MICROBIAL ABUNDANCE, DIVERSITY AND PHYSICOCHEMISTRY OF SEDIMENTS OF IKO RIVER ESTUARY, AKWA IBOM STATE

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ABSTRACT

Microbial abundance, diversity and physicochemistry of the sediments of Iko River Estuary in Akwa Ibom State, Nigeria were evaluated using standard plate count, analytical, whole bacterial community analyses and DNA sequencing techniques. The total hetrotrophic bacteria ranged from 2.1×10^6 to 3.6×10^6 CFU/g and sulphate reducing bacteria (SRB) from 2.1×10^1CFU/g to 4.1×10^1CFU/g. Culture dependent analysis revealed 16 bacterial genera with *Bacillus subtilis*, *Kleibsiella* sp, *Pseudomonas aeruginosa* and *P. flourescens* as the most abundant species (100%). Metagenomic analysis showed that the phyla Proteobacteria and Acidobacteria had the highest and lowest counts respectively for bacterial species. The two top spots were occupied by Unknown organisms with read counts of 582.0 (33.88%) and 562 (33.26%). Top known bacteria in sediments were *Thiomicrospira frisia* 20.0 (1.36%), *Fusibacter* 15.0 (1.02%), *Thiomicrospira chilensis* 13.0 (0.88%) and *Sulfurimonas* 13.0 (0.88%). Physicochemical analyses revealed slight decrease in sediment pH (6.20) upstream, (6.40) midstream, (6.50) downstream, Temperature (28^{o}C for upstream and 29^{o}C for downstream) and Electrical conductivity (130µScm^{-1}) towards the downstream. The rich organic matter and microbial population in the sediments of Iko River Estuary provide nutrients and niches for commercially and ecologically important flora and fauna. These data may form the baseline during future ecological evaluation, monitoring and assessment of estuaries in the Niger Delta

INTRODUCTION

Rivers that feed estuaries deposit sediments rich in nutrients, which settle on the sand and mud of the estuary floor, providing unique ecological niches to different microbes which play various roles in nutrient recycling as well as various environmental activities These conditions create unique habitats for microorganisms and provide an environment for biological diversity (fish, shrimp, crabs, and oysters) that are able to adapt to the brackish conditions (Wolanski, 2007).

Estuaries are amongst the most heavily populated areas in the world with about 60% of the world's population living along estuaries and the coasts (Agorye *et al.*, 2014). The high population leads to the ultimate degradation of the environment resulting from several factors, such as sedimentation from soil erosion, deforestation, overgrazing, and other poor farming practices, overfishing, drainage and filling of wetlands, eutrophication due to excessive nutrients from sewage and animal wastes, pollutants including heavy metals, radionuclides and hydrocarbons from sewage inputs (Eke, 2002). Important variable characteristic of estuarine water are the spatial variability in salinity, with a range of near zero at the tidal limit of the tributary river(s) to 34% at the estuary mouth. At any one point, the salinity will vary considerably over time and seasons, making it a harsh environment for organisms (Kaiser, 2005). Microbial communities readily respond to environmental changes thus playing key roles as indicators of environmental pollution. According to Charles *et al*. (2000), estuaries are subjected to both marine influences, such as tides, wave and the flux of saline water, and riverine influences, such as flow of fresh water and sediment. As a result, they may contain many biological niches within a small area and so are associated with high biological diversity.

As anthropogenic activities continually impact our environments, it underscores the need for constant environmental surveillance and reporting in order to foster environmental protection, economic stability and sustainability. Estuarine communities in Akwa Ibom State (an oil rich state in the Niger Delta region of Nigeria) have had more than a fair share of pollution and degradation.

A recent primary factor responsible for estuarine degradation in Akwa Ibom State is oil spill, which has continually posed environmental threat in the Niger Delta region (Udotong *et al*., 2015). This condition exists especially as a result of over seven decades (1950-2018) of oil and gas exploration and production (Udofia, 2017). History of oil spillage, improper waste management practices and pipeline vandalism in the estuaries in Akwa Ibom State indicates gross environmental contamination of water, soil, sediment and aquatic resources of the entire ecosystem (Kabari *et al*., 2017).

Understanding the drivers of species diversity and composition is a central goal of ecology, as it provides a window into how microbial communities form and why diversity varies across ecosystems (Joshi *et al.*, 2016). The presence and persistence of species in a given location or habitat is dependent on a suite of factors, including resource availability, biotic and abiotic interactions (Anderson *et al*., 2005). Just as the ecosystem influences the distribution of species, species diversity and composition can increase the likelihood of emergence of species capable of utilizing complementary resources and a much more efficient specie

in the community (Cleland, 2011). Thus, understanding what may impact geographical distribution, or biogeography of species is essential for understanding ecology as well as understanding the functioning of ecosystems, especially in the face of increasing anthropogenic stress.

Our understanding of the biogeography of microbes is less advanced than that for macroorganisms (Hanson *et al*., 2012). Such an understanding for patterns of microbial biogeography and the processes driving these patterns are incredibly important due to ecosystem services provided by the highly abundant and diverse organisms. Microbes are ubiquitous on earth, inhabiting lakes, soils, oceans, as well as more extreme environments including hot springs, deep sea hydrothermal vents, and saline pools (Anton *et al*., 2000); glacial ice (Boyd *et al*., 2011) and acid mine tailings (Baker and Banfield, 2003).

Once a knowledge base exists of patterns of bacterial communities across environmental gradients, we will then be able to better understand the processes underlying microbial distribution and potentially formulate predictions of how microbial communities might respond to changing environmental conditions. However, a strong need still exists for further characterizing microbial communities across ecologically important gradients such as sediments of estuaries. This study will provide information on the microbial diversity and physicochemistry of sediment of Iko river estuary in Akwa Ibom State.

