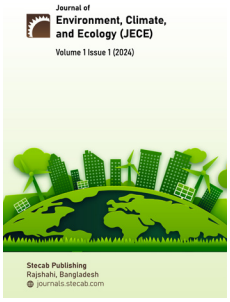

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Research Article

Utilization of Humic Substances by Bacteria Isolated From Humic Freshwater Sediment Ecosystem

*[1,2]Egbomuche, R. C., [2]Ekwenye, U. N., [3]Udofia, G. E., [2]Ikeh, C. E., [4]Ikeh, A. O.

About Article

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About Author

[1] Department of Microbiology, Faculty of Natural and Applied Science, Obong University, ObongNtak, EtimEkpo LGA, AkwaIbom State, Nigeria.

[2] Department of Microbiology, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria.

[3] Department of Microbiology, Faculty of Science, University of Uyo, Uyo – Nigeria.

[4] Department of Crop Science, Faculty of Agriculture, University of Agriculture and Environmental Sciences, Umuagwo, Imo State, Nigeria.

Contact @ Egbomuche, R. C.
iykeh2007@yahoo.com

ABSTRACT

A study on the utilization of humic substances by bacteria isolated from the sediment of humic freshwater, Eniong River ecosystem, a tributary of middle course of Cross River in Southern Nigeria was conducted using different microbiological and analytical techniques. Benthic sediments were collected from three sample stations known as upstream, midstream and downstream using an Eck sediment grab. The humic sediment of Eniong River contains diverse bacteria assemblages. The results showed that the heterotrophic bacterial counts ranged from 5.92 $\log_{10}$cfu/g downstream to 5.98 $\log_{10}$cfu/g upstream with mean count of 5.95±0.41 $\log_{10}$cfu/g while nitrogen-fixing bacteria ranged from 5.23 $\log_{10}$cfu/g midstream to 5.52 $\log_{10}$cfu/g downstream with a mean count of 5.36±0.67 $\log_{10}$cfu/g. Phosphate solubilizing bacteria counts on the other hand ranged from 4.30 $\log_{10}$cfu/g downstream to 4.54 $\log_{10}$cfu/g upstream with a mean count of 4.41±0.62 $\log_{10}$cfu/g. The characterization analysis studies of the isolates from the humic freshwater sediment ecosystem revealed 30 genera and 37 species of bacteria and 2 genera and 2 species of Actinomycetes. Growth profiles of the humic substance-utilizing bacteria on humic substance supplemented mineral salts medium revealed remarkable variations in species capabilities to utilize humic substances. The actual capability to utilize HS as a sole source of carbon and energy was indicated by increase in cell density overtime. Pseudomonas ($\log_{10}$7.93cfu/g and $\log_{10}$7.83cfu/g) exhibited the highest increase in cell density in HS of concentration 0.05%. However, the least cell density was observed by Bacillus subtilis ($\log_{10}$2.48cfu/g) in HS of concentration 0.25%. The pH and the optical density of culture of isolates exposed to different concentrations of HS indicating growth of isolates showed that Pseudomonas aeruginosa (pH 4.99 OD 2.00) of HS concentration 0.05% showed the best growth. HS utilization by the test isolates was characterized by increase in viable cell numbers as well as concomitant increase in substrate acidity (low pH) and increase in attenuance levels of the optical density (OD) as concentration of HS decreases.

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1. INTRODUCTION

1.1. Background of the Study

Bacteria diversity is defined as the variability among bacteria and this includes diversity within species, between species and of ecosystems. The main key of bacteria diversity on earth is due to evolution which accounts for most of the diversity of life on our planet. Diversity of bacteria in freshwater is influenced by some important physicochemical factors such as pH, dissolved oxygen (DO), total dissolved solids (TDS), total suspended solids (TSS), conductivity, temperature, salinity, oxidation and autotrophic nutrients as well as some biological aspects like plasmids, phages and transposons. DNA that influence the genetic properties and in most cases, the phenotypes of their host have great influence on bacteria diversity (Zhao *et al.*, 2012). In addition, protozoans are also reported to influence the bacteria diversity (Clarholm, 1994). Bacteria diversity represents a very large but mostly underexplored biological and genetic store house, which can be harnessed for novel genes, their products and metabolic pathways.

Diversity of bacteria can be employed as a sensitive pollution indicator in the environment and change because bacteria respond quickly to changes in the ecosystem (Seckbach & Kluwer, 2000). Since bacteria communities are involved in many sediment processes, they may also be used as a reliable indicator of ecosystem integrity. In sediment, high species richness diversity promotes interspecies relationships and interpopulation interactions. More diverse bacteria communities can better cope with disturbance and stress than low diversity in what is known as co-metabolism. Bacteria play an indispensable role in the environment and many organisms are dependent on their relationships with bacteria (Berman-Frank & Parker, 2003). They provide essential ecosystem services and are very crucial for all kinds of lives on the planet. They are critical in central biogeochemical processes, nutrient recycling, decomposition of organic matter, and consequently influence the composition of the atmosphere and affect the climate (Madigan *et al.*, 2003; Nagata, 2008). Bacteria community processes include reduction of sulphate, fixation of nitrogen, production of carbohydrate from sunlight, removal of nitrogen, methane production, reduction of metals and phosphate solubilization (Paerl & Pickney, 1996). Many of these processes result in the detoxification of noxious and other harmful contaminants that pose health risks to humans. This wide range of metabolic plasticity allows bacteria to inhabit many habitats, including habitats under extreme environmental conditions (Head *et al.*, 1998). An assessment of bacteria community diversity and structure is therefore an important step for understanding their role in ecosystem functions.

Humic freshwater ecosystem is a freshwater ecosystem that contains dissolved humic substances (HS). These are components that are present in the ecosystem and may be extracted readily from nearly all types of soil, water (ground and surface water) and sediment. They are complex organic molecules that make up most (50-80%) of dissolved organic matter (DOM) in freshwater ecosystem (Thurman, 1985; Wetzel, 2001). The substances are quantitatively important in all aquatic systems, ranging from 25% of the dissolved organic carbon (DOC) in ground water to as much as 90% of that in marshes and swamps (Thurman, 1985). There are many possible sources of HS including partially degraded materials from vascular plants, algae, microbial product and animal's remains. Structural polymers of vascular plants, collectively called lignocellulose-derived compound, particularly the lignin-derived fraction, have long residence times (Moran & Hodson, 1990a) and are likely sources of humic substances (Moran & Hodson, 1989b). The relative importance of lignocellulose as reservoir of humic compounds is expected to vary among aquatic ecosystems, however, according to the magnitude of vascular plant production relative to phytoplankton production. They originate from two main sources, in situ microbial production and terrestrial plants, and are usually referred to as autochthonous and allochthonous humic substances, accordingly. The allochthonous humic substances are dominant in freshwaters where they receive their main organic materials through surface runoff and leaching of the soil. Humic substances (HS) of terrestrial origin contain high levels of lignin and lignin degradation products, which are derived from the decomposition of vascular land plants and give terrestrial humic substances their pronounced aromatic character (Malcolm, 1990). Autochthonous humic substances dominate the DOM pool in the oceans, eutrophic lakes, and receiving limited terrestrial organic input and are highly aliphatic with lower aromatic character (Mcknight *et al.,* 1991).

HS, estimated at 1.6x1018 g C represent an important source of carbon in the biosphere. Due to their crucial role in reductive and oxidative reactions, sorption, complexation and transport of pollutants, minerals and trace elements, sustaining plant growth, soil structure and formation, and control of the biogeochemistry of organic carbon in the world ecosystem, HS are extremely important to environmental processes (Grinhut, Hadar & Chen, 2007). HS moderate bacteria activities through trace-element chelation effect on P cycling and effect on enzyme activity (Steward & Wetzel, 1982; Visser, 1985; Jones *et al.*, 1988). However, it is co-metabolized during bacteria utilization of high and sensitive substrates (De Haan, 1974, De Haan, 1997). It is possible also that some fraction of aquatic humic substances is available as primary bacteria substrate and if so, could play a significant role in supporting bacteria secondary production in aquatic ecosystems. Thus far, this possibility is consistent with observed correlation between humic concentrations and bacteria biomass and activity in a range of aquatic environment (Hessen, 1985; Tranvik & Hofle, 1987; Tranvik, 1988a) with observed increases in bacteria biomass and activity following addition of humic substances at concentrations above those naturally present (Tranvik & Sieburth, 1989) and in laboratory studies of bacteria isolates grown on humic substances (De Haan, 1997; Sederholm *et al.*, 1993).

