ASSESSMENT OF CRUDE OIL DEGRADATION AND HYDROCARBON UTILIZING BACTERIA ASSOCIATED WITH SOME BEACH SOILS IN GHANA

BY

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JULY, 2017
DECLARATION

I do hereby declare that this thesis has been written by me and that it is the record of my own research work. It has not been presented for another degree elsewhere. Works of other researchers have been duly cited by references to the authors. All assistance received has also been acknowledged.

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DEDICATION

This work is affectionately and humbly dedicated to my lovely wife, Mary Ama Serwah, my children, Derrich, Emma, Henrietta and Rexford, my caring Mother Emma Adjei and late Father Anthony William Andoh, my siblings and to all those who took an interest and encouraged me in my academic pursuit.
ACKNOWLEDGEMENT

Glory be to God for bringing me this far in my academic pursuit. I am highly grateful to my family, for every form of support and sacrifice throughout all these years of my education. I also want to specifically show much appreciative to my principal supervisor Dr. Innocent Y. D. Lawson who never gave up on me during this challenging period but kept guiding and encouraging me. I also acknowledge the contributions of Dr. Stella Asuming-Brempong my co-supervisor towards the shaping of this thesis. Not forgetting all the other lecturers in the Department of Soil Science who in one way or the other contributed to the outcome of this thesis.

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ABSTRACT

Ghana discovered oil in 2007 at Cape Three Point in the Western Region. It experienced its first low-based mud oil spillage in 2009 and 2010. Ghana is therefore exposed to possible oil spillage due to oil production in commercial quantities. It is against this background that the present study is aimed to assess the degradation along the coast and identify hydrocarbon utilizing bacteria (HUB) associated with some beach soils. The beach soils were taken from eight coastal towns; Aflao, Anloga, Ada, Accra, Winneba, Cape Coast, Takoradi and Axim. The soils were contaminated with crude oil at 10 g oil /kg soil and incubated under room temperature for a period of 60 days. The contaminated soils were sampled at ten (10) days interval for HUB population and quantity of crude oil degraded. The results showed a cumulative significantly (p < 0.05) increase in the amount of crude oil degraded with time in all the soils. There was variation in degradation potential along the coast. Ada showed significantly (p < 0.05) highest degradation potential followed by Anloga. However, Takoradi and Axim soils showed the least degradation potential but was significantly (p < 0.05) lower than the others. The hydrocarbon utilizing bacteria genera associated with the soils are Bacillus, Pseudomonas, Staphylococcus and Yersinia. The dominant HUB identified was Bacillus. Further research work should be conducted to determine the diversity of HUB and enhancement of oil degradation along the coast.
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<tbody>
<tr>
<td>AAS</td>
<td>Atomic Absorption Spectrophotometer</td>
</tr>
<tr>
<td>API</td>
<td>American Petroleum Institute</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>CNS</td>
<td>Carbon Nitrogen and Sulphur analyzer</td>
</tr>
<tr>
<td>DMAB</td>
<td>P-dimethylamino Benzaldehyde</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organisation</td>
</tr>
<tr>
<td>GERMP</td>
<td>Ghana Environmental Resource Management Project</td>
</tr>
<tr>
<td>LECO</td>
<td>LECO Corporation</td>
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<tr>
<td>LSD</td>
<td>Least Significant Difference</td>
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<tr>
<td>MI</td>
<td>Michigan</td>
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<tr>
<td>PAH</td>
<td>Polycyclic Aromatic Hydrocarbon</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethanolamine</td>
</tr>
<tr>
<td>USDA</td>
<td>United State Development Agency</td>
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<tr>
<td>USA</td>
<td>United State of America</td>
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CHAPTER ONE

INTRODUCTION

1.1 Background
Industrialized countries and the most powerful economies of the world today rely mostly on crude oil and its derivatives for use as fuel for their existence (Plohl et al., 2002). This in effect has led to a huge reliance on crude oil as energy source thereby leading to the gradual depletion of natural oil reserves. According to Medjor et al. (2012), the world demand for crude oil stood at 94.3 million barrels per day in the year 2010, and was expected to reach 101.6 million barrel per day in 2015. A report by the US National Research Council (2002) stated that products of hydrocarbon are the most used energy source for agriculture, transportation, construction and industries with an estimated annual production of over 2 billion tons worldwide. Out of this estimate, an annual release into our natural environment worldwide is over 1,300,000 thousand for a period between 1990 and 1999 alone, well over 260,000 tons of oil was released into the natural environment in North America from various sources each year (US National Research Council, 2002).

According to Henry (2001), out of the total oil released into the environment, 90% involves human activities such as deliberate waste disposal and about 30% of the spill enters water bodies. According to Abu and Dike (2008), about 1.7 and 8.8 million metric tons of oil are released into the world’s water and soil, respectively, every year.

There have been major oil spills over the world. For example, the Exxon Valdez oil tanker that hit a reef in Prince William Sound, Alaska, released over 40 million liters of crude within five hours in March 1989 and this spillage spread to over 1,500 km of coastal land with devastating effect (Hinton, 1995). Also in the Gulf of Mexico there was oil spill which occurred in the USA in April
Other oil spills that occurred were the Mumbai oil spill in India in August 2010 and the Persian Gulf oil spill in Kuwait in January 1991. In 2010, Nigeria experienced several forms of oil spill but the major one was the spill in Niger Delta from Exxon Mobil oil (Moss, 2010). Environmental pollution with petroleum and petrochemical products has been identified as a serious threat and a major problem to the environment and it is of concern to environmentalist (Alexander 1995, 2000). The study of distribution, fate and behavior of oil and its derivatives in the environment has gained interest in the world because of the toxic nature of most components of hydrocarbon product as it is easily incorporated into the food chain (Alexander, 1995, 2000; Semple et al., 2001, 2003; Stroud et al., 2007, 2009). Environmental pollution with hydrocarbon also results in a myriad of hydrocarbons causing different kinds of problems, for example long-term damage to aquatic and soil ecosystems, human health and natural resources. Atlas and Philip (2005), and Trevors and Saier (2010) noted that some crude oil constituents possessed carcinogenic and neurotoxic organic pollutants. The result of releasing hydrocarbon and its derivatives into the surroundings amid accident or by anthropogenic means antecedent water and soil contamination (Bankar et al., 2009). The effect of hydrocarbon contamination on farmland and crops cannot be over emphasized. Erhieguren (2017) noted that in Nigeria, a high percentage of oil spills that happened on the dry land within 1978 and 1979 simulated farm lands usually where food crops like rice cassava, plantain and many others are cultivated (Nwakanma et al., 2016). Onwurah (1999) stated that the yield of hydrocarbon contaminants affects sprout and neoplasm of some plants and affects soil fertility but the probability of the influence rides on the dosage and the kind of oil spillage. Joo et al. (2008) and Chaîneau et al. (2000) documented that petroleum contaminant on land influence unspecified physiochemical properties of the soil like the mineral and organic matter content, calcium, potassium, sodium, magnesium, redox properties.
and pH value (Onwurah et al., 2007). This change in physicochemical parameters of the soil usually occurs as a result of anaerobic condition created by the introduction of hydrocarbon contaminant resulting in waterlogging and acidic metabolites which release high amount of aluminum and manganese ions into the soil that become toxic to plant growth (Jaesche et al., 2006). Most microbes can use hydrocarbons as a source of carbon and energy for their metabolic activities, but their utilization rely on the chemical constituent of compounds inside the hydrocarbon and on surrounding determinants and biodegradability of these compounds (Van Hamme et al., 2003). These microbes are referred to as hydrocarbon utilizing bacteria and this form of degradation is known as biodegradation. According to Hamdi et al. (2007) biodegradation is known to be less costive and without influence on the environment with it application and has been found to be the favorable water and soil remediation approach for befoul area with hydrocarbon spills. Bioremediation can rely on the native microbes in the soil or water bodies, or bioaugmentation but the most effective method is biostimulation that concern the addition of nourishment and fertilizers to a polluted matrix (Tyagi et al., 2011). However, a number of limiting factors such as site characteristics, environmental factors (e.g., temperature, pH, electron acceptor, nutrient), the nature of the contamination, whether appropriate biodegradable genes are present, and the bioavailability of the contaminants to degrading microorganisms within the site have been recognized to affect the biodegradation of crude oil (Sabate et al., 2004).

1.2 Problem Statement

Ghana discovered crude oil in 2007 at Cape Three Points in the Western Region and started commercial production in the late 2010. The country experienced its first spillage in December 26, 2009, of about 584 barrels of low-based mud drilling fluid and another mud spill of 7 barrels occurred on March 23, 2010 (Lawson et al., 2012 and Ghanaian Daily Graphic, 2010). As an oil
producing country, the country is exposed to oil spill due to tons of oil being produced and transported along the coast and its environs.

1.3 Justification

From past records, when there is an offshore oil production there is possibility of oil spillage which spread to contaminate coastal lands. Hence, Ghana is exposed to possible oil spills due to tons of oil being produced and transported around the coasts and its environs as a result of commercial production of oil at offshore. Similar studies conducted in Ghana by both Lawson (2012) and Abeka (2014) on microbial degradation potential of some inland soils in Ghana contaminated with diesel oil. These previous research work did not address the degradation potential of all the beach soils in Ghana and also they both concentrated their research on diesel oil degradation. No work has been done to identify hydrocarbon utilizing bacteria and crude oil degrading potential of the soils along the coast of Ghana. Hence, the unavailability of preliminary information or data on potential degradation due to limited research and hydrocarbon utilizing bacteria in the coastal soils in Ghana could delay or holdback remediation in the event of oil contamination.

1.4 Main objective of the Study

There is the need to undertake a scientific study along the coast of Ghana to assess degradation and presence of hydrocarbon utilizing bacteria. It is against this background that the present study is aimed at assessing the degradation of crude oil and identifying hydrocarbon utilizing bacteria in some selected beach soils along the coast of Ghana.
Specific objectives:

- To determine crude oil degradation potential of soils along the coast of Ghana
- To isolate and identify hydrocarbon utilizing bacteria in the soils along the coast.

1.5 Hypothesis:

**HO:** Coastal soils in Ghana harbours hydrocarbon utilizing bacterial and has potential to degrade crude oil.

**HA:** Coastal soils in Ghana do not harbour hydrocarbon utilizing bacteria and do not have the potential to degrade crude oil.
CHAPTER TWO
LITERATURE REVIEW

1.0 Introduction.
In order to understand the assessment of crude oil degradation and hydrocarbon utilizing bacteria mostly associated with beach soils, one would first define crude, their types and implications for degradation. Further discussion would be done on sources and impact of crude oil contamination on agriculture, soil, coastal and other land communities. Further, various types of remediation methods, conditions that affect remediation of crude oil contaminated soils and some microorganisms and other factors that associated with crude oil degradation shall also be discussed.