MATERIALS AND METHODS

Site Description

Figure 1: Iko River Estuary

This study was carried out in Iko River Estuary, Eastern Obolo Local Government Area, Akwa Ibom State (Figure 1). The hydrology of the estuary is directed by tides, although seasonal influences which are related to the climatic regime are evident and are directly influenced by processes in the Atlantic coastal waters (Ekpe *et al*., 1995).

The study location (Okoroete – upstream, Utapete – midstream and Edonwhii – downstream) lies within latitude 7° 30' N and 7° 45' N and longitude 7° 30' E and 7° 30' E. The Iko River takes its rise from Qua Iboe River and drains into the Atlantic Ocean at the Bight of Bonny. The River estuary is formed by adjourning tributaries, creeks and channels. These constitute natural sites for petroleum exploration activities, fishing as well as breeding for diverse aquatic resources in the area (Ekpe *et al*., 1995).

Sample Collection

Sediment samples were collected from three (3) points viz; Okoroete (upstream) located at coordinate 04^0 30' 50" N and 007^0 44' 32.4" E; Utapete (midstream) located at coordinate 04^0 30' 48.7" N and 007^0 44' 59.8" E and Edonwhii (downstream) located at coordinate 04^0 30' 47.4" N and 007^0 45' 42.6", along the river estuary. This was carried out using Van-veen grab Sampler (Loving and Rantala, 1992) and stored in sterile containers. Samples were properly labeled and stored in ice-packed coolers and immediately transported to the laboratory for analyses. The sediments were further homogenized into composite samples for metagenomic analyses.

Culture Dependent Methods

Microbiological studies

Serial dilution

Serial dilutions of the sediment samples were done according to the method of Cheesbrough (2000). Precisely 10 g of sediment were measured and introduced into beakers containing 90 ml of sterile distilled water. These were shaken for even distribution and thereafter 1 ml aliquot was aseptically transferred into sterile test tubes containing 9 ml of diluents to give a dilution of 10^{-1}. This was repeated as a ten-fold serial dilution.

Estimation of Loads of Microbial Species in Sediment Samples

Standard microbiological techniques described by Harrigan and McCance (1990) were employed for the microbiological analysis of the sediment samples.

Densities of Heterotrophic Bacteria, Autotrophic bacteria and Fungi.

The total heterotrophic bacterial and fungal counts in sediment were estimated by the pour plate method using Nutrient agar (NA) and Sabouraud Dextrose Agar (SDA) as the analytical media respectively. The density of actinomycetes in sediment were enumerated after 7 days of incubation at 28°C $\pm$ 2°C using acidified Nutrient agar /Starch nitrate agar (Essien and Udosen, 2000).

Microbiological analyses were carried out to determine loads of Nitrate Reducing Bacteria (NRB), Sulphate Reducing Bacteria (SRB), Phosphate Solubilizing Bacteria (PSB), Nitrogen Fixing Bacteria (NFB), Hydrocarbon Utilizing Bacteria (HUB), Total Fungal (TF), Actinomycetes (AC), Total Coliform (TC) and Feacal Coliform (FC).

Bacterial nitrate reduction and denitrification
Nitrate broth adopted in accordance with the formula published in the Society of American Bacteriologist was used. The composition involved was achieved by dissolving 5.0 g of Peptic digest of animal tissue, 3.0 g of Meat extract, 1.0 g of Potassium nitrate and 30 g of Sodium chloride into 1000 ml of distilled water to give a pH7.0

The medium was heated to dissolve completely, dispensed into tubes and sterilized at 15 psi pressure at 121ºC for 15 minutes. The broth was inoculated with 1 g of sediment and then incubated aerobically at 20-24°C for 24-48 hours. After incubation, about 5 drops of + alpha-naphthylamine and sulfanilic acid were added to the medium and shaken gently in order to mix the reagents. Red color indicated the presence of nitrate reducing microorganisms or organism capable of denitrification. When color development in the broth after addition of + alpha-naphthylamine and sulfanilic acid failed, then a small amount of zinc dust was added to confirm the absence of nitrate in the medium. (MacFaddin, 2000).

Sulphate reducing bacteria
Sulphate API Broth was prepared according to the formulation described in the American Petroleum Institute Recommended Practice for detection of sulphate reducing bacteria (Tran *et al.*, 2021). Yeast extract (1.0 g), Magnesium sulphate (0.2 g), Dipotassium phosphate (0.01 g), Ferrous ammonium sulphate (0.1 g), Sodium chloride (10 g) and Ascorbic acid (0.1 g) were suspended in (1000 ml) distilled water and 4 ml of sodium lactate were added.

The medium was heated to dissolve completely. It was dispensed in screw-capped tubes in 9 ml amounts and sterilized by autoclaving at 15 psi and 121°C temperature for 10 minutes. The caps were immediately closed and the medium allowed to cool before inoculated with 1 g of sediment and incubated for 7-14 days. Sulphate-reducing bacteria converts sulphate to sulphide indicated by black coloration

Phosphate solubilizing bacteria
Pikovskaya media modified from Joe and Narendrakumar (2018) was used to detect the population of phosphates solubilizing bacteria (PSB) in the sediment samples. f Yeast extract (0.5 g), 10 g of Dextrose, 5.0 g of calcium phosphate, 0.5g of ammonium sulphate, 0.2 g of potassium chloride, 0.1g of Magnesium sulphate, 0.0001 of Manganese sulphate, 0.0001 of Ferrous sulphate and 15g agar were dissolved in 1000 ml of distilled water.

The solution was heated to boil to dissolve the medium completely and sterilized by autoclaving at 15 psi and 121°C temperature for 15 minutes. After proper shaking to mix, it was poured into sterile Petri plates. The presence of Phosphate-solubilizing bacteria showed formation of clear zone around the colony.

