Detection of Pools of Bacteria with Public Health Importance in Wastewater Effluent from a Municipality in South Africa Using Next Generation Sequencing and Metagenomics Analysis

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Abstract. Wastewater effluents are always accompanied with possibilities for human health risks as diverse pathogenic microorganisms are harboured in them, especially if untreated or poorly treated. They allow the release of pathogens into the environment and these may find its way into food cycle. This paper reports the findings of our research work that focused on the characterization of microorganisms from a municipal final wastewater effluent that receives bulk of its spent water from a research farm. High throughput sequencing using Illumina MiSeq apparatus and metagenomics analysis showed a high abundance of microbial genes, which was dominated by Bacteria (99.88%), but also contained Archaea (0.07%) and Viruses (0.05%). Most prominent in the bacterial group is the Proteobacteria (86.6%), which is a major phylum containing wide variety of pathogens, such as Escherichia, Salmonella, Vibrio, Helicobacter, etc. Further analysis showed that the Genus Thauera occurred in largest amounts across all 6 data sets, while Thiomonas and Bacteroides propionicifaciens also made significant appearances. The presence of some of the detected bacteria like

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Corynebacterium crenatum showed degradation and/or fermentation in the effluent, which was evidenced by fouling during sampling. Notable pathogens classified with critical criteria by World Health Organization (WHO) for research and development including *Acinetobacter* sp., *Escherichia coli,* and *Pseudomonas* sp. in the effluent were being released to the environment. Our results suggest a potential influence of wastewater effluent on microbial community structure of the receiving water bodies, the environment as well as possible effects on the individuals exposed to the effluents. The evidences from the results in this study suggest an imminent public health problem that may become sporadic if the discharged effluent is not properly treated. This situation is also a potential contributor of antimicrobial resistance genes to the natural environments.

Keywords: Metagenomics · Illumina · MiSeq · Genus Thauera · *Acinetobacter* sp. · Human health risks

1 Introduction

Microorganisms are ubiquitous and they exist in diverse communities. These microbes play very important roles in proper functioning of the ecosystem and they are central in the flow of biogeochemical cycles. On the other hand, some of them are implicated in several diseases that plague the human population. Microbial pathogens that cause diseases in humans include; viruses, bacteria, fungi, protozoa and helminthes [1, 2]. Wastewater provides a safe haven for such pathogens to multiply because it contains various materials that are rich to support microbial growth [3]. Illustrative examples include the death of several humans exposed to Vibrio Cholerae bearing wastewater in London in mid-19th century [3]. Epidemiological evidence in Norway also linked the outbreaks of legionnaires disease to contaminated wastewater [4]. Although, wastewater and animal farm have been reported to contain useful bacterial diversities, they have also been reported as the source of various pathogens of immense public health importance [5–7].

Adegoke and Okoh [6] reported the detection of Methicillin Resistant Staphylococcus Species (MRSS) bearing mecA genes from some animal farms in Nkonkobe municipality of South Africa. Stevik et al. [5] as well as Cai and Zhang [8] also reported the presence of Escherichia coli, Mycobacterium tuberculosis, Pseudomonas aeruginosa, Salmonella enterica, Shigella flexneri, Staphylococcus aureus and Vibrio cholerae, etc. in wastewater. Furthermore, several viruses have also been detected by some researchers as reported in the literature. Thus, the detection of these pathogens may provide a source of surveillance that gives early warnings ahead of disease outbreaks, which may be consequent on the release of wastewater to the environment [9, 10].

It is apparent that the general assessment of microbial population that are inherent in municipal wastewater is highly imperative [8]. In codicil, the methods that are deployed for such assessment should allow broad detection of both cultured and uncultured microbes. However, most of the existing methods target one or few pathogens, consequently providing less than the required information on the diversities of microorganisms in wastewater samples [11]. Methods such as colony count [12, 13], PCR [13, 14], qPCR [15] and Microarray [16] are frequently deployed albeit their limitations in assessing uncultured microbes and providing comprehensive information on the microbial population.

