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Bacterial composition and antibiotic resistome profile of water in the Manila Bay South Harbor

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ABSTRACT

Anthropogenic activities in Manila Bay, the oldest and busiest international port in the Philippines, may cause changes in flora and fauna within its harbors. Specifically, the ballasting and deballasting of ships may accidentally introduce new populations of microorganisms, plants, and animals. Ballasting (and deballasting), or the process of carrying in/out of seawater from ship tanks in major ports/harbors, are causing major disturbances, particularly in marine ecosystems. Previous studies have shown that marine microorganisms comprise 98% of the primary production and regulation of the ocean's biogeochemical processes. However, with the continuous community disturbances due to human influence, potential marine bacterial populations with antibiotic resistomes may occur, and impact marine diversity. Since data on the microbial communities in Manila Bay South Harbor are limited, this study determined the bacterial composition and antibiotic resistome profiles of water samples from the area. Next-generation sequencing techniques and culture-based methods were utilized to elucidate the bacterial and functional diversity, as well as the antibiotic resistome of the water samples from the Manila Bay South Harbor. The bacterial diversity of the water sample was made up primarily of members of Bacteroidetes, Firmicutes, and

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Prevotella. The Clusters of Orthologous Group (COG) Level 1 identified metabolism as the most dominant functional predicted metagenome. The characterization of antibiotic resistance genes (ARGs) from water samples revealed that ARGs encoding resistance to multidrug was the most abundant. On the other hand, the identified antibiotic-resistant bacteria included ampicillin-resistant *Escherichia coli*, multidrug-resistant *Klebsiella pneumoniae*, ampicillin- and cefuroxime-resistant *Vibrio alginolyticus* and ampicillin resistant *Vibrio furnissii*. The above results provide a baseline marine bacterial community and antibiotic resistome profile of the Manila Bay South Harbor. This information is critical in gaining a better understanding of the potential roles of shifts in marine microbiomes, and in turn, help in developing appropriate solutions for managing the undesirable consequences of ballast water.

INTRODUCTION

Shipping is the most efficient mode of goods and passenger transport worldwide. International Maritime Organization reported that ships carry almost 80% of world trade. In an archipelagic country like the Philippines, thousands of ships navigate the Philippine waters on both inter-island and international routes. The country's most prominent and main shipping port is the port of Manila, situated on the eastern shore of Manila Bay. The port comprises the North and South Harbors, with the North Harbor for local inter-island shipping and the South Harbor for international shipping (Jacinto et al. 2006). Manila Bay supports the food, employment, livelihood, and recreation needs of 23 million Filipinos residing on its coasts (Prudente et al. 1997; Kim et al. 2011; Jacinto et al. 2006; PEMSEA and MBEMP-MBIN 2007; Greenpeace 2013).

Like many ports, the port of Manila is prone to pollution. The ballasting and deballasting of ships had been reported as one of the contributory factors to marine pollution (PoPa 2009). During the voyage, when ships are not carrying heavy enough cargo or more stability is required due to rough seas, commercial ships use ballast water to provide stability and maneuverability. An individual vessel carries approximately 150,000 metric tons of ballast water and sediments, and it can contain a diverse community of organisms, including invasive species, which are carried across the globe (Deacutis and Ribb 2002). Ships can carry about 3,000 to 4,500 marine species through the global ballasting and deballasting processes. The deballasting of water from ships acts as a vehicle for the global distribution of pathogens, waterborne diseases, and possible antibiotic-resistant forms, which may adversely impact humans, marine animals, and the aquatic ecosystem (Ruiz et al. 2000). Therefore, untreated ballast water can potentially impose an environmental and public health threat to coastal regions where ballast waters are released.

In a previous study by Balolong et al. (2020), *Bacillus sp.* (S23) isolate from Baseco Beach, Manila Bay was susceptible to four classes of antibiotics namely: ampicillin, cefazolin, cefuroxime, and ceftazidime. *Klebsiella pneumoniae* isolate was resistant to ampicillin while *Vibrio alginolyticus* (S6, S20, and S22) isolates were resistant to ampicillin, cefazolin, and cefuroxime (Balolong et al. 2020). In another study by Ng et al. (2015), antibiotic resistance profiles were screened and the result showed that the ballast water samples in the port of Singapore encode resistance to sulfonamides, trimethoprim, and chloramphenicol-florfenicol antibiotics. In particular, ARGs can be transferred via horizontal gene transfer (HGT) between microbes, including pathogens. Therefore, ARGs are spreading rapidly and becoming global environmental and public health issues (Pruden et al. 2006). If ballast water containing ARGs is discharged into new destination ports, these ARGs may proliferate in the receiving waters and threaten both environmental and human health (Lv et al. 2020). To date, few studies regarding the abundance and profiles of ARGs in ship ballast water have been reported (Lv et al. 2020). To reduce the risk of introducing an alien species via ballast water, several measures have been established by the International Maritime Organization (IMO), such as instituting the International Convention for the Control and Management of Ships' Ballast Water and Sediments (Lv et al. 2020). Of the proposed solutions, ballast water exchange (BWE) is an important and widely used method that involves the replacement of coastal water from ballast tanks with open-ocean water (Balaji et al. 2014; Gray et al. 2007; Seiden and Rivkin 2014).

On June 8, 2018, the Philippines acceded to the IMO Ballast Water Management and Anti-Fouling Conventions. These management and conventions oblige the Philippines to ensure its ports and maritime transport industry comply with the agreements to lessen the risk of biological invasion, public health threats, and the transport and translocation of harmful marine organisms and pathogens. A key program in the Ballast Water Management Convention (BWMC) is to come up with port ecological baselines that will provide a basis for assessing risks in ballast water species translocation.

