BIOREMEDIATION OF CRUDE OIL AND HEAVY METALS POLLUTED SOIL WITH CONSTRUCTED WETLANDSYSTEM

BY

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CERTIFICATION

This is to certify that this work "Bioremediation of crude oil and heavy metals polluted soil with constructed wetland system" was carried out by Ikeanumba, Michael Okwudiri (Reg. No. 20164026898) in partial fulfilment of the requirements for the award of Doctor of Philosophy (PhD) degree Environmental Microbiology in the Department of Microbiology, postgraduate School, Federal University of Technology, Owerri, Imo State.

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DEDICATION

I dedicate this work to my wife Mrs. Ingibo Malachi Michael for always being there for me; my children – Belema, Nimi, Hephzibah, Beulah and to the family of Prof. and Dr. (Mrs.) Julian Chukwuma Iwuagwu for their priceless supports.

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ABSTRACT

Thepreliminary part of this study was carried out to determine the tolerance limit of eight (8) eight native plant species in soil polluted with crude oil (3%, 7%, and 10% concentrations) and fixed levels of heavy metals (Zn: 39.4 mg/L, Ni: 10.2 mg/L, Cu: 29.4 mg/L, Pb: 11.2 mg/L). The selected plants—*Brachiaria distachyoides Stapf*, *Paspalum conjugatum P.J. Bergius*, *Cyperus dichrostachyus Hochst. ex A. Rich.*, *Kalanchoe pinnata (Lam.) Pers.*, *Panicum maximum Jacq.*, *Mimosa pudica L.*, *Mariscus ligularis L.*, and *Mariscus rotundus*—were sourced from crude oil-impacted sites.Results showed that *Paspalum conjugatum P.J. Bergius*gave the best result, thriving in 10% crude oil and heavy metal-contaminated soil for five months followed by*Mariscus ligularis L.* and *Brachiaria distachyoides Stapf*which survived for 30 days under the same conditions. Other species were only able to tolerate 7% crude oil heavy metals. Toxicology studies of amendments revealed that a 1% native soap solution provided optimal growth stimulation for hydrocarbon-utilizing microorganisms (bacteria and fungi), followed by 1% poultry manure and a 1% combination of both. This was followed by 10%, while higher amendment concentrations of 30% significantly inhibited microbial growth.The study shows the effectiveness of hybrid constructed wetlands for the remediation of soil and water polluted with crude oil and heavy metals. Microbial analysis identified *Pseudomonas xiamenensis*, *Acinetobacter baumannii*, *Alcaligenes cloacae*, *Enterobacter cloacae*, *Pantoea dispersa*, *Lysinibacillus fusiformis*, and *Kocuria palustris* as hydrocarbon-degrading bacterial species in the soil. Among these, *Pseudomonas xiamenensis* was the most prolific biosurfactant producer, while the others showed moderate production levels.These findings provide valuable insights into the development of sustainable strategies for bioremediation of crude oil and heavy metal-polluted soils.

Key words: Phytoremediation, phytoaccumlation, biosurfactant, remediation and constructed wetland

CHAPTER ONE

INTRODUCTION

1.1 Background information

The necessity for sustainable wastewater and land treatment technologies that are environmentally friendly, easy to operate, less energy-intensive, and cost-effective is increasingly critical in light of growing environmental crisis. These include pollution, water shortages, climate change (Hartemink, 2006), rapid population growth, and other pressing issues. Constructed wetlands (CWs) have emerged as a viable solution for achieving wastewater treatment objectives by harnessing natural components and processes. This approach minimizes reliance on energy-intensive mechanical systems and reduces technological complexity. Additionally, CWs leverage natural processes to effectively transform hazardous chemicals (Yergeau, Sanschagrin, Beaumier, & Greer, 2012).

Wetlands are now acknowledged as ecologically significant systems that provide habitats for diverse species and support their survival. They contribute to groundwater aquifer replenishment, flood management, carbon dioxide sequestration, heat regulation, sediment trapping, and other ecological benefits (Stefanakis, Akratos, and Tsihrintzis, 2014). The advantages of wetlands can be articulated in environmental, social, cultural, and economic contexts (De Groot, Stuip, Finlayson, & Davidson 2006).

Historically, natural wetlands have been capable of filtering water and improving water quality; however, the pervasive nature of industrial pollution has necessitated a reassessment of their purification capabilities. For centuries, natural wetlands have served as disposal sites for secondary or tertiary wastewater effluents. During the Minoan period on the Greek island of Crete, advanced sewerage systems were constructed at the Zakros and Knossos Palaces, with nearby rivers and marshes utilized as disposal sites ((Angelakis, Koutsoyiannis, & Tchobanoglous, 2005;Stefanakis, Akratos, & Tsihrintzis, 2014). The transition from natural wetlands to constructed wetlands (CWs) in modern times is based on the potential to harness naturally occurring processes in a controlled environment for beneficial human and environmental purposes while ensuring the preservation of the natural ecosystem.

Constructed wetlands are designed to replicate and enhance the functions of natural wetlands, providing similar benefits but often exhibiting greater biological diversity. Research has shown that constructed wetlands can significantly improve flood and stormwater management, enhance water quality, and restore biodiversity (Ghermandi, Van den Bergh, Brander, De Groot, & Nunes, 2010). This effectiveness may stem from the ease with which urban planners, engineers, and landscape architects can integrate them into developed areas. For instance, Alexander (2018) noted that using engineered wetlands for bioremediation of crude oil-contaminated soil led to a reduction in soil pH, suggesting this method can mitigate the hydrocarbon effects of oil spills in wetland environments. Today, "Constructed Wetlands"—also known as "Treatment Wetlands" or "Reed Beds"—refers to these artificial systems designed to utilize natural processes within regulated settings (Stefanakis et al., 2014). They fall under a broader category of natural treatment systems that employ natural processes or components for wastewater treatment while minimizing reliance on external energy sources. Natural treatment systems are classified into three types: terrestrial, aquatic, and wetland. Examples include stabilization or oxidation ponds for aquatic systems and slow rate systems or soil aquifer treatment for terrestrial systems (Stefanakis et al., 2014).

Constructed wetlands (CWs) occupy a unique position between natural and traditional wastewater treatment systems. The technology for constructed wetlands began in the 1950s with research conducted at the Max Planck Institute in Germany. The first CW systems were established in Europe during the 1960s, followed by implementations in the United States in the 1970s and 1980s. However, despite these initial developments, constructed wetlands did not gain widespread testing or usage until the late 1980s. Early challenges to their adoption included failures of some of the first systems due to design flaws, reflecting a lack of expertise, alongside competition from conventional treatment methods that had been in place for over 80 years.

In the past 20 years, there has been a significant rise in environmental awareness and sensitivity, leading to increased interest in alternative treatment methods. As a result, the technology surrounding constructed wetlands has experienced substantial growth in both research and practical applications. According to Stefanakis et al. (2014), the number of published studies on constructed wetlands more than doubled from 2000 to 2010 compared to the previous decade (1990-2000).

Constructed wetlands represent one of the most intriguing and attractive advancements in environmental and ecological engineering. Unlike traditional biological treatment systems, which typically follow a centralized approach, constructed wetlands operate on a decentralized model. This innovative method for treating pollution introduces new dimensions and perspectives, emphasizing sustainability and the overall environmental impact.

1.2 Problem statement

Over the past 50 years, the manufacturing, petroleum, and gas industries in Nigeria have discharged significant quantities of hydrocarbons and related pollutants, including heavy metals, into the Niger Delta and other regions from both refined and unrefined petroleum products (Obot, Antonio, Braide, Dore, Wicks, & Steiner, 2006; UNEP 2011).

Pollution levels have severely impacted the environment, particularly in soil and water bodies, leading to significant declines in both terrestrial and aquatic biodiversity, as well as disruptions to public health and the life support systems of local communities. Despite some advancements made in this area, efforts to establish a clear and effective bioremediation process for petrochemicals and heavy metals have not gained widespread recognition, resulting in ongoing inconsistencies (Ameh, Mohammed-Dabo, Ibrahim, & Ameh, 2013; Schaefer & Filser 2007; Singer, Jury, Leupromchai, Yahng, Crowley, 2001).

Natural systems like constructed wetlands (CWs) are utilized to meet wastewater treatment objectives by leveraging natural components such as plants and microorganisms. These systems employ processes like bioremediation and phytoremediation, which help reduce reliance on energy-intensive mechanical devices and minimize technological complexity. CWs facilitate natural processes that effectively transform hazardous chemicals (Yergeau et al., 2012). Natural wetlands are recognized as the most biologically diverse ecosystems, performing essential functions such as nutrient storage and recycling, providing habitats for wildlife, stabilizing shorelines, managing and buffering natural floods, recharging groundwater, and treating water pollutants. Constructed wetlands are engineered systems designed to mimic the functions of natural wetlands in a specific area, allowing for management and manipulation to achieve desired environmental goals. Research indicates that constructed wetlands can effectively remove nitrogen and phosphorus through a combination of physical, chemical, and biological processes. They act as "nature's kidneys," filtering out pollutants while also transforming and sequestering nutrients as water flows through them. This multifaceted approach enhances their ability to improve water quality while providing various ecosystem services.

1.3 Objectives of the Study

The main objective of this study is the use of constructed wetland systems in bioremediation of soil contaminated with crude oil and heavy metals. The specific objectives are to;

i. Screen various native plants for their potential to tolerate crude oil and heavy metals in an impacted ecosystem.
ii. Determine genera of crude oil-utilizing microorganisms associated with phytoremediator plants
iii. Determine the effect of native black soap, poultry litter, or their combination in enhancing bioremediation of heavy metal and crude oil polluted soils.
iv. Investigate the efficacy of constructed wetland technology in phytoremediation of polluted soil.
v. Determine the efficacy in TPH reduction under different amendments in plants, native soap and poultry manure.

1.4 Justification of the study

Crude oil spills and heavy metal contamination pose significant threats to drinking water supplies and agricultural lands, adversely affecting aquatic life, animals, and humans. When these pollutants are ingested, they can lead to carcinogenic and mutagenic effects; unfortunately, cultivated plants and animals can absorb these toxins, transferring them through the food chain. Consequently, it is imperative to remediate contaminated sites promptly. While physical and chemical remediation methods have been commonly employed, they often lack environmental sustainability and can result in further ecological harm. Constructed wetlands are environmentally friendly bioremediation methods that utilize microorganisms and phytoremediation techniques involving plants and their associated rhizosphere bacteria. Additionally, they are aesthetically pleasing and contribute to the preservation of soil structure.

Constructed wetlands utilize natural systems to rehabilitate ecosystems damaged by pollution by providing habitats for wildlife, enhancing biodiversity, and accelerating the restoration process. The use of poultry manure and black soap (a native soap) serves as organic, biodegradable, and socially acceptable options. Phytoremediation of contaminated sites with poultry litter and black soap can expedite the recovery of polluted soil and improve its environmental health. The nutrient-rich poultry manure promotes plant growth and ecological recovery in contaminated wetland areas, while the soap emulsifies crude oil, thereby increasing the rate of bioremediation.

1.5 Scope of the study

This study focuses on the application of constructed wetland systems as an innovative and sustainable approach to bioremediate soils contaminated with crude oil and heavy metals. The study identified and evaluate the potential of native plant species to tolerate and survive in soils contaminated with crude oil and heavy metals. This screening emphasized on species that are resilient under such environmental stress and capable of promoting bioremediation. Genera of crude oil-degrading microorganisms associated with identified phytoremediator plants will be isolated and characterized. The role of these microorganisms in hydrocarbon degradation and their interactions with plant roots was also explored. The effects of native black soap, poultry litter, and their combinations for the enhancement of crude oil and heavy metal bioremediation was studied. This componentassessed the amendments' roles in improving the efficiency of hydrocarbon degradation and heavy metal stabilization.The efficacy of constructed wetland systems as a technology for the remediation of polluted soils was investigated. The study include evaluations of plant-microbe interactions, pollutant removal efficiencies, and the long-term sustainability of the constructed wetland system in diverse environmental conditions. The effectiveness of various combinations of plants, native soap, and poultry manure in reducing Total Petroleum Hydrocarbons (TPH) was determined. Comparisons of TPH reduction rates under different treatment scenarios provided insights into optimal remediation strategies.

1.6 Limitations of the study

The study was limited to specific native plant species, crude oil, and heavy metals impacted soil. The focus was on evaluating bioremediation performance under controlled conditions, with findings potentially requiring further validation in broader field applications.By addressing these areas, this research aims to advance knowledge in bioremediation techniques and contribute to sustainable soil restoration practices.

CHAPTER TWO

LITERATURE REVIEW

Over the past 50 years, the petroleum and gas industries in Nigeria have released significant quantities of hydrocarbons and associated pollutants, including heavy metals, into the Niger Delta environment from both refined and unrefined petroleum products (Obot et al., 2006; UNEP, 2011).

Pollution levels have severely impacted the environment, particularly in soil and water bodies, leading to a significant decline in both terrestrial and aquatic biodiversity, as well as disruptions to public health and the life support systems of local communities.

The dynamic field of bioremediation has emerged as a mainstream method for repairing and restoring contaminated environments, driven by the global demand for environmentally friendly solutions. Over the years, numerous studies have been published in this area, showcasing significant advancements in the treatment of various contaminants through both laboratory and field research **(**Barker & Bryson 2002; Ceccanti, Masciandaro, Garcia, Macci, Doni, 2006; Cheng & Wong 2002; Chris 2007; Hongjian 2009; Iordache & Borza 2012; Singer et al., 2001; Singleton, Hendrix, Coleman, & Whitemann, 2003; Tharakan, Addagada, & Tomlinson, 2004; Tomoko, Toyota, & Shiraishi, 2005; Zorn, Van, Geste, & Eijsackers, 2009).

Despite the progress made in the field, establishing a clear and effective bioremediation procedure for petrochemicals and heavy metals has not gained widespread recognition and remains inconsistent (Ameh et al., 2013; Schaefer & Filser, 2007; Singer et al., 2001). The variability in the composition, geological formations, and types of hydrocarbons present in different regions of the world makes it challenging to adopt a standardized strategy for bioremediation of crude oil and its associated components. This understanding highlights the need for researchers to identify relevant, accepted, and suitable ecologically based approaches tailored to the bioremediation of petroleum hydrocarbons specific to each region.

2.1 History of hydrocarbon pollution of the environment

Modern society continues to depend on petroleum hydrocarbons to meet its energy needs. Despite advancements in technology, leaks of crude oil and refined products frequently occur during extraction, transportation, storage, refining, and distribution processes. It is estimated that between 1.7 and 8.8 million metric tons of oil are released into the world's waters each year (ITOPF, 2001), with over 90% of these incidents directly linked to human activities such as intentional waste dumping. Contrary to popular belief, tanker accidents account for only about one-eighth of the oil discharged into marine environments. Additionally, approximately 30% of the spilled oil is believed to enter freshwater systems (Ameh et al., 2013). These figures mentioned above are highly speculative and can vary significantly from year to year based on different estimation sources and spill events.

The International Tanker Owners Pollution Federation's (ITOPF) oil spill database was utilized to create a detailed summary of oil leaks and the quantities released from 1970 to 1999. This data encompasses spills from tankers, combination carriers, and barges weighing over seven tons. While the statistics suggest a decline in oil spills, this trend may merely reflect a temporary decrease that is part of a larger, irregular cyclical pattern over time.

The Exxon Valdez oil spill and similar catastrophic events have significantly heightened public awareness regarding the risks associated with the storage and transportation of oil and its products. This increased awareness led Congress to enact stricter regulations, notably the Oil Pollution Act of 1990. Despite these regulatory measures, the ongoing reliance on oil suggests that spills and leaks are likely to persist in the future. Therefore, it is essential to establish effective countermeasures to address this ongoing issue.

2.2 Causes of oil spillage in Nigeria

Oil spills in Nigeria arise from a combination of natural and human factors, along with a third category often referred to as "mysterious causes."

2.2.1Natural cause

Natural oil spills, also referred to as natural seepage, occur when crude oil and natural gas leak from geological formations beneath the ocean floor. These events can result from various natural phenomena, including tectonic shifts and geological processes.

Human-induced oil spills are primarily the result of actions taken by individuals or organizations, often stemming from negligence or intentional misconduct. Key contributing factors include:

1. Pipeline Vandalism: Deliberate acts of sabotage on oil pipelines can lead to significant spills. This is particularly prevalent in Nigeria's Niger Delta, where such vandalism and disruptions in production are common occurrences (Nwachukwu, 2006).

2. Operational Carelessness: Negligence by workers during oil extraction and maintenance processes can result in spills. This includes failing to follow safety protocols or mishandling equipment.
3. Tanker Driver Errors: Accidents involving tanker drivers during the transportation of oil can also cause spills. These incidents often occur during loading and unloading operations.
4. Illegal Activities: Practices such as oil bunkering and siphoning contribute to environmental degradation by tapping into pipelines without authorization, leading to uncontrolled leaks.

The Niger Delta is particularly vulnerable to these human-induced spills due to its extensive network of pipelines connecting various flow stations. The region's high incidence of pipeline sabotage underscores the urgent need for improved security and regulatory measures to address these challenges (Nwilo, Peters, & Bodeji, 2000).

2.2.2 Mysterious causes

Unknown causes of oil spills refer to incidents where the precise origin of the leak cannot be identified. An illustrative case is the mystery leak that occurred in Quebec in 1999, as described by Wang, Fingas, and Sigouin (2001). This spill was discovered along the banks of the St. Lawrence River, near the Thermex Company. Although initial investigations suggested that the spill originated from a nearby facility, further analysis revealed that the chemical composition of the leaked oil did not match that of the facility's output, containing less fuel than expected.

In Nigeria, there are also numerous instances of mystery spills. For example, Shell data indicated estimated spill volumes of 0.1 barrels on July 2, 2017, and 5 barrels on August 16, 2016. Other notable projected leak volumes included 0.5 barrels on November 6, 2015, and 30 barrels on September 19, 2015. Among these incidents, one of the most significant was reported on January 17, 1980, when a blowout at the Funiwa 5 offshore station led to a massive spill of approximately 37 million liters (Nwilo & Badejo, 2005).

Table 2.1 provides an overview of some of Nigeria's most significant oil spills, detailing the date of each incident, the terrain and location, estimated spill volumes, and their causes. Notably, some of these predicted spill volumes are considerably larger than those recorded in the 1980s.

Table 2.1: Oil spill incident in Nigeria in September 2018

Date reported	Incident site	Terrain	Cause	Estimated spill volume(bbl)
Sept.03, 2018	24" Nkpoku-Bomu Pipeline at Bera.	Land	Sabotage	19
Sept. 04, 2018	Imo River 2 Well 31L Flowline at Odagwa Umuadeokwara	Land	Sabotage	0.1
Sept. 06, 2018	14" Okordia Rumuekpe Pipeline at Akaramini	Land	Sabotage	81
Sept. 08, 2018	20" Otumara - Escravos Pipeline at Ugboegungun	Swamp	Sabotage	172
Sept. 09, 2018	36"Nkpoku - Bomu Pipeline at Rumuesara Eneka	Land	Sabotage	622
Sept. 11, 2018	Imo River Well 59T Flowline at Igiriukwu- Owaza	Land	Sabotage	0.2
Sept. 14, 2018	6'' Obigbo North- Ogale Pipeline at Assa	Land	Sabotage	72
Sept. 15, 2018	16''Egbema – Assa Rumuekpe Pipeline at Assa	Land	Sabotage	34

Source: https://www.shell.com.ng/sustainability/environment/oil-spills.html

2.3 The behavior of oil in the environment

2.3.1 Weathering processes

When oil is released into the environment, it undergoes a series of physical, chemical, andbiological transformations that significantly alter its composition and properties. These changes, collectively referred to as weathering, can impact the effectiveness of spill response strategies.

Weathering processes include;

i. Physical Changes:

Initial processes include spreading, evaporation, and dispersion, which occur shortly after the spill. Over time, oil can emulsify, forming water-in-oil emulsions that increase in viscosity and complicate cleanup efforts.

ii. Chemical Changes:

Chemical weathering involves reactions such as oxidation and hydrolysis, which can break down oil into more soluble products or create persistent compounds like tars. These processes are influenced by environmental factors such as temperature and sunlight exposure.

iii. Biological Changes:

Biodegradation is a critical component of weathering, where microorganisms break down hydrocarbons into less harmful substances. This process can vary significantly based on the type of oil and environmental conditions, including nutrient availability (e.g., nitrogen and phosphorus) that can enhance microbial activity.

Bioremediation

Bioremediation is often employed as a final treatment method after other cleaning techniques have been exhausted. This process can be lengthy, as the remaining target oil may be extensively weathered before bioremediation techniques are applied. The effectiveness of bioremediation depends on several factors, including:

- The composition of the residual oil.
- The presence of suitable microorganisms capable of degrading the weathered hydrocarbons.
- Environmental conditions such as temperature and nutrient levels.

Research has shown that while natural weathering processes can remove many hydrocarbons over time, they may take months or even years to achieve significant reduction in contamination levels (Tarr, Zito, Overton, Olson, Adhikari, & Reddy, (2016).). Understanding these weathering processes is crucial for developing effective spill response strategies and enhancing bioremediation efforts.

2.3.2 Spreading

If the oil pour point is lower than the ambient temperature, spreading oil on water is one of the most significant activities during the initial hours of a spill. Gravity, inertia, friction, viscosity, and surface tension are the main forces that influence oil spreading. This process increases the spill's overall surface area, allowing for faster mass transfer through evaporation, dissolution, and biodegradation (IPIECA, 2015; Xueqing, Albert, Makram, & Kenneth, 2001).

2.3.3 Evaporation

Evaporation is a crucial weathering process during the initial stages of an oil spill, significantly impacting the environment by removing a substantial portion of the oil, especially the more hazardous, lower molecular weight components (Xueqing et al., 2001). Within 1 to 10 days, evaporation can eliminate nearly all typical alkanes smaller than C15 from oil on water surfaces. It can also rapidly remove volatile aromatic compounds, such as benzene and toluene, from an oil slick. However, when oil becomes trapped in sediments, these volatile components may persist longer. Crude oil typically contains 20-50 percent volatile components, while No. 2 fuel oil contains about 75 percent, and gasoline and kerosene consist entirely of volatile substances. Consequently, the physical properties of the remaining slick, such as density and viscosity, change significantly. Factors like the oil's composition, wave action, wind speed, and water temperature play crucial roles in determining the rate of evaporation (Aghajanloo & Pirooz, 2011).

2.3.4 Dissolution

While dissolution contributes less to overall mass loss after an oil spill, the concentration of dissolved hydrocarbons in water is critical, as it can affect bioremediation processes and the toxicity impact on biological systems. The extent of dissolution depends on the solubility of the spilled oil, weather conditions, and the characteristics of the spill site. Low molecular weight aromatics are the most soluble and toxic components in both crude and refined oils. Although many of these compounds can be removed through evaporation, their environmental impact is far greater than what simple mass balance calculations might suggest (Xueqing et al., 2001). Additionally, photochemical and biological processes can influence dissolution rates.

2.3.5 Photooxidation

Photooxidation is another weathering process that can have biological impacts. Natural sunlight, in the presence of oxygen, provides enough energy to transform complex petroleum molecules, such as high molecular weight aromatics and polar compounds, into simpler substances through a series of free-radical chain reactions. This process generates polar molecules like hydroperoxides, aldehydes, ketones, phenols, and carboxylic acids, which can increase the solubility of oil in water, thereby enhancing its bioavailability. While this increased solubility can boost the biodegradation of petroleum—particularly at low concentrations where acute toxicity is less significant—it also produces hazardous compounds that can have negative ecological effects (IPIECA, 2015; Nicodem, Fernandes, Guedes, & Correa, 1997).

2.3.6 Dispersion

Dispersion, or the formation of oil-in-water emulsions, involves breaking the oil into small droplets and distributing them throughout the water column, which increases the oil's surface area. Generally, oil-in-water emulsions are not stable. However, continuous agitation, interactions with dispersed particles, and the use of chemical dispersants can help maintain their stability. Dispersion can enhance oil biodegradation rates by increasing the contact between oil and microorganisms and/or by accelerating the dissolution of more soluble oil components (IPIECA, 2015).

2.3.7 Emulsification

Emulsification of oils involves the transformation from an oil-on-water slick or oil-in-water dispersion into a water-in-oil emulsion, eventually forming a thick, sticky mixture that can contain up to 80% water, commonly known as "chocolate mousse." The chemical composition of the oils significantly influences emulsion formation and stability, with waxes and asphaltic components playing key roles. Additionally, surface-active compounds generated through photochemical and biological processes contribute to emulsion creation. This emulsification process complicates oil spill clean-up efforts by reducing the efficiency of physical recovery methods and slowing down the natural biodegradation rates of the oil (IPIECA, 2015).

2.3.8 Sedimentation

The concentration of suspended solids, including fine sediments and other particles, in the water column significantly influences the fate and effects of dispersed oil. Dispersed oil droplets can attach to these suspended particles, altering their physical properties. Chemically dispersed droplets may be less likely to bind with suspended solids compared to physically dispersed ones until the dispersion undergoes biodegradation. These suspended solids can settle on the seabed, becoming absorbed into muddy areas with active sedimentation, or can spread more widely as loose aggregates (flocs) of oiled particles, or a combination of both. In a worst-case scenario, high concentrations of both oil droplets and suspended sediments can result in substantial deposition of contaminated particles, leading to heavily oiled seabed sediments that may persist for years, causing long-term environmental impacts. A notable example occurred in two estuaries on France's northwestern coast following the 1978 Amoco Cadiz oil spill. Fortunately, such situations are rare, as most dispersed oil is widely distributed and biodegrades before it can settle into seabed sediments. However, the presence of loose flocs of oiled particles (flocculent material formed by the aggregation of suspended oil and sediment particles) can expose filter-feeding seafloor organisms to elevated hydrocarbon concentrations (IPIECA, 2015).

2.3.9 Sinking

Sinking is often discussed alongside sedimentation, but it is ecologically distinct as it does not create plumes or flocs of oiled particles. When spilled oil is denser than seawater, it sinks, leading to long-term accumulations on the seabed, where it may sometimes become buried. Although the area of the seabed affected by sunken oil is typically smaller compared to that impacted by dispersed oil sedimentation, the presence of sunken oil can result in prolonged suffocation and habitat loss. However, even after significant weathering, few oils become dense enough to sink (IPIECA, 2015).

2.4 Environmental impact of hydrocarbon spill

2.4.1 Destruction of the ecosystem

A significant portion of the mangrove ecosystem in Nigeria's coastal region has been devastated. Once a vital source of fuel and a habitat for the area's diverse wildlife, the mangrove is now unable to withstand the oil toxicity in its environment. Oil spills have severely impacted marine life, leading to heavy contamination that poses health risks for humans who consume affected seafood. Additionally, the spills have damaged crops, polluted groundwater and drinking water, and disrupted coastal fishing activities. Twumasi and Merem (2006) used geospatial data processing and analysis to study the Niger Delta forest area, utilizing two Landsat Thematic Mapper I and Enhanced Thematic Mapper Plus (ETM+) images from 1985 to 2005. Their findings revealed a slight reduction in water bodies, from 343,654 to 343,513 hectares. The mangrove and closed forest areas also saw a significant decline, with mangrove coverage dropping from 55,410 hectares in 1985 to 37,117 hectares, and closed forest shrinking from 250,161 hectares to 175,609 hectares over the same period.

2.4.2 Gas explosion

(a) **Burning of Forests**

In 2004, a leak in the Nigerian Liquefied Natural Gas pipeline that runs through Kala Akama caused a fire in the Okrika mangrove forest, burning for three days and devastating the local plant and animal life (Nenibarini, 2004). This incident is one of several well-documented cases where fires have resulted in significant human fatalities over the years.

(b) Acid Rain and Heat Effects

Research by Salau (1993) and Adeyemo (2002) on the impact of gas flaring on agriculture found a direct correlation between gas flaring and reduced agricultural productivity. Major oil spills also negatively affect human health and well-being, a critical aspect of environmental sustainability. Inhabitants of oil-producing regions may be forced to consume water contaminated with residual oil for years, even after clean-up efforts. Additionally, chemical dispersants used during clean-up can have lasting health effects. For instance, many residents of the Niger Delta have reported conditions such as asthma, respiratory issues, headaches, nausea, throat irritation, and chronic bronchitis. These health concerns can result in substantial toxic tort claims due to exposure to hazardous substances and chemicals.

2.4.3 Clean-up obligations and costs

Cleaning up a major oil spill can take many years. For instance, the United Nations estimates that restoring Nigeria's Niger Delta could take up to 30 years, with costs reaching $1 billion (USD) within the first five years. This highlights the extensive and far-reaching costs associated with clean-up, restoration, and reclamation efforts. Consequently, international environmental law mandates that multinational corporations bear the financial responsibility for these clean-up expenses.

2.4.4 International liability issues

Major oil spills can negatively impact the seas and environmental quality of neighboring countries. For instance, Mexico argues that the BP oil disaster had cross-border effects on its marine environment. This situation violates Article 194(2) of the Law of the Sea Convention, which obligates states to "take all measures necessary to ensure that activities under their jurisdiction or control do not cause pollution to other states or their environment and that pollution arising from incidents or activities under their jurisdiction or control does not spread beyond the areas where they exercise sovereign rights in accordance with this Convention."

2.4.5 Loss of business profits and subsistence rights

Oil spills have severe economic impacts on individuals and businesses in the commercial fishing, shrimp, and oyster industries, as they lose their income and means of livelihood. Those affected include fishermen and women, charter boat operators, hotel owners, tourist management companies, rental property owners, and other coastal businesses. Consequently, oil spills can violate international and national laws aimed at protecting people's rights to their means of subsistence. For instance, firms and workers in the Gulf region have filed over 30,000 individual claims against BP, seeking compensation for lost profits and income resulting from the oil spill disaster (from the crude oil or hydrocarbon folder, *Review of the Impacts of Oil Exploration and Production in the Niger Delta, Nigeria*).

One of the key factors influencing the impact of an oil spill is its location. Spills that occur closer to shore or near populated areas tend to have greater economic impacts and are more expensive to clean up. For example, despite the ABT Summer accident in 1991 and the Atlantic Empress spill in 1979 each involving over 250,000 tonnes of oil, their distance from shore meant they did not directly affect human populations (White & Molloy, 2003). Additionally, large offshore cleanups can cost as much as $300,000 per tonne, while smaller nearshore spills may cost around $29,000 per tonne (Kontovas, C. A., Psaraftis, H. N., and Ventikos N. P., 2010). Another study indicated that cleaning oil from beaches is 4-5 times costlier than collecting it at sea, and 100 times more expensive than pumping oil from a wrecked vessel, based solely on location (Nyman, 2009).

The volume of oil spilled and the rate at which it leaks also significantly influence the severity of the impact. According to Alló and Loureiro (2013), a 1% increase in spill size results in an additional US$0.718 million in damages. Slow, continuous leaks, such as those from immobilized tankers that continue to release oil, can exacerbate the damage by requiring repeated responses. Notable examples include the Prestige and Betelgeuse incidents, where oil continued to leak for months, leading to prolonged and costly recovery efforts (Loureiro, Loomis, & Vázquez, 2009; Punzón, Trujillo, Castro, Perez, Bellido, Abad, Villamor, Abaunza, & Velasco, 2009; White & Molloy 2003).

2.4.6 Ecosystem impact

The secondary effects of an oil spill are related to its impact on the ecosystem and the process of recovery. Marine ecosystems are structured by many interacting species, each of which can be affected differently by an oil spill. While each ecosystem is unique, previous oil spills have highlighted some key factors. The chemical composition and quantity of the oil to which organisms are exposed play a critical role in determining how populations respond to the contamination.

Certain biological characteristics, especially the habitat and depth at which species reside, influence their likelihood of exposure to oil. Since most spilled oil floats on the water's surface, it generally affects only those species that come into contact with the surface. Subtidal species are less likely to be exposed, with notable exceptions being species like kelp and seagrass, whose canopies reach the water's surface. Marine mammals and birds face heightened risks from oil exposure, as they frequently cross the air-water interface to breathe (Peterson, Rice, Short, Esler, Bodkin, Ballachey, & Irons, 2003). In contrast, pelagic fish species are less exposed to oil. The intertidal zone is especially vulnerable in spills where oil floats, as the rising and falling tides bring the oil into close contact with the species inhabiting these areas.

Oil exposure can lead to a range of toxic effects, including ingestion of oil, accumulation of contaminants in tissues, DNA damage, immune dysfunction, cardiac issues, mass mortality of eggs and larvae (such as in fish), loss of buoyancy and insulation in birds, and inhalation of toxic vapors (Aguilera et al., 2010; Incardona et al., 2009; Judson et al., 2010; Kazlauskiene et al., 2008; Liber, & MacKinnon, 2002; Ma et al., 2003; Major & Wang, 2012; Ormseth & Ben-David, 2000; Rogers, Wickstrom,). Species' responses to oil exposure can vary greatly due to their morphological and physiological differences, which are often influenced by genetic variations. This means that understanding how related species have responded to past spills can help predict the reactions of local species to a new oil spill. For instance, barnacle populations tend to show resilience even when directly exposed to oil, whereas amphipod populations often experience significant and prolonged declines after oil exposure. However, even closely related species can exhibit different reactions to oiling events due to subtle differences in their biology.

2.4.7 Societal impact

A third level of the framework addresses the impact of oil spills on human society, encompassing effects on individual health, community well-being, and the economy. Webler and Lord (2010) identified categories of human-related processes, impacts, and vulnerabilities, outlining three primary ways oil spills can affect people. First, oil can disrupt ecological processes, leading to direct harm, such as health risks from consuming seafood contaminated with bioaccumulated oil toxins. Second, oil spill stressors can indirectly affect people by altering intermediary processes, like the economic losses fishermen face due to impacts on fish populations. Third, stressors can cause direct harm, such as respiratory health issues from inhaling oil vapors. Under this framework, oil spills create economic, health, and societal impacts through various pathways. This review focuses on key empirical factors and their role in determining the severity of these socioeconomic effects.

2.4.8 Economy

Estimating the economic impact of an oil spill is challenging due to limitations in baseline data, long-term forecasting techniques, and the complexity of evaluating nonmarket costs. While immediate property damage can be more easily identified, establishing a clear link between oil spills and wider economic losses, such as reductions in income or market share, is far more complex. Additionally, the valuation process is highly context-dependent, shaped by socio-cultural factors that vary across different situations. Historical oil spills have demonstrated that certain industries consistently experience losses from both direct damage and market disruptions. For instance, the destruction of resources due to direct mortality or loss of habitat, along with restricted access from harvest bans or area closures, has significantly impacted commercial fisheries and aquaculture operations (Punzón, Trujillo, Castro, Perez, Bellido, Abad, Villamor, Abaunza, & Velasco, 2009). Moreover, market demand often decreases as consumers perceive a risk of contamination in affected products (Cheong, 2012; Garza-Gil, Surís-Regueiro, & Varela-

Lafuente, 2006), leading to further financial losses. These losses ripple through the supply chain, affecting docks, processors, and suppliers that support the fishing industry (García, Villasante, Carballo, & Rodríguez, 2009).

2.5 Hydrocarbon degradation in the soil

Biodegradation plays a crucial role in petroleum toxicology by altering the nature and concentration of chemical components. As a form of bioremediation, it is widely used to treat soils, water, and sediments contaminated with polycyclic aromatic hydrocarbons (PAHs). For effective biodegradation, microorganisms should ideally be native to the contaminated site (Das & Chandran, 2011). Various microorganisms have the capacity to clean up hydrocarbon-polluted environments by converting chemical substances into energy, cell mass, and biological waste products (Rahman, Thahira-Rahman, Lakshmanaperumalsamy, & Banat, 2002). Hydrocarbon-degrading microorganisms are abundant in soil ecosystems, with certain bacteria—primarily from the genera *Pseudomonas* and *Mycobacterium*—demonstrated to transform and degrade PAHs under aerobic conditions (Mrozik, Piotrowska-Seget, & Labuzek, 2003). Increasing evidence shows that some microorganisms, such as *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Torulopsis bombicola*, can produce bioremediation surfactants like surfactin, rhamnolipid, and sophorolipid. These surfactants enhance the biodegradation process by solubilizing PAHs in water, thus improving their bioavailability (Cottin & Merlin, 2007). Additionally, cyanobacteria, molds, and yeasts capable of hydrocarbon degradation have been identified across various ecosystems, contributing significantly to the breakdown of contaminants (Chaillan, le Fleche, BuryPhantavong, Grimont, Saliot, & Oudot, 2004).

2.5.1 Bacteria

Most hydrocarbon-utilizing bacteria metabolize either aliphatic or aromatic hydrocarbons, and they can use a broad range of hydrocarbons as an energy source. Naphthalene, the simplest and most soluble polycyclic aromatic hydrocarbon (PAH), is particularly useful for isolating bacteria capable of degrading it (Mrozik et al., 2003). These researchers explored the metabolic sequences and enzymatic processes involved in the breakdown of naphthalene. Some naphthalene-degrading bacteria include *Pseudomonas* sp., *Vibrio* sp., *Mycobacterium* sp., *Marinobacter* sp., *Sphingomonas* sp., *Rhodococcus* sp., and *Micrococcus* sp. (Pawar, Ugale, More, Kokani, & Khandelwal, 2013). The breakdown of aromatic substrates often begins with the oxygenation of the aromatic ring, forming a diol (a compound with two alcohol groups).

According to Pawar et al., (2013), many PAH-degrading bacteria possess genes that are highly similar to the naphthalene-degrading gene (nah gene) found in the NAH7 plasmid of *Pseudomonas putida* strain G. *Pseudomonas* is particularly known for its ability to degrade three- and four-ring PAHs (Bamforth & Singleton, 2005). For example, *Pseudomonas* sp. strain PP2 breaks down phenanthrene through a dioxygenase-initiated pathway, eventually converting it into intermediates that follow the naphthalene degradation pathway (Parales & Haddock, 2004). Phenanthrene's bay and K regions can form an epoxide, a compound that is considered carcinogenic (Bamforth & Singleton, 2005). Phenanthrene is often used as a model compound to study the catabolic pathways for breaking down other carcinogenic substances found in the bay and K-regions, such as benzo[a]pyrene, benzo[a]anthracene, and chrysene (Bamforth & Singleton, 2005). Various bacterial species involved in these biodegradation processes are listed in Table 2.2.

Table 2.2: Bacterial species. which are involved in Biodegradation

Compounds	Microorganisms
Alkanes	*Pseudomonas* sp., *Bacillus* sp., *Acinetobacter calcoaceticus and Micrococcus* sp., *Candida Antarctica, Nocardia erythroplis, Ochrobactrum* sp*. and Acinetobacter* sp., *Serratia marcescens, Candida tropicalis, Alcaligene sodorans, Arthrobacter* sp. *andRhodococcus* sp
Mono-aromatic hydrocarbons	*Brevibacillus* sp., *Pseudomonas* sp., *Bacillus* sp., *B. stereothermophilus and Vibrio* sp., *Corynebacterium* sp., *Ochrobactrum sp. and Achromobacter* sp
Poly-aromatic hydrocarbons	*Alcaligenes odorans, Sphingomonas paucimobilis, Achromobacter* sp*. and Mycobacterium* sp.,*Pseudomonas* sp., *Mycobacterium flavescens, Rhodococcus* sp., *Arthrobacter* sp*. and Bacillus* sp.,*Burkholderia cepacia, Xanthomonas sp. and Alcaligenes*
Resins	*Burkholderia cepacia, Xanthomonas* sp*. and Alcaligenes Pseudomonas* sp., *Members of* Vibrionaceae, *Enterobacteriaceea and Moraxella* sp.

Source: Bamforth & Singleton, 2005

The degradation of PAH by thirteen deuteromycete ligninolytic fungus strains was studied by Clemente, Anazawa, & Durrant, (2001), who discovered that the degree of degradation is dependent on the activity of lignolytic enzymes. The strain 984 with Mn-peroxidase activity showed the highest rate of naphthalene breakdown (69%) followed by strain 870 (17%) with lignin peroxidase and laccase activities. With Mn-peroxidase and laccase activity, strain 870 showed a 12 percent degradation of phenanthrene. The strain 710 was discovered to have a high amount of anthracene degradation (65%). *Aspergillus terreus* was found to be superior for the production of ligninolytic enzymes by Ali, Akhtar, Khan, Khan, Rasul, Zaman, Khalid, Waseem, Mahmood, & Ali, (2012). Optimal temperatures for lignin peroxidase and manganese peroxidase synthesis are 33.6°C and 33.1°C, respectively, and pH is 4.1 and 5.8. In soil models, it was able to breakdown 98.5 percent of naphthalene and 91% of anthracene under ideal conditions.

2.5.2 Algal degradation

Numerous studies have highlighted the role of fresh algae, such as *Chlorella vulgaris*, *Scenedesmus platydiscus*, *Scenedesmus quadricauda*, and *Selenastrum capricornutum*, in the degradation of polycyclic aromatic hydrocarbons (PAHs) (Wang & Zhao, 2007). The metabolism of naphthalene by both prokaryotic and eukaryotic photoautotrophic marine algae, including cyanobacteria, green algae, and diatoms, has been well-documented (Haritash & Kaushik, 2009). In their research, Haritash and Kaushik specifically examined the role of cyanobacteria (blue-green algae) in breaking down naphthalene. At non-toxic concentrations, cyanobacteria metabolize naphthalene into four primary metabolites: 1-naphthol, 4-hydroxy-4-tetralone, *cis*-

naphthalene dihydrodiol, and *trans*-naphthalene dihydrodiol. Additionally, the potential of algal-bacterial microcosms, specifically involving *Pseudomonas migulae* and *Sphingomonas yanoikuyae*, was explored for the degradation of phenanthrene (Haritash & Kaushik, 2009). The degradation of fluoranthene, pyrene, and their combination using *Chlorella vulgaris*, *Scenedesmus platydiscus*, *Scenedesmus quadricauda*, and *Selenastrum capricornutum* was studied by Ueno, Wada, & Urano (2008). After seven days of treatment, *S. capricornutum* and *C. vulgaris* removed 78% and 48% of PAHs, respectively.

2.5.3 Fungal degradation

Several fungi have been identified as capable of degrading persistent pollutants (Haritash & Kaushik, 2009). As noted by Spellman (2008), fungi, like bacteria, can metabolize dissolved organic matter, playing a critical role in carbon decomposition within the biosphere. Similarly, Matavulj & Molitoris (2009) found that fungi possess extracellular multi-enzyme complexes, enabling them to break down natural polymeric materials through their hyphal systems. These hyphal structures can rapidly colonize and penetrate substrates, allowing for efficient nutrient transport and redistribution throughout the mycelium.

PAH degradation can be carried out by two types of fungi: non-ligninolytic and ligninolytic fungi (Bamforth & Singleton, 2005). Non-ligninolytic fungi such as *Chrysosporium pannorum*, *Cunninghamella elegans*, and *Aspergillus niger* utilize a cytochrome P450 monooxygenase enzyme-mediated oxidative pathway for breaking down PAHs. On the other hand, ligninolytic fungi like *Pleurotus ostreatus* and *Antrodia vaillantii* produce ligninolytic enzymes that help degrade lignin in wood and other organic materials (Bamforth & Singleton, 2005). The ligninolytic enzyme system consists of lignin peroxidases (LP), manganese-dependent peroxidases (MnP), and laccases (Haritash & Kaushik, 2009).

The degradation of phenanthrene involves initial oxidation by cytochrome P450 enzymes, followed by further breakdown using lignin peroxidase enzymes (Bezalel, Hadar, & Cerniglia, 1997). Additionally, *Penicillium janthinellum* SFU 403, a strain isolated from petroleum-contaminated soils, has been shown to metabolize pyrene (Leitao, 2009). In the early stages of degradation, it produces compounds such as monophenols, diphenols, dihydrodiols, and quinones. Pyrene is further broken down through hydroxylation to form 1-pyrenol, which is subsequently converted into 1,6- and 1,8-pyrenequinones (Wang & Zhao, 2007).

2.5.4 Yeast degradation

Several yeast species can use aromatic compounds as growth substrates, but their ability to transform aromatic molecules through cometabolism is particularly noteworthy. For example, some species, like the soil yeast *Trichosporon cutaneum*, have specialized energy-dependent absorption systems for aromatic compounds such as phenol. Additionally, yeasts are capable of utilizing aliphatic hydrocarbons present in crude oil and petroleum products (Miranda, de Souza, Gomes, Lovaglio, Lopes, & de Queiroz Sousa, 2007). Notable alkane-utilizing yeasts include *Candida lipolytica*, *Candida tropicalis*, *Rhodotorula rubra aurantiaca*, and *Aureobasidium* (formerly *Trichosporon*) *pullulans*. Both *Rhodotorula aurantiaca* and *C. ernobii* have shown the ability to degrade diesel oil.

According to Leelaruji et al., (2013), *Aureobasidium pullulans* var. *melanogenum* is a lipolytic yeast capable of breaking down naphthalene (24.4%), anthracene (37.3%), pyrene (27.3%), and benzo[a]pyrene (45.95%) through the synthesis of laccase enzymes. Similarly, Hesham et al.

(2006) identified a yeast strain, AEH, that could degrade naphthalene (5.36 mg L–1), phenanthrene (5.04 mg L–1), and chrysene (1.54 mg L–1) within 2, 10, and 10 days, respectively. In a binary system, these three PAHs can serve as carbon sources for the cometabolic breakdown of benzo[a]pyrene, with naphthalene being the most efficient.

Yeasts have also been extensively studied for their ability to remove heavy metals, particularly through biosorption. For example, *Schizosaccharomyces pombe* has been shown to effectively remove copper (Subhashini et al., 2011). According to Wang and Chen (2006), yeasts can accumulate heavy metals like Cu(II), Ni(II), Co(II), Cd(II), and Mg(II), often outperforming bacteria in this regard. Both live and dead cells of yeast species such as *Cyberlindnera fabianii*, *Wickerhamomyces anomalus*, and *C. tropicalis* can biosorb Cr(VI) (Bahafid et al., 2011, 2013). *Pichia anomala* is another example of a yeast capable of removing Cr(VI).

Additionally, yeast strains like *S. cerevisiae*, *P. guilliermondii*, *Rhodotorula pilimanae*, *Yarrowia lipolytica*, and *Hansenula polymorpha* have been shown to reduce Cr(VI) to the less toxic Cr(III) (Ksheminska, Honchar, Gayda, & Gonchar, 2006). The chromate resistance of *P. guilliermondii*, in particular, is linked to its ability to reduce Cr(VI) and facilitate Cr(III) chelation outside the cell (Ksheminska et al., 2008). Numerous studies have highlighted the effectiveness of immobilized yeast cells in metal removal, such as the use of *Schizosaccharomyces pombe* for copper removal (Subhashini, Kaliappan, & Velan, 2011).

2.5.5 Protozoa degradation

Fungi, bacteria, and algae are more effective biodegraders than protozoa. However, the presence of protozoa has been shown to reduce the number of bacteria available for hydrocarbon degradation, suggesting that their presence in a biodegradation system may not always be beneficial (Stapleton & Singh, 2002). Consequently, protozoa do not play as significant an ecological role in the breakdown of hydrocarbons in the environment compared to algae and fungi.

2.6. Mechanisms of microbial bioremediation

2.6.1 Enzymatic degradation

Cytochrome P450 hydroxylases play a crucial role in the microbial degradation of chlorinated oil, polycyclic hydrocarbons (PHs), and other compounds (Van Beilen & Funhoff, 2007). These enzymes have been isolated from various *Candida* species, including *Candida apicola*, *C. maltosa*, and *C. tropicalis* (Scheller et al., 1998). Alkane oxygenases, such as cytochrome P450 enzymes, integral membrane di-iron alkane hydroxylases (e.g., alkB), membrane-bound copper-containing methane monooxygenases, and soluble di-iron methane monooxygenases, are widely distributed in both prokaryotes and eukaryotes. They actively participate in alkane degradation under aerobic conditions (Van Beilen & Funhoff, 2005).

Fungi are effective in PH degradation and offer several advantages over bacteria due to their ability to grow on diverse substrates. They produce extracellular enzymes that can penetrate contaminated soil and degrade pollutants (Messias et al., 2009). The efficiency of fungal enzyme-mediated biodegradation depends on factors such as nutrient availability, oxygen levels, and optimal enzymatic conditions, including pH, temperature, chemical structure, partitioning in growth media, and cellular transport properties (Singh & Ward, 2004). Fungi, having evolved to break down the irregular structure of lignin, have enhanced their capacity to degrade and mineralize various organic pollutants. Their extracellular peroxidases initiate PH oxidation (Zhang et al., 2015).

Fungal lignin peroxidases directly oxidize certain PHs, whereas manganese peroxidases co-oxidize them via enzyme-mediated lignin peroxidation (Li et al., 2014). Novotný et al. (2004) studied the enzymatic activities of lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase in the degradation of pyrene and anthracene by various ligninolytic fungal species in liquid and soil cultures. They found that the degradation of these compounds by *Trametes versicolor*, *Pleurotus ostreatus*, and *Phanerochaete chrysosporium* depends on MnP and laccase secretion in the soil. While fungal degradation of polycyclic aromatic hydrocarbons (PAHs) is generally slower and less efficient than bacterial degradation, fungi exhibit broad substrate specificity and the ability to hydroxylate various xenobiotics.

In addition to LiP, MnP, and laccase, other fungal enzymes such as epoxide hydrolases, cytochrome P450 monooxygenases, dioxygenases, proteases, and lipases have been extensively studied for their PAH degradation capabilities (Balaji, Arulazhagan, & Ebenezer, 2014). The extracellular enzyme system of six *Aspergillus* species isolated from crude oil-contaminated soil has demonstrated efficiency in crude oil degradation, highlighting their potential for oil recovery (Zhang et al., 2016). Jové et al. (2016) evaluated anthracene degradation by three fungi, both ligninolytic and non-ligninolytic, and found that *Phanerochaete chrysosporium* exhibited higher degradation efficiency than *Pleurotus ostreatus* and *Irpex lacteus*. Balaji et al. (2014) also analyzed the ability of various fungal species to produce extracellular enzymes such as laccase, lipase, protease, and peroxidase, further demonstrating their potential in bioremediation.

2.6.2 Redoxreaction

Enzymatic oxidation is a process in which pollutant compounds are transformed from a higher oxidation state to a lower one, reducing their toxicity. During this process, heavy metals lose electrons, making them less harmful. This method relies on oxidoreductase enzymes released by microbes and is particularly effective for remediating dyes, phenols, and other pollutants that are resistant to bacterial degradation (Unuofin, Okoh, & Nwodo, 2019). Oxidative enzymes generate radicals that break down pollutants into smaller fractions, ultimately forming high-molecular-weight compounds (Unuofin et al., 2019).

One key example of an oxidoreductase enzyme is laccase, which catalyzes the oxidation of aromatic amines (Gangola, Sharma, Bhatt, Khati, & Chaudhary, 2018). Other oxidoreductases, such as those acting on phenols and polyphenols, facilitate the reduction of molecular oxygen to water (Kushwaha et al., 2018; Sahay, 2021). Laccase production has been reported in *Pycnoporus* sp. and *Leptosphaerulina* sp., where it has been shown to contribute to heavy metal degradation (Copete-Pertuz et al., 2018; Tian et al., 2020).

In contrast, enzymatic reduction involves converting pollutants to a reduced state, making them insoluble. This process is primarily carried out by obligate and facultative anaerobes and is highly effective in bioremediating persistent compounds such as polychlorinated dibenzo-p-dioxins and dibenzofurans (Zacharia, 2019). Specific enzymes, such as chrome reductase, catalyze the reduction of hexavalent chromium to its less toxic trivalent form, while azoreductase breaks down azo compounds by cleaving azo bonds (Saxena, Kishor, & Bharagava, 2020).

Further research is needed to identify additional microorganisms capable of bioremediating environmental pollutants and to enhance our understanding of their potential applications.

2.6.3 Hydrolysis

Hydrolysis plays a crucial role in the detoxification of contaminants. Hydrolytic enzymes, such as esterases and lipases, break down ester bonds in persistent pollutants, reducing their toxicity. This characteristic makes lipases and esterases promising candidates for the biodegradation of plastic waste, organophosphates, and pesticides.

Aryloxyphenoxy propionate (AOPP) herbicides are a highly effective class of herbicides widely used in agriculture. This group includes fenoxaprop-ethyl (FE), cyhalofop-butyl (CB), haloxyfop-R-methyl (HM), quizalofop-p-ethyl (QE), and clodinafop-propargyl (CP). The FE hydrolase (Feh) from *Rhodococcus* catalyzes the initial step in FE biodegradation by hydrolyzing its ester bond, converting fenoxaprop-ethyl into fenoxaprop acid (Hou et al., 2011). Feh is also capable of hydrolyzing CB, HM, and QE into their respective acids. Similarly, the esterase ChbH, found in *Pseudomonas azotoformans* QDZ-1, hydrolyzes cyhalofop-butyl (CB) into cyhalofop acid (CA) (Nie et al., 2011).

For amide herbicides, the enzyme arylamidase AmpA, purified from *Paracoccus* sp. FLN-7, catalyzes the cleavage of amide bonds in herbicides such as propanil, propham, and chlorpropham (Zhang et al., 2012).

Pyrethroids, a widely used class of insecticides, are known for their high efficacy and low toxicity to mammals. They are commonly applied in household pest control and agricultural production. Several pyrethroid-degrading enzymes, including PytY, PytH, EstP, and Sys410, have been cloned and characterized. However, none of these enzymes have demonstrated efficient and stable pyrethroid degradation (Wang et al., 2009; Li, Wang, & Liu, 2008; Fan et al., 2012). To address this limitation, Liu et al. (2017) used random mutagenesis and secretory expression techniques to develop a mutant variant of Sys410 with enhanced activity and thermostability. This improved enzyme achieved a hydrolysis rate exceeding 98%, demonstrating significant potential for pyrethroid degradation.

2.6.4 Conjugation

Horizontal Gene Transfer (HGT) and Plasmid Efficiency in Biodegradation

Conjugation enables donor bacteria to transfer plasmids carrying degradation pathway genes (e.g., *bphC* for PAH degradation) directly to indigenous soil bacteria. This process eliminates the need for introducing external microbial strains, as native bacteria are already well-adapted to local environmental conditions (Liu et al., 2017).*Plasmid Backbone and Transfer Efficiency* The structure of the plasmid backbone plays a crucial role in determining conjugation rates. For instance, RSF1010 plasmids, which possess independent replication systems, facilitate gene transfer at rates four orders of magnitude higher than host-dependent pUC19 plasmids in soil bacteria such as *Pseudomonas putida* (Crosby & Stadler, 2025).

2.6.5 Hydroxylation

Hydroxylation is a vital biochemical process in microbial bioremediation, where enzymes introduce hydroxyl groups (-OH) into organic pollutants. This modification enhances their solubility and reactivity, making them more susceptible to further degradation. This mechanism is particularly effective in breaking down complex aromatic hydrocarbons, such as polycyclic aromatic hydrocarbons (PAHs), and other persistent environmental contaminants.

Mechanism of Hydroxylation

Microbial enzymes, including dioxygenases and cytochrome P450, play key roles in hydroxylation:

1. Dioxygenase-Mediated Hydroxylation

    Dioxygenases incorporate two oxygen atoms into aromatic rings, forming *cis*-dihydrodiol intermediates (Karigar & Rao, 2011; Dell'Anno et al., 2021).

    This destabilization initiates further degradation through dehydrogenation and ring-cleaving pathways.

2. Cytochrome P450 Hydroxylation

    Cytochrome P450 enzymes mediate alternative oxidation pathways, producing *trans*-dihydrodiols, which undergo further metabolism into less toxic compounds (Dell'Anno et al., 2021).

Functional Impact

Hydroxylation increases the water solubility of hydrophobic pollutants, facilitating microbial uptake and enzymatic degradation (Karigar & Rao, 2011; Dell'Anno et al., 2021).

This process is a critical step in breaking down recalcitrant compounds, directing them into central metabolic pathways such as the citric acid cycle.

Examples in Bioremediation

Pseudomonas aeruginosa and *Klebsiella pneumoniae* utilize dioxygenase-mediated hydroxylation to degrade PAHs in marine sediments (Dell'Anno et al., 2021).

Fungal oxidoreductases, such as laccases, hydroxylate phenolic pollutants, leading to polymerization or binding with soil organic matter (Ayilara & Babalola, 2023; Karigar & Rao, 2011).

By modifying pollutant structures, hydroxylation enhances their susceptibility to complete mineralization, making it a key microbial strategy for environmental detoxification (Karigar & Rao, 2011; Dell'Anno et al., 2021).