The emergence of metagenomics analysis has addressed the limitations of the stated traditional approaches for the assessment of both cultured and uncultured microbial populations [17, 18]. It is becoming easier to carry out direct genetic analysis of genomes in an environmental sample with a view to determining the potential health hazards they can constitute [19]. The beauty in assessing hidden diversity of microscopic life as well as the huge potentials in understanding the entire biosphere make metagenomics analysis a widely sort after tool [20–22]. Besides the determination of diversities, it is possible to determine the role of each of the microorganisms in their niche environments [23]. As both taxonomic and functional diversities of microbes are assessed, the inference drawn forthwith could be relevant for diagnosis and therapy administration especially when human pathogens are involved [18, 24–27]. This kind of dependable procedure for monitoring pathogens is imperative for setting guidelines on restricted and unrestricted treated wastewater reuse [28]. This is important because treated wastewater to be released to the environment (for purposes of irrigation and drinking water production) is expected to be pathogen-free. This paper presents a report on the metagenomics analysis of wastewater effluent from a municipal site in South Africa. In Sect. 2, we outline the materials and methods, Sect. 3 presents the result and discussion while the conclusion is drawn in Sect. 4.

2 Materials and Methods

2.1 Study Site and Sample Collection

The sample site is close to the Agricultural Research Council- Animal Production (ARC-AP), South Africa. The site is situated about 25 km south of Pretoria (25°52′S 28°13′E/25.867°S 28.217°E/− 25.867; 28.217 in Gauteng) adjacent to the village of Irene in the suburb of Centurion. The area has a typical Highveld climate (altitude 1,523 m), with hot days and cool nights in summer and moderate winter days with cold nights. Winter maximum daytime temperature is around 20 °C dropping to a crisp average minimum of 5 °C, and warm to hot summers (October to April) tempered by late-afternoon showers often accompanied by extreme thunder and lightning. Hailstorms are not uncommon, but a serious hailstorm has not happened for many years. Soil structure in the area is mostly coarse, with pores of 0.7 mm to 2 mm in size. Soil in ARC-AP is loamy with mostly silt soil, which is richer in nutrients and is able to retain water for long periods.

Expended water was collected as a composite sample from the final wastewater effluent. The effluent contains wastewaters from the residential surrounding suburbs and a research farm. Water sample (1000 mL) was collected in glass bottle cleaned with dilute Nitric acid (HNO3) and detergent followed by rinsing with sterile distilled water. The sample was transported on ice to the laboratory and processed.

2.2 Collection of Samples and Extraction of Environmental DNA

The sample was collected according to the protocol described in Gonzalez-Martinez et al. [29] with modifications. 76 subsets of wastewater effluent samples were collected

to form a composite sample. The sample was kept at 4 °C until they reached the laboratory. Then mixed sample of about 5000 mL was centrifuged in batches at 3500 rpm during 10 min at room temperature until a mixture of 1000 mL was achieved. Further centrifugation under the same condition was done for separation of biomass and water. The pelleted biomass was kept at −20 °C for subsequent DNA extraction procedure. The DNA extraction was done using the FastDNA SPIN Kit for water (MP Biomedicals, Solon, OH, USA) and the FastPrep apparatus following the instructions given by the manufacturer. The integrity of the DNA was determined using Thermo NanoDrop 1000 Spectrophotometer. The DNA pools were then kept at −20 °C and sent to ARC- Biotechnology Platform Laboratory for sequencing.

2.3 Illumina MiSeq Sequencing

The DNA pools were subjected to sequencing procedure using the Illumina MiSeq apparatus and the Illumina MiSeq Reagent v3. This protocol was done three times for each DNA pool to independently identify Bacteria, Archaea, Viruses and Fungi OTUs. The primer pairs 28FF-519R (5′-GAGTTTGATCNTGGCTCAG-3′ and 5′-GTNTT-ACNGCGGCKGCTG-3′), 519F-1041R (5′-CAGCMGCCGCGGTAA-3′ and 5′-GGC-CATGCACCWCCTCTC-3′) and ITS1F-ITS2 (5′-CTTGGTCATTTAGAGGAAG-TAA-3′ and 5′-GCTGCGTTCTTCAT CGATGC-3′) were chosen for the amplification of the hypervariable regions V1-V3 of 16S rRNA gene of Bacteria, the hypervariable regions V4-V6 of 16S rRNA gene of Archaea, and ITS region of Fungi, respectively. The raw reads of this study has been deposited in the Sequence Read Archive (SRA) under the accession number SRP159184.

2.4 Data Analysis

MetaPhlAn2 was utilised to analyse and identify the microbial composition of the microbial communities as depicted in the sequenced wastewater effluent data [30]. The reads were analysed and combined to form a merged abundance table. This table was edited and viewed using LibreOffice Calc. A heatmap showing the abundance profiles of the most abundant 50 microbes was generated using Hclust2. A cladogram showing taxonomic relatedness was captured using GraphlAn [31]. This was done by rendering trees and annotating them with microbial names and relative abundances. Several charts, showing specific comparisons based on various clade were generated using Krona [32].

