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Antibiotic Resistance: A Tale of Two Environments

Assessment of the Emerging Ecological Risk of Antibiotic Resistance Released from The Cleveland Bay Sewage Treatment Plant onto the Great Barrier Reef Marine Park.

Submitted by

Kezia Ellyse Drane

In fulfilment of the requirements for the degree of

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Declaration of Ethics

The research presented and reported in this thesis was conducted within the guidelines for research and ethics outlined in the Joint NHMRC/AVCC Statement and Guidelines on Research Practice (1997), the James Cook University Policy on Experimental Ethics, Standard Practices and Guidelines (2001), and the James Cook University Statement and Guidelines on Research Practice (2001). The Proposed research methodology received clearance from the James Cook University Experimentation Ethics Review (Approval number A2733). Additionally, all environmental samples were acquired under the federal GBRMPA permit 46568, and the Queensland state Department of Environment and Science permit P-SPP-100143721.

Abstract

Dissemination of antibiotic resistance in marine environments is a global concern with a propensity to affect public health and many ecosystems worldwide. Wastewater treatment plants (WWTPs) are well-characterised points of convergence for antibiotics, antibiotic resistant bacteria (ARB), and their causative antibiotic resistance genes (ARGs) due to the high antibiotic and resistance load incorporated within their influents. However, there are currently no legislative requirements globally to reduce the quantity of antibiotic compounds or ARGs in wastewater effluent. Antibiotics, ARBs, and ARGs have recently been identified as contaminants of emerging concern from WWTPs due to their risk to both public and environmental health. Interest is growing in the need to identify accurate assessment scaffolds to predict the risk of ARB and ARGs released from WWTPs to public health, animals, and the environment (One Health). There is concern in the scientific community, however, that the current state of knowledge on disseminating ARBs and ARGs from WWTPs cannot be accurately applied to predict the risk to One Health. Furthermore, different WWTPs employ different technologies to treat and process their influents, which may reduce ARGs' release to varying degrees. Surveillance data of many different forms of treatment is required for interactions of ARGs and ARBs in various affected environments to assess this risk accurately. However, there is little continuity in the literature regarding the effect of different forms of wastewater treatment on the presence and abundance of antibiotics, ARB, and ARGs, both within the treatment process and after it. Methods used to characterise ARBs and ARGs in wastewater and their associated ecosystems are not standardised, with previous studies relying on differing combinations of bacterial culture, genetic, and genomic identification methods. This study provides much-needed information using whole genome sequencing methods to assess antibiotic resistance throughout a wastewater treatment process and its dissemination in the receiving environment. This thesis provides a standardised data collection method of a single WWTP that may be utilised to assess further individual WWTPs (or other sources of antibiotic resistance pollution). This information will aid in creating a standardised One Health risk assessment of antibiotic resistance pollution and its surveillance in surrounding ecosystems.

The consensus within the literature does exist on the application of membrane bioreactors (MBR) as a form of tertiary wastewater treatment and their ability to reduce the load of ARBs and some ARGs within WWTPs and their treated effluents, though many assessed plants were found to have individual resistomes and bacterial interactions. However, the knowledge surrounding the capability for antibiotics, ARB, and ARGs to reach marine environments from the effluents of WWTPs that employ MBRs is limited and even less known are the effects of these contaminants on the environment or their inhabitant flora and fauna. Previous studies on marine turtles residing within

the Great Barrier Reef Marine Park, Australia, have implied that these tertiary treated effluents may contribute to increased ARBs identified within these environments and their sentinel species.

WWTPs associated with the Great Barrier Reef Marine Park have unique legislative and regulatory pressures with respect to WWTP effluents discharged in the environment. Such is demonstrated by one of the largest points of discharge along the GBR, the Cleveland Bay Sewage Treatment Plant (CBSTP), which has been described as one of the largest MBR plants in the southern hemisphere. This novel study utilised whole genome sequencing techniques to define the distinct resistome and microbiome within the influent, biosolids, and effluent of the CBSTP and highlighted the microbiome and resistome shifts observed within the treatment steps of the CBSTP. The CBSTP was demonstrated to have a high removal efficiency of antibiotic resistance; however, the findings highlight that distinct ARGs are present within the effluent of this MBR plant. Furthermore, the possibility of horizontal gene transfer (HGT) of ARGs between treatment steps of the CBSTP may provide additional opportunities for antibiotic resistance proliferation in previously non-resistant bacterial species, which may have ongoing impacts on aspects of public and environmental health.

The Great Barrier Reef relies heavily on its microbial diversity, and the discharging of antibiotics, ARB, and ARG into this environment may pose a significant health risk to this world heritage site by creating selective pressures for specific bacterial communities and reducing bacterial diversity. This novel investigation utilised whole genome sequencing to characterise the dissemination patterns of ARGs from the treated CBSTP effluent through comparison with an uninhabited environment in the Great Barrier Reef Marine Park known as the Howick Group of Islands. This was done by a cross-sectional analysis of the interactions between a select group of anthropogenically used antibiotics, ARGs, and environmental microbiomes. The resistome within the immediate area (Near-Field) of the CBSTP was represented largely by CBSTP-associated ARGs, and correlations to the microbiome indicated possible instances of out-competition of functionally important bacteria due to this dissemination. Furthermore, some ARGs within the microbiome in the Near-Field environment and the presence of macrolide antibiotics at above no predicted effect concentrations within the Cleveland Bay sediments strongly correlate with changes in the functional microbiome of the Near-field. This strongly suggests the likely occurrence of horizontal ARG transfer.

Increased stress markers of other areas within Cleveland Bay (the Mid and Far-Fields) compared to that of the Howicks or the Near-Field suggest that consideration should be given to other sources of antibiotic resistance factors, and their modes of dissemination within marine ecosystems. This study highlights that ARGs and antibiotics from membrane bioreactor plants may affect associated marine receiving ecosystems. This is the first detailed comprehensive

characterisation via whole genome sequencing, to identify microbiomes and resistomes within various environmental sample matrices (sediment, seawater, and seagrass) within two distinct Great Barrier Reef sites. Additionally, this study is novel in its approach to identifying ARGs as specified genetic material from treated wastewater effluent, and providing insight into the effect antibiotics and ARGs may provide on marine receiving environments.

Overall, this thesis provides valuable insight into the effect of anthropogenic activities associated with wastewater processing on the ecological health of the Cleveland Bay coastal area and the Great Barrier Reef on the Northeast coastline of Australia. The findings of this study will inform the design and development of environmental risk assessment procedures and the implementation of strategies to mitigate anthropogenic pollution emerging from wastewater treatment plants throughout the highly valuable ecosystem of the Great Barrier Reef.

Table of Contents

Acknowledgements ..... **i**
Statement of the Contribution of Others ..... **iii**
Declaration of Ethics ..... **iv**
Abstract ..... **v**
Table of Contents ..... **viii**
List of Tables ..... **xii**
List of Figures ..... **xiii**
List of Publications ..... **xviii**
List of Communications ..... **xix**
List of Abbreviations ..... **xx**
Chapter 1: Introduction **1**
1.1 An Introduction to Antibiotic Resistance and One Health 1
1.1.1 Contributing Components of Antibiotic Resistance 1
1.2 Antibiotic Resistance Pollution 2
1.3 Effects of Antibiotic Resistance Pollution on the Receiving Environment 3
Chapter 2: Marine Testudines as Sentinels for Monitoring the Dissemination of Antibiotic Resistance in Marine Environments: An Integrative Review **6**
2.1 Introduction 6
2.1.1 Commonly Used Antibiotics, Their Modes of Action, and Mechanisms of Antibiotic Resistance 7
2.1.2 Antibiotics and Antibiotic Resistant Bacteria in Marine Environments 8
2.2 Methods used to Evaluate Turtles as Sentinels for Monitoring Antibiotic Resistance in Marine Environments 10
2.2.1 Systematic Literature Search Strategy, Inclusion and Exclusion Criteria, and Quality Assessment 10
2.2.2 Quantitative Evaluation of Compiled Data 10
2.3 Results and Discussion of Data on Using Turtles for Monitoring Antibiotic Resistance in Marine Environments 11
2.3.1 An overview of the Systematic Search of the Literature on Using Sea Turtles for Monitoring Antibiotic Resistance 11
2.3.2 Distribution of Studies on Antibiotic Resistance in Marine Environments by Geographical Site and Turtle Species 12
2.3.2.1 Antibiotic Resistance in Turtles in Marine Environments and the Potential Influence of Geographical Location 12
2.3.3 Antibiotic Resistance in Marine Environments and the Potential Influence of Differences in Turtle Species 17

2.3.4 Detection and Quantification of Antibiotic Resistance Profiles in Marine Environments 18
2.3.4.1 Present Challenges in Detecting ARB in Marine Turtles 18
2.3.4.2 Quantitative Evaluation of Phenotypic Antibiotic Resistance Patterns in Marine Turtles 20
2.3.4.3 Quantitative Evaluation of Anthropogenic Sources of Antibiotic Resistance in Marine Turtles 23
2.4 Conclusions 24
Chapter 3: The Role of Wastewater Treatment Plants in Dissemination of Antibiotic Resistance: Source, Measurement, Removal and Risk Assessment 27
3.1 Introduction 27
3.1.1 Antibiotic Resistance in Wastewater 28
3.2 Approaches for Identifying Antimicrobial Resistance Factors in WWTPs and the Environment 29
3.2.1 Identifying ARB and ARGs 29
3.2.2 Identifying Antibiotics 31
3.3 Effects of Wastewater Treatment Processes on ARGs Released from WWTPs 33
3.3.1 Primary Wastewater Treatment 34
3.3.2 Secondary Wastewater Treatment 34
3.3.3 Tertiary Wastewater Treatment 36
3.3.3.1 Chlorination Disinfection 36
3.3.3.2 Ultra-Violet Disinfection 37
3.3.3.3 Ozone Treatment 38
3.3.3.4 Membrane Filtration 38
3.4 Assessing the Risk of Antibiotic Resistance from WWTPs in the Environment 39
3.4.1 Interactions between ARB, ARGs, MGEs and Co-Occurrence Genes for Risk Scafolding 39
3.4.2 A Proposed Pathway to Informed Risk Scaffolds for Antibiotic Resistance from WWTPs 40
Chapter 4: Concordance in Molecular Methods for Detection of Antimicrobial Resistance: A Cross Sectional Study of the Influent to a Wastewater Plant 42
4.1 Introduction 42
4.2 Materials and Methods 44
4.2.1 Study Site and Sampling 44
4.2.2 Nucleic acid extraction and preservation 44
4.2.3 Using qPCR to detect and quantify ARGs in pooled influent samples for a WWTP 45
4.2.4 Metagenomic DNA or RNA sequencing to determine bacterial population and ARG Profiles 45
4.2.5 16S rRNA gene sequencing to determine the bacterial population 49
4.2.6 Data Analysis 49

4.2.6.1 Analysis of ARG expression by qPCR 49
4.2.6.2 Analysis of ARG expression by metagenomic DNA and RNA sequencing 49
4.2.6.3 Analysis of bacterial population profiles 50
4.3 Results 50
4.3.1 Characterisation of the ARG profile by a customised microbial DNA qPCR array . 50
4.3.2 ARG characterisation by illumina sequencing and probing of annotated datases 51
4.3.2.1 DNA Sequencing and ARG annotation in MEGARes database 51
4.3.2.2 DNA Sequencing and ARG annotation in CARD database 51
4.3.2.3 Comparison of qPCR and metagenomic DNA sequencing for detection of ARGs 56
4.3.2.4 Comparison of RNA sequencing and ARG annotation in MEGARes database 58
4.3.3 Profiling the microbiome by 16S rRNA sequencing and WGS using Kraken2 database 64
4.4 Discussion 68
4.5 Conclusions 72
Chapter 5: Antimicrobial Resistance Profiling in Sewage Processed by a Membrane Bioreactor Plant: Implications for Release into the Great Barrier Reef 73
5.1 Introduction 73
5.2 Materials and Methods 74
5.2.1 Sampling Locations and Sampling 74
5.2.2 Nucleic Acid Extraction 77
5.2.3 Metagenomic Sequencing 77
5.2.4 Data Analysis 78
5.3 Results 80
5.3.1 Metagenome Sequencing Overview 80
5.3.2 Daily Temporal Variation of the CBSTP Microbiome 80
5.3.3 Spatial Variation of the Microbiome and Resistome within the CBSTP 81
5.3.3.1 Community Diversity of Sample Types 81
5.3.3.2 Community Diversity Between Sample Types 83
5.3.4 Indications of horizontal movement of resistance genes throughout the Cleveland Bay Sewage Treatment Plant 87
5.3.5 Proportion of Bacteria to Resistance Genes 90
5.3.6 Monthly Temporal Patterns 91
5.4 Discussion 93
Chapter 6: Dissemination of Antibiotics and Antibiotics Resistance Genes from a Membrane Bioreactor Wastewater Plant on the Great Barrier Reef Australia: Implications for Marine Microbial Communities 97
6.1 Introduction 97
6.2 Methods 99

6.2.1 Sampling Locations 99
6.2.2 Sampling Method 104
6.2.3 Nucleic Acid Extraction 105
6.2.4 Composite Sampling Strategy 105
6.2.5 Antibiotic Residue Concentrations 106
6.2.6 Shotgun Metagenomic sequencing 106
6.2.7 Data Analysis 106
6.3 Results 107
6.3.1 Defining the Microbiome and Resistome of Environmental Samples by Sample Type 107
6.3.2 Defining the Microbiome and Resistome of Environmental Samples by Sampling Location 110
6.3.3 Dissemination of Antibiotic Resistance from the Cleveland Bay Sewage Treatment Plant 116
6.3.4 Dissemination of Antibiotics from the Cleveland Bay Sewage Treatment Plant . 124
6.4 Discussion 130
6.5 Conclusions 137
Chapter 7: General Discussions and Concluding Remarks 138
7.1 Conclusion 146
References 148
Appendix A 168
Appendix B 171
Appendix C 159

List of Tables

Table 2-1: Global distribution of geographical sites where different sea turtle species have been examined as sentinel species for Antibiotic Resistance. The plus sign (+) denotes the isolation of multi-drug resistant bacterial isolates..... 16

Table 6-1: Concentrations of antibiotic compounds identified in liquid sample types (effluent and seawater)..... 125

Table 6-2: Concentrations of antibiotic compounds identified in sediment samples. 125

Table 6-3: Correlations between concentrations of clarithromycin and azithromycin compared to core sediment bacteria..... 127

List of Figures

Figure 2-1:Summarised process for the literature search indicating detection, screening, and eligibility of included publications. Fourteen articles were retained for final analysis but only 12 of these were direct studies on the abundance and effect of ARB in marine turtles. Twelve studies were included for analysis of the metadata..... 12

Figure 2-2: Map of geographical location and clustering of studies on the monitoring of antibiotic resistance in marine environments using sea turtles as an animal sentinel species (red pins). Represented areas are the Mediterranean and Arabian seas, the Pacific Ocean along the east coast of Australia, and the South Atlantic Ocean along the west coast of Africa and the east coast of South America (Modified from Google maps under the terms and conditions of the Creative Commons Attribution (CC BY) license)..... 14

Figure 2-3: Frequency distribution of antibiotic resistance against commonly used antibiotic drugs stratified by turtle species (*Chelonia mydas* and *Caretta caretta*) irrespective of geographical marine environment, biological specimen considered or tested bacterial isolates (panel A) and, frequency distribution of antibiotic resistance stratified by considered biological specimen irrespective of geographical marine environment, turtle species or tested bacterial isolates (Panel B). Amikacin = AMI; amoxicillin = AMO; Amoxicillin-clavulanic acid = AMC; Ampicillin = AMP; Carbenicillin = CAR; Ceftazidime = CAZ; Ceftiofur = CEF; Cephalexin = CLX; Chloramphenicol = CHL; Doxycycline = DOX; Enrofloxacin = ENR; Erythromycin = ERY; Gentamicin = GEN; Kanamycin = KAN; Nalidixic acid = NAL; Neomycin = NEO; Streptomycin = STR; Tetracycline = TET; Tobramycin = TOB; Trimethoprim-sulfamethoxazole = TMS 21

Figure 2-4: Frequency distribution of antibiotic resistance against commonly used antibiotic drugs stratified by geographical marine environment irrespective of biological specimen considered, bacterial isolates or turtle species for beta-lactams (panel A) and other antibiotic classes (panel B). Amikacin = AMI; Amoxicillin = AMO; Amoxicillin-clavulanic acid = AMC; Ampicillin = AMP; Ampicillin-salbactam = APS; Aztreonam = AZT; Carbenicillin = CAR; Ceftazidime = CAZ; Ceftiofur = CEF; Cefepime = CEP; Cefazolin = CFZ; Ciprofloxacin = CIP; Cefalexin = CLX; Chloramphenicol = CHL; Colistin = COL; Ceftriaxone = CTN; Cefotaxime = CXM; Cefoxetin = CXT; Doxycycline = DOX; Enrofloxacin = ENR; Erythromycin = ERY; Rifampicin = RIF; Gentamicin = GEN; Imipenem = IMI; Lomefloxacin = LOM; MDRB = percentage of multi-drug resistant isolates; Meropenem = MER; Minocycline = MIN; Kanamycin = KAN; Nalidixic acid = NAL; Netlimicin = NET; Nitrofurantoin = NFT; Neomycin = NEO; Norfloxacin = NOR; Penicillin = PEN; Oxytetracycline = OXY; Piperacillin = PIP; Piperacillin-tazobactam = PPT; Streptomycin = STR; Sulfamethoxazole = SMX; Ticarcillin-clavulanic acid = TCA; Tetracycline =

TET; Ticarcillin = TIC; Tobramycin = TOB; Trimethoprim = TRM; Trimethoprim/sulfamethoxazole = TMS; Vancomycin = VMC. 22

Figure 2-5: Percentage distribution of the frequency for antibiotic resistance to common antibiotics in bacteria isolated throughout the 12 studies globally, and stratification by the potential sources of exposure as postulated by each original study. This includes: rehabilitation centres for sick or injured turtles (grey), treated wastewater from WWTPs (blue) and, other unidentified sources of anthropogenic pollutants (orange). Abbreviations are defined as: AMC = amoxicillin-clavulanic acid; AMP = ampicillin; CHL = chloramphenicol; DOX = doxycycline; ENR = enrofloxacin; GEN = gentamicin; NAL = nalidixic acid; STR = streptomycin; TRS = trimethoprim-sulfamethoxazole. 23

Figure 4-1: A simplified overview of different methods and analyses that were used. This outlines the workflow from sampling to the final qualitative or quantitative results including antibiotic resistance gene copy numbers and microbiome identification and distribution. 48

Figure 4-2: Average gene copy number of antibiotic resistance genes represented on a qPCR array. DNA was extracted from 18 samples of influent to a wastewater plant. The samples were pooled into a composite sample and assayed in duplicates on two different arrays..... 53

Figure 4-3: Distribution of ARGs stratified by antimicrobial class against which they encode resistance after whole genome sequencing and gene annotation for ARGs in MEGARes databases. This graph denotes ARGs and corresponding antimicrobial classes common to MEGARes and CARD databases for comparison. DNA was extracted from 18 influent samples and then pooled into a composite sample that was analysed by whole genomic sequencing. 54

Figure 4-4: Distribution of ARGs stratified by antimicrobial class against which they encode resistance after whole genome sequencing and gene annotation in the CARD database. This graph denotes ARGs and corresponding antimicrobial classes common to MEGARes and CARD databases for comparison. DNA was extracted from 18 influent samples, pooled into a composite sample, and then analysed by whole genome sequencing. 55

Figure 4-5: Z-score for normalised gene copy numbers of ARGs identified by qPCR (blue) and whole genome sequencing followed by gene annotation in the MEGARes (orange) and CARD (grey) databases. Bars represented by lighter, faded colours indicate Z-scores equal to zero actual abundance. DNA was extracted from 18 influent samples, pooled into a composite sample, and then analysed by qPCR and the respective metagenomic DNA sequencing approaches. 57

Figure 4-6: Distribution of ARG sequences stratified by antimicrobial class against which they encode resistance after whole genome sequencing and gene annotation in the CARD and MEGARes database. (A) Relative ARG sequence abundance by antimicrobial class and (B), Alpha diversity measure of observed richness and evenness of ARG distribution..... 60

Figure 4-7: Distribution of ARG sequences stratified by antimicrobial class against which they encode resistance after whole genome sequencing, and RNA sequencing with gene annotation in the MEGARes database. (A) Relative ARG abundance stratified by antimicrobial class in DNA and RNA sequences, (B) Relative abundance of specific ARGs in DNA and RNA sequences. 62

Figure 4-8: Visualised model coefficients to illustrate differences in relative ARG expression in DNA compared to RNA (A) and, the antimicrobial class against which resistance is encoded (B). Values above zero indicate relatively higher abundance in DNA than RNA, and values below zero indicate relatively higher abundance in RNA than DNA. 63

Figure 4-9: A comparison of the distribution and relative proportion for classes of bacteria constituting the top 100 genera following 16S rRNA gene sequencing with taxonomic annotation in GreenGenes and SILVA, and after whole genome sequencing with taxonomic annotation using Kraken2 and the NCBI nucleotide database (A), and alpha diversity measure of observed richness and evenness (B). DNA was extracted from 18 influent samples, pooled into a composite sample, and then analysed. 66

Figure 4-10: Relative abundance and distribution of bacterial class in 18 individual samples and the pooled composite sample following 16S rRNA gene sequencing and bacterial class annotation using SILVA. 67

Figure 5-1: Visualised sampling timetable of CBSTP samples collected between October 2021 and July 2022. 76

Figure 5-2: Visualised flowchart of DNA extracted from CBSTP samples, describing the analysis of genomic data obtained through whole genome sequencing. 79

Figure 5-3: Alpha diversity measures of Richness and Evenness for the microbiome and resistome communities identified within various areas of the CBSTP, depicted as whisker plots with medians and the interquartile range. 82

Figure 5-4: Principle Component Analysis (PCA) depicting differences in microbiome and resistome community distributions within three separate sampling locations of the CBSTP. 84

Figure 5-5: Venn diagram of core (A) resistome and (B) microbiome per sample type. 86

Figure 5-6: Heatmap describing the correlations between proportions of influent (left) and effluent (right) core microbiomes compared to the shared resistome of both locations. Where red colours annotate positive correlations, blue colours annotate negative correlations (colour depth varies based on the strength of the correlation), and ‘x’ annotates correlations found to be statistically significant. 90

Figure 5-7: The ratio of ARG OTUs to bacterial OTUs identified between the three tested locations of the CBSTP, depicted via a box and whisker plot with medians and the interquartile range. 90

Figure 5-8: Antibiotic resistant genes (representing multiple sequences) found to be significantly differentially expressed in the (A) influent, (B) Effluent, and (C) biosolids over the sampling period of the CBSTP, depicted as temporal line graphs. 93

Figure 6-1: Sampling distance categories utilised in Cleveland Bay as the receiving environment of the Cleveland Bay Sewage Treatment Plant, where the three Near Field Sample locations are indicated in yellow, the 15 Mid-Field Sample locations are indicated in green, and the 29 Far Field Sample locations are indicated in red. Samples from locations indicated in blue could not be acquired. Map lines delineate study areas and do not necessarily depict accepted national boundaries..... 102

Figure 6-2: Locations from which samples were acquired in the Howick Group of Islands to be utilised as a reference area. Specific sampling locations are indicated in yellow. Map lines delineate study areas and do not necessarily depict accepted national boundaries. 104

Figure 6-3: Alpha diversity measures of Richness and Evenness for the microbiome and resistome communities identified within different sample types Effluent, seagrass, seawater, and sediment (x-axis), Data are presented as medians with interquartile range and depicted as box and whisker plots. Sample colours correspond to sample type, as annotated in the legend where (A) represents the richness of the resistome, (B) represents the evenness of the resistome, (C) represents the richness of the microbiome, and (D) represents the evenness of the microbiome identified in separate sample matrices. 109

Figure 6-4: Bar graph depicting the relative abundance of antibiotic resistant gene operational taxonomic units of each sample type in each distance category (Near-Field, Mid-Field, Far-Field, and Howicks), stratified by antibiotic class to which ARGs encode resistance..... 111

Figure 6-5: Principal Component Analysis (PCA) depicting differences in resistome (A) and microbiome (B) community distributions within the sediment composite samples from separate distance categories, with Near-Field represented in blue, Mid-Field in yellow, Far-Field in green, and Howicks in red. 112

Figure 6-6: Venn diagram with a visual representation of the similarities and differences in core microbiome operational taxonomic units and resistome operational taxonomic units expressed within each sample type (sediment, seawater, and seagrass) across all distance categories (Near-Field, Mid-Field, Far-Field, and Howicks). 115

Figure 6-7: An illustration of the ratio of antibiotic resistant genes operational taxonomic units to bacterial operational taxonomic units in sediments at different distance categories (Near-Field, Mid-Field, Far-Field, and Howicks). Data are presented as medians with interquartile ranges and depicted as box and whisker plots. 116

Figure 6-8: Bar graph depicting the number of antibiotic resistant genes operational taxonomic units defined in the Cleveland Bay Sewage Treatment Plant effluent and their presence in the environmental samples (sediment, sweater and seagrass inclusive) of each distance category (Near-Field, Mid-Field, Far-Field, and Howicks). 117

Figure 6-9: Bar graph depicting the relative abundance of Cleveland Bay Sewage Treatment Plant effluent associated antibiotic resistant operational taxonomic units identified in each distance category (Near-Field, Mid-Field, Far-Field, and Howicks) as stratified by antibiotic class for which the antibiotic resistant genes encode resistance. 118

Figure 6-10: Heatmap describing the correlations between effluent Antibiotic resistant genes operational taxonomic units and effluent core bacterial operational taxonomic units. Where red colours annotate positive correlations, blue colours annotate negative correlations (colour depth varies based on the strength of the correlation), and ‘x’ annotates correlations found to be statistically significant. Example 1 is indicated in yellow, and Example 2 is indicated in orange. 122

Figure 6-11: Heatmap describing the correlations between effluent antibiotic resistant genes operational taxonomic units and Near-Field core bacterial operational taxonomic units. Where red colours annotate positive correlations and blue colours annotate negative correlations (colour depth varies based on the strength of the correlation) and ‘x’ annotates correlations found to be statistically significant. Example 1 is indicated in yellow, and Example 2 is indicated in orange. 124

List of Publications

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DRANE, K. 2021. Marine Testudines As Sentinels For Monitoring The Dissemination Of Antibiotic Resistance in Marine Environments (Best Paper Winner). *Australian Water Association – North Queensland Regional Conference – Reefs, Catchments and Resilience.* July 29-30, 2021. Mackay, Queensland, Australia.

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List of Abbreviations

AGRF - Australian Genome Research Facility

ARB – Antibiotic resistant bacteria

ARG - Antibiotic resistance gene

CARD – Comprehensive Antibiotic Resistance Database

CBSTP – Cleveland Bay Sewage Treatment Plant

CEC – Contaminant of emerging concern

eARG – Environmental/ extracellular antibiotic resistant gene

eDNA - Environmental/ extracellular Deoxyribonucleic acid

GCN – Gene copy number

GF – Gene fraction

HGT – Horizontal gene transfer

iARG – Intracellular antibiotic resistance gene

ICP/MS - Inductively coupled plasma mass spectrometry

MBR – Membrane bioreactor

MDRB – Multi-drug resistant bacteria

MGE – Mobile genetic element

MLS - Macrolides, lincosamides, streptogramines

qPCR - quantitative polymerase chain reaction

SM-Seq – shotgun metagenomic sequencing

WWTP – Wastewater treatment plant

. Chapter 1: Introduction

1.1 An Introduction to Antibiotic Resistance and One Health

Antibiotics (which are a subset of antimicrobials) are a comprehensive class of pharmaceuticals. There has been a constant discovery and modification of both naturally occurring and synthetically made antibiotics for public health, veterinary, and agricultural uses (Panthee et al., 2016), which has indirectly led to the current clinical antibiotic resistance pandemic affecting humans, animals, and environmental ecosystems in a One Health continuum. The World Health Organisation describes One Health as "an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals, and ecosystems". The effect of antibiotic resistance on public health has been extensively studied and is estimated to have caused nearly 1.3 million deaths in 2019 alone (Murray et al., 2022). Most of the global burden of antibiotic resistance is attributed to antibiotic resistant bacteria (ARBs) in clinical settings (French, 2005, Li et al., 2015b, Mbhele et al., 2021). However, there are resistant infections that have been attributed to ARBs acquired from environmental settings (Aminov, 2021, Balasa et al., 2021, Leonard et al., 2022). How humans are exposed to ARBs in the environment is poorly understood and varies greatly, but some examples include recreational water (Leonard et al., 2022), beach sand (Akanbi et al., 2017), consumable produce (Ghosh and Mandal, 2010), and the air (Gwenzi et al., 2022). There is a need, therefore, for a One Health approach when assessing antibiotic resistance, as the literature is dominated by consequences of ARBs to public health, and as such, there is historically less emphasis on antimicrobial resistance in environmental microbiomes and the horizontal gene transfer (HGT) of that resistance to human pathogens. Similarly, even less is known about the implications of ARBs and contamination with antibiotics, on the natural environment.

1.1.1 Contributing Components of Antibiotic Resistance

Some forms of antibiotics are not only naturally occurring, but the process of microbes creating and utilising antibiotics is both natural and ancient; therefore, the presence of bacteria that are resistant to these antibiotics is just as ancient (Perry et al., 2016). ARBs that are naturally resistant to antibiotics are generally referred to as intrinsically resistant, with the resistance gene (or multiple genes) often located on the bacterial chromosome. Additionally, bacteria may be unaffected by an antibiotic due to the absence of the targeted pathway, or natural mutations at binding sites of critical molecular targets. However, the current escalation of resistance to these compounds is predominantly attributed to acquired resistance (Karkman et al., 2018).

There are multiple mechanisms that bacterial cells utilise to protect against exposure to antibiotics, all of which are encoded in DNA as genes, otherwise, known as antibiotic resistance genes (ARGs). ARGs can be encoded for by extracellular or environmental DNA (eDNA); referred to as extracellular or environmental ARGs (eARGs). In contrast, "intracellular" ARGs (iARGs), are chromosomal genes or genes on other genetic material (e.g. bacterial plasmids) contained within the bacterial cell. These iARGs can be shared through HGT mechanisms to produce new ARBs. Additionally, new ARBs can be produced through HGT mechanisms when ARGs are acquired by bacteria via bacterial transformation, transduction, and conjugation. The HGT of resistance genes is often mediated by mobile genetic elements (MGEs), with the most significant MGEs for the HGT of resistance genes being plasmids and integrons (Leungtongkam et al., 2018). These principles are important for understanding this concept from a One Health perspective, as the interactions between ARGs, ARBs, and antibiotics are crucial for the dissemination of antibiotic resistance in the environment, animals, and humans.

1.2 Antibiotic Resistance Pollution

The term antibiotic resistance pollution is used broadly to describe the release of material (solid or liquid) containing antibiotics, ARBs, ARGs, or any combination thereof. However, for this thesis, the term antibiotic resistance pollution refers to the unnatural production and release of ARBs and ARGs into the environment (whether they are iARGs contained within ARBs or as eARGs). Antibiotic resistance pollution often, but not always occurs in tandem with antibiotic pollution, i.e., the release of antibiotic-laden effluent into the natural environment. Whilst these two terms are similar and often occur simultaneously, antibiotics and ARGs should be considered separately because of differences in physical properties, identification methods, and dissemination ability (Sabri et al., 2020, Sanderson et al., 2016, Sanderson et al., 2019, Hong et al., 2018, Östman et al., 2017). Individual antibiotics can be tested for and quantified directly using analytical methods such as liquid chromatography with the mass analysis capabilities of tandem mass spectrometry (Östman et al., 2017). By contrast, iARGs within ARBs can be identified through the presence of functional ARGs by using culture and antibiotic sensitivity tests (Hong et al., 2018, Karkman et al., 2018, Harnisz et al., 2020). Additionally, specific ARGs can be identified by searching for corresponding DNA sequences among all other DNA sequences within the sample through a variety of different testing methods (Harnisz et al., 2020, Karkman et al., 2018).

The release of antibiotics, ARBs, and ARGs into the natural environment is attributed to a multitude of sources. Generally, these sources include hospitals and animal health clinics, industrial

waste, agriculture production facilities, and domestic sewage which is often laden with antibiotics from general medical use within the local population (Kümmerer, 2009, Almakki et al., 2019). It is important to preface this background, by stating that most wastewater treatment plants (WWTPs) effluents are not the largest sources of antibiotics or resistant bacteria, they are simply a hub. There is extensive literature to suggest that levels of ARGs, antibiotics, and ARB in WWTPs effluent are significantly lower than that of other known sources, such as direct untreated hospital waste (Alexander et al., 2020), waste from the pharmaceutical industry (Guardabassi et al., 1998, Binh et al., 2018), abattoirs (Foyle et al., 2023), and in some cases agricultural runoff (Lu et al., 2022). However, WWTPs are often the destination of these types of waste and act as a site for ARGs, ARBs, and antibiotics from multiple sources to combine and interact. As such, WWTP influents often have high comparable levels of ARG, ARB, and antibiotics and this allows for the mixing of resistance originating from different sources (Alexander et al., 2020, Foyle et al., 2023, Guardabassi et al., 1998).

1.3 Effects of Antibiotic Resistance Pollution on the Receiving Environment

Though ARB, antibiotics, and ARGs should be considered as separate contaminants, they do often occur in tandem, and the co-occurrence of these contaminants can cause a greater impact on the environmental microbiome in an effluent-receiving environment, through selective pressure. Selection pressure reduces the abundance of susceptible bacterial species and changes the composition of the microbiome to be heavily dominated by ARBs, thus potentially resulting in a loss of key bacterial groups responsible for critical functions within an ecosystem (Martinez, 2009, Li et al., 2021). Limited documentation exists on the effect of antibiotic resistance on inhabitants of marine environments and most of this information has relied on robust detection systems using animal sentinel species as bioindicators. Bioindicator species allow for early indications of ecological threats by natural and human activities, such as antibiotic resistance pollution (Al-Bahry et al., 2009b, Paulson et al., 2016). Marine turtle species have been utilised as a sentinel species for monitoring the health of marine ecosystems (Figgener et al., 2019, Fraga et al., 2018), and antibiotic resistant bacteria associated with these species have been utilised as a bio-indicator of anthropogenic pollution within the marine environment (Al-Bahry et al., 2009a).

In general, this brief background defines potential effects, but it also outlines some of the critical factors affecting the dissemination of antibiotic resistance between humans, animals, and environmental ecosystems in a One Health continuum. A pertinent example of a human-animal-environment interaction that may influence the dissemination of antibiotic resistance is a sewage

treatment plant in Cleveland Bay (CBSTP), that discharges treated effluent into the general use zone of the Great Barrier Reef Marine Park (GBRMP) on the Northeast coastline of Australia in Townsville. The role of the CBSTP in dissemination of antimicrobial resistance in the receiving marine environment and its effects have not been studied previously. However, previous studies on marine turtles in the area have postulated the effects of wastewater effluents on its receiving marine habitat (Ahasan et al., 2018).

The aim of this study was to determine the prevalence, and distribution pattern of ARGs as well as the microbiome in the CBSTP influent, effluent, and in the immediate receiving environment adjacent to the GBRMP. The putative impacts of the antibiotics and ARGs released from the CBSTP on environmental microbial diversity in Cleveland Bay were examined. The specific research questions with corresponding aims, and a general outline of the thesis are provided below.

Chapter two is entitled “Testudines as sentinels for monitoring the dissemination of antibiotic resistance in marine environments: An integrative review”. This literature review examines the feasibility, knowledge gaps, and limitations of using turtles as bioindicators for the presence and dissemination of antimicrobial resistance in marine environments, as well as defined WWTP as a potential source of antibiotic resistance pollution identified in marine turtle species previously.

Chapter three is titled “The Role of Wastewater Treatment Plants in Dissemination of Antibiotic Resistance: Source, Measurement, Removal and Risk Assessment”. This is a scoping literature review on ARBs and ARGs identified in WWTPs. This chapter covers different forms of wastewater treatment and their ability to remove antibiotic resistance, as well as outlines the knowledge gaps for defining ARG dissemination in receiving environments. Herein, a possible framework for identifying the risk of ARGs in WWTP to public health and the environment is also discussed.

Chapter four is titled “Concordance in molecular methods for detection of antimicrobial resistance: A cross-sectional study of the Influent to a wastewater plant”. This chapter provides a detailed description and validation of core molecular biology methods that were used throughout the thesis.

Chapter five is titled “Antimicrobial Resistance Profiling in Sewage Processed by a Membrane Bioreactor Plant: Implications for Release into the Great Barrier Reef”. This chapter addressed the specific research question of whether the CBSTP significantly reduces the relative ARG load in the effluent compared to the influent. The specific aim was to determine the intra-plant spatial (influent, biosolids, and effluent) and temporal distribution of ARGs and associated microbiomes.

Chapter six is titled “Dissemination of Antibiotics and Antibiotics Resistance Genes from a Membrane Bioreactor Wastewater Plant on the Great Barrier Reef, Australia: Implications for Marine Microbial Communities”. The specific research question was, the extent of ARG spread in the receiving environment and whether ARGs in the effluent from the CBSTP affect the environmental microbiome. The specific aim was to determine the spatial distribution of ARGs and associated microbiomes in the immediate (near), mid-, and far-fields of the receiving environment.

Chapter seven presents the general thesis discussion, conclusions, and future directions.

Collectively, this study provides valuable insight into the effect of anthropogenic activities associated with wastewater processing on the ecological health of the Cleveland Bay coastal area that is adjacent to the Great Barrier Reef on the Northeast coastline of Australia. This work will assist in informing environmental risk assessments and in designing mitigation strategies for antibiotic resistance pollution in this highly valuable ecosystem.

. Chapter 2: Marine Testudines as Sentinels for Monitoring the Dissemination of Antibiotic Resistance in Marine Environments: An Integrative Review

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Note: Variation from the published manuscript exists in the description of whole genome sequencing, which is adapted to shot-gun metagenomic sequencing for continuity purposes with the remaining thesis body of work.

Dissemination of antibiotic resistance in marine environments is a global concern with a propensity to affect public health and many ecosystems worldwide. We evaluated the use of sea turtles as sentinel species for monitoring Antibiotic resistance in marine environments. In this field, antibiotic resistant bacteria have been commonly identified by using standard culture and sensitivity tests, leading to an overrepresentation of specific, culturable bacterial classes in the available literature. Antibiotic resistance was detected against all major antibiotic classes, but the highest cumulative global frequency of resistance in all represented geographical sites was against the beta-lactam class by a two-fold difference compared to all other antibiotics. Wastewater facilities and turtle rehabilitation centers were associated with higher incidences of multidrug-resistant bacteria accounting for an average of 58% and 49% of resistant isolates, respectively. Furthermore, a relatively similar prevalence of multi-drug resistant bacteria (MDRB) was seen in all studied locations. These data suggest that anthropogenically driven selection pressures for the development of Antibiotic resistance in sea turtles and marine environments are relatively similar worldwide. There is a need however, to establish direct demonstrable associations between Antibiotic resistance in sea turtles in their respective marine environments with wastewater facilities and other anthropogenic activities worldwide.

2.1 Introduction

Antibiotics have provided effective and safe treatments for bacterial infections and are an essential tool in modern medicine (Hawkey, 2008). Unfortunately, the rise in antibiotic resistance among common bacterial pathogens is of increasing concern, and infections produced by multiple drug-resistant bacteria (MDRB) are associated with an increased frequency of mortality (Sydnor and Perl, 2011, Pacios et al., 2020). To address this problem, the World Health Organisation has commissioned a series of action plans including increasing the awareness of the magnitude and impact of antibiotic resistance through communication, training, education, surveillance, and research (Collignon et al., 2018). Dissemination and propagation of antibiotic resistance in the

marine environment is also an increasing concern, as this may affect previously unpolluted microbial communities within the world's oceans (Deng et al., 2020, Pruden et al., 2006). In effect, a higher prevalence of MDRB in marine environments threatens marine wildlife but also serves as a platform for potential transmission of environmental antibiotic resistant genes (ARGs) to human pathogens. For instance, antibiotic resistance and virulence genes that are characteristically linked with methicillin-resistant *Staphylococcus aureus* were isolated in healthy edible marine fish in South Africa (Fri et al., 2020). Similarly, an additional review (Aguirre et al., 2006) reported examples of infection and disease caused by *Vibrio* spp. and *Salmonella* spp. in humans following the consumption of sea turtle meat and eggs.

Despite the existence of these specific examples pertaining to the threat of antibiotic resistance in the marine environment, our comprehensive understanding of ecological and anthropologic factors that contribute to the development and dissemination of antibiotic resistance in such environments is lacking. To study and elucidate factors that contribute to antibiotic resistance in otherwise very diverse and complex marine ecosystems, sentinel animal species whose health is considered to reflect the wellbeing of ecosystems should be used. Sea turtles are considered a good sentinel species for monitoring the health of marine ecosystems having been used in many toxicological and trophic ecology studies (Figgener et al., 2019, Fraga et al., 2018). This value as sentinels is because of their long life span and the tendency to bioaccumulate contaminants and pathogens (Ahasan et al., 2018, Kraemer et al., 2019, Paulson et al., 2016); furthermore, antibiotic resistant bacteria have been described previously as a bio-indicator of anthropogenic pollution within the marine environment (Al-Bahry et al., 2009a). However, no study has provided a critical integrative evaluation of the role played by sea turtles in monitoring the prevalence, or dissemination of antibiotic resistance in marine environments globally. In this review, we provide a critical appraisal on the utilization of turtles as sentinels in determining causes, prevalence, and distribution patterns of antibiotic resistance in marine ecosystems.

2.1.1 Commonly Used Antibiotics, Their Modes of Action, and Mechanisms of Antibiotic Resistance

Many of the antibiotic compounds used in medicine and agriculture are naturally occurring, and, therefore, co-existing bacteria have evolved mechanisms to survive exposure to antibiotics or other environmental threats (Kraemer et al., 2019). This means that bacterial populations are often intrinsically resistant to one or more antibiotics (Munita and Arias, 2016). General mechanisms by which bacteria become intrinsically resistant to antibiotics include the alteration of the target sites,

adjustment or destruction of the antibiotic molecule, and removal of the antibiotic molecule via efflux transporters, or reduced influx of the antibiotic through decreased membrane permeability (Munita and Arias, 2016). Mutation in genes that code for these molecular targets of antibiotic compounds allows the mutated bacterial cell to survive exposure to the antibiotic compound where other cells would be susceptible (Hawkey, 2008, Munita and Arias, 2016). These mechanisms of resistance may be present in a single bacterial cell simultaneously, allowing for resistance to multiple antibiotic molecules (Nikaido, 2009). Acquired resistance is of greatest concern, as it almost always results from the acquisition of ARGs from intrinsically resistant bacteria (Kraemer et al., 2019). A prominent mechanism of propagating resistance from one bacterial cell to another is the acquisition of foreign ARGs through horizontal gene transfer (HGT), these ARGs may then be cloned to subsequent daughter cells (vertical gene transfer). HGT accounts for the majority of antibiotic resistance that develops in previously susceptible bacteria (Raleigh and Low, 2013).

2.1.2 Antibiotics and Antibiotic Resistant Bacteria in Marine Environments

Marine ecosystems are home to the largest and most diverse communities of flora and fauna, presenting different habitats that cover 75% of the earth's surface and harbour over 90% of all life on earth, including bacteria (Belkin and Colwell, 2005). Marine dwelling bacteria can be located in seawater (estimated at 10^6 cells/mL), sediment on the seafloor (estimated at 10^8 cells/mL) and, on or within macroscopic organisms such as turtles, fish, and sea mammals (Belkin and Colwell, 2005, Whitman et al., 1998, Schloss and Handelsman, 2006). Furthermore, the gastrointestinal systems of sea vertebrates represent a highly diverse microbial community. For example, a study on the gut bacterial flora in green turtles determined that there were over 174 identifiable bacterial families within the gastrointestinal site (Ahasan et al., 2018).

The dissemination of antibiotics and resistance-causing genes into marine environments is a major global concern, as it allows for the selection of antibiotic resistant bacteria (ARB) within these highly concentrated and diverse bacterial populations (Kraemer et al., 2019). Research efforts to address this problem have identified wastewater treatment plants (WWTPs) and run-off from agricultural industry as major contributors to the dissemination of antibiotics and resistant bacteria in marine environments (Cowieson and Kluenter, 2019, Kümmerer, 2009). Globally, influents into WWTPs and WWTP treatment processes vary greatly. Effluent of varying quality is discharged into highly diverse marine environments. From the point of view of antibiotic resistance, the most significant sources of influents into WWTPs are hospital effluents, industry effluents, particularly intensive agricultural practices, and domestic wastewater or sewage created by the general populace

(Kümmerer, 2009). This type of waste is usually enriched with antibiotics and other antimicrobial agents, bacteria including antibiotic resistant pathogens, and other biological molecules such as free plasmids and "naked DNA" which may be vectors for ARGs and allow for HGT (Chen and Zhang, 2013b). Collectively, WWTPs have the propensity to generate, concentrate, and disseminate ARGs and MDRB in the immediate receiving marine environments (Kraemer et al., 2019, Chen and Zhang, 2013b). It is worth noting, however, that different WWTPs across geographical locations employ different technologies to treat and process sewage; this may restrain the release of ARGs to varying degrees (Rizzo et al., 2013, Pazda et al., 2019). This issue is complicated further by the fact that policies that govern the implementation of processes and technologies differ internationally (Chen and Zhang, 2013b, Diehl and LaPara, 2010, Reinthaler et al., 2003). The global difference in WWTP policy and technology may allow some geographic areas to have a higher burden of antibiotic resistance pollution disseminated into their environment by WWTPs. Furthermore, other countries with more restrictive policies and advanced treatment technologies may be capable of reducing antibiotic resistance from their influent sources through their wastewater treatment process.

Run-off from the agricultural industry that may not be directly associated with WWTPs is another important source of antibiotics, bacteria, and ARGs in marine environments. There is extensive antibiotic use in the agricultural industry of many countries not to just treat present infections, but to utilise broad-spectrum antibiotics as growth promoters, which pre-emptively prevent infection in the short term. This standard agricultural practice of long-term utilisation of broad-spectrum antibiotics can provide the perfect environment for cultivating ARB and propagating ARGs (Cowieson and Kluenter, 2019, Chee-Sanford et al., 2009, Woolhouse et al., 2015). To put this into context, the cumulative global usage of antibiotics in agricultural facilities exceeds the usage in hospitals (Chee-Sanford et al., 2009). To counteract this problem, a few countries and particularly those in the European Union are now implementing stricter regulations on antibiotic use in agriculture. Nonetheless, this practice has persisted in many countries such as India, China, Spain, the USA, and Italy that have a high agriculture turnover (Ronquillo and Hernandez, 2017, Cuong et al., 2018). This exposure to different antibiotic compounds affects off-target bacterial populations, exerts intensive selection pressure within the microbiome of treated animals and, it results in the shedding of ARB and antibiotics in manure which then contaminates surface and groundwater (Grenni et al., 2018, Pruden et al., 2006). This too, creates antibiotic resistance "hot spots" providing pathways for contamination of marine environments with antibiotics, ARB and ARGs.

WWTPs and run-off from the agricultural industry are important "hot spots" for the antibiotic resistance pollution of marine environments. These sources of contamination provide the mechanisms and processes that underlie the development and dissemination of antibiotic resistance

in aquatic environments. Additionally, the presence of antibiotic resistance in these marine environments from these sources creates potential harmful effects for an indiscriminate spread of antibiotic resistance on marine ecosystems as well as public health. It is important, therefore, to develop a robust, reliable, and universally applicable system for monitoring the spread of antibiotic resistance in marine ecosystems. Herein, we sought to evaluate sea turtles as sentinel species for monitoring as well as reporting incidences, dissemination, and distribution patterns of antibiotic resistance in marine environments.

2.2 Methods used to Evaluate Turtles as Sentinels for Monitoring Antibiotic Resistance in Marine Environments

2.2.1 Systematic Literature Search Strategy, Inclusion and Exclusion Criteria, and Quality Assessment

Relevant literature was gathered systematically from three bibliographic databases: Medline (Ovid), Scopus, and Web of Science. Search terms used were as follows: "Antibiotic Resistance Genes" or "Antibiotic Resistance"; and "turtle" or "Testudines". This allowed for the identification of older documents analysing antibiotics by culture to obtain resistance identification, as well as newer documents identifying antibiotic resistance genes through metagenomic methods. Eligibility criteria were not limited to publication types, including journal articles, reviews, and case studies. The eligibility criteria did not include language limitations and coincidentally, all articles found to fit the eligibility criteria were publications in English. The exclusion criteria included papers that were not focused on the study of (or previous study of) antibiotic resistance in marine turtles. There was no limitation on publication dates and all used databases were searched from time of inception to December 2020. To allow for a critical review of the literature, subsequent citations from the results of the previously found articles were also appraised for suitability and inclusion in this review. Quality appraisal was ensured by using the CASP tool checklist relevant to each piece of literature, which allowed for the evaluation of the quality of each publication (Long et al., 2020). The literature search process is summarised in Figure 2.1.

2.2.2 Quantitative Evaluation of Compiled Data

All relevant data were extracted from text, figures, and Tables. The data were then stratified according to predetermined variables including turtle species involved, anatomical sampling sites on turtles, the most prevalent bacterial isolates, classification of tested antibiotics, geographical locations and, the potential source of antibiotic resistance. Quantitative data on antibiotic resistance

was presented as mean percentages of observed antibiotic resistance in confirmed bacterial isolates. Where applicable, statistical comparisons between groups were done using Kruskal-Wallis test, and $P < 0.05$ was considered significant.

2.3 Results and Discussion of Data on Using Turtles for Monitoring Antibiotic Resistance in Marine Environments

2.3.1 An overview of the Systematic Search of the Literature on Using Sea Turtles for Monitoring Antibiotic Resistance

Fourteen published studies focusing on antibiotic resistance, ARB, and ARG isolated in sea turtle species (Figure 2.1). Two of these literature pieces were case studies of specific antibiotic resistance isolates in a singular diseased animal, and the remaining 12 publications were peer-reviewed journal articles describing studies on this theme. The two case studies on individual turtles were not included for statistical analysis, as their focus was on the identification of specific resistance against one antibiotic and thus were not representative of a studied population. Twelve published studies were included in statistical analyses for the metadata. These were: (Ahasan et al., 2017, Al-Bahry et al., 2009a, Al-Bahry et al., 2011, Al-Bahry et al., 2012, Alduina et al., 2020, Delli Paoli Carini et al., 2017, Foti et al., 2009, Oliveira et al., 2017, Goldberg et al., 2019, Pace et al., 2019, Prichula et al., 2016, Zavala-Norzagaray et al., 2015).

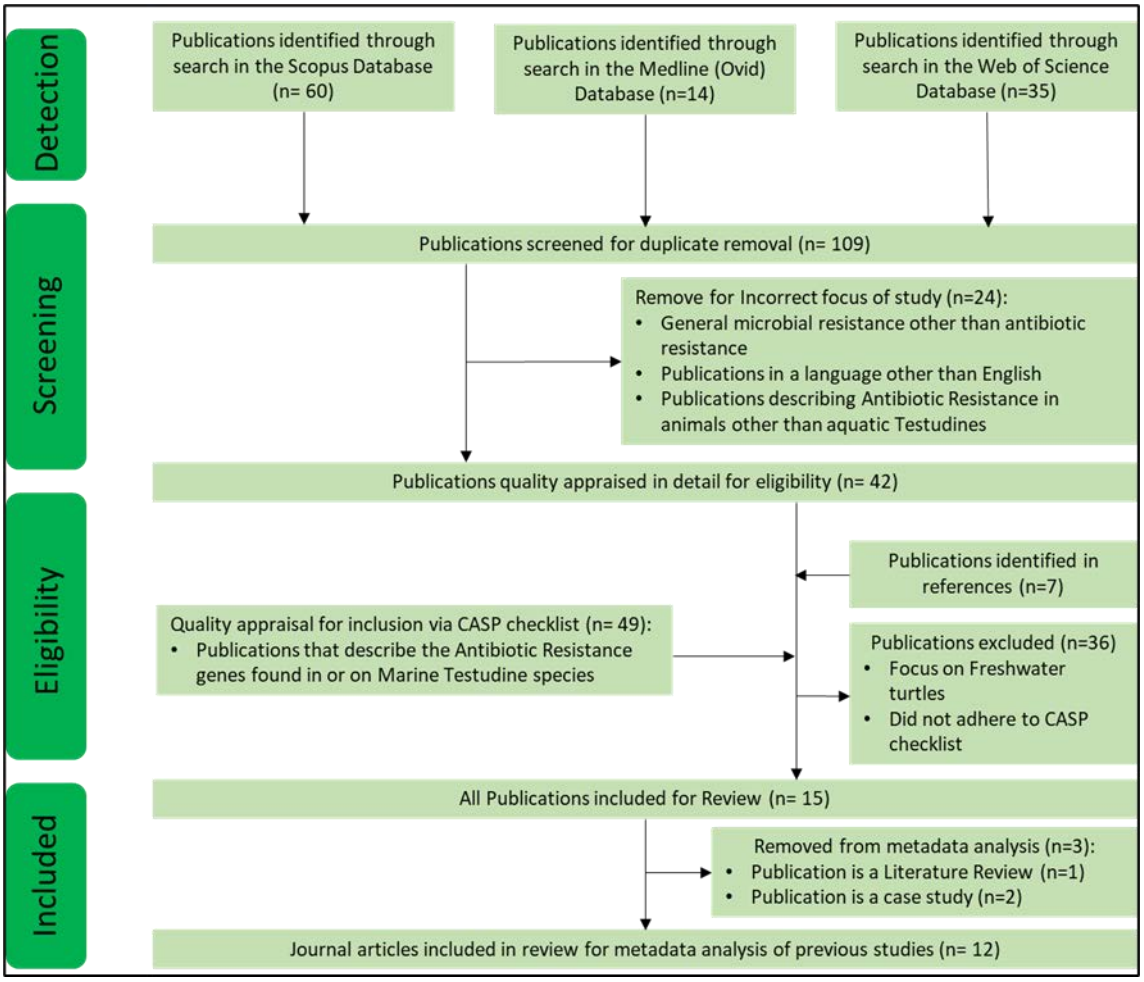


Figure 2-1:Summarised process for the literature search indicating detection, screening, and eligibility of included publications. Fourteen articles were retained for final analysis but only 12 of these were direct studies on the abundance and effect of ARB in marine turtles. Twelve studies were included for analysis of the metadata.

2.3.2 Distribution of Studies on Antibiotic Resistance in Marine Environments by Geographical Site and Turtle Species

2.3.2.1 Antibiotic Resistance in Turtles in Marine Environments and the Potential Influence of Geographical Location

The geographic distribution of marine turtles is worldwide with an exception of the Arctic and Antarctic waters (Hirth, 1997). The highly migratory nature allows sea turtles to utilize foraging areas that often exceed thousands of kilometres during migration every few years (Witherington et al., 2009). To date, studies on the use of turtles to monitor antibiotic resistance in marine environments have examined at least five turtle species including green, loggerhead, leatherback, olive ridley, and hawksbill sea turtles, but the green and loggerhead turtles represent the majority of

these studies in vastly different geographical sites. While antibiotic usage rates and patterns of ARB also vary markedly across geographical areas (Brasg et al., 2017), currently available studies on turtles only represent a very limited geographical distribution including the Mediterranean and Arabian seas, the Pacific Ocean along the east coast of Australia, and the South Atlantic Ocean along the west coast of Africa and the east coast of South America (Figure 2.2 and Table 2.1). Considering that wastewater through WWTPs and run-off of industry and agricultural waste are the major contributors to antibiotic resistance in marine environments, it is important to take the international policies of these antibiotic resistance "hot spots" into account when describing sources of pollution in geographical areas.

The coastal areas of the Mediterranean and Arabian Seas are shared by 21 and 6 nations respectively (Figure 2.2). These countries have marked variations in policies on antibiotic usage and efficiency in wastewater treatment. For example, up to 51% of the treated wastewater in countries around the Mediterranean and Arabian seas is recycled and utilised as agricultural irrigation (Sato et al., 2013), which may further increase the propagation of antibiotic resistance by renewing the cycle of ARB presence and exposure (Collignon et al., 2018). This argument is supported by the fact that similar antibiotic resistance patterns were observed in bacterial isolates from a WWTP in Oman, and marine turtles in the Arabian Sea and the Gulf of Oman, suggesting that the resistance in the marine turtles and wastewater effluent are directly linked (Al-Bahry et al., 2009a, Al-Bahry et al., 2011, Al-Bahry et al., 2012). Similar anthropogenic waste disseminated from WWTPs was described as the putative point source of ARB in turtles in the Mediterranean sea in Italy (Foti et al., 2009, Pace et al., 2019), and the South Atlantic ocean in Brazil (Prichula et al., 2016). Furthermore, on the east coast of the Pacific Ocean in Australia, sea turtles were tested for antibiotic resistance in turtle rehabilitation centres, as well as the surrounding bay area's (Ahasan et al., 2017, Delli Paoli Carini et al., 2017). This site is of note, as it has many resident green turtles that inhabit the receiving environment of WWTPs, and this receiving environment is also situated on the Great Barrier Reef. WWTP's associated with the world's largest coral reef system have unique legislative and regulatory pressures with respect to the environment. However, there are currently no legislative requirements either in this region, or world-wide, to reduce the quantity of antibiotic compounds and ARGs in wastewater effluent. The Great Barrier Reef relies heavily on its diverse bacterial communities, and the discharging of both antibiotic compounds and ARG into this environment may provide a significant health risk to this world heritage site by creating selective pressures for specific bacterial communities and reducing the bacterial diversity. Fortunately, cities along this coast line are taking innovative steps to determine the potential effects of both antibiotic compounds and resistance isolates from WWTP's (Kuskopf et al., 2020), including the Townsville region where this ARB in

marine turtles was observed by both Australian studies (Ahasan et al., 2017, Delli Paoli Carini et al., 2017).

Many other studies describe the presence of ARB in turtles in the wild, with no clear identifiable source of antibiotic resistance (Ahasan et al., 2017, Alduina et al., 2020, Oliveira et al., 2017, Zavala-Norzagaray et al., 2015, Dutton et al., 2013). The Mediterranean Sea is surrounded by a heterogeneous group of 21 countries representing Western Europe, the Middle East, and North Africa. European Union countries surrounding this area, such as Spain, France, Italy, Greece, Croatia, and Slovenia have banned the use of antibiotics as growth promoters for agricultural farming since 2006 and, they have implemented a One Health action plan against antibiotic resistance since 2017 (Collignon et al., 2018). These EU countries have also instituted high standards requiring the treatment of at least 71% of all wastewater through WWTPs before discharging into the sea (Sato et al., 2013). However, the North African side of the Mediterranean sea is much less stringent in regulating the general use of antibiotic agents and the discharge of wastewater through WWTPs (Sato et al., 2013). This makes it difficult to attain an accurate and reliable assessment of the geographical origins of antibiotic resistance observed within turtles in marine environments across different jurisdictions in the world.



Figure 2-2: Map of geographical location and clustering of studies on the monitoring of antibiotic resistance in marine environments using sea turtles as an animal sentinel species (red pins). Represented areas are the Mediterranean and Arabian seas, the Pacific Ocean along the east coast of Australia, and the South AtlanticOcean along the west coast of Africa and the east coast of South America (Modified from Google maps under the terms and conditions of the Creative Commons Attribution (CC BY) license)

Table 2-1: Global distribution of geographical sites where different sea turtle species have been examined as sentinel species for Antibiotic Resistance. The plus sign (+) denotes the isolation of multi-drug resistant bacterial isolates, and the hyphen (-) denotes that multi-drug resistant strains were not identified.

Turtle species	Food sources in marine environments	Geographical site & presumed ARB source, biological sample, and dominant bacterial isolate	MDRB
Green *(Chelonia mydas)*	Juveniles: Shrimp, crabs, clams, mussels, fish, squid. Adults: Seagrass, algae	NW Indian Ocean & Arabian sea, fresh eggs, *Psuedomonas spp*, (n =30). Al-Bahry et al., 2009.	+
		Gulf of Oman Arabian sea, oviductal fluid, *Citrobacter spp*, (n = 40). Al-Bahry et al., 2011.	+
		Gulf of Oman Arabian sea, oviductal fluid, *Citrobacter spp*, (n = 20). Al-Bahry et al., 2012.	+
		Pacific Ocean (Rehabilitation centres, NE Australia), ocular swabs, *Vibrio spp*, (n = 7). Carini et al., 2017.	+
		Atlantic Ocean (South coast of Brazil), cloacal or rectal swabs, *Enteroccus spp*, (n = 6). Prichula et al., 2016.	+
		Pacific Ocean (Baja California & Sinaloa Mexico), nasopharyngeal & oral swabs, *Vibrio spp*, (n = 42). Zavala-Norzagaray et al., 2015.	+
		Pacific Ocean (Rehabilitation centres & wild, NE Australia), cloacal swabs, *Citrobacter spp*, (n = 73). Ahasan et al., 2017.	+
Loggerhead *(Caretta caretta)*	Juveniles & adults: Shrimp, crabs, clams, mussels, fish, squid.	Mediterranean Sea (Rehabilitation centre in Italy), internal organs, cloacal, oral & skin swabs, *Aeromonas* & *Citrobacter spp*, (n = 20). Alduina et al., 2020.	+
		Central Mediterranean Sea (Italy), cloacal, oral & skin swabs, *Proteus* & *Citrobacter spp*, (n = 19). Foti et al., 20	+
		West Mediterranean Sea (Italy), oral & cloacal swabs, *Pseudomonas* & *Citrobacter spp*, (n = 35). Pace et al., 2019.	+
Leatherback *(Dermochelys coriacea)*	Juveniles & adults: Jellyfish, other soft-bodied animals, algae.	Atlantic Ocean (Gulf of Guinea, Principe Island), oral & cloacal swabs, *Pseudomonas spp*, (n = 10 Leatherback & n = 2 Green). Oliveira et al., 2017.	-
Olive Ridley *(Lepidochelys olivacea)*	Juveniles & adults: Shrimp, crabs, clams, mussels, fish, squid.	Pacific Ocean (Sinaloa Mexico), nasopharyngeal & oral swabs, *Vibrio spp*, (n = 22). Zavala-Norzagaray et al., 2015.	+
Hawksbill *(Eretmochelys imbricate)*	Juveniles and adults: Sponges, squid, shrimp, anemones.	Altantic ocean (South coast of Brazil), cloacal or rectal swabs, *Enteroccus spp*, (n = 2). Prichula et al., 2016.	+

2.3.3 Antibiotic Resistance in Marine Environments and the Potential Influence of Differences in Turtle Species

In all documented, peer-reviewed studies on the use of turtles to monitor antibiotic resistance in marine environments herein, green turtles (*Chelonia mydas*) represented the majority (~75%) of the publications (Table 2.1). The complex life-history traits and dietary variations of green turtles make them particularly vulnerable to a wide range of anthropogenic threats, including antibiotic resistance pollution (Paulson et al., 2016). Dietary variations throughout a green turtle's life, from omnivores as juveniles to herbivores as adults, along with their highly migratory nature exposes them to a diverse range of microbial communities and sources of pollution in different marine environments (Witherington et al., 2009). Based on the diversity in dietary requirements of sea turtles (summarised in Table 2.1), different sea turtle species may represent the level of contamination throughout their food chains thus providing information on the prevalence and relative abundance of ARB and ARGs in their food sources. It is conceivable that marine turtle species, like the leatherback (*Dermochelys coriacea*), which feeds almost exclusively on jellyfish, may accumulate antibiotics, ARGs, and ARB from any area in which the food source has travelled. Thus, turtle species depending on a more protein-rich diet may not represent antibiotic resistance profiles within their immediate environment, but possibly other geographical regions.

In contrast, more mature green turtles primarily feeding on seagrasses and algae in times of non-migration, are likely to provide more accurate and site-specific information on the bioaccumulation of antibiotics, ARGs, and ARB due to the stationary nature of seagrass and algae within near-shore environments. Certainly, the ability of seagrass to bioaccumulate other chemical compounds and pollutants has been documented previously (Faridahanim et al., 2014). However, distinguishing the time of last migration in adult green turtles is not possible without constant and costly satellite tracking, which was not noted in any of the literature studied for this review. Furthermore, some studies specifically utilised migrating and breeding adults, and specifically egg-laying females for research purposes (Al-Bahry et al., 2009a, Al-Bahry et al., 2012, Al-Bahry et al., 2011). Though this class of adults is easier to capture when in high numbers at breeding and egg-laying sites, the nature of migration may lead to the possibility that resistance within their microbiome is not representative of their current location. The antibiotic resistance within these adults of migrational age (i.e. sexual maturity) may represent any location passed between their residential feeding grounds and their breeding sites, which may be up to 2,600 kilometres away and contain any number of possible antibiotic resistance pollution dissemination points (Ahasan et al., 2017). The sub-adult class of green turtles may be a more effective animal model for assessing

antibiotic resistance within a specific geographical area. This age class of green turtles are resident to a particular area and have the same diet as adults of predominantly seagrass, though they have not reached sexual maturity, and therefore, do not migrate (Hancock et al., 2018, Shimada et al., 2014). However, age class and migration status were not consistently stated for each green turtle sampled throughout the seven studies focusing on this species (Ahasan et al., 2017, Al-Bahry et al., 2009a, Al-Bahry et al., 2012, Delli Paoli Carini et al., 2017, Prichula et al., 2016, Zavala-Norzagaray et al., 2015). Furthermore, two studies that included green turtles did mention that samples were taken from different age classes (from juvenile and adult), but did not differentiate antibiotic resistance results based on age class (Prichula et al., 2016, Ahasan et al., 2017). This potential variation in microbiome and antibiotic resistance compared to age, and sexual maturity may also be true for other marine turtle species; however, due to declining populations and elusiveness of other species, much less is known about their complex life-history traits.

Bacteria can colonise different anatomical regions of the turtle including the respiratory and reproductive tracts, the plastron or carapace, and more predominantly, the gastrointestinal tract (Wyneken et al., 2013). It is also likely that the dietary requirements for different turtle species determine the microbiota composition in their gastrointestinal systems. This factor is pertinent in monitoring antibiotic resistance using different turtle species because anatomic sites involved in the gastrointestinal tract are often utilised, such as cloacal or oral cavity swabs (Table 2.1). It was argued that ocular surface samples of turtles more accurately represent the surrounding environment, not affected by feeding or migratory habits (Delli Paoli Carini et al., 2017). Similarly, analyses of freshly hatched eggs and oviduct fluid may reflect the ability of marine turtles to serve as vectors for vertical transmission of antibiotic resistance from mother to hatchling and consequently serving as a carrier and spreader of antibiotic resistance in marine environments worldwide (Al-Bahry et al., 2009a, Alduina et al., 2020). These observations indicate that it is rational to recommend that any analyses on effects of exposure to antibiotic agents and ARB should account for resident bacteria in anatomical sampling sites, turtle species in question and geographical locations to ascertain significance.

2.3.4 Detection and Quantification of Antibiotic Resistance Profiles in Marine Environments

2.3.4.1 Present Challenges in Detecting ARB in Marine Turtles

The identification of ARB in biological samples obtained from turtles in marine environments is heavily dependent on our knowledge of the detectable resident microbiome in the different turtle species and more importantly, the methods used to isolate, detect, and characterise ARB. In all

published studies on antibiotic resistance in sea turtles to date, resistance has been identified in at least 48 different bacterial species. Gram-negative coliforms and particularly *Citrobacter spp* were the predominant bacteria representing 25% of all identified isolates. *Citrobacter spp* constitutes part of the normal gut flora of sea turtles but it has also been associated with severe infections in humans as well as captive and wild sea turtles (Ahasan et al., 2018, Goldberg et al., 2019, Foti et al., 2009, Huang et al., 2015). Sea turtles were also found to harbour other ARB such as *Salmonella spp* and *Pseudomonas spp,* representing an average of 11% and 16% of isolated ARB (Al-Bahry et al., 2009a, Al-Bahry et al., 2011, Alduina et al., 2020, Foti et al., 2009)(Table 2.1). *Salmonella spp* and *Pseudomonas spp* have the potential to cause severe zoonotic infections that are also resistant to treatment in humans. Indeed, severe salmonellosis associated with the consumption of turtle meat or eggs in some coastal locations has been documented previously (Oliveira et al., 2017, O'Grady and Krause, 1999). Zoonotic transmission of *Pseudomonas spp*. from turtles to humans linked to diseases including urinary and respiratory infections, meningitis, and endocarditis has also been reported (Warwick et al., 2013). From a One Health perspective, the proliferation of these types of ARB within marine turtles can have equally detrimental effects on the growth, maturation, and overall health of the host (Belkin and Colwell, 2005, Gibbons and Steffes, 2013, Sarmiento-Ramirez et al., 2014). Additionally, pathogenic ARB in sea turtles might signify a risk for other marine creatures and especially through predation (Alduina et al., 2020, Pace et al., 2019). This provides a mechanism for spreading ARB throughout the food chain (Bogomolni et al., 2008, Higgins, 2003, Spencer and Thompson, 2005). All these studies indicate that sea turtles are a good sentinel model for monitoring antibiotic resistance including the detection of potentially pathogenic ARB in marine environments. However, the true magnitude of ARB in entire microbiomes in turtles (resistome) has remained unknown.