HS are composed of three components which include; humic acids (HAs), fulvic acids (FAs) and humins which are defined based on their stability. HA comprised of high molecular weight organic substances soluble in water at a high pH value. HA can be extracted from soil by various reagents which are not soluble in acid. They are dark brown to black in color, whereas FAs are comprised of moderate molecular weight organic substances which dissolve in water at all pH value (Trump *et al.*, 2006). They remain in solution after removal of HA by acidification.

They are light yellow to yellow-brown in colour. Humins are the components of humic substances that do not dissolvable in water at all pH. Their colour is black (Bruns & Rice, 2000). In oligotrophic freshwater ecosystem with dissolved organic carbon (DOC) concentration between 1 and 100mg/l, HS exceed the organic carbon in all living organisms by roughly one order of magnitude (Thurman 1985; Wetzel, 2001; Steinberg and Munster, 1985). Jones (1998 & 2005) emphasized that all freshwater ecosystems contain some HS of allochthonous and at least autochthonous origin. Although, dissolved HS make up≏ 50% or more (DOC) in freshwater ecosystem their trophodynamic roles remain unresolved. HS make up most of the organic matter (OM) in freshwater environments and were thought to be inert or refractory, except for photolytic degradation. Accordingly, proof abounds showing an improved interaction of HS with organisms from aquatic ecosystems and weak man-made chemicals with specific and non-specific effects. An example of specific effect has to do with the hormone- like effect, such as the modulation of the number of offsprings which was first described with the nematode Caenorbaditiselegans. However, a hormone effect is not limited to only the nematode (William & Scott, 2004). Dissolved humic materials can affect the availability and toxicity of environmental chemicals (William & Scott, 2004). Due to the fact that they are everywhere and as a result of their different functional groups, humic substances have the ability to influence nearly all biogeochemical pathways in freshwater organisms and its ecosystems. The function of humic substances in indirectly powering the autotrophs in freshwater systems is outstanding. It is worthy to note that humic substances are equally active environmental chemicals, allowing the survival of fishes in extremely soft waters and due to internal or external mechanisms, stop iono-regulatory disturbance. Because of the low molecular mass of the building blocks of HS, (≏0.5 k Da), they appear to possess the ability to penetrate bio-membranes. Inside the organisms and behaving as natural chemicals, they are metabolized like xenobiotics. Microbial metabolism and diversity are mainly driven by the concentration and quality/composition of DOM and dissolved inorganic nutrients (Eiler *et al*., 2003b). Natural solar radiation, especially ultraviolet radiation (UV-B [280-315nm], UV-A [315-400nm]), are observed to induce chemical transformation of DOM with the production of a variety of photo-products, including carbon dioxide, carbon monoxide and ammonium phosphate (Mopper and Kieber, 2002). The origin and chemical composition of DOM strongly influence its photo-reactivity. The light-absorbing fraction of DOM, chromophoric dissolved organic matter (CDOM) from both terrestrial and autochthonous origins, is the primary absorber of sunlight in aquatic ecosystem and plays an important role for most photo-chemically mediated activities in water surfaces (Mopper and Kieber, 2002). This photo-transformation of DOM on bacteria growth is probably due to two alternate processes: photo and chemical reduction intake of freely produced algal carbon (Obernoster & Benner, 2004; Obernosterer *et al*., 1999; Tranvik & Bertilsson, 2001).

Therefore, the purpose of this study was to investigate the utilization of humic substances by bacteria isolated from humic freshwater sediment ecosystem of Eniong River.

2. LITERATURE REVIEW

2.1. DOC as the domain of bacteria in aquatic ecosystem

In aquatic ecosystem, DOC turnover is almost exclusively the domain of bacteria because of their high numbers, large surface area to volume ratios and transport systems efficient at low substrate concentration. Studies of bacteria utilization of DOC have indicated the existence of two distinct pools of dissolved compounds, one labile and one refractory, in most aquatic systems (Ogura, 1975). The labile DOC account for <20% of the total dissolved organic carbon (Allen, 1976; Ogura, 1975) and transforms very fast and therefore believed to support the bulk of DOC based bacteria secondary production (Allen, 1976). The refractory pool is larger but turns over more slowly on the order of weeks to months (Geller, 1986) and therefore may be relatively unimportant as a substrate for bacteria growth. However, it is worthy to note that none of this dissolved organic carbon components have been analyzed chemically in respect to chemical composition. Although it is thought that the labile pool is made up of amino acids, peptides, sugars and perhaps other minute compounds (Thurman, 1985), whereas the refractory DOC is composed of molecules mostly humic in nature (Fenchel & Blackburn, 1979), and polymerized from simple compounds.

2.2. Humic Substances

Humic substances (HS) are the most widely-spread natural complexing ligands occurring in nature and therefore contribute the bulk of the organic substances in nature. They represent bulk of the organic materials in soil, peat, lignites, brown coals, sewage, natural waters and their sediments. HS are sub-divided into 3 components: fulvic acids (FAs), humic acids (HAs) and humin. Humic acids and fulvic acids represent alkali-soluble humus fragments while humin represents the insoluble residue. As a result of the molecular structure of HS, they provide numerous benefits to crop production. They assist in the degradation of clay and compacted soils, help in moving those nutrients needed in minute quantities from the soil to the plant (Senesi *et al*., 1991), improve water retention, enhance seed germination rates and penetration and stimulate the microbial community development in the ecosystems.

2.3. Humic substances structure

It is crystal clear that humic substances are composed of heterogeneous combination of compounds for which no one structural formula can represent (Stevenson, 1982). HAs are presumed to be a complex aromatic macromolecules containing peptides, aliphatic compounds, sugars and amino acids that are linked with aromatic groups. The HA typical structure was freely attached and bound with phenolic (OH) groups, nitrogen, oxygen as well as quinone while carboxyl (COOH) groups are differently positioned on aromatic rings (Stevenson, 1982). It is currently believed by most researchers that every black coloured humic substance are not totally like high molecular weight polymers but part of closely related system. As a result of this observation, differences that exist between fulvic and humic acids can be described differently in their numbers of functional groups (carboxyl, phenolic OH),

molecular weight and the extent of polymerization (Stevenson, 1982). There is a lower carbon contents but higher oxygen in low molecular weight fulvic acids than the high molecular weight humic acids. It's obvious to note that there is more functional groups of an acidic property particularly carboxyl (COOH) that exist in fulvic acids. The acidic composition of fulvic acids which is (900-1400meq/100g) is clearly higher than that obtainable in humic acids (400-870meq/100g). It is however very important to state that while oxygen` present in FAs may largely be observed in specific molecular groups like C=O, COOH and OH, a large part of the oxygen found in humic acids normally exist as foundational composition of the nucleus. It was observed in electron microscope that HAs of various soils appear as clusters, chains and rings as a result of their polymeric structure. Their macromolecular structure sizes ranges from 60-500A (Stevenson, 1982).

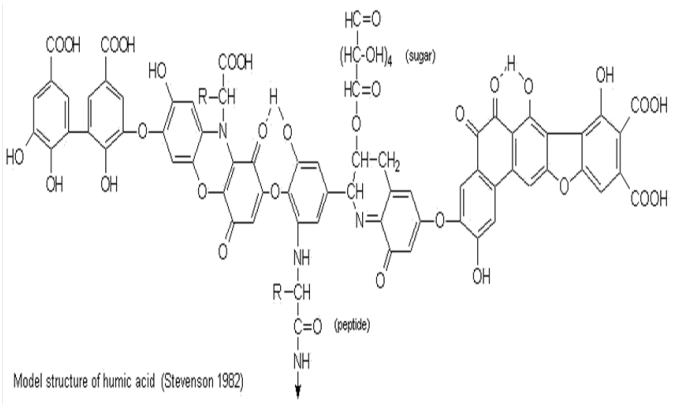

Figure 1. Model structure of humic acid (Stevenson, 1982)