2.6.6 Methylation

Methylation in Metal(loid) Detoxification

Methylation is a key biological mechanism for detoxifying toxic metal(loid)s such as mercury (Hg) by converting them into methylated derivatives, which are subsequently removed through volatilization (Adriano, Wenzel, Vangronsveld, & Bolan, 2004). This process alters the volatility, solubility, toxicity, and mobility of elements like arsenic (As), mercury (Hg), and selenium (Se), influencing their environmental behavior.

Chemical vs. Biological Methylation

While both abiotic (chemical) and biotic (biological) processes contribute to metal(loid) methylation, biomethylation—the microbial-mediated process—is dominant in soils and aquatic environments. Biomethylation can facilitate metal(loid) detoxification by:

Converting toxic elements into methylated forms that can be readily excreted from microbial cells.

Producing volatile compounds that are released into the atmosphere.

Generating organoarsenicals, which are often less toxic than their inorganic counterparts.

Role of Microorganisms and Organic Matter

Soil and sediment microorganisms serve as active methylators of metal(loid)s (Lamb, Ming, Megharaj, & Naidu, 2009; Mason, 2012). Organic matter acts as a crucial **methyl donor**, supporting both biological and abiotic methylation processes. Specifically:

Low molecular weight fractions of fulvic acid regulate Hg methylation in soils.

Organic matter and alternative electron acceptors significantly influence Hg methylation in sediments.

Environmental Impact of Biomethylation

Biomethylation effectively transforms arsenic (As) into volatile alkylarsines, allowing for their release into the atmosphere (Frank, 2020; Thayer, 2012). In both aerobic and anaerobic conditions, benthic microbes methylate arsenic, forming methylarsines and other methylated arsenic compounds, which contribute to the natural cycling of arsenic in aquatic and terrestrial ecosystems.

This process plays a crucial role in reducing the toxicity and environmental persistence of metal(loid)s, highlighting its importance in natural and engineered bioremediation strategies.

This revision enhances clarity, flow, and readability while maintaining scientific accuracy. Let me know if you need further refinements!

2.6.7 Adsorption

Adsorption in Microbial Biodegradation

Adsorption is a fundamental mechanism in microbial biodegradation, where microorganisms or immobilized carriers bind pollutants to their surfaces before enzymatic breakdown. This process plays a crucial role in the initial stages of bioremediation by concentrating and stabilizing contaminants, making them more accessible for microbial metabolism.

Mechanism of Adsorption in Microbial Biodegradation

1. Surface Binding

Microbial cells and immobilized carriers adsorb pollutants through chemical interactions such as hydrogen bonding, ionic bonding, and van der Waals forces. For example, bacteria immobilized on natural carriers like cinnamon and peanut shells effectively adsorb diesel via hydrogen bonds (Fu, Wang, Bai, Xue, Gao, Hu, ... & Sun, 2020).

This adsorption process typically follows a **pseudo-second-order kinetic model**, indicating that chemical interactions primarily govern the adsorption rate (Fu et al., 2020).

2. Facilitation of Biodegradation

Adsorption enhances biodegradation by

i. Concentrating pollutants near microbial cells, improving their accessibility for enzymatic degradation.
ii. Enabling stepwise breakdown, where pollutants are first adsorbed, then metabolized into smaller molecules, and eventually mineralized into CO_2 and H_2O (Betsholtz, Falås, Svahn, Cimbritz, & Davidsson, 2024; Fu et al., 2020).
iii. Synergizing with filtration systems, such as granular activated carbon filters, where adsorption and biodegradation may act independently or cooperatively depending on the pollutant type and environmental conditions (Betsholtz et al., 2024).

Applications in Waste Bioremediation

i. Immobilized Microbial Systems: Bio-carriers enhance both adsorption and biodegradation efficiency. For instance, immobilized bacteria degraded up to 69.94% of diesel waste, with adsorption facilitating subsequent enzymatic breakdown (Fu et al., 2020).

ii. Radioactive Waste Remediation: Microbial adsorption is employed for removing radioactive nuclides from contaminated solutions. Optimizing biosorbent properties improves pollutant binding efficiency, enabling safe removal without generating secondary pollution (Wang, Zhang, Qiao, Jiang, Xiao, Duan, & Zhao, 2024).

Advantages of Adsorption in Bioremediation

i. High efficiency in pollutant capture, particularly in the early degradation stages. Stabilization of contaminants, promoting microbial uptake and enzymatic breakdown.

ii. Cost-effective and eco-friendly, offering a sustainable alternative to conventional remediation techniques.

iii. By serving as a foundational step in microbial biodegradation, adsorption enhances pollutant removal and transformation, making it a key strategy in environmental bioremediation.

2.6.8 Solubilization

Solubilization in Microbial Biodegradation

Solubilization is a key process in microbial biodegradation, enhancing the breakdown of hydrophobic pollutants by increasing their water solubility. This mechanism is particularly crucial for degrading persistent contaminants such as polyaromatic hydrocarbons (PAHs) and heterocyclic compounds, which have inherently low aqueous solubility and are resistant to microbial uptake (Stucki & Alexander, 1987).

Mechanisms of Solubilization

1. Biosurfactant Production

Microorganisms produce biosurfactants—such as glycolipids and lipopeptides—which emulsify hydrophobic pollutants, forming micelles that disperse them into aqueous solutions. This process is especially critical for hydrocarbons and petroleum-derived pollutants, facilitating their microbial degradation.

2. Enzymatic Modification

Microbial enzymes, including oxygenases and hydrolases, chemically modify pollutants by introducing polar functional groups (e.g., hydroxyl or carboxyl groups), increasing their solubility. For instance, Burkholderia xenovorans employs oxygenases to oxidize aromatic rings, transforming them into water-soluble derivatives suitable for further degradation.

3. Bioavailability Enhancement

Solubilization counteracts the adsorption of pollutants onto soil particles or clays, which can hinder biodegradation. Studies indicate that compounds such as 2-picoline are less degradable when adsorbed onto clays, highlighting the importance of solubilization in improving microbial access to contaminants (Stucki & Alexander, 1987).

Significance in Bioremediation

i. Increases microbial access to hydrophobic pollutants.

ii. Enhances degradation efficiency of persistent contaminants.

iii. Overcomes adsorption barriers, promoting biodegradation in soil environments.

By improving pollutant solubility, solubilization serves as a crucial strategy in microbial bioremediation, enabling the efficient breakdown of environmental contaminants.

2.6.9 Cometabolism

Cometabolic Bioremediation

Cometabolic bioremediation occurs when a microbial enzyme or cofactor, originally produced for degrading a primary metabolic substrate, also incidentally degrades target contaminants. This approach is specifically designed to enhance the breakdown of contaminants of concern (COCs) by stimulating or augmenting natural microbial processes.

Distinction from Simultaneous Catabolism

Unlike simultaneous catabolism, where multiple compounds are degraded concurrently to generate energy, cometabolism involves the unintended transformation of contaminants without providing the microbe with energy or carbon benefits.

Advantages of Cometabolic Bioremediation

i. Effective at low contaminant concentrations, even below levels required to sustain microbial growth.
ii. Capable of reducing contaminants to non-detect levels, making it useful for stringent remediation goals.
iii. Targets persistent pollutants, including chlorinated solvents and hydrocarbons, that might not be easily degraded through direct metabolism (Hazen, 2010).
iv. By leveraging the fortuitous enzymatic activity of microbes, cometabolic bioremediation provides an efficient and sustainable strategy for the removal of environmental contaminants.

2.6.10 Biostimulation

Biostimulation in Bioremediation

Biostimulation enhances the activity of indigenous microbes by supplementing the environment with essential nutrients (e.g., nitrogen, phosphorus, potassium), electron donors, electron acceptors, biosurfactants, enzymes, and other limiting factors. This method accelerates microbial degradation of pollutants, making it an efficient, cost-effective, and environmentally friendly approach (Ojuederie & Babalola, 2017; Ayangbenro & Babalola, 2018).

Advantages of Biostimulation

More effective than bioaugmentation, as indigenous microbes are naturally adapted to the environment and outcompete introduced strains (Sayed, Baloo, & Sharma, 2021).

Preserves microbial diversity, maintaining ecological balance in the remediation site (Nivetha et al., 2023).

Proven effectiveness in heavy metal remediation, with microbes such as Bacillus sp., *Rhodococcus* sp., Staphylococcus sp., Klebsiella sp., Pseudomonas sp., and *Citrobacter* sp. successfully used in various studies (Nivetha et al., 2023).

Potential Environmental Concerns

Eutrophication risk – Excess nutrients can stimulate harmful algal blooms, disrupting aquatic ecosystems.

Chemical pollution – If synthetic additives are used, they may introduce secondary pollutants, counteracting the benefits of bioremediation.

Despite these challenges, biostimulation remains a widely preferred strategy due to its efficiency and sustainability in enhancing microbial-driven pollutant degradation.

2.6.11 Syntrophy

Syntrophy, derived from the Greek words *syn* (together) and *trophe* (nourishment), is a metabolic partnership in which one microbial species relies on the metabolic byproducts of another for survival. This mutualistic interaction enables microorganisms to efficiently break down environmental pollutants by sharing biochemical tasks, preventing the accumulation of toxic intermediates and maintaining thermodynamically favorable conditions for degradation.

Mechanism of Syntrophy

Cross-feeding: One microbe produces a metabolic product that serves as a substrate or growth factor for another. Nutritional Interdependence: The survival and growth of one species depend on the byproducts, enzymes, or co-metabolites of its partner. Enhanced Degradation: This cooperation optimizes pollutant breakdown by facilitating sequential or parallel metabolic pathways.

Dolfing (2014) described syntrophy as the "critical interdependency between producer and consumer," emphasizing its role in microbial metabolism. Morris et al. (2013) referred to it as "obligately mutualistic metabolism," highlighting the essential nature of this relationship in microbial communities. Goodier (2008) and Morris et al. (2013) further explored how syntrophy underpins bacterial symbiosis and biodegradation processes.By fostering cooperative interactions, syntrophic relationships play a crucial role in environmental remediation, anaerobic digestion, and microbial ecology, making them a fundamental aspect of sustainable bioremediation strategies.

2.6.12 Biosurfactants production

Bacteria, yeasts, and filamentous fungi can produce a diverse array of chemical molecules with surface activity, each characterized by different structures. These amphiphilic compounds consist of a hydrophilic portion, such as acids, peptide cations or anions, or mono-, di-, or polysaccharides, along with a hydrophobic portion that may include unsaturated or saturated hydrocarbon chains, fatty acids, or lipids (Banat et al., 2010). Examples of biosurfactants include lipopeptides, glycolipids, and proteins. Notable glycolipids include rhamnolipids, trehalose lipids, sophorolipids, and mannosylerythritol lipids, while lipopeptides include compounds like surfactin and fungicin (Banat, 2010; Banat, Satpute Cameotra, Patil & Nyayanit, 2014; Dobler, Vilela, Almeida, & Neves, 2016; Franzetti, Bestetti, Caredda, Colla, & Tamburini, 2008a; Franzetti, Tamburini, & Cameotra & Singh, 2009; Luna, Santos & Sarubbo, 2016; Nguyen & Sabatini, 2011; Santos, Rufino, Luna, Santos & Sarubbo, 2016). Additionally, bioemulsifiers, which are high-molecular-weight polymers made up of lipopolysaccharides, polysaccharides, proteins, or lipoproteins, include examples like the lipopolysaccharide emulsan and the polysaccharide-protein complex alasan (Neu, 1996; Uzoigwe et al., 2015).

While biosurfactants are effective in reducing surface and interfacial tensions, bioemulsifiers are more effective at stabilizing oil-in-water emulsions, although they have a lesser ability to lower surface tension than biosurfactants (Smyth et al., 2010a; Smyth et al., 2010b). Microbial surfactants can enhance bacterial growth on petroleum hydrocarbons (PHCs) by increasing the surface area between oil and water through emulsification and by improving pseudosolubility through micelle partitioning (Volkering, Breure, & Rulkens, 1997). This process can increase the bioavailability of contaminants to degrading microbes in certain conditions. Recent reviews have highlighted successful applications of biosurfactants in bioremediation processes (Mulligan, 2009; Pacwa-Plociniczak et al., 2011; Lawniczak et al., 2013). Lipopeptides from *Bacillus circulans* (Das et al., 2008) and lipopeptides and protein-starch-lipid mixtures from two strains of *Pseudomonas aeruginosa* (Bordoloi & Konwar, 2009) have been shown to enhance the biodegradation of PAHs

2.6.13 Biofilms formation

Biofilms are bacterial communities encased in self-produced polymeric matrices that are reversibly attached to inert or biotic surfaces (Costerton, Lewandowski, Caldwell, Korber, & Lappinscott, 1995). This adaptive mechanism enables microorganisms to better withstand harsh physical and chemical conditions, facilitates the exchange of metabolites, enhances horizontal gene transfer, and regulates the redox state of their environment (Gorbushina & Broughton, 2009; Shemesh, Kolter, & Losick, 2010). The biofilm matrix is composed of extracellular polysaccharides (EPSs), proteins, and DNA (Sutherland, 2001; Branda, Vik, Friedman, & Kolter, 2005; Rinaudi & Gonzalez, 2009). EPSs play a crucial role in determining the porosity, density, water content, charge, hydrophobicity, and mechanical stability of biofilms (Sutherland, 2001; Branda et al., 2005; Rinaudi & Gonzalez, 2009; Flemming & Wingender, 2010).

Biofilms can be particularly beneficial for the bioremediation of petroleum hydrocarbons (PHCs) by increasing the bioavailability of pollutants (Wick, Colangelo, & Harms, 2002; Johnsen & Karlson, 2004). The formation of biofilms is closely linked to the secretion of polymers by microorganisms; thus, when biofilms develop on the surface of insoluble hydrocarbons, the microorganisms involved become highly effective in treating recalcitrant compounds. This is due to the higher microbial biomass within biofilms compared to dispersed cultures and their ability to immobilize compounds through adsorption. Additionally, the biofilm lifestyle supports degradation processes by maintaining optimal conditions near the cells, such as stable pH, localized solute concentrations, and redox potential (Singh, DeMarini, Dick, Tabor, Ryan, Linak, Kobayashi, & Gilmour, 2004).

2.6.14 Chemotaxis

Bacterial chemotactic behavior can be either positive (toward a chemical) or negative (away from a chemical) in response to a gradient. This chemotaxis is believed to serve as a balancing mechanism, enabling bacteria to function optimally by enhancing pollutant bioavailability while also protecting them from harmful substances. This balance might explain why the naphthalene-degrading *Pseudomonas putida* PpG7 was repelled by vapor-phase naphthalene at steady-state concentrations that were much lower than those causing positive chemotaxis (Hanzel, Harms, & Wick, 2010).

Chemotaxis, the directed movement of microorganisms in response to chemical gradients to find favorable conditions for growth and survival (Eisenbach & Caplan, 1998; Wadhams & Armitage, 2004; Baker et al., 2006a, b), plays a crucial role in microbial utilization of petroleum hydrocarbons (PHCs) in soil and water environments (Eisenbach & Caplan, 1998; Wadhams & Armitage, 2004; Parales & Haddock, 2004; Ford & Harvey, 2007; Strobel et al., 2011). For instance, the ability of bacteria to detect and move towards n-hexadecane, gas oil, various monocyclic aromatic hydrocarbons (MAHs), polycyclic aromatic hydrocarbons (PAHs), and their nitro-, amino-, or chloro-substituted derivatives has been shown to enhance the degradation of these PHCs (Grimm & Harwood, 1997; Parales, Ditty, & Harwood, 2000; Olson, Castro, Joern, DuTeau, Pilon-Smits, 2008; Jeong et al., 2010).

2.7 Bioremediation of petroleum hydrocarbons

Bioremediation is the process of using biological mechanisms to detoxify, degrade, or transform contaminants into harmless substances (Azubuike et al., 2016). It is a valuable method for treating environments contaminated with petroleum hydrocarbons (PHCs), both on land and in marine settings (Atlas, 1995; Atlas and Cerniglia, 1995; Almeida et al., 2013; Xue et al., 2015; Scoma et al., 2016; Wang et al., 2016). For microbes to effectively utilize these energy-rich compounds for growth and energy, they must first access PHC substrates while mitigating harmful effects.

The extent to which PHCs are susceptible to biodegradation is influenced by three main factors:

1. Microbial properties: This includes genetic composition, gene regulation and expression, surface hydrophobicity, metabolic diversity and adaptability, substrate uptake or adhesion

mechanisms, tolerance to metals and other toxic substances, chemotaxis, and biofilm formation (Martinez-Checa et al., 2007; Bordoloi and Konwar, 2009; Calvo et al., 2009; Banat et al., 2010).

2. Environmental factors: These include the availability of terminal electron acceptors, nutrients, salinity, pressure, temperature, pH, water availability, and osmotic stress (Botalova et al., 2009; Couling et al., 2010).
3. Hydrocarbon substrate characteristics: This category considers the solubility, concentration, hydrophobicity, volatility, and molecular weight of the hydrocarbons (Couling et al., 2010).

The process of PHC removal by microbial communities is largely dependent on bioavailability and bioaccessibility. Bioavailability refers to the amount of a contaminant that is freely available to pass through an organism's cellular membrane from its environment, while bioaccessibility indicates the potential of a contaminant to cross into an organism from its surroundings (Semple et al., 2007; Dandie et al., 2010). Bioavailability can be assessed through two primary approaches:

- Chemical methods: These involve selective extraction to identify the accessible fraction of specific contaminants (Harmsen, 2007).
- Biological methods: These involve exposing organisms to contaminated media to evaluate the extent of bioavailability.

Numerous studies have suggested that PHC bioavailability is a limiting factor in the effectiveness of bioremediation (Schwartz & Scow, 2001; Tabak et al., 2003; Hamdi et al., 2007b). However, given the diversity in biological systems, it is important to note that such generalizations may not apply universally to all situations (Huesemann, Hausmann, & Fortman, 2004). (Huesemann, Hausmann, & Fortman, 2004).

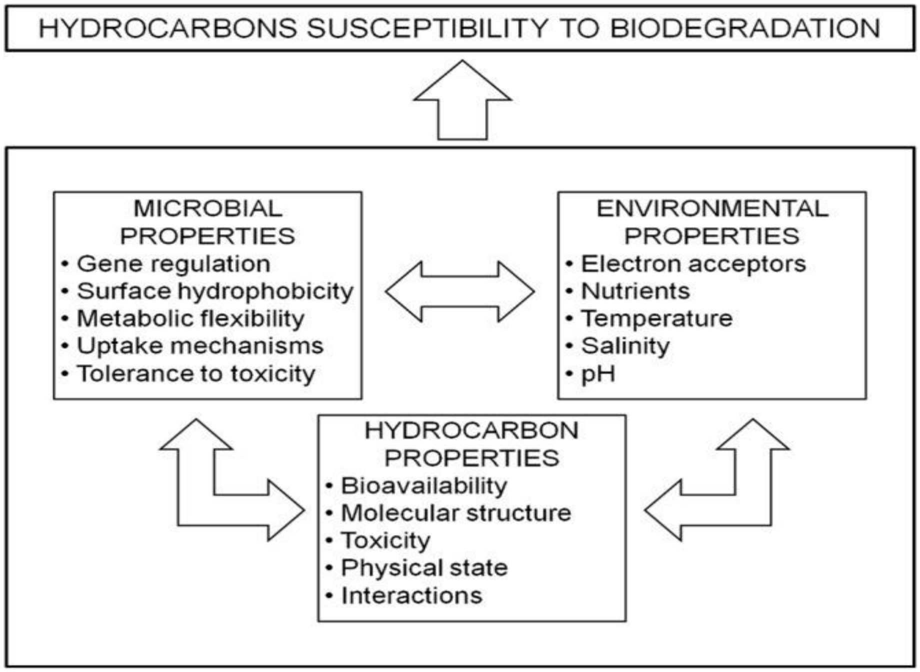

Figure 2.1: Main factors affecting biodegradation of petroleum hydrocarbons (PHCs).

Source: Gkorezis et al., 2016

2.7.1 Remediation Strategies

Ex situ and in situ bioremediation techniques have both been applied to restore environments contaminated with petroleum hydrocarbons (PHCs) (Stroud et al., 2007). However, in situ approaches have gained greater popularity due to their generally lower costs and minimal disruption to natural landscapes compared to ex situ methods (Romantschuk et al., 2000). The assessment of ecological sustainability in in situ bioremediation processes has been thoroughly reviewed (Pandey et al., 2009), with particular attention given to natural attenuation (Scow & Hicks, 2005), as well as biostimulation and bioaugmentation strategies.

2.7.2 Natural attenuation

Natural attenuation has emerged as a viable remediation strategy for soils, estuarine sediments, and groundwater contaminated with petroleum hydrocarbons (PHCs), supported by extensive research including modeling and field trials (Verginelli & Baciocchi, 2013; Khan & Husain, 2003). Numerous studies have highlighted the critical role of subsurface natural attenuation processes in bioremediation efforts (Devaull, 2007; Lundegard et al., 2008; Pasteris et al., 2002). This approach has proven effective for the long-term bioremediation of diesel-oil-contaminated sites, even in challenging conditions like low temperatures (Margesin & Schinner, 2001).

The recovery of the Gulf of Mexico following the Deepwater Horizon disaster illustrates the potential success of in situ bioremediation through natural attenuation after large-scale oil spills. The native microflora of the deep marine ecosystem rapidly adapted to the oil contamination, leading to the dominance of Oceanospirillales bacteria within the class of Proteobacteria, which includes known psychrophilic hydrocarbon degraders and microorganisms adapted to hydrocarbon-rich environments (Hazen et al., 2010).

2.7.3 Biostimulation

Biostimulation is a method used to enhance the degradation of petroleum hydrocarbons (PHCs) by adding nutrients (such as nitrogen, phosphorus, poultry litter, horse manure, domestic sewage, rice straw biochar, and crop residues) and other supplementary agents like biosurfactants and electron acceptors (e.g., oxygen, chelated Fe(III), nitrates, and sulfate). These additions create a more favorable environment for hydrocarbon-degrading bacterial communities (Coles et al., 2009; Gallego et al., 2001; Molina-Barahona et al., 2004). The effectiveness of these components is tied to their ability to either enhance the metabolic activity of the indigenous degrading bacteria or increase the bioavailability of PHCs. Nutrient enrichment, in particular, has been shown to significantly boost the degradation capacity of native microbial communities among various biostimulants (Delille et al., 2004; Garcia-Blanco et al., 2007; Thomassin-Lacroix et al., 2002;).

Several studies have demonstrated that adding biosolids, including inorganic fertilizers rich in nitrogen and phosphorus, and organic fertilizers to PHCs (such as diesel oil, pyrene, and phenanthrene) accelerates their degradation both in laboratory settings and field applications (Carmichael & Pfaender, 1997; Margesin et al., 2003; Sarkar et al., 2005; Xu & Obbard, 2003). Margesin et al., (2007) observed that the positive impact of fertilization on PHC degradation was more pronounced with higher levels of initial PHC contamination. Similar results have been reported for aquatic environments, though caution is necessary to prevent ecological issues like eutrophication due to excessive nutrient levels (Nikolopoulou & Kalogerakis, 2009).

For oil spill cleanup, an application of 1–5% nitrogen by weight of the oil, with a nitrogen to phosphorus (N) ratio of 5–10:1, is generally recommended. Theoretically, converting 1 gram of hydrocarbon into microbial biomass requires around 150 mg of nitrogen and 30 mg of phosphorus. OptimalC: N ratios for in situ bioremediation have been explored in various studies, with proposed ratios such as 100:9:2, 100:10:1, 100:10:5, and 250:10:3 being identified as ideal for enhancing hydrocarbon degradation in soil (Zawierucha & Malina, 2011).

2.7.4 Bioaugmentation

Bioaugmentation is a bioremediation strategy that involves introducing a sufficient number of bacteria with the necessary catabolic capabilities to facilitate the breakdown of petroleum hydrocarbons (PHCs) (Paliwal, Puranik, & Purohit, 2012). This process often requires the addition of one or more of the following: (a) a pre-adapted bacterial strain, (b) pre-adapted microbial consortia, (c) genetically modified bacteria, or (d) catabolic genes carried in a vector for delivery into native microorganisms through conjugation (El Fantroussi & Agathos, 2005; Singer et al., 2005; Thompson et al., 2005). When implementing bioaugmentation, it is important to consider local regulations regarding:

1. The introduction of a single strain or a known mixed microbial consortium.
2. The re-inoculation of an autochthonous bacterial consortium that has been previously enriched from polluted soil and cultured with hydrocarbons as the carbon source.
3. The re-inoculation of an allochthonous bacterial consortium derived from a different PHC-polluted environment (Ueno et al., 2007).

Introducing microbial communities capable of PHC biodegradation to newly contaminated soils can significantly aid the bioremediation process (Greenwood et al., 2009). Studies have shown that bioaugmentation can accelerate the bioremediation of soils contaminated with diesel oil and various heavy metals when appropriate native strains are used (Alisi et al., 2009). Further research on the potential of indigenous and exogenous microorganisms for remediating diesel-contaminated clayey and silty soils revealed that native consortia were most effective for silty soil, while a mix of native and exogenous consortia proved more effective for clayey soils (Moliterni, Rodriguez, Fernandez, & Villasenor, 2012).

2.7.5 Plant-bacteria synegy for the remediation of petroleum hydrocarbons

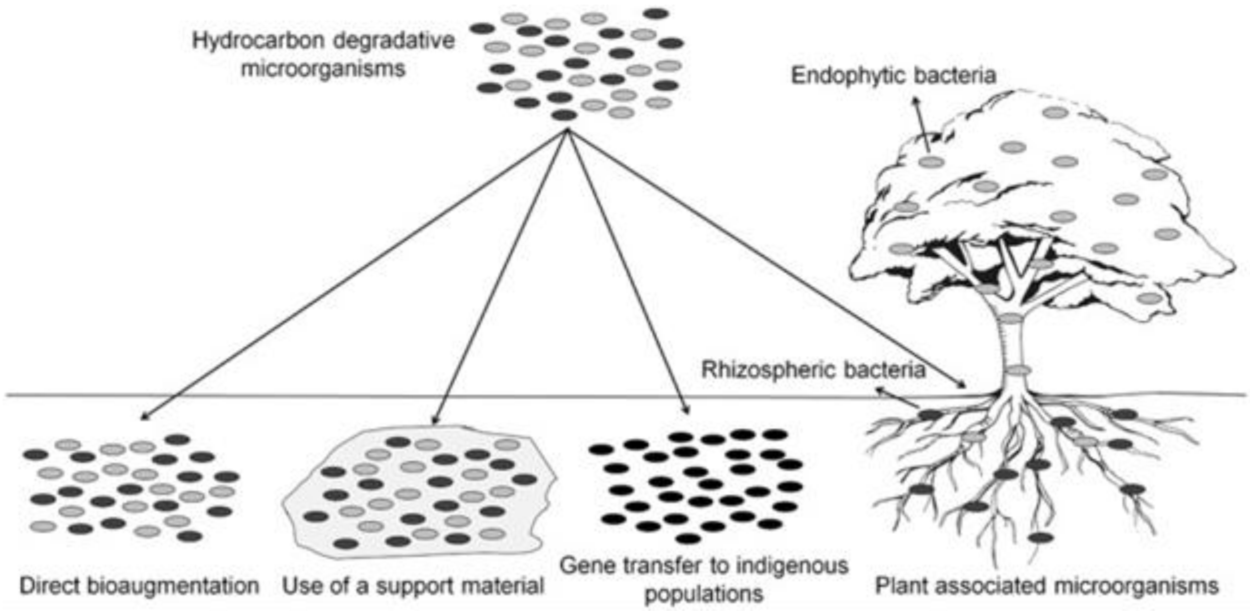

Figure 2.2: Plant-Bacteria synergy for the Remediation of Petroleum Hydrocarbons

Source: Gkorezis et al., (2016)

Phytoremediation involves the use of plants and their associated microorganisms to assimilate, transform, metabolize, detoxify, and degrade various toxic inorganic and organic compounds, such as petroleum hydrocarbons (PHCs), pesticides, dyes, and solvents, in soil, water, groundwater, and air (Kabra, Khandar, Waghmode, & Govindwar, 2012; Prasad, Freitas, Fraenzle, Wuenschmann, & Markert, 2010; Wenzel, 2009). Plant-associated bacteria, including endophytic, phyllospheric, and rhizospheric types, interact with plants in various ways, ranging from active pathogens and opportunistic pathogens to symbiotic relationships that benefit both the plant and the bacteria (Newman & Reynolds, 2004; Weyens et al., 2009).

Plant-associated bacteria, including endophytic, phyllospheric, and rhizospheric types, interact with plants in diverse ways. These interactions range from acting as active or opportunistic pathogens, to residing within the plant for physical protection, and even forming mutually beneficial relationships with their host (Newman & Reynolds, 2004; Weyens, van der Lelie, Taghavi, Newman, & Vangronsveld, 2009c). Bacteria equipped with catabolic genes and enzymes can utilize complex compounds present in petroleum mixtures as energy sources, enabling the decomposition of petroleum hydrocarbons (PHCs) (Das & Chandran, 2010; Rojo, 2009). Several bacterial strains, such as *Pseudomonas*, *Acinetobacter*, *Mycobacterium*, *Haemophilus*, *Rhodococcus*, *Paenibacillus*, and *Ralstonia*, have been extensively studied for their capacity to degrade hydrocarbons (Tyagi, da Fonseca, & de Carvalho, 2011).

Although these bacteria can metabolize many hydrocarbons independently, their efficiency is often limited in bulk soil due to their low abundance. However, microbial activity in the rhizosphere is 10 to 1,000 times higher than in bulk soil, highlighting the crucial role of plants in enhancing this process (Palmroth, Pichtel, & Puhakka, 2002). Thus, plants and their associated bacteria work together to drive the ongoing degradation of PHCs.

PHCs pose significant threats not only to soil but also to estuarine sediments, which are biologically rich and sensitive to pollutants (Chapman & Wang, 2001; Daane, Harjono, Zylstra, & Haggblom, 2001). The ecological importance and vulnerability of these ecosystems have led researchers to investigate the role of plant–microorganism interactions in degrading PHCs in estuarine environments. Recent studies have shown that partnerships between salt marsh plants and bacteria can significantly enhance PHC degradation by increasing the functional diversity of the PHC-degrading bacterial community (Oliveira, Gomes, Almeida, Silva, Silva, & Cunha, 2014).

2.8 Phytoremediation mechanisms

The four key mechanisms of phytoremediation include phytostabilization, phytodegradation, phytovolatilization, and rhizodegradation (Germida, Frick, & Farrell, 2002). Phytostabilization involves immobilizing pollutants within the soil by preventing erosion, leaching, or dispersion, or by converting contaminants into less bioavailable forms through precipitation in the rhizosphere. Phytodegradation and rhizodegradation work synergistically, as both plants and microbes use their enzymatic capabilities to break down pollutants. Phytovolatilization is particularly promising, as it removes pollutants from the site entirely by converting them into gaseous forms, thus eliminating the need for plant harvesting and disposal (Pilon-Smits, 2005). Additionally, studies have shown that phyllosphere bacteria can metabolize both gaseous and deposited petroleum hydrocarbons (PHCs) (Ali, Khalil, N.M. & El-Ghany, 2012; Al-Awadhi, Al-Mailem, Dashti, Hakam, Eliyas, & Radwan, 2012; Waight, Pinyakong, & Luepromchai, 2007; Yutthammo, Thongthammachat, Pinphanichakarn, & Luepromchai, 2010); the latter has great potential in air clean-up by opening up a new direction of air phyllo-remediation, which is actually the exploitation of air remediation (Weyens et al., 2015).

Phyllospheric bacteria can establish stable communities after their initial recruitment, despite constant exposure to airborne populations (Whipps, Hand, Pink, & Bending, 2008). This suggests that these bacteria undergo specific selection processes (Rastogi, Sbodio, Tech, Suslow, Coaker, Leveau & 2012; Vokou, Vareli, Zarali, Karamanoli, Constantinidou, Monokrousos, Halley, & Sainis, 2012; Vorholt, 2012). Factors such as plant species, leaf age, season, geographical location, and other environmental conditions are thought to influence the organization of these communities (Muller & Ruppel, 2014; Vokou et al., 2012). Given the diversity in phyllospheric community structures, further research is needed to investigate the bacterial communities associated with different plant species across varying conditions, to better understand their role in air bioremediation. In these close plant–bacteria interactions, plants provide resources and a habitat for bacteria, which can enhance the effectiveness of phytoremediation in environments contaminated with petroleum hydrocarbons (PHCs).

A recent review (Thijs, Sillen, Rineau, Weyens, & Vangronsveld, 2016) emphasizes that considering meta-organisms in their natural contexts—i.e., the host and its microbiome together—can deepen our understanding of plant-microbial interactions, leading to more effective and predictable phytoremediation strategies. The following sections will highlight selected case studies that shed light on PHC degradation through the collaboration of plants, microorganisms, and their close interactions.

2.8.1 Mechanisms for the phytoremediation of petroleum hydrocarbon degradation

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Figure: 2.3 Plant-microbe interactions and plant-growth-promoting effects of rhizosphere and endosphere bacteria

Source: Olivera et al., 2014

Degradation is the transformation of a formerly hazardous chemical into a less harmful or harmless form. Plants and microbes are both directly and indirectly engaged in the breakdown of petroleum hydrocarbons. Alcohol, acids, carbon dioxide, and water are among of the end-products, and they are typically less harmful and persistent in the environment than the parent molecules (Ojuederie & Babalola, 2017). Despite the fact that plants and microorganisms may both degrade petroleum hydrocarbons,**)** claim that the interaction between plants and microorganisms (i.e., the rhizosphere effect) is the fundamental mechanism responsible for petrochemical degradation in phytoremediation efforts

The rhizosphere is the area of soil nearest to a plant's roots, and it is therefore directly influenced by the root system Al-Hawash, (2018)**.**Plants give carbon, energy, nutrients, enzymes, and occasionally oxygen to microbial communities in the rhizosphere through root exudates. Sugar, alcohol, and acid exudates from the roots can account for 10-20% of annual plant photosynthesis and supply enough carbon and energy to support a significant number of microorganisms (example, approximately 10^8-10^9 vegetative microbes per gram of soil) in the rhizosphere.Microbial populations and activity in the rhizosphere are 5 to 100 times higher than in bulk soil (i.e. soil not in touch with plant roots) due to these exudates.The rhizosphere effect **(**Liu, 2020) is a plant-induced increase in microbial population that is thought to result in increased breakdown of organic material in the rhizosphere.

2.8.2 Indirect degradation

In contrast to the scanty evidence available on plants' direct degradation of petroleum hydrocarbons, there is a substantial body of information accessible on plants' indirect functions in petroleum hydrocarbon degradation. These are some of them

• The rhizosphere impact and accelerated cometabolic breakdown are caused by the availability of root exudates.

• The release of enzymes linked with roots that might convert organic contaminants

• Plants and their root systems' physical and chemical effects on soil conditions

2.8.3 Root exudates

The rhizosphere effect is caused by a relationship between plants and bacteria called root exudates **(**Vives-Peris, De Ollas, C., Gómez-Cadenas, & Pérez-Clemente, 2020)**.** The kind and amount of root exudate produced is determined by the plant's species and stage of growth. Badri, & Vivanco, (2009)discovered that the release of total phenolics by the roots of red mulberry (*Morus rubra.*) rose steadily throughout the plant's life, with a huge release near the end of the season, coinciding with leaf senescence.

The kind of root exudate is also likely to vary by location and time (Siciliano & Germida, 1998). Variables including soil types, nutritional levels, pH, water availability, temperature, oxygen status, light intensity, and atmospheric carbon dioxide concentration all have an impact on the kind and quantity of root exudates produced (Baetz, & Martinoia, 2014)

2.8.4 Cometabolism

When another growth-supporting substrate is available, cometabolism occurs, and a molecule that cannot sustain microbial development on its own is transformed or destroyed (. Plant exudates, for example, can offer enough energy to sustain a population of bacteria that co-metabolize petroleum hydrocarbons. Plant exudates, for example, may have functioned as co-metabolites during the biodegradation of (^{14}C) Pyrene in the rhizosphere of crested wheatgrass, according to Badri, & Vivanco, (2009).

2.8.5 Plant enzymes involved in phytoremediation

Another indirect function that plants play in the breakdown of petroleum hydrocarbons is the release of enzymes from their roots. These enzymes can catalyze chemical processes in soil to convert organic pollutants (Kvesitadze, Gordeziani, Khatisashvili, Sadunishvili, & Ramsden, 2001). Plant enzymes were discovered as the causal agents in the transformation of pollutants combined with silt and soil by Schäffner, Messner, Langebartels, & Sandermann, (2002). Dehalogenase, nitroreductase, peroxidase, laccase, and nitrilase were among the enzyme systems isolated. These findings imply that plant enzymes may have major geographical and temporal impacts that reach beyond the plant itself after it has perished (Schäffner, Messner, Langebartels, & Sandermann, (2002).).

2.8.6 Endophytic Remediation

Bacteria residing within the internal tissues of plants (such as roots, stems, and leaves) have an advantage over rhizosphere bacteria, as they face less competition for nutrients and space, and are better protected from harsh environmental conditions (Schulz, Boyle, & Sieber, 2006).

A variety of culturable endophytic bacteria have been isolated from different plant species, including crop plants like sugar cane (Loiret, Ortega, Kleiner, Ortega-Rodés, Rodes, & Dong, 2004), wheat (Loiret et al., 2004), and maize (Gutierrez-Zamora & Martınez-Romero, 2001).

Endophytes have also been found in the metal hyperaccumulator alpine pennycress (*Thlaspi caerulescens*) (Lodewyckx, Mergeay, Vangronsveld, Clijsters, & Van Der Lelie, 2002), tall Fescue (Malinowski, Alloush, & Belesky, 2000), and *Arabidopsis* seeds (Truyens, Beckers, Thijs, Weyens, Cuypers, & Vangronsveld, 2015a; Truyens, Beckers, Thijs, Weyens, Cuypers, & Vangronsveld, 2015b). Additionally, they have been identified in various grass species (Dalton et al., 2004; Thijs et al., 2014b), woody trees such as oak, ash (Weyens et al., 2009a), sycamore (Thijs et al., 2014a), poplar (Porteous-Moore, Barac, Borremans, Oeyen, Vangronsveld, van der Lelie, Campbell, & Moore, 2006; Van der Lelie, Taghavi, Monchy, Schwender, Miller, Ferrieri, Rogers, Wu, Zhu, Weyens, Vangronsveld, &Newman, 2009), *Mimosa pudica* (Pandey, Chauhan, & Jain, 2009), pine seeds (Cankar, Kraigher, Ravnikar, & Rupnik, 2005), and other forest trees (Pirttilä & Frank, 2011).

2.8.7 Transfer of petroleum hydrocarbons to the atmosphere (phytovolatilization)

A plant's natural ability to volatilize pollutants absorbed through its roots can be harnessed to create a natural air-stripping pump system. Pollutants like BTEX (benzene, toluene, ethylbenzene, and xylene), TCE (trichloroethylene), vinyl chloride, and carbon tetrachloride are examples that can be effectively treated with traditional air-stripping methods. These substances have a Henry's constant (KH) greater than 10 atm·m^3 water·m^{-3} air, which allows them to volatilize efficiently. In contrast, chemicals with lower volatility and a KH less than 10 atm·m^3 water·m^{-3} air, such as phenol and pentachlorophenol (PCP), are not suitable for the air-stripping mechanism (Zhang, Daprato, Nishino, J.C. Spain & Hughes, 2001).

2.9. Factors affecting plant-bacteria partnership

The effectiveness of the plant-bacteria relationship is influenced by the genetic diversity of both the bacteria and the host plant, as well as environmental factors like climate, soil type, and competing microbial communities. Understanding the intricacies of this relationship is crucial for optimizing its potential. One approach to enhance this synergy is bioaugmentation, which involves adding bacterial cultures to accelerate the biodegradation process. However, the success of bioaugmentation depends significantly on the ability of the bacterial species to colonize the plant's root system, as this is essential for forming strong associations and maximizing the benefits of the remediation process. Additionally, the interaction between plants and bacteria is shaped by biotic factors (such as pathogens, protozoa, and fungi) and abiotic factors (including pH, temperature, moisture, and nutrient availability)..

2.9.1 Method of Inoculation

The method of inoculation can significantly influence a microorganism's ability to survive, colonize, and its potential for bioremediation. The colonization, persistence, and activity of an introduced microbe in the soil, rhizosphere (root zone), and various parts of the plant (roots and shoots) are all affected by the inoculation technique used (Afzal, Yousaf, Reichenauer, & Sessitsch, 2012; Afzal, Khan, Iqbal, Mirza, & Khan, 2013). There are four primary methods of inoculation: (i) seed inoculation, which involves coating seeds with a peat-based slurry in a sugar solution; (ii) soil inoculation**,** where a bacterial liquid culture is spread on the soil surface along with irrigation water; (iii) rhizosphere inoculation, which entails dripping or injecting bacteria directly into the rhizosphere; and (iv) foliar inoculation**,** where bacterial culture is sprayed onto the surface of the plant or soil.

Several researchers have evaluated these inoculation methods and shared their findings. Afzal et al., (2013) conducted a study using ryegrass grown in hydrocarbon-contaminated soil, employing various inoculation techniques to introduce *Burkholderia phytofirmans* PsJN. They discovered that soil inoculation was particularly effective in promoting plant growth, enhancing hydrocarbon degradation, and improving phytoremediation outcomes. Additionally, compared to seed imbibition, soil inoculation of *Pantoea* sp. in conjunction with *Pseudomonas sp.* yielded the most significant increases in hydrocarbon biodegradation rates and supported better ryegrass growth (Afzal, 2012). Furthermore, the inoculation of beneficial bacteria into the roots or seeds of metal-accumulating plants, such as spinach and maize, improved the bioavailability of metals, thereby enhancing their uptake by the plants (Ahmad, Nadeem, Naveed, & Zahir, 2016; Ali et al., 2013).

2.9.2 Root Colonization by Bacteria

The relationship between plants and bacteria is highly specialized (Hussain et al., 2014a, b). Plants release flavonoids to attract specific bacteria, while bacteria produce nod factors to recognize their host plants. This interaction fosters a mutualistic relationship, wherein plants provide carbon substrates (root exudates) to microbes (Bais, Broeckling, & Vivanco, 2008; Shukla et al., 2011; Thimmaraju, Czymmek, Pare, & Bais, 2008;). In return, microbes exhibit plant growth-promoting (PGP) characteristics that enhance plant growth under both normal and stressful conditions. To optimize bioremediation processes and promote host plant growth, it is essential to increase the population of beneficial bacteria in the plant's rhizosphere or on the surface of its roots.

As previously mentioned, the injection method influences a bacterium's ability to colonize plants, and a robust population of bacteria in the rhizosphere is crucial for bacterial-assisted phytoremediation of contaminants (Afzal et al., 2013). Shukla et al. (2011) noted that beneficial bacteria need to effectively degrade specific soil contaminants and colonize plant roots to accelerate the rhizoremediation process, which involves the remediation of rhizosphere soil through degradation or transformation of contaminants. However, different bacteria exhibit varying capacities to colonize plant roots or their internal tissues. For instance, the ability of *Pseudomonas putida* strain PCL1444 to colonize the rhizosphere was found to be 100 times greater than that of *Pseudomonas fluorescens* strain WCS365, attributed to the former's proficiency in utilizing root exudates from *Lolium multiflorum* and degrading heavily contaminated naphthalene soil (Kuiper, Kravchenko, Bloemberg, & Lugtenberg, 2002). Additionally, Germaine et al. (2009) explored a similar relationship in plants inoculated with *Pseudomonas putida*.

They found that *Pseudomonas putida* strain PCL1444 was an excellent colonizer of both the exterior and interior of roots, capable of degrading naphthalene by up to 40%. Moreover, inoculated plants exhibited higher seed germination and transpiration rates compared to uninoculated control plants. Ahmad et al. (2016) reported that the seed inoculation of *Klebsiella* and *Enterobacter* strains CIK-518 and CIK-521R, respectively, enhanced maize growth in cadmium (Cd)-contaminated soils, as these bacteria demonstrated greater tolerance to high Cd concentrations and more effective root colonization. Additionally, Cavalca, Corsini, Bachate, & Andreoni, (2013) observed a 53% increase in arsenic uptake in sunflowers following inoculation with *Alcaligenes* sp. strain Dhal-L. They noted higher colonization of injected bacteria in the rhizosphere soil of sunflowers. In another study, molecular analysis revealed microbial community diversity in rhizosphere soil contaminated with Cd (Moreira, Marques, Franco, Rangel, & Castro, 2014).

The microbial community was found to be effective in the short-term phytostabilization of Cd in soil. The above research indicated a high association between root colonization and pollutant degradation/stabilization/extraction, hence this section focuses on root colonization of bacterial strains utilized in the remediation process. Root colonization is an effective method for identifying and testing efficient bacterial strains that aid in the phytoremediation process.

2.10 None oil related heavy metals environmental pollution

Heavy metals are typically defined as metallic elements with atomic numbers greater than 20, generally excluding alkali and alkaline earth metals. These naturally occurring compounds possess a high density, typically greater than 5 g/cm^3, which is at least five times denser than water. They are among the most persistent pollutants found in soil and water. Heavy metals can be categorized into two groups based on their roles in living organisms: essential and non-essential. Essential heavy metals, such as manganese (Mn), iron (Fe), nickel (Ni), and zinc (Zn), are required for the growth, development, and physiological functions of living organisms. In contrast, non-essential heavy metals, including cadmium (Cd), lead (Pb), mercury (Hg), and arsenic (As), are not necessary for any physiological functions in living organisms (Gohre et al., 2006).

However, anthropogenic activities such as mining have resulted in elevated levels of these contaminants in the environment. By definition, any toxic metal may be called a heavy metal, irrespective of its atomic mass or density. The classification includes some metalloids, transition metals, basic metals, lanthanides and actinides and metals of groups III to V of the periodic table

Examples include As, Pb, Hg, Cd, Cr, Co, Ni, Cu, Zn, Se, Al, Cs, Mn, Mo, Sr, U, Be and Bi (Dietz, & Schnoor, 2001).

Natural and anthropogenic/human sources of heavy metals in soil are the two main sources. Soil erosion, volcanic activity, urban runoff, and particle aerosols are examples of natural variables, while metal polishing and electroplating processes, mine extraction operations, textile industries, and nuclear power are examples of human factors. The presence of these heavy metals in soil and water bodies is known to degrade the quality of those soils and waters dramatically. The presence of metals in soils and streams is thought to be caused by a number of volcanic rocks and volatiles. This is because the hydrological material transfer in volcanic strata is aided by the diffusion of acidic volcanic gases through water permeable rocks. Volcanic activity has been blamed for the emission of metals like arsenic, mercury, aluminum, rubidium, lead, magnesium, copper, zinc, and a variety of others (Amarlal, Cruz, Cunha, & Rodrigues, 2006).

Soil erosion has also been identified as a source of heavy metal pollution. Wind and water are the two main causes of soil erosion. Sediment-bound heavy metals are transferred to the soil during rainfall. While causing erosion, water containing agrochemicals with dangerous metal concentrations drops this sediment-bound metal in the soil. Furthermore, some aerosol particles (fine colloidal particles or water droplets in the air; in other situations, they can be gas) can contain various contaminants, such as smoke cloud and heavy metals. These heavy metal-containing aerosols often form small particles on leaf surfaces and can penetrate the leaves through stomata (Sardar, Ali, Hameed, Afza, Fatima, Shakoor, Bharwana, & Tauqeer, 2013).

Metal finishing and electroplating, mining and extraction operations, textiles industries, and nuclear power are some of the anthropogenic sources of heavy metals in soil. Electrochemical methods are used to deposit thin protective layers onto prepared metal surfaces during metal finishing and electroplating. Toxic metals may be discharged into wastewater effluents if this happens. This might happen through product rinsing or spillage and dumping of process baths. It's also been suggested that cleaning process tanks and wastewater treatment can result in large amounts of wet sludge containing significant levels of hazardous metals **(**Kumar, Jeena, Gangola, & Singh,.2019).

Mining operations can also release toxic metals into the environment. Heavy metals are commonly found in the environment as a result of metal mining and smelting. A substantial number of harmful metals deposits have been identified in the water, soil, crops, and vegetables of locations where these operations take place (Wei et al., 2008). Textile industries have also been identified as important contributors of heavy metal pollution in soil and water.

The dyeing process, which is a major step in such sectors, is considered to be the source of most of this. Copper, chromium, nickel, and lead, which are all poisonous and carcinogenic, are among the substances utilized in these dyeing procedures (coloration). Nuclear power plants have been implicated in the release of heavy metals such as copper and zinc into surface soil and water in some circumstances. Because nuclear power plants take a lot of water to operate, the nuclear effluent containing heavy metals is discharged into surface and groundwater bodies after the operation, polluting soil and aquatic systems (Hagberget, 2007; Wuana, & Okieimen, 2011).

2.11 Dangers of heavy metal pollutants to plant and human life

2.11.1 Effect on human

When untreated or improperly treated heavy metal-contaminated wastewater is released into soil and water bodies, it can have numerous health and environmental consequences. Heavy metals can decrease the diversity of living organisms in aquatic habitats and adversely affect the growth of aquatic species, leading to significant issues in biological wastewater treatment systems. The presence of heavy metal pollution poses a serious threat to soil quality and the plants that grow in such contaminated soils. As animals and humans consume these plants, heavy metals can biomagnify and bioaccumulate in the food chain, resulting in severe health impacts (Saidi, 2010). Toxic metals in vegetables and maize products can accumulate in the kidneys, potentially leading to organ dysfunction. Below are some reported effects of heavy metals on human health:

i. Selenium

High levels of selenium, as reported in some studies, have been linked to skeletal deterioration (osteoporosis) in humans (Abdullahi, 2013).

ii. Cadmium

Cadmium is a highly toxic heavy metal that poses significant health risks even at low concentrations in humans. It has been identified as a carcinogen and a cumulative toxin (Lin et al., 2005). Long-term exposure to cadmium can lead to renal dysfunction, while higher levels may result in obstructive pulmonary disease, cadmium pneumonitis, bone disorders, osteomalacia, osteoporosis, spontaneous fractures, elevated blood pressure, and heart failure (Duruibe, Ogwuegbu, & Egwurugwu, 2007). The severity of symptoms—including nausea, vomiting, stomach cramps, dyspnea, and muscle weakness—can vary depending on the level of exposure to cadmium compounds. Excessive exposure may lead to pulmonary edema and even death (Duruibe et al., 2007; INECAR, 2000; Young, 2005).

iii. Copper

Although copper is an essential nutrient for human health, high concentrations in drinking water have been associated with liver cirrhosis, anemia, and damage to the liver and kidneys. Children consuming copper-contaminated water may develop anemia, along with symptoms such as diarrhea, abdominal pain, vomiting, headaches, and nausea (Salem, Eweida, & Farag, 2000; Nolan, 2003).

iv. Zinc

Zinc is a crucial component of various enzymes in the human body, including alkaline phosphatase, superoxide dismutase, alcohol dehydrogenase, and carbonic anhydrase. However, excessive intake can disrupt physiological systems, leading to growth and reproductive issues. Symptoms of zinc toxicity include diarrhea, vomiting, jaundice (yellowing of mucous membranes), bloody urine, anemia, renal failure, and liver failure (Duruibe et al., 2007; Nolan, 2003; INECAR, 2000).

2.11.2 Effect on Soil

Heavy metals have been associated with significant alterations in soil ecosystems, particularly affecting plants that grow in contaminated environments. These pollutants can lead to various detrimental effects, including reduced seed germination, lower lipid content, decreased enzyme activity, and stunted plant growth. Specifically, heavy metals such as cadmium, chromium, copper, mercury, nickel, and lead can inhibit photosynthesis, diminish chlorophyll production, and impede overall plant development (Gardea-Torresdey, Peralta-Videa, Rosa, & Parsons, 2005). High concentrations of heavy metals in soil can further hinder plant growth and disrupt uptake processes, leading to physiological and metabolic dysfunctions. This includes symptoms such as chlorosis, root tip damage, and reduced water and nutrient absorption, as well as impaired enzyme activity (Sardar et al., 2013).

2.11.3 Effect on water body

Additionally, wastewater effluents contaminated with heavy metals can significantly impact the quality of receiving water bodies, with effects varying based on the volume and composition of the discharged effluent (Owyli, 2003; Akpor & Muchie, 2011). In aquatic environments, elevated lead levels can reduce dissolved oxygen availability, making juvenile aquatic species, such as young fish, particularly vulnerable to lead exposure compared to adult fish. Lead poisoning in these young fish can result in tail blackening and spiral malformations (European Commission, 2002).

2.12 Mechanisms of metal resistance and detoxification by plant

Heavy metal contamination in soil leads to the accumulation of these metals in plants. Although heavy metals are toxic to plants, they can resist this toxicity through mechanisms of avoidance and tolerance (Guo, Jianchu, Pingping, Jieyue, Junyu, Shixuan, & Zhiyong, 2019).

In response to heavy metal stress, many plants have developed strategies of avoidance and tolerance. Avoidance strategies include altering heavy metal mobility and influencing microbial activity through root exudates, as well as isolating and compartmentalizing heavy metals within cell walls, cell membranes, and vacuoles (Anjum, Hasanuzzaman, Hossain, Thangavel Roychoudhury. Gill, Rodrigo, Merlos, Fujita, Duarte, Pereira, & Ahmad, 2014; Wang, Shen, Xu, Zhu, Li, Zhang, Xie Gong, & Liu, 2015). The primary aim of these mechanisms is to reduce the amount of heavy metals entering the plant. Additionally, plants have mechanisms to mitigate the toxicity of heavy metals once inside their tissues, such as chelation, (Javed, Akram, Tanwir, Chaudhary, Ali, Stoltz, & Lindberg, 2017).

2.12.1 Root exudates

i. Organic acid

When exposed to heavy metals, plants secrete organic acids such as oxalic acid, citric acid, malic acid, tartaric acid, and succinic acid in increased amounts. These organic acids typically contain one or more carboxyl groups, allowing them to chelate heavy metals and form non-toxic compounds that prevent metal uptake by the plant. Research has shown that organic acids released by the roots of *Phyllostachys pubescens* can enhance soil phosphorus levels (Chen, Shafi, Wang, Wu, Ye, Liu, Zhong, Guo, He, & Liu, 2016). Specifically, phosphate ions can react with lead to form precipitates, effectively cementing the lead in the soil and preventing its absorption by roots. Furthermore, during cadmium stress, cadmium-tolerant rice varieties secrete 1.76 to 2.43 times more organic acids than cadmium-sensitive varieties (Fu, Yu, Li & Zhang, X. 2018). Similarly, cadmium-tolerant pepper varieties exhibit significantly higher concentrations of tartaric acid, oxalic acid, and acetic acid in their roots compared to cadmium-sensitive types (Xin, Huang, Dai, Liu, Zhou, & Liao, 2014).

ii. Amino acid

The secretion of amino acids from plant roots significantly increases in response to heavy metal exposure (Xin et al., 2014). For instance, the levels of methionine, lysine, and histidine in rice roots rise dramatically with increased cadmium (Cd) content (Wang, Kuo, Hong, Chang, & Kao, 2016). These root amino acid secretions serve as nutrient sources for rhizosphere microorganisms, including bacteria, fungi, yeasts, and sulfur bacteria (Wang et al., 2016). In turn, these microorganisms utilize their own secretions and metabolites to help protect plant roots from heavy metal absorption. For example, sulfur bacteria can react with heavy metals to form sulfide precipitates, which inhibit metal entry into the plant (Sallah-Ud-Din, Farid, Saeed, Ali., Rizwan, Tauqeer,& Bukhari , 2017). Additionally, root amino acid secretions can directly chelate heavy metals, thereby mitigating their toxic effects (Xie, Weiss, Weng, Liu, Lu, & Yan, 2013).

iii. Soluble sugar and soluble protein

Under heavy metal stress, plant cells actively uptake certain soluble solutes, including soluble proteins and soluble carbohydrates, to reduce intracellular osmotic potential and support normal physiological functions (Jia, Yang, & Li, 2012). For instance, in ryegrass and Timothy grass, the concentrations of soluble sugars and soluble proteins initially increased with rising cobalt (Co) levels, before subsequently declining (Patade, Bhargava, & Suprasanna, 2012). This indicates that soluble sugar and protein content increases as a response to heavy metal stress.