In all documented studies to date (Table 2.1), identified common and predominant bacterial isolates were used to detect AR, but these are not representative of entire resistomes likely to be hosted and disseminated by sea turtles. This is primarily because all studies in this area have utilized many different methods to isolate and characterize ARB. Culturing bacteria followed by antibiotic sensitivity testing was the most commonly used method for monitoring antibiotic resistance in sea turtles. This approach limits the discovery of ARB only to bacteria that grow under laboratory conditions thus creating a bias that may over-represent specific genera of bacteria such as *Citrobacter*, *Pseudomonas*, *Enterococcus,* and *Vibrio*. It has been shown that microbiomes identified via culture methods account for only ~1% of the true microbial diversity (Edet et al., 2017). This is especially true for animals inhabiting marine environments where diverse bacterial communities exist (Hilton et al., 2016). This problem is likely to be resolved by extensive application of modern

molecular biology methods such as sequencing 16s ribosomal RNA gene and metagenomic sequencing of entire microbiomes in identifying ARB and ARGs in marine environments (Alcock et al., 2020). However, these newer approaches require relatively expensive facilities and highly skilled technical expertise but may start to become more readily available with advancements in these technologies. For this review, because of the outlined methodological limitations associated with isolating bacteria and identifying antibiotic resistance in sea turtles, reported frequencies and the distribution patterns of ARB were not stratified and analysed by genus or species of the bacterial isolates.

2.3.4.2 Quantitative Evaluation of Phenotypic Antibiotic Resistance Patterns in Marine Turtles

To identify and quantify the functional nature of acquired antibiotic resistance in sea turtles in marine environments, our analyses revealed that tests have been done against all major antibiotic classes including beta-lactams, tetracyclines, aminoglycosides, and quinolones. Only two turtle species (green and loggerhead) were represented by multiple studies at different geographical sites (Table 2.1). Irrespective of geographical site, biological specimen, and bacteria isolated, there were no statistically significant differences in reported frequencies of resistance against all tested antibiotics stratified by turtle species (Figure 2.3). Similarly, no differences in reported frequencies of resistance were observed when antibiotic resistance profiles were stratified by the anatomical origin of the specimen for bacterial isolation (Figure 2.3). Our analyses of the global distribution frequencies show that antibiotic resistance in turtles in marine environments is overrepresented by beta-lactams (penicillins, cephalosporins, monobactams, and carbapenems) by a two-fold difference compared to all other antibiotic classes. However, resistance was reported against all major antibiotic agents (Figure 2.4). While not all antibiotic agents have been tested for antibiotic resistance in all reported studies included in this review, there was no apparent difference in reported frequencies of resistance to commonly used, first-line drugs such as ampicillin, which was tested at multiple sites. Moreover, a relatively similar incidence of MDRB across all major antibiotic classes was observed in all represented geographical locations in the currently available studies (Figure 2.4). The analysis of this data when stratified by geographical area further suggests that the predominant age class of marine turtle that has been utilised for these previous studies (i.e., adults who are capable of migration, or currently migrating) may not adequately represent antibiotic resistance within an immediate area unless assurances can be made towards its residency and time of last migration. Furthermore, the occurrence of resistance to reserved antibiotic classes such as quinolones and aminoglycosides is truly worrying as these drugs are usually the last line of defence in

many life-threatening infections. Collectively, data collected for this review seems to suggest that irrespective of geographical location, the selection pressures that have driven the development of antibiotic resistance in sea turtles and marine environments are similar.

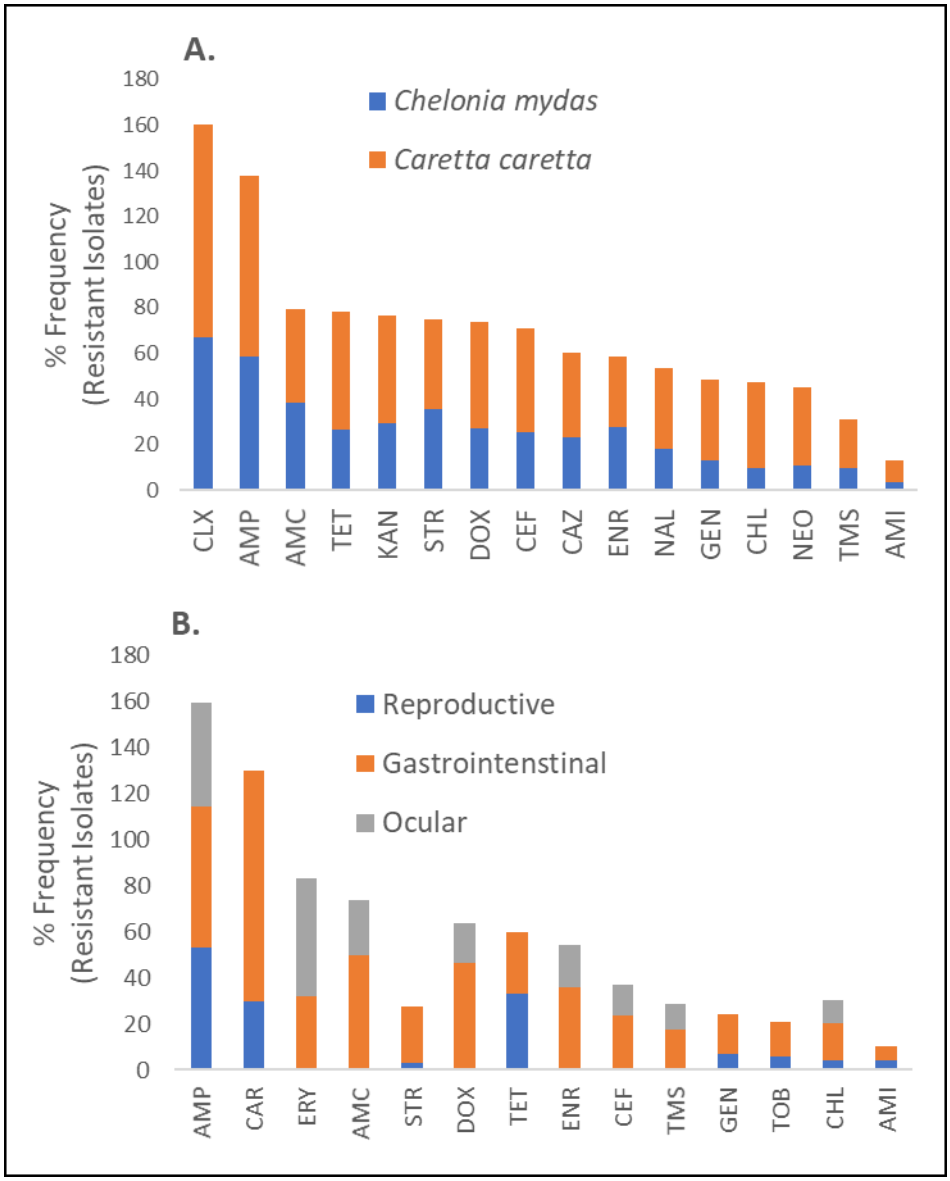


Figure 2-3: Frequency distribution of antibiotic resistance against commonly used antibiotic drugs stratified by turtle species (*Chelonia mydas* and *Caretta caretta*) irrespective of geographical marine environment, biological specimen considered or tested bacterial isolates (panel A) and, frequency distribution of antibiotic resistance stratified by considered biological specimen irrespective of geographical marine environment, turtle species or tested bacterial isolates (Panel B). Amikacin = AMI; amoxicillin = AMO; Amoxicillin-clavulanic acid = AMC; Ampicillin = AMP; Carbenicillin = CAR; Ceftazidime = CAZ; Ceftiofur = CEF; Cephalexin = CLX; Chloramphenicol = CHL; Doxycycline = DOX; Enrofloxacin = ENR; Erythromycin = ERY; Gentamicin =

GEN; Kanamycin = KAN; Nalidixic acid = NAL; Neomycin = NEO; Streptomycin = STR; Tetracycline = TET; Tobramycin = TOB; Trimethoprim-sulfamethoxazole = TMS

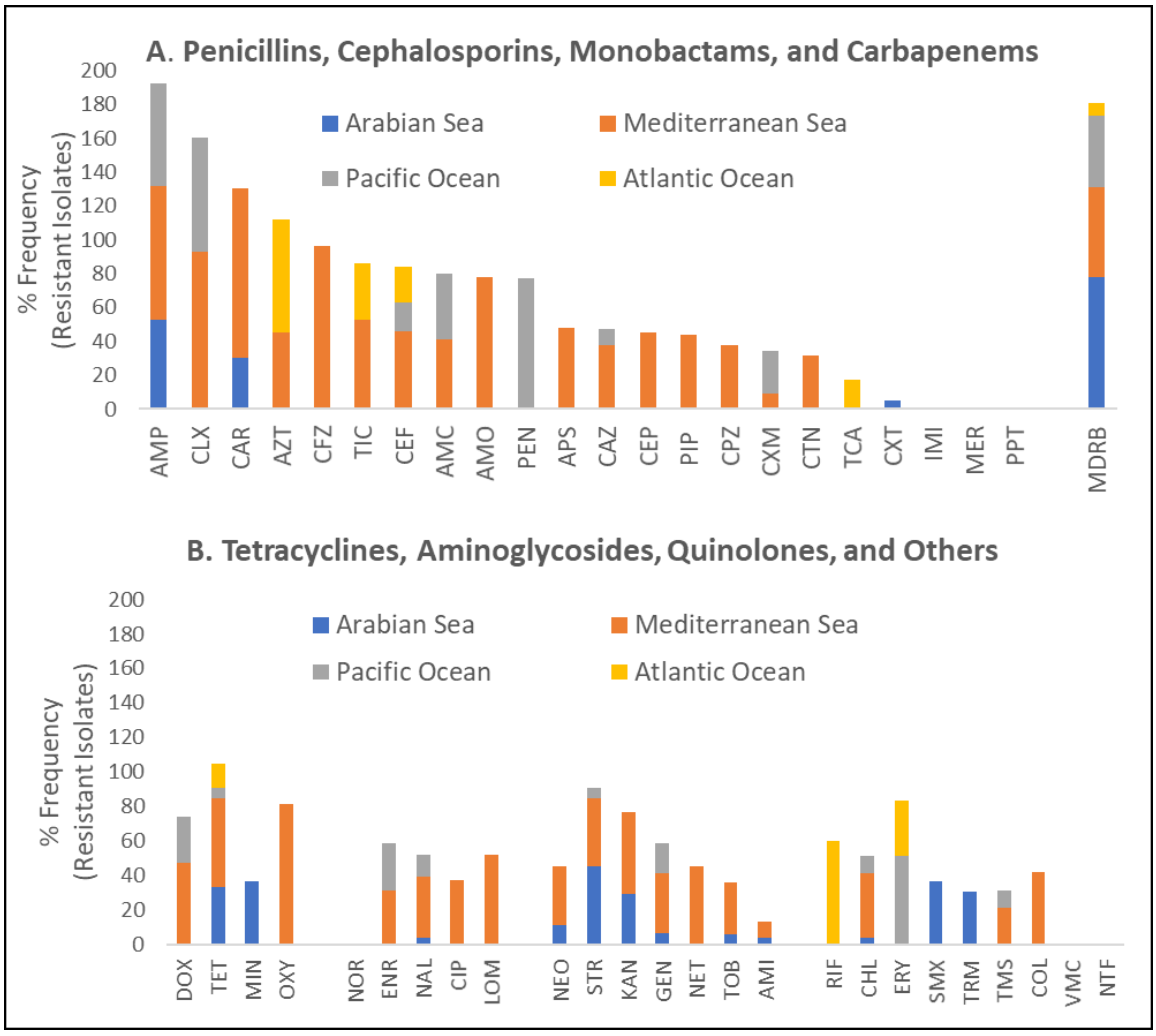


Figure 2-4: Frequency distribution of antibiotic resistance against commonly used antibiotic drugs stratified by geographical marine environment irrespective of biological specimen considered, bacterial isolates or turtle species for beta-lactams (panel A) and other antibiotic classes (panel B). Amikacin = AMI; Amoxicillin = AMO; Amoxicillin-clavulanic acid = AMC; Ampicillin = AMP; Ampicillin-salbactam = APS; Aztreonam = AZT; Carbenicillin = CAR; Ceftazidime = CAZ; Ceftiofur = CEF; Cefepime = CEP; Cefazolin = CFZ; Ciprofloxacin = CIP; Cefalexin = CLX; Chloramphenicol = CHL; Colistin = COL; Ceftriaxone = CTN; Cefotaxime = CXM; Cefoxetin = CXT; Doxycycline = DOX; Enrofloxacin = ENR; Erythromycin = ERY; Rifampicin = RIF; Gentamicin = GEN; Imipenem = IMI; Lomefloxacin = LOM; MDRB = percentage of multi-drug resistant isolates; Meropenem = MER; Minocycline = MIN; Kanamycin = KAN; Nalidixic acid = NAL; Netlimicin = NET; Nitrofurantoin = NFT; Neomycin = NEO; Norfloxacin = NOR; Penicillin = PEN; Oxytetracycline = OXY; Piperacillin = PIP; Piperacillin-tazobactam = PPT; Streptomycin = STR; Sulfamethoxazole = SMX; Ticarcillin-

clavulanic acid = TCA; tetracycline = TET; Ticarcillin = TIC; Tobramycin = TOB; Trimethoprim = TRM; Trimethoprim/sulfamethoxazole = TMS; Vancomycin = VMC.

Future studies in this area should focus on not only identifying resistance but also ascertaining the definitive source of AR, as well as devising mitigation strategies. It is of significant note that the current literature in this field has not compared the levels of antibiotic compounds at the site of capture for each sea turtle used as an animal model. However, in the current study, we were able to stratify the frequencies of observed antibiotic resistance with putative sources of antibiotic compounds, ARGs, and ARB as predominantly defined by the primary authors of included studies. We show that the highest prevalence of MDRB was associated with sources, such as WWTPs and rehabilitation centres for sick and injured sea turtles, which are both considered as sources of exposure to antibiotic agents and ARB (Figure 2.5).

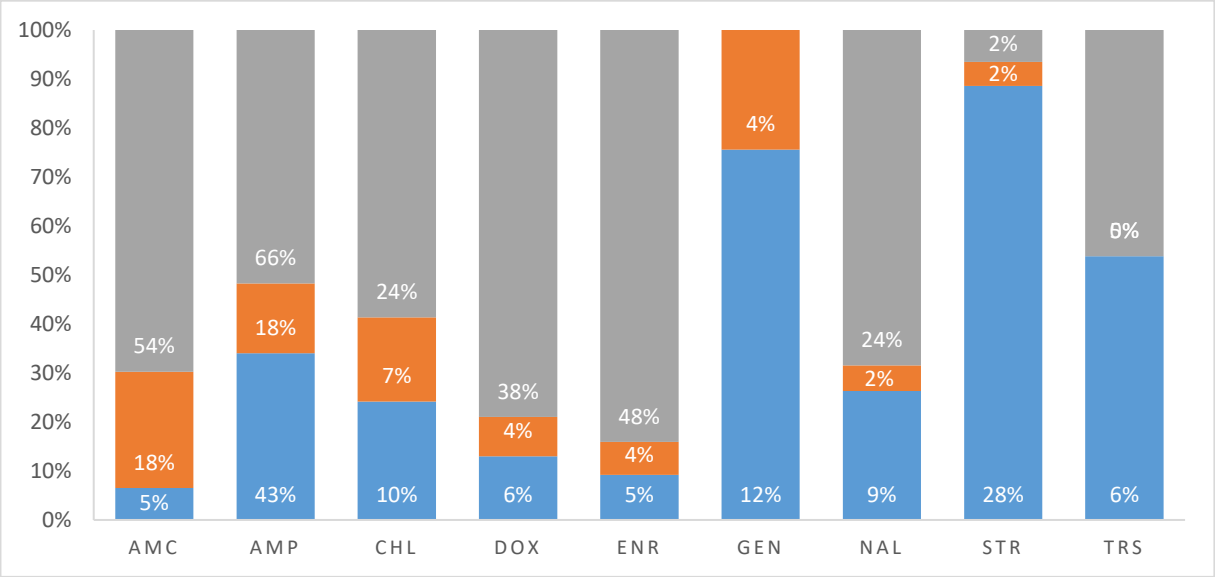


Figure 2-5: Percentage distribution of the frequency for antibiotic resistance to common antibiotics in bacteria isolated throughout the 12 studies globally, and stratification by the potential sources of exposure as postulated by each original study. This includes: rehabilitation centres for sick or injured turtles (grey), treated wastewater from WWTPs (blue) and, other unidentified sources of anthropogenic pollutants (orange). Abbreviations are defined as: AMC = amoxicillin-clavulanic acid; AMP = ampicillin; CHL = chloramphenicol; DOX = doxycycline; ENR = enrofloxacin; GEN = gentamicin; NAL = nalidixic acid; STR = streptomycin; TRS = trimethoprim-sulfamethoxazole.

2.3.4.3 Quantitative Evaluation of Anthropogenic Sources of Antibiotic Resistance in Marine Turtles

On average, WWTPs and turtle rehabilitation centres accounted for 58% and 49% of MDRB isolates in published studies. This result is not surprising because the rehabilitation of sick or injured turtles for purposes of conservation is usually associated with marked broad-spectrum antibiotic usage, as methods for diagnosis in wild animals are sorely lacking. Antibiotic agents classified as quinolones (nalidixic acid and enrofloxacin), beta-lactams (ampicillin and amoxicillin-clavulanic acid), and tetracyclines (doxycycline) showed the highest frequency of resistance concerning WWTPs and turtle rehabilitation centres. This directly correlates with the increased usage of these antibiotic classes at rehabilitation centres because they have a broad spectrum of activity and are safe to use in reptiles. These arguments on the observed antibiotic resistance patterns are supported by the fact that there was no resistance against aminoglycosides (gentamicin and streptomycin) when tested for at turtle hospitals or rehabilitation centres. Coincidentally, aminoglycosides are severely nephrotoxic in reptiles and as such, the application of this class to treat infections in turtles is very limited. In contrast, chloramphenicol is not a drug of primary choice in treating sick turtles, but it showed a relatively high frequency of resistance. This may be attributable to generalized non-specific mechanisms of resistance such as the increased expression of efflux pumps by bacteria. The antibiotic resistance pattern associated with WWTPs or other unidentified sources was more diverse, affecting all the major antibiotic classes. It is plausible that this is because WWTPs are points of convergence for a diverse mix of antibiotic agents, ARGs and MDRB. However, specific mechanisms by which the observed antibiotic resistance patterns manifest in turtles in marine environments are not known. It is reasonable to assume, however, that similar pools of ARGs and means of antibiotic resistance acquisition seen in other ecosystems are involved here as well. For instance, the respective genes encoding for some of the reported resistance patterns against tetracyclines (TETA), sulphonamides (*SULII*), beta-lactams (BLATEM and BLACTXM), and quinolones (QNRS), were also isolated in wild sea turtles (Alduina et al., 2020). In addition, this same study (Alduina et al., 2020), identified a higher percentage of the INT1 gene, encoding for the mobile element class 1 Integron. This may suggest that the propensity for HGT of ARGs which may enhance the spread of ARB in sea turtles in marine environments.

2.4 Conclusions

This review summarises and evaluates sea turtles as sentinel species for monitoring as well as reporting incidences, dissemination, and distribution patterns of antibiotic resistance in marine environments. The high geographic distribution and long-life span of sea turtles have allowed them to be utilised as sentinel species for antibiotic resistance in previous literature. To date, studies on the use of turtles to monitor antibiotic resistance in marine environments have examined at least five

turtle species including green, loggerhead, leatherback, olive ridley, and hawksbill sea turtles. Though there were no statistically significant differences in reported frequencies of resistance when stratified by turtle species or anatomical origin of the specimen, it is hypothesised that species with higher protein-rich diets may contain representative antibiotic resistance elements from their food sources. Conversely, species that rely on more stationary food sources may provide a more accurate representation of their immediate feeding grounds.

All studies reviewed state that sea turtles are a good sentinel model for monitoring antibiotic resistance including the detection of potentially pathogenic ARB in marine environments. However, the majority of marine turtles utilised as animal models for these studies were adults capable of migration or currently migrating, meaning that their microbiome may not be representative of the geographical location in which they were sampled. These adult animal models may instead represent the marine environment on a more global scale. It is hypothesised that the sub-adult class of green turtles may be a more effective animal model for assessing antibiotic resistance within a specific geographical area. Unfortunately, the current literature does not provide insight into the differences in antibiotic resistance or microbiome between size classes of marine turtle species, and thus should be a point of focus for this research area in the future. Future studies in this area should also focus on not only identifying resistance but also ascertaining the definitive source of antibiotic resistance, as well as devising mitigation strategies. As with the current literature, it is difficult to attain an accurate and reliable assessment of the geographical origins of antibiotic resistance observed within turtles in marine environments across different jurisdictions in the world; and the potential environmental effects of the various WWTP and agricultural runoff policies, both of which are the major contributors to antibiotic resistance in marine environments. This review has highlighted the need for more comprehensive understanding of how antibiotic resistance is obtained by marine turtles, and how anthropogenic sources of resistance contribute to the propagation of antibiotic compounds, ARB, and ARG in the marine environment. This research group will be pursuing this line of inquiry in a region where WWTP's were considered the likely contributor of antibiotic resistance in marine turtles (Townsville, Queensland, Australia) (Ahasan et al., 2017, Delli Paoli Carini et al., 2017).

Furthermore, the true magnitude of ARB in entire microbiomes in marine turtles has remained unknown. All available literature identified common and predominant bacterial isolates via culture and sensitivity testing, but these results are not representative of entire resistomes that likely to be hosted and disseminated by sea turtles. This approach limits the discovery of ARB only to bacteria that grow under laboratory conditions thus creating a bias that may over-represent specific genera of bacteria such as *Citrobacter*, *Pseudomonas*, *Enterococcus,* and *Vibrio*. However, our analyses of resistance within the identified bacteria show that antibiotic resistance in marine turtles

is overrepresented by beta-lactams by a two-fold difference compared to all other antibiotic classes. Furthermore, the occurrence of resistance to reserved antibiotic classes such as quinolones and aminoglycosides is truly worrying as these drugs are usually the last line of defence in many life-threatening and multiple-drug resistant infections. All studies evaluated stated a relatively similar incidence of MDRB across all major antibiotic classes. This suggests that irrespective of geographical location, the selection pressures that have driven the development of antibiotic resistance in sea turtles and marine environments are similar and may pose a serious threat to both environmental and public health.

. Chapter 3: The Role of Wastewater Treatment Plants in Dissemination of Antibiotic Resistance: Source, Measurement, Removal and Risk Assessment

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Note: Variation from the published manuscript exists in the description of whole genome sequencing, which is adapted to shot-gun metagenomic sequencing for continuity purposes with the remaining thesis body of work.

Antibiotic Resistance Genes (ARGs) are important Contaminants of Emerging Concern (CEC), and the potential impact on public health and environmental health is significant. This review aims to discuss factors that influence the presence and abundance of ARGs within Wastewater Treatment Plants (WWTPs) and environmental effects that may eventuate from the dissemination of ARGs from treated effluents. Both antibiotic resistant bacteria (ARBs) and resistance genes have been detected in the influent and the effluent of WWTPs worldwide. Different levels of wastewater treatment (Primary, Secondary, and Tertiary) show different degrees of removal efficiency of ARGs, with further differences being observed when ARGs are captured as intracellular or extracellular forms. Furthermore, different molecular methodologies that are routinely used to characterise ARGs such as quantitative polymerase chain reaction or whole genome sequencing may vary in identifying the resistomes, and ARG removal efficiencies from WWTPs. This scoping review critically analysed the literature surrounding antibiotic resistance pollution and the factors that influence the identification, prevalence, growth, and dissemination of antibiotic resistance within WWTPs). Additionally, there is an emphasis on the emerging One Health risk assessment framework, and future strategies on how WWTPs can be assessed for environmental risk and public health impacts are discussed. Understanding mechanisms of removing ARBs and ARGs from wastewater is important for decelerating the worldwide spread of antibiotic resistance and its dissemination into natural environments that rely on complex and diverse microbiomes.

3.1 Introduction

Chemical pollutants that are collectively termed Contaminants of Emerging Concern (CEC) are classified as non-traditional unmeasured pollutants, distinguished by characteristics such as potentially high environmental risk at very low concentrations (Rizzo et al., 2019). WWTPS are traditionally associated with disseminating CEC into receiving environments, specifically fresh and marine aquatic ecosystems (Vidal-Dorsch et al., 2012). Antibiotics, ARBs, and ARGs have recently

been identified as CEC from WWTPs (Rizzo et al., 2019), and these pose enormous risk to both public and environmental health. Interest is growing in the need to identify accurate assessment scaffolds to predict the risk of ARB and ARGs released from WWTPs to public health, animals, and the environment. There is concern in the scientific community, however, that the current state of knowledge on the dissemination of ARB and ARGs from WWTPs cannot be accurately applied to predict the risk to One Health.

3.1.1 Antibiotic Resistance in Wastewater

The identifying trait of an antibiotic is to kill a bacterial cell or disable its ability to replicate. This is a natural process that organisms have used for millions of years to evolve in hostile environments and compete for resources. Antibiotic resistance (which is a subset of antimicrobial resistance) has already been identified as contributing to tens of thousands of human deaths per year in Europe and the USA alone (Talebi Bezmin Abadi et al., 2019). To date, there are over 100 clinically used antibiotics, which are either developed from microbial isolates (e.g., erythromycin from *Saccharopolyspora erythraea*), or made synthetically (e.g., ciprofloxacin) (Panthee et al., 2016). Alternatively, some antimicrobial compounds are synthetic derivatives of naturally produced antibiotics (e.g., amoxicillin is a semi-synthetic derivative of penicillin). Each of these naturally occurring or synthetic antibiotics generally fit within specific classes including penicillins, macrolides, cephalosporins, fluoroquinolones, carbapenems, tetracyclines, sulphonamides, aminoglycosides, phenicols, lincosamides, glycopeptides, oxazolidinones and rifampicin. For each of these antimicrobial classes, general mechanisms of action and resistance have been reviewed previously (Hassan et al., 2022). It is known that genes encoding for antimicrobial resistance are continuously evolving and are significantly more varied than the number of existing antimicrobial compounds. The extensive variability and overlap in mechanisms of resistance has led to marked interest in characterizing the risk factors for resistance against each class of compounds.

The majority of currently available scaffolds for assessing risks for the highly diverse antimicrobial resistance pollution from WWTPs have suggested that the quantity and quality of antibiotics, ARB, ARGs as well as non-antibiotic determinants of resistance should be established from the sources of pollution for a proper risk evaluation in the One Health context (Guo et al., 2017, Liu et al., 2019a). Additionally, the available risk assessments indicate that vastly different microbiomes and resistomes exist within different WWTPs and their receiving environments, possibly due to a multitude of factors including marked diversity in influent sources and wastewater treatment processes, suggesting a need for an individualized risk assessment for each WWTP (Guo et

al., 2017, Liu et al., 2019a). The most common sources of antibiotics and ARB in urban environments include hospitals and animal health clinics, industrial agriculture production facilities, and domestic sewage that is often laden with antibiotics from general medical use within the local population (Kümmerer, 2009). Most antibiotics are poorly biotransformed after consumption, and in some cases, up to 90% of the dose can be expelled un-metabolized or as active conjugates through urine and faeces (Liu et al., 2019a). WWTPs provide an opportunity to monitor and contain antibiotic resistance as all major sources and drivers of resistance tend to converge in urban WWTPs. As such, urban WWTPs are often considered hotspots for antibiotics and ARB and have been the subject of much investigation (Kümmerer, 2011). The processes involved in some forms of wastewater treatment have been observed to lead to significant variations in the microbial composition of WWTP effluents, and these rearrangements are reflected in the composition of antibiotic resistance harboured by the changing microbial communities (Alexander et al., 2016, Novo et al., 2013, Varela et al., 2014, Ye and Zhang, 2013). Furthermore, in most jurisdictions globally, the dissemination of microbial contaminants within the effluent is generally not controlled or regulated, and no currently implemented wastewater treatment options are capable of total elimination of ARGs in effluents (Li et al., 2018, Osińska et al., 2019). As a result, therefore, wastewater treatment facilities are still considered hot spots for potential amplification and dissemination of antibiotic resistance (Al-Asheh et al., 2021), despite a large reduction in the abundance of ARB and ARGs in some tertiary forms of wastewater treatment. Even relatively small abundances of ARGs, ARB, or antibiotics released into receiving environments can provide both the means and opportunities for the perpetuation of antibiotic resistance within environmental bacterial communities (Osińska et al., 2019).

This review briefly outlines factors that influence the presence and relative abundance of ARGs in effluents that are discharged from WWTPs. More specifically, elements that lead to the acquisition of ARGs by susceptible bacteria, as well as effects of influent quality and intra-plant secondary and tertiary wastewater treatment processes, are described. In addition, a framework for assessing the risk to the environment from dissemination of ARGs associated with WWTPs is highlighted.

3.2 Approaches for Identifying Antimicrobial Resistance Factors in WWTPs and the Environment

3.2.1 Identifying ARB and ARGs

Vastly different methods are used to identify and quantify antibiotic resistance within WWTPs, WWTP-effluents and receiving environments. Most risk assessment scaffolds agree that testing for antibiotic and antibiotic resistance pollution within WWTPs should be standardised

(Garner et al., 2021, Huijbers et al., 2019). Consistency in methods for the identification of ARB, ARGs, and antibiotics is necessary, so that quantitative or qualitative values may be accurately compared between different WWTPs and associated receiving environments.

Although bacterial culture and specific antibiotic sensitivity testing is less common, some studies have used this technique to quantify ARB and putative corresponding intracellular ARGs (iARGs) in wastewater (Akanbi et al., 2017, Balsalobre and de la Campa, 2008, Guardabassi et al., 1998). Bacterial culture-based approaches can be helpful when assessing wastewater for specific bacterial species/strain and especially pathogenic strains and their associated resistance mechanisms. These approaches provide the added benefit of observing the effects of specific stressors under controlled conditions, as well as identification of functional ARGs and virulence factors. Culture methods have also been used in tandem with gene sequencing techniques, which can detect ARGs harboured by specific bacteria (Adekanmbi et al., 2020, Wang et al., 2014). However, cultivation-based approaches limit whole resistome and microbiome assessments, as only a small fraction of environmental bacteria are capable of growth under known laboratory conditions (Al-Awadhi et al., 2013). Additionally, bacterial culture does not provide information on the identification or quantification of environmental eARGs. This, therefore, may represent a selective bias as opportunities to characterise the full scale of ARGs and the associated microbiome within WWTP samples are lost.

Currently, the bulk of knowledge on evaluation of scope and frequency of antibiotic resistance in WWTPs has largely relied on the use of quantitative Polymerase Chain Reaction (qPCR) and Shot-gun metagenomic sequencing (SM-Seq) approaches. With qPCR, specified ARGs can be detected and quantified in environmental samples, and recent advances in high-throughput qPCR arrays have enabled the characterisation of hundreds of ARGs simultaneously (Hultman et al., 2018, Karkman et al., 2016, Muurinen et al., 2017, Muziasari et al., 2016, Stedtfeld et al., 2008). The use of the qPCR method for the detection of ARGs from wastewater also provides the opportunity for high-resolution quantification of specified sequences of ARGs within an array. However, the selectivity of primers for qPCR assays limits the identification of ARGs because the breadth of a resistome often encompasses more ARG sequences than could be feasibly tested via qPCR. This may provide further bias to the characterisation of WWTP resistomes. This bias is notable in this scientific field, with observable reliance on a few specific genes in several publications, and these include ARGs encoding for resistance against beta-lactams (blaCTX-M, blaTEM, and blaSHV), tetracyclines (TETM, TETO, TETQ, and TETW), sulphonamides (SULI and SULII), and class 1 integron-integrase (INTI1), which indicate the presence of mobile genetic elements (MGEs) (Bueno et al., 2020, Czekalski et al., 2012, Neudorf et al., 2017, Harnisz et al., 2020, Lu et al., 2020).

More recently, SM-Seq has been used extensively for the identification of ARGs in WWTPs and environmental samples (Conco et al., 2022, Garner et al., 2021, Li et al., 2015a, Yang et al., 2014). This approach is helpful for not only identifying known ARGs (Conco et al., 2022, Li et al., 2015a, Yang et al., 2014), but also for its potential to identify novel ARGs (Tansirichaiya et al., 2022), while simultaneously identifying the microbiome within WWTPs (Conco et al., 2022, Yang et al., 2014). SM-Seq methods are expensive, require a significant amount of bioinformatic skills, and gene identification is heavily reliant on reference databases and bioinformatic platforms and software to process raw reads, to generate interpretable data (Lakin et al., 2017, Yin et al., 2018, de Nies et al., 2021). Furthermore, the quantification of gene expression by SM-Seq is only determined as relative abundance because it is limited by gene characteristics such as nucleotide sequence length and abundance within the profiled resistome, and both qPCR and SM-Seq approaches do not provide immediate information on the functional capability of identified ARGs. To gain an insight into whether ARGs identified by SM-Seq have a functional capability, SM-Seq is often coupled with advanced transcriptomic and proteomic analyses. A benefit of SM-Seq is the volume of data generated, and it can facilitate the identification of different ARG variants as well as novel ARGs and bacterial chromosome sequences (Liu et al., 2013). Given the strengths and limitations of each of the approaches used to characterise ARB and ARGs in wastewater and WWTPs, a clear set of guidelines for each of these techniques and consistent reporting of such details would enable better data comparisons across scientific fields and facilitate the acquisition of data that is necessary to accurately predict risks to the environment from sources of antibiotic resistance pollution. This argument has also been advanced by others in this field (Garner et al., 2021).

3.2.2 Identifying Antibiotics

Assessing WWTPs and associated receiving environments for antibiotics as selective agents for antimicrobial resistance is inconsistent across a number of studies. While some studies have tested for, identified, and quantified specific antibiotics of interest in wastewater and WWTPs (Huijbers et al., 2019, Wang et al., 2020a), in other studies, this has not been done at all (Bueno et al., 2020, Chen and Zhang, 2013a). The presence and abundance of antibiotics provides an opportunity for ARGs to spread horizontally and/or vertically in bacterial communities, creating novel ARB. Analysing these compounds and their effective concentrations in conjunction with ARB and ARGs can markedly improve ecological risk assessments (Garner et al., 2021, Huijbers et al., 2019).

The presence and concentrations of antimicrobial compounds in wastewater and WWTPs have been used as indicators for conditions under which selection pressure would lead to the

development and spread of ARB. To evaluate the efficacy and clinical applicability of antibiotics in laboratory settings, the concept of Minimum Inhibitory Concentrations (MICs) that inhibit bacterial growth in culture has been established. MICs are commonly applied to determine resistance or susceptibility to specific antibiotics (Zhang et al., 2021a, Tello et al., 2012). By contrast, there is also increased interest in determining effects of exposing bacteria to antibiotic concentrations below respective MIC values (Bengtsson-Palme and Larsson, 2016, Fajardo and Martínez, 2008). Lower concentrations (<MICs) of antibacterial compounds have multiple effects, including the stimulation of Horizontal Gene Transfer (HGT) of ARGs through conjugation (Xia et al., 2008, Shun-Mei et al., 2018) and changing the transcription profiles of virulence factors for some pathogenic bacteria (Shen et al., 2008). Furthermore, low levels of antibiotics have also been linked to the activation of the SOS response signalling and uptake of eDNA (Memar et al., 2020), and changing gene regulation through quorum sensing has been linked to increase bacterial cell variability (Carette et al., 2020, Liu et al., 2013). Given that myriad effects are attributed to antibiotics at sub-lethal concentrations for bacteria, recent studies have focused on determining Predicted No-Effect Concentrations (PNECs), as concentrations at which different types of antibiotics may be present but have no predicted effect on the microbiome (Murray et al., 2021, Huijbers et al., 2019, Bengtsson-Palme and Larsson, 2016). PNECs are expected to become crucial in the monitoring and control of antibiotic pollution, and this would streamline risk assessment approaches with measures that are used for other CECs. It is important to note, however, that the PNEC of an antibiotic is likely to vary with the bacterial composition against which an antibiotic is tested, and the laboratory culture conditions for this validation process may further bias this scientific field. Furthermore, the assessment of antibiotics alone does not provide context for the risk of transmission of ARGs within the microbiome in receiving environments, and as such should not be the sole basis for the assessment of antibiotic resistance and the risk it may pose to an environment. There are other challenges to accurately reporting levels of antibiotics in WWTPs and environmental samples. For instance, the sorption of antibiotics to sediments in aquatic ecosystems, and marked variations in limits of detection or quantification of antibiotics by different testing methods will limit accurate assessment (Harrower et al., 2021). Antibiotics are not the only known selective agent for antimicrobial resistance, and, in effect, antibiotics may act synergistically or additively with heavy metals, biocides, pharmaceuticals and other emerging organic contaminants in wastewater to increase the abundance of specific ARB (van Hamelsveld et al., 2023). It is still highly debated whether this is due to selection and induction of the expression of co-resistance genes, enhanced adaption of resistance gene cassettes, or altered physiological/biochemical properties that upregulate ARG expression (Harrower et al., 2021, Alderton et al., 2021). Nevertheless, the availability of known selective agents other than antibiotics

should be considered when assessing the risk of antibiotic resistance pollution in WWTP-receiving environments.

3.3 Effects of Wastewater Treatment Processes on ARGs Released from WWTPs

Antibiotics contained within influent sources can enter WWTPs and allow resistant bacterial populations to propagate with less competition from susceptible bacteria strains. This is evidenced by positive correlations between specific antibiotics in the influent and their cognate ARGs in WWTPs (Ju et al., 2019, Zhang et al., 2019). For example, antibiotics of the macrolide class (clarithromycin and azithromycin) correlated positively with a macrolide resistance gene (MACB) in wastewater (Ju et al., 2019), and an increase in the total abundance of ARGs identified by qPCR in the activated sludge was observed after the addition of tetracycline (Zhang et al., 2019). Furthermore, it has been noted that up to 50% of antibiotic concentrations in influents are retained throughout the treatment process and the retained antibiotics are detectable in WWTP effluents (Mao et al., 2015). Despite these specific examples, drawing broad and generalizable conclusions on the link between the presence of antibiotics and their associated ARGs remains difficult. For example, a positive correlation was observed between the concentration of tetracyclines and the number of cultured bacteria resistant to tetracycline, but there was no significant correlation between seven other antibiotics and their related culturable ARB (Li et al., 2015b). Additionally, Xu et al. (2015) observed no link between the presence of sulfonamides and common sulfonamide resistance genes (SUL1 and SUL2) identified by qPCR (Xu et al., 2015). By contrast, a negative correlation was observed when the abundance of a quinolone resistance gene (QNRC) decreased as the concentrations of enrofloxacin (a quinolone) increased in the effluent from a WWTP (Xu et al., 2015). This phenomenon suggests that the relative abundance of an antibiotic may affect the presence but not necessarily the abundance of genes associated with resistance to that specific antibiotic (Liu et al., 2019a). Comparisons between different studies and identification of trends in the literature surrounding the interaction of ARB, ARGs, and antibiotics is also made difficult by the inconsistency in methods used to investigate these correlations. In addition, treatment type and the operational processes of individual WWTPs are also influential factors in reducing total bacteria and, by extension, the abundance of ARB and ARGs in effluents (Bouki et al., 2013). In this review, emphasis is placed on the impact of wastewater treatment processes on ARGs in the effluent (liquid phase) released from WWTPs (Appendix A Table 8-1). At most modern WWTPs, many different technological approaches are used in processing wastewater in what is classified as primary, secondary, and tertiary treatment (Liu et al., 2019a).

3.3.1 Primary Wastewater Treatment

Primary treatment generally involves physical screening and removal of large solid, particulate matter. Despite there being limited literature on the effect of primary treatment on ARGs, the available evidence seems to indicate that primary physical treatment does not significantly remove ARGs, as noted for TETA, TETO, TETW, SULI, SULII, blaCTX-M, blaTEM and blaSHV following identification by qPCR (Wen et al., 2016, Yang et al., 2019). It should be noted, however, that some forms of eARGs and eDNA can generally sorb to solid particles; speculation exists that a limited amount of such DNA molecules could still be removed from influents into WWTPs during the primary treatment phase (Bouki et al., 2013).

3.3.2 Secondary Wastewater Treatment

Secondary wastewater treatment is usually characterised by a biological treatment step, where specific conditions are applied to the wastewater to explicitly cultivate specific bacterial species to break down biological material and nutrients. The biological step in secondary treatment may include an aerobic process and/or an anaerobic process as well as anoxic and/or oxic processes. The composition of bacterial communities throughout a WWTP are intricate (Wagner et al., 2002), and the artificially changed oxygen and nitrogen availabilities throughout these processes drastically rearranges the wastewater bacterial community. Many studies, utilising both SM-Seq and qPCR methods, have noted that this process results in a reduction in ARBs and their iARGs (Narciso-da-Rocha et al., 2018, Ju et al., 2019, Osińska et al., 2019, Wen et al., 2016). Similarly, in studies utilising both SM-Seq and bacterial culture paired with qPCR, the total abundance of ARGs was found to decrease throughout WWTPs as the abundance of bacteria declined (Yang et al., 2014, Wang et al., 2020c, Karkman et al., 2018, Bengtsson-Palme and Larsson, 2016). Therefore, this reduction is likely because ARGs follow a similar distribution to that of total bacteria during wastewater treatment since the major removal of bacteria via sludge separation occurs after the anaerobic and aerobic processing (Narciso-da-Rocha et al., 2018). By contrast, there are instances where qPCR-based methods identified ARB and ARGs increasing in relative abundance when compared to total bacterial and gene loads (Hultman et al., 2018, Mao et al., 2015). To date, there has been very limited focus on the link between the composition and distribution of specific bacterial hosts of ARGs (i.e., ARB) within critical sections of WWTPs and whether this distribution is similar in both the influent and the effluent. Using qPCR, emulsion paired isolation, and concatenation PCR to detect bacterial hosts of ARGs, Hultman et al. (2018) observed that the distribution of four ARGs and the bacterial cells, which harboured these genes, varied between the WWTP influent and effluent (Hultman et al., 2018). A subsequent study found that the composition of the WWTP microbiome changed markedly

throughout the WWTP and that bacterial hosts of ARGs and MGEs monitored by qPCR also changed throughout the plant (Osińska et al., 2019). These observations are likely to be attributed to the high incidence of HGT and sharing of ARGs on MGEs within WWTPs, and this speculation was based on identification by qPCR of many ARGs, including INTI1, blaTEM, blaOXA-A, blaSHV, blaCTX-M, SULI, SULII, and QNRS (Narciso-da-Rocha et al., 2018).

Further evidence of HGT of ARGs in WWTPs can be seen on an individual gene level. For example, the removal efficiency of a specific sulfonamide resistance gene (SULI) in biological treatment was investigated in four separate studies (Gao et al., 2012, Munir et al., 2011, Rodriguez-Mozaz et al., 2015). All four studies utilised the same qPCR primer and observed varied changes in the absolute abundance of other cumulatively measured ARGs as well as SULI. No difference in the relative abundance of SULI to bacterial load after biological treatment or a significant increase in the relative abundance of SULI after treatment was observed (Rodriguez-Mozaz et al., 2015), while a decrease in the relative abundance of SULI was observed after treatment (Munir et al., 2011). This inconsistency in specific gene removal by secondary treatment processes may be attributed to two plausible factors. First, ARGs such as SULI may be conserved within some bacterial species, and this would account for a similar rate of reduction in absolute abundance of bacteria as well as ARGs following secondary treatments in WWTPs (Gao et al., 2012, Rodriguez-Mozaz et al., 2015). Secondly, the increase in the relative abundance of SULI after treatment (Gao et al., 2012, Rodriguez-Mozaz et al., 2015) may be due to the SULI gene also existing as an eARG, outside the host antimicrobial-resistant bacterial cells (Rodriguez-Mozaz et al., 2015). This is further substantiated by the link of SULI to MGE and high rates of HGT (Wang et al., 2014, Adekanmbi et al., 2020, Griffith et al., 2018). Furthermore, the association of various ARGs with multiple host ARB during wastewater processing has been documented previously (Machado et al., 2023, Zhang et al., 2023). However, with the exception of bacterial culture or utilisation of sophisticated gene sequencing approaches, it is difficult to determine the definitive bacterial host of ARGs in wastewater.

Dynamic shifts in ARG relative abundance across secondary treatment processes in WWTPs have also been demonstrated in other studies. For example, Osińska et al. (2019) noted more than 90% reduction in total ARG load across secondary treatment processes, but they also showed significant increases in the relative abundance of specific ARGs, including: TETM (a gene for tetracycline resistance) and blaTEM (an extended spectrum beta-lactamase gene) identified via PCR methods (Osińska et al., 2019). Additionally, the levels of free or adsorbed eARGs may not always be significantly affected by secondary treatment processes (Lee et al., 2017, Pazda et al., 2020, Sui et al., 2019, Yuan et al., 2019). Some authors have noted differences in antibiotic resistance profiles between aerobically and anaerobically digested sludge. Anaerobic digesters might reduce ARG

abundance and decrease horizontal transfer of ARGs that is facilitated by MGEs including plasmids (Guo et al., 2017). Nonetheless, some paradoxical effects have been seen in anaerobic sludge digesters. While some ARGs were undetectable after anaerobic digestion, other unique ARGs appeared to emerge only after this form of treatment, and this suggests that anaerobic digestion may induce or specifically select for some forms of ARGs (Guo et al., 2017, Miller et al., 2016) . It is conceivable, therefore, that this conflicting evidence is a direct result of changes in microbial communities and associated ARGs, following anaerobic treatments. This implies that the net effect of anaerobic reactors in the secondary phase of wastewater treatment is dependent on influent quality and its microbial composition, types and concentrations of antibiotics and other chemicals as well as other factors such as the seasonal changes in temperature, humidity, and rainfall. Thus, the impact of anaerobic treatments in the secondary phase of wastewater treatment cannot be generalised from individual case studies in specific plants. The environmental impact of each WWTP as relates to the spread of antimicrobial resistance should be assessed individually.

3.3.3 Tertiary Wastewater Treatment

Tertiary treatment contributes to wastewater disinfection and is used to remove components, which are not reduced by the secondary treatment, such as pathogenic or non-pathogenic microorganisms and associated iARGs, as well as eARGs. The effectiveness of tertiary treatment and its impact on ARGs varies between tertiary wastewater treatment processes. The commonly used tertiary processes include disinfection via ultra-violet radiation, chlorination, ozone treatment, and the use of Membrane-Bioreactors (MBR).

3.3.3.1 Chlorination Disinfection

The use of chlorination processes as a disinfectant in WWTP has shown varying outcomes on ARB and ARGs. Studies on this form of disinfectant have reported that chlorination either does not significantly reduce ARGs or in some cases favours the growth of ARB and an increase in the relative abundance and conjugative transfer of ARGs between bacteria (Conco et al., 2022, Guo et al., 2015). For example, investigations using culture methods showed that approximately 40% and 80% of erythromycin-resistance and tetracycline-resistance genes could not be removed by chlorination at a dose up to 300 mg Cl_2 min/L, respectively (Yuan et al., 2015). Furthermore, a WWTP in northern China reported that ARB identified by culture methods were more resistant to disinfection by chlorination than were total bacteria (Mao et al., 2015). Mao et al. (2015) demonstrated that 12 ARGs identified by qPCR and encoding for resistance against tetracyclines (TETA, TETB, TETE, TETG,

TETH, TETS, TETT, TETX), sulfonamides (SULI, SULII), quinolones (QNRB) and macrolides (ERMC) were released from the WWTP at higher concentrations in the effluent than the influent following a tertiary chlorine disinfection dose of 5 mg/L for 30 min (Mao et al., 2015). Similarly, treatment of wastewater with 8–9 mg/L of chlorine dioxide for 30 min resulted in up to an 8-fold increase in the abundances of iARGs and up to a 4-fold preferential increase in the abundances of eARGs against macrolides (ERMB), tetracyclines (TETA, TETB, TETC), sulfonamides (SULI, SULII, SULIII), beta-lactams (AMPC), aminoglycosides (APH(2')-LD), rifampicin (KATG) and vancomycin (VANA) (Liu et al., 2018b). Collectively, these studies suggest that disinfection of wastewater with chlorine may enhance environmental pollution with eARGs and iARGs, thereby compounding the spread of antimicrobial resistance. These observations need to be substantiated in large-scale studies covering a wider geographical distribution.

3.3.3.2 Ultra-Violet Disinfection

Multiple studies have investigated the efficiency of ultra-violet light (UV) to reduce the load of ARGs in WWTPs. At a UV dose of 27 mJ/cm^2, a 34–75% removal efficiency of ARBs was observed with no obvious reduction in eight ARGs identified by qPCR (Lee et al., 2017). From this study, Lee et al. (2017) suggested that although UV disinfection may be appropriate for the treatment and removal of bacterial cells and ARB, it cannot effectively disassemble eARGs, and it may even facilitate the release of iARGs from bacterial cells during lysis and, as a result, create eARGs. This notion is supported by other studies, which have observed a lack of reduction in ARGs when using UV disinfection (Chen and Zhang, 2013a, Rafraf et al., 2016). Using a UV disinfector with a 45% transmittance of 900 kW and light intensity greater than 1 mW/mm^2, only a slight change in ARG concentrations was observed between the pre- and post-UV disinfected effluent samples via qPCR, with ~0.5–0.7 orders of magnitude for TET genes and 0.3 orders of magnitude for SUL and INTI1 genes (Chen and Zhang, 2013a). In a comprehensive review, however, it was shown that the removal of ARB and ARGs from wastewater depended on several factors, including the nature of bacteria (Gram-positive or Gram-negative), whether targeted ARGs were harbored on plasmids or, chromosomally, whether UV treatment was coupled with other treatments such as advanced oxidation with hydrogen peroxide or chlorination, and the dose and duration of UV treatment (Umar, 2022). Overall, UV disinfection alone is unlikely to be highly effective in the removal of ARGs from wastewater on a large scale.

3.3.3.3 Ozone Treatment

Preliminary research on the effectiveness of ozone in reducing ARGs and ARB in wastewater is promising, and this technology may be effective, but studies on this issue are limited, and most have relied on using a mixture of specified bacterial culture and qPCR methods. In laboratory-scale experiments, the intracellular genes of a wastewater bacterial community were disrupted at a specific ozone dose (0.55 g of O_3 per dissolved organic carbon) (Czekalski et al., 2016). This dose was feasible for full-scale application but had not been applied anywhere, practically, at the time of the initial publication (Czekalski et al., 2016). Another related study demonstrated that at a concentration of 2 milligrams per litre, ozone provided higher removal efficiency of ARGs than UV or chlorination (Zheng et al., 2017). However, Czekalski et al. (2016) also found that some ARGs within *Escherichia coli* cultures, including the SULI gene, were unaffected by ozonation, suggesting that ozone at these doses may not eliminate all ARGs and may be selective in ARG removal (Czekalski et al., 2016). Furthermore, both UV and ozone disinfection may result in cell lysis, with bacterial DNA being released into the environment and the resulting ARGs ending up as free DNA in the treated wastewater (Zheng et al., 2017, Yang et al., 2019).

3.3.3.4 Membrane Filtration

The comparably recent implementation of high-efficiency membranes in some WWTPs has significantly reduced microbial concentration in wastewater effluent (Purnell et al., 2015). Membrane Bioreactor (MBR) plants have several advantages when compared to the conventional use of activated sludge clarification. MBRs have been proven to be effective in removing organic and inorganic matter (Al-Asheh et al., 2021). MBRs are suggested to be among the most effective removal systems for ARB and their accompanying ARGs (Wang et al., 2020b, Lin et al., 2021, Zhu et al., 2021, Nnadozie et al., 2017). Wang et al. (2020) stated that for some eARGs, including INT1, blaTEM, ERMB, TETO, and TETW, the average removal through a full-scale MBR process approached 100%, as quantified by qPCR (Wang et al., 2020b). Similarly, utilising high-throughput qPCR to investigate 319 ARGs and 57 MGEs, a 98.4% decrease in ARGs within an MBR plant was reported by Lin et al. (2021). However, this study by Lin et al. (2021) also observed that 35 ARGs and 14 MGEs were persistent in all samples, including in the MBR effluent (Lin et al., 2021). Furthermore, MGEs in the form of plasmids have been documented to pass through ultra-filtration membrane pores that are smaller than their DNA diameter possibly due to supercoiling (Arkhangelsky et al., 2011). This supercoiling of plasmid DNA is also enhanced by the hydrodynamic pressure caused by transmembrane gradient, and this may be the cause of many eARGs crossing ultra-filtration

membrane barriers. Collectively these observations suggest that WWTPs with MBR plants may be effective in the removal of iARGs and/or eARGs, but they are not a perfect system, and effluents from such treatment plants may still contain eARGs at biologically significant concentrations (Lin et al., 2021). Further studies on the elucidation of the specific roles of MGEs, iARGs and eARGs in the dissemination of antimicrobial resistance from WWTPs with MBRs are warranted.

3.4 Assessing the Risk of Antibiotic Resistance from WWTPs in the Environment

Many of the currently suggested antimicrobial resistance risk assessment scaffolds have predominantly focussed on public health and include measures of antibiotics, pathogenicity of ARB and their correlations with high risk ARGs (Garner et al., 2021, Zhang et al., 2021a, Ben et al., 2019, O'Flaherty and Cummins, 2017, Hu et al., 2021, Bondarczuk et al., 2016, Ashbolt et al., 2013). A thorough One Health risk assessment in this field must consider the impact on public health and environmental ecosystems in the context of other factors such as microbial diversity and functions performed by environmental microbiomes, the functionality of ARGs, and effects on sentinel environmental animal or plant species (Berendonk et al., 2015, Korzeniewska and Harnisz, 2020). Identifying ARGs and how ARGs spread is crucial to understanding the potential risk for resistance within an environment. However, ARB do ultimately determine the implications of antibiotic resistance either to public health via altered pathogenicity and morbidity or altered microbial diversity and function (Xu et al., 2020, Isles et al., 2022, Francino, 2016).

Direct effects of antibiotic use on microbial diversity in humans and animals have been well documented (Cycoń et al., 2019, Grenni et al., 2018, Jiang et al., 2019, Louca et al., 2016, Moran, 2015), with indications of functional effects to the immune system, metabolism, and other important biological functions. Whilst a similar level of functional effect has been hypothesised to contribute to loss of microbial diversity in environmental microbiomes (Cycoń et al., 2019, Grenni et al., 2018), very limited information is available on the functional impact of this dysbiosis within natural ecosystems (Moran, 2015). As such, frameworks on the true functional risks to environmental microbiomes are under-informed. Nonetheless, some progress has been registered in this domain, and the contribution of microbial diversity to environmental effects such as carbon dioxide production, nitrogen fixation, and pH stabilisation have been suggested (Jiang et al., 2019, Louca et al., 2016, Moran, 2015).

3.4.1 Interactions between ARB, ARGs, MGEs and Co-Occurrence Genes for Risk Scafolding

An appropriate antibiotic resistance risk assessment framework should also incorporate the characterisation of the fate and transport of ARBs, ARGs and MGEs. Many complex interactions lead to HGT and development of antibiotic resistance within specific bacterial cells. One such critical interaction involves co-occurrence genes that describe ARGs that are located on the same MGE or chromosome as genes encoding for other forms of resistance such as metal or biocide resistance (Li et al., 2017). This can create a situation where antibiotic and heavy metal resistance increase in abundance when the afflicted ARB are exposed to either the specified metal, antibiotic, or both stressors (Li et al., 2017). The effect of this specific type of interaction when categorising risk to One Health components is largely unknown. However, accurate genomic approaches can be used to characterise shifts in abundances and diversity of microbial communities and accompanying ARGs; preliminary studies suggest that the combination of ARGs with MGEs plays an important part in the dissemination of antimicrobial resistance in environmental ecosystems (Ahmad et al., 2022, Sun et al., 2021, Zhang et al., 2018, Zhao et al., 2017). ARGs associated with the co-occurrence of heavy metals or biocide resistance genes have been found to increase in proportion with high abundances of these compounds (Zhang et al., 2018, Zhao et al., 2017, Sun et al., 2021). It is conceivable that the co-occurrence of genes related to resistance to other stressors may impact the capability and accuracy of a risk scaffold used to predict the effect of antibiotic resistance pollution within any environmental ecosystem. All these aspects should be included in risk assessments.

3.4.2 A Proposed Pathway to Informed Risk Scaffolds for Antibiotic Resistance from WWTPs

Long-term surveillance of varied WWTPs and their upstream influent sources and downstream receiving environments may provide the insight necessary to make future investment decisions on infrastructure used to manage wastewater based on One Health risk scaffolds. In programs for the systematic surveillance of WWTPs for antimicrobial resistance specifically, risk assessments must be individualised to eliminate bias that may be attributed to quantitative and qualitative differences in influents to WWTPs and differences in wastewater treatment processes. Additionally, analyses of samples from WWTPs and environments into which effluents are discharged should account for the spatial as well as temporal distribution of resistomes. This provides an insight into how wide the antibiotic resistance is spread from sources of pollution and within different matrices, including water, sediment, flora, and fauna. Insight into how this form of pollution may be influenced by seasonal variations or climate change is also provided. The three pillars of One Health (Public Health, Animal Health, and Environmental Health) have been identified as potentially at risk from the indiscriminate dissemination of ARGs (Ahmad et al., 2022), with human and animal health threats related to unknown alterations of microbiomes and subsequent dysbiosis, as well as

environmental hazards relating to selection pressure and the further rise of environmental antibiotic resistance.

In summary, we provide a structured outline of some of the critical elements required to develop a risk assessment framework for antimicrobial resistance associated with wastewater processing. Herein, we have emphasised the following critical components: (i) accurate identification of the varied sources for antibiotic resistance in wastewater; (ii) characterisation of pathways and fate of the different determinants of antibiotic resistance, including ARB, ARGs, co-occurrence genes, antibiotics and other biocides; (iii) assessing exposure and quantification of risk by determining pathways through which humans, animals, and the environment may be exposed and using antimicrobial MICs or probabilistic PNECs as well as changes in abundance or diversity of ARGs and ARB to estimate the likelihood of adverse outcomes; (iv) identifying mitigation strategies by evaluating wastewater treatment processes and technologies. These risk scaffolding components should be used to establish monitoring programs to track incidences of antimicrobial resistance in WWTPs and receiving environments, but it is also critically important to periodically evaluate the effectiveness of any instituted antimicrobial resistance control and monitoring programs.

. Chapter 4: Concordance in Molecular Methods for Detection of Antimicrobial Resistance: A Cross Sectional Study of the Influent to a Wastewater Plant

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Methods that are used to characterise microbiomes and antimicrobial resistance genes (ARGs) in wastewater are not standardised. We used shotgun metagenomic sequencing (SM-Seq), RNA sequencing (RNA-seq) and targeted qPCR to compare microbial and ARG diversity in the influent to a municipal wastewater treatment plant in Australia. ARGs were annotated with CARD-RGI and MEGARes databases, and bacterial diversity was characterised by 16S rRNA gene sequencing and SM-Seq, with species annotation in SILVA/GreenGenes databases or Kraken2 and the NCBI nucleotide database respectively. CARD and MEGARes identified evenly distributed ARG profiles but MEGARes detected a richer array of ARGs (richness = 475 vs 320). Qualitatively, ARGs encoding for aminoglycoside, macrolide-lincosamide-streptogramin and multidrug resistance were the most abundant in all examined databases. RNA-seq detected only 32% of ARGs identified by SM-Seq, but there was concordance in the qualitative identification of aminoglycoside, macrolide-lincosamide, phenicol, sulfonamide and multidrug resistance by SM-Seq and RNA-seq. qPCR confirmed the detection of some ARGs, including OXA, VEB and EREB, that were identified by SM-Seq and RNA-seq in the influent. For bacteria, SM-Seq or 16S rRNA gene sequencing were equally effective in population profiling at phyla or class level. However, SM-Seq identified a significantly higher species richness (richness = 15,000 vs 3,750). These results demonstrate that SM-Seq with gene annotation in CARD and MEGARes are equally sufficient for surveillance of antimicrobial resistance in wastewater. For more precise ARG identification and quantification however, MEGARes presented a better resolution. The functionality of detected ARGs was not confirmed, but general agreement on the putative phenotypic resistance profile by antimicrobial class was observed between RNA-Seq and SM-Seq.

4.1 Introduction

The dissemination of antibiotics and ARGs into aquatic ecosystems is a major global concern, as it promotes the selection of antibiotic resistant bacteria (ARB) within complex microbial populations (Guo et al., 2017). WWTPs are a well characterised point of convergence of antibiotics, bacteria and ARGs, and their subsequent dissemination into aquatic ecosystems (Adekanmbi et al., 2020, Liu et al., 2018b, Muurinen et al., 2017, Wang et al., 2014). Monitoring of raw sewage is particularly promising for large-scale surveillance of ARG dissemination, as it contains pooled faecal

bacteria, antibiotics, and other chemicals from large populations as well as agricultural and industrial sources (Larsson and Flach, 2022). Currently, many different molecular techniques are used to quantify as well as describe qualitative features of bacteria and ARGs in waste water treatment plants (WWTPs) , but there is no standardised approach and cross-validation of methods is rarely done. This makes it difficult to qualitatively and quantitatively compare, or validate results from different studies. Using customised primers or commercially provided arrays, together with polymerase chain reaction (PCR) or quantitative PCR (qPCR), are commonly used to detect and quantify specific ARGs and this method can detect genes at low copy numbers thus providing very high sensitivity (Liu et al., 2019b, Rocha et al., 2019). However, PCR and qPCR assays can take time to optimise and the scope of ARG detection is usually limited by experimental design or primer sets provided on commercial arrays (Gaviria-Figueroa et al., 2019, Muurinen et al., 2017).

By contrast, sequencing of the 16S ribosomal ribonucleic acid (16S rRNA) gene and SM-Seq approaches have been used to identify entire microbiomes or a much wider array of ARGs in experimental samples (Christgen et al., 2015, Guo et al., 2017, Majeed et al., 2021). SM-Seq or RNA sequencing and probing of known DNA or RNA libraries of ARG databases is the method of choice for the global surveillance of markers for antimicrobial resistance in sewage and other environmental matrices (Hendriksen et al., 2019, Larsson and Flach, 2022). SM-Seq is now readily available since the cost has declined through economy of scale and it is a robust and efficient method because all major steps of the process can be automated. SM-Seq can also be used to retrospectively analyse archived data to identify novel ARGs or ARG mutations (Boolchandani et al., 2019). Nonetheless, some limitations to the applicability of SM-Seq for ARG detection still exist. Highly technical knowledge and expertise is required for several steps involved in SM-Seq approaches, and detection of ARGs using this method is limited by the scope of reference ARG sequences in probed databases such as the Comprehensive Antibiotic Resistance Database (CARD), Antibiotic Resistance Gene-annotation (ARG-ANNOT), an antimicrobial resistance database for high throughput sequencing (MEGARes), and an open online resource for identification of antimicrobial resistance genes in next-generation sequencing data (ResFinder) (Hendriksen et al., 2019). In addition, neither DNA sequencing nor PCR/qPCR provides a direct link to the functional nature of identified genetic determinants unless it is coupled with other forms of identification such as culture and sensitivity testing or transcriptomic analyses (Boolchandani et al., 2019, Fok et al., 2018). For example, in a comparative study targeting specific ARGs against defined antimicrobial classes in faecal coliforms, qPCR data strongly correlated with culture based evaluations in wastewater, recycled water, and tap water (Rocha et al., 2019). Likewise, high concordance was observed between three different SM-Seq approaches for ARG or virulence detection, and phenotypic expressions in culture-based methods (Mason et al., 2018).

However, data validation based on bacterial culture is only effective for specific target ARGs and bacterial isolates. Its application to validate data for samples from complex environmental matrices, with a diverse bacterial population, and a much bigger ARG pool is not practical. As such, robust molecular methods including qPCR, SM-Seq, and RNA sequencing are more likely to be used for high throughput screening of complex environmental samples, but direct information on agreement in different molecular methods is scarce. The primary objective of this study was to compare qPCR, SM-Seq and RNA sequencing methods in the detection and relative quantification of ARGs, and the associated bacterial population in the influent to a metropolitan WWTP. Influent samples are ideal for these comparisons because of the likely accurate representation of microbial and ARG diversity, free of wastewater processing influences such as biological treatment reactions and chemical additions.

4.2 Materials and Methods

4.2.1 Study Site and Sampling

Samples of influent were obtained from the Cleveland Bay Sewage Treatment Plant (CBSTP), Queensland, Australia, with the help of the Townsville City Council. The city of Townsville lies on the eastern coast of Australia adjacent to the boundary of the Great Barrier Reef Marine Park (GBRMP). The CBSTP is a membrane bioreactor plant that discharges processed effluent directly into the Queensland State Marine Park and within the general use zone of the Great Barrier Reef Marine Park located at -19.288744118517624 and 146.8551011782239. The CBSTP is Townsville's largest municipal sewage treatment plant, and currently services up to approximately 110,000 equivalent persons and it utilises the largest membrane bioreactor in the southern hemisphere. The catchment for the influent into the CBSTP includes commercial, industrial, and domestic sources as well as a multitude of medical and veterinary sources all of which may influence the ARG and ARB load. Samples of CBSTP influent were collected daily over 18 consecutive days and then used to create a composite sample that captures any short-term temporal fluctuation in the influent. Samples were collected in sterile one litre containers using a flow proportional autosampler and then transported to the laboratory within 1 hour at 5°C for further processing.

4.2.2 Nucleic acid extraction and preservation

Samples (1-2 mL aliquots) were centrifuged at 10,000 ×g for 2 min at 25˚C. The supernatant was then discarded, and the pellet was preserved with 750 µl of DNA /RNA shield. The pelleted 2 mL aliquots were pooled into two composite samples and DNA or RNA was extracted using

ZymoBIOMICS™ Miniprep kits (Zymo Research Corp., Irvine, CA, USA) according to the manufacturer's instructions. The concentration and purity of DNA or RNA was determined using a NadoDrop™ ND-2000c spectrophotometer (Thermo Fisher Scientific Inc., MA USA). Extracted nucleic acids were stored at -20°C (DNA) or -80°C (RNA) until further analyses. As environmental based nucleic acids are notoriously difficult to extract at workable quantities, extracted DNA and RNA was considered suitable for downstream applications at a concentration ≥ 10 ng/µL. All samples fell within the acceptable quality parameters for DNA and RNA; these were a 260/280 nm ratio = 1.80 ± 0.2 and 2.00 ± 0.2 respectively, and a 260/230 ratio $\geq$ 2 for both DNA and RNA. Extracted DNA was used for qPCR, 16S rRNA gene sequencing, and SM-Seq, while extracted RNA was subjected to RNA sequencing for transcriptomic analysis.

4.2.3 Using qPCR to detect and quantify ARGs in pooled influent samples for a WWTP

Extracted DNA was pooled into two composite samples for these analyses. Microbial DNA qPCR analysis for ARGs was done using customized arrays (Qiagen, Valencia, CA USA). These arrays provide a high-throughput profiling of 84 genes that represent major classes of ARGs including aminoglycosides, beta-lactams, fluoroquinolones, macrolides-lincosamides-streptogramins, tetracyclines, vancomycin, and other drug classes. Thermal cycling was performed on an applied Biosystems™ QuantStudio™ 3 Real-Time PCR System thermocycler (Waltham, MA, USA). Five nanograms of template DNA was used with a HotStart DNA polymerase and an initial PCR activation at 95°C for 10 mins followed by 40 cycles consisting of denaturation for 15 sec at 95 °C and annealing and extension for 2 min at 60°C. A threshold cycle (C_T) value of 37 was used and baselines were manually set for cycles 8–20 with a threshold fluorescence setting of 0.24 and 0.35 respectively for each duplicate. The qPCR array data was evaluated according to the manufacturer's criteria and as previously published (Adekanmbi et al., 2020, Liu et al., 2018a, Liu et al., 2018b, Looft et al., 2012, Muurinen et al., 2017).

