MICROBIAL COMMUNITY DYNAMICS WITHIN IMPACTED COASTAL ECOSYSTEMS

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CERTIFICATE OF ORIGINAL AUTHORSHIP

I, Richard Linley Carney declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Life Sciences at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise reference or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

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Thesis by compilation

This thesis is formatted in accordance with the requirements of a thesis by compilation.

A thesis by compilation is structured as a single manuscript that comprises a combination of chapters and published/publishable works.

It should be noted that data chapters two through to five, within this thesis, are structured according to the requirements of each respective journal where the manuscripts has, or will be submitted, however formatting has been kept consistent throughout as much as possible.

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Abstract

The microbes that inhabit coastal environments are responsible for high rates of primary productivity and nutrient cycling, and are amongst the first organisms to respond to changes in their habitat. This means that microbes are often the key biological initiators of positive (eg. bioremediation) or negative (eg. algal blooms) outcomes in response to environmental perturbations associated with natural or anthropogenic disturbances. Until now, most investigations of coastal microbiology have only focused on a narrow set of organisms that indicate faecal pollution, and consequently, have overlooked the 99% of environmental microbes that are yet to be cultured. Given this shortfall in our understanding of coastal microbiology, this project aimed to identify the key environmental and biological processes that influence the microbial ecology of coastal ecosystems, including potentially harmful species. To achieve this, a 24 month, high temporal resolution sampling regime was conducted at two urban beaches that experience contrasting hydrology and exposure to anthropogenic influences. Water samples were analysed in several ways using a suite of molecular, computational and statistical approaches, to elicit patterns and interactions between climate, water chemistry and microbial community assemblages. Each of my thesis chapters focus on a particular ecological aspect of the microbial communities extracted from water collected at two beach sample locations. In Chapter 1, I provide a background and overview of microbial ecology in coastal ecosystems. In Chapter 2, the first of my data chapters, 16S rRNA sequencing was combined with threshold indicator taxa analysis and network analysis to capture the dynamics of bacteria at the community-scale, which

revealed contrasting temporal patterns in whole bacterial communities, and seasonal switching between closely related taxa. Broad screening of bacterial communities also detected an interesting pattern in a population of emerging pathogens within the genus Arcobacter. This became the focus of Chapter 3, where large peaks were detected in Arcobacter abundance during inputs of stormwater and WWSO at the impacted beach, which persisted in high abundance for a further week following rainfall. In Chapter 4, the focus shifted from enteric microbes, to genes that confer resistance to antibiotics, which found 100-fold increases in several antibiotic resistance genes that were strongly correlated with potentially pathogenic enteric bacteria. In Chapter 5, this project closely tracked populations of pathogenic species within the genus Vibrio, providing new evidence that directly links blooms in pathogenic Vibrio in coastal surface waters with marine heatwave events. Overall, the findings of this project provide new and transformative insight into the microbial ecology of coastal systems, which has highlighted several potential implications for ecological and public health. Specifically, the detection of genes associated with antibiotic resistance and potential pathogens, that persist in beach water even when standard fecal indicators have diminished, has highlighted a need for broader screening tools in the monitoring and management of water quality in swimming beaches.

1. Chapter 1 - Introduction

1.1 Coastal Environments are Important, and Highly Dynamic Ecosystems

Coastal ecosystems are a pivotally important source of biodiversity and function, which underpins ecosystem products and services, and supports the health of both terrestrial and marine environments (Alongi, 1998). Although coastal habitat represents only 7% of the total ocean volume, they account for 19% of ocean primary production, 80% of organic matter burial, and 90% of sediment nutrient remineralisation, meaning that coasts have a disproportionately large influence on ocean function (Azam, 1998, Teeling et al., 2012, Alongi and McKinnon, 2005). Coastal ecosystems accommodate a diverse assemblage of habitats, including coral reefs, seagrasses, mangroves, salt marshes and sand beaches. These habitats provide valuable ecosystem services, estimated to be worth over $12 trillion USD per year (Costanza et al., 1997). Coastal ecosystems provide protection from erosion forces (by wave and wind action), water catchment and purification, and carbon sequestration, and yield products of high economic value, including 90% of fisheries catch (Pauly et al., 2002) and a rich source of raw materials used in building and industry (Baba et al., 2013).

In comparison to open ocean environments, coastal ecosystems are characterised by extremely dynamic physical, chemical and biological conditions (Alongi, 1998, Wu et al., 2010). The highly heterogeneous nature of coastal ecosystems arises from natural hydrological processes, including the influence of waves, tidal flows and freshwater river-plumes (Nash and Moum, 2005), and oceanographic processes, including coastal currents, eddies and up-welling events (Suthers et al., 2011). In addition, the

interconnection of coasts with terrestrial habitats results in localised allochthonous impacts, including diffuse runoff, and point-source inputs from stormwater and sewage outlets (Ahn et al., 2005). These inputs modify coastal water chemistry by transporting anthropogenic pollutants from terrestrial and urban environments into natural coastal receiving waters (Gnecco et al., 2005, Howarth et al., 2000, Islam and Tanaka, 2004). Collectively, these features act at multiple spatial and temporal scales, translating into ecological impacts that are manifested across both short (eg. days to weeks) and prolonged (eg. months to years) periods, and across spatial scales of meters to kilometers from the shoreline (Defeo et al., 2009, Halpern et al., 2008a).

1.2 Coastal ecosystems are heavily impacted by anthropogenic forces

More than 50% of the global human population lives near to coastal ecosystems (Shuval, 2006). As a consequence, human activities are highly concentrated within coastal zones and are having negative impacts on ecosystem health (Lee et al., 2006, Moritz-Zimmermann et al., 2002). In particular, urban impacts within coastal watersheds have profound influence over coastal water quality (Halliday and Gast, 2010). Both intermittent and constant inputs of organic and inorganic pollutants into coastal systems, via urban and industrial run-off, storm-water and sewage discharges, can markedly shift the chemical status of coastal waters (Birch and Rochford, 2010, Ahn et al., 2005). Furthermore, there is emerging evidence that climate change, driven by anthropogenic carbon emissions, is amplifying the negative impacts caused by direct human activities within coastal regions (Halpern et al., 2008b, Paerl et al., 2011). In particular, chronic rises in sea surface temperatures (SSTs) (Lima and Wethey, 2012),

and the increased frequency and severity of extreme high temperature anomalies - marine heatwaves (MHWs) (Oliver et al., 2018), further compromise the sustainability of coastal ecosystem products and services (Barbier et al., 2011, Oliver et al., 2018, Heron et al., 2016).

Recent estimates suggest that approximately 50% of salt marshes, 35% of mangroves, 30% of coral reefs, and 29% of seagrasses are either lost or degraded worldwide (Valiela et al., 2001, MEA, 2005, Orth et al., 2006, UNEP, 2006, FAO, 2007, Waycott et al., 2009). This has led to markedly diminished output of coastal products and services, including a 33% decline in viable fisheries and a 69% decline in nursery habitats for oyster reefs, sea grass beds and wetlands (Worm et al., 2006). Within this changing scenario, there is an urgent need to understand the interactions of organisms at the base of coastal ecosystems food webs, to develop strategies for curbing the current trajectory of ecosystem degradation.

1.3 Coastal Microbiology

The marine environment is the largest biome on Earth, harboring approximately 10^{29} microbial cells, which comprise an estimated 2×10^{6} species (Curtis et al., 2002). Collectively, marine microbes, including bacteria, archaea, phytoplankton and viruses, make up the majority of all marine biomass, and contribute the greatest portion of metabolic activity (Curtis and Sloan, 2004). As well as being amazingly abundant and diverse, marine microbes form the foundation of aquatic food webs, and govern the biogeochemical processes that ultimately underpin nutrient recycling and ecosystem productivity (Gasol and Kirchman, 2018). Within coastal environments, microbes

contribute up to 50% of global photosynthesis (Sarmiento and Bruber, 2006), but also perform important roles in the decomposition of organic matter and remineralisation of nutrients (Arnosti, 2011, Anderson and Ducklow, 2001).

Microbial activities can either mitigate or exacerbate environmental impacts within coastal ecosystems. For example, some aquatic microbes have the capacity to biodegrade petrochemical pollutants, as was seen in the rapid microbial degradation of the BP Deep Water Horizon oil spill in 2010 (Reddy et al., 2012). In addition, some microbes have been utilised strategically in a wide range of bioremediation applications, including the removal of heavy metals from industrial wastewater (Saranraj and Sujitha, 2013, Naser, 2013). Conversely, some coastal microbes can exacerbate negative human impacts. For example, urban and agricultural nutrient runoff has led to eutrophication in marine environments, leading to blooms of sometimes noxious microorganisms (Deegan et al., 2012, Wallace et al., 2014). In some extreme cases, high rates of respiration, as a result of the microbial response to the influx of nutrients resources, has led to the establishment of hypoxic “dead zones”, and resulted in mass death of fish and crustaceans (Howarth et al., 2011), and even complete ecosystem collapse (Howarth et al., 2011, Paerl et al., 1998, Zhang et al., 2010). Finally, microbes can also be useful as indicators of water quality or changes to aquatic ecological health (Stewart et al., 2008), as a consequence of their rapid growth rates and sensitivity to even subtle environmental perturbations (King et al., 2011).

1.4 Coastal Microbes Experience a Highly Heterogeneous Habitat

In the relatively stable conditions of open ocean environments, microbial communities are generally numerically dominated by only a few bacterial groups, which typically shift between seasons (Giovannoni, 2017, Morris et al., 2002). In contrast, the dynamics of coastal microbial populations are often influenced by localised, natural and anthropogenic processes that often incorporate very rapid shifts in conditions (Caron et al., 2017, Heba El-Swais, 2014, Zhang et al., 2009), often resulting in boom or bust population cycles among the resident microbial communities. These cycles are likely to play a significant role in defining microbial assemblage structure and function, and the ecological health and functionality of coastal ecosystems.

Marine microbes generally employ one of two, broad life-strategies, that offer advantages dependent on the conditions of their habitat (Lauro et al., 2009). Marine oligotrophs grow slowly, but are adapted to grow under low nutrient concentrations, and are hence most abundant within stable oligotrophic environments, like the open ocean (Yooseph et al., 2010). This strategy has enabled oligotrophic marine bacterial groups, such as *Pelagibacter*, *Prochlorococcus* and *Synechococcus* to dominate across large regions of oligotrophic ocean (Flombaum et al., 2013, Giovannoni, 2017). Conversely, marine copiotrophs typically maintain low abundance populations, but are capable of rapid growth and exploitation of short-lived bursts of high resource availability (Lauro et al., 2009). Marine copiotrophs generally dominate in dynamic coastal environments that are exposed to terrestrial runoff and subsequent inputs of allochthonous nutrients. Many copiotrophs include members of the “rare biosphere”,

which represents a seedbank of typically rare organisms that can exploit specific environmental shifts to periodically emerge as dominant members of the community (Campbell et al., 2011). Importantly, some of these rare organisms are also potentially harmful, including microbes that can negatively impact their surrounding habitat during bloom events, or exhibit pathogenicity and pose a risk to animal and human health (Thompson and Polz, 2006). Consequently, relative to within more stable open ocean environments, the composition and function of coastal microbial assemblages can shift rapidly following environmental perturbations (Nogales et al., 2011), which may re-shape the ecology of entire habitats and lead to blooms of harmful microbes.

Coastal ecosystems are at the interface of terrestrial and aquatic biomes, and are therefore key receiving bodies for terrestrial runoff, and allochthonous chemicals and microbes (Ahn et al., 2005, Birch and Rochford, 2010). Distinguishing anthropogenic drivers of change over and above variation related to natural drivers of change requires 1) an understanding of slight and incremental changes that occur over time periods of years and decades, and 2) data that captures microbial and environmental changes before, during and after anthropogenic perturbations. Allochthonous microbes that are transported into coastal ecosystems derive from a variety of sources, including urban surfaces (roads, roofs and parks) (Herve et al., 2018), stormwater conveyance infrastructure (pipes and open channels) (Badin et al., 2012, McLellan et al., 2015), or from sewage (ocean outfalls and wet weather overflows) (Newton et al., 2011, Sidhu, 2013). Many of these microbes have adapted to niche urban environments and chronic exposure to anthropogenic chemicals (Rizzo et al., 2013). Therefore, coastal incursions

by allochthonous microbes via runoff and waste streams also introduce their genetic features and metabolisms, that potentially modify the microbiology of coastal ecosystems (Sidhu et al., 2013). This could have significant implications for the ecology of coastal ecosystems, and can potentially result in negative ecological and public health outcomes.

1.5 Environmental perturbations can have negative environmental and human health impacts

Several harmful micro-algae and bacteria are indigenous to coastal habitats such as rivers, estuaries and beaches. These organisms generally persist in low abundances, until specific environmental stimuli, such as changes in water temperature or chemistry, support their emergence as numerically dominant members of the community (Sjostedt et al., 2012). For example, input of inorganic nutrients can stimulate blooms in cyanobacteria, dinoflagellates and diatoms, which can alter the physico-chemical state of their habitat, and lead to detrimental ecological outcomes (Paerl et al., 2016). Some marine algae produce toxins, and blooms in these species (associated with nutrient runoff and elevated temperatures) have resulted in harm to marine organisms and humans (Gobler et al., 2017). Algal blooms can themselves provide resources (eg. algal exudates) and opportunities for other, potentially harmful microbes to persist and increase numerically in marine waters. Bacteria from the *Vibrio* genus, typically occur at low levels, but can increase rapidly in response to ephemeral resources, such as chitin derived from some diatoms (Frischkorn et al., 2013). Evidence from studies monitoring Vibrio dynamics in coastal waters confirmed that increases in Vibrio abundance

sometimes coincide with diatom-dominated phytoplankton blooms (Asplund et al., 2011). This is significant as some species of Vibrios exhibit highly pathogenic traits, including *V. cholerae*, which causes cholera in humans (Ali et al., 2012), and *V. vulnificus* and *V. parahaemolyticus*, which cause acute skin infections and gastrointestinal illness (Ralph and Currie, 2007, CDC, 1998).

The incidence of Vibrio associated infections has increased in northern hemisphere regions such as the Mediterranean Sea (Paz et al., 2007), and the North Atlantic Ocean (Vezzulli et al., 2016b), that are also experiencing rising SST and extreme heat anomalies (Di Lorenzo and Mantua, 2016, Oliver et al., 2018). Several Vibrio outbreaks have been associated with consumption of shellfish (CDC, 1998) or direct exposure by humans in recreational waters (Ralph and Currie, 2007, Strom and Paranjpye, 2000, Wise and Newton, 1992).

In addition to these natural marine pathogens, several harmful, non-indigenous microbes, including *Escherichia coli* and *Salmonella* spp. (Shuval, 2006), entero-viruses (including noroviruses, adenoviruses, echoviruses and hepatitis A (Sinclair et al., 2009)), and protozoan pathogens (Collier et al., 2012) can be introduced to coastal habitats via sewage and stormwater, often posing serious public health risks. Waterborne infections contracted from coastal waters are estimated to cause 120 million cases of gastrointestinal illness and over 50 million cases of serious respiratory disease annually (Shuval, 2006). Accordingly, much of the effort to monitor the microbiology of coastal environments is focused on these organisms. In addition, new and emerging human pathogens are more frequently being detected within natural and urbanised aquatic

environments, leading to an increasing recognition of their potential ecological importance and public health relevance (Fisher et al., 2015, Sharma et al., 2003). For instance, several sewer associated bacteria, including *Acinetobacter*, *Arcobacter*, *Cloacibacterium*, *Pseudomonas* and *Shewanella* have recently gained increased attention as emerging pathogens (Ramees et al., 2017, Janda, 2014), due to their increased rate of detection within recreational waters and isolation from clinical samples, coupled with their association with disease in a broad range of hosts (Douidah et al., 2012). The mixing of these enteric microorganisms with indigenous marine populations can also lead to the exchange of genetic material, including genes encoding antibiotic resistance (AbR), virulence and toxicity, from enteric to indigenous marine microbes. Indeed, the increased global incidence of untreatable infections in humans due to antibiotic resistance has heightened interest in the role of natural environments in the dissemination of AbR genes (Baquero et al., 2008).

From both an ecological and public health perspective, understanding the function and potential for pathogenicity in coastal microbial assemblages is imperative. However, we currently only have a piece-meal perception of the microbial ecology of these ecosystems and an incomplete understanding of the microbiological factors that drive large and rapid shifts in the chemical status of coastal environments or the emergence of noxious organisms. As a consequence, coastal environments represent promising, yet poorly characterised model systems for studying microbial ecology.

1.6 Coastal microbial ecology remains poorly characterised

Human health implications related to the introduction of enteric pathogens has been the primary focus for most microbiological investigations within coastal environments. As a consequence, most coastal studies are conducted within water quality monitoring programs that often only focus on a narrow range of indicator organisms of public health significance (Boehm and Sassoubre, 2014). Although important for detecting fecal pollution and minimising risk of pathogen exposure to humans using recreational waters (Boehm and Sassoubre, 2014), these studies often ignore the >99% of the microbial community that are missed by culture-based approaches and as a consequence potentially overlook significant microbial interactions, including the emergence of non-targeted endemic and introduced pathogens. Consequently, our understanding of microbial dynamics in coastal ecosystems at the community scale is less developed than in terrestrial and open ocean environments.

Relative to open-ocean environments, there have been far fewer long-term, high temporal resolution studies of microbial ecology in coastal ecosystems. The few notable timeseries investigations that have been conducted in coastal environments have reported interesting ecological dynamics, including seasonal switching between closely related marine oligotrophs (Ward et al., 2017), repeatable seasonal patterns (Holbrook and Bindoff, 1997) in microbial assemblages (Gilbert et al., 2012), and disproportionate levels of activity and recruitment from the rare biosphere (Campbell et al., 2011).

The eastern coastline of Australia represents an ideal, yet understudied, region to examine dynamic patterns in microbial ecology, as it has been identified as a “climate

change hotspot" (Wu et al., 2012). In this region, seawater temperatures are rising three to four times more rapidly than in most other parts of the ocean (Ridgway and Hill, 2009), and coastal habitats are experiencing a high frequency of extreme weather events such as cyclones and high inflows and floods (Pepler and Rakich, 2010). The east coast of Australia is also where over 85% of the Australian population lives (Australian Bureau of Statistics, 2006), and where impacts from urban infrastructure and activities are most intensely impacting Australian coastal ecosystems (Clark and Johnston, 2017, Mayer-Pinto et al., 2015). Therefore, two urban beach locations were chosen to study patterns in microbial ecology at the near-shore in Sydney, situated within the temperate region of the east Australian coast, where impact from climate and anthropogenic activities tightly converge (Church et al., 2006, Mayer-Pinto et al., 2015)

1.7 Aims and objectives

The over-arching goal of this project is to comprehensively characterise bacterial communities collected from the near-shore waters of coastal marine environments, and in doing so, tease-out the environmental and biological interactions that drive shifts in community assemblages, and influence ecological and public health. This project will track community-scale patterns in both natural coastal marine bacteria and microbes introduced from terrestrial habitats and anthropogenic waste streams. In addition to a general assessment of microbial ecology of these coastal regions, there will be a specific focus on the dynamics of pathogenic microbes that are both (i) indigenous to coastal environments, and (ii) introduced via urban waste streams, with an over-arching goal of identifying the key environmental and anthropogenic processes that govern their

temporal patterns within coastal marine ecosystems. ***Within this framework, my specific Aims are to:***

1. Perform a high-resolution examination of the temporal dynamics of natural coastal microbial communities exposed to intermittent fluctuations in seawater chemistry influenced by natural and anthropogenic inputs using 16S rRNA gene sequence analysis.
2. Use a quantitative molecular technique call quantitative PCR to assess the occurrence and spatiotemporal dynamics of potentially harmful *Vibrio* bacteria in coastal environments.
3. Pair quantitative molecular techniques with correlation statistical approaches including Local Similarity Analysis (Xia, 2011) and network analysis (Faust et al., 2015) to track the abundance of antibiotic resistance genes in coastal marine ecosystems that are exposed to contrasting levels of anthropogenic inputs.

1.8 Study design

The sampling design of this thesis is based around a two-year time-series. Two urban beach locations within Sydney were selected to closely analyse bacterial community dynamics under contrasting hydrological and water quality conditions. We analysed the microbiology of water samples using a suite of molecular-based methods, , with focus on a diverse suite of microbiological features including;

- Amplicon sequencing, a next generation sequencing tool that allows broad screening of whole bacterial communities, and

- quantitative PCR, used to measure of the absolute abundance of genes that infer the presence of microbial groups of interest (eg. a species of pathogen), or genes that confer virulence or resistance to antimicrobial agents.

Important ecological patterns were teased out using several statistical and data visualisation methods, including;

- Multivariate statistics such as analysis of similarity (ANOSIM), similarity percentage (SIMPER) analysis, canonical correspondence analysis (CCA) and permutational analysis of variance (PERMANOVA) were used to describe patterns in microbial community assemblages and link these patterns to potential environmental drivers,
- Threshold indicator taxa analysis (TITAN 2) was used to detect bacterial taxon-specific changes of abundance and occurrence frequency that occur along an environmental gradient, and
- Network analysis, which combines correlation testing (local similarity analysis, Pearson's correlation coefficient) with a graphical tool to visualise and interpret co-occurrence and co-variation between organisms and their environment, that could be of ecological or public health significance.

In **Chapter 2**, flow cytometry and sequencing of the 16S rRNA amplicon was used to compare the abundance and composition of whole bacterial communities between sample locations. In this chapter, patterns observed in bacterial assemblages are described, and multivariate statistics and threshold indicator analysis is used to identify thresholds in environmental parameters that mark significant shifts in bacterial

community composition. In **Chapter 3**, screening of bacterial communities using 16S rRNA sequencing revealed the relative importance of Arcobacter, an emerging pathogen, that dominated communities following rainfall at the impacted beach. In **Chapter 4**, genes that confer resistance to antibiotics critical to human health were tracked and compared between beach locations. In **Chapter 5**, the focus shifted to indigenous marine pathogens belonging to the Vibrio genus and the influence of increasingly recurrent marine heatwave events in leading to blooms of these pathogens.

The development of a better understanding of the ecology of coastal microbial assemblages is important because these microbes govern the biogeochemical processes that both underpin the health and stability of coastal ecosystem and potentially impact human health. It is anticipated that through the application of novel approaches that combine molecular-based techniques with computational and correlative statistical methods, the work presented in this thesis will deliver a transformative understanding of coastal microbial ecology in urbanised beaches.

2. Chapter 2

Highly dynamic bacterial assemblage structure and contrasting environmental thresholds within coastal habitats

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Chapter 2 - Highly dynamic bacterial assemblage structure and contrasting environmental thresholds within coastal habitats

2.1 Preface

This chapter explores and contrasts the ecological dynamics of bacteria inhabiting one less impacted beach and one heavily urbanized beach. The focus of this work was to screen whole bacterial communities at high temporal resolution, with the objective of capturing community scale shifts across a temporal continuum, that could have important ecological implications.

2.2 Abstract

Microbes play a pivotal role in controlling the ecological and biogeochemical function of coastal ecosystems and are also highly sensitive indicators of natural oscillations and anthropogenic disturbances in highly dynamic coastal settings. We sought to understand how physical and biological processes within coastal marine habitats elicit temporal changes in microbial community assemblage structure, with a specific focus on the different effects of natural, seasonal variability and ephemeral perturbation events such as stormwater input. We tracked the abundance and composition of bacterioplankton communities at weekly intervals over 24 months at two beaches near to Sydney, Australia; one beach that is frequently subjected to stormwater input, and another less impacted site. From our 16S rRNA timeseries datasets, the core, transient and rare microbiome populations within bacterial communities were discriminated, and temporal patterns were linked to environmental drivers. The two beaches harbored strikingly similar core microbiome assemblages, which exhibited a distinct marine signature. However, both sites hosted very dissimilar transient and rare microbiomes, including more than two-fold higher species richness at the less impacted beach.

Chapter 2 - Highly dynamic bacterial assemblage structure and contrasting environmental thresholds within coastal habitats

Extended Local Similarity Analysis enabled inference of direct relationships between environmental variables and bacterial members based on time-lagged and co-occurrence patterns, while using the threshold indicator taxa analysis pipeline allowed detection of environmental change point thresholds. Using these approaches, we determined that temperature best explained the seasonal shifts in core and transient microbiomes at the less impacted site. Conversely, at the impacted site, in instances where stormwater and wet weather sewer overflows were significant, the marine signature of the core microbiome diminished, and was shifted to one temporarily dominated by enteric microbes washed in from stormwater and sewer environments. We also detected seasonal switching between *Pelagibacter* and *Synechococcus*, the two most dominant marine OTUs in this study, and opposing temperature preferences and change point thresholds between closely related OTUs. Overall, this study has revealed that contrasting environmental influences elicit different processes across core, transient and rare components of bacterial communities. These seasonal and episodic processes that influence the assemblage structure of bacterial communities could also influence community function, which would have implications for ecosystem productivity and health.

2.3 Introduction

Coastal ecosystems represent only 7% of total ocean volume, but they account for disproportionately high levels of primary production, nutrient cycling and biodiversity (Azam, 1998, Alongi and McKinnon, 2005, Teeling et al., 2012), meaning that the coasts have a strong influence on ocean function. They are also highly dynamic relative to other environments because coastal marine environments are influenced by a broad range of natural and anthropogenic terrestrial inputs (Clark and Johnston, 2017), and dynamic hydrological processes. Relative to other, comparatively stable environments such as the open ocean and many terrestrial habitats, coastal environments support a high diversity and temporal over-turn of environmental niches and communities (Blum and Mills, 2012). Therefore, there are contrasting and competing influences from longer term perturbations and episodic events like urban runoff and stormwater input. Within this context, coastal habitats make excellent dynamic model environments for examining microbial ecology.

Microbes play a pivotal role in coastal ecosystems, where they have the capacity to exacerbate negative environmental changes, such as diseases of benthic animals (Tout et al., 2015) or mitigate anthropogenic impacts, as demonstrated by the rapid degradation of oil spills by natural bacterial populations (Kostka et al., 2011). Furthermore, microbes are fast growing, sensitive to changes in their habitat and respond rapidly to perturbations, making them promising candidates as indicators of environmental change, which could provide early warning of imminent ecological or public health hazards (Stewart et al., 2008).

Chapter 2 - Highly dynamic bacterial assemblage structure and contrasting environmental thresholds within coastal habitats

The assemblage structure of coastal microbial communities is influenced by a multitude of complex ecological interactions, incorporating selection for specific populations or phenotypes imposed by environmental factors that change on different time-scales (Gienapp et al., 2008), closely coupled competition and predator-prey interactions (Fuhrman et al., 2015) and successional dynamics (Teeling et al., 2012). As a consequence the structure and function of coastal microbial assemblages is often highly nuanced, heterogenous and difficult to predict (Fuhrman et al., 2015, Fuhrman and Steele, 2008). At a broad scale, meteorology and oceanography influence the composition and temporal dynamics of coastal microbes, leading to geographical and seasonal partitioning of communities (Fuhrman et al., 2008). However, recent evidence suggests that climate change processes, which can cause higher frequency of extreme weather events and rising sea surface temperatures, are blurring some of these distinctions (Flombaum et al., 2013). In addition to broad-scale regional and seasonal forces, terrestrial influences and anthropogenic impacts drive further variability. For instance, river plumes transport terrestrial and lotic substrate, nutrients and microbes into coastal environments, shifting the productivity and composition of coastal microbial assemblages (Fortunato et al., 2013). In addition, anthropogenic infrastructure, including urban stormwater systems and sewage overflow inputs introduce both enteric microorganisms (Ahn et al., 2005) and a suite of anthropogenic chemicals including antibiotics, detergents and heavy metals into coastal habitats (Sidhu

et al., 2013), which can strongly govern the nature of microbial communities (Nogales et al., 2011).

As a consequence of the diversity of ecological drivers, coastal microbial assemblages are likely to display meaningful variability in abundance, activity, composition and function over a continuum of time-scales. Seasonal fluctuations in day-length, light levels, temperature and terrestrial run-off will lead to distinct shifts in the assemblage structure of microbial communities (Gilbert et al., 2012, Giovannoni and Vergin, 2012). Ecological processes, such as algal blooms lead to rapid, and drastic changes in water chemistry, such as oxygen depletion (Howarth et al., 2011), breakdown of trophic structure and food web collapse (Anderson et al., 2002), and the release of toxins harmful to animals and humans (O'Neil et al., 2012, Paerl et al., 2016). Episodic external stimuli, such as heatwaves (Smale et al., 2017) or rainfall events (Ackerman and Weisberg, 2003) will often lead to immediate biological responses, as often seen by the transport of allochthonous runoff following heavy precipitation (Ackerman and Weisberg, 2003), or occurring with a significant time lag, where the response of endemic microbial assemblages can take days to weeks.

Microbial community dynamics in coastal settings are intrinsically difficult to capture, because anthropogenic driven perturbation events may confound more predictable, seasonal patterns and oscillations, because they are episodic, occur outside of seasonal bounds, and drastically alter the trophic state of coastal marine habitat. Furthermore, examinations of the microbiology of coastal habitats are often biased towards indicator

organisms (Boehm and Sassoubre, 2014), which miss the vast majority of natural species (Staley, 1985). Here we aim to deliver a more complete understanding of microbial community dynamics within a highly heterogenous and dynamic coastal setting by employing network analysis and community threshold analysis to disentangle seasonal climate influences from more stochastic perturbations caused by stormwater and sewage terrestrial inputs and anthropogenic impacts.

2.4 Materials and Methods

2.4.1 Study sites, data and sample collection

Patterns in planktonic microbial community dynamics were examined at two study sites, in Sydney (Australia), that were selected according to different hydrological regimes and contrasting levels of exposure to urban impacts, including storm-water and sewage pollution, according to ongoing monitoring by the state beach water quality monitoring department, BeachWatch. Within this context, we defined these urban beaches as one that is highly impacted, and one relatively less impacted. The heavily impacted site at Foreshore Beach, is situated within the partially enclosed waters of Botany Bay (33.95 ˚S, 151.9 ˚E) (SI Fig 1) and subject to the influence of multiple storm-water outlets, resulting in frequent exposure to high levels of urban storm-water discharge and periodic sewer overflows from upstream overflow outlets (NSW Office of Environment and Heritage, 2015). In contrast, the second, less impacted site at Maroubra Beach (33.95˚S, 151.25 ˚E) is directly exposed to the Tasman Sea and experiences higher levels of hydrodynamic flushing and substantially less impact from stormwater inputs (NSW Office of Environment and Heritage, 2015) (SI Fig 1). At each site, we conducted weekly

sampling over 24 months (May 2, 2014 – April 29, 2016) to track patterns in bacterial community dynamics.

Rainfall data was retrieved from the Australian Bureau of Meteorology website (www.bom.gov.au/climate/data), using the nearest gauge station to each sampling site. Storm-water inputs were identified according to decreases in water salinity, measured here as electrical conductivity (EC), which allowed for separation of sample time points into either stormwater impacted or non-impacted samples. Additionally, at Foreshore Beach, modelled overflow data derived from the Model for Urban Sewers (MOUSE) wastewater hydraulic system model (DHI inc., PA, U.S.A), was provided by Sydney Water. This was used to estimate the total weekly volume of wet weather sewer overflow (WWSO) released at Mill Pond, an upstream sewer overflow discharge point, which allowed for further distinction of WWSO events from stormwater-only events. Here, we define WWSOs as the release of excess, dilute wastewater, containing a mixture of stormwater and sewage, from the wastewater system in heavy rain. Modelled WWSO volumes were obtained from Sydney Water (the water authority responsible for the operation of Sydney's wastewater systems) as rates of WWSO (measured in m^3 15 min^{-1}). These values were summed across the week preceding each sampling event. WWSO events were defined as samples collected within 7 days of a modelled WWSO from the Mill Pond overflow point into Botany Bay.

At each site, all samples were collected in triplicate from near to the shore-line (<1.5 m depth). Physico-chemical parameters, including temperature, pH, electrical conductivity

(EC) and dissolved oxygen (DO) were measured *in situ* using a WTW Multiprobe meter (Multi 3430, Germany). For analysis of dissolved inorganic nutrients, including NH_4, NO_x and PO_4, triplicate 50 mL samples were collected and filtered through a 0.45 µm filter (Sartorius Stedim Biotech) and immediately transported to the laboratory on ice. Samples were stored at -80 °C within 2 hours of sample collection for later nutrient analysis. For bacterial enumeration, triplicate 1 mL water samples were collected from each 5 L water sample, filtered through a 100 µm mesh, fixed with glutaraldehyde (2% final concentration) and snap frozen in liquid nitrogen for subsequent flow cytometric (FCM) analysis. Microbial DNA was retrieved by filtering triplicate 2.5 L samples through 0.22 µm pore-size membrane filters (Millipore) using a peristaltic pump. Filters were stored at -80 °C for subsequent DNA extraction.

2.4.2 Nutrient analysis

Concentrations of NH_4, NO_x and PO_4 were determined via a colorimetric analysis using the Lachat Quickchem QC8500 Automated Ion Analyser (LACHAT Instruments, USA) in accordance with the manufacturer's guidelines. Internal controls, including addition of spiked recovery samples and blanks were included to monitor the consistency of instrument performance. The resulting data were transferred and interpreted using Omnion version 3 software (LACHAT Instruments, USA) as per manufacturer's guidelines.

2.4.3 Bacterial cell enumeration

Prior to analysis, samples were quick-thawed in warm water and stained with SYBR Green I nucleic acid stain (1:10,000 final dilution; Molecular Probes, Eugene, OR, USA) (Marie et al., 1997, Gasol and Del Giorgio, 2000, Seymour et al., 2007). Fluorescent reference beads (1 µm in diameter, yellow–green; Molecular Probes) were added to each sample as an internal concentration and fluorescence standard in a final concentration of 10^5 mL^{-1}. Samples were analysed using an LSRII flow cytometer (Becton Dickinson, San Jose, CA, USA) and bacterial populations were discriminated according to cell side scatter (SSC) and SYBR Green fluorescence (Seymour et al., 2007). Data-files were analysed using WinMDI 2.9 software (WinMDI, Joseph Trotter, Salk Institute for Biological Studies, La Jolla, CA, USA).

2.4.4 DNA extraction and 16S rRNA-amplicon sequencing

Microbial DNA was extracted from 0.22 µm membrane filters using the Powerwater DNA isolation Kit (MoBio Laboratories, Inc) in accordance with the manufacturer's instructions. DNA quantity and purity was evaluated using a Nanodrop-1000 Spectrophotometer. Patterns in bacterial community diversity and composition throughout the two year time-series were characterised using 16S rRNA amplicon sequencing. The V1–V3 variable region of the 16S rRNA gene was amplified using the primers 27f 5'-AGRGTTTGATCMTGGCTCAG-3' and 519r 5'-GTNTTACNGCGGCKGCTG-3'. Sequencing was performed using the Illumina MiSeq platform following the manufacturer's guidelines. Raw data files in FASTQ format were deposited in the NCBI

Sequence Read Archive (SRA) with the study accession number SRP143624 under Bioproject number PRJNA4209011.

16S rRNA gene sequences were analysed using the QIIME pipeline (Caporaso et al., 2010, Kuczynski et al., 2012). Briefly, paired-end DNA sequences were joined and *de novo* Operational Taxonomic Units (OTUs) were defined at 97% sequence identity using UCLUST (Edgar et al., 2011). Chimeric sequences were detected using ChimeraSlayer (Haas et al., 2011) and filtered from the dataset. Taxonomy was assigned against the SILVA v128 database using the BLAST option in QIIME. Sequences were rarefied to the same depth to remove the effect of sampling effort upon analysis, and Shannon's Index was calculated within QIIME to quantify alpha-diversity. All chloroplast OTUs were filtered out of the bacterial data-set.

2.4.5 Bacterial community biomes

To reveal underlying patterns in microbial community dynamics, that are otherwise masked by the inherent enormity and complexity of microbial OTU datasets, OTUs within the 16S rRNA bacterial relative abundance profiles at each site were partitioned into sub-groups, based on occurrence frequency (ie. how often each OTU was present), across the 24 month timeseries. OTUs present in > 90% of time points were categorised as members of the "core population". Whereas OTU's present in < 90% of time points, but contributing > 1% of the total community in at least one time point, were classed as "transient and conditionally rare" members. We defined the low abundance (rare)

populations as OTUs that each contributed < 0.01% of all sequence counts, and did not exceed > 1% of the community at any given time point. As the 16S rRNA sequence dataset was rarefied (to exclude even the sequence depth across all samples), it is possible that some or many other rare bacterial taxa were present but excluded from this study.

2.4.6 Identifying potential environmental drivers of microbial community assemblages

Canonical Correspondence Analysis (CCA) (Ter Braak, 1986) was used inside the Paleontological Statistics 3.20 (PAST) software to identify the key environmental variables that best explain patterns in microbial assemblages at Maroubra and Foreshore beaches. The implementation of CCA in PAST follows the eigenanalysis algorithm provided in *Legendre and Legendre*, 1998. The ordinations (OTU relative abundance values) were given as site scores, and environmental variables were plotted as correlations with site scores. Prior to analysis, OTU relative abundance values were square-root transformed to minimise the effect of outliers while maintaining proportionality, and environmental parameters were standardised using LOG (x+1) transformation. CCA was performed for 16S rRNA bacterial OTU community profiles at each site.

Exploration of statistically significant correlation patterns between bacteria and environmental parameters was conducted within each site, across the 104 week time-series using Extended Local Similarity Analysis (eLSA) (Xia et al., 2011). eLSA was used to identify statistically significant correlations, including time-lagged interactions, between

OTUs, and environmental variables, for subsequent construction of microbial networks. Only pairwise interactions that occurred across 10% or more of the time points were included, and each remaining interaction was subjected to 1000 permutations to calculate statistical significance (p value) and the false detection rate (q value). Interactions where $p > 0.001$ and $q > 0.01$ were excluded to reduce the risk of Type I and Type II errors. Resultant data defined all statistically significant co-occurrence events, including those that occurred with -2 to +2 time-interval time lags. eLSA data was ultimately visualised using the edge-weighted spring embedded function in Cytoscape 3.4.0 software package, using the local similarity (LS) value of pairwise correlations to define the length of edges connecting nodes.

2.4.7 Seasonal microbial community dynamics

Tracking the composition of whole bacterial communities at weekly intervals over two years allowed statistical testing for significant difference between seasons, and difference between varying water conditions. Using the methods described in (Anderson, 2001), permutational analysis of variance (PERMANOVA) was employed in PAST to test for significant difference between community assemblages across seasons (24 replicates per season), and contrasting physico-chemical conditions (dry, stormwater or sewer overflow impacted). Canonical correspondence analysis (COA) was also performed to confirm the significance of differences, given the repeated measures in the study design. Similarity percentage (SIMPER) (Clarke, 1993) was subsequently

used to identify specific taxonomic groups principally responsible for the seasonally and conditionally driven distinction between assemblages.

2.4.8 Identification of taxon-specific change points along novel environmental gradients

CCA results were also used to inform the selection of environmental parameters for inclusion in the Threshold Indicator Taxa Analysis (TITAN). We used the TITAN2 pipeline, developed by Baker and King (2010), in R.3.4.3, to detect the key transitional points, also referred to as change points or zones of rapid change, in taxon abundance (16S rRNA bacterial OTU profiles in this study), that occur in response to small, continuous increases in an environmental parameter. TITAN2 uses the indicator value (IndVal), described in (Dufrene and Legendre, 1997), which was calculated for all OTUs, for all possible change points along an environmental gradient, with permutation tests to assess the uncertainty in these scores. Permutated IndVal scores are standardised as Z scores and summed for positive [sum(z+)] and negative [sum(z-)] value responses for each possible change point. Peaks or dips in sum(z) indicate locations within the environmental variable gradient where many taxa exhibit strong directional changes in abundance and occurrence frequency, termed “community thresholds”. Indicator reliability (proportion of bootstrap replicates with maximum IndVal reaching a specified p value), purity (proportion of bootstrap replicates matching group assignment in the original data) and uncertainty requires that 95% of the results from bootstrap replicates have a IndVal p-value less than or equal to 0.05, indicating a response magnitude at a

given change point location that differs significantly from what would be expected from random permutations. The location of individual taxon and community change points were estimated using bootstrapping (1000 repetitions). Taxa displaying < 0.95 purity, reliability < 0.95, p > 0.05, or 100% frequency were excluded from this analysis.

2.5 Results

2.5.1Water chemistry and physical conditions

In situ water temperature followed seasonal patterns at Foreshore and Maroubra beaches, where mean temperatures were highest in Summer (23.7 ± 1.9 and 22.0 ± 1.8 °C respectively) and lowest in Winter (16.3 ± 1.3 and 17.3 ± 1.0 °C respectively), and temperature range was higher at Foreshore Beach (13.4 °C) compared to Maroubra Beach (9.4 °C). Concentrations of dissolved inorganic nutrients, including NH^3, NOx and PO^4 were significantly higher at Foreshore Beach, compared to Maroubra Beach (p = < 0.05), with mean concentrations highest at both locations in Summer compared to other seasons. Rainfall had a stronger impact on electrical conductivity at Foreshore Beach compared to Maroubra Beach, due to Foreshore Beach's frequent exposure to stormwater runoff from a highly urbanised and industrial watershed. The input of stormwater following heavy precipitation correlated with drops in electrical conductivity (EC) (p < 0.05) (SI Fig 2). EC values returned to baseline levels (ranging between 52 – 54 mS cm^2 during dry periods) by the subsequent sampling occasion, with the exception of periods impacted by prolonged rainfall. Sixteen stormwater events were observed during the 24 month study period, of which, 10 events involved wet

weather sewer overflows (WWSO). These WWSO events ranged in magnitude from 4,193 m^3 $week^{-1}$ to 465,438 m^3 $week^{-1}$ (SI Fig 3). Conversely, the less impacted site at Maroubra Beach experienced only two isolated weeks where stormwater input was sufficient to reduce salinity below marine baseline levels (SI Fig 2). Accordingly, water chemistry was notably dissimilar and mean concentrations of nutrient levels were significantly lower compared to Foreshore Beach (SI Fig 2).

The input of stormwater at Foreshore Beach led to drops in dissolved oxygen (DO) (PEARSON 2.54, $p < 0.05$), from concentrations > 8mg L^{-1} prior to input, down to concentrations as low as 2.4mg L^{-1}. Stormwater inputs also led to significantly higher concentrations of NH_4 (ANOVA; F = 6.65, d*f* = 1,76, p = 0.01) (SI Fig 2), while WWSOs led to significantly higher levels of NH_4 (ANOVA; F = 9.51, d*f* = 1,80, p = < 0.01) and PO_4 (ANOVA; F = 8.05, d*f* = 1,80, p = < 0.01) relative to dry periods. The highest nutrient concentrations were recorded following WWSOs, when up to 22-fold higher concentrations of NH^4 were observed relative to baseline conditions (SI Fig 3). Conversely, all measured nutrients remained relatively stable and did not vary significantly between seasons ($p > 0.05$) at Maroubra Beach. Mean concentrations of NH_4 (23.2 mg L^{-1} $\pm$ 10.4 mg L^{-1}), PO_4 (5.5 mg L^{-1} $\pm$ 2.4 mg L^{-1}), and NOx (26.7 mg L^{-1} $\pm$ 10.9 mg L^{-1}) at Maroubra Beach were not significantly different from concentrations measured during dry periods at Foreshore Beach ($p > 0.05$), but were significantly lower compared to stormwater and WWSO impacted periods at Foreshore Beach (SI Fig 3).