2.12.2 Subcellular structure

i. Cytoderm

It has functional groups on its surface, including as carboxyl, hydroxyl, amino, and aldehyde, that can be coupled with metal ions to limit heavy metal transmembrane transit. The lateral root cell of the radish accounted for 71.08 percent to 80.40 percent of the total lead in the lateral root under lead stress (Wang et al., 2015). The concentration of zinc in the root cytoderm dropped dramatically after hemicellulose was removed from the root walls of common cabbage, leaf lettuce, pepper, tomato, and rice, whereas the accumulation in the shoot increased. Furthermore, cysteine-rich proteins are found in the cytoderm of rice roots to fix lead. Exogenous administration of nitrogen oxides improves rice and Kandelia cadmium tolerance by increasing hemicellulose and pectin content in the root cytoderm.

ii. Cytomembrane

In plant cytomembranes, various transport proteins are involved in heavy metal transport, including Heavy Metal ATPase (P-ATPase), ATP-Binding Cassette (ABC) transporters, and Cation Diffusion Facilitators (CDF). A study by Sasaki, Yamaji, and Ma (2014) found that overexpressing OsHMA3, a subclass of the HMA family of P-ATPases, enhanced cadmium tolerance in rice roots. In contrast, overexpression of NtHMA3a and NtHMA3b did not improve tobacco's tolerance to mercury, even though ABC transporters are known to play a significant role in the plant's response to toxicity (Chang & Shu, 2015).

2.12.3 Chelation

i. Metallothionein

Metallothionein (MT) is a low molecular weight protein rich in cysteine that binds metals. When plants experience heavy metal stress, it triggers the transcription of mRNA, resulting in the direct synthesis of MT (Cobbett & Goldsbrough, 2002). The transfer of the SaMT3 gene into *Escherichia coli* enhanced the cells' tolerance to copper and lead (Gong, Zhou, Yu, Mao, Zhang, Cheng, & Zhu, 2009).

Phytochelatins (PC) are characterized by large sulfhydryl groups and a strong affinity for heavy metals. They can be produced in corn and wheat in response to exposure to various substances, including cadmium, copper, mercury, lead, zinc, silver, strontium, gold, tin, nickel, arsenic, and selenium. Among these, cadmium, lead, zinc, antimony, silver, mercury, arsenic, copper, tin, gold, and strontium exhibit the strongest binding capacities to PC (Cobbett & Goldsbrough, 2002). Metal ions can form stable chelates with PC, which helps isolate these ions in the vacuole, thereby reducing the concentration of free metal ions within plant cells.

ii. Reduced glutathione

Reduced glutathione (GSH) is a derivative of the amino acids glutamic acid, cysteine, and glycine. It can chelate heavy metals and mitigate their toxicity by functioning as a ligand (Geng, Wang, Wu, Wang, Wu, Yang, Chen, Wen, & Liu, 2018). Exogenous GSH has been shown to enhance the synthesis of phytochelatins in *Dianthus chinensis* and poplar seedlings, forming a non-toxic chelate with cadmium that reduces toxicity in plants (Ding, Ma, Shi, Liu, Lu, Liu, & Luo, 2017).

2.13 Constructed wetlands (CWS)

Natural wetlands are among the most biologically diverse ecosystems, playing crucial roles such as nutrient storage and recycling, providing habitats for wildlife, stabilizing shorelines, managing and buffering natural floods, recharging groundwater, and treating pollutants in water. Constructed wetlands (CWs) are artificial systems designed to replicate the functions of natural wetlands in specific areas, allowing for management and manipulation to achieve desired outcomes. CWs offer a cost-effective alternative for wastewater treatment, effectively improving water quality through various physical, chemical, and biological processes. While natural wetlands have been utilized for water filtration for thousands of years, dating back to ancient Chinese and Egyptian practices, the first artificial wetland was established in Australia in 1904. Currently, the primary use of CWs is for river flow management (Wu & Hu, 2007), with limited research conducted on their application for treating contaminated river water (Jing, Lin, Lee, & Wang, 2001; He, Yan, Kong, Liu, Wu, & Hu, 2007; Zhou & Hosomi, 2008).

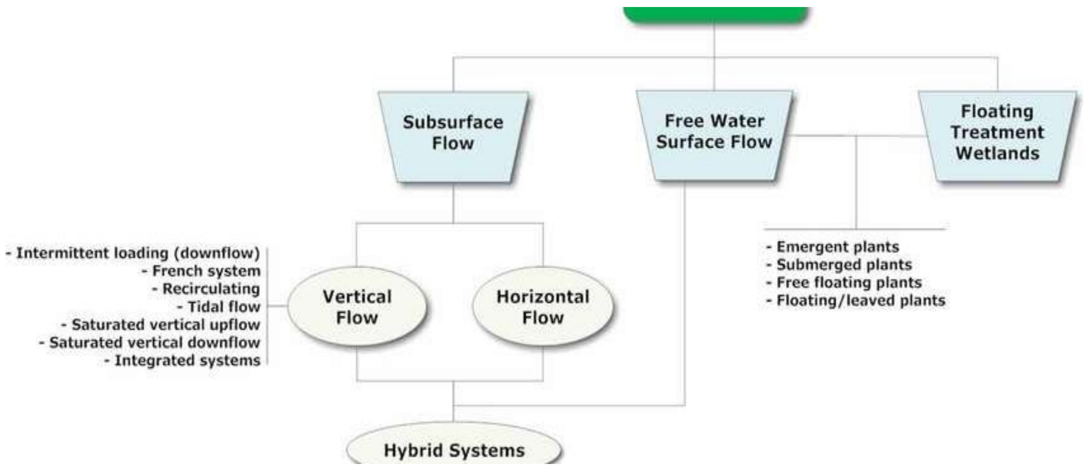

Figure 2.4: Classification of constructed wetlands

Source: Stefanakis, (2017)

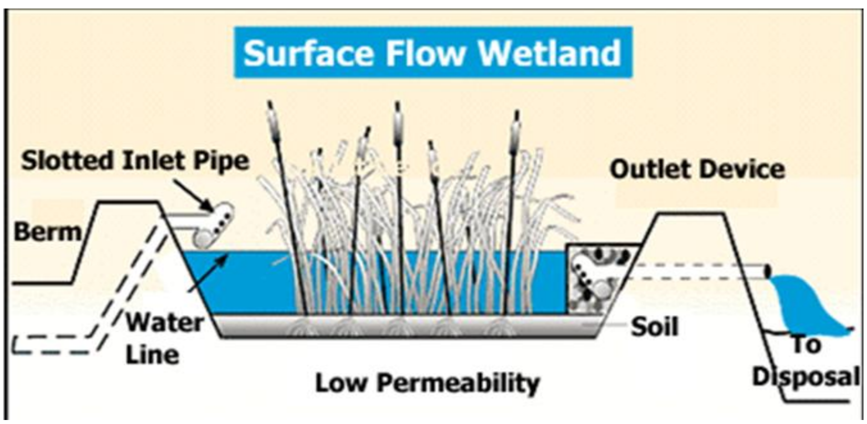

Figure 2.5: Surface flow wetland

Source: https://www.mdpi.com/2073-445X/11/2/174

Figure 2.6: Surface flow constructed wetlands with *Eichhornia crassipes* (water hyacinth) in Langtou near Guangzhou, China.
Source: Vymazal, 2022

2.13.1 Brief History and development of constructed wetlands

Constructed wetlands (CWs) for wastewater treatment are engineered systems designed to harness natural processes for the removal of pollutants from wastewater. While CWs utilize many of the same mechanisms found in natural wetlands, they do so within a more controlled environment (Vymazal, 2022).

The first documented designed treatment wetland system was patented in 1901, as noted by Wallace and Knight (2006). This system was a standard vertical flow constructed wetland (CW), but there is no record of its subsequent development. The concept of built wetland treatment systems did not reemerge in Germany until the early 1950s, primarily due to the work of K. Seidel (Vymazal, 2022). In the early 1960s, Seidel conducted experiments with macrophytes to improve the efficiency of rural wastewater treatment systems, such as septic and Imhoff tanks. She utilized highly permeable substrates in modified basins planted with various macrophytes. The initial stage of her design was vertical and aerobic to enhance the oxygenation of septic effluent, while the second stage was horizontal. In the mid-1960s, her collaboration with R. Kickuth resulted in the development of a horizontal flow CW known as the "Root Zone Method." In this system, *Phragmites australis* was planted in a filtration bed filled with heavy clay-containing soil. The first full-scale horizontal flow CW was established in Liebenburg-Othfresen in 1974 for the treatment of municipal wastewater. Although much of the research on constructed treatment wetlands in the 1960s concentrated on subsurface systems, full-scale surface (free water surface) constructed wetlands were also developed in the Netherlands.

In the 1970s, research on artificial wetlands for wastewater treatment in Europe was predominantly focused on the Root Zone Method, with most studies taking place in Germany. Meanwhile, in the United States, the emphasis shifted towards surface flow constructed wetlands, although subsurface flow technologies were also explored. Notable large-scale surface flow projects included constructed wetlands designed for refinery wastewaters in North Dakota and municipal wastewaters in the cold climate of Michigan (Kadlec, 2009).

The 1980s and 1990s marked a significant period for the rapid expansion of engineered wetlands for wastewater treatment worldwide. Initially, the technology spread gradually, primarily through personal sharing of experiences until the early 1980s. However, in the late 1980s and early 1990s, a series of international conferences dedicated to this technology were organized in Europe, Asia, Australia, and both North and South America, facilitating its broader adoption and dissemination.

By the end of the 20th century, constructed wetland technology had proliferated across all continents, encompassing a variety of constructed wetland types. Research in the early 21st century focused on various design and operational features that could enhance pollutant removal efficiency (Vymazal, 2021; Wu, Kuschk, Brix, Vymazal, & Dong, 2014). Key areas of investigation included:

- Operating techniques: This encompasses methods such as aeration, microbial fuel cells, and bioaugmentation.
- Electron donors: Identifying suitable electron donors to assist in the removal of specific inorganic anions.

- Filter materials: Selecting materials with enhanced sorption capacity and facilitating microbial biofilm formation.
- Bacterial activity: Studying the roles of different bacterial groups in pollution removal.
- Macrophyte selection: Choosing plant species that can improve pollutant removal.
- Greenhouse gas emissions: Evaluating how constructed wetlands impact greenhouse gas emissions.
- Pharmaceuticals and personal care products: Assessing the effectiveness of constructed wetlands in removing these contaminants.

In the context of sustainable water management in urban areas, constructed wetlands are gaining attention as part of the circular economy and "sponge" city concept. According to Masi, Rizzo, and Regelsberger (2018), constructed wetlands can effectively treat sewage, greywater, stormwater runoff, and overflow, while also facilitating water recycling within urban settings. Additionally, they play a significant role in Sustainable Urban Drainage Systems (SUDS). Stefanakis (2019) further highlighted the potential of artificial wetlands for wastewater treatment, noting their promising integration into urban and peri-urban environments, aligning seamlessly with the concepts of sponge cities and the circular economy.

2.13.2 Types of constructed wetlands

Fonder and Headley (2010) provided the most comprehensive classification of constructed treatment wetlands, identifying three key characteristics of these systems: the presence of macrophytes, water-logged or saturated substrate conditions for at least part of the time, and the inflow of contaminated water containing constituents to be removed. Constructed wetlands can be categorized into surface flow CWs (also known as free water surface CWs) and subsurface flow

CWs, based on the primary location of water within the system. Within subsurface flow systems, the flow direction further classifies them into horizontal or vertical types.

2. 13.3 Surface flow wetlands free or water surface constructed wetlands (FWS CWS)

A surface flow (SF) wetland consists of a shallow basin, a substrate (such as soil or another medium) to support the roots of vegetation, and a water control device to maintain a shallow depth of water (see Figure 5). In these systems, the water surface is higher than the substrate. SF wetlands resemble natural marshes, offering habitat for wildlife, aesthetic benefits, and effective water treatment. The near-surface layer of SF wetlands is typically aerobic, while the deeper waters and substrate are usually anaerobic. These wetlands are commonly used for stormwater management, as well as for remediating mine drainage and agricultural runoff. SF wetlands are also referred to as free water surface wetlands or aerobic wetlands when utilized for mine drainage.

The advantages of SF wetlands include low capital and operational costs, along with ease of installation, management, and maintenance. However, a notable drawback is that SF systems typically require a larger land area compared to other wetland systems.

2.13.4 CWs with Free-Floating Macrophytes

Free-floating macrophytes vary significantly in size and habitat, ranging from large plants with extensive leaves and roots, such as *Eichhornia crassipes* (water hyacinth; see Figure 1), to tiny plants with microscopic roots, like members of the *Lemnaceae* family (duckweeds, including *Lemna* spp., *Sporadela polyrhiza*, and *Wolffia* spp.) (Osti, Henares, & Camargo, 2018). These free-floating plants are highly prolific and are among the fastest-growing plants globally. While frost-sensitive species like *E. crassipes* and *Pistia stratiotes* are limited to tropical and subtropical regions and do not thrive in temperate or cold climates, *Lemnaceae* (see Figure 6) have a much broader geographic distribution due to their ability to withstand mild cold (Kumar & Dutta, 2019).

In the late 1970s and early 1980s, extensive research on constructed wetlands featuring free-floating macrophytes revealed that these systems had high operating and maintenance costs. Additionally, the frequent need for plant harvesting and disposal limited their widespread use (Ozimek & Czupry, Nski, 2003). Due to their ability to be easily dispersed by wind or birds, duckweed can naturally establish itself in all types of surface flow artificial wetlands

2.13.5 CW with submerged macrophytes

The roots of submerged macrophytes (see Figure 5) are anchored in the substrate, with the entire plant submerged in water. While these plants primarily obtain nutrients from the sediments, research has indicated that some species can also absorb nutrients directly from the water column (Kochi, Freitas, Maranho, Juneau, & Gomes, 2020).). Submerged macrophytes thrive only in well-oxygenated environments with low concentrations of suspended solids, as high levels can hinder the penetration of photosynthetically active radiation (PAR), which is essential for effective photosynthesis. Therefore, submerged macrophytes are recommended for use in artificial wetlands designed for tertiary treatment (Jiang, Wang, He, ZhuYang, Fang, & Yang, 2024).

2.13.6 CWs with emergent macrophytes

A typical surface flow constructed wetland (CW) with emergent macrophytes (see Figure 9) consists of a shallow basin or a series of basins featuring 20–30 cm of rooting soil and a water depth ranging from 10 to 60 cm, along with a dense growth of macrophytes (Figure 9). The most commonly used plants in this type of CW include *Phragmites australis* (common reed), *Typha* spp. (cattails), and *Scirpus/Schoenoplectus* spp. (bulrushes) (Vymazal, 2013). The configuration of plant stalks and litter, combined with the shallow water depth and low flow velocity, regulates water flow and maintains plug-flow conditions, particularly in long, narrow channels.

Most surface flow constructed wetlands feature aerated zones, particularly near the water surface, due to air diffusion and the oxygen produced by the photosynthetic activities of algae and cyanobacteria. Anoxic and even anaerobic conditions may develop at the bottom, especially within the layer of decomposing plant material.

.2.13.7 CWs with floating mats of emergent macrophytes

This represents the latest evolution in constructed wetland (CW) technology, integrating a traditional CW system with a pond (Van de Moortel, Meers, De Pauw, & Tack, 2010). These systems feature a floating platform, typically made of plastic, on which plants are anchored, giving them the appearance of floating islands from a distance. Similar to other types of CWs, the plants develop deep and extensive root systems within the underlying water column (Stefanakis et al., 2014; Tanner & Headley, 2011). The design allows the system to remain unaffected by fluctuations in water levels, as the porous plastic base and the plants float on the water surface. Floating treatment wetlands (FTWs) have been effectively used to purify water in rivers, channels, lakes, and other aquatic environments, as well as for treating runoff, residential, and municipal wastewater (Van de Moortel et al., 2010; Stefanakis et al., 2014).

2.13.8 CWs with trees

The use of constructed wetlands with trees for wastewater treatment is uncommon; yet, there are several excellent instances of such treatment wetlands. *Taxodium distichum* (bald cypress) *Melaleuca quinquenervia* (paper bark tea tree) or mangroves, which may be utilized to treat salty (waste)waters (Huang, Yuan, Yang, & Yang, 2019), were the tree species employed in created wetlands.

2.13.9 Horizontal subsurface flow constructed wetlands (HSF CWs)

Constructed wetlands with a subsurface flow can be divided into horizontal (HF CWs) and vertical (V CWs) categories based on the flow direction (VF CWs). The HF CWs are fed constantly, whereas the VF CWs are supplied on a more irregular basis. In HF CWs, the feeding mode provides anoxic/anaerobic redox conditions in the filter media, whereas in VF CWs, the redox conditions are aerobic (Vymazal, 2022).In Europe, HSF CWs are more widely utilized than in the United States (Stefanakis & Tsihrintzis, 2009a). Basins with gravel material are generally planted with common reeds (*Phragmites australis*) or other species like Typha (*latifolia, angustifolia*) and Scirpus (e.g., *lacustris, californicus*) (Vymazal, 2011). Rocks of various origins and compositions are used as the substrate. Unlike FWS CWs, there is no water surface exposed to the atmosphere in this CW type; the water level is kept 5-10 cm below the surface of the gravel layer, and water runs via the pores of the substrate media, coming into touch with the media grains, plant roots, and associated biofilm (Stefanakis et al., 2014). As a result, in this CW type, the health hazards associated with probable human contact with the wastewater and mosquito difficulties are minimal (Kadlec & Wallace, 2009). The thickness of the substrate layer varies from 30 to 80 cm (Akratos & Tsihrintzis, 2007; Kadlec & Wallace, 2009).

2.13.10 Horizontal Flow Constructed Wetlands

Mechanically processed wastewater runs slowly under the surface of a porous filter bed seeded with emergent macrophytes in HF CWs (Figures 14 and 15). The wastewater passes through a network of aerobic, anoxic, and anaerobic zones as it passes through the filter material. The aerobic zones are confined to a small area proximal to the roots and rhizomes, which leak oxygen into the ground (Vymazal, 2001).

To avoid leaking into the groundwater, the filter bed is separated from the surrounding area by an impermeable barrier; in most cases, a plastic liner. Swiveling elbows or flexible hoses, or plastic pipes that may be kept in place by a chain, are used to keep the water level in the filter bed in the outflow sump.

The bed of a constructed wetland is typically lined with an impermeable geo-membrane and sloped (1-3%) to facilitate water flow. In Free Water Surface (FWS) constructed wetlands, achieving uniform wastewater distribution across the width at the inflow point is essential for optimal system function (Akratos & Tsihrintzis, 2007). Additionally, implementing step-feeding of wastewater along the length of the wetland has been shown to enhance performance (Akratos & Tsihrintzis, 2007; Stefanakis, Akratos, and Tsihrintzis, 2011a). Compared to FWS constructed wetlands, Horizontal Subsurface Flow (HSF) systems require less space but may incur higher initial costs (Kadlec & Wallace, 2009; Stefanakis et al., 2014).

This type of constructed wetland (CW) has proven highly effective for treating municipal wastewater, achieving significant removal of suspended solids (SS) and organic matter (BOD5), while offering moderate removal of nutrients such as nitrogen and phosphorus (Akratos & Tsihrintzis, 2007; Vymazal, 2007; Kadlec & Wallace, 2009; Stefanakis et al., 2014). Several strategies have been proposed to enhance system performance, including effluent recirculation (Stefanakis & Tsihrintzis, 2009a), step-feeding of wastewater (Stefanakis et al., 2011a), raising water levels (Stefanakis & Tsihrintzis, 2009a), and using zeolite gravity filters for effluent treatment (Stefanakis, Akratos, Gikas, & Tsihrintzis, 2009a). Horizontal Subsurface Flow (HSF) CWs have also been applied to treat various industrial wastewaters, including mine drainage, dairy, swine, olive mill effluents, landfill leachate, cork processing effluent, contaminated groundwater, hydrocarbons, and more (Vymazal, 2009).

2.13.11 Vertical Flow constructed wetlands (VFCWs)

Flow in the vertical direction

Constructed wetlands are typically made out of a porous material bed through which water flows vertically. This category of CWs encompasses a wide range of hydrologic properties. Vertical subsurface flow built wetlands may be divided into three types: down flow, up flow, and fill and drain (Fonder, & Headley, 2010).

The most common type of vertical flow constructed wetland (CW) is the free-drainage downflow unit, where the outlet is located at the base of the filter bed. Wastewater is intermittently supplied in batches to the surface of the filter bed, with a new batch introduced only after the previous one has fully percolated through the bed. This intermittent supply allows for air to disperse through the empty bed, making the filter primarily aerobic. To prevent short-circuiting, the wastewater is evenly distributed across the filter surface using a network of pipes with multiple diffusers. The influent distribution pipes can be positioned on or above the filter bed's surface, buried within the coarse filter material, or placed behind an insulating mulch layer in colder climates (Fonder & Headley, 2010). This technique was developed by Dr. Seidel in Germany in the mid-1960s, originally to oxidize the anaerobic effluent from a septic tank.

2.13.12 Free drain down flow cws

Free drain downflow constructed wetlands (CWs) require less surface area compared to horizontal flow (HF) CWs, with a typical requirement of 4 m^2 per population equivalent, as specified in Standard DWA-A 262A (2018) and ÖNORM B 2505 (2009). In France, downflow vertical flow (VF) systems are used for treating raw sewage through a two-stage VF approach, known as the "French system." The first stage focuses on partial removal of organic matter and nitrification while also treating sludge. The second stage further removes organics and enhances nitrification. This system is designed with an area of 1.2 m^2 per population equivalent for the first stage and 0.8 m^2 for the second stage (Molle, Boutin, Merlin, & Iwema, 2005).

2.13.13 Vertical up flow

The up flow system is the second most common form of vertical subsurface flow system. The wastewater is dispersed to the bottom of the filter and then travels up to the filtration bed surface in this system. The outflow might be below or above the surface of the bed. Upflow vertical built wetlands were first used in Brazil in the 1980s as "filtering soil". Due to the saturation of the filter bed, this system is employed considerably less often than down flow systems and, in general, offers the similar treatment conditions as horizontal flow CWs.

Combining the advantages of different systems, various types of artificial wetlands can achieve higher treatment efficiency. Most hybrid constructed wetlands incorporate both vertical and horizontal filtration stages. The vertical-horizontal filter system was first developed in the late 1950s and early 1960s, though hybrid systems were initially adopted in only a few cases. In the 1980s, hybrid artificial wetlands were introduced in France and the United Kingdom, and today, they are in use in many countries worldwide. While hybrid constructed wetlands require expert design, they can largely be built using locally available materials, with communities trained for operation and maintenance. The treated effluent can be safely discharged into receiving water bodies or reused for purposes like irrigation and aquaculture.

2.13.14 Advantages of Constructed Wetlands

1. CWs are generally straightforward to construct and run, and they provide treatment that is resistant to flow changes and pollutant concentrations. They look to be a perfect solution, particularly for small communities and settlements, as well as for onsite treatment, where the current need for effective wastewater treatment is great. General effluent discharge standards (e.g., BOD5 30 mg/L, COD 100 mg/L, total nitrogen 15 mg/L) can be met with CWs plants, and even higher-quality effluents can be produced (Kadlec & Wallace, 2009; Stefanakis, et al., 2014).
2. Although traditional treatment methods have been providing successful wastewater treatment for decades, they do have some drawbacks. Because these facilities have an industrial aspect and are unpleasant, they are typically located distant from residential areas. Large and complicated mechanical equipment, including as aeration ventilators and pumps, are used extensively in centralized facilities, while non-renewable resources (concrete and steel) are used extensively in the construction of basins, tanks, and

equipment. This means that, despite the fact that the goal is to be environmentally friendly, the building of such facilities might be costly and non-environmentally friendly (wastewater treatment).

3. Pumps are the sole mechanical equipment used to carry water from one stage to the next, which can be avoided with correct design and use of the installation area's topography. Furthermore, typical treatment facilities are energy-intensive devices that require a significant amount of energy to operate.
4. Facilities with a lot of complicated mechanical parts and equipment may have a lot of maintenance requirements. As a result, such facilities' operating costs are similarly substantial.
5. On the contrary, the principal advantage of CW plants is likely to be their low operational costs. The energy input required in CWs is very low, typically merely for the pumps that may be present in the facility. Renewable energy sources are employed by plants to meet the energy requirements for the treatment processes (solar, wind energy). At the same time, maintenance requirements are modest because the operation of a CW facility is virtually self-contained; at full operation, a typical maintenance scheme is one monthly visit to the site.
6. In contrast to conventional facilities, where specialized people is required, the CW facility may require significantly fewer skilled workers. Operating costs of CW facilities can be up to 90% lower than conventional plants, according to global experience from numerous countries and professionals. Furthermore, unlike conventional treatment plants, CWs do not require the addition of any chemical ingredients to function.

7. Furthermore, there are no by-products from the process in CWs. Within the system, produced sludge accumulates. Conventional treatment plants, on the other hand, produce significant amounts of extra sludge on a regular basis. In order to be further used, this extra sludge must be handled, dewatered, and stabilized. Despite the fact that sludge accounts for just a tiny percentage of total treated volume (1-3%), its management and processing can account for up to 50% of total facility expenditures (Stefanakis et al., 2014).

2.13.15 Disadvantages of Constructed Wetlands

Despite the many advantages of constructed wetlands (CWs), there are certain drawbacks that must be considered. A key challenge is that CWs require a larger land area compared to traditional treatment facilities. Although ongoing research aims to reduce the footprint of CW systems and improve their design—such as through the use of vertical flow systems and, more recently, aerated systems—CWs still require significantly more space (approximately 3 to 10 times more) than conventional systems (Stefanakis et al., 2014).

Additionally, poor design can lead to issues such as odor problems and the emergence of a water surface in subsurface systems. However, it's important to note that when CWs are properly designed and constructed, they do not produce odors. Since wetland technology is still relatively new, CW systems are sometimes perceived as a "black box," with simple regression equations often used for design purposes (Button, Nivala, Weber, Aubron, & Müller, 2014).

However, it is widely recognized that the majority of pollutant removal processes in CWs rely on microbial activity (Faulwetter et al., 2009). This suggests that a deeper understanding of the fundamental processes occurring within these systems is essential for achieving optimal design and performance (Stefanakis et al., 2014). As a result, CW design continues to rely heavily on empirical data and professional expertise, contributing to the lack of universally accepted design guidelines for CW systems.

Optimal performance is typically reached after one or two years of operation, once the plants are fully established, though seasonal variations in efficiency may occur. It is worth noting that the scientific community has been actively addressing these challenges, and increased research over the past 10-15 years has helped mitigate many of these issues, with further improvements anticipated.

2.13.16 The importance of plants in CWs environment

In constructed wetlands (CWs), the growing biomass, particularly aquatic macrophytes, plays a crucial role in interacting with the CW environment to remove pollutants and purify wastewater. The aerial parts of these plants, including roots, stems, and leaves, provide substrates and attachment sites for metabolically active microbial communities within CW systems. In subsurface flow constructed wetlands (SSF-CWs), especially in horizontal subsurface flow systems (HSSF-CWs), the roots of emergent macrophytes facilitate oxygen transfer to the rhizosphere through diffusion. This is particularly important in HSSF-CWs, where oxygen levels are often limited, which can constrain key oxidative processes such as nitrification and nutrient transformations (Sun, Xu, Wu, Wang, Zhuang, G. & Bai, 2019).

Constructed wetland (CW) plants can transfer between 5 to 45 g of O_2 per day per m², depending on oxygen demand and plant density (Bia, Davies-Albuquerque, & Randerson, 2012; Sun et al., 2019). These plants offer protection by providing insulation against freezing in winter, preventing substrate clogging—especially in vertical subsurface flow CWs (VSSF-CW)—and ensuring bed stability. They also create optimal conditions for physical processes like filtration, regulate hydrodynamic properties such as flow rate, support wildlife, and enhance the aesthetic appeal of CW systems. When selecting plant species for CWs, criteria such as resilience to harsh climates and tolerance to eutrophic, acidic, hypoxic, and waterlogged conditions are important considerations (Wu, Zhang, Hao, Guo, Hu, Liang & Fan, 2015). Plants contribute to contaminant treatment through processes like absorption, bio-immobilization, and biosorption. The dynamic role of vegetation highlights the significance of phytoremediation (botanical bioremediation) in removing heavy metals from CW ecosystems (Zhu, Huang, Ho, Wang, L. & Yang, 2017).

Figure 2.7. Constructed wetland (Ironbridge, Florida) planted
with *Nuphar lutea* (spatterdock), designed for tertiary treatment
of 800,000 PE in Orlando, Florida.
Source: Vymazal, 2022

Figure 2:8. Surface flow CW with submerged macrophytes (mostly *Myriophyllum spicatum*, water milfoil) in Montréal, Canada.

Source: Vymazal, 2022

Figure 2.9. Surface flow CW with emergent plants (*Eleocharis sphacelata*, tall spikerush). Otorohanga, New Zealand.

Source: Vymazal, 2022

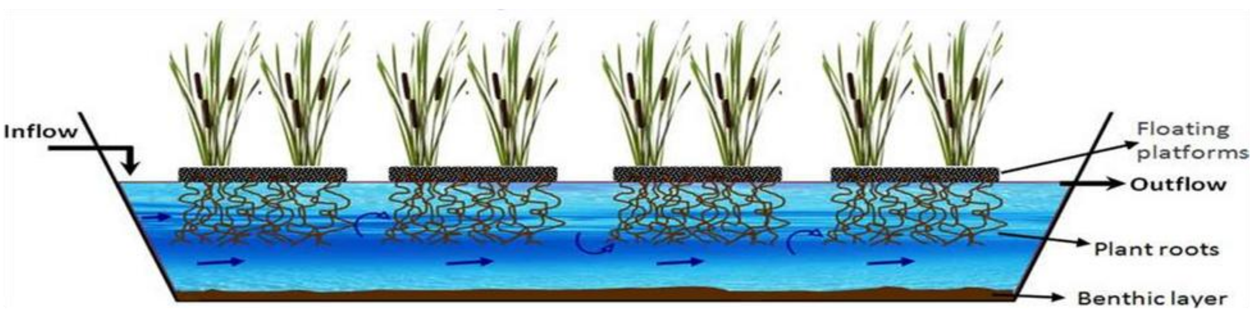

Figure 2.10: Schematic representation of floating treatment wetlands.

Source: Stefanakis, 2016

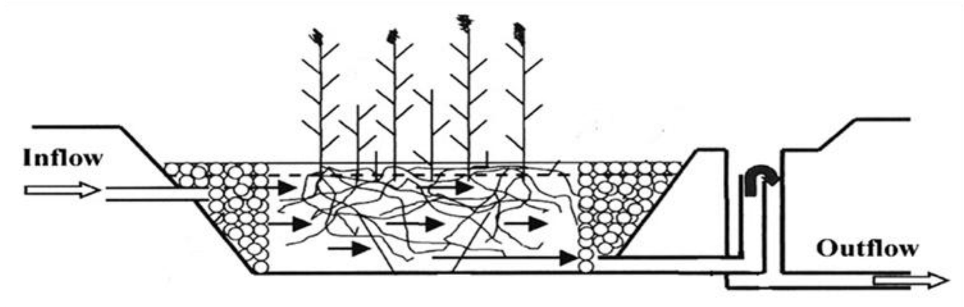

Figure 2.11: horizontal flow constructed wetland

Source: Rajitha et al., (2019)

Figure 2.12: Distribution zone filled with large stones and the filtration bed filled with crushed rocks (4–8 mm) before planting. HF CWCˇ ejkovice, Czech Republic.

Source: Vymazal, 2022

Figure 2.13: Constructed wetland with a horizontal subsurface flow. Roseˇc, Czech Republic.

Source: Vymazal, 2022

Figure 2.14. Horizontal subsurface flow CW, Freethorpe, United Kingdom, for 900 PE. The early CWs were built as a single bed.

Source: Vymazal, 2022

Figure 2.15. Surface flow CW at Ironbridge. Tertiary treatment for the city of Orlando, Florida.

Source: Vymazal, 2022

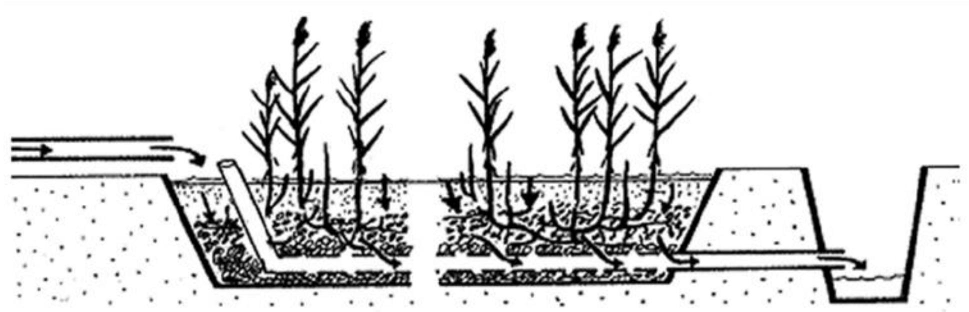

Figure 2.16: vertical flow constructed wetland

Source: Rajitha *et al.*, (2019)

2.14. Sustainable character of constructed wetlands

Based on the above discussion, it is evident that constructed wetland (CW) systems possess various properties that enhance their ecological value as a treatment approach. Often regarded as long-term solutions, CWs are characterized by low energy consumption and the use of natural materials such as gravel, soil, sand, and plants. One of the largest initiatives employing wetland technology is the Everglades restoration project, which utilizes CWs to filter nutrients from agricultural runoff into the Everglades (Wu, Zhang, Ngo, Guo, Hu, Liang, S,FanJ.,& Liu, 2015).

2.14 .1 Health

Health considerations are essential as they influence the risk associated with exposure to pathogenic microorganisms and hazardous chemicals. Constructed wetland (CW) systems have demonstrated effectiveness in pathogen removal (Kadlec & Wallace, 2009; Stefanakis et al., 2014). In vertical flow constructed wetlands (VFCWs), elimination rates can reach up to 99.99 percent (Stefanakis et al., 2014), while high removal rates in free water surface (FWS) constructed wetlands are attributed to direct UV radiation and disinfection (Kadlec & Wallace, 2009).

2.14 .2 Environmental protection

Environmental protection and the preservation of natural resources are key priorities in constructed wetland (CW) systems. By ensuring effective treatment and high removal efficiency, these systems minimize the pollution that reaches final recipients, such as surface and groundwater bodies, thereby reducing the risk of degradation to ecosystems and aquatic life.

2.14.3 Energy viability

Minimizing energy consumption in constructed wetland (CW) facilities conserves natural resources and decreases pollution, particularly when the energy source is non-renewable, such as fossil fuels. This approach contributes to lower greenhouse gas emissions (CO_2, CH_4, and N_2O), enhancing the overall environmental benefits of CW systems (Langergraber, 2013; Stefanakis et al., 2014).

2.14.4 Ecological friendly

Assessing the ecological impact and global warming potential of various wastewater treatment methods requires quantifying gas emissions throughout both the construction and operational phases. Research indicates that constructed wetland (CW) systems have a slightly lower environmental impact during construction compared to conventional technologies like activated sludge and trickling filters, but show a significantly reduced impact during operation (Georges, Thornton, & Sadler, 2009).

2.14.5 Cost effective

The cost of a treatment method is a crucial factor in assessing its sustainability. During the construction phase of constructed wetlands (CWs), key expenses include earthworks (such as basin excavation and fill), filter media used as substrates, plants, and the bottom liner (e.g., geomembrane/geotextile) (Kadlec & Wallace, 2009; Stefanakis et al., 2014).

Labor costs can vary significantly from one country to another. Generally, CW facilities are comparable in cost or somewhat cheaper to construct than traditional treatment plants (Langergraber, 2013; Stefanakis et al., 2014). However, the most significant economic advantage of CWs lies in their substantially lower operating and maintenance costs, which result from reduced energy consumption and less reliance on technical equipment (Brix, 1999; Dixon et al., 2003; Langergraber, 2013; Stefanakis et al., 2014).

2.14.6 Public acceptibity

Constructed wetland (CW) facilities are often more favorably received by the public due to their green, aesthetically pleasing design compared to conventional treatment plants. Many organizations, including industries and municipal-private partnerships, opt for CW technology to treat wastewater generated on-site. This choice enhances their environmental profile and aligns with their corporate social responsibility strategies by promoting sustainable practices.

2.15. Removal of oil from water using constructed wetlands

For many years, experts have grappled with the challenge of removing oil from water. However, recent scientific advancements have made it possible to address long-standing issues. By combining various types of root-colonizing endophytic bacteria, phytoremediation has demonstrated potential in eliminating oil content and hydrocarbons from soils (Olson, Ford, Smith, & Fernandez, 2004; Pilon-Smits, 2005; Dzantor, 2007).

Constructed wetlands have proven effective in removing not only oils but also pesticides and heavy metals (such as copper, zinc, and nickel), as well as various organic and inorganic wastes from both soil and water bodies. Phytoremediation has recently been implemented in Pakistan to address oil contamination from industrial discharges of diesel fuels and crude oil, leveraging the plant-microbe interactions present in constructed wetlands (Afza et al., 2012; Afza et al., 2013; Fatima, Afzal, Imran, & Khan, 2015).

Researchers in China (Ji, Sun, & Ni, 2007), the United States (Murray-Gulde, Heatley, Karanfil, Rodgers & Myers, 2003), Malaysia (Darajeh, Idris, Masoumi, Nourani, Truong, & Sairi, 2016), and other countries have successfully utilized constructed wetlands to remove oil from water. In China, surface flow constructed wetlands have shown remarkable potential in reducing mineral oil content in water by promoting the growth of various oxygen-loving bacteria due to the oxygen generated near plant roots (Ji et al., 2007). Additionally, researchers at the National Institute for Biotechnology and Genetic Engineering (NIBGE) in Faisalabad, Pakistan, have effectively removed diesel oil from contaminated soils.

Afzal (2013) demonstrated that using ryegrass plants in combination with the plant growth-promoting bacterial strain Burkholderia phytofirmans PsJN can significantly enhance the growth of ryegrass in soils contaminated with various pollutants, facilitating the degradation of these contaminants (Afzal et al., 2013).

In the United States, a specific type of constructed wetland system known as reverse osmosis constructed wetlands has been employed to remove salt from water for irrigation purposes. Similarly, in Malaysia, a novel phytoremediation device called the floating Vetiver system has been utilized to treat palm oil mill effluents Darajeh et al., 2016). Darajeh et al., (2016) used Vetiver grass as the plant source for the removal of palm oil effluents, capitalizing on its superior ability to absorb and break down water pollutants (Truong, 2008). In Singapore, subsurface flow constructed wetlands have been used to treat effluents from wastewater.

2.16. Heavy metal uptake by wetlands plants and micro-organisms

2.16.1 Plants

Wetlands are defined by Amler, Schmidt, & Menz (2015) as areas where water remains long enough for plants and animals to adapt to saturated conditions. Mahdavi, Salehi, Granger, Amani, Brisco, & Huang (2018) categorized wetland plants into four groups: emergent, surface floating, rooted leaves, and submerged macrophytes. According to Tiner (2016) and Mahdavi et al. (2018), emergent and surface-floating plants absorb heavy metals through their roots, while euhydrophytes (plants with completely submerged leaves or both floating and submerged leaves) absorb heavy metals through both their leaves and roots.

Mahdavi et al. (2018) also observed that rooted floating-leaved taxa depend more on their roots for heavy metal absorption, whereas submerged taxa have a lesser reliance on roots. As plants become more submerged and their shoot structure simplifies, the tendency to utilize shoots for heavy metal absorption increases. Submerged rooted plants can collect metals from both water and sediments, while rootless plants can only extract metals from water. In cases of heavy metal foliar absorption, passive aqueous phase transfer occurs through cuticle fissures or stomata to the cell wall and ultimately the plasmalemma (Peralta-Videa, Lopez, Narayan, Saupe, & Gardea-Torresdey, 2009).

High concentrations of copper were identified in active growth areas, such as stem apices and immature leaves, which act as copper sinks. Copper is an essential trace element in photosynthesis, particularly within the biochemical processes of photosystem I and cytochrome, further indicating copper translocation in plants (Peralta-Videa et al., 2009). The researchers concluded that heavy metals are absorbed and translocated by plants before being excreted.

This phenomenon explains why wetland plants can accumulate heavy metals in their tissues at concentrations up to 200,000 times greater than those found in their surrounding environment (Hu, Niu, & Chen, 2017; Schreck, Xiong, & Niazi, 2017; Shahid, Dumat, Khalid, S., Schreck, Xiong, & Niazi, (2017.). These findings align with those of Peralta-Videa et al. (2009), who reported that metals accumulated in aquatic plants at levels exceeding those in the external medium under experimental conditions. Myriophyllum spicatum has been shown to absorb mercury when grown in sediments containing organic or inorganic mercury compounds (Shahid et al., 2017).

Shahid et al. (2017) summarized the metal absorption process in the shoots and leaves of submerged plants as follows: (I) The Apparent Free Space (AFS), comprising Water Free Space (WFS) and Donnaan Free Space (DFS), allows for passive ion penetration (primarily cation exchange) into the peripheral area (DFS). (ii) Active ion absorption occurs in the cytoplasm, including autonomous mobility of various ions. (iii) Active secretion of ions takes place from the cytoplasm into the vacuole. (iv) Ion translocation occurs in the symplasm, an active mechanism that transports ions from cell to cell via plasmodesmata in the cytoplasm.

Peralta-Videa et al. (2009) further demonstrated that a natural papyrus wetland between Lake George, Uganda, and the river carrying heavy metals from cobalt tailings upstream at the Kilembe mines effectively prevented heavy metals from reaching the lake. Consequently, this protection helped avoid heavy metal accumulation in the biota through the food web, safeguarding the lake's fisheries. Heavy metals were retained in the wetland's sediments, water, and vegetation.

While heavy metals are predominantly absorbed by sediments, macrophytes can also absorb these metals through their roots and shoots. During the growth season, macrophyte communities can accumulate a substantial metal burden, which is released when the plants reach senescence and die. Some macrophytes can tolerate high metal concentrations in their body mass without suffering growth issues. According to Tangahu, Sheikh, Abdullah, Basri, Idris, Anuar, and Mukhlisin (2011), species such as *Typha* and *Schenoplectus* exhibit greater tolerance than others. Although the mechanisms underlying metal tolerance and absorption are not fully understood, sediment chemistry—specifically pH, redox potential, and organic matter—has been found to influence the entire process.

2.16.2 Phytoplankton

Bastian and Hammer, (2020) discovered that phytoplankton significantly influences heavy metal dynamics in wetlands. For instance, zinc absorption by cyanobacteria reduced concentrations from 21 to 8 mg Zn/l in a 15-meter area (Rauf, Javed, & Jabeen, 2019). Under alkaline conditions, algae have the capacity to accumulate uranium (Ur), zinc (Zn), copper (Cu), nickel (Ni), and radium-226 (Ra-226) in their tissues (Rauf, Javed, & Jabeen, 2019).

2.16.3 Microorganisms

Microorganisms eliminate heavy metals from wetlands through two primary mechanisms. The first mechanism involves a metabolism-dependent uptake of metals into their cells at low concentrations, as some toxic metal ions serve as micronutrients for the microbes. The second mechanism is bio-sorption, a passive adsorption process that binds metal ions to extracellular charged materials or cell walls.

Hydrophilic heavy metal ions are thought to traverse the hydrophobic region of a biomembrane through a "shuttle" process known as facilitated diffusion (or host-mediated transport) in microorganisms. In this process, a receptor molecule, such as a protein on the outer membrane surface, binds to a metal ion (Chaalal, Zekri, & Islam, 2005).

The metal ion is then released into the cytosol, where it is likely retained through interaction with a thiol molecule, as the hydrophilic metal-receptor complex diffuses across the membrane. The receptor subsequently diffuses back to the opposite surface of the membrane, where it can bind to another metal ion. However, if the metal complex is lipid-soluble, a much faster process of direct diffusion can occur. This direct diffusion differs from assisted diffusion not only in its speed but also in the fact that the ligand is delivered directly into the cytosol (Chaalal, et al., 2005).

2.16.4 Fauna

Heavy metals are eliminated through the stomach or detoxified in the liver, kidney, and spleen by a group of high-sulfur proteins called metallothioneins. These proteins are produced in organisms in response to heavy metal exposure (Yin, Wang, Lv, & Chen, 2019). Such defenses allow organisms to cope with relatively high levels of heavy metals in the food chain and sediments. Toxicity occurs when the intake of metals exceeds the body's capacity to produce metallothionein, a phenomenon referred to as overflow.

2.16.5 Animals

In contrast, animals have not evolved the ability to tolerate free metal ions in water that come into contact with their gills or other exposed biomembranes, such as skin. Copper (Cu(II)) ions bind to marine phytoplankton with a log b1 stability constant in the range of 10-12, indicating that complexation occurs via protein and carboxylic acid groups (Rehman, Nazir, Irshad, Tahir, Rehman, Islam, & Wahab, 2021). Cu is then transported across the biomembranes by a carrier protein through facilitated diffusion, where it binds to a thiol (possibly glutathione) in the cytosol or on the inner surface of the membrane to form Cu^{2+} .

Heavy metal removal from polluted wastewater in a wetland can be aided by plant uptake, microorganisms associated with the surface of roots and sediments, and immobilization mechanisms. These mechanisms include adsorption on ion-exchange sites, chelation with organic matter, incorporation into lattice structures, and precipitation into insoluble compounds.

2.17. The mechanism and fate of heavy metals in constructed wetland system

Once a heavy metal enters a wetland, it can undergo a variety of dynamic changes, regardless of whether the water is stagnant or flowing (Šíma, Svoboda, Šeda, Krejsa, & Jahodová, 2019). The metal can transition between different compartments, moving from water to sediments, biota, or suspended particles, and vice versa.

2.17.1 Sedimentation and flocculation

The sedimentation process is closely linked to the hydrological flow patterns of wetlands. Particles that are denser than water will settle in calm waters. Sedimentation rates can be measured using vertical accretion (cm/year) and mass accumulation (g/m^3/year). Observations have shown that wetland accretion rates can range from nearly zero in wetlands with minimal sediment input to over 1.5 cm/year in those that receive substantial sediment. In floodplain wetlands and those receiving agricultural runoff, accumulation rates exceeding 5000 g/m^3/year have been reported (Pourhakkak, Taghizadeh, Taghizadeh, Ghaedi, & Haghdoust, 2021).

Flocs are formed when clay and organic mineral particles, which possess an electrical charge on their surfaces, combine. These flocs settle more rapidly in wetlands than individual particles (Fox, Hill, Milligan, & Boldrin, 2004). Other types of suspended particles, including heavy metals, can also be adsorbed by flocs. Factors such as increased pH, turbulence, suspended matter concentration, ionic strength, and high algal density facilitate flocculation in wetlands. Under conditions of high pH, low turbulence, and high concentrations, smaller particles tend to flocculate more readily than larger ones. Additionally, brackish waters and salinity play a significant role in enhancing sedimentation and flocculation due to their higher surface area. The positive electrical charge of iron and aluminum hydrous oxides is essential for neutralizing the negative charges of colloidal particles, leading to aggregation and sedimentation.

Sedimentation is not a straightforward physical response to comprehend. It is preceded by other processes, such as complexation, precipitation, and co-precipitation. Essentially, sedimentation is a physical process that occurs after these mechanisms have aggregated heavy metals into sufficiently large particles that can sink. In this manner, heavy metals are removed from wastewater and trapped in wetland sediments, ultimately protecting the aquatic ecosystem.

2.17.2 Adsorption

Heavy metals are electrostatically attracted to clay and organic materials found in sediments (Patrick, Gambrell, & Khalid, 1990). Once adsorbed onto humic or clay colloids, heavy metals remain as metal atoms, unlike organic contaminants, which degrade over time. Their speciation may change as the organic compounds that bind them decompose or as sediment conditions fluctuate. Various factors determine the quantity of metal ions adsorbed through cation exchange or non-specific adsorption, including the properties of the metals themselves (such as valence, radius, degree of hydration, and coordination with oxygen) and the physico-chemical environment (including pH and redox state). The nature of the adsorbent medium (which includes both permanent and pH-dependent charge complex-forming ligands) and the amounts and characteristics of other metals and soluble ligands also play critical roles (Dąbrowski, 2001).

More than 50% of pollutants, such as heavy metals, can be readily adsorbed onto particulate matter in wetlands, thus removing them from the water component through sedimentation (Muller, 1988). In wetland soils and sediments, the selectivity of clay minerals and hydrous oxide adsorbents for divalent metals typically follows the order Pb>Cu>Zn>Ni>Cd, although some variations exist among different minerals and under changing pH conditions. For peat, the selectivity order has been shown to be Pb>Cu>Cd=Zn>Ca. Generally, Pb and Cu are the most firmly adsorbed metals, while Zn and Cd are retained less strongly, indicating that the latter metals are more labile and bioavailable (Dąbrowski, 2001).

The adsorption of metal ions onto solids is often described by the Langmuir or Freundlich adsorption isotherm equations. The Langmuir equation can be used to characterize metal adsorption onto manganese oxide for a limited range of metal concentrations (approximately one order of magnitude) (Dąbrowski, 2001). However, these isotherms do not provide insights into the underlying adsorption mechanisms, as they both assume a uniform distribution of adsorption sites on the adsorbent and no interactions between adsorbed ions (Pourhakkak, Taghizadeh, Taghizadeh, Ghaedi, & Haghdoust, 2021).

Wetland plants utilize their internal gas spaces, known as aerenchyma, to transport oxygen from their shoots to their root rhizomes. This oxygen is subsequently released into the reduced environment through the roots and rhizomes. Such oxidized conditions promote the precipitation of Fe^{3+} and Mn^{2-} oxyhydroxides, which can also absorb other phytotoxic heavy metals present in the wetland's water compartment (Pourhakkak et al.,2021).

2.17.3 Co-precipitation

Heavy metal co-precipitation with secondary minerals, such as Fe, Al, and Mn hydrous oxides, plays a crucial role in the adsorptive processes within wetland sediments. In the presence of Fe oxides, metals like Cu, Mn, Mo, Ni, V, and Zn tend to co-precipitate, while Mn oxides facilitate the co-precipitation of Co, Fe, Ni, Pb, and Zn. Initially, Fe(III) precipitates as stable, gelatinous forms, primarily goethite. However, ferrihydrite is more prone to dissolution under conditions of reduced Eh or pH. As a scavenger, ferrihydrite co-precipitates with various ions and effectively sorbs both cations and anions, particularly heavy metals, due to its extensive surface area, including ions like HPO_4^{2-} or H_2PO^{4+} and AsO_4^{3-} .

In reducing circumstaneess, the reduction of sulfate to sulfide results in the formation of pyrite (FeS_2), releasing H_2S, which subsequently interacts with Fe^{2+} to produce FeS and $FeS_{2.}$ The oxidation of sulfides, such as pyrite, leads to the acidification of wetland soils, causing the reintroduction of heavy metals into the solution.

The biogeochemical transformations of Fe and Mn are facilitated by specialized bacteria, such as *Thiobacillus ferroxidans* and *Metallogenum* spp. Coatings on soil particles, void fills, and concentric nodules are examples of Fe and Mn oxides. Although these oxides are mineralogically distinct, they are often intermingled with clay and humus colloids, forming part of the clay-sized fraction.

The following heavy metals are commonly observed in soil sediments co-precipitated with secondary minerals (Siposito, 1983):

- Fe oxides: V, Mn, Ni, Cu, Zn, Mo
- Mn oxides: Fe, Co, Ni, Zn, Pb
- Ca carbonates: V, Mo, Fe, Ni, Co, Cd
- Clay minerals: V, Ni, Co, Cr, Zn, Gu, Pb, T, Mn, Fe

When hydrous Mn and Fe oxides dissolve under reducing conditions, the concentrations of various additional elements in the sediment solution are likely to increase. In gleyed (periodically flooded) soils, elements like Cu, Co, Ni, Fe, V, and Mn exhibit greater bioavailability compared to drained wetland soils. In contrast, metals such as Cu, B, Co, Mo, and Zn do not undergo redox reactions; instead, they are coprecipitated by hydrous oxides. Additionally, in wetlands draining limestone catchment areas, heavy metal co-precipitation on carbonates, predominantly $CaCO_{3,}$ is significant, with Cd undergoing chemisorption in the calcite crystal structure, substituting for Ca.

2.17.4 Precipitation

Precipitation is one of the most important methods for removing metals from wetlandwater and depositing them in sediments. One of several mechanisms limiting heavy metal bioavailability in many aquatic habitats is the production of insoluble heavy metal precipitates. The solubility product Ksp of the metal species involved, the pH of the wetland, and the concentration of metal ions and related anions all influence precipitation. The dynamic equilibrium MX_2(s) M^{2+} (aq) + X can be used to depict precipitation from a saturated solution of a sparingly soluble heavy metal salt (aq). KSP = [M^{2+}] is the constant that governs this equilibrium.

$[X-]^2$, i.e., the rate of metal ion removal in the form of a precipitate equals the rate of metal ion dissolution from the precipitate at equilibrium. Precipitation happens when the cation and anion concentrations are sufficient that their product surpasses Ksp.

Metal carbonates, hydroxides, and sulphides precipitate in reducing circumstances, and their precipitation is pH-dependent. Sulphides are distinguished by the fact that they are insoluble at neutral pH and hence accumulate in freshwater wetlands sediments. The partial pressure of CO_2 has an effect on the solubility of carbonates. In the presence of CO_2, the solubility of $PbCO_3$ can be raised several fold (Bourgouin, 2000).

2.17.5 Cation and Anion Exchange

Between the counter ions that balance the surface charge on sediment colloids and the ions present in wetland water, ion exchange can occur. Cation exchange, which involves the substitution of a hydrogen ion for a metal, is driven by the negative charges on the sediment colloids. The cation exchange capacity (CEC), measured in cmolc/kg, defines the extent to which sediment elements can function as cation exchangers. Although sediment organic matter is present in much smaller amounts (1-10%) than clays, it has a higher capacity and plays a crucial role in adsorption processes in most soils (80 percent).

There are two types of negative charges found on the surfaces of sediment colloids:

(a) Permanent charges resulting from the isomorphic substitution of a lower-valence ion for a component of a clay mineral.

(b) Charges on Fe, Al, Mn, Si, and organic colloids that are positive at pH levels below their isoelectric points and negative at pH levels above their isoelectric points, making them pH-dependent. Generally, clay and organic colloids are negatively charged in alkaline conditions, while hydrous Fe and Al oxides have high isoelectric points (> pH 8) and thus tend to be positively charged in most situations. Increasing soil pH, at least up to neutrality, usually raises the CEC of most colloids. The dissociation of protons from carboxyl and phenolic groups in humic polymers within the sediment organic matter fraction leads to a negative charge.

The term "cation exchange" refers to the movement of ions between the colloid surface (double diffuse layer) of the wetlands and the surrounding water. With the exception of H^+, which behaves like a trivalent ion, the relative replacing power of an anion on the cation exchange complex is influenced by its valence, hydrated diameter, and the type and concentration of other ions in the water. Higher valence ions exhibit a greater degree of adsorption. For instance, while K^+ and Na^+ have the same valence, K^+ is more likely to replace Na+ due to Na^+'s larger hydrated size.

On the cation exchange complex of metal cations, the generally reported relative order of replaceability is Li+=Na+>K+=NH4 +>Rb+>Cs+>Mg2+>Ca2+>Sr2+=Ba2 +>La3+=A13+>Tn4+. The heavy metals are replaced in the following sequence for particular sediment components **(**Bergaya, Lagaly, & Vayer, 2006)

Ca>Pb>Cu>Mg>Cd>Zn Montmorillonite clay

Pb>Cu>Zn>Ni>Cd>Co>SPMg ferrihydrite

Pb>Cu>Cd>=Zn>Ca in peat

When anions are attracted to positive charges on sediment colloids, anion exchange occurs. Because Fe and Al hydrous oxides are frequently positively charged, they are the most common sites for anion exchange in sediments. Anion exchange capacity in most sediments is lower than cation exchange capacity. Some anions, such as NO_3 and Cl, are little adsorbed, whereas others, such as H_2PO_4 are heavily adsorbed. At typical sediment pH levels, some organic pesticides, such as phenoxyalkanoic acid herbicides, exist as ions and are adsorbed to some extent through hydrous oxides and H_2 bonding to humic polymers.

2.17.6 Complexation

Heavy metal ions replace one or more coordinated water molecules in the coordination sphere during the complexation process, often with additional nucleophilic groups or ligands. This complexation process regulates heavy metal ion speciation in water, influencing metal reactivity in organic materials like humic, tannic, and fulvic acids (HA, TA, and FA).