4.2.4 Metagenomic DNA or RNA sequencing to determine bacterial population and ARG Profiles

DNA extracted from 18 samples based on 18 days of sampling was pooled into a composite sample and then subjected to SM-Seq at the Australian Genome Research Facility (AGRF), Melbourne Australia. Library preparation and SM-Seq was performed according to AGRF's methodology. Briefly, the Shotgun library was constructed by using an Illumina DNA preparation kit (San Diego, CA, USA). DNA was fragmented to a size of 150 bp, and the fragments were then ligated with index barcode sequences, end-polished, A-tailed and ligated with adaptors for Illumina sequencing. The library was

sequenced using Illumina NovaSeq S4 sequencing platform (San Diego, CA, USA) with a single lane for this sample. The raw read sequences were processed through Trim Galore (version 0.4.5) to identify and remove sequencing adapters and low-quality stretches of base quality below 20 as outlined previously (Martin, 2011). Kraken2 (version 2.0.8) (Wood et al., 2019), and Bracken (version 2.5) (Lu et al., 2017) were applied to profile the composition of microbial communities using NCBI genome and nucleotide (nt) database (downloaded 15/04/2021). Gene fraction was defined as the minimum proportion of nucleotides in a reference sequence that had to be aligned by at least one read to be considered as 'identified' in the sequenced data. A threshold of 80% was used to minimize potential false classifications and removing accessions with sparse alignment and functional profiles were generated using HUMAnN2 (version 2.8.1).

ARGs were identified from SM-Seq data using two separate analysis pipelines as outlined in Figure 4-1. AMR++ 3.0 was used for quality assessment of raw reads, followed by filtering, trimming and adapter removal as previously described (Bonin et al., 2023). This pipeline also facilitated the removal of human DNA. Two separate databases were then probed to identify ARGs. Using AMR++ 3.0 facilitated the probing of MEGARes database using bwa-mem as described previously (Bonin et al., 2023). ResistomeAnalyzer was then applied to process the alignments using a minimum gene fraction threshold of 80%. Further ARG analysis was also completed using the CARD resistance gene identifier (RGI) (Alcock et al., 2023). RGI aligned short DNA sequences in FASTQ format using KMA and then the FASTQ sequences were aligned to the in silico predicted allelic variants in CARD database (Clausen et al., 2018). The gene fraction threshold was set at 80% to minimize potential false classifications. Finally, a resistome matrix was built using the two databases to get final results. These two databases were selected based on their comprehensiveness of ARG coverage, frequency of maintenance and update, and being publicly accessible. MEGARes contains approximately 9,000 ARGs and the CARD database contains 5,010 ARG reference sequences, and this is updated regularly (Alcock et al., 2023, Bonin et al., 2023).

RNA was extracted from a composite sample comprising of 18 days of sampled influent, corresponding to the same 18 days used for DNA analysis described above. The extracted RNA composite sample was then subjected to RNA sequencing at the AGRF. Library preparation and sequencing was performed according to AGRF's methodology. Briefly, a library was created using Illumina Stranded Total RNA with Ribo-Zero Plus kit (San Diego, CA, USA) using 150 ng input, 16 PCR cycles and a 0.8X bead ratio for the final library clean-up.

The quality of the library was assessed using the LabChip GX Touh II DNA High Sensitivity Assay (Perkin Elmer, Hamburg, Germany), and the concentration of the library was determined using

NEB's qPCR kit and sequenced on Illumina's NovaSeq 6000 (San Diego, CA, USA) on a lane of an S4 300 flow cell. Data was processed through AMR++3.0 for identification of functional ARGs in the sample as described previously (Bonin et al., 2023).

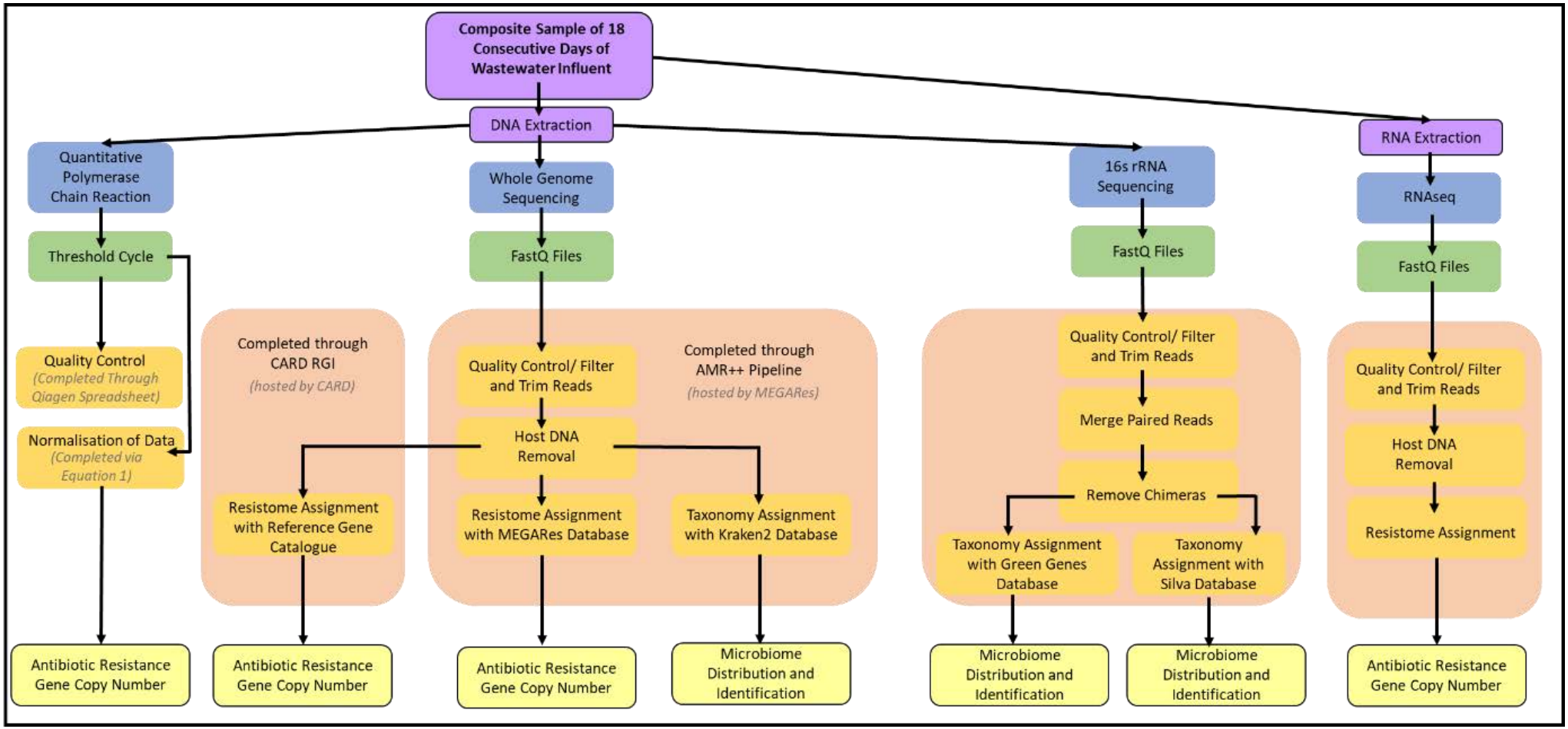


Figure 4-1: A simplified overview of different methods and analyses that were used. This outlines the workflow from sampling to the final qualitative or quantitative results including antibiotic resistance gene copy numbers and microbiome identification and distribution.

4.2.5 16S rRNA gene sequencing to determine the bacterial population

16S rRNA gene sequencing was performed according to AGRF's methodology. Briefly, the V3-V4 hypervariable region of the 16S rRNA gene was amplified using universal primers 341-Forward (5'CCTACGGGNGGCWGCAG) and 806-Reverse (5' GGACTACHVGGGTWTCTAAT). DNA quality control secreening was done via PCR and indexed using fluorometry prior to diversity profiling of the 16S rRNA primer targets. Amplicons were sequenced on the Illumina MiSeq platform (San Diego, CA, USA) for the production of a 300 bp paired end run. Diversity profile analysis was performed with QIIME 2 2019.7 (Bolyen et al., 2019). The demultiplexed raw reads were primer trimmed and quality filtered using the cutadapt plugin followed by denoising and removal of chimeras via q2-dada2 as previously described (Callahan et al., 2016). Taxonomy was assigned from the SILVA database (Quast et al., 2012) and GreenGenes database (DeSantis et al., 2006) to amplicon sequence variants using the q2-feature-classifier (Bokulich et al., 2018), and sequence variant set at 97% identity.

4.2.6 Data Analysis

4.2.6.1 Analysis of ARG expression by qPCR

The criteria for qPCR data analysis were: (1) the sample was identified as positive for a gene if the C_T was below 34, (2) detection for a gene was inconclusive if the C_T value was between 34 and 37, and (3) the sample was identified as negative for a gene if the C_T value was greater than 37. These guidelines were based on the C_T values obtained with the negative control sample prepared with molecular grade water. The C_T value for each positively identified gene was converted to gene copy number using Eq. 1 as previously described (Adekanmbi et al., 2020, Liu et al., 2018a, Liu et al., 2018b, Looft et al., 2012, Muurinen et al., 2017).

Equation 1 $$\text{Relative Gene Copy} = 10^{(34-C_T)\div(\frac{10}{3})}$$

Where C_T refers to the number of cycles required to exceed background fluorescence for each probed gene, 34 refers to the detection limit, and 10/3 refers to the 10-fold difference in gene copy numbers (GCN) at 100% efficiency.

4.2.6.2 Analysis of ARG expression by metagenomic DNA and RNA sequencing

Cumulative read counts of each ARG were normalized to gene length as reads per kilobase (RPK) as outlined previously (Honda et al., 2023). The relative proportion of each ARG in the sample was evaluated as the ratio of RPK for each ARG to the sum of RPK for all ARGs. Data were processed

and presented using the "ggplot2" package version 3.4.1 (Wickham, 2011). Statistical analysis of diversity and richness of ARG expression was assessed using the "Phyloseq" package version 3.16 (McMURDIE and Holmes, 2012). Alpha diversity of ARGs included the evaluation of Richness and Eveness. Differences in detection methods for relative ARG expression in the composite sample were also analysed by a correlation coefficient model (R software version 4.1.1) and Z-scores.

4.2.6.3 Analysis of bacterial population profiles

The microbiome composition identified by probing the NCBI nucleotide database using SM-Seq Kraken2 software, and two 16S rRNA sequence databases (GreenGenes and SILVA) were also compared using R software (v4.1.1) as previously described (Odom et al., 2023). Read counts of 16S rRNA gene were also normalized as RPK and the relative proportions of different classes of bacteria was evaluated as the ratio of RPK for each class to the sum of RPK for the sample and presented as bar plots using "ggplot2" package version 3.4.1 (Wickham, 2011). "Phyloseq" package (version 3.16) was used for alpha diversity assessments as outlined above.

4.3 Results

4.3.1 Characterisation of the ARG profile by a customised microbial DNA qPCR array

Bacterial phosphoenolpyruvate carboxylase gene (*PPC*) was used as a positive control for each qPCR array and it was amplified within the recommended C_T of 22 ± 2 for the duplicate assays. Duplicate DNA qPCR arrays identified a similar ARG profile in the composite sample. The averages of C_T values from duplicate arrays showed that 44 out of 83 individual ARGs and variants were positively expressed while the expression of 12 genes was categorised as inconclusive and 27 gene were not expressed (Appendix Table A.B-1). For the 44 ARGs and variants expressed in the composite influent sample, there was no difference in duplicate C_T values from two separate assays. The average difference in C_T values was 0.42 with a range of 0.04 (for CCRA gene) to 1.17 (for QNRB-1 gene). Average C_T values were used to determine gene copy number as outlined in (Eq. 1). Of the 83 ARGs screened in duplicate qPCR assays, 10 were represented multiple times either as specific variants of the same gene e.g., CTX-1, CTX-8 and CTX-9, or sequences of the same gene with reference to different bacterial strains of origin e.g., SHV(238G240E) and SHV(238G240K) (Appendix Table A.B-1). Copy numbers of variants of the same gene were aggregated and represented as a single gene and the type of resistance it encodes for. The 10 most abundant ARGs were OXA (31.0%), ERMB (19.1%), GES (15.2%), MEFA (15.0%), VEB (4.6%), TETA (4.0%), AADA1 (3.9%), AAC6 (2.6%), QNRS (1.3%) and MOX (0.8%), collectively making up 97.5% of all detected ARGs (Figure 4-2). Resistance genes

associated with beta-lactamases had the highest percentage of GCN (53%) and this may be attributable to high representation (28 out of 50) ARG primer sets to probe the expression of beta-lactamases. In contrast, the combined GCN for five ARGs encoding for resistance against macrolides, lincosamides and streptogramins (MLS) accounted for 35% of all detected GCN. Other represented antimicrobial classes were fluoroquinolones, tetracyclines and aminoglycosides.

4.3.2 ARG characterisation by illumina sequencing and probing of annotated datases

4.3.2.1 DNA Sequencing and ARG annotation in MEGARes database

The MEGARes database identified 184 ARGs out of 487,068 reads and each ARG sequence matched at least 80% of the individual gene fraction (GF) in the database. ARGs encoding for resistance against the aminoglycosides class of antimicrobials were the most abundant representing 33%, but other major antimicrobial classes including MLS (31%), biocides (11%), beta-lactams (6%), fluoroquinolone (4%), and tetracyclines (7%) were also represented (Figure 4-3). To provide context, genes encoding for aminoglycoside resistance represented 19% of all ARGs in MEGARes database while ARGs against MLS represented less than 5% of ARGs in the database. A single gene, A16S encoding for an aminoglycoside-resistant 16S ribosomal subunit protein accounted for a large proportion (17%) of the total ARGs in the composite influent sample. Similarly, MLS23S encoding for macrolide resistance 23S rRNA mutation accounted for 16% of the total ARGs in the composite influent sample.

4.3.2.2 DNA Sequencing and ARG annotation in CARD database

The CARD database identified 317 unique ARG sequences from a total of 53,411 reads, and all ARG sequences matched 80-100% of the individual GF. In this database, genes encoding for multi-drug resistance represented the highest fraction (~40%) of all ARGs identified in the composite influent sample (Figure 4-4). In the distinct antimicrobial classes, resistance against MLS represented the highest fraction (15%) of all identified ARGs and resistance against other major antimicrobial classes was represented by aminoglycosides (10%), sulfonamides (7%), beta-lactams (5%) and tetracyclines (4%). Interestingly, this database also identified genes encoding for resistance against disinfectants and antiseptics representing ~11% of ARGs in CARD and noted herein as drug and biocide resistance (Figure 4-4). The ACRB gene that encodes for a cell membrane drug antiporter and multi-drug resistance was significantly more abundant representing 32% of ARG sequences and others included SUL1 (a sulfonamide resistant dihydropteroate synthase), SAT4 (streptothricin

acetyltransferase) and QACG (quaternary ammonium compound gene) representing 6.4%, 6.0% and 4% of total ARGs respectively.

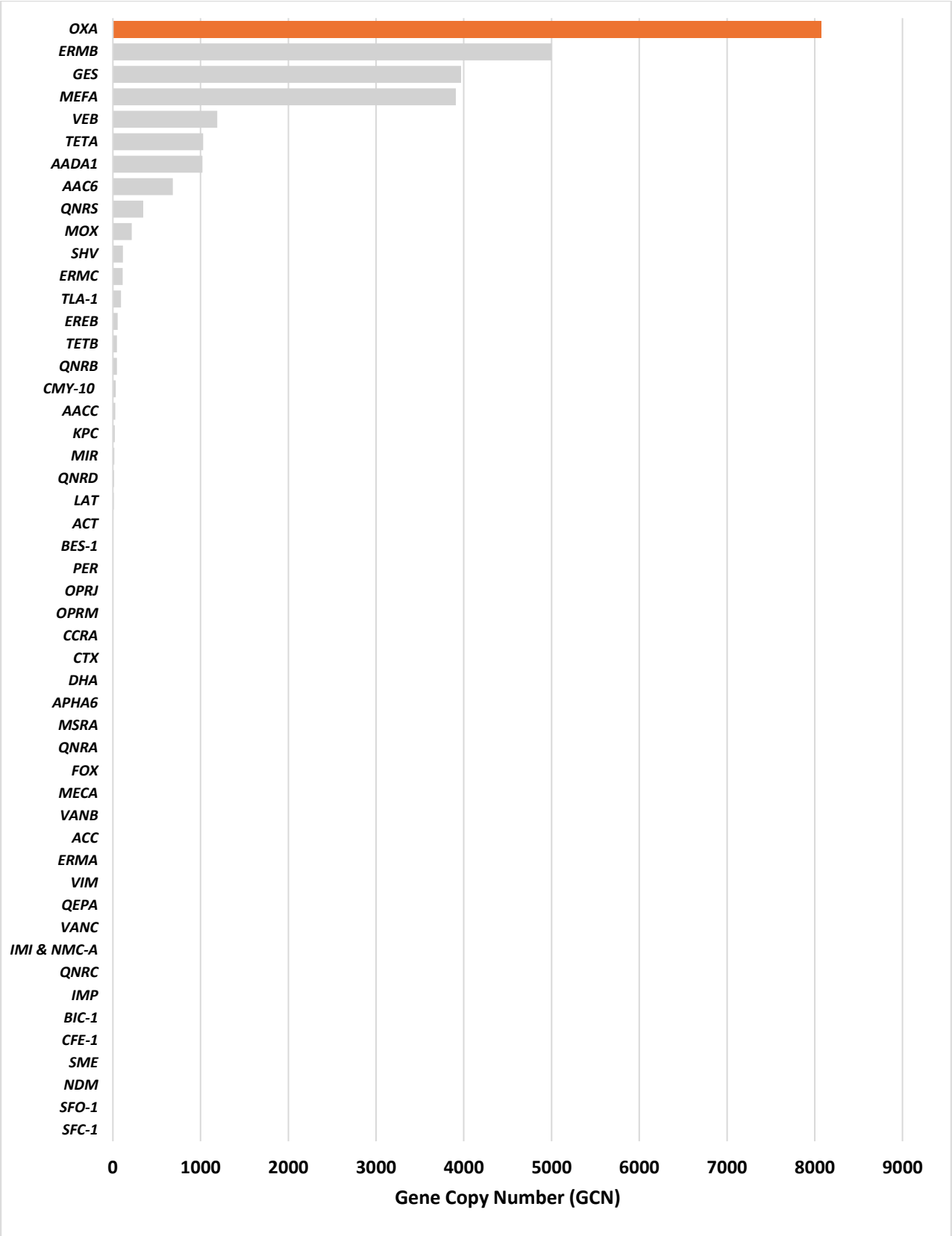


Figure 4-2: Average gene copy number of antibiotic resistance genes represented on a qPCR array. DNA was extracted from 18 samples of influent to a wastewater plant. The samples were pooled into a composite sample and assayed in duplicates on two different arrays.

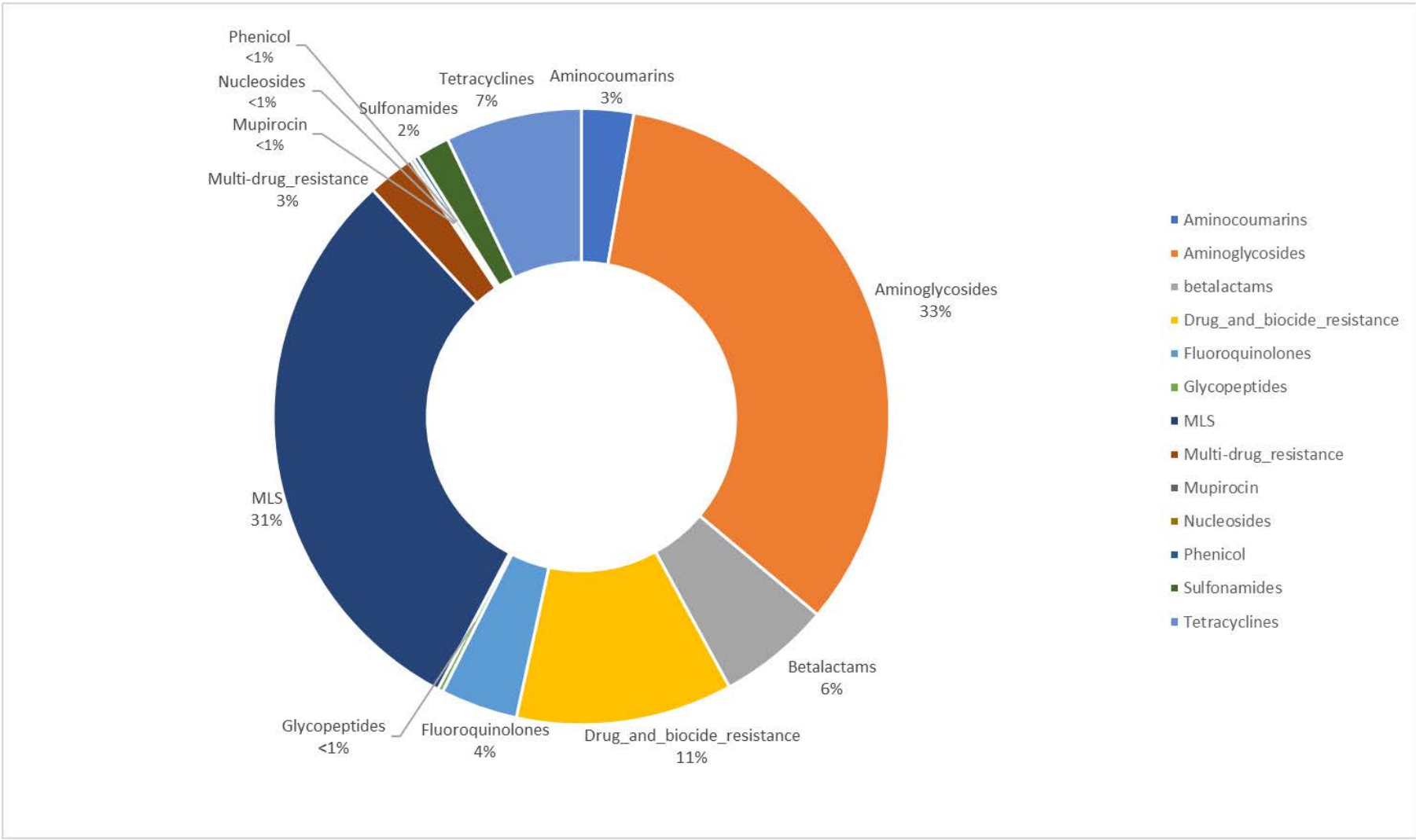


Figure 4-3: Distribution of ARGs stratified by antimicrobial class against which they encode resistance after whole genome sequencing and gene annotation for ARGs in MEGARes databases. This graph denotes ARGs and corresponding antimicrobial classes common to MEGARes and CARD databases for comparison. DNA was extracted from 18 influent samples and then pooled into a composite sample that was analysed by whole genomic sequencing.

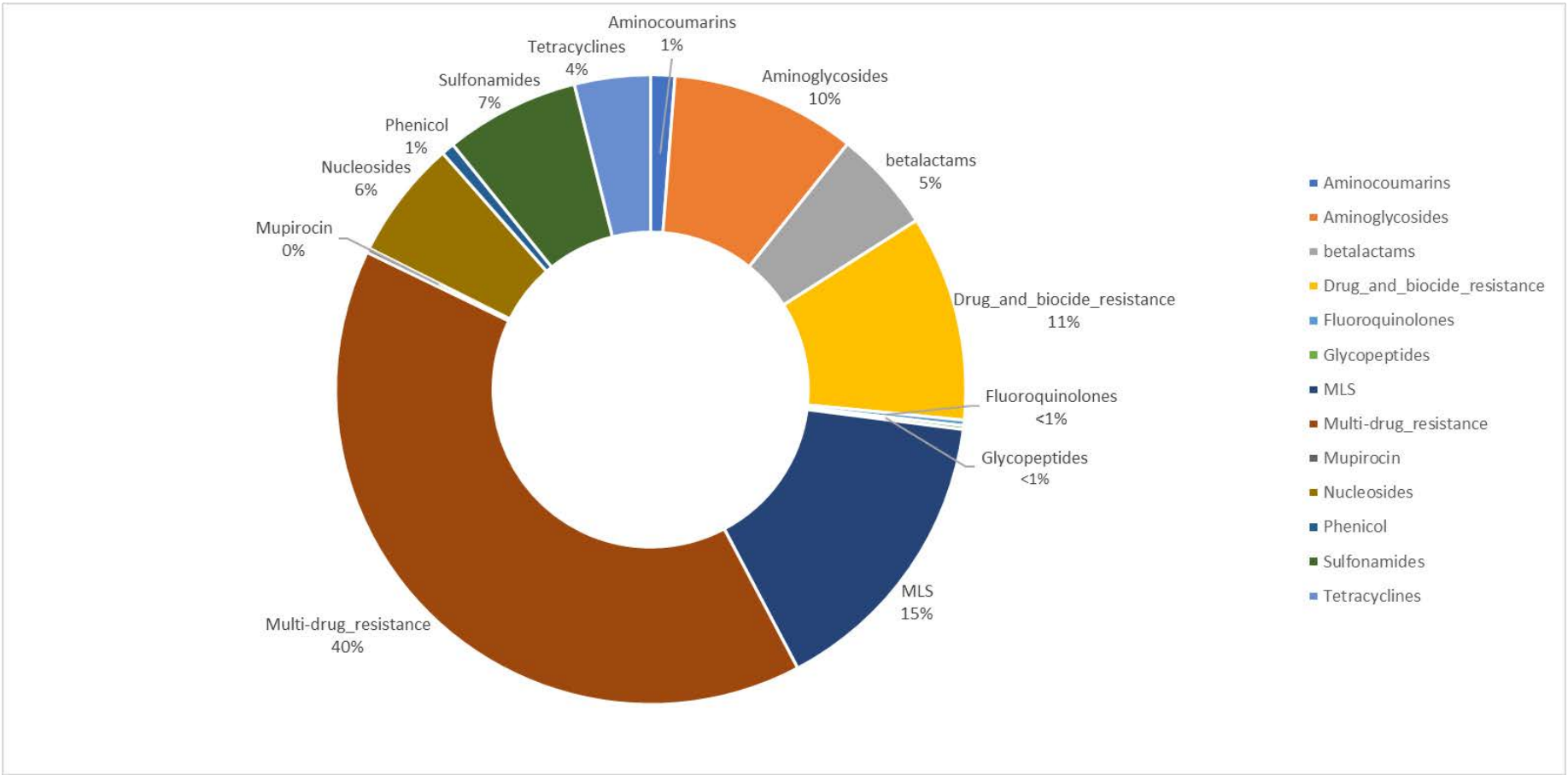


Figure 4-4: Distribution of ARGs stratified by antimicrobial class against which they encode resistance after whole genome sequencing and gene annotation in the CARD database. This graph denotes ARGs and corresponding antimicrobial classes common to MEGARes and CARD databases for comparison. DNA was extracted from 18 influent samples, pooled into a composite sample, and then analysed by whole genome sequencing.

4.3.2.3 Comparison of qPCR and metagenomic DNA sequencing for detection of ARGs

The qPCR array was customised to probe a total of 83 ARGs; out of this, 46 ARGs were in the MEGARes database and 47 ARGs were in the CARD database. Only 3 ARGs (AAC6, AACC, APHA1) were not in the MEGAREs and CARD databases at the time of analysis. Only 11 distinct ARGs were identified in qPCR assays and the MEGARes, and 13 ARGs were identified in qPCR and CARD database (Figure 4-5). Interestingly, the ERMA gene that encodes for MLS resistance was detected by MEGARes and CARD at a relatively high abundance but it was not detected by qPCR. The three methods based on SM-Seq and qPCR yielded markedly different GCN, but GCN normalisation using Z-scores showed very good agreement in ARG detection and relative quantification by MEGARes, CARD and qPCR (Figure 4-5). For instance, all 3 methods indicate that OXA, GES and MEFA were some of the specific ARGs with the highest relative abundance in respective datasets. Very limited discordance was observed for some ARGs that had very low GCN. The TETB and MOX genes for example, were detected by qPCR but not via either SM-Seq database (along with an additional 14 ARGs), while ERMA and SME were detected by one or both SM-Seq methods and not the qPCR array (Figure 4-5).

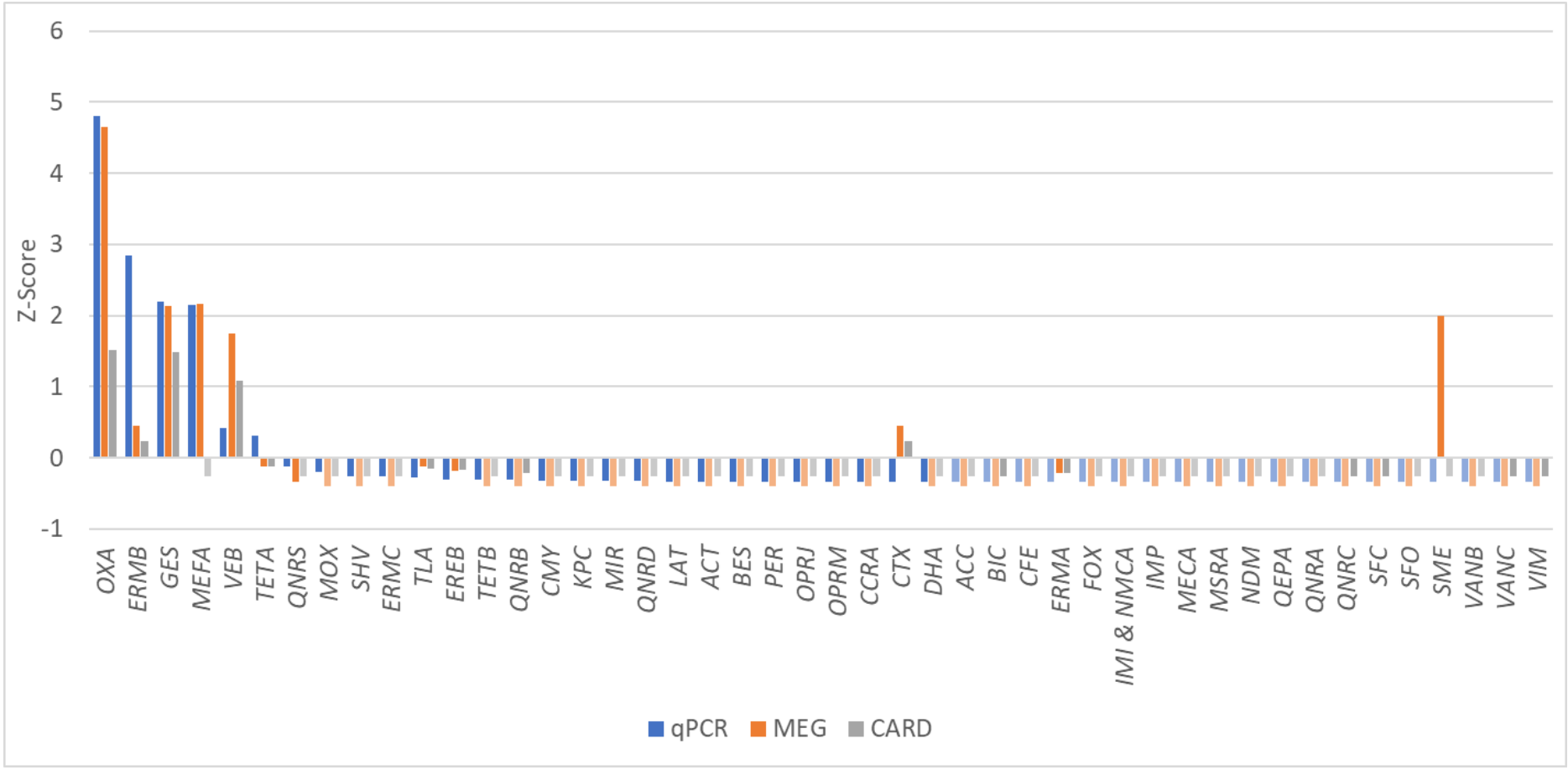
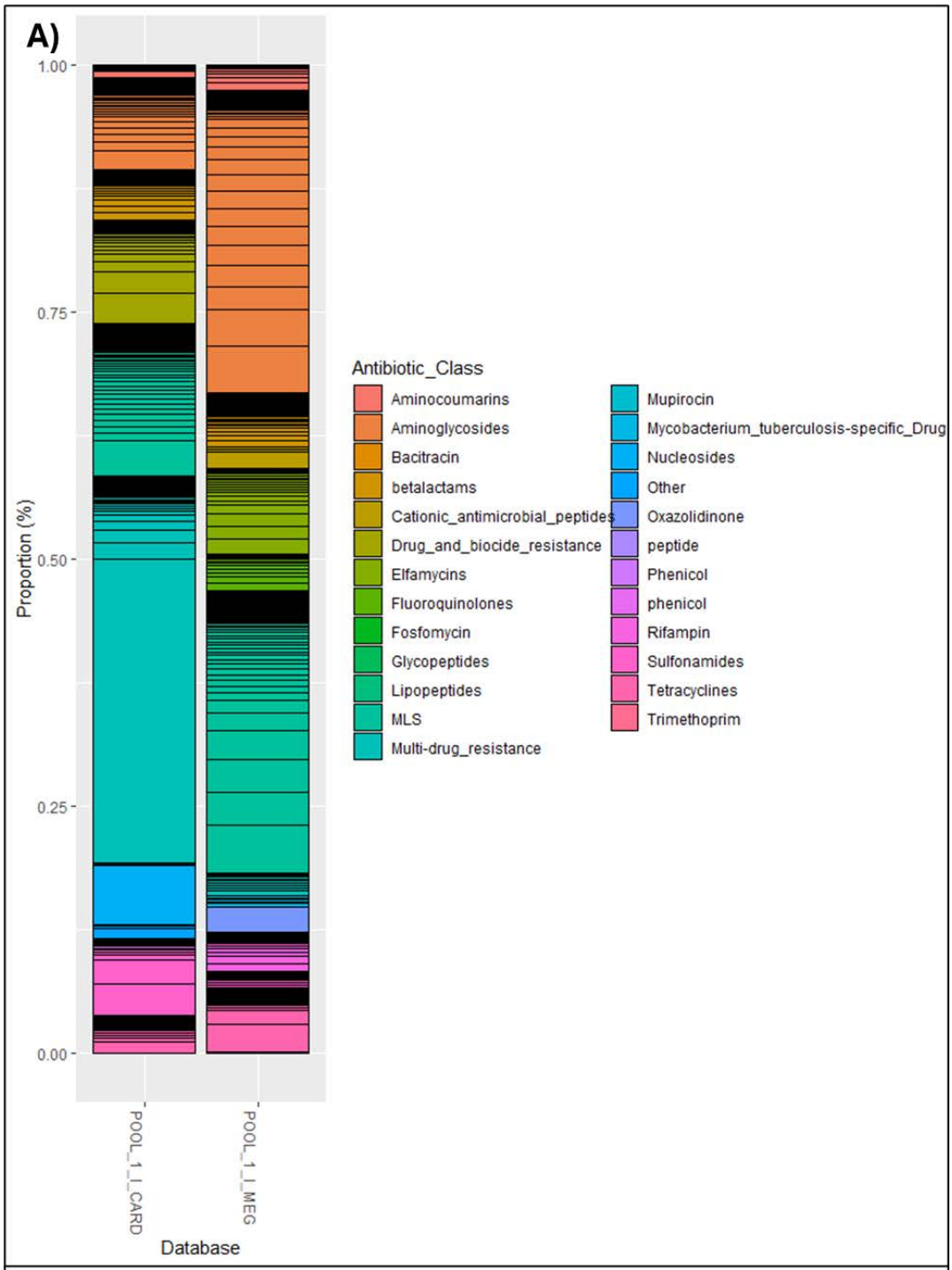


Figure 4-5: Z-score for normalised gene copy numbers of ARGs identified by qPCR (blue) and whole genome sequencing followed by gene annotation in the MEGARes (orange) and CARD (grey) databases. Bars represented by lighter, faded colours indicate Z-scores equal to zero actual abundance. DNA was extracted from 18 influent samples, pooled into a composite sample, and then analysed by qPCR and the respective metagenomic DNA sequencing approaches.

Qualitatively, SM-Seq and gene annotation in MEGARes or CARD identified a similar distribution of ARGs against the major antimicrobial classes albeit at different relative abundance (Figure 4-6A). For example, genes encoding for multi-drug resistance represented the highest proportion of ARGs detected in CARD while genes encoding for aminoglycoside and MLS represented the highest proportion of ARGs detected in MEGARes (Figure 4-6A). These observations were also supported by further in depth analysis of ARG distribution in MEGARes and CARD databases (Figure 4-6B). Expressed ARGs were evenly distributed in the MEGARes and CARD databases (Evenness = 0.72 vs 0.75 respectively). In contrast, MEGARes presented a richer distribution of ARGs than CARD (observed richness = 475 vs 320 ARG sequences respectively).

4.3.2.4 Comparison of RNA sequencing and ARG annotation in MEGARes database

A total of 59 ARGs were identified from RNA sequencing followed by probing of MEGARes in the composite influent sample. This represented 32% of the total number of ARGs identified by DNA sequencing and gene annotation in MEGARes. All 59 ARGs identified by RNA sequencing were also detected in the DNA sample but the relative proportion of matched reads in RNA for each ARG was generally lower (Figure 4-7A). The distribution of all 59 specific ARGs that were identified by sequencing and gene annotation of both DNA and RNA is illustrated in (Figure 4-7B). ARGs with the highest abundance in both RNA and DNA sequences were MLS23S and A16S, encoding for resistance against MLS and aminoglycosides respectively. The magnitude of differences in relative abundance for ARGs expressed in RNA and DNA sequences was illustrated using visualised model coefficients (Figure 4-8). These data also show that some uncommon ARGs including P16S encoding for pactamycin resistance, and P23S encoding for pleuromutilin resistance were represented by a high proportion in RNA sequences as compared to their expression in the DNA sequences. By contrast however, there was no significant difference in the relative abundance between RNA and DNA sequences, for genes encoding for phenicol, sulfonamide, and multi-drug resistance (Figures 4-7 and 4-8).


A)
1.00
0.75
0.50
0.25
0.00
Proportion (%)
POOL_1_I_CARD
POOL_1_I_MEG
Database
Antibiotic_Class
Aminocoumarins
Aminoglycosides
Bacitracin
betalactams
Cationic_antimicrobial_peptides
Drug_and_biocide_resistance
Elfamycins
Fluoroquinolones
Fosfomycin
Glycopeptides
Lipopeptides
MLS
Multi-drug_resistance
Mupirocin
Mycobacterium_tuberculosis-specific_Drug
Nucleosides
Other
Oxazolidinone
peptide
Phenicol
phenicol
Rifampin
Sulfonamides
Tetracyclines
Trimethoprim

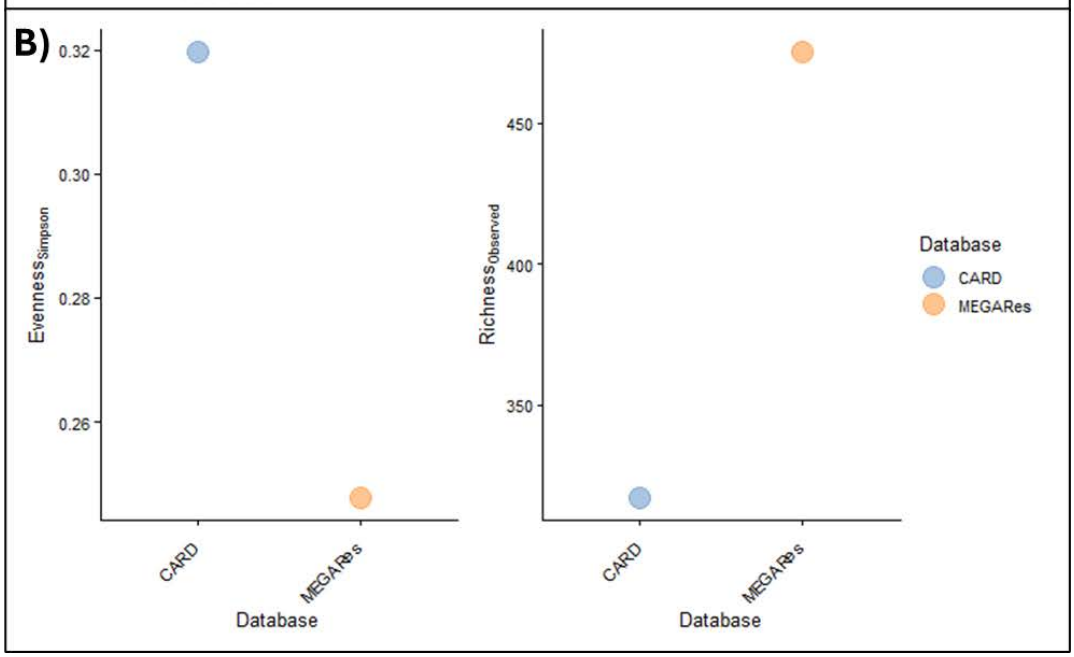

B)
0.32
0.30
0.28
0.26
Evenness Simpson
CARD
MEGARes
Database
450
400
350
Richness Observed
CARD
MEGARes
Database
Database
CARD
MEGARes

Figure 4-6: Distribution of ARG sequences stratified by antimicrobial class against which they encode resistance after whole genome sequencing and gene annotation in the CARD and MEGARes database. (A) Relative ARG sequence abundance by antimicrobial class and black borders depicting the proportion of individual resistance genes and (B), Alpha diversity measure of observed richness and evenness of ARG distribution.

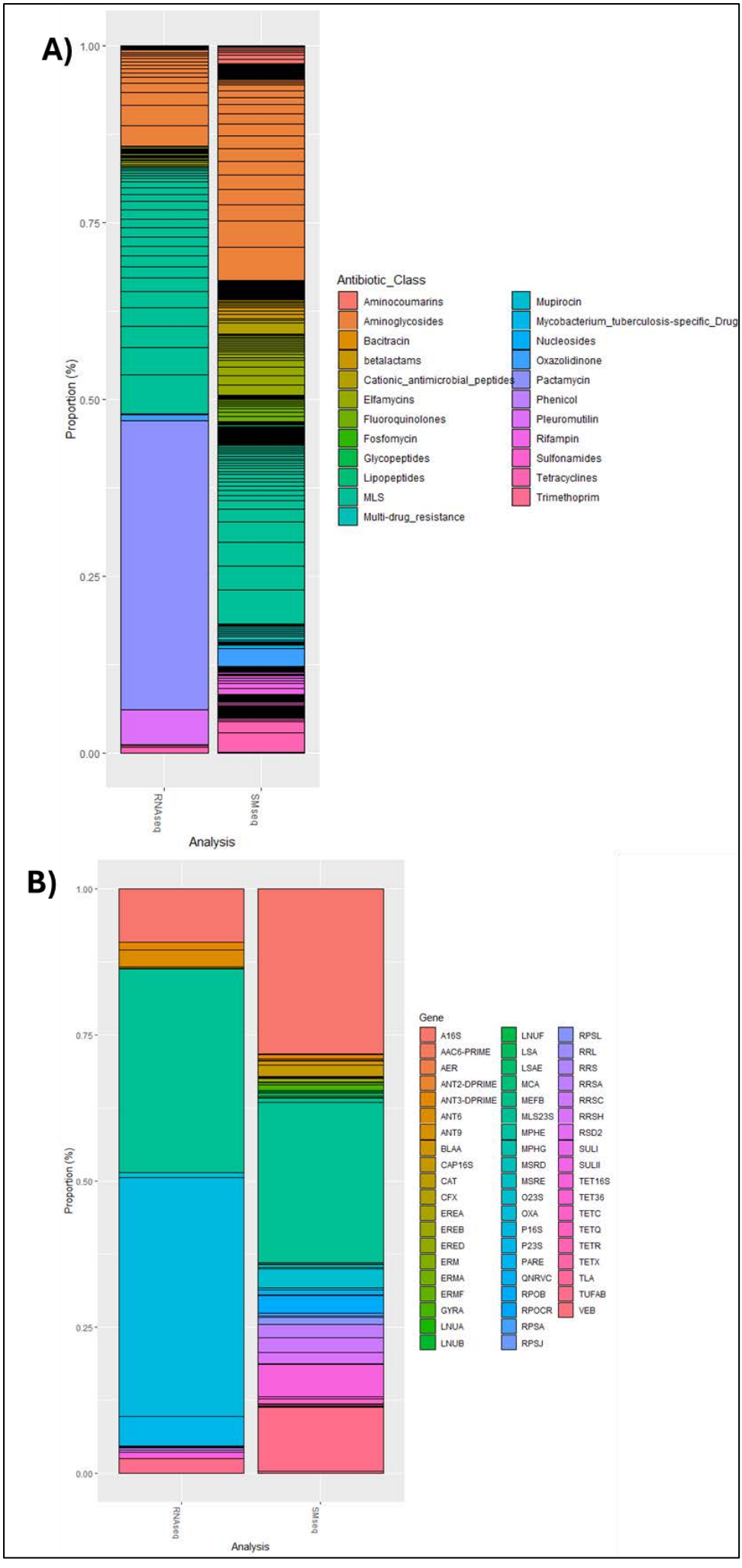

A)
1.00
0.75
0.50
0.25
0.00
Proportion (%)
RNAseq
SMseq
Analysis
Antibiotic_Class
Aminocoumarins
Aminoglycosides
Bacitracin
betalactams
Cationic_antimicrobial_peptides
Elfamycins
Fluoroquinolones
Fosfomycin
Glycopeptides
Lipopeptides
MLS
Multi-drug_resistance
Mupirocin
Mycobacterium_tuberculosis-specific_Drug
Nucleosides
Oxazolidinone
Pactamycin
Phenicol
Pleuromutilin
Rifampin
Sulfonamides
Tetracyclines
Trimethoprim
B)
1.00
0.75
0.50
0.25
0.00
Proportion (%)
RNAseq
SMseq
Analysis
Gene
A16S
AAC6-PRIME
AER
ANT2-DPRIME
ANT3-DPRIME
ANT6
ANT9
BLAA
CAP16S
CAT
CFX
EREA
EREB
ERED
ERM
ERMA
ERMF
GYRA
LNUA
LNUB
LNUF
LSA
LSAE
MCA
MEFB
MLS23S
MPHE
MPHG
MSRD
MSRE
O23S
OXA
P16S
P23S
PARE
QNRVC
RPOB
RPOCR
RPSA
RPSJ
RPSL
RRL
RRS
RRSA
RRSC
RRSH
RSD2
SULI
SULII
TET16S
TET36
TETC
TETQ
TETR
TETX
TLA
TUFAB
VEB

Figure 4-7: Distribution of ARG sequences stratified by antimicrobial class against which they encode resistance after whole genome sequencing, and RNA sequencing with gene annotation in the MEGARes database. (A) Relative ARG abundance stratified by antimicrobial class in DNA and RNA sequences, and black borders depicting the proportion of individual resistance genes, (B) Relative abundance of specific ARGs in DNA and RNA sequences and black borders surrounding depicting the proportion of individual sequences.

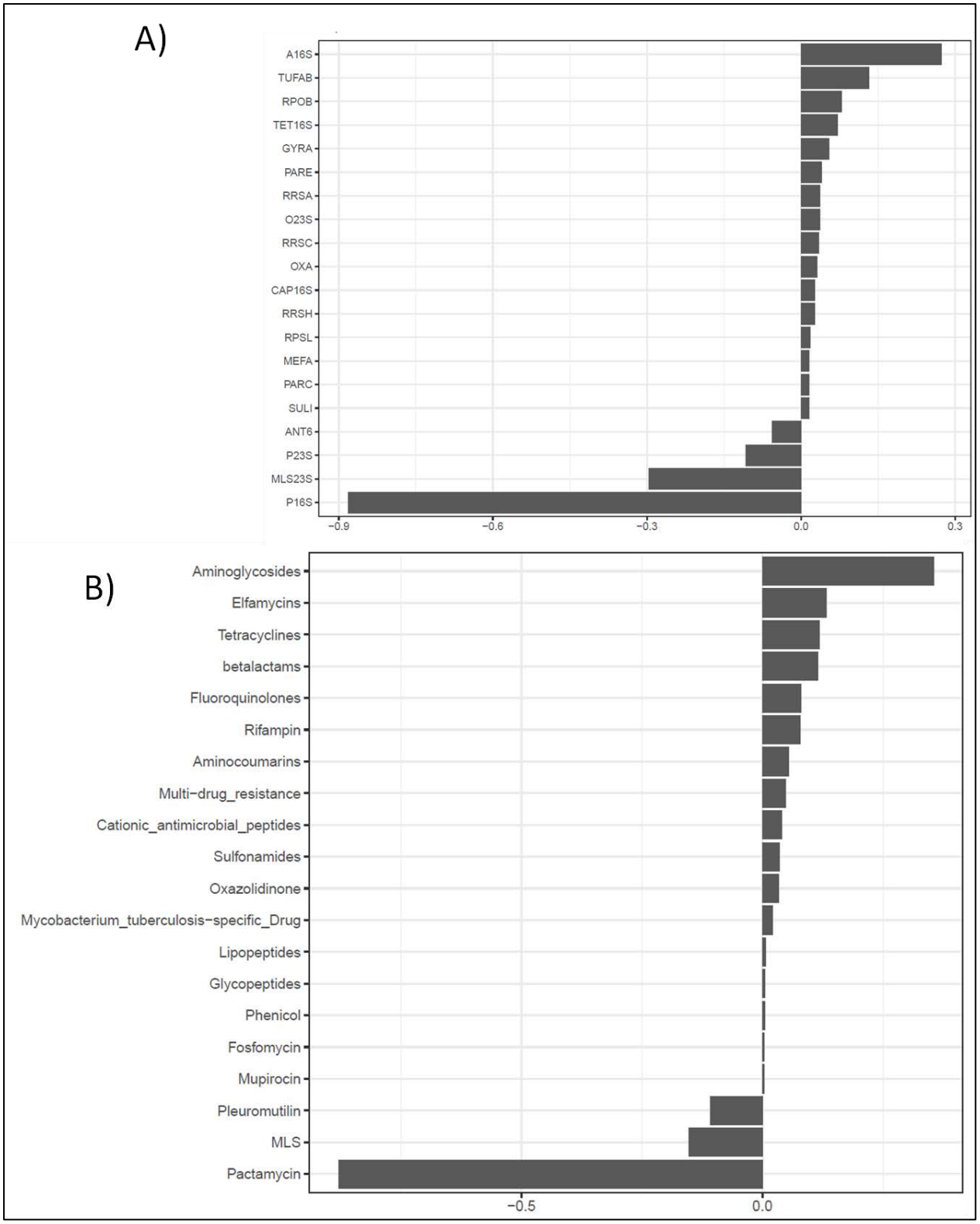
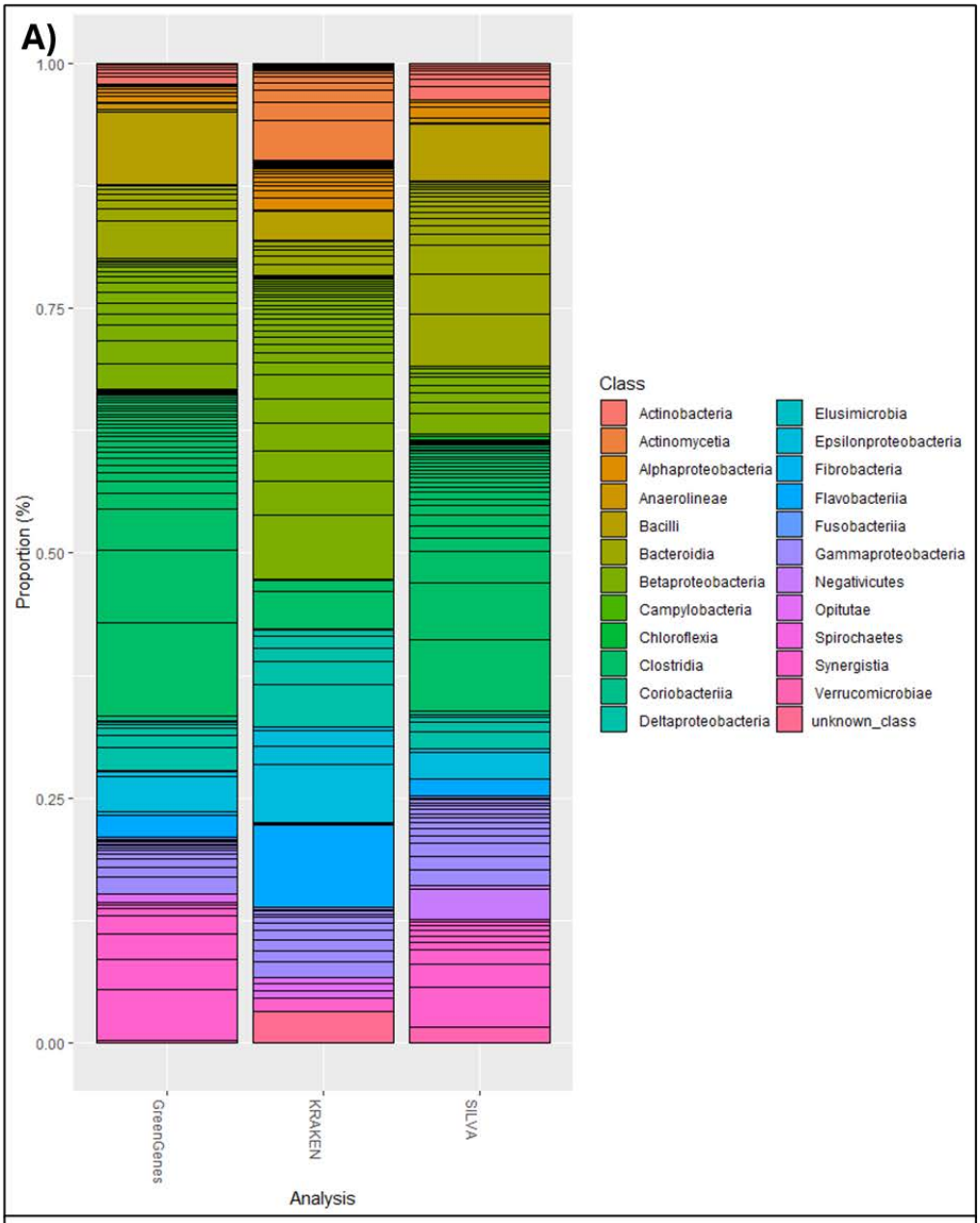


Figure 4-8: Visualised model coefficients to illustrate differences in relative ARG expression in DNA compared to RNA (A) and, the antimicrobial class against which resistance is encoded (B). Values above zero indicate relatively higher abundance in DNA than RNA, and values below zero indicate relatively higher abundance in RNA than DNA.

4.3.3 Profiling the microbiome by 16S rRNA sequencing and SM-Seq using Kraken2 database

Profiling of the 16S rRNA amplicon in SILVA database identified 1471 bacterial species while the GreenGenes database identified 1460 bacterial species in the composite influent sample (Figure 4-9A). The distribution and relative abundance of bacterial classes was similar for SILVA_16S and GreenGenes_16S methods and this was further illustrated by evaluated alpha diversity for observed population richness and evenness (Figure 4-9B). In comparison, a SM-Seq approach coupled with bacterial species profiling in the NCBI nucleotide database using Kraken2 software (SM-Seq _Kraken2) yielded more mapped reads (17,560,817) and bacterial taxonomic sequences (36,255). The relative proportion of some of the most prevalent bacterial classes including Betaproteobacteria, Actinobacteria, Deltaproteobacteria, Fibrobacteria and Flavobacteria was higher in the sample analysed by SM-Seq followed by profiling in Kraken2 (Figure 4-9). This difference was also highlighted by profiling bacterial population diversity where SM-Seq _Kraken2 had a markedly higher observed richness of bacterial species (richness = 15,000 for SM-Seq _Kraken2 vs 3,750 for 16S rRNA gene metabarcoding). The observed evenness in bacterial species distribution were 0.865 for 16S rRNA gene metabarcoding versus 0.765 for SM-Seq _Kraken2). Further analysis of the 18 individual samples that constituted the composite sample revealed a similar microbial profile as detected in the composite sample using the SILVA_16S rRNA gene metabarcoding approach (Figure 4-10). Bacteriodia, Clostridia, Gammaproteobacteria, and Synergistia were the bacterial classes with highest abundance across all 18 days of sampling albeit in different proportions.

The biggest variation in individual days was in the sample collected on day 15 in which Gammaproteobacteria constituted more than 80% of the identified species. Analysis of the 18 individual samples as well as the composite sample by a non-parametric, permutational multivariate analysis (PERMANOVA) revealed no significant differences ($P = 0.941$), in the microbial composition of all samples.


A)
1.00
0.75
0.50
0.25
0.00
Proportion (%)
GreenGenes
KRAKEN
SILVA
Analysis
Class
Actinobacteria
Actinomycetia
Alphaproteobacteria
Anaerolineae
Bacilli
Bacteroidia
Betaproteobacteria
Campylobacteria
Chloroflexia
Clostridia
Coriobacteriia
Deltaproteobacteria
Elusimicrobia
Epsilonproteobacteria
Fibrobacteria
Flavobacteriia
Fusobacteriia
Gammaproteobacteria
Negativicutes
Opitutae
Spirochaetes
Synergistia
Verrucomicrobiae
unknown_class

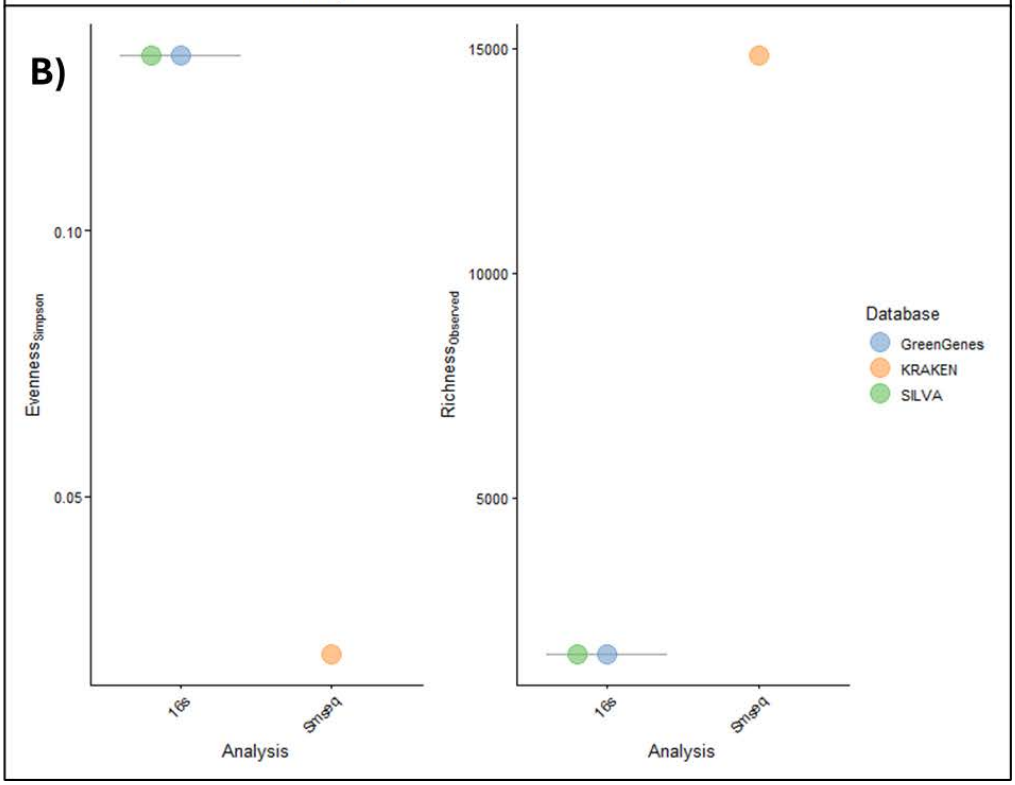

B)
0.10
0.05
Evenness Simpson
16s
Shotgun
Analysis
15000
10000
5000
Richness Observed
16s
Shotgun
Analysis
Database
GreenGenes
KRAKEN
SILVA

Figure 4-9: A comparison of the distribution and relative proportion for classes of bacteria constituting the top 100 genera following 16S rRNA gene sequencing with taxonomic annotation in GreenGenes and SILVA, and after whole genome sequencing with taxonomic annotation using Kraken2 and the NCBI nucleotide database and black borders depicting the proportion of individual bacterial order (A), and alpha diversity measure of observed richness and evenness (B). DNA was extracted from 18 influent samples, pooled into a composite sample, and then analysed.

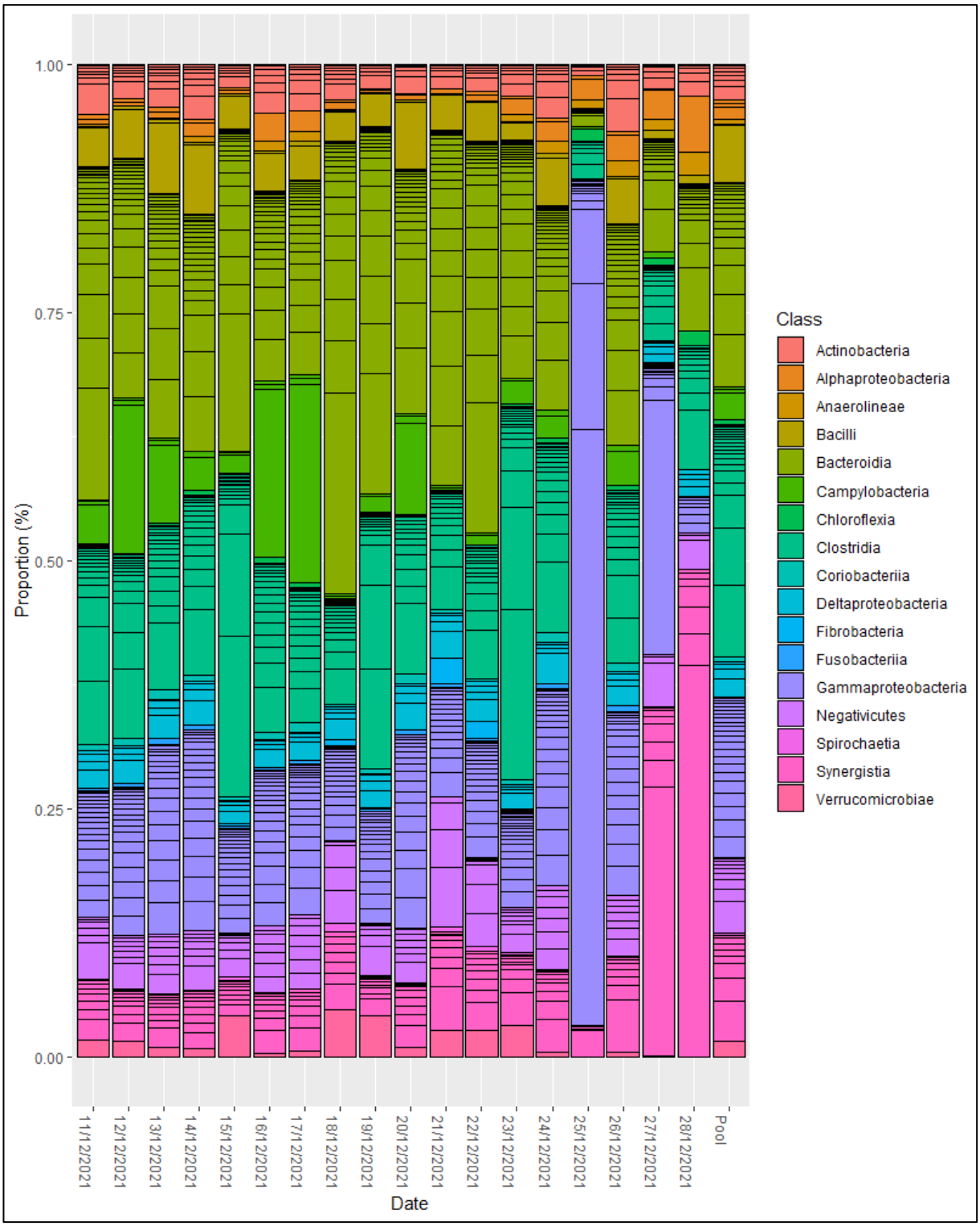


Figure 4-10: Relative abundance and distribution of bacterial class in 18 individual samples and the pooled composite sample following 16S rRNA gene sequencing and bacterial class annotation using SILVA and black borders depicting the proportion of individual bacterial order within each class.

4.4 Discussion

In this study, influent samples into a municipal wastewater treatment plant (WWTP) were collected over 18 consecutive days, pooled into a composite sample, and then used to compare molecular methods for detection and relative quantification of antimicrobial resistance genes (ARGs). The influent was considered because it is likely to represent the diversity in ARGs and microbiome from individual contributors to the WWTP without the complications of the biological treatment process itself, or the addition of treatment plant chemicals. In addition, it has been shown that pooling microbiome samples before DNA amplification and metagenomic sequencing is a viable approach to evaluating diversity in population-level association studies (Ray et al., 2019). We also show that the bacterial populations detected in individual influent samples could be accurately predicted by the composite pooled sample. The other main observations was that SM-Seq and qPCR yielded markedly different GCN for ARGs, but standardisation of these data using z-scores demonstrated very good concordance in ARG detection and relative quantification by the two approaches. RNA sequencing identified only 32% of specific ARGs detected by SM-Seq, and these ARGs were identified at a lower sequencing depth than that for DNA. However, when the distribution of ARGs detected by RNA sequencing were stratified by antimicrobial class against which resistance is encoded, the putative phenotypic AMR profile was not different from that determined by SM-Seq. This is because specific ARGs detected via RNA sequencing were likely to be functional with the predicted phenotypic expression matching that of similar ARGs detected via SM-Seq in the composite DNA sample. Based on this limited dataset though, it is also apparent that a larger portion of ARGs identified via SM-Seq were possibly not transcribed into RNA transcripts at the time of sample collection.

Antimicrobial resistance gene qPCR arrays with customised amplification conditions can detect target genes with very low copy numbers (Rocha et al., 2019). This may explain the relatively higher GCN for ARGs detected by qPCR compared to SM-Seq or RNA sequencing in this study. In addition, 16 ARGs that are detectable in CARD and 18 ARGs that are detectable in MEGARes were identified by qPCR arrays in the composite sample, but were not detected by the respective SM-Seq approaches. The specific cause of this qPCR versus SM-Seq mismatch is not known. It is possible this represents false positive identification of unknown origin in qPCR since all affected ARGs were expressed at a relatively low GCN. Conversely, two ARGs including ERMA encoding for MLS resistance and SME encoding for carbapenem resistance were detected in the composite sample by SM-Seq and gene annotation in CARD or MEGARes but not via qPCR. Although not confirmed, a plausible explanation for this mismatch is that qPCR assays are highly specific with an ability to detect gene variants in a manner that is dependent on the targeted nucleotide sequences via specifically

designed primers. This implies that ARG variants with subtle differences in nucleotides are easily detected by reference gene sequences in metagenomic databases but could be missed by qPCR. In the MEGARes database for example, the ERMA gene is linked to five separate DNA sequences (Bonin et al., 2023). Furthermore, it is well known that DNA extracted from environmental samples may contain qPCR inhibitors (Stults et al., 2001). For the composite WWTP influent sample studied here however, inhibition of qPCR was unlikely because the same DNA template for ARG detection and qPCR positive controls had the same amplification efficiency. Collectively, these data show that qPCR arrays used in the current study had a limited selection of detectable ARGs, but the arrays were suitable for identification, relative quantification and validation of ARGs in the composite influent sample. In contrast, SM-Seq methods provided a more comprehensive coverage of ARG detection and these methods were well suited for screening and prediction of antimicrobial resistance profiles.

Currently available databases for ARG screening were created and are curated differently with many fundamental differences in the type and proportional representation of ARGs, gene variants or mutations (Papp and Solymosi, 2022). This study sought to cross validate SM-Seq and ARG annotation using either the CARD or MEGARes databases. In the composite WWTP influent sample examined here, multi-drug resistance and MLS resistance had the highest proportion of ARGs in CARD while MLS and aminoglycoside resistance had the highest proportion of ARGs in MEGARes. This is likely due to inherent differences in composition of specific ARGs in the gene pool provided in the two databases. The original CARD database was built around an ontology-driven framework which allows for only one version of an ARG sequence to exist in the database while MEGARes may have multiple sequences associated with the same gene or sub-group of gene but it utilises a cyclic form of gene annotation to minimise false identification and linking of the same gene with resistance to multiple classes of antimicrobial compounds (Alcock et al., 2023, Bonin et al., 2023, Papp and Solymosi, 2022). Based on these differences, it is conceivable that CARD would provide a more even distribution of ARG sequences while MEGARes would provide a more comprehensive assessment of ARGs within the sample. Qualitatively, results from the current study indicate that ARG annotation in CARD or MEGARes identified different proportions of an overlapping set of ARGs that code for similar resistance patterns across the major antimicrobial classes. This is because many different bacterial genes can code for resistance against multiple antimicrobial classes creating different gene-antimicrobial class permutations that may manifest as phenotypic antibiotic resistance. A relevant example of this combination is the ACRB gene that codes for a multi-drug efflux pump against tetracyclines, phenicols, MLS, rifampicin and fluoroquinolones (Hobbs et al., 2012). These data suggest that CARD and MEGARes are equally suited for surveillance of known ARGs in environmental samples. In addition, the evenness of ARG distribution in MEGARes and CARD was similar but the

observed ARG richness was higher in the MEGARes database. This is primarily because of the comprehensive coverage of currently known ARGs and ARG-variants in the MEGARes database. Thus, for large scale studies aimed at specific ARG identification and elucidation of molecular mechanisms for ABR, the direct comparison of CARD and MEGARes is not recommended. For comparative validation of detailed metagenomic analyses for ARG expression, the choice of ARG database to consider should be consistent.

