculated as statistically significant, although a strong linear relationship isn't apparent. However, ANOVA deemed the relationship between TPH removal and organic matter to be non-significant.

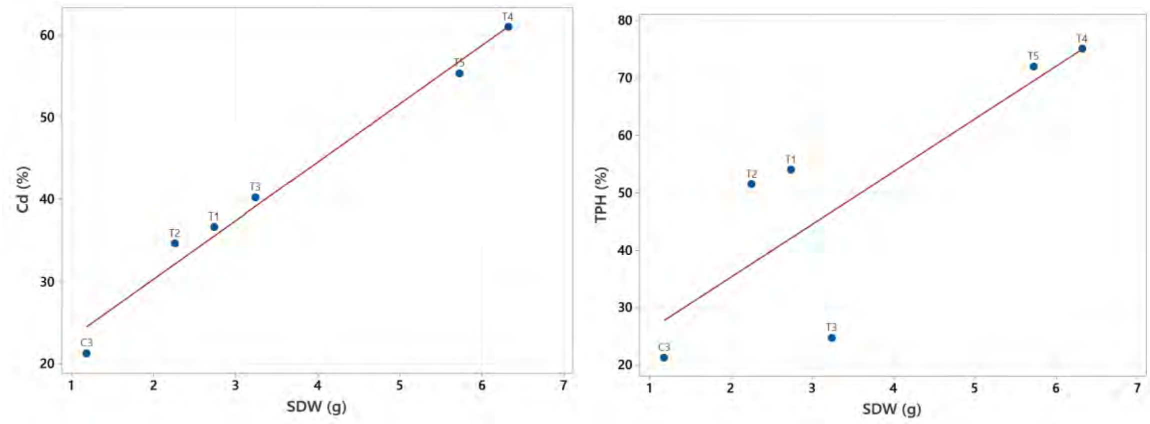


a. b.

Fig 3.17 Regression analysis of Cd (a) and TPH (b) removal with soil organic matter

The regression analysis and ANOVA test were also applied to understand the relationships between contaminant removal and the plant parameters selected after the Pearson correlation analysis.

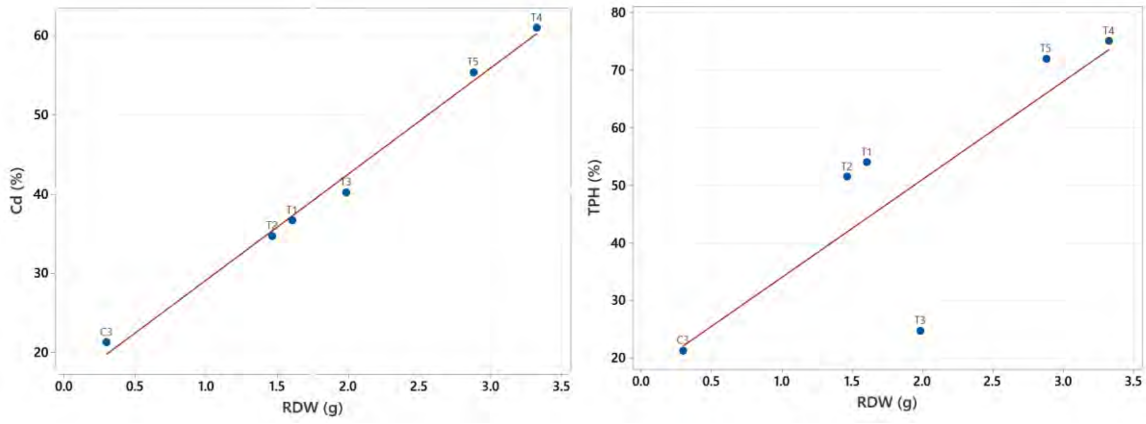
Subsequently, it was observed that Cd removal has a significant and linear relationship with Cd removal. A p-value of 0.048 is just below the conventional significance level of 0.05. This suggests that the observed relationship is likely to be statistically significant at the 0.05 significance level. Therefore, there is evidence to suggest that TPH removal and shoot dry weight might have a relationship.



a. b.

Fig 3.18 Regression analysis of Cd (a) and TPH (b) removal with shoot dry weight

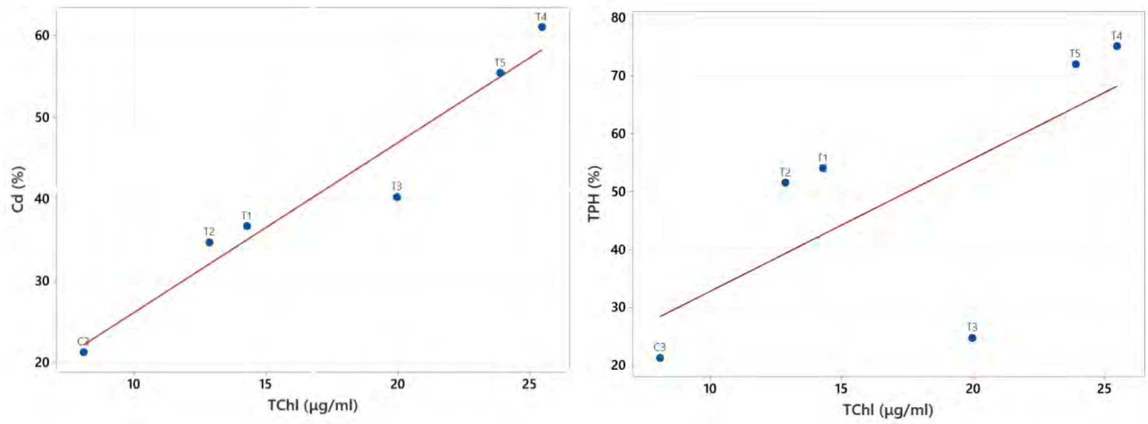
Similarly, a significant and linear relationship is observed between Cd removal and root dry weight. However, in the case of TPH removal, a p-value of 0.052 is slightly above the conventional significance level of 0.05. While not meeting the strict threshold for statistical significance, this p-value indicates that the observed relationship has a moderate likelihood of being significant. It suggests that changes in root dry eight might be associated with changes in TPH removal.



a. b.

Fig 3.19 Regression analysis of Cd (a) and TPH (b) removal with root dry weight

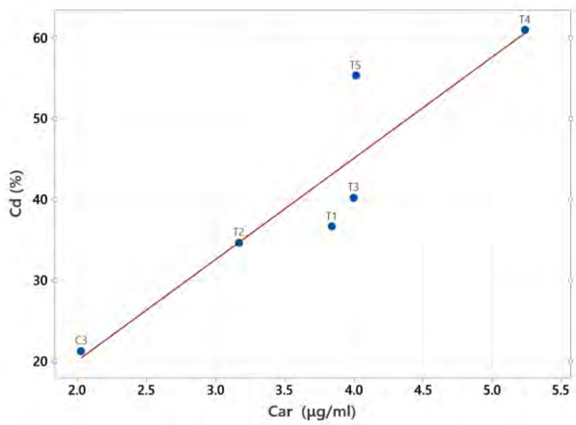
The total chlorophyll content in plant leaf samples also has a significant and linear relationship with Cd removal. However, the ANOVA test showed that there was no statistically significant relationship.



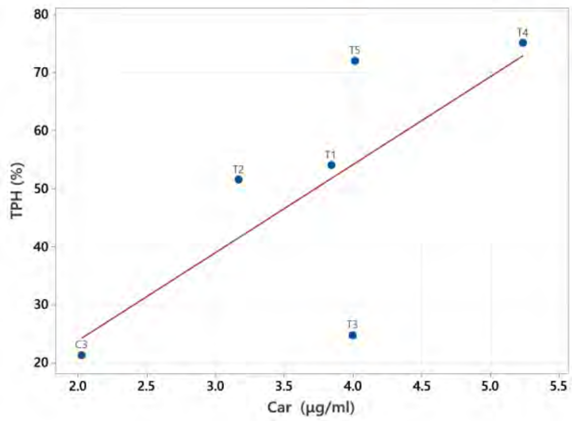
a. b.

Fig 3.20 Regression analysis of Cd (a) and TPH (b) removal with total chlorophyll content

Similarly, a significant and linear relationship was observed between Cd removal and carotenoid content. However, a statistically significant relationship was not seen in the case of TPH removal.



