JUMBO, RAPHAEL BUTLER

ENHANCING BIOREMEDIATION EFFICIENCY OF ACIDIC WETLANDS CONTAMINATED WITH CRUDE OIL

SCHOOL OF WATER, ENERGY AND ENVIRONMENT
ENERGY AND POWER

PhD
Academic Year: 2019 - 2023

Supervisor: Dr Ying Jiang
Associate Supervisors: Dr Imma Bortone
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ABSTRACT

Crude oil exploration and exploitation has significantly impacted the Niger Delta, Nigeria wetlands and its ecosystems. Studies suggest that acidification is ongoing with several acid forming and acid tolerant microbes identified in the Niger Delta wetlands. The efficient remediation of the crude oil contaminants in the acidified wetlands is the only alternative left to the Niger Delta for effective ecological restoration of the environment. In this research, different combinations of bioremediation strategies were investigated to enhance the remediation of simulated crude oil contaminated acidic wetlands similar to the Nigeria Niger Delta wetlands contamination conditions. A series of mesocosm experiments subjected to wetland condition and a combination of treatments were evaluated as follows: for biostimulation experiment, Food waste anaerobic digestate (FWAD), and Tween 80 surfactant were individually added to the mesocosms at 10%, 20% and 30% w/w respectively with soil in the mesocosm experiments. For bioaugmentation experiments, mesocosms were enriched with *Pseudomonas aeruginosa*, *Bacillus subtilis*, or microbes indigenous to the crude oil spiked soil. Sequel to the results of these experiments, an optimised combination of FWAD (30% w/w) plus Tween 80 (30% w/w), Tween 80 (30% w/w) plus indigenous microbes, and digestate (30% w/w) plus Tween 80 (30% w/w) plus indigenous microbes were investigated. For each set of the experiments, pristine soil, acidified soil, and crude oil spiked acidified soil were maintained as controls. Total petroleum hydrocarbon (TPH) contents, soil basal respiration, and soil microbial communities' dynamics were measured over 112 days of the experiments. For the biostimulation experiment, the FWAD and Tween 80 each at 30% (w/w) resulted in the highest petroleum hydrocarbons degradation (> 87% removal in 49 days). Augmentation with indigenous microbes enhanced the extent of degradation of the petroleum hydrocarbons (up to 80% in 49 days). For the optimised combined strategies, digestate (30% w/w) plus Tween 80 (30% w/w) plus indigenous microbes resulted in degradation of the hydrocarbons by > 98%. The correlation between basal respiration, microbial community and hydrocarbons showed that the more the biogenic CO_2 produced by the relevant microbial community, the faster the rate of the hydrocarbons degradation. Gram

positive bacteria were the dominant microbial group in the FWAD, Tween 80 surfactant, indigenous microbes, and combined digestate (30% w/w) plus Tween 80 (30% w/w) plus indigenous microbe mesocosms. This research has demonstrated that acidified wetlands contaminated by petroleum hydrocarbons can be effectively remediated using low carbon biomaterials and indigenous microbial consortia. This conclusion was further confirmed by the more than 90% maize germination and undetectable bioavailable hydrocarbons recorded at the end of the experiment in these mesocosms. Potential exists for further studies in low carbon remediation of weathered hydrocarbons contaminants in various types of wetlands and sediments using FWAD, Tween 80 surfactant, and indigenous microbes.

Keywords:

Bioremediation, wetlands, hydrocarbons, microbes, digestate.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF EQUATIONS

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AD	Anaerobic digestate
B. subtilis	*Bacillus subtilis*
C: N: P	Carbon: Nitrogen: Phosphorus
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
FWAD	Food waste anaerobic digestate
HCs	Hydrocarbons
HYPREP	Hydrocarbons Pollution Remediation Project
IM	Indigenous microbes
NDPR	Nigeria Department of Petroleum Resources
NPK	Nitrogen, phosphorus, potassium
P. aeruginosa	*Pseudomonas aeruginosa*
PAHs	Polycyclic aromatic hydrocarbons
TOC	Total organic carbon
TK	Total potassium
TN	Total nitrogen
TP	Total phosphorus
TW80	Tween 80
UNEP	United Nations Environment Programme
Bio A	Bioaugmentation
D	Digestate

1 INTRODUCTION

1.1 Background

Wetlands pollution by petroleum hydrocarbons is a global environmental issue prevalent in the Niger Delta, Nigeria. The Niger Delta is one of the most important wetland ecosystems in Africa (Konne, 2014). Crude oil exploration in the Niger Delta wetlands commenced six decades ago (Kadafa, 2012), causing contamination and a subsequent alteration of the wetland ecosystems (Ruley et al., 2020; Sam et al., 2016). The continued oil exploration, alongside gas flaring, and increased industrial activities leading to persistent acid rainfall, which is gradually increasing the acidity of the wetlands (Rehan & Mohammad, 2021; Jeffries et al., 2003; Elum et al., 2016). Acidic wetlands have a pH ranging from 4 to 6.5 (Mitsch, & Gosselink, 2015; Tiner, 2017). The pH of wetlands increase over time due to the presence of chemical compounds from acid rain, including sulfuric (H_2SO_4) and nitric acids (HNO_3), carbonic acid (H_2CO_3) from industrialization, and hydrocarbons from oil exploration and spills (Driscoll et al., 2001; Baird, & Cann, 2012; Karimian et al., 2023). The consistent occurrences of petroleum hydrocarbons spillages have led to severe economic and ecological damages in the wetlands (Zhu et al., 2004; Osuji et al., 2006), shown in Figure 1.1.

Figure 1.1. Map of Niger Delta, Nigeria and wetlands contaminated with crude oil.

Several research studies have well documented how petroleum hydrocarbon contamination in Niger Delta acidified wetlands is a severe and widespread problem, causing environmental, ecological, and public health concerns (Sam et al., 2016; Nwaichi & Uzazobona, 2011; Osuji et al., 2004). Certain groups of petroleum hydrocarbons, particularly the medium to heavy molecular weight hydrocarbons, in the acidic wetlands are made bioavailable to the soil ecosystem through contact and sorption to the soil, soil microbes, and groundwater (Brown et al., 2017; Prokop et al., 2016). In an attempt to remediate the hydrocarbons contaminated, acidified wetlands, several 'conventional' remediation techniques have been adopted. These include physical and chemical techniques, bioaugmentation, phytoremediation, and biostimulation (Lu et al., 2019; Ngene & Tota-maharaj, 2019; Okoye et al., 2020). However, the extent of petroleum hydrocarbons degradation achieved after remediation was limited (Jørgensen et al., 2000; Canet et al., 2001; Guirado et al., 2021). This limitation was associated with neglecting the petroleum hydrocarbons sorption and bioavailability processes, combined with inadequate information of the acidic wetlands systems in the design of remediation strategies (Nwaichi & Uzazobona, 2011; Chikere et al., 2017; Okoye et al., 2020). Limited information on the soil/oil interaction and understanding of petroleum hydrocarbons sequestration mechanisms is another cause of deficient degradation of the hydrocarbons.

In the quest to improve petroleum hydrocarbon degradation efficiencies, ecologically low risk biostimulation and bioaugmentation strategies were adopted for the remediation of the contaminated acidic wetlands (Chikere et al., 2017; Oyetibo et al., 2016; John et al., 2011). Biostimulation is a bioremediation technique that aims to enhance the activity and growth of indigenous microorganisms in a contaminated environment by providing them with the necessary nutrients, electron acceptors, or other growth-promoting substances (Chikere et al., 2017; Thomas et al., 2020). Prominent stimulants adopted for bioremediation include organic and inorganic fertilisers, compost, and activated carbons (Sojinu et al., 2010; Shekwolo & Igbuku, 2014). Selected microbes (such as *Pseudomonas aeruginosa, Acinetobacter sp., Bacillus subtilis,* and *Pusillimonas sp.*) were considered for bioaugmentation strategies (Okafor et al.,

2021; Ejechi & Ozochi, 2015; Nkereuwem et al., 2020). Bioaugmentation is a bioremediation technique that involves the introduction of specific microorganisms or microbial consortia into a contaminated environment to enhance the degradation or transformation of pollutants.(Varjani & Upasani, 2019; Okafor et al., 2021). The application of *Pseudomonas aeruginosa,* and *Acinetobacter sp.*, on wetlands with 45,000 mg/kg of total petroleum hydrocarbon (TPH) showed a degradation of 50% and 40% (after 140 days) respectively (Jin et al., 2017; Ejechi & Ozochi, 2015; Abdulsalam et al., 2011). The application of microbes indigenous to the wetlands under similar contaminant concentration yielded 55% of the TPH degradation at 130 days (John et al., 2011; Thomas et al., 2020; Okafor et al., 2021). These incomplete remediations have been linked to the acidic conditions of the wetlands, poor understanding of the intermediate reactions, and deficient experimental and field designs for the remediation of contaminants in the wetlands.

The application of inorganic fertilisers to acidified wetlands contaminated with 50,000 mg/kg total petroleum hydrocarbon resulted in hydrocarbons degradation efficiencies not greater than 40% in 160 days (Ezekoye et al., 2018; Aghalibe et al., 2017). Dhadli, and Brar, (2016) monitored the soil emission of carbon dioxide under similar conditions, and observed that at day 100, carbon dioxide was increased from 563 CO_2-C kg/ha in the control soil to 819 CO_2-C kg/ha when NPK fertilizer was applied to the soil. The research of Wu et al., (2021) on the life-cycle assessment of inorganic fertiliser production and application to soil from 1998 to 2016, showed that the overall carbon dioxide emissions from chemical fertiliser production and application to soil increased from 1.3 $\times 10^8$ tonnes CO_2-equivalent to 1.8×10^8 tonnes CO_2-equivalent. As a result, the overall carbon dioxide emissions per unit area increased from 9.8 $\times 10^2$ tonnes CO_2-equivalent per hectare to 1.2×10^3 tonnes CO_2-equivalent per hectare in 2016. Yuan et al. (2022) studied the average emission of carbon dioxide from organic and inorganic fertilizer from production to application in soil. Yuan et al. showed that the average carbon dioxide emissions from biomass waste-derived organic fertiliser were 5.5 kg CO_2-equivalent/kg fertilizer, while inorganic fertiliser (NPK-based fertiliser) emissions were 14.0 kg CO_2-equivalent/kg fertilizer. These studies provide

valuable insights into the potential environmental benefits of biomass/waste-derived biofertilizers, specifically in terms of reducing CO_2 emission. The biomass/waste that are derived from renewable resources that has a reduced carbon footprint throughout its life cycle and designed to minimize carbon dioxide emissions, contribute to sustainable development, and mitigate the impacts of climate change are called low carbon biomaterials (Zhou et al., 2023; Feng et al., 2021). Low carbon biomaterials had been recently adopted in bioremediation. Bioremediation strategy that leads to less emission of carbon dioxide is known as low carbon bioremediation strategy (Priya et al, 2022; Sui et al., 2021). The adoption of low carbon biostimulants and the combination of biostimulants in acidic wetlands remediation was led by organic nutrients, compost, and biochar. At 100,000 mg/kg of total petroleum hydrocarbons, the degradation efficiency reported was up to 65% at 120 days (Orji et al., 2013; Osadebe et al., 2022). Despite the remediation successes, the increase in metals and metalloids contents, and emission of greenhouse gases have posed another challenge (Herath et al., 2013; Zhang et al., 2021). These issues can be avoided by replacing compost and biochar and other low carbon stimulants with digestate during low-carbon bioremediation of contaminated soils (Andrew, 2012; Gielnik et al., 2021).

Digestate from anaerobic digestion (AD) of organic feedstock is a by-product of the AD process (Peng and Pivato, 2019). Digestate is a high-quality bio-fertilizer, low in metals and carbon contents, cost-effective (compared to the conventional fertilizers) with readily available nutrients for the soil (Vaneeckhaute et al., 2017; Koszel & Lorencowicz, 2015). The food waste feedstock produces digestate of higher quality (in terms of nutrient value) when compared to the other feedstocks (such as sewage and animal waste) (Andrew, 2012; Opatokun et al., 2015). Food waste digestate as a biofertilizer can be adopted for bioremediation or combined with other stimulants such as surfactants for wetlands decontamination.

Surfactants have also been adopted for soil remediation; however, there use was mainly for soil washing but high energy cost and release of harmful by-product (from the soil washing activities) to the environment limits the use (Akpoveta et

al., 2012; Ceschia et al., 2014). Tween 80 surfactant (Figure 1.2), a non-ionic ecologically low risk surfactant has rarely been adopted as a stimulant in acidic wetland soils remediation irrespective of its ability to increase the bioavailability of petroleum hydrocarbons.

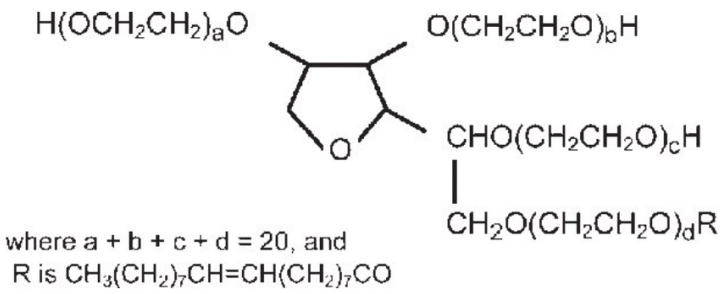

Figure 1.2. Chemical structure of Tween 80.

In bioremediation of wetlands, the combination of Tween 80 surfactant and digestate as stimulants was rarely considered since surfactant were prominent for soil washing. Thus, low carbon biomaterials such as digestate (which are readily available and affordable), and non-ionic, ecological low-risk surfactant such as Tween 80 which can promote the biodegradation of petroleum hydrocarbons were often neglected.

Finally, the enrichment of the acidic wetland soil with microbial consortia and the simultaneous optimised combination with low carbon biostimulants have either been neglected or wrongly applied in the remediation of petroleum hydrocarbon contaminants in acidic wetlands (Ceschia et al., 2014; Priya et al, 2022; Ezekoye et al., 2018).

1.2 Aim and Objectives

The aim of this research is to develop new, sustainable, approaches to accelerate the degradation of petroleum hydrocarbons from acidic wetlands in the Niger Delta.

The objectives of the research are:

1. To critically review existing trends towards low carbon, sustainable remediation approaches and recent progress on innovative bioremediation strategies.
2. To investigate the effects of food waste anaerobic digestate fibre and non-ionic surfactants on the fate, degradation, and behaviour of hydrocarbons in acidic wetland soils.
3. To assess the efficacy of indigenous bacterial consortia on hydrocarbon biodegradation in acidic wetlands.
4. To evaluate the efficacy of optimised combined bioremediation strategies and define endpoints of bioremediation for acidic wetlands.

1.3 Structure of the Thesis

This thesis is presented in paper format. Each of the following papers is self-contained research which together form this PhD research:

Chapter 2: A critical review on existing trends toward low carbon, sustainable remediation approaches, and recent progress on innovative bioremediation strategies. This chapter covers the theories and trends in bioremediation, low carbon and sustainable approaches and recent progress made on innovative bioremediation strategies. This paper critically spotlights challenges encountered during remediation of soil, wetlands and acidic wetlands and the areas for further research.

Chapter 3: Evaluating different soil amendments as bioremediation strategy for wetlands contaminated by crude oil. This chapter evaluates low carbon, ecological low risk bioremediation strategies of petroleum hydrocarbon degradation in acidic wetlands using food waste digestate fibre and Tween 80 surfactants. The petroleum hydrocarbon degradation rate, the microbial communities' dynamics, the basal respiration, and baseline information were monitored for the digestate and surfactants respectively at 10, 20, and 30% w/w.

Chapter 4: Assessing the efficacy of bioaugmentation strategies for remediating oil impacted wetlands. This chapter evaluates the bioremediation of petroleum hydrocarbons contaminants in acidic wetlands using indigenous microbes prominent in the Niger Delta acidic wetland soils. Indigenous microbes that survived the crude oil stress in the experimental soil were also assessed. Single cultures of *Pseudomonas aeruginosa,* and *Bacillus subtilis*, along with enriched indigenous microbial consortia and three different controls were adopted for the analysis. Petroleum hydrocarbon degradation rate, microbial communities' dynamics, basal respiration, and baseline information were monitored using mesocosms, and remediation end points determined.

Chapter 5: Analysing the simultaneous use of biostimulation and bioaugmentation as optimised strategies for remediating oil impacted wetlands. This chapter evaluates the bioremediation of petroleum hydrocarbons contaminated acidic wetlands using three optimised treatments: i) digestate plus Tween 80 surfactants, ii) enriched microbial consortia plus Tween 80 surfactants, iii) digestate plus Tween 80 surfactants plus enriched microbial consortia. Petroleum hydrocarbon degradation rate, microbial communities' dynamics, basal respiration, and baseline information were monitored, and the remediation end point determined.

Chapter 6: Conclusion and Recommendation. This chapter covered the summary of conclusions made from this study and the recommendations for further studies. The chapter summarized key findings of the research. From the key findings, digestate was justified as a sustainable low carbon biomaterial suitable for use in remediation of acidic wetlands. Indigenous microbial consortia were identified to degrade petroleum hydrocarbon contaminants in acidic wetlands faster than other microbial consortia. The combined bioremediation strategies showed that combined biostimulation and bioaugmentation strategies improved the rate and extent of biodegradation of petroleum hydrocarbons and it is effective for ecological risk reduction in contaminated acidified wetlands.

Based on these findings, some recommendations were suggested for further research. The efficacy of indigenous microbes to degrade petroleum

hydrocarbons in contaminated coastal and estuarine sediments should be considered for further studies. The application of the various strategies in this studies was recommended for field scale application.

The schematic diagram of the thesis structure is shown in Figure 1.3.

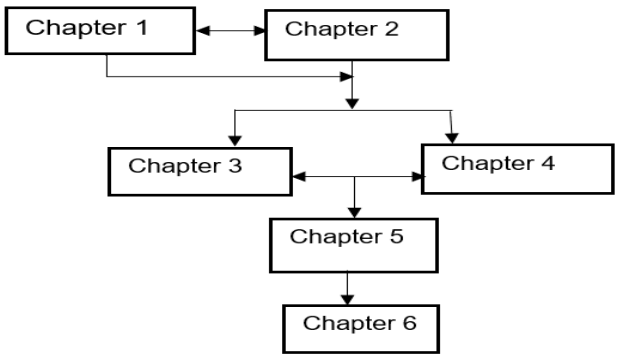

Figure 1.3. The schematic diagram of the structure of the thesis.

1.4 Publications

1.4.1 Conference Papers

Raphael B. Jumbo, Frederic Coulon, Imma Bortone, & Ying Jiang (2022, May 4 - 5). Low carbon remediation of oil impacted acidic wetlands using fibre food-based digestate and eco-friendly surfactant (Abstract Presented). NICOLE Conference on Technical Solutions for Climate Resilience in Industrial Land Management 2022.

Raphael B. Jumbo, Frederic Coulon, Imma Bortone, & Ying Jiang (2022, October 21-23). Optimised low carbon remediation of oil impacted acidic wetlands. 10th International Conference on Sustainable Environment and Agriculture (ICSEA 2022). Can Tho, Vietnam.

1.4.2 Journal Papers

Raphael B. Jumbo, Frederic Coulon, Tamazon Cowley, Ikeabiama Azuazu, Emmanuel Atai, Imma Bortone, & Ying Jiang (2022). Evaluating different soil amendments as bioremediation strategy for wetland soil contaminated by crude oil. Sustainability 2022, 14 (24), 1 - 22. https://doi.org/10.3390/ su14241656.

Raphael B. Jumbo, Frederic Coulon, Imma Bortone, & Ying Jiang (2022). Assessing the efficacy of bioaugmentation strategies for remediating oil impacted wetlands. Microorganisms 2022. (In press).

1.4.3 Presentations

Raphael B. Jumbo, Frederic Coulon, Imma Bortone, & Ying Jiang (2020, November 18 - 19). Bioremediation of acidic wetlands contaminated by weathered hydrocarbons: challenges and opportunities (Poster Presentation). International Symposium on Risk Assessment, 2020.

1.4.4 Other Dissemination Output

Raphael B. Jumbo, Frederic Coulon, Imma Bortone, & Ying Jiang (2020, November 10). Improving the efficiency of bioremediation of acidic wetlands impacted by petroleum hydrocarbons. Cranfield, SWEE, Energy & Power, PhD and MRes Student Seminar.

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2 A CRITICAL REVIEW ON EXISTING TRENDS TOWARDS LOW CARBON, SUSTAINABLE REMEDIATION APPROACHES AND RECENT PROGRESS MADE ON INNOVATIVE BIOREMEDIATION STRATEGIES

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2.1 Abstract

Acidic wetlands and their subsequent pollution especially by petroleum hydrocarbons are fast becoming issues of global concern. Studies have shown various negative impacts of hydrocarbons on the wetland soils and their ecology. To address these impacts, remediation methods, including physical, chemical, and bioremediation processes have been adopted over the years to clean contaminated acidic wetlands. Physical methods, commonly practiced, are soil replacement, physical encapsulation, and thermal method, while chemical methods include soil washing with surfactants, chemical immobilization, and oxidation processes. Prominent bioremediation techniques practiced for wetland remediation include biostimulation, remediation by enhanced natural attenuation, and bioaugmentation. However, the efficiency of field contaminant degradation obtained from these methods were between 40 – 50% at 160 – 180 days. In attempts to improve the efficiency of degradation, several modifications were proposed on the microbial augmentation and stimulation with 55 – 65% of contaminants degradation at 130 – 150 days. This led to the adoption of a low carbon bioremediation approach as an alternative to remediate acidified wetlands contaminated by petroleum hydrocarbons. This paper provides an overview of remediation methods commonly used for acidic wetlands soils by analysing Niger Delta wetland (Nigeria) as a case study. Literature research studies, improvements, and inherent pitfalls from acidic wetlands remediation were reviewed. This review investigates the trends in remediation techniques used for contaminated wetland soils and various successful, sustainable low carbon remediation techniques. Finally, this

study examines the suitability and sustainability of using digestate for bioremediation of wetlands contaminated by petroleum hydrocarbons.

Keywords: wetland, soils, bioremediation, acidic, hydrocarbons.

2.2 Introduction

Wetlands (WLs) and their subsequent pollution by petroleum hydrocarbons (PHCs) are fast becoming issues of global concern. Wetlands are poorly drained areas subject to permanent or periodic water saturation (Drake et al., 2009), which are usually found in lowlands areas (González-Alcaraz et al.,2013). Most wetlands are on the transition between the terrestrial and aquatic ecosystem (Bodelier & Dedysh, 2013; Morse et al., 2012; Ly et al., 2019). Wetlands exhibit close proximity of oxic-anoxic conditions and facilitate simultaneous activities of anaerobic as well as aerobic microbial communities (Bodelier & Dedysh, 2013; Kolb & Horn, 2012). They are both ecologically and economically important because of their high agricultural productivity, complex biogeochemistry, and nutrient recycling ability (Morse et al., 2012; Nwankwoala & Okujagu, 2021). The Figure 2.1 shows wetlands used for agricultural production.

Figure 2.1. Wetlands used for agricultural productivity.

However, wetlands acidity is of primary concern because of its effects on associated ecosystems, dissolved metal ions, conductivity, water quality, and agriculture (Andrew et al., 2022; Singh & Chakraborty, 2020; Johnston et al., 2014). Economic issues of concern associated with increasing wetlands acidification include the decline of property values, recreation activities, and corrosion of structures near to the wetlands (Ly et al., 2019). The acidity of wetlands can be caused by continuous acid rainfall, oil field gas flaring, poor quality fertilizers, and high level of organic matter (Johnston et al., 2014; Ruiz-Halpern et al.,2015; McKee et al., 2015). Additionally, wetlands and

their ecosystems have been considerably impacted by human activities, particularly by petroleum hydrocarbon industries (Johnston et al., 2014). The contamination of wetlands, by the petroleum hydrocarbons industries, can alter the wetlands ecosystems if the hydrocarbons contaminants are not urgently remediated (Agbonifo, 2020).

2.3 Acidic Wetlands Pollution by Petroleum Hydrocarbons

Wetlands and acidified wetlands pollution by petroleum hydrocarbons is a prevalent environmental issue in Nigeria. The Nigerian petroleum industry (NPI) has been one of the largest producers of crude oil in Africa since 1958 (Olayinka & Ogbonna, 2013; Adati, 2012; Economou & Agnolucci, 2016). Despite contributing significantly to the Nigerian economy, this output has raised concerns over environmental pollution and public health risks. For over 60 years, the Nigeria petroleum industry has significantly and consistently polluted the acidic wetlands in the oil-bearing region of the country called the Niger Delta (Sam et al., 2016). As a result, the Niger Delta has been rated as one of the most vulnerable areas in the world for crude oil hydrocarbons spills (Zabbey et al., 2017). Reports published respectively by the Organisation of the Petroleum Exporting Countries (OPEC) (2022), and the Nigerian Department of Petroleum Resources (NDPR) (2022) showed that Nigeria produced about 1.6 million barrels of crude oil per day (out of 31.7 million barrels OPEC daily production) in 2021, while approximately 27 million litres of crude oil were spilled into the soil environment between 2010 – 2018. Reports by Friends of the Earth International (2019) stated that between 1976 to 1991, over 2 million barrels of crude oil polluted the area of Ogoniland in Niger Delta. When these pollution incidences occur, the contaminated sites are usually left unattended pending evaluation by relevant government agencies. In addition, inadequate workforces, delayed release of funds (Agbonifo, 2020; Olayinka & Ogbonna, 2013); systemic negligence caused by inadequate monitoring facilities and internal politics; and intra-government agencies bureaucracy (Olayinka & Ogbonna, 2013b; Adati, 2012) further impede remediation of the polluted acidic wetlands. This delay eventually leads to changes in the petroleum hydrocarbon composition, toxicity, distribution, and availability in the environment (Oualha et al., 2019; Bento et al., 2005). The extent of the environmental devastation of wetlands depends on the type of hydrocarbons present, the nature of the wetlands,

environmental biotic and abiotic factors, susceptibility, and hydrocarbons bioavailability (Jiang et al., 2016; Oualha et al., 2019).

Crude oil spills in wetlands do not only destroy the wetland soil and ecosystem, but they also have serious consequences on wildlife, and other organisms which relies on these wetlands as habitat, nursery grounds and agriculture (Zhu et al., 2004). Wetlands are prominently used for farming activities (Igoni, 2018; Akpa et al., 2014), and their pollution has led to a decreased agricultural livelihood, low-quality water discharge, and forced rural dwellers to search for non-existent source of livelihood. Consequently, food production and income generation by local farmers are significantly lower when compared with those in non-polluted areas (Elum et al., 2016; Osuji et al., 2005). Agricultural products, such as root vegetables (for example, carrot, and cocoyam), tuber crops (such as cassava, and potato), and cereal crops (such as maize) which are common in grain foods in the Niger Delta, are prone to uptake petroleum hydrocarbons in the contaminated soil sites (Abdel-shafy & Mansour, 2016; Bansal & Kim, 2015).

2.3.1 Petroleum Hydrocarbons in Plants and Remediation Funding in Niger Delta

Petroleum hydrocarbons are adsorbed into plants either through the root suberin cortical zones (that is the lipophilic constituents) or root cells (Perrin-ganier et al., 2002). When agriculture products grown in these contaminated wetlands are consumed, the adsorbed petroleum hydrocarbons accumulate in the lipid tissue causing stomach cancer and DNA adducts in the lungs (Garrido et al., 2010; Abdel-shafy & Mansour, 2016; Campo-Daza et al., 2022). Hydrocarbons can also cause neurological symptoms such as drowsiness, poor coordination, stupor, or seizures (Agbonifo, 2020; Abdel-shafy & Mansour, 2016). Figure 2.2 shows some examples of medium and heavy molecular weight hydrocarbons.

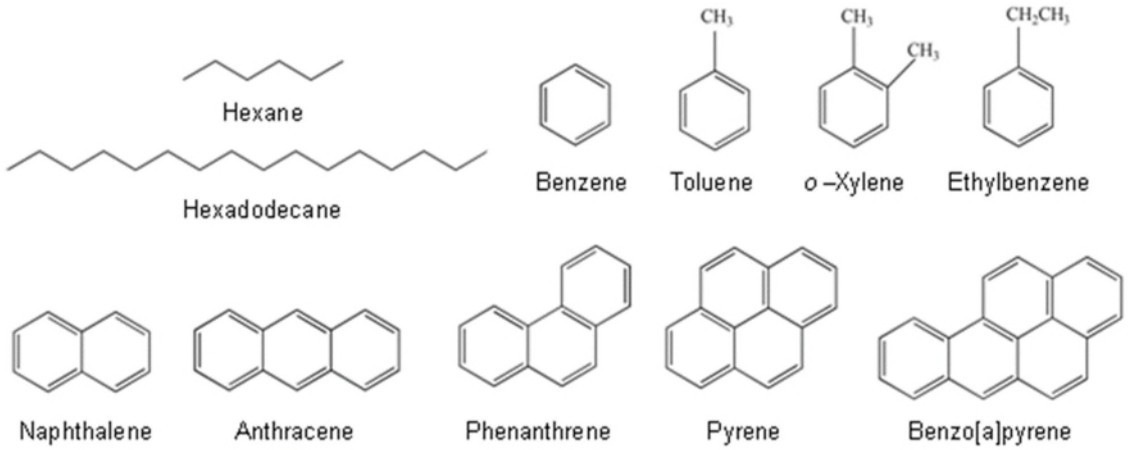

Figure 2.2. Some representatives of medium and heavy molecular weight hydrocarbons (Agbonifo, 2020; Garrido et al., 2010; Abdel-shafy & Mansour, 2016).

Studies on soils contaminated by petroleum hydrocarbons have shown severe toxic impacts for plants, and animals (including humans), particularly in the Nigeria oil-producing hub of Niger Delta (Sam et al., 2016; Nwaichi & Uzazobona, 2011; Osuji et al., 2004). Hydrocarbons inhibit plant-microbe interactions and decrease microbes' ability to digest organic substances that plants require as nutrition (Camila et al., 2020). The medium (C10 – C18) to heavy (C19 – C40) molecular weight petroleum hydrocarbons in soil can be bioavailable to the soil ecosystem (Prokop et al.,2016) and humans, through contact and sorption, soil microbes, groundwater, or farm products (Bolan et al., 2008; Brown et al., 2017). Bioavailable hydrocarbons reduce plant transpiration rate, crop yield, and damage cell membranes of crops and animals (including humans) (Khan et al., 2018). These issues have raised serious public health concerns in the Niger Delta and subsequent pressure from local and the international communities on the Nigerian government to remediate petroleum hydrocarbon polluted sites (Olawoyin, 2016; UNEP, 2011). In this context, the Nigerian government initiated the Hydrocarbons Pollution Remediation Project (HYPREP). The primary interest of HYPREP was the remediation of hydrocarbons polluted soils in the region using available and applicable environmentally friendly remediation techniques. Funding delays have severely slowed down the remediation activities in the Niger Delta region. For example, between 2010 - 2018, the recorded number of sites with crude oil spillages within the Niger Delta were over 5,800 (Department of Petroleum Resources, 2018). However, until February 2021, only about 21 sites had undergone remediation within the region due to insufficient funds approved for the remediation

activities (Hydrocarbons Pollution Remediation Project, 2021). In 2021, only about 0.0026% of 13.08 trillion-naira (about 30 billion USD) appropriation act of Nigeria, was budgeted for remediation-related activities in Niger Delta (Federal Ministry of Finance, Nigeria, 2021). Whereas the United Nations Environment Programme (UNEP) in 2011 proposed annual budgeting of 76 billion Nigerian Naira (about 174 million USD) for the Niger Delta clean-up for 30 years. The remediation techniques adopted by HYPREP have been prevalently physical and chemical methods, enhanced natural attenuation, biostimulation and bioaugmentation (Edema et al., 2011).

This critical review appraises the remediation methods commonly used for acidic wetlands impacted by petroleum hydrocarbons. Literature lessons learnt, limitations, and improvements on these methods were examined in this review. Additionally, the review aims to identify trends in sustainable, low carbon, bioremediation techniques for contaminated wetlands. The use of food-based digestate and other sustainable low carbon biomaterials for the degradation of hydrocarbons contaminants in wetlands is also reviewed. Finally, adequate recommendations are made for improving the rate and efficiency of sustainable low carbon bioremediation of petroleum hydrocarbons in acidic wetland soils.

2.4 Overview of the Remediation Methods Commonly used for Acidic Wetlands Soils: Lessons Learnt, Pitfalls and Improvements

Remediation of contaminated soils by physical methods was one of the commonly adopted techniques for remediation, for its simplicity of use (Adekola & Mitchell, 2011). The physical method involves a manipulation of the contaminated soil to immobilize or detoxify its contaminants (Sakshi et al., 2019), and include soil replacement, soil washing, physical encapsulation and immobilization, vitrification, and thermal methods (Lu et al., 2019; Sam & Zabbey, 2018; Scanferla et al., 2009). The physical methods commonly practiced in acidic wetlands are soil replacement and thermal method.

Soil replacement methods involved the removal of the contaminated soil and its mixing with clean soil for alternative use (Zabbey et al., 2017). The excavated contaminated area was filled with uncontaminated soils (Swati et al., 2018). Douay et al. (2008) also used this technique for remediating contaminated soils in kitchen gardens near a former smelter. The soil area was not more than 100 m^2 and was contaminated by

lead (3300 mg/kg) and cadmium (24 mg/kg). The concentration of lead in the contaminated soil required a large volume of clean soil to be mixed to attain the European commission safe limit of 30 mg/kg for lead. In addition, the impact of heavy metals on the environment was not considered in Douay et al. research. Soil replacement methods are ineffective for large contaminated sites, because it is expensive and disruptive of the donor site, labour intensive, and causes secondary pollution during excavation and transportation to treatment site (Ruley et al., 2020; Zabbey et al., 2017). The high treatment cost alongside the poor funding of the Niger Delta remediation programme by the Nigerian government (Mmom & Igbuku, 2015) seriously limits the use of soil replacement methods in the region. Importantly, this method would not solve the problem as it only dilutes the concentration of the soil pollutants with clean soil and it does not degrade the displaced contaminants, which would still be biologically available to the environment.

2.4.1 Thermal Treatment Method

Thermal treatment methods have been used to remediate soil contaminated by petroleum hydrocarbons, and include incineration, microwave frequency heating, and thermal desorption (Lim et al., 2016; Sakshi et al., 2019). The incineration technique is the most practiced thermal method because it is effective in destroying soil contaminants (O'Brien et al., 2018; Vidonish et al., 2016). Incineration effectively destroys Polycyclic aromatic hydrocarbons (PAHs) in contaminated soils using temperatures ranging between 900 – 12,000 ^{o}C (Kuppusamy et al., 2017). Shekwolo and Augustine (2014) adopted incineration technique in the remediation of contaminated wetlands from Ejama Ebubu (Nigeria). After incineration at 1,000 ^{o}C, the petroleum hydrocarbon content of the soil was within the Nigeria Department of Petroleum Resources (NDPR) standard of 5,000 mg/kg. When compared with the World Health Organisation (WHO) standard of 5 mg/kg (WHO, 1998), the remediated soil (with 5,000 mg/kg of total hydrocarbon content) was potentially harmful to the soil ecosystem. Soils treated by incineration up to 5 mg/kg of petroleum hydrocarbons are often mixed with agricultural soils use for growing crops (Pape et al., 2015). In the Niger Delta, incinerated soils are often crushed, mixed with clean soil, water, and cement for the production of concrete cement blocks (Shekwolo & Augustine, 2014). The leachability of the remaining contaminants from the blocks is not usually considered, even if the contaminants are potentially harmful. Andrew et al. (2015)

investigated the impact of thermal remediation on the contaminated soil ecology. The contaminated soil was incinerated at 500 and 1,000 ^{o}C. After the incineration, the soils were amended with compost and then used for growing crops. It was observed that the 500 ^{o}C incinerated soils recovered and supported plant growth while the 1,000 ^{o}C treated soil had poor plant growth with minimal microbial recolonization. It was also observed that most research on the use of incineration method in remediating contaminated soils pay little attention to the hydrocarbons and soil biological diversity before incineration, because at high temperature all the contaminants and microbes in the soil were destroyed. Besides, the incineration method requires a skilled workforce, and suitable for small-area-contaminated sites (Khalid et al., 2017; Nwankwo, 2014). The research of Araruna et al. (2004) on the oil spill clean-up by using incineration showed that at 4,500 ^{o}C the total petroleum hydrocarbon contents reduced by 95% in 2 hours, while in 8 hours the hydrocarbon contents reduced by 98%. Incineration produces high carbon footprint, and it is expensive, as it required a steady power supply, which is not feasible in most developing countries like Nigeria. The available power supply in Nigeria is about 4,500 MW with only 45% of the 210 million Nigerian population having access to the power, and only 30% of their power demand is met (Chigozie & Oluchukwu, 2013; Clinton & Chinago, 2018).

2.4.2 Chemical Remediation Method

The chemical remediation methods involve the use of chemical reagents to bind or immobilize petroleum hydrocarbons in the soil (that is, chemical immobilization methods) or degrade them (that is, chemical oxidation methods) (Calace et al., 2005). Chemical oxidation methods using oxidants injected into the soil (Kuppusamy et al., 2017), have been effective at increasing remediation rates. Two prominent oxidants used for remediation of petroleum hydrocarbons contaminated soils are hydrogen peroxide (H_2O_2) and Fenton reagent ($FeH_4O_6S^{+2}$) (Mohamed et al., 2002; Stuart et al., 2001). The research of Ojinnaka and Osuji (2012) on the remediation of soil contaminated with 25,000 mg/kg of total petroleum hydrocarbons (TPH) using Fenton's reagents showed that the oxidant (Fenton's reagents) reduced polycyclic aromatic hydrocarbons (PAH) in the soil by 96% in seven days. The method involved temperatures of 60 – 300 ^{o}C which invariably heats the surrounding air and emits greenhouse gases as by-products into the atmosphere. However, the success recorded at a field scale was very low when compared to that at a laboratory scale.

Furthermore, the method is not easy to operate, and it is expensive at the scale required. The research of McAlexander et al. (2015) on the treatability testing of petroleum hydrocarbons in soils using the oxidation method showed variation in extractable petroleum hydrocarbons as the oxidant doses increased. After the remediation processes, about 5,500 mg/kg of the initial 40,867 mg/kg total petroleum hydrocarbons were still available in the soil when hydrogen peroxide was used as oxidant. During the decomposition of the oxidant, heat and off-gases were released into the atmosphere, which serve as a secondary pollutant to the environment. Given the above mentioned limitations, a further chemical method of using surfactants was tested.