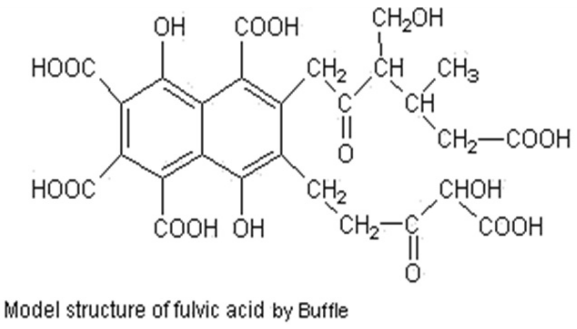

Figure 2. Model structure of fulvic acid (Stevenson, 1982)

2.4. Humic substances composition

2.4.1 Humic Acid (HA)

Humic acids are comprised of weak carbon chains and rings. They are organic acids that are not soluble in aqueous solution at low pH states, but are soluble, under basic (alkaline) conditions. When the pH is decreased below 2 in water-based solutions, humic acids solidify and fall out of solution, becoming that tiny fraction of concretions (precipitates) visible at the base of the test tube. On average 35% of the humic acid molecules are rings of carbon atoms (aromatic) while the remaining components (65%) are molecules that exist as carbon chains (aliphatic). The molecular weights of humic acids range from approximately 10,000 to 100,000. Humic acid macromolecules quickly combine with clay thereby forming strong organic clay components. When there is no clay or humic colloids, the cations may be lost either through fixation or leaching, and thereby lost to the plant root. Humic acids are crucial to living things in this planet, but they are susceptible to depletion via oxidation, and may become deactivated by sodium or aluminum. This creates soil problems which may impact the linear feeding relationship all the way up. Humic substances have been observed to stimulate seed germination of several varieties of crops. The plant characteristic that the introduction of humic substances has consistently enhanced more than any other is root length, especially on sandy soils. Top growth, vigor, and trunk cross-sectional area are also increased in response to stimulation by humates, but the effect is usually more prominent in the roots. A proliferation in root growth, resulting in an efficient root system, is a likely cause of higher plant yields seen in response to humic acid treatment. Humic acids readily form minerals with non-organic minor elements. A scientific study of extracts from naturally-occurring humic acids may show about five dozen or more of different mineralized elements present. This shows that these micronutrients are adsorbed to humic acids in a way that could be assimilated by different organisms. Because of this very important observation, we now realize why HAs play an important role in complexing of metals or chelating systems.

2.4.2. Fulvic Acids (FA)

FA, from "fulvus" means yellow. The colour of fulvic acids ranges from light yellow to yellowish-brown. They are that component of humic substances mixable in water at every pH levels. FA remain in the aqueous mixture after HA has been eliminated by addition of acid. FA is a low molecular weight natural organic substance soluble in water, derived from humus and normally present at water surface. Amongst the 3 major humic substances previously outlined, FAs are perhaps the most interesting for nutritional purposes. The size of FA molecules is smaller than HAs, with molecular weights ranging between 1,000g - 10,000g. Because of their relatively small size, fulvic acid molecules can readily penetrate into roots, stems and plant leaves. Therefore, fulvic acids are key ingredients of high quality foliar fertilizers. As they penetrate parts of plants, FAs conduct micronutrients from the surfaces of plant to the plant interiors or tissues. Fulvic acids immediately move micronutrients straight to their processing points within plants cells on application to the leaves. Hence, foliar spray introductions at particular plant growth levels, containing mineral chelates, may be employed as an elementary method for improving plants' productive capacity. When applied at relatively low concentrations they are completely non-toxic and 100% plant compatible. Fulvic acids maintain double oxygen constituent as compared to HAs. Because of the presence of several hydroxyl (OH) and carboxyl groups, fulvic acids are chemically reactive than other humic substances. It is equally interesting to note that fulvic acids possess a cationic exchange capacity that is higher than double of that of HAs. It has also been revealed that FAs could be the most prominent and effective carbon-containing compounds with chelating properties known. Scientific researchers have observed FAs as being the elements that actually make nutrients absorbable. This gives it the power to have effect on all kinds of diseases

and health problems that afflict the world today (Stevenson, 1994). Fulvic acids are very potent such that its single molecule has the capacity to take into plant cells several of vitamins, minerals and micronutrients. Scientists also tell us that fulvic acid is one of the most powerful natural electrolytes known to man. It is also amongst the most potent natural antioxidants and free radical scavengers known. Fulvic acids are known to accumulate heavy metals and carry out detoxification of contaminants or pollutants. It is manufactured by the activities of millions of beneficial microbes, working within an adequately oxygenated soil environment (Stevenson, 1994).

2.4.3. Humin

Humins are commonly black in colour. They are that group of HS that are neither soluble in high basic/alkaline (pH>7) nor in low acidic (pH<7) solutions. Humin cannot be dissolved in aqueous solution at any pH. Humin complexes are considered to be the largest of the so-called organic substances (macro) because of their high molecular weight (100,000g-10,000,000g). Humins physical and chemical characteristics are not well understood. of all the humic substances known, humins that are found within the soil are the most resistant to decomposition. Some of their main functions in the soil structure are the maintenance of the soil stability in order to enhance the soil's otherwise water-holding capacity. They also improve soil content while generally improving soil fertility. Humin has been revealed to be an essential key component of soil fertility because of the important functions it performs (Stevenson, 1994).

2.4.4. Humification

The process in which dead organic matters are converted into smaller states with concurrent release of energy and inherent bio-nutrients as well as different inorganic element is known as decomposition. The remnants can be assimilated by microorganisms (Lavelle & Pashanasi, 1989). This conversion of dead organic matters into forms that can be assimilated involves the complementary and simultaneous humification and mineralization processes. Whereas mineralization has to do with the conversion of elements that exist in organic form within bio-tissues to inorganic forms such as sulphate, nitrate and phosphate ions, humification is however, a biosynthetic process in which organic molecules are compacted into recalcitrant organic polymers, that may remain undegraded for several years. It is very essential to know that decomposition is a biological occurrence and uptake of nutrients by plants is basically due to a process of decomposition. However, it is clear that microorganisms are the most important contributors to respiration in the sediment and are therefore in charge of about 85-95% of the overall CO2 respired (Satchell, 1971; Lamotte, 1989). Hence, the interactions of three factors are responsible for decomposition process. These factors include the organisms, the decomposing resources quality and environmental factors (Swift, Heal and Anderson, 1979; Anderson and Flanagan, 1989). Furthermore, these factors that affect decomposition function at diverse spatial and temporal levels (Lavell, 1981). Living things are composed of many compounds and will be decomposed in sediment at the death of these organisms. As a result of this decomposition process, the organic materials in the sediments are gently converted such that they are no longer recognizable as the initial organic matter.

2.4.5. Humus in ecosystems

Due to organic carbon accumulation, humus possesses a characteristic dark-brown colouration. Humus is an organic matter that has reached a stable point where it cannot be degraded further (Whitelead & Tinsely, 2006). It can also be used to describe compost that has matured which is used in soil amendment. Humus is equally employed to explain the top soil that constitutes the organic material (Chertov *et al.*, 1997). The benefit of chemically stabilized humus is because of the nutrients it gives soil in both chemical and physical senses (Hargitai, 1993). Some agriculturists focus more on other aspects of humus, such as disease suppressiveness (Hoitink & Farley, 1986), improving soil retention of water by expanding microporosity (De Macedo *et al.*, 2002) and enhancing the formation of improved soil aggregates or arrangements (Hempfling *et al.*, 1990). Several active, anionic sites that bind to cations of plant nutrient are generated because of incorporation of oxygen into molecular assemblages thereby increasing their availability by ion exchange (Szalay, 1964). It enhances the ability of microbes to carry out feeding and reproduction process (Elos *et al.*, 2006; Vreeken-Buijs *et al.*, 1998). Humus has always been termed as the life force of soil. Yet, it has not been easy to describe it in precise terms due to its complexity. Humus is different from decomposing organic matter because of the rough looks of the organic matter. However, humified organic material, when observed under the microscope without any chemical treatment, could show small bit clearly identifiable microbial, animal or plant remnants which have already been mechanically degraded (Bernier and Ponge, 1994). This points to fuzzy limits between humus and organic matters. In most recent literature, humus is clearly considered as part and parcel of soil organic material (SOM). The breakdown of materials of plant origin ensures complex compounds are gradually oxidized (lignin-like humus) and converted further into biomass of microorganisms or are reorganized into humic assemblages (humic substances) which bind to metal hydroxides and clay minerals. It has been under debate whether plants can adsorb humic substances from their root systems and metabolize them. However, there is now a general agreement about humus playing a normal function rather than nutritional role in plant metabolism (Eyheraguibel *et al.*, 2008). During the natural conversion of organic matter into humic substances, microorganisms (bacteria and fungi) release sticky gums and mucilage; which contribute to the soil structure thereby bringing the soil particles together and enhancing soil aeration (Caesar – Tonthat, 2002). Furthermore, substances such as high metal and nutrients concentration that are toxic can be chelated and stopped from movement into the ecosystem, thereby detoxifying it (Huang *et al.*, 2008).