Hydrocarbonoclastic Bacteria
The vapor phase transfer method as described by Okpokwasili and Okorie (1988) was employed in this analysis. Hydrocarbon utilizing bacteria in the sediments were estimated by the viable plate count method using the surface spreading technique. After a ten-fold serial dilution of the sediment (10^{-1} to 10^{-4}), 0.1 ml of the various dilutions were plated in triplicates unto mineral salt agar medium supplemented with nystatin to inhibit fungal growth. Sterile filter paper (Whatman no.1) was aseptically placed in the inside of the cover lid and saturation with 2.0 ml of filtered Bonny light crude oil and then sealed around with a masking tape. This was to ensure the supply of hydrocarbons by vapor phase transfer as the sole source of carbon and energy for growth of the organisms that developed on the agar surfaces. The plates were incubated at room temperature (28±2ºC) for 9 to 12 days before the colonies that developed were counted and expressed as colony forming units per gram (cfu g^{-1}) of the sediment samples. Discrete colonies which developed were picked and purified by repeated sub-culturing and then stored on nutrient agar slants at 4ºC in a refrigerator for further studies.

Estimation of Total Yeast (TY)
The total yeast counts in the sediment samples were performed on Potato dextrose agar (PDA). Sterile streptomycin (50 mg/ml) was added to the PDA to suppress bacterial growth (Okorentugba and Ezereonye, 2003). The spread plate technique as described by Prescott *et al*. (2005) was adopted. An aliquot (0.1 ml) of the serially diluted samples were inoculated in triplicates onto sterile pre-dried PDA plates and then spread evenly with a sterile glass spreader. The plates were incubated at room temperature for about 3-5 days after which the colonies were counted and the mean count recorded accordingly. Test organisms were identified under standard procedures by observing their morphological, cultural and physiological characteristics (Udofia, 2008).

Actinomycetes
The density of actinomycetes from sediment and soil samples collected from the estuary were also enumerated after 7 days of incubation at 28^0C $\pm$ 2^0C using acidified Nutrient agar /Starch nitrate agar (Essien and Udosen, 2000).

Total coliform counts
The method of Prescott *et al.* (2005) was adopted where 0.1 milliliter of the serially diluted samples were inoculated onto sterile MacConkey agar plates in triplicates. The inoculum was spread evenly on the surface of the media using a sterile spreader. This was followed by incubation at 37^0C for 24 hours, after which the colonies were counted and the mean total coliform count expressed as cfu/g.

Purification and Maintenance of Bacterial Isolates
Representatives or Discrete colonies from culture plates were picked for characterization. Bacterial colonies were repeatedly sub-cultured into freshly prepared Nutrient agar plates by streaking methods and incubated for growth at 28ºC for 24 hours before transferring them to agar slant (Cheesbrough, 2000). The pure isolates of bacteria were maintained on agar slant as stock and preserved in the refrigerator for further use.

Characterization and identification of fungi
Pure cultures of the fungal isolates were characterized and identified on the basis of their cultural as well as morphological features of the vegetative and reproductive structures (Sampson *et al*., 1984).

Characterization and Identification of Bacterial Isolates
Bacterial isolates were characterized and identified presumptively based on their morphological, cultural and physiological characteristics confirmatory identification was based on biochemical reactions. The following biochemical tests were carried out. Gram straining, motility, coagulase, catalase, spore staining, oxidase, urease, citrate, starch hydrolysis, Methyl Red – Voges Proskaures (MR/VP) test, indole and sugar fermentation (lactose, glucose, mannitol, maltose, Galactose and sucrose). The results derived from the test for various isolates were collected and the identification was carried out by comparing the characteristics of known taxa using the scheme of Holt *et al*. (1994).

Determination of the Physicochemical Properties of Sediments
Fresh sediments samples were used for the determination of pH, temperature, total organic carbon, available phosphorus organic Nitrogen, salinity, Nutritive salts, particle size. Acidity, chloride content, sulphates and nitrates (AOAC, 1975).

Metagenomic Studies
Metagenomic analysis of sediment from Iko River estuary employed in this investigation to determine the microbial diversity include:

i. Community DNA extraction and quantification
DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen,). The quantity of the DNA fragments extracted was quantified by recording its UV absorption spectrum using NanoDrop 2000 spectrophotometer (Thermo Fisher, United States).

ii. DNA sequencing
Samples were sequenced on the sequel system by PacBio (www.pacb.com). Raw subreads were processed through the SMRTlink (v7.0.1) Circular Concensus Sequences (CCS) algorithm to produce highly accurate reads (>QV40). These accurate reads were then processed through vsearch (https://github.com/torogens/vsearch) and taxanomic information was determined based on QIMME2. Report generation command used: Screate_vsearch_single_sample_pdf_report_report_pacbio.py 200131_Cell_ccs-lbc28-UB2.otu_table.tsv LBC28 UB2 r200131 16s

iii. Bioinformatics Analysis of Metadata
General bioinformatics analyses were done using NCBI-BLAST-2.2.24 and CLC BioGenomics workbench v7.5.1 package.

RESULTS

Microbiological Analysis

Microbial Densities of the samples
The microbial density of sediments revealed the presence of high heterotrophic bacterial count. This high load was followed by total coliforms. The heterotrophic bacterial load in the sediment samples ranged between 2.1 x 10^6 and 3.6 x 10^6 CFU/g while, the sulphate reducing bacterial count ranged from 2.1 x 10^1 to 4.1 x 10^1 CFU/g. there was however, no nitrogen fixing bacteria detected from the midstream and downstream sediment samples while the upstream had a mean count of 1.1x 10^2 CFU/g. A summary of the result is presented in Table1.

