Molecular microbiological approaches reduce ambiguity about the sources of faecal pollution and identify microbial hazards within an urbanised coastal environment

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Keywords

Water Quality, Microbial Source Tracking, qPCR, 16S rRNA gene amplicon sequencing, Antibiotic Resistance Genes, Arcobacter.

Abstract

Urbanised beaches are regularly impacted by faecal pollution, but management actions to resolve the causes of contamination are often obfuscated by the inability of standard Faecal Indicator Bacteria (FIB) analyses to discriminate sources of faecal material or detect other microbial hazards, including antibiotic resistance genes (ARGs). We aimed to determine the causes, spatial extent, and point sources of faecal contamination within Rose Bay, a highly urbanised beach within Sydney, Australia's largest city, using molecular microbiological approaches. Sampling was performed across a network of transects originating at 9 stormwater drains located on Rose Bay beach over the course of a significant (67.5 mm) rainfall event, whereby samples were taken 6 days prior to any rain, on the day of initial rainfall (3.8mm), three days later after 43mm of rain and then four days after any rain. Quantitative PCR (qPCR) was used to target marker genes from bacteria (i.e., *Lachnospiraceae* and *Bacteroides*) that have been demonstrated to be specific to human faeces (sewage), along with gene sequences from *Heliobacter* and *Bacteriodes* that are specific to bird and dog faeces respectively, and ARGs (*sulI, tetA, qnrS, dfrA1 and vanB*). 16S rRNA gene amplicon sequencing was also used to discriminate microbial signatures of faecal contamination. Prior to the rain event, low FIB levels (mean: 2.4 CFU/100ml) were accompanied by generally low levels of the human and animal faecal markers, with the exception of one transect, potentially indicative of a dry weather sewage leak . Following 43 mm of rain, levels of both human faecal markers increased significantly in stormwater drain and seawater samples, with highest levels of these markers pinpointing several stormwater drains as sources of sewage contamination. During this time, sewage contamination was observed up to 1000 m from shore and was significantly and positively correlated with often highly elevated levels of the ARGs *dfrA1, qnrS, sulI* and *vanB*. Significantly elevated levels of the dog faecal marker in stormwater drains at this time also indicated that rainfall led to increased input of dog faecal material from the surrounding

catchment. Using 16S rRNA gene amplicon sequencing, several indicator taxa for stormwater contamination such as *Arcobacter spp*. and *Comamonadaceae spp*. were identified and the Bayesian SourceTracker tool was used to model the relative impact of specific stormwater drains on the surrounding environment, revealing a heterogeneous contribution of discrete stormwater drains during different periods of the rainfall event, with the microbial signature of one particular drain contributing up to 50% of bacterial community in the seawater directly adjacent. By applying a suite of molecular microbiological approaches, we have precisely pinpointed the causes and point-sources of faecal contamination and other associated microbiological hazards (e.g., ARGs) at an urbanised beach, which has helped to identify the most suitable locations for targeted management of water quality at the beach.

1.0 Introduction

Beaches and estuaries used for recreational purposes deliver substantial societal and economic benefits (Bockstael et al., 1987; Lau et al., 2019). However, particularly when located within urbanised settings, these environments often experience reduced water quality, which can sometimes cause substantial public health risks (McLellan et al., 2015). Contamination of coastal waters can, often simultaneously, be derived from diverse sources, including urban and industrial runoff (Nevers et al., 2018), faeces from native (Nguyen et al., 2018) and domesticated animals (Green et al., 2014b), and sewage (McLellan et al., 2015). However, pinpointing the principal causes of contamination and subsequent degradation of water quality can often be challenging, impeding effective management.

Urban stormwater, which is washed into coastal environments in large volumes after rainfall events (Tsihrintzis and Hamid, 2001), can contain potential pathogens delivered into the environment from numerous sources, such as the surfaces of built infrastructure, soils and, animal faeces (McLellan et al., 2015). Stormwater pipes are also often contiguous to sewerage infrastructure and can experience sewage inputs during dry weather due to pipe blockages and leaks, and following rainfall during wet weather sewer over-flow events. As a result, untreated sewage can often be introduced to aquatic environments through stormwater infrastructure (Olds et al., 2018).

Contamination of urbanised beaches by sewage is a major public health concern (WHO, 2009) because sewage is enriched in pathogens, including viruses such as *Rotavirus*, *Norovirus* and *Adenovirus* (Strubbia et al., 2019) protistan parasites such as *Giardia duodenalis* and *Cryptosporidium* (Efstratiou et al., 2017), and bacteria such as *Escherichia coli* (Anastasi et al., 2012) and *Arcobacter* species (Fisher et al., 2014). The global economic impact of human illness linked to faecal contamination of coastal waters has been estimated to exceed $12 billion a year (Shuval, 2003).

