Molecular evaluation of spatial and temporal variation in bacterial communities and clinically relevant antibiotic resistance genes in an aquatic system

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Fahad Alshabrmi

2/05/2019

Dedication

I dedicate this work to my dear father Mohammad Alshabrmi, who sadly passed away during my PhD journey. I also dedicate this work to my mother Latifa Alshabrmi. Both of them have given me unconditional love and support throughout my life and I will not be where I am today without them. I also dedicate this work to my brothers and sisters for their love, support and prayers for my success. My special heart-felt thanks goes to my sister Hessah, who made so much effort to ensure that I get proper education from a tender age till now that I am an adult.

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ABBREVIATION	DESCRIPTION
BA	Ballan
AP	April
AUG	August
AWARH	The Australia-wide Assessment of River health
aadA	Streptomycin 3"-adenylyltransferase
ANOSIM	Analysis of Similarities
ANOVA	Analysis of one-way variance
aa	Amino acid
ARGs	Antibiotic resistance genes
BM	Bacchus Marsh
BLAST	Basic local alignment search tool
bp	Base pair
Bod	Biochemical Oxygen Demand Riversdale
CF	Cobbledicks
°C	Degrees Celsius
CTns	Conjugative transposons
Cat	Chloramphenicol acetyltransferase
DO	Dissolve Oxygen
dec	December
DOM	Dissolve organic matter
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
EC	Electrical Conductivity

ERM	Erythromycin
FunGene	Functional gene pipeline
FEB	February
GHAP	Greenfield hybrid analysis pipeline
HTS	High-throughput sequencing
HGT	Horizontal gene transfer
H'	Shannon diversity
HVRS	Hyper variable regions
jun	June
LPS	Lipopolysaccharides
L	Liter
MCR-1	Mobilized colistin resistance
MecA	Methicillin
MGEs	Mobile genetic elements
ml	Milliliter
mM	Millimole
MRSA	Methicillin-resistant *Staphylococcus aureus*
µM	Micromole
µl	Microlitre
NCBI	National Centre for Biotechnology Information
NO_2^-	Nitrite
NH_4^+	Ammonium
NO_3	Nitrates
NDM-1	New Delhi metallo-beta-lactamase
NGS	Next generation sequencing
OCT	October

OTU	Operational taxonomic units
PCR	Polymerase chain reaction
PBPs	Penicillin binding proteins
POC	particulate organic carbon
Q-PCR	Quantitative PCR
rRNA	Ribosomal RNA
RIF	Rifampicin
RDP	Ribosomal database project
RefSeq	Reference sequence database
RNA	Ribonucleic acid
Rd	Riversdale
SEM	Standard mean error
Sul	Sulfonamide
TET	Tetracycline
T_m	Melting point
tRNA	Transfer ribonucleic acid
U	Unit
van	Vancomycin
VRSA	Vancomycin- resistant Staphylococcus aureus
VRE	Vancomycin resistant enterococci
WWTPs	Wastewater Treatment Plants
WGS	Whole genome shotgun sequencing
%	Percent
µm	Micrometer

Executive Summary

Freshwater ecosystems such as rivers and lakes contain up to 10% of all species and are an important repository of genetic information. They are important sources of drinking water and food. They are also extensively used for different agricultural and industrial processes. One major health concern on the use of water resources from rivers is the presence of antibiotics resistance genes (ARGs). ARGs are found in bacterial genomes and can spread between environmental bacteria and ultimately into the human microflora. ARGs can render antibiotics ineffective, increasing the morbidity and mortality rates of human diseases. Unfortunately, this health concern is poorly assessed in rivers systems including those used for agricultural processes, such as the Werribee River, Victoria which is the focus of the research in this thesis. Therefore, this study aims to study the bacterial community structure, diversity and composition and the influence of physico-chemical factors on this community in the Werribee River in Melbourne using 16S rRNA-based Next-Generation Sequencing (NGS) tool. The prevalence of ARGs using PCR, qPCR and ARG-based targeted Next-Generation Sequencing approaches was also investigated to explore variation in the antibiotic resistance gene pool along the river and over time.

Variation in bacterial community structure, composition and diversity along the river was investigated at four selected sites; Ballan and Bacchus Marsh (upstream sites) and Cobbledicks Ford and Riversdale (downstream sites). At these sites, both spatial and/or temporal variation was observed in the numbers of Operational Taxonomic Units (OTUs), Shannon diversity (*H'*) and taxonomic composition (phylum, class and genus levels) in most samples. The highest Chao1 OTU richness value of 2332 was recorded in Cobbledicks Ford while the lowest value of 1645 was observed in Bacchus Marsh. Similarly, the lowest Shannon diversity (*H'*) of 2.5 was reported at Bacchus Marsh while the highest diversity (*H'* of 4) was recorded in Ballan. Bacterial diversity (*H'*) (December-February; 4.2 and 3.9) and Chao1 OTU richness

(December; 2408, February; 2976) were significantly higher ($P \leq 0.05$) in the summer months when compared to most other months. Three key phyla (*Proteobacteria, Actinobacteria* and *Bacteroidetes*) were observed at all sites with the *Proteobacteria* being the most dominant phylum across all sites and at all sampling months. At the class level, four different classes, *Actinobacteria, Alphaproteobacteria, Betaproteobacteria and Cytophagia,* were the dominant classes based on SIMPER analysis. The *Betaproteobacteria* was the most abundant class at all sites except at Riversdale where it was replaced by the *Actinobacteria*. Different bacterial genera were important at different sites. At upstream sites, the key genera were *Polynucleobacter, Arcicella, Limnohabitans, Acidovorax and Methylotenera,* whilst in downstream sites, the key genera were *Acidovorax, Candidatus* Pelagibacter, *Limnohabitans* and *Demequina.* Temperature and dissolved oxygen concentrations were commonly associated with changes in the bacterial community at upstream and downstream sites. Bacterial diversity and OTU numbers were strongly and positively correlated to water temperature but negatively correlated to dissolved oxygen concentrations. Other factors such as turbidity, suspended solids, electrical conductivity, total bound nitrogen and ammonia concentrations were also important at the (downstream) Cobbledicks Ford site.

The prevalence of 12 ARGs (*^{bla}NDM-1, mecA, tet(M), ampC, VanA, mcr-1, tet(B), erm(B), aac (6')-Ie-aph (2'')-Ia, SulII, catII* and *dfrA1*) was assessed using PCR in upstream and downstream samples from the Werribee River. Out of these ARGs, only three ARGs, *^{bla}NDM-1*, *tet(B)* and *catII* were detected. Substantial spatial and temporal variation in the frequency of detection of these ARGs was observed. These ARGs were detected more frequently at the downstream sites of Cobbledicks Ford and Riversdale (detection frequency of 12.5%) than at upstream sites Ballan (4.2 %) and Bacchus Marsh (1.4 %). Only the *catII* gene was detected at Bacchus Marsh. There was, therefore, a greater risk of exposure to ARGs from water samples in downstream sites than in upstream sites.

The relative abundance of detected ARGs as evaluated using quantitative PCR showed limited spatial variation. This was because apart from the abundance of the ARGs in Riversdale samples being significantly higher ($P \leq 0.05$), than at Bacchus Marsh, there was no significant difference in ARGs relative abundance at the remaining sites. However, there was substantial temporal variation in ARG abundance at most sampling times. This variation was significant ($P \leq 0.05$) in some instances, for example, when the relative abundance was higher in summer (December and February) compared to autumn (April). The detection of ARGs, especially at downstream sites highlights a potential health risk of using water from Werribee River for recreational and agricultural purposes. However, this study did not determine whether the greater frequency of detection and increasing ARG relative abundance at the downstream sites was a natural occurrence or due to anthropogenic sources.

The application of targeted ARG-based NGS to selected samples was partly successful. The ARGs evaluated were ^{bla}*NDM* (sample from Riversdale) and *catII* (samples from Cobbledicks Ford, Riversdale, Ballan and Bacchus Marsh). Data analysis showed a significant similarity of ^{bla}*NDM* sequence from Riversdale to a ^{bla}*NDM* gene from *Escherichia coli* indicating that the target gene was successfully amplified in the Riversdale sample. However, the target gene/enzyme (chloramphenicol O-acetyltransferase) was identified at only one site (from Cobbledicks Ford). At the remaining sites, chloramphenicol resistance genes were not identified and instead, other acetyltransferase genes were detected, namely; acetyl-CoA C-transferase (Bacchus Marsh and Riversdale) and 3-oxoadipyl-CoA thiolase (Ballan) which are not associated with chloramphenicol resistance.

This study has therefore shown substantial spatial and temporal variation in the bacterial community structure and diversity in the Werribee River with temperature and DO levels being some of the significant drivers of changes in the bacterial community structure. This spatial and temporal variation was reflected in the frequency of detection of the important ARGs

(*bla*NDM, *tet(B)* and *catII*) in the Werribee River. Finally, the targeted ARG-based NGS approach used in this study is a promising approach for determining the diversity, identity and relative abundance of ARG sequences in environmental samples but requires more optimizations with more samples from different sources in future investigation.

1 Chapter 1: Literature Review

1.1 The freshwater environment

Freshwater is vital for all terrestrial life and an important resource for several human activities such as those involving the industrial production of goods and materials, farming (irrigation), recreational activities and for domestic uses (Hahn, 2006). However, freshwater can also be a means of transmitting communicable diseases, especially in resource-poor countries lacking adequate water treatment facilities (Özler and Aydın, 2008). Approximately, 2.5% of the planet's water is freshwater, but a significant portion (>60%) of this is locked up in the form of ice in glaciers or tundra (USGS, 2018). This makes proper management of the available freshwater resources more important.

Whilst freshwater is ubiquitous in daily life, there has been less emphasis on the microbiological research of freshwater when compared to similar research efforts by marine microbiologists (Debroas et al., 2009). Freshwater is important because it is home to taxonomically- and functionally-diverse microorganisms which are impacted by water quality. Changes in the chemical or physical properties of freshwater can have dramatic impacts on the structure, composition and function of these microorganisms. Freshwater microorganisms respond differently to the changes in their environment. Whilst some can survive in a range of conditions, others that are particularly sensitive to pollutants are eliminated potentially causing changes in microbial-diversity and activities of the concerned ecosystem (Raibole and Singh, 2011, Tlili et al., 2017, Hu et al., 2017).

1.2 The ecology of rivers

Rivers are generally composed of an upper, middle and lower course, each providing unique chemical and physical features to support a diversity of aquatic life (Rosgen, 1996). The upper course of a river tends to be V-shaped in profile with high water velocity, high dissolved oxygen content due to rapid mixing by rapids and low nutrient content. The middle course has a U-shaped cross-section with medium water velocity and increasing quantities of dissolved solids. In the lower course, the river is broad and U-shaped due to extensive lateral erosion and water moves slowly and carries a high burden of particulate matter and ions, thus increasing turbidity, reducing light penetration and increasing conductivity (Rosgen, 1996).

A river ecosystem consists of interactions between both biotic and abiotic factors and these interactions influence the microbial activities in the ecosystem (Boyero et al., 2016). Biotic factors may include plants, fish, insects and other invertebrates and microorganisms (Angelier, 2003) while abiotic factors can include factors such as temperature, light intensity and pH. Urban rivers are often used as a source of freshwater for domestic purposes by society including for drinking water (Zhang et al., 2012). These rivers also provide water for farming and industrial activities (Kenzaka et al., 2001). However, as rivers are open systems that are connected to their environment, the ecology of a river is vulnerable to outside interferences (Velimirov et al., 2011). Effluents, both treated and untreated, are frequently deposited directly into river systems (Minh et al., 2016, Mandaric et al., 2018, Zhang et al., 2012). Such practices can inundate a river with pollutants, rendering it non-potable and creating public health issues to the relevant urban population (Olayemi, 1994, Imre et al., 2017, Gothwal and Thatikonda, 2017, Minh et al., 2016).

Rivers and input stream relationship are well understood with rivers thought to receive mineral nutrients through surface run-offs from their input streams (Kengnal et al., 2015, Sadro and

Melack, 2012). However, aquatic ecosystems now face increasing pressures due to increased release of anthropogenic waste which can influence the biomass and community composition in these systems (Tlili et al., 2017, Wu et al., 2019)).

In order to address this issue, and to reverse the contamination of a river system, improved understanding of river microbiology and microbial ecology is required. Rivers are considered to be dynamic, active ecosystems; organic material enters and leaves the river system and ultimately into the sea (Skorczewski and Mudryk, 2009). The variance in dissolved organic matter (DOM) concentrations makes rivers a perfect ecosystem in which to study the link between bacterial communities and DOM composition and microbial utilization (Brailsford et al., 2017, Kirchman et al., 2004). Bacterial communities are highly sensitive to DOM and can use or process it in a variety of ways (Yamakanamardi and Goulder, 1995, Kamjunke et al., 2016) with specific microbial clades associated with different types of DOM (Amaral and Abelho, 2016).

1.3 Microbial carbon and nutrient cycling

Rivers provide food, water, shelter and space, essential for the survival of both terrestrial and aquatic animals, plants invertebrates and microorganisms (Wishart et al., 2000) with microorganisms playing a fundamental role in the maintenance of the health of aquatic ecosystems. Photosynthetic microorganisms such as C*yanobacteria* spp. and microalgae utilize energy from sunlight to convert carbon dioxide into organic matter (Rodrigues et al., 2008) while other heterotrophic microorganisms mineralise organic carbon via respiration. Other microorganisms are involved in the nitrogen cycle (e.g. blue-green algae) (Cottingham et al., 2015). Atmospheric nitrogen is fixed by nitrogen-fixing bacteria. It is initially converted from ammonium ions (NH_4^+) to nitrite (NO_2^-) by chemoautotrophic ammonia-oxidizing bacteria and archaea (Jetten, 2008, Prosser and Nicol, 2008) and then from NO_2^- to nitrates (NO_3^-) by *Nitrobacter* sp. with NO_3^- then available for assimilation by aquatic plants (Rick and Thomas,

2001). Conversely, nitrates can be converted into atmospheric nitrogen via the anaerobic process of denitrification by heterotrophic denitrifying bacteria such as *Pseudomonas denitrificans* and *P. aeruginosa* (Holmes et al., 2019). Nitrates can also be reduced to ammonia through dissimilatory nitrate reduction processes (Holmes et al., 2019). In addition, multiple microbial groups are involved in the recycling of organic matter (Falkowski et al., 2008) in rivers. A river ecosystem cannot maintain a healthy state without the appropriate mix of microorganisms to support the various nutrient cycles. Changes in an aquatic system's physical and chemical properties will directly affect the relative abundance of different microorganisms.

Studying carbon cycling is important to the understanding of the dynamics of food webs and how biotic communities are structured and supported in aquatic ecosystems. Streams and rivers are known to play important roles in the storage and cycling of the organic matter (Cole et al., 2007, Withers and Jarvie, 2008, Battin et al., 2009). There are two views of carbon cycling in rivers: one is the "neutral pipe" which states that carbon from the terrestrial ecosystem is delivered to oceans by rivers (Cole et al., 2007). The second view is that the active components of the global carbon cycle store terrestrially derived carbon in the sediments and emit CO_2 to the atmosphere (Cole et al., 2007).

Inorganic carbon is found in the atmosphere in the form of carbon dioxide (CO_2) at concentrations now exceeding 415 ppm. The increase in the concentrations of atmospheric CO_2 leads to a greenhouse effect which will undoubtedly influence the aquatic ecosystems (Hutchin et al., 1995, Magnuson et al., 1997). CO_2 dissolved in water can exist in different forms such as carbon dioxide, carbonic acid, bicarbonate and carbonate depending on the receiving system. The form of dissolved CO_2 determines its availability for photosynthetic processes in a river ecosystem and affects the buffering capacity of rivers (Butler et al., 1991).

The organic form of carbon is divided into two forms: a) dissolved organic carbon (DOC) (< 0.5 µm in size) and b) particulate organic carbon (POC) (>0.5 µm in size) (Hedges et al., 1997). The amount of organic carbon in ecosystems is important for the heterotrophic organisms. One common method of estimating the amount of organic carbon is through the determination of the biochemical oxygen demand (BOD) or the total demand for oxygen by oxidative reactions in a river (Wetzel, 2001). The dissolved organic carbon can be divided into a) humic and b) non-humic compounds. Humic compounds are the ones which confer a brown colouration to the water while the non-humic compounds include sugars, carbohydrates, pigments, lipids, amino acid and proteins.

Photosynthesis and aerobic respiration are the two main fluxes of carbon cycling in river water. However, the carbon cycle is a complex one due to associated anaerobic cycling processes and the uptake of organic compounds in anoxic and oxic conditions. Furthermore, microorganisms continuously adapt and are able to utilize multiple forms of organic carbon compounds released from anthropogenic waste, allowing energy from these wastes to be used for beneficial purposes (Cho et al., 2018, Han et al., 2018). In the absence of oxygen, other electron acceptors such as nitrate, sulfate and iron permit the oxidation of organic carbon (Lovley, 1997), with such processes (especially nitrate reduction) mediated by facultative anaerobes.

Microorganisms play important roles in nitrogen, phosphorous and sulphur cycling and by so doing, influence the biological structures and processes in the ecosystem of rivers (Pakulski et al., 2000). Owing to the rapid growth or proliferation of freshwater microorganisms, they form an essential component of nutrient cycling, globally. Due to industrial, municipal and agricultural activities, compounds containing nitrogen, sulphur, phosphorus are released into rivers (Baldwin and Mitchell, 2000, Sabater et al., 2002, Hope et al., 1994). This may lead to eutrophication (Zhang et al., 2017). Eutrophication occurs when nutrients in the aquatic system facilitate the growth of photosynthetic algae which are lysed by algal phage releasing organic

carbon. The released organic carbon is used by bacteria growing aerobically which depletes oxygen causing an alteration in the distribution and diversity of aquatic species, including extreme events such as mass fish kills (Meziti et al., 2016). The role of aquatic environments such as rivers in cycling the nutrients and the study of aquatic microbial systems is now gaining increasing interest from the scientific community because of their impacts on the health of aquatic environments (Benstead and Leigh, 2012, Wilson et al., 2013).

1.4 Microbial community structure in aquatic environments

Microbial communities are essential components of aquatic systems as microorganisms play a critical role in regulating the energy fluxes and the biogeochemical cycle therein (Falkowski et al., 2008). In river ecosystems, the majority of microorganisms are present in the benthic regions (Harris et al., 2014) and are involved in biogeochemical cycling, mineralisation, nutrient uptake, transfer of nutrients to higher trophic levels and transformation or immobilization of contaminants in the benthic area (von Schiller et al., 2016, Buchkowski et al., 2015). In streams, various autotrophic and heterotrophic taxa are closely linked with one another through trophic interactions (Fitter and Hillebrand, 2009). In river ecosystems, microbial taxa have extremely high levels of genetic diversity in terms of species (Newton et al., 2011, Huang et al., 2017).

The microbial composition of rivers can be influenced by natural and anthropogenic factors. Seasonal variations which include changes in temperature, precipitation and water depth have been shown to influence changes in the microbial community composition in freshwater systems (Wuellner et al., 2010, Fortunato and Crump, 2011). The composition of bacterial communities can also be highly variable due to variations in other factors such as hydraulic water flow rate and bedrock material (Hunt and Parry, 1998).

Anthropogenic effects are often associated with the usage of surrounding catchment area either for agriculture, domestic and industrial activities associated with urbanization and pollution resulting from agricultural and industrial activities (Bumunang et al., 2015, García-Armisen et al., 2014, Beale et al., 2017). Nutrient inputs and faecal pollution can impact bacterial community composition by introducing coliforms and causing eutrophication as shown in a study conducted upstream and downstream of an urban area on the Mooi River in the North West Province of South Africa (Jordaan and Bezuidenhout, 2016). In addition, various pharmaceuticals, including antibiotics, can enter the environment in many ways and greatly influence the composition of bacterial communities (especially with respect to antibiotic resistant bacterial groups) found in rivers (Marti et al., 2013). Given that the focus of this thesis is on antibiotics resistance factors in freshwater systems, reviews of antibiotics and their impacts on freshwater ecosystem are presented below.

1.5 Introduction to antibiotics

Antibiotics are a class of compounds that possess antimicrobial properties which allows them to inhibit or kill bacteria and are widely used to treat bacterial diseases in humans and animals. Antibiotics can occur naturally (produced by certain fungi or bacteria) or may be chemically synthesized. Antibiotics fall into two broad categories: bacteriostatic, which inhibit the reproduction or function of bacteria; or bactericidal, which kill bacteria (Soares et al., 2012). In addition to vaccines, antibiotics are one of the most effective pharmaceutical treatment (that humanity has ever produced, preventing the deaths of millions. However, the widespread use of antibiotics has led to the development of and increased selection for bacterial resistance to antibiotics, and there is a fear that medicine could return to the pre-antibiotic era (Davies and Davies, 2010).

1.5.1 History of antibiotics

The history of antibiotics dates back thousands of years, well before what is considered as the era of modern medicine. For example, trace amounts of tetracycline were detected in human skeletons from Nubia (modern-day Sudan) that dated back to 550-350CE (Aminov, 2010). The 'antibiotic era' began with the discovery of penicillin, a compound synthesised by the fungus *Penicillium chrysogenum* (Walsh, 2003a). In July 1928, Scottish microbiologist Alexander Fleming was the first person to observe the inhibition of the bacterium *Staphylococcus* by a mould called *Penicillium notatum* (Hare, 1982, Derderian, 2007). Fleming called the compound produced by this mould penicillin (Sykes, 2001). A brief timeline of the discovery of penicillin and other antibiotics, their use and the detection of resistance to these antibiotics is presented in **Figure 1.1**.

Antibiotics discovery	Some diseases treated	Antibiotics resistance identified
Penicillin (1943)	Pneumonia, meningitis, bone, joint, blood and other tissue infections	1945 (penicillin) 1965 (penicillin-R Pneumococcus)
Tetracycline (1950)	Pneumonia, acne, ulcers, genital and urinary tracts infections	1959 (tetracycline-R Shigella)
Erythromycin (1953)	Bronchitis, rheumatic fever, whooping cough, pneumonia, legionnaires disease	1968 (erythromycin-R Streptococcus)
Methicillin (1960)	Staph infections	1962 (methicillin-R Staphylococcus)
Gentamicin (1967)	Lung, skin, bone, blood infections	1979 (gentamicin-R Enterococcus)
Vancomycin	Colitis	1988 (vancomycin-R Enterococcus) and 2002 (vancomycin-R Staphylococcus)
Imipenem & ceftazidime (1985)	Lung, skin, bone, blood, stomach and urinary tract infections	1987 (ceftazidime-R Enterobacteriaceae) & 1998 (imipenem-R Enterobacteriaceae)
Levofloxacin (1996)	Chronic bronchitis, pneumoniae, kidney & prostrate infections	1996 (levofloxacin-R Pneumococcus)
Linezolid (2000)	Pneumonia. Skin and blood infections	2001 (linezolid-R Staphylococcus)
Ceftaroline (2010)	Skin infections and pneumonia	2011 (ceftaroline-R Staphylococcus

Figure 1.1: Timeline of antibiotic's development and resistance (adapted from (FERN, 2018))

1.5.2 Classification of antibiotics

The classification of antibiotics is typically based on the antibiotics' origins, chemical structures, and mechanisms of actions. Antibiotics inhibit key bacterial metabolic processes such as cell wall formation, protein synthesis and cellular division, thereby causing the death of target microorganisms (Kapoor et al., 2017, Davies and Davies, 2010).

1.5.2.1 Biosynthetic/chemical origin-based classification

Antibiotics are classified into three distinct groups based on their origins, namely: natural antibiotics, semi-synthetic antibiotics and synthetic antibiotics (Walsh, 2003a). The sources and types of natural antibiotics and the differences between these three groups of antibiotics are shown in Table 1.1.

Table 1.1 Classification of antibiotics based on origin (adapted from Korzybski et al., 2013).

Classification	**Sources**	**Examples**	
Natural antibiotics	Fungi and bacteria	**Antibiotics**	**Source microorganisms**
		Penicillin	*Penicillium chrysogenum*
		Cephalosporins	*Acremonium chrysogenum*
		Thienamycin	*Streptomyces cattleya*
		Carbapenem	*Erwinia* spp
		Erythromycin	*Saccharopolyspora erythraea*
Semi-synthetic antibiotics	Modifications of various natural compounds	**Antibiotics**	**Original natural compounds**
		Beta-lactam antibiotics	Penicillin G
		Doxycycline minocycline	Tetracycline
		Azithromycin	Erythromycin
Synthetic antibiotics	From structures that are not found in nature	The sulfonamides, the quinolones and the oxazolidinones-are solely produced by chemical synthesis.	

1.5.2.2 Classification based on the mode of action of antibiotics

Antibiotics have different modes of actions and these modes can be used for their classification. The four major mechanisms of actions used for the classification of antibiotics include those relating to the inhibition of the synthesis of i) cell walls and membranes, ii) proteins, iii) folate coenzymes and iv) DNA replication and repair (Walsh, 2003a, Soares et al., 2012, Walsh, 2000, Conn et al., 2019).

1.5.2.2.1 Inhibitors of cell wall synthesis

Most antibiotics such as Penicillin that inhibit cell wall synthesis inhibitors have a β-lactam ring in their molecular structures (Dowling et al., 2017). Penicillin is a five-membered thiazolidine ring (sulphur ring) linked to a four-membered β-lactam ring with an acyl side chain (Figure. 1.2).

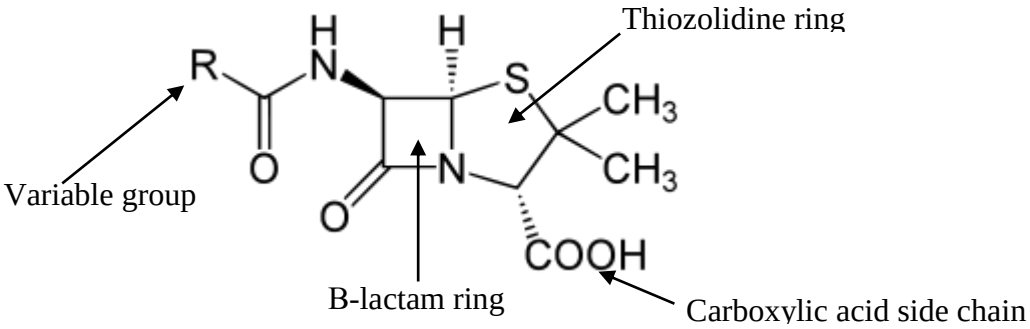


Figure 1.2: Penicillin core structure (Mucsi et al., 2013). R variable group can be phenoxymethyl (penicillin V), benzyl (penicillin G) or other chemical groups.

Penicillin and its derivatives are bactericidal drugs killing bacteria by inactivating the peptidoglycan-cross-linking transpeptidases. Transpeptidases mistake β-lactam antibiotics for the substrate needed for the synthesis of the cross-linked peptidoglycan chain. In addition, there are other active sites for penicillin and these are collectively termed penicillin-binding proteins (PBP) (Soares et al., 2012). A representation of this process is shown in Figure 1.3 (Ramachandran et al., 2006). The Glycopeptides (e.g. vancomycin and teicoplanin) is another class of cell wall inhibitor. These antibiotics cannot penetrate the pores on the outer membrane

Gram-negative bacteria and hence are typically applied against Gram-positive pathogens. They prevent transpeptidation by interacting with peptidoglycan units that have pentapeptidyl tails terminating in D-Ala$_4$-D-Ala (Kohanski et al., 2010b, Walsh, 2003b) which prevents cross-linkage and peptidoglycan synthesis (Figure 1.4).

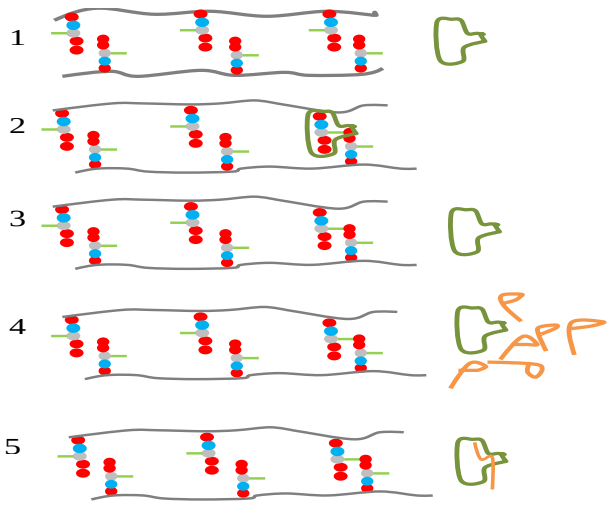


Figure 1.3: Cross-link formation in bacterial cell wall mediated by penicillin-binding proteins (PBP enzymes) and consequent penicillin inhibition of cell wall formation.

1. Cell wall of bacteria. 2. PBP forms cross-links by binding to peptide side chains in the absence of penicillin. Binding facilitated by the removal of a molecule of D-Alanine from a peptide side chain. 3. Cross-link formation triggers the dissociation of PBP from the cell wall. 4. Introduction of penicillin to the system. 5. Penicillin's beta-lactam ring of penicillin ("P") is opened in interactions with PBP, an irreversible process. Penicillin and PBP are covalently bonded, blocking the active site and inhibiting any cross-linking.

Alanine (L or D); D- glutamate; D-lysine; Pentaglycine Chain; Bacterial cell wall; Penicillin-binding protein

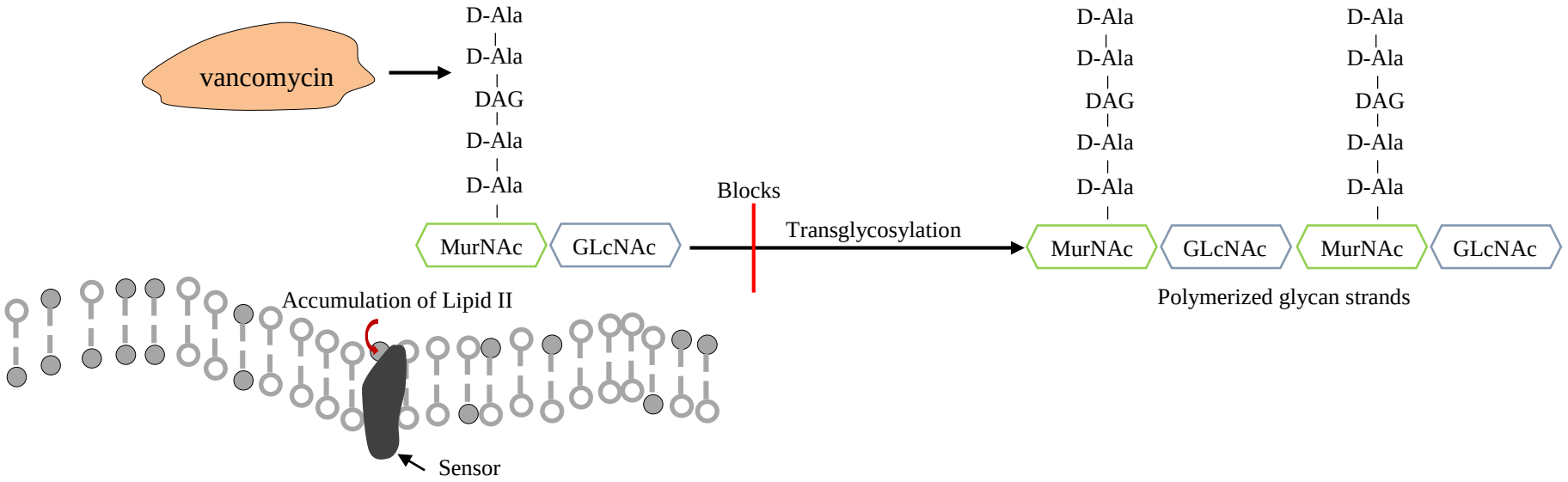


Figure 1.3: Schematic diagram of vancomycin acting on the cell wall of Gram-positive bacteria. Binding of vancomycin to the peptidyl-D-Ala-D-Ala blocks transglycosylation, by accumulating the bacterial cell wall precursor Lipid II, triggering a sensor protein, and allowing replacement of D-alanine by D-lactate at the C terminus of peptidoglycan precursors (Arthur, 2010).

1.5.2.2.2 Protein synthesis inhibitors

Protein synthesis inhibitors include various classes of antibiotics that are bacteriostatic or bactericidal by blocking one or more protein biosynthetic steps (Arenz and Wilson, 2016, Walsh, 2000). Inhibition of protein translation can occur on the 30S subunits of the bacterial ribosome in antibiotics such as tetracycline, spectinomycin, aminoglycosides kanamycin and streptomycin (Thaker et al., 2010, Böttger, 1994, Wimberly et al., 2000, Soares et al., 2012, Brodersen et al., 2000). It can also occur on the 50S subunits in clindamycin, chloramphenicol, linezolid, erythromycin, clarithromycin, azithromycin, and tylosin (Walsh, 2003a). Some other antibiotics such as rifampicin (Rif) (Campbell et al., 2001), Fidaxomicin (Artsimovitch et al., 2012) and Streptolydigin (Temiakov et al., 2005) can act at the protein transcription stage as inhibitors of RNA polymerases (Campbell et al., 2001). The Aminoglycosides such as streptomycin, tobramycin, gentamicin, amikacin and linezolid are active against pathogenic bacteria by initiating conformational changes on RNA nucleotides leading to the misreading of codons and incorrect mRNA translations (Figure. 1.5) (Perry et al., 1986, Walsh, 2003a, Serio et al., 2017, Moazed and Noller, 1987, Recht et al., 1999).

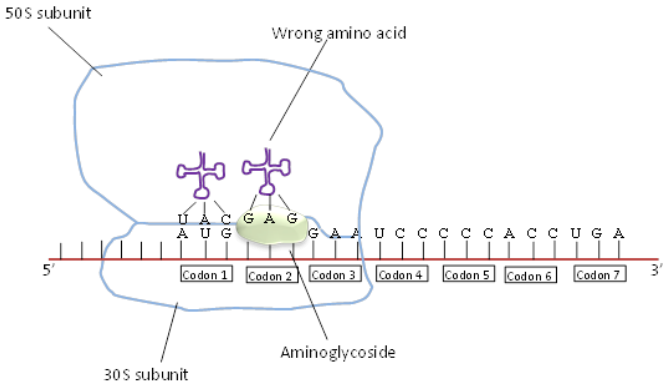


Figure 1.4: Binding of Aminoglycosides to 16S rRNA.

The aminoglycosides are irreversibly combined to the 16S rRNA in the 30S subunit of bacterial ribosomes and interfere with the proofreading process. The antibiotics reduce the rejection rate for tRNAs that are close matches for the codon. Codon misreading or early (premature) protein termination subsequently occurs (Schroeder et al., 2000).

1.5.2.2.3 Nucleic acid inhibitors

Some classes of antibiotics can inhibit nucleic acids synthesis with such antibiotics being assigned to two major sub-classes designated as DNA and RNA inhibitors (Etebu and Arikekpar, 2016). The mechanisms of action of these two sub-groups are, however, highly similar. For example, Quinolone drugs are DNA synthesis inhibitors that impede the function of DNA gyrase, a type II topoisomerase, a key bacterial enzyme in *Escherichia coli* and *Neisseria gonorrhoea* (Kohanski et al., 2010a). However, some topoisomerase subtypes are not found in higher eukaryotes and this makes them ideal targets for use as antibacterial agents (Chowdhury and Majumder, 2019).

1.6 Bacterial antibiotic resistance

The widespread use of antibiotics because of their proven efficacy against pathogens has resulted in frequent bacterial contact with antibiotics, leading to the development of resistance by previously susceptible bacterial groups. Bacterial antibiotic resistance mechanisms include the development of antibiotic resistant cellular components (e.g. efflux pumps etc.) (Banin et al., 2017, Caniça et al., 2019) which is propagated through vertical gene and plasmid transfer. These resistant genes can also be transferred to other bacterial groups by horizontal gene transfer (Wright, 2011, Povolo and Ackermann, 2019). The collection of all the bacterial antibiotics' resistance genes within a microbiome is referred to as the antibiotic resistome (Wright, 2007, Lanza et al., 2018). It can also refer to antibiotic resistant genes in a specific microbiome.

Antibiotic resistomes not only consist of antibiotic resistance genes but also of the different genes such as proto-genes that codes for proteins which are precursors of resistance elements and the silent genes which are only expressed by the mutation or mobilization of regulatory elements (Perry et al., 2014, Wright, 2007). Resistomes are adaptable and expand in the presence or absence of selection pressure and are found in pathogenic and non-pathogenic

bacteria, with the composition of resistomes varying and dependent on the microbiome being studied (Wright, 2010), However, for this review, the focus is on the genes responsible for antibiotics resistance.

Research on the genetics, biochemistry and evolution of antibiotic resistance (D'Costa et al., 2011, Davies and Davies, 2010) has indicated that bacterial antibiotic resistance can be intrinsic (Taber et al., 1987, Handal and Olsen, 2000, Maisuria et al., 2019) or acquired through gene mutations and horizontal gene transfer (Nakata et al., 2012, Daum et al., 2002, Bird et al., 2019). Intrinsic resistance is the natural ability of a bacterial genus, species, or strains to resist the action of a specific antibiotic. This could be due to the absence of a target for a specific as observed in vancomycin which is only active against Gram-positive pathogens (Reynolds, 1989). Alternatively, antibiotic resistance can occur through an active process; this could be through the alteration or modification of antibiotic targets, acquisition of antibiotic resistance from other bacteria through horizontal gene transfer or the removal of antibiotics from the bacterial cytoplasm through active pumps (Wright, 2011). The mechanisms of antibiotic resistance in bacteria are based on four major strategies. These are, (i) inactivation of the antibiotics through structural modifications or degradation, (ii) alteration of the antibiotic target site in bacteria, (iii) reduction in the antibiotics' cellular membrane permeability rates, and (iv) utilization of efficient efflux systems for removal of antibiotics from the cell.

1.6.1 Modification and degradation of antibiotics

One method utilized by some bacteria to limit or prevent the entry of antibiotics into their cells is by modifying the active components of antibiotics (Sander et al., 2018). For example, β-lactamases mediate the hydrolytic deactivation of the β-lactam ring in penicillin and cephalosporin (Drawz and Bonomo, 2010). This results in the antibiotic being unable to bind to PBPs (penicillin-binding proteins) rendering it ineffective and protecting bacterial cell wall synthesis. Some Gram-positive and -negative bacteria also neutralize aminoglycosides via this

mechanism through different biochemical processes such as acetylation and phosphorylation (Jana and Deb, 2006). Aminoglycoside antibiotics are usually composed of large molecules with naked (exposed) hydroxyl and amide groups (Norris and Serpersu, 2013). This makes them more amenable to structural modifications mediated by bacterial enzymes as observed in the pathogen *Campylobacter coli* which can be resistant to gentamicin (Qin et al., 2012).

Some resistant bacteria evade antibiotics by the modification of the target site to avoid recognition by the antibiotic (Mishra et al., 2013, Vester and Douthwaite, 2001, Sander et al., 2018). Therefore, no subsequent binding will take place as the active antibiotic molecule is unable to fit into the active site of the target enzyme. This strategy has been adopted by Staphylococci, which are protected from β-lactams by the acquisition and expression of genes encoding different PBPs to which β-lactams are unable to sufficiently bind to, allowing bacterial cell wall synthesis to go on largely unhindered (Villegas-Estrada et al., 2008).