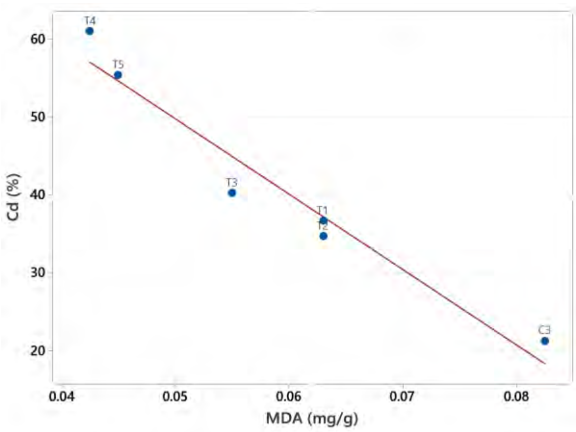
a.



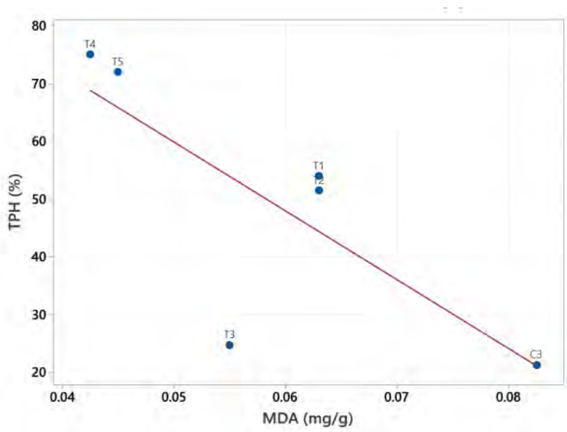
b.

Fig 3.21 Regression analysis of Cd (a) and TPH (b) removal with carotenoid content

Finally, when the ANOVA and regression tests are applied to the stress indicators, we see that MDA, H2O2, APX, and GPX have a significant, linear, and negative relationship with Cd removal. With CAT, a significant relationship was observed at a p-value of 0.027. A linear relationship was not strongly suggested, however, the trendline showed a positive relationship instead of a negative one as in the case of other stress indicators. With TPH removal, the ANOVA test showed that the conditions for a statistically significant relationship were not met. However, the trendlines in the regression plot for TPH removal against stress indicators, albeit non-significant, are similar to the trendlines observed for Cd removal against stress indicators. The regression trendlines for Cd and TPH removal against the five stress indicators are provided below.

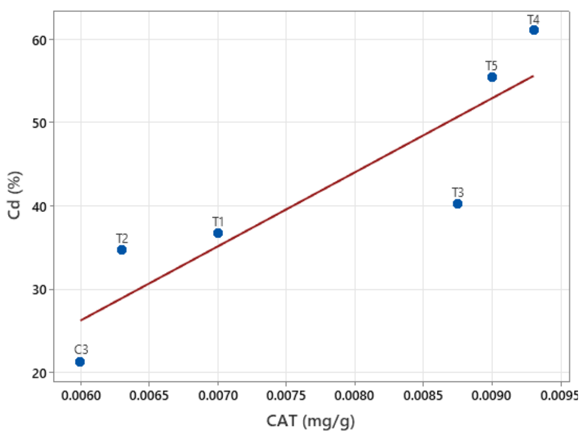


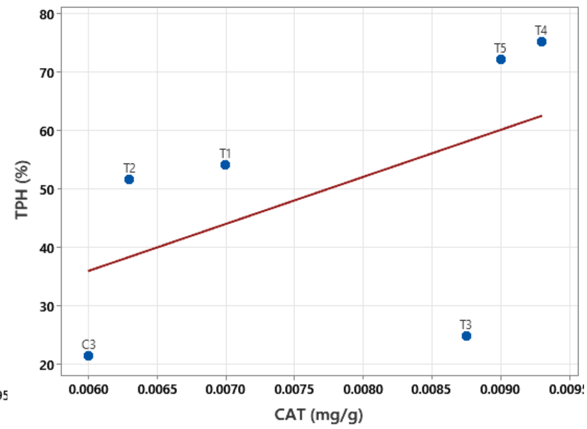
a



b.

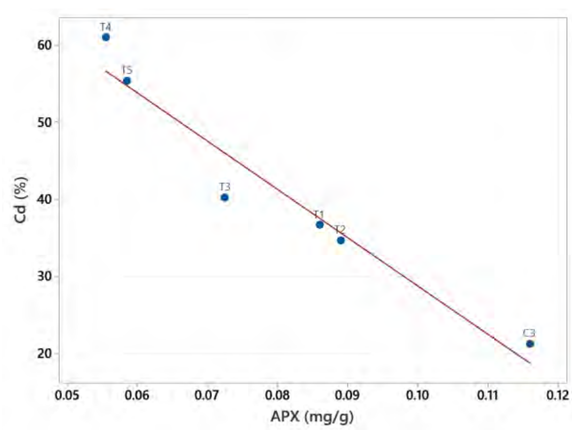
Fig 3.22 Regression analysis of Cd (a) and TPH (b) removal with MDA

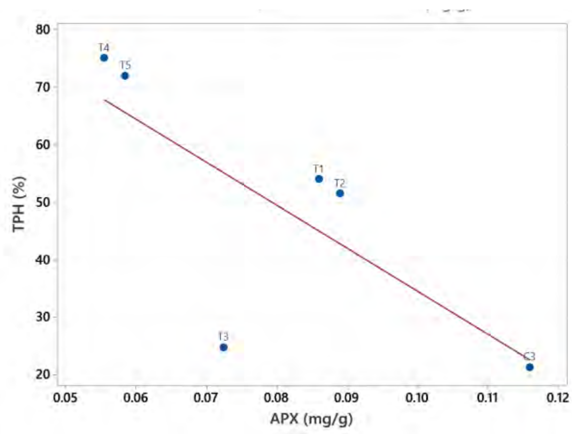




a. b.

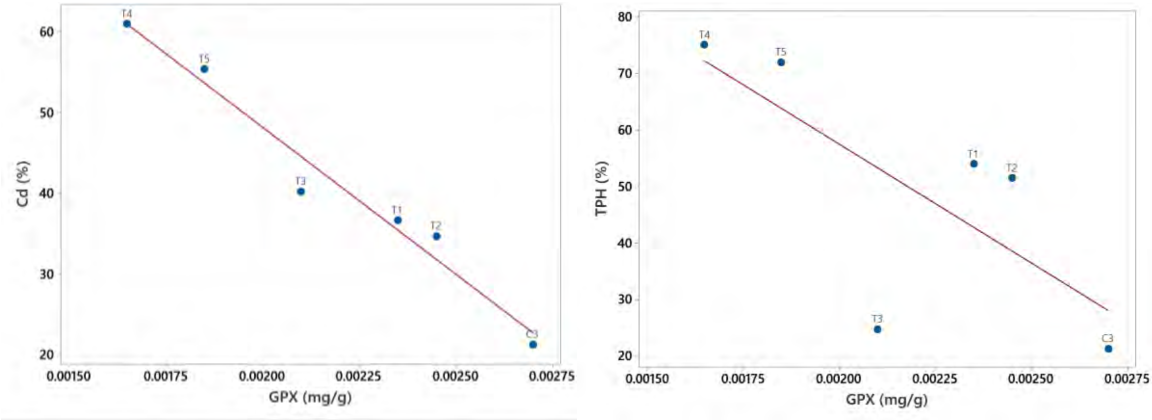
Fig 3.22 Regression analysis of Cd (a) and TPH (b) removal with CAT





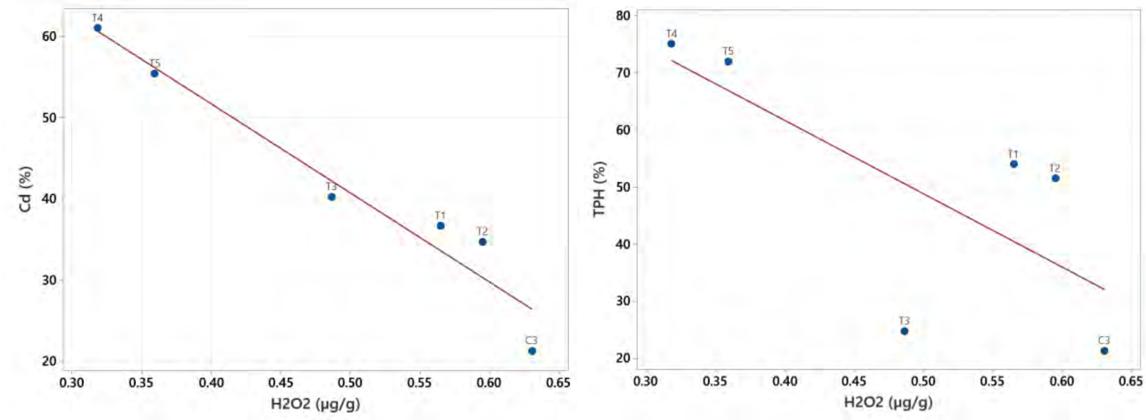
a. b.

