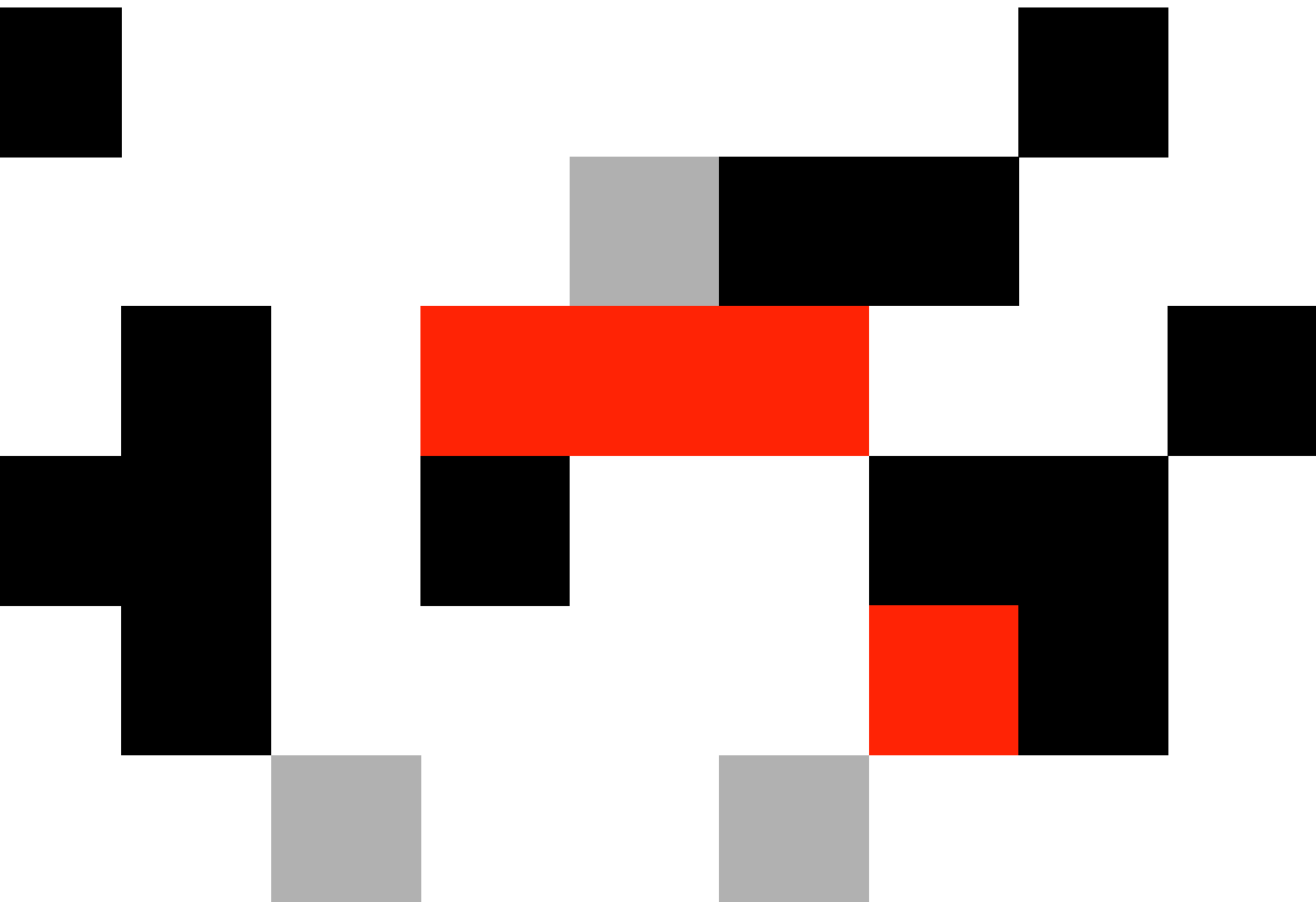

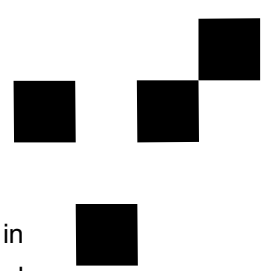
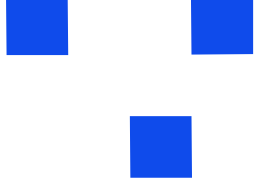




Climate Change Cluster, Faculty of Science
University of Technology Sydney

Microbial source-tracking in NSW coastal habitats FINAL REPORT





This Research Report has been prepared by the University of Technology Sydney (“UTS”) in good faith as part of a collaboration with the NSW Department of Planning, Industry and Environment (DPIE). Central Coast Council were also a collaborating partner in this research and provided Enterococci level data and assisted with planning and performing sampling at Terrigal.

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EXECUTIVE SUMMARY

The research presented in this report was commissioned by the NSW Department of Planning, Industry and Environment, and performed by the Ocean Microbiology Group at the University of Technology Sydney (UTS). The principal goal of the research was to develop and apply new molecular microbiological approaches to assist efforts in defining the causes of poor water quality at two beaches in NSW - Terrigal Beach and Rose Bay. Samples from stormwater drains and seawater samples at each site were analysed using a suite of assays targeting microbial indicators of human, bird and dog faecal material as well as microbial markers for anthropogenic impact and urban waste-water infrastructure.

Under dry weather conditions, seawater samples collected from within Terrigal Haven and the southern end of Terrigal Beach generally exhibited low levels of Enterococci bacteria, which is the standard measure for water quality used by monitoring authorities. However, Enterococci levels in the outlets of stormwater drains discharging water into Terrigal Beach were often very high. These drain samples also consistently had elevated levels of three microbial markers indicative of human faecal material (sewage), with these markers sometimes also observed in seawater samples collected from Terrigal Beach. A microbial indicator for dog faeces was below detection limit in all samples, implying a negligible impact from this potential source of contamination. The microbial indicator for bird faeces generally occurred in levels consistent with those observed in a pristine control site, but was elevated in two samples collected from one of the stormwater drains. These results are indicative of a regular presence of human wastewater and faecal material in the stormwater outlet system at Terrigal Beach, with one drain at the southern end of Terrigal Beach consistently exhibiting high levels of contamination from these sources. Notably, there is evidence that even under dry weather conditions, microbial signatures of human impact were in some instances dispersed into the seawater at this site.

During a moderate (40 mm) rainfall event during June 2019, Enterococci levels within all drain discharge points and seawater samples within Terrigal Haven increased to very high levels, significantly exceeding the threshold for human health risk. Relative to dry weather conditions, the three microbial markers for sewage also occurred in substantially elevated levels within the stormwater drain discharge points and in seawater samples. Highest levels were observed in a drain in the southern-most corner of Terrigal Beach, with seawater samples collected adjacent to its location also exhibiting high levels of the human faeces markers. Levels of the human faecal markers also increased in seawater adjacent to the mouth of Terrigal Lagoon after the lagoon entrance was opened during the rainfall event, indicating that Terrigal Lagoon may be a further source of contamination under some conditions. Cumulatively, the outcomes of the dry and wet weather studies at Terrigal are indicative of the input of human sources of faecal contamination with discharge from stormwater drains in the southern end of Terrigal Beach potentially having the greatest impact.

During dry weather conditions in Rose Bay, Enterococci levels were generally within the lower range microbial risk categories within seawater samples, but were often very high within stormwater drain discharge points. Microbial markers for human faeces were

EXECUTIVE SUMMARY (Continued)

detected in 97% of samples within Rose Bay and occurred in significantly higher concentrations than the control site in 95% of cases. Highest levels of the human faecal markers were observed in the drain samples, but significant levels were also intermittently observed in seawater samples, particularly following a moderate rain event. The microbial marker for bird faeces was detected in all samples, but did not differ significantly to levels observed in the pristine control site or shift measurably between sampling sites, suggesting it occurred in natural base-line levels across Rose Bay. The marker for dog faeces was detected within 17% of samples, with highest levels observed in stormwater drain discharge points, potentially pointing to an external (i.e. off-beach) input of this signal from the catchment.

The cumulative outcomes of this research provide insights into the causes and sources of poor water quality Terrigal and Rose Bay. Within Terrigal, specific stormwater discharge drains are a source of human faecal bacteria that are likely to be present in sewage, with the impact of this input greatly amplified under rainfall conditions. With the exception of high levels of bird faeces-associated bacteria in one drain on two dry weather sampling occasions, the impact of animal-associated faecal bacteria appears to negligible at this site. Within Rose Bay, there was also a clear signature for human wastewater infrastructure and sewage within a very high proportion of samples, in particular those associated with stormwater drains.

1.0 BACKGROUND

Coastal ecosystems have considerable economic and intrinsic value to NSW. According to a 2016 report, it was estimated that the value of beach use by Sydney residents exceeds \$1.2 billion per year¹, while the Marine Estate Management Authority highlighted improving water quality at beaches as one of three key priority areas identified during an extensive community engagement program². However, like many urbanised coastal environments³, a number of beaches and estuarine ecosystems in NSW are regularly characterised by poor water quality⁴, which in some cases could have profound implications for both ecosystem and human health⁵.

Within NSW, water quality at coastal beaches is impacted by a range of factors, including stormwater inputs and sewage overflow events. Water quality is monitored locally by several city councils, while a state-wide monitoring program conducted by Beachwatch, rates swimming beaches according to safety for recreational use⁴. These monitoring programs generally use the global standard for water quality assessment, which involves enumeration of the faecal indicator bacteria *Enterococci*. This approach is employed as a proxy measure for sewage pollution in natural aquatic environments and is implemented according to standardised international guidelines⁶. *Enterococci* enumeration is also relatively inexpensive and simple to perform, yet has two significant short-comings. Firstly, it cannot precisely discriminate the origin of the enterococci target bacteria between human (i.e. sewage) and animal sources, sometimes leading to ambiguity about the true cause of elevated enterococci counts within an environment⁷. Secondly, it is insensitive to a range of other microbial hazards, including endemic aquatic pathogens⁸, emerging pathogens associated with human waste water⁹ and potentially harmful microbial genetic features, including antibiotic resistance¹⁰. As a consequence, there has been increasing demand from environmental scientists and managers for more sophisticated molecular biological approaches for assessing water quality and the microbiological state of natural aquatic ecosystems¹¹.

Among the beaches assessed by the Beachwatch program, a handful of sites consistently receive poor results⁴, when *Enterococci* levels exceed *Microbial Assessment Category* thresholds (Table 1)¹². Among these locations, consistently poor results at Terrigal Beach and Rose Bay have led to particular concern given the substantial use of these beaches for recreation, with water quality becoming a significant local issue for residents^{13,14}. However, the factors influencing water quality at both Terrigal and Rose Bay are not straightforward. Both sites receive inflows from complex stormwater and wastewater infrastructure, with multiple stormwater outlet pipes, each with differing (and often unknown) levels of contamination from wet weather sewage overflows, at each location. Furthermore, at both beaches the potential influence of animal (i.e. dog and seabird) faecal

material is equivocal, making it very difficult to precisely discriminate the cause of high enterococci counts during Beachwatch monitoring. This in turn restricts capacity to design and implement management strategies to resolve water quality issues at these sites.

The principal goal of this project was to aid the NSW Department of Planning Industry and Environment's (DPIE) efforts to improve water quality at coastal beaches in NSW, by delivering an enhanced understanding of the causes of poor water quality ratings (i.e. high Enterococci counts) at Terrigal and Rose Bay beaches. We aimed to achieve this through the development and application of a new set of molecular microbiological tools that will provide greatly enhanced precision in defining the sources of coastal contamination and identifying microbial hazards in impacted environments. Specifically, our goals were to define the origin (e.g. human sewage or animal faeces), source (i.e. input point [pipe, lagoon etc]) and unforeseen implications (i.e. microbial hazards not detected by standard monitoring approaches) of water contamination at Terrigal and Rose Bay.

2.0 OBJECTIVES

The over-all objective for the planned work is to develop and apply new approaches for defining the probable causes and sources of high Enterococci levels in coastal waters at Rose Bay and Terrigal Beach. The specific objectives of the project are:

- Establish a suite of new molecular biological assays for determining the sources of faecal contamination in coastal environments
- Perform dry and wet-weather sampling programs at Terrigal Beach and Rose Bay to link high Enterococci levels to microbial markers for human or animal faecal material to determine likely origin of contamination
- Develop and implement a sampling strategy to define the likely sources (e.g. stormwater pipes, lagoons etc) of high Enterococci counts at Terrigal and Rose Bay

3.0 METHODOLOGY

This project was structured around the characterisation of water quality issues and microbial threats at Terrigal Beach and Rose Bay. Dry weather water sampling was conducted at both beaches over five weeks, during the period of 28/3/19 – 2/5/19 and a wet weather sampling program was conducted at Terrigal Beach in early June 2019. In each of these three studies, triplicate samples were collected for analysis of a range of physicochemical and microbiological parameters from multiple points at each beach. The sampling design was developed in close consultation with DPIE, with the goal of identifying the most likely sources of contamination at each beach.

3.1 Terrigal Beach Dry Weather Sampling

Sampling at Terrigal Beach was focussed on the southern end of the beach within the region known as 'the Haven'. This region is where the bulk of recreational activities take place and near to where Beachwatch sampling is routinely performed between the swimming flags. This part of Terrigal beach is believed to be impacted by inputs from a network of stormwater infrastructure that drains directly onto the beach. Samples were collected from 10 locations chosen according to proximity to potential points of contamination (Figure 1). These included three stormwater drains (D1, D2 and D4 in Figure 1) that have previously been suspected by Central Coast Council to be sources of faecal contamination, and from 50 cm depth seawater at points immediately adjacent to these drains. Drain 1 is located in the south-eastern corner of Terrigal haven, and is believed to collect water from the Broken Head dog park. A visual survey of this park immediately prior to sampling, indicated the presence of dog, rabbit, seagull and pelican faeces on the grass slope adjacent to the beach. Drain 2 collects water from the region surrounding Terrigal Haven playing field, while drain 4 represents the output from a junction of drains that are exposed to run-off from Terrigal's urban center and potential wet weather overflow points. In addition to these 6 drain and adjacent seawater sampling points, samples were also taken from within Terrigal Lagoon and from 50cm depth seawater directly adjacent to the mouth of the lagoon, which was closed at the time of dry weather sampling. Finally, samples were also collected from two reference sites, including the point where routine Beachwatch sampling is conducted in 50cm depth water immediately in front of the Terrigal Surf Club and from a relatively un-impacted 'Control' site at Forresters Beach. This Control site is situated on the same stretch of beach as the Terrigal sampling points, but is approximately 9 Km north of the haven and is surrounded by a relatively un-developed area of bushland with little exposure to urban runoff. Samples from this point are anticipated to represent baseline levels of microbial contaminants sourced from urban infrastructure.

3.2 Terrigal Beach Wet Weather Event Sampling

Water quality within many coastal habitats is often diminished following rainfall as a consequence of allochthonous inputs of urban stormwater and sewage overflow events. To examine the influence of rainfall events on water quality within Terrigal Haven, a wet weather event sampling regime was conducted in June 2019 when a substantial rainfall event, resulting in 40 mm of rain over the course of 3 days, occurred. Samples were collected from the 10 locations described above (3.1) on 6 occasions corresponding to 2 weeks (20/5/19) and 5 days (31/5/19) prior to the rainfall event, the day of 20 mm rainfall (4/6/19), two days later when a further 20 mm of rain occurred (6/6/19), a second sample on 6/6/19 after the entrance to Terrigal Lagoon was opened to the ocean, and 1 week following the rain event (11/6/19). Samples were processed and analysed in the same manner as the dry weather sampling regime (3.1).