Surfactants can be used to facilitate the removal of contaminants from soil during soil washing operations (Ceschia et al., 2014). The application of surfactants to petroleum hydrocarbon contaminated soils can be carried out either *ex-situ* or *in-situ*. Anacletus et al. (2017) studied the effect of surfactants on crude oil impacted soil and concluded that the application of surfactants improved the soil properties having reduced the petroleum hydrocarbon content by 77% in six hours. The method failed to consider the peculiar ecological conditions (such as the microbial communities, and the pH) of the contaminated wetland during the washing processes, the discharge and treatment of wastewater after the soil washing processes were not given attention in the research. Additionally, Kalali et al. (2011) also showed that soil washing techniques using surfactants are an effective method of remediating petroleum hydrocarbon contaminated soils. However, the efficiency of the techniques reduces as the exposure period of the contaminants in the soil increases. In the research, 800 mg/L of surfactants were used to wash a 20-day old, contaminated soil, and the total PHCs removal efficiency obtained was 97%. The soil washing techniques are not sustainable for *in-situ* applications in wetlands. Soil washing is ecologically unfriendly (since the microbial diversities are given no consideration in the washing activities), varies with pollutants, and causes secondary pollution (Liu et al., 2019; Khalid et al., 2017; Chima & Vure, 2014). The mean cost for soil washing in Niger Delta including the cost of power to drive the system is about 243.75 million Nigerian Naira (about 560 thousand USD) for 5,000 m^2 plot of land at 1 m depth (Postle et al., 1999; Pearl, 2007; Clinton & Chinago, 2018). Besides, the fact that the method is expensive to use in large areas

of petroleum hydrocarbon contaminated soils, it is not easily applied in Niger Delta acidic wetlands.

2.5 Current Bioremediation Techniques for Hydrocarbon Contaminated Site and its Suitability for Nigeria

The issues mentioned against physical and chemical remediation methods alongside the very limited funds released by the Nigerian government led to the quest for alternative and cost-effective techniques. Bioremediation being potentially cost-effective, relatively easy to manipulate together with its potential to restore the acidic wetlands ecosystems to their previous status before the petroleum hydrocarbon contamination, by degrading the contaminants to carbon dioxide and water. Therefore, bioremediation has been proposed as the most suitable technique for the Niger Delta. Bioremediation techniques involve microbial degradation of the petroleum hydrocarbon contaminants (Ezenne et al., 2014). This method has shown to be relatively environmentally friendly, potentially economical, efficient, and highly accepted by the public (Delgado et al., 2019; Redfern et al., 2019). For instance, in the Gio community of Niger Delta, the bioremediation methods adopted for remediating the crude oil polluted wetlands reduced the total petroleum hydrocarbons from 36,776 mg/kg to 24,274 mg/kg in 30 days (Okoye et al., 2020). These techniques showed to be more environmentally friendly when compared with the physical and chemical methods of remediating petroleum hydrocarbon contaminated soils. However, the remediated site with a concentration of 24,274 mg/kg was still potentially harmful to the soil and its ecosystem (after the 30 day remediation) if compared with the WHO safe limit of 5 mg/kg PHCs in soil.

Remediation techniques commonly adopted in the Niger Delta region, and the prominent cause of failures of the techniques are outlined in Figure 2.3. Some of the causes of major setbacks in bioremediation of wetlands particularly include neglecting the sorption and availability of the petroleum hydrocarbon contaminants, acidity of wetlands, and soil type (Essien & John, 2011; John & Okpokwasili, 2012; Ngene & Tota-maharaj, 2019). Other setbacks are limited information on the soil/oil interaction and sequestration mechanisms of the petroleum hydrocarbons in the acidic wetlands, and availability of cost effective biostimulants suitable for acidic wetlands (Orji et al., 2013; Brown et al., 2017; Okoye et al., 2020).

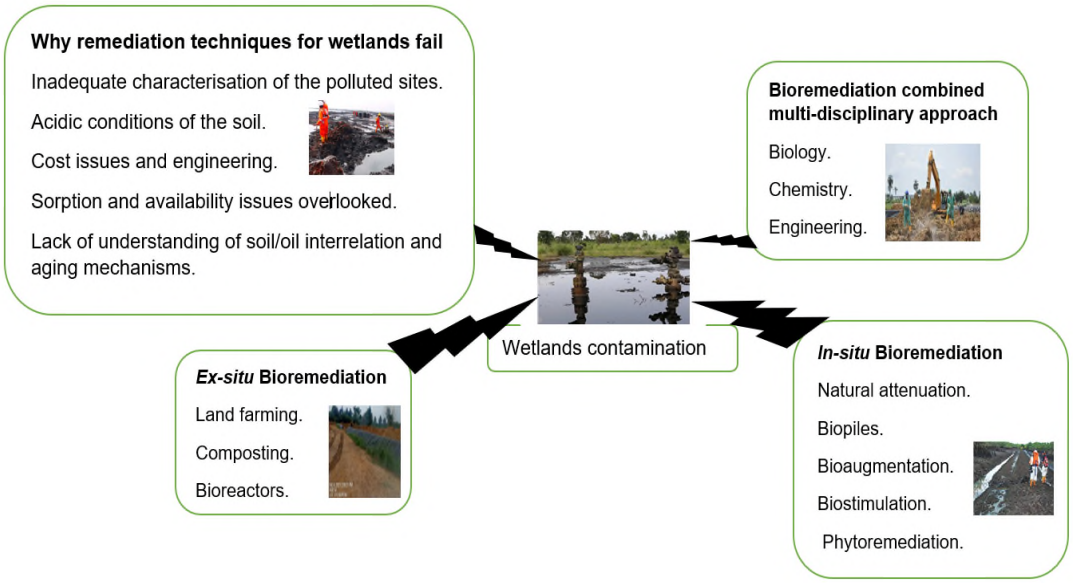

Figure 2.3. Remediation methods adopted in Nigeria and their causes of failure.

Sources: Essien & John, 2011; John & Okpokwasili, 2012; Orji et al., 2013; Ngene & Tota-maharaj, 2019; Brown et al., 2017; Okoye et al., 2020.

In the research of Orji et al., 2012, the soil hydrocarbons were degraded from 14,000 mg/kg to 5,200 mg/kg in 70 days, leaving the soil still potentially hazardous to the ecosystem at the end of the 70-day experiment. In an attempt to achieve complete petroleum hydrocarbon degradation, several modifications have been made on the microbial augments and stimulants with little success (Ngene and Tota-maharaj, 2019). Some of these modifications applied in Niger Delta are shown in Table 2.1. Prominent bioremediation techniques practiced within the region include biostimulation, bioaugmentation, and enhanced natural attenuation (Chikere et al., 2019; Oyetibo et al., 2010; John et al., 2012). Table 2.1 shows that hydrocarbons remediation of contaminated wetlands and non-wetlands. From Table 2.1, research on the efficacy of inorganic fertilizers to remediate polluted soils and wetlands were prominently led by the use of nitrogen, phosphorus, and potassium (NPK) based fertilizers (Nwaichi et al., 2011; Nkereuwem et al., 2020). The results obtained from these bioremediation processes was an average of 60% degradation efficiency for not more than 40,000 mg/kg total petroleum hydrocarbons (TPH) in 120 – 160 days, for newly contaminated non-wetland soils. Whereas in-situ, bioremediation of petroleum hydrocarbons in wetlands soils gave an average of 45% degradation for not greater than 55,000 mg/kg TPH in similar timescale (Nwankwegu et al., 2016; Fubara-Manuel

et al., 2017; Brown et al., 2017). Figure 2.4 shows hydrocarbons degradations in wetlands and non-wetlands with linear extrapolations.

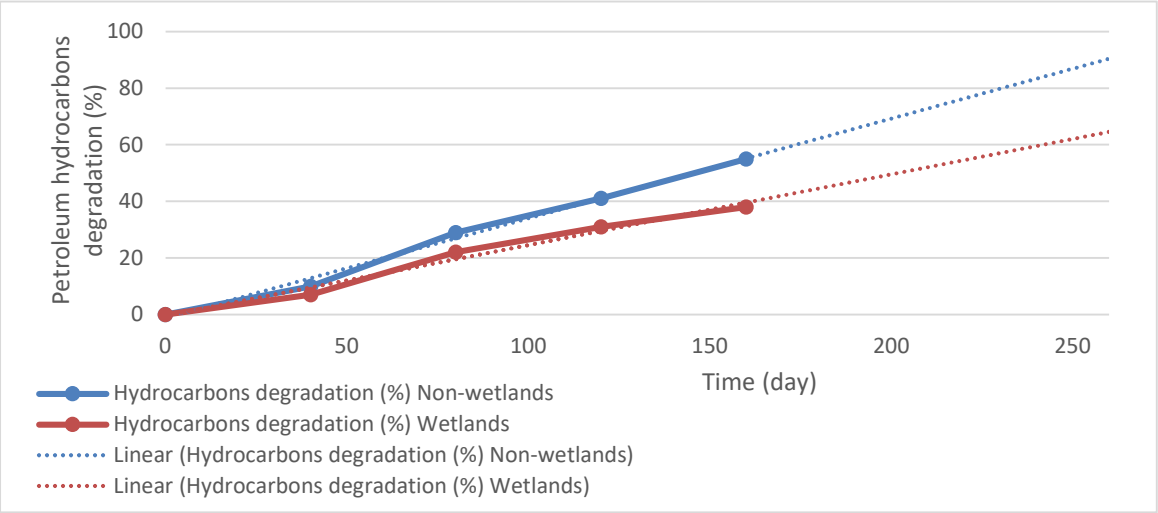

Figure 2.4. Graph of petroleum hydrocarbons degradation (%) versus time (day)

Source: Nwaichi et al., (2011), Nkereuwem et al., (2020), Fernández-Bayo et al. (2017), Nwankwegu et al., (2016), Fubara-Manuel et al., (2017), Brown et al., (2017).

The extrapolations of Figure 2.4 shows that more than 90% and 65% petroleum hydrocarbons degradation, for non-wetlands and wetlands respectively, can be achieved at 250th day of remediation. The application of inorganic fertilizers for decontaminating acidic wetlands resulted in remediation efficiencies up to 35% petroleum hydrocarbons degradation 45,000 mg/kg total petroleum hydrocarbons (TPH) in 130 – 180 days (Ngene & Tota-maharaj, 2020; Lee et al., 2001). The application of inorganic fertilizers practiced in the region as biostimulation is mainly through direct application of NPK fertilizer with or without a bulking agent (which provides more aeration), or indirect application of the fertilizer through irrigation (Saha et al., 2019; Nkereuwem et al., 2020; Fubara-Manuel et al., 2017). These methods, when adopted in the Niger Delta paid little attention to the acidity of the wetlands in the region. The current high purchasing cost of NPK fertilizer in Nigeria for the remediation activities was another limiting factor (Camila et al., 2020). An in-depth baseline analysis of the contaminated soil is scarcely done, and no clear post-remediation plan was provided (Zabbey et al., 2017; Fubara-Manuel et al., 2017). The limited results obtained from the process are also attributable to the deficient modifications made on existing biostimulation methods due to an inadequate

understanding of the soil-oil interaction, the site microbial community, and the sequestration of the petroleum hydrocarbons in the soil.

2.5.1 Biostimulation

Research on the biostimulation for remediating petroleum hydrocarbon contaminated soils mainly involves the use of farmyard manure, and other biodegradable nutrients and wastes such as cow and poultry manure. The use of poultry and cow manure, and municipal biodegradable waste in bioremediation of petroleum hydrocarbon contaminated soils yielded an average of 65% contaminant reduction with not more than 45,000 mg/kg total petroleum hydrocarbon (TPH) in soils in 100 – 120 days (Table 2.1) (Udosen et al., 2001; Ubochi et al., 2006; Nwankwegu et al., 2016; Oghoje et al., 2020). For the *in-situ* application of the organic nutrients on soils with petroleum hydrocarbon contaminants, the reduction in hydrocarbon content averaged at 40% depending on the extent and age of the contamination in 120 – 130 days (Table 2.1) (Asquith et al., 2012; Demelza et al., 2007; Nwankwegu et al., 2016). On wetlands, the efficiency obtained in *in-situ* biostimulation of petroleum hydrocarbon contaminated wetlands using organic wastes was an average of 45% degradation for 60,000 mg/kg TPH content in 80 – 160 days (Abu and Dike, 2008; Orji et al., 2013; Lee et al., 2001). The application of organic manures on contaminated wetlands rarely considers the soil acidity, and the hygiene of the animal manures are neglected. The degradation results obtained from these methods is mainly due to the biotransformation of the petroleum hydrocarbon contaminants in the wetland environment. The acidic nature of the wetlands, which are mostly not considered in the designing or modification of existing methods is another cause of concern since most microbial activities are reduced in acidic soil (Gazey, 2018). Additionally, the limited knowledge of the sorption and availability of the contaminants also leads to deficient modifications made on the methods for the remediation of the petroleum hydrocarbon contaminated wetlands. For instance, the bioavailability of hydrocarbons to the soil microbes is an influencing factor for the hydrocarbon degradation (Nwankwegu et al., 2016; Yang et al., 2021).

2.5.2 Bioaugmentation

To overcome the challenges from biostimulation, bioaugmentation techniques were also adopted for the remediation of wetlands. Bioaugmentation involves the inoculation of the contaminated soil with exogenous microbes (Kuppusamy et al.,

2016). This is usually practiced when the soil indigenous microbes do not achieve the required microbial metabolic activities to degrade the hydrocarbons in the soil. Table 2.1 shows that microbes frequently used in the Niger Delta for bioaugmentation include *Achromobacter species, Pseudomonas aeruginosa, Acinetobacter species, Alcaligenes species, Azospirillus species, Bacillus subtilis, Lysinibacillus species, Ochrobactrum species, Proteus species, and Pusillimonas species* (Akpoka et al., 2019; Chikere et al., 2017; Okoye et al., 2020). The results obtained from bioaugmentation of hydrocarbons averaged at 60% of 40,000 mg/kg TPH degradation in 70 – 150 days (Puntus et al., 2019; Chikere et al., 2017). For petroleum hydrocarbon contaminated wetlands, about 63,000 mg/kg of TPH were reduced to an average of 45% using bioaugmentation in 90 – 150 days (Nkereuwem et al., 2020; Kuppusamy et al., 2016). For acidic wetlands, the results obtained from microbial inoculation with petroleum hydrocarbon contaminants (at similar period) was not more than 55% degradation efficiencies under similar contaminant levels. As shown in Table 2.1, the degradation highly depends on the ability of the microbes to adapt to a hostile environment. (Okoye et al., 2020; Okoro 2010; Olukunle, 2013). The genes encoding degradation activities in the inoculated microbes could be transferred across the microbial communities through lateral genes transfer, helping the bacterial community to adapt and fit into the contaminated environment (Gielnik et al., 2021). The adaptation observed from the microbes varies depending on soil type, pollutants, pH, and availability of oxygen. In most bioaugmentation of petroleum hydrocarbons contaminated soils in the Niger Delta, the presence of the soil established indigenous microbial community is usually not recognized. This means there is still limited understanding of the hostility and competition between the inoculated microbes and the indigenous microbes.

The use of indigenous microbes, such as *Pseudomonas aeruginosa, Bacillus subtilis, Bacillus polymyxa,* and *Azotobacter* for the remediation of contaminated acidic wetlands was adopted due to the microbial adaptability to the acidic wetlands, high ecological and environmental friendliness. The application of *Pseudomonas aeruginosa* for the reduction of benzo[a]pyrene in acidic wetlands was carried out by Jin et al. (2017). The microbes reduced the contaminants from 40 mg/kg to 11.6 mg/kg in 40 days. The combination of *Clostridium pasteurianum, Bacillus polymyxa,* and *Pseudomonas aeruginosa* to degrade petroleum hydrocarbons was carried out by

John et al. (2011). After 100 days of activities, the TPH were reduced from 5200 mg/kg to 1040 mg/kg. Furthermore, indigenous microbes from freshwater wetlands were used for the remediation of TPH by Ugochukwu et al. (2018). After 90 days of remediation, the TPH were reduced from 62,388 mg/kg to 15,122 mg/kg. The inability of the microbes to completely remediate the contaminants in the soil may be due to some biotic (such as microbial predation by protozoa and bacteriophages) and/or abiotic stress (such as acidity, temperature, and nutrient availability) not tested during the design of the experiment. Furthermore, the presence of microbial predators (of the inoculated microbes) has been given little concern in most bioaugmentation of petroleum hydrocarbons contaminated wetlands. The predator microbes can kill and eat the inoculated microbes leading to limited degradation of the target petroleum hydrocarbon contaminants in the soil (Gazey, 2018; Okoye et al., 2020). Modifications made on bioaugmentation in the Niger Delta are the basic substitution of the microbial genera with little attention given to the aforementioned factors influencing the survival of the microbes in the inoculated soils. The various modifications done on bioremediation (including biostimulation and bioaugmentation) in the Niger Delta have yielded to a scarce in-situ degradation of the petroleum hydrocarbon contaminants in wetlands (Eze & Orjiakor, 2020; Wokem & Madufuro, 2020). Some of the degradation efficiencies obtained from bioremediation in the region were attributed to the long-time changes in environmental conditions, sequestration, and biotransformation of the petroleum hydrocarbon contaminants in the wetlands. Also, the intermediate metabolites (such as catechol 2,3-dioxygenases, and gentisate) from the petroleum hydrocarbons remediation, in the acidic wetlands, are another neglected factor. For example, catechol 2,3-dioxygenases, and gentisate, are responsible key intermediate metabolites for the biodegradation of hydrocarbons (Figure 2.5), and these metabolites functions optimally at pH of 7.5 – 8 (Tavakoli & Hamzah, 2017; Nilanjana & Preethy, 2011). These limitations from bioremediation have led to the quest for sustainable and efficient low carbon bioremediation techniques.

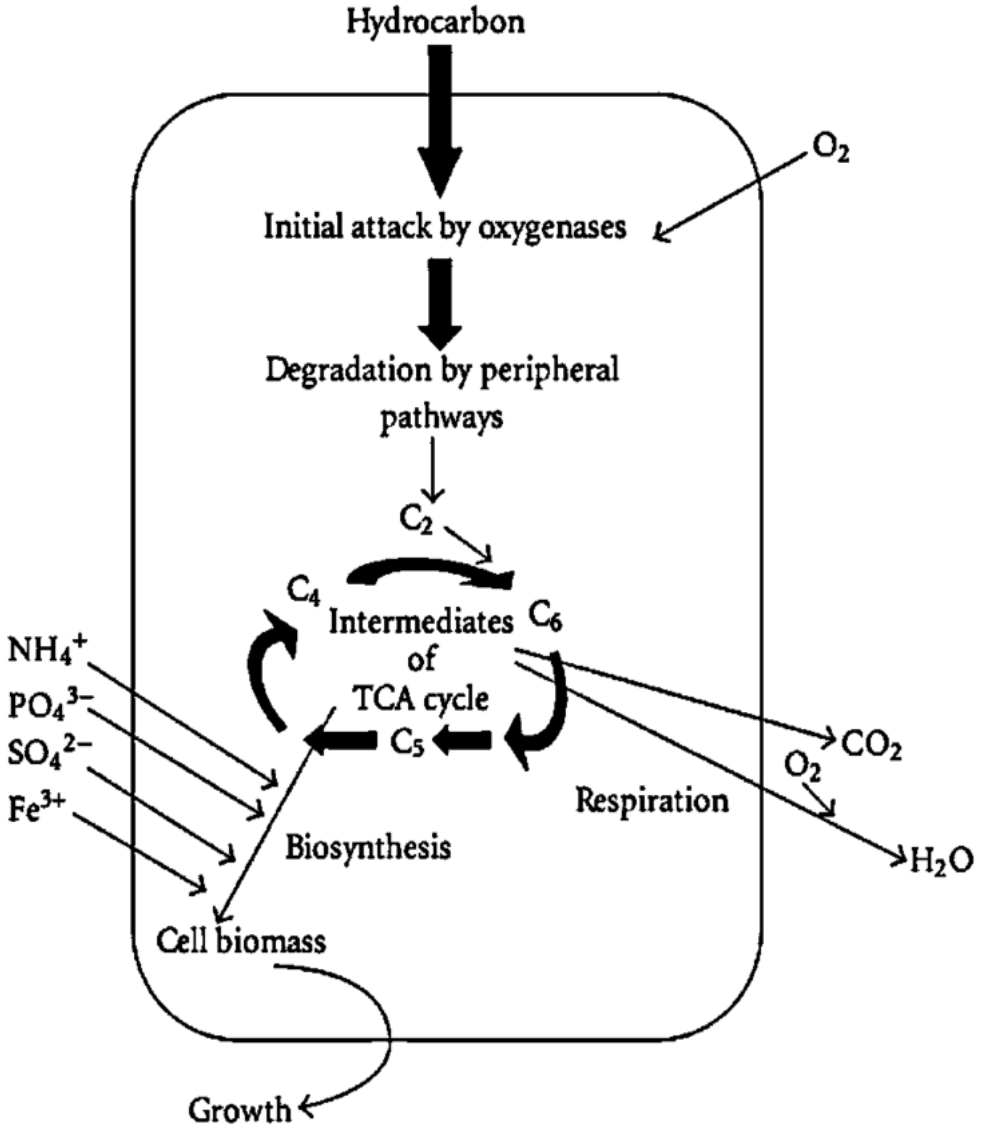

Figure 2.5. Intermediate reactions in hydrocarbons degradation from wetland bacteria.

Source: Tavakoli & Hamzah, (2017); Nilanjana & Preethy (2011).

Table 2.11. Remediation techniques for hydrocarbons contaminated wetlands and non-wetlands.

Remediation method	Materials used	Soil type	Indicator	Time (day)	Contaminant concentration before remediation (mg/kg)	Contaminant concentration after remediation (mg/kg)	Research needs	Reference
Biostimulation	Poultry manure	Not verified	TPH	42	0.96	0.83	Secondary pollution, limited degradation	Ezenne et al., 2014
Biostimulation	Cow manure	Wetland	TPH	70	12934.75	5222.99	Secondary pollution, low degradation rates	Orji et al., 2012
Biostimulation	NPK fertilizer	Clay	TPH	56	88820	25310	Un-sustainable, contaminants potentially available after remediation	Nwankwegu et al., 2016
Biostimulation	Nutrient from spent water hyacinth	wetland	TPH	60	12517	3083	Limited species of water hyacinth in the region, prolonged degradation period	Feng et al., 2021

Biostimulation	Saline (brackish) water amended with NPK	Sandy loam	TPH	84	64,494	28474	Contaminants bioavailable after remediation, unsustainable at field scale	Ayotamuno et al., 2011
Biostimulation	NPK fertilizer, tillage, and irrigation	Not verified	TPH	42	16618	2493	Unsuitable for in-situ wetlands, contaminants bioavailable after remediation,	Chikere et al., 2017
Biostimulation	Nitrogen with phosphorus nutrient	wetland	TPH	140	160	19.2	Microbial communities' activities reduced	Garcia-blanco et al., 2007
Biostimulation	Poultry manure	wetland	TPH	112	3000	700	Wetland potentially toxic, altered microbial community	Egobueze et al., 2019
Biostimulation and phytoremediation	*Oryza longistaminata* and cow dung	wetland	TPH	120	75000	6100	Increased soil toxicity, and reduced plant growth	Ruley et al., 2020

Bioaugmentation (Rapid test technique)	Indigenous microbes	Sandy loam	TPH	3	22,107	16580	Extensive microbial investigation required	Okafor et al., 2021
Bioaugmentation	Heterotrophic bacteria and hydrocarbon utilizing bacteria	Not verified	TPH	56	8635.68	677.2	Limited remediation below 30 cm and prolonged remediation period	Chikere et al., 2019
Bioaugmenta-tion	Bacterial consortium and *sophorolipid*	Not verified	TPH	30	1025	565	Soil bacterial species not identified	Feng et al., 2021
Bioaugmentation	*Pseudomonas sp., Bacillus sp., Achromobacter sp., Proteus sp. and Serratia sp.*	Wetland	PAH	45	12,210	4273.5	Contaminants bioavailable after remediation	Okoye et al., 2020
Bioaugmentation	*Pseudomonas songnenensis, Nocardioides solisilvae*	Not verified	TPH	150	98,857.10	20,760	Unsuitable for wetlands, soil potentially polluted after remediation, the	Ali et al., 2020

							soil ecology was not considered in the design	
Bioaugmentation	Bacteria consortium	Sediments	PAH	28	490	220	The method only considered coastal wetland sediments, soil ecotoxicity was not investigated.	Tiralerdpanich et al., 2018
Bioaugmentation	Mixed microbial consortium	Not verified	TPH	84	700,000.00	700.00	Bacteria consortia not specified, bacteria used is unsuitable for the anaerobic environment and ecotoxicological aspect overlocked.	Poi et al., 2017
Bioaugmentation	Hydrocarbons utilizing bacterial consortium and nutrients	Clay	TPH	60	321,196.84	94,038.68	Impact of the nutrients on soil neglected, unfit for wetlands, potentially harmful to environment	Varjani & Upasani, 2019

Bioaugmentation	Hydrocarbons utilizing bacterial consortium	Sediments	Alkanes	35	2,158	Undetectable	The impact of PAHs on the sediments were not investigated	Thomas et al., 2020
Bioaugmentation	Reed rhizosphere microbes	Wetland	TPH	14	16000	9760	Soil potentially toxic, altered microbial structure	Cao et al., 2012
Bioaugmentation	*Clostridium pasteurianum, Bacillus polymyxa, Azotobacter sp*	Wetland	TPH	100	5200	1040	Negatively impacted microbial structure	John et al., 2011
Bioaugmentation	*Pseudomonas sp*.	Wetland	benzo[a]pyrene	40	40	11.6	Reduction in soil microbial activities	Jin et al., 2017
Bioaugmentation	*Clostridium pasteurianum, Bacillus polymyxa and Pseudomonas aeruginosa*	Wetland	TPH	100	5200	1040	Soil potentially toxic after remediation, reduced microbial population	John et al., 2011

Bioaugmentation	Indigenous microbes and microbes from cow dung	Wetland	TPH	90	62388	15122	Polluted food chain, reduced agricultural land use	Ugochukwu et al., 2018
Bioaugmentation	*Lactobacillaceae sp.*	Wetland	TPH	49	50	25	Negatively impacted microbial community	Shaoping et al., 2021
Bioaugmentation	Indigenous microorganisms	wetland	PAH	1095	0.0021	0.0002	Toxins in the food chain, reduced recreational use	Han et al., 2019
Low carbon biostimulation remediation	Compost	Clay soil	TPH	60	88820	5850	Microbes in compost not verified, contaminants bioavailable after remediation	Nwankwegu et al., 2016
Low carbon biostimulation remediation	Mycorrhizal spent fungi	Sandy	TPH	84	65750	53840	Suitable for small area, unsustainable, contaminants bioavailable after remediation	Nkereuwem et al., 2020

Low carbon biostimulation remediation	Mushroom and algae	Sandy loam	PAH	63	166400	4992	Nutrients not readily available for large scale use produced poor results at wetlands	Edema et al., 2011
Low carbon biostimulation remediation	Biochar, rhamnolipid, biosurfactant and nitrogen	Sediment	TPH	50	9000	2500	Method is unsuitable for acidic wetland conditions, secondary pollution issues not considered.	Wei et al., 2020
Low carbon biostimulation remediation	Compost	Wetland	PAH	50	17	7	Low degradation rates, secondary pollution from treatment chemicals	Cottin & Merlin, 2008
Low carbon biostimulation remediation	Fungi	Wetland	Diesel	112	20,000	5000	Soil ecotoxicity was neglected, contaminants potentially harmful after remediation	Zou et al., 2013

Low carbon biostimulation remediation	Root exudates and gel-beads/ reeds combination	Wetland	Pyrene	20	0.178	0.035	Increased soil toxicity, reduced recreational land use, shift in microbial community	Tian et al., 2017
Low carbon biostimulation remediation	Spent water hyacinth nutrients	Wetland	TPH	70	14187.03	4119.52	Increased soil toxicity, shift in microbial community	Orji, et al., 2013
Low carbon biostimulation remediation	Biochar, nitrogen and *rhamnolipid* biosurfactant	Wetland	TPH	50	540	102.6	Sediments pollution, ecological value reduction, altered microbial structure and diversity	Wei et al., 2020
Phytoremediation	*Scirpus triqueter*	Wetland	Pyrene	80	80	28.32	Increased soil toxicity on plants and microbes	Zhang et al., 2011
Phytoremediation	*Lemna paucicostata*	Wetland	TPH	120	3651.77	500	Impacts on microbial communities unverified	Ekperusi et al., 2020

Phytoremediation	*Calamagrostis angustifolia*	wetland	TPH	153	7400	3800	Increased soil toxicity, reduced plant growth	Ying et al., 2013
Phytoremediation	Microbes and enzymes from *Scirpus triqueter rhisosphere*	Wetland	TPH	335	712	470	Soil toxicity and estuarine water pollution	Wei et al., 2018
Soil washing	surfactants	Loamy sand	TPH	1	1800	200	Energy consuming, secondary pollution issue, and unsustainable	Akpoveta et al., 2012
Chemical oxidation	Fenton reagent	Sandy	PAH	7	137.014	0.61	Unsuitable for wetlands and ex-situ application, secondary pollution issue imminent	Ojinnaka & Osuji, 2012
Chemical oxidation	Hydrogen peroxide	Clay	PAH	60	17,000	7000	Contaminants bioavailable after remediation,	Rosik-Dulewska et al., 2015

							secondary pollution by reagent	
Incineration method	heat	Not verified	PAH	1	8.9	0.19	Air pollution, high energy consumption, and high cost	Edema et al., 2011

2.6 Trends in Sustainable Low Carbon Remediation Techniques for Contaminated Wetland Soils

The need for increased petroleum hydrocarbons degradation rates and efficiency has led to the search for an alternative sustainable remediation techniques. This search led to the adoption of low carbon bioremediation techniques. Low carbon bioremediation techniques are bioremediation techniques that use biological based materials which emits less carbon dioxide (Khan et al., 2004). Biological materials (also called biomaterials) used in remediation of hydrocarbons are cheap and readily available as biodegradable wastes (Zou et al., 2013). These techniques, when compared to other soil remediation methods, are highly economical and ecologically more friendly (Edema et al., 2011). Additionally, they improve soil fertility and tends to eliminate or reduce the greenhouse gases emission during remediation activities in wetlands (Al-Mutairi et al., 2008; Liang et al., 2020). A schematic representation for bioremediation of petroleum hydrocarbons contaminated wetlands is as shown in Figure 2.6.

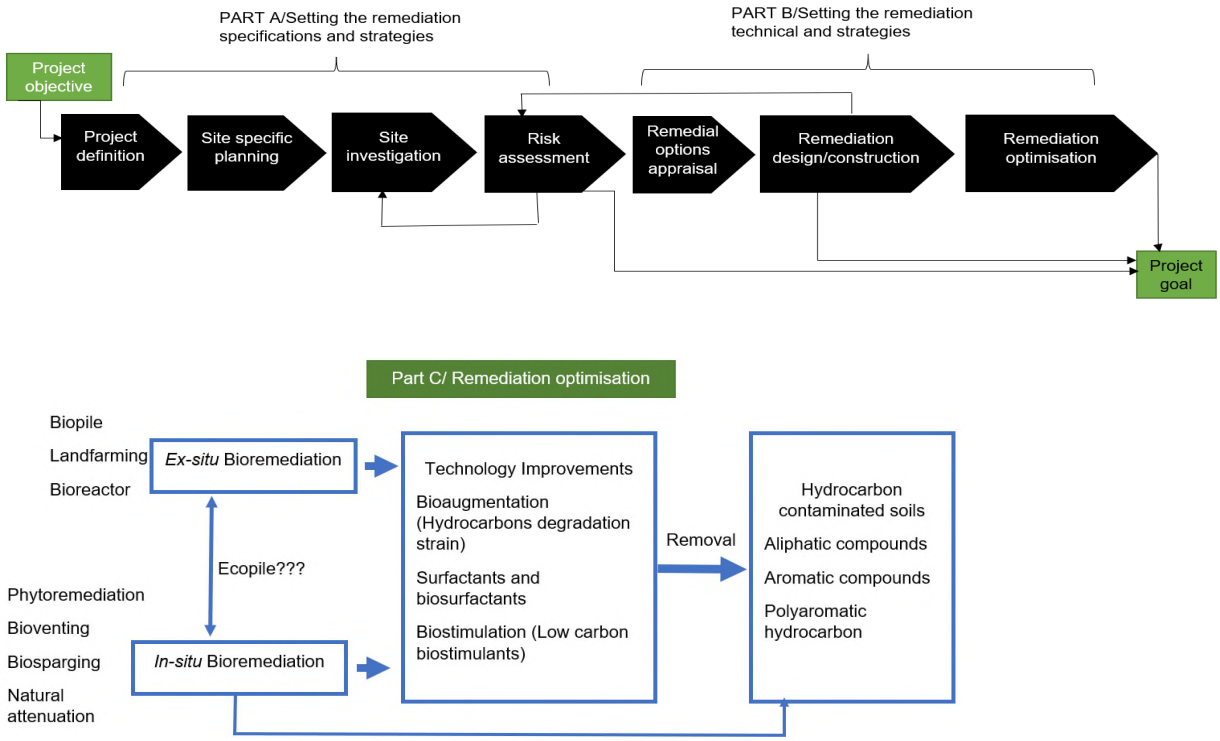

Figure 2.6. Schematic representation for bioremediation of hydrocarbons contaminated wetlands (Adopted from CL:AIRE, 2022; Yap et al., 2021).

The focus of low carbon bioremediation techniques is towards sustainability and efficiency. For low carbon remediation techniques to be sustainable, the techniques should meet the present needs of remediating petroleum hydrocarbons polluted wetlands and acidic wetlands without compromising the ability of the future to meet agricultural, other economic, environmental, and people-oriented needs. Emerging trends in sustainable low carbon remediation techniques are tending towards increasing contaminants biodegradation rate, increasing soil biomass, reducing nutrient leaching, eliminating carbon footprints, improving soil ecology and quality, and using low-cost eco-friendly biomaterials. Low carbon, eco-friendly and cost-effective nutrients are readily available either as waste or organic nutrients in most regions of the world (Chima & Vure, 2014). Examples of successful sustainable low carbon remediation previously done include the use of compost, fungi, algae, and biochar (Ibeto et al., 2020; Ye et al., 2019). From Table 2.1, various low carbon remediation methods were identified, and the extent of degradations obtained from these remediation approaches indicates that most remediated soils still possess harm to the environment and the remediated sites cannot be used for agricultural purposes.

The adoption of a low carbon biostimulation approach in acidic wetland remediation is dominated by such nutrients as biochar, compost, and few organic manures like spent water hyacinth nutrients and combinations of farmyard manure with other low carbon manure (Table 2.1). The pros and cons of these nutrients is shown in Table 2.2, while the degradation of petroleum hydrocarbons using various remediation techniques is shown in Figure 2.7. From the Figure 2.7, the use of low carbon biostimulants (such as compost, biochar, and spent mushrooms) degraded the petroleum hydrocarbons in the wetlands faster than other remediation methods.

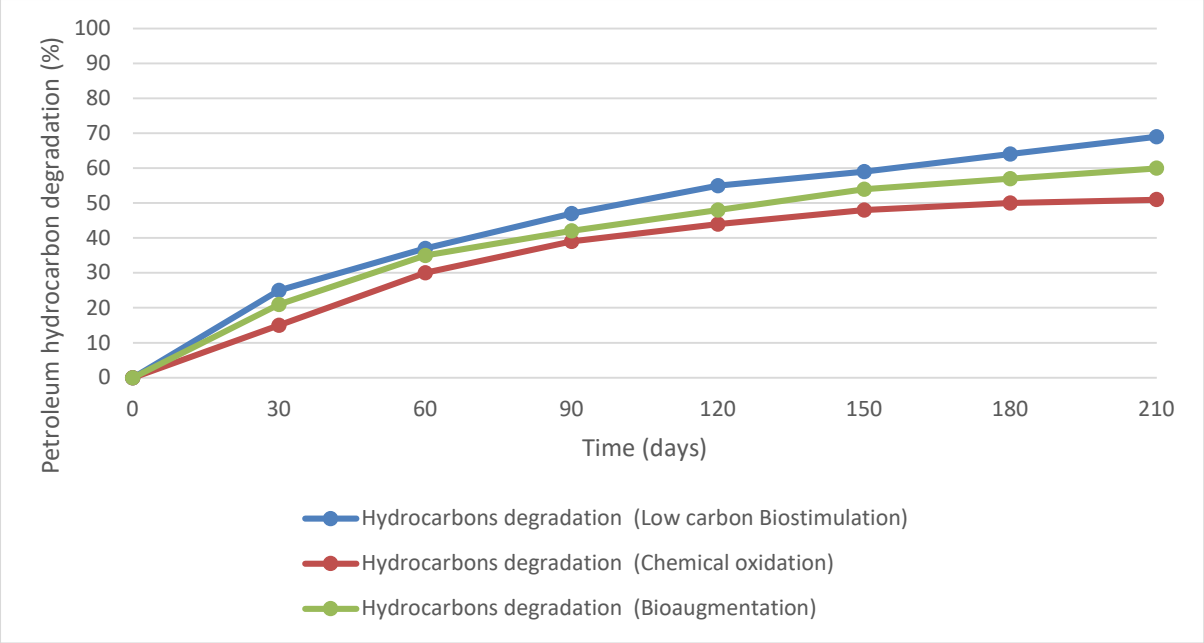

Figure 2.7. Degradation of petroleum hydrocarbons (%) in wetlands versus time(days).

Source: Guo et al., (2020), Wei et al., (2020), Jie et al. (2020), Ruley et al., (2020), Gentry et al., (2010), Awari et al. (2020), Thomas et al., (2020), Liu et al., (2022), Shaoping et al., (2021), Rosik-Dulewska et al., (2015).

The use of biochar, compost, and their combinations with various surfactants yielded to petroleum hydrocarbons degradation efficiencies averaged at 67% degradation for not more than 50,000 mg/kg TPH within 150 days (Wei et al., 2020; Tian et al., 2017; John et al., 2011). The response obtained from composts for the bioremediation of petroleum hydrocarbons produced a degradation efficiency of 60% at about 120 days under similar contaminants levels (Taiwo et al., 2016; Battaglia et al., 2007; Poi et al., 2017). The research of Delgado et al. (2013) on the bioremediation of soils contaminated with crude oil under tropical humid forest using compost, showed that the addition of compost to the polluted soil increased the efficiency of remediation of 25,000 mg/kg TPH from 18% to about 58% in 60 days. The results indicated that a 42% of the petroleum hydrocarbons remained in the wetland after the remediation process. The combination of compost and biochar carried out by Beesley et al., (2010) yielded a reduction of PAH from 55 mg/kg to 8 mg/kg in 60 days. In the investigation of Ye et al., (2019) using compost and biochar to degrade petroleum hydrocarbons in acidic wetlands, the combined nutrients reduced PAH from 200 mg/kg to 25 mg/kg in 45 days. These reductions in the petroleum hydrocarbon content were possible

because the compost decreased the surface area of the biochar due to clogging of the micropore by adsorption of compost-derived organic matter. The compost-derived organic matter then caused an increased surface reactions of the biochar for the sorption of the petroleum hydrocarbons and its subsequent degradation by microbes (Haipeng et al., 2017; Ye et al., 2019). The evolution of greenhouse gases, increased metals (such as nickel, arsenic, and lead) and carbons footprints after remediation was observed with these nutrients. Farmyard manure, spent water hyacinth, and algae nutrients were adopted as alternative low carbon biostimulants.