Due to the ballasting and deballasting operations of ships and other human activities, Manila Bay may support a diverse array of marine ecosystems that include vertebrates, invertebrates, and large groups of microorganisms, including bacteria, viruses, archaea, protists, and fungi. However, only a few baseline reports exist on these microbial groups in Manila Bay South Harbor. This under-explored marine community can result in the most crucial environmental issues due to the emergence of possible pathogenic bacteria and antibiotic-resistant forms. Hence, this study provides baseline information on bacterial composition and antibiotic resistome profiles of water in the Manila Bay South Harbor. Specifically, this study describes the bacterial diversity of water in the Manila Bay South Harbor and the bacterial community's functional diversity, characterizes the antibiotic resistance genes (ARGs) of bacteria from the site, and identifies the clinically relevant culturable antibiotic-resistant bacteria (ARB).

MATERIALS AND METHODS

A. Study Site

Three sampling sites in Manila Bay, with entry points shown in Figure 1, were selected as these sites receive international vessels that could carry non-indigenous species from large ocean-going vessels. Global Positioning System (GPS) coordinates at each sampling points were obtained.

B. Sampling and Monitoring of Water Quality Parameters

Two water sample collection periods, eight weeks apart, were done in the dry season of 2021. Environmental parameters, such as dissolved oxygen, temperature, pH, total dissolved solids, and conductivity of the water sample, were measured using the Horiba water quality checker U-5000 (Horiba Ltd., Kyoto, Japan). Two liters of harbor water were collected from the three sampling sites and were pooled as one sample. The sample was obtained using a pre-cleaned bucket with a rope that was submerged twelve inches below the surface water. The water sample was transferred into a sterile plastic container, which was then labeled and sealed. The sample containers were transported in tightly-packed, sealed coolers to the laboratory and were filtered immediately.

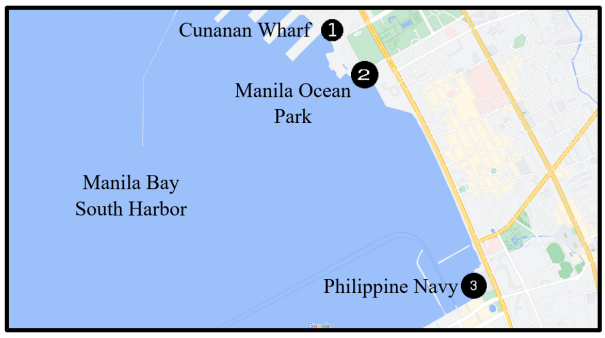

Figure 1: Sampling sites (labeled 1-3) of the study

C. Water Filtration and Genomic DNA Extraction

A one-liter water sample from each sampling site was filtered using a 0.22 um sterile nylon filter membrane. The filter membranes containing the residue were stored for 12 hours at -80°C in a centrifuge tube containing RNAlater, a tissue storage reagent, until further use.

The genomic DNA from the filter membrane containing the residue was extracted with the DNeasy® PowerWater® DNA Isolation kit (Qiagen, Germany) according to the manufacturer's procedures. DNA yields and purity were measured using a Thermo Scientific Nanodrop 2000c spectrophotometer (Wilmington, DE 19810, USA).

D. Shotgun Metagenomic Sequencing and Bioinformatics Analysis

Extracted bacterial genomic DNA was sent to Macrogen, Inc. in South Korea for fragment library construction and shotgun sequencing. The concentration of at least 5ng/ul and 260/280 purity ratio of at least 1.7 required by Macrogen, Inc. were satisfied, and the samples were subjected to Quality Control. Qualified samples proceeded to the library construction.

The sequencing library was prepared by random fragmentation of the DNA or cDNA sample, followed by 5' and 3' adapter ligation. Adapter-ligated fragments were then PCR amplified and gel purified. The library was loaded into a flow cell for cluster generation. When the cluster generation was completed, the templates were prepared for sequencing using Illumina SBS technology.

Sequencing data were converted into raw data for analysis. All raw reads were functionally and taxonomically classified using the metagenomics Rapid Annotation using Subsystem Technology (MG-RAST) server version 4.0.3. The taxonomic classification and functional characterization were performed with the MG-RAST server using the Reference Sequence (RefSeq) and Clusters of Orthologs Group (COG) databases, respectively, with default parameters.

E. Antibiotic-Resistance Genes (ARG) Characterization

Antibiotic resistance genes (ARGs) were identified using the data obtained from the shotgun metagenomic sequencing and bioinformatics analysis. Read quality was first checked using the FastQC quality control checker, and low-quality reads were trimmed using the Trimmomatic flexible read trimming tool. Trimmed reads were then assembled using the de novo type Megahit assembler into contigs. The quality of the contigs was then checked using Benchmarking Universal Single-Copy Orthologs (BUSCO). Finally, contigs were mapped against the ARG-miner database (VirginiaTech) with the Diamond aligner tool (Buchfink et al. 2021). The shotgun reads were compared with databases of known functional and resistance genes to determine the relative abundance of resistance genes.

F. Identification and Antibiotic Susceptibility Test (AST) of Clinically Relevant Antibiotic-Resistant Bacteria (ARB)

One liter from each sampling site was mixed well to a total volume of three liters. A volume of 120 ml of the combined water sample was transferred to a sterile sample container. The samples were placed in a cold shipping container and brought to the Microbiology Laboratory of Makati Medical Center, Department of Pathology and Laboratories for identification and antibiotic susceptibility test (AST) of antibiotic-resistant bacteria (ARB) using the VITEK® Automated System. The antibiotics tested were cefepime, amikacin, gentamicin, amoxicillin/ clavulanic acid, piperacillin/ tazobactam, cefoxitin, ceftazidime, ceftriaxone, cefuroxime, trimethoprim/ sulfamethoxazole, ampicillin, ertapenem, imipenem, meropenem, and ciprofloxacin.