Fig 3.21 Regression analysis of Cd (a) and TPH (b) removal with APX



a. b.

Fig 3.24 Regression analysis of Cd (a) and TPH (b) removal with GPX



a. b.

Fig 3.25 Regression analysis of Cd (a) and TPH (b) removal with GPX

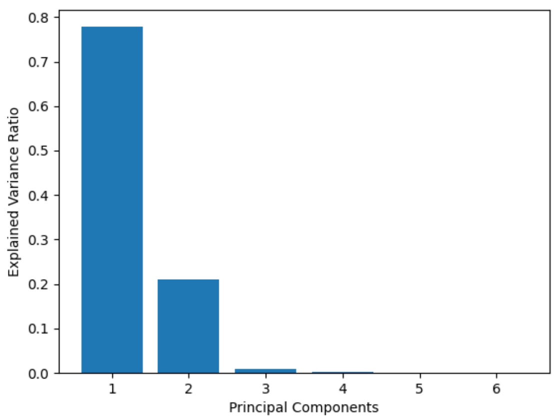
3.5.3 Revealing Hidden Trends and Explaining Anomalies

In our analysis, we've encountered a scenario where certain soil and plant parameters exhibit limited statistical significance or weak significance with respect to TPH removal when subjected to ANOVA. Despite this, a discernible pattern becomes apparent when we take a look at the bar graphs that pair each soil/plant parameter with corresponding control/treatment groups. Furthermore, when TPH removal is graphed against soil/plant parameters in regression analysis, the scatterplots notably show a pattern in how treatment/control groups are positioned. This intriguing pattern implies the potential existence of a relationship that may not be captured by ANOVA and related tests alone. An important observation arises when we consider the influence of a particular treatment group: T3. This treatment group exhibits relatively high Cd (Cd) removal but markedly low TPH removal. Notably, T3 is the only treatment group that lacks a bacterial strain inoculation. This insight brings to light the possibility that the presence of T3 might be introducing complexity into the ANOVA results and other associated analyses including regression.

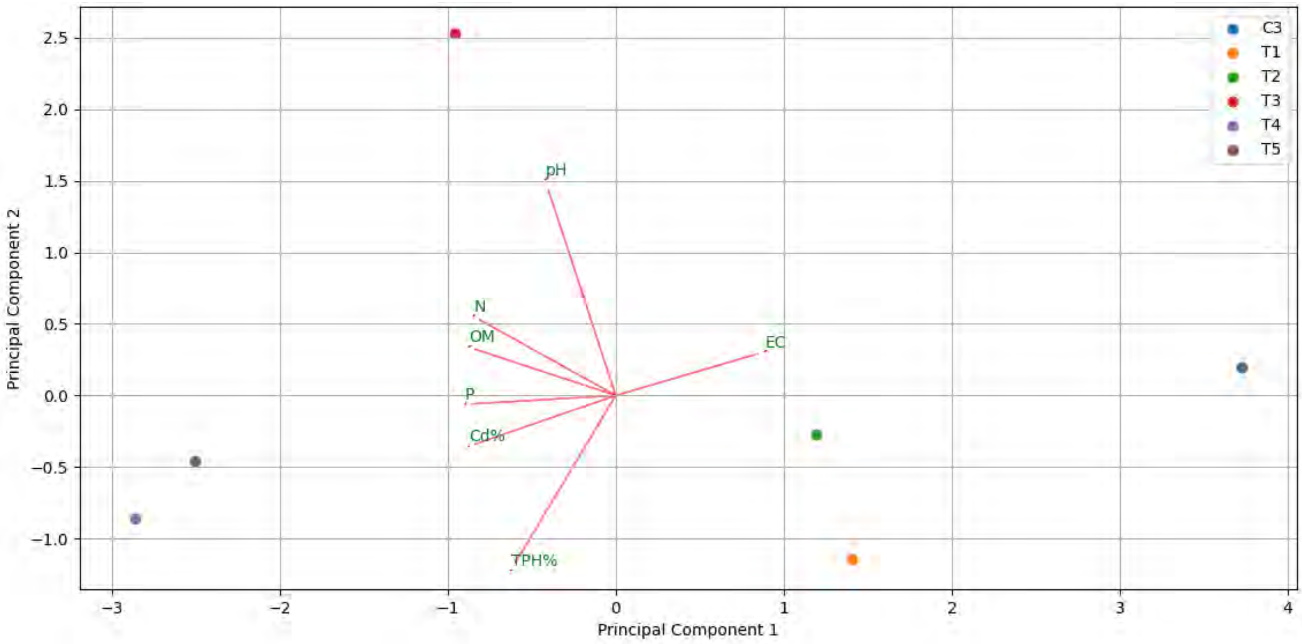
To confirm and account for the complexity that T3 presents, we will proceed with the principal component analysis (PCA). PCA is particularly suited for capturing underlying trends and relationships within complex datasets. Using this technique, we can visualize clusters of similar treatment/control groups in a reduced dimensional space. By examining how treatment/control groups cluster in the PCA space, we can uncover relationships that might not be apparent in traditional analyses. Moreover, PCA provides insight into which original parameters contribute most to the variance captured by each principal component. This can help us identify the key parameters that drive the observed trends in Cd and TPH removal.

Therefore, PCA will offer us a "birds-eye view" of the interplay between various soil and plant parameters and their relationship with Cd and TPH removal. Using this approach, we can identify underlying trends, potentially mitigating the effects of the anomaly introduced by T3 and enhancing our understanding of the complex dynamics at play in our experiment.

The PCA biplots and associated scree plots incorporating Cd and TPH removal with soil and plant parameters are shown below.



a. Scree plot



b. PCA biplot

Fig 3.27 Scree plot and PCA biplot of soil parameters incorporating Cd and TPH removal

The scree plot for the PCA biplot in Figure 3.24 shows that a majority of the variance is captured by the first principal component (PC1), and to a lesser extent, by the second principal component (PC2). Therefore, the PCA biplot shown for this data is valid and further principal components do not need to be accounted for.

Furthermore, the biplot showing soil parameters along with contaminant removal percentages confirms our earlier observation and speculation. Most treatments and control groups are spread out across PC1 and near the lower end of PC2. However, T3 is situated near the upper end of PC2 and much further away from the rest of the experimental groups. This observation suggests are T3 exhibits distinct characteristics compared to other groups. In addition, T1 and T2 are close to each other and can be clustered together, while T4 and T5 can be clustered together.

C3 is situated at the far left of PC1 and is closer to the T1 and T2 cluster, suggesting that these three experimental groups have similar characteristics. T3 lies in the middle while the T4 and T5 cluster is situated on the far right of PC2. This pattern again confirms our previous observations that experimental groups T4 and T5 have similar characteristics.

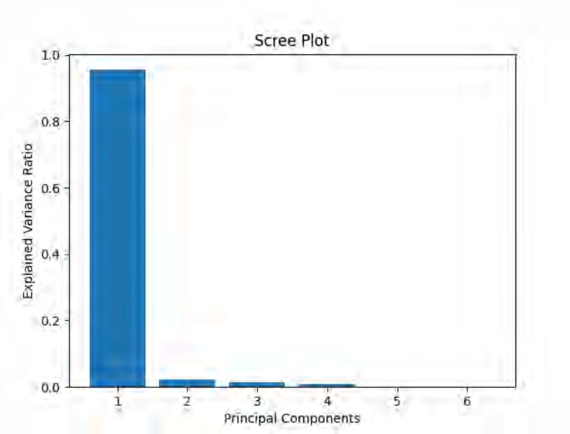
We can also make certain conclusions by looking at the directions and angles of the vectors in the PCA biplot. In general, when two vectors are in close proximity, creating a narrow angle between them, the variables they symbolize tend to exhibit positive correlation. If the vectors intersect at a right angle of 90°, their correlation is likely to be negligible. As the vectors diverge and form a wide angle (approaching 180°), they indicate a negative correlation between the variables.

Keeping the above points in mind, we can see that the vectors for Cd removal and EC are in opposite directions, with an angle approaching 180°, indicating a negative relationship. Cd removal and pH intersect at about 90°, indicating a negligible correlation. TPH removal intersects with pH at an angle that is close to 90°. Additionally, it has a wide angle with the EC vector, which indicates that the relationship is likely to be negative, but doesn't indicate an exactly negative correlation.