The use of spent water hyacinth nutrients, algae, farmyard manure, and their combinations yielded 50% degradation of the petroleum hydrocarbons in the acidic wetlands within 100 days of remediation (Garcia-blanco et al., 2007; Egobueze et al., 2019; Ruley et al., 2020). The results produced from bioremediation of petroleum hydrocarbons using various farmyard manures gave 45% TPH degradation for similar contaminants level in 90 days (Ibekwe et al., 2006; Ezenne et al., 2014; Adesodun & Mbagwu, 2008). The research of Orji et al. (2013) on bioremediation of petroleum hydrocarbon contaminated wetlands using cow manure showed 55% TPH maximum reduction after 70 days of bioremediation. The issue of secondary pollution from heavy metals contained in the cow manures were not considered in the research. The heavy metals can inhibit the activities of soil enzymes, disturb organic matter transformation, and reduce microbial biodiversity and biomass in the soil (Guo et al., 2020; Yang et al., 2021). The undetected 45% TPH for microbial remediation can be potentially harmful to the soil and its ecosystem. Furthermore, the research of Ejechi and Ozochi (2015) on the assessment of the degradation of crude oil contaminated soils using poultry and cow manure showed that the petroleum hydrocarbons reduced from 100 - 30% within 243 days of remediation. This research was limited to loamy soils, and the metal contents of the cow manure was not investigated. Research by Awari et al. (2020) on the bioremediation of crude oil-polluted soil using goat manure showed a 99.2% reduction of TPH at the 56th day of remediation. Even though the research was carried out on dry topsoil, the soil type was not considered. In addition, the content of the goat manure was not examined, so the impact of the waste on the soil and the environment was unknown in the research.

Algae and fungi have been used as an alternative nutrients for environmental remediation since they have reduced metal contents and low greenhouse gas

emission when compared to animal manure (Kandasamy et al., 2021; Kuppusamy et al., 2017). Algae are autotrophic organisms that exist on water bodies or high moisture environments and fungi are heterotrophic organisms that exist on dead organic matter. The prominent mechanisms in fungal degradation of petroleum hydrocarbons and other contaminants are enzymatic transformation by intracellular cytochrome P450 enzymes and extracellular ligninolytic enzymes (Cerniglia,1997; Gupta et al., 2015). Figure 2.8 shows the mechanisms adopted by fungi for bioremediation of toxic, recalcitrant compounds such as hydrocarbons using intracellular cytochrome P450 enzymes and extracellular ligninolytic enzymes.

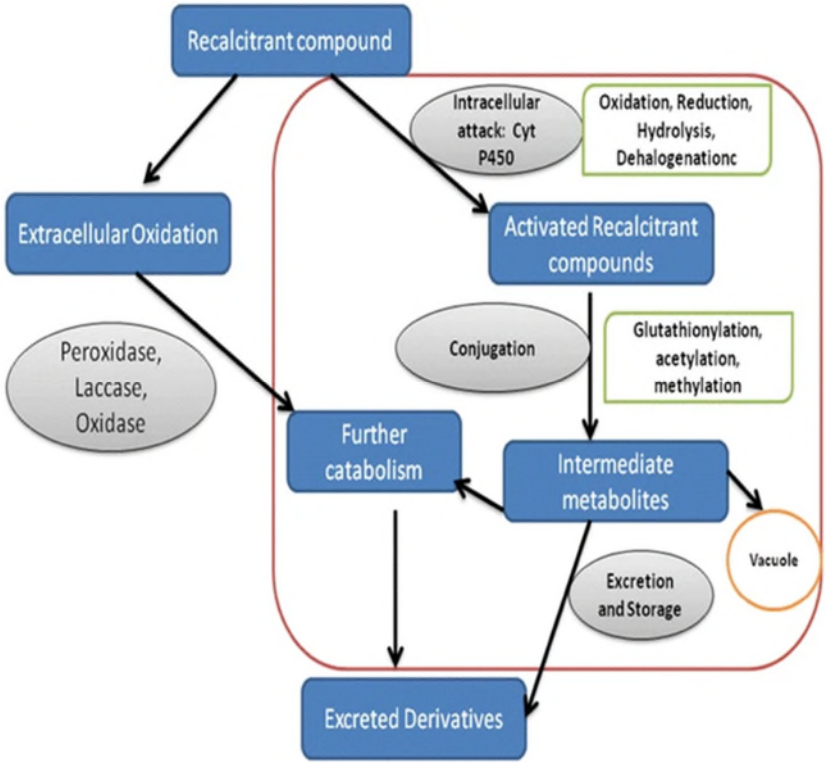

Figure 2.8. Mechanisms adopted by fungi for bioremediation of toxic, recalcitrant compounds.

Source: Deshmukh et al. (2016).

Edema et al., (2011) investigated the use of mushroom (*Agaricus spp*.) and algae, which reduced PAH concentrations in soil respectively by 98% and 97% in 120 days. In the research, the soil samples were polluted with fresh crude oil and the soil type was sandy loam. Environmental conditions such as the frequent acidic rainfall, and acidic wetlands conditions common to the Nigerian terrain were neglected in the experiments. Similarly, the application of fungi (*Termitomyces*) on crude oil

contaminated acidic wetland soil by Orji et al. (2013) showed a 28% reduction in TPH content after 60 days. The inability of the *Termitomyces* to adapt to the acidic wetland soil was attributed to the slow remediation rates observed in the study. Additionally, limited nutrients in the contaminated soil can reduce the microbial degradation rates of the hydrocarbons. Ibeto et al. (2020) stated that a more readily available and sustainable form of low carbon nutrients is abundant in digestate.

Table 2.22. Advantages and disadvantages of some nutrients commonly used in bioremediation of petroleum hydrocarbons contaminated wetlands.

Remediation nutrient	**Advantages**	**Disadvantages**
Biochar	Reduces greenhouse gas emissions during remediation.	Causes an increase in soil pH.
	Good soil amendment and improves soil nutrient value after bioremediation.	Biochar immobilizes beneficial elements like nitrogen in the soil matrix which may pose a risk to crop productivity.
	Increased microbial activities for effective hydrocarbons degradation.	Biochar changes soil microbial communities and their abundance thereby altering the ecosystem.
	Source of renewable bioenergy and easy to apply in soil.	Requires high energy for production.
	Readily available, cheap, and sustainable.	Biochar generated from sewage sludge has heavy metal contents.
	Effective for use in sorption of organic contaminants such as hydrocarbons	When applied in the soil, biochar changes the natural soil albedo (amount of light reflected from the earth to space)

Compost	Reducing greenhouse gas emissions during remediation.	Increases the soil electrical conductivity (measure of salt) in soil.
	Good soil amendment and improves soil nutrient and organic matter value after remediation.	Compost, depending on feedstocks such as sewage and animal manure, increases metals contents in soil.
	Increased microbial activities for effective hydrocarbons degradation.	Some composts contain pathogenic microbes.
	Compost increases aeration and aggregate stability (which increases oxygen supply to microbes) in soils, and it is easy to apply.	Inadequate odour control and hygiene is common in composting
	Readily available, cheap, and sustainable.	Composting is time consuming and requires space, it also needs initial investment.
	Compost is effective for wetlands reclamation, and hydrocarbons degradation in wetlands	Compost efficiency depends on the amount of organic waste present.
Organic manure	Organic matter present in the manure improves soil structures, water holding capacity and nutrient value.	Organic manures deliver nutrients at slow rates.
	Improves soil microbial activities which subsequently increases biodegradation of contaminants.	Organic manures, such as animal manure, increases metals contents in soil.

	Organic manures rarely upset the balance in the soil because they do not deposit any artificial compounds in soils.	The level of nutrients present in organic manures are often inconsistent.
	Readily available, increases carbon storage, and sustainable for gardens and small farms.	For large farms, organic fertilizer is not readily available.
	The process of decomposition requires no chemical intervention.	Natural manures are slow to break down into the nutrients.
	The organic manure can be used in any type of soil and can be applied throughout the year.	Organic manure may not contain primary nutrients like nitrogen, phosphorous, or potassium.
Inorganic fertilizers	Inorganic fertilizers are designed to give plants all the nutrients-Nitrogen, Phosphorous, and Potassium, in the appropriate proportions and amounts.	Inorganic fertilizers are not entirely composed of the nutrients, It also contains salts and other compounds which can alter the microbial communities structures.
	They are easy to handle and store because they come in convenient packages.	Some inorganic fertilizers tend to lower soil pH, making it more acidic.
	Readily available nutrient to facilitate microbial activities for contaminants degradation.	Adding more inorganic fertilizer leaches toxic chemicals into the soil and other areas, pollutes water sources.

	Suitable for large farms and large area remediation activities.	Manufactured industrially thereby contributing to emissions of greenhouse gas.
	Inorganic fertilizers improve crop yield and quality by improving soil nutrients and provides nutrients for bacterial growth and metabolism.	The continues use of inorganic fertilizer reduces the soil nitrogen, and organic carbon, and alters soil ecosystem.

2.7 Digestate as a Sustainable Low Carbon Biomaterial for Bioremediation in Acidic Wetlands

Digestate is produced by the anaerobic digestion of biodegradable feedstocks (Peng & Pivato, 2019). The feedstocks to produce digestate are low-cost, sustainable, and biodegradable materials such as food waste, farmyard manure, municipal waste, and sewage (Le-Hyaric et al., 2012; Gielnik et al., 2020; Bustamante et al., 2012) with the food-waste feedstock producing digestate of better quality (in terms of nutrients) when compared to the other feedstocks (Andrew, 2012). Digestate has often shown to have greater agricultural value than the parent material because of its higher nitrogen and organic matter contents (Bustamante et al., 2012). Thus, digestate is a high-quality low carbon bio-fertilizer that is cost-effective with readily available nutrients to the soil (Nkoa, 2014; Vaneeckhaute et al., 2017; Fernández-Bayo et al., 2017;). The production and use of digestate is cost effective compared to that of conventional fertilizers (such as NPK fertilizers) (Peng and Pivato, 2019). Digestate typically can be found in three forms; whole digestate (mostly in a slurry form with 5% dry matter), liquor digestate (having all the solid material separated), and solid (also called fibre) digestate (solid fractions separated from the whole) (Andrew, 2012; Gielnik et al., 2020). The digestate fibre has shown to be a better bio-fertilizers, more hygienic, and stabilized when compared to the other two digestate forms (Peng & Pivato, 2019; Gielnik et al., 2019).

Food-waste anaerobic digestate fibre (FWAD) having over 80% of its nutrients readily available to the soil is a good biofertilizer and soil amendment when compared with digestate of other feedstocks (Andrew, 2012; Chen et al., 2019). The high availability of nutrients from FWAD allows its use as a direct replacement for inorganic and organic fertilizers. Therefore, for proper management, sustainability, and easiness of use of digestate to be achieved, the digestate must satisfy quality criteria which include stability, cost, environmental friendliness, and readily available (Alburquerque et al., 2012; Koszel & Lorencowicz, 2015). Some of the hygienic and stability characteristics that limit the direct usage of digestate in agricultural or other land-related activities include

odour, viscosity, emission of greenhouse gases, and high levels of volatile fatty acids (Bustamante et al., 2013). Digestate can also be a source of pathogens (Vaneeckhaute et al., 2017) if the digestion did not proceed under optimal thermophilic conditions. To overcome these issues, digestate usually undergoes a cost-effective refinement after its production (Chen et al., 2019; Bustamante et al., 2012).

A refined digestate applied as soil fertilizer or amendment can cause changes in the soil physical, chemical, and biological properties (Fernández-Bayo et al., 2017). These changes, which particularly include an increase in the amount of soil nitrogen and phosphorus, tend to decrease with time leading to scarce residual effects. Bustamante et al. (2012) in their research on the co-composting of the solid fraction of anaerobic digestate observed that the available nitrogen, phosphorus, and potassium value from the composted digestate had concentrations respectively of 28 – 32 g/kg, 6 – 8 g/kg, and 15 – 20 g/kg. The research of Bustamante et al. (2012) corroborated with that of Kratzeisen et al. (2010) where the researchers found that digestate contains 20 – 27% phosphorus and 8 – 15% potassium. These results indicated that digestate could make a good nutrient for soil quality improvement. According to Nkoa (2014) and Teglia et al. (2011) for any material to be considered as a soil amendment, it must improve or maintain the soil physicochemical and biological properties. Therefore, digestate can be called a soil amendment since, it can improve the soil properties.

Digestate also causes an increase in soil microbial biomass (Alburquerque et al., 2012). Koutra et al. (2018) observed a biomass yield of 570 and 1,117 mg/l for *Chlorella vulgaris* and *Acutodesmus obliquus*, respectively, when fibre digestate was used as a culture medium. Also, Dickinson et al. (2015) observed maximum growth rates of 1.84 ± 0.04, 1.82 ± 0.12, and 1.92 ± 0.10 d^{-1} for wastewater, wastewater plus 1.6 times anaerobic digestate fibre and wastewater plus 2.4 times anaerobic digestate fibre respectively, within 2 – 3 growth days for *Scenedesmus species*. Furthermore, Bjornsson et al. (2013) recorded a maximum biomass yield of 0.21 – 0.27 gdw/l from anaerobic digestate fibre used as a nutrient in cultivation. Finally, the research of Gielnik et al. (2019) on the

effect of digestate on soil microbial respiration showed that the presence of digestate increased *Proteobacteria* concentrations from 9.3% to 15.8% and *Aminicenantes* from 7.3% to 7.9%. These results imply that a properly refined anaerobic digestate increases soil microbial biomass.

Despite its positive influence on increasing soil microbial biomass (Gielnik et al., 2019b), digestate has been of little used for bioremediation of contaminated soils. The use of digestate on bioremediation of hydrocarbons contaminated soils was recently investigated by Gielnik et al. (2019), in their research on the bacterial seeding potential of digestate in bioremediation of diesel contaminated soil. After 21 days of bioremediation, 78% of the starting 13,200 mg/kg TPH had been degraded in soil amended with digestate; whereas amendment with compost resulted in only 46% TPH degradation from its initial 9,163 mg/kg after 180 days (Cipullo et al., 2019). These results indicate that digestate has a higher potential to degrade hydrocarbon contaminants in soil if compared to compost and biochar. To date, the effect of digestate on bioremediation on acidic wetlands contaminated with petroleum hydrocarbons is unknown. Therefore, research on the potentials of digestate alongside improved eco-friendly bioaugmentation and chemical methods in degrading petroleum hydrocarbon contaminated acidic wetlands could be of great impact and useful for Niger Delta environment. The effectiveness of digestate on the remediation of petroleum hydrocarbon contaminated acidic wetlands can be ascertained using bioassays as remediation end points determinants.

2.8 Hydrocarbons Toxicity Impact on Bioremediation Endpoint

Demonstrating that the original contaminants has been remediated does not necessarily mean a subsequent reduction in soil toxicity (Poi et al., 2017). Toxicity in remediated soils could be caused by production of intermediate metabolites (Philips et al., 2000). Ecotoxicity studies are used for the estimation of the contaminant's toxicity levels in soils (Plaza et al., 2005) and often considered as a reliable determinant of remediation endpoint at the close of remediation. Remediation end points, also called remediation clean-up criteria, are targets that need to be achieved to demonstrate the treatment efficacy (National Remediation

Framework, 2018). These targets can be assessed by using numerical values, or by a qualitative approach, and include methods such as phytotoxicity assays, Microtox assay, and the use of model invertebrates such as earthworms for bioassays. The use of model invertebrates (such as *Lumbricus rubellus*, *Aporrectodea longa*, and *Eisenia fetida)* for the bioassays of contaminated and/or remediated soils allows for estimates of ecological toxicity (Hankard et al., 2004). However, the technique is deficient for validation and monitoring purposes, due to fibre instability and varying sensitivity of the invertebrates on the contaminated and/or remediated soil (Hankard et al., 2004; Chen et al., 2022).

Microtox assay was used as an alternative techniques for soil toxicity assessment. Microtox assay measures the bioluminescence response of a marine bacterium (*Vibrio fisheri*). *Vibrio fischeri* is a marine microbe that operates at optimal pH range of 7.8 – 9 and bioluminescence in response to the contaminants toxicity as shown in Figure 2.9. The pH correction factor applied to the soil samples to ensure that *Vibrio fisheri* functions optimally could alter the bioavailability of the contaminants to the bacteria since pH alters the bioavailability of metals and other contaminants, and subsequently generate false toxicity response (Palmer et al., 1998; Nkereuwem et al., 2020; Lajoie et al., 2002). Microtox is cost intensive and require the relevant technical skills to have excellent results.

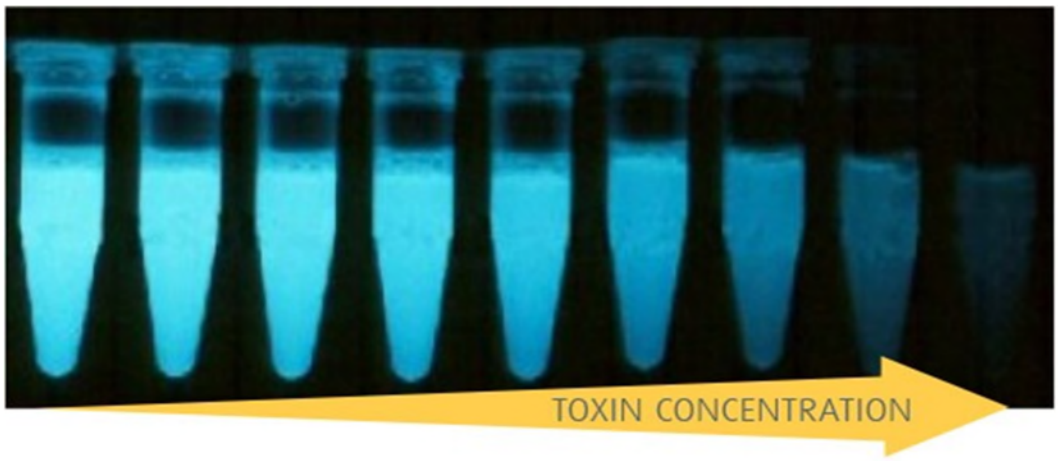

Figure 2.9. Mocrotox assay showing toxin concentration.

The search for cost effective and efficient techniques led to adopting phytotoxicity techniques as an alternative. Phytotoxicity is the use of plants to assess the ecological health of contaminated and/or remediated soil. Symptoms of toxicity on plants include inhibition in seed germination and seedling development, reduced photosynthesis activity, stunted growth, and chlorosis (Haider et al., 2021; Zhang et al., 2019). Using plants as a measure of toxicity is ecologically low risk and can be cost effective if compared to alternative techniques such as microtox assay (Wuana & Okieimen, 2010). The soil samples to be bioassessed are used as growing or germinating medium for the plants and the response is monitored (Cipullo et al., 2019; Chiwetalu et al., 2020). The plants response to the toxicants can be monitored through percentage of germination, number of days for germination to occur, sprouting height, leaf area index, plant biomass, and stem length (Haider et al., 2021; Ren et al., 1996). Commonly adopted plants for phytotoxicity in the Niger Delta include mustard (*Brassica spp.*), pea (*Cajanus cajan*), and maize (*Zea mays*) (Ekperusi et al., 2020).

Maize (*Zea mays*) exhibits high phytotoxicity sensitivity to high, medium, and low molecular weight hydrocarbons based on shoots, germination delays, and root biomass (Baek et al., 2004; Maliszewska-kordybach & Smreczak, 2003). Maize was introduced to Nigeria in the 10th century and since then has overtaken the raffia palms (which were native to the Niger Delta and dominant species in the 10th century) to become one of the prominent crops in the country (Ayotamuno et al., 2011; Osim & Oniah, 2023). Maize is the second most important cereal crop in Nigeria, ranking behind sorghum (*Sorghum bicolor*) and is the most consumed cereal crop within the Niger Delta (Fubara-Manuel et al., 2017). The method of seeding include broadcasting, dibbling, drilling, sowing behind the country plough and transplanting. Dibbling requires less seeds and, gives rapid and uniform germination with good seedling vigour and yield. This method is most suited for laboratory-based experiments, and it is commonly practiced among Nigeria local farmers of maize crops (Masoni et al., 2002). The research of Wuana and Okieimen, (2010) using maize in phytotoxicity showed that at 1,500 mg/kg contaminants concentration, a significant decrease of biomass was recorded in the maize roots and shoots. Similar results were obtained by Khan et al. (2018)

where diesel contamination inhibited crop germination. The researchers concluded that maize crops were better suited for phytotoxicity monitoring and early detection of remediation endpoint in hydrocarbons remediated soils if compared with pea and wheat.

2.9 Conclusion

This review reveals that the bioremediation techniques employed for the petroleum hydrocarbon remediation in acidic wetlands were inadequate, leaving harmful contaminants in the soil available to the surrounding environment. Over the years, low carbon remediation techniques have been adopted for contaminated wetlands. The review examined the efficacy of low carbon remediation in petroleum hydrocarbon contaminated wetlands. It was discovered that limited knowledge of the contaminated wetlands ecosystem (before and after contamination), and negligible attention given to the nature of the contaminants were the primary factors enhancing the deficient degradation of the contaminants. These factors lead to inadequate decisions on the low carbons nutrients to be used for remediation and influence the improvements made on the techniques. It was concluded that for sustainable low carbon bioremediation techniques to achieve the required efficiency and remediation endpoint during bioremediation of petroleum hydrocarbons in acidic wetlands, sustainable biostimulants such as digestate, with readily available nutrient and high biomass seeding potentials, should be adopted.

Digestate, which is a by-product of anaerobic digestion, is a sustainable low carbon biomaterial to use for remediation in acidic wetlands. It is a good biofertilizer and cost-effective soil amendment. Food-waste digestate has been justified in this review as the most valuable digestate in terms of nutrient quality and availability of its nutrients to the soil and can lead to increased biomass in wetlands. The efficacy of surfactants in enhancing the rate of bioremediation in acidic wetlands was also studied. Tween 80 surfactant, being ecologically low risk, can increase the bioavailability of petroleum hydrocarbons in acidic wetlands if used as a stimulant, making the contaminants bioavailable for degradation by the microbial communities in the soil. The effects of bioaugmentation on acidic

wetlands contaminated with petroleum hydrocarbons was also reviewed. Indigenous microbial consortia were identified that degrade petroleum hydrocarbon contaminants in acidic wetlands faster than non-indigenous microbial consortia. Limited bioavailability of the petroleum hydrocarbon contaminants, and deficient bioaugmentation strategies contribute to the limitations of effective bioaugmentation.

To overcome relatively slow and inefficient remediation of petroleum hydrocarbons contaminated acidic wetlands, sustainable strategies to accelerate hydrocarbons degradation such as combinations of improved eco-friendly bioaugmentation, and low carbon biostimulation should be adopted. The efficacies of the proposed techniques can be ascertained using maize to determine the remediation endpoint.

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3 EVALUATING DIFFERENT SOIL AMENDMENTS AS BIOREMEDIATION STRATEGY FOR WETLANDS CONTAMINATED BY CRUDE OIL

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3.1 Abstract

This study evaluated the efficacy of using Tween 80 surfactant (TW80) and food-waste anaerobic digestate fibre (FWAD) as soil amendments for the remediation of wetlands contaminated by crude oil. A 112-day mesocosms experiment was carried out to simulate hydrocarbon degradation under typical acidified wetland conditions. Soil was spiked with 50,000 mg kg^{-1} crude oil and TW80 and FWAD were added to mesocosms at 10%, 20% and 30% w/w. The soil basal respiration, microbial community dynamics, environmental stress, alkanes, and PAHs degradation were monitored throughout the mesocosm experiment. Amending the mesocosms with FWAD and TW80 enabled the recovery of the soil microbial activities. This result was evidenced by soil basal respiration which was highest in the 30% FWAD and 30% TW80 mesocosms and translated into increased degradation rates of 32% and 23% for alkanes, and 33% and 26% for PAHs compared to natural attenuation, respectively. Total hydrocarbon degradation was achieved in soil mesocosms with 30% FWAD and 30% TW80 at 90% and 87%, respectively after 49 days. Following the FWAD and TW80 treatment, seed germination rates increased significantly from 29% to over 90%.

Keywords: remediation, wetlands, food-base digestate, surfactant, hydrocarbons.

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3.2 Introduction

Wetlands are poorly drained areas subject to permanent or periodic water saturation (Drake et al., 2009). Wetlands are both ecologically and economically important because of their high agricultural productivity, complex biogeochemistry, and nutrient

cycling ability (Morse et al., 2012; Nwankwoala & Okujagu, 2021). The Niger Delta, Nigeria is one of the most important and biodiverse wetland ecosystems in the world (Konne, 2014). However, the wetlands of the Niger Delta house most of the crude oil fields in Nigeria. The exploration and exploitation of crude oil in the wetlands often leads to contamination (both small (<100 m^2) and large scale (>100 m^2)) through spillages and subsequent alteration in the wetland's ecosystems (Ruley et al., 2020). Studies have shown that acidification is occurring along with high sulphate, and nitrate concentration in the Niger Delta wetlands (Ohimain, 2003; Johnston et al., 2014). Acid rainfall, caused by oil field gas flaring and continual industrialization has been linked to the acidification of the wetlands (Jeffries et al., 2003). The acidification of wetlands is of primary concern because of its effects on wetland ecosystems, and agriculture (Singh & Chakraborty, 2020).

Persistent petroleum hydrocarbons spillages on the wetlands in the Niger Delta Region has led to severe public health concerns, economic, and ecological risk (Zhu et al., 2004; Osuji et al., 2006), which has been well documented (Sam et al., 2016; Nwaichi & Uzazobona, 2011; Osuji et al., 2004). Certain groups of petroleum hydrocarbon contaminants in the acidified wetlands, mainly medium and heavy molecular weight alkanes, and polycyclic aromatics hydrocarbons (PAHs) (Figure 2.2) are of concern due to their high soil and water mobility, bioavailability, recalcitrant to degradation, and carcinogenic nature (Robichaud et al., 2019; Yu et al., 2019; Brown et al., 2017). Remediation of these contaminants in acidified wetlands using conventional remediation methods including soil excavation and physiochemical treatments were not suitable due to cost, emission of greenhouse gases, and secondary pollutions (Scanferla et al., 2009; Ngene & Tota-maharaj, 2019; Okoye et al., 2020).

Previous studies suggested biostimulation using nutrient rich soil amendments to increase soil microbial activities and thus improve biodegradation of contaminants (Ossai et al., 2022). Cipullo et al. (2019) reported using compost as a nutrient amendment in the bioremediation of petroleum hydrocarbon contaminated soils which resulted in total hydrocarbons (TPH) degradation up to 46% from its initial 9,163 mg/kg after 180 days. Typically, biostimulation as a remediation strategy is less intrusive to the environment than physicochemical techniques. The overall remediation costs and

carbon emissions during remediation process can be significantly reduced when using non-commercial nutrient supplement, including compost, farmyard manure or digestate, (Ngene & Tota-Maharaj, 2020; Aghalibe et al., 2017; Abdulyekeen et al., 2021; Smidt et al., 2011; Lee et al., 2017).

Digestate from the anaerobic digestion (AD) of organic feedstock is a by-product of the AD process (Peng & Pivato, 2019). It contains high levels of nutrients including nitrogen, phosphorus, and potassium, and when applied to land, is a high-quality bio-fertilizer which provides readily available nutrients to the soil (Nkoa, 2014; Vaneeckhaute et al., 2012; Fernández-Bayo et al., 2017). The solid fraction of the digestate (digestate fibre) are better bio-fertilizers, more hygienic, and stabilized when compared with the whole or liquid fraction of digestate (Peng & Pivato, 2019; Gielnik et al., 2019). The feedstock to produce digestate includes biodegradable materials such as food waste, farmyard manure, municipal waste, and sewage (Silva et al., 2012; Gielnik et al., 2021; Bustamante et al., 2012). Sewage digestate has been successfully applied as biostimulant for the remediation of diesel contaminated soils (Gielnik et al., 2019). However, sewage digestates can have high available metal and metalloids content and can also introduce pathogenic bacteria into the remediated soils (Yu et al., 2022; Andrew, 2012). Therefore, there has been understandable environmental and public health concerns related to land application of sewage digestate (Gielnik et al. 2019; Bustamante et al., 2012). Food waste anaerobic digestate (FWAD) possess higher nutrients contents when compared to the digestate of other feedstocks (Andrew, 2012; Opatokun et al., 2015). Food waste anaerobic digestate is known to have low metal and metalloids contents, high nitrogen, phosphorus, and potassium content, which could increase soil nutrient levels after hydrocarbon remediation, and can be suitable to large scale remediation of the wetlands of Niger Delta (Peng & Pivato, 2019; Opatokun et al., 2015; Ruley et al., 2020).

While the addition of anaerobic digestates, composts, or biochar have all delivered promising results in enhancing the degradation of petroleum hydrocarbons, the extent of degradation can be highly variable and this is often related to the accessibility of the hydrocarbons compounds to the microbes, in other word the bioavailability of the hydrocarbons (Sung et al., 2013; Wang et al., 2018). Enhancing the bioavailability of

the contaminants using surface-active substance like non-ionic surfactant has shown to be more suitable for soil remediation than the cationic and amphoteric surfactants (Figure 1.2) (Cheng et al., 2018). The non-ionic surfactants are also cost-effective with minimal toxicity to the soil microbial communities (Seo & Bishop, 2007). Tween 80 (TW80) a non-ionic surfactant, increases the solubility and mass transfer of hydrophobic organic compounds including hydrocarbons, low in ecological toxicity, and readily available, can be suitable for remediating the numerous small-scale contaminations in the wetlands of Niger Delta (Ceschia et al., 2014; Sam et al., 2016). Despite these benefits, Tween 80 has been used primarily for *ex-situ* soil washing (Cheng et al., 2018), and rarely been considered as a supplement during *in-situ* hydrocarbon remediation. The focus of this research is evaluating the potential of TW80 or digestate as soil amendments for remediating petroleum hydrocarbons contaminants in acidified wetlands and the subsequent establishment of the corresponding remediation endpoints.

3.3 Materials and Methods

3.3.1 Mesocosm Soil and Experimental Design

Pristine soil, with no record of petroleum hydrocarbons contamination, was collected from a construction site in Cranfield University (52.0746 N, 0.6283E). Soil was collected from 0 to 30cm soil depth, the soil was air dried at room temperature, sieved through 2 mm aperture sieve (model: BS410 manufactured by: Endecotts, London, England), and stored for 4 days at 20 ^{o}C before use. Triplicate soil mesocosms were set up using 1 kg soil in 2.5 litre transparent polytetrafluoroethylene (PFTE) containers. Nine different mesocosms conditions were evaluated as summarised in Table 3.1 and Figure 3.1.

Figure 3.1. Experimental setup for biostimulation treatment of hydrocarbon contaminated acidified wetland soil.

Table 3.11. Overview of the biostimulation treatments evaluated along with the controls.

Mesocosm conditions	**Abbreviations**
Pristine soil (freshly collected from field)	Control
Pristine soil acidified at pH 5.8 (using HNO_3)	Acidified
Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg with crude oil	Crude oil
Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg with crude oil + 10% (w/w) FWAD digestate	10% FWAD
Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg with crude oil + 20% (w/w) FWAD digestate	20% FWAD
Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg with crude oil + 30% (w/w) FWAD digestate	30% FWAD

Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg with crude oil + 10% (w/w) Tween 80	10% TW80
Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg with crude oil + 20% (w/w) Tween 80	20% TW80
Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg with crude oil + 30% (w/w) Tween 80	30% TW80

The mesocosms, except the pristine soils (control), were all acidified to pH of 5.8 using HNO_3 (PrimarPlus- trace analysis grade, supplied by Fisher Scientific UK, Limited). The HNO_3 was adopted because rainfall in most part of the Niger Delta wetlands were weak nitric acid of pH between 5.7 - 5.9 (Onu et al., 2014; Nduka et al., 2008), the soil mesocosms were spiked with 60 ml of crude oil (<0.5% sulphur) (SDS, Regulation 1907/2006/EC) to achieve a target hydrocarbon concentration of 50,000 mg/kg. The adopted concentration fell within the range of recorded petroleum hydrocarbon contaminated wetlands in the Niger Delta (Ugochukwu et al., 2018; Chidinma et al., 2021). The mesocosms were incubated at 28 °C and sandy soil was adopted to mimic the mean temperature and prominent soils of the Niger Delta (Fubara-Manuel et al., 2017). Three controls (pristine soil, acidified soil (without PHCs) and crude oil spiked acidified soil with no treatment (natural attenuation)) were maintained throughout the experiment. The soil moisture content of 13.75% was increased and maintained at saturation with 54.54% (Table 3.4) moisture increase. Moisture at saturation was maintained to depict the wetland condition in all mesocosms. Deionised water was used to maintain moisture in all mesocosm. The deionised water was added at 7 days intervals to maintain the soil saturation.

FWAD was air dried and particles larger than 2 mm were removed using a 2 mm aperture sieve. Dried FWAD was thoroughly mixed with crude oil spiked acidified soil at 10, 20 and 30% (w/w) in triplicates following the methods described in Nwankwo (2014).

The TW80 (Polyoxyethylene(20)sorbitan monooleate) was applied at 10, 20 and 30% (w/w) and thoroughly mixed with the crude oil spiked acidified soil samples in triplicates. The application of the non-ionic surfactants (TW80) was as described by Trinchera and Baratella (2018).

3.3.2 Soil and FWAD Physicochemical Properties Determination

Soil moisture content and dry matter content were determined according to BS 7755: Section 3.1:1994 standard operating procedure (SOP). 50 g of air-dried soil samples were oven dried at 105 °C for 24 hours. The weight difference before and after oven drying of the samples and the weight of the empty crucible were recorded and moisture content and dry matter content estimated using formulae 3.1 and 3.2 respectively:

$$W_{wc} = \frac{M_1 - M_2}{M_2 - M_0} \times 100\% \qquad 3\text{-}11$$

$$W_{dm} = \frac{M_2 - M_0}{M_1 - M_0} \times 100\% \qquad 3\text{-}22$$

W_{dm} is the dry matter content expressed as a percentage of the original sample mass.

W_{wc} is the water content expressed as a percentage of the oven-dried mass of sample.

M_0 is the mass, in grams, of the empty crucible.

M_1 is the mass, in grams, of the crucible plus field-moist soil.

M_2 is the mass, in grams, of the crucible plus oven-dried soil.

Organic matter content was determined through loss on ignition (LOI). 50g of the soil samples were dehydrated at 105 ^oC for 24 hours, then combusted in a furnace (Carbolite Eurotherm, model 3216, Scientific Laboratory Supplies, UK) at 450 ^oC for 4 hours (as stated in BS EN 13039: 2000). The LOI was determined using the formular 3.3 and expressed as a percentage of the furnace dried mass of the sample.

$$\boldsymbol{LOI = \frac{M_2 - M_3}{M_2 - M_0} \times 100\%} \qquad 3\text{-}33$$

M_3 is the mass, in grams, of the crucible plus furnace - dried soil.

The water holding capacity at saturation and field capacity were determine using BS 7755 Section 5.5:1999 (which is identical to ISO 11274:1998) method. Air-dried soil samples were packed in a 45.80 cm^3 soil core with mesh and oven dried at 105°C for 24 hours. The setup was then placed in a foam wetting-up bath. During the procedure, the foam was kept continually saturated with water. The mass of the sample, tin, mesh, and elastic band was repeatedly recorded until an equilibrium was established. All measurements for water release data were first expressed in terms of mass of oven-dry soil. The water holding capacity was determined through equation 3.4:

$$\boldsymbol{WHC_{ma}} = \frac{\boldsymbol{WHC_b - WHC_c}}{\boldsymbol{WHC_c - WHC_d}} \times \mathbf{100}\% \qquad 3\text{-}44$$

WHC_b is the mass, in grams, of the saturated sample, tin and mesh.

WHC_C is the mass, in grams, of the oven-dried sample, tin and mesh.

WHC_d is the mass, in grams, of the tin and mesh.

Particle size distribution was carried out by sedimentation according to ISO 11277 (1998) using a Laser Analyzer Master-sizer (MS3000, Malvern Instruments Ltd, Worcestershire, UK) equipped with a hydro adaptor. Soil measurements were taken in the range of 0.02 – 2,000µm based on the standard operating manual of the machine. The samples refractive index was 1.7, with absorption coefficient of 3.5. The sample and background measurement time were 20 seconds with 5 measurement cycles at no delay between measurements. Six independent measurements were taken for each soil sample. Soil textural classification was based on percentage clay, silt, and sand using the United State Department of Agriculture soil textural classification scheme.

The pH values of the mesocosm samples were measured with a pH meter (Jenway 3540, Cole-Palmer, Staffordshire, UK). Samples were first mixed with deionised water slurry (at a ratio of 1:5 w/w). The mixture was shaken for 60 minutes at 300 rpm using orbital shaker (model: SSL1, manufacturer: Barloworld Scientific, United Kingdom) before measurement was taken. The phosphorus content of the samples was determined using British Standard BS 7755: Section 3.6:1995. 5g of soil was added to 100ml sodium hydrogen carbonate reagent in a 250-polypropylene bottle. The setup

was shaken on a side-to- side shaker set at 300 min^{-1} for 30 minutes, the 1 ml of 1.5 mol/l sulfuric acid added to 5ml of the filtrate and swirled, after which 20 ml of 0.15% m/v ammonium molybdate reagent and 5 ml of ascorbic acid solution added. Extracts in the flasks were analyzed using a Jenway 6850 spectrophotometer (manufacturer: Jenway, United Kingdom) at 880 nm absorbance. Potassium and magnesium were extracted by adding 50 ml of 1 M ammonium nitrate solution to 10 g of the soil samples according to according to British Standard BS 3882. The mixture was shaken for 30 minutes at 300 rpm using an orbital shaker. Filtrates were analyzed using an atomic absorption spectrometer (model: A Analyst 800, manufacturer: Perkin Elmer, Bath, UK). Total organic carbon (TOC), soil organic matter (SOM) and total nitrogen (TN) were determined according to British Standard BS 7755 Section 3.8:1995. The carousel of the automatic sample feeder (model: Vario EL cube and manufacturer: Elementar, Germany) was used for the analysis.

3.3.3 Soil Biological Properties Determination

Soil basal respiration is the measurement of the steady rate of microbial respiration in soil (He & Xu, 2021). Soil basal respiration was measured at the onset of the experiment and, 7, 14, 28, 49, 77 and 112^{th} day of the mesocosm experiment. Soil samples basal respiration were determined using the Rapid automated bacterial impedance technique (RABIT) (Don Whitley Scientific, UK) as a respirometer. 1 g of soil samples, moistened to saturation in a glass boat, was inserted into a clean dry cell as described by Pawlett et al. (2013) for the determination of soil basal respiration. The microbial respiratory response ran for 48 hours at 25 °C. Changes in conductivities (micro siemens) were determined and quantified to CO_2 according to Ritz et al. (2006). The RABIT software (RABIT version 2.31, 01-1999) was used for quantification of the conductivities.