1. Isolation and Purification

A volume of 10 ml water samples was planted in five test tubes of Lactose Broth and incubated for 24 hours at 35ºC. Subculture was done with all turbid lactose tubes in Blood Agar Plate (BAP), and MacConkey (MAC) using a 10 ul loop and incubated at 35-37ºC for 24 hours. All growth from subculture plates was picked and re-isolated in another MAC and BAP media to ensure growth purity.

2. Partial Characterization

For gram-negative bacteria, differentiation was done in MacConkey agar and an oxidase test was performed. For gram-positive microorganisms, differentiation was done by hemolysis reading on a blood agar plate and a catalase test was conducted.

3. Identification

The VITEK® MS target slides were used in the VITEK® MS for the identification of the isolated colonies.

4. Antibiotic Susceptibility Testing (AST)

VITEK® 2 AST-N261 ID Card was used for the rapid, accurate species-level AST of clinically relevant bacteria and are also validated to be used for non-clinical samples (Pecson et al. 2021, Park et al. 2021, and Nakamura et al. 2020). The antibiotics tested were cefepime, amikacin, gentamicin, amoxicillin/ clavulanic acid, piperacillin/ tazobactam, cefoxitin, ceftazidime, ceftriaxone, cefuroxime, trimethoprim/ sulfamethoxazole, ampicillin, ertapenem, imipenem, meropenem, and ciprofloxacin. The selection of antibiotics in the VITEK® 2 XL is based on the Laboratory Standards Institute (CLSI) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST). Every Minimum Inhibitory Concentration (MIC) result was checked against a database of more than 3,500 phenotypes and 30,000 MIC distributions to determine consistency with previously defined wild or resistant phenotypes. Results were certified by a clinical pathologist from the Microbiology Section of the Makati Medical Center.

RESULTS AND DISCUSSION

The amount of data obtained through next-generation shotgun sequencing, bioinformatics analysis, and culture-dependent antibiotic susceptibility test, provides baseline information on bacterial composition and antibiotic resistome of water samples from Manila Bay South Harbor. The sampling was performed during the dry season to capture all the bacterial composition and antibiotic resistome present in Manila Bay. Results of the first and second sampling collections were consolidated to provide a more explicit description of the bacterial composition and antibiotic resistome in Manila Bay.

A. Water Quality Parameters

Generally, tropical regions' marine water temperatures range from about -2°C to 30°C (Webb, 2022). The study's surface water temperature values have an average value of 30.15°C. The result suggests that the temperature in Manila Bay is generally ambient for microorganisms to thrive. Meanwhile, the ideal pH value for marine and natural freshwater bacteria ranges from 2 to 13.1 (EMB DENR, 2008). The average pH value of the sample is 6.22, suggesting that the pH level for the bacterial community in Manila Bay is ideal for their survival. The dissolved oxygen level in the sampling area is 6.05 milligrams per liter (mg O2/L). Levels above five mg/L are considered optimal for the survival of marine organisms (EMB DENR, 2008). Thus, the water quality in Manila Bay can support desirable aquatic life. Too high or too low values of these water quality parameters may negatively impact the water's biological and chemical activities.

B. Bacterial Diversity

The shotgun data set generated a total average of 3,232,667 sequences and 794,655,193 base pairs with an average length of 245.5 bps. After quality filtering, 2,759,361 (85.36%) unique sequences, with an average length of 249 base pairs, passed the Quality Control pipelines. Taxonomic affiliations were assigned to sequences with predicted proteins and rRNA genes based on a comparison with the M5NR database. Alpha diversity value and rarefaction curve were obtained using the Metagenomics Rapid Annotation using Subsystem Technology (MG-RAST) tool. Alpha diversity based on Shannon's index for the sample was 155. The Shannon's diversity index combines richness and diversity; thus, this value is the number of species with well-balanced abundances in the sample. The sample's rarefaction curve was asymptotic, suggesting that most taxonomic diversity was recovered and that a more intensive sampling will yield only a few additional species (Figure 2). The sampling curve ascended quickly and then leveled off toward an asymptote as fewer new species per unit of individuals were collected, indicating that the bacterial communities in the metagenome were well-represented.

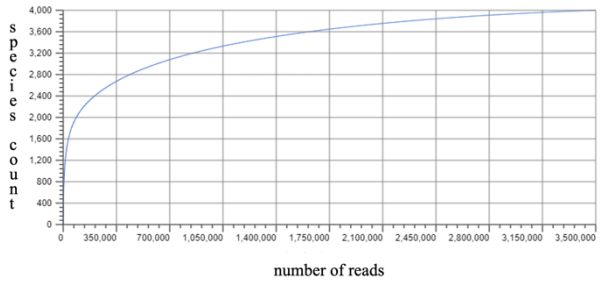

Figure 2: Rarefaction curve of annotated species richness of bacterial community isolated from water samples.

The metagenomic sequencing of Manila Bay South Harbor water samples revealed that ~ 85% of the total assigned reads for taxonomic classification are bacteria (Figure 3A). A total of 27 bacterial phyla were identified in the sample, and these were further classified into 42 classes and 86 orders (Table 1). The bacterial community was diverse, representing 206 families and 502 genera.

