MASTER'S THESIS

# Metagenomic analysis reveals wastewater treatment plants as hotspots of antibiotic- and biocide-metal resistome and mobilome

SACHIN KUMAR GUPTA

DEPARTMENT OF BIOTECHNOLOGY

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

FEBRUARY 2019

MASTER'S THESIS

# Metagenomic analysis reveals wastewater treatment plants as hotspots of antibiotic- and biocide-metal resistome and mobilome

SACHIN KUMAR GUPTA

(Supervised by Professor Tatsuya Unno)

DEPARTMENT OF BIOTECHNOLOGY

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

FEBRUARY 2019

# Metagenomic analysis reveals wastewater treatment plants as hotspots of antibiotic- and biocide-metal resistome and mobilome

**SACHIN KUMAR GUPTA**

**(Supervised by Professor Tatsuya Unno)**

**A thesis submitted in partial fulfillment of the requirement**

**for the degree of Master of Science**

**February 2019**

**This thesis has been examined and approved by**

**Soo-Je Park, Ph.D., College of Natural Life Sciences, Jeju National University**

**Dukki Han, Ph.D., College of Applied Life Sciences, Jeju National University**

**Tatsuya Unno, Ph.D., College of Applied Life Sciences, Jeju National University**

**Department of Biotechnology**

**GRADUATE SCHOOL**

**JEJU NATIONAL UNIVERSITY**

# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# NOMENCLATURE

| | |
|---|---|
| ARG | Antibiotic Resistance Gene |
| BMRG | Biocide-metal Resistance Gene |
| ARB | Antibiotic resistant Bacteria |
| WWTP | Waste Water Treatment Plant |
| HGT | Horizontal Gene Transfer |
| ICE | Integrative and Conjugative Element |
| HTS | High Throughput Sequencing |
| DDD | Defined Daily Doses |
| MDR | Multi-drug resistance |
| VF | Virulence Factor |
| CARD | Comprehensive Antibiotic Resistance Database |
| BacMet | Antibacterial biocide and Metal resistance genes Database |
| DNA | Deoxyribonucleic acid |
| GJ | Gwangju |
| JS | Jangseong |
| JR | Jungrang |
| ORFs | Open Reading Frames |
| Prodigal | Prokaryotic Dynamic Programming Gene finding Algorithm |
| MLS | Macrolide-Lincosamide-Streptogramin |
| HPM/ml | Hits per million per milli-liter |
| QACs | Quaternary Ammonium Compounds |
| BLAST | Basic Local Alignment Search Tool |

# ABSTRACT

The intensive use of antibiotics due to their broad applications and incessant release into the environment has led to the widespread emergence of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs). Antibiotic resistance has turned into a genuine threat to human health. Wastewater treatment plants (WWTPs) have been identified as one of the major sources of ARGs and biocide-metal resistance genes (BMRGs), known to provide numerous environmental conditions potentially supporting the selection of ARGs and their dissemination into the environment mostly via horizontal gene transfer (HGT). Even though the distribution of antibiotic- and biocide-metal resistome (the ensemble of resistance genes) in WWTPs has been profoundly investigated, in-depth knowledge of the prevalence and abundance of mobilome (transposable elements and integrative and conjugative elements) and their association to ARGs is still lacking. Moreover, little is known regarding the pool of antibiotic- and biocide-metal resistome along with the mobilome in the chromosomal and plasmid metagenome. In this study, using Illumina high-throughput sequencing based metagenomic approach, we investigated the prevalence and abundance of antibiotic- and biocide-metal resistome and mobilome in the influent, effluent, upstream and downstream samples of three different WWTPs located at Seoul, Gwangju, and Jangseong city of South Korea. Our results showed that 21 out of 24 ARG types were detected in all the WWTPs. Moreover, BMRG abundance was found to be approximately 2 times higher than that of ARGs. Genes conferring resistance to multi-drug and rifampicin were detected high in all the samples. Additionally, our results suggested that mobile MCR-5 colistin resistance gene may be present on a plasmid in the influent sample of Gwangju WWTP. Metal resistance gene abundance was found to be higher than that of the biocide resistance gene, while genes conferring resistance to both biocides and metals were a few. The influent samples showed higher antibiotic and biocide-metal resistome abundance than those of the effluent (10 times and 5-7 times, respectively), suggesting the successful elimination of antibiotic- and biocide-metal resistome by the WWTPs. Similar trends were observed for the mobilome abundance in all the WWTPs. Nevertheless, the downstream

showed a higher abundance of antibiotic- and biocide-metal resistome and mobilome than the upstream in the two WWTPs.

On the other hand, we detected a higher abundance of integrative and conjugative elements (ICE) than that of the transposable elements in all the samples. Furthermore, a large number of mobile ARGs were associated with ICE and located on the chromosomes, while several mobilizable and conjugative plasmids were found to carry mobile ARGs along with BMRGs and virulence genes. It has been reported that plasmids carrying mobile ARGs were designated to various human pathogenic bacteria such as *Vibrio cholerae, Salmonella enterica subsp. enterica serovar Heidelberg, Klebsiella pneumoniae, Salmonella enterica subsp. enterica serovar Corvallis,* and *Pseudomonas syringae*. Therefore, regardless the abundance, those plasmids should be monitored with caution. Our results showed that the plasmids can persist the treatment processes in the WWTPs, depending on the total abundance of plasmids. On analyzing the microbial community, the phylum Proteobacteria dominated all the WWTP samples. At the genus level, there was a substantial difference between the influent and effluent samples although the downstream and upstream samples did not show any significant difference in microbial community composition. Moreover, various potentially pathogenic species belonging to genera *Pseudomonas* and *Acinetobacter* were detected in all the WWTPs.

Overall, our results suggest that the WWTPs may serve as a potential hotspot for antibiotic- and biocides-metal resistome and mobilome, although a substantial amount of resistome was successfully removed. Yet, WWTPs likely contribute to the dissemination of these genes into the surrounding environment, thus resistome near WWTPs should be monitored continuously and intensively.

# CHAPTER 1

# INTRODUCTION

Antibiotics are an important group of pharmaceuticals that are either natural or artificial compounds. While the first antibiotics were of natural origin, most of them are synthesized chemically nowadays. They belong to several classes such as aminoglycosides, beta-lactams, tetracyclines, macrolides, sulfonamides and others. Because of their diverse applications, they are used extensively in treating human, animal and plant infections, enhancement of growth rate of animals as well as prophylaxis in aquaculture. From the past few decades, the usage of antibiotics has tremendously increased across the globe. A recent report on antibiotic use, covering 76 countries between 2000 to 2015 showed that the antibiotic consumption, expressed in defined daily doses (DDD), increased 65% (21.1–34.8 billion DDDs), and the antibiotic consumption rate increased 39% (11.3–15.7 DDDs per 1,000 inhabitants per day) [1]. A similar study conducted in South Korea between 2007 to 2014 reported that DDD increased from 23.5 to 27.7 which is still much higher than the average for Organization for Economic Co-operation and Development (OECD) countries (18 DDD) as of 2012 [2].

Following administration of antibiotics, a large amount of antibiotics gets excreted out either unaltered or as active metabolites depending on pharmacokinetic and pharmacodynamic parameters in the organism [3]. Therefore, an intensive use of antibiotics in various above-mentioned fields often leads to the massive accumulation of residual antibiotics into the environment. Upon entering the ecosystems, its presence may induce the selection of antibiotic resistance genes (ARGs) and thus leading to the emergence of antibiotic-resistant bacteria (ARB), which may pose serious threats to humans and animals. The development and proliferation of resistance were seen immediately, even for the very first antibiotics developed during the 1940s, including penicillin (beta-lactam) and the clinical impact was detectable within a couple of years

[4]. These rapidly emerging resistant bacteria debilitate the phenomenal medical advantages that have been accomplished with antibiotics.

Antibiotic resistance is expanding around the globe at an alarming rate and moreover, antibiotic-resistant infections correlate with the level of antibiotic consumption [5]. This increased antibiotic resistance in pathogenic bacteria may lead to untreatable nosocomial infections due to their reduced susceptibility to antibiotics [6]. Thus, antibiotic resistance can have a potential impact on global population health, Gross Domestic Product and increased economic burden mainly due to reduced treatment options [7, 8]. Furthermore, this continued antibiotic resistance among pathogenic bacteria by the year 2050 could result into a post-antibiotic era where even trivial injuries or diseases might become difficult to cure and therefore may lead to high human mortality rates [9]. Several pathogens, such as *Acinetobacter baumannii* and multi-drug resistant (MDR) *Klebsiella pneumoniae*, are almost untreatable with the currently available antibiotics [10, 11]. Every year around 25,000 people die because of drug-resistant tuberculosis as reported by the World Health Organization (WHO) [12]. Additionally, WHO launched Global Antimicrobial Resistance Surveillance System (GLASS) in 2015 and has recently focused on antibiotic resistance as one of the most serious human health challenges and heralded the need for a global strategy to overcome ARB, and also suggested a list of critically important antimicrobials, such as carbapenems, quinolones, cephalosporins [13].

The spread of ARB and ARGs is a globally epidemiological challenge, being focused by several international institutions. Even though researches on antibiotic resistance have been concentrated on clinical uses, understanding the resistance genes and its dissemination in the environment has been considered to be essential for the management and prevention of health issues. In the environment, ARGs have been suggested to be ubiquitous and, in particular, the aquatic environment such as lakes, rivers, streams and even coastlines can act as a reservoir of ARGs and ARB. These aquatic bodies receive effluent coming from wastewater treatment plants (WWTPs) which have been suggested to play an important role in

disinfection of wastewater originating from hospitals, households, agriculture or livestock, therefore WWTPs serve as a unique interface between anthropogenic activity and both the aquatic and soil environment. The conditions in WWTPs have been reported to be favourable for the development of ARB [14]. In WWTPs, microbes are repeatedly mixed with sub-inhibitory concentrations of antibiotics during biological treatment process in WWTPs, providing an environment potentially appropriate for ARG and ARB emergence and their spread between pathogenic and enteric bacteria [15]. Not to mention that the emergence of multidrug-resistant pathogenic microbes is even more troublesome.

A WWTP usually receives several million tons of sewage every day and constantly operates a huge variety of pollutants. They are mainly aimed at reducing physical and chemical pollutants but they are not tailored specifically to remove biological contaminants such are ARGs and ARB [16]. Reports have shown that WWTPs can significantly lessen the threat of water-borne diseases [17] but antibiotics residues and ARGs in disinfected effluents can still be discharged to the environment [18]. Fortunately, recent studies have reported the advanced disinfection processes in WWTPs to reduce ARGs in effluents [19].

WWTPs role in disseminating ARGs and ARB in rivers is an active field of research [20]. The addition of ARGs and ARB in rivers by WWTP becomes more problematic, especially if it is reused for cattle, irrigation, or drinking water production. A recent study reported that higher antibiotic resistance level was observed in the river receiving effluent discharge as compared to upstream [20]. Therefore, there is a dire need for clean and safe water, free from antibiotic residues, ARGs and ARB.

With the advancement of high-throughput sequencing (HTS) technology such as 454 pyrosequencing and Illumina sequencing, analyzing metagenome obtained from complex environments such as human guts, WWTPs, fresh water, sea water, and soil has become more precise, time-saving and cost-effective. The HTS methods have an advantage over culture-dependent methods as it can be used for unculturable or yet-to-be cultured bacteria with its unprecedented sequencing depth [21]. While the culture-independent PCR-based methods do not allow enough resolution to grasp quantification of most of the genes and microbes

due to the limitation of dedicated primers [22], HTS methods offer a far more extensive comprehension about the prevalence and abundance of a wide range of genes and organisms within a complex sample.

In addition to ARGs, various studies revealed the prevalence of biocide/metal resistance genes (BMRGs) and mobilome (Integrons, transposases, insertion sequences or integrative and conjugative elements) in WWTPs [23, 24]. Scientific Committee on Emerging and Newly Identified Health Risks suggested that the stresses such as metals and antibacterial biocides have the tendency to co-select for ARB [25]. This usually takes place when biocide/metal and antibiotic share a common resistance mechanism, or if the genes for the resistance of these types are carried by a bacterium [26, 27]. Several reports have suggested that the certain concentrations of heavy metals in WWTP effluents might promote antibiotic resistance via co-selection among pathogenic and clinical bacteria [28]. Studies found that the burden of toxic heavy metals such as Zn, Hg, Pb, Cd and Cu, etc. applied a strong selection pressure as a complementary factor for ARG abundance [28, 29]. The discharge of antibiotics together with heavy metals from various sources such as agricultural runoff and animal farms into the WWTP can provide a much favourable condition for the selection of ARGs in bacteria. And the other concern regarding the heavy metals is that they can persist in the environment for periods [30]. Ahemad *et al.* reported that the samples from the agricultural soil where wastewater was re-used for irrigation purposes were a rich source of resistant bacteria to both antibiotics and heavy metals [31].

Furthermore, the mechanism of co-selection of resistant genes is highly favoured when various resistance genes are carried by the same mobilome [24]. If both biocide/metals and antibiotic resistance genes are physically located on the same plasmid, biocide/metal exposure can promote horizontal gene transfer (HGT) of antibiotic resistance [24]. Studies have suggested WWTP as hotspots for HGT, allowing even wider propagation of ARGs [32]. HGT is a process of exchanging of genetic material among bacteria at different taxonomies. This can be achieved by transformation where a cell directly uptake exogenous DNA from surroundings, transduction by bacteriophages which can transfer DNA from one bacterium to another, or

conjugation through the cell to cell contact. Plasmid-mediated conjugation is the most efficient method for HGT and therefore, the frequent presence of ARGs on plasmids leads to their dissemination. The plasmid-borne ARGs receiving bacteria can persist in the environments, facilitating their propagation [33]. In 2017, carbapenem resistance gene, NDM-9, a variant of New Delhi metallo-β-lactamases, was detected from *Klebsiella variicola* in the river of South Korea, incorporating eight antibiotic resistance genes. It indicated that NDM-9 located on the mobilome has been spreading in the environment. [34]. Studies reported that the plasmids could play a crucial role in the ARG dissemination as they could be quite persistent in the environment [35].

Of significance, bacteria may be intrinsically resistant to few antibiotics or acquire resistance through HGT (through plasmids) but antibiotic resistance can also be chromosomally acquired through mutations [36]. Moreover, all bacteria have MDR efflux pumps encoding genes located on chromosomes, few of them have been mobilized; that is, they have been deployed onto plasmids that can spread between bacteria [36]. And the question arises as for how the resistance genes located on chromosomes get transferred to the plasmids. This can be done by mobilome which includes transposable elements comprising of insertion sequences, transposases and integrons that promote intracellular DNA mobility i.e. between plasmids or from chromosome to a plasmid as shown in Figure 1. The insertion sequences and transposases facilitate the movement, assembly and rearrangement of associated resistance genes haphazardly to new locations in the same or different DNA segment within a cell. While an integron is a genetic element which uses site-specific recombination to move antibiotic resistance genes (gene cassettes) between defined sites with a cell. These type of mobilome are often available in multiple copies in various locations in a genome and can facilitate homologous recombination. Therefore, resistance genes located on chromosomes along with the mobilome, can be easily transmitted to plasmids and further to other bacterial cells depending on the mobility or conjugation ability of plasmids. And an evidence from a study reveals that plasmids carrying mobile ARGs (ARGs associated with mobilome) are frequently mobilizable [37]. This arises a stronger

need to check whether a plasmid is mobile or can conjugate with other bacteria because the evidence provided by studies revealed that plasmid mobility plays a crucial role in the evolution and dissemination of ARGs in bacteria found in the various environment [38]. And moreover, the highest degree of risk arises if the plasmids carrying mobile ARGs can be transmitted to other bacteria due to their conjugation ability, thereby introducing ARGs to clinically important bacteria.

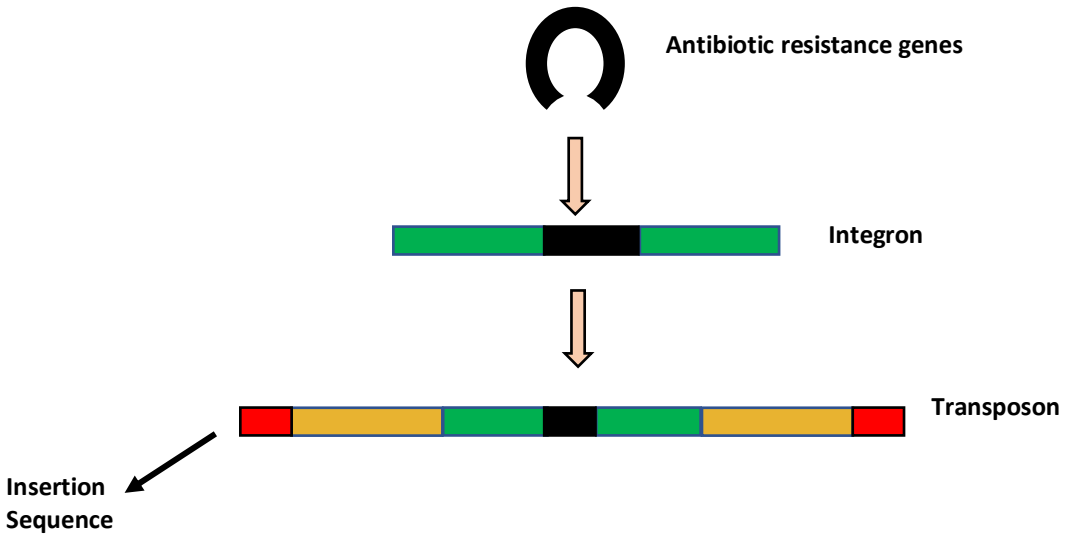


**Figure 1: A schematic of antibiotic resistance gene transfer with integrons and transposons.**

Some other evidence proves that there are widespread specific mobile DNA elements which are integrated into chromosomes and can get transferred independently and are neither plasmid nor phage [39]. One of these is integrative and conjugative elements (ICE) which can transmit both horizontally and vertically as they can remain in two states, either integrated to chromosomes or in the conjugative state [40]. These elements contain genes for integration, excision, regulation and conjugation and in addition, the cargo genes of ICEs can code for the antibiotic resistance determinants and virulence factor (VF) thus acting as a vital driving force for transfer of ARGs located on chromosomes directly to between bacterial cells [40] as shown in Figure 2. Furthermore, interactions between different sorts of mobilome support the quick development of assorted MDR pathogens in the face of antimicrobial chemotherapy [41]. The emergence of MDR pathogens is even more problematic. Due to increasing antibiotic intervention, the problem of antibiotic resistance is becoming more challenging, hence it might be possible that there be no defence against infections in the future [42]. Although various independent studies have been performed to investigate the distribution and abundance of antibiotic- and biocide-metal resistome and mobilome (ICE and transposable elements), still a more combined comprehensive study is required to provide a fundamental understanding in this field. In the present study, we investigated the prevalence and abundance of resistome i.e. ARGs and BMRGs and mobilome comprising of transposable elements and integrative conjugative elements in the three WWTPs located at the Gwangju, Jangseong, and Seoul city of South Korea. Based on the sequence annotation, we analyzed the presence of mobile ARGs, estimated the mobility or conjugation ability, and pathogenicity of plasmids as they pose the highest risk to human beings. The persistence of plasmids in WWTPs samples was also investigated to see whether plasmids in downstream were originated from WWTP. Lastly, we investigated the microbial community composition to investigate if WWTPs selectively discharge specific bacteria. Our study should provide a comprehensive understanding of the prevalence and abundance of antibiotic- and biocide-metal resistome and mobilome in WWTP.

