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A Metagenomes-Based Investigation of the Impact of Natural Run-offs and Anthropogenesis on a Freshwater Ecosystem at Points of Use in Niger Delta, Nigeria

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Abstract:
A metagenomes-based approach was applied to investigate the impact of anthropogenic activities and run-offs to the diversity and relative abundance of bacterial community structure in a freshwater ecosystem at points of use across three referenced sites (RIK, RTM and RSW) in Yenagoa Metropolitan area of Bayelsa State, Nigeria. High throughput sequencing (HTS) on Illumina Miseq of the V_1 -V_3 hyper variable regions of the 16s rRNA gene revealed high bacterial diversity. In all, a total of 291,757 and 204,179 quality filtered reads describing ten distinct bacterial phyla from water and environmental soils respectively were generated at the top eight classifications. Proteobacteria was the modal abundant phylum in all communities with Betaproteobacteria as the most common class in all water samples and Alphaproteobacteria in the environmental soils. Differences in community structure were noted especially among the rare population. Proteobacteria, Actinobacteria, unclassified bacteria and Firmicutes were the most common and abundant phyla in the freshwater Metagenome. A comparative analysis of the environmental soils from settlements situated at river banks revealed Proteobacteria, Actinobacteria, unclassified bacteria, Firmicutes and Bacteriodetes as the most common and abundant phyla. The degree of abundance of the unclassified bacteria at phylum level significantly increased down to the specie level taxonomic categories in all the water and soil metagenomes across the sampling sites. The genera; Burkholderia, Leptothrix, Hebaspirillium, Novosphingobium and Thaurea detected in water samples are of public health risk. A progressive increase in the abundance of the Actinobacteria members in both water and environmental soil metagenomes across the three study sites indicates higher anthropogenic disturbance at the downstream site. The dominance of Betaproteobacteria in water samples and Alphaproteobacteria in soil samples further suggests that runoff discharges significantly impacted the community structure. This study reveals that run-off discharges and anthropogenesis from metropolitan activities could lead to variation in microbial community structure, diversity and distribution of pathogenic bacteria at points of use of a freshwater ecosystem. The study also reveals an abundance of unclassified bacterial sequences which may give rise to novel bacteria with unexploited microbial genetic diversity within the freshwater ecosystem in the Niger Delta region.

Keywords: *Anthropogenesis, bacteria, high-throughput sequencing, metagenomes, run-offs.*

1. Introduction

The freshwater ecosystem in the Niger Delta region of Southern Nigeria is the largest mangrove swamp and wetland in Africa, third largest drainage basin in the continent and third largest wetland in the world after Holland and Mississippi; it runs approximately 11,700km from the lower River Niger to the Imo River entrance and draining an area of approximately 70,000km^2 (Ekubo and Abowei, 2011) into the Atlantic Ocean. The rivers and creeks are used by many communities along the water courses as their main source of drinking water and for a variety of recreational, transportation, agricultural, anthropogenic and metropolitan activities which introduce nutrients into the water bodies. High anthropogenic activities (e.g. boating, fishing, swimming, washing, bathing, dredging, refuse dumping, effluent and sewage discharge from sewers) along water courses and run-off discharges (e.g. from roofs, oil exploration sites, dumpsites, farmlands, settlements, bushes, markets and drainage systems) into water courses during the wet seasons are common phenomena. The combination of anthropogenic activities and run-off discharges into water bodies could attract high load of microbial contamination and subsequent deterioration of water quality. Exposure to drinking water contaminants and its resultant health effect is a major concern to public health. The presence of contaminants in water, above the recommended standard set by water quality regulating bodies like EPA, WHO and the Nigerian Standards Organization (NSO) could result in serious health hazards

(USEPA, 2002). Different illnesses, mostly acute gastro intestinal illness, are associated with the contamination of drinking water by pathogens (Colford et al., 2006; Hoffman et al., 2009). Reports have shown that water pollution accounts for the deaths of 14,000 people daily and with five million deaths annually (Hogan, 2010). Different sources of pollution which contribute to the presence or increase in nutrients, pharmaceuticals, heavy metals, herbicides and non indigenous bacteria (Woudneh et al. 2009; Schuler and Rand 2008; Topp et al. 2008; Rada et al. 1990) may alter the microbial community structure (Staley et al. 2013) and presence or abundance of specific taxa in a source-dependent manner (Zampella et al. 2007; Tu 2011).