2.1 Crude oil
Crude oil has been defined by many scientists usually on the bases of their origin, component, presences of hydrocarbon and their chemical compositions. According to Wiehe and Kennedy (2000), crude oil consists of a blend of fossil material, mainly of plant origin and consists of thousands of organic and a little several of inorganic compounds. Rodgers et al. (2005) further explained that crude oils differed immensely in chemical composition relative concentrations of different chemicals, and in physical properties. Rodger et al., further stated that no two components are similar and such crude oils are advanced to generate a wide variety of refined and residual yield. However, Balba et al. (1998) defined crude oil as a natural product that developed from an anaerobic transformation of biological matter beneath sea bed with high temperature and pressure. Kvenvolden (2006) and Yemashova et al. (2007) defined crude oil as a combination of compounds in petroleum products that are all composed completely from carbon and hydrogen thus denoting
“hydrocarbon”; and combination of crude oil consisting of different heteroatomic and non-heteroatomic hydrocarbons, respectively (Olajire and Essien 2014). Crude oil is inherently occurring compound, made up of basically hydrocarbon with different elements like oxygen, Sulphur, nitrogen and others, coming out in a form of organic compound which in some cases form complexes with metals (Bland and Davidson, 1983).

2.2 Types of crude oil and their implications for degradation

Crude oil is a baroque combinations of dissimilar hydrocarbons involving aliphatic (linear or branched), cycloalkanes, mono- and polyaromatics, asphaltenes and resins and bulks of these compounds are sturdy, poisonous, and carcinogenic (Philip et al., 2005; Yemashova et al., 2007). Degradation in any form relies on the type of crude oil involved with the spillage, crude oil compounds like alkanes, benzene, toluene, ethyl benzene, xylene and others like polycyclic hydrocarbons (PAHs) are biodegradable under correct environmental form (Chaillana et al., 2006; Van Hamme, 2003). Most crude in proper environment in which carbons are arranged in a straight chain usually break down faster than those whose carbons are arranged in branched or multiple chains; example polycyclic aromatic compounds (Vidali, 2001). However, the utmost recusant fragment crude oil such as resins and asphaltenes can exist for millennia (Vidali, 2001).

Crude oil with a single bulky slick create a less surface area for the microbes to become access to the crude oil, making degradation slower (Merkl et al., 2005). On the other hand, heavy and viscous type of crude oil, enhance the diffusing biodegradable constituent into a thick matrix to the crude oil-water interface for microbes to have access to them II the oil is heavy and viscous, the biodegradable components must first diffuse through the thick matrix to the oil-water interface so that the microbes can access (Chaillana et al., 2006; Merkl et al., 2005). This implies that the lighter
the crude oil, the faster its diffusion, making the biodegradable compounds more available to the microbes (Chaillana et al., 2006; Merkl et al., 2005).

2.3 The Mechanisms of crude oil Degradation

Degradation of organic compounds consists of numerous mechanisms. In the course of crude oil degradation, bacterial are selected based on their capability to breakdown substance molecule involving several mechanisms (Das and Chandran, 2001). The mechanisms for degradation of crude oil components is based on two procedures namely: (a) co-metabolism and (b) growth, with the bacteria being electron acceptor for degradation of crude oil molecules (Fritsche and Holfrischter, 2005). According to Fritsche and Holfrischter (2005), co-metabolism involves the breakdown of an organic compounds with the company of growth substrate that serves as a primary source of carbon and energy and further deduced that, the mechanism of degradation relies on crude oil enzymes system. Angelidaki and Sanders (2004) also explained growth mechanism as the way bacteria utilize organic carbon as a source of energy and carbon; also refers to as mineralization of organic contaminant. Das and Chandran (2011) mentioned other two mechanisms as: production of surfactant and attachment of microbial cells to the substrate. Hydroxylases and dioxygenase monooxygenase enzymes play vital function in degradation of crude oil by microorganisms (Fritsche and Holfrischter 2005). The breakdown of crude oil in contaminated soils involves as consortium of microorganism with different enzymatic abilities to breakdown crude oil contaminated soils site (Fritsche and Holfrischter 2005). This is because crude oil consists of complex mixture of organic compound which needs a microbial community with individual species having different genetic information required to breakdown the complex mixture in crude oil (Fritsche and Holfrischter 2005).
2.4 Sources of crude oil contamination

Relatively there is over five million tons of hydrocarbon contaminants step into the surroundings annually as a results of antecedent of crude oil contamination by human influence such as oil spills (Hinchee and Kitte, 1995). Other sources of hydrocarbon pollution include leakage from reservoirs, refueling of vehicles, rubbles of hydrocarbon tankers conveying oil and inappropriate discard by mechanics working on damaged tankers (Ofoegbu, 2014; Hill and Moxey, 1980). Crude oil spill contamination could also emanate from marketing of crude oil products, pipeline overflow, breakage, and reservoirs tank spill (Ofoegbu, 2014; Henry and Heinke, 1996; Obire and Wemedo, 1996).

2.4.1 Impact of crude oil contamination on soil ecosystem and agriculture

Soil is a key receptor of crude oil spill as well as many diverse kinds of products and chemicals. As soon as the crude oil enters the soil, crude oil form part of the biological cycle, thereby influencing all shapes of life supported by that surrounding (Mbah et al., 2009). The premise influence of crude oil contamination on soil ecosystem is the loss of soil fertility. This effect decreases the productive capacity of the polluted soil, creating nutritional imbalances at the spilled sites (Chorom et al., 2010). The aftermath of crude oil contamination affect plant reproduction, removes most vegetative cover and in the end set off soil erosion (Merkl et al., 2005). There is a general reduction in plant growth due to direct toxic effect on plants as a result of oil spill on agriculture land and it causes reduction in germination of plant seeds (Udo and Fayemi, 1975).

Olajire and Essien (2014) documented that crude oil pollution in soil ecosystem precipitate an imbalance in the carbon-nitrogen ratio (C: N/ratio) at the crude oil contaminated area, this in effect creates a nitrogen deficiency in oil impregnated soil, slowing the neoplasm of bacteria and the usage of carbon sources. In addition, voluminous absorption of biodegradable organics in the top
layer soil devour oxygen reserves in the soil and retard the rates of oxygen diffusion into deeper layers (Barathi and Vasudevan, 2001).

### 2.4.2 Impact of crude oil contamination on the environment

Most components of crude oil especially diesel oil is found to cause cancer, mutagenic and it is a potent immuno-toxicants serving as hazard to human and animal health (Lawson et al., 2012; Boonchan et al., 2000 and Samanta et al., 2002). Consequently, affecting all forms of life including living organisms and on the economy of the inhabitants (Etuk et al., 2013). There is also a major concern over the various contaminants that are released into the environment in the cause of crude oil spill; a combination of pollutants like as salts, organics, alcohols, phenols, acid, radionuclides and heavy metals such as zinc, cadmium, mercury, copper, chromium, lead etc. at differing amounts are released into soil, groundwater, and or waste water (Olajire and Essien, 2014; Pathak et al., 2010 and Olajire et al., 2008).

Usually volunteers of local communities involved themselves in the mobilization of clean-up of oil spill spillage occurs in other to minimize the effect on natural and economic resources and to regain the environment in earliest time. This in effect exposed the in habitants to health risk of the noxious properties of the oil (Orion and Lotanna 2013; Aguilera et al, 2010). Crude oil toxicity affects the liver congestion of the liver, fat degeneration, and dissociation of hepatocytes (Odion and Lotanna 2013; Sathishkumar et al., 2008).
2.5 Remediation

Remediation of crude oil contaminated environment is a serious concern to an environmentalist and can be very expensive when using standard systems of oil clean-up example, the use of dispersants, manual removal, use of chemicals, burning, cutting vegetation, passive collection of sorbents, debris removal, trenching, disposal of sediment, slurry and blasting, excavating and many others (Odion and Lotanna 2013 and Michel et al., 2010).

2.5.1 Volatilization

Naturally occurring volatilization influence spontaneous delineation of air releasing through the soil. An air is made to come out or release from the soil by causing a draft fan into the soil through a slotted or screened pipe, for air to whiles the entrainment of soil particles is defined. (Sparks, 2003). Certain treatments, e.g., activated carbon, are employ to regain the volatilized pollutant. This method is restricted to volatile organic carbon substance (Sparks, 2003)

2.5.2 Phytoremediation

Plants are said to (phytoremediation) effectively decontaminate soils and water that has been contaminated with hydrocarbon or crude oil. It is an emerging technology that pledges productive, affordable, and less interfering means of remediation of oil-contaminated surrounding (Onwurah et al., 2007 and Stomp et al., 1993). According to Onwurah et al., (2007) and Schnoor et al. (1995) and there are known crude oil toxicity resistance plants that are known to have the capacity to remediate hydrocarbon contaminated soil; among them are miscanthus grass (elephant grass), black poplar and willows. According to Spark, (2003) Sunflowers have been found to be hyper accumulators of uranium, on the other hand, some ferns lofty habitude for arsenic, alpine herbs absorb Zn, mustards can take up lead, clovers absorb oil, and poplar trees devastate dry-cleaning solvents and some hundreds of plant species that can detoxify pollutants (Spark, 2003). Onwurah
(2007) documented that source uptake of crude oil into plant tissues; loose an enzyme, exudates simulating the condition of hydrocarbon clastic microbes and direct biochemical conversion (enzymes) of crude oil. Onwurah (2007) documented three research mechanisms by which plants decontaminate crude oil contaminated environments and these are enhanced in the degradation of the pollutants in the rhizosphere due to mycorrhizal fungi and the condition of soil microbial consortia (Onwurah (2007 and Schnoor et al., 1995). One major hindrance in this form of remediation is that the plants in effect compete with the hydrocarbonoclastic bacteria groups for accessible nitrogen and phosphorus (Vega-Jarquin et al. 2001 and Onwurah 2007).

2.5.3 Leaching

According to Sparks (2003), for a leaching technique to be efficient, it requires the soil to be porous, very permeable, and homogenous. Spark (2003) stated that the method of leaching also comprises of leaching the soil with water and often with a surfactant to extract the contaminant and the leachate is then received downstream of the area, applying a library of methods for handling and or dumping. The disadvantage of the use of this approach has been restricted because considerable amount of water is applying to extract the pollutants or the contaminants and, at the end waste stream becomes very huge making disposal very costly to undertake (GOK, 2012 and Sparks, 2003).

2.5.4 Vitrification

In situ vitrification (ISV) is a process of applying heat treatment into earth at a temperature of between 1400°C and 2000°C with maximum depth of soil, approximately 6.09 m, in a single treatment (Oppelt, 1995). Sparks (2003) also elaborated that with vitrification process hydrocarbon pollutant are hardened with an electric current. This causes the immobilization the pollutants for
as long as 10,000 years. However, because the system employs huge amount of electricity, the technique is regarded as being costly (GOK 2012).

2.5.5 Isolation or Containment

This method involves the utilization of surface physical barriers for instance clay liners and slurry walls to deprecate lateral migration thereby holding the contaminant in place (Xu et al., 1997). Environmentalist and other researchers have come out with surfactant addition to clay minerals (organo-clay) which ease the retention of hydrocarbon compounds or the organic pollutants (Sundararajan et al., 1990).

2.5.6 Passive remediation

The technique involves the use of natural courses, such as biodegradation, aeration, volatilization and photolysis to naturally effect decontamination of the polluted site. It is simple inexpensive and demands keeping an eye on the area (Spark 2003). According to GOK (2012) soil permeability, groundwater depth, infiltration, and the nature of the pollutant, biodegradation, adsorption, volatilization, leaching, and photolysis are conditions that this method depends on (Leavin and Gealt, 1993).