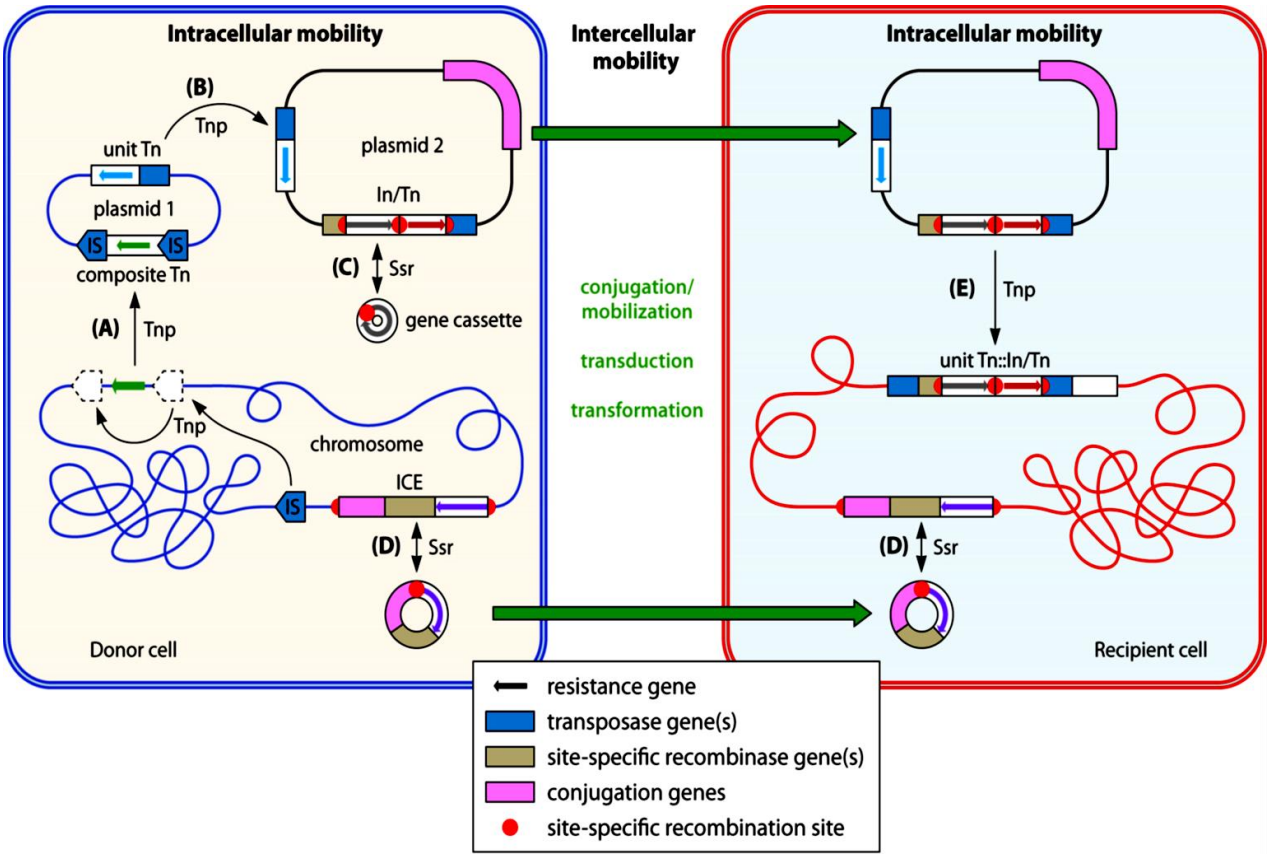


**Figure 2: Examples of mobilome and processes involved in intracellular mobility or intercellular transfer of antibiotic resistance genes.**

Two cells of different strains or species are represented, with one acting as a donor (envelope and chromosome shown in blue; contains two plasmids) and the other as the recipient (shown in red). Various mobilomes are shown, with the functions of the genes they carry colour-coded as shown in the key. Different resistance genes associated with different mobile are represented by small arrows of various colours. Thin black arrows indicate intracellular processes, with those mediated by a transposase protein, labelled Tnp and those mediated by a site-specific recombinase protein labelled Ssr. Thick green arrows represent intercellular (horizontal) transfer. Successive insertions of the same IS on both sides of a resistance gene may allow it to be captured and moved to another DNA molecule (e.g., from the chromosome to a plasmid) as part of a composite Tn (A). A unit Tn carrying a resistance gene may transpose between plasmids (B) or from a plasmid to the chromosome or vice versa. A gene cassette may move between In (a class 1 In/Tn structure is represented here) via a circular intermediate (C). An ICE can be integrated into the chromosome or excised as a circular element that can then conjugate into a recipient cell and integrate (reversibly) into the chromosome at a specific recombination site (D). A plasmid may be able to mediate its own intercellular transfer by conjugation or, if it lacks a conjugation region, be mobilized by another plasmid (or, alternatively, move horizontally by phage transduction or transformation). Tn and/or In and associated resistance genes on an incoming plasmid may move into the chromosome or other plasmid(s) in the recipient cell (E), as illustrated here for class 1 In/Tn, which are known to target unit Tn. (Source: Partridge, S. R., Kwong, S. M., Firth, N., & Jensen, S. O. (2018). Mobile genetic elements associated with antimicrobial resistance. *Clinical Microbiology Reviews*)

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Site description

Water samples (i.e. influent, effluent, upstream and downstream) were obtained from the three WWTPs located at the Gwangju, Seoul, and Jangseong city of South Korea (Figure 3). The Gwangju (GJ) WWTP (35.091126° N, 126.79789° E) collects wastes from a population coverage of 198,991 people and 306 hospitals/pharmacies while the Jangseong (JS) WWTP (35.279124° N, 126.761090° E) gets influent from a population coverage of 17,808 people and 45 hospitals/pharmacies. On the other hand, the Jungrang (JR) WWTP (37.560307° N, 127.062753° E) located at the Seoul city receives wastewater from 2,808,966 people and 6,361 hospitals/pharmacies. The water samples collected from all the four sites included 4 litres wastewater from the influent, 20 litres from the effluent and 6 litres each from upstream and downstream. The water samples were collected in sterile 4-litre packs and placed at 4 °C for transport to the laboratory within 6 hours.

## 2.2 DNA extraction and sequencing

Prior to extraction, the water samples were vacuum filtered through 10 µm pore-size membranes and further through 0.22 µm pore-size membranes (ADVANTEC). DNA was extracted using the PowerWater DNA Isolation Kit (MOBIO Laboratories) according to the manufacturer's instruction. Spectrophotometer (NanoDrop ND-200, BIOAND) was used to determine the concentration of DNA isolated from the samples. The DNA samples for all the WWTPs were sent to Macrogen Inc. (South Korea) for high-throughput sequencing using Illumina Hiseq 4000 platform (2 × 150 bp). Paired-end high-throughput sequencing of all the metagenomic DNA samples was performed using TruSeq Nano DNA kit.

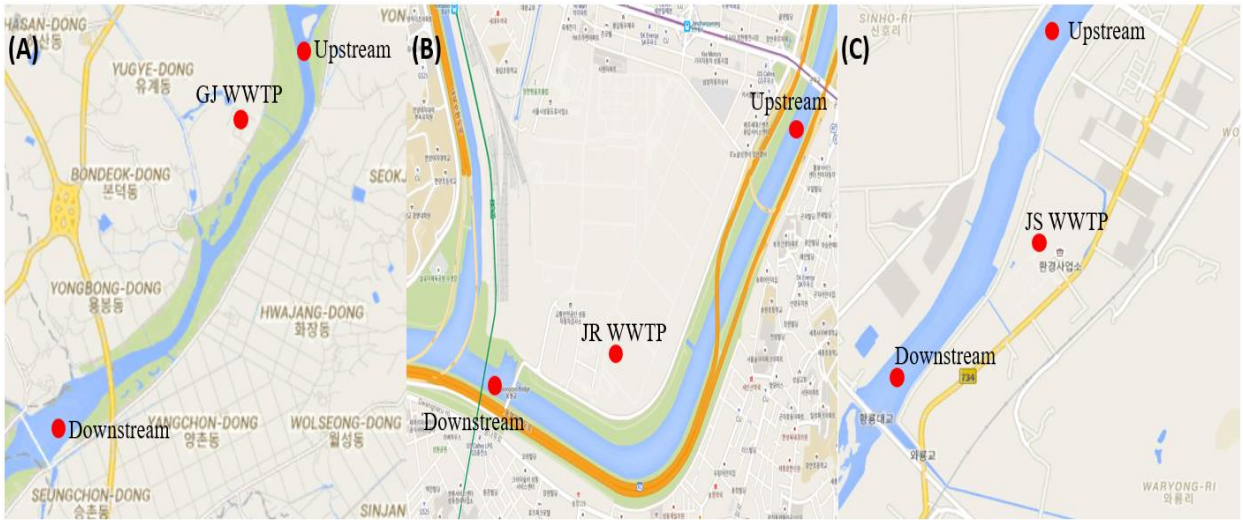


**Figure 3: Location of water sampling sites belonging to Gwangju (GJ) WWTP (A), Jungrang (JR) WWTP (B), and JangSeong (JS) WWTP (C).**

### 2.3 Sequencing data analysis

The metagenomic datasets received from Macrogen Inc. were used to remove the duplicate reads by using BBmap tool [43]. Further, the reads were quality trimmed and filtered before the downstream processing. This was performed by FastQ Quality Control Software (FaQCs) [44] with the following parameters: (1) removal of reads with 3 or more ambiguous nucleotide 'N': (2) trimming the reads containing Illumina adapters/primers: (3) average quality cutoff for filtering the reads was set at 30: (4) removal of trimmed reads shorter than 50 bp: (5) cutoff 0.85 as maximum fraction of mono-/di-nucleotide sequence. The number of clean reads in all the samples are shown in Table 1 and were used for further analysis.

After quality filtering, the recovered clean reads can either be passed directly for further annotations or can be assembled. Therefore, both unassembled reads or assembled contigs can be utilized for the functional annotation depending on the research question. However, due to the large data size of short reads and they may lack resolution for functional annotations, the usage of assembled contigs is more suitable [45]. Assembly is the computational process of connecting reads to retrieve long contiguous contigs. MEGAHIT, a *de novo* assembler was used to assemble the clean reads into contigs with default parameters [46]. Contigs with a minimum size of at least 200 bp were used for further analysis.

**Table 1: Amount of DNA and number of metagenomic sequences and number of assembled contigs used in this study**

| WWTP | Site | DNA amount used for HiSeq (ug) | Number of raw reads | Duplicate removal | Number of Clean Reads | Number of contigs | Number of plasmids contigs | Number of chromosomes contigs | Number of unclassified contigs |
|---|---|---|---|---|---|---|---|---|---|
| GJ | Influent | 0.06 | 137,941,884 | 119,149,634 | 118,638,902 | 1,261,290 | 53,664 | 76,335 | 84,806 |
| | Effluent | 1.49 | 118,122,276 | 114,472,260 | 111,391,512 | 905,013 | 52,391 | 65,359 | 79,815 |
| | Upstream | 0.96 | 121,745,268 | 117,629,360 | 113,224,810 | 1,533,570 | 57,098 | 81,686 | 102,190 |
| | Downstream | 0.42 | 128,111,680 | 123,247,504 | 118,907,452 | 1,052,633 | 41,387 | 67,448 | 75,744 |
| JS | Influent | 3.34 | 125,602,606 | 121,677,604 | 116,745,852 | 848,469 | 35,688 | 50,768 | 56,414 |
| | Effluent | 0.29 | 136,941,826 | 133,718,384 | 131,519,676 | 1,089,338 | 60,976 | 68,402 | 93,329 |
| | Upstream | 0.85 | 115,040,328 | 110,969,652 | 107,087,648 | 1,302,657 | 57,263 | 61,811 | 88,207 |
| | Downstream | 3.45 | 144,536,146 | 138,983,846 | 133,771,888 | 1,616,951 | 65,433 | 88,335 | 110,201 |
| JR | Influent | 0.70 | 93,774,628 | 88,123,904 | 86,075,316 | 1,095,598 | 52,474 | 71,409 | 83,115 |
| | Effluent | 1.01 | 96,094,846 | 91,179,932 | 88,861,820 | 697,850 | 35,490 | 44,102 | 51,425 |
| | Upstream | 0.84 | 173,994,668 | 164,391,344 | 161,018,870 | 1,744,132 | 85,213 | 80,421 | 115,074 |
| | Downstream | 0.82 | 155,217,456 | 146,963,212 | 143,472,124 | 1,576,972 | 94,783 | 90,121 | 124,803 |

Open reading frames (ORFs) were predicted from contigs using Prodigal with the option "-p meta" for metagenomic samples and -c option for closed ends of ORFs [47]. Both, nucleotide and translated ORFs were predicted from contigs. Regarding ARGs annotation, ARG sequences (2241) (amino acid sequences) were downloaded from Comprehensive Antibiotic Resistance Database (CARD) (version 2.0.3) and categorized into 24 types such as aminocoumarin, aminoglycoside, sulfonamide, multi-drug , etc. [48]. For BMRGs identification, a local database of BMRGs (amino acid sequences) was created by downloading experimentally confirmed biocide/metal resistance gene sequences (753) from BacMet database (version 2.0) [49]. These genes were further classified into three types – biocide resistance genes, metal resistance genes and biocide/metal resistance genes (genes conferring resistance to both biocide and metal).

For determining mobilome, sequences from a recently developed Mobile Genetic Elements database were downloaded from https://github.com/KatariinaParnanen/MobileGeneticElementDatabase [50]. The nucleotide sequences of the genes encoding transposable elements (transposases, insertion sequences and integrons) were used for making the local database. Another database, namely ICEberg for ICE annotation was used to download all the experimentally confirmed ICEs with intact sequences which were 7493 amino acid sequences (version 2.0) [51]. All the translated ORFs predicted from the contigs in this study were aligned to CARD, BacMet, and ICEberg database using local BLAST (BLASTp) with an E value threshold of $1e^{-10}$, bit score > 50, and sequence similarity cutoff > 70% [22]. While the nucleotide ORFs retrieved from the contigs were used to blast (BLASTn) against Mobile Genetic Elements database with an E value cutoff $1e^{-10}$, bit score > 50, and a cutoff threshold greater than 70% sequence identity [52] to identify transposable elements in our metagenomic datasets.

Plasmid-mediated dissemination of ARGs is broadly perceived to occur in numerous ecological compartments yet stays difficult to study because of the absence of exhaustive tools and the complexity of the environmental matrices. Moreover, the high-throughput sequencing provides substantially more information in a more rapid manner at a lower cost compared to the traditional methods and makes it

feasible to survey the plasmid metagenomes with culture-independent approaches [53]. Therefore, the contigs belonging to the whole metagenome samples were classified into plasmid- and chromosome-like contigs using recently developed PlasFlow tool (version 1.1) which uses a neural network approach to recover plasmids sequences from assembled metagenomes [54]. As PlasFlow recommends using contigs greater than 1000 bp in length thus these contigs (>1000 bp) were retrieved from whole metagenome samples using a python script and were then used for the prediction of chromosome and plasmids with default parameters. Similarly, nucleotide and translated ORFs were predicted using Prodigal for the chromosome and plasmid-like contigs and were aligned against CARD, BacMet, Mobile Genetic Elements, and ICE database using the same BLAST parameters as discussed previously. As ICEs are present either on chromosomes or independently within a cell, therefore, we considered whole metagenome and chromosomes for their presence.

Additionally, we checked the prevalence of mobile ARGs (ARGs associated with transposable elements or ICE) in our metagenomic datasets. To evaluate mobile ARGs, we manually checked the contigs where the antibiotic-resistant ORFs and the transposable element or ICE ORFs were detected concurrently on the same contig [55]. Moreover, estimation of mobility or conjugation ability of plasmids was also performed for the plasmids carrying mobile ARGs using a recently developed MOB-suite tool having a MOB-typer method which can predict the plasmids as conjugative, mobilizable or non-mobilizable [56]. As the plasmid-contigs predicted from the whole metagenome using PlasFlow were usually not full-length plasmids but merely a fragment of large-sized plasmids, therefore, we aligned the plasmid-contigs against NCBI RefSeq plasmids database (ftp://ftp.ncbi.nlm.nih.gov/genomes/refseq/plasmid/) using BLAST (BLASTn) with an E value cutoff $1e^{-10}$, bit score > 50, and a strict cutoff threshold greater than 80% sequence identity. The retrieved full-length plasmids from RefSeq plasmids database were used by MOB-suite. Moreover, we checked for the presence of VFs on the plasmids carrying mobile ARGs as bacteria can induce various diseases by the expression of different combinations of VFs which is often mediated by the plasmids. For investigating the VFs, amino acid sequences were downloaded from the PATRIC

database which has curated sequences from Virulence Factor and Victor database [57, 58, 59]. Translated ORFs predicted from the plasmid contigs were aligned using BLAST (BLASTp) with E-value < $1e^{-10}$ and 60% coverage and similarity [60]. Additionally, to check the taxonomic profile of the plasmid contigs carrying mobile ARGs we used Kaiju, which can also classify contigs based on the alignment against the NCBI BLAST nr database and uses the Burrows-Wheeler transform (BWT) of the protein database, which enables exact string matching [61].