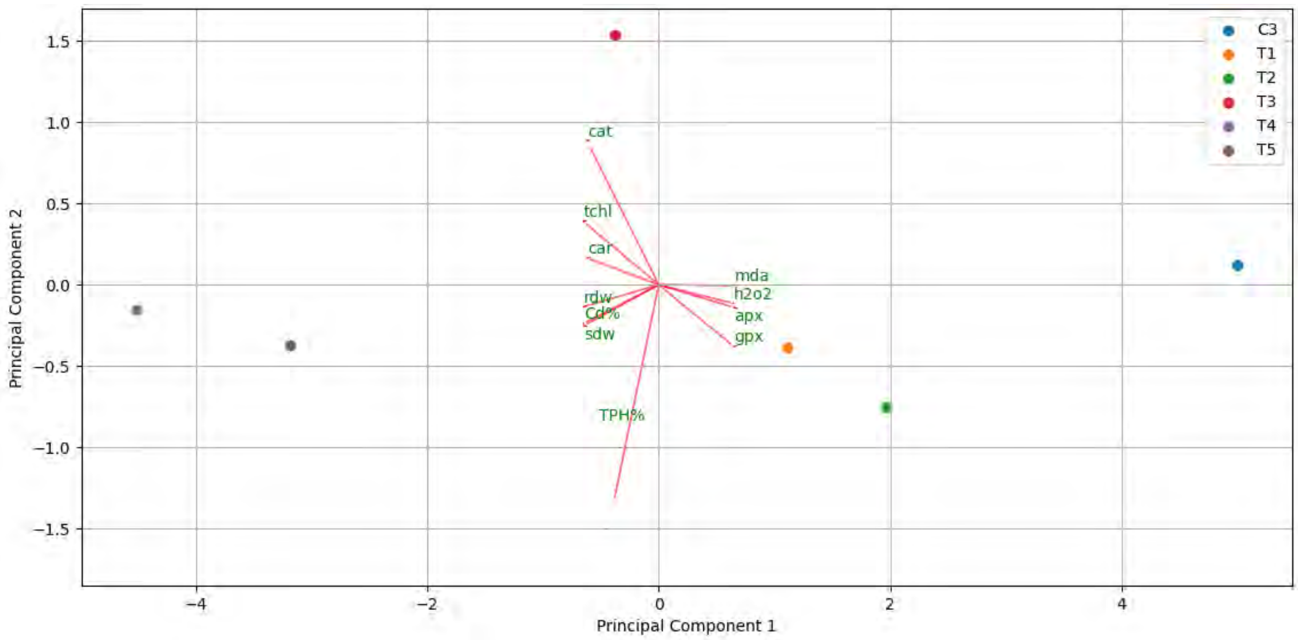
The Cd removal vector has the narrowest angle with phosphates, then organic matter, and then nitrates. This represents that Cd removal has the strongest positive relationship with soil phosphate levels and so on. The TPH removal vector has the smallest angle with Cd removal, followed by phosphates. A relationship is also indicated between TPH and organic matter. However, the angle between TPH removal vector and the vectors for nitrates is close to 90°.

Another conclusion that can be made from the PCA biplot is about the direction of the vectors and the positions of the experimental groups. The vectors for Cd removal, TPH removal, phosphates, organic matter, and nitrates point closely toward the clusters T4 and T5. They are also on the left side of the 0 mark on PC1. Conversely, the vector for EC points closely toward C3, with the clusters T1 and T2 nearby. Additionally, they are on the right side of the 0 mark of PC1. Since PC1 explains most of the relationships occuring in the PCA technique, the positions can tell us about the influence that these opposing vectors have on each experimental group. Notably, the vector for TPH points in the opposite direction of T3.

A PCA biplot for plant parameters and contaminant removal can be interpreted in the same way and is shown below.



a. Scree plot



b. PCA biplot

Fig 3.28 Scree plot and PCA biplot of plant parameters incorporating Cd and TPH removal

The scree plot in Figure 3.24 shows that a majority of variance is captured by PC1 and to a lesser extent, PC2. Exploring further principal components is not necessary. Moreover, the experimental groups dispersed in the PCA biplot for plant parameters show a similar pattern to the one we observed in the biplot with soil parameters. The treatment groups T1 and T2 along with T4 and T5 are closest to each other, respectively, and therefore can be clustered. All groups except T3 are near or below the 0 unit in PC2 while T3 is positioned much farther above them.

The vector for Cd removal has small angles with shoot dry weight, root dry weight, carotenoids, and total chlorophyll. The vector for TPH removal has small angles shoot and root dry weight as well, but they are relatively larger than the ones Cd removal has. The vectors for these parameters, along with the one for CAT, have opposing directions to the vectors for APX, GPX, MDA, and H2O2 along PC1. Furthermore, the vectors for the four stress indicators point in the direction of C3 and near the T1/T2 cluster. The remaining vectors point toward or near the direction of the T4/T5 cluster. As in the case of the biplot for soil parameters, in this biplot, the TPH removal vector points in an opposing direction to the group T3.

The overarching pattern for both PCA biplots is that similar treatments are clustered together: T4 with T5 and T1 with T2. C3 is positioned further away, but T3 was the most distinct treatment. This pattern makes sense when we link it with the distinctions among each treatment/control group. T4 and T5 consisted of bacterial strain and compost application and they show the highest removal for both TPH and Cd. T1 and T2 consisted of bacterial strain application only, and both show similar Cd and TPH removal. In T3, Cd removal is high (less than T4 and T5, but more than T1 and T2), but TPH removal is only slightly more than in the control group C3. Finally, C3 has the lowest removal percentages for both contaminants.

In addition, the biplots showed that Cd removal had stronger relationships, be it positive or negative, with soil and plant parameters than TPH removal. This was observed in the Pearson correlation analysis as well. Importantly, the PCA technique showed us that a relationship between TPH reduction and certain soil/plant parameters might exist, even if it is not as strong as their relationship with Cd removal. Therefore, this analysis propositions a different conclusion than the ANOVA test, which declared that most of the relationships are non-significant.

Chapter 4
Discussion

The present study takes a look at the remediation of Cd and TPH co-contamination in soil. The approach used to execute this task is phytoremediation with the plant *Sorghum bicolor*, and various combinations of the two Cd and TPH-resistant strains, *B. cereus* and *S.bozhouensis*, along with compost made from animal manure and plant waste.

The results of this study indicate that the maximum level of remediation for both Cd and TPH was achieved by the treatment group, T4. This group included both compost and the bacterial strain *S.bozhouensis.* T5 exhibited similar but slightly lower levels of Cd and TPH removal. This treatment included the application of *B. cereus* and compost. The PCA technique also clusters these treatment groups together, indicating their similarity in terms of contaminant removal and relationships with soil and plant attributes.

The next treatment that showed relatively high levels of co-contaminant removal was T1. The treatment group T2 showed similar, but relatively lower levels of removal. T2 included the application of *B.cereus* while T1 included the application of *S.bozhouensis*. Compost was not included in these treatments. In the case of T3, a Cd removal percentage higher than T1 and T2 was achieved, however, the TPH removal was much lower. The control group C3 with spiked soil and plant had the lowest removal percentages for both Cd and TPH.

These results are consistent with other studies in the literature. For instance, Ogundiran et al. (2018) observed that compost addition enhances plant root and shoot production, thereby, enhancing the heavy metal phytoextraction efficiency of the plant. Chirakkara and Reddy (2015) also made similar observations but with co-contaminated soil containing heavy metals and hydrocarbons; the application of compost improved the remediation of heavy metals due to an increase in plant biomass. Additionally, Saqib et al. (2021) demonstrate that incorporating animal manure-derived compost significantly enhanced plant biomass and chlorophyll content. This insight is also consistent with our observations that show higher biomass and photosynthetic pigment levels in treatments with compost application.

In another study, Ahmad et al. (2018) showed that a combined application of compost and a Bacillus spp. improved plant biomass, chlorophyll contents, and antioxidant homeostasis under heavy metal stress, more than compost or bacterial application alone. This observation is consistent with the improvement of plant growth parameters and antioxidant defense mechanisms in our treatments with combined compost application and bacterial inoculation.

Taking another look at the plant-bacteria synergism, Liu et al. (2020) observed that the inoculation of a Serratia spp. improved Cd translocation within the selected plant, thereby improving Cd remediation from the soil. In addition, this effect was enhanced when organic amendments were combined with bacterial inoculation. Similarly, Kumar, et al. (2022) showed that a combined application of organic manure and bacterial species resulted in greater Cd uptake due to an improvement in plant biomass, photosynthetic pigments, MDA content, and activity of antioxidant enzymes including GPX and APX. Again, the effect was more pronounced than with individual bacterial inoculation and amendment application.