1.6.2 Reducing permeability to antibiotics

Antimicrobial compounds are almost always required to pass through porin protein channels (Livermore, 1992) to cross the bacterial outer membrane and then to bind to cytoplasmic target sites and inhibit key bacterial metabolic processes in the cell. By reducing the permeability of these antimicrobials, access to cytoplasmic targets is reduced (reduced uptake) or denied and bacterial processes and proliferation continue unaffected (Fernández et al., 2017). This is an intrinsic mode of bacterial antibiotic resistance, commonly observed in Gram-negative bacteria. This mode of protection has been observed in *Pseudomonas aeruginosa*, *Enterobacter aerogenes* and *Klebsiella* spp. against imipenem (Livermore, 1992) and in *Escherichia coli* and *Enterobacter* spp. against cephalosporins and carbapenems (Lavigne et al., 2013, Tangden et al., 2013). Gram-negative bacteria are intrinsically less permeable to many more antibiotics than Gram-positive bacterial species (Lambert, 2002) due to their differing cell wall configurations.

1.6.3 Efflux of the antibiotics

The removal of the antibiotics from the cytoplasm of cells results in low intracellular concentrations, well below the concentration required to inhibit bacterial metabolic activities. Cellular expulsion of antibiotics is, therefore, another mechanism of bacterial resistance to antibiotics. This involves the use of efflux pumps which pump antibiotics out of the cell (Wright, 2011). These pumps can be specific in action selectively pumping out specific antibiotics such as lincosamides, macrolides, tetracyclines and streptogramins or broad spectrum in action, expelling structurally diverse antibiotics (Poole, 2007, Viveiros et al., 2005). The latter types of efflux pumps are designated as multiple drug resistance pumps and have been found for example, in *E. coli* (tetracycline) (Viveiros et al., 2005), and in Staphylococci (macrolides and streptogramins) (Poole, 2007). New antibiotic efflux pumps in different bacterial species or strains continue to be reported. Examples of such pumps include MedA in *Streptococcus mutans*, KexD in *Klebsiella. pneumonia* and LmrS in *S. aureus* (Floyd et al., 2010, Ogawa et al., 2012, Kim et al., 2013). Some of these pumps are plasmid-borne and are transferable to other bacteria (Dolejska et al., 2013, Mazel, 2006, Piddock, 2006). Figure 1.6 shows the interaction of drug molecules with the Gram-negative cell envelope.

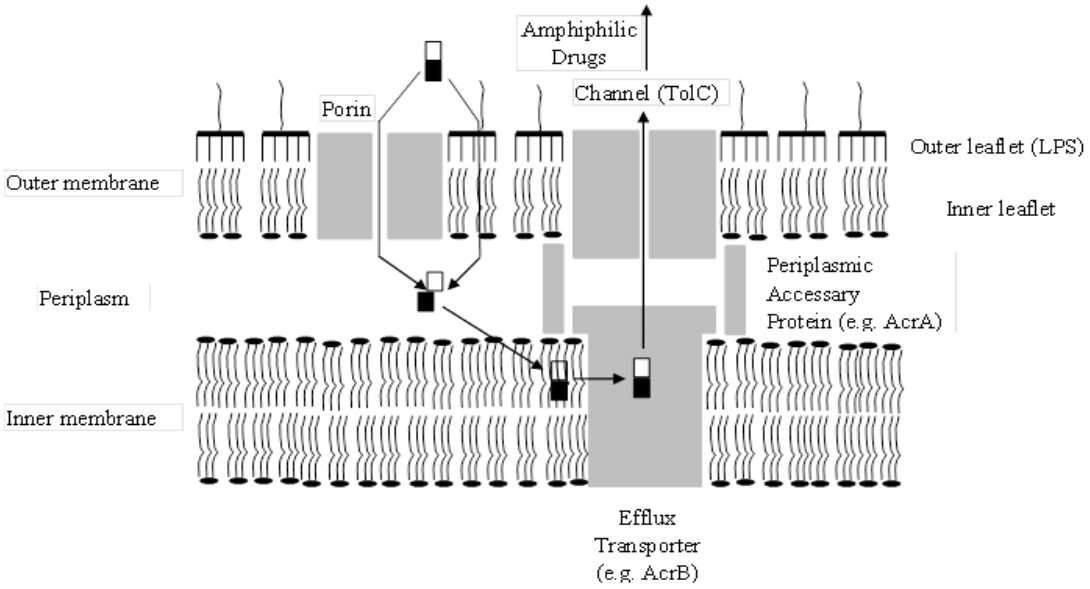


Figure 1.5: Representations of outer membrane permeability barrier and the mode of action of the multidrug efflux pump in Gram-negative bacteria. TolC is a type of outer membrane channel while AcrA and AcrB are types of efflux pumps.

Drug molecules pass through the outer membrane at a slow rate. Passage of hydrophilic molecules through the narrow porin channel is limited but lipophilic molecules penetrate the lipid bilayer domain. This is because of the low fluidity of the outer layer (devoid of unsaturated fatty acids (shown as straight lines in contrast to the wavy lines denoting the unsaturated fatty acids of phospholipids; Figure 1.6). This outer layer is comprised entirely of lipopolysaccharides (LPS). In the periplasm, amphiphilic drugs are rapidly partitioned into cytoplasmic membrane, picked up by transporters (e.g. AcrB) and then pumped out of the cell and back into the medium. This is mediated by a multisubunit complex equipped with OM channel (e.g. TolC) and periplasmic accessory proteins (such as AcrA).

1.7 Acquisition of antibiotic resistance

Acquired resistance to antibiotics by bacterial species can occur by many routes. These include both mutation of genes (e.g. antibiotic-targets) within the genome and secondly the acquisition of antibiotic resistance genes carried on plasmids (and by transposons and integrons on these plasmids) which are then transferred to recipient bacteria by horizontal gene transfer (Rodríguez-Rojas et al., 2013). Conjugation in bacteria is the key mechanism for the transfer of antibiotic resistance genes and plasmids on which these genes reside between bacteria. The different mechanisms of acquired antibiotic resistance are described in Tables 1.2 and 1.3.

Table 1.2 Mutation driven antibiotic resistance

Mutations	**General Traits**	**Examples**	**References**
Spontaneous Mutations	The chromosomal mutation rate is one in a population of 10^6-10^8 microorganisms.	Quinolones resistant *E. coli* is resulted from at least 7 amino acid mutations in the *gyrA* gene and three amino acid mutations in the *pacA* gene	(Džidić et al., 2008, Hawkey, 1998, Levy, 1998)
Hyper-mutators	A small bacterial population achieves a high rate of mutation due to prolonged exposure to nonlethal selection. The mutation rate can increase 10 to 50 up to 10,000 times	Most hypermutators happen in the populations of *E. coli*, *S. enterica*, *Neisseria meningitidis*, *S. aureus*, *Streptococcus pneumonia*, and *P. aeruginosa*	(Džidić et al., 2008)
Adaptive Mutagenesis	Mutations occur only during the nonlethal selection of microorganisms, mainly happen in dividing cells.	Streptomycin causes a hypermutable phenotype in *E. coli*, and some antibiotics can induce the SOS mutagenic response, hence induce resistance in host microorganisms	(Erill et al., 2006, Guerin et al., 2009)

Table 1.3 Mechanisms of horizontal gene transfer

Horizontal Gene Transfer		**General Traits**	**Examples**	**References**
Transformation		Foreign DNA is incorporated into the recipient chromosome by recombination or transposition.	Transformation of Tn916 demonstrated in oral biofilms	(Wu and Wu, 1987)
Conjugation	Plasmids	Double-stranded circular DNA, size 1-100kb, mobilizable and can replicate independently.	Resistance (R) plasmids, which contain genes that provide resistance against antibiotics or poisons. Histori cally, Known as R factors.	(Stone, 1974)
	Conjugative transposons	Promotes self-transfer and integrates into chromosomes	Tn916: Tet and Mino Tn1545: Tet, Mino. Ery, and Kan	(Clewell et al., 1995)
Transduction	Bacteriophage	Bacterial chromosome packaged into phage cell, lysis occurs, and phages are released.	Carriage of antibiotic resistance genes in bacteriophage	(Haapa-Paananen et al., 2002)

1.8 Antibiotic resistance genes (ARGs) in the environment

Pharmaceuticals, including antibiotics, can enter the environment including into lakes, rivers and streams in many ways. These include through industrial waste products; animal or human waste; improper disposal; runoff from antibiotics sprayed onto agricultural produce; the spreading of manure (containing antibiotics) onto farmland and the output from sewage treatment plants (Rodriguez-Mozaz et al., 2015, Proia et al., 2016, Gothwal and Shashidhar, 2015, Gothwal and Thatikonda, 2017, Zhao et al., 2019). Municipal sewage treatment, especially in areas using simple or inadequate treatment systems, are important sources of antibiotic contamination of aquatic systems. This is because of the pharmacological properties of some antibiotic compounds, which may be excreted in their active form in human and animal waste. These properties allow them to survive in aquatic systems or remain intact in the soil until they seep into the water table and potable water sources (Kümmerer, 2009, Kümmerer, 2008). In the environment, elimination of sensitive bacterial population and selection of microbial groups resistant to the contaminating antibiotics can occur. The increasing presence of drivers of resistance such as the increased scale of human and antibiotics usage and discharge into the environments and the increased use of reclaimed water and sludge has resulted in elevated levels of antibiotics in the environment (Singer et al., 2016). This potentially accelerates the development of bacterial resistance to these anti-microbials, shortening the time frame from introduction to the development of resistance genes to antimicrobials. A good example is the development of resistance to penicillin by strains of *Streptococcus* within 7 weeks of the use of penicillin in culture-based assays (Abraham et al., 1941). These resistance genes or ARGs can be propagated through vertical and horizontal transfer.

Apart from anthropogenic sources, earlier investigations have revealed that the antibiotics resistance and by extension ARGs that encode these resistances can exist naturally in the environment. Research into non-agricultural and non-clinical environments has demonstrated the presence of resistance to tetracycline and beta-lactam antibiotics soils from these environments using PCR and cloning based

assays (Allen et al., 2010). Similarly, a reservoir of antibiotic resistance genes including those responsible for resistance to novobiocin, aminoglycosides, tetracycline, and macrolides was detected in samples from remote pristine Antarctic soils shotgun metagenomics (Van Goethem et al., 2018).

1.8.1 Antibiotic resistance genes from clinical sources and environments

One of the most well-known and important causes of antibiotic resistance is their clinical use, via appropriate or inappropriate prescriptions. Different antibiotic resistant bacteria and genes have been identified from clinical sources. One bacterium, *Klebsiella pneuomoniae* is recognized worldwide as one of the major sources and transfer of antibiotic resistance, acquiring ARGs via mutations and/or acquisition of plasmids carrying resistance genes leading to the development of multidrug-resistant bacteria (Navon-Venezia et al., 2017). For example, phenicol-oxazolidinone-tetracycline (*poxtA*) resistance genes have been detected in an MRSA strain (Antonelli et al., 2018). Linezolid resistance mediated by *cfr*, *poxtA* and *optrA* genes has been observed in enterococci strains of clinical origins (Bender et al., 2019). Other ARGs such as *bla* (beta-lactams) (Rahbarnia et al., 2020), *mecA* (methicillin) and *vanA* (vancomycin) (Saeed et al., 2020), *qnr* and *aac(6')-Ib-cr* (quinolone) (El-Badawy et al., 2019) and *tet* (tetracycline) (Amoako et al., 2019) have been identified in clinical bacterial isolates. In addition to culture-based assays, different ARGs have been detected in clinical wastes using PCR and next-generation sequencing approaches based assays. ARGs such as *bla* NDM-1, *bla* CTX, *bla* OXA and *int1* have been detected in hospital wastes or wastewaters from India using PCR and Q-PCR assays (Lamba et al., 2017). These ARGs are often present in clinical wastes and play important roles in the dissemination of antibiotics resistance when these wastes are discharged into environmental systems.

Environmental contamination in both terrestrial and aquatic environments by clinical wastes that are rich in antibiotics can lead to the selection and proliferation of antibiotic-resistant microbial groups (Alam et al., 2018). Therefore, the development and spread of antibiotic resistance has been exacerbated by the widespread use of broad-spectrum compounds and their extensive use in

agriculture, (Ventola, 2015). These factors either promote increased use (and environmental exposure) to antibiotics leading to a selection of resistant strains and/or increased horizontal gene transfer of antibiotic resistant genes and/or increased human exposure to potential antibiotic resistant bacteria. Co-selection of resistance to antibiotics and heavy metals (both are plasmid-borne) can also occur when plasmids with these genes are transferred to new bacterial groups. These are important factors to consider, as resistant bacterial pathogens have the potential to cause increased rates of human morbidity and mortality. Additionally, this places greater demands on the healthcare system with associated increases in public health costs due to increased length of hospital stay and treatment costs (Lipsitch et al., 2000, Klein et al., 2018, Friedman et al., 2016, Ventola, 2015).

1.8.2 Antibiotic resistance genes in the agricultural environment

Antibiotics have been used in agriculture as prophylactics for several decades in many countries including the United States, United Kingdom and Australia although regulations have now been developed to regulate (limit) their use (Shaban et al., 2014, Centner, 2016). This widespread use of antibiotics in animal feed has led to the development and spread of resistant genes amongst animal-borne bacteria with increasing numbers of animals being infected with resistant strains of common pathogens (McEwen and Fedorka-Cray, 2002, Makary et al., 2018). In addition, the use of reclaimed wastewater in agriculture can be a source of ARGs (Christou et al., 2017). The use of manure and treated sewage (biosolids) can also be a source of ARGs in agricultural systems (Yang et al., 2018, Zhao et al., 2017, Dungan et al., 2019, Zhao et al., 2019, Heuer et al., 2011). Therefore, there is a considerable concern that antibiotic resistance associated with agricultural-activities and -produce could lead to increased antibiotic resistance of human-borne bacteria via horizontal gene transfer. Consequently, many countries now recommend the use of a sub-therapeutic antibiotic dose for animals or the use of antibiotics not used in human clinical settings (Aarestrup et al., 2010).

ARGs for beta-lactamase, tetracycline, vancomycin, sulphonamide, chloramphenicol and MLSB have been detected in bovine, pig and chicken manure (Qian et al., 2018). Evaluation of the diversity

of ARGs in lettuce plants (soil amended with biosolids) showed that dominant ARGs detected therein were *cat, tet, php, vanB,* and macrolide-lincosamide-streptogramin B resistance genes (MLSB). The abundance of these ARGs was positively related to the applied biosolid concentration (Yang et al., 2018). NDM1 genes have also been detected in isolates from fresh lettuce from farms in which biosolids or reclaimed water had presumably been used for irrigation (Wang et al., 2018b). The application of animal wastes and biosolids as manure to farmland is thought to an important means for the spread of antibiotic resistance genes (Heuer et al., 2011). Research has shown that IncP-1 plasmids (carrying resistance genes such as *intI*1 and *sul*1) abound in manure and are important vectors of ARG in agriculture (Heuer et al., 2012). Apart from manure, the use of wastewater for urban agriculture has been shown to increase the abundance of EBSLs, with ARGs encoding for amphenicol and tetracycline resistance being detected in irrigated soils (Bougnom et al., 2020). Indeed, one of the challenges of or impediments to re-using urban wastewater is the presence of ARGs (Fatta-Kassinos et al., 2019). ARGs have also been detected in aquaculture. Recent investigations of *Eriocheir sinensis* (Chinese mitten crab) aquaculture showed that bacitracin (*bacA*) and multidrug (*smeE, acrB* and *macB*) ARGs were the most dominant resistance genes detected in bacteria therein, with other ARGs such as *tet, van* and *bla* were also detected (Fang et al., 2019).

1.8.3 Antibiotic resistance genes in wastewater treatment plants

Wastewater treatment plants (WWTPs) collect sewage from a large area, normally encompassing residential areas, industrial sites and hospitals. WWTPs aim to remove biological contaminants from wastewater so that it might be deemed sufficiently safe for prescribed downstream uses (Reinthaler et al., 2003, Racz and Goel, 2010). Wastewater is frequently contaminated with human and animal pathogens; this makes WWTPs an ideal location for antibiotic resistant bacteria (ARB) and antibiotic resistance gene (ARG) to flourish and for resistance gene transfer from one bacterial group to another (Bouki et al., 2013, Guo et al., 2017, Svobodová et al., 2018, Bueno et al., 2020). Often antibiotic resistance will first develop in the gastrointestinal tracts of humans or animals, with waste-water

containing these resistant strains then draining to a local WWTP where such bacteria can multiply, pass on the resistance factors and go on to contaminate local aquatic environments. Consequently, many studies have focused on faecal coliform bacteria detection conducted alongside assays for antibiotic resistance genes, as these microorganisms are the most prevalent group in sewage (Carnelli et al., 2017, Lyimo et al., 2016). Several studies of WWTPs have found that outflow pipes or wastewater from these facilities contain resistant bacteria (Cacace et al., 2019, Turolla et al., 2018). Furthermore, unprocessed waste may enter water sources either intentionally or unintentionally, or via combined sewer overflows (CSOs) (West et al., 2011). Due to these factors, WWTPs are both a breeding ground and a reservoir for new antibiotic resistance in human pathogens. It is interesting to note that, due to the use of antibiotic-type agents in the waste treatment process, processed sewage may have higher rates of resistance than unprocessed sewage (Guardabassi et al., 2002, Reinthaler et al., 2003).

Multiple ARGs have been identified from sewage, wastewater and soil samples from wastewater treatment plants (Voolaid et al., 2018). A metagenomics based assessment of urban sewage review from 60 different countries showed that the sewage was a hotspot for antibiotic resistance genes, with the prevailing ARGs in each country determined by factors related to the socio-economic status, environmental use and overall health of the concerned populace (Hendriksen et al., 2019). ARGs such as *ermB* (macrolide)*, aph(3')-IIIa* (aminoglycosides)*, tetM* and *tetL* (tetracycline resistance) have been detected in *Enterococcus* isolates from wastewater treatment plants (Hamiwe et al., 2019). Indeed, wastewater treatment plants and environments can be hotspots for ARG carriage with effluents from these plants disseminating ARGs into receiving aquatic systems (Bueno et al., 2020). Assays for the diversity of ARG in aerobic activated sludge and anaerobically digested sludges from WWTP using high throughput methods have shown that ARGs mediating resistance to tetracycline, fluoroquinolones, sulfonamides, beta-lactams, phenicols, trimethoprim, macrolide-lincosamide-streptogramin (MLS), aminoglycosides and glycopeptides were commonly detected (Guo et al.,

2017). The relative abundance of multidrug (antibiotics) resistant genes, *tet* and *sul* genes were high in both samples (Guo et al.). An excellent review of ARGs in the influent, sludge and effluent of WWTP assessed using traditional and molecular microbiological tools has conclusively demonstrated the prevalence of ARGs in these samples. These include multiple variants of *bla* (beta-lactams resistance), *ere*, *mef*, *mel* and *erm* (macrolides resistance), *gyr*, *par* and *qnr* (quinoline resistance), *dfr*, *dhfr* and *sul* (sulphonamide resistance), tet (tetracycline resistance) and *acr*, *amr*, *mdt* and *mex* (multidrug efflux pumps) genes (Pazda et al., 2019).

1.8.4 Antibiotic resistance genes in the aquatic environment

Aquatic environments are particularly amenable to harbouring bacteria with antibiotic resistance as they receive multiple contaminated inputs (Gothwal and Thatikonda, 2017). As it is difficult to directly assess mixed populations of bacteria found in aquatic environments, researchers tend to assess pathogen indicators in the water such as total faecal Coliforms, *Enterococci, Escherichia coli and Clostridium.* The most commonly measured microbial indicators of faecal pollution are *E. coli* and *Enterococcus* (Cabelli et al., 1982, Prüss, 1998). Several researchers have found that these pathogens can survive outside their hosts for long periods, even re-acclimatising to sand, algae or soil (Byappanahalli et al., 2006, Ishii et al., 2006a, Ishii et al., 2006b, Ishii et al., 2007, Yan et al., 2007).

The ubiquitous use of antibiotics has resulted in extensive resistance in bacteria (both commensal and non-commensal) in aquatic ecosystems. These resistant bacteria are now recognized as contaminants in their own rights, as they have the potential to transmit resistance through the use of plasmids or transposons to pathogenic bacteria (Rysz and Alvarez, 2004, Snow et al., 2009, Martínez, 2008). Several researchers have demonstrated that aquatic environments are becoming basins of pathogens and resistant genes (Boon and Cattanach, 1999, Chee-Sanford et al., 2001, Le Quesne et al., 2018, Carvalho and Santos, 2016).

A recent study found that *Enterococcus* bacteria were evolving in response to medical waste discharged through a plant-river continuum. These populations demonstrated both novel antibiotic resistance and resistance passed on from incoming bacteria (Leclercq et al., 2013). Furthermore, antibiotic resistant genes (ARGs) are particularly worrying as they have been detected in wastewater by multiple researchers (Pruden et al., 2006, Watkinson et al., 2007, Karkman et al., 2018) and could be potentially transferred to recipient microbes. For example, ARGs were noted in large quantities in reclaimed water in the Werribee Basin (Australia). This basin provides water for irrigation, meaning that resistance may spread to an agricultural environment (Barker-Reid et al., 2010). Similarly, ARGs such as *tetM*, *vanA* and *vanB* have been predominantly detected in animal husbandry facilities and in subtropical waters used for recreation and other domestic purposes (Rivera et al., 1988, Byappanahalli et al., 2003, Agersø et al., 2006, Roberts et al., 2009, Akanbi et al., 2017, Li et al., 2018a).

The ubiquity of antibiotics has engendered an ever-evolving presence of antibiotic resistance. While this resistance represents a looming public health crisis, there is comparatively limited surveillance of the prevalence of environmental antimicrobial resistance in rivers. Factors that reduce or shorten the persistence of antibiotics such as biodegradation by bacterial species, adsorption and potential inactivation by sludge and colloidal materials and sunlight mediated degradation have been reported (Singer et al., 2016, Maki et al., 2006, Engemann et al., 2006). Nevertheless, these factors are not widely studied and it would be highly challenging to exploit these processes to seek to reduce antibiotic persistence in the environment. The problem is especially troubling as aquatic ecosystems are an ideal environment for the transmission of mobile genetic elements (MGEs) that pass on antibiotic resistance. In order to head off this crisis, decisive steps must be taken to limit the use, dispersal and discharge of antibiotics into the environment. Incorporating ARG screening steps into a biomonitoring system for rivers would, therefore, be greatly advantageous for monitoring the success of such a strategy.

1.9 Analysis of bacterial communities in the natural environment

1.9.1 16S ribosomal RNA (rRNA) gene analysis

The 16S rRNA gene encodes the 16S rRNA in the 30S small subunit of ribosome in bacteria and archaea. The 16S rRNA gene is the most widely used molecular marker for identification and classification of different bacterial groups in complex environments because it is present all bacteria (and archaea) (Janda and Abbott, 2007, Tringe and Hugenholtz, 2008). Structurally, the 16S rRNA gene encompasses conserved regions interleaved with hypervariable regions (HVRs) and primers used for PCR are designed to target the conserved regions. These conserved regions, therefore, serve as anchor points for the designed primers and the subsequent sequencing of the hypervariable regions between the conserved regions allows for accurate bacterial identification (Fuks et al., 2018). Therefore, PCR amplification of target sequences using universal primers facilitates the identification of a single bacterial species and or differentiation between a number of different species or genera (Becker et al., 2004, Maynard et al., 2005). These distinct features of the16S rRNA gene make it a suitable genetic marker for the taxonomic characterization of microbial species isolated or directly sequenced from natural environments (Hao and Chen, 2012, Dethlefsen et al., 2008, Ortiz-Estrada et al., 2019). 16S rRNA gene sequencing is now widely utilized in the identification of pathogen and antibiotics resistance bacteria in samples from various environments such as lakes, rivers, wastewater and wastewater treatment plants (Khan et al., 2019, Srinivasan et al., 2015, Su et al., 2018, Quintela-Baluja et al., 2019).

Within the last few decades, 16S rRNA gene-based techniques such as the Polymerase Chain Reaction (PCR), quantitative PCR, metagenomics and other culture-independent assays (which allow for the detection of culturable and non-culturable microorganisms) have been developed. These 16S rRNA based methods can be used to identify bacterial cultures that have already been determined via functional assays to be resistant to antibiotics (Armalytė et al., 2019, Ullmann et al., 2019). These methods are not usually used to detect ARGs in environmental samples as these genes (ARGs) are

not found on the 16S rRNA gene. 16S rRNA gene-based methods are now routinely used for comprehensive analysis of the taxonomy, diversity and function of microorganisms in environmental samples. Such approaches allow the analysis of the more than 98% of the bacteria which cannot be cultured using traditional methods but by culture-independent techniques (Wooley et al., 2010).

1.9.2 Qualitative and quantitative detection of antibiotic resistance genes and resistant bacteria using PCR and qPCR

The presence and abundance of antibiotic resistance genes can be investigated using endpoint PCR and quantitative PCR (Q-PCR). Direct detection of ARGs in clinical and environmental samples by PCR involves the use of custom-designed primers targeting antibiotic resistance genes of interest. This approach has been used to target ARGs in pristine freshwater samples from rivers (Avşar, 2019, Lima-Bittencourt et al., 2007), polluted rivers (Mittal et al., 2019, Wang et al., 2020), from aquaculture (Zhao et al., 2020, Xiong et al., 2015), freshwater lakes and sediments (Czekalski et al., 2014, Ohore et al., 2020). Plasmid-borne antibiotic resistance genes (such as tigecycline, chloramphenicol, β lactams, sulphonamide and macrolide resistance genes) in bacterial cultures and in bacterial communities from waste treatment plants have also been detected using PCR (Ji et al., 2020, Szczepanowski et al., 2009). However, while bacteria with ARGs can be detected and identified using PCR-sequencing based approaches, their abundance cannot be determined as end-point PCR is not a quantitative method.

Quantitative PCR (qPCR) is a reliable method for both the detection and quantification of specific genes (including) ARG by quantifying the target DNA based on the number of copies amplified during the cycles of the polymerase chain reaction. qPCR remains a robust and reliable, cost-effective method for quantifying target genes and biomarkers with excellent reviews of the basic principles, application and data analysis already presented (VanGuilder et al., 2008). Binding of fluorescent SYBR Green to amplicons during the Q-PCR is the most commonly used approach in qPCR assays (Cao and Shockey, 2012). SYBR Green based qPCR assays have been used to detect and quantify

ARGs such as *tet, mph* and *aph(3')* (Morsczeck et al., 2004), *erm* (Ouyang et al., 2020), *int1* (Zheng et al., 2020) and *mef* genes (Reinert et al., 2004) in environmental samples. The prevalence of other ARGs including s*ul, aph(3')* and *npt* ARGs in rivers (Paulus et al., 2019, Pei et al., 2006) and drinking water (Zhang et al., 2020), *tetA, B, E,* M and Z genes in a polluted river (Xu et al., 2015) and ampC, *bla TEM, SHV* and *NDM* genes in tropical rivers and water systems in oil fields (Chen et al., 2020b, Devarajan et al., 2016) have also been quantified with qPCR. Alternatively, TaqMan probes can be used to provide increased specificity during Q-PCR. This approach has been used to determine and quantify prevalent ARGs such as *tetO, tetW* and *tetQ* (Smith et al., 2004), *vanA*, *mecA* and *ampC* (Volkmann et al., 2004) in wastewater and *bla, sul, tet* and *mec* genes in contaminated rivers (McConnell et al., 2018). Quantitative PCR is now routinely used to detect and quantify ARGs freshwater systems (Czekalski et al., 2015, Devarajan et al., 2015).

Furthermore, qPCR can also be used to compare the effects of pollution on aquatic systems by quantifying ARGs (e.g. *sul* genes or *npt* genes) in pristine and polluted systems (Pei et al., 2006). In this instance, the concentration of resistance genes was higher at the impacted sites as compared to the more pristine sites along the course of the river (Pei *et al*., 2006). Reports have shown that the frequency of *tetM, tetQ,* tetO and tetW genes was higher in swine lagoon polluted wells than in clean wells (Mackie et al., 2006). Higher bla_{TEM} *a*nd bla_{NDM} abundance were observed in effluent polluted Cauvery River in India compared to unpolluted control sites (Devarajan et al., 2016) with pollution generally leading to elevated ARG concentrations in most river systems (Cacace et al., 2019). qPCR while being an excellent tool for quantifying the abundance of specific genes, it provides no information on the diversity of these genes hence the need for the use of sequencing approaches such as next-generation sequencing method.

1.9.2.1 16S rRNA based next-generation sequencing (NGS) approaches

One of the most commonly used NGS approaches for studying bacterial communities is 16S-rRNA based NGS or targeted sequencing (Fuhrman, 2012). Targeted sequencing involves the sequencing

of individual marker genes such as 16S rRNA, ITS regions and specific genes of interest to predict the diversity and relative abundance of microorganisms or targeted genes within environmental samples. Next-generation sequencing approaches (also sometimes called high-throughput sequencing approaches) by nature are sometimes culture-independent approaches used for "massive parallel DNA sequencing read production" (Mardis, 2013, Mardis, 2008). However, they can also be used to sequence the genome of bacterial cultures. This is typically performed on sequencing platforms such as the Ion Torrent PGM and Proton (Thermo Fischer), Roche 454 FLX platform (pyrosequencing), Illumina's MiSeq and HiSeq, Applied Biosystem's SOLiD, and PacBio's SMRT systems (Mardis, 2013, Liu et al., 2012, Mardis, 2008).

Due to the absence of ARGs on the 16S rRNA gene, there is no report of the use of this marker for direct assay for ARG using amplicon sequencing or gene-centric analysis (Prosser, 2015). While widely used in PCR, primers targeting different ARGs are rarely used in targeted gene (amplicon-based) NGS based approaches to evaluate the prevalence and diversity of antibiotic resistance genes in environmental and clinical samples. When used, the results have sometimes been presented in peer-reviewed literature as proof of concept publications. For example, an amplicon-based sequencing of *Mycobacterium tuberculosis* DNA from sputum samples of patients was successfully used to generate patients' drug susceptibility profiles based on specific antibiotic resistance genes (*inhA* and *katG* (isoniazid resistance), *rpoB* (rifampicin resistance), *gyrA* (quinolone resistance), *eis* (kanamycin resistance)) in a proof of concept study. The limited use of ARG-specific oligonucleotides in gene-centric analysis may be due to problems with specificity of primer being used for the PCR amplification due to technical difficulties associated with NGS. If these primers are highly (too) specific, then there is limited diversity in the obtained sequences but if they were less conserve or specific, non-target amplicons are obtained. Nevertheless, this is a promising research area. However, ARGs have been widely studied using metagenomics and this is discussed in the next section.

1.9.2.2 Non 16S rRNA based next-generation sequencing (NGS) approaches

Metagenomics is the analysis of the genomic DNA of the organisms present within a community through cloning or random shotgun sequencing of environmental DNA in order to study gene diversity inherent in microbial communities (Venter et al., 2004). This can be performed via two different but complementary approaches: functional metagenomics and sequence-based metagenomics (Simon and Daniel, 2011). Functional metagenomics involves cloning and heterologous expression of environmental DNA in a heterologous host combined with activity-based screening to discover gene functions. Sequence-based metagenomics involves extracting and randomly sequencing of DNA directly from the environment including the DNA of unculturable bacteria on the desired NGS sequencing platforms.

A review of the literature showed random shotgun sequencing approaches have in recent years been widely applied to the study of the prevalence of antibiotic resistance genes (which is the focus of this study) in different environments (Parsley et al., 2010, Li et al., 2015, Amos et al., 2014, Wang et al., 2018a). This approach has been used to study the prevalence of ARGs in environmental systems, through high-throughput sequencing of total extracted DNA, with the generated reads, quality filtered and annotated. Thereafter, ARG sequences of interest retrieved from suitable databases such as ARDB (Antibiotic Resistance Genes Database) and MGEs (Mobile Genetic Elements) can then be compared (for example using blast) against these reads to detect them in the metagenomic data (Zhang et al., 2018). The prevalence of detected ARGs can be expressed as a percentage of the total reads or relative to other detected ARGs or other genes such as 16S rRNA genes, enabling the comparison of relative abundance (Knapp et al., 2017). For example, a high-throughput sequencing-based metagenomic approach combined with data analyses using a structured ARG database was used to identify ARGs in samples from different environments including river water (Li et al., 2015). In this study, ARGs responsible for resistance to bacitracin, aminoglycoside, tetracycline and beta-lactam antibiotics were detected and quantified. This approach was also used to identify 18 ARGs types such

as those responsible for resistance to beta-lactam, sulphonamide, tetracycline, vancomycin, chloramphenicol and quinolone resistance in samples from a sewage treatment plant (Yang et al., 2013). A follow-up study using a metagenomic approach to detect the fate of ARGs in a full-scale sewage treatment plant (STP) (Yang et al., 2014), revealed a high removal efficiency of 99.82% for total ARGs in wastewater after sewage treatment. Also, this approach has been used to demonstrate that activated sludge and waste treatment plants, in general, can be hotspots of ARGs and MGEs (Guo et al., 2017, Liu et al., 2019). It has also been used to detect antibiotics resistance genes in water reservoirs (Dang et al., 2020) and ARG prevalence in freshwater systems and recycled water (Chopyk et al., 2020).

However, there are inherent limitations in the use of the 16S amplicon sequencing (and also shotgun metagenomics sequencing approaches). These include misleading results from sample or reagent contaminants which are sequenced alongside target molecules (Salter et al., 2014), bias associated with DNA extraction and library preparation methods and also amplification bias and inability to determine whether the extracted DNA is from viable or non-viable microorganisms (Bardy and Psaltis, 2016). Besides, the results of data analysis may vary depending on the choice of clustering algorithms and pipeline used (Bardy and Psaltis, 2016, Prodan et al., 2020).

In conclusion, this review has demonstrated that endpoint and quantitative PCR approaches are reliable and widely used methods for studying ARGs in aquatic and other environmental systems. Primers designed to target different ARGs of interest also exist in literature. This is why these two approaches have been selected for use in this study. However, gene-centric next-generation sequencing-based approaches have not been applied to the study of antibiotic resistance genes. Instead, an indirect approach usually involving the sequencing of extracted DNA on NGS platforms, and a subsequent sequence-based assay for the prevalence of ARGs of interest in the sequence data is usually used. Therefore, useful information on ARG that can be derived from a targeted sequencing approach is unavailable and this represents a limitation in the present NGS-based approach to the

study of these genes. The scientific understanding of the distribution and prevalence of ARGs in environmental systems derived from data obtained from a direct approach (targeted sequencing) is therefore unavailable and represents a gap in our knowledge of ARGs. This is why this study, in addition to using the traditional endpoint PCR and qPCR assays, has incorporated a targeted-next generation sequencing (amplicon-based) approach using the same qPCR antibiotic resistance genes primers to detect and quantify ARGs in samples from a river in Melbourne.

1.10 Aims and Objectives

This study aims to investigate the structure and diversity of bacterial communities and the presence, abundance and diversity of antibiotic resistance prevalence therein at multiple locations along the Werribee River, Victoria using culture-independent approaches; endpoint PCR, quantitative PCR and Next Generation Sequencing (NGS) (random shotgun sequencing and a targeted sequencing approach). This is premised on the fact that using a combination of these methods will present a more comprehensive picture of the diversity of these genes in the Werribee River than that presented by each of the individual methods. The three specific objectives of this research were.

1. To assess the spatial and temporal variation in the structure, composition and diversity of bacterial communities along the Werribee River.
2. To investigate the spatial and temporal variation in the presence and abundance of antibiotic resistance genes (ARG) at selected sites along the Werribee River, Australia using PCR and qPCR detection assays.
3. To assess the suitability of an amplicon-based next-generation sequencing approach to the study of antibiotics resistance genes in surface water samples from the Werribee River.

2 Chapter 2: Materials and Methods

2.1 Experimental design and sample location

2.1.1 Sampling sites

Samples of water used in this study were collected from four selected sites along the Werribee River, in Victoria (Figure 1). These sites were Ballan (BA) and Bacchus Marsh (BM) located in the upstream sections and Cobbledicks Ford (CF) and Riversdale (RD) located in the downstream sections of the Werribee River. Water samples were collected from these in April, June, August, October, and December of 2015 and February 2016.

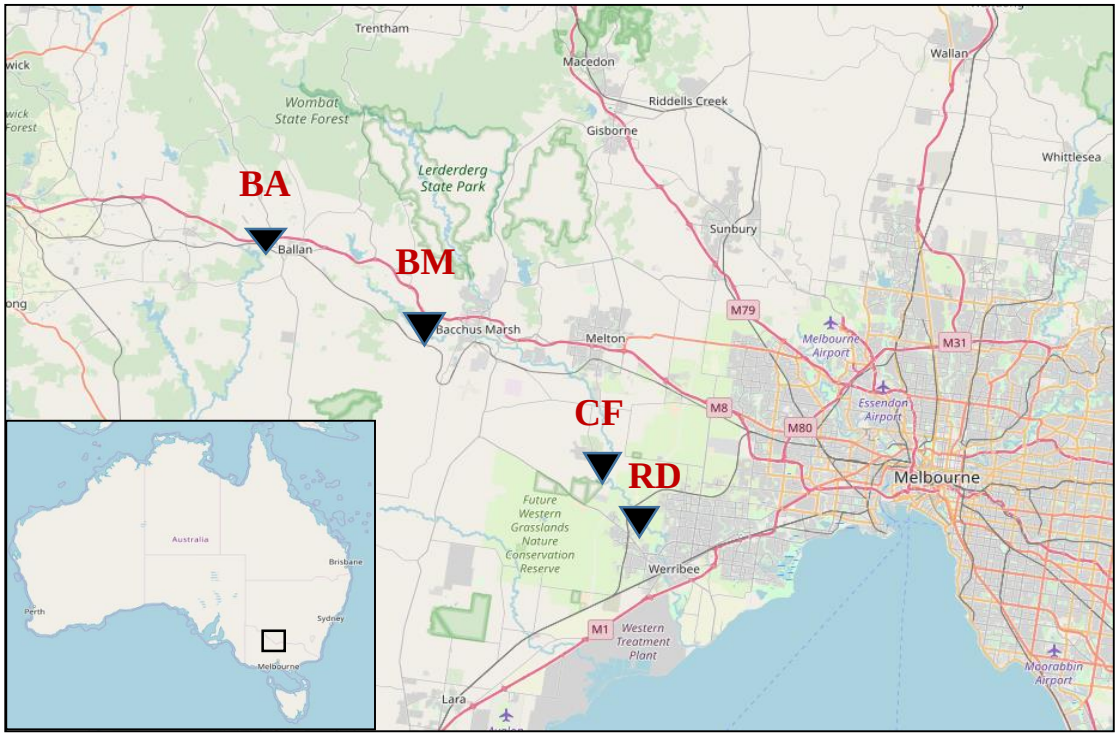


Figure 2.1 Location of sampling sites on the Werribee River. Mill Park Reserve Ballan (BA) (37°35'51.2"S 144°13'51.0"E**)** and Miekle Rd Bacchus Marsh (BM) (37°40'56.7"S 144°25'56.5"E**)** are upstream sites while the downstream sites Cobbledick Ford (CF) (37°49'13.3"S 144°34'51.3"E) Wyndham Vale and Riversdale Drive (RD) (37°52'48.4"S 144°38'50.8"E). (Source: Open map).

2.1.2 Collection and preparation of samples

Triplicate water samples were collected from each of the four sites along the Werribee River at two-monthly intervals, from April 2015 to February 2016, totalling 72 samples across sites and months. At each site, triplicate samples were collected relative to one another along a 6 m reach in the river. The physico-chemical parameters such as water temperature, pH, dissolved oxygen concentration and electrical conductivity were measured with Aquaread AP-2000 multi-parameter water quality equipment (Aquaread, UK) according to the manufacturer's instruction. Surface water samples were collected at a depth of approximately 0.5 m (Khan *et al*., 2016). These water samples were collected in 300 mL sterile Schott bottles and transported on ice to the RMIT research laboratory in Melbourne, Victoria. Multiple analyses to determine the nutrient composition of the samples were carried out using the appropriate methodologies. Turbidity and suspended solids were determined using the APHA 2130 and APHA 2540D methods (APHA, 2012), ammonia with APHA 4500-NH3 (APHA, 1998b), nitrites and nitrates with APHA NO2 B (APHA, 1998a), phosphates with APHA 4500-PF, total Kjehldahl nitrogen(TKN), total nitrogen and total phosphorous with APHA, 4500-N C and 4500-P J. (APHA, 2012).