2.5. Humic substances utilization by organisms

Arguments have been raised concerning whether humic substances are bioavailable. For the time being, observable evidence revealed that they are really bioavailable. Wang *et al.* (1999) however revealed that humic substances or its

fractions were discovered to be present in the cells and DNA of microorganisms. Very recently, it was disclosed by (Nardi *et al*., 200) that metabolic effects of HS on plants are dependent on its origin, molecular mass and concentration. Evidence was presented by scientists that humic substance less than 3.5KD can easily penetrate the cell membranes which were accordingly absorbed. Evidence was presented by Steinberg (2003) that 14C- labeled HS-like substances such as caffeic acid oxidized products were absorbed and bioconcentrated by organisms found in freshwater. It was equally observed that the invertebrate, Gammarus pulex, the vertebrate, tadpoles of the Moor frog, Rana arvalis and the Macrophyte ceratophyllum demersum were observed to have bioconcentrated considerable quantities of 14C in their bodies during short-period of exposure. Recently, familiar results were obtained (Nardi *et al*., 2017) with 14C-labelled natural organic matter from compost and water flea Daphina magna. Argument has it that it's never the whole of caffeic acid oxidation product or NOM, but photo-degradation productions that is responsible for the biocoencetration of 14C (Hogue *et al*., 2003; Cooper *et al*., 2004; Hatcher *et al*., 2004).

2.6. Biodegradation of HS

HS are heterogeneous and high molecular mass organic materials that are everywhere in terrestrial and aquatic environments. They are not easily degraded by microorganisms (Mcknight, 1990) and therefore not particularly and dynamically considered to be involved in the metabolism, most importantly in anaerobic environments. However, it has been reported that some microbes present in sediments have the capacity to utilize HS as acceptor of electrons for anaerobic oxidation of hydrogen and organic compounds. This transportation of electron produces energy to support the growth of microorganisms. Other less accessible acceptors of electrons such as non-soluble Fe (iii) oxide enhances the capacity of microorganisms to increase microbial humic reduction. The discovery that microbes can donate electrons to humic acids has significant roles for the mechanism by which microbes oxidize both natural and contaminated organics in anoxic sediments and soils, thereby suggesting a biological origin of electrons for humic-mediated reduction of contaminant organics and metals (Mcknight, 1990). Several processes have been reported for the degradation of HS by using fungi (Kim & Wetzel, 2002) cultivation (Bongiovanni and Lobartini, 2006). A highly advanced oxidation processes is the Fenton technique that has been of considerable interest for contaminated soils remediation. The Fenton reaction involves the formation of hydroxyl radicals (OH-) from the catalytic decomposition of hydrogen peroxide (H2O2) by the soluble ferrous ion (Fe2+). OH- radical is a strong oxidizing agent for the destruction of organic pollutants (Villa and Nogueria, 2006). The Fenton technique is a promising chemical oxidation reaction as a result of its high efficiency and low cost (Sun and Yan, 2007). Its reagent involves a mixture of hydrogen peroxide and ferrous ions which generates hydroxyl radicals.

Moreover, microorganisms have a basic role in cycling of element and soil structure formation (Bastida *et al*., 2007). Current evidence suggests that in aquatic sediment ecosystem microorganisms are the chief agents for the biodegradation of substances of environmental concern. Thus, the microbiological techniques are seen as the most important and promising for application with different types of organic contaminant. The employment of microbiological techniques in the detoxification of contaminants typically is less expensive than the physical and chemical methods (Balba *et al*., 1998) and is also environmentally friendly. Organic matters from the soil are complex mixture which affect some of the soil characteristics and nutrients movements. The humic substances degradation is related to ecological processes (Mishra and Srivastava, 1986; Mackowiak *et al*., 2001; Katsumata *et al*., 2008; Badis *et al*., 2009). However, the information available on the characteristics of soil HAs and their degradation by microorganisms are little.

3. MATERIALS AND METHODS

3.1. Study Area

The study area is a humic freshwater ecosystem of Eniong River, a tributary of the Middle course of the Cross River (Figure 1.0) located in South-Eastern coast of the Niger Delta, AkwaIbom State, Nigeria. Eniong River is located in the South-Eastern coast of Niger Delta between latitude 05o 151 and 56.011 N and longitude 05o 121 N and 05o 05411 E.

3.1.1. Sample collection and preparation

Benthic sediment samples were collected from three sample stations designated ST3 (Upstream), ST2 (Midstream) and ST1 (Downstream) of the River as described by Nweke *et al*. (2007). A total of 9 sediment samples were collected for microbiological and humic substance analysis. Benthic sediment samples were aseptically collected using Eckman sediment grab into 95% ethanol-sterilized plastic containers and transported to the Microbiology laboratory within 24 h of collection for further analysis. Samples were kept at lower temperature by being placed in ice packed coolers. On arrival to the laboratory, the samples were immediately transferred into the refrigerator where they were stored at 4oC until required.

3.2. Microbiological Analysis

3.2.1. Isolation of diverse groups of bacteria from sediment samples of Eniong River

Ten-fold serial dilution of the sediment samples was carried out with known volume of sterile distilled water before enumeration of densities of the different microbial groups. Serial dilution of sediment samples was done according to the method of Cheesbrough (2006). Precisely 10g of sediment samples were measured and introduced into conical flasks containing 90ml of sterile distilled water (Cheesbrough, 2006). These were shaken for even distribution and thereafter 1ml of the aliquot was aseptically transferred into sterile test tubes containing 9ml of diluents to give a dilution of 10-1. This was repeated until a tenth (10-10) dilution factor was attained.

3.2.2. Determination of densities of different Bacteria groups in Eniong River sediment ecosystem

The densities of different bacterial groups were determined using standardized analytical procedures. These included the estimation of the heterotrophic bacteria as well as those of

phosphate solubilizing and nitrogen fixing bacteria.

3.3. Densities of heterotrophic bacteria from Eniong River sediment

The counts of total heterotrophic bacteria (HB) in the samples were respectively determined by the pour plate techniques using Nutrient agar (NA) as the analytical media.

3.4. Densities of nitrogen fixing bacteria from Eniong River sediment

The estimation of densities of nitrogen fixing bacteria (NFB) was carried out by pour plate technique using nitrate agar (Winogradsky medium) as the analytical medium. The medium was composed of (NH4)2SO4 -2.0g; K2HPO4 – 1g; MgSO4.7H2O-0.5g; NaCl-2.0g; FeSO4.7H2O-0.4g; CaCO3-0.01g, agar-15.0g and distilled water-1000ml. One milliliter (1 ml) each of the serially diluted samples was pour-plated and incubated in duplicates at a temperature of 28oC±2oC for a period of about 4 days (Essien & Udosen, 2000).

3.5. Densities of phosphate solubilizing bacteria from Eniong River sediment

The population of phosphates solubilizing bacteria (PSB) in the samples was estimated by the methods of Lu and Huang (2010). In this case, the diluted samples were introduced into compounded medium (Pikovskaya's (PKV) medium). The medium was composed of yeast extract -0.1g; glucose – 5g; Na2HPO4 – 1g; NaCl – 0.04g; NH4Cl– 0.04g; KCl – 0.04g; FeSO4 – 3 drops; Agar agar – 4g and 200ml of distilled water. One ml (1 ml) each of the serially diluted samples was pour-plated and incubated anaerobically at room temperature for a period of 3 days. The bacterial colonies showing clear zones around them were considered as phosphate solubilizing bacteria (PSB) (De Freitaset *et al.*, 1997) and were recorded appropriately.

3.6. Maintenance of Pure Cultures of Bacteria Isolates

Distinct cultures of the representative colonies of the different bacteria isolates observed on the culture plates were purified by repeated sub-culturing on freshly prepared nutrient agar medium. The medium used and conditions originally used for their isolation were adopted (Cowan, 1985). Stock cultures of the pure bacteria isolates were maintained on nutrient agar slants in McCartney bottles and incubated appropriately before preservation in the refrigerator for further use at 4oC.