In Nigeria, phenotypic, culture-based methods are commonly used to characterize drinking water bacterial community. This method would underestimate the microbial diversity within the water samples since most extant microbial genetic diversity are not yet exploited. These culture-based microbiological methods (Abu and Egeonu, 2008) and some culture-independent approaches (McCoy and VanBriesen, 2012) which do not give a complete assessment of the diverse microbial communities have been used to assess bacterial communities in freshwater ecosystems, treatment plants and distribution systems. Recently, metagenomics has been considered as the most promising approach that gives a complete environmental assessment of the diverse, complex microbial communities in various environmental compartments. Thus, it averts the limitation of culture dependent genetic exploitation that gives incomplete information (Cowan et al. 2005). High throughput sequencing (HTS) is a powerful metagenomics tool for comprehensive overview of microbial communities (Kwon et al., 2011; Liao et al., 2013) and functional genes (Zhang et al., 2011) in various ecosystems through the amplification of hyper variable regions of the 16s rRNA genes targeted with specific primers to allow for an unprecedented sequencing depths and identification of rare populations in low abundance (Caporaso et al. 2011; Kysela et al. 2005; Sogin et al. 2006). This technique has been deployed in investigating microbial structure and /or functions in various ecosystems such as sea water (Delong et al., 2006), fresh water (Breitbart et al., 2009), soil (Yergeau et al., 2012), human guts (Gregoracci et al., 2012), activated sludge (Zhang et al., 2011), sediments (Kristiansson et al., 2011), sand filters (Bai et al., 2013) and tannery waste water treatment plant (Wang et al., 2013).

Since high anthropogenic activity and run-offs discharges are common phenomena in the freshwater ecosystem of the Niger delta region, we hypothesized that using Metagenomic-based approach could give rise to novel organisms with unexploited genetic diversity since previous studies on characterization of bacterial communities in water samples within the region were centered on phenotypic methods. Furthermore, we also hypothesized that impacts of run-offs and anthropogenic activity could influence the community structure along the water course. The run-offs are a source of potential chemicals of concern (PCOC), while the anthropogenic activities are sources of potential chemicals of concern (PCOC) as well as potential microbes of concern (PMOC).

In the present study, we aimed at using High throughput sequencing (HTS) on Illumina Miseq of the V_1-V_3 hyper variable regions of the 16s rRNA gene to investigate the influence of anthropogenic activities and run-offs to the diversity and relative abundance of bacterial community structure in a freshwater ecosystem at points of use across three reference sites (RIK, RTM and RSW) in Yenagoa Metropolitan area of Bayelsa State, Nigeria

2. Materials and Methods

2.1. Study Area

The study area is Yenagoa, which is the capital city and the most commercial and industrialized section of Bayelsa State (Fig.1). It is located within the lower delta plain with an elevation between 3-7m above mean sea level (Wizor and Agbabou, 2014) and drained with rivers and creeks among which are Epie and Taylor Creek, Nun and Ekole Rivers. The area lies approximately between latitudes 4° 59′N to 5° 15′N and longitudes 6° 15′ E to 6° 30′ E within the Niger Delta region of Southern Nigeria. It has a humid climate with relative humidity of above 80 %, an average annual temperature of 27 °C (Nwankwoala et al., 2011) and vegetation of freshwater swamp and lowland forests (Wizor and Agbabou, 2014). It is characterized by long wet and short dry season duration with the wet season spanning between April and mid-December with a peak in October and the dry season occurring between mid-December and March with a peak in Mid-January and February.

