Diversity of Prokaryotic Microbiota in Iloilo River, Iloilo Province, Philippines Through Partial 16S rRNA Gene Sequence Analysis

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Abstract

Rivers are vital components of the biosphere and an excellent habitat for many of the Earth's organisms. The prime focus of this study is the Iloilo River located in the City of Iloilo, Panay Island, Philippines. It is classified as Class C body of water. In spite of the unique abundance of biodiversity in Iloilo River, the quality of water in the area has been severely affected by rapid industrialization and urbanization. This study focused on the characterization of bacterial microbiota in the river using 16S rRNA sequencing. Based on 16S rRNA gene sequencing eighteen bacterial family and 19 species were taxonomically identified and classified across the sampling sites. At the family level *Microbacteriacea* accounted for the most abundant family (8.41%) belonging to the phylum *Actinomycetota* followed by *Flavobacteriaceae* (6.34%) under the phylum *Bacteroidetes*. On the species level the two most dominant species across all sites were *Marichromatium gracile* (3.62%) and *Candidatus aquiluna rubra* (3.42%). Most of the identified bacteria were novel and recognized as important human and aquatic pathogens; hence, provide evidence that Iloilo River harbors bacterial population, which might pose health and environmental risks to humans and aquatic lives. There was low microbial diversity index across all sites. Analyses of the selected physicochemical parameters such as temperature, turbidity, salinity and total suspended solids (TSS) pH and dissolved oxygen (DO), indicate that the values were within the national standard limit for Class C Water Body Classification.

Keywords: bacteria, microbial diversity, public health, river systems, sequencing

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Introduction

River is a vital component of the biosphere containing less than one percent of the world's freshwater with its higher ecological and social significance which are being polluted by indiscriminate disposal of sewage waste, indiscriminate industrial waste, and by human activities that affect their physical and chemical characteristics and lead to various damaging effects on aquatic organisms [1, 2]. This present study focuses on Iloilo River a water reservoir located in the City of Iloilo, Panay Island. It is a seawater inlet of the Iloilo Strait. The river supports fisheries for crabs, shrimps and small demersal fishes with yield estimates of 35.1-48.0 metric ton/km² and hosts about 600 ha of fishponds [3, 4]. Iloilo River is classified as Class C body of water, suitable for growth of fish and other aquatic resources; recreational water Class II for boating, fishing or similar activities and for industrial water supply, Class I. The river also passes through some of the city's most densely populated areas where plenty of establishments nearby and many informal settlers are situated, as well as economic and commercial centers [4, 5].

Iloilo River is a valuable resource and is vital to all living organisms, however, the rapid industrialization and urbanization in the area had continuously impacted the quality of water which threatens the sustainability of the river ecosystem. The vanishing quality of water is most likely due to anthropogenic activities which enters into the river and affect the microbial communities. Bacteria, archaea, microbial eukaryotes and viruses are the aquatic microbial communities that represent practically immeasurable levels of the aquatic biodiversity [6, 7]. The activities carried out by these microbial communities within the aquatic environment may directly impact the quality of human life, the survival of animals and plants population, and the productivity level of the habitat [8, 9]. Improperly treated sewage discharges can contaminate the river with microorganisms that may give rise to waterborne diseases [10, 11].

Today, issues in terms of the quality of water in the river becomes of great concern worldwide due to the proliferation of pathogenic bacterial microbiome that may pose an environmental and epidemiological threat to human's health. The development of current technology significantly improved the identification and role of microbes in aquatic biomes. Moreover, comprehensive evaluations of microbial quality of water require survey of all the pathogens that have potential for human infections [12, 13]. Metagenomics is used to describe microbial populations in water and sediment to understand community structure and the role of microorganisms in ecological processes [14, 15]. It has also been used to examine water quality to protect public health [16, 17]. Additionally, some researchers [18-20] pointed out that information generated using genetic approaches enables the ability to understand the microbial composition, their distribution in the environment and the pollution mechanisms. In the case of Iloilo River, no study using metagenomic sequencing have been done. In previous studies done on the habitat health of Iloilo River using sediment samples, the presence of dominant opportunistic macrobenthic taxa were noted [21, 5], however; there was no mention about specific characteristics of bacteria.