A recently published study reported that lessening antibiotic usage alone is likely inadequate for reversing resistance as many resistance genes can persist for long periods in the absence of antibiotics and showed that common conjugal plasmids can persist by transferring at higher rates of HGT [62]. Therefore, we further checked the persistence of plasmids in WWTPs. For investigating the persistent plasmids, we aligned the plasmid contigs belonging to all sites in a WWTP using psi-cd-hit which works similar to the CD-HIT tool but it uses BLASTn to cluster the DNA sequences [63, 64]. The clustering cutoff used was 90% nucleotide identity and alignment coverage along with global sequence identity parameter. A plasmid-contig present both in effluent/influent and downstream and absent in upstream was counted as a persistent plasmid. Furthermore, the abundance of microbial composition and taxonomic profiling for the whole metagenome was investigated using Metagenomic Phylogenetic Analysis (MetaPhlan; version 2.0) [65] which detects bacterial composition based on unique clade-specific marker genes identified from reference genomes.

### 2.4 Statistical analysis

To determine the abundance of all assembled contigs, metagenomic reads were mapped to assembled contigs using Burrows-Wheeler transform (BWA) with default cutoff parameters [66]. The abundance was normalized based on the length of the gene (kb) per one million reads along with the amount of water sampled from each site. The abundance (Hits per million per ml) of each contig was computed using the following formula –

$$\boldsymbol{Abundance} = \left(\frac{\boldsymbol{Number\ of\ reads\ mapped\ to\ contig}}{\boldsymbol{Total\ number\ of\ reads}} \times \frac{\mathbf{1}}{\boldsymbol{Length\ of\ contig(kb)}} \times \mathbf{1,000,000}\right) / \boldsymbol{Amount\ of\ water\ sampled\ (ml)}$$

All the graphs and line plots were prepared by using Origin 9.0 while the heatmap was prepared by using R packages such as ggplot, ggplot2 and Rcolorbrewer.

# CHAPTER 3

# RESULTS AND DISCUSSION

## 3.1 Prevalence and abundance of the antibiotic- and biocide-metal resistome in WWTPs

### 3.1.1 Antibiotic- and biocide-metal resistome analysis in the whole metagenomes

To investigate the profile of ARGs and BMRGs in the collected metagenomic samples, we conducted BLAST analysis of the metagenomic datasets against the CARD and BacMet database, respectively.

The analysis clearly revealed the prevalence of ARGs in the WWTPs, as 21 out of 24 antibiotic resistance genes types were detected in all the three WWTPs. Furthermore, genes conferring resistance to multi-drugs were most abundant in the influent, effluent, upstream, and downstream samples followed by other prominent resistance genes belonging to rifampicins, MLS (Macrolide-Lincosamide-Streptogramin), aminocoumarins, peptides and aminoglycosides as shown in Figure 4,5,6.

In the influent samples, several types of ARGs with varied abundance were detected, although the genes conferring resistance to multi-drug, peptide, MLS, rifampicin, and aminoglycosides were found to be most abundant in all the three WWTP influents. The reason for detecting various kinds of ARGs may rely upon the collection of wastes from the different sources (domestic, industries, hospitals, etc.) to the WWTP influent which is also supported by previous studies as they also detected a diverse range of ARGs in the WWTP influent [67, 68]. Interestingly, all the other water samples (effluent, upstream and downstream) from three WWTPs showed a similar pattern with respect to the top two most abundant ARGs detected in these samples - multi-drug and rifampicin resistance genes, although the downstream of GJ WWTP was an exception to this. These two most abundant ARGs accounted for approximately 56 % (average) of the total ARG abundance in these samples. The abundance of MDR genes was due to the efflux pumps and moreover, these pumps are mostly encoded by the chromosomes. Our results seem to be consistent with the previous studies which showed a significant increase in the genes conferring resistance to rifampicin and multi-drug

in a diverse environmental samples (aquaculture farm and river sediments, activated sludge, biofilm, anaerobic digestion sludge, and river water) [22, 69]. Moreover, a recent finding showed that the aminocoumarin and rifampicin antibiotics can be used for plasmid curing (the process by which plasmids are removed from the bacterial population) and may be useful in reducing plasmid-mediated ARG dissemination [70]. Although, the high abundance of rifampicin and aminocoumarin resistance genes may prove to cause hindrance in plasmid curing and thus limiting the use of these compounds in plasmid curing processes [70].

The total abundance (HPM/ml) of the antibiotic resistome in the influent samples was found to be much higher as compared to the effluent samples in all the three WWTPs. Moreover, the trend of decreasing ARGs abundance from the effluent to influent showed a 10-fold decline in ARGs abundance in all WWTPs. This clearly suggests that the WWTPs were able to eliminate large amounts of dissolved ARGs during the treatment process. Nevertheless, the total abundance of ARGs in downstream samples was found to be elevated as compared to the upstream samples. On comparing the WWTPs, it was clearly observed that the samples from JS WWTP carried a higher abundance of antibiotic resistome than the other WWTP samples (Figure 5). In the case of GJ WWTP, the total abundance of antibiotic resistome in the downstream depicted similar abundance with that of influent and moreover it showed a higher abundance as compared to upstream (Figure 4). While the downstream of JR and JS WWTPs depicted an approximately similar abundance of antibiotic resistome as compared to the upstream (Figure 5 and 6).

Furthermore, the higher abundance of antibiotic resistome in the downstream of GJ and JS WWTPs than that of the upstream samples may delineate the possible role of WWTPs effluent in upraising the antibiotic resistome in downstream either through the introduction of genes or by altering the chemical environment as supported by a past study [22]. Moreover, a recent study conducted in the Netherlands reported that the WWTP effluent significantly increased the prevalence of ARGs in the receiving river as compared to the upstream [20]. Although, a more comprehensive statistical study is required to reach to this conclusion

along with the analysis of sediment samples as the distribution of antibiotic resistome in sediments may provide a better insight into the role of WWTP effluents on the downstream rivers.

We also checked the resistance genes at the subtype level (i.e., gene names) as shown in Figure 7 and found that the *rpob2* (rifampicin resistance), *novA* (aminocoumarin resistance), *msbA, mexK* (MDR) genes were the most abundant resistant genes in all the samples. The high abundance of *rpob2* and *novA* resistance genes in all the samples is also supported by a previous study which revealed the high abundance of these genes in a diverse sample [69].

Additionally, we investigated the abundance of BMRGs in all the samples and the analysis showed that the metal resistance genes were more significant in all the three WWTPs followed by biocide resistance genes while genes conferring resistance to both biocide and metal resistance genes were least abundant as shown in Figure 4, 5 and 6.

Furthermore, we found that the BMRGs were more abundant as compared to the ARGs and their total abundance was approximately 2 times higher than the total ARGs abundance. Like the observations in the case of ARGs, the BMRGs too decreased from influent to effluent (5-10 folds) while a further increase in their abundance was seen in the downstream samples as compared to the upstream samples. Moreover, the distribution pattern of ARG and BMRG abundance in all the samples was much alike. The possible reason could be the co-resistance mechanism (both ARGs and BMRGs co-located together in the same cell) which has been previously reported by several studies [35, 71]. A previous study revealed the significant correlation between ARG and metal resistance gene composition [72]. As several studies revealed the potential of metal and biocide resistance genes in the spread of ARGs, therefore, detecting higher abundance antibiotic- and biocide-metal resistome in our study may suggest the risk that the ARGs and BMRGs selected in the treatment processes may further get disseminated into the aquatic or soil environments [27, 73, 74]. Therefore, this may express the need to pay more attention to study their co-occurrence distribution and further dissemination in WWTPs.

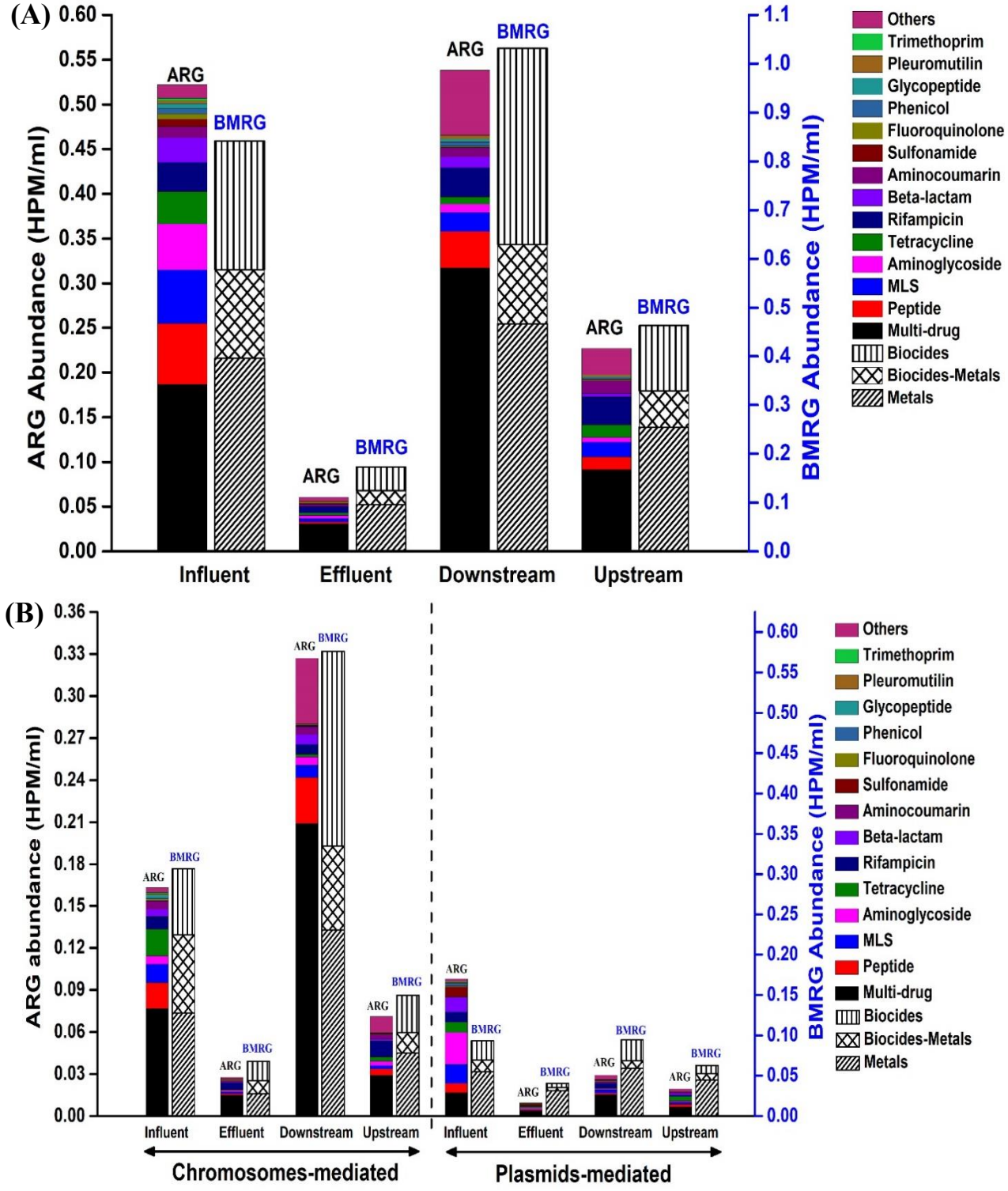


**Figure 4: Relative abundance of antibiotic- and biocide-metal resistome in the whole metagenome (A) and chromosomal and plasmid metagenome (B) of GJ WWTP**

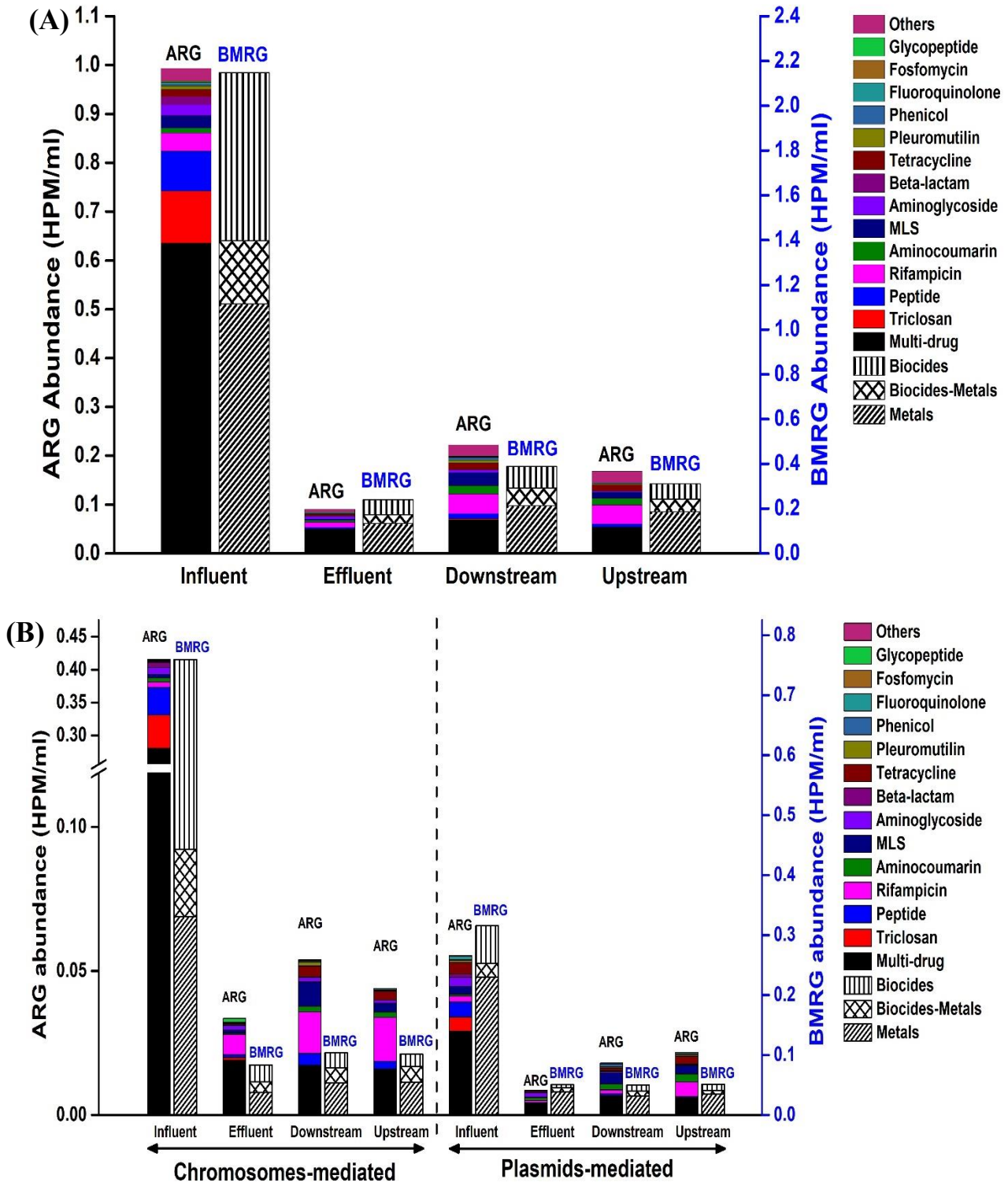


**Figure 5: Relative abundance of antibiotic- and biocide-metal resistome in the whole metagenome (A) and chromosomal and plasmid metagenome (B) of JS WWTP**

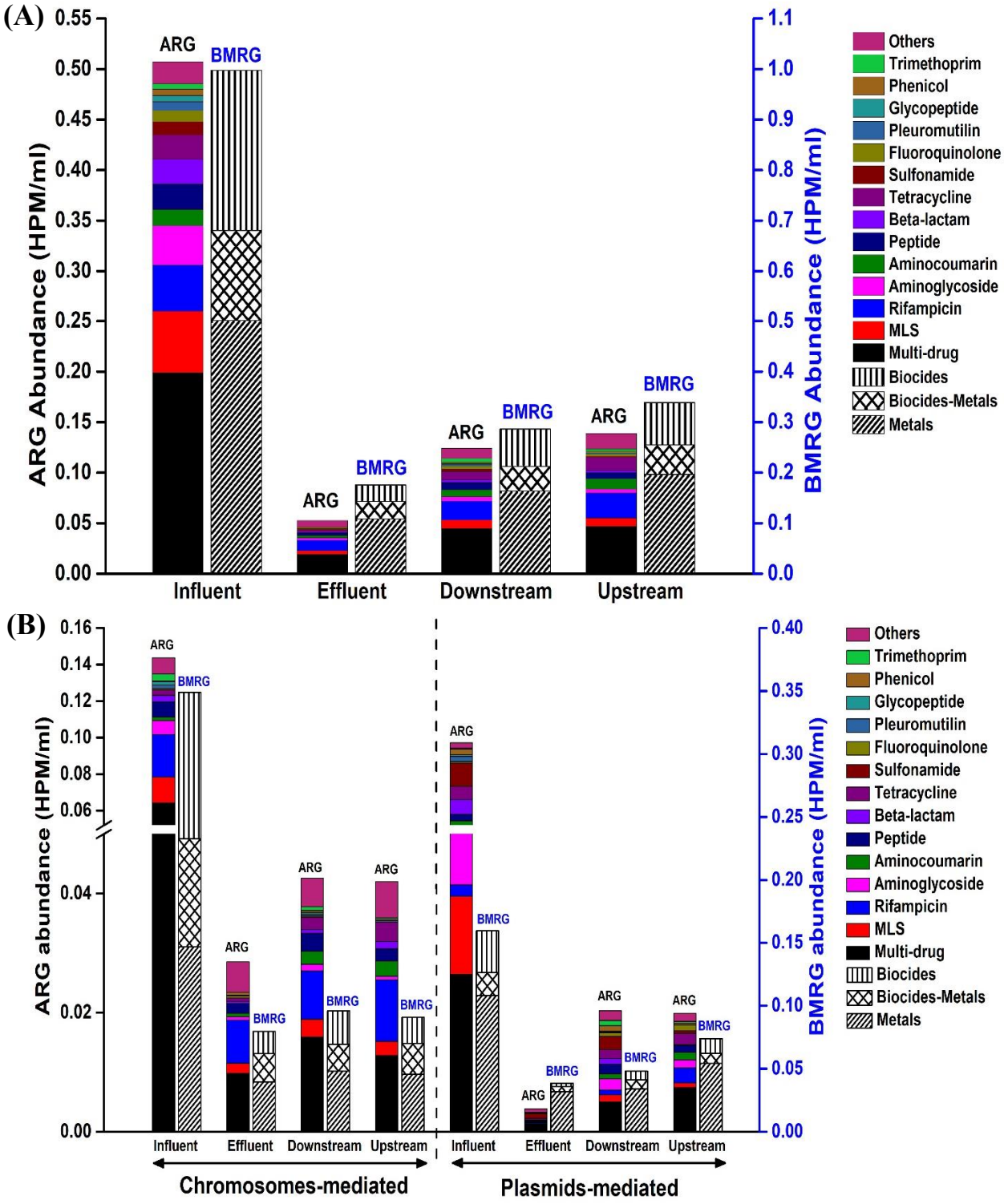


**Figure 6: Relative abundance of antibiotic- and biocide-metal resistome in the whole metagenome (A) and chromosomal and plasmid metagenome (B) of JR WWTP**