Furthermore, Lee et al. (2023) showed that compost application improved TPH degradation in heavy metal and diesel co-contaminated soil as it resulted in the bio stimulation of heavy-metal resistant, hydrocarbon-degrading bacteria. These studies support our findings which show the highest removal of the co-contamination, along with an improvement in the Cd translocation factor when a combined application of compost and bacterial inoculation is used.

Uyizeye et al. (2019) also demonstrate that compost can prove to be very beneficial for TPH removal. In addition, they explain that compost needs to be accessible for microbial communities that possess the ability to degrade hydrocarbons. An effective remedy for this is phytoremediation as the plant’s root system can support TPH-degrading microbial communities by providing nutrients and growth promoters through root exudates.

However, in soil with high levels of heavy metal and TPH co-contamination, these microbial communities also need to be heavy metal resistant. It’s difficult to find such native microbial communities in fresh soil, which we later spiked with Cd and TPH for our experiment. This can explain why there wasn’t a noteworthy amount of TPH degradation in T3, which contained compost, but no bacterial inoculation. Nevertheless, in both C3 and T3, there was slightly greater TPH reduction than in C1, which in the absence of the plant, only showed natural attenuation of TPH. Some studies suggest that plants can potentially uptake hydrocarbons in their roots (Chen et al., 2018b; Balasubramaniyam, 2015). However, without additional studies, this effect cannot be confirmed.

Moving forward, we may be tempted to believe that the treatment differences (combinations of compost and bacterial strains) that explained Cd and TPH removal can also explain variations in soil and plant parameters. However, it should be noted that examining soil/plant parameters in a co-contamination scenario, as in our case with both Cd and TPH spiked soil, is a complex phenomenon on its own. This complexity exists because of the unique impact each contaminant has on distinct soil or plant parameters. Therefore, the remediation of one contaminant inherently affects associated parameters, and when we also apply treatments to remediate the other contaminant at the same time, it becomes harder to confidently determine which specific changes are caused by either contaminant.

For instance, by observing our results, we can say that Cd removal from the soil and subsequent uptake by the plant reduces EC in the soil. However, when we see a drop in EC in treatments with high TPH removal, it becomes difficult to pinpoint TPH as a responsible factor for this decline, without properly acknowledging that Cd might also be contributing to the result. Without the presence of a treatment like T3, where one contaminant gets markedly remediated but the other doesn't, it might have been difficult for us to reach conclusions on the relationship between soil/plant parameters and individual contaminants in a co-contamination scenario.

One conclusion that we can draw from our data is that the Cd contamination in our experiment has a greater effect on all relevant soil/plant parameters than the TPH contamination. We can observe this by comparing T3 with T1 and T2. Treatments T1 and T2 only included bacterial inoculation. They performed better than the control group C3, but not better than the treatments with compost in terms of Cd removal. This can be attributed to lesser nutrient enrichment, less ideal soil structure, and a lower level of bio stimulation. However, despite a lower Cd removal, these treatments did show greater TPH removal owing to the presence of hydrocarbon-degrading bacteria. The TPH content in these treatments was, therefore, lower than in T3. Despite this, T3 still performed better and had better plant growth indicators such as greater biomass, higher photosynthetic pigments, and lower stress marker levels. This difference between T3 and the groups T1 and T2 was not very high but still enough to be statistically significant.

Statistical tests like PCA and Pearson correlation also show a stronger relationship between Cd removal and relevant soil/plant parameters than that with TPH removal. These observations suggest that in the relationships between TPH removal and soil/plant parameters, Cd acts as a confounding variable (Kamangar, 2012). TPH removal may also be a confounding variable for relationships between Cd removal and soil/plant parameters. However, it does not appear to be as potent as Cd. Therefore, it can be suggested that the individual relationships we may see between TPH and soil/plant parameters may not be true representatives of their actual relationships, as they are confounded by the presence of Cd.

The above dilemma can be mitigated when don't look toward tests like ANOVA to prove statistical significance between Cd/TPH removal and soil/parameters. Such tests cannot offer us sound conclusions, as we observed in Section 3, where the ANOVA test showed nonsignificance between TPH and most parameters, even though intuitively and through an analysis of the literature, we know that couldn't be the case. Techniques like PCA offer helpful alternatives by clustering similar experimental groups and parameters together and allowing us to insights based on the data that we have and available literature.

In our analysis, we couldn't establish a relationship between pH and contaminant removal. However, this may be due to the complex interactions between bacteria, compost, and soil (Naz et al., 2022). Soil EC and TDS are shown to decrease proportionally with heavy metal uptake and this trend existed in our data as well (Salimi et al., 2012). However, there is no evidence of a straightforward relationship between EC, TDS, and TPH removal so far. Nevertheless, in one study on heavy metal and TPH co-contamination, the addition of TPH in heavy metal spiked soil is linked to a decrease in EC (Hammami et al., 2018). In our study however, we can't establish a strong link between TPH removal and EC due to the confounding presence of Cd removal.

Furthermore, Bai et al. (2021) suggest that increasing nitrate levels can enhance Cd accumulation in sorghum. An increase in phosphates has also been linked with better phytoextraction (Maqbool et al., 2022). Optimal levels of nitrates and phosphates also support microbial activity, which is inexplicably linked with TPH removal. The increase in nitrates and phosphates in T3, T4, and T5 can be attributed to compost addition. Furthermore, the slightly higher phosphate levels in inoculation treatments may suggest the phosphate solubilizing activities of the bacterial inoculated (Owhonka & Obire, 2019b).

The increase in organic matter and total organic carbon can be attributed to compost in the associated treatments as well. An increase in organic matter is beneficial to both plant and microbial health and activities. This can explain the improved plant parameter observations due to increased organic matter as a result of compost application (Grobelak, 2016).

In the case of plant parameters, as we have discussed above, the introduction of plant-growth-promoting bacteria and organic amendments like compost has a positive effect on plant physiology and photosynthesis abilities. Since Cd and TPH removal are highly correlated with increased biomass, they have a positive correlation with the former parameters as well (Hou et al., 2015).

Treatments that increase plant biomass and improve plant physiology not only lead to greater removal of contaminants, they also show a trend with stress markers. For instance, Guarino et al. (2018) show that microbial inoculant treatments which resulted in greater phytoextraction also had lower MDA levels than non-inoculated treatments. MDA is a by-product of lipid peroxidation from free radical damage. H2O2 is also a similar stress marker which also results from free radical damage.

Oxidative or free radical damage in plants occurs due to the stressful conditions created by the presence of contaminants in the plant's environment. As a response to these conditions, the plant upregulates the production of antioxidant enzymes which neutralize free radicals. APX, GPX, and CAT are examples of these enzymes in plants. Guarino et al. (2018) also note that with a high level of MDA, a high level of GPX and APX as well. This indicates the response and homeostasis that antioxidant enzymes have with free radical levels.

In our data, we have observed that CAT does not follow the predictable trend that other antioxidant enzymes follow. However, this trend can be explained through the observations of Sidhu et al. (2020) and Słomka et al. (2008). They found that CAT appears to have a complementary relationship with APX. Both enzymes are produced in response to H2O2, however, the amount in which they are produced differs from organelle to organelle. One part of a plant in stress may have high CAT content, but low APX content while in another part, the opposite may be true. This might also be occurring in our case, as we only measured stress markers and antioxidant enzymes in the plant leaf. The levels of CAT can be possibly higher in another part of our plant with complementary levels of APX.

Apart from trends in soil/plant parameters and contaminant removal, our data reveals some additional insights. Firstly, our selected plant, sorghum was not indicated as a hyperaccumulator. Even though a high translocation factor was observed in some treatments, it was not greater than one. For a plant to be a hyperaccumulator of a certain metal, it must possess a translocation factor that is greater than 1. Therefore, phytostabilization of Cd in *Sorghum* roots is indicated (Takarina & Pin, 2017).

Another important observation was in the context of the bacterial strains selected: *S.bozhouensis* and *B.cereus*. While *B.cereus* strains have been reported to have significant potential for Cd and TPH phytoremediation (Patowary et al., 2023; Wang et al., 2023), studies with *S.bozhouensis* have not been reported so far. Therefore, the present study can add to the literature on the benefits of this bacterial species for phytoremediation of Cd and TPH co-contamination.