Water samples were filtered using vacuum pumps (Chemker 411, Taipei, Taiwan) equipped with 0.2 µm pore size filters (Millipore Merck, Melbourne, Australia). The 0.2 µm pore size filters were aseptically removed from the vacuum pump with sterile tweezers and placed directly in PowerWater Bead tubes (MoBio Laboratories, West Carlsbad, USA). The tubes were stored at -80 °C until further processing.

2.2 Bacterial strains:

Ten bacterial strains were selected for use as positive controls for in PCR and qPCR assays. They were selected based on their resistance to some target antibiotics and carriage of the respective antibiotic resistance gene (Table 2.1).

Table 2.1 Bacterial strains with antibiotic resistance genes

Bacterial Name	RMIT Strain Number	Antibiotic resistance phenotype
Enterobacter cloacae	100/2-3	Chloramphenicol
Enterobacter cloacae	100/2-3	Sulphonamide
Klebsiella sp.	18/-	Carbapenem
Staphylococcus saprophyticus	344/1-1	Colistin
Serratia marcescens (strain: T10)	342/1-7	Ampicillin
Staphylococcus aureus	344/2-3	Erythromycin
Staphylococcus aureus	344/2-7	Methicillin
Staphylococcus aureus	344/2-10	Tetracycline
Enterococcus faecium	345/19-1	Vancomycin
Staphylococcus aureus	344/2-16	Aminoglycoside

2.3 Oligonucleotides

Different sets of oligonucleotides were used to target the hypervariable regions of the 16S rRNA gene and antibiotic resistance genes using polymerase chain reaction. The first set of oligonucleotides used was a modified primer pair of 515F (5'-AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3') and 806R (5'-CAAGCAGAAGACGGCATACGAGATAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3') (Caporaso et al., 2011). The primers were modified for use in next-generation sequencing procedures performed on Illumina's MiSeq instrument, to investigate the structure, diversity and composition of bacterial communities in water samples from the Werribee River. The second primer pair used was the 341F (5'-CCTACGGGAGGCAGCAG-3') and 518R (5'- ATTACCGCGGCTGCTGG-3') and the use of this primer pair resulted in the generation of a 200 bp amplicon (Muyzer et al., 1993). The 341F-518R primer set was used in quantitative PCR assays of 16S rRNA genes in selected water samples. The prevalence of twelve antibiotic resistance genes, (^{bla}*NDM-1, mecA, tet(M), ampC, vanA, mcr-1, tet(B), erm(B), aac (6')-Ie-aph (2'')-Ia, sulII, catII* and *dfrA1*) was also investigated in water samples from the Werribee River using specific oligonucleotides. These oligonucleotides and the antibiotic resistance genes they target are shown in Table 2.2. In addition, the primer sets targeting ^{bla}*NDM* and *catII* genes were modified with Illumina tags (Table 2.3) and used for next generation sequencing of these PCR amplicons based on the fact that they were detected in Werribee River samples in the initial endpoint PCR based assay (results in Chapter 4).

Table 2.2 Oligonucleotides used in PCR and Q-PCR amplification of antibiotic resistance genes.

Target Gene	**Primer name**	**Nucleotide sequence (5' to 3') ***	**Amplicon size (bp)**	**PCR Annealing temperature (°C)**	**Reference**
^{bla}NDM-1	NDM1-F	GGTTTGGCGATCTGGTTTTC	621	52	(Nordmann et al., 2011)
	NDM1-R	CGGAATGGCTCATCACGATC			
mecA	mecA F 1282	AAAATCGATGGTAAAGGTTGGC	533	54	(Murakami et al., 1991)
	mecA R 1793	AGTTCTGCAGTACCGGATTTGC			
tet(B)	tet(B)-F	CCTTATCATGCCAGTCTTGC	774	51	(Maynard et al., 2003)
	tet(B)-R	GGAACATCTGTGGTATGGCG			
tet(M)	tetM-FW	ACAGAAAGCTTATTATATAAC	171	49	(Aminov et al., 2001)
	tetM-RW	TGGCGTGTCTATGATGTTCAC			
ampC	ampC-F	TTCTATCAAMACTGGCARCC	550	52	(Schwartz et al., 2003)
	ampC-R	CCYTTTTATGTACCCAYGA			
VanA	VanA1	GGGAAAACGACAATTGC	732	54	(Dutka-Malen et al., 1995)
	VanA2	GTACAATGCGGCCGTTA			

mcr-1	CLR5-F	CGGTCAGTCCGTTTGTTC	309	52	(Liu et al., 2016)
	CLR5-R	CTTGGTCGGTCTGTAGGG			
erm(B)	ermB-1	CATTTAACGACGAAACTGGC	405	53	(Gevers et al., 2003)
	ermB-2	GGAACATCTGTGGTATGGCG			
aac(6')-Ie-aph(2'')-Ia	aac6-aph2F	CAGAGCCTTGGGAAGATGAAG	348	53	(Vakulenko et al., 2003)
	aac6-aph2R	CCTCGTGTAATTCATGTTCTGGC			
SulII	Sul2-F	CGGCATCGTCAACATAACC	722	51	(Maynard et al., 2003)
	Sul2-R	GTGTGCGGATGAAGTCAG			
CatII	CatII-F	CCTGGAACCGCAGAGAAC	495	50	(Vassort-Bruneau et al., 1996)
	CatII-R	CCTGCTGAAACTTTGCCA			
dfrA1	dhfrI-F	AAGAATGGAGTTATCGGGAATG	391	50	(Maynard et al., 2003)
	dhfrI-R	GGGTAAAAACTGGCCTAAAATTG			

F = forward primer; **R** = reverse primer; **bp** = base pair (Table.2.2), * **M**=A or C, **H**=A or C or T, **V**=A or G or C, **W**=A or T, **R**= A or G, **Y**= C or T.

Table 2.3 Oligonucleotides with Illumina tags used for sequence analysis of antibiotic resistance genes.

Target Gene	Primer name	Nucleotide sequence (5' to 3') *	PCR Annealing temperature (°C)	Reference
***bla*NDM-1**	NDM1-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGTTTGGCGATCTGGTTTTC	52	(Nordmann et al., 2011)
	NDM1-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CGGAATGGCTCATCACGATC		
CatII	*CatII*-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTGGAACCGCAGAGAAC	50	(Vassort-Bruneau et al., 1996)
	CatII-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CCTGCTGAAACTTTGCCA		

F = forward primer; **R** = reverse; Note: Sequences in red refer to the attached Illumina tags.

2.4 Molecular analysis

2.4.1 DNA extraction and purification

DNA was extracted from the filters stored in PowerWater Bead Tubes using the PowerWater DNA isolation kit (MoBio Laboratories, West Carlsbad, USA), Briefly, 1 ml aliquots of solution PW1 (warmed at 55 °C for 5-10 mins) was added to each of the PowerWater Bead Tubes. The tubes were attached to a MoBio Vortex Adapter and vortexed at maximum speed for 5 min. The tubes were centrifuged at 4000 × g for 1 min after which the rest of the protocol as described in the PowerWater DNA isolation kit's manual was followed. At the final step in the protocol, extracted DNA was eluted into 100 µL of sterile deionized water added to the center of a spin filter and placed in a fresh collection tube. The concentration of the eluted DNA was determined using a Nanodrop spectrophotometer (Thermo Scientific™ NanoDrop Lite Spectrophotometer, Thermo Fisher Scientific, Australia). Extracted DNA was stored at -20 °C until required for further analysis.

2.4.2 PCR amplification

2.4.2.1 Amplification of 16S rRNA genes

PCR amplification with primers 314F and 518R was performed using of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 94° C for 30 s, 55 °C for 30 s and 72° C for 60 and a final extension at 72 °C for 10 min. Bacterial 16S rRNA genes were also amplified with modified (barcoded) universal bacterial primer pair of 515F and 806R. The PCR was carried out as followed; 94 °C for 3 min; 35 cycles of 94 °C for 45 s, 50 °C for 60 s and 72 ° C for 90 s; and a final extension at 72 °C for 10 min.

All Polymerase Chain Reactions (PCR) were conducted in total volumes of 25 µL. This consisted of 16.5 µL of sterile water, 2.5 mM $MgCL_2$, x10 GoTaq Flexi Buffer, 0.2 mM dNTPs, 0.5 pmol of each forward and reverse primer, 1.25 U of GoTaq Polymerase and 1 µL of template DNA.

2.4.2.2 Amplification of antibiotic resistance genes

PCR assays for the different antibiotic resistance genes in water samples from the Werribee River were performed using primers specific for each target gene as presented in Table 2.2. The PCR conditions used were as described in the research in which these primers were designed and/or used (Table 2.2) for each target gene and largely consisted of initial denaturation at 94 ° C for 3 min; 35 cycles of 94° C for 45 s, annealing temperature based on the target gene (Table 2.2) for 60 s and 72°C for 90 s and a final extension at 72° C for 10 min. The reaction volume for all the polymerase chain reaction (PCR) was 25 µL. The PCR contained 17.5 µL of sterile water, 2.5 mM $MgCL_2$, x10 GoTaq Flexi Buffer, 0.2 mM dNTPs, 0.5 pmol of each forward and reverse primers, 1.25 U of GoTaq Polymerase and 1 µL of template DNA from the target water sample

2.4.3 Agarose gel electrophoresis

PCR products were visualised using agarose gel electrophoresis. Electrophoresis was performed using 1.2% agarose gel (Oxoid, UK) in 1 X TAE buffer. Thawed SYBR safe (Invitrogen, Australia) reagent was added to 100 ml of molten 1.2% agarose-1X TAE solution. For electrophoresis, 5 µl of

PCR products and 2 µl loading dye (Bioline, Australia) were mixed together and loaded into the agarose gel and electrophoresed at 85 Volts for 60 minutes. Gels were visualized on a GelDoc system (Bio-Rad, Melbourne, Australia) at 302 nm.

2.4.4 Quantitative PCR (qPCR)

PCR standards were prepared for the three ARGs (^{bla}NDM1, *tet(B)* and *catII*) detected in samples from the Werribee River using endpoint PCR. PCR amplicons were generated using the appropriate primers (Table 2.2) and the thermocycling conditions already described each of the target ARG from the bacterial strains used as positive strains (Table 2.1). The amplicons obtained from these PCR assays were purified using a Geneclean Turbo™ Kit (MP Biomedicals, LLC., Australia) based on the manufacturer's protocol and quantified with a Nanodrop spectrophotometer (Thermo Scientific™ NanoDrop Lite Spectrophotometer, Thermo Fisher Scientific, Australia). The purity of the DNA amplicons was evaluated using the ratio of absorbance at 260nm and 280nm. Purified DNA was serially diluted (down to 10^{-8}) and used as template DNA to generate qPCR standards for each of the ARGs. Triplicate standards were generated for each dilution and used for qPCR assays.

qPCR assays were performed using triplicate samples (1 µL of DNA from water samples, 1 µL of qPCR standards, and 1 µL of negative controls (sterile molecular grade water)) for ^{bla}*NDM1*, *tet(B)*, *catII* genes and 16S rRNA genes. For each target gene, a master mix of 19 µL per reaction was prepared based on the recommendations of Bioline as shown in Table 2.4. qPCR was carried out on the Rotor-Gene Q System (QIAGEN Melbourne, Australia) for the detection and quantification antibiotic resistance genes.

Table 2.4; Components of qPCR Master-mix

Reagent	Volume	Final concentration
2x SensiFAST SYBR No-ROX Mix	10 μL	1x
10 μM forward primer	0.8 μL	400 nM
10 μM reverse primer	0.8 μL	400 nM
PCR-grade water	7.4 μL	N/A

The qPCR cycling conditions used were as described in Shahsavari *et al*., (2016). This consisted of an initial denaturation step of 95 °C for 5 min, followed by 40 cycles of 95° C denaturation for 10 s, annealing at the different temperatures for each primer set (Table 2.2) used for 30 s, 72 °C for 30 s, 80 °C for 10 s for primer dimer removal and signal acquisition. Signal acquisition was carried out at 78–80 °C to minimize the formation of primer dimer. The generated melting curves generated were subject to further data analysis to confirm the absence of peaks belonging to non-specific products (Shahsavari et al., 2016).

Standard curves were generated for each ARG and for each 16S rRNA gene using multiple diluents (up to 10^8) using template DNA from relevant bacterial isolates. These curves were generated by plotting the CT values against the DNA concentrations of standards used for the qPCR assays. Standard curves with $R^2 > 0.95$ were selected for use in this study. PCR efficiency (PE) was calculated using the formula, $PE = 10^{(-1/slope)} - 1$, with the slope determined from the standard curve. PE between 0.9-1.1 was acceptable for use in this study. Gene copy numbers were calculated using the formula Ymolecules/ μl= (X g/μl DNA/ [PCR product length in base pairs × 660]) × 6:022 × 10^{23} where Y is gene copy numbers and X is the DNA concentration (Shahsavari et al., 2016).

2.4.4.1 Quality control (QC) measures for qPCR

Specific steps were taken to ensure that the qPCR assays were free of contaminants. These included having two separate preparation areas or PCR cabinets for the assay, each with its own dedicated set of pipettes. The first area or cabinet was for the preparation of the master-mix and was essentially DNA or nucleic acid free. The second area or cabinet was solely for the addition of nucleic acid (or template DNA). These cabinets were kept cleaned and decontaminated with 70% ethanol and the use of UV light before carrying out any assay.

For each PCR assay, internal controls were included for QC purposes. These included positive control replicate wells inoculated with exogenous DNA, known to be amplified by the primer pair being used for the qPCR assay. This allowed for the validation of the successful amplification of DNA in the assay. Negative controls in the form of No Template Controls (in replicates) were also included. No DNA was added to these controls and the absence of amplification in these wells indicated the absence of extraneous DNA contamination. Given that SYBR green was used for the qPCR assays in this study, the No Template Controls also served as an important control for detecting the formation of primer-dimers and false positives. The melt curve was evaluated after each qPCR run to detect and eliminate false positives as a result of primer-dimer formation.

2.4.4.2 Normalization of ARG gene copy numbers to 16S rRNA gene copy numbers

For each sample analysed, both the target ARG and 16S rRNA gene copy numbers were determined using the appropriate primers by qPCR assays. The gene copy numbers were calculated using the appropriate standard curves as already described and expressed as Log_{10} values. The mean of the replicate samples at each time point and site was determined and used for further calculations. Log_{10} ARG copy numbers were normalized to Log_{10} 16S rRNA gene copy numbers by dividing the selected ARG copy numbers with the corresponding 16S rRNA gene copy numbers from the same sample. The results were expressed in graphical forms.

2.5 Illumina-based next-generation sequencing (chapters 3 and 5)

2.5.1 Amplicon library preparation and DNA sequencing

16S rRNA gene sequencing libraries were prepared using a Nextera XTv2 Index Kit (Illumina, San Diego, CA, USA) as described in the 16S Metagenomic Sequencing Library Preparation protocol (Illumina). The DNA from the library was quantified using a Qubits 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol. Subsets of five random amplicons of desired samples were selected from each plate. These samples were electrophoresed on a 1.5 % agarose gel before and after the indexing PCR was performed to visualise the incorporation of Nextera Index primers (i.e. larger PCR products). Amplicons were then pooled together to a final concentration of 4 nM, loaded together with 15% PhiX before being sequenced on a MiSeq platform (Illumina, San Diego, CA, USA) at RMIT University. The same procedure was followed for the preparation of libraries made from amplicons generated from modified ^{bla}*NDM1* and *catII* primers.

2.6 Data and statistical analysis

Data generated by the Illumina MiSeq were initially analysed using the Illumina BaseSpace application, 16S Metagenomics software. Quality filtered 16S rRNA gene sequence reads were then processed and annotated through the GHAP pipeline (Greenfield, 2017). Using the GHAP amplicon pipeline, split reads were de-multiplexed and later merged by paired reads. After this, the reads that required trimming were determined from a created read-length histogram with these reads being trimmed at 250 bp. Further quality control was carried out using Usearch in the GHAP amplicon pipeline. USearch is a tool for analysing sequences which utilizes clustering algorithms for quality controls procedures including those used to identify and remove chimeric sequences (Edgar, 2017). Quality filtered gene sequences were annotated through the referral to the reference sequences on the Ribosomal Database Project (RDP) (Wang et al., 2007, Cole et al., 2014). Four analysis pipelines were used in this study to analyse NGS data from the Illumina MiSeq platform. These were PRIMER v7

(Clarke and Gorley, 2015), MEGAN v6 (Huson et al., 2016), R packages (Oksanen, 2018) and iNEXT (Hsieh et al., 2016) in R-Studio. Square-root transformation of the OTU tables generated from sequenced data was carried out on PRIMER v7. Analysis of Similarities (ANOSIM) (Clarke and Green, 1988) was carried out on the transformed data.

Multivariate statistical analysis was carried out by using the PRIMER-7 software package with the PERMANOVA^{+} add-on (Clarke and Gorley, 2015). An Operational Taxonomic Units (OTU) genus-level table was used and the data was standardized and transformed using square root. SIMPER (similarity percentage) analysis (using Bray-Curtis similarities and 90% cut-off for low contributions) was used to identify the most significant taxa in different sites. Besides, principal coordinate analysis (PCoA) was used for sequencing data over the months and sites. Also, the canonical analysis of principal coordinates (CAP) analysis was performed to observe the main groups and clusters in microbial communities based on SIMPER results (highest % contribution taxa were selected). Distanced based linear model analyses/Distance-based redundancy analysis (DistLM /dbRDAs) was used for environmental and molecular samples to identify the environmental drivers.

The Shannon diversity (Shannon and Weaver, 1949) and Pielou evenness (Pielou, 1966) were generated using the R package, vegan. Chao1 species richness (Chao, 1984, Chao, 1987) was calculated using iNEXT in the R package. T-Tests and one-way ANOVA statistical tests were employed to determine whether there were significant differences in bacterial diversity (OTU assigned at 97% identity) and Shannon diversity (*H*')) between different sampling sites and over time. Relationships between OTUs, Shannon diversity and physicochemical parameters of water samples (temperature, pH, dissolved oxygen concentration and electrical conductivity) were evaluated using Pearson correlation efficiency.

The antibiotic resistance gene sequence reads were processed through the Functional Gene Pipeline and Repository (FunGene, http://fungene.cme.msu.edu/FunGenePipeline/) on the RDP server (Cole,

2013, Fish et al., 2013). Quality filtering procedures were initially carried out. This involved trimming of primer sequences and the removal of amplicons of low quality. Reads > 200 bp with bases with a quality or a Phred score of at least q20 were selected using Fastx-toolkit via command lines. Reads in fastq file format were then converted to fasta file formats and reads with primer sequences extracted using the "grep" command line. Quality-filtered sequences were subsequently translated into their complementary amino acid sequences using FrameBot on the FunGene Pipeline (Fish et al., 2013). All subsequent downstream analyses were carried out using these amino acid sequences.

Alignment and clipping of amino acid sequences were performed at specific reference positions (from 60 aa to 80 aa) based on a 0.4 identity cut-off. Classification of OTUs was carried out and rarefaction curves were produced using distance matrices of amino acid sequences at three similarity levels These were performed at 0.05 (95%), 0.2 (80%) and 0.5 (50%) similarity levels using the mcClust and rarefaction tools available on the FunGene Pipeline (Fish *et al.,* 2013). The relative abundance of sequence groups in selected samples was determined using the sumif command and expressed as percentages of the total number of sequences in each sample. Based on the 0.4 similarity level, representative sequences were selected and used for identification of possible taxonomic origin for each translated resistance gene sequence, with the five most abundant clusters being assigned. Additionally, polypeptide sequences were identified following translation from DNA sequences using ORF-FINDER to determine the putative identities of sequences, translated sequence reads were compared against the reference proteins (refseq_protein) database at the National Center for Biotechnology Information NCBI GenBank using Blastp (protein-protein BLAST) (Altschul et al., 1997)using the *E*-value cut-off 10^{-5} (best hit used).

3 Chapter 3: Spatial and temporal variation in bacterial community structure, diversity and composition in the Werribee River, Melbourne, Australia.

3.1 Introduction

Rivers act as the interface of and transport system to link terrestrial and marine environments (Carmack et al., 2016). They provide food, water, shelter and space, essential for the survival of freshwater animals, plants and microorganisms (Wishart, 2000). At a local level, discrete micro-environments are found at the air/water interface, within the water column, and in the river bed (Naiman and Decamps, 1997). These micro-environments are created by multiple physico-chemical and environmental factors, including the rate of water flow, topography and composition of the river bed, dissolved oxygen content and turbidity of the water. Additionally, nutrient load, pH, biological oxygen demand, light penetration as well as the chemical composition of the water are also important factors in river micro-environments. River ecology can also be influenced by seasonal factors such as temperature, length of days and rainfall patterns (Miller and Miller, 2007). Overlaid upon these factors is the influence of humans which may cause an increase in the severity of river pollution (Páll et al., 2013).

Although some pollution does occur as a result of natural activities, most river pollution is due to anthropogenic activities. For example, extensive amounts of river water may be diverted to support agricultural and domestic uses, with adverse effects on the river ecosystem. Wastewater and runoffs from human activities can introduce a large load of nutrients and/or pathogens to pollute rivers (Templar et al., 2016, Kirschner et al., 2017, Wen et al., 2017). Pesticides and other agricultural wastes can also pollute rivers through run-off and by both accidental or deliberate discharges (Strokal et al., 2016, Wen et al., 2017). Industrial activities and wastes are other common sources of river pollution (Raptis et al., 2016, Wright et al., 2017, Ali et al., 2018) and pollution from these sources

can dramatically affect the flow rates, temperature and nutrient content of rivers and hence have a profound effect on river ecology.

Historically, the first attempts at monitoring the health of rivers were motivated by concerns for human health (De Pauw and Hawkes, 1994). Monitoring techniques were initially focused on chemical indicators, followed by the application of indicator bacteria such as *Escherichia coli* (associated with human wastes) and later to eukaryotic species such as algae, fungi and protozoa to assess microbiological risks (De Pauw and Hawkes, 1994). Guidelines for water quality assessment and pathogen detection issued by the World Health Organization (WHO) promote the use of indicator organisms for assessment purposes (Fewtrell and Bartram, 2001). This involves observing changes in the numbers, growth rates, reproduction rates or mortality of these indicator organisms.

Microorganisms play fundamental roles in maintaining the health of aquatic ecosystems. Photosynthetic microorganisms such as *Cyanobacteria* and microalgae utilize energy from sunlight to fix carbon from carbon dioxide into biomass, helping to drive the carbon cycle. A river ecosystem cannot maintain its good health without the appropriate mix of microorganisms to support the various nutrient cycles (Falkowski et al., 2008). Changes in an aquatic system's physical and chemical properties will directly affect the relative abundance of different microorganisms (Axmanovà et al., 2006) with some specific microbial groups associated with specific pollutants. For example, coliform bacteria are usually present in the digestive systems of human and warm-blooded animals and consequently in human faecal wastes. Therefore, their presence in any aquatic system is credible evidence of pollution from these sources.

Several studies have investigated the impact of environmental factors on the diversity of aquatic microbiota. Water temperature (Zeglin, 2015), pH levels (Liu et al., 2015), salinity (Yoshimura et al., 2018), quantity and type of organic matter (Lønborg et al., 2016) and dissolved oxygen concentrations (Hernandez et al., 2015) can affect bacterial community diversity and activities in riverine systems.

Seasonal variations which include changes in temperature and rainfall abundance have been shown to have significant impacts upon the microbial community composition of freshwater systems (Jordaan and Bezuidenhout, 2016, Collins and Kipling, 1957).

In recent years, high-throughput DNA sequence analyses of PCR-amplified 16S rRNA genes has been used to investigate microbial community structure and composition in aquatic systems (Sinclair et al., 2015). These high-resolution next-generation sequencing methods have been applied to the investigation of microbial communities in freshwater reservoirs (Iliev et al., 2017), lakes (Toyama et al., 2016) and freshwater systems with organic contaminants (fertilizers) (Meneghine et al., 2017). Significantly, two microbial studies from two geographically distant rivers (the Sinos River in Brazil and the Mississippi River in the United States), using 16S rRNA gene amplicon sequencing showed that the five most abundant phyla detected were *Proteobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria* and *Verrucomicrobia*. In the Mississippi River, the composition of the bacterial communities in the upper and downstream regions varied principally in relation to changes in the relative abundance, (rather than the presence or absence) of operational taxonomic units (OTUs). Besides and similar to the findings in the Sinos River, *Proteobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria* and *Verrucomicrobia* accounted for 93.6% of all sequence reads (Staley et al., 2013). Therefore, in freshwater ecosystems, the major globally distributed phyla indigenous to freshwater bacterial communities include the *Proteobacteria* (∝, β and γ) *Bacteroidetes*, *Cyanobacteria*, *Actinobacteria, Verrucomicrobia* and *Planctomycetes* (Ghai et al., 2011, Oh et al., 2011). Analyses of freshwater microbiota have shown that bacterial communities, generally, were similar at the level of phylum but differ in terms of genus and species composition. These differences were dependent on factors such as the physico-chemical properties and spatial and temporal variation between sampling sites as well as on the impact of allochthonous nutrient input into the ecosystem (Zwart et al., 2002, Oh et al., 2011).

The Werribee River catchment covers an area of 2,700 square kilometres to the west of Melbourne, Australia. It originates in the foothills of the Great Dividing Range near the town of Ballan, crosses a high basalt plain, then descends into a lower coastal plain via a series of gorges. It follows a south-easterly direction until it enters the waters of Port Philip Bay to the South East of the city of Werribee. Around a quarter of the catchment retains native vegetation and 67% of the catchment area is used for agriculture and 5% for urban development. Rainfall across the catchment varies from 1000 mm in the upper reaches to 450 mm on the dry coastal plain https://www.melbournewater.com.au/water/health-and-monitoring/river-health-and-monitoring/werribee-catchment.

The upper catchment originates in the Wombat State Forest before encountering agricultural land and the small urbanized area of Ballan as it passes over a high basalt plain. The river enters the coastal plain at Bacchus Marsh, another urban development area, which was formerly marshland before drainage and is now heavily used for horticulture, before encountering Melton reservoir. The Melton reservoir provides water to the urban developments of Bacchus Marsh and Melton. Much of this area is designated an Urban Water Protection Area State. The lower part of the Werribee runs through the city of Werribee and along the edge of the Western Treatment Plant before entering https://www.melbournewater.com.au/water/health-and-monitoring/river-health-and-monitoring/werribee-catchment.

Most parts of the Werribee River Area have been cleared for agricultural use since the onset of European settlement with the dominant land use being dryland grazing. However, the catchment also includes urban settlements such as Bacchus Marsh Werribee, Melton and Werribee. Therefore, the availability and quality of water from the river also plays an important part in determining people's quality of life (Snee, 2015). Moreover, the water quality of the river is a highly significant concern for ecologists, as water from rivers is used for both agriculture and human consumption (Barmuta, 2003).

The Australia-wide Assessment of River Health (AWARH), initiated under the National River Care Program is used to assess the health of Australia's rivers (EPA, 2000). Physical and chemical assessments (salinity, turbidity, pH, the presence of toxic substances, amount of dissolved oxygen and nutrient levels) of water samples from rivers are fundamental aspects of assessing the health of the river (EPA, 2000). These measurements provide a general overview of environmental conditions at the time of sampling in relation to seasonal and spatial conditions. They also allow for the determination of the water quality of the river and its species biodiversity especially, and the effects of biota on the environment (EPA, 2000).

More recently, scientific research has focused on the use of a microbiological component of rivers, to assess and monitor water quality. Concerning the Werribee River, only a single peer-reviewed report on the microbiology of the river is known. This study focused on the presence of antibiotic resistance genes (ARGs) in the river (Barker-Reid et al., 2010), although the investigators did not study the structure and composition of bacterial communities within the river. There is, therefore, no information available on the bacterial communities in the Werribee River and how these may vary over different seasons at different sites on the river.

Consequently, the research presented in this chapter is concerned with the analysis of bacterial communities at selected sites on the Werribee River. An evaluation of the physico-chemical qualities at these sites (impacted and relatively un-impacted sites) was carried out. Samples used for scientific investigations were collected from upstream and downstream sections of Werribee River over 12 months. The data on the physico-chemical parameters (supplied by Melbourne water) and the microbial communities (obtained from this study) would provide baseline information on the Werribee River. This should allow for clarity on any allochthonous input of nutrients into the river system and the role of environmental drivers in any observed change in the bacterial communities of the Werribee River. The baseline data on the microbial structure and composition obtained in this study would be beneficial to a proposed biomonitoring system of the Werribee River by the

government of the state of Victoria in Australia. If for example, nutrient inputs were found to be the drivers of changes in the structure and composition of the bacterial communities in the river, then investigations can be carried out to determine the source of these nutrients and appropriate steps taken to prevent their introduction into the Werribee River. However, given that most of the land in the river's catchment area is presently used for livestock grazing, it is believed that any observed change in the bacterial communities in the Werribee River would be related to sampling sites and the time (months) the samples were collected. Specifically, it is hypothesized that bacterial community structure, composition and diversity will show substantial variation between the upstream and downstream sites based on their different physico-chemical properties (e.g. pH, temperature, conductivity, nutrient composition etc.) within the Werribee River. As these physico-chemical properties can change over time (months), substantial variation in bacterial structure, composition and diversity should be observed based on the time (season) of sample collection form the Werribee River.

Therefore, the overall aim of this chapter was to assess the spatial and temporal variation in the structure, composition and diversity of bacterial communities along the Werribee River.

Specifically, this research aimed to:

1. determine how bacterial community structure, diversity and composition vary along the Werribee River.
2. determine how the bacterial communities vary between seasons.
3. identify the most abundant bacterial taxa at each site.
4. determine how variation in bacterial community structure and diversity relates to variation in the physico-chemical environment.

3.2 Results

Variation in bacterial community, structure, diversity and composition were investigated at four sampling sites along the Werribee River with 72 water samples collected from these sites along the course of the river. Two of these sites, namely Ballan and Bacchus Marsh, were located in the upstream reaches of the river. The remaining two sites, Cobbledicks Ford and Riversdale were in the downstream section of the river. Three samples (biological replicates) were collected from each location at each of the six-time points sampled at two-monthly intervals (April, June, August, October and December 2015 and February 2016). DNA was extracted and purified from filtered microbial biomass. Bacterial 16S rRNA gene sequences were amplified from DNA isolated from the water samples and sequenced in two runs. The total reads of the two runs were 36.63 million and 17.52 million reads, respectively (raw sequences) (Table 3.1). The samples used for further analysis after passing the Illumina Chastity filter spanned a range of 11,871 to 516,916 reads. The Illumina chastity filter is an internal quality filter that is designed to filter out (remove) the least reliable clusters of reads from image analysis results. It is based on the ratio of the brightest base intensity divided by the sum of the brightest and second brightest base intensities.

Table 3.1. Illumina MiSeq DNA sequencing run statistics for 16S rRNA gene sequences

Sequence Run	Total Reads	Reads passed filter	Mean^* Reads passed filter Per samples	% ≥ Q30[a]	Yield (Gbp)	Aligned to PhiX control (%)	Error of Aligned Phix control
1	36,631,086	36,110,700	168,345± 5880	70.27	11.09	7.14	2.8
2	18,639,396	17,521,568	74508 ± 7667	75.05	10.76	10.72	2.29

Q30[a] = Base call accuracy of 99.9%

3.2.1 Spatial and temporal variation in the bacterial communities along the Werribee River

Spatial and temporal variation in the bacterial communities at the four sampling sites from April 2015 to February 2016 was investigated through the use of principal component analyses based on a Bray-Curtis similarity matrix (Figure 3.1). With respect to spatial variation, the bacterial communities were grouped by site (Figure 3.1) There were two clusters on the PCoA plots; the first cluster was composed of upstream sites (Ballan and Bacchus Marsh) and the second cluster contained samples from the downstream sites of Cobbledick Ford and Riversdale (Figure 3.1). However, when the bacterial communities were analysed with respect to temporal variation (Figure 3.2), the trend was not as clearly defined as was observed in the PCoA plot of spatial variation. In Ballan, triplicate samples from June, August and October were grouped to form two distinct clusters while samples obtained in December, February and April formed a different distinct cluster. At Bacchus Marsh, April, June, August and October samples were grouped, forming a distinct cluster which was different from the cluster formed largely by December and February samples (Figure 3.2). However, in Cobbledicks Ford, a downstream site, the bacterial communities in samples from October were different from those in samples from the other months. At the second downstream sites (Riversdale), a different trend was obtained with the bacterial communities in samples from April and December being more closely related to one another (a cluster) than to communities from the remaining months (a different cluster) (Figure 3.2).

Analysis of similarities (ANOSIM) was performed between sampling sites in a pair-wise manner (Table 3.2). The R statistic values ranged from 0.204 (Ballan and Bacchus Marsh) to 0.506 (Ballan and Cobbledicks Ford). ANOSIM R values between the bacterial communities within the two upstream sites (R=0.204; Ballan and Bacchus Marsh) and within the two downstream sites (R=0.24; Cobbledicks Ford and Riversdale) was lower than the variation between upstream and downstream

sites (R statistic value range of 0.414 to 0.506) (Table 3.2). The upstream sites bacterial communities were therefore significantly different from downstream communities ($P < 0.001$).

Analysis of similarities (ANOSIM) was also used to investigate differences in community structure across all the bacterial communities in the Werribee River over time (between months; Table 3.3) Bacterial communities in April were significantly different across all sites from those present in August, October, December and February ($P < 0.05$). Conversely, the communities in April did not differ significantly ($P=0.184$) from those in June. Additionally, significant temporal variation was seen overall (across all sites) between communities in the summer months (December and February) when compared to, for example, those in autumn, winter and spring (R ranges from 0.185 to 0.352; $P < 0.05$). Finally, the communities in December and February were slightly different from each other ($R=0.07$).

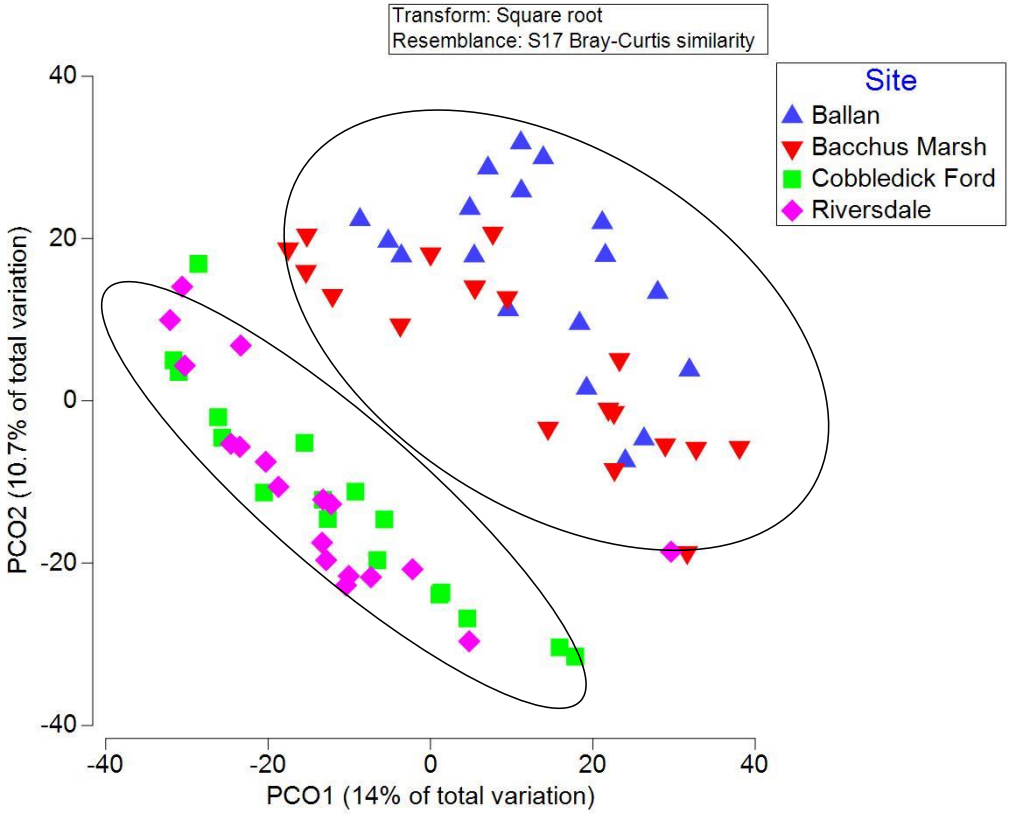


Figure 3.1: Principal coordinate analysis (PCoA) showing the spatial variation of the bacterial community at genus level based on 16S rRNA gene sequencing at upstream and downstream sites along the Werribee River. PCoA was generated with Primer 7 software. Ballan and Bacchus Marsh are upstream sites while Cobbledicks Ford and Riversdale are downstream sites.

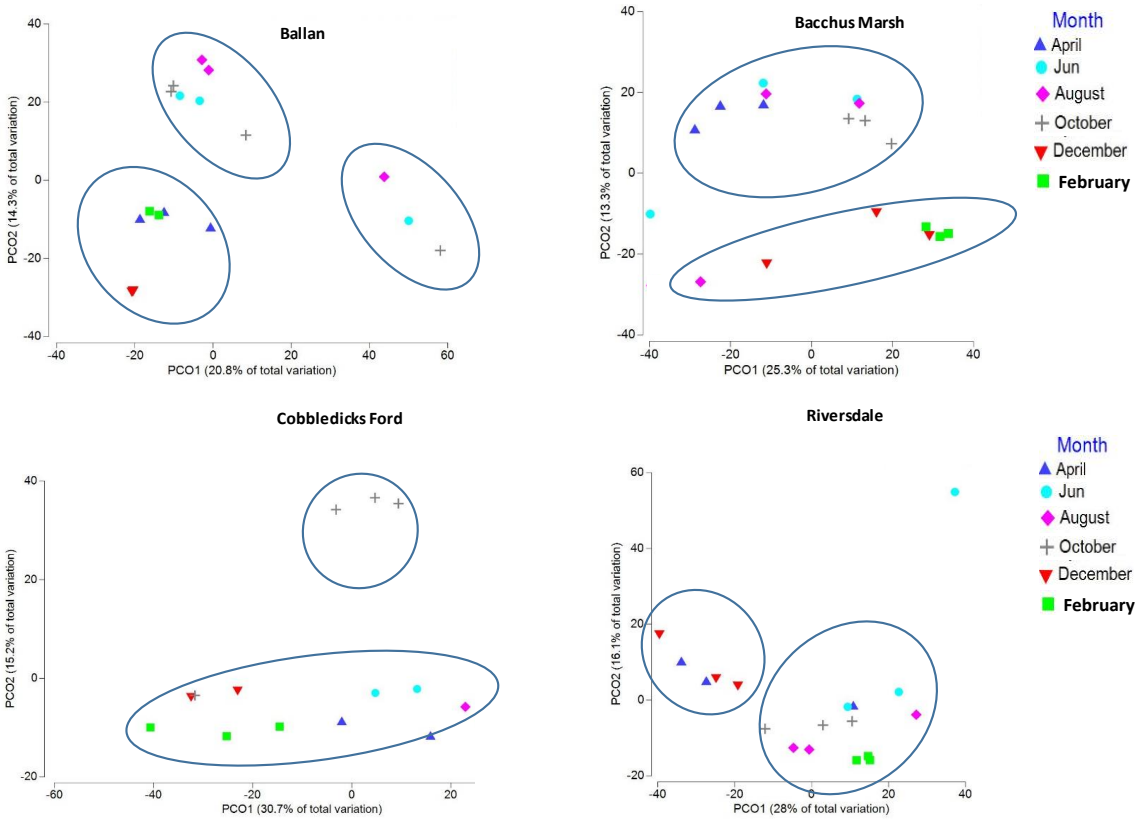


Figure 3.2: Principal coordinate analysis (PCoA) (based on Bray Curtis similarity matrix) showing temporal variation in bacterial community structure at genus level over six-time points in the upstream sites, Ballan and Bacchus Marsh and downstream sites, Cobbledicks Ford and Riversdale sites along the Werribee River. PCoA was generated with Primer 7 software. Months studied are April, June, August, October, December and February. (n= 3 per time point for each site).