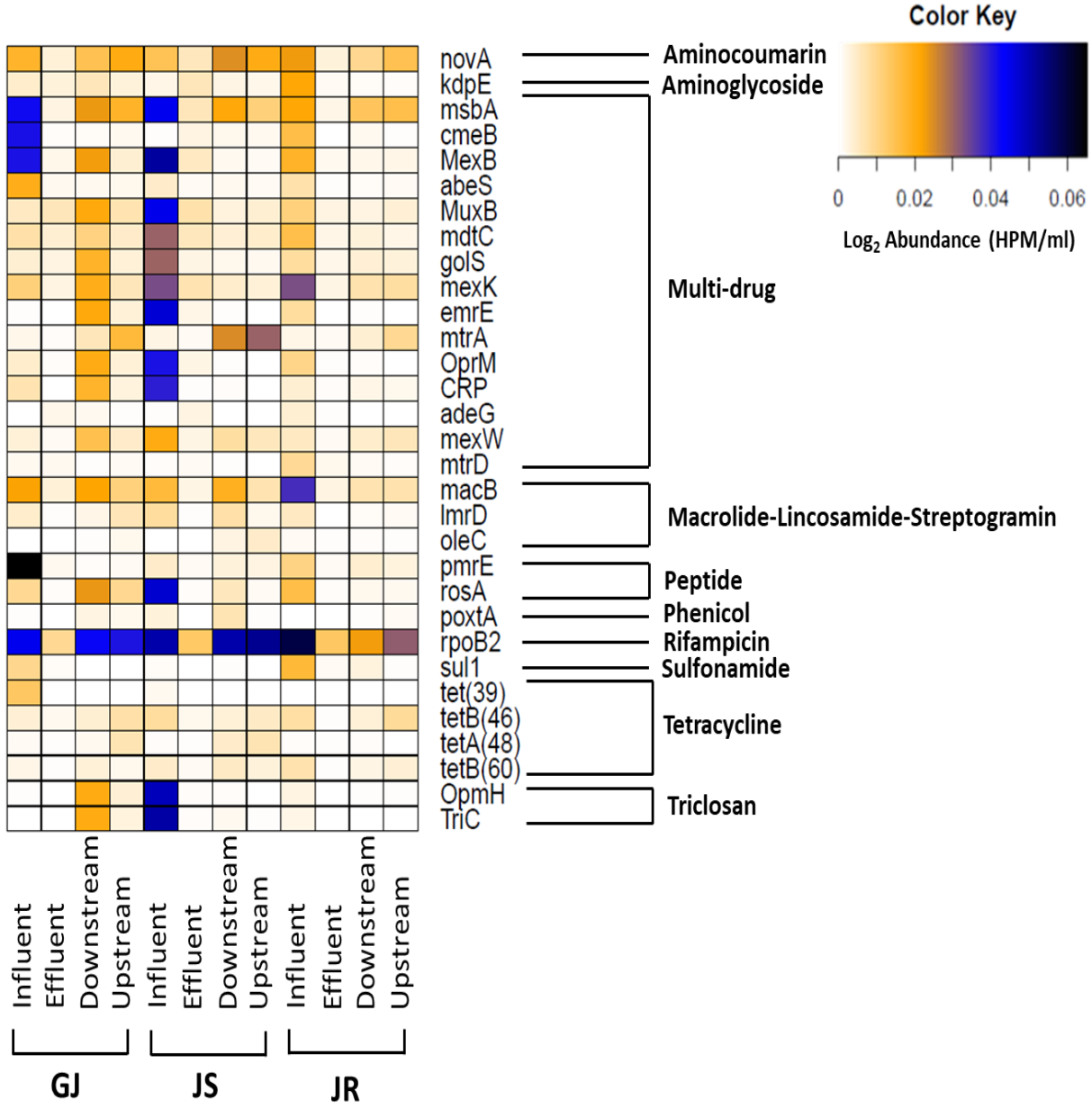


**Figure 7: Relative abundance of top 10 antibiotic resistance gene subtypes detected in the whole metagenome at each WWTP**

### 3.1.2 Antibiotic- and biocide-metal resistome analysis in the chromosomal and plasmid metagenomes

To investigate the distribution of antibiotic- and biocide-metal resistome in the chromosomal and plasmid metagenome, we used recently developed tool PlasFlow to predict plasmid and chromosome contigs from the assembled contigs in the metagenomic samples. Because of the selected default cut-off (0.7), many contigs remained unclassified while the others were classified either chromosome or plasmid depending on the genomic signatures as shown in Table 1. ORFs predicted from chromosome and plasmid-contigs using Prodigal were aligned against the CARD and BacMet databases for detecting ARGs and BMRGs, respectively. Surprisingly, we observed that the abundance of ARGs was higher on chromosomes as compared to the plasmids although the plasmids are known to encode more ARGs. One of the possible reasons for this may be the greater number of chromosome-contigs retrieved as compared to plasmids-contigs by PlasFlow as shown in Table 1 and this can be supported by the fact that the total DNA was extracted from all the samples and no plasmid-safe DNA kit was used so probably the metagenome DNA carried more chromosomes. Furthermore, a similar pattern in the abundance of antibiotic- and biocide-metal resistome was observed in the chromosomal and plasmid metagenomes to that of whole metagenome samples with influent having more antibiotic- and biocide-metal resistome abundance and decreased abundance in effluent and additionally higher abundance of antibiotic- and biocide-metal resistome in the downstream than that of upstream in all the WWTPs. Moreover, the distribution of BMRGs and ARGs total abundance in chromosomes and plasmids was also seen to follow the same trend as previously discussed with BMRGs having a higher abundance (approximately 2 times) as compared to the total abundance of ARGs. The likely explanation could be the bifurcation of the whole metagenome antibiotic- and biocide-metal resistome into chromosomes and plasmids carrying resistance genes.

On further analysis, we observed that the abundance of plasmids carrying resistance genes in GJ WWTP showed the different pattern as compared to whole metagenome and chromosomes carrying resistance genes. We found that the plasmids carrying resistance genes were more abundant in influent as compared

to the effluent and downstream although chromosomes (similar to the whole metagenome) showed a higher proportion of antibiotic- and biocide-metal resistome abundance in the downstream of GJ WWTP. Additionally, we observed that genes conferring resistance to Streptothricin antibiotic were only detected on chromosomes and were totally absent on plasmids. Although Streptothricin resistance genes (SAT-3 and SAT-4) are usually encoded on plasmids, their absence in our study may be due to the limitation of detecting ARGs with negligible abundance or absence of plasmids carrying these genes in our metagenome samples. Notably, BMRGs were highly abundant on the chromosomes which is supported by a previous study revealing chromosomes may carry more BMRGs as compared to the plasmids [24]. A previous study revealed a strong correlation between ARGs diversity and plasmids diversity [69]. Studies revealed that the occurrence of ARGs and BMRGs on the plasmids pose a more serious risk of disseminating these resistance genes into a different environment with the help of HGT especially through conjugation [35]. Therefore, we further conducted a more comprehensive analysis of plasmids carrying ARGs in our study.

## 3.2 Prevalence and abundance of the mobilome (transposable elements and integrative conjugative elements) in WWTPs

### 3.2.1 Mobilome analysis in the whole metagenomes

The mobility of ARGs depends on whether these genes are either located on the plasmids or carried by another mobilome, such as transposable elements (integrons, transposases, insertion sequences) and the integrative and conjugative elements. In this study, we analyzed the metagenomic samples to investigate the prevalence and abundance of these mobilomes. We used Mobile Genetic Elements and ICEberg database for the detection of transposable elements and ICEs, respectively. The analysis results clearly showed the prevalence and abundance of mobilome in WWTPs as shown in Figure 8. In the case of transposable elements, genes encoding transposases were the most abundant in all the samples followed by insertion sequences and integrons. On an average, approximately 65% of total abundance of transposable elements was contributed by the transposases. On further examining at the gene level, we found that the

three genes namely *tnpA* (transposase), *IS91* and *istA* (insertion sequence) were the most abundant genes encoding transposable elements. The high abundance of *tnpA* has been previously reported by a study on landfills in China [75] and samples of urban park soil with reclaimed water irrigation [76]. A previous study suggested a significant positive correlation between transposases and ARGs indicating the importance of transposase in the propagation of ARGs [76]. Furthermore, genes encoding ICE were found to be in much higher abundance than the transposable elements in all the samples. Previous studies also revealed that the ICEs were the most abundant conjugative elements in almost all the prokaryotes and therefore they can even outnumber the plasmids present in a cell [77]. Although limited studies have been performed on the occurrence of ICE in the WWTPs whereas various reports have suggested the high abundance of transposable elements in the WWTPs [78, 79]. The high abundance of ICE in our samples may require further supervision and more understanding of their dissemination in the WWTPs. Collectively, the distribution of ICE and transposable elements was found to be similar in the metagenome samples of all three WWTPs. Moreover, the pattern of total abundance of mobilome was much alike to that of antibiotic- and biocide-metal resistome abundance (previously discussed) in all the samples as influent showed the higher abundance of the mobilome with a much-decreased abundance in effluent samples, thereby suggesting that the WWTPs were also able to successfully get rid of a large amount of mobilome. Additionally, the downstream samples too exhibited a similar trend as previously discussed with an elevated abundance of mobilome as compared to the upstream in all the WWTPs. A culture-independent shotgun metagenomics study performed by Kristiansson *et al.* showed a much higher levels of transposases and integrons (class 1) in the downstream sediments as compared to the upstream samples [80]. Thus, similar results from our study may suggest that the mobilome may get added up by the effluent of WWTPs to the receiving downstream.

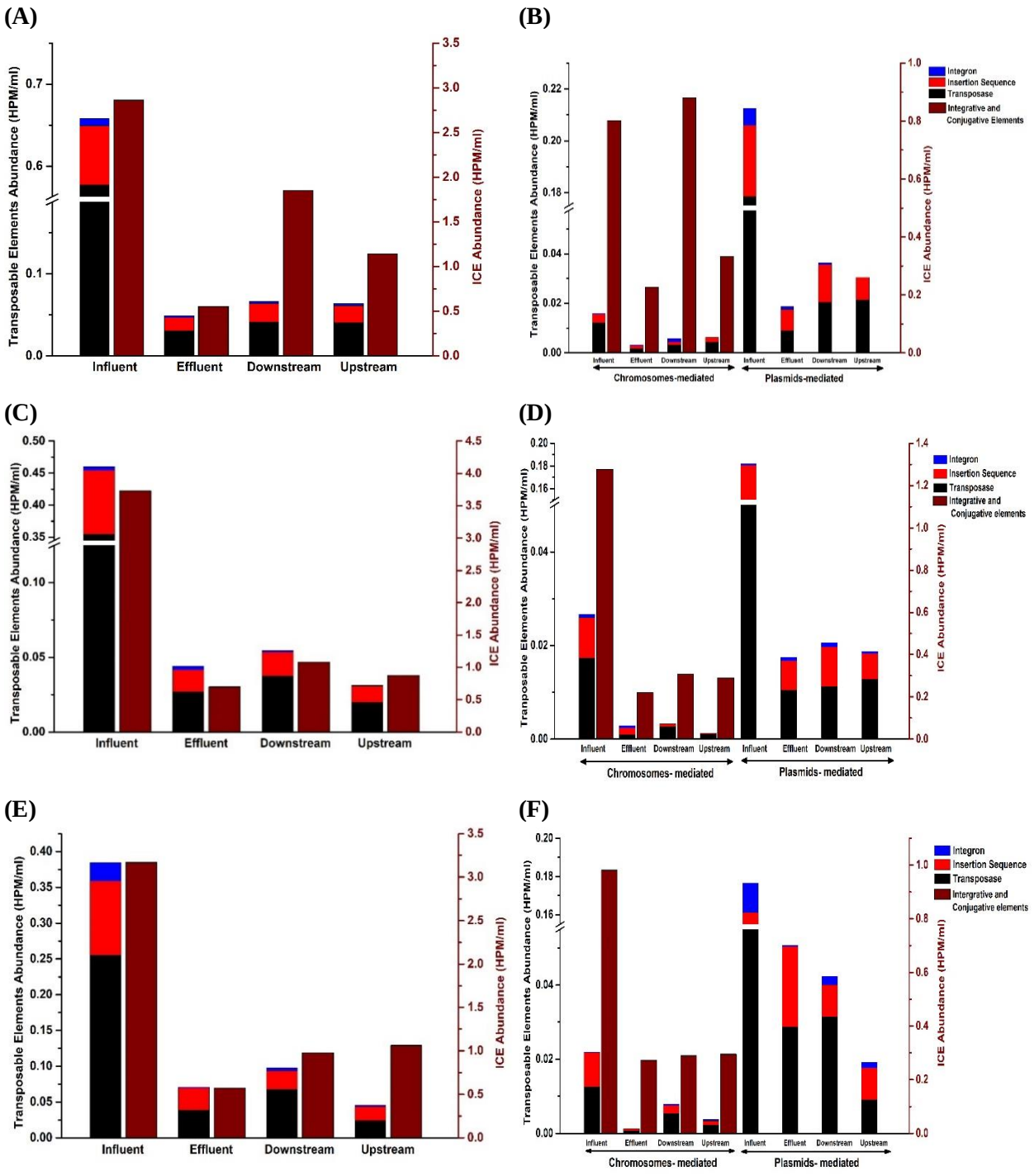


**Figure 8: Relative abundance of mobilome (transposases, integrons, insertional sequences and integrative and conjugative elements) in WWTPs.**

(A), (C) and (E) shows the relative abundance of mobilome in whole metagenome samples of GJ, JS and JR WWTP, respectively. (B), (D) and (F) shows the relative abundance of mobilome in chromosomal and plasmid metagenome of GJ, JS, and JR WWTP, respectively.

### 3.2.2 Mobilome analysis in the chromosomal and plasmid metagenomes

As the transposable elements can be located either on the chromosomes or plasmids, therefore we further checked the distribution and abundance of the mobilome on them. As seen in the case of the whole metagenome, we detected a high abundance of genes encoding transposases followed by insertion sequences and integrons on the chromosomes and plasmids. Further, we observed a higher abundance of transposable elements on the plasmids (average of 7.5 times) as compared to the chromosomes and in the case of the effluent of JR WWTP, the abundance of plasmids carrying transposable elements was 40 times higher than the abundance of transposable elements on chromosomes. Moreover, various insertion sequences and integrons (*int1*, *int2* and *int3*) were also detected on the chromosomes and plasmids. Higher abundance of transposable elements on the plasmids was supported by a previous study which also revealed a higher density of these elements in plasmid metagenomes [79].

Additionally, ICEs can be found integrated to chromosomes or exist independently in a bacterium, therefore, we uncovered their abundance on chromosomes. Although the pattern of distribution of chromosomes carrying ICE was much alike to that of ICEs found in the whole metagenome but the total abundance of ICEs carried by chromosomes was lower as compared to total ICEs abundance in the whole metagenome, therefore this may be suggested that the remaining ICEs possibly existed independently (not integrated to chromosomes) in the microbes. Also, the abundance of chromosomes carrying ICE was much higher than the abundance of chromosomes carrying transposable elements on them.

### 3.3 Percentage of Mobile ARGs in the WWTPs

#### 3.3.1 Percentage of mobile ARGs in the whole metagenomes

To take advantage of the maximum capacity of metagenomic datasets and infer information eventually essential to evaluate the real hazards by the ARGs to the environment and human beings, detecting antibiotic resistome and mobilome is not enough, thus, we further identified the mobile ARGs (ARGs associated with mobilome) in our samples as they can be transferred within the chromosomes and plasmids and ultimately to human pathogens from their original hosts. To evaluate mobile ARGs in our samples, we checked the presence of the genes belonging to antibiotic resistance and mobilome simultaneously on the same contig. In our study, we observed a fair proportion of mobile ARGs in our samples, ranging from 26% to 35 % of the total identified ARGs in metagenome samples as shown in Figure 9, 10 and 11. No significant variation in the percentage of mobile ARGs among different WWTPs was observed in this study as all the WWTPs showed a similar range. Interestingly, the effluent samples coming from GJ and JS WWTP showed a higher percentage of mobile ARGs as compared to other samples although the abundance was still lower than the influent as shown in Figure 9 and 10. Furthermore, the abundance of mobile ARGs in the downstream of GJ and JS WWTP was higher than that of upstream samples although they witnessed a similar percentage of mobile ARGs. On further analysis, we observed that mobile ARGs were greatly contributed by the ICEs as compared to the transposable elements as their contribution ranged from 350-750 mobile ARGs in all the samples whereas the mobile ARGs contributed by the transposable elements ranged from 5-15 in each sample. This may suggest that the greater number of ARGs are associated with ICE rather than transposable elements although a more comprehensive statistical analysis is required to confirm this evidence. These findings may be alarming as the ARGs associated to ICEs can be directly transferred between bacteria via conjugation, however, the transposable elements (except conjugative types) only allows the movement of ARGs within a cell. Additionally, we found that the mobile ARGs mostly

belonged to multi-drug, aminocoumarin, MLS and tetracyclines types, although this is supported by the findings of most abundant antibiotic resistome in our study (MDR, rifampicin and MLS), surprisingly rifampicin resistance genes were not detected in high number as the mobile ARGs. This may be suggested that the mobility of a particular ARG (association to mobilome) does not totally depend on their total abundance. Collectively, our study suggests that the mobile ARGs can be prevalent in WWTPs as favored by a recent study [22] and as far as the correlation of mobile ARGs and ARGs abundance is concerned, it is difficult to withdraw any conclusion without any statistical analysis. Although greater association of ARGs to ICE should be monitored with caution.