Soil microbial community profiles and dynamics were determined based on phospholipid fatty acids (PLFA) analysis using a modified method from Frostegård and Bååth (1993). The PLFA were measured at 30 days intervals. Lipids were extracted from 10 g of freeze-dried soil samples using 1: 2: 0.8 (v/v/v) of chloroform: methanol: citrate buffer and 30 mg of Butylhydroxytoluene (BHT). The extracted lipids were fractionated by solid phase extraction, and phospholipids fraction derivatized by mild alkaline methanolysis according to Dowling and White (1986). Resulting fatty acids

methyl esters (FAMEs) were analyzed by gas chromatography as described by Pawlett et al. (2013). Fatty acids were used as an indicator of the presence of groups of microbes (biomarkers). Biomarkers were categorized into gram positive bacteria, gram negative bacteria, actinobacteria and fungi according to Quideau et al. (2016) and Frostegård and Bååth (1993).

Soil microbial count were determined using colony forming unit (CFU) plate counting technique (Varjani & Upasani, 2019). Soil suspensions were prepared by 10-fold serial dilutions with 1g of soil in triplicates, using deionized water as diluents. The plates were incubated for a period of 24 hours in an incubator (Heraeus Incubator, Thermos Scientific, Germany) at 37 ^{o}C.

3.3.4 Hydrocarbons Analysis

Total petroleum hydrocarbons (TPH) were extracted and analysed using the procedure described by Risdon et al. (2008). Readily available hydrocarbons fraction was extracted using 15 ml of methanol (HPLC grade, Merck Life Science Limited, UK) while the bioavailable hydrocarbons fraction was extracted using 50 ml of 50m M of 2-hydroxypropyl-β-cyclodextrin (HPCD; Merck Life Science Limited, UK) according to Cipullo et al. (2018). The hydrocarbons fractions of each extraction were analyzed using a TQ8040 Shimadzu (manufacturer: CTC Analytics, Switzerland) gas chromatography–mass spectrometer (GCMS) equipped with an AOC 6000 auto-sampler (manufacturer: CTC Analytics, Switzerland) and operated in positive ion mode at +70eV with scan speed of 2,500 in time range of 3 – 36.33 minutes. The ion source temperature was 200 ^{o}C, with interface temperature of 300 ^{o}C. The initial temperature was 60 ^{o}C (0 min hold time), ramped at 20 ^{o}C/min to 220 ^{o}C (0 min hold time), and a final ramp of 6 ^{o}C/min to 300 ^{o}C (15 min hold time) and a column flow of 2ml/min. During injection, the system ran split-less with 1µL injection volume. The column type and dimension were Rtx-5 30 m x 0.25 mm x 0.25 µm. The mass spectrometer was operated using the full scan mode (range m/z 50-500) for quantitative analysis of target aliphatic and aromatic hydrocarbons using the Schimadzu TQ 8040 software. Hydrocarbons compounds were identified by retention time and m/z. Each compound was quantified by integrating the peak at specific m/z. The external multilevel calibration (level 1- 5) was carried out using alkanes standard solution (C8 - C40) (Sigma Aldrich, Merck Life Science Limited**,** Dorset, UK) and the PAH standard

solution (EPA525 PAH Mix A) (Sigma Aldrich, Merck Life Science Limited**,** Dorset, UK). The multilevel concentration ranged from 0.5µg/ml - 5µg/ml. Quality control and assurance procedure were carried out with the whole procedure blank, clean soil matrix spike recovery and comparison with reference materials. The TPH and readily available hydrocarbons were analysed at day 0, 7, 14, 28, 49, 77 and 112 while the bioavailable hydrocarbons were measured at day 0, 28 and 112. Alkanes were grouped into C11-C18 which are prominently liquids and are medium molecular weight, made up of undecane, dodecane to octadecane. The other group was C19 – C37 which are prominently wax and heavy molecular weight, made up of nonadecane, octadecane to heptatriacontane. The petroleum PAHs were similarly grouped into C10 – C18 (which include naphthalene to chrysene) and C19 - C22 (benzo[b]fluoranthene, to indeno(123) [cd]pyrene).

3.3.5 Metal(loid)s Analysis

Metal(loid)s including molybdenum, chromium, nickel, arsenic, cadmium, lead, and mercury in the soil samples were determined using US EPA Method 3051 and British Standard BS 7755: Section 3.13:1998. Soil acid digestion was carried out using 0.5g of the soil samples added with 6ml of hydrochloric acid (1.18 specific gravity) and 2ml of nitric acid (1.42 specific gravity) in the liner of a pressure vessel. Vessels were loaded into a microwave machine (model: Mars 240/50, manufacturer: CEM corporation, USA) for digestion, and 10ml of the filtrate used for a flame atomic absorption spectrophotometer analysis (model: Jenway 6850, manufacturer: Jenway, United Kingdom).

3.3.6 Ecotoxicity Assay

Germination assays were carried out using maize (*Zea mays*). Maize was chosen for the ecological risk assessment since it exhibits high toxicity sensitivity to high and low molecular weight hydrocarbons based on shoots, and germination delays and root biomass compared to mustard, pea, and sorghum (Baek et al., 2004; Maliszewska-kordybach & Smreczak, 2003). Maize is the second most important cereal crop in Nigeria ranking behind sorghum and most consumed cereal crop within the Niger Delta region of Nigeria (Fubara-Manuel et al., 2017). The maize was planted using the dibbling method which requires less seeds and, gives rapid and uniform germination and good yield (Nyagumbo et al., 2016). This method is most suited for laboratory-

based experiments, and it is commonly practiced among local farmers (Masoni et al., 2002). Five seeds were planted per cell and the germination response and days of germination after planting were recorded at the onset and 112th day of the experiment.

3.3.7 Statistical Analysis

Descriptive statistical analysis was carried out including mean, standard deviation, standard error using Microsoft Excel (Version 2111 Build 16.0.14701.20278). Standard error was used to evaluate the variability across germination assays and the applied environmental stress while standard deviation was used to ascertain the variability within sample measurements and applied to the metal(loids) data. The JMP pro (version 16) software was used for Spearman correlation and models. Differences in respiration, hydrocarbons concentrations between treatments were compared using spearman correlation at 99.99 percent confidence level, the difference was considered significant if probability (p) <0.01.

3.4 Results and Discussion

3.4.1 Soil Characterisation

The pristine soil was a sandy silt loam soil with a pH of 8.7 and moisture content of 13.75% (Table 3.3). The C: N: P ratio of soils are important indicators of soil fertility and soils with high C: N: P ratios are referred to as organic rich soils (Chen & Chen, 2021). The optimal soil C: N: P ratio for effective biodegradation of contaminants by microbes has been recommended at 100:10:1(US EPA, 1994). The C: N: P ratio of 60:2:1 for the pristine soil samples suggest low carbon and nitrogen (Table 3.3 & 3.4). When soil C: N: P ratios are below the optimal value, it can result in limited microbial activities (Griffiths et al. 2012), supplementation can restore this. FWAD with C: N: P ratio of 250:13:1 indicated that the digestate had higher quantity of N and organic carbons to improve C: N: P ratio in the pristine soil, and potentially stimulate microbial activities. This was confirmed by the higher degradation rates of petroleum HCs (shown in Table 3.5 and 3.6) after addition of FWAD. The soil total and organic carbons for the pristine soil were 3.09% and 2.25% respectively (Table 3.3). The total carbon was increased by spiking of the soil with crude oil to 5.08%. Acidifying the soil increased the availability of the soil metals and metalloids (Table 3.2). This observed increment in availability of the metals and metalloids agreed with the findings of

Chintala et al. (2014) and Ning et al. (2016). These researchers concluded that the availability of metals increased as the soil becomes more acidic.

Table 3.22. Baseline concentrations of metal and metalloid and standard deviation in soil samples.

Metal(loid) (mg/kg)	**Control**	**Acidified**	**Crude oil**	**FWAD**
Mo	0.93 ± 0.08	1.27 ± 0.13	1.21 ± 0.07	1.20 ± 0.49
Cr	45.24 ± 1.23	59.56 ± 2.94	50.87 ± 1.50	36.05 ± 2.17
Ni	29.65 ± 1.24	42.60 ± 0.93	35.25 ± 0.79	23.79 ± 1.58
As	14.83 ± 0.98	20.21 ± 0.74	17.18 ± 0.78	10.13 ± 0.52
Cd	0.59 ± 0.09	0.83 ± 0.09	0.73 ± 0.07	0.72 ± 0.10
Pb	17.00 ± 0.91	23.31 ± 0.45	21.41 ± 0.8	15.62 ± 0.94
Hg	0.29 ± 0.01	0.12 ± 0.01	0.12 ± 0.02	0.14 ± 0.04

Table 3.33. Physical characteristics of the pristine soil used in the mesocosm experiment.

Soil Physicochemical characteristics	
Soil Moisture content (%)	13.75
Loss on ignition (%)	3.66
Dry matter content (%)	86.25
Water holding capacity (%)	54.54
TOC (%)	3.09
Org C (%)	2.25
TN (%)	0.12
TP (mg/kg)	5.58
TK (mg/kg)	236.00
Soil Particle size distribution	
Sand (%)	46.67
Silt (%)	45.89
Clay (%)	7.44
FWAD characteristics	
TOC (%)	17.22
Org C (%)	4.97
TN (%)	0.98
TP (mg/kg)	300.25
TK (mg/kg)	8107.50
C: N: P	250:13:1

Table 3.44. Mean chemical properties and bacteria count of soils in the various mesocosms.

Mesocosm	Treatment	K (Mg/kg)	C: N: P	Bacteria count (10^5) (CFU/g)
FWAD	10% FWAD	1310.00	128:9:1	20
	20% FWAD	1694.17	167:10:1	10
	30% FWAD	1806.67	180:9:1	30
TW80	10% TW80	224.75	60:2:1	1
	20% TW80	151.58	65:2:1	3
	30% TW80	141.50	75:3:1	4
Controls	Control	236.00	60:2:1	102
	Acidified	243.83	58:4:1	2
	Crude oil	157.08	60:2:1	7

3.4.2 Soil Basal Respiration and CO_2 Effects on Hydrocarbons Degradation

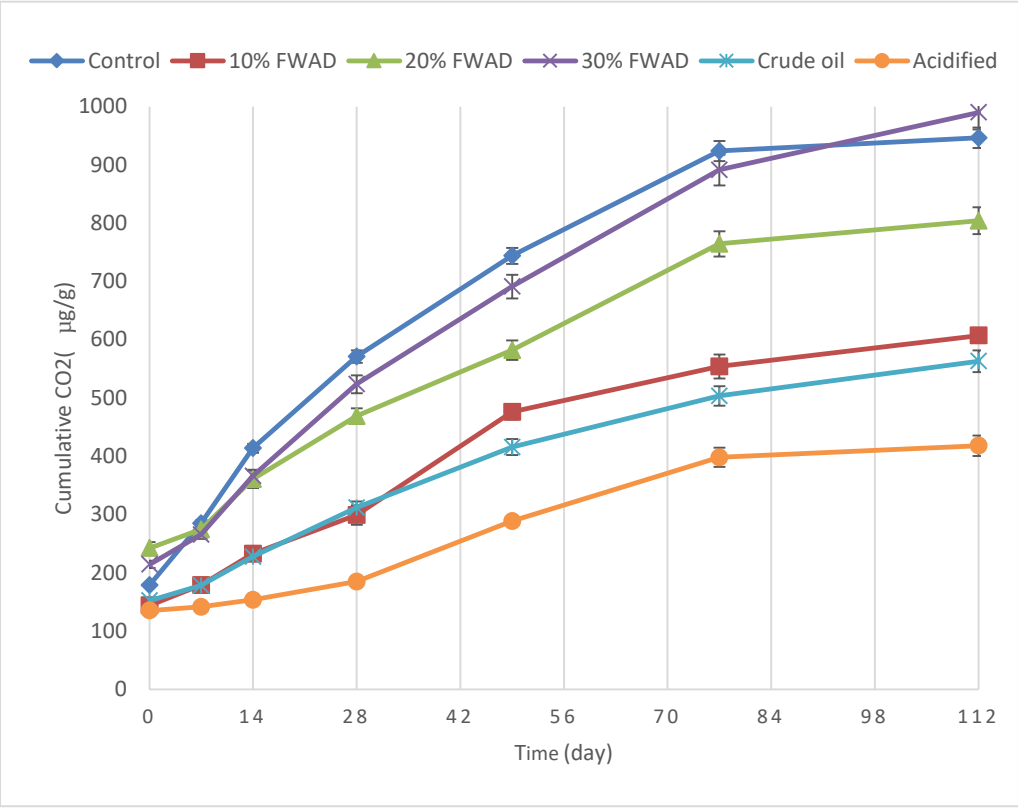
The pristine soil cumulative respiration rate was 946 µg CO_2/g soil by day 112. Acidification of the pristine soil reduced its cumulative respiration rate by 56% (Figure 3.1 a & b). The poor respiration can be attributed to the stress induced by the acidification on the soil microbial community. Similar conditions have been reported in several studies in the Niger Delta (Idzi et al., 2013; Abu & Dike, 2008). Kaur et al. (2005) reported that environmental stresses such as soil acidification can limit

microbial communities' performance. The acidified soil with crude oil (crude oil mesocosm) had a higher respiration than acidified pristine soil (Figure 3.1 a & b). The observed increment, which is statistically significant (Table 3.7), could be linked to the biodegradation of petroleum hydrocarbons by the surviving soil microbes.

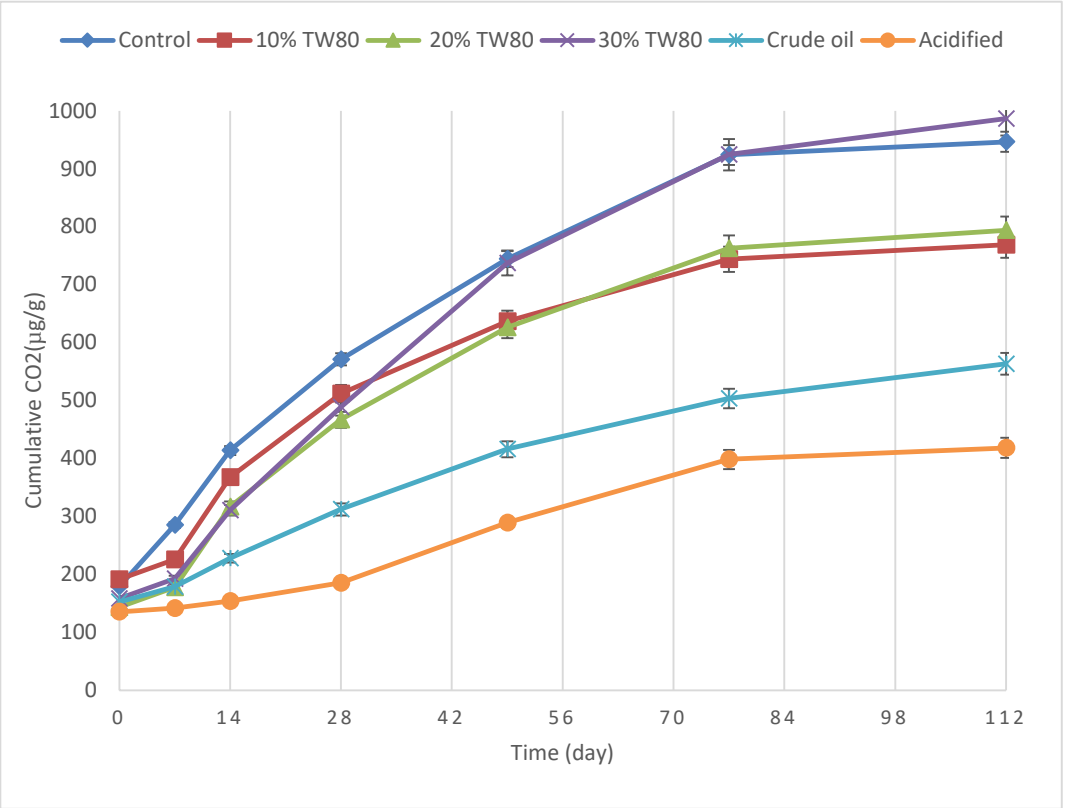
The application of digestate and Tween 80 surfactant to the spiked soils caused an increase in the soil respiration (Figure 3.1 a & b). This increase indicated that the TW80 and FWAD resuscitated the microbial communities by providing the required nutrients (shown in the C: N: P ratio) for improved microbial activities. The 30% FWAD and 30% TW80 mesocosms showed 44% and 43% increment in respiration rates respectively when compared to the crude oil mesocosms. Similar trends were also observed with the 20% and 10% FWAD and TW80 mesocosms respectively (Figure 3.1 a & b). The increments in CO_2 production rate can be linked to increased activities of hydrocarbon degrading microbial communities that used the hydrocarbons as carbon and energy sources under the thriving environment provided by the digestate (Figure 3.1 (a & b), and Figure 3.5). This finding agreed with the research of Sándor (2020) where the researcher posited that when the soil nutrients quality is improved, it stimulates the activities and stability of the soil microbial community. The level of biogenic CO_2 (CO_2 from biomass or organic matter) evolution is an indication of the organic level in the soil after effective remediation of organic contaminants from the soil and indicate the extent of subsequent plant germination, growth, and yield (St.Clair & Lynch, 2010; Henryson et al., 2018). This hypothesis corroborates the increased germination percentages recorded in the remediated samples of TW80 and FWAD mesocosms which showed higher cumulative CO_2 values when compared with the natural attenuation sample (Figure 3.2 and 3.6). Tween 80 surfactant can aid in changing the microbial cell surface hydrophobicity and improving the cell surface absorbing ability of the available hydrocarbons (Cheng et al., 2018). This can subsequently cause more petroleum hydrocarbons to be degraded, leading to an increment in the CO_2 generation rate (Cheng et al., 2018; Song et al., 2021).

There was an inverse relationship between the soil basal respiration and the hydrocarbon degradation for all the mesocosms spiked with crude oil (Table 3.5 & 3.6). Negative degradation rates were established for the FWAD and TW80 mesocosms which implied that the higher the degradation rate the more CO_2 produced and the

greater the reduction in soil PAHs and alkanes. The highest degradation rates were the 30% FWAD and 30% TW80 mesocosms (Table 3.5 and 3.6). A strong positive correlation ($p<0.01$) was established in all the mesocosms between the respiration rate and hydrocarbons degradation rates using Spearman correlation. At $p<0.01$, Spearman coefficient (ϒ) is considered significant when greater than absolute p but less than 1 (Table 3.7). This strong correlation demonstrated that the more the respiration rates the more the hydrocarbons that were degraded by the active microbial communities. These findings supported by Jiang et al. (2016) who stated that the higher the numbers of hydrocarbons degraders, the more the CO_2 is produced in mesocosms.



a. Soil cumulative respiration per day for FWAD mesocosms



b. Soil cumulative respiration per day for TW80 mesocosms

Figure 3.2. Cumulative CO_2 µg/g soil per day for various mesocosms.

Where number of observation (n) for triplicate samples is n = 3 ± standard deviation (SD).

Table 3.55. Soil basal respiration versus PAHs degradation models and degradation rates.

Mesocosms	Treatment	models	R^2	Degradation rates ($mgCO_2$/mg PAHs/day)
FWAD	10% FWAD	y = -0.2741x + 513.83	0.98	-0.27
	20% FWAD	y = -0.3282x + 688.55	0.97	-0.33
	30% FWAD	y = -0.445x + 819.33	0.97	-0.45
TW80	10% TW80	y = -0.3672x + 721.46	0.92	-0.37
	20% TW80	y = -0.412x + 726.83	0.97	-0.41
	30% TW80	y = -0.5206x + 867.58	0.98	-0.52
Control	Acidified HCs	y = -0.2433x + 469.52	0.90	-0.24

Where y = basal respiration rate and x = PAHs degradation rate.

Table 3.66. Soil basal respiration versus alkanes degradation models and degradation rates.

Mesocosms	Treatment	models	R^2	Degradation rates ($mgCO_2$/mg Alkanes/day)
FWAD	10% FWAD	$y=2203.5x^{-0.265}$	0.97	-0.55
	20% FWAD	$y=2280.8x^{-0.217}$	0.97	-0.64
	30% FWAD	$y=3741.4x^{-0.275}$	0.98	-0.82
TW80	10% TW80	$y=3129.7x^{-0.257}$	0.96	-0.37
	20% TW80	$y=4455x^{-0.317}$	0.96	-0.59
	30% TW80	$y=6130.5.4x^{-0.345}$	0.96	-0.68
Control	Acidified HCs	$y=2182.9x^{-0.266}$	0.99	-0.22

Where y = basal respiration rate and x = alkanes degradation rate.

Table 3.77. Correlation between basal respiration and TPH degradation.

Treatment	Spearman coefficient (ɤ)	Prob>|p|	correlation strength
Control	0.8104	<0.0001	++++++++
Acidified	0.87	<0.0001	+++++++++
Crude oil	0.8805	<0.0001	+++++++++
10% TW80	0.8395	<0.0001	++++++++
20% TW80	0.8732	<0.0001	+++++++++
30% TW80	0.8949	<0.0001	+++++++++
10% FWAD	0.8588	<0.0001	+++++++++
20% FWAD	0.8358	<0.0001	++++++++
30% FWAD	0.8327	<0.0001	++++++++

3.4.3 Soil Microbial Community Dynamics, and Environmental Stress

Phospholipid fatty acid (PLFA) analysis is a biochemical technology that provides an effective, non-culture-based method for fingerprinting soil microbial communities (Zhang et al., 2021). Phospholipids reflect the soil biomass and community structure of living microorganisms; the microbial cell membranes are rapidly degraded and metabolised after cell death (Trögl et al., 2016; Lewe et al., 2021). At the onset of the experiments the soil microbial community was composed of 42% Gram-positive bacteria, 30% Gram-negative bacteria, 15% Actinobacteria and 13% Fungi (Figure 3.5). Crude oil contamination and acidification induced a shift in the soil microbial community towards the Gram-positive and Gram-negative bacteria (Figure 3.5). The Gram-positive bacteria lack an outer membrane (which contains lipopolysaccharide) but are surrounded by layers of peptidoglycan many times thicker than is found in the Gram-negative bacteria (Figure 3.3), which increases its cell rigidity and bioaccessibility of the hydrocarbon contaminants (Silhavy et al., 2010). The application of FWAD and TW80 to the soils further induced the shift towards the Gram-positive bacteria (Figure 3.5 a & b). The observed dominance by the Gram positive and negative bacteria could be linked to the degradation of the long chain alkanes and PAHs (Lazaroaie, 2010; Zhang et al., 2021). Studies by Cipullo et al. (2019) correlated hydrocarbons degradation to PLFA-specific to the microbial communities that survived the environmental stress induced by the hydrocarbon contamination. The dominant microbial communities (Figure 3.5 a & b) survived and adjusted to the applied environmental stress from both the acidification and crude oil spike. Dunfield (2007) and Lewe et al. (2021) stated that resistant microbial groups can survive higher environmental stresses from contamination when compared to the less resistant groups.

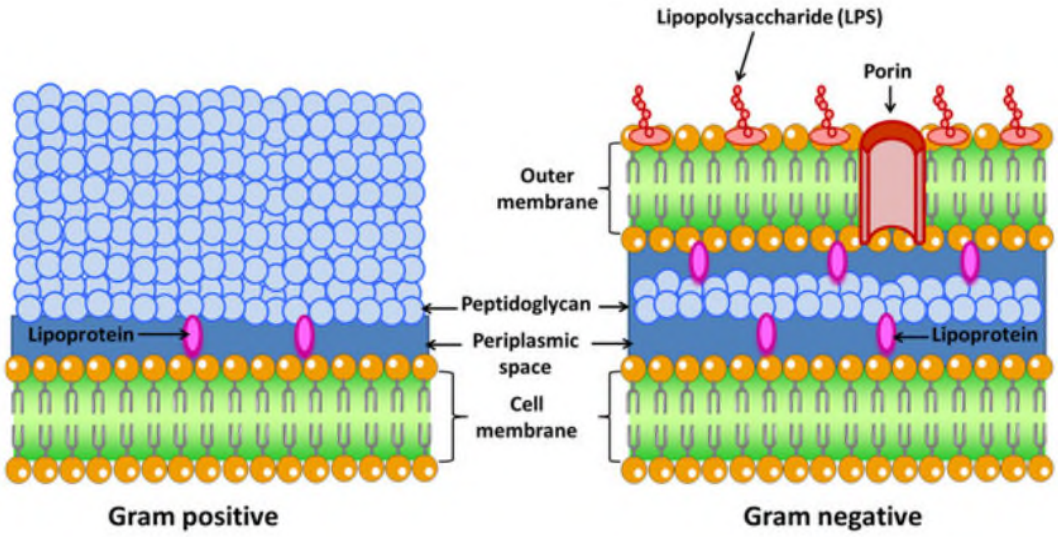


Figure 3.3. Gram positive and Gram-negative bacteria description, showing Gram-positive bacteria without outer membrane but are surrounded by layers of peptidoglycan many times thicker than is found in the Gram-negative bacteria.

Source: Jiménez-Jiménez et al. (2022).

The applied environmental stress was examined using trans/cis ratio (Figure 3.6) from PLFA of Figure 3.5 (a & b). The high environmental stresses observed at the day 30 of remediation dropped across the mesocosms at the day 112 of remediation. The conversion from cis to trans (Figure 3.4) of unsaturated fatty acids causes a reduction in microbial membranes fluidity, which counteracts against induced stress (Fischer et al., 2010; Kaur et al., 2005). However, the trans/cis ratio (Figure 3.5) for the acidified and crude oil mesocosms were greater than 10:1 (suggesting a nutrient pressure) and implying that the microorganisms in the acidified and crude oil mesocosms experienced nutrient starvation. This finding is in agreement with the research of Zhang et al. (2021) on the characteristics analysis of PLFA in sediments. The researchers concluded that at trans/cis ratio >10:1, the sediments bacteria were experiencing severe starvation due to the applied environmental stress. The reduction in environmental stress (trans/cis ratio) at the FWAD and TW80 mesocosms could be linked to the observed shift in the dominant Gram-positive microbial communities. This shift subsequently implied that there was a drop in soil toxicity and improvement in the

soil ecological quality (Frostegård et al., 2011; Zhang et al., 2021), which can be attributed to the FWAD and TW80. The trans/cis ratio have higher predicting efficiency for environmental stress when compared with percentage actinobacterial PLFA and G+/G- ratio (Trögl et al., 2016).

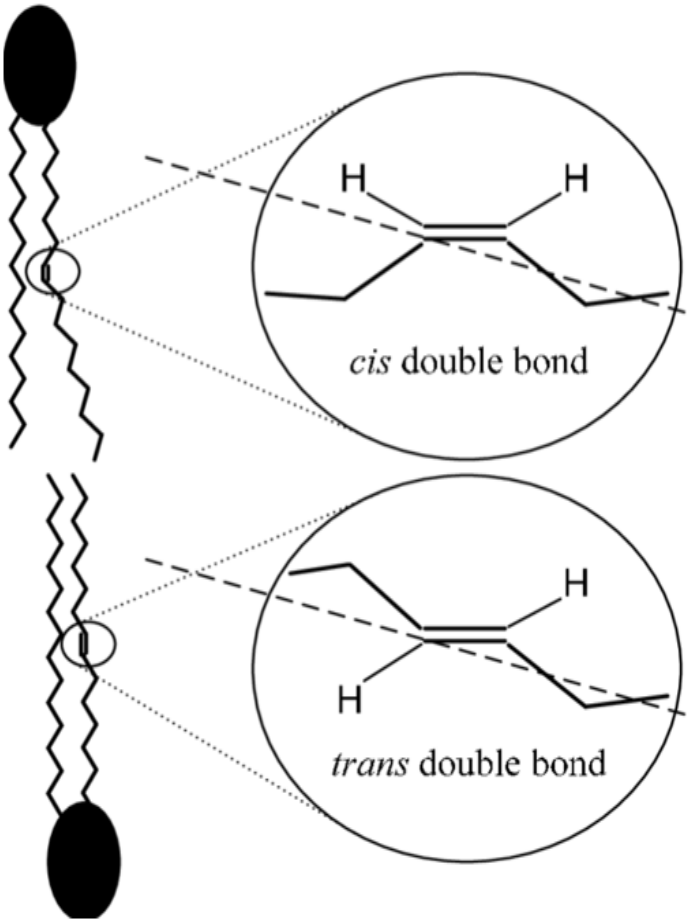
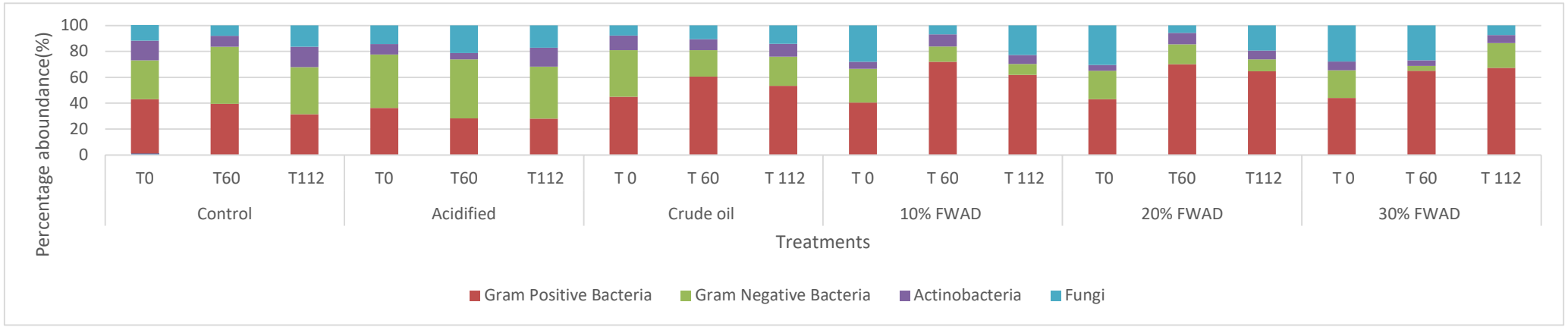
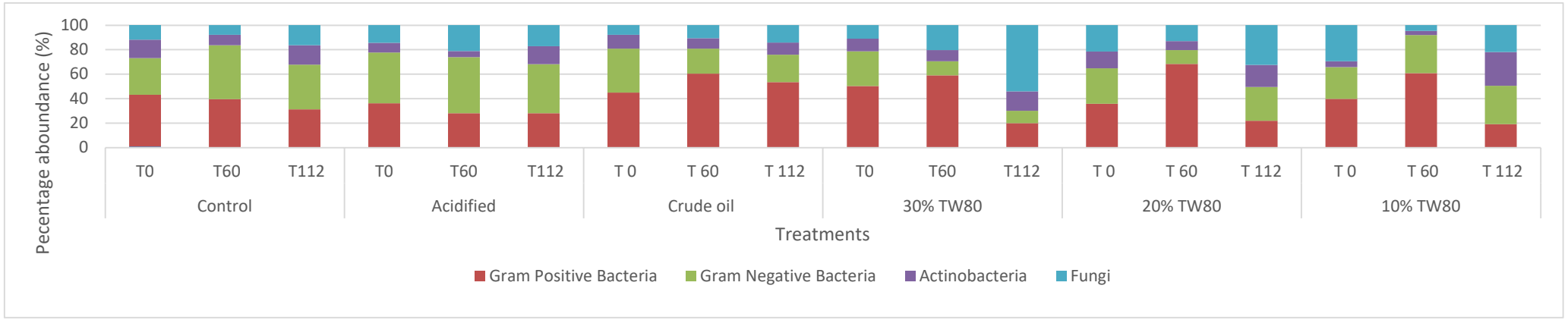


Figure 3.4. Cis and trans configuration of PLFA.

Source: Maia (2010).



a. FWAD mesocosms



b. Tween 80 mesocosms

Figure 3.5. PLFA for soil microbial communities' dynamics. Number of observation, $\boldsymbol{n = 3}$ (triplicate samples), T= Time (days), Measured at onset, middle, and end of experiments.

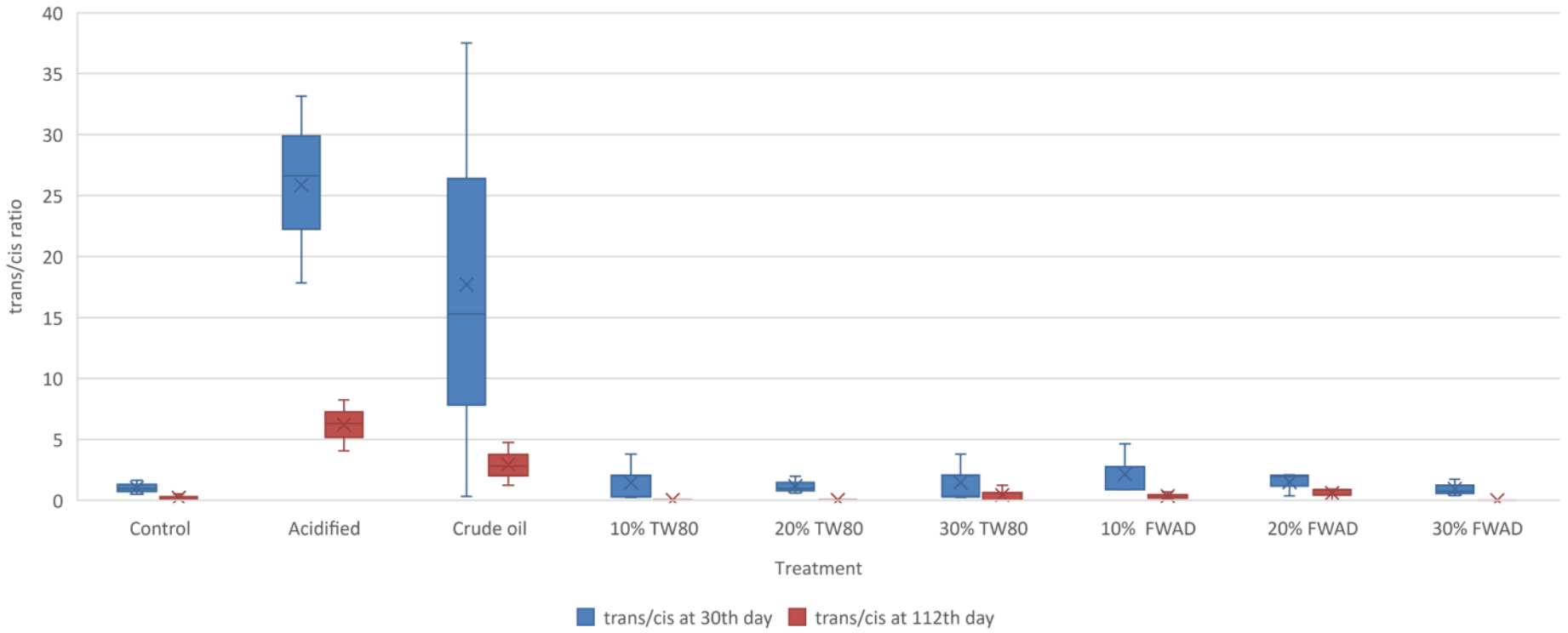


Figure 3.6. Cis/trans ratio for FWAD and Tween 80 mesocosms. Number of observation, $n = 3 \pm SD$, T= Time (days), Measured at day 30, and end of experiments.

3.4.4 Crude Oil Degradation

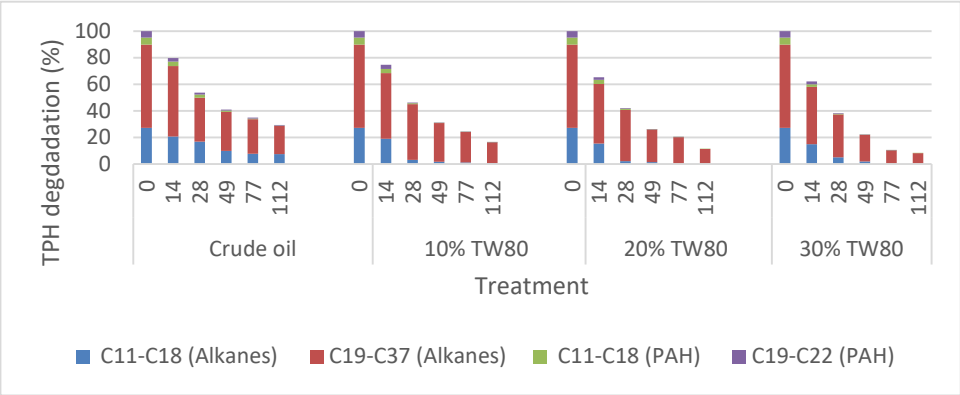
The degradation of medium molecular weight alkanes (which include undecane to octadecane) and PAHs (such as naphthalene to chrysene), and heavy molecular weight alkanes (that is pristane to heptatriacontane) and PAHs (including benzo[b]fluoranthene, to indeno(123) [cd]pyrene) were monitored from onset to day 112 (Figure 3.7). From Figure 3.7a, alkane and PAH degradation were greatest in soil mesocosms with 30% TW80 in comparison with 20% and 10% TW80, and this strategy could be suitable for pockets of small-scale (<100 m^2 area) contaminations in the Niger Delta wetlands. At day 49, 76% of alkanes and 98% of PAH in the 30% TW80 mesocosm were degraded (Figure 3.7 a). These results agreed with the findings of Feng et al. (2021) who found that surfactant increases dissolution of petroleum hydrocarbons in the aqueous phase aiding bioaccessibility of the contaminants to the microbes for degradation. Ceschia et al. (2014) demonstrated that surfactants in wet soils reduced the interfacial tension and attraction between the contaminants, soil particles and soil moisture. These actions make the contaminants more accessible to the bacteria, leading to mineralisation of the hydrocarbons. The availability of the contaminant in the TW 80 mesocosms decreased as the hydrocarbons were degraded (Figure 3.7 a & c). The medium molecular weight hydrocarbons (C11 – C18) with initial available hydrocarbons of 13,426 mg/kg degraded faster than the heavy molecular weight hydrocarbons (C19 – C37) (which was 2.3 times the initial quantity of the medium weight hydrocarbons).