3.3 Rose Bay Sampling

Sampling at Rose Bay was conducted using a similar strategy to the Terrigal sampling, whereby samples were obtained from the mouth of stormwater drains and in immediately adjacent seawater. Samples were collected from the outlets of three

drains (D4, D5, D6 in Figure 2), which were chosen due to their regularly high flow rates (even under dry weather conditions) and evidence that they may contribute to the periodically high Enterococci counts recorded at this beach by Beachwatch⁶. These drain outlets are believed to mainly be conduits for urban storm-water, but in some instances may be impacted from wet weather sewer overflows. Samples were also collected from reference points, including the Beachwatch sampling site located at the western end of Rose Bay and from a relatively pristine 'Control' site. The Control site within Nielsen Park is part of the Sydney Harbour National Park and void of any urban stormwater infrastructure.

Figure 1



Figure 1. Map of Terrigal Haven. 'B1,2,4,7' represents bay seawater samples and 'D1,2,4' represents drain samples, while 'L' represents a sampling site inside Terrigal Lagoon. Samples were also collected from the Beachwatch sampling location (B) located between the swimming flags. The un-impacted Control site at Forrester's Beach is situated approximately 8 km north of this image.

Figure 2



Figure 2. Map of Rosebay sampling points. D4-D6 represents drains 4-6 and B4-6 represents bay samples 4-6. "B" represents the Beachwatch sampling site.

3.4 Sample Processing and Analyses

At each sampling site, triplicate 2 L water samples were collected using individual plastic containers. Within 2 hours, these samples were filtered through 0.22 µm pore-size membrane filters (Merk-Millipore) using a peristaltic pump (100 rpm). Filters were transported to the laboratory on ice and stored at -20 °C for DNA extraction, which was performed within two weeks of collection.

A suite of environmental parameters were collected along-side all microbiological samples. Physico-chemical parameters including temperature, pH, salinity and dissolved oxygen were measured *in situ* using a ProDSS Multiparameter Water Quality Meter. For nutrient analysis (Ammonia, Nitrate, Nitrite and Phosphate), 60 ml of seawater was collected, with 40 ml filtered through a 0.45 µm Minisart® syringe filter and 20ml kept unfiltered. Nutrient samples were transported on ice to the laboratory where they were stored at -20°C until analysed.

3.5 Microbiological Analysis

Enterococci levels were derived using standard membrane filtration techniques at a commercial diagnostic laboratory following the Australian standard (AS/NZS 4276.9:2007). The NHMRC Microbial Assessment Categories were used to relate Enterococci levels to degree of potential human health risk (Table 1).

Table 1: Microbial Assessment Categories (NHMRC 2008)¹²

Category	95 th percentile of enterococci (cfu/100 mL)	Basis of derivation	Estimation of probability
A	< 40	No illness seen in most epidemiological studies	GI illness risk < 1% AFRI risk < 0.3%
B	41- 200	Upper limit is above the threshold of illness transmission reported in most studies	GI illness risk < 1-5% AFRI risk < 0.3 – 1.9%
C	201- 500	Represents a substantial elevation in the probability of adverse health outcomes	GI illness risk >5 – 10% AFRI risk < 1.9-3.9%
D	> 500	Above this level there may be a significant risk of high levels of illness transmission	GI illness risk > 10% AFRI risk > 3.9%

GI = gastrointestinal

AFRI = acute febrile respiratory illness

For molecular microbiological analysis, DNA was extracted from filters using a bead beating and chemical lysis kit (DNeasy PowerWater Kit, QIAGEN). Quantitative PCR (qPCR) was then used as the principal analytical technique. This molecular biological approach delivers precise quantification of a specific target DNA sequence that can be selected as a marker for microbial phylogenetic identity or a functional gene (e.g.

toxin or antibiotic resistance genes). We assembled a set of qPCR primers designed to target several bacterial groups that provide: (i) unambiguous discrimination of potential human and animal sources of faecal material; (ii) indicators for anthropogenic contamination of natural aquatic ecosystems; (iii) markers for emerging pathogens and other microbial hazards. The selected targets for qPCR analysis are presented in Table 2.

Table 2: Quantitative PCR assays used in this study

Target Organism or Gene	qPCR Primers Used	Rationale	Ref
<i>Bacteroides</i> 16S rRNA (human)	HF183	A major component of the human gut microbiome and an excellent discriminator of human faecal material. Indicative of human sewage, allowing discrimination from animal faecal material signals.	15
<i>Lachnospiraceae</i> 16S rRNA	Lachno3	A major component of the human gut microbiome and a highly specific marker for human faecal contamination. Indicative of human sewage, allowing discrimination from animal faecal material signals.	16
<i>Lachnospiraceae</i> 16S rRNA	Lachno12	A major component of the human gut microbiome and a highly specific marker for human faecal contamination. Indicative of human sewage, allowing discrimination from animal faecal material signals.	16
Integron-integrase gene (IntI1)	intI1	Bacterial gene shown to be an excellent proxy for anthropogenic pollution, due to its links to antibiotic and heavy metal resistance genes. Indicative of human contamination.	17
<i>Arcobacter</i> 23S rRNA	ARCO1	Bacterial genus containing emerging enteric pathogens and species believed to inhabit waste-water infrastructure (i.e. the pipe environment). Indicative of input from stormwater/sewage pipes.	18
<i>Bacteroides</i> (Dog)	DG3	A dog faeces specific marker targeting <i>Bacteroides</i> bacteria dominating the dog faecal microbiome	19
Enterococci (Bird)	GFD	A 100% avian specific bacterial marker, which targets bird-specific Enterococci and is present in the faeces of gulls, geese, chickens, and ducks.	20

4.0 Results

4.1 Terrigal Beach Dry Weather Conditions

4.1.1 SUMMARY OF RESULTS

A detailed description of the results for this section of the project are provided below, but a brief synopsis of the major findings is presented here:

- **Enterococci levels** during the dry weather sampling period at Terrigal Haven were generally low (mean < 27 CFU 100 mL⁻¹) within seawater samples, including the Beachwatch sampling site, indicating a generally low level of health risk according to the NHMRC Microbial Assessment guidelines. However, Enterococci levels were consistently very high within the Drain samples (mean across Drain 2 and 4: 5,095 CFU 100mL⁻¹), where they regularly exceeded the NHMRC maximum threshold for significant risk of illness. Elevated Enterococci levels were also observed in some lagoon samples and seawater samples (Bay 4) following a moderate rainfall event.
- **Levels of the human sewage markers** used here (HF183, Lachno3, Lachno12), varied significantly between sampling sites. In all but 36% of seawater (Bay, Beachwatch, Control) samples, these markers were at low or undetectable levels. In contrast, significant levels were observed in 89% of Drain samples, with highest levels observed in Drain 4. Across the entire data-set, average levels of the HF183 and Lachno12 markers were significantly correlated with Enterococci levels, but no significant correlation was observed between the Lachno3 marker and Enterococci levels. These data are suggestive of a significant contribution of human faecal material to the Enterococci measurements during dry weather conditions within Terrigal Haven.
- **The marker for dog faeces (DG3)** was below detection limit in 100% of samples, indicating a negligible impact of dog faeces in the sampled environment during the dry weather period.
- **Bird associated enterococci** indicated by the GFD marker were detectable in all samples, but, with the exception of Drain 2, levels of this marker were not significantly higher than the pristine control site. These patterns indicate that natural base-line levels of bird faecal material occurred in all sites, except in Drain 2, where significantly higher levels of bird associated Enterococci were observed on two occasions (12th and 18th of April, coinciding with highly elevated Enterococci plate counts
- **The wastewater infrastructure-associated bacteria, *Arcobacter*, and the microbial marker for anthropogenic impact, *intl1***, were elevated in Drains 2 and 4, and the Bay 2 and 4 samples. The elevated presence (relative to the pristine control site) of these markers in the Bay samples is indicative of the presence of a human wastewater signature in Terrigal Haven, even under dry weather conditions.

4.1.2 Enterococci Levels

During dry weather conditions, average (across the 5 weeks of sampling) Enterococci levels within the sampled sites in Terrigal Haven differed significantly ($p < 0.05$). With the exception of one sample taken from Bay 4 and adjacent to Terrigal Lagoon, Enterococci levels within ocean waters in Terrigal Haven and Forresters Beach were generally low (mean < 27 CFU 100 mL^{-1}) and below the lowest health risk level in the NHMRC Microbial Assessment Categories¹² (Table 2). At the Beachwatch water quality monitoring site, Enterococci counts did not exceed $10 \text{ CFU } 100 \text{ mL}^{-1}$ during this dry weather sampling period. However, Enterococci levels in the drains were consistently very high. Highest enterococci levels were observed in Drain 2, where average concentrations of $10,093 \text{ CFU } 100 \text{ mL}^{-1}$ were observed, with a maximum level of $24,195 \text{ CFU } 100 \text{ mL}^{-1}$ reached. In Drain 4, Enterococci levels in all samples also exceeded the 500 CFU mL^{-1} threshold for significant risk of illness (Table 2), reaching a maximum of $2,351 \text{ CFU } 100 \text{ mL}^{-1}$. While generally low ($< 40 \text{ CFU } 100 \text{ mL}^{-1}$), on one occasion (4/4/19) Enterococci counts were very high and exceeding the NHMRC maximum threshold for significant risk of illness within Terrigal Lagoon ($12,033 \text{ CFU } 100 \text{ mL}^{-1}$) and within the seawater immediately adjacent to the lagoon ($1,917 \text{ CFU } 100 \text{ mL}^{-1}$). Notably, these high counts occurred after 24 mm of rain in the preceding two days.

Figure 3

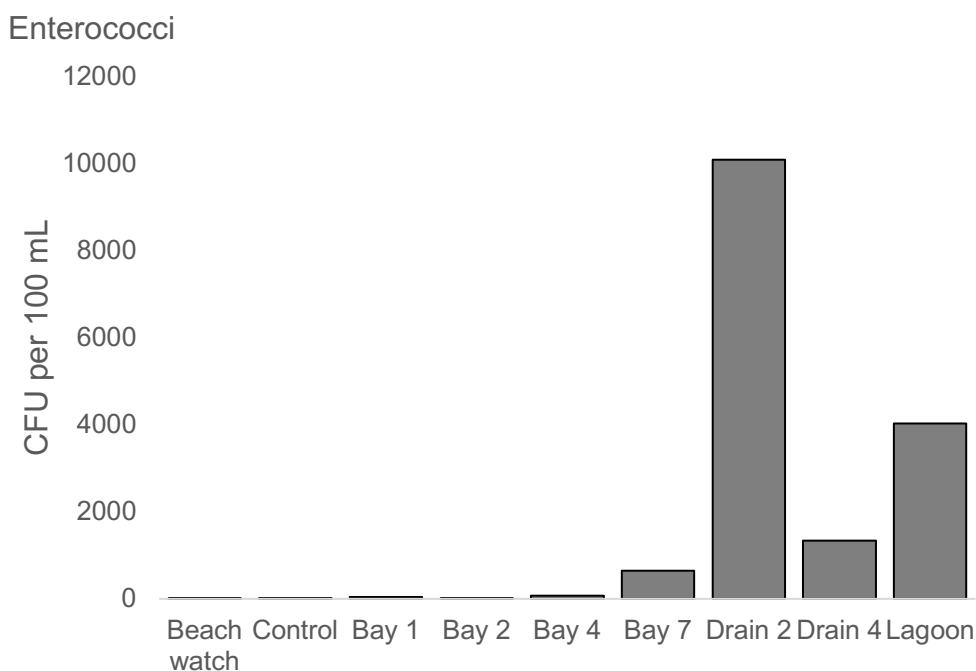


Figure 3: Average enterococci levels ($\text{CFU } 100 \text{ mL}^{-1}$) within the Terrigal Haven sampling sites over the five week dry weather sampling regime. Enterococci levels were derived using standard membrane filtration at a commercial diagnostic laboratory following the Australian standard (AS/NZS 4276.9:2007).

4.1.3 Human Faecal markers

During dry weather conditions, average concentrations of the three human faecal marker genes employed here, Lachno3, Lachno 12 and HF183, indicative of human gut microbiome associated *Lachnospiraceae* and *Bacteriodes*, differed significantly ($p < 0.001$; Kruskal-Wallis test) across the 9 sampling locations (Figure 4). Across the entire data-set, average abundances of the HF183 and Lachno 12 markers both displayed significant ($p < 0.01$; Spearman) correlations with Enterococci levels, but no significant correlation was observed between the Lachno3 marker and Enterococci levels.

Consistent with the very low levels of Enterococci observed at the 'pristine' control site at Forresters Beach, all three human faeces markers were below the detection limit within all samples collected at the control site. Similarly, levels of these human faecal markers were either below detection levels or very low in 64% of samples collected from the seawater sites (i.e. Bay 1, 2, 4, 7 and the Beachwatch sampling site), indicating a generally low level of contamination by human sewage at these sites during most periods of this dry weather period. However, when elevated levels of Enterococci were recorded in these seawater samples, significant levels of the three human faeces markers were also observed. Specifically, when Enterococci levels reached 250 CFU 100 mL⁻¹ in the Bay 4 sample on April 12, all three human faeces markers were detected in significant levels.

In contrast to the seawater sites, the drain and some lagoon samples consistently exhibited significant ($P < 0.05$) levels of the human faeces markers, which generally coincided with elevated Enterococci levels. Within Drain 4, where mean Enterococci levels during the 5 weeks of dry weather sampling were 1,341 CFU 100 mL⁻¹, significant concentrations of the human faeces markers were observed in 93% of samples. Across all 3 human faecal markers, Drain 4 exhibited the highest levels observed in any sample sites. Within Drain 2, where mean Enterococci levels were 10,093 CFU 100 mL⁻¹, elevated concentrations of the human faeces markers were observed in 75% of samples. We interpret these patterns as indicative of human sewage contamination in these stormwater pipe discharge point (Drain) samples, even during dry weather periods.

There were, however, some instances where the human faeces markers did not correspond with Enterococci levels. These included the Terrigal Lagoon sample on the 4th of April, when Enterococci levels exceeded 10,000 CFU 100 mL⁻¹ after a moderate rain event, but none of the 3 human markers were detected. Given that none of the animal faecal markers tested here were elevated in this sample either (4.1.4), the reason for this discrepancy is unclear, and currently being further investigated. It is notable, however, that the levels of all 3 human markers were elevated within the lagoon sample in the two weeks following this date. Another notable discrepancy between the Enterococci levels and human faecal markers occurred within the Bay 1 and 2 samples on the 2nd of May, when levels of both of the *Lachnospiraceae* markers (Lachno3 and Lachno12) were elevated, despite Enterococci levels being relatively low.