Table 1: Microbial Load in Sediment Samples from Iko River Estuary

Microbial Group		THBC x 10^6	NRB x 10	SRB x 10	PSB x 10^2	NFB x 10^2	HUB x 10^1	TFC x 10^3	AC x 10	TCC x 10^3	FCC x 10^3
Sediment	SUO	2.7	5.1	2.1	2.4	1.1	2.1	2.1	2.5	3.5	1.1
	SMU	2.1	2.4	3.5	2.0	0	2.0	2.0	1.6	3.3	0
	SDE	3.6	2.0	4.1	2.5	0	2.6	0	1.1	3.2	0

Key: SUO –Upstream sediment (Okoroette), SMU – Midstream sediment (Utapete), SDE – downstream sediment (Edonwhii), THBC – Total Heterotrophic Bacterial count, NRB – Nitrate Reducing Bacteria, SRB – Sulphate Reducing Bacteria, PSB – Phosphate Solubilizing Bacteria, NFB – Nitrogen Fixing Bacteria, HUB – Hydrocarbon Utilizing Bacteria, TFC – Total Fungal Count, AC – Actinomycetes Count. TCC – Total Coliform Count; FCC – Feacal Coliform Count.

Morphological and Biochemical Characterization of Culturable Microbial Isolates
The cultural, morphological and biochemical attributes of the bacterial and fungal isolates from the samples are presented on Table 2 and Table 3. The bacterial species identified include species of *Bacillus*, *Streptococcus*, *Klebsiella*, *Proteus*, *Escherichia*, *Vibrio*, *Shigella*, *Salmonella*, *Clostridium*, *Micrococcus*, *Serratia*, *Pseudomonas*, *Staphylococcus*, *Enterobacter*, *Chromatium* and *Actinomycetes*. On the other hand, the fungal species comprised of *Aspergillus* sp., *Geotrichum* sp, *Penicilluim* sp., *Epicoccum* sp., *Rhizopus* sp., *Mucor* sp, *Trichoderma* sp., *Cladiosporium* sp., *Trichophyton* sp. and *Microsporium* sp.

Distribution and Occurrence of Culturable Bacterial Species in Sediment Samples

Analysis of the distribution and occurrence of bacterial isolates among the seven studied samples are as shown in Table 4. From the results, upstream sediment sample (Okoroette), harbor 13 (76.5) bacterial species as the highest count recorded, followed by downstream sediment (Edonwhii) 10 (64.7) and the least bacterial species of 9 (62.2) recorded for midstream sediment (Utapete). Of the total seventeen (17) bacterial species isolated in this study, *Bacillus subtilis, Klebsiella* sp., *P. aeruginosa* and *P. fluoresens* were the most abundant.

Phylum Classification

Table 5 shows the phylum classification of the bacterial community in the sediment samples from Iko River Estuary. The result revealed that Proteobacteria had the highest read counts of 990.0 (67.39%). Bacteroidetes came a distant behind with read counts of 168.0 (11.44%) in second place. The next ranking phyla with their corresponding read counts were Planctomycetes 63.0 (4.29%), Chlorofexi 51.0 (3.47%) Firmicutes 50.0 (3.40%), Unknown 26.0 (1.77%), Acidobacteria 23.0 (1.57%).

Table 2: Cultural, morphological, biochemical characteristics and identification of bacteria from Iko River sediments.

Gram Reaction	Shape	Catalase	Coagulase	Motility	Starch Hydrolysis	Citrate Test	Urease	MR	Voges-Proskauer	Spore formation	H_2S	Oxidase	Indole	Glucose	Lactose	Fructose	Sucrose	Mannitol	Galactose	Probable Organism
+	Thick rod	+	-	-	+	+	+	-	+	+	+	-	-	A	-	A	A	-	-	*Bacillus subtilis*
+	Cocci in chain	+	-	-	-	+		+	-	-	+	-	-	AG	-	AG	A	-	AG	*Streptococcus* sp.
-	Rod	+	-	+	-	+	+	+	-	+	+	-	-	AG	AG	-	AG	-	AG	*Klebsiella* sp.
-	Rod	+	-	+	+	+	+	+	-	-	+	-	-	AG	AG	AG	AG	-	AG	*Proteus* sp.
-	Short rod	+	-	-	+	-	-	-	+	-	+	-	+	AG	AG	AG	AG	-	AG	*Escherichia coli*
-	Comma	+	-	+	-	+	-	+	-	-	+	+	-	A	AG	AG	A	-	A	*Vibrio* sp.
-	Rod	+	-	+	-	-	+	-	+	-	+	-	-	AG	AG	AG	AG	-	AG	*Shigella* sp.
-	Rod	+	-	+	+	-	-	+	-	-	+	-	-	AG	AG	A	A	-	A	*Salmonella* sp.
+	Short rod	-	-	-	+	+	+	+	-	+	-	-	-	A	-	A	A	-	A	*Clostridium* sp.
+	cocci	+	-	-	+	-	+	+	-	-	+	+	-	A	-	A	A	-	A	*Micrococcus* sp.
-	Rod	+	-	+	-	+	+	-	+	-	+	-	-	AG	AG	AG	AG	-	AG	*Serratia* sp.
+	Cocci	+	+	-	-	-	-	-	+	-	-	-	-	A	-	AG	A	AG	A	*Staphylococcus aureus*
-	Rod	+	-	+	-	+	-	+	-	-	+	+	-	A	-	AG	AG	-	AG	*Pseudomonas aeruginosa*
-	Rod short	+	-	-	+	-	-	+	-	-	+	-	+	AG	AG	AG	AG	-	AG	*Klebsiella aerogenes*
+	Rod	+	-	+	-	+	+	-	+	-	+	-	-	AG	A	AG	AG	-	AG	*Chromatium* sp.
+	Rod	+	-	+	+	-	-	+	-	-	-	-	-	A	A	A	A	-	A	*Actinomycetes* sp.
-	Rod	+	-	+	-	-	-	+	-	-	-	+	-	AG	-	-	-	-	-	*Pseudomonas fluorescens*

Key: A = Acid; AG = Acid and Gas; + = Positive; - = Negative.

