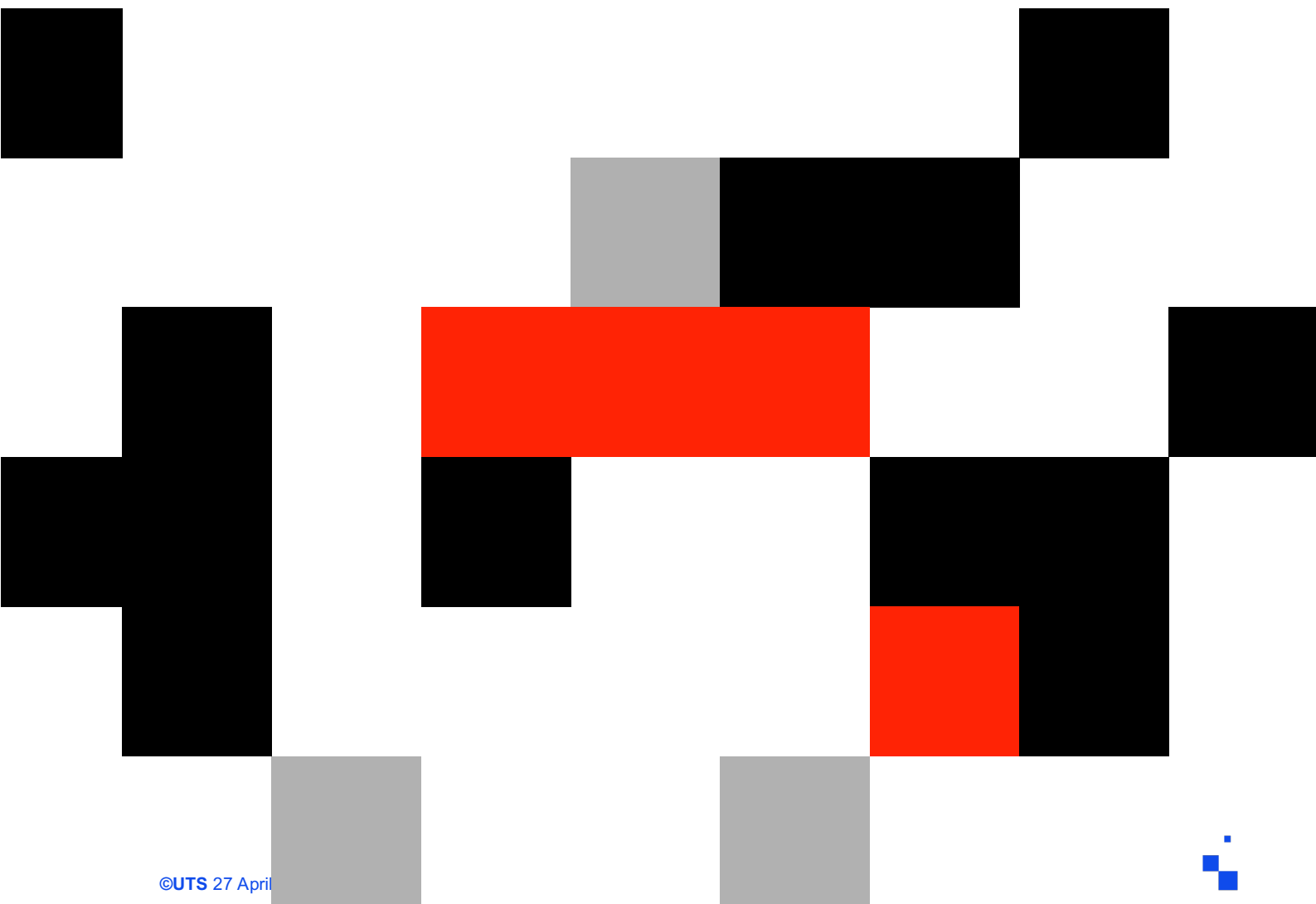
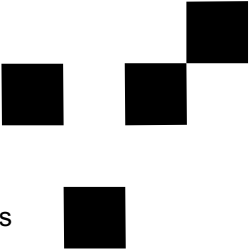
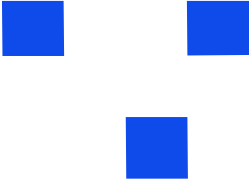


Climate Change Cluster, Faculty of Science,  
University of Technology Sydney

# Microbial source-tracking to assess water quality issues at Rose Bay





This report has been prepared by the University of Technology Sydney (“UTS”) in good faith as part of a collaboration with Beachwatch and the NSW Department of Planning, Industry and Environment (DPIE). Beachwatch provided *Enterococci* data and assisted with planning and performing sampling.

## FOR THE ATTENTION OF:

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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 Rose Bay (NSW). Samples from stormwater drains and seawater samples were analysed using a suite of assays targeting microbial indicators of human, bird and dog faecal material. Sampling was conducted before, during and after a significant rainfall event (66.8 mm over 5 days), with the goal of understanding to what extent sewage vs animal sources of faecal contamination influence water quality at Rose Bay and identifying the principal point-sources of contamination input.

Under dry weather conditions, water quality within Rose Bay was generally good, with Enterococci levels remaining within the lowest health risk level in the NHMRC Microbial Assessment Categories. Markers for dog and bird faecal material were detected in 22% and 90% of samples respectively, but levels of the bird faecal marker were not significantly different to those observed at a 'pristine' control environment and detections of the dog faecal marker did not correspond with elevated Enterococci levels. We therefore conclude that animal faeces have a negligible impact on Rose Bay water quality during dry weather.

Dry weather samples taken from the network of stormwater drains located at Rose Bay revealed elevated Enterococci levels and the presence of markers for human faecal material (sewage) in several drains. Drain 5 in particular exhibited high levels of two human faecal markers, indicating a potential dry weather incursion of sewage into this drain. Notably, following a small (3.8 mm) rainfall event, levels of the human faecal markers increased further within this drain and were detected in high levels within adjacent seawater samples, indicating that this drain is a potential point-source of sewage contamination in Rose Bay, even under low levels of rainfall.

Following a significant (43 mm) rainfall event, water quality in Rose Bay declined significantly, with Enterococci levels in drains and several near shore water samples substantially exceeding the highest health risk level in the NHMRC Microbial Assessment Categories. Extremely high Enterococci levels in Drains 3, 4, 8 and 10 and adjacent seawater samples were mirrored by very high levels of two microbial markers for human faeces, indicative of sewage contamination. Drain 3 displayed extremely high levels of the human faecal markers, potentially indicative of a major impact of wet weather sewage overflow within this drain. Notably, seawater samples adjacent to this drain displayed high levels of human faecal markers even several metres from shore, indicating that during significant rainfall, Drain 3 has the most pronounced effect on the water quality of Rose Bay by introducing sewage contamination. Notably, in several drains an increase in the marker for dog faeces co-occurred with the human faecal markers, indicating an additional influence of dog faeces on water quality in Rose Bay. However, given the concentration of this marker in stormwater drains, we hypothesise that the source of this signal is dog faeces from the catchment serviced by the stormwater drains, rather than Rose Bay beach. It is also noteworthy that concentrations of the dog faecal marker were up to an order of magnitude lower than the human faecal markers, implying a smaller contribution of faecal contamination from dogs.

In conclusion, recreational water quality at Rose Bay is primarily influenced by sewage contamination from a network of stormwater drains. Under dry weather conditions, Drain 5 displays a signature for sewage contamination, which enters Rose Bay following low to moderate rainfall. However, following heavy rainfall, human faecal contamination of several drains, but in particular Drain 3, substantially diminishes water quality at Rose Bay.

## 1.0 BACKGROUND

The economic value of recreational beach use by Sydney residents exceeds \$1.2 billion per year<sup>1</sup>, with substantial further 'social value' and personal wellbeing derived from Sydney's beaches<sup>2</sup>. Sydney Harbour hosts more than 20 beaches that are suitable for recreational use, providing a relatively unique opportunity for beach use within a major city. However, like many highly urbanised coastal environments<sup>3</sup>, several Sydney Harbour beaches are regularly impacted by compromised water quality<sup>4</sup>, which in some cases could have negative implications for human health<sup>5</sup>.

Within NSW, a state-wide water-quality monitoring program conducted by *Beachwatch* rates swimming beaches according to safety for recreational use<sup>4</sup>. Several Sydney Harbour beaches, including Rose Bay, are regularly reported to have poor water quality. In fact, in the last three *State of the Beaches* reports, prepared by Beachwatch, Rose Bay has been rated as one of the poorest NSW beaches for recreational water quality<sup>4</sup>, resulting in substantial community concern<sup>5</sup>. Yet the principal causes of poor recreational water quality at this beach remain uncertain.

Water quality at Rose Bay is often poorest following rainfall<sup>4</sup>, likely because of inputs from a substantial network of stormwater outlet pipes, each with differing, but largely undefined levels of contamination from the surrounding catchment and impact from wet weather sewage overflow points. Further ambiguity about the source of contamination at this site is created by the potential influence of animal (e.g. dog) faecal material, which cannot be discriminated from sewage using standard water-quality monitoring approaches. Uncertainty about the causes and sources of contamination has impeded capacity to design and implement management strategies to resolve water quality issues at Rose Bay.

Standard water-quality monitoring programs, including Beachwatch, generally use global benchmarks for water quality assessment, which involve enumeration of faecal indicator bacteria (FIB), such as Enterococci. This type of analysis is employed as a proxy measure for sewage pollution in natural aquatic environments and is implemented according to standardised international guidelines<sup>6</sup>. However, FIB approaches cannot precisely discriminate the origin of the target bacteria between human (i.e. sewage) and animal sources, often leading to ambiguity about the true cause of elevated Enterococci counts within an environment<sup>7</sup>. Recently, more sophisticated 'microbial source tracking' approaches that can precisely identify specific indicator organisms or microbiological features (e.g. toxin genes) based on DNA signatures have shown great utility in identifying the causes and sources of aquatic pollution<sup>8</sup>.

The principal goal of this project was to apply these microbial source tracking strategies to determine: (i) the relative impact of sewage vs animal faecal material and (ii) the principal input sources of water contamination at Rose Bay, in order to facilitate future management actions to improve water quality at this site.

## 2.0 OBJECTIVES

The over-arching objective of this project was to apply novel molecular microbiological source tracking approaches to define the probable causes and sources of poor water quality at Rose Bay following wet-weather. The specific objectives of the project were to:

- 1) Determine whether high Enterococci levels at Rose Bay are primarily caused by human (i.e. sewage) or animal (dog or bird) sources of faecal contamination. !
- 2) Identify the primary points of contamination input by sampling the network of stormwater drain outlets and adjacent seawater sites within Rose Bay.
- 3) Understand the spatial and temporal dynamics of water contamination within Rose Bay during a wet weather event.