2.5.2 Bacterial abundance

Bacterial abundance patterns at Maroubra Beach followed a strong seasonal trend (ANOVA; $F = 7.14$, $df = 3,99$, $p = < 0.001$), attributed to higher concentrations in Spring and Summer, which were twice the mean concentrations measured in Autumn and Winter (Fig 1). Significant seasonal variation in bacterial abundance was not detected at Foreshore Beach. However, In addition to nutrient augmentation, stormwater and WWSO events were also correlated with bacterial abundance (Fig 1). The mean concentrations of bacterial cells at Foreshore Beach were significantly different between periods experiencing dry, stormwater and WWSO's conditions (Kruskal-Wallis; $H = 8.837$, $p = 0.01$), attributed to the increase in mean bacterial cell concentrations during stormwater input and WWSO events, compared to dry periods (Fig 1). Conversely, stormwater input had insignificant influence at Maroubra Beach ($p > 0.05$).

2.5.3 Bacterial diversity

Over the study period, Maroubra Beach exhibited greater bacterial community richness compared to Foreshore Beach, with each site harboring 725 and 388 OTUs respectively, matching to 45 and 35 different bacterial genera respectively (Fig 2a,b). Peaks and troughs in OTU richness and diversity tracked consistently at both beaches, with the exception of one period at Maroubra Beach, where Shannon's diversity dropped sharply to 1.1, but richness only decreased modestly. This was during a summer 2015 when the relative abundance of Synechococcus peaked. Bacterial richness and Shannon's diversity did not vary significantly between seasons at either Maroubra or Foreshore beaches (p

> 0.05) (Fig 2b). However, at Foreshore Beach, stormwater input and modelled WWSO events led to sharp, temporary increases in bacteria richness and Shannon's diversity at the OTU level ($p < 0.05$), when compared to dry periods (Fig 2a).

2.5.4 Bacterial community composition

Throughout the study period, a total of 614 water samples were collected from Foreshore and Maroubra beaches, comprising 104 time points, sampled in triplicate, across a 24 month time series. Sequencing of the 16S rRNA gene yielded 15,832,356 bacterial sequences from Foreshore and Maroubra beaches. After OTU clustering at 97% similarity, rarefication to 1931 sequence reads per sample was performed, and sequence quality control (discard of potentially erroneous OTUs and those contributing < 100 sequences across the entire timeseries).

At Foreshore Beach, 5 OTUs were defined as "core" organisms, occurring in > 90% of samples across the time-series (Fig 3). These included OTUs exhibiting closest affinity to *Pelagibacter,* S*ynechococcus,* and *Rhodobacter*. A further 205 bacterial OTUs were detected in < 90% of samples and therefore classified as transient and conditionally rare (Fig 3). The most abundant members of the transient microbiome included bacteria commonly associated with stormwater and sewer environments, such as *Thiomicrospira*, *Anaerospora*, *Arcobacter* and *Cloacibacterium* (Fig 3). with this category comprising 178 OTUs matching to 21 different genera. The most temporally persistent

of the rare genera included OTUs displaying high affinity for *Phaeobacter*, *SargSea-WGS* and *Turicibacter*, which were present in 26%, 16% and 12% of sample occasions respectively (Fig 1c). Several other OTUs that did not have representation in the transient or core populations were only present in the rare microbiome, including genera notable for their involvement in human illness, including *Vibrio*, *Streptococcus* and *Staphylococcus*. *Vibrio* was one of the most prevalent rare OTUs, detected in 34% of samples, but at low abundances below 1% of the total community, whereas *Streptococcus* and *Staphylococcus* were comparatively less prevalent, detected in 3.9 and 0.2% of samples.

Although seasonality did not lead to significant quantitative changes in total bacterial abundance or alpha diversity at Foreshore Beach, significant differences in bacterial community composition occurred between all seasons (PERMANOVA; $F = 10.31$, perm = 9999, $p = < 0.001$). Summer and Winter bacterial assemblages exhibited the highest level of dissimilarity between each other (PERMANOVA; $F = 14.42$, perm = 9999, $p = < 0.001$), which was mainly attributed to the higher relative abundances of core OTUs matching marine bacterial groups *Pelagibacter* and *OCS155* (from the *Acidomicrobiales* family) during Winter and Spring (Fig 3). According to SIMPER, these organisms contributed 6.4 and 4.8% respectively to seasonal dissimilarity in community composition. Temperature was the strongest potential driver of this pattern, with the relative abundance of both OTUs negatively correlated with temperature, with a 1 week time-lag (*Pelagibacter*_C1: eLSA; LS = -0.31, d -1, $p = 0.007$; *OCS155* eLSA; LS = -0.42, d = -1, $p < 0.001$). In contrast,

OTUs closely related to *Synechococcus* were the most abundant in Summer and Autumn, accounting for 4.2% (SIMPER) of the average seasonal dissimilarity, with *Synechococcus* OTUs positively correlated with temperature – *Synechococcus*_C1 (eLSA; LS = 0.66, d = 0, p = 0.007).

Although clear seasonal shifts in bacterial community structure was apparent at Foreshore Beach, the input of stormwater and WWSO were by far the strongest driver of temporal heterogeneity in community assemblage structure (PERMANOVA; F = 14.16, perm = 9999, p = < 0.001) (Fig 4a). The significant dissimilarity (PERMANOVA; F = 10.26, perm = 9999, p = < 0.001) between community structure during dry periods and storm-water impacted samples was mainly attributed to reduced presence of the core OTUs *Pelagibacter* (6.7% average dissimilarity), *OCS155* (4.7% average dissimilarity) and *Synechococcus* (4.0% average dissimilarity), and a concomitant increase in the abundance of transient bacterial groups, including 3 *Arcobacter* OTUs (4.1% average dissimilarity) and 17 other OTUs as determined using SIMPER. These OTUs all exhibited negative correlations to EC (LS scores from -0.59 to -0.35, d = 0, p < 0.001), indicating a link to stormwater input. The dissimilarity between microbial assemblages occurring during dry-periods and WWSO-periods was even more pronounced (PERMANOVA; F = 22.93 perm = 9999, p = < 0.001), and similarly characterized by a decreased relative abundance of presence of core marine bacterial groups, and an increase in a suite of transient and conditionally rare putatively sewage-associated (Collado et al., 2008) enteric bacteria, including 4 OTUs matching *Arcobacter, which* contributed up to 7.7%

dissimilarity according to SIMPER analysis. In addition to these Arcobacter OTUs, another 18 bacterial OTUs were positively correlated with WWSO inputs (Fig 5).

The core component of the bacterial assemblage inhabiting the less impacted site at Maroubra Beach comprised a marine signature similar to the core community observed at Foreshore Beach, with five taxa closely related to *Synechococcus*, OCS155, *Pelagibacter*, *Rhodobacter*, and *Lesingera* (Fig 6). A further 226 OTU's were classified as transient and conditionally rare (Fig 6). The most abundant bacterial members within this component included OTUs closely matching members of the *Arcobacter*, *Ulvibacter*, *Formosa* and *Acidovorax* (Fig 6). Maroubra Beach harboured a far richer rare bacterial microbiome compared to Foreshore Beach, comprising 494 OTUs matching to 21 bacterial genera (Fig 1d). Furthermore, the rare microbiome of Maroubra Beach was very dissimilar to Foreshore Beach, with only 3 out of 21 rare genera (including *Desulfotalea*, *Phaeobacter* and *Psychrobacter*) being common between locations. The most persistent rare organisms at Maroubra Beach included *Balneola*, *Polynucleobacter* and *Trichococcus*, which were present at 25%, 23% and 22% of sample occasions respectively (Fig 1d).

Significant seasonal variation in the composition of bacterial communities was observed at Maroubra Beach (PERMANOVA; F = 3.91 perm = 9999, p = < 0.001) (Fig 5b), although to a lesser extent than the strong shifts seen at Foreshore Beach. Seasonal dissimilarity

in microbial assemblage structure at Maroubra Beach resembled the patterns observed at Foreshore Beach, and was attributed to variation in the abundance of core OTUs as determined using SIMPER; *Pelagibacter*_1 and *Thiomicrospira_1* (average dissimilarity 5.8 and 3.6% respectively) (Fig 7). *Pelagibacter*_1 correlated negatively with temperature (eLSA; LS = -0.38, d = 0, p = 0.007), and dominated in Winter, while *Thiomicrospira*_1 also correlated negatively with temperature (eLSA; LS = -0.21, d = 1, p = 0.02) (Fig 7) but was most dominant in Spring. In-line with observations at Foreshore Beach, two core OTUs matching *Synechococcus* were the most abundant bacterial members in Summer and Autumn, and correlated significantly with temperature (eLSA; LS = 0.74, d = -1, $p < 0.01$, and LS = 0.59, d = -1, $p < 0.001$) (Fig 7).

Although rainfall had considerably less influence over water quality at Maroubra Beach relative to the impact seen at Foreshore Beach, Maroubra Beach did experience two occasions where stormwater input was significant. At these times, *Pelagibacter* increased two-fold in relative abundance, contributing the most to community dissimilarity (14.8% - SIMPER), and the relative abundance of *Synechococcus* decreased 50%, between stormwater impacted and dry periods.

2.5.5 Bacterial community threshold responses along two environmental gradients

Using the TITAN2 pipeline we evaluated the bacterial response to different environmental stressors, at both individual OTU and community scales. Following the

determination of the strongest factors influencing patterns in bacterial community composition at each site, using CCA, we next employed TITAN2 to distinguish the strength and direction of each OTU response to EC at Foreshore Beach, and temperature at Maroubra Beach. By tracking the positive (z+) and negative (z-) responses of each OTU, at each increment along the EC and temperature gradients, change thresholds for individual OTUs were determined. Using the same approach, TITAN2 was used to calculate the sum of all bacterial taxa responses when they increased sum(z+) or decreased sum(z-) in response to their respective environmental gradient, to identify community response thresholds.

Given that stormwater was the strongest environmental influence over patterns in microbial assemblages at Foreshore Beach (confirmed using CCA) (Fig 4a), TITAN2 was used to identify bacterial community change points (or thresholds) along an EC gradient. The outcomes revealed a filtered sum (z+) value of 40.8 mS cm^2, indicating this is the threshold for the strongest bacterial responses to shifting EC (Fig 8a). A total of 22 out of 50 of the most abundant OTUs were identified as pure and reliable z+ taxa, meaning their abundance patterns met the assumptions of the TITAN2 test, and were appropriate for inclusion. The strongest indicator z+ OTUs, including 3 OTUs matching the *Flavobacteria: Flavobacterium_1* (IndVal = 92.8), *Flavobacterium_2* (IndVal 90.8) and *Flavobacterium_3* (IndVal 88.4), become more abundant when EC values reached 34.5, 32.3 and 40.5 mS cm^2respectively (Fig 9a). Other important z+ taxa included 3 OTUs matching *Pelagibacter*, 2 OTUs matching *Synechococcus*, and 2 OTUs matching *Arcobacter* (Fig 9a). A total of 10 out of 50 most abundant OTUs were identified as pure

(requiring that 95% of the results from bootstrap replicates agree with the observed response direction) and reliable z- taxa, which comprised a suite of quintessential sewage bacterial genera, including *Anaerospora*, *Arcobacter*, *Bacteroides*, *Cloacibacterium* and *Tenacibaculum*. The frequency of most z- OTU's was highest when EC values were below the filtered sum (z-) value of 35.5 mS cm^2 (Fig 9a). The z- OTUs determined to be the strongest indicator taxa for OTUs that prefer low EC values were *Roseovarius* (IndVal = 95.23), *Arcobacter_2* (IndVal = 88.32) and *Cloacibacterium* (IndVal = 79.86), which became more abundant at EC values of 18.1, 35.5 and 40.8 mS cm^2 respectively (Fig 9a).

Temperature was revealed, via CCA, to correlate strongest with temporal patterns in bacterial communities at Maroubra Beach (Fig 4b), and was therefore examined further using TITAN2, under the same rationale employed for Foreshore Beach samples. A total of 34% of the 50 OTUs were removed as they did not pass purity, reliability or frequency checks. Of the remaining 34 OTUs, 16 were identified as z+ taxa, meaning they increased in occurrence frequency (proportion of samples where OTU was present) and abundance beyond the threshold of 18.85 °C (Fig 8b). The strongest positive indicator OTUs, defined as z+ OTUs that display the highest IndVal scores, included *Flavobacterium*, *Persicirhabdus* and *Ulvibacter*, which exhibited thresholds of 19.35, 19.15 and 18.6 °C (Fig 9b). The remaining 18 OTUs were identified as z- taxa that decreased in abundance and occurrence frequency at temperatures below the sum (z-) value of 20.35 °C (Fig 9b). The highest IndVal scores were associated with OTUs closely

matching *Thiomicrospira* (IndVal 83.1), *Arcobacter_6* (IndVal 81.7) and *Synechococcus_4* (IndVal 80.4), which displayed change point values of 19.45 °C, 18.85 °C, and 18.95 °C respectively (Fig 9b). Notably, several OTUs closely affiliated with the same genera exhibited opposing preference for temperature. For example, OTUs *Synechococcus, Synechococcus*_4 and *Synechococcus*_5 increased in response to temperature, but at temperatures ranging from 17 – 20 °C, while closely related *Synechococcus*_2 decreased in response to temperature. A similar pattern was also observed for OTUs affiliated to *Arcobacter*, *Fluviicola* and *Tenacibaculum*.

2.6 Discussion

Microbial communities inhabiting coastal waters play a key role in near shore ecosystem function (Cloern et al., 2014, Dang et al., 2008), and contribute greater levels of productivity and nutrient cycling relative to pelagic ocean environments (Field et al., 1998). Coastal environments are also where humans and animals come into direct contact with marine microbes that inhabit coastal surface waters, and enteric microbes of public health relevance, derived from waste streams (McLellan et al., 2015). For the latter reason, much of our understanding of coastal microbiology is biased towards beach monitoring programs that track the presence of fecal pollution using indicator organisms such as *E. coli* and Enterococci (Boehm and Sassoubre, 2014). However, these approaches ignore more than 99% of microbes that do not grow on selective media (Fuhrman, 2007). Furthermore, most investigations of coastal microbial ecology lack the temporal resolution and duration required to capture rapid shifts in community

composition, which are difficult to anticipate but typical of coastal environments. Here we combined 16S rRNA sequencing with network analysis and community threshold testing to disentangle the complex interactions between microbes and their environment in near-shore marine environments.

2.6.1 Seasonal trends in bacterial community composition

Coastal bacterioplankton communities exhibit high levels of spatiotemporal heterogeneity (Herlemann et al., 2011, Kirchman et al., 2005), but several recent studies demonstrate repeatable and predictable patterns in community assemblage (Fuhrman et al., 2006, Fortunato et al., 2013). Patterns that are repeatable are typically those that follow seasonal trends in association with temperature, day-length and wind (Gilbert et al., 2012, Heba El-Swais, 2014). In this study, bacterioplankton communities at Maroubra and Foreshore beaches shared a similar core microbiome, consisting of archetypal marine bacteria, which typically followed seasonal patterns. The most relatively abundant OTU at both beach locations was an OTU matching *Pelagibacter*, a member of the SAR11 clade - the most abundant heterotrophic prokaryote in the ocean (Morris et al., 2002). The most abundant *Pelagibacter* OTU displayed a strong affinity for cooler conditions in Winter and early Spring, but were succeeded in Summer and Autumn by two OTUs matching *Synechococcus*, which became the most relatively abundant OTUs at those times. Their large increase in relative abundance resulted in a profound decrease in community diversity, but not richness. *Synechococcus* is a ubiquitous marine cyanobacteria and important contributor to primary production and

nutrient cycling (Bertilsson et al., 2003), providing nutrient resources to other bacterial species. It is also interesting to note that several less abundant *Pelagibacter* and *Synechococcus* OTUs exhibited contrasting seasonal dynamics to their dominant counterparts, demonstrating that closely related bacteria can exhibit dissimilar environmental preferences and can occupy different ecological niches. Understanding ecological patterns in the micro-diversity of microbial communities is important because these subtle differences in phylogeny might have broad implications for net microbial function and productivity (Fuhrman, 2009).

2.6.2 The rare coastal microbiome

The diversity and significance of the rare microbiome of marine microbial communities has only relatively recently been comprehensively examined (Sogin et al., 2006, Campbell et al., 2011, Sjostedt et al., 2012). Although the bacterial members that constitute the rare microbiome maintain low abundances as individual species, collectively they can contribute over 20% of total bacterial productivity (Campbell et al., 2011). The rare microbiome may also function as an important seedbank and recruitment pool, where members of the rare biosphere can become abundant and restore ecological balance after disturbance (Sjostedt et al., 2012). These bacteria could have important roles in ecosystem processes, and support community resilience against environmental stressors (Sjostedt et al., 2012).

In this study, it was found that most of the dissimilarity in bacterial community composition between Maroubra and Foreshore beaches was attributed to differences in low abundance populations. Maroubra Beach harbored twice the richness within its low abundance populations, and comprised a broad suite of bacterial genera, from those typical of marine waters, to others more commonly associated with sediments or sewage. On the other hand, the low abundance populations at Foreshore Beach was comprised almost exclusively of OTUs matching stormwater, sewage and human associated bacterial genera. When considering the potential ecological processes responsible for this apparent disparity between beaches in rare microbiome species richness, we derived the following, two-part explanation: 1) Lower species richness in the microbiome of Foreshore Beach is the result of high frequency disturbances caused by stormwater and WWSO inputs . These events elicit strong selective force for a narrow breadth of bacterial guilds, requiring that these bacteria must be both resilient to anthropogenic pollutants, and capable of rapid uptake of ephemeral nutrient inputs to avoid being out-competed; 2) The higher levels of stability in physicochemical conditions at Maroubra Beach better permit the establishment of prolonged environmental niches, which support the persistence of a more diverse array of rare species, whereas the extremely dynamic conditions at Foreshore Beach are constantly leading to local extinction of rare species within the rare microbiome.

2.6.3 Allochthonous inputs fuel microbial abundance and diversity

The coastal environment is an interface between terrestrial and marine biomes, where microbes that inhabit urbanised coastal ecosystems often experience heavy influence from allochthonous runoff. Foreshore Beach experienced 16 stormwater and 10 WWSO events across the 24 month time series. Weekly sampling provided the temporal resolution needed to study the impact of these events, and understand how the coastal microbial community responds to the change in physical conditions and introduction of non-indigenous microbes. Input of allochthonous water from the stormwater and sewer system transport a rich and diverse community of bacteria into coastal receiving waters (Sidhu et al., 2013), derived from humans and animals (McLellan et al., 2013), various surfaces across the urban watershed (roofs, parks and streets) (Herve et al., 2018), and stormwater and sewer infrastructure (waste water treatment plants and pipes) (McLellan et al., 2015). In this study, local similarity testing detected strong correlations between stormwater input and the relative abundances of enteric bacterial genera belonging to *Arcobacter*, *Acinetobacter*, *Bacteroides*, and *Cloacibacterium*. Notably, different levels of stormwater intensity (indicated by the level of EC) corresponded with increased abundance in distinct enteric bacterial groups. For instance, two OTUs matching *Arcobacter*, and one OTU matching *Acidovorax* required only 2 – 3 $\mu S\ cm^2$ drops in EC before increasing in occurrence frequency and relative abundance. These bacterial groups live within sediments and adhere to surfaces in stormwater conveyance systems (Badin et al., 2012), and their propensity to increase in abundance following relatively minimum stormwater input may suggest that these two OTUs are being scoured and advected from the stormwater system near to outflow points (Assanta et

al., 2002). Whereas, OTUs matching *Cloacibacterium* and *Acinetobacter* only became dominant when EC dropped below 40 μS cm^2. The association of these two genera with stormwater has been previously reported (Vandewalle et al., 2012), and the patterns seen here may suggest that they are being carried via stormwater flow from deeper within the stormwater system or the urban environment. Moreover, two OTUs closely related to *Arcobacter*, and one to *Lachnospiraceae* correlated exclusively with WWSO, indicating that these bacteria are sourced directly from sewage overflow, rather than stormwater or the stormwater system.

Monitoring by Beachwatch (a local state water quality monitoring department) indicates that for the past 23 years, rainfall has had very little impact on water quality at Maroubra Beach. However, prior to this period (approximately 27 years ago), fecal pollution was a serious issue due to nearby ocean sewage outfalls. Much of the water quality problems related to this infrastructure were resolved after the outfalls were extended from the coastline. During the 24 month study period, Maroubra Beach experienced only two occasions where stormwater input led to substantial drops in EC. However, during these periods bacterial assemblages harbored several OTUs matching genera often associated with stormwater, including *Acinetobacter* and *Arcobacter* (Vandewalle et al., 2012), and *Tenacibaculum*, a genus that harbors several species of marine fish pathogens (Avendaño-Herrera et al., 2006), albeit at much lower relative abundances than were observed at Foreshore Beach.

Waste stream (stormwater and sewage) microbial communities are a consortium of bacteria derived from humans and animals, wastewater treatment facilities, and effluent conveyance infrastructure including pipes and open channels (McLellan et al., 2015). Many of these microbes perform nutrient cycling processes such as nitrification, denitrification and decomposition of organic compounds, which are necessary for the treatment of wastewater, but may modify the chemical status of natural receiving waters, and influence the function of the indigenous microbial populations (Fisher et al., 2015). The survival and persistence of enteric bacteria in natural aquatic environments depends on both their physiological features, and the physico-chemical conditions of the receiving environment (Anderson et al., 2005, Rozen and Belkin, 2001). Cultivation-ability decreases sharply only days after enteric microbes enter natural environmental waters, which is accelerated in cooler, and higher salinity conditions (Rozen and Belkin, 2001). However, some enteric microbes employ physiological strategies for survival, including reduced cell size and metabolic output, and can remain viable but not culturable (VBNC) for many months (Hibbing et al., 2010). Our data shows that stormwater and sewage inputs have immediate and pronounced impact on the composition of bacterial communities at Maroubra and Foreshore beaches, which could also, potentially have chronic consequences that influence community function.

2.6.4 Environmental selection and ecological thresholds

This work indicates that coastal ecosystems have different environmental drivers which elicit varying ecological effects, that occur on different scales of magnitude and time.

The relationship between water temperature and bacterioplankton assemblages at Maroubra Beach explained much of the temporal variability in bacterial communities, with precise temperature thresholds separating bacterial taxa between those that exhibited preference for either warm or cool water. Interestingly, several closely related OTUs exhibiting high IndVal scores (suggesting that variation in the abundance of these bacterial species indicate broader shifts in community structure), displayed opposing temperature preferences at Maroubra Beach, and dominated in alternate seasons. These included highly abundant, core marine OTUs matching *Pelagibacter* and *Synechococcus*, but also transient OTUs including *Arcobacter*, *Fluviicola* and *Tenacibaculum*. This is consistent with previous studies that have observed marine microbial community shifts driven by seasonal switching between closely related strains (Ward et al., 2017), and evidence for a strong regulating role of temperature in governing the abundance of bacterial ecotypes (Fisher et al., 2014, Brown et al., 2012). Furthermore, evidence from metagenomic studies (Ward et al., 2017) suggest that only modest shifts in phylogenetic composition (observed in this study) is needed to lead to drastic changes in the net function of microbial communities.

2. 7 Conclusion

By tracking bacterial abundance, diversity and community composition in coastal water weekly across 24 months to compare microbial dynamics under contrasting levels of urban impact, this study found that two closely located beaches harbored strikingly similar core microbiomes, but high levels of dissimilarity between their respective

transient and low abundance microbiomes. Furthermore, we identified distinct environmental processes that best explained shifts in bacterioplankton assemblages at each site. Seasonal variability in temperature played the largest role in structuring bacterial assemblages at Maroubra Beach, whereas, stormwater input and WWSO had the strongest influence at Foreshore Beach. These findings highlight the exceptionally variable nature of microbial assemblages in coastal ecosystems, and reinforce the importance of high temporal resolution sampling in order to capture rapid and temporary patterns in coastal microbial dynamics.

2.8 Acknowlegements

This study was funded by Australian Research Council project FT130100218 to J.R.S. We thank OEH Beachwatch for providing advice on sampling locations, and Sydney Water for making modelled WWSO data available.

2.9 Tables

Table 1. Rare bacterial OTUs combined by taxa.

Maroubra Beach			
Taxa	**Mean**	**Percent Non Zero Observations**	**Max Value**
Bacillus	0.003	0.935	0.333
Corynebacterium	0.006	1.869	0.333
Crenothrix	0.069	4.673	2.667
Delftia	0.059	9.346	2.333
Ectocarpus	0.012	3.738	0.333
Erythrobacter	0.188	29.907	3.000
Leucothrix	0.053	9.346	1.667
Persicirhabdus	0.028	6.542	0.667
Phaeobacter	0.114	26.168	1.000
Photobacterium	0.072	11.215	2.000
Planomicrobium	0.009	1.869	0.667
Propionibacterium	0.053	7.477	2.333
Pseudoalteromonas	0.058	11.215	1.500
Psychrilyobacter	0.040	9.346	1.333
Psychrobacter	0.022	4.673	0.667
Rubritalea	0.055	6.542	2.667
Sphingobium	0.045	7.477	1.000
Sphingopyxis	0.051	12.150	1.000
Turicibacter	0.072	14.953	1.333
Winogradskyella	0.040	10.280	1.000

Foreshore Beach			
Taxa	**Mean**	**Percent Non Zero Observations**	**Max Value**
Acidaminococcus	0.026	3.922	1.333
Balneola	0.199	25.490	2.667
Blautia	0.177	23.529	2.000
Xiphinematobacter	0.057	8.824	1.333
Chryseobacterium	0.033	3.922	1.000
Coprococcus	0.143	23.529	2.000
Dechloromonas	0.051	11.765	0.750
Desulfotalea	0.310	44.118	3.000
Faecalibacterium	0.108	17.647	1.500
Fibrobacter	0.025	4.902	1.000
Hydrogenophaga	0.336	39.216	4.000
Lachnospira	0.038	5.882	1.500
Limnobacter	8.033	57.843	180.000
Limnohabitans	0.028	5.882	1.333
Mycobacterium	0.050	5.882	3.000
Oleispira	0.013	3.922	0.500
Phaeobacter	0.265	36.275	3.333
Polynucleobacter	0.196	21.569	4.667
Psychrilyobacter	0.339	42.157	2.000
Ruminococcus	0.095	13.725	4.000
Trichococcus	0.194	21.569	3.500

2.10 Figures

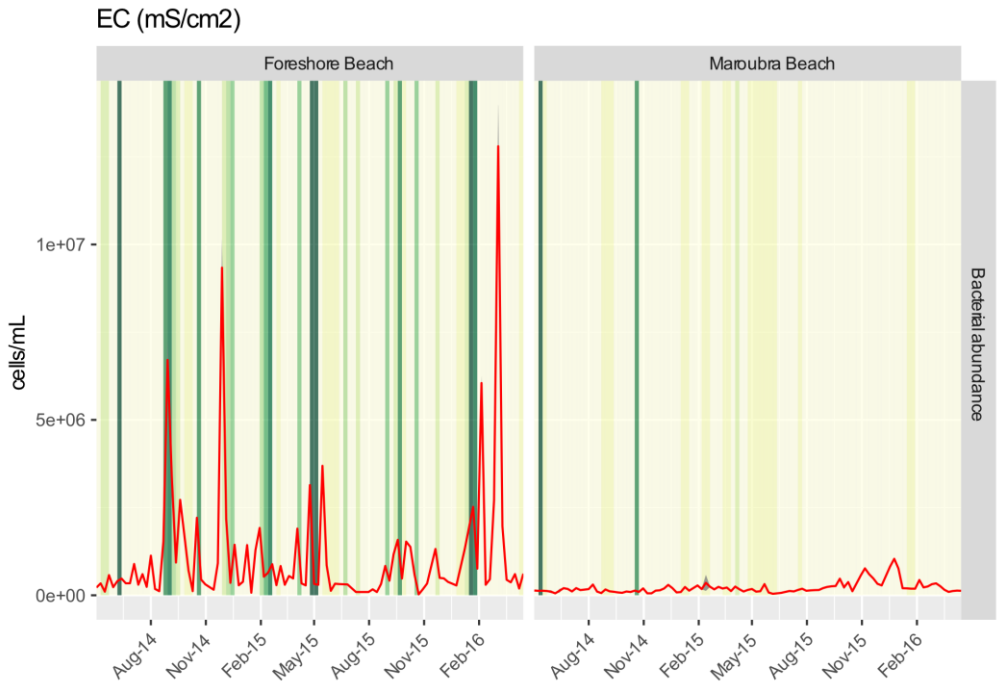
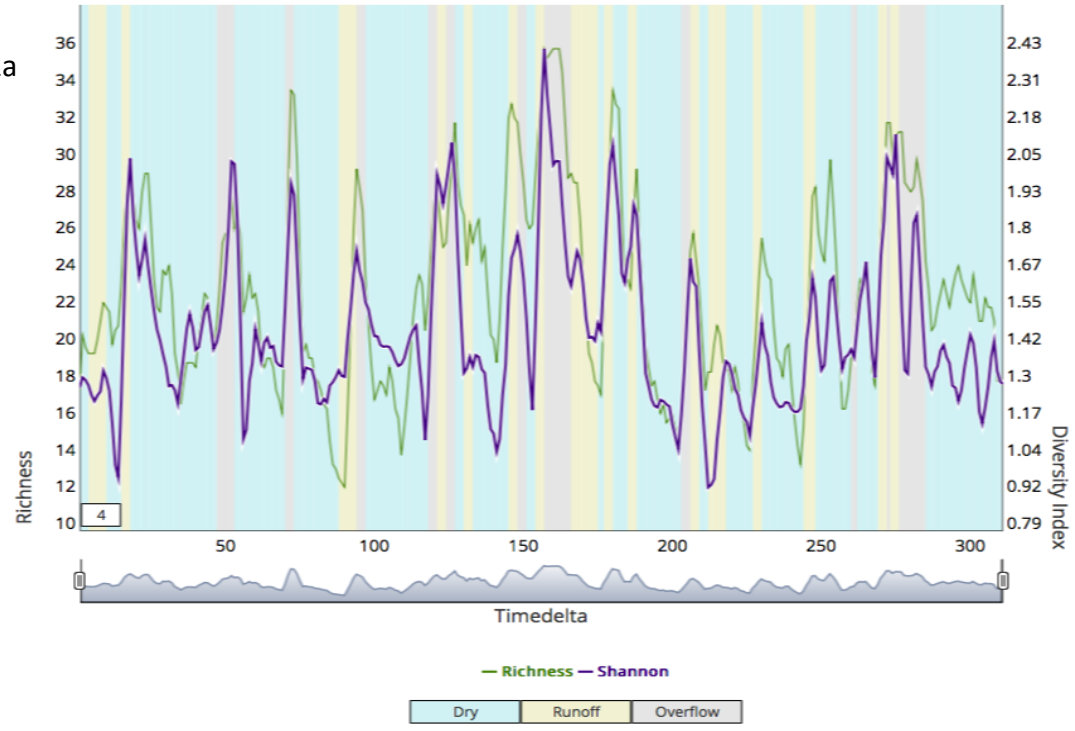


Figure 1. *104 weekly bacterial cell abundance concentrations at Foreshore and Maroubra beaches, measured using flow cytometry. Background colours represent the level of electrical conductivity (EC) at each given timepoint, where dark green indicates reduced EC, which is indicative of stormwater input.*

2a



2b

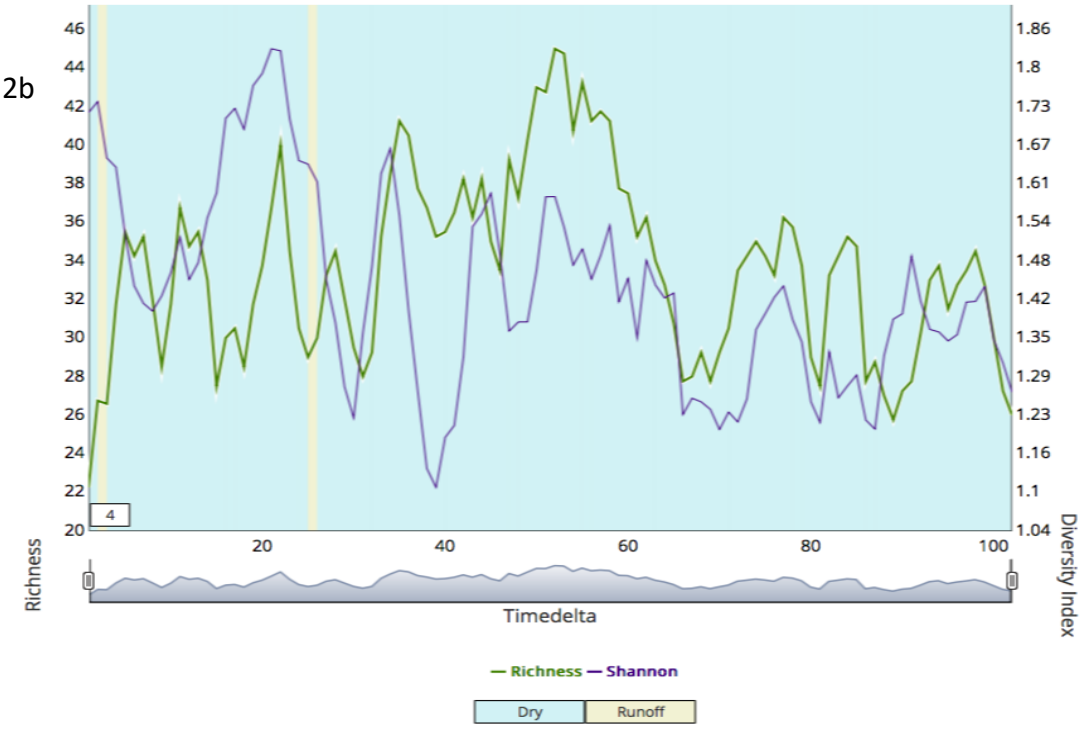


Figure 2. *Bacterial community diversity and rare microbiomes of Foreshore and Maroubra beaches. Vertical background colours distinguish sample periods by environmental condition - dry (blue), stormwater (yellow) and wet weather overflow(grey). Four week smoothing of 104 timepoint datasets was applied to enhance interpretability of plot.* ***a)*** *Foreshore Beach - bacterial OTU diversity (Shannon's index) and richness,* ***b)*** *Maroubra Beach - bacterial OTU diversity (Shannon's index) and richness,* ***c)*** *Foreshore Beach – Rare OTUs grouped into closest affiliated genera,* ***d)*** *Maroubra Beach - Rare OTUs grouped into closest affiliated genera.*

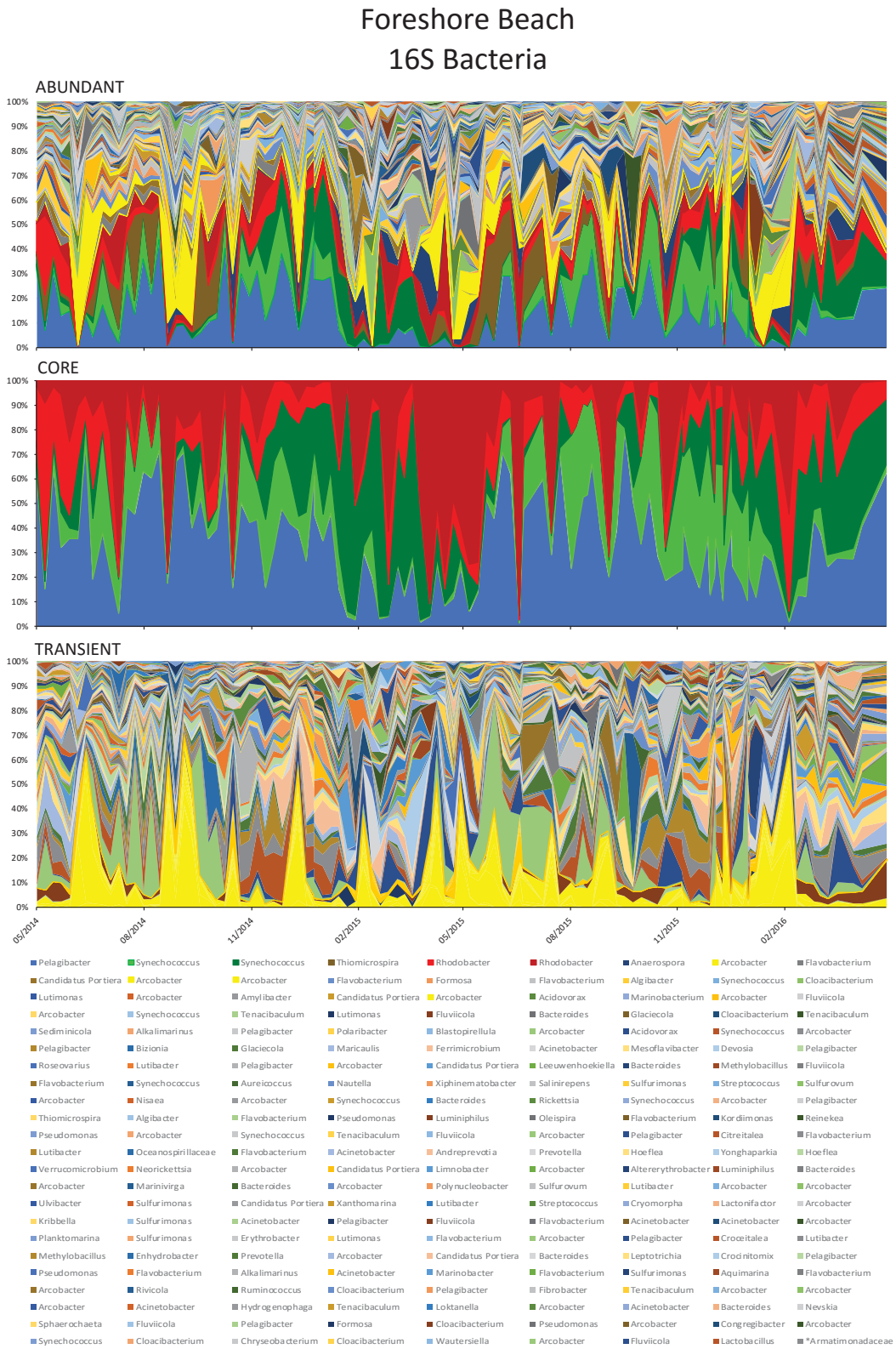


Figure 3. *Weekly timeseries profiles of bacterial communities produced from 16S rRNA amplicon sequencing, depicting the relative abundances of bacterial OTUs inhabiting Foreshore Beach. The top panel shows all bacterial OTUs detected across the timeseries. The middle panel shows bacterial OTUs belonging to the "abundant" population that*

were present across >90% of sample occasions. The bottom panel shows bacterial OTUs belonging to the "transient" population, defined as OTUs that contributed > 1% of 16S sequences for at least one sample across the timeseries.