2.2. Sample Collection

Water and environmental soils from three geo referenced sites; Ikorama (IK) community (upstream with very low anthropogenic activity), Tombia (TM) community (midstream with high anthropogenic activity) and Swali (SW) community (downstream with higher anthropogenic activity) along the water course within the metropolitan area of Bayelsa State were sampled at the peak of wet season (October) in 2014.The distance from IK to TM is about13 km while the distance from TM to SW is about 3 km. At every site of collection, about 1L of near-surface river water at communities' points of use was aseptically collected into sterile screw capped plastic containers. Environmental soils were sampled from settlements within a 10-meters distanceto the river bank and at a depth within 0-15cm. All samples were transported to the laboratory in coolers packed with ice blocks for analysis within 6 hours of collection.

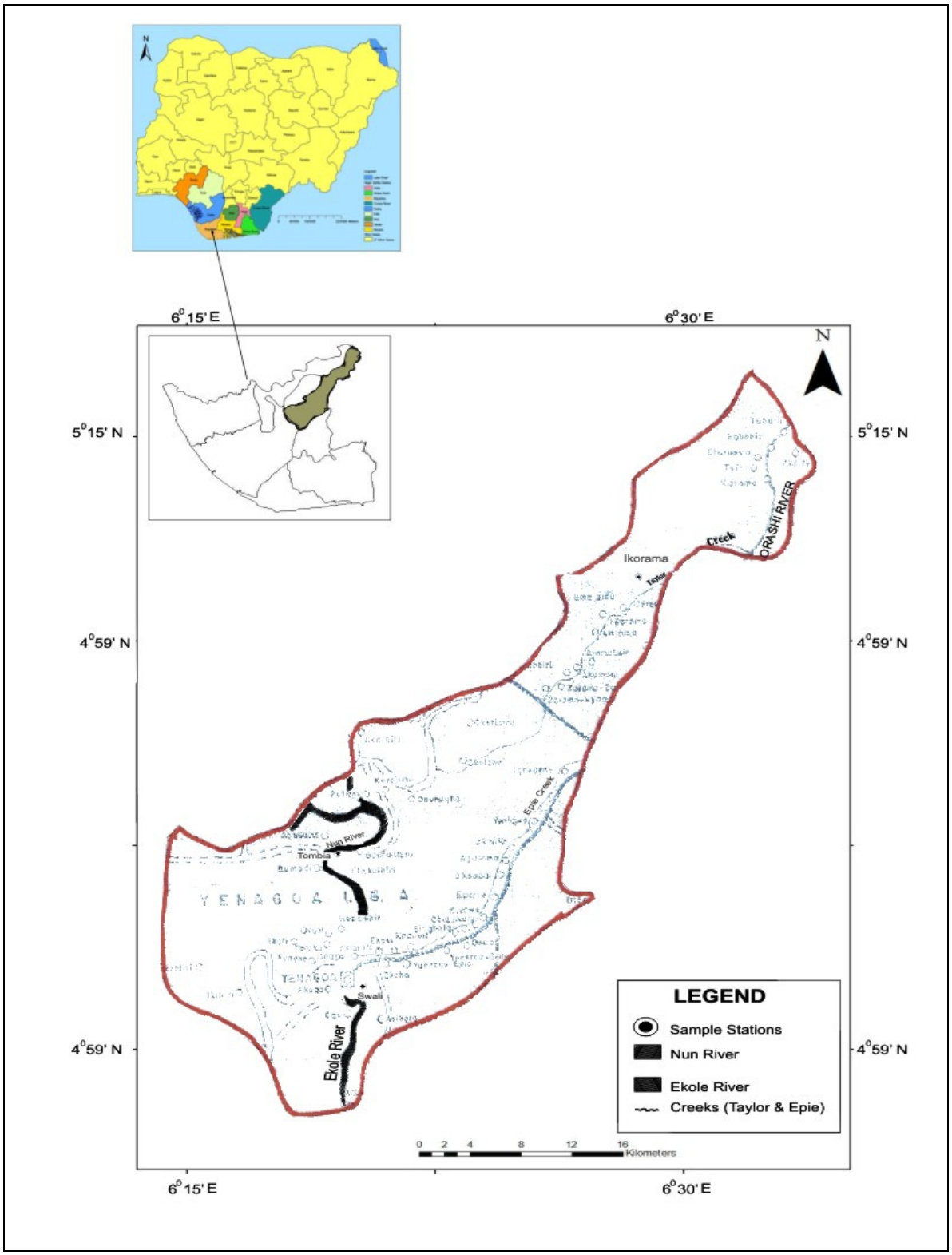

Figure 1: Map of Yenagoa Metropolitan area of Bayelsa State in the Niger Delta region showing the study locations

2.3. Molecular Procedures

2.3.1. DNA extraction

Genomic DNA extractions were conducted on a total of 6 samples collected from river water (RW) and environmental soils around the river (RES), across 3 geographical locations during the peak of wet season (October) of 2014 using ZR Fungal/bacterial miniprep™ soil DNA kit in accordance with the manufacturer's instruction (Zymo Research Corp.). For the water samples,

approximately 100ml were first centrifuged at 16,000rpm for 3 minutes each to concentrate the bacterial cells. About 1ml of the concentrated sample was then used for the genomic DNA extraction following the protocol of the ZR soil DNA kit. The DNA concentration and purity were measured by microspectrometry (NanoDrop ® ND-1000, Thermo Scientific).

2.3.2. PCR Amplification