## 3.0 METHODOLOGY

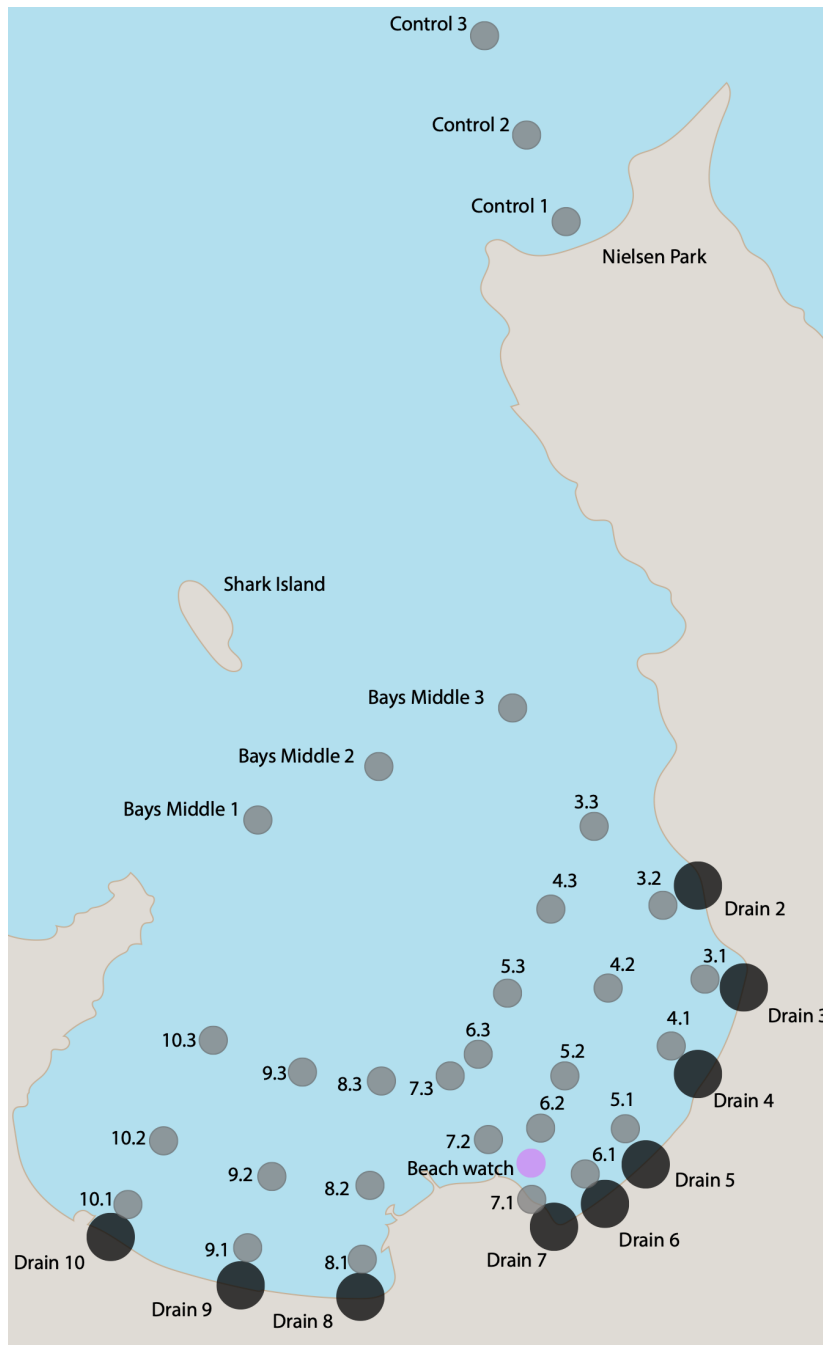
Sampling of stormwater drain outlets and seawater samples was conducted at Rose Bay before, during and after a wet weather event in August 2019. The sampling design was developed in close consultation with DPIE and Beachwatch, with the goal of identifying the most likely sources of contamination at Rose Bay.

Samples were collected from 41 locations chosen according to proximity to potential points of contamination (Figure 1). Samples were collected from the outlets of 9 stormwater drains, which were chosen due to their regularly high flow rates (often even under dry weather conditions) and evidence that they may contribute to the periodically high Enterococci counts recorded at Rose Bay by Beachwatch<sup>4</sup>. These drain outlets are believed to mainly be conduits for urban stormwater, but in some instances may be impacted from wet weather sewer overflows. Seawater samples were also collected from points located along a shore-to-sea transect adjacent to each of these drains, to examine the extent of dispersal of contamination from drains into Rose Bay (Figure 1). These transects comprised surface seawater samples collected from immediately adjacent to drains at the shoreline in water of 50cm depth (RBT\_1 samples), 250m offshore (RBT\_2 samples) and 500m offshore (RBT\_3 samples). Finally, samples were collected from reference points including the Beachwatch sampling site located at the western end of Rose Bay, a deep-water transect across the entrance to Rose Bay from Woollahra Point to Hermit Point and from a relatively pristine 'Control' site, within Nielsen Park (Sydney Harbour National Park), which is void of any urban stormwater infrastructure.

Sampling was conducted during the course of a significant rainfall event, which resulted in a total of 69.8 mm of rain over the course of 5 days, including 43 mm

over 24 hours. Samples were collected from the locations described above on four occasions, corresponding to 6 days before rain (21/8/19), a light rainfall event (3.8 mm) (27/8/19), the peak rainfall event (43 mm) (30/8/19), and 4 days after rain (3/9/19).

**Figure 1**



**Figure 1.** Map of Rosebay sampling points. RBD refers to drain samples, RBT refers to seawater transect samples collected from surface seawater from the shoreline (1), and 250m (2) and 500m (3) offshore. RBC samples correspond to Control samples collected from the pristine site at Nielsen Park and RBE samples refer to a deep-water transect across the entrance to Rose Bay.



### 3.4 Sample Processing and Analyses

At each sampling site within Rose Bay, triplicate 2 L water samples were collected using 10L plastic containers from which triplicate samples were filtered. Within 2 hours, samples were transported to the lab and filtered through 0.22 µm pore-size membrane filters (Merk-Millipore) using a peristaltic pump (100 rpm). Filters were stored at -80 °C for DNA extraction, which was performed within two weeks of collection.

### 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)<sup>9</sup>

Category	95 <sup>th</sup> 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. We assembled a set of qPCR primers designed to target several bacterial groups that provide unambiguous discrimination of potential human and animal sources of faecal material (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.	10
<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.	11
<i>Bacteroides</i> (Dog)	DG3	A dog faeces specific marker targeting <i>Bacteroides</i> bacteria dominating the dog faecal microbiome	12
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.	13

### 3.6 Statistical Analysis

To test for differences in levels of qPCR markers between sites and time points, the nonparametric Kruskal-Wallis test was used in conjunction with Mann-Whitney pairwise comparisons, whereby Bonferroni corrected p values were used. In order to test correlations between Enterococci plate counts (single replicate) and the qPCR samples (three biological replicates) average values for qPCR data were used. Correlations between Enterococci counts and data derived from qPCR assays were determined using Pearson's Linear Correlation analysis.



## 4.0 Results

### 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 dry weather conditions, Enterococci levels within Rose Bay were low at all sites, except immediately adjacent to Drain 5, where moderate levels were present. Elevated Enterococci levels were observed in several stormwater drains, with highest levels recorded in Drain 3. Following a light (3.8 mm) rainfall event, a sharp increase in Enterococci levels occurred in several drains (most notably Drains 3, 4 and 10), but levels remained relatively low within seawater samples within Rose Bay. After a more substantial rainfall event (49 mm), Enterococci levels increased to extremely high levels within all drains and within seawater samples collected from immediately adjacent to drains, with seawater samples from near to drains 3 and 10 exhibiting the highest levels. Following 4 days without further rainfall, Enterococci counts generally returned to low levels across all sampling points, with the exception of Drains 3 and 5.
- **The human sewage markers** used here, Lachno3 and HF183, indicative of human gut microbiome associated *Lachnospiraceae* and *Bacteroides* bacteria, were detected in 79 and 61% of samples respectively, with both markers displaying statistically significant correlations to total Enterococci counts. During dry weather conditions, levels of both human faecal markers were low within Rose Bay seawater samples, where they were comparable to levels observed at the pristine control site. However, high levels of both markers were observed in Drain 5, indicative of a sewage signal within this drain even under dry weather conditions. Following the rainfall event, average levels of human faecal markers increased by over an order of magnitude, indicating a significant input of sewage-contaminated water into Rose Bay during rainfall. Both drain and seawater samples exhibited high levels of both human faecal markers, although spikes in seawater levels were generally restricted to samples taken immediately adjacent to drains at the shoreline. Extremely high levels of both human faecal markers were observed in Drain 3 and adjacent seawater samples, suggesting a significant impact of sewage within this drain following rainfall. Notably, relative to Enterococci counts, levels of the human faecal markers, in particular HF183, remained elevated (in comparison to initial dry weather conditions) in many samples four days after rainfall.
- **Bird associated enterococci** measured using the GFD marker were detectable in 79% of samples but were not correlated with total Enterococci levels and did not differ significantly between Rose Bay and the pristine control site. There was no trend for increasing GFD levels following rain or in association with drains. Measured levels of this marker were consistent with those previously recorded at NSW beaches, which we conclude are indicative of natural base-line levels of bird faeces.
- **The marker for dog faeces (DG3)** was detected within only 37% of samples collected from Rose Bay and was always below detection limit within the control site. A moderate, but statistically significant correlation was observed between DG3 levels and total Enterococci counts. During dry weather conditions, moderate levels of the DG3 marker were detected in 22% of samples, with detections limited to nearshore seawater samples, in particular samples near to Drains 3 and 5. Following the rainfall event, average levels of this marker increased by an order of magnitude, with highest levels observed in drain samples, in particular drains 3, 5 and 8. During this time, seawater samples adjacent to Drains 3, 5, 8 and 9 also displayed increased levels of the dog faecal marker. Four days after the rainfall event, the proportion of samples that the DG3 marker was detected in and average DG3 levels decreased, although significant levels persisted in several seawater sample sites within Rose Bay.

#### 4.1 Enterococci Analysis

Substantial changes in Enterococci levels were observed between sites and over time (Figure 2). Prior to rainfall, with the exception of one sampling point (Transect 5-1), Enterococci levels (mean: 2.4 CFU 100 mL<sup>-1</sup>) within all seawater samples collected from within Rose Bay and the reference points were well below the lowest health risk level (Category A) in the NHMRC Microbial Assessment Categories<sup>9</sup> (Table 2). However, Enterococci levels within the 5-1 sample, located immediately adjacent to Drain 5 reached 180 CFU 100 mL<sup>-1</sup> (Category B). Notably, these Enterococci levels were even higher than those observed in Drain 5 (75 CFU 100 mL<sup>-1</sup>) at the time of sampling. Consistent with previous measurements at Rose Bay<sup>17</sup>, Enterococci levels were elevated within all sampled stormwater drains (mean: 154 CFU 100 mL<sup>-1</sup>), with the highest levels observed in Drain 3 (470 CFU 100 mL<sup>-1</sup>) [NB: during this period Drains 2, 7, 9 and 10 could not be sampled due to insufficient water flow].