Bacteroidetes (60.98%) and Firmicutes (26.37%) were found to be the two predominant phyla, representing 87.28% of the total bacterial community (Figure 3B). Bacteroidetes is one of the most abundant bacterial groups in aquatic ecosystems, contributing approximately 4% to 22% of total bacteria (Acinas et al. 2015; Alonso-Sáez and Gasol 2007; Cottrell and Kirchman 2000). Bacteroidetes is reported to be pivotal in processing organic matter in the oceans (Kirchman 2002). The phylum Bacteroidetes is also known to be inhabitants of fecal material and have been used to discern fecal source types (Jeong et al. 2011). Similar studies by Unno et al. (2010) revealed that the phylum Firmicutes is also one of the significant human fecal bacteria detected in watersheds. Sun et al. (2017) reported the detection of high levels of Firmicutes attributed to the fecal bacteria in the effluent discharged from a nearby wastewater treatment plant. The high abundance of fecal bacteria phyla in the sample may be due to human interference and the direct discharge of human excreta from the surrounding environments into Manila Bay.

The bacterial class with the highest relative abundance was Bacteroidia (58.15%). The relative abundance of the dominant bacterial groups at the class level is presented in Figure 3C. Bacteroidia, belonging to the phylum Bacteroidetes, was primarily detected in municipal sewage samples (Tiwari et al. 2021). The genus under class Bacteroidia has been reported to exhibit pathogenicity to marine organisms (Reza et al. 2018). The most abundant order in the metagenome is Bacteroidales accounting for almost 58.15% of the total bacterial population (Figure 3D). The order Bacteroidales belongs to the class Bacteroidia and phylum Bacteroidetes. Bacteroidales are fecal anaerobic bacteria commonly observed in the wastewater collection system (Bae and Wuertz 2009) and most surface water (Schriewer et al. 2010). The most dominant bacterial family in the metagenome is Bacteroidaceae (Figure 3E). Bacteroidaceae includes gram-negative, anaerobic, non-spore-forming rod (Moore et al. 1976) species that are recovered mostly from clinical specimens (Brook 2017) and are resistant to penicillins, mainly through the production of β-lactamase (Brook 2017). They are part of the normal gastrointestinal flora and predominate in intra-abdominal infections and other infections originating from the gut flora (Brook 2017).

The topmost abundant bacterial genera isolated from the water sample of Manila Bay are Bacteroides and Prevotella (Figure 3F). *Bacteroides* species are anaerobic, bile-resistant, non-spore-forming, gram-negative rods and are significant clinical pathogens (Wexler 2007). *Bacteroides* are the most abundant organism in human feces (Cabral 2010) and are reported to exhibit resistance to a wide variety of antimicrobials (Vedantam 2009). *Bacteroides* are naturally resistant to aminoglycosides and can acquire resistance to the beta-lactams, tetracyclines, macrolide-lincosamide-streptogramin (MLS) drugs, nitroimidazoles, and quinolones (Vedantam 2009). A previous study showed that certain *Bacteroides* species in the environment always indicate fecal pollution since they are strict anaerobe and are not expected to grow in oxic environments (Bakir et al. 2006). Since *Bacteroides* were detected from water samples of Manila Bay, this suggests that fecal pollution around the sampling areas is likely due to a high anthropogenic influence on the area's coastal water. The second most abundant bacterial genus was *Prevotella* within the family Prevotellaceae of the phylum Bacteroidetes. *Prevotella* species are rod-shaped, gram-negative, obligate anaerobic bacteria often found in the human oral, intestinal, and urogenital floras (Jousimies-Somer 1995). This anaerobe is considered an opportunistic pathogen (Könönen et al. 1994) and several studies have demonstrated the increasing resistance among *Prevotella* species against β-lactam antibiotics. Some have shown partial resistance to metronidazole (Sandoe 2001, Könönen et al. 1997). Earlier studies have reported that members of the *Prevotella* genus have been detected in feces-contaminated surface waters (Ponce-Terashima et al. 2014). *Prevotella* was also reported as the most abundant genus in human fecal samples and sewage samples (Koskey et al. 2014). The high prevalence of this genus in the water sample may be caused by the transmission of waste belonging to human and animal normal flora into the bodies of water in Manila Bay.