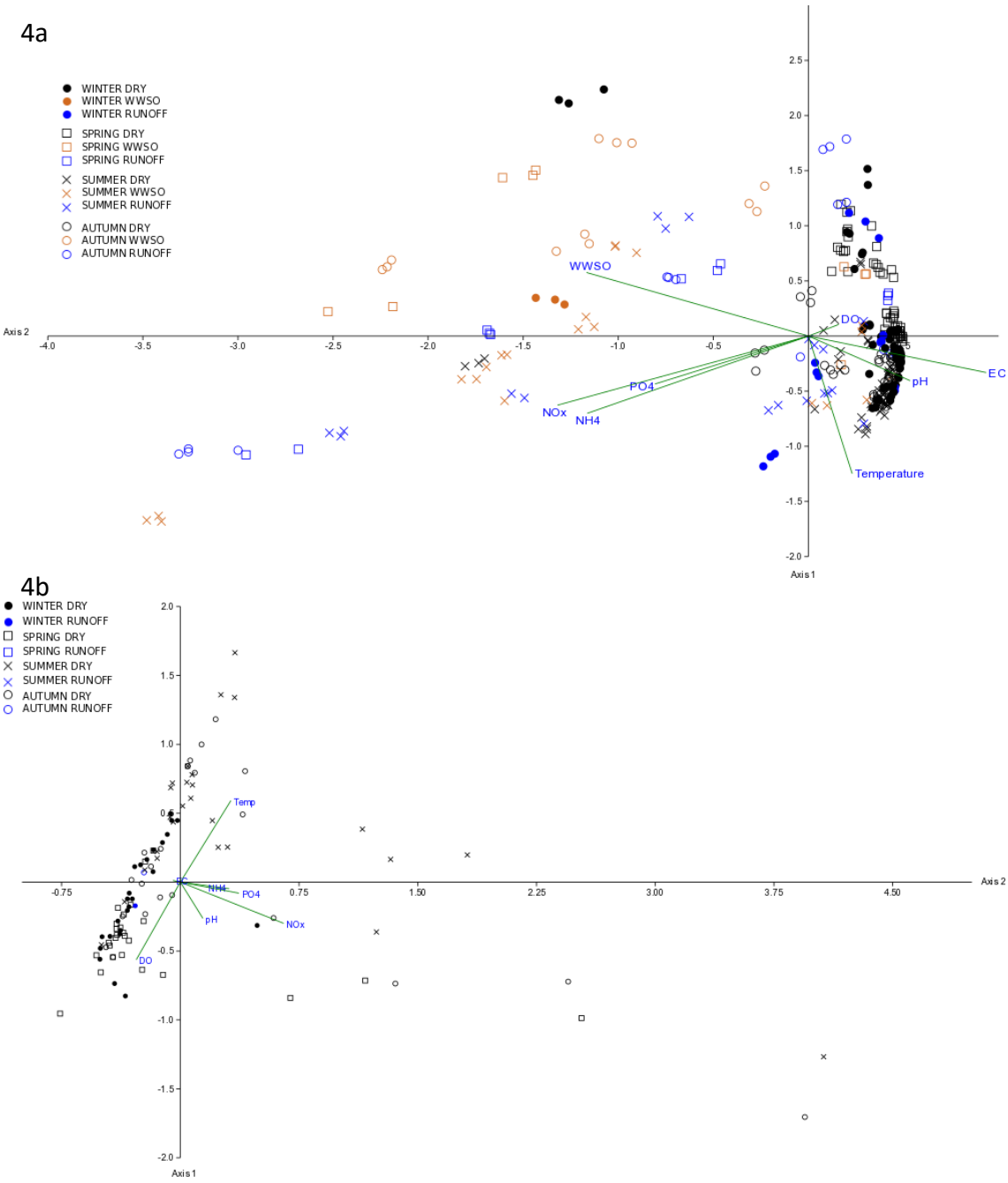


Figure 4. *Canonical correspondence analysis was used to assess the relationship between gradients in environmental variables and patterns in the composition of bacterial communities at a) Foreshore Beach, which included seven water chemistry parameters plus modelled volumes of wet weather sewer overflows, and b) Maroubra Beach, which included the same seven water chemistry parameters.*

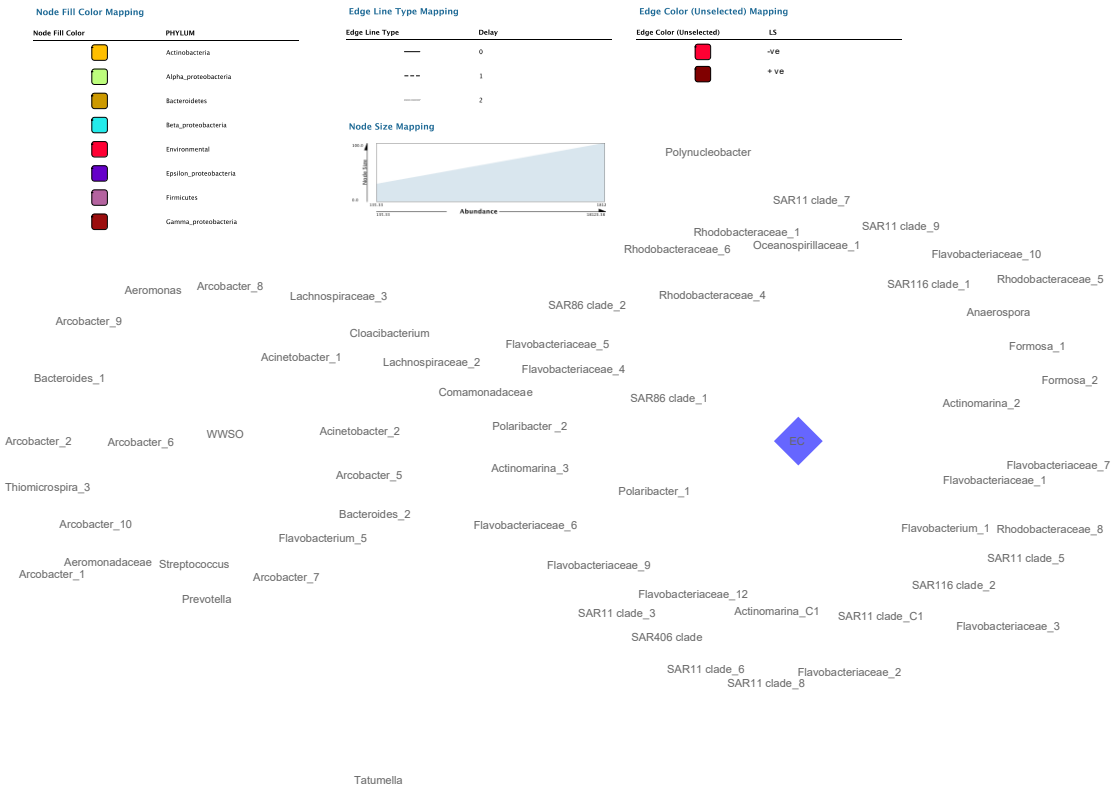


Figure 5. *Foreshore Beach network analysis, displaying significant positive (black edges) and negative (red edges) correlations between electrical conductivity - EC (blue diamond) and bacterial OTUs, and wet weather sewer overflows - WWSO (red diamond) and bacterial OTUs. Due to the inverse relationship between EC and stormwater input, bacterial OTUs connected to by red edges to EC are interpreted as OTUs that increase with stormwater input. The length of connecting edges relate to the local similarity correlation score, whereby strongly positive correlations bring nodes (circles representing bacterial OTUs) closer, and strongly negative correlation push nodes further apart. The node size relates directly to the overall abundance of the OTU across the entire time series dataset.*

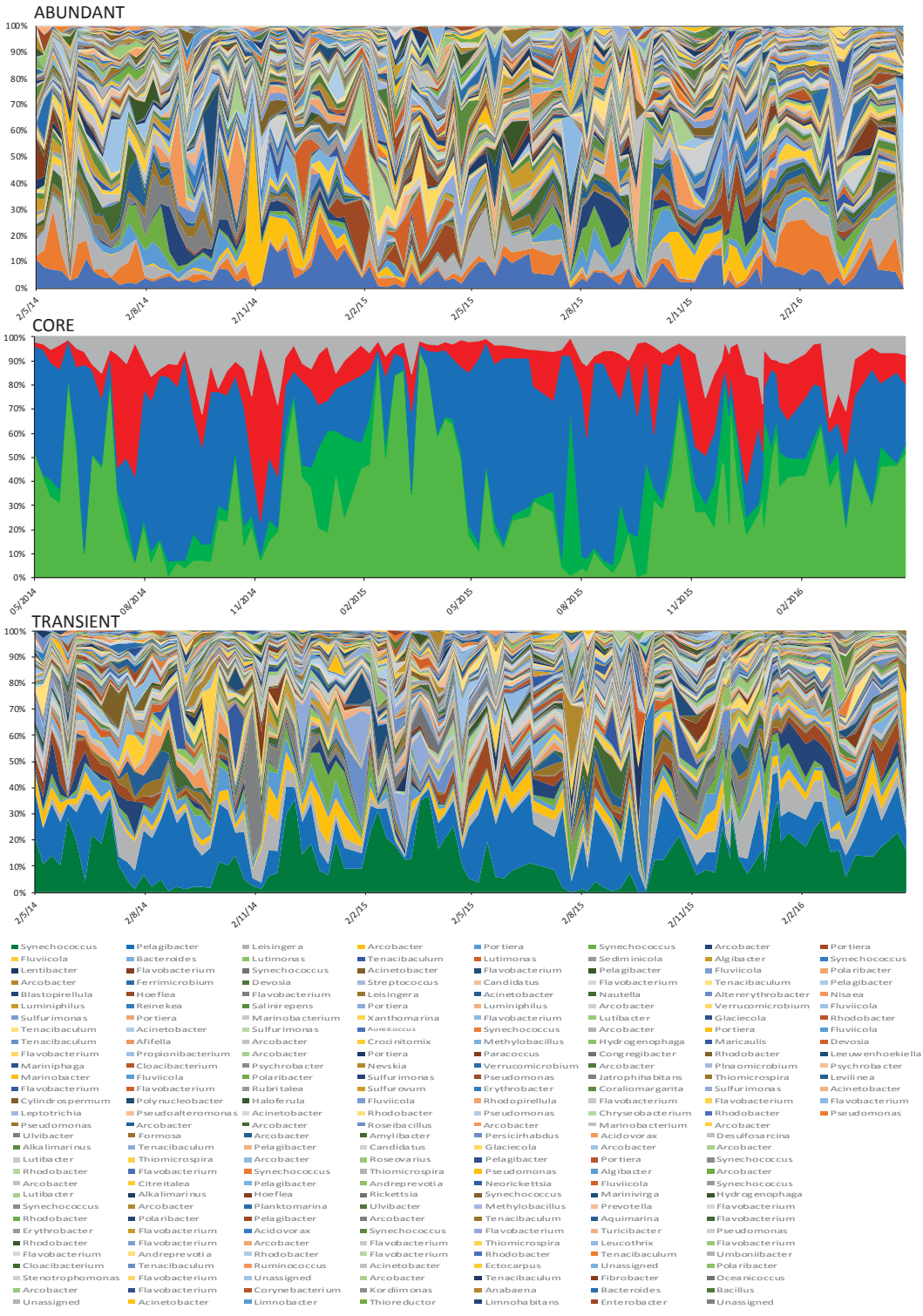


Figure 6. *Weekly timeseries profiles of bacterial communities produced from 16S rRNA amplicon sequencing, depicting the relative abundances of bacterial OTUs inhabiting Maroubra Beach. The top panel shows all bacterial OTUs detected across the timeseries. The middle panel shows bacterial OTUs belonging to the "abundant" population that were present across >90% of sample occasions. The bottom panel shows bacterial OTUs belonging to the "transient" population, defined as OTUs that contributed > 1% of 16S sequences for at least one sample across the timeseries.*

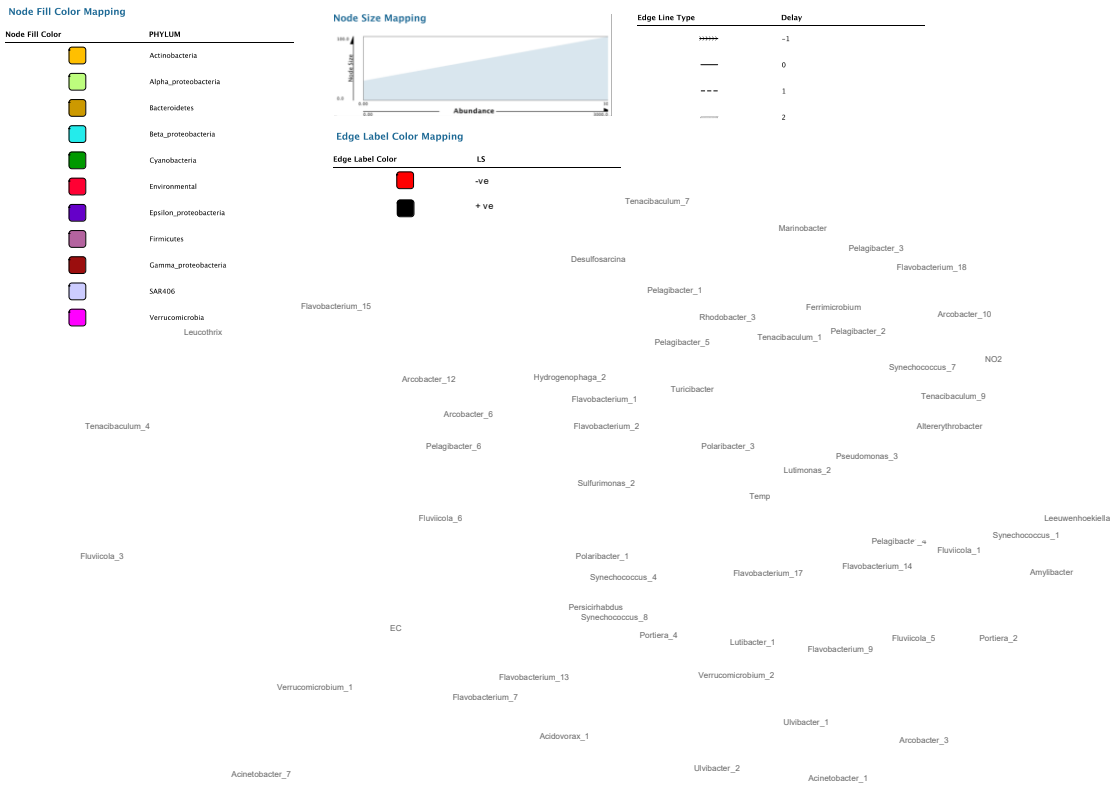
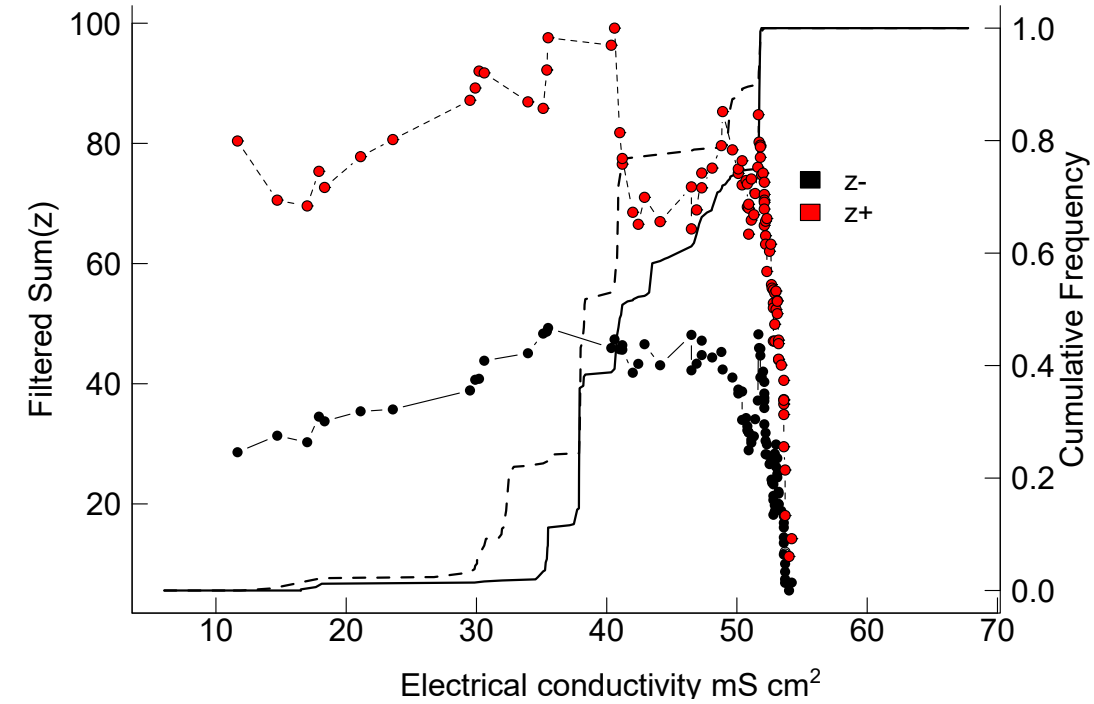


Figure 7. *Maroubra Beach network analysis, displaying significant positive (black edges) and negative (red edges) correlations between electrical conductivity - EC (left hand side blue diamond) and bacterial OTUs, and temperature (right hand side red diamond) and bacterial OTUs. Due to the inverse relationship between EC and stormwater input, bacterial OTUs connected to by red edges to EC are interpreted as OTUs that increase with stormwater input. The length of connecting edges relate to the local similarity correlation score, whereby strongly positive correlations bring nodes (circles representing bacterial OTUs) closer, and strongly negative correlation push nodes further apart. The node size relates directly to the overall abundance of the OTU across the entire time series dataset.*



8b

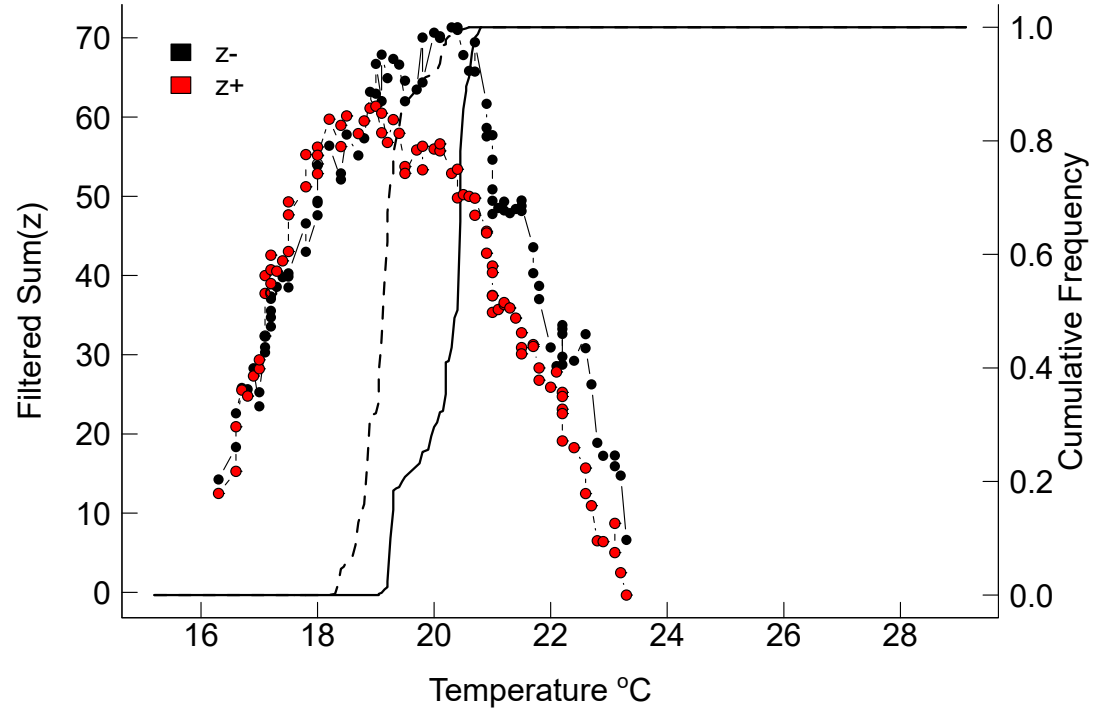


Figure 8. *Threshold Indicator Taxa ANalysis (TITAN) and change-point analysis (nCPA, Bray–Curtis distance) of bacterial community response to the a) Electrical conductivity (EC) gradient (n = 104) at Foreshore Beach, and b)*

temperature gradient (n = 104) at Maroubra Beach. TITAN sum(z−) and sum(z+) values corresponding to all candidate change points along the EC (mS/cm^2) and Temperature (°C). Solid black and dashed black vertical lines represent the cumulative frequency distribution of change points among 1000 bootstrap replicates for sum(z−) and sum(z+), respectively.

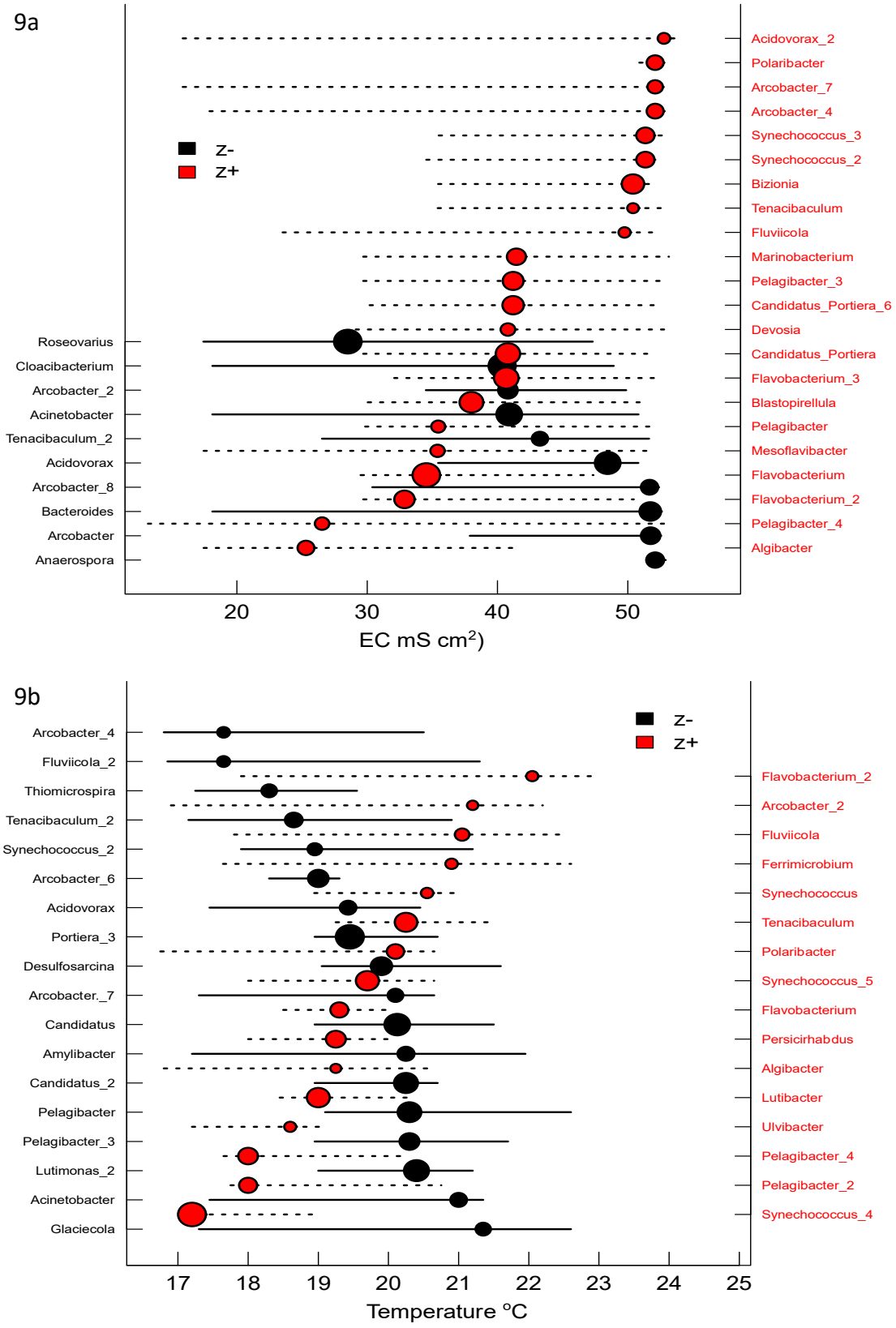
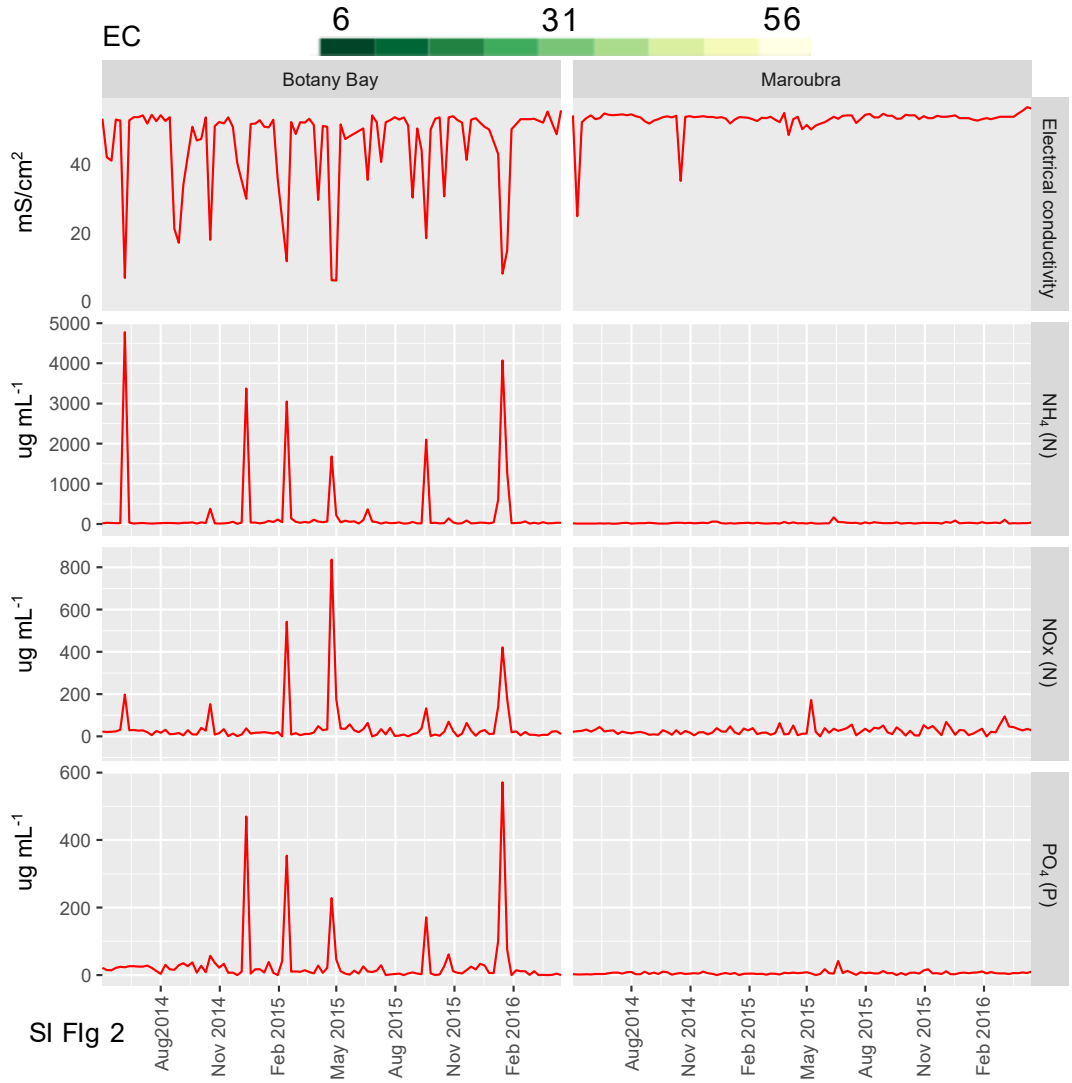


Figure 9. *Significant (purity ≥ _0.95, reliability ≥ _0.95, and p ≤ _0.05) bacterial indicator taxa are plotted in increasing order of their observed change points. Black symbols correspond to negative (z−) indicator taxa, whereas red symbols correspond to positive(z+) indicator taxa. Symbols are sized in proportion to magnitude of the response (z scores).*

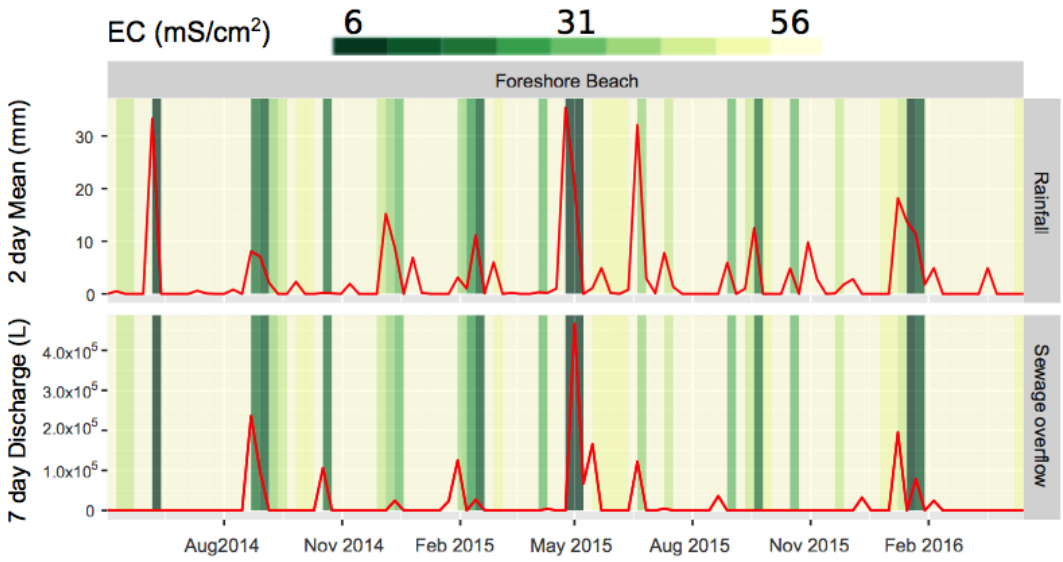
Horizontal lines overlapping each symbol represent 5th and 95th percentiles among 1000 bootstrap replicates. Indicator taxa plotted against a) electrical conductivity at Foreshore Beach, and b) temperature at Maroubra Beach.

2.11 Supplementary Figures

SI Fig 1. *Map of study sites – Foreshore Beach (red), Maroubra Beach (green) and Millpond Sewage overflow point (brown).*



SI Fig 2. *Electrical conductivity (EC) and inorganic nutrient concentrations measured at Foreshore and Maroubra beaches, weekly from 02/04/2014 to 29/04/2016. Background vertical colours depict levels of EC, where dark green indicates the lowest EC levels, indicative of stormwater input. Orange vertical bars indicate time points where wet weather sewer overflows occurred. From top; electrical conductivity (EC), concentrations of ammonia, oxides of nitrogen and phosphorous.*



SI Fig 3.The top panel tracks rainfall (mm) at Foreshore Beach, and bottom panel tracks mean total weekly volume of modelled wet weather sewer overflow at Foreshore Beach. Background vertical colours depict levels of EC, where dark green indicates the lowest EC levels, indicative of stormwater input.

3. Chapter 3

Coastal stormwater and sewage fuel spikes in the abundance and diversity of potentially pathogenic Arcobacter

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Current status of paper:

- Not yet submitted

Author contributions:

- Richard Carney conducted the sampling, laboratory and data analysis. Mark Brown and Timothy Kahlke assisted with bioinformatics work. All authors contributed to the writing of this manuscript.

Chapter 3 - Coastal stormwater and sewage fuel spikes in the abundance and diversity of potentially pathogenic Arcobacter

3.1 Preface

In chapter 2 we observed evidence for an increase in the relative abundance of putative storm-water microbes, including Arcobacter, Acinetobacter, Aeromonas and others. In this chapter we have performed a more focussed analysis on the dynamics of Arcobacter, an emerging aquatic pathogen that is putatively associated with intrusions of stormwater and sewage.

3.2 Summary

We characterised the temporal dynamics of potentially pathogenic bacteria at an urban beach subject to significant stormwater input and wet weather sewer overflows (WWSO). Weekly monitoring of bacterial communities over 24 months using 16S rRNA amplicon sequencing revealed large, intermittent peaks in the relative abundance of the emerging pathogen Arcobacter, which regularly increased from < 1% up to 70% of the total bacterial assemblage following inputs of stormwater and modelled WWSO. Quantitative PCR was subsequently employed to track absolute abundance of Arcobacter 23S rRNA gene copies, revealing that peaks in Arcobacter abundance reached up to 10^8 gene copies L^{-1}, with these increases statistically correlated with stormwater intrusion and modelled WWSO. Notably, peaks in Arcobacter abundance were poorly indicated by enterococci plate counts, and remained elevated for one week following heavy rainfall. Using oligotyping we discriminated single nucleotide variants (SNVs) within the Arcobacter population, revealing 10 distinct clusters of SNVs that we defined as Arcobacter “ecotypes”, which displayed differing temporal dynamics. The most abundant ecotype during stormwater and modelled WWSO events closely

matched *A. cryaerophilius*, a species previously implicated in human illness. Our findings point to a potentially important, yet overlooked exposure risk of these potential pathogens to water users in coastal surface waters.

3.3 Originality-Significance Statement

Arcobacter, an emerging pathogen in both clinical and environmental settings, may pose a significant risk to water users as they are not routinely monitored for, are capable of persisting in a wide range of environments, and are increasingly associated with illness in humans and animals. This study coupled a high temporal-resolution time-series design with oligotyping and network analysis to deliver a detailed characterisation of Arcobacter population diversity and abundance in a coastal marine setting, allowing us to link Arcobacter population dynamics to stormwater and modelled WWSO inputs, revealing the potential for human exposure to a key emerging pathogen.

3.4 Introduction

Fifty percent of the human population lives within 100 Km of the coastline (Shuval, 2006) and as a consequence coastal environments are heavily impacted by anthropogenic activities (Clark and Johnston, 2017, Stewart et al., 2008). In particular, intensive urbanisation has amplified the need for stormwater drainage and wastewater removal, which has led to many estuaries and beaches becoming key receiving points for a range of anthropogenic pollutants including domestic and industrial chemicals, pharmaceuticals, and human faecal waste (Sidhu, 2013).

Chemicals from human waste-streams, including organic and inorganic nutrients, washed into marine receiving waters may alter the physico-chemical state of marine habitats and disrupt balance in the composition of indigenous microbial communities (Zhang et al., 2009), potentially leading to blooms of harmful algae (Howarth et al., 2000, Hallegraeff, 1993) and pathogenic bacteria (e.g. *Vibrio spp.*) (Thompson and Polz, 2006). However, from a human health perspective, the major microbiological consequence of urban waste-streams involves the input of enteric bacteria, including *Escherichia coli* and *Salmonella* spp. (Shuval, 2006), viruses (including noroviruses, adenoviruses, echoviruses and hepatitis A (Sinclair et al., 2009)), and protozoan pathogens (Collier et al., 2012). This allochthonous input of enteric microbes has significant consequences for human health, with waterborne infections contracted from coastal waters causing approximately 120 million cases of gastrointestinal illness and over 50 million cases of serious respiratory disease annually (Shuval 2003) (www.who.int). In the USA alone,

these impacts have an associated economic cost of upwards of 300 million dollars yr^{-1} (Collier et al., 2012).

The public health risks from aquatic pathogens are often enabled or exacerbated by coastal water management strategies. Stormwater infrastructure introduces water from urban surfaces and drains into environmental waters. In many urban centres, stormwater systems are also used to divert wet weather sewer overflows (WWSO) into natural waterways during periods of heavy rainfall or following sewer system failures (Bickford et al., 1999). As a consequence, stormwater systems represent a key conduit for the spread of human pathogens into natural aquatic settings (Sidhu et al., 2013). Notably, stormwater infrastructure (e.g. pipes and drains) also provides a niche for the environmental persistence and transmission of some enteric microbes, whereby specific physiological features (e.g. biofilm production) enable adherence to pipe surfaces (Assanta et al., 2002) and resistance to anthropogenic chemicals, including biocides, antibiotics and disinfectants (McLellan et al., 2015).

Public health and environmental monitoring practices typically involve the detection of key indicator microbes as a measure of impact from stormwater and sewage overflows. Specifically, the cultivation of faecal indicator bacteria (FIB), such as faecal coliforms and enterococci, is routinely used to detect the presence of faecal contamination in coastal waters and estimate the risk of exposure to human health (Boehm and Sassoubre, 2014). However, culture-based approaches can miss the occurrence of other, non-targeted pathogens that are not reliably predicted by FIB, including microbes that can be virulent even at low concentrations (Ralph and Currie, 2007) or have physiological

capacity to persist in marine habitats for longer periods than FIBs, such as Acinetobacter or Arcobacter (Newton, 2013). In light of these limitations, it has become apparent that culture-independent molecular methods have the advantages of identifying community-level signatures of faecal pollution (Sauer et al., 2011), detecting the presence of pathogens that are not easily cultured (Newton, 2013) and potentially discovering new or previously overlooked emerging pathogens.

New and emerging human pathogens are being detected more frequently within natural and urbanised aquatic environments, leading to an increasing recognition of their potential ecological importance and public health relevance. For instance, bacteria belonging to the genus *Arcobacter* have recently gained increased attention as emerging pathogens (Ramees et al., 2017), largely due to their association with sewage (Fisher et al., 2014), food contamination (Girbau et al., 2015) and disease in a broad range of hosts (Douidah et al., 2012). As a consequence of a growing body of literature implicating *Arcobacter spp.* in illness and disease, the identification of the sources, transport vectors and reservoirs of Arcobacter within natural environments has public health relevance (Ramees et al., 2017). In particular, coastal waters represent a key interface for direct human contact with these potential pathogens. Here, after observing the intermittent prominence of Arcobacter in 16S rRNA surverys, we tracked weekly changes in the abundance and diversity of Arcobacter spp. at a public beach, frequently subjected to allochthonous inputs from stormwater and sewage overflow discharge.

3.5 Results

Water samples were collected weekly for 24 months at Foreshore Beach (33.95 ˚S, 151.9 ˚E), Botany Bay on the east coast of Sydney, Australia. Foreshore Beach is frequently subject to inputs of stormwater from a highly urbanised and industrial watershed, which were detected following periods of intense rainfall and correlated with drops in electrical conductivity (EC) (Supplementary Fig 1). In most cases, EC values returned to baseline levels (seen during dry periods) by the subsequent sampling occasion. A total of 16 stormwater events were observed during the two year time-series, of which 10 events involved modelled wet weather sewer overflows (WWSO). These modelled WWSO events represent the volume of water estimated to have been discharged from an overflow outfall situated in Millpond, and ranged in magnitude from 4,193 m^3 $week^{-1}$ to 465,438 m^3 $week^{-1}$ (Supplementary Fig 2).

The input of stormwater at Foreshore Beach led to significantly higher levels of NH_4 ($F = 6.65$, $df = 1,76$, $p = 0.01$) (Supplementary Fig 3), while modelled WWSOs led to significantly higher levels of NH_4 ($F = 9.51$, $df = 1,80$, $p = < 0.01$) and PO_4 ($F = 8.05$, $df = 1,80$, $p = < 0.01$) relative to dry periods. The highest nutrient concentrations were recorded following modelled WWSOs, when up to 22-fold higher concentrations of NH^4 were observed when compared to baseline conditions (Supplementary Fig 3). Stormwater input and modelled WWSO events also led to 1 – 2 order of magnitude increases in total bacterial abundance (Supplementary Fig 3). Peaks in enterococci counts also coincided with these events, with counts increasing from < 40 CFU ml^{-1}

during dry periods to > 200 CFU ml^{-1} during all 10 modelled WWSO events and 4 stormwater-only events (Fig 4).

Stormwater and modelled WWSO events led to pronounced changes in the diversity and composition of bacterial assemblages at Foreshore Beach, as determined using 16S rRNA amplicon sequencing. Input of stormwater and modelled WWSO following precipitation led to compositional shifts in bacterial assemblages from a characteristic marine assemblage (i.e. dominated by organisms such as SAR11 and *Synechococcus*) during dry periods, to a community that often included a high proportion of enteric bacteria transported via the stormwater system. Notably, WWSO events led to a significant increase in diversity compared to dry periods (ANOVA; $F = 4.8$, $df = 1,80$, $p = 0.03$) (Shannon's Diversity Index; Supplementary Fig. 4) but not stormwater input alone ($p > 0.05$).The most notable shifts in bacterial assemblage structure involved the presence of large spikes in the relative abundance of OTUs assigned to the Arcobacter genus (Fig 1). During dry periods, Arcobacter OTUs comprised on average only 5% of all 16S sequences, but increased significantly ($p < 0.01$) in relative abundance following input of stormwater and modelled WWSO (Supplemtary Fig 5), with the stongest spikes reaching maximums of 37 and 66% respectively.

We confirmed the significance of these large increases in relative abundance of Arcobacter OTUs using qPCR, whereby Arcobacter was detected in 97 of 104 samples.

Consistent with the patterns observed in the amplicon sequencing data, absolute abundance of Arcobacter was lowest during dry periods, when mean background concentrations were 2.2 x 10^6 copies L^{-1} ($\pm$ S.D 6.36 x 10^6), but increased during stormwater input to 7.8 x 10^6 gene copies L^{-1} ($\pm$ S.D 1.25 x 10^6) and modelled WWSO to 1.8 x 10^7 copies L^{-1} ($\pm$ S.D 2.45 x 10^7) (Fig 2). CCF analysis confirmed that peaks in Arcobacter abundance generally persisted for an additional week after intense rainfall (Fig 3a) and the subsequent input of stormwater and modelled WWSO (Fig 3b).

An oligotyping approach (Eren et al., 2013) was used to discriminate the strain-level diversity of Arcobacter populations in the 16S rRNA sequencing data. We detected the presence of 181 discrete Arcobacter oligotypes during the 2 year study period, with network analysis clustering these oligotypes into 10 distinct modules, each containing between 5-31 closely coupled nodes (Fig 5). Oligotypes within these nodes exhibited highly correlated temporal dynamics and relationships with environmental parameters, and we therefore consider these clusters of oligotypes to be distinct groups of "ecotypes" that occupy a common environmental niche (Fig 6).

Ecotype 1 comprised 31 tightly clustered oligotypes (Fig 5), with a closest phylogenetic match (95% sequence similarity) to *A. cryaerophilus* (NCBI Accession no. U25805) (Fig 7). Ecotype 1 was the most abundant Arcobacter ecotype during the study period, representing 56% of the Arcobacter community when relative Arcobacter sequence

abundances were normalised to Arcobacter gene copies defined using qPCR (Fig 6). However, the abundance of Ecotype 1 was highly variable across the time-series, ranging between < 1% and 98% of all Arcobacter sequences (Fig 6b), reaching a maximum abundance of 1.62 x 10^8 gene copies L^{-1}. Ecotype 1 was most abundant during the summer, when water temperatures ranged between 19.5 - 26.5 ˚C and there was a high frequency of stormwater and modelled WWSO events. Indeed, Ecotype 1 abundance was statistically correlated with stormwater events (MIC = 0.47, $p < 0.01$), when it comprised 33% of Arcobacter sequences, and modelled WWSO input (MIC = 0.35, $p < 0.01$), when it comprised 66% of Arcobacter sequences (Fig 8).

Ecotype 2 comprised 18 oligotypes, with all most closely matching (96% sequence similarity) *A. nitrofigilis* (NCBI Accession no. L14627) (Fig 7). This ecotype represented a modest contribution to total Arcobacter abundance (3.9% of normalised sequence reads), but was most prevalent during dry periods (2.15 x $10^5 \pm$ S.D 5.63 x 10^5 gene copies L^{-1}) and during the winter (5.63 x $10^5 \pm$ S.D 6.65 x 10^5 gene copies L^{-1}) (Fig 6a), when the abundance of Arcobacter populations were low relative to other periods. Ecotype Groups 3, 4, and 5 (Fig 5), were all most closely phylogenetically related to *A. molluscorum* (each with 95% sequence similarity to NCBI Accession no. 117055) (Fig 7), but displayed contrasting occurrence and abundance to one another between seasons and under different environmental conditions. For example, Ecotype 3 was the second most abundant group of Arcobacter over the course of the study, contributing over 24% of normalised sequence reads, and most abundant in Winter (5.02 x $10^6 \pm$ S.D 9.05 x 10^6

gene copies L^{-1}) and during dry periods (1.83 x 10^6 ± S.D 5.03 x 10^6 gene copies L^{-1}). while Ecotype 4 and 5 were the least abundant, each contributing under 1% of all Arcobacter sequence reads, or 1 – 2 orders of magnitude less gene copies L^{-1} compared with Ecotype 3, and were conditionally rare (not detected from > 50% of samples). Ecotype 3 and 4 were most abundant during dry periods in Winter, with their abundance negatively correlated to temperature (MIC = 0.67 $p < 0.01$) and highest abundances occurring when water temperatures and inorganic nutrient concentrations were low (temperature: 13.1 – 18.8 ˚C). In contrast, the majority of normalised sequence reads belonging to Ecotype 5 (58.24%) occurred during stormwater impacted periods in Spring.

Ecotypes 6, 7, 8 and 9 (Fig 5) all displayed highest phylogenetic affiliation with *A. venerupis* (95% sequence similarities to NCBI Accession no. 117569) (Fig 6), and were each comprised of between 5 - 23 tightly clustered Arcobacter oligotypes (Fig 5). Ecotype 8 was the third most abundant ecotype, contributing 10% of sequence reads across the entire time-series, due to it's prominence during periods impacted by WWSO (2.90 x 10^6 ± S.D 4.13 x 10^6 gene copies L^{-1}). Conversely, within the context of relative abundance, Ecotypes 6, 7 and 9 were less abundant (0.9%, 2.3% and 1.5% of sequence reads respectively)and differed significantly ($p = 0.05$) across seasons (Fig 7b), with Ecotypes 7, 8 and 9 most abundant in Spring (2.24 x 10^5 ± S.D 5.53 x 10^6, 1.41 x 10^6 ± S.D 8.40 x 10^5, and 1.44 x 10^5 ± S.D 8.14 x 10^4 gene copies L^{-1} respectively) and Ecotype 6 most abundant in Summer (1.24 x 10^5 ± S.D 7.65 x 10^4. These ecotypes were also most prominent during periods impacted by WWSO, increasing by 1 – 2 orders of magnitude

compared to dry periods (10^4 – 10^5 and 10^6 gene copies L^{-1})as indicated by strong correlations with WWSO volume (MIC = 0.31, 0.30, 0.43 and 0.38 respectively, $p < 0.01$) (Fig 8). Notably, however, these groups were not correlated with stormwater events alone.

All four ecotypes affiliated with *A. venerupis* (groups 6-9) correlated significantly with Ecotype 10 (MIC = 0.36 – 0.55; $p < 0.01$), which consisted of 11 oligotypes (red nodes; Fig 5). However, Ecotype 10 had a highest taxonomic match to *A. cloacae* (97% sequence similarity) (Fig 6). This group coincided almost exclusively with modelled WWSO, with over 90% of normalised sequence reads belonging to samples collected during modelled WWSO events. Across the entire time-series, Ecotype 10 was the least abundant and most rare Arcobacter ecotype, comprising less than 1% of all Arcobacter sequence reads (Fig 7b), and not detected across > 50% of samples. However following stormwater input on 3 occasions during Spring, this ecotype spiked in abundance by 3 orders of magnitude, reaching concentrations > 10^6 gene copies L^{-1} (Fig 7a,b). Notably, there was no statistically significant correlation between either absolute Arcobacter abundance or the relative abundance of any Arcobacter oligotypes and enterococci counts.

3.6 Discussion

Urbanised coastal habitats are often exposed to high levels of terrestrial runoff (Ferguson et al., 2003), including stormwater and WWSO following rainfall (Sidhu et al.,

2013, Newton et al., 2011), that can lead to shifts in microbial community composition (Cunha and Almeida, 2006) and the transport of human pathogens from waste stream environments into marine surface waters (Sauer et al., 2011). Standard approaches for monitoring these events include the enumeration of FIBs, such as faecal coliforms, *Escherichia coli*, and enterococci (Boehm and Sassoubre, 2014). However, while these approaches quantify the extent of contamination according to the detection of specific marker microbes, other potentially pathogenic microbes can display different levels of occurrence and persistence to FIBs (Bradshaw et al., 2016) .

In our high temporal resolution coastal time-series, 16S rRNA amplicon sequencing revealed dramatic shifts in the composition of bacterial communities at a stormwater impacted beach located near to Sydney, Australia. Sydney is the most densely populated city in Australia, with more than 5 million people as of 2017 (Australian Bureau of Statistics, 2017). Sydney's climate is warm-temperate to sub-tropical, with precipitation patterns characterised by intermittent periods of intense and heavy rainfall, which is often strongest during the late summer, leading to large and intense inputs of runoff and stormwater discharge into the coastal marine environment (Office of Environment and Heritage NSW, 2014).