The medium molecular weight alkanes showed more than 99% degradation by day 112 for the 30% TW80 mesocosms (Table 3.8 and Figure 3.7 a). The heavy molecular weight hydrocarbons degraded at a reduced rate with the heavier molecular weights showing lesser degradation and availability. At day 112, 85% degradation was achieved in the 30% TW80 mesocosms. Wartell et al. (2021) stated that medium weight alkanes are more easily degraded by microorganisms than heavy molecular weight alkanes. The degradation observed can be linked to the presence of TW80 which changed both soil surface and bacteria cell surface hydrophobicity by absorbing the surfactants molecules to the bacteria cell surface improving the transmembrane import of the hydrocarbons into the bacterial cell (Cheng et al., 2018). The degradation pattern observed with the alkanes was similar to that of the soil PAHs. The medium

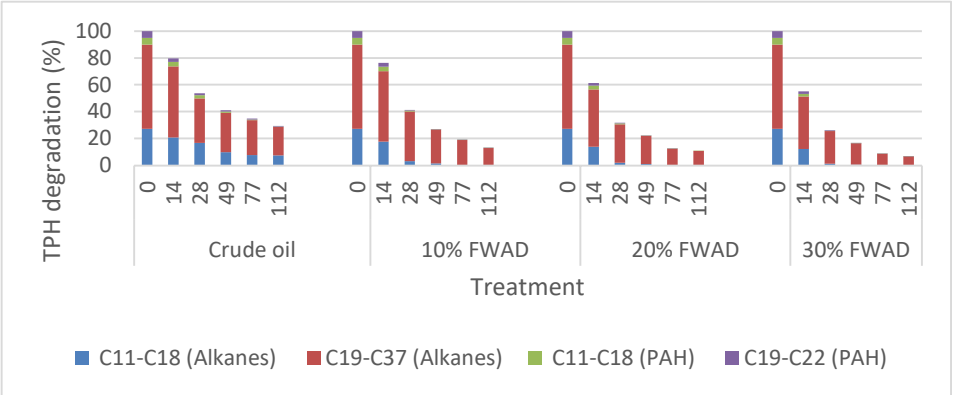
molecular weight compounds degraded on average 1.25 times faster than the heavier molecular weight PAHs for the TW80 mesocosms. At day 49, the medium and heavy molecular weight PAHs showed 99% and 96% hydrocarbon degradation respectively (Table 3.10 and 3.11; Figure 3.7a) for 30% TW80. At day 112, both medium and heavy molecular weight PAHs showed more than 99% degradation. 20% and 10% TW80 mesocosms showed similar degradation trends at reduced rates. Wang et al. (2018) researched on the surfactant enhanced remediation of PAHs in farmlands. The researchers concluded that surfactant weakens soil-contaminants sorption thereby enhancing PAHs desorption from soil.

The fastest hydrocarbons degradation rate for the FWAD mesocosms was in the 30% FWAD mesocosms (Figure 3.7 b). At day 49, 82% of alkanes and 98% of PAHs were degraded compared to the natural attenuation (crude oil mesocosms) which had less than 65% for both alkanes and PAHs. Gielnik et al. (2021) hypothesized that the metabolic potential of soils can be enriched by the bacteria contained in digestate which provide new hydrocarbon-degrading taxa and increase the *alkB* gene content. The *aIkB* genes encoding alkane hydroxylases belonging to monooxygenases family and are effective in alkanes degradation (Powell et al., 2006; Pawlett et al., 2013; Jin & Kim, 2017). The hypothesis corroborates with the high CFU/g count in the FWAD mesocosms (Table 3.4), which can be linked to the high metabolization of the petroleum hydrocarbons by the dominating Gram positive bacteria communities. At day 49, the medium molecular weight hydrocarbons were degraded faster than the heavy molecular weight hydrocarbons. The medium molecular weight hydrocarbons showed 99.5% degradation at day 112 for the 30% FWAD mesocosms while 20% and 10% FWAD mesocosms showed 99.3% and 99.2% degradation respectively (Table 3.9 and Figure 3.7 b). The increased degradation could be linked to the bioavailability of the medium weight hydrocarbons alongside increased microbial activities from nutrients supplied by FWAD (Table 3.4 and Figure 3.7 d). However, the degradation of the heavy molecular weight hydrocarbons decreased with increase in the hydrocarbons molecular weight. At day 112, the heavy molecular weight hydrocarbons achieved 88.6% degradation for 30% FWAD while 20% and 10% FWAD had 81% and 78% degradation respectively (Table 3.9 and Figure 3.7 b & d). The PAHs in the FWAD mesocosms degraded faster than the alkanes (Figure 3.7 b & d). At day 112, more

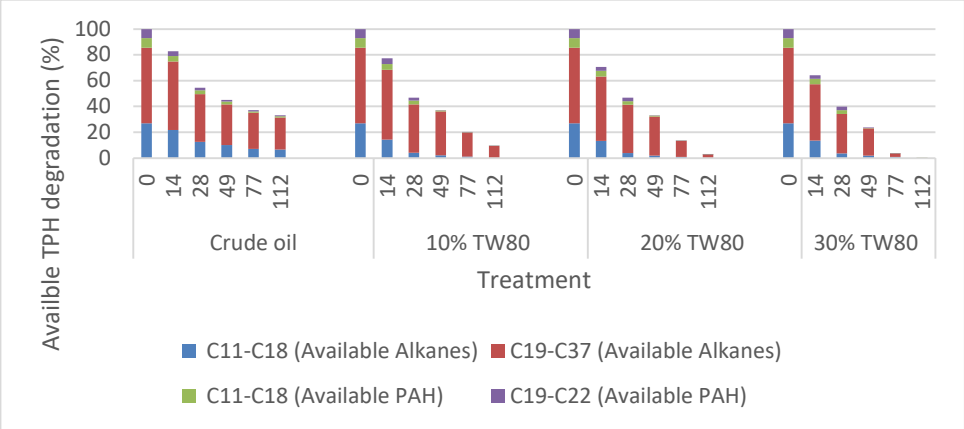
than 99% of the PAHs were degraded (Table 3.10 & 3.11). Iqbal et al. (2010) and Huang et al. (2021) stated the application of stimulants (such as FWAD) could cause an increase in surface area of the samples which could allow for increased microbial attacks on the PAHs and are suitable from small to large scale remediation.



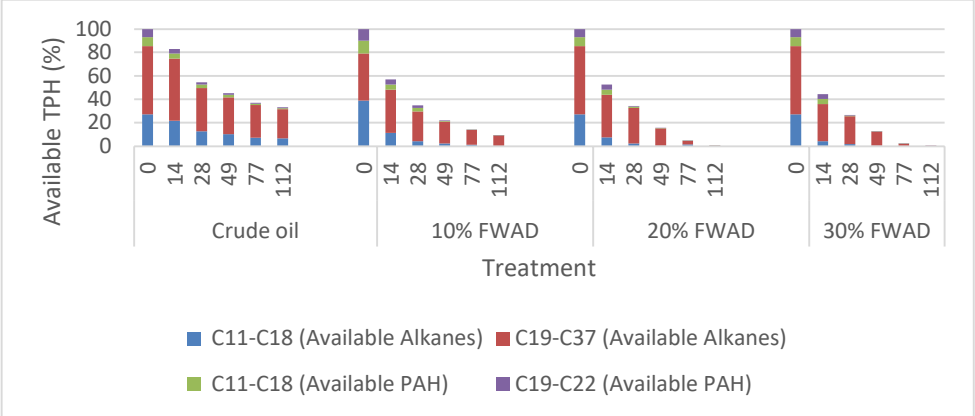
a. Total TPH degradation for TW80 mesocosms



b. Total TPH degradation for FWAD mesocosms



c. Available TPH for TW80 mesocosms



d. Available TPH for FWAD mesocosms

Figure 3.7. Total and available hydrocarbons degradation for FWAD and TW80 mesocosms. Observations were done in triplicates (n = 3), measured from onset to day 112.

Table 3.88. Mean alkanes concentrations and percentage degradations for C11 - C18 alkanes group.

Alkanes group	**Initial alkanes**		**Percentage degradation (%) at 112th day**					
C11-C18	concentration (mg/kg)	Crude oil	10% FWAD	20% FWAD	30% FWAD	10% TW80	20% TW80	30% TW80
Undecane	2339.0	93.7	99.7	99.8	99.8	99.7	99.7	99.8
Dodecane	1226.2	59.8	99.3	99.5	99.7	99.3	99.4	99.6
Tridecane	1710.5	74.8	99.7	99.7	99.8	99.6	99.7	99.8
Tetradecane	1658.9	68.5	99.6	99.6	99.7	99.5	99.5	99.6
Pentadecane	1669.2	67.4	99.5	99.5	99.5	99.3	99.4	99.6
Hexadecane	1597.1	62.8	99.2	99.2	99.3	98.2	99.2	99.3
Heptadecane	1648.6	75.1	98.7	98.7	99.3	98.7	98.2	98.8
Octadecane	1576.5	65.2	98.2	98.2	98.5	98.4	98.4	98.5
Percentage summary		70.9	99.2	99.3	99.5	99.1	99.2	99.4

Table 3.99. Mean alkanes concentrations and percentage degradations for C19 - C37 alkanes group.

Alkanes group	**Initial alkanes**		**Percentage degradation (%) at 112th day**					
C19-C37	concentration (mg/kg)	Crude oil	10% FWAD	20% FWAD	30% FWAD	10% TW80	20% TW80	30% TW80
Pristane	1100.0	80.0	90.8	91.7	96.4	91.8	94.9	96.3
Phytane	1151.8	82.5	91.0	92.2	96.4	90.4	95.5	96.6
Nonadecane	1296.9	75.8	91.9	94.6	96.1	91.4	93.8	96.7
Eicosane	1416.3	75.5	91.7	92.3	95.6	91.3	94.3	97.2
Henicosane	1714.9	81.2	93.0	93.6	95.3	89.5	94.5	96.5
Dosocane	1808.8	75.4	90.4	93.2	96.6	90.6	94.3	95.0
Trisocane	1798.9	79.4	90.2	92.4	96.1	90.0	94.4	95.0
Tetracosane	1775.7	77.7	89.8	92.6	93.1	89.3	95.0	96.5
Pentacosane	1743.9	79.3	89.6	93.0	94.5	89.1	94.2	95.9

Hexacosane	1890.3	77.3	88.7	93.2	94.2	86.5	94.4	94.9
Heptacosane	1070.9	69.7	71.8	87.8	90.5	68.9	90.5	92.3
Octacosane	1936.8	79.5	83.1	91.7	94.1	80.0	89.8	94.9
Nonacosane	1953.8	78.4	83.5	87.7	89.7	74.5	89.3	94.9
Triacontane	1612.5	67.7	74.8	79.2	87.3	67.3	86.2	93.8
Hentriacontane	1569.9	66.9	73.9	75.3	81.0	65.0	77.0	84.7
Dotriacontane	1313.9	45.2	60.6	62.6	81.8	55.4	61.6	81.0
Tritriacontane	1058.0	22.3	51.1	52.8	72.7	43.5	51.6	78.0
Tetratritacontane	1183.6	38.6	56.5	58.7	81.2	44.6	48.5	65.0
Pentatriacontane	1245.7	35.6	58.6	60.0	81.1	45.7	50.3	55.9
Hexatriacontane	1176.4	32.3	56.2	57.7	78.9	42.3	43.9	44.9
Heptatriacontane	1254.6	20.4	59.3	61.7	68.4	44.3	46.6	48.1
Percentage summary		63.8	77.9	81.1	88.6	72.9	80.0	85.4

Table 3.1010. Mean alkanes concentrations and percentage degradations for C10 - C18 PAHs group.

PAHs group	Initial PAHs		Percentage degradation (%) at 112th day					
C10 - C18	concentration (mg/kg)	Crude oil	10% FWAD	20% FWAD	30% FWAD	10% TW80	20% TW80	30% TW80
Naphthalene	224.1	91.4	99.9	99.9	99.9	99.9	99.9	99.9
Fluorene	458.5	91.6	99.1	99.9	99.9	99.9	99.9	99.9
Phenanthrene	931.1	95.5	99.9	99.9	99.9	99.9	99.9	99.9
Anthracene	297.2	96.7	99.7	99.9	99.9	99.3	99.1	99.9
Pyrene	67.9	87.9	98.5	99.9	99.9	97.0	98.1	99.9
Benz(a)anthracene	212.3	90.4	98.6	99.9	99.9	98.1	98.9	99.5
Chrysene	356.6	88.8	98.6	99.9	99.9	98.0	99.4	99.0
Percentage summary		91.8	99.2	99.9	99.9	98.9	99.1	99.7

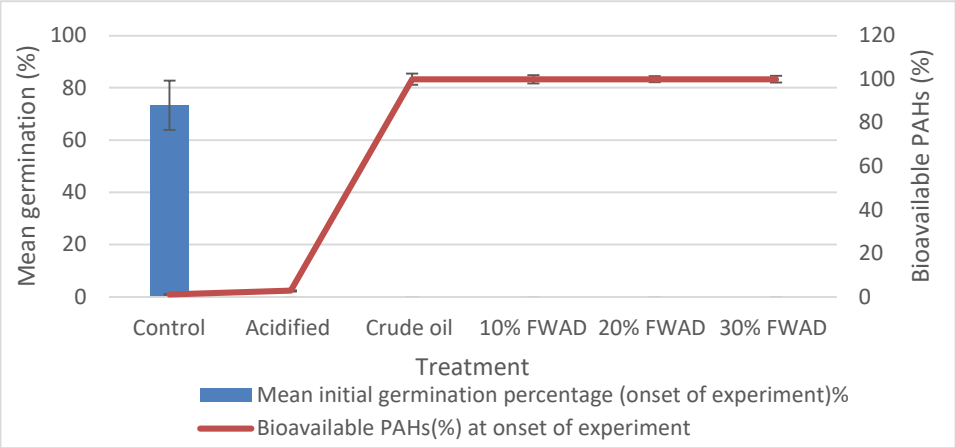
Table 3.1111. Mean alkanes concentrations and percentage degradations for C19 - C22 PAHs group.

PAHs group	**Initial PAHs**		**Percentage degradation (%) at 112th day**					
C19-C22	concentration (mg/kg)	Crude oil	10% FWAD	20% FWAD	30% FWAD	10% TW80	20% TW80	30% TW80
Benzo[b]fluoranthene	334.8	93.0	99.1	99.9	99.9	99.0	99.9	99.9
Benzo[k]fluoranthene	186	93.5	99.3	99.6	99.7	99.0	99.2	99.9
Benz(a)pyrene	176.7	87.3	99.1	99.7	99.9	98.4	99.4	99.9
Benzo(ghi)perylene	818.4	89.4	99.1	99.7	99.7	98.1	99.1	99.6
Benzo[b)triphenylene	604.5	89.8	99.3	99.4	99.7	98.0	99.1	99.7
Indeno(123)[cd]pyrene	344.1	86.5	98.5	99.2	99.8	97.9	98.9	99.1
Percentage summary		89.9	99.1	99.6	99.8	98.4	99.3	99.7

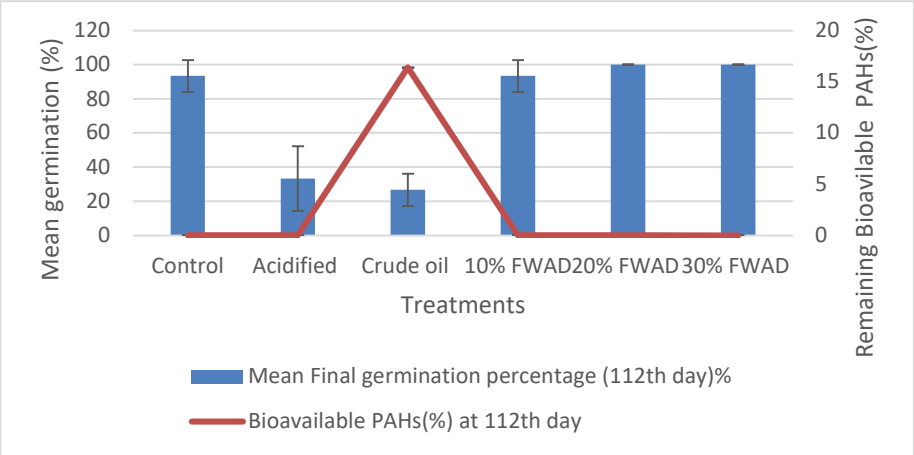
3.4.5 Remediation Endpoint

At the onset of the experiment, germination level was only recorded in the control (pristine soil). The acidification of the soil to pH of 5.8 and spiking with crude oil increased the soil toxicity, thus inhibiting the germination of the maize (Figure 3.8). This result corroborates the research of Sierra et al. (2003) on the response of plants to soil acidity. The researchers concluded that soil acidity severely affects plant root development and germination. The bioavailability of the PAHs and alkanes, from the spiked crude oil, could have increased the soil toxicity level leading to the no-germination recorded at the onset of the experiments (Figure 3.8). Bioavailability, the freely available fraction of contaminants in soil, is an important feature in risk assessment as it explains contaminants partitioning and degradation in environment (Cipullo et al., 2019). Seed germination and bioavailability assays have the potential to cost effectively evaluate the establishment of remediation endpoint (Wang et al., 2016; Cipullo et al., 2019; Khaled & Fawy, 2011).

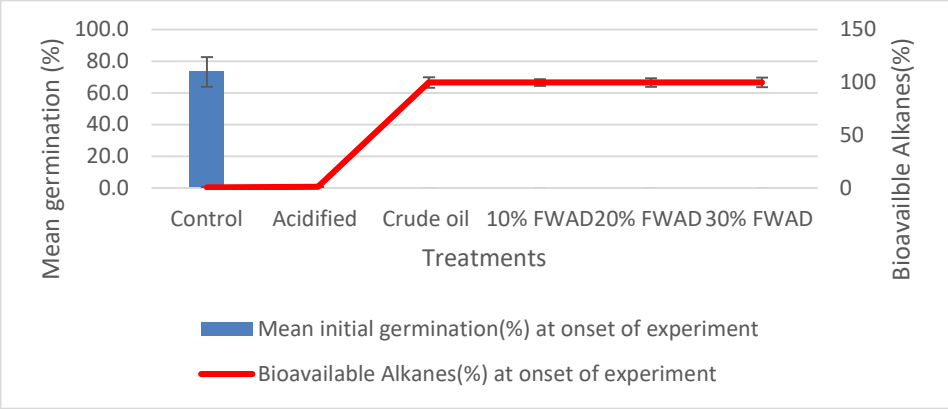
At day 112 of the experiment, the highest germination, 100% recorded was in the 30% and 20% FWAD mesocosms while the 30% TW80 and the control had 93% germination whereas the crude oil mesocosms had 26% germination (Figure 3.8 a, b, f & h). The greater than 90% germination rates in the various remediated mesocosms corroborates with the low bioavailable PAHs and alkanes in the mesocosms. This implied that the FWAD and TW80 treatments aided in the recovery of the soil contaminated with crude oil. These findings are in agreement with the ecotoxicity evaluation research of Nwankwegu et al. (2016) who stated that the response of plants to germination on polluted soils varies with ability of the nutrient used to remediate contaminants from the soil. On the whole, the extent of recovery shown by the soils through the maize germination and the low bioavailable alkanes and PAHs was an indication that remediation endpoint was achieved at day 112.



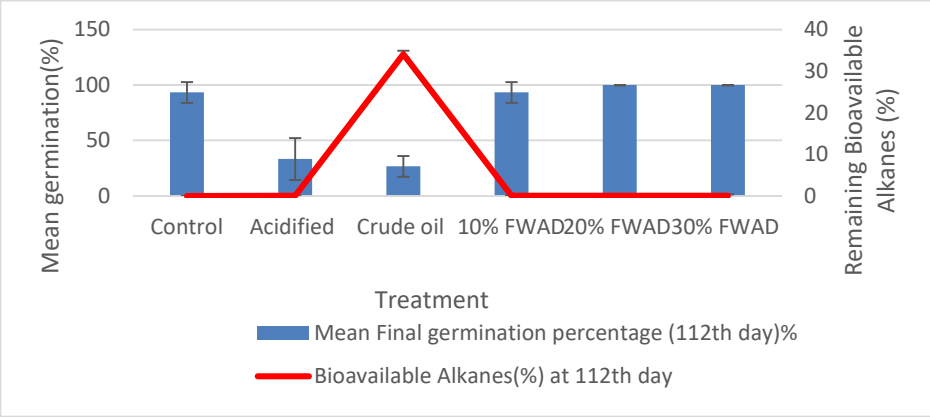
a. Mean initial germination and bioavailable PAHs for FWAD



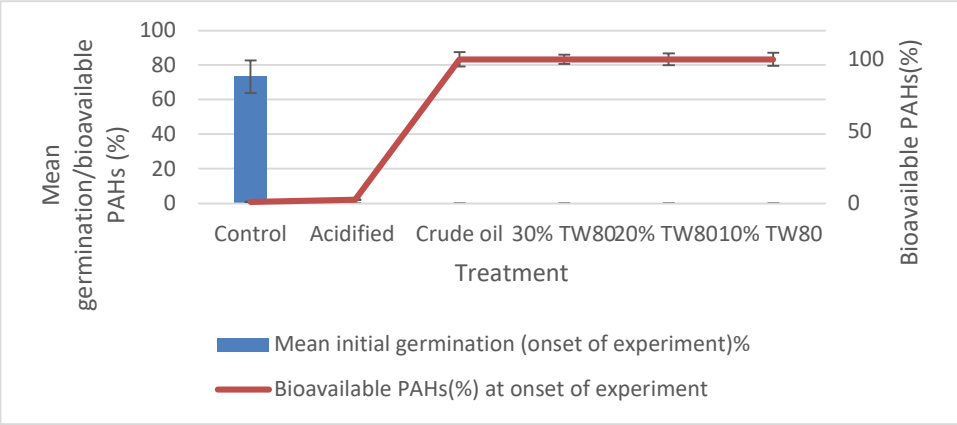
b. Mean final germination and bioavailable PAHs for FWAD



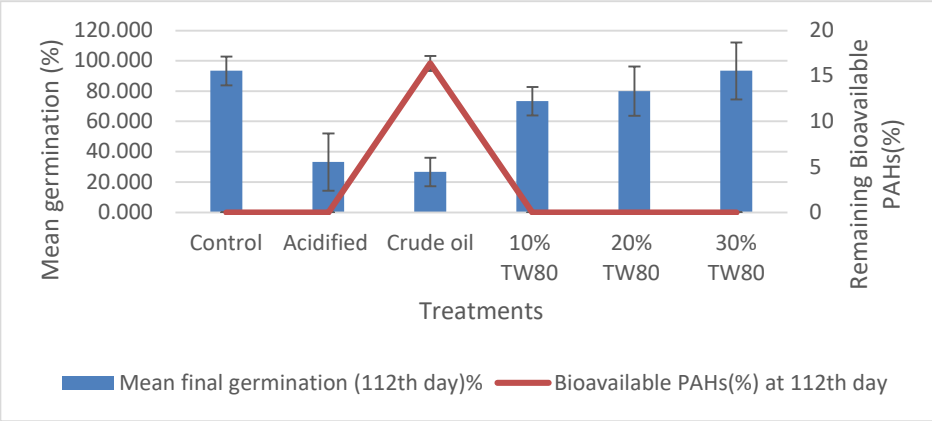
c. Mean initial germination and bioavailable alkanes for FWAD



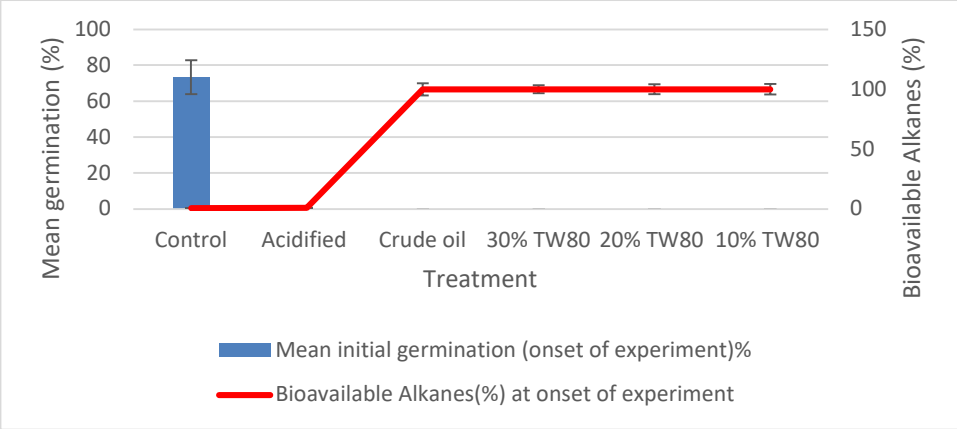
d. Mean final germination and bioavailable alkanes for FWAD



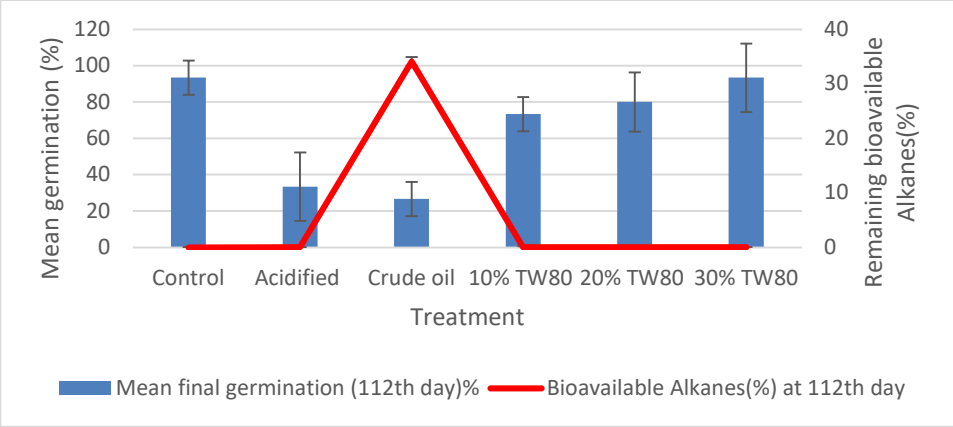
e. Mean initial germination and bioavailable PAHs for TW80



f. Mean final germination and bioavailable PAHs for TW80



g. Mean initial germination and bioavailable alkanes for TW80



h. Mean final germination and bioavailable alkanes for TW80

Figure 3.8. Mean germination of maize crops and bioavailable hydrocarbons for FWAD and TW80 mesocosms. Number of observation, $\mathbf{n = 3 \pm SD}$, Measured at onset and day 112.

3.5 Conclusion

This research had shown that in laboratory conditions acidified wetland soil contaminated by petroleum hydrocarbons can be effectively remediated using low carbon stimulants such as FWAD and TW80 surfactant. This research justified FWAD and TW80 surfactant to be suitable for large- and small-scale remediation respectively in the Niger Delta. The application of 30% FWAD, and 30% TW80 degraded the total petroleum hydrocarbon contaminants in the acidified wetlands by 90%, and 87% in 49 days respectively. At the end of remediation, when compared with the other mesocosms, the 30% FWAD was the least metabolically stressed mesocosm followed by 30% TW80. Therefore, 30% FWAD and 30% TW80 mesocosms showed the least environmental toxicity to the soil ecosystems and achieved remediation endpoints faster. This conclusion was confirmed by the high (> 90%) maize germination alongside no detectable bioavailable hydrocarbons recorded at the end of the experiment for these treatments. As hydrocarbons were mineralised by the microbes to generate the CO_2, the extent, and rate of hydrocarbons degradation was dependent on the CO_2 generation rate from the basal respiration of the soil microbial communities. The Gram-positive bacteria were the dominant microbial group in the FWAD and TW80 mesocosms. In summary, the multiple lines of evidence shown through spatiotemporal changes during the bioremediation strategies, including physical, chemical, and biological characteristics, defined the establishment of the remediation end point in wetlands.

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4 ASSESSING THE EFFICACY OF BIOAUGMENTATION STRATEGIES FOR REMEDIATING OIL IMPACTED WETLANDS

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4.1 Abstract

The performance of three, bioaugmentation strategies on oil impacted acidified wetlands was investigated using a series of mesocosms over 16 weeks. The first strategy consisted of increasing the indigenous microbial abundance to 9 x10^5 cells/g, the second was adding 5.58 x10^6 cells/g of *Pseudomonas aeruginosa* NCTC 10332, and the third, adding 6.3 x10^5 cells/g of *Bacillus subtilis* NCTC 3610. Hydrocarbon degradation, soil basal respiration and microbial community dynamics were monitored over 16 weeks. After 7 weeks, 80% of the initial 50,000 mg/kg total hydrocarbon contents, in mesocosms with boosted indigenous microbial abundance, was degraded. Degradation of the mid-distillate aliphatic fraction (ranging C12 and C22) was > 92%, while the aromatic fraction, was reduced by 93% of its original concentration. In contrast, neither the addition of *P. aeruginosa* nor *B. subtilis* enhanced the degradation of total hydrocarbons from the soil, by more than 86% at week 7 despite both strains being known hydrocarbons degraders. Gram-positive bacteria dominated the indigenous microbes and *B. subtilis* enriched mesocosms while the gram-negative bacteria dominated the *P. aeruginosa* mesocosms. A positive corelation was established between CO_2 generation and hydrocarbons degradation and was considered significant at $p \leq 0.05$.

Keywords: wetlands, microbes, bioaugmentation, hydrocarbons, degradation.

4.2 Introduction

Bioaugmentation is the addition of microorganisms to enhance the degradation of target contaminants in the environment (Oladipo & Ogunsona, 2020). However, the effectiveness of bioaugmentation depends on some prominent environmental factors

such as oxygen, nutrient availability, temperature, pH, and soil physicochemical characteristics (Ejechi & Ozochi, 2015; Jiang et al., 2016). Other factors which have direct influence on the rate of degradation of the contaminants are the contaminant bioavailability and concentration, presence of predators and interspecies competitions, and the presence of active indigenous microbes (Feng et al., 2021).

The success of a bioaugmentation strategy is dependent on the survival of the inoculated microbial consortia, the genetic content of the microbes, availability of micronutrients and energy source (Sam & Zabbey, 2018; Vogel, 1996). The main rationale of bioaugmentation is to augment the microbial biomass of the contaminants degraders, to increase the rate of biodegradation process, and sustain the microbial activity (Bajagain et al., 2018; Coulon et al., 2012). Bioaugmentation has shown some success in remediating soils and wetlands contaminated with hydrocarbons. The addition of specialised degraders such as *Enterococcus faecium* to soil contaminated with hydrocarbons was investigated by Feng et al., (2021). The researchers observed that after 30 days, about 44.5% of the original 1000 mg/kg total petroleum hydrocarbons (TPH) was degraded. Further studies also showed that in 90 days, 55% of 60,000mg/kg TPH in a wetland was degraded by bioaugmentation compared to the natural attenuation which had 35% degradation (Mohajeri et al., 2017). The limited degradation observed in the bioaugmentation could be linked to the recalcitrant nature of the heavy molecular weight hydrocarbons and lack of adequate micronutrients to stimulate microbial activities (Zabbey et al., 2017). However, studies where the indigenous microbial communities were augmented, have shown that the extent of hydrocarbons degradation was improved by two–fold compared to the non-indigenous microbes counterpart (Asquith et al., 2012; Chidinma et al., 2021). Pérez et al. (2017) investigated the remediation of hydrocarbons contaminated soils using indigenous microbial consortia. In their study, 120,000 mg/kg TPH was degraded to 20,000 mg/kg in 18 months compared to the control (contaminated soil without treatment) which had 65,000 mg/kg.

Limited successes have been recorded on the use of microbial consortia to remediate petroleum hydrocarbons in the Niger Delta acidified wetlands (Sam & Zabbey, 2018; Nkanang et al., 2018). Bioaddition can be outcompeted or cannibalized by the indigenous microbes, or the environmental conditions not optimised (Ataikiru &

Okerentugba, 2018; Orji et al., 2013). Feng et al. (2021a) and Orji et al. (2013) suggested that for efficient bioaugmentation of hydrocarbons contaminants from wetlands to be achieved, the contaminated soil should be enriched with the surviving indigenous microbial communities. Studies have found *Pseudomonas aeruginosa* and *Bacillus subtilis* to comprise some of the prominent microbial communities in the Niger Delta contaminated wetlands (Lazaroaie, 2010; Oladipo & Ogunsona, 2020; Udofia et al., 2018). Though several species of *Pseudomonas, Bacillus* and other indigenous microbes have been used in the remediation of hydrocarbons in the Niger Delta, their application on the acidified wetlands have not yet been well studied (Jacques et al. 2009; Udosen et al., 2001; Olukunle et al., 2015). Therefore, for enriched indigenous microbial consortia to be effective in the bioaugmentation of hydrocarbons in acidified wetlands, environmental factors, and extent of dominance of the inoculating microbes should be given prominent attention. The objective of this study was to assess the efficacy of indigenous bacterial consortia on hydrocarbons biodegradation in acidic wetlands.

4.3 Materials and Methods

4.3.1 Soil Mesocosms Conditions

Pristine soil, with no record of petroleum hydrocarbons contamination, was collected from a construction site in Cranfield University (52.0746 N, 0.6283E). Soil was collected from 0 to 30cm soil depth, the soil was air dried at room temperature, sieved through 2 mm aperture sieve (model: BS410 manufactured by: Endecotts, London, England), and stored for a week at 20 ^{o}C before use. Triplicate soil mesocosms were set up using 1 kg soil in 2.5 litre transparent polytetrafluoroethylene (PFTE) containers. Six different mesocosms conditions were evaluated as summarised in Table 4.1 and Figure 4.1. Soil characteristics and properties are described in Chapter 3.

Table 4.3Mesocosm experimental design for bioaugmentation strategies with all treatments in triplicates.

Mesocosm	Treatment	Abbreviation
Pseudomonas aeruginosa	Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg crude oil + *Pseudomonas Aeruginosa (NCTC 10332)* culture at 5.58 $x10^6$ cells/g	*P. aeruginosa*
Bacillus subtilis	Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg crude oil + *Bacillus subtilis* (*NCTC 3610*) culture at 6.3 $x10^5$ cells/g	*B. subtilis*
Indigenous Microbes	Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg crude oil + indigenous microbial culture at 9 $x10^5$ cells/g	IM
Controls	Pristine soil (freshly collected from field)	P
	Pristine soil acidified at pH 5. (using HNO_3)	A
	Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg crude oil	Oil

Figure 4.1. Experimental setup for bioaugmentation treatment of hydrocarbon contaminated acidified wetland soil.

The mesocosms except the pristine soils (control) were all acidified to pH of 5.8 using HNO_3 (PrimarPlus- trace analysis grade, supplied by Fisher Scientific UK, Limited). The acidified soil mesocosms were spiked with 60 ml of crude oil sweet (<0.5% sulfur) (SDS, Regulation 1907/2006/EC) to achieve a target hydrocarbons concentration of 50,000 mg/kg. The mesocosms were incubated at 28 °C and sandy soil was adopted to mimic the mean temperature and prominent soils of the Niger Delta (Fubara-Manuel et al., 2017).

Three controls (pristine soil, acidified soil (without HCs) and hydrocarbons spiked acidified soil with no treatment (natural attenuation)) were maintained throughout the experiment. The soil moisture content of 13.8% was increased by 21% to maintained saturation at the mesocosms. Deionised water was used to maintain moisture at saturation in all mesocosms. The deionised water was added at 7 days intervals to maintain the soil saturation.

4.3.2 Bioaugmentation Strategies

Pseudomonas aeruginosa and *Bacillus subtilis* cultures were purchased from the UK Health Security Agency. *Pseudomonas aeruginosa* was reconstituted with 0.5 ml of LB broth. 10 minutes was allowed for rehydration, then the mixture was mixed to avoid

aerosols. 125µl of the rehydrated culture was then dispatched in 50 ml sterile centrifuge tubes containing 10 ml LB broth, incubated for 24 hours at 150 rpm and 37 ^{o}C. A similar procedure was adopted for *Bacillus subtilis*. Indigenous microbes were cultured from the crude oil spiked acidified soil using a dilution series approach. The LB broth was used as a medium, and the indigenous microbes' pure culture incubated for 24 hours at 150 rpm for 37 ^{o}C. Microbial cell counting was performed using a microscope (Leica DM4000B, magnification 6x, Breckland, UK).

To confirm the genes of the microbes added to the mesocosms, DNA sequencing was carried out in the mesocosms. The DNeasy Power soil Pro kit (Qiagen, Maryland, USA) was used for DNA extraction following the manufacturer's protocol. DNA extracts were stored at -80 °C prior the quantification. The concentration and purity of DNA extracts was measured using the Jenway Genova Nano Spectrophotometer (Cole-Parmer, UK).

PCR was used to amplify the bacterial 16S rRNA gene for the dominant bacterial strains. Each PCR reaction (50 µL) contained: 1 µL template DNA, 0.25 µL DreamTaq DNA polymerase (5U/µL) (Thermo Fisher Scientific), universal primers (2 µL each) (Lane, 1991), 27F and 1492R (10 µM) (Sigma-Aldrich), 1 µL dNTP Mix (10 mM each) (Thermo Fisher Scientific), 5 µL DreamTaq buffer (10x) and 38.75 µL nuclease-free water. The thermal cycling program was: 94 °C/4 min; 94 °C/30 s, 50 °C/30 s and 72 °C/90 s (30 cycles), and 72 °C/10 min.

The 16S gene PCR amplicons were purified using the QIAquick PCR purification kit (Qiagen, Maryland, USA) according to the manufacturer's instructions. The concentration of the purified DNA amplicons was quantified using the Jenway Genova Nano spectrophotometer (Cole-Parmer, UK). Amplicons were checked by electrophoresis on 1%(w/v) E-gel Agarose Gel on the samples using Invitrogen E-Gel Power Snap Electrophoresis System (Supplier: Life Technology limited, Paisley, UK). The purified samples were sent to Eurofins Genomics, UK, for Sanger sequencing on an ABI 3730xl DNA Analyzer. Forward and reverse reads for each strain were assembled using DNA Baser Assembler 5.15.0. and compared to the EZBiocloud and the NCBI 16S rRNA identification databases.

4.3.3 Hydrocarbons Analysis

The hydrocarbons analysis was as described in chapter 3. The soil petroleum hydrocarbons were grouped into mid-ranged distillates and the heavy distillates fractions for both alkanes and PAHs.

4.3.4 Soil Respiration

Soil basal respiration was used to quantify the CO_2 generation rate (He & Xu, 2021), and was measured as described in chapter 3. Changes in conductivity (micro siemens) were used to quantify CO_2 release according to Ritz et al. (2006).

4.3.5 Soil Microbial Abundance

Soil microbial abundance was determined as described in chapter 3. The soil microbial count were determined using colony forming unit (CFU/g) plate counting technique by Varjani and Upasani (2019). This techniques indicates the viable microbes in the mesocosms (Oladipo & Ogunsona, 2020; Tiralerdpanich et al., 2018).

4.3.6 Soil Microbial Community Profiles and Dynamics

Soil microbial community profiles and dynamics were determined as described in chapter 3. Fatty acids were used as an indicator of the presence of groups of microbes (biomarkers). The biomarkers were categorized into Gram-positive bacteria, Gram-negative bacteria, actinobacteria and fungi according to Quideau et al. (2016) and Frostegård and Bååth (1993).

4.3.7 Ecotoxicity Assay

The ecotoxicity assay adopted as described in chapter 3. Germination response and days of germination after planting were recorded at the onset (day 0) and day 112 of the experiment (Figure 4.2).

Figure 4.2. Some germinations response from some mesocosms.

4.3.8 Statistical Analysis

Statistical analysis carried out using Microsoft Excel (Version 2111 Build 16.0.14701.20278). Standard error was used to evaluate the variability across germination assays and the applied environmental stress while standard deviation was used to ascertain the variability within sample measurements. Regression analysis was performed on soil respiration versus available hydrocarbons and p was considered significant if less than or equal to 0.05. Regression analysis was used to ascertain the corelation between the available hydrocarbons and soil basal respiration.