Table 3.2: Analysis of Similarities (ANOSIM) comparing similarities in bacterial communities between sites ($P < 0.001$ for all comparisons).

	Group	R- statistic
Sites	Ballan and Bacchus Marsh	0.204
	Ballan and Cobbledicks Ford	0.506
	Ballan and Riversdale	0.46
	Bacchus Marsh, Cobbledicks Ford	0.438
	Bacchus Marsh, Riversdale	0.414
	Cobbledicks Ford, Riversdale	0.24
Sites Mean		0.366

Table 3.3 Analysis of Similarities (ANOSIM) comparing similarities in bacterial communities between months.

	Group	R- statistic	P-Value
Months	April, February	0.324	0.001*
	April, December	0.352	0.001*
	April, October	0.119	0.043*
	April, August	0.111	0.032*
	April, June	0.041	0.184
	June, October	0.035	0.181
	August, October	0.062	0.081
	August, June	0.004	0.382
	December, February	0.07	0.0007*
	December, August	0.282	0.002*
	December, June	0.285	0.001*
	December, October	0.185	0.0018*
	February, October	0.26	0.0026*
	February, August	0.289	0.001*
	February, June	0.286	0.001*
Of all months		0.168	

*indicates a significant difference between months

3.2.2 Spatial variation in bacterial diversity within bacterial communities along the Werribee River

The observed numbers of operational taxonomic units (OTUs assigned at 97% identity, approximating to species level) were determined at the four sites along the Werribee River as averaged over six-time points between April 2015 and February 2016 (Figure 3.3). One-way ANOVA showed no significant differences when the number of OTUs of the four different sampling sites were compared ($P > 0.05$). The highest number of OTUs was found at Cobbledicks Ford (a downstream site) and Ballan (an upper stream site) with mean numbers of OTUs 1733.52 and 1559.61 OTUs, respectively. The lowest number of OTUs was found at Riversdale (the second downstream site) with a mean number of 1411.23 OTUs identified.

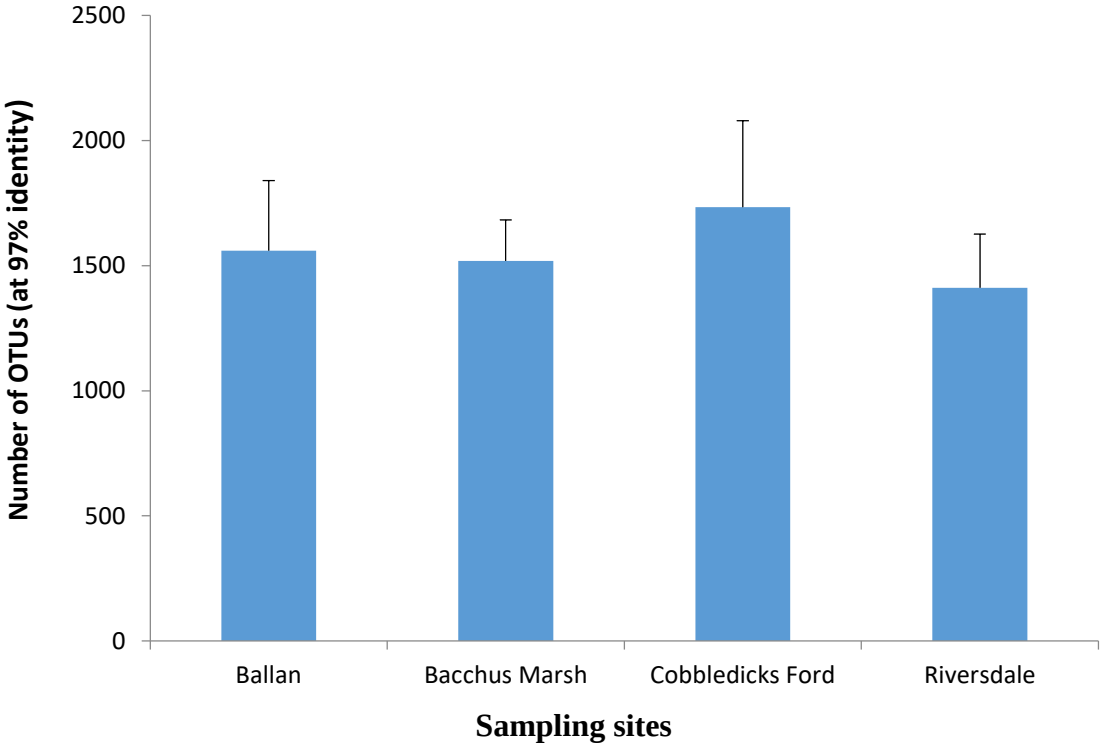


Figure 3.3 Spatial (location-specific) variation in numbers of Operational Taxonomic Units (classified at 97% identity, approximating to species-level) along the Werribee River based on 16S rRNA gene sequencing. No significant difference was observed between the means ($P \geq 0.05$). Bars indicate standard error of means (Mean ± SEM, $n \geq 8$).

Variation in the number of observed OTUs present within sampling sites can be influenced by the number of sequence reads generated for each sample. The number of sequence reads per sample varied in some cases by more than one order of magnitude between different samples in this study (Table 3.1). Therefore, Shannon diversity indices were calculated as an estimate of relative bacterial taxonomic diversity between the four sampling sites (Figure 3.4). Overall, a one-way ANOVA revealed that Shannon diversity varied significantly between Bacchus Marsh and the three other sites ($P \leq 0.05$). The bacterial community in Ballan, an upstream site was the most diverse ($H' = 4.03$) followed by the community in Riversdale ($H' = 3.82$), a downstream site. The bacterial community in Bacchus Marsh, another upstream site, was the least diverse of all the four sites ($H' = 2.6$ (Figure. 3.4). However, the Shannon diversity was significantly lower ($P \leq 0.05$) at Bacchus Marsh when compared to the other three sites (Ballan, Cobbledicks Ford and Riversdale). Chao1 estimates of bacterial diversities were also determined. The Chao1 values ranged from 1645 (Bacchus Marsh) and 1671 (Ballan) to 1787 (Riversdale) and 2332 at Cobbledicks Ford (Table 3.4). Generally, downstream sites had higher Chao1 richness values. Evenness of the bacterial communities was similar at the four sites (J value ranged from 0.471 to 0.54) (Table 3.4). However, one-way ANOVA showed no significant difference overall between sites for either Chao1 or Pielou J evenness ($P \geq 0.15$).

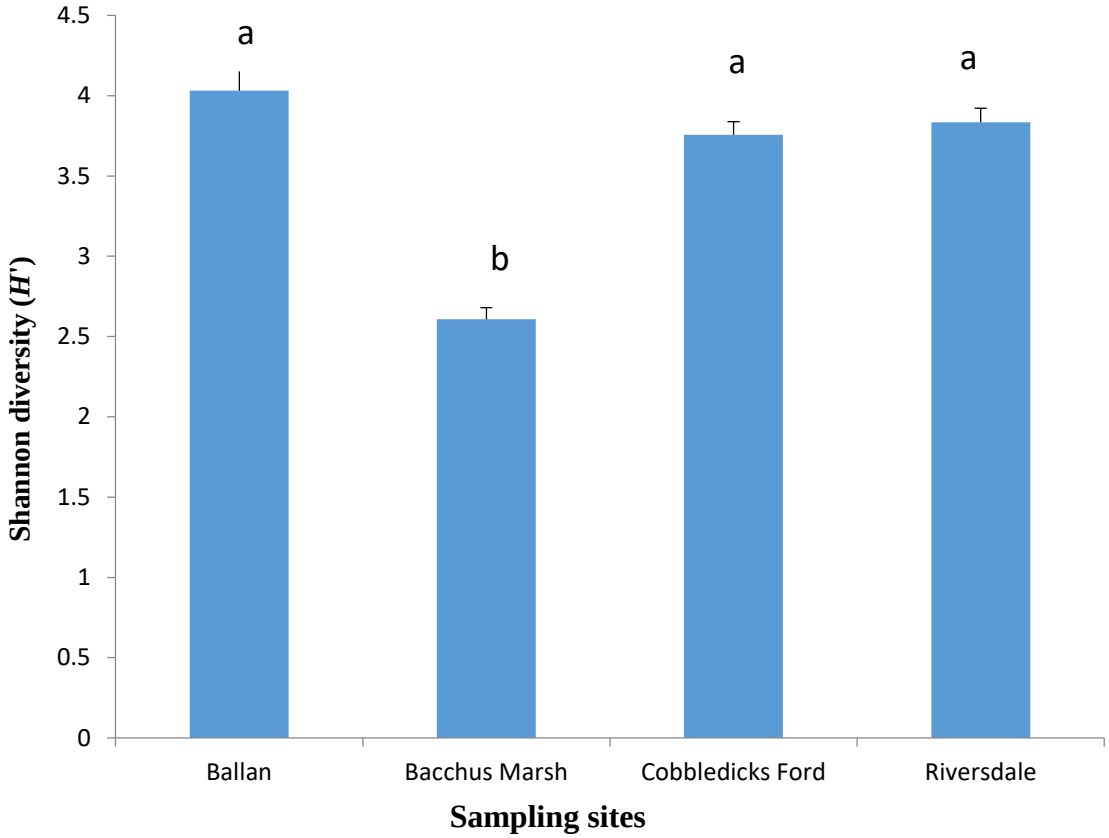


Figure 3.4: Spatial variation in Shannon diversity indices based on OTU (assigned at 97% identity) based on 16S rRNA gene sequencing at Ballan, Bacchus Marsh, Cobbledicks Ford and Riversdale along the Werribee River sampled at the six-time points. Significant differences between Shannon indices are indicated by different letters ($P < 0.05$). Bars indicate standard error of means (Mean ± SEM, $n \geq 8$).

Table 3.4: Spatial variation in the estimates of Chao1 OTU richness (as assigned at 97% identity) and taxon evenness (Pielou J) for bacterial communities at four sites along the Werribee River. Mean values ± SEM (n ≥ 8) are shown.

Sites	**Chao1 OTU Richness**	**Evenness Pielou J'**
Ballan	1670.55 ± 249	0.47 ± 0.03
Bacchus Marsh	1645.30 ± 237	0.48 ± 0.02
Cobbledicks Ford	2331.69 ± 299	0.48 ± 0.25
Riversdale	1786.58 ± 207	0.54 ± 0.13
Mean of four sites	1858.53 ± 125	0.50 ± 0.12

3.2.3 Temporal variation in bacterial diversity within bacterial communities along the Werribee River

The observed number of operational taxonomic units (at 97% identity, approximating to species level) were determined at four sites along the Werribee River between April 2015 and February 2016 (Figure 3.5). A one-way ANOVA showed significant overall variation in the number of OTUs between sampling months ($P < 0.05$). The highest number of OTUs was found in February 2016 followed by December 2015 with means of 2339.31 and 1956 OTUs respectively. The lowest number of OTUs was found in April 2015 with a mean of 1027 OTUs. In general, the number of bacterial OTUs increased overall between April 2015 and February 2016. The only exception was the samples obtained in October 2016 that had lower OTU numbers when compared to the previous month of August (Figure 3.5). The numbers of OTUs in December (2015) and February (2016) were significantly higher than in April, June and October (2015) ($P < 0.05$). Moreover, OTU numbers in February were also significantly higher than in August (2015) (Figure 3.5). There was no significant difference in OTU numbers between August and December.

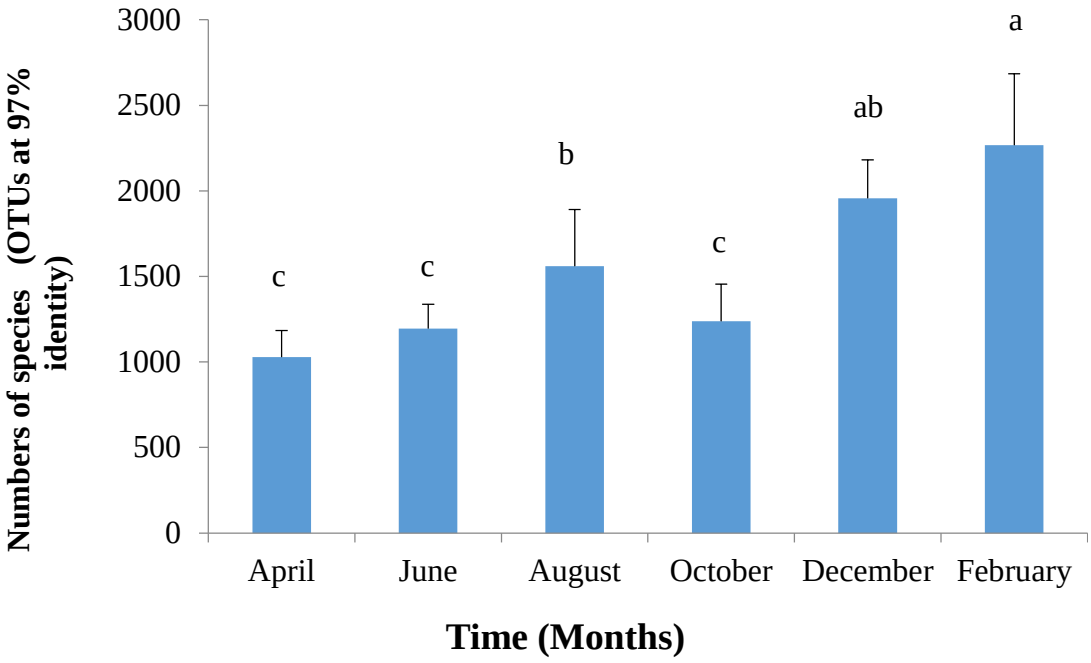


Figure 3.5. Temporal variation in numbers of Operational Taxonomic Units (classified at 97% identity approximating to species-level) along the Werribee River based on 16S rRNA gene sequencing. Sample means with different letters are significantly different ($P \leq 0.05$) from each other. Data are presented as Mean ± SEM ($n \geq 6$).

Overall, significant variation was observed in Shannon diversity indices between sampling months in the Werribee River based on one-way ANOVA ($P < 0.05$). The highest diversity in bacterial communities was in December 2015 ($H' = 4.15$) and followed by February 2016 ($H' = 3.92$) whilst the lowest Shannon diversity was seen in June 2015 ($H' = 2.92$) (Figure 3.6). Bacterial community diversity was significantly higher in summer months (December 2015 and February 2016) ($P < 0.05$) (Figure 3.6) than in the Autumn (April) and winter (June and August) months. Additionally, Shannon diversity was significantly higher in December than in October. Chao1 estimates of bacterial diversity were also compared between sampling months. Highest Chao1 values were similarly recorded in December (2407) and February (2976) and the lowest values in August and October (both 1515). One-way ANOVA showed that there were significant differences ($P \leq 0.05$)

between the Chao1 values observed in February, where they were higher than those in April, June, August and October. Evenness (Pielou J (varied between 0.43 (June) and 0.57 (December) with the highest values in summer months (December and February; Table 3.5). Evenness in June was significantly lower in December and February ($P \leq 0.05$).

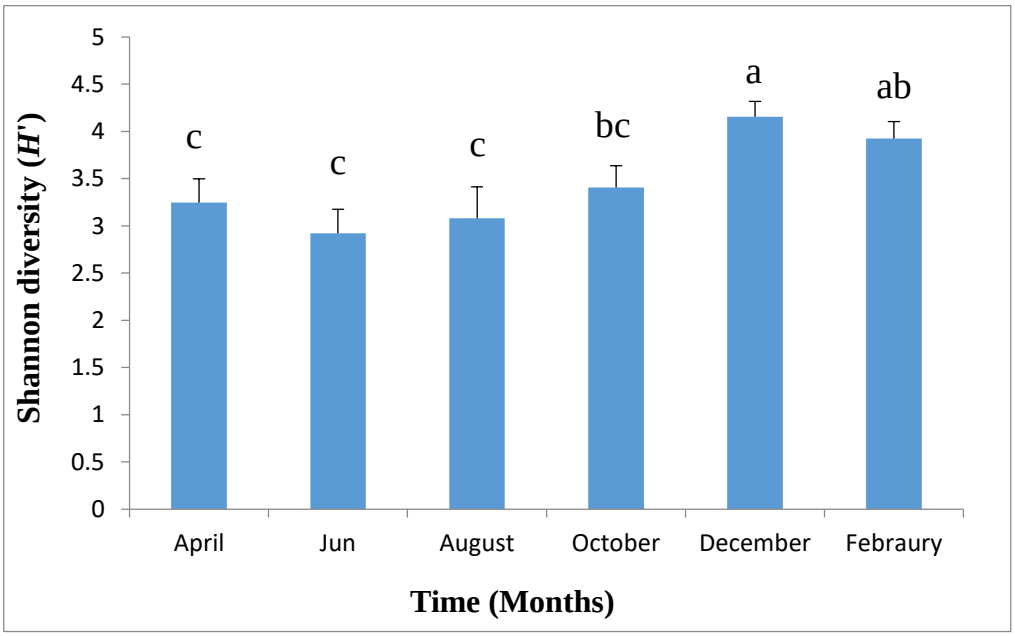


Figure 3.6: Temporal variation in Shannon diversity indices based on operational taxonomic units (OTUs) (classified at 97% identity approximating to species level) based on 16S rRNA gene sequencing between different time points (months) at Ballan, Bacchus Marsh, Cobbledicks Ford and Riversdale along the Werribee River. Sample means with different letters are significantly different ($P \leq 0.05$) from each other. Data are presented as Mean ± SEM ($n \geq 6$).

Table 3.5: Temporal variation in estimates of Chao1 OTU richness (as assigned at 97% identity) and taxon evenness (Pielou J) for bacterial communities along the Werribee River. Mean values ± SEM (n ≥ 6) are shown.

Seasons	Chao1 OTU Richness	OTU J'
April 2015	1259.20 ± 195	0.49 ± 0.02
June 2015	1521.36 ± 196	0.43 ± 0.02
August 2015	1514.90 ± 301	0.46 ± 0.03
October 2015	1514.93 ± 219	0.46 ± 0.04
December 2015	2407.85 ± 269	0.57 ± 0.01
February 2016	2976.25 ± 355	0.55 ± 0.01
Mean of months	1865.738 ± 124	0.49 ± 0.01

3.2.4 Spatial and temporal variation in bacterial taxonomic composition within bacterial communities along the Werribee River.

3.2.4.1 Spatial and temporal variations at the phylum level

At the phylum level, the three phyla (*Proteobacteria, Actinobacteria* and *Bacteroidetes*) with the highest relative abundances as determined using SIMPER analysis were evaluated between sites and over different months (Figure 3.7). The most dominant phylum across all the four sites was the phylum *Proteobacteria*. At the upper-stream sites (Ballan and Bacchus Marsh), the overall relative abundance of the identified bacterial phyla was as follows: *Proteobacteria* (up to 37%), *Bacteroidetes* (up to 24%) and *Actinobacteria* (up to ~11%). However, at the downstream sites, Cobbledicks Ford and Riversdale, whilst *Proteobacteri*a were again most abundant, (up to 28%), the second most abundant phylum was *Actinobacteria* (up to 17%) and then *Bacteroidetes* (up to ~5%) Members of the phylum Proteobacteria were detected in high

numbers throughout the sampling time-frame (April, June, August, October, December and February) irrespective of the sampling site

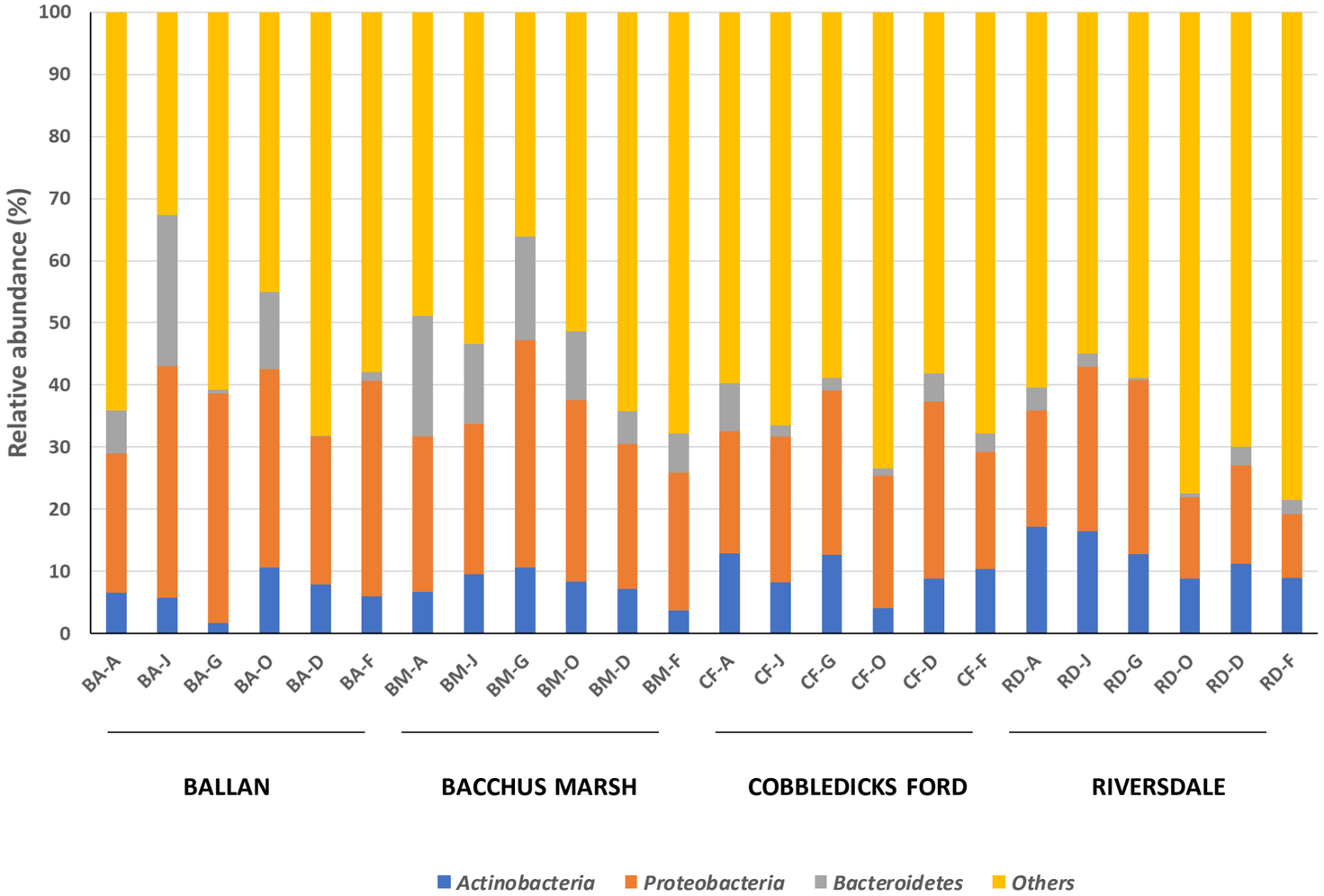


Figure 3.7 Spatial and temporal variation of bacterial phyla (with the highest % contribution based on similarity percentage (SIMPER) analysis) in upstream sites (Ballan and Bacchus Marsh) and downstream sites (Cobbledicks Ford and Riversdale) along the Werribee River. BA refers to Ballan, BM refers to Bacchus Marsh, CF refers to Cobbledicks Ford and RD refers to Riversdale. A refers to April, J refers to June, G refers to August, O refers to October, D refers to December and F refers to February. SIMPER analysis was carried out with PRIMER 7.

3.2.4.2 Spatial and temporal variation at class level

Analysis of the spatial and temporal variation trend at class level showed distinct differences between upstream and downstream sites. In Ballan and Bacchus Marsh, *Betaproteobacteria* was, in general, the most abundant class irrespective of the sampling months (Figure 3.8). *Cytophaga* was usually the second most abundant class at the two sites except in August, December and February in Ballan and in December at Bacchus Marsh. For these months, the class *Actinobacteria* was the second most abundant class. *Alphaproteobacteria* were only observed in June (Bacchus Marsh), August (Ballan and Bacchus Marsh) and October to February (Bacchus Marsh).

In Cobbledicks Ford, *Betaproteobacteria* was the most dominant class in April (~16%), June (14%), October (20%) and December (25%) while *Alphaproteobacteria* was the most abundant class in in August (15%) and February (~11%). At Riversdale, the *Actinobacteria* was the most abundant class in April (17%), June (~16%), October (~9%), December (~11%) and February (~9%) while *Alphaproteobacteri*a was the most abundant class in August (~18%) (Figure 3.8).

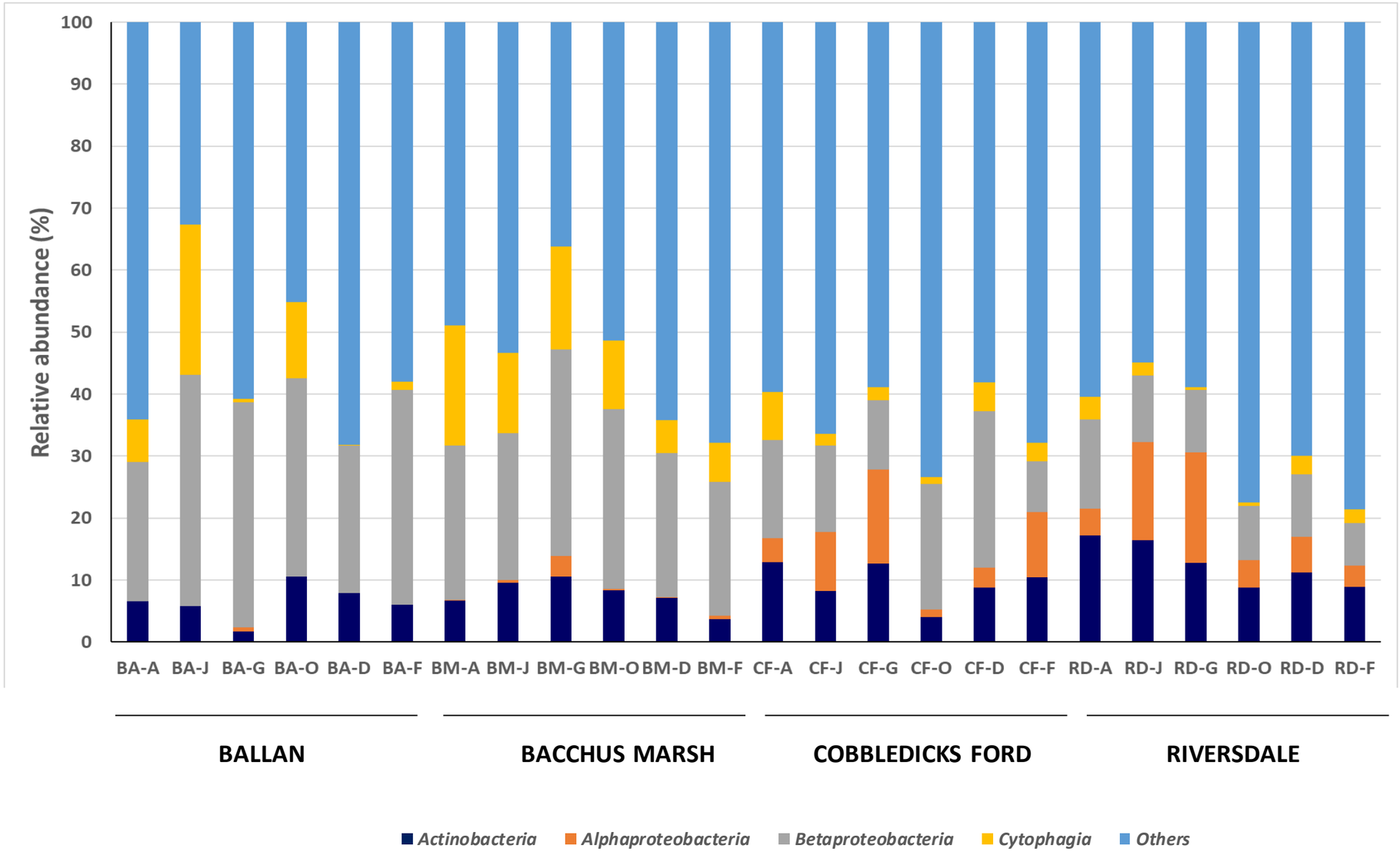


Figure 3.8 Spatial and temporal variation of bacterial classes (with the highest % contribution based on similarity percentage (SIMPER) analysis) in upstream sites (Ballan and Bacchus Marsh) and downstream sites (Cobbledicks Ford and Riversdale) along the Werribee River. BA refers to Ballan, BM refers to Bacchus Marsh, CF refers to Cobbledicks Ford and RD refers to Riversdale. A refers to April, J refers to June, G refers to August, O refers to October, D refers to December and F refers to February. SIMPER analysis was carried out with PRIMER 7.

3.2.4.3 Spatial and temporal variation at the genus level

At the genus level, substantial spatial and temporal variation in the relative abundance of the eleven taxa with the highest relative abundances (as determined with SIMPER analysis) were observed across all sites. These eleven genera were *Polynucleobacter, Terrabacter, Arcicella, Limnohabitans, Acidovorax, Demequina, Nakamurella, Undibacterium, Methylotenera, Candidatus Pelagibacter* and *Leptothrix* species. At Ballan, the first upstream site, the most abundant genus was *Polynucleobacter* in April (8.4%) (autumn), August (17%) (winter), October (16%) (spring) and February (25%) (summer) while *Methylotenera* was the most abundant genus in June (11%) (winter) and *Limnohabitans* in December (11%) (summer) (Figure 3.9). In the second upstream site (Bacchus Marsh), *Arcicella* was the most abundant genus in April (19%) (autumn), June (13%), August (17%) (winter) and October (11%) (spring) while in December (summer), *Methylotenera* was (8%) dominant and *Acidovorax* was the most abundant genus in February (10%) (summer).

In downstream sites, the genus *Acidovorax* had the highest relative abundance of 10% in April (autumn), *Candidatus Pelagibacter* (9.5%) in June, August (15%) (winter) and February (10.5%) (summer), *Limnohabitans* (12%) in October and December (11%) (spring/summer) at Cobbledicks Ford. In contrast at Riversdale, *Demequina* had the highest relative abundance of 9.4% in April (winter) and December (6%) (summer). *Candidatus Pelagibacter* was the most abundant genus in June (16%), August (18%) (winter) and October (4%) (spring). While at upstream sites the key genera were *Polynucleobacter, Arcicella, Limnohabitans, Acidovorax and Methylotenera,* the key genera in downstream sites were *Acidovorax, Candidatus Pelagibacter, Limnohabitans* and *Demequina* (Figure 3.9).

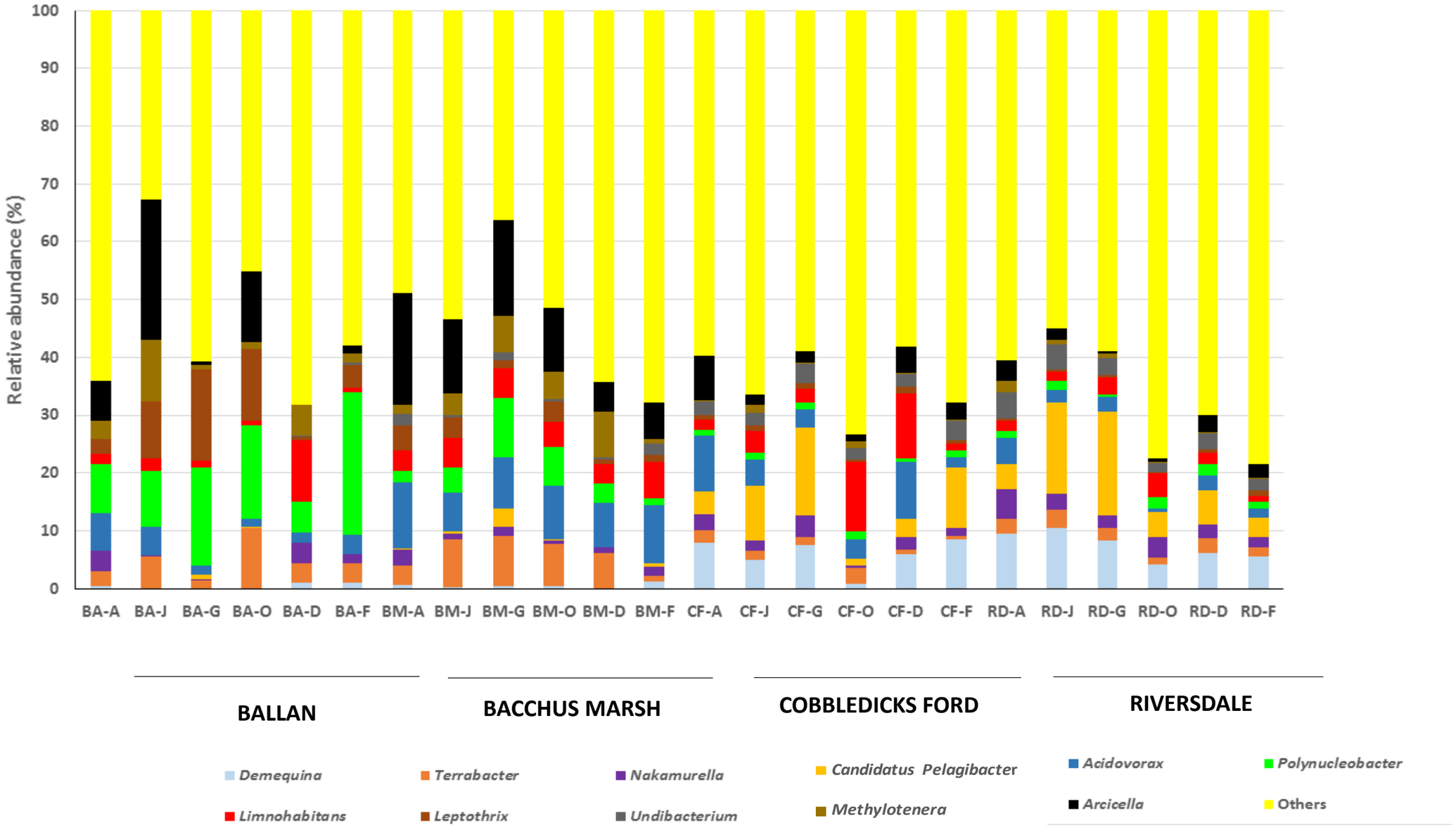


Figure 3.9 Spatial and temporal variation of bacterial genera (with the highest % contribution based on similarity percentage (SIMPER) analysis) in upstream sites (Ballan and Bacchus Marsh) and downstream sites (Cobbledicks Ford and Riversdale) along the Werribee River. BA refers to Ballan, BM refers to Bacchus Marsh, CF refers to Cobbledicks Ford and RD refers to Riversdale. A refers to April, J refers to June, G refers to August, O refers to October, D refers to December and F refers to February. SIMPER analysis was carried out with PRIMER 7.

3.2.5 Inter-relationships between bacterial diversity and key physico-chemical parameters at different sites and months on the Werribee River

The relationships between environmental factors such as temperature, dissolved oxygen concentrations (DO), electrical conductivity, pH, turbidity, suspended solids, bacterial diversity (OTU numbers and Shannon diversity) and nutrients (Table 3.6) were examined using distance-based redundancy analysis (db-RDA) in water samples from the Werribee River. The nutrients used for these analyses included nitrites, nitrates, ammonia, total Kjehldahl nitrogen (nitrogen bound in organic matter) and total nitrogen (free and bound), phosphates and total phosphorous. This analysis was carried out to investigate the inter-relationships between these factors by site and month. Physico-chemical (including nutrient) data were supplied by Melbourne Water.

The dbRDA for the microbiological and environmental data showed differences in their effects on sites on the Werribee River. No relationship or association was evident between microbiological and physico-chemical data at Ballan, an upstream site. However, at the second upstream site, Bacchus Marsh, dbRDA plots showed that dissolved oxygen concentration and temperature were important factors which were associated with changes in the bacterial community at this site ($P \leq 0.005$). (Figure 3.10A). At downstream sites, factors such as turbidity, suspended solids, electrical conductivity, total bound nitrogen (TKN), ammonia, temperature were associated with variation in the bacterial community in Cobbledicks Ford ($P \leq 0.005$). However, at Riversdale, dissolved oxygen was a key factor associated with variation in the bacterial community at this site ($P=0.001$) (Figure 3.10A).

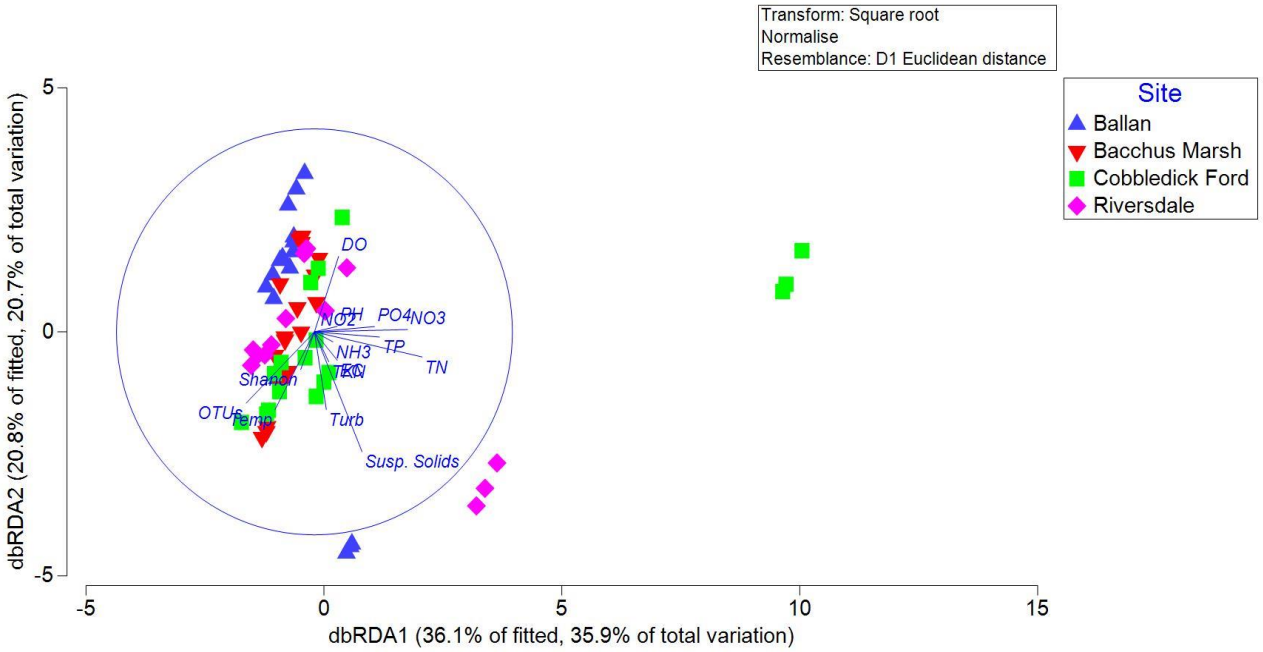
With respect to months, different factors were associated with changes in the bacterial community of the Werribee River ($P \leq 0.005$). These factors were dissolved oxygen, suspended solids, turbidity and temperature factors in April and dissolved oxygen concentrations in June, (Figure 3.10B). While none of the factors was important in August, temperature, turbidity, suspended solids and TKN (total bound nitrogen) were the important factors associated with bacterial community changes in October ($P \leq$

0.005). In December and February, the key factor was the temperature ($P \leq 0.005$) (Figure 3.10B). The cumulative proportions of changes due to each factor are shown in Table 3.7.