Figure 4

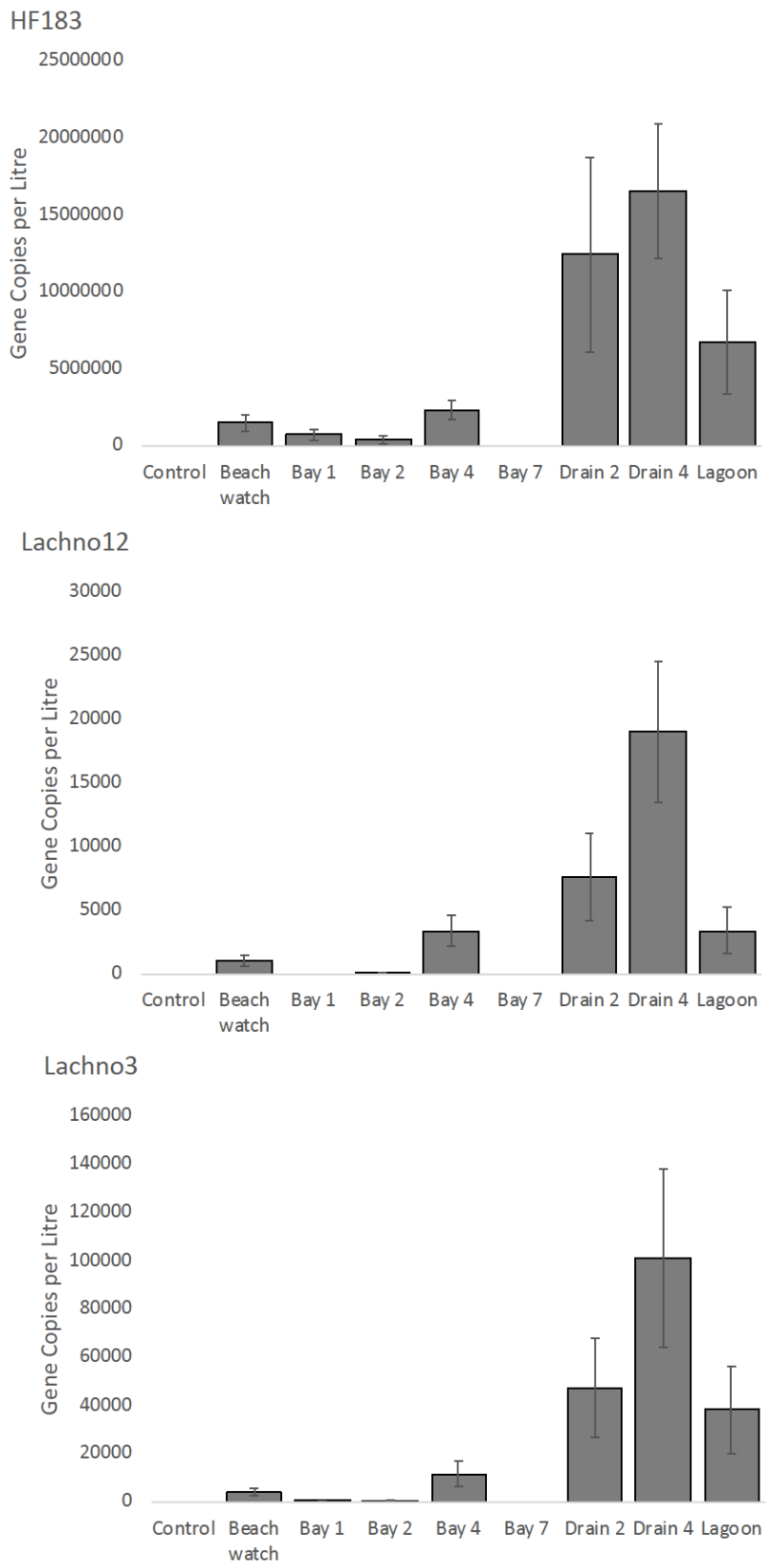


Figure 4: Quantitative PCR analyses of the human faecal bacterial markers HF183 (*Bacteroides*), Lachno3 and Lachno12 (*Lachnospiraceae*), which are indicative of sewage contamination, in the Terrigal Haven sampling locations. Data derived from triplicate samples. Error bars represent Standard Error (SE).

4.1.4 Animal Faecal Markers

Levels of the DG3 marker for dog faeces-associated *Bacteroides* were below the detection limit in all samples collected from Terrigal Haven during the five weeks of dry weather sampling, indicating a negligible influence of dog faeces during this study period. On the other-hand, bird associated *Enterococci* indicated by the GFD marker were detectable in all samples. However, gene copies for this bird faeces marker were not significantly higher ($p > 0.05$ Kruskal-Wallis test) than those observed at the control site in all but one of the sampling locations. Our interpretation of these results is that natural base-line levels of bird faecal material occurred in all sites, except the Drain 2 site, where significantly higher gene copy numbers ($p < 0.05$) were observed on two occasions (12th and 18th of April). Notably, these high gene copy counts corresponded with very high *Enterococci* counts observed in this drain on April 12, potentially suggesting a contribution of bird faecal material to the high *Enterococci* counts observed in this sample (NB: abundances of the two *Lachnospiraceae* human faecal markers [*Lachno3* and *Lachno12*] were also elevated within this sample). It is unclear however, if these high levels of the bird faeces indicator were sourced from the catchment serviced by this stormwater drain or the mouth of the drain itself, as this region of Terrigal Haven often has very high levels of seabird (seagull and pelican) activity.

Figure 5

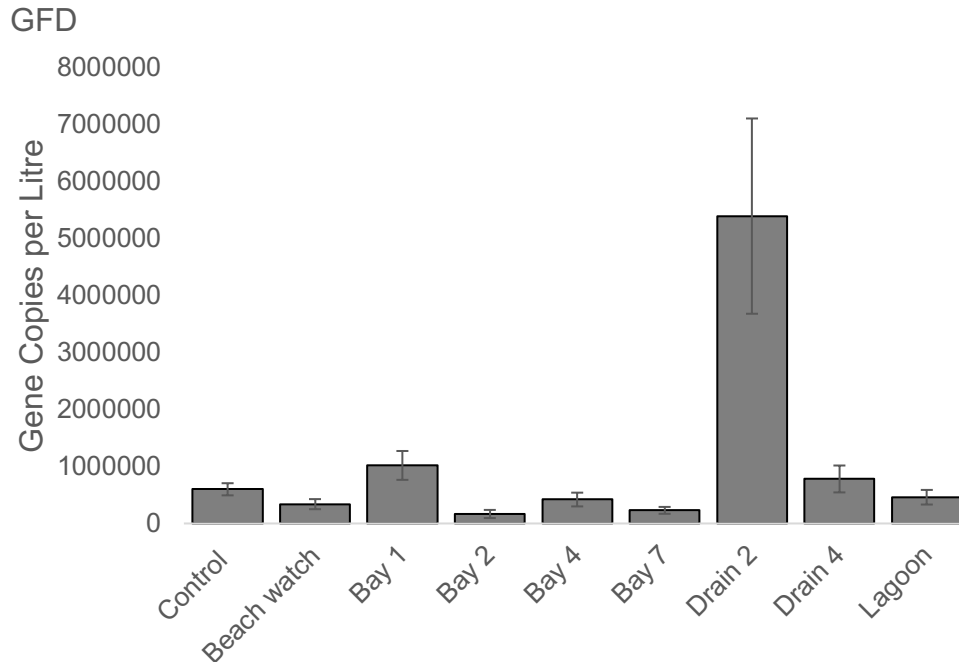


Figure 5: Quantitative PCR analyses of the avian *Enterococci* marker GFD, which is indicative of bird faecal material, in the Terrigal Haven sampling locations. Data derived from triplicate samples. Error bars represent Standard Error (SE).

4.1.5 Other Indicators of Anthropogenic Impact

Arcobacter are a genus of bacteria that include species associated with human sewage and are thought to inhabit urban storm-water and sewage pipe infrastructure²¹, and their occurrence in coastal waters is suggestive of inputs from anthropogenic water infrastructure²². Relative to the control site at Forresters Beach, significantly ($p < 0.05$) higher mean abundances of *Arcobacter* 23S rRNA genes were observed in Drains 2 and 4 and Bays 2 and 4 (Figure 6). Not surprisingly, given the expected pipe-habitat of *Arcobacter*, highest abundances of these organisms were generally observed in Drain samples, but the elevated abundances of *Arcobacter* in the Bay 2 and 4 samples is indicative of a signature from the stormwater infrastructure within these seawater samples. There was not a significant correlation ($p > 0.05$) observed between *Arcobacter* and Enterococci levels during this dry weather period.

The Class 1 Integron-integrase gene (*Int11*) has been identified as an excellent microbial measure of anthropogenic contamination in aquatic habitats²³. This gene was observed in 98% of samples collected during the dry weather sampling, but similarly to the patterns in *Arcobacter* abundance was significantly ($p < 0.01$) elevated within Drain 2 and 4 and Bay 2 and 4 (Figure 7). Also consistent with *Arcobacter*, there was no significant correlation ($p > 0.05$) between the *Int11* marker and Enterococci levels during this dry weather period.

Figure 6

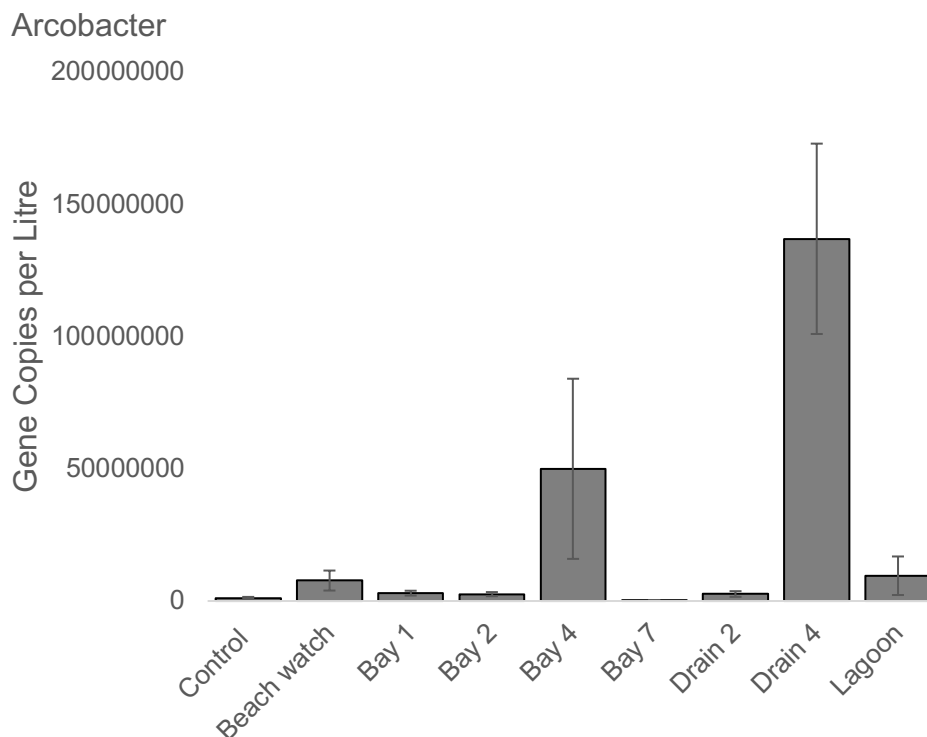


Figure 6: qPCR analyses of the *Arcobacter* 23S rRNA gene in the Terrigal Haven sampling locations. Data derived from triplicate samples. Error bars represent Standard Error (SE).

Taken together, the results of the *Arcobacter* and Integron-integrase gene quantification are suggestive of a significant signature of human wastewater infrastructure in Terrigal Haven particularly within the Bay 2 and Bay 4 samples.

Figure 7

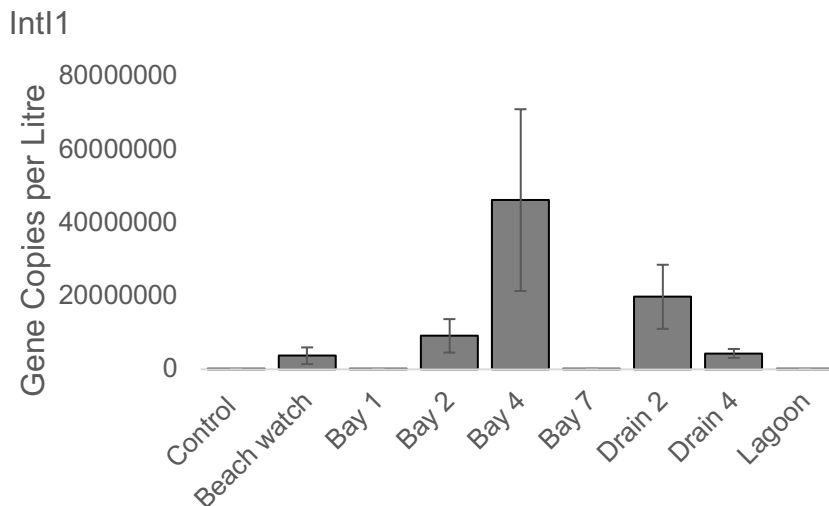


Figure 7: qPCR analyses of the Class 1 Integron Integrase gene *Int1* in the Terrigal Haven sampling locations. Data derived from triplicate samples. Error bars represent Standard Error (SE).

4.2 Terrigal Wet Weather Sampling

4.2.1 SUMMARY OF RESULTS

A detailed description of the results for this section of the project are provided below, but a brief synopsis of the major findings is presented here:

- **Enterococci levels** were low (~ 10 CFU 100 mL⁻¹) in all sampling sites prior to the rainfall event, but increased significantly in all sites following 20 mm of rain. During the rain event, highest Enterococci levels were observed in Drains 1, 2, and 4, where they exceeded 10,000 CFU 100 mL⁻¹, which is indicative of a very high risk to human health. Very high Enterococci levels (often $> 1,000$ CFU 100 mL⁻¹) were also observed in several seawater (e.g. Bay, Beachwatch site) samples on the day of the rain event, with highest levels reached in the water samples adjacent to Drain 4, but these rapidly decreased to pre-rainfall levels by the following day.

- **Levels of the microbial indicators of human sewage** used here (HF183, Lachno3, Lachno12) all increased by 1-3 orders of magnitude during the rain event. Highest levels of these markers were observed in Drain 4, followed by Drains 1 and 2. Within the seawater samples collected from Terrigal Haven, highest levels of the human faecal markers were observed in Bay 4, concomitant with the high levels of these markers observed in Drain 4 and suggestive of this drain being a major source of sewage within Terrigal Haven. While human faecal marker levels within the control site at Forrester's beach and the Beachwatch sampling point increased significantly

4.2.1 SUMMARY OF RESULTS (Continued)

during the rain event, levels at these sites remained 1-3 orders of magnitude lower than within the Drain and associated Bay samples, further supporting the notion that Drains 1, 2 and 4 are sources of human faecal material in Terrigal Haven. During the second day of the rain event, the mouth of Terrigal Lagoon was opened, resulting in an order of magnitude increase in the levels of the HF183 and Lachno3 markers in the Bay 7 seawater sample adjacent to the mouth of the lagoon, providing a signature of contamination from Terrigal Lagoon. Notably, elevated levels of human faecal bacteria in some instances persisted for 5 days after the rainfall event, even though enterococci levels, used as a standard measure of faecal contamination, had immediately returned to base-line levels several days prior.