3.7. Characterization and Identification of Bacteria Isolates

The bacteria isolated from humic freshwater were characterized by using standard procedures as described by Cowan (1985) and Holt *et al.*, 1994). The tests that were conducted include Gram's stain, spore stain, motility test, catalase test, urease test, coagulase test, oxidase test, citrate test, sugars utilization test, methyl red and voges-proskauer (MR-VP test) etc.

3.8. Extraction of Humic Acids (Ha) from Sediment Sample of Eniong River

Humic acid was extracted from the sediment samples from Eniong River by a modified Essington method (Jones, 1992). Fifty gram (50g) sediment sample was placed in conical flasks and washed with 50ml of 0.1M HCl. The HA in the sediment sample was extracted by 100ml of 0.5M NaOH in an orbital shaker at 120 rpm for 2 hours. Subsequently, extracted solution was filtered and adjusted approximately to pH 1 with concentrated 6M HCl. As a result of pH adjustment, a solid precipitate was produced representing the crude HA fraction, while the crude fulvic acid (FA) fraction remain in the filtrate. The crude HA fraction was re-dissolved again in 0.5M NaOH and then re-precipitated by acidification with 6M HCl. Additionally, the filtrate containing the crude FA was combined with the earlier fraction. The solid precipitate was re-dissolved in 0.5M NaOH to obtain the humic acid (HA) solution (Trumet *et al.*, 2006).

3.9. Purification of extracted humic acid

The yellow coloured supernatants containing the fulvic fractions were filtered and discarded leaving the humic acid which was finally washed with double distilled water until the last washing gave a negative chloride test with silver nitrate (Inam, 2005). The humic acid obtained was centrifuged at 4000 rpm for 15 minutes to further separate the humic acid from fulvic acid fractions which was then decanted and dried in an oven at 110oC. The dried humic acid was preserved for further use.

3.10. Determination of humic acid yield

The average weight of dried river sediment was obtained and recorded. The average weight of extracted humic acid was equally obtained and recorded. The percentage yield of humic acid extracted from river sediment was calculated by the formula below.

3.11. Utilization of Humic Substance (HS) from Eniong River by the Bacteria Isolates from Eniong

3.11.1. River Sediment

Different concentrations 0.25%, 0.125% and 0.05% of humic acid (HA) were measured and mixed with mineral salt medium (MSM) dissolved in 100ml of distilled water. The mixture was autoclaved at 121oC for 15 minutes. Thereafter, 1ml each of broth cultures of different bacteria species isolated from Eniong River sediments was separately inoculated into the humic substances supplemented mineral salt media (MSM) and incubated accordingly at room temperature in an aerobic shaker at120 m/s revolution for a period of 14 days. Inoculated flasks containing un-supplemented MSM was also prepared to serve as controls.

The total viable cell (TVC) counts of the inocula in the MSM were determined 48 hourly by the pour plate method. Enumeration was done after incubation at room temperature. The pH of the different media was measured by means of a Scott Gerate pH meter, which was calibrated in the laboratory. The optical density (O.D) of the media was measured with the aid of a spectrophotometer. The determination of O.D was based on the transmission of monochromatic incident light which decreases with an increase in microbial density of the medium. The equation below was employed for the calculation of optical density (O.D) (Trumet *et al.*, 2006).

OD: -log T
Where OD: Absorbance or Optical density
T: Transmittance

4. RESULTS AND DISCUSSION

4.1. Enumeration of different bacterial groups in Eniong River sediment (freshwater ecosystem)

The humic sediment of Eniong River harbours remarkable bacteria loads which are presented in (Figures 1 - 3). Three (3) different bacterial groups were isolated. The results showed that the heterotrophic bacterial counts ranged from 5.92 log10cfu/g downstream to 5.98 log10cfu/g upstream with mean count of 5.95±0.41 log10cfu/g (Figure 1) while densities of nitrogen-fixing bacteria ranged from 5.23 log10cfu/g midstream to 5.52 log10cfu/g downstream with a mean count of 5.36±0.67 log10cfu/g (Figure 2). Phosphate solubilizing bacteria counts ranged from 4.30 log10cfu/g downstream to 4.54 log10cfu/g upstream with a mean count of 4.41±0.62 log10cfu/g (Figure 3).

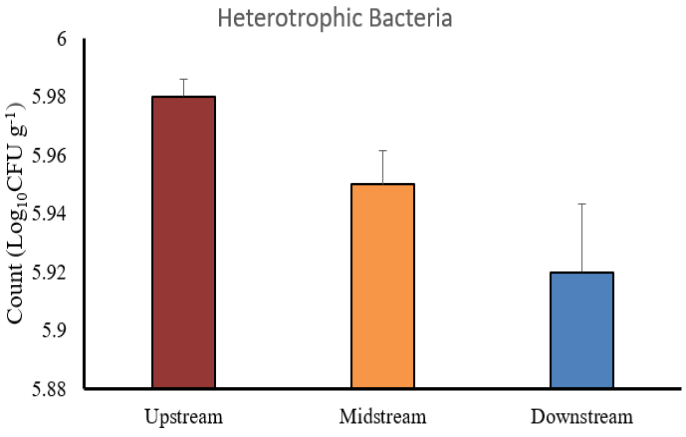

Figure 1. Heterotrophic bacteria counts in upstream, midstream and downstream of Eniong River

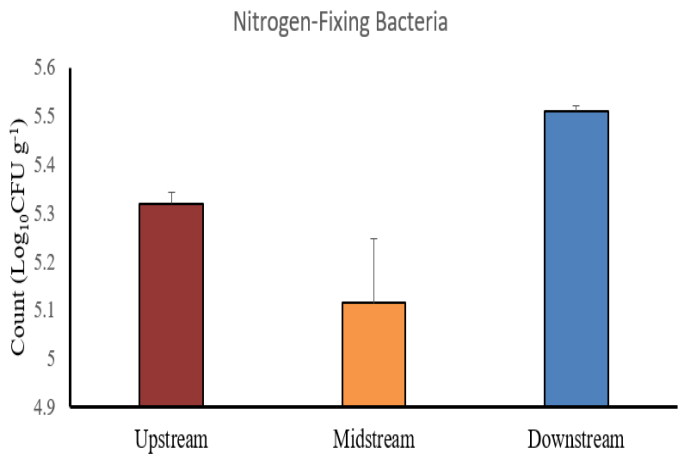

Figure 2. Nitrogen fixing bacteria counts in upstream, midstream and downstream of Eniong River

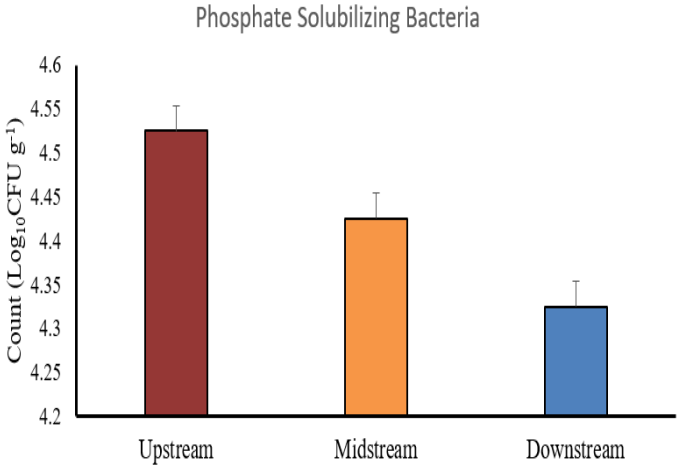

Figure 3. Phosphate solubilizing bacteria in upstream, midstream and downstream of Eniong

Table 1. Morphological, cultural and biochemical characteristics of isolates from Eniong River sediment ecosystem