Conclusion and Future Recommendations

The current study sought to address the complex challenge of co-contamination involving Cd and TPH in soil through phytoremediation. Employing the plant *Sorghum bicolor*, in conjunction with two Cd and TPH-resistant bacterial strains, *B. cereus* and *S. bozhouensis*, and supplemented by compost derived from animal manure and plant waste, this research aimed to assess the effectiveness of different treatment combinations in achieving efficient co-contaminant removal.

The findings of this study reveal that the treatment group T4, incorporating both compost and the bacterial strain *S. bozhouensis*, exhibited the highest levels of Cd and TPH removal. T5, involving the application of *B. cereus* and compost, also demonstrated similar albeit slightly lower rates of co-contaminant removal. Notably, the introduction of compost and bacterial strains in these treatments synergistically enhanced contaminant degradation and plant metal accumulation, demonstrating the promising potential of this approach which combines bioaugmentation and biostimulation.

Another important observation is that when compost application and bacterial inoculation were compared individually, we found that the treatment group with compost (T3) performs better than both treatment groups with bacterial inoculation alone (T1 and T2) in terms of Cd removal. However, for effective TPH remediation, the co-contaminated soil should be inoculated with heavy-metal resistant, hydrocarbon degrading microbes.

Our observations imply that organic amendments like compost contribute to enhanced nutrient availability and organic matter content in the soil, along with stimulating bacterial activity. These improvements positively impact both plant growth and contaminant remediation. Plant parameters such as biomass and physiological indicators, as well as stress markers are significantly improved by application of organic amendments, and enhanced further when plant growth promoting bacteria are applied as well.

Furthermore, this research highlights that Cd contamination plays a more dominant role in influencing soil and plant parameters than TPH contamination. While correlations between TPH removal and parameters exist, variations in Cd levels can confound these relationships.

In future research, it is recommended to explore the intricate interactions between contaminants, soil parameters, and plant responses in co-contamination scenarios. To establish a clearer cause-and-effect relationship, more controlled studies could isolate the impact of individual contaminants and then compare them with co-contaminated scenarios. Additionally, investigating the unique potential of bacterial strains like *S. bozhouensis* for Cd and TPH co-contamination phytoremediation, as well as exploring the role of different plant species, can further contribute to the development of effective remediation strategies. On the whole, this study provides valuable insights into the complex dynamics of co-contaminant remediation and opens avenues for further investigation into sustainable soil restoration methods.

References

Agamuthu, P., & Dadrasnia, A. (2013). Dynamics phytoremediation of Zn and diesel fuel in co-contaminated soil using biowastes. *J Bioremed Biodeg S*, *4*, 2.

Abbas, T., Rizwan, M., Ali, S., Adrees, M., Mahmood, A., Zia-ur-Rehman, M., ... & Qayyum, M. F. (2018). Biochar application increased the growth and yield and reduced Cd in drought stressed wheat grown in an aged contaminated soil. *Ecotoxicology and environmental safety*, *148*, 825-833.

Adedapo, A. A., & Adeoye, B. O. (2014). Cd, an environmental pollutant: a review. *West African Journal of Pharmacology and Drug Research*, *29*, 1-11.

Adieze, I. E., Orji, J. C., Nwabueze, R. N., & Onyeze, G. O. C. (2012). Hydrocarbon stress response of four tropical plants in weathered crude oil contaminated soil in microcosms. *International Journal of Environmental Studies*, *69*(3), 490-500.

Afegbua, S. L., & Batty, L. C. (2018). Effect of single and mixed polycyclic aromatic hydrocarbon contamination on plant biomass yield and PAH dissipation during phytoremediation. *Environmental Science and Pollution Research*, *25*, 18596-18603.

Agarry, S. E., Aremu, M. O., & Aworanti, O. A. (2013a). Biostimulation and phytoremediation treatment strategies of Gasoline-Nickel Co-Contaminated soil. *Soil and Sediment Contamination: An International Journal*, *23*(3), 227–244.

Agarry, S. E., Aremu, M. O., & Aworanti, O. A. (2013b). Biostimulation and phytoremediation treatment strategies of Gasoline-Nickel Co-Contaminated soil. *Soil and Sediment Contamination: An International Journal*, *23*(3), 227–244.

Agnello, A. C. (2014). *Potential of alfalfa for use in chemically and biologically assisted phytoremediation of soil co-contaminated with petroleum hydrocarbons and metals* (Doctoral dissertation, Université Paris Est; Università degli Studi di Cassino e del Lazio Meridionale; UNESCO-IHE).

Ahmad, I., Akhtar, M. J., Mehmood, S., Akhter, K., Tahir, M., Saeed, M. F., Hussain, M., & Hussain, S. (2018). Combined application of compost and Bacillus sp. CIK-512 ameliorated the lead toxicity in radish by regulating the homeostasis of antioxidants and lead. *Ecotoxicology and Environmental Safety*, *148*, 805–812.

Alaee, S., Talaiekhozani, A., Rezaee, S., Alaee, K., & Yousefian, E. (2014). Cd and male infertility. *Journal of Infertility and Reproductive Biology*, *2*(2), 62-69.

Ali, H., Khan, E., & Sajad, M. A. (2013). Phytoremediation of heavy metals—concepts and applications. *Chemosphere*, *91*(7), 869-881.

Anyanwu, B. O., Ezejiofor, A. N., Igweze, Z. N., & Orisakwe, O. E. (2018). Heavy metal mixture exposure and effects in developing nations: an update. *Toxics*, *6*(4), 65.

Asgher, M., Khan, M. I. R., Anjum, N. A., & Khan, N. A. (2015). Minimising toxicity of Cd in plants—role of plant growth regulators. *Protoplasma*, *252*, 399-413.

Ayesha Ameen, Jalil Ahmad and Shahid Raza (2018); Determination of total organic matter of mature compost prepared by using municipal solid waste; Int J Sci Res Publ 6(5) (ISSN: 2250-3153). http://www.ijsrp.org/research-paper-0516.php?rp=P535375

Bai, Z., Li, D., Zhu, L., Tang, X., Wang, Y., Mao, R., & Wu, J. (2021). Nitrate increases Cd accumulation in sweet sorghum for improving phytoextraction efficiency rather than ammonium. *Frontiers in Plant Science*, *12*.

Balasubramaniyam, A. (2015). The influence of plants in the remediation of petroleum hydrocarbon- contaminated sites. *Pharmaceutical Analytical Chemistry*, *01*(01).

Baltrons, O., López-Mesas, M., Vilaseca, M., Gutiérrez-Bouzán, C., Le Derf, F., Portet-Koltalo, F., & Palet, C. (2018). Influence of a mixture of metals on PAHs biodegradation processes in soils. *Science of the total environment*, *628*, 150-158.

Bashir, S., Gulshan, A. B., Iqbal, J., Husain, A., Alwahibi, M. S., Alkahtani, J., Dwiningsih, Y., Bakhsh, A., Ahmed, N., Khan, M. J., Ibrahim, M., & Diao, Z. (2021). Comparative role of animal manure and vegetable waste induced compost for polluted soil restoration and maize growth. *Saudi Journal of Biological Sciences*, *28*(4), 2534–2539.

Bernhoft, R. A. (2013). Cd toxicity and treatment. *The Scientific World Journal*, *2013*.

Bora, M. S., & Sarma, K. P. (2021). Anatomical and ultrastructural alterations in Ceratopteris pteridoides under Cd stress: A mechanism of Cd tolerance. *Ecotoxicology and Environmental Safety*, *218*, 112285.

Branca, J. J., Fiorillo, C., Carrino, D., Paternostro, F., Taddei, N., Gulisano, M., ... & Becatti, M. (2020). Cd-induced oxidative stress: focus on the central nervous system. *Antioxidants*, *9*(6), 492.

Chen, C., Wang, X., & Wang, J. (2019). Phytoremediation of Cd-contaminated soil by Sorghum bicolor and the variation of microbial community. *Chemosphere*, 235, 985–994.

Chen, L., Larson, S. L., Ballard, J. H., Ma, Y., Zhang, Q., Li, J., ... & Han, F. X. (2019). Laboratory spiking process of soil with various uranium and other heavy metals. *MethodsX*, *6*, 734-739.

Chen, S., Wang, J., Waigi, M. G., & Gao, Y. (2018a). Glomalin-related soil protein influences the accumulation of polycyclic aromatic hydrocarbons by plant roots. *Science of the Total Environment*, *644*, 465–473.

Chen, S., Wang, J., Waigi, M. G., & Gao, Y. (2018b). Glomalin-related soil protein influences the accumulation of polycyclic aromatic hydrocarbons by plant roots. *Science of the Total Environment*, *644*, 465–473.