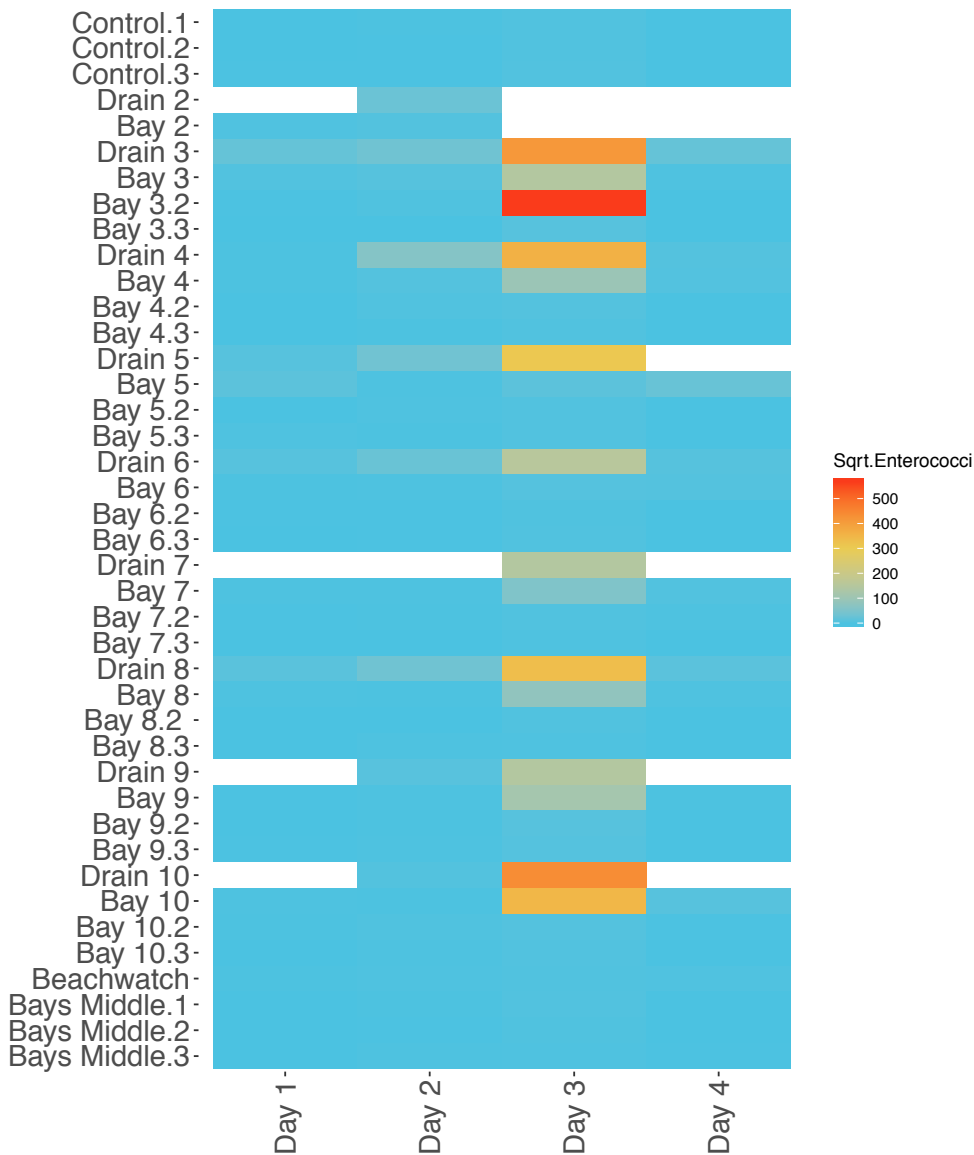
Following a light rainfall (3.8 mm) event on 27/8/19, Enterococci levels within all drains increased substantially, with average levels reaching 1,072 CFU 100 mL<sup>-1</sup> and exceeding the NHMRC maximum threshold for significant risk of illness (Category D; Table 2). However, substantial variability in Enterococci counts occurred between drains (Figure 2), with highest levels observed in Drains 3, 4 and 5. While sharp increases in Enterococci levels were observed in several drains, average levels generally remained very low (8.6 CFU 100 mL<sup>-1</sup>) within the seawater samples collected from Rose Bay, indicating minimal impact from the drains during this low rainfall event.

On 30/8/19, following 43 mm of rain, Enterococci levels within most samples increased substantially. Within drains, Enterococci levels became extremely high (average: 95,250 CFU 100 mL<sup>-1</sup>), with levels substantially exceeding the NHMRC maximum threshold for significant risk of illness (Category D; Table 2) in all tested drains. Across all drains, highest Enterococci levels were observed in Drains 10 (190,000 CFU 100 mL<sup>-1</sup>) and 3 (170,000 CFU 100 mL<sup>-1</sup>). During this period, Enterococci levels also increased dramatically within Rose Bay seawater samples, with average levels (18,268 CFU 100 mL<sup>-1</sup>) exceeding the NHMRC maximum threshold for significant risk of illness (Category D; Table 2) and significantly greater than levels observed at the 'pristine' control site at Nielsen Park (average: 21 CFU 100 mL<sup>-1</sup>). However, there was substantial spatial variability in the extent of impact within Rose Bay, with Enterococci levels significantly higher near to stormwater drains, relative to the offshore points in transects (Figure 3). Specifically, while average Enterococci levels within the most off-shore transect points only reached 26 CFU 100 mL<sup>-1</sup>, at the sampling points closest to the shore and drain outlet points, average Enterococci levels reached 21,590 CFU 100 mL<sup>-1</sup>, substantially exceeding the NHMRC maximum threshold for significant risk of illness (Category D; Table 2). Highest Enterococci levels were observed in samples collected between Drains 2 and 3 (320,000 CFU 100 mL<sup>-1</sup>; sample 3 -2) and adjacent to Drain 10 (120,000 CFU 100 mL<sup>-1</sup>).

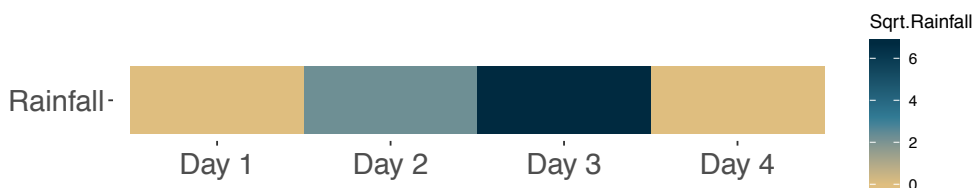
On the 3/9/19, following a period of 72 hours without further rainfall, Enterococci levels within Rose Bay dropped substantially. Within seawater samples, the average Enterococci levels were 30 CFU 100 mL<sup>-1</sup> (i.e. below the lowest health risk level (Category A) in the NHMRC Microbial Assessment Categories<sup>9</sup>). Among drains that could be sampled at this time (NB: drains 2, 7, 9 and 10 could not be sampled due to low flow levels), average Enterococci levels dropped to 269 CFU 100 mL<sup>-1</sup>, but remained high in Drains 3 (480 CFU 100 mL<sup>-1</sup>) and 5 (600 CFU 100 mL<sup>-1</sup>).

**Figure 2**

**A**



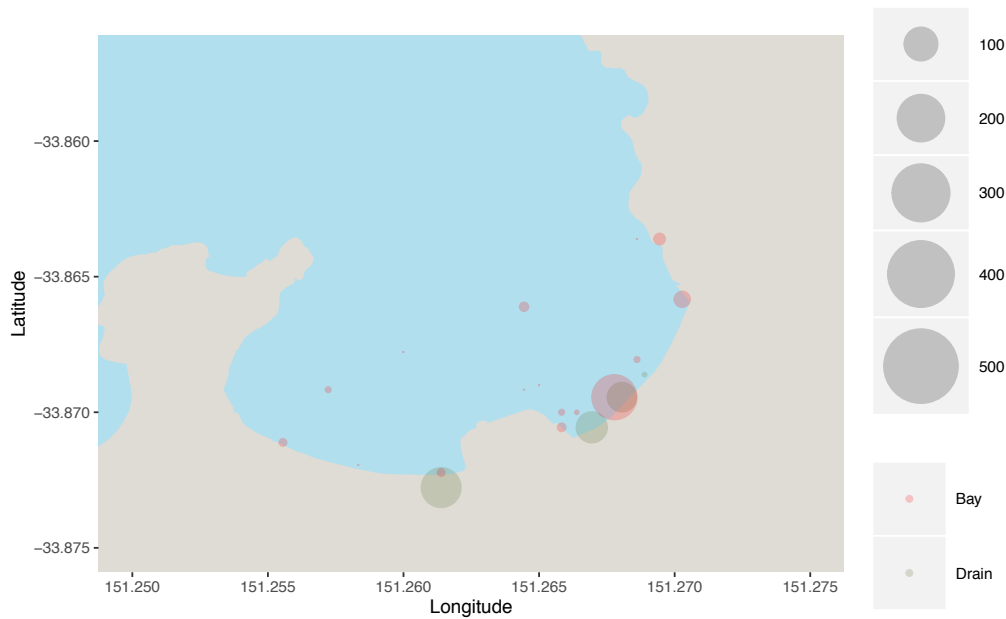
**B**



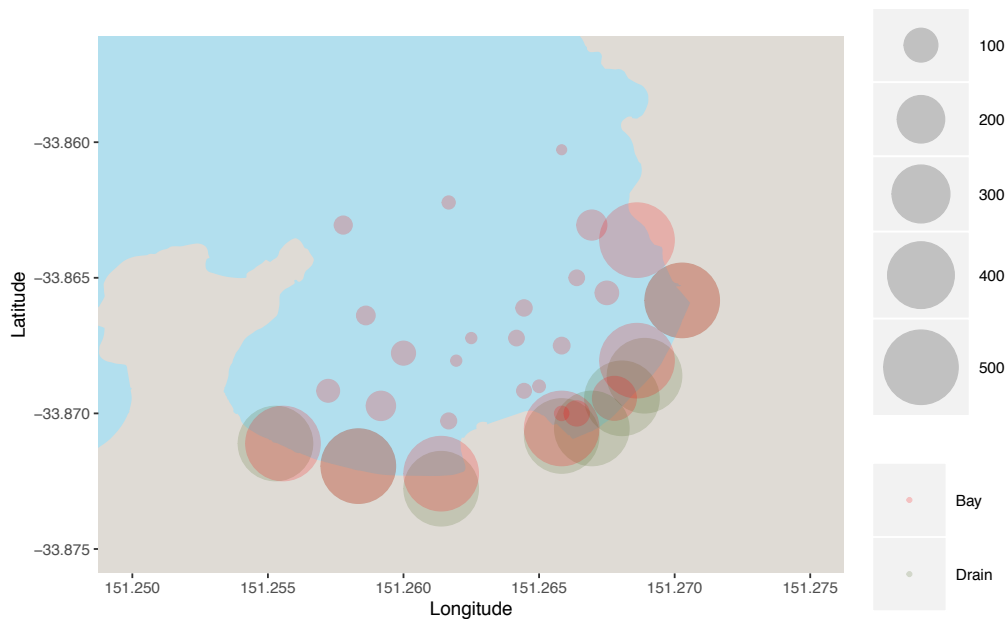
**Figure 2: (A)** Heatmap displaying distribution of Enterococci levels determined using standard membrane filtration techniques (AS/NZS 4276.9:2007) across sampling locations (Y axis) and days (x axis). Colour scale corresponds to square root of Enterococci count data. Blank cells represent samples not collected either due to lack of water flow in drains or low safety levels during the rainfall event. **(B)** Heatmap displaying intensity of rainfall on each sampling day. Colour scale corresponds to square root of rainfall data (mm).