PCR amplification was conducted using a PCR thermal cycler; GeneAmp PCR system 9700 (Applied Biosystems).The bacterial 16S rRNA gene was amplified with a set of primers targeting the hyper variable of the V_1-V_3 region. The primers were 27F (GAGTTTGATCCTGGCTCAG) and 518R (ATTACCGCGGCTGCTGG). PCR was conducted in a reaction system (50 µl) for the genomic DNA extracted from both the water and soil samples. For all the soil samples, each 50 µl reaction mixture consisted of 25 µl of master mix (i.e. a combination of $MgCl_2$, dNTPs and Taq DNA polymerase), 0·8 µl of 100 µm primer 27F, 0·8 µl of 100 µm primer 518 R, 7 µl of template DNA and 16·4 µl double distilled water. For the water samples, each 50 µl reaction mixture consisted of 25 µl of master mix (i.e. a combination of $MgCl_2$, dNTPs and Taq DNA polymerase), 0·8 µl of 100 µm primer 27F, 0·8 µl of 100 µm primer 518 R, 10 µl of template DNA and 13·4 µl double distilled water. The PCR cycle conditions were as follows: an initial denaturation at 95 °C for 3.13 min, followed by 35 cycles at 95 °C for 30s, annealing at 52 °C for 30s, and extension at 72°C for 30s, and a final extension at 72 °C for 5 min.To confirm the PCR product formation, PCR amplicons were first purified using a fast gene PCR extraction kit (Nippon Genetics Co, Ltd). The concentration and quality of the cleaned PCR products were checked using a micro spectrophotometer (NanoDrop ® ND-1000 Thermo Scientific). The purity of the cleaned amplicons was further confirmed using agarose gel electrophoresis in 1% agarose (Nippon Genetics Co, Ltd) at 100 V for 40 min using Ethydium bromide as the DNA staining dye (Nippon Genetics Co, Ltd).

2.3.3. Illumina Sequencing

Cleaned PCR products (10µg each) generated from the 6 genomic DNA extracted from the environmental samples were subjected to high throughput sequencing using 2×3000-bp PE strategy (Inqaba Biotech, Pretoria, South Africa) on the Illumina Miseq (Illumina, USA) platform to generate ~ 20 Mb of data per sample. The library preparation kit used for the amplicons indexing wasNEBNext® Ultra™ DNA Library Prep Kit for Illumina® and the sequencing kit used was Miseq v3, 600 cycles (Illumina, USA).