To develop realistic models of heavy metal speciation in natural waters, a comprehensive understanding of heavy metal-organic interactions is essential. The overall negative charge of colloidal particles significantly influences cation adsorption on organic compounds. Various parameters, such as redox potential and pH, affect this process. However, the nature of HA and FA presents significant challenges due to their polydispersity and chemical variability. Consequently, multiple theories have been proposed to address their interactions with heavy metals (Loftsson, Hreinsdóttir, & Másson, 2007).

Our capacity to measure, represent, and interpret complexation equilibria varies from a simple 'Scatchard' model with a 1:1 metal site stoichiometry and no site/site interactions to more complex 1:1 and 1:2 complexes that account for electrostatic site/site interactions and explicitly consider the characteristics of the solution and complex phases. Heterogeneous complexants exhibit at least three distinct characteristics:

(i) Their polyfunctionality, meaning they possess multiple complexing sites of varying characteristics on the same physical structure.

(ii) The polyelectrolytic nature of their enzymes, which can lead to high electric charge densities due to numerous dissociable functional groups per physical entity.

Varma, Deshpande, and Kennedy (2004) demonstrated that the extremely alkaline organic and saline waters of the wetland-lake ecotone of the Lake George-Edward System in Western Uganda mitigated heavy metal contamination from a dormant copper mine at Kilembe and from cobalt stockpile tailings in Kasese. The primary ions in wetland-lake water are Na^+, $CO_3^{2-,}$ and Cl^-, alongside significant concentrations of K^+ and Mg^{2+} (Filella, 2008). Consequently, total dissolved solids, conductivity, and salinity—which quantitatively assess ionic species in water—are elevated, as are water hardness ($CaCO_3$) and alkalinity.

The comparatively high ion concentration increases the ionic strength of the water, which reflects the electrical field present. The "chemical activity" is calculated as the product of the ionic concentration and the "activity coefficient." In such water, activity coefficients are typically less than 1 (whereas they are 1 in dilute solutions), resulting in chemical activities that are lower than the ionic concentration for any given ionic species (e.g., $Cu^{2+).}$ When the activity of a species in water is lower than its concentration, it suggests that the species cannot function independently and is influenced by the presence of other ions in the water.

As a result, the presence of other ions reduces the effective concentration of the species. Thus, the dissolved ionic species in the wetlands-lake ecotone can create ionic interference with $Cu^{2+,}$ decreasing its effective concentration. The water also exhibits high levels of iron (Fe, 4.83 mg/l) and organic matter (COD, 307 mg/l). Copper interacts with iron oxide/organic colloids, leading to its precipitation in micromole per kg amounts (Varma, Deshpande, & Kennedy, 2004). The precipitation of iron oxide/hydroxide on colloids and suspended particles plays a crucial role in reducing the concentration of metal ions (e.g., $Cu^{2+).}$ The water has a pH range of 8 to 10, within which Cu^{2+} hydrolysis products are formed.

$Cu^{2+} + H_2O) = CuOH^+ + H^+$

$Cu^{2+} + 2H_2O = Cu(OH)0\ ^{2+}\ 2H_2^+$

$2Cu^{2+} +2H_2O = Cu_2(OH)O_2 +2H^+$

The effective concentration of Cu is further diminished by the hydrolysis processes mentioned earlier. Copper exhibits a strong affinity for organisms, solid phases, and organic materials (Varma, Deshpande, & Kennedy, 2004). Consequently, the lake experiences eutrophication, characterized by a high concentration of organic molecules that reduces the chemical activity of metallic ions through chelation, a specific type of complexation. Chelation involves the binding of a single metal ion to a single ligand that possesses two or more electron donor sites. The most robust interactions formed by cupric ions occur with intermediate electron donors (O, N, P) present in dissolved organic matter. This complexation significantly influences the chemical and biological behavior of copper.

2.17.7 Oxidation/Reduction

The redox state of heavy metals in solution is a crucial speciation parameter, as it can significantly influence their toxicity, adsorptive behavior, and transport **(**Szlachetko, Ferri, Marchionni, Kambolis, Safonova, Milne, & Sá, 2013). This redox state is contingent upon the presence of either anoxic or oxic conditions within the wetlands. Microorganisms, such as *Thiobacillus* spp., facilitate the oxidation of sulfides. In instances of pollution resulting from tailings of metalliferous mining, particles of ore minerals in the soils, such as PbS, ZnS, and

CuFeS2, oxidize and release metal cations like Pb^{2+}, Cu^{2+}, Zn^{2+}, and Cd^{2+} into the sediment upon adsorption.

Certain organic pollutant molecules on the soil surface may undergo photolytic degradation due to exposure to UV wavelengths during the daytime, which can release the metals that were initially deposited on them. The activity of oxygenase enzymes secreted by microorganisms promotes the oxidation of organic contaminants. Under reducing conditions, fluctuations in the redox potential (Eh) facilitate the precipitation of metals as metal sulfides.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection of samples

3.1.1 Soil

The soil samples used for this study are (i) uncontaminated soil (ii) contaminated soil through simulation.

3.1.2 Uncontaminated soil sample

The uncontaminated soil sample was collected from the back of Okemini Naval barrack, Rumuolumini, in Obio Akpor Local Government area of Rivers State (this site is not known to be polluted with petroleum hydrocarbon or by any other industrial pollutants. Surface soils (0-20 cm) were randomly collected using a shovel. It was bulked to form a composite sample and then transported to laboratory. The soil sample was dried, and sieved through a 2 mm mesh to ensured uniformity. Sieved samples were stored in plastic bottles and covered with stoppers until analysis

3.1.3 Source of crude Oil

The crude oil type that was used for this work, was Bonny light crude oil obtained from the Port Harcourt Refinery Company Limited Eleme, Rivers State. The crude oil was collected in twenty-five liters (25 L) plastic container and stored at room temperature in the laboratory until when it was used.

3.2 Sample pollution

3.2.1 Water sample pollution with heavy metals

A liter (1 L) of dionized water was polluted with fixed concentrations of heavy metals (Zn, Cu, Ni and Pb) by dissolving their hydrated salts $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, $Ni(NO_3)_2.6H_2O$ and $Pb(NO_3)_2$ respectively to achieve concentrations above the maximum permissible limits for discharge waste water effluent into Nigerian environment as shown in Table 3.1 below, (Nigeria Federal Ministry of Environment (NFME), 1992). An equivalent of 10 L was obtained and stored in a 10 L clean and dried container. A 100 ml of this solution was added to a kg of the crude oil polluted soil, thoroughly mixed with a hand trowel to create a heavy metal and crude oil polluted soil.

Table 3.1: Respective concentrations of heavy metals in constituted polluted soil samples and their FME permissible limits in soil

Metals	Exp conc. (mg/l)	Limit for agric. land (mg/l)* (NFME, 1992)	Limit for nonagric. land (mg/l)* (NFME, 1992)
Zn	39.4	5.0	0.03
Ni	10.2	0.2	0.2
Cu	29.4	1.0	4.0μ/l
Pb	11.2	0.05	1.8μ/l

Key: Exp. conc – experimental concentration

3.2.2 Soil pollution

Uncontaminated soil obtained as stated in Section 3.1 above was polluted using method adopted by Adieze, Orji, Nwabueze, & Onyeze, (2012). The crude oil was dissolved in acetone (3:1), and mixed with 10% of total soil. The crude oil laddered soil which served as the stock was added to the bulk of the soil and mixed with heavy metals laddered distilled water to obtain the final concentrations of 3% (30 g/kg), 7% (70 g/kg) and 10% (100 g/kg) crude oil and heavy metals (Zn = 39.4 mg/l, Ni = 10.2 mg/l, CU = 29.4 mg/l and PB = 11.2 mg/l) in the soil. The mixed crude oil enriched soil and heavy metals was stirred several times for 2 days to remove acetone (Adieze et al., 2012).

3.3 Plant selection

A total of eight (8) native plant species, selected based on their prevalence and survival in contaminated sites were screened for their ability to tolerate crude oil (i.e., experimental concentrations), heavy metals pollution (concentration as shown in table 3.1) and for their tolerance to wetland environment (Preliminary stage). Of the screened plants, the best two (2) were selected for further studies. The criteria for selection of potential phytoremediator and heavy metals accumulator plant species were based on frequency of occurrence in contaminated sites and densities of the species in re-vegetating contaminated sites, ease of propagation (ability of their seedlings to grow in a contaminated soil, vegetative propagation), and root structures.

3.3.1 Plant identification

Selected plants for preliminary studies were taken to University of Portharcourt, Faculty of Science, Department of Plant Science and Biotechnology. The plants were identified by Dr.

Ekeke, Chimezie a specialist in plant taxonomy in charge of University of Port Harcourt reference herbarium for research and germplasm conservation section of the department.

Table 3.2 Plant species used for the study and their scientific and English name

Scientific name	English name
Brachiaria distachyoides Stapf,	Signalgrass
Cyperus dichrostachyus Hochst. ex A. Rich	African nut sedge
Kalanchoe pinnata (Lam.) Pers	Cathedral bells, Air plant, Life plant, Miracle leaf, Goethe plant or love bush
Panicum maximum Jacq	Guinea grass
Mimosa pudica L	Sensitive Plant, Sleepy plant, Touch-me-not orShameplant
Paspalum conjugatum P.J.Bergius	Crab grass
Mariscus rotundus	Purple nutsedge, Nut grass or Red nut sedge
Mariscus ligularis (L.)	Swamp flat sedge

3.3.2 Seedlings preparation

Seedlings of plants to be screen for their heavy metals accumulation and phytoremediation of crude oil pollution abilities were planted into an uncontaminated soil (100 g) in a perforated transparent polythene bag of 10.5 x 25 cm and then transferred into a black ornamental polyethylene bag measuring 29 ×59 cm containing 3 kg of soil contaminated with heavy metals and crude oil of various experimental concentrations.

The concentrations of crude oil pollution were 3%, 7% and 10% and while that of heavy metals were as stated in table 3.1 respectively. The eight plants species under investigation were planted each in a separate ornamental polythene bag containing soil of different concentrations of crude oil pollutant but the same concentrations of heavy metals as stated in table 3.1 above. The total experimental setup was made up of twenty-eight (28) ornamental polythene bag with twenty-four (24) containing eight (8) plants in three (3) different treatments with different concentrations (3%, 7% and 10%) of crude oil and heavy metal pollutants, while eight (8) were unpolluted control for each of the eight (8) plants and three (3) containing polluted soil only without any plant and one control (1) ornamental bag without polluted soil). This set up served to determine the effect of microorganisms and natural attenuation on the pollutants.

3.3.3 Preliminary screening of plants species for tolerance to crude oil and heavy metals in wetland soil

The preliminary study was carried out in a greenhouse and involved the screening of eight (8) different species of plants for tolerance to different concentrations of crude oil and heavy metals pollutants in the CW soil. Two (2) plants were selected from the preliminary study based on their tolerance to contaminants (heavy metals and crude oil). The protocols for selection of heavy metals and crude oil tolerant plant species involved the determination of the following plant growth indices;

1. Plants' shoot height,
2. Plants' leaf width and,
3. Plants' total biomass (wet weight)

3.3.4 Determination of crude oil and heavy metal concentrations' tolerance limit for the selected plants

The plants that exhibited the best tolerance for pollutants in experimental set up section 3.3.3 were further subjected to soil sample polluted with heavy metals and 12% crude oil. The experimental protocols are shown below in Table 3.3.

Table 3.3: Experimental set up for the preliminary study for the selection of test plants in response to hydrocarbon and heavy metals pollution

Treatment	Purpose	Number of pots
Unpolluted soil +plants	Control	1
Crude oil polluted soil (3%) + plants+ heavy metals	Effect of 3% crude oil and heavy metals on the different species of plants	8
Crude oil polluted soil (7%) + plants+ heavy metals	Effect of 7% crude oil and heavy metals on the different species of plants	8
Crude oil polluted soil (10%) + plants+ heavy metals	Effect of 10% crude oil and heavy metals on the different species of plants	8
Crude oil polluted soil (12%) + plant + heavy metals	Effect of 12% crude oil + heavy metals on the selected plant from the above experimental set up	1

3.4 Microbiological analysis

Soil samples were obtained from the set up in Table 3.3 on the eight (8) week of study for determination of total heterotrophic microorganisms and hydrocarbon utilizing species.

(i) Enumeration of total heterotrophic microorganisms

Microbial populations in the soil samples were assayed by standard plate count technique using the soil from control as shown in Table 3.3. The total aerobic heterotrophic culturable microbial populations present in the soil samples during the study were estimated by spread plate techniques according to Pelczar& Chan (1977).

A 10-fold serial dilution of soil sample suspension was prepared by weighing out 10 g of soil sample into 90 ml of sterile distilled water in sterile 20 ml test tube. This constituted a 10^{-1} dilution. The soil suspension was vigorously shaken for 3 minutes by hand and was allowed to stand for 30 seconds. Then using a sterile 1 ml pipette, 1 ml was removed from the middle of the suspension and transferred into a 9 ml sterile distilled water to achieve 10^{-2} dilution. This process was repeated until 10^{-7} dilution was obtained. After the serial dilutions, aliquots (0.1 ml) of dilutions 10^{-6} to 10^{-8} were plated in duplicate on nutrient agar (Oxoid) plates supplemented with fulcin (500 mg/l) an antifungal agent, and on sabourauds dextrose agar (SDA) plates supplemented with streptomycin (5 μg/ml) for the determination of aerobic heterotrophic bacterial and total fungal counts, respectively. The plates were incubated at 30 ± 2^oC respectively for 24 hrfor bacterial counts and three (3) days for fungal counts after which colonies were counted and average counts recorded and used for the calculation of colony forming units per gram (cfu/g) of soil.

(ii) Isolation and Enumeration of hydrocarbon utilizing species

This was done by obtaining one (1) gram of soil sample from each ornamental bag containg polluted soil with different treatments as shown in Table 3.3, mixed and stirred for homogenenity.Aliquot (0.1 ml) of appropriate dilutions (10^{-6} to 10^{-8}) of soil samples were plated in duplicate onto a mineral salts (MS) medium using the method of Okpokwasili & Amanchukwu, (1988). Sterile filter paper (Whatman No. 1) was aseptically saturated with crude oil (Bonny light) and placed unto the covers of the inoculated inverted plates and then incubated for 3-7 days at 30±2^{o}C. Hydrocarbon from the crude oil saturated filter paper will supply in vapour phase to the surface of the agar plate as sole source of carbon. Colonies were counted from duplicate plates and the average counts were recorded and used for the calculation of colony forming units per gram (Cfu/g) of the soil. Colonies of hydrocarbon utilizing species from the contaminated soil and rhizosphere of the selected plants were selected based on their colonial characteristics from the mineral salts (MS) agar plates. The isolates were purified by streaking on nutrient agar (NA) and sabround dextrose agar (SDA) plates for bacterial and fungal isolates respectively, which were then transferred onto nutrient agar and SDA slant in Bijou bottles and stored at 4^{o}C in a refrigerator for further studies.

3.4.1 Biochemical characterization and identification of hydrocarbon utilizing bacteria and non-hydrocarbon utilizing species

Hydrocarbon utilizing bacterial species isolated from the mineral salt agar plates with crude oil as sole carbon source were stored on nutrient agar slants in Bijou bottles and kept in a refrigerator for further studies. They were examined for their biochemical and morphological characteristics.

The colonial appearance of the isolates was examined and noted. Gram staining procedure as described by Gerhardt et al., (1981**)** was performed to determine the cellular morphologies and gram reactions of the isolates. Other tests that were performed includes motility test, catalase test, citrate utilization test, indole test, oxidase test, oxidative fermentative utilization of glucose, urea test, methyl red (MR) test and voges-proskauer (VP) test.

Characterization of the isolates followed the procedures in the Bergey's manual of determinative bacteriology (Holt, 1994).

The various biochemical tests were carried out following the procedures outlined below:

(i) Motility test

A semi solid agar medium was stab inoculated with the test organism; the inoculated medium was incubated at $30 \pm 2^{o}C$ for 48 hrs. The medium was examined every 6 hrs. Diffuse hazy growth that spread throughout the medium indicated positive result for motility, while a negative result showed growth confined to the line of inoculation.

(ii) Catalase test

A few colonies of the test organism were emulsified in three drops of distilled water on a clean glass slide placed in a petri dish. Two drops of hydrogen peroxide H_2O_2 (3%) were added to the emulsified colonies. Immediate generation of gas bubbles indicated a positive reaction.

(iii) Citrate utilization test

Simmon's citrates agar slant on a screw capped test tube was surface inoculated with a suspension of 24h culture of the test organism using a wire loop, and incubated for seven days. A change of colour in the agar from green to blue colour indicated alkaline reaction arising from citrate utilization.

(iv) Indole test

The test organism was grown in peptone water at 30^oC. After 24h growth, a few drops of Kovac's reagent were added to the 24h culture in peptone water. Observation of a red ring above the peptone water showed that the test is positive for insole production.

(v) Oxidase test

A few drops of one percent aqueous solution of tetramethyl-P-phenylenediamine hydrochloride was used to moisten a filter paper in a petri dish. With a glass rod, a colony of the test culture was collected and smeared on the moistened filter paper. Purple colouration produced within 5-10 seconds indicated a positive oxidase test.

(vi) Oxidative/fermentative (O/F) utilization of glucose

Aseptically, 1 ml of sterile 10% glucose solution (sterilized by filtration) was added to four test tubes each containing 9 ml sterile Hugh and Leifson's medium to obtain a final concentration of 1% glucose. The tubes were then stab-inoculated in duplicate with bacterial culture, retaining two un-inoculated tubes as control tubes. Vaseline was used to cover one of the inoculated duplicate tubes and one control to discourage oxidative utilization of sugar. The tubes were incubated for 4 days at 37^oC. Production of acid in the culture indicated by change of the medium from blue to yellow was observed daily. Yellow colouration in the open tubes only, suggested oxidative utilization of the sugar while acid production in the sealed and open tubes suggested a fermentative reaction.

(vi) Methyl red (MR) test

The test organism was grown in a medium containing peptone and Di-potassium hydrogen phosphate K_2HPO_4 at 30^oC. After five days' growth, a few drops of the indicator were added to the culture. Observation of a red colour indicated a positive (acid) reaction.

(vii) Urease test

The entire slant surface of the Christensen's urea on a screw capped test tube was inoculated with a suspension on 24 h culture of the test organism using a wire loop, and incubated at 30^oC for 24 h. A change of colour to red-pink indicated a positive test.

(viii) Voges-proskauer (VP) test

About 2ml of the medium used for methyl red test was inoculated with the test organism and incubated at 30^oC. After 48 h, 1 ml of 10% potassium hydroxide was added and then left at room temperature for 1 h. Development of pink colour indicated a positive result, while no colour development was negative.

3.4.2 Fungal isolation and identification

The fungal isolates obtained from section 3.4 (ii) were identified based on their cultural and morphological characteristics, including colony growth patterns, conidial morphology, and pigmentation, as described by Tafinta, Shehu, Abdulganiyyu, Rabe, & Usman (2013). Additionally, the identification process utilized the method outlined byOyeleke& Manga, (2008), employing cotton blue in lactophenol stain. A drop of the stain was placed on a clean slide, and a small portion of aerial mycelia from representative fungal cultures was carefully removed using a mounting needle. The mycelia were transferred into the stain and evenly spread on the slide. A cover slip was gently placed over the preparation with light pressure to remove any air bubbles.

The slide was then mounted and examined under a light microscope using ×10 and ×40 objective lenses. The observed morphological features of the fungi were identified with reference to the guidelines provided by Adebayo-Tayo, Odu, Esen, & Okonko(2012);Onuorah, Ifeanyi, & Ugochukwu (2015) and Samson, & Varga (2007).

3.4.3 Molecular identification of hydrocarbon utililzing bacterial isolates

3.4.3.1 Bacterial genomic DNA extraction

Total DNA extraction and isolates identification on hydrocarbon utilizing isolates obtained from section 3.4 (ii) were carried out at Nucleometrix Molecular Laboratory, Yengoa, Bayelsa State.

The method as described by Saitou & Nei, (1987**),** was adopted for molecular identification of isolates. Five milliliters of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) was spun at 14000 rpm for 3 min. The cells were re-suspended in 500 µl of normal saline and heated at 95^{0}C for 20 min. The heated bacterial suspension was cooled on ice and spun for 3 min at 14000 rpm. The supernatant containing the DNA was transferred to a 1.5 ml microcentrifuge tube and stored at -20^{o}C for other downstream reactions.

3.4.3.2 DNA quantification

The extracted genomic DNA obtained as described above was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was lunched by double clicking on the Nanodrop icon. The equipment was initialized with 2 ul of sterile distilled water and blanked using normal saline. Two microlitre of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the “measure” button.

3.4.3.3 16S rRNA Amplification

The 16s RRNA region of the rRNA genes of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3’ and 1492R: 5'-CGGTTACCTTGTTACGACTT-3’ primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microlitres for 35 cycles. The PCR mix included: The X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a UV transilluminator.

3.4.3.4 Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10 ul, the components include 0.25 ul BigDye® terminator v1.1/v3.1, 2.25 ul of 5 x BigDye sequencing buffer, 10 uM Primer PCR primer, and 2-10 ng PCR template per 100 bp. The sequencing conditions were as follows 32 cycles of 96°C for 10 s, 55°C for 5 s and 60°C for 4 min.

3.4.3.5 Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of the taxa analysed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes & Cantor 1969).

3.4.4 Preparation and standardization of bacterial inocula

Suspension of the hydrocarbon utilizing bacterial species isolates obtained as described above, were inoculated into 10ml sterile normal saline contained in 20 ml sterile test tubes. The suspension was shaken for five minutes to evenly distribute the organisms and then transferred into a sterile 500 ml conical flask containing 190 ml sterile salt broth (Okpokwasili & Amanchukwu, 1988) containing 1% Bonny light crude oil, this mixture was incubated on a rotary shaker (150 rpm) at room temperature of $28\pm2^{o}C$ for five days (Odokuma & Dickson, 2003). The pH of the cultures was monitored and maintained at between 7-7.2 by adjusting the culture with standard phosphate buffer.

To obtain the inocula, aliquots of the final cultures was centrifuged at 10,000 x g for 20 minutes. The supernatants were discarded and the cell pellets collected and washed twice in 20ml of sterile tap water. After washing, the cell pellets were resuspended in 100 ml sterile distilled water and adjusted to final OD of 0.40 at 660 nm (population between 10^6-10^8 cfu/ml). The suspension was used for toxicity assay for the soil amendments (native soap, poultry droppings and combination of both) used for the work.

3.4.5 Toxicity assay for native soap, poultry manure and combination of both on microbial growth

The concentration of native soap that supported the optimal growth of hydrocarbon utilizing bacterial isolates was determined by preparing different concentrations (1%,10% and 30%)of the native soap in six (6) different conical flask containing 10% crude oil v/v in deionized water polluted with heavy metals as stated in table 3.1. The setups were inoculated with standardized isolates of hydrocarbon utilizing isolates from section 3.4 (ii) This mixture was incubated on a rotary shaker (150 rpm) at room temperature of 28±2^{o}C for ten (10) days (Odokuma and Dickson, 2003). The pH of the cultures was monitored and maintained at between 7-7.2 by adjusting the culture with standard phosphate buffer.

Optical densites of the cultures were taken and recorded at day 0, 2, 4, 6 and 8 respectively. The same procedure was adopted for hydrocarbon and heavy metals tolerating fungi for 12 days and their cfu/l taken at intervals of 0, 3, 6, 9 and 12 days respectively. The same procedure that was adopted for section 3.4.5 above was applied for the determination of the concentration of poultry droppings and combination of poultry droppings and native soap that supported the maximum microbial growth.

3.4.6 Biosurfactant production

3.4.6.1 Screening of bacterial isolates for biosurfactant production.

i. *Preparation and standardization of microbial inocula*

Hydrocarbon utilizing bacterial obtained in section 3.4 were screened for biosurfactant production. The isolates (*Pseudomonas* spp, *Acinobacter* spp, *Enterobacter* spp, *Alicaligenes spp., Pantoea* spp., *Bacillus* spp., *and Microccus* spp..), stored on agar slants were subcultured on nutrient broth and incubated on a shaker (150 rpm) at 30^{o}C for 24 h and aliquots of the cultures were centrifuged at 10,000 x g for 20 minutes after which the supernatants were discarded, the cell pellets was harvested and washed twice in 20ml of sterile tap water. After washing, the cell pellets were resuspended in 10 ml sterile physiological saline (0.85% NaCl) contained in 20 ml sterile test tubes. The procedure was used to standardized the bacterial inocula.

ii. Inoculation of culture broth for biosurfactant production

Suspension of 24 h culture of the hydrocarbon utilizing bacterial species was dispensed into a 10 ml sterile physiological saline (0.85% NaCl) contained in a 20ml sterile test tubes. The suspension (about 10^6 CFU/ml) was shaken for five minutes to evenly distribute the organisms and then transferred into a sterile 250ml conical flasks containing 90ml of sterile modified mineral salt medium (Okpokwasili &Amanchukwu, 1988) with 1.0% (v/v)/ Bonny light crude oil as sole carbon source. This mixture was placed on a shaker (150 rpm) and incubated at room, temperature of 28±2^{o}C for seven days. To obtain the crude biosurfactant, aliquots of the final cultures were centrifuged at 10,000 x g for 10 minutes. The supernatants obtained were subsequently used in a drop-collapsing test.

3.4.6.2 Drop collapse assay

Screening of biosurfactant production was performed using the qualitative drop-collapse test (Bodour &Miller-Maier ,1998; Batista, Mounteer, Amorim, & Totola, 2006.). Bonny light crude oil was used in this test and were all carried out in duplicates. The wells of a polystyrene 96 well micro-plate lid (Corning Incorporated, United States) was coated with 2 μL of polluted soil sample and left to dry for 24 h at 25°C. Filtered cell-free supernatant (5 μL) was transferred to the center of the oil coated well. The results were recorded after 1–2 min and considered positive for biosurfactant production when the oil drop will be flat. Those that gave rounded drops were scored negative, an indication of the absence of biosurfactant production (Youssef, Duncan, Nagle, Savage, Knapp, & McInerney, 2004).

3.5 THE SECOND PHASE

3.5.1 Plants response to various polluted soil amendments

The plant species that showed the best tolerance to 10% crude oil and heavy metals, after experimental set as described in table 3.3, was selected for further studies in 12% crude oil and heavy metals polluted soil. Where none of the plants survived, the one that exhibited the highest survival with time in the highest pollutant concentration was selected. The selected plant was subjected to various amendment to determine their effects in enhancing bioremediation of the polluted soil samples. The protocols to determine the effect of various amendments to crude oil and heavy metal polluted CW soils are as shown in Table 3.4 below.

Table 3.4: Different amendment on 10% crude oil and heavy metals polluted soils and their purpose

Treatment	Purpose
UPS + SP Only	control
PS (CO + HM+S) Only	effect of microorganisms on remediation of the polluted soil
PS +SP	Effect of crude oil and heavy metals on the selected plants growth.
PS+ NS+ SP	Effect of amendment with natural soap on indigenous hydrocarbon utilizing microorganisms and phytoremediation of crude oil and bioaccumulation of heavy metals by selected plants
PS+ PD + SP	Effect of poultry droppings on indigenous hydrocarbon utilizing microorganisms and phytoremediation of hydrocarbons and bioaccumulation of heavy metals by selected plants
PS+ NS +PD + SP	Combined effects of native soap and poultry droppings on indigenous hydrocarbon utilizing microorganisms and phytoremediation of hydrocarbons and bioaccumulation of heavy metals in selected plants
	Effect of native soap on bioremediation of

PS + NS	hydrocarbons and bioaccumulation of heavy metals by microorganisms.
PS + PD	Effect of poultry droppings on bioremediation of hydrocarbons and bioaccumulation of heavy metals by microorganism.

KEY:

UPS → Unpolluted soil, PS → Polluted soil with crude oil and heavy metals, CO → Crude oil. HM → Heavy metal, SP → Selected plants, NS → Native soap. PD → Poultry droppings (manure). OB → Ornamental bag

3.5.2 Experimental design

3.5.3 Constructed hybrid wetland (CHW) design and operation

A vertical surface flow hybrid constructed wetlands system (CWS) was designed for this study, comprising two cylindrical container troughs. Each trough represents a constructed wetland system, incorporating essential components like polluted soil, gravel, and waterlogged soil, simulating real-world CWS conditions. The first trough is positioned 128 cm above the second, allowing gravitational water flow when the connecting tap is opened. Each container has a capacity of 25 liters. The first CWS contains polluted soil amended with 1% natural soap solution, while the second contains unpolluted soil, also treated with 1% natural soap solution and bioaugmented with hydrocarbon-utilizing microorganisms (bacteria and fungi) previously isolated, as described in Section 3.7.2, subsection ii. Both CWS units were planted with *Paspalum conjugatum*, the plant that demonstrated the most desirable traits in preliminary tests as shown in Tables 3.2 and 3.4.

A 50-liter reservoir was constructed to manually control and distribute equal volumes of heavy metal-laden water into the constructed wetlands. Water flow was regulated through inlet and outlet valves, allowing for controlled water movement in and out of the vertical surface constructed wetlands via a 50 mm polyvinyl chloride (PVC) pipe. The retention time for the remediation process in each CWS was 30 days. After this period, the treated water was directed into the second CWS for further treatment by opening the second inlet valve. Following an additional 30 days of treatment, the outlet valve was opened to collect the remediated water, which had undergone microbial and phytoremediation, into a conical flask.

The collected water was transferred into a dry glass container with a screw cap and sent to the laboratory for analysis. Every 30 days, both the treated water and 5 g of soil sediment samples were taken from various parts of the first constructed wetland. These samples were thoroughly mixed to create a homogeneous sediment mixture, accurately representing the soil sediment in the CWS. The samples were then taken to the laboratory for analysis of residual crude oil and heavy metal concentrations.

The samples analysis for this study were carried out by Aigberua Ayotunde of Analytical Concept LTD, headquartered at Poultry Road, near the 2nd Railway, Odani Green City, Elelenwo, Port Harcourt, Rivers State. The diagram of the constructed wetland is displayed below:

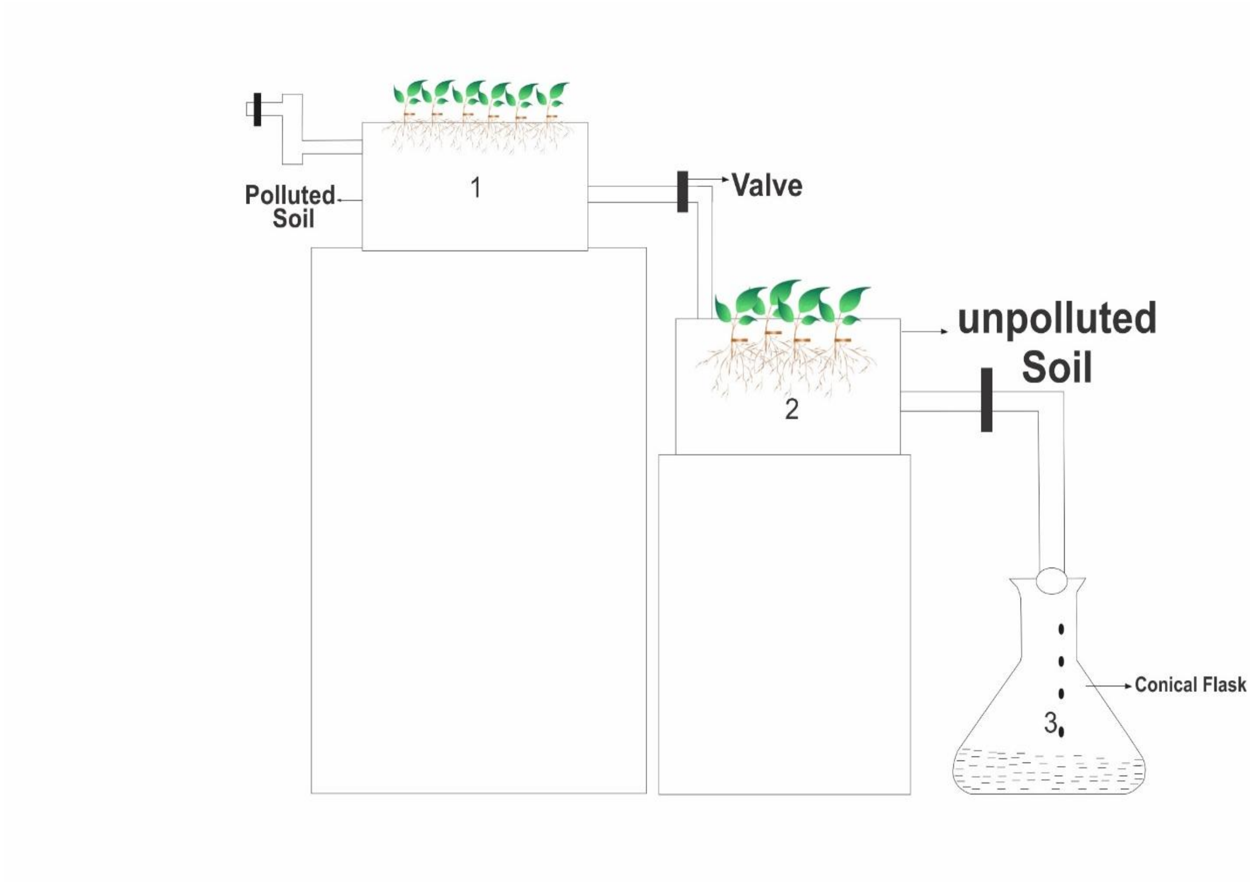

Figure. 3.1: Constructed hybrid wetland system

3.6. SAMPLE ANALYSIS

Plant samples destructively harvested and soil samples both from constructed hybrid wetland in section 3.5.3 were assayed for the following;

i. Plant growth characteristics performance was determined at every four (4) weeks for twenty-four (24) weeks

ii. Crude oil and heavy metal removal was also determined as stated above.

3.6. 1. Physicochemical analysis of soil samples

The soil samples for physicochemical analysis were first air dried after collection, and sieved through a 2 mm mesh, stored in covered plastic bottles until ready for analysis. Methods that were used for the determination of the properties are as follows:

(i) Particle size

This was carried out using standard sieves for sand and gravel fractions and pipette analysis for the mud (silt and clay) fraction according to the procedures outlined by Folk (1974).

(ii) Soil pH

pH of the soil was determined based on the modified method of Mc Lean (1982). 5 g of soil sample was weighed into 20ml test tube containing 12.5 ml distilled water, the suspension was stirred and allowed to stand for 30 minutes after which the pH of the supernatant liquid was determined using Corning pH meter (model 7)

(iii) Phosphorus

Available phosphorus was determined using the method of ISO Standard No. 3696:1987 of International Organization for Standardization(2018).

(iv) Total nitrogen

Total nitrogen was determined using the macro Kjeldahl method of Walkley & Black (1934). Nitrogen here was oxidized and converted into ammonium with an acid and quantity of ammonium produced was determined by distillation with an alkali. The process was carried out in two stages.

(1) Digestion of soil with H_2SO_4 to convert nitrogen to ammonium.

(2) Determination of quantity of ammonium using the medthod of EPA, (1993)

Into a dry 500 ml macro-kjeldahl flask was weighed 10 g of air dried soil. To the flask, 20 ml of water was added and swirled for 5 mins. The flask was then allowed to stand for 30mins. Then, 10 g K_2SO_4, 1 g $CuSO_4.5H_20$, 0.1 g Se and 30 ml conc. H_2SO_4 were added and mixed by swirling motion. The flask was heated cautiously in a fume chamber with inter5mittent swirling motion. The flask was then heated cautiously in a fume chamber with intermittent swirling until the digest turns light green or gray in colour. Heating was continued from this stage for another one hour. The flask then was allowed to cool and then slowly, while shaking flask, 100 ml of tap water was added, and solution was transferred to a clean flask for distillation. As much as possible, sand residues were retained in the digestion flask during transfer. The sand residues were washed with 50 ml aliquots until 250 – 300 ml solution was obtained.

Into a 500 ml flask on which the 150 ml level has been marked, 50 ml of 4% boric acid (H_3BO_3) solution and 3 drops of mixed indicators were added. The flask was then placed under the condenser of the distillation apparatus so that the end of the condenser dipped into the solution inside. A small piece of litmus paper was dropped into the flask containing the diluted digest. Then 125 ml of 45% of NaOH was added to the flask carefully by pouring down the side of the flask so that alkali reached the bottom of the flask without mixing appreciably with the digest. The flask was then attached to condenser and swirled to mix the contents. The litmus paper will now show that the solution is alkaline. The solution was then distilled, regulating the heat to minimize bumping, until about 150 ml of the distillate was collected. The distillate was then titrated with 0.05 N H_2SO_4. The colour changed from green through grayish-blue to pink. A blank was prepared and titrated as adopted by the method of Behzadimoghadam & Feizi, (2019)

Calculation:

Milliequivalents of N in the sample = ml 0.05 N $H2SO_4$ used – blank value x normality of H_2SO4.

Total % N =ml 0.05 N H2SO4 x normality x F

Where F = Correction factor = 0.14

(v) Organic carbon

Organic carbon was measured using modified Walkley & Black method (1938), which is based on the exothermic heating and oxidation of organic matter with potassium dichromate and concentrated sulphuric acid, followed by back-titration with ferrous ammonium sulphate using phenyl-amine as indicator. The soil samples were first grinded and sieved through a 100µm mesh sieve. Then 1 g of soil was weighed into 500ml conical flask, into which 10 ml $K_2Cr_2O_7$ wasadded and gently swirled, immediately followed by addition of 20ml of concentrated H_2SO_4 with about 250ml distilled water. To the content of the conical flask was added 10ml of concentrated H_3PO_4 and 1ml of 0.5% diphenyl amine which gave a blue colouration. The chromic acid not used up in the oxidation was titrated with 0.4 N $FeSO_4.7H_2O$. A colour change from deep blue to green indicated the end point. A blank which contained all the reagents but without soil was prepared. The blank usually takes 25 ml of 0.4 N $FeSO_4.7H_2O$ to reach the end point.

Calculation:

% organic carbon = (V_1- V_2 x 0.003/W) x 100 x F

V_1 = volume of N. K_2CrO_7 (i.e. 10 ml)

V_{2} = Volume of N. FeSO4.7H_2O used in titration

W = weight of air dried soil

F = correction factor (usually 1.33)

Organic matter = Percentage (%) organic matter is equal to organic carbon (%) x 1.724

3.6.2 Plants analysis from preliminary studies

Samples of potted plants from Table 3.3 were destructively harvested from the ornamental bags, and were assayed at various times during the study as described below to determine plant's performances from the polluted soil samples.

3.6.2.1 Estimation of plant's performance

(i) Selections of plants tolerant to different concentrations of crude oil and heavy metals pollutions

Each of the eight different species of plant was grown for studies in a separate ornamental polyethylene bag. The same eight (8) species of plants were employed for the selection studies based on the protocols as previously described in Table 3.3.

The seedlings that survived and showed the highest tolerance to various concentrations of crude oil and heavy metal polluted soil (3%, 7% and 10%) were selected 150 days after planting (DAP) and were subjected to further studies (percentage survival) in 12% crude oil pollution with the concentrations of heavy metals remaining the same as in previous cases.

(ii) Measurement of plants' height

Plant's shoot height was measured from the shoot base to the apical tip using a meter rule at intervals of 30 to 150 DAP. All the measurements were carried out at the same intervals and concurrently

(iii) Measurement of plants' biomass

Plants whose shoot height had been measured were from the soil level carefull uprooted and washed with tap water. The wet weight (g) of each plant was measured and recorded. (Merkl et al., 2005). This exercise was performed using two (2) replicate potted plants each chosen at random.

(iv) Evaluation of plant's response to stress

The method adopted by Kage, Kochler, & Stützel, (2004) was employed in evaluating the response of the plants to the stress from the crude oil and heavy metals contaminants soil. Plants whose weights were less than 25% of their control plant's weight were considered strongly susceptible, and higher than 50% as tolerant.

3.6.2.2 Percentage performances of shoot length, root length, wet weight and leaf size of test plants

1. Plant growth characteristics/ performance was determined at ever four (4) weeks for twenty-four (24) weeks

2. Crude oil and heavy metal removal was also determined as stated above

The formula below was used to calculate the percentage performance of the measured parameters in comparison to that of the seedlings of the different plants at day 0;

$$\frac{Xi - Xo}{Xo} \times 100$$

Where Xo – seedling (shoot length, root length, wet weight and leaf size) at day 0

Xi - plant (shoot length, root length, wet weight and leaf size) at day t

3.6.3 Sample analysis from the constructed hybrid wetland

Paspalum conjugatum which was the plant that gave the best tolerance during the preliminary sturdies was distructively harvested from the constructed hybrid wetland (CHW) in section 3.12.1 and the following parameters were detetermined as outlined below;

3.6.3.1 Metals in the soil and plant tissue

(i). Soil sample preparation and digestion procedure

Soil samples from constructed hybrid wetland from section 3.5.2 were prepared and digested according to USEPA Method 3050b, as described by Aktaruzzaman, Fakhruddin, Chowdhury, Fardous, & Alam (2013). A 25 g soil sample was oven-dried at 95°C for 48 hours and then ground into a fine powder using a pestle and mortar. Next, 15 g of the powdered sediment sample was placed in a conical flask, and 15 ml of 1M HNO_3 was added. This was followed by the addition of 30 ml of distilled water, and the mixture was left to stand for 24 hours. After the 24-hour period, additional distilled water was added to bring the total weight of the solution to 150 g.

The sample was then centrifuged and filtered using Whatman No. 41 filter paper. The resulting filtrates were analyzed using Atomic Absorption Spectroscopy (AAS).

(ii) Plant sample preparation and digestion procedure

Plant samples were digested with nitric acid for heavy metal determination following the method of DB53/T 288-2009, as described by Allen, Grimshaw, & Rowland (1986). Upon collection, the samples were first rinsed with tap water. The cleaned samples were then air-dried, cut into small pieces, and placed in an oven at 60°C for 24 hours until fully dried. After drying, the samples were ground and homogenized into a fine powder using an electric grinder. The powdered samples were stored in airtight containers to prevent exposure to moisture.

3.6.3.2 Residual heavy metals in water sample from hybrid constructed wetland

Treated water from the constructed hybrid wetland in Section 3.5.2, was collected in a 1000 mL conical flask at 30-day intervals over a period of 150 days. For each water sample, 100 mL was acidified with 20 mL of nitric acid. The acidified mixture was digested in a fume cupboard at 100ºC for one hour until a clear solution formed and the volume reduced to 20 mL. After cooling, the mixture was transferred to a 100 mL volumetric flask, diluted with deionized water, and made up to the 100 mL mark. The solution was filtered using filter paper and analyzed for lead, copper, zinc, and nickel using an Atomic Absorption Spectrophotometer(ASJ,2012).

3.6.3.3 Heavy metal concentrations in plant samples

Atomic Absorption Spectroscopy (AAS) (Model: AA-6401F, Shimadzu, made in Australia), was used for the determination of heavy metals in plant tissues. To provide element specific wavelengths, a light beam from a lamp whose cathode is made of the element being determined was passed through the flame.

i. Extraction and determination of residual crude oil concentration in soil samples.

Soxhlet extraction, following EPA Method SW-846 3540 as described by Adeniji, Okoh, & Okoh (2017), was used for extracting and determining residual crude oil in soil samples. This analysis was conducted at intervals of 0, 30, 60, 90, 120, and 150 days after planting (DAP) for both vegetated and non-vegetated polluted soil samples. 2 g of dried, crushed soil sample, sieved through a 0.5 mm sieve, was weighed into a clean extraction container. Then, 10 ml of pentane was added to the sample, thoroughly mixed, and allowed to settle. The mixture was carefully filtered into a clean extraction bottle using filter paper placed in a Buchner funnel. The extract was then concentrated to 2 ml before being transferred for further cleanup and separation.

ii. Column cleanup and separation

The technique of column cleanup was employed to separate organic analytes from interfering substances of varying polarity that may have been co-extracted with the analytes as adopted by Zemo, O'Reilly, Mohler, Tiwary-Magaw, Synowiec, (2013). This step is crucial when using infrared-based and gravimetric methods, as these are highly sensitive to non-petroleum interferences. However, it is less commonly applied in gas chromatography-based methods because skilled analysts can typically identify the presence of interfering compounds.

The cleanup protocol involved preparing a slurry of 2 g of activated silica gel in 10 ml of methylene chloride, which was then placed into a chromatographic column (10 mm I.D. x 250 mm length) containing 1 cm of moderately packed glass wool at the bottom. A 0.5 cm layer of sodium sulfate was added to the top, and the column was washed with 10 ml of methylene chloride. Pre-elution of the column was done using 20 ml of pentane, which was allowed to flow through until the liquid level was just above the sodium sulfate layer.

Next, 1 ml of the extracted sample was applied to the column. The stopcock was opened, and the eluant was collected using a 10 ml graduated cylinder. Pentane was added in 1-2 ml increments before the sodium sulfate layer was exposed to air, and 8-10 ml of the eluant containing the aliphatic fraction was collected. The column was then further eluted with 1-2 ml increments of a 1:1 mixture of acetone and methylene chloride. A precisely measured volume of 8-10 ml of the eluant containing the aromatic fraction was collected.

For polycyclic aromatic hydrocarbons (PAHs) analysis using gas chromatography, the aromatic fraction was concentrated to 1 ml. The residual oil in the soil sample was determined using Gas Chromatography with a Flame Ionization Detector (GC-FID), utilizing a GC recorder connected to an HP Pentium III MMX computer.

iii. GC analysis

The concentrated aromatic fraction obtained was transferred into a labeled glass vial with a Teflon rubber crimp cap for gas chromatography (GC) analysis, following the method described by Frysinger, Gaines, & Reddy (2002). Using a Hamilton syringe, 1 μL of the concentrated sample was injected through a rubber septum into the GC column. Separation occurred as the vaporized components partitioned between the gas and liquid phases. The samples were detected automatically as they emerged from the column by the Flame Ionization Detector (FID), which measured the retention time—defined as the time in minutes between the sample injection and the recording of the chromatographic peak. The FID response was dependent on the composition of the vapor.

3.6.3.4 TPH in hybrid constructed wetland water

The U.S. Environmental Protection Agency (UEPA) Method 1664 was used to determine Total Petroleum Hydrocarbons (TPH) in water from a constructed hybrid wetland. A 250 ml water sample was measured into a separatory funnel, and the container was rinsed with dichloromethane. Next, 25 ml of dichloromethane was added to the 250 ml water sample, and the mixture was vigorously shaken to ensure thorough extraction of organic materials. The organic extract was then collected into a receiving container (vial) by passing it through a column containing cotton wool, silica gel, and anhydrous sodium sulfate.

The silica gel facilitated the cleanup of the extract by preventing the passage of debris, while the anhydrous sodium sulfate acted as a dehydrating agent, removing any remaining moisture from the sample since the two liquids were immiscible. The collected organic extract was then injected into a gas chromatograph. Using a hypodermic syringe, 1 µL of the concentrated sample extract was introduced through a rubber septum into the chromatograph column. The various fractions of aliphatic compounds (C8-C40) were detected automatically as they emerged from the column by the Flame Ionization Detector (FID), which responded based on the composition of the vapor. The results were reported in ppm or mg/L, which are equivalent units.

3.5 Statistical analysis

Statistical tools used for this study are SPSS version 22 and Microsoft Excel 2010.The mean and standard error (SE) values of two (n = 2) or (n = 3) replicates was calculated and the difference between treatments tested by a one-way ANOVA. If the difference was significant, the student's t-test comparisons was carried out to determine where the difference in sample means lie. The expression 'significant', as used in the text, refers to statistical significance at $p \leq 0.05$

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Physicochemical and microbiological characteristics of unpolluted soil sample

Table 4.1 Shows the result of the physicochemical and microbiological characteristics of the unpolluted soil sample.

4.1.2 Preliminary assessment of plants tolerance to polluted wetland soil

The Results of assessment of plants' tolerance to wetland soil polluted with varying concentrations of crude oil and heavy metals (Zn =8mg/l, Ni = 1mg/l, Cu = 3mg/l, Pb = 0.2mg/l) are shown in Tables 4.2 – 4.7. Table 4.2 shows that *Brachiaria distachyoides* Stapf was not affected much when its experiment indices are compared to the control. This means that there was no significant difference between the experiment and control study

When subjected to 7% crude oil and heavy metals pollution as shown in Table 4.3, *B. distachyoides* Stapf exhibited less tolerance to the toxic effect of the pollutants. As the time of planting increases the difference between the experiment indices and control become more apparent and at day 150, the colour of the leave became yellowish green indicating that the plant is overwhelmed by the toxic effect of the pollutants.

Table 4.4 shows that at 10% crude oil and heavy metal pollution, *B. distachyoides* Stapf stopped growing from the time of planting and died within 30 days after planting which indicated that it was not a plant of choice for the study

Table 4.1a: Physicochemical and microbiological characteristics of unpolluted soil sample

Soil parameters	Values in soil
(i) physicochemical	
Sand	47.3%
Silt	27.7%
Clay	23.0%
Texture	Loamy soil
Organic matter	4,500 mg/kg or 0.45%
Organic carbon	6,500 mg/kg or 0.65%
Total nitrogen	1,800 mg/kg or 0.18%
Available phosphorous	7,982 mg/kg or 0.80%
pH	7.16

Table 4.1bMicrobiological characteristics of unpolluted soil sample

Soil parameters	Values in soil
Total heterotrophic bacterial count	3.4×10^{8} Cfu/g soil
Hydrocarbon utilizing bacterial count	4.6×10^{6} Cfu/g soil
% heterotrophic bacterial that are hydrocarbon utilizers	1.35%
Total fungal count	1.5×10^{4} Cfu/g soil
Hydrocarbon utilizing fungal count	0.9×10^{3} Cfu/g soil
% fungi that are hydrocarbon utilizers	6%

Table 4.1cMicrobiological characteristics of polluted soil sample

Soil parameters	Values in soil
Total heterotrophic bacterial count	3.4×10^8 Cfu/g soil
Hydrocarbon utilizing bacterial count	4.6×10^6 Cfu/g soil
% heterotrophic bacterial that are hydrocarbon utilizers	1.35%
Total fungal count	1.5×10^4 Cfu/g soil
Hydrocarbon utilizing fungal count	0.9×10^3 Cfu/g soil
% fungi that are hydrocarbon utilizers	6%

Table 4.2: Effect of crude oil (3%) and heavy metals polluted soil on *B. distachyoides* Stapf growth indices in wetland

Time	SL (%)		RL (%)		Wt (%)		LW (%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.
30	23.66	51.28	16.67	61.53	16.67	33.33	0.00	100	G	G
60	79.39	158.16	75.00	176.92	75.00	133.33	100	150	G	G
90	127.77	208.57	141.67	269.24	125	326.67	150	150	G	G
120	194.66	397.14	158.33	376.92	216.67	440.00	150	150	G	G
150	251.90	401.43	216.67	384.61	308.33	373.33	150	150	G	G

Key: Exp. – Experimental, Cont. – Control, SL – Shoot length, RL (%) – Percentage Root length, Wt (%) – Percentage Wet weight, LW (%) – Percentage Leave width, LC – Leave colour, G – Green colour

Table 4.3: *B. distachyoides* Stapf in 7% crude oil heavy metals polluted wetland soil

Time	SL(%)		RL(%)		Wt (%)		LW(%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont
30	19.08	51.28	33.33	61.54	25.00	33.33	0.00	100	G	G
60	41.22	158.16	33.33	176.92	58.33	133.33	100	150	G	G
90	89.31	283.57	75.00	269.27	133.33	326.67	150	150	G	G
120	140.45	397.14	135.33	276.92	158.33	440.00	150	150	G	G
150	148.70	401.43	138.33	384.61	161.00	373.33	150	150	YG	G

Table 4.4: *B. distachyoides* Stapf in 10% crude oil heavy metals polluted wetland soil

Time	SL(%)		RL(%)		Wt (%)		LW(%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.
30	1.53	51.29	0.00	61.53	8.33	33.33	0.00	100	GY	G
60	1.53	158.17	8.33	176.92	8.13	133.33	0.00	150	D	G
90	1.53	283.57	8.33	269.24	6.11	326.67	0.00	150	D	G

Tble 4.5 shows that *Cyperus dichrostachyus* Hochst. ex A. Rich has a high tolerance to 3% crude oil and heavy metals pollution. The percentage growth indices of the experimental and control study showed no significant difference since the ratio of both is within 50% range.

When subjected to 7% concentration of crude oil and fixed heavy metal as shown in Table 4.6, the toxic effect of the pollutants seem to be overbearing on *C. dichrostachyus* Hochst. ex A. Rich 30 days after planting. After 90 days, it started showing signs of dying by the leaves turning greenish yellow

The results from Table 4.7 shows that the seedlings of *C. dichrostachyus* Hochst. ex A. Rich couldn't grow in 10% crude oil and fixed concentration of heavy metals pollution and as a result not suitable as the right choice of plant for study.

Table 4.5: *C. dichrostachyus* Hochst. ex A. Rich. in 3% crude oil heavy metals polluted wetland soil

Time	SL (%)		RL (%)		Wt (%)		LW (%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont
30	8.40	43.70	18.15	18.18	58.37	87.50	0.00	0.00	G	G
60	53.79	120.57	75.00	54.75	314.29	350.00	0.00	0.00	G	G
90	128.03	213.48	118.75	109.10	385.71	487.51	25.00	25.00	G	G
120	188.64	263.83	118.75	136.64	457.14	625.00	150	150.00	G	G
150	253.33	340.43	118.75	200.00	557.14	762.50	275	275	G	G

Table 4.6: *C. dichrostachyus* Hochst. ex A. Rich. in 7% crude oil heavy metals polluted wetland soil

Time	SL(%)		RL(%)		Wt (%)		LW(%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont
30	17.61	43.70	0.00	18.18	28.57	87.50	0.00	25	G	G
60	36.39	120.57	54.55	54.75	68.21	350.00	0.00	25	G	G
90	58.73	213.48	93.75	109.10	142.86	487.50	25	25	G	G
120	92.12	263.83	118.75	136.36	160.00	625.00	25	150	GY	G
150	136.21	340.43	118.25	200.00	169.00	762.50	25	275	GY	G

Key: GY – Greenish yellow colour

Table 4.7: *C. dichrostachyus* Hochst. ex A. Rich. in 10% crude oil heavy metals polluted wetland soil

Time	SL(%)		RL(%)		Wt (%)		LW(%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont
30	2.27	43.93	0.00	18.18	1.29	87.50	0.00	25	D	G
60	2.27	120.57	0.00	54.75	0.57	350.00	0.00	25	D	G

Key: D – Dead

In Table 4.8, *Kalanchoe pinnata (Lam.) Pers* was able to grow in 3% crude oil and heavy metal pollution. The percentage growth indices of the experimental and control study showed no significant difference since the ratio of both is within 50% range.

At exposure to 7% crude oil and fixed heavy metals as shown in Table 4.9, *K. pinnata (Lam.) Pers* showed poor percentage growth indices when the experimental and control studies were compared. Growth stop and the plant leaves turned greenish yellow from day 120 after planting.

Table 4.10 shows that 30 days after planting the seedlings of *K. pinnata (Lam.) Pers* in 10% crude oil and fixed heavy metals polluted soil, it died which indicated that it's not a plant of choice for the study

Table 4.8*: K. pinnata* (Lam.) Pers in 3% crude oil heavy metals polluted wetland soil

Time	SL(%)		RL(%)		Wt (%)		LW(%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.
30	10.71	47.37	12.24	47.06	6.94	32.35	33.33	33.33	G	G
60	22.62	76.81	15.78	64.79	15.39	51.32	33.33	33.33	G	G
90	53.74	126.41	24.18	107.25	28.01	72.47	33.33	66.57	G	G
120	79.01	201.18	48.21	174.42	53.10	1O3.74	33.33	66.67	G	G
150	91.24	284.37	71.53	209.22	74.21	186.41	66.57	66.67	G	G

Table 4.9*: K. pinnata (Lam.) Pers* in 7% crude oil heavy metals polluted wetland soil

Time	SL(%)		RL(%)		Wt (%)		LW(%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.
30	9.82	47.37	6.24	47.06	5.94	32.35	33.33	33.33	G	G
60	16.62	76.81	15.78	64.79	12.74	51.32	33.33	33.33	G	G
90	24.31	126.41	21.82	107.25	31.01	72.47	33.33	66.57	G	G
120	49.01	201.18	32.21	174.42	38.35	1O3.74	33.33	66.67	GY	G
150	63.62	284.37	71.53	209.22	47.21	186.41	66.57	66.67	GY	G

Table 4.10: *K. pinnata (Lam.) Pers* in 10% crude oil heavy metals polluted wetland soil

Time	SL(%)		RL(%)		Wt (%)		LW(%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.
30	3.41	47.37	0.00	47.06	2.41	32.35	0.00	33.33	D	G
60	3.41	76.81	0.00	64.79	1.17	32.13	0.00	33.33	D	G

Table 4.11 shows that at 3 % crude oil and heavy metals pollution, *Panicum maximum* Jacq percentage growth indices showed no significant difference between the control and experimental studies. This is an indication that it was able to withstand toxic effects of the pollutants

P. maximum Jacq started showing sign of stress 30 days after planting when exposed to 7% crude oil and heavy metals pollution as shown in Table 4.12 and by day 120 after planting, percentage growth indices became significant when the experimental and control studies are compared

In Table 4.13, *P. maximum* Jacq died within 30 days of exposure to 10% crude oil and heavy metals pollution, which is an indication that this plant is not suitable as a choice for this study

Table 4.11: *P. maximum* Jacq in 3% crude oil heavy metals polluted wetland soil

Time	SL(%)		RL(%)		Wt (%)		LW(%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.
30	26.25	58.50	3.85	24.62	5.61	49.60	2.0	2.0	G	G
60	125.62	190.71	59.62	71.78	80.80	147.60	2.0	2.0	G	G
90	167.50	381.42	71.32	135.21	258.43	311.50	2.0	2.0	G	G
120	240.06	410.20	73.51	138.33	302.80	357.59	2.0	2.0	G	G
150	282.31	432.51	76.72	138.35	321.42	407.47	2.0	2.0	G	G

.