Figure 3 \*

A



B



**Figure 3:** Distribution of Enterococci counts determined using standard membrane filtration techniques (AS/NZS 4276.9:2007) across sampling locations on **(A)** 21/8/19 - dry weather sample and **(B)** 30/8/19 – following 43 mm of rain. Bubble size scales to data magnitude bins (refer to side scale). Red bubbles correspond to seawater samples collected in Rose Bay; green bubbles correspond to samples collected in Drains.



## 4.2 Human Faecal Markers

Within Rose Bay seawater samples, the two human faecal marker genes employed here, Lachno3 and HF183, indicative of human gut microbiome associated *Lachnospiraceae* and *Bacteriodes* bacteria<sup>10,11</sup>, were detected in 79 and 61% of samples respectively. Across the entire dataset, concentrations of both markers were significantly ( $p < 0.01$ ) higher within Rose Bay water samples than at the 'pristine' control site at Nielsen Park, indicative of a significant impact of sewage within Rose Bay. However, the occurrence of human faecal marker genes changed markedly with both space and time (Figures 4 and 5), with the highest proportion of detections and highest concentrations of the marker genes observed close to stormwater drains and after rainfall. Both markers displayed moderate, but statistically significant correlations to total Enterococci counts (Lachno3:  $r = 0.363$ ,  $p = 0.012$ ; HF183:  $r = 0.365$ ,  $p = 0.0163$ ).

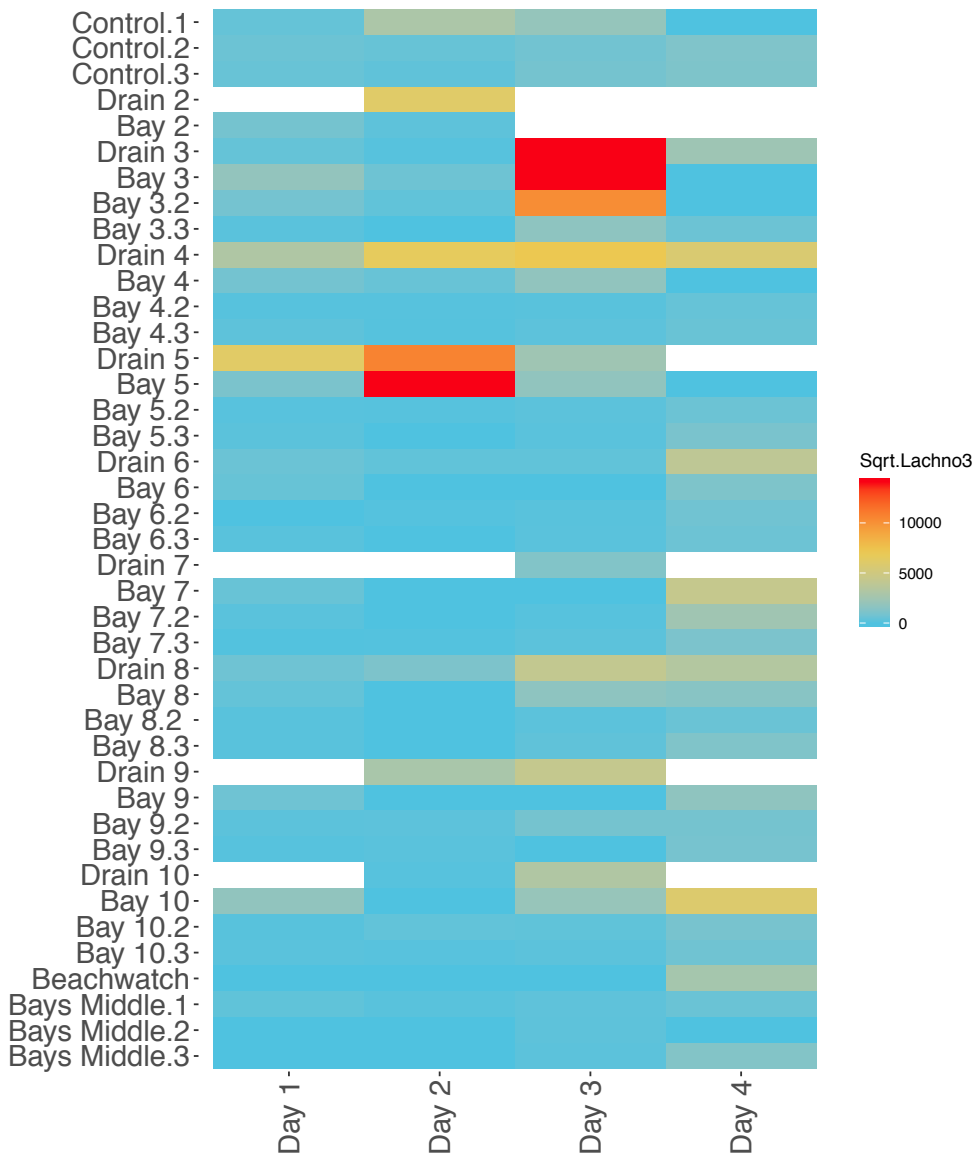
Under dry weather conditions, the Lachno3 and HF183 human faecal marker genes were detected in 90 and 50% of Rose Bay seawater samples respectively, but detectable concentrations were only 2.1 and 0.7 times, and not significantly ( $p > 0.05$  and 0.08, respectively) higher than, those observed within the Nielsen Bay control site. These relatively low concentrations are consistent with the low levels of Enterococci quantified within dry water samples. However, with the exception of HF183 in the Drain 9 transect, highest seawater concentrations of these human faecal markers were always observed in samples immediately adjacent to drains (Figures 4, 5, 6, 7). This pattern was in-line with the significantly higher ( $p < 0.002$ ) concentrations of both human faecal marker genes within the drain samples, where average concentrations of Lachno3 and HF183 were 24 and 23 times greater than in the seawater samples, with highest levels of both markers observed in Drain 5.

Following the light rainfall (3.8 mm) event on 27/8/19, concentrations of the Lachno3 and HF183 human faecal markers in drain samples increased by 2.6 and 22 times respectively, with highest concentrations again observed in Drain 5. Among Rose Bay seawater samples, Lachno3 increased by 17-fold relative to dry weather conditions, with highest concentrations observed in samples adjacent to Drain 5. Consistent with patterns observed in the Enterococci counts, concentrations of both markers were generally very low beyond the immediate shoreline (i.e. >250m offshore).

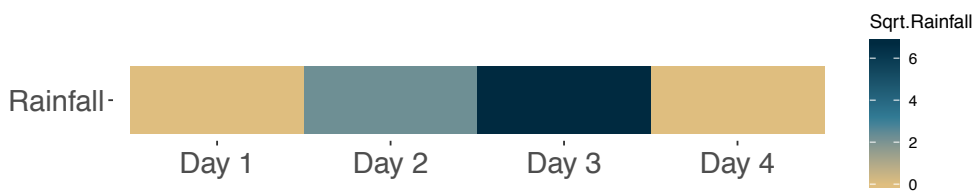
Significant ( $p < 0.006$ ) increases of both human markers occurred within drains and adjacent seawater samples following a significant rainfall event (49 mm) on 30/8/19. Across all drain samples, concentrations of Lachno3 and HF183 increased significantly ( $p < 0.03$ ) by 109 and 76 times relative to dry weather conditions, with highest concentrations of both markers observed within Drain 3 (Figures 6 and 7). The high concentrations of human faecal markers in Drain 3, were reflected within the Rose Bay seawater samples, with highest concentrations of both markers observed in Rose Bay transect samples adjacent to Drain 3 (Figures 6 and 7), where the highest seawater concentrations of human faecal markers recorded during this study period were observed. While a clear gradient in Lachno3 and HF183 was observed across the transect adjacent to Drain 3, in most other transects there was an immediate decay in human faecal marker levels beyond the sample collected