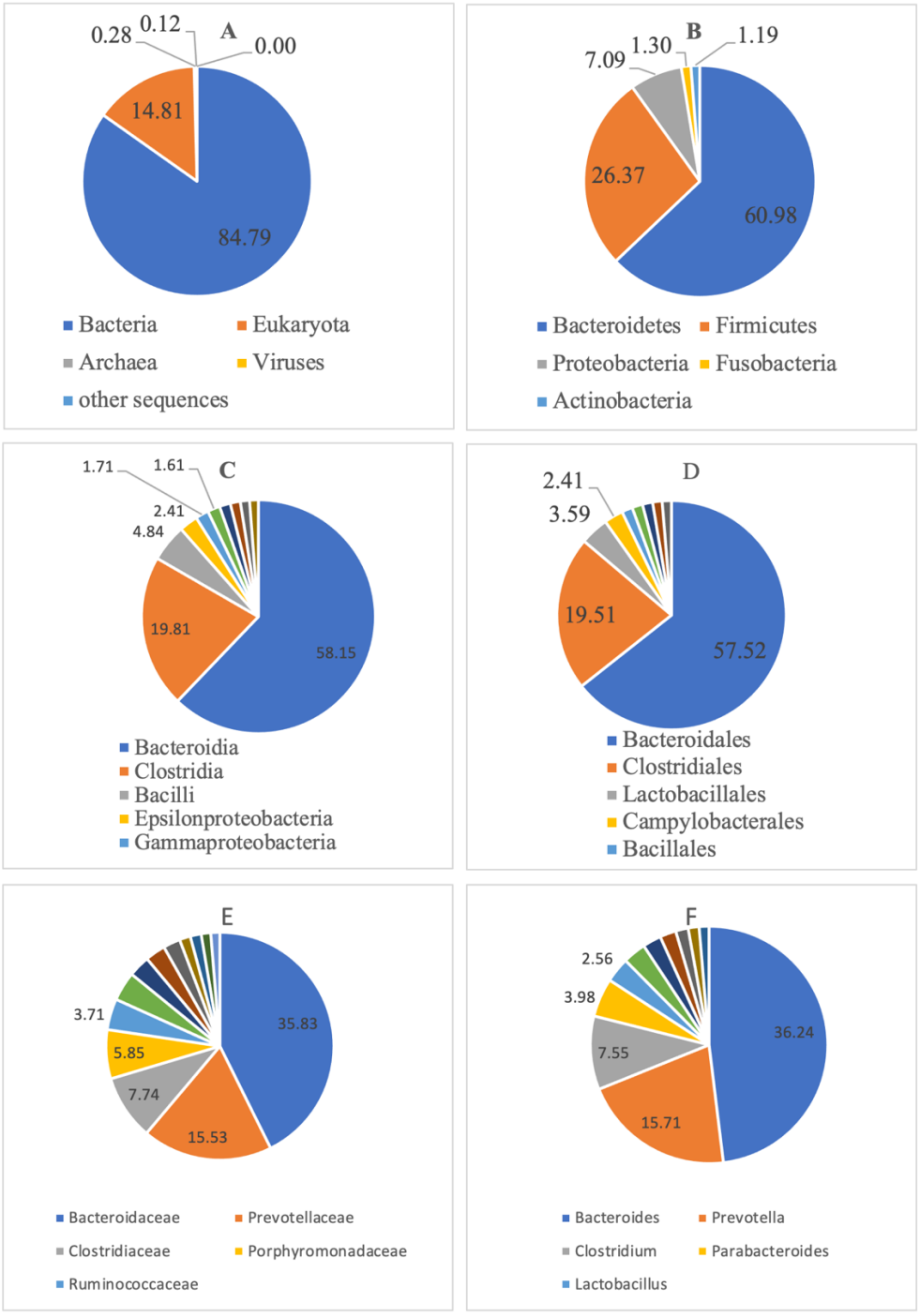

Figure 3: Bacterial domain distribution based on metagenomic analysis of bacterial genomic DNA isolated from water samples, A – Domain, B – Phylum, C – Class, D – Order, E – Family, and F - Genus

Table 1: Physicochemical characteristics of water samples.

Environmental Parameters	Mean of Two Sampling Collection Periods
Temperature °C	30.15±0.93
pH level	6.22±0.90
Conductivity (mS/cm)	37.63±1.62
Dissolved oxygen (mg/L)	6.05±0.03
Total dissolved solids (g/L)	22.67±1.24

Of the potential bacterial pathogens in the water, a total of 17 were detected from the samples. The potential pathogens include *Bacillus, Escherichia, Enterococcus, Mycoplasm, Pseudomonas, Vibrio, Neiserria, Salmonella, Shigella, Arcobacter, Mycobacterium, Leptospira, Corynebacterium, Legionella, Enterobacter, Aeromonas,* and *Serratia*. The direct exposure of humans to pathogens can contribute to developing health threats. The interaction of the pathogen and the susceptibility of the host and its environment are factors to the threats and risks to the community near the Manila Bay South Harbor.

Ballast water containing ARBs and ARGs has the potential to invade the receiving waters' indigenous ecosystems. Because of the abundance of ARGs and potential pathogens in ballast tanks or destination ports after discharge, it is possible that ARGs was transmitted among bacteria via HGT. Ship ballast water should

be considered a fast-moving carrier of antibiotic resistance due to the high abundances of the ARGs found in the sample.

Based on the results of the taxonomic composition, the study site may be constantly exposed to fecal bacteria. Fecal bacteria can contribute to the accumulated load of biological, organic, and chemical contaminants in water environments (Paruch et al. 2019). These contaminants additionally create different stresses on the central environmental processes in ecosystems, such as nutrient flow and availability, biodegradation, and disturbances in the water and energy cycle (Paruch et al. 2019). Because of these factors, fecal pollution can cause negative impacts on the structure of the aquatic community (Paruch et al. 2019). Fecal input can also constitute an extra selective pressure on the aquatic microbial community, selectively promoting the growth of some beneficial microbes and thus resulting in the extinction of other microbial populations (Paruch et al. 2019). Direct or indirect sewage or domestic waste disposal from nearby facilities may have introduced clinical strains in the environment. Aside from the marine pollution due to ballasting and deballasting processes, reports from the Department of Health (DOH) Environmental and Occupational Health Cluster revealed that wastes from nearby households and healthcare facilities like clinics and hospitals contribute to the contamination of the Manila Bay waters. The polluted water in Manila Bay may pose potential health risks to the environment; thus, quality issues should become the core concern for public and regulatory authorities.