Table 4.12.: *P. maximum* Jacq in 7% crude oil heavy metals polluted wetland soil

Time	SL(%)		RL(%)		Wt (%)		LW(%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.
30	21.72	58.50	3.25	24.62	5.01	49.60	2.0	2.0	G	G
60	39.11	190.71	25.46	71.78	68.12	147.60	2.0	2.0	G	G
90	62.31	381.42	41.32	135.21	105.27	311.50	2.0	2.0	G	G
120	84 93	410.20	41.51	138.33	152.41	357.59	2.0	2.0	GY	G
150	96.41	432.51	48.22	138.35	223.42	407.47	2.0	2.0	GY	G

Table 4.13: *P. maximum* Jacq in 10% crude oil heavy metals polluted wetland soil

Time	SL(%)		RL(%)		Wt (%)		LW(%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.
30	5.47	58.50	0.00	24.62	3.43	49.60	0.00	2.0	D	G
60	5.47	190.71	0.00	71.78	2.17	147.60	0.00	2.0	D	G

Table 4.14 shows that throughout the planting duration of 150 days, percentage growth indices showed no significant difference between the control and experimental studies. Almost all the experimental measured parameters showed 50% ratio when the experimental and control studies were compared

Mimosa pudica L could not stand the stress due to the combined toxic effect of the pollutants at a concentration of 7% crude oil and heavy metals as shown by Table 4.15. This plant started showing extreme stress within 30 days after planting under this environmental conditions and this indicated by the colour of the leaves which turned yellowish green. They died from 120 days after planting which shows that it is not a suitable choice for this study

Table 4.14: *M. pudica* L. in 3% crude oil heavy metals polluted wetland soil)

Time	SL(%)		RL(%)		Wt (%)		LW(%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont
30	22.58	46.15	7.70	21.42	23.07	35.29	20	20	G	G
60	89.94	102.88	46.15	64.28	115.38	141.18	20	20	G	G
90	154.84	345.19	57.69	101.79	223.08	335.29	20	20	G	G
120	272.04	496.15	88.46	141.07	292.23	423.52	20	20	G	G
150	465.60	638.46	125.00	198.93	392.31	588.24	20	20	G	G

Table 4.15: *M. pudica* L. in 7% crude oil heavy metals polluted wetland soil

Time	SL(%)		RL(%)		Wt (%)		LW(%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont
30	13.98	46.15	7.69	21.42	15.38	35.29	20	20	YG	G
60	30.17	102.88	13.45	64.28	30.77	141.18	20	20	YG	G
90	32.84	345.19	13.08	101.79	30.52	335.29	20	20	YG	G
120	32.85	496.15	13.69	141.07	23.54	423.52	20	20	D	G
150	32.91	638.46	13.70	198.93	21.72	588.24	20	20	D	G

D – death

M. pudica L. in 7% crude oil heavy metals polluted wetland soil could not survive. At day 120, it died and stiopped growing, while the control grew and was ever green

Table 4.16 shows like other plants used for this study that *Paspalum conjugatum* P.J. Bergius thrived excellently at a pollutants concentration of 3% crude oil and heavy metals pollution. The percentage growth indices showed no significant difference between the control and experimental studies because almost all the experimental measured parameters showed 50% ratio when the experimental and control studies are compared.

The experimental plants show consistently lower growth in terms of shoot length, root length, plant weight, and leaf width compared to control plants. However, the leaf color remains green in both groups, indicating no severe nutrient deficiencies. The observed differences highlight the importance of optimal growing conditions for plant development and suggest that the experimental conditions are suboptimal for maximizing plant growth. This is an indication that at an increased pollutant concentration of 7% crude oil and heavy metals pollution, the percentage growth indices which is approximately 50% and above, showed that this plant can tolerate pollutants at the above stated concentration.

The table above showed that the experimental conditions resulted in significantly lowering growth rates across all measured parameters compared to the control group. The data suggests that the experimental plants are subjected to stress factors (10% crude oil and heavy metals pollution) limiting their growth potential. However, the persistent green color of the leaves indicates that these conditions are not causing severe nutrient deficiencies. The data from the LC suggested that at this pollutants concentration, though other measured parameters were affected, *Paspalum conjugatum* P.J. Bergius met the requirements of choice for this study

Table 4.16: *P. conjugatum* P.J. Bergius in 3% crude oil heavy metals polluted wetland soil

Time	SL(%)		RL(%)		Wt (%)		LW(%)		LC (%)	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont
30	17.70	32.28	13.04	23.08	15.38	23.08	0.00	0.00	G	G
60	74.34	189.76	26.09	38.48	27.27	61.53	9.10	9.10	G	G
90	168.40	270.87	56.52	61.53	81.81	146.15	26.67	26.27	G	G
120	283.05	343.31	69.57	96.15	127.27	176.92	26.67	26.67	G	G
150	322.12	469.29	69.57	96.15	166.67	223.08	26.67	26.67	G	G

Table 4.17: *P. conjugatum* P.J. Bergius in 7% crude oil heavy metals polluted wetland soil

Time	SL(%)		RL(%)		Wt (%)		LW(%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.
30	15.31	32.28	13.04	23.08	18.18	23.08	0.00	0.00	G	G
60	51.33	189.76	13.04	38.46	36.36	61.53	9.10	9.10	G	G
90	137.17	270.87	34.78	61.53	54.54	146.15	27.67	27.67	G	G
120	194.69	343.31	47.87	96.15	109.10	176.91	27.67	27.67	G	G
150	252.21	469.29	65.22	96.15	107.69	223.08	27.67	27.67	G	G

Table 4.18: *P. conjugatum* P.J. Bergius in 10% crude oil heavy metals polluted wetland soil

Time	SL(%)		RL(%)		Wt (%)		LW(%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.
30	7.07	32.28	8.04	23.08	9.10	23.08	3.15	0.00	G	G
60	39.82	189.76	13.04	38.46	11.18	61.53	5.10	9.10	G	G
90	51.76	270.87	16.78	61.53	36.27	146.15	8.18	27.67	G	G
120	72.79	243.31	18.83	96.15	48.23	176.92	11.18	27.67	G	G
150	91.93	469.26	18.82	96.15	63.55	223.08	11.18	27.63	G	G

Like all previously assayed plants, *Mariscus rotundus* was able to show positive response to 3% crude oil and heavy metals pollution within 150 days after planting. The percentage growth indices at this pollution concentration between the experimental and control is 50% and above and indication that this plant can stand the stress of the pollutant at this concentration

From the above table, *Mimosa pudica* L could not stand the stress due to the combined toxic effect of the pollutants at a concentration of 7% crude oil and heavy metals. This plant started showing extreme stress within 90 days after planting and this in indicated by the colour of the leaves which turned yellowish green. After this time, growth stopped due to the toxic effect of the pollutant which seem to have overwhelmed the plant. The growth indices dropped far below the accepted value

The above table shows that *Mariscus rotundus* died within 30 days of exposure to 10% of the above stated pollutants which is an indication that this plant is not suitable as a choice for this study

Table 4.19: *M. rotundus* in 3% crude oil heavy metals polluted wetland soil

Time	SL(%)		RL(%)		Wt (%)		LW(%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont
30	46.43	110.81	13.51	29.73	120.83	188.46	25	25	G	G
60	136.43	187.84	81.08	94.59	180.76	261.54	25	25	G	G
90	210.00	331.10	137.84	175.68	291.67	350.00	25	25	G	G
120	367.14	431.08	145.95	202.70	379.17	480.77	25	25	G	G
150	457.14	602.70	197.80	202.70	525.00	715.38	25	25	G	G

.

Table 4.20: *M. rotundus* in 7% crude oil heavy metals polluted wetland soil

Time	SL(%)		RL(%)		Wt (%)		LW(%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp	Cont
30	17.14	110.81	8.11	29.73	70.83	188.42	25	25	G	G
60	87.86	187.84	48.65	94.59	111.67	261.54	25	25	G	G
90	89.51	331.40	54.57	175.68	115.00	350.00	25	25	YG	G
120	91.00	431.08	57.84	202.70	118.33	480.00	25	25	YG	G
150	91.86	602.70	58.75	202.70	119.00	715.38	25	25	YG	G

Table 4.21: *M. rotundus* in 10% crude oil heavy metals polluted wetland soil

Time	SL(%)		RL(%)		Wt (%)		LW(%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.
30	12.86	110.81	1.11	29.73	13.10	188.46	5.0	25	D	G
60	12.82	187.84	1.12	94.59	8.06	261.54	5.0	25	D	G

Table 4.22 shows like other plants used for this study that *Mariscus ligularis* (L.) tolerated at a pollutants concentration of 3% crude oil and heavy metals pollution. The percentage growth indices showed no significant difference between the control and experimental studies because almost all the experimental measured parameters showed 50% ratio when the experimental and control studies are compared

The above table showed that at an increased pollutant concentration of 7% crude oil and heavy metals, *M. ligularis* (L.) started to exhibit non tolerance to the pollutant toxicity. This is marked by reduction in the growth percentage index which is far below 50% when the experimental data are compared to the control. After day 120 of planting, the leaves of the plants started showing greenish yellow coloration, which is an indication of stress due to toxic impact of the pollutants on the plants.

The above table shows that *s M. ligularis* (L.) died within 60 days of planting them in 10% of the above stated pollutants concentration which is an indication that this plant is not suitable as a choice for this study

Table 4.22: *M. ligularis* (L.) in 3% crude oil heavy metals polluted wetland soil

Time	SL(%)		RL(%)		Wt (%)		LW(%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont
30	10.70	57.14	8.70	34.52	15.00	38.89	0.25	0.25	G	G
60	16.56	81.37	10.97	57.42	20.33	64.44	0.25	0.25	G	G
90	53.00	126.67	37.74	80.64	48.33	94.44	0.25	0.25	G	G
120	91.70	290.47	87.10	96.77	92.00	138.89	0.25	0.25	G	G
150	129.58	382.54	87.10	103.23	102	288.89	0.25	0.25	G	G

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Table 4.23: *M. ligularis* (L.) in 7% crude oil heavy metals polluted wetland soil

Time	SL(%)		RL(%)		Wt (%)		LW(%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont
30	10.07	57.14	38.71	34.52	13.81	38.89	0.25	0.25	G	G
60	17.30	81.39	11.36	57.42	21.02	64.44	0.25	0.25	G	G
90	46.38	126.67	35.74	80.64	44.26	94.44	0.25	0.25	G	G
120	52.96	290.97	36.26	96.77	45.33	138.89	0.25	0.25	G	G
150	59.48	382.00	36.26	103.23	47.00	288.89	0.25	0.25	GY	G

Table 4.24: *M. ligularis* (L.) in 10% crude oil heavy metals polluted wetland soil

Time	SL(%)		RL(%)		Wt (%)		LW(%)		LC	
(days)	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.	Cont
30	2.11	57.14	0.00	34.52	3.25	6.89	0.00	0.25	GY	G
60	5.90	81.39	1.67	57.42	5.63	18.44	0.25	0.25	D	G
90	5.90	126.67	1.67	80.64	3.85	19.02	0.25	0.25	D	G

4.1.3 Percentage performances of shoot length, root length, wet weigh and leave size of study plants at 10% crude oil and heavy metal polluted soil.

Figure 4.1 illustrates percentage (%) shoot length of eight plants (A to H) over a 150-day period, with measurements taken at 30-day intervals. The data reveals distinct growth patterns among the plants. Plant F displays the most dramatic percentage shoot length increase growth followed by Plant A which shows minimal and consistent increase in shoot length. Similarly, plant B mirrors this pattern but both could not tolerate the toxic effects of the pollutants for a long growth period. Plant C, D, G and H show initial growth in shoot length but the growth ceases, remaining flat at 0 for the rest of the period. Plant E shows no growth throughout the entire period, as the value remains at 0 from the beginning to the end.

Figure 4.2 represents the % Root length of eight plants (Plant A to Plant H) subjected to 10% and fixed concentrations heavy metals polluted soil over five time intervals: 30, 60, 90, 120, and 150 days. The recorded values show measurements, and zero values indicate either no measurable growth or that the plants are no longer growing. Plant A, B, C, D, E, F, G and H initially showed no root growth but Plant F shows significant growth across all time points. Plant B, Plant C, Plant D, and Plant E did not show any significant growth throughout the entire period, as all recorded values are zero. The line graph created from this data, time (in days) on the x-axis and the growth measurements on the y-axis. The line for Plant F show a steady upward trend, indicating its continuous growth over the observation period, while the lines for Plants A, G, and H rise initially and then flatten out, indicating the cessation of growth. Plants B, C, D, and E would have flat lines at zero, showing no growth at all throughout the period. The graph visually demonstrate Plant F's strong growth compared to the other plants, which either stopped growing early or did not grow at all.

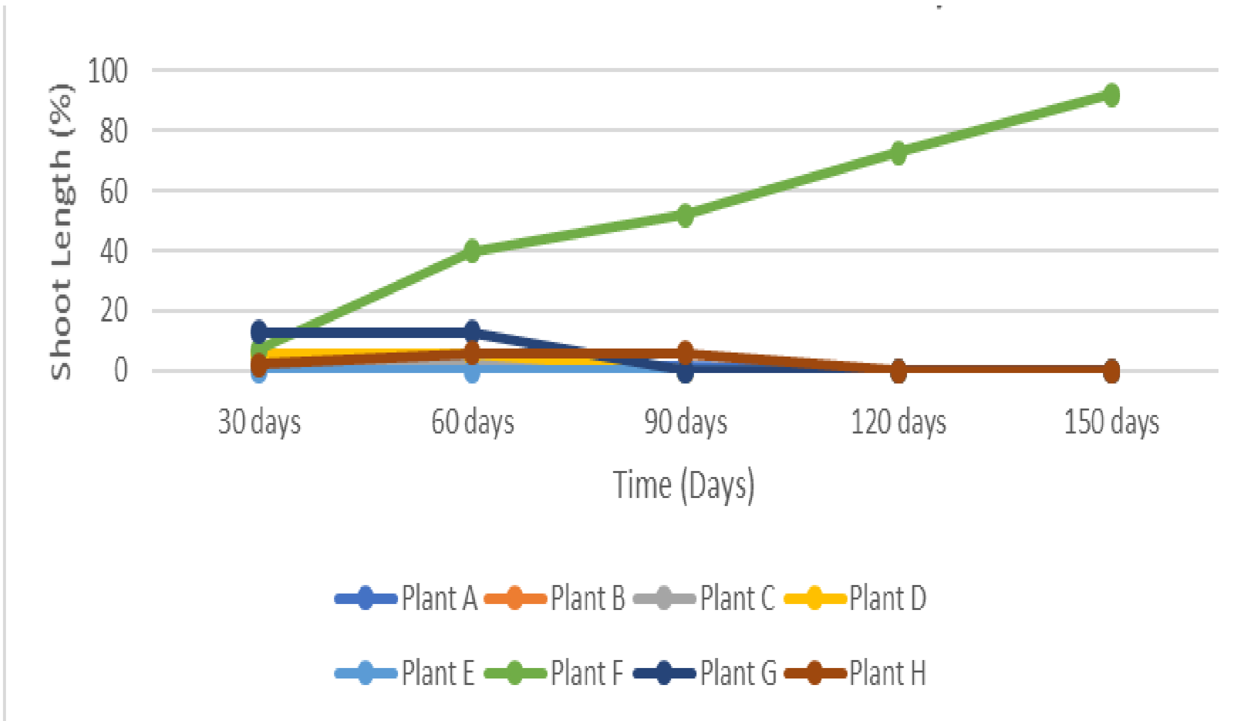

Figure 4.1: Shoot length (%) of the study plants performance in 10% crude oil with fixed concentration of heavy metals

Key: Plant (A–Brachiaria *distachyoides* Stapf, B–Cyperus *dichrostachyus* Hochst. ex A. Rich, C–Kalanchoe *pinnata* (Lam.) Pers, D–Panicum *maximum* Jacq, E–Mimosa *pudica* L, F–*Paspalum conjugatum* P.J. Bergius, G–Mariscus *rotundus*, H–Mariscus *ligularis* L.)

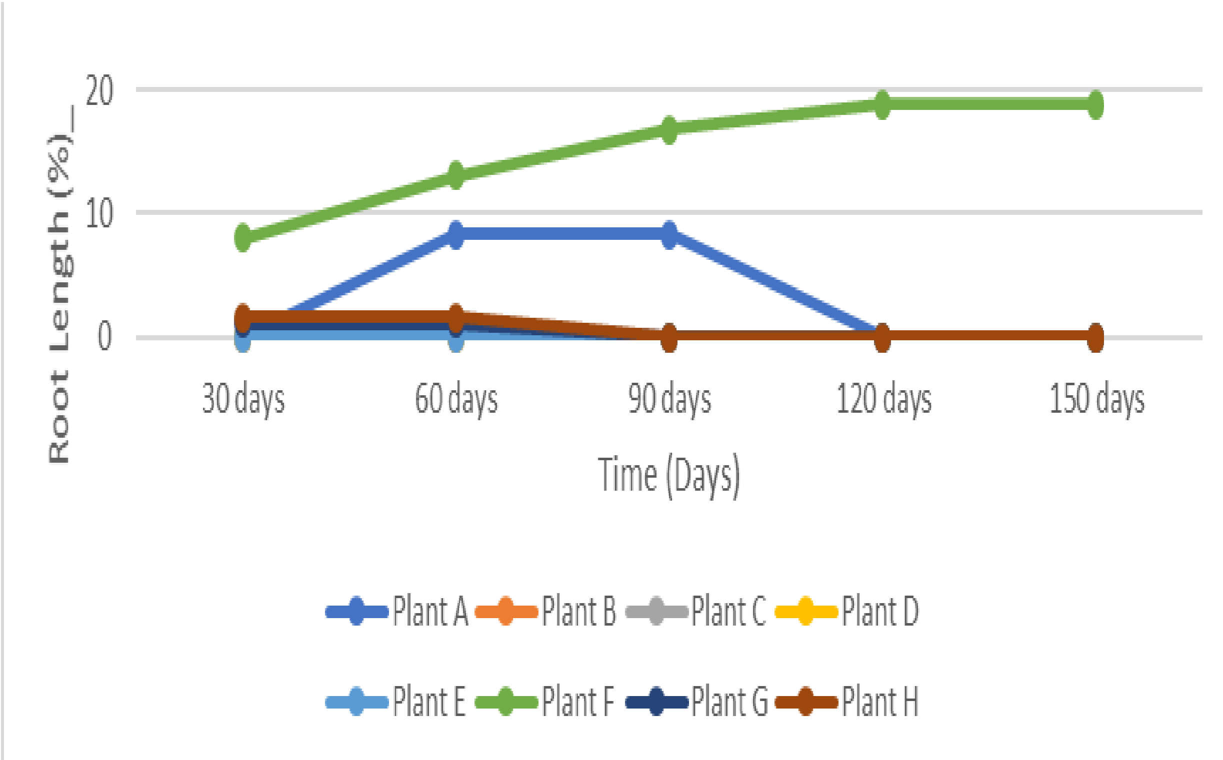

Fig. 4. 2: Root length (%) of the study plants performance in 10% crude oil with fixed concetration of heavy metals.

Figure 4.3 illustrates the % wet weigh gain patterns of eight different plants (A to H) over a period of 150 days. Each plant exhibits distinct growth behavior, which is tracked at five time intervals: 30, 60, 90, 120, and 150 days. The y-axis represents the growth (possibly height or biomass), and the x-axis represents the time in days.

Plant A and G shows similar wet weight with plant A showing better tolerance but both showed no weight gain at day 120 an indication that they were overwhelmed by the toxic effect of the pollutants. Plant B displays very limited weight gain and by day 90, it stoped growing entirely, as shown by a flat line at zero for the remainder of the period. Plant C exhibits a similar trend to Plant B. Plant D follows a comparable pattern as exhibited by A and B. Plant E showed no growth throughout the observation period, and indication that it could not survive the toxic effect of the pollutant at 7%. Plant F, on the other hand, exhibits continuous and substantial weight gain. This plant shows the most significant growth out of all the plants in the dataset. The flat lines of Plants A, B, C, D, G, and H after day 90, and Plant E throughout the period, indicate the cessation or lack of growth. This suggests a varied response among the plants, with Plant F likely being the most tolerant or adaptable to these conditions.

The growth patterns of eight plants (A to H) in figure 4.4 over a 150-day period shows that plant F demonstrates the best performance in terms of leaf width growth in the presence of crude oil and heavy metals, showing a significant and sustained increase over time. Plant A and Plant E initially show some growth but then decline to 0%, indicating possible sensitivity or inability to sustain growth after an initial response. Plants B, C, D, G, and H show no growth in leaf width, suggesting they may not tolerate the combination of crude oil and heavy metals or are not suitable for growth in such conditions. This suggests a varied response among the plants, with Plant F likely being the most tolerant or adaptable to these conditions.

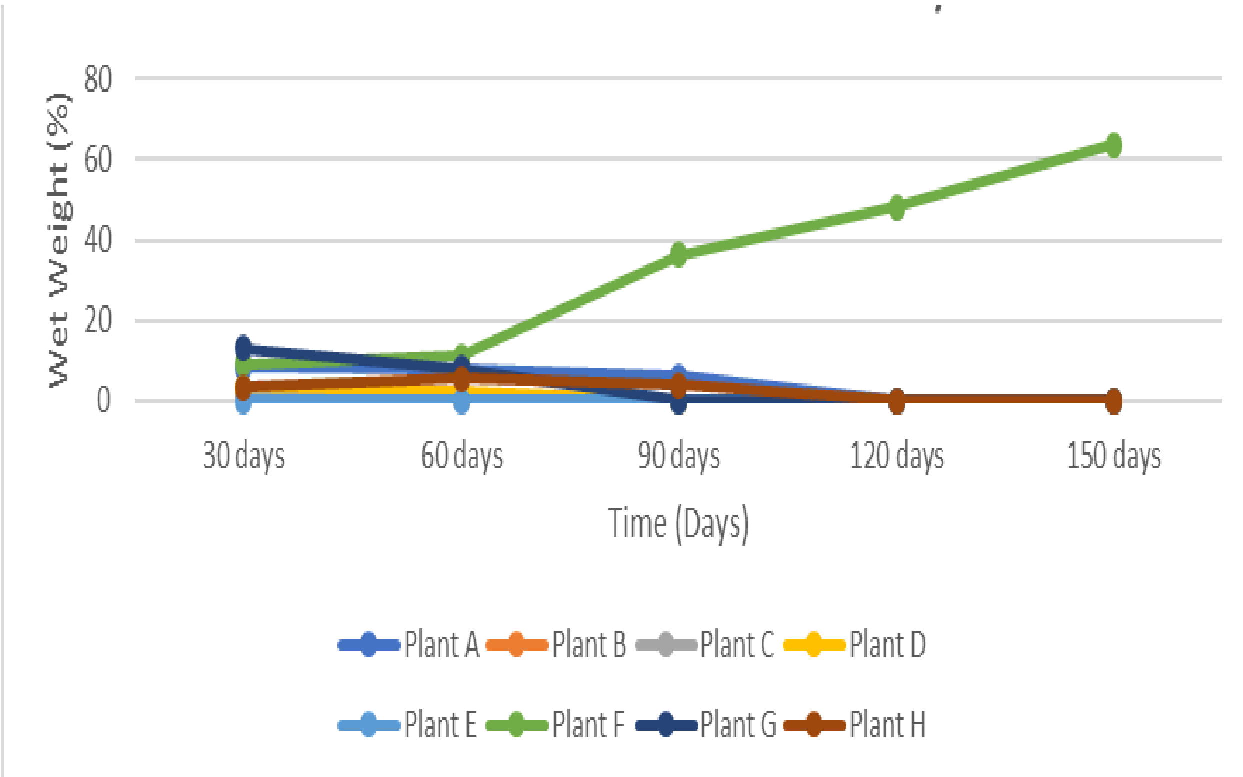

Figure 4.3: wet weigh (%) of the study plants performance in 10% crude oil with fixed concentration of heavy metals

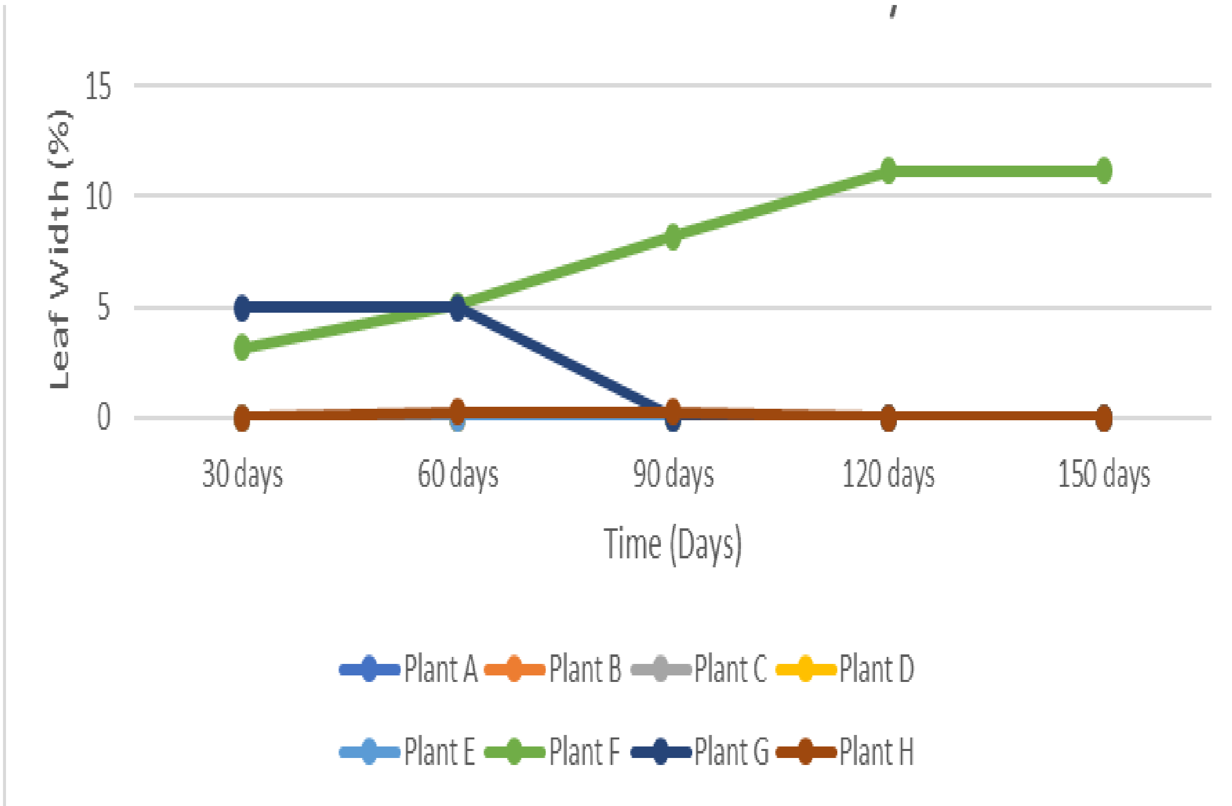

Figure 4.4: Leaf width (%) of the study plants performance in 10% crude oil with fixed concentration of heavy metals

4.1.4 Plant of choice for the study

Based on the outcome of the preliminary study, *Paspalum conjugatum* P.J. Bergius was the plant of choice. Table 4.16-18 showed that although the experimental conditions resulted in significantly lowering growth rates across all measured parameters compared to the control group. However, the persistent green color of the leaves indicated that these conditions are not causing severe nutrient deficiencies though measured parameters were affected but their tolerance to the maximum pollutant concentration used for this study superseded that of other plants species used for this study.

4.1.5 Biochemical identification of bacterial and fungal isolates

In this section, the Tables (Tables 4.25-4.29) list the names of bacterial and fungal genera inferred from traditional biochemical tests. These tests involve studying the metabolic properties of bacteria, such as their ability to metabolize specific substrates or produce certain enzymes, to narrow down the potential identification of the bacteria. Fungal identification was based on hyphal and spore observation, pigmentation and ability to ferment some carbohydrates.

Table 4.25: Biochemical characterization and probable identity of heterotrophic bacterial isolates

Code	organisms	Grams reaction	Shp	Pg	Es	Mt	Ct	Ox	Vp	Mr	Ur	O/F	Ci	In
M1	*Arthrobacter* spp.	+	rod	-	-	-	+	-	-	-	+	+/-	-	-
M2	*Agrobacterium* spp.	-	rod	-	-	+	+	+	-	-	+	+/-	+	+
M3	*Flavobacterium* spp.	-	rod	+	-	-	+	+	-	-	-	+/-	+	-
M4	*Corynebacterium* spp.	-	plm	+	-	-	+	-	-	-	-	-/+	+	-
M5	*Mycobacterium* spp.	-	rod	+	-	-	+	+	-	-	-	+/-	-	-
M6	*Rhodococcus* spp.	+	sph	+	-	-	+	+	-	-	-	+/-	+	-
M7	*Flavobacterium* spp.	-	rod	+	-	-	+	+	-	-	-	+/-	+	-
M8	*Agrobacterium* spp.	-	rod	-	-	+	+	+	-	-	+	+/-	+	+
M9	*Corynebacterium spp*	-	plm	+	-	-	+	-	-	-	-	-/+	+	-
M10	*Rhodococcus* spp.	+	sph	+	-	-	+	+	-	-	-	+/-	+	-

M11	*Agrobacterium* spp.	-	rod	-	-	+	+	+	-	-	+	+/-	+	+
N12	*Corynebacterium* spp.	-	plm	+	-	-	+	-	-	-	-	-/+	+	-
M13	*Flavobacterium* spp.	-	rod	+	-	-	+	+	-	-	-	+/-	+	-
M14	*Flavobacterium* spp.	-	rod	+	-	-	+	+	-	-	-	+/-	+	-
M15	*Rhodococcus* spp.	+	sph	+	-	-	+	+	-	-	-	+/-	+	-
M16	*Arthrobacter* spp.	+	rod	-	-	-	+	-	-	-	+	+/-	-	-
M17	*Acinetobacter* spp.	-	rod	w	-	-	+	-	-	-	-	-	+	+
M18	*Enterobacter* spp.	+	rod	w	-	+	+	+	-	+	+	+	+	-
M19	*Arthrobacter* spp.	+	rod	-	-	-	+	-	-	-	+	+/-	-	-
M20	*Rhizobium* spp.	-	rod	y	-	+	+	+	-	-	+	-	+	-
M21	*Alcaligenes* spp.	-	rod	c	-	+	+	+	-	+	+	+	+	-
M22	*Rhodococcus* spp.	-	sph.	y	-	+	-	-	+	-	-	+	+	-
M23	*Arthrobacter*	-	rod	c	+	+	+	+	-	-	+	-	-	-

	spp.													
M24	*Micrococcus* spp.	+	cocci	y	-	-	+	-	-	+	-	+	-	-
M25	*Rhizobium* spp	-	rod	y	-	+	+	+	-	-	+	-	+	-
M26	*Rhizobium* spp	-	rod	y	-	+	+	+	-	-	+	-	+	-
M27	*Arthrobacter* spp.	-	rod	y	-	+	-	-	+	-	-	+	+	-
M28	*Micrococcus* spp.	+	cocci	y	-	-	+	-	-	+	-	+	-	-
M29	*Pseudomonas* spp.	-	rod	y	-	+	+	+	-	-	+	-	+	-
M30	*Pseudomonas* spp.	-	rod	y	-	+	+	+	-	-	+	-	+	-
M31	*Alcaligenes* spp.	-	rod	c	-	+	+	+	-	+	+	+	+	-
M32	*Bacillus* spp.	-	rod	c	+	+	+	+	-	-	+	-	-	-
M33	*Rhizobium* spp.	-	rod	y	-	+	+	+	-	-	+	-	+	-
M34	*Erwinia* spp.	-	rod	y	-	+	-	-	+	-	-	+	+	-
M35	*Enterobacter* spp.	+	rod	w	-	+	+	+	-	+	+	+	+	-

Key: Es. – Endospore; Gs. – Gram stain; Shp. – Shape; Pg. – Pigmentation; Mt. – Mortality; Ct. – Catalase test; Ox. – Oxidase; Vp. – Voges-proskauer; MR. – Methyl Red; Ur. – Urease;

O/F. − Oxidation/Fermentation; Ci. − Citrate utilization; In. − Indole; C − Cream; W − White; Y − Yellow; plm − Pleomorphic shape, sph − Spherical shape

Table 4.26: Biochemical characterization and probable identity of hydrocarbon utilizing bacterial (HUB) isolates

S/N	organisms	GS	Shp	Pg	Es	Mt	Ct	Ox	VP	MR	Ur	O/F	Ci	In
M1	*Pseudomonas* spp.	-	rod	y	-	+	+	+	-	-	+	-	+	-
M2	*Acinetobacter* spp.	-	rod	w	-	-	+	-	-	-	-	-	+	+
M3	*Enterobacter* spp.	+	rod	w	-	+	+	+	-	+	+	+	+	-
M4	*Alcaligenes* spp.	-	rod	c	-	+	+	+	-	+	+	+	+	-
M5	*Erwinia* spp.	-	rod	y	-	+	-	-	+	-	-	+	+	-
M6	*Bacillus* spp.	-	rod	c	+	+	+	+	-	-	+	-	-	-
M7	*Micrococcus* spp.	+	cocci	y	-	-	+	-	-	+	-	+	-	-
M9	*Pseudomonas* spp.	-	rod	y	-	+	+	+	-	-	+	-	+	-
M10	*Erwinia* spp.	-	rod	y	-	+	-	-	+	-	-	+	+	-
M11	*Micrococcus*	+	cocci	y	-	-	+	-	-	+	-	+	-	-

	spp.													
M12	*Pseudomonas* spp.	-	rod	y	-	+	+	+	-	-	+	-	+	-
M13	*Pseudomonas* spp.	-	rod	y	-	+	+	+	-	-	+	-	+	-
M14	*Acinetobacter* spp.	-	rod	w	-	-	+	-	-	-	-	-	+	+
M15	*Acinetobacter* spp.	-	rod	w	-	-	+	-	-	-	-	-	+	+
M16	*Alcaligenes* spp.	-	rod	c	-	+	+	+	-	+	+	+	+	-
M17	*Bacillus* spp.	-	rod	c	+	+	+	+	-	-	+	-	-	-
M18	*Pseudomonas* spp.	-	rod	y	-	+	+	+	-	-	+	-	+	-
M19	*Alcaligenes* spp.	-	rod	c	-	+	+	+	-	+	+	+	+	-
M20	*Pseudomonas* spp.	-	rod	y	-	+	+	+	-	-	+	-	+	-
M22	*Erwinia* spp.	-	rod	y	-	+	-	-	+	-	-	+	+	-

Table 4.27 Morphological and biochemical characteristics and probable identity of fungal isolates

Code	Isolate	Type of hyphae	Type of spore	Sucrose fermenter	Maltose fermenter	pigmentation
F1	*Penicillium* spp.	sepatate	Smooth conidiophore	−	−	green
F2	*Mucor* spp.	aseptate	smooth	−	−	brown
F3	*Aspergillus* spp.	septate	Smooth chain conidiophore	−	−	black
F4	*Fusarium* spp.	septate	oval	−	−	white
F5	*Candida* spp.	Yeast form	absent	+	+	cream
F6	*Microsporum* spp.	septate	Smooth macrocondia	−	−	reddish-brown.

Table 4.28: Morphological and biochemical characteristics and probable identity of hydrocarbon utilizing fungal isolates from polluted soil sample

S/N	Isolate	Type of hyphae	Type of spore	Sucrose fermenter	Maltose fermenter	pigmentation
G1	*Penicillium* spp.	sepatate	Smooth conidiophore	−	−	green
G2	*Aspergillus* spp.	septate	Smooth chain conidiophore	−	−	black
G3	*Fusarium* spp.	septate	Oval	−	−	white

Table 4.29: Percentage occurrence of different heterotrophic bacteria isolates

S/N	Organism	Frequency	% Occurrence
1	*Arthrobacter* spp.	iiii	14.29
2	*Agrobacterium* spp.	iii	8.57
3	*Flavobacterium* spp.	iiii	11.42
4	*Corynebacterium* spp.	iii	8.63
5	*Mycobacterium* spp.	i	2.86
6	*Rhodococcus* spp.	iiii	11.42
7	*Acinetobacter* spp.	i	2.85
8	*Enterobacter* spp.	ii	5.71
9	*Pseudomonas* spp.	ii	5.71
10	*Alcaligens* spp.	ii	5.71
11	*Erwinia* spp.	i	2.85
12	*Bacillus* spp.	i	2.85
13	*Micrococcus* spp.	ii	5.71
14	*Rhizobium* spp.	iiii	11.42
	Total	35	100.00

4.1.6 Molecular identified of isolated (bacteria) strains

The obtained 16s rRNA sequence from the isolate produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The 16S rRNA of the isolates showed a percentage similarity to other species at 100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolates within the *Pseudomonas, Acinetobacter, Enterobacter*, *Pantoa,* and *Lysinibacillus, Kocuria sp* and revealed a closely relatedness to *Pseudomonas xiamenensis, Acinetobacter baumanii*, *Enterobacter cloacae, Pantoa dispersa,* and *Lysinibacillus fusiformis* and *Kocuriapalustris* (Figure. 4.5).

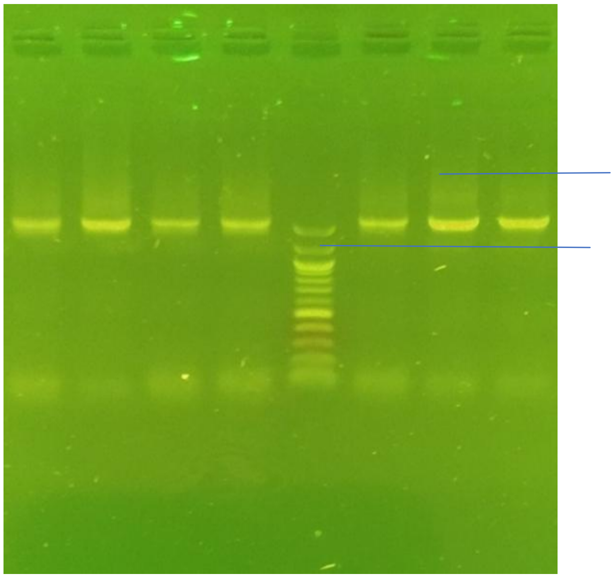

Figure 4.5 Agarose gel electrophoresis showing the plasmid bands. Lane 1-7 showing the 16SrRNA bands at 1500bp while lane L represents the 100bp molecular ladder

4.1.7 Genome of each hydrocarbon utilizing bacterial isolate

>M1_27-F_H08 P_23.ab1

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TGGGGAATATTGGACAATGGGGGCAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAA

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AACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTA

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CAGCCATCTGGCCTAATACTGACGCTGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC

ATGCCGTAA

Key: M1-M7 — Isolates identification number

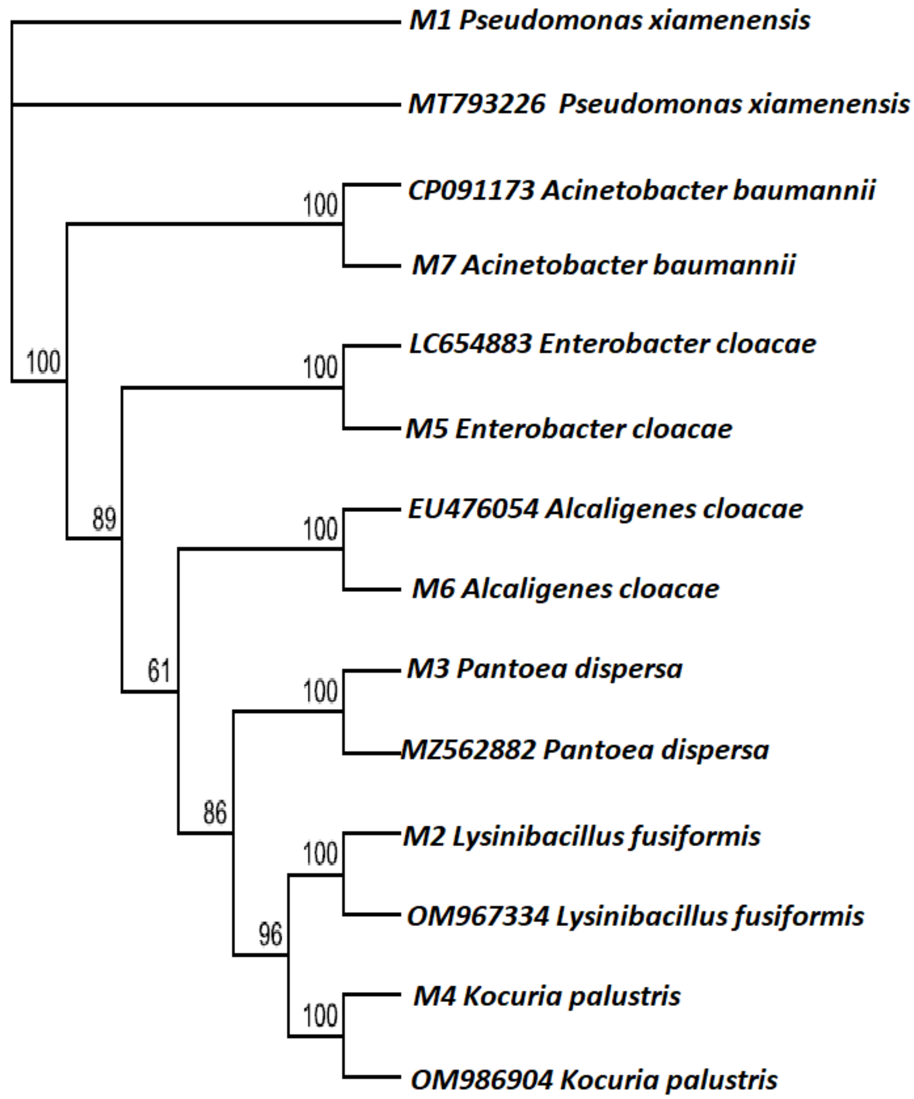

Figure 4.6 phylogentic tree

In Table 4.30 lists the actual molecular identities of the isolates. Molecular identification techniques, such as DNA sequencing, provide a more accurate and precise way of identifying bacteria by analyzing their genetic material. This Table highlights the potential discrepancies between traditional biochemical identification methods and more accurate molecular identification techniques. It suggests that while biochemical tests may provide an initial indication of the identity of bacterial isolates, molecular methods are often necessary for precise and reliable identification

Table 4.30: Molecular equivalent of biochemical identified hydrocarbon utilizing bacteria (HUB) isolates

IIN	Probable identity of isolates from biochemical tests	Molecular identity of isolate
M1	*Pseudomonas* spp.	*Pseudomonas xiamenensis*
M2	*Bacillus* spp.	*Lysinibacillus fusiformis*
M3	*Erwinia* spp.	*Pantoa dispersa*
M4	*Micrococcus* spp	*Kocuria palus*
M5	*Enterobacter* spp.	*Enterobacter cloacae*
M6	*Alcaligens* spp.	*Alcaligenes cloacae*
M7	*Acinetobacter* spp	*Acinetobactor baumanii*

Key

IIN – Isolate identification number

Table 4.32 detailed the biosurfactant production capabilities of various organisms, along with the degree of production and the average size of the collapse drop they produce. The column lists the different bacterial species or organisms being studied. The presence of a "+" sign indicates that the organism is capable of producing biosurfactants. Biosurfactants are molecules produced by microorganisms that have surfactant properties, meaning they can reduce surface tension between two liquids or a liquid and a solid.

This column describes the level of biosurfactant production by each organism. It's categorized as high, moderate, or low. This column provides the average size of the collapse drop produced by each organism, measured in millimeters. The collapse drop refers to the size of the drop formed when a biosurfactant collapses a bubble or foam.

Pseudomonas xiamenensis produces biosurfactants at a high degree and generates collapse drops with an average size of 3.4 mm. *Acinetobacter baumannii* also produces biosurfactants, but at a low degree, and the collapse drops it produces are smaller, with an average size of 1.6 mm. *Lysinibacillus fusiformis* produces biosurfactants at a low degree as well, with collapse drops slightly smaller than those of *Acinetobacter baumannii*, averaging 1.5 mm.

4.1.8 Drop collapse assay

Table 4:31: Biosurfactant production ability of different hydrocarbon utilizing bacterial isolates from the polluted soil sample

Organism	Biosurfactant Production	Degree of Production	Av. Size of Collapse Drop (mm)
Pseudomonas xiamenensis	+	High	3.4
Acinetobacter baumannii	+	Low	1.6
Alcaligenes cloacae	+	Moderate	2.2
Enterobacter cloacae	+	Moderate	2.0
Pantoea dispersa	+	Moderate	2.4
Lysinibacillus fusiformis	+	Low	1.5
Kocuria palus	+	Moderate	2.6

Table 4.32 provides insight into how pollutants, specifically heavy metals and crude oil, influence the populations of heterotrophic bacteria and fungi in soil samples over a period of six months.

At the beginning of the experiment (time 0), the average THBC count in the polluted soil (Exp.) is 2.8×10^6 cfu/g, while in the unpolluted soil (Cont.), it is 3.4×10^8 cfu/g. Over the course of six months, the THBC count in the polluted soil decreases, indicating a negative impact of pollutants. However, it's notable that the THBC count in the unpolluted soil also decreases over time. The TFC count in the polluted soil shows fluctuations over time, while the TFC count in the unpolluted soil remains relatively stable.

Table 4.32: Effect of pollutants (heavy metals and crude oil (10%)) on mean total heterotrophic bacterial count (THBC) and mean total fungal counts (TFC) in polluted soil samples

Time	Mean THBC		Time	Mean TFC	
(days)	Exp. $\times 10^6$cfu/g	Cont. $\times 10^8$cfu/g	(days)	Exp$\times 10^3$cfu/g	Cont.$\times 10^4$cfu/g
0	2.8	3.4	3	1.2	1.2
2	2.4	2.8	6	0.9	1.5
4	1.5	3.1	9	0.6	0.8
6	1.3	3.6	12	1.1	1.3
8	1.6	3.3	15	1.3	3.2

4.1.9 Toxicology assay of native soap, poultry droppings and combination of both on HUB and HUF isolates from polluted soil

Figure 4.7 highlights that HUB counts were consistent across all concentrations (1%, 10% and 30%) of natural soap (NS) during Day 1 and 2. However, starting from Day 3 and continuing until Day 6, concentration (A representing 1%) exhibited the highest HUB counts, indicating its superiority in supporting HUB growth over other concentrations during this period. The figure suggests that the 1% concentration of NS (native soap) yielded the highest and significantly different hydrocarbon utilizing bacterial count compared to concentrations B and C.

In summary, these results highlight the effectiveness of the 1% concentration of NS in promoting higher hydrocarbon utilizing bacterial counts, as evidenced by its significant difference from other concentrations.

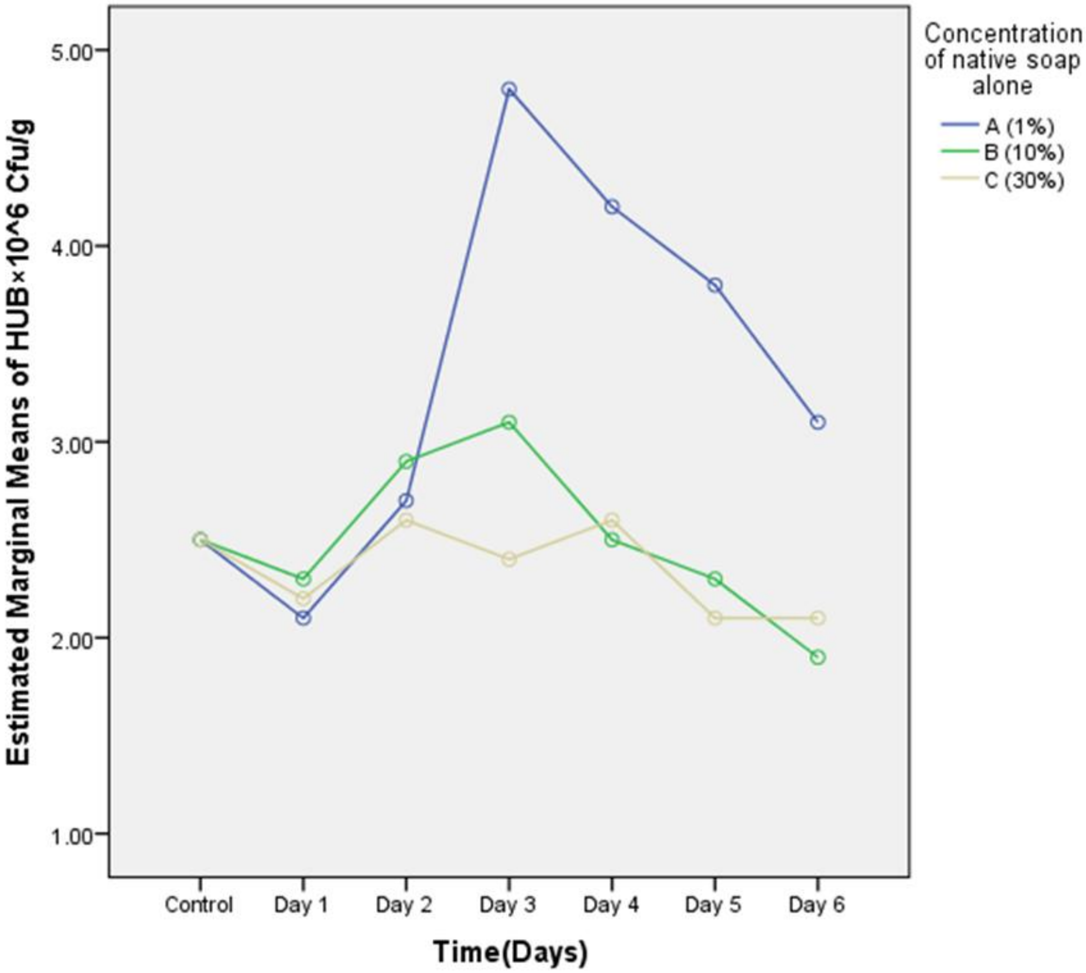

Figure 4.7 Growth response of hydrocarbon utilizing bacterial (HUB × 10^6 Cfu/g) in different concentrations of native soap solution

Table 4.33 indicates that all the concentrations gave significant different effects on the THUF counts. However, by size, concentration A (1% NS) had the highest effect (that is resulted to highest THUFC counts).

Figure 4.8 illustrates the variation in Heterotrophic Utilizing Fungal (HUF) counts across three different concentrations over the course of several days. Upon critical examination of the figure, it becomes apparent that the HUF counts fluctuated with an increase and subsequent decrease as the duration of time (days) progressed.

Initially, from Day 4 to Day 6, the HUF counts exhibited distinct differences among the three concentrations. This suggests that the concentrations had varying effects on fungal growth during this period. The fluctuations observed in HUF counts over time indicate dynamic changes in fungal populations, with counts increasing initially and then decreasing.

The critical examination of this figure underscores the dynamic nature of fungal growth in response to different concentrations of the natural soap (NS) over time. It suggests that the effectiveness of each concentration in promoting fungal growth may vary depending on the duration of exposure. Further analysis and interpretation of such trends are crucial for understanding the temporal dynamics of fungal populations and optimizing conditions for their growth.

Table 4.33: Effect of different Concentrations of native soap on hydrocarbon utilizing fungi isolates from soil polluted with crude oil and heavy metals

Tukey HSD Test for Effect of Concentration of Native soap on THUFC×10^4 Cfu/g

Concentration of native soap alone	N	Subset 1	2	3
C (30%)	21	1.7286		
B (10%)	21		2.6429	
A (1%)	21			3.4000
Sig.		1.000	1.000	1.000

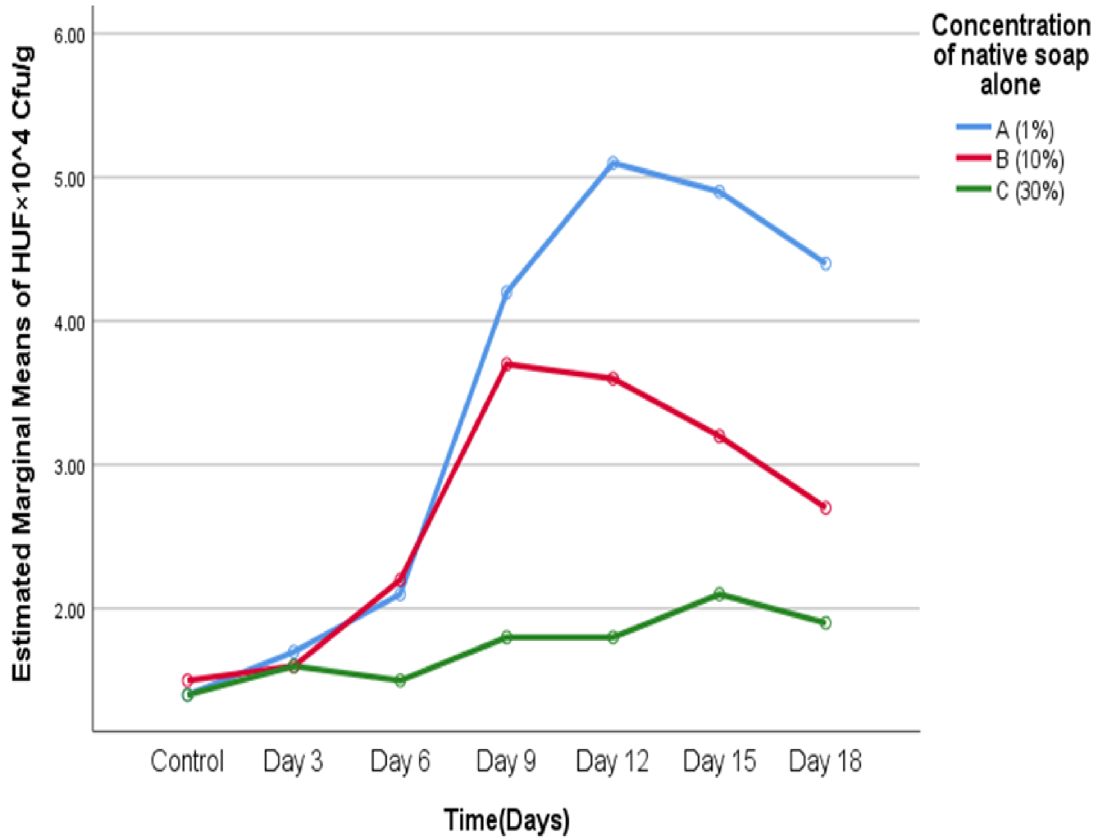

Figure 4.8 Growth response of Hydrocarbon utilizing Fungi (HUF × 10^4 Cfu/g) in different concentrations of native soap solution

Table 4.34 presents the results of a Tukey Honestly Significant Difference (HSD) test for the effect of different time points (days) on Total Heterotrophic Utilizing Bacterial Counts (HUBC) measured in units of 10^6 CFU/g for samples treated with Poultry Dropping (PD). The table allows for comparative HUBC counts between different time points. For example, there is no significant difference in HUBC counts between Day 1 and Day 6 ($p = 0.109$), but there is a trend towards significance between Day 1 and Day 2 ($p = 0.068$). The results suggest a trend where HUBC counts may increase over time, with some potential differences between specific time points. However, further investigation or additional data may be needed to confirm these trends conclusively.