The most notable shifts in the composition of bacterial communities at Foreshore Beach were associated with large and rapid increases in the relative abundance of bacteria belonging to the Arcobacter genus, with sequences assigned to this group increasing from background levels of <1% of 16S rRNA sequences up to 70%. These increases in

Arcobacter relative abundance generally followed large stormwater and modelled WWSO intrusions that occurred as a result of heavy rainfall events. This is consistent with observations from other regions, that have demonstrated high levels of Arcobacter within aquatic environments, including Lake Eerie (Lee et al., 2012) and Lake Michigan (Newton, 2013), following periods of significant rainfall. These observations are of public health concern, given the status of this genus as an emerging environmental pathogen (Ferreira et al., 2016).

3.6.1 Stormwater and WWSO fuels peaks in Arcobacter abundance

Investigations into the occurrence of Arcobacter in human and animal gut microbiomes indicate that this genus generally comprises less than 0.001% of the total microbial consortium (Gevers et al., 2012). However, in wastewater treatment facilities, where imposed treatment conditions supress all but the most resilient enteric microbes, the relative abundance of Arcobacter can be much higher (5 – 11% of bacterial communities) (Fisher et al., 2014). Within natural marine and freshwater environments, Arcobacter generally occur in low abundances (below 1%) (Lee et al., 2012), but may increase in abundance in environments subject to influence from human and animal waste streams. However, to date investigations to determine whether intrusions of wastewater can lead to increases in the environmental abundance of this group are lacking.

Five orders of magnitude (from 10^3 to 10^8 gene copies L^{-1}) increase in the absolute abundance of Arcobacter occurred at Foreshore Beach during and following rainfall. Notably, the highest concentrations of Arcobacter detected in this study were 100-fold greater than those reported by Lee *et al. (2012*), whom employed the same qPCR assay to investigate Arcobacter abundance at Lake Eerie beach; an environment similarly impacted by faecal contamination. While peaks in Arcobacter abundance were tightly coupled with the input of stormwater, and modelled WWSO discharge, it is notable that Arcobacter abundance were not generally well predicted by FIB (in this case Enterococci) count data (Fig 4). In particular, four of the highest observed peaks in total Arcobacter abundance corresponded with low enterococci counts, which would not on it's own trigger warnings to swimmers or beach closures.

3.6.2 Arcobacter diversity is temporally dynamic

Understanding the ecology of Arcobacter in coastal marine environments is important because Arcobacter are increasingly associated with disease in animals and humans (Collado and Figueras, 2011). Levels of virulence and niche dynamics vary between Arcobacter species (Douidah et al., 2012), but the temporal dynamics of this group are not well understood within coastal environments where contact with humans may be significant. Using an oligotyping approach (Eren et al., 2013), we found a high level of micro-diversity within Arcobacter populations, and clear shifts in the assemblage structure over time and in association with stormwater and modelled WWSO events. A total of 181 oligotypes were distributed across 10 dominant ecotypes, with these groups

showing clearly different temporal dynamics and environmental preferences to one another. This approach allowed for the further partitioning of the Arcobacter community into 2 distinct populations; marine ecotypes that persist in surface waters at low abundances during dry conditions, and waste stream ecotypes that dominate during periods impacted by stormwater and WWSO input, but occur in low abundances or disappear completely outside of these events.

Several environmental species of Arcobacter have been isolated from marine organisms, including *A. nitrofigilis,* which was first identified as a nitrogen fixing symbiont of seagrass (Pati et al., 2010), and Arcobacter species isolated from bivalves (clams, mussels and oysters), including *A. elissii* (Figueras et al., 2011b), *A. molluscorium* (Figueras et al., 2011a*)* and *A. bivavlorium (Levican et al., 2012)*. A number of other Arcobacter species, including *A. butzleri, A. skirrowi* and *A. cryaerophilus,* have been isolated from organisms harvested for seafoods such as shell fish (Mottola et al., 2016) and have also been implicated in gastrointestinal diseases (Nieva-Echevarria et al., 2013). In fact, it appears that many species of Arcobacter share a propensity for inhabiting shellfish, which may be attributed to genes that confer production of adhesion enzymes that facilitate adherence and invasion of gut epithelial cells (Girbau et al., 2015). Additionally, physiological characteristics that allow for tolerance across a broad range of temperatures and oxygen levels enables Arcobacter to survive in environmental waters for longer periods than other enteric microbes , which provides increased opportunity to encounter and colonise filter feeders. This is important

because humans often consume shellfish that is under-cooked or raw and may therefore be exposed to emerging pathogens from the Arcobacter genus that are not routinely tested for (Ramees et al., 2017).

Although detection of Arcobacter in marine organisms has been reported (Figueras et al., 2011a, Figueras et al., 2011b), the origin, distribution and persistence of these Arcobacter species in the surface waters of coastal marine environments remains poorly characterised. In this study, strong seasonal partitioning of dry period Arcobacter communities was observed. For example, 3 arcobacter ecotypes matching *A. molluscorium* were the dominant Arcobacter populations during dry periods in Winter (when total Arcobacter abundance was lowest). Alternatively, ecotype 10 (most closely matched to *A. cloacae*, which has been previously isolated and associated with sewage), was not correlated with stormwater or modelled WWSO input, and instead was one of the more dominant ecotypes during dry periods in Summer.

Other Arcobacter populations enter the surface waters of coastal habitats in high abundances via fecal pollution (Collado et al., 2008). In this study, ecotypes 6, 7 and 8, which exhibited closest sequence identity to *A. venerupis*, were strongly correlated with both stormwater input and modelled WWSO. This was an unexpected observation because to date *A. venerupis* has not previously been linked to sewage from human waste streams. On the other hand, the strong correlation observed between Ecotype 1

and stormwater and modelled WWSO inputs is consistent with this species' documented association with sewage pollution. This ecotype contributed the highest absolute abundance of all 10 ecotypes. When normalised to qPCR data, this group peaked in abundance when more than 24,000 m^3 of modelled WWSO was discharged from the upstream Mill Pond overflow point. The high levels of occurrence of this species in the coastal environment is significant, because *A. cryaerophilus* is the second most (after *A. butzleri*) reported Arcobacter species to be associated with water and food-borne diseases (Van den Abeele et al., 2014), with evidence that this species is responsible for outbreaks of gastroenteritis and clinical cases of bacteraemia (Collado and Figueras, 2011). Notably, oligotypes matching *A. cryaerophilus* were most abundant during the Summer periods when more water users, particularly swimmers, are at risk of exposure (Leonard et al., 2015).

Curiously, the influence of sewage pollution at Foreshore Beach did not result in detection of the Arcobacter species most frequently isolated from sewage and clinical samples – *A. butzleri* (Arguello et al., 2015). Indeed, none of the Arcobacter oligotypes identified in this study exhibited close sequence resemblance to this species. Similarly, Fisher *et al.* (2014) noted in their study of Arcobacter populations across 37 sewer locations in the U.S.A and Spain, that *A. butzleri* was only the eleventh most abundant oligotype detected. The previous apparent prominence of *A. butzleri* in sewage communities may have been an overrepresentation related to the use of culture based

approaches, a phenomonem noted in previous studies (Fisher et al., 2014, Gilbert et al., 2014)

3.6.3 Implications for pathogen monitoring in coastal environments

There are several important implications to consider in light of the observations made in this study. Firstly, our data demonstrates that weekly sampling resolution, at least, is required to fully capture rapid shifts in the abundance and diversity of Arcobacter, and potentially other enteric microbes, in coastal waters subject to the influence of stormwater and WWSO. Secondly, discrete rainfall events and waste-stream inputs that lead to peaks in Arcobacter abundance may result in variable levels of risk to public health, because the composition and diversity of Arcobacter communities shifts between seasons and perturbation events. For instance, our data indicates that at Foreshore Beach, not only were total peaks in Arcobacter abundance highest during the summer, when beaches are most frequented by swimmers and other water users, but the Arcobacter community also comprised the highest proportion of pathogenic species such as *A. cryaerophilus* at this time. Thirdly, our data revealed that peaks in the abundance of Arcobacter can remain elevated for up to 1 week following intense rainfall and, importantly, can remain high for periods that significantly exceed the time that FIB levels are elevated. This could have serious implications for public health, as standard recommendations by water quality organisations suggest avoiding water use for 1-3 days after rainfall. Given that we detected numerous peaks in the abundance of Arcobacter (> 10^7 gene copies L^{-1}) that occurred within 3 days of Category A (low risk)

enterococci counts, these patterns indicate that water users may be exposed to high levels of Arcobacter and other sewage and pipe bacteria during periods when FIB based monitoring suggest low levels of risk, placing swimmers at greater risk of contracting a water-borne illness.

3.7 Conclusion

This study has resolved the fine-scale temporal dynamics of Arcobacter entering and persisting within the marine environment, as a result of stormwater input and release of modelled WWSO. Given that many Arcobacter species are (i) human pathogens, (ii) have the capacity to accumulate in marine organisms that are consumed by humans (oysters, clams and mussels), (iii) are rarely monitored in swimming locations, and (iv) survive in marine waters longer than other enteric microbes targeted in standard FIB based testing routines, these organisms could pose a significant, yet largely overlooked risk to the ecological health of coastal habitats, and the safety of water users. In this study, the application of 16S rRNA sequencing and other molecular based approaches allowed us to initially identify, and then focus our analyses on a potentially concerning microbial pathogen, which would have been overlooked using standard culture dependant methods, highlighting the potential utility of modern molecular microbiological tools for environmental monitoring programs.

3.8 Materials and Methods

3.8.1 Study sites and Sampling Protocols

A high resolution microbial time-series site was established at Foreshore Beach (33.95 ˚S, 151.9 ˚E), Botany Bay on the east coast of Sydney, Australia. Foreshore Beach is frequently subject to inputs of stormwater from a highly urbanised and industrial watershed. Electrical conductivity (EC) was used as a proxy for water salinity, with decreases in EC by 10% below mean levels used as a criteria for identifying periods of significant stormwater input. Using this approach, we discriminated stormwater impacted and dry period sampling points. Water quality at Foreshore Beach is also frequently impacted by faecal pollution from sewage overflow released at Mill Pond, a discharge point situated upstream from Foreshore Beach. The Mill Pond overflow point is one of over 1000 sewage overflow points in Sydney's wastewater system, which diverts incompletely treated wastewater into environmental waterways during heavy rainfall (OEH, 2017). Modelled wet weather sewage overflow (WWSO) rates obtained from Sydney Water (the water authority responsible for operation of Sydney's wastewater systems). Rates of sewage overflow quantified in m^3 15 min^{-1} were converted to total m^3 and summed across each sample date and the six preceding days to provide a measure of modelled WWSO integrated over the previous week. Total modelled WWSO was used to differentiate stormwater events from modelled WWSO events.

Water samples were collected from < 1.5m depth, on a weekly basis (every Friday morning between 0800-1100 AEST) for 24 months, beginning on the 2nd May 2014, providing a high temporal resolution time-series comprising 104 time points. Physicochemical parameters, including temperature, pH, electrical conductivity and dissolved oxygen, were measured *in situ* using a WTW Multiprobe meter (Multi 3430, Germany). Triplicate 5 L samples were collected to provide material for nutrient, flow cytometry and molecular analysis. From these, triplicate 50 ml samples were filtered through 0.45 µm Sartorius Minisart filters (Sartorius stedim Biotech), kept in the dark and on ice in falcon tubes during immediate transport to the laboratory, where they were stored at -80 ˚C before analysis for nutrients including NH_4, NO_x and PO_4. Triplicate 1 mL water samples were filtered through a 100 mm mesh, fixed with glutaraldehyde (2% final concentration) and snap frozen in liquid nitrogen for subsequent flow cytometric (FCM) analysis of bacterial cell abundance. Microbial biomass was retained from triplicate 2.5 L samples by filtration through 0.22 µm pore-size membrane filters (Millipore) using a peristaltic pump. Filters were stored at -80 ˚C prior to DNA extraction.

3.8.2 Water chemistry analysis

Concentrations of NH_4, NO_x and PO_4 were determined via a colorimetric analysis using the Lachat Quickchem QC8500 Automated Ion Analyser (LACHAT Instruments, USA) in accordance with the manufacturer's guidelines. Internal controls, including addition of spiked recovery samples and blanks, were included to monitor the consistency of

instrument performance. The resulting data were transferred and interpreted using Omnion version 3 software (LACHAT Instruments, USA).

3.8.3 DNA extraction and 16S rRNA gene amplicon sequencing

DNA was extracted from filters using the Powerwater DNA isolation Kit (MoBio Laboratories, Inc) in accordance with the manufacturer's instructions. DNA quantity and purity was evaluated using a Nanodrop-2000 Spectrophotometer (Thermo Fisher Scientific, D.E, U.S.A). We employed 16S rRNA amplicon sequencing to characterise patterns in bacterial community diversity and composition throughout the 24 month time-series. The V1–V3 variable region of the 16S rRNA gene was amplified using the 27f 5'-AGRGTTTGATCMTGGCTCAG-3' and 519r 5'-GTNTTACNGCGGCKGCTG-3' primer pair (Nadkarni et al., 2002), with a barcode on the forward primer. Sequencing was performed using the Illumina MiSeq platform (at Molecular Research LP; Shallowater, TX, USA) following the manufacturer's guidelines. Raw data files in FASTQ format were deposited in NCBI Sequence Read Archive (SRA) with the study accession number SRP143624 under Bioproject number PRJNA420901.

16S rRNA gene sequences were analysed using the QIIME pipeline (Caporaso et al., 2010, Kuczynski et al., 2012). Briefly, paired-end DNA sequences were joined and *de novo* Operational Taxonomic Units (OTUs) were defined at 97% sequence identity using UCLUST (Edgar et al., 2011). Chimeric sequences were detected using ChimeraSlayer

(Haas et al., 2011) and filtered from the dataset. Taxonomy was assigned against the SILVA v128 database using BLAST (Altschul et al., 1990). Samples were then rarefied to the same sequencing depth to remove the effect of uneven sampling effort upon downstream analyses.

3.8.4 Quantitative PCR (qPCR)

Peaks observed in the relative abundance of 16S rRNA amplicon sequences matching Arcobacter suggested that members of this genus were of quantitative importance following stormwater events, prompting further investigation. Quantitative PCR (qPCR) was used to track patterns in the absolute abundance of bacteria belonging to the *Arcobacter* genus. The oligonucleotide primers ARCO1 (5'-GTCGTGCCAAGAAAAGCCA-3') and ARCO2 (5'TTCGCTTGCGCTGACAT-3') were used to amplify a 331-bp segment of 23S rRNA gene (Bastyns et al., 1995). rRNA samples for qPCR were run in technical triplicate, amounting to a total of 936 reactions measured across the 24 month time-series. The efficiency of amplification of the 23S rRNA gene varied between 95 – 102%, and the limit of quantification was determined to be 10^3 gene copies mL water. Quantification of the absolute abundance of Arcobacter was achieved by normalisation to volume of water filtered.

PCR product standards for the Arcobacter qPCR assay were created by amplification of the 23S rRNA segment using the ARCO1 and ARCO2 primers from an Arcobacter strain

isolated from Foreshore Beach on a modified marine agar medium of 0.01g peptone, 0.01g yeast extract, 2.8g sea salts into 240 mL of filtered, purified water. DNA from the isolate was extracted using a phenol chloroform extraction method. Briefly, Arcobacter cells grown on a marine agar plate were chemically lysed using a lysis buffer solution containing 0.5% Lysozyme. DNA was isolated and purified using phenol/chloroform, precipitated using isopropanol and eluted into 30 µl TE buffer solution. The isolate was confirmed to be a strain of Arcobacter using Sanger sequencing performed at the Australian Genomics Research Facility (NCBI submition number of this isolate), with the closest taxonomic match (97% similar) to this isolate in the NCBI database determined to be *Arcobacter Venerupis*.

qPCR was performed using the BIO-RAD CFX384 Touch™ Real-Time PCR Detection System™ and Arcobacter gene copies were calculated by plotting against a standard curve using BIO-RAD CFX Manager software. Each sample was run using three technical replicates, consisting of 5 µl reaction volumes containing 2.5 µl iTaq UniverSYBR Green SMX 2500®, 0.1 µl nuclease free water, 0.2 µM for each forward and reverse primers and 2 µl of diluted (1:10) DNA template. Calibration curves were run within every plate, with all reagent and plate preparation conducted using a epMotion® 5075l Automated Liquid Handling System. To quantify temporal patterns in the Arcobacter 23S rDNA gene, the qPCR cycling parameters described in (González and Suski, 2010) were used. To confirm that the primer pair produced a single specific product, a melting curve was added to the end of the first plate.

3.8.5 Characterisation of Arcobacter OTU diversity using Oligotyping

Oligotyping is a computational method for measuring subtle, single nucleotide variations among 16S rRNA gene sequences, allowing for the discrimination of otherwise concealed strain level diversity within OTUs (Eren et al., 2013). We used this approach to track patterns in Arcobacter community diversity throughout the time-series. The q2oligo.py python pipeline inside the QIIME v1.9 environment was used to filter 410,427 sequence reads matching the Arcobacter genus from the 16S rRNA dataset. Supervised minimum entropy decomposition was employed using the Oligotyping 2.0 pipeline (available from https://pypi.python.org/pypi/oligotyping/2.0), until each oligotype had converged – whereby the remaining entropy was attributable only to noise. The mean was taken for each sample triplicate set, leaving 90 sample time points across the time-series containing a sufficient quantity of Arcobacter sequences for inclusion. From these samples, a further 47,538 sequences were discarded as noise, resulting in 3,076 oligotype raw nodes, which were further refined to 181 final oligotypes after removing rare nodes (< 500 total sequences across all 90 samples). Upon completion of oligotyping analysis, observation counts (number of sequences of each oligotype expressed as a percentage of all Arcobacter sequences) were multiplied by the absolute abundance of total Arcobacter gene copies, measured using qPCR, to produce estimates of absolute abundance for each Ecotype. Both relative and absolute abundances were used in downstream analyses of Arcobacter diversity and abundance.

3.8.6 Network Analysis

The maximal information-based nonparametric exploration (MINE) pipeline (Reshef et al., 2011) was used to measure co-occurrence patterns between Arcobacter oligotypes, which were subsequently visualised using the edge-weighted, spring embedded function in Cytoscape 3.4.0 (Doerks et al., 2002). This allowed for Arcobacter oligotypes to be clustered into discrete "ecotype" modules based on co-occurrence. Representative sequences within each ecotype module were chosen using the pick_rep_set.py command in QIIME 1.9 and taxonomy was assigned to these sequences by referencing against a curated database of Arcobacter sequences extracted from the SILVAv128 database. A phylogenetic tree was created using Figtree (Rambaut, 2007) using Campylobacer as an outgroup, and representative oligotypes from each ecotype module were compared to the closest matching species from the curated database.

To uncover significant correlations between environmental conditions and Arcobactor ecotype modules, the MINE pipeline was further employed to link ecotypes to measured environmental parameters. Statistically significant correlation patterns between Arcobacter ecotypes, absolute Arcobacter abundance, water chemistry parameters, stormwater input and sewage discharge volume were measured across the 104 week time-series. Only pairwise interactions that occurred across 10% or more of the time points were included, and interactions with a maximal information coefficient (MIC) value below 0.379 (estimated to be equivalent to $p = < 0.001$ after correction for

multiple tests) were removed. Remaining interactions were visualised using the edge-weighted spring embedded function in Cystoscape 3.4.0.

Selected pairwise co-occurrence and time-lagged correlations were measured in R using cross correlation factor (CCF) to determine temporal relationships between input of stormwater and runoff, and the absolute abundance of Arcobacter using qPCR (quantitative abundance) data. Resultant data was visualised in a CCF graph, showing the temporal correlation relationships across the 104 week time-series.

3.8.7 Phylogenetic relationship of Arcobacter oligotypes

A maximum likelyhood phylogeny was built to investigate the relationship of representative arcobacter oligotypes to known arcobacter strains. A data set of 20 selected arcobacter oligotypes from this study was aligned with the 16S ribosomal nucleotide sequences of 19 representative arcobacter strains previously used in a similar study (Fisher et *al*., 2014). Additionally, the 16S sequence of *Campylobacter jejuni* was included in the data set as an outgroup. The representative arcobacter sequences as well as the sequence of *C. jejuni* were extracted from the SILVA v128 (Quast et *al.,* 2013). The complete data set was aligned using Mafft v7.03 (Katoh and Standley, 2013) with default parameters. The alignment was subsequently curated and positions with more than 2 gaps were removed, resulting in a final alignment of 374 positions in all 40

sequences. The approximate maximum-likelyhood phylogeny was inferred using FastTree 2 (Price et *al.,* 2010).

3.8.8 Enterococci counts

Historical Enterococci colony forming unit (CFU) count data from Foreshore Beach was retrieved from the NSW Office of Environment and Heritage – Beachwatch website http://www.environment.nsw.gov.au/beachapp/report_enterococci.aspx. This data was used to compare a standard FIB monitoring approach to our qPCR measurements of Arcobacter. However, samples for measurement of Enterococci were often collected on different days and at varied time intervals compared to our data, prohibiting a direct statistical comparison of these datasets. Therefore, Pearson's Correlation was used to test for significant correlations between Enterococci counts and Arcobacter abundance and population composition, using the closest preceding or proceeding Enterococci value (within three days) to compare with each Arcobacter absolute abundance and oligotype relative abundance value.

Enterococci counts were partitioned into 4 microbial assessment categories (MAC), A, B, C and D. The MAC is a method established by the World Health Organisation for calculating the risk of illness in swimmers, dependant on the concentration of a FIB (NSW Office of Environment and Heritage, 2015). Each category represents a different, escalating level of risk of illness based upon epidemiological studies in comparable

environments. For example, enterococci counts < 40 CFU mL^{-1} are assigned to Category A, whereby the risk of a water user contracting a gastrointestinal illness (GI) is estimated to be < 1 %, or an acute fever or rash (AFR) < 0.3 %. Estimated risk escalates with higher Enterococci counts values, up to Category D (see Table 1).

3.8.9 Acknowledgements

This study was funded by Australian Research Council project FT130100218 to J.R.S. We thank OEH Beachwatch for providing advice on sampling locations, and Sydney Water for making modelled WWSO data available.

3.9 Figures

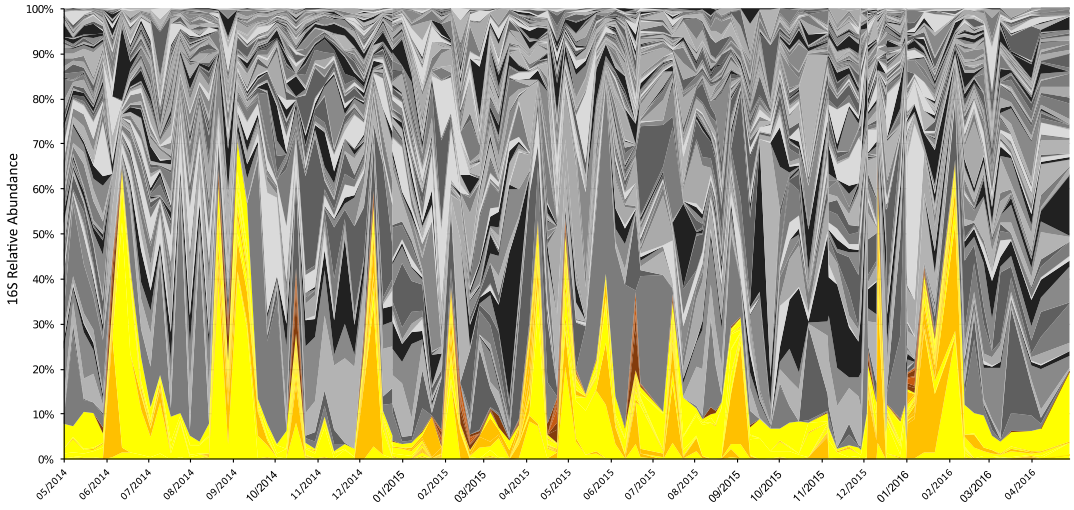


Figure 10 *16S rRNA amplicon sequence profile of the abundant bacterial community (OTU's contributing > 0.1% of total 16S sequence reads) at Foreshore beach, from 02/05/2014 – 29/04/2016. Yellow coloured OTU's along the base of the profile represent the relative abundance of OTU's assigned to Arcobacter using the SILVA v128 library. Across 104 time points at weekly intervals, Arcobacter relative abundance ranges from < 1% to approximately 70% of the whole bacterial community.*

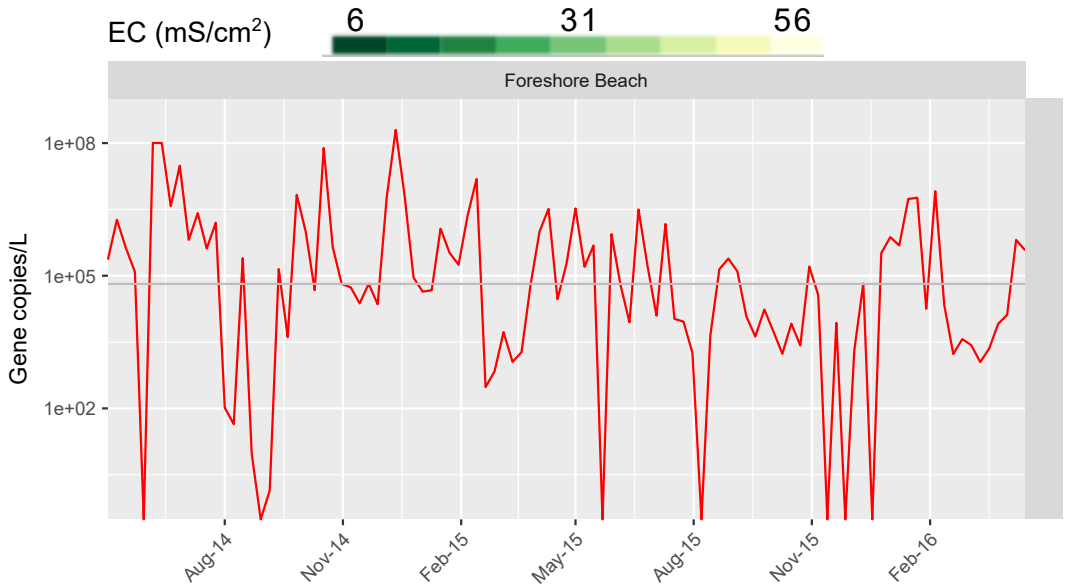


Figure 2. *The absolute abundance of total Arcobacter was measured using qPCR to enumerate a 23S amplicon (Arco1) conserved across the Arcobacter genus, at weekly time intervals from the 02/05/2014 to 29/04/2016. Gene copy numbers per litre of sea water are represented by the red line, which is the mean of 3 sample replicates, each determined using 3 technical replicates. The grey shading indicates standard deviation. The grey horizontal line*

represents the mean abundance across the time-series. Green vertical bars indicate values of electrical conductivity, where drops in EC are represented by darker shades of green are indicative of runoff, and stormwater input.

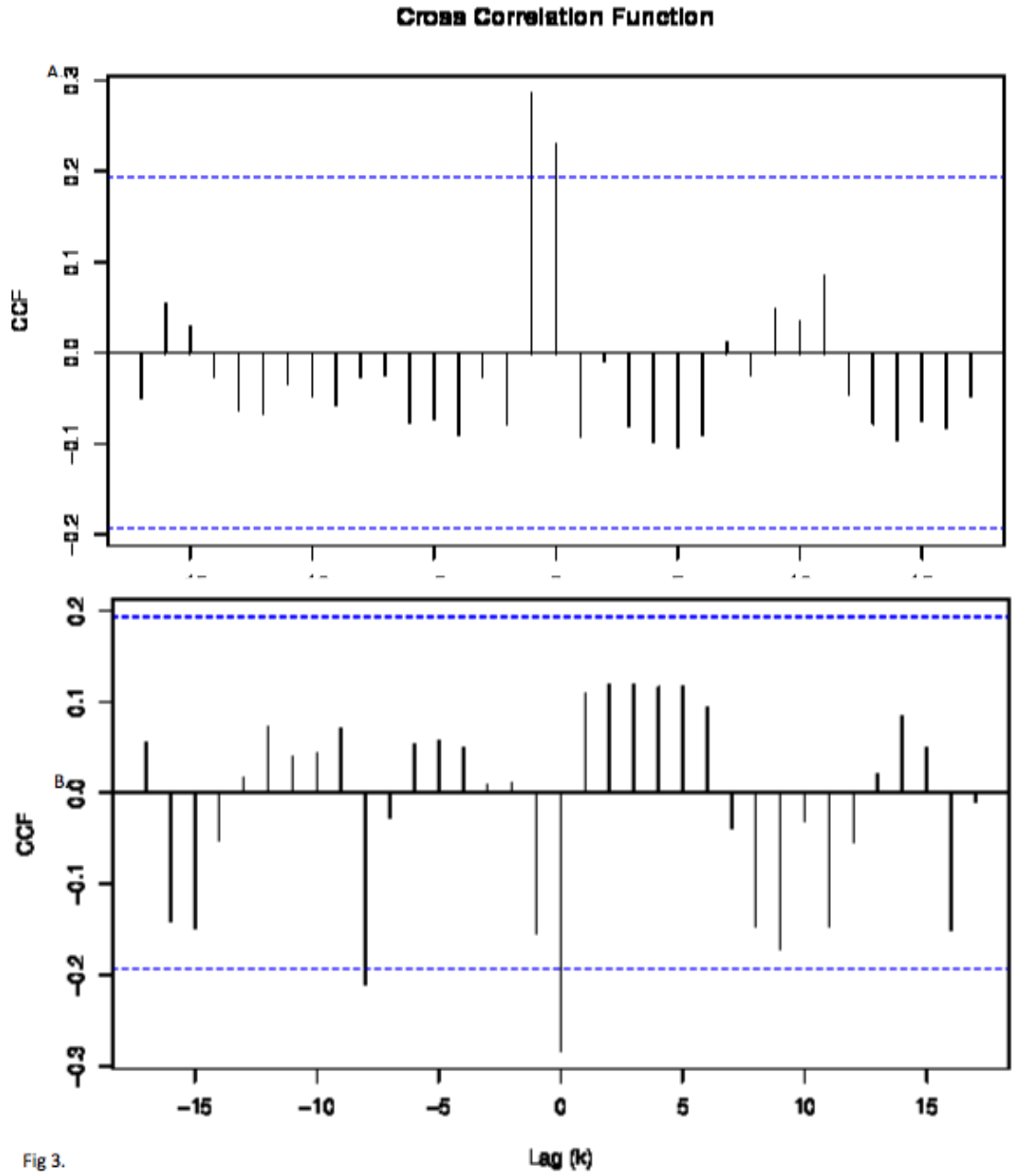


Figure 3. *Cross Correlation Function was employed to measure the co-occurrence and time lagged association between a) rainfall (variable 1) and the absolute abundance of Arcobacter (qPCR gene copy numbers (variable 2); and b) electrical conductivity (variable 1) and the absolute abundance of Arcobacter (variable 2). Values across the x axis represent the time lag (in weeks), where 0 is co-occurrence, negative values represent weeks at which variable 1 predicts variable 2, and positive values represent correlations where variable 2 predicts variable 1. Values across the y axis represent the strength of the correlation, and its orientation as positive or negative (above or below the central horizontal line respectively). Instances where horizontal lines intersect the perforated blue lines (the 95% confidence threshold) indicate a significant (< 0.05) correlation. The top panel shows a significant positive correlation at time lag 0 and -1, indicating that rainfall events lead to elevated Arcobacter abundance during and 1 week following the rainfall. The bottom panel reveals a significant and strong negative co-occurrence between EC and Arcobacter abundance, suggesting that when EC levels drop, Arcobacter abundance is increased.*

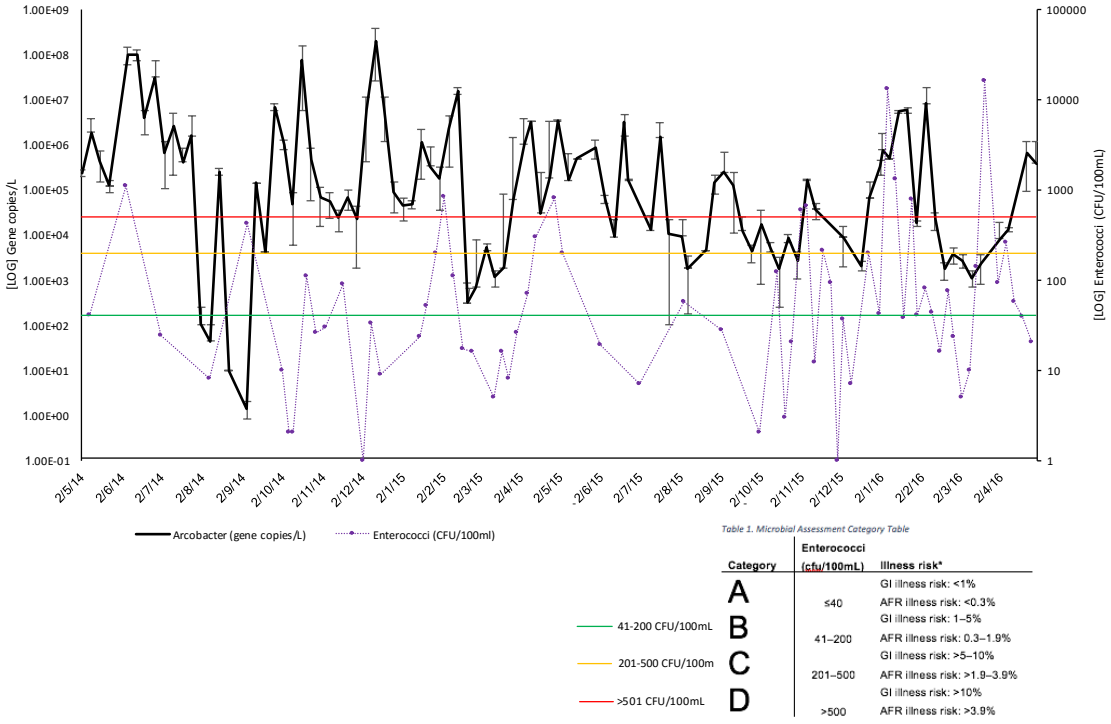


Figure 4. *Patterns are compared between qPCR measurements of the absolute abundance of Arcobacter (primary vertical axis) and enterococci colony plate counts (secondary vertical axis), across the 2-year time-series. Background colours are representative of ascending levels of risk of a swimmer contracting a waterborne illness in association with enterococci count data.*

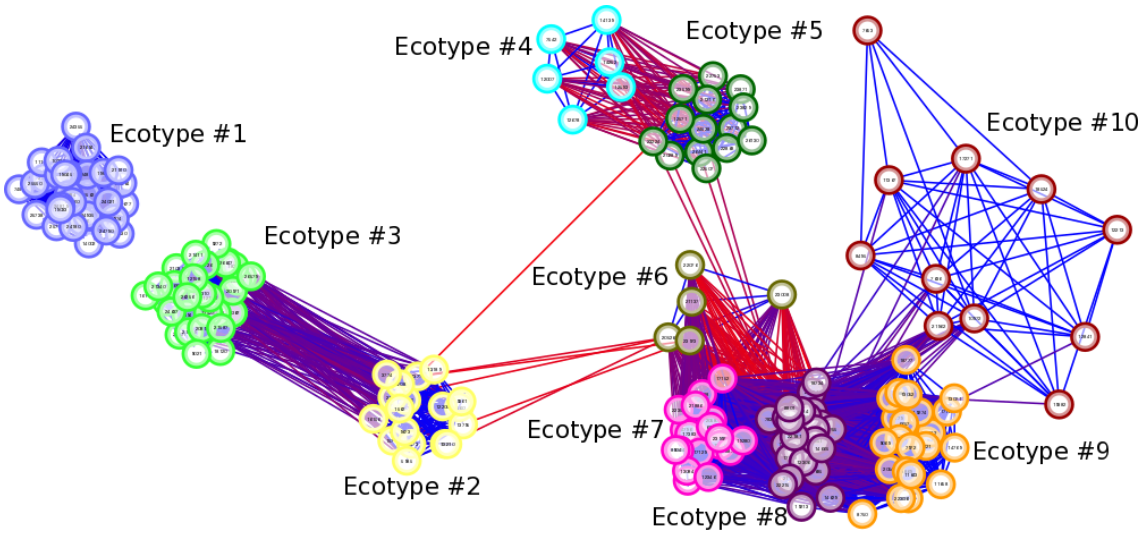


Figure 5. *Network analysis, depicting the co-occurrence associations measured by the maximal information coefficient (MIC) (seen as edges/lines connecting circles) between 181 Arcobacter oligotypes (represented by nodes/circles). Correlations were measured using the MINe pipeline. Blue edges indicate a positive correlation, and red indicate a negative correlation. Nodes that are interconnected with positive edges and cluster together represent Arcobacter oligotypes that co-occur closely, and likely occupy the same temporal niches across the time-series, and have been termed an "ecotype".*

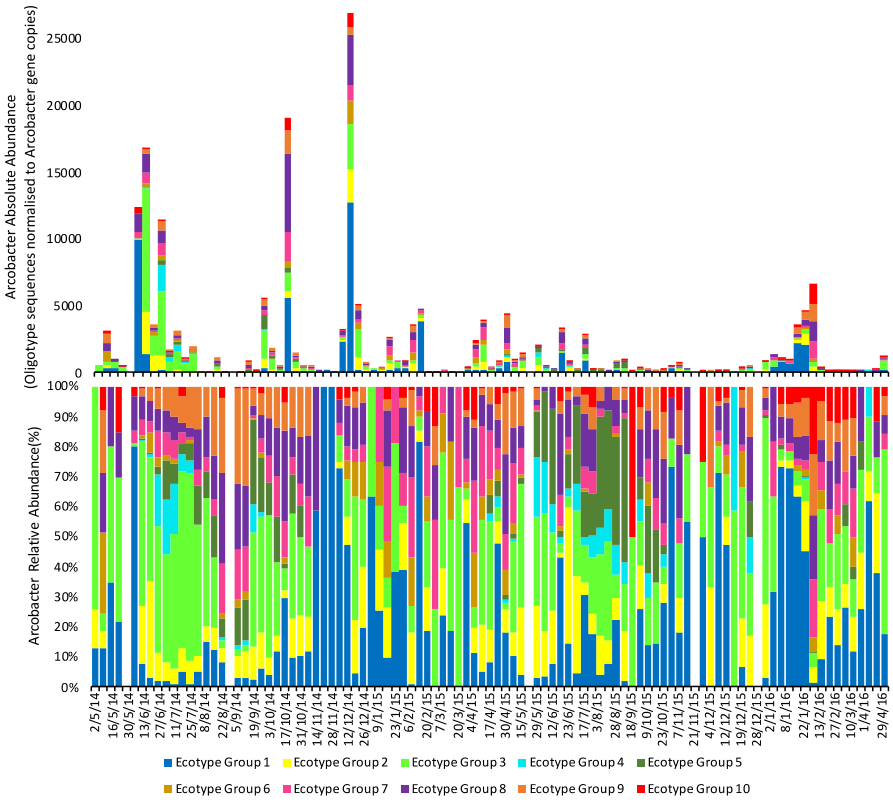


Figure 6. *Relative abundance of Arcobacter ecotypes (colour coded consistently with Fig 5.) across 32 of the 104 time points at Foreshore beach, where the 181 oligotypes were present. Time points omitted from this figure also contained Arcobacter oligotypes, which were removed from the analysis due to their very low abundances. Relative abundance was calculated by normalising the number of Arcobacter sequence reads by the total number of 16S amplicon sequence reads for each sample.*

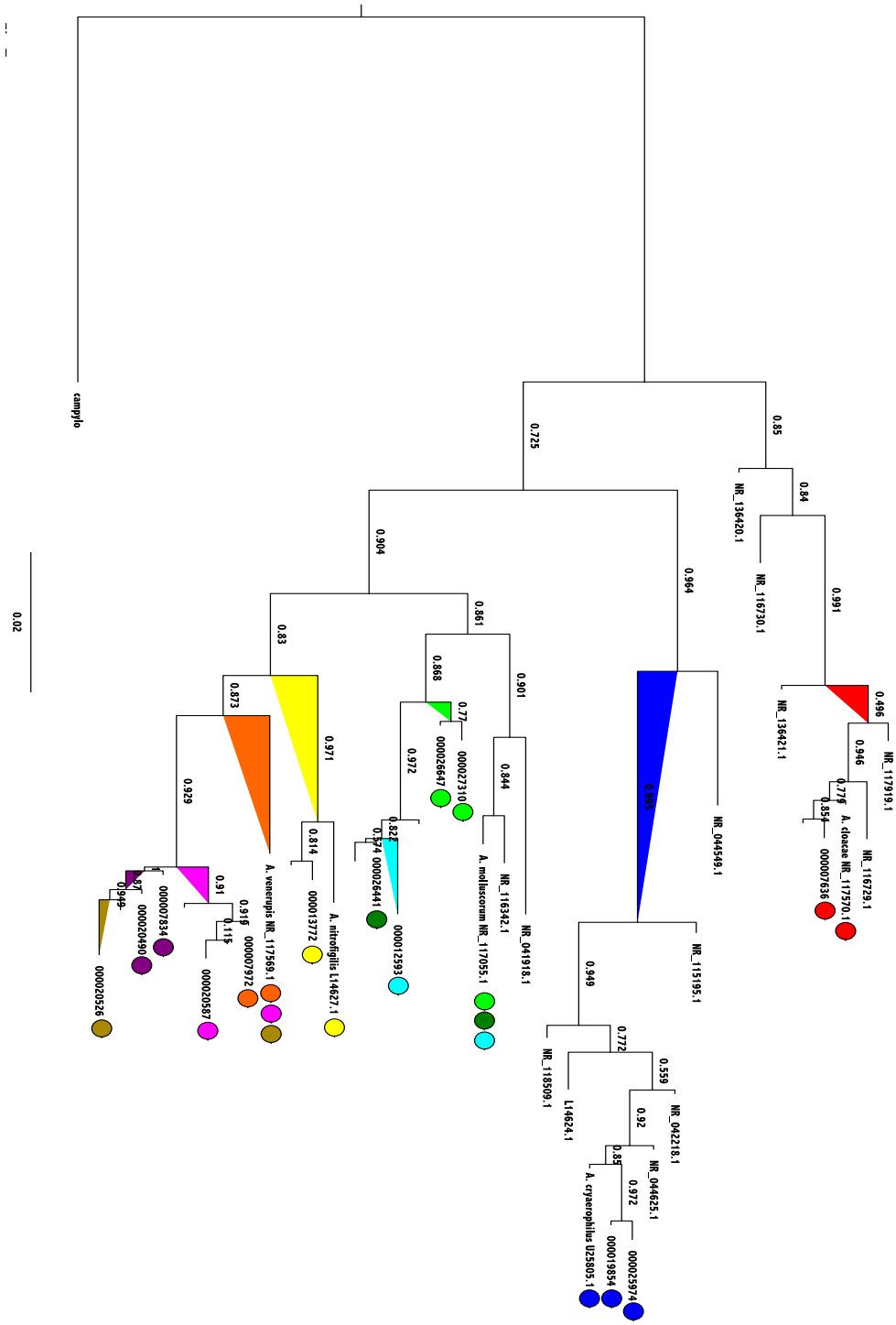


Figure 7. *Maximum likelihood phylogeny of representative Arcobacter oligotypes and Arcobacter sequences from the SILVA ribosomal database. Branch labels represent Fasttree support values. Phylogeny was visualised using the Figtree program. Representative Arcobacter oligotypes are identifiable by numbers beginning in 0, and reference sequences from the SILVA v128 library are identifiable by beginning with NR_. Colour coding of representative Arcobacter oligotypes is consistent with Figures 4 and 5.*