The ARG profile identified by MEGARes and CARD databases in this composite WWTP influent sample was consistent with SM-Seq assessments of WWTP influents in some previous studies (Honda et al., 2023, Shi et al., 2023). These studies demonstrated that WWTP influents were dominated by ARGs that encode for resistance against macrolides, aminoglycosides, tetracyclines, beta-lactams, and multiple antimicrobial classes. It is worth noting however, that marked variation in the relative abundance of specific ARGs is observed across different studies and this may be attributed to qualitative differences in WWTP influent sources. The type and volume of antibiotic usage, and the presence of other factors such as the co-selection for resistance induced by metals and biocides are among the important factors that determine the microbiome and specific ARGs in WWTP influents. In the current study, a relatively high proportion of specific ARGs including AAC6, OXA, VEB, QNRVC were identified and these encode for resistance against aminoglycosides (AAC6), beta-lactam drugs (OXA and VEB), and fluoroquinolones (QNRVC). These antimicrobial classes are commonly used in healthcare facilities in the catchment area for the studied WWTP influent.

RNA sequencing was done to assess the putative expression and functionality of detected ARGs using the MEGARes database. MEGARes was considered for this comparison because it presented a higher ARG richness in its database compared to CARD. Fifty-nine ARGs were identified via RNA sequencing of the composite influent sample examined in this study. This however, only represented 32% of the total number of ARGs identified by DNA sequencing and the relative abundance of genes expressed in RNA was low. This DNA-RNA sequencing mismatch is a common phenomenon (Pitt et al., 2020, Liu et al., 2019a) and may be attributable to two main factors. Primarily, RNA is composed of ribose sugars instead of deoxyribose and this makes RNA unstable compared to DNA; prone to degradation in environmental samples, by biochemical and physical elements. Degradation of messenger RNA by endonucleases and exonucleases is an important process for controlling gene expression in bacteria. It is possible that the standard protocol for nucleic acid extraction used in this study may not have sufficiently prevented RNA degradation. Secondly, RNA sequencing is only likely to represent actively expressed RNA transcripts for ARGs with a concurrent exposure to sub-lethal concentrations of corresponding antibiotics at the time of sampling. For example, in *E. coli* harbouring tetracyline resistance genes, mRNA gene expression for

TETA and TETR was tightly regulated by the concentration of tetracycline (Møller et al., 2016). Herein we show that the relative abunadance of tetracycline resistance genes was markedly lower in RNA compared to the DNA, but determination of qualitative and quantitative features of antibiotics in samples was beyond the scope of this study. The chemical composition of the composite sample evaluated in this study may also explain why some uncommon ARGs including P16S encoding for pactamycin resistance, and P23S encoding for pleuromutilin resistance were markedly upregulated in RNA sequences. Whenever feasible therefore, real-time correlations of antimicrobial composition and concentrations with ARG transcriptomic evaluations should be considered. All these factors notwithstanding, ARGs with the highest abundance in both RNA and DNA sequences were MLS23S and A16S, encoding for resistance against MLS and aminoglycosides respectively. Similarly, there was no difference in the relative abundance between RNA and DNA sequences, for some ARGs encoding for phenicols, sulfonamides and multi-drug resistance. This suggests concordance in the characterisation of resistance against these specific antimicrobial classes. Similar results were observed via metagenomic and transcriptomic analyses of activated sludge from wastewater processing previously (Liu et al., 2019b).

Two methods were used to characterise the microbiome associated with the ARG profile described above; these were sequencing of 16S rRNA gene paired with bacterial class annotation in GreenGenes or SILVA databases and, bacterial genome sequencing coupled with class annotation using Kraken2 bioinformatics pipeline. Sequencing of the the 16S rRNA gene is a conventional method because it is cost effective and has large reference databases. This approach utilises defined operational taxonomic units that are more accurately analysed at phyla and class level but less precisely at species level. For the composite influent sample considered here, GreenGenes and SILVA bioinformatics pipelines yielded similar microbial profiles with no differences in observed evenness or richness in the bacterial population. In contrast, SM-Seq coupled with bacterial species annotation using Kraken2 pipeline identified more bacterial classes and at much higher relative abundance with a significantly higher measure of richness in the bacterial population. This is likely due to deeper sequencing and species identification provided by SM-Seq. These data and the work elucidated by others (Durazzi et al., 2021, Lin and Liu, 2023), indicate that 16S rRNA sequencing or SM-Seq could effectively be used for bacterial population profiling at phyla and class level in surveillance studies. For more accurate speciation of bacteria in more diverse populations and identification of bacterial genes, SM-Seq would be a recommended approach. In the composite influent sample examined here, Proteobacteria, Bacteriodia and Clostridia were the predominant classes identified by both 16S rRNA sequencing and SM-Seq. This profile is very similar to that documented in influents into WWTPs in other cosmopolitan cities in the United Kingdom, Japan and China (Chau et al., 2023, Honda et al.,

2023, Shi et al., 2023). This strongly suggests that influents into WWTPs in urban settings are likely to be dominated by enteric anaerobic bacteria.

4.5 Conclusions

Methods that are commonly used for surveillance of the microbiome and ARGs in environmental samples are not standardised; very limited data on comparative robustness exists. We compared qPCR, SM-Seq and RNA sequencing for the qualitative and quantitative detection of ARGs and bacteria in WWTP influent. SM-Seq coupled with ARG annotation in two known databases (CARD and MEGARes) provided comprehensive ARG detection that is well suited for surveillance and mapping of antimicrobial resistance profile to different antimicrobial classes. However, the detection and relative quantification of specific ARGs by this approach is limited by the size and quality of the probed ARG database. Thus, a direct comparison of CARD and MEGARes is not recommended. It was also apparent that gene amplification using pre-selected qPCR arrays was adequate for identification, relative quantification and validation of ARGs represented in the CARD or MEGARes database. RNA sequencing identified only 32% of the ARGs that were recognised by DNA sequencing followed by gene annotation in MEGARes. This emphasises the need to undertake phenotypic characterisations to validate ABR data determined by bacterial whole genome sequencing. Interestingly, all ARGs detected by RNA-seq were also detected via SM-Seq albeit at varying relative proportions. For example, there was concordance in the detection and relative quantification of some ARGs encoding for resistance against aminoglycosides, MLS, phenicols, and sulfonamides, providing a reliable indication of ABR patterns from RNA or DNA sequencing. Lastly, we show that sequencing of the 16S rRNA gene or SM-Seq could effectively be used for general bacterial population profiling at phyla or class level. However, SM-Seq identified more bacterial classes and at much higher relative abundance with a significantly higher measure of richness in the bacterial population. For comprehensive and precise speciation of bacteria in complex environmental samples therefore, SM-Seq would be a recommended approach. All recommendations for general methodological and technical approaches in this chapter were applied for data collection and analysis for chapters five and six.

. Chapter 5: Antimicrobial Resistance Profiling in Sewage Processed by a Membrane Bioreactor Plant: Implications for Release into the Great Barrier Reef

Submitted to Environmental Pollution on the 31st of January 2025

Wastewater treatment plants (WWTPs) provide an opportunity to monitor and perhaps limit the dissemination of antibiotic resistance pollution into marine environments as they often receive mixed sources of medical, agricultural, and industrial waste, all of which contain aspects of antibiotics, antibiotic resistant bacteria (ARB), and antibiotic resistant genes (ARGs). This study aimed to characterise the ARG profile (resistome) and bacterial profile (microbiome) in the influent, biosolids, and effluent associated with one of the largest membrane bioreactor WWTPs in the southern hemisphere, the Cleveland Bay Sewage Treatment Plant (CBSTP), which is situated on the Great Barrier Reef, Australia. Results showed that whilst ARG sequences are still present within biosolid and effluent of the CBSTP, the processes employed by the CBSTP are effective in reducing the load of resistance from the influent. Additionally, bacterial cells were retained in the biomass (with an ultimate fate in the biosolids) and excluded from the effluent via ultra-filtration membranes. Conversely, the general profile of ARGs of the influent primarily followed the path of liquid waste to the effluent. Possible instances of horizontal gene transfer of ARGs within the CBSTP were also identified. As the CBSTP is one of the largest points of discharge along the Great Barrier Reef Marine Park future studies should investigate the potential implications of the effluent resistome dissemination and HGT in the marine receiving environment microbiome, and its resident fauna.

5.1 Introduction

The widespread use of antibiotics in human and animal healthcare significantly contributes to the emergence of ARGs and their persistence in public health and agricultural systems. ARGs are also emerging as contaminants of concern within the natural environment (Alygizakis et al., 2020, Anand et al., 2021, Hutinel et al., 2022). Antibiotics can alter microbial community structure through the application of selective pressure for ARB, which promotes the use and transfer of ARGs; however, the effect of these interactions on the environment is still not sufficiently researched (Hirsch et al., 1999, Karthikeyan and Meyer, 2006). WWTPs are a known source for the dissemination of antibiotics and ARGs into the marine environment (Kümmerer, 2009, Narciso-da-Rocha et al., 2018, Osińska et al., 2020) . These treatment facilities often receive mixed sources of medical, agricultural, and industrial waste, and as such they provide an opportunity to monitor and perhaps limit the dissemination of antibiotic resistance pollution into marine environments. However, less is known about the effect these emerging contaminants (antibiotics, ARBs, and ARGs) from WWTPs have on

the environment, specifically within delicately balanced ecosystems, such as the Great Barrier Reef, that rely on diverse and complex microbiome communities.

The Great Barrier Reef Marine Park extends over 2,300 km along the Coral Sea coast of Queensland, Australia, and is regarded as one of the seven natural wonders of the world. One WWTP within the GBRMP, the CBSTP, services the largest population centre in this region (approximately 190,429 in June 2018). This plant discharges within the general use zone of the GBRMP, as well as directly into the Queensland State Marine Park. The CBSTP is a MBR and utilises ultra-filtration membranes of 0.04 µm pore size, which decreases further to 0.01 µm with membrane fouling. As it currently stands, the CBSTP is considered one of the largest membrane bio-reactors (MBRs) in the southern hemisphere and comprises a 4-stage biological process with both primary and secondary sludge removal. MBRs have been suggested to be among the most effective removal systems for ARBs and their accompanying ARGs (Wang et al., 2020b, Lin et al., 2021, Zhu et al., 2021, Nnadozie et al., 2017). Wang et al (2020), stated that the average ARG removal approached 100% through a full-scale MBR process. Similarly, a 98.4% decrease in ARGs within an MBR plant was reported by Lin et al (2021). Collectively these observations suggest that MBR plants are effective in the reduction of ARGs but, they are not a perfect removal system and effluents from such treatment plants do still contain ARGs (Lin et al., 2021). Additionally, whilst MBR may significantly reduce ARBs from influent wastes, questions remain surrounding the profile of the limited ARGs that are released. The effect of MBR processes on the horizontal gene transfer (HGT) of ARGs within WWTPs is also of interest in this scientific field, due to the implications of susceptible bacteria acquiring resistance to antibiotics, and the downstream effects this may have for One Health (public, animal, and environmental health) and the "core" microbiome of the CBSTP.

This study aimed to characterise the ARG profile (resistome) and bacterial profile (microbiome) in the influent, biosolids, and effluent associated with the CBSTP and its critical processing units within the plant. This study also aimed to assess the effects of the key processing units on changes to the waste resistome throughout the plant, as well as any temporal shifts that may be observed over a daily and monthly period.

5.2 Materials and Methods

5.2.1 Sampling Locations and Sampling

The geographical location and the description of the CBSTP are detailed in the Materials and Methods section of Chapter 3. To assess monthly temporal patterns of the treated waste microbiome and resistome, as well as the effect of treatment processes on the resistome, samples of influent and effluent were collected from the CBSTP over the course of a 10-month period. Influent

and effluent samples were obtained every 18 days +/- 2 days to account for any variation due to the 21-day sludge retention time for 10 months from 11/10/2021 to 22/7/2022, resulting in 16 sample points over the total period. Preserved CBSTP influent and effluent samples were separately pooled based on the month of sampling, providing an approximate profile of the DNA obtained from both sample types (influent and effluent) at the CBSTP throughout October 2021 to July 2022 for a total of ten sample points for each sample type. In addition, six biosolids samples were taken sporadically between March 2022 and July 2022. The CBSTP is unusual in that it is a twin sludge plant, where it has both primary and secondary sludge that is digested anaerobically and are dewatered separately. The primary and secondary sludge also have differing profiles in that the secondary sludge typically represents a greater bacterial load whilst the primary sludge may have a higher inert load originating from environmental sediments that elude the primary screenings and grit removal process. Biosolid samples were collected post anaerobic digestion as a representation of the secondary treatment process of bioreactors and oxidation ditches. These biosolid samples were analysed as singular grab samples rather than monthly pools to provide information relating to the biomass resistome and the sludge retention time.

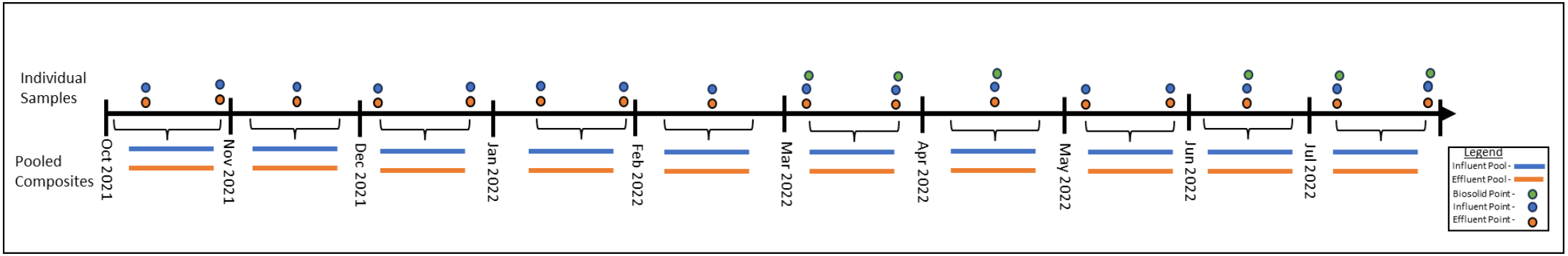


Figure 5-1: Visualised sampling timetable of CBSTP samples collected between October 2021 and July 2022.

Samples of wastewater effluent were collected via a filtration pump on site. Filtration of water samples is the most prominent and reliable method to obtain a functional yield of DNA, specifically from sample types containing low volumes of DNA (Wong et al., 2020). Grover Scientific peristaltic pumps were selected for this study as they were specifically designed for DNA filtration. For each sample point, five litres of effluent from the CBSTP storage tank were filtered through sterile 0.22 µm mixed cellulose ester filters made by Sartorius Australia PTY LTD. Membrane filters composed of mixed cellulose ester have been previously found to obtain a higher yield of DNA from water samples and provide a more consistent DNA community composition than other filter materials (Majaneva et al., 2018).

Samples of CBSTP influent were collected as per sampling methods in Chapter 3 Materials and Methods. Samples of CBSTP biosolids were taken from the biosolids evacuation bins using sterile forceps and placed in a sterile 150 ml sampling jar. All CBSTP sample types were transported in separated sections of a sterilised cold box to James Cook University below 5°C within 1 hour of sampling. Samples were analysed at the Molecular Ecology and Evolution Laboratory at James Cook University Campus (Building 145, level 3).

5.2.2 Nucleic Acid Extraction

Samples were processed for DNA extraction and preserved as previously described in the materials and methods of Chapter 3.

5.2.3 Metagenomic Sequencing

Duplicate twenty microlitres of each purified DNA sample (average DNA concentration of 50.5 ng/µl) were sent to the Australian Genome Research Facility (AGRF), Melbourne, Australia. Library preparation and SM-Seq was performed according to AGRF's methodology described in Chapter 4.2.4 (Drane et al., 2024a). The libraries were sequenced using the Illumina NovaSeq S4 sequencing platform (San Diego, CA, USA). Note, the March 2022 influent pool sample failed to reach suitable quality control parameters of library preparation, and thus was not included in data analysis procedures. Additionally, within the daily temporal variation experiment, three samples (of 33 total) failed to reach suitable quality control parameters of library preparation and these were, influent sample of 25/10/2021, and effluent samples from 11/10/2021 to 13/10/2021.

5.2.4 Data Analysis

The raw read sequences were processed by AGRF through Trimgalore (version 0.4.5; (Martin, 2011) to identify and remove sequencing adapters and low-quality stretches of base quality below 20. Kraken2 (version 2.0.8; (Wood et al., 2019) and bracken (version 2.5(Lu et al., 2017) were applied for profiling the composition of microbial communities using NCBI genome and nucleotide (nt) database. Further details of which are described in materials and methods of Chapter 3 and as illustrated in Figure 5-2. These raw reads were utilised for further downstream analysis related to ARG detection; this is described in the section on data analysis. ARGs were identified from the raw SM-Seq data (average raw read count of 67,743,602 bp) using AMR++ 3.0 through quality control measures, such as filtering and trimming inaccurate reads, and removing human DNA (average quality-controlled coverage count of 13,657,808 bp and sequencing depth of 23 GB/sample), prior to annotation of the reads via the MEGARes and Kraken2 databases. Finally, filtered reads were compared to the MEGARes database for the resistome profile and Kraken2 for the microbiome profile (Bonin et al., 2023). These methodological details are described in materials and methods of Chapter 3.

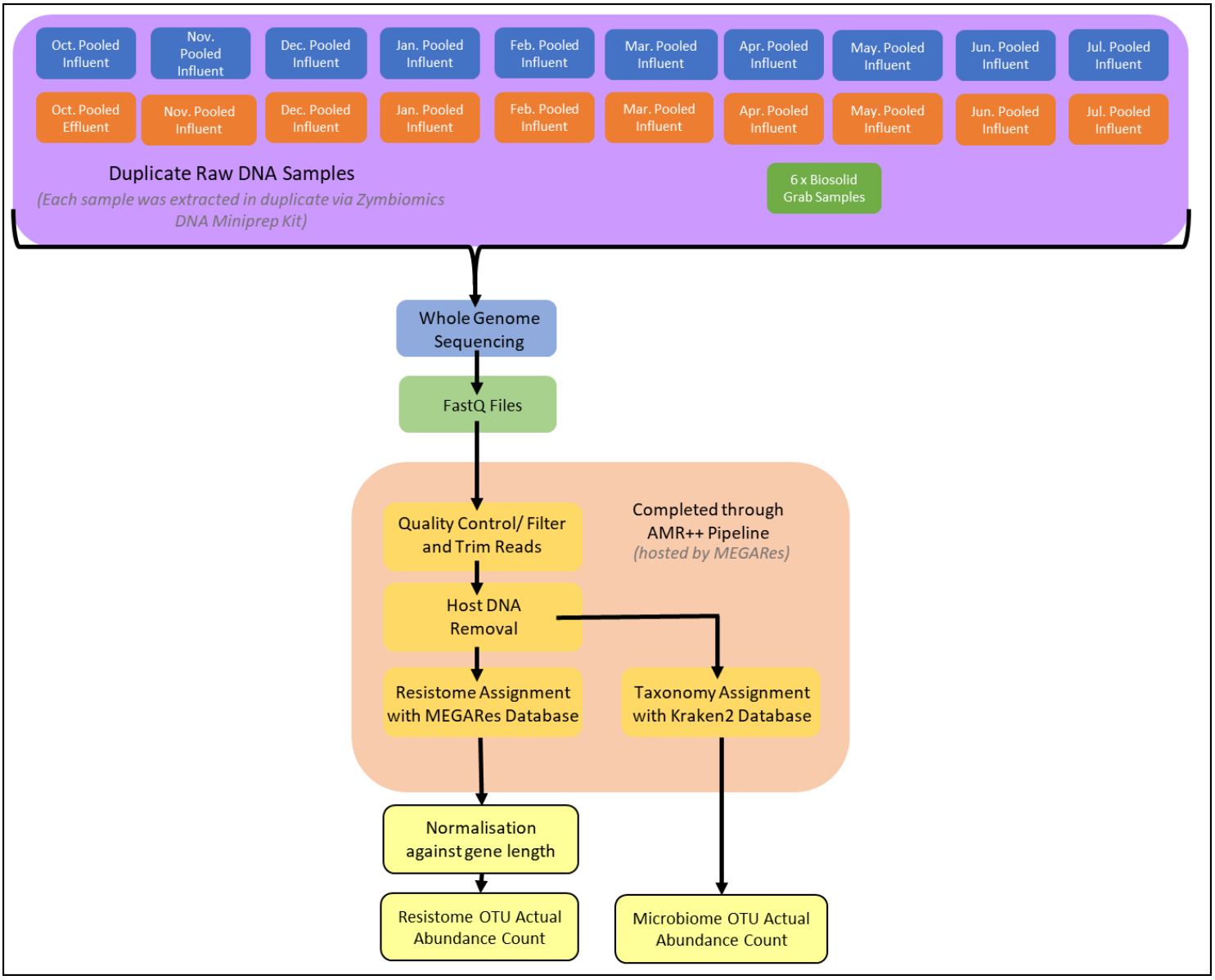


Figure 5-2: Visualised flowchart of DNA extracted from CBSTP samples, describing the analysis of genomic data obtained through whole genome sequencing.

To account for the incorrect identification of bacterial OTU sequences to the Kraken2 reference database, all bacterial OTU data was filtered to remove those with a sequence sum of less than 20. The relative abundance of these filtered microbiome OTU copies was then assessed using R (v4.1.1) (Liu et al., 2021, Mammola et al., 2021). The OTU count of ARGs identified by MEGARes was normalised to eliminate gene length bias by calculating a ratio of each OTU by dividing its count by its length (bp). The details regarding this process are described in the materials and methods of Chapter 3.

Alpha-diversity metrics and visualization of microbiome and resistome proportions were analysed using the “mia” package (version 1.6.0; (Borman T, 2024) and “ggplot2” package (version 3.4.1; Wickham, 2011). Comparison of beta-diversity metrics and visualization of Principal component analyses (PCA) were done using the “phyloseq” R-package (version 3.16). Differential expression analysis was done through "DESeq2” (version 1.38.3; (Love et al., 2014) and “mia” R-packages. Core microbiome and resistome OTUs were analysed using the “microbiome” (version 1.20.0), “microbiomeutilities” (version 1.0.17), and “ggplot2” R-packages. Spearman correlation analyses were conducted using the ‘mia’ package and facilitated by transforming the data on a logarithmic scale. To assess differences between microbiomes and resistomes concerning centroid and dispersion, a Permutational multivariate analysis of variance statistical test was performed using the "DESeq2” R-package. The Permutational multivariate analysis of variance was based on Euclidean distances between samples. Furthermore, a permutation test was used to determine the significance of the result, allowing all samples to be randomly mixed throughout the groups. Additionally, the core microbiome and resistome of a bacterial community for this study was defined as an OTU that occurs in at least 90% of samples and has a relative abundance of at least 0.01.

5.3 Results

5.3.1 Metagenome Sequencing Overview

The relative abundance of 1,122 normalised resistome OTUs were assessed using R (v4.1.1) (Liu et al., 2021, Mammola et al., 2021). Using the R software “Phyloseq” package (v3.16; (McMURDIE and Holmes, 2012), the normalised resistome OTU copies were filtered to remove OTUs that had a total sum of less than 0.001% of all samples to account for incorrect identification from the MEGARes database, resulting in a total of 844 OTUs. The proportion of OTUs was used to filter incorrect identification of sequences in the instance of the MEGARes resistome data, due to the normalisation of count data implemented and the subsequent inability to accurately represent count sums, such as in the instance of the microbiome data.

5.3.2 Daily Temporal Variation of the CBSTP Microbiome

The daily temporal variation experiment revealed that the bacterial distribution within the microbiome of influent and effluent daily samples were significantly different from each other ($p = 0.01$). The bacterial distribution throughout the effluent samples did not fluctuate significantly daily ($p = 0.196$). Furthermore, an additional Permutational multivariate analysis of variance test showed that daily effluent samples were not significantly different from the October effluent composite ($p =$

0.333) used for the monthly temporal variations experiment. Conversely, the daily influent samples within the short-term temporal variations experiment were found to fluctuate significantly within the 18 days ($p = 0.003$), and daily influent samples were not significantly different from the October influent composite ($p = 0.111$) used for the monthly temporal variations experiment.

5.3.3 Spatial Variation of the Microbiome and Resistome within the CBSTP

5.3.3.1 Community Diversity of Sample Types

The resistome richness of ARG OTUs was markedly different across the three different sample types including influent, biosolids, and effluent (see Figures 5-3 and 5-4). These different resistome profiles may be influenced by dissimilar diversity of ARGs in each sample type. Such as, influent samples showed higher diversity (with high variability) in ARGs identified via the MEGARes database than that of the biosolid and influent samples (Figure 5-3A and 5-3B). Additionally, influent samples have a less even distribution of ARGs than that of the other sample types (Figure 5-3A). Effluent samples have the least diversity within their resistome and vary markedly less in proportion than the influent samples (Figure 5-3A and 5-3B). However, the less diverse ARGs in the effluent are distributed in a more even manner than that of the influent. These disparities in evenness are reflected in effluent samples (Figure 5-3B), which showed higher proportions of resistance to aminocoumarins, fluoroquinolones, and multidrug resistance than other locations within the CBSTP. Biosolid samples have a higher diversity of ARGs than effluent samples (Figure 5-3B); however, the evenness by which these ARGs are distributed is akin to that of the effluent samples (Figure 5-3A).

The bacterial OTU richness and evenness in the influent samples followed a similar trend to that of the resistome data, with high richness and low evenness (Figures 5-3C and 5-3D). In contrast to this, both biosolid samples and effluent samples did not show similar richness and evenness trends for the microbiome as they did in the resistome (Figures 5-3C and 5-3D). Biosolid samples showed a high diversity of bacterial chromosomes within the richness index (Figure 5-3D) and effluent samples represented a highly uneven distribution of bacterial relative abundances (Figure 5-3C).

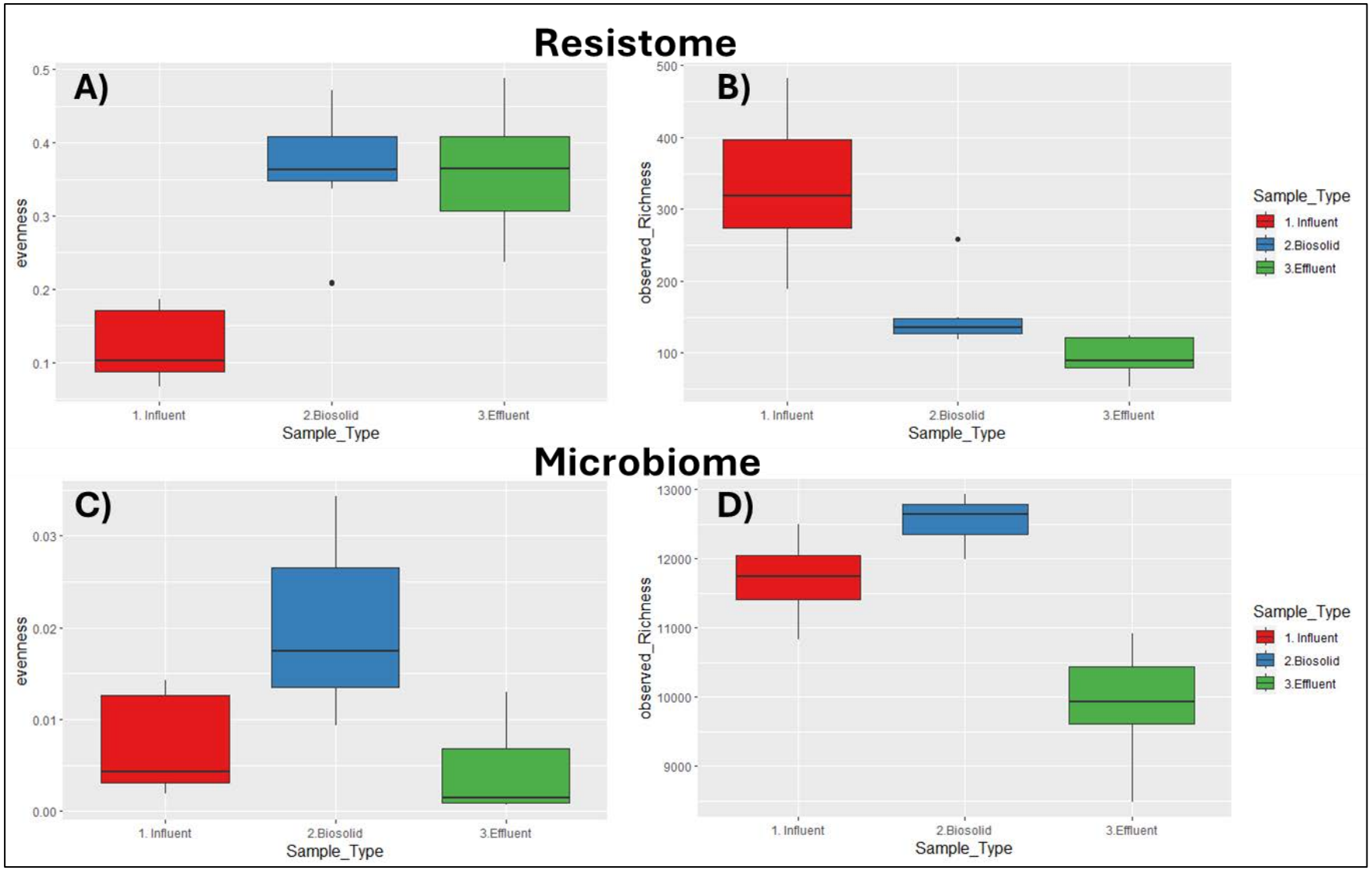


Figure 5-3: Alpha diversity measures of Richness and Evenness for the microbiome and resistome communities identified within various areas of the CBSTP, depicted as whisker plots with medians and the interquartile range.

5.3.3.2 Community Diversity Between Sample Types

The microbiome of the three separate sample types were found to be significantly different (Permutational multivariate analysis of variance with Euclidean distances, p=0.001), this is observed most prominently via PCA plot (Figure 5-4). Furthermore, as observed by a Wilcox Rank Sum test, the median OTU abundance of each sample type was found to be significantly different between influent and biosolids (p= 0.0018), between biosolids and effluent (p= 0.0004), and between influent and effluent samples (p=0.0493) (Figure 5-5). In total 8,365 unique bacterial OTUs of the initial 15,547 bacterial OTUs were found to be significantly different (via a differential expression analysis) between the sample types, with the majority (6,856 OTUs) found to be significantly different between the influent and effluent microbiomes. Additionally, 6,059 OTUs were found to be significantly different between the effluent and biosolid samples, and 5,624 OTUs were found to be significantly different between the influent and biosolid samples. With respect to bacterial phyla, *Proteobacteria* had the most significant variations within microbiomes of the different sample types.

The resistome also varied significantly across the different sample types (Permutational multivariate analysis of variance with Euclidean distances, p=0.001), and was observed via PCA plot (Figure 5-4). Additionally, the median ARG OTU abundance of each sample type was found to be significantly different between influent and biosolids (p= 0.0026), between biosolids and effluent (p= 0.021), and between influent and effluent samples (p=0.0427) via a Wilcox rank sum test (Figure 5-4). In total, 771 unique bacterial OTUs of the initial 844 ARG OTUs were found to be significantly different between the sample types, with 579 OTUs found to be significantly different between the influent and effluent microbiomes. Additionally, 598 OTUs were found to be significantly different between the effluent and biosolid samples, and 438 OTUs were found to be significantly different between the influent and biosolid samples. ARGs associated with the MLS class of antibiotic resistance varied most prominently across the three sample types, closely followed by tetracycline-resistance ARGs.

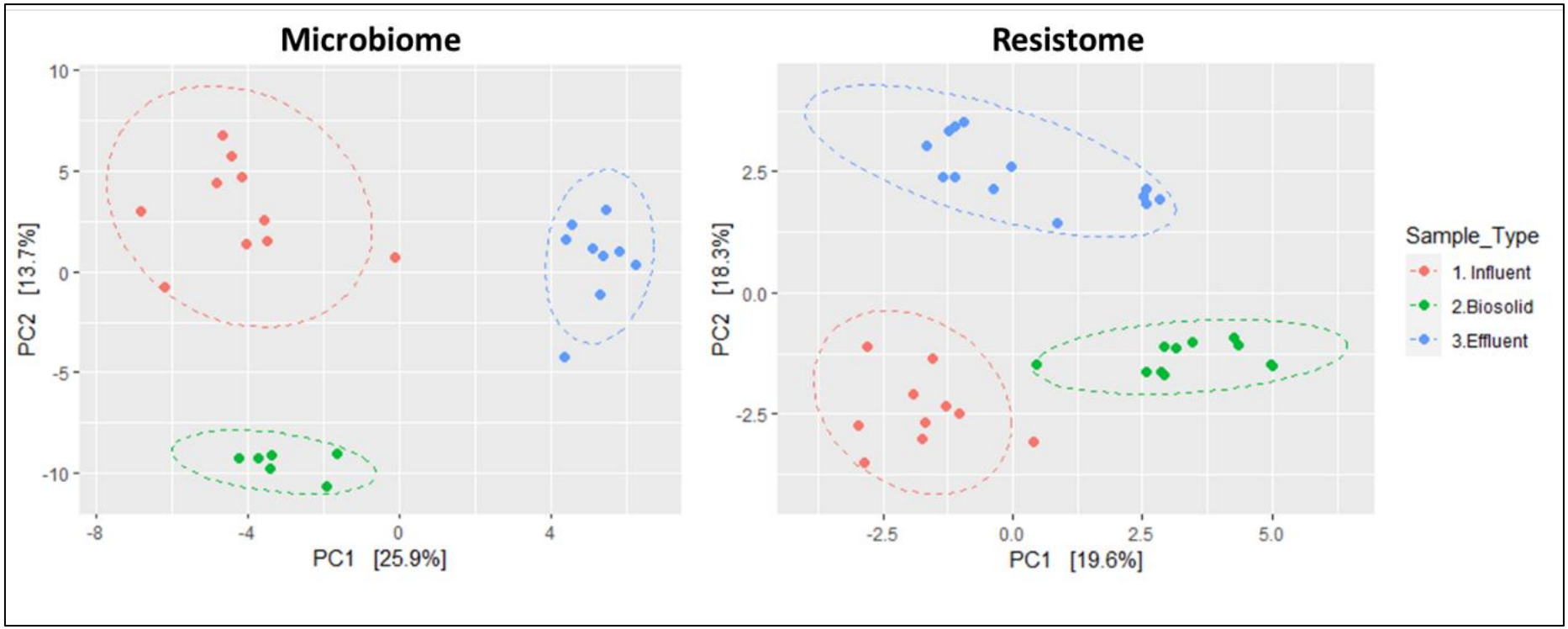


Figure 5-4: Principal Component Analysis (PCA) depicting differences in microbiome and resistome community distributions within three separate sampling locations of the CBSTP.

The core resistome of the influent contained 76 ARG OTUs (Figure 5-5A), with specific resistance genes representing different antimicrobial classes including aminocourmarins (e.g. PARE), aminoglycosides (e.g. A16S, APH3-DPRIME, RPSL, RRSA, RRSC, RRSH), beta-lactams (e.g. BKC, BLAA, GES, OMPR, OXA), cationic antimicrobial peptides (e.g. ARNA, BASRS), elfamycin (e.g. TUFAB), flouroquinalones (e.g. GYRA, PARC), lipopeptides (e.g. PHOP, PHOQ), MLS (e.g. MLS23S), Multi-drug resistance (e.g. EMRE, MUXA, MUXB, MUXC, NFXB, OPMB, OPMD, OPMH), Mycobacterium-tuberculosis specific drug (e.g. RPSA), oxazolidinones (e.g. O23S), rifampin (e.g. RPOB), sulfonamides (e.g. FOLP, SUL1) and tetracyclines (e.g. TET16S). OTUs that facilitate resistance to aminoglycoside antibiotics were the most frequently represented forms of resistance with 14 OTUs in total and 9 separate OTU sequences for the A16S genes present within the influent core resistome, closely followed by MLS resistance with 12 separate sequences encoding for the MLS23S gene.

The effluent core resistome had 39 OTUs (Figure 5-5A), with resistance to the following antibiotic classes represented: aminocourmarins (e.g. PARE), aminoglycosides (e.g. A16S, RRSA, RRSC, RRSH), betalactams (e.g. CAU, AIM), cationic antimicrobial peptides (e.g. CAP16S, BASRS), elfamycin (e.g. TUFAB), flouroquinolones (e.g. GYRA, PARC), lipopeptides (e.g. PHOP), MLS (e.g. MLS23S), multi-drug resistance (e.g. MUXB, MUXC, OPMB, OPMH), Mycobacterium-tuberculosis specific drug (e.g. RPSA), oxazolidinones (e.g. O23S), rifampin (e.g. RPOB), tetracyclines (e.g. TET16S). OTUs that facilitate resistance to aminoglycoside antibiotics were the most frequently represented forms of resistance with 11 OTUs in total and 8 separate OTU sequences for the A16S genes were present within the effluent core resistome.

The biosolid core resistome consisted of 62 OTUs (Figure 5-5A), with resistance to the following antibiotic classes represented: aminocourmarins (e.g. PARE), aminoglycosides (e.g. A16S, APH3-DPRIME, RPSL, RRSA, RRSC, RRSH), beta-lactams (e.g. BKC, BLAA, GES, OMPR, OXA), cationic antimicrobial peptides (e.g. ARNA, BASRS), elfamycin (e.g. TUFAB), flouroquinolones (e.g. GYRA, PARC), glycopeptides (e.g. PHOP, PHOQ), MLS (e.g. MLS23S), multi-drug resistance (e.g. EMRE, MUXA, MUXB, MUXC, NFXB, OPMB, OPMD, OPMH), Mycobacterium-tuberculosis specific drug (e.g. RPSA), oxazolidinones (e.g. O23S), rifampin (e.g. RPOB), tetracyclines (e.g. TET16S). OTUs that facilitate resistance to aminoglycoside antibiotics were the most frequently represented forms of resistance with 14 OTUs in total and 9 separate OTU sequences for the A16S genes were present in the biosolid core resistome, closely followed by MLS resistance with 12 separate sequences encoding for the MLS23S gene.

A total of 96 unique OTUs were identified as being part of the core resistome of one or more sample types within the CBSTP (Figure 5-5A). Many of these core ARGs are described as multi-drug

resistant (MDR) ARGs, and harbour resistance against multiple different antibiotic classes, though these MDR ARGs are not collectively represented in high proportion. However, ARGs with resistance against the beta-lactam and tetracycline class of antibiotics were heavily featured.

A total of 29 ARG OTUs were common in the core resistome across all three sample types, with aminoglycoside-resistant ARGs representing 11 of these overlapping core ARGs. This included eight separate OTUs encoding for the A16S gene, two RRSA genes, and the RRSH gene. Resistance against the following classes of antibiotics were also represented within this overlapping core resistome: aminocoumaines (two PARE genes), elfamycins (two GYRA genes, and PARC), cationic antimicrobial peptide (CAP16S), fluoroquinolones (four TUFAB genes), MLS (MLS23S), rifampin (three RPOB genes), tetracyclines (TET16S), multi-drug resistance (MUXB), and a Mycobacterium-tuberculosis specific drug-resistant OTU (RPSA).

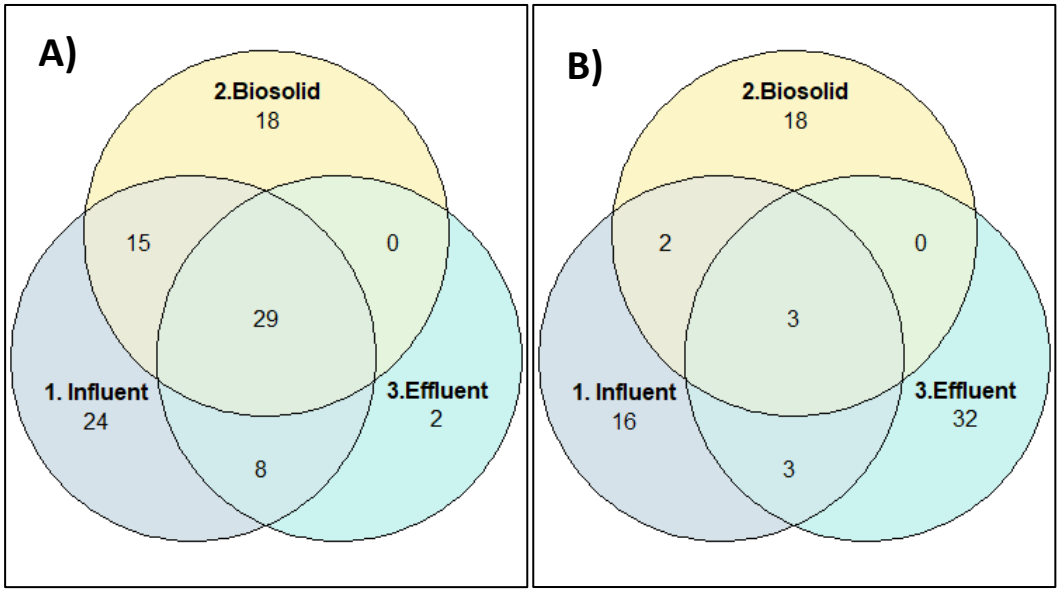


Figure 5-5: Venn diagram of core (A) resistome and (B) microbiome per sample type.

A total of 24 bacterial taxa constituted the core microbiome of the influent (Figure 5-5B), and this was heavily dominated by the phyla assigned to Proteobacteria (20/24 taxa). This is in addition to single taxa belonging to each of the following phyla: Bacteroidetes (*Cloacibacterium normanense* – OTU 237258), Zixibacteria (uncultured bacterium – OTU 77133), Firmicutes (*Acetoanaerobium sticklandii* – OTU 1511), and Synergistetes (*Aminomonas paucivorans* – OTU 81412).

The core microbiome of the biosolids was constituted by 23 taxa (Figure 5-5B), a large portion of these were phyla assigned to Proteobacteria (12/23 taxa). Other phyla within the biosolid

core microbiome were: Zixibacteria (uncultured bacterium – OTU 77133), Actinobacteria (*micropruina glycogenica* – OTU 75385, uncultured bacterium – OTU 1883427), Chloroflexi (*caldilinea aerophile* – OUT 133453, *candidatus promineofilum breve* – OTU 1806508, *tepidiforma bronchosmolovskayae* – OTU 2601677), Planctomycetes (*gemmata obscuriglobus* – OTU 114, *urbifossiella limmaea* – OTU 2528023, *limnoglobus roseus* – OTU 2598579, *frigoriglobus tundricola* – OTU 2774151).

A total of 38 bacterial taxa made up the core microbiome of the effluent (Figure 5-5B), which was almost exclusively dominated by Proteobacteria (37/38). The only exception to this was the presence of Zixibacteria (uncultured bacterium – OTU 77133). A total of 74 unique bacterial OTUs were found to be the core microbiome of one or more sample types within the CBSTP (Figure 5-5B). However, less overlap of the core microbiome between sample types was noted when compared to the resistome, with only three (3) common core microbiome OTUs between the sample types. These three common core microbiome taxa were uncultured bacterium (OTU 77133) from the Zixibacteria phyla, and two Proteobacteria phyla *rhodopseudomonas palustris* (OTU 1076) and *variovorax paradoxus* (OTU 34073).

5.3.4 Indications of horizontal movement of resistance genes throughout the Cleveland Bay Sewage Treatment Plant

Of the 29 core ARG OTUs that overlap between biosolids, influent, and effluent samples, 25 showed either positive or negative correlations correlated with at least one core bacterial OTU in the influent and effluent samples (Figure 5-6), and no overlapping core ARG OTUs were correlated with any of the core bacterial OTUs in the biosolid samples. Whilst the same number (25) of ARG OTUs were correlated with core bacteria in the influent and effluent, the bacteria of which they are correlated to changes in some instances between the sample types (Figure 5-6). For example, a core bacterium that was present in both influent and effluent core microbiomes *pseudomonas aeruginosa* (OTU# 287) was positively correlated with a single ARG OTU in the influent OTU# 4101:MUXB (MDR), which is intrinsically linked to the chromosome of *Pseudomonas aeruginosa.* Yet, in the effluent, it was positively correlated with several additional ARGs including OTU# 4101:MUXB (MDR), OTU# 6127:RPOB (for rifampin resistance), OTU# 6147:RRSH (for aminoglycoside resistance), OTU#
490:CAP16S (for cationic antimicrobial peptide) and OTU# 6149:RRSC (for aminoglycoside resistance) (Figure 5-6). Another example is that of ARG OTU# 7305:TUFAB (elfamycin resistance), which was observed to be correlated with a number of bacteria in the influent, These were primarily of the alpha-proteobacteria class, including*: Sphingopyxis sp.* (*QXT-3, OPL*, and *PAMC25046*), *Sphingopyxi*

fribergensis, and *Nordella sp. HKS 07* (Figure 5-6). However, instances of OTU# 7305:TUFAB were not positively correlated with any core bacteria within the effluent (Figure 5-6).

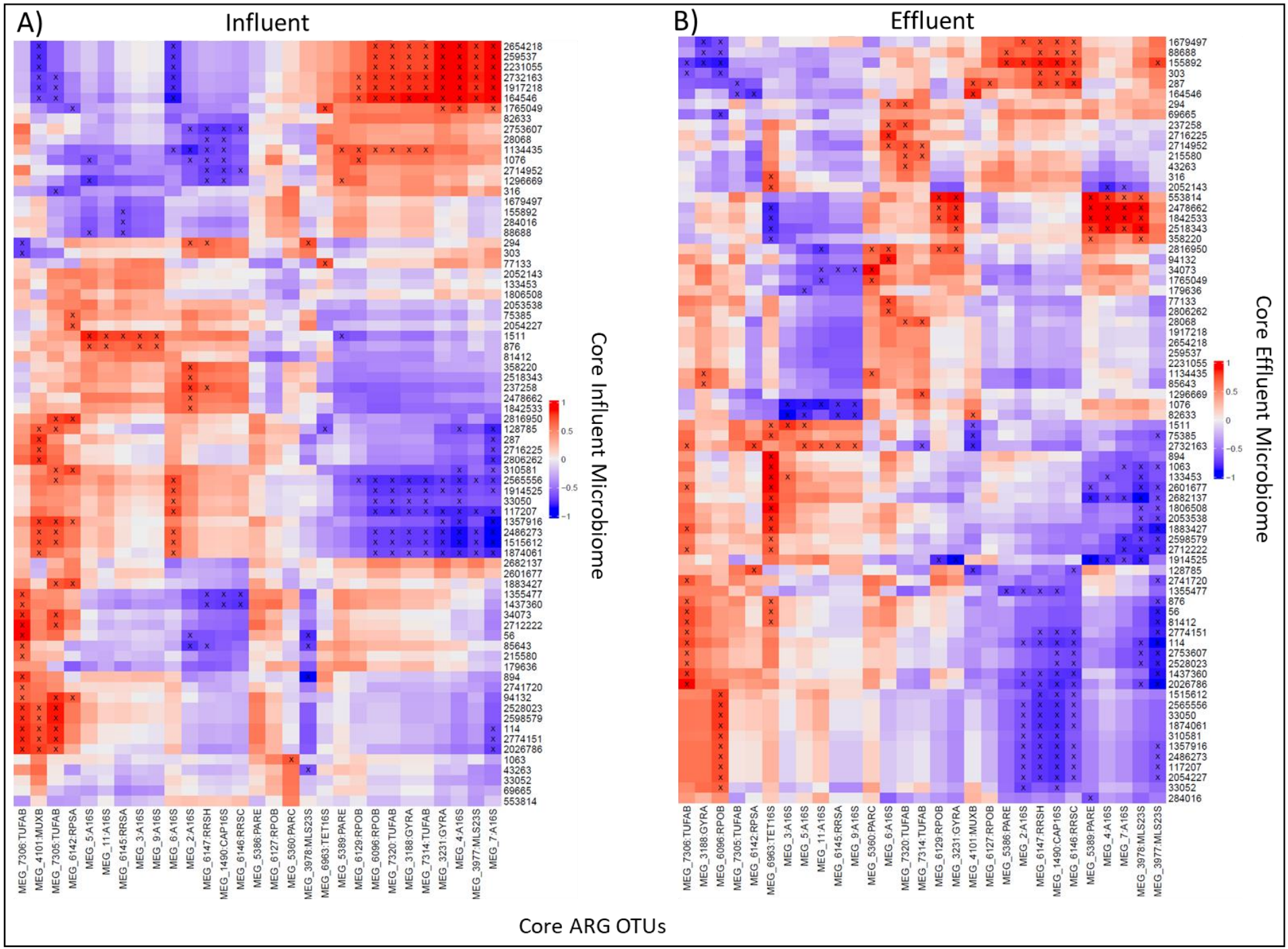

A)
Influent
Core Influent Microbiome
B)
Effluent
Core Effluent Microbiome
Core ARG OTUs

Figure 5-6: Heatmap describing the correlations between proportions of influent (left) and effluent (right) core microbiomes compared to the shared resistome of both locations. Where red colours annotate positive correlations, blue colours annotate negative correlations (colour depth varies based on the strength of the correlation), and 'x' annotates correlations found to be statistically significant.

5.3.5 Proportion of Bacteria to Resistance Genes

The ratio of ARG OTUs to bacterial OTUs represents the incidence of resistance to the occurrence of identified bacterial chromosomes (Miller et al., 2016). Significant differences in this ratio were identified between the three tested locations of the CBSTP ($p = 6.66 \times 10^{-11}$) through a single factor analysis of variance of the numerical ratio (Figure 5-7). Furthermore, a single factor analysis of variance comparing this proportion of resistance to bacteria in the effluent (average of 0.024 ARG OTU/ Bacterial OTU) and biosolid (average of 0.0099 ARG OTU/ Bacterial OTU) locations found the ratio of the effluent to be significantly higher ($p = 7.12 \times 10^{-5}$) (Figure 5-7). Additionally, the incidence of resistance genes per bacterial OTU was identified to be significantly higher in the influent (average of 0.0316 ARG/OTU) than in the effluent (Figure 5-7).

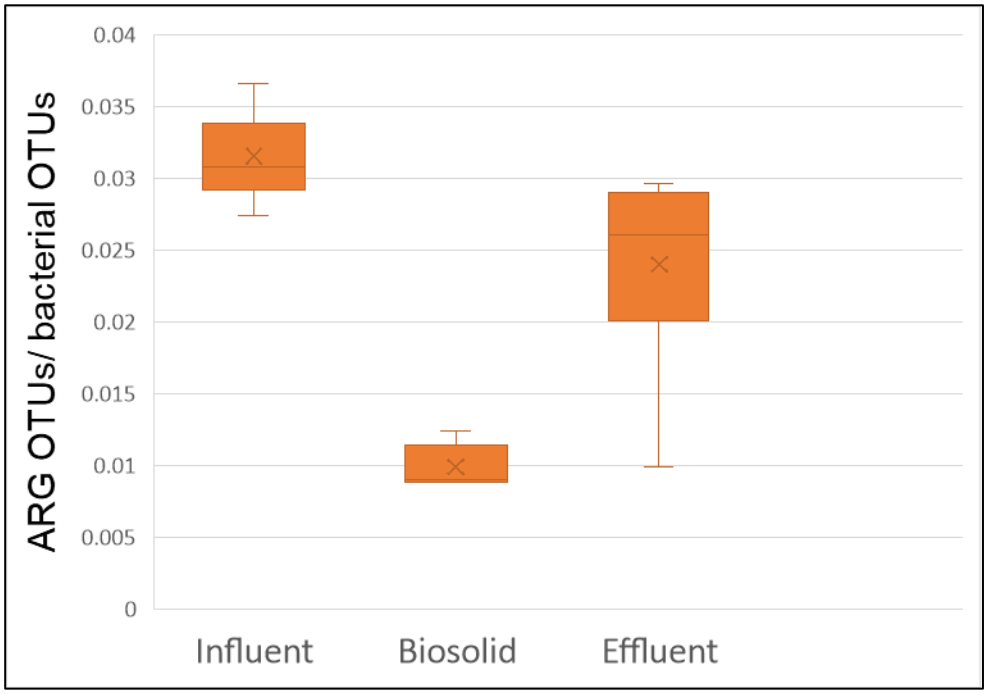


Figure 5-7: The ratio of ARG OTUs to bacterial OTUs identified between the three tested locations of the CBSTP, depicted via a box and whisker plot with medians and the interquartile range.

5.3.6 Monthly Temporal Patterns

An analysis of ARG OTUs that were differentially expressed over the ten months showed that the resistome of all different sample types fluctuated significantly over time. The influent resistome showed significant differences in the relative abundance of 93 ARG OTUs, corresponding to 46 ARGs, over the length of the temporal experiment (Figure 5-8A). Figure 5-11 depicts that these ARGs encoded for resistance against several different antibiotic groups, including aminocoumarins (PARE, GYRBA), aminoglycosides (AAC3, ANT3-DPRIME, APH3-DPRIME, APH6), bacitacin (BACA), beta-lactams (BLAA, BLAF, BLASGM, CARB, CTX, GCP, OXA), elfamycins (TUFAB), flouroquinolones (UHPT, PTSL, PARC, GYRA, GYRB), fosfomycin (MURA), lipopeptides (LPXC), MLS (LNUF, OLEC, ERM), multi-drugs (NALC, ASMA, MSBA), mupirocin (ILES), *Mycobacterium tuberculosis*-specific drug (EMBA, EMBC, FOLC), phenicols (FLOR, CATB, CMLB), Rifampin (RPOB), sulfonamides (FLOP, SULI, SULII, SULIV), tetracyclines (TET39, TTC, TETR, TETM, TETV).

The biosolid resistome showed significant differences in the relative abundance of 104 ARG OTUs, corresponding to 71 ARGs, over the length of the temporal experiment. Figure 5-8B depicts that these ARG correspond resistance to several different antibiotic classes, including aminocoumarins (NOVA, GYRBA), aminoglycosides (A16S, AAC3, AAC6-DPRIME, ANT3-DPRIME, ANT9, APH3-DPRIME, APH6), betalactams (AER, AIM, BKC, GES, LCR, OXA), elfamycins (TUFAB), flouroquinolones (QNRD, PARC, GYRA, GYRB), fosfomycin (MURA), glycopeptides (RPOC), lipopeptides (PHOP), MLS (ERED, ERM, ERMF, ERMG, LNUF, MLS23S, MPHE, MPHG, MSRE), multi-drugs (DRRA, EMRE, IRFA, LSAC, MUXA, OPMB, OPMH, OPMD, TAP), mupirocin (ILES), *Mycobacterium tuberculosis*-specific drug (TYHA, NDH, MSHA, KASA, INIC, FOLC, ETHA, EMBC, EMBA), oxazolidinone (RPLC), phenicol (CATB), pleuromutilin (P23S), rifampin (RPOB), sulfonamides (FLOP, SULI, SULII, SULII, SULIV), tetracycline (TET39, TET64, TETBP, TETC, TETG, TETR, TETT, TETV, TETX), trimethoprim (DFRA, DFRB).

The effluent resistome showed significant differences in the relative abundance of 65 ARG OTUs, corresponding to 42 ARGs, over the length of the temporal experiment (Figure 5-8C). These ARGs encoded for resistance against several different antibiotic groups, including: aminocoumarins (PARE), aminoglycosides (A16S, APH3-DPRIME, RPSL, RRSA, RRSC, RRSH), beta-lactams (CAU, OMPR, OPRD, OXA), catioic antimicrobial peptides (ARNA, BASRS, CAP16S), elfamycins (TUFAB), flouroquinolones (CRPP, GYRA, PARC), fosfomycin (FOSA), lipopeptides (PHOQ), MLS (MLS23S, OLEC, TLRC), multi-drugs (EMRE, MTRAD, MUXA, MUXB, NALD, NFXB, OPMB, PARS), *Mycobacterium tuberculosis*-specific drug (RPSA), oxazolidinones (O23S), phenicols (CAT, ESTD), rifampin (RBPA, RPOB, RPOCR), sulfonamides (SULII), tetracyclines (TET42, TETR).

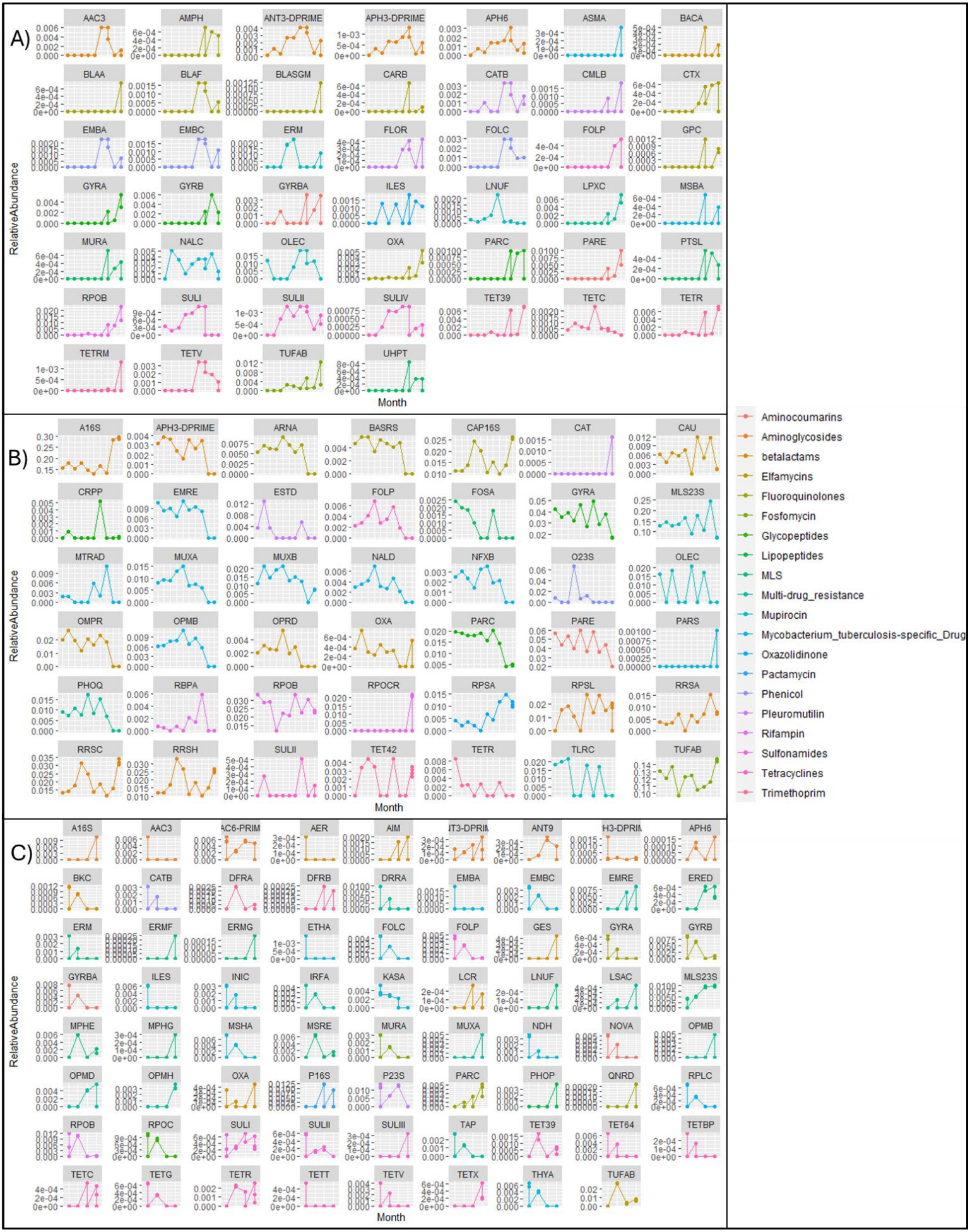

A)
B)
C)
RelativeAbundance
Month
AAC3
AMPH
ANT3-DPRIME
APH3-DPRIME
APH6
ASMA
BACA
BLAA
BLAF
BLASGM
CARB
CATB
CMLB
CTX
EMBA
EMBC
ERM
FLOR
FOLC
FOLP
GPC
GYRA
GYRB
GYRBA
ILES
LNUF
LPXC
MSBA
MURA
NALC
OLEC
OXA
PARC
PARE
PTSL
RPOB
SULI
SULII
SULIV
TET39
TETC
TETR
TETRM
TETV
TUFAB
UHPT
A16S
APH3-DPRIME
ARNA
BASRS
CAP16S
CAT
CAU
CRPP
EMRE
ESTD
FOLP
FOSA
GYRA
MLS23S
MTRAD
MUXA
MUXB
NALD
NFXB
O23S
OLEC
OMPR
OPMB
OPRD
OXA
PARC
PARE
PARS
PHOQ
RBPA
RPOB
RPOCR
RPSA
RPSL
RRSA
RRSC
RRSH
SULII
TET42
TETR
TLRC
TUFAB
AAC3
AC6-PRIM
AER
AIM
IT3-DPRIM
ANT9
H3-DPRIM
APH6
BKC
CATB
DFRA
DFRB
DRRA
EMBA
EMBC
EMRE
ERED
ERM
ERMF
ERMG
ETHA
FOLC
FOLP
GES
GYRA
GYRB
GYRBA
ILES
INIC
IRFA
KASA
LCR
LNUF
LSAC
MLS23S
MPHE
MPHG
MSHA
MSRE
MURA
MUXA
NDH
NOVA
OPMB
OPMD
OPMH
OXA
P16S
P23S
PARC
PHOP
QNRD
RPLC
RPOB
RPOC
SULI
SULII
SULIII
TAP
TET39
TET64
TETBP
TETC
TETG
TETR
TETT
TETV
TETX
THYA
TUFAB
Aminocoumarins
Aminoglycosides
betalactams
Elfamycins
Fluoroquinolones
Fosfomycin
Glycopeptides
Lipopeptides
MLS
Multi-drug_resistance
Mupirocin
Mycobacterium_tuberculosis-specific_Drug
Oxazolidinone
Pactamycin
Phenicol
Pleuromutilin
Rifampin
Sulfonamides
Tetracyclines
Trimethoprim

Figure 5-8: Antibiotic resistant genes (representing multiple sequences) found to be significantly differentially expressed in the (A) influent, (B) Effluent, and (C) biosolids over the sampling period of the CBSTP, depicted as temporal line graphs.

5.4 Discussion

Analysis of the microbiome within daily effluent samples were shown to not fluctuate significantly within the short-term temporal variations experiment, though daily influent samples were found to fluctuate significantly over this same period. Despite this, results showing the insignificant variability between daily influent samples and the corresponding October pool indicate that the method of pooling influent samples monthly with a ~20-day sampling regime allows the short-term variations between daily samples to be accounted for. This time period accounts for the sludge retention time of CBSTP. Sludge retention time refers to an operating parameter of treatment involving activated sludge, where the average length of time an organism spends within the bioreactor is accounted for (Jagaba et al., 2021). This is approximately 21 days within the bioreactor of the CBSTP. As such, microorganisms and their accompanying ARGs within the CBSTP bioreactor are likely to be accounted for within the monthly temporal experiment.

The analysis of monthly composites of CBSTP samples showed that the influent samples have a significantly higher ratio of ARG OTUs to bacterial OTUs than that of the effluent and biosolids, suggesting that this sample type had a higher occurrence of resistance genes to bacterial chromosomes. This, in conjunction with the differences in sample volume represented by each sample type, suggests that whilst ARG sequences are still present within biosolid and effluent of the CBSTP, the processes employed by the CBSTP as an MBR are effective in reducing the load of resistance from the influent. This is supported by other studies, which have stated that the incidence of ARGs are greatly reduced throughout the MBR treatment process (Wang et al., 2020b, Lin et al., 2021, Zhu et al., 2021, Nnadozie et al., 2017). This study also supports the notion that whilst ARG occurrence is reduced, ARGs are still present within the effluent samples of the CBSTP, as is also reported within the literature (Lin et al., 2021, Wang et al., 2020b).

Further analysis of the monthly CBSTP composite samples showed the bacterial richness and evenness in the influent follow a similar trend to that of the resistome data, with high richness and low evenness. This is similar to what is described in other WWTP publications (Zarei-Baygi et al., 2020) with ARGs in the influent following similar trends to that of the bacterial community, suggesting that the majority of ARGs in the influent are harboured within bacterial cells, rather than presenting as free MGE. In contrast to this, both biosolid samples and effluent samples do not show

similar richness and evenness trends for the microbiome as they did in the resistome. Biosolid samples showed a high diversity of bacterial OTUs within the richness index and effluent samples had a highly uneven distribution of bacterial OTU relative abundances. Conversely, Biosolid samples showed comparatively low richness of resistance OTUs present, and effluent resistance OTUs presented in even proportions throughout the study. These instances seem to further allude to the possibility of ARGs existing as free MGE such as eDNA plasmids, or bacteriophages (Leão et al., 2023, Ju et al., 2019). Furthermore, this supports the theory that the biomass of the CBSTP bioreactors (which eventuate to biosolids) may not retain the ARGs observed within the initial influent, as previously reported in the literature (Zeng et al., 2022).

Significant microbiome and resistome shifts observed from the influent to biosolids and effluent respectively, are indicative of different treatment processes within the CBSTP. Where the microbiome of the biosolids is representative of the wastewater processing infrastructure through the purposeful culture of bacterial cell lineages within activated sludge through anaerobic digesters and oxidation ditches. These bacterial phyla include *Actinobacteria*, *Proteobacteria*, and *Chloroflexi*. These taxa have been observed previously with biosolids and activated sludge (Zeng et al., 2022, Schlatter et al., 2019, Matar et al., 2017), along with *Planctomycetes*, which though less frequent, have been observed previously (Schlatter et al., 2019, Matar et al., 2017). *Actinobacteria*, *Proteobacteria*, *Chloroflexi*, and *Planctomycetes* were observed to make up the majority of the biosolids core microbiome within the CBSTP. Conversely, the microbiome of the effluent is likely influenced predominantly by the biofilm harboured within the ultrafiltration membranes. This is evidenced by the core microbiome of the CBSTP effluent having a high degree of familiarity with the biofilm core microbiome established in other research works. This is observed by the dominance of *Proteobacteria* identified in membrane biofilm previously (Rehman et al., 2020), where the classes of *Alphaproteobacteria* and *Betaproteobacteria* are the most dominant within this phylum (Matar et al., 2017). This distribution in the core microbiome of membrane biofilm is reflected in the distribution of core bacterial taxa in the effluent of the CBSTP. Further investigation is required to ascertain if the cleaning cycle of the ultrafiltration membrane biofilm and alteration of the pore size through various stages of biofouling would affect the release and profile of CBSTP resistome.

Whilst bacterial cells were likely retained in the biomass (with an ultimate fate in the biosolids) and excluded from the effluent via ultra-filtration membranes, this process may not be true for ARGs. The general profile of CBSTP ARG within this study was shown to primarily follow the path of liquid waste. As evidenced by the large proportion of shared core resistome between the influent and effluent sampling locations and the comparatively low ratio of resistance to bacteria within the biosolids when compared to the effluent. This is also evidenced by the high proportion of

ARGs exclusive to the core resistome of the biosolids, rather than other sample locations, suggesting that these ARGs are primarily observed in the portion of the microbiome purposefully cultured within the activated sludge treatment process. However, the majority of core ARGs were shared across all sample types, suggesting that this portion of the resistome is ubiquitous in the CBSTP. The possibility exists of the effluent core resistome being influenced by bacteria retained in the biofilm of the ultrafiltration membrane (similarly to the microbiome), though this is likely a small representation of the resistome as suggested by the two ARGs exclusive to the core effluent resistome. As the shared microbiome between influent and effluent sample locations is comparatively small, likely, the majority of ARGs within the effluent resistome (despite originating in the influent) are present through various instances of HGT and eDNA.