Another set of parameters to evaluate the quality of water is through the analyses of the physicochemical parameters. It is important to get an exact idea about the quality of water because changes in the chemical and physical properties of water are the major causes of water pollution [22, 23]. These are used as indicators of water quality [24]. The quality of water can be regarded as a network of variables such as pH, oxygen concentration, temperature, etc. and any changes in these physical and chemical variables can affect aquatic biota in a variety of ways [25, 26]. Previous reports demonstrated that high fluctuations in the physicochemical parameters indicates the intensity of pollution [27, 28]. Thus, this study was conceptualized because of the following reasons: to date there is no available data on the characterization of bacterial microbiota in Iloilo River. Second, most of the microbial parameters currently employed focused on total coliforms only. Finally, result of this study would provide valuable information on the quality of water in order to review and strengthen existing water quality enhancement and rehabilitation policy of Iloilo River in order to promote tourism and intensify preventive measures to deter the development of diseases and possible outbreaks in the future. Hence, to meet these objectives, partial sequencing of the 16S rRNA of bacterial isolates from Iloilo River was conducted to assess the bacterial diversity in this body of water.

Materials and Methods

Sample Collection

Water sampling was done during the dry season when the sea was at its lowest level. A triplicate water sample was collected from each of the three sampling sites. The water collection started upstream (Carpenters Bridge), midstream (Benigno Aquino Bridge) and downstream (Parola-Muelle Loney Bridge). The center portion of the bridge was marked as the reference point. Approximately 3 meters from the center, a plastic container was dropped about 1 meter below the water surface to collect the water . The screw cap of the conical tube was removed and dipped into the pail and about fifty (50) milliliters of water was filled. Water samples were properly labelled, sealed and transported in a cooler with frozen gel packs and brought to the DNA Sequencing Core Facility, Philippine Genome Center for metagenomic sequencing.

DNA Extraction

Samples were submitted in 50 mL conical tube. Three molar (3M) sodium acetate (NaOAc) and absolute ethanol were added to the samples and were incubated in -20ºC for 4 days. Precipitated samples were then extracted using DNeasy Blood and Tissue Kit. Extracted samples were then processed for library preparation.

Library Preparation, Amplification and 16s Metagenomic Sequencing

All samples were processed for library preparation. The samples were prepared following 16S Metagenomic Sequencing Library Preparation. The gene-specific sequences used target the 16S V3 and V4 region. They are selected as the most promising bacterial primer pair [29, 30]. Index used in library preparation is Nextera XT v2 Index kit set B. Libraries were check for size and concentration using Tape Station 2200 and Qubit dsDNA HS assay. All libraries were pooled, loaded and sequenced using MiSeq Reagent Nano Kit v2, 500 cycles. Illumina adapter overhang nucleotide sequences are added to the gene-specific sequences. The full length primer sequences, using standard IUPAC nucleotide nomenclature, to follow the protocol targeting this region are:16S Amplicon PCR Forward Primer = 5' TCGTCGGCAGCGTCA GATGTGTATAAGAGACAGCCTACGGGNGGCWGC AG 16S Amplicon PCR Reverse Primer = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAG ACAGGACTACHVGGGTATCTAATCC The Illumina overhang adapter sequences to be added to locus-specific sequences are: Forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG -[locus- specific sequence] Reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG -[locus- specific sequence]

Sequence Analysis

Raw data were processed using MiSeq Reagent Nano Kit v2, 500 cycles. The loading concentration is 6pM, % PhiX is 15 with an estimated yield of 499.9 with Q-score distribution suggests sequence reads are predominantly above Q30

Microbial Diversity Index Determination

Shannon-Wiener diversity index was used to determine the microbial diversity index across sampling sites. It tells how diverse the species in a given community are. The mathematical formula is as follows:

$$H' = -\sum_{i=1}^{s} p_i \ln p_i$$

where H' is the species diversity index, s is the number of species, and pi is the proportion of individuals of each species belonging to the ith species of the total number of individuals [31, 32].