C. Functional Characterization

The Clusters of Orthologous Group (COG) database revealed different patterns of functions from the bacterial community in water samples of Manila Bay. Level 1 indicates the broadest set of functions to which sequences are assigned, while level 2 refers to a more specific functional assignment within level 1 categories. The three metagenomes predicted functions classified using Clusters of Orthologous Group (COG) level 1 are metabolism (48.03%), information storage and processing (26.93%), and cellular processes and signaling (25.04%) (Table 2), suggesting the dominant role of these functional categories in all samples. Within the level 1 metabolism category, sequences associated with level 2 functional categories such as carbohydrate transport and metabolism, amino acid transport and metabolism, and energy production and conversion were dominant in the sample (Table 3). This finding corresponds well with the expected higher content of carbohydrates in water samples because these compounds can serve as carbon and energy sources for microbial activity. Amino acid transport and metabolism were also detected with high abundance in the water sample. This might be due to the presence of free and combined amino acids (e.g., oligopeptides and proteins), which are essential sources of carbon, nitrogen, and energy for marine heterotrophic bacteria. The presence of energy production and conversion are significant in the water sample since they are responsible for many fundamental biological processes of most heterotrophic marine bacteria. The top functional genes appeared to be consistent with previous reports, where genes involved in carbohydrate, protein, and amino acid metabolism were observed in the Pacific and Atlantic Oceans (Venter et al. 2004; Rusch et al. 2007; Hewson et al. 2009). This result implies that the prokaryotes that dominate surface water environments possibly share a core set of genes critical to these communities' adaptation and survival (Hewson et al. 2009). For more specific functional assignments within level 1 information storage and processing (ISP), functions affiliated with level 2 were cell wall/membrane/envelope biogenesis, replication, recombination, repair transcription, and Chromatin structure and dynamics. Biogenesis of cell wall/membrane/envelope is a cellular process that results in the synthesis of constituent macromolecules, assembly, and arrangement of constituent parts of the cellular component (Smith et al. 2019). This process is also essential for marine bacteria to maintain their shape and protect them from osmotic lysis (Smith et al. 2019).

Table 2: Percent relative abundance of sequences belonging to COG level 1 category.

COG Level 1	Percent Relative Abundance
Metabolism	48.03
Information Storage And Processing	26.93
Cellular Processes And Signaling	25.04

Functional analysis of the metagenomic data explored what the microbes that are present are doing, and how they are doing it. The functional analysis provides essential clues about functional diversity and variation to better understand the molecular function, cellular component, and biological processes of bacterial communities in the water sample. Determining subtle differences in metabolic potential will allow environmental changes at the early stages of perturbation and identify previously unknown pathways for therapeutics (Dinsdale et al. 2008). Understanding the capabilities of bacterial communities in water will aid the industry and its regulators improve environmental and economic sustainability through informed water management decisions.

D. ARG Characterization

The relative abundance of the dominant ARGs (greater than 1% abundance) is presented in Table 4. The ARGs encoding resistance to multidrug was the most abundant in water samples. Multidrug-ARGs were also previously reported as the dominant ARG types in sewage and activated sludge (Li et al. 2015). As expected, these dominant ARGs were usually associated with antibiotics that have been extensively used in human or veterinary medicine (Li et al. 2015). Land-based human activities, including the discharge of municipal, industrial and agricultural wastes and runoff, and ballasting and deballasting, can be the leading cause of this pollution in Manila Bay.

E. Antibiotic-Resistant Bacteria Identification

Culture-dependent identification and susceptibility testing using VITEK® MS and VITEK® 2 XL automated system are only limited to analyzing the most clinically relevant culturable bacteria. However, some previous publications demonstrate that VITEX®2 XL is a versatile platform that can be used for detecting pathogens in a variety of non-clinical samples; thus manual validation using other method(s) is/are not needed. A study by Pecson et al. (2021) demonstrated the use of VITEX® 2 XL for detecting SARS-CoV-2 in wastewater sample. Another study by Park et al. (2021) validated the use of VITEX®2 platform for detecting Norovirus in fresh produce wash water. Nakamura et al. (2020) also demonstrated the use of VITEX® 2 XL for detecting foodborne pathogens in food samples. The antibiotics tested in this study were cefepime, amikacin, gentamicin, amoxicillin/ clavulanic acid, piperacillin/ tazobactam, cefoxitin, ceftazidime, ceftriaxone, cefuroxime, trimethoprim/ sulfamethoxazole, ampicillin, ertapenem, imipenem, meropenem, and ciprofloxacin.

Twelve bacterial isolates were identified in the sample (Table 5). Four isolates were recognized as antibiotic-resistant bacteria, namely, multidrug (cefepime, ceftazidime, ceftriaxone, cefuroxime, trimethoprim/sulfamethoxazole, ciprofloxacin, and amoxicillin/clavulanic) resistant *Klebsiella pneumoniae*, ampicillin-resistant *Escherichia coli*, ampicillin-resistant *Vibrio furnissi*, and cefuroxime and ampicillin-resistant *Vibrio alginolyticus*. The presence of antibiotic-resistant bacteria may serve as source for the transmission of resistant determinants to susceptible bacteria, including human pathogens. Some of the

Table 3: Percent relative abundance of sequences belonging to COG level 2 functional categories within associated level 1 categories.

COG Level 1	COG Level 2	Percent Relative Abundance
Metabolism	Carbohydrate transport and metabolism	11.38
Metabolism	Amino acid transport and metabolism	10.22
ISP	Cell wall/membrane/envelope biogenesis	8.65
CPS	Cell cycle control, cell division, chromosome partitioning	5.62
Metabolism	Energy production and conversion	5.18
Metabolism	Coenzyme transport and metabolism	5.18
ISP	Translation, ribosomal structure and biogenesis	5.12
CPS	Cell motility	4.91
ISP	Chromatin structure and dynamics	4.23
CPS	Defense mechanisms	3.77

Legend: ISP-Information, Storage and Processing; CPS-Cellular Processes and Signaling

Table 4: List of bacterial ARGs representing >1% of the total community identified by metagenomic analysis.