3 Results and Discussions

3.1 Microbial Composition of Wastewater Sample

The analysis of the microbial samples showed that the environment was dominated by Bacteria (99.88%). However, the sample also showed that Archaea (0.07%) and Viruses (0.05%) were present in very small proportion. Figures 1 shows graphical illustrations of the wastewater sample. This illustration was generated using krona with the microbes' distributions shown in Table 1 for clarity.

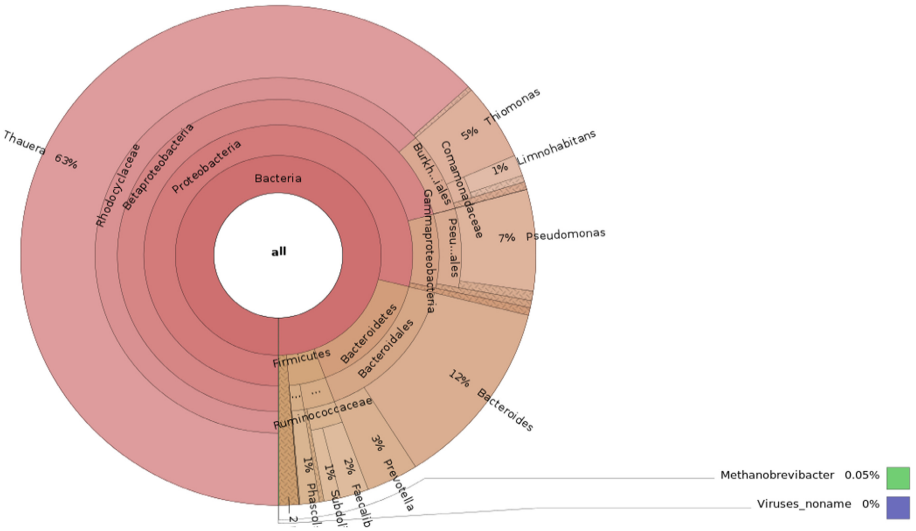

Fig. 1. Microbial composition of the wastewater sample

Table 1. Microbial abundance by Kingdom based on MetaPhlAn2 Analysis

ID	Set 1(%)	Set 2(%)	Set 3(%)	Set 4(%)	Set 5(%)	Set 6(%)
k_Bacteria	99.84544	99.88687	99.89423	99.94428	99.46088	100
k_Archaea	0.15456	0.11313	0	0.05572	0.15536	0
k_Viruses	0	0	0.10577	0	0.38376	0

Upon further analysis, it was shown that about 5 phyla were present within the Bacterial population namely: Actinobacteria (0.5%), Bacteroidetes (9%), Firmicutes (3.3%), Proteobacteria (86.6%) and Spirochaetes (0.6%). Proteobacteria is a major phylum containing wide variety of pathogens, such as Escherichia, Salmonella, Vibrio, Helicobacter and etc. [33]. Their large detection in this analysis is a pointer to the fact that large numbers of pathogens in the effluents are being released from the farm site to the environment.

A total of 40 different genera were identified with the genera Thauera making up 74% of the entire population (Table 2). This comes as no surprise as Thauera is a denitrifying bacteria playing a crucial role in the wastewater ecosystem [34]. Thauera plays an important role in the removal of nitrogen nitrate and other aromatic compounds [35]. For this reason, Thauera is usually detected in most wastewater treatment samples [36, 37]. The composition of the 10 most abundant species in the effluent is shown in Table 2.

Another notable genera worth noting among the bacterial population is the genera Thiomona making about 5% of the bacterial population (Fig. 2). Studies by Ryoki et al. [38] have shown that thiomona is useful for hydrogen sulfide removal through oxidation [38, 39].