Table 3.6: Physico-chemical parameters at Werribee River by site and months

Sample Designation	Site Location	Site and Month	Temperature	DO	EC	PH	Turbidity	Susp. Solids (mg/L)	NO3 (mg/L)	NO2 (mg/L)	NH3 (mg/L)	TKN (mg/L)	Total N (mg/L)	PO4 (mg/L)	Total P (mg/L)
BA1A	Upper stream	Ballan (April)	12.7	7.68	1200	7.4	3.5	2	0.006	0.002	0.025	0.65	0.66	0.0015	0.02
BA1J	Upper stream	Ballan (Jun)	8.2	9.71	1200	7.8	1.5	1	0.0015	0.001	0.005	0.42	0.42	0.0015	0.007
BA1G	Upper stream	Ballan (August)	8.3	10.41	496	7.8	8	4	0.0015	0.001	0.006	0.58	0.58	0.004	0.019
BA1O	Upper stream	Ballan (October)	13.8	7.18	610	7.8	3	1	0.0015	0.001	0.007	0.58	0.58	0.003	0.016
BA1D	Upper stream	Ballan (December)	20.3	3.6	1300	7.7	23	27	0.004	0.001	0.006	2.1	2.1	0.003	0.14
BA1F	Upper stream	Ballan (February)	20.1	3.6	900	7.2	4	5	0.0015	0.001	0.039	0.96	0.96	0.005	0.047
BM1A	Upper stream	Bacchus Marsh (April)	13.9	8.98	1400	7.7	7.3	6	0.046	0.001	0.011	0.31	0.36	0.0015	0.01
BM1J	Upper stream	Bacchus Marsh (Jun)	10.4	9.23	1700	7.6	9.3	6	0.12	0.001	0.007	0.26	0.39	0.0015	0.013
BM1G	Upper stream	Bacchus Marsh (August)	10	9.69	1500	7.8	14	14	0.063	0.001	0.026	0.32	0.38	0.004	0.015
BM1O	Upper stream	Bacchus Marsh (October)	16	6.55	1600	7.6	22	19	0.038	0.002	0.011	0.37	0.41	0.0015	0.017
BM1D	Upper stream	Bacchus Marsh (December)	24.3	8.21	2700	8.1	9	8	0.0015	0.001	0.001	0.43	0.44	0.0015	0.021
BM1F	Upper stream	Bacchus Marsh (February)	22.5	6.13	1800	7.6	21	20	0.0015	0.001	0.004	0.42	0.42	0.0015	0.031
CF1A	Down stream	Cobbledick Ford (April)	14.8	8.5	1700	8	11	13	0.0015	0.001	0.001	0.59	0.59	0.007	0.05
CF1J	Down stream	Cobbledick Ford (Jun)	10	10.23	2800	8.1	5.9	7	0.0015	0.001	0.003	0.5	0.5	0.0015	0.044
CF1G	Down stream	Cobbledick Ford (August)	9.8	10.58	1800	8.1	6	8	5	0.083	0.008	0.95	6	2.6	2.6
CF1O	Down stream	Cobbledick Ford (October)	14.9	5.73	3500	7.8	13	14	0.004	0.001	0.001	0.61	0.62	0.061	0.15
CF1D	Down stream	Cobbledick Ford (December)	22.7	6.6	1500	8	8	15	0.0015	0.001	0.001	0.57	0.57	0.007	0.057
CF1F	Down stream	Cobbledick Ford (February)	22.1	6.74	1200	8	9	10	0.0015	0.001	0.003	0.48	0.48	0.004	0.041
RD1A	Down stream	Riversdale (April)	15.2	5.71	1300	7.6	6.1	7	0.023	0.004	0.068	0.58	0.61	0.011	0.044
RD1J	Down stream	Riversdale (Jun)	10.6	7.82	1900	7.7	5.5	4	0.062	0.005	0.073	0.62	0.68	0.009	0.029
RD1G	Down stream	Riversdale (August)	9.5	9.45	2700	8	4	4	0.024	0.001	0.003	0.44	0.47	0.0015	0.027
RD1O	Down stream	Riversdale (October)	13.6	7.08	2200	8.4	39	38	0.021	0.008	0.13	1.2	1.2	0.41	0.54
RD1D	Down stream	Riversdale (December)	22.1	8.1	1600	8.2	4	6	0.0015	0.001	0.001	0.45	0.45	0.024	0.051
RD1F	Down stream	Riversdale (February)	22.7	4.48	1500	7.7	3	2	0.0015	0.001	0.005	0.48	0.48	0.042	0.069

A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Site
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Riversdale
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TKN
Shannon
OTUs
Temp
Turb
Susp. Solids
dbRDA2 (20.8% of fitted, 20.7% of total variation)
dbRDA1 (36.1% of fitted, 35.9% of total variation)
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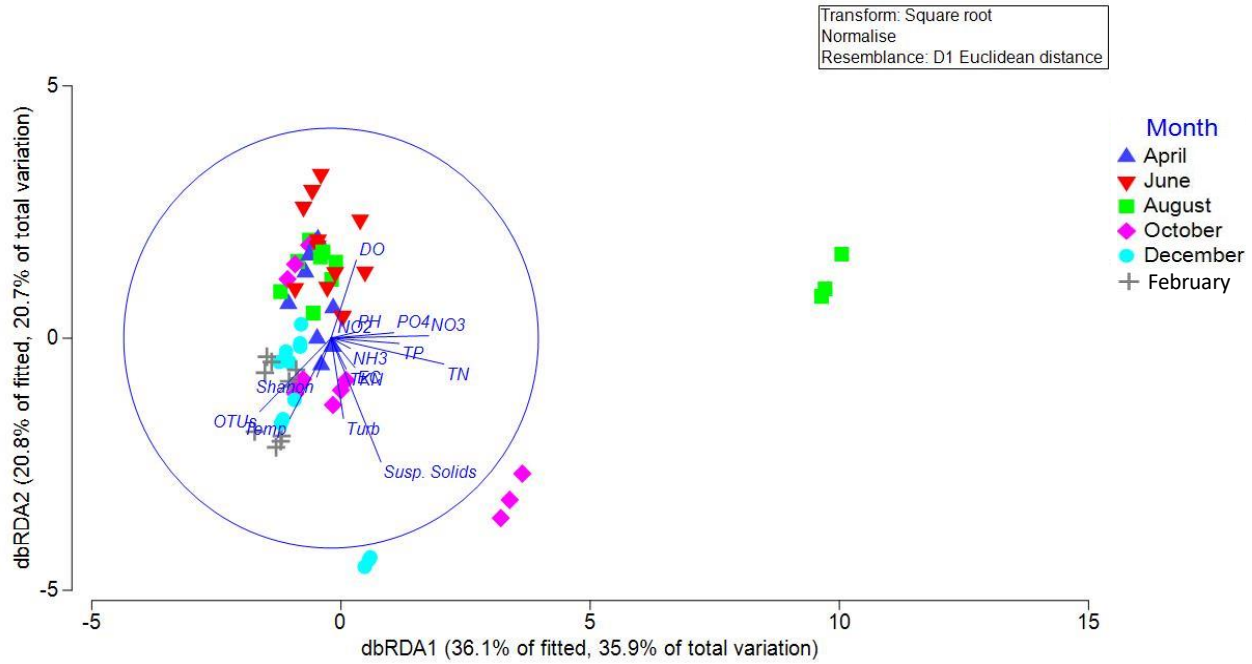
B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TN
TKN
Shannon
OTUs
Temp
Turb
Susp. Solids
dbRDA2 (20.8% of fitted, 20.7% of total variation)
dbRDA1 (36.1% of fitted, 35.9% of total variation)
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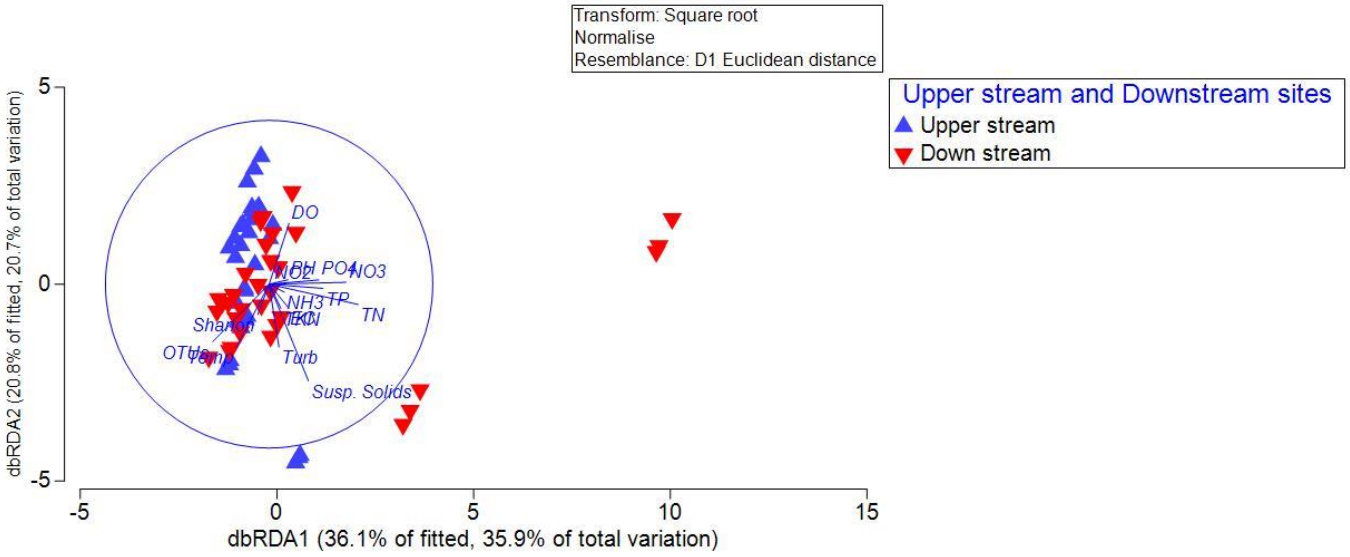


Figure 3.10: Distance-based RDA ordination of first and second fitted axes relating the microbiological data and environmental variables to (A) sampling sites, (B) sampling months and (C) the cumulative upper stream and downstream sites on the Werribee River. Variables shown to explain significant amounts of variation in the bacterial community as determined with DISTLM (Table 3.7) were included in the model.

Table 3.7: DistLM sequential tests of factors on bacterial community structure in samples from different sites and months at Werribee River.

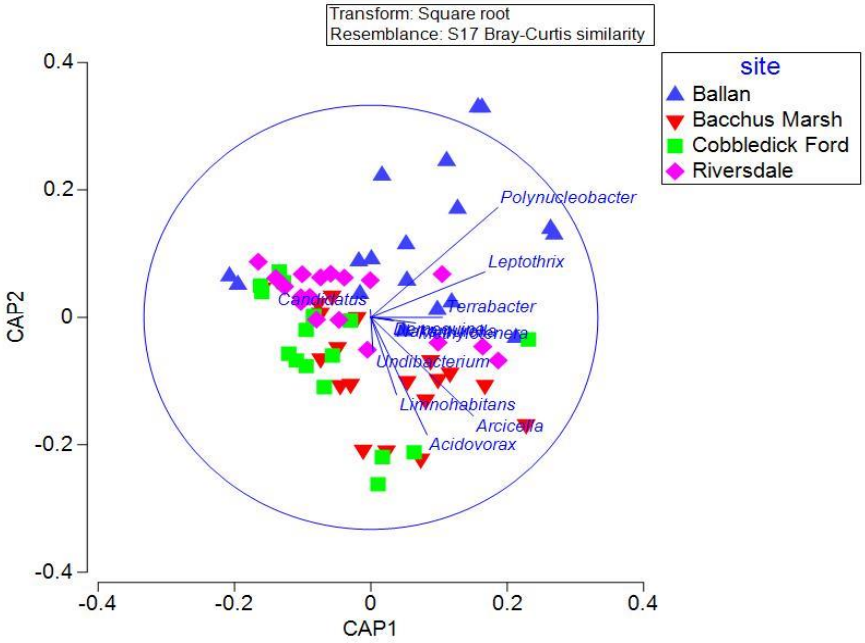
Variable	**R^2**	**SS(trace)**	**Pseudo-F**	**P**	**Proportion**	**Cumulative**
Shannon	0.15935	162.54	12.7	**0.001**	0.15935	0.15935
OTUs	0.21218	53.88	4.4253	**0.005**	0.052823	0.21218
Temperature	0.28478	74.052	6.5979	**0.001**	0.0726	0.28478
Dissolved Oxygen	0.38465	101.88	10.388	**0.001**	0.099878	0.38465
Electrical conductivity	0.45807	74.888	8.5351	**0.002**	0.073419	0.45807
pH	0.56408	108.13	15.078	**0.001**	0.10601	0.56408
Turbidity	0.65576	93.507	16.245	**0.001**	0.091674	0.65576
Suspended Solids	0.68869	33.592	6.3474	**0.006**	0.032934	0.68869
NO_3	0.92456	240.59	184.47	**0.001**	0.23587	0.92456
NO_2	0.95441	30.442	37.967	**0.001**	0.029845	0.95441
NH_3	0.96486	10.663	16.957	**0.001**	0.010454	0.96486
TKN	0.98902	24.639	123.15	**0.001**	0.024156	0.98902
Total Nitrogen	0.99008	1.0805	5.8705	**0.001**	0.001059	0.99008
PO_4	0.99325	3.2367	25.38	**0.001**	0.003173	0.99325
Total Phosphorous	0.99423	0.99986	9.0021	**0.001**	0.00098	0.99423

Note: Pseudo F values were from permuted tests (n = 9999) while bold texts (P) indicates statistical significance as derive with Benjamini-Hochberg adjustment.

These different factors led to the selection of different key taxa at different sites and months. The key taxa were evaluated with Canonical Analysis of Principal (CAP) Coordinates and the results are presented in Figure 3.10. At Ballan, genera such as *Polynucleobacter, Leptothrix, Candidatus Pelagibacter, Terrabacter* and *Methylotenera* were the important or key genera at this site while genera such as *Limnohabitans, Arcicella, Undibacterium, Candidatus Pelagibacter* and *Acidovorax* were equally important at Bacchus Marsh (Figure 3.11A). At the two downstream sites

(Cobbledicks Ford and Riversdale), genera such as *Candidatus Pelagibacter*, *Methylotenera* and *Demequina* were important at these two sites (Figure 3.11A). Different bacterial genera had different abundance at different months. For example, *Methylotenera* and *Demequina* were present at high relative abundances in April, *Undibacterium* and *Limnohabitans* in June, *Arcicera* in October, *Polynucleobacter* in December and *Leptothrix* and *Polynucleobacter* in February (Figure 3.11B and 3.11C).

A



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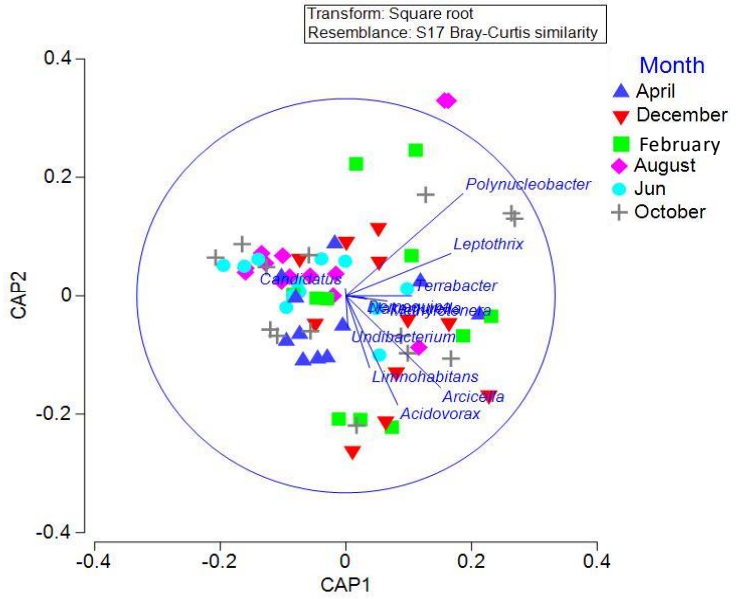

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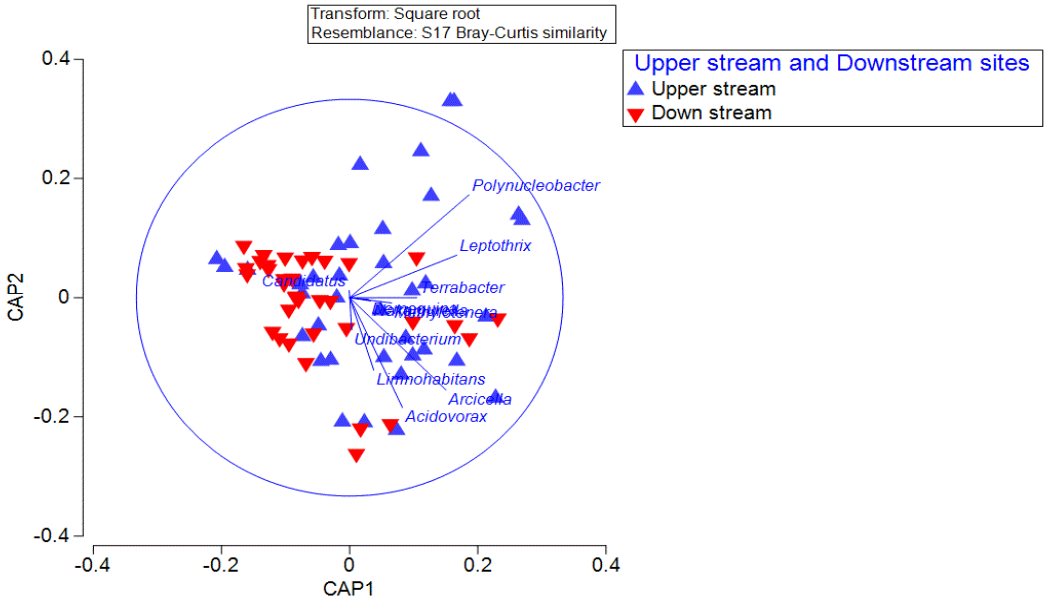


Figure 3.11: Ordination plots generated by Canonical Analysis of Principal (CAP) coordinates of selected bacterial genera in relation to (A) site, (B) months and (C) cumulative upstream and

downstream sites in samples from the Werribee River. Spearman correlation vectors were generated from OTUs with the highest percentage contribution as determined by similarity percentage (SIMPER) analysis and included in the plot. Note that *Candidatu*s refers *to Candidatus Pelagibacter*.

3.2.6 Discussion

This study was carried out on water samples obtained from four selected sampling sites on the Werribee River for which water quality monitoring was conducted (by Melbourne Water) to evaluate the spatial and temporal variation in the bacterial community structure and diversity at these sites. The relationship between the bacterial community and selected physico-chemical factors was also studied. These investigations were conducted using an amplicon-based next-generation sequencing approach.

PCoA plots showed that the bacterial community structure formed site-specific clusters. This indicated that, for example, the bacterial communities in Ballan and Bacchus Marsh (upstream sites) were more closely related to each other than to the communities in the two downstream sites (Cobbledicks Ford and Riversdale). Similar trends have been reported in several of bacterial communities in aquatic ecosystems (Staley et al., 2015a, Luo et al., 2016, Staley et al., 2015b). For example, spatial variation was observed in bacterial communities from different sampling sites located in alpine ponds in New Zealand (Lear et al., 2014) with substantial differences observed in bacterial community composition (up to 38%) between samples that were more than 20 m apart in the ponds. Spatial variation was also observed in the bacterial communities from 11 different sampling sites along the Mississippi River (Staley et al., 2015b). In this case, the observed spatial variation was thought to be due to shifts in the relative abundance of OTUs at these sites and anthropogenic activities such as the construction of dams on the rivers system. In this current study,

spatial variation was also observed when the OTU numbers at the four sampling sites were compared, although this variation was not statistically significant.

Temporal variation in the bacterial community was also observed within each sampling site along the Werribee River (Ballan, Bacchus Marsh, Cobbledicks Ford and Riversdale) over the different sampling months (April, June, August, October, December 2015 and February 2016). For example, at Ballan, the bacterial communities in June, August and October formed a distinct cluster which was different to the cluster formed by bacterial from the communities in December, February and April (Fig 3.2). Similarly, in downstream sites and using Riversdale as an example, the bacterial communities in April and December were clustered together, and different from clusters formed by the communities obtained at remaining months. The observed temporal variation in bacterial community composition was also reflected in the numbers of OTU with significant differences observed in OTU numbers of some of the sites over the different months. For example, the numbers of OTUs in April and June were significantly lower ($P < 0.05$) from those in December or February. Other research findings have indicated that strong seasonal (temporal) variation can occur in the bacterial composition of sites in aquatic ecosystems. Temporal variation has been reported in the bacterial composition of samples obtained from May to October in lake Wisconsin (US) (Yannarell and Triplett, 2005). Strong temporal variation was also observed in epilithic bacterial biofilm communities of a river ecosystem over time when samples collected in 2001, 2002 and 2003 were compared (Anderson-Glenna et al., 2008). This variation was thought to be due to differences in inter-annual changes in weather and environmental conditions such as temperature. Temporal changes were also reported in freshwater bacterial communities in the Mississippi which were thought to be due to seasonal (annual) variation in the abundances of specific OTUs (Staley et al., 2015b). Additionally, the abundance of bacterial cells can also fluctuate in response to environmental changes (temperature and nutrient load) associated with different seasons (Staley et al., 2015b).

There was significant spatial variation in bacterial Shannon diversity indices between the four sites, with significantly lower diversity at Bacchus Marsh than at other sites (Figure 3.4). Other river-based studies have reported a similar trend. Bacterial diversity as assessed by next-generation sequencing in the Yenisei River in the Arctic showed substantial variation between some of the sites with *H'* values ranging from 6.89 to 8.25 (Kolmakova et al., 2014). Spatial variation was also observed in bacterial Shannon diversity at six different sites along the Yellow River in China in water and in surface sediment samples as assessed using a PCR-clone library approach (Xia et al., 2014). Spatial variability was also higher in the bacterial community in sediment samples. Differences in the concentrations of suspended particulate organic matter between the sampling sites were thought to be responsible for this variation (Xia *et al*., 2014).

Significant temporal variation was also observed in the Shannon diversity of some of the water samples obtained from April to February in this study. The highest *H* values were recorded in December and February which were significantly higher than the *H* values in April, June and August (Figure 3.6). The Chao1 OTU richness ranged from 1259 in April to 2976 in February (summer month). As observed with the Shannon diversity values, the two highest Chao1 values of 2408 and 2976 were recorded in the summer months of December and February. A similar trend was observed in the Shannon diversity and Chao indices in a study of the Ganjiang River in China (Wang et al., 2016), in which samples were collected in April, May, June, July and August and subject to NGS based analysis. The Shannon diversity values ranged from 3.01 to 4.70 and differed significantly between the months with the highest diversity values observed in summer months (June and July) (Wang et al., 2016). DGGE-based bacterial Shannon diversity analysis of bacterial community from nine sampling sites along the Haihe River in China revealed different *H* values. These values ranged from 2.31 to 3.12, reflecting significant seasonal variation in autumn, winter, spring and summer

samples. The bacterial community diversity was substantially higher in the autumn period compared to most other months (Ma et al., 2016).

The 16S rRNA gene next-generation sequencing of samples from Werribee River revealed that the most abundant phyla were *Proteobacteria, Bacteroidetes* and *Actinobacteria*. Other studies on river systems from different parts of the world have also identified some of these phyla as the core phyla in aquatic systems. These include studies on the Thames River in England (Read et al., 2015), the Sinos River in Brazil (de Oliveira and Margis, 2015), the Danube River in Europe (Savio et al., 2015), freshwater lakes in Bulgaria (Iliev et al., 2017), the Mississippi River in the United States (Staley et al., 2013) and the Yellow River in China (Xia et al., 2014).

While there was only minor spatial and temporal variation in bacterial phyla, some spatial and temporal variation in the significant bacterial taxa at class and genus level were observed at the four sampling times and over different months. For example, at Ballan and Bacchus Marsh, which are upstream sites on the Werribee River, *Betaproteobacteria* were, in general, the most abundant and usually followed by the *Cytophaga* or the *Actinobacteria*. In the downstream sites, *Betaproteobacteria* were again the most abundant class at Cobbledicks Ford but in contrast at Riversdale, *Actinobacteria* was the most abundant class. Different bacterial genera were important at different sites. For example, at upstream sites, the key genera were *Polynucleobacter, Arcicella, Limnohabitans, Acidovorax and Methylotenera,* whilst in downstream sites, the key genera were *Acidovorax, Candidatus Pelagibacter, Limnohabitans* and *Demequina.* The detection of *Candidatus Pelagibacter* in freshwater systems is especially interesting given that it is commonly detected in marine environments (Nimnoi and Pongsilp, 2020). However, *Candidatus Pelagibacter* has been detected in the metagenome datasets from freshwater systems in the Amazon (Brazil) and from Lake Gatun (Panama) sharing up to 64% of its protein sequence identity with the marine equivalent (Ghai et al., 2011).

The trend observed in this study has been reported in other studies. For example, the spatial variation observed in the taxonomic composition between seven sites on the Yellow River (China) were associated with differences in taxa type and abundance of the aquatic bacterial community (Xia et al., 2014). Some sites on the Yellow River had unique groups such as *Firmicutes* and *Nitrospira* when compared to other sites. Also, some sites had the same taxa such as *Beta-* and *Gamma-Proteobacteria* but at varying abundance; for example, *Gammaproteobacteria* were more abundant at one site while the *Betaproteobacteria* abundance was higher at the second site (Xia *et al*., 2014). Similarly, differences in the relative abundance of different phyla such as *Bacteroidetes, Planctomycetes, Proteobacteria* and *Firmicutes* were thought to be responsible for the spatial variation in the bacterial communities from ten different sites along the Yenisei River (Kolmakova et al., 2014).

Differences in taxa and taxa abundance were also responsible for the temporal variation observed at each of the four different sites over the 12-month experimental time-frame of this study. Using the upstream site Ballan, as an example, some bacterial taxa and abundance changed over time. OTUs classified into the *Actinobacteria, Betaproteobacteria* and *Cytophaga* classes were detected in April and June but by August, taxa within the class Cytophaga were almost absent whilst members of the *Alphaproteobacteria* were now detectable. By December, members of the class *Cytophaga* were no longer significantly abundant leaving only the *Actinobacteria* and *Betaproteobacteria.* Similarly, at the three remaining sites, the relative abundance of these key classes fluctuated over time. The trend observed in this study is not unusual as some other studies of microbial composition within aquatic systems over time have shown similar trends. For example, temporal changes in the abundance of taxa (reflective of the changes in the abundance of Alpha-, Beta- and Gammaproteobacteria, and Epsilonbacteria) over a 12-month time-frame have been reported in an urban river ecosystem, Zenne River in Belgium Zenne River in Belgium(García-Armisen et al., 2014).

Several studies have shown that spatial and temporal variability in bacterial OTU, community, diversity and taxonomic composition can be associated with multiple environmental and physico-chemical factors such as water temperature, salinity, water depth, dissolved organic matter, the concentration of pollutants, dissolved oxygen concentration and tributary confluences (Fortunato et al., 2012a, Payne et al., 2017a, Bouskill et al., 2010, Staley et al., 2015b). However, these environmental factors vary in relation to their location within a specific river or at different times of the year. They influence bacterial community structure by enhancing the growth of individual taxa while suppressing the growth of others (Crump et al., 2004, Fuhrman et al., 2006, Lozupone and Knight, 2007, Nemergut et al., 2011, Staley et al., 2015b). In this current study, multiple environmental factors were studied at each sampling site. These were temperature, dissolved oxygen concentrations, electrical conductivity (EC), suspended solids, turbidity and pH. In addition, NO_2 (nitrites), NO_3 (nitrates), NH_3 (ammonia), total nitrogen (TN), Total bound Nitrogen (TKN), PO_4 (phosphates), and total phosphorous (TP) contents of samples were determined. The observed impacts of these factors on bacterial OTUs and Shannon diversity at different sites and months are discussed below.

In this study, the different environmental factors were important at different sites. At Ballan (an upstream site) none of the listed factors showed any strong or direct relationship to variation in the bacterial community at this site. This meant that whatever bacterial community changes that were observed at this site, these occurred independently of the variables analysed in this study. Nevertheless, bacterial genera such as *Polynucleobacter, Leptothrix, Candidatus Pelagibacter, Terrabacter* and *Methylotenera* were found to be the important genera in Ballan over this study's sampling period. In contrast, at the second upstream site (Bacchus Marsh), temperature and dissolved oxygen concentrations were important factors associated with the changes in the bacterial community ($P \leq 0.005$, Table 3.7). In Cobbledicks Ford, the key factors were temperature, turbidity, suspended solids, electrical conductivity, total bound nitrogen and ammonia concentrations while dissolved

oxygen concentrations were important in Riversdale community ($P \leq 0.005$, Table 3.7). A selected set of key factors are subsequently discussed below.

In some upstream and downstream sites, the temperature was an important factor that affected bacterial taxon composition and diversity. The maximum water temperature in the Werribee River was 24.3 °C during the summer (December) with a mean of 2266 OTUs (Figure 3.5). The minimum water temperature was 10.2 °C during the winter season in June during and a mean value of 1196 OTUs was observed. The estimated number of OTUs (Chao1) and bacterial diversity (Shannon *H'*) also increased in December and February (summer months) when compared to winter months (June and August) showing possible temperature effects. In a study of bacterial communities on the Ganjiang River in China, temperature (in addition to flow rate) was one the main factors influencing bacterial community composition, causing variations in OTU abundance (Wang et al., 2016). Higher air temperatures were associated with higher OTU numbers in some months. For example, samples obtained in June (32.5 ^{o}C) and July (30.5 ^{o}C) had higher mean numbers of OTUs (475.8 and 492 respectively) compared to in April (21.5 ^{o}C;377.4 OTUs) (Wang et al., 2016). Water temperature is a significant environmental parameter that impacts bacterial diversity in aquatic ecosystems, through its effects on the metabolic rate and biological activity of all aquatic organisms (Dallas, 2008). Water temperature can vary in temperate river systems because of changing the seasons and anthropogenic inputs (pollutants) (Fortunato et al., 2012b) and is a strong driver of bacterial community composition (Crump and Hobbie, 2005, Staley et al., 2015b).

In addition, changes in the relative abundance in phyla, class and genus compositions were observed at both upstream and downstream sites based on seasons (temperature changes). For example, at the genus level, *Polynucleobacter* was the most abundant genus in early autumn (April) and early spring (October) and late summer (February) while *Methylotenera* was the most abundant genus in early winter (June) and *Limnohabitans* in early summer in December at Ballan. In contrast at Riversdale,

Demequina was the most abundant genus in early winter (April) and early summer (December) while *Candidatus Pelagibacter* was the most abundant genus in winter (June and August) (18%) and early spring (October). Therefore, fluctuations in bacterial community structure and diversity which were reflected in changes in bacterial abundance (and the dominant genera) were observed across seasons (i.e. with different temperatures). Similarly, in the Ganjiang River (China), there was a strong positive correlation between Shannon bacterial diversity and temperature (Wang et al., 2016).

Dissolved oxygen (DO) concentrations were another important environmental factor in this study. Dissolved oxygen concentrations in water may sometimes decline with increasing water temperature (as cold water tend to hold more oxygen than warm water). The effects of dissolved oxygen concentrations in aquatic systems are variable. Higher DO concentrations have been correlated with increases in the proportion of *Proteobacteria* and *Firmicutes* in some studies (Kaevska et al., 2016). Increases in bacterial OTUs and diversity may translate into increased metabolic activities and increased biochemical oxygen demand (more DO is consumed), which will invariably lead to a lower DO over time. Dissolved oxygen concentrations have previously been correlated with bacterial diversity, with increasing DO associated with increasing diversity (Mohiuddin et al., 2019).

Turbidity and suspended solids are important factors that can affect bacterial composition (OTU numbers, diversity and taxa structure) in aquatic ecosystems such as rivers and the marine environment. Turbidity refers to the degree of water clarity while suspended solids refer to particles in water that are at least 2 μm in size. The higher the concentration of suspended solids the higher the water turbidity. Recent reports on the urban water system have shown a significant correlation between bacterial composition and water turbidity with samples having the highest turbidity also have the highest diversity of bacterial groups (Jin et al., 2018). This is not unexpected as the suspended materials in water may include nutrients and acts as attachment points for aquatic bacteria and this is known to drive increases in bacterial diversity (Luo et al., 2020). Therefore, changes (increase or decrease) in bacterial OTU numbers and diversity as observed, for example, in

Cobbledicks Ford might have been driven by changes in the turbidity and suspended solids in Werribee River.

In conclusion, spatial and temporal variation was observed in numbers of OTUs, Shannon bacterial diversity and in the composition of bacterial communities especially at class and genus levels in the Werribee River. Irrespective of site and months, the three most abundant phyla were the *Proteobacteria*, the *Actinobacteria* and the *Bacteroidetes*. At upstream sites, the key genera were *Polynucleobacter, Arcicella, Limnohabitans, Acidovorax and Methylotenera,* while the key genera in downstream sites were *Acidovorax, Candidatus Pelagibacter, Limnohabitans* and *Demequina.* Site and time-related differences were observed in the OTU numbers, bacterial diversity and bacterial composition in upstream and downstream sites on the Werribee River. For example, bacterial OTU numbers were highest at Cobbledicks Ford (a downstream site), Shannon diversity highest at Ballan (an upstream site) while the highest OTU numbers and Shannon diversity values were observed in February and December, respectively. Bacterial diversity and OTU numbers were strongly and positively correlated to temperature but negatively correlated to dissolved oxygen concentrations. In this study factors such as water temperature, dissolved oxygen concentrations, turbidity and suspended solids were some of the important drivers of changes in bacterial community structure and diversity in the Werribee River. The data generated in this study would be useful in monitoring the health of the Werribee river system as it receives increasing pressure from urban expansion. The next chapter will explore spatial and temporal variation in the antibiotic resistance gene pool in these bacterial communities.

4 Chapter 4: Spatial and temporal variation in the presence and abundance of clinically relevant antibiotic resistance genes in the Werribee River, Melbourne, Victoria, Australia,

4.1 Introduction

Pathogenic bacteria are a major cause of infection and human mortality. However, the discovery and subsequent widespread use of antibiotics for therapeutic purposes has resulted in substantial reductions in human mortality due to bacterial infections, improving human health and increasing life expectancy by up to 15 years (Hayes et al., 1993, Keeney et al., 2014, Murray et al., 2005). This widespread use of antibiotics and their synthetic derivatives has resulted in significant environmental contamination with widespread antibiotic usage resulting in increasing cases of antibiotics resistance in pathogens (Larsson, 2014). While antibiotic resistance can occur naturally, environmental contamination by sewage often results in the selection of antibiotic-resistant microorganisms. Antibiotic resistance factors in these groups can then be subsequently transferred to other bacterial taxa (Ishii, 2013, Baquero et al., 2008, Martinez, 2009, Zhang et al., 2019, Lin et al., 2019).

Antibiotic resistance is now a major threat to human health with the resistance mechanisms for most antibiotics well known (Blair et al., 2015). This resistance is linked to the expression of antibiotic resistant genes (ARGs) found both in bacterial chromosomes and on extrachromosomal mobile genetic elements such as plasmids. The distribution and dissemination of ARGs have been widely studied in different environments such as sediments (Chen et al., 2019b), urban rivers (Huang et al., 2019) and lakes (Stange et al., 2019) with the molecular mechanisms underpinning antibiotic resistance well described (Banin et al., 2017, Blair et al., 2015). Bacteria can be naturally resistant to antibiotics and /or develop resistance through mutation and/ or horizontal gene transfer following the input of antibiotics or antibiotics-containing wastes into environmental systems such as soil and water (Burmeister,

2015, Woodford and Ellington, 2007, Sun et al., 2019). However, this transfer and/or development of antibiotic resistance can at times be adversely affected by environmental conditions. For example, there have been reports of the loss of *tet* resistance genes in wastewater originating from feedlots following exposure to sunlight over 7 days presumably due to photolytic effects (UV damage) on bacteria carrying these genes (Engemann et al., 2006). In some instances, the loss of antibiotic resistance genes can occur when the selective pressure of specific antibiotics is alleviated. For example, in clinical samples, the loss of resistance to the antibiotic carbapenem has been reported in a *Klebsiella pneumoniae* strain once the selective pressure of meropenem was removed (Simner et al., 2018)

Environmental exposure to antibiotics can occur from multiple sources. These include through the discharge of materials or wastes containing synthetic antibiotics following excretion from the human body, and from industries, hospitals, sewage and sludge from waste treatment processes and as a consequence of their widespread use in agriculture (Stoll et al., 2012, Pruden et al., 2013, Lau et al., 2017, Bougnom and Piddock, 2017, Martin-Laurent et al., 2019, Karkman et al., 2019). The development of antibiotic resistance in humans has been linked to exposure to environments and food materials containing bacteria carrying ARGs (Cabello, 2006). High rates of antibiotic resistance in humans including to broad-spectrum antibiotics have been reported (Ventola, 2015) and are a significant health concern.

Multiple studies have shown correlations between biosolids use and effluent discharge (especially those containing antibiotics) and the concentration of ARGs. These correlations show that increased use of biosolids and or effluent discharge can lead to increases in the abundance of ARGs. For example, investigations in the Cauvery River in India have shown a high concentration of ARGs that confer resistance to broad-spectrum antibiotics (Streptomycin

3"-adenylyltransferase (*aadA),* beta-lactamase (*bla**TEM*) and the New Delhi Metallo-1 (*NDM1*) resistance genes) in the river sediments surrounding hospital waste outlet pipes (Devarajan et al., 2016). A similar correlation between the levels of ARGs in river water and their exposure to hospital and urban wastes has been reported in Spain (Rodriguez-Mozaz et al., 2015). The abundance of ARGs such as *sul*1, *tet*(A), *int*II and *qac*E was proposed to have significantly increased in the sediments of Sava River, in Croatia as a consequence of this river receiving effluents from drug formulation facilities (González-Plaza et al., 2019). Similarly, the abundance of *tet* and *sul* genes in soils has also been positively correlated with antibiotics and metal concentrations (Cu, As and Zn) from applied manure (Guo et al., 2018). Increasing fecal pollution (as monitored via faecal markers such as crAssphage) has also been linked to increased abundance of antibiotic resistance genes in polluted environments in several countries including India, Singapore, Spain and the US (Karkman et al., 2019).

The aquatic environment when exposed to antibiotics containing wastes provides an ideal environment for horizontal gene exchange and transfer of mobile genetic elements carrying resistance genes among bacterial species (Zhang et al., 2015). Recent studies have shown that aquatic ecosystems can serve as a reservoir for antibiotic-resistant bacteria (ARB) and therefore, a reservoir of ARGs (Stoll et al., 2012, Reddy and Dubey, 2019). This has been observed in the Yarra River in Australia using a culture-based approach with the detection of indigenous bacteria resistant to commonly used antibiotics such as ampicillin, chloramphenicol and kanamycin (Boon and Cattanach, 1999). High levels of bacterial antibiotic resistance (ARGs detected) have also been reported in the Yellow River in China (Zhang et al., 2015), in the Rhine and Danube Rivers in Germany and in the Brisbane River in Australia (Stoll et al., 2012), River Ganges in India (Reddy and Dubey, 2019) and Yangtze River in China (Wang et al., 2019).