4.4 Results and Discussions

4.4.1. Influence of the Bioaugmentation Strategies on Soil Respiration and Hydrocarbon Degradation

Soil respiration is a reliable indicator of microbial activity (Sándor, 2020). From Figure 4.3, soil acidification reduced the basal respiration by 56% from the original 946µg CO_2 / g soil. Spiking of the acidified soil with crude oil caused a 26% increase in soil basal respiration at week 16 (Figure 4.3). The reduction in CO_2 generation rate could be linked to the stress induced on the microbial communities by the acidification and crude oil spillage (Song et al., 2021). In the bioaugmentation strategy, the mesocosms enriched with indigenous microbes showed the highest CO_2 generation rate with about 35% increment when compared with the natural attenuation (oil mesocosms) (Figure 4.3). The *B. subtilis* and *P. aeruginosa* mesocosms showed 14% and 28% increments respectively when compared with the natural attenuation. The increments in CO_2

production among the bioaugmentation strategies were all significant at $p \leq 0.05$ (Table 4.5). The CO_2 generated is linked to the availability of the hydrocarbons for microbial degradation (Nwankwo, 2014).

The relationship between the CO_2 generation and the available total petroleum hydrocarbons (TPH) in the bioaugmentation mesocosm showed that significant difference was established at $p \leq 0.05$ (Table 4.2 & 4.3). From the regression model (Table 4.4), the indigenous microbes-enriched mesocosms showed higher TPH degradation rate, which subsequently resulted into increased CO_2 generation rate. This finding agreed with those of Gielnik et al. (2021) and Randy et al. (2002) on respiration of microbes in contaminated soils.

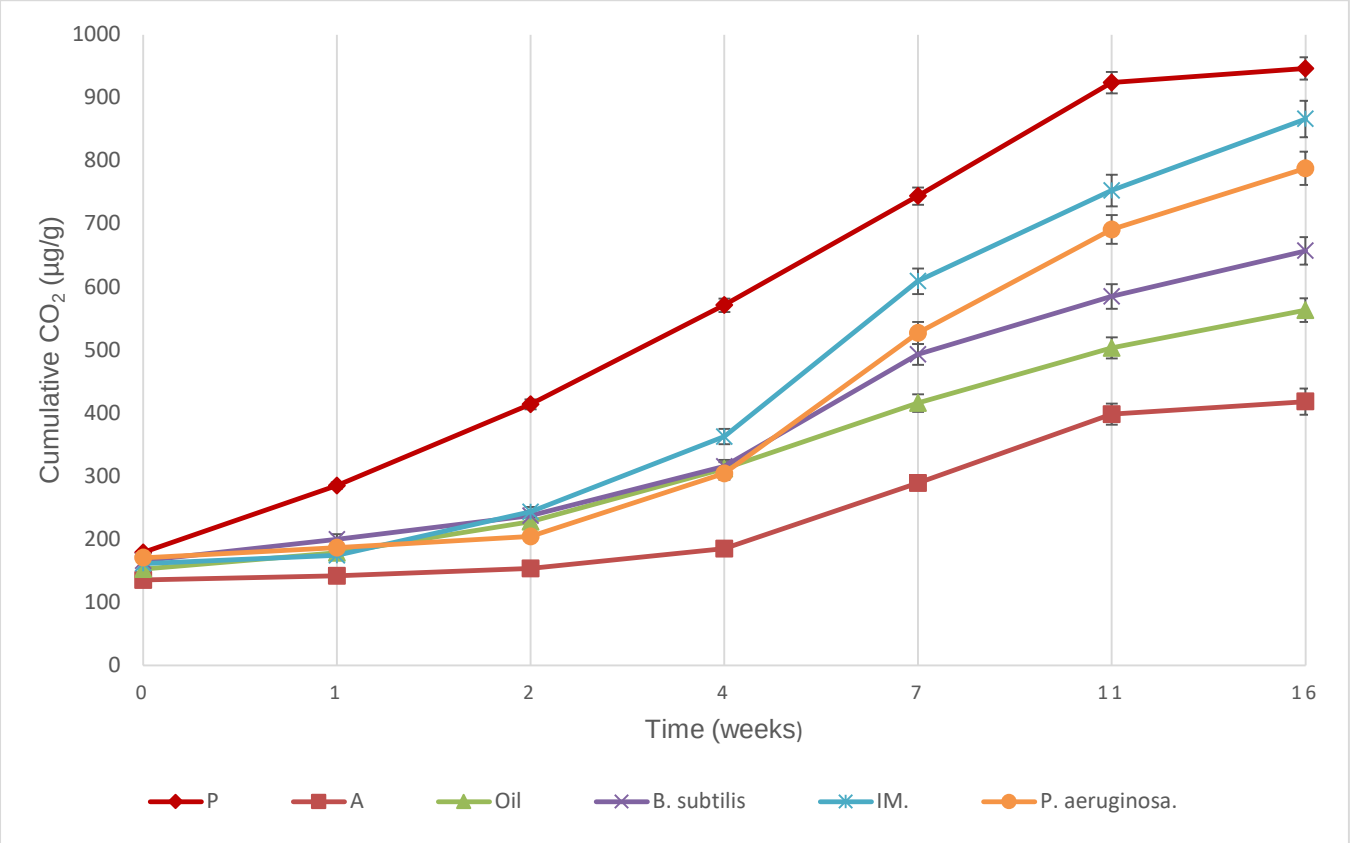


Figure 4.3. Cumulative CO_2 (µg/g soil) per week for bioaugmentation strategy.

Where P, A, Oil, *B. subtilis,* IM and *P. aeruginosa* mesocosms are as stated in Table 4.1, and number of observation (n) for triplicate samples is n = 3 ± standard deviation (SD).

Table 4.11. Regression summary for bioaugmentation strategies (respiration versus available hydrocarbons).

Mesocosms	**Multiple R**	**R Square**	**Adjusted R Square**
Oil	0.949	0.902	0.869
B. subtilis	0.948	0.900	0.865
IM	0.949	0.902	0.869
P. aeruginosa	0.957	0.915	0.872

Where Oil, *B. subtilis,* IM and *P. aeruginosa* mesocosms are as stated in Table 4.1.

Table 4.22. Regression ANOVA Table showing significance level for bioaugmentation strategies (respiration versus available hydrocarbons).

Mesocosms	**Item**	**df**	**SS**	**MS**	**F**	**Significance F**
Oil	Regression	1	67637.036	67637.036	27.74283	0.013338
	Residual	3	7314.0004	2438.0001		
	Total	4	74951.036			
B. subtilis	Regression	1	113388.55	113388.55	26.64134	0.014104
	Residual	3	12768.338	4256.1128		
	Total	4	126156.89			
IM	Regression	1	245912.37	245912.37	27.69156	0.013372
	Residual	3	26641.222	8880.4073		
	Total	4	272553.59			
P. aeruginosa	Regression	1	216319.91	216319.91	21.89389	0.018442
	Residual	3	29641.14	9880.3801		
	Total	4	245961.05			

Where Oil, *B. subtilis,* IM and *P. aeruginosa* mesocosms are as stated in Table 4.1.

Table 4.33. Regression model and p-value table for bioaugmentation strategies (respiration versus available hydrocarbons).

Mesocosms		**Standard Error**	**t Stat**	**P-value**	**Regression model**
Oil	Intercept	64.12125	11.2543	0.001504	Y= 721.6 -0.017x
	Available TPH	0.003162	-5.26715	0.013338	
B. subtilis	Intercept	50.23207	13.31428	0.000916	$Y_1 = 668.8 - 0.016x_1$
	Available TPH	0.003159	-5.16153	0.014104	
IM	Intercept	69.69604	12.32705	0.00115	$Y_2 = 859.1 - 0.025x_2$
	Available TPH	0.00471	-5.26228	0.013372	
P. aeruginosa.	Intercept	76.0858	10.40951	0.001892	$Y_3 = 792 - 0.023x_3$
	Available TPH	0.004921	-4.67909	0.018442	

Where Oil, *B. subtilis,* IM and *P. aeruginosa* mesocosms are as stated in Table 4.1, and Y = respiration rate, x = available hydrocarbons degradation rate.

Table 4.44. Regression summary table for bioaugmentation strategies respiration.

Mesocosm	Multiple R	R Square	Adjusted R Square	Standard Error	F	Significance F
B. subtilis versus IM	0.999	0.999	0.999	4.969	5104.575	6.04E-06
P. aeruginosa versus IM	0.999	0.998	0.997	12.690	1524.155	3.7E-05
IM versus Oil	0.997	0.995	0.993	21.496	586.7891	0.000154
P. aeruginosa versus *B. subtilis*	0.998	0.996	0.995	17.091	839.035	9.035E-05
B. subtilis versus oil	0.995	0.992	0.989	18.578	362.483	0.000316
P. aeruginosa versus oil	0.996	0.994	0.992	22.655	476.201	0.00021

Where Oil, *B. subtilis,* IM and *P. aeruginosa* mesocosms are as stated in Table 4.1.

After 7 weeks, the indigenous microbial abundance had increased by 91%, and 77% and 90% of the total alkanes and PAHs respectively were degraded (Table 4.6 and Figure 4.4). Further to this, the degradation rates of the alkanes and PAHs were 1.35 times faster than the bioaugmented mesocosm with the single species (*Pseudomonas aeruginosa,* and *Bacillus subtilis*) (Table 4.8 & 4.9, and Figure 4.4). Ferraro et al. (2021) stated that for inoculated microbes to establish and degrade contaminants, the enriched inoculum must be capable of tolerating and thriving on the concentration of the hydrocarbons in the samples. In the medium molecular weight hydrocarbons, undecane to hexadecane showed increased degradation with average degradation of about 97% degradation, while heptadecane to hexacosane showed 88% degradation

for the indigenous microbes mesocosms after week 7 (Table 4.8). The heavier molecular weight hydrocarbons such as heptacosane to heptatriacontane showed about 53% (Table 4.8). The reduced degradation observed for the heavier molecular weight alkanes compounds could be linked to the recalcitrant nature of these compounds and the limited soil nutrients. This result corroborates the soil C: N: P ratio of 60: 2: 1 (Table 3.4) which showed an unfavourable ratio among the principal nutrients. The optimal soil C: N: P ratio for effective biodegradation of contaminants by microbes has been recommended at 100:10:1(US EPA, 2002). The PAHs compounds from naphthalene to indeno(123)[cd]pyrene showed a degradation range of 99% - 81% after week 7 respectively (Table 4.9), whereas the indigenous microbes degraded the PAHs 1.2 times faster than the *aeruginosa* and *subtilis*. The results obtained from the mesocosms inoculated with indigenous microbial consortia agreed the conclusions of Feng et al. (2021). The researchers stated that reinoculation of soils with indigenous microbes enhanced bioremediation of organic contaminants. The increased degradation rates of the PAHs (Table 4.9), could be linked to the hydrocarbons bioavailability to the microbial cells and its subsequent transport to the cytochrome P450 enzyme which is effective for PAHs degradation (Ataikiru & Okerentugba, 2018). The cytochrome P450 monooxygenases detoxifies xenobiotics (such as PAHs), and by oxidizing PAHs to phenols it subsequently conjugates with sulfate, glucuronic acid, or glucose (Figure 2.5) (Peng et al. 2008; Tiralerdpanich et al., 2018).

Between the onset and week 7 of the experiment, 85% of the medium molecular weight available alkanes and PAHs were degraded (Figure 4.5). The higher molecular weight alkanes and PAH tended to persist as the remediation proceeds (Figure 4.4 and 4.5). The results are in agreement with the research of Xiao et al. (2014) on toxic levels of hydrocarbons in wetlands. Xiao et al. stated that reduced degradation was observed with the higher molecular weight hydrocarbons due to inaccessibility of the hydrocarbons for microbial degradation. The observed reduction in available hydrocarbons corroborates the degradation of the alkanes and PAHs within the period (Figure 4.5). After 7 weeks, the available hydrocarbons fractions were reduced by more than 90% in the indigenous microbes enriched mesocosms, which translated into a plateaued CO_2 generation rate at week 11 (Figure 4.4 and Table 4.4). From the

regression models, the degradation rate of TPH showed a positive correlation, significant at $p \leq 0.05$, with the CO_2 generation rate within the periods (Table 4.7). The research of Robichaud et al. (2019) confirmed that CO_2 is dependent on hydrocarbons degradation by microorganisms. Though the pattern of degradation of TPH and the corresponding evolution of CO_2 in the bioaugmentation strategies are similar, the indigenous microbes enriched mesocosms was 1.3 times better than the *aeruginosa* and *subtilis* enriched mesocosms from week 4 of the experiment (Figure 4.4 and Table 4.4).

Table 4.55. Microbial abundance for various mesocosms.

Mesocosm	**Bacteria count (× 10^5CFU/g) at onset**	**Bacteria count (× 10^5CFU/g) at day 30**	**Bacteria count (× 10^5CFU/g) at day 112**
P	96 ± 1.2	102 ± 0.82	111 ± 0.16
A	1 ± 0.88	2 ± 0.47	3 ± 0.41
Oil	5 ± 0.92	7 ± 0.47	29 ± 0.52
IM	28 ± 0.9	297 ± 1.3	169 ± 0.8
P. aeruginosa	31 ± 0.7	180 ± 2.1	98 ± 1.1
B. subtilis	13 ± 1.2	152 ± 0.9	92 ± 1.3

Where P, A, Oil, *B. subtilis,* IM and *P. aeruginosa* mesocosms are as stated in Table 4.1., and number of observation (n) for triplicate samples is n = 3 ± SD.

Table 4.66. Regression models for CO_2 and TPH degradation rates for bioaugmentation strategies.

Mesocosms	**Multiple R**	**R Square**	**Adjusted R Square**	**Standard Error**	**F**	**Significance F**	**model**
Oil	0.962	0.925	0.875	9.837	18.445	0.021	$r = 67.96+0.0013h -0.216t$
IM	0.981	0.962	0.938	3.228	38.899	0.0071	$r1 = 196.5 - 0.0085h_1 - 0.56t_1$
P. aeruginosa	0.992	0.984	0.972	2.056	90.169	0.0021	$r2 = 190.38- 0.0014h_2 -0.598t_2$
B. subtilis	0.992	0.985	0.976	3.071	104.908	0.00167	$r3 = 120.82 + 0.0003h_3 -0.369t_3$

Where Oil, *B. subtilis,* IM and *P. aeruginosa* mesocosms are as stated in Table 4.1., and r = respiration (μg/g), h = TPH (mg/kg), t= time (days).

Table 4.8. Alkanes degradation for bioaugmentation strategies.

Total alkanes group	**Treatment**	**Oil (%) degradation**		**IM (%) degradation**		***P. aeruginosa* (%) degradation**		***B. subtilis* (%) degradation**	
	Initial concentration (mg/kg)	week 7	week 16	week 7	week 16	week 7	week 16	week 7	week 16
Undecane	2339.0	87.1	93.7	97.1	99.3	97.8	99.1	98.1	99.0
Dodecane	1226.2	49.1	59.8	97.0	99.8	94.4	97.8	95.5	98.4
Tridecane	1710.5	60.1	74.8	96.9	99.7	94.1	98.2	95.2	97.9
Tetradecane	1658.9	57.9	68.5	97.1	99.4	88.0	97.6	88.6	97.1
Pentadecane	1669.2	64.6	67.4	97.0	99.3	86.6	97.1	81.6	95.8
Hexadecane	1597.1	54.8	62.8	97.4	98.0	80.8	94.5	77.1	93.9
Heptadecane	1648.6	67.0	75.1	94.9	97.3	79.5	94.6	76.8	93.9
Octadecane	1576.5	55.7	65.2	90.4	95.3	76.7	93.7	76.0	93.1
Pristane	1100.0	79.2	80.0	88.7	97.3	85.5	96.5	90.5	97.1
Phytane	1151.8	77.6	82.5	89.0	97.1	88.7	96.1	89.6	96.6

Nonadecane	1296.9	72.1	75.8	90.0	96.2	88.9	96.0	89.8	97.1
Eicosane	1416.3	71.6	75.5	88.9	95.6	89.0	95.0	87.4	96.1
Henicosane	1714.9	72.7	81.2	90.7	96.1	86.0	93.0	88.4	96.4
Dosocane	1808.8	60.6	75.4	90.2	96.0	84.0	92.4	84.7	89.8
Trisocane	1798.9	61.0	79.4	85.7	95.5	88.1	92.0	84.0	89.4
Tetracosane	1775.7	55.8	77.7	84.9	94.8	87.4	91.2	82.3	86.8
Pentacosane	1743.9	64.3	79.3	83.3	93.3	82.4	90.3	80.3	85.9
Hexacosane	1890.3	63.5	77.3	79.8	93.3	79.2	85.6	77.9	80.9
Heptacosane	1070.9	52.5	69.7	57.4	87.6	57.5	73.5	55.0	75.2
Octacosane	1936.8	59.2	79.5	76.3	91.6	75.9	84.8	75.3	85.1
Nonacosane	1953.8	53.7	78.4	73.7	90.8	69.2	83.8	67.7	79.4
Triacontane	1612.5	41.5	67.7	66.5	86.8	62.9	81.3	60.1	73.8
Hentriacontane	1569.9	40.9	66.9	60.4	85.5	58.5	72.3	54.9	68.2
Dotriacontane	1313.9	29.2	45.2	47.5	82.3	46.9	65.9	42.7	61.6

Tritriacontane	1058.0	19.1	22.3	34.5	77.6	33.7	47.9	26.1	50.9
Tetratritacontane	1183.6	36.6	38.6	42.4	72.7	40.9	58.9	49.2	50.7
Pentatriacontane	1245.7	34.0	35.6	43.9	65.1	41.5	53.9	37.4	49.8
Hexatriacontane	1176.4	29.2	32.3	37.6	57.6	36.0	50.6	31.5	44.3
Heptatriacontane	1254.6	17.8	20.4	39.7	58.0	38.9	51.1	37.7	50.0

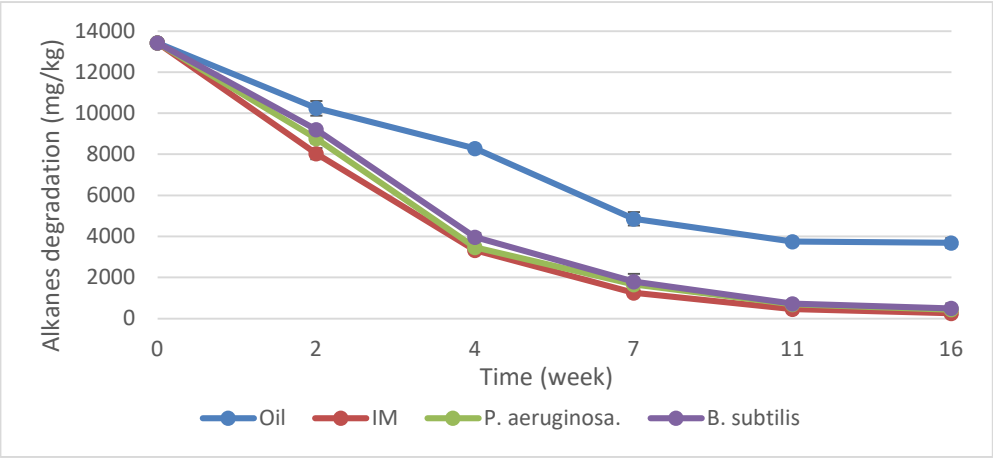
Where P, A, Oil, *B. subtilis,* IM and *P. aeruginosa* mesocosms are as stated in Table 4.1

Table 4.9. PAHs degradation for bioaugmentation strategies.

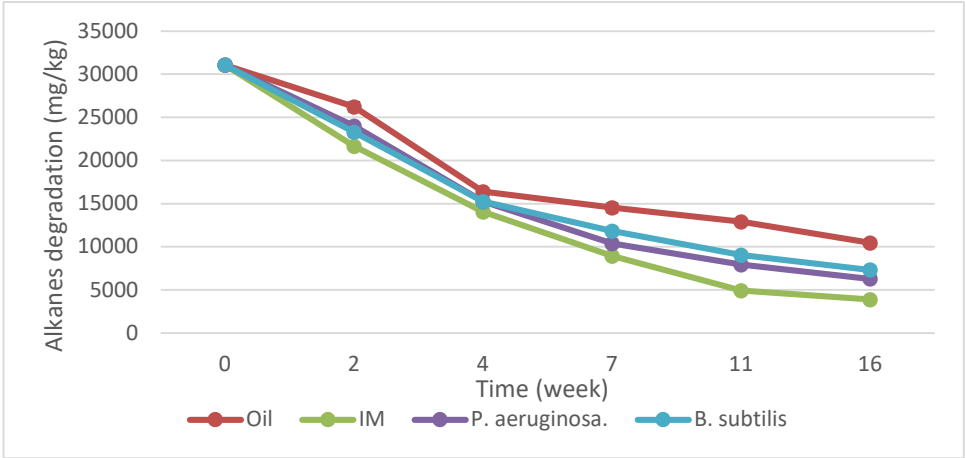
Total PAH group	**Treatment**	**Oil (%) degradation**		**IM (%) degradation**		***P. aeruginosa* (%) degradation**		***B. subtilis* (%) degradation**	
	Crude oil	week 7	week 16	week 7	week 16	week 7	week 16	week 7	week 16
Naphthalene	224.1	79.3	91.4	98.9	99.4	80.3	96.4	86.9	95.3
Fluorene	458.5	84.2	91.6	98.3	99.2	93.2	98.0	91.8	97.6
Phenanthrene	931.1	91.2	95.5	97.7	99.3	91.4	98.7	90.5	98.0
Anthracene	297.2	71.8	96.7	93.4	98.3	87.0	97.0	82.8	94.2
Pyrene	67.9	66.9	87.9	86.0	93.8	68.6	88.0	64.8	86.2
Benz(a)anthracene	212.3	60.1	90.4	83.3	94.7	62.7	90.6	62.1	89.2
Chrysene	356.6	72.5	88.8	81.0	95.6	74.8	92.9	72.2	89.7
Benzo[b]fluoranthene	334.8	92.2	93.0	97.2	99.0	96.2	98.3	95.9	98.3
Benzo[k]fluoranthene	186.0	77.7	93.5	95.1	97.9	92.9	95.8	84.4	93.0
Benz(a)pyrene	176.7	72.7	87.3	94.2	97.7	87.6	93.5	80.7	90.6
Benzo(ghi)perylene	818.4	88.1	89.4	97.7	98.4	94.9	96.7	93.9	96.4

Benzo[b]triphenylene	604.5	81.9	89.8	93.5	97.5	91.3	95.3	89.7	94.7
Indeno(123)[cd]pyrene	344.1	74.0	86.5	86.6	94.2	80.1	90.6	80.6	87.8

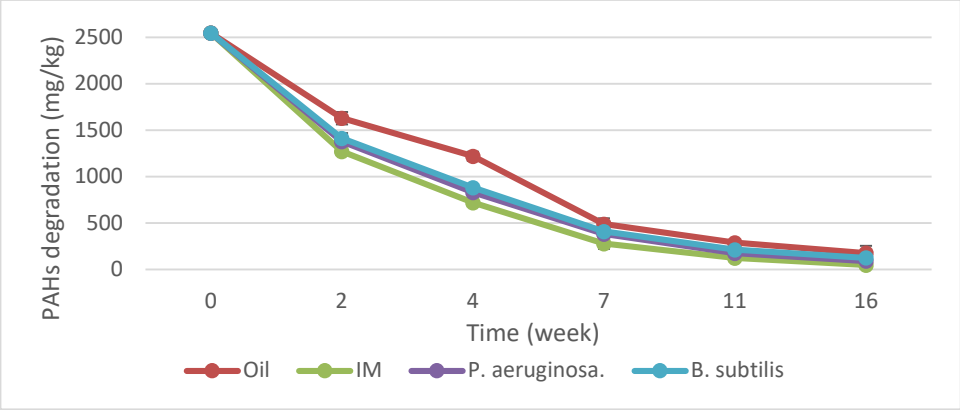
Where P, A, Oil, *B. subtilis,* IM and *P. aeruginosa* mesocosms are as stated in Table 4.1



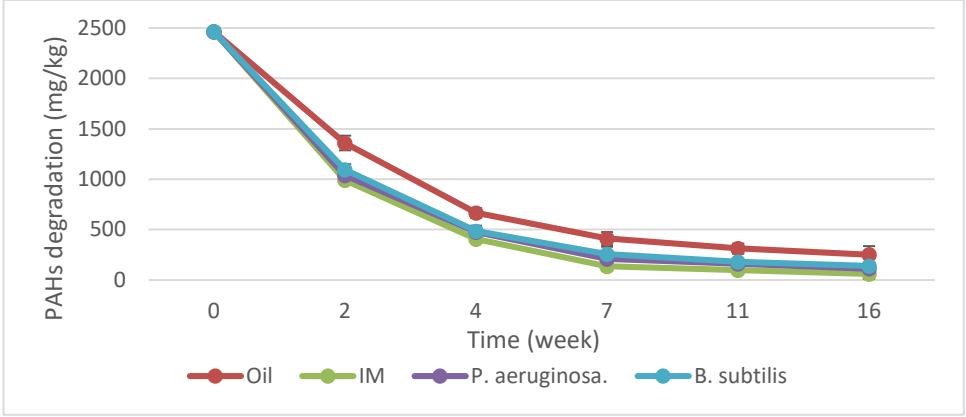
a. Medium molecular weight alkanes (C11 – C18) degradation



b. High molecular weight alkanes (C19 -C37) degradation

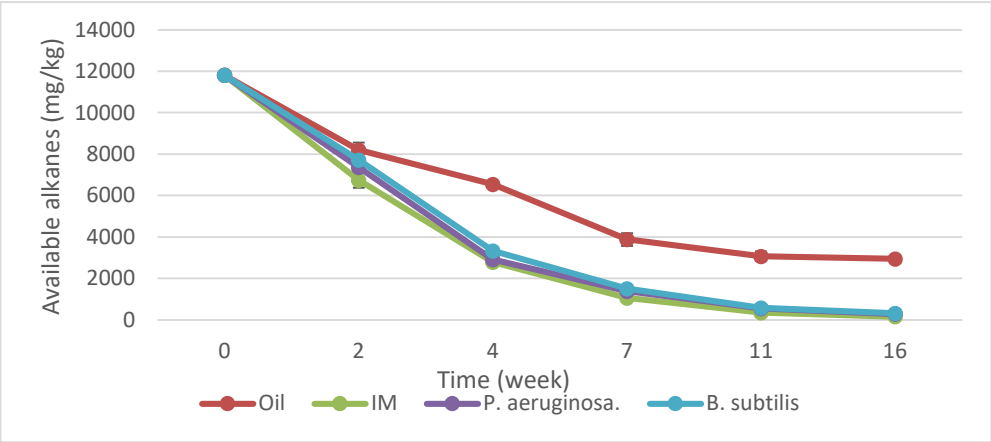


c. Medium molecular weight PAH (C10 -C18) degradation

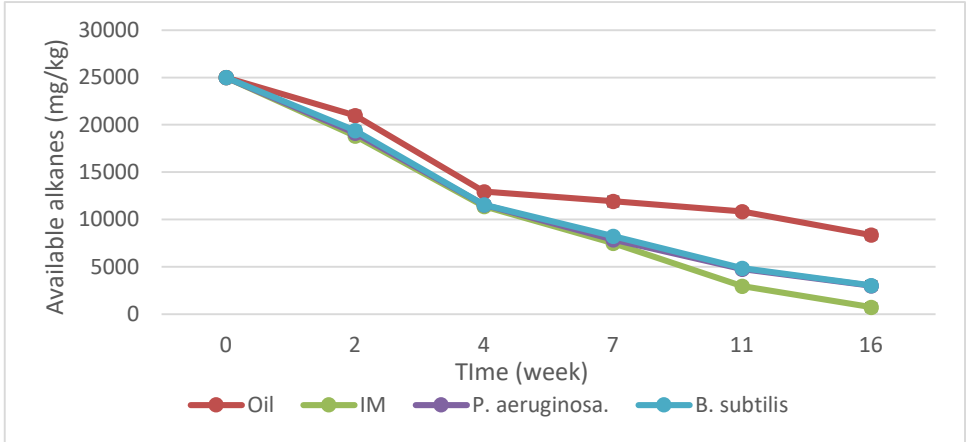


d. High molecular weight PAHs (C19 – C22) degradation

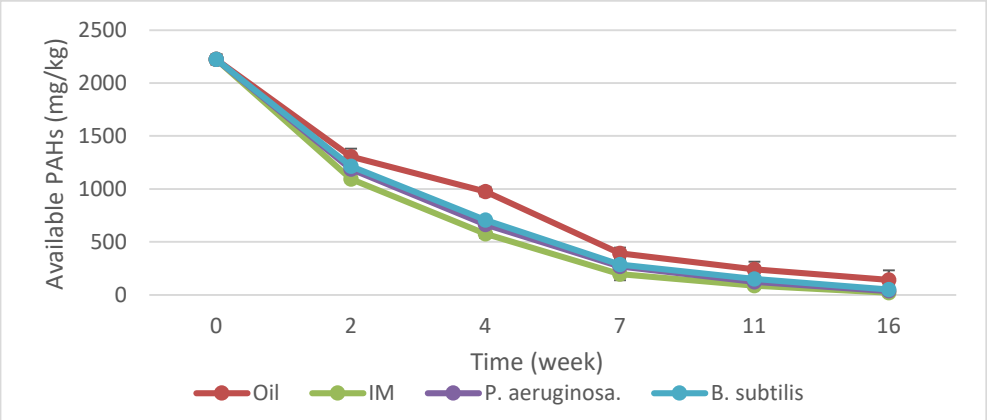
Figure 4.4. Alkanes and PAHs degradation. Number of observation (n) for triplicate samples is n = 3 ± SD. Where P, A, Oil, *B. subtilis,* IM and *P. aeruginosa* mesocosms are as stated in Table 4.1



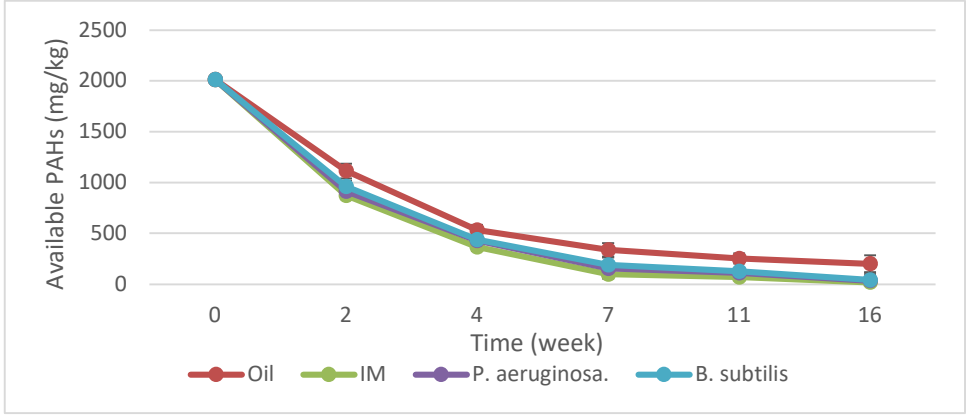
a. Medium molecular weight available alkanes (C11 – C18).



b. High molecular weight available alkanes (C19 – C37).



c. Medium molecular weight available PAHs (C10 – C18).



d. High molecular weight available PAHs (C19 – C22).

Figure 4.**5**. Available Alkanes and PAHs. Number of observation (n) for triplicate samples is n = 3 ± SD**.** Where P, A, Oil, *B. subtilis,* IM and *P. aeruginosa* mesocosms are as stated in Table 4.1.

4.4.2. Identification of the Dominant Indigenous Genera and Soil Microbial Dynamics

The dominant microbial colonies in the bioaugmentation mesocosms were identified to ascertain the species of microorganisms leading the degradation of the hydrocarbons in the various mesocosms (Ossai et al., 2022). Dominant colonies from the mesocosms enriched with indigenous microbes (from the experimental soil) were *Bacillus toyonensis* BCT-7112(T) having 99.22% with EZBiocloud and 99.64% with NCBI blast. For the *P. aeruginosa* and *B. subtilis* mesocosms, the identified dominant species were as expected, *Pseudomonas aeruginosa* JMC 5962, and *Bacillus subtilis* A29 having 99.3% and 98.6% with EZBioclud and 99% with NCBI blast respectively.

At the onset of the experiment, the pristine soil was composed of 42% Gram-positive bacteria, 30% Gram-negative bacteria, 15% actinobacteria and 13% fungi (Figure 4.6). The acidification and crude oil spillage shifted the microbial dominance to the Gram-positive bacteria (45% by composition). Enrichment of the mesocosms with the indigenous microbes further tilted the microbial dominance to the Gram-positive bacteria (58% by composition) while *P. aeruginosa* and *B. subtilis* mesocosms had 49% and 46% Gram-positive bacteria respectively (Figure 4.6). However, at day 60 the Gram-positive bacteria continued to dominate the indigenous microbes and *B. subtilis* mesocosms with about 77% and 78% respectively while the *P. aeruginosa* mesocosms shifted to Gram-negative bacteria (about 70% by composition) (Figure 4.6). Wang et al. (2016) posited that increment in bacterial PLFA should corelate with the rate of hydrocarbons degradation. Extracellular enzymes such as oxidases, dehydrogenases, and hydrolases are produced by these bacteria, which catalyse the breakdown of hydrocarbons by oxidation, reduction, or hydrolysis processes (Teng et al., 2020; Peng et al., 2019).

From the established trends in the bioaugmentation of microbial community dynamics and hydrocarbon degradation (Figure 4.6 and 4.4), It was observed that petroleum hydrocarbon degradation activities in the indigenous microbes and

B. subtilis mesocosms were led by the Gram-positive bacteria while the Gram-negative bacteria led the degradation of hydrocarbons in *P. aeruginosa* mesocosms. This observation agreed with the research of Dunfield (2007) and Lewe et al. (2021). Where the researchers agreed that in most acidified soils, the Gram-positive and Gram-negative bacteria dominate contaminants degradation, as opposed to the contribution of actinobacteria and fungi.

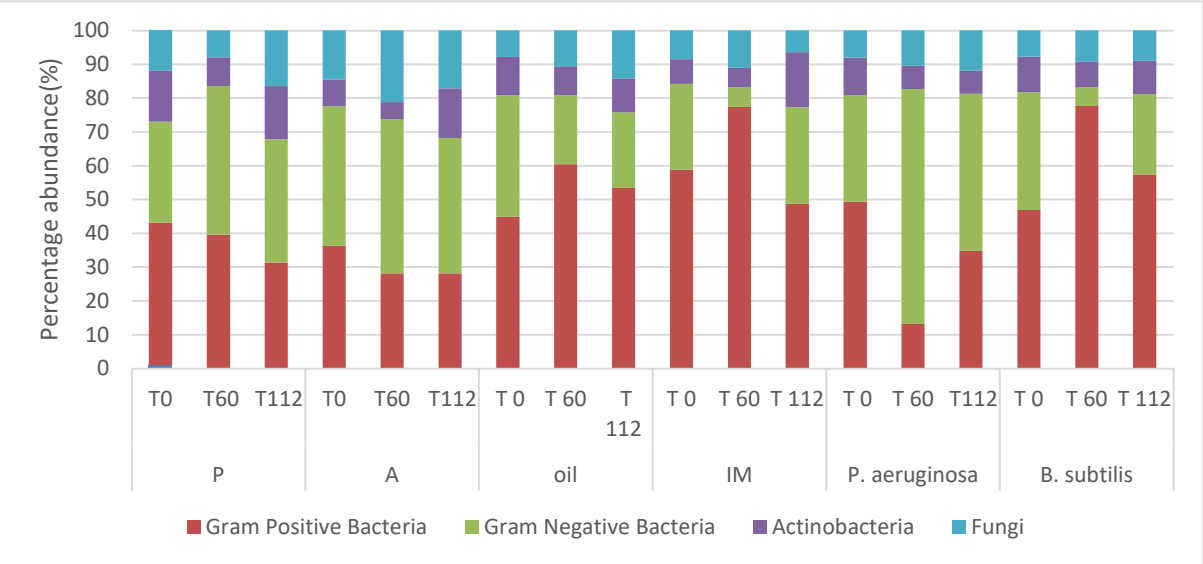


Figure 4.6. Microbial communities' dynamics for bioaugmentation mesocosms. Measured by PLFA at time (T) in day. Where P, A, Oil, *B. subtilis,* IM and *P. aeruginosa* mesocosms are as stated in Table 4.1.

At day 112, a reduction in the percentage abundance of the dominant microbial groups in all the inoculated mesocosms were observed (Figure 4.6). The trends in total PLFAs is an important indicator of the biomass of living microbes in the mesocosms (Lewe et al., 2021). The reduction in the percentage abundance observed can be linked to starvation due to the limited availability of the hydrocarbons for microbial mineralisation. The effect of such starvation could lead to a decrease in membrane permeability of the surviving microbes, thereby reducing microbial viability (Kaur et al., 2005). There was a positive correlation, significant at $p \leq 0.05$, between the available hydrocarbons, CO_2 generation and PLFA abundance (Table 4.2, 4.3 and Figure 4.6). This relationship shows that at high microbial abundance more hydrocarbons are degraded, reducing soil toxicity

and enabling microbial numbers to further increase. The environmental stress on the microbial cells of the various inoculated mesocosms showed a reduction in stress between day 30 and 112, when compared to the natural attenuation (crude oil spiked acidified soils) (Figure 4.7). The trans/cis ratio shift after day 30 showed the sensitivity of the microbial communities to established environmental stresses such as pH, and toxicity reduction. Kimura et al., (2001) stated that the trans/cis ratio is a good indicator of environmental stress. The adaptative shift shown by the trans/cis ratio counteracts the toxic effects of the petroleum hydrocarbons and maintains the functionality of the membrane to normal activities (Fischer et al., 2010). Restoration of the microbial cells to normal functions indicate recovery of the soils in the mesocosms. These findings agreed with the studies of Frostegård et al. (2011) and Zhang et al. (2020).

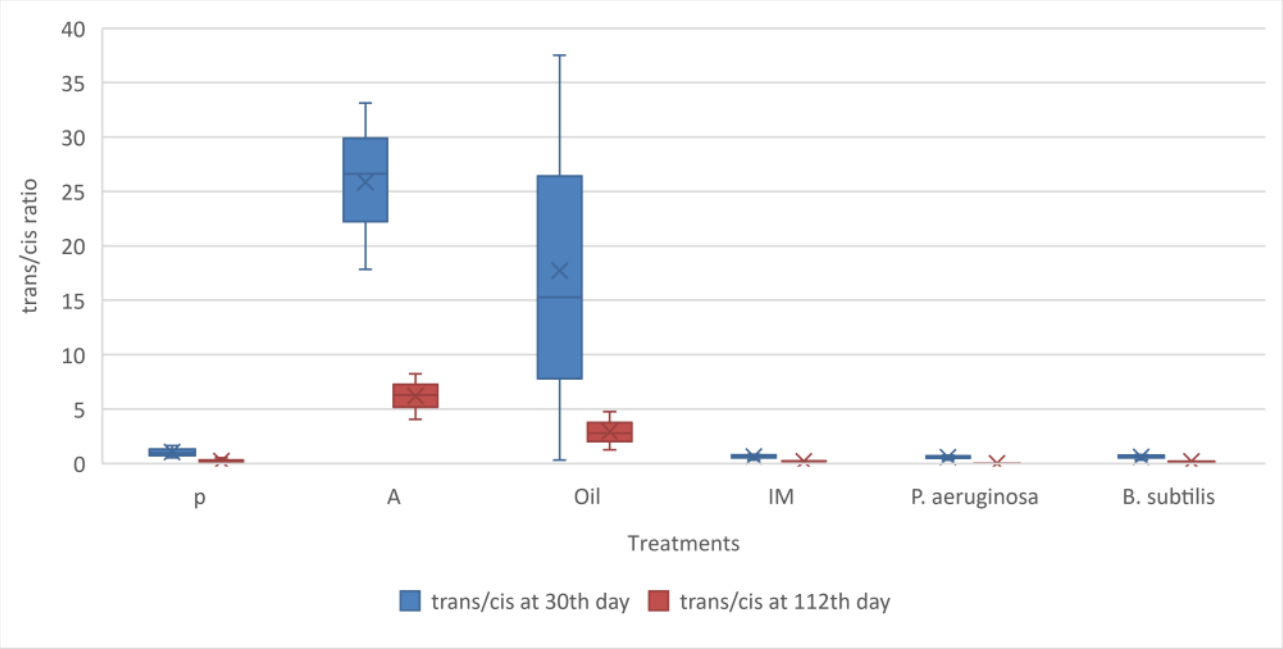


Figure 4.7. Environmental stress for bioaugmentation mesocosms. Measured by PLFA at day 30 and 112. Number of observation (n) for triplicate samples is n = 3 ± SD. Where P, A, Oil, *B. subtilis,* IM and *P. aeruginosa* mesocosms are as stated in Table 4.1.