- **Bird associated enterococci** levels, indicated by the GFD marker, increased moderately in all sampling sites during the rain event, but to a lower degree than the increases in the human faecal markers. Unlike the human faecal markers, levels of the GFD marker were not elevated within the Drain samples relative to seawater samples. Notably GFD levels were highest within samples from the pristine control site at Forresters Beach relative to all other sampling locations, likely ruling out an allochthonous input of bird enterococci into Terrigal Haven during this event.

4.2.2 Enterococci Levels

Prior to the rainfall event on June 4, Enterococci levels in all sampling sites were low (~ 10 CFU 100 mL^{-1}) indicative of a negligible base-line level of contamination. However, following 20 mm of rain, Enterococci levels in all sites, except Bay 1 (where they remained < 30 CFU 100 mL^{-1}) increased dramatically to between 1000 to $> 10,000$ CFU 100 mL^{-1} (Figure 8) - well above the threshold for the maximum health risk level in the NHMRC Microbial Assessment Categories (Table 2). Highest Enterococci levels were observed in Drains 1, 2 and 4, where they reached 24,196 CFU 100 mL^{-1} . However, it is notable that Enterococci levels also reached comparable levels within the Bay 2 and 4 samples. In all sites except Terrigal Lagoon, Enterococci levels decreased rapidly after the initial spike on June 4, with levels dropping to low, pre-rain event conditions on the following day, even though a further 20 mm of rain occurred during this period.

Figure 8

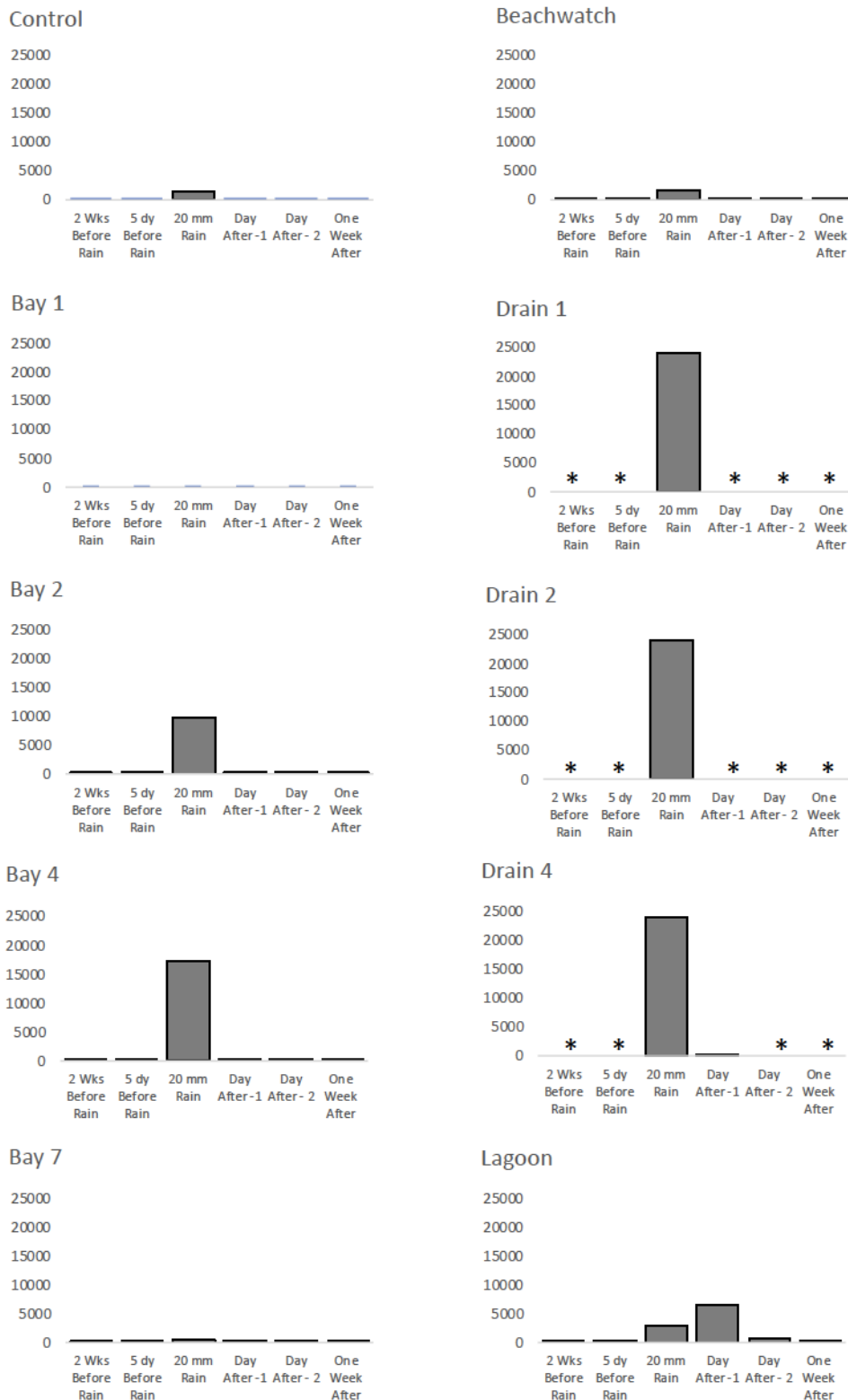


Figure 8: Average Enterococci levels (CFU 100 mL⁻¹) within the Terrigal Haven sampling sites during the wet weather sampling campaign. Enterococci levels were derived using standard membrane filtration according to the Australian standard (AS/NZS 4276.9:2007). Asterisks represent Drain samples that could not be sampled due to inadequate flow and water volume for sample acquisition.



4.2.3 Human Faecal Indicators

All three human faecal indicators (HF183, Lachno3 and Lachno12) exhibited substantial – often multiple order of magnitude – increases in abundances (relative to dry weather conditions) following the rainfall event, although the extent and nature of these increases varied between sampling site and qPCR assay. Across all 3 human faecal markers, highest rain event levels were observed in Drain 4, with levels within this drain often 100-1000 times higher than the other sampling sites. This pattern is in accordance with the dry weather sampling study, where Drain 4 exhibited among the highest levels of human faecal markers (4.1.3).

Concomitant with patterns in the adjacent Drain, the Bay 4 site also exhibited significant ($p < 0.05$) increases in levels of all three human faecal markers following the rain event. With the exception of the HF183 human Bacteroides marker, Drains 1 and 2 displayed similar dynamics to Drain 4, with levels of all three markers increasing above dry weather levels by 1-2 orders of magnitude during the rain event. The adjacent seawater samples in Bay 1 and 2 also displayed substantial increases in the levels of all 3 markers during the rainfall, yet levels within these locations were generally lower than those observed in Bay 4.

Both the Control site at Forresters Beach and the Beachwatch sampling site also exhibited increases in the levels of the three human faecal markers during the rainfall event relative to dry weather conditions. However, levels of all three human faecal markers were generally significantly lower at these sites than within the Drain and other Bay samples. The substantially lower levels of these human faecal markers at the Beachwatch site are particularly notable given that it is located within a few hundred metres of the Bay 1, 2 and 4 sites, and potentially provide further evidence for the localised roles of Drains 1, 2 and 4 as sources of these markers.

Within Terrigal Lagoon, levels of all three human faecal markers increased during the rainfall event, with levels within this site reaching 1-2 orders of magnitude higher levels during this event than the dry weather sampling period. However, levels within Terrigal Lagoon were generally an order of magnitude or more lower than were observed in Drain 4. During the second day of the rain event, the mouth of Terrigal Lagoon was opened, allowing for exchange of water from the lagoon with seawater in the adjacent coastal environment. While the sampling site within Terrigal Haven immediately adjacent to the opening of Terrigal Lagoon displayed significant increases in the human faecal markers during the course of the rain event, a further increase and potential signature of Terrigal Lagoon water was subsequently observed with the HF183 and Lachno3 markers, which both increased by approximately an order of magnitude after the opening of the lagoon's mouth. Levels of the Lachno12 marker did not notably increase after this lagoon-opening event. Notably, while Enterococci levels decreased to pre-rain levels by the second day of the rainfall event in all sites except Terrigal Lagoon, the human faecal markers often remained significantly elevated, sometimes to extremely high levels (Lachno3 and Lachno12 in Drain 4) in several sampling sites for 5 days after the rainfall event. This is potentially indicative of environmental persistence of human faecal bacteria beyond the time-frame indicated by standard monitoring approaches.

Figure 9

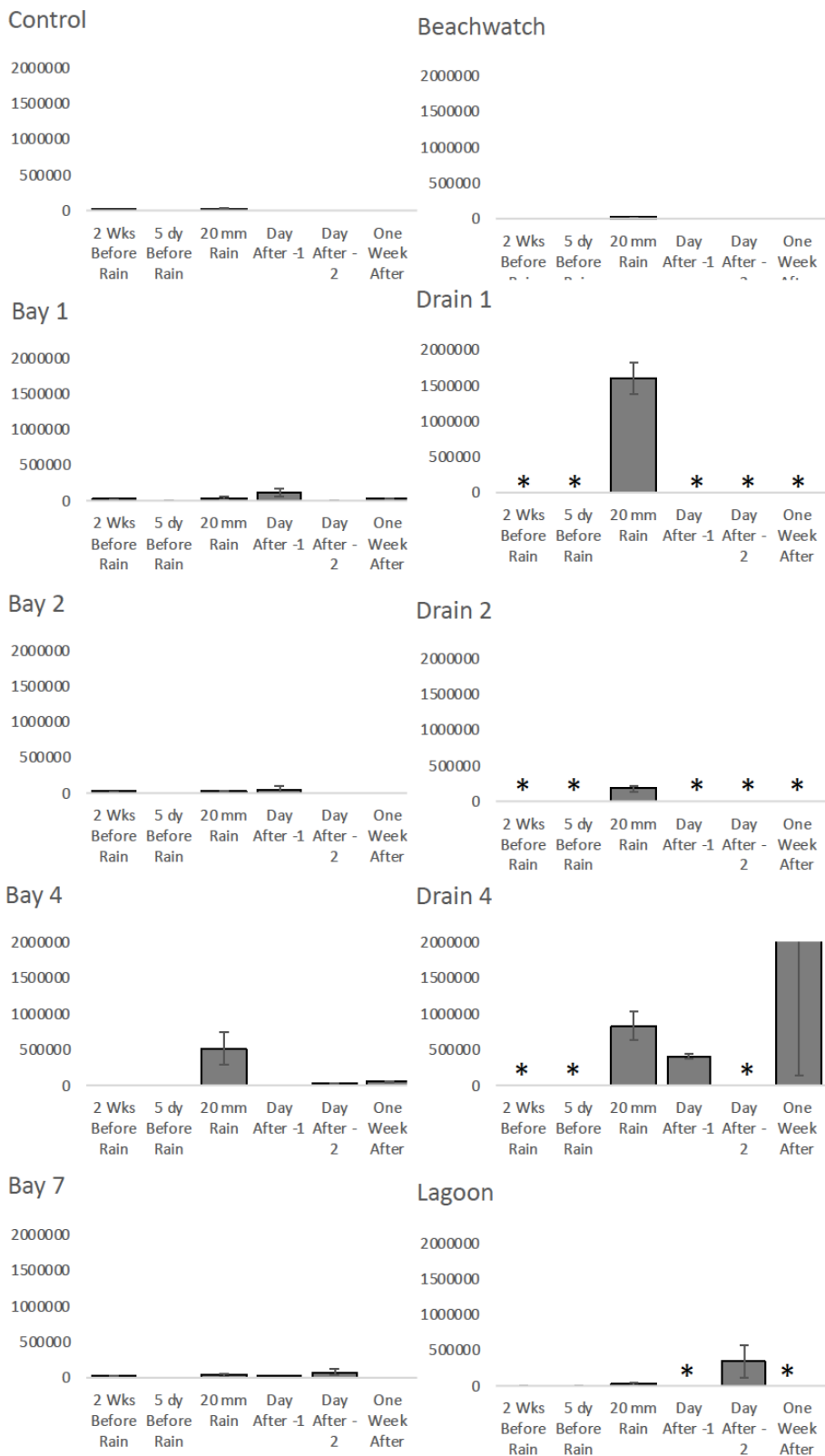


Figure 9: Quantitative PCR analyses of the human faecal bacterial marker *Lachno3* (*Lachnospiraceae*), across the 10 sites sampled during the Terrigal Haven wet weather sampling regime. Data derived from triplicate samples. Error bars represent Standard Error (SE). Asterisks represent Drain samples that could not be sampled due to inadequate flow and water volume for sample acquisition.

Figure 10

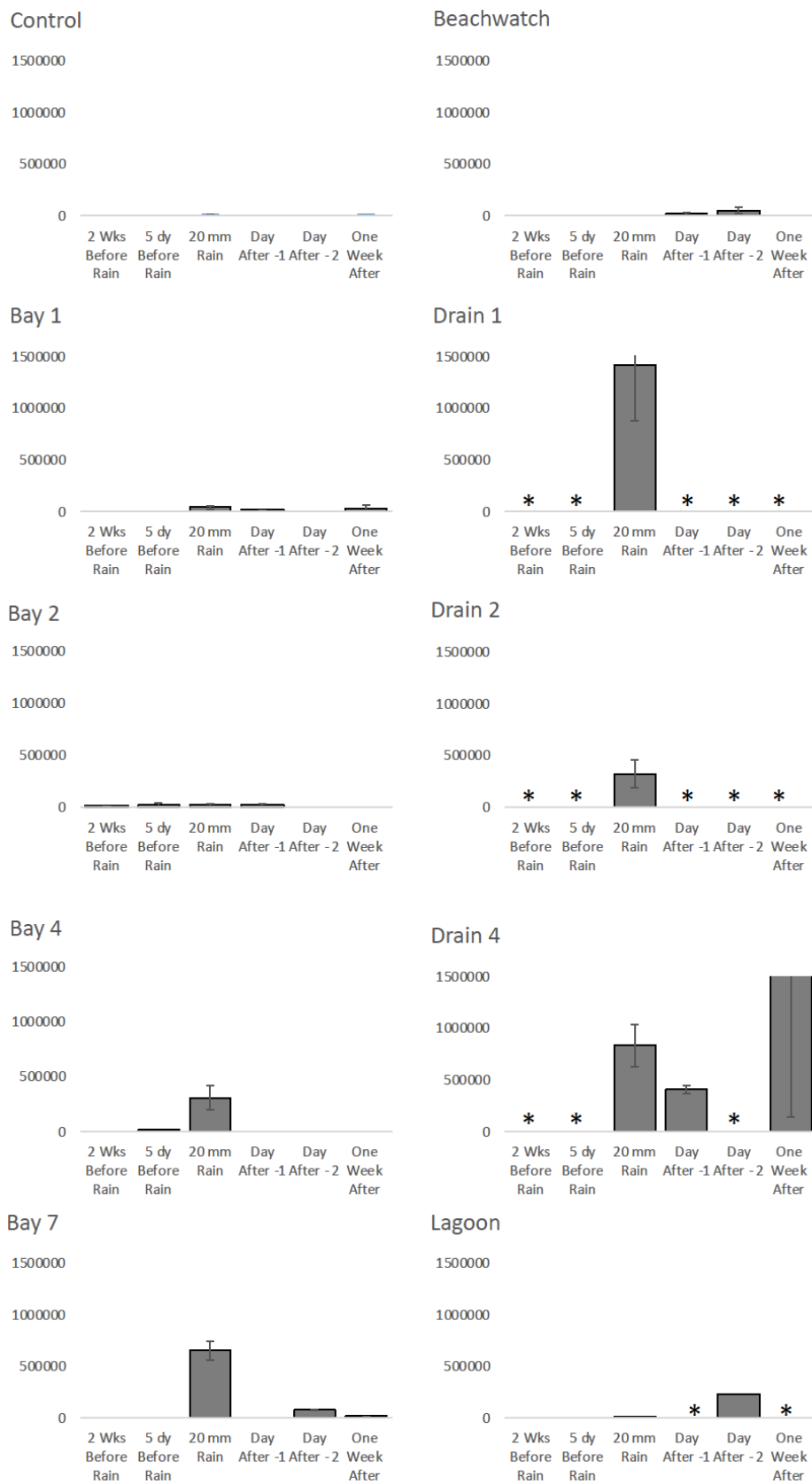


Figure 10: Quantitative PCR analyses of the human faecal bacterial marker Lachno12 (*Lachnospiraceae*) across the 10 sites sampled during the Terrigal Haven wet weather sampling regime. Data derived from triplicate samples. Error bars represent Standard Error (SE). Asterisks represent Drain samples that could not be sampled due to inadequate flow and water volume for sample acquisition.