2.3.4. Metagenomic Analyses

After sequencing, the pre-processing of the raw reads and taxonomic classification to assess the microbial community membership was carried out using the Miseq Reporter (MSR) Software (i.e. Analysis Software Version: 2.4.60.80). MSR Software is an in-built pre-installed bioinformatics pipelines in the Illumina-Miseq Sequencing machine that performs secondary data analysis.

3. Results

3.1. Bacterial Community Composition and Diversity in the Freshwater

The Metagenomic data was extracted with a total read of 325,563 and 221,692 corresponding, respectively to the sequences for water and environmental soils across the three geo-referenced sites representing the upstream (RIK), midstream (RTM) and downstream (RSW) of the freshwater ecosystem in Yenagoa metropolis; a total of 291,757 and 204,179 quality filtered reads were generated from the respective reads (Table 1).

Source	Sample ID	Total raw reads	Total clean reads	%clean reads	Domain (%)			Bacterial Phyla taxonomic categories
					Bacteria	Unclassified	Viruses	
Surface water	RIK-W	135,713	121,975	89.9	94·64	5·35	0·01	26
	RTM-W	103,550	90,764	87.7	57·94	42·04	0·02	50
	RSW-W	86,300	79,018	91.6	95·31	4·67	0·01	25
Environmental soils	RIK-E	80,347	74,335	92.5	96·32	3·68	0	28
	RTM-E	45,348	40,718	89.8	87·09	12·88	0·03	27
	RSW-E	959,976	89,126	92.8	94.25	5·74	0·01	27

Table 1: Metagenome summary of the freshwater ecosystem in Yenagoa Metropolis
Note: RIK: Upstream site; RTM: Midstream site; RSW: Downstream site; W: Water; E: Environmental soils

Although, viral and unclassified reads were detected in both water and soil samples, most of the reads were related to the bacterial domain. In the bacterial domain, clean reads belonging to a total of 101 phyla represented in the freshwater and 82 in the environmental soils of the river were observed (Table 1). Based on the percentage total reads generated in the bacterial domain and taxonomic categories at phylum level, results revealed that the freshwater Metagenome of the midstream site; RTM-W at points of use had the least community composition and more diversity than their corresponding water metagenomes at the upstream and downstream sites of the freshwaters within the metropolitan area of Bayelsa State. This implies that as water flows from the upstream (i.e. which reflects the indigenous community structure (Staley, et al. 2013)) to the downstream, the bacterial diversity at RTM-W significantly increased; indicating variation in the indigenous community structure. Previous studies have shown that anthropogenic

impacts (Staley et al. 2013) and pollution sources (Unno et al. 2011) can lead to a significant variation in microbial community structure.

The bacterial community at the top eight taxonomic classifications presented Proteobacteria as the most abundant phylum in all bacterial communities (Fig. 2) found in water and soils metagenomes from all sites at human communities' points of use with beta-Proteobacteria as the most abundant class in the freshwater followed by alpha-, gamma- and delta subdivisions. The abundance of Proteobacteria decreased as water flowed from the upstream (RIK-W) (69%) to Midstream (RTM-W) (28%) and slightly increased at the downstream (RSW-W) (46%). For the environmental soils, the relative abundance of Proteobacteria followed the same pattern with the water Metagenome (i.e. (RIK-E) (49.43%) >RTM-E (24.12 %) <RSW-E (37.8%)) indicating that water flow was not responsible for the variation in the rate of abundance of the Proteobacteria structure in the freshwater.