These instances of HGT can be observed in the CBSTP microbiome which correlated with incidences in the core resistome of the influent and effluent. Similar instances of HGT have been documented within WWTPs previously (Wei et al., 2021, Yin et al., 2019). For example, an elfamycin resistance ARG (TUFAB) shared in the core resistome of all sampling locations, was associated with a number of alpha-proteobacteria in the influent; however, was not associated with any core bacteria within the effluent (Figure 9). This instance is suggestive of HGT, as this sequence of TUFAB remained within the core resistome of the effluent without being associated with core bacteria, and thus may exist as eDNA. Furthermore, a specific strain of *pseudomonas aeruginosa* was correlated to a single MDR ARG (MUXB) in the influent, likely as this gene is intrinsically coded to the Pseudomonas aeruginosa chromosome. Yet, this strain was found in the effluent to be correlated with the additional presence of ARGs corresponding to resistance against other classes of antibiotics (rifampin, aminoglycoside, cationic antimicrobial peptide, and aminoglycoside). Suggesting that this bacterial strain may have gained additional ARGs through HGT methods throughout the treatment process. Additionally, the higher proportion of resistance to certain antibiotics observed within the effluent in comparison to the influent, including multidrug resistance may be exacerbated by the time spent in the sewage treatment plant reactor, where HGT is likely to occur most frequently. The efflux pump gene MUXB correlated with Pseudomonas aeruginosa in both the influent and effluent of the CBSTP, likely as this gene is intrinsically encoded on the Pseudomonas chromosome. However, MUXB encodes a single component of putative resistance nodulation cell division (RND)-type multidrug efflux pump system, along with MUXA and MUXC. These two genes (MUXA and MUXC) were not identified to be correlated with *Pseudomonas aeruginosa*, suggesting ambiguity in the use of correlation analysis for identifying the presence of ARGs within bacterial species.

The CBSTP resistome was observed to fluctuate significantly within the sampling period, with some similarities observed in the fluctuating ARGs of all sampling locations. These similarities presented in the form of ARGs related to beta-lactam resistance (OXA), rifampin resistance (RPOB), and elfamycin resistance (TUFAB), all of which were found to fluctuate significantly overtime in the

three sampling locations. Additionally, fluoroquinolone resistance genes GYRA and PARC were observed to fluctuate over time in both the influent and the effluent. Despite these similar instances of temporal fluctuations, the fluctuating resistome was generally different in each of the sampling locations. As evidenced by the large proportion of resistance to *Mycobacterium tuberculosis*-specific drugs (TYHA, NDH, MSHA, KASA, INIC, FOLC, ETHA, EMBC, EMBA) found to fluctuate in the biosolids. Glycopeptides and pleuromutilin resistance were found to fluctuate in the biosolids, but not either the effluent or the influent. Furthermore, fluctuations were observed in resistance to trimethoprim antibiotics (DFRA and DFRB) in the biosolids, despite these ARGs not being identified in influent and effluent samples. Additionally, the composition of MDR genes in the effluent and biosolids was observed to fluctuate more vigorously than in the influent. Whilst significant fluctuations of ARGs were identified in each of the sample types, and some consistency in antibiotic class were observed, the pattern of these significant ARG fluctuations is likely to be attributable to varied sources and explanations, including weather, seasonality, treatment regimes, and fluctuations to varied influent sources. Further analysis is required to fully elucidate the cause of these temporal resistome fluctuations.

This study defined the distinct resistome and microbiome within the influent, biosolids, and effluent of the CBSTP and highlighted the microbiome and resistome shifts observed within the treatment steps of the CBSTP. Additionally, despite the CBSTP removal efficiency of antibiotic resistance, this study highlights that ARGs are present within the effluent of this MBR. Furthermore, the possibility of HGT of ARGs between treatment steps of the CBSTP may provide additional opportunities for antibiotic resistance proliferation in previously non-resistant bacterial species which may have ongoing impacts on aspects of public and environmental health. As the CBSTP is one of the largest points of discharge along the Great Barrier Reef Marine Park, future studies should investigate the potential implications of the effluent resistome dissemination and HGT in the marine receiving environment microbiome, and its resident fauna, and this was the focus for chapter six.

. Chapter 6: Dissemination of Antibiotics and Antibiotics Resistance Genes from a Membrane Bioreactor Wastewater Plant on the Great Barrier Reef Australia: Implications for Marine Microbial Communities

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Cleveland Bay is situated on the boundary of the Great Barrier Reef Marine Park (GBRMP) and is the receiving environment for treated effluent produced by the Cleveland Bay Sewage Treatment Plant (CBSTP). This study characterised the dissemination patterns of antibiotics and antibiotic resistance genes (ARGs) from the CBSTP through comparison with an uninhabited environment in the Great Barrier Reef Marine Park known as the Howick Group of Islands. This was done by a cross-sectional analysis of the interactions between a select group of anthropogenically used antibiotics, ARGs, and environmental microbiomes. The resistome within the Near-Field of Cleveland Bay was represented largely by CBSTP-associated ARGs, and correlations to the microbiome indicated instances of out-competition of functionally important bacteria. Furthermore, implications of horizontal gene transfer of CBSTP-associated ARGs were identified between the CBSTP effluent and the Near-Field microbiome. Additionally, the presence of macrolide antibiotics above predicted no-effect concentration within Cleveland Bay sediments were correlated with changes in the functional microbiome of the Near-Field. Increased stress markers of the Mid and Far-Fields compared to that of the Howicks, or the Near-Field suggest that consideration should be given to other sources of resistance dissemination within marine ecosystems. However, this study highlights that ARGs and antibiotics from membrane bioreactor plants may affect associated marine receiving ecosystems.

6.1 Introduction

Antibiotic resistant bacteria (ARB) are the natural product of the enduring relationship between antibiotics and environmental microbiomes since the earliest existence of bacteria (Kümmerer, 2009). Whilst antibiotics and antibiotic resistance genes (ARGs) are both natural, the interference in this relationship, through the use of antibiotics in various sectors including public health, veterinary science, and agriculture, has exponentially increased the prevalence of ARB in nature (Panthee et al., 2016). This has implications for all aspects of One Health (public, animal, and environmental health). Consequences of the dynamic interactions between antibiotics, ARB, and their associated ARGs in public health have been well-documented in the literature (Bouki et al., 2013, Osińska et al., 2019, Sabri et al., 2020), but less information exists on the consequences of

similar interactions within many of the world's natural ecosystems. This is specifically true for delicate marine ecosystems, such as the Great Barrier Reef.

Wastewater treatment plants (WWTPs) are a source (or "hot spot") for antibiotic resistance to reach aquatic ecosystems (Drane et al., 2024b). This is due to their influent sources comprising unmetabolised antibiotics and ARB from hospitals, the public, veterinary practices, as well as other catchment sources such as pharmaceutical industries and agricultural enterprises (Drane et al., 2024b). Common forms of wastewater treatment are not specifically designed for antibiotic removal, and consequently, these molecules and associated ARB can be released directly into the receiving environment (Drane et al., 2024b, Halling-Sørensen et al., 1998, Kümmerer, 2003, Zuccato et al., 2010). Unfortunately, environmental studies examining the microbial effect of antibiotics generally relate to agricultural applications in land-based soils (Liu et al., 2014, Yang et al., 2009, Liu et al., 2012, Unger et al., 2013, Schauss et al., 2009, Underwood et al., 2011), with less focus given to aquatic matrices and their associated sediments.

Insight into the dissemination of antibiotic resistance stressors in aquatic ecosystems may be obtained by comparing upstream and downstream ARBs and ARGs in river-based receiving environments relative to anthropogenic sources (Li et al., 2011, Laverman et al., 2015, Proia et al., 2013). However, the literature regarding the implications of marine coastal receiving environments is limited. This is despite antibiotics and their associated ARGs being defined as indicators of anthropogenic pollution (Yang et al., 2021, Palacios et al., 2021, Tan et al., 2018, Eckert et al., 2018, Hou et al., 2015, Näslund et al., 2008), and the reported sensitivity of marine environments' microbiome (Hou et al., 2015, Näslund et al., 2008). Such delicate microbiomes may be directly impacted by the dissemination of ARGs and antibiotics from anthropogenic sources, including WWTP effluents (Hou et al., 2015, Näslund et al., 2008).

The Great Barrier Reef Marine Park (GBRMP) extends over 2,300 km along the Coral Sea coast of Queensland, Australia, and is regarded as one of the world's seven natural wonders. A portion of the Coral Sea coast known as Cleveland Bay straddles the boundary of the federally managed Great Barrier Reef Marine Park and the tidal Queensland State Marine Park and is the receiving environment for treated effluent produced by the Cleveland Bay Sewage Treatment Plant (CBSTP). The CBSTP services the largest population centre in North Queensland and is regarded as one of the largest membrane bioreactors (MBR) in the southern hemisphere. Treatment of wastewater via MBRs is an example of current wastewater treatment infrastructure capable of removing ARBs and their accompanying ARGs through small pore filtration membranes (Wang et al., 2020b, Lin et al., 2021, Zhu et al., 2021, Nnadozie et al., 2017). Wang et al (2020) stated that the

average ARG removal approached 100% through a full-scale MBR process (Wang et al., 2020b). Similarly, a 98.4% decrease in ARGs within an MBR plant was reported by Lin et al (2021) (Lin et al., 2021). Collectively, these observations suggest that MBR plants may be effective in the removal of ARGs, but they are not a perfect system, and effluents from such treatment plants do still contain ARGs (Lin et al., 2021).

Preliminary work described the bacteria and ARGs present within the treated effluent from the CBSTP and showed that although ARGs were removed, distinctive ARGs and bacteria were still present within the effluent, as described in Chapter 5. Due to the lack of applicable risk assessment scaffolds, there is limited understanding of the implications of these ARGs. This raises concern regarding the potential effects on Cleveland Bay's ecosystem and fundamental microbiome from ARGs within the CBSTP effluents.

This study aimed to provide further insight into the changes in the environmental microbiome and its potential association with the presence of antibiotics, ARGs, and bacteria in the marine receiving environment of the effluent from CBSTP. This information will contribute to closing the knowledge gap in assessing the effects of antibiotic resistance factors in marine ecosystems subject to anthropogenic influences such as treated WWTP effluent. Specifically, this study aimed to categorise the dissemination patterns of antibiotics and ARGs from a wastewater treatment plant situated near the Great Barrier Reef in North Queensland, Australia, by a cross-sectional characterisation of the interactions between a select group of anthropogenically used antibiotics, ARGs, and environmental microbiomes.

6.2 Methods

All samples were acquired under the federal GBRMPA permit 46568, and the Queensland state Department of Environment and Science permit P-SPP-100143721. Samples of seagrass were obtained within guidelines set by the Queensland Department of Agriculture and Fisheries, as described in "Accepted development requirements for operational work that is the removal, destruction or damage of marine plants" (QPW/2017/3931 v2).

6.2.1 Sampling Locations

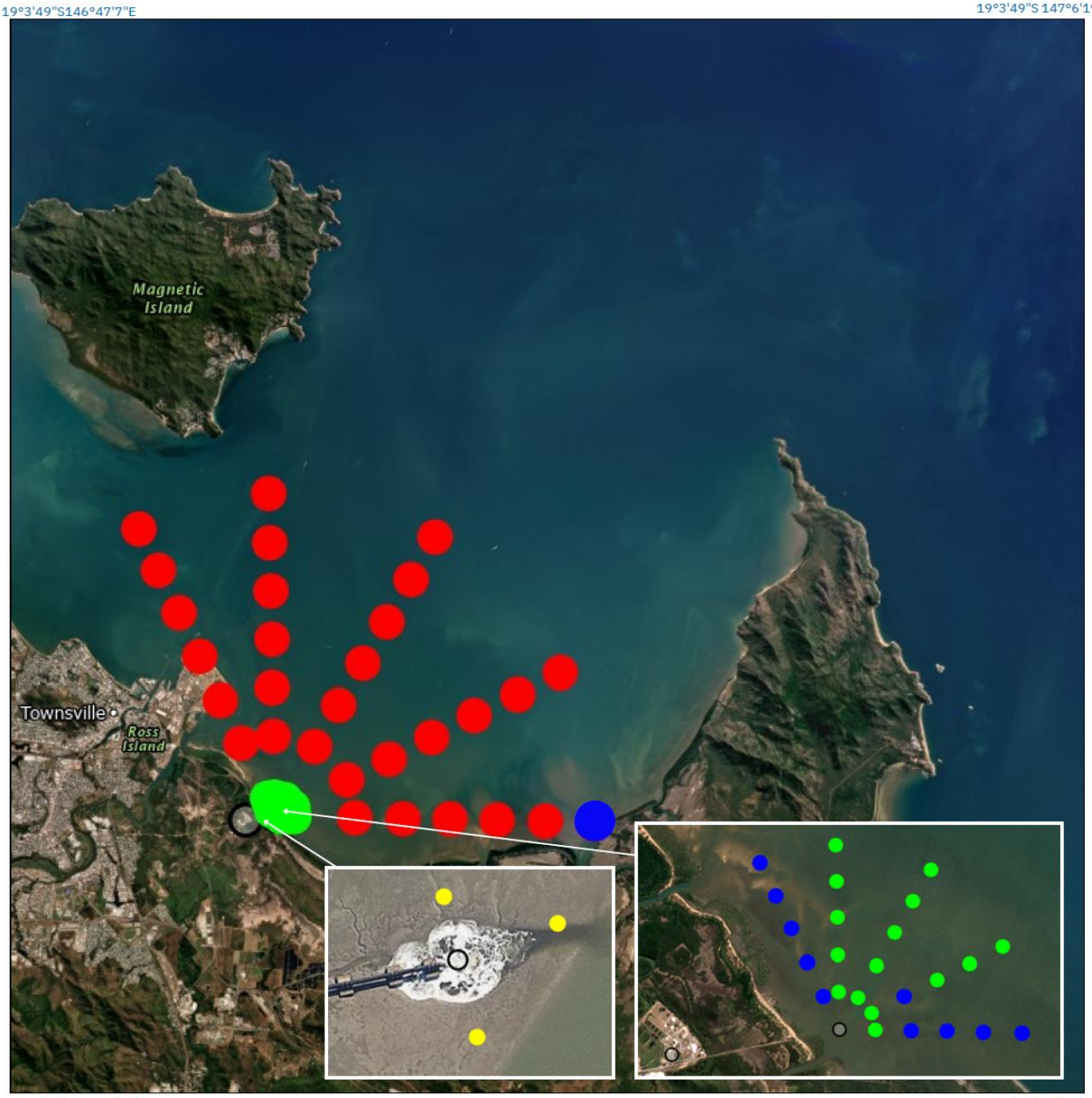
Locations for environmental sample collection were chosen throughout the Cleveland Bay site based on their distance from the CBSTP. These locations were classified into three distance categories, comprising Near, Mid, and Far-Fields, as described in monitoring programs and previous

literature on dissemination patterns of contaminants in marine environments (2014, Inan, 2019). The Near-Field was described as three distinct geographic locations closest to the CBSTP-treated effluent outfall pipe (hereon referred to as outfall), approximately 10 meters distant (seaward) from the outfall (Figure 6-1 – points shown in yellow). The Mid-Field was described as 15 geographic locations between approximately 200 meters and 1 km approximately from the outfall (Figure 6-1 – points shown in green). The Far-Field was described as 29 geographic locations between approximately 2.5 km and 10 km from the outfall (Figure 6-1 – points shown in red). Both the Near and Mid-Filed were comprised primarily of a tidal mud-flat zone, whereas the Mid-Field and Far Field outer locations comprised coastal seagrass fields and coral reefs. Tidal heights at the time of sampling limited access to 10 locations within the Mid-Field and one location within the Far-Field.

Cleveland Bay

Sampling Locations

19°3'49"S146°47'7"E 19°3'49"S 147°6'19"E



19°21'57"S 146°47'7"E 19°21'57"S 147°6'19"E

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Printed at: A4

Print date: 20/9/2024

Not suitable for accurate measurement.

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Queensland Government

Department of Resources

Figure 6-1: Sampling distance categories utilised in Cleveland Bay as the receiving environment of the Cleveland Bay Sewage Treatment Plant, where the three Near Field Sample locations are indicated in yellow, the 15 Mid-Field Sample locations are indicated in green, and the 29 Far Field Sample locations are indicated in red. Samples from locations indicated in blue could not be acquired. Map lines delineate study areas and do not necessarily depict accepted national boundaries.

The Howick Group of Islands was chosen as a reference site due to its limited direct anthropogenic impacts and human inhabitants, and lack of proximity to sewage treatment facilities. The Howick Group of Islands is a remote, uninhabited group of islands in the northern Great Barrier Reef Marine Park; additionally, it is subject to low frequencies of visitation and provides low instances of anthropogenic-associated stormwater and agriculture run-off. Sampling within this site comprised four locations at the perimeter of Ingram Island (-14.418031, 144.880146) and three locations around the perimeter of Combe Island (-14.400946, 144.902234) (Figure 6-2 – points shown in yellow). The perimeter of both Combe and Ingram Islands is comprised of coral reefs and coastal seagrass fields. The geographical location and the description of the CBSTP are detailed in Chapter 4.

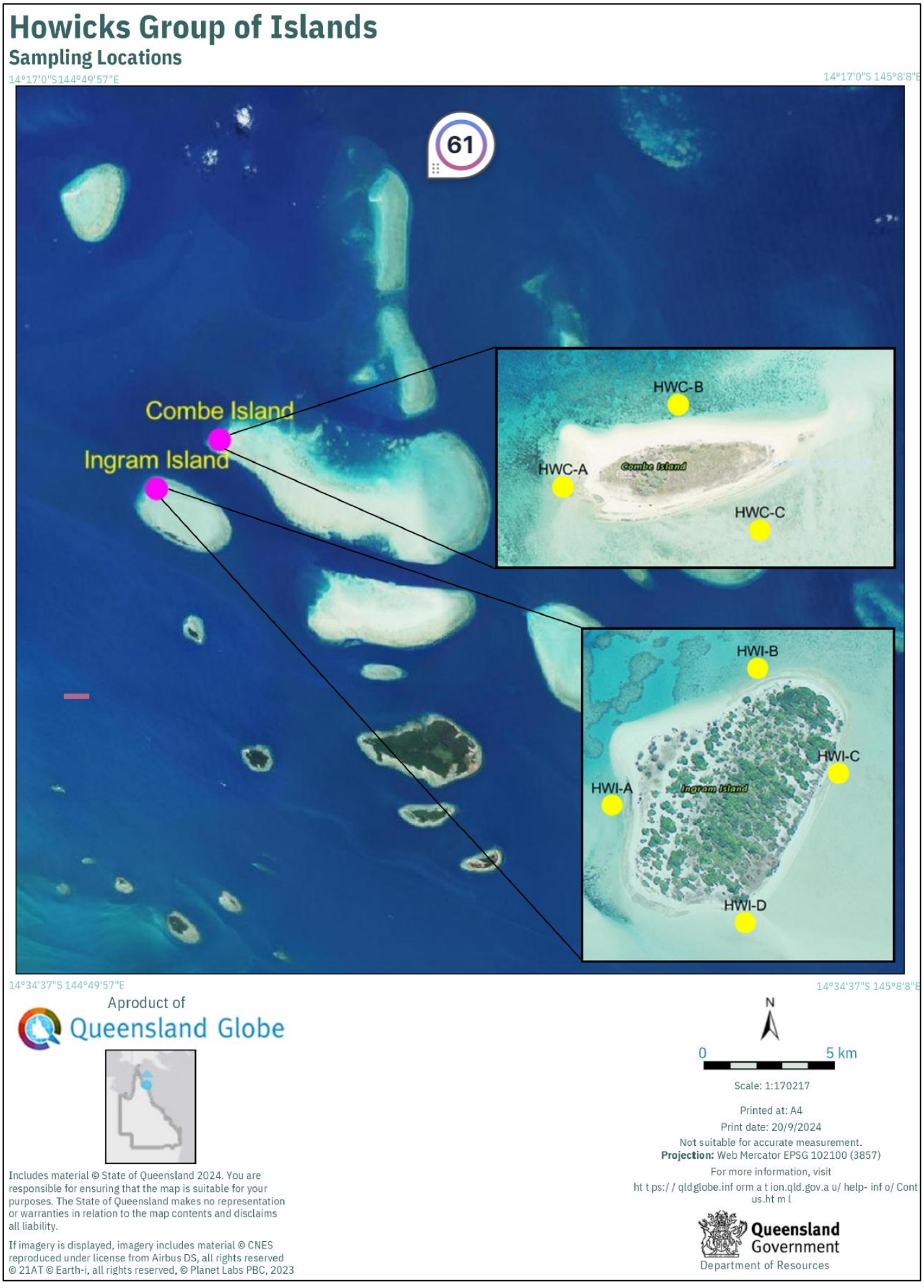

Howicks Group of Islands
Sampling Locations
14°17'0"S144°49'57"E
14°17'0"S 145°8'8"E
61
Combe Island
Ingram Island
HWC-B
HWC-A
Combe Island
HWC-C
HWI-B
HWI-C
HWI-A
Ingram Island
HWI-D
14°34'37"S 144°49'57"E
14°34'37"S 145°8'8"E
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Printed at: A4
Print date: 20/9/2024
Not suitable for accurate measurement.
Projection: Web Mercator EPSG 102100 (3857)
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Department of Resources

Figure 6-2: Locations from which samples were acquired in the Howick Group of Islands to be utilised as a reference area. Specific sampling locations are indicated in yellow. Map lines delineate study areas and do not necessarily depict accepted national boundaries.

6.2.2 Sampling Method

Separate sample types were taken within each location at both the Cleveland Bay and Howicks sites. Samples of Cleveland Bay locations were acquired between the 18th -31st of July 2022, and samples of Howick locations were acquired on the 10th and 11th of August 2022. These dates were chosen for the acquisition of Cleveland Bay environmental samples as well as the use of effluent samples collected in the preceding months to provide an accurate representation of possible temporal changes exhibited within the effluent and its contained antibiotic resistance preceding collection. Furthermore, the collection of environmental samples from both sites within the regions dry season was intended to reduce the severity of other contaminant sources providing large areas of dissemination. Samples of Near and Mid-Field locations at the Cleveland Bay site were taken at high tides to allow for appropriate water depth to facilitate transportation via marine vessels. The sample types comprised of sediment, seawater, and seagrass (where this was available). Seawater samples were acquired utilising an aluminium sampling pole with an attached open sterile 400 ml reservoir. The sterile reservoir was submerged at each sample location to an approximate depth of 10 cm and closed using an aseptic technique (with no external interference of the sample and sterile gloved hands) upon filling.

Sediment samples were acquired utilising a 2 L Van Veen Grab Sampler (Eijkelkamp, Netherlands), where surface sediments were captured at each Mid and Far-Field site and brought to the surface; additionally, tidal heights and the prospect of contamination limited sediment sampling of the Near-Field environment to low tides. The contents of the grab sampler were then released into a sterile 15 L tub, which allowed for visual inspection of the seagrass present. Upon inspection of the Van Veen contents, a portion of the sediment contents (~100 grams) was then collected in a 150 ml sterile jar utilising an aseptic technique, and seagrass present within the sample was collected in a separate 150 ml sterile jar utilising sterile forceps, though seagrass was not present within the Near-Field sample locations. All samples were immediately placed in separate sterile bags and then in a sterilised cold box at ≤ 5°C. Samples were subsequently preserved onshore at -20°C within 12 hours of sampling. Samples were stored and processed at the Molecular Ecology and Evolution Laboratory at James Cook University Campus (Building 145, Level 3).

The effluent of CBSTP was obtained every 18 days +/- 2 days to account for any variation due to the 21-day sludge retention time for 10 months from 11/10/2021 to 22/7/2022, resulting in 16 sample points over the total period. Samples of wastewater effluent were collected via a filtration pump on-site (Wong et al., 2020) utilising Grover Scientific peristaltic pumps. For each sample point, five litres of effluent from the CBSTP storage tank were filtered through sterile 0.22 µm mixed cellulose ester filters made by Sartorius Australia PTY LTD (Majaneva et al., 2018). Preserved CBSTP effluent samples were separately pooled based on the month of sampling, providing an approximate profile of the DNA obtained from the effluent throughout October 2021 to July 2022 for a total of ten sample points. This seasonal examination of the CBSTP effluent provided identification of antibiotic resistance factors that may have fluctuated on a temporal basis and allowed the characterisation of the CBSTP effluent resistome at the time of environmental sampling as well as in the months preceding, which may have also been represented in the receiving environment.

6.2.3 Nucleic Acid Extraction

Seawater samples were processed for DNA extraction by filtering 30 ml of liquid through sterile 0.22 µm mixed cellulose ester filters made by Sartorius Australia PTY LTD via Grover Scientific peristaltic pumps. The subsequent seawater sample filter papers, along with sediment, seagrass and effluent samples, were processed for DNA extraction and preserved utilising ZymoBIOMICS™ Miniprep kits (Zymo Research Corp., Irvine, CA, USA) according to the manufacturer's instructions as per materials and methods of Chapter 4.

6.2.4 Composite Sampling Strategy

Extracted DNA from each seawater and seagrass sample collected from Cleveland Bay and the Howicks was pooled into representative composites per distance category. Extracted DNA from each sediment sample from the Mid and Far-Field of Cleveland Bay was pooled into three representative composites per distance category, and as three sampling locations existed for the Near-Field, sediment samples were not pooled for this distance category. Extracted DNA from each sediment sample from the Howicks was pooled into two representative composites. These described composites of DNA created a final volume of 200 µl and preserved in sterile 250 µl centrifuge tubes at -20°C.

These extracted DNA composite samples were utilised for SM-Seq, where duplicate sequencing was conducted on one composite for each sample type from each distance category to account for sequencing methodology errors. The remaining bulk sample volumes of sediment from

all locations were pooled into representative composites based on each distance category of approximately 200 g. The remaining bulk seawater samples from the Mid and Far-Fields were pooled into representative composites based on each distance category of approximately 500 ml. These bulk composite samples were utilised for antibiotic residue testing. Due to unavailable sample volumes of remaining bulk seawater samples in the Howicks and the Near-Field antibiotic residue concentrations could not be obtained from these seawater samples.

6.2.5 Antibiotic Residue Concentrations

Representative composite samples of sediment and seawater from the Howicks (sediment only), Near-Field (sediment only), Mid-Field, Far-Field, and effluent were analysed by Eurofins (Eurofins Australia Pty Ltd) for antibiotic residue testing via Liquid Chromatography with tandem mass spectrometry. Additionally, representative samples of CBSTP effluent of approximately 1000 ml obtained in July 2022 were also used for this analysis and analysed in duplicate. Antibiotics tested for were azithromycin, ciprofloxacin, clarithromycin, norfloxacin, sulfamethoxazole, and trimethoprim. These antibiotics were targeted due to their possible persistence in marine environments and the capability of current scientific practices to identify these compounds accurately at concentrations at or below their predicted no-effect concentration (PNEC). These antibiotics were described previously in a preliminary screening program of contaminants of emerging concern from the CBSTP which incorporated elements of chemical screening in line with European Union, United States and Canada, and United States Environmental Protection Agency's Persistent, bio-accumulative and or toxic nature profiler, as well as other systematic methods (Kuskopf et al., 2020). Further data analysis was only done for antibiotics that were detected at concentrations above their PNEC as defined previously (Bengtsson-Palme and Larsson, 2016).

6.2.6 Shotgun Metagenomic sequencing

Twenty microlitres of each purified DNA composite sample (average DNA concentration of 26.4 ng/µl) were sent to the Australian Genome Research Facility (AGRF), Melbourne, Australia. Library preparation and SM-Seq were performed according to AGRF's methodology as described in Chapter 4.2.4 (Drane et al., 2024a). The libraries were sequenced using the Illumina NovaSeq S4 sequencing platform (San Diego, CA, USA).

6.2.7 Data Analysis

Details on the processing of raw read sequences through Trimgalore, Kraken2, and bracken utilised in this study, as well as profiling and trimming the composition of microbiome and resistome communities through AMR++ 3.0 are described in materials and methods of Chapter 4.

Alpha-diversity metrics and visualization of the microbiome and resistome proportions were analysed using the “mia” package (version 1.6.0) (Borman T, 2024) and “ggplot2” package (version 3.4.1) (Wickham, 2011). Comparison of beta-diversity metrics and visualization of Principal component analyses (PCA) was done using the “phyloseq” R-package (version 3.16). Differential expression analysis was done through "DESeq2” (version 1.38.3) (Love et al., 2014) and “mia” version 1.6.0 R-packages. (mia, 2022) R-packages. Core microbiome and resistome operational taxonomic unit (OTUs) were analysed using the “microbiome” (version 1.20.0) (Leo Lahti, 2012), microbiomeutilities (version 1.0.17) (Shetty, 2021), and “ggplot2” packages in R software (v4.1.1) (Wickham, 2011), where the core microbiome and resistome of a bacterial community for this study were defined as an OTU that occurs in at least 90% of samples and has a relative abundance of at least 0.01. Spearman correlation analyses were conducted using the ‘mia’ package and facilitated by transforming the data on a logarithmic scale. To assess differences between microbiomes and resistomes concerning centroid and dispersion, a Permutational multivariate analysis of variance statistical test was performed using the "DESeq2” R-package. The Permutational multivariate analysis of variance was based on Euclidean distances between samples. Furthermore, a permutation test was used to determine the significance of the result, allowing all samples to be randomly mixed throughout the groups.

6.3 Results

6.3.1 Defining the Microbiome and Resistome of Environmental Samples by Sample Type

Increased microbial diversity within environmental samples has been previously linked with the health of ecosystems, with increased ecological stressors linked to decreases in the diversity of microbiomes (Rocca et al., 2019). Samples collected from the different Cleveland Bay matrices showed differing resistome richness trends between distinct sampling distance categories (Figure 6-3A). The sediment samples were shown to have a richer median resistome than that of other sample types; however, this was found to be more variable than other sample types (Figure 6-3A). Further analysis of these data suggests that the variability of sediment resistome richness was likely attributed to high richness found in sediment collected from the Mid (~200 – 250) and Far fields (~150 – 300) and comparatively low resistome richness exhibited in the sediment of the Howicks (~50) and Near Field (~50 – 100) of Cleveland Bay. This contrasts with the richness of the resistome

observed for seawater and seagrass samples, which was found to have little variation across all sample distance categories (~75-85 and ~50-75, respectively). However, an outlier was observed for the Howicks resistome richness index, with a singular seawater sample showing considerably higher richness than other samples from the same area.

Resistome profiles of the separate sample types were shown to have different evenness distributions (Figure 3B). Seagrass samples were found to have the least evenness in distribution (~0.09 - 0.2), followed by seawater (~ 0.19 – 0.27). Sediment samples were found to have a similar evenness distribution to that of the effluent (~0.31-0.39 and ~0.32-0.41, respectively), with the median evenness of distribution in the effluent higher (~0.37) than that of the sediment (~0.34) (Figure 3B). Two outliers were observed for sediment samples from the Howicks site, which were significantly above the interquartile ranges at >0.6 evenness.

Similar to the resistome, sediment sample types were the richest, with a median of ~120000 and an interquartile range of ~10900 – 12300 (Figure 3C). The median richness of the microbiome of the seagrass and seawater was similar to the effluent, ranging from ~9400 – 10000 (Figure 6-3C). However, the variation in bacterial richness was found to be much more prominent in seagrass samples than other sample types (~7900 – 9700), this is attributable to the comparatively low microbiome richness of seagrass observed from the Mid-Field (~7400) when compared to that of the Far-Field (~10000), and Howicks (~9500).

The evenness of OTU distribution in the microbiome data was found to be consistently low for seagrass (median ~0.0025) and seawater (median ~0.002) sample types, and these had very minimal variation from the medians (~ 0.0025 – 0.0035, and ~0.002 – 0.0027 respectively) (Figure 6-3D). The effluent samples were found to have a lower median for evenness of OTU distribution (~0.0015) than the seagrass and seawater. However, the effluent sample evenness fluctuated more, with interquartile ranges from ~0.001 – 0.007 (Figure 6-3D). Additionally, the sediment sample types had the highest evenness of OTU distribution (median ~0.013) and fluctuated significantly with interquartile ranges from ~0.012 – 0.018 (Figure 6-3D). This extensive range of sediment microbiome evenness is attributable to the very even microbiome sediment data from the Mid-Field (>0.02).

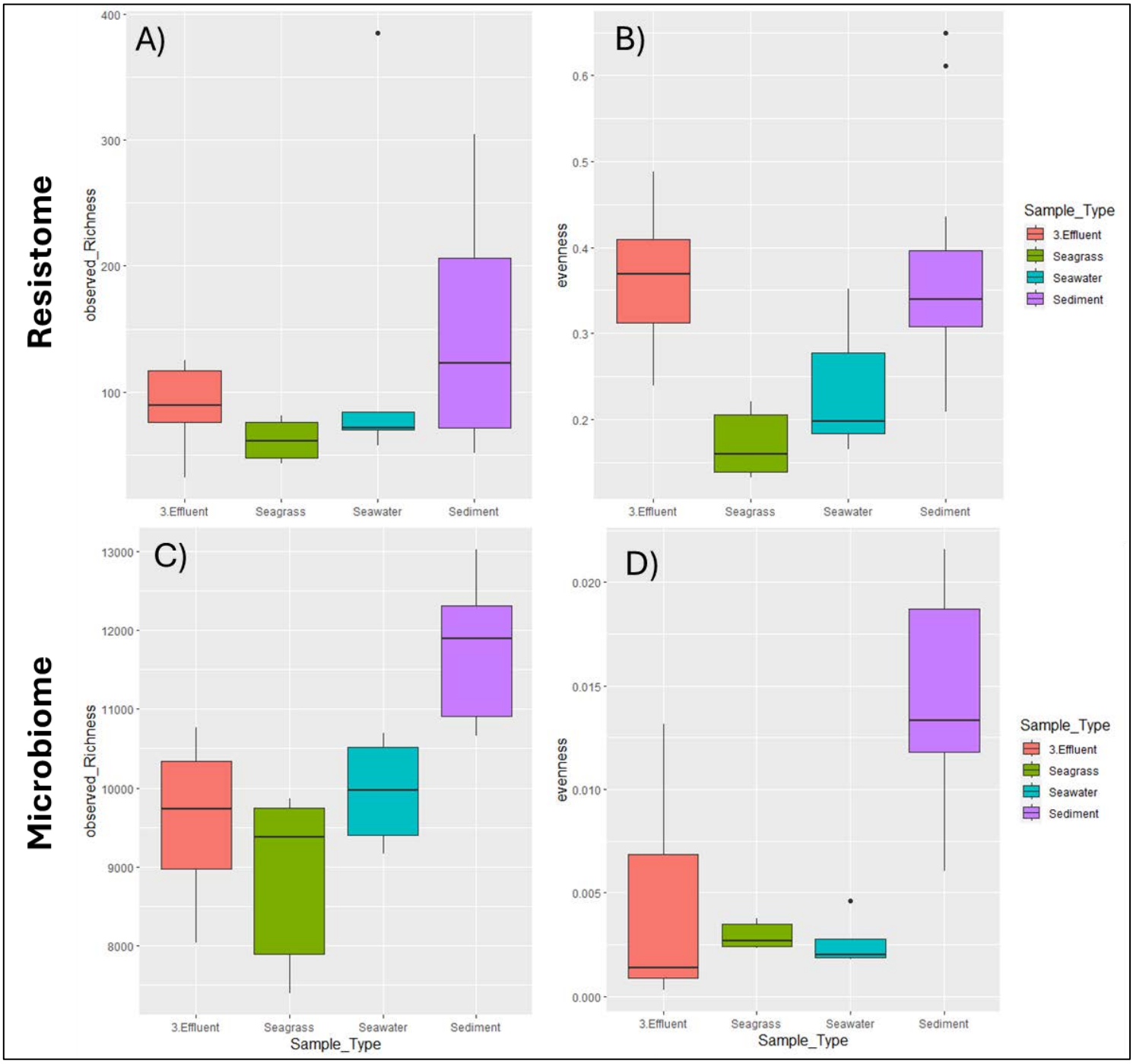


Figure 6-3: Alpha diversity measures of Richness and Evenness for the microbiome and resistome communities identified within different sample types Effluent (n = 24), seagrass (n = 6), seawater (n = 8), and sediment (n = 15) (x-axis), Data are presented as medians with interquartile range and depicted as box and whisker plots. Sample colours correspond to sample type, as annotated in the legend where (A) represents the richness of the resistome, (B) represents the evenness of the resistome, (C) represents the richness of the microbiome, and (D) represents the evenness of the microbiome identified in separate sample matrices. Where n represents the number of sequencing runs conducted for each sample type.

6.3.2 Defining the Microbiome and Resistome of Environmental Samples by Sampling Location

A similar distribution was observed when OTUs were stratified by class of antibiotic against which resistance was encoded throughout differing fields (Figure 6-4). High proportions of ARGs encoding for aminoglycoside and MLS resistance were found in most sample types across all distance categories. However, the population of specific resistance genes that confers these forms of resistance were found to differ substantially across sample types and distance categories (Figure 6-5A).

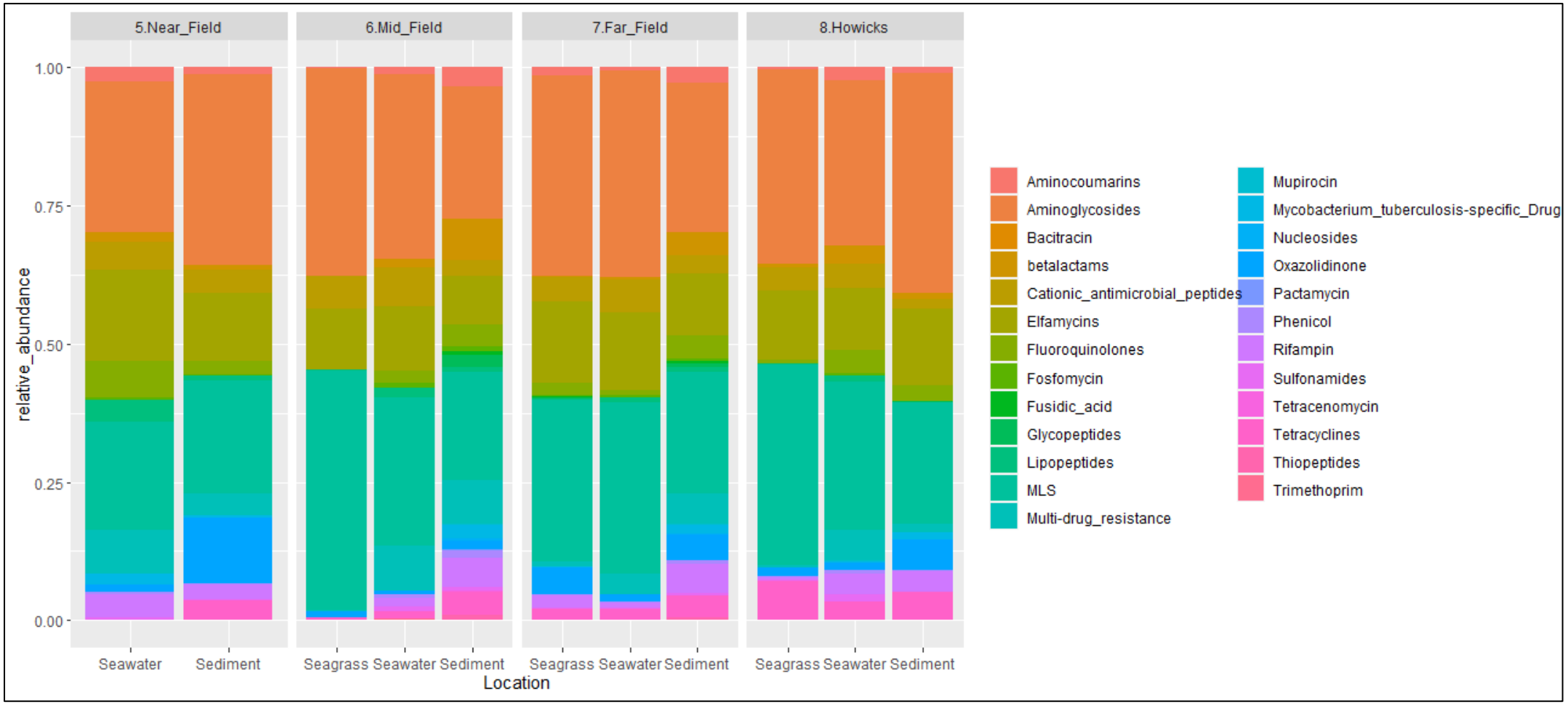


Figure 6-4: Bar graph depicting the relative abundance of antibiotic resistant gene operational taxonomic units of each sample type in each distance category (Near-Field, Mid-Field, Far-Field, and Howicks), stratified by antibiotic class to which ARGs encode resistance.

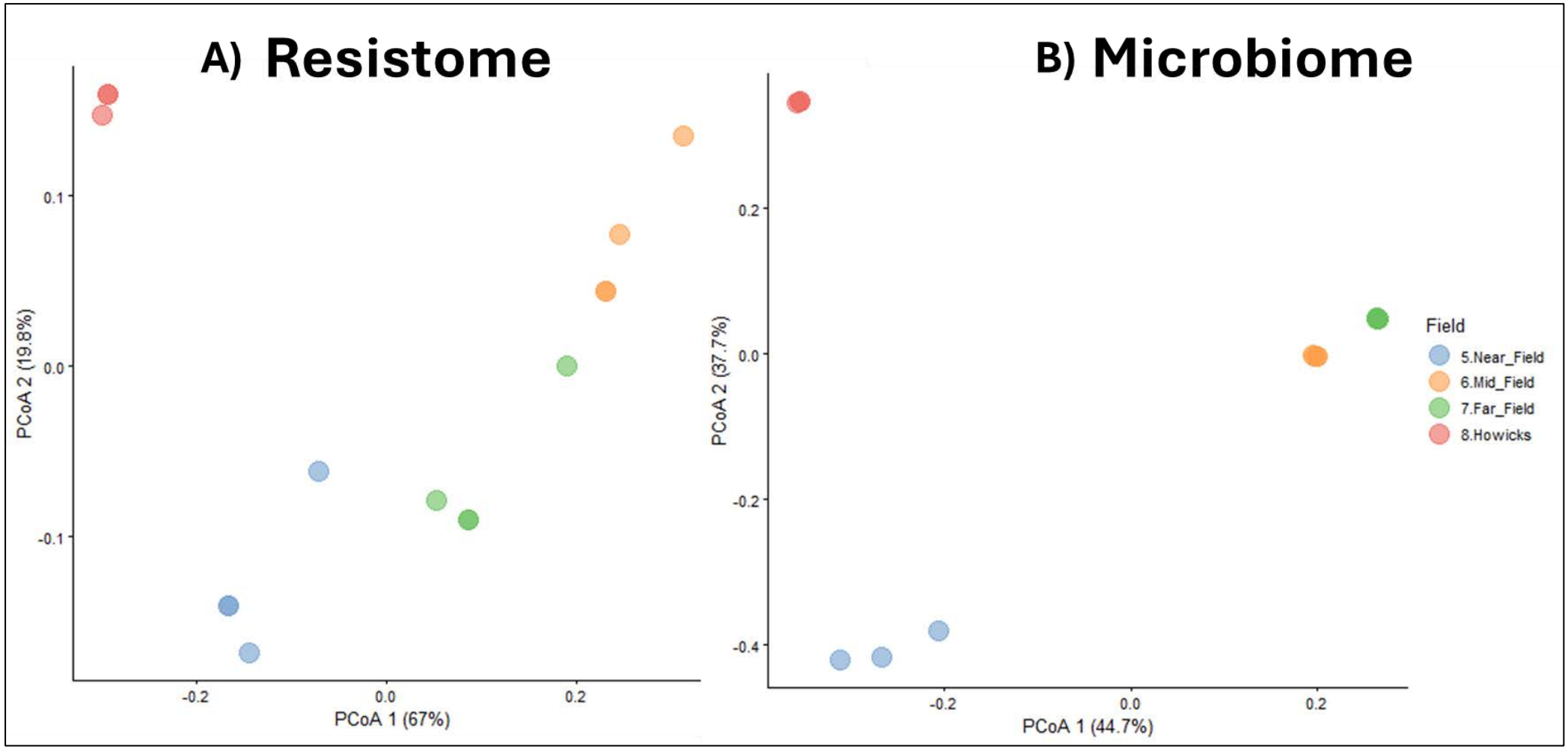


Figure 6-5: Principal Component Analysis (PCA) depicting differences in resistome (A) and microbiome (B) community distributions within the sediment composite samples from separate distance categories, with Near-Field represented in blue, Mid-Field in yellow, Far-Field in green, and Howicks in red.

Redundancy analysis of the resistome data acquired from all environmental sample types obtained from the different distance categories of Cleveland Bay and the Howick Group of Islands showed that sample type accounts for 20% of the variance, whilst the distance categories only accounted for 10% of the variance. This suggested that the type of sample acquired (sediment, seagrass, and seawater) had a different resistome inherent to that sample type. However, this redundancy analysis also indicated that when sample types were analysed in isolation, the variances between distance categories become abundant. This can be most prominently observed in a PCA plot where the resistome of sediment obtained from the different distance categories was observed to be significantly different from one another ($p = 0.001$) (Figure 6-5A). A similar trend was observed for the microbiome data of these distance categories, with a redundancy analysis showing that sample type accounted for 19% of the variance, whilst the distance category only accounted for 11% of the variance. A PCA of this microbiome data within the sediment samples also showed a significant difference between the distance categories ($p = 0.001$) (Figure 6-5B).

The stability of an environmental ecosystem has been linked to the diversity of its core microbiome (Björk et al., 2018). These differences in each sample type and the effective differences observed between the distance categories described above are also reflected in the core microbiome. The core microbiome for the sediment samples involved a total of 193 unique bacterial OTUs (Figure 6-6A). However, only 7 distinct bacterial OTUs were found to be shared across the core microbiomes of sediment in each distance category, and the number of core bacterial OTUs unique to each distance category was 19 in the Near Field, 24 in the Mid-Field, 29 in the Far Field, and the Howicks represented the richest unique core microbiome with 51 bacterial OTUs not identified in other core microbiomes (Figure 6-6A).

The core microbiome of each of the seagrass samples involved a total of 163 unique bacterial OTUs, with, only 5 distinct bacterial OTUs found to be shared across the core microbiomes of seagrass in each distance category. The Howicks was identified to have the richest seagrass core microbiome (97 bacterial OTUs), and the most unique core bacterial OTUs (62) when compared to that of the Mid-Field (36 core OTUs and 10 unique core OTUs) and Far-Field (75 core OTUs and 51 unique core OTUs) (Figure 6-6C).

The core microbiome of each of the seawater samples involved a total of 217 unique bacterial OTUs, with only 3 distinct bacterial OTUs shared across the core microbiomes of seawater in each distance category. Furthermore, the near-Field and the Howicks were identified to have the richest core microbiome (90 and 88 core bacterial OTUs respectively) and most unique core

microbiomes (71 and 62 unique core bacterial OTUs respectively) when compared to the Mid-Field (59 core OTUs and 8 unique core OTUs) and Far-Field (60 core OTUs and 14 unique core OTUs).

Similarly, the core resistome of the different sample types varied across the different distance categories. The sediment samples shared a portion of these core resistance genes in all distance categories (33 ARG OTUs), though a relatively large number was only represented in Mid-Field samples (71). There were 67 unique ARG OTUs in seagrass samples, with 16 identified in the Mid-Field, 60 in the Far Field, and 44 in the Howicks (Figure 6-6F). The seawater samples were defined by a total of 87 unique ARG OTUs, and the number of ARG OTUs identified for each distance category changed, with 68 in the Near Field, 65 identified in the Far Field, 48 identified in the Howicks, and the Mid-Field represented the richest seawater core resistome with 73 ARG OTUs identified (Figure 6-6E).

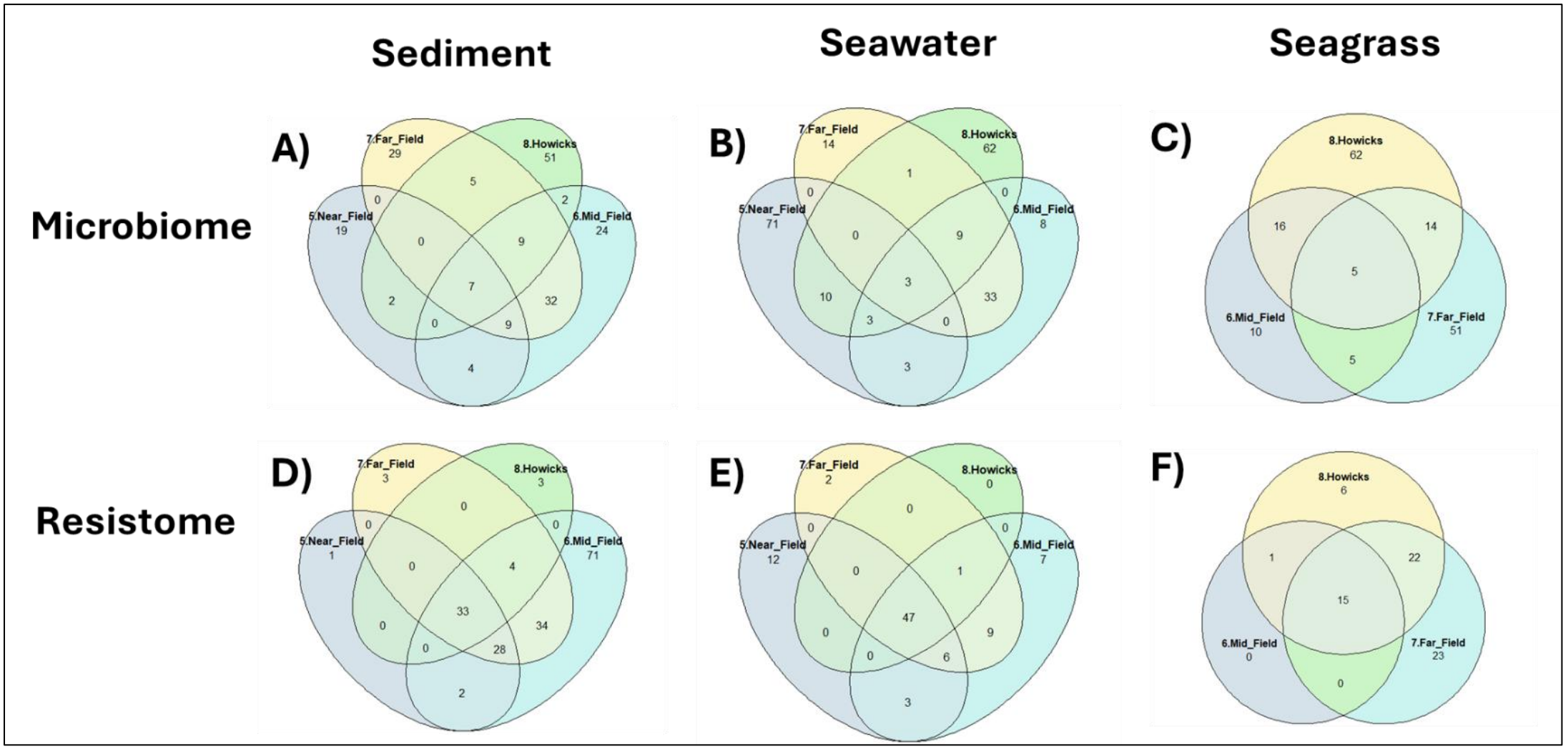


Figure 6-6: Venn diagram with a visual representation of the similarities and differences in core microbiome operational taxonomic units and resistome operational taxonomic units expressed within each sample type (sediment, seawater, and seagrass) across all distance categories (Near-Field, Mid-Field, Far-Field, and Howicks).

The ratio of resistome OTUs to microbiome OTUs defined in the study provides insights into the resistance load in different distance categories. When all sample types were accounted for, no significant difference was observed in the ARGs to bacteria ratio between any distance categories. However, when sediment was analysed in isolation, a significant difference was observed with a pairwise t-test between the Near-Field and the Howicks. The Howicks had a significantly lower ratio than the Near-Field ($p = 0.0039$), and the Near-Field had a significantly lower ratio than that of the Mid-Field ($p = 0.018$) and Far-Fields ($p = 0.0041$), but there were no differences between Mid-Field and Far-Fields ($p = 0.87$) (Figure 6-7).

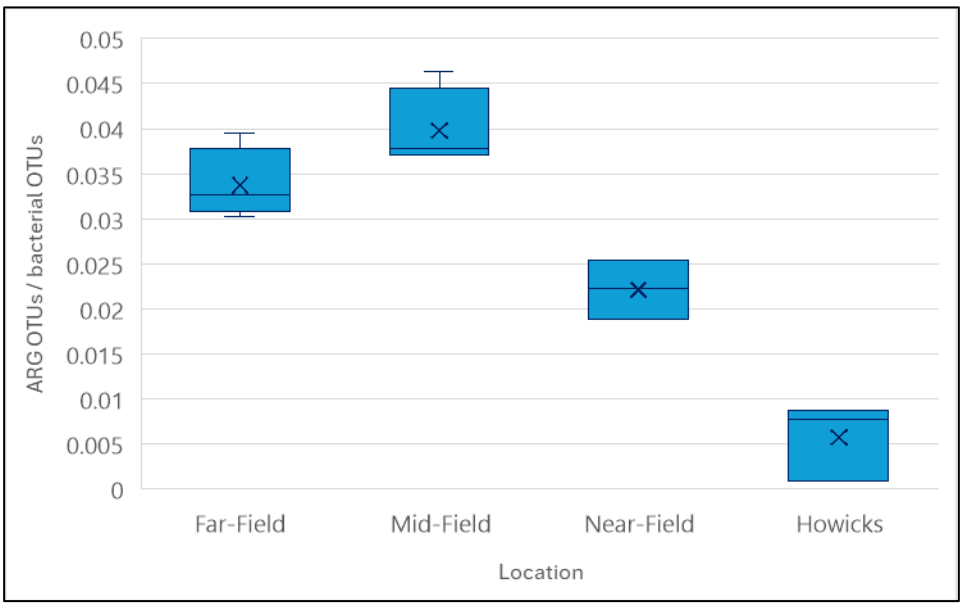


Figure 6-7: An illustration of the ratio of antibiotic resistant genes operational taxonomic units to bacterial operational taxonomic units in sediments at different distance categories (Near-Field, Mid-Field, Far-Field, and Howicks). Data are presented as medians with interquartile ranges and depicted as box and whisker plots.

6.3.3 Dissemination of Antibiotic Resistance from the Cleveland Bay Sewage Treatment Plant

A 10-month study identified 288 distinct ARG OTUs within the treated effluent of the CBSTP. Of these ARGs, 98 were present in the Near-Field and represented 86.7% of ARGs within samples from this distance category (Figure 6-8). A relatively larger number of ARG OTUs were identified in the Mid-Field, Far-Field, and Howicks (130, 134, and 171, respectively), but the ARG OTUs identified in these distance categories were more diverse. This implies that effluent-associated ARG OTUs did not represent a large proportion of the resistome (42.8%, 41.9%, and 41.8%, respectively) (Figure 6-

8). Additionally, a similar distribution was observed in the different distance categories when effluent associated ARG OTUs were classified by antimicrobial class against which resistance is encoded (Figure 6-9). Large proportions of effluent-associated ARG OTUs represented aminoglycosides and MLS (Figure 6-9).

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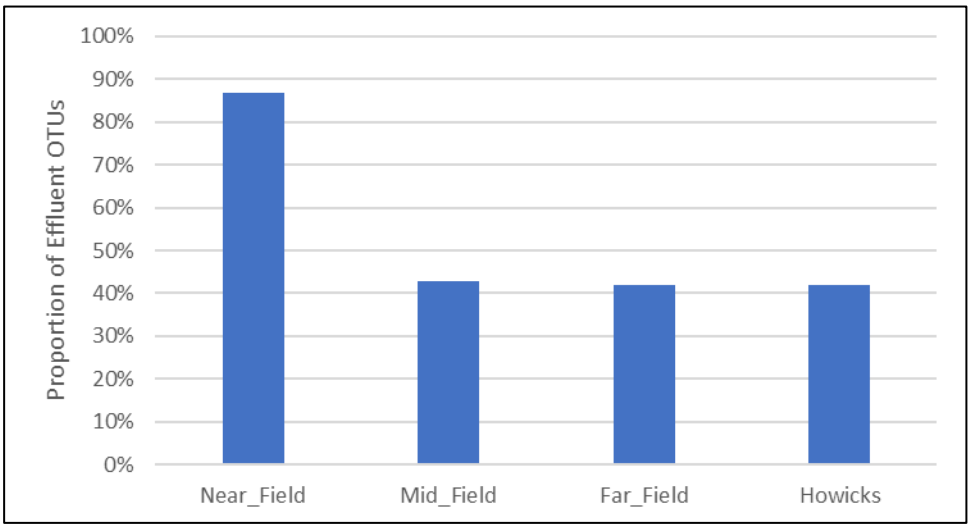


Figure 6-8: Bar graph depicting the number of antibiotic resistant genes operational taxonomic units defined in the Cleveland Bay Sewage Treatment Plant effluent and their presence in the environmental samples (sediment, sweater and seagrass inclusive) of each distance category (Near-Field, Mid-Field, Far-Field, and Howicks).

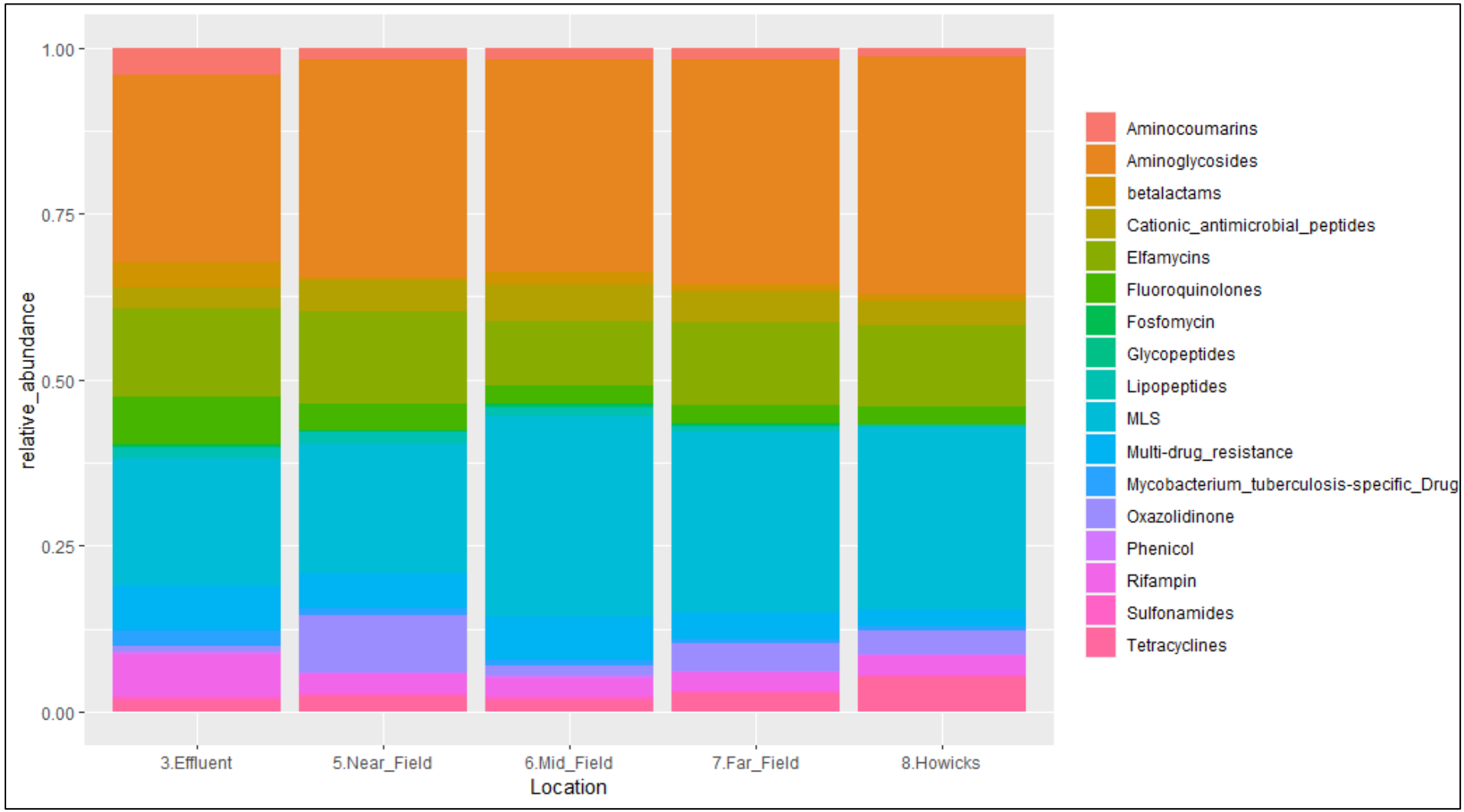


Figure 6-9: Bar graph depicting the relative abundance of Cleveland Bay Sewage Treatment Plant effluent associated antibiotic resistant operational taxonomic units identified in each distance category (Near-Field, Mid-Field, Far-Field, and Howicks) as stratified by antibiotic class for which the antibiotic resistant genes encode resistance.

Whilst a large proportion of the resistome within the Near-Field was associated with ARG OTUs found in the CBSTP effluent, this observation did not extend to the microbiome and only 2 bacterial OTUs were observed to overlap in the core microbiome of the effluent and the Near-filed. These were identified as *Candidatus-Vampirococcus* uncultured bacterium (OTU #77133) (Figure 6-10 and Figure 6-11 - Example A) and *Pseudomonas aeruginosa* (OTU #287) (Figure 6-10 and Figure 6-11 - Example B). The core microbiome of the effluent and Near-Field are described in the x-axis of Figure 6-10 and Figure 6-11, respectively.

A correlation analysis was conducted of the effluent resistome compared to the core microbiome of the effluent to assess the bacterial strains associated with each ARG OTU. This identified that *Candidatus-Vampirococcus* uncultured bacterium (OTU #77133) was positively correlated with 22 ARG OTUs (Figure 6-10 – Example A), and *Pseudomonas aeruginosa* (OTU #287) was also positively correlated with 22 ARG OTUs in the effluent (Figure 6-10 – Example B).

Conversely, a correlation analysis was conducted of the 98 effluent-associated ARGs found to be present in the Near-Field compared to the core microbiome of the Near-Field to assess the bacterial strains associated with each of the effluent-associated ARG OTUs. Of the 98 effluent-associated ARGs in the Near-Field, 68 were associated with 22 core bacteria, including *Candidatus-Vampirococcus* uncultured bacterium (OTU #77133) and *Pseudomonas aeruginosa* (OTU #287). In the Near-Field *Candidatus-Vampirococcus* uncultured bacterium (OTU #77133) was positively correlated with 4 ARG OTUs (Figure 6-11 – Example A), and *Pseudomonas aeruginosa* (OTU #287) was positively correlated with 3 ARG OTUs (Figure 6-11 – Example B).

Two of the effluent ARGs that correlated with *Candidatus-Vampirococcus* uncultured bacterium (OTU #77133) in the Near-Field were also positively correlated with it in the effluent. These were 3206: GYRA and 4276: OLEC, whilst 3188: GYRA and 4290: OMPR were not (Figure 6-11 – Example A). These two remaining ARGs (3188: GYRA and 4290: OMPR) were previously (i.e. in the effluent) positively correlated with the Proteobacteria species, including *Sphingopyxis terrae, Sphingopyxis sp. QXT-31, Sphingopyxis lindanitolerans, Sphingopyxis macrogoltabida*, *Sphingopyxis fribergensis* and, *Alicycliphilus denitrificans and Variovorax paradoxus,* respectively. Additionally, all three effluent-associated ARG OTUs positively correlated with *Pseudomonas aeruginosa* (OTU #287) in the Near-Field (Figure 6-11 – Example B) and were also positively correlated with *Pseudomonas aeruginosa* (OTU #287) in the effluent samples (Figure 6-10 – Example B).

Of the 22 core Near-Field bacteria that correlated with effluent-associated ARGs, four bacteria (*Marinobacter sp.LQ44* [OTU #1749259], *Marinobacter sp.LV10R510.11A* [OTU #1415568], *Marinobacter sp.Arc7.DN.1* [OTU #2304594]*, Marinobacter sp.es.042* [OTU #1671721]) were only

negatively correlated with the effluent ARGs (namely 6144:RPSL, and 7302:TUFAB) (Figure 6-11). The remaining 18 core Near-Field bacteria were all observed to be positively correlated with ARGs associated with the CBSTP effluent (Figure 6-11).

A differential expression analysis was utilised to exhibit significant differences in the proportion of bacteria identified in the Near-Field and the Howicks. This differential expression analysis showed that 15 of the bacteria positively correlated with effluent ARGs were also observed in significantly higher proportions (via a Wilcox rank sum test) in the Near-Field than in the Howicks ($p = 0.005430801$).

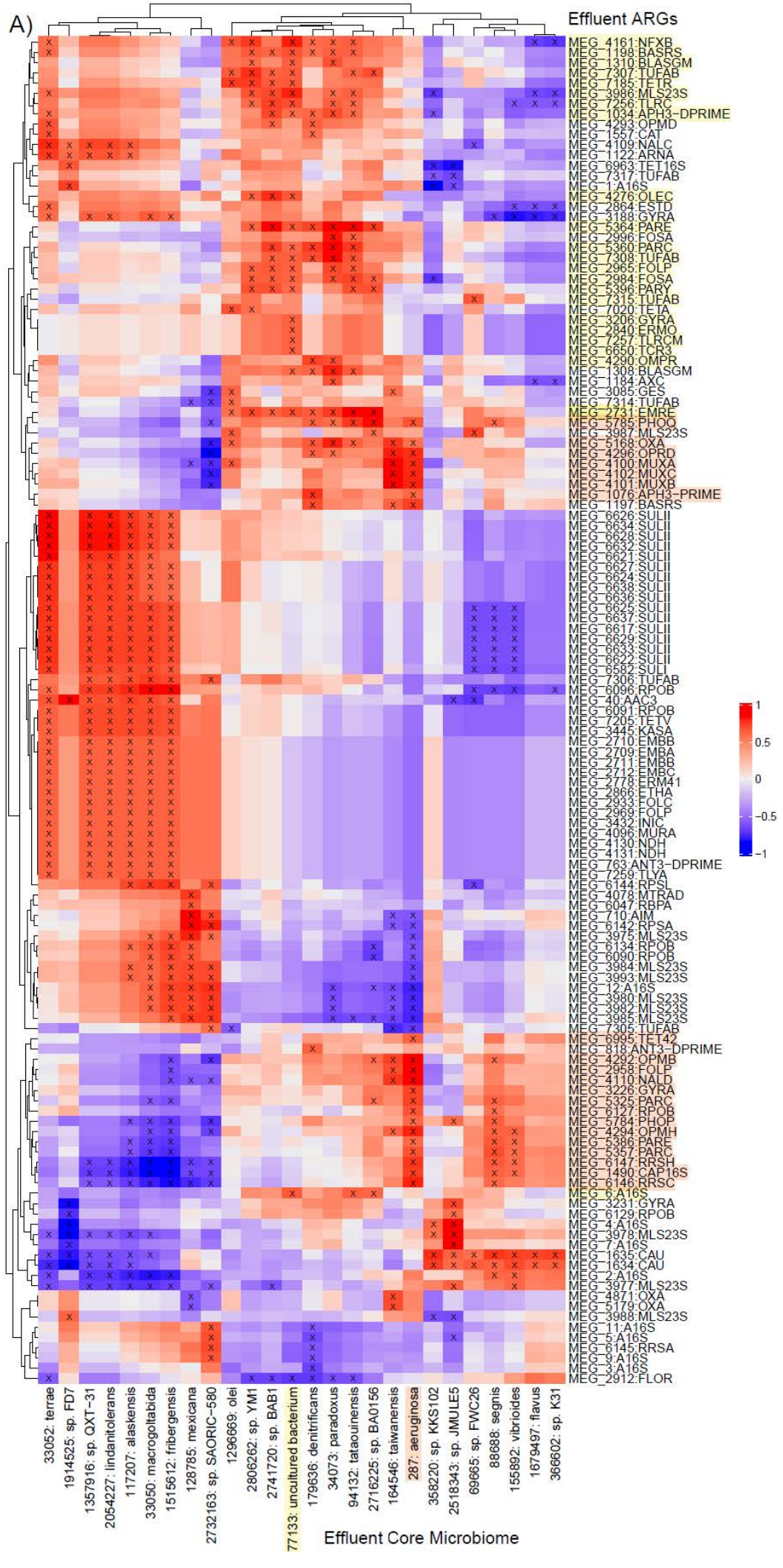

A)
Effluent ARGs
MEG_4161:NFXB
MEG_1198:BASRS
MEG_1310:BLASGM
MEG_7307:TUFAB
MEG_7185:TETR
MEG_3986:MLS23S
MEG_7256:TLRC
MEG_1034:APH3−DPRIME
MEG_4293:OPMD
MEG_1557:CAT
MEG_4109:NALC
MEG_1122:ARNA
MEG_6963:TET16S
MEG_7317:TUFAB
MEG_1:A16S
MEG_4276:OLEC
MEG_2864:ESTD
MEG_3188:GYRA
MEG_5364:PARE
MEG_2996:FOSA
MEG_5360:PARC
MEG_7308:TUFAB
MEG_2965:FOLP
MEG_2984:FOSA
MEG_5396:PARY
MEG_7315:TUFAB
MEG_7020:TETA
MEG_3206:GYRA
MEG_2840:ERMO
MEG_7257:TLRCM
MEG_6650:TCR3
MEG_4290:OMPR
MEG_1308:BLASGM
MEG_1184:AXC
MEG_3085:GES
MEG_7314:TUFAB
MEG_2731:EMRE
MEG_5785:PHOQ
MEG_3987:MLS23S
MEG_5168:OXA
MEG_4296:OPRD
MEG_4100:MUXA
MEG_4102:MUXC
MEG_4101:MUXB
MEG_1076:APH3−PRIME
MEG_1197:BASRS
MEG_6626:SULII
MEG_6634:SULII
MEG_6628:SULII
MEG_6632:SULII
MEG_6621:SULII
MEG_6627:SULII
MEG_6624:SULII
MEG_6638:SULII
MEG_6636:SULII
MEG_6625:SULII
MEG_6637:SULII
MEG_6617:SULII
MEG_6629:SULII
MEG_6633:SULII
MEG_6622:SULII
MEG_6582:SULI
MEG_7306:TUFAB
MEG_6096:RPOB
MEG_40:AAC3
MEG_6091:RPOB
MEG_7205:TETV
MEG_3445:KASA
MEG_2710:EMBB
MEG_2709:EMBA
MEG_2711:EMBB
MEG_2712:EMBC
MEG_2778:ERM41
MEG_2866:ETHA
MEG_2933:FOLC
MEG_2969:FOLP
MEG_3432:INIC
MEG_4096:MURA
MEG_4130:NDH
MEG_4131:NDH
MEG_763:ANT3−DPRIME
MEG_7259:TLYA
MEG_6144:RPSL
MEG_4078:MTRAD
MEG_6047:RBPA
MEG_710:AIM
MEG_6142:RPSA
MEG_3975:MLS23S
MEG_6134:RPOB
MEG_6090:RPOB
MEG_3984:MLS23S
MEG_3993:MLS23S
MEG_12:A16S
MEG_3980:MLS23S
MEG_3982:MLS23S
MEG_3985:MLS23S
MEG_7305:TUFAB
MEG_6995:TET42
MEG_818:ANT3−DPRIME
MEG_4292:OPMB
MEG_2958:FOLP
MEG_4110:NALD
MEG_3226:GYRA
MEG_5325:PARC
MEG_6127:RPOB
MEG_5784:PHOP
MEG_4294:OPMH
MEG_5386:PARE
MEG_5357:PARC
MEG_6147:RRSH
MEG_1490:CAP16S
MEG_6146:RRSC
MEG_6:A16S
MEG_3231:GYRA
MEG_6129:RPOB
MEG_4:A16S
MEG_3978:MLS23S
MEG_7:A16S
MEG_1635:CAU
MEG_1634:CAU
MEG_2:A16S
MEG_3977:MLS23S
MEG_4871:OXA
MEG_5179:OXA
MEG_3988:MLS23S
MEG_11:A16S
MEG_5:A16S
MEG_6145:RRSA
MEG_9:A16S
MEG_3:A16S
MEG_2912:FLOR
1
0.5
0
−0.5
−1
33052: terrae
1914525: sp. FD7
1357916: sp. QXT−31
2054227: lindanitolerans
117207: alaskensis
33050: macrogoltabida
1515612: fribergensis
128785: mexicana
2732163: sp. SAORIC−580
1296669: olei
2806262: sp. YM1
2741720: sp. BAB1
77133: uncultured bacterium
179636: denitrificans
34073: paradoxus
94132: tataouinensis
2716225: sp. BA0156
164546: taiwanensis
287: aeruginosa
358220: sp. KKS102
2518343: sp. JMULE5
69665: sp. FWC26
88688: segnis
155892: vibrioides
1679497: flavus
366602: sp. K31
Effluent Core Microbiome

Figure 6-10: Heatmap describing the correlations between effluent Antibiotic resistant genes operational taxonomic units and effluent core bacterial operational taxonomic units. Where red colours annotate positive correlations, blue colours annotate negative correlations (colour depth varies based on the strength of the correlation), and ‘x’ annotates correlations found to be statistically significant. Example 1 is indicated in yellow, and Example 2 is indicated in orange.