Figure 11

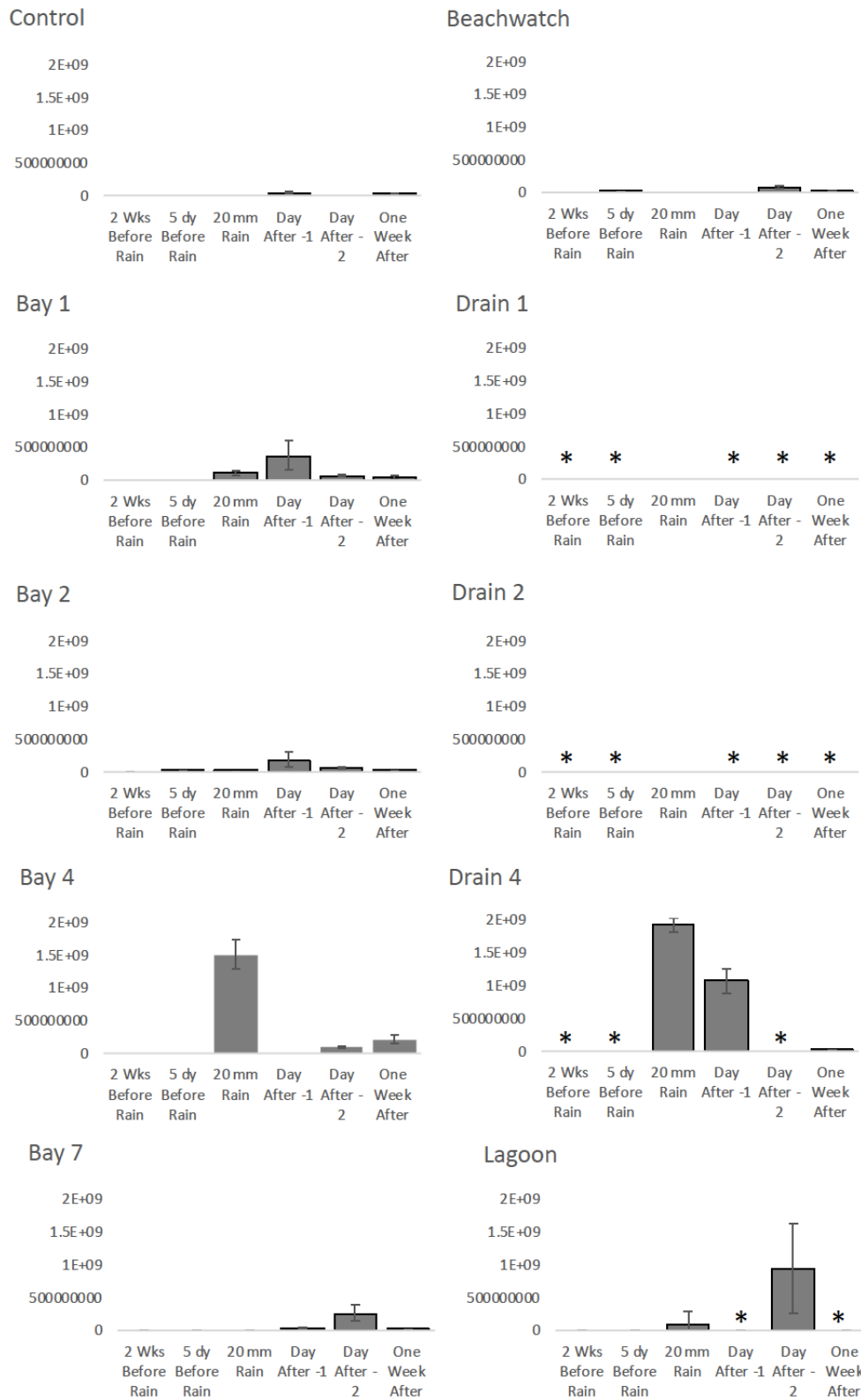


Figure 11: Quantitative PCR analyses of the human faecal bacterial marker HF183 (*Bacteroides*) across the 10 sites sampled during the Terrigal Haven wet weather sampling regime. Data derived from triplicate samples. Error bars represent Standard Error (SE). Asterisks represent Drain samples that could not be sampled due to inadequate flow and water volume for sample acquisition.

4.2.4 Bird Enterococci Marker

Relative to samples collected 5 days prior to the rain event, levels of the GFD avian enterococci marker displayed moderate increases during the rainfall event in all sampling sites, although these were not as marked as the increases observed in the human faecal markers. In contrast to the human faecal markers, the GFD marker was not markedly elevated within Drain samples relative to Bay samples. Perhaps more notably, levels of the GFD marker within the pristine control site at Forresters Beach sampling site, which displayed substantially lower levels of the human faecal markers than the other sites, were significantly higher than those observed in the Drain and Bay samples. We interpret this pulse in levels of the GFD marker at the control site to be indicative of natural seabird faeces being washed off the beach and into the ocean during the rainfall event. While the patterns in the human faecal markers indicate the drains in Terrigal Haven are a source of sewage-associated bacteria during rain events, the GFD marker patterns indicate a relatively uniform level of avian Enterococci across this environment during periods of heavy rainfall.

Figure 12

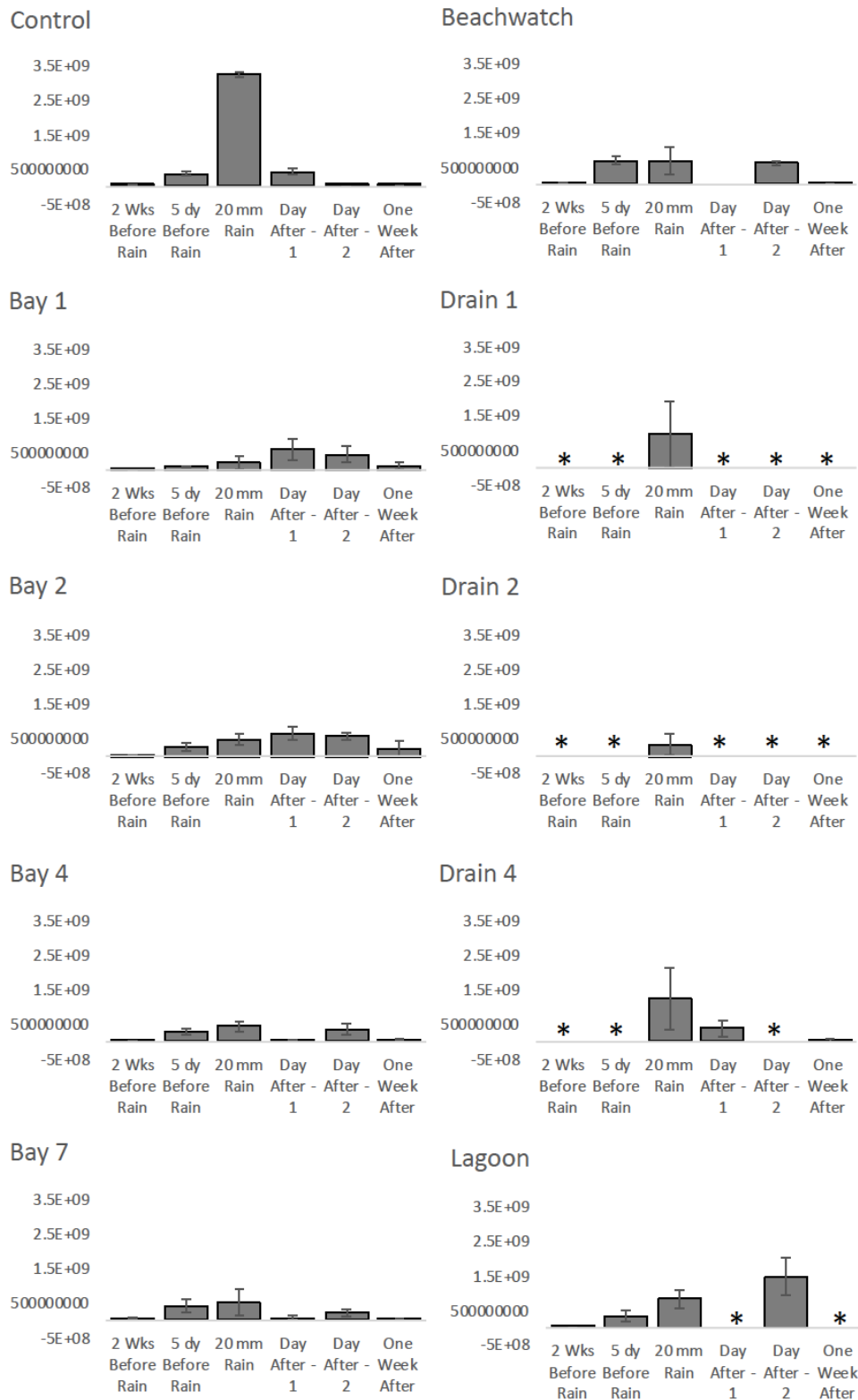


Figure 12: Quantitative PCR analyses of the bird enterococci marker GFD across the 10 sites sampled during the Terrigal Haven wet weather sampling regime. Data derived from triplicate samples. Error bars represent Standard Error (SE). Asterisks represent Drain samples that could not be sampled due to inadequate flow and water volume for sample acquisition.

4.3 Rosebay Dry Weather Sampling

4.3.1 SUMMARY OF RESULTS

A detailed description of the results for this section of the project are provided below, but a brief synopsis of the major findings is presented here:

- **Enterococci levels** during the dry weather sampling period within Rose Bay were significantly higher than within the control site within the Sydney Harbour National Park. Average Enterococci levels within seawater samples (i.e. Bay 4, 5, 6 and the Beachwatch sampling point) were higher (81 CFU 100 mL⁻¹) than those observed at Terrigal beach during the dry weather monitoring program, yet still generally within the lower two NHMRC microbial risk categories. Enterococci levels were, however, substantially elevated within the Drain samples, with average levels in Drains 4 and 5 (586 and 630 CFU 100mL⁻¹) exceeding the NHMRC maximum threshold for significant risk of illness.
- **The human sewage markers** used here (HF183, Lachno3, Lachno12) were detected in 97% of samples within Rose Bay and occurred in significantly higher concentrations than the control site in 95% of cases. Highest levels of the human faecal markers were observed in the Drain samples, but significant levels were also intermittently observed in the Bay samples, particularly during the sampling period coinciding with 6mm of rain. Across the entire data-set, all three human markers were significantly correlated with Enterococci levels, yet in a small proportion of Drain samples there was poor correspondence between the human faecal markers and elevated Enterococci levels.
- **Bird associated enterococci** indicated by the GFD marker were detectable in all samples in Rose Bay, but did not differ significantly to levels observed in the pristine control site, which we suggest are indicative of natural base-line levels of bird faecal material in marine environments.
- **The marker for dog faeces (DG3)** was detected within 17% of samples collected from Rose Bay, but was below detection limit within the control site. The majority of detections of this marker were within Drain 4 and 5, with elevated levels often coinciding with high Enterococci levels, which was reflected in a positive correlation between the DG3 marker and Enterococci measurements. However, levels of the DG3 marker were highly heterogenous over time, suggestive of a temporally uneven impact of dog faeces within Rose Bay.
- **The wastewater infrastructure-associated bacteria, *Arcobacter*, and the microbial marker for anthropogenic impact, *intl1***, were detected in 99% of samples collected in Rose Bay and were both correlated to Enterococci levels. Highest levels of these markers were typically observed in the Drain samples, but significant levels were also intermittently observed in the Bay samples, particularly during the sampling period coinciding with 6mm of rain. The significantly elevated presence of these markers relative to the pristine control site is indicative of the presence of a human wastewater signature in Rose Bay, even under dry weather conditions.

4.3.2 Enterococci Levels

During dry weather conditions, average Enterococci levels across the 5 weeks of samples collected at Rose Bay generally significantly ($p < 0.05$) exceeded levels at the control site at Nielsen Park, within the Sydney Harbour National Park (where concentrations never exceeded $10 \text{ CFU } 100 \text{ mL}^{-1}$) (Figure 13). This trend is indicative of a higher level of impact from faecal contamination within Rose Bay than within the 'pristine' control site. Within the Rose Bay sampling sites, Enterococci levels reached maximum levels of $> 1000 \text{ CFU } 100 \text{ mL}^{-1}$ in some drain samples. However, it is notable that Enterococci levels within the Beachwatch reference location in Rose Bay averaged only $37 \text{ CFU } 100 \text{ mL}^{-1}$ and only exceeded $50 \text{ CFU } 100 \text{ mL}^{-1}$ on one occasion, indicating that swimming conditions at the Beachwatch reference station were not problematic during this dry weather period. In contrast, some of the drains that were sampled during this period were characterised by consistently high Enterococci levels.

Within Drain 4, Enterococci levels exceeded $200 \text{ CFU } 100 \text{ mL}^{-1}$, which is indicative of an increased probability of adverse health effects (Table 2), in 100% of samples, while the $500 \text{ CFU } 100 \text{ mL}^{-1}$ threshold for significant risk of illness was exceeded in 60% of samples. Similarly, Enterococci levels in Drain 5 substantially exceeded the highest NHMRC Microbial Assessment risk category¹² on three of the sampling days (60% of samples). Drain 6 levels exceeded this threshold on one occasion (20% of samples), but were generally significantly lower than the other two drains. High Enterococci levels were not restricted to the drain samples, with the $200 \text{ CFU } 100 \text{ mL}^{-1}$ threshold exceeded in the Bay samples in 10% of samples, but notably, high levels of Enterococci in the bay sites did not always coincide with high levels within the adjacent drains.