Other dominant bacterial phyla in the water samples after Proteobacteria include; Bacteriodetes (12.26 %), unclassified phylum (8.06 %), Actinobacteria (3.83 %), and Firmicutes (3.01 %) at the upstream site (RIK-W); unclassified bacteria (47.75 %), Actinobacteria(19.40 %) and Firmicutes (2.29 %) at the Midstream site (RTM-W) and Actinobacteria (20.70 %), unclassified bacteria (14.16 %), Firmicutes (6.62 %) and Cyanobacteria (6.68 %) at the downstream site (RSW-W). For the corresponding environmental soils of the river, unclassified phylum (13.12 %), Firmicutes (10.30 %), Acidobacteria (9.33 %) and Actinobacteria(8.69 %) at the upstream soil (RIK-E); unclassified phylum (22.47 %), Firmicutes(18.61 %), Cyanobacteria (11.05 %), Chloroflexi (9.01 %) and Actinobacteria (9.23 %) at Midstream soil (TM-E) and unclassified phylum (15.59 %), Actinobacteria (18.65 %), Chloroflexi (11.96 %) and Firmicutes (8.82) at the downstream soil (SW-E), dominated after Proteobacteria.

Members of the rare population observed in the freshwater include; Acidobacteria, Chloroflexi, Nitrospirae, Verrucomicrobia, Spirochetes and Cyanobacteria. They occurred in low abundance and varied considerably across sampling sites.

Our study also revealed that there was a progressive increase in the relative abundance of Actinobacteria in water from upstream to the downstream (3.83 %, 19.40 %, and 20.70 %). The corresponding environmental soils of the river at different sites where the water samples were taken equally show the relative abundance of Actinobacteria phyla increase from the upstream to the downstream (8.69 %, 9.23 %, and 18.65 %) indicating that water flow was not responsible for the accumulation of Actinobacteria members downstream but may be attributed to anthropogenic impacts. Actinobacteria members are widely distributed in aquatic ecosystems and play a major role in decomposition and recycling of biomaterials. They have different lifestyles and can be pathogens, soil inhabitants, plant commensals or gastrointestinal commensals (Ventura et al. 2007).

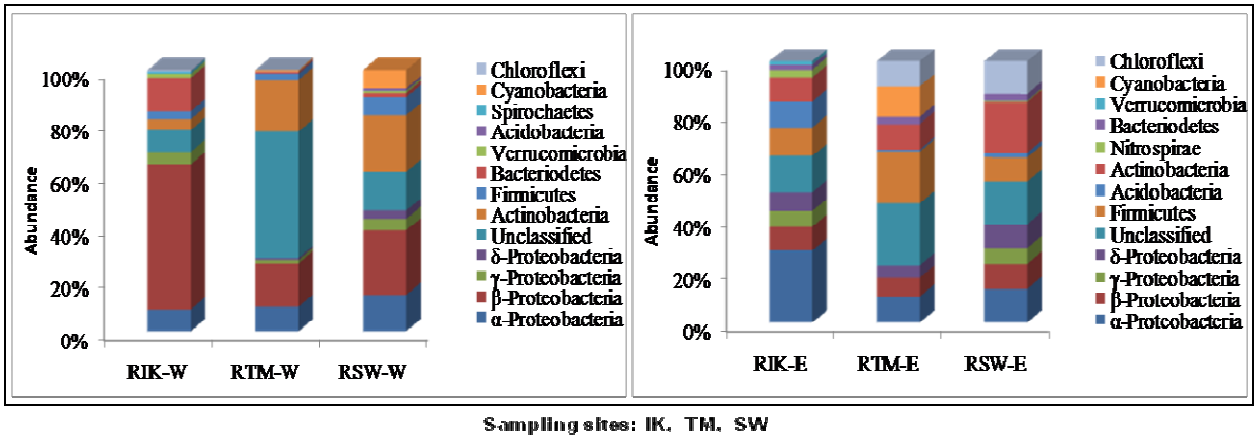

Figure 2: Distribution of the most abundant bacterial communities at Phyla level in (a) water (W) and (b) environmental soils (E) collected at points of use among sampling sites