In addition to pathogens, sewage and impacted water infrastructure also contains other microbiological hazards, including high levels of antibiotic resistant microbes (Karkman et al., 2019). Widespread use of antibiotics in medicine and agricultural settings (Kunin et al., 1973) results in large quantities of these chemicals in wastewater, which are difficult to remove during wastewater treatment (Ahmed et al., 2015). As a result, microbial assemblages inhabiting human waste-streams are exposed to consistently high levels of antibiotics (Michael et al., 2013), resulting in selection for antibiotic resistance (Bougnom et al., 2019). In addition, antimicrobial resistance can also accumulate in the guts of medicated patients, who then shed resistant bacteria into sewage (Steinbakk et al., 1992). These resistant bacteria can be introduced into coastal environments during stormwater and sewage over-flows (Carney et al., 2019), where they can pose a public health risk (Leonard et al., 2018).

Faecal contamination of aquatic habitats can also originate from agricultural (Dwight et al., 2005), domestic (Ervin et al., 2014) and native animals (Araújo et al., 2014), and can enter the environment in urban and agricultural (Lewis et al., 2019) runoff or via animals excreting their faeces directly into the environment(Araújo et al., 2014; Lewis et al., 2019). While often not given the same attention as sewage contamination, high levels of contamination by animal faeces can also potentially pose a human health hazard because animal faeces also harbour both pathogens (Sobsey et al., 2011) and antibiotic resistance bacteria (Ortega-Paredes et al., 2019).

Most coastal water quality monitoring programs quantify the presence of faecal contamination in the environment using standardised approaches to enumerate specific microbes, referred to as faecal indicator bacteria (FIB). FIB include bacterial species (e.g., *Escherichia coli*, *Clostridium perfringens* and *Enterococci* (WHO, 2009) that reside in the faeces of warm-blooded animals (Layton et al., 2010), but rarely exist in significant numbers within uncontaminated waters, meaning they can be used as indicators of faecal contamination (Byappanahalli et al., 2012). FIB-based assessments of coastal water quality, however, have

two significant limitations. Firstly, most FIB are not restricted to human faeces (sewage) but are present in the faeces of many other warm-blooded animals (Byappanahalli et al., 2012), which can create ambiguity around the true source of faecal contamination (i.e., human vs animal) within an environment. The second limitation of FIB methods is that they are insensitive to other microbial hazards in the environment, including endemic aquatic pathogens (Fisher et al., 2014), antibiotic resistance genes (ARGs) (Carney et al., 2019) and other emerging pathogens present in wastewater infrastructure (McLellan and Roguet, 2019). These limitations can both obfuscate the source of contamination and overlook potential health hazards.

To overcome the shortcomings of FIB analyses, Microbial Source Tracking (MST) approaches, have been increasingly adopted to deliver more precise information on the sources of faecal contamination in natural environments (Ahmed et al., 2020). MST typically employs molecular microbiological methods, such as quantitative PCR (qPCR) (Feng et al., 2018; Green et al., 2014a, 2014b, 2012; Templar et al., 2016), and more recently DNA sequencing approaches (Brumfield et al., 2021; Newton et al., 2013a), to quantify specific microbial marker genes. Whilst one potential caveat of DNA-based approaches is that they may sometimes detect a signal from unviable cells, potentially leading to over-estimates of impact, they have continuously been able to unambiguously identify sources of faecal contamination within an environment (Ahmed et al., 2020, 2019; Alm et al., 2018; Green et al., 2019; Li et al., 2021; Shrestha et al., 2020).

Urbanised coastal ecosystems are regularly negatively impacted by a wide variety of contamination sources, which can enter the environment from multiple potential sources, and it is often very difficult for managers to pinpoint both the cause and point sources of contamination. Here we used an urbanised beach as a model environment to demonstrate the utility of microbial source tracking and DNA sequencing approaches to elucidate the source

(i.e., animal faeces vs sewage) and location of faecal contamination. Our principal goals were to determine the source and location of input of fecal contamination using a combination of FIB and MST qPCR techniques, and then to detail the spatial and temporal distribution of contamination using 16S rRNA amplicon sequencing.

2.0 Methods

2.1 Sampling Sites

Rose Bay is an urban beach located in the Sydney Harbour estuary and near to the centre of Sydney, which is Australia's largest city, with a population of over 5 million people. This site was targeted based on consistently poor water quality ratings, according to regularly high FIB levels, within the regional water quality monitoring program *Beachwatch* (DPIE, 2020). The beach receives stormwater runoff from nine drain networks, which feed directly onto the beach and is also a popular dog-walking beach.