Figure 13

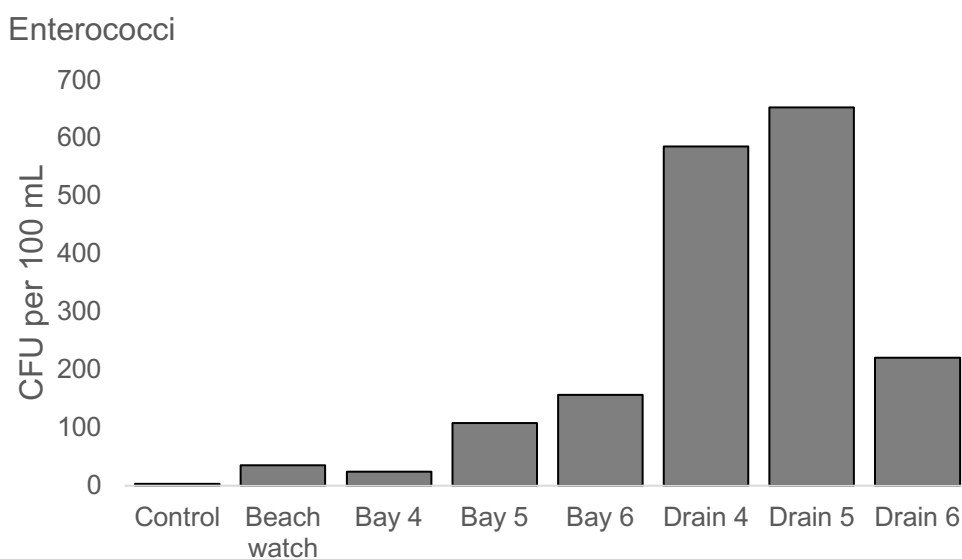


Figure 13: Average enterococci levels ($\text{CFU } 100 \text{ mL}^{-1}$) within the Rose Bay sampling sites over the five week dry weather sampling regime. Enterococci levels were derived using standard membrane filtration following the Australian standard (AS/NZS 4276.9:2007).

4.3.3 Human Faecal markers

The three human faecal marker genes employed here, Lachno3, Lachno 12 and HF183, indicative of human gut microbiome associated *Lachnospiraceae* and *Bacteriodes* bacteria¹⁵⁻¹⁶ were detected in 97% of samples, indicating a consistent influence of human faeces in Rose Bay. Of the 4 samples where concentrations of these markers were below the detection limit of the assay, 3 occurred in the Control site at Nielsen Park, confirming the 'pristine' condition of this location relative to Rose Bay. Average concentrations of the three human faeces markers within the Rose Bay sampling sites occurred in significantly higher levels than the control site at Nielsen Park in 95% of cases. Across the entire data-set, all three human markers tracked the patterns in Enterococci counts (Figure 14), exhibiting statistically significant positive correlations with the Enterococci levels ($p < 0.05$).

In Drain 6, all three human markers tracked the major patterns in Enterococci levels relatively closely. Specifically, on the two occasions that Enterococci levels were highly elevated within this drain, on the 2nd and 8th of April, concentrations of the HF183, Lachno3 and Lachno 12 markers were also significantly elevated ($p < 0.05$). Given the lack of correspondence between these high Enterococci levels and the patterns in both the dog and bird markers quantified within this drain (4.3.4), we conclude that the high Enterococci counts observed in Drain 6 were very likely associated with human faeces.

In Drains 4 and 5, the agreement between patterns in Enterococci counts and the human markers was not always as clear as that found within Drain 6. For instance, within Drain 4, the very high Enterococci counts observed on the 2nd (600 CFU 100mL⁻¹) and 8th of April (750 CFU 100mL⁻¹), did not correspond to substantially elevated gene copy abundances in any of the three human markers (Figure 15). Notably, Drain 4 samples on the 2nd of April were instead characterised by significantly elevated ($p < 0.05$) levels of the DG3 dog faeces marker (4.3.4). However, the elevated Enterococci levels within Drain 4 on the 26th of April (600 CFU 100mL⁻¹), did clearly correspond to significantly ($p < 0.05$) elevated levels of all three human faecal markers. Our interpretation of these patterns is that: (i) the contribution of human and animal faecal material to the high Enterococci levels observed in Drain 4 is heterogeneous over time, and (ii) the Lachno3 marker is a less sensitive measure of human faecal material than the other qPCR markers employed here, which is consistent with previous findings¹⁶.

In Drain 5, where the highest Enterococci levels observed during this study were recorded, the mean levels of the three human faecal markers were significantly elevated above the control sample ($P < 0.05$). However, a clear link between high Enterococci counts and elevated levels of these markers was not always apparent. On the 26th of April, when the highest Enterococci levels observed in any sample during the entire study occurred (1400 CFU 100mL⁻¹) in Drain 5, very high abundances of gene copies were reported using the HF183 and Lachno 12 assays, yet the Lachno3 assay again showed little correspondence with these patterns.

Figure 14

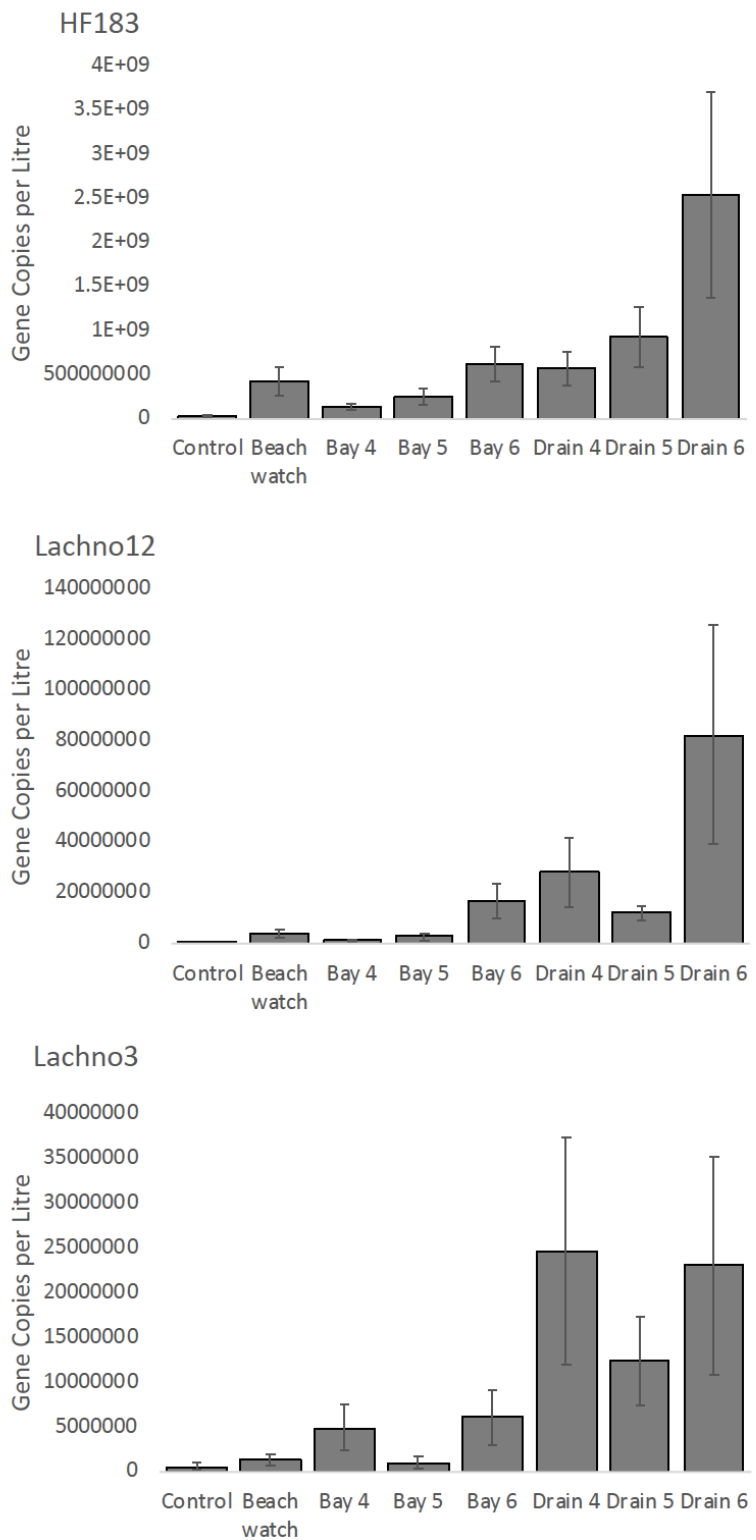


Figure 14: qPCR analyses of the human faecal bacterial markers HF183 (*Bacteroides*), Lachno3 and Lachno12 (*Lachnospiraceae*), which are indicative of sewage contamination, in the Rose Bay sampling locations. Data derived from triplicate samples. Error bars represent Standard Error (SE).



On the 2nd and 8th of April, when enterococci counts exceeded 800 CFU 100mL⁻¹, all 3 human marker assays were significantly elevated relative to the control, but were often not markedly higher than levels observed in other samples, perhaps implying a less sensitive tracking of the elevated enterococci levels. Notably, on April 8, the high enterococci levels in Drain 5 were accompanied by a more pronounced increase in the abundance of the DG3 dog *Bacteroides* marker (see below) than was observed in any of the human markers.

Figure 15

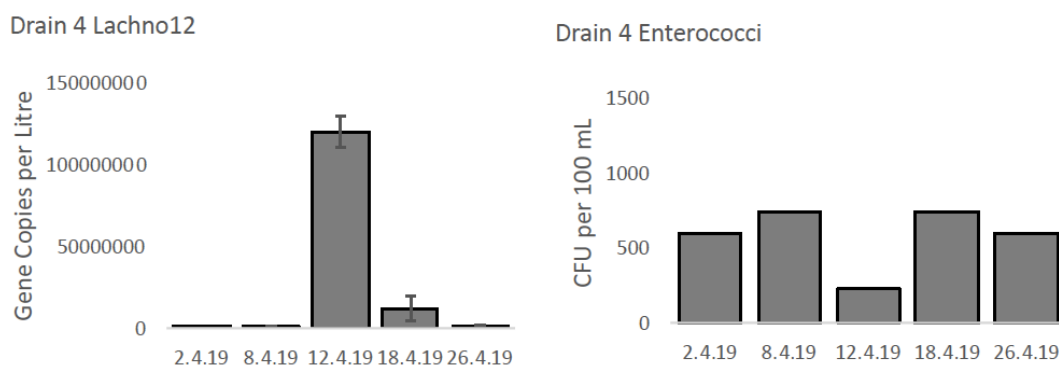


Figure 15: Patterns in the Lachno12 human faecal marker within Drain 4 at Rose Bay over the 5 week dry sampling campaign (left) and enterococci counts from corresponding samples (right). Levels of the human faecal bacterial markers did not correspond with the high levels of enterococci recorded on 2/4/19 and 8/4/19. Data derived from triplicate samples. Error bars represent Standard Error (SE).

Levels of the human faeces markers were generally substantially higher within the drain than the bay samples, with the exception of the 2nd of April, when all three markers occurred in significantly higher levels within the Bay 4, 5 and 6 samples than the corresponding drain samples. Notably, this pattern occurred during the only period of rain (6 mm) that occurred during this study (Figure 16).



Figure 16

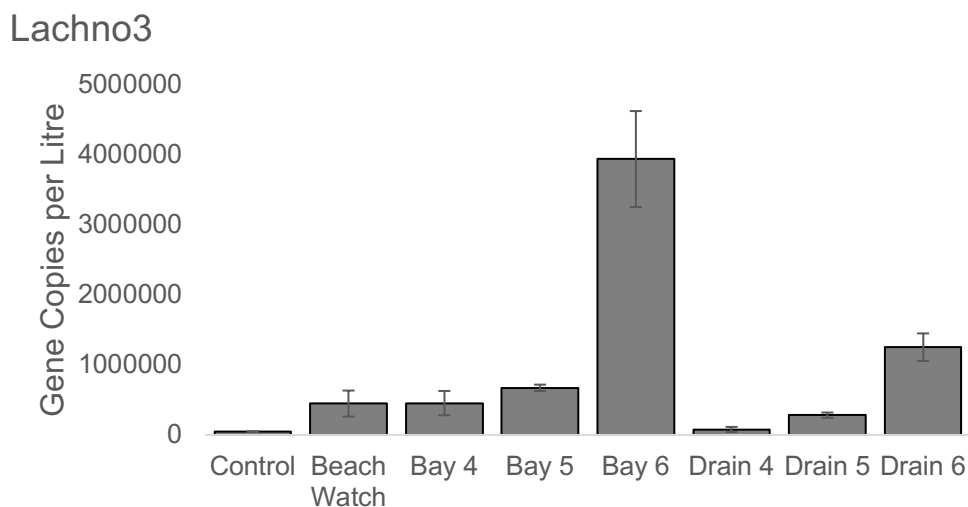


Figure 16: qPCR analyses of the human faecal bacterial marker Lachno3 (*Lachnospiraceae*) on the 2nd of April 2019, when levels of this marker for human faeces was recorded in higher levels in the Bay samples than Drain samples. This followed a period of rain (6 mm). Data derived from triplicate samples. Error bars represent Standard Error (SE).

Although Enterococci levels at the Beachwatch sampling point were generally below 75 CFU 100 ml⁻¹ during this dry weather sampling period, it is notable that levels of all three human markers were significantly ($p < 0.05$) elevated compared to the control samples, suggesting a consistent impact of human faeces at this reference point relative to the pristine environment in Nielsen Park.

4.3.4 Animal Faecal Markers

The bird-specific Enterococci marker GFD was observed within 100% of samples collected within Rose Bay, but in contrast to the patterns observed at Terrigal Beach, where this marker was highly elevated within specific drain samples, relatively uniform concentrations were observed across the sampled sites. Relative to the control site at Nielsen Park, levels of the GFD marker were not significantly ($p > 0.05$) elevated in any of the Rose Bay samples. Furthermore, there was no significant ($p > 0.05$) correlation between Enterococci levels and the GFD marker. Notably, copies of the GFD marker within Rose Bay were also comparable to those observed in most samples at Terrigal Beach, which we interpret to be indicative of natural baseline levels of bird faecal bacteria within marine environments, suggesting that the impact of bird faeces on water quality in Rose Bay was negligible during the time of sampling.