3.2. Unclassified Bacterial Sequences at Phylum Level Taxonomic Categories

Sequencing results revealed that some unclassified bacterial sequences occupied a large portion in both water and soil metagenomes of the freshwater ecosystem (Fig. 3). This suggests that certain amount of novel sequences was captured. The degree in abundance of the unclassified bacterial sequences in the different water Metagenomes increased from the phylum to the specie level taxonomic classification, and was significantly different among the samples especially at the specie level. The percentage abundance of the unclassified bacterial sequences in both the water and environmental metagenomes had the highest percentage abundance at the Midstream sites (47.75 %, 22.47 %) suggesting that the community where the midstream is situated is driven by both natural and anthropogenic processes. Conducting further analysis using other bioinformatics pipelines will be necessary towards the identification of these bacterial sequences which may give rise to novel organisms with novel functional genes. According to Wu and Sun (2009), novel functional genes have been identified in underexplored metal-contaminated freshwater sediment environments. There is also a possibility that these unclassified bacteria are members of the 'rare biosphere' that may account for differences in community structure among sites (Sogin *et al.* 2006).

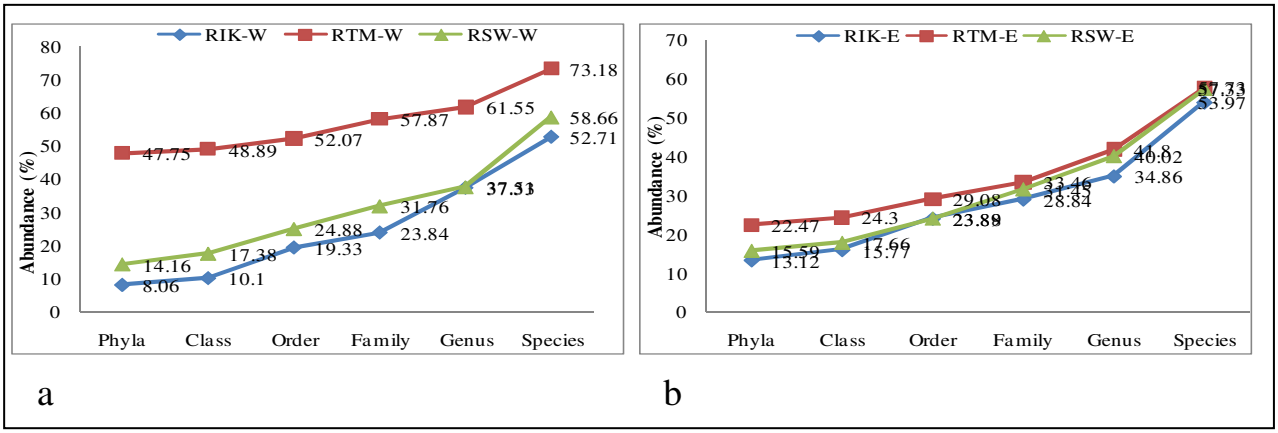

Figure 3: Distribution of the abundance of unclassified bacterial sequences at Phyla level in (a) water (W) and (b) environmental soils (E) among sampling sites

3.3. Public Health Implications

At the genus-level distribution, the top 8 classification revealed that some sequences retrieved from the surface water metagenomes communities' points of use are associated with bacterial groups with public health risk (Table 1). For example, *Burkholderia, Leptothrix, Hebaspirillium, Novosphingobium* and *Thaurea* are all different genera of pathogenic bacteria detected that have the capacity to cause disease in humans. Since taxonomic identification of annotated reads detected various pathogenic bacteria in water samples collected at points of use, further work on the clean reads generated by bioinformatics algorithms could determine the virulence genes associated with the different water metagenomes.

Genus	Pathogenic species	Disease	References	Water samples (%)		
				IK-W	TM-W	SW-W
Hebaspirillium	*H. huttiense*	Cystic Fibrosis?	Spilker et al. 2008	2.70	ND	ND
Leptothrix	*L. discophora*	Leptotrichia		4.60	ND	ND
Novosphingobium	*N. yangbajingensis*	Chronic Obstructive Pulmonary disease	Rutebemberwa et al. 2014	ND	ND	1.90
Thaurea	*T. aromatica*	Tumor promoter	Bhandore et al. 2006	ND	ND	3.30
Burkholderia	*B. ubonensis*	Melioidosis	Levy et al. 2008	ND	2.41	1.40

Table 2: Distribution of the most abundant pathogenic bacteria in water at communities' points of use as determined by taxonomic identification of annotated proteins
NB: ND-Not detected; RIK-W, RTM-W, RSW-W: representing upstream, midstream and downstream sites where water samples were collected.