Table 3: Cultural and morphological characteristics of fungi from Iko River sediments.

Colony Color	Type of Soma	Nature of hyphae	Special Vegetative structure	Asexual spore	Special Reproductive Structure	Conidial head	Vesicle shape	Probable Organisms
Smoky or white colony	Filamentous	Septate	Footcell	Globose conidia	Short condiophores	Typically Columnar	dome shaped broadly clavate	*Aspergillus candidus*
Smoky or gray green colony	Filamentous	Septate	Footcell	Globose conidia	Short condiophores	Typically Columnar	dome shaped broadly clavate	*Aspergillus fumigatus*
White smooth membranous colony	Filamentous	Septate dichotomously branched	Absent	Cylindrical conidia	__	Arthrosporous	__	*Geotrichum Candidum*
Dense felt gray-green Spreading Colony	Filaments	Septate	Broom-like appearance	Globose condia produced in long columns	Erect conidiophores terminating in whorl of phialides	__	__	*Penicillium frequentans*
Cotton yellow to Brownish olive or black	Filaments	__	Crowded coniviophores	Subglobose	Short branch conidiophores	__	__	*Epicoccum* sp
White becoming grayish brown	Filamentous	Coenocytic	Stolons rhizoids	Ovoid Sporangiospors	tall sporargiohores in groups, brown black Sporangia	__	__	*Rhizopus stolonifer*
Compact white or yellow basal dark colony	Filaments	Septate	Footcell	Globose conidia	Smooth walled erect conidiophores	Globose	Globose	*Aspergillus Niger*
Creamish yellow	Filamentous	Coenocytic	-	Sporangiospore	Sympodially branched sporangiophore	-	-	*Mucor* sp
White colony with blue green shapes	Filamentous	Septate	-	Globose conidia	Pyramidally branched conidiophores	-	-	*Trichoderma* sp.
Powdery olivaceous brown	Filamentous	Septate	-	Acropetal branched conidia chains	Short conidiophores	-	-	*Cladosporium* sp.
Creamy white with reddish center and reddish-brown reverse	Filamentous at 28 $\pm 2^0$C	Septate	-	Micronidia, clavate thick-walled smooth marcroconidia	-	-	-	*Trichophyton* sp.
Glabrous white	Filamentous at 28 $\pm 2^0$C	Septate	-	Microconidia, spindle shaped marcroconidia	Poorly differentiated conidiophores	-	Terminal vesicle	*Microsporium* sp.
grayish green	Filamentous	Septate	Footcell	subglobose conidia	hyaline conidiophores	radiate	Subglobose	*Aspergillus glaucus*
hyaline mycelium becoming gray to grayish brown	Filamentous	Septate	sclerotia, swollen conidiogenous	abundant short conidiophores	-	-	-	*Botrytis* sp.
creamy milk white colony	pseudohypha e at 28± 2^0c	Septate	well developed	blastoconidia, chlomydospores	-	-	-	*Candida albicans*

Table 4: Occurrence of Bacterial Isolates in sediments of Iko River Estuary

Isolates	SUO	SMU	SDE	Occurrence rate (%)
Actinomycetes sp	-	+	+	2 (66.6)
Bacillus subtilis	+	+	+	3 (100)
Chromatium sp.	+	+	-	2 (66.6)
Clostridium sp.	+	-	-	1 (33.3)
Escherichia coli	+	-	-	1 (33.3)
Enterobacter aerogenes	-	+	-	1 (33.3)
Klebsiella sp.	+	+	+	3 (100)
Micrococcus sp.	-	-	+	1 (33.3)
Proteus sp.	+	-	+	2 (66.6)
P. aeruginosa	+	+	+	3 (100)
P. fluorescens	+	+	+	3 (100)
S. aureus	+	-	-	1 (33.3)
Streptococcus sp	+	-	+	2 (66.6)
Salmonella sp.	+	-	-	1 (33.3)
Shigella sp.	-	+	+	2 (66.6)
Serratia sp.	+	-	+	2 (66.6)
Vibrio sp.	+	+	-	2 (66.6)
Species Richness	13 (76.5)	9 (52.9)	10 (58.8)	

Key: SUO –Upstream sediment (Okoroette), SMU – Midstream sediment (Utapete), SDE – Downstream sediment (Edonwhii)

Table 5: Top 7 Phyla Classification in Sediment sample from Iko River Estuary

Phyla Classification	Read Count	%
Proteobacteria	990.0	67.39
Bacteroidetes	168.0	11.44
Planctomycetes	63.0	4.29
Chloroflexi	51.0	3.47
Firmicutes	50.0	3.40
Unknown	26.0	1.77
Acidobacteria	23.0	1.57

Table 6: Top 7 Bacterial Class Classifications in the sediment sample from Iko River Estuary

Class	Read Count	%
Gammaproteobacteria	569.0	38.73
Alphaproteobacteria	167.0	11.37
Deltaproteobacteria	119.0	8.10
Unknown	81.0	5.51
Cytophagia	61.0	4.15
Bacteroidia	52.0	3.54
Clostridia	46.0	3.13