Different molecular methods have been used to study the prevalence of ARGs in aquatic systems. These include DNA hybridization methods (Fluorescence in situ hybridization and Southern blot methods) (Werner et al., 2007), PCR (endpoint and multiplex PCR) (Strommenger et al., 2003, Canizalez-Roman et al., 2019), quantitative PCR (Rodriguez-Mozaz et al., 2015, Al Salah et al., 2019) and DNA microarrays (Biswal et al., 2014, Bogaerts et al., 2016). Endpoint PCR and quantitative PCR approaches are more commonly used due to their relatively low costs, ease of application and high accuracy

Molecular (DNA-based) detection techniques (PCR and qPCR) have been previously applied to the global study of ARGs at the Brisbane, Rhine and Danube Rivers (Stoll et al., 2012). The prevalence of resistance to eight clinically significant antibiotic classes: β-lactams, aminoglycosides, glycopeptides, chloramphenicol, tetracycline, macrolides, trimethoprim, and sulphonamides was demonstrated in surface water samples. A higher prevalence of chloramphenicol resistance *catII* genes and *β*-lactam resistance *ampC* gene was observed in the Brisbane River when compared to the Rhine and Danube rivers in Germany. However, macrolide (*ermB*) resistance genes were more prevalent in rivers in Germany (68%) when compared to the Brisbane River (18%). Interestingly, both Australian and German rivers showed a high prevalence of *sul*I, *sul*II (100-77%) and *dfrA1* (55−43%) genes which code for bacterial resistance to the antibiotics sulfonamide and trimethoprim, which are a clinically significant class of antibiotics broadly used in human therapies (Stoll et al., 2012).

Similarly, a PCR-based approach was used to investigate the prevalence of ARGs such as *tet*(A), *tet*(B), *tet*(C), *tet*(D), *sul*1, *erm*F, *lin*A, *aac*(6')-Ib-cr, *int*l1, *blaTEM* and *blaCTX-M* in autumn and winter samples from Pilica River in Poland (Koniuszewska et al., 2019a). ARGs were observed to be more diverse and frequently detected in samples from winter months than in the autumn. Specific ARGs, (*blaTEM, tet*(A), *tet*(E), *erm*F and *int*l1) encoding resistance to multiple human antibiotics were detected in most samples irrespective of sampling time and

site demonstrating their ubiquity in the river samples, with anthropogenic inputs thought to be responsible for this prevalence. Most of these ARGs (including *int*l1) are subject is to horizontal gene transfer processes as a means of ARG dissemination (Koniuszewska et al., 2019b).

Antibiotic resistance genes (ARGs) are found in the natural environment largely as a result of anthropogenic inputs. Scientific investigations of their interactions in soils have shown positive correlations between these antibiotics resistance genes and the concentrations of some metals. In Australia, soil metals such as Al, Mn and Pb have shown significant positive relationships with ARGs with their presence being associated with higher numbers of bacteria with ARGs in soils from Western Australia residential areas (Knapp et al., 2017). Examination of aquaculture isolates and samples isolates from different states in Australia has shown that ARGs such as *tet*, *sul*1, *aadA* and *Int1* are prevalent in these samples (Akinbowale et al., 2007, Ndi and Barton, 2011). The prevalence of ARGs can be exacerbated by the use of reclaimed water. For example, increased abundance of ARGs such as those coding for Beta lactams resistance in soils from urban parks in Victoria has been associated with the irrigation of these parks with reclaimed water (Han et al., 2016). In Queensland, investigations have shown a significant positive correlation between *E. coli* resistant to the antibiotic ciproflaxin isolate from some river systems and WWTP discharge volume into these rivers such as the lower Brisbane, Maroochy, Pine, Logan and Noosa Rivers (Watkinson et al., 2017). Indeed, in Australia and other parts of the world, WWTPs are well-recognized hotspots and sources of ARGs and antibiotic resistant bacteria (ARB) (Voolaid et al., 2018).

River systems in Australia are a valuable source of water for irrigation in agriculture (crop production) in Australia. The Werribee River (Melbourne), in particular, is a major source of water for farmland in the Werribee basin. The river could therefore be potentially impacted by anthropogenic activities including exposure to antibiotic-containing wastes and run-offs.

However, there are limited reports on the prevalence of ARGs in this aquatic system. Barker-Reid *et al*., (2010) investigated the occurrence of ARGs in the Werribee River Basin in Australia over a one-year period demonstrating the low prevalence of ARGs in water samples using PCR-based techniques.

In the previous chapter, microbial community analysis of samples from the Werribee River, showed that there was considerable variation in the bacterial communities at different sites (and at different time points) on the Werribee River. This chapter now explores variation in the prevalence of antibiotic resistance genes along the river. Therefore, this study aimed to investigate spatial and temporal variation in the presence and abundance of antibiotic resistance genes (ARG) at selected sites along the Werribee River, Australia, using PCR and qPCR detection assays. The sites selected for this investigation are Ballan, Bacchus Marsh, Cobbledicks Ford and Riversdale sampled at six-time periods over a one-year period. Molecular approaches were used to determine the distribution and abundance of 12 selected clinically relevant antibiotic resistant genes (^{bla}*NDM-1, mecA, tet(M), ampC, vanA, mcr-1, tet(B), erm(B), aac (6')-Ie-aph (2'')-Ia, SulII, catII* and *dfrA1*), encoding resistance to antibiotics that are widely used in clinical medicine and/or in veterinary treatment, including in agriculture. It is hypothesized that there will be substantial spatial and temporal variation in the relative abundance of clinically important ARGs in samples from the Werribee River.

4.2 Results

4.2.1 Detection of antibiotic resistance genes (ARGs) along Werribee River

Surface water samples (72) from both upstream (Ballan and Bacchus Marsh) and downstream (Cobbledicks Ford and Riversdale) sites on the Werribee River were subject to PCR-based analyses to assess the prevalence of ARGs. The prevalence of a total of twelve antibiotic

resistance genes (**Table 4.1**) in these water samples was investigated using different primer combinations in PCR-based assays. These twelve antibiotic resistance genes, (*^{bla}NDM-1, mecA, tet(M), ampC, VanA, mcr-1, tet(B), erm(B), aac (6')-Ie-aph (2'')-Ia, sulII, catII* and *dfrA1*) were screened by agarose gel electrophoresis after PCR and agarose gels in which PCR products were detected are presented in Figures 4.1, 4.2 and 4.3. The results of the screening assays showed that nine ARG (*mecA, tet(M), ampC, VanA, mcr-1, erm(B), aac (6')-Ie-aph (2'')-Ia, SulII,* and *dfrA1*) were not detected (irrespective of site and season) by PCR (Table 4.1). However, three ARGs (*^{bla}NDM-1, tet(B)* and *catII*) were each detected and in at least three sites on the Werribee River.

With respect to the *catII* gene, Figure 4.1 shows detected *catII* genes after PCR and visualization by agarose gel electrophoresis. The *catII* gene was detected in both the upstream and downstream sites along the Werribee River. The gene was occasionally detected at upstream sites, specifically at Ballan in April and at Bacchus Marsh in October. The *catII* gene was more frequently detected in downstream sites, being detected at Cobbledicks Ford in August, October and February and at Riversdale in August, December and February (Table 4.1). The *catII* gene was detected at an overall frequency of 33.3% along the river (Figure 4.4).

Table 4.1 Detection of antibiotic resistance genes (ARGs) at four sites along the Werribee River at different months. Detection of ARGs along Werribee River at both upstream (Ballan & Bacchus Marsh) and downstream sites (Cobbledicks Ford & Riversdale) at six-time points from April 2015 to February 2016 are shown. Apr, Jun, Aug, Oct, Dec and Feb refer to April, June, August, October, December and February, respectively. The red plus sign (+) indicates the detection of the resistance gene by PCR at each site and month. The minus sign (-) indicates the absence of detection by PCR of the resistance gene at each site and month. At each site, the

total number PCR amplifications performed was 72 (with individual PCR amplification of each of the 12 resistance genes conducted for DNA isolated from each site for each of the six months.

ARGs / Time	Ballan Site						Bacchus Marsh Site						Cobbledicks Ford Site						Riversdale Site					
	Apr	Jun	Aug	Oct	Dec	Feb	Apr	Jun	Aug	Oct	Dec	Feb	Apr	Jun	Aug	Oct	Dec	Feb	Apr	Jun	Aug	Oct	Dec	Feb
^{bla}NDM-1	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	-
mecA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
tet(M)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ampC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VanA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
mcr-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
tet(B)	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	-	-	+	-	-	+
erm(B)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
aac(6')-Ie-aph(2'')-Ia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SulII	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
catII	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	-	+	-	+	+
dfrA1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

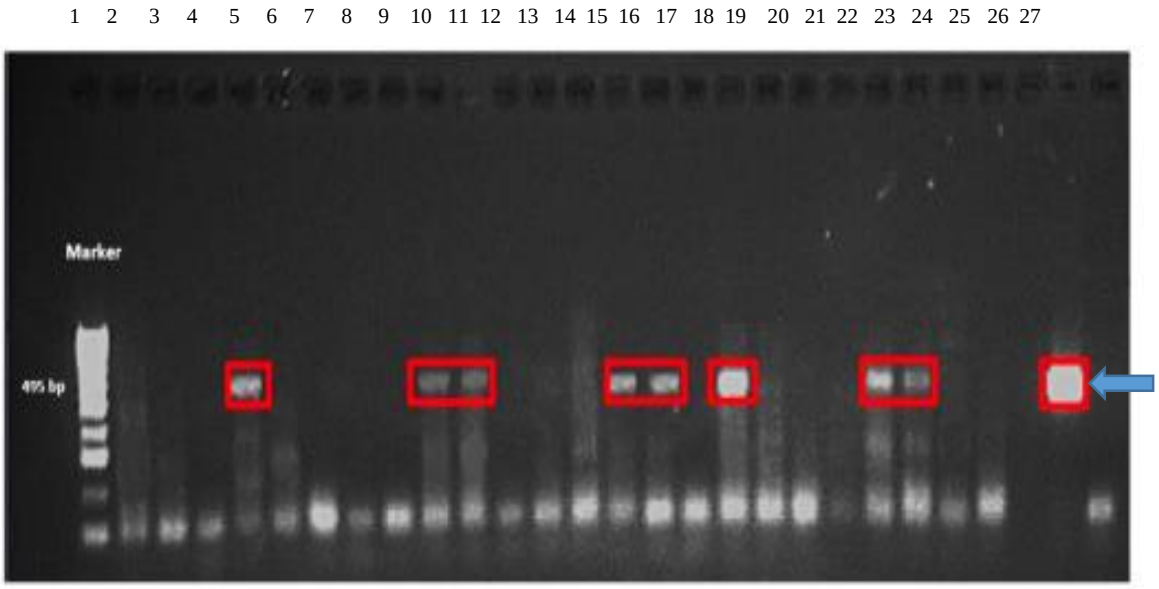


Figure 4.1. Agarose gel electrophoresis of PCR-amplified *catII* antibiotic resistance gene sequences along the Werribee River. Lanes 1-25 are DNA samples from river water (1 RD April, 2-CF April, 3-BM April, 4-BA April, 5-June, 6-CF June, 7-BM June, 8 BA June, 9-RD August, 10-CF August, 11-BM August, 12-BA August, 13-RD October,14 CF October, 15BM October, 16-BA October, 17-RD December, 18-CF December, 19-BM December, 20-BA December, 21-RD February, 22CF February, 23-BM February, 24-BA February); 26 positive control; 27 sterile distilled water, M= hyperladder 100 bp marker. BA=Ballan, BM=BacchasMarsh, CF=CobbledicksFord, RD=Riversdale, AP=April, Jun=June, Aug=August, Oct=October, Dec=December, Feb= February

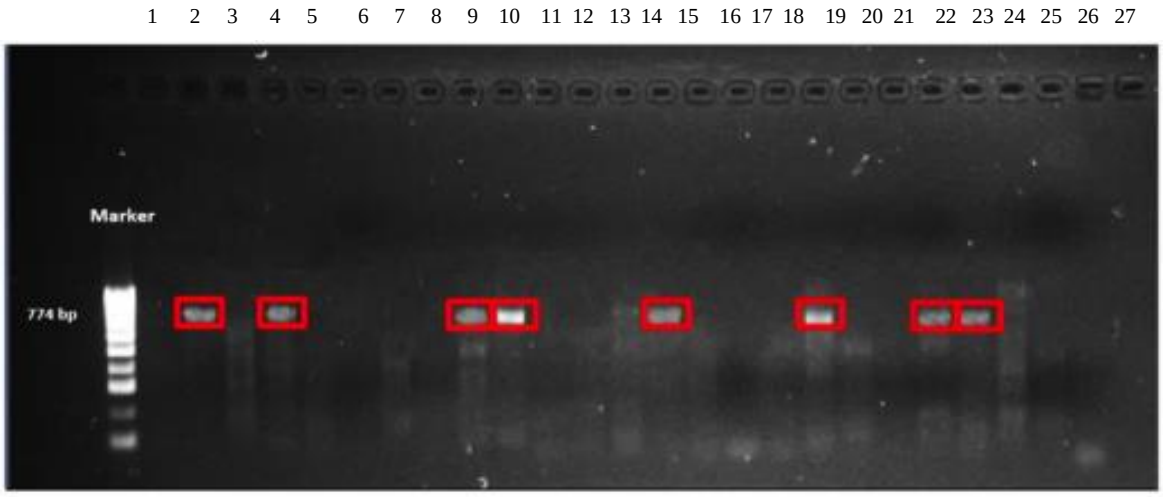


Figure 4.2. Agarose gel electrophoresis of PCR-amplified *tet(B)* antibiotic resistance gene sequences along the Werribee River. Lanes 1-25 are DNA samples from river water (1-RD April, 2-CF April, 3-BM April, 4-BA April, 5-RD June, 6-CF June, 7-BM June, 8-BA June, 9-RD August, 10-CF August, 11-BM August, 12-BA August, 13-RD October, 14-CF October, 15BM October, 16-BA October, 17-RD December, 18-CF December, 19-BM December, 20-BA December, 21-RD February, 22CF February, 23-BM February, 24-BA February) 26 steril distilled water, M= hyperladder100bp marker. BA= Ballan, BM= Bacchas Marsh, CF= Cobbledicks Ford, RD= Riversdale, AP= April, Jun= June, Aug= August, Oct= October, Dec= December, Feb= February.

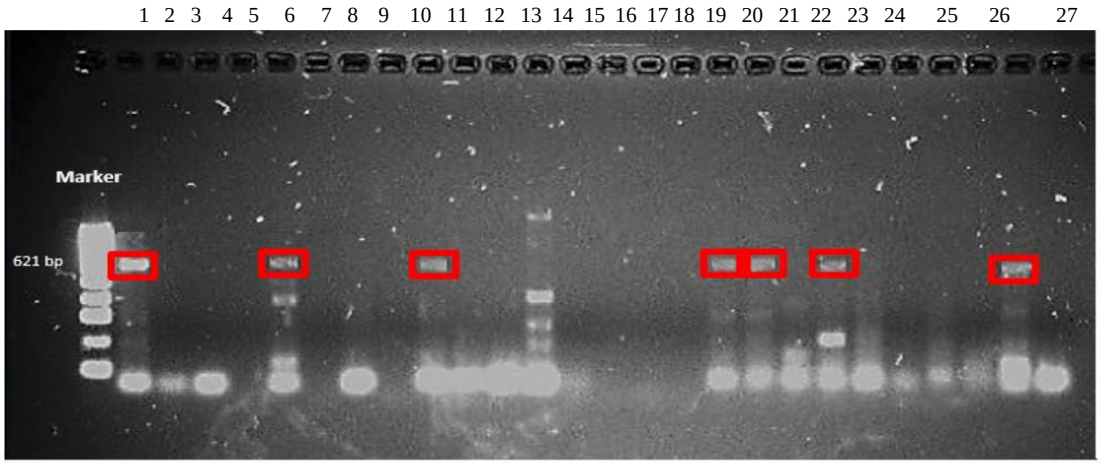


Figure 4.3. Agarose gel electrophoresis of PCR-amplified *bla*NDM-1 antibiotic resistance gene sequences along the Werribee River. Lanes 1-25 are DNA samples from river water (1-RD April, 2-CF April, 3-BM April, 4-BA April, 5-RD June, 6-CF June, 7-BM June, 8-BA June, 9-RD August, 10-CF August, 11-BM August, 12-BA August, 13-RD October, 14-CF October, 15BM October, 16-BA October, 17-RD December, 18-CF December, 19-BM December, 20-BA December, 21-RD February, 22CF February, 23-BM February, 24-BA February); 26 positive control; 27 sterile distilled water, M = hyperladder 100 bp marker. BA= Ballan,BM= BacchasMarsh, CF= Cobbledicks Ford, RD= Riversdale, AP= April, Jun= June, Aug= August, Oct=October, Dec= December, Feb= February

The *tet(B)* antibiotic resistance gene was only detected at Ballan (upstream site) in April and was not detected at Bacchus Marsh at any time point (Table 4.1 and Figure 4.2). However, *tet(B)* genes were more frequently detected in downstream sites namely at Cobbledicks Ford in the months of April, August, October, December and February Ford and at Riversdale in August and October. Figure 4.2 shows the detected *tet(B)* gene band after PCR and visualization using agarose gel electrophoresis. The *tet(B)* gene was detected at an overall frequency of 33.3% along the Werribee River (Figure 4.4).

The third detected ARG was the ^{bla}*NDM-1* gene. This gene was only detected in the upstream site at Ballan in December and was not detected at Bacchus Marsh (Table 4.1). In the downstream sites, ^{bla}*NDM-1* was detected at Cobbledicks Ford in December and at Riversdale in April, June, August and December. This gene was not detected at any site in February (2016) (Table 4.1 and Figure 4.3). Figure 4.3 shows the ^{bla}*NDM-1*gene band after PCR and agarose gel electrophoresis. Overall, the ^{bla}*NDM-1* gene was detected at an overall frequency of 25% along the Werribee River (Figure 4.4).

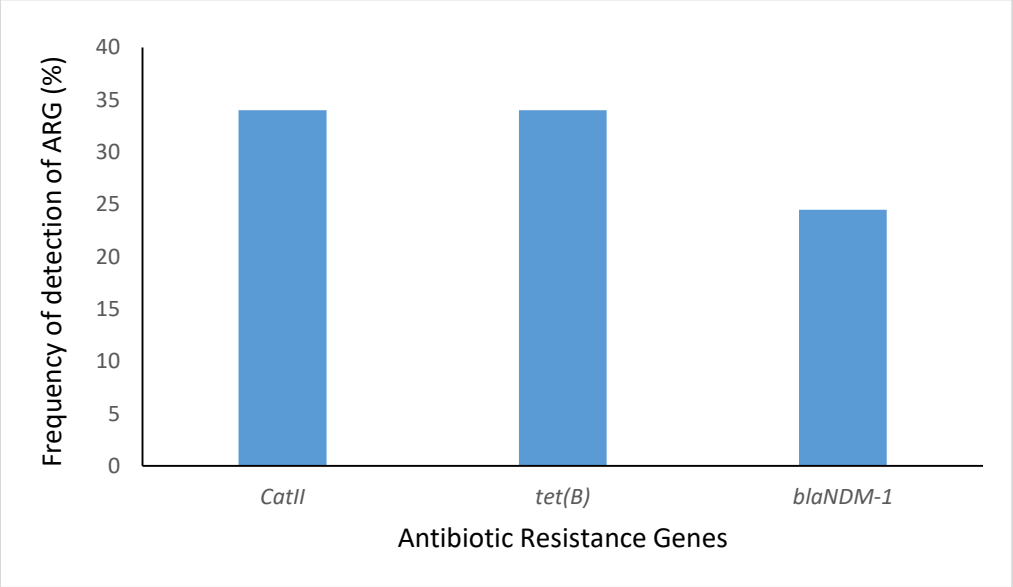


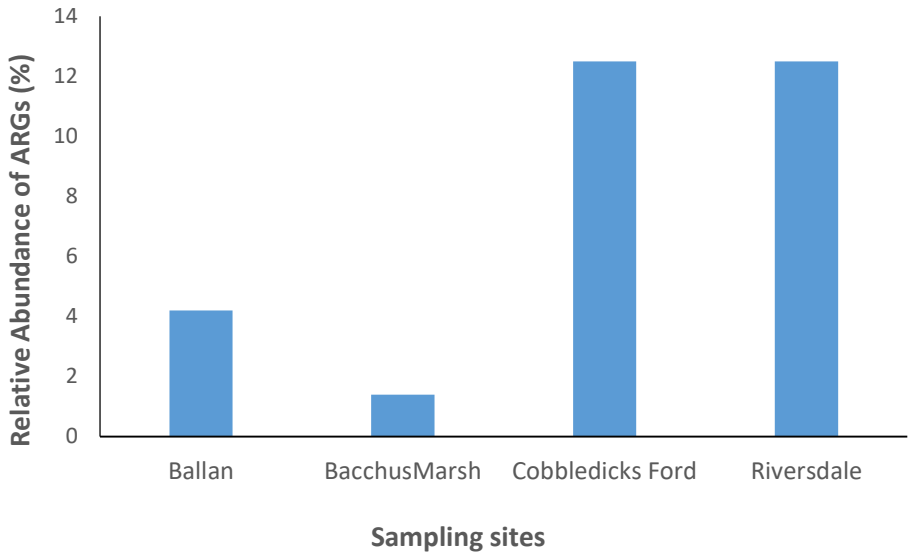
Figure 4.4; Frequency of detection of three antibiotic resistance genes (ARGs) detected across all sites along the Werribee River**.**

4.2.2 Spatial and temporal variation in the frequency of detection (relative abundance) of ARGs between sites and over time along the Werribee River

Spatial and temporal variation in the abundance of ARGs in the Werribee River were assessed by considering the relative abundance (generated from the frequency of detection) of all the ARGs identified in samples obtained from the Werribee River. Spatial variation in the distribution of the three ARGs between the four sampling sites; Ballan and Bacchus Marsh (upstream) and Cobbledicks Ford and Riversdale (downstream) along the Werribee River are shown in Figure 4.5a. The highest frequencies of ARG detection in the river samples were recorded at the downstream sites of Cobbledicks Ford and Riversdale with an overall frequency of 12.5% at both sites. In contrast, ARGs were less frequently detected at the upstream sites; Ballan (4.2%) and Bacchus Marsh (1.4%). Significant variation in the distribution of ARGs across Werribee River between upstream and downstream sites was also observed with upstream sites having a significantly higher frequency of detection of ARGs than at downstream sites ($P < 0.05$).

Temporal variation in the distribution of ARG along the Werribee River between different sampling months is shown in Figure 4.5b. The highest detection frequencies of ARGs along the Werribee River were recorded in August and December (both 10.4%), followed by April and February (both 8.3%) and October (6.3%). The lowest detection frequency of ARGs in the Werribee River was observed in June (2.1%). The frequencies of detection of ARGs in both August and December were significantly higher ($P < 0.05$) than in June.

a



b

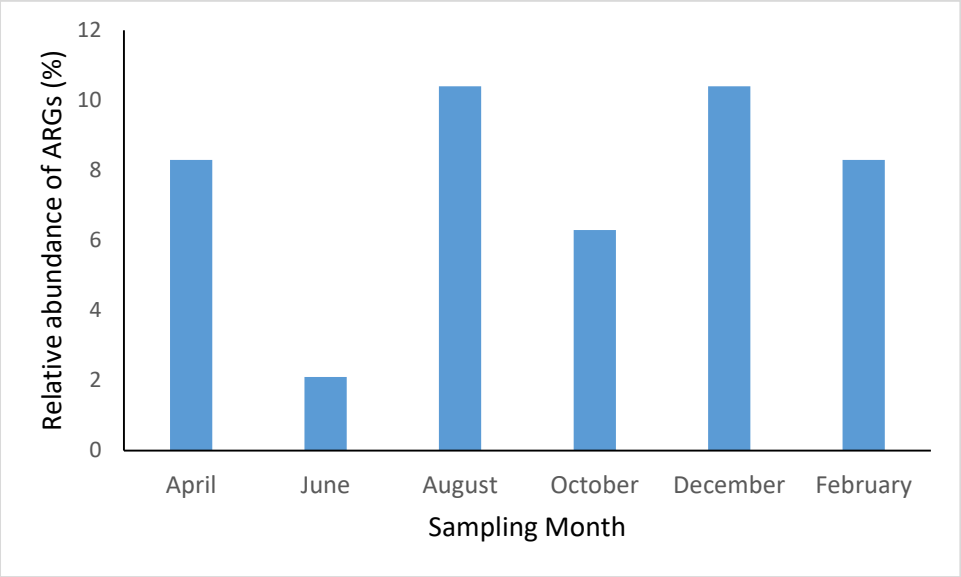


Figure 4.5: Spatial and temporal variation in the frequency of detection of ARGs at a) different sites in the Werribee River and b) over time (different months. Relative abundance refers to the overall abundance of the three ARGs (*catII*, *tet(B)* and ^{bla}*NDM-1*) detected in this study. Ballan and Bacchus Marsh sites are upstream sites while Cobbledicks Ford and Riversdale sites are downstream sites.

Figure 4.6 shows the spatial and temporal variations in ARG distributions over time (months) and between upstream and downstream sites along the Werribee River. At the upstream sites, ARGs were occasionally detected, twice at Ballan (April and December) and once at Bacchus Marsh (October). Overall, ARGs were detected at more time points and a higher frequency of detection in the downstream sites of the Werribee River than in the upstream sites (Figure 4.6). The highest frequency of detection of ARG in the Werribee River was recorded at Riversdale in August (10.4%). At Cobbledicks Ford, ARG detection frequency was the same in August, October, December and February (all at 16.6%) whilst frequencies of detection were lower in April (8.3%) and ARGs were not detected in June. At Riversdale, frequency of detection of ARGs was similarly highest in August, December and February, although in contrast to Cobbledicks Ford, ARGs were not detected at Riversdale in October.

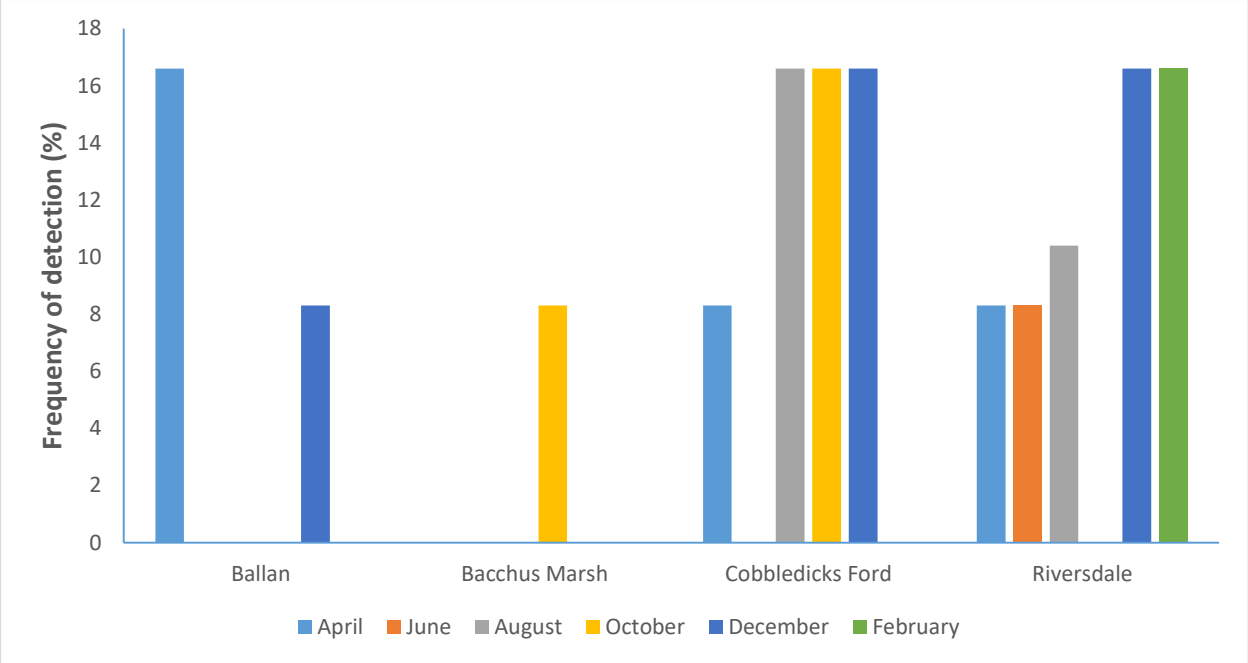
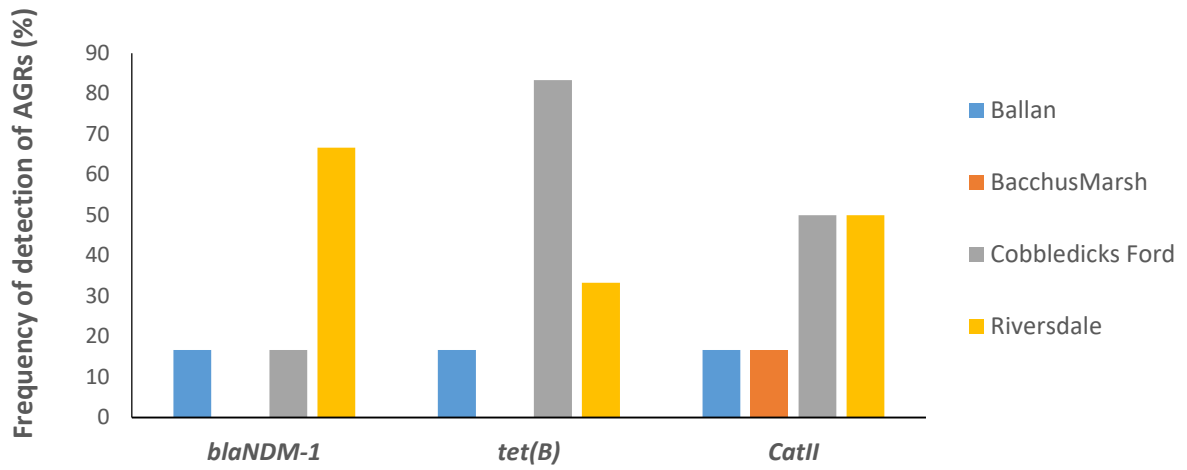


Figure 4.6**.** Spatial and temporal variations in the frequency of detection of at individual sites and in each month along the Werribee River. Ballan and Bacchus Marsh sites are upstream sites while Cobbledicks Ford and Riversdale sites are downstream sites. Months are as indicated in the key

Figure 4.7 shows the variation in the frequency of detection of the three ARGs (*^{bla}NDM-1*, *tet(B)* and *catII*) that were detected in the Werribee River. All three ARGs were detected in the two downstream sites but at Ballan, only *^{bla}NDM-1* and *tet(B)* genes were detected and at Bacchus Marsh, only the *catII* gene was detected. The relative abundance of the *^{bla}NDM-1* genes was highest at the Riversdale site (66.7%) and lowest (where detected) at the second downstream site, Cobbledicks Ford and at the upstream Ballan site (both at 16.7%). In contrast, the *tet(B)* gene was most frequently detected at Cobbledicks Ford (83.3%) and least frequently detected (where detected) at Ballan (16.7%). The *catII* gene was also detected less frequently at the upstream sites (16.7%) compared to the two downstream sites (50%) (Figure 4.7a).

Distinct temporal variation was also observed in the relative abundance (detection and distribution) of these ARG. The *^{bla}NDM-1* gene was most frequently detected in December (75%) with lower frequencies of detection in April, June and August (25%), and it was not detected in October and February. The *tet(B)* gene was most frequently detected in April, August and February (50%) and was detected less frequently at other months except in June when it was not detected at all. The frequency of detection of the *catII* gene was the same and highest in August, October and February (50%) with lowest frequencies of detection, when detected, in April and December (25%) (Figure 4.7b).

a



b

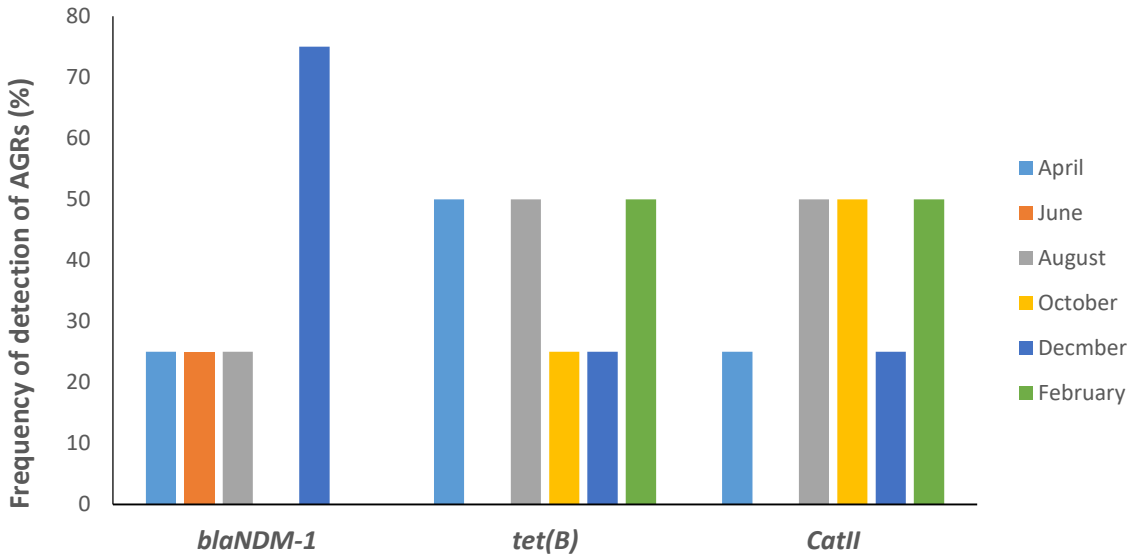


Figure 4.7**.** Spatial and temporal variation in the frequency of detection of the different types of ARGs at (a) different sites in the Werribee River and (b) over time (different months). Ballan and Bacchus Marsh sites are upstream sites while Cobbledicks Ford and Riversdale sites are downstream sites. Sites and months are as indicated in the keys.

4.2.3 Spatial and temporal variation of the abundance of 16S rRNA and antibiotic resistance genes

4.2.3.1 Variation in the spatial and temporal abundance of 16S rRNA genes in the Werribee River

In the previous sections, endpoint PCR was used to assess the frequency of detection and was then expressed as a percentage of the frequency of detection of selected ARGs. In this section, quantitative PCR was applied to explore variation in the abundance of ARGs previously detected by endpoint PCR. Firstly, absolute gene copy numbers of individual ARGs were determined and subsequently, ARG gene abundances were normalised by comparison to the abundance of 16S rRNA genes amplified from each DNA sample. This latter approach was undertaken, in consideration of potential differences in numbers of bacteria (as estimated via Q-PCR of 16S rRNA genes) between individual samples. The results of the analysis of qPCR data are presented in Figures 4.8 to 4.12.

Figure 4.8 shows the variation in the abundance of 16S rRNA genes (ml^{-1} water) between sites and months in water samples from the Werribee River. The 16S rRNA gene numbers varied across the four sampling sites and were lowest at Bacchus Marsh (upstream site) and highest at Riversdale (downstream site) with 16S rRNA gene numbers significantly higher at Riversdale than at Bacchus Marsh ($P \leq 0.05$) (Figure 4.8a). 16S rRNA gene was lowest in April and increased over the sampling period to be highest in February (Figure 4.8b) with significant differences observed only between samples obtained in April and December, April and February, June and February, August and December and October and February ($P \leq 0.05$).

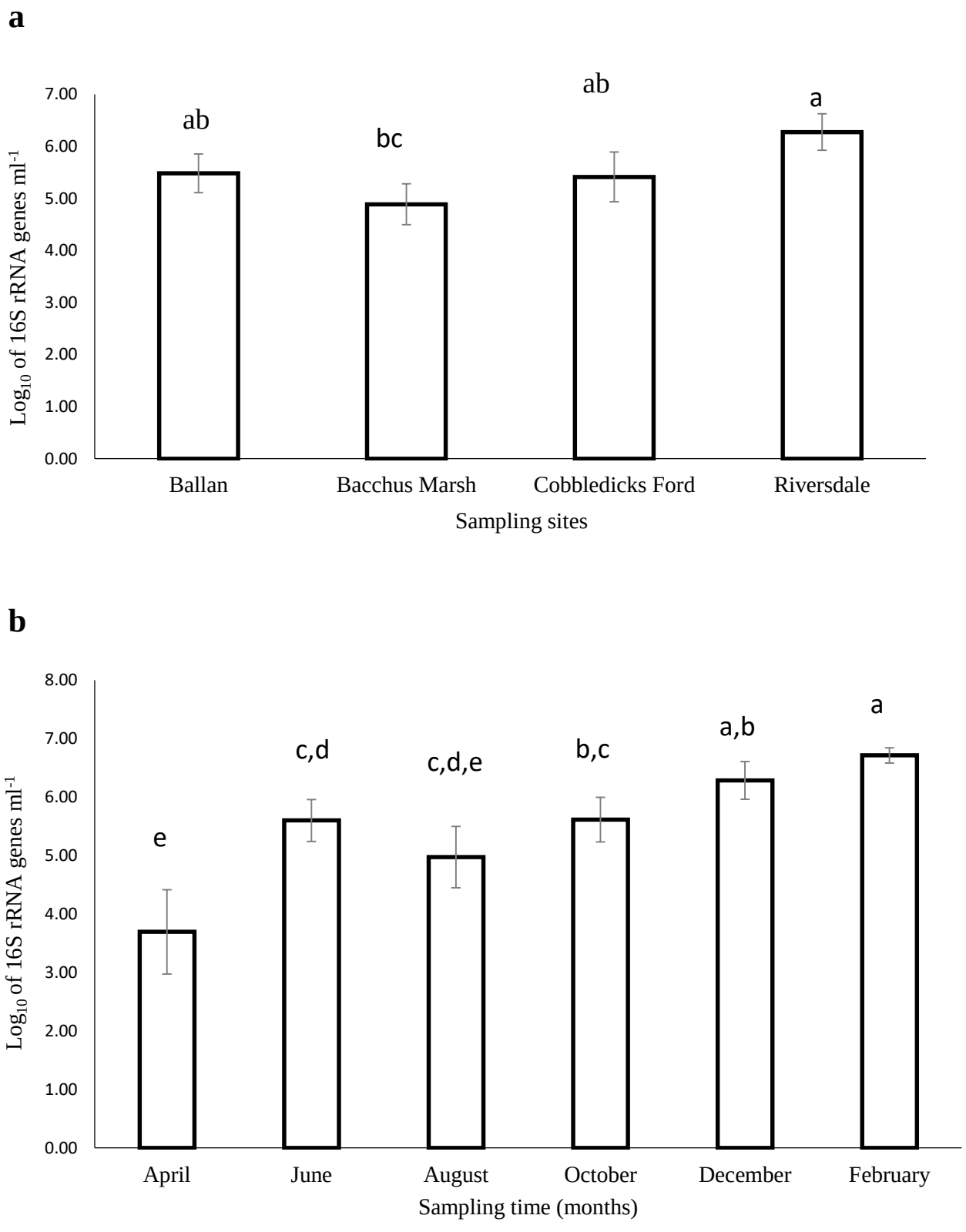


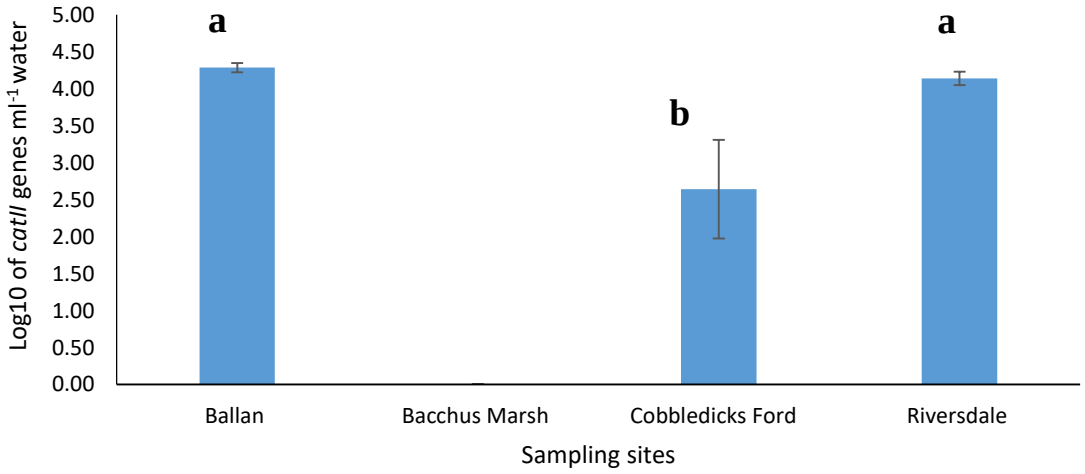
Figure 4.8**.** Spatial and temporal variation in 16S rRNA gene numbers (expressed as Log_{10}) between (a) different sites and (b) months. Ballan and Bacchus Marsh sites are upstream sites while Cobbledicks Ford and Riversdale sites are downstream sites. For (a), significant

differences in 16S rRNA gene numbers were observed between samples (sites) with the different letters ($P \leq 0.05$). For (b), significant differences in 16S rRNA gene numbers were observed between samples (months) with a different letter ($P \leq 0.05$). For sites (panel a), n = 12 (4 sites with 3 replicates each) and for months (panel b), n=18 (6-time points with 3 replicates each)

4.2.3.2 Variation in the spatial and temporal abundance of antibiotic resistance genes in the Werribee River

The *catII* genes were most abundant at Ballan and Riversdale (Figure 4.9a) and numbers of *catII* genes at three sites were both significantly higher than at Cobbledicks Ford ($P \leq 0.05$) whilst *catII* genes were not detected at Bacchus Marsh (Fig 4.10a). The number of c*atII* genes ranged from a $\log_{10}$ count genes ml^{-1} of 3.8 in February to a $\log_{10}$ count of 4.3 in December (where detected). Significant differences in the abundance of *catII* genes abundance were only found when comparing samples obtained in February in which *catII* genes were at a lower gene abundance than in April, August, October and December ($P \leq 0.05$), whilst c*atII* gene was not detected in June (Fig 4.9 b).