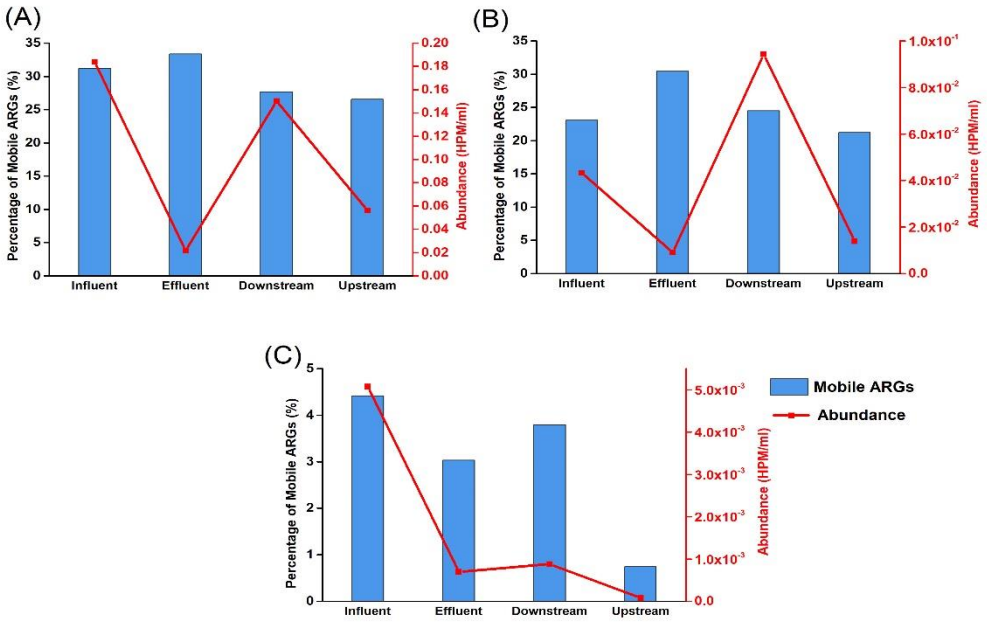


**Figure 9: Percentage and abundance of mobile ARGs in the whole metagenome (A), chromosomal (B), and plasmid metagenome (C) of GJ WWTP**

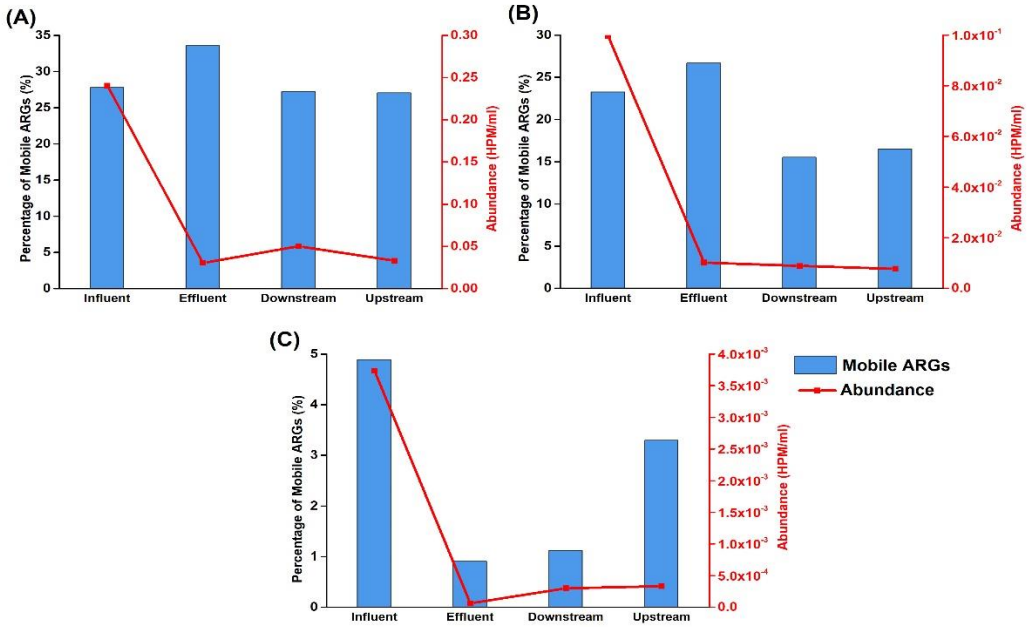


**Figure 10: Percentage and abundance of mobile ARGs in the whole metagenome (A), chromosomal (B), and plasmid metagenome (C) of JS WWTP**

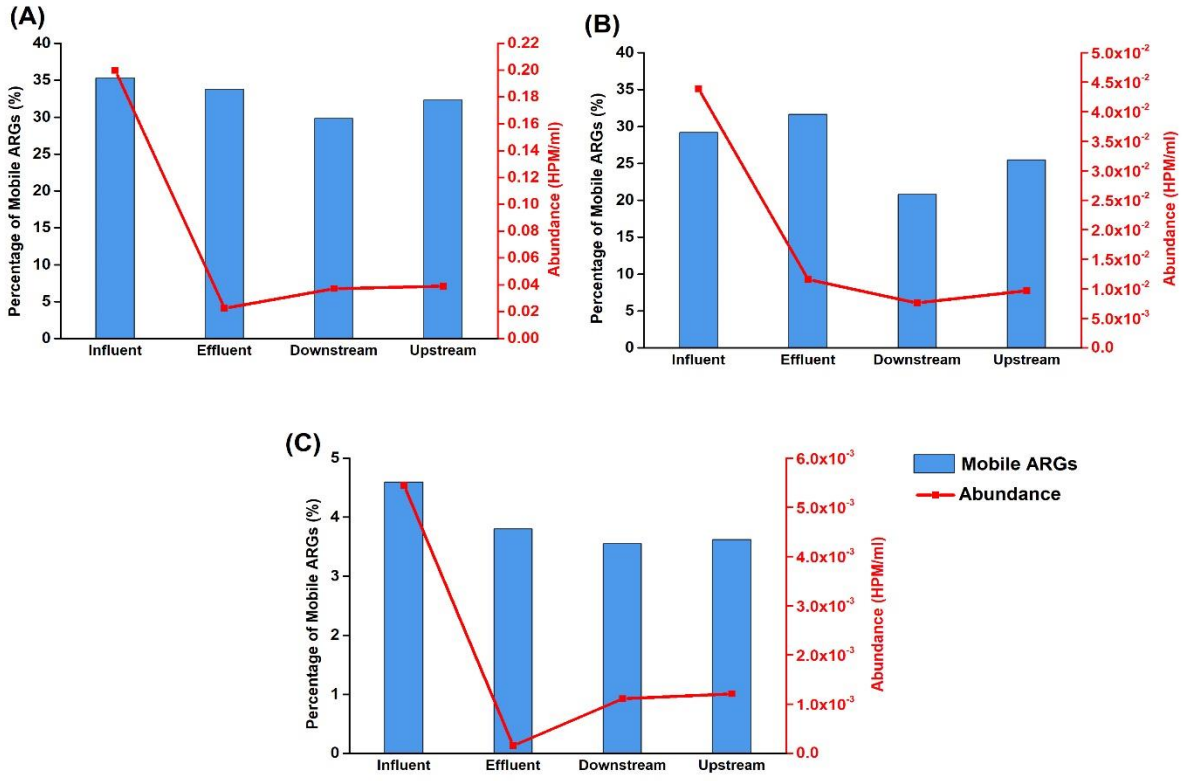


**Figure 11: Percentage and abundance of mobile ARGs in the whole metagenome (A), chromosomal (B), and plasmid metagenome (C) of JR WWTP**

### 3.3.2 Percentage of mobile ARGs in the chromosomal and plasmid metagenomes

Furthermore, we analyzed the chromosomes and plasmid sequences to check their contribution to the mobile ARGs in the samples. We calculated the percentage of mobile ARGs on the chromosomes and plasmids with respect to the total ARGs carried by the chromosomes and plasmids, respectively. Our results clearly showed that the plasmids exhibited a much lower abundance of mobile ARGs as compared to the abundance of mobile ARGs on chromosomes. The possible reason may be the presence of a large number of ICEs on the chromosomes, while in the case of plasmids, the mobile ARGs were only contributed by transposable elements. On further analysis, we found that the number of ARGs associated with transposable elements was almost negligible on the chromosomes while on the plasmids it was much higher. Therefore, a high number of mobile ARGs on the chromosomes were found to be solely due to the presence of ICEs on them while the transposable elements were the reason for mobile ARGs on the plasmids.

The mobile ARGs contributed by the ICEs located on the chromosomes can exhibit intercellular mobility by excising themselves as circular elements and conjugating to the recipient cells, but the mobile ARGs contributed by the transposable elements on the chromosomes can just move within the cell i.e. between chromosomes and plasmids. However, plasmids can serve this purpose of transferring these mobile ARGs from the chromosomes of the host cell to the other cells. Therefore, we performed further analysis on the plasmids carrying these mobile ARGs and checked their conjugation ability as it may empower the plasmids to disseminate the ARGs to other bacterial cells. Moreover, the mobile ARGs on the plasmids can be easily transferred to the chromosomes of recipient cell and may subsist for an extended period in bacteria without the risk of elimination due to the burden of fitness cost and application of various plasmid curing strategies [70].

### 3.4 Estimation of mobility or conjugation ability of the plasmids carrying mobile ARGs

We used MOB-suite tool having a MOB-typer method which can classify the plasmids as conjugative, mobilizable or non-mobilizable. Therefore, we analyzed the 23, 16 and 28 plasmids carrying mobile ARGs detected in GJ, JS, and JR WWTP, respectively as shown in Table 2, 3 and 4. The results showed that out of the 23 plasmids carrying mobile ARGs in GJ WWTP, 15 plasmids were either conjugative (10) or mobilizable (5) as shown in Table 2. In the case of JS WWTP, we detected 10 conjugative, 3 mobilizable and 3 non-mobilizable plasmids carrying mobile ARGs (Table 3). While 14 conjugative, 5 mobilizable and 9 non-mobilizable plasmids were retrieved in the JR WWTP as shown in Table 4. Moreover, we conducted this study only on the plasmids carrying mobile ARGs which covers a small share of total plasmids carrying ARGs. Therefore, these results may suggest that many mobilizable or conjugative resistance plasmids may be found in WWTPs which may encourage substantial dissemination of ARGs within microbial communities. Studies reported that if an ARG is located on a conjugative or mobilizable plasmid then this ARG has the potential to be transferred among bacteria and can eventually spread in a given ecosystem [81, 82]. Furthermore, the genes conferring resistance to tetracyclines were found to be higher in number followed by aminoglycosides, beta-lactams and fluoroquinolones on these plasmids which is supported by a past study (urban WTP) which also detected conjugative and mobilizable plasmids carrying genes coding resistance to tetracycline, beta-lactams [37]. Additionally, several plasmids were found carrying multiple mobile ARGs on them, mostly belonging to fluoroquinolones, sulfonamides and aminoglycosides. Although MDR genes were found to be highly abundant in our samples, only three plasmids were found to carry mobile MDR genes.

Interestingly, a recently discovered colistin resistance gene (MCR-5) belonging to peptide antibiotic type was also found in the influent of GJ WWTP. The MCR-5 colistin resistance gene was found to be associated with transposase tnpA2, tnpA on a plasmid and this finding is supported by the recent identification of

transposon-associated phosphoethanolamine transferase gene, MCR-5, conferring colistin resistance in d-tartrate fermenting *Salmonella enterica subsp. enterica serovar Paratyphi B* [83]. In our study, the mobile MCR-5 gene was found on the plasmid although it might have been transferred from chromosome to plasmid as suggested by a previous study [83]. As colistin is considered one of the last-resort antibiotics against the infections caused by multi-drug gram-negative bacteria including *Acinetobacter baumannii*, carbapenemase-producing Enterobacteriaceae and *Pseudomonas aeruginosa*, therefore occurrence of the mobile-colistin resistance gene in the WWTPs may pose serious threats to the connecting environments. This is the first case to report the detection of the mobile MCR-5 resistance gene in a WWTP. Furthermore, we carried a more extensive study and checked these plasmids for the presence of BMRGs along with the mobile ARGs as the presence of BMRGs may help in the co-selection of ARGs under biocide or metal burden [24]. Several plasmids were found to carry genes conferring resistance to biocides especially belonging to Quaternary Ammonium Compounds (QACs) as shown in Table 2, 3 and 4. Other biocide resistance genes belonging to hydrogen peroxide, ethidium bromide and several dyes were also found on the plasmids along with mobile ARGs. A previous WWTP study also found several mobilizable and conjugative plasmids carrying mobile ARGs along with QACs and heavy metals [37]. Additionally, we checked the presence of VFs on the plasmids along with mobile ARGs as bacteria are able to induce various diseases by expression of different combinations of VFs which is often mediated by plasmids. We found few plasmids carrying various VFs such as *hupA, htpB* (adherence), *ibeB* (invasion), *lysR* (transcriptional regulator), etc. as shown in Table 2, 3 and 4. However, these VFs encoded a range of products, not all of which are exclusively pathogenic (e.g., those related to host association), therefore we further investigated the taxonomy of these plasmids to investigate whether or not these plasmids belonged to some pathogenic bacteria.

For taxonomy classification, we used Kaiju, a metagenomic profiling tool which can be used to classify metagenomic contigs. Although Kaiju was able to classify the plasmid contigs, most of the classifications remained up to domain level (bacteria) while some plasmids were designated to species level as shown in

Table 2, 3 and 4. These plasmids mainly belonged to the phylum Proteobacteria and further to Gamma proteobacteria at the class level. We observed that the plasmids carrying mobile ARGs were assigned to various pathogenic bacteria taxon. Various human pathogenic bacteria species such as *Vibrio cholerae, Salmonella enterica subsp. enterica serovar Heidelberg, Klebsiella pneumoniae, Salmonella enterica subsp. enterica serovar Corvallis,* and *Pseudomonas syringae* (plant pathogen) were detected and many bacteria were classified up to genus level namely *Pseudomonas, Achromobacter* (opportunistic pathogens). A conjugative plasmid carrying mobile beta-lactamase gene, PDC-7 conferring resistance to cephalosporin, monobactam, carbapenem along with tellurium resistance genes and two virulence factor genes (*hupA, recA*) and designated to *Pseudomonas saponiphila* (xenobiotic degrading strain) was found in the influent of the JR WWTP [84]. A conjugative plasmid belonging to V*ibrio cholerae* was found to carry genes resistant to fluoroquinolone, sulfonamide and QACs on it. Moreover, all the plasmids belonging to *Salmonella enterica subsp. enterica serovar Corvallis* were found to carry sulfonamide resistance genes on them. *Pseudomonas syringae*, a plant pathogen was found to carry a plasmid conferring resistance to multi-drug (OprM) associated with transposase along with ethidium bromide resistance genes and a virulence factor (ibeB) encoding a protein for invasion. Collectively, in this study, the plasmids carrying mobile ARGs were classified to various pathogenic bacteria and moreover, many plasmids carried various BMRGs along with mobile ARG which was supported by a previous study on the WWTP [37]. This requires urgent mitigation efforts as the cumulative potential for mobile ARGs to occur on plasmids and in pathogens poses the real hazard to human beings and the associated environment [85].

**Table 2: Prediction of BMRGs, VFs and taxonomy of plasmids carrying mobile ARGs in GJ WWTP**

| Site | Transposable Elements | Antibiotic Resistance | | Typing | Other Genes (BMRGs/VFs) | Taxa |
|---|---|---|---|---|---|---|
| **Influent** | tnpA | Tetracycline | tetC | Conjugative | - | Bacteria |
| | tnpA | Beta-lactam | CfxA6 | Non-mobilizable | - | Bacteria |
| | tniB | Phenicol | cmx | Mobilizable | - | Bacteria |
| | tnpA | Beta-lactam | OXA-58 | Mobilizable | - | Gamma proteobacteria |
| | tnpAcp2 | Aminoglycoside | AAC (3)-IIa | Conjugative | - | Gamma proteobacteria |
| | tnpA | Beta-lactam | TEM-1, CTX-M-3 | Conjugative | - | Bacteria |
| | tnpA2, tnpA | Peptide | MCR-5 | Non-mobilizable | - | Proteobacteria |
| | tnpA-3, tnpA | Beta-lactam | AER-1 | Mobilizable | - | Bacteria |
| | Tn916-orf9 | Tetracycline | tetM | Conjugative | - | Bacteria |
| | tnpA | Aminoglycoside | AAC (6')-Ie-APH (2'')-Ia | Mobilizable | - | Firmicutes |
| | tnpA4 | Beta-lactam | NPS-1 | Mobilizable | - | Bacteria |
| **Effluent** | tnpAN, tnpA | Aminoglycoside | APH (6)-Id, APH (3'')-Ib | Non-mobilizable | - | Proteobacteria |
| | qacEdelta | Fluoroquinolone | qacH, sul1 | Conjugative | qacEdelta (Biocide) | Bacteria |
| | intI1 | Aminoglycoside | AAC (6')-Ib7 | Conjugative | xerD (VF) | *Vibrio cholerae* |
| | tnpA | Tetracycline | tetC | Conjugative | - | Bacteria |
| | tnpA | Tetracycline | tetC | Conjugative | - | *Salmonella enterica subsp. enterica serovar Heidelberg* |
| **Downstream** | qacEdelta | Fluoroquinolone | qacH | Non-mobilizable | qacF (Biocide) | Bacteria |
| | qacEdelta | Tetracycline | tet(G) | Conjugative | - | Proteobacteria |
| | tnpA, tniB, qacEdelta | Fluoroquinolone, Sulfonamide | sul1, qacH | Conjugative | qacEdelta (Biocide) | Bacteria |
| | tnpA | Tetracycline | tetC | Non-mobilizable | - | *Pseudomonas* |
| | tnpA | Multi-drug | OprM | Non-mobilizable | emhC (Biocide), ibeB (VF) | *Pseudomonas syringae* |
| | tnpA, tnpAN | Aminoglycoside | APH (3'')-Ib, APH (6)-Id | Non-mobilizable | - | *Achromobacter* |
| **Upstream** | tnpA | Aminoglycoside | APH (3'')-Ib | Non-mobilizable | - | Proteobacteria |