Water samples were collected from a network of 10 transects, comprising a total of 41 sampling locations (Figure 1), which were chosen according to proximity to potential points of contamination. These included sampling points within the outlets of 9 stormwater drains, which are mainly conduits for urban stormwater, but in some instances may be impacted by wet weather sewer overflow events. Water samples were collected from points located along transects originating at points adjacent to each of these drains, to examine the extent of dispersal of contamination from drains into Rose Bay. Surface seawater samples were collected along the transect from immediately adjacent to drains at the shoreline in water of 50cm depth [x.1], 250m offshore [x.2] and 500m offshore [x.3]. Samples were also collected from a suite of reference points, including the 'Beachwatch' routine monitoring site located at the western end of Rose Bay, and a deep-water transect across the entrance to Rose Bay (x.4), located 1000m offshore and used here to determine the broader spatial extent of faecal pollution from

Rose Bay and the background contamination in Sydney Harbour. Finally, samples were also collected from a 'Control' site, within Nielsen Park (Sydney Harbour National Park), which is located 2km from Rose Bay, has no urban stormwater infrastructure and dogs are prohibited on the beach.

Sampling was conducted over the course a significant rainfall event, which involved a total of 69.8 mm of rain falling over a period of five days, including 43 mm within one 24-hour period. Samples were collected from the locations described above on four occasions, corresponding to six 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 four days after rain (3/9/19). Of note, drains 2, 7, 9 and 10 did not have sufficient flow to be sampled on the 21/8/19, nor did drain 7 on the 27/8/19 or drains 2, 5, 7, 9 and 10 on the 3/8/19.

2.2 Sample Processing and Analyses

Water samples were collected using 10L pre-sterilised plastic containers and filtered through 47mm, 0.22 µm pore-size membrane filters (Millipore, DURAPORE PVDF .22UM WH PL) using a peristaltic pump (100rpm), within 2hrs of sample collection. Before each sample was filtered 250ml 10% bleach was run through the pump, followed by 500ml MiliQ water, and then 1L of sample. Filters were stored at -80°C, until DNA was extracted using the PowerWater DNA isolation Kit (QIAGEN). DNA extractions were performed in batches of either 48 or 96, with every batch including 3 kit blanks, which were subsequently included in in all qPCR analyses. Physiochemical parameters including temperature, dissolved oxygen, salinity, and pH were measured in situ using a WTW multiprobe meter (Multi3430, Germany). Salinity ratio was calculated as per methods described by (Ho et al., 2021) (Supplementary Material Section 1.2).

To quantify Chl-a concentrations, a 110ml water sample was taken at each site and filtered through a 0.45 μm glass fibre filter. The filter was immediately frozen and returned to the laboratory for analysis of Chl-a concentration using a modified APHA Method 10200-H (Eaton and Franson, 2005).

At all sites, three water samples were collected for nutrient analysis. One unfiltered sample was collected using a disposable syringe and transferred directly into a 30ml sterile vial for total nutrient analysis. Two additional samples were collected and passed through a 0.45mm cellulose acetate syringe filter into two additional tubes for dissolved total and inorganic nutrient analysis. All nutrient samples were immediately frozen prior to analysis using standard methods; Nitrate and Nitrite (APHA 4500-NO3-I -Cadmium reduction method), Ammonium N (APHA 4500-NH3-H: Phenate method), filterable reactive phosphorus (FRP) (APHA 4500-P-E-Ascobic acid method), Total Nitrogen (TN), Total Phosphate (TP), Total Dissolved Nitrogen (TDN), Total Dissolved Phosphate (TDP) (APHA 4500-P-J: Persulfate digestion method) (Eaton and Franson, 2005).

2.3 Enterococci analysis

Levels of the FIB *Enterococci* were measured using standard membrane filtration techniques at a commercial diagnostic laboratory following the Australian standard (AS/NZS 4276.9:2007).

2.4 Quantitative PCR (qPCR) analysis of MST markers

Quantitative PCR (qPCR) targeting the bacterial 16S rRNA gene, using the BACT1369F and PROK1492R primer pair and the TM1389F probe was used to provide a measure of bacterial abundance within each sample (Suzuki et al., 2000). To detect the presence of human faeces, we employed two qPCR analyses, including the HF183 assay (Templar et al.,

2016), which targets human gut microbiome-associated HF183 *Bacteroidales* cluster and the Lachno3 assay (Feng et al., 2018), which targets human gut microbiome-associated *Lachnospiraceae*. To detect faeces from dogs, we used the DG3 assay (Green et al., 2014b), which targets dog-specific *Bacteroidales,* and to detect faeces from birds we used the GFD assay (Green et al., 2012), which targets bird-specific *Heliobacter* (Table 1, Supplementary Material).