2.5.7 Heat treatment

Gan et al. (2009) reported different types of thermal or heat treatment that can be applied to soil polluted with hydrocarbons. These heat treatments include electric heating with temperature above 100°C injection of hot water to reduce viscosity of the oil, injection of hot air to evaporate compounds in soil with high pressure and steam injection to reduce viscosity of oil. Ron et al. (2000) noted that, the heat treatment is a model for contamination treatment technology that involves the combustion of contaminants and is a convenient method to minimize pollution and
harmful emissions in the environment. According to Leavin and Gealt (1993) heat treatment creates a very high temperature or heat (about 500°C) in the excavated soil employing a heat incinerator (Spark 2003). This large temperature separates the pollutant, brings out the volatiles which are then received, pushed through an afterburner and combusted or recaptured with solvents Spark (2003).

2.5.8 Asphalt incorporation

The application of this technique, contaminated or tainted the soils are excavated and placed in hot asphalt mixers and then applied in paving (GOK (2012 and Spark 2003). The asphalt and contaminated soil are heated while they are in the mixer. This creates volatilization or decomposition of certain of the pollutants. The remaining pollutants are then immobilized in the asphalt (GOK (2012 and Leavin and Gealt, 1993).

2.5.9 Solidification or Stabilization

Kumar et al. (2011) explained the technique as addition of an additive to excavated hydrocarbon or crude oil contaminated soil in order for contaminants to encapsulate and then disposed them to a landfill site. Hence, the pollutants become immobilized; hence, not able to devastated. The technique has been used to reduce inorganic contaminant polluting the soil ecosystem (Spark 2003).

2.5.10 Chemical extraction

Spark (2003) and Riser-Robert (1998) reported the use of solvent, surfactant, or solvent/surfactant mixture to mix with an excavated polluted soil to extract contaminants or pollutants from hydrocarbon or other organic polluted environment. The soil is then bathed or aerated to remove the solvent/surfactant and the latter is then filtered for fine particles and treated to take off the
pollutant. The method is very costly because of the cost involved and is not often used or practiced (GOK 2012 and Weans and Okieimen, 2011).

2.5.11 Excavation

According to Selberg (2013) and Kumar et al. (2011) first line of this method is the discarding and disposal of the contaminated soil from the tainted area to a landfill site. The landfill site must be located in an area which does not pose any hazard to human and must hold lining material like clay, that reduces the mobility of the pollutants, or where the soil permeability is low (GOK 2012 and Kumar et al. 2011). The various demerits of this method include requirement of a sizeable land area often posing hazard problems for the community in vicinity, high cost of disposal and also drawback challenge, protection treats, odour yield, possible runoff and groundwater pollution challenges.

2.6 Bioremediation

According to Odion (2013) and Diaz (2004) bioremediation is a mechanism by which organic substances are separated into fine component by the enzymes produced by indigenous organisms converting the substance through metabolic or enzymatic procedures. The biogeochemical complement of microorganisms seem almost confines, and it is often said that microorganisms are “Earth’s greatest chemists” (Madigan et al., 2012). The conditions of these “little chemists” have been exploit in many ways. Bioremediation is becoming one of the most auspicious technologies for the disposition of petroleum hydrocarbons contaminant in the environment. In view of this, the screening of potential crude oil degrading organisms is one of the fundamental measures to degrade crude oil contaminated soil, hence screening for hydrocarbon utilizing bacterial was carried out by DCPIP (Dichlorophenolindophenol) method as described earlier by Hanson et al.
(1993) and Bidoia et al. (2010). Youssef et al. (2010) reported that the initial approach of defense against oil pollution in the environment is the microbial population presence.

2.6.1 Indigenous microbial community in the environment

There are adequate indigenous microbial communities (regular soil, beach soil and sea soil) suitable for broad degradation of hydrocarbons with favorable conditions that help in their oil-degrading metabolic activity (Gerson, 1985). Relying on indigenous microorganisms to degrade hydrocarbons rather than biostimulating (addition of microbes) is far advantageous. To do this, the indigenous microbial population must evolve for many years and must be acclimatized for survival and booming in that environment Olajire and Essien (2014). Also, the capacity to use hydrocarbons as carbon source is distributed amidst a diverse microbial population. These diverse microbial populations come about in natural environment and either independently metabolize various hydrocarbons or form a consortium (Gerson, 1985). In many cases, if the quantity of microbial population is high in the contaminated environment, microbial seeding may not be necessary. Microorganisms (bacteria and fungi) have different rates at which they utilize and degrade hydrocarbons in the soil or water (Olajire and Essien 2014). The application of microorganisms to break down petroleum hydrocarbon developing from oil spillage has been a serious concern for environmentalist (Atlas 1981; Olajire and Essien 2014).

2.6.2 Microorganisms associated with the degradation of crude oil

The frequent use of crude oil-based contaminants has precipitated in improved microbial consortium capable of surviving toxic contamination (Chikere 2009). Several research findings have it that bacterial community composition in hydrocarbon-polluted sediments usually comprises of mostly bacteria that are outstanding to conform source of use by crude oil as carbon sources (Chikere 2009; Engelhardt et al., 2001; Iwabuchi et al., 2002; Kasai et al., 2002). These
hydrocarbon utilizing microbes are sensitive to changes in their surroundings, that is anytime the chemical or physical environment changes suddenly the community adapts to the change or new condition by entering into a lag period or acclimation period that enhances the micro-organisms to cultivate the metabolic reservoir require for their survival (Leahy and Colwell, 1990; Chikere 2009 and Okpokwasili, 2004; Nweke and Okpokwasili, 2004). Studies by Wackett (2003) stated that hydrocarbon utilizing bacteria are found everywhere in the surrounding, specifically in the oil-contaminated environment. Microorganisms have been found to be applicable in biodegradation systems, even though many studies have been on bacteria in the lately. Although a wide phylogenetic diversity of microorganisms is capable of aerobic degradation of pollutants, Pseudomonas species and closely related organisms have been the most researched widely due to their capability to degrade many diverse pollutants. The oil-degrading populations are widely spread in the lands and water bodies (Olajire and Essien 2014). Rosenberg and Ron (1996); Abed et al. (2002); Head et al. (2006); Yakimov et al. (2007) also indicated that bacterial communities in sediments are significant in the marine food web, where they are responsible for re-cycling of nutrients and degradation of pollutants. Alex (2012); Bordenave et al. (2004) and Edlund and Head et al. (2006), also researched on oil-impacted marine surrounding also showed that some of these bacteria are multiplying and speedily chosen when crude oil degradation is spiked by addition of nutrients. The documentation on the number of the bacterial populations in a contaminated area is of distinct importance in order to assess the self-purification abilities of the ecosystem and the attainability of biological decontamination if engineered bioremediation should be acclimatized (Iwabuchi et al., 2002; Kasai et al., 2002; Xu and Obbard 2003; Xu et al., 2004; Head et al., 2006; Allen et al., 2007; Said et al. 2008). Wolfgang (2007) research certain characteristic bacteria with wide crude oil biodegradative capacities both Gram negative and Gram
positive bacteria as *Pseudomonas spp*, *Acinetobacter spp*, *Alcaligenes spp*, *Flavobacterium spp*, *Xanthomonas spp*; and *Norcardia spp*, *Mycobacterium spp*, *Corynebacterium spp*, *Arthobacter spp* *Bacillus spp*. For instance, a publication by Roy et al. (2014) suggested that up to 39 indigenous hydrocarbon degrading bacteria can be found at polluted area and that these were dominated by the *Pseudomonas* genus. The work of Suja et al. (2014) also documented *Pseudomonas* bacteria and other strains. In virtually all cases of field tests of bioremediation in the literature either indigenous or naturally occurring bacteria added in the bioremediation treatment have been utilized. The notion of utilizing “genetically engineered” micro-organisms still envisage regulatory challenge because of the unknown outcome that may ensue the release in nature of such manipulated micro-organisms (Megharaj et al., 2014).

2.7 Soil ecosystem

Whenever there is occurrence of crude oil spillage, petroleum components bind to soil constituent, and they become very hard to be disposed or degraded (Barathi and Vasudevan, 2001). According to Dhaker and Jain (2011), crude oil spillage in soil develops in an imbalance in the carbon-nitrogen ratio at the polluted site, since carbon and hydrogen are the main component of hydrocarbon and in effect nitrogen becomes deficiency in crude oil contaminated soil, thus slowing down the growth of bacteria and the utilization of carbon sources. However, Jain et al. (2010) elaborated that considerable concentrations of biodegradable organics in the top layer soil run out of oxygen reserves in the soil and retard the rates of oxygen diffusion into deeper layers. Many indigenous microorganisms in water and soil are capable of degrading petroleum contaminants. (Bajpai et al., 2011) reported that degradation of complex mixtures of hydrocarbons such as crude oil and metals in soil demands a hybrid population with universal enzyme capacities. Compounds of hydrocarbons are known to be degraded by diverse group of microorganisms in soil that have
the ability to use hydrocarbon as carbon source; but bacteria are the most functional agents that works as primary degraders in crude oil contaminated soils (Das and Chandran 2011; Rahman et al., 2003).

2.7.1 Aquatic ecosystem

Aquatic ecosystem has been contaminated as a consequence of oil spills and the public health is delicately threatened. Researchers have estimated that, to recover all the damage that crude oil spill has caused, clean-up will take up to 30 years (Okwoche, 2011). There is a finite surface area accessible for microbial activity in aquatic ecosystem due to considerable masses of mousse, tar balls or high concentrations of oil in sleepy environments that tend to persist, since dispersion and emulsification of oil in slicks appear to be requirement for quick biodegradation (Okwoche, 2011). This makes microbial degradation a major mechanism for the removal of spilled oil and dispersants from aquatic environment (Hamzah et al., 2013). Yanto and Tachibana (2013) demonstrated that *Fusarium sp.* has the capability to degrade chrysene and the aliphatic fragments of crude oil contaminating liquid culture with artificial sea water. The micro aromatics (benzene, toluene and xylene) evaporated quickly occurred after the 4th day of incubation (Kumari and Abraham, 2012). Microorganism isolated from crude oil contaminated water that were found to be effective in degrading petroleum compounds were yeast species of *Candida lipolytica, Rhodotorula mucilaginosa, Geotrichum sp, and Trichosporon mucoides* (Bouguslawski and Dabrowski 2001; Kumari and Abraham 2012).

Different forms of reclamation, disposal and the containment of oil are undertaken after crude oil spill occurs on aquatic ecosystem. Conventionally different methods are used to remove oil from aquatic ecosystems and these include chemical clean up and microbial degradation mechanical clean up (Hamzah et al. 2013). The mechanical removal of spilled oil and dispersant is nearly
unattainable in “protected” ecosystems; hence the major process of eliminating crude oil spill and microbial degradation is the major mechanism for the elimination of spilled oil and dispersants from aquatic environment (Hamzah et al., 2013).