**Table 3: Prediction of BMRGs, VFs and taxonomy of plasmids carrying mobile ARGs in JS WWTP**

| Site | Transposable Elements | Antibiotic Resistance | | Typing | Other Genes (BMRGs/VFs) | Taxa |
|---|---|---|---|---|---|---|
| Influent | Xis-Tn916, Tn916-orf8 | Tetracycline | tetM | Non-mobilizable | - | Bacteria |
| | intI1, IS91, qacEdelta | Tetracycline | tet(G) | Conjugative | htpB (VF) | Bacteria |
| | qacEdelta, tniB, tnpA | Fluoroquinolone, Sulfonamide | qacH, sul1, sul2 | Conjugative | qacEdelta (Biocide) | Bacteria |
| | IS91 | Sulfonamide | sul2 | Conjugative | glmM (VF) | *Salmonella enterica subsp. enterica serovar Corvallis* |
| | tnpA | Multi-drug | abeM | Mobilizable | abeM (Biocide) | *Acinetobacter sp. LCT-H3* |
| | tnpA | Aminoglycoside | AAC (6')-Ie-APH (2")-Ia | Conjugative | - | *Bacilli* |
| | tnpA | Tetracycline | tetC | Conjugative | - | Bacteria |
| | tnpA-3, tnpA | Beta-lactam | AER-1 | Mobilizable | - | *Klebsiella pneumoniae* |
| | tnpA | Aminoglycoside | APH (3")-Ib, APH (6)-Id | Non-mobilizable | - | Bacteria |
| Effluent | tnpAB, tnpA | Tetracycline | tetC | Conjugative | - | Bacteria |
| | tnpA | Multi-drug | MexB | Conjugative | kexD (Biocide) | Bacteria |
| Downstream | qacEdelta | Tetracycline | tet(G) | Conjugative | - | Proteobacteria |
| | qacEdelta | Aminoglycoside, Fluoroquinolone, Sulfonamide | aadA, qacH, sul1 | Non-mobilizable | qacEdelta (Biocide) | Bacteria |
| Upstream | intI1 | Aminoglycoside | aadA3 | Conjugative | - | Gamma proteobacteria |
| | qacEdelta, tniB | Fluoroquinolone, Sulfonamide | qacH, sul1 | Mobilizable | qacEdelta (Biocide) | Bacteria |
| | qacEdelta | Tetracycline | tet(G) | Conjugative | oxyRkp (Biocide), lysR (VF) | Bacteria |

**Table 4: Prediction of BMRGs, VFs and taxonomy of plasmids carrying mobile ARGs in JR WWTP**

| Site | Transposable Elements | Antibiotic Resistance | | Typing | Other Genes (BMRGs/VFs) | Taxa |
|---|---|---|---|---|---|---|
| **Influent** | qacEdelta | Tetracycline | tet(G) | Conjugative | - | Bacteria |
| | tnpA | Aminoglycoside, Fluoroquinolone | AAC (6')-Ib7, qacH | Non-mobilizable | qacF (Biocide) | Bacteria |
| | tnpA | Beta-lactam | TEM-1 | Conjugative | - | Enterobacteriaceae |
| | tnpA | MLS | lnuF | Conjugative | - | Bacteria |
| | IS91 | Sulfonamide | sul2 | Conjugative | glmM (VF) | *Salmonella enterica subsp. enterica serovar Corvallis* |
| | tnpA | Aminoglycoside | APH (3')-Ia | Conjugative | - | Bacteria |
| | tnpA, tnpA-3 | Beta-lactam | AER-1 | Mobilizable | - | *Klebsiella pneumoniae* |
| | qacEdelta | Fluoroquinolone | QnrVC4, qacH | Non-mobilizable | qacF (Biocide) | Gammaproteobacteria |
| | Xis-Tn916, Tn916-orf8 | Tetracycline | tetM | Non-mobilizable | - | Firmicutes |
| | tniA, tnpA | Beta-lactam | PDC-7 | Conjugative | terC (Metal), hupA, recA (VF) | *Pseudomonas saponiphila* |
| | tnpA | MLS | ErmB | Mobilizable | - | *Bacilli* |
| | Tn916-orf14 | Aminoglycoside | ANT (9)-Ia, ANT (6)-Ia | Mobilizable | - | *Anaerobium acetethylicum* |
| | tnpA | Tetracycline | tetC | Conjugative | - | Bacteria |
| **Effluent** | tnpA | Beta-lactam | AER-1 | Mobilizable | - | *Klebsiella pneumoniae* |
| | tnpA | Tetracycline | tetC | Conjugative | - | Proteobacteria |
| | tnpAN, tnpA | Aminoglycoside | APH (6)-Id, APH (3")-Ib | Conjugative | - | *Achromobacter* |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Downstream** | tnpA | Aminoglycoside | APH (3'')-Ib, APH (6)-Id | Non-mobilizable | - | Proteobacteria |
| | qacEdelta | Fluoroquinolone | qacH | Non-mobilizable | qacF (Biocide) | Bacteria |
| | qacEdelta | Phenicol, Tetracycline | floR, tetG | Non-mobilizable | - | Proteobacteria |
| | IS91 | Sulfonamide | sul2 | Conjugative | arsH (Biocides/Metals), guaA, glmM (VF) | *Salmonella enterica subsp. enterica serovar Corvallis* |
| | tnpA | Tetracycline | tetC | Conjugative | - | Bacteria |
| | tnpA | Beta-lactam | AER-1 | Mobilizable | - | *Klebsiella pneumoniae* |
| **Upstream** | qacEdelta | Fluoroquinolone | qacH | Conjugative | qacE (Biocide) | *Polynucleobacter sp. GWA2_45_21* |
| | tnpA | Phenicol | floR | Conjugative | - | Proteobacteria |
| | qacEdelta | Tetracycline | tet(G) | Non-mobilizable | - | Proteobacteria |
| | tnpA | Tetracycline | tet(G) | Non-mobilizable | - | *Rhodobacter sp. LPB0142* |
| | qacEdelta | Fluoroquinolone | qacH | Non-mobilizable | qacF (Biocide) | Bacteria |
| | tnpA | Tetracycline | tetC | Conjugative | - | Bacteria |

### 3.5 Investigating the persistence of plasmids in WWTPs

To investigate the potentially persistent plasmids in the WWTP samples, psi-cd-hit was used to cluster the plasmid-contigs detected in the influent, effluent, downstream and upstream samples of each WWTP. The plasmids contigs which were shared by all the samples excluding upstream were considered to be persistent. Moreover, we also investigated the persistence of plasmids carrying ARGs for all the samples. Table 5 summarizes the total number of plasmids and plasmids carrying ARGs in each sample along with their abundance. Interestingly, many plasmids persisted in all the WWTPs. In GJ WWTP, 449 persistent plasmids were detected while 2,564 and 3,028 persistent plasmid contigs were witnessed in the JS and JR WWTPs, respectively.

**Table 5: Number of potentially persistent plasmids in each WWTP**

| WWTP | Plasmid type | Number of plasmids (HPM/ml) in | | | |
|---|---|---|---|---|---|
| | | Influent | Effluent | Downstream | Persistent |
| GJ | All Plasmids | 54,060 (10.72) | 51,718 (1.61) | 40,429 (5.59) | 449 |
| | Plasmids carrying ARGs | 229 (0.09) | 155 (0.01) | 149 (0.03) | 3 |
| JS | All Plasmids | 33,824 (10.64) | 58,513 (2.00) | 61,478 (7.34) | 2,564 |
| | Plasmids carrying ARGs | 164 (0.05) | 209 (0.01) | 162 (0.02) | 8 |
| JR | All Plasmids | 52,036 (10.59) | 32,586 (1.46) | 89,431 (10.67) | 3,038 |
| | Plasmids carrying ARGs | 253 (0.09) | 69 (0.004) | 143 (0.02) | 13 |

Furthermore, we checked the persistence of plasmids carrying ARGs as reports suggested that plasmids carrying ARGs tend to persist in the environment [62]. We found that only 3 plasmids carrying ARGs persisted in the GJ WWTP while JS and JS WWTPs showed the persistence of 8 and 13 plasmids carrying ARGs, respectively as shown in Table 5.

We also investigated the abundance of total and potentially persistent plasmids in all the samples. Results in Figure 12 showed that the abundance of total and potentially persistent plasmids followed similar pattern between influent, effluent and downstream, except for the abundance of potentially persistent plasmids in the influent of JR WWTP. Likewise, the abundance of total and potentially persistent plasmids carrying ARGs showed a similar trend between influent, effluent and downstream of all the WWTPs. Additionally, the abundance of all plasmids affiliated to the influent in all WWTPs was found to be similar with an average of 10.6 HPM/ml and likewise, the effluent of all WWTPs also showed comparable abundances within a range of 1.4 – 2. It can be suggested that the plasmids can persist depending on the abundance of plasmids.

A previous study reported that plasmids associated with ARGs were seen to persist in soil following application of sewage sludge [86]. Moreover, studies suggested that the plasmids could persist in the environment without continued antibiotic selection and additionally, even if they bear fitness cost still they can be maintained with higher conjugation rates [37, 62]. Collectively, the results in our study showed that the plasmids may persist the treatment process of the WWTPs and may further be added to the downstream rivers or other aquatic environments. Moreover, even subtle persistent plasmids carrying ARGs may contribute dissemination of ARB in the environment.

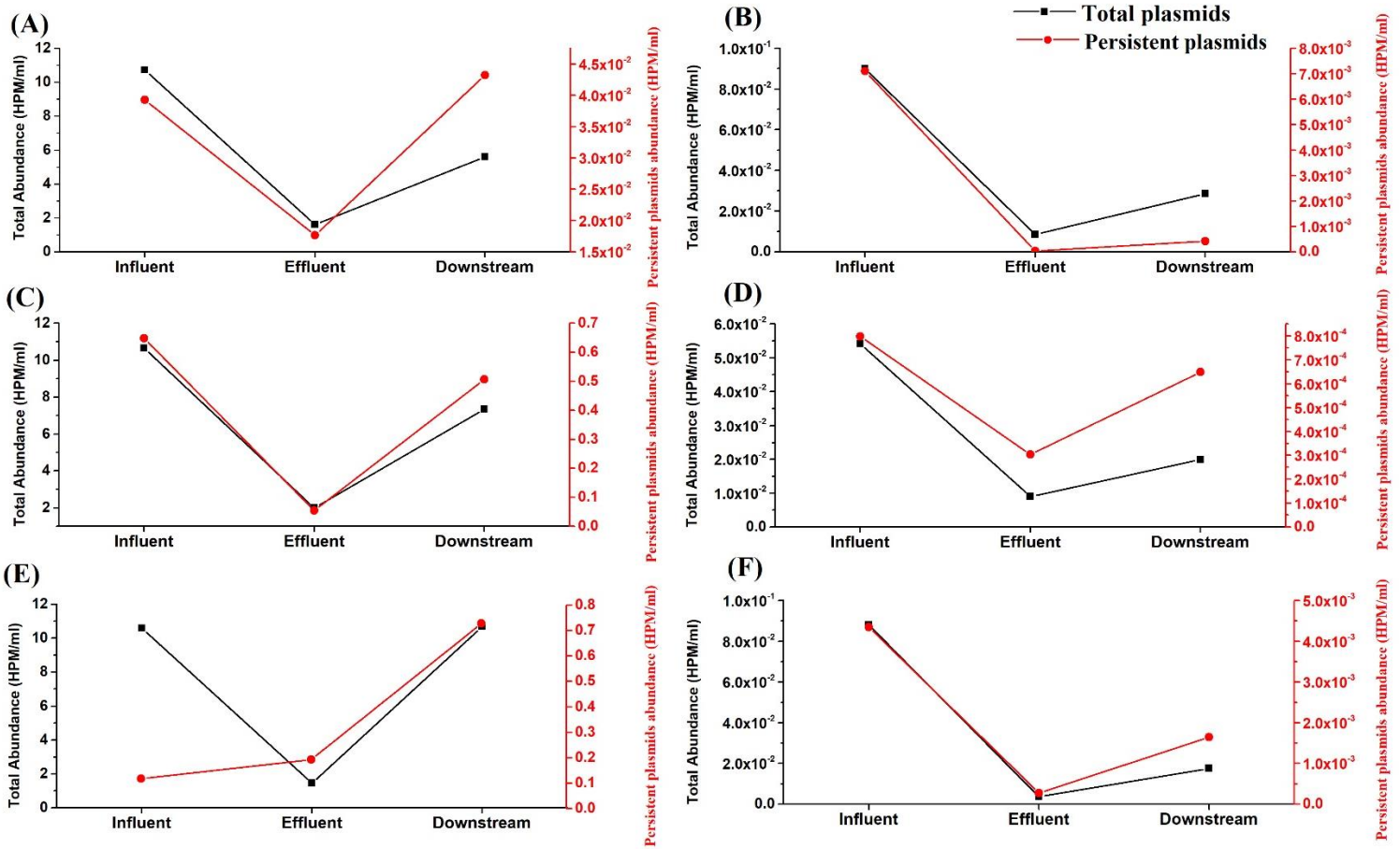


**Figure 12: Abundance of persistent and total plasmids in the WWTPs.**
(A), (C) and (E) shows the abundance of total and persistent plasmids in the GJ, JS, and JR WWTP, respectively. (B), (D), and (F) shows the abundance of total and persistent plasmids carrying ARGs on them in the GJ, JS, and JR WWTP, respectively.

### 3.6 Bacterial Composition Analysis

We next explored the bacterial community structure, derived from the genetic composition of the metagenomic samples in all the WWTPs. The taxonomic profiling was investigated using MetaPhlan2. The bacterial composition at the Phylum level in the samples showed that Proteobacteria were dominant in all the samples followed by Actinobacteria, Bacteroidetes, Firmicutes, etc. as shown in Figure 13. The abundance of Proteobacteria varied in between the range of 55% to 97%. The high abundance of Proteobacteria is supported by many previous studies on the WWTPs [22, 87]. A previous study revealed that the ARGs are more inclined to subsist in phylum Proteobacteria [88]. In the case of effluent samples belonging to JS WWTP, we observed that the phylum Chloroflexi accounted for approximately 40%, although they were not detected in the influent samples. A study conducted in a WWTP located in South Korea reported a high abundance of Chloroflexi in the WWTP receiving hospital waste than the domestic water receiving WWTP [89]. Although, previous studies revealed that the Chloroflexi are highly abundant in the activated sludge samples and their abundance get reduced in the effluent but in our study, it was abundant in one effluent sample [90]. The probable reason could be the problem of sludge bulking in the JS WWTP as the filamentous bacteria belonging to Chloroflexi are known to promote operational problem of impaired sludge settle ability known as bulking [91].

The results of bacterial composition at the genus level clearly showed the difference between the samples of all WWTPs as shown in Figure 14 (A). The influent sample of the GJ WWTP carried highly abundant *Arcobacter* (45%), followed by *Acinetobacter* (15%), *Bacteroidetes* (3%), *Bifidobacterium* (3%) and *Prevotella* (3%). While in the effluent sample, genus *Methyloversatilis* accounted for 73% followed by *Thiomonas (4%)* and *Caulobacter (3%). Arcobacter* spp. are reported to be high in abundance in untreated (raw) sewage water in the WWTPs and are an indicator of human fecal pollution as suggested by the previous studies [92]. Although 41% of *Arcobacter* spp. remained unclassified but the remaining 4% of them were classified as *Arcobacter butzleri* which is a reported as an enteropathogen and caused an

outbreak of recurrent abdominal cramps in Italy [93]. Moreover, *Arcobacter* was not detected in the effluent, upstream and downstream of this WWTP, suggesting the successful elimination of this genus by the GJ WWTP. On the other hand, *Pseudomonas* accounted for 86% and 50% in the downstream and upstream samples, respectively. Most of the *Pseudomonas* found in the downstream (59%) and upstream (34%) were not taxonomically classified at the species level as shown in Figure 14 (B). Furthermore, we detected many pathogenic species of genus *Pseudomonas* such as *P. syringae* (plant pathogen), *P. putida*, *P. monteii* (human pathogens), *P. tolaasii* (mushroom pathogen) in the downstream and upstream samples. Similarly, various pathogenic *Acinetobacter* species were also detected in the GJ WWTP including *A. pittii*, *A. nosocomialis*, *A. johnsonii*, *A. iwoffii*, *A. soli*, and *A. junii*. Moreover, a previous study suggested that WWTPs may selectively add ARGs to *Acinetobacter* spp. [94].

The taxonomy profiling of the JS WWTP samples also witnessed substantial variation at the genus level. In the influent, the most abundant genus detected was *Pseudomonas* (83%) followed by *Acinetobacter* (10%) and *Sphingomonas* (2%). Although in the effluent sample, genus *Pseudomonas* and *Acinetobacter* accounted merely 7% and 0.05% as the most abundant genera in the effluent were *Herpetosiphon* (40%) and *Methyloversatilis* (26%). Similar multiple pathogenic *Pseudomonas spp.* and *Acinetobacter* spp. were detected in the JS WWTP samples as that of GJ WWTP, although most prevalent human pathogen in the *Acinetobacter* genus, *Acinetobacter baumannii* were detected in the influent and downstream samples which are reported to cause serious nosocomial infections [95]. Other less pathogenic *Acinetobacter* species included *Acinetobacter ursingii*, and *Acinetobacter schindleri* [96]. The downstream and upstream samples in this WWTP showed a similar pattern in the bacterial composition although with different relative abundances. In downstream, most abundant genera *Polynucleobacter*, *Pseudomonas* and *Acinetobacter* accounted for 35%, 27% and 8%, respectively, whereas upstream witnessed Sphingomonas (20%) followed by *Rhodococcus* (16%), *Polynucleobacter* (14%) and *Actinobacteria unclassified* (14%).

In the case of JR WWTP, the influent sample showed higher abundance of *Pseudomonas* (28%) accompanied by *Arcobacter* (15%) and *Caulobacter* (12%) while the abundant genera belonged to *Limnohabitans* (32%), *Polynucleobacter* (30%), and *Rhodococcus (20%)* in the effluent samples. Genera belonging to *Limnohabitans, Candidatus Saccharimonas* and *Polynucleobacter* accounted for 31%, 19% and 17% in the downstream sample, respectively and moreover genera *Limnohabitans (76%)* and *Polynucleobacter (14%)* were also found to be abundant in upstream samples. Therefore, we observed that Genera *Limnohabitans* dominated in the JR WWTP except in its influent sample. Unfortunately, the species level of genus *Limnohabitans* was not identified in any of the samples and remained unclassified although species *Polynucleobacter necessaries* were designated to the second most abundant genus *Polynucleobacter* in all the samples. Similar to the above results, various pathogenic *Acinetobacter* species were detected such as *A. pittii, A. nosocomialis, A. johnsonii,* A. *juni,* and *A. ursingii* in all the samples except the upstream.

In all the samples, we detected more than 50 types of different genera, however, due to the low abundance of several genera, we grouped them into "others" group. Moreover, we focused our investigation mainly on the abundant bacterial communities in our samples. Collectively, we witnessed a broad range of genera in our samples along with various types of pathogenic bacterial species. Although we observed differences in the relative abundance of some genera (JR WWTP) between the effluent, downstream and upstream samples, it is difficult to conclude from these relative abundances alone that WWTP influenced the bacterial composition in the downstream samples. However, previous studies suggested the possible influence of WWTPs on the prevalence of bacterial community composition in a receiving river as compared to the upstream [90, 97]. Moreover, a research suggested that some WWTPs processes may sustain microbial communities that productively bolster the spread of the plasmids carrying multiple ARGs [98]. Additionally, evidence from previous research proves that the transfer of ARGs to the enteric bacteria could be witnessed in the WWTPs [15]. Therefore, WWTPs may have the potential to spread the pathogenic antibiotic-resistant bacteria and may have an impact on the microbial composition of the receiving aquatic environment.