Table 6 shows result of the identified classes of organism found in the sediment sample from Iko river estuary. The result shows Gammaproteobacteria occupying top spot with 569.0 (38.73%) read counts. The next top six classes recorded were Alphaproteobacteria, Deltaproteobacteria, Unknown, Cytophagia, Bacteroidia and Clostridia with read counts of 167.0 (11.37%), 119.0 (8.10%), 81.0 (5.51%), 61.0 (4.15%), 52.0 (3.54%) and 46.0 (3.13%) respectively. Table 7 shows the order classification in the sediment samples of Iko River Estuary. The analysis revealed that unknown bacterial orders also constituted the highest read count of 287.0 (19.54%) and 177.0 (12.05%). The top five orders were Chromatiales, Thiotrichales, Rhodobacterales, Cytophagales and Alteromonadales 114.0 (7.76%), 112.0 (7.62%), 82.0 (5.58%), 61.0 (4.15%), and 57.0(3.88%) respectively. Table 8 shows the family classification in the sediment samples of Iko River Estuary. The results indicate that unknown family had the highest read counts of 756.0 (51.46%). The next six abundant families were Piscirickettsiaceae, Rhodobacteraceae, Flammeovirgaceae, Helicobacteraceae, Marinilabiaceae, Nitrosomonadaceae. Table 9 shows top 7 bacterial genera in sediment samples and their percentages.

Table 7: Top 7 Bacterial Orders Classification in the Sediment sample from Iko River Estuary

Order	Read Count	%
Unknown	287.0	19.54
Unknown	177.0	12.05
Chromatiales	114.0	7.76
Thiotrichales	112.0	7.62
Rhodobacterales	82.0	5.58
Cytophagales	61.0	4.15
Alteromonadales	57.0	3.88

Table 8: Top 7 Bacterial Family Classification in the Sediment Sample

Family	Read Count	%
Unknown	756.0	51.46
Piscirickettsiaceae	90.0	6.13
Rhodobacteraceae	80.0	5.45
Flammeovirgaceae	45.0	3.06
Helicobacteraceae	34.0	2.31
Marinilabiace	29.0	1.97
Nitrosomonadaceae	29.0	1.97

Metagenomics analysis of the sediments of Iko river estuary reveal at the genus level the following; Unknown 1095.0 (74.54%), *Thiomicrospira* 41.0 (2.79%), *Sulfurimonas* 21.0 (1.43%), *Nitrosomonas* 20.0 (1.36%), *Fusibacter* 15.0(1.02%), *Marinobacter* 14.0(0.95%), *Cytophaga* 13.0 (0.88%).

Table 9: Top 7 Bacterial Genera Present in the Sediment samples

Genus	Read Count	%
Unknown	1095.0	74.54
Thiomicrospira	41.0	2.79
Sulfurimonas	21.0	1.43
Nitrosomonas	20.0	1.36
Fusibacter	15.0	1.02
Marinobacter	14.0	0.95
Cytophaga	13.0	0.88

Further analysis identified a total of 148 organisms at the Species levels in the sediment sample. The top two spots were occupied by Unknown species with close read counts of 5710 (38.87%) and 562.0 (38.26%). Read counts for the next top ten species fell far behind with *Thiomicrospira frisia* 20.0 (1.36%) at third, *Fusibacter* 15.0 (1.02%) at fourth, *Thiomicrospira chilensis* 13.0 (0.88%) at fifth, *Sulfurimonas* 13.0 (0.88%) at sixth, *Cytophaga fermentans* 12.0 (0.82%) at seventh, *Oceanisphaera* 12.0 (0.82%) at eighth, *Mariprofundus* 8.0 (0.54%) at ninth and *Arcobacter*7.0(0.48%) at tenth.

Physicochemical characteristics of sediment

The physicochemical characteristics of sediment from Iko River Estuary are presented on Table 10. Temperature stood at 28^0 C for the upstream and midstream sediment but slightly higher downstream with 29^0 C while the pH was 6.20, 6.40 and 6.50 for the upstream, midstream and downstream benthic sediment respectively. Electrical Conductivity was highest for the downstream sediment (130µscm^{-1}) and least for midstream (124µscm^{-1}). Chloride content was consistent for the upstream (309.3mg/l), midstream and downstream (308.6mg/l) respectively. Nitrate content was 8.03 mg/l, 8.11 mg/l and 8.13 mg/l for upstream, midstream and downstream respectively.

Nitrite concentrations were 0.090mg/l; 0.083mg/l; 0.085mg/l for upstream, midstream and downstream respectively. Phosphate concentrations recorded 7 mg/l for the three stations, Sulphate concentrations maintained a value of 84 mg/l for the upstream, midstream and downstream sediments. Values of TOC recorded close percentages values of 3.044%, 3.039% and 3.035% for the upstream, midstream, and downstream sediments. The Particle size were dominated by sand (78.42%), followed by clay (20.14%) and silt at 1.44%.

DISCUSSION

Microorganisms are distributed in the biosphere based on natural selection which shapes interaction, but dependent on environmental context (Holly *et al.*, 2018).

Table 10: Physicochemical characteristics of Sediment Sample from Iko River Estuary

Parameter	**Benthic sediment (upstream)**	**Benthic sediment (midstream)**	**Benthic sediment (downstream)**
Temperature (^{0}C)	28	28	29
pH	6.20	6.40	6.50
E. Conductivity (µscm^{-1})	126	124	130
Chloride (mg/l)	309.3	308.6	308.3
Nitrate (mg/l)	8.03	8.11	8.13
Nitrite (mg/l)	0.090	0.083	0.085
Phosphate (mg/l)	7.130	7.062	7.066
Sulphate (mg/l)	84.04	84.12	84.14
TOC (%)	3.044	3.036	3.035
Organic matter (%)	71.093	71.091	71.092
Particle size			
Silt	1.46%	1.44%	1.44%
Clay	20.11%	20.14%	20.14%
Sand	78.41%	78.42%	78.42%

In sediment ecosystem these microorganisms play important roles in the decomposition of organic matter, mineralization, element cycling and transformation due to their versatile metabolic abilities. Micro-organisms, including bacteria, archaea, protists and fungi, dominate sediments exhibiting varying abundance and biomass. They represent a large and diverse pool of species and exhibit a vast array of metabolic functions. Some of them are key players in biogeochemical processes (Falkowski *et al.*, 2008), participating in controlling water and sediment quality (Yu *et al.*, 2009).