Figure 17

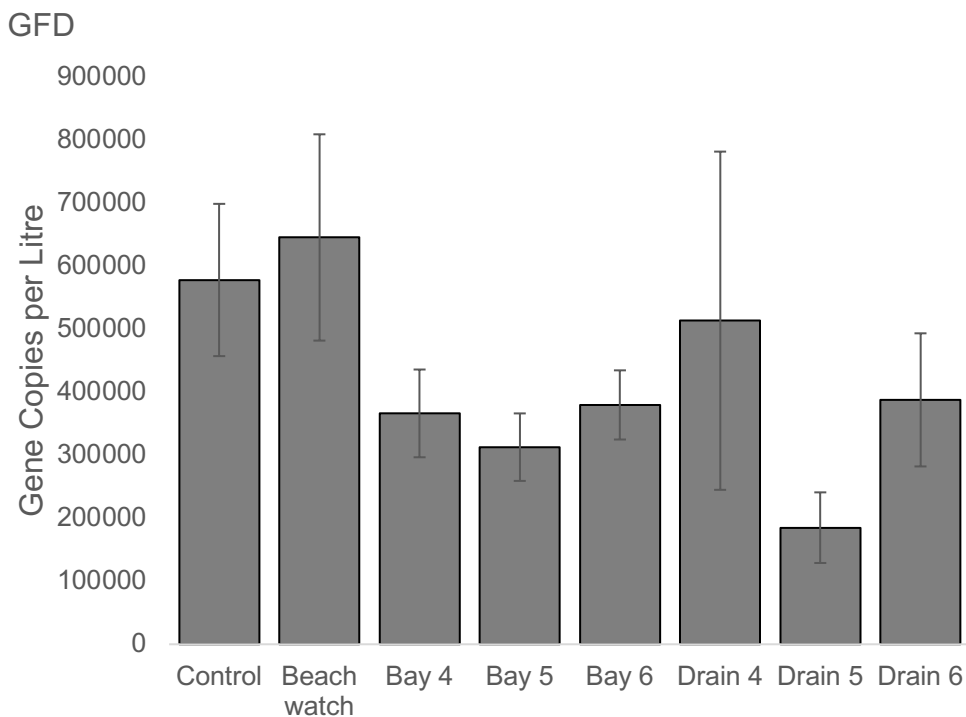


Figure 17: Average levels of the avian enterococci marker GFD in the Rose Bay sampling locations during dry weather sampling. Data derived from triplicate samples. Error bars represent Standard Error (SE).

In contrast to the dry-weather results observed at Terrigal Beach, where the DG3 marker for dog-faeces associated *Bacteroides* was not detected in any sample, this marker was observed in 17% of samples collected from Rose Bay. Notably, this dog faeces marker was below detection limit within the control site at Nielsen Park.

Within Rose Bay, the highest concentrations of the DG3 dog marker were observed within the Drain 4 and Drain 5 samples, with samples immediately in front of these drains in Bay 4 and Bay 5 also sometimes exhibiting elevated numbers of dog *Bacteroides* gene copies. A significant correlation between Enterococci counts and DG3 copy numbers was observed ($p < 0.05$). However, similarly to the patterns observed with the human faeces markers described above, the correspondence between elevated levels of the DG3 marker and high enterococci levels was variable in time.

Figure 18

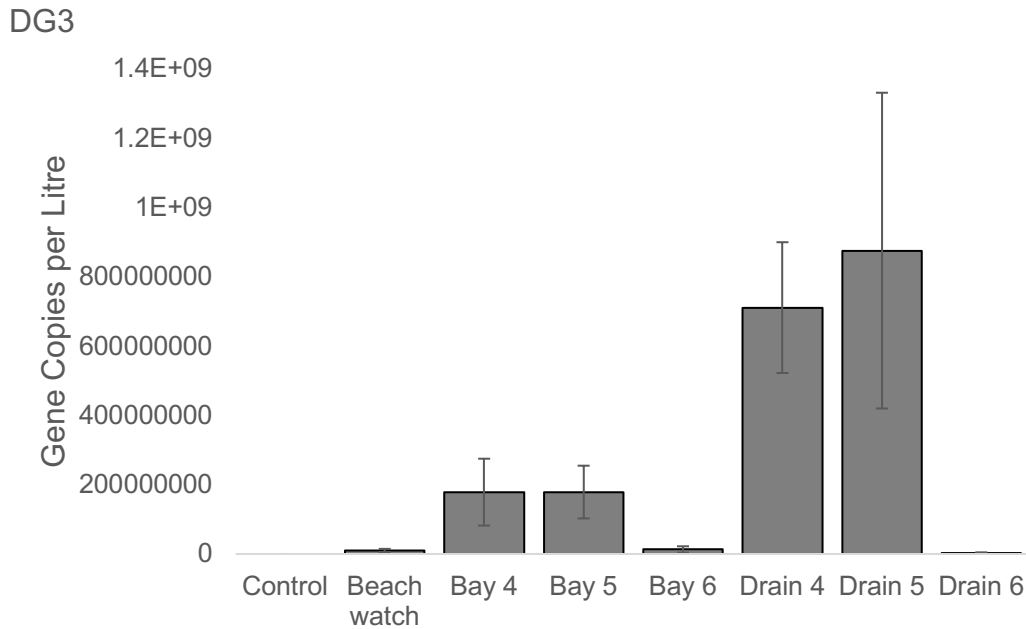


Figure 18: qPCR analyses of the canine *Bacteroides* marker DG3 in the Rose Bay sampling locations. Data derived from triplicate samples. Error bars represent Standard Error (SE).

In Drain 4, gene copies of the DG3 dog *Bacteroides* marker were significantly ($p < 0.05$) elevated on the 2nd, 8th and 12th of April, when *Enterococci* levels were high, but were below the detection limit on April 18 and 26 when *Enterococci* counts were also very high. Similarly, high levels of the DG3 marker were observed in Drain 5 on April 8, but were very low or undetectable on all other occasions, including periods where *Enterococci* levels were very high (Figure 19). Our interpretation of these patterns is that the occurrence of dog faecal material, and its contribution to the measured *Enterococci* levels, at the sampled locations is highly variable with time. The patterns in the DG3 data, whereby highest levels occurred within Drain 4 and 5 samples, also lead us to suspect that the source of the dog faecal material is from within the catchment serviced by these stormwater drains, rather than off the beach.

Figure 19

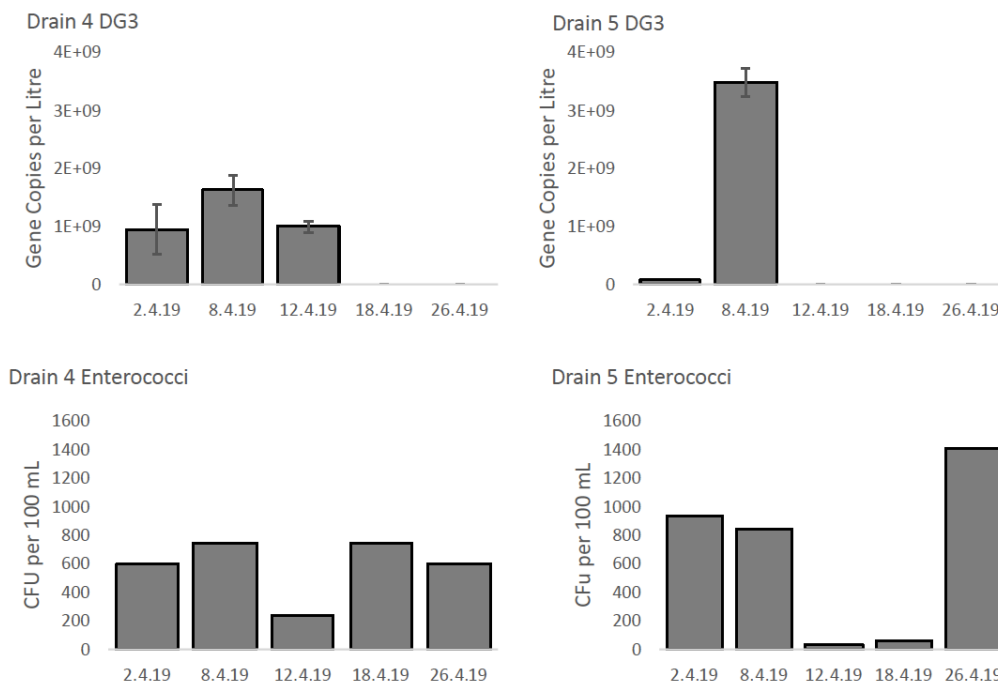


Figure 19: Patterns in the DG3 marker for dog *Bacteriodes* in Drain 4 and 5 at Rose Bay over the 5 week sampling regime (top) and corresponding enterococci levels (bottom). Elevated levels of DG3 corresponded with higher enterococci levels on April 2 and 4, but not April 18 and 26. Data derived from triplicate samples. Error bars represent Standard Error (SE).

4.3.5 Other Indicators of Anthropogenic Impact

Of the qPCR assays employed throughout this Rose Bay dry weather study, the Class 1 Integron integrase gene (*IntI1*), which has elsewhere been proposed as an excellent proxy for anthropogenic impact²³, displayed the strongest correlation ($P < 0.001$) to Enterococci levels, with average gene copies tracking patterns in Enterococci levels closely (Figure 20). This pattern provides evidence that the Enterococci levels measured at the Rose Bay sites were significantly influenced by anthropogenic sources of contamination.

Relative to the control site at Nielsen Bay, significantly ($p < 0.001$) higher mean copies of the *IntI1* gene were observed in all sites within Rose Bay. Levels of *IntI1* were significantly elevated within all drains, but notably were also higher than the Control within the seawater samples collected from Rose Bay, indicating a significant anthropogenic signature across this site.

Figure 20

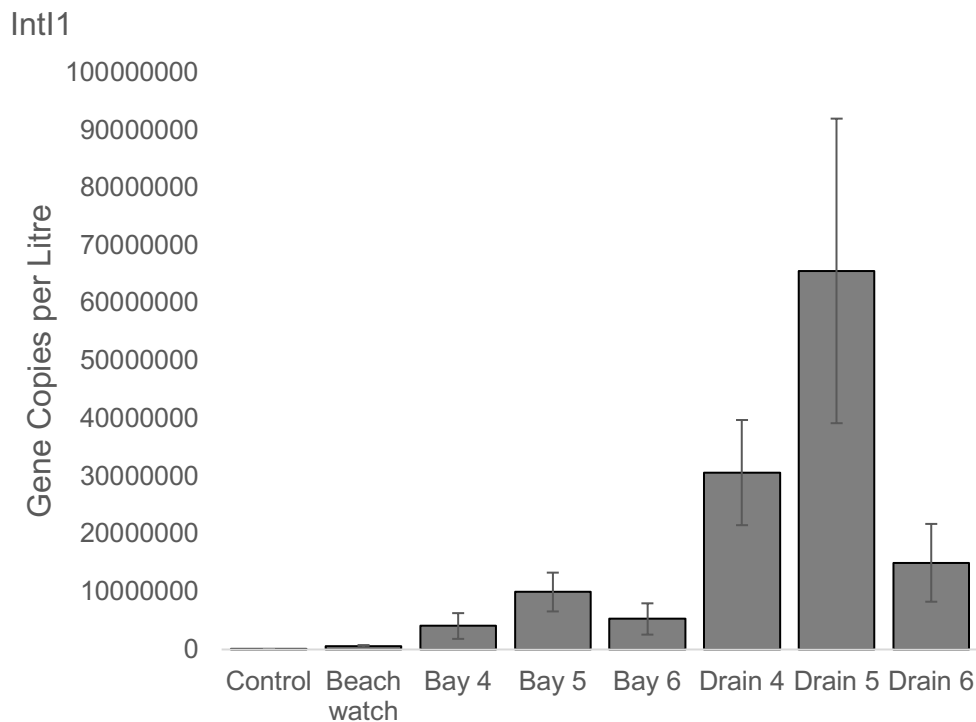


Figure 20: qPCR analyses of the Class 1 Integron Integrase gene *IntI1* in the Rose Bay sampling locations. Data derived from triplicate samples. Error bars represent Standard Error (SE).

The *Arcobacter* 23S rRNA gene was significantly ($p < 0.01$) elevated relative to the Control site in the Drain 4 and 5 samples, but was not significantly different to the control in the other sampling sites. A significant correlation ($p < 0.001$) between *Arcobacter* 23S rRNA gene copies and Enterococci levels was observed, further implying a link between the measured Enterococci levels and urban pipe infrastructure. However, throughout this study period, elevated levels of *Arcobacter* were generally restricted to the Drain samples, with the exception of the 2nd of April, when significantly ($p < 0.05$) elevated *Arcobacter* levels were observed in the Bay 4 and Bay 5 samples. Notably, this followed the only period of moderate rainfall (6 mm) during this part of the study, potentially leading to a flushing of pipe bacteria into Rose Bay.

Figure 21

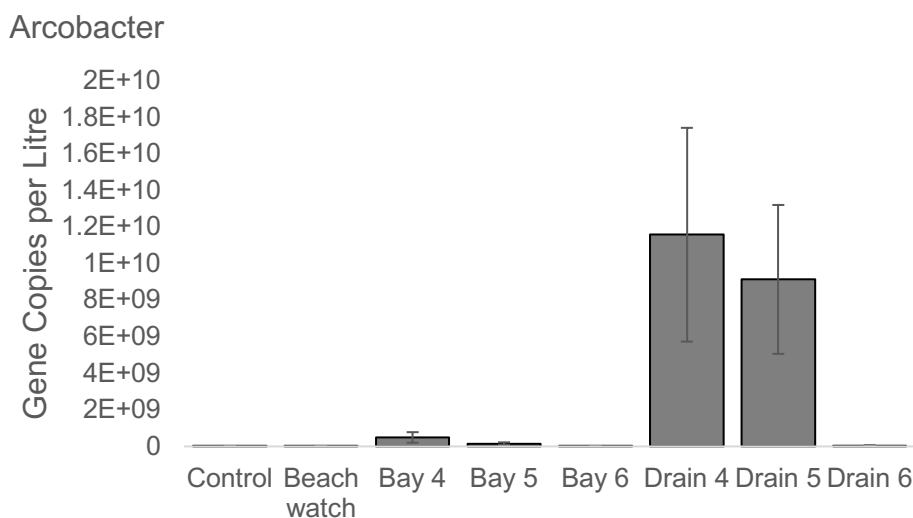


Figure 21: qPCR analyses of the *Arcobacter* 23S rRNA gene in the Rose Bay sampling locations. Data derived from triplicate samples. Error bars represent Standard Error (SE).

5.0 DISCUSSION & INTERPRETATION

5.1 Terrigal Beach – Dry Weather Conditions

The standard marker for microbial contamination of coastal environments, Enterococci levels, remained relatively low in most seawater samples collected from Terrigal Beach and Terrigal Haven during the dry weather sampling period. However, concentrations within the tested stormwater drain discharge points were often extremely high and substantially exceeded the maximum threshold for human health risk. Highest Enterococci levels were consistently observed within the stormwater discharge points at Drain 4, at the southern end of Terrigal Beach and Drain 2, located within Terrigal Haven. Using a suite of molecular microbiological markers targeting human and animal faecal bacteria and indicators of urban waste-water infrastructure it was possible to deliver a more nuanced insight into the microbiological conditions of these drains and other locations at Terrigal Beach to provide insights into the likely causes and sources of contamination observed at this site.

Drain 4 was characterised by the highest levels of the three markers for human faeces (sewage) used here, which target specific bacterial groups (*Bacteroides* and *Lachnospiraceae*), previously identified as major components of the human faecal microbiome¹⁵⁻¹⁶. We suggest that the observed patterns in the HF183, Lachno3 and Lachno12 markers are indicative of a sewage signal within the water discharged from Drain 4. It is notable that levels of these human faecal markers also became elevated within the Bay 4 sample (which represents seawater collected at Terrigal Beach from a location adjacent to Drain 4) on April 4, following 24 mm of rain in the preceding two days. This provides evidence that Drain 4 may also represent a potential source of sewage contamination to ocean waters of Terrigal Beach following rainfall. Significantly, although the qPCR markers detected a signature of human sewage

within Bay 4 on this occasion, the standard Enterococci monitoring method did not detect increased levels in this sample.