Physicochemical Analysis

The physical properties of water were determined based on temperature, turbidity, salinity and total suspended solids (TSS) while chemical parameters were determined based on pH and dissolved oxygen (DO). Temperature was measured using glass thermometer, turbidity was measured by turbidimeter, salinity using salinity meter and total suspended solids using nephelometer. pH was measured using a digital pH meter, dissolved oxygen was measured using an oxygen meter. Results were compared with the reference limit for Class C Water Body Classification [10].

Results and Discussion

Bacterial Characterization and Diversity of Water in Iloilo River

Fig. 1 shows the eighteen bacterial families common in all the sample sites in the Iloilo River. The top five were *Microbacteriaceae* accounted for the most abundant (8.41%) followed by *Flavobacteriaceae* (6.34%), *Clostridiaceae* (4.28%), *Chromatiaceae* (3.60%) and *Rhodobacteraceae* (3.38%). Interestingly, *Microbacteriaceae, Flavobacteriaceae and Clostridiaceae* have been largely connected to the gut microbiota of humans and animals and have been proposed as an indicator of fecal contamination. The findings of this study were in agreement with Brown et al. [15] who mentioned that these bacteria are indicators of fecal contamination and their presence are due to human activities such as disposal of human waste, dumping of bits and pieces of household items in the vicinity. As noted, the Iloilo River bank is a densely populated area; thus, the presence of these bacteria are most likely high. *Flavobacteriaceae,* is an important fish pathogen that have recently emerged in association with fish disease outbreaks [33]. This bacterium is recently considered as a community-acquired opportunistic pathogen [34, 33]. An earlier assessment done by Adedire et al. [35] in India, demonstrated the universality of *Microbacteriaceae* and *Flavobacteriaceae* and *Clostridiaceae* in the rivers. The presence of these bacterial families poses threat to human health and fishes due to their pathogenic nature, and these findings were also in agreement with the results obtained in the present study.

Notably, *Rhodobacteraceae* (3.38%) and *Chromatiaceae* (3.60%) are free-living non-pathogenic microorganisms that thrive in marine environments. Their presence has a significant impact in Iloilo River. *Rhodobacteraceae* a Gram-negative
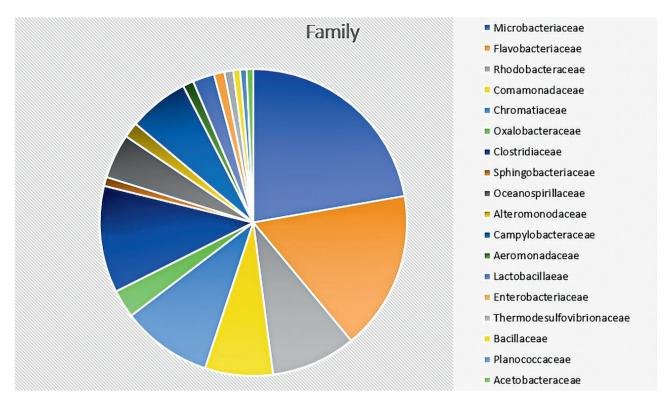
Fig. 1. Bacterial composition based on Family-Level.

bacterium and previously known as photoheterotrophs and chemoheterotrophs purple non-sulfur bacteria deeply involved in sulfur and carbon biogeochemical cycling and symbiosis [36, 37]. On the other hand, *Chromatiaceae* is a group of purple sulfur bacteria, and their occurrence signifies the presence of elemental sulfur in the environment [38, 39].