Figure 4.9 allows the comparison of HUB counts between different time points. For example, there is no significant difference in HUBC counts between Day 1 and Day 6 ($p = 0.109$), but there is a trend towards significance between Day 1 and Day 2 ($p = 0.068$). The results suggest a trend where HUB counts may increase over time, with some potential differences between specific time points.

Table 4.34: Effect of Different Concentrations of Poultry Droppings On Hydrocarbon Utilizing Bacteria Isolates from soil polluted with crude oil and heavy metals

Tukey HSD Test for Effect of Time (Days) on THUBC×10^6 Cfu/g for Poultry Dropping

		Subset	
Time (Days)	N	1	2
Control	9	2.4667	
Day 1	9	3.2000	3.2000
Day 6	9	3.5000	3.5000
Day 5	9	3.8667	3.8667
Day 2	9		4.0333
Day 4	9		4.3000
Day 3	9		4.5000
Sig.		.068	.109

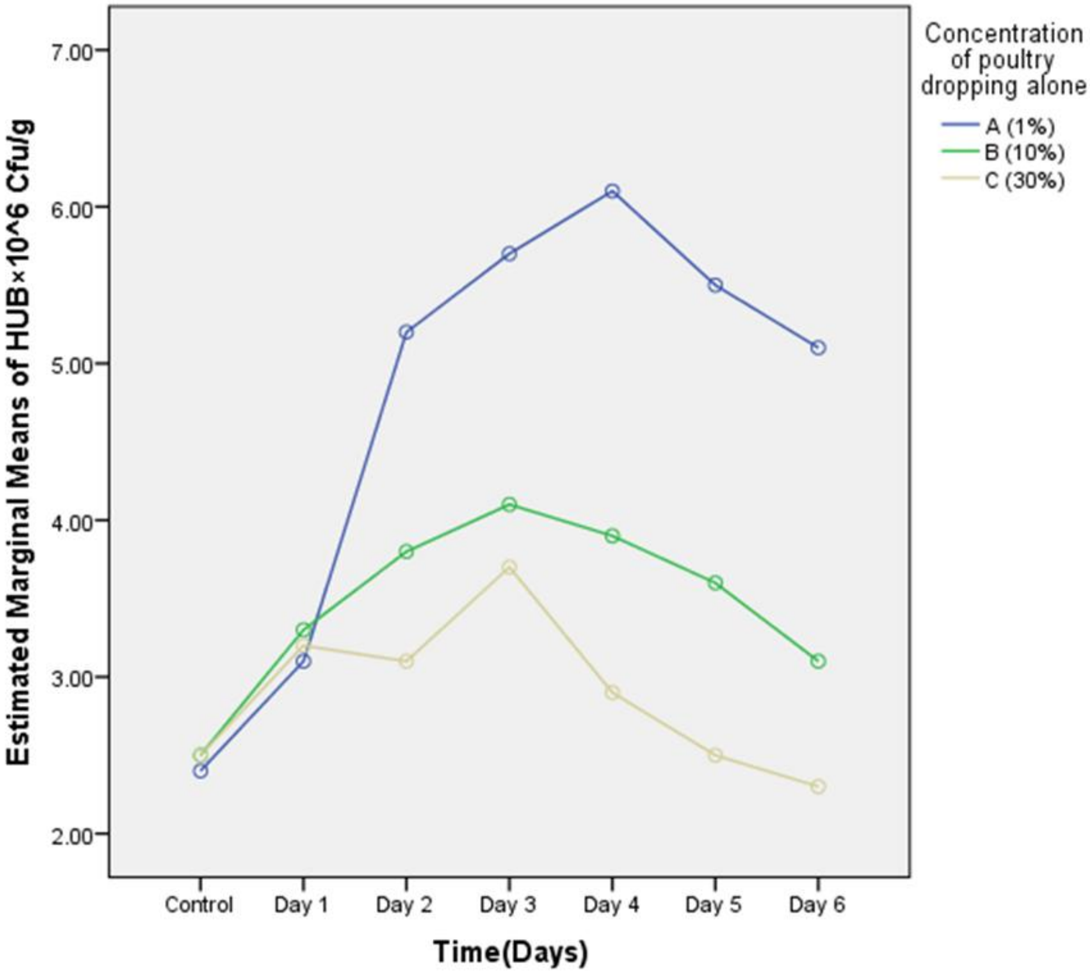

Figure 4.9 Growth response of Hydrocarbon utilizing Bacteria (HUB × 10^6 Cfu/g) in different concentrations of poultry dropping

Table 4.35 presents the results of a Tukey Honestly Significant Difference (HSD) test for the effect of different time points (days) on Total Heterotrophic Utilizing Fungal Counts (THUFC) measured in units of 10^4 CFU/g for samples treated with Poultry Dropping (PD). The table allows you to compare THUFC counts between different time points. For example, there is no significant difference in THUFC counts between Day 1 and Day 6 ($p = 0.622$), but there is a trend towards significance between Day 1 and Day 3 ($p = 0.127$).

In summary, this table provides insights into the variation in THUFC counts over different time points, suggesting potential trends that may not reach statistical significance.

Figure 4.10 shows consistent competition among the three different concentrations to produce the highest average THUB counts per day. However, concentration A (1%) remained the best concentration over time. The results show that at lower concentrations, the environment is milder and will encourage more growth but at higher concentration, microbial count increases but due to heat and change in environment, some will either stop growing, go into non cultural state or die.

The effect of PD concentration is statistically significant for HUBC ($p < 0.05$) but not for THUFC ($p > 0.05$). This suggests that varying the concentration of PD significantly affects bacterial counts but not fungal counts. The effect of time is significant for both THUBC and THUFC ($p < 0.05$). This indicates that the duration of time significantly influences both bacterial and fungal counts.

The interaction between time and PD concentration is not statistically significant for either THUBC or THUFC ($p > 0.05$). This suggests that the combined effect of PD concentration and time does not significantly influence bacterial or fungal counts.

Table 4.35: Effect of Different Concentrations of Poultry Droppings On Hydrocarbon Utilizing Fungi Isolates from soil polluted with crude oil and heavy metals

Tukey HSD Test for Effect of Time (Days) on THUFC×10^4 Cfu/g for Poultry Dropping

		Subset	
Time (Days)	N	1	2
Control	9	1.4333	
Day 6	9	2.2667	2.2667
Day 2	9	2.3667	2.3667
Day 1	9	2.6000	2.6000
Day 5	9	2.7000	2.7000
Day 3	9		3.0000
Day 4	9		3.0667
Sig.		.127	.622

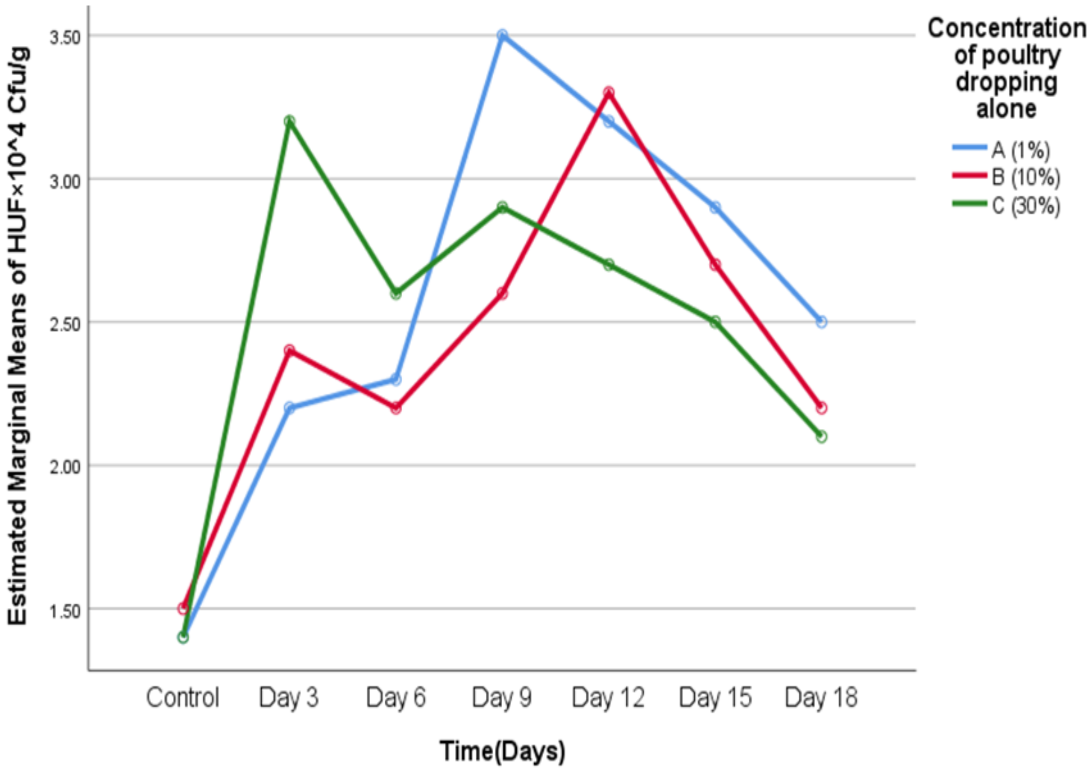

Figure 4.10 Growth response of Hydrocarbon utilizing Fungi (HUF × 10^4 Cfu/g) in different concentrations of poultry droppings

Table 4.36 presents the results of a Tukey Honestly Significant Difference (HSD) test for the effect of different concentrations of a combined mixture of Poultry Dropping (PD) and Natural Soap (NS) on Total Heterotrophic Utilizing Bacterial Counts (THUBC) measured in units of 10^6 CFU/g.

The Tukey HSD test results reveal significant differences in THUBC counts between the different concentrations of the combined mixture of PD and NS. Specifically, concentration B (5% PD + 5% NS) has a significantly higher mean THUBC count compared to concentration A (0.5% PD + 0.5% NS). However, there is no significant difference between concentrations C (15% PD + 15% NS) and A (0.5% PD + 0.5% NS).

In summary, the results suggest that the concentration of the combined mixture of PD and NS significantly affects THUBC counts, with concentration B resulting in higher counts compared to concentration A.

Table 4.36: Effect of different combined concentrations of natural soap (NS) and poultry droppings (PD) on hydrocarbon utilizing bacteria isolates from soil polluted with crude oil and heavy metals

Tukey Test for the Effect of Concentration of PD and NS Combined for THUBC$\times 10^6$Cfu/g

Combined concentration of poultry dropping and natural soap	N	Subset 1	2
C (15% + 15%)	21	2.1286	
A (0.5% + 0.5%)	21	2.5929	
B (5% + 5%)	21		3.4000
Sig.		.299	1.000

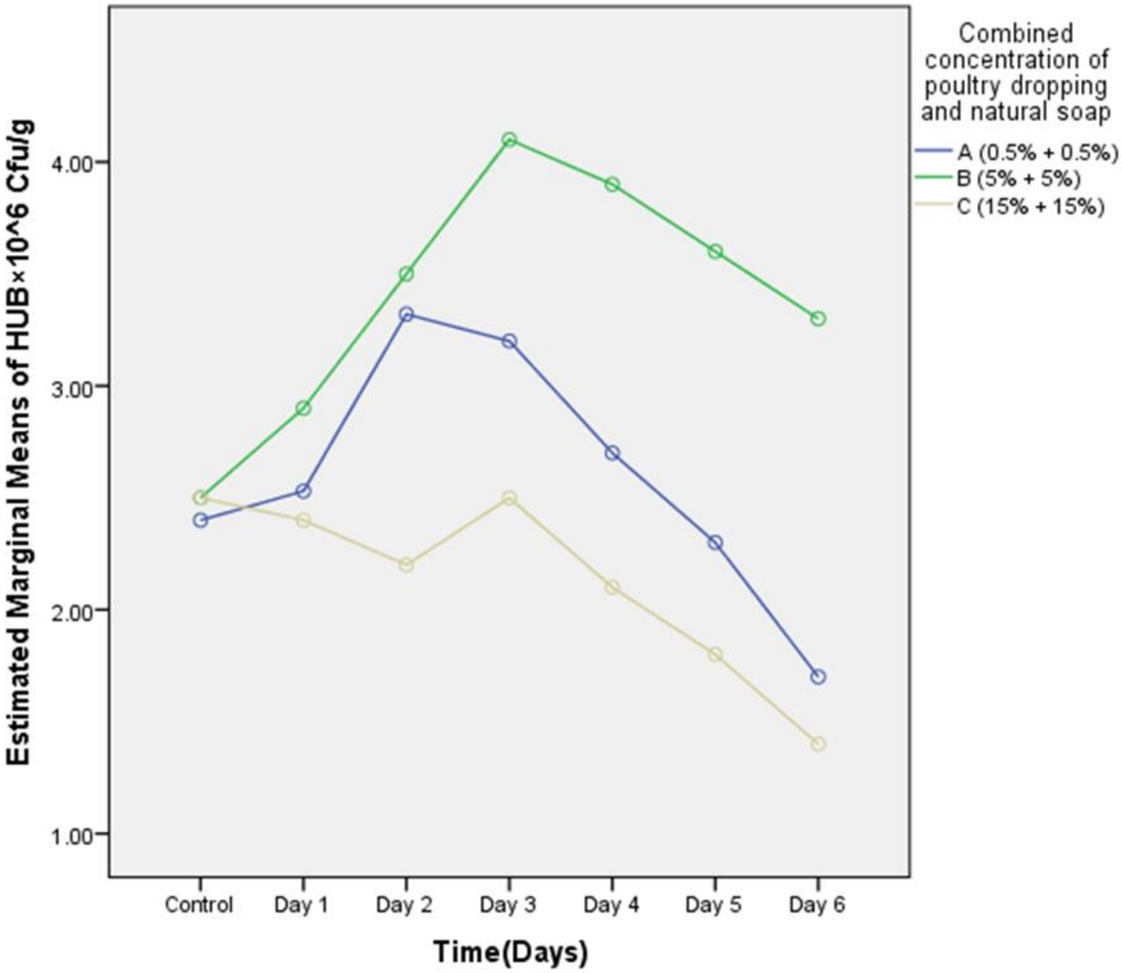

Figure 4.11 Growth response of Hydrocarbon utilizing Bacteria (HUB × 10^6 Cfu/g) in different concentrations of combined native soap and poultry droppings

Table 4.37 presents the results of a Tukey Honestly Significant Difference (HSD) test for the effect of different time points (Days) on Total Heterotrophic Utilizing Fungal Counts (THUFC) measured in units of 10^4 CFU/g for samples treated with a combination of Poultry Dropping (PD) and Natural Soap (NS). The table allows you to compare THUFC counts between different time points. For example, there is no significant difference in THUFC counts between Day 1 and Day 6 ($p = 0.071$), Day 1 and Day 5 ($p = 0.093$), or Day 2 and Day 3 ($p = 0.286$). The results suggest that there may be some variation in THUFC counts over time, but these differences are not statistically significant at the chosen significance level (0.05).

In summary, this table provides insights into the variation in THUFC counts over different time points for samples treated with a combination of PD and NS, suggesting potential trends that may not reach statistical significance.

Table 4.37: Effect of Different Combined Concentrations of natural soap (NS) and Poultry Droppings (PD) On Hydrocarbon Utilizing Fungi Isolates from Soil Polluted with Crude Oil and Heavy Metals

Tukey HSD Test for the Effect of Time (Days) on THUFC×10^4 Cfu/g for PD and NS combined

		Subset		
Time (Days)	N	1	2	3
Control	9	2.0433		
Day 6	9	2.5333	2.5333	
Day 1	9	2.9333	2.9333	2.9333
Day 5	9	3.0333	3.0333	3.0333
Day 2	9	3.4333	3.4333	3.4333
Day 4	9		3.8667	3.8667
Day 3	9			4.0000
Sig.		.071	.093	.286

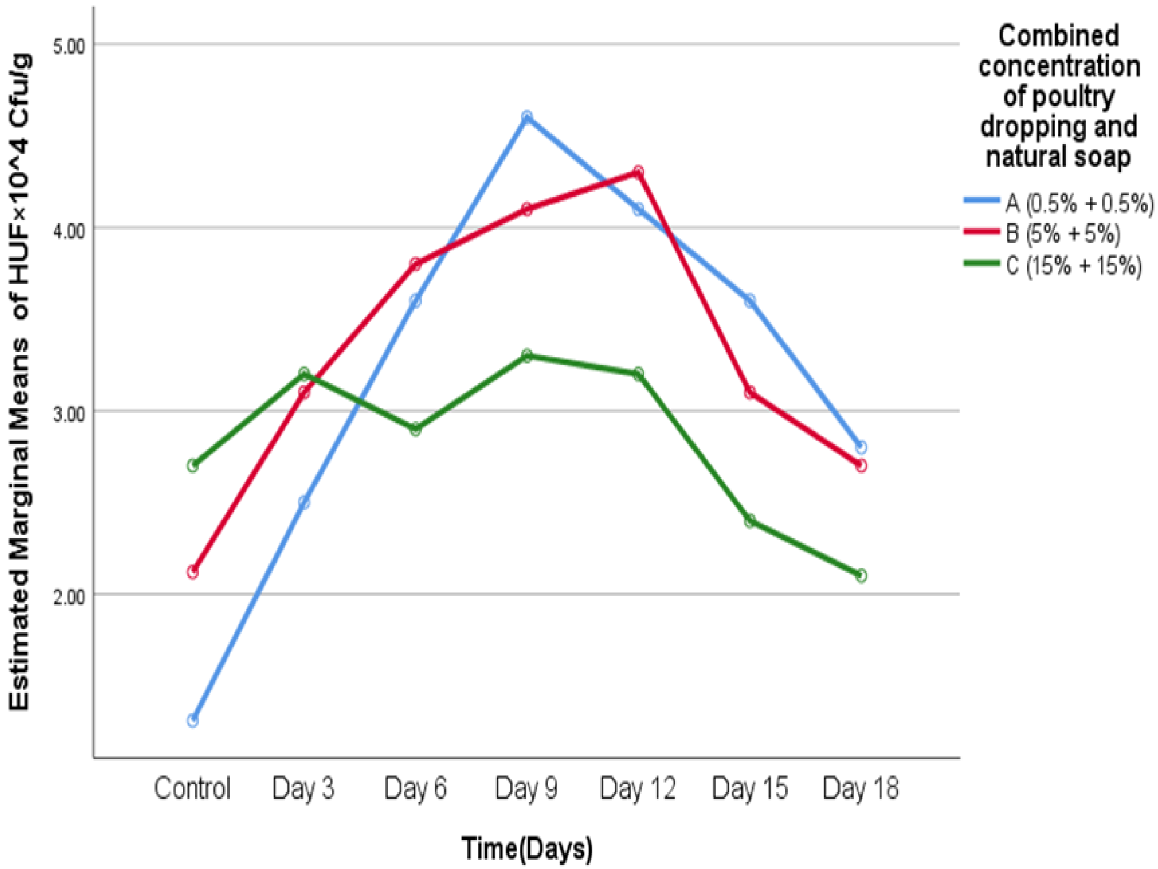

Figure 4.12 Growth response of Hydrocarbon utilizing Fungi (HUF × 10^4 Cfu/g) in different concentrations of combined native soap and poultry droppings

4.1.10 *Paspalum conjugatum* P.J. Bergius responses to wetland polluted soil amended with 1% poultry droppings, 1% native soap and combined 1% poultry droppings and 1% native soap

Figure 4.13 highlights that the combination represented by PS+NS+SP shows the most significant effect on shoot length growth, while PS+PD+SP has less pronounced growth, and PS+NS+PD+SP shows moderate but consistent growth. From the figure, the PS+NS outperformed the other combinations over the 6 months' experimental period. Its efficiency in increasing the shoot length increased with time.

Figure 4.14indicates that treatment set up of PS+NS+SP resulted in the most consistent and positive root length growth by the end of the 6-month period, while the other two treatments experience more fluctuations but recover to similar levels.

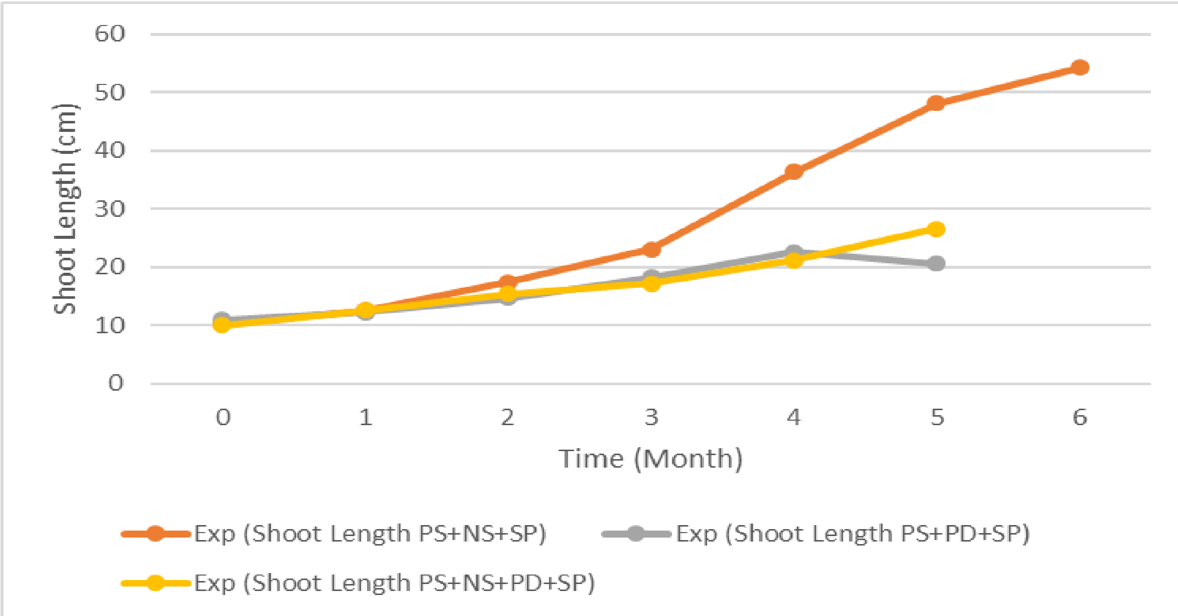

Figure 4.13*P. conjugatum* P.J. Bergius shoot response to wetland soil polluted with 10% crude oil and heavy metals (Cu, Pb, Zn and Ni) with different amendments of poultry droppings, natural soap and combination of both

Key: PS–Polluted soil , NS– Natural soap , SP–Selected plant (*Paspalum conjugatum* P.J. Bergius), PD– Poultry droppings

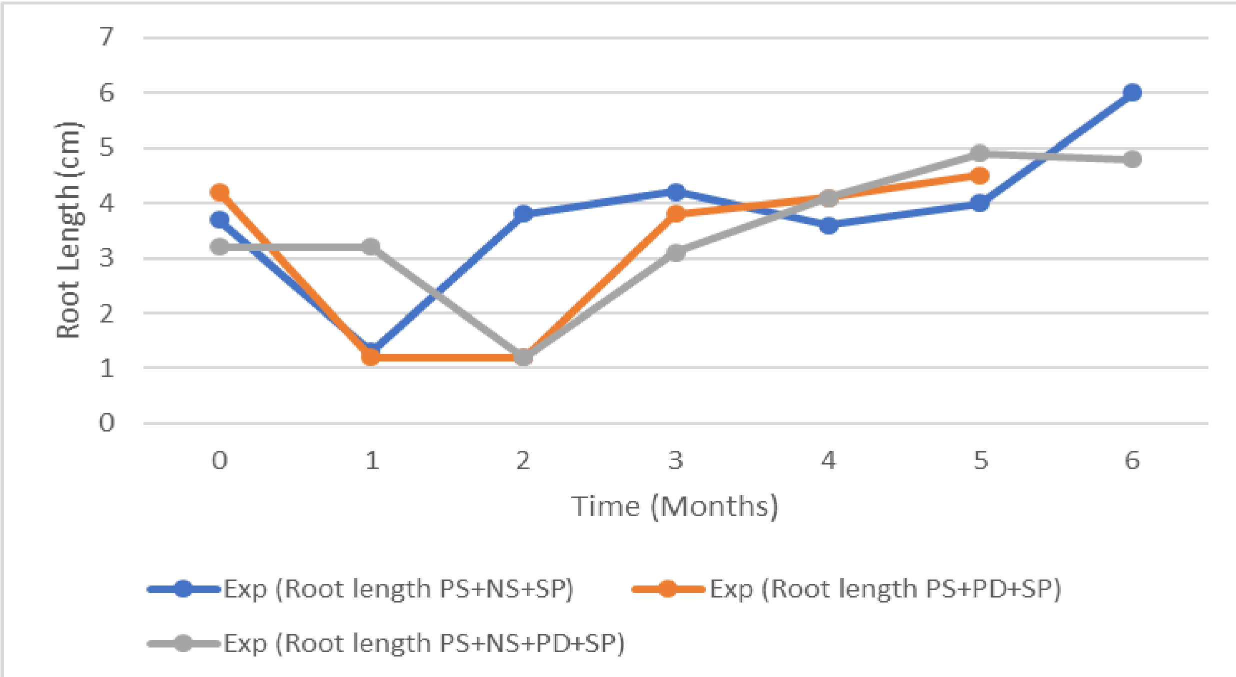

Figure. 4.14 *P. conjugatum* P.J. Bergius roots' length response to wetland soil polluted with 10% crude oil and heavy metals (Cu, Pb, Zn and Ni) with different amendments of poultry droppings, natural soap and combination of both

Figure 4.15shows similar PS+NS+SP performed significantly better in the leaf size at the second and third month but all combinations performed equally from fourth month until the end of the experiment. It is important to note that PS+PD+SP and PS+NS+PD+SP combinations produced same leaf sizes for the various months. Overall, the treatment PS+NS+SP seems to result in the most consistent and positive root length growth by the end of the 6-month period, while the other two treatments experience more fluctuations but recover to similar levels.

Figure 4.16 shows that PS+NS+SP treatment combination has the strongest effect on wet weight of the plant, while the other two treatments demonstrate more variability and slower overall increase in wet weight over time. From the figure, PS+NS+SP performed significantly better in the wet weight increase than other treatment combinations.

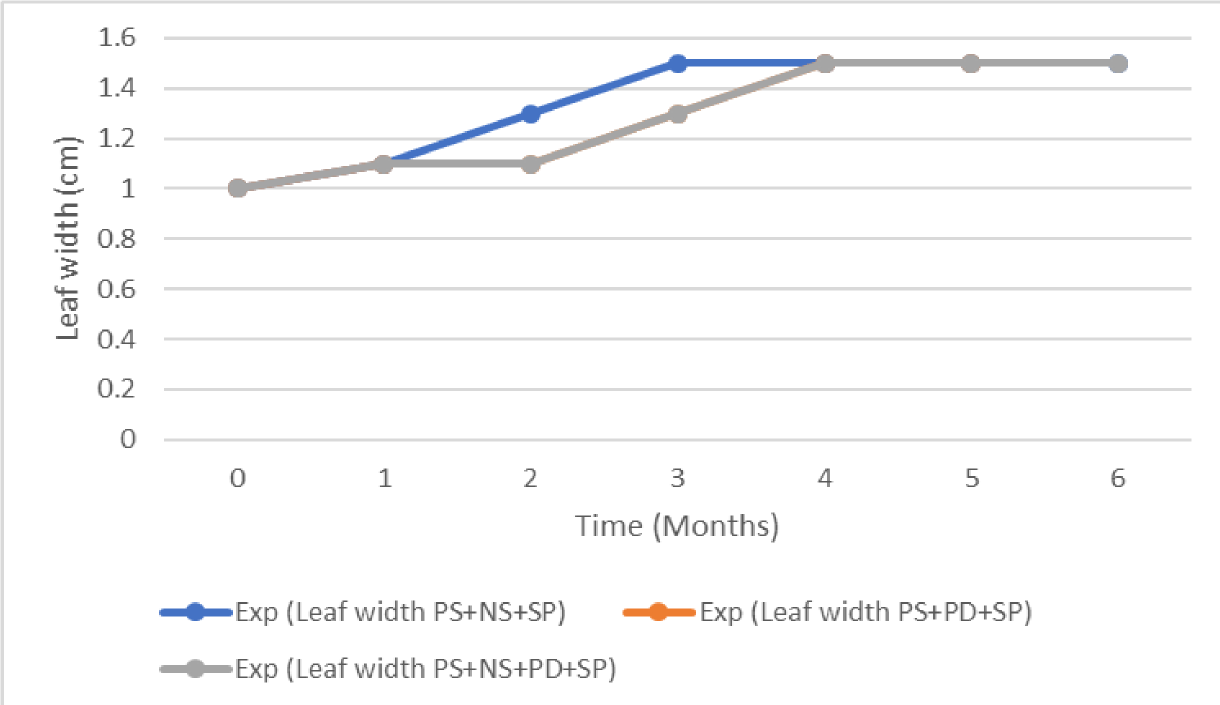

Figure 4.15: *Paspalum conjugatum* P.J.Bergius leave size response to wetland soil polluted with 10% crude oil and heavy metals (Cu, Pb, Zn and Ni) with different amendments of poultry droppings, natural soap and combination of both

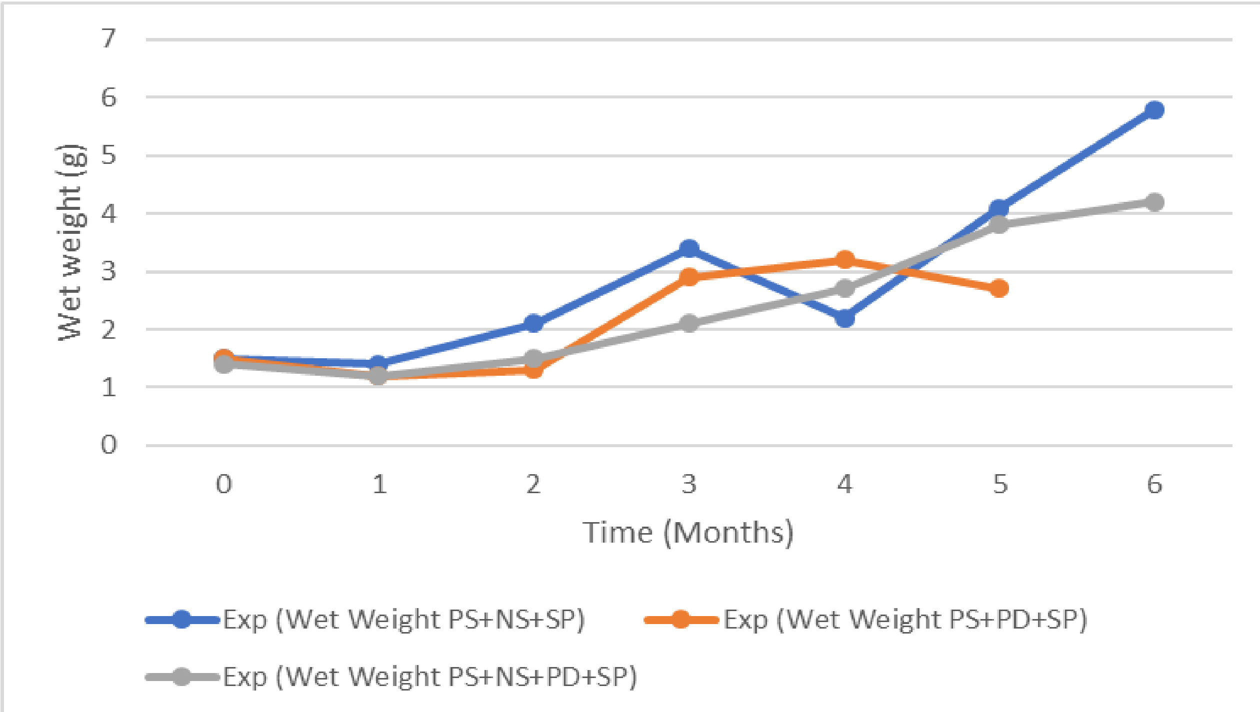

Figure 4.16: *P. conjugatum* P.J. Bergius wet weights' response to wetland soil polluted with 10% crude oil and heavy metals (Cu, Pb, Zn and Ni) with different amendments of poultry droppings, natural soap and combination of both

4.1.11 Sediment sample(s) from hybrid construction wetland with different soil amendments

Based on Table 4.38, the Tukey HSD test results for Total Petroleum Hydrocarbons (TPH) content in sediment samples from the hybrid construction wetland analysis compares TPH levels across different time points. The table indicates that there are significant differences in TPH content between any of the time points (Days 30, 60, 90, 120, 150) and the Control group (baseline) because the means appeared separately in distinct subsets.

In summary, the Tukey HSD test results suggest that there are significant differences in TPH content among sediment samples collected at different time points (Days 30, 60, 90, 120, 150) compared to the Control group. This indicates variability in TPH levels in the sediment samples over the observed time period.

Figure 4.17 shows that all treatments result in a decline in TPH concentrations over time, indicating effective remediation. The control group shows the highest TPH concentrations throughout, confirming the effectiveness of the amendments. PS + NS + PLT (Yellow): This combination shows the most significant reduction in TPH, reaching the lowest residual levels, suggesting a synergistic effect of multiple amendments. This is followed byPS + NS +PD + PLT (Grey): PS + NS (Green) and PS + PD + NS (Orange): These combinations also show substantial TPH reduction, with PS + NS performing slightly better than PS + PD + NS. PS + PD (Yellow) and PS + PD + PLT (Purple): These treatments are moderately effective, with PS + PD + PLT showing a gradual decrease in TPH. PS + PLT (Red) and PS (Blue): These treatments show less TPH reduction compared to others, indicating that plants alone or polluted soil alone are less effective.

Table 4.38: Residual concentration of total petroleum hydrocarbon (TPH) in the soil sediments from hybrid constructed wetland

Tukey HSD Test for TPH (mg/kg) content in sediment sample from hybrid construction wetland

		Subset					
Time (Days)	N	1	2	3	4	5	6
Day 150	24	246.0325					
Day 120	24		429.5375				
Day 90	24			651.3963			
Day 60	24				801.7487		
Day 30	24					951.1813	
Control	3						1361.2500
Sig.		1.000	1.000	1.000	1.000	1.000	1.000

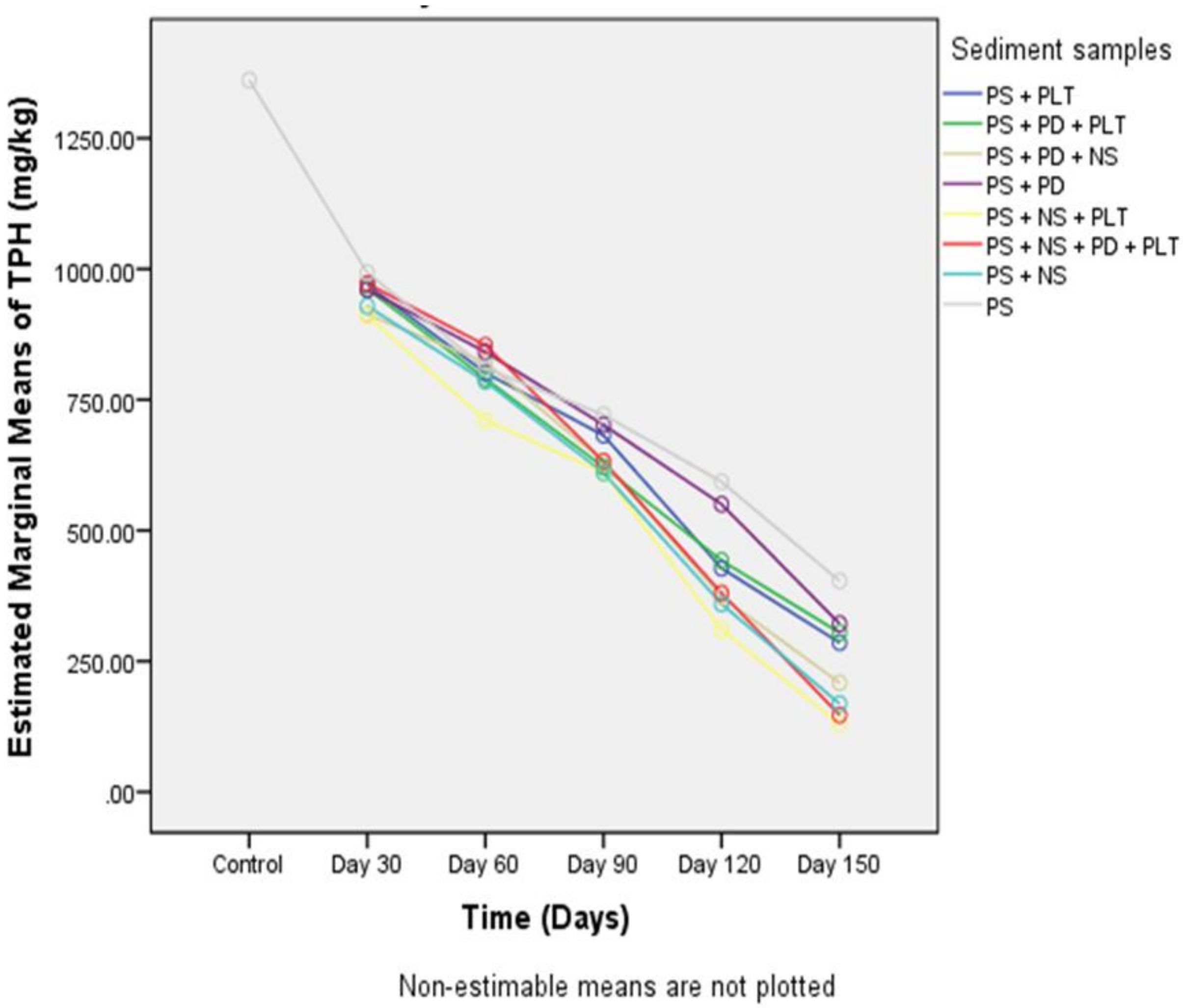

Figure 4.17: Residual concentration of total petroleum hydrocarbon (TPH) in the soil sediments from hybrid constructed wetland with different soil amendments.

Table 4.39 shows the Tukey HSD test results for Zinc (Zn) content in sediment samples from the hybrid construction wetland. The analysis compares Zn levels across different time points (Day 30, Day 60, Day 90, Day 120, and Day 150) as well as a Control group. The p-values for the comparisons between different time points and the Control group vary. For Day 150 and Day 120, the p-values are 0.172 and 0.233, respectively, which are greater than the typical significance level of 0.05. This indicates that Zn levels at these time points are not significantly different from the Control group. However, for the other time points (Day 90, Day 60, and Day 30), the p-values are all 1.000, indicating no statistically significant differences in Zn content among them.

The Tukey HSD test results suggest that means within the same subsets (Day 150 and Day 120) have no significant difference between them, but they are significantly different from means in other subsets. This implies that there are significant differences in Zn content between Day 150 and Day 120 compared to the other time points and the Control group, while no significant differences are observed among the other time points

Figure 4.18 shows that all treatments, except the control, show a decline in zinc concentration over time, indicating effective remediation. The control group has the highest zinc concentrations throughout, emphasizing the impact of the amendments. PS + NS + PLT (Yellow): This combination initially shows a decline, but later concentrations stabilize, suggesting most effectiveness reduction over time. PS + NS + PD +PLT (Cyan): This treatment shows a consistent reduction, reaching the lowest zinc levels by day 150, indicating effective long-term remediation. PS + PD + NS (Red) and PS + PD + PLT (Purple): These combinations show moderate reductions, with PS + PD + PLT showing some fluctuations. PS+ PD (Yellow) and PS + NS (Blue): These treatments also show a decline, with PS + PD reaching lower zinc levels than PS + NS. PS + PLT (Green): Shows a gradual decline in zinc concentration, suggesting the effectiveness of plants alone. PS (Orange): The polluted soil alone shows the least reduction, highlighting the necessity of amendments for effective zinc remediation.

Table 4.39: Residual concentration of Zinc (Zn) in the soil sediments from hybrid constructed wetland

Tukey HSD Test for Zn (mg/kg) content in sediment sample from hybrid construction wetland

		Subset				
Time (Days)	N	1	2	3	4	5
Day 150	24	42.2338				
Day 120	24	43.2363	43.2363			
Day 90	24		44.1750			
Day 60	24			47.4588		
Day 30	24				50.4625	
Control	3					56.7500
Sig.		.172	.233	1.000	1.000	1.000

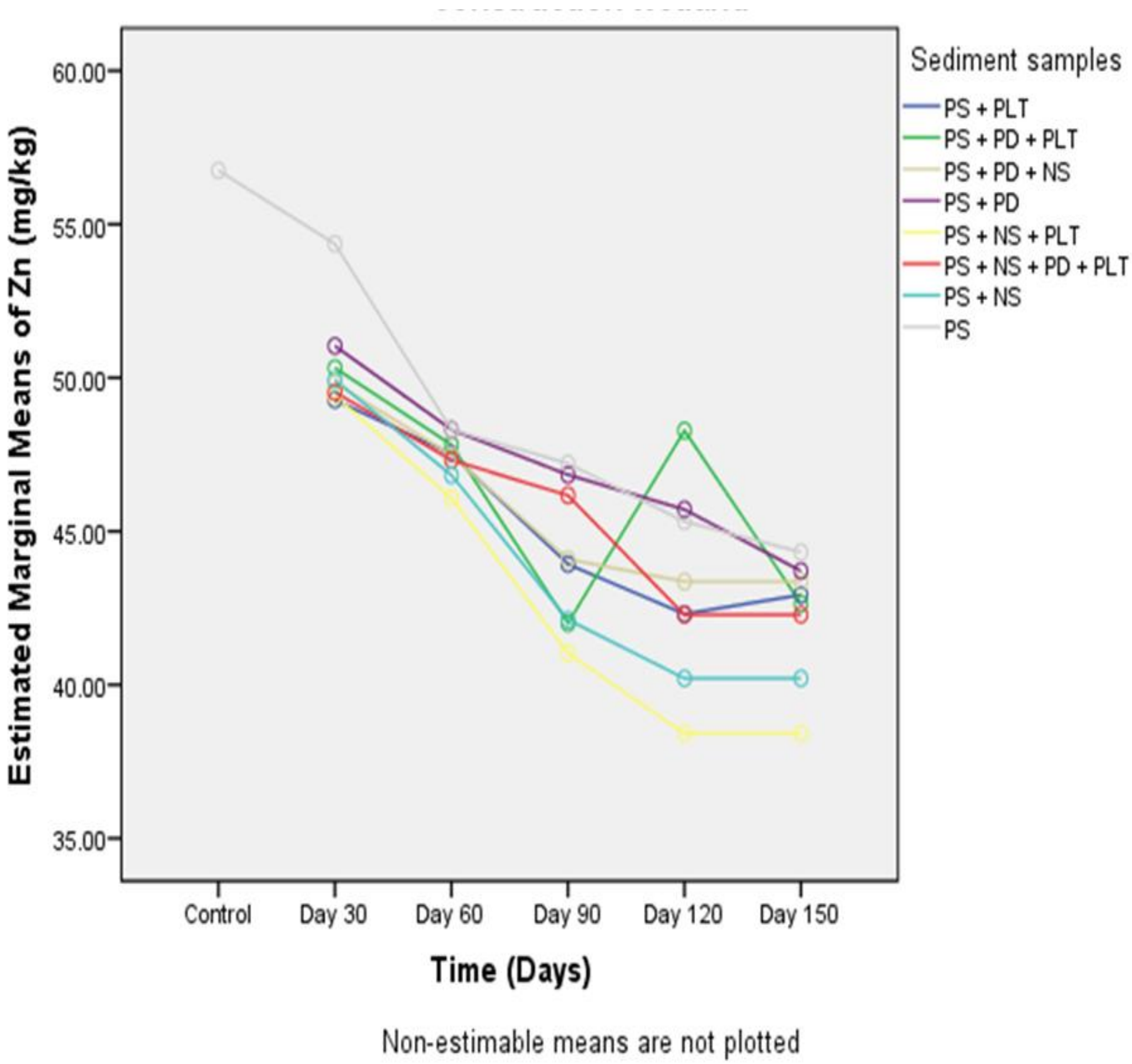

Figure 4.18: Residual concentration of Zinc (mg/kg) in the soil sediments from hybrid constructed wetland

Table 4.40 indicates that the Tukey HSD test results for Lead (Pb) content in sediment samples from the hybrid construction wetland. The p-values for the comparisons between different time points and the Control group vary. For Day 150, Day 120, and Day 90, the p-values are 0.175, 0.247, and 0.428, respectively, which are greater than the typical significance level of 0.05. This indicates that Pb levels at these time points are significantly different from the Control group. However, for Day 30 is not significantly different from Day 60 but are significantly different from the control

In figure 4.19 all treatments result in a decline in Pb concentrations over time from the wetland soil sediment, indicating effective lead remediation. The control group (polluted soil without amendments) consistently shows the highest Pb concentrations, highlighting the necessity of amendments. PS + PD + NS (Orange): This combination shows a significant and steady decrease in Pb concentration, reaching the lowest levels by day 150, suggesting a highly effective remediation strategy. PS + PD + PLT (Green): This treatment also shows a consistent reduction in Pb concentration, although not as pronounced as PS + NS + PLT. PS + PD + NS + PLT (Cyan): This combination shows a notable reduction, particularly after day 60, indicating that the synergy between poultry droppings and natural soap is beneficial. PS + NS + PLT (Yellow**)**: Shows a moderate reduction, but not as effective as combinations including poultry droppings. PS **+** PD (Purple): Demonstrates a significant decline, suggesting that poultry droppings alone are quite effective in reducing Pb concentrations. PS + NS (Skey blue): Shows a gradual decrease in Pb concentration, but less effective compared to combinations including plants or poultry droppings. PS + PLT (Navy blue): Shows the least reduction among the combined amendments, indicating plants alone are less effective than other combinations. PS (Grey): The polluted soil alone shows the least reduction in Pb concentration, emphasizing the need for amendments for effective remediation.

Table 4.40: Residual concentration of Lead (pb) in the soil sediments from hybrid constructed wetland

Tukey HSD Test for Pb (mg/kg) content in sediment sample from hybrid construction wetland

		Subset			
Time (Days)	N	1	2	3	4
Day 150	24	10.3088			
Day 120	24	11.3088	11.3088		
Day 90	24		12.2338		
Day 60	24			13.4613	
Day 30	24			14.2463	
Control	3				15.6100
Sig.		.175	.247	.428	1.000

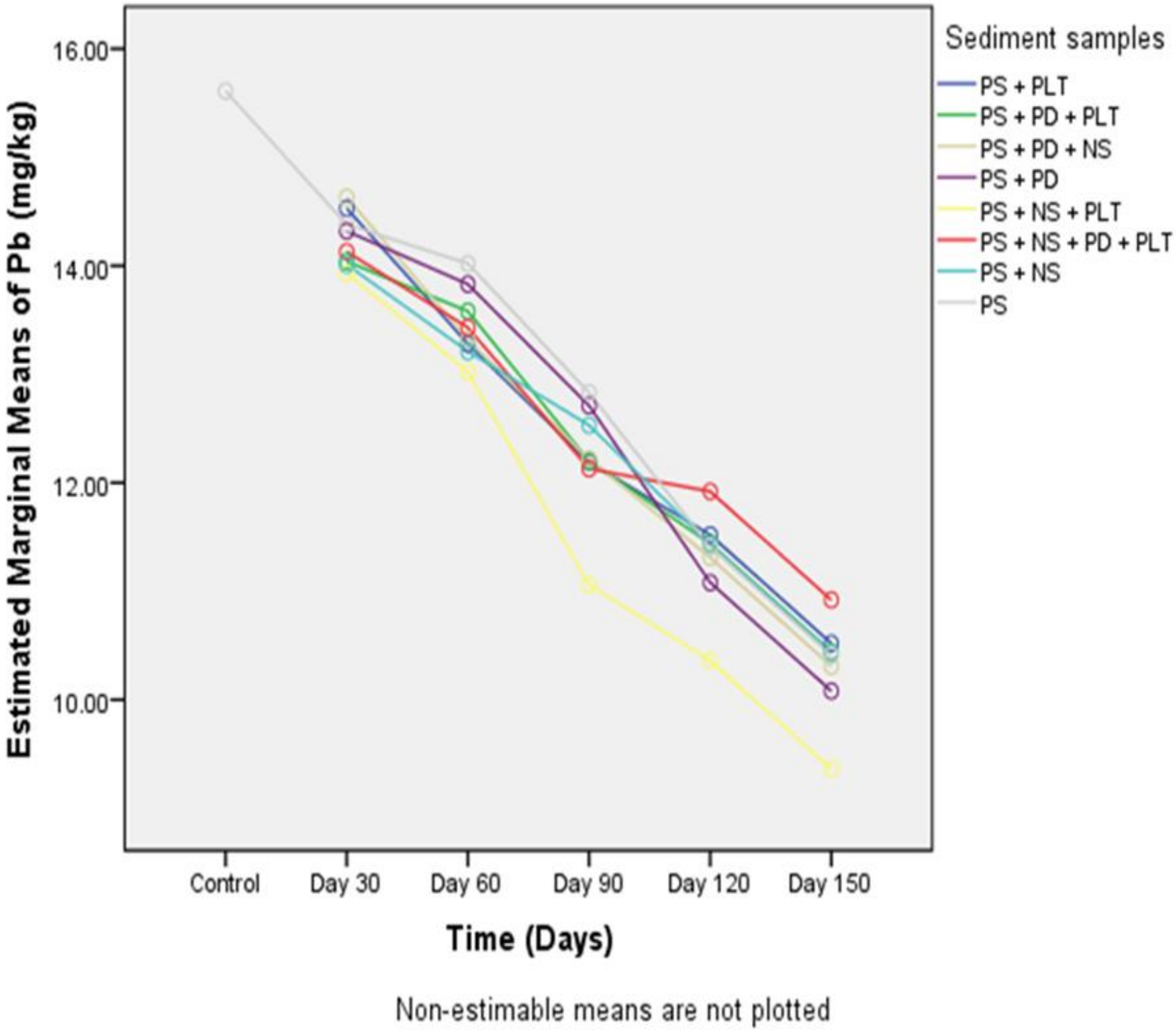

Figure 4.19: Residual concentration of Lead (mg/kg) in the soil sediments from hybrid constructed wetland

Table 4.41 shows that based on the Tukey HSD test results for Nickel (Ni) content in sediment samples from the hybrid construction wetland. Day 150 and Day 90 have the same effect but Day 120 is significantly different from Day 150. All days have significantly different effects from the control.

In figure 4.20, all treatments show a decline in Ni concentrations over time, indicating effective remediation. The control group (polluted soil without amendments) consistently shows the highest Ni concentrations, highlighting the impact of the various amendments.

PS + NS + PLT (Green): This combination shows a significant and steady decrease in Ni concentration, reaching the lowest levels by day 150, suggesting a highly effective remediation strategy. PS + PD + PLT (Purple): This treatment also shows a consistent reduction in Ni concentration, although not as pronounced as PS + PD + NS + PLT. PS + PD + NS + PLT (Cyan): This combination shows a notable reduction, particularly after day 60, indicating that the synergy between poultry droppings and natural soap is beneficial. PS + PD + NS (Brown): Shows a moderate reduction, but not as effective as combinations including poultry droppings. PS + PD (Purple): Demonstrates a significant decline, suggesting that poultry droppings alone are quite effective in reducing Ni concentrations. PS + NS (Green): Shows a gradual decrease in Ni concentration, but less effective compared to combinations including plants or poultry droppings. PS + PLT (Blue): Shows the least reduction among the combined amendments, indicating plants alone are less effective than other combinations. PS (Grey): The polluted soil alone shows the least reduction in Ni concentration, emphasizing the need for amendments for effective remediation.

Table 4.41: Residual concentration of nickel (ni) in the soil sediments from hybrid constructed wetland

Tukey HSD Test for Ni (mg/kg) content in sediment sample from hybrid construction wetland

		Subset				
Time (Days)	N	1	2	3	4	5
Day 150	24	7.2150				
Day 90	24	8.1038	8.1038			
Day 120	24		8.5237	8.5237		
Day 60	24			9.4387	9.4387	
Day 30	24				10.1600	
Control	3					11.7500
Sig.		.289	.916	.258	.524	1.000

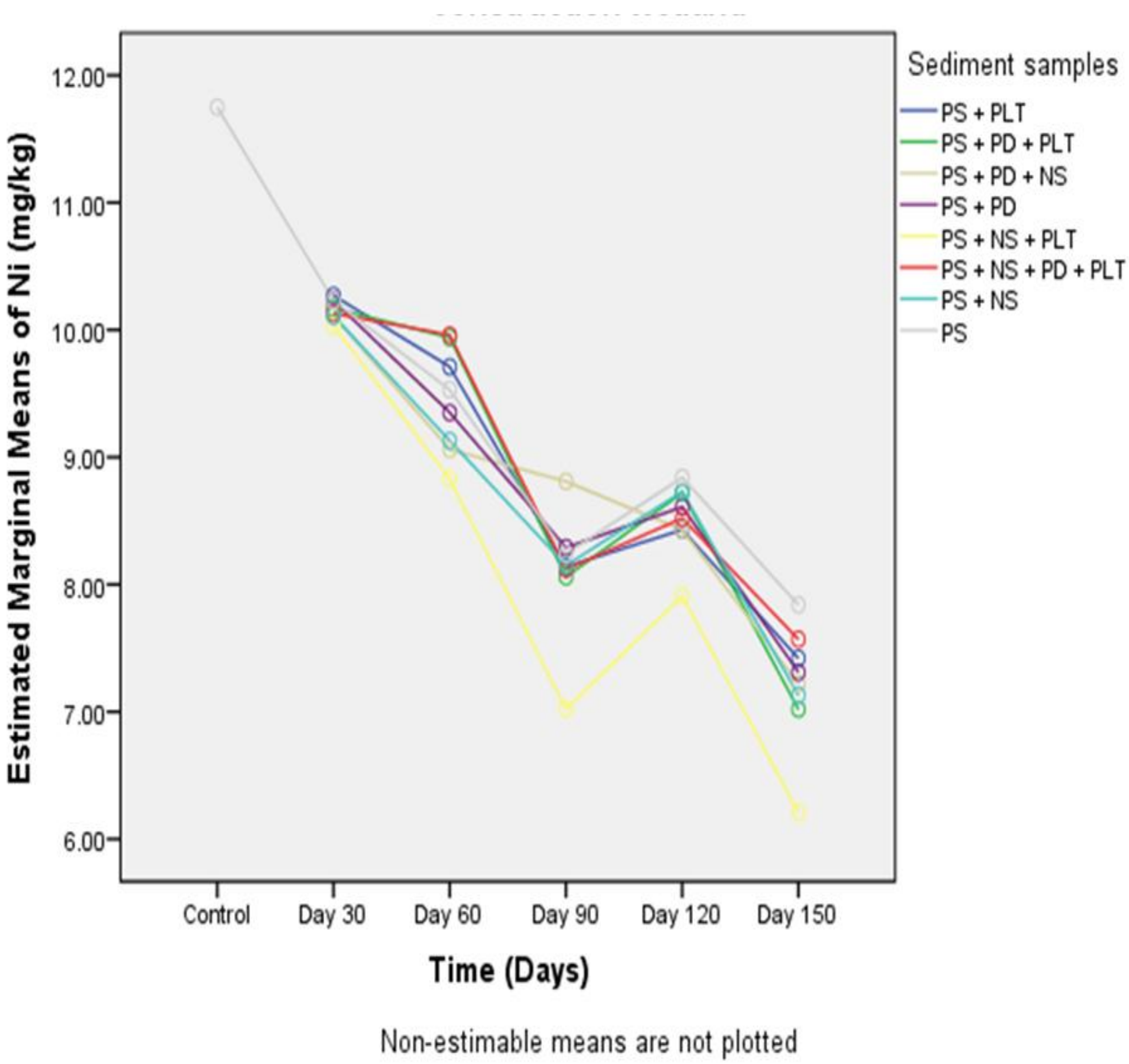

Figure 4.20: Residual concentration of Nickel (mg/kg) in the soil sediments from hybrid constructed wetland

Table 4.42 highlights that based on the Tukey HSD test results for Copper (Cu) content in sediment samples from the hybrid construction wetland. The Copper content was not significantly different on Days 90, 120, and 150. Day 60 and 90 had no significant difference but significantly different from Day 120 and 150 to the control.