Chirakkara, R. A., & Reddy, K. R. (2015). Biomass and chemical amendments for enhanced phytoremediation of mixed contaminated soils. *Ecological Engineering*, *85*, 265–274.

Choden, D., Pokethitiyook, P., Poolpak, T., & Kruatrachue, M. (2020). Phytoremediation of soil co-contaminated with zinc and crude oil using *Ocimum gratissimum* (L.) in association with *Pseudomonas putida* MU02. *International Journal of Phytoremediation*, *23*(2), 181–189.

Ding, Y. (2019). Heavy metal pollution and transboundary issues in ASEAN countries. *Water Policy*, *21*(5), 1096-1106.

Estefan, G. (2013). Methods of soil, plant, and water analysis: a manual for the West Asia and North Africa region.

Girolkar, S., Thawale, P., & Juwarkar, A. (2021). Bacteria-assisted phytoremediation of heavy metals and organic pollutants: Challenges and future prospects. *Bioremediation for environmental sustainability*, 247-267.

Gobetti, S., Kumor, A., Płociniczak, T., Prach, M., Sinkkonen, A., & Pacwa-Płociniczak, M. (2022). The multiway support of phytoremediation efficiency of soil historically co-contaminated with petroleum hydrocarbons and heavy metals using Zea mays. *Research Square (Research Square)*.

Gouda, A. H., El-Gendy, A. S., El-Razek, T. M. A., & El-Kassas, H. I. (2016). Evaluation of phytoremediation and bioremediation for sandy soil contaminated with petroleum hydrocarbons. *International Journal of Environmental Science and Development*, *7*(7), 490-493.

Grifoni, M., Rosellini, I., Angelini, P., Petruzzelli, G., & Pezzarossa, B. (2020). The effect of residual hydrocarbons in soil following oil spillages on the growth of Zea mays plants. *Environmental Pollution*, *265*, 114950.

Grobelak, A. (2016). Organic Soil Amendments in the Phytoremediation Process. In *Phytoremediation: Management of Environmental Contaminants*.

Guarino, C., Paura, B., & Sciarrillo, R. (2018). Enhancing phytoextraction of HMS at real scale, by combining Salicaceae trees with microbial consortia. *Frontiers in Environmental Science*.

Haider, F. U., Ejaz, M., Cheema, S. A., Khan, M. I., Zhao, B., Liqun, C., ... & Mustafa, A. (2021). Phytotoxicity of petroleum hydrocarbons: Sources, impacts and remediation strategies. *Environmental Research*, *197*, 111031.

Hammami, H., Alaie, E., & Dastgheib, S. M. M. (2018). The ability of *Silybum marianum* to phytoremediate cadmium and/or diesel oil from the soil. *International Journal of Phytoremediation*, *20*(8), 756–763.

Harikumar, P. S., & Sreedevi, M. A. (2023). Occurrence, distribution, and ecological risk of heavy metals and persistent organic pollutants (OCPs, PCBs, and PAHs) in surface sediments of the Ashtamudi wetland, south-west coast of India. *Regional Studies in Marine Science*, 103044.

Hoang, S. A., Sarkar, B., Seshadri, B., Lamb, D., Wijesekara, H., Vithanage, M., ... & Bolan, N. S. (2021). Mitigation of petroleum-hydrocarbon-contaminated hazardous soils using organic amendments: a review. *Journal of Hazardous Materials*, *416*, 125702.

Hou, J., Liu, W., Wang, B., Wang, Q., Luo, Y., & Franks, A. E. (2015). PGPR enhanced phytoremediation of petroleum contaminated soil and rhizosphere microbial community response. *Chemosphere*, *138*, 592–598.

Hseu, Z. Y., Chen, Z. S., Tsai, C. C., Tsui, C. C., Cheng, S. F., Liu, C. L., & Lin, H. T. (2002). Digestion methods for total heavy metals in sediments and soils. *Water, air, and soil pollution*, *141*, 189-205.

Huang, M., Zhu, Y., Li, Z., Huang, B., Luo, N., Liu, C., & Zeng, G. (2016). Compost as a soil amendment to remediate heavy metal-contaminated agricultural soil: mechanisms, efficacy, problems, and strategies. *Water, Air, & Soil Pollution*, *227*, 1-18.

Ibeto, C., Onyekachi, O., & Aju, E. (2023). Environmental and health risks assessment of n-alkanes and BTEX in Eze Iyi River at oil spill site in Isuikwuato, Abia State, Nigeria. *Environmental Monitoring and Assessment*, *195*(6), 717.

Jampasri, K., Pokethitiyook, P., Poolpak, T., Kruatrachue, M., Ounjai, P., & Kumsopa, A. (2019). Bacteria-assisted phytoremediation of fuel oil and lead co-contaminated soil in the salt-stressed condition by *chromolaena odorata* and *Micrococcus luteus*. *International Journal of Phytoremediation*, *22*(3), 322–333.

Kamangar, F. (2012, August 1). *Confounding variables in Epidemiologic Studies: Basics and beyond*. http://journalaim.com/Article/348

Kapoor, D., Singh, S., Kumar, V., Romero, R., Prasad, R., & Singh, J. (2019). Antioxidant enzymes regulation in plants in reference to reactive oxygen species (ROS) and reactive nitrogen species (RNS). *Plant Gene*, *19*, 100182.

Khan, S., Afzal, M., Iqbal, S., & Khan, Q. M. (2013). Plant–bacteria partnerships for the remediation of hydrocarbon contaminated soils. *Chemosphere*, *90*(4), 1317-1332.

Koohkan, H., Mortazavi, M. S., Golchin, A., Saraji, F., & Akbarzadeh-Chomachaei, G. (2023). Comparison of Native Bacterial and Fungal Bioaugmentation in the Removal of Petroleum from Soil in the Presence of Sorghum. *Water, Air, & Soil Pollution*, *234*(5), 309.

Kumar, H., Ishtiyaq, S., Favas, P. J., Varun, M., & Paul, M. S. (2022). Effect of Metal-resistant PGPB on the Metal Uptake, Antioxidative Defense, Physiology, and Growth of Atriplex lentiformis (Torr.) S.Wats. in Soil Contaminated with Cd and Nickel. *Journal of Plant Growth Regulation*, *42*(6), 3868–3887.

Lee, S. Y., Lee, Y., & Cho, K. (2022). Effect of Novosphingobium sp. CuT1 inoculation on the rhizoremediation of heavy metal- and diesel-contaminated soil planted with tall fescue. *Environmental Science and Pollution Research*, *30*(6), 16612–16625.

Lee, Y. Y., Lee, S. Y., & Cho, K. S. (2023). Phytoremediation and bacterial community dynamics of diesel-and heavy metal-contaminated soil: Long-term monitoring on a pilot scale. International Biodeterioration & Biodegradation, 183, 105642.

Lee, Y., Lee, S. Y., & Cho, K. (2023). Long-term comparison of the performance of biostimulation and phytoextraction in soil contaminated with diesel and heavy metals. *Chemosphere*, *337*, 139332.

Li, H., Yang, Z., Dai, M., Diao, X., Dai, S., Fang, T., & Dong, X. (2020). Input of Cd from agriculture phosphate fertilizer application in China during 2006–2016. *Science of the Total Environment*, *698*, 134149.

Liu, H., Xie, Y., Li, J., Zeng, G., Li, H., Xu, F., Su, F., & Xu, H. (2020). Effect of Serratia sp. K3 combined with organic materials on Cd migration in soil-vetiveria zizanioides L. system and bacterial community in contaminated soil. *Chemosphere*, *242*, 125164.

Liu, M., Sun, J., Li, Y., & Xiao, Y. (2017). Nitrogen fertilizer enhances growth and nutrient uptake of Medicago sativa inoculated with Glomus tortuosum grown in Cd-contaminated acidic soil. Chemosphere, 167, 204-211.

Lu, H., Xia, C., Chinnathambi, A., Nasif, O., Narayanan, M., Shanmugam, S., ... & Anupong, W. (2023). Optimistic influence of multi-metal tolerant Bacillus species on phytoremediation potential of Chrysopogon zizanioides on metal contaminated soil. *Chemosphere*, *311*, 136889.