2.7.2 Marine ecosystem

McGenity (2012) numerated some conditions that are required for microbial response on crude oil spill at sea and these are oil composition and degree of weathering, as well as environmental conditions, specifically temperature and nutrient level. Notwithstanding, there are some typical patterns including considerable multiplication in abundance of Alcanivorax spp., that degrade straight-chain and branched alkanes (Kostka et al., 2011; Coulon et al., 2007), followed by Cycloclasticus spp., which degrade PAHs (Head, 2006). Nine bacteria isolate namely Pseudomonas fluorescens, P. aeruginosa, Bacillus subtilis, Bacillus sp., Alcaligenes sp., Acinetobacter Iwoffi, Flavobacterium sp., Micrococcus roseus, and Corynebacterium sp. from polluted tropical stream in Lagos, Nigeria were reported by Adebusoye et al., (2007) as being responsible for the degradation of the crude oil. Acinetobacter spp. are the most commonly isolated from crude oil-contaminated marine ecosystem with a diverse array of alkane hydroxylase network that enhance them to breakdown both short- and long-chain alkanes (Ron and Rosenberg, 2010; Rojo 2009). Alcanivorax borkumensis also has a host of diverse adaptations to access oil and to survive in open marine environment (e.g. synthesis of emulsifiers and biofilm formation (Schneider et al., 2006; Coulon et al., 2007).

2. 8 Aerobic bioremediation of hydrocarbons

Wolfgang (2007) defines aerobic biodegradation as the breakdown of organic pollutants by microorganisms in the presence of oxygen. He elaborated as microbial catalytic process occurring only in presence of oxygen; hence, the chemistry of the system, environment or organism is
characterized by oxidative factors. Most organic pollutants are increasingly degraded under aerobic conditions. Aerobic microorganisms Some have an oxygen based metabolism where aerobes through cellular respiration, use oxygen to oxidize substrates in order to obtain energy. Before cellular respiration begins, glucose molecules are broken into smaller molecules in the cytoplasm. Oxygen of the cells is used in the chemical reactions that breakdown organic molecules into H₂O and CO₂ in a reaction that releases energy. Microorganisms involve in utilizing aerobic condition to biodegrade hydrocarbon include *Rhodococcus sp.*, *Burkholderia xenovorans*, *Pseudomonas spp* (McLeod and Eltis, 2008). The important characteristics of aerobic microorganisms degrading organic pollutants are metabolic steps for optimizing the contact between the microbial cells and the organic pollutants, the chemicals must be accessible to the organisms having biodegrading activities; for instance, hydrocarbons are water-insoluble and their degradation requires the production of biosurfactants and the initial intracellular attack of organic pollutants is an oxidative process. The activation and incorporation of oxygen is the enzymatic key reaction catalyzed by oxygenases and peroxidases. Sugars required for various biosynthesis and growth must be synthesized by gluconeogenesis (Wentzel et al., 2007). The most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic conditions. The process of aerobic degradation differs based on the microbes involved in the transformation pathways (Das and Chandran, 2010). Bacterial metabolism is different from that of fungi which is either lignolytic or non-lignolytic (Wentzel et al., 2007). Degradation of benzene (aromatic) by bacteria, the ring of the Polycyclic Aromatic Hydrocarbons (PAH) is oxidized to cis-dihydrodiols by the action of dioxygen as enzymes, followed by the dehydrogenation of these dihydrodriols by dehydrogenase enzymes to form dihydroxylated intermediates and with further
metabolism using catechols, carbon-dioxide and water are formed (Cerniglia, 1993, Cerniglia, 1997 and Juhasz and Naidu, 2000)

### 2.8.1 Microbial enzyme activity

Microbial enzymes may appear from biotic (viable cells) or abiotic (extracellular) constituent and their very distinct reactions may only permit a small fragment of the total population being determined in the test (Parson, 2004). In effect, soil enzyme activities may not always show a strong relation with other soil biological parameters (Albiach et al. 2000). Microbial enzyme activity assay is a unique and fast approach to determine the cycling of nutrients in soil. Dehydrogenase is involved in the biological oxidation of organic compounds and has been found in relation with the oxygen uptake and organic substrate removal rates in aerobic systems (Pepper et al., 1995). In unnaturally contaminated soils, abiotic processes such as volatilization and adsorption on soil colloids (clay minerals and humus particles) play a vital role in the decontamination of petroleum hydrocarbons (Bollag, 2008). Part of the mineral oil hydrocarbons added to contaminated soils remained undetected (Mehrasbi et al. 2003). N-alkenes with the intermediate chain length (C10 - C24) are degraded most rapidly (Vashishtha 2011; Mehrasbi et al. 2003). Very long chain alkenes like gas oil are increasingly resistant to biodegradation (Atlas, 1998). Microorganisms have enzymes that degrade and utilize diesel oil as a source of carbon and energy (Ezeji et al., 2005). Aliphatic and aromatic hydrocarbon compounds could be biodegraded under both aerobic and anaerobic conditions. (Van Hamme 2003).

### 2.9 Conditions for bioremediation

The main conditions that affect bioremediation of crude oil contaminated soils are the pH, soil moisture, oxygen supply, the nutrient level, bacterial diversity and the temperature, among which
the impact of the petroleum hydrocarbon degrading bacteria on the effect is critical (Chaillan et al., 2006; Annupama et al., 2009)

### 2.9.1 pH range

The rate of biodegradation is highest at pH near neutrality and extreme pH range (pH 7.5-10.6) alkaliphilic bacteria are mostly efficient in degrading phenols from waste water and highly alkaline lake and industrial effluents at this optimal range (Alexander, 1999). The importance of soil pH to microorganism cannot be overemphasized because most microbial species can only survive within a certain pH range since soil pH can affect nutrient availability and is found to be very variable, ranging from 2.5 in mine soil to 11.0 alkaline desert soil (Bossert and Bartha, 1984). Roberts (1998) reported an optimum pH for biodegradation between 6 and 8. Notwithstanding, effective biodegradation can be also found outside this range. Soil pH may affect the solubility, mobility, and ionized forms of contaminants. Microbial activity in the soil is greatly affected by pH, through the availability of nutrients and toxicants and the tolerance of organisms to pH variations (Bossert and Bartha, 1984).

Soil pH can affect the solubility or availability of macro- and micronutrients, the mobility of potentially toxic materials, and the reactivity of minerals. A near doubling of rates of biodegradation of gasoline in an acidic (pH 4.5) soil then the pH adjusted to 7.4 (Verstraete et al., 1976). Hydrocarbon contaminants and soil nutrients can often reduce the pH of the soil. This is because during aerobic degradation of organic molecules, carbonic acid, organic acid intermediates, nitrate and Sulphate may accumulate and this can lower the soil pH and inhibit biological activity (Dibble and Bartha, 1979).
2.9.2 Presence of moisture

U.S. National Research Council (1993) has noted that mostly all soil microorganisms need soil moisture for cell growth, development and function. They further elaborated that accessibility of water influences diffusion of water and soluble nutrients into and out of microorganism cells. Notwithstanding, oversupply of moisture, example in saturated soil, is undesirable because it reduces the amount of available oxygen for aerobic respiration (Chaillana et al., 2006). Chaillana et al. (2006) elaborated further that an anaerobic respiration which gives out less energy for microorganisms more than aerobic a source of water is a necessity for life. Every microorganism needs specific amount of moisture for their survival and growth. Hydrocarbon or crude oil contaminated soil that contains moisture status below 50% turn out to hinder degradation and slows the rate of biodegradation (Trindade et al.2005). Soil moisture contents between 45 and 85 percent of the water-holding capacity (field capacity) of the soil or about 12 percent to 30 percent by weight‖ are optimal for petroleum hydrocarbon degradation (Chaillana et al., 2006). According to Trindade et al. (2005) most microbes are limited to soluble materials that are transported across their cell membranes through the interior of the cell, and moisture solubilizes the substrate and permits the substrate to enter the cell.

2.9.3 The presence of adequate nutrient

In water and soil, the growth of petroleum-hydrocarbon-utilizing cells is limited if mineral nutrients, especially N and P, are in short supply (Bartha, 1986). Iron was found to be limiting in clean, offshore seawater, but should not be a limiting factor in most cases (Dibble and Bartha, 1976). To avoid nutrient from depleting in any environment and for biological treatment processes to sustain microbial activities, the ratio of C: N: P is adjusted at 120:10:1 based on the organic carbon content of the feed (Sims et al., 1989; Thomas et al., 1992). For bioremediation to be
effective, an enhancement with organic nitrogen-rich nutrients are required and this can be achieved by (biostimulation) i.e. adjustment of C: N: P ratios by the addition of nitrogen and phosphorus fertilizers like urea-phosphate, N-P-K fertilizers, and ammonium and phosphate salts (Dibble and Bartha 1979; Jamison et al., 1975; Jobson et al., 1974; Verstraete et al., 1976). Agricultural wastes or left over and a native tropical South America cassava also known as tapioca has been found to be highly potential sources of bio-stimulating agents in bioremediation of crude oil in the tropics (Agarrya et al., 2010; Adams et al., 2014; Omoni et al., 2015, Romanus et al., 2015)

2.9.4 The presence of oxygen

The presence of molecular oxygen is of utmost importance in bioremediation of hydrocarbons, since the availability of oxygen in soils, ecosystem and other ecosystems is dependent on rates of microbial oxygen consumed, the type of soil (e.g. waterlogged) and the presence of consumable substrates that can lower oxygen content (Leahy and Colwell, 1990; Balba et al., 1998).

The concentration of oxygen has been identified as the rate-limiting variable in the biodegradation of petroleum in soil and of gasoline in groundwater (Leahy and Colwell, 1990). To achieve an effective biodegradation of petroleum hydrocarbons, aerobic conditions are required since anaerobic degradation of these hydrocarbon compounds has been studied to be quite slow (Hambrick et al., 1980). The activities of anaerobic bacteria such as sulphate-reducing bacteria, metal-reducing bacteria, methanogens and denitrifies are known by researchers to contribute significant reduction in crude oil contamination and play important role in the transformation of petroleum hydrocarbon contamination in anaerobic environment despite occurring in limited oxygen (Beller et al., 1996).
2.9.5 Optimal temperature range

Temperature influences both chemical nature of hydrocarbon compounds and affects the metabolic activities of microorganism hence low bioremediation of hydrocarbons is related to low temperature range and high organic residues or substrate (Alexander 1999). At low temperatures, the viscosity of oil increases, thereby lowering the rate of oil spreading in water and soil, retarding the volatilization of short chain alkanes (<10 C atoms) and hence maximizing their solubility and quantity in water and soil, respectively in effect their toxicity is enhanced (Leahy and Colwell 1990). Nester et al. (2001) also reported of enzymatic reactions rate within microorganisms being influenced and controlled by temperature. Thermophilic bacteria which are usually found in hot springs and compost pile lived indigenously in cool soil ecosystem and can be enhanced to degrade hydrocarbons with rise in temperature to 60 °C as used in thermally enhanced bioremediation techniques in temperate soils (Perfumo et al., 2007).