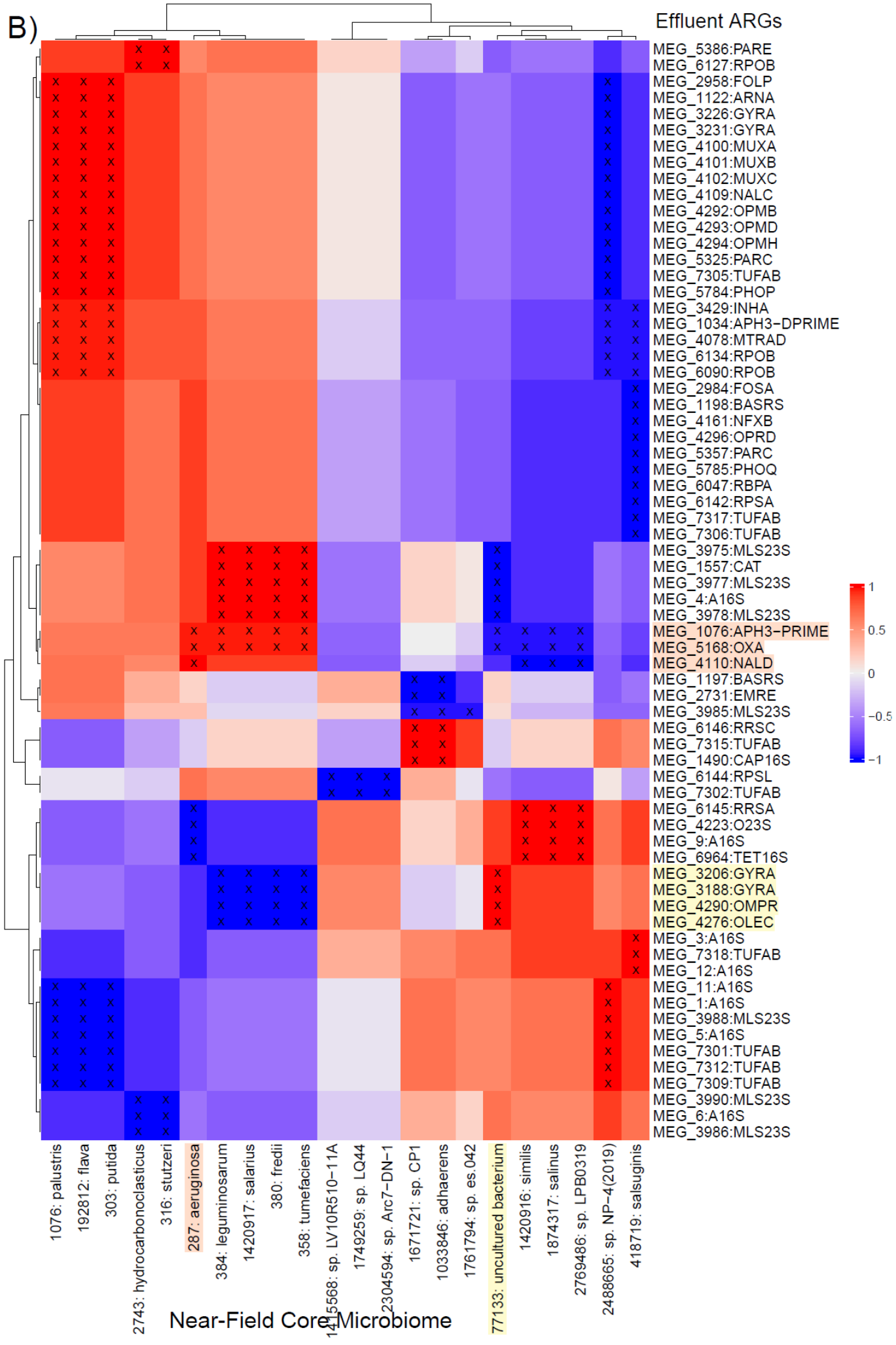

B)
Effluent ARGs
MEG_5386:PARE
MEG_6127:RPOB
MEG_2958:FOLP
MEG_1122:ARNA
MEG_3226:GYRA
MEG_3231:GYRA
MEG_4100:MUXA
MEG_4101:MUXB
MEG_4102:MUXC
MEG_4109:NALC
MEG_4292:OPMB
MEG_4293:OPMD
MEG_4294:OPMH
MEG_5325:PARC
MEG_7305:TUFAB
MEG_5784:PHOP
MEG_3429:INHA
MEG_1034:APH3−DPRIME
MEG_4078:MTRAD
MEG_6134:RPOB
MEG_6090:RPOB
MEG_2984:FOSA
MEG_1198:BASRS
MEG_4161:NFXB
MEG_4296:OPRD
MEG_5357:PARC
MEG_5785:PHOQ
MEG_6047:RBPA
MEG_6142:RPSA
MEG_7317:TUFAB
MEG_7306:TUFAB
MEG_3975:MLS23S
MEG_1557:CAT
MEG_3977:MLS23S
MEG_4:A16S
MEG_3978:MLS23S
MEG_1076:APH3−PRIME
MEG_5168:OXA
MEG_4110:NALD
MEG_1197:BASRS
MEG_2731:EMRE
MEG_3985:MLS23S
MEG_6146:RRSC
MEG_7315:TUFAB
MEG_1490:CAP16S
MEG_6144:RPSL
MEG_7302:TUFAB
MEG_6145:RRSA
MEG_4223:O23S
MEG_9:A16S
MEG_6964:TET16S
MEG_3206:GYRA
MEG_3188:GYRA
MEG_4290:OMPR
MEG_4276:OLEC
MEG_3:A16S
MEG_7318:TUFAB
MEG_12:A16S
MEG_11:A16S
MEG_1:A16S
MEG_3988:MLS23S
MEG_5:A16S
MEG_7301:TUFAB
MEG_7312:TUFAB
MEG_7309:TUFAB
MEG_3990:MLS23S
MEG_6:A16S
MEG_3986:MLS23S
1
0.5
0
−0.5
−1
1076: palustris
192812: flava
303: putida
2743: hydrocarbonoclasticus
316: stutzeri
287: aeruginosa
384: leguminosarum
1420917: salarius
380: fredii
358: tumefaciens
1415568: sp. LV10R510−11A
1749259: sp. LQ44
2304594: sp. Arc7−DN−1
1671721: sp. CP1
1033846: adhaerens
1761794: sp. es.042
77133: uncultured bacterium
1420916: similis
1874317: salinus
2769486: sp. LPB0319
2488665: sp. NP−4(2019)
418719: salsuginis
Near-Field Core Microbiome

Figure 6-11: Heatmap describing the correlations between effluent antibiotic resistant genes operational taxonomic units and Near-Field core bacterial operational taxonomic units. Where red colours annotate positive correlations and blue colours annotate negative correlations (colour depth varies based on the strength of the correlation) and 'x' annotates correlations found to be statistically significant. Example 1 is indicated in yellow, and Example 2 is indicated in orange.

6.3.4 Dissemination of Antibiotics from the Cleveland Bay Sewage Treatment Plant

Trimethoprim and ciprofloxacin were only observed at detectable levels in the effluent and were found to be diluted upon dissemination to the receiving environment below the laboratory limits of reporting (LOR) (Table 6-1 and Table 6-2). Norfloxacin was not observed in concentrations above the laboratory LOR in sediment, seawater, or CBSTP effluent (Table 6-1 and Table 6-2). Azithromycin was found to have high concentrations in the sediment of the Near-Field immediately outside the CBSTP and detectable concentrations in the effluent, Mid-Field, and Far-Field (Table 6-1 and Table 6-2). Concentrations were measured above PNEC (0.25 µg/L) and MIC (4 µg/L) in only the Near (25 µg/kg) and Mid-Field (0.9 µg/kg) sediments (Table 6-1 and Table 6-2). However, azithromycin was not observed at detectable concentrations in any of the tested seawater samples. Clarithromycin was observed above detectable levels in the effluent, as well as the sediment of both the Near-Field (0.47 µg/kg) and the Howicks (0.17 µg/kg) (Table 6-1 and Table 6-2). However, clarithromycin was not detected above PNEC (0.25 µg/L) and MIC (2 µg/L) within Howicks and was not measured above the limit of reporting in any seawater samples (Table 6-1 and Table 6-2). Sulfamethoxazole was found in concentrations above detectable limits (though below that of calculated PNEC = 16 µg/L) in the seawater of the Mid-Field but was not detected in any other environmental samples (Table 6-1 and Table 6-2).

Table 6-1: Concentrations of antibiotic compounds identified in liquid sample types (effluent and seawater).

Antibiotic	PNEC [1]	MIC [1]	LOR	Effluent Duplicates		Mid-Field Seawater	Far-Field Seawater
azithromycin	0.25	4	< 0.0025	0.15	0.15	< 0.0025	< 0.0025
ciprofloxacin	0.064	1	< 0.0025	0.008	0.0093	< 0.0025	< 0.0025
clarithromycin	0.25	2	< 0.0025	0.0092	0.0094	< 0.0025	< 0.0025
norfloxacin	0.5	4	< 0.0025	<0.0025	<0.0025	< 0.0025	< 0.0025
sulfamethoxazole	16	125	< 0.0025	0.25	0.31	0.0087	< 0.0025
trimethoprim	0.5	8	< 0.0025	0.05	0.051	< 0.0025	< 0.0025

[1] Denotes parameters referenced from Bengtsson-Palm and Larsson, 2016.

Note: All values shown are in µg/L.

Table 6-2: Concentrations of antibiotic compounds identified in sediment samples.

Antibiotic	PNEC [1]	MIC [1]	LOR	Near Field	Mid-Field	Far-Field	Howicks
azithromycin	0.25	4	0.05	27	0.9	0.2	0.2
ciprofloxacin	0.064	1	1	< 1	< 1	< 1	< 1
clarithromycin	0.25	2	0.05	0.47	< 0.05	< 0.05	0.17
norfloxacin	0.5	4	1	< 1	< 1	< 1	< 1
sulfamethoxazole	16	125	0.05	< 0.05	< 0.05	< 0.05	< 0.05
trimethoprim	0.5	8	0.05	< 0.05	< 0.05	< 0.05	< 0.05

[1] Denotes parameters referenced from Bengtsson-Palm and Larsson, 2016.

Note: All values shown are in µg/kg.

A correlation analysis was conducted for the concentrations of azithromycin and clarithromycin detected in sediments throughout the different distance categories compared to the sediment core microbiome of the different distance categories to ascertain perspective inhibitory effects. Of the 142 shared core bacteria of the Near, Mid, and Far-Field sediment, 32 were significantly positively correlated with the concentrations of clarithromycin, and no core bacteria were observed to be significantly positively correlated with azithromycin. Conversely, A single core bacteria (150340: *antiquarius*) was observed to be significantly negatively correlated with clarithromycin, whilst 107 bacterial OTUs were significantly negatively correlated with the concentrations of azithromycin (Table 6-3).

Differential expression analysis was utilised to exhibit significant differences in the proportion of bacteria identified in different distance categories of Cleveland Bay and the Howicks. Through differential expression analyses, three (all genus *marinobacter*) of the 32 OTUs that were significantly positively correlated with the concentrations of clarithromycin were found to be in significantly lower proportion in the Howicks compared to the Near-Field. These were also all found to be in significantly higher proportions in the Near field than in the Mid-Field and Far-Field. Additional differential expression analyses showed 25 of the 107 OTUs that significantly negatively correlated with the concentrations of azithromycin were found to be in significantly higher proportion in the Howicks compared to the Near-Field.

Table 6-3: Correlations of concentrations of clarithromycin and azithromycin to core sediment bacteria, where negative correlation may indicate susceptibility to the antibiotic and positive correlation may indicate increased proliferation of bacterial species in conjunction with the antibiotic due to out-competition of susceptible species.

Sediment Microbiome OTUs	*Clarithromycin*	*Azithromycin*
1038856: plakortidis	+	
1761794: sp. es.042	+	
2488665: sp. NP−4(2019)	+	
1671721: sp. CP1	+	
1033846: adhaerens	+	
1620421: sp. IMCC20628	+	
244596: phototrophica	+	
182270: coralicida	+	
651183: manganoxydans	+	
279359: echinicola	+	
411153: forsetii	+	
1250205: sp. MAR_2010_147	+	
1913577: salexigens	+	
2126553: fulva	+	
1486245: flava	+	
143223: salegens	+	
1729720: sp. T436	+	
2058175: flavum	+	
2585771: arcticum	+	
398743: profunda	+	
1871037: Flavobacteriaceae bacterium	+	
1336796: sp. Hel1_33_143	+	
2719912: sp. ZYFB001	+	
1176255: dechangensis	+	
2562285: sp. ALE3EI	+	
2769486: sp. LPB0319	+	
2304594: sp. Arc7−DN−1	+	
1420916: similis	+	
1874317: salinus	+	
1420917: salarius	+	
358: tumefaciens	+	
384: leguminosarum	+	-
2292766: caeni		-
1897729: aestuarii		-
418719: salsuginis		-
1402135: pseudonitzschiae		-
2742145: thermophila		-
2304600: sp. L−A4		-
192812: flava		-
2170729: sp. wino2		-
1384459: caenitepidi		-
79883: horikoshii		-
2779776: sp. HD4P25		-
255247: arsenicus		-
28108: macleodii		-
2789776: tropica		-
116092: sp. A25		-

359303: loihica	-
1190813: shioyasakiensis	-
48076: georgiense	-
303: putida	-
1548547: oceani	-
40324: maltophilia	-
696485: owensii	-
190895: rotiferianus	-
669: harveyi	-
691: natriegens	-
689: mediterranei	-
170679: chagasii	-
1295392: gaetbulicola	-
511062: sp. GK1	-
1913989: Gammaproteobacteria bacterium	-
1076: palustris	-
60890: gallaeciensis	-
379347: mobile	-
74318: maris	-
34073: paradoxus	-
56: cellulosum	-
927083: amylolyticus	-
80816: ochraceum	-
2569540: sp. IMCC35004	-
54571: venezuelae	-
1883427: Actinobacteria bacterium	-
467094: coccineus	-
2527978: panareensis	-
2605989: chilikensis	-
2608982: benthica	-
77133: uncultured bacterium	-
1660251: Acidobacteria bacterium Mor1	-
2478486: Holophagales bacterium	-
170673: kanaloae	-
43660: undina	-
663: alginolyticus	-
670: parahaemolyticus	-
1792508: Yangia sp. CCB−MM3	-
294: fluorescens	-
316: stutzeri	-
540747: indicus	-
2605946: ceae bacterium SH−1	-
89184: pomeroyi	-
1063: sphaeroides	-
42444: algicola	-
53501: gelatinovorus	-
2785907: sp. A30−3	-
2739433: mangrovi	-
2182384: aureus	-
1904441: ceae bacterium	-
44012: balearica	-
287: aeruginosa	-

2745199: sp. YPW1		-
2681547: sp. SH−1		-
1516059: sp. ALW1		-
2587856: sp. THAF38		-
359370: sp. A4B17		-
2591606: sp. GL−2		-
1769779: aggregans		-
48074: hydrolyticus		-
252514: thermotolerans		-
260552: agarilyticus		-
47960: halophila		-
680: campbellii		-
50719: diabolicus		-
2751178: sp. B1FLJ16		-
2021862: sp. VG12		-
1229727: profundus		-
311180: pacificus		-
2587853: sp. THAF33		-
2293862: sp. AD91A		-
1396826: aquaemixtae		-
133924: methylohalidivorans		-
1549850: electrotropha		-
561184: alba		-
1826607: algicola		-
2009329: sp. P11		-
1250539: abyssi		-
2782568: sp. 6D36		-
2502843: sp. HKB08		-
2587851: sp. THAF29		-
2011159: sp. KY5		-
39960: litoralis		-
266951: seohaensis		-
2604537: sp. BO−6		-
476157: ishigakiensis		-
361183: epoxidivorans		-
2795526: sp. V3		-
218284: vietnamensis		-
129985: jeotgali		-
2806989: sp. cB07		-
221822: inhibens		-
150340: antiquarius	-	+

+ Denotes statistically significantly positive correlation

- Denotes statistically significantly negative correlation

Note: Data utilised included microbiome and antibiotic concentration data from all sediment locations. Core sediment bacteria not significantly correlated with the two antibiotic concentrations are not shown.

A further correlation analysis was conducted for the concentrations of azithromycin and clarithromycin detected in sediments throughout the different distance categories compared to the sediment resistome of the different distance categories to ascertain perspective resistance gene acquisition. Correlation analysis between concentrations of azithromycin and ARGs identified in the sediment of the Near, Mid, and Far-Field show 13 OTUs (that encode for 10 separate ARGs: A16S, BLAECM, GYRA, MLS23S, O23S, OLEC, OMPR, P16S, PH23S, and RBPA) were significantly positively correlated to azithromycin. Three of these ARGs (MLS23S, OLEC, and O23S) encode for resistance to MLS class of antibiotics in which azithromycin falls. Correlation analysis between concentrations of clarithromycin and ARGs identified in the sediment of the Near, Mid, and Far-Field show 7 OTUs (that encode for 4 separate ARGs: A16S, PH23S, RRSA, and TET16S) were significantly positively correlated to clarithromycin. None of these encoded for resistance to MLS class of antibiotics in which clarithromycin also falls.

6.4 Discussion

Antibiotic resistance is both a natural phenomenon (Larsson and Flach, 2022) and an indicator of anthropogenic pollution (Yang et al., 2021, Al-Bahry et al., 2009b). Antimicrobial compounds are widespread throughout the globe due to, in small part, the natural occurrence of these genes, though primarily due to the use of antibiotics in public health, agriculture, and veterinary science (Tan et al., 2018, Yang et al., 2021). For the purpose of this study, the Howick Group of Islands was utilised as an area potentially containing a natural background antibiotic resistance profile available within a comparable ecosystem to Cleveland Bay. This site had minimal direct anthropogenic activities, and specific resistance identified was likely to reflect the extent of pollution in untouched global locations, as described previously (Tan et al., 2018, Yang et al., 2021). In many cases, it is impossible to differentiate between the natural and background occurrences caused by anthropogenic impact. However, certain synthetic antibiotics do not have known equivalent natural components and can be used as indicators for anthropogenic pollution. The presence of this widespread background pollution is further supported by the detection of two semi-synthetic antibiotics (azithromycin and clarithromycin) within the sediment of this site. As derivatives of the natural antibiotic compound erythromycin, azithromycin and clarithromycin are synthesized for use in public health or veterinary medicine and do not occur naturally (Alvarez-Elcoro and Enzler, 1999). The detection of azithromycin and clarithromycin within the Howick Group of Islands suggests that anthropogenic pollution by antibiotics may penetrate various regions of the

Great Barrier Reef environment. However, whilst the MIC and PNEC for pharmaceutical compounds vary based on the microorganism tested, effective concentrations of these antibiotics in environmental samples have been previously calculated based on the European Committee on Antimicrobial Susceptibility Testing scale, with azithromycin having a MIC of 4 µg/L and a PNEC of 0.25 µg/L, and clarithromycin having a MIC of 2 µg/L and a PNEC of 0.25 µg/L (Bengtsson-Palme and Larsson, 2016). As azithromycin and clarithromycin were measured in the Howicks sediment at concentrations of 0.2 µg/kg and 0.17 µg/kg, respectively, these concentrations are below that of the calculated PNEC described previously (Bengtsson-Palme and Larsson, 2016), and as such, are not expected to have created an effect on the microbiome of the Howick Group of Islands.

The core microbiome observed in the Howick Group of Islands was distinctly diverse for all sampled matrices (sediment, seagrass, and seawater) with a richer and more unique core microbiome identified in these sample types when compared to their Cleveland Bay counterparts. As mentioned previously, increased microbial diversity within environmental samples has been previously linked with the health of ecosystems, with increased ecological stressors linked to decreases in the diversity of microbiomes (Rocca et al., 2019), and the stability of an environmental ecosystem is also linked to the diversity of its core microbiome (Björk et al., 2018). Although these conclusions are sporadically contended in the literature (Nogales et al., 2011). Additionally, minimal literature exists on resistomes in marine environments, with some previous studies identifying that aquatic resistomes become constant with increased anthropogenic disturbances (Corno et al., 2019), and that high resistome diversity may be linked to areas of highly stressed ecosystems (Di Cesare et al., 2023). The Howicks ecosystem may be less stressed in comparison to Cleveland Bay, due to incidences of low ARG to bacteria ratios identified in the Howicks in conjunction with its diverse core microbiome and low diversity in core resistome, in comparison to Cleveland Bay. This lends credence to the hypothesis that whilst anthropogenic linked antibiotics were present in small amounts, the microbiome and resistome of the Howick Group of Islands were generally reflective of a less stressed ecosystem than that of Cleveland Bay. However, it must be acknowledged that different ecosystems may inhabit different benthic zones of the Near, Mid, and Far-Fields, which may impact their functional microbiome and, this may not be represented in the in-shore reef samples of the Howick Group of Islands that were considered for the current study.

The Mid-Field and Far-Field distance categories showed numbers of effluent-associated ARGs comparable to those of the Howicks. Conversely, the Near-Field was represented by predominantly effluent-associated ARGs (86.7%). This is suggests that ARGs from the treated CBSTP effluent are disseminated into the Cleveland Bay Near-Field, and indicates that the most likely influence of the resistome in the Near-Field samples was the CBSTP. Whilst this dominance of

effluent-associated ARGs is observed for the Near-Field resistome, a similar observation was not made for the core microbiome, with only two bacterial OTUs observed in the core microbiome of both the effluent and the Near-field. These two bacterial strains retained in both the core microbiome of the CBSTP effluent, and the Near-Field were found to be correlated with a number of CBSTP ARGs in both locations. This suggests that small amounts of these effluent-associated ARGs identified in the Near-Field may be disseminated within some ARBs. Nevertheless, the question remains of how the majority of CBSTP ARGs may have proliferated in the Near-Field without effluent-associated bacteria. This is likely to be attributed to horizontal gene transfer and mobile genetic elements.

Bacterial DNA containing ARGs can exist as environmental DNA (eDNA) or within bacterial cells. ARGs contained within bacterial cells can be shared to new unrelated bacterial lineages or acquired by new bacterial cell from eDNA within their environment through horizontal gene transfer methods (e.g. transformation, transduction, and conjugation). The horizontal gene transfer of resistance genes is often mediated by mobile genetic elements, with the most significant mobile genetic elements for the horizontal gene transfer of resistance genes being plasmids and integrons (Leungtongkam et al., 2018). Previous literature has described the prominence of mobile genetic elements and ARGs within forms of eDNA in wastewater effluents (Zhou et al., 2019, Drane et al., 2024b, Qin et al., 2020). This is of relevance for the CBSTP, which utilises 0.4 µm ultrafiltration membranes (reducing to 0.1 with membrane fouling) as its primary form of tertiary treatment. Whilst this form of tertiary treatment has been previously described as the most capable of ARG removal, it does not have a perfect removal capacity (Drane et al., 2024b). As such, mobile genetic elements harbouring ARGs have been documented in ultra-filtration wastewater effluent previously (Drane et al., 2024b). Studies of other ecosystems show these mobile genetic elements harbouring ARGs can disseminate into the receiving environment and facilitate resistance to bacteria that did not originate from the WWTP via horizontal gene transfer (Proia et al., 2016, Milaković et al., 2019, Milaković et al., 2020, Zhang et al., 2021b). This is reflected in this current marine study, where the presence of effluent-associated ARGs in the Near-Field was significantly correlated with the presence of functional core bacteria within the Near-Field. Of the 98 effluent-associated ARGs identified to be released into the Near-Field, 68 were associated with 22 Near-Field core bacteria. This is despite only two core bacteria having been identified in the core microbiome of the effluent and the Near-Field. This, in conjunction with the high incidence of effluent-associated ARGs identified in the Near-Field compared with other distance categories, suggests that it is possible that these ARGs were transferred to core bacteria of the Near-Field upon dissemination into Cleveland Bay.

Incidences of possible over-stimulation of some core bacteria, which were correlated with effluent-associated ARGs within the Near-Field were identified. A large portion of the Near-Field microbiome (15 of 22 bacterial OTUs) was observed to be in significantly higher proportions in the Near-Field than their proportion in the Howick Group of Islands; these bacterial lineages were also positively correlated with effluent-associated ARGs. The overstimulation of these bacterial lineages in proportions higher than that identified in the reference area of the Howicks suggests that the release of these effluent-associated ARGs and their possible acquisition in the Near-Field of Cleveland Bay may have functional effects on necessary environmental processes facilitated by these bacteria and may also have implications of out-competition of other functionally important bacteria within the ecosystem.

Examples of possible out-competition were detected through correlation analysis of effluent-associated ARGs and the Near-Field Microbiome, where four of the core Near-Field bacteria were solely negatively correlated with two effluent-associated ARGs (namely, RPSL for aminoglycoside resistance and TUFAB for resistance to elfamycins). These correlations indicate instances of potentially out-competing functionally important bacteria in the Near-Field, by bacteria that may harbour RPSL and TUFAB. The bacteria identified to be negatively correlated with these specific ARG OTUs of RPSL and TUFAB were of the *Marinobacter* genus. *Marinobacter* species have been identified as providing necessary functions within marine ecosystems (Handley and Lloyd, 2013), such as their ability to biodegrade hydrocarbons (Raddadi et al., 2017), and proposed functions in algal bloom control through their production of bioavailable forms of iron and interactions with phytoplankton (Yarimizu et al., 2018). This example is suggestive of further changes to the core microbiome diversity, with the potential to facilitate out-competition of functionally important bacteria through the increased survival outcomes of bacteria harbouring these effluent-associated. Similar instances to this have been described previously, such as the correlation of ARGs to possible host genera within agricultural wastes, some of which were found to have implications for public health in the form of antibiotic resistant pathogens and water eutrophication processes (Yuan et al., 2018, Wang et al., 2022, Zhao et al., 2023, Shi et al., 2013, Novo et al., 2013, Tao et al., 2016, Jia et al., 2017). However, the reliance of this analysis on correlation-based statistics provides latitude for alternative explanations for the cause of these microbiome shifts.

Despite the Near-Field representing the closest location to the CBSTP, many other potential sources of pollution exist within the Cleveland Bay area. Other potential ecosystem stressors to the Mid-Field and Far-Fields include a metal refinery, a busy port, agricultural land and general stormwater run-off. Many such sources may hydrologically discharge via Sandfly Creek, the mouth of which is located within the Mid-Field sampling site. These pollutant sources may influence the

resistome and microbiome observed between Cleveland Bay distance categories. This theory is supported, in part, by the observed relatively low ratios of ARG abundance per bacteria for the Near-Field (0.022 ARGs/bacteria) versus the Mid (0.04 ARGs/bacteria) and Far-Fields (0.034 ARGs/bacteria) in Cleveland Bay, suggesting that the Mid-Field and Far-Field may represent a more stressed ecosystem than that of the Near-Field. Additionally, it was observed for both sediment and seawater matrices that the Mid-Field presented the highest representative core resistome and the lowest diversity of unique core microbiome OTUs. These results suggest that the Mid-Field may be most reflective of a highly stressed ecosystem, aligning with our understanding of where Sandfly Creek intersects the locations adopted in this paper.

Antibiotic compounds tested throughout this study were also observed to have potential effective changes on the Cleveland Bay microbiome. Azithromycin and clarithromycin were primarily observed in sediment samples due to their affinity to sorb to sediments (Hanamoto and Yamamoto-Ikemoto, 2022, Gravesen and Judy, 2020). Despite concentrations of clarithromycin being measured below that of MIC, possible indirect effects in the form of inhibited growth and out-competition were observed within the Near-Field sediments of Cleveland Bay. Core bacteria of Cleveland Bay sediments were positively correlated with the increased concentrations of clarithromycin (0.47µg/L), with only one bacterial species observed to be negatively correlated (*Vibrio antiquarius*). Low resistance of Vibrio species to Clarithromycin has been documented previously in clinical settings (Yu et al., 2023), suggesting that despite Clarithromycin being measured at concentrations below MIC (2ug/L), some restrictive effects of this antibiotic may be effective on *Vibrio antiquarius* at these concentrations. Vibrio species have been identified previously as providing important functions within marine ecosystems, such as contributing to nutrient cycling (Sampaio et al., 2022). Though some species (including *Vibrio antiquarius*) are pathogenic to humans (Turner et al., 2018, Sampaio et al., 2022), the pathogenicity of *Vibrio antiquarius* may also serve a fundamental ecological role in marine environments, such as symbiotic relationships with macroaquatic species (Hasan et al., 2015). These indicative restrictive correlations suggest that concentrations of some antibiotics below MIC yet above PNEC may provide functional changes to core species of marine ecosystem microbiomes. Future investigations and frameworks on inhibitory or effective concentrations of antibiotics and other bactericidal agents should consider these possible effects on environmental microbiomes.

Furthermore, possible over-stimulation of less susceptible bacterial lineages was also postulated, with three *marinobacter* species positively correlated with the concentrations of clarithromycin in the Cleveland Bay sediment. They were also observed in higher proportions in the Near-Field than in the Howick Group of Islands sediments. This is suggestive of the possibility of

clarithromycin at 0.47 µg/L which is below MIC (2 µg/L) yet above PNEC (0.25 µg/L), promoting the proportional growth of functional microbiome species within the Near-Field sediments in Cleveland Bay. Despite *marinobacter* species having been documented previously as providing positive functions to marine sediment ecosystems (Handley and Lloyd, 2013), over-expression of any bacterial species will out-compete with other functional bacteria for available resources. The discharge of macrolide antibiotics has been shown to create bacterial community shifts in river-based receiving environments and also contributed to increased enrichment of ARGs (Milaković et al., 2019). Furthermore, a previous study of macrolide antibiotics in MBR effluents postulated that both azithromycin and clarithromycin should be classified as a moderate risk for ecological impact due to their broad-spectrum abilities and toxicity to varied bacterial genera (Monahan et al., 2023).

The proportion of most of the shared core bacteria of Cleveland Bay sediments were negatively correlated with the concentration of azithromycin observed in sediments throughout Cleveland Bay (27 – 0.2 µg/L). A portion of these negatively correlated core bacteria were in significantly higher proportions in the Howicks sediment microbiome than that of the Near-Field sediment of Cleveland Bay. As this concentration is above the MIC (4 µg/L), it suggests that these functional bacterial species' growth has likely been inhibited in the Near-Field by azithromycin concentrations at 27 µg/L. Additionally, this concentration of azithromycin is likely to have facilitated the increased proportion of two ARGs (OLEC and MLS23S) that encode resistance against the MLS class of antibiotics. An additional 10 ARGs were positively correlated with the concentration of azithromycin in Cleveland Bay sediments, none of which provide resistance to macrolide antibiotics.

Instances of antibiotic correlation to non-specific resistance genes have been documented previously (Wang et al., 2022, Wang et al., 2021) and have been attributed to the co-occurrence of other forms of resistance (other antibiotics, biocides, metals) in conjunction with the long-term interaction of effective agents (including the shifting antibiotics and heavy metals). As both azithromycin and clarithromycin were detected at concentrations above PNEC in the Near-Field sediments, considerations should be made for co-resistance in these core bacteria for other antibiotic compounds and untested biocidal agents. Furthermore, the sub-inhibitory concentration of clarithromycin likely explains the lack of macrolide-related ARGs observed in correlation with its presence. However, an alternative explanation for these results is through the use of correlation-based statistics, this study is unable to define the actual causative link of these microbiome shifts in the sediment of the Near Field. As such, azithromycin, clarithromycin, or neither antibiotic may be the significant functional driver in these community changes.

Nevertheless, these detected microbiome shifts correlated with the dissemination of CBSTP effluent-associated ARGs and antibiotics in the Near-Field may have severe consequences for the function of this bacterial community. Microbial communities play a key role in fundamental ecological processes, such as climate regulation (e.g., biological transformation of greenhouse gasses) and environmental quality regulation (e.g., sewage and waste treatment) (Kraemer et al., 2019). Furthermore, functions such as biological nitrification, denitrification, and anaerobic ammonium oxidation are crucial for environmental quality regulation and can be partially or entirely inhibited by antibiotics, even at very low concentrations (Wang et al., 2020b).

Globally, no limits are currently implemented for antibiotic concentrations or the abundance of ARG disseminated from WWTP. In the last decade, the European Union has set environmental quality standards for contaminants of emerging concern, where both clarithromycin and azithromycin were included (Grenni et al., 2018). Studies have also suggested that ARGs should be considered contaminants of emerging concern in conjunction with antibiotics (Ahmed et al., 2021, Niu et al., 2022). Australia, like many countries in the European Union, have taken strides to reduce antibiotic use and the production of ARGs through the prohibition of some antibiotics in aquaculture and agriculture (Carrique-Mas et al., 2023) and, through the legislation of tertiary treatment of wastewater in the vicinity of the Great Barrier Reef (Waterhouse and Johnson, 2002). In many other countries, however, antibiotics are still widely used as growth promoters in agriculture (Chattopadhyay, 2014). Unfortunately, this is a global problem, and the complete elimination of antibiotic use in public health, veterinary science, and agriculture is unlikely to occur on a worldwide scale (Cabello et al., 2016, Pallecchi et al., 2008). Therefore, pursuits for decreased and ethical antibiotic use in all aspects of One Health must continue with haste worldwide.

In the interim, future strategies should improve monitoring capabilities for antibiotics and antibiotic resistance genes, focusing on quantitative and qualitative data on the abundance of resistance genes in different ecosystems. This will facilitate a better understanding of the interactions between antibiotics and environmental bacteria, which is necessary to develop adequate risk assessments (Grenni et al., 2018, Pauwels and Verstraete, 2006, Drane et al., 2024b). Additionally, whilst WWTPs, as so-called “hot spots”, bear the brunt of criticism globally for antibiotic resistance pollution within waterways, consideration should be given to other sources of resistance dissemination. As evidenced by the Mid and Far-Fields of Cleaveland Bay, the CBSTP effluents are unlikely to have been the sole or primary resistance pollution source within these areas. This has led to further questions on other influences of resistance within this anthropogenically impacted area.

This study highlights the implications of antibiotic resistance on delicate microbiomes, such as those in the Great Barrier Reef, and the harm associated with higher-order ecosystems from disseminated ARGs and antibiotics. As such, future monitoring studies should provide equal scrutiny to WWTP and other sources of antibiotics and antibiotic resistance pollution to make informative conclusions for pollution source control and management.

6.5 Conclusions

This study highlights that ultra-filtration is not entirely effective at removing ARGs and antibiotics from the CBSTP, which are disseminated into Cleveland Bay. This study emphasises the putative impact that ARGs and antibiotics released from the CBSTP may have on the functional microbiome of the Near-Field area of Cleveland Bay. Furthermore, whilst azithromycin was observed at levels above PNEC in the Mid-Field of Cleveland Bay before dilution, the majority of parameters measured throughout this study suggest that antibiotics and antibiotic resistance originating from the CBSTP are likely to have an effective radius between 10 m (Near-Field) to 200 m (Mid-Field) from the effluent source of the WWTP. Whilst the compelling correlations described in this study are yet to be confirmed as causations, this study also highlighted that WWTPs with high ARG removal efficiency may have a profound effect on their receiving environment through changes in the core microbiome and their necessary functions.

. Chapter 7: General Discussions and Concluding Remarks

The three pillars of One Health (Human Health, Animal Health, and Environmental Health) are at risk from the indiscriminate dissemination of ARGs (Ahmad et al., 2022). While human and animal health threats of antibiotic resistance genes (ARGs) are often related to alterations of microbiomes and subsequent dysbiosis, little information exists on ARGs, antibiotic resistant bacteria (ARBs), and antibiotics as environmental hazards relating to selection pressure and the further rise of environmental antibiotic resistance. Wastewater treatment plants (WWTPs) provide an opportunity to monitor and contain antibiotic resistance as many major sources and drivers of resistance tend to converge in urban WWTPs. As such, urban WWTPs are often considered "hotspots" for antibiotics and ARB and have been the subject of much investigation. This project aimed to characterise the antibiotic and antibiotic resistance pollution released from a WWTP situated in proximity to the Great Barrier Reef, the Cleveland Bay Purification Plant (CBSTP), and identify the distribution and microbial effect of these contaminants of emerging concern within the receiving environment of Cleveland Bay.

Previous studies show the bulk of knowledge on the evaluation of the scope and frequency of antibiotic resistance in WWTPs has largely relied on the use of qPCR and limited shotgun metagenomic sequencing (SM-Seq) approaches to identify both the microbiome and resistome. Through comparative techniques, this study identified that recent advances in high-throughput qPCR arrays enabled the quantification of many CBSTP influent ARGs simultaneously. However, the selectivity of primers for qPCR arrays limited the identification of many ARGs, as the breadth of the CBSTP influent resistome encompassed more ARG sequences than could be feasibly tested via qPCR. The use of these qPCR arrays to characterise ARGs has provided selective bias within this scientific field, as is notable in previous publications, with observable reliance on specific ARGs resistant to clinically significant classes of antibiotics. These include ARGs encoding for resistance against beta-lactams (blaCTX-M, blaTEM, and blaSHV), tetracyclines (TETM, TETO, TETQ, and TETW), and sulphonamides (SULI and SULII) (Bueno et al., 2020, Czekalski et al., 2012, Neudorf et al., 2017, Harnisz et al., 2020, Lu et al., 2020). Two of these beta-lactam-resistant genes were analysed in this study using a qPCR array, where blaSHV was not identified in the CBSTP influent, and CTX-M was found to be present. Whilst the emphasis on ARGs in WWTPs that have clinical importance for public health is warranted, the need to characterise ARGs that may have significant environmental health effects must also be emphasised. It is recommended that future studies incorporate techniques that show a broader array of ARGs when establishing WWTP resistomes, such as SM-Seq, as the lack of selectivity shown through the use of SM-Seq methods allowed for a more diverse resistome to be

identified. This is exemplified within Chapter 3, which showed that ARGs characterised by this qPCR array may indicate a resistome overrepresented by beta-lactam resistance. However, when the DNA of the influent was compared to a database of approximately 9,000 known ARG sequences, the influent resistome was established to have a much smaller proportion of beta-lactam resistance compared to other classes of antibiotics, such as MLS and aminoglycoside resistance.

The microbiome data characterised in this current study was consistent with previous work elucidated by others (Lin and Liu, 2023), which indicated that the high-throughput sequencing analysis of the V3-V4 hypervariable region of the 16S rRNA gene or SM-Seq could both effectively be used for bacterial population profiling at phyla and class level in WWTP surveillance studies. However, for more accurate speciation of bacteria in the diverse populations of wastewater and identification of bacterial genes, SM-Seq was the recommended approach. As such, SM-Seq methods were utilised throughout this project, with sequencing providing data necessary for both resistome and microbiome identification. It is, however, postulated that future surveillance strategies at the CBSTP may employ targeted qPCR for significant ARGs identified within this whole resistome assessment, as the use of the qPCR method for the detection of ARGs from wastewater provided the opportunity for high-resolution quantification of the specified sequences.

Through the use of SM-Seq methodology, the CBSTP effluent was found to be in agreement with previous studies (Lin et al., 2021, Nnadozie et al., 2017, Wang et al., 2020b, Zhu et al., 2021), to have decreased in ratio of ARG operational taxonomic units (OTUs) to bacterial OTUs compared to that of the influent. This suggests that the CBSTP as an MBR is effective in reducing the load of ARGs throughout the treatment process. However, ARGs are still present within the effluent samples of the CBSTP, as reported in previous studies on MBR effluents (Lin et al., 2021, Wang et al., 2020b). The resistome of the CBSTP influent was suggested to present as predominantly intracellular ARGS (iARGs) within ARBs and follow trends of the microbiome, with the majority of present ARG OTUs changing in proportion with correlated core bacterial OTUs. Bacteria within the CBSTP were identified to likely have been excluded from the effluent via ultra-filtration membranes; however, the findings of Chapter 5 suggest this process may not be true for ARGs. ARGs identified in biosolids and effluent samples did not follow the same trend as the influent, with many ARGs not correlated with core bacteria, and alluded to the likelihood of ARGs existing as free environmental or extracellular ARGs (eARGs) within the effluent. Furthermore, the general profile of CBSTP ARGs within this study was shown to follow the path of liquid waste primarily. As evidenced by the large proportion of shared core resistome between the influent and effluent and the comparatively low ratio of ARG OTUs to bacterial OTUs within the biosolids when compared to the effluent. This is also evidenced by the high proportion of ARGs exclusive to the core resistome of the biosolids rather

than the influent and effluent, suggesting that these unique core biosolid ARGs are primarily observed in the portion of the microbiome purposefully cultured within the activated sludge treatment process.

Direct effects of antibiotic resistance on microbial diversity in humans and animals have been well documented, with indications of functional effects on the immune system, metabolism, and other important biological functions. Whilst a similar level of functional effect has been hypothesised to contribute to the loss of microbial diversity in environmental microbiomes (Cycoń et al., 2019, Grenni et al., 2018), very limited information is available on the functional impact of this dysbiosis within natural ecosystems (Moran, 2015). Insight into the dissemination of antibiotic resistance stressors from WWTPs in aquatic environments have been heavily dominated by comparisons from upstream and downstream ecosystems in river-based receiving environments (Laverman et al., 2015, Li et al., 2011, Proia et al., 2013). However, literature regarding implications in marine coastal receiving environments is limited. This is despite antibiotics and their associated ARGs being defined as indicators of anthropogenic pollution within delicate marine environments (Eckert et al., 2018, Hou et al., 2015, Näslund et al., 2008, Palacios et al., 2021, Tan et al., 2018, Yang et al., 2021). This study is novel in delivering a full metagenomic assessment of the WWTP in question as well as a full assessment of the marine receiving environment, it is also the first instance of characterisation of the microbiome and resistome within this at-risk ecosystem of the Great Barrier Reef.

Characterisation of the resistome of the Cleveland Bay environment nearest the CBSTP was identified as consisting of predominantly ARGs associated with the CBSTP effluent (86.7%), in contrast to the areas further from the CBSTP (42.8 - 41.9%) or the reference environment of the Howicks (41.8%). This suggests that despite other anthropogenic sources of pollution existing in Cleveland Bay (e.g. a metal refinery, a busy port, agricultural land, and general stormwater run-off), the most significant influence on the resistome in the Near-Field samples is likely the CBSTP. This also indicates that CBSTP effluents not only contain ARGs, but that these ARGs are identifiable once reaching the receiving environment. However, this study also revealed that possibly only 32% of ARGs identified within the DNA of influent samples were also found in the transcriptome. Since genes need to be expressed as mRNA to function, this suggests that not all CBSTP-associated ARGs are actively transcribed by bacteria, even though they are present in their genome. Nevertheless, this study shows that some of these ARGs (such as sequences of GYRA, OLEC, APH3-PRIME, OXA, and NALD) were likely contained within ARBs upon dissemination into the Near-Field. This is exemplified by an uncultured species of *Candidatus-Vampirococcus* and *Pseudomonas aeruginosa*, which were shown to be correlated with the same effluent-associated ARGs in the effluent; and the Near-Field

environment. However, as established previously, the majority of ARGs in the CBSTP were likely present as eARGs. Furthermore, the increase in the proportion of some species within the environmental microbiome of the Near-Field, in conjunction with the increase in the CBSTP-associated ARGs, may be a causative factor for functional changes in the core Near-Field microbiome in comparison to that of the Howicks reference area. Such changes may allude to potential dysbiosis of this ecosystem, with changes to the core microbiome diversity and potential increased survival outcomes of bacteria harbouring these CBSTP-associated ARGs. This may facilitate the out-competition of other species and have severe consequences for the function of this bacterial community. Future studies are encouraged to include the use of Hi-C proximity ligation sequencing to identify the presence of bacterial MGEs (such as plasmids) containing ARGs within the correlated bacterial lineage or its possible presence within the correlated bacterial chromosome to further ascertain the causation of this out-competition.

Antibiotic compounds identified in the CBSTP-treated effluent in this study were also observed to have potential effective changes on the Cleveland Bay microbiome, with the two macrolide antibiotics, azithromycin and clarithromycin, observed above the PNEC within sediments in the Near-field. Core functional bacteria within the sediment of Cleveland Bay were found to be reduced in the Near-Field when compared to their proportion within the Howicks. This reduction correlated with the increase of azithromycin above the minimum inhibitory concentration (MIC) (4 µg/L), suggesting that these functional bacterial species may have been inhibited in the Near-Field by azithromycin at 27 µg/L. Incidences in macrolide resistance genes (OLEC and MLS23S) were also found to increase in conjunction with azithromycin concentrations in the Near-Field. However, an additional 10 ARGs were also found to be positively correlated with the concentration of azithromycin in Cleveland Bay sediments, none of which are known to provide resistance to macrolide antibiotics, which may be indicative of unmonitored effective variable, and the possibility of laboratory based MIC and predicted no-effect concentrations (PNEC) parameters unpredictability with environmental bacterial and genetic compositions. Similar observations were made for concentrations of clarithromycin (0.47µg/L) in the Near-Field, which were below the MIC (2 µg/L) and were found to be correlated with the increase in a number of ARGs, none of which express resistance to macrolide antibiotics. However, in times of high influent volumes (such as during high rainfall and flooding events), the CBSTP bypasses many of the treatment steps and provides only coarse primary screening. These bypass effluents may contain higher volumes of antibiotics, ARGs, ARBs, and other resistance stressors. Future studies seeking to characterise antibiotic resistance pollution associated with these bypass events should provide the additional context of excessive

runoff from other anthropogenic sources during high rainfall events (including agriculture and infrastructure industries).

Instances of the presence and concentration of antibiotics correlating to non-specific resistance genes have been documented previously (Wang et al., 2022, Wang et al., 2021) and have been attributed to the co-occurrence of other forms of resistance (other antibiotics, biocides, metals) in conjunction with long-term interaction of effective agents (including the shifting antibiotics and heavy metals). It is still highly debated whether this is due to selection and induction of the expression of co-resistance genes, enhanced adaption of resistance gene cassettes, or altered physiological/biochemical properties that upregulate ARG expression (Harrower et al., 2021). These instances of non-macrolide-related ARGs may be reflective of the presence of these co-resistance genes within the microbiome of Cleveland Bay. As analyses within this study were predominantly based on correlation-based statistics, considerations should be made for either azithromycin, clarithromycin, or neither antibiotic to be the significant functional driver in these community changes. However, future research strategies should build on these foundational works to identify co-resistance genes and characterise other effective pollutants within the Near-field environment. Further possible direct effects of clarithromycin were observed in the form of inhibited growth of the core bacteria *Vibrio antiquaries* and additional overrepresentation of three *marinobacter* species, suggesting instances of out-competition in the Near-Field sediments of Cleveland Bay. These indicative restrictive correlations suggest that concentrations of some antibiotics below MIC yet above PNEC may provide functional changes to core species of marine ecosystem microbiomes. Further works are required by the scientific field to provide conclusive evidence as to the causation of these described correlations; however, such findings highlight that inhibitory concentrations of antibiotics established for culturable bacteria may not be attributable to complex environmental microbiomes.

Exposing bacteria to antibiotic concentrations below respective MIC values, as is documented in this study with clarithromycin, has been previously documented to have multiple effects, including the stimulation of horizontal gene transfer (HGT) of ARGs through conjugation (Shun-Mei et al., 2018, Xia et al., 2008). Furthermore, low levels of antibiotics have also been linked to the activation of the SOS response signalling and uptake of eDNA (Memar et al., 2020) and changing gene regulation through quorum sensing has been linked to increased bacterial cell variability (Carette et al., 2020, Liu et al., 2013). As the majority of the ARGs released from the CBSTP were likely present as eDNA, the presence of antibiotics above PNEC and eARGs within the CBSTP effluents likely facilitated further HGT of these ARGs within the natural environment microbiome. Future works are encouraged to utilise Hi-C proximity ligation sequencing to establish the presence

of these ARGs within extracellular MGE (such as plasmids and eDNA) and their subsequent HGT to additional bacterial cell lineages.

Additional stressors, such as those experienced by the microbiome during wastewater treatment, may also have an effect on the HGT of ARGs, as was observed in the CBSTP. The effluent core resistome within the CBSTP was likely predominantly influenced by HGT occurring throughout the treatment process, as many shared ARGs were present between the core resistome of both the influent and effluent, yet the shared core microbiome of the two sample locations is comparatively small, suggesting that these ARGs reside within different bacterial cell lineages or as eDNA. These instances of HGT were observed in the CBSTP microbiome and correlated instances to the core resistome of the influent and effluent; similar instances of HGT have been documented within WWTPs previously (Wei et al., 2021, Yin et al., 2019). Additionally, the higher proportion of resistance to certain antibiotics observed within the effluent in comparison to the influent, including multidrug resistance, may be exacerbated by the approximately 21 days (sludge retention time) spent in the treatment plant reactor, where HGT is likely to occur most frequently.

Similarly to occurrences within the CBSTP, interactions between cell lineages and ARGs from the effluent to the Near-Field of Cleveland Bay suggest that HGT incidences may have occurred. This is exemplified by correlations of CBSTP-associated ARGs to bacteria within the Near-Field core microbiome, many species of which were not present within the effluent microbiome. These putative instances of HGT are supported by observations in other ecosystem studies that showed mobile genetic elements (MGE) harbouring ARGs that disseminated into the receiving environment of treated waste and provided resistance to bacteria that did not originate from the WWTP (Milaković et al., 2020, Milaković et al., 2019, Proia et al., 2016, Zhang et al., 2021b). These instances are indicative of potentially out-competing functionally important bacteria in the Near-Field by bacteria that may harbour CBSTP-associated ARGs. This suggests that not only is there possible horizontal gene transfer of the CBSTP effluent resistome to the environmental microbiome in the Near-Field of Cleveland Bay, but that these horizontal gene shifts may create an effective change to the Near-Field functional microbiome when compared to the Howicks natural reference. Future works are encouraged to utilise transcriptomic sequencing and lab-scale bacterial culture experiments in tandem with metagenomics to assess the utilisation capability of these ARGs to be functional within the receiving environment. Such experiments may include culture and antibiotic sensitivity testing to ensure both the function and use of these effluent-ARGs within the environmental ARBs, as well as the use of Hi-C proximity ligation sequencing to identify the presence of bacterial MGEs (such as plasmids) containing the specific ARGs within the correlated bacterial lineage.

The possibility of HGT of ARGs between treatment steps of the CBSTP, as well as between the CBSTP and the receiving environment, may provide additional opportunities for antibiotic resistance proliferation in previously non-resistant bacterial species, as well as increased instances of clinically relevant multi drug resistant bacteria (MDRB). This may have ongoing impacts on environmental health. Similar instances to this have been described previously, such as the correlation of ARGs to possible host genera within agricultural wastes, some of which were found to have implications for public health in the form of antibiotic resistance in clinically relevant pathogens and increased multi-drug resistance (Jia et al., 2017, Novo et al., 2013, Shi et al., 2013, Tao et al., 2016, Wang et al., 2022, Yuan et al., 2018).

Whilst the Near-Field environment may have been most significantly impacted by the CBSTP, many other sources of anthropogenic pollution that may contain antibiotics, ARGs, and ARBs exist in Cleveland Bay, including a metal refinery, a busy port, agricultural land and general stormwater run-off. The influence of these sources was likely reflected in the microbiome and resistome of other areas of Cleveland Bay, through increased ratios of ARGs to bacteria, low core microbiome diversity, and high diversity of core resistomes. The increased microbial diversity within environmental samples has been linked to ecosystem health, and increased instances of ecological stress linked to decreases in the diversity of microbiomes (Rocca et al., 2019). The stability of an environmental ecosystem has also been previously linked to the diversity of its core microbiome (Björk et al., 2018). The Cleveland Bay Mid-Field presented the highest representative core resistome and the lowest diversity of unique core microbiome OTUs. These results suggest that the Mid-Field may be most reflective of a highly stressed ecosystem, aligning with our understanding of where Sandfly Creek intersects the locations adopted in this study. Furthermore, whilst the Howicks environment may have represented the least stressed microbiome compared to other marine areas investigated in this study, azithromycin and clarithromycin were detected within the sediment from the Howicks at concentrations above PNEC. The same concentration of azithromycin was also measured in the Far-Field of Ceveland Bay. These semi-synthetic macrolide antibiotics are not known to occur naturally and are often used in public health or veterinary medicine (Alvarez-Elcoro and Enzler, 1999). The presence of these antibiotics within the Howicks group of islands as well as the Far-Field at the same magnitude may reflect the widespread nature of antibiotic pollution, which may penetrate various regions of the Great Barrier Reef environment and affect various flora and fauna that inhabit it. This widespread nature of antibiotic pollution has been demonstrated previously with synthetic antibiotics measured in in untouched global locations (Tan et al., 2018, Yang et al., 2021).

Understanding the spread of antibiotic resistance into organisms within the marine ecosystem is vitally important to assessing the potential risk of anthropogenic antibiotic resistance pollution. To address the validity of using turtle species as sentinel markers of antibiotic resistance, some preliminary work has been done on the consequence of pollution in Cleveland Bay and its effect on the gut microbiome and ARG profile of a sentinel animal species (green sea turtles, *Chelonia mydas*). For comparison, green sea turtles from the Howicks, at the same maturity status as Cleveland Bay turtles, were studied. The preliminary evaluations indicate that the gastrointestinal resistome of these species was likely influenced by the ingestion of seawater and seagrass within their respective environments (Appendix C: Figure A.C-10 and Figure A.C-11). However, little variation was observed between the resistome composition of the green turtle populations in the Howicks and Cleveland Bay (Appendix C: Figure A.C-4), and small distinctions in the functional core resistome of each population (Appendix C: Figure A.C-7) could not be identified as distinctions specific to each environment (Appendix C: Figure A.C-12). However, credence should be given to preliminary assessments of the gastrointestinal microbiome of the two green turtle populations, which were identified to be significantly different (Appendix C: Figure A.C-3) and may be reflective of dysbiosis. However, the tracked range of the Sub-adult Green Sea turtles was found to not exceed 2 km (Appendix C: Figure A.C-1), and the location of Sub-adult Green Sea turtles sampled in Cleveland Bay was over 10 km from the CBSTP. This, in conjunction with the dissemination range of the antibiotic and antibiotic resistance pollution identified from the CBSTP to be within 10 m (Near-Field) to 200 m (Mid-Field) from the effluent source, suggests that any dysbiosis identified in either population is likely contributed to by other sources of anthropogenic pollution. This preliminary assessment suggests that further research is required to identify the capability of Sub-adult Green sea turtles to act as sentinels for antibiotic resistance within a defined geographical area or, by extension, for antibiotic resistance disseminated from a specific point source. As such, further research is recommended on turtle populations inhabiting the seagrass field in the directly affected area (Near-Field) of the CBSTP to deduce whether this form of antibiotic resistance pollution may impact these sentinel species. Furthermore, current evidence is not sufficient to elucidate the effects of anthropogenic pollution within marine environments on marine sentinel species, such as Sub-adult Green Sea turtles. Further research is also required to define the effect that microbiomes of environmental matrices (e.g. sediment, seawater, and seagrass) may have on the microbiome of green turtle gastro-intestinal system. Such analysis on microbiome variations may also clarify the small variations observed between the two green turtle populations' resistomes due to larger-scale datasets and provide causations for such variations.

7.1 Conclusion

The processes involved in the treatment of influent throughout the CBSTP were observed to lead to significant variations in the microbial composition of the effluents, and these rearrangements were reflected in the composition of antibiotic resistance harboured by the changing microbial communities, as well as through changes facilitated by HGT. Furthermore, despite the CBSTP capability of reducing antibiotic resistance throughout the treatment process, tangible volumes of ARGs, ARB, and antibiotics are released into the marine receiving environment of Cleveland Bay. These instances of antibiotic and antibiotic resistance pollution did provide both the means and opportunities for the perpetuation of antibiotic resistance within the Great Barrier Reef microbiome. However, this dissemination distance could not be established further than the Near-Field of Cleveland Bay. Furthermore, whilst WWTPs, are criticised globally for antibiotic resistance pollution within waterways, consideration should be given to other sources of resistance dissemination. Ecological stress established in the Mid and Far-Field microbiomes of Cleaveland Bay was not related to high incidences of effluent-associated ARGs or antibiotics, and other forms of anthropogenic pollution are likely to have facilitated stress within these microbiomes. This has led to further questions on other influences of resistance within this anthropogenically impacted area.

This thesis utilised a novel take on the use of whole genome sequencing methods for the identification of ARGs and ARBs originating from a WWTP and effects of dissemination within the receiving marine environment. This represents a proof of concept for future risk assessment scaffolds to provide quantitative and qualitative assessments of ARGs and antibiotics, which may have functional effects on the microbiome of receiving environments. Further surveillance and comprehensive analysis of additional WWTPs and their corresponding receiving environments are required to fully elucidate the true effect of antibiotic and antibiotic resistance pollution released from WWTPs globally through various environments and utilising different forms of treatment. This will facilitate a better understanding of the interactions between antibiotics and environmental bacteria, which is necessary for the development of adequate risk assessments. Future research should build on this information to provide an informed risk assessment scaffold for One Health risks associated with antibiotic resistance dissemination from WWTPs, and investigations from other sources of antibiotic resistance pollution should also be highly considered.

This study highlights the implications of antibiotic resistance on delicate microbiomes, such as those in the Great Barrier Reef, and the harm associated with their higher-order ecosystems from disseminated ARGs and antibiotics. As such, future monitoring studies of the CBSTP should provide equal scrutiny to WWTP and other sources of antibiotics and antibiotic resistance pollution so that

informative conclusions can be made for pollution source control and management within this ecosystem.

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Appendix A

Table A.A 1: Commonly identified ARGs in WWTPs and methods of detection.

Publication	Study Focus	Treatment in WWTP	Location	ARG Detection Method	ARGs Identified in Influent	Changes to ARG Relative Abundance in Effluent
Chen and Zhang, 2013	Effect of treatment on ARGs	Activated Sludge	China	qPCR (abundance relative to 16s)	*TETM*, *TETO*, *TETQ*, *TETW*, *SULI*, *SULII*, *INTI1*	*TETM*, *TETO*, *TETQ*, *TETW*, *SULI*, *SULII*, *INTI1*
		constructed wetland		qPCR (abundance relative to 16s)	*TETM*, *TETO*, *TETQ*, *TETW*, *SULI*, *SULII*, *INTI1*	*TETM*, *TETO*, *TETQ*, *TETW*, *SULI*, *SULII*, *INTI1*
		ultraviolet (UV) disinfection		qPCR (abundance relative to 16s)	*TETM*, *TETO*, *TETQ*, *TETW*, *SULI*, *SULII*, *INTI1*	*TETM*, *TETO*, *TETQ*, *TETW*, *SULI*, *SULII*, *INTI1*
Conco, et al, 2022	ARGs identified in hospital waste	Chlorination	South Africa	WGS	Resistome identified	*SULI*, *AADA4–5*, *TEM-1D* Several ARGs were downregulated
Czekalski, et al, 2012	Multi-drug resistant bacteria in wastewater stream	Activated Sludge	Switzerland	Bacterial culture, antibiotic sensitivity testing, and qPCR	*SULI*, *SULII*	*SULI*, *SULII*
Czekalski, et al., 2016	Escherichia coli	ozonation	Germany	Bacterial culture, and qPCR	*SULI*	*SULI*
Gao, et al., 2012	tetracycline and sulfonamide resistance genes	Activated Sludge	U.S.A.	qPCR (abundance relative to 16s)	*TETO*, *TETW*, *SULI*	*SULI*, *TETO*, *TETW*
Ju, et al., 2019	Resistome	Activated Sludge	Switzerland	WGS (abundance relative to total sequences)	Resistome identified	Several ARGs for multi-drug, beta-lactam, aminoglycoside, tetracycline, trimethoprim, vancomycin, bacitracin, chloramphenicol, rifamycin, sulfonamide, macrolide.
Karkman, et al., 2016	Resistome	Activated Sludge and biofilters	Finland	qPCR (abundance relative to 16s)	*ERMF*, *blaTEM*, *TETA*, *TNPA*, *ERMB*, *SULII*, *AACC*, *TNPA*, *MEXF*, *TNPA*, *TETO*, *TETW*, *STRB*, *AADA*	*blaTEM*, *TETA*, *TNPA*, *ERMB*
Lee, et al., 2017	Impact of UV disinfection on ARGs and ARB	ultraviolet (UV) disinfection	China	qPCR (abundance relative to 16s)	*TETX*, *TETM*, *TETA*, *SULI*, *SULII*, *ERMB*, *QNRD*, *blaTEM*	*TETA*, *TETX*, *TETM*, *SULI*, *SULII*, *ERMB*, *QNRD*, *blaTEM*
Li, et al., 2015	Relationship of Antibiotics to ARGs	ultraviolet (UV) disinfection and constructed wetland	China	qPCR (abundance relative to 16s)	*TETA*, *TETB*, *TETC*, *TETG*, *TETL*, *TETM*, *TETO*, *TETQ*, *TETW*, *TETX*, *SULI*, *SULII*	*TETC*, *TETG*, *TETM*, *TETX*, *SULI*, *INTI1*, *TETA*, *TETB*, *TETL*, *TETO*, *TETQ*, *TETW*, *SULII*
Li, et al., 2018	pharmaceutical wastewater treatment plant	Activated Sludge	China	qPCR (abundance relative to 16s)	*TETB*, *TETW*, *SULI*, *SULII*, *GYRA*, *QEPA*, *ERMB*, *ERMF*, *INTI1*, *INTI2*	*TETB*, *SULI*, *SULII*, *GYRA*, *INTI1*, *ERMB*, *ERMF*, *QEPA*, *INTI2*
Lin, et al., 2021	Relationship of Antibiotics to ARGs	Membrane bioreactors	China	qPCR (abundance relative to 16s)	Unspecified 319 ARGs	*APHA3*, *MERA*, *DFRA14*, *APH3-III*, *ERMF*, *ARR-2*, *TET32*, *CEFA*, *blaTEM*, *AACC2*, *DFRA1*, *CN1A5*, *AADA16*, *AADA6*, *AAC(6)-LB*, *COPA*, *MEFA*, *ERMQ*, *INU(F)*, *AADA_99*, *AAC(6)*, *CATB3*, *QACH_351*, *AADA5*, *blaVEB*, *AADA21*, *EREB*, *AADA17*, *MSRE*, *ERMB*, *TETM*, *TETW*, *TETA*, *SULI*, *MPHA*, Expression of all other ARGs was maintained or downregulated

Liu, et al., 2019	Relationship of Antibiotics to ARGs	ultraviolet (UV) disinfection	China	qPCR (abundance relative to 16s)	*TETA, TETC, TETQ, TETW, TETX, SULI, SULII, SULIII, INTI 1*	*TETA, TETC, TETQ, TETW, TETX, SULI, SULII, SULIII, INTI1*
Liu, et al., 2018	Intracellular and extracellular ARGs	Chlorination	China	qPCR (abundance relative to 16s)	*VANA, DFRA1, CATA1, KATG, RPOB1, APH(2')-ID, AADA, QNRA, GYRA, AMPC, blaTEM, ERMB, ERMA, SULIII, SULII, SULI, TETX, TETQ, TETM, TETC, TETB, TETA*	*ERMB, TETA, TETB, TETC, SULI, SULII, SULIII, AMPC, APH(2')-ID, KATG, VANA, DFRA1, CATA1, KATG, RPOB1, AADA, QNRA, GYRA, blaTEM, ERMA, TETX, TETQ, TETM*
Mao, et al., 2015	Relationship of Antibiotics and Heavy metals to ARGs	Chlorination	China	qPCR (abundance relative to 16s)	*TETA, TETB, TETC, TETD, TETE, TETG, TETH, TETJ, TETK, TETL, TETM, TETO, TETQ, TETT, TETW, TETX, TETZ, SULI, SULII, SULIII, SULA, QNRA, QNRB, QNRD, QNRS, ERMB, ERMC*	*TETA, TETB, TETE, TETG, TETH, TETS, TETT, TETX, SULI, SULII, QNRB, ERMC, TETC, TETD, TETJ, TETK, TETL, TETM, TETO, TETQ, TETW, TETZ, SULIII, SULA, QNRA, QNRD, QNRS, ERMB*
Machado, et al., 2023	Effect of different treatment types	Activated Sludge anaerobic sludge blanket Reactor ultraviolet (UV) disinfection	Brazil	Bacterial culture, antibiotic sensitivity testing, and qPCR	*INT1, blaTEM, TETA, SULI, QNRB, EMRB*	*INT1, blaTEM, TETA, SULI, QNRB, EMRB* *blaTEM, TETA, QNRB, INT1, SULI, EMRB* *QNRB, EMRB, INT1, blaTEM, TETA, SULI*
Munir, et al., 2011	Effect of different treatment types	Activated Sludge Oxidative Ditch Membrane bioreactors	U.S.A.	qPCR (abundance relative to 16s)	*TETW, TETO, SULI*	*TETW, TETO, SULI* *TETW, TETO, SULI* *TETW, TETO, SULI*
Narciso-da-Rocha, et al., 2018	Effect of treatment on ARGs	ultraviolet (UV) disinfection	Portugal	qPCR (abundance relative to 16s)	*INTI1, blaTEM, blaOXA-A, blaSHV, blaCTX-M, SULI, SULII, QNRS*	*blaTEM, blaOXA-A, QNRS, INTI1, SULI, blaSHV, blaCTX-M, SULII*
Neudorf, et al., 2017	Effect of treatment type	constructed wetland	Canada	qPCR (abundance relative to 16s)	*INT1, SULI, SULII, TETO, ERMB, MECA, blaCTX-M, blaTEM, QNRS*	*SULI, SULII, MECA, TETO, QNRS*
Osińska, et al., 2019	Culture-based identification	Activated Sludge, Plus Anerobic-aerobic reactor	Poland	Bacterial culture, antibiotic sensitivity testing, and qPCR	*blaTEM, blaSHV, blaOXA, TETA, TETM*	TETA, TETM, blaTEM, blaSHV, blaOXA
		Plus mechanical & biological system	Poland	Bacterial culture, antibiotic sensitivity testing, and qPCR	*blaTEM, blaSHV, blaOXA, TETA, TETM*	*TETM, TETA, blaTEM, blaSHV, blaOXA*
		Plus Sequencing batch reactor	Poland	Bacterial culture, antibiotic sensitivity testing, and qPCR	*blaTEM, blaSHV, blaOXA, TETA, TETM*	*TETM, TETA, blaTEM, blaSHV, blaOXA*
		Plus mechanical & biological nutrient removal	Poland	Bacterial culture, antibiotic sensitivity testing, and qPCR	*blaTEM, blaSHV, blaOXA, TETA, TETM*	*TETM, TETA, blaTEM, blaSHV, blaOXA*
Pazda, et al., 2020	Effect of different treatment types on tetracycline and sulfonamide resistance genes	Activated Sludge constructed wetland	Poland	qPCR (abundance relative to 16s)	*TETA, TETB, TETC, TETG, TETL, TETM, TETO, TETQ, TETX, SULI, SULII, SULIII*	*TETB, TETG, TETH, TETS, TETT, TETX AND SULI, SULII* *TETB, TETK, TETL, TETO, SULIII*
Rafraf, et al., 2016	Effect of different treatment types on resistance genes	Activated Sludge constructed wetland	Tunisia	qPCR (abundance relative to 16s)	*blaCTX-M, blaTEM, QNRA, QNRS, SULI, ERMB, INTI1*	*QNRS, ERMB* *blaCTX-M, blaTEM, AND QNRA, INTI1*
Rodriguez-Mozaz, et al., 2015	hospital-urban wastewater system	Activated Sludge	Spain	qPCR (abundance relative to 16s)	*blaTEM, ERMB, QNRS, SULI, TETW*	*blaTEM, SULI, QNRS, ERMB, TETW*

Sui, et al., 2019	Intracellular and extracellular ARGs	Activated Sludge	China	qPCR (abundance relative to 16s)	*TETM*, *TETW*, *TETG*, *TETX*, *ERMB*, *ERMF*, *MEFA*, *EREA*, *SULI*, *SULI*, *blaTEM*, *INTI1*	*MEFA, TETM, TETW, SULI, TETG, TETX, ERMB, ERMF, EREA, SULI, blaTEM, INTI1*
Wang, et al., 2020	Intracellular and extracellular ARGs	Membrane bioreactors	China	qPCR (abundance relative to 16s)	*INTI*, *blaTEM*, *ERMB*, *TETO*, *TETW*	*INTI1, blaTEM, ERMB, TETO, TETW*
Wen, et al., 2016	Effect of treatment on ARGs	ultraviolet (UV) disinfection	China	qPCR (abundance relative to 16s)	*TETA*, *TETO*, *TETW*, *SULI*, *SULII*, *blaCTX-M*, *blaTEM*, *blaSHV*, *INTI1*	*TETA, TETO, TETW, SULI, SULII, blaCTX-M, blaTEM, blaSHV, INTI1*
Xu, et al., 2015	Relationship of Antibiotics to ARGs	Activated Sludge	China	qPCR (abundance relative to 16s)	*TETA*, *TETB*, *TETE*, *TETW*, *TETM*, *TETZ*, *SULI*, *SULII*, *SULIII*, *GRYA*, *PARC*, *QNRC*, *QNRD*	*QNRC, TETM, QNRD, PARC, GYRA, TETA, TETB, TETE, TETW, TETZ, SULI, SULII, SULIII*
Yang, et al., 2014	Resistome	Activated Sludge	China	WGS (abundance relative to total sequences)	Resistome identified	ARGs for Sulfonamide, Quinolone and Chloramphenicol resistance. Beta-lactams, Tetracyclines, Aminoglycosides
Yuan, et al., 2019	Intracellular and extracellular ARGs	Activated Sludge	China	qPCR (abundance relative to 16s)	*TETA*, *TETC*, *TETM*, *TETX*, *SULI*, *SULII*, *blaTEM*, *EREA*, *ERMB*	*TETA, TETC, TETM, TETX, SULI, SULII, blaTEM, EREA, ERMB*

Note: ARGs highlighted in green were identified as having significantly increased in relative abundance in the effluent of WWTPs compared to the relative abundance in the influent. ARGs highlighted in red were observed to either significantly decrease or not change in relative abundance in the effluent compared to the relative abundance in the influent.