(a)



(b)

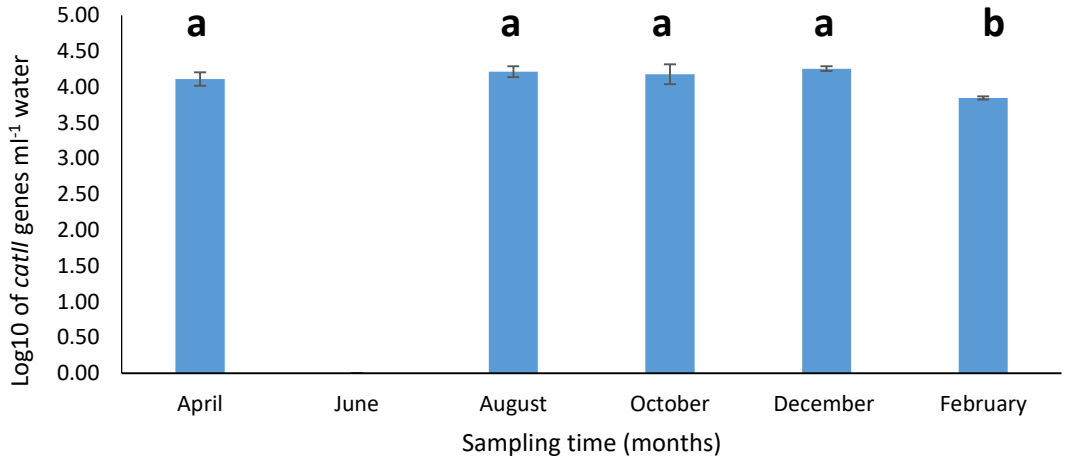
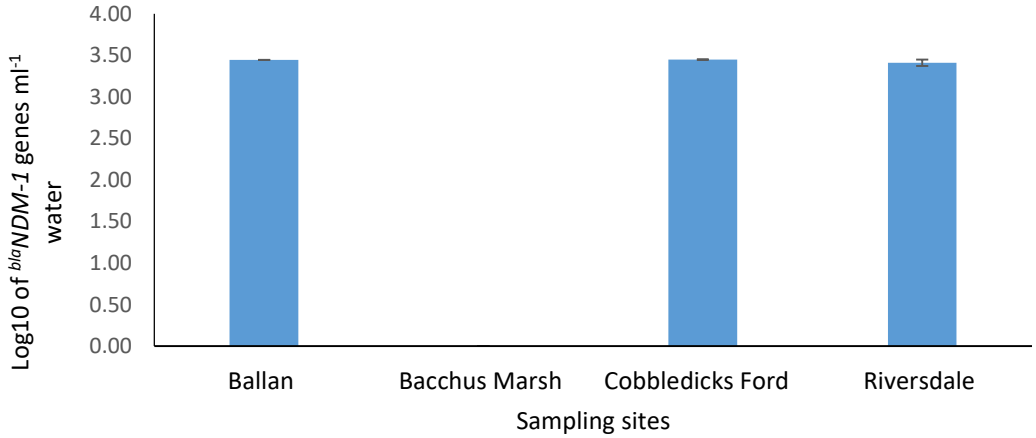


Figure 4.9**.** Spatial and temporal variation in the abundance of c*atII* at (a) different sites and (b) seasons. Note: Ballan and Bacchus Marsh sites are upstream sites while Cobbledicks Ford and Riversdale sites are downstream sites. Significant differences in *catII* gene numbers were observed between samples (site or months) with different letters.

The numbers of ^{bla}*NDM*-1 genes, where detected and quantified, did not vary significantly between sites (Ballan, Cobbledicks Ford and Riversdale; $P \geq 0.05$; Figure 4.10a) or between months, where ^{Bla}*NDM*-1 genes were detected ($P \geq 0.05$; Figure 4.10b).

(a)



(b)

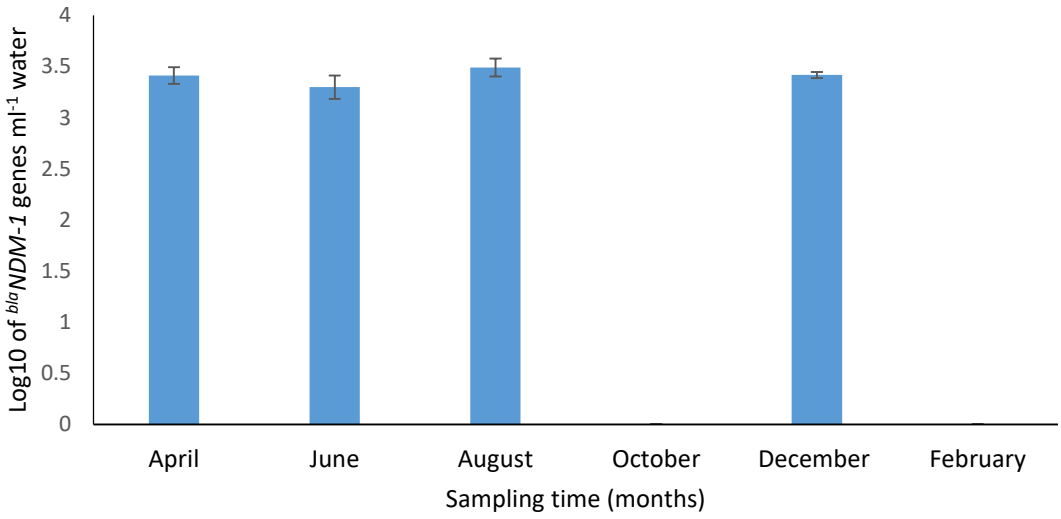
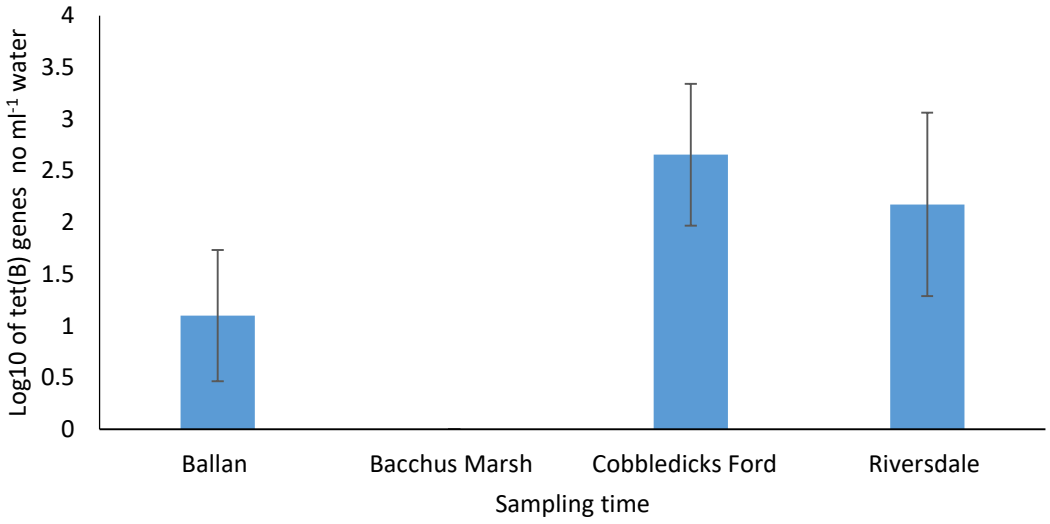


Figure 4.10**.** Spatial and temporal variation in the abundance of ^{bla}*NDM-1* genes at (a) different sites and (b) months. Ballan and Bacchus Marsh sites are upstream sites while Cobbledicks Ford and Riversdale sites are downstream sites. No significant difference was observed between samples from different sites or months ($P \geq 0.05$).

The abundance of *tet(B)* genes was highest, where detected, at Cobbledicks Ford and lowest at Ballan, although differences in *tet(B)* gene numbers were not significantly ($P \geq 0.05$) (Fig. 4.11a). Similarly, the abundance of *tet(B)* genes did not differ significantly between months (in which this gene was detected; $P \geq 0.05$) (Fig. 4.11b).

(a)



(b)

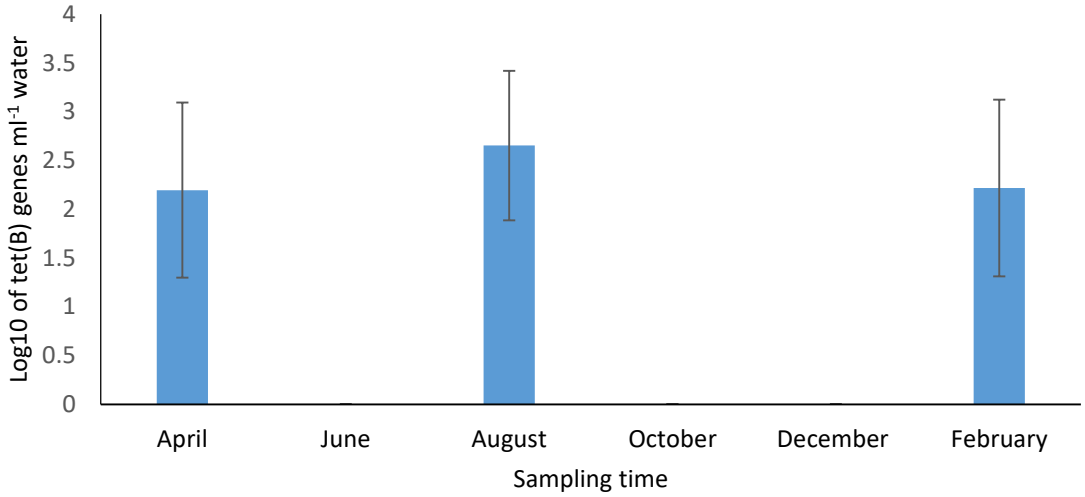
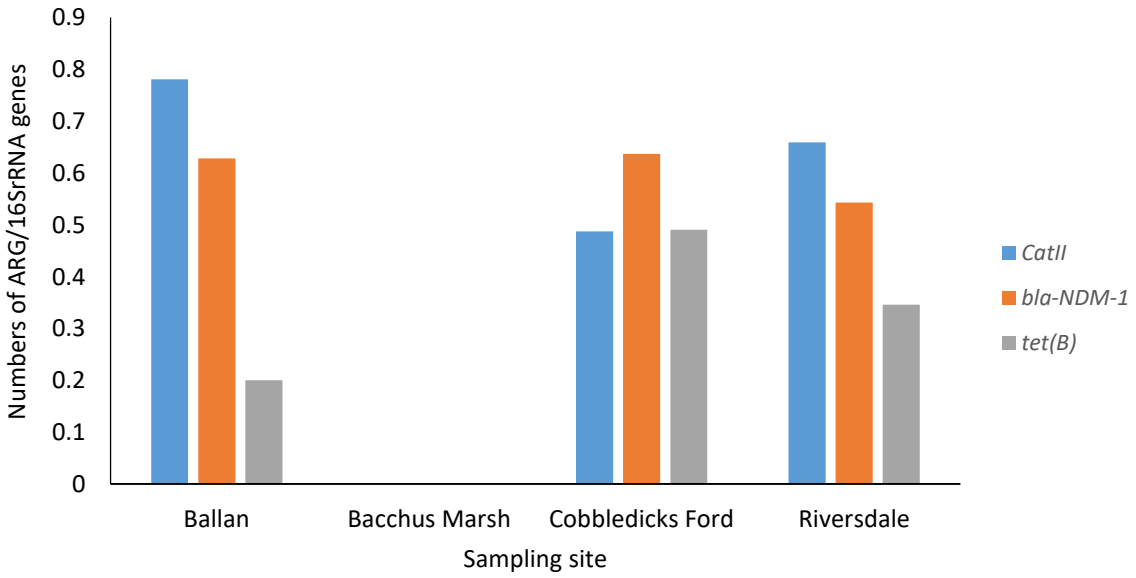


Figure 4.11**.** Spatial and temporal variation in the abundance of *tet(B)* gene copy numbers at (a) different sites and (b) months. Ballan and Bacchus Marsh sites are upstream sites while Cobbledicks Ford and Riversdale sites are downstream sites. No significant difference was observed between samples from either different sites or months ($P \geq 0.05$).

Numbers of ARGs were normalised to the numbers of 16S rRNA genes to take into account any variation between sites in the numbers of bacteria present, using 16S rRNA genes as a proxy for bacterial numbers.

For *catII* genes, their relative abundance (where detected) was highest at Ballan and lowest at Cobbledicks Ford, whilst the relative abundance of ^{bla}NDM-1 genes was the same at Ballan and Cobbledicks Ford and slightly lower at Riversdale. The relative abundance of *tet(B)* genes at Cobbledicks Ford was 2.5 times higher than at Ballan (Figure 4.12a). Considerable temporal variation in the relative abundance of the three genes was observed (Figure 4.12b). Most notably, the relative abundance of *catII* genes declined by approximately 55% between April 2015 and February 2016.

(a)



(b)

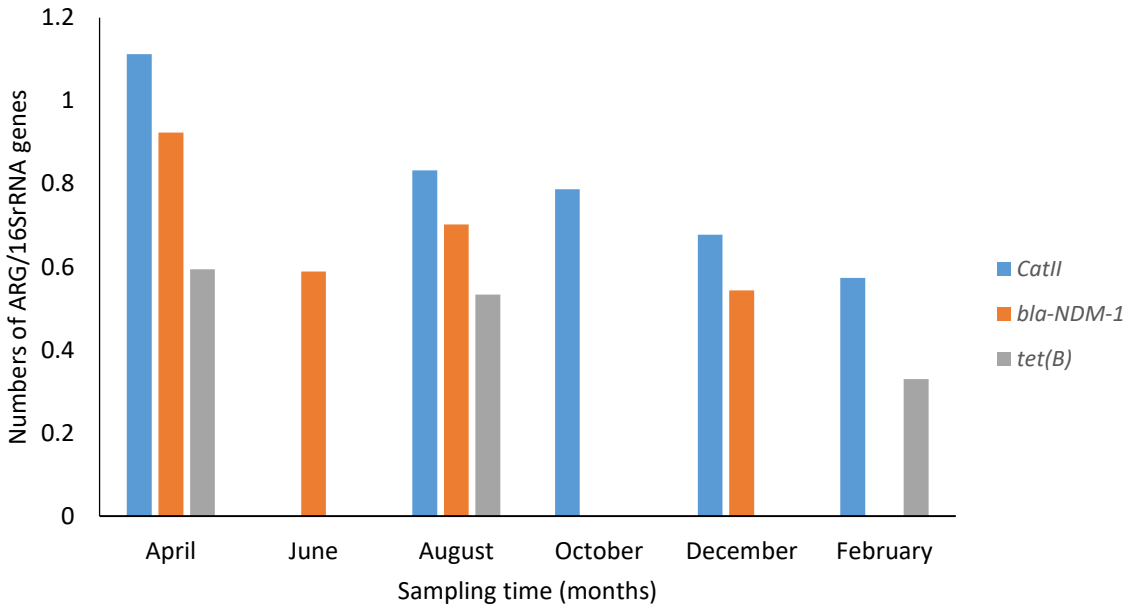


Figure 4.12**.** Relative abundance of *catII,* ^{bla}*-NDM-1* and *tet (B)* genes in the Werribee River at different (a) sites and (b) months. Antibiotic resistance gene numbers were normalised to the numbers of 16S rRNA genes at each site and month. Ballan and Bacchus Marsh sites are upstream sites while Cobbledicks Ford and Riversdale sites are downstream sites

4.2.4 Discussion

The widespread development of antibiotic resistance among pathogenic bacteria mediated by ARG is now considered to be a major threat to public health (Blair et al., 2015). The aquatic environment, which allows dynamic interactions between bacteria can be a major reservoir for ARGs and facilitate the spread of ARGs through horizontal gene transfer (Proia et al., 2016, Rodriguez-Mozaz et al., 2015, Sabri et al., 2020, Xu et al., 2016). These ARGs can also be transferred to human microflora (including pathogens) with adverse health effects (Stoll et al., 2012)). This is why studies such as the one reported here, designed to investigate the presence of antibiotic resistance genes in the aquatic environment (in this case, Werribee River) are important.

In this study, the prevalence of twelve antibiotic resistance genes was determined in surface samples water from the Werribee River from both upstream and downstream sites using PCR detection. These genes were *^{bla}NDM-1, mecA, tet(M), ampC, VanA, mcr-1, tet(B), erm(B), aac (6')-Ie-aph (2'')-Ia, SulII, catII* and *dfrA1*. Three out of twelve ARGs were identified in samples from the Werribee River, a 25% detection rate for the twelve ARGs surveyed. These genes were *catII, tet(B)* and *^{bla}NDM-1*; encoding resistance to chloramphenicol, tetracycline and beta-lactam antibiotics respectively. In a prior study on the Werribee River that targeted five ARGs (methicillin (*mecA*), gentamicin (*aac(3)-I),* vancomycin (*vanA* and *vanB*) and sulfonamide (*sul(I)*), only two ARGs, *mecA* and *sul(I)* genes were detected (Barker-Reid *et al*., 2010) and *mecA* genes were not detected in this current study.

However, unlike the earlier study where the frequency of detection of *mecA* and *sul(I)* genes was very low (~4% for both) (Barker-Reid et al., 2010), the frequency of detection of the ARGs screened in this current study was higher. Frequencies of detection were 25% for *$^{bla-}$NDM-1* and 33.3% for both *tet(B)* and *catII* genes (Figure 3.4) and up to ~70% for *$^{bla-}$NDM-1*, ~85% for *tet(B)* and ~50% for *catII* when detection rates were evaluated by sampling sites (Figure 4.7). This indicated that these three ARGs were more prevalent in the Werribee River than the *mecA* and *sul(I)* genes that had previously been detected in the river by Barker-Reid *et al*. (2010).

The frequency of detection of ARGs in aquatic systems can be affected by anthropogenic activities (and waste input arising from such activities) along the course of any aquatic system (Pruden et al., 2013). Therefore, different river systems may have different prevalent ARGs and varying frequencies for detection of these ARGs. For example, *tetA* and *tetB* have been widely detected in the Pearl River in China at frequencies of up to 43% as a result of sewage input from anthropogenic sources (Tao et al., 2010). High concentrations of ARGs such as those that confer resistance through Streptomycin 3"-adenylyltransferase (*aadA),* beta-

lactamase (*^{bla}TEM*) and the New Delhi Metallo-1 beta-lactams (*NDM1*) in the Cauvery River in India have been linked to hospital wastes contaminating the river (Devarajan et al., 2016).

Sulfonamide and tetracycline ARGs have also been successfully tracked and linked to anthropogenic sources using molecular tools from waste-treatment plants and agricultural activities (Storteboom et al., 2010). ARGs such as *catII* (64%) and *ampC* (36%) were more frequently detected in the Brisbane River when compared to the Rhine River in Germany and reflected greater usage of beta-lactams in humans and in animal husbandry in Brisbane (Stoll et al., 2012). Therefore, high detection rates of ARGs in different environments may be associated with the exposure of such environments to wastes from urban waste-treatment plants, clinical (hospital wastes) and agricultural (use of antibiotics in animals) activities (Shao et al., 2018, Li et al., 2018a, He et al., 2020, Titilawo et al., 2015, Müller et al., 2018). In addition to the type of human activities close to the river, the type of wastes, source of wastes and their entry points into the rivers can also affect the types of ARGs detected and also the location in which they are detected (Xu et al., 2015{Rodriguez-Mozaz, 2015 #143, Pei et al., 2006, Xu et al., 2016)}.

Over the sampling period, substantial spatial and temporal variation in the frequency of detection was observed, with higher frequencies of ARG detection recorded in the downstream sites of Cobbledicks Ford and Riversdale (3-7 fold higher) than in the upstream sites (Ballan and Bacchus Marsh). This finding was not unusual as spatial and temporal variation in ARG detection frequency had been reported in an earlier 12-month study of selected ARGs in the Werribee River (Barker-Reid *et al.*, 2010). The downstream area of Werribee River is used for irrigated horticulture using reclaimed effluent water that itself is rich in ARGs and for intensive animal production (Barker-Reid *et al.*, 2010). Wastes and runoffs from these activities could have played some role in the observed trend of higher ARG frequencies of detection at downstream sites. In addition, the higher frequencies of ARG detection at the downstream sites

could be related to the presence of higher bacterial diversity at the Werribee River downstream sites when compared to upstream sites (see Chapter 3). Higher microbial abundance and diversity could increase the potential for a greater selection of antibiotic resistant groups and horizontal transfer of ARGs leading to more ARGs being detected. Exposure of sections of an aquatic system to runoffs from farms (containing manure and animal waste) using antibiotics has also been shown to increase the frequency of detection of some ARGs (Heuer et al., 2012). Contamination by wastewater from waste-water treatment plants and cattle feedlots and ARG rich sources may also contribute to increased detection of ARGs depending on the content of the wastewater (Schwartz et al., 2003, Kümmerer, 2009, He et al., 2020, Titilawo et al., 2015, Sabri et al., 2020). Multiple studies have reported spatial variation in the abundance of ARG in aquatic ecosystems. Spatial variation in antibiotic (streptomycin and kanamycin) resistance in stream bacteria has also been reported in streams exposed to industrial pollutants (e.g. mercury, tritium, nitrates) (McArthur and Tuckfield, 2000). The resistant bacterial population was substantially higher in the mid-reaches of this polluted stream when compared to other sections and unpolluted streams. The proximity of sampling sites to the source of contamination can also influence spatial variation in the abundance of ARGs. Investigations of the spatial abundance of *sul1*, *tet* and *qnrA* ARGs in Lake Geneva, Switzerland, showed up to a 200-fold higher abundance close to the source of sewage contamination when compared to reference sites in the middle of the lake (Czekalski et al., 2014).

Analysis of temporal ARG distribution data indicated that overall, there were significant variations in their prevalence across both upstream (Ballan & Bacchus Marsh) and downstream (Cobbledicks Ford & Riversdale) sites of the Werribee River in relation to different sampling months (Figures 4.5). The highest prevalence (detection) of ARGs (10%) was during August and December and lowest in June.

Quantitative PCR was used to evaluate the abundance of the three ARGs spatially and over different months. A review of prior literature did not identify prior research that had specifically compared the abundance of ARG and their frequency of detection in aquatic systems. Nevertheless, the results of this study indicated that a higher frequency of detection of ARG may not necessarily translate to a higher abundance of a particular gene. For example, *catII* genes were up to 4.2-fold more abundant at the upstream Ballan site (highest relative abundance recorded amongst all ARGs and sites over time; Figure 4.12a) than at Cobbledicks Ford (a downstream site) despite this gene being more frequently detected at downstream sites (Cobbledicks Ford and Riversdale) (Table 4.1). However, in contrast for the *tet(B)* genes, a higher frequency of detection did translate to a higher relative abundance with downstream sites having up to 2.6-fold more *tet(B)* genes than upstream sites (Fig 4.12b). For $^{bla\text{-}}$*NDM-1* genes, their relative abundance was about the same or slightly lower at the downstream sites when compared to Ballan.

This study also showed substantial spatial and temporal variation in the abundance of the three detected ARGs. The *catII* genes had the highest abundance followed by $^{bla\text{-}}$*NDM-1* genes at Ballan and Riversdale, while $^{bla\text{-}}$*NDM-1* abundance was highest at Ballan and Cobbledicks Ford. The relative abundance of the three ARGs largely decreased from April to February indicating a strong seasonal effect with the abundance of *catII* and *tet(B)* genes in February about half of their relative abundance in April (Figure 4.12b). Studies of the Almendares River in Cuba showed substantial temporal and spatial variation in ARG abundance (*tet M, Q, O* and *W, erm B* and *E* and ^{bla}*TEM*, ^{bla}*SHV* and ^{bla}*OXA-1*). ARG abundance was higher in wet months compared to the dry months (with little or limited rainfall) as a result of high-water flow caused by rainfall and associated greater input of wastes during these months (Knapp et al., 2012). However, greater spatial variation in the abundance of ARG in water columns was observed in the dry season and this was thought to be related to the proximity of waste outfalls into the

river systems (Knapp et al., 2012). In some aquatic systems, the abundance of ARGs can be lower in the wet months when there is a lot of rainfall than in the dry months because of dilution effects in rivers that regularly receive wastes (Yang et al., 2011). In some instances, higher ARG (*sul1*, ^{Bla}*TEM* and *tetA*) abundances in autumn and winter months were observed when compared to other months in some river-based studies (Son et al., 2018). In this particular case, bacterial responses to oxidative and other environmental stress associated with cold weather were thought to be significant drivers for the horizontal gene transfer of ARGs.

Environmental factors, type of pollutant, proximity to a source of contamination and seasonal changes in water flow all appear to play important roles in the spatial and temporal variation in the abundance of ARGs in a river ecosystem. It is possible that some of these factors would have played some role in the variations in ARG abundance observed in this study, but this will require further investigation. Nevertheless, the risk of human exposure to ARGs (*catII, tet(B)* and $^{bla\text{-}}$*NDM-1*) from Ballan was high but only in April and December respectively, whereas in Cobbledicks Ford (August, October, December and February) and Riversdale (April, June, August, December and February) carriage and presence of ARGs in the bacterial communities were consistent over multiple months. Therefore, the risk of exposure to ARGs was higher and present across more months at downstream sites when compared to upstream sites.

This study has demonstrated the widespread prevalence of three ARGs, $^{bla\text{-}}$*NDM-1*, *tet(B)* and *catII* qualitatively and quantitatively in the Werribee River. Substantial spatial and temporal variation was observed with the frequency of detection of ARGs higher at downstream sites (Cobbledicks Ford and Riversdale) than at upstream sites of Ballan and Bacchus Marsh. However, this high frequency of detection was only associated with higher relative gene abundance for *tet(B)* genes (through qPCR). In contrast, the *catII* gene was infrequently detected but present at a higher abundance at Ballan when compared to other sites. The next chapter Chapter 5) will utilize molecular (next-generation sequencing) approaches to

investigate the diversity and likely taxonomic affiliation of antibiotic resistance genes from within the river.

5 Chapter 5: Next-generation sequencing analysis of PCR-amplified antibiotic resistance genes from the Werribee River

5.1 Introduction

The over-prescription, over-use and misuse of antibiotics for human and veterinary medical applications can result in the introduction of antibiotics into the natural environment at elevated concentrations. This means that soils, surface and underground water systems can become contaminated with antibiotics from sewage (Rodriguez-Mozaz et al., 2015), farm runoffs (Storteboom et al., 2010)and farm manure used as fertilizers (Heuer et al., 2011). The presence of antibiotics in these environments selects bacterial groups resistance to the contaminating antibiotics with this resistance being mediated by antibiotic resistance genes or ARGs. These ARGs can also be transferred to other bacterial groups through horizontal gene transfer, thereby spreading antibiotics resistance in the environment (Marti et al., 2014). Although antibiotic resistance can occur naturally in bacteria (Olivares Pacheco et al., 2013), environmental contamination by antibiotics from anthropogenic origins substantially contributes to the increased levels of antibiotics resistance presently observed in some human pathogens (Marti et al., 2013, Marti et al., 2014)

Aquatic systems such as lakes and surface rivers and sediments which are easily contaminated by wastes and run-offs can be reservoirs of antibiotic resistance genes, providing conducive environments for the dissemination of antibiotics resistance factors to aquatic microorganisms. The type of contaminating antibiotics determines the range of ARGs detected in the environment. Multiple studies have demonstrated the presence of different ARGs such as *tet, sul, bla-TEM, ermB* and *qnrS* in different aquatic systems (Amos et al., 2014, Rodriguez-Mozaz et al., 2015, Marti et al., 2013, Rowe et al., 2016, Marti et al., 2014). Antibiotic-containing wastes can also pass through soils and contaminate groundwater systems resulting in ARGs being subsequently detected in groundwater samples (Böckelmann et al., 2009). For example, lagoon seepages from swine farms can pass through the soil layers, contaminating

the aquifer under these farms, with the detected *tet(M)* ARG in these aquifer samples identical to the ones detected in the surface lagoons (Chee-Sanford et al., 2001).

Assays for antibiotic resistant microorganisms can be carried out using culture-dependent techniques. This involves bacterial isolation and subsequent evaluation of the growth of these isolates in the presence of specific antibiotics and at different antibiotic concentrations. This allows for the determination of the sensitivity or resistance of isolates to the target antibiotics using broth, agar dilution, agar disk diffusion and E-test-based assays (McLain et al., 2016). However, this approach is limited by the fact that the assays can only be performed on culturable isolates, while a vast number of non-culturable but viable and antibiotic resistant bacteria remains untouched. The process can be labour intensive and repeated culturing of isolates on non-selective media can lead to loss of antibiotic resistance in some isolates over time. This has led to concerns about the validity of the results of culture-based antibiotics resistance assays performed on bacterial isolates from long-term storage (Ludwig et al., 2012).

The application of culture-independent molecular methods allows for a more comprehensive study of antibiotic resistant culturable and non-culturable bacterial population. Different molecular approaches such as PCR-cloning, PCR-DGGE, and Next Generation Sequencing methods have been utilized to assess the diversity of antibiotic resistance genes (bacteria) in soils, rivers and groundwater. However, sometimes a molecular approach is combined with culture-dependent approaches for a more detailed screening exercise. For example, clone libraries have been constructed by cloning soil DNA fragments into different host-range expression vectors such as pCF430, PBeloBAC11 and pJN105 (encoding for tetracycline, kanamycin and gentamicin resistance respectively), with clones from these libraries screened for resistance to multiple antibiotics. After screening in liquid media, clones resistant to apramycin, butirosin, kanamycin and tetracycline were detected (Riesenfeld et al., 2004). BAC

libraries have been constructed from DNA fragments, screened on LB agar plates with clones' resistant to chloramphenicol being identified (Koike et al., 2017).

In addition to qPCR, Next Generation Sequencing (NGS) approaches have been successfully used to evaluate the diversity and concentration of ARGs in aquatic systems usually using a DNA shotgun sequencing approach. A 2016 study of ARGs in the River Cam Catchment in Cambridge, U.K. was conducted using an NGS based approach. In this case, extracted DNA from water samples (effluents from WWTP flowing into the river) was subject to shotgun sequencing. The obtained metagenomic reads were then subject to bioinformatic analyses with ARGs identified using the Search Engine for Antimicrobial Resistance and appropriate online databases such as ARDB and NCBI GenBank. An array of genes was identified with *tetC, tetW* and *sul2* being the most abundant ARGs in the analysed samples (Rowe et al., 2016). A pyrosequencing approach has also been applied to the characterization of ARGs such as *qnr* (quinolines), *sul* (sulphonamide) and *strA* (streptomycin) in sediments of antibiotics contaminated river in Hyderabad, India (Kristiansson et al., 2011).

The previous result chapters have described the structure and diversity of bacterial communities (Chapter 3) and the prevalence of ARGs in surface water samples (Chapter 4) from four different sites along the Werribee River. Three ARGs, ^{bla}*NDM1*, *catII* and *tet(B)* were detected in these samples. A review of the literature shows that these ARGs have been detected in wastewater, sewage, natural and drinking water systems from different parts of the world (Zhang et al., 2009). For example, a combination of a culture-based approach and qPCR was applied to the study of the prevalence of NDM, VIM and OXA genes in German surface water and wastewater and *NDM* genes were detected in clinical wastewater (Müller et al., 2018) and can potentially contaminate or be disseminated in any wastewater receiving aquatic systems. ^{bla}*NDM1* genes have also been detected in contaminated rivers in Hanoi, Vietnam (Isozumi et al., 2012) and in the Ganges River in India (Ahammad et al., 2014) using a

combination of culture and PCR-based approaches. Similarly, *tet(B)* genes have been quantified using qPCR in river systems polluted by livestock wastes in Zhaodong, China ((Li et al., 2018b). Sections of the Poudre River, in Colorado (USA) that are impacted by urban and agricultural activities, were observed to have higher concentrations of *tet* ARGs (based on qPCR assays) compared to pristine sections in which no *tetB* genes were detected (Pei et al., 2006). Investigations of surface water and sediment samples from seawater and lakes have revealed the presence of *cat* genes in these samples (Dang et al., 2008, Dong et al., 2019).

Investigations of the prevalence and diversity of ARGs in environmental systems conducted with next-generation sequencing typically involve the use of shotgun sequencing approaches after which the data is analysed for the presence of ARGs of interest (Li et al., 2015, Guo et al., 2018, Wang et al., 2018a). Targeted (gene-based) next-generation amplicon sequencing has been successfully applied to 16S rRNA genes for bacteria and Internal Transcribed Spacer regions for fungi (Sinclair et al., 2015, Vaz et al., 2017) providing more detailed and specific information on bacterial taxa (up to species level) than the typical shotgun sequencing approach. However, this approach has not been readily applied to the study of antibiotic resistance genes in the environment.

Therefore, this part of this research sought to assess the potential suitability of an amplicon-based next-generation sequencing approach for the study of genetic variation within specific antibiotic resistance genes in surface water samples from the Werribee River. The application of this approach would seek to determine whether there was variation in these antibiotic resistance genes either between different locations and/or between genes amplified at different times. For each gene, the hypothesis was proposed that gene sequences for the selected ARGs (detected previously by PCR) varied between different sites along the river and/or over time. If supported, this hypothesis would suggest diversity exists within individual resistance genes within the wider antibiotic resistome that is present in the Werribee River.

Scientific investigations were carried out using water samples from the Werribee River from which the antibiotic resistance genes (*catII* and ^{bla}*NDM1)* had been amplified. Primers targeting *catII* and ^{bla}*NDM1* ARGs were modified by the attachment of Illumina sequencing linker sequences after which they were used to generate amplicons from selected samples, which were sequenced on an Illumina MiSeq instrument. These primers had been used in earlier studies (Chapter 4) in PCR and qPCR-based assays. Blast analysis of the sequences obtained from the Werribee River was carried out to determine the likely relationship of these antibiotic resistance genes to those from sequences in the National Center for Biotechnology Information (NCBI).

5.2 Results

PCR-amplified antibiotic resistance genes from the Werribee River were sequenced to investigate their diversity and relationship to those from previously characterized resistance genes. Five selected samples were subject to targeted amplicon-based NGS sequencing. PCR amplicons of *catII* genes from Ballan, Bacchus Marsh, Cobbledicks Ford and Riversdale genes, together with ^{BLA}*NDM1* amplicons from Riversdale were analysed.

Table 5.1 shows the sequencing run statistics of the amplicon-based NGS carried out in this study. A total of 55,020,592 reads were obtained from the sequencing run which corresponded to approximately 14.5 Gb of data. Out of these reads, 47,196,880 reads or approximately 87% of the reads passed the pre-set quality filter and ~68% of the reads had a Phred score of ≥ Q30 (Table 5.1).

Table 5.1. Illumina MiSeq amplicon sequence run statistics of *catII* and ^{bla}*NDM1* antibiotic resistance gene amplicons in selected samples.

Total Reads	**Reads passed filter**	**% ≥[a]Q30**	**Yield (Gbp)**	**Aligned to PhiX Control (%)**	**Error of Aligned Phix control**
55,020,592	47,196,880	67.67	14.49	7.72	3.23

[a]Q30= Base call accuracy of 99.9%

The selected sequences were analysed using the FunGene pipeline (Fish et al., 2013) to investigate the relationships of the PCR-amplified sequences to those of previously characterised antibiotic resistance genes from reference sequences which have been curated as a dataset for comparison purposes. Following the translation of DNA sequences to protein

sequences, multiple different protein sequences were identified with varying relative abundances of each sequence type for each gene and/or at each site.

For *catII* gene sequences, the relative abundance of the ten most abundant sequence types is presented in Table 5.2. This relative abundance for *catII* genes ranged from 6.3% in sequence type BA10 to 20.72% in sequence type BA 1 at Ballan site. At Bacchus Marsh site, the sequence type BM10 had the lowest abundance of 4.5% while sequence type BM1 with 28.3% was the most abundant. Similarly, at Cobbledicks Ford and Riversdale sites, sequence types CF10 and RD10 at 3.3% and 2.6% respectively were the least abundant sequence types. At these two sites, CF1 and RD1 at 26.8% and 32.6% respectively, were the most abundant sequence types (Table 5.2).

Using the FunGene pipeline, *catII* sequences from the most abundant sequence types were then translated into their protein equivalents and compared to a library of chloramphenicol acetyl transferase (cat) proteins using FunGene. For the sample from Cobbledicks Ford, Seq CF1, having the highest relative abundance (29.8%) (Table 5.2) was used for this analysis. The most abundant protein sequences were related to chloramphenicol O-acetyltransferase genes (Table 5.3) from Gammaproteobacteria and in particular, to *catII* protein sequences in *Enterobacteriaceae* (*Escherichia coli* or *Klebsiella pneumoniae*) isolates with identities of >90% (Table 5.3). Figure 5.1 shows an alignment of the translated *catII* gene sequence from CF1 to its closest related sequence (*Escherichia coli*).

Table 5.2: The relative abundance of the ten most frequent *catII* gene sequence types across the four sites (Ballan, Bacchus Marsh, Cobbledicks Ford and Riversdale) along the Werribee River.