Table 4.45 indicates significant effects for all contaminants (TPH, Zn, Pb, Ni, Cu) across the factors Time (Days). This significance is denoted by the p-values being less than 0.05 (the chosen significance level). Not all main effects (Sample) and interaction effects (Days * Sample) are statistically significant for all contaminants. Specifically, the p-values for Nickel (Ni) and Copper (Cu) in the main effect of Sample and their interaction with Days (* Sample) are both above 0.05, indicating that these effects are not statistically significant. The non-significant p-values suggest that the levels of Nickel and Copper in sediment samples are not significantly influenced by the specific samples tested or the interaction between sample types and time.

The partial eta squared values indicate the proportion of variance in the dependent variables explained by the significant independent variables and their interactions. Higher values suggest larger effect sizes. For instance, TPH shows a particularly strong effect size with a partial eta squared of 1.000, indicating that the variables and interactions accounted for all the variance in TPH levels.

Figure 4.21 presents the result of tests of between-subjects effects for sediment samples from a hybrid construction wetland, focusing on various contaminants including Total Petroleum Hydrocarbons (TPH), Zinc (Zn), Lead (Pb), Nickel (Ni), and Copper (Cu), measured in units of mg/kg.

In summary, while significant effects are observed for most contaminants, Nickel and Copper levels in sediment samples from the hybrid construction wetland are not significantly influenced by the specific samples tested or the interaction between sample types and time. This underscores the variability in contaminant dynamics and highlights the need for further investigation into the factors affecting Nickel and Copper concentrations in the wetland sediment.

Table 4.42: Residual concentration of copper (cu) in the soil sediments from hybrid constructed wetland

Tukey HSD for Cu (mg/kg) content in a sediment sample from hybrid construction wetland

		Subset			
Time (Days)	N	1	2	3	4
Day 120	24	25.3700			
Day 150	24	25.5913			
Day 90	24	26.0713	26.0713		
Day 60	24		27.2950		
Day 30	24			29.6325	
Control	3				32.1433
Sig.		.555	.050	1.000	1.000

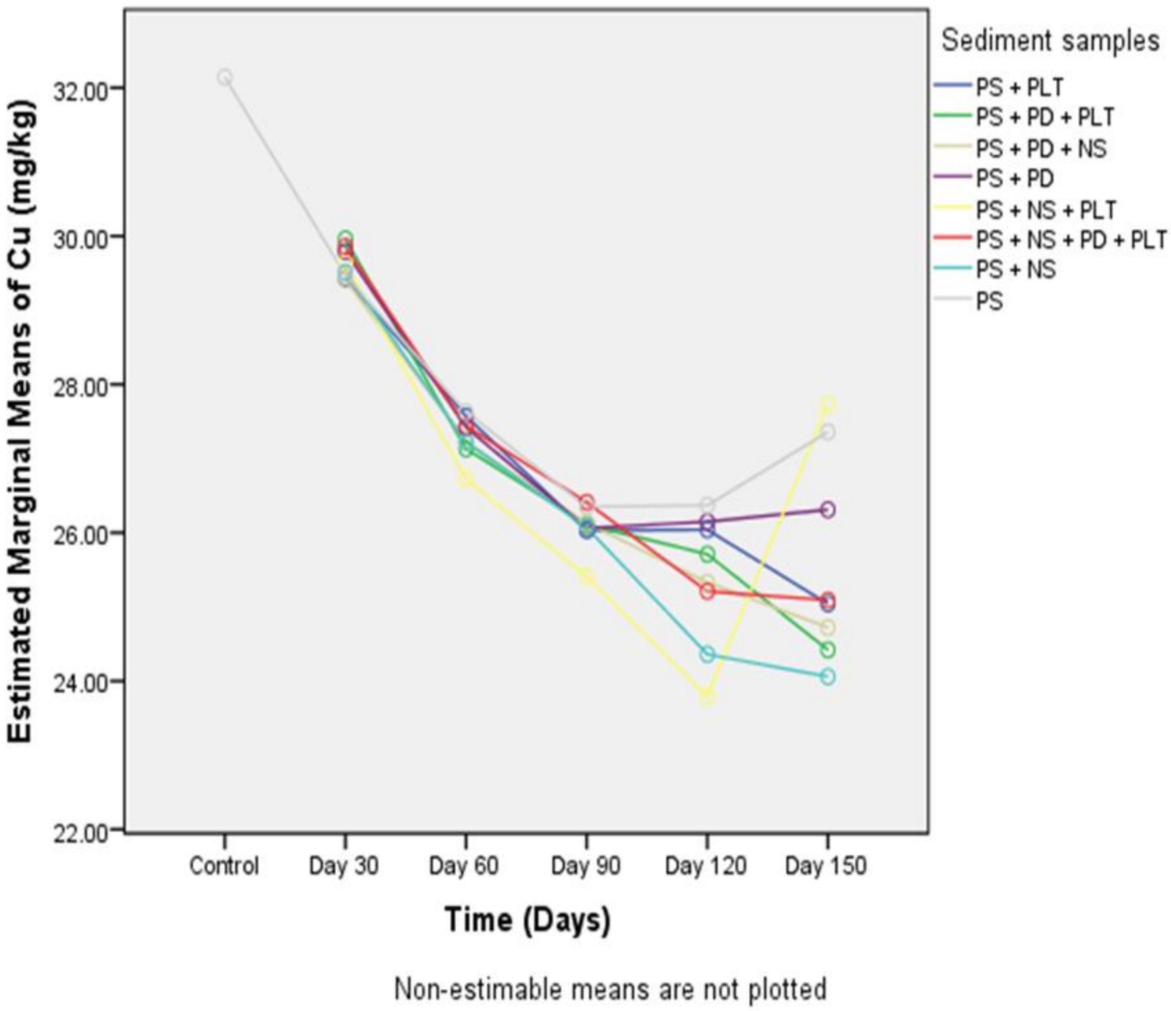

Figure 4.21: Residual concentration of copper (cu) in the soil sediments from hybrid constructed wetland

4.1.12Water samples from constructed hybrid wetland

Table 4.43 presents the HSD (Honestly Significant Difference) test for THC (Total Hydrocarbon) content in water samples from the hybrid construction wetland, analyzed across different days, yielded the following results: These results suggest that there are significant differences in THC content across the different days analyzed in the water samples from the hybrid construction wetland

Figure 4.22 shows the Total Hydrocarbon Content (THC) in water samples from a constructed hybrid wetland over time, ranging from the control day to 180 days. The decrease in THC content slows down, with the values stabilizing around 100 mg/L. This suggests that while the system continues to reduce THC, the rate of removal declines as the remaining hydrocarbons become harder to degrade or are present in trace, suggesting significant differences in THC content across the different days analyzed in the water samples from the hybrid construction wetland

Table 4.43: THC (mg/kg) content in water sample from hybrid construction wetland

HSD Test for THC(mg/kg) content in water sample from hybrid construction wetland

Days for analysis of water samples from constructed hybrid wetland

	N	Subset						
		1	2	3	4	5	6	7
Day 150	3	14.8700						
180.00	3		16.6200					
Day 120	3			86.8200				
Day 90	3				163.4600			
Day 60	3					433.2700		
Day 30	3						685.2600	
Control	3							1211.2600
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000

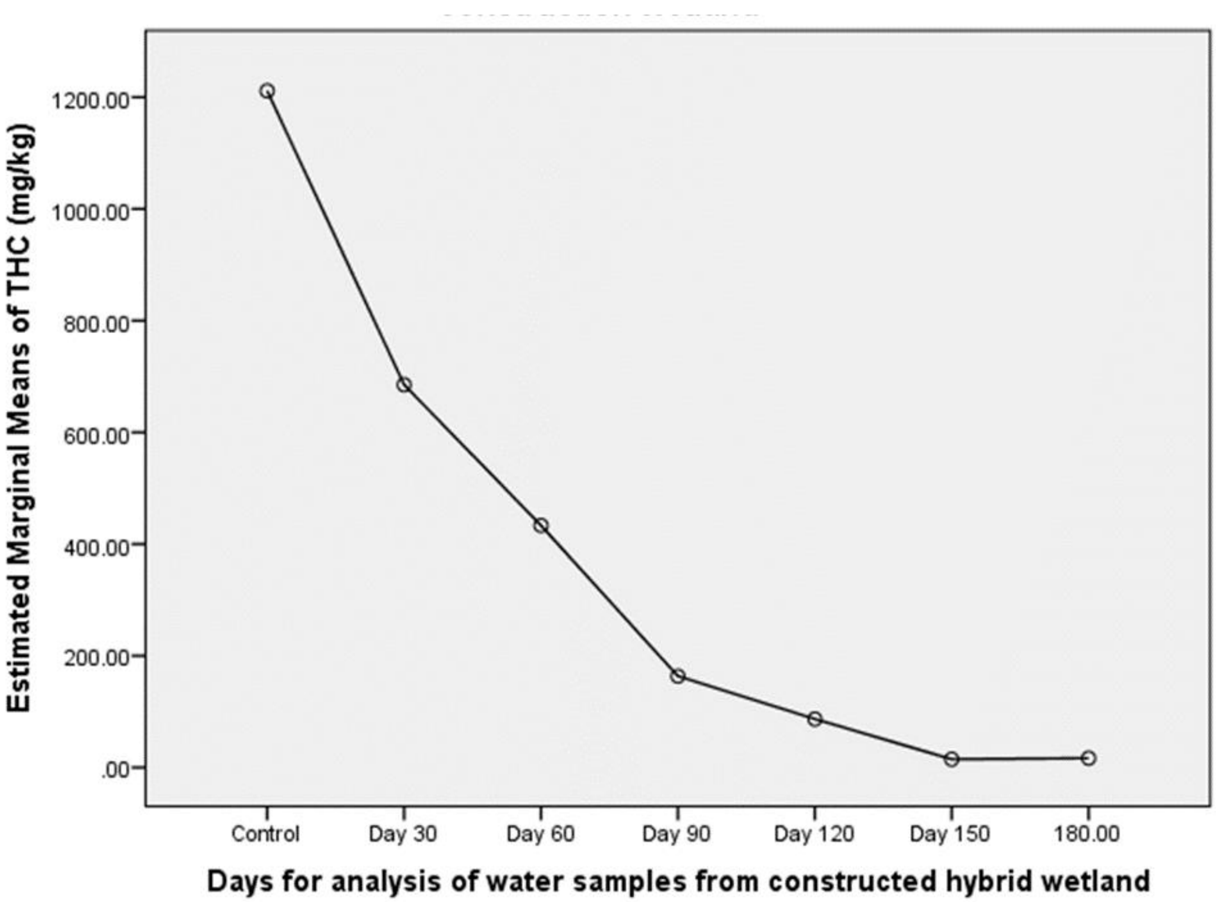

Figure 4.22: THC content in water sample from hybrid construction wetland

Table 4.44 presents the Tukey HSD (Honestly Significant Difference) test for Pb (Lead) content in water samples from the hybrid construction wetland, analyzed across different days, yielded the following results: there are significant differences in Pb content between the subsets analyzed on different days in the water samples from the hybrid construction wetland. Pb content in the samples on Days 150 and 180 are significantly lower than those of Days 30 and control. This simply shows that Pb content declines with time.

Figure4.23shows that Pb concentration starts at the highest level with a significant and steady decline in Pb concentration over time. This suggests that the hybrid wetland system is effectively reducing the Pb content in the water through mechanisms such as phytoremediation, sedimentation, and microbial action. This final phase of the curve indicates that the constructed wetland has achieved a near-complete removal of Pb from the water. The consistent decline in Pb concentration across time points suggests that there is a significant difference in Pb content from the control to the final sampling point (Day 180). The steep drop, especially between Days 30 and 90, shows marked changes that is statistically significant.

Table 4.44: Concentration of Pb content in water sample from hybrid construction wetland

Tukey HSD Test for Pb (mg/kg) content in water sample from hybrid construction wetland

Days for analysis of water samples from constructed hybrid wetland	N	Subset	
		1	2
Day 180	3	.0010	
Day 150	3	.0020	
Day 90	3	.3480	.3480
Day 120	3	.3510	.3510
Day 60	3	.4220	.4220
Day 30	3		.6870
Control	3		.8100
Sig.		.205	.137

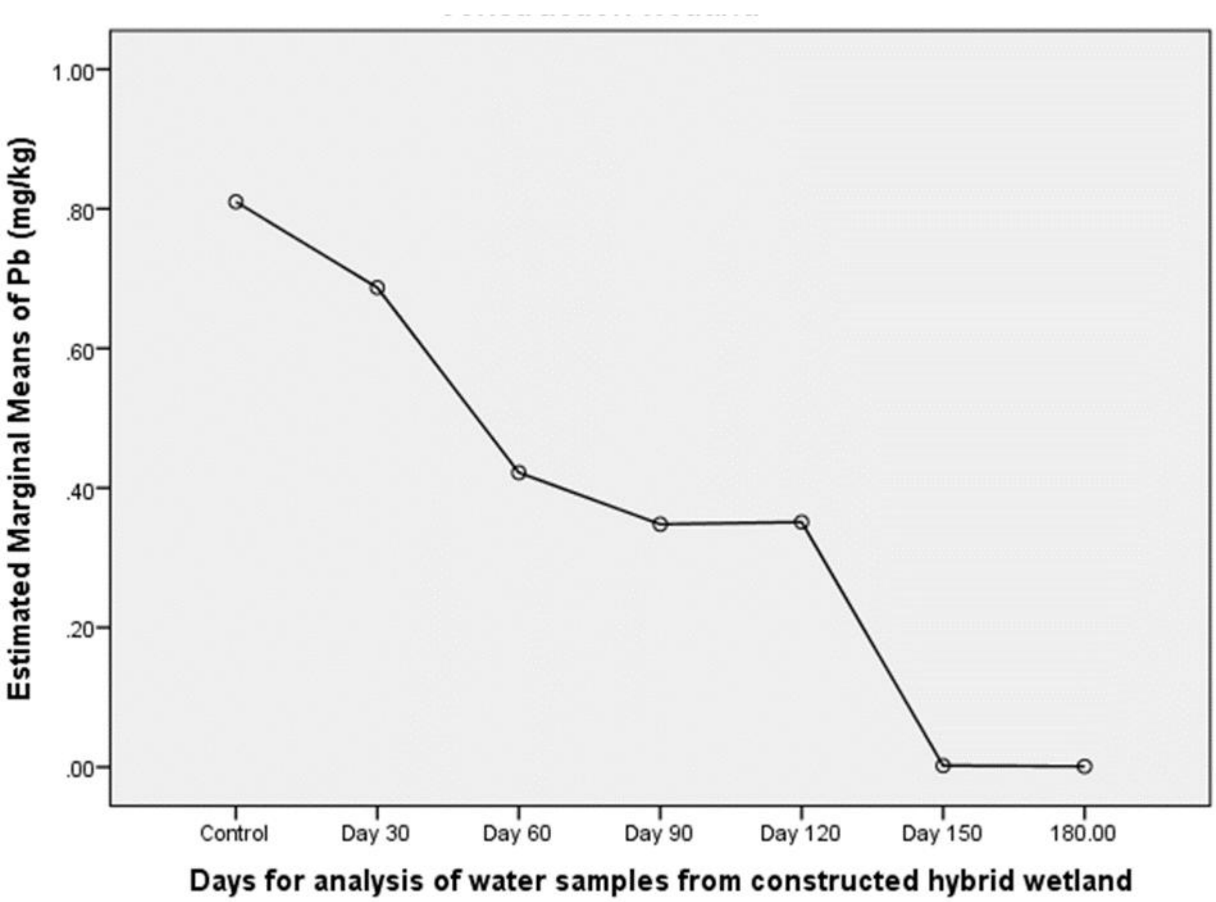

Figure 4.23: Concentration of Pb (mg/kg) content in water sample from hybrid construction wetland

In table 4.45 the Tukey HSD (Honestly Significant Difference) test for Cu (Copper) content in water samples from the hybrid construction wetland, analyzed across different days, yielded the following results:

The p-values for the comparisons between subsets are 0.989, 0.059, and 0.994, respectively. These p-values indicate that there are significant differences in Cu content between subset 1 and subset 2 for Day 120, with subset 1 having a higher Cu content. However, there are no significant differences between subsets for Day 90 and Day 30. Therefore, there are significant differences in Cu content between subsets analyzed on Day 120, but not on Day 30 and Day 90 in the water samples from the hybrid construction wetland.

Figure 4.24represents the concentration of Cu (Copper) in water samples from a hybrid constructed wetland over time, shows fluctuations in Cu levels which could be due to experimental error. The concentration drops significantly. This indicates an initial sharp decrease in Cu levels, likely due to the early effectiveness of the hybrid wetland in reducing Cu content through processes like sorption, phytoremediation, or sedimentation. The figure shows both significant decreases and increases in Cu concentration over time, with notable shifts on Day 30 (sharp decrease) and Day 150 (sharp increase). While the data presents substantial variability, the early decline and late recovery suggest potential significant differences across time points.

Table 4.45: Concentration of Cu content in water sample from hybrid construction wetland

Days for analysis of water samples from constructed hybrid wetland	N	Subset		
		1	2	3
180.00	3	.1140		
Day 120	3	.1580		
Day 60	3	.1630		
Day 90	3	.1980	.1980	
Day 30	3	.2310	.2310	
Day 150	3		.7400	.7400
Control	3			.8430
Sig.		.989	.059	.994

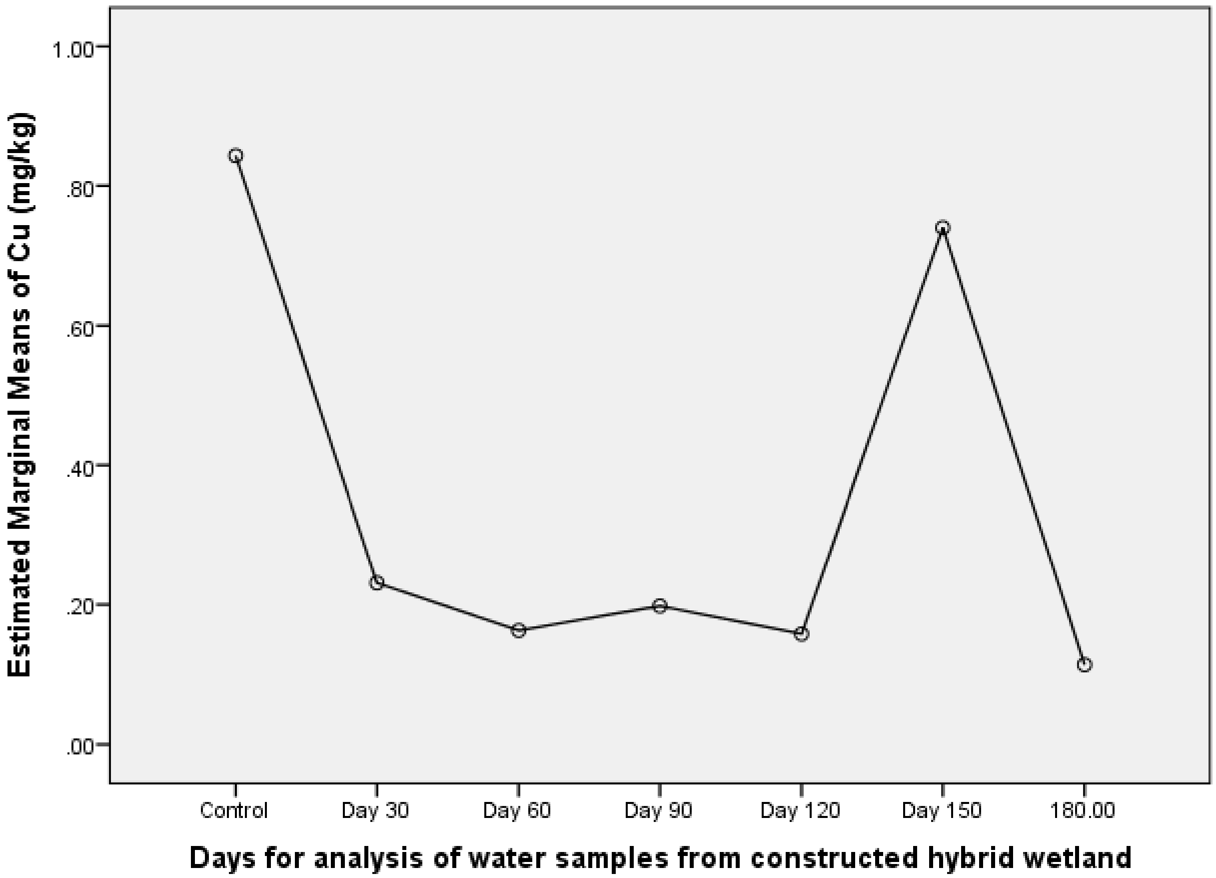

Figure 4.24: Concentration Cu (mg/kg) content in water sample from hybrid construction wetland

Table 4.46 shows the Tukey HSD test results for Nickel (Ni) content in sediment samples from the hybrid construction wetland. Day 150 and Day 90 have the same effect but Day 120 is significantly different from Day 150. All days have significantly different effects from the control.

Figure 4.25represents the concentration of Ni (Nickel) in water samples from a hybrid constructed wetland over time, shows consistent decline of Ni with little fluctuation. The concentration drops significantly which is an indication of the efectiveness of the hybrid wetland in reducing Ni content through processes like sorption, phytoremediation, or sedimentation. The figure shows significant decreases Ni concentration over time, with potential significant differences across time points

Table 4.46 Concentration of Ni (mg/kg) content in water sample from hybrid construction wetland

Days Days for heavy metal concentration in plant tissues used for phytoremediation	N	Subset 1	2	3	4	5
Day 150	24	7.2150				
Day 90	24	8.1038	8.1038			
Day 120	24		8.5237	8.5237		
Day 60	24			9.4387	9.4387	
Day 30	24				10.1600	
Control	3					11.7500
Sig.		.289	.916	.258	.524	1.000

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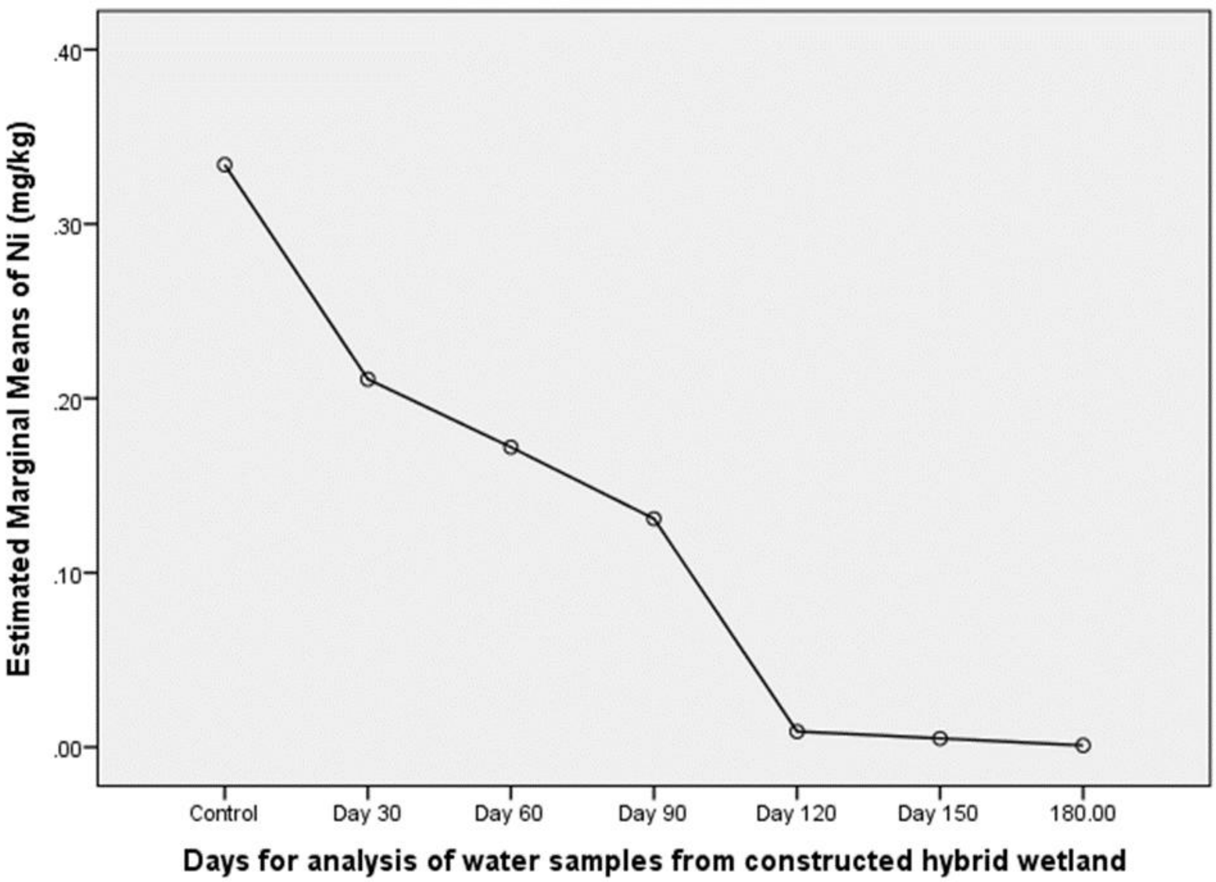


Figure 4.25 Concentration of Ni (mg/kg) content in water sample from hybrid construction wetland

4.1.13Heavy metal concentration in plant tissue used for phytoremediation in constructed hybrid wetland system microcosm studies.

Table 4.47 shows Tukey HSD Test for Zn (mg/kg) content in plant tissue used for phytoremediation in hybrid construction wetland. Zn (Zinc) Content in*P. conjugatum* P. J. Bergius tissue used for phytoremediation microcosm studies in hybrid constructed wetland. There are no significant differences in Zn content among the different days of heavy metal concentration analysis in plant tissues used for phytoremediation (Days 30 and 60) when compared to the control group. The control group is not in the same subset with Days 90, 120, 150, and 180 indicating that there is a significant difference in Zn content between any pair of days and the control group. These results suggest that the Zn content in plant tissues used for phytoremediation significantly vary across some days of heavy metal concentration analysis in the hybrid construction wetland.

Figure 4.26 shows This suggests that the plants are progressively accumulating more zinc over time. The figure shows significant increase in Zn content of the plant tissue over time, with potential significant differences across time points

Table 4.47: Zn content in *Paspalum conjugatum* P.J. Bergius tissue used for phytoremediation microcosm studies in hybrid constructed wetla

Days for heavy metal concentration in plant tissues used for phytoremediation	N	Subset				
		1	2	3	4	5
Control	3	48.2100				
Day 30	3	48.7500				
Day 60	3	49.3600				
Day 90	3		52.3800			
Day 120	3			58.2000		
Day 150	3				62.0100	
Day 180	3					64.7300
Sig.		.140	1.000	1.000	1.000	1.000

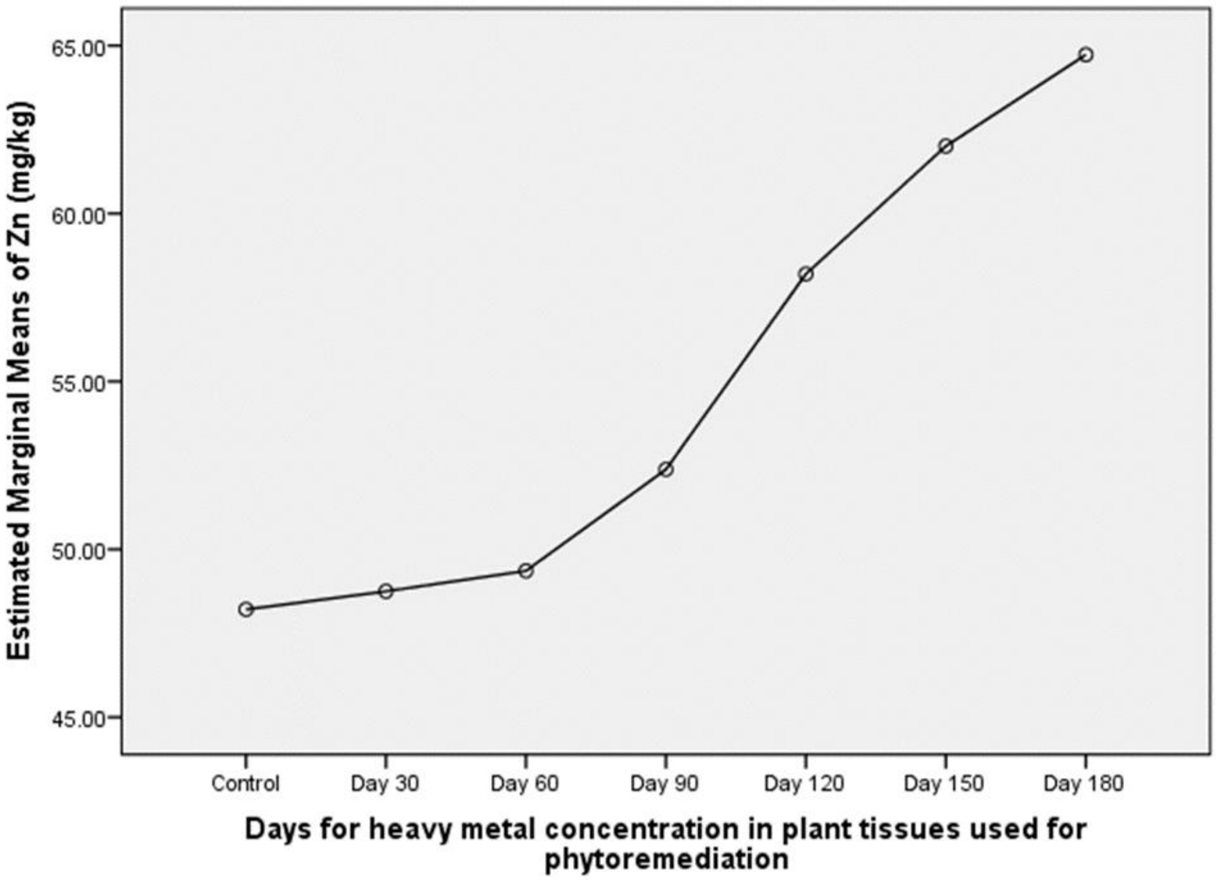


Figure 4.26: Zn (mg/kg) content in plant tissue used for phytoremediation microcosm studies in a hybrid constructed wetland

Table 4.48 shows Tukey HSD Test for Pb (mg/kg) content in plant tissue used for Phytoremediation in Hybrid Construction Wetland. The Tukey HSD Test for Lead (Pb) content in plant tissue used for phytoremediation in the hybrid construction wetland yielded the following results: There are no significant differences in Pb content among Day 30 and control, Day 30 and 60 and Day 120 and 150 of heavy metal concentration analysis in plant tissues used for phytoremediation. However, Day 180 and 90 are significantly different from every other day while the highest Pb content was remediate on Day 180.

Figure 4.27 highlights that Pb content in plant (*P conjugatum* P.J. Bergius)tissues used for phytoremediation significantly increase with longer days of heavy metal concentration analysis in the hybrid construction wetland.

Table 4.48: Pb content in *P conjugatum* P.J.Bergius tissue used for phytoremediation microcosm studies in hybrid constructed wetland

Days for heavy metal concentration in plant tissues used for phytoremediation	N	Subset				
		1	2	3	4	5
Control	3	9.0200				
Day 30	3	10.2800	10.2800			
Day 60	3		11.2100			
Day 90	3			12.6200		
Day 120	3				14.6800	
Day 150	3				15.7300	
Day 180	3					17.5100
Sig.		.089	.318	1.000	.207	1.000

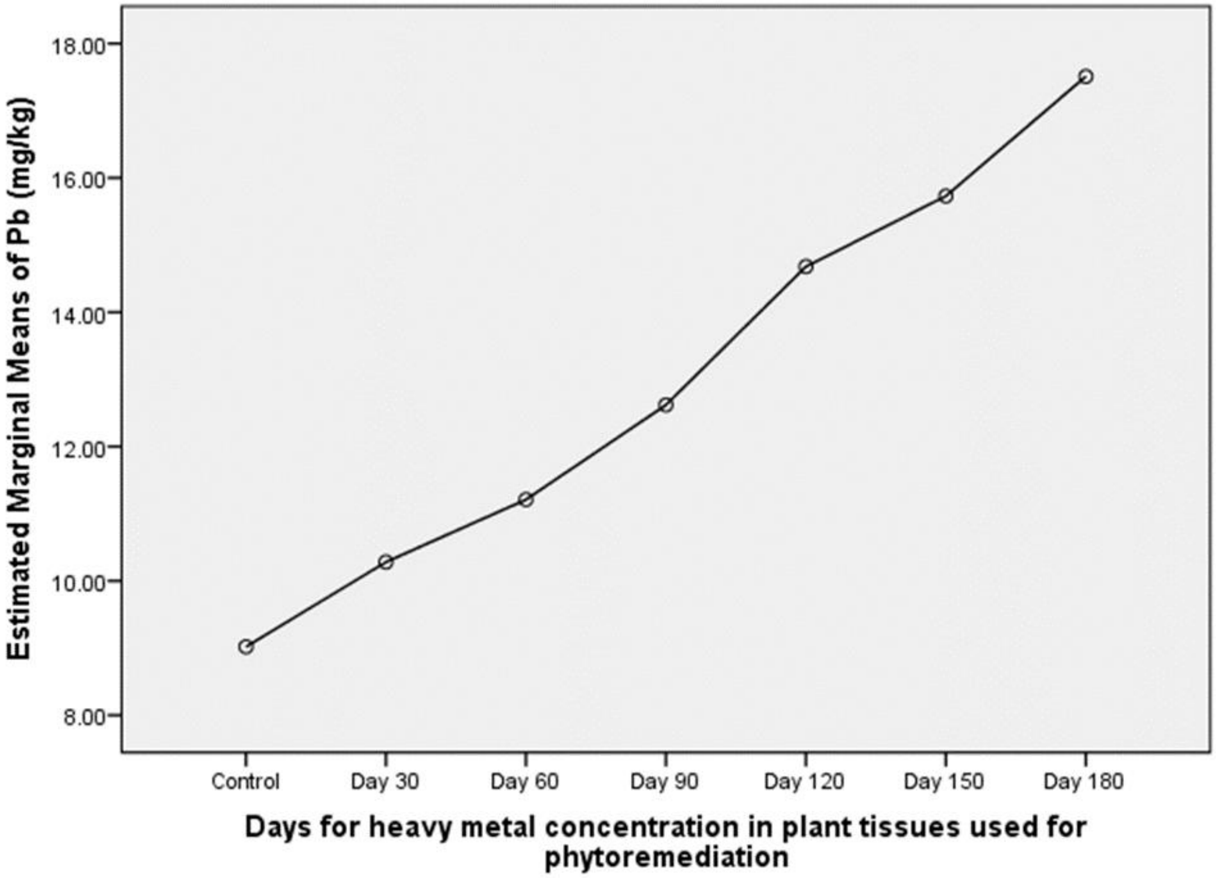
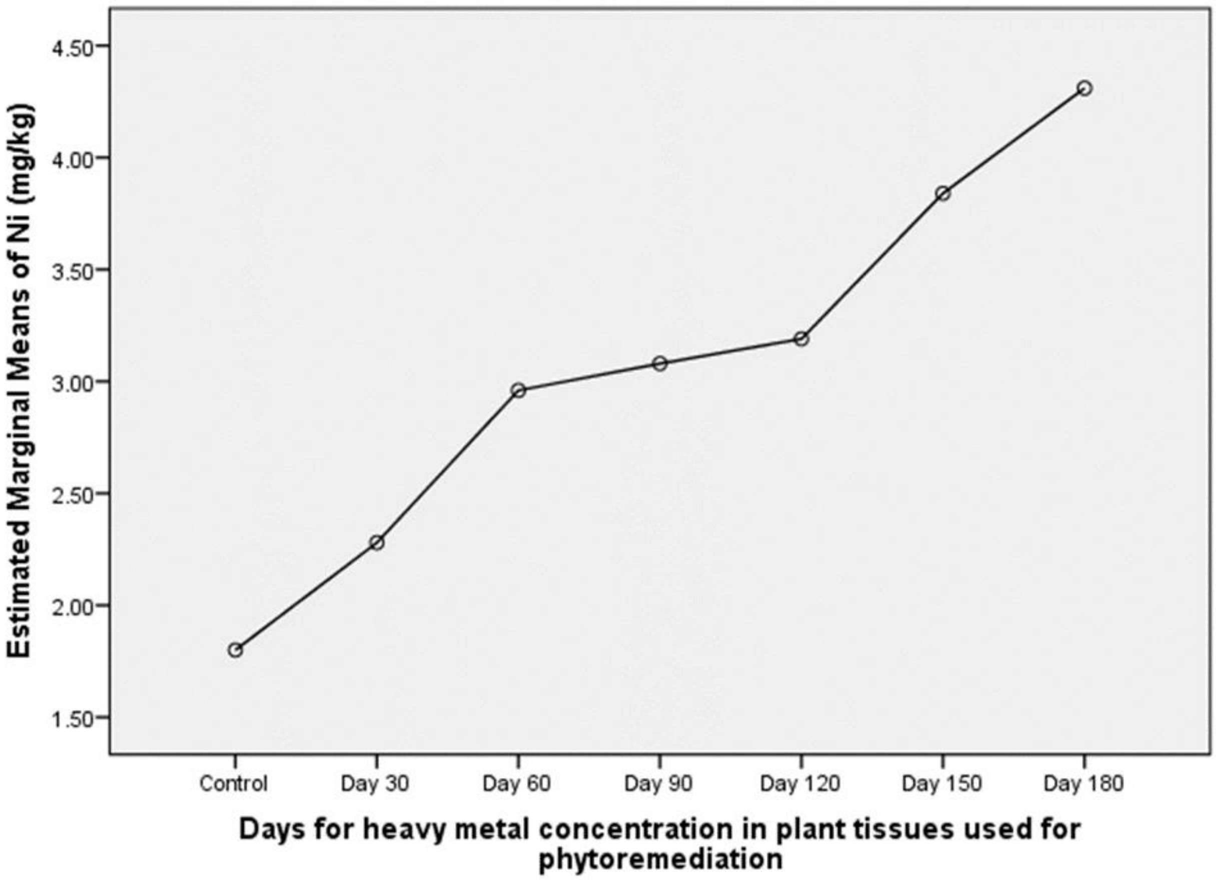


Figure 4.27: Pb (mg/kg) content in *P. conjugatum* P.J.Bergius tissue used for phytoremediation microcosm studies in hybrid constructed wetland

Table 49 presents Tukey HSD Test for Ni (mg/kg) content in plant tissue used for phytoremediation in hybrid construction wetland. The results show that there was significant difference in the amount of Ni remediated on Days 150 and 180 compared to control. This also indicates that time has significant effect on quantity of Ni absorbed by the plant tissuesTable 4.49: Ni content in *P. conjugatum* P.J. Bergius tissue used for phytoremediation microcosm studies in hybrid constructed wetland

Table 4.49: Ni (mg/kg) content in *P conjugatum* P.J.Bergius tissue used for phytoremediation microcosm studies in hybrid constructed wetland

Days for heavy metal concentration in plant tissues used for phytoremediation	N	Subset 1	2
Control	3	1.8000	
Day 30	3	2.2800	
Day 60	3	2.9600	2.9600
Day 90	3	3.0800	3.0800
Day 120	3	3.1900	3.1900
Day 150	3		3.8400
Day 180	3		4.3100
Sig.		.051	.061



Fiure 4.28: Ni (mg/kg) content in*P. conjugatum* P.J. Bergius tissue used for phytoremediation microcosm studies in hybrid constructed wetland

Table 50 shows Tukey HSD Test for Cu (mg/kg) content in *P. conjugatum* P.J. Bergius tissue used for phytoremediation in hybrid construction wetland The Tukey HSD Test for Copper (Cu) content in plant tissue used for phytoremediation in the hybrid construction wetland yielded the following results: There is significant difference in Cu content between the control group and Day 30 ($p = 0.146$), Day 30, 60 and 90 ($p = 0.170$), Day 90, 120 and 150 ($p = 0.277$), Day 120, 150 and 180 ($p = 0.164$). These results suggest that there are no significant differences in Cu content in plant tissues across different close time points but significant different exists when compared to the control group.

Table 4.50: Cu Content in *P. conjugatum* P.J. Bergius tissue used for phytoremediation microcosm studies in hybrid constructed wetland

Days for heavy metal concentration in plant tissues used for phytoremediation	N	Subset				
		1	2	3	4	5
Control	3	7.3800				
Day 30	3	8.5200	8.5200			
Day 60	3		9.3800	9.3800		
Day 90	3		9.6200	9.6200	9.6200	
Day 120	3			10.3500	10.3500	10.3500
Day 150	3				10.9200	10.9200
Day 180	3					11.4600
Sig.		.146	.170	.277	.075	.164

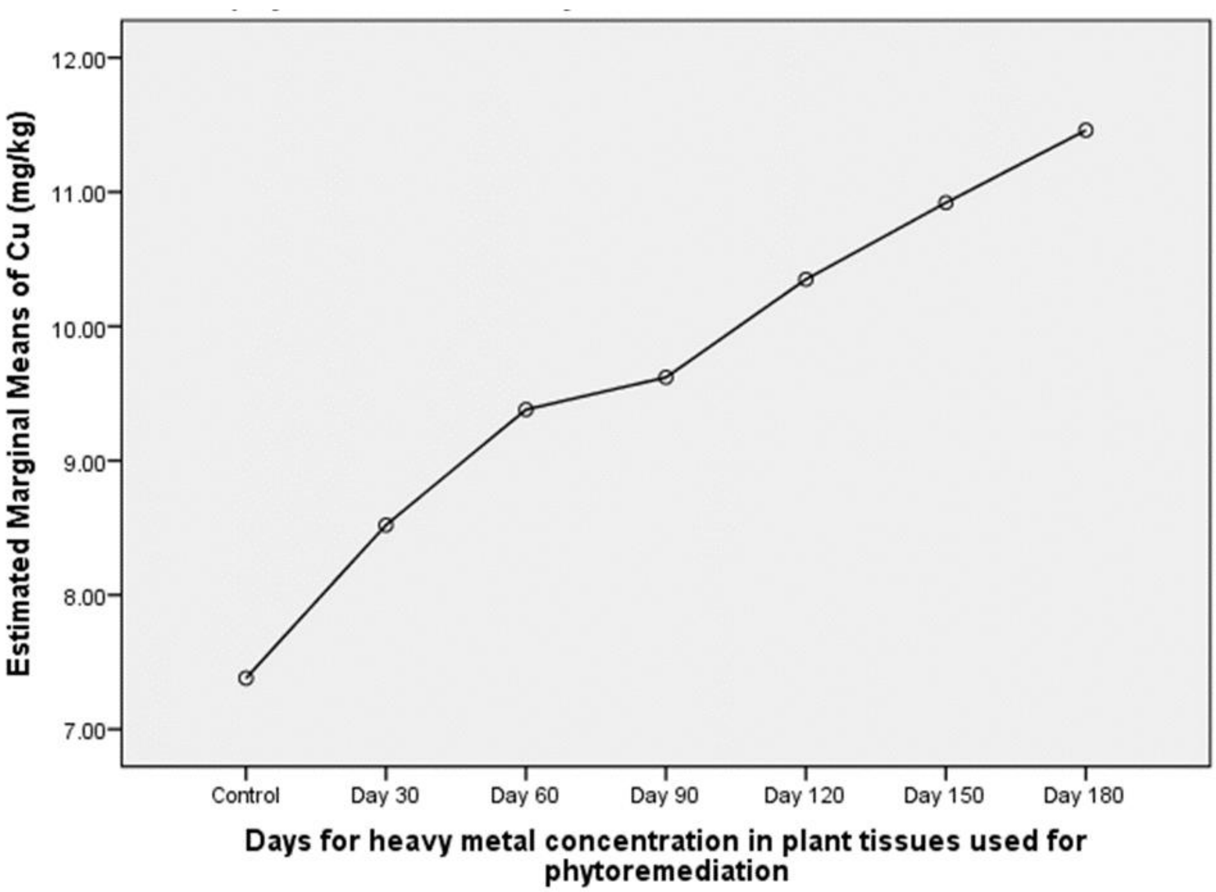


Figure 4.29: Cu (mg/kg) content in *P. conjugatum* P.J. Bergius tissue used for phytoremediation microcosm studies in hybrid constructed wetland

4.2 Discussion

The analysis of the unpolluted soil sample used in this study revealed the following composition: sand content of 47.3%, silt content of 27.3%, and clay content of 23%. Additionally, the soil had an organic carbon content of 0.65%, organic matter content of 0.45%, total nitrogen of 0.18%, available phosphorus content of 0.80%, and a pH level of 7.16.

These findings align with previous reports by Ogundola, Bvenura & Afolayan, (2018); Lindsay, (2001); Mohammed and Yusif, (2020); Umeugokwe, Ugwu, Umeugochukwu, Uzoh, Obalum, Ddamulira, & Alenoma (2021); Jin, Hu, Khan, Zhang, Yang, Jia & Sun (2021). They have all documented varying levels of sand, silt, clay, organic matter, organic carbon, total nitrogen, and available phosphorus in loamy soil. The study also indicates that the percentage of hydrocarbon-utilizing bacteria in the soil sample is 1.35%. This result is consistent with the earlier work of Ogbonna, Douglas, & Awari (2020), who observed that unpolluted soils generally have a lower percentage of hydrocarbon-utilizing bacteria compared to polluted soils.

The soil's average total heterotrophic bacteria (THB) count was 3.4×10^8 cfu/g, while the average hydrocarbon-utilizing bacteria (HUB) count was 4.6×10^6 cfu/g. Additionally, the total fungal count was 1.5×10^4 cfu/g, and the average hydrocarbon-utilizing fungi (HUF) count was 0.9×10^2 cfu/g. These findings align with the previous studies by Soludo, Orji, Anaukwu, Anyaoha, Ajogwu, & Eze (2024) and Eze, Owunna, & Avoaja (2013). They reported that heterotrophic bacterial and fungal counts in unpolluted soil were higher than those in polluted soil. Both studies, along with others, have documented varying average THB, HUB, THF, and HUF counts in unpolluted and hydrocarbon-polluted soils.

The ability of various hydrocarbon-utilizing bacteria isolated from experimental soil samples polluted with crude oil and heavy metals was assessed for biosurfactant production. Each isolate was assigned a "+" sign, indicating its capacity to produce biosurfactants. The number of "+" signs given to an isolate reflects the relative amount of biosurfactant it produced compared to other isolates. This assessment revealed that all isolates from the crude oil-polluted soil demonstrated the ability to produce biosurfactants.

Biosurfactant production was classified into three levels, high, moderate, low and based on qualitative or quantitative assessments conducted in laboratory experiments. Each bacterium is categorized according to its respective level of biosurfactant production (an indirect assessment) which is based on the size of collapse drop size and assigned '+' for low, '++' for moderate and '+++' for high. The result obtained from this assay showed that *Pseudomonas xiamenensis* has the highest collapse drop size indicating highest producer of biosurfactant among the hydrocarbon utilizer isolates. This is in agreement with the previous works of Parthipan, Preetham, Machuca, Rahman, Murugan, & Rajasekar (2017);Soberón-Chávez, Lépine, & Déziel (2005); Kaskatepe, Yildiz, Gumustas, & Ozkan (2015); Sarafin, Donio, Velmurugan, Michaelbabu, & Citarasu (2014); They all agreed that *Pseudomonas* spp. exhibited a higher level of biosurfactant production compared to most bacteria. They also affirmed that biosurfactants produced by *Pseudomonas* species were more effective in crude oil degradation.Padhi & Gokhale (2016); Okore, Nwaehiri, Mbanefo, Ogbulie, Ugenyi, Ogbuka, Ejele. & Okwujiako (2017);although no comparative study was carried out by these authors, they all agreed that *Acinetobacter baumannii, Pantoea dispersa, Enterobacter cloacae and Kocuria palus* produces moderate biosurfactants. The study by Padhi & Gokhalesuggested that although *Pantoea dispersa* possess a significant potential for biotechnological applications, that these species are less commonly studied for biosurfactant production compared to the others mentioned. However, some studies indicate their potential in specific environmental conditions, highlighting the diversity in biosurfactant-producing capabilities among different bacterial genera. Padhi and Gokhale, (2016); in their study identified *Enterobacter cloacae* as a predominant microorganism in a mixed culture isolated from petrochemical sludge. Their research highlighted its ability to degrade hydrocarbons, suggesting its potential role in the bioremediation of crude oil-contaminated environments. Result from this study indicated that *Lysinibacillus fusiformis* and *Acinetobacter baumannii* showed low drop size which is in line with the works of Pacwa-Płociniczak, Płaza, Piotrowska-Seget, & Cameotra, 2011; Ekprasert, Kanakai, & Yosprasong, 2020; Vasileva-Tonkova & Gesheva, 2007). Their research highlighted that while *Acinetobacter baumannii* can produce biosurfactants, the production levels were not as high as those observed in other bacterial species such as *Bacillus subtilis* and *Pseudomonas aeruginosa* but this finding is in contrast with the reports of Ndlovu, Rautenbach, Vosloo, Khan, & Khan, (2017); they reported that *Acinetobacter baumannii* was capable of producing substantial amounts of biosurfactants, particularly under optimized conditions, and effectively degraded hydrocarbons while Rosenberg & Ron, (1999) found that certain strains of *Acinetobacter baumannii* were prolific biosurfactant producers, particularly emulsan, which was effective in hydrocarbon degradation.

Preliminary study on *Brachiaria distachyoides* Stapf (African foxtail grass) shows that it was tolerant to 3% crude oil and heavy metals polluted soil. At 7% crude oil and heavy metals polluted wetlandsoil *Brachiaria distachyoides* Stapf exhibited reduced tolerance to this concentration of pollution, although there was a substantial growth but the measured values reduced when the experimental setup was compared to the control. This observation is in agreement with the study by Merkl, Schultze-Kraft, & Infante, (2004). They found that *Brachiaria* specie could tolerate crude oil concentrations within the range of 0%, 3%, and 5% without significant adverse effects. According to their findings, concentrations above this threshold resulted in decreased photosynthetic efficiency and biomass production. The result from this study shows that *Brachiaria distachyoides* Stapf could not survive 10% crude oil and heavy metals pollution but the study by Robson *et al*. (2013), reported that *Brachiaria* specie showed high potential on heavy metal phytoextraction indicating that the inability of this plant to grow at the stimulated pollutants concentrations may not be the result of heavy metals but rather the 10% concentration of crude oil. These findings make *Brachiaria distachyoides* Stapf not to be a plant of choice for this study.

The preliminary studies show that *Cyperus dichrostachyus* Hochst. ex A. Rich. was tolerant to 3% crude oil and fixed heavy metal polluted soil. This observation is in contrast with the previous findings of Chakravarty & Deka, (2021); Budhadev, Rubul, & Sabitry, (2012); Ighovie & Edwin, (2012) They found that *Cyperus dichrostachyus* was effective in remediating crude oil and heavy metals at different levels of crude oil and heavy metals concentrations. Results from this studies showed that *Cyperus dichrostachyus* Hochst. ex A. Rich (nutsedge) is slightly tolerant to 7% and highly susceptible to 10% crude oil and heavy metals and as a result, could not survive at this concentration. Though studies on *Cyperus dichrostachyus* Hochst. ex A as a phytoremediator plant for the remediation of crude oil and heavy metals polluted soil is sketchy, but a study by Sunday & Aboh, (2012) shows that crude oil amended with *Cyperus* sp., and *Axonopus* sp. worked together to reduce hydrocarbon by 59%. Nevertheless, *Axonopus* sp. and *Cyperus* sp. were responsible for 47% and 48% of the hydrocarbon reduction, respectively. Also review by

Ariyachandra, Alwis, & Wimalasiri, (2023), showed that *Cyperus rotundus* can extract and accumulate As, Cd, Pb, Rb, Sn, and Zn in its roots and shoots when the soil is heavily contaminated with the aforementioned heavy metals. Furthermore, *Cypreus rotundus* highlights the potential for heavy metal remediation using this plant species while highlighting the significant importance of bioconcentration factors and translocation factors to various heavy metals. For future phytoremediation research, Cyperus rotundus may be found to be a potential hyperaccumulator and phytostabilizer for the majority of heavy metals.

The inability of *C. dichrostachyus* to grow at above 7% concentration of crude oil and heavy metals could be attributed to the overwhelming toxic effect of crude oil at above 7% and not heavy metals present in the pollution. This is in agreement with the review by Ariyachandra et al. (2023) as described above. Though a good phytoaccumulator and phytoremediator plant, the inability to grow in 10% crude oil and hevy metals polluted wetland soil could be as a result of toxic effect of the hydrocarbon instead of heavy metals.

The studies on growth response of *Kalanchoe pinnata (Lam.) Pers* (Cathedral bells, Air plant, Life plant, Miracle leaf, Goethe plant or Love bush) to 3% crude oil and heavy pollution showed that the experimental to control ratio at this concentration was approximately above 50% indicating good tolerance to the toxic effects of the pollutants. At exposure of 7% pollutant concentration, a lower tolerance was observed and growth stopped. This showed that *Kalanchoe pinnata (Lam.) Pers* couldn't survive the toxicity of 10% crude oil concentration and heavy metals pollution. Research specifically on *Kalanchoe pinnata (Lam.) Pers*. as a phytoremediator plant for the remediation of crude oil-polluted soil appears to be less commonly documented compared to its use for heavy metal remediation. These observations are in agreement with the previous study of Ubogu & Odokuma, (2019). They screened the following plant species,*Zea mays*,*Telfairaoccidentalis, Saccharum officinarum, Kalanchoe pinnata, Phaseolus vulgaris, Arachis hypogaea, Phragmitis australis, Azolla pinnata* and *Eichornia crasssipes* for their ability to grow and tolerate 0, 1, 3 and 6% w/w crude oil contamination for a 120-day period to determine the influence of crude oil on plant germination, height, root length, leaf area growth and survival/death time. Among the nine plants tested only *E. crassipes, P. australis* and *S. officinarum* survived for the 120-day period of the study at 6% w/w contamination. The survival of these plants in oil-contaminated soils indicates that they could be used for rhizoremediation in the Niger Delta. The fact that *Kalanchoe pinnata* was among the screened plants and could not grow when subjected to 6% crude oil pollution, shows that it is not a good phytoremediatior plant. The fact that the studies by Odoemelam & Ukpe, (2008); Villarreal Romero, Robles Camargo, & Costa, (2023); collectively highlight the potential of *Kalanchoe pinnata* as an effective phytoremediator for various heavy metal-contaminated soils, shows that its inability to tolerate 10% crude oil and heavy metals pollution may not be attributed to the presence of heavy metals pollution but rather its ability to tolerate the toxic effect of crude oil at that concentration. For not being able to survive the 10% concentration of crude oil and heavy metals, *Kalanchoe pinnata* was not a plant of choice for bioremediation.

The results obtained when *Panicum maximum* Jacq (Guinea grass) was subjected to concentrations of 3%, 7% and 10% crude oil and heavy metals polluted soil during the preliminary studies show that the plant was tolerant to 3% pollutant concentration, but non tolerant to higher concentrations of the pollutants. The study on *Panicum maximum* Jacq as a phytoremediator plant for remediation of crude oil polluted soil is sketchy but Messou, Ouattara,

& Coulibaly, (2020) focused on the phytoextraction capacity of *Panicum maximum* to accumulate heavy metals such as lead (Pb), cadmium (Cd), nickel (Ni), zinc (Zn), and copper (Cu) in a controlled environment. The results indicated that *Panicum maximum* effectively accumulates these metals, suggesting its potential for use in phytoremediation strategies but no mention of crude oil was made. Therefore, high crude oil contamination significantly inhibited the growth and physiological activities of *Panicum antidotale*, a species within the *Panicum* genus which is in agreement with the findings of this study. Although the effect of heavy metals on the plants were not studied, but the study by Fakayode & Onianwa (2002) found that *P. maximum* had high accumulation factors for Cr, Cd, Ni, and Mn in the Ikeja Industrial Estate in Lagos, Nigeria. Messou et al. (2020); Nwadinigwe & Ugwu, (2018); Coulibaly, (2020), all agreed that *P. maximum* accumulate Pb, Cd, Ni, Zn, and Cu in a controlled environment and demonstrated that *P. maximum* has the capacity to clean up sites contaminated with these heavy metals. This indicates its potential for use in cleaning up heavy metal-polluted environments therefore its non-survival in 7% and 10% crude oil and heavy metal pollution cannot be attributed to the presence of heavy metals but rather the high concentration of crude oil. Based on the findings from the preliminary study on *Panicum maximum* Jacq, death of plant when subjected to higher concentrations of the pollutants, was due to its non-tolerance to high concentration of crude oil rather their heavy metals and as a result, it was not a plant of choice for bioremediation.