It is worth noting, that majority of the identified families were known to be opportunistic pathogens, which most likely pose a threat to human health and fishes. Findings of this study were in agreement with previous studies done in India [35] and China [40].

Fig. 2 shows the diversity index of bacterial families in the different sections of Iloilo River. The highest
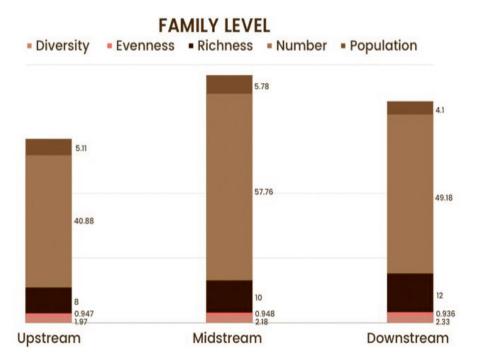

Fig. 2. Taxonomic bar plots depicting diversity of bacterial family in Iloilo River.

diversity index of 57.76 % was seen in the midstream section of Iloilo River, while the upstream section had the lowest diversity index of 40.88%. The diversity index in the upstream, midstream and downstream sections of Iloilo River were 1.97, 2.18 and 2.33, respectively, suggesting a low relative index across all sites.

There were nineteen bacterial species identified in all sampling sites (Fig. 3). The five most common species across all sites were *Marichromatium gracile* (3.62%), *Candidatus aquiluna rubra* (3.42%), *Peptoniphilus coxii* (1.85%), *Polynucleobacter cosmopolitanus* (1.45%) and *Clostridium cadaveris* (0.93%). The majority of the species were novel and some are pathogenic to humans and aquatic marine. *M. gracile* is a purple sulfur non-pathogenic bacterium capable of using reduced sulfur compound, nitrogen compounds or molecular hydrogen as electron donors for growth which was consistent with the native environments in inshore mangrove systems [41].

C. aquiluna rubra the family of *Microbacteriaceae* of the *Actinobacteria* was initially proposed as an indigenous freshwater species obtained from saline environment [42, 43]. *Polynucleobacter cosmopolitanus* is a novel species and free-living planktonic bacteria inhabiting freshwater lakes and rivers [44]. At some point, *Peptoniphilus coxii* a gram-positive coccus, commensals in human vagina and gut its presence in the river signifies fecal contamination and is considered as pathogenic to human and aquatic marine resources

[15]. Of further interest, *C. cadaveris*, is a non-toxin producing, enteric, gas-forming anaerobe and the most prominent bacteria present during human decomposition [45].

As shown in Fig. 4 the highest relative number (23.33%) of species was observed in the midstream while the upstream had the lowest number (16.85%). The diversity index obtained for upstream, midstream and downstream were 2.0, 2.29 and 2.39, respectively suggesting a low relative index across all sites based on Shannon -Wiener index reference value. A study done by Jumat et al. [16] supports the finding of this study, wherein, bacterial communities in physically stressed ecosystems, characteristically have low species diversity and the decrease in diversity was accompanied by a decrease in sample evenness [46].