QPCR was also used to quantify a suite of antibiotic resistance genes (ARGs) that have previously been detected in high abundances at two Sydney beaches exposed to wet weather associated sewage incursions (Carney et al., 2019), including the genes *sulI* which confers resistance to sulfonamide antibiotics in gram-negative bacteria (Huovinen et al., 1995), *tetA* which encodes an inner membrane protein antiporter (Allard 1992) which aids resistance to tetracycline, *qnrS* encodes a pentapeptide protein that defends DNA gyrase and topoisomerase IV from inhibition by quinolone (Berglund et al., 2014), *dfrA1* which encodes a dihydrofolate reductase that confers resistance to the antibiotic trimethoprim (Lombardo et al., 2017) and *vanB* which encodes resistance to vancomycin (Berglund et al., 2014), which is a last line of defence antibiotic (Berglund et al., 2014). Each qPCR assay was performed using a BIO-RAD CFX384 Touch™ Real-Time PCR Detection System™. (For QPCR assay details see Table 1, Supplementary Material).

In each case, gene copies were calculated for each target, using a (6-7 point) standard curve using BIO-RAD's CFX MAESTROTM software version 1.1. Standard curves were generated from known concentrations of a synthesised DNA fragment of each targeted gene, with a standard curve run with each plate. Samples outside of the calibration curve were considered below the limit of detection and included in the analysis as 0. Each DNA fragment for the standard curve was checked using MEGA7 to ensure they matched both primers, the probe (if applicable) and target gene and blasted in the NCBI database to ensure it was from

the correct target gene. Along with standard curves, a no template control (NTC) was added to each qPCR run. For further details on qPCR analysis, see supplementary material section 1.1.

2.5 16S sequencing and analysis

To characterise bacterial community composition in seawater and stormwater drain samples, the V3–V4 region of the bacterial 16S rRNA gene was amplified using the 341f/805r primer set (Suzuki et al., 2000), with the following cycling conditions: 95°C for 3 minutes followed by 25 cycles of: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and then 72°C for 5 minutes with a final hold at 4°C (Illumina, 2013). Amplicons were subsequently sequenced using the Illumina MiSeq platform (300 bp paired-end analysis at the Ramaciotti Institute of Genomics, University of New South Wales). For sequencing analysis details, refer to supplementary material. Raw sequences were uploaded to NCBI, BioProject ID PRJNA766238.

Paired R1 and R2 reads were subsequently processed using the DADA2 pipeline (Callahan et al., 2016). Reads with any 'N' bases were removed and bacterial V3-V4 primers were truncated using cutadapt (Martin, 2011). Reads were trimmed to remove low quality terminal ends (trunc (R1= 280; R2= 250)). To produce the highest number of merged reads after learning error rate and removing chimeric sequences, we used the dada2 removeBimeraDenovo program at the default threshold stringent minFoldParentOverAbundance=1. ASVs were annotated against the SILVA v138 database with a 50% probability cut-off. The ASV table was subsequently filtered to remove ASVs not assigned as kingdom Bacteria, as well as any ASVs classified as chloroplast or mitochondria. Finally, the dataset was rarefied to 30,000 reads using vegan (Dixon, 2003).

2.6 Statistical Analysis

To test for differences in abiotic variables, *Enterococci* counts and qPCR copies/100 ml, the nonparametric Kruskal-Wallis test was used, followed by Mann-Whitney pairwise comparisons with Bonferroni corrected p-values. Correlations between *Enterococci* counts, and data derived from qPCR assays were determined using Spearman's RS, with Bonferroni corrected p values. These statistics were performed in Past Version 4 (Hammer et al., 2001). To test for differences in microbial community composition and alpha diversity (16S rRNA data) between samples we used the Adonis function from *Vegan* (Dixon, 2003) and the pairwise.adonis function from the *PairwiseAdonis* (Arbizu, 2020) R package. To determine which ASVs were responsible for the dissimilarity between dry weather (before and after rain event) and wet weather (both days of rain), performed a SIMPER analysis on square root transformed data. To determine which ASVs represented 'indicator taxa' for drain communities, the multipatt function within the *indicspecies* package (de Caceres and Jansen, 2018) was implemented using the drain and seawater communities. Identified ASVs with an average relative abundance <0.01% were filtered from the resulting dataset. Finally, we used the 16S rRNA bacterial community profiles as a tracer of contamination from each stormwater drain experienced at the Beachwatch reference site by applying the predict function within the R package *SourceTracker* (Knights et al., 2011). This was performed by measuring the extent of microbial signature from source samples, which included each of the stormwater drains as well as a seawater community "control" (Rose Bay Entrance 1km offshore) within a sink sample (the 'Beachwatch' site). We also used the output of *SourceTracker* to calculate and display the spatial extent of impact from specific stormwater drains across Rose Bay. In this case, the source samples were again, drains, and the sink samples were the Rose Bay seawater samples. We analysed the contribution of Drain 5 during dry weather, Drain 5 after 3.8mm of rain, Drain 3 after 43mm of rain and Drain 6 post rain event. For each analysis we created a