Table 2. Microbial composition of the 10 most abundant species

ID	Set 1(%)	Set 2(%)	Set 3(%)	Set 4(%)	Set 5(%)	Set 6(%)	Set 7(%)	Set 8(%)	Set 9(%)
Thauera unclassified	74.78797	73.7054	74.45052	72.75747	73.01384	75.59471	73.71421	73.76752	71.86082
Bacteroides propionicifaciens	6.24441	5.90474	6.0248	6.35372	6.09813	7.02906	8.09416	6.17204	6.34544
Thiomonas unclassified	3.78366	5.52679	5.62429	6.79345	5.4099	3.39223	10.7756	5.32803	5.73392
Pseudomonas caeni	3.53141	3.66291	3.75899	3.76593	3.62164	3.84445	2.92225	3.6477	3.78147
Limnohabitans unclassified	1.43182	1.2971	1.39118	1.27389	1.29692	1.23933	0.32113	1.35369	1.56769
Prevotella copri	1.42776	1.22748	1.5482	1.27939	1.37822	1.34788	1.26741	1.14799	1.29424
Subdoligranulum unclassified	1.32637	0.83955	1.02524	1.28996	0.86505	1.44284	0	0.7471	1.19192
Faecalibacterium prausnitzii	0.98493	0.96129	1.01895	0.95755	1.03276	0.91465	0	0.83229	1.03483
Acinetobacter unclassified	0.88567	0.93844	0.78184	0.34917	1.18873	0.61385	0	0.67223	0.68754
Phascolarctobacterium succinatutens	0.81715	0.77118	0.58456	0.59888	0.61381	0.56615	0	0.66996	0.70073

The viruses present in the effluent are composed majorly of the genus Siphoviridae and Gammaretrovirus. The Kingdom Archaea was found to consist of only the genera Methanobrevibacter. To aid further analysis, a heat map was generated using Hclust2 (Fig. 3). This made it possible to view the various organisms and their relative abundances in one glance. With the map showing the degree of abundance, it became easy to trace the most populous species present. The map was shortened to the 50 most abundant species to allow for visibility. The results are the same as those earlier presented - the Genus Thauera occurred in largest amounts across all the data sets. Thiomonas and Bacteroides_propionicifaciens also made significant appearances.