Physicochemical Parameters

Physicochemical parameters were measured in all sampling sites using standard methods and results were compared to the standard reference limit for Class C Water Body Classification [9]. As shown in Table 1 the results of the physicochemical parameters in Iloilo River revealed that temperature is within the standard limit (26.40ºC). The salinity (0.01) is within the permissible limit. Moreover, turbidity of water samples is within the acceptable limit (0.33 TCU). The value of pH (7.36), is within the standard limit; Total suspended solids (TSS) of water samples is within the allowable
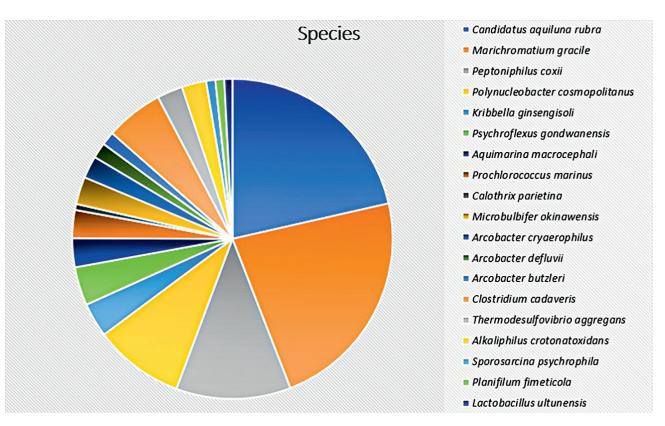

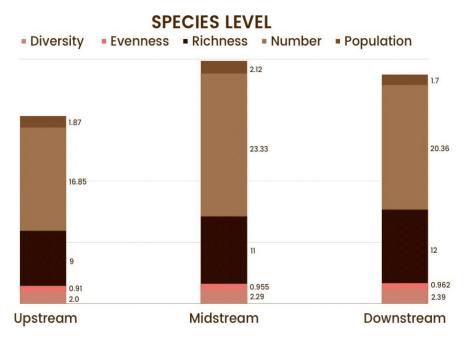Fig. 3. Bacterial composition based on Species-Level.

SPECIES LEVEL

■ Diversity ■ Evenness ■ Richness ■ Number ■ Population

Upstream
- 1.87
- 16.85
- 9
- 0.91
- 2.0

Midstream
- 2.12
- 23.33
- 11
- 0.955
- 2.29

Downstream
- 1.7
- 20.36
- 12
- 0.962
- 2.39

Fig. 4. Taxonomic bar plots depicting diversity of bacterial species in Iloilo River.

Table 1. Physicochemical Parameters in the Iloilo River.

Physicochemical Parameters	Total Mean	Standard reference limit for Class C Water Body Classification (DENR, 2016)
Temperature (ºC)	26.40	25-32
Salinity (ppt)	0.01	0.5
Turbidity (TCU)	0.33	75
Total Dissolved Solids (mg/L)	77.78	80
pH	7.36	6.9-7.6
Dissolved Oxygen (mg/L)	4.82	5

limit (77.78 mg/L). The dissolved oxygen is also within the reference limit (4.82 mg/L). The result of this study implies that the physicochemical parameters of the Iloilo River is not essentially polluted and results falls within permissible limits which is suitable for irrigation, fish culture, industrial use or any human use. The physicochemical parameters of Iloilo River were within the allowable limit set by the Department of Environment and Natural Resources (DENR) [4, 5] and that Iloilo River is saline because it is near the sea.

Conclusions

The 16S rRNA sequence analyses presented in this study show a detailed study of the bacterial microbiota in Iloilo River, Philippines. The majority of the identified bacterial family and species are novel. Most of which are known as human pathogens, while some are of emerging interest for fecal contamination. This approach also revealed several non-pathogenic bacteria that are known as degraders and reducers which are vital in the nutrient cycle in the aquatic environment. There is a relatively low microbial diversity index across all sites. Commercial establishments and informal settlers abound along the river, sewage pollution from human and animal waste are major sources of contaminants that greatly influenced the bacterial abundance and diversity thereby reducing the water quality. This study unveiled the condition of Iloilo River which might pose health and environmental risks to human and aquatic lives. There should be routine monitoring of bacterial communities in the water. An information dissemination campaign on the state of water in the Iloilo River for public awareness should be made. Application of relevant legal laws with regard to the proper handling of industrial and household wastes before entering the river course must be enforced. Further study may be conducted using the raw reads data to identify bacterial gene markers that are pathogenic, virulent, and antimicrobial resistant.

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Conflict of Interest

The authors declare no conflict of interest.

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