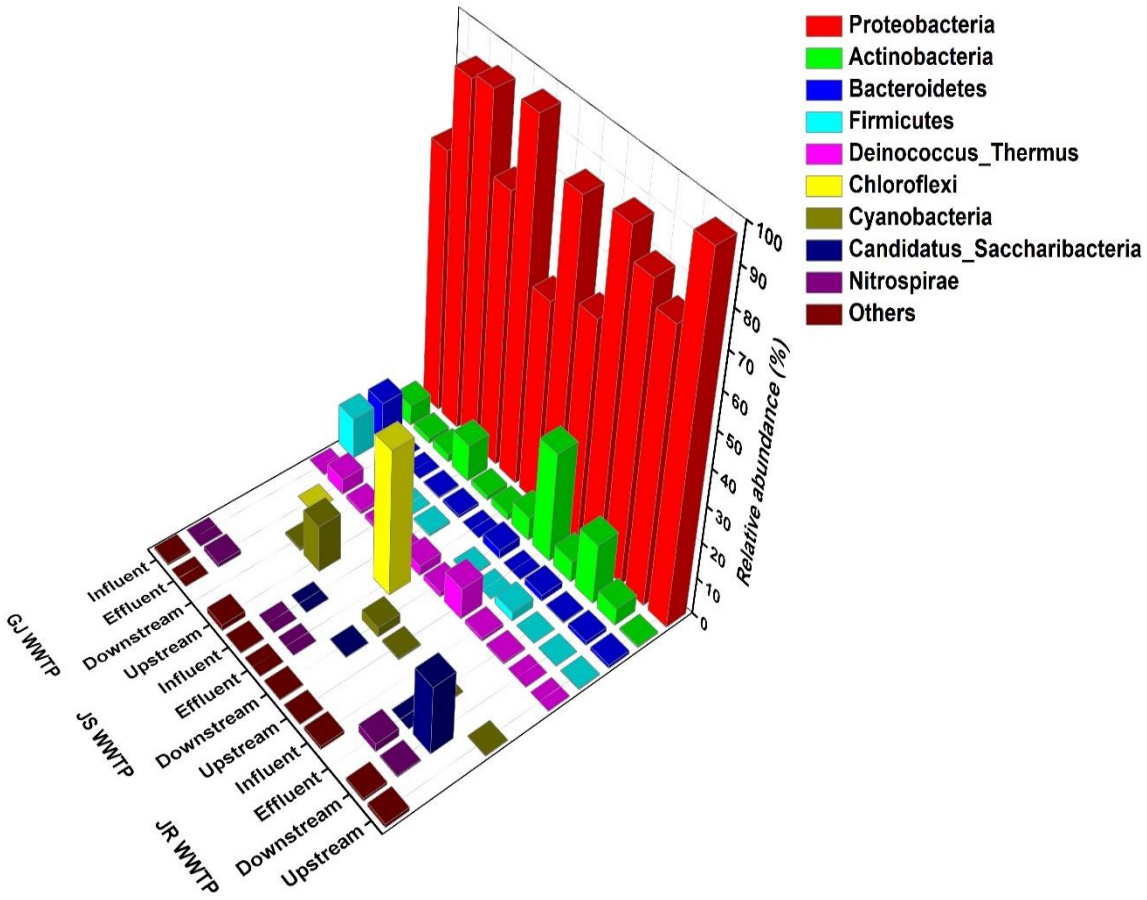


**Figure 13: Taxonomic assignment of metagenomic data from all the WWTPs at the phylum level**

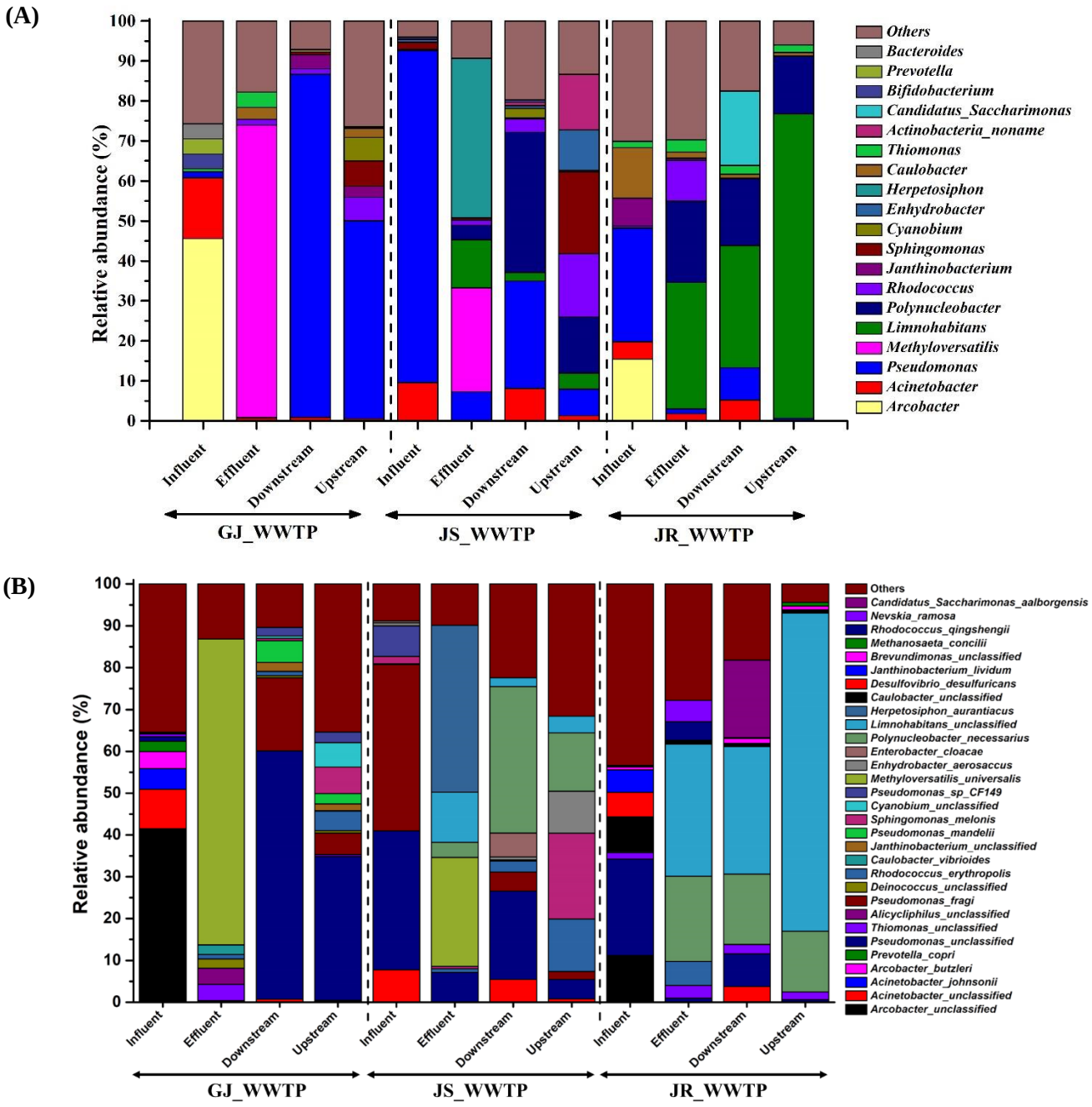


**Figure 14: Taxonomic assignment of metagenomic data from all the WWTPs at the genus level (A) and the species level (B).**

# CHAPTER 4

# CONCLUSION

In this study, we investigated the prevalence and abundance of antibiotic- and biocide-metal resistome and mobilome in three WWTPs using metagenomics. Additionally, we also revealed the occurrence of mobile ARGs, persistent plasmids and determined bacterial community composition. The results clearly revealed the prevalence of ARGs and BMRGs in the WWTPs along with the genes encoding mobilome (transposable elements and integrative conjugative elements). The abundance of antibiotic- and biocide-metal resistome and mobilome got immensely reduced in the effluent samples as compared to the influent suggesting the successful elimination of these genes by the treatment processes. Nevertheless, the higher abundance of resistome and mobilome in the downstream samples as compared to their upstream may delineate the possible role of WWTPs effluent in upraising these genes in downstream either by altering the chemical environment or through the introduction of genes. Moreover, the high abundance of BMRGs along with ARGs and their similar distribution pattern in our study may require additional supervision as it may suggest the possibility of co-resistance mechanism in the microbial communities. Therefore, this arises the requirement of a more comprehensive study to get insight into their co-occurrence distribution. Additionally, the occurrence and high abundance of mobile ARGs may advocate the probability of higher chances of their dissemination to other bacterial communities through HGT. As far as mobilome is concerned, the high abundance of integrative and conjugative elements may need to be studied more comprehensively in the WWTPs as their substantial association with ARGs may pose a serious threat like those of conjugative resistance plasmids. Furthermore, the occurrence of mobilizable or conjugative resistance plasmids along with BMRGs on them and moreover belonging to various human pathogenic bacteria may earnestly require mitigation efforts, as the aggregate potential for mobile ARGs to occur on plasmids and in pathogens represent the genuine risk to human beings and associated environment. The study also revealed that the

plasmids with higher abundance may persist the treatment process of WWTPs and may further be added to the receiving downstream. Similarly, the plasmids carrying ARGs may also persist and can be disseminated to pathogenic bacteria from their host cells.

Overall, the detection of several human pathogenic bacteria and prevalence and persistence of antibiotic- and biocide-metal resistome and mobilome in all the examined WWTPs highlights the fact that WWTPs may serve as a potential hotspot for antibiotic- and biocide-metal resistome and mobilome. Although the effective elimination of mobilome and resistome was witnessed in the WWTPs yet the subtle persistent resistome or off plasmids may pose a risk of the dissemination of these genes between the environmental bacteria and human enteric bacteria. However, the recent advancement in treatment processes may have potential to remove a larger section of resistome, mobilome and bacterial pathogens but it is evident from our study that the plasmids carrying ARGs may persist the treatment process. Our result suggests that a more comprehensive research should be performed on the mobile ARGs and persistent plasmids in addition to the development of novel disinfection system to reduce these emerging pollutants.

# ACKNOWLEDGEMENT

I am grateful to Sustainable Agriculture Research Institute (SARI) at Jeju National University for providing the experimental facilities. This research was supported by the Korea Ministry of Environment (MOE) as "the Environmental Health Action Program" (2016001350006).

I would like to express my deep gratitude to my supervisor Professor Tatsuya Unno. This work would not have been possible to achieve without his guidance, support and supervision. During my initial days in the lab, I was apprehensive about my decision to join an alien field, where things were new to me. But he came to my rescue with his intelligent ideas and teaching style which really encouraged me and ultimately, I got to broaden my boundary of knowledge in the field of metagenomics. His encouragement and belief to let me handle this work throughout my master course is something I will be in debt of. It was his extensive workaholic and grounded nature which always stimulated me to work hard and push my limits. I am deeply grateful to him for providing kind and generous financial assistance to carry out my study. It has been an honor to have him as my supervisor and to have got a chance to know him in my life.

I would like to thank my respected referees, Professor Soo Je Park and Dr. Dukki Han for providing constructive comments and suggestions which helped me in improving my thesis research. I would like to thank my all the lab members including Jungman Kim, Nakwon Hwang, Gwangpyo Ko, Hyejun Jo, and Adeel Farooq who always came forward positively whenever I needed their help. Especially thanks to Jiwan Hong and Shahbaz Raza whose support really helped in carrying out the research without any constraints. Special thanks to my lab member and senior Mr Vineet Singh for always been a major source of support when things would get a bit discouraging. He cannot realize how easy his presence had made my stay away from home.

The life in a foreign land would not have been so easier without the support of Dr Anil Kumar Khambampati who always guided and supported in every respect like a guardian. I would always be grateful to him for

his overwhelming care and support. I express my heart-felt gratitude to Dr Nagamaleshwar Rao Alluri and Mrs Latha Rao Alluri, Mr Gaurav Khandelwal and Mrs Juhi Khandelwal for making me feel like home and supporting me all through this journey. I am very fortunate to have amazing friends at Jeju National University. The presence of friends like Mr Sravan Kumar Konki, Mr Vivekananthan Venkateshwar, Mr Rajagopalan Pandey, Mr Suresh Rai, Mr Sunam Kumar Sharma, Mr Rahat Ahmed, Mr Divakar Ravi and many other friends always who made my everyday life comfortable and pleasant in Jeju. My heartiest thanks to all my friends from Nepal, Sri Lanka, Bangladesh, Philippines and Pakistan. Special thanks to Dr Reniel Pamplona for being a great room-mate cum friend and keeping a healthy atmosphere and bearing with me all these days.

Nevertheless, I am deeply grateful to have friends like Mr Roshan Mangal Bhattarai and Mrs Shirjana Bhattarai who always provided a home to go in Jeju and moreover their kind nature and companionship will always be cherished. Special thanks to Mrs Shirjana Bhattarai for teaching the basics of origin tool which helped me in attaining appealing graphs and plots.

Finally, I must express my very profound gratitude to my parents Mr Rajendra Gupta and Mrs Rekha Gupta and my sisters for providing me with unfailing moral and emotional support and continuous encouragement throughout my years of study. This accomplishment would not have been possible without them. Thank you.

# REFERENCES


[1] E. Y. Klein *et al.*, "Global increase and geographic convergence in antibiotic consumption between 2000 and 2015," *Proc. Natl. Acad. Sci.*, Mar. 2018.

[2] J. Park, E. Han, S. O. Lee, and D.-S. Kim, "Antibiotic use in South Korea from 2007 to 2014: A health insurance database-generated time series analysis.," *PLoS One*, vol. 12, no. 5, p. e0177435, 2017.

[3] N. Kemper, "Veterinary antibiotics in the aquatic and terrestrial environment," *Ecol. Indic.*, vol. 8, no. 1, pp. 1–13, Jan. 2008.

[4] K.-F. Kong, L. Schneper, and K. Mathee, "Beta-lactam antibiotics: from antibiosis to resistance and bacteriology," *APMIS*, vol. 118, no. 1, pp. 1–36, Jan. 2010.

[5] S. Bin Zaman, M. A. Hussain, R. Nye, V. Mehta, K. T. Mamun, and N. Hossain, "A Review on Antibiotic Resistance: Alarm Bells are Ringing," *Cureus*, vol. 9, no. 6, pp. e1403–e1403, Jun. 2017.

[6] N. Noguchi *et al.*, "Antimicrobial susceptibilities and distribution of resistance genes for beta-lactams and macrolides in Streptococcus pneumoniae isolated between 2002 and 2004 in Tokyo.," *Int. J. Antimicrob. Agents*, vol. 29, no. 1, pp. 26–33, Jan. 2007.

[7] J. O'Neill, "Antimicrobial resistance: tackling a crisis for the health and wealth of nations," *Rev. Antimicrob. Resist*, vol. 20, pp. 1–16, 2014.

[8] O. R. Sipahi, "Economics of antibiotic resistance.," *Expert Rev. Anti. Infect. Ther.*, vol. 6, no. 4, pp. 523–539, Aug. 2008.

[9] M. Helms, P. Vastrup, P. Gerner-Smidt, and K. Mølbak, "Excess mortality associated with antimicrobial drug-resistant Salmonella typhimurium," *Emerg. Infect. Dis.*, vol. 8, no. 5, pp. 490–

495, May 2002.

[10] J. E. McGowan, “Resistance in Nonfermenting Gram-Negative Bacteria: Multidrug Resistance to the Maximum,” *Am. J. Med.*, vol. 119, no. 6, Supplement 1, pp. S29–S36, 2006.

[11] E. J. Giamarellos-Bourboulis *et al.*, “Clarithromycin is an effective immunomodulator in experimental pyelonephritis caused by pan-resistant Klebsiella pneumoniae,” *J. Antimicrob. Chemother.*, vol. 57, no. 5, pp. 937–944, May 2006.

[12] World Heath Organization, “Antibacterial agents in clinical development: An analysis of the antibacterial clinical development pipeline, including tuberculosis,” 2017.

[13] World Health Organization, "Global antimicrobial resistance surveillance system: manual for early implementation," 2015.

[14] C. Bouki, D. Venieri, and E. Diamadopoulos, “Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: a review,” *Ecotoxicol. Environ. Saf.*, vol. 91, pp. 1–9, 2013.

[15] M. Z. Alam, F. Aqil, I. Ahmad, and S. Ahmad, “Incidence and transferability of antibiotic resistance in the enteric bacteria isolated from hospital wastewater,” *Braz. J. Microbiol.*, vol. 44, no. 3, pp. 799–806, Jan. 2014.

[16] S. Suzuki, A. Pruden, M. Virta, and T. Zhang, “Editorial: Antibiotic Resistance in Aquatic Systems,” *Front. Microbiol.*, vol. 8, p. 14, Jan. 2017.

[17] USEPA, "Primer for Municipal Waste water Treatment Systems," *Document no. EPA 832-R-04-001,* 2004.

[18] L. Rizzo *et al.*, “Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review,” *Sci. Total Environ.*, vol. 447, pp. 345–360, 2013.

[19] Y. Zhuang *et al.*, “Inactivation of antibiotic resistance genes in municipal wastewater by chlorination,

ultraviolet, and ozonation disinfection," *Environ. Sci. Pollut. Res.*, vol. 22, no. 9, pp. 7037–7044, 2015.

[20] N. A. Sabri *et al.*, "Prevalence of antibiotics and antibiotic resistance genes in a wastewater effluent-receiving river in the Netherlands," *J. Environ. Chem. Eng.*, 2018.

[21] J. G. Caporaso *et al.*, "Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample," *Proc. Natl. Acad. Sci.*, vol. 108, no. Supplement 1, p. 4516 LP-4522, Mar. 2011.

[22] B. T. T. Chu *et al.*, "Metagenomics Reveals the Impact of Wastewater Treatment Plants on the Dispersal of Microorganisms and Genes in Aquatic Sediments," *Appl. Environ. Microbiol.*, vol. 84, no. 5, Mar. 2018.

[23] J. Guo, J. Li, H. Chen, P. L. Bond, and Z. Yuan, "Metagenomic analysis reveals wastewater treatment plants as hotspots of antibiotic resistance genes and mobile genetic elements," *Water Res.*, 2017.