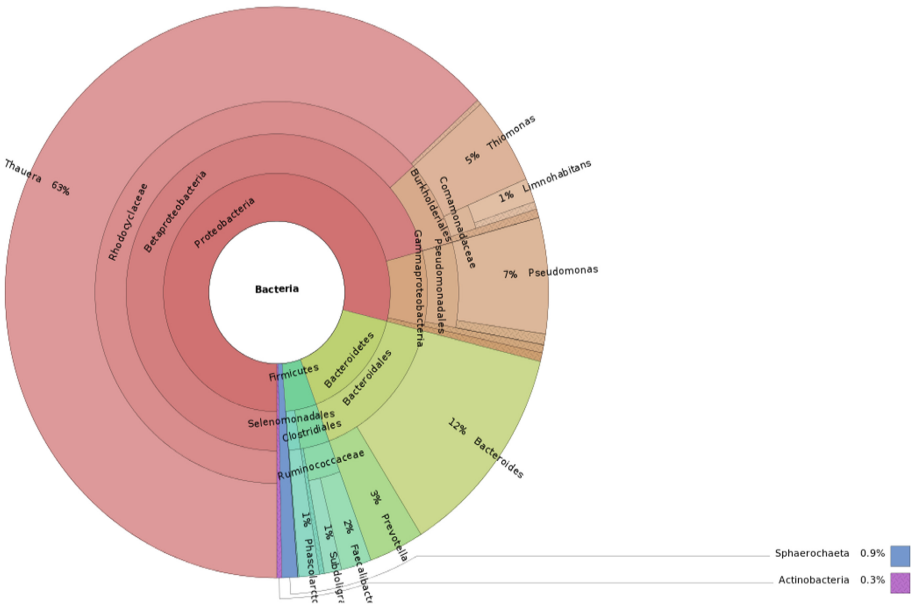

Fig. 2. Microbial composition of Bacteria present using Krona

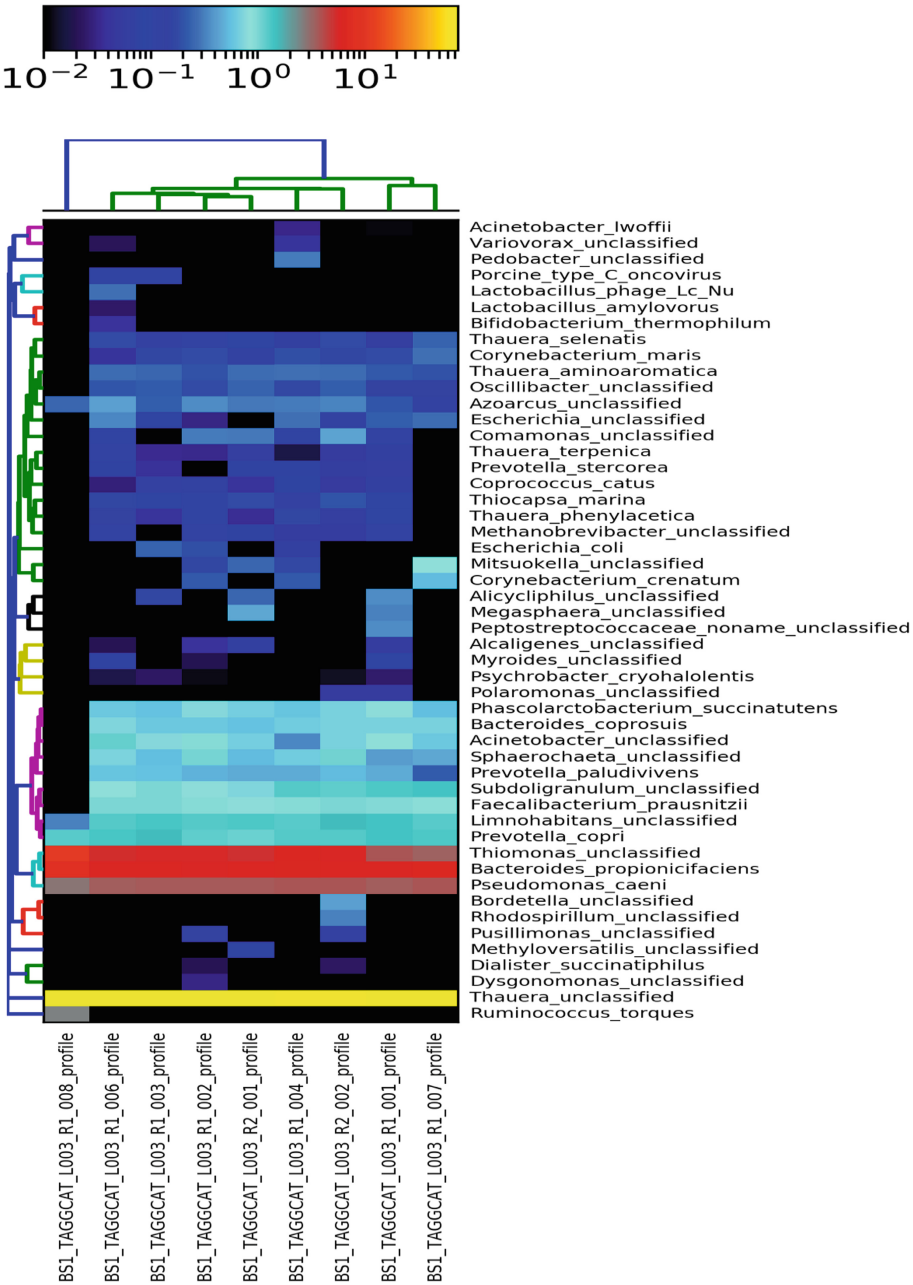

Fig. 3. Heat map showing microbial abundance of topmost 50 species using Hclust

The detection of Acinetobacter sp., Escherichia coli, Pseudomonas sp, etc. are significant as strains of these bacteria have been listed with critical criteria for research and development by WHO [40]. Besides that, Corynebacterium crenatum detected in these effluent samples shows there would be active fermentation and fouling of the environment where these effluent is introduced, since Corynebacterium crenatum is notorious for outstanding fermentation [41].

WHO [28] clearly highlights the health risks to humans that are associated with the release of wastewater effluent containing pathogens for reuse on farmers, farm workers, residents in the neighbouring households where the wastewater is reused, as well as the sellers and consumers of crops irrigated with the wastewater. Both WHO [28] and United States Environmental Protection Agency (USEPA) [42] disallow the presence of pathogens in wastewater that are meant for reuse. These pathogens could be taken up by crops, get internalized and then passed to consumers of such crops [13, 43].

4 Conclusion

Using metagenomic analysis, this study was able to provide insight into the microbial community present in the wastewater effluent. We were also able to use various bioinformatics tools to provide graphical illustrations that aided our interpretation. The results revealed that a large percentage of the microorganisms present were bacteria and we were able to view their diversity. A huge part of this bacterial presence was directly involved in the wastewater ecology and had major roles in the breakdown of compounds present. The presence of potential opportunistic pathogens showed that the effluents did not meet both WHO and USEPA guidelines for final effluents, which may be reused in the environment, especially when the sampling location is surrounded by highly cultivated farmlands. This is because the presence of these pathogens may constitute high health risks to exposed individuals.

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