2.9.6 Bioavailability of oil in the soil

Accustomed with the intricate relations between soil biomass, crude oil contaminants and other soil nutrients, there has been a great deal of questions about the word “bioavailability” of nutrients. This is of the reason that crude oil or hydrocarbon and their derivatives are known as hydrophobic compounds and usually not able to incorporate adequately to microorganisms as compared to hydrophilic nutrients (Betancur et al.2014). However, there are some additives that ease the transport of nutrient within the soil matrix for bioremediation to take place. These were perpetually surfactants, basically nonionic and occasionally bioemulsifiers (Calvo et al.2009).
CHAPTER THREE
MATERIALS AND METHODS

3.1 Soil sampling and collection

The soils used in this study were sampled from coastal areas of four regions of Ghana (Volta, Greater Accra, Central and Western). Two sites from each region were selected for the sampling. Soils from the Volta Region were sampled from Aflao beach (06° 06.630’N; 001º 11.678’W and 8m) and Anloga beach (05°47.146’N; 000º 55.143’W and 15m). In Greater Accra Region soil samples were taken from Ada (05º 46.518’N; 000º37.813’W and 12m) and Accra (N 05º30.110; 000º20.719’W and 10m). Soils from the Central Region were sampled at Winneba (N 05º19.856; 000º 37.596’W and 9m) and Cape Coast (N05º 06.019, 001º 16.798’W and 11m). Western Region soil samples were taken from Tarkoradi (N 04º52.705; 001º45.219’W and 9m) and Axim (N04º51.067; 002º14.004’W and 19m). The sampling sites are indicated in Fig. 3.1. The soils from Aflao, Anloga, Ada, Accra and Winneba are Keta series and were classified Haplic Arenosol according to FAO (2014) system of classification whiles soils from Cape Coast, Takoradi and Axim are Goi series and classified as Gleyic Arenosol according to FAO (2014) system of classification. The soils were sampled within the plough depth of 0-20 cm at different sample points, mixed and composite sample were collected. The soils were transported to the Department of Soil Science Laboratory, University of Ghana. The soils were air-dried, debris were removed by hand picking and sieved through a 2 mm sieve.
Fig. 3.1 Study area and Location map

3.2 Laboratory analysis of soils

3.2.1 Soil pH

The pH of the soil was determined at a ratio of 1:1 soil to water. An MV 88 Pracitronic pH glass electrometer was used. Ten (10) g of soil sample was weighed into a 50 mL beaker and 10 mL of distilled water was added. A paste (solid-liquid suspensions) was then shaken for 30 minutes and
was then allowed at room temperature to equilibrate. A buffer solution of pH 4.0 and 7.0 was used to standardize the pH electrometer. The pH of the soil sample was then measured by immersing the standardized electrode into the supernatant of the suspension.

3.2.2 Determination of total C, N and S

The total carbon, nitrogen and Sulphur were determined by using a LECO Trumac version 3.1 CNS analyzer (LECO Corporation, St. Joseph. MI, USA.

An amount of soil sample was passed through a 0.5 mm sieve and 0.25 g of the sample was weighed and mixed with Concat reagent. It was then placed in the combining chamber of the analyzer by using helium as a carrier gas and highly purified oxygen for the combustion. The carbon, nitrogen and the Sulphur were then analyzed and results produced for respective element were recorded as their respective percentages.

3.2.3 Exchangeable cations

Five (5) g soil was weighed into a 50 ml extraction bottle. 50 mL solution of 1 M ammonium acetate (NH₄OAc at pH 7) was added. The bottle and its content were then shaken for one hour and filtered through a Whatman No. 42 filter paper. The extract was collected into a clean plastic container. Atomic Absorption Spectrometer (AAS) was used to determine the exchangeable calcium and magnesium and exchangeable Na and K were determined by flame photometer.

3.2.4 Potassium (K) determination

The flame photometer was standardized such that 10 mg/kg of K gave 100 full scale deflections. The flame photometer after standardization was used to determine the concentration of potassium in 10 ml aliquot. The result was used in the calculation of the amount of potassium present in the soil as shown in the formula below:
Exchangeable K (cmol/kg Soil) = \( R \times V \times \frac{100}{39.1} \)

Where \( V \) = Volume of extract (100ml)

39.1 = molecular weight of potassium

\( R = \) Flame photometer reading for K (ppm)

### 3.2.5 Sodium (Na) determination

The flame photometer was standardized in a way that 10 mg/kg of Na gave 100 full scale deflections. After the standardization of the photometer, the concentration of sodium in 10mL aliquot was determined. The result was then used in the calculation of the amount of sodium (Na) present in the soil as shown by the formula below.

Exchangeable Na (cmol/kg) = \( R \times V \times \frac{100}{23} \)

Where \( V = \) Volume of extract (100ml)

23 = molecular weight of Sodium

\( R = \) Flame photometer reading for Sodium (ppm)

### 3.2.6 Calcium (Ca) determination

Ten (10) ml aliquot of the soil extract was taken and 10 ml of 10% KOH and 1ml triethanolamine (TEA) were added. About three drops of 1M KCN solution and pinch of crystals of cal-red indicator were then added and was titrated with 0.02M EDTA solution. The end point colour change was from red to blue. The titre value recorded was used in the calculation of calcium as shown below.

\[
\text{Ca (cmol/kg)} = \text{Titre Value} \times N \times \text{Volume of extract} \times 100 \text{ meq/100g}
\]

\[
\times \text{Volume of aliquot} \times \text{Weight of Soil}
\]

Where \( N = \) Normality of EDTA
3.2.7 Magnesium (Mg) determination

Ten 10 ml aliquot of the filtrate solution was added to a 5 ml of ammonium chloride – ammonium hydroxide buffer solution and 1 ml of triethanolamine. About three drops of 1M KCN solution and drops of Eriochrome black T solutions were added. The mixture was then titrated with 0.02M EDTA solution and the end point (red to blue) titre values was used to determine the amount of calcium and magnesium in the solution. The titre value of magnesium was then determined by subtracting the value obtained for calcium above from the new titre value obtained. The titre value of magnesium was then used for the calculation of the concentration of magnesium (Mg) as shown below:

\[
\text{Mg (cmol/kg)} = \frac{\text{Titre value} \times N \times \text{Volume of extract} \times 100 \text{ (meq/100g)}}{\text{Volume of aliquot} \times \text{weight of Soil}}
\]

Where \(N\) = Normality of EDTA

3.2.8 Particle size distribution

Soil particle size distribution was determined by the modified Bouyoucos Hydrometer method described by Day (1965). Forty (40) g of soil was taken into a beaker followed by the addition of 60 mL of 6% \(\text{H}_2\text{O}_2\) in order to destroy the organic matter in the soil. One hundred (100) mL of 5% calgon (sodium hexametaphosphate) solution was added. The suspension was allowed to stand for approximately 10 minutes and stirred with a mechanical stirrer for 30 minutes.

The suspension was then transferred into a graduated sedimentation cylinder using distilled water from a wash bottle and made up to the 1-liter mark with distilled water. The temperature of the suspension was recorded after equilibration. The content of the cylinder was then mixed thoroughly with the help of a plunger and hydrometer readings taken 5 minutes and 5 hours thereafter. The suspension was then poured into a 47-\(\mu\)m sieve and the particles retained on the
sieve washed with water and dried in an oven at 105 °C for 24 hours. The dried samples were then weighed to represent the sand fraction. Blank hydrometer readings of sodium hexametaphosphate solution at 5 minutes and 5 hours were taken. The particle size distribution was then determined using the formulae below:

\[(\text{Clay + Silt}) \% = \frac{5 \text{ minutes’ hydrometer reading} \times 100}{\text{Sample mass}} \] …… (1)

\[(\text{Clay}) \% = \frac{5\text{-hour hydrometer reading} \times 100}{\text{Sample mass}} \]……………… (2)

\[(\text{Silt}) \% = (1) - (2) \]……………… (3)

\[(\text{Sand}) \% = \frac{\text{Oven dry mass (g) of particle retained on 47 μm Sieve}}{\text{Sample mass (g)}} \]……………… (4)

### 3.3 Incubation experiment

One (1) kg of the air dried soil was weighed into plastic containers and contaminated with crude oil at 10 g oil/kg soil as described by Ekpo and Ebeagwu (2009). The soils were then mixed thoroughly with the crude oil and moisture content was maintained at 16% (wt: wt basis). The contaminated soils were incubated in the dark at room temperature for 60 days in a randomized complete design with four replicate. Samples were taken at an interval of 10 days for determination for quantity of crude oil degraded and hydrocarbon utilizing bacterial (HUB) population.

#### 3.3.1 Crude oil used

The crude oil used in this study was obtained from Jubilee Oil field at Cape Three Point, Western Region of Ghana through the National Petroleum Authority. The Jubilee field oil used had the following physicochemical properties: Density at 15 °C was 849.3 kg/m³, Specific gravity was 0.8493, Gravity (°API) was 35, Pour point (°C) was -3, Total Sulphur 0.237% wt, Salt in crude
8.07 ptb, Viscosity at °C 6.53 cSt, Reid vapour pressure at 37.8 °C was 0.18 kg/mg, Total acid number 0.20 mg KOH/g, Ash weight 0.016% wt, Water and sediments 0.1% V and Water by distillation 0.05% V and Light and sweet oil as documented by Appenteng et al. (2012).

Fig. 3.2 Experimental set-up of incubation experiment

3.3.2 Determination of crude oil degraded

Residual crude oil in the contaminated soils was extracted using a modified method of Abu and Ogiji (1996). Soil samples taken from the contaminated soil were air dried to constant weight and 1.0 g was placed into small plastic containers and 10 mL chloroform was added. Residual oil was extracted by gently shaking the plastic containers for 5 minutes. Each extract was filtered through cotton wool in a funnel and collected in a clean glass container, closed immediately and analyzed.
for crude oil content. Quantitative determination of crude oil extracts was employed as described by Udeme and Antai (1988). A standard curve of absorbance at 520 nm against varying concentrations of crude oil in chloroform was drawn after taking readings from a Pharo.300 Spectrophotometer. The crude oil concentrations were then calculated from the standard curve.

3.3.3 Determination of hydrocarbon utilizing bacterial population

The hydrocarbon utilizing bacterial count was estimated using the modified mineral salts agar medium of Mills et al. (1978) and modified vapour phase transfer technique of Okpokwasili and Amanchkwu (1988). One (1) g of contaminated soil was placed in a test tube containing 9 mL of sterilized distilled water containing 0.6% NaCl and serial dilution was prepared. One (1) mL of the serial dilution prepared above was plated onto the modified mineral salts medium containing 10 g NaCl, 0.42 g MgSO$_4$.7H$_2$O, 0.29 g KCl, 0.53 g KH$_2$PO$_4$, 0.42 g NH$_4$NO$_3$, and 15 g agar in 1L distilled water (adjusted pH= 6.8). The modified vapour transfer technique involves spreading 0.5 mL of crude oil (serving as carbon source) onto the mineral salts agar medium after setting and allowed the crude oil to diffuse into the agar medium for about 1 hour before incubated at room temperature for 5 to 7 days. The number of colonies formed was counted and used to estimate the hydrocarbon utilizing bacterial population.

3.3.4 Isolation and Identification of hydrocarbon utilizing bacteria

Culturally different colonies of hydrocarbon utilizing bacteria were picked from the modified mineral salts agar. These colonies were streaked over the mineral salts agar medium supplemented with crude oil and grown at 28°C for up to 7 days. Isolates were maintained on Nutrient agar slants which were sub cultured at 15 days’ interval at 37°C for 24-42 hours and then stored at 4°C. The identification was done morphologically by Simple staining, Gram staining and Spore staining,
whiles biochemical analysis was carried out by Catalase test, Citrate test, oxidase test, Indole test, urease test, Hydrogen Sulphur test and TSI (Slant and Butt) test.

3.4 Morphological test

3.4.1 Preparation of a smear and heat fixing
A sterilized inoculating loop was used to transfer a loopful of an isolated colony from a culture plate to a slide with a water drop. A thin smear was made by spreading the drop over an area, the size of a dime. The smear was then allowed to dry thoroughly. Heat was used to fix the smear by cautiously passing it underside of the slide through the burner flame two or three times. The bacteria cell was then fixed to the slide. It was ensured that the slide was not over heated to avoid distortion of the bacterial cells.