**Figure 4**

**A**



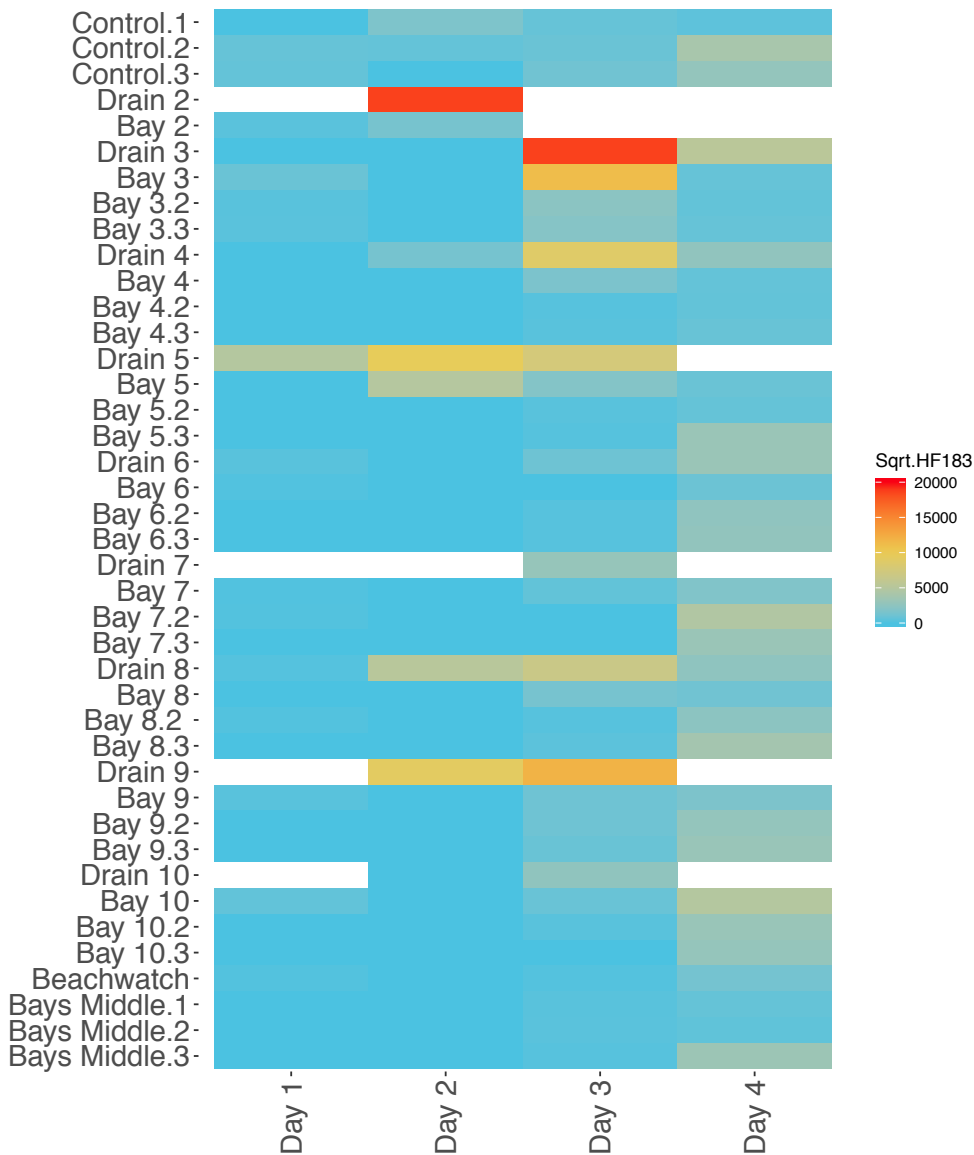
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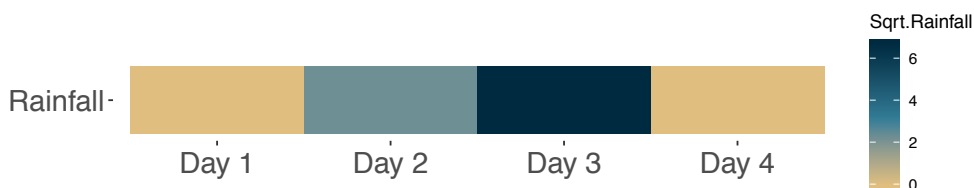
**Figure 4: (A)** Heatmap displaying distribution of *Lachno3* marker for the human faecal bacteria *Lachnospiraceae* (sewage marker) across sampling locations (Y axis) and days (x axis). Colour scale corresponds to square root of copy numbers defined using qPCR. Blank cells represent samples not collected either due to lack of water flow in drains or low safety levels during the rainfall event. **(B)** Heatmap displaying intensity of rainfall on each sampling day. Colour scale corresponds to square root of rainfall data (mm).

**Figure 5**

**A**



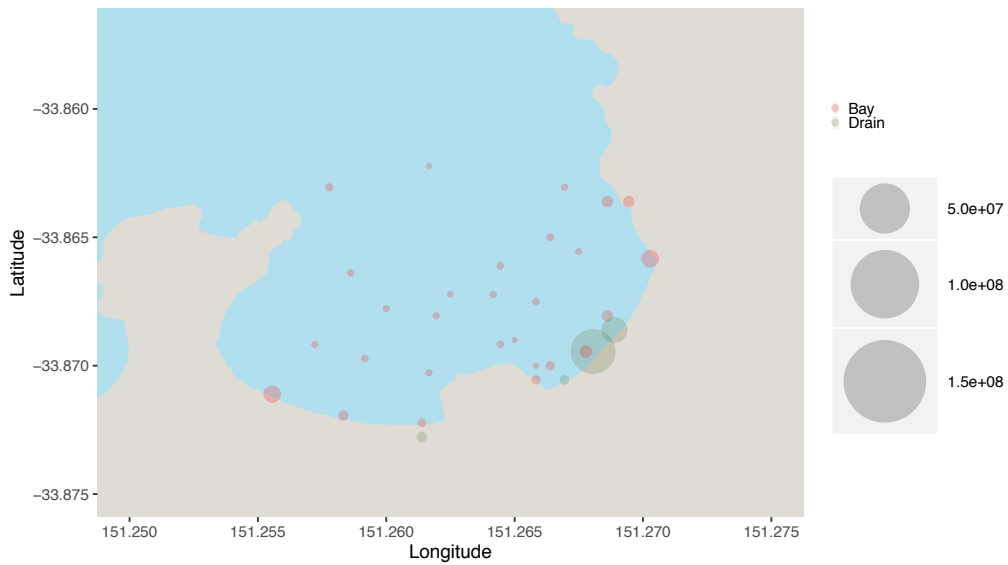
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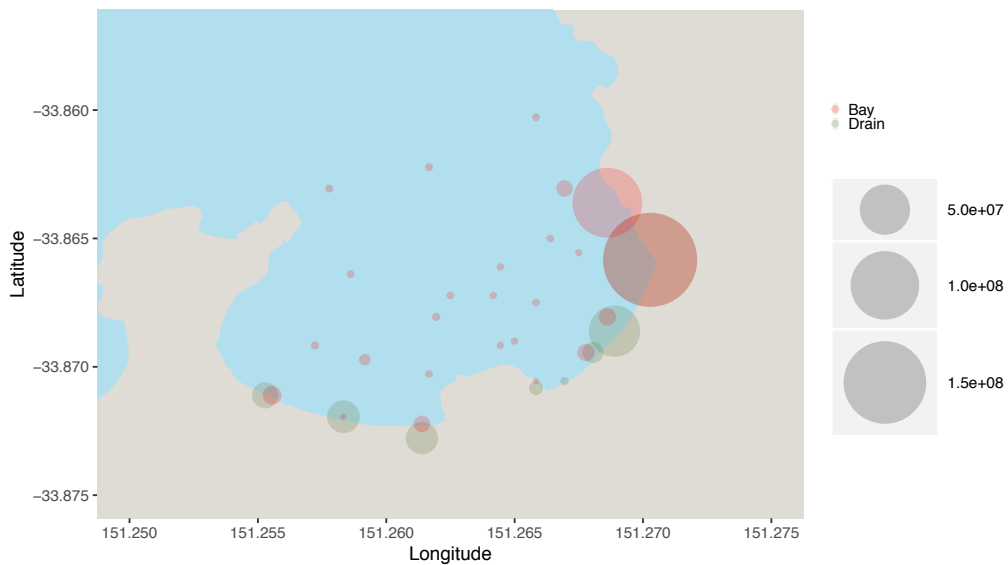
**Figure 5: (A)** Heatmap displaying distribution of HF183 marker for the human faecal bacteria *Bacteroides* (sewage marker) across sampling locations (Y axis) and days (x axis). Colour scale corresponds to square root of copy numbers defined using qPCR. Blank cells represent samples not collected either due to lack of water flow in drains or low safety levels during the rainfall event. **(B)** Heatmap displaying intensity of rainfall on each sampling day. Colour scale corresponds to square root of rainfall data (mm).

**Figure 6**

**A**



**B**



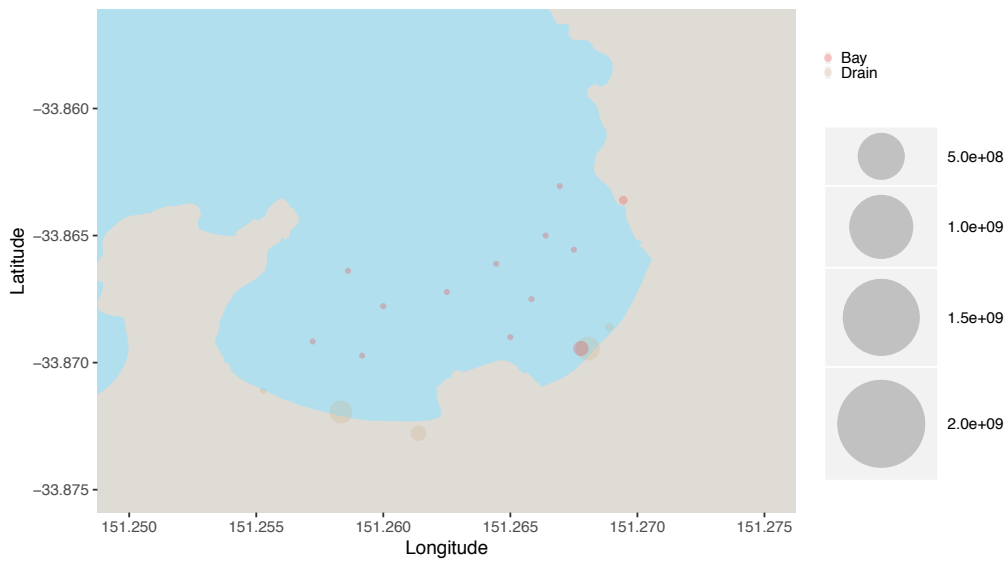
**Figure 6:** Distribution of *Lachno3* marker for the human faecal bacteria *Lachnospiraceae* (sewage marker) across sampling locations on **(A)** 21/8/19 - dry weather sample and **(B)** 30/8/19 – following 43 mm of rain. Bubble size scales to data magnitude bins (refer to side scale). Red bubbles correspond to seawater samples collected in Rose Bay; green bubbles correspond to samples collected in Drains.



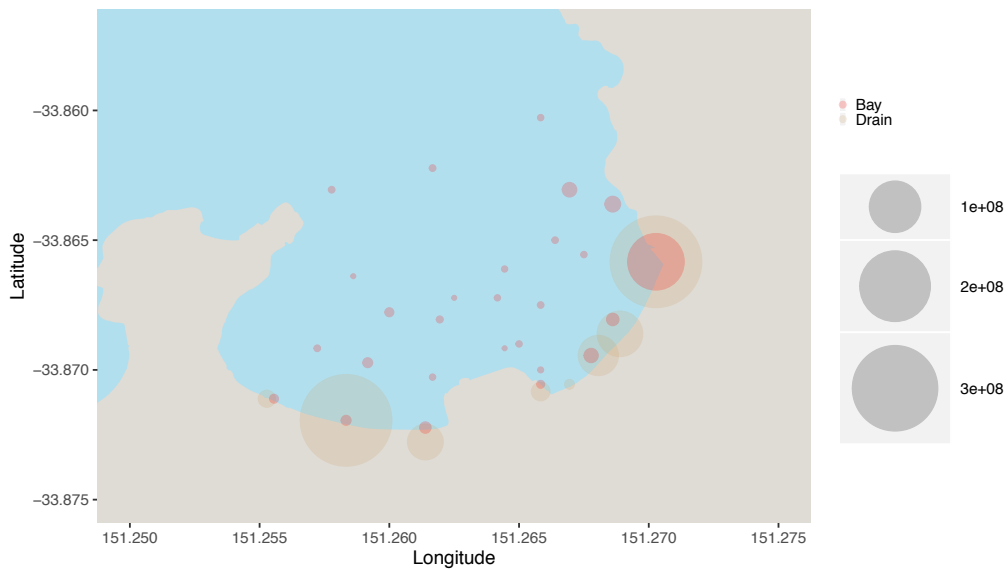


Figure 7 \*

A



B



**Figure 7:** Distribution of HF183 marker for the human faecal bacteria *Bacteroides* (sewage marker) across sampling locations on **(A)** 21/8/19 - dry weather sample and **(B)** 30/8/19 – following 43 mm of rain. Bubble size scales to data magnitude bins (refer to side scale). Red bubbles correspond to seawater samples collected in Rose Bay; green bubbles correspond to samples collected in Drains.



from proximate to the drain, which was consistent with the patterns observed in the Enterococci analysis.