Sequence type	***catII* genes % (Ballan)**	**Sequence type**	***catII* genes % (Bacchus Marsh)**	**Sequence type**	***catII* genes % (Cobbledicks Ford)**	**Sequence type**	***catII* genes % (Riversdale)**
BA1	20.72	BM1	28.27	CF1	29.77	RD1	32.58
BA2	13.36	BM2	18.71	CF2	21.04	RD2	16.14
BA3	12.57	BM3	11.26	CF3	13.28	RD3	13.28
BA4	9.10	BM4	8.74	CF4	9.55	RD4	10.58
BA5	8.82	BM5	7.33	CF5	6.27	RD5	6.70
BA6	8.21	BM6	6.17	CF6	5.30	RD6	5.65
BA7	7.60	BM7	5.60	CF7	4.58	RD7	5.39
BA8	6.85	BM8	4.79	CF8	3.47	RD8	3.52
BA9	6.46	BM9	4.60	CF9	3.44	RD9	3.30
BA10	6.31	BM10	4.51	CF10	3.31	RD10	2.85

Table 5.3: Similarity of the translated *catII* gene sequence from Cobbledicks Ford (CF1 sequence) to Cat proteins from Proteobacteria.

Related sequence	Accession number	Identity (%)
Type A chloramphenicol O-acetyltransferase [*Escherichia coli]*	WP_072093947.1	91
Type A chloramphenicol O-acetyltransferase [*Klebsiella pneumoniae*]	WP_074185479.1	91
MULTISPECIES: type A-2 chloramphenicol O-acetyltransferase CatII [*Gammaproteobacteria]*	WP_001011939.1	91
Type A chloramphenicol O-acetyltransferase [*Klebsiella pneumoniae*]	WP_096925670.1	90
Type A chloramphenicol O-acetyltransferase *[Morganella morganii]*	WP_123568436.1	88
MULTISPECIES: type A-2 chloramphenicol O-acetyltransferase CatII [*Proteobacteria*]	WP_012477888.1	88
Type A chloramphenicol O-acetyltransferase [*Vibrio vulnificus]*	WP_103307714.1	88
Type A chloramphenicol O-acetyltransferase [*Morganella morganii]*	WP_123568436.1	88
Type A-2 chloramphenicol O-acetyltransferase CatII *[Aeromonas salmonicida]*	WP_032490795.1	87
Type A chloramphenicol O-acetyltransferase [Escherichia coli	WP_101135477.1	87

CF1 1 WNRREHFALYRQQIKCGFSLTTKLDITALRTALAKTGYKFYPLMIYLISRAVNQFPXFRM 60
WNRREHFALYRQQIKCGFSLTTKLDITALRTALAKTGYKFYPLMIYLISRAVNQFP FRM
E coli WNRREHFALYRQQIKCGFSLTTKLDITALRTALAKTGYKFYPLMIYLISRAVNQFPEFRM 70

CF1 61 AMKDNELIYWEQSDPVFTVFHKETEXXSAXXXRYXXXLS 99
AMKDNELIYWEQSDPVFTVFHKETE SA RY LS
E coli 71 AMKDNELIYWEQSDPVFTVFHKETETFSALSCRYFPDLS 109

Figure 5.1: Sequence alignment of translated *catII* amplicon sequence from Cobbledicks Ford (CF1 sequence) to the most closely related Cat protein sequence from *Escherichia coli* (accession number WP_072093947.1).

FunGene analysis did not identify any similarities between the translated *catII* sequences from Ballan, Bacchus Marsh and Riversdale to previously characterised cat proteins. Therefore, the *catII* sequences from these sites were converted to protein sequences using ORF-finder and analysed by BLASTP with comparison to the Ref-Seq database in the NCBI. Protein sequences of translated *catII* genes from Riversdale and Bacchus Marsh were found to be most closely related to the acetyl-Co-A-transferase sequences from multiple *Polynucleobacter* sp. (Table 5.4) rather than to chloramphenicol acetyl-Co-A transferase sequences. Other bacterial groups that this transferase was also detected in include *Polynucleobacter duraquae*, *P. acidiphobus, P. sinensis* and *P. campilacus* at Riversdale (Table 5.4). At Baccus Marsh, this enzyme was also present in related bacterial groups such as *Polynucleobacter difficilis, P duraquae, P. acidiphobus, P. sinensis* and *P. campilacus* (Table 5.5). Translated *catII* gene sequences from the Ballan sampling site from Werribee River were most closely related to those of a different enzyme, namely 3-oxoadipyl-CoA thiolase from *Hydrogenophaga* sp (Table 5.6). Sequence alignments carried out on these samples are shown in Figures 5.2 to 5.4.

Table 5.4: Similarity of translated *catII* gene sequences in samples from Riversdale (RD1 sequence) to acyltransferase (acetyl-CoA C-acyltransferase).

Related sequence	Accession number	Identity (%)
acetyl-CoA C-acyltransferase [*Polynucleobacter difficilis*]	WP_108469024.1	92
acetyl-CoA C-acyltransferase [*beta proteobacterium CB*]	WP_015421096.1	92
acetyl-CoA C-acyltransferase [*Polynucleobacter duraquae*]	WP_046330162.1	90
acetyl-CoA C-acyltransferase [*Polynucleobacter acidiphobus*]	WP_108509336.1	90
acetyl-CoA C-acyltransferase [*Polynucleobacter sinensis*]	WP_062308706.1	92
acetyl-CoA C-acyltransferase [Polynucleobacter campilacus]	WP_088526134.1	90
acetyl-CoA C-acyltransferase [*Polynucleobacter sp. UB-Domo-W1*]	WP_100378527.1	89
acetyl-CoA C-acyltransferase [*Polynucleobacter sphagniphilus*]	WP_076023320.1	90
acetyl-CoA C-acyltransferase [*Polynucleobacter aenigmaticus*]	WP_088527615.1	90
MULTISPECIES: acetyl-CoA C-acyltransferase [*Polynucleobacter*]	WP_112294655.1	88

Table 5.5: Similarity of translated *catII* gene sequences in samples from Bacchus Marsh (BM1 sequence) to acyltransferase (acetyl-CoA C-acyltransferase).

Related sequence	Accession number	Identity (%)
acetyl-CoA C-acyltransferase [*Polynucleobacter difficilis*]	WP_108469024.1	92
acetyl-CoA C-acyltransferase [*beta proteobacterium CB*]	WP_015421096.1	92
acetyl-CoA C-acyltransferase [*Polynucleobacter duraquae*]	WP_046330162.1	90
acetyl-CoA C-acyltransferase [*Polynucleobacter acidiphobus*]	WP_108509336.1	90
acetyl-CoA C-acyltransferase [*Polynucleobacter sinensis*]	WP_062308706.1	92
acetyl-CoA C-acyltransferase [*Polynucleobacter campilacus*]	WP_088526134.1	90
acetyl-CoA C-acyltransferase [*Polynucleobacter sp. UB-Domo-W1*]	WP_100378527.1	89
acetyl-CoA C-acyltransferase [*Polynucleobacter sphagniphilus*]	WP_076023320.1	92
acetyl-CoA C-acyltransferase [*Polynucleobacter aenigmaticus*]	WP_088527615.1	90
MULTISPECIES: acetyl-CoA C-acyltransferase [*Polynucleobacter*] *yangtzensis*	WP_112294655.1	88

Table 5.6: Similarity of translated *catII* gene sequences in samples from Ballan (BA1 sequence) to acyltransferase (3-oxoadipyl-CoA thiolase).

Related sequence	Accession number	Identity (%)
3-oxoadipyl-CoA thiolase [*Hydrogenophaga sp. IBVHS2*]	WP_086118994.1	84
MULTISPECIES: 3-oxoadipyl-CoA thiolase [*Hydrogenophaga*]	WP_009518871.1	80
3-oxoadipyl-CoA thiolase [*Burkholderiales bacterium JOSHI_001*]	WP_009553073.1	81
3-oxoadipyl-CoA thiolase [*Comamonas granuli*]	WP_042429075.1	83
3-oxoadipyl-CoA thiolase [*Polaromonas sp. CF318*]	WP_007863120.1	80
3-oxoadipyl-CoA thiolase [*Hydrogenophaga sp. LA-38*]	WP_116957729.1	80
3-oxoadipyl-CoA thiolase [*Aquincola tertiaricarbonis*]	WP_046110924.1	79
3-oxoadipyl-CoA thiolase [*Polaromonas sp. CG9_12*]	WP_036812480.1	80
3-oxoadipyl-CoA thiolase [*Hydrogenophaga sp. PML113*]*palleronii*	WP_070398036.1	80
3-oxoadipyl-CoA thiolase [*Methylibium sp. CF059*]	WP_047489147.1	79

```
RD1 3    TAENLAEKWKITREEQDAFAVESHRRAALAIKEGRFKSQIVPITIKTRKGDVVFDTDEHC 62
         TAENLAEKWK+TREEQDAFAVESHRRAA+AIKEGRFKSQIVPITIK+RKGDVVFDTDEHC
PD* 161 TAENLAEKWKLTREEQDAFAVESHRRAAVAIKEGRFKSQIVPITIKSRKGDVVFDTDEHC 220
RD1  63   KPDTTMETLAKFQ  75
          KPDTTMETL+K +
PD* 221  KPDTTMETLSKMK  233
```

***PD refers to *Polynucleobacter difficilis*

Figure 5.2: Sequence alignment of translated *catII* amplicon sequences from Riversdale (RD1 sequence) to the most closely related Cat protein sequence from *Polynucleobacter difficilis* (accession number WP_108469024.1).

```
BM1 3    TAENLAEKWKITREEQDAFAVESHRRAAL  AIKEGRFKSQIVPITIKTRKGDVVFDTDEHC
62
         TAENL EKWK+TREEQDA  AVESHRRAA    AIKEGRFKSQIVPITIKTRKGDVVFDTDEHC
CB* 161  TAENLVEKWKLTREEQDALAVESHRRAAHAIKEGRFKSQIVPITIKTRKGDVVFDTDEHC
220

BM1 63  KPDTTMETLAKFQ  75
         KPDTTMETLAK +
CB* 221  KPDTTMETLAKMK  233
```

***CB refers to *Proteobacterium* CB

Figure 5.3: Sequence alignment of translated *catII* amplicon sequence from Bacchus Marsh (BM1sequence) to the most closely related Cat protein sequence from Beta Proteobacterium (accession number WP_108469024.1).

```
BA 1     PGTAENVATDHKIEREAQDRMALSSQLKAVAAQKAGYLANEITPVSIAQKKGDPLLVSQD  60
         PTAENVA  TDH  IEREAQDRMAL+SQ+KAVAAQKAG +LA  EI  PVSI QKKGDPLLVS  D
HS*164   PETAENVATDHGIEREAQDRMALASQMKAVAAQKAGHLAREIVPVSIPQKKGDPLLVSAD  223

BA1 61   EHPRETSLEALAKFQ  75
         EHPRETSLEALA+ +
HS* 224  EHPRETSLEALARLK  238
```

*****HS refers to *Hydrogenophaga* sp IBVHS2

Figure 5.4: Sequence alignment of translated *catII* amplicon sequence from Ballan ((BA1 sequence) to the most closely related Cat protein sequence from *Hydrogenophaga* sp. IBVHS2 (accession number WP_086118994.1).

*bla**NDM1* sequences PCR-amplified from DNA from Riversdale were analysed with the FunGene pipeline and compared to a library of blaNDM1 protein sequences. The most abundant *bla**NDM1* gene at Riversdale was found to be most closely related (40% identity) to a *bla**NDM1* protein sequence from *Escherichia coli* isolated from a hospital in Switzerland in 2018 albeit over a short sequence length (63 amino acids). The alignment of these two sequences is shown in Figure 5.5.

```
RD1 1  TSDVVGSNIGVKNLRLECCLKVRDLR----AFSVLGSLTALPVSSKAIGISFPS  ASK   IVM  56
       TSD +  I  ++   CL +R    A SV+  +L+ P+A      G +                  FP AS   IVM
E. coli TSDNITVGIDGTDIAFGGCL-IRTARPSRSAISVMPTLSTTP-RQRAFGAAFPKASMIVM  247
RD1 57      SHS  59
E. coli 248 SHS  250
```

Figure 5.5: The alignment of the Riversdale blaNDM1 sample sequence compared to *Escherichia coli* blaNDM1 sequence.

5.3 Discussion

The three enzymes, Chloramphenicol acetyltransferase, Acetyl-CoA C-transferase and 3-oxoadipyl-CoA thiolase identified in this study belong to the same transferase enzyme family. However, only chloramphenicol acetyltransferase (*catII*) has been directly associated with resistance to chloramphenicol in bacteria. In the current study, chloramphenicol acetyltransferase sequences related to those from *E. coli* were detected in water from Cobbledicks Ford using this approach. Previously, chloramphenicol resistance has been detected in *E. coli* isolates from Dongjiang River catchment in China (Su et al., 2012). The use of *cat* gene-specific oligonucleotides in PCR assays has shown the presence of these genes in E. coli from Jiaozhou Bay in China (Dang et al., 2008). Some prior next-generation shotgun sequencing-based studies have identified *cat* genes in metagenomes from environmental samples (soil, ocean and faecal materials) (Nesme et al., 2014).

The *cat* gene is a trimeric enzyme with two structural classes (A and B) (Wright, 2005). It can facilitate the transfer of the acetyl groups from acetyl CoA to chloramphenicol molecules when expressed and is responsible for resistance to this antibiotic in bacterial groups that carry and express this gene. This is because the attachment of acetyl CoA molecule to chloramphenicol prevents it from binding to the ribosomes (structural modification via acetylation) (Schwarz et al., 2004).

The protein sequences amplified using primers targeting *catII* genes from the three remaining sites were most closely related to an Acetyl-CoA C-transferase in *Polynucleobacter difficilis* and to a beta proteobacterium in samples from Riversdale and Bacchus Marsh while in Ballan, a 3-oxoadipyl-CoA thiolase found in *Hydrogenophaga* sp. was detected. The genus *Polynucleobacter* are commonly found in freshwater rivers (Nakayama et al., 2017) and drinking water sources (Jia et al., 2015). Members of the genus *Polynucleobacter* alongside

Hydrogenophaga sp. (3-oxoadipyl-CoA thiolase in Ballan samples) have been shown to carry Bacitracin (Ahn and Choi, 2016) resistance genes. They can also be carriers of MCR (resistance to Colistin antibiotic) and NDM (resistance to Carbapenems) antibiotic resistance genes (Wang et al., 2019). Interestingly, the bacterial community composition analysis carried out in Chapter 3 of this study had shown that the genus *Hydrogenophaga* was the most abundant genus across the four sampling sites in the Werribee River and over time.

The Acetyl-CoA C-transferase identified in this study is an enzyme that is involved in multiple biochemical reactions. In the Kreb's cycle, the enzyme catalyses the condensation of two molecules of Acetyl-CoA resulting in the formation of Acetoacetyl-CoA. The enzyme is also involved in the final fatty acid oxidation step resulting in the release of acetyl-CoA molecules. The other enzyme, 3-oxoadipyl-CoA thiolase, also belongs to the larger family of acetyl transferases and is involved in the condensation of a molecule of succinyl-CoA and a molecule of acetyl-CoA to form a 3-oxoadipyl-CoA molecule. It can also form acetoacetyl-CoA from acetoacetate ((Stirrett et al., 2009).

The *catII* gene primers used in this study were originally designed by Vassort-Bruneau *et al.* (1996) and exhaustively tested and shown to specifically target *catII* genes in test strains. Nevertheless, in this study, the *catII* primers have been shown to amplify non-target sequences in samples from Riversdale, Bacchus Marsh and Ballan. The reason for this is unclear, although the attachment of Illumina tags to these primers could have caused annealing to the wrong target in these samples and requires further investigations in future studies.

Based on the limited data obtained in this study, the ^{bla}*NDM1* protein sequence from Riversdale was found to be most closely related to a ^{bla}*NDM1* gene in *E. coli* from a clinical sample from Switzerland. The ^{bla}*NDM* gene is a well characterized antibiotic resistance gene in *E. coli* isolates from clinical (Ho et al., 2011, Mushtaq et al., 2011, Pfeifer et al., 2011) and water

samples from rivers and sewage plants in India (Akiba et al., 2016, Ahammad et al., 2014). Variants of this beta-lactam resistant gene (^{bla}TEM and ^{bla}Z) have been identified in *E .coli* isolates from different rivers in Nigeria using PCR based assays (Titilawo et al., 2015). Other variants such as ^{bla}RSA1 and ^{bla}RSA have also been detected in river sediments and samples from waste treatment plants in Hyderabad, India through functional genomics using Sanger and PacBio RS sequencing (Marathe et al., 2018). The *^{bla}NDM1* primers used in this study had been previously used to successfully detect and quantify *^{bla}NDM1* genes using qPCR (Poirel et al., 2011) but not with amplicon-based next-generation sequencing. Therefore, the results from this study suggest that this approach while promising requires further investigations and possible optimizations before it can be considered suitable for use for the sequence analysis of *^{bla}NDM1* genes from environmental samples"

The modification of *catII* primers resulted in a successful amplicon-based NGS process based on the limited number of samples used in this pilot study and only in samples from Cobbledicks Ford. This is because whilst this resulted in the amplification of the target chloramphenicol acetyltransferase gene from Cobbledicks Ford, these primers also amplified sequences that were most closely related to acetyl-CoA C-transferase and 3-oxoadipyl-CoA thiolase genes rather than only the target chloramphenicol acetyltransferase gene. For *^{bla}NDM*1 sequences from Riversdale low percentage identity was found between sequences amplified from DNA isolated from river water to ^{bla}NDM1 sequences from an *Escherichia coli* isolate. Collectively, this approach has suggested this amplicon-based sequencing method has some value, with detection of the target gene in one of four sites (Cobbledicks Ford) for *catII* genes (and detection of related acetyl-co-A transferase sequences) at two sites, whilst an NDM-1 related sequence was identified at Riversdale. Further research to develop this approach is required and warranted.

One approach would be testing the specificity of the modified *catII* or ^{bla}*NDM1* primers used in this study (or other credible candidate primer sets) under controlled conditions and on more bacterial isolates containing diverse *catII* or ^{bla}*NDM1* genes rather than using these primers directly on environmental samples. Additionally, this could entail spiking of environmental DNA from (soil and water) with DNA isolated from bacterial strains carrying these ARGs to test the efficacy and the sensitivity of these primers to amplify these target sequences. The optimized primers and procedures can be subsequently applied for use in environmental samples. Therefore, while the applied functional gene-based NGS was partly successfully applied for *catII* and ^{bla}*NDM* genes in samples from Cobbledicks Ford and Riversdale, respectively in this study, further future work is required before it used in environmental studies. Another alternative approach that can be used is shotgun metagenomics (Chen et al., 2020a) and this could approach could be used to study antibiotic resistance gene pools in environmental samples.

In conclusion, given that variations in sequences generated from different sites for *catII* sequences were observed, the hypothesis that the *catII* gene sequences for the selected ARGs varied between different sites along the river and/or over time is therefore supported.

6 Chapter 6: General Discussion

Aquatic environments such as freshwater systems are important because they provide valuable services to human society. They are sources of potable (drinking) water while also supporting multiple processes in agriculture, transportation and power generation (Covich *et al*., 2004). Freshwater environments are critically important ecosystems as they contain 6-10% of all species (Dudgeon *et al*., 2006; Strayer and Dudgeon, 2010). Due to the importance of freshwater systems in the provision of drinking water and food production, any process (such as pollution) that adversely affects the health of freshwater systems constitute risks to human health.

Aquatic microorganisms play important roles in maintaining and or restoring the health of streams and rivers through metabolic processes, nutrient cycling and photosynthesis (Falkowski et al., 2008). However, some aquatic microorganisms can pose significant risks to human health. These include human pathogens (enteric and coliform bacteria such as *E. coli* and *Salmonella* sp) (Axmanovà et al., 2006) and bacteria which have antibiotic resistance genes or ARGs (Nnadozie and Odume, 2019). ARGs are important because they mediate the development of resistance to antibiotics by pathogens, thereby endangering the efficacy of most widely used antibiotics (Ventola, 2015). This may reverse the current significant reduction in human morbidity and mortality worldwide which is associated with the use of antibiotics for therapeutic purposes (Ventola, 2015).

Rivers and other freshwater systems receiving urban and agricultural run-off and waste discharges from sewage treatment plants and clinical sources can be reservoirs and sources of antibiotic resistance genes for transfer to other bacterial groups as a result of this pollution (Stoll et al., 2012, Rodriguez-Mozaz et al., 2015, Zhang et al., 2015). This is why studies such as the current study, which was carried out on a major river, the Werribee River (in Melbourne),

are important. The Werribee River and its catchment area covers approximately 2700 km^2 and depending on location, is a resource for agriculture (irrigation) and urban development. Unfortunately, despite the crucial services that this river provides, there are limited reports on the microbiology and the prevalence of ARGs in this freshwater system.

Therefore, this study has focused on investigating spatial and temporal variability in bacterial communities and their associated antibiotic resistances in the Werribee River. Firstly, this study investigated the effects of physico-chemical parameters such as temperature, pH, dissolved oxygen, electrical conductivity, turbidity and suspended solids upon the bacterial community at selected sites along the river. The effects of nutrients such as nitrites, nitrates, ammonia, total Kjehldahl nitrogen (nitrogen bound in organic matter) and total nitrogen (free and bound), phosphates and total phosphorous were also assessed. This was carried out using samples from Ballan and Bacchus Marsh (upstream sites) and Cobbledicks Ford and Riversdale (downstream sites) and over time (April 2015 to February 2016). A 16S rRNA-based Next Generation Sequencing approach was used to investigate variation in the distribution, structure and diversity of the bacterial community in the Werribee River and to identify environmental factors of ecological importance.

Secondly, the prevalence of selected antibiotic resistance genes was determined in the bacterial communities at these selected sites using endpoint and quantitative PCR approaches. This was performed because an understanding of the prevalence ARGs is critical to assessing the health risks associated with the present and future use of the Werribee River's water resources. The data obtained would allow for proper characterization of health risks associated with the use the river water resources at different sites and months.

The third aim of this study was to evaluate the suitability of a gene-targeted (amplicon-based) Next-Generation Sequencing approach for the simultaneous detection (identification) and

quantification (relative abundance) of ARGs within the river. Presently, the detection and identification of bacteria groups with antibiotic resistance genes are usually carried out through endpoint PCR while the quantification these groups is typically performed with quantitative PCR; a two-phase approach. If successful, amplicon-based Next Generation Sequencing could be a more efficient approach to evaluate the prevalence and diversity of ARGs in rivers.

This study found that the four sites had substantial spatial variation in the structure and composition of their bacterial communities, varying both in OTU numbers and Shannon diversity. The bacterial communities within each of the four sites on the Werribee River were more closely related to one another (and over time) than to those from other sites. A similar trend has been reported in other studies carried out in aquatic systems such as ponds and rivers (Lear *et al.*, 2014; Staley *et al*., 2015; Luo *et al* 2016). In addition, the bacterial communities in the upstream sites were substantially different from the communities in the downstream sites. Spatial variation was also observed in the numbers of OTU and in Shannon diversity at each site. Substantial spatial variation with respect to Shannon diversity has been reported in other studies on aquatic ecosystems such those on the different sites on the Arctic Yenisei River (Kolmakova *et al*., 2014) and in the Yellow River in China (Xia *et al*., 2014).

Similarly, temporal variation was observed in the structure and composition of the bacterial community and in diversity (OTU numbers and Shannon diversity) of these four sites. For example, both Chao1 OTU richness and Shannon diversity were highest in the summer months (December and February) when compared to the winter period (June). This variation reflects seasonal effects (changes in environmental conditions) on the Werribee River's bacterial community and showed that the bacterial community was more diverse in the summer period than at other times. These time-related effects on the bacterial community can be variable depending on geographical location and the river system being studied. While a similar increase in bacterial diversity was reported in the summer months in sites on the Ganjiang River

in China (Wang *et al* 2016), bacterial community diversity was higher in autumn months when compared to other months in sites on the Haihe River in China (Ma *et al*., 2016).

Spatial and temporal variation was also observed in the bacterial composition at phylum, class and genus levels in this study for most bacterial groups (except with the *Proteobacteria*) across sites and over time. The predominant bacterial phylum was the Proteobacteria (especially of the class *Betaproteobacteria*) with Betaproteobacterial groups being detected at most sites, irrespective of the sampling months. Other dominant phyla detected in samples from the river include the *Bacteroidetes* and *Actinobacteria*. These different phyla have been identified as being dominant in other studies on the Thames River in England (Read et al., 2015), the Danube River in Europe (Savio et al., 2015) and the Mississippi River in the United States (Staley et al., 2013).

In addition to the *Betaproteobacteria* (mot dominant class), bacterial groups belonging to the class *Cytophaga, Actinobacteria* and *Alphaproteobacteria* were also detected at sites on the Werribee River. Bacteria groups belonging to the *Betaproteobacteria* (Nuy et al., 2020), *Cytophag*a (Pitt et al., 2019), *Actinobacteria* (Protasov et al., 2020) and *Alphaproteobacteria* (Guo et al., 2020) have been isolated or detected in water samples or sediments from rivers and lakes in other scientific investigations of the microbial diversity of freshwater systems.

The most commonly detected genus was *Polynucleobacter* which was the most dominant genus in April, August, October and February in the upstream site of Ballan while *Arcicella* was the most abundant genus in April, June, August and October in Bacchus Marsh. This finding showed that there was variation in the dominant genera over time and with sites in the upstream reaches of the Werribee River. Other important genera showing both spatial and temporal variation at these sites were *Limnohabitans, Acidovorax and Methylotenera*. At downstream sites, *Candidatus* Pelagibacter was the most dominant bacterial genus in Cobbledicks Ford

(June, August and February) and Riversdale (June, August and October). Other important genera detected at significant levels in downstream sites were *Acidovorax, Limnohabitans* and *Demequina.* Changes in bacterial composition between sites and over time in aquatic ecosystems are not unusual and have been reported in the Zenne River in Belgium (Garcia-Armisen *et al*., 2014). It is possible that differences in the relative abundance of these bacterial groups were responsible for the variation in the OTU numbers, OTU richness and Shannon diversity (Kolmakova *et al*., 2014) observed in this study.

Spatial and temporal variation in the bacterial community composition, structure and diversity reflect site characteristics, exposure to anthropogenic inputs, the inflow of tributaries and differences in physico-chemical factors across sites and over time (months/seasons) (Fortunato et al., 2012b, Payne et al., 2017b, Bouskill et al., 2010, Staley et al., 2015b). In this study, the two of the most important environmental factors were water temperature and dissolved oxygen concentration at most sites although other factors such as pH, turbidity, suspended solids, EC, ammonia and total bound nitrogen (TKN) were only important at one site, Cobbledicks Ford. Water temperature was a significant driver of bacterial community structure and diversity in the Werribee River with both Shannon diversity and OTU numbers strongly and positively correlated to temperature. Water temperature has been reported to be a strong driver of bacterial composition in other freshwater-based studies (Crump and Hobbie, 2005; Staley *et al*., 2015) probably because of its effects on the metabolic activities of aquatic bacteria (Dallas, 2008). Shannon diversity and OTUs were however strongly negatively correlated to dissolved oxygen concentrations. This is in contrast to some other findings which showed that dissolved oxygen concentrations were positively correlated with alpha bacterial diversity in aquatic systems (Mohiuddin *et al*., 2019).

For this chapter, it was hypothesized that bacterial community structure, composition and diversity would show substantial variation between the upstream and downstream sites over

time based on their different physico-chemical properties (e.g. pH, temperature, conductivity, nutrient composition etc.) within the Werribee River. The results obtained supported this hypothesis with temperature and dissolved oxygen concentration assessed to be the two most important environmental factors. Nutrients were only identified as an important factor at one site (Cobbledicks Ford). This suggested that nutrient input from possible anthropogenic sources may not be an important driver of changes in the bacterial community at the remaining sites.

Having observed substantial spatial and temporal variation in the bacterial communities in samples from upstream and downstream sites at Werribee River, the prevalence of ARGs of ecological relevance and their relative abundance was assessed. Out of the twelve ARGs assayed for (*^{bla}NDM1, mecA, tet(M), ampC, VanA, mcr-1, tet(B),* er*m(B), aac (6')-Ie-aph (2'')*, *SulII*, *catII*, and *dfrA1*), only three were detected, namely *^{bla}NDM1* (responsible for resistance to beta-lactam antibiotics), *catII* (responsible for resistance to chloramphenicol) and *tet(B)* (responsible for resistance to tetracycline). Spatial and temporal variation in the frequency of ARG detection of these three ARGs was observed. Previous research on the Werribee River had also demonstrated spatial and temporal variation in the frequency of detection of different ARGs such as *mecA* and *sul1* (Barker-Reid *et al* 2010) and has been reported in other studies on freshwater systems (Knap et al., 2012; Zhao *et al*., 2016).

The three ARGS were also more frequently detected in the downstream sites (Cobbledicks Ford and Riversdale) than in upstream sites (Ballan and Bacchus Marsh). Therefore, there was a greater health risk of exposure to these ARGs from water samples from downstream sites (at most times of the year) than in upstream sites. For example, potential exposure to ^{bla}*NDM-1*, *tet(B)* and *catII* ARGs was only identified in April and December at Ballan and to *catII* ARG only in October at Bacchus Marsh.

Interestingly, while ARGs were less frequently detected in upstream sites compared to downstream sites, there was no significant difference in the relative abundance of ARGs between most of these sites. However, the relative abundance of ARGs varied over seasons with increases in abundance observed in most months from April to February (significant increase in summer months (December-February), when compared to the autumn month (April).

Therefore, the spatial and temporal variation observed in the total bacterial community was reflected in the detection of ARGs and the variation in the relative abundance of these ARGs (observed in samples from different sites and times). Therefore, the hypothesis that there will be substantial spatial and temporal variation in the relative abundance of clinically important ARGs in samples from the Werribee River was supported in the three ARGs (^{bla}*NDM-1*, *tet(B)* and *catII*) detected in this study. However, the reason for the increased frequency of detection of ARGs at the downstream sites was not investigated in this study. However, it could be a natural occurrence or be related to possible anthropogenic inputs into the Werribee River. There are agricultural activities along the course of the Werribee River (especially at the downstream sites). The reclaimed water used for irrigation in the Werribee River basin had previously been shown to contains ARGs such as those responsible for resistance to methicillin and sulfonamide antibiotics (Barker-Reid et al., 2010). These are potential sources of inputs into the Werribee River.

Research has shown that river inputs in the form of effluents from waste treatment plants, run-off from agriculture and pollution from hospital wastes (Devarajan et al., 2016) can lead to increases in the number and abundance of ARGs in rivers and other freshwater systems. The exposure of some sections of streams to industrial wastes has been shown to lead to elevated levels of bacterial species resistant to streptomycin and kanamycin when compared to other

sections (McArthur and TuckField, 2000). Spatial variation has also been observed in the abundance of *tet* genes due to sewage contamination (Czekalski *et al*., 2014).

Currently, PCR based methods do not allow for simultaneous identification (taxonomic description of bacteria species with ARG) and the quantification of the relative abundance of antibiotic resistance genes and different sequence types. For example, endpoint PCR, using primers targeting ARGs of interest can be used to detect whether the target ARGs are present in environmental samples. The amplicons can be sequenced, and the sequences blasted against a suitable database to determine the putative identities of the bacterial isolates in the amplicons. Endpoint-PCR-sequencing methods cannot be used to quantify the abundance of target ARGs. Quantification of ARGs is usually performed with qPCR, using specific primers in SYBR Green or TaqMan Probe-based assays. Amplicons from qPCR assays cannot be sequenced and the quantification data is only reflective of target ARG or target genus and no comparative taxonomic information on the abundance of other non-target ARG bearing bacteria can be generated.

Shotgun random next-generation sequencing approaches provide an opportunity to simultaneously determine the taxonomic diversity of bacterial with ARGs and the relative abundance of these bacteria species in environmental samples. It involves random sequencing of metagenomic DNA on suitable or desired NGS platforms. The sequence data is then compared with a curated database of ARGs (bacteria with ARGs) such as ARDB (Zhao et al., 2018). However, the fact that metagenomic DNA is randomly sequenced means that the process does not specifically target bacteria with ARGs but all bacterial species. Therefore, the specificity/sensitivity that is needed for picking up ARGs present in environmental samples at low concentrations is often compromised. Therefore, a targeted next-generation sequencing approach that is specific for ARG of interest should be sensitive enough whilst providing detailed information on different taxa with the target ARG in environmental samples.

Consequently, the third result chapter was focused on determining whether a targeted next-generation sequencing approach could be used to identify and quantify the relative abundance of target ARGs in environmental samples. Two ARGs, *catII* and ^{bla}*NDM1* were used for this investigation using specific primers previously used in PCR and qPCR assays. The target sequences were not modified but linker DNA and barcodes were added to these sequences to enable sequencing on the Illumina MiSeq platform.

When the sequence data were analysed using a functional gene database (FunGene) and Blast analysis, variable results on primer specificity were obtained, although this was dependent on the sample analysed. The target gene, chloramphenicol O-acetyltransferase was only detected in samples from Cobbledicks Ford (a downstream site) that were related to *catII* sequence from *E. coli* and other enterobacteria have been shown from literature to carry *cat* genes (Su *et al*., 2012; Williams *et al*., 2019; Charnock *et al*., 2018). The same trend was observed in the single ^{bla}*NDM1* sample as the sequence analyses showed similarity to a protein sequence of ^{bla}*NDM1* gene from *E. coli*. In samples from Bacchus Marsh and Riversdale, a different enzyme (Acetyl-CoA C-transferase) was detected and 3-oxoadipyl-CoA thiolase identified in Ballan samples when subject to *catII* gene-based analyses.

The results obtained, indicated limited success in the application of targeted ARG-based NGS approach. Therefore, the proposed hypothesis that gene sequences for the selected ARGs (detected previously by PCR) varied between different sites along the river and/or over time could not be effectively evaluated. It was difficult to assess the diversity that may exist within individual resistance genes within the wider antibiotic resistome in the Werribee River because of the observed instances of lack of specificity with some of the primers used. The reason for this is unclear because the target sequences and primers used have been well validated for the detection of *cat* and *BLA-NDM* ARGs using PCR (Poirel *et al.*, 2011; Vassort-Bruneau *et al*., 1996). With the amplicon-based NGS approach used in this study, the lack of target specificity

because of poor primer design would cause these primers to amplify a suite of similar genes, as observed with *cat*-gene based assays. Poor primer design can result in the amplification of non-specific target genes, including genes encoding enzymes that have related sequences and functions but are not the target gene. A good example of this is the amplification of acetyl-co-A gene sequences in some instances, by the *cat* gene primers used in this study with the resulting amplicon sequences related to but distinct from the targeted chloramphenicol acetyl-co-A genes. This may be the major factor responsible for the limited success observed with the use of these PCR primers in this study. Therefore, future studies should be carried out using primers specifically designed and optimized for use in amplicon-based next-generation sequencing approaches. In addition, shotgun metagenomics could also be used to investigate ARGs as has been reported in some recent studies (Chen et al., 2019a, Bai et al., 2019).

The detection of the target *catII* gene in one out of the four samples tested could also indicate that the source of the sample may be influencing the specificity of this targeted sequencing approach. The source of the sample may also affect the relative abundance of DNA from particular genera driving the amplification of non-target genes from DNA from this genus. Alternatively, it is possible that the modifications that were carried out on the primers i.e. to allow for barcoding could have resulted in primer mis-annealing.

This study has therefore successfully improved the understanding of the bacterial community composition and diversity in the Werribee River which should be useful for future research work on the microbial ecology of this river. Potentially increased health risks of using river water from downstream sites compared to upstream sites were also reported because some ARGs were more frequently detected at downstream sites. Variable results were observed with respect to the specificity of targeted ARG-based NGS approach used in this study with the applied functional gene-based NGS successfully amplifying target *catII* and ^{bla}*NDM1* genes only in samples from Cobbledicks Ford and Riversdale respectively

This study has also identified gaps in the knowledge of ARGs found in the Werribee River. For example, the prevalence of only 12 ARGs was evaluated with no information available on most other known ARGs. Future work should be carried out to determine the prevalence of variants of these 12 ARGs and other ARGs not assessed in this study. This would lead to an improved understanding of the ARG resistome in the Werribee River. The detection of ARGs does not automatically mean that these ARGs are expressed or readily transferred to other bacterial species or groups (especially the human microflora). Future investigations could also be complemented by using culture-based assays to determine whether the observed elevated frequency of detection and abundance of ARG bearing bacteria is reflected in demonstrable resistance by bacterial isolates from selected sites to the target antibiotics.

Although the frequency of detection of ARGs was higher in downstream sites when compared to upstream sites, the reason for this was not investigated in this study. Therefore, it was not possible to determine whether this trend was stochastic or due to anthropogenic inputs. Future investigations would be needed to assess anthropogenic activities (agricultural activities) and inputs from urban run-off along the course of the Werribee River (especially at downstream sites) to determine their possible contribution (if any) to the presence of ARGs in Werribee River. These activities may be curtailed in the future if they are found to be sources of ARGs for the river.

Whilst the ARG-based next-generation sequencing approach yielded varying results, including sequencing of non-target gene sequences, there are further experiments that can be carried out to fully understand the reasons for these results. Firstly, future investigations would need to be carried out with more samples from Cobbledicks Ford (*catII*) and Riversdale (^{bla}*NDM1*) to confirm that the chloramphenicol O-acetyltransferase and ^{bla}*NDM* gene can be detected in these samples to validate the preliminary results obtained in this study. It would also be useful to analyse freshwater samples from other sites known to have the target ARG (*catII*) to determine

whether a similar result can be obtained. Finally, variants of the *cat* and *^{bla}NDM* gene primers could be assessed using a targeted ARG-gene based NGS approach to determine whether these show more specificity for the chloramphenicol O-acetyltransferase and *^{bla}NDM* gene than observed in the primer sequences used for this study.

Once optimized, the ARG-based next-generation sequencing approach used in this study can also be applied to the analysis of clinical samples or the study of the human microbiome (for example, of the gut microbiome). A profile of bacterial taxa in clinical samples, the prevalent antibiotic resistant genes and the relative abundance of these antibiotic resistant genes can be accurately generated. This information on the antibiotic resistance profiles of clinical samples (patients) can be used for diagnostic and therapeutic purposes (i.e. prescribing antibiotics that the patients are sensitive to). Also, patients with multi-drug resistant bacteria can be quickly identified and isolated to curb the spread of the ARG in hospital wards. Therefore, the application of an ARG-based next-generation sequencing would be beneficial to the healthcare sector.

The specific aims of this project which included the assessment of the spatial and temporal variation in the structure, composition and diversity of bacterial communities and antibiotic resistance genes (ARG) along the Werribee River using amplicon-based next-generation sequencing and PCR and qPCR detection assays were achieved. However, further work is required with respect to the third aim "to assess the suitability of an amplicon-based next-generation approach to the study of antibiotics resistance genes in surface water samples from the Werribee River" as limited success was obtained probably largely due to primer specificity issues.

In conclusion, this study has improved understanding of the bacterial communities in the Werribee River and the effects of physico-chemical factors on the composition and structure

of these communities. The bacterial communities were more diverse in summer months when compared to other months, spatially and temporally variable and this variation was primarily correlated to water temperature (positively) and dissolved oxygen concentration (negatively). Three ARGs (*catII, tet(B)* and ^{bla}*NDM1*) were identified in water samples and the risk of exposure to these ARGs were higher in downstream sites when compared to upstream sites. Target gene-based next-generation sequencing was successfully applied for the identification and quantification of *catII* and ^{bla}*NDM1* genes in some samples and remains a promising approach for the analysis of environmental and clinical samples when fully optimized.

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