Ma, D., Xu, J., Zhou, J., Ren, L., Li, J., Zhang, Z., ... & Wu, T. (2023). Using Sweet Sorghum Varieties for the Phytoremediation of Petroleum-Contaminated Salinized Soil: A Preliminary Study Based on Pot Experiments. *Toxics*, *11*(3), 208

Madariaga-Navarrete, A., Rodríguez-Pastrana, B. R., Villagómez-Ibarra, J. R., Acevedo-Sandoval, O. A., Perry, G., & Islas-Pelcastre, M. (2017). Bioremediation model for atrazine contaminated agricultural soils using phytoremediation (using Phaseolus vulgaris L.) and a locally adapted microbial consortium. *Journal of Environmental Science and Health, Part B*, *52*(6), 367-375.

Maqbool, A., Rizwan, M., Yasmeen, T., Arif, M. S., Hussain, A., Mansha, A., Ali, S., Alshaya, H., & Okla, M. K. (2022). Phosphorus Fertilizers Enhance the Phytoextraction of Cd through Solanum nigrum L. *Plants*, *11*(3), 236.

Marchal, G., Smith, K. E., Mayer, P., de Jonge, L. W., & Karlson, U. G. (2014). Impact of soil amendments and the plant rhizosphere on PAH behaviour in soil. *Environmental pollution*, *188*, 124-131.

Naz, M., Dai, Z., Hussain, S., Tariq, M., Danish, S., Khan, I. U., Qi, S., & Du, D. (2022). The soil pH and heavy metals revealed their impact on soil microbial community. *Journal of Environmental Management*, *321*, 115770.

Ogundiran, M. B., Mekwunyei, N. S., & Adejumo, S. A. (2018). Compost and biochar assisted phytoremediation potentials of Moringa oleifera for remediation of lead contaminated soil. *Journal of Environmental Chemical Engineering*, *6*(2), 2206–2213.

Oriomah, C., Adelowo, O. O., & Adekanmbi, A. O. (2015). Bacteria from spent engine-oil-contaminated soils possess dual tolerance to hydrocarbon and heavy metals, and degrade spent oil in the presence of copper, lead, zinc and combinations thereof. *Annals of microbiology*, *65*, 207-215.

Owhonka, A., & Obire, O. (2019a). Effect of soil microbes on nitrate, phosphate and total hydrocarbon content of various concentrations of oilfield wastewater. *Journal of Advances in Microbiology*, 1–5.

Owhonka, A., & Obire, O. (2019b). Effect of soil microbes on nitrate, phosphate and total hydrocarbon content of various concentrations of oilfield wastewater. *Journal of Advances in Microbiology*, 1–5.

Patowary, K., Bhuyan, T., Patowary, R., Mohanta, Y. K., Panda, B. P., Deka, S., Islam, N. F., Joshi, S., & Sarma, H. (2023). Soil treatment using a biosurfactant producing bacterial consortium in rice fields contaminated with oily sludge— a sustainable approach. *Environmental Research*, *220*, 115092.

Rehman, R., Ali, M. I., Qureshi, U. M., & Jamal, A. (2019). Characterization of brownfield: ex-situ detection of hydrocarbon degrading and biosurfactants producing microflora. *Pakistan Journal of Agricultural Sciences*, *56*(4).

Salimi, M., Amin, M. M., Ebrahimi, A., Ghazifard, A., & Najafi, P. (2012). Influence of electrical conductivity on the phytoremediation of contaminated soils to $Cd^{2+}$and Zn^{2+}. *International Journal of Environmental Health Engineering*, *1*(1), 11.

Sarathchandra, S. S., Rengel, Z., & Solaiman, Z. M. (2023). A Review on Remediation of Iron Ore Mine Tailings via Organic Amendments Coupled with Phytoremediation. *Plants*, *12*(9), 1871.

Sarma, N., Goswami, M., Rabha, S., Patowary, R., & Devi, A. (2023). Baseline study of water, soil, and identification of potential native phytoremediators of total petroleum hydrocarbon from oil-contaminated areas in the vicinity of Geleky oilfield of Assam. *Environmental Monitoring and Assessment*, *195*(7), 1-24.

Sekar, A., Katzman, S., O'Donnell, M., Velani, S., & Conway, K. S. (2022). Suspected Fanconi syndrome from Cd toxicity exacerbated by heavy kratom use. A rare occurrence. *Clin. Toxicol*.

Shi, Y., Huang, J., Zeng, G., Gu, Y., Chen, Y., Hu, Y., ... & Shi, L. (2017). Exploiting extracellular polymeric substances (EPS) controlling strategies for performance enhancement of biological wastewater treatments: an overview. *Chemosphere*, *180*, 396-411.

Shirisha, K., Sahrawat, K. L., Devi, B. P., & Wani, S. P. (2014). Simple and accurate method for routine analysis of heavy metals in soil, plant, and fertilizer. *Communications in soil science and plant analysis*, *45*(16), 2201-2206.

Sidhu, G. P. S., Bali, A. S., Singh, H. P., Batish, D. R., & Kohli, R. K. (2020). Insights into the tolerance and phytoremediation potential of Coronopus didymus L. (Sm) grown under zinc stress. *Chemosphere*, *244*, 125350.

Słomka, A., Libik-Konieczny, M., Kuta, E., & Miszalski, Z. (2008). Metalliferous and non-metalliferous populations of Viola tricolor represent similar mode of antioxidative response. *Journal of Plant Physiology*, *165*(15), 1610–1619.

Smolders, E., & Six, L. (2013). Revisiting and updating the effect of phosphate fertilizers to Cd accumulation in European agricultural soils. *Division of SoilandWater Management, Heverlee, Belgium*.

Su, Q., Yu, J., Fang, K., Dong, P., Li, Z., Zhang, W., ... & Cai, J. (2023). Microbial Removal of Petroleum Hydrocarbons from Contaminated Soil under Arsenic Stress. *Toxics*, *11*(2), 143.

Suciu, N. A., Devivo, R., Rizzati, N., & Capri, E. (2022). Cd content in phosphate fertilizer: which potential risk for the environment and human health?. *Current Opinion in Environmental Science & Health*, 100392.

Takarina, N. D., & Pin, T. G. (2017). Bioconcentration factor (BCF) and translocation factor (TF) of heavy metals in mangrove trees of Blanakan Fish Farm. *Makara Journal of Science*, *21*(2).

Timofeeva, A., Galyamova, M., & Sedykh, S. (2022). Prospects for using phosphate-solubilizing microorganisms as natural fertilizers in agriculture. *Plants*, *11*(16), 2119.

Uyizeye, O. C., Thiet, R. K., & Knorr, M. A. (2019). Effects of community-accessible biochar and compost on diesel-contaminated soil. *Bioremediation Journal*, *23*(2), 107–117.

Villalobos, M., Avila-Forcada, A. P., & Gutierrez-Ruiz, M. E. (2008). An improved gravimetric method to determine total petroleum hydrocarbons in contaminated soils. *Water, air, and soil pollution*, *194*, 151-161.

Wang, X., Luo, S., Chen, Y., Zhang, R., Ling, L., Lin, K., Qiu, C., & Xu, H. (2023). Potential of Miscanthus floridulus associated with endophytic bacterium Bacillus cereus BL4 to remediate Cd contaminated soil. *Science of the Total Environment*, *857*, 159384.

Wu, J., Zhao, N., Zhang, P., Zhu, L., Lu, Y., Lei, X., & Bai, Z. (2023). Nitrate enhances Cd accumulation through modulating sulfur metabolism in sweet sorghum. *Chemosphere*, *313*, 137413.

Ye, S., Zeng, G., Wu, H., Zhang, C., Dai, J., Liang, J., ... & Zhang, C. (2017). Biological technologies for the remediation of co-contaminated soil. *Critical reviews in biotechnology*, *37*(8), 1062-1076.

Younis, U., Malik, S. A., Rizwan, M., Qayyum, M. F., Ok, Y. S., Shah, M. H. R., ... & Ahmad, N. (2016). Biochar enhances the Cd tolerance in spinach (Spinacia oleracea) through modification of Cd uptake and physiological and biochemical attributes. *Environmental Science and Pollution Research*, *23*, 21385-21394.

Zhang, C., Chen, Y., Xu, W., Chi, S., Li, T., Li, Y., ... & Feng, D. (2019). Resistance of alfalfa and Indian mustard to Cd and the correlation of plant Cd uptake and soil Cd form. *Environmental Science and Pollution Research*, *26*, 13804-13811.

Zulfiqar, U., Jiang, W., Xiukang, W., Hussain, S., Ahmad, M., Maqsood, M. F., ... & Mustafa, A. (2022). Cd phytotoxicity, tolerance, and advanced remediation approaches in agricultural soils; a comprehensive review. *Frontiers in Plant Science*, *13*, 773815.