Following a period of 72 hours without further rainfall (3/9/19), average concentrations of HF183 and Lachno3 dropped by over 11- and 60-times respectively (Figures 4 and 5). However, this pattern was highly variable among sampling locations and the two assays, with Lachno3 and HF183 levels in 63% and 44% of samples differing significantly ( $p < 0.05$ ) to the preceding time-point. Highest levels of the human faecal markers persisted in Drain 3, 4 and 6 (NB: drains 2, 5, 7, 9 and 10 could not be sampled at this time due to low flow) and water samples immediately adjacent to Drains 7 and 10. It is noteworthy, that levels of the Lachno3 and HF183 markers remained elevated in several seawater samples for 4 days after rainfall, and after Enterococci levels had decreased. Furthermore, a more pronounced spatial gradient in the occurrence of the HF183 marker in particular occurred at this time-point than the preceding time-points.

### 4.3 Animal Faecal Markers

#### 4.3.1 Bird Enterococci Marker

Across the study period, the GFD avian enterococci marker was observed in 79% of samples, but levels of this marker were not significantly (Pearson linear correlation  $p > 0.39$ ) correlated to Enterococci counts. Furthermore, levels of the GFD marker were not significantly elevated in Rose Bay seawater samples relative to the 'pristine' control site at Nielsen Bay during either dry ( $p > 0.7$ ) or wet ( $p > 0.14$ ) conditions. Unlike the human faecal markers, there was no trend of increasing levels of the GFD marker following rainfall, in either drains or seawater samples, with concentrations of this marker in fact decreasing following rainfall (Figures 8 and 9). Finally, concentrations of this marker remained within a range consistent with previous measurements within both pristine and impacted environments in NSW<sup>14</sup>, indicating that the levels detected here represent natural base-line concentrations of bird faecal material within coastal habitats.

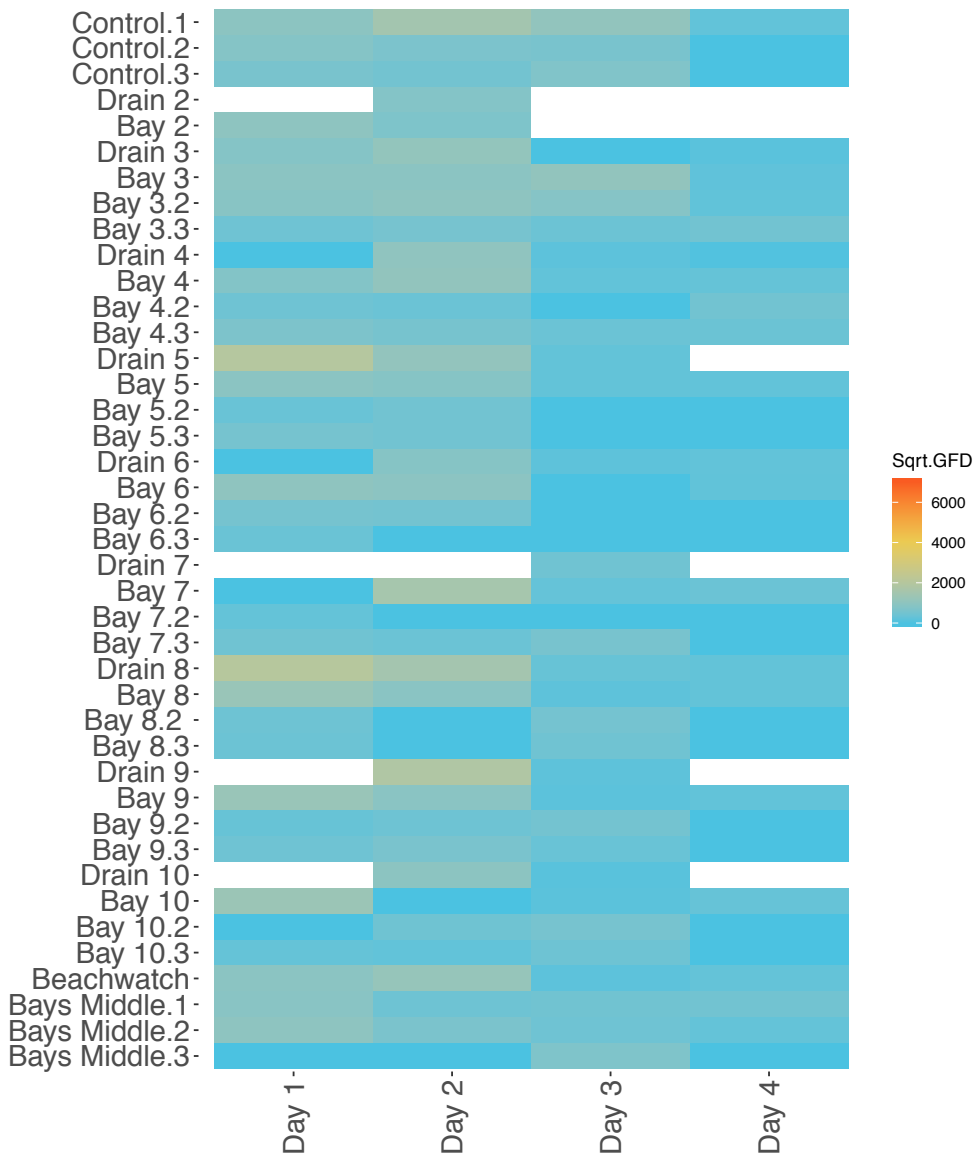
#### 4.3.2 Dog Bacteroides Marker

The DG3 marker for dog-faeces associated *Bacteroides* was detectable in quantifiable levels in only 37% of samples, but was significantly ( $r = 0.47$ ,  $p < 0.0025$ ) correlated to Enterococci counts. Under dry weather conditions (21/8/19), the DG3 marker was undetectable at the Nielsen Bay control site and was only observed in 22% of seawater samples at Rose Bay, but notably these were all near-shore sampling points (RBT3\_1 RBT4\_1 RBT5\_1 RBT6\_1 RBT9\_1 RBT10\_1). This dog faeces specific marker was not detected in any of the tested drain samples under dry weather conditions.

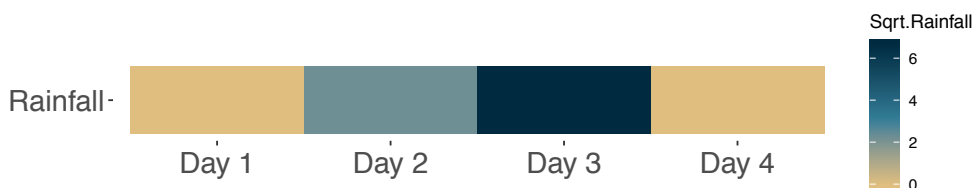
After the light rainfall (3.8 mm) event, the proportion of samples that the DG3 marker was detected in remained low (18%), yet the average concentration of the marker increased significantly ( $p < 0.0054$ ) by 6-fold. Notably, there was also a shift in the location of DG3 detections, with the bulk of detections observed in drain samples (highest concentrations observed in Drains 8 and 6).

Figure 8

A



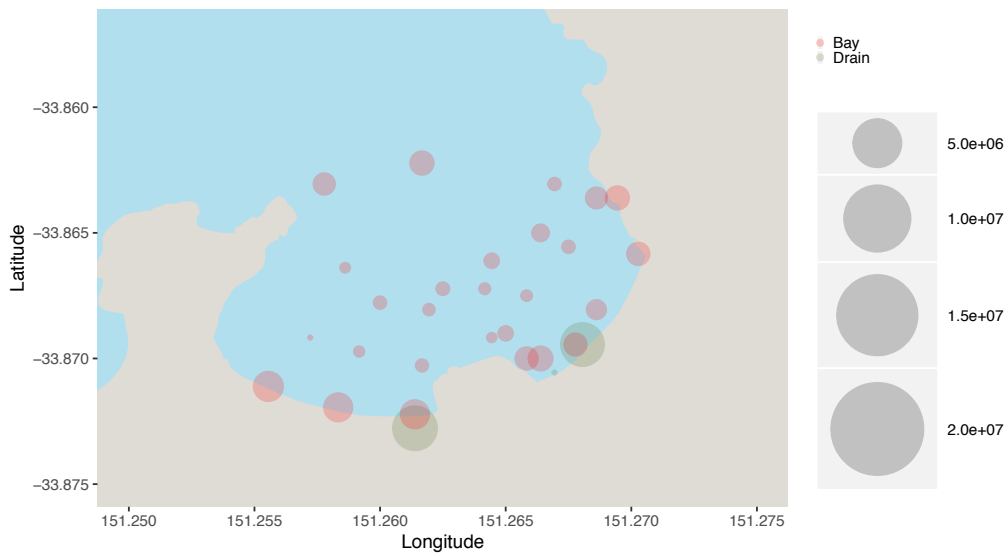
B



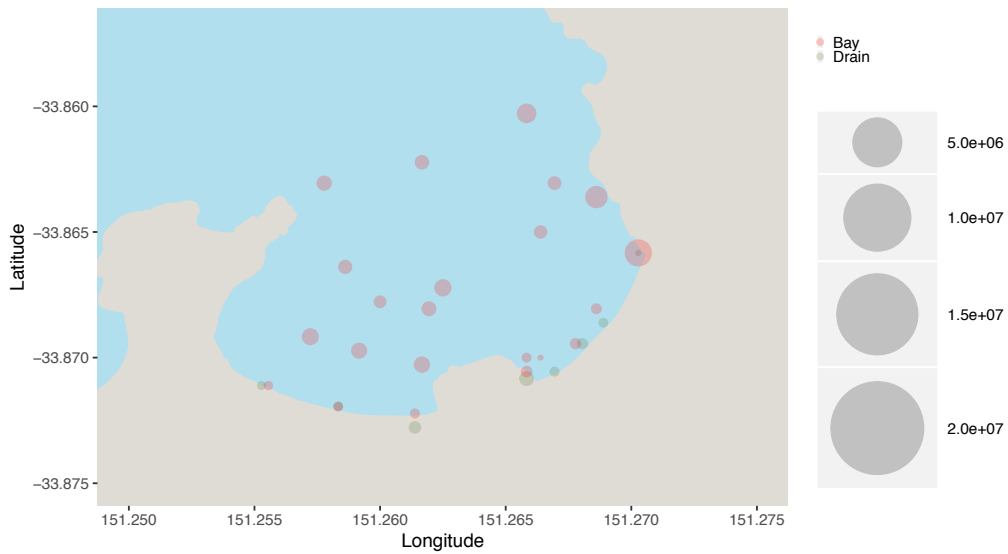
**Figure 8: (A)** Heatmap displaying distribution of GFD marker for the avian *Enterococci* (bird faeces) across sampling locations (Y axis) and days (x axis). Colour scale corresponds to square root of copy numbers defined using qPCR. Blank cells represent samples not collected either due to lack of water flow in drains or low safety levels during the rainfall event. **(B)** Heatmap displaying intensity of rainfall on each sampling day. Colour scale corresponds to square root of rainfall data (mm).