Morphology	Gram Reaction	Catalase	Citrate	Coagulase	Indole	Motility	Oxidase	Methyl Red	VogesProskauer	Urease	Glucose	Sucrose	Lactose	Mannitol	Maltose	Nitrate Reduction	H2S Production	Gas Production	Probable Organisms
Rod	-	-	-	-	+	+	+	+	+	-	A	A	-	A	A	-	-	-	Vibrio cholera
Rod	-	-	+	-	+	+	+	+	-	-	A	-	-	A	A	-	-	-	Vibrio parahaemolyticus
Rod	-	+	-	-	+	-	-	+	-	-	A	-	-	A	A	+	-	+	Escherichia coli
Rod	-	+	+	-	-	+	+	+	-	-	AG	-	-	A	A	-	+	+	Salmonella typhi
Rod	-	+	-	-	-	-	-	+	-	-	A	-	-	A	A	+	-	+	Shigella sonnei
Rod	-	+	+	-	-	+	-	-	+	+	A	+	V	+	+	+	-	-	Enterobacter agglumerans
Cocci	+	+	+	+	+	-	+	+	+	+	+	+	Aww	A	A	+	-	-	Staphylococcus aureus
Cocci	+	+	+	+	-	+	+	+	+	+	+	+	-	A	A	-	-	-	Staphylococcus epidermidis
Rod	-	+	-	-	+	+	+	+	-	+	AG	+	-	-	+	+	+	+	Proteus vulgaris

Rod	+	+	+	-	-	+	-	-	+	+	A	A	A	A	A	+	+	+	Bacillus cereus
Rod	-	+	+	-	-	+	+	-	+	-	-	-	-	+	-	+	-	-	Pseudomonas aeruginosa
Rod	-	+	-	-	-	+	+	-	+	+	AG	A	A	AG	-	-	+	+	Desulfovibrio vulgaris
Rod	-	+	+	-	-	+	+	-	+	-	A	A	A	A	A	+	+	-	Desulfuromonassp.
Rod	-	-	+	-	-	-	-	-	+	-	AG	A	A	A	AG	+	+	+	Desulfobactersp.
Cocci	+	+	-	-	-	-	+	+	+	-	A	A	AG	AG	A	-	-	+	Micrococcus sp.
Rod	-	+	+	+	+	-	-	+	+	-	AG	A	A	AG	A	+	-	+	Burkholderiasp.
Rod	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	Lactobacillus casei
Rod	-	+	+	-	-	+	+	-	-	-	+	-	-	-	+	-	-	-	Sphingomonas sp.
Rod	+	-	-	-	-	+	+	-	-	+	+	-	+	-	-	+	-	-	Streptomyces griseus
Rod	-	+	+	-	-	+	-	-	+	-	+	-	-	+	-	+	-	-	Serretiamarcescens
Rod	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	+	+	Bacillus subtilis
Rod	+	-	-	-	-	+	+	-	-	-	+	-	-	-	+	-	+	-	Clostridium botulinum
Rod	+	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	Clostridium perferingens
Rod	+	+	-	-	-	-	+	-	-	+	-	-	+	+	-	+	+	-	Nocardiasp.
Rod	+	+	-	-	-	-	-	+	+	+	-	-	-	+	+	-	-	-	Mycobacterium sp.
Rod	-	+	+	-	-	-	+	-	-	-	+	+	+	-	+	+	-	-	Flavobacteriumaquatile
Rod	+	+	-	-	-	-	+	-	-	-	+	+	+	-	+	-	-	-	Brevibacteriumsp.
Rod	-	+	+	-	-	+	-	-	-	+	+	+	-	-	+	-	-	-	Achromobactersp.
Rod	-	+	-	-	-	+	-	+	-	+	-	+	-	+	+	-	-	-	Nitrosomonas sp.
Rod	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-	+	-	Desulfovibriohydrophilus
Rod	-	+	+	-	-	+	-	+	-	+	AG	A	A	+	+	+	+	-	Citrobacterfreundii
Rod	-	+	+	-	-	+	+	-	-	+	+	-	-	-	+	+	-	-	Pseudomonas fluorescens
Rod	-	+	+	-	-	+	+	-	-	-	+	-	-	+	-	+	+	-	Pseudomonas putida
Rod	-	+	-	-	-	+	-	+	-	-	AG	AG	-	-	-	+	-	-	Acetobacteraceti
Cocci	+	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	-	-	Enterococcus faecalis
Rod	-	+	+	-	-	+	-	-	+	+	+	-	+	+	-	+	-	+	Klebsiella pneumonia
Rod	-	+	-	-	+	-	-	+	-	+	A	A	-	+	+	+	-	+	Yersinia pestis
Cocci	+	-	+	-	-	+	+	+	-	-	+	+	+	-	+	-	-	+	Streptococcus sp
Rod	-	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	-	-	Aeromonashydrophila

Key:A: Acid, G: Gas, AG: Acid and Gas, + Present, - Absent

4.2. Identification of diverse species of bacteria isolated from Eniong River sediment humic freshwater ecosystem

Results of the characterization studies of the isolates from the humic freshwater sediment ecosystem are presented in Table 1. These involve 30 genera and 37 species of bacteria and 2 genera and 2 species of Actinomycetes.

4.3. Utilization of humic Substance (HS) by bacteria isolated from Eniong River sediment

The results of the growth of humic substance (HS)-utilizing bacteria on extracted humic substance incorporated into mineral salts medium are presented in Figures (4-6). Growth profiles of the humic substance-utilizing bacteria on humic substance supplemented mineral salts medium (HMSM) illustrated in Figures (7-14) revealed remarkable variations in species capabilities to utilize humic substance. The actual capability to utilize HS as sole source of carbon and energy was indicated by increase in cell density overtime Figures (4-6). Pseudomonas (log107.93cfu/g and log107.83cfu/g) exhibited the highest increase in cell density in HS of concentration 0.05% (Figure 4). This was followed by Micrococcus (Log107.78cfu/g) (Figure 5) in HS of concentration 0.125%. However, the least cell density was observed by Bacillussubtilis (log102.48cfu/g) (Figure 6) in HS of concentration 0.25%.

The pH and the optical density of culture of isolates exposed to different concentrations of HS indicating growth of isolates are shown in Figures (7-14). Pseudomonasaeruginosa (pH 4.99 OD 2.00) and Bacillussubtilis (pH 5.08 OD 2.00) of HS concentration 0.05% showed the best growth (Figures 7 and 8). This was followed by Bacillus (pH 6.0 OD 1.99) and Micrococcus (pH 5.05 OD 1.55) in HS concentration 0.125% Figures (12 and 11). The least growth was shown by Pseudomonas aeruginosa (pH

6.48 OD 0.37) in HS concentrations of 0.125% Figure (10). HS utilization by the test isolates was characterized by increase in viable cell numbers as well as concomitant increase in substrate acidity (low pH) and increase in attenuance levels of the optical

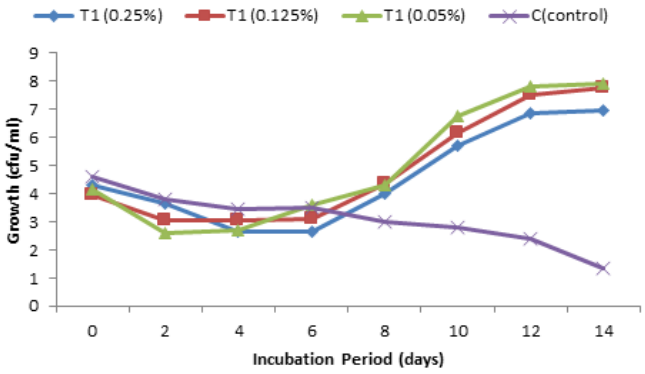

Figure 4. Density of Pseudomonas aeruginosa in mineral salt medium supplemented with different concentrations of HS

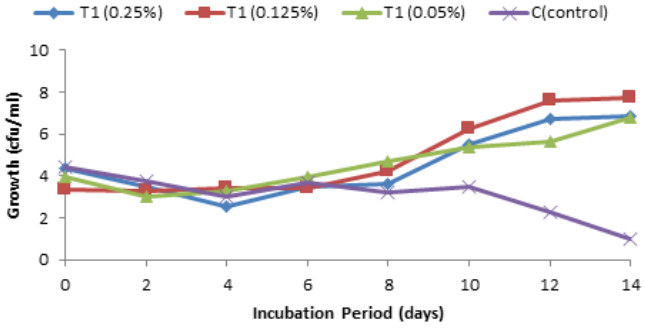

Figure 5. Density of Micrococcus sp. in mineral salt medium supplemented with different concentrations of HS