The growth response of *Mimosa pudica* L. (Sensitive Plant, Sleepy Plant, Touch-me-not orShame plant) to 3%,7% and 10% concentrations of crude oil and heavy metals showed that it gave a positive response to 3% but 7% and 10% exhibited a negative response to phytotoxocity. Thirty (30) days after planting in 7% pollutant concentration, the plant stopped responding to measured growth indices. This observation is in agreement with the previous work of Kamble & Bhosle, (2012). They found that *Mimosa pudica* could tolerate crude-oil contamination up to 6.2% (w/w). Also Ahalya, Ramachandra, & Kanamadi, (2003), evaluated the effectiveness of *Mimosa pudica* in the phytoremediation of heavy metals. They reported that the plant demonstrated significant uptake of metals such as lead (Pb), cadmium (Cd), and zinc (Zn) and showed a tolerance to metal concentrations up to 100 mg/kg for Pb, 50 mg/kg for Cd, and 200 mg/kg for Zn. Toxic Effects of Hydrocarbons, Nutrient imbalance and phototoxicity among other factors could be responsible for *Mimosa pudica* not being able to tolerate high level of crude oil and heavy metals polluted soil therefore was not a plant of choice for bioremediation.

The results of the growth response of *Paspalum conjugatum* P.J.Bergius (Crab grass) to 3%, 7% and 10% of crude oil and heavy metals polluted soil during preliminary studies show that they were tolerant to the toxic effect of the pollutants at the applied study concentrations although at 10% crude oil and heavy mental concentration, there were impairment to growth indices but observation from the study, showed that the leaves of the plant were still green throughout the study period but could not grow when subjected to a higher concentration of 12%. These observations are in agreement with the previous studies by Fadliah, Yadi, Didy, & Mohamad, (2020):Adesuyi, Njoku, Akinola, & Jolaoso, (2018); in their comparative studies involving different plants they found that *Paspalum conjugatum* significantly reduced the total petroleum hydrocarbons (TPH) in the soil even at a 15% crude oil concentration. The plant showed good growth and resilience, contributing to the degradation of hydrocarbons. The study also observed that *Paspalum conjugatum* is a good phytoacumulator of heavy metals. Also the study by Ogbo, Zibigha, & Odogu, (2009); showed that at varying degrees of crude oil contamination (0.00, 2.50, 5.00, 7.50, 10.00, 12.50, and 15.00%) on the growth of *Paspalum scrobiculatum*, a prevalent weed in Nigeria. Plant height, wet weight, and leaf area were significantly decreased as a result of the varying degrees of crude oil pollution. The effect grew as the contamination level rose (for example, the leaf area decreased from 68.47 cm2 in the control to 34.07 cm2 in the 15.00% level of contamination). The plant's dry weights did not significantly decrease as a result of the pollution. Erute, Zibigha, & Odogu, (2009), reported that 15% crude oil pollution had insignificant reduction in the dry weights of *Paspalum scrobiculatum.* In contrast, the study by Paz-Alberto, Sigua, Baui, & Prudente, (2007) reported that *Paspalum conjugatum* L. was the least phtytoaccumulator of Pb amongst four plants studied, while the study by Adesuyi et al. (2019) who carried out a comparative study involving many plant species to monitored the distribution of Cd, Cr, Cu, Ni, Pb and Zn in plants of Lagos lagoon wetlands in Nigeria reported that *Paspalum vaginatum's* root had the highest Cu concentration and also a good phytoaccumulator plant for the above mentioned heavy metals. *Paspalum conjugatum* P.J. Bergius was the plant of choice for this study because of its ability to tolerate 10% crude oil and heavy metal concentration as applied to the study.

The results of the growth response of *Mariscus rotundus* (Nutgrass or Purple nutsedge) during the preliminary studies shows that *Mariscus rotundus* exhibited the tolerance to 3%, 7% crude oil and heavy metal pollution but could not tolerate the toxic effect of 10% concentration of crude oil and heavy metals. This is in contrast with the previous work of Basumatary, Saikia, & Bordoloi, (2012). They examined the use of *Cyperus rotundus* (nut grass) from the family Cyperaceae for the remediation of crude oil (2.05, 4.08, 6.1, 8.15, and 10.2%) impacted soil and at the end of 180 days, result showed that plant biomass and heights decreased to 26 and 21.9%, respectively, in the presence of crude oil. *C. rotundus* could cause a reduction in TOG content and tolerate crude oil pollution up to the concentration of 10.2%. The reductions in TOG for the unvegetated pots were 4.4%, 5.6%, 6.6%, 7.6%, and 9.6% for treatments A, B, C, D, and E, in that order, demonstrating the plant species' suitability for phytoremediation. Also Nwaichi, Chukwuere, Abosi, & Onukwuru, (2021), demonstrated that purple nutsedge is a good pytoremediator and pytoaccumulator plant after planting it on a crude oil and heavy metals impacted (though the concentration of the pollutant was not stated) soil samples collected from Kom-Kom community, Oyigbo, Rivers state, Nigeria. The result also indicated that *Mariscus rotundus* is a good phytoaccumulator plant which is in agreement with study by Nafea & Šera, (2020); Ariyachandra et al. (2023); Chukwuma, Aruorivwooghene, Nwaichi, & Monanu, (2020). Their review highlighted that *C. rotundus* among other plants has a high capacity for absorbing and accumulating heavy metals from contaminated soils. They found significant differences in metal concentrations between control and tested samples, indicating the plant's sensitivity to heavy metal pollution. The study emphasizes that *C. rotundus* can be used for bioremediation in polluted habitats. Aryalet al.(2016) reported that *cyperus vaginatus*, from the family Cyperaceae have been used in constructed wetlands to alter the biogeochemistry of waterlogged soils through removal of heavy metals from the water bodies. Ajuru & Nmom, (2020); in their review, they reported that *Mariscus rotundus* amongst other plant species are widely studied and recommended for phytoremediation for crude oil and heavy metals polluted soils.

The results of the growth response of *Mariscus ligularis* L. (umbrella sedge) to 3%, 7% and 10% of crude oil and heavy metals polluted soil in a microcosm hybrid constructed wetland system during preliminary studies showed that showed that *Mariscus ligularis* L. like other plant species used for this study was tolerant to 3% crude oil and heavy metals pollution.

Results obtained from the growth response studies further showed that the growth indices of the experimental setup have negative trends when compared to the control at 7% crude oil and heavy metal pollution. The toxic effect of the pollutants overwhelmed *Mariscus ligularis* L at 120 and 150 days after planting under 7% crude oil and heavy metal pollution become more noticeable through the colour of the plant leaves. At 10% concentration of the pollutants, *Mariscus ligularis* (L.) could not grow. This is in agreement with the studies by Abednego, Pappoe, Armah, & Ato, (2013). In their study investigating the phytoremediation potential of indigenous Ghanaian grass and grass-like species grown on used motor oil contaminated soils, they found that *Mariscus ligularis* had the lowest total hydrocarbon content (THC) uptake and percentage degradation of hydrocarbons compared to other tested plants like *Bothriochloa bladhii* and *Torulinium odoratum*. They did, however, come to the conclusion that *Mariscus ligularis* was less successful than the other plants at phytoremediating soil contaminated with used motor oil. Four plant species—*Mariscus alternifolius, Fimbristylis ferruginea, Schwenkia americana,* and *Spermacoce ocymoides*—were found to be successful in remediating heavy metal contamination in soil polluted by crude oil, according to a study on heavy metals tolerance conducted by Chukwuma et al., (2020). *F. ferruginea* removed cadmium and chromium the best after the cleanup period, with removal rates of 70.3% and 93.8%, respectively. *M. alternifolius* had the highest clearance rate of lead (89.0%). *S. ocymoides* restored lead levels by 65.07%, while *M. alternifolius, F. ferruginea*, and *S. americana* recovered lead levels by 145.47%, 27.18%, and 353.36%, respectively. All four plants helped to restore the contaminated soil. The study concludes that these plants are effective for heavy metal removal in crude oil-polluted soils and are recommended for future remediation efforts. Due to these findings, *M. ligularis* like the other seven plants with the exception of *Paspalum conjugatum* P.J. Bergius could not be plants of choice for this study.

The response of hydrocarbon utilizing bacterial in different concentrations of native soap solution was assessed for a period of six days. The result suggests that lower concentration (1%) of native soap supported the growth of the test organisms more significantly than higher concentrations greater than 10%. This is in agreement with the previous work of Olajuyigbe, Adeoye-Isijola, & Adedayo, (2017); whose study on Black soap concluded that the minimum inhibitory concentration for *Klebsiella pneumoniae* and *Enterococcus faecalis* ranged between 0.125 mg/mL and 2 mg/mL, *Staphylococcus aureus* (0.25–4) mg/mL, *Escherichia coli* (0.125–4) mg/mL This means that higher concentration of the soap results in the death of the studied bacteria. Also the study by Wemedo, Amadi, Nedie, & Olaolu, (2018), agreed that the high concentration of soap leads to higher significant mortality rate in the study organisms. Also the previous study by Anoliefo, Ikhajiagbe, Okoye, & Omoregie, (2016). Anoliefo et al. (2019), found that 1% w/v local soap-in-water caused an increase in rise of surface-active chemicals following the application of soap treatments, including rhamnolipids, trehalolipids, sophorolipids, emulsan, liposan, and surfactin which led to increase in the polluted soil's microbial consortia resulting to a general decrease in the soil's total petroleum hydrocarbon (TPH) concentrations.

The assessment of growth response of hydrocarbon utilizing fungi in different concentrations of the native soap was also carried out. The result illustrates that the efficacy of native soap on fungal growth varies with concentration and time, showing the highest growth at lower concentrations followed by a decline, while higher concentrations maintain a stable but lower effectiveness on fungal growth. These observations are in contrast with the previous study by Fasola, Aponmade, & Aponjolosun, (2020); The result from their study shows that at a native soap concentration of 0.5, 1.0, 1.5 and 2.0 mL were found to have significant antifungal effect on all the four fungi at all volumes. The result highlights the importance of optimizing concentration for maximum fungal growth while avoiding potential toxicity or inhibitory effects.

The growth response of hydrocarbon utilizing bacterial (HUB) species in different concentrations of poultry dropping (poultry manure) was assessed. It was observed that the treatment with 1% poultry dropping concentration resulted in a significant increase in bacterial species growth. The results indicate that the 1% concentration of poultry droppings is the most effective treatment for increase in bacterial species growth. The 10% concentration also shows a beneficial effect but is less effective than the 1% concentration. The 30% concentration has the least effect, indicating that higher concentrations might not proportionally increase efficacy and could potentially have adverse effects. These observations are in contrast with the previous works of Zhang, Sun, Wang, Peng, Wang, Lin, Yang, Hua, & Wu, (2023). They observed that high concentrations of chicken manure resulted in distinct microbial profiles. Also Jin, H., Zhang, Yan, Yang, Fang, Li, Shao, Wang , Yue, Wang , Cheng, Shi, & Qin, (2022); Yang, Ashworth, DeBruyn, Willett, Durso, Cook, & Owens, (2019); Acosta- Martínez, and Harmel, (2006),they all studied the impact of poultry manure application on soil microbial communities and found that high concentrations of poultry manure altered the complexity and structure of both bacterial and fungal networks, enhancing the abundance of certain keystone taxa which play crucial roles in nutrient cycling. Also the study by Okafor, Orji, Agu, Awah, Okeke, Okafor, & Okoro, (2016) contrasted the findings of this work. They studied the impact of poultry droppings in the bioremediation of crude oil-polluted soil was evaluated. Different concentrations of the poultry droppings (10%, 30%, and 50%) were also studied and the result obtained from this study shows that the microbial growth rate increased as the concentration of the poultry droppings increased. The reason for the contrast in observed results could be among other factors due to concentration of the pollutants at which this study was carried. At 10% crude oil with heavy metals pollution, 30% percent poultry manure could be detrimental to the growth of HUB due to heat released to the environment from its breakdown which might result in additional stress on the bacteria resulting to their death.

Growth response of Hydrocarbon utilizing Fungi (HUF) in different concentrations of poultry droppings showed that all responses decline, with the higher concentration group showing a more rapid decrease. The graph shows that low concentrations (1%) of poultry droppings lead to the highest and most sustained response, whereas higher concentrations (10% and 30%) show an initial positive response followed by a decline. This pattern is consistent with the findings from Naowasarn & Leungprasert, (2016) Their research demonstrated that the addition of poultry droppings though the concentration was not highlighted, at low concentrations crude of oil pollution (5%) improved the degradation rates of hydrocarbons in contaminated soil. Their study also noted an increase in the population of hydrocarbon-degrading organisms, suggesting that poultry droppings provide essential nutrients that stimulate microbial growth. The findings by Okafor et al. (2016); Coo, Oviasogie, & Ikhajiagbe, (2022).; Ekpo & Nya, (2012) contrast to the above findings. In their study they found that high concentrations of organic amendments, including poultry droppings, significantly enhanced the growth and activity of hydrocarbon-degrading microorganisms.

The growth response of hydrocarbon utilizing bacteria (HUB and hydrocarbon utilizing fungi (HUF) in different combined concentrations of natural soap (NS) and poultry droppings (PD) over six days for bacteria and 21days for HUF, shows that all groups showed a decline, the study shows that the combination (0.5% + 0.5%) to most significantly support the growth of HUB followed by (5% +5%) combination, with the highest concentration (15% + 15%) showing the steepest drop. Although the analysis of the growth response of hydrocarbon-utilizing bacteria (HUB) in various combined concentrations of natural soap (NS) and poultry droppings (PD) is limited, the findings of Akpokodje & Hilary (2019) align with this observation. In their study, "Bioremediation of Hydrocarbon Contaminated Soil: Assessment of Compost Manure and Organic Soap," they reported that the growth and activity of hydrocarbon-degrading bacteria significantly increased when low to moderate concentrations of poultry manure were combined with natural soap.

Paspalum conjugatum P.J. Bergius growth response to measured parameters (shoot length, root length, weight and leave size) in hybrid constructed wetland soil polluted with 10% crude oil and heavy metals (Cu, Pb, Zn and Ni) treated with different amendments of poultry droppings (1%), natural soap (1%) and combination of both (1%). Result obtained indicates that combination of natural soap and *Paspalum conjugatum* P.J. Bergius (PS + NS + PLT) at the pollutants concentrations show a continuous increase reaching the highest *Paspalum conjugatum* P.J. Bergius measured growth parameters, suggesting the best performance among the treatments. This implies that natural soap in combination with *Paspalum conjugatum* P.J. Bergius significantly aids in mitigating the pollutants' negative effects. In contrast, although poultry manure was not used, but the study by Akpokodje and Hilary demonstrated that the combination of compost manure and organic soap effectively degraded total hydrocarbon content (THC) from 957.21 mg/kg to 154.36 mg/kg in contaminated soil. The results indicated significant improvements in soil physical characteristics and vegetative growth in treated samples compared to controls.

The combination of natural soap, poultry droppings and *Paspalum conjugatum* P.J. Bergius (PS + NS + PD + PLT) shows steady growth, indicating that the combination of natural soap and poultry droppings works well but slightly less effectively than natural soap alone and *Paspalum conjugatum* P.J. Bergius combined. This observation is in agreement with the previous studies by Akpokodje &Hilary, (2019) who demonstrated that the combination of compost manure and organic soap effectively degraded total hydrocarbon content and vegetative growth in treated samples compared to controls. The benefits of combining natural soap and organic amendments to enhance nutrient availability, improve soil structure, and promote microbial activity, thereby supporting better plant growth in contaminated soils. This contrast could be because of the difference in the concentrations of pollutants in which the phytoremediation plants were subjected. A 10% crude oil and heavy metals polluted soil by all standard is a heavily polluted soil environment and the addition of poultry manure may add to the toxicity of the soil initially of which may wane off with time due to natural attenuation, after which addition of organic manure will play a significant role in all measured plant growth parameters. This is in agreement with the review by Saharan, Singh, Goyat, Umar, Ibrahim, Akbar, & Baskoutas, (2023); who stated that the choice of cleanup technique depends on the types of contamination, since a poor choice may hinder high removal efficiency.

The residual concentration of Total Petroleum Hydrocarbon (TPH) in soil sediments from hybrid constructed wetlands polluted with crude oil and heavy metals and phytoremediated over 150 days with various amendments: poultry droppings (PD), natural soap (NS), plant (PLT), and combinations of these shows that all treatments resulted in a decline in TPH concentrations over time with phytoremediation of the polluted soil sample with combined effect of natural soap and plant (*Paspalum conjugatum* P.J.Bergius) (PS + NS + PLT) shows the most significant reduction in TPH than all other treatments, suggesting a synergistic effect of multiple amendments. This finding is in line with the previous studies by Hoang, Lamb, Sarkar, Seshadri, Lam, Vinu, Bolan, (2022). This study assessed the effects of a synthetic surfactant (Triton X-100) and a triterpenoid saponin (from red ash leaves, Alphitonia excelsa) on plant growth and TPH biodegradation in the rhizosphere of two native wild species (a shrub, Hakea prostrata, and a grass, Chloris truncata). Results obtained shows that at high concentration of Triton X-100 dramatically inhibited the growth of the two plants under study (reducing biomass and photosynthesis) and the microbial activity in the rhizosphere. In contrast, saponin administration greatly boosted TPH elimination (up to 60% in C. truncata at 1000 mg/kg due to greater plant growth and related microbial activity in the rhizosphere). Their study demonstrated that natural soap reduced TPH concentrations more effectively than poultry manure in soil contaminated with high levels of crude oil; plants treated with natural soap showed improved physiological parameters, including increased chlorophyll content and better overall health and enhanced degradation of hydrocarbons was attributed to the improved solubilization of oil by natural soap, facilitating microbial degradation processes.

Phytoremediation treatment with combination of poultry dropping and natural soap (PS + PD + NS)and natural soap alone (PS + NS) resulted in substantial TPH reduction, with PS + PD + NS performing slightly better PS + NS. This finding is in agreement with the studies of Akpokodje &

Uguru, (2019). They reported that combined application of poultry manure and natural soap showed a more substantial decrease in TPH levels than natural soap alone.

Treatment with poultry droppings alone (PS + PD) and with combination of plant and poultry droppings (PS + PD + PLT), results showed that these treatments are moderately effective, with PS + PD + PLT showing a more gradual decrease in TPH than the former. This is in agreement with the previous work of White, Wolf, Thoma, & Reynolds, (2006). According to their research on plant selection and soil amendments from a field study, the TPH levels in vegetated fertilized plots at six months were considerably lower than those in non-vegetated, non-fertilized plots. Elevated numbers of bacteria, fungi, and PAH degraders were seen following the growth of vegetation and the addition of fertilizer.

Treatment with plant alone (PS + PLT) and without amendment (PS alone), showed the least TPH reduction compared to others, indicating that plants alone or polluted soil (hydrocarbon degrading microorganisms present in polluted soil and natural attenuation) alone are less effective. This finding is in line with the previous work of Chikere, Azubuike, & Fubara, ((2017). The study assessed the natural attenuation of crude oil-polluted soil without any organic or inorganic amendments while the results indicated that there was minimal reduction in TPH levels over time in the untreated soil. The limited degradation was attributed to the lack of nutrient availability and microbial activity that typically aid in the breakdown of hydrocarbons.

Determination of residual concentration of zinc (Zn), Nickel (Ni) Copper (Cu) and Lead (Pb) (mg/kg) in the soil sediments from hybrid constructed wetland showed that phytoremediation with combined treatment of poultry manure and natural soap (PS + PD + NS + PLT) initially shows a decline, but later concentrations stabilize, suggesting initial effectiveness but potential limitations over time. Phytoremediation with natural soap alone (PS + NS + PLT), gave the most significant reduction among all treatment options, indicating effective long-term remediation. This finding is in contrast with the study of Wuana, Okieimen, & Imborvungu, (2010); Akpokodje & Uguru, (2019); Ogunwande, Adebayo, & Akinpelu, (2020). Their studies examined the impact of combining poultry manure and natural soap on the phytoremediation efficiency of heavy crude oil-polluted soil using different pytoremdiator plants and the results demonstrated that the combination treatment significantly reduced heavy metal concentrations, including arsenic (As), mercury (Hg), and cadmium (Cd), nickel (Ni), zinc (Zn), and copper (Cu) in the soil compared to other treatments. The combination of organic nutrients from poultry manure and the surfactant properties of natural soap improved the bioavailability of heavy metals, facilitating their uptake by the plants and leading to effective remediation.

Phytoremediation treatment with poultry droppings only (PS + PD + PLT) and remediation with combined poultry droppings and natural soap (PS + PD + NS) showed moderate reductions, with PS + PD + PLT showing some higher reduction. Treatment of the soil sample with poultry droppings alone (PS + PD) and natural soap alone (PS + NS), these treatments also showed a decline, with PS + PD reaching lower zinc levels than PS + NS. Treatment with *Paspalum conjugatum* P.J. Bergius alone (PS + PLT), Shows a gradual decline in heavy metals concentration, suggesting the effectiveness of plants alone which is in agreement with the studies by Sherameti &Varma (2011);Morel, Chaineau, Schiavon, & Lichtfouse, (1999). In their chapter "The Role of Plants in the Remediation of Contaminated Soils," the authors discuss how plants can significantly alter the fate of pollutants in soils. They highlight that phytoremediation, through the uptake of pollutants and the release of root exudates, can enhance the degradation of organic pollutants, leading to a reduction in contaminant levels over time.

Polluted soil without any treatment (PS), showed the least reduction, highlighting the necessity of amendments for effective zinc remediation. This is in agreement with the previous studies by Gao, Faheem, & Yu, (2022), whose work on bibliometric analysis of contaminated soil remediation research found that amendments are crucial for enhancing soil health, stabilizing contaminants, and improving soil structure. Lacalle, Becerril, & Garbisu, (2020); Li, Han, Liu, & Wang, (2022); Michael-Igolima, Abbey, & Ifelebuegu, (2022); Morel et al., (1999) concluded that biological remediation methods like phytoremediation and vermiremediation, often combined with organic amendments, are effective in recovering soil health and ecosystem service

Total hydrocarbon (THC) content in water sample from microcosm hybrid construction wetland was determined and the results showed that THC levels drop significantly over time, with a sharp decrease between the control and day 30 (down to around 800 mg/kg), continuing to decrease steadily to near zero by day 150 and maintaining low levels up to day 180. The sharp decline in THC concentration suggests effective phytoremediation. The combination of plants and natural soap significantly enhanced the degradation and removal of hydrocarbons from the water. This is in agreement with the previous studies of Uloaku, Abbey, & Ifelebuegu, (2022). This systematic review discusses various remediation methods for oil-contaminated soils, emphasizing the effectiveness of combining plant-based treatments with natural surfactants like soaps. Hoang Son et al., (2022), in their study titled "Plant-derived saponin enhances biodegradation of petroleum hydrocarbons in the rhizosphere of native wild plants," the authors found that the combination of plant roots and saponins significantly improved the biodegradation of petroleum hydrocarbons, highlighting the synergistic effect of these natural surfactants in remediation processes. The authors highlight that such combinations can significantly enhance the degradation of hydrocarbons in contaminated environments. This is consistent with the rapid decrease in THC observed in the figure.

The results indicate that natural amendments, such as natural soaps and organic matter (poultry droppings), can enhance microbial activity and hydrocarbon degradation, supporting the observed trend of decreasing THC levels. This finding aligns with the study by Eze & Nwankwo (2023), who evaluated the effectiveness of compost manure and organic soap on hydrocarbon degradation in petroleum-contaminated soil. It is also consistent with the research by Al-Mallah, Goutx, Mille, & Bertrand (1990), which investigated the production of emulsifying agents by marine bacteria, emphasizing the role of surfactants in enhancing hydrocarbon biodegradation. Although not directly focused on plant-soap combinations, this earlier work helps to understand how surfactants can facilitate the degradation process. Their findings showed that the combination of compost manure and organic soap significantly reduced total hydrocarbon content, highlighting the potential of this approach for effective soil remediation.

Furthermore, the results demonstrate that combining plants with natural amendments substantially improves the efficiency of hydrocarbon removal from contaminated sites, supporting the enhanced treatment efficacy observed in the graph. This is in line with the findings of Uloaku et al. (2022). In their systematic review, they discuss various methods for remediating oil-contaminated soils, including the use of plant-derived surfactants, and their effectiveness in promoting hydrocarbon degradation. The authors emphasize the role of microbial consortia in the rhizosphere for breaking down hydrocarbons, which can be further boosted by plant exudates and natural amendments. This supports the observed steady decline in THC levels as microbial activity facilitates hydrocarbon breakdown.

The concentration of lead (Pb mg/kg) content in water sample from hybrid construction wetland was determined and results showed that there was a significant decline in Pb concentration, the steady decline in Pb concentration demonstrates the effectiveness of the phytoremediation by hybrid constructed wetland in removing lead from crude oil and heavy metals polluted water and soil. This agreed with the previous study of Kadlec & Wallace (2008); Sheoran & Sheoran, (2006); they demonstrated that the dynamics of heavy metal removal in wetlands often show rapid initial uptake, followed by a slower, more stable phase as the system reaches equilibrium.The significant reduction in Pb levels within the first 90 days suggests that the phytoremediation process is most effective during the initial period. The marked drop between Day 120 and Day 150 indicates a critical phase in the remediation process, possibly due to enhanced microbial activity, plant growth, or other biogeochemical processes. By Day 180, Pb levels are almost undetectable, showing the long-term effectiveness and sustainability of the phytoremediation system in reducing heavy metal contamination in water. The above observation aligned with the previous works of Vymazal, (2010); Matagi, Swai, & Mugabe, (1998); Marchand, Mench, Jacob, & Otte, (2010);Ye, Whiting, Lin, Lytle, Qian, & Terry, (2001); Gikas, Ranieri, & Tchobanoglous, (2013); O'Sullivan, Moran, & Otte, (2004). They all demonstrated that Long-term studies indicate that sustained phytoremediation efforts can lead to significant reductions in contaminant levels, although the rate of removal may decrease over time as contaminant availability diminishes. The study by Faulwetter, Gagnon, Sundberg, Chazarenc, Burr, Brisson, & Stein, (2009). also demonstrated that the interaction of plants, microbes, and the abiotic environment in constructed wetlands facilitates complex biogeochemical processes that contribute to the removal of heavy metals.

The concentration of copper (Cu) (mg/kg) in water samples from a hybrid constructed wetland was measured, revealing a significant decrease in Cu levels within the first 30 days, indicating rapid initial uptake and removal of Cu from the water. However, around Day 150, a noticeable spike occurred, with Cu concentration rising to approximately 0.70 mg/kg. This increase could be due to several factors, such as the release of Cu from sediment, disturbances within the wetland system, or seasonal effects that influence biological activity in the wetland. This observation aligns with the findings of Kadlec & Wallace (2008), who noted that heavy metals might be released from sediments under certain conditions, leading to temporary spikes in metal concentrations in the water column. After the spike, a sharp decline followed, with Cu concentration dropping to around 0.05 mg/kg by Day 180. This trend is consistent with the studies of Almuktar, Abed, & Scholz (2018) and Pilon-Smits (2005), who conducted long-term research on phytoremediation. They demonstrated that constructed wetlands can significantly reduce heavy metal concentrations over time, though regular monitoring and maintenance are required to manage fluctuations.

The concentration of nickel (Ni) (mg/kg) in water samples from a hybrid constructed wetland was monitored over a period of 180 days, showing a slow but steady decline, reaching near zero by the end of the study. This trend indicates that the phytoremediation process in the constructed wetland was effective in reducing Ni contamination over time. Constructed wetlands are well-known for their capacity to remove heavy metals from contaminated water through mechanisms such as sedimentation, adsorption, and plant uptake. This finding aligns with previous studies, including those by Vymazal et al. (2010), Ali et al. (2013), Kadlec and Wallace (2009), and Marchand et al. (2010), which reported that hybrid constructed wetlands exhibit high removal efficiency for heavy metals like Cd, Ni, Pb, Cu, Cr, and Zn.

Zinc (Zn mg/kg) content in plant tissue used for phytoremediation microcosm studies in the hybrid constructed wetland shows that Zn concentration in plant tissues increases from approximately 48 mg/kg at the control stage to around 65 mg/kg by day 180. This trend indicates that the plants are progressively accumulating zinc over time, which is a characteristic outcome of effective phytoremediation. Phytoremediation is a well-documented method for the removal of heavy metals from contaminated environments. Plants used in this process absorb metals through their roots and accumulate them in their tissues which was documented through the previous work of Vymazal, (2010); Vymazal et al. (2010) which discussed the mechanisms by which plants absorb and accumulate heavy metals, supporting the observed increase in Zn concentration in plant tissues over time. Hybrid constructed wetlands combine the benefits of different types of wetlands, enhancing their capacity to treat contaminants, including heavy metals. The previous work of Vymazal, (2010); Wiessner, Kuschk, Buddhawong, Stottmeister, Mattusch, & Kästner, (2006); Kröpfelová, Vymazal, Švehla, & Štíchová, (2009);Lesage, Rousseau, Meers, Tack, & De Pauw, (2007); Maine, M. A., Sune, N., Hadad, H., Sánchez, G., and Bonetto, C. (2006); Maine, Sune, Hadad, Sánchez, & Bonetto, (2006); Maine, Suñe, Hadad, Sánchez & Bonetto, (2009); highlighted the effectiveness of hybrid constructed wetlands in treating various pollutants, including heavy metals like zinc, through plant uptake which is in line with observation from this study. The progressive increase in Zn concentration in plant tissues is consistent with the plants' ability to accumulate heavy metals over time, which is crucial for effective phytoremediation, this is in agreement with the previous study of Yadav, (2010). This article discussed how plants tolerated and accumulated heavy metals, emphasizing the increase in metal concentration in plant tissues as observed in the study. The time-dependent increase in heavy metal concentration in plant tissues is typical in phytoremediation studies, as plants gradually absorb and accumulate metals from the environment. The previous study of Samecka-Cymerman, Stepien, & Kempers, (2004), supports the time-dependent accumulation of heavy metals in plant tissues, which is consistent with the trend observed in the study, Different plant species have varying capacities to accumulate heavy metals, and the choice of species can significantly impact the phytoremediation process. Ali, Khan, & Sajad, (2013) in their review of 'Phytoremediation of heavy metals—concepts and applications' highlighted the importance of selecting appropriate plant species for effective phytoremediation, supporting the trend of increasing Zn concentration in plant tissues as observed in this study.

The lead (Pb mg/kg) content in *Paspalum conjugatum* P.J. Bergius tissue used for phytoremediation microcosm studies in hybrid constructed wetland polluted with crude oil and heavy metals was determined and the results shows that the concentration of Pb in the plant tissues increases significantly from the control stage to day 180 of the study. This trend indicates that the plants are progressively accumulating lead over time, which is consistent with effective phytoremediation. This observation can be explained from the fact that phytoremediation involves using plants to absorb, concentrate, and remove contaminants from the environment. The observed increase in Pb concentration in plant tissues over time aligns with the plants' natural capacity to take up and sequester heavy metals from contaminated soils and water. This agreed with the previous studies of Samecka-Cymerman et al, (2004) who agreed that the capacity of plants to accumulate lead is well-documented stating that certain plant species are particularly effective at uptake of lead from contaminated environments and storing it in their tissues. Also the study by Peijnenburg, Baerselman, De Groot, Jager, Leenders, Posthuma, & Van Veen, (2000); provides evidence of the time-dependent accumulation of heavy metals in plant tissues, supporting the trend observed in the graph while the study by Aliet al., (2013*)* which reviewed and discussed the importance of plant selection in phytoremediation projects, highlighted the species that are particularly effective at accumulating heavy metals such as lead.

Nickel (Ni) content in *Paspalum conjugatum* P.J. Bergius tissue used for phytoremediation microcosm studies in hybrid constructed wetland polluted with crude oil and heavy metals was determined and the results showed that Ni (mg/kg) content in plant tissue increases with time is consistent with the findings from several studies on the phytoremediation of heavy metals in constructed wetlands. The studies by Mihailović, Niketić, & Tomović, (2020) and other authors reported that certain hyperaccumulator plants show significant increases in nickel concentration in their tissues over time, particularly in the later stages of growth. Tangahu, Sheikh Abdullah, Basri, Idris, Anuar, & Mukhlisin, (2011) highlighted that plants like *Brassica juncea* and *Pteris vittata* accumulate metals progressively, with peak accumulation often occurring just before flowering or during full vegetative growth. McNear Jr, Chaney, & Sparks, (2010), found that *Alyssum murale* and other nickel hyperaccumulators increase metal concentration in their tissues consistently over time, with the highest levels typically observed at maturity. Sour *et al.* (2019) emphasized the importance of monitoring metal accumulation at different growth stages to optimize harvest times for maximum metal uptake while Souri, Hatamian, & Tesfamariam, (2019); Song, Wang, Zhai, Ge, Hao, Shi, Lian, Chen, Shen, & Chen, (2022); showed that species like Sedum *alfredii* and *Phytolacca acinosa* demonstrate steady increases in heavy metal concentrations in their tissues over time, suggesting that extended growth periods enhance phytoremediation efficiency.

The ability of plants to absorb and translocate heavy metals from contaminated water or sediment into their above-ground biomass leads to the gradual increase in Ni concentration within their tissues. Research has shown that certain macrophyte species, such as *Pistia stratiotes* and *Eichhornia crassipes*, are particularly effective in removing heavy metals like nickel from wastewater through phytoremediation. Additionally, the hybrid nature of constructed wetlands—which integrates different wetland types, such as vertical and horizontal flow—provides a variety of environmental conditions and supports diverse plant species. This combination enhances the overall efficiency of heavy metal removal; as different plants can accumulate a broader range of contaminants.

The copper (Cu) content in *Paspalum conjugatum* P.J. Bergius tissues was measured during phytoremediation microcosm studies in a hybrid constructed wetland polluted with crude oil and heavy metals. The results showed a progressive increase in Cu (mg/kg) concentration in plant tissues over time. This finding aligns with previous studies, which demonstrated that Cu content in plant tissues tends to rise throughout the phytoremediation process in hybrid constructed wetlands. This trend can be attributed to the nature of phytoremediation, where plants continuously absorb heavy metals from the contaminated environment, leading to cumulative uptake by the roots and translocation to aerial parts of the plant. As the plants continue to extract

Cu from the soil or water, the concentration within their tissues increases. This observation is consistent with the work of Ali et al. (2013), whose review on the mechanisms of heavy metal uptake by plants supports the idea that metal accumulation in plant tissues typically increases over time during phytoremediation.

Certain plants are capable of hyperaccumulating heavy metals, and their efficiency may improve with prolonged exposure. As the phytoremediation process continues, these plants can adapt to the contaminated conditions, leading to higher concentrations of heavy metals, including Cu, within their tissues. This is in agreement with studies by Chaney, Angle, Broadhurst, Peters, Tappero, & Sparks (2007); Pilon-Smits (2005); Kumar (1995); and Salt, Blaylock, Kumar, Dushenkov, Ensley, Chet, & Raskin (1995). These studies collectively enhance our understanding of how specific plant species can be used to remediate heavy metal-polluted soils through hyperaccumulation. They also emphasize the importance of selecting suitable plant species and optimizing conditions to maximize the effectiveness of phytoremediation.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

This study shows that hybrid constructed wetlands are a promising technology for the remediation of environments contaminated with crude oil and heavy metals, offering a balance of effectiveness, sustainability, and cost-efficiency through enhanced contaminant removal, the combination of different types of wetland systems (e.g., vertical and horizontal flow) in hybrid constructed wetlands improves the efficiency of contaminant removal compared to single-system wetlands. Synergistic Effects; The integration of various physical, chemical, and biological processes in hybrid systems enhances the degradation and uptake of pollutants, leading to better overall treatment performance. Utilizing a variety of plant species in hybrid wetlands supports the breakdown and absorption of both organic and inorganic pollutants, improving the phytoremediation potential. Hybrid wetlands promote diverse microbial communities that contribute to the biodegradation of hydrocarbons and the transformation and immobilization of heavy metals. These systems are considered sustainable and cost-effective solutions for the treatment of polluted soils, leveraging natural processes to achieve high levels of pollutant removal.

It also shows that plants differ in their susceptibility or otherwise to crude oil and heavy metals polluted soil. These studies collectively highlight the effectiveness of *P. conjugatum P.J. Bergius* among the eight (8) plants selected for this study as the most effective phytoremediator and pytoaccumultor plant because of its ability to tolerate combined effects of 10% crude oil and heavy metals pollution, demonstrating its potential and insynegy with microorganisms able to reduce hydrocarbon contamination and its ability to tolerate varying levels of crude oil pollution, thereby promoting soil recovery.Molecular studies show that *Pseudomonas xiamenensis, Acinetobacter baumanii, Alcaligenes cloacae, Enterobacter cloacae, Pantoa dispersa, Pantoa dispersa, Lysinibacillus fusiformis, Kocuriapalus were* the hydrocarbon utilizers isolated from the impacted soil used for this study, while the fungi are *Penicillium* spp., *Aspergillus* spp., *Fusarium* spp. The study highlighted that *Pseudomonasxiamenensis* gave the best result for biosurfactant production, other bacteria like *Acinetobacter, Enterobacter,* and *Pantoea* also show significant capabilities, albeit with varying efficiencies. Each bacterium has unique traits that make them suitable for specific applications in bioremediation and industrial processes.

The results from the study shows that amendment with lower concentrations of poultry droppings (1% and 10%) are more effective in supporting the growth of tested bacterial species. Higher concentration (30%) showed a less effectiveness in the support of the growth of tested bacterial species, this is likely due to potential toxicity or its inhibitory effects. This highlights the importance of optimizing poultry dropping concentrations for effective use in agricultural or environmental applications. These studies collectively demonstrate that while natural soap and poultry manure each have beneficial effects on the growth of remediator plants in crude oil and heavy metals polluted soil, their combined application generally will show synergistic effects, leading to significant improvements in plant weight, shoot length, root length, and leaf size. The addition of plants to the combined treatment provides the best overall results for both plant growth and soil remediation but these studies highlight the effectiveness of using natural soap and plants in a constructed wetland system to enhance the phytoremediation process, particularly in reducing TPH levels in heavy crude oil-polluted soils. The combination of natural soap and plant roots improves hydrocarbon solubility, bioavailability, and microbial activity, leading to more effective remediation outcomes. The study also points to the fact that although the combined synergistic of poultry manure, natural soap and plants are well documented, the concentration of the pollutants can impose a limiting factor to the growth of the plants and microorganisms. This is because the result from the study shows a negative response from the plant and microorganisms at a high concentration (10%) of crude oil and heavy metals when amended with poultry manure.

The study reveals the effectiveness of using combined treatment of natural soap and plants in a constructed wetland system to enhance the phytoremediation process, particularly in reducing TPH levels in high concentration (10%) of crude oil and heavy metals polluted soil. The combination of natural soap and plant roots improves hydrocarbon solubility, bioavailability, and microbial activity, leading to more effective remediation outcomes. These studies highlight the significant role of natural soap in enhancing the phytoremediation process, particularly in the reduction of heavy metals in crude oil-polluted soils. The surfactant properties of natural soap improve the bioavailability and mobility of heavy metals, facilitating their uptake by plants and leading to more effective remediation outcomes. Also illustrated by the study was the effective reduction of total hydrocarbon (THC) content in water samples from a hybrid constructed wetland phytoremediated with plants and natural soap. The significant decrease in THC levels over time highlights the efficiency of this combined treatment strategy. The observed trends are supported by recent literature that emphasizes the benefits of phytoremediation, especially when combined with natural amendments, for enhancing the degradation and removal of hydrocarbons from contaminated environments.

Increase in heavy metals (Ni, Cu, Zn and Pb) content in the plant tissues used for phytoremediation microcosm studies was observed and this highlights the effectiveness of the hybrid constructed wetland system in removing these heavy metals from the contaminated environment. However, it also raises concerns about their toxicity, biomass production, and the proper disposal of the harvested plant biomass.

5.2 Recommendations

The results obtained from this study recommends the use of hybrid constructed wetland for the treatment of soil and water bodies polluted with crude oil and heavy metals. The study also suggests the use of *P. conjugatum* P.J. Bergiusas a phtoremediator plant due to its numerous advantages over some grasses which includes ability to tolerate high concentration (10%) crude oil and heavy metals pollution, high growth rate, easy to propagate and ability to tolerate dual environmental conditions because it can thrive well in both normal and waterlogged soils and requiring little or no fertilizer for its proliferation.

This study recommends that every industry in Niger Delta and Nigeria as a whole involved in production of goods and services should adopt the methodologies applied in this study for the treatment of their waste and effluents before it's discharge into the environment be it soil or water bodies.

In alignment with the Millennium Development Goals (MDGs) established by the United Nations in 2000, which aimed to address global challenges by 2015, this study focuses on Goal 7 of the MDG agenda. It is recommended that policymakers in this country implement and enforce environmental regulations requiring all production industries to adopt the use of hybrid constructed wetland systems for treating waste and effluents before their release into the environment. These systems are cost-effective, environmentally friendly, easy to operate and maintain, require minimal technical expertise, and support sustainable development. Enforcing such a policy would contribute to achieving Goal 7 of the MDGs, which seeks to ensure environmental sustainability by integrating sustainable development principles into policies, reducing biodiversity loss, and improving access to safe drinking water and sanitation (U.N., 2015).

This study also tends to suggest that in event of pollution of the environment (land or water) with high concentration of crude oil and or heavy metals, amendment of the impacted site with native soap should be the first procedure followed by other amendments such as biostimulation through the use of organic fertilizer such as poultry manure and planting. Further research is needed to optimize the phytoremediation process and address these potential implications.

5.3 Contributions to knowledge

i. These studies collectively demonstrate that hybrid constructed wetland system is highly effective in treating various types of waste pollution, including effluent discharge from production industries as well as hydrocarbon- and heavy metal-contaminated soils. It is, therefore, recommended that every industry discharging effluents into the environment should install such a system before releasing wastewater into water bodies or soil.

ii. This comprehensive study has revealed that *Paspalum conjugatum* P.J. Bergius is an effective phytoremediator and phytoaccumulator plant, capable of thriving in both wetland and dryland conditions.

iii. Molecular studies from this work reveals that *Pseudomonas xiamenensis*, *Acinetobacter baumannii*, *Alcaligenes cloacae*, *Enterobacter cloacae*, *Pantoea dispersa*, *Lysinibacillus fusiformis*, and *Kocuria palustris* were the hydrocarbon-degrading bacteria isolated from the impacted soil and plant rhizosphere. Many of these microorganisms are rare in the environment and produce high yields of biosurfactants, which are crucial for the remediation of crude oil-contaminated soil. These rare bacteria, if properly harnessed could be of immense importance in remediation of impacted ennvironments.

iv. This study indicates that concentrations of soap and poultry manure above 10% should be avoided in the biostimulation process, as they may inhibit the growth of many microorganisms involved in bioremediation. The findings also reveal that 1% native soap and 1% poultry manure resulted in the optimal microbial growth, followed by 10% native soap and 10% poultry manure.

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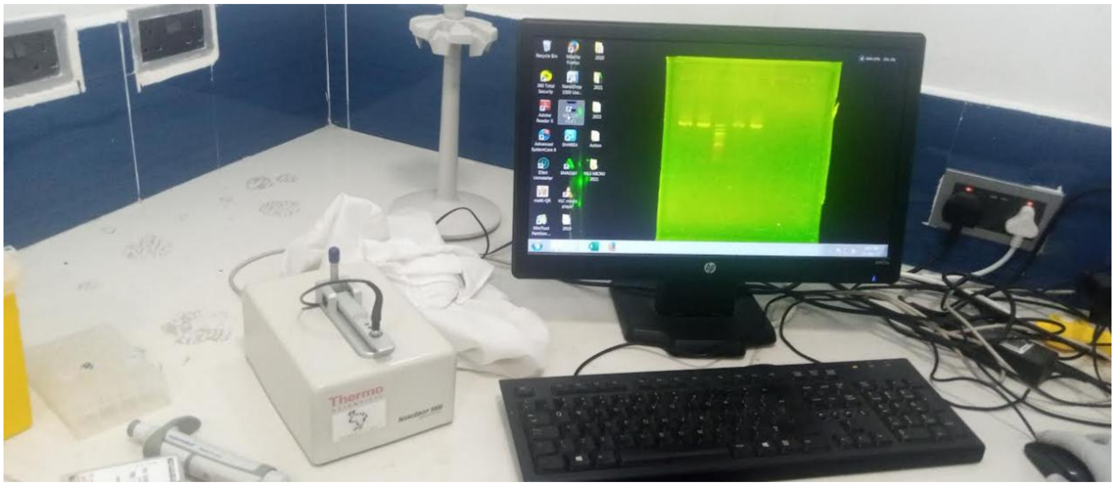
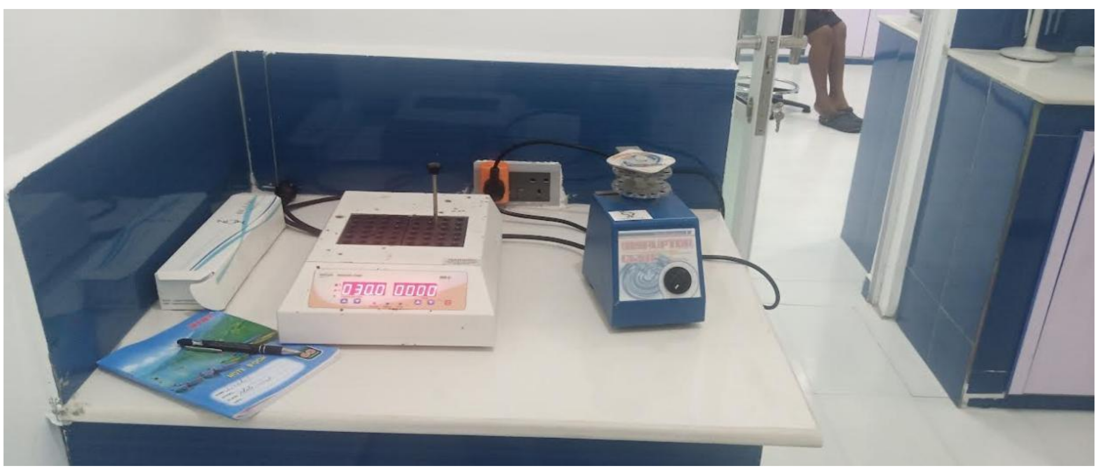
APPENDICES

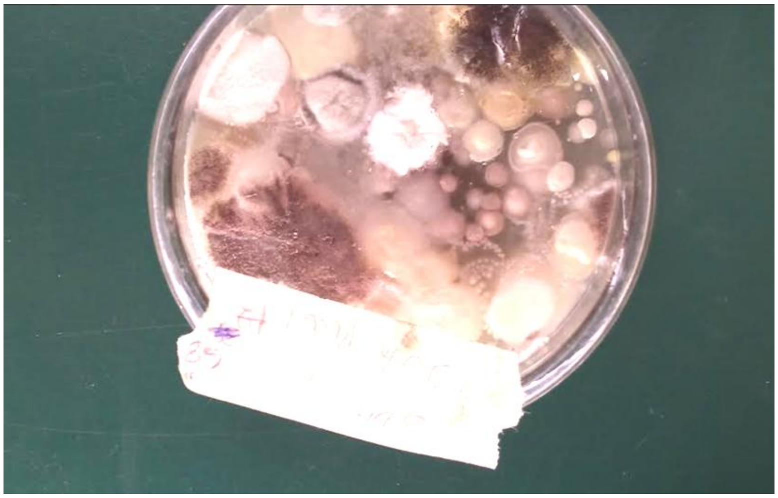


PART OF NUCLEOMETRIX LAB SHOWING UV TRANSILLUMINATOR SCREEN

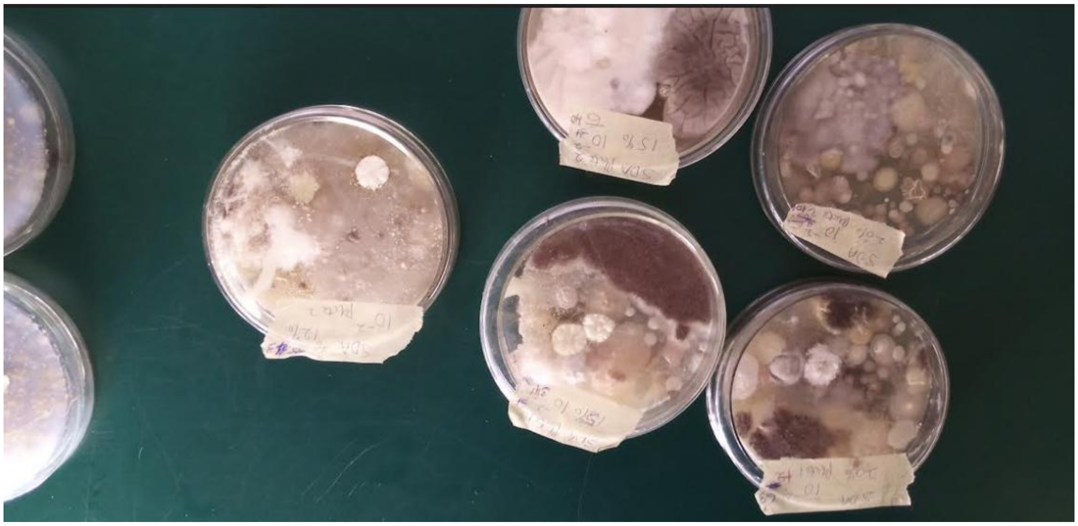


PART OF NUCLEOMETRIX LAB FOR MOLECULAR STUDIES IN BAYELSA STATE

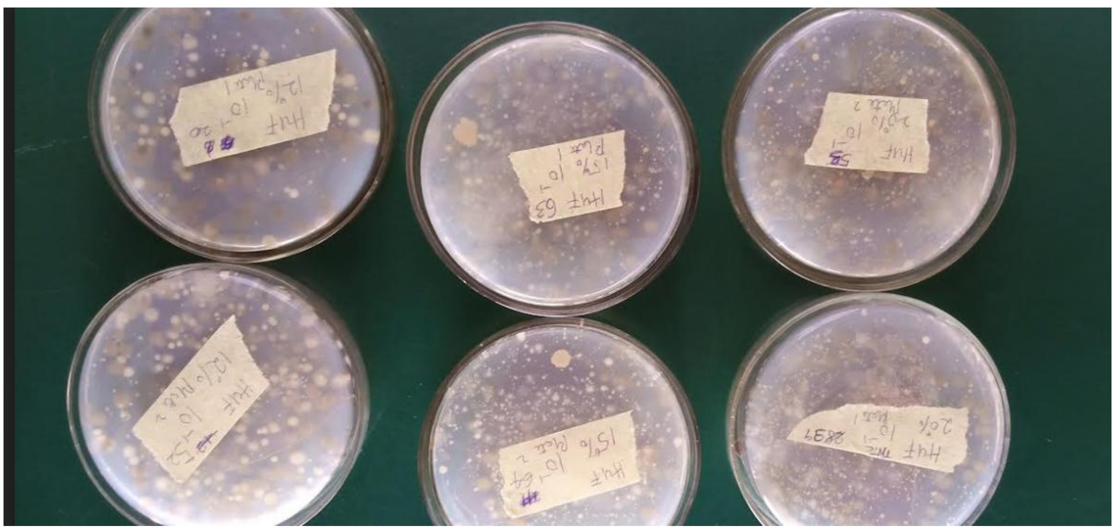
PURE CULTURE OF HUB MICROBIAL ISOLATES ON A SLANT OF BIGUO BOTTLE READY TO BE SENT FOR MOLECULAR ANALYSIS AT NUCLEOMETRIX MOLECULAR RESEARCH LAB., YENEGOA , BALYELSA STATE



CULTURE PLATE SHOWING DIFFERENT FUNGAL ISOLATES FROM THE STUDY POLLUTED SOIL SAMPLE IN AN SDA MEDIUM



HUF ISOLATES



HYDROCARBON UTILIZING FUNGI ISOLATES FROM POLLUTED SOIL SAMPLE

WORKING IN ENVIRONMENTAL MICROBIOLOGY LAB IN UNIVERSITY OF PORTHARCOURT

MICROBIAL ANALYSIS OF SOIL SAMPLES IN UNIPORT POSTGRADUATE ENVIRONMENTAL LAB

EFFECT OF POLLUTANT ON ONE OF THE PLANTS AFTER 3 MONTHS DURING THE PRELIMINARY STUDIES RESULTING TO THEIR DEATH

PRELIMINARY STUDIES AT 10% POLLUTANT CONCENTRATION

PRELIMINARY STUDIES AT CONCENTRATION OF 7% AT 3 MONTH

PRELIMINARY GROWTH IN 3% CRUDE OIL AND FIXED HEAVY METALS CONCENTRATIONS STUDIES AT 3 MONTHS.

THE SAME SECTION OF THE GREENHOUSE BUT IN DIFFERENT POLLUTANT CONCENTRATION

PART OF THE PRELIMINARY STUDIES IN THE GREENHOUSE

PLANT GROWTH DURING PRELIMINARY STUDIES

PLANTS GROWTH IN THE GREENHOUSE DURING THE PRELIMINARY STUDIES

ONE OF THE PLANTS USED FOR THE STUDY FROM A POLLUTED WETLAND AT THE BACK OF AGIP OIL COMPANY IN PORTHARCOURT

ONE OF THE PLANTS FOR STUDY HARVESTED FROM A HYDROCARBON POLLUTED WETLAND AT THE BACK OF AGIP OIL COMPANY PORTHARCOURT

HYDROCARBON POLLUTED SOIL WHERE ONE OF THE PLANTS FOR STUDY WAS HARVESTED FOR STUDY

SEEDLING PREPARATION BEFORE INSERTING INTO THE ORNAMENTAL BAGS CONTAINING THE POLLUTED SOIL

PREPARED SEEDLING ON A GLASS RAISED PLATFORMS

CONSTRUCTED HYBRID VERTICAL WETLAND SYSTEM EMPLOYED FOR THE TREATMENT OF THE POLLUTANTS (10% CRUDE OIL WITH FIXED

CONCENTRATIONS OF HEAVY METALS). IN THE FIGURE, THE UPPER WETLAND IS MORE POLLUTED THAN THE LOWER WETLAND BECAUSE IT RECEIVED THE FRESH POLLUTED SOIL WHICH RESULTED TO THE DEATH OF SOME OF THE PLANTS , BUT AFTER THE INITIAL TREATMENT THROUGH PHYTOREMEDIATION AND ACCUMULATION HAVE TAKEN PLACE OVER TIME, THE LOWER WETLAND WHICH CONTAINED UNPOLLUTED SOIL, RECEIVED THE EFFLUENTS WHICH UNDERGO FURTHER PHYTOREMEDIATION TREATMENTS RESULTING IN THE CLEAN UP. THE RESERVOIR SERVES TO COLLECT THE FINAL EFFLUENT FROM THE SECOND TREATMENT WILL BE TAKEN TO THE LAB FOR ANALYSIS OF TPH AND PRESENCE OF HEAVY METALS

PREPARED ORNAMENTAL BAGS READY FOR PLANTING

BAGED 12% CRUDE OIL WITH FIXED CONCENTRATION OF HEAVY METALS

SOIL SAMPLE SHOWING 12% CRUDE OIL POLLUTION

SOIL SAMPLE SHOWING 12% AND 15% CRUDE OIL POLLUTION . THE CONCENTRATIONS OF THE POLLUTANT IS AN INDICATION OF THE COLOUR INTENSITY DIFFERENCE

SOIL SAMPLE SHOWING 3% CRUDE OIL POLLUTION

SOIL SAMPLE SHOWING 10% CRUDE OIL POLLUTION

ELECTRONIC WEIGHING MACHINE WITH THE SALTS OF THE HEAVY METALS USED FOR THE STUDY

POLLUTED SOIL SAMPLES SHOWING DIFFERENT CRUDE OIL AND FIXED HEAVY METAL POLLUTANT CONCENTRATION

SOIL WEIGHING FOR POLLUTION SIMULATION

PREPARED SOIL SAMPLES CONTAINING DIFFERENT CONCENTRATIONS OF POLLUTANTS (3%, 7% AND 10% CRUDE OIL WITH FIXED CONCENTRATION OF HEAVY METALS)

ORNAMENTAL BAGS CONTAINING SOIL SAMPLE POLLUTED WITH 7% CRUDE OIL WITH FIXED CONCENTRATIONS OF HEAVY METALS

BAGGING OF 3% POLLUTED SOIL SAMPLE WITH BLACK ORNAMENTAL NYLON BAGS

MY WORKING TOOLS

CONSTRUCTED WETLAND MICROCOSM STUDIES

THESE ARE THE PLANTS USED FOR THE STUDY