Resistance	Type	Percent Relative Abundance
Multidrug	evgS	2.41
macrolide-lincosamide-streptogramin	macB	2.10
Quinolone	Mfd	2.03
beta lactam	OXA-34	1.69
Oxazolidinone	optrA	1.53
macrolide-lincosamide-streptogramin	desR	1.31
Pleuromutilin	TaeA	1.41
Mupirocin	mupB	1.03

Table 5: Antibiotic Susceptibility Assay of Isolates using VITEK® 2 XL.

Antibiotic	**Isolates**											
	1	2	3	4	5	6	7	8	9	10	11	12
Cefepime	S	R	S	S	S	S	S	S	S	S	S	S
Amikacin	S	S	S	S	S	S	S	S	S	S	S	S
Gentamicin	S	S	S	S	S	S	S	S	S	S	S	S
Amoxicillin/ Clavulanic Acid	S	S	S	S	S	S	S	S	S	S	S	S
Piperacillin/ Tazobactam	S	S	S	S	S	S	S	S	S	S	S	S
Cefoxitin	S	S	S	S	S	S	S	S	S	S	S	S
Ceftazidime	S	R	S	S	S	S	S	S	S	S	S	S
Ceftriaxone	S	R	S	S	S	S	S	S	S	S	S	S
Cefuroxime	S	R	S	S	S	S	S	S	S	S	R	S
Trimethoprim/ Sulfamethoxazole	S	R	S	S	S	S	S	S	S	S	S	S
Ampicillin	R	R	S	S	S	R	S	S	S	S	R	S
Ertapenem	S	S	S	S	S	S	S	S	S	S	S	S
Imipenem	S	S	S	S	S	S	S	S	S	S	S	S
Meropenem	S	S	S	S	S	S	S	S	S	S	S	S
Ciprofloxacin	S	R	S	S	S	S	S	S	S	S	S	S

Table Legend: S-Susceptible; I-Intermediate; R-Resistant; 1-*Escherichia coli;* 2-*Klebsiella pneumoniae;* 3-*Photobacterium damselae;* 4-*Sphingomonas paucimobilis;* 5-*Aeromonas hydrophila;* 6-*Vibrio furnissii;* 7-*Aeromonas punctate;* 8-*Vibrio parahaemolyticus;* 9-*Proteus penneri;* 10-*Aeromonas enteropelegenes;* 11-*Vibrio alginolyticus;* 12-*Vibrio fluvialis*

bacterial strains identified are of clinical importance and may pose treatment failures in the host during antibiotic therapy. The ability to disseminate of multidrug-resistant *Klebsiella pneumoniae* strains from hospitals to the environment was previously demonstrated (Mahon et al. 2017; Khan et al. 2018; Lepuschitz et al. 2019) highlighting the ability of these strains to survive and persist in environmental conditions. Multidrug-resistant *Klebsiella pneumoniae* is a major nosocomial pathogen, causing infections with high morbidity and mortality rates of up to 50% (Bassetti et al. 2018), caused by limited treatment options. This pathogen harbors a wide resistome that could evolve under antibiotic selective pressure, leading to extremely drug-resistant or high-risk clones with great epidemic potential (Navon-Venezia et al. 2017) if not correctly treated. Members of the *Vibrio* genus were also detected to have in this sample. This genus is an autochthonous inhabitant of aquatic environments and plays a vital role in sustaining the aquatic milieu. A recent study in Baseco Beach, Manila Bay, revealed that some *Vibrio* species harbor resistance toward ampicillin, cefazolin, cefuroxime, and gentamicin (Balolong et al. 2020). The development of resistance in *Vibrio* species can be due to frequent usage of antibiotics as part of the *Vibrio* infection treatment regimen (Sudha et al. 2014). *Escherichia coli* isolated from this sample showed resistance against ampicillin. In the previous study of *Escherichia coli* isolates from irrigation water in Metro Manila, the highest resistance was also observed in ampicillin, and tetracycline (Vital et al. 2018). The presence of ampicillin-resistant *Escherichia coli* in Manila Bay water is alarming, as ampicillin is widely available and extensively used in the management and treatment of certain infections that are caused by bacteria.

F. Bacterial Composition and Antibiotic Resistome in the Manila Bay South Harbor

The water bacterial diversity is mainly composed of phylum Bacteroidetes and members of bacterial taxa under it. However, in the culture-dependent bacterial identification and antibiotic

susceptibility tests using the VITEK® automated system, the predominant bacterial isolates were enteric bacteria from phylum Proteobacteria, because they were specifically selected through the culture conditions employed. The enteric bacteria, members of Enterobacteriaceae family that were detected by culture-dependent methods, were found lower in abundance in the metagenomic studies. As expected, many bacteria detected by the shotgun metagenomic sequencing were not isolated by the conventional culture-dependent methods. However, culturing complemented the metagenomic data results as clinically-relevant bacteria of concern were detected. Therefore, culture-dependent methods and metagenomics approaches are needed for a full insight into resistomes, and the combination of both methods significantly advanced our understanding of the role of the bacteria in the environmental samples.