This study has revealed that sediments of Iko river estuary harbor a wide diversity of both cultivable and non-cultivable microbial diversity. This may be attributed to the fact that sediment ecosystems serve as sink for pollutants and waste from both the atmosphere and the lithosphere. The densities of heterotrophic bacteria obtained from the estuarine sediment in the present study are slightly lower than values reported in Australia where the numbers ranged from 2.0 x 10^8 cells to 3.6 x 10^{10} cells g^{-1} dry weight of sediment (Alongi, 1994), but similar to the population recorded in estuaries around the region of Xuande Atoll off the South China Sea (Zhang *et al*., 2020). It has been stated that heterotrophic activities among microorganisms permit them to obtain many of the benefits of multicellular life. Interaction between microorganisms permits activities such as co-metabolism and diverse populations are less affected by environmental changes and can recover from adverse conditions more rapidly than ecosystem of less diversity (Varnam and Evans, 2000).

Autotrophic bacterial groups including sulfate reducing bacteria, nitrogen fixing bacteria and phosphate solubilizing bacteria were encountered in the studied sediments. Sulfate occurs widely in sediments and water rich in decaying organic material and sulfate reducing bacteria are common in anaerobic environments utilizing the ions as terminal electron acceptors, reducing it to hydrogen sulfide. Toxic hydrogen sulfide is a metabolic product and its rotten egg odor is often a marker for the presence of sulfate reducing bacteria in nature (Dexter, 2003). Sulfate-reducing bacteria are responsible for the sulfurous odors of salt marshes and mud flats. Much of the hydrogen sulfide produced react with metal ions in sediment, water and soil to produce metal sulfides. These metal sulfides are responsible for the dark color of sludge (Ernst-Detlef and Mooney, 1993) and the sulfides being water insoluble and heavier than water settle down in the water as precipitates (Hussain *et al.*, 2016). Several species reduce small amounts of sulfates in order to synthesize sulfur-containing cell components in a process of assimilatory sulfate reduction. Others reduce sulfate in large amounts to obtain energy and expel the resultant sulfide as waste in a dissimilatory sulfate reduction process. Sulfate-reducing bacteria are ubiquitous in anoxic sediment habitats, where they play important role in both sulphur and carbon cycles.

Nitrogen fixing (nitrifying or denitrifying) organisms are autotrophs and use carbon dioxide as their carbon source for growth. Free-living nitrogen fixers including *Pseudomonas, Klebsiella, Nocardia, Bacillus, Micrococcus* and *Enterobacter* sp and confirmed diazotrophs like *Nitrosomonas* and *Nitrobacter* were isolated from the estuarine sediments in the present study. 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 to nitrite (Marsh *et al.,* 2005). Their presence in the estuarine sediments contributes to nitrogen inputs and fluxes in the sediment system, thus, enhancing and maintaining adequate nitrogen supply which is an essential component of proteins, nucleic acid and other cellular constituents. Though there is an abundant supply of nitrogen in the earth's atmosphere (79%) in the form of nitrogen gas, however, nitrogen is unavailable for use by most organisms because there is a triple bond between the two nitrogen atoms, making the molecule almost inert. In order for nitrogen to be used for growth, it must be fixed or combined in the form of ammonium or nitrate ions. The processes of weathering release these ions from rocks in a slow manner that it has a negligible effect on the availability of fixed nitrogen. Thus, nitrogen is often the limiting factor for growth and biomass production in all environments.

Bacteria have a central role in almost all aspects of nitrogen availability and thus for life support on earth. This group of organisms are present in numbers in the studied sediment of Iko River.

Phosphate solubilizing bacteria (PSB) are beneficial bacteria capable of solubilizing inorganic phosphates from insoluble compounds (Chen *et al.,* 2006). P-solubilization ability of microorganism 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. In this study, diverse phosphate solubilizing bacterial species were observed. Application of PSB in soil appears to be an efficient way to convert insoluble phosphate compounds to useable form for plant use, resulting in enhanced crop plant productivity and increases efficiency of nitrogen fixation. Their application is an ecologically and economically sound approach (Kalayu, 2019).

Coliform bacteria are enteric bacteria that are used as indicators of the likelihood of the presence of bacterial pathogen. Although fecal coliform on their own are usually not harmful to humans, their presence indicates the presence of fecal wastes which may contain pathogens (Fajri *et al*., 2017). The high incidence of coliforms observed for the sediment in this study may be attributed to human impact and a pointer to the inherent risk of disease outbreak if the contaminated water is deliberately or accidentally consumed. This assertion is again confirmed by the equally high densities of *Escherichia coli, Salmonella, Shigella* and *Vibrio* in the estaurine sediment. This finding is in agreement with the report that reduction in fecal coliforms often correlates with reduction in *Salmonella* species and other pathogenic microorganisms, consequently, coliform monitoring maybe predictive for the presence of fecal pathogen such as *Salmonella* in human-impacted sediments (Lyimo *et al.,* 2016). Humans and animals could be exposed to pathogens directly by coming in contact with contaminated sediments and water or indirectly by consuming or drinking water or seafood contaminated by the pathogens. Despite the well-studied association between fecal contamination of water and the acute enteric and skin disease (USEPA, 2012). Correlation between these bacterial proxies and specific disease-causing organisms has been difficult to demonstrate in the absence of a point-source such as sewage outflows (Wu *et al.,* 2011). Known limitation that could explain this weak association is the short survival of some fecal indicator organisms in water. However, the pathogenicity of the isolates was not determined in the present study.