[24] C. Pal, J. Bengtsson-Palme, E. Kristiansson, and D. G. J. Larsson, "Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential," *BMC Genomics*, vol. 16, no. 1, p. 964, Nov. 2015.

[25] Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), "Assessment of the antibiotic resistance effects of biocides," *Eur. Comm. Heal. Consum. Prot.*, 2009.

[26] J. S. Chapman, "Disinfectant resistance mechanisms, cross-resistance, and co-resistance," *Int. Biodeterior. Biodegradation*, vol. 51, no. 4, pp. 271–276, Jun. 2003.

[27] C. Baker-Austin, M. S. Wright, R. Stepanauskas, and J. V McArthur, "Co-selection of antibiotic and metal resistance," *Trends Microbiol.*, vol. 14, no. 4, pp. 176–182, 2006.

[28] C. Seiler and T. Berendonk, "Heavy metal driven co-selection of antibiotic resistance in soil and

water bodies impacted by agriculture and aquaculture ," *Frontiers in Microbiology* , vol. 3. p. 399, 2012.

[29] X. Ji *et al.*, "Antibiotic resistance gene abundances associated with antibiotics and heavy metals in animal manures and agricultural soils adjacent to feedlots in Shanghai; China.," *J. Hazard. Mater.*, vol. 235–236, pp. 178–185, Oct. 2012.

[30] Z. Yu, L. Gunn, P. Wall, and S. Fanning, "Antimicrobial resistance and its association with tolerance to heavy metals in agriculture production," *Food Microbiol.*, vol. 64, pp. 23–32, 2017.

[31] M. Ahemad and A. Malik, "Prevalence of Heavy Metal and Antibiotic Resistance in Bacterial Isolates from Metal Polluted Soils," 2013.

[32] T. U. Berendonk *et al.*, "Tackling antibiotic resistance: the environmental framework.," *Nature reviews. Microbiology*, vol. 13, no. 5. England, pp. 310–317, May-2015.

[33] S. Suhartono, M. Savin, and E. E. Gbur, "Genetic redundancy and persistence of plasmid-mediated trimethoprim/sulfamethoxazole resistant effluent and stream water Escherichia coli," *Water Res.*, vol. 103, pp. 197–204, 2016.

[34] D. Y. W. Di, J. Jang, T. Unno, and H. G. Hur, "Emergence of Klebsiella variicola positive for NDM-9, a variant of New Delhi metallo-β-lactamase, in an urban river in South Korea," *J. Antimicrob. Chemother.*, vol. 72, no. 4, pp. 1063–1067, 2017.

[35] A.-D. Li, L.-G. Li, and T. Zhang, "Exploring antibiotic resistance genes and metal resistance genes in plasmid metagenomes from wastewater treatment plants," *Front. Microbiol.*, vol. 6, p. 1025, Sep. 2015.

[36] J. M. A. Blair, M. A. Webber, A. J. Baylay, D. O. Ogbolu, and L. J. V Piddock, "Molecular mechanisms of antibiotic resistance," *Nat. Rev. Microbiol.*, vol. 13, p. 42, Dec. 2014.

[37] T. O. Rahube, L. S. Viana, G. Koraimann, and C. K. Yost, “Characterization and comparative analysis of antibiotic resistance plasmids isolated from a wastewater treatment plant,” *Front. Microbiol.*, vol. 5, p. 558, Oct. 2014.

[38] C. Smillie, M. P. Garcillán-Barcia, M. V. Francia, E. P. C. Rocha, and F. de la Cruz, “Mobility of plasmids,” *Microbiol. Mol. Biol. Rev.*, vol. 74, no. 3, pp. 434–452, Sep. 2010.

[39] T. E. Roos and M. W. J. van Passel, “A quantitative account of genomic island acquisitions in prokaryotes.,” *BMC Genomics*, vol. 12, p. 427, Aug. 2011.

[40] F. Delavat, R. Miyazaki, N. Carraro, N. Pradervand, and J. R. van der Meer, “The hidden life of integrative and conjugative elements,” *FEMS Microbiol. Rev.*, vol. 41, no. 4, pp. 512–537, Jul. 2017.

[41] S. R. Partridge, S. M. Kwong, N. Firth, and S. O. Jensen, “Mobile genetic elements associated with antimicrobial resistance,” *Clin. Microbiol. Rev.*, vol. 31, no. 4, pp. e00088-17, 2018.

[42] C. W. McKinney and A. Pruden, “Ultraviolet disinfection of antibiotic resistant bacteria and their antibiotic resistance genes in water and wastewater.,” *Environ. Sci. Technol.*, vol. 46, no. 24, pp. 13393–13400, Dec. 2012.

[43] B. Bushnell, “BBMap: a fast, accurate, splice-aware aligner,” 2014.

[44] C.-C. Lo and P. S. G. Chain, “Rapid evaluation and quality control of next generation sequencing data with FaQCs.,” *BMC Bioinformatics*, vol. 15, p. 366, Nov. 2014.

[45] F. P. Breitwieser, J. Lu, and S. L. Salzberg, “A review of methods and databases for metagenomic classification and assembly.,” *Brief. Bioinform.*, Sep. 2017.

[46] D. Li, C.-M. Liu, R. Luo, K. Sadakane, and T.-W. Lam, “MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph.,” *Bioinformatics*, vol. 31, no. 10, pp. 1674–1676, May 2015.

[47] D. Hyatt, G.-L. Chen, P. F. Locascio, M. L. Land, F. W. Larimer, and L. J. Hauser, "Prodigal: prokaryotic gene recognition and translation initiation site identification," *BMC Bioinformatics*, vol. 11, p. 119, Mar. 2010.

[48] B. Jia *et al.*, "CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database.," *Nucleic Acids Res.*, vol. 45, no. D1, pp. D566–D573, Jan. 2017.

[49] C. Pal, J. Bengtsson-Palme, C. Rensing, E. Kristiansson, and D. G. J. Larsson, "BacMet: antibacterial biocide and metal resistance genes database," *Nucleic Acids Res.*, vol. 42, no. D1, pp. D737–D743, Jan. 2014.

[50] K. Pärnänen *et al.*, "Maternal gut and breast milk microbiota affect infant gut antibiotic resistome and mobile genetic elements," *Nat. Commun.*, vol. 9, no. 1, p. 3891, 2018.

[51] J. Cury, M. Touchon, and E. P. C. Rocha, "Integrative and conjugative elements and their hosts: composition, distribution and organization," *Nucleic Acids Res.*, vol. 45, no. 15, pp. 8943–8956, Sep. 2017.

[52] W. R. Pearson, "An Introduction to Sequence Similarity ('Homology') Searching," *Curr. Protoc. Bioinformatics*, vol. 0 3, p. 10.1002/0471250953.bi0301s42, Jun. 2013.

[53] V. Sentchilo *et al.*, "Community-wide plasmid gene mobilization and selection.," *ISME J.*, vol. 7, no. 6, pp. 1173–1186, Jun. 2013.

[54] P. S. Krawczyk, L. Lipinski, and A. Dziembowski, "PlasFlow: predicting plasmid sequences in metagenomic data using genome signatures.," *Nucleic Acids Res.*, vol. 46, no. 6, p. e35, Apr. 2018.

[55] M. Oh, A. Pruden, C. Chen, L. S. Heath, K. Xia, and L. Zhang, "MetaCompare: a computational pipeline for prioritizing environmental resistome risk," *FEMS Microbiol. Ecol.*, vol. 94, no. 7, p. fiy079, Apr. 2018.

[56] J. Robertson and J. H. E. Nash, “MOB-suite: software tools for clustering, reconstruction and typing of plasmids from draft assemblies,” *Microb. genomics*, vol. 4, no. 8, p. e000206, Jul. 2018.

[57] L. Chen, D. Zheng, B. Liu, J. Yang, and Q. Jin, “VFDB 2016: hierarchical and refined dataset for big data analysis--10 years on,” *Nucleic Acids Res.*, vol. 44, no. D1, pp. D694–D697, Jan. 2016.

[58] S. Sayers *et al.*, “Victors: a web-based knowledge base of virulence factors in human and animal pathogens,” *Nucleic Acids Res.*, pp. gky999-gky999, Oct. 2018.

[59] A. R. Wattam *et al.*, “Improvements to PATRIC, the all-bacterial Bioinformatics Database and Analysis Resource Center.,” *Nucleic Acids Res.*, vol. 45, no. D1, pp. D535–D542, Jan. 2017.

[60] S. Sahay *et al.*, “Insights into the genome sequence of ovine Pasteurella multocida type A strain associated with pneumonic pasteurellosis,” *Small Rumin. Res.*, 2018.

[61] P. Menzel, K. L. Ng, and A. Krogh, “Fast and sensitive taxonomic classification for metagenomics with Kaiju,” *Nat. Commun.*, vol. 7, p. 11257, Apr. 2016.

[62] A. J. Lopatkin, H. R. Meredith, J. K. Srimani, C. Pfeiffer, R. Durrett, and L. You, “Persistence and reversal of plasmid-mediated antibiotic resistance,” *Nat. Commun.*, vol. 8, no. 1, p. 1689, 2017.

[63] W. Li, L. Fu, B. Niu, S. Wu, and J. Wooley, “Ultrafast clustering algorithms for metagenomic sequence analysis,” *Brief. Bioinform.*, vol. 13, no. 6, pp. 656–668, Nov. 2012.

[64] W. Li and A. Godzik, “Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences.,” *Bioinformatics*, vol. 22, no. 13, pp. 1658–1659, Jul. 2006.

[65] D. T. Truong *et al.*, “MetaPhlAn2 for enhanced metagenomic taxonomic profiling,” *Nat. Methods*, vol. 12, p. 902, Sep. 2015.

[66] H. Li and R. Durbin, “Fast and accurate short read alignment with Burrows-Wheeler transform.,” *Bioinformatics*, vol. 25, no. 14, pp. 1754–1760, Jul. 2009.

[67] Y. Yang, B. Li, S. Zou, H. H. P. Fang, and T. Zhang, “Fate of antibiotic resistance genes in sewage treatment plant revealed by metagenomic approach.,” *Water Res.*, vol. 62, pp. 97–106, Oct. 2014.

[68] J. Xu *et al.*, “Occurrence of antibiotics and antibiotic resistance genes in a sewage treatment plant and its effluent-receiving river.,” *Chemosphere*, vol. 119, pp. 1379–1385, Jan. 2015.

[69] L. Ma, B. Li, and T. Zhang, “Abundant rifampin resistance genes and significant correlations of antibiotic resistance genes and plasmids in various environments revealed by metagenomic analysis.,” *Appl. Microbiol. Biotechnol.*, vol. 98, no. 11, pp. 5195–5204, Jun. 2014.

[70] M. M. C. Buckner, M. L. Ciusa, and L. J. V Piddock, “Strategies to combat antimicrobial resistance: anti-plasmid and plasmid curing,” *FEMS Microbiol. Rev.*, vol. 42, no. 6, pp. 781–804, Jul. 2018.

[71] C. Pal *et al.*, “Chapter Seven - Metal Resistance and Its Association With Antibiotic Resistance,” in *Microbiology of Metal Ions*, vol. 70, R. K. B. T.-A. in M. P. Poole, Ed. Academic Press, 2017, pp. 261–313.

[72] G. Luo, B. Li, L.-G. Li, T. Zhang, and I. Angelidaki, “Antibiotic Resistance Genes and Correlations with Microbial Community and Metal Resistance Genes in Full-Scale Biogas Reactors As Revealed by Metagenomic Analysis,” *Environ. Sci. Technol.*, vol. 51, no. 7, pp. 4069–4080, Apr. 2017.

[73] A. Di Cesare *et al.*, “Co-occurrence of integrase 1, antibiotic and heavy metal resistance genes in municipal wastewater treatment plants,” *Water Res.*, vol. 94, pp. 208–214, May 2016.

[74] J. L. Romero, M. J. Grande Burgos, R. Pérez-Pulido, A. Gálvez, and R. Lucas, “Resistance to Antibiotics, Biocides, Preservatives and Metals in Bacteria Isolated from Seafoods: Co-Selection of Strains Resistant or Tolerant to Different Classes of Compounds,” *Front. Microbiol.*, vol. 8, p. 1650, Aug. 2017.

[75] Z. Yu, P. He, L. Shao, H. Zhang, and F. Lü, “Co-occurrence of mobile genetic elements and antibiotic resistance genes in municipal solid waste landfill leachates: A preliminary insight into the

role of landfill age," *Water Res.*, vol. 106, pp. 583–592, Dec. 2016.

[76] F.-H. Wang, M. Qiao, J.-Q. Su, Z. Chen, X. Zhou, and Y.-G. Zhu, "High Throughput Profiling of Antibiotic Resistance Genes in Urban Park Soils with Reclaimed Water Irrigation," *Environ. Sci. Technol.*, vol. 48, no. 16, pp. 9079–9085, Aug. 2014.

[77] J. Guglielmini, L. Quintais, M. P. Garcillán-Barcia, F. de la Cruz, and E. P. C. Rocha, "The Repertoire of ICE in Prokaryotes Underscores the Unity, Diversity, and Ubiquity of Conjugation," *PLoS Genet.*, vol. 7, no. 8, p. e1002222, Aug. 2011.

[78] A. Karkman *et al.*, "High-throughput quantification of antibiotic resistance genes from an urban wastewater treatment plant," *FEMS Microbiol. Ecol.*, vol. 92, no. 3, p. fiw014, Mar. 2016.

[79] T. Zhang, X.-X. Zhang, and L. Ye, "Plasmid Metagenome Reveals High Levels of Antibiotic Resistance Genes and Mobile Genetic Elements in Activated Sludge," *PLoS One*, vol. 6, no. 10, p. e26041, Oct. 2011.

[80] E. Kristiansson *et al.*, "Pyrosequencing of Antibiotic-Contaminated River Sediments Reveals High Levels of Resistance and Gene Transfer Elements," *PLoS One*, vol. 6, no. 2, p. e17038, Feb. 2011.

[81] P. M. Bennett, "Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria.," *Br. J. Pharmacol.*, vol. 153 Suppl, pp. S347-57, Mar. 2008.

[82] A. van Hoek, D. Mevius, B. Guerra, P. Mullany, A. Roberts, and H. Aarts, "Acquired Antibiotic Resistance Genes: An Overview ," *Frontiers in Microbiology* , vol. 2. p. 203, 2011.

[83] M. Borowiak, J. Fischer, J. A. Hammerl, R. S. Hendriksen, I. Szabo, and B. Malorny, "Identification of a novel transposon-associated phosphoethanolamine transferase gene, mcr-5, conferring colistin resistance in d-tartrate fermenting Salmonella enterica subsp. enterica serovar Paratyphi B," *J. Antimicrob. Chemother.*, vol. 72, no. 12, pp. 3317–3324, Dec. 2017.

[84] E. Lang, M. Burghartz, S. Spring, J. Swiderski, and C. Sproer, “Pseudomonas benzenivorans sp. nov. and Pseudomonas saponiphila sp. nov., represented by xenobiotics degrading type strains.,” *Curr. Microbiol.*, vol. 60, no. 2, pp. 85–91, Feb. 2010.

[85] J. L. Martínez, T. M. Coque, and F. Baquero, “What is a resistance gene? Ranking risk in resistomes,” *Nat. Rev. Microbiol.*, vol. 13, p. 116, Dec. 2014.

[86] T. O. Rahube *et al.*, “Persistence of antibiotic resistance and plasmid-associated genes in soil following application of sewage sludge and abundance on vegetables at harvest,” *Can. J. Microbiol.*, vol. 62, no. 7, pp. 600–607, Mar. 2016.

[87] Z. Wang *et al.*, “Metagenomic Profiling of Antibiotic Resistance Genes and Mobile Genetic Elements in a Tannery Wastewater Treatment Plant,” *PLoS One*, 2013.

[88] Y. Hu *et al.*, “Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota,” *Nat. Commun.*, vol. 4, p. 2151, Jul. 2013.

[89] Y. Ahn and J. Choi, “Bacterial communities and antibiotic resistance communities in a full-scale hospital wastewater treatment plant by high-throughput pyrosequencing,” *Water*, vol. 8, no. 12, p. 580, 2016.

[90] B. Drury, E. Rosi-Marshall, and J. J. Kelly, “Wastewater Treatment Effluent Reduces the Abundance and Diversity of Benthic Bacterial Communities in Urban and Suburban Rivers,” *Appl. Environ. Microbiol.*, vol. 79, no. 6, p. 1897 LP-1905, Mar. 2013.

[91] M. Nierychlo, A. Miłobędzka, F. Petriglieri, B. McIlroy, P. H. Nielsen, and S. J. McIlroy, “The ecology of the Chloroflexi in full-scale activated sludge wastewater treatment plants,” *bioRxiv*, Jan. 2018.

[92] L. Collado, I. Inza, J. Guarro, and M. J. Figueras, “Presence of Arcobacter spp. in environmental waters correlates with high levels of fecal pollution,” *Environ. Microbiol.*, vol. 10, no. 6, pp. 1635–

1640, Jan. 2008.

[93] P. Vandamme *et al.*, "Outbreak of recurrent abdominal cramps associated with Arcobacter butzleri in an Italian school.," *J. Clin. Microbiol.*, vol. 30, no. 9, pp. 2335–2337, Sep. 1992.

[94] Y. Zhang, C. F. Marrs, C. Simon, and C. Xi, "Wastewater treatment contributes to selective increase of antibiotic resistance among Acinetobacter spp.," *Sci. Total Environ.*, vol. 407, no. 12, pp. 3702–3706, Jun. 2009.

[95] A. Y. Peleg, H. Seifert, and D. L. Paterson, "Acinetobacter baumannii: emergence of a successful pathogen," *Clin. Microbiol. Rev.*, vol. 21, no. 3, pp. 538–582, Jul. 2008.

[96] J. F. Turton, J. Shah, C. Ozongwu, and R. Pike, "Incidence of Acinetobacter species other than A. baumannii among clinical isolates of Acinetobacter: evidence for emerging species," *J. Clin. Microbiol.*, vol. 48, no. 4, pp. 1445–1449, Apr. 2010.

[97] E. Marti, J. Jofre, and J. L. Balcazar, "Prevalence of Antibiotic Resistance Genes and Bacterial Community Composition in a River Influenced by a Wastewater Treatment Plant," *PLoS One*, vol. 8, no. 10, p. e78906, Oct. 2013.

[98] C. Merlin, S. Bonot, S. Courtois, and J.-C. Block, "Persistence and dissemination of the multiple-antibiotic-resistance plasmid pB10 in the microbial communities of wastewater sludge microcosms.," *Water Res.*, vol. 45, no. 9, pp. 2897–2905, Apr. 2011.