grid with a high resolution of co-ordinates (53578 points) and used the R package "Raster" (R. Hijmans et al., 2010) to predict the data at these 53578 points which were then plotted onto the map of Rose Bay. For further details on indicator species and *SourceTracker* analysis see R scripts on GitHub (https://github.com/Nwilliams96/Rose-Bay-Wet-Weather-2019). To test for differences between the contribution of individual drains on the 'Beachwatch' site and then differences between the contribution of single drains at the Rose Bay seawater sites, we used the nonparametric Kruskal-Wallis test followed by Dunn's Post Hoc pairwise comparisons with Bonferroni corrected p-values (Hammer et al., 2001). Drain 3 (21/8/19, 27/8/19), Drain 9 (27/8/19) and Drain 10 (27/8/19) had evidence of seawater washing into them and were removed from this specific analysis.

3.0 Results

3.1 Environmental Conditions

Six days before the rain event (21/8/19), mean seawater temperature, salinity and turbidity levels at Rose Bay were 15.5°C ± 0.3 [n = 31], 35ppt ± 0.5 [n=31] and 0.70 NTU ± 0.56 [n=31]respectively. Notably, on this day, freshwater from the drains [mean salinity: 0.25 ± 0.06 ppt, n = 4] impacted salinity levels nearshore, which were significantly lower [$p<0.01$] when compared to samples that were taken 500m offshore. On the 27/8/19 (second day of sampling), a total of 3.8mm of rainfall was recorded and on the 30/8/19 (third day of sampling) 43mm of rain was recorded. Following this rain, inputs of significantly [$p<0.01$] more turbid [mean: 18.3 NTU, ± 9.04, n = 8] and fresh [mean: 3ppt, ± 0.05, n = 8] stormwater led to significant drops [$p<0.01$] in both salinity and optical density. Of note, after 43mm of rain, the samples nearshore were most impacted by stormwater, with the salinity at these sites being significantly lower [$p<0.01$] than sites 250m and 500m offshore, with a mixing ratio of 1:34 (one part freshwater to 34 parts seawater). Three days after the rain event (3/9/19), with 4/9

stormwater drains still running, the salinity nearshore was again significantly lower nearshore [$p<0.01$] compared to sites 250m and 500m offshore.

Inputs of stormwater also led to a significant rise [$p<0.01$] in chlorophyll, filterable reactive phosphorus, and total dissolved phosphate levels. In contrast to the patterns observed within Rose Bay, the rainfall event did not lead to a significant change within any of the tested environmental variables at the control site at Nielsen Park. Three days after the rain event, levels of total phosphate in Rose Bay decreased significantly [$p<0.01$] relative to during the rainfall event. Levels of nitrate-nitrite, total dissolved nitrogen and total nitrogen did not change significantly when comparing samples taken before the rain event (21/8/19) to samples taken during rainfall (27/8/19 and 30/8/19), but were significantly elevated [$p<0.01$] after three days of no rain (Supplementary Table 4 and 5).

3.2 Bacterial Abundance

Prior to the rainfall event levels of the 16S rRNA gene, used here as a proxy for bacterial abundance, were significantly lower within the drain samples [$p<0.01$] compared to the Rose Bay seawater. After 3.8mm of rain the levels of the 16S gene (mean: 7.64 x 1010 copies/100ml ± 3.87 x1010, n = 79) increased by 95% within the Rose Bay seawater samples (mean: 3.91 x 1010 copies/100ml ± 3.74 x1010, n = 86), and increased significantly [$p<0.01$] within the drain water, where levels were an order of magnitude higher than those observed in the seawater. After 43mm of rain, mean levels of the 16S rRNA gene decreased by 37% within Rose Bay seawater samples, but in contrast increased significantly [$p<0.01$] within the drain samples relative to the preceding sampling date, where again, levels of the 16S gene were an order of magnitude higher than those observed in the seawater. Three days after the rain event (3/9/19), 16S rRNA gene concentrations with the drains were not statistically distinguishable to those

observed during the rainfall event but were significantly elevated within the seawater [$p<0.01$] relative to the other sampling time-points.