4.4.3 Remediation Endpoint

At the onset of the experiment with 50,000 mg/kg TPH, no germination was reported in all the crude oil spiked acidified mesocosms (Figure 4.8). However, the pristine soil recorded about 73% germination while the sudden environmental stress caused by the fresh acidification on mesocosms A (acidified with no crude oil) inhibited germination completely. The acidification and oil also reduce soil fertility (Table 3.2 & 3.4), and reduces nutrient availability to plants (Essien & John, 2011). At week 16, pristine soil and *P. aeruginosa* bioaugmented soil recorded the highest germination with 93% while the indigenous microbes and *B. subtilis* mesocosms recorded more than 87% germination (Figure 4.8).

The high germination percentages were recorded when the medium molecular weight available alkanes and PAHs fractions were reduced by about >99% and the high molecular weight alkanes and PAHs reduced by about >95% (Figure 4.5). This result implied that at week 16 after spiking, the remediation end point was reached, and is further supported by the low trans/cis ratio recorded at week 16 (Figure 4.7). Cipullo et al. (2019) stated that medium molecular weight hydrocarbons fractions could define the remediation endpoint since they are easier to degrade by microbes and potentially constitute drivers of toxicity reduction in the environment.

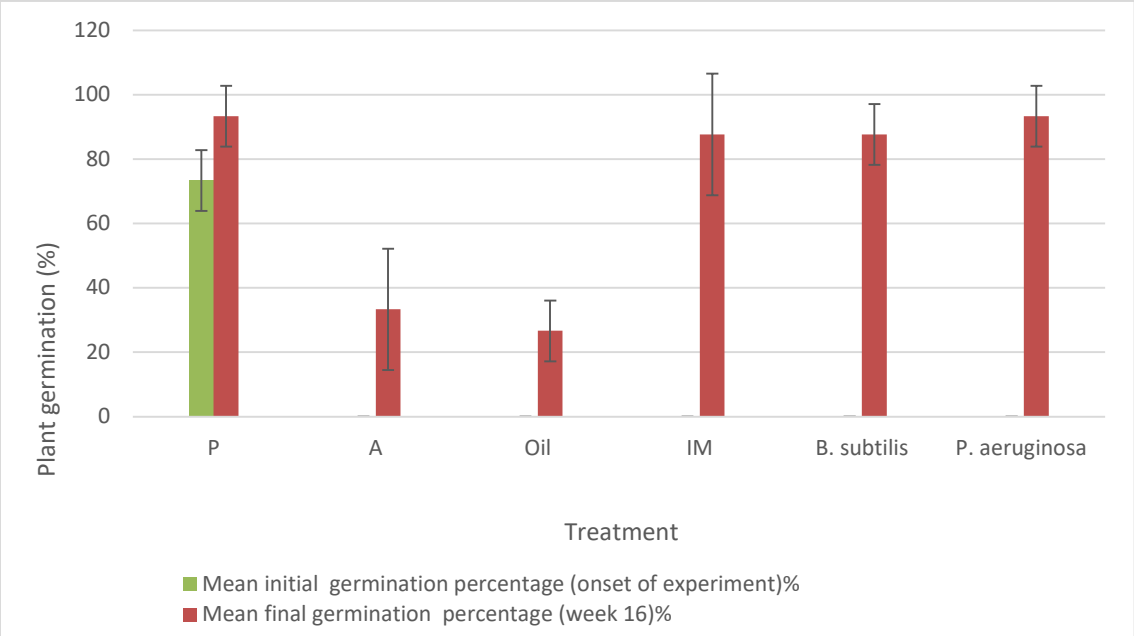


Figure 4.8. Plant germination for bioaugmentation mesocosms. Number of observation (n) for triplicate samples is n = 3 ± SD. Where P, A, Oil, *B. subtilis,* IM and *P. aeruginosa* mesocosms are as stated in Table 4.1.

4.5 Conclusion

The research had shown that in laboratory conditions, acidified wetland soil contaminated by petroleum hydrocarbons can be effectively remediated using bioaugmentation strategies. The indigenous microbial consortia degraded the alkanes and PAHs in the acidified wetland soil by 77%, and 91% respectively after week 7. The indigenous microbes were effective in degrading the medium molecular weight (C11 – C16) alkanes and PAHs (C10 – C16) with about 97% alkanes (C11 – C16) and 95% PAHs (C10 – C16) degradation after week 7 respectively. The Gram-positive bacteria were the dominant microbial communities for the indigenous microbes and *B. subtilis* enriched mesocosms while the Gram-negative bacteria formed the dominant microbial communities in the *P. aeruginosa* enriched mesocosms. After 16 weeks of bioremediation, the indigenous microbes-enriched mesocosms had the least environmental stress and least available hydrocarbons, achieving remediation endpoint faster. This result was confirmed by high germination rates (almost 90% germination)

recorded in the indigenous mesocosms. A positive corelation, significant at $p \leq 0.05$, was established between CO_2 generation and hydrocarbon degradation in all the bioaugmentation mesocosms. This research has shown that the indigenous microbes (*Bacillus toyonensis* BCT-7112(T)), are effective in degrading petroleum hydrocarbons in acidified wetlands having outperformed bioaugmentation with known petroleum hydrocarbon degrading species, *aeruginosa* and *subtilis*. Further studies on hydrocarbons degradation by *toyonensis* BCT-7112(T), and combinations of *toyonensis* BCT-7112(T) with *aeruginosa and subtilis* should be considered in crude oil contaminated coastal and estuarine sediments since such polluted sites are also prominent in the Niger Delta.

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5 SIMULTANEOUS USE OF BIOSTIMULATION AND BIOAUGMENTATION AS OPTIMISED STRATEGIES FOR REMEDIATING OIL IMPACTED WETLANDS

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5.1 Abstract

The performance of combined biostimulation and bioaugmentation strategies was investigated for acidic wetlands contaminated with crude oil. Stimulants and bioadds used in the investigation include Tween 80 surfactant, food-waste digestate fibre, and enrichment of the soil indigenous microbial community. Hydrocarbon degradation, soil basal respiration and microbial communities' dynamics were monitored over 112 days. On average all combined strategies showed increased hydrocarbon degradation rates of 32% compared to natural attenuation mesocosms after 112 days. Fastest degradation (> 98%) was obtained when 30% digestate, 30% Tween 80 were added along with augmentation of indigenous microbes with 9 $\times 10^5$ cells/g. Further to this, the degradation rate of aliphatic and aromatic hydrocarbons was improved by factor 1.6 especially for the medium and heavy molecular weight hydrocarbons (> C11-C36 alkanes and C10 – C22 PAHs) when 30% digestate, 30% Tween 80, and 9 $\times 10^5$ cells/g indigenous microbes were added. The dominancy in microbial communities shifted from fungi to the Gram-positive bacteria over 112 days. A positive correlation was established for CO_2 generation rate and total petroleum hydrocarbon (TPH) degradation for all the optimised mesocosms. At the end of the experiment, the seed germination response with respect to the available TPH and environmental stress assessed using trans/cis technique (from PLFA analysis) showed that at day 112 remediation endpoint was established.

Keywords: bioremediation, wetlands, respiration, microbes, hydrocarbons.

5.2 Introduction

The Niger Delta wetlands is one of Africa largest wetlands and is considered one of the world richest wetland in terms of biodiversity (Nwankwoala & Okujagu, 2021; Adekola & Mitchell, 2011). In 2021, Nigeria produced about 1.6 million barrels of crude oil per day, making it one of the largest crude oil producers in the world (OPEC, 2022). Most of the crude oil fields are located in the wetlands of Niger Delta, Nigeria. Crude oil exploration and exploitation has significantly negatively impacted the Niger Delta wetlands and its ecosystems. This impact has led to calls for proper management and remediation of the contaminated wetlands (Chidumeje et al., 2015). Between 2010 – 2018, approximately 27 million litres of crude oil were spilled into the wetlands (NDPR, 2022). Studies suggest that acidification is ongoing on account of low pH, high sulphate and nitrate concentration with a number of acid forming and acid tolerant microbes been identified in the Niger Delta wetlands (Ohimain, 2003).

Delays in remediation of the hydrocarbons in the acidified wetlands eventually lead to changes in the petroleum hydrocarbons composition, increased toxicity, increased distribution, and decreased availability in the environment (Oualha et al., 2019; Bento et al., 2005). Delays in soil remediation either *in-situ* or through long soil storage cause severe environmental stress on the soil microbial communities (Fischer et al., 2010; Huang et al., 2021). Remediation techniques used so far include physical and chemical methods, enhanced natural attenuation, bioremediation and low carbon remediation techniques (Edema et al., 2011; Adejumo et al., 2010). However, the results reported for these techniques have been inadequate, causing secondary pollution, increased available metal content, or increased soil pathogens abundance (Chiwetalu et al., 2020; Wuana & Okieimen, 2010).

The combined use of biostimulant and bioaugmentation for remediating contaminated wetlands has shown some success. For example, Wei et al. (2020) reported total petroleum hydrocarbons (TPH) degradation > 80% after 50 days when biochar was added in combination with rhamnolipid and compost to wetlands contaminated with crude oil at 350 mg/kg, compared to 39% degradation in untreated control. However, several studies have reported issues associated with combined biostimulation and bioaugmentation strategies such as increased metal(loid)s content in soil, increased

soil carbon and greenhouse gases as the remediation progresses (Herath et al., 2013; Taiwo et al., 2016; Zhang et al., 2021). Further to this, the pathogenic content and toxicity of most of the wetlands increased after remediation due to incomplete contaminants degradation and production of intermediate metabolites (Philips et al. 2000; Chikere et al., 2017). Thus, to overcome these challenges, biostimulants and other low carbon stimulants can be replaced with digestate in low-carbon bioremediation of contaminated wetlands (Andrew, 2012; Gielnik et al., 2020).

Low carbon biostimulants are organic stimulants that, when added to soil, promote the growth and activity of microorganisms without significantly increasing carbon dioxide (CO_2) evolution (Alori et al., 2017). They are rapidly degraded and metabolized by soil microorganisms, leading to a lower net CO_2 emission, and are more sustainable option for improving soil health while minimizing negative impacts on the environment (Huang & Li, 2017). Digestate, a low carbon biostimulant, is from anaerobic digestion of by-product of biodegradable feedstock (Peng & Pivato, 2019). Digestate from food waste feedstock produces higher quality digestate in terms of nutrient contents with very low heavy metals and metalloid contents, when compared to feedstock from other waste streams (Andrew, 2012; Opatokun et al., 2015). The food waste digestate fibre despite improving the nutrient value of soils, also possess the required nutrients needed for optimal microbial activities (Yu et al., 2022; Gielnik et al., 2021). Therefore, if applied to acidified wetlands for remediation of petroleum hydrocarbon contaminants, digestate could boost the remediating ability of the surviving indigenous microbes by supplementing the lacking nutrients needed for optimal microbial performance. The potentials of the surviving indigenous microbes in the contaminated acidified wetlands can also be strengthened by enriching the contaminated soils with the surviving indigenous microbes. The enriched indigenous microbes act as an optimised biocatalyst to degrade large quantities of the target contaminants (Oladipo & Ogunsona, 2020; Tiralerdpanich et al., 2018). Lladó and Baldrian (2017) stated that indigenous microbial consortia represent a more efficient and cost-effective strategy for the bioremediation of hydrocarbon contaminated soil than single culture. Indigenous microbial consortia co-evolve with the polluted environment, establish relationships and interactions with one another, which enables them to function synergistically in the degradation of contaminants found in that environment (Liu et al.,

2019). Activities of the indigenous microbes can be further boosted by enhancing the bioavailability of the contaminants using surfactants (Sung et al., 2013). An ecological low risk surfactants such as Tween 80 can form an active layer on the outside of the cell, acting as a bridge to assist in the assimilation of hydrophobic contaminants such as hydrocarbons (Liu et al., 2003). Despite these promising alternatives, to date, few studies have investigated combined simultaneous biostimulation and bioaugmentation strategies using digestate in contaminated acidified wetlands. Further to this, the endpoint for such remediated acidified wetlands is scarcely established.

5.3 Materials and Methods

5.3.1 Mesocosms Soil and Experimental Design

Pristine soil, with no record of petroleum hydrocarbons contamination, was collected from a construction site in Cranfield University (52.0746 N, 0.6283E). Soil was collected from 0 to 30cm soil depth, the soil was air dried at room temperature, sieved through 2 mm aperture sieve (model: BS410 manufactured by: Endecotts, London, England), and stored for four months at 20 ^{o}C before use. Triplicate soil mesocosms were set up using 1 kg soil in 2.5 litre transparent polytetrafluoroethylene (PFTE) containers. Six different mesocosms conditions were evaluated as summarised in Table 5.1 and Figure 5.1. Soil characteristics and properties are described in Chapter 3.

Table 5.11. Mesocosm experimental design with all treatments in triplicates.

Mesocosm condition	Treatment	Abbreviation
1	Pristine soil (freshly collected from field)	Control
2	Pristine soil acidified at pH 5. (using HNO_3)	Acidified
3	Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg crude oil	Crude oil
4	Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg crude oil + 30% digestate + 30% Tween 80	TW80 + D

5	Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg crude oil + 30% Tween 80 + Indigenous microbial culture at 9×10^5 cells/g	TW80 + BioA
6	Pristine soil acidified at pH 5.8 and spiked at 50,000 mg/kg crude oil + 30% Digestate + 30% Tween 80 + indigenous microbial culture at 9×10^5 cells/g	TW80 + D + BioA

Figure 5.1. Experimental setup for optimised combined biostimulation and bioaugmentation treatment of hydrocarbon contaminated acidified wetland soil.

The mesocosms except the pristine soils (control) were all acidified to pH of 5.8 using HNO_3 (PrimarPlus- trace analysis grade, supplied by Fisher Scientific UK, Limited). The acidified soil mesocosms were spiked with 60 ml of crude oil sweet (<0.5% sulfur) (SDS, Regulation 1907/2006/EC) to achieve a target hydrocarbons concentration of 50,000 mg/kg. The mesocosms were incubated at 28 °C and sandy soil was adopted to mimic the mean temperature and prominent soils of the Niger Delta (Fubara-Manuel et al., 2017).

Food waste digestate was air dried and particles larger than 2 mm were removed using a 2 mm aperture sieve. The dried digestate was thoroughly mixed with crude oil spiked

acidified soil at 30% (w/w) in triplicates following the methods described in Nwankwo (2014) (Table 5.1). Tween 80 (TW80 (also called Polyoxyethylene(20)sorbitan monooleate)) was applied at 30% (w/w) and mixed with the crude oil spiked acidified soil samples in triplicates (Table 5.1). The application of the non-ionic surfactants (TW80) was as described by Trinchera and Baratella (2018). The LB broth was used as a medium, and the indigenous microbes' pure culture (cultured from the experimental contaminated soil using 10-fold dilution) was incubated for 24 hours at 150 rpm for 37 ^{o}C. Microbial cell counting was performed using a microscope (Leica DM4000B, magnification 6x, Breckland, UK). 9 x 10^5 cells/g of the indigenous microbes were applied to the mesocosms (Table 5.1).

Three controls (pristine soil, acidified soil (without HCs) and hydrocarbons spiked acidified soil with no treatment (natural attenuation)) were maintained throughout the experiment. The soil moisture content of 13.8% was increased by 21% to maintained saturation at the mesocosms. Moisture saturation was maintained to depict the wetland condition in all mesocosms. Deionised water was used to maintain moisture in all mesocosm. The deionised water was added at 7 days intervals to maintain the soil saturation.

5.3.2 Hydrocarbons Analysis

The hydrocarbons analysis was as described in chapter 3. The soil petroleum alkanes were grouped into C11-C13, C14 – C20, C21 – C30, C31 – C37. The petroleum PAHs were similarly grouped into C10 – C13, C14 – C18 and C19 - C27.

5.3.3 Soil Respiration

Soil basal respiration was used to quantify the CO_2 generation rate (He & Xu, 2021), and was measured as described in chapter 3. Changes in conductivity (micro siemens) were used to quantify CO_2 release according to Ritz et al. (2006).

5.3.4 Soil Microbial Abundance

Soil microbial abundance was determined as described in chapter 3. The soil microbial count were determined using colony forming unit (CFU/g) plate counting technique by Varjani and Upasani (2019).

5.3.5 Soil Microbial Community Profiles and Dynamics

Soil microbial community profiles and dynamics were determined as described in chapter 3. Fatty acids were used as an indicator of the presence of groups of microbes (biomarkers). The biomarkers were categorized into Gram-positive bacteria, Gram-negative bacteria, actinobacteria and fungi according to Quideau et al. (2016) and Frostegård and Bååth (1993).

5.3.6 Ecotoxicity Assay

The ecotoxicity assay adopted as described in chapter 3. Germination response and days of germination after planting were recorded at the onset (day 0) and day 112 of the experiment.

5.3.7 Statistical Analysis

Statistical analysis carried out using Microsoft Excel (Version 2111 Build 16.0.14701.20278). Standard error was used to evaluate the variability across germination assays and the applied environmental stress while standard deviation was used to ascertain the variability within sample measurements. The JMP pro (version 16) software was used for the Spearman correlation. The spearman correlation was carried out for the respiration rate and total petroleum hydrocarbons (TPH) degradation rates. At $p<0.01$ Spearman coefficient (ϒ) is considered significant if it is greater than absolute p but less than 1. Regression analysis was done to ascertain the corelation between the hydrocarbons and soil basal respiration.

5.4 Results and Discussions

5.4.1 Effect of Environmental Stress on Soil Microbial Diversity

The pristine soil is a sandy silt loam soil with pH of 8.7 and moisture content of 13.7%. The pristine soil C: N ratio was 19: 1 suggesting nutrients stress of the microbial community (Table 5.2 & 5.3). The addition of stimulants can compensate for inadequate nutrients in the soil (Wang et al., 2021), with the optimum soil C: N to stimulate effective microbial activities being 24: 1(USDA, 2011).

Heavy metals in the soils can also inhibit the soil microbial activities (Table 5.2). Li et al. (2020) stated that heavy metals could penetrate the cell membranes of microbes and replace the cell essentials metal ions thereby inhibiting enzyme activities needed

for effective microbial cell metabolisms. For example, Zhang et al. (2017) found that cadmium significantly reduced soil microbial biomass and enzyme activity, while Yang et al. (2019) stated that heavy metals inhibit carbon and nitrogen metabolism enzymes such as sucrase and urease. Heavy metals also affect microbial metabolism by disrupting membrane integrity, which can lead to leakage of intracellular contents and reduced metabolic activity (Xu et al., 2020). Chai et al. (2021) stated that heavy metals significantly inhibited soil microbial activity, and the inhibition was accompanied by a decrease in the diversity and abundance of soil microorganisms. Heavy metals inhibition of microbes can therefore reduce decomposition and biodegradation of contaminants and other material elements in the soils (Chu, 2018).

The pristine soil was stored at 20 ^{o}C for about four months before use for the experiment, which altered the microbial dominance of the soil biomarkers from bacteria (chapters 3 & 4) to fungi at the onset of the experiment (Figure 5.2). The shift in dominance from bacteria to fungi could be linked to the changes in temperature (from varying room temperature to a constant storage temperature) over the four months storage period. This observation is in agreement with Ding et al. (2009) and Zhang et al. (2021). Both have shown that microbial communities' dominance shift with temperature and nutrients availability changes. The acidification and spiking of the soils with crude oil diverted the soil microbial community shift towards the Gram-positive group (Figure 5.2).

In all conditions evaluated, Gram-positive bacteria became dominant after day 60 indicating that they are leading the petroleum hydrocarbons degradation (Figure 5.2). Trögl et al. (2016) as well as Zhang et al. (2021) observed similar responses in their soil mesocosm studies, reinforcing the important role of Gram-positive microbial group in the biodegradation of hydrocarbons in contaminated soil. However, at the end of this experiment several microbial groups showed diverse levels of increments, especially fungi and Gram-negative bacteria which showed 23% and 11% increment respectively when digestate and TW80 were added. Similarly, Gram-negative and Actinobacteria increased by 11% and 19% respectively at day 112, when soil mesocosms where bioaugmented with the soil indigenous microbes + TW80 (Figure 5.2). In contrast, combined addition of digestate, TW80 and indigenous microbes contributed to an increase of 7% of the fungal group at the end of the experiment. All

these observed shifts could have been caused by starvation of the Gram-positive bacteria due to limitation of available alkanes and PAHs which it used as source of energy and carbons (Zhang et al., 2021). Similar microbial shift trends were observed in chapters 3 and 4. Also, the observed shifts at day 112 corroborate the establishment of remediation end point due to limited available PAHs and alkanes (Figure 5.2 and 5.7).

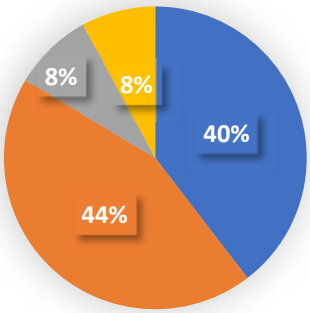
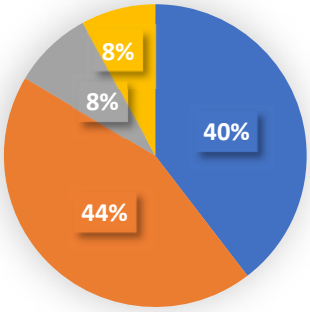
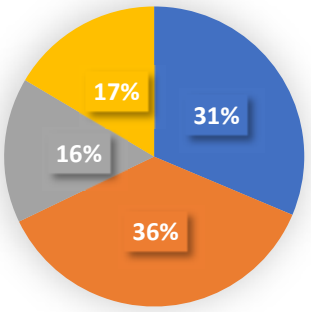
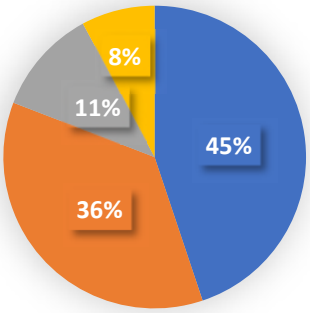
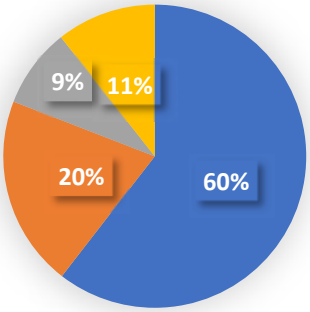
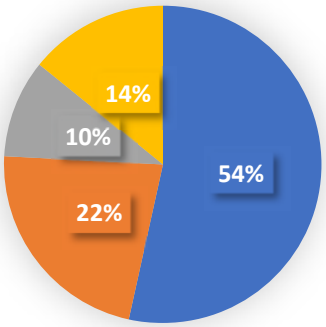
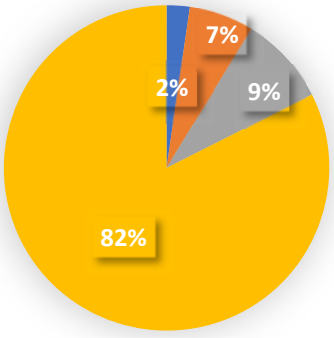
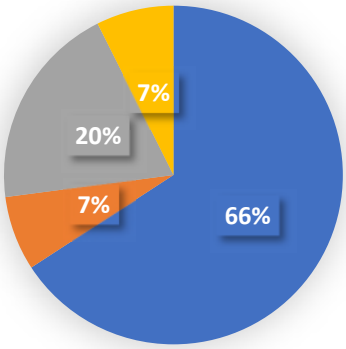
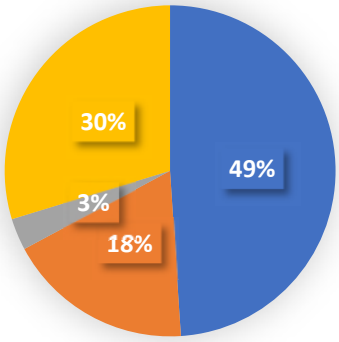
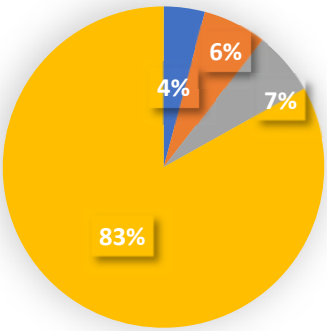
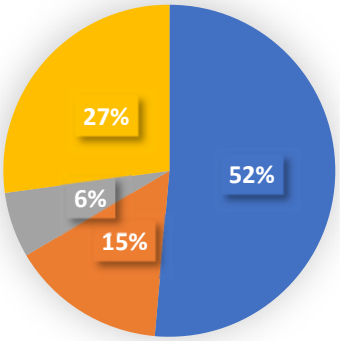
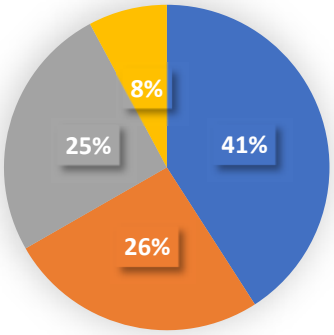
The extent of toxicity or environmental stress on any sample microbial communities for PAHs or alkanes polluted or remediated soil can be evaluated using trans/cis ratio (Fischer et al., 2010; Zhang et al., 2021). The trans/cis isomerisation ratio describes the urgent response of the microbial community to toxic compounds (Fischer et al., 2010; Trögl et al., 2016). At the day 30, the trans/cis isomerisation ratio was >10:1, indicating that the microbial communities of the mesocosms were under severe environmental stress (Figure 5.3). Zhang et al. (2021) and Trögl et al. (2016) stated that at trans/cis isomerisation ratio >10:1, the microbial communities were in an unhealthy situation due to applied environmental stress such as increased acidity, and high concentration of hydrocarbons. The trans/cis isomerisation ratio at day 112 showed that the environmental stress across the optimised combined mesocosms dropped and was less than 2.5:1 (Figure 5.3). The least stressed mesocosm at the end of the experiment was the optimised TW80 + D + BioA. The stress reduction observed indicated low risk and toxicity to the soil ecosystem (Kimura et al., 2012) at the end of the experiment.

Table 5.22. Baseline concentrations of nutrients and metal(loid) in the soil samples.

Nutrients/ metal(loid)	**Control**	**Acidified**	**Crude oil**
Total C (%)	3.09 ±0.11	2.81±0.08	5.08±0.09
Organic C (%)	2.25±0.13	1.44±0.08	1.93±0.23
Total N (%)	0.12±0.02	0.31±0.02	0.11±0.0
Total P (mg/kg)	5.58 ± 0.28	5.47 ± 0.21	4.91 ± 0.16
Total K(mg/kg)	236.00 ± 2.2	236.00 ± 2.2	236.00 ± 2.2
C: N	19:1	5:1	18:1
Mo (mg/kg)	0.93 ± 0.08	1.27 ± 0.13	1.21 ± 0.07
Cr (mg/kg)	45.24 ± 1.23	59.56 ± 2.94	50.87 ± 1.50
Ni (mg/kg)	29.65 ± 1.24	42.60 ± 0.93	35.25 ± 0.79
As (mg/kg)	14.83 ± 0.98	20.21 ± 0.74	17.18 ± 0.78
Cd (mg/kg)	0.59 ± 0.09	0.83 ± 0.09	0.73 ± 0.07
Pb (mg/kg)	17.00 ± 0.91	23.31 ± 0.45	21.41 ± 0.8
Hg (mg/kg)	0.29 ± 0.01	0.12 ± 0.01	0.12 ± 0.02

Table 5.33. Physical characteristics of the pristine soil used in the mesocosm experiment.

Soil Physical characteristics	
Soil Moisture content (%)	13.70
Loss on ignition (%)	3.66
Dry matter content (%)	86.25
Water holding capacity (%)	54.54
Soil Particle size distribution	
Sand (%)	46.67
Silt (%)	45.89
Clay (%)	7.44

	T0	day 60	day 112
Control			
Crude oil			
TW80 + D			
TW80 + BioA			

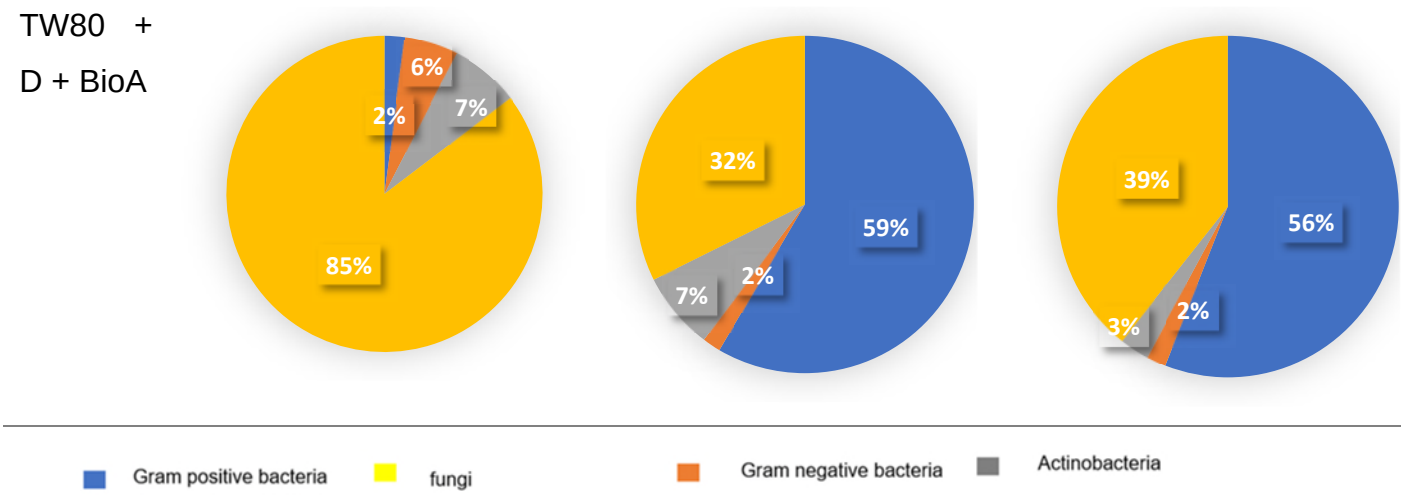


Figure 5.2. Microbial dynamics for optimised combined strategies mesocosms. Number of observation (n) for triplicate samples is n = 3. Where control, crude oil, TW80+BioA, TW80+D and TW80+D+BioA mesocosms are as stated in Table 5.1

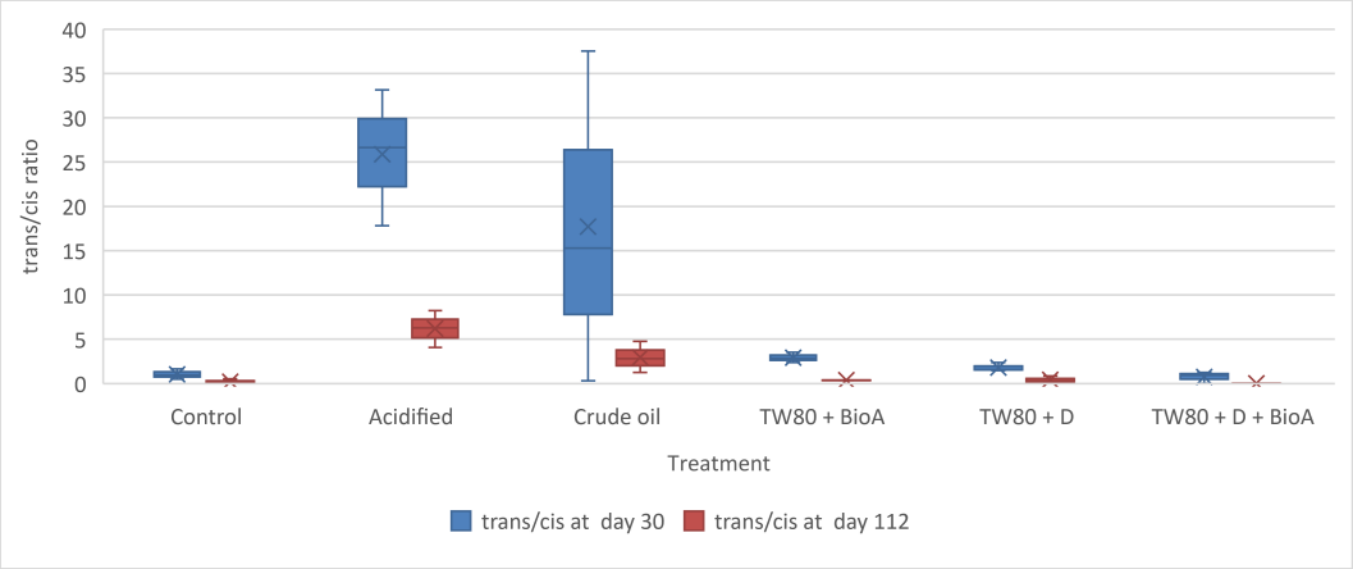


Figure 5.3. Environmental stress at day 30 and 112 after spiking with crude oil. Number of observation (n) for triplicate samples is n = 3 ± SD. Where control, acidified, crude oil, TW80+BioA, TW80+D and TW80+D+BioA mesocosms are as stated in Table 5.1.

5.4.2 Soil Microbial Activity and Degradation Rate

The highest soil respiration was recorded in the mesocosms containing TW80 + D, and TW80 + D +BioA, with 52% and 47% respectively increase in CO_2 generation at day 112 compared to the natural attenuation (Figure 5.4). Only 6% increment in CO_2 (μg/g) was observed between day 77 and 112 when TW80, digestate and BioA were used in combination. The lower increment observed could be attributed to the limited remaining amount of petroleum hydrocarbons for degradation (Figure 5.4, 5.5 & 5.6). Robichaud et al. (2019) stated that at higher hydrocarbons degradation rates, greater amounts of CO_2 are correspondingly produced. The hypothesis corroborates the high degradation rate observed at the TW80 + D + BioA mesocosms when compared to the other mesocosms (Table 5.6). The gradients of the regression models (the degradation rates ($mgCO_2$ /mg TPH /day)) for CO_2 generation rate versus total petroleum hydrocarbon (TPH) degradation rate indicates that the more CO_2 generated, the more TPH is degraded (Table 5.6).

The TW80 + D mesocosms showed similar trends in CO_2 production with the TW80 + D +BioA mesocosms but at a reduced degradation rate. The TW80 + BioA mesocosms showed the least performance in CO_2 generation when compared to TW80 + D and TW80 + D +BioA mesocosms. The increments in CO_2 production in the combined mesocosms can be attributed to mineralization of the petroleum hydrocarbons due to the increased availability of the contaminant caused by Tween 80 (Asquith et al., 2012). The fact also agreed with the research of Ma et al. (2018) and Philben et al. (2020) where the researchers concluded that an increased availability of degradable contaminants and nutrients led to a corresponding increase in CO_2 generation by the microbes. This hypothesis was in agreement with increment and reduction in biomass (Table 5.8) and the corresponding increment and reduction in CO_2 production from onset of the experiment to day 112 (Figure 5.4). Similar results were obtained when Tween 80 and food waste digestate were used as stimulants (Chapter 3) and in bioaugmentation when indigenous microbes, *Pseudomonas*, and *Bacillus* were used to degrade hydrocarbon contaminants in the mesocosms (Chapter 4).

The respiration rate of the optimized combined mesocosms experiments showed a positive correlation, significant at $p \leq 0.05$, between soil CO_2 generation and TPH degradation rate using regression correlation F and p values (Table 5.5 & 5.4). The observed correlation was reaffirmed using the Spearman correlation at $p \leq 0.01$. At $p \leq 0.01$, Spearman coefficient ($\mathfrak{r}$) is considered significant if it is greater than absolute p but less than 1 (Table 5.7). These findings agreed with the research of Gielnik et al. (2021) on functional potential of sewage digestate on hydrocarbons degradation. The researchers stated that CO_2 evolution correlated with the removal of petroleum hydrocarbons from the soil.

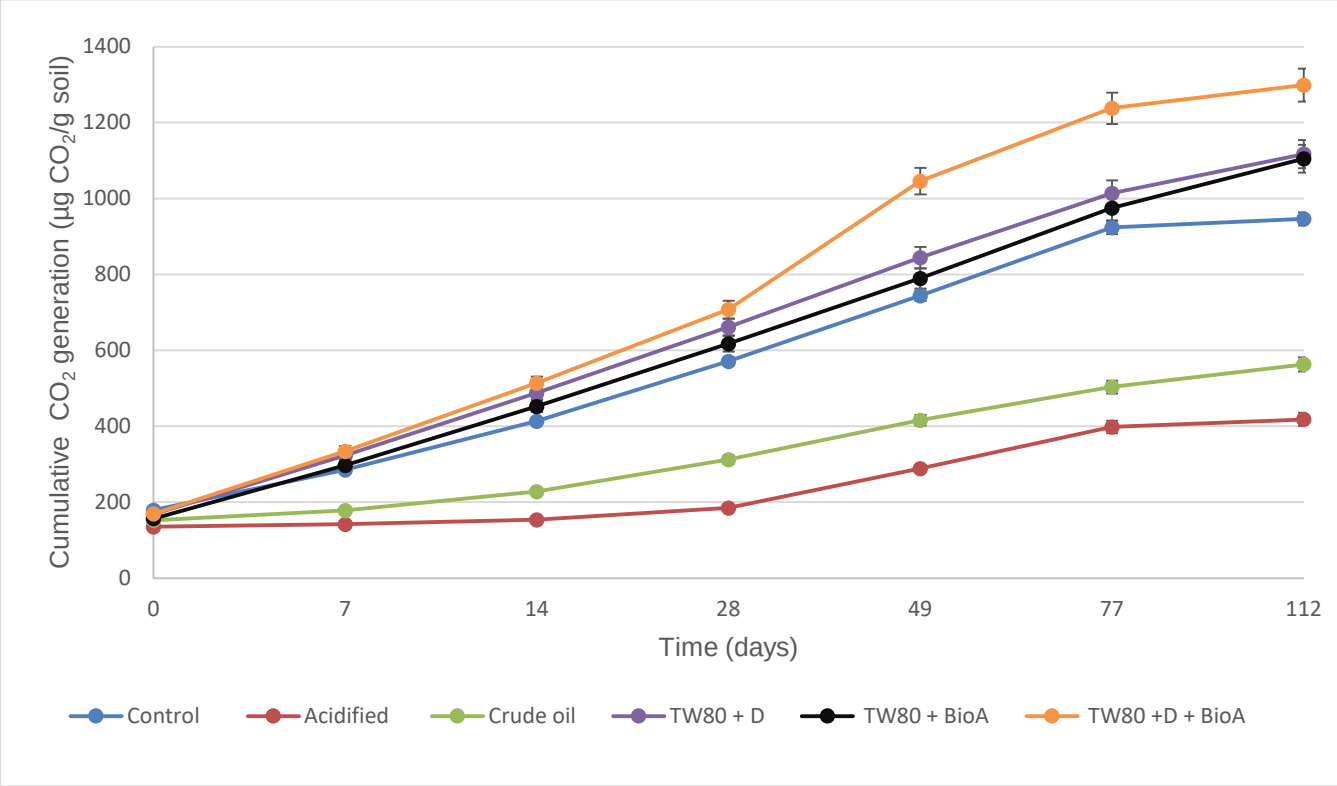


Figure 5.4. Cumulative CO_2 (µg/g soil) per day for optimised combined strategies mesocosms.