Drain 2, and in some cases Terrigal Lagoon, also exhibited elevated levels of the three markers for human faeces, with levels in Drain 2 sometimes exceeding those in Drain 4, indicating that these sites also exhibit a sewage signature during dry weather conditions. However, the apparent influence of Drain 2 on the seawater quality in Terrigal Haven was less pronounced than Drain 4 during this dry weather sampling period, with the adjacent Bay 2 sample generally exhibiting negligible levels of the human faecal markers. Inconsistencies and temporal shifts in the relative levels of the human faecal markers between Drains 2 and 4 highlight the complex nature of stormwater contamination within Terrigal. However, across the entire dry weather data set, two of the human faecal markers (HF183 and Lachno12) displayed statistically significant correlations with Enterococci levels, implying a significant contribution of human sources (sewage) to the Enterococci measurements used to monitor water quality at Terrigal Beach.

With one exception, animal contributions to the Enterococci levels observed within Terrigal Beach appear to have been minimal. The dog Bacteroides marker, indicative of dog faeces, was not detected in any sample, ruling out an influence from dogs on water quality at this site. The avian Enterococci marker, indicative of bird faeces, was detected across all samples, but levels of this marker did not differ significantly to those observed in the pristine Control site at Forresters Beach, in all but two samples. These two exceptions occurred within Drain 2, where levels of the bird Enterococci marker were an order of magnitude higher than other samples. It is currently unclear whether these isolated cases of elevated levels of the bird faeces marker were sourced externally from the catchment captured by Drain 2, or whether the high levels of seabird (seagulls, pelicans) activity within the eastern corner of Terrigal Haven were responsible for this pattern. However, the isolated occurrence of these two events across the entire data-set, and the general pattern of a relatively uniform background signature of bird faeces, lead us to conclude that animal sources of contamination generally had a small impact on water quality within Terrigal beach during this dry weather study.

While elevated levels of Enterococci and human faecal markers were largely restricted to the Drain samples, it is notable that two other indicators for wastewater contamination were often elevated within the seawater (Bay) samples collected during this dry weather study. Although often in lower levels than observed within Drain samples, 96% of Bay and Beachwatch samples were characterised by significant levels of *Arcobacter*, which is a genus of bacteria indicative of urban water infrastructure (i.e. pipes). The *Arcobacter* genus incorporates several species that are considered emerging human pathogens⁹ and occur within sewage²⁴. Notably, there is evidence that *Arcobacter* populations can colonise sewage and stormwater pipe infrastructure²¹, and that following rainfall events these bacteria are flushed into coastal habitats²², providing a contamination signature. We also observed elevated levels of the Class 1 Integron Integrase gene (*Int1*) within samples from Terrigal Beach (particularly Bay 4), relative to the control site. This bacterial gene has been proposed to be a very good marker for anthropogenic impact within natural environments, due to its links to genes associated with antibiotic, heavy metal and

disinfectant resistance and its occurrence in pathogenic enteric bacteria²³, and has elsewhere been shown to be a very sensitive tracer of stormwater contamination within coastal environments²⁵. The observation of elevated levels of *Arcobacter* and *Int11* within Terrigal seawater samples provides further indication of microbial contamination of this environment from urban stormwater infrastructure, even during dry weather periods.

In summary, the main outcomes from the dry weather characterisation of Terrigal Beach include evidence that: (i) sewage (as determined by 3 human faecal microbiome markers) generally influences Enterococci levels measured within this environment to a greater extent than other animal sources of faecal pollution; (ii) principal sources of this sewage signature are stormwater discharge points at the southern end of Terrigal Beach (Drain 4) and within Terrigal Haven (Drain 2), and (iii) even during dry weather periods signatures of both sewage and urban waste water infrastructure are apparent within seawater samples at Terrigal beach, even when measured Enterococci levels are low, (iii) two isolated incidences of very high levels of the bird faecal marker were observed in one stormwater outlet drain (Drain 2), but it is currently unclear whether this is a signature of bird populations on Terrigal Beach, or bird faecal material washed into the stormwater system from the catchment.

5.2 Terrigal Beach – Wet Weather Event

Following rainfall, urbanised coastal environments often experience heightened levels of contamination as a consequence of discharges from stormwater infrastructure^{22,25}. In some instances, this can involve an increased impact from sewage, following wet-weather sewer overflows and pipe blockages²⁵. As a consequence, levels of Enterococci are regularly elevated at coastal beaches immediately following rainfall⁴. To examine the influence of rainfall events on the microbiological contamination of Terrigal Beach and further elucidate the causes and sources of poor water quality at this site, we conducted a wet weather sampling program.

Immediately following 20 mm of rainfall, Enterococci levels within all Drain and Bay samples (with the exception of Bay 1) increased sharply to exceed the maximum NHMRC threshold for significant human health risk. Consistent with patterns in the dry weather sampling (5.1), Drains 4 and 2 exhibited the highest Enterococci levels, although very high levels were also observed in Drain 1. Notably, and in-line with evidence from the dry weather sampling, the seawater sites immediately adjacent to the Drain 2 and 4 stormwater discharge points (i.e. Bay 2 and 4) also experienced rapid and intense increases in Enterococci levels, indicating a clear impact of stormwater discharges on coastal water quality. However, with the exception of Terrigal Lagoon, Enterococci levels decreased to pre-rain levels very rapidly (i.e. within 1 day), even though further rainfall fell during the ensuing two days. Possible explanations for this pattern are: (i) a build-up of contaminated (i.e. Enterococci) water within stormwater drains was completely flushed into the environment during the first day of the rainfall event; (ii) potential sewage overflow events within the stormwater infrastructure were restricted to the first day of heavy rainfall; (iii) the very large surf conditions during the second part of the rainfall event led to dispersal and/or dilution of allochthonously introduced Enterococci.

Relative to during dry weather conditions, the three human faecal (sewage) markers were substantially elevated (often by 1-2 orders of magnitude) within the stormwater discharge drains. Consistent with dry weather conditions, highest levels of these markers were observed in Drain 4, along with Drain 1. The increases of these markers, relative to dry weather conditions, were even more pronounced in the seawater samples adjacent to these stormwater discharge points (i.e. Bay 1, 2 and 4), where during the rainfall event, levels were sometimes comparable to those observed in the Drain samples. Consistent with the patterns observed during the dry weather sampling, Bay 4 exhibited the highest levels of the human faecal markers, indicating the substantial influence of Drain 4 on the microbiology of seawater within Terrigal Beach.

Levels of the human faecal markers also became highly elevated within Terrigal Lagoon during the rainfall event. An impact of the lagoon on water quality of Terrigal Beach was observed when the connection between the lagoon and ocean was opened two days after the start of the rainfall event and a concomitant increase in levels of the human faecal markers was observed in the adjacent Bay 7 sample. This pattern indicates that Terrigal Lagoon, as well as the stormwater discharge points, is a potential source of microbial contamination of Terrigal Beach under rainfall conditions.

Moderate increases in the levels of the bird Enterococci marker were observed across all sampling locations during the wet weather event. However, these increases were relatively uniform across all locations and were far less pronounced than those observed among the human faecal markers. Furthermore, the most substantial increases in the levels of this marker occurred in the pristine control site at Forresters Beach. Our interpretation of these patterns is that the moderate increases in the bird faeces marker during the rainfall event were likely a consequence of natural beach run-off of seabird faeces²⁶ and that relative to the multiple order of magnitude changes in human faecal markers observed, these likely made a negligible contribution to the large increases in total Enterococci levels observed during the rain event.

In summary, a moderate (40 mm total) rainfall event led to significant increases in Enterococci levels within Terrigal Beach, with levels exceeding the maximum threshold for human health risk. These patterns were likely driven by substantial increases in human faecal bacteria, presumably associated with input of sewage. All three stormwater drains sampled exhibited very high levels of human faecal markers, although highest levels were observed in Drain 4. The influence of these drains on water quality in Terrigal Beach was apparent from the substantial increases in human faecal markers within adjacent seawater samples. In addition to the influence of the stormwater drains, the opening of the mouth of Terrigal Lagoon also led to the input of human faecal bacteria into Terrigal Beach.

5.3 Rose Bay – Dry Weather Conditions

Average Enterococci levels within seawater samples collected from Rose Bay were higher than those observed at Terrigal Beach during the dry weather monitoring programs, yet were still generally within the lower two NHMRC microbial risk categories. In-line with the patterns observed at Terrigal, Enterococci levels were, however, substantially elevated within the stormwater discharge (Drain) samples, where they regularly exceeded the NHMRC maximum threshold for significant risk of

illness. Our goal was to use molecular microbiological approaches to develop a greater understanding the causes of these Enterococci patterns.

Relative to Terrigal, the three human faecal markers were observed in a much higher proportion (97%) of samples collected from Rose Bay. Highest levels of these markers were observed in the Drain samples, but significant levels were also intermittently observed in the Bay samples, particularly during the sampling period coinciding with 6mm of rain. Consistent with this pattern, significant levels of the *Arcobacter* and *Int11* markers for urban wastewater and anthropogenic impact were also intermittently elevated in the Rose Bay seawater samples, particularly after the rainfall event. We hypothesise that two potential explanations for the greater occurrence of this anthropogenic signature (human faecal markers, *Arcobacter*, *Int11*) in Rose Bay than Terrigal Beach are: (i) Rose Bay is part of a large highly urbanised estuary (Sydney Harbour) where background levels of anthropogenic contaminants will likely be higher, and (ii) levels of hydrodynamic flushing of the highly protected Rose Bay are likely be substantially lower than the open-ocean facing Terrigal Beach, leading to reduced dispersal and dilution of allochthonous contaminants.

Across the entire data-set, all three human faecal markers were significantly correlated with Enterococci levels, yet in some Drain samples there was poor correspondence between these two measures, and relative to Terrigal Beach, the links between Enterococci counts and the human faecal markers were not always as clear cut. A potential explanation for this discrepancy is the influence of animal faecal material. The canine *Bacteroides* marker for dog faeces was detected within 17% of samples collected from Rose Bay, with the majority of detections within Drain 4 and 5 in samples characterised high Enterococci levels. This pattern was further reflected by a positive correlation between the dog faecal marker and Enterococci measurements. Although dog walking is permitted on Rose Bay, given that high levels of the dog faecal marker were generally restricted to the stormwater discharge (Drain) samples, we speculate that the dog faeces signature was likely sourced from urban run-off in the catchment, rather than off the beach. While the avian Enterococci marker was observed in all samples within Rose Bay, levels of this marker were relatively uniform and did not differ significantly to levels observed in the pristine control site, which we suggest is indicative of natural base-line levels of bird faecal material in marine environments.

In summary, during dry weather conditions, Rose Bay is characterised by low-moderate levels of Enterococci, but the discharge points from several stormwater drains depositing into the bay often have elevated Enterococci levels that regularly exceed the threshold for human health risk. Our suite of qPCR assays revealed that seawater samples within Rose Bay regularly exhibit the signature of urban waste water infrastructure and sewage, presumably sourced from stormwater discharge drains, where levels of these contaminants are further amplified. However, while human faecal markers exhibited statistically significant correlations with measured Enterococci levels, in some instances there appeared to be a combined influence of human and dog faecal material. We suggest that to more completely detangle the microbiological dynamics within Rose Bay, a wet weather sampling study would be

highly beneficial in helping to define the sources and causes of contamination within this site.

6.0 CONCLUSIONS & RECOMMENDATIONS

The main conclusions arising from this project are:

1) Molecular microbiological techniques, including quantitative PCR, exhibit great utility for reducing ambiguity about the causes and sources of elevated Enterococci levels within contaminated aquatic environments. The suite of assays that were employed here included three microbial markers for human faeces and specific markers for dog and bird faeces, and in most instances were capable of pin-pointing the most likely cause of high Enterococci counts (i.e. human vs animal faecal material). There was some redundancy in the use of human faecal markers in this study and we suggest that in future studies, the number of these markers could be reduced to include only the marker for human *Bacteroides* (HF183) and one of the *Lachnospiraceae* markers. *Lachno12* has elsewhere been shown to have greater specificity than *Lachno3* and throughout the analyses performed here *Lachno12* generally showed better correlations with Enterococci counts and displayed higher levels of fidelity across replicates. We therefore propose that future studies could solely use the *Lachno12* marker as an indicator of human *Lachnospiraceae*.

In addition to the faecal indicator assays, we believe that the *Arcobacter* and *Int11* assays employed here provide valuable further evidence for an anthropogenic / waste-water signature within natural environments. While the detection of these markers in drain samples is perhaps obvious, they provide an excellent tracer for wastewater influence in seawater samples that compliments the faecal indicators.

For the two urban coastal environments that were the focus of this research, we made the *a priori* decision that bird and dog markers were the most relevant targets for animal faecal material, and the assays selected proved to be highly sensitive in detecting animal faecal signatures. However, in other systems additional or alternative animal markers may be required. For instance, the assessment of water quality in rural environments will often benefit from the application of markers specific to faecal material from agricultural animals (e.g. cows, pigs, chicken) or other wildlife (e.g. bats, marsupials, ducks, rabbits). Microbial markers for faeces from many of these organisms already exist, or boutique markers could be created with relatively minor development time. We suggest that the careful selection of suitable animal markers should be a key consideration during the planning of further research of this type in other environments.

2) At Terrigal Beach, water quality within seawater samples was generally not problematic during dry weather periods, but even during dry weather periods water discharged from stormwater drains is often characterised by high levels of Enterococci contamination. Our analysis indicates that high Enterococci levels within these drains are most commonly linked to human (rather than animal) faecal contamination (sewage), with levels of the three microbial markers for human faeces elevated within these drains. In particular Drain 4, at the southerly end of Terrigal Beach and Drain 2

within Terrigal Haven, consistently exhibited a pronounced human sewage signature, suggesting that these are sites that may warrant further investigation. However, the very high levels of the bird Enterococci GFD marker observed in two Drain 2 samples are notable, yet the source of this material is unclear and may also warrant further examination.

3) During wet-weather conditions at Terrigal Beach, the sewage signature within the stormwater discharge points becomes further amplified, sometimes by over 100-fold, with the microbial markers for human faeces also observed in much higher concentrations within seawater samples. Highest seawater levels of the human faecal markers occurred in sites adjacent to stormwater drains (Bay 4 in particular), essentially confirming these drains as the source for human faecal contamination of the seawater at Terrigal Beach. The opening of Terrigal Lagoon during this event also led to a spike in the levels of human faecal markers in Terrigal beach water, indicating this activity is another source of faecal contamination, although it appears to have a lower impact than the stormwater drains.

4) At Rosebay, levels of the microbial markers for human faeces (HF183, Lachno3, Lachno12), wastewaters infrastructure (Arcobacter) and anthropogenic impact (IntI1) were all elevated relative to the pristine control site. Like Terrigal, levels of these markers were substantially greater within stormwater drains, pinpointing these as sources of contamination. Clear links between Enterococci levels and the human faecal markers were apparent in Drain 6, indicating sewage contamination within this stormwater drain. However, within Drains 4 and 5 there appears to be a combined signal from human and dog faeces, with the influence of dog faeces variable, but sometimes significant. While Rose Bay is a dog beach, we propose that the occurrence of highest levels of the microbial marker for dog faecal material within the stormwater drains points to an external input from the catchment, rather than from dogs on the beach. However, some ambiguity around the key factors influencing water quality at Rose Bay remains and we suggest that a wet-weather sampling study performed at this site will likely provide greater clarity.

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