3.4.2 Simple staining
The smear was gently covered with methylene blue and was allowed to remain in the smear for approximately one minute. The excess methylene blue was washed off from the slide by directing a gentle stream of water over the surface of the slide. The smear was then saturated with iodine. Excess iodine was washed off gently with running tap water and rinse thoroughly. The back of the slide was then wiped with a paper towel. The slide was then observed under microscope stage with smear side up by focus using the 10X objective in the area of the smear in which the bacteria cells were well spread in a monolayer. Immersion oil was applied directly to the smear, and was focused under oil with the 100x objective.

3.4.3 Gram’s staining procedure
A smear of the cells was made on the slide. The cells were then fixed to the slide with flame and stained with crystal violet. The slide was then allowed to stand for about 2-3 minutes and washed
off again with iodine. The iodine was allowed to stain for a minute to form a crystal violet iodine complex. Acetone alcohol was then used to decolorize the stains and subsequently washed off with water. Safranin was applied and washed with water immediately. The slides were then observed under oil immersion (100x) using a bright field microscope.

3.4.4 Spore Staining

The spore staining was done using Brachman and Freeley method (1970) and Conklin’s modification of Whirtz’s method. A sterilized loop was used to pick a cell from a single or pure colony onto a moist glass slide. The cell was heated to fix and stained with Malachite’s green. The saturated Malachite green was flamed intermittently for 7 to 10 minutes and rinsed with tap water. Safranin stain was then applied for 15 seconds and washed off with water. The slide was then observed under oil immersion on the microscope with 100X objective.

3.5 Biochemical test

3.5.1 Composition and Procedure for Triple Sugar Iron Agar (TSI)

The TSI agar consists of Lactose, Sucrose and Glucose in the concentration of 10:10:1 (i.e. 10 part Lactose 1%), 10-part Sucrose (1%) and 1-part Glucose (0.1%). TSI is similar to Kligler’s iron agar (KIA), except that Kligler’s iron agar contains only two carbohydrates: glucose (0.1%) and lactose (1%). A sterilized straight inoculating needle was used to touch the top of a well isolated colony. The inoculation of TSI agar was done by first stabbing through the center of the medium to the bottom of the tube and then streaking on the surface of the agar slant. The cup of the TSI tube was left loose and was incubated at 35°C for 18 to 24 hours.
5.2 Principle and Procedure for H$_2$S (Hydrogen Sulphide) test

The test is used to determine whether the microorganism can reduce Sulphur- containing compounds to sulphide during the process of metabolism. Two TSI slants were used at a time, one served as an uninoculated negative control. A sterilized inoculating needle was used to pick colony that is 18 to 24 hours old to make a stab inoculation into one of the TSI slants. It was then incubated at 35°C for 18 to 24 hours. After incubation the slants were observed for color and gas production.

3.5.3 Preparation of reagents and Procedure for indole test

The tryptone broth was prepared by dissolving 10 gm of tryptone and 5 gm of sodium chloride in 1 litter of sterile water. Four mils (4ml) of the reagent was dispensed into test tubes caped and autoclaved at 121°C under 15 psi pressure for 15 minute. The tube and its content was then stored in refrigerator at 4°C. on the hand Kovac’s reagent was prepared by taken ten grammes (10g) p-dimethylaminobenzaldehyde (DMAB) was dissolved in 150 ml Amyl alcohol and 150 ml of conc. HCl was then added to the aldehyde alcohol mixture. A sterilized test tube containing 4 ml of tryptophan broth aseptically inoculated with colony that was 18 to 24 hrs old. It was then incubated at 37°C for 24-28 hours. Half a milliliter (0.5 ml) of Kovac’s reagent was added to the broth culture. It was then observed for the presence or absence of ring.

3.5.4 Principles and Procedure for Catalase test

The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. The presence of the enzyme in a bacterial isolate is evident when a small inoculum is introduced into hydrogen peroxide, and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is
evident by a lack of or weak bubble production. The culture should not be more than 24 hours old. Bacteria thereby protect themselves from the lethal effect of Hydrogen peroxide which is accumulated as an end product of aerobic carbohydrate metabolism. Four (4) to 5 drops of 3% \( \text{H}_2\text{O}_2 \) (Hydrogen peroxide) was added to test tube. A wooden applicator stick was used to collect a small amount of bacteria from a well-isolated 18- to 24-hour colony and was placed into the test tube. The tube and its content was placed against a dark background and observed for immediate bubble formation at the end of the wooden applicator stick.

**3.5.5 Preparation of Reagents and Procedure for Citrate Utilization Test**

The Simmon’s Citrate Agar contains sodium chloride 5.0g, sodium citrate (dehydrate) 2.0 g, ammonium dihydrogen phosphate 1.0 g, dipotassium phosphate 1.0 g, magnesium sulfate (heptahydrate) 0.2 g and bromothymol blue 0.08 g. These were dissolved in deionized water with pH adjusted to 6.9. The agar and the measured bromothymol blue were then mixed and was top up with deionized water to 1L. The solution was gently heated to boiling until the agar dissolved to effect mixing. Four to five mils was dispensed into test tubes. It was then autoclaved at 121°C under 15 psi pressure for 15 minutes. A slant was made from Simmon’s citrate agar prepared above by touching the tip of a needle to a colony that is 18 to 24 hours old. It was then incubated at 35°C to 37°C for 18 to 24 hours. A green to blue change in colour indicates conversion of citrate to pyruvate.

**3.5.6 Principle and Procedure for Oxidase Test**

The oxidase test is based on the principle that certain bacteria produce indophenol blue from the oxidation of dimethyl-p-phenylenediamine and \( \alpha \)-naphthol. In presence of the enzyme cytochrome oxidase (gram-negative bacteria), the \( N, N \)-dimethyl-p-phenylenediamine oxalate and \( \alpha \)-naphthol react to indophenol blue. A filter paper was soaked with the substrate tetramethyl-p-
phenylenediamine dihydrochloride and it was moistened with sterile distilled water. A platinum loop was used to pick a colony to be tested and it was smeared in the filter paper. It was then observed for colour change within 10-30 seconds.

3.5.7 Principle and Procedure for Urease Test

Urea is a diamide of carbonic acid. It is hydrolyzed with the release of ammonia and carbon dioxide. Many organisms especially those that infect the urinary tract, have a urease enzyme which is able to split urea in the presence of water to release ammonia and carbon dioxide. The ammonia combines with carbon dioxide and water to form ammonium carbonate which turns the medium alkaline, turning the indicator phenol red from its original orange yellow colour to bright pink. An equation below indicates hydrolysis of urea by urease enzyme

\[(NH_2)_2CO + 2H_2O \xrightarrow{Urease} CO_2 + H_2O + 2NH_3\]

3.5.8 Preparation of Reagents and Procedure for Procedure for urease test

The ingredients used in the preparation of Christensen’s urea agar are urea 20 g, sodium chloride 5 g, monopotassium phosphate 2 g, peptone 1 g, dextrose 1 g, phenol red 0.012 g and agar 15 g. The ingredients were dissolved in 100 ml of distilled water, filtered and sterilized (0.45-mm pore size). The agar was suspended in 900 ml of distilled water and was boiled to dissolve completely. The boiled agar was then autoclaved at 121°C and 15 psi for 15 minutes. While the agar was in molten state, 100ml of filtered-sterilized urea base was then aseptically added to cool the agar solution and was mixed thoroughly. Four to five (5) mL of the solution was distributed into sterile tubes (13 x 100 mm) and the tubes were slanted during cooling until solidified. Four (4) to 5 mL of Christensen’s urea agar prepared above was used to prepare the slants in test tube and was
allowed to cool and solidify. A well-isolated colony was picked and used to inoculate the slant by streaking on the surface of the urea agar slant. The cap on the tubes were left loosely and incubated at 35°-37°C in ambient air for 48 hours to 7 days. It was then examined for the development of a pink colour after 7 days of incubation.

3.6 Statistical analysis

The population of Hydrocarbon utilizing bacterial count obtained from the crude oil contaminated soil was log transformed and later subjected to analysis of variance (ANOVA) using Genstat statistical software package (12th edition, 2009). Analysis of variance (ANOVA) was also performed for amount of crude oil degraded. The means were separated using the least significant difference (LSD) test, considering a significant level of p< 0.05.
CHAPTER FOUR
RESULTS

4.1 Physicochemical and Biological Characteristics of the Soils

The physical, chemical and biological properties of the eight coastal soils used in the present study are shown in Tables 4.1 & 4.2. The soils were sandy in texture according to the (USDA, 2003) system of classification. The soil pH values were between 7.5 and 8.4 and electrical conductivity was between 3.20 and 4.75 dS/m. The organic carbon content was between the values of 0.03 and 0.84%. Winneba soil had the highest percentage carbon content of 0.84% with Axim soil having the lowest value of 0.03%. For total nitrogen, it was in the range of 0.01 to 0.02%. Except for Ada and Winneba whose Sulphur contents were not detected, the other soils had Sulphur contents in the range of 0.01 to 0.02%. The soils also had available phosphorus values between 0.35 and 1.35 mg/kg, with Ada soil having the highest value. Total exchangeable base values for all the soils studied were between 4.71 and 14.02 cmol/kg. Aflao and Accra soil had the highest values of 14.02 cmol/kg and 11.6 cmol/kg, respectfly. The Aflao soil had the highest value for calcium (8.60 cmol (+)/kg), followed by Winneba. All the soils recorded low values for Magnesium with a range value of 0.23 to 1.24 cmol (+)/kg. Aflao and Accra recorded high content of Sodium (4.50 cmol/kg). Potassium values recorded were low with a range value of 0.44 to 0.97 cmol (+)/kg. The results obtained for the initial hydrocarbon utilizing bacteria HUB population count for the various soils are presented in Table 4.2. Ada soil had the highest mean value of 4.61 and Takoradi soil recorded the lowest mean value of 3.37. The other soils had HUB population in the range of 4.10 and 4.51.
Table 4.1 Some chemical characteristics of the soils used

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<tr>
<th>Sites</th>
<th>pH</th>
<th>Total C (1:1H₂O)</th>
<th>Total N (%)</th>
<th>Total S (mg/kg)</th>
<th>Av. P (dS/m)</th>
<th>EC (cmol (+)/kg)</th>
<th>Exchangeable bases</th>
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AF = Aflao, AN = Anloga, AD = Ada, AC = Accra, WI = Winneba, CC = Cape-Coast, TD = Takoradi, AX = Axim
Table 4.2 Some physico-biological characteristics of the soils used

<table>
<thead>
<tr>
<th>Sites</th>
<th>Sand (%)</th>
<th>Silt (%)</th>
<th>Clay (%)</th>
<th>Texture Class</th>
<th>HUB Log (cfu/g)</th>
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</thead>
<tbody>
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<td>5.00</td>
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<td>10.00</td>
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<td>7.50</td>
<td>Sandy</td>
<td>4.31</td>
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</tbody>
</table>

HUB = hydrocarbon utilizing bacteria, AF = Aflao, AN = Anloga, AD = Ada, AC = Accra, WI = Winneba, CC = Cape-Coast, TD = Takoradi, AX = Axim
4.2 Amount of Crude oil degraded