Appendix B

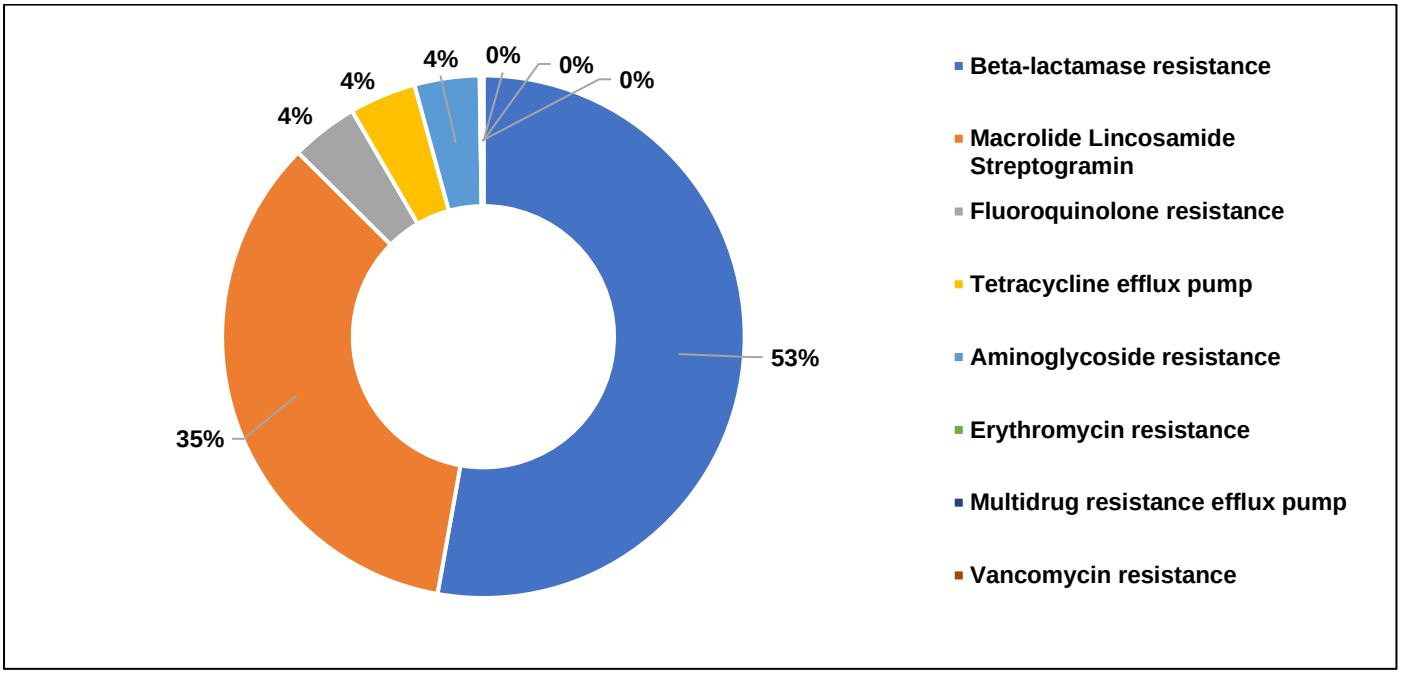


Figure A.B- 1: An illustration of the distribution and relative abundance of ARGs in the composite influent sample as identified by duplicate qPCR assays.

Table A.B- 1: ARG detection using a qPCR array.

Gene Name	Gene Group	Gene Sub-Group	Ct result	Detection result
VIM-7	Fluoroquinolone resistance		35.81399	+/-
VIM-13	Aminoglycoside resistance		0	-
VIM-1	Aminoglycoside resistance		36.5659	+/-
VEB	Aminoglycoside resistance		20.74906	+
VANC	Aminoglycoside resistance		36.45095	+/-
VANB	Beta-lactamase resistance	Class C	34.26411	+/-
TLA-1	Beta-lactamase resistance	Class C	27.46056	+
TETB	Beta-lactamase resistance	Class C	28.45476	+
TETA	Beta-lactamase resistance	Class C	23.95714	+
SME	Aminoglycoside resistance		0	-
SHV(238S240K)	Beta-lactamase resistance	Class A	32.89501	+
SHV(238S240E)	Beta-lactamase resistance	Class A	38.83601	-
SHV(238G240K)	Beta-lactamase resistance	Class B	0	-
SHV(238G240E)	Beta-lactamase resistance	Class C	28.74067	+
SHV(156G)	Beta-lactamase resistance	Class C	28.55219	+
SHV(156D)	Beta-lactamase resistance	Class A	33.19384	+
SHV	Beta-lactamase resistance	Class A	29.11503	+
SFO-1	Beta-lactamase resistance	Class A	0	-
SFC-1	Beta-lactamase resistance	Class C	0	-
QNRS	Erythromycin resistance		25.53808	+
QNRD	Macrolide Lincosamide Streptogramin		30.35037	+
QNRC	Macrolide Lincosamide Streptogramin		0	-
QNRB-8	Macrolide Lincosamide Streptogramin		32.07501	+
QNRB-5	Beta-lactamase resistance	Class C	28.72834	+
QNRB-4	Beta-lactamase resistance	Class A	33.3113	+
QNRB-31	Beta-lactamase resistance	Class A	0	-
QNRB-1	Beta-lactamase resistance	Class B	33.39205	+
QNRA	Beta-lactamase resistance	Class B	35.28001	+/-
QEPA	Beta-lactamase resistance	Class B	38.5537	-
PER-2	Beta-lactamase resistance	Class B	31.91074	+

PER-1	**Beta-lactamase resistance**	**Class A**	**0**	**-**
OXA-60	**Beta-lactamase resistance**	**Class C**	**0**	**-**
OXA-58	**Beta-lactamase resistance**		**26.42179**	**+**
OXA-55	**Macrolide Lincosamide Streptogramin**		**0**	**-**
OXA-54	**Beta-lactamase resistance**	**Class C**	**0**	**-**
OXA-51	**Beta-lactamase resistance**	**Class C**	**31.52856**	**+**
OXA-50	**Macrolide Lincosamide Streptogramin**		**30.42367**	**+**
OXA-48	**Beta-lactamase resistance**	**Class B**	**37.4855**	**-**
OXA-45	**Multidrug resistance efflux pump**		**0**	**-**
OXA-24	**Multidrug resistance efflux pump**		**30.36774**	**+**
OXA-23	**Beta-lactamase resistance**	**Class D**	**38.35823**	**-**
OXA-2	**Beta-lactamase resistance**	**Class D**	**24.25962**	**+**
OXA-18	**Beta-lactamase resistance**	**Class D**	**34.05982**	**+/-**
OXA-10	**Beta-lactamase resistance**	**Class D**	**21.17794**	**+**
OPRM	**Beta-lactamase resistance**	**Class D**	**32.70294**	**+**
OPRJ	**Beta-lactamase resistance**	**Class D**	**31.99009**	**+**
NDM	**Beta-lactamase resistance**	**Class D**	**0**	**-**
MSRA	**Beta-lactamase resistance**	**Class D**	**34.95159**	**+/-**
MOX	**Beta-lactamase resistance**	**Class D**	**26.2317**	**+**
MIR	**Beta-lactamase resistance**	**Class D**	**29.92176**	**+**
MEFA	**Beta-lactamase resistance**	**Class D**	**22.02614**	**+**
MECA	**Beta-lactamase resistance**	**Class D**	**36.75798**	**+/-**
LAT	**Beta-lactamase resistance**	**Class D**	**30.62147**	**+**
KPC	**Beta-lactamase resistance**	**Class A**	**29.55468**	**+**
IMP-5	**Beta-lactamase resistance**	**Class A**	**0**	**-**
IMP-2	**Fluoroquinolone resistance**		**0**	**-**
IMP-12	**Fluoroquinolone resistance**		**0**	**-**
IMP-1	**Fluoroquinolone resistance**		**0**	**-**
IMI & NMC-A	**Fluoroquinolone resistance**		**0**	**-**
GES	**Fluoroquinolone resistance**		**22.00411**	**+**
FOX	**Fluoroquinolone resistance**		**35.51235**	**+/-**
ERMC	**Fluoroquinolone resistance**		**27.1829**	**+**
ERMB	**Fluoroquinolone resistance**		**21.66958**	**+**
ERMA	**Fluoroquinolone resistance**		**38.02143**	**-**
EREB	**Fluoroquinolone resistance**		**28.24108**	**+**

DHA	**Beta-lactamase resistance**	**Class A**	**33.34134**	+
CTX-9	**Beta-lactamase resistance**	**Class A**	**33.89955**	+
CTX-8	**Beta-lactamase resistance**	**Class A**	**35.82237**	+/-
CTX-1	**Beta-lactamase resistance**	**Class A**	**34.25129**	+/-
CMY-10	**Beta-lactamase resistance**	**Class A**	**29.01085**	+
CFE-1	**Beta-lactamase resistance**	**Class A**	**0**	-
CCRA	**Beta-lactamase resistance**	**Class A**	**32.71236**	+
BIC-1	**Beta-lactamase resistance**	**Class A**	**0**	-
BES-1	**Beta-lactamase resistance**	**Class A**	**31.47344**	+
APHA6	**Beta-lactamase resistance**	**Class A**	**34.82659**	+/-
ACT-1	**Tetracycline efflux pump**		**32.06015**	+
ACT -5/7	**Tetracycline efflux pump**		**32.76105**	+
ACC-3	**Beta-lactamase resistance**	**Class A**	**37.4961**	-
ACC-1	**Vancomycin resistance**		**0**	-
AADA1	**Vancomycin resistance**		**23.97005**	+
AACC4	**Beta-lactamase resistance**	**Class A**	**37.0436**	-
AACC2	**Beta-lactamase resistance**	**Class B**	**32.2031**	+
AACC1	**Beta-lactamase resistance**	**Class B**	**29.41765**	+
AAC6	**Beta-lactamase resistance**	**Class B**	**24.55377**	+

Note: + Denotes gene presence

- Denotes gene absence

Appendix C


Howicks Group of Islands
Turtle Tracking Locations
14°17'0"S144°49'57"E
14°17'0"S 145°8'8"E
Ingram-Beanley Reef
QA95305
QA49397
QB24974
QA70009
QA95306
14°34'37"S 144°49'57"E
14°34'37"S 145°8'8"E
N
Printed at: A4
Print date: 20/9/2024
Not suitable for accurate measurement.
Image sourced from: https://earth.google.com/web

Figure A.C- 1: Map depicting the coordinates gathered of the five turtles tracked in the Howicks Group of Islands and the tracked locations from each turtle, where data from the individual turtles are colour-coded by line colour. The specified home range locations for each turtle are depicted as points within each sub-map titled with the individual turtle tag, where colour range corresponds to time variations, with green colours representing the start of tracking, and red corresponding to the end of tracking data.

Analysis of the tracking coordinates show that after leaving the tracker application site (Ingram Island) and over the course of the tracking period, the sub-adult sea turtles were found to each have a maximum home radius of 1.98 km^2 (QA95305), 1.74 km^2 (QA49397), 1.16 km^2 (QB24974), 0.74 km^2 (QA95306), 0.45 km^2 (QA70009). This data establishes that the turtles returned to their feeding grounds and encompassed a residential area of less than 2 km^2 where they were observed to stay within the remainder of the tracking period. This tracking period covered the general migration season of October to March. Suggesting that green sea turtles within this size class are resident to their feeding grounds and have not reached sexual maturity, thus do not migrate.

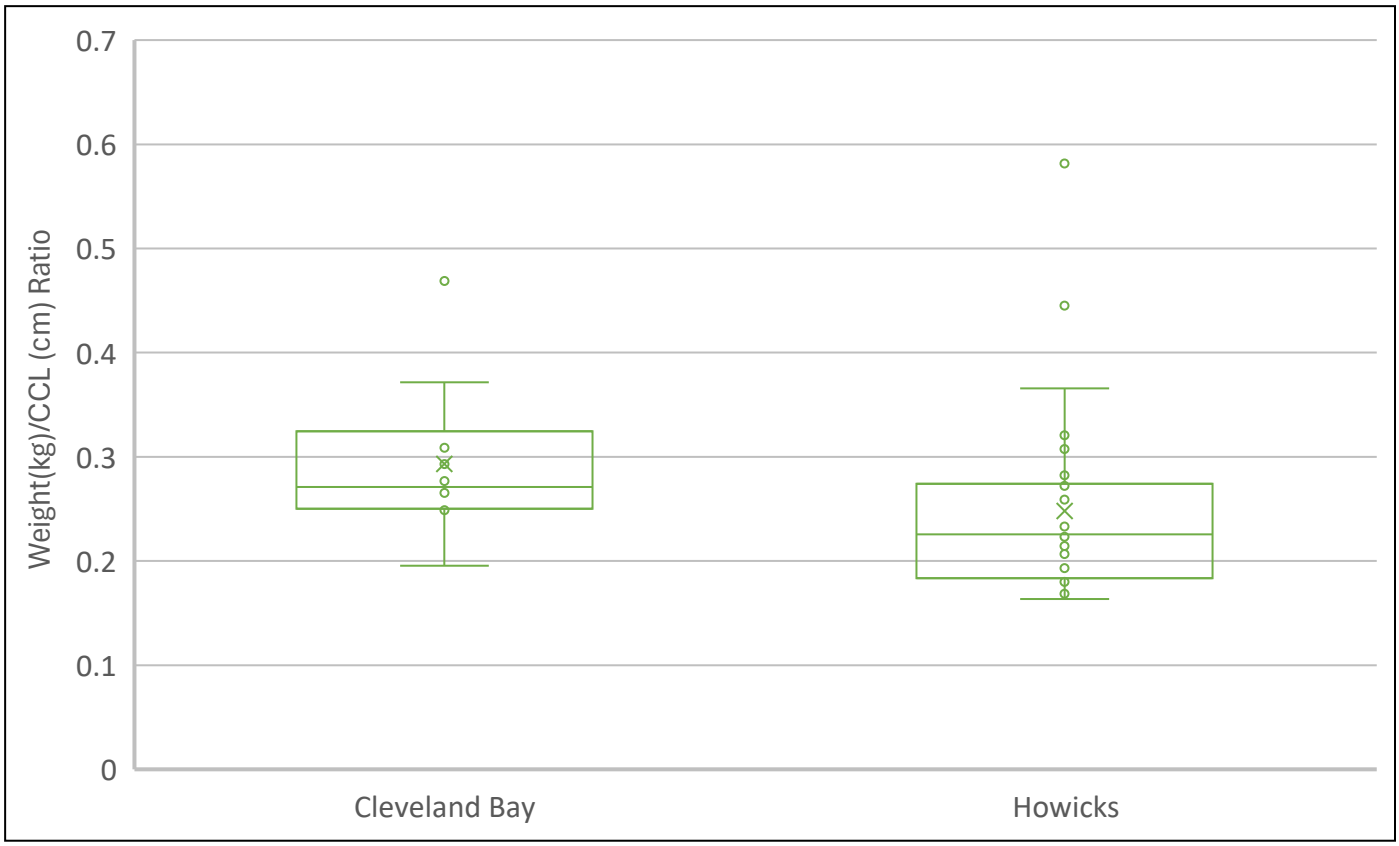


Figure A.C- 2: Box and whisker plots depicting the Physiological traits of the two turtle populations were analysed with the weight of an individual to curved carapace length (CCL) utilised as a standardised measurement for body condition and stratified by population as a standardised measurement for body condition.

The body condition of both populations was not found to be significantly different ($p=0.069$). Suggesting that the health conditions of both populations are similar; this is also supported by a recent study that analysed health parameters in both populations and established that the populations fell within previously defined health ratios for

tested analytes (Kophamel et al., 2022). This small population analysis suggests that whilst the two locations differ in levels of anthropogenic pollution, it has not significantly affected the body condition of the sub-adult green turtles.

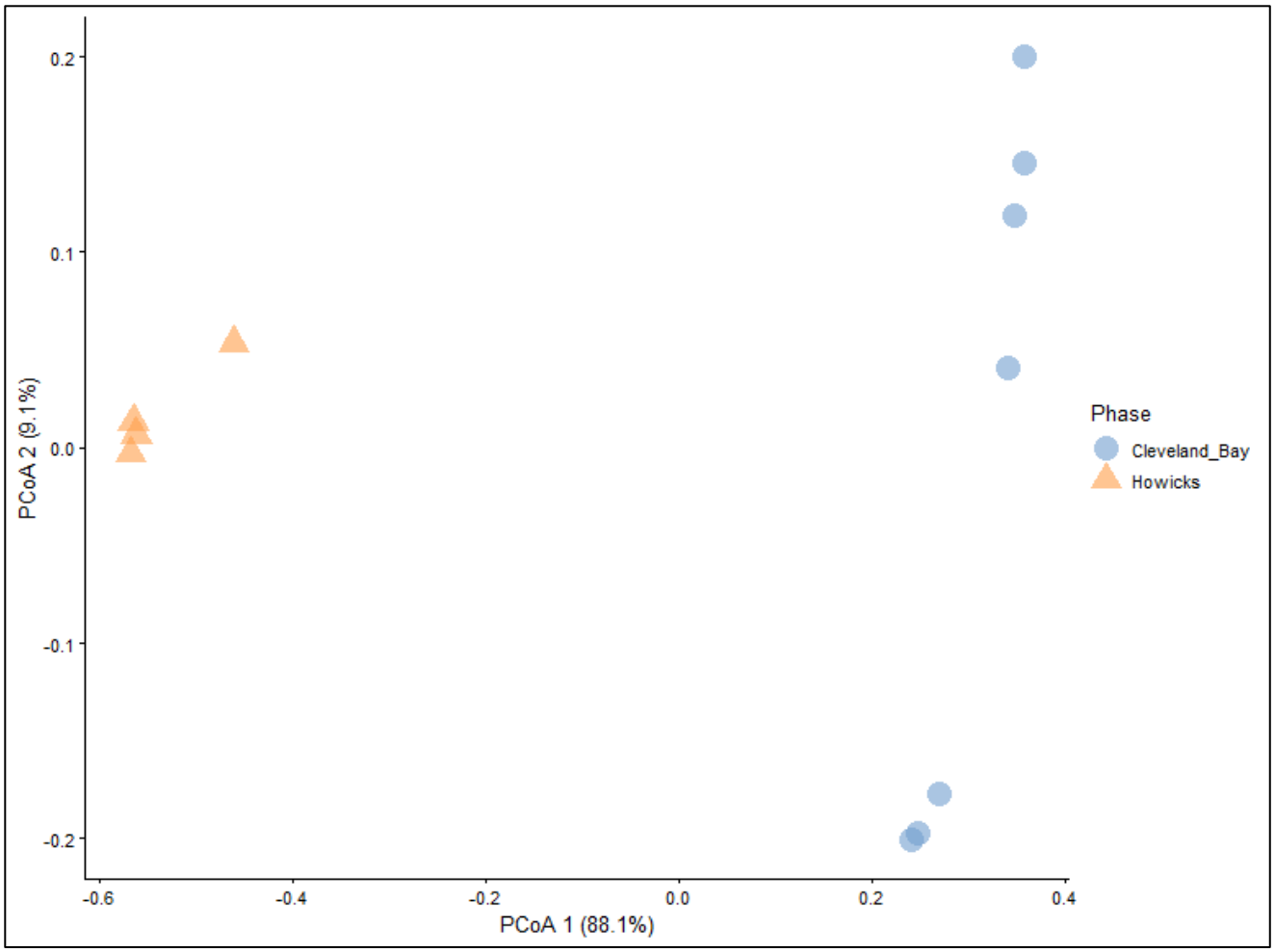


Figure A.C- 3: Principal component analysis (PCA) plot of the microbiome composition stratified by population location. The microbiome composition of the two separate populations were found to be significantly different (p=0.039)

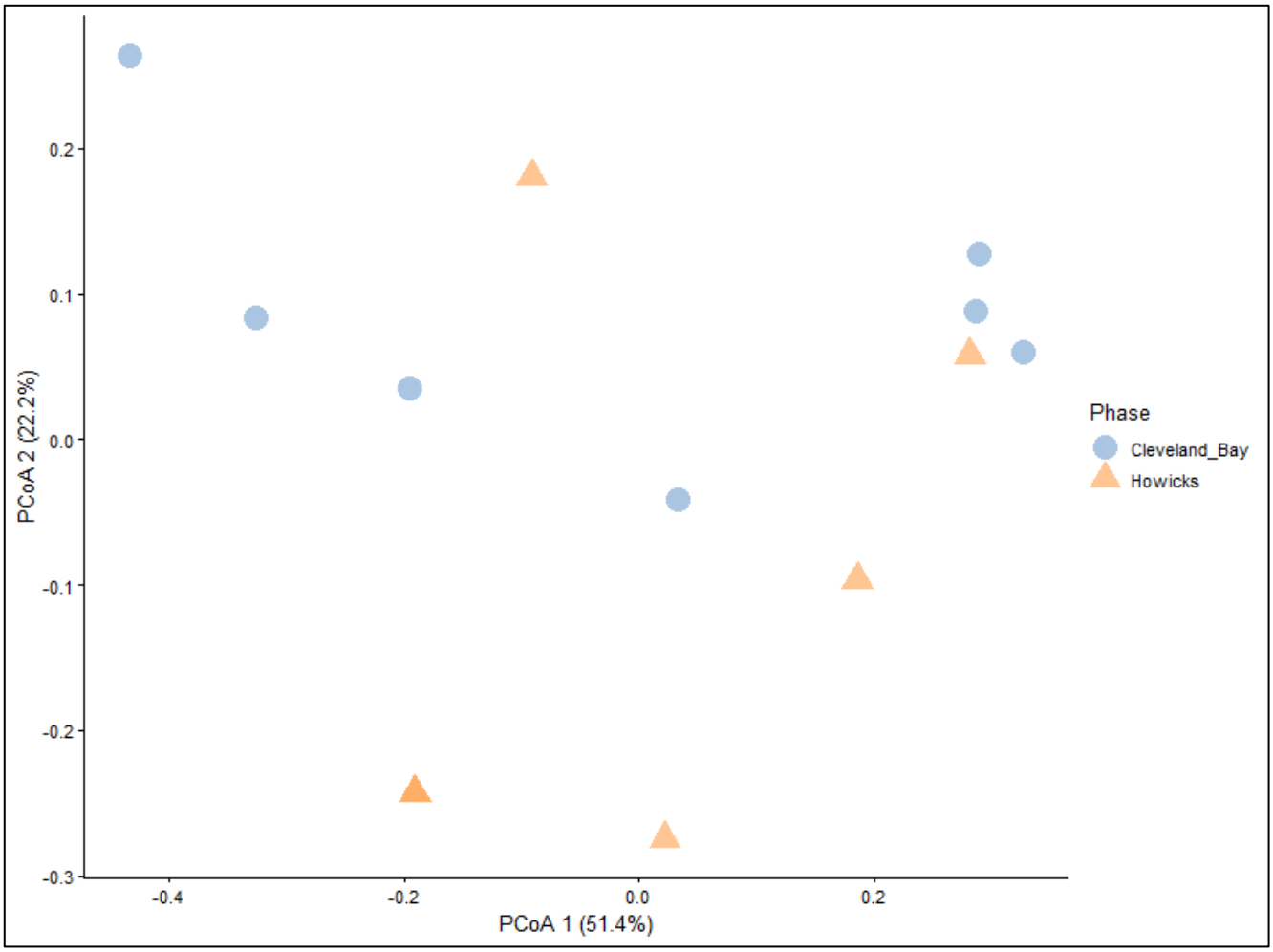


Figure A.C- 4: Principal component analysis (PCA) plot of the resistome composition stratified by population location. The resistome of the two populations were not found to be significantly different (p=0.153)

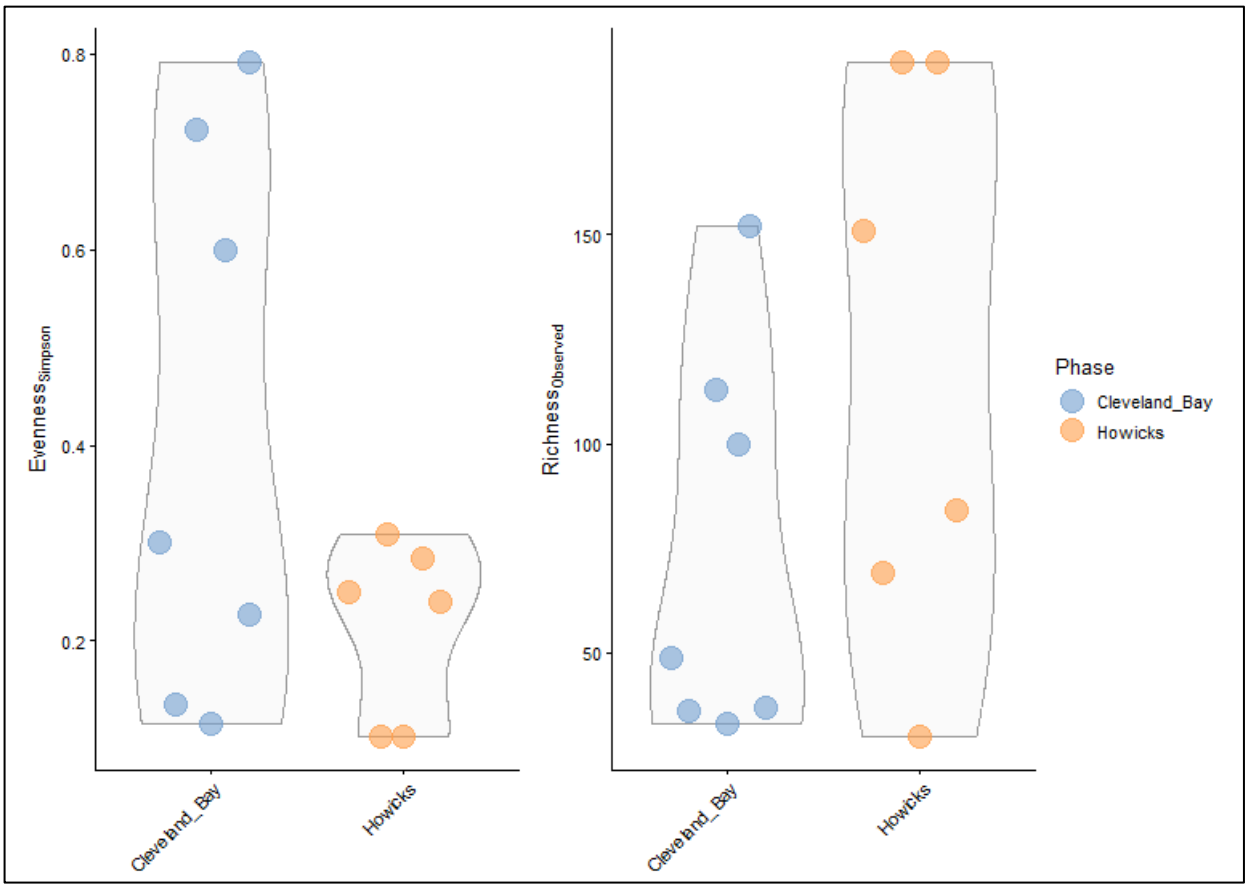


Figure A.C- 5: Evenness and richness of resistome data stratified by population location and depicted as a dot plot.

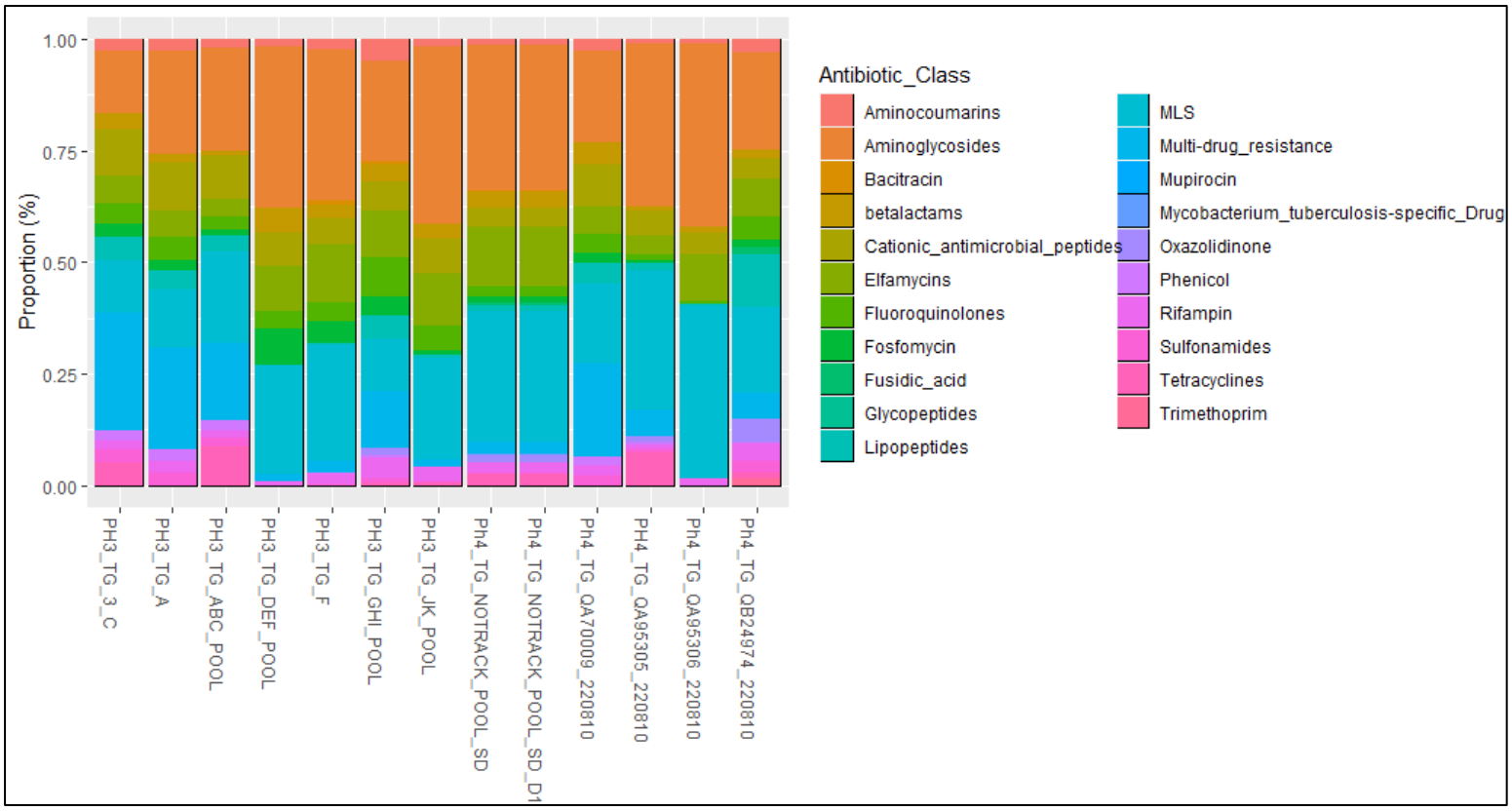


Figure A.C- 6: The distribution of the resistome of each pooled sample, which identifies the antibiotic class by which each antibiotic gene encodes resistance, was found to be predominantly composed of aminoglycoside resistance, MLS resistance, and tetracycline resistance in both populations.

This contrasts with previous literature, which suggested that beta-lactam resistance is highly expressed in sea turtle resistomes, as discussed in Chapter 2. Though beta-lactam resistance was identified in all samples, it did not dominate the distribution of the resistome. Suggesting that the use of bacterial culture and genetic assays utilised in previous studies may present inherent bias towards this class of antibiotic resistance due to its less comprehensive capabilities in the identification of the resistome composition when compared to WGS.

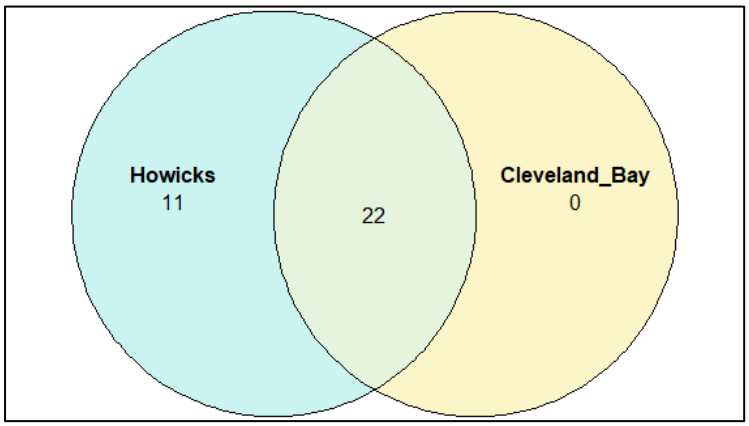
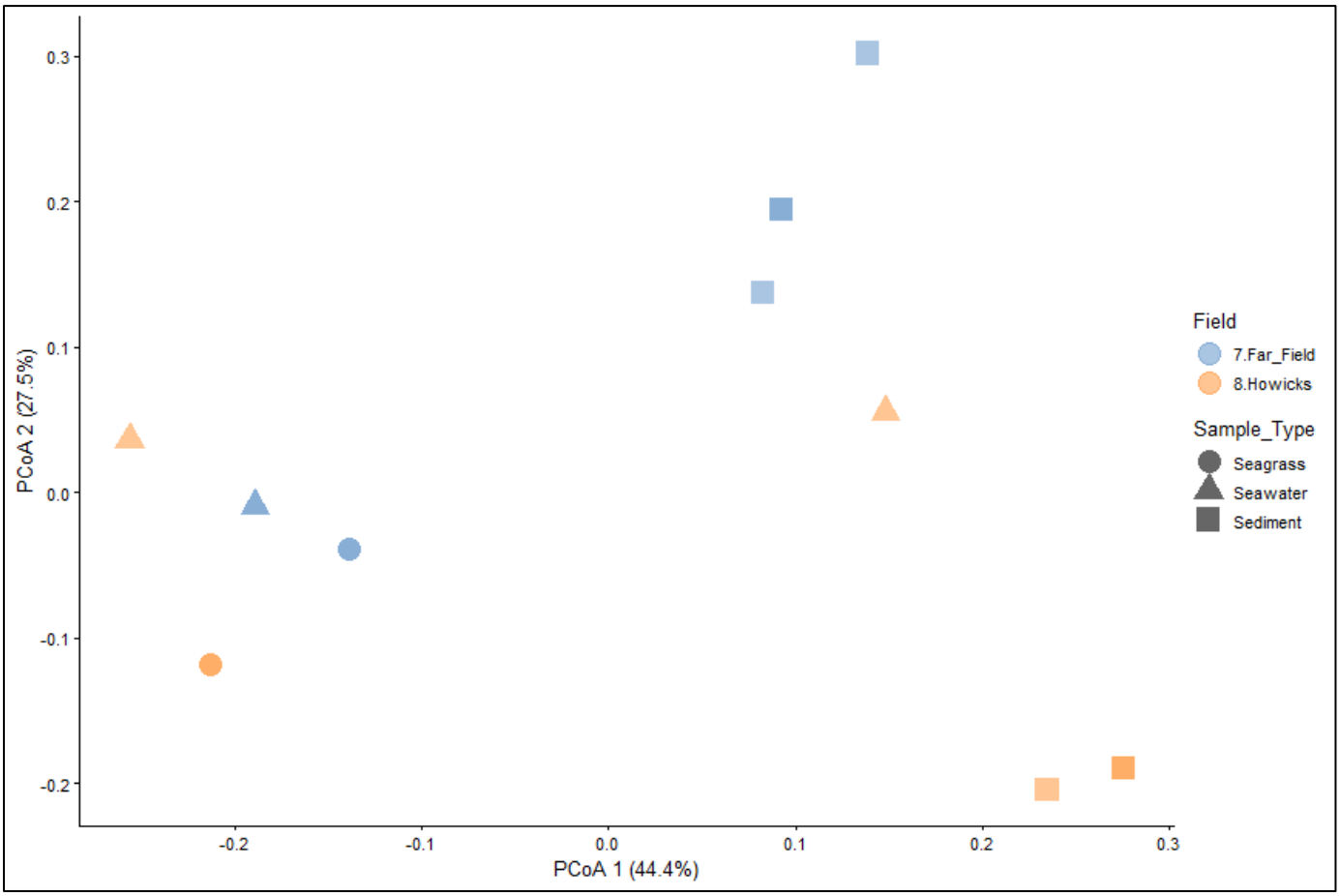


Figure A.C- 7: Depiction of the core resistome of the GIT of the two separate populations of Green turtles via Venn diagram.

The core resistome of both locations did not comprise the same core resistance genes, suggesting some functional differences exist between the two populations GIT resistome. The two populations shared 22 core ARGs, but the Howicks population contained an additional 11 distinct core ARGs. Suggesting that although the general resistome composition between the two sites are similar, functional differences may exist in the GIT resistome between populations. This may be an extension of smaller data volumes captured for resistome rather than microbiome, where large volumes of data can identify the small differences between the populations to a significant value.

Figure A.C- 8: PCA depicting the resistome composition of environmental samples obtained from the two separate



locations. The resistome of seawater and seagrass were found to be significantly different between locations (p=0.039). The resistome composition of seawater and seagrass in the separate locations were not found to be significantly different from each other (p=0.13), though they were found to be significantly different from their respective location's sediment (p=0.015).

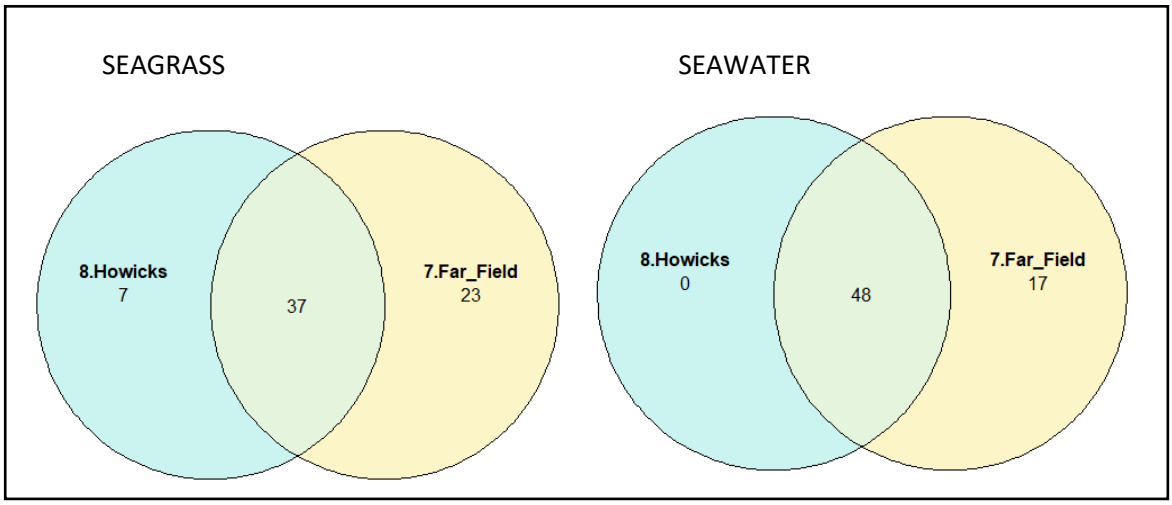


Figure A.C- 9: Depiction of the core resistome of the seawater and seagrass of the two separate locations via Venn diagram. the core resistome identified for the seawater and seagrass of the two separate locations was observed to be distinct.

The seagrass samples of the Far-Field of Cleveland Bay were identified to have 37 identical core ARGs in common with that of the Howicks, though an additional 23 ARGs were found to be distinct to the core resistome of the Cleveland Bay seagrass and a further 7 ARGs distinct to the seagrass of the Howicks. The core resistome of the seawater in both locations showed similar resistance compositions with 48 identical core ARGs; however, an additional 17 ARGs were identified in the core resistome of the Howicks seawater samples. These variations in environmental core resistome between the two sample sites are likely to be reflective of the separate conditions under which each location exists, with high anthropogenic use within the Cleveland Bay area and low levels of anthropogenic activities in the Howick Group of Islands.

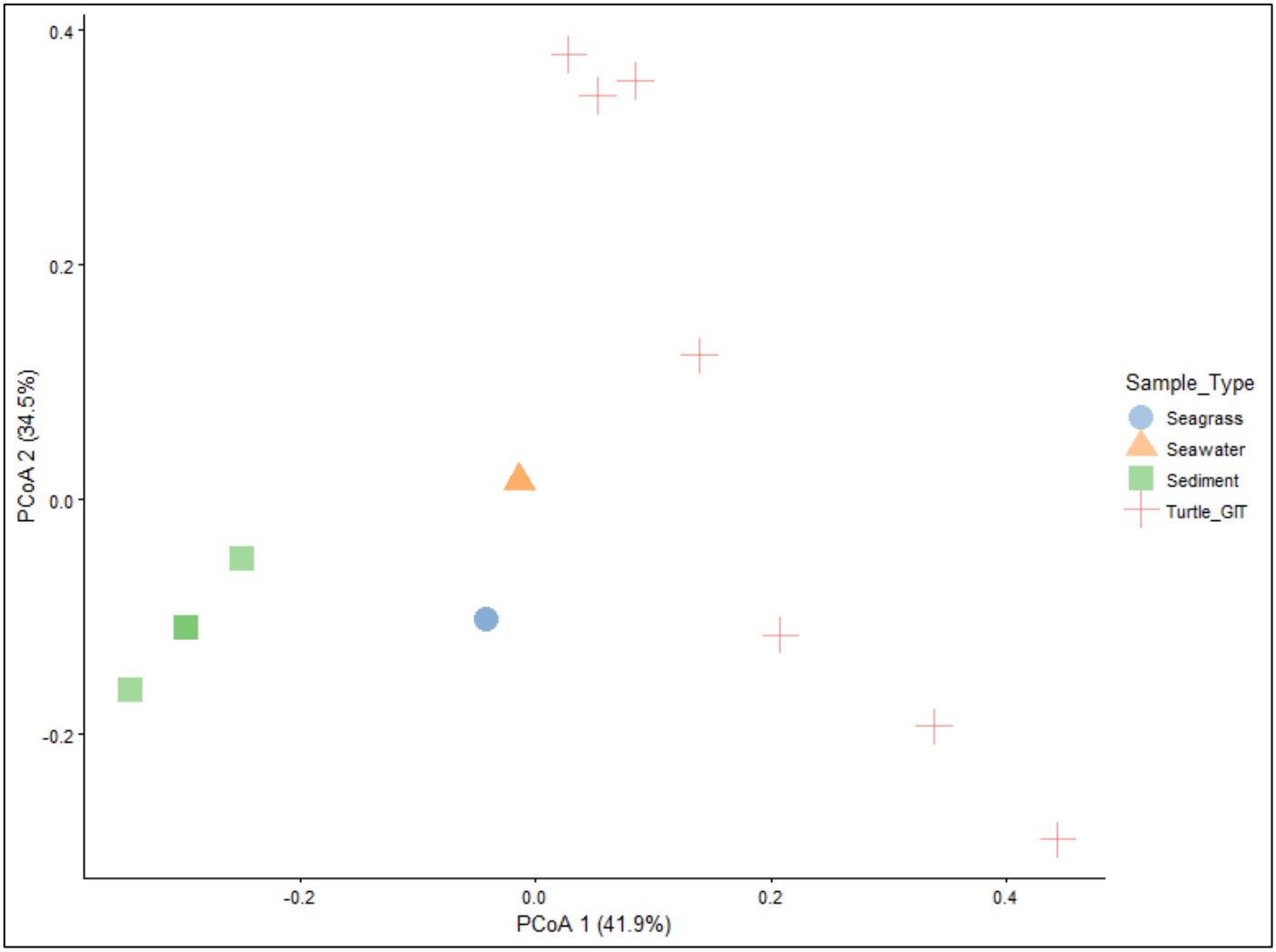


Figure A.C- 10: PCA depicting the resistome composition of environmental samples and turtle GIT samples obtained from Cleveland Bay. The GIT resistome of turtles in each location were found to be significantly different than the resistome composition in sediments of Cleveland Bay (p =0.031). The GIT resistome of turtles in Cleveland Bay were found to not be significantly different from the resistome identified in seawater and seagrass (p =0.151).

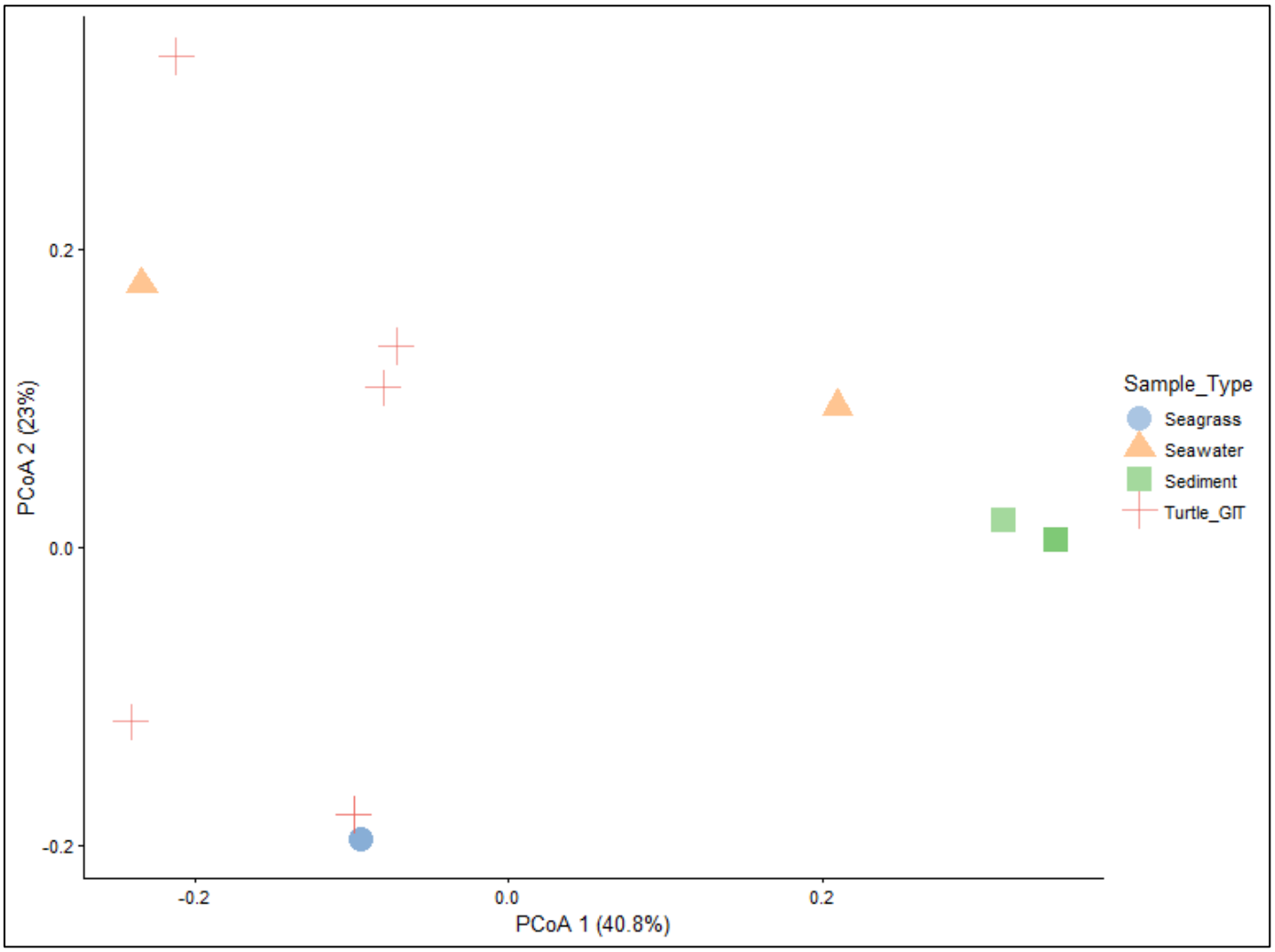


Figure A.C- 11: PCA depicting the resistome composition of environmental samples and turtle GIT samples obtained from Howicks. The GIT resistome of turtles in each location were found to be significantly different than the resistome composition in sediments of the Howicks ($p = 0.037$). The GIT resistome of turtles in the Howicks were found to not be significantly different from the resistome identified within seawater and seagrass ($p = 0.112$). Suggesting that the composition of turtle GIT resistomes is more representative of that of seawater and seagrass than the resistome within corresponding sediments.

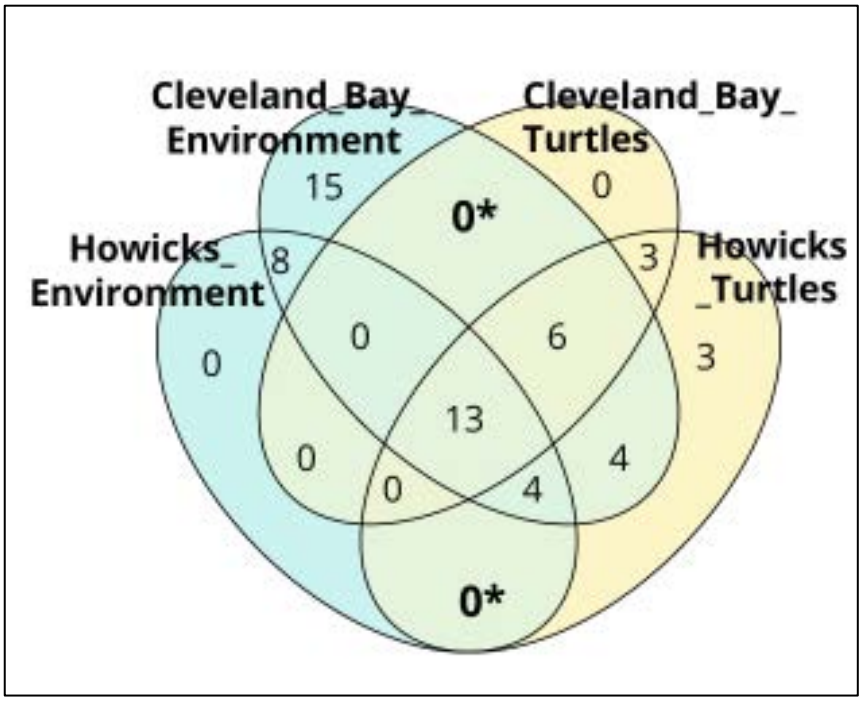


Figure A.C- 12: Depiction of the core resistome of the environmental sample (comprising of both seawater and seagrass resistomes) of the two separate locations as well as core resistome of the GIT of the two separate turtle populations via Venn diagram.

The core resistome of the Howicks population of turtles was fully represented by ARGs present in the core resistome of the Howicks seagrass and seawater, including the 11 exclusive ARGs in Howicks population of turtles. These 11 distinct ARGs were not represented by the 7 exclusive ARGs in the Howicks seagrass core resistome and were only present within the overlapping core environmental resistome of both locations. The core resistome of the Cleveland Bay turtle population are representative of the Cleveland Bay core resistome identified in the seawater and seagrass. None of these ARGs were represented exclusively in Cleveland Bay environmental samples. This data suggests that turtle GIT resistomes are similar to that of the core resistome of seawater and seagrass. However, the subtle differences observed for these environmental samples in each location are not represented in the distinct core resistome of the two turtle populations. A redundancy analysis identified that location is more influential on the turtle resistome (44.51%), than other captured physiological factors, such as weight (26.12%), or whether the turtle harboured a tracking device (0.0%). Further suggesting that location of the population of turtles is influential in their resistome composition, despite not being reflective of differences in the core resistome of tested environmental samples. This is suggestive of other external influences exclusive to population on the resistome of Green turtles that may not have been captured in this current environmental sampling dataset. Previous studies have investigated the influence of the mother's microbiome and egg laying beach on turtle hatchling microbiomes, which may have lasting effects on populations (Al-Bahry et al., 2009a, Al-Bahry et al., 2012, Al-Bahry et al., 2011).

Appendix D

Table D. 1: Antibiotic Resistance gene sequences identified via the MEGARes database throughout this study.

OTU_ID	*Resistance_Type*	*Resistance_Class*	*Mechanism_of_Resistance*	*Gene*
MEG_1	Drugs	Aminoglycosides	Aminoglycoside-resistant_16S_ribosomal_subunit_protein	A16S
MEG_1000	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT6
MEG_1016	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH2-DPRIME
MEG_1034	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH3-DPRIME
MEG_1036	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH3-DPRIME
MEG_1037	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH3-DPRIME
MEG_1039	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH3-DPRIME
MEG_1040	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH3-DPRIME
MEG_1042	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH3-DPRIME
MEG_1045	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH3-DPRIME
MEG_1046	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH3-DPRIME
MEG_1049	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH3-DPRIME
MEG_1053	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH3-DPRIME
MEG_1054	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH3-DPRIME
MEG_1055	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH3-DPRIME
MEG_1056	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH3-DPRIME
MEG_1057	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH3-DPRIME
MEG_1058	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH3-DPRIME
MEG_1064	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH3-PRIME
MEG_1068	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH3-PRIME
MEG_1071	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH3-PRIME
MEG_1073	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH3-PRIME
MEG_1076	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH3-PRIME
MEG_1084	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH6
MEG_1085	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH6
MEG_1086	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH6
MEG_1087	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH6
MEG_1088	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH6
MEG_1089	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH6
MEG_1091	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH6
MEG_1093	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH6
MEG_1095	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH6
MEG_1096	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH6
MEG_1097	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH6
MEG_1098	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH7-DPRIME
MEG_1099	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH9
MEG_11	Drugs	Aminoglycosides	Aminoglycoside-resistant_16S_ribosomal_subunit_protein	A16S
MEG_1101	Drugs	Aminoglycosides	Aminoglycoside_O-phosphotransferases	APH9
MEG_1104	Drugs	betalactams	Class_C_betalactamases	AQU
MEG_1105	Drugs	betalactams	Class_C_betalactamases	AQU
MEG_1106	Drugs	betalactams	Class_C_betalactamases	AQU
MEG_1121	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	ARMR
MEG_1122	Drugs	Cationic_antimicrobial_peptides	Polymyxin_B_resistance_regulator	ARNA
MEG_1127	Drugs	Rifampin	Rifampin_ADP-ribosyltransferase_Arr	ARR
MEG_1128	Drugs	Rifampin	Rifampin_ADP-ribosyltransferase_Arr	ARR
MEG_1129	Drugs	Rifampin	Rifampin_ADP-ribosyltransferase_Arr	ARR
MEG_1135	Drugs	Rifampin	Rifampin_ADP-ribosyltransferase_Arr	ARR

MEG_1179	Drugs	Multi-drug_resistance	Multi-drug_RND_efflux_regulator	ASMA
MEG_1180	Drugs	Multi-drug_resistance	Multi-drug_RND_efflux_regulator	ASMA
MEG_1183	Drugs	betalactams	Class_A_betalactamases	AST
MEG_1184	Drugs	betalactams	Class_A_betalactamases	AXC
MEG_1185	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	AXY
MEG_1186	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	AXY
MEG_1187	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	AXYZ
MEG_1189	Drugs	Bacitracin	Undecaprenyl_pyrophosphate_phosphatase	BACA
MEG_1190	Drugs	Bacitracin	Undecaprenyl_pyrophosphate_phosphatase	BACA
MEG_1192	Multi-compound	Drug_and_biocide_and_metal_resistance	Drug_and_biocide_and_metal_RND_efflux_regulator	BAER
MEG_1193	Multi-compound	Drug_and_biocide_and_metal_resistance	Drug_and_biocide_and_metal_RND_efflux_regulator	BAES
MEG_1195	Multi-compound	Drug_and_biocide_and_metal_resistance	Drug_and_biocide_and_metal_RND_efflux_regulator	BAES
MEG_1197	Drugs	Cationic_antimicrobial_peptides	Polymyxin_B_resistance_regulator	BASRS
MEG_1198	Drugs	Cationic_antimicrobial_peptides	Polymyxin_B_resistance_regulator	BASRS
MEG_12	Drugs	Aminoglycosides	Aminoglycoside-resistant_16S_ribosomal_subunit_protein	A16S
MEG_1203	Drugs	betalactams	Class_B_betalactamases	BCII
MEG_1209	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	BCR
MEG_121	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_1210	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	BCR
MEG_1211	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	BCR
MEG_1212	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	BCR1
MEG_1216	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	BCRCM
MEG_1217	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	BCRCM
MEG_1218	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	BCRCM
MEG_1225	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	BEPE
MEG_1234	Drugs	betalactams	Class_B_betalactamases	BJP1
MEG_1235	Drugs	betalactams	Class_B_betalactamases	BJP1
MEG_1236	Drugs	betalactams	Class_A_betalactamases	BKC
MEG_124	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_1240	Drugs	betalactams	Class_A_betalactamases	BLAA
MEG_1246	Drugs	betalactams	Class_A_betalactamases	BLAA
MEG_1274	Drugs	betalactams	Class_B_betalactamases	BLAEAM
MEG_1275	Drugs	betalactams	Class_C_betalactamases	BLAEC
MEG_1276	Drugs	betalactams	Class_C_betalactamases	BLAEC
MEG_1277	Drugs	betalactams	Class_C_betalactamases	BLAEC
MEG_1285	Drugs	betalactams	Class_B_betalactamases	BLAECM
MEG_1286	Drugs	betalactams	Class_A_betalactamases	BLAF
MEG_1287	Drugs	betalactams	Penicillin_binding_protein_regulator	BLAI
MEG_1290	Drugs	betalactams	Class_A_betalactamases	BLAL
MEG_1292	Drugs	betalactams	Class_A_betalactamases	BLAL
MEG_1295	Drugs	betalactams	Class_A_betalactamases	BLAPAM

MEG_13	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC2-PRIME
MEG_1304	Drugs	betalactams	Class_A_betalactamases	BLAS
MEG_1305	Drugs	betalactams	Class_A_betalactamases	BLASGM
MEG_1308	Drugs	betalactams	Class_A_betalactamases	BLASGM
MEG_1310	Drugs	betalactams	Class_A_betalactamases	BLASGM
MEG_1311	Drugs	betalactams	Class_A_betalactamases	BLASGM
MEG_1312	Drugs	betalactams	Class_A_betalactamases	BLASGM
MEG_137	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_1457	Drugs	Glycopeptides	Bleomycin_resistance_protein	BLE
MEG_1458	Drugs	Glycopeptides	Bleomycin_resistance_protein	BLE
MEG_1459	Drugs	Glycopeptides	Bleomycin_resistance_protein	BLE
MEG_1461	Drugs	Glycopeptides	Bleomycin_resistance_protein	BLM
MEG_1462	Drugs	Glycopeptides	Bleomycin_resistance_protein	BLM
MEG_1472	Drugs	betalactams	Class_D_betalactamases	BPU
MEG_1490	Drugs	Cationic_antimicrobial_peptides	Cationic_peptide-resistant_16S_ribosomal_subunit_protein	CAP16S
MEG_1491	Drugs	Multi-drug_resistance	Multi-drug_ABC_efflux_pumps	CARA
MEG_1497	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1498	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1503	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1504	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1505	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1507	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1512	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1518	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1519	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1522	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1523	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1525	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1527	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1528	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1529	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1532	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1533	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1534	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1538	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1540	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1541	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1542	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1544	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1545	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1546	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1547	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_1553	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CAT
MEG_1554	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CAT
MEG_1555	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CAT
MEG_1557	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CAT
MEG_1558	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CAT
MEG_1560	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CATA
MEG_1561	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CATA
MEG_1577	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CATA
MEG_1583	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CATA
MEG_1596	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CATB
MEG_1603	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CATB

MEG_1605	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CATB
MEG_1613	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CATB
MEG_1614	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CATB
MEG_1618	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CATB
MEG_1620	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CATB
MEG_1624	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CATB
MEG_1625	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CATB
MEG_1626	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CATB
MEG_1627	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CATB
MEG_1628	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CATB
MEG_1629	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CATB
MEG_1632	Drugs	Phenicol	Chloramphenicol_acetyltransferases	CATP
MEG_1634	Drugs	betalactams	Class_B_betalactamases	CAU
MEG_1635	Drugs	betalactams	Class_B_betalactamases	CAU
MEG_1636	Drugs	betalactams	Class_A_betalactamases	CBLA
MEG_1643	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	CEOA
MEG_1644	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	CEOB
MEG_1685	Drugs	Multi-drug_resistance	MDR_23S_ribosomal_RNA_methyltransferase	CFRC
MEG_1687	Drugs	betalactams	Class_A_betalactamases	CFX
MEG_1689	Drugs	betalactams	Class_A_betalactamases	CFX
MEG_1690	Drugs	betalactams	Class_A_betalactamases	CFX
MEG_1691	Drugs	betalactams	Class_A_betalactamases	CFX
MEG_1692	Drugs	betalactams	Class_A_betalactamases	CFX
MEG_1693	Drugs	betalactams	Class_A_betalactamases	CFX
MEG_1694	Drugs	betalactams	Class_A_betalactamases	CFX
MEG_1696	Drugs	betalactams	Class_A_betalactamases	CFX
MEG_1697	Drugs	betalactams	Class_A_betalactamases	CFX
MEG_1698	Drugs	betalactams	Class_A_betalactamases	CFX
MEG_1723	Drugs	Multi-drug_resistance	MDR_23S_ribosomal_RNA_methyltransferase	CIPA
MEG_1726	Drugs	Multi-drug_resistance	MDR_23S_ribosomal_RNA_methyltransferase	CLBA
MEG_1731	Drugs	Lipopeptides	Daptomycin-resistant_mutant	CLS
MEG_1755	Drugs	Phenicol	Phenicol_resistance_MFS_efflux_pumps	CMLA
MEG_1761	Drugs	Phenicol	Phenicol_resistance_MFS_efflux_pumps	CMLA
MEG_1767	Drugs	Phenicol	Phenicol_resistance_MFS_efflux_pumps	CMLA
MEG_1769	Drugs	Phenicol	Phenicol_resistance_MFS_efflux_pumps	CMLA
MEG_1770	Drugs	Phenicol	Phenicol_resistance_MFS_efflux_pumps	CMLA
MEG_1772	Drugs	Phenicol	Phenicol_resistance_MFS_efflux_pumps	CMLA
MEG_1775	Drugs	Phenicol	Phenicol_resistance_MFS_efflux_pumps	CMLB
MEG_1776	Drugs	Phenicol	Phenicol_resistance_MFS_efflux_pumps	CMLR
MEG_1777	Drugs	Phenicol	Chloramphenicol_phosphotransferase	CMLV
MEG_1778	Drugs	Phenicol	Chloramphenicol_phosphotransferase	CMLV
MEG_18	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC2-PRIME
MEG_1809	Drugs	betalactams	Class_C_betalactamases	CMY
MEG_182	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_1833	Drugs	betalactams	Class_C_betalactamases	CMY
MEG_1834	Drugs	betalactams	Class_C_betalactamases	CMY
MEG_1883	Drugs	betalactams	Class_C_betalactamases	CMY
MEG_1884	Drugs	betalactams	Class_C_betalactamases	CMY
MEG_19	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC2-PRIME
MEG_191	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_1914	Drugs	betalactams	Class_C_betalactamases	CMY