Figure 9 \*

A

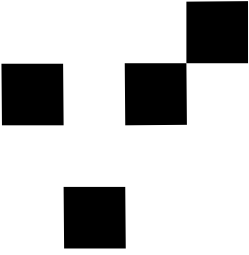




B




**Figure 9:** Distribution of GFD marker for the avian *Enterococci* (bird faeces) across sampling locations on **(A)** 21/8/19 - dry weather sample and **(B)** 30/8/19 – following 43 mm of rain. Bubble size scales to data magnitude bins (refer to side scale). Red bubbles correspond to seawater samples collected in Rose Bay; green bubbles correspond to samples collected in Drains.





Following the significant rainfall event on 27/8/19, the proportion of samples that the DG3 marker was detectable in increased to 74%, with average concentrations of this marker also increasing significantly ( $p < 0.0146$ ) relative to each of the preceding sampling days, with levels over 12 times higher than those observed during dry weather conditions (Figure 10). Highest concentrations of DG3 were observed in Drains 3, 5 and 8 (Figures 10 and 11).

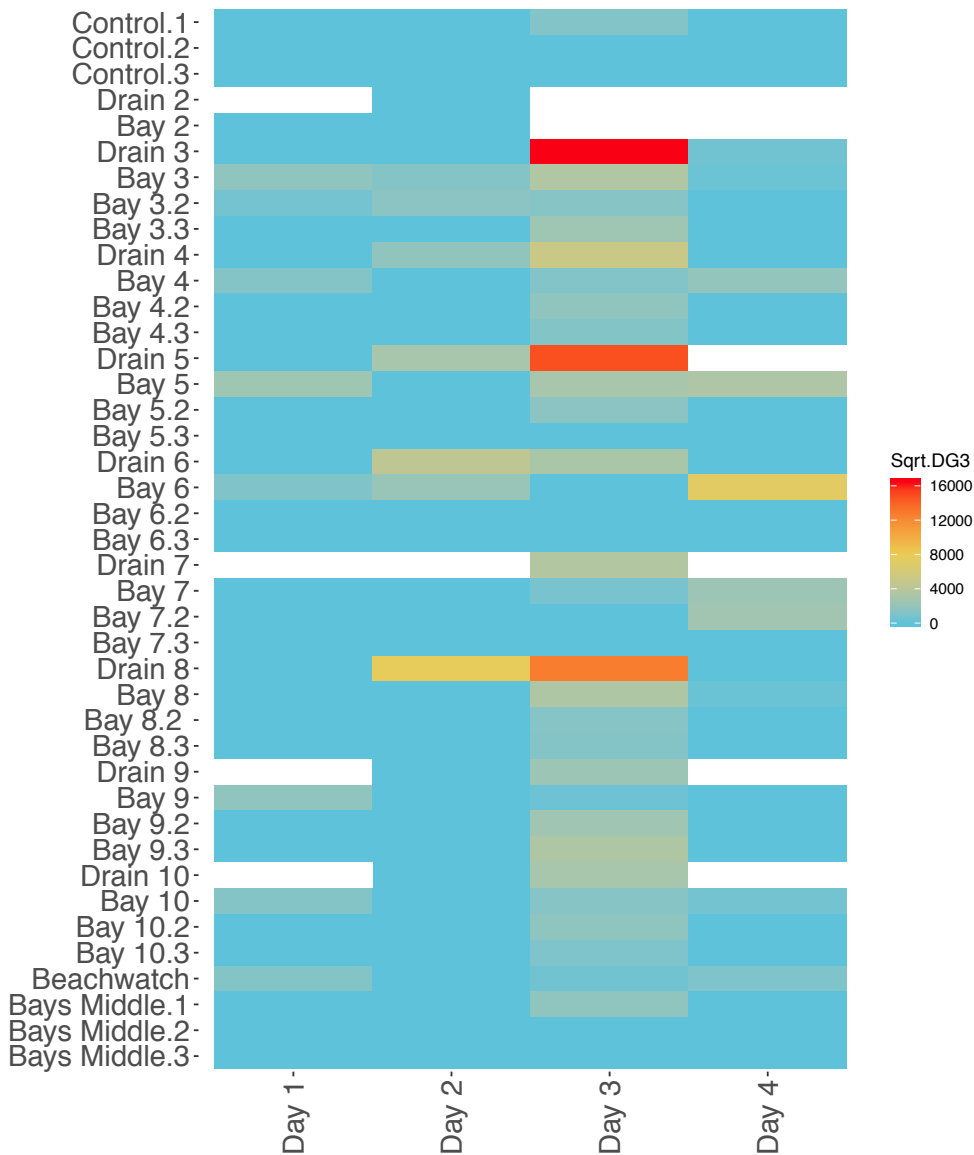


Three days after the rain event, the proportion of samples that the DG3 marker was detectable in quantifiable levels within decreased to 36%, but average levels were not significantly ( $p > 0.125$ ) different from those observed during the rainfall.

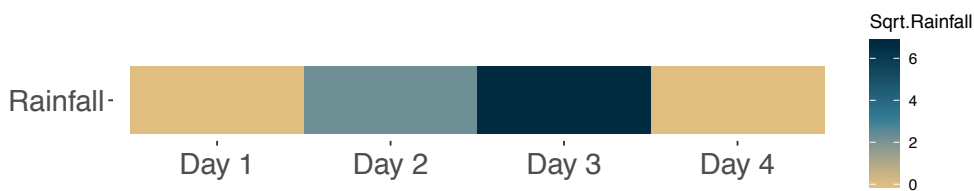


**Figure 10**

**A**



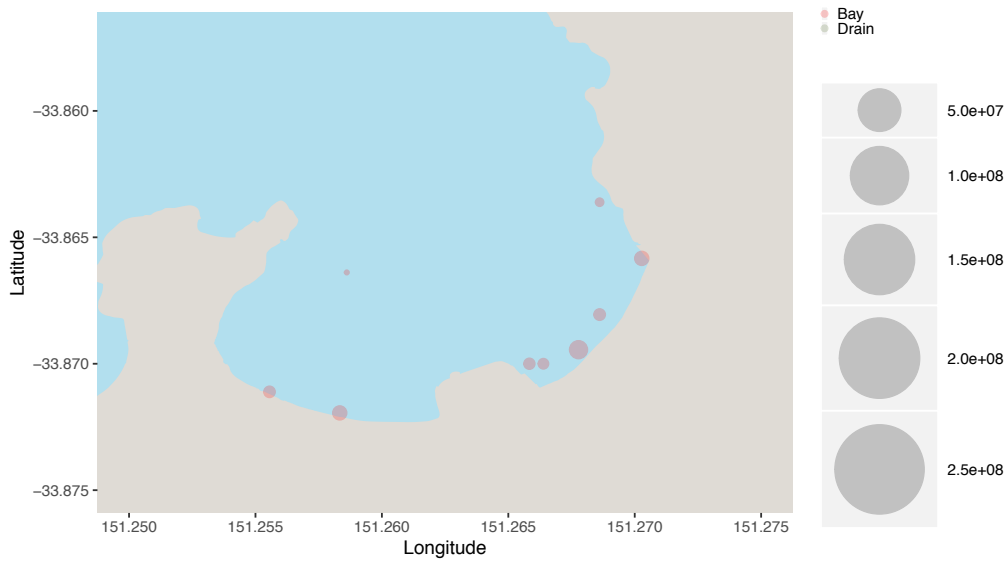
**B**



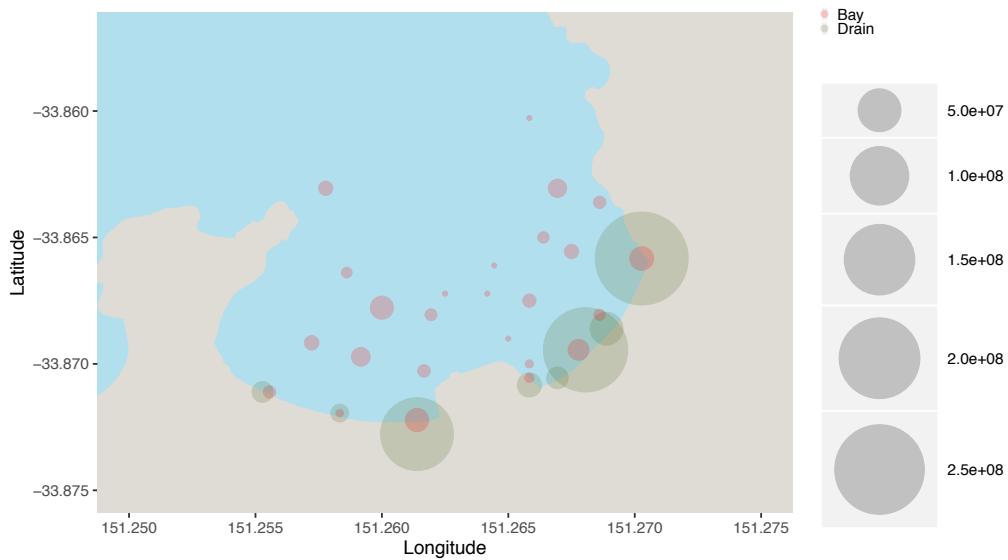
**Figure 10: (A)** Heatmap displaying distribution of DG3 marker for canine *Bacteroides* (dog faeces) across sampling locations (Y axis) and days (x axis). Colour scale corresponds to square root of copy numbers defined using qPCR. Blank cells represent samples not collected either due to lack of water flow in drains or low safety levels during the rainfall event. **(B)** Heatmap displaying intensity of rainfall on each sampling day. Colour scale corresponds to square root of rainfall data (mm).