The results of amount of crude oil degraded in the various contaminated soils are presented in Fig. 4.1 above. There was a general significant (p < 0.05) increase in the amount of crude oil degraded from day 10 up to day 60 for all the soils as indicated by the cumulative amount of crude oil degraded in the soils. Ten (10) days after incubation, the Ada soils tended to degrade more oil than the other soils. This trend (0.060 g) continued throughout the sixty 60-day period. Anloga recorded the second highest (0.048 g) degrading soil and Takoradi soil recorded the lowest (0.039 g) degrading potential. The overall degradation was in the order of Ada > Anloga > Aflao > Takoradi > (Accra, Winneba, Cape Coast) > Axim.
4.4 Hydrocarbon utilizing bacteria population in different soil samples

The growth of hydrocarbon utilizing bacteria (HUB) in the contaminated soils during the incubation period is represented in Fig. 4.2 above. Ada (AD) and Takoradi (TD) had the highest and lowest population 4.91 and 4.18 log cfu/g soil respectively, at the initial period of incubation. There was sharp increase in the microbial growth 10 days after incubation within the contaminated soils. The HUB attained peak growth in the respective soils 40 days after incubation. At the peak level, Ada and Takoradi recorded the highest and lowest growth 5.09 and 4.61 cfu/g soil,
respectively. Microbial growth in all the soils declined 40 days after incubation and at 60 days after incubation Ada and Takoradi still maintained the highest and lowest growth, respectively. At 60 days after incubation growth was in the order Ada > Anloga > Cape Coast > Accra > Winneba > Aflao > Axim > Takoradi.
Table 4.3 Morphological characteristics of different isolates obtained from the soils

<table>
<thead>
<tr>
<th>Sites</th>
<th>Simple staining</th>
<th>Gram staining</th>
<th>Spore formation</th>
<th>Spore position</th>
</tr>
</thead>
<tbody>
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<td>Size (µm)</td>
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<td>AF₅</td>
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</tr>
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AF= Aflao, AN=Anloga, AD= Ada, AC= Accra, WI= Winneba, CC= Cape Coast, TD= Takoradi, AX= Axim
Table 4. 4 Continued

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<thead>
<tr>
<th>Site</th>
<th>Simple staining</th>
<th>Gram staining</th>
<th>Spore formation</th>
<th>Spore position</th>
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</tbody>
</table>

AF= Aflao, AN=Anloga, AD= Ada, AC= Accra, WI= Winneba, CC= Cape Coast, TD = Takoradi, AX= Axim
4.5 The morphological characteristics

The morphological characteristics observed for the isolates obtained from the different soil types are shown in Table 4.3 above. Results showed that the hydrocarbon utilizing bacteria (HUB) isolated from the soils were dominated by Gram positive rod shaped bacteria. The HUB were also dominated by spore bearing bacteria characterized by central and terminal positions.
Table 4.5 Biochemical characteristics of the isolates from the different soil types

<table>
<thead>
<tr>
<th>Sites</th>
<th>Triple Sugar Iron test</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slant</td>
<td>Butt</td>
<td>CO₂</td>
<td>H₂S</td>
<td></td>
</tr>
<tr>
<td>AF₃</td>
<td>Red</td>
<td>Red</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>AF₅</td>
<td>Red</td>
<td>Red</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>AD₂</td>
<td>Red</td>
<td>Red</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>AD₄</td>
<td>Red</td>
<td>Red</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>AN₁</td>
<td>Red</td>
<td>Red</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>AN₅</td>
<td>Red</td>
<td>Red</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>AC₄</td>
<td>Red</td>
<td>Red</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CC₃</td>
<td>Red</td>
<td>Red</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CC₄</td>
<td>Red</td>
<td>Red</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>AX₄</td>
<td>Red</td>
<td>Red</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.4 Continued

<table>
<thead>
<tr>
<th>Sites</th>
<th>Indole test</th>
<th>Catalase test</th>
<th>Citrate Test</th>
<th>Oxidase Test</th>
<th>Urease test</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF₃</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>AF₅</td>
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<td>+</td>
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<tr>
<td>AD₂</td>
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<td>+</td>
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<td>+</td>
<td>-</td>
</tr>
<tr>
<td>AD₄</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>AN₁</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>AN₅</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>AC₄</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CC₃</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CC₄</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>AX₄</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Red: glucose, lactose and sucrose non fermenter

AF= Aflao, AN = Anloga, AD= Ada, AC = Accra, WI = Winneba, CC= Cape Coast, TD = Takoradi, AX= Axim
4.6 The biochemical characteristics of isolates obtained from different soil types

The biochemical characteristics of the isolates are represented in Table 4.4 above. Results showed that the isolates do not ferment sugars (lactose, sucrose and glucose) under aerobic and anaerobic conditions. Besides, the isolates do not produce \( \text{H}_2\text{S} \) in TSI agar test and only one (1) isolate from soil sample from Cape Coast (CC4) was not able to produce \( \text{CO}_2 \) in TSI agar test. Results also showed that the isolates contained catalase and citrate permease enzymes. Isolates from Aflao (AF3 and AF5), Ada (AD2), Anloga (AN1) and Accra (AC4) contained oxidase enzyme. Urease enzyme was detected in isolates obtained from Ada (AD4), Anloga (AN5), Cape Coast (CC3 and CC4) and Axim (AX4). Finally, only isolates from Ada (AD4), Anloga (AN5), Cape Coast (CC3) and Axim (AX4) contained tryptophanase enzymes as shown by the Indole test.
Table 4. 6 Possible HUB associated with the sampling sites

<table>
<thead>
<tr>
<th>Sites</th>
<th>Genera</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFLAO</td>
<td>Bacillus and Pseudomonas</td>
</tr>
<tr>
<td>ANLOGA</td>
<td>Bacillus, Pseudomonas, Yersinia</td>
</tr>
<tr>
<td>ADA</td>
<td>Bacillus, Pseudomonas and Yersinia</td>
</tr>
<tr>
<td>ACCRA</td>
<td>Bacillus and Pseudomonas</td>
</tr>
<tr>
<td>WINNEBA</td>
<td>Bacillus</td>
</tr>
<tr>
<td>CAPE COAST</td>
<td>Bacillus, Staphylococci and Yersinia</td>
</tr>
<tr>
<td>TARKORADI</td>
<td>Bacillus</td>
</tr>
<tr>
<td>AXIM</td>
<td>Bacillus and Yersinia</td>
</tr>
</tbody>
</table>

AF= Aflao. AN= Anloga, AD= Ada, AC=, Accra, WI= Winneba, CC=Cape Coast, TD=Takoradi and AX= Axim

4.7 Possible hydrocarbon utilizing bacteria

The possible genera identified in the soils used in this study are presented in Table 4.5. The HUB identified based on the morphological and biochemical tests were Bacillus, Pseudomonas, Yersinia and Staphylococci. Among these microorganisms, Bacillus was the dominant HUB. Staphylococci genus was identified in only the soil from Cape Coast. Soils from Ada and Anloga contained the genera Bacillus, Pseudomonas and Yersinia. Besides, Pseudomonas was associated with soils from Ada, Anloga, Aflao and Accra.
CHAPTER FIVE
DISCUSSIONS

5.1 Growth of hydrocarbon Utilizing Bacteria

The results of the growth of hydrocarbon utilizing bacteria population (HUB) in all the contaminated soils under study showed an initial increase with incubation time up to day 40. The increase in the hydrocarbon utilizing bacterial population conforms to the work done by Odeyemi (2014), Antai (1993) and Lawson et al. (2012) who documented that exposing hydrocarbon to soil environment rapidly cause increase in the population of indigenous microorganisms, especially HUB by enhancing their adaptive mechanism to selectively enrich their genetic makeup. This initial increase observed, support the findings documented by Lawson et al. (2012) and Nsobilaatibila (2013) who stated that optimum growth rates were observed within 30 and 40 days. In the present study there was decline in growth after 40 days. This observation conforms to the work done these researchers who stated that reduction in HUB growth, beyond day 40 could be attributed to depletion of the hydrocarbon in the soil. They also documented that when hydrocarbon component within a soil is depleted, the hydrocarbon utilizing bacteria are left with less substrate to survive and could be devoured by bigger predators. Schaefer and Juliane (2007) reported that, with time, soil resistant components with high chain and less remaining nutrients, cause decrease in oil degradation and bacteria growth. At the end of the experiment, results indicated that there was increase in hydrocarbon utilizing bacteria in all the contaminated soils when compared to the initial growth. This implies that the HUB’s were identified are able to survive, reproduce and tolerate anaerobic condition created by crude oil contaminated site. They also use crude oil as carbon source (nutrient) to increase their biomass. This was also observed by
other researchers such as Baker (1989), Lee and Levy (1991), Fought and Westlake (1992) and Abu and Ogiji (1996).

5.2 Possible hydrocarbon utilizing bacteria associated with the soils

The possible hydrocarbon utilizing bacterial genera identified in the soils used were Bacillus, Pseudomonas, Yersinia and Staphylococcus. Amongst these, the dominant genus was Bacillus. This is evidence of characteristics of Bacillus being spore bearers with vegetative cell, can tolerate high salt and high pH environment; their ability to strive well in anaerobic condition such as the one created by the presence of crude oil in soil environment. Lawson et al. (2012) also identified Staphylococci and Yersinia in four inland soils of Ghana and the dominant genus was Bacillus. Varjiani et al. (2013) and Obiakalaije et al. (2015) also identified among other HUB genera Bacillus and Pseudomonas in oil contaminated soils from Gujarat in India and Isaka mangrove in Nigeria, respectively. Ghazali et al. (2004) and Van-Hamme and Odumeru (2002) further identified Bacillus and Yersinia as dominant bacteria amongst hydrocarbon utilizing bacteria characterized from oil contaminated soil samples and bacteria capable of degrading crude oil in a contaminated site.

5.3 Amount of crude oil degraded

In the present study, there was no lag phase which usually indicates adaption period of microorganisms to the oil. This absence of adaption period is an indication that the HUB in the soils might already be adjusted to oil in their various soils. If this is true, then the beach soils might be experiencing or had experienced oil contamination. Ghanaian Daily Graphic (2010) reported that Ghana experienced mud oil spillage during the early oil production. There was cumulative significantly (p < 0.05) increase in amount of oil degraded in all the soils with time of incubation. Atibila (2013) also observed similar increment in beach sand. Wackket (2003) and Al-Aubaidy
(2004) also documented cumulative increase in amount of oil degraded crude contaminated soils. The cumulative increase in the amount of oil degraded might be due to presence of high population of HUB observed in this study. Okoh et al. (2003) suggested that, the important factor in the degradation of petroleum oil is adequate bacterial biomass. The cumulative increase in the amount of oil degraded could also be attributed to the class of crude oil used in this study which was classified as light crude oil, since the lighter crude oil, diffuse faster, making the biodegradable compounds more available to the microbes (Chaillana et al., 2006; Merkl et al., 2005).