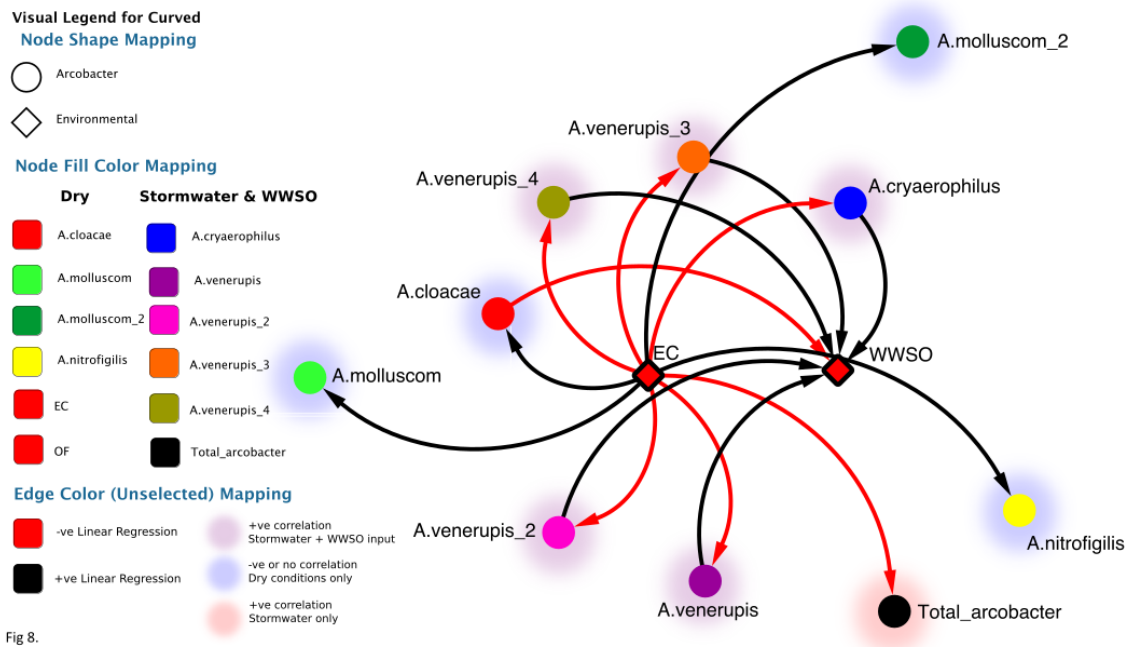
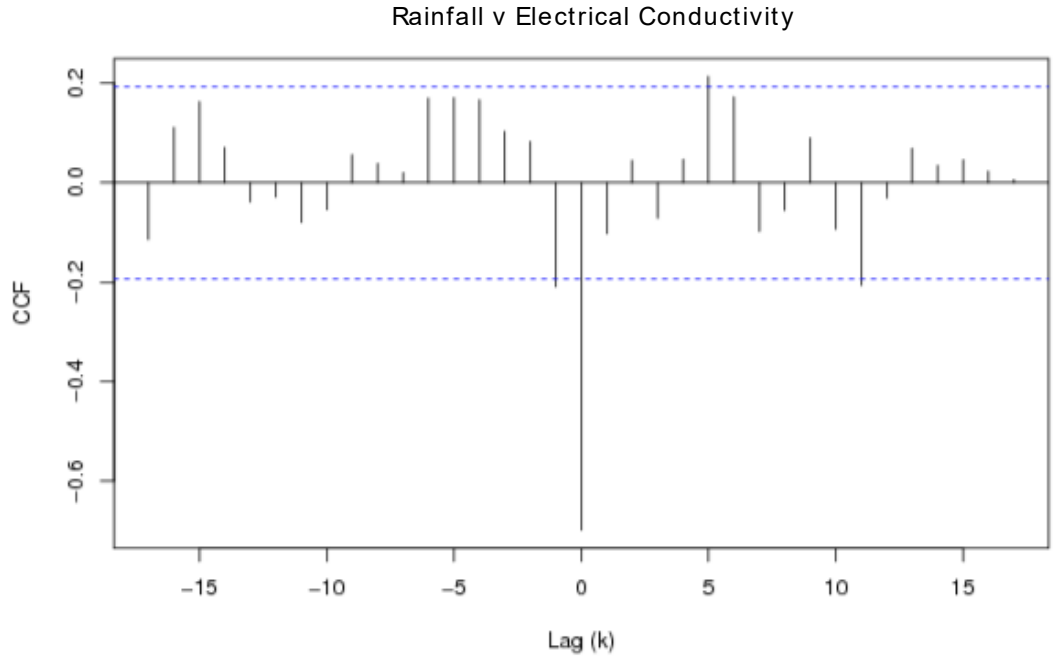


Figure 8. *Network analysis, depicting the co-occurrence associations measured by the maximal information coefficient (MIC) (seen as edges/lines connecting circles) between Arcobacter ecotypes (represented by nodes/circles) and environmental perturbations (red diamonds - runoff and stormwater represented by EC, and WWSO represented by OF). Correlations were measured using the MINe pipeline. Black edges indicate a positive correlation, and red indicate a negative correlation. The shorter the edge connecting 2 nodes, the stronger the correlation, and the longer the connecting edge, the weaker the correlation. Arcobacter ecotypes that are connected to EC with a red edge are more abundant during runoff and stormwater impacted periods (when EC drops), and Arcobacter ecotypes that are connected to OF by black edges are more abundant during periods impacted by WWSO. Glow effect has been used for ease of identification of putative stormwater and WWSO ecotypes (red), and dry condition, putative environmental ecotypes (blue).*

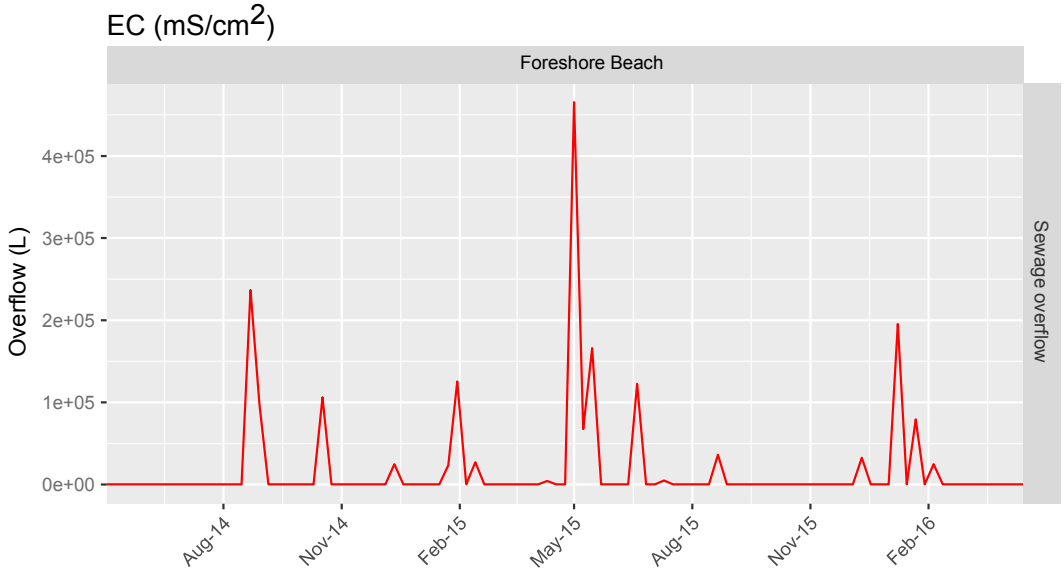
Table 2. Microbial Assessment Category Table

Category	Enterococci (cfu/100mL)	Illness risk*
A	≤40	GI illness risk: <1% AFR illness risk: <0.3%
B	41–200	GI illness risk: 1–5% AFR illness risk: 0.3–1.9%
C	201–500	GI illness risk: >5–10% AFR illness risk: >1.9–3.9%
D	>500	GI illness risk: >10% AFR illness risk: >3.9%

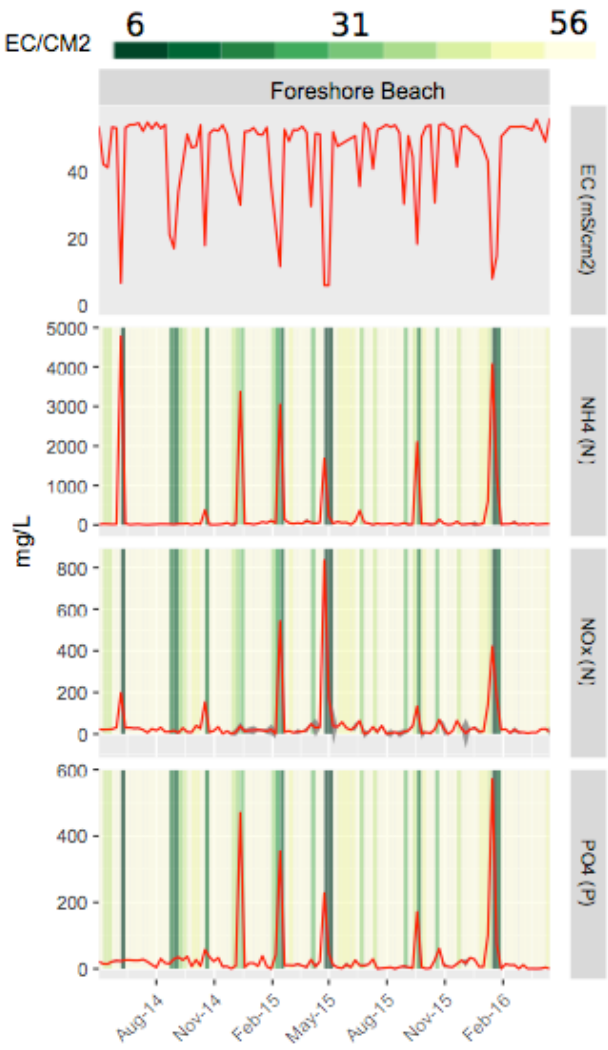
3.10 Supplementary Figures



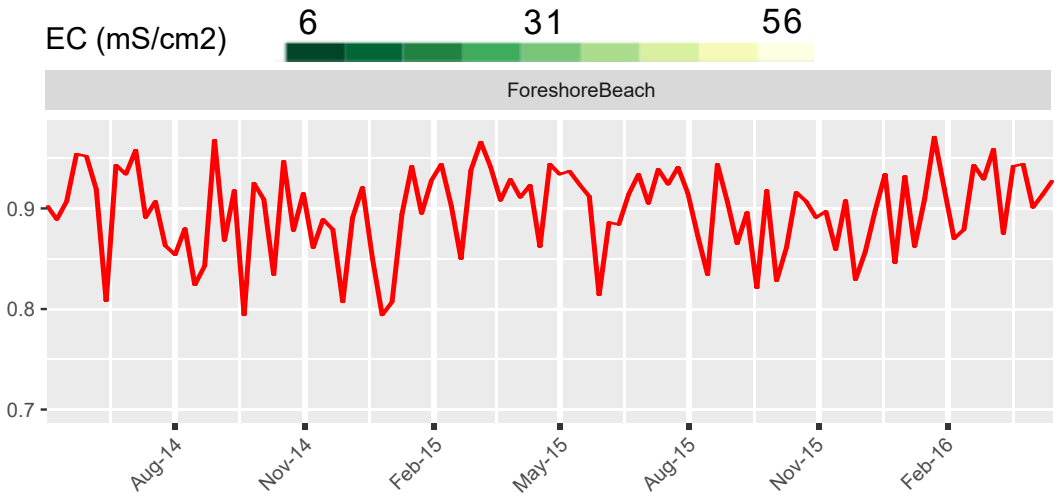
SI Fig 1. *Cross Correlation Function (CCF) was used to measure patterns of co-occurrence and time-shifted correlation between two variables, in this case rainfall (series 1) and electrical conductivity (EC) (series 2) at Foreshore Beach. Weekly time intervals were plotted across the horizontal axis (where 0 is co-occurrence or no time lag, negative values represent time intervals where rainfall precedes EC (negative time lag), and positive values represent time intervals where rainfall proceeds EC (positive time lag). Values on the vertical axis represent the cross-correlation function (CCF) of each time lag, which is considered as a correlation co-efficient between the two series, at different time lags. Where a vertical line value crosses the broken blue horizontal line (95% confidence interval), this indicates a statistically significant correlation at this given time lag. A strong, negative and significant co-occurrence at time lag 0 is evident, as well as a weaker, but significant -1 week time lag, suggesting that rainfall leads to decreases in EC at Foreshore Beach, lasting for up to 1 week following the rainfall.*



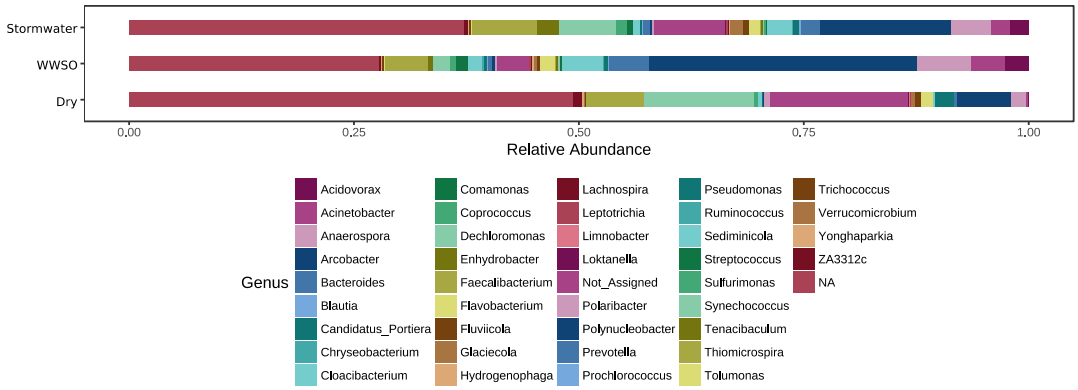
SI Fig 2. *Rainfall and wet weather sewage overflow (WWSO) across the 2 year time series at Foreshore Beach. Vertical bars indicate level of electrical conductivity (EC) values with dark green bars showing significant drops in EC that are indicative of storm-water input. Rainfall was measured as the average quantity of precipitation (mm) on the day of sampling plus the preceding day as retrieved from the Sydney International Airport weather gauge. The red line tracks the volume of WWSO released into Foreshore beach from the Millpond overspill point, 1.06 km upstream from Foreshore Beach, across the two year time series. Sewage released from Millpond was measured as the total (m3) on the day of sampling plus the six preceding days (data provided by Sydney Water).*



SI Fig 3. *Line plots of electrical conductivity (EC) and dissolved nutrients at Foreshore Beach across the two year time series. In all panels, red lines represent values of EC, NH^4, NOx and PO^4 respectively (from top to bottom). Green vertical bars in the nutrient panels represent EC with dark green bars showing a decrease in EC (indicative of storm-water input).*



SI Fig 4. *Line plot of bacterial diversity (Shannon's Index) at Foreshore Beach across the two year time series. Background green vertical bars represent electrical conductivity (EC), where dark green bars indicate a decrease in EC (indicative of storm-water input).*



SI Fig 5. Bacterial community relative abundance profile, generated from 16S rRNA amplicon sequencing, and used for comparison of Arcobacter relative abundance (dark blue) between dry, stormwater and wet weather sewer overflow impacted periods.

4. Chapter 4

Urban beaches are environmental hotspots for antibiotic resistance following rainfall

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Chapter 4 - Urban beaches are environmental hotspots for antibiotic resistance following rainfall

4.1 Preface

In chapter 3, having we closely interrogated the population dynamics of Arcobacter, an enteric bacteria that enters natural marine environments via stormwater and sewage runoff. In this chapter we have switched the focus of analysis onto the detection and quantification of microbial genes that confer resistance to antibiotics that are critical for human health.

4.2 Abstract

To reveal the occurrence and mechanisms for dispersal of antibiotic resistance (AbR) within coastal settings, we performed a weekly, two-year duration time-series study at two urban beaches between 2014 - 2016. Our approach combined quantitative PCR and multiplex PCR/reverse line blot techniques to track patterns in the occurrence of 31 AbR genes, including genes that confer resistance to antibiotics that are critically important antimicrobials for human medicine. Patterns in the abundance of these genes were linked to specific microbial groups and environmental parameters using 16S rRNA amplicon sequencing and network analysis. We observed up to 100-fold increases in the abundance of several AbR genes, including genes conferring resistance to both high use front line antibiotics (e.g. quinolones, trimethoprim, sulfonamides and tetracycline) and last resort antibiotics (e.g. vancomycin and carbapenams), following storm-water and modelled wet-weather sewer overflow events. The abundance of AbR genes strongly and significantly co-occurred with several potentially pathogenic bacterial OTU's regularly associated with wastewater infrastructure, such as *Arcobacter*, *Acinetobacter*, *Aeromonas* and *Cloacibacterium*. These high-resolution observations provide clear links

between storm-water and sewer overflow and the occurrence of AbR in the coastal microbial assemblages inhabiting urban beaches, highlighting a direct mechanism for potentially profound AbR exposure risks to humans.

4.3 Introduction

The rapid global rise of antibiotic resistance (AbR) has primarily occurred as a consequence of the misuse and over-use of therapeutic antibiotics in medicine and agriculture (Van Boeckel et al., 2014, Sarmah et al., 2006, Witte, 1998). As a result, the efficacy of many life-saving antibiotics has diminished (Wright, 2007, O'Neill, 2016), leading to increased incidence of untreatable infections in humans (Michael et al., 2014, Hawkey and Jones, 2009). While the bulk of research into the spread of AbR has focused on clinical settings (Wright, 2010), AbR has become increasingly considered a "One Health" issue - the concept that human health is connected to the health of animals and the natural environment (Robinson et al., 2016). This is because AbR can emerge and move between humans, animals and wider environments (Pehrsson et al., 2016), with the global spread of AbR facilitated by the movement of the human population, the transport of livestock and food products (Sarmah et al., 2006, Foley and Lynne, 2008) and insufficiently treated waste streams (Baquero et al., 2008).

Anthropogenic waste streams contribute to the environmental spread of AbR in two ways. First, excreted therapeutic antibiotics present in the urine and faeces of animals and humans (Rang, 2016), not destroyed during wastewater treatment processes (Michael et al., 2013), select for AbR microbes when introduced into the environment (Baquero et al., 2008). Second, anthropogenic waste streams deposit clinically important AbR microbes and their associated genes and genetically-linked mobile genetic elements (MGEs) such as plasmids, transposons and integrons directly into the

environment (Gillings et al., 2008, Marti et al., 2014, Chamosa et al., 2017, Kummerer, 2009). This creates a scenario where transmission of resistance genes from human and animal pathogens into endemic aquatic microbial communities (Taylor et al., 2011) or into the microbiomes of exposed humans (Hehemann et al., 2010) can occur.

Urban coastal beaches represent an important interface for human exposure to microbial contaminants. These environments are heavily used by humans for recreation and food supply (Clark and Johnston, 2017), but are also potentially subject to large quantities of human and animal waste streams (Stewart et al., 2008). The transport of potentially pathogenic enteric microbes from urban waste streams into coastal waters is a major public health concern due to high incidence of waterborne infections, and associated healthcare costs (Collier et al., 2012). There are four primary mechanisms by which waste streams transport enteric microbes into coastal surface waters. Firstly, sewage overflow (including dry and wet weather events) enter the storm-water system, bypass wastewater sanitation processes and directly transport enteric microbes into coastal waters (McLellan et al., 2015). Secondly, untreated sewage can enter into environmental waters due to sewer blockages and leaks. Thirdly, following heavy rainfall, runoff from urban surfaces such as streets, roofs and parks wash enteric microbes into drains that discharge into beaches. Fourthly, enteric microbes that persist in storm-water infrastructure can be scoured from stormwater surfaces during intense flow and subsequently flushed into beaches (McLellan et al., 2015, Vandewalle et al., 2012). Notably, because storm-water environments are frequently exposed to a range

of antimicrobial anthropogenic pollutants, including trace concentrations of antibiotics, genes that confer resistance to antibiotics are often selected for in waste stream microbes (Marchaim et al., 2012, Czekalski et al., 2012). Therefore, while the negative influence of enteric pathogens has typically been considered the central human health issue within coastal environments (Turgeon et al., 2011), the transmission of AbR genes poses an additional threat, with emerging evidence that regular users of coastal ecosystems (e.g. surfers) can assimilate AbR microbes into their microbiome (Leonard et al., 2015, Leonard et al., 2018).

Coastal water quality monitoring programs typically only target a narrow selection of indicator organisms (Boehm and Sassoubre, 2014), and thus generally overlook antibiotic resistant microbes and AbR genes that can enter natural waterways via storm-water systems. However, the dissemination of AbR genes from waste streams into coastal habitats could have profound consequences for human health (Leonard et al., 2018). Investigations into the reservoirs and pathways of AbR across different environments are therefore needed to understand the processes that influence the movement of AbR microbes and genes, and to develop strategies for combating the growing global AbR crisis (Wright, 2010). Within this context, we performed a two year duration, high-resolution microbial time-series study at two coastal sites in Sydney, Australia's largest city, with the goal of defining the temporal dynamics and mechanisms for input of AbR genes in urban beach environments.

4.4 Materials and Methods

A comprehensive account of study sites, methods for sample acquisition, and data processing and analysis are contained within the *Supporting Information*. Briefly, two study sites were selected according to contrasting levels of exposure to storm-water and sewage pollution, with one highly impacted and one relatively unaffected site selected (SI Fig 1). Sampling was conducted at each site on a weekly basis (every Friday morning between 0800-1100 AEST) over the course of 24 months, leading to the establishment of two time-series, each comprising 104 time points. A suite of water physico-chemical parameters were measured using a WTW Multiprobe meter (Multi 3430, Germany), and dissolved inorganic nutrients using the Lachat Quickchem QC8500 Automated Ion Analyser (LACHAT Instruments, USA) in accordance with the manufacturer's guidelines. Flow cytometry was used to enumerate bacterial cells as described in *Seymour et al.* 2015 (Seymour et al., 2005). Microbial DNA was extracted from 0.22 µm membrane filters using the Powerwater DNA isolation Kit (MoBio Laboratories, Inc) in accordance with the manufacturer's instructions. Sequencing of the 16S rRNA amplicon was performed on an Illumina MiSeq platform following the manufacturer's guidelines. Raw data files in FASTQ format are deposited in the NCBI Sequence Read Archive (SRA) with the study accession number SRP143624 under Bioproject number PRJNA4209011. Quantitative PCR was used to quantify the abundance of 5 AbR genes using assays, efficiencies and limit of quantification reported in SI material. Finally, DNA samples collected from Foreshore Beach were screened for 26 additional AbR genes of regional clinical relevance using multiplex polymerase reaction with reverse line blot (mPCR/RLB)

hybridization. Multivariate statistical approaches including analysis of similarity (ANOSIM) and similarity percentage (SIMPER) analysis were used to compare bacterial communities between stormwater, WWSO and dry periods, and identify and rank the most abundant bacterial OTU's. Correlations between environmental parameters, the relative abundance of bacterial OTUs, and concentrations of AbR genes were measured and visualized using eLSA (Xia et al., 2011) and network analysis using Cytoscape 3.4.0.

4.5 Results & Discussion

4.5.1 Storm-water and wet weather sewage overflow events alter physicochemical conditions and microbial community abundance and structure

Every year, an estimated three trillion litres of storm-water is discharged from Australian urban centres into aquatic habitats (SECRC and Committee), 2015). Here, we defined storm-water events at the Foreshore Beach and Maroubra Beach sites as periods where a 10% reduction in EC from mean levels was recorded. Moreover, in many urbanised settings, intense rainfall events sometimes result in WWSO events. In Sydney, Australia's largest city, it is estimated that 143 WWSO's occurred across 10 coastal WWTP's from 2014 to 2015, resulting in approximately 27,964 ML of WWSO, as modelled by the Trunk Wastewater System Model Update, Re-calibration and Annual Reporting Procedure (Sydney Water Corporation, 2015). The WWSO events that occurred at Foreshore Beach during the course of this study were characterised by the modelled total volume of WWSO released from an upstream overspill point within seven days of each sample occasion.

Storm-water events were observed at both sampling sites, although at varying levels of intensity and duration. CCF analysis revealed that rainfall correlated significantly with a decline in EC at the impacted Foreshore Beach site (Supplementary Fig 2), during the 16 storm-water events observed during the two-year study (Fig 1). The duration of impact of storm-water events at this site ranged between 1 to 4 weeks (Fig 1). In contrast, rainfall did not correlate significantly with declines in EC at Maroubra Beach (Supplementary Fig. 2), with only 2 notable storm-water events recorded at this site. Notably, the modelled WWSO events coincided with 10 of the 16 storm-water events at Foreshore Beach (Fig 1A), with modelled WWSO volume ranging in magnitude from 4,193 m^3 $week^{-1}$ to 465,438 m^3 $week^{-1}$. The highest recorded nutrient concentrations coincided with sewage overflow events (Supplementary Fig. 3).

At Foreshore Beach, storm-water events led to significant increases in the concentration of all measured inorganic nutrients (NH_4, NOx and PO_4); Supplementary Fig. 3; $r = 0.429$, $p = 0.001$). However, this pattern was not observed at Maroubra Beach ($p > 0.05$), where fewer storm-water events occurred, and mixing and tidal flushing likely disperses runoff rapidly. Similarly, both bacterial abundance and diversity (Shannon's Index) remained relatively stable at Maroubra Beach ($p > 0.01$), but varied significantly ($p < 0.01$) at Foreshore Beach, with highest bacterial abundances and diversity corresponding with periods of rainfall that resulted in storm-water input and modelled WWSO (Supplementary Fig. 4). We propose that the rapid increases in bacterial diversity

recorded at Foreshore Beach during storm-water inputs are reflective of the allochthonous input of enteric and pipe-dwelling microbes during storm-water and WWSO events, suggesting that the observed increases in abundance at this site during these times occurred as a consequence of these inputs rather than the growth of endemic marine microbes.

The composition of bacterial communities changed significantly between dry periods and storm-water input events at Foreshore Beach ($r = 0.184$, $p = 0.017$, perm = 999), and even more strongly between dry periods and modelled WWSO events ($r = 0.999$, $p = 0.010$, perm = 999). However, significant changes in bacterial composition were not observed at Maroubra Beach ($p > 0.05$). Following storm-water events, bacterial communities at Foreshore Beach were 53.2% dissimilar to those observed during dry periods, while input from WWSO led to further dissimilarity (63.2%) among microbial assemblages.

While bacterial communities at Maroubra Beach largely retained a marine signature throughout the 2 year time-series, where archetypal marine groups including *Pelagibacter* and *Synechococcus* dominated, storm-water events at Foreshore Beach led to a marked shift in bacterial assemblage structure from one dominated by marine microbes to one resembling enteric and storm-water pipe associated communities (Millar and Raghavan, 2017). During dry periods, the Foreshore Beach assemblage was

predominantly comprised of marine bacterial groups, including *Pelagibacter* (8.18%), *Rhodobacteraceae* (4.94%) and *Synechococcus* (3.79%). The significant ($p < 0.05$) dissimilarity between bacterial assemblages during the dry period and storm-water events was principally attributed to reduced relative abundance of some of these marine groups, including *Pelagibacter* (3.21%), *Actinomarina* (3.17%), *Synechococcus* (2.47%) and *Rhodobacteraceae* (2.22%). Bacterial communities present during modelled WWSO periods were most dissimilar to dry period communities, due to increased presence of bacterial groups that are often associated with human waste streams (Sauer et al., 2011, Fisher et al., 2014) including two *Arcobacter* OTUs (contributing 4.14% and 3.57% respectively to dissimilarity), *Cloacibacterium* (3.01%), *Thiomicrospira* (2.76%), *Anaerospora* (1.79%) and *Acinetobacter* (1.05%).

The shift in bacterial assemblage structure away from a marine community following storm-water events at Foreshore Beach was further confirmed by network analysis. Using data generated from the eLSA pipeline, we detected statistically significant co-occurrence and time lagged relationships between specific bacterial OTUs and periods of storm-water and modelled WWSO input. At Foreshore Beach, storm-water input negatively correlated with 151 OTUs assigned to key marine taxa, including 35 phototrophic eukaryote OTUs, 13 *Flavobacteraceae* and 11 *Rhodobacteraceae* OTUs (Fig 2A). In contrast, 64 OTUs were positively correlated with storm-water events, with the strongest of these correlations involving archetypal sewage and pipe-dwelling bacterial groups (Fisher et al., 2014, Vandewalle et al., 2012), including (in descending order of

correlative strength) *Lachnospiraceae*, *Bacteroides*, *Cloacibacterium*, *Acinetobacter* and *Aeromonadaceae* (Fig 2A). The composition of bacterial communities during periods of WWSO also exhibited a similarly weakened marine signature (Fig 2A), with strong correlations between the extent of modelled WWSO volume and the occurrence of OTUs within wastewater associated taxa including *Acinetobacter*, *Bacteroides*, *Cloacibacterium* and *Arcobacter*. These results demonstrate a clear temporal partitioning of microbial communities at Foreshore Beach, where distinct microbial assemblage structure was observed following storm-water and modelled WWSO events relative to dry periods. Notably, these temporal shifts in community structure were much less evident at Maroubra Beach, where storm-water events were less significant.

4.5.2 Storm-water and WWSO events increase the abundance of AbR genes

A significant co-occurrence and one week time-lag between storm-water and the absolute abundance (gene copies L^{-1}) of *intI1* was observed at both Maroubra Beach and Foreshore Beach (Supplementary Fig. 4) and modelled WWSO input and *intI1* at Foreshore Beach, indicating that concentrations of *intI1* remained above base line levels for up to one week after storm-water and WWSO input. This gene has previously been employed as a molecular marker for anthropogenic pollution in terrestrial, aquatic and marine environments (Hardwick et al., 2008), where its presence can be indicative of contamination by sewage, heavy metals and disinfectants, and it can act as a proxy for AbR gene occurrence (Koczura et al., 2016, Gillings et al., 2015).

Across the two-year time-series, mean *intI1* concentrations were an order of magnitude higher at Foreshore Beach (8.18 x 10^4 gene copies L^{-1}) than Maroubra Beach (4.37 x 10^3 gene copies L^{-1}), with this pattern largely driven by the significant peaks in abundance of this gene at Foreshore Beach during storm-water and modelled WWSO events (Fig 3). These patterns indicate that a background level of *intI1*-carrying microbes is a consistent component of marine bacterial communities at both sites, possibly as a result of prior exposure. However, storm-water and modelled WWSO input events at Foreshore Beach led to a 1-2 order of magnitude increase in the abundance of this gene, which is consistent with evidence that it is an accurate indicator of anthropogenic impact (Gillings et al., 2015).

eLSA revealed strong correlations between *intI1* abundance and storm-water and modelled WWSO events, as well as to the abundance of AbR genes including *sul1* (LS = 0.52, p = <0.001), *tetA* (LS = 0.49, p = <0.001), *dfrA1* (LS = 0.44, p = <0.001) and *qnrS* (LS = 0.43, p = <0.001). The correlations between *intI1* and AbR genes detected in this study support the notion that *intI1* is a good proxy for the presence of antibiotic resistance genes (Gillings et al., 2008).

Weekly patterns in the abundance of five AbR genes that confer resistance to several classes of antibiotics that are included in the World Health Organisation's "most critically important antimicrobials for human medicine list" (WHO, 2017) were tracked during the

2 year time series. Notably, all AbR genes, with the exception of *vanb*, were detectable at base-line levels, in concentrations between 10^2 to 10^4 gene copies L^{-1} , at both beaches. Importantly, the occurrence of each of these AbR genes increased dramatically in conjunction with storm-water input and modelled WWSO events, where 1-2 order of magnitude spikes in abundance occurred (Fig 3). Infrequent spikes in AbR gene abundance associated with drops in EC were observed at Maroubra Beach, particularly for *dfra1* and *teta* (Fig 4). However, at Foreshore Beach, where a substantially greater influence of storm-water input occurred (Fig 1), spikes in AbR gene abundance were much more frequent and intense, with all five genes, including *vanb*, showing pronounced spikes in abundance, although notably these did not always coincide with each other (Fig 4).

While the correspondence between AbR gene abundance and storm-water events was qualitatively clear (Fig 4), we also observed a statistically significant co-occurrence between storm-water input and the concentration of all but one gene (*vanb*) (Supplementary Fig. 4). These immediate increases in AbR abundance during storm-water events are consistent with the concept that AbR genes are abundant and selected for in waste stream microbes, which, as our bacterial community profile data above confirm, are washed into marine environments via storm-water infrastructure.

The *teta* gene confers resistance to tetracycline antibiotics, which are used widely in human and animal medicine as first line treatment and prophylactic control in a range of bacterial and protozoal infections (Chopra and Roberts, 2001, Schnappinger and Hillen, 1996). These antibiotics are also commonly detected in wastewater environments, and wastewater receiving environments (Lin et al., 2015). Concentrations of *teta* increased 10-fold above background levels at both Maroubra Beach and Foreshore Beach, with the highest concentrations detected during one storm-water event (1.51×10^5 gene copies L^{-1}) and WWSO events (maximum peak of 8.11×10^4 gene copies L^{-1}) at Foreshore Beach (Fig 4), where statistically significant co-occurrence with storm-water and modelled WWSO events were detected ($p < 0.05$) (Supplementary Fig. 4). Peaks in *tetA* persisted during storm-water and modelled WWSO events, but generally returned to baseline concentrations within 7 days.

The *sul1* and *dfrA1* genes confer resistance to sulfonamides and trimethoprim, respectively (Huovinen et al., 1996), which are typically used in combination to treat common bacterial infections in humans (Stegeman et al., 1996). Sulfonamides are resistant to removal by wastewater treatment processes, and persist in wastewater effluent (Pehrsson et al., 2016), which in turn exerts selective pressure for sulphonamide-resistant bacteria in effluent streams and marine receiving waters (Czekalski et al., 2012). This is consistent with our observations of 10-fold increases in the abundance of these genes above background concentrations during both storm-water-only and modelled WWSO events at Foreshore Beach (Fig 4). Maroubra Beach

experienced two peaks in *dfrA1* and one peak in *sul1* abundance, however, only one of the *dfrA1* peaks corresponded with a very modest drop in EC, while the others occurred during dry periods. Notably, *sul1* and *dfrA1* displayed a statistically significant co-occurrence and 1 week time-lag with storm-water input events at Foreshore Beach ($P < 0.01$; Supplementary Fig. 4), indicating that elevated occurrence of these genes can persist in the environment for prolonged periods after an initial input event.

The *qnrS* gene confers non-susceptibility to quinolone antibiotics, which are used extensively in human and veterinary medicine, and are commonly detected in wastewater treatment plant (WWTP) influent and effluent (Karthikeyan and Meyer, 2006). The *qnrs* gene displayed a statistically significant co-occurrence with storm-water and modelled WWSO events ($P < 0.05$). Increases of 10-30-fold above background occurred during storm-water events and releases of WWSO, with gene abundance rising above 10^4 gene copies L^{-1} on 11 occasions at Foreshore Beach, of which 4 co-occurred with storm-water-only input, while 7 co-occurred with sewage overflows, reaching a maximum peak of 2.0×10^6 (Fig 4). The abundance of *qnrs* peaked above 10^5 on 3 occasions at Maroubra Beach, of which only one co-occurred in association with rainfall and runoff, with a maximum peak of 2.47×10^5 (Fig 4). Peaks in *qnrs* subsided one week after storm-water events at both sites and returned to background levels below 10^4 gene copies L^{-1}.

In contrast to the other AbR genes that were generally present across the entire time series, we detected the presence of *vanb* in only 4 of 104 sample occasions at Maroubra Beach, and 13 of 104 samples at Foreshore Beach (Fig 4). *vanb* confers resistance to vancomycin, an important last resort treatment for multi-drug resistant *Staphylococcus aureus* (Koch et al., 2014, Roberts et al., 2009). The single, modest peak in *vanb* abundance at Maroubra Beach (1.9×10^3 gene copies L^{-1}) occurred outside of storm-water input, and persisted for only 1 week (Fig 4). Similarly, at Foreshore Beach, 11 small peaks in *vanb* abundance (relative to peaks seen in other AbR genes) occurred during dry periods. However, the two largest increases, each lasting one week, occurred during storm-water input (in the absence of WWSO) reaching concentrations of 2.67×10^4 and 1.57×10^4 (Fig 3). The general lack of correlation between *vanb* occurrence and storm-water events is intriguing, allowing for the possibility of a co-selection process being responsible, however the source of input of this gene is currently unclear.

In addition to the direct quantification of five key AbR genes, a mPCR/RLB hybridization approach, which has formerly only been applied to clinical bacterial isolates (Kong and Gilbert, 2006), was applied. This approach detected the presence of 7 out of 26 other genes associated with resistance to key antibiotics (Fig 5), which encode for production of carbapenemase enzymes that are important for de-activation and resistance to clinically important carbapenems (Poirel and Nordmann, 2006, Evans and Amyes, 2014). Carbapenem antibiotics are particularly important medications in cases where limited therapy options exist for treatment of infections by multi-drug resistant bacteria (WHO,

2017) (Nordmann et al., 2012, Poirel and Nordmann, 2006). The presence of OXA ß-lactamases, including OXA_{-10} $OXA_{,48}$, correlated significantly with storm-water input (LS = -0.81, p = <0.001 and LS = 0.51, p = <0.001 respectively), and modelled WWSO volume (LS = 1.29, p = <0.001 and LS = 0.74, p = <0.001 respectively), but OXA_{-23} or $OXA_{,30}$, although detected on seven and three occasions respectively, did not correlate significantly with stormwater or modelled WWSO events. Other genes that confer resistance to ß-lactam antibiotics, including bla_{TEM} and bla_{VEB}, plus a carbapenemase gene bla_{VIM}, were also detected (Fig 5), but at only one time point each. bla_{VIM} occurred during a dry period, while bla_{VEB} and bla_{TEM} co-occurred with a storm-water input event. These observations are notable, given that detection of bla_{VIM} and bla_{VEB} outside of clinical environments is uncommon, perhaps due to the restricted application of some ß-lactam antibiotics to kerb rates of resistance (Gruson et al., 2000). However, this indicates that even when used at low levels, the degree of resistance to these antibiotics can still become elevated in the natural environment.

4.5.3 Network analysis identifies potential bacterial vectors for antibiotic resistance genes

Extended Local Similarity Analysis confirmed that the presence of several AbR genes was statistically linked to storm-water and modelled WWSO events. Specifically, *qnrs*, *sul1* and *teta* occurrence was significantly correlated with drops in EC, while *qnrs* and *sul1* occurrence was also correlated with modelled WWSO events (Fig 2A). The correlations between storm-water and modelled WWSO events and increases in both bacterial

diversity and the abundance of AbR genes described indicate that specific bacterial vectors are involved in the deposition of these genes in coastal environments. Storm-water and sewage overflow events will likely lead to the allochthonous input of both bacteria present within the source waters and those inhabiting storm-water and sewage pipe infrastructure (McLellan et al., 2015). Both groups are predicted to function as reservoirs for AbR genes and agents for their dissemination into coastal habitats (Millar and Raghavan, 2017).

The strongest positive co-occurrence patterns occurred between AbR genes that confer resistance to quinolone (*qnrs*), carbapenem ($bla_{\text{OXA-48}}$) and ß-lactam (bla_{DHA}) antibiotics, and 22 bacterial OTU's, of which 17 closely matched species common to sewage effluent and storm-water pipe infrastructure microbiomes (McLellan et al., 2013, Vandewalle et al., 2012) (Fig 2B). The most prominent of these sewage and pipe bacterial groups include OTUs classified as *Arcobacter nitrofigilis, Cloacibacterium repunse, Arcobacter butzleri, Arcobacter skirowi, Aeromonas caviacae* and *Acinetobacter johnsonii*. Importantly, many bacteria belonging to these groups are also human pathogens, and have been implicated in multi-drug resistant, nosocomial and community borne infections (Douidah et al., 2012, Poirel and Nordmann, 2006). These patterns confirm our proposition that the most likely vehicles for AbR flux into natural coastal habitats are bacteria associated with wastewater and those that live within the wastewater infrastructure environment (e.g. pipes). AbR genes from pipe sources present a significant, yet largely overlooked challenge for managing water quality and safety. This

is because AbR genes may continue to be released into marine coastal environments after faecal indicator bacteria, which are typically used as a proxy for contamination (Boehm and Sassoubre, 2014), have fallen below thresholds that trigger beach closure, potentially exposing water users to high concentrations of AbR genes, which may ultimately be assimilated into human microbiomes (Leonard et al., 2015, Leonard et al., 2018).

4.5.4 Conclusions

By coupling culture-independent molecular approaches with a high temporal frequency sampling regime, this study has demonstrated that the occurrence and persistence of AbR genes in urban coastal habitats are closely linked to inputs of storm-water from an urban watershed, and modelled WWSO. Peaks in the abundance of clinically important AbR genes, including genes that confer resistance to both high usage front line antibiotics (e.g. quinolones, trimethoprim, sulfonamides and tetracycline), as well as last resort antibiotics (e.g. vancomycin, carbapenems), occurred in association with storm-water input and modelled WWSO events, whereby AbR gene abundances increased by 1-2 orders of magnitude over periods of 1 to 2 weeks. These observations strongly point to sewage and urban waste-stream infrastructure as potential reservoirs and conduits for the dissemination of AbR genes into coastal waters. Importantly, we have highlighted the role of storm-water and WWSO as a means for potentially pathogenic bacteria carrying AbR genes to cross habitat boundaries and augment the resistomes of coastal marine habitats. Thus, waste stream driven AbR in coastal waters poses a possible threat

to human, animal and ecosystem health by: exposing users of coastal habitats to allochthonous AbR pathogens, facilitating the integration (e.g. through horizontal gene transfer) of AbR into the microbiomes of humans and marine animals, or leading to the assimilation of AbR by endemic marine pathogens.

4.5.5 Acknowledgements

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4.6 Supporting Information

4.6.1 Study sites, data and sample collection

Two study sites were selected according to contrasting levels of exposure to storm-water and sewage pollution, with one highly impacted and one relatively unaffected site selected. Foreshore Beach, situated within the partially enclosed waters of Botany Bay (33.95 ˚S, 151.9 ˚E) (Supplementary Fig 1), is subject to the influence of multiple storm-water outlets and is frequently exposed to high levels of urban storm-water discharge as well as periodic sewage overflows from upstream overflow outlets (NSW Office of Environment and Heritage, 2015). In contrast, Maroubra Beach (33.95˚S, 151.25 ˚E) is directly exposed to the Pacific Ocean and has higher levels of hydrodynamic flushing, while also experiencing substantially less impact from storm-water inputs (NSW Office of Environment and Heritage, 2015) (Supplementary Fig 1).

During the study period (2nd May, 2014 to 29th April, 2016), rainfall data was retrieved from the Australian Bureau of Meteorology website (www.bom.gov.au/climate/data) using the nearest gauge station to each sampling site. Storm-water inputs were identified according to decreases in water salinity, measured here as electrical conductivity (EC), which allowed for separation of sample time points into either storm-water impacted or non-impacted samples. Additionally, at Foreshore Beach, modelled data was used to estimate the total weekly volume of wet weather sewer overflow (WWSO) (released at Mill Pond, an upstream sewage overflow discharge point), which

allowed for further distinction of WWSO events from storm-water-only events. Here, we define WWSOs as the release of excess, dilute wastewater (containing a mixture of stormwater and sewage) from the wastewater system in heavy rain thereby avoiding backflow into residential and commercial buildings. Modelled WWSO volumes were obtained from Sydney Water (the water authority responsible for the operation of Sydney's wastewater systems) as rates of WWSO (measured in m^3 15 min^{-1}). These values were summed across the week preceding each sampling event. WWSO events were defined as samples collected within 7 days of a modelled WWSO from the Mill Pond overflow point into Botany Bay.