The presence of enteric bacteria indicates fecal pollution and bacteria under Bacteroidetes have also been found in wastewater thus, Manila Bay might be highly contaminated by human/animal wastes. Considering the harsh environment in Manila Bay due to the shipping activities that disturb the marine habitat, functional genes that dominate surface water are important to the communities' adaptation and survival. The ARB present in the sample are members of the phylum Proteobacteria. Although they are present in lower abundance in the samples, there is still a tendency for horizontal gene transfer in the aquatic environment, hence a cause for concern for the environment's ecology. Acquisition of foreign DNA material through horizontal gene transfer is one of the most critical contributors to bacterial evolution and is often responsible for developing antimicrobial resistance. In this study, the antibiotic resistance genes encoding resistance to multiple drugs were the most abundant genes in the water sample; thus, the isolation of the multidrug-resistant bacteria phenotypically confirms the presence of the multidrug resistance genes in the sample. Antibiotics used in human and animal medicine are released through wastewater and subsequently to bodies of water, which is a major concern in the development of ARB. Even though the released antibiotics are diluted and degraded in the aquatic environment, trace level concentrations can potentially select for and preserve ARB. The detection of ARB in the sampling area will serve as a good contamination marker that will benefit human health risk evaluation. Although no data is available about medical cases of infections from Manila Bay due to bathing and other recreational activities done by people in Manila Bay or ingestion of contaminated seafood, the DOH warned the public that swimming or bathing in or near the polluted bodies of Manila Bay could lead to infections. Few studies have been conducted to estimate human health risks associated with ARB in recreational waters during various activities such as swimming, surfing, diving, boating, wading/splashing, and fishing. Exposure to ARB is possible during these recreational activities; however, further studies are necessary to draw public health conclusions. The direct exposure of humans to pathogens can contribute to developing health threats. The interaction of the pathogen and the susceptibility of the host and its environment are factors to the threats and risks to the community near the Manila Bay South Harbor.

In a study by Ng et al., (2015), they observed differences in the bacterial community composition, indicator organisms (*Escherichia coli, Enterococcus, Pseudomonas aeruginosa, Salmonella spp.,* and *Vibrio spp*.), and the concentrations of ARB and ARGs in the ballast water of 3 ships and harbor waters at their port of call. They were able to find correlations between the concentrations of indicator organisms (*Escherichia coli, Vibrio cholera, Vibrio vulnificus,* and *P. aeruginosa*) and ARGs, which suggest that these species in ballast and harbor waters may be carriers of ARGs. Generally, the culturable marine microbial communities in ballast and harbor waters were most resistant to trimethoprim, sulfanilamide, lincomycin, and tetracycline (Ng et al., 2015).

In this study, the high abundance of ARB and ARGs found in port water samples may act as a carrier for international ARGs transfer if these water samples are to be used for ship ballast water. Because of the large carrying capacities and high speeds of ocean vessels, ARGs in water samples should be given greater consideration. Although Ballast Water Exchange (BWE) can reduce the levels of several ARGs in ballast water, it is not a perfect protective strategy because residual ARGs are a risk (Lv et al. 2020). Disinfection techniques that remove ARGs may aid in the management of these emerging contaminants. As a result, more research on the removal of ARGs from ship ballast water is required to mitigate their negative effects. The ecological risks posed by emerging contaminants introduced by ship ballast water must be adequately addressed.

The identification of members of Bacteroidetes, Firmicutes, and Prevotella, as well as ARGs encoding resistance to multidrug, in ballast water samples is crucial for developing appropriate solutions for managing the undesirable consequences of BWE. The development of effective ballast water treatment technologies that can eliminate harmful microorganisms and reduce the spread of antibiotic resistance. This study can inform policies and regulations that aim to reduce the impact of BWE on the environment and public health. The findings of such study can help identify the sources and routes of transmission of antibiotic-resistant bacteria. By identifying the fecal bacterial and antibiotic resistome in ballast water, researchers can determine the types and prevalence of antibiotic-resistant bacteria in different ports and shipping lanes. This information can then be used to develop appropriate solutions for managing the spread of antibiotic resistance, such as the implementation of ballast water treatment technologies, regulation of ballast water exchange, or the use of alternative methods for managing ship ballast.

CONCLUSION

The bacterial diversity of water from the Manila Bay South Harbor was primarily composed of Bacteroidetes and members of bacterial taxa under it. Potential bacterial pathogens in the sample were detected, implying that Manila Bay is a reservoir of these potentially harmful bacteria. The functional diversity profiles of the bacterial community from the said water sample were dominated by carbohydrate and amino acid transport and metabolism genes. The characterization of antibiotic resistance genes (ARGs) from water samples revealed that ARGs encoding resistance to multidrug, macrolide-lincosamide-streptogramin, and quinolone was the most abundant. These dominant ARGs are usually associated with antibiotics that have been extensively used in human or veterinary therapy. The antibiotic-resistant bacteria (ARB) present in the sample were multidrug (cefepime, ceftazidime, ceftriaxone, cefuroxime, trimethoprim/sulfamethoxazole, ciprofloxacin, amoxicillin/clavulanic, and ampicillin) resistant *Klebsiella pneumoniae*, ampicillin-resistant *Escherichia coli*, ampicillin-resistant *Vibrio furnissii,* and ampicillin- and cefuroxime-resistant *Vibrio alginolyticus.* Detecting these microorganisms will help prevent the widespread dissemination of invasive species and possible antibiotic-resistant forms. Findings from this study provide baseline data for future studies on marine bacterial community and will serve as a good platform for many prospective areas of study. These data may be used to prevent widespread dissemination of ARB and ARGs in port water,

ensure proper ballast water management programs, and provide safe water to the surrounding environment. Based on the water sample's bacterial composition and antibiotic resistome, Manila Bay harbor contains potentially opportunistic pathogens. This contaminated harbor water may pose health risks to humans and the environment, and could possibly be spread in other areas through ballasting thus, water management decisions must be regulated in the area surrounding Manila Bay.

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