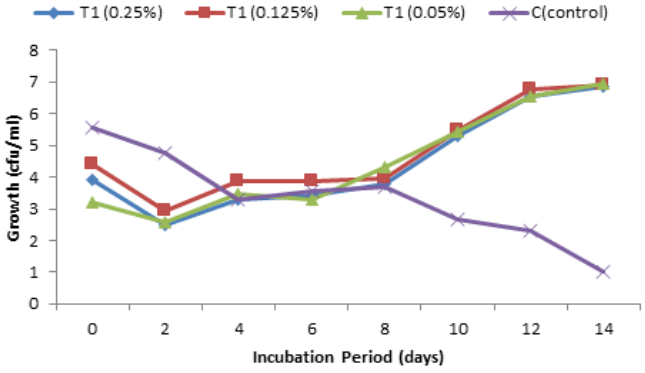

Figure 6. Density of Bacillus subtilis in mineral salt medium supplemented with different concentrations of HS

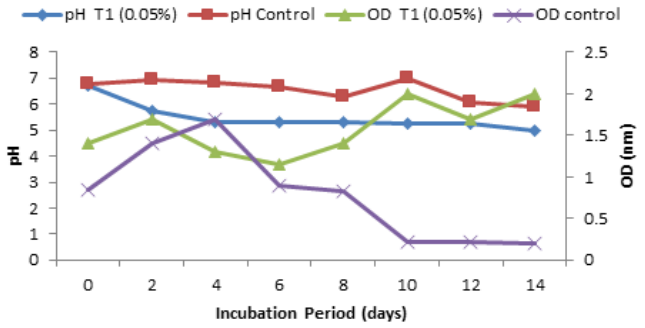

Figure 7. Growth profile (pH and OD) of Pseudomonas aeruginosa cultured on 0.05% humic substance supplemented mineral salt medium

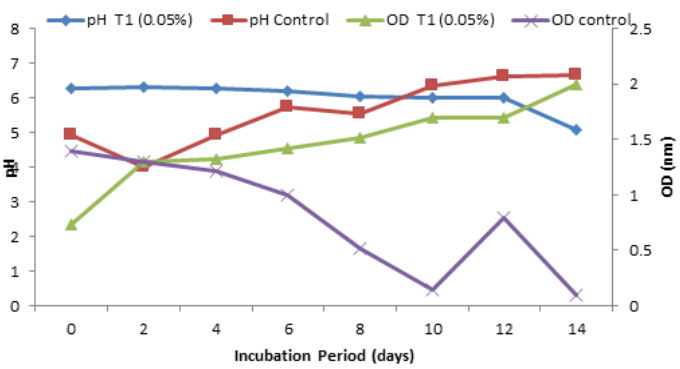

Figure 8. Growth profile (pH and OD) of Bacillus subtilis cultured on 0.05% humic substance supplemented mineral salt medium

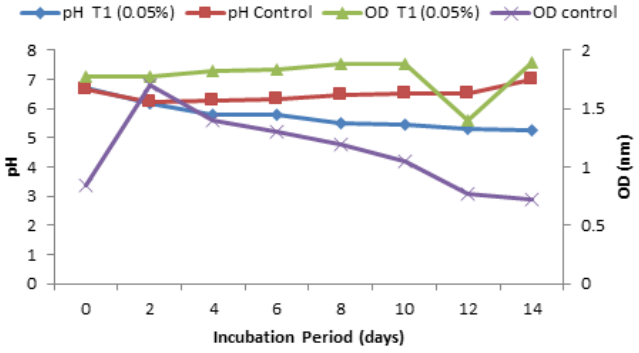

Figure 9. Growth profile (pH and OD) of Micrococcus sp cultured on 0.05% humic substance supplemented mineral salt medium

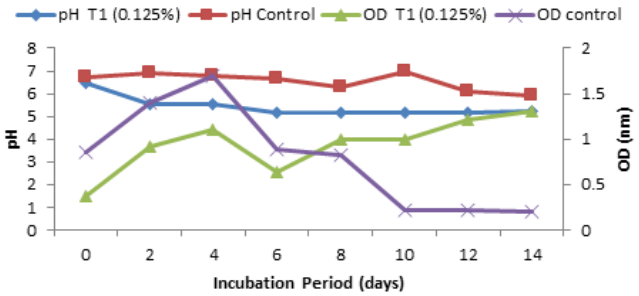

Figure 10. Growth profile (pH and OD) of Pseudomonas aeruginosa cultured on 0.125% humic substance supplemented mineral salt medium

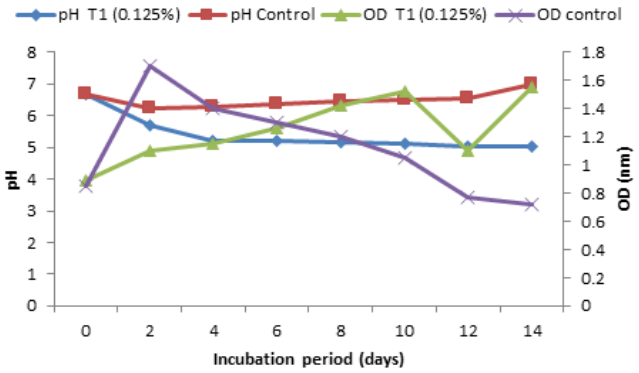

Figure 11. Growth profile (pH and OD) of Micrococcus sp cultured on 0.125% humic substance supplemented mineral salt medium

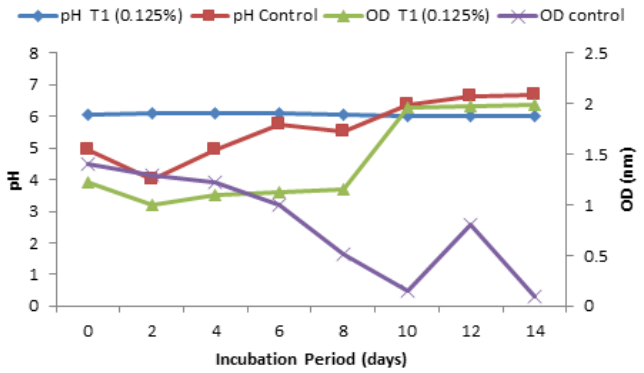

Figure 12. Growth profile (pH and OD) of Bacillus subtilis cultured on 0.125% humic substance supplemented mineral salt medium

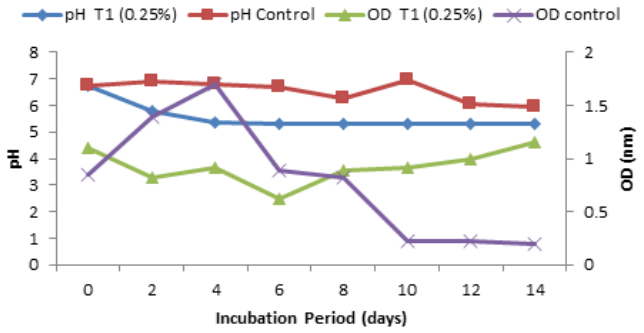

Figure 13. Growth profile (pH and OD) of Pseudomonassp cultured on 0.25% humic substance supplemented mineral salt medium

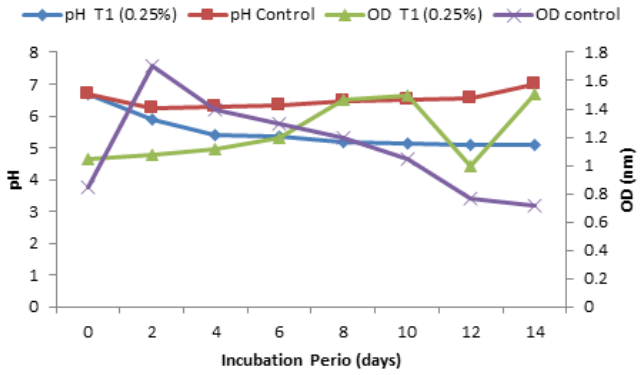

Figure 14. Growth profile (pH and OD) of Micrococcussp cultured on 0.25% humic substance supplemented mineral salt medium

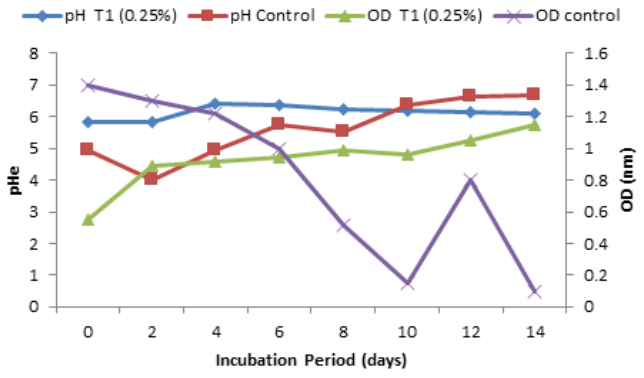

Figure 15. Growth profile (pH and OD) of Bacillus subtilis cultured on 0.25% humic substance supplemented mineral salt medium

density (OD) as concentration of HS decreases.