Sampling was conducted at each site on a weekly basis (every Friday morning between 0800-1100 AEST) over the course of 24 months, leading to the establishment of two time-series, each comprising 104 time points. At each site, all samples were collected in triplicate from near to the shore-line (<1.5 m depth). Physico-chemical parameters, including temperature, pH, electrical conductivity (EC) and dissolved oxygen (DO) were measured *in situ* using a WTW Multiprobe meter (Multi 3430, Germany). For analysis of dissolved inorganic nutrients, including NH_4, NO_x and PO_4, triplicate 50 mL samples were collected and filtered through a 0.45 μm filter (Sartorius Stedim Biotech) and immediately transported to the laboratory on ice. Samples were stored at -80 °C within 2 hours of sample collection for later nutrient analysis. For bacterial enumeration, triplicate 1 mL water samples were collected from each 5 L water sample, filtered through a 100 μm mesh, fixed with glutaraldehyde (2% final concentration) and snap

frozen in liquid nitrogen for subsequent flow cytometric (FCM) analysis. Microbial DNA was retrieved by filtering triplicate 2.5 L samples through 0.22 µm pore-size membrane filters (Millipore) using a peristaltic pump. Filters were stored at -80°C for subsequent DNA extraction.

4.6.2 Nutrient analysis

Concentrations of NH_4, NO_x and PO_4 were determined via a colorimetric analysis using the Lachat Quickchem QC8500 Automated Ion Analyser (LACHAT Instruments, USA) in accordance with the manufacturer's guidelines. Internal controls, including addition of spiked recovery samples and blanks were included to monitor the consistency of instrument performance. The resulting data were transferred and interpreted using Omnion version 3 software (LACHAT Instruments, USA).

4.6.3 Bacterial cell enumeration

Prior to analysis, samples were quick-thawed in warm water and stained with SYBR Green I nucleic acid stain (1:10,000 final dilution; Molecular Probes, Eugene, OR, USA) (Marie et al. 1997; Gasol and Del Giorgio 2000; Seymour et al. 2007). Fluorescent reference beads (1 µm in diameter, yellow–green; Molecular Probes) were added to each sample as an internal concentration and fluorescence standard in a final concentration of 10^5 mL^{-1}. Samples were analysed using an LSRII flow cytometer (Becton Dickinson, San Jose, CA, USA) and bacterial populations were discriminated according to cell side scatter (SSC) and SYBR Green fluorescence (Seymour et al. 2007). Data-files

were analysed using WinMDI 2.9 software (WinMDI, Joseph Trotter, Salk Institute for Biological Studies, La Jolla, CA, USA).

4.6.4 DNA extraction and 16S rRNA-amplicon sequencing

Microbial DNA was extracted from 0.22 µm membrane filters using the Powerwater DNA isolation Kit (MoBio Laboratories, Inc) in accordance with the manufacturer's instructions. DNA quantity and purity was evaluated using a Nanodrop-1000 Spectrophotometer. Patterns in bacterial community diversity and composition throughout the two year time series were characterised using 16S rRNA amplicon sequencing. The V1–V3 variable region of the 16S rRNA gene was amplified using the primers 27f 5'-AGRGTTTGATCMTGGCTCAG-3' and 519r 5'-GTNTTACNGCGGCKGCTG-3'. Sequencing was performed using the Illumina MiSeq platform following the manufacturer's guidelines. Raw data files in FASTQ format are deposited in the NCBI Sequence Read Archive (SRA) with the study accession number SRP143624 under Bioproject number PRJNA4209011.

16S rRNA gene sequences were analysed using the QIIME pipeline (Caporaso et al., 2010, Kuczynski et al., 2012). Briefly, paired-end DNA sequences were joined and *de novo* Operational Taxonomic Units (OTUs) were defined at 97% sequence identity using UCLUST (Edgar et al., 2011). Chimeric sequences were detected using ChimeraSlayer (Haas et al., 2011) and filtered from the dataset. Taxonomy was assigned against the SILVA v128 database using the BLAST option in QIIME. Sequences were rarefied to the

same depth to remove the effect of sampling effort upon analysis, and Shannon's Index was calculated within QIIME to quantify α diversity. All Chloroplast OTU's were filtered out of the bacterial data-set and transferred to a separate OTU table, where taxonomy was subsequently assigned against the PhytoREF database (Needham and Fuhrman, 2016, Decelle et al., 2015) using QIIME (Caporaso et al., 2010). The relative abundance of each chloroplast OTU was calculated after normalising chloroplast sequences to total sequence reads within each sample.

4.6.5 Quantitative PCR (qPCR)

Quantitative PCR (qPCR) was used to track patterns in the absolute abundance of the class 1 integron integrase gene (*intI1*), which has been shown to be an excellent proxy for anthropogenic pollution in natural habitats (Gillings et al., 2015), as well as five genes that confer resistance to broad spectrum, first line and last resort antibiotics. These included *teta*, *sul1*, *qnrs, dfra1, and vanb,* which confer resistance to tetracyclines, sulfonamides, fluoroquinolones, trimethoprim, and vancomycin respectively (Supplementary Table 1). qPCR was run in triplicate for each assay, amounting to a total of 3,744 analyses across the two 24 month time-series. Quantification of the relative abundance of *intI1* and the five AbR genes was achieved by normalising to total volume of water sample filtered for DNA extraction.

To create qPCR standards for *intI1, dfra1, qnrs, sul1, teta and vanb,* the respective amplicon was PCR amplified from genomic DNA acquired from Foreshore Beach and

cloned using the pGEM-T Easy Vector System (Promega) according to the manufacturer's instructions. To confirm that correct inserts were cloned, fragment sizes were confirmed by gel electrophoresis. Purified plasmid (GenElute Plasmid Miniprep Kit, Sigma) or PCR products (Isolate II PCR and Gel Kit, Bioline) were quantified using Qubit (Invitrogen) and stored at -20°C.

qPCR was performed using the BIORAD CFX384 Touch™ Real-Time PCR Detection System™ and gene copies were calculated for each amplicon by plotting against their respective standard curve using BIORAD CFX Manager software. All qPCR tests were run using three technical replicates, consisting of 5 µl reaction volumes containing 2.5 µl iTaq UniverSYBR Green SMX 2500®, 0.1 µl nuclease free water, 0.2 µM of each forward and reverse primer and 2 µl of diluted (1:10) DNA template. Negative control samples were run with every plate for detection of contamination, and a 16S rRNA qPCR assay (Nadkarni et al., 2002) was also run for all samples to confirm that inhibitors were not preventing amplification of genes. Calibration curves were run with every plate, with all reagent and plate preparation conducted using an epMotion® 5075l Automated Liquid Handling System. Cycling parameters for each qPCR assay are provided in Supplementary Table 1. To confirm that each primer pair produced only a single specific product, a melting curve was added to the end of every qPCR assay for each set of primers.

4.6.6 Multiplex PCR (mPCR) and Reverse Line Blot (RLB) Hybridization

DNA samples collected from Foreshore Beach were screened for additional AbR genes (see supplementary Table 1 for complete list of primers and targeted AbR genes) of regional clinical relevance using multiplex polymerase reaction with reverse line blot (mPCR/RLB) hybridization (Kong and Gilbert, 2006, Ginn et al., 2013, Agyekum et al., 2016). Briefly, amine-labelled oligonucleotide probes targeting 26 AbR genes were covalently bound to a nylon membrane in a 45-lane miniblotter. Biotin-labelled mPCR products plus blanks and positive controls were loaded perpendicularly, followed by hybridization at 60 °C. Streptavidin-peroxidase conjugate and chemiluminescent substrate were used for detection of the membrane-bound mPCR amplicons.

4.6.7 Statistical analysis

Bacterial abundance, diversity and community composition was compared using Analysis of Similarity (ANOSIM) in the PRIMER6 + PERMANOVA package (Anderson et al., 2007, Anderson, 2001). Similarity percentage analysis (SIMPER) was employed to identify and rank the top 25% of bacterial OTU's that contributed most to storm-water and sewage impacted and dry period communities at both beaches.

Exploration of statistically significant correlation patterns between bacteria, phytoplankton, environmental parameters and AbR genes was conducted within each site, across the 104 week time-series using Extended Local Similarity Analysis (eLSA) (Xia, 2011). eLSA was used to identify statistically significant correlations, including time-

lagged interactions, between OTUs, AbR gene abundances and environmental variables, for subsequent construction of microbial networks. Only pairwise interactions that occurred across 10% or more of the time points were included, and each remaining interaction was subjected to 1000 permutations to calculate statistical significance (p value) and the false detection rate (q value). Interactions where $p > 0.001$ and $q > 0.01$ were excluded to reduce the risk of Type I and Type II errors. Resultant data defined all statistically significant co-occurrence events, including those that occurred with -2 to +2 time-interval time lags. eLSA data was ultimately visualised using the edge-weighted spring embedded function in Cytoscape 3.4.0 software package, using the local similarity (LS) value of pairwise correlations to define the length of edges connecting nodes.

To determine temporal relationships between environmental variables and the abundance of AbR genes measured using qPCR (absolute abundance) and mPCR/RLB (presence/absence), selected pairwise co-occurrence and time-lagged correlations were measured in R using the cross correlation factor (CCF) (Wessa, (2017)). Resultant data was visualised in CCF graphs showing the temporal correlation relationships across the 104 week time-series.

4.7 Figures

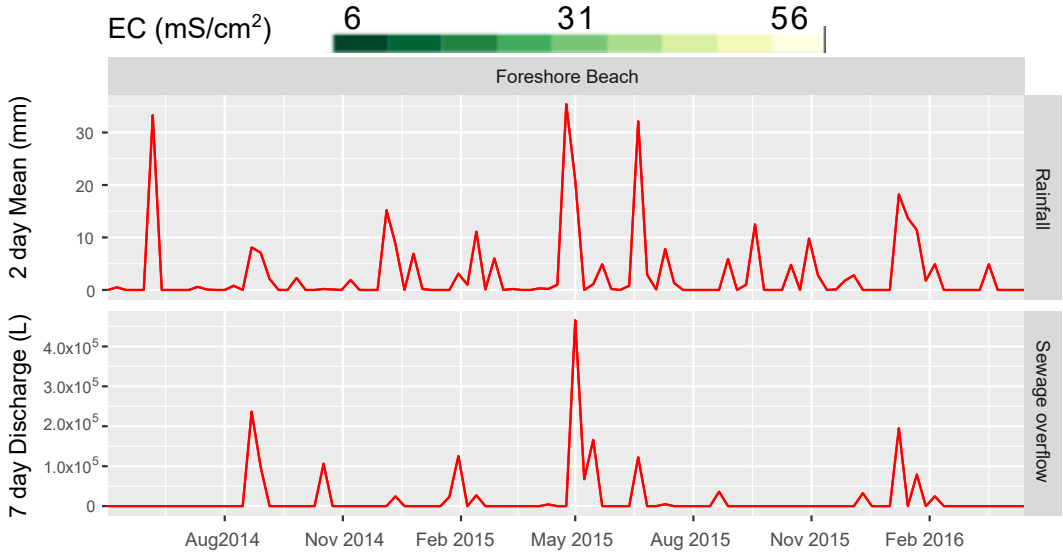


Figure 11 *Rainfall (A) and wet weather overflow (B) across the two year time series at Foreshore Beach. Vertical bars indicate level of EC values with dark green bars showing significant drops in EC that are indicative of storm-water input. A) Depicts weekly rainfall (red line) at Foreshore Beach across the two-year time series. Rainfall was measured as the average quantity of precipitation (mm) on the day of sampling plus the preceding day as retrieved from the Sydney International Airport weather gauge. (B) The red line tracks the volume of wet weather sewer overflow (WWSO) released into Foreshore Beach from the Millpond overspill point, 1.06 km upstream from Foreshore Beach, across the two year time series. WWSO released from Millpond was measured as the total (m3) on the day of sampling plus the six preceding days (data provided by Sydney Water).*

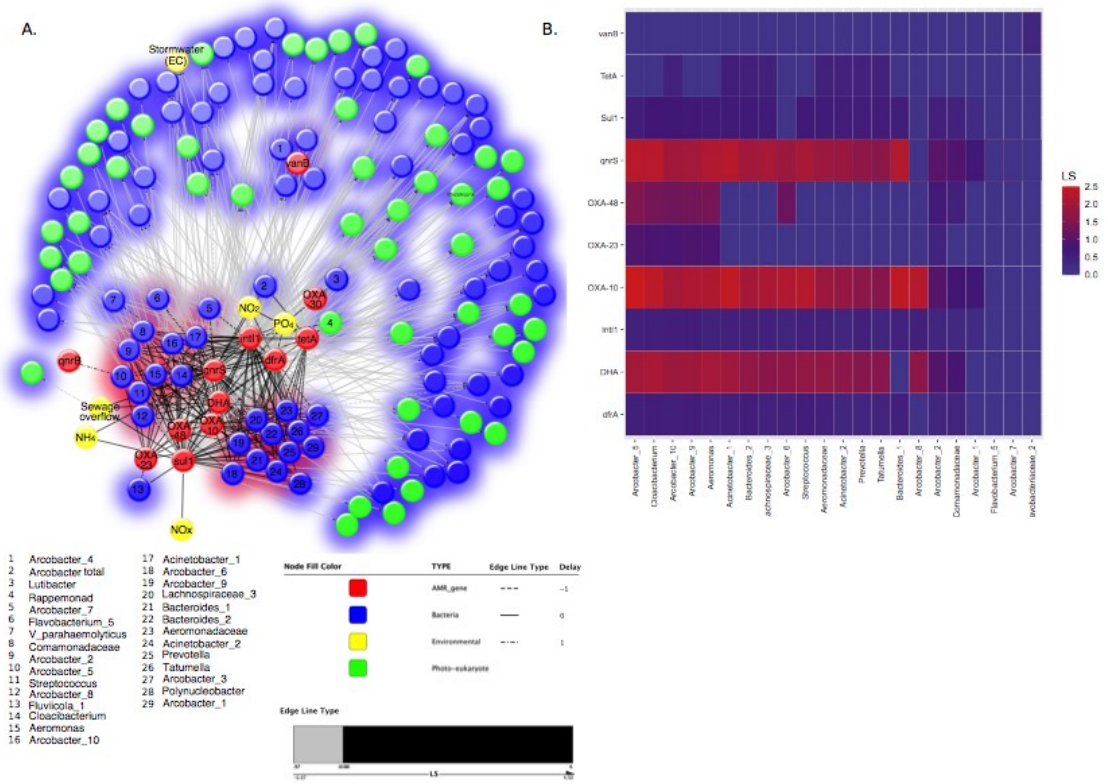


Figure 2. A) Edge-weighted, spring embedded sub-network of all bacterial and photoeukaryote OTU's at Foreshore Beach that were significantly correlated with AbR genes. Resultant network incorporates 150 nodes (circles), representing 83 bacterial OTUs blue), 49 photoeukaryotic OTUs (green), 6 environmental parameters (yellow) including EC (indicative of storm-water input), OF - "overflows" (representing the weekly volume of sewage overflow discharge), and dissolved inorganic nutrients, 12 AbR genes (red), connected by 444 edge connections (lines). Edge length represents local similarity (LS) value of connected nodes, where strong positive correlations bring nodes closer together, and strong negative correlations force nodes further apart. All edges assigned a positive LS value are black, and edges assigned to a negative LS value are grey. Broken line edges represent a time lagged correlation (see legend), and unbroken lines represent co-occurrence. Node glow represents the sum LS of all edges connected to an AbR node, where the highest sum LS connected nodes have a red glow, and nodes with a negative sum of LS have a blue glow. B) Heatmap depicting 22 bacterial OTUs that share a strong, positive correlation to AbR genes, identified using eLSA and network analysis. Bacterial OTUs are positioned in descending order of sum LS connections to AbR genes, from left to right. Assignment of OTU taxonomy to species level achieved using BLASTn, matching OTU representative sequence to the closest match in the NCBI database.

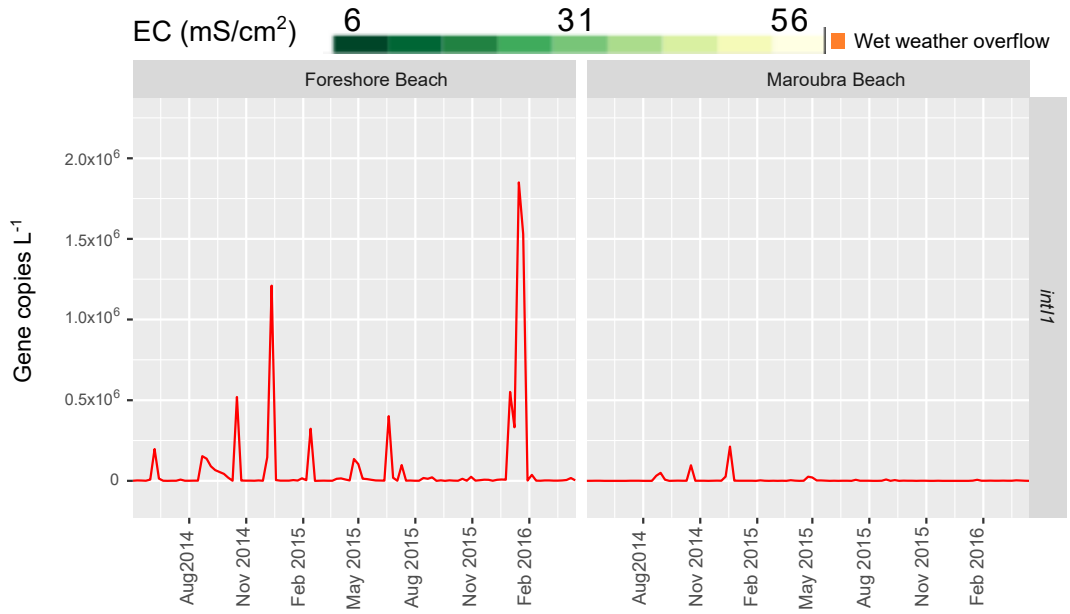


Figure 3. *Abundance of intI1 (red line) over the two year time series at Maroubra Beach and Foreshore Beach. Grey shading represents standard deviation across . Green vertical bars represent electrical conductivity (EC) with dark green bars showing a significant drop in EC and indicative of storm-water input. Orange vertical bars indicate periods of wet weather sewer overflow (WWSO).*

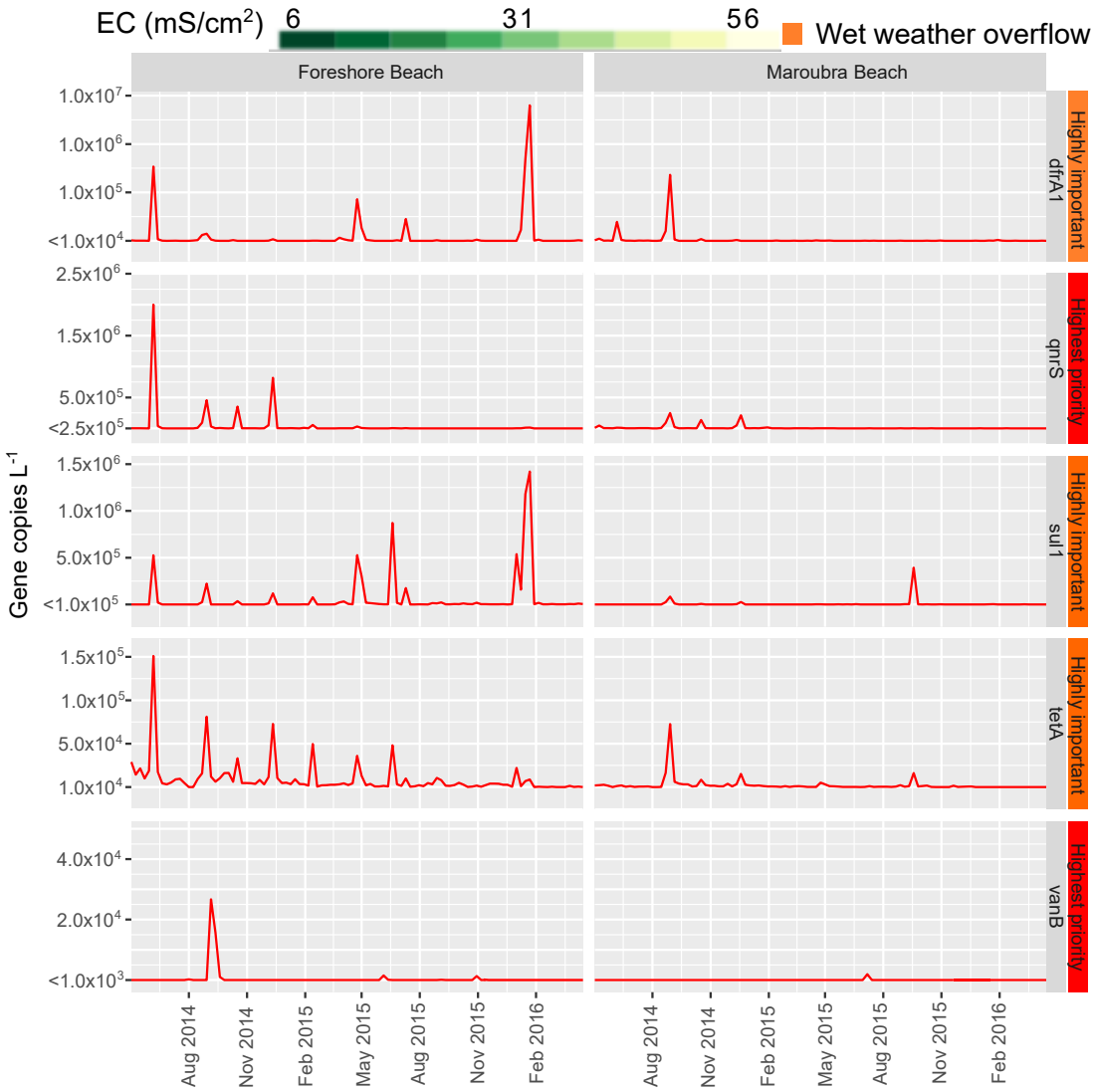


Figure 4. *Abundance of antibiotic resistance (AbR) genes over the two year time series at Maroubra Beach and Foreshore Beach. Grey shading represents standard deviation across three triplicates. Green vertical bars represent electrical conductivity (EC) with dark green bars showing a significant drop in EC and indicative of storm-water input. Orange vertical bars represent weeks where the level of rainfall resulted in wet weather sewer overflow (WWSO).*

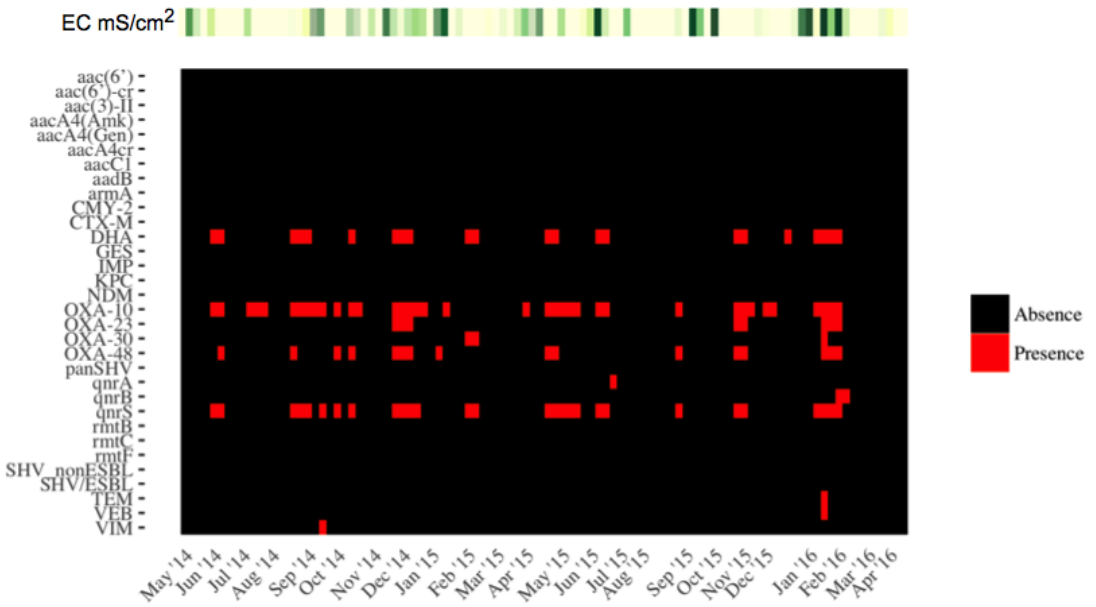
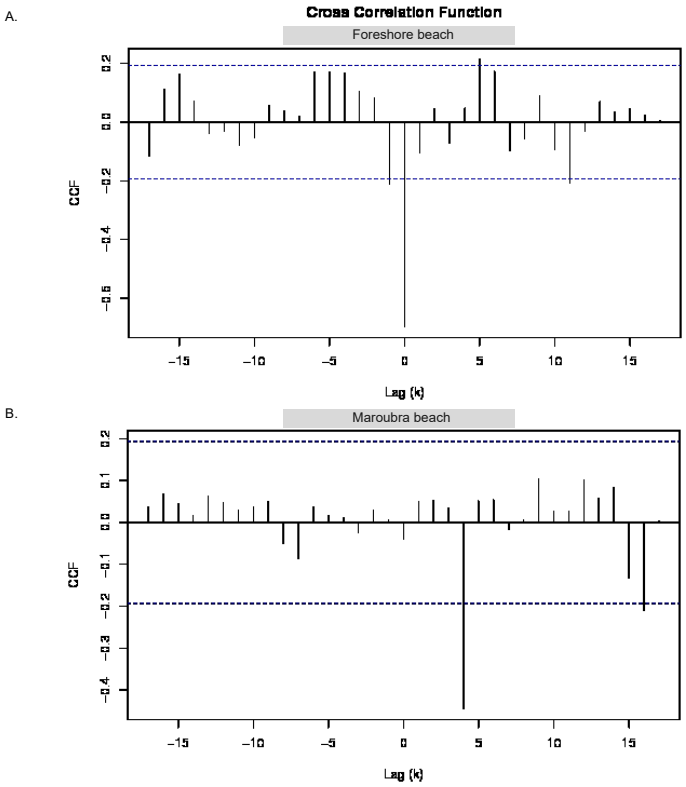


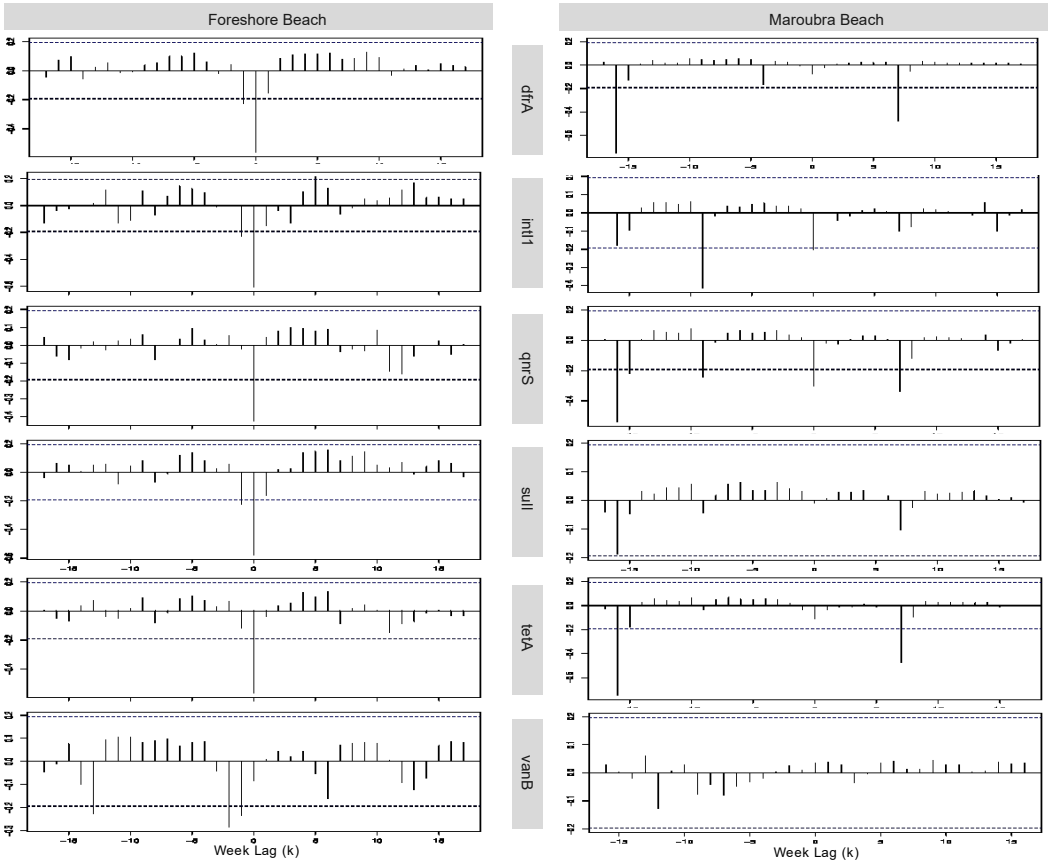
Figure 12 *Presence and absence of 26 AbR gene targets at Foreshore Beach across the two year time series as measured using mPCR/RLB hybridization. Green vertical bars represent electrical conductivity (EC) with dark green bars showing a significant drop in EC (indicative of storm-water input). AbR genes detected include blaDHA, blaOXA-10, blaOXA-23, blaOXA-30, blaOXA-48, qnra, qnrb, qnrs, blaTEM, blaVEB and blaVIM.*

4.8 Supplementary Figures

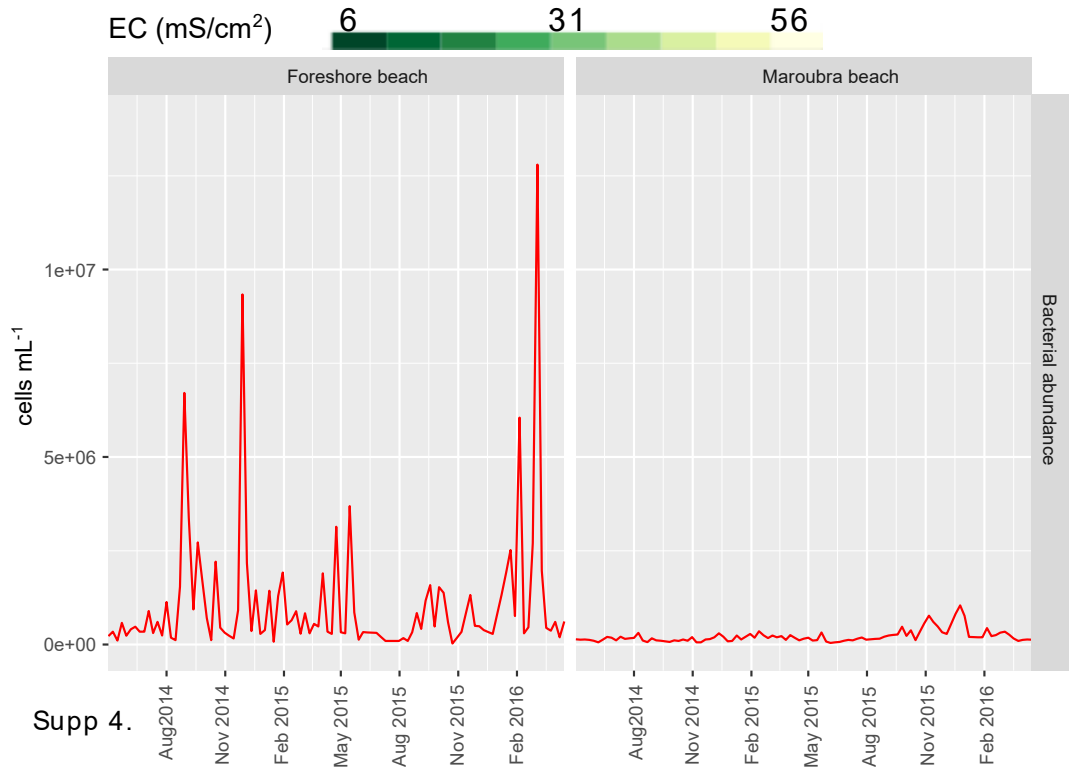
SI Fig 1. *Map of study sites – Foreshore Beach (red), Maroubra Beach (green) and Millpond Sewage overflow point (brown).*



SI Fig 2. *Cross Correlation Function (CCF) was used to measure patterns of co-occurrence and time-shifted correlation between two variables, in this case rainfall (series 1) and electrical conductivity (EC) (series 2) at Foreshore Beach (A) and Maroubra Beach (B). Weekly time intervals were plotted across the horizontal axis (where 0 is co-occurrence or no time lag, negative values represent time intervals where rainfall precedes EC (negative time lag), and positive values represent time intervals where rainfall proceeds EC (positive time lag). Values on the vertical axis represent the cross-correlation function (CCF) of each time lag, which is considered as a correlation co-efficient between the two series, at different time lags. Where a vertical line value crosses the broken blue horizontal line (95% confidence interval), this indicates a statistically significant correlation at this given time lag. A) CCF of rainfall and EC at Foreshore Beach. A strong, negative and significant co-occurrence at time lag 0 is evident, as well as a weaker, but significant -1 week time lag, suggesting that rainfall leads to decreases in EC at Foreshore Beach, lasting for up to 1 week following the rainfall. B) CCF of rainfall and EC at Maroubra Beach. No significant co-occurrence or time lagged correlation as seen at Foreshore Beach (statistically significant positive time lags at 4 and 17 weeks are not ecologically relevant).*

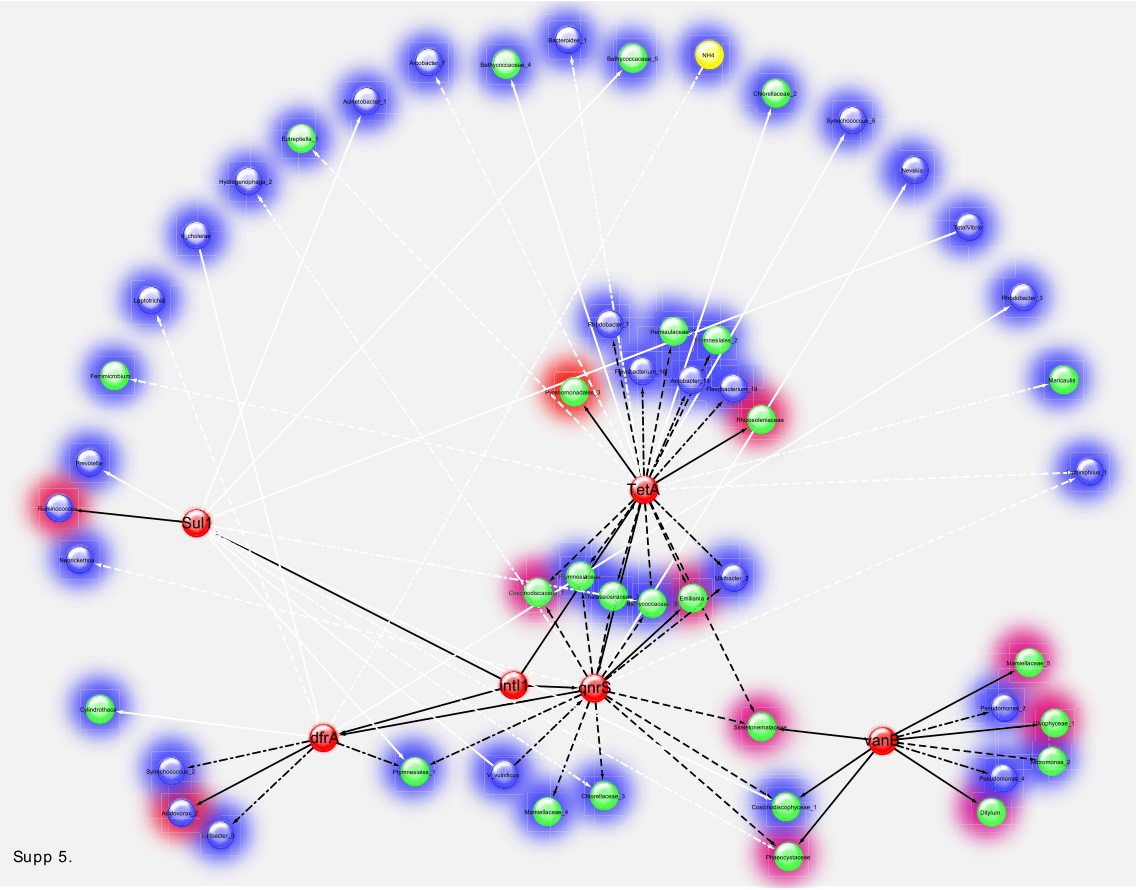


SI Fig 3. *Cross Correlation Function (CCF) was used to measure patterns of co-occurrence and time-shifted correlation between two data series, in this case rainfall (series 1) and AbR and intI1 genes (series 2) at Foreshore Beach (left panels) and Maroubra Beach (right panels). Time intervals are plotted across the horizontal axis (where 0 is co-occurrence or no time lag, negative values represent time intervals where rainfall precedes AbR and intII genes (negative time lag), and positive values represent time intervals where rainfall proceeds AbR and intII genes (positive time lag). Values on the vertical axis represent the cross-correlation function (CCF) of each time lag, which is considered as a correlation co-efficient between the two series, at different time lags. Where a vertical line value crosses the broken blue horizontal line (95% confidence interval), this indicates a statistically significant correlation at this given time lag. CCF of electrical conductivity (EC) and dfra1 at Foreshore Beach and Maroubra Beach show a strong, negative and significant co-occurrence at time lag 0, as well as a weaker, but significant -1 week time lag, suggesting that drops in EC lead to increases in dfra1 at Foreshore Beach, lasting for up to 1 week. No ecologically relevant time lag relationship detected between EC and dfra1. EC and intI1 at Foreshore Beach show strong, negative and significant co-occurrence at time lag 0, as well as a weaker, but significant -1 week time lag, suggesting that drops in EC lead to increases in intI1 at Foreshore Beach, lasting for up to 1 week. EC and intI1 at Maroubra Beach show a weak, but significant co-occurrence between EC and intI1. EC and qnrs at Foreshore Beach show a strong, negative and significant co-occurrence at time lag 0, suggesting that drops in EC lead to a short duration rise in qnrs at Foreshore Beach, which returns to base line levels the following week. EC and qnrs at Maroubra Beach show a weak, but significant co-occurrence between EC and qnrS. EC and sul at Foreshore Beach show a strong, negative and significant co-occurrence at time lag 0, as well as a weaker, but significant -1 week time lag, suggesting that drops in EC lead to increases in sull at Foreshore Beach, lasting for up to 1 week. The absence of a detectable correlation between EC and sull at Maroubra Beach indicate that there is no ecologically relevant time lag relationship. EC and tetA at Foreshore Beach show a strong, negative and significant co-occurrence at time lag 0, suggesting that drops in EC lead to short lived increases in tetA at Foreshore Beach, which return to base line levels the following week. EC and tetA at Maroubra Beachdo not show ecologically relevant time lag relationship detected between EC and tetA. EC and vanB at Foreshore Beach show a moderate, negative and significant -1 and -2 week time lag evident, suggesting that drops in EC lead to delayed increase in vanB that begins 1 week following an initial drop in EC at Foreshore Beach, which returns to base line levels 3 weeks following storm-water input. EC and vanB at Maroubra Beach reveal no ecologically relevant time lag relationship.*



Supp 4.

SI Fig 4. *Total bacterial abundance (red line) at Foreshore Beach (left) and Maroubra Beach (right) as measured using flow cytometry over the 2 year time series. Vertical green bars indicate levels of electrical conductivity, where dark green bars represent very low electrical conductivity, indicative of storm-water input. Orange vertical bars represent periods that coincided with the release of sewage overflow from the Millpond discharge point, that flushes into Foreshore Beach.*



SI Fig 5. *Edge-weighted, spring embedded sub-network of all bacterial and photoeukaryote OTU's at Maroubra Beach that were significantly correlated with AbR genes. Resultant network incorporates 58 nodes (circles); representing 25 bacterial OTU's (blue), 26 photoeukaryotic OTU's (green), 1 environmental parameter (yellow) and 6 AbR genes (red), connected by 76 edge connections (lines). Edge length represents local similarity (LS) value of connected nodes, where strong positive correlations bring nodes closer together, and strong negative correlations force nodes further apart. All edges assigned a positive LS value are black, and edges assigned to a negative LS value are grey. Broken line edges represent a time lagged correlation, and unbroken lines represent co-occurrence. Node glow represents the sum LS of all edges connected to an AbR node, where the highest sum LS connected nodes have a red glow, and nodes with a negative sum of LS have a blue glow.*

Table 1. qPCR targets, standards, primers and cycle conditions used for each mPCR and qPCR assay in this study.