Number of observation (n) for triplicate samples is $n = 3 \pm SD$. Where control, acidified, crude oil, TW80+BioA, TW80+D and TW80+D+BioA mesocosms are as stated in Table 5.1.

Table 5.44. Regression summary of the generated CO_2 (µg/g soil) versus TPH (mg/kg) for the various combined mesocosms.

Mesocosms	Multiple R	R Square	Adjusted R Square	Observations
Crude oil	0.96	0.92	0.91	6
TW80 + D	0.97	0.94	0.93	6
TW80 + BioA	0.96	0.93	0.92	6
TW80 + D + BioA	0.96	0.92	0.90	6

Number of observation (n) for triplicate samples is n = 3. Where crude oil, TW80+BioA, TW80+D and TW80+D+BioA mesocosms are as stated in Table 5.1.

Table 5.55. Regression analysis of variance (ANOVA) of the generated CO_2 (µg/g soil) versus TPH (mg/kg) showing significance level.

Mesocosms	Item	df	SS	MS	F	Significance F
Crude oil	Regression	1	118272.04	118272.04	49.53	0.0021
	Residual	4	9551.94	2387.99		
	Total	5	127823.98			
TW80 + D	Regression	1	584712.70	584712.70	63.37	0.0013
	Residual	4	36905.18	9226.30		
	Total	5	621617.88			
TW80 + BioA	Regression	1	567980.40	567980.40	55.42	0.0017
	Residual	4	40992.68	10248.17		
	Total	5	608973.08			
TW80 + D + BioA	Regression	1	904242.92	904242.92	45.40	0.0025
	Residual	4	79665.81	19916.45		
	Total	5		983908.73		

Table 5.66. Regression ANOVA table showing degradation rates for the various mesocosms.

Mesocosms		**Standard Error**	**t Stat**	**P-value**	**Regression model**	**Degradation rate (slope) (mg CO_2/mg TPH/day)**
Crude oil	Intercept	48.49347	13.89139	0.000156	y=673.64 - 0.0111x	- 0.0111
	TPH	0.001581	-7.03761	0.002149		
TW80 + D	Intercept	57.60023	18.25639	5.29E-05	y=1051.57 - 0.0189x	- 0.0189
	TPH	0.002386	-7.96082	0.001349		
TW80 + BioA	Intercept	63.82587	16.37145	8.15E-05	y=1044.92 - 0.0191x	- 0.0191
	TPH	0.002565	-7.44464	0.001739		
TW80 + D + BioA	Intercept	82.22822	14.89103	0.000118	y= 1224.46 - 0.0258x	- 0.0258
	TPH	0.003836	-6.73809	0.002528		

Y = generated CO_2 and x = TPH degradation

df= degree of freedom, SS= sum of squares, MS = Mean square.

Number of observation (n) for triplicate samples is n = 3. Where crude oil, TW80+BioA, TW80+D and TW80+D+BioA mesocosms are as stated in Table 5.1.

Table 5.77. Spearman correlation between basal respiration and TPH degradation.

Treatment	Spearman coefficient ($\varkappa$)	Prob>|p|	correlation strength
Control	0.8104	<0.0001	++++++++
Acidified	0.87	<0.0001	+++++++++
Crude oil	0.8805	<0.0001	+++++++++
TW80 + D	0.9175	<0.0001	+++++++++
TW80 + D + BioA	0.9706	<0.0001	++++++++++
TW80 + Bio A	0.8214	<0.0001	+++++++

Number of observation (n) for triplicate samples is n = 3. Where control, acidified, crude oil, TW80+BioA, TW80+D and TW80+D+BioA mesocosms are as stated in Table 5.1.

Table 5.88. Microbial abundance for various mesocosms.

Mesocosm	**Bacteria count (× 10^5CFU/g) at onset**	**Bacteria count (× 10^5CFU/g) at day 30**	**Bacteria count (× 10^5CFU/g) at day 112**
Control	96 ± 1.2	102 ± 0.82	111 ± 0.16
Acidified	1 ± 0.88	2 ± 0.47	3 ± 0.41
Crude oil	5 ± 0.92	7 ± 0.47	29 ± 0.52
TW80 + D	29 ± 1.4	251 ± 0.47	157 ± 0.14
TW80 + BioA	17 ± 1.2	270 ± 1.25	159 ± 0.82
TW80 + D + BioA	35 ± 0.99	450 ± 0.47	272 ± 0.41

Number of observation (n) for triplicate samples is n = 3 ± SD. Where control, acidified, crude oil, TW80+BioA, TW80+D and TW80+D+BioA mesocosms are as stated in Table 5.1.

5.4.3 Determination of the Remediation Endpoint

The highest petroleum hydrocarbons degradation was achieved in the presence of optimised TW80 + D + BioA. About 98% of alkanes and PAHs were degraded at day 49 (Figure 5.5 & 5.6). The extent of degradation was 1.7 times faster and 1.2-fold higher in alkanes and PAHs degradation respectively than the natural attenuation (Figure 5.5 & 5.6). This degradation rates are higher when compared to single stimulants and bioadds (used in chapters 3 & 4). For example, at day 49, the hydrocarbons degradation with 30% TW80 treatment only was 1.47 and 1.16 times faster for alkanes and PAHs respectively while 30% digestate only was 1.56 and 1.18-fold faster for alkanes and PAHs respectively when compared to the natural attenuation (Figure 3.6, & Tables 3.8 – 3.11). Within the same periods, the indigenous microbes enriched mesocosm was 1.4 and 1.16 times faster for alkanes and PAHs respectively when compared to the natural attenuation (Figure 4.4, 7 Tables 4.8 – 4.9). These degradation rates can be linked to the increased availability of the contaminants by the Tween 80, increased concentration of *alkB* genes, and the inoculation with an indigenous consortium which enhanced soil microbial communities (Poi et al., 2017; Gielnik et al., 2021). At day 112, in TW80 + D + BioA mesocosms, C11 – C13 (undecane to tridecane) showed more than 99% degradation and C14 – C20 (tetradecane, pentadecane to phytane and eicosane) degraded by about 99% (Figure 5.5 and Table 5.9 & 5.10). The heavy alkanes compounds which include C21 – C30 (henicosane, dosocane to nonacosane) and the C31 – C37 (triacontane, hentriacontane to heptatriacontane) groups degraded by about 98.6% and 97.5% respectively (Figure 5.5 and Table 5.10). The aromatic hydrocarbons degraded faster with more than 99% degradation for C10 – C13, C14 – C18 and C19 – C22 which include naphthalene, fluorene to indeno(123)[cd]pyrene (Figure 5.6 and Table 5.11 & 5.12). The TW80 + D, and TW80 + BioA combinations showed similar trends in alkanes and PAHs degradation.

Tween 80 increases cell transmembrane transport of bioavailable hydrocarbons for intracellular biodegradation (Cheng et al., 2018; Wang et al., 2018). The digestate is high in C: N: P ratio (Table 3.5), therefore, it can provide the required nutrient needed for optimal microbial activities and reproduction, which could lead to the increased mineralisation of the petroleum hydrocarbons, and release of biogenic CO_2 generated

from the process. This finding agreed with that of Gielnik et al. (2021) and the researchers concluded that petroleum hydrocarbons degradation can be monitored through CO_2 evolution, and microbial metabolic activities. The comparison of the CO_2 evolution rate with the hydrocarbon degradation and microbial community dynamics showed a significant ($p<0.01$) positive correlation for all optimised mesocosms (Table 5.7).

At the onset of the experiment with available TPH of about 49,250 mg/kg, there was no germination in the acidified crude oil spiked mesocosms (Figure 5.7a). However, the pristine soil (control) recorded about 73% germination which can be linked to the saturated condition of the soil (Figure 5.7a, & Table 3.5). At the 112^{th} day, growth was recorded in all the mesocosms. 100% germination were recorded at optimised TW80 + D + BioA, and TW80 + D mesocosms while the pristine soil (control) had about 93% compared to the crude oil mesocosms with 26% germination (5.7 b).

The 100% percentage germination recorded at the optimised TW80 + D + BioA mesocosms where the availability of the TPH had reduced by more than 99% (Figure 5.7b) shows that the soil toxicity is acceptable for maize germination, and indicates that at day 112, remediation endpoint was established. The highly reduced environmental stress (Figure 5.3) recorded at day 112 further supports the establishment of the remediation endpoint in the experiment. Sonali et al. (2019) concluded that the most significant part of endpoint is its detection.

Table 5.99. Mean alkanes concentrations and percentage degradations for medium molecular weight alkanes.

Alkanes compounds	**Initial alkanes**	**Percentage degradation at day 112**			
	concentration (mg/kg)	Crude oil	TW80 + D	TW80 + BioA	TW80 + D + BioA
Undecane	2339.8	93.7	99.8	99.9	99.9
Dodecane	1226.2	59.8	99.6	98.8	99.6
Tridecane	1710.5	74.8	99.8	98.7	99.8
Tetradecane	1658.9	68.5	99.6	98.6	99.6
Pentadecane	1669.3	67.4	99.3	98.1	99.5
Hexadecane	1597.1	62.8	99.3	97.9	99.4
Heptadecane	1648.6	75.1	99.2	96.9	99.3
Octadecane	1576.5	65.2	99.1	96.4	99.2

Table 5.1010. Mean alkanes concentrations and percentage degradations for heavy molecular weight alkanes.

Alkanes compounds	**Initial Alkanes**	**Percentage degradation at day 112**			
	Concentration (mg/kg)	Crude oil	TW80+D	TW80 + BioA	TW80 + D + BioA
Pristane	1100.0	80.0	97.7	97.3	99.2
Phytane	1151.8	82.5	97.5	96.8	99.6
Nonadecane	1296.9	75.8	97.3	96.6	98.9
Eicosane	1416.3	75.5	97.3	96.1	98.8
Henicosane	1714.9	81.2	97.1	96.5	98.6
Dosocane	1808.8	75.4	97.2	96.4	98.4
Trisocane	1798.9	79.4	96.7	95.8	98.2
Tetracosane	1775.7	77.7	96.2	94.9	98.6
Pentacosane	1743.9	79.3	95.3	94.5	98.9
Hexacosane	1890.3	77.3	95.3	93.7	97.7
Heptacosane	1070.9	69.7	91.4	88.0	97.9

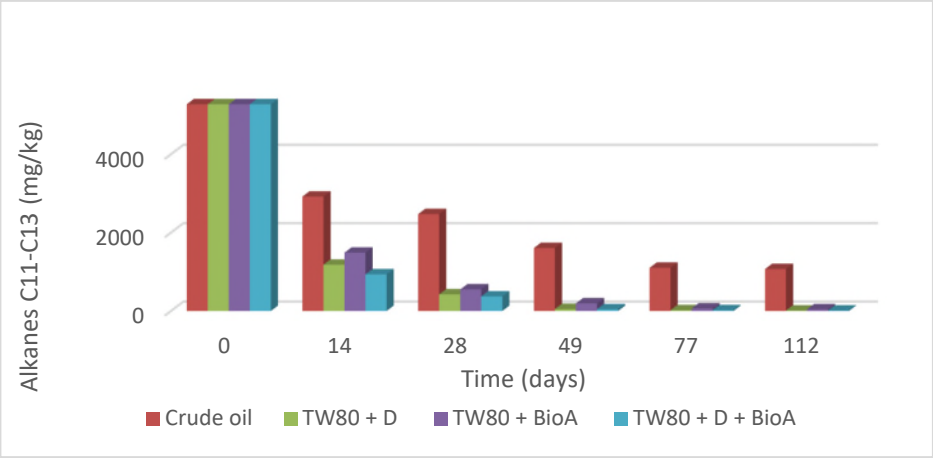
Octacosane	1936.8	79.5	94.8	91.9	97.9
Nonacosane	1953.8	78.4	93.4	91.0	97.8
Triacontane	1612.5	67.7	91.6	87.9	97.9
Hentriacontane	1569.9	66.9	88.7	87.3	97.6
Dotriacontane	1313.9	45.2	85.6	83.5	96.9
Tritriacontane	1057.9	22.3	78.3	78.8	97.8
Tetratritacontane	1183.6	38.6	76.7	73.1	97.6
Pentatriacontane	1245.7	35.6	74.9	65.3	97.4
Hexatriacontane	1176.4	32.3	67.2	57.8	94.9
Heptatriacontane	1254.6	20.4	64.9	58.4	94.2

Table 5.1111. Mean PAHs concentrations and percentage degradations for medium molecular weight PAHs compounds.

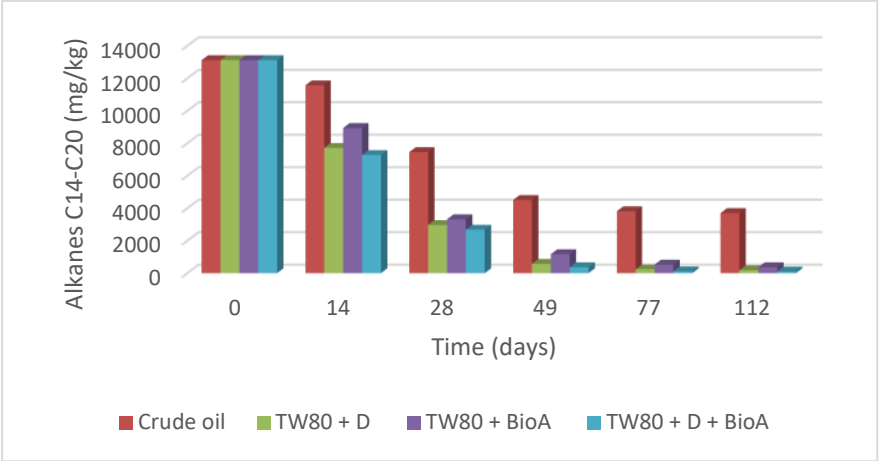
PAHs compounds	Initial PAHs	Percentage degradation (%) at 112th day			
	Concentration (mg/kg)	Crude oil	TW80 + D	TW80 + BioA	TW80 + D + BioA
Naphthalene	224.136	91.4	99.8	99.4	99.9
Fluorene	458.46	91.6	99.9	99.5	99.9
Phenanthrene	931.07	95.5	99.9	99.7	99.9
Anthracene	297.15	96.7	99.8	98.1	99.7
Pyrene	67.92	87.9	98.9	88.5	99.8
Benz(a)anthracene	212.25	90.4	99.5	93.8	99.6
Chrysene	356.58	88.8	99.7	95.8	99.8

Table 5.1212. Mean PAHs concentrations and percentage degradations for heavy molecular weight PAHs compounds.

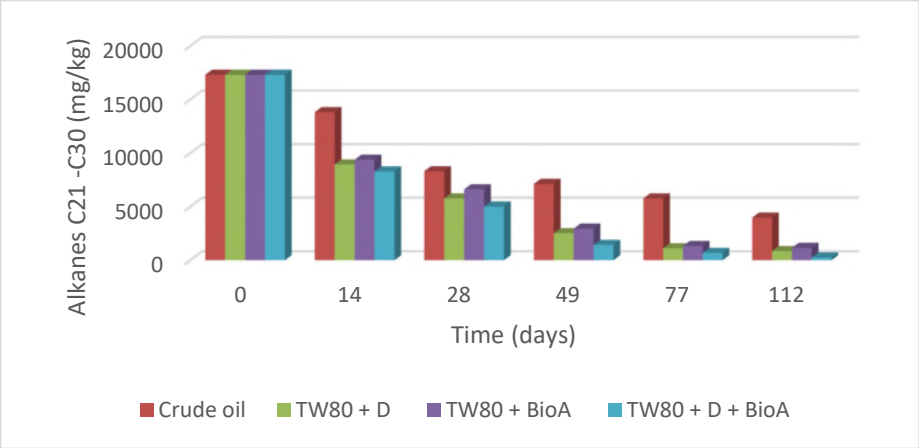
PAHs compounds	**Initial PAHs**	**Percentage degradation (%) at 112th day**			
	Concentration (mg/kg)	Crude oil	TW80 + D	TW80 + BioA	TW80 + D + BioA
Benzo[b]fluoranthene	334.8	93.0	99.7	99.2	99.9
Benzo[k]fluoranthene	186.0	93.5	99.4	98.3	99.9
Benz(a)pyrene	176.7	87.3	99.3	97.0	99.8
Benzo(ghi)perylene	818.4	89.4	99.9	98.5	99.9
Benzo[b)triphenylene	604.5	89.8	99.8	97.6	99.9
Indeno(123)[cd]pyrene	344.1	86.5	99.4	94.3	99.8



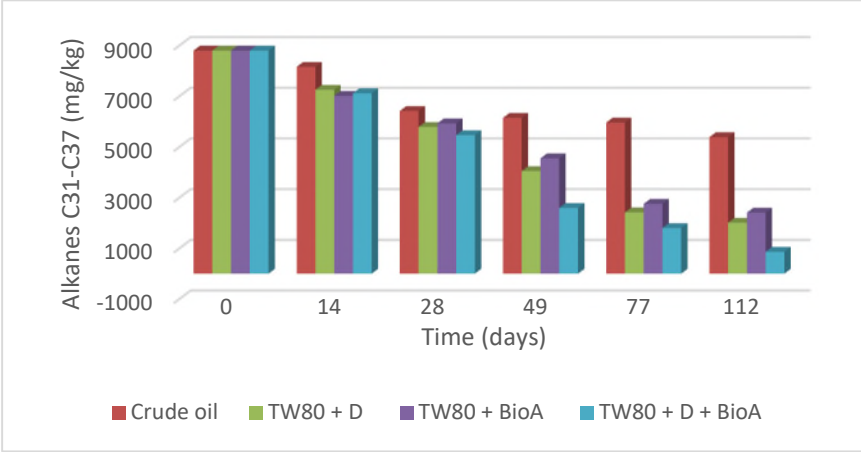
a. Alkanes C11 – C13 degradation



b. Alkanes C14 – C20 degradation

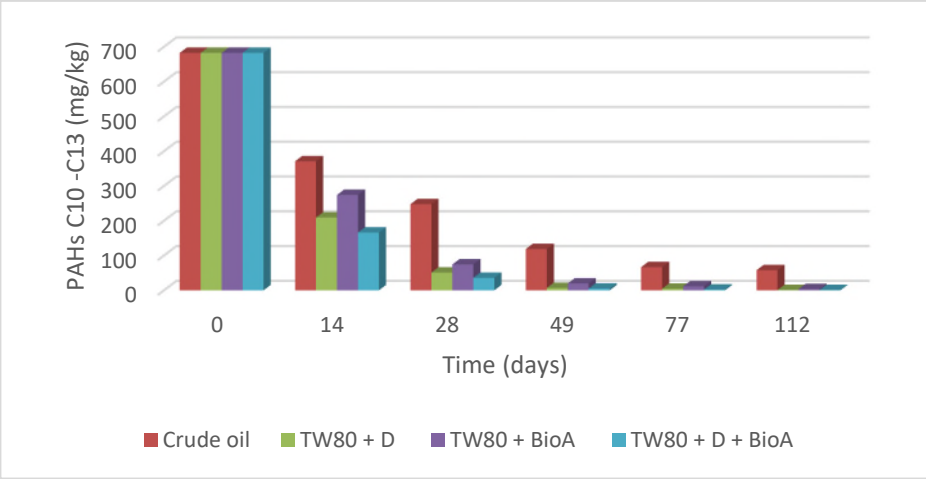


c. Alkanes C21 -C30 degradation

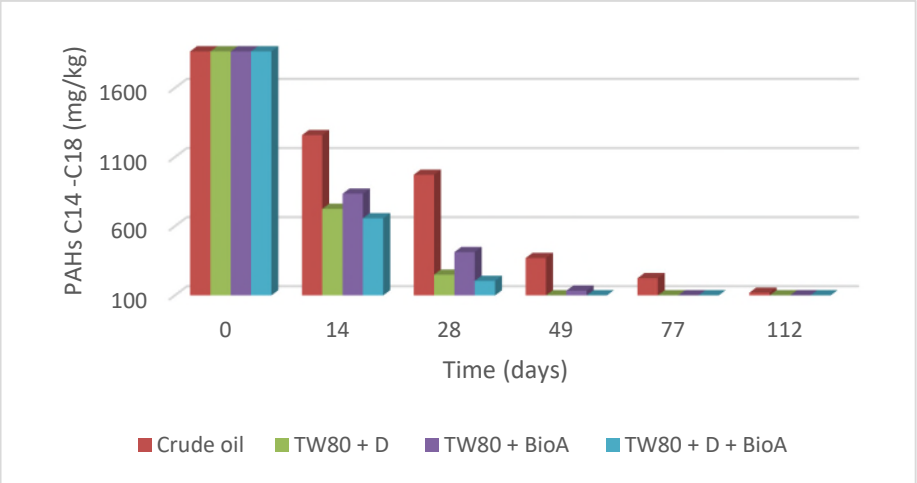


d. Alkanes C31 – C37 degradation

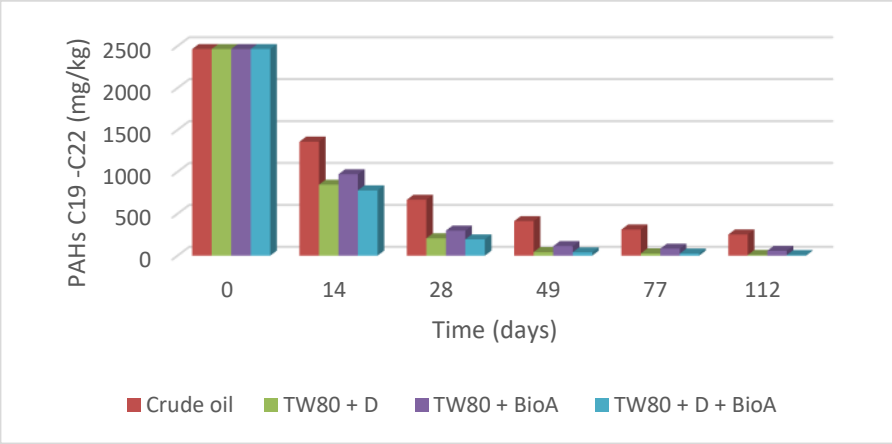
Figure 5.5. Alkanes degradation



a. PAHs C10 – C13 degradation

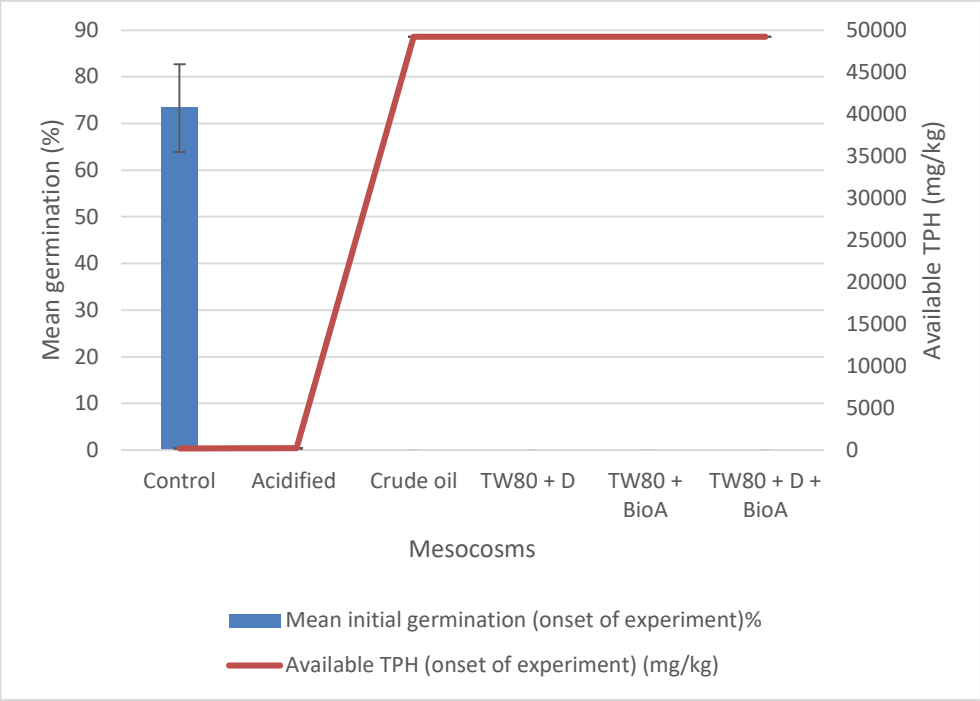


b. PAHs C14 – C18 degradation

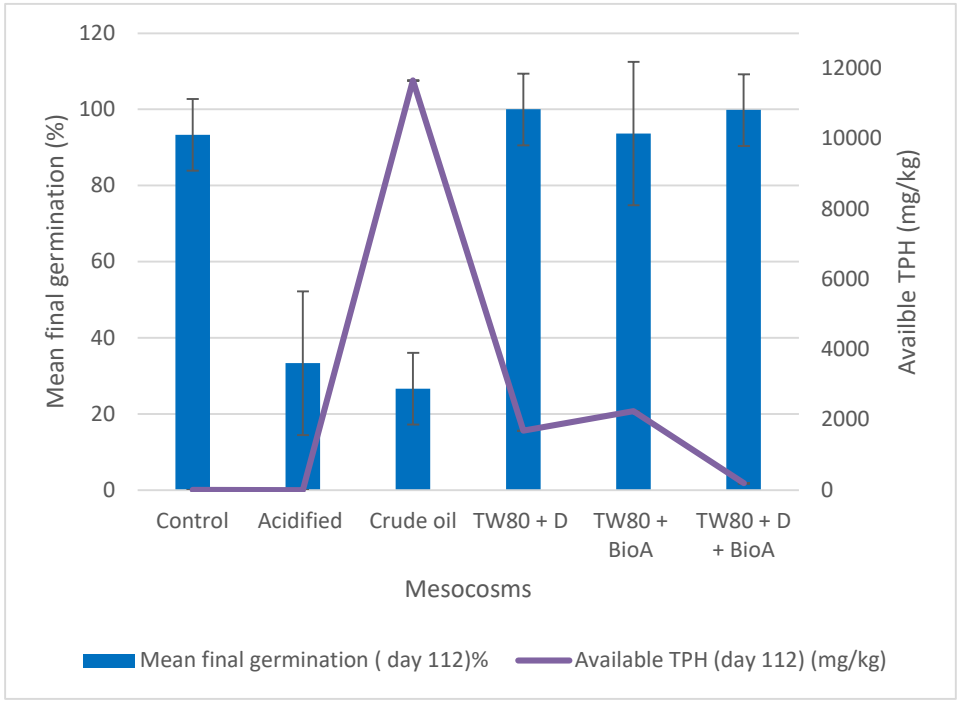


c. PAHs C19 - C22

Figure 5.6. PAHs degradation.



a. Plant germination versus available TPH at onset of experiment



b. Plant germination versus available TPH at day 112

Figure 5.7. Plant mean germination (%) versus available TPH (mg/kg).

5.5 Conclusion

This research had shown that petroleum hydrocarbon contaminants in acidified wetlands can be effectively remediated using low carbon combined strategies. The optimised TW80 + D + BioA mesocosms reduced the TPH content to > 98% of its original concentration of 50,000 mg/kg within 49 days. This reduction also translated into the least metabolic stressed mesocosms at day 112. The 100% maize germination in the optimised TW80 + D + BioA and no-detection of available TPH indicated that the mesocosm had the least environmental toxicity to the soil ecosystem and achieved remediation endpoint faster than the other treatments. The respiration rate (CO_2 production rate) of the combined mesocosms experiments positively correlated to the TPH degradation rate. Fungi dominated the optimised mesocosms at the onset of the experiment while the Gram-positive bacteria were the dominant microbial communities by the end of the experiment. This indicates that the Gram-positive led the hydrocarbons degradation in the mesocosms. Multiple evidence obtained from the optimised combined bioremediation strategies showed that biostimulation combined with bioaugmentation strategies improved the rate and extent of biodegradation of petroleum hydrocarbons and it is effective for ecological risk reduction in contaminated acidified wetlands. Following on from the achievements gained through these studies, there is now the need to investigate the use of this strategies under variable temperature and at field scale.

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6 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The aim of this research was to develop sustainable remediation approaches to accelerate the remediation of acidic wetlands impacted by petroleum hydrocarbons in the Niger Delta. This has been achieved through a series of studies from critical literature review to laboratory experiments. Given below are the conclusions drawn from the overview of this research.

6.1.1. A Critical Review on Existing Trends Towards Low Carbon, Sustainable Remediation Approaches and Recent Progress Made on Innovative Bioremediation Strategies

The research on the bioremediation of petroleum hydrocarbons in acidic wetlands soils was reviewed, by analysing its successes and failures. This review has shown that the current bioremediation techniques employed for the petroleum hydrocarbons remediation in acidic wetlands were inadequate, leaving the contaminants in the remediated soil still harmful to the surrounding environment. Over the years, low carbon remediation techniques have been adopted. This review examined the causes of failures of low carbon remediation in petroleum hydrocarbons contaminated wetlands. It was observed that inadequate characterization, limited knowledge of the contaminated wetlands ecosystem, and the negligible attention given to the nature of the contaminants (such as bioavailability and extent of weathering) were the primary factors enhancing the failures. These factors led to inadequate decisions on the low carbons nutrients to be used for remediation and influence the improvements made on the techniques. It was concluded that for sustainable, low carbon bioremediation techniques to achieve the required efficiency, and for remediation endpoint to be quickly established during bioremediation of petroleum hydrocarbons in acidic wetlands, sustainable biostimulants such as digestate with readily available nutrient and high biomass seeding potentials should be adopted.

This review demonstrated that digestate, which is a by-product of anaerobic digestion, is a sustainable low carbon biomaterial to use for remediation in acidic wetlands. The

review showed that digestate is an efficient biofertilizer and cost-effective soil amendment. Food waste digestate has been justified in this review as the most valuable digestate in terms of quality and availability of its nutrients to the soil. The review showed that digestate can lead to increased biomass in wetlands. The review further studied the efficacy of surfactants in enhancing the rate of bioremediation in acidic wetlands. It was revealed that Tween 80 surfactant is ecologically low risk and can increase the bioavailability of petroleum hydrocarbons in acidic wetlands, making the contaminants bioavailable for degradation by the microbial communities in the soil. The effects of bioaugmentation on acidic wetlands contaminated with petroleum hydrocarbons were also reviewed. Indigenous microbial consortia were identified to degrade petroleum hydrocarbon contaminants in acidic wetlands faster than other microbial consortia. Limited bioavailability of the petroleum hydrocarbon contaminants and inadequate modifications on bioaugmentation strategies are some of the causes of the limitations encountered in bioaugmentation. Therefore, to overcome relatively slow and inefficient remediation of petroleum hydrocarbons contaminated acidic wetlands, sustainable strategies to accelerate hydrocarbons degradation such as combinations of improved eco-friendly bioaugmentation, and low carbon biostimulation should be adopted. The efficacies of the proposed techniques can be ascertained using maize to determine the remediation endpoint.

6.1.2. Investigating the Effects of Food Waste Anaerobic Digestate Fiber and Non-ionic Surfactants on the Fate, Degradation, and Behavior of Hydrocarbons in Acidic Wetland Soil

This research had shown that acidified wetlands contaminated by petroleum hydrocarbons can be effectively remediated using low carbon stimulants such as Food waste anaerobic digestate (FWAD) and Tween 80 (TW80) surfactant. The Gram-positive bacteria were the dominant microbial group in the FWAD and TW80 surfactant mesocosms. The application of 30% FWAD, and 30% TW80 degraded the hydrocarbon contaminants in the acidified wetlands by 90%, and 86.8%, of TPH in 49 days respectively. The 30% FWAD was the least metabolic stressed mesocosms followed by 30% TW80 at the end of remediation when compared with the other mesocosms.

Therefore, 30% FWAD and 30% TW80 mesocosms showed the least environmental toxicity to the soil ecosystems and achieved remediation endpoints faster. This conclusion was further confirmed by the more than 90% maize germination alongside no bioavailable hydrocarbons detected at the end of the experiment in the 30% FWAD and 30% TW80 mesocosms. The extent and rate of hydrocarbons degradation was dependent on the CO_2 generation rate from the basal respiration of the soil microbial communities since the hydrocarbons were mineralised by the microbes to generate the CO_2. Multiple lines of evidence, shown through the spatiotemporal changes during the bioremediation strategies which include physical, chemical, and biological characteristics, defined the establishment of remediation end point in the wetlands.

6.1.3. Assessing the Efficacy of Indigenous Bacterial Consortia on Hydrocarbons Biodegradation in Acidic Wetlands

The research had shown that acidified wetlands contaminated by petroleum hydrocarbons can be effectively remediated using bioaugmentation strategies. The indigenous microbial consortia degraded the alkanes and PAHs in the acidified wetlands by 77%, and 91% respectively after week 7. The indigenous microbes were very effective in degrading the medium molecular weight (C11 – C16) alkanes and PAHs (C10 – C16) with about 97% alkanes (C11 – C16) and 95% PAHs (C10 – C16) degradation after week 7 respectively. The Gram-positive bacteria were the dominant microbial communities for the indigenous microbes and *B. subtilis* enriched mesocosms while the Gram-negative bacteria formed the dominant microbial communities in the *P. aeruginosa* enriched mesocosms. After 16 weeks of bioremediation, the indigenous microbes enriched mesocosms had the least environmental stress and least available hydrocarbons thereby achieving remediation endpoint faster. This was confirmed by the high germination (almost 90% germination) recorded in the indigenous mesocosm. A positive corelation, significant at $p \leq 0.05$, was established between CO_2 generation and hydrocarbons degradation in all the bioaugmentation mesocosms. This research has shown that the indigenous microbes (*Bacillus toyonensis* BCT-7112(T)), is effective in degrading petroleum hydrocarbons in acidified wetlands having outperformed the *Pseudomonas aeruginosa* and *Bacillus subtilis* which are prominently known for degrading petroleum hydrocarbons.

6.1.4. Evaluating the Efficacy of Optimised Combined Bioremediation Strategies and Defining Endpoints of Bioremediation for Acidic Wetlands

This research had shown that petroleum hydrocarbon contaminants in acidified wetlands can be effectively remediated using optimised low carbon combined strategies. The optimised Tween 80 plus digestate plus indigenous microbes (TW80 + D + BioA) mesocosms reduce the TPH content to > 98% of its original concentration of 50,000 mg/kg within 49 days. This also translated into the least metabolic stressed mesocosms at day 112. The 100% maize crop germination in the optimised TW80 + D + BioA and the scarce availability of the TPH indicated that the mesocosm had the least environmental toxicity to the soil ecosystem and achieved remediation endpoint faster than the other mesocosms. The respiration rate (CO_2 production rate) of the optimised combined mesocosms experiments positively correlated to the TPH degradation rate in all the optimized combined mesocosms. Fungi dominated the optimized mesocosms at the onset of the experiment while the Gram-positive bacteria were the dominant microbial communities by the end of the experiment. Multiple evidence obtained from the optimised combined bioremediation strategies showed that optimised biostimulation cum bioaugmentation strategies improved the rate and extent of biodegradation of petroleum hydrocarbons and it is effective for ecological risk reduction in contaminated acidified wetlands.

6.2 Recommendation for Further Studies

This research has improved the knowledge on remediating petroleum hydrocarbon contaminants in acidic wetlands. The extent of research done navigated through environmental engineering, microbiology, analytical chemistry, soil mechanics and biotechnology. The output presented here have led to some challenges which provides the framework for further studies. The suggestions for further studies are given below.

Studies on the efficacy of FWAD to remediate weathered hydrocarbons in wetlands should be investigated. The efficacy of FWAD in degrading petroleum hydrocarbon contaminants in saline sediments in the Niger Delta, Nigeria should be analysed to gain

insight into FWAD applicability in such challenging environments, as well as its ability to enhance the ability of indigenous microbes in saline sediments to degrade hydrocarbon contaminants.

Further studies on efficacy of indigenous microbes to degrade petroleum hydrocarbons in contaminated coastal and estuarine sediments should be considered. Research on combination of indigenous microbes (*toyonensis* BCT-7112(T)), *Pseudomonas aeruginosa* and/or *Bacillus subtilis* to degrade weathered petroleum hydrocarbons in wetlands and sediments should be considered. This will help to determine the synergistic capacities of these single microbes in order to improve the overall remediation efficacy by building on the natural capabilities of indigenous microbial communities in such difficult environment. Studies on the effect of indigenous microbes (*toyonensis* BCT-7112(T)) and TW80 on acidic wetlands contaminated with petroleum hydrocarbons should be investigated under seasonal variations in the Niger Delta since the behaviour and fate of petroleum hydrocarbons and the performance of remediation strategies are influenced by seasonal variation. Also, because we have pockets of small areas contaminated by weathered hydrocarbons in the Niger Delta's saline sediments and/or wetlands, research on the effect of TW80 on weathered hydrocarbon degradation in saline ecosystem should be considered to understand its potential as a remediation agent (by improving hydrocarbon solubility and dispersal, bioavailability), and facilitating hydrocarbon degradation by indigenous microorganisms, and/or its role in risk assessment and wetlands ecological restoration.

Despite the achievements gained through these studies, there is the need for application of these strategies at field scale. The effect of the optimised combined strategies in weathered petroleum hydrocarbons in wetlands and sediments under seasonal variation of the Niger Delta should not be neglected.

Finally, there is the need for improvement in the remediation policy and soil hydrocarbons targets in the Niger Delta from the current 5,000 mg/kg TPH to World Health Organisation standards for soil hydrocarbons. For effective remediation of hydrocarbon contaminants from the wetlands of the Niger Delta, the United Nations Environment Programme (UNEP) report on funding hydrocarbons remediation projects in Niger Delta, Nigeria should be adopted by the government.

7 APPENDIX