4.4. Discussion

The humic sediment of Eniong River harbours remarkable bacterial loads. Three (3) different bacterial groups were isolated. The best growth was observed by heterotrophic bacteria. Heterotrophic bacteria derive energy from organic compounds. They are widely distributed and could be ether aerobic or anaerobic. Heterotrophic bacteria are ubiquitous meaning that they could be found everywhere including food, soil, air and water.

Similarly, the density of nitrogen-fixing bacteria is moderately high in the Eniong River sediment. Nitrogen fixing bacteria are autotrophs and use carbon dioxide as their carbon source for growth. Some possess the enzyme, urease, which catalyzes the conversion of the urea molecule to two ammonia molecules and one carbon dioxide molecule. Free-living nitrogen fixers such as Pseudomonas, Klebsiella, Nocardia, Bacillus, Micrococcus and Enterobacter sp and confirmed diazotrophs like Nitrosomonas and Nitrobacterwere isolated from the freshwater sediment. They are known to assimilate the carbon dioxide released by the reaction to make biomass via the Calvin Cycle, and harvest energy by oxidizing ammonia (the other product of urease) to nitrite (Marsh *et al.*, 2005).

Remarkable loads of phosphate solubilizing bacteria are substantial in the sediment ecosystem. Phosphate solubilizing bacteria (PSB) are beneficial bacteria capable of solubilizing inorganic phosphorus from insoluble compounds (Chen *et al.*, 2006). Phosphate solubilization ability of rhizosphere microorganisms is considered to be one of the most important traits associated with plant phosphate nutrition. It is generally accepted that the mechanism of mineral phosphate solubilization by PSB strains is associated with the release of low molecular weight organic acids, through which their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble forms. Pseudomonas putida strains isolated from Eniong River sediment in this study are highly efficient insoluble phosphate solubilizers. Recently, researchers at Colorado State University demonstrated that a consortium of four bacteria synergistically solubilized phosphorus at a much faster rate than any single strain alone. However, there is a limit on the amount of phosphate which can be added to the environment due to the issue of eutrophication (Park, 2011).

Autotrophic bacterial groups including nitrogen fixing bacteria, sulphate reducing bacteria and phosphate solubilizing bacteria are very significant in the freshwater sediment.

The Niger Delta wetlands are recognized as a significant area of high bacterial diversity. The humic freshwater ecosystem native micro-flora characterized comprises 37 species of bacteria and 2 species of Actinomycetes. The diagnostic features of the bacterial isolates revealed the presence of diverse species of bacteria and Actinomycetes in humic freshwater. The bacterial isolates encountered were Proteus vulgaris, B. cereus, P. aeruginosa, Desulfovibrio vulgaris, Desulfuromonassp., Desulfobactersp., Micrococcus sp., Burkholderiasp., Lactobacillus casei, Sphingomonas sp.,Serretiamarcescens, B. subtilis, C. botulinum, C. perferingens, Mycobacterium sp., Flavobacteriumaquatile. The results have shown that like

other natural environments, the humic freshwater ecosystem investigated is inhabited by a diverse population of bacteria. These encompass a wide range of physiological and nutritional types, from autotrophs to heterotrophs. Heterogeneous communities are of major importance in aquatic ecosystems because of the considerable advantages gained by members of the population. It has been reported that spatial organization in biofilms and similar situations permits microorganisms to obtain many of the benefits of multicellular life (Varnam and Evans, 2000). The same authors reported that interaction between microorganisms permits activities such as co-metabolism and cross feeding, while diverse populations are less affected by environmental change and can recover from disaster more rapidly than ecosystems of lower diversity. This leads to long-term stability, although it should be appreciated that most microbial ecosystems are dynamic communities and are subject to continual short-term changes.

The river sediment properties have a major impact on bacterial distribution and adaptations. Some of the characteristics are silt, pH, cation exchange capacity (CEC), nitrate ion (NO-3) level, presence of humic substances and low oxygen level tension. The results of crude extraction of humic acid from the sediment of the Eniong River and adjoining non-humic sediment from the main Cross River has confirmed Eniong River sediments as richly laden with natural humic acid content, whereas the substance was below detectable level in the sediment of Cross River, a non-humic ecosystem. These findings differ slightly from those of Jones (1998 and 2005) who emphasized that all freshwater ecosystems contain some humic substances of allochthonous origin and at least autochthonous origin. There are several possible sources of these dissolved humic substances in this ecosystem. These may include partially degraded materials from vascular plants, algae, microbial products and animal remains that occur under low oxygen tension prevailing in such sediment. Structural polymers of vascular plants collectively called lignocellulose derived fractions are known to have long residence times and are additional sources of humic substances in the ecosystem (Moran and Hodson, 1990). The rich humic substrate ecosystem of Eniong River represents a new frontier of poorly understood ecosystem that holds critical value for life on earth. Although, a few estimates of total species numbers and biogeographic pattern have been attempted in humic sediment, the present study provides information on novel species from the ecosystem to suggest that humic sedimentary microorganism significantly impact major ecological processes.

5. CONCLUSION

The results of the growth of humic substance (HS)-utilizing bacteria on extracted humic substances incorporated into mineral salts medium indicates the profile which is based on changes in the total viable cell counts (TVC cfu/ml), the optical density (OD at 560 nm wavelength) and pH of cultures at room temperature (28 ± 2oC). The profiles of the humic substance-utilizing bacteria on humic substance supplemented mineral salts medium (HMSM) revealed a remarkable variation in species capabilities to utilize humic substance for growth. The actual capability to utilize HS as sole source of carbon and energy was indicated by increase in cell density overtime. Pseudomonas exhibited the highest increase in cell density in HS of concentration 0.05% at day 14 while the least growth was observed by Bacillussubtilis in HS of concentration 0.25% at day 2. The lowest pH and the highest optical density (OD) which indicates the highest growth are shown by Pseudomonas and Bacillus of HS concentration 0.05% at day 14. The least growth was shown by Pseudomonas in HS concentrations of 0.25% and 0.125% at day 0 respectively. HS utilization by the test isolates was characterized by increase in viable cell numbers as well as concomitant increase in substrate acidity (low pH) and increase in attenuance levels of the optical density (OD) as concentration of HS decreases. The breakdown of HS resulted in their growth and consequent production of acidic metabolic products. The acidic metabolites are responsible for the decrease in the pH of the growth medium from acidic pH 6.7 to more acidic levels over time. The effect on pH decreases with time but however varied with the microbial species involved. The capabilities of the humic substance-utilizing isolates to actually degrade the complex humic substances are intrisic. The HS-degrading isolates utilize the HS for biomass production. On HS-supplemented MSM (HMSM), the biomass of the HS-utilizing bacteria increased with increase in incubation periods. The HS supplement in the MSM provides the energy and carbon source for the proliferation of test organisms unlike the controls which do not contain HS. The isolates on MSM (control) may multiply using the endogenous energy base but eventually enters the death phase associated with lack of energy and carbon source for proliferation. These findings showed that the ability to utilize HS is genetically endowed and varied with the different microbial species, and its breakdown is concentration- and time-dependent. The growth and proliferation of selected species of bacteria; Bacillus subtilis, Pseudomonas aeruginosa and Micrococcus on extracted humic substances has confirmed that indeed, microorganisms can actually utilize humic substrate for growth. The question as to whether or not HS are taken up by organisms has been argued intensely in the literatures. Currently, empirical evidence has been revealed that HS are indeed taken up. Wang *et al.* (1999) showed that HS or at least a fraction thereof, were found inside the cells and even in the DNA in a cell culture study. Nardi *et al.* (2002) showed that physiological effects of HS on terrestrial plants depend on the source, concentration and molecular mass of HS. The researchers presented evidence that HS < 3.5kDa has the ability to pass the cell membrane of higher plant cells and were absorbed. Steinberg (2003) showed an evidence that 14C- labeled HS- like substances were assimilated and bioconcentrated by freshwater organisms.

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