Figure 11 \*

A



B



**Figure 11:** Distribution of DG3 marker for canine *Bacteroides* (dog faeces) across sampling locations on **(A)** 21/8/19 - dry weather sample and **(B)** 30/8/19 – following 43 mm of rain. Bubble size scales to data magnitude bins (refer to side scale). Red bubbles correspond to seawater samples collected in Rose Bay; green bubbles correspond to samples collected in Drains.



## 5.0 Interpretation of Results & Conclusions

This project applied a microbial source tracking approach to understand the causes and sources of poor recreational water quality at Rose Bay (NSW) by performing a targeted sampling campaign during the course of a significant wet-weather event. A previous microbial source tracking study performed at this location during dry weather conditions revealed that both sewage and dog faecal material are likely to contribute to poor water quality at Rose Bay<sup>14</sup>, but it was concluded that further sampling during a wet weather event would be informative for disentangling the relative importance of these impacts.

In the present study, significant increases in Enterococci counts were observed at Rose Bay following rainfall, which is consistent with previous observations<sup>4,14</sup>. Elevated Enterococci levels during the rain event were accompanied by increases in microbial markers for both human faecal material (sewage) and dog faeces. Highest levels of both enterococci counts and these markers were generally observed in stormwater drain outlets, with specific drains appearing to be key conduits of faecal contamination, which we address in detail below within the context of our 3 primary research objectives.

### ***Are high Enterococci levels at Rose Bay primarily caused by human (i.e. sewage) or animal sources of faecal contamination?***

Across the entire dataset, levels of both markers for human faeces (sewage) and the dog faeces marker displayed moderate, statistically significant correlations to Enterococci counts. This could imply either that both sewage and dog faeces contribute to the measured Enterococci levels within Rose Bay, or that the dynamics of the human and dog faecal markers are driven by the same environmental processes (e.g. same input point), with one contaminant governing Enterococci counts.

The marker for avian Enterococci (GFD) did not display a statistically significant correlation to total Enterococci counts, nor an increase associated with either rainfall or proximity to drains. Furthermore, given that (i) the levels of this bird faecal marker were not higher in Rose Bay than the pristine control site at Nielsen Park and (ii) GFD levels were always within the range of those observed in other NSW coastal habitats during dry weather conditions, we conclude that bird faeces played a minimal role in driving the elevated total Enterococci levels observed during rainfall.

During dry weather conditions, Enterococci levels within Rose Bay water samples were generally within the lowest health risk level (Category A) in the NHMRC Microbial Assessment Categories<sup>9</sup>, indicating good water quality. The single exception to this pattern was the sample collected adjacent to Drain 5 (RBT5\_1), where Enterococci levels were 180 CFU 100 mL<sup>-1</sup> (Category B). Notably, these levels were higher than those recorded in the adjacent drain. During this time, both human markers were highly elevated (relative to all other samples) within Drain 5.



However, the dog faecal marker DG3 was also elevated within RBT5\_1, but absent with the Drain 5 sample. These patterns imply one of two explanations for the moderate Enterococci levels observed in the RBT5\_1 sample under dry weather conditions: (1) A combination of sewage and dog faecal material sourced from Drain 5 has impacted this location; (2) Dog faecal material sourced from the beach has contributed to the high Enterococci levels measured at this location. Given that comparable levels of DG3 were recorded at other near-shore sites (RBT3\_1 RBT4\_1 RBT5\_1 RBT6\_1 RBT9\_1 RBT10\_1) that did not exhibit elevated Enterococci levels at this time, we propose that the elevated Enterococci levels within this sample were the result of a combinatory effect of human (sewage from Drain 5) and dog faecal material.

Following a substantial rainfall event, Enterococci levels increased significantly within both drain samples and Rose Bay water samples immediately adjacent to some drains. Within the drain and seawater samples where the highest Enterococci levels occurred (i.e. Drain 3 and adjacent seawater samples, Drains 4, 8 and 10), increases in both human faecal markers and the dog faecal marker were observed. In Drain 3, a substantial peak in both human faecal markers was observed, while the elevated seawater Enterococci levels spanning the Drain 3 transect into Rose Bay were mirrored by increases in the human faecal markers. We conclude that Drain 3 and the surrounding waters within Rose Bay experience the most pronounced influence of sewage during rainfall events. However, in this drain, as well as several of the other drains experiencing high Enterococci levels during the rainfall event (specifically Drains 4, 5, 6 and 8), significant peaks in the dog faeces marker DG3 co-occurred with peaks in the human faecal markers. This indicates that both sewage and dog faeces potentially contribute to the high Enterococci levels observed in stormwater drains during rainfall at Rose Bay, which is a pattern consistent with reports from other coastal environments in NSW<sup>15</sup>. We propose that a useful avenue for future research will be to identify the source of dog faeces occurring within the stormwater network (i.e. run-off from parks, roads or from the sewage system). However, it is notable that the average concentration of the dog faecal marker in these drain samples was substantially lower than the human faecal markers (12.1 and 3.6 times lower than the Lachno3 and HF183 markers, respectively), suggesting a smaller contribution to faecal contamination and Enterococci levels than sewage<sup>15</sup>.

In conclusion, the results of this microbial source tracking exercise indicate that both sewage and dog faeces contribute to high Enterococci counts observed at Rose Bay during periods of significant rainfall. The main points of input of both forms of faecal material are stormwater drains, which appear to experience contamination from sewage and dog faecal material from the catchment.

### ***What are the primary points of contamination within Rose Bay?***

During both dry and wet weather conditions it is clear that the network of stormwater drains at Rose Bay are the key source of seawater contamination, rather than the surrounding beach environment. However, some drains had a greater influence than others, with the level of impact also varying according to whether sampling was conducted during dry or wet weather periods.

Under dry weather conditions, Drain 5 exhibited elevated levels of both of the human faecal markers, with these levels increasing further and extending into the adjacent seawater sample after the first moderate (3.8 mm) rainfall event. We suggest that these patterns are potentially indicative of a dry weather sewage leak into Drain 5, which may have contributed to the slightly elevated Enterococci levels within the RBT5\_1 seawater sample during dry weather. Notably, Drain 5 is adjacent to a sewage pumping station behind Rose Bay beach, which may contribute to these patterns and is potentially worthy of further examination.

Following the major rainfall event, highly elevated Enterococci levels occurred in all drains, with highest levels within Drains 3, 4, 8 and 10. Notably, the bay samples adjacent to several of these drains also showed highly elevated Enterococci levels, indicating a substantial impact on seawater quality in Rose Bay. Both human faecal markers were highly elevated within each of these drains, and adjacent bay samples, with Drains 3 and 10 clearly hotspots of sewage contamination. Given that concentrations of the human faecal markers became significantly elevated within these drains following the major rainfall event, we suggest that these two drains potentially represent sites most influenced by wet weather sewage overflows. However, it is notable that flow rates within these drains are generally lower than those in Drains 4 and 8 following rainfall, meaning that total microbial loads from Drains 4 and 8 might in fact have a larger impact on over-all water quality at Rose Bay. Future research coupling measurements of faecal marker concentrations with flow rate measurements will therefore be illuminating.

### ***What are the spatial and temporal dynamics of water contamination within Rose Bay during a wet weather event?***

The sampling design employed during this study permitted a detailed investigation of the spatial and temporal patterns of multiple markers for faecal contamination over the course of dry weather conditions, a moderate rainfall event, an intense rainfall event and dry 'recovery' period, several days after a rainfall event. This analysis revealed that Enterococci levels within Rose Bay increase significantly following rainfall and with proximity to stormwater drains, with this pattern largely driven by sewage contamination of the drains, with a further contribution from dog faecal material likely sourced from the catchment serviced by these drains.

The sampling regime, involving on-shore to off-shore transects, revealed that for the most part, very high Enterococci counts and levels of the human and dog faecal markers were restricted to near-shore samples. A notable exception for this pattern was the transect adjacent to Drain 3, which displayed highly elevated levels of the human faecal markers and Enterococci levels across several samples extending away from the shoreline. This is indicative of a substantial influence of Drain 3 on water quality within Rose Bay following rainfall. It should, however, be noted that the high levels of contamination within the Drain 3 transect samples may have also been influenced by Drain 2, which is located adjacent to this transect, but could not be safely sampled during the wet weather conditions. We suggest that, if possible, an increased focus on Drain 2 would be desirable in future studies.

Four days after the major rainfall event, Enterococci levels within Rose Bay decreased to the low levels observed prior to the rainfall event, indicating that water quality was suitable for swimming. However, it is notable that slightly elevated levels of both of the human faecal markers (in particular HF183) persisted within the environment. There are two potential explanations for this pattern: (i) The *Lachnospiraceae* and *Bacteriodes* bacteria targeted by the Lachno3 and HF183 markers can persist for longer periods in seawater than Enterococci, or (ii) The DNA-based, rather than culture-dependent, qPCR based approach used to quantify *Lachnospiraceae* and *Bacteriodes* detects unviable bacteria that would not grow via a culture-based approach.

### **Conclusions**









This research reveals that water quality at Rose Bay is detrimentally impacted by faecal contamination, largely linked to a network of stormwater drains servicing the surrounding catchment, with the level of impact substantially amplified following rainfall.

Under dry weather conditions, animal faecal material is omni-present, but given the low Enterococci levels generally observed during dry periods, probably has minimal impact on water quality at the site. Levels of bird faecal material at Rose Bay were not significantly higher than the pristine control site and are likely representative of natural background levels experienced along the NSW coastline. Bacteria associated with dog faeces were detectable in low levels in several near-shore water samples, but did not drive elevated Enterococci counts, which we conclude indicates a negligible influence of dog faeces on water quality at Rose Bay during dry weather conditions. Under dry weather conditions, Drain 5 exhibited relatively high levels of both human faecal markers, which were also present in adjacent seawater samples after a moderate rainfall event, which we conclude is indicative of sewage contamination of this drain even under dry weather conditions.

Following a significant rainfall event, substantial increases in Enterococci counts, occurred in both drains and adjacent seawater samples, where levels exceeded the NHMRC maximum threshold for significant risk of illness. These increases were mirrored by substantial increases in human faecal markers, indicating the potential influence of wet weather sewage overflow into several drains, in particular Drains 3 and 10. Occurrence of these human faecal markers within seawater samples collected adjacent to drains confirm that sewage impacts water quality within Rose Bay during wet weather. Co-occurrence of elevated levels of the dog faeces marker with human faeces markers in several drains during rain is indicative of an impact of animal faeces within stormwater derived from the surrounding catchment. However, given that concentrations of the dog faeces marker were substantially lower than the human faeces markers, the influence of this source of contamination appears to be less than the impact of sewage.

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