3.3 Faecal Indicator Bacteria

Significant changes in *Enterococci* levels were observed between sites and over time (Figure 2, A). Throughout the dataset *Enterococci* levels were negatively correlated with salinity [$p<0.05$, $r_s = -0.60$], but positively correlated with pH, turbidity, FRP, NH^4, NO_x, TDN, TDP and TN [$p<0.05$, $r_s > 0.46$]. Prior to rainfall, *Enterococci* levels were low (mean: 2.4 ± 31.7 CFU/100ml, n = 30) within all seawater samples, with the exception of the sample located immediately adjacent to Drain 5 (site 5.1), which reached 180 CFU/100ml. *Enterococci* levels were elevated within all sampled stormwater drains (mean: 154 ± 183.2 CFU/100 ml, n = 5), with the highest levels observed in Drain 3 (470 CFU/100ml). Following a light rainfall (3.8mm, 27/8/19) event, *Enterococci* levels within all drains increased by an order of magnitude, with levels reaching [mean: 1,072 ± 1113.7 CFU/100ml, n = 8]. However, marked spatial variability in *Enterococci* counts occurred between drains (Figure 2, A), with highest levels observed in Drains 3, 4 and 5. While notable increases in *Enterococci* levels were observed in several drains, levels generally remained very low (mean: 8.6 ± 14.4 CFU/100ml, n = 31) within the seawater samples collected from Rose Bay, indicating minimal impact from the drains during this low rainfall event.

Following 43mm of rain (30/8/19), *Enterococci* levels within all samples were elevated relative to samples taken after 3.8mm of rain and before the rain event (21/8/19). Within stormwater drains, *Enterococci* levels became extremely high (mean:95,250 ± 67711.0 CFU/100ml, n=8), with highest levels observed in Drains 10 (190,000 CFU/100ml) and 3 (170,000 CFU/100ml). During this period, *Enterococci* levels also increased significantly [$p<0.01$] within Rose Bay seawater samples relative to seawater samples taken before the rain

event (21/8/19), with levels reaching (mean: 18,268, ± 60468.7 CFU/100ml, n=31). *Enterococci* levels within the seawater samples were also significantly greater [$p<0.01$] than those observed at the control site at Nielsen Park (mean: 20.7 CFU/100ml, SD ± 2.9, n=3). 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 2, A). Specifically, while *Enterococci* levels within the most offshore samples only reached 26 CFU/100ml, *Enterococci* levels reached (mean: 21,590 CFU/100ml ± 40382.9 CFU/100ml, n = 8) at the sampling points closest to the shore and drain outlet points. Highest *Enterococci* levels were observed in samples collected between Drains 2 and 3 (320,000 CFU/100ml; sample 3.2) and adjacent to Drain 10 (120,000 CFU/100ml). Following a period of 72 hours without further rainfall, *Enterococci* levels within Rose Bay seawater samples dropped by two orders of magnitude (mean: 30 ± 107.8 CFU/100ml, n=31). Among drains that could be sampled at this time, *Enterococci* levels also dropped (mean: 269 ± 254.1 CFU/100ml, n=4), but remained high in Drains 3 (480 CFU/100ml) and 5 (600 CFU CFU/100ml).

3.4.0 Microbial Source Tracking

3.4.1 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 (Feng et al., 2018;Templar et al., 2016), were detected in 79% (n=95/120) and 61% (n=74/120) of samples respectively. Across the data set, both Lachno3 and HF183 levels were negatively correlated with salinity [$p<0.05$, $r_s = -0.53$] and positively correlated with turbidity, FRP, NH4, NOx, TDN and TDP [$p<0.05$, $r_s > 0.23$]. Levels of both markers across the entire dataset (HF183 – mean: $33.35 \times 10^4 \pm 1.35 \times 10^5$ copies/100ml, n = 284.

Lachno3 – mean: 1.71 x 10^5 ± 1.28 x 10^5 copies/100ml, n = 271) were significantly [$p<0.01$] higher within Rose Bay than within the control site at Nielsen Park (HF183 – mean: 2.39 x 10^4 ± 4.90 x 10^4 copies/100ml, n = 29. Lachno3 – mean: 1.62 x 10^4 ± 3.15 x 10^4 copies/100ml, n = 30). Both markers displayed moderate, but statistically significant correlations to total *Enterococci* counts (Lachno3: $r_s = 0.363$, $p = 0.012$; HF183: $r_s = 0.365$, $p = 0.0163$).