Metagenomics of Iko River Estuary Sediment Samples

This study confirmed that cultural method for enumerating microorganisms in the ecosystem is limited as it only accounts for >1% of the total microbial community in the ecosystem (Su *et al.,* 2012). Though much research has been conducted so far, information is still limited about the prokaryotic distribution and community structures in the sediment of Iko river estuarine environment. This is in spite of the fact that sediments represent a substantial part of the biosphere and sediment microorganisms play an important role in carbon cycling and global geochemistry (Parkes *et al*., 1994). It has been estimated that the number of

prokaryotic cells in the largely unexplored sediments represents 10–30 % (D'Hondt *et al*., 2002). Metagenomic analyses of Iko river estuary sediment samples revealed it harbor a total of 15 bacterial phyla. The top bacterial phyla associated with the sediment samples were Proteobacteria, Actinobacteria, Chloroflexi, Unkinown, Acidobacteria, Planctomycetes, Bacteroidetes while the remaining phyla made the remaining 8.27%.

The high reads of bacteria obtained in this study agrees with several reports that points to it being the dominant microbial domain in the sediments (Meneghine *et al.*, 2017). Meneghine *et al.* (2017) in their study reported the presence of 22 bacterial phyla with a large proportion of the sequences being unclassified at the phylum level. According to the authors, the phyla Actinobacteria, Firmicutes, Planctomycetes, Gemmatimonadetes, Chloroflexi, and Acidobacteria were in relative abundance. Similarly, Mahmoudi *et al*. (2015) detected a total of fifty-five bacterial phyla in Caspian Sea sediment with Proteobacteria (33% of bacterial reads on average) being the most abundant, followed by Planctomycetes (14%) and Chloroflexi (12%). In the sediment of the studied site, Gammaproteobacterial had the highest reads followed by Alphaproteobacteria and Deltaproteobacteria.

Gammaproteobacteria have been reported to be one of the abundant bacterial groups in sediments (Ruff *et al.*, 2013). The presence of uncultured bacterial phylum Acidobacteria in the sediment samples in this study also agrees with Barns *et al*. (2007). However, the subgroups are rarely cultured, and consequently, the Acidobacteria remains a poorly studied phylum. In this study, it was discovered that only two acidobacterial subgroups (Gp22 and Gp23) predominated, with similar abundances in sediment types. Wang *et al.* (2012) reported that the acidobacterial subgroups Gp10 and Gp22 were higher abundance ranked taxa in marine sediments. Studies have reported that the abundance and/or diversity of Acidobacteria is affected by environmental factors including pH, soil types, and other soil abiotic factors (avarrete *et al.*, 2013). In the class Actinobacteria, *Ilumatobacter* and *Propionibacterium* were identified as a major and a minor genus respectively in sediments.

Consistent with previous studies of sediments (Blazejak and Schippers, 2010), the relative abundance of Chloroflexi and Planctomycetes increased with sediment depth. Little is known about the physiology of Chloroflexi, although they are presumed to be heterotrophic (Webster *et al.*, 2011). Members are key bacterial representatives associated with methane hydrates and commonly co-occur with Candida JSI in anoxic sediment zones (Jørgensen *et al*., 2012). In this study, sequences belonging to JS1 comprised a small proportion (1–5%) of the microbial community. Even though Chloroflexi and JS1 often co-occur, it has been suggested that Chloroflexi dominate organic-rich seafloor sediments while JS1 dominate strictly anoxic, organic-rich but poor-quality recalcitrant carbon muddy sediments with low sulfate concentrations (Webster *et al.*, 2007).

Physicochemical Analyses of sediment of Iko River Estuary

Physicochemical parameters of sediment are important factors affecting the nutrient retention and aquatic resources of these ecosystems (Rao and Rao, 2014). The results of the physicochemical analysis show that pH levels for sediment was near neutral meaning that, the combined effects of slight changes in the temperature, pH, and electrical conductivity for sediment may have been responsible for the repressed microbial count towards the downstream sediment at Edonwhii

In summary, Iko River Estuary, Akwa Ibom State harbors a very robust microbial diversity. The key physicochemical parameters of the sediment of Iko River Estuary like pH, Electrical Conductivity, Total Organic Carbon and Organic matter supports normal functioning of the sediment environmental processes and health. Metagenomics continually indicates the comprehensive microbial diversity and is a precursor of the biodiversity functionality of any ecological niche. Environmental monitoring must be constant and remains key in understanding environmental health, microbial ecological functions and their relationship. The data generated from this work will form a useful tool for further ecological assessment and monitoring of the coastal ecosystem of Iko River Estuary, Akwa Ibom State.

CONCLUSION

This study revealed that the coastal sediment of Iko River Estuary was rich in organic matter and was a beehive for heterotrophic activities. The major environmental attributes recorded are at tolerant levels in respect to microbial processes and environmental health. Despite increasing anthropogenic activities in and around the study environment, findings from this work corroborates that Iko River Estuary harbors very robust microbial diversity pointing to an excellent capacity for heterotrophic activity. This is very important as it upholds the ability of microorganisms to adapt under unfavorable environmental conditions. The metagenomic assay also validates the diversity of microorganisms in sediments of the estuary which is critical to the bio-geochemical cycling and maintenance of environmental health and quality. More so, the data generated in this work will form a useful tool for further ecological assessment and monitoring of the coastal ecosystem of Iko River Estuary and other tropical estuaries.

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