4. Discussion

Almost all community members in both water and soil samples across the three different sites originated from the bacterial domain and there was an even distribution of Proteobacteria, Actinobacteria, Unclassified bacteria, Firmicutes and Bacteriodetes phyla across the different metagenomes and with Proteobacteria as the most dominant phylum.This result supports earlier reports that Proteobacteria is the most predominant phylum within bacterial communities found in different environmental compartments (Huang et al. 2014; Oh et al. 2011; Staley et al. 2013; Wang et al. 2012 and Zhang et al. 2011). There was an uneven distribution of some phyla (i.e. rare population) in the water metagenomes (i.e. *Chloroflexi, Nitrospirae, Verrucomicrobia, Spirochetes, Cyanobacteria* and *Acidobacteria*) and the soil metagenomes (i.e. *Cyanobacteria, Chloroflexi, Nitrospirae* and *Verrucomicrobia*) which may be attributed to differences in the number of operational taxonomic units (OTUs) present in the different metagenomes across different sites. Previous studies on the characterization of bacterial community on the upper Mississippi river revealed that shifts in community structure among sites were attributed to changes in the presence and relative numbers of less abundant OTUs as well as shifts in the relative abundance of the dominant lineages (Staley et al. 2013).

Within the Proteobacteria phylum, its classes showed different patterns. For instance, beta-Proteobacteria dominated the freshwater samples while the environmental soils of the surface water were enriched with alpha-Proteobacteria across the three study sites; there was a gradual shift in the Proteobacteria structure; from alpha-, beta- and gamma- Proteobacteria in the upstream site to alpha-, beta-, gamma- and delta- Proteobacteria at both the midstream and downstream sites. Previous study (Gonzalez et al. 2000) indicated that deeper waters (200-500m) of the freshwater ecosystems are more likely to harbor delta-Proteobacteria than shallow water. In our study, the depth profile of downstream (RSW-W) and midstream (RTM-W) freshwater sites are projected to be higher than the upstream freshwater site (i.e. RSW-W > RTM-W > RIK-W) and this might have contributed towards the rate of distribution and abundance of delta-Proteobacteria (i.e. RIK-W (0), RTM-W (0.76) and RSW-W (3.09)) in the sites. Thus, the degree of the freshwater

depth is related to the distribution and relative abundance of delta-Proteobacteria. In comparison, the environmental soils at the three sites all harbored delta-Proteobacteria indicating that there is no correlation between environmental factors and the distribution of delta- Proteobacteria in the freshwater. Furthermore, there was a significant variation in the relative abundance of Proteobacteria community structure across the three sites with the upstream site having a higher abundance (68.27 %) than the two other sites (28.32 % and 46.05 %) respectively. The significant drop of the Proteobacteria at the midstream site was due to a sharp drop in the abundance of beta- (from 53.28% to 16.34%) and gamma-Proteobacteria subdivision (from 4.67% to 1.23%).

The study has revealed unclassified bacterial sequences, a spatial distribution of beta- Proteobacteria in water samples and alpha-Proteobacteria subdivisions in all environmental soils of surface water across the three sites, and presence of pathogenic bacteria at points of use. Thus, anthropogenic activities and run-off discharges significantly impacted the freshwater ecosystem at communities' point of use.

5. Conclusion

This is the first study of its kind to characterize bacterial community in a freshwater ecosystem at communities' points of use in a Niger Delta Metropolis of Southern Nigeria using high throughput sequencing on Illumina Miseq. The unclassified bacterial sequences with unexploited genetic diversities may give rise to novel organisms /genes.

6. Acknowledgements

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