Before the rain event (21/8/19), the Lachno3 and HF183 human faecal marker genes were detected in 90% (n = 27/30) and 50% (15/30) of Rose Bay seawater samples respectively, but detectable concentrations were only 2.1 and 0.7 times higher, and not statistically distinguishable from those observed within the Nielsen Bay control site. Except for HF183 in the Drain 9 transect, highest seawater concentrations of these human faecal markers were always observed in samples immediately adjacent to drains (Figure 2 B, C). This pattern was in-line with the significantly higher [$p<0.01$] concentrations of both human faecal marker genes (HF183 – mean: 6.09 x 10^4 ± 1.12 x 10^5 copies/100ml, n = 12, Lachno3 – mean: 9.86 x 10^4 ± 31.57 x 10^5 copies/100ml, n = 15) within the drain samples, which were 24 and 23 times greater than in the seawater samples (HF183 – mean: 1.02 x 10^3 ± 2.07 x 10^3 copies/100ml, n = 82. Lachno3 – mean: 4.79 x 10^3 ± 9.40 x 10^3 copies/100ml, n = 77), with highest levels of both markers observed in Drain 5.

Following a light rainfall (3.8mm, 27/8/19) event, 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 levels (mean: 1.15 x 10^4 copies/100ml ± 4.80 x 10^4, n =77) increased by 17-fold relative to seawater samples samples taken before the rain event (21/8/19), 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).

Further significant [$p<0.01$] increases of both human faecal markers occurred within drains and adjacent seawater samples following a larger (43mm, 30/8/19) rainfall event. Across all drain samples, concentrations of Lachno3 and HF183 increased significantly [$p<0.01$] by 109 and 76 times relative to conditions before the rain event on the 21/8/19, with highest concentrations of both markers observed within Drain 3 (Figure 2 B, C). 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 (Figure 2 B, C), where the highest seawater concentrations of human faecal markers recorded during this study period were observed. While clear gradients in both Lachno3 and HF183 were 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 from immediately proximate to the drain, which was consistent with the patterns observed in the *Enterococci* analysis.

Following a period of 72 hours without further rainfall on the 3/9/19, concentrations of HF183 and Lachno3 (HF183 – mean: $6.4 \times 10^4 \pm 7.27 \times 10^4$ copies/100ml, n=74. Lachno3 – mean: $3.07 \times 10^4 \pm 7.94 \times 10^4$ copies/100ml, n = 74) dropped by over 11- and 60-times respectively (Figure 2 B, C). However, this pattern was highly variable among sampling locations and the two assays, with Lachno3 and HF183 (detected in 100% (n= 34/34) and 82% (n = 28/34) of samples) levels being significantly lower within Rose Bay seawater [$p<0.01$] in comparison to the preceding time point. Highest levels of the human faecal markers persisted in Drain 3, 4 and 6 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.

3.4.2 Dog Faecal Marker

Across the entire dataset, the dog faeces marker, DG3 was detected in only 40% (n = 60/148) of samples, but was significantly correlated to *Enterococci* levels [$r_s = 0.47$, $p < 0.05$]. DG3 levels were also positively correlated with turbidity, FRP, NH4, NOx, TDN, TDP and TN [$p<0.05$, $r_s > 0.32$] and negatively correlated with salinity [$p<0.05$, rs = -0.51]. Prior to the rainfall event on the 21/8/19, DG3 was detected in only 22% (n= 8/36) of samples, with all detections in nearshore samples (except BW.2 and 3.2) (Figure 2, D). This dog faeces specific marker was not detected in any of the tested drain samples during this time.

After 3.8mm rain (27/8/19), detection levels of the dog faeces marker remained low [20% (n = 8/39)], yet concentrations of the marker (mean: $1.76 \times 10^3 \pm 7.04 \times 10^3$ copies/100ml, n = 81)increased significantly [$p<0.01$] by 6-fold. Notably, there was also a clear shift in the location of DG3 detections (Figure 2, D), with half (50%, n = 4/8) of detections observed in drain samples (highest concentrations observed in Drains 8 and 6) rather than seawater samples.

Following 43mm of rain (30/8/19), DG3 levels (mean: 8.91×10^5, $\pm 1.08 \times 10^6$ copies/100ml, n=24) were significantly elevated [$p<0.01$] within the drains (highest in Drains 3, 5 and 8) compared to conditions before the rain event (21/8/19). Within nearshore seawater samples, DG3 levels (mean: $5.25 \times 10^4 \pm 5.44 \times 10^4$ copies/100ml, n=21) were statistically indistinguishable compared to levels recorded prior to rainfall (mean: $1.59 \times 10^4 \pm 2.40 \times 10^4$ copies/100ml, n = 23), but in offshore samples were significantly elevated [$p<0.01$] relative to conditions before the rain event and highest along transects 3, 5 and 8 (Figure 2, D).