The order of oil degradation in the soils was significantly (p < 0.05) different from each other in this order Ada > Anloga > Aflao > Accra > Cape Coast > Winneba > Takoradi > Axim. Considering the HUB present in these soils, the difference is that the first four study sites (Ada, Anloga, Aflao and Accra) contained Pseudomonas which was absent in the other four sites which had low degradation potential. The difference in degradation potential of the former four soils and latter four soils could be due to difference in presence and absence, respectively, of Pseudomonas, because Wolfgang (2007) documented that Pseudomonas has high degradative potential in oil contaminated soils. It could also be attributed to
CHAPTER SIX

CONCLUSION AND RECOMMENDATION

In the present study there was cumulative increase in the amount of crude oil degraded with time in all the soils used. This cumulative increase was attributed to the presence of high population of HUB observed in this study which use crude oil as carbon source (nutrient) to increase their biomass and this finding supported the fact that, the important factor in the degradation of petroleum oil is adequate bacterial biomass and can be serve as potential microbes for remediation of crude oil. There was no lag phase during the degradation and this was attributed to possible contamination of oil along the coast of Ghana. There was variation in oil degradation along the coast. The order of degradation was Ada > Anloga > Aflao > Accra > Cape Coast > Winneba > Takoradi > Axim. The study also revealed that the dominant HUB was Bacillus and was attributed to characteristics of Bacillus being spore bearers with vegetative cell, having ability to tolerate high salt and high pH environment; strive well in anaerobic condition such as the one created by the presence of crude oil in soil environment. Other HUB identified were Pseudomonas, Yersinia and Staphylococcus. Further research work should be conducted to assess oil contamination along the coast, the diversity of HUB and identification of effective HUB along the coast of Ghana and enhancement of oil degradation because there was low degradation in some of the soils used.
REFERENCES


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Gupta and V. Bajpai (Eds.). LAP lambert academic publishing AG and Co. KG, Germany, 189-204.


GÖK G. (2012). The factors effecting the remediation of non-aqueous phase liquid (napl) contaminants in soils. dokuz eylül university graduate school of natural and applied sciences.


APPENDIX A

ANOVA TABLE FOR CRUDE OIL DEGRADATION

Table for Day 10

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trr</td>
<td>7</td>
<td>3.68E-04</td>
<td>5.26E-05</td>
<td>7.47</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Residual</td>
<td>24</td>
<td>1.69E-04</td>
<td>7.04E-06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>5.37E-04</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table for Day 20

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<th>Source of variation</th>
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<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
</tr>
</thead>
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<tr>
<td>Treatments</td>
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Table for Day 30

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<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
</tr>
</thead>
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<td>2.25E-06</td>
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<td></td>
</tr>
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<td>Total</td>
<td>31</td>
<td>5.35E-03</td>
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<td></td>
<td></td>
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</table>
Table for Day 40

<table>
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<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
</tr>
</thead>
<tbody>
<tr>
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<td>31</td>
<td>2.49E-02</td>
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<td></td>
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</tbody>
</table>

Table for: Day 50

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<th>Source of variation</th>
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<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
</tr>
</thead>
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<tr>
<td>Trr</td>
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<td>3.39E-03</td>
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<tr>
<td>Residual</td>
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<td></td>
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<td>2.38E-02</td>
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</tbody>
</table>

Table for Day 60

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<th>Source of variation</th>
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<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
</tr>
</thead>
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</tbody>
</table>
## APPENDIX B

### ANOVA TABLE FOR HYDROCARBON UTILIZING BACTERIA

#### Log of CFU/g Day 0

<table>
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<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
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<td>2.78E-04</td>
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<td>69.25</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Residual</td>
<td>24</td>
<td>1.38E-05</td>
<td>5.73E-07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>2.92E-04</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Log of CFU/g Day 10

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
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<td>3.53E-04</td>
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<td>&lt;.001</td>
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<td>7.19E-07</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>3.70E-04</td>
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</table>

#### Log of CFU/g Day 30

<table>
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<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
</tr>
</thead>
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<tr>
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<td>2.76E-03</td>
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<td>&lt;.001</td>
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<td></td>
</tr>
<tr>
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</table>
### Log of CFU/g Day 40

<table>
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<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
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<td>&lt;.001</td>
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</tr>
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<td>2.44E-03</td>
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</tr>
</tbody>
</table>

### Log of CFU/g Day 50

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
</tr>
</thead>
<tbody>
<tr>
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<td>&lt;.001</td>
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<td>Residual</td>
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</tr>
<tr>
<td>Total</td>
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<td>1.76E-03</td>
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</tr>
</tbody>
</table>

### Log of CFU/g Day 60

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<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
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<tbody>
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<tr>
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<td>1.26E-03</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX C

Standard curve for determining the amount of crude oil

\[ y = 7.9778x + 0.0758 \]

\[ R^2 = 0.9951 \]

<table>
<thead>
<tr>
<th>Weight of crude oil (g)</th>
<th>Absorbance (abs)</th>
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</thead>
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<td>0</td>
</tr>
<tr>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>0.2</td>
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<tr>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>0.35</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Standard curve for determining the amount of crude oil using Spectrophotometer
APPENDIX D

Effect of incubation period on crude oil degradation

Table 4.3

<table>
<thead>
<tr>
<th>Towns</th>
<th>Day 10</th>
<th>Day 20</th>
<th>Day 30</th>
<th>Day 40</th>
<th>Day 50</th>
<th>Day 60</th>
</tr>
</thead>
<tbody>
<tr>
<td>AX</td>
<td>0.002494a</td>
<td>0.00624a</td>
<td>0.01099a</td>
<td>0.01224a</td>
<td>0.01849a</td>
<td>0.02149a</td>
</tr>
<tr>
<td>TD</td>
<td>0.002889a</td>
<td>0.00614a</td>
<td>0.011664c</td>
<td>0.02289c</td>
<td><strong>0.04239c</strong></td>
<td>0.05664cd</td>
</tr>
<tr>
<td>WI</td>
<td>0.004864a</td>
<td>0.00744ab</td>
<td>0.01044a</td>
<td>0.02269c</td>
<td>0.02594b</td>
<td>0.03544b</td>
</tr>
<tr>
<td>AF</td>
<td>0.005389a</td>
<td>0.01214cd</td>
<td>0.03364d</td>
<td>0.04539e</td>
<td>0.04864d</td>
<td>0.05564cd</td>
</tr>
<tr>
<td>AD</td>
<td>0.0021139a</td>
<td>0.0136d</td>
<td>0.04589e</td>
<td>0.04939f</td>
<td>0.05664e</td>
<td>0.06064d</td>
</tr>
<tr>
<td>AN</td>
<td>0.013194b</td>
<td>0.02194e</td>
<td>0.03619d</td>
<td>0.03944d</td>
<td>0.04594cd</td>
<td>0.049946c</td>
</tr>
<tr>
<td>AC</td>
<td>0.002694a</td>
<td>0.00994bc</td>
<td>0.01244ab</td>
<td>0.10519g</td>
<td>0.10994f</td>
<td>0.11544e</td>
</tr>
<tr>
<td>CC</td>
<td>0.005694a</td>
<td>0.00894ab</td>
<td>0.01519bc</td>
<td>0.01819b</td>
<td>0.02269b</td>
<td>0.02619a</td>
</tr>
<tr>
<td>LSD (5%)</td>
<td>0.0039</td>
<td>0.0019</td>
<td>0.0022</td>
<td>0.0021</td>
<td>0.0023</td>
<td>0.0054</td>
</tr>
<tr>
<td>CV</td>
<td>5.3</td>
<td>11.9</td>
<td>6.6</td>
<td>4.4</td>
<td>3.4</td>
<td>7.1</td>
</tr>
</tbody>
</table>

Means with the same letter in common within each column do not have any significant difference at 5% probability.
APPENDIX E

Log values of hydrocarbon utilizing bacteria (HUB) count during incubation period.

Table 4.3

<table>
<thead>
<tr>
<th>Study</th>
<th>Day 0</th>
<th>Day10</th>
<th>Day 20</th>
<th>Day30</th>
<th>Day 40</th>
<th>Day 50</th>
<th>Day 60</th>
</tr>
</thead>
<tbody>
<tr>
<td>TD</td>
<td>3.727a</td>
<td>4.153a</td>
<td>4.243a</td>
<td>4.573a</td>
<td>4.607a</td>
<td>4.47a</td>
<td>4.177a</td>
</tr>
<tr>
<td>AX</td>
<td>4.31b</td>
<td>4.523b</td>
<td>4.613b</td>
<td>4.77b</td>
<td>4.787b</td>
<td>4.67b</td>
<td>4.36b</td>
</tr>
<tr>
<td>CC</td>
<td>4.193c</td>
<td>4.57c</td>
<td>4.71c</td>
<td>4.93c</td>
<td>4.977c</td>
<td>4.913c</td>
<td>4.75c</td>
</tr>
<tr>
<td>AF</td>
<td>4.51d</td>
<td>4.66d</td>
<td>4.74c</td>
<td>4.89c</td>
<td>4.870c</td>
<td>4.76d</td>
<td>4.18c</td>
</tr>
<tr>
<td>WI</td>
<td>4.50d</td>
<td>4.65d</td>
<td>4.72c</td>
<td>4.87d</td>
<td>4.910d</td>
<td>4.847e</td>
<td>4.64c</td>
</tr>
<tr>
<td>AC</td>
<td>4.29e</td>
<td>4.663d</td>
<td>4.80d</td>
<td>4.92d</td>
<td>4.940e</td>
<td>4.82e</td>
<td>4.65d</td>
</tr>
<tr>
<td>AD</td>
<td>4.61e</td>
<td>4.76e</td>
<td>4.90e</td>
<td>5.05e</td>
<td>5.090h</td>
<td>5.00e</td>
<td>4.91e</td>
</tr>
<tr>
<td>AN</td>
<td>4.10f</td>
<td>4.84f</td>
<td>4.91e</td>
<td>5.00f</td>
<td>5.02g</td>
<td>4.94h</td>
<td>4.84f</td>
</tr>
</tbody>
</table>

| LSD   | 0.03115| 0.0256| 0.02563| 0.0106| 0.01287| 0.01287| 0.01804|
| CV%   | 0.5    | 0.4   | 0.4    | 0.1   | 0.2    | 0.2    | 0.3    |

* Means having subscript in common within each column do not have any significant difference at 5% probability.
APPENDIX F

Some plate showing morphological characteristics of the various isolates

AF1 rod like (G+)

AF 2 rod like (G+)
AF3 rod like (G+)

AF4 short rod (G−)
AF 5 short rod (G −)

AN1 short rod (G -)
AN 2 central spore (G+)

AN 3 terminal spore (G+)
AN4 central spore (G+)

AN 5 central spore (G+)
AD 1 fusiform rod (G+)

AD2 short rod (G -)
AD3 sub-terminal rod (+)

AD 4 short rod (G -)
AD 5 central spore (G +)

AC1 central spore (G+)

University of Ghana  http://ugspace.ug.edu.gh
AC 2 central spore (G +)

AC 3 terminal spore (G +)
AC 4 short rod (G -)

AC 5 central spore (G+)
WI 1 central spore (G+)

WI 2 central spore (G+)
WI 3 terminal spore (G+)

WI 4 terminal spore (G+)
WI 5 terminal spore (G+)

CC 1 short rod (G -)
CC 2 central spore (G+)

CC 3 central spores (G+)
CC 4 cocci (G+)

CC 5 terminal spores (G+)
TD 1 terminal spores (G+)

TD 2 terminal spores (G+)
TD 3 central spores (G+)

TD 4 central spores (G+)
TD 5 central spores (G+)

Ax 1 central spore (G+)
AX 2 terminal spore (G+)

AX 3 terminal spores (G+)
AX 4 diplococci (G -)

AX 5 diplococci (G+)_