SupplementaryTable 1. A) List of 26 primer and probe sets used to detect the presence of 26 AbR gene targets at Foreshore Beach from 02/05/2014 - 26/04/2016

Targets	Reference	Forward primer	Reverse primer	Probe	Alternative nam
aac(3)-II	Ginn 2013	CGTATGAGATGCCGATGC	AAGATAGGTGACGCCGAAC	AAGCAATCGAGAATGCCGTT	
aacA4	Ginn 2013	CACATHGTYGARTGGTGGG	AABCCBGCCTTCTCRTAGCA	GTYTCDTCYTCCCACCDKCCRYC	aac(6')-Ib
aacA4-cr	Ginn 2013			GTYTCDTCYTCCCACCAKCCRYC	aac(6')-Ib-cr
aacC2	Ginn 2013	ATCATTCGCACATGTAGGC	CGGAGACTGCGAGATCATA	CGACCGAAAAGATCAAGAGC	
aadB	Ginn 2013	ACACAACGCAGGTCACATTd	CTAAGAATCCATAGTCCAACTCC	ATTTCGCTCATCTGCCGCAGCTA	
armA	Ginn 2013	GCATCAAATATGGGGGTCTTA	TTGAAGCCACAACCAAAATCT	CGAATGAAAGAGTCGCAACA	
CMY-2-like	Ginn 2013	CGATATCGTTAAYCGCACCA	CGGATCGCTGAGCTTRATTT	GAATAGCCTGCTCCTGCATC	
CTX-M	Ginn 2013	ATGTGCAGYACCAGTAARGTKATGGC	CCVCCVASVTGRGMAATCARYTTRTTCAT	ACGTGYTTYTCVGCAATSGGRTTRTAGTTA	
DHA-like	Ginn 2013	ACACTGATTTCCGCTCTGCT	ATTTTCAGTGACCGGCTGTT	GGAATATCCTGCTGTGCCAT	
IMP	Ginn 2013	ACAYGGYTTGGTGGTTCTTGT	AGTTCATTKGTTAATTCAGANGCATA	GGAATAGAGTGGCTTAAYTCTCRATC	
KPC	Ginn 2013	CAGCTCATTCAAGGGCTTTC	GGCGGCGTTATCACTGTATT	ATCTGACAACAGGCATGACG	
NDM	Agyekum 2016	GAATGTCTGGCAGCACACTT	ATACCGCCCATCTTGTCCTG	AGACCGCCCAGATCCTCAACT	
OXA-10-like	Ginn 2013	GGGAACTGAGTCAAATCCTG	GATTTTCTTAGCGGCAACTTA	TTTACTTTTTCGCCTTTAACATGGAT	
OXA-23-like	Ginn 2013	CCCAATTAGCACATACACAGC	ATTTCTGACCGCATTTCCAT	AGCAGCCAGATGGAAAAATTGT	
OXA-30-like	Ginn 2013	CAAATTCAATTCCTGCGTAA	AATAAACCCTTCAAACCATCCc	AAACTGTATGGGAAAACTGGTGC	
OXA-48-like	Agyekum 2016	CATGCTGACCGAAGCCAAT	GGCATATCCATATTCATCGC	CATTATCATCAAGTTCAACCCAACC	
qnrA	Ginn 2013	TTCAGCAAGAGGATTTCTCAC	ATCGCAYTCCCTGAACTCTAT	AAACTGCAATCCTCGAAACTGGC	
qnrB	Ginn 2013	GATCGTGAAAGCCAGAAAGG	CCACAGCTCACACTTTTCCA	TTTRAAAATGGCATCTTTCAGCAT	
qnrS	Ginn 2013	TGAACAGGGTGATATCGAAGG	AGTTTGYTCGGGAAAAGTTGG	GATGCAAGTTTCCAACAATGC	
mtlB	Agyekum 2016	TAGTTTAGGCGGGCGTGGA	ATAAGTTCTGTTCCGATGGTCTT	CTACGCCGCATGGTTCGAGG	
mtlC	Ginn 2013	CCCAGAGGGTATTGTTGTCA	ATCCCAACATCTCTCCCACT	ACTCTACTCGATCGGCAGGA	
mtlF	Agyekum 2016	CTTCTGATGAAGCTCCTGCC	GAACCAGTCGGCATAGTGCT	CCCCAGGCTGGTCGTGACCT	
SHV ESBL (-5/-12)	Ginn 2013	GCTGGGAAACGGAACTGA	CACRATSCGCTCTGSTTTGTTATT	ACCGGAGCTAGCAAGCGG	
TEM	Ginn 2013	ACAGCGGTAAGATYCTTGAG	CTCCGATCGTTGTCAGAAGT	GATGCTTTTCTGTGACTGGTGAG	
VEB	Ginn 2013	CCCTCAAGACCTTTTGCCTA	TTCCAATCCTTGTGCATTTG	TTCAATCAAAGCAAACGAAGAA	
VIM	Ginn 2013	TTTGGTCGCATATCGCAAC	TGCTTYTCAATCTCGCCGA	CGTCATGRAAGTGCGTRGA	

SupplementaryTable 1. B) List of 7 primers and/or probes used in qPCR assays

	qPCR Standard	Size (BP)	Primers	Sequence	qPCR Conditions	Source of assay
16S	E coli bacteria, PCR product	466	331f	5'-TCCTACGGGAGGCAGCAGT-3'	95.0c 3min, 40cycles (95c 30s, 60c 30s, 72c 60s)	Nadkarni *et al.* 2002
			797r	5'-GGACTACCAGGGTATCTAATCCTGTT-3'		
			Probe	(6-FAM)-5'-CGTATTACCGCGGCTGCTGGCAC-3'-(TAMRA)		
IntI1	Plasmid insert amplified from Foreshores Beach sample	484	Int1.F	GGG TCA AGG ATC TGG ATT TCG	95.0c 3min, 39cycles (95c 15s, 60c 30s)	Mazel *et al.* 2000
			Int1.R	ACA TGC GTG TAA ATC ATC GTC G		
sulI	Plasmid insert amplified from Foreshores Beach sample	163	sul-FW	CGC ACC GGA AAC ATC GCT GCA C	95.0c 3min, 39cycles (95c 15s, 65c 30s)	Pei *et al.* 2006
			sul-RV	TGA AGT TCC GCC GCA AGG CTC G		
tetO	Plasmid insert amplified from Foreshores Beach sample	96	tetA-F2-L	CAG CCT CAA TTT GCT GAC GGG CtG a	95.0c 3min, 39cycles (95c 15s, 58c 30s)	Borjesson *et al.* 2010
			tetA-R2	GAA GCG AGC GGG TTG AGA G		
vanB	Plasmid,bacteria w/plasmid pgem, topo10 cells	3300	VB-F	AAT CTT AAT TGA GCA AGC GAT TTC	95.0c 3min, 39cycles (95c 15s, 60c 30s)	Berglund *et al.* 2014
			VB-R	CCT GAT GGA TGC GGA AGA TA		
qnrs	Plasmid,bacteria w/plasmid pgem, topo10 cells	3200	QnrS-F	ATC AAG TGA GTA ATC GTA TGT ACT	95.0c 3min, 39cycles (95c 15s, 61c 30s)	Berglund *et al.* 2014
			QnrS-R	CAC CTC GAC TTA AGT CTG AC		
dfrA1	Plasmid,bacteria w/plasmid pgem, topo10 cells	3500	dfr1a-f	ATG GAG TGC CAA AGG TGA AC	95.0c 3min, 39cycles (95c 15s, 62c 30s)	Grape *et al.* 2007
			dfr1a-r	TAT CTC CCC ACC ACC TGA AA		

5. Chapter 5

Marine heatwaves stimulate blooms of pathogenic bacteria in coastal habitats

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Chapter 5 - Marine heatwaves stimulate blooms of pathogenic bacteria in coastal habitats

5.1 Preface

This chapter shifts the focus from allochthonous microbes, to a group of endemic marine bacteria that include several pathogenic species of high public health concern. We also explore the potential influence of meteorological forces on the abundance of Vibrio bacteria.

5.2 Introductory paragraph

Climate change is causing increased sea surface temperatures (SST), which are not only expressed as chronic rises in average global SST (Cane et al., 1997), but have led to intense, albeit transient, spikes in localised temperatures, termed marine heatwaves (MHWs) (Hobday et al., 2016). MHW events are increasing in occurrence and extent (Oliver et al., 2018) and are an emergent cause of marine ecosystem disruption and catastrophic losses of key-stone marine communities (Oliver et al., 2016). Rising global temperatures have also been implicated in the spread of pathogens and infectious diseases in both terrestrial and aquatic habitats (Baker-Austin et al., 2013, O'Neil et al., 2012), with evidence that increasing average SSTs are responsible for increases in the abundance and virulence of some marine pathogens (Paz et al., 2007). Here, we explored the role of MHWs in the occurrence of blooms of an important group of marine pathogens within the Vibrio genus, at two urban beaches on the eastern coastline of Australia, a region identified as a climate change hotspot due to rapidly increasing SST (Last et al., 2008) and recurrent MHW events (Oliver et al., 2018). By coupling culture-independent molecular techniques with extended local similarity analysis (eLSA) and threshold autoregression approaches we identified strong links between blooms of

Vibrio and both increased SST and the occurrence of MHWs. Increases in SST of 1.02 – 1.85 °C preceded modest increases in total vibrio and Vibrio cholerae abundance. However, these increases were overshadowed by episodic bloom events of the three key human pathogens, *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*, which occurred during MHW periods. These findings suggest that MHW could play a more important role in the development of vibrio blooms in coastal ecosystems than chronic increases in average SST, and highlight a new implication of the increasing occurrence of MHW events.

Over 90% of excess heat generated from human induced global warming is sequestered by the oceans (Yan et al., 2016), which has caused global mean sea surface temperatures (SSTs) to increase by 0.6 °C over the past 100 years (Pachauri, 2007) and led to more frequent and severe marine heatwaves (MHWs) (Oliver et al., 2018). MHWs are defined as localised regions of ocean surface water experiencing prolonged spells of exceptionally high temperature, which can be characterised by the duration, intensity, rate of evolution, and spatial extent of impact (Hobday et al., 2016). Over the past century, MHWs have increased in frequency and duration by 34% and 17% respectively, which has resulted in a 54% increase in annual global MHW days (Frolicher and Laufkotter, 2018). MHWs are responsible for dramatic shifts in the ecology of marine ecosystems, with recent events resulting in the devastation of populations of habitat-supporting marine organisms, including corals and macro-algae (Hughes et al., 2017,

Heron et al., 2016, Wernberg et al., 2012), and mass mortalities among marine mammals (Cavole et al., 2016).

While MHWs are ephemeral events, their impacts can often be more severe than chronic climate change-induced shifts in environmental conditions. For example, long term increases in SSTs have led to gradual restructuring of coral communities (Hoegh-Guldberg and Bruno, 2010) and declining resilience to other environmental stressors such as eutrophication (Bruno et al., 2007). However, episodic MHWs often lead to the most extreme coral bleaching events (Moore et al., 2012, Hughes et al., 2017) resulting in mass loss of corals and biodiversity in reef systems (Oliver et al., 2016).

While the impact of climate change is often detrimental at a whole ecosystem scale, not all organisms are immediate losers, with some benefiting from changing climatic conditions (Kelly et al., 2016). This is particularly true for some microorganisms (Smale et al., 2017), including harmful species (Paerl and Paul, 2012). In fact, the World Health Organization has identified climate change as a major driver of emerging infectious diseases (World Health Organization, 2002). In the marine environment, increasing SST has been implicated in the increasing abundance of pathogenic bacteria within the *Vibrio* genus (Vezzulli et al., 2011) and subsequent increased incidence of infections of marine organisms (Tout et al., 2015, Lewis et al., 2017) and humans (Paz et al., 2007, Martinez-Urtaza et al., 2010).

Chapter 5 - Marine heatwaves stimulate blooms of pathogenic bacteria in coastal habitats

Vibrios are indigenous marine bacteria, with several species responsible for a range of infections in marine animals and humans (Austin, 2010). The most prominent human pathogens include *V. cholerae* , *V. parahaemolyticus* and *V. vulnificus*, with initial infections most commonly acquired through either direct exposure from seawater or the consumption of undercooked seafood (Ralph and Currie, 2007, Summer et al., 2001). Human health costs related to infections by these organisms exceed $30M USD yr^{-1} in the US alone (Ralston et al., 2011). Vibrio species generally thrive in warm-water coastal environments, with their abundance and virulence directly coupled to temperature (Chávez et al., 2005, Julie et al., 2010, Kelly, 1982, Randa et al., 2004). As a consequence of these links to temperature, there is an emerging body of evidence suggesting that increasing SSTs are expanding the geographic range of *Vibrios* (Vezzulli et al., 2012) and increasing the incidence of *Vibrio*-associated human infections (Vezzulli et al., 2016a, Baker-Austin et al., 2013). These expansions in Vibrio distribution and influence have the potential to threaten human health (Tantillo et al., 2004) and coastal industries, including aquaculture (Austin and Zhang, 2006).

Given that blooms of marine microbes are often short-lived and stimulated by episodic, rather than chronic, shifts in environmental conditions (Baker-Austin et al., 2016), we considered the influence of MHWs on the abundance of pathogenic Vibrio, within the east coast of Australia, a region defined as a hotspot for climate change and experiencing increasing occurrence of MHW events (Kelly et al., 2016). We tracked

patterns in the occurrence of pathogenic Vibrio species and examined links to MHW events within this region over the course of a 24 month, weekly sampling resolution time-series, conducted at two beaches near to Sydney, Australia.

We selected two beach locations based on their contrasting patterns in Vibrio abundance. Foreshore Beach is situated within the partially enclosed waters of Botany Bay (33.95 ˚S, 151.9 ˚E), while Maroubra Beach (33.95˚S, 151.25 ˚E) is directly exposed to the Tasman Sea and experiences higher levels of hydrodynamic flushing and substantially less impact from stormwater inputs (NSW Office of Environment and Heritage, 2015). During the two year time-series, Foreshore Beach experienced a broader seasonal range in water temperatures (14.1 - 26.5 °C) compared to Maroubra Beach (15.6 – 24.8 °C) (SI Fig 1). While concentrations of inorganic nutrients remained relatively stable across all seasons at Maroubra Beach, at Foreshore Beach periodic peaks in nutrient concentrations were linked to drops in electrical conductivity, indicative of stormwater inputs. With the exception of a significant correlation between NOx and *V. vulnificus* at both beach locations, no statistically significant influence of nutrients, chlorophyll α or salinity on the abundance of any *Vibrio* group was observed at either site, during the two year time-series. Temperature, on the other hand, had a strong and consistent effect.

Quantitative PCR analysis (qPCR), coupled with a multipronged statistical approach, incorporating correlation, similarity, variance and threshold testing (Xia, 2011, Clarke, 1993, Hammer et al., 2001, Lo and Zivot, 2001), was used to track temporal patterns in total Vibrio spp. and three pathogenic species; *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. The absolute abundance of total *Vibrio* spp. was not statistically different between the two study sites ($p > 0.05$) over the course of the time-series. Total *Vibrio* abundance exhibited significant seasonal variability at both Maroubra Beach (ANOVA, $F_{(3,93)} = 8.54$, $p < 0.0001$) and Foreshore Beach (ANOVA, $F_{(3,93)} = 6.45$, $p < 0.0001$), with highest mean abundances at both sites occurring during the Summer (Fig 1). However, sharp peaks in Vibrio abundance occurred at intermittent intervals at both locations (Fig1). Highest *Vibrio* abundance at Maroubra Beach, where gene copy abundance increased by up to an order of magnitude above background levels, occurred in conjunction with 7 discrete blooms in *Vibrio* abundance during both the 2014-15 and 2015-16 summer periods (Fig 1). During these events, the abundance of Vibrio spp. reached up to 14% of total 16S gene copies, compared to a base-line of < 1% at other times (SI Fig 3). At Foreshore Beach, Vibrio abundance exceeded 10^6 gene copies mL^{-1} during 8 discrete bloom events (Fig 1), that occurred across all seasons, including a total of 8 weeks in Summer, 3 weeks in Winter, 2 weeks in Spring and 1 week in Autumn. The largest bloom occurred in Summer, when Vibrio abundances reached up to 32% of total 16S gene copies. Extended Local Similarity Analysis (eLSA), which detects distinct periods of significant correlation within a time-series (Xia, 2011), revealed that increases in total *Vibrio* abundance were directly correlated with *in situ* water temperature at Maroubra Beach (eLSA, LS = 0.37, d = 0, $p < 0.001$) between weeks 12 to 97 of the study

period, with this correlation also occurring with a one week time-lag at Foreshore Beach (eLSA, LS = 0.24, d = -1, $p < 0.05$) for the entire time-series.

Given the apparent importance of temperature in controlling total Vibrio abundance in each of the studied environments, we next used a Bivariate Threshold Autoregressive model (TVAR) (*Lo and Zivot,* 2001) to examine the temperature thresholds explaining significant increases in *Vibrio* abundance above baseline levels. Using this approach, which tests for threshold co-integration between two variables, *in situ* water temperatures of 22.9 °c (Fig 2a) and 24.5 °c (Fig 2b), occurring 3 weeks before a bloom, were shown to best predict increases in total *Vibrio* abundance at Maroubra and Foreshore beaches respectively. This is consistent with observations in other marine environments, where Vibrio blooms have followed increases in temperature above 20 °C (Kelly, 1982, Greenfield et al., 2017).

We next targeted three pathogenic *Vibrio* spp. of significance to the health of humans. *V. cholerae* is the causative agent of cholera, which is responsible for 2.2 million human mortalities per year (WHO, 2014). Notably, not all strains of *V. cholerae* cause cholera, with only O1 and O139 serogroups responsible (Levine and Eduardo, 2006). However, other, non-toxigenic strains of *V. cholerae* are responsible for severe skin infections (Andersson and Ekdahl, 2006, Lukinmaa et al., 2006, Stypulkowska-Misiurewicz et al., 2006) and gastroenteritis (Luo et al., 2013, Dutta et al., 2013). Given the significant

human health risk associated with this species, and emerging evidence that *V. cholerae* infections are increasing in frequency and global distribution as a consequence of climate change (Patz et al., 2005), we targeted the *ompW* gene (Gubala, 2006, Gubala and Proll, 2006a) to quantify abundance of this species. *V. cholerae* was detected in 95% of samples collected at Maroubra and Foreshore beaches. At both sites, baseline concentrations were in the range of $10^2 – 10^3$ gene copies mL^{-1} (Fig 1). Consistent with total Vibrio abundances, *V. cholerae* abundance varied significantly between seasons at both sites (Maroubra Beach ANOVA, $F_{(3,93)} = 12.56$, $p < 0.0001$; Foreshore Beach (ANOVA, $F_{(3,93)} = 3.49$, $p < 0.01$), with higher abundances during the Summer periods. Five peaks in *V. cholerae* abundance were observed at Maroubra Beach during the two year study (Fig 1), while four ephemeral blooms occurred at Foreshore Beach, with the largest bloom in the 2014-15 Summer (Fig 1). At both sites, a 1 week time-lagged correlation was detected between *V. cholerae* abundance and *in situ* water temperature (eLSA, LS = 0.37, d = -1, $p < 0.05$ at Maroubra between weeks 25 - 104; LS = 0.39, d = -1, $p = 0.003$ at Foreshore Beach, between weeks 29 - 103). No statistically significant correlations between *V. cholerae* abundance and any other environmental variables were detected.

V. parahaemolyticus is the leading cause of seafood poisoning (Fleming et al., 2018), causing acute gastroenteritis in humans when raw or insufficiently cooked shellfish are consumed (CDC, 1998). *V. parahaemolyticus* can also cause serious wound infections following direct seawater exposure (e.g. swimming), with an increased occurrence of

these types of infection previously linked to rising SST (Martinez-Urtaza et al., 2010). Targeting the thermostable direct haemolysin gene (*TdhS*) to quantify the abundance of pathogenic strains of *V. parahaemolyticus*, we detected this species in 57% and 67% of samples collected from Maroubra and Foreshore beaches respectively. However, abundances of *V. parahaemolyticus* were typically low (10^1 - 10^2 gene copies mL^{-1})(Fig 1), remaining below 1% of 16S sequences across the entire study period (SI Fig 3). At both beaches, peaks in *V. parahaemolyticus* abundance were confined to the 2014-15 Spring and Summer period (Fig 1), with a direct correlation between abundance and temperature observed at Foreshore Beach (eLSA, LS = 0.38, d = 0, $p < 0.05$) between weeks 26 – 104 and a 1-week time-lagged correlation to temperature occurring at Maroubra Beach between weeks 42 – 103 of the time series. No correlation between *V. parahaemolyticus* abundance and any other measured physical or water chemistry variables was observed.

V. vulnificus is a significant marine pathogen, capable of causing necrotizing wound infections and primary septicaemia in humans (Ralph and Currie, 2007) and high levels of seafood-related mortality (Linkous and Oliver, 1999). Between 1992 - 2007, this species was responsible for 459 human deaths in the US alone (Jones and Oliver, 2009). Like other vibrio species, the prevalence of *V. vulnificus* infections are increasing globally, with this pattern believed to be associated with increasing SST (Martinez-Urtaza et al., 2010). Detection of the virulence gene *vvhA* confirmed the presence of pathogenic *V. vulnificus* in 83% and 81% of samples collected from Maroubra and

Foreshore beaches respectively, albeit generally at low baseline concentrations between 10^1 - 10^2 gene copies mL^{-1} (Fig 1). However, intermittent bloom events resulted in *V. vulnificus* abundance exceeding 10^3-10^4 gene copies mL^{-1} in 32 and 39% of samples at Maroubra Beach and Foreshore Beach respectively (Fig 1), with abundances remaining above baseline concentrations for 1 – 2 weeks. Across the time-series, *V. vulnificus* abundance was significantly correlated with concentrations of NOx at both Maroubra Beach (eLSA, LS = 0.52, d = -1, $p < 0.05$) between weeks 9 – 35 and Foreshore Beach (eLSA, LS = 0.23, d = -1, $p < 0.05$) between weeks 1 – 64. While no direct correlation with *in situ* water temperature was observed, the highest concentrations of *V. vulnificus* (between 10^3 – 10^4 gene copies mL^{-1}) occurred during May 2014, which followed a prolonged marine heatwave that lasted 41 days between March and April 2014 (Fig 3).

To further evaluate the influence of SST on Vibrio abundance patterns in coastal environments, the mean weekly variation of SST above or below climatological average was tracked and correlations with *Vibrio* qPCR abundance measurements were measured (Fig 4). This revealed significant positive correlations between periods when SST was above climatological average and abundances of Vibrio populations. Specifically, a 3 week time-lagged correlation with total *Vibrio* abundance was observed at both sites (Maroubra; eLSA, LS = 0.27, d = -3, $p < 0.05$, Foreshore eLSA, LS = 0.29, d = -3, $p < 0.05$) between weeks 21 – 81 and 4 – 98 respectively, while 4 week time-lagged correlations with *V. cholerae* abundance (Maroubra; eLSA, LS = 0.33, d = -4, $p < 0.01$,

Foreshore eLSA, LS = 0.32, d = -4, $p < 0.01$) between weeks 10 – 98 at both sites, *V. parahaemolyticus* (Maroubra; eLSA, LS = 0.28, d = -4, $p < 0.05$, Foreshore eLSA, LS = 0.27, d = -4, $p < 0.05$) between weeks 10 – 90 at both sites, and *V. vulnificus* (Maroubra; eLSA, LS = 0.24, d = -4, $p < 0.05$, Foreshore eLSA, LS = 0.23, d = -4, $p < 0.05$) between weeks 10 – 81 and 9 – 95 respectively, were also observed at both locations. Furthermore, TVAR revealed that an increase in SST of 1.02 and 1.85 °C above climatological average, occurring 1 and 3 weeks prior, provided the best early prediction of a *Vibrio* bloom at Maroubra and Foreshore beaches respectively.

While periodic peaks, or blooms, in the abundance of Vibrio pathogens were generally restricted to warm-water summer months and were consistently correlated with SST at both sites, they did not persist for entire periods of elevated temperatures, implying the involvement of some other intrinsic, and potentially temporally-localised driver. Notably, positive Vibrio – SST relationships have been observed in low temperature waters (Vezzulli et al., 2016a), suggesting that localised and more abrupt deviations above the mean condition might influence bloom initiation as well as, or instead of, longer term seasonal variability. To more closely assess temperature anomaly-dependent dynamics of these peaks in Vibrio abundance, we considered the influence of the MHW events that have been demonstrated to be a common and increasingly significant feature within the study region (Oliver et al., 2018).

During the study period, 9 discrete MHW events, defined according to the criteria described in Hobday *et al.* (2016), occurred. These accounted for 15 MHW weeks during the 104 week time-series, and notably occurred across all seasons, including 6 weeks of MHW in Summer, 5 MHW weeks in Autumn, and 2 MHW weeks each in Winter and Spring. The mean duration of these MHW events was 7.6 $\pm$ 3.5 days, with a mean intensity of 2.2 $\pm$ 0.4 °C above the climatological average, and a maximum intensity of 3.5 °C above average conditions (Fig 5). To explore a direct link in the establishment of *Vibrio* blooms with MHWs, we compared the abundance of total vibrio, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* sampled during MHWs, and outside of MHWs. Mean abundances for total Vibrio and all pathogenic species measured were 2 – 10 times higher when SST was above the climatological average, compared to periods when SST was below the climatological average (SI Table 1). Notably, the highest mean abundance of Vibrios occurred in samples collected during MHW events (SI Table 1). Strong and significant differences were detected in total Vibrio abundance between MHW and non-MHW periods, at Maroubra and Foreshore beaches (SI Table 2). Additionally, abundances of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* were significantly higher during MHW events compared to non-MHW periods at both locations (SI Table 1 and SI Table 2). These patterns indicate that MHW events are a potentially more important mechanism for the development of ephemeral blooms in pathogenic vibrio species, than the chronic rise in SST alone. Furthermore, our detection of Vibrio blooms associated with MHWs in cooler seasons suggest that MHW events can extend the seasonal range of vibrio blooms beyond the temporal boundary of warm seasons.

Given the apparent importance of MHW events in driving Vibrio dynamics within the study region, we next focused our analysis on SSTs in coastal waters nearby to Maroubra Beach to assess the frequency and dynamics of MHW events in this region. SST satellite readings from January 1992 – August 2018, from a location approximately 30 km east of Maroubra and Foreshore beaches, revealed the occurrence of 64 marine heatwaves (MHW's) between 1992 – 2018 (Fig 3), with a mean duration of 9.8 days, and intensity of 2.3 °C above climatological average. Furthermore, MHWs in this region have increased in regularity, with a four-fold increase in the frequency of MHWs during the last 5 years (Fig 3). In fact, while 33 MHWs, with mean duration of 8.7 $\pm$ 5.3 days and mean intensity of 2.3 $\pm$ 0.9 °C , occurred in the 20 years between 1992 – 2012 (Fig 3), there were 31 MHWs in only the 5 years between 2013 – 2018, with mean duration of 10.9 $\pm$ 8.7 days, and mean intensity of 2.4 $\pm$ 0.5 °C (Fig 3). This observed increase in the occurrence of MHWs in the temperate region of the Tasman Sea is consistent with the increased incidence of MHWs occurring in other Australian coasts (Wernberg et al., 2012) and regions in the northern hemisphere (Jacox et al., 2016, Di Lorenzo and Mantua, 2016). Given our observations of clear links between MHW occurrence and Vibrio bloom dynamics within this region, it is therefore likely that Vibrio blooms will become more frequent and potentially intense in this region of coastline, which also coincides with Australia's highest population density and use of coastal waters (Australian Bureau of Statistics, 2006). Although rare in Australia, infections by *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* acquired from Australian waters have

led to serious illness and mortalities (Ralph and Currie, 2007, Wise and Newton, 1992). The increasing influence of MHW may result in higher rates of Vibrio infections in this region, which would be consistent with patterns observed in the northern hemisphere (Andersson and Ekdahl, 2006, Paz et al., 2007).

By closely tracking *Vibrio* population dynamics using a high temporal resolution sampling regime within a coastal region subject to high levels of climate change induced warming (Wernberg et al., 2011), this study has revealed strong and significant links between the occurrence of marine heatwave events and blooms of pathogenic marine bacterial species. While significant correlations between vibrio populations and increasing SST were observed, MHWs led to the highest elevations in vibrio abundance. Our observations reveal an additional important environmental impact of the rising global occurrence of MHWs (Frolicher and Laufkotter, 2018), which has potentially profound implications for human health and coastal industries affected by Vibrio outbreaks, including aquaculture (Lafferty et al., 2015).

5.3 Materials and methods

5.3.1 Study sites, data and sample collection

Two study sites were selected according to contrasting hydrological features, including Foreshore Beach, situated within the partially enclosed estuarine waters of Botany Bay (33.95 ˚S, 151.9 ˚E), and Maroubra Beach (33.95˚S, 151.25 ˚E), which is open to the

Pacific Ocean, has higher levels of hydrodynamic flushing, and is directly exposed to oceanographic forces such as tides and wind. Sampling was conducted at each site on a weekly basis (every Friday morning between 0800-1100 AEST) over the course of 24 months, from 02/05/2014 – 29/04/2016, leading to the establishment of two time-series, each comprising 104 time points. At each site, samples were collected in triplicate from near to the shore-line (<1.5 m depth). Physico-chemical parameters, including temperature, pH, electrical conductivity (EC) and dissolved oxygen (DO) were measured *in situ* using a WTW Multiprobe meter (Multi 3430, Germany). For analysis of dissolved inorganic nutrients, including NH_4, NO_x and PO_4, triplicate 50 mL samples were collected and filtered through a 0.45 µm filter (Sartorius Stedim Biotech) and immediately transported to the laboratory on ice. Samples were stored at -80 °C within 2 hours of sample collection for later nutrient analysis. Microbial DNA was retrieved by filtering triplicate 2.5 L samples through 0.22 µm pore-size membrane filters (Millipore) using a peristaltic pump. Filters were stored at -80 °C for subsequent DNA extraction.

5.3.1 Nutrient analysis

Concentrations of NH_4, NO_x and PO_4 were determined via a colorimetric analysis using the Lachat Quickchem QC8500 Automated Ion Analyser (LACHAT Instruments, USA) in accordance with the manufacturer's guidelines. Internal controls, including addition of spiked recovery samples and blanks were included to monitor the consistency of instrument performance. The resulting data were transferred and interpreted using Omnion version 3 software (LACHAT Instruments, USA).

Chlorophyll *a* (Chl *a*) concentrations were determined by filtering 200 mL of surface water onto 0.75 µm pore-size glass microfiber filters and analysing Chl *a* concentrations using the acetone extraction method described in (Eaton and Franson, 2005).

5.3.2 DNA extraction and quantitative PCR

Microbial DNA was extracted from 0.22 µm membrane filters using the Powerwater DNA isolation Kit (MoBio Laboratories, Inc) in accordance with the manufacturer's instructions. DNA quantity and purity was evaluated using a Nanodrop-2000 Spectrophotometer.

Quantitative PCR (qPCR) was used to track patterns in the abundance of the total *Vibrio* community, as well as the human pathogens *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus*. qPCR was performed using the BIORAD CFX384 Touch™ Real-Time PCR Detection System™ and gene copies were calculated for each amplicon by plotting against their respective standard curve using BIORAD CFX Manager software. All qPCR tests were run using three technical replicates, consisting of 5 µl reaction volumes containing 2.5 µl iTaq UniverSYBR Green SMX 2500®, 0.1 µl nuclease free water, 0.2 µM of each forward and reverse primer and 2 µl of diluted (1:10) DNA template. Negative control samples were run with every plate for detection of contamination, and a 16S rRNA qPCR assay (Nadkarni et al., 2002) was also run for all samples to confirm that inhibitors were not preventing amplification of genes. Calibration curves were run with

every plate, with all reagent and plate preparation conducted using an epMotion® 5075l Automated Liquid Handling System. Cycling parameters for each qPCR assay are provided in Supplementary Table 1. To confirm that each primer pair produced only a single specific product, a melting curve was added to the end of every qPCR assay for each set of primers. Amplification efficiencies ranged from 95 – 105%, and limits of quantification were from 10^2 gene copies mL^{-1}.

The primer pair Vib1-f and Vib2-r (Thompson et al., 2004, Vezzulli et al., 2012) were used to amplify 16S rRNA genes specific to the *Vibrio* genus. *V. cholerae* abundance was measured by targeting the outer-membrane protein *ompW* gene (Gubala, 2006, Gubala and Proll, 2006b). This *ompW* gene allows discrimination between *V. cholerae* and other *Vibrios*, but does not necessarily identify toxigenic strains of *V. cholerae*. To quantify *V. vulnificus* we targeted the hemolysin A *vvhA* gene (Panicker and Bej, 2005, Panicker et al., 2004) using a set of primers designed by *Siboni et al. 2016*. To specifically target *V. parahaemolyticus*, the thermostable direct haemolysin *tdhS* gene was targeted (Rizvi and Bej, 2010). All qPCR cycling parameters, and cultures used for standard curve creation followed the methods and cycle conditions described in *Siboni et al. 2016*.

5.3.3 Correlation and threshold testing of Vibrio abundance and in situ water temperature

The extended Local Similarity Analysis pipeline was used to detect significant correlations between *Vibrio* abundance, *in situ* water temperature and deviation of sea surface temperature (SST) from climatological average, which also included the detection of time-lagged relationships. Estimation of *in situ* water temperature thresholds that best predict blooms in *Vibrio* was achieved using the Threshold Autoregression (TVAR) function, within the TsDyn package in R (Di Narzo et al., 2018). Briefly, the Augmented Dickey-Fuller and Ljung-Box tests were performed to test whether temperature and *Vibrio* abundance data met the assumptions of stationarity and independence from autocorrelation respectively. Detrending of *in situ* temperature data was performed where autocorrelation was detected. Extended Local Similarity Analysis (eLSA) was then used to detect distinct periods within the timeseries where statistically significant time-lagged relationships between *Vibrio* abundances, *in situ* water temperature and sea surface temperature (SST) occurred. The TVAR function in R was subsequently used to estimate *in situ* water temperature and SST thresholds that best predicted ephemeral blooms in *Vibrio* abundance.

5.3.4 Calculation of Marine Heatwaves

Daily sea surface temperature (SST) data was retrieved from the Australian Ocean Data Network (https://portal.aodn.org.au/search), using the IMOS Australian National Mooring Network (ANMN) Facility Temperature an Salinity Timeseries. Analysis of this data enabled detection of SST anomalies, or marine heatwaves as defined by *Hobday et*

al. 2016, impacting the east coast of Sydney, Australia. The closest available location to the sample collection sites was selected to track SST from the earliest available date of 01/01/1992 to 20/08/2018, located at -33.95, 151.89, approximately 30 km east of Maroubra and Foreshore beaches. We used the Rmarineheatwave package in R to characterise the frequency, intensity and duration of MHW's across a 26 year timeseries. A MHW was defined using the criteria explained in *Oliver et al.* 2018, which requires that an event be a discrete, prolonged anomalously warm water event of duration of at least 5 days, whereby 'anomalously warm' is defined by reference to the climatology baseline. Heatwave events were confirmed by identifying periods when daily SSTs were above the 90th percentile of climatology average for at least five consecutive days. Two events with a break of less than 3 days were considered as a single event.

5.3.5 Statistical testing for differences between vibrio populations in association with MHW events

To test for a statistical difference in the abundance of vibrio populations within and outside of MHW events, one-way ANOVA using an unbalanced design was used in PaST (Hammer et al., 2001). Vibrio qPCR gene copy data-series were first checked against the assumptions of ANOVA (independence, normality and homogeneity of variance). LOG transformation was performed to achieve normality in data series that were not normally distributed. Where variance from means were not equal between groups, the Welch F test (which is less sensitive to different variance and outliers) was used.

5.4 Figures

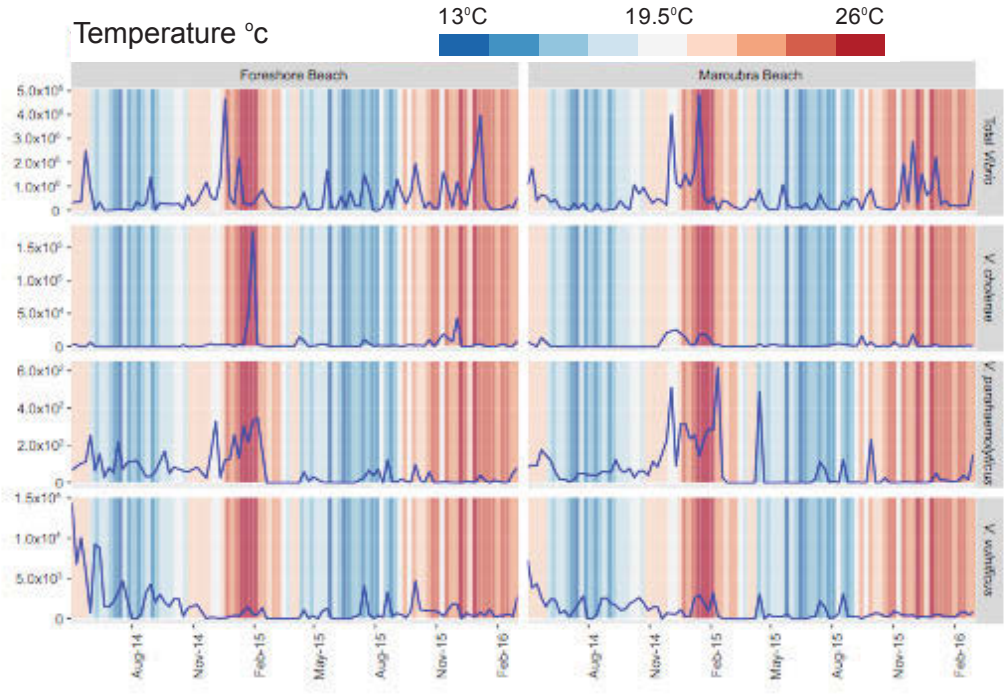


Figure 1. *Absolute abundance of total Vibrio, V. cholerae, V. parahaemolyticus and V. vulnificus gene copies, measured across the two year time series at Maroubra Beach and Foreshore Beach using qPCR are represented by red lines. Grey shading represents standard deviation. Vertical bars represent in situ water temperature, with red bars showing higher temperatures, and blue bars representing lower temperatures across the temperature scale.*

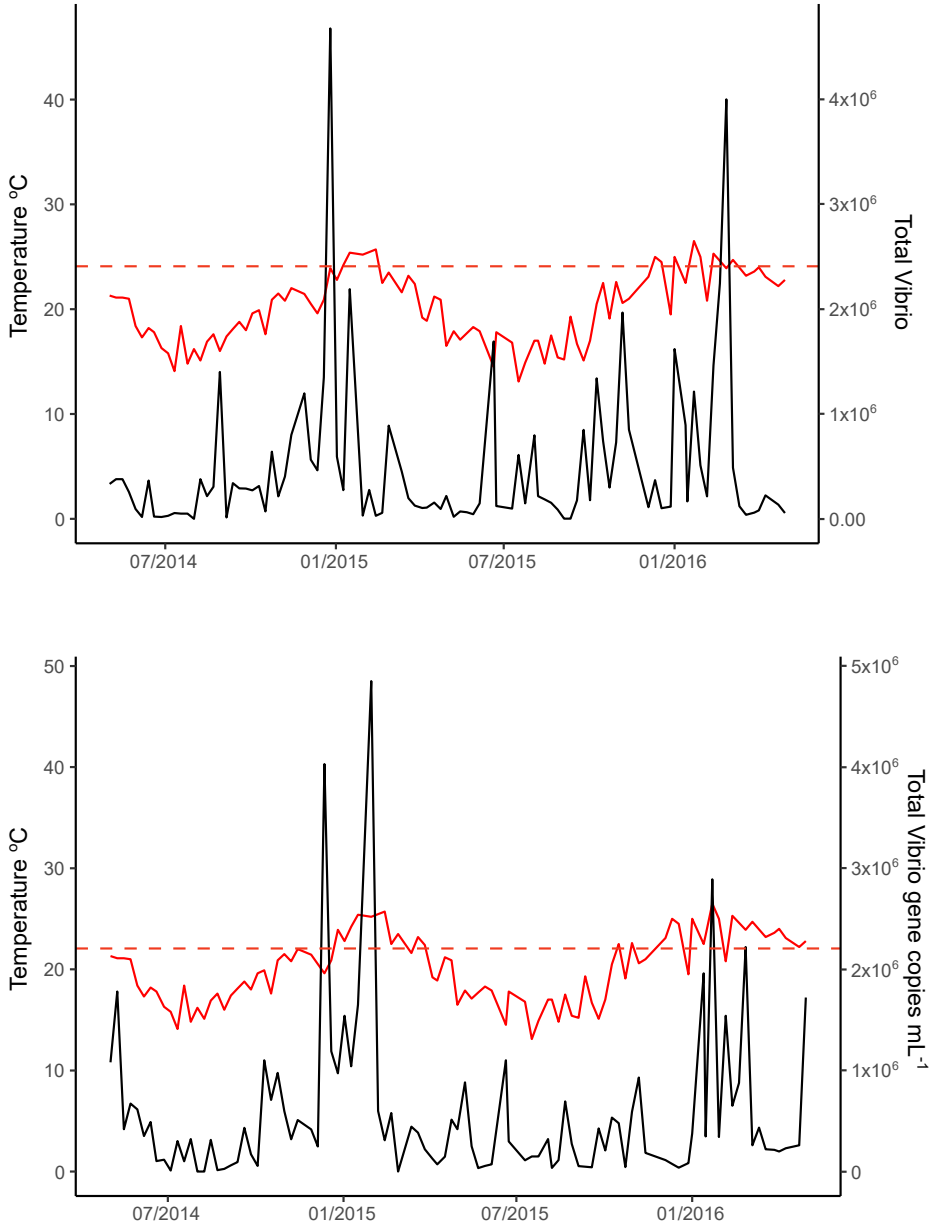


Figure 2. *a) Abundance of total Vibrio across the 24 month time-series at Maroubra Beach, represented by black line, and in situ water temperature represented by the red line. The orange perforated line represents the temperature threshold determined using threshold autoregression analysis, to best predict blooms in total Vibrio. b) Abundance of total Vibrio across the 24 month timeseries at Foreshore Beach, represented by black line, and in situ water temperature represented by the red line. The orange perforated line represents the temperature threshold determined using threshold autoregression analysis, to best predict blooms in total Vibrio.*

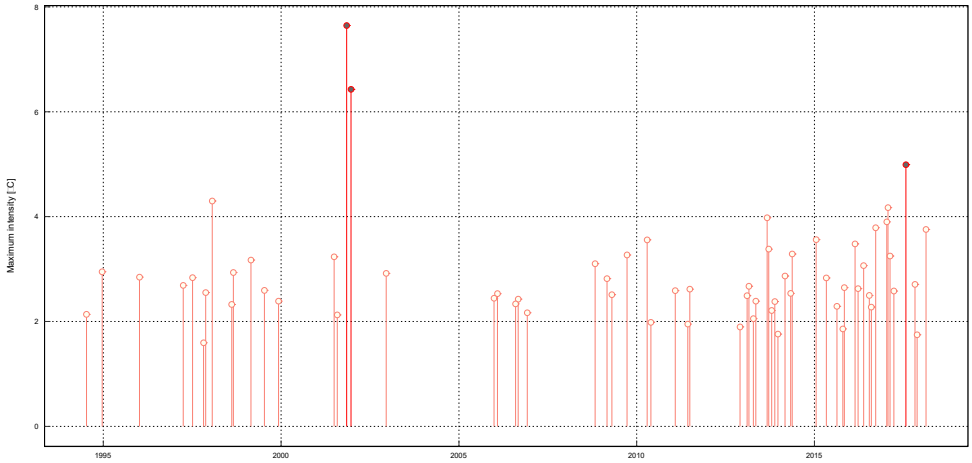


Figure 3. *Lollipop plot depicting 64 marine heatwaves that occurred in the Tasman Sea, at coordinate -33.95, 151.89, approximately 30 km east of Maroubra and Foreshore beaches. The height of each vertical line represents the maximum temperature intensity of that given event.*

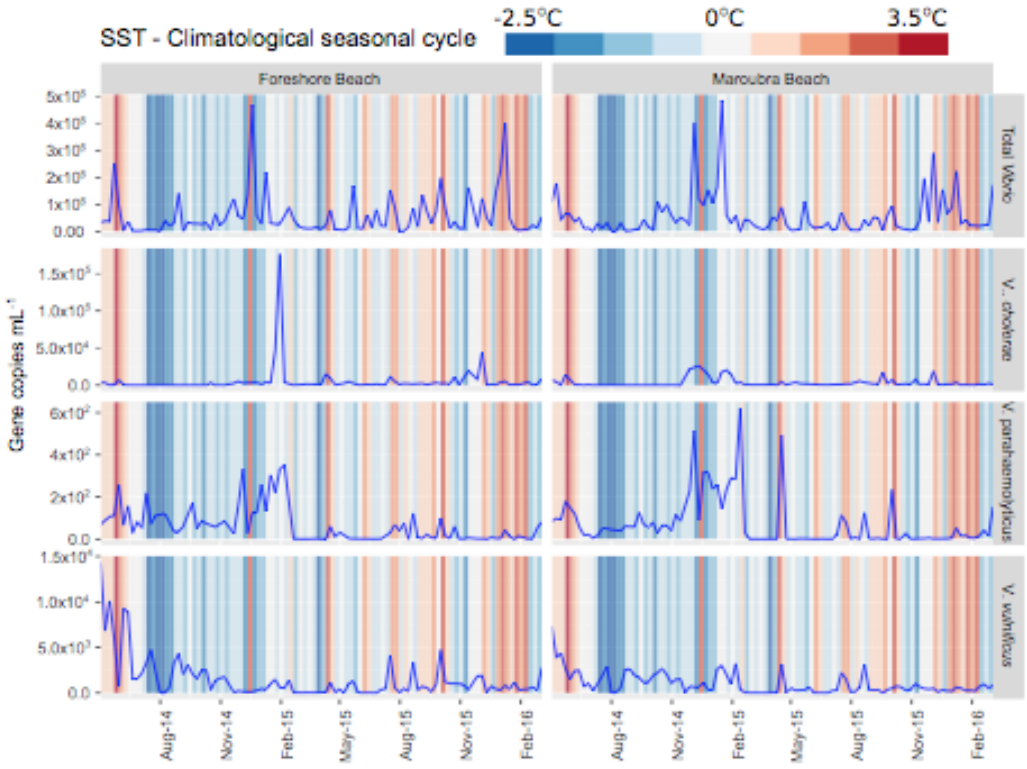


Figure 4. *The abundance of total Vibrio and V. cholerae gene copies across the two-year time series at Maroubra Beach and Foreshore Beach are represented by red lines. Grey shading represents standard deviation. Vertical bars represent the intensity of sea surface temperature (SST) above climatological average, at coordinate -33.95, 151.89, approximately 30 km east of Maroubra and Foreshore beaches. Red bars indicate SST's above climatological average, and blue bars represent SST's below climatological average.*