MEG_1915	Drugs	betalactams	Class_C_betalactamases	CMY
MEG_1916	Drugs	betalactams	Class_C_betalactamases	CMY
MEG_1925	Drugs	betalactams	Class_C_betalactamases	CMY
MEG_193	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_1932	Drugs	betalactams	Class_C_betalactamases	CMY
MEG_195	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_196	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_1969	Drugs	betalactams	Class_C_betalactamases	CMY
MEG_197	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_1970	Drugs	betalactams	Class_C_betalactamases	CMY
MEG_1971	Drugs	betalactams	Class_C_betalactamases	CMY
MEG_198	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_1983	Drugs	betalactams	Class_C_betalactamases	CMY
MEG_1989	Drugs	betalactams	Class_C_betalactamases	CMY
MEG_1992	Drugs	betalactams	Class_C_betalactamases	CMY
MEG_1997	Drugs	betalactams	Class_C_betalactamases	CMY
MEG_2	Drugs	Aminoglycosides	Aminoglycoside-resistant_16S_ribosomal_subunit_protein	A16S
MEG_205	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_206	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_209	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_210	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_2100	Drugs	betalactams	Class_B_betalactamases	CPHA
MEG_2103	Drugs	betalactams	Class_B_betalactamases	CPHA
MEG_2106	Drugs	betalactams	Class_B_betalactamases	CPHA
MEG_2107	Drugs	betalactams	Class_B_betalactamases	CPHA
MEG_2108	Drugs	betalactams	Class_B_betalactamases	CPHA
MEG_2109	Drugs	betalactams	Class_B_betalactamases	CPHA
MEG_2111	Drugs	betalactams	Class_B_betalactamases	CPHA
MEG_2113	Drugs	Phenicol	Chloramphenicol_phosphotransferase	CPT
MEG_2114	Drugs	Phenicol	Chloramphenicol_phosphotransferase	CPT
MEG_2115	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	CPXAR
MEG_2116	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	CPXAR
MEG_2117	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	CPXAR
MEG_2118	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	CPXAR
MEG_2119	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	CPXAR
MEG_212	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_2120	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	CPXAR
MEG_2121	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	CPXAR
MEG_2122	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	CPXAR
MEG_2132	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	CRP
MEG_2133	Drugs	Fluoroquinolones	Fluoroquinolone_resistance_phosphotransferase	CRPP
MEG_23	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC3
MEG_2378	Drugs	betalactams	Class_A_betalactamases	CTX
MEG_242	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_2430	Drugs	betalactams	Class_A_betalactamases	CTX

MEG_2435	Drugs	betalactams	Class_A_betalactamases	CTX
MEG_250	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_251	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_2517	Drugs	Trimethoprim	Dihydrofolate_reductase	DFRA
MEG_252	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_253	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_254	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_256	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_2563	Drugs	Trimethoprim	Dihydrofolate_reductase	DFRB
MEG_2566	Drugs	Trimethoprim	Dihydrofolate_reductase	DFRB
MEG_2577	Drugs	Trimethoprim	Dihydrofolate_reductase	DFRE
MEG_2578	Drugs	Trimethoprim	Dihydrofolate_reductase	DFRF
MEG_2617	Drugs	Trimethoprim	Dihydrofolate_reductase	DHFR
MEG_2619	Drugs	Trimethoprim	Dihydrofolate_reductase	DHFR
MEG_2623	Drugs	Trimethoprim	Dihydrofolate_reductase	DHFR
MEG_2624	Drugs	Trimethoprim	Dihydrofolate_reductase	DHFR
MEG_263	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_2630	Drugs	Trimethoprim	Dihydrofolate_reductase	DHFR
MEG_2632	Drugs	Trimethoprim	Dihydrofolate_reductase	DHFR
MEG_2633	Drugs	Trimethoprim	Dihydrofolate_reductase	DHFR
MEG_2648	Drugs	betalactams	Class_B_betalactamases	DHT
MEG_2670	Drugs	Multi-drug_resistance	Multi-drug_ABC_efflux_pumps	DRRA
MEG_2703	Drugs	betalactams	Class_B_betalactamases	EFM
MEG_2705	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	EFPA
MEG_2706	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_ABC_efflux_pumps	EFRA
MEG_2707	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_ABC_efflux_pumps	EFRB
MEG_2709	Drugs	Mycobacterium_tuberculosis-specific_Drug	Ethambutol_resistant_arabinosyltransferase	EMBA
MEG_2710	Drugs	Mycobacterium_tuberculosis-specific_Drug	Ethambutol_resistant_arabinosyltransferase	EMBB
MEG_2711	Drugs	Mycobacterium_tuberculosis-specific_Drug	Ethambutol_resistant_arabinosyltransferase	EMBB
MEG_2712	Drugs	Mycobacterium_tuberculosis-specific_Drug	Ethambutol_resistant_arabinosyltransferase	EMBC
MEG_2717	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	EMHC
MEG_2718	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	EMHC
MEG_2721	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	EMRA
MEG_2722	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	EMRA
MEG_2723	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	EMRASM
MEG_2724	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	EMRB
MEG_2725	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	EMRB
MEG_2726	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	EMRBSM
MEG_2727	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	EMRCSM
MEG_2729	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	EMRD
MEG_273	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME

MEG_2730	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	EMRD
MEG_2731	Drugs	Multi-drug_resistance	Multi-drug_SMR_efflux_regulator	EMRE
MEG_2732	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	EMRK
MEG_2733	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	EMRK
MEG_2734	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_regulator	EMRR
MEG_2735	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_regulator	EMRRSM
MEG_2736	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	EMRY
MEG_2737	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	EMRY
MEG_2739	Drugs	Cationic_antimicrobial_peptides	Lipid_A_modification	EPTA
MEG_274	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_2740	Drugs	MLS	Macrolide_esterases	EREA
MEG_2741	Drugs	MLS	Macrolide_esterases	EREA
MEG_2742	Drugs	MLS	Macrolide_esterases	EREA
MEG_2743	Drugs	MLS	Macrolide_esterases	EREA
MEG_2744	Drugs	MLS	Macrolide_esterases	EREA
MEG_2745	Drugs	MLS	Macrolide_esterases	EREA
MEG_2746	Drugs	MLS	Macrolide_esterases	EREA
MEG_2747	Drugs	MLS	Macrolide_esterases	EREA
MEG_2748	Drugs	MLS	Macrolide_esterases	EREA
MEG_2749	Drugs	MLS	Macrolide_esterases	EREA
MEG_2750	Drugs	MLS	Macrolide_esterases	EREA
MEG_2751	Drugs	MLS	Macrolide_esterases	EREA
MEG_2752	Drugs	MLS	Macrolide_esterases	EREB
MEG_2753	Drugs	MLS	Macrolide_esterases	EREB
MEG_2754	Drugs	MLS	Macrolide_esterases	ERED
MEG_2755	Drugs	MLS	23S_rRNA_methyltransferases	ERM
MEG_2756	Drugs	MLS	23S_rRNA_methyltransferases	ERM
MEG_2759	Drugs	MLS	23S_rRNA_methyltransferases	ERM
MEG_2761	Drugs	MLS	23S_rRNA_methyltransferases	ERM
MEG_2762	Drugs	MLS	23S_rRNA_methyltransferases	ERM
MEG_2763	Drugs	MLS	23S_rRNA_methyltransferases	ERM
MEG_2768	Drugs	MLS	23S_rRNA_methyltransferases	ERM
MEG_2778	Drugs	MLS	23S_rRNA_methyltransferases	ERM41
MEG_278	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_2787	Drugs	MLS	23S_rRNA_methyltransferases	ERMA
MEG_2789	Drugs	MLS	23S_rRNA_methyltransferases	ERMB
MEG_2793	Drugs	MLS	23S_rRNA_methyltransferases	ERMB
MEG_2794	Drugs	MLS	23S_rRNA_methyltransferases	ERMB
MEG_2795	Drugs	MLS	23S_rRNA_methyltransferases	ERMB
MEG_2796	Drugs	MLS	23S_rRNA_methyltransferases	ERMB
MEG_2797	Drugs	MLS	23S_rRNA_methyltransferases	ERMB
MEG_2799	Drugs	MLS	23S_rRNA_methyltransferases	ERMB
MEG_2801	Drugs	MLS	23S_rRNA_methyltransferases	ERMB
MEG_2803	Drugs	MLS	23S_rRNA_methyltransferases	ERMB
MEG_2804	Drugs	MLS	23S_rRNA_methyltransferases	ERMB
MEG_2805	Drugs	MLS	23S_rRNA_methyltransferases	ERMB
MEG_2806	Drugs	MLS	23S_rRNA_methyltransferases	ERMB

MEG_2826	Drugs	MLS	23S_rRNA_methyltransferases	ERME
MEG_2827	Drugs	MLS	23S_rRNA_methyltransferases	ERME
MEG_2829	Drugs	MLS	23S_rRNA_methyltransferases	ERMF
MEG_2831	Drugs	MLS	23S_rRNA_methyltransferases	ERMF
MEG_2832	Drugs	MLS	23S_rRNA_methyltransferases	ERMF
MEG_2833	Drugs	MLS	23S_rRNA_methyltransferases	ERMF
MEG_2834	Drugs	MLS	23S_rRNA_methyltransferases	ERMG
MEG_2835	Drugs	MLS	23S_rRNA_methyltransferases	ERMG
MEG_2836	Drugs	MLS	23S_rRNA_methyltransferases	ERMG
MEG_2838	Drugs	MLS	23S_rRNA_methyltransferases	ERMN
MEG_2839	Drugs	MLS	23S_rRNA_methyltransferases	ERMO
MEG_2840	Drugs	MLS	23S_rRNA_methyltransferases	ERMO
MEG_2842	Drugs	MLS	23S_rRNA_methyltransferases	ERMQ
MEG_2843	Drugs	MLS	23S_rRNA_methyltransferases	ERMR
MEG_2845	Drugs	MLS	23S_rRNA_methyltransferases	ERMS
MEG_2848	Drugs	MLS	23S_rRNA_methyltransferases	ERMT
MEG_2863	Drugs	betalactams	Class_B_betalactamases	ESP
MEG_2864	Drugs	Phenicol	Chloramphenicol_hydrolase	ESTD
MEG_2865	Drugs	Phenicol	Chloramphenicol_hydrolase	ESTD
MEG_2866	Drugs	Mycobacterium_tuberculosis-specific_Drug	Ethionamide-resistant_mutant	ETHA
MEG_2868	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_regulator	EVGS
MEG_2869	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_regulator	EVGS
MEG_2870	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_regulator	EVGS
MEG_2872	Drugs	betalactams	Class_A_betalactamases	EXO
MEG_2880	Drugs	Elfamycins	Elfamycin_efflux_pumps	FACT
MEG_2881	Drugs	betalactams	Class_A_betalactamases	FAR1
MEG_2882	Drugs	betalactams	Class_A_betalactamases	FAR1
MEG_2907	Drugs	betalactams	Class_B_betalactamases	FEZ
MEG_2912	Drugs	Phenicol	Phenicol_resistance_MFS_efflux_pumps	FLOR
MEG_2915	Drugs	Phenicol	Phenicol_resistance_MFS_efflux_pumps	FLOR
MEG_2917	Drugs	Phenicol	Phenicol_resistance_MFS_efflux_pumps	FLOR
MEG_2920	Drugs	Phenicol	Phenicol_resistance_MFS_efflux_pumps	FLOR
MEG_2922	Drugs	Phenicol	Phenicol_resistance_MFS_efflux_pumps	FLOR
MEG_2923	Drugs	Phenicol	Phenicol_resistance_MFS_efflux_pumps	FLOR
MEG_2926	Drugs	Phenicol	Phenicol_resistance_MFS_efflux_pumps	FLOR
MEG_2932	Drugs	Aminoglycosides	16S_rRNA_methyltransferases	FMRO
MEG_2933	Drugs	Mycobacterium_tuberculosis-specific_Drug	Para-aminosalicylic_acid_resistant_mutant	FOLC
MEG_2941	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	FOLP
MEG_2942	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	FOLP
MEG_2947	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	FOLP
MEG_2952	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	FOLP
MEG_2954	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	FOLP
MEG_2955	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	FOLP
MEG_2958	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	FOLP
MEG_2959	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	FOLP
MEG_2961	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	FOLP
MEG_2964	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	FOLP
MEG_2965	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	FOLP
MEG_2969	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	FOLP
MEG_297	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME

MEG_2972	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	FOLP
MEG_2973	Drugs	Fosfomycin	Fosfomycin_phosphorylation	FOMA
MEG_2975	Drugs	Fosfomycin	Fosfomycin_phosphorylation	FOMB
MEG_2984	Drugs	Fosfomycin	Fosfomycin_thiol_transferases	FOSA
MEG_2987	Drugs	Fosfomycin	Fosfomycin_thiol_transferases	FOSA
MEG_2991	Drugs	Fosfomycin	Fosfomycin_thiol_transferases	FOSA
MEG_2993	Drugs	Fosfomycin	Fosfomycin_thiol_transferases	FOSA
MEG_2994	Drugs	Fosfomycin	Fosfomycin_thiol_transferases	FOSA
MEG_2995	Drugs	Fosfomycin	Fosfomycin_thiol_transferases	FOSA
MEG_2996	Drugs	Fosfomycin	Fosfomycin_thiol_transferases	FOSA
MEG_3	Drugs	Aminoglycosides	Aminoglycoside-resistant_16S_ribosomal_subunit_protein	A16S
MEG_3000	Drugs	Fosfomycin	Fosfomycin_thiol_transferases	FOSA
MEG_3001	Drugs	Fosfomycin	Fosfomycin_thiol_transferases	FOSA
MEG_3002	Drugs	Fosfomycin	Fosfomycin_thiol_transferases	FOSA
MEG_3008	Drugs	Fosfomycin	Fosfomycin_thiol_transferases	FOSB
MEG_301	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_3022	Drugs	Fosfomycin	Fosfomycin_thiol_transferases	FOSG
MEG_3025	Drugs	Fosfomycin	Fosfomycin_thiol_transferases	FOSX
MEG_305	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_3065	Drugs	Fusidic_acid	Fusidic_acid-resistant_mutation	FUSA
MEG_3069	Drugs	Fusidic_acid	Ribosomal_zinc-binding_protein	FUSD
MEG_307	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_3070	Drugs	Fusidic_acid	Ribosomal_zinc-binding_protein	FUSD
MEG_3071	Drugs	Fusidic_acid	Fusidic_acid-resistant_mutation	FUSE
MEG_3074	Drugs	Fusidic_acid	Fusidic_acid_esterases	FUSH
MEG_3081	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	GADW
MEG_3082	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	GADW
MEG_3083	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	GADX
MEG_3085	Drugs	betalactams	Class_A_betalactamases	GES
MEG_309	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_3090	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3091	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3092	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3093	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3094	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3096	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3097	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3099	Drugs	betalactams	Class_A_betalactamases	GES
MEG_31	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC3
MEG_3100	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3102	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3103	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3104	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3105	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3106	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3107	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3108	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3109	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3110	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3111	Drugs	betalactams	Class_A_betalactamases	GES

MEG_3116	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3117	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3118	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3119	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3123	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3124	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3127	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3128	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3129	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3130	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3131	Drugs	betalactams	Class_A_betalactamases	GES
MEG_3139	Drugs	MLS	Macrolide_glycosyltransferases	GIMA
MEG_3144	Drugs	Fosfomycin	Fosfomycin_target_mutation	GLPT
MEG_316	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_3169	Drugs	betalactams	Class_A_betalactamases	GPC
MEG_3174	Drugs	Aminoglycosides	16S_rRNA_methyltransferases	GRM
MEG_3175	Drugs	Lipopeptides	Daptomycin-resistant_mutant	GSHF
MEG_3178	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3180	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3183	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3187	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3188	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3189	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3192	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3193	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3197	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3200	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3201	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3205	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3206	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3209	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3210	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3211	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3215	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3216	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3217	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3219	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3220	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3226	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3227	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3228	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3231	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3232	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3234	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3237	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_3238	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRB
MEG_3243	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRB
MEG_3244	Drugs	Aminocoumarins	Aminocoumarin-resistant_DNA_topoisomerases	GYRBA
MEG_3245	Drugs	Aminocoumarins	Aminocoumarin-resistant_DNA_topoisomerases	GYRBA
MEG_3249	Drugs	Aminocoumarins	Aminocoumarin-resistant_DNA_topoisomerases	GYRBA
MEG_3250	Drugs	Aminocoumarins	Aminocoumarin-resistant_DNA_topoisomerases	GYRBA
MEG_3271	Drugs	Multi-drug_resistance	Multi-drug_RND_efflux_pumps	HNS
MEG_3293	Drugs	Lipopeptides	Colistin_phosphoethanolamine_transferase	ICR

MEG_3295	Drugs	Mupirocin	Mupirocin-resistant_isoleucyl-tRNA_synthetase	ILES
MEG_3296	Drugs	Mupirocin	Mupirocin-resistant_isoleucyl-tRNA_synthetase	ILES
MEG_333	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_34	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC3
MEG_3429	Drugs	Mycobacterium_tuberculosis-specific_Drug	Isoniazid-resistant_mutant	INHA
MEG_3431	Drugs	Mycobacterium_tuberculosis-specific_Drug	Ethambutol-resistant_mutant	INIB
MEG_3432	Drugs	Mycobacterium_tuberculosis-specific_Drug	Ethambutol-resistant_mutant	INIC
MEG_3433	Drugs	Multi-drug_resistance	Multi-drug_MFS_efflux_pumps	IRFA
MEG_344	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_3442	Drugs	Aminoglycosides	16S_rRNA_methyltransferases	KAMB
MEG_3443	Drugs	Aminoglycosides	16S_rRNA_methyltransferases	KAMB
MEG_3444	Drugs	Aminoglycosides	16S_rRNA_methyltransferases	KAMB
MEG_3445	Drugs	Mycobacterium_tuberculosis-specific_Drug	Isoniazid-resistant_mutant	KASA
MEG_3446	Drugs	Mycobacterium_tuberculosis-specific_Drug	Isoniazid-resistant_mutant	KATG
MEG_3447	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	KDEA
MEG_3448	Drugs	Aminoglycosides	Aminoglycoside_efflux_pumps	KDPE
MEG_3450	Drugs	Aminoglycosides	16S_rRNA_methyltransferases	KGMB
MEG_3459	Drugs	Aminoglycosides	16S_rRNA_methyltransferases	KMR
MEG_346	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_3460	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	KMRA
MEG_35	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC3
MEG_3512	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	KPN
MEG_3513	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	KPN
MEG_3514	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	KPNE
MEG_3515	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	KPNF
MEG_3516	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	KPNO
MEG_352	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_3525	Drugs	betalactams	Class_B_betalactamases	L1
MEG_3532	Drugs	betalactams	Class_B_betalactamases	L1
MEG_3533	Drugs	betalactams	Class_B_betalactamases	L1
MEG_3538	Drugs	betalactams	Class_D_betalactamases	LCR
MEG_3541	Drugs	betalactams	Class_A_betalactamases	LEN
MEG_3548	Drugs	betalactams	Class_A_betalactamases	LEN
MEG_355	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_3558	Drugs	betalactams	Class_A_betalactamases	LEN
MEG_3560	Drugs	betalactams	Class_A_betalactamases	LEN
MEG_3568	Drugs	betalactams	Class_A_betalactamases	LEN
MEG_3569	Drugs	betalactams	Class_A_betalactamases	LEN
MEG_3570	Drugs	betalactams	Class_A_betalactamases	LEN
MEG_3574	Drugs	betalactams	Class_A_betalactamases	LEN
MEG_3576	Drugs	betalactams	Class_A_betalactamases	LEN
MEG_3579	Drugs	betalactams	Class_A_betalactamases	LEN
MEG_3586	Drugs	Lipopeptides	Daptomycin-resistant_mutant	LIAFSR
MEG_3587	Drugs	Lipopeptides	Daptomycin-resistant_mutant	LIAFSR
MEG_3588	Drugs	Lipopeptides	Daptomycin-resistant_mutant	LIAFSR
MEG_3593	Drugs	MLS	23S_rRNA_methyltransferases	LLMA
MEG_3594	Drugs	Multi-drug_resistance	Multi-drug_ABC_efflux_pumps	LMRA

MEG_3596	Drugs	Multi-drug_resistance	Multi-drug_ABC_efflux_pumps	LMRC
MEG_3601	Drugs	MLS	Lincosamide_nucleotidyltransferases	LNUA
MEG_3606	Drugs	MLS	Lincosamide_nucleotidyltransferases	LNUA
MEG_3609	Drugs	MLS	Lincosamide_nucleotidyltransferases	LNUB
MEG_3610	Drugs	MLS	Lincosamide_nucleotidyltransferases	LNUC
MEG_3611	Drugs	MLS	Lincosamide_nucleotidyltransferases	LNUC
MEG_3613	Drugs	MLS	Lincosamide_nucleotidyltransferases	LNUF
MEG_3615	Drugs	MLS	Lincosamide_nucleotidyltransferases	LNUF
MEG_3616	Drugs	MLS	Lincosamide_nucleotidyltransferases	LNUF
MEG_3617	Drugs	MLS	Lincosamide_nucleotidyltransferases	LNUF
MEG_3618	Drugs	MLS	Lincosamide_nucleotidyltransferases	LNUF
MEG_3619	Drugs	MLS	Lincosamide_nucleotidyltransferases	LNUF
MEG_3621	Drugs	MLS	Lincosamide_nucleotidyltransferases	LNUP
MEG_3626	Drugs	Lipopeptides	Colistin-resistant_mutant	LPXA
MEG_3627	Drugs	Lipopeptides	Colistin-resistant_mutant	LPXC
MEG_3630	Drugs	betalactams	Class_B_betalactamases	LRA
MEG_364	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_3641	Drugs	MLS	MLS_resistance_ABC_efflux_pumps	LSA
MEG_3642	Drugs	MLS	MLS_resistance_ABC_efflux_pumps	LSA
MEG_3643	Drugs	MLS	MLS_resistance_ABC_efflux_pumps	LSA
MEG_3644	Drugs	MLS	MLS_resistance_ABC_efflux_pumps	LSA
MEG_3645	Drugs	MLS	MLS_resistance_ABC_efflux_pumps	LSA
MEG_3647	Drugs	Multi-drug_resistance	Multi-drug_ABC_efflux_pumps	LSAC
MEG_3648	Drugs	Multi-drug_resistance	Multi-drug_ABC_efflux_pumps	LSAE
MEG_3656	Drugs	betalactams	Class_A_betalactamases	M1
MEG_3659	Drugs	betalactams	Class_A_betalactamases	MAL
MEG_3660	Drugs	betalactams	Class_A_betalactamases	MAL
MEG_3661	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	MARA
MEG_3662	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	MARA
MEG_3663	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	MARR
MEG_3664	Drugs	betalactams	Class_C_betalactamases	MCA
MEG_3677	Drugs	Lipopeptides	Colistin_phosphoethanolamine_transferase	MCR
MEG_369	Drugs	Fosfomycin	Fosfomycin_MFS_efflux_pump	ABAF
MEG_370	Drugs	Fosfomycin	Fosfomycin_MFS_efflux_pump	ABAQ
MEG_3705	Drugs	Lipopeptides	Colistin_phosphoethanolamine_transferase	MCR
MEG_372	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MATE_efflux_pumps	ABEM
MEG_3724	Drugs	Lipopeptides	Colistin_phosphoethanolamine_transferase	MCR
MEG_373	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MATE_efflux_pumps	ABEM
MEG_3736	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	MDFA
MEG_3737	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	MDFA
MEG_3738	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	MDFA
MEG_3746	Multi-compound	Drug_and_biocide_and_metal_resistance	Drug_and_biocide_and_metal_RND_efflux_pumps	MDTA
MEG_3747	Multi-compound	Drug_and_biocide_and_metal_resistance	Drug_and_biocide_and_metal_RND_efflux_pumps	MDTB
MEG_3748	Multi-compound	Drug_and_biocide_and_metal_resistance	Drug_and_biocide_and_metal_RND_efflux_pumps	MDTB

MEG_3749	Multi-compound	Drug_and_biocide_and_metal_resistance	Drug_and_biocide_and_metal_RND_efflux_pumps	MDTC
MEG_3750	Multi-compound	Drug_and_biocide_and_metal_resistance	Drug_and_biocide_and_metal_RND_efflux_pumps	MDTC
MEG_3751	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MDTE
MEG_3752	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MDTF
MEG_3753	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	MDTG
MEG_3754	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	MDTG
MEG_3755	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	MDTH
MEG_3756	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	MDTI
MEG_3757	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	MDTJ
MEG_3758	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MATE_efflux_pumps	MDTK
MEG_3759	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MATE_efflux_pumps	MDTK
MEG_3761	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	MDTM
MEG_3762	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	MDTM
MEG_3763	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MDTN
MEG_3764	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MDTO
MEG_3765	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MDTP
MEG_380	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	ABES
MEG_3809	Drugs	MLS	MLS_resistance_MFS_efflux_pumps	MEFA
MEG_3812	Drugs	MLS	MLS_resistance_MFS_efflux_pumps	MEFA
MEG_3813	Drugs	MLS	MLS_resistance_MFS_efflux_pumps	MEFA
MEG_3815	Drugs	MLS	MLS_resistance_MFS_efflux_pumps	MEFA
MEG_3817	Drugs	MLS	MLS_resistance_MFS_efflux_pumps	MEFA
MEG_3818	Drugs	MLS	MLS_resistance_MFS_efflux_pumps	MEFA
MEG_3819	Drugs	MLS	MLS_resistance_MFS_efflux_pumps	MEFA
MEG_3820	Drugs	MLS	MLS_resistance_MFS_efflux_pumps	MEFA
MEG_3821	Drugs	MLS	MLS_resistance_MFS_efflux_pumps	MEFA
MEG_3822	Drugs	MLS	MLS_resistance_MFS_efflux_pumps	MEFA
MEG_3824	Drugs	MLS	MLS_resistance_MFS_efflux_pumps	MEFA
MEG_3825	Drugs	MLS	MLS_resistance_MFS_efflux_pumps	MEFA
MEG_3826	Drugs	MLS	MLS_resistance_MFS_efflux_pumps	MEFA
MEG_3827	Drugs	MLS	MLS_resistance_MFS_efflux_pumps	MEFB
MEG_3828	Drugs	MLS	MLS_resistance_MFS_efflux_pumps	MEFC
MEG_3830	Drugs	MLS	MLS_resistance_MFS_efflux_pumps	MEFE
MEG_3899	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXA
MEG_3900	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXA
MEG_3901	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXB

MEG_3902	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXB
MEG_3903	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXB
MEG_3904	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXC
MEG_3905	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXC
MEG_3906	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXC
MEG_3907	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXD
MEG_3908	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXE
MEG_3909	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXE
MEG_3911	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXF
MEG_3912	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXF
MEG_3913	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXG
MEG_3914	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXH
MEG_3915	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXI
MEG_3916	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXJ
MEG_3917	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXK
MEG_3918	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	MEXL
MEG_3919	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXN
MEG_3920	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXN
MEG_3921	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXQ
MEG_3922	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXQ
MEG_3923	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXQ
MEG_3924	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	MEXR
MEG_3925	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	MEXR
MEG_3926	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	MEXR
MEG_3927	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	MEXR
MEG_3928	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	MEXR
MEG_3929	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	MEXR
MEG_3930	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	MEXR
MEG_3931	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	MEXS

MEG_3932	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	MEXT
MEG_3933	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	MEXT
MEG_3934	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	MEXT
MEG_3937	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXX
MEG_3938	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXX
MEG_3939	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXY
MEG_3940	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	MEXY
MEG_3941	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	MEXZ
MEG_3944	Drugs	MLS	Macrolide_glycosyltransferases	MGT
MEG_3945	Drugs	MLS	Macrolide_glycosyltransferases	MGT
MEG_3975	Drugs	MLS	Macrolide-resistant_23S_rRNA_mutation	MLS23S
MEG_3976	Drugs	MLS	Macrolide-resistant_23S_rRNA_mutation	MLS23S
MEG_3977	Drugs	MLS	Macrolide-resistant_23S_rRNA_mutation	MLS23S
MEG_3978	Drugs	MLS	Macrolide-resistant_23S_rRNA_mutation	MLS23S
MEG_3980	Drugs	MLS	Macrolide-resistant_23S_rRNA_mutation	MLS23S
MEG_3982	Drugs	MLS	Macrolide-resistant_23S_rRNA_mutation	MLS23S
MEG_3984	Drugs	MLS	Macrolide-resistant_23S_rRNA_mutation	MLS23S
MEG_3985	Drugs	MLS	Macrolide-resistant_23S_rRNA_mutation	MLS23S
MEG_3986	Drugs	MLS	Macrolide-resistant_23S_rRNA_mutation	MLS23S
MEG_3987	Drugs	MLS	Macrolide-resistant_23S_rRNA_mutation	MLS23S
MEG_3988	Drugs	MLS	Macrolide-resistant_23S_rRNA_mutation	MLS23S
MEG_399	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	ACRA
MEG_3990	Drugs	MLS	Macrolide-resistant_23S_rRNA_mutation	MLS23S
MEG_3992	Drugs	MLS	Macrolide-resistant_23S_rRNA_mutation	MLS23S
MEG_3993	Drugs	MLS	Macrolide-resistant_23S_rRNA_mutation	MLS23S
MEG_3994	Drugs	MLS	Macrolide-resistant_23S_rRNA_mutation	MLS23S
MEG_4	Drugs	Aminoglycosides	Aminoglycoside-resistant_16S_ribosomal_subunit_protein	A16S
MEG_40	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC3
MEG_400	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	ACRA
MEG_401	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	ACRB
MEG_402	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	ACRB
MEG_4024	Drugs	betalactams	Class_C_betalactamases	MOX
MEG_4027	Drugs	MLS	Macrolide_phosphotransferases	MPH
MEG_4029	Drugs	MLS	Macrolide_phosphotransferases	MPHA
MEG_403	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	ACRC
MEG_4030	Drugs	MLS	Macrolide_phosphotransferases	MPHA
MEG_4033	Drugs	MLS	Macrolide_phosphotransferases	MPHB
MEG_404	Multi-compound	Drug_and_biocide_and_metal_resistance	Drug_and_biocide_and_metal_RND_efflux_pumps	ACRD
MEG_4042	Drugs	MLS	Macrolide_phosphotransferases	MPHE
MEG_4043	Drugs	MLS	Macrolide_phosphotransferases	MPHE
MEG_4044	Drugs	MLS	Macrolide_phosphotransferases	MPHE

MEG_4045	Drugs	MLS	Macrolide_phosphotransferases	MPHG
MEG_4046	Drugs	MLS	Macrolide_phosphotransferases	MPHH
MEG_405	Multi-compound	Drug_and_biocide_and_metal_resistance	Drug_and_biocide_and_metal_RND_efflux_pumps	ACRD
MEG_4052	Drugs	MLS	Macrolide_phosphotransferases	MPHO
MEG_406	Multi-compound	Drug_and_biocide_and_metal_resistance	Drug_and_biocide_and_metal_RND_efflux_pumps	ACRD
MEG_4061	Drugs	Multi-drug_resistance	Multi-drug_ABC_efflux_pumps	MSBA
MEG_4062	Drugs	betalactams	Class_B_betalactamases	MSI
MEG_407	Multi-compound	Drug_and_biocide_and_metal_resistance	Drug_and_biocide_and_metal_RND_efflux_pumps	ACRD
MEG_4073	Drugs	MLS	MLS_resistance_ABC_efflux_pumps	MSRD
MEG_4074	Drugs	MLS	MLS_resistance_ABC_efflux_pumps	MSRD
MEG_4075	Drugs	MLS	MLS_resistance_ABC_efflux_pumps	MSRE
MEG_4078	Drugs	Multi-drug_resistance	Multi-drug_RND_efflux_regulator	MTRAD
MEG_408	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	ACRE
MEG_409	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	ACRF
MEG_4095	Drugs	Fosfomycin	Fosfomycin_target_mutation	MURA
MEG_4096	Drugs	Fosfomycin	Fosfomycin_target_mutation	MURA
MEG_410	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	ACRF
MEG_4100	Drugs	Multi-drug_resistance	Multi-drug_RND_efflux_pumps	MUXA
MEG_4101	Drugs	Multi-drug_resistance	Multi-drug_RND_efflux_pumps	MUXB
MEG_4102	Drugs	Multi-drug_resistance	Multi-drug_RND_efflux_pumps	MUXC
MEG_4103	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	MVRC
MEG_4106	Drugs	MLS	23S_rRNA_methyltransferases	MYRA
MEG_4109	Drugs	Multi-drug_resistance	Multi-drug_RND_efflux_pumps	NALC
MEG_411	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	ACRR
MEG_4110	Drugs	Multi-drug_resistance	Multi-drug_RND_efflux_regulator	NALD
MEG_412	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	ACRR
MEG_413	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	ACRR
MEG_4130	Drugs	Mycobacterium_tuberculosis-specific_Drug	Isoniazid-resistant_mutant	NDH
MEG_4131	Drugs	Mycobacterium_tuberculosis-specific_Drug	Isoniazid-resistant_mutant	NDH
MEG_4132	Drugs	Mycobacterium_tuberculosis-specific_Drug	Isoniazid-resistant_mutant	NDH
MEG_414	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	ACRR
MEG_415	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	ACRS
MEG_4161	Drugs	Multi-drug_resistance	Multi-drug_RND_efflux_pumps	NFXB
MEG_4207	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	NORA
MEG_4212	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MATE_efflux_pumps	NORM
MEG_4214	Drugs	Aminocoumarins	Aminocoumarin_efflux_pump	NOVA
MEG_4216	Drugs	betalactams	Class_D_betalactamases	NPS
MEG_4222	Drugs	Thiopeptides	Thiostrepton_23S_rRNA_methyltransferases	NSHR
MEG_4223	Drugs	Oxazolidinone	Oxazolidinone-resistant_23S_rRNA_mutation	O23S
MEG_4227	Drugs	betalactams	Class_C_betalactamases	OCH
MEG_4229	Drugs	betalactams	Class_C_betalactamases	OCH

MEG_4230	Drugs	betalactams	Class_C_betalactamases	OCH
MEG_4231	Drugs	betalactams	Class_C_betalactamases	OCH
MEG_4232	Drugs	betalactams	Class_C_betalactamases	OCH
MEG_4275	Drugs	MLS	MLS_resistance_ABC_efflux_pumps	OLEB
MEG_4276	Drugs	MLS	MLS_resistance_ABC_efflux_pumps	OLEC
MEG_4277	Drugs	MLS	Macrolide_glycosyltransferases	OLED
MEG_4278	Drugs	MLS	Macrolide_glycosyltransferases	OLEI
MEG_4279	Drugs	betalactams	Mutant_porin_proteins	OMP36
MEG_4280	Drugs	betalactams	Mutant_porin_proteins	OMP36
MEG_4281	Drugs	betalactams	Mutant_porin_proteins	OMP36
MEG_4282	Drugs	betalactams	Mutant_porin_proteins	OMP36
MEG_4283	Drugs	betalactams	Mutant_porin_proteins	OMP37
MEG_4286	Drugs	Multi-drug_resistance	MDR_mutant_porin_proteins	OMPF
MEG_4289	Drugs	betalactams	Mutant_porin_proteins	OMPFB
MEG_4290	Drugs	betalactams	Mutant_porin_proteins	OMPR
MEG_4291	Drugs	Multi-drug_resistance	Multi-drug_RND_efflux_pumps	OPCM
MEG_4292	Drugs	Multi-drug_resistance	Multi-drug_RND_efflux_pumps	OPMB
MEG_4293	Drugs	Multi-drug_resistance	Multi-drug_RND_efflux_pumps	OPMD
MEG_4294	Drugs	Multi-drug_resistance	Multi-drug_RND_efflux_pumps	OPMH
MEG_4295	Drugs	Multi-drug_resistance	Multi-drug_RND_efflux_pumps	OPRA
MEG_4296	Drugs	betalactams	Mutant_porin_proteins	OPRD
MEG_4301	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	OPRN
MEG_4302	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	OPRN
MEG_4303	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	OPRZ
MEG_4325	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	OQXA
MEG_4326	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	OQXA
MEG_4327	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	OQXA
MEG_4329	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	OQXA
MEG_4330	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	OQXA
MEG_4331	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	OQXA
MEG_4332	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	OQXA
MEG_4333	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	OQXA
MEG_4335	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	OQXA
MEG_4336	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	OQXB
MEG_4345	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	OQXB
MEG_4346	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	OQXB
MEG_4347	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	OQXB
MEG_4359	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	OQXB
MEG_4378	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	OTRA

MEG_4379	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	OTRA
MEG_4380	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	OTRB
MEG_4382	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	OTRB
MEG_4383	Drugs	Tetracyclines	Tetracycline_resistance_ABC_efflux_pumps	OTRC
MEG_4408	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4411	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4412	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4417	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4418	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4419	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4420	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4421	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4422	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4423	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_45	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC3
MEG_4513	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4515	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4533	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4541	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4542	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4544	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4595	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4598	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4600	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4601	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4605	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4608	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4621	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4624	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4627	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4671	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4672	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4673	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4674	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4675	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4676	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4677	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4678	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4720	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4727	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4729	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4730	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4731	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4732	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4734	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4739	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4745	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4747	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4748	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4749	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4750	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4751	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4757	Drugs	betalactams	Class_D_betalactamases	OXA

MEG_4758	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4762	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4763	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4765	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4770	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4771	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4773	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4774	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4775	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4776	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4777	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4781	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4787	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4791	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4792	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4797	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_48	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC3
MEG_4809	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4810	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4835	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4841	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4842	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4845	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4848	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4849	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4851	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4867	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4868	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4871	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4886	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4889	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4911	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4919	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4921	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4923	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4926	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4932	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4934	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4965	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_497	Drugs	betalactams	Class_C_betalactamases	ACT
MEG_4971	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4973	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4974	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4975	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4976	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4977	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4985	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_4986	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5	Drugs	Aminoglycosides	Aminoglycoside-resistant_16S_ribosomal_subunit_protein	A16S
MEG_5004	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5005	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5008	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5044	Drugs	betalactams	Class_D_betalactamases	OXA

MEG_5045	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5060	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5064	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5085	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5086	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5090	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5095	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5145	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5148	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5149	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5150	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5154	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5156	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5157	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5159	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5160	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5168	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5171	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5172	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5175	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5178	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5179	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5180	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5181	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_52	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC3
MEG_5204	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5207	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5208	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5209	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5212	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5214	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5217	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5218	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5223	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5224	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5249	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5259	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5260	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5261	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5262	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5266	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5267	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5269	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5277	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_5288	Drugs	betalactams	Class_A_betalactamases	OXY
MEG_53	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC3
MEG_5313	Drugs	betalactams	Class_A_betalactamases	OXY
MEG_5322	Drugs	Pactamycin	Pactamycin-resistant_16S_ribosomal_subunit_protein	P16S
MEG_5325	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	PARC
MEG_5332	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	PARC
MEG_5333	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	PARC
MEG_5334	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	PARC
MEG_5337	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	PARC

MEG_5341	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	PARC
MEG_5342	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	PARC
MEG_5348	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	PARC
MEG_5350	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	PARC
MEG_5353	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	PARC
MEG_5357	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	PARC
MEG_5360	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	PARC
MEG_5361	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	PARC
MEG_5363	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	PARC
MEG_5364	Drugs	Aminocoumarins	Aminocoumarin-resistant_DNA_topoisomerases	PARE
MEG_5365	Drugs	Aminocoumarins	Aminocoumarin-resistant_DNA_topoisomerases	PARE
MEG_5367	Drugs	Aminocoumarins	Aminocoumarin-resistant_DNA_topoisomerases	PARE
MEG_5368	Drugs	Aminocoumarins	Aminocoumarin-resistant_DNA_topoisomerases	PARE
MEG_5369	Drugs	Aminocoumarins	Aminocoumarin-resistant_DNA_topoisomerases	PARE
MEG_5373	Drugs	Aminocoumarins	Aminocoumarin-resistant_DNA_topoisomerases	PARE
MEG_5374	Drugs	Aminocoumarins	Aminocoumarin-resistant_DNA_topoisomerases	PARE
MEG_5380	Drugs	Aminocoumarins	Aminocoumarin-resistant_DNA_topoisomerases	PARE
MEG_5381	Drugs	Aminocoumarins	Aminocoumarin-resistant_DNA_topoisomerases	PARE
MEG_5382	Drugs	Aminocoumarins	Aminocoumarin-resistant_DNA_topoisomerases	PARE
MEG_5386	Drugs	Aminocoumarins	Aminocoumarin-resistant_DNA_topoisomerases	PARE
MEG_5388	Drugs	Aminocoumarins	Aminocoumarin-resistant_DNA_topoisomerases	PARE
MEG_5389	Drugs	Aminocoumarins	Aminocoumarin-resistant_DNA_topoisomerases	PARE
MEG_5393	Drugs	Aminocoumarins	Aminocoumarin-resistant_DNA_topoisomerases	PARE
MEG_5394	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	PAREF
MEG_5396	Drugs	Aminocoumarins	Aminocoumarin-resistant_DNA_topoisomerases	PARY
MEG_54	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC3
MEG_5400	Drugs	betalactams	Penicillin_binding_protein	PBP2
MEG_5435	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5437	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5441	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5458	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5464	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5467	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5469	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5470	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5481	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5485	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5487	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5488	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5490	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5499	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_55	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC3
MEG_5502	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5518	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5530	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5531	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5537	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5540	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5541	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5557	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5561	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5563	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5566	Drugs	betalactams	Class_C_betalactamases	PDC

MEG_5568	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5569	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5570	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5571	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5574	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5576	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5580	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5584	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5585	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5587	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5588	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5589	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5591	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5592	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5593	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5594	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5595	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5596	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5597	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5600	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5601	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5602	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5606	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5607	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5608	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5609	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5610	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5611	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5612	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5614	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5621	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5624	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5625	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5630	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5631	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5633	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5634	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5635	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5636	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5637	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5639	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5640	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5641	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5642	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5643	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5644	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5660	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5664	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5667	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5669	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5670	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5675	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5676	Drugs	betalactams	Class_C_betalactamases	PDC

MEG_5677	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5687	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5688	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5689	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5690	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5691	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5692	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5694	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5695	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5696	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5698	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5700	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5701	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5703	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5704	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5705	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5724	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5732	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5733	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5737	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5738	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5740	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5743	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5744	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_5751	Drugs	betalactams	Class_A_betalactamases	PENA
MEG_5752	Drugs	betalactams	Class_A_betalactamases	PENA
MEG_5753	Drugs	betalactams	Class_A_betalactamases	PENI
MEG_5754	Drugs	betalactams	Class_A_betalactamases	PENI
MEG_5755	Drugs	betalactams	Class_A_betalactamases	PENI
MEG_5756	Drugs	betalactams	Class_A_betalactamases	PENI
MEG_5781	Drugs	Phenicol	Phenicol-resistant_23S_rRNA_mutation	PH23S
MEG_5782	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_ABC_efflux_regulator	PHOB
MEG_5783	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_ABC_efflux_regulator	PHOB
MEG_5784	Drugs	Lipopeptides	Colistin-resistant_mutant	PHOP
MEG_5785	Drugs	Lipopeptides	Colistin-resistant_mutant	PHOQ
MEG_5786	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_regulator	PHOR
MEG_5793	Drugs	betalactams	Class_A_betalactamases	PME
MEG_5794	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MATE_efflux_pumps	PMPM
MEG_58	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC3
MEG_5801	Drugs	Cationic_antimicrobial_peptides	Lipid_A_modification	PMRF
MEG_5804	Drugs	betalactams	Class_B_betalactamases	PNGM
MEG_5805	Drugs	betalactams	Class_B_betalactamases	POM
MEG_5806	Drugs	betalactams	Class_B_betalactamases	POM
MEG_5807	Drugs	betalactams	Class_B_betalactamases	POM
MEG_5810	Drugs	betalactams	Class_A_betalactamases	PSE
MEG_5811	Drugs	betalactams	Class_A_betalactamases	PSE
MEG_5819	Drugs	Fosfomycin	Fosfomycin_target_mutation	PTSL
MEG_5825	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACC

MEG_5826	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACC
MEG_5827	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACE
MEG_5828	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACE
MEG_5829	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACEDELTA1
MEG_5830	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACEDELTA1
MEG_5831	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACEDELTA1
MEG_5832	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACF
MEG_5833	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACF
MEG_5834	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACF
MEG_5835	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACF
MEG_5836	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACF
MEG_5837	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACF
MEG_5838	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACF
MEG_5839	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACG
MEG_5840	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACG
MEG_5841	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACG
MEG_5842	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACG
MEG_5843	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACG
MEG_5844	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACG
MEG_5845	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACG
MEG_5848	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACH
MEG_5849	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACJ
MEG_5850	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACK
MEG_5851	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACL
MEG_5852	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACL
MEG_5853	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACL
MEG_5854	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACL
MEG_5855	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACPB
MEG_5858	Drugs	Fluoroquinolones	Quinolone_active_efflux	QEPA
MEG_5869	Drugs	Fluoroquinolones	Quinolone_resistance_protein_Qnr	QNRA

MEG_5870	Drugs	Fluoroquinolones	Quinolone_resistance_protein_Qnr	QNRA
MEG_5871	Drugs	Fluoroquinolones	Quinolone_resistance_protein_Qnr	QNRA
MEG_5872	Drugs	Fluoroquinolones	Quinolone_resistance_protein_Qnr	QNRA
MEG_5874	Drugs	Fluoroquinolones	Quinolone_resistance_protein_Qnr	QNRA
MEG_5875	Drugs	Fluoroquinolones	Quinolone_resistance_protein_Qnr	QNRA
MEG_5876	Drugs	Fluoroquinolones	Quinolone_resistance_protein_Qnr	QNRA
MEG_5877	Drugs	Fluoroquinolones	Quinolone_resistance_protein_Qnr	QNRA
MEG_5904	Drugs	Fluoroquinolones	Quinolone_resistance_protein_Qnr	QNRB
MEG_5949	Drugs	Fluoroquinolones	Quinolone_resistance_protein_Qnr	QNRB
MEG_5952	Drugs	Fluoroquinolones	Quinolone_resistance_protein_Qnr	QNRB
MEG_5998	Drugs	Fluoroquinolones	Quinolone_resistance_protein_Qnr	QNRB
MEG_6	Drugs	Aminoglycosides	Aminoglycoside-resistant_16S_ribosomal_subunit_protein	A16S
MEG_6013	Drugs	Fluoroquinolones	Quinolone_resistance_protein_Qnr	QNRD
MEG_6021	Drugs	Fluoroquinolones	Quinolone_resistance_protein_Qnr	QNRS
MEG_6034	Drugs	Fluoroquinolones	Quinolone_resistance_protein_Qnr	QNRVC
MEG_6035	Drugs	Fluoroquinolones	Quinolone_resistance_protein_Qnr	QNRVC
MEG_6037	Drugs	Fluoroquinolones	Quinolone_resistance_protein_Qnr	QNRVC
MEG_6040	Drugs	betalactams	Class_D_betalactamases	R39
MEG_6044	Drugs	Multi-drug_resistance	Multi-drug_RND_efflux_regulator	RAMA
MEG_6045	Drugs	Multi-drug_resistance	Multi-drug_RND_efflux_regulator	RAMR
MEG_6047	Drugs	Rifampin	RNA-polymerase_binding_protein	RBPA
MEG_6051	Drugs	betalactams	Class_A_betalactamases	RCP
MEG_6054	Drugs	Rifampin	Rifampin_glycosyltransferase	RGT
MEG_6055	Drugs	Mycobacterium_tuberculosis-specific_Drug	Para-aminosalicylic_acid_resistant_mutant	RIBD
MEG_6059	Drugs	betalactams	Class_B_betalactamases	RM3
MEG_6082	Multi-compound	Drug_and_biocide_and_metal_resistance	Drug_and_biocide_and_metal_RND_efflux_pumps	ROBA
MEG_6085	Drugs	Rifampin	Monooxygenase	ROX
MEG_6086	Drugs	Rifampin	Rifampin_phosphotransferase	RPH
MEG_6088	Drugs	Rifampin	Rifampin_phosphotransferase	RPH
MEG_6090	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6091	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6092	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6093	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6094	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6096	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6097	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6099	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6100	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6101	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6102	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6104	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6105	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6106	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6107	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6108	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6109	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6113	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6115	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6116	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6118	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6120	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6121	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB

MEG_6122	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6123	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6125	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6126	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6127	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6129	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6130	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6131	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6132	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6134	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6135	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOB
MEG_6138	Drugs	Glycopeptides	Vancomycin-resistant_mutation	RPOC
MEG_6139	Drugs	Lipopeptides	Daptomycin-resistant_beta-subunit_of_RNA_polymerase_RpoC	RPOCL
MEG_6142	Drugs	Mycobacterium_tuberculosis-specific_Drug	Pyrazinamide-resistant_mutant	RPSA
MEG_6143	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	RPSJ
MEG_6144	Drugs	Aminoglycosides	Aminoglycoside-resistant_16S_ribosomal_subunit_protein	RPSL
MEG_6145	Drugs	Aminoglycosides	Aminoglycoside-resistant_16S_ribosomal_subunit_protein	RRSA
MEG_6146	Drugs	Aminoglycosides	Aminoglycoside-resistant_16S_ribosomal_subunit_protein	RRSC
MEG_6147	Drugs	Aminoglycosides	Aminoglycoside-resistant_16S_ribosomal_subunit_protein	RRSH
MEG_6149	Drugs	betalactams	Class_A_betalactamases	RSA
MEG_6150	Drugs	betalactams	Class_A_betalactamases	RTG
MEG_6151	Drugs	betalactams	Class_A_betalactamases	RTG
MEG_6166	Drugs	Nucleosides	Streptothricin_acetyltransferase	SAT
MEG_6167	Drugs	Nucleosides	Streptothricin_acetyltransferase	SAT
MEG_6168	Drugs	Nucleosides	Streptothricin_acetyltransferase	SAT
MEG_6173	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	SDEB
MEG_6177	Drugs	betalactams	Class_A_betalactamases	SED
MEG_64	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC3
MEG_6521	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	SME
MEG_6522	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	SME
MEG_6523	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	SME
MEG_6524	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	SME
MEG_6525	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	SME
MEG_6526	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	SME
MEG_6527	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	SME
MEG_6528	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	SME
MEG_6529	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	SME
MEG_6530	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	SMET
MEG_6532	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	SMR
MEG_6533	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	SMR
MEG_6534	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	SMR

MEG_6547	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_ABC_efflux_regulator	SOXR
MEG_6551	Multi-compound	Drug_and_biocide_and_metal_resistance	Drug_and_biocide_and_metal_resistance_regulator	SOXS
MEG_6552	Multi-compound	Drug_and_biocide_and_metal_resistance	Drug_and_biocide_and_metal_resistance_regulator	SOXS
MEG_6553	Drugs	betalactams	Class_B_betalactamases	SPG
MEG_6555	Drugs	betalactams	Class_B_betalactamases	SPN
MEG_6558	Drugs	Multi-drug_resistance	Multi-drug_ABC_efflux_pumps	SRMB
MEG_6580	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6581	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6582	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6583	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6584	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6585	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6586	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6587	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6588	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6589	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6590	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6591	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6592	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6593	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6594	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6595	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6596	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6597	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6598	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_66	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC3
MEG_6601	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6604	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6606	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6607	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6608	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6609	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6610	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6611	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6612	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6613	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6614	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6615	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6616	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULI
MEG_6617	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULII
MEG_6618	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULII
MEG_6620	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULII
MEG_6621	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULII
MEG_6622	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULII
MEG_6624	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULII
MEG_6625	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULII
MEG_6626	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULII
MEG_6627	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULII
MEG_6628	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULII
MEG_6629	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULII

MEG_6630	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULII
MEG_6632	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULII
MEG_6633	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULII
MEG_6634	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULII
MEG_6635	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULII
MEG_6636	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULII
MEG_6637	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULII
MEG_6638	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULII
MEG_6639	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULIII
MEG_6640	Drugs	Sulfonamides	Sulfonamide-resistant_dihydropteroate_synthases	SULIV
MEG_6643	Drugs	Multi-drug_resistance	Multi-drug_ABC_efflux_pumps	TAEA
MEG_6644	Drugs	Multi-drug_resistance	Multi-drug_MFS_efflux_pumps	TAP
MEG_6649	Drugs	Tetracenomycin	Tetracenomycin_MFS_efflux_pump	TCMA
MEG_6650	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TCR3
MEG_677	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	ADEA
MEG_678	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	ADEB
MEG_682	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	ADEF
MEG_684	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	ADEG
MEG_685	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	ADEH
MEG_69	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC3-PRIME
MEG_692	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	ADEJ
MEG_6963	Drugs	Tetracyclines	Tetracycline-resistant_16S_ribosomal_subunit_protein	TET16S
MEG_6964	Drugs	Tetracyclines	Tetracycline-resistant_16S_ribosomal_subunit_protein	TET16S
MEG_6970	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TET32
MEG_6971	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TET32
MEG_6976	Drugs	Tetracyclines	Tetracycline_inactivation_enzymes	TET34
MEG_6977	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TET35
MEG_6978	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TET35
MEG_6979	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TET36
MEG_6987	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TET39
MEG_6988	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TET39
MEG_6989	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TET39
MEG_6990	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TET39
MEG_6995	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TET42
MEG_6997	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TET44
MEG_6998	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TET44
MEG_7	Drugs	Aminoglycosides	Aminoglycoside-resistant_16S_ribosomal_subunit_protein	A16S
MEG_700	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	ADEL
MEG_7002	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TET49
MEG_7009	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TET56
MEG_7010	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TET57
MEG_7011	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TET58
MEG_7014	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETA
MEG_7016	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETA
MEG_7018	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETA
MEG_7019	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETA

MEG_702	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	ADEN
MEG_7020	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETA
MEG_7023	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETA
MEG_7024	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETA
MEG_7025	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETA
MEG_7029	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETA
MEG_703	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	ADER
MEG_7032	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETA
MEG_7033	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETA
MEG_7039	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETB
MEG_704	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	ADES
MEG_7041	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETB
MEG_7042	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETB
MEG_7048	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETB
MEG_7049	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETB
MEG_705	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	ADET1
MEG_7052	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETB
MEG_7055	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETBP
MEG_7056	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETC
MEG_7057	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETC
MEG_7058	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETC
MEG_7059	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETC
MEG_7062	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETC
MEG_7064	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETC
MEG_7065	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETC
MEG_7066	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETC
MEG_7067	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETC
MEG_707	Drugs	betalactams	Class_A_betalactamases	AER
MEG_7076	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETG
MEG_7077	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETG
MEG_7078	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETG
MEG_7079	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETG
MEG_708	Drugs	betalactams	Class_A_betalactamases	AER
MEG_7080	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETG
MEG_7081	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETG
MEG_7082	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETH
MEG_7083	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETH
MEG_7084	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETH
MEG_7085	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETH
MEG_7086	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETH
MEG_710	Drugs	betalactams	Class_B_betalactamases	AIM
MEG_7112	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TETM
MEG_7113	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TETM
MEG_7139	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TETM
MEG_7149	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TETO
MEG_7154	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TETO
MEG_7156	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TETO
MEG_7161	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TETO
MEG_7165	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TETO

MEG_7178	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TETQ
MEG_718	Drugs	betalactams	Class_B_betalactamases	ALG
MEG_7183	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TETQ
MEG_7184	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TETQ
MEG_7185	Drugs	Tetracyclines	Tetracycline_transcriptional_repressor	TETR
MEG_7186	Drugs	Tetracyclines	Tetracycline_transcriptional_repressor	TETR
MEG_7189	Drugs	Tetracyclines	Tetracycline_transcriptional_repressor	TETR
MEG_719	Drugs	betalactams	Class_B_betalactamases	ALG
MEG_7190	Drugs	Tetracyclines	Tetracycline_transcriptional_repressor	TETR
MEG_7192	Drugs	Tetracyclines	Tetracycline_transcriptional_repressor	TETR
MEG_7193	Drugs	Tetracyclines	Tetracycline_transcriptional_repressor	TETR
MEG_7194	Drugs	Tetracyclines	Tetracycline_transcriptional_repressor	TETR
MEG_7195	Drugs	Tetracyclines	Tetracycline_transcriptional_repressor	TETR
MEG_7196	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_regulator	TETRM
MEG_7205	Drugs	Tetracyclines	Tetracycline_resistance_MFS_efflux_pumps	TETV
MEG_7219	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TETW
MEG_7220	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TETW
MEG_7221	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TETW
MEG_7222	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TETW
MEG_7225	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TETW
MEG_7226	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TETW
MEG_7227	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TETW
MEG_7229	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TETW
MEG_7233	Drugs	Tetracyclines	Tetracycline_resistance_ribosomal_protection_proteins	TETW
MEG_7238	Drugs	Tetracyclines	Tetracycline_inactivation_enzymes	TETX
MEG_7240	Drugs	Tetracyclines	Tetracycline_inactivation_enzymes	TETX
MEG_7243	Drugs	Tetracyclines	Tetracycline_inactivation_enzymes	TETX
MEG_725	Drugs	betalactams	Class_C_betalactamases	AMPC
MEG_7250	Drugs	Mycobacterium_tuberculosis-specific_Drug	Para-aminosalicylic_acid_resistant_mutant	THYA
MEG_7251	Drugs	betalactams	Class_A_betalactamases	TLA
MEG_7252	Drugs	betalactams	Class_A_betalactamases	TLA
MEG_7253	Drugs	betalactams	Class_A_betalactamases	TLA
MEG_7255	Drugs	Glycopeptides	Bleomycin_resistance_protein	TLMB
MEG_7256	Drugs	MLS	MLS_resistance_ABC_efflux_pumps	TLRC
MEG_7257	Drugs	Multi-drug_resistance	Multi-drug_ABC_efflux_pumps	TLRCM
MEG_7258	Drugs	Aminoglycosides	Aminoglycoside-resistant_arabinosyltransferase	TLYA
MEG_7259	Drugs	Aminoglycosides	Aminoglycoside-resistant_arabinosyltransferase	TLYA
MEG_7285	Drugs	Thiopeptides	Thiostrepton_23S_rRNA_methyltransferases	TSNR
MEG_7286	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	TTGA
MEG_7287	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	TTGA
MEG_7288	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	TTGB
MEG_7289	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	TTGB
MEG_729	Drugs	betalactams	Penicillin_binding_protein	AMPH
MEG_7290	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	TTGC
MEG_7296	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	TTGR
MEG_73	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_730	Drugs	betalactams	Penicillin_binding_protein	AMPH
MEG_7301	Drugs	Elfamycins	EF-Tu_inhibition	TUFAB

MEG_7302	Drugs	Elfamycins	EF-Tu_inhibition	TUFAB
MEG_7303	Drugs	Elfamycins	EF-Tu_inhibition	TUFAB
MEG_7304	Drugs	Elfamycins	EF-Tu_inhibition	TUFAB
MEG_7305	Drugs	Elfamycins	EF-Tu_inhibition	TUFAB
MEG_7306	Drugs	Elfamycins	EF-Tu_inhibition	TUFAB
MEG_7307	Drugs	Elfamycins	EF-Tu_inhibition	TUFAB
MEG_7308	Drugs	Elfamycins	EF-Tu_inhibition	TUFAB
MEG_7309	Drugs	Elfamycins	EF-Tu_inhibition	TUFAB
MEG_731	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	AMRA
MEG_7310	Drugs	Elfamycins	EF-Tu_inhibition	TUFAB
MEG_7312	Drugs	Elfamycins	EF-Tu_inhibition	TUFAB
MEG_7313	Drugs	Elfamycins	EF-Tu_inhibition	TUFAB
MEG_7314	Drugs	Elfamycins	EF-Tu_inhibition	TUFAB
MEG_7315	Drugs	Elfamycins	EF-Tu_inhibition	TUFAB
MEG_7316	Drugs	Elfamycins	EF-Tu_inhibition	TUFAB
MEG_7317	Drugs	Elfamycins	EF-Tu_inhibition	TUFAB
MEG_7318	Drugs	Elfamycins	EF-Tu_inhibition	TUFAB
MEG_7319	Drugs	Elfamycins	EF-Tu_inhibition	TUFAB
MEG_732	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	AMRB
MEG_7320	Drugs	Elfamycins	EF-Tu_inhibition	TUFAB
MEG_7321	Drugs	Elfamycins	EF-Tu_inhibition	TUFAB
MEG_7328	Drugs	Cationic_antimicrobial_peptides	Lipid_A_modification	UGD
MEG_733	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_MFS_efflux_pumps	AMVA
MEG_7330	Drugs	Fosfomycin	Fosfomycin_target_mutation	UHPT
MEG_7336	Drugs	Glycopeptides	VanA-type_resistance_protein	VANA
MEG_734	Drugs	betalactams	Class_B_betalactamases	ANA
MEG_739	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT2-DPRIME
MEG_740	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT2-DPRIME
MEG_743	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT2-DPRIME
MEG_7443	Drugs	Glycopeptides	VanK-type_resistance_protein	VANK
MEG_746	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT2-DPRIME
MEG_7485	Drugs	Glycopeptides	VanO-type_regulator	VANRO
MEG_75	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_751	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT2-DPRIME
MEG_753	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT2-DPRIME
MEG_754	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT2-DPRIME
MEG_7556	Drugs	Glycopeptides	VanA-type_resistance_protein	VANXA
MEG_757	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT2-DPRIME
MEG_758	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_759	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_7593	Drugs	Glycopeptides	VanA-type_accessory_protein	VANYA
MEG_762	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_763	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_7647	Drugs	betalactams	Class_A_betalactamases	VEB
MEG_7648	Drugs	betalactams	Class_A_betalactamases	VEB
MEG_7649	Drugs	betalactams	Class_A_betalactamases	VEB
MEG_7650	Drugs	betalactams	Class_A_betalactamases	VEB
MEG_7651	Drugs	betalactams	Class_A_betalactamases	VEB
MEG_7652	Drugs	betalactams	Class_A_betalactamases	VEB
MEG_7654	Drugs	betalactams	Class_A_betalactamases	VEB

MEG_7655	Drugs	betalactams	Class_A_betalactamases	VEB
MEG_7657	Drugs	betalactams	Class_A_betalactamases	VEB
MEG_7658	Drugs	betalactams	Class_A_betalactamases	VEB
MEG_7659	Drugs	betalactams	Class_A_betalactamases	VEB
MEG_7660	Drugs	betalactams	Class_A_betalactamases	VEB
MEG_7665	Drugs	betalactams	Class_A_betalactamases	VEB
MEG_7666	Drugs	betalactams	Class_A_betalactamases	VEB
MEG_7667	Drugs	betalactams	Class_A_betalactamases	VEB
MEG_7669	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	VEXB
MEG_769	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_7695	Drugs	betalactams	Class_A_betalactamases	VHW
MEG_770	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_774	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_775	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_779	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_780	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_7804	Drugs	Mycobacterium_tuberculosis-specific_Drug	Viomycin_phosphotransferases	VPH
MEG_7807	Drugs	betalactams	Class_A_betalactamases	WHH
MEG_7842	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_ABC_efflux_pumps	YOGI
MEG_7843	Drugs	Lipopeptides	Daptomycin-resistant_mutant	YYBT
MEG_7844	Drugs	Glycopeptides	Bleomycin_resistance_protein	ZBMA
MEG_7883	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	ACRB
MEG_7884	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	ACRR
MEG_7976	Drugs	Lipopeptides	Colistin_phosphoethanolamine_transferase	ARNT
MEG_7979	Drugs	betalactams	Class_A_betalactamases	AXC
MEG_798	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8	Drugs	Aminoglycosides	Aminoglycoside-resistant_16S_ribosomal_subunit_protein	A16S
MEG_8011	Drugs	betalactams	Class_A_betalactamases	CARB
MEG_8027	Drugs	betalactams	Class_C_betalactamases	CMY
MEG_8035	Drugs	Lipopeptides	Colistin_phosphoethanolamine_transferase	CPRR
MEG_8036	Drugs	Lipopeptides	Colistin_phosphoethanolamine_transferase	CPRS
MEG_8068	Drugs	Trimethoprim	Dihydrofolate_reductase	DFRA
MEG_8077	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_ABC_efflux_pumps	EFRA
MEG_8080	Drugs	Lipopeptides	Colistin_phosphoethanolamine_transferase	EPTB
MEG_813	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8144	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_8146	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRA
MEG_8150	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRB
MEG_8151	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	GYRB
MEG_816	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_817	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_818	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_819	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_821	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8215	Drugs	betalactams	Class_A_betalactamases	LEN
MEG_8217	Drugs	betalactams	Class_A_betalactamases	LEN
MEG_822	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8226	Drugs	betalactams	Class_A_betalactamases	LEN
MEG_8227	Drugs	betalactams	Class_A_betalactamases	LEN

MEG_823	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8232	Drugs	Multi-drug_resistance	Multi-drug_ABC_efflux_pumps	LPTD
MEG_824	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8249	Drugs	MLS	Macrolide-resistant_23S_rRNA_mutation	MLS23S
MEG_825	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8250	Drugs	MLS	Macrolide-resistant_23S_rRNA_mutation	MLS23S
MEG_8251	Drugs	MLS	Macrolide-resistant_23S_rRNA_mutation	MLS23S
MEG_8252	Drugs	MLS	Macrolide-resistant_23S_rRNA_mutation	MLS23S
MEG_8253	Drugs	MLS	Macrolide-resistant_23S_rRNA_mutation	MLS23S
MEG_8254	Drugs	MLS	Macrolide-resistant_23S_rRNA_mutation	MLS23S
MEG_826	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_827	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8287	Drugs	betalactams	Mutant_porin_proteins	OMP36
MEG_8288	Drugs	betalactams	Mutant_porin_proteins	OMPA
MEG_8289	Drugs	Multi-drug_resistance	MDR_mutant_porin_proteins	OMPK
MEG_8290	Drugs	betalactams	Mutant_porin_proteins	OMPK36
MEG_830	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8301	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_8317	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_8319	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_832	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8367	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_837	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8371	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_8372	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_8373	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_8379	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_838	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_840	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8412	Drugs	betalactams	Class_D_betalactamases	OXA
MEG_842	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_844	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8446	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	PARC
MEG_8447	Drugs	Fluoroquinolones	Fluoroquinolone-resistant_DNA_topoisomerases	PARC
MEG_8449	Drugs	Aminocoumarins	Aminocoumarin-resistant_DNA_topoisomerases	PARE
MEG_8451	Drugs	Multi-drug_resistance	MDR_regulator	PARR
MEG_8452	Drugs	Multi-drug_resistance	MDR_regulator	PARS
MEG_846	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8464	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8468	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8469	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8471	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_848	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8482	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8486	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8489	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_849	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8490	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8503	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8504	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8505	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8506	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_851	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME

MEG_8510	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8515	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8516	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8518	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8521	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8523	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8524	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8532	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8536	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8542	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8544	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8545	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_855	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8550	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8551	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8555	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_856	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8576	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8582	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8583	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8586	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_859	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8594	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8596	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8598	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8600	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8603	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8604	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_861	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8614	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8615	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8616	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8617	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_862	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8620	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8622	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_8623	Drugs	betalactams	Class_C_betalactamases	PDC
MEG_863	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_864	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8640	Drugs	betalactams	Class_C_betalactamases	PRC
MEG_865	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8651	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_SMR_efflux_pumps	QACL
MEG_8659	Drugs	Multi-drug_resistance	Multi-drug_RND_efflux_regulator	RAMR
MEG_866	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8675	Drugs	Rifampin	Rifampin-resistant_beta-subunit_of_RNA_polymerase_RpoB	RPOCR
MEG_8677	Drugs	Aminoglycosides	Aminoglycoside-resistant_16S_ribosomal_subunit_protein	RPSL
MEG_8678	Drugs	Aminoglycosides	Aminoglycoside-resistant_16S_ribosomal_subunit_protein	RPSL
MEG_8680	Drugs	Aminoglycosides	Aminoglycoside-resistant_16S_ribosomal_subunit_protein	RRS
MEG_8686	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_pumps	RSMA
MEG_869	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8725	Drugs	Glycopeptides	VanR-type_regulator	VANRS

MEG_874	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_8741	Multi-compound	Drug_and_biocide_resistance	Drug_and_biocide_RND_efflux_regulator	YAJC
MEG_875	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_876	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_878	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_88	Drugs	Aminoglycosides	Aminoglycoside_N-acetyltransferases	AAC6-PRIME
MEG_882	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_892	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_896	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_9	Drugs	Aminoglycosides	Aminoglycoside-resistant_16S_ribosomal_subunit_protein	A16S
MEG_900	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_901	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_906	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_907	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_910	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_915	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_916	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_917	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_919	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_921	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_926	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_927	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_928	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_929	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_931	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_932	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_934	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_939	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_943	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_945	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_947	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_948	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_949	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_951	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_952	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_955	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_957	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_959	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_961	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_963	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_965	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_966	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_967	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_970	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_971	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_972	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_974	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_975	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT3-DPRIME
MEG_987	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT6
MEG_988	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT6
MEG_997	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT6
MEG_998	Drugs	Aminoglycosides	Aminoglycoside_O-nucleotidyltransferases	ANT6