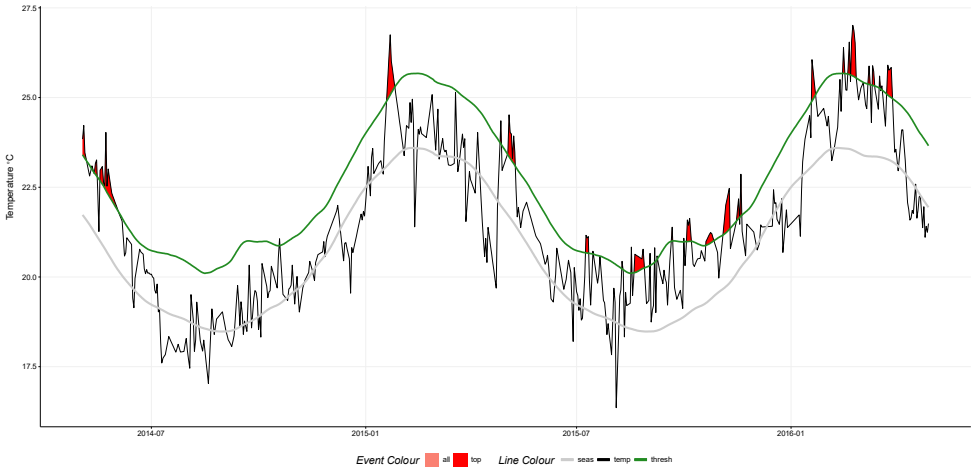


Figure 5. *The sea surface temperature (SST) climatology average is represented by the black line, the 90th percentile marine heatwave (MHW) threshold is represented by the green line. Where the SST time series (black line) exceeds the MHW threshold for >3 days, the area under the curve is shaded with red. The red filled area indicates the period of time associated with the identified MHW.*

SI Table 1. *Comparison of Vibrio abundance summary statistics.*

Foreshore Beach

	Total vibrio		*V. cholerae*		*V. parahaemolyticus*		*V. vulnificus*	
	MHW	nonMHW	MHW	nonMHW	MHW	nonMHW	MHW	nonMHW
N	15	82	15	82	15	82	15	82
Min	2.76E+05	3.15E+01	5.38E+02	7.54E+00	2.94E+01	0.00E+00	3.56E+02	2.45E+00
Max	4.00E+06	4.68E+06	1.76E+05	4.37E+04	3.53E+02	3.31E+02	1.44E+04	1.01E+04
Sum	1.80E+07	3.75E+07	2.89E+05	2.14E+05	2.28E+03	3.94E+03	4.27E+04	1.04E+05
Mean	1.20E+06	4.57E+05	1.93E+04	2.60E+03	1.52E+02	4.81E+01	2.84E+03	1.27E+03
Std. error	2.74E+05	7.28E+04	1.16E+04	6.33E+02	2.85E+01	7.13E+00	9.45E+02	2.21E+02
Variance	1.13E+12	4.35E+11	2.01E+09	3.28E+07	1.22E+04	4.17E+03	1.34E+07	4.01E+06
Stand. dev	1.06E+06	6.60E+05	4.48E+04	5.73E+03	1.10E+02	6.46E+01	3.66E+03	2.00E+03
Median	8.49E+05	2.17E+05	4.84E+03	1.00E+03	1.13E+02	1.68E+01	1.12E+03	4.68E+02
25 prcntil	3.33E+05	1.00E+05	2.62E+03	7.02E+01	6.79E+01	1.65E+00	6.10E+02	1.02E+02
75 prcntil	1.97E+06	5.71E+05	9.41E+03	2.47E+03	2.55E+02	7.37E+01	4.12E+03	1.55E+03
Skewness	1.523807	3.825696	3.507386	5.243171	0.7951056	1.992601	2.548855	2.922693
Kurtosis	2.193958	20.5205	12.70874	33.61696	-0.9019425	4.793564	7.476304	9.171394
Geom. mean	8.52E+05	1.90E+05	5.95E+03	5.53E+02	1.17E+02	0.00E+00	1.60E+03	3.76E+02
Coeff. var	88.64521	144.4362	232.6086	219.9887	72.76787	134.2372	128.6124	157.4611

Maroubra Beach

	Total vibrio		*V. cholerae*		*V. parahaemolyticus*		*V. vulnificus*	
	MHW	nonMHW	MHW	nonMHW	MHW	nonMHW	MHW	nonMHW
N	15	82	15	82	15	82	15	82
Min	2.73E+05	7.19E+01	1.05E+03	6.49E+00	5.14E+01	1.76E-01	5.23E+02	1.50E+00
Max	4.85E+06	4.03E+06	2.01E+04	2.61E+04	4.91E+02	6.22E+02	7.32E+03	4.40E+03
Sum	1.75E+07	3.71E+07	1.08E+05	2.36E+05	2.87E+03	4.30E+03	3.52E+04	6.42E+04
Mean	1.17E+06	4.53E+05	7.22E+03	2.88E+03	1.91E+02	5.25E+01	2.35E+03	7.83E+02
Std. error	3.00E+05	7.07E+04	1.65E+03	6.34E+02	2.90E+01	1.16E+01	4.34E+02	1.02E+02
Variance	1.35E+12	4.10E+11	4.08E+07	3.30E+07	1.26E+04	1.10E+04	2.82E+06	8.56E+05
Stand. dev	1.16E+06	6.40E+05	6.39E+03	5.74E+03	1.12E+02	1.05E+02	1.68E+03	9.25E+02
Median	6.94E+05	2.55E+05	4.31E+03	6.60E+02	1.54E+02	1.08E+01	2.22E+03	3.96E+02
25 prcntil	5.78E+05	9.08E+04	2.42E+03	7.60E+01	1.13E+02	1.42E+00	9.58E+02	2.04E+02
75 prcntil	1.65E+06	4.95E+05	1.40E+04	1.88E+03	2.59E+02	5.82E+01	3.11E+03	1.05E+03
Skewness	2.574871	3.286762	0.9902906	2.814593	1.320312	3.596336	1.855039	1.798542
Kurtosis	7.572602	13.65389	-0.377953	7.112895	2.440632	14.75469	5.165737	3.208714
Geom. mean	8.56E+05	1.83E+05	4.81E+03	5.22E+02	1.64E+02	8.54E+00	1.87E+03	2.83E+02
Coeff. var	99.61436	141.4288	88.43267	199.3834	58.64412	199.7512	71.61044	118.2412

SI Table 2. *One-way analysis of variance, testing for significant differences between Vibrio abundance at time points experiencing sea surface temperatures (SSTs) below and above climatological average, and marine heatwaves.*

MAROUBRA BEACH

Total Vibrio

	Sum of sqrs	df	Mean square	F	p (same)
Between groups:	6.45296	2	3.22648	5.83	0.004105
Within groups:	52.0234	94	0.553441	Permutation p (n=99999)	
Total:	58.4764	96	0.00565		

Components of variance (only for random effects):

Var(group):	0.116011	Var(error):	0.553441	ICC:	0.173293

omega2: 0.09057

Levene´s test for homogeneity of variance, from means	p (same):	0.01488
Levene´s test, from medians	p (same):	0.04525

Welch F test in the case of unequal variances: F=14.88, df=29.4, p=3.434E-05

Dunn's Post-hoc	MHW	Positive_SST	Negative_SST
MHW		0.0001279	0.00125
Positive_SST	0.0001279		0.768
Negative_SST	0.00125	0.768	

V. cholerae

	Sum of sqrs	df	Mean square	F	p (same)
Between groups:	14.8283	2	7.41415	9.31	0.0002047
Within groups:	74.8577	94	0.796359	Permutation p (n=99999)	
Total:	89.686	96	0.00024		

Components of variance (only for random effects):

Var(group):	0.287215	Var(error):	0.796359	ICC:	0.265063

omega2: 0.1463

Levene´s test for homogeneity of variance, from means	p (same):	0.0003545
Levene´s test, from medians	p (same):	0.009008

Welch F test in the case of unequal variances: F=19.68, df=29.64, p=3.649E-06

Dunn's Post-hoc	MHW	Positive_SST	Negative_SST
MHW		0.0002156	0.0001796
Positive_SST	0.0002156		0.2762
Negative_SST	0.0001796	0.2762	

V. parahaemolyticus

	Sum of sqrs	df	Mean square	F	p (same)
Between groups:	25.9487	2	12.9743	16.86	5.536E-07
Within groups:	72.3405	94	0.76958	Permutation p (n=99999)	
Total:	98.2892	96	0.00001		

Components of variance (only for random effects):

Var(group):	0.529692	Var(error):	0.76958	ICC:	0.407684

omega2: 0.2464

Levene´s test for homogeneity of variance, from means	p (same):	0.00005268
Levene´s test, from medians	p (same):	0.0002352

Welch F test in the case of unequal variances: F=56.95, df=33.42, p=1.718E-11

Dunn's Post-hoc	MHW	Positive_SST	Negative_SST
MHW		1.705E-07	0.01421
Positive_SST	1.705E-07		0.03623
Negative_SST	0.01421	0.03623	

V. vulnificus

	Sum of sqrs	df	Mean square	F	p (same)
Between groups:	8.70425	2	4.35213	7.343	0.001088
Within groups:	55.7123	94	0.592684	Permutation p (n=99999)	
Total:	64.4165	96	0.00142		

Components of variance (only for random effects):

Var(group):	0.163161	Var(error):	0.592684	ICC:	0.215866

omega2: 0.1157

Levene´s test for homogeneity of variance, from means	p (same):	0.01708
Levene´s test, from medians	p (same):	0.09193

Welch F test in the case of unequal variances: F=22.66, df=32.39, p=7.002E-07

Dunn's Post-hoc	MHW	Positive_SST	Negative_SST
MHW		9.487E-06	0.009737
Positive_SST	9.487E-06		0.261
Negative_SST	0.009737	0.261	

FORESHORE BEACH

Total Vibrio

	Sum of sqrs	df	Mean square	F	p (same)
Between groups:	6.80318	2	3.40159	6.87	0.001641
Within groups:	46.5411	94	0.495119	Permutation p (n=99999)	
Total:	53.3443	96	0.00209		

Components of variance (only for random effects):

Var(group):	0.126142	Var(error):	0.495119	ICC:	0.203042

omega2: 0.108

Levene´s test for homogeneity of variance, from means	p (same):	0.07795
Levene´s test, from medians	p (same):	0.1118

Welch F test in the case of unequal variances: F=12.9, df=27.7, p=0.0001099

Dunn's Post-hoc	MHW	Positive_SST	Negative_SST
MHW		0.000554	0.0002913
Positive_SST	0.000554		0.2387
Negative_SST	0.0002913	0.2387	

V. cholerae

	Sum of sqrs	df	Mean square	F	p (same)
Between groups:	19.8465	2	9.92326	13.16	0.000009179
Within groups:	70.9072	94	0.754332	Permutation p (n=99999)	
Total:	90.7537	96	0.00002		

Components of variance (only for random effects):

Var(group):	0.397935	Var(error):	0.754332	ICC:	0.34535

omega2: 0.2004

Levene´s test for homogeneity of variance, from means	p (same):	0.003359
Levene´s test, from medians	p (same):	0.0254

Welch F test in the case of unequal variances: F=16.56, df=26.89, p=2.051E-05

Dunn's Post-hoc	MHW	Positive_SST	Negative_SST
MHW		0.0001351	0.00001536
Positive_SST	0.0001351		0.08727
Negative_SST	0.00001536	0.08727	

V. parahaemolyticus

	Sum of sqrs	df	Mean square	F	p (same)
Between groups:	5372.75	2	2686.37	7.906	0.0006706
Within groups:	31940.2	94	339.79	Permutation p (n=99999)	
Total:	37313	96	0.00258		

Components of variance (only for random effects):

Var(group):	101.843	Var(error):	339.79	ICC:	0.230605

omega2: 0.1246

Levene´s test for homogeneity of variance, from means	p (same):	3.081E-13
Levene´s test, from medians	p (same):	0.000653

Welch F test in the case of unequal variances: F=6.916, df=30.9, p=0.003295

Dunn's Post-hoc	MHW	Positive_SST	Negative_SST
MHW		0.3422	0.00572
Positive_SST	0.3422		0.009805
Negative_SST	0.00572	0.009805	

V. vulnificus

	Sum of sqrs	df	Mean square	F	p (same)
Between groups:	5.04315	2	2.52157	4.002	0.02148
Within groups:	59.2311	94	0.630118	Permutation p (n=99999)	
Total:	64.2742	96	0.0208		

Components of variance (only for random effects):

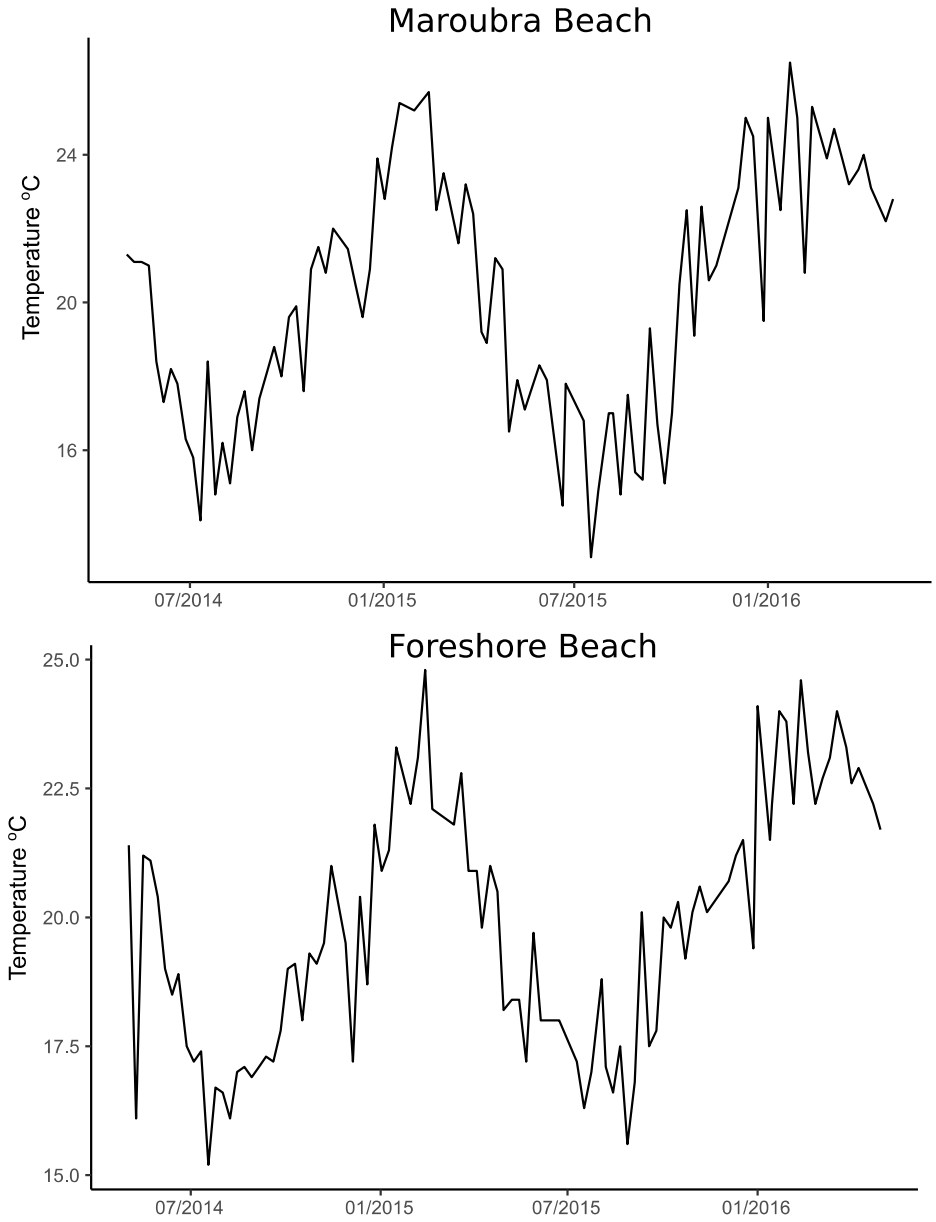
Var(group):	0.0820901	Var(error):	0.630118	ICC:	0.115261

omega2: 0.05828

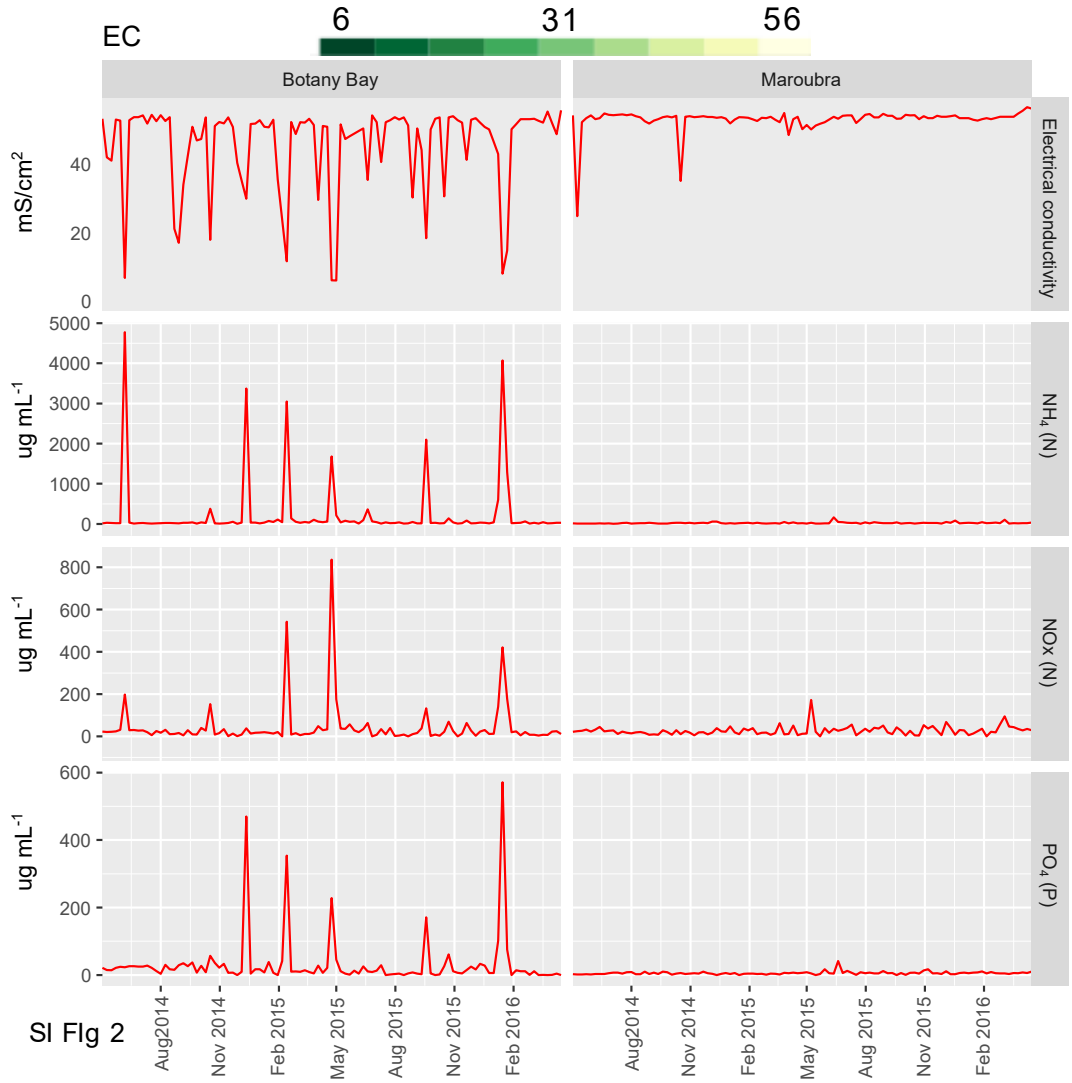
Levene´s test for homogeneity of variance, from means	p (same):	0.06235
Levene´s test, from medians	p (same):	0.08636

Welch F test in the case of unequal variances: F=8.37, df=29.24, p=0.001336

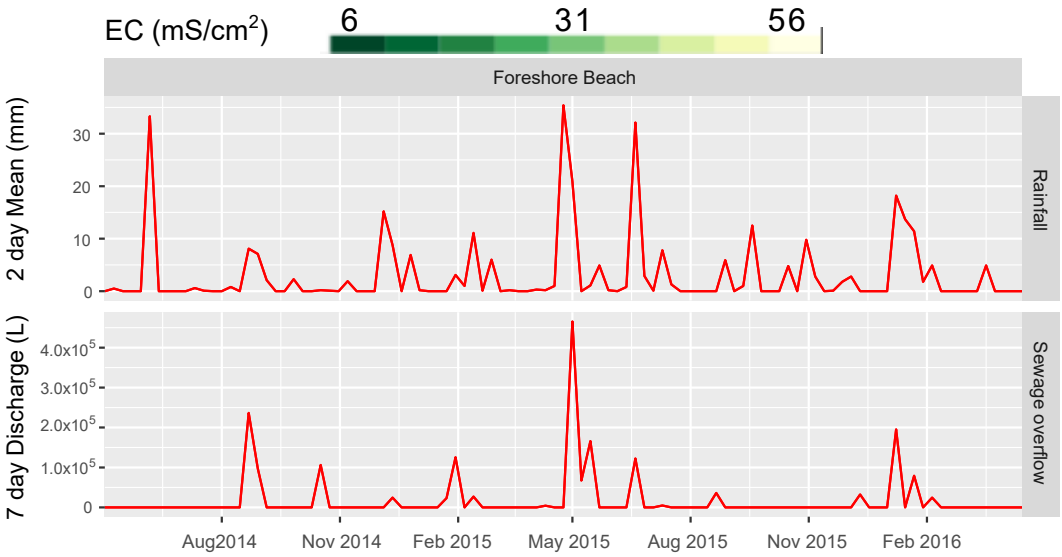
Dunn's Post-hoc	MHW	Positive_SST	Negative_SST
MHW		0.007324	0.05049
Positive_SST	0.007324		0.8559
Negative_SST	0.05049	0.8559	



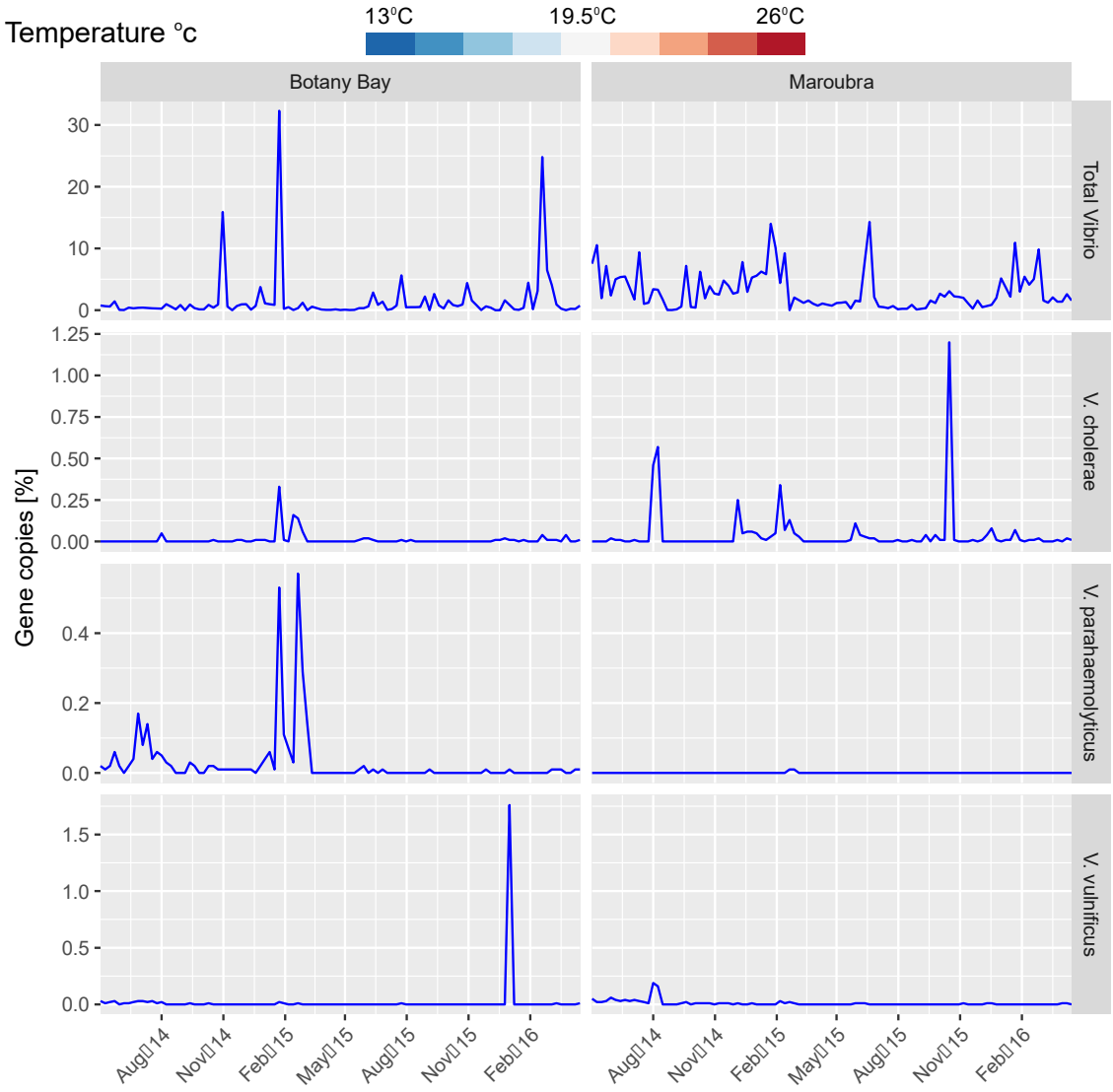
SI Fig 1. *Weekly, in situ temperature readings at Maroubra and Foreshore beaches, from 02/05/2014 – 29/04/2016.*



SI Fig 2. *Weekly measurements of electrical conductivity (EC), and dissolved nutrient NH4, NOx and PO4, represented by red line plots. Green vertical bars represent EC, whereby darker shades of green indicate low EC, which is indicative of stormwater input, and lighter green bars represent dry periods with no detected impact on EC from stormwater. Orange vertical bars represent occasions when wet weather sewer overflow (WWSO) occurred at Millpond overflow point, that discharges into Botany Bay. Modelled WWSO data provided by Sydney Water.*



SI Fig 3. *Relative abundance of total Vibrio, V. cholerae, V. parahaemolyticus and V. vulnificus gene copies (normalized to 16S gene copies, and expressed as a percentage), measured weekly across the two year time series at Maroubra Beach and Foreshore Beach using qPCR, are represented by red lines. Grey shading represents standard deviation. Vertical bars represent in situ water temperature, with red bars showing higher temperatures, and blue bars representing lower temperatures across the temperature scale.*



SI Fig 4. *The gene copy abundance of total Vibrio, V. cholerae, V. parahaemolyticus and V. vulnificus was normalised to 16S gene copies to produce relative abundance values. These were measured across the two year time series at Maroubra Beach and Foreshore Beach using qPCR are represented by blue lines. Grey shading represents standard deviation. Vertical bars represent in situ water temperature, with red bars showing higher temperatures, and blue bars representing lower temperatures across the temperature scale.*

6. Chapter 6 – Discussion

Coastal marine ecosystems are characterised by highly dynamic and heterogeneous conditions, that support disproportionately high rates of productivity and nutrient cycling relative to terrestrial and open ocean environments (Azam, 1998, Alongi and McKinnon, 2005, Teeling et al., 2012). Coasts also provide essential ecosystem products and services (Agardy et al., 2005, Baba et al., 2013), but human-related impacts have eroded the health and stability of many coastal ecosystems, which has diminished the output of ecosystem products and services (Deegan et al., 2012, Halpern et al., 2008a, Islam and Tanaka, 2004). To monitor the impact from human activities, most investigations of coastal microbiology have focussed only on a narrow selection of indicator organisms (Boehm and Sassoubre, 2014), hence our understanding of microbial ecology in coastal ecosystems at the scale of whole communities is less developed. To address this knowledge gap, this project has focussed on closely studying the bacterioplankton communities at the base of coastal food webs - organisms that underpin the geochemical processes that drive productivity and the generation of ecosystem benefits, but also have the potential to exacerbate negative human impacts (Tout et al., 2015), and harbour pathogens of public health significance (Stewart et al., 2008).

In this study, water chemistry and patterns in the abundance and assemblage structure of bacterial communities were tracked weekly, and compared between two beach environments that experienced contrasting hydrodynamics and levels of anthropogenic influence. Most notably, water chemistry remained relatively stable at Maroubra Beach,

our less impacted site. Whereas, input of stormwater and wet weather sewer overflow (WWSO) led to decreased electrical conductivity (EC) and dissolved oxygen (DO), and spikes in nutrients at the frequently impacted Foreshore Beach.

6.1 Potential Environmental drivers of temporal shifts in bacterial assemblages

Although each beach experienced very different environmental dynamics, both sites were characterised by a high level of weekly variation in bacterial composition, reflecting the variety of environmental determinants of microbial processes within coastal ecosystems. Additionally, both beaches harboured strikingly similar core microbiomes, comprised of cosmopolitan marine bacteria. Within the core microbiomes, we detected seasonal switching between *Pelagibacter* and *Synechococcus* at Maroubra Beach, where *Pelagibacter* dominated communities during Winter and Spring, and *Synechococcus* in Summer and Autumn, which correlated significantly with temperature, as has been noted in previous studies (Giovannoni and Vergin, 2012). We also observed opposing temperature preferences between closely related OTUs within these two key bacterial genera, which are consistent with observations in another time-series study conducted in at the Pivers Island Coastal Observatory, off the east coast of the U.S (Ward et al., 2017), indicating that the determinant role of temperature is wide-spread across microbial communities inhabiting coastal ecosystems.

While the major members of the core microbiomes at each site were consistent, the temporal dynamics of the core microbiome differed between sites. At Foreshore beach,

rainfall associated perturbations, including stormwater input and WWSO, led to sharp, temporary decreases in the relative abundance of all core bacterial members and spikes in the relative abundance of stormwater and sewage-associated microbes, comprising the 'transient microbiome'. This rapid shift was typically followed by re-establishment of marine core members one to two weeks after the rainfall ceased. These findings indicate that localised perturbations from terrestrial and urban features can supersede gradual, seasonal transitions in bacterial community assemblages, which may have important implications for net bacterial community function.

Differences in the composition and temporal dynamics of bacterial communities at Foreshore and Maroubra beaches were most evident in the transient and rare microbiomes, driven by contrasting environmental factors. Maroubra Beach retained a highly diverse transient and rare microbiome, which shifted seasonally and correlated most strongly with water temperature. On the other hand, a consortia of putative enteric microbes were washed into the receiving waters at Foreshore Beach following heavy rain, and subsequent inputs of stormwater and WWSO. Some of these allochthonous microbes dominated bacterial communities for only short periods of one or two weeks following rainfall, while others persisted for much longer, but at relatively lower abundances. These observations indicate that stormwater and WWSO events not only elicit immediate and sometimes severe impacts on the ecology of coastal microbial communities, by depositing enteric microbes into marine waters (which has been noted widely throughout the literature) (McLellan et al., 2015, Sidhu et al., 2013, Cunha and Almeida, 2006), but that some of these microbes persist in marine environments for

several weeks. At Foreshore Beach, some of these transient and rare members included OTUs closely related to potentially pathogenic species, including members of the *Acinetobacter, Arcobacter, Aeromonas, Streptococcus* and *Staphylococcus*. Therefore, the establishment of potential pathogens within rare microbiomes of coastal microbial communities could be widespread, but overlooked by typical, culture-based investigations.

6.2 Incursions of allochthonous Arcobacter from the urban environment

From the 16S rRNA community profiles used to track patterns in bacterial community composition, we detected multiple large peaks in the relative abundance of one particular group of bacteria following intense rainfall at the impacted beach, namely bacteria from the Arcobacter genus. This group shifted sharply from low relative abundances of less than 10% of the community, to the most abundant bacterial OTUs (up to 70 %) over the space of one week. While not an initial foci of this thesis, detection of regular spikes in the abundance of Arcobacter prompted further investigation, and became a key focus in this project.

Stormwater systems and WWSOs convey microbes from humans and the urban environment, including enteric bacteria, viruses and protozoan, into natural waterways and coastal receiving water (Okoh et al., 2010, Rozen and Belkin, 2001, Sauer et al., 2011, Stewart et al., 2008). Most of these enteric microbes have relatively short survival times in marine environments (Rozen and Belkin, 2001), and can be tracked adequately using faecal indicator bacteria (FIB) such as *E. coli* or enterococci (Boehm and Sassoubre,

2014). However, a few enteric microbes are capable of persisting and growing in marine waters and sediments, including some species associated with human and animal illnesses (Anderson et al., 2005). These emerging environmental pathogens may pose a potential exposure risk to humans, as they can persist beyond the time indicated by FIBs, and are not routinely screened for in most water quality monitoring programs. Chapter 2 of this thesis focussed on the temporal dynamics and diversity of Arcobacter, which represent one of these groups of emerging environmental pathogens. Infections by pathogenic species of Arcobacter have resulted in gastrointestinal illness and serious wound infections in humans (Arguello et al., 2015, Figueras et al., 2014). While Arcobacter have been isolated from coastal marine environments and organisms (Fera et al., 2004, Mottola et al., 2016), relative to other enteric bacterial pathogens (Foote et al., 2017, Miller et al., 2006), little is currently known about the ecology of Arcobacter, including their distribution, persistence and diversity in the natural environment.

In Chapter 2, qPCR was used to track the weekly absolute abundance of Arcobacter at the highly impacted Foreshore beach. Patterns in Arcobacter abundance measured using our qPCR assay mirrored relative abundance patterns seen in 16S rRNA profiles, whereby Arcobacter increased in abundance with inputs of stormwater and WWSO. Given that several studies have identified Arcobacter as common members of human and animal gut microbiomes (Van den Abeele et al., 2014), which are selected for in wastewater treatment plant environments (Fisher et al., 2014), it is most probable that the peaks in Arcobacter are due to advection from the stormwater and sewer system, rather than blooming of species inhabiting the coastal receiving waters. Our data

revealed that Arcobacter remained present at Foreshore Beach between stormwater events, but at low abundances, hinting that different species within the Arcobacter population could be exploiting different environmental niches within the urban and coastal marine ecosystems.

Oligotyping, a computational method for resolving the diversity of closely related but distinct bacterial organisms from within OTU (Eren et al., 2013), revealed a high level of diversity within Arcobacter populations, that could be grouped into ten ecotypes based on temporal co-occurrence. We found that some ecotypes were present exclusively during dry periods, suggesting that strains within these ecotypes are possibly environmental strains. Additionally, ecotypes matched to two Arcobacter species, *A. venerupis* and *A. cryaerophilus*, strongly correlated with input of stormwater and WWSO. *A. venerupis* has been isolated from shellfish (Levican et al., 2012), but has not previously been associated directly with stormwater, sewage or human illness. On the other hand, multiple virulence genes have been detected in *A. cryaerophilus* isolates (Douidah et al., 2012), and several strains within this species have been associated with bacteraemia and gastroenteritis in humans (Vandenberg et al., 2004), with this species consequently considered an important emerging pathogen (Ramees et al., 2017). Our findings indicated that *A. cryaerophilus* was most prevalent in Summer, when beaches are most frequently visited by water users (Moreno et al., 2008), and often at times when enterococci counts would not suggest that water quality is unsafe, suggesting that *A. cryaerophilus* could sometimes pose a serious exposure risk to swimmers at some Sydney beaches.

6.3 The dissemination of antibiotic resistance genes in coastal habitat

Indicator organisms that denote the presence of enteric pathogens are intensely monitored in coastal waters where humans swim (Boehm and Sassoubre, 2014). However, relatively less attention has been given to the, sometimes problematic, genetic features that these enteric microbes transport into marine receiving waters, or how these might then be further disseminated through natural environments. Of particular concern, the rise of antibiotic resistance (AbR) has been described as a global health emergency (O'Neill, 2014). While the bulk of research in this area has been focused on clinical and agricultural settings (Wright, 2010), emerging evidence suggests that the human microbiome can acquire antibiotic resistance from environmental reservoirs, including beaches (Leonard et al., 2015, Leonard et al., 2018). Coastal environments are indeed both a key interface for human exposure to environmental microbiological elements and a potential hotspot for AbR as a consequence of influences from human waste streams, including storm-water and sewage systems that select for AbR (Czekalski et al., 2012, Gaze et al., 2011, Rizzo et al., 2013).

The few detailed examinations of AbR in natural aquatic settings have largely involved *ad hoc* sampling regimes, a reliance upon culture-dependent methods, which can miss up to 99% of natural microbial assemblages, and a focus on a very narrow range of AbR genes (Deshpande et al., 2006, Ingti et al., 2017, Lee et al., 2011). As a consequence, there is still a significant paucity in knowledge regarding the true extent of anthropogenic augmentation of the resistome of coastal habitats and the underlying

drivers and dynamics of AbR inputs. In Chapter 3 of this thesis, my high temporal resolution sampling design was utilised to track the abundance of the Class 1 *integron/integrase* gene, which has been proposed to be a very good molecular marker for anthropogenic pollution in terrestrial, aquatic and marine environment (Gillings et al., 2015) and an important vector for horizontal transfer of virulence and AbR genes (Gaze et al., 2011). Additionally, 31 AbR genes that confer resistance to antibiotics included in the *World Health Organisation's list of critically important antibiotics (WHO, 2017)* were tracked, including genes that encode for production of carbapenemase enzymes, that are important for de-activation and resistance to carbapenems (Poirel and Nordmann, 2006), of which several are important last resort antibiotics. With the aid of 16S rRNA amplicon sequencing and network analysis, patterns in the abundance of AbR gene could be related to specific members within the microbial communities of the coastal marine environment, with the intention of identifying the most likely bacteria to carry these genes.

Throughout the coastal time-series study, I identified up to 100-fold increases in the abundance of the *intI1* gene and several AbR genes. These spikes in abundance were statistically correlated with the input of stormwater and WWSO, confirming these rises in AbR genes above background levels were due to the advection of AbR genes from the stormwater and sewer environment. Using network analysis, I next disentangled the complex interactions between meteorologically driven perturbation events, urban water quality management and the dynamics of AbR within coastal habitats. Specifically, I observed strong statistical links between the occurrence of AbR genes and the bacterial

members that are most likely carrying these genes, including bacteria closely related to potentially pathogenic *Acinetobacter, Arcobacter, Aeromonas* and *Cloacibacterium*. This work has disentangled the complex relationships between stormwater intrusion, sewer overflows, and the occurrence of genes that confer resistance to antibiotics that are both of regional importance, and critically important on the world-scale. Here, I have provided strong evidence for the role of storm-water and WWSO as a means for potentially pathogenic bacteria carrying AbR genes to cross habitat boundaries and augment the resistomes of coastal marine habitats.

6.4 Influence of marine heatwaves on endemic marine pathogens

While Chapters 2 and 3 of this project focussed on the role of rainfall and episodic stormwater events in disseminating enteric pathogens and genes derived from human wastes streams, Chapter 4 has examined patterns in the abundance of indigenous marine bacteria, some of which can also be very harmful, both to humans and our fisheries (Paerl and Paul, 2012, Austin, 2010). Among these are several members of the genus *Vibrio*, which are responsible for a range of infections in marine animals and humans (Goarant et al., 2000, Jones and Oliver, 2009, Linkous and Oliver, 1999). Human health costs directly associated with Vibrio exposure exceed $30M annually in the US (Ralston et al., 2011), where skin infections by *V. vulnificus* kill more bathers and fisherman each year, than shark-attacks world-wide (Forres, 2008). Within Australia, a number of serious (including fatal) cases of *V. vulnificus*, *V. cholerae* and *V.*

parahaemolyticus infections have been reported in recent years (Ralph and Currie, 2007).

In the northern hemisphere, there is an emerging concern that increasing sea water temperatures linked to climate change have led to shifts in the distribution of Vibrios in the environment and increases in the numbers of human infections (Baker-Austin et al., 2013). This pattern may also be particularly pertinent to the east coast of Australia, which has been classified a “climate change hotspot”, because seawater temperatures in this region are rising more rapidly than in most other parts of the ocean (Ridgway and Hill, 2009). Furthermore, in the past 7 years this region has experienced extreme warming anomalies, or “marine heatwaves”, that have caused coral bleaching, loss of seagrasses, and mass mortality of marine mammals (Moore et al., 2012, Pearce and Feng, 2013). Emerging evidence suggests that temperature elicits a strong influence on the distribution of pathogenic Vibrio species (Baker-Austin et al., 2013, Vezzulli et al., 2016b). In Chapter 4 of this thesis, I hypothesised that elevated sea surface temperatures (SST), and extreme marine heatwaves (MHV) events, occurring along the Sydney coast, would lead to blooms in pathogenic species of Vibrios, including *V. cholerae, V. parahaemolyticus*, and *V. vulnificus*. Accordingly, Vibrio abundances were tracked weekly over two years at Maroubra and Foreshore beaches, which allowed for correlations between the abundance of potentially pathogenic Vibrios to be statistically tested against physico-chemical parameters, and the occurrence of MHW events. This approach revealed that MHWs led to blooms in pathogenic Vibrios, with the occurrence

of MHW shown to exceed the influence of seasonal and gradual rises in SST, providing novel evidence for an additional negative impact of MHW events in coastal regions.

6.5 Conclusion

By tracking patterns in whole bacterial communities, and distinct pathogenic populations and genes of public health significance, at high temporal resolution from two beach environments that experience contrasting hydrology and anthropogenic impact, this project advances our understanding of important ecological processes that occur in coastal ecosystems. Through 16S rRNA sequencing, my analyses identified key local environmental features such as temperature, and allochthonous inputs of stormwater and sewage, as processes that drive seasonal switching between genera and closely related OTUs, and large incursions of enteric microbes, some of which persist beyond the range of faecal indicator bacteria. These processes have been previously overlooked in coastal waters due the focus on only a narrow selection of microbes, or due to insufficient temporal resolution to capture events that are brief, but have profound impact on microbial dynamics. These ephemeral events clearly have profound impact on the assemblage structure of bacterial communities, which may also have implications that influence the net function of microbial communities. Additionally, this project identified several AbR genes, which were correlated strongly with potentially pathogenic enteric bacteria, that could pose an exposure risk to water users in beaches. Given the recent evidence that AbR genes in natural environments can be assimilated into the microbiomes of surfers, this project's findings highlight the need for further investigation of the occurrence and drivers of AbR dissemination in coastal beaches. Finally, coastal habitats are amongst the most vulnerable ecosystems to climate change,

and associated increasing sea surface temperatures and frequency in the occurrence of MHW events. This project closely tracked populations of pathogenic Vibrios, and is the first study to provide evidence that directly links blooms in pathogenic Vibrio in coastal surface waters with MHW events. Given that MHW events are on the rise, and considering the significance of Vibrio as environmental pathogens, these findings make a strong case for further investigation of Vibrio dynamics in response to the manifestations of climate change that occur in the coastal marine environment.

Coastal ecosystems are experiencing unprecedented pressure from the combined force of urbanisation and climate change, which put at risk the continued supply of important ecosystem products and services. To conclude, this project has identified key ecological processes that are driven by these stressors, and described the way in which they influence the temporal dynamics of microbes within coastal ecosystems. As microbes underpin productivity and biodiversity in their coastal habitat, the findings of this project provide new and transformative insight into the microbial ecology of coastal systems, which has highlighted several potential implications for ecological and public health.

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