Following the rainfall event (3/9/19), the DG3 marker was only detectable in one drain (Drain 3), at significantly lower levels [$p<0.01$] than during the rainfall event (both 27/8/19 and 30/8/19), but was detected in all nearshore seawater samples (except 9.1) and in 37% (n=6/13) of offshore samples (Figure 2, D), although concentrations of this marker were significantly lower [$p<0.01$] than during rainfall periods.

3.4.3 Bird Faecal Marker

The GFD avian faecal marker was detected in 88% (n=130/148) of samples, but levels of this marker were not significantly correlated [r_s=0.1, p>0.05] with *Enterococci* counts and were statistically indistinguishable between Rose Bay and the control site, during both dry (before rain on the 21/8/19 and after rain on the 3/9/19) and wet weather conditions (3.8 mm rain on the 27/8/19 and 43 mm on the 30/8/19). No trend of increasing levels of GFD following rainfall was observed, in either drains or seawater samples, with concentrations of this marker often in fact decreasing following rainfall (Figure 2, E).

3.4.6 Genes conferring Antibiotic resistance:

Throughout the sampling period, levels of the antibiotic resistance genes *dfrA1, qnrS, sulI* and *vanB* were correlated with the human faecal marker HF183 [$r_s > 0.25$, $p < 0.01$]. Before the rain event *sulI* was detected in 19% (n=6/30) of seawater samples. However, *sulI* levels were an order of magnitude lower within the seawater samples (mean: $7.96 \times 10^1 \pm 2.16 \times 10^2$ copies/100ml, n = 29) than within the drain samples (mean: $2.07 \times 10^4 \pm 3.19 \times 10^4$ copies/100ml, n = 15). *qnrS* was detected in 43% (n=13/30) of seawater samples. However, levels of this gene within the drains (mean: $9.77 \times 10^2 \pm 2.52 \times 10^3$ copies/100ml, n = 14) and seawater samples (mean: $1.38 \times 10^3 \pm 3.23 \times 10^3$ copies/100ml, n = 67) could not be statistically distinguished. *tetA* was detected within 100% (n=30/30) of seawater samples. The levels of this gene displayed another discrete, but notable, pattern, whereby significantly higher [p<0.01] levels occurred within the samples 250m, 500m and 1000m offshore (mean: $2.87 \times 10^5 \pm 2.50 \times 10^5$ copies/100ml, n =57) relative to both drain (mean: $9.56 \times 10^3 \pm 7.95 \times 10^3$ copies/100ml, n = 15) and nearshore (mean: $9.98 \times 10^3 \pm 3.03 \times 10^3$, n = 30) samples. *dfrA1* was only present in one drain sample (drain 5), and *vanB* was not detected either before or after the rain event.

Following 3.8mm of rain, levels of *tetA* (mean: 2.32 x 10^5 ± 3.61 x 10^5 copies/100ml, n=30), *qnrS* (mean: 7.26 x 10^3 ± 9.34 x 10^3 copies/100ml, n = 23) and *sulI* (mean: 7.62 x 10^3 ± 1.16 x 10^4 copies/100ml, n = 28) all increased significantly [$p < 0.01$] in nearshore samples. Similarly, levels of these ABR genes also increased significantly [$p < 0.01$] within the stormwater drain samples, with highest levels observed within drains 6 and 8 (Figure 3 A, B, C). There was, however, no statistically distinguishable impact of rain on ABR gene levels either 250m, 500m or 1000m offshore on this day. However, the number of samples that ABR were detected within increased. *sul1* was detected in 46% (n=14/30) of samples, *qnrS* was detected in 70% (n=21/30) of samples and *tetA* again was detected in 100% (n=30/30) of samples (Figure 3 A, B and D). Rainfall also did not impact *dfrA1* levels within the drains. Rainfall did however impact the spatial dynamics of *dfrA1* in Rose Bay seawater samples, with the proportion of samples this gene was detected in increasing from 1% (n= 1/30) to 16% (n=5/30) of samples (Figure 3 E). Notably, following this rainfall event *vanB* was detected in Drain 9.

Following 43mm of rain, levels of all ABRs within the drains increased by an order of magnitude. Within Rose Bay seawater samples, however, the ABRs followed one of two trends: (i) increasing by an order of magnitude (*qnrS* and *vanB*) or (ii) remaining statistically indistinguishable from the preceding measurements (*tetA* and *sulI*) (Figure 3 A-E). The only ABR gene that did not follow one of these trends was *dfrA1*, with levels decreasing by two orders of magnitude after heavy rain. Spatially, patterns varied between genes, with *sulI* detected in 48% (n=14/30) of samples and *tetA* detected in one less sample relative to the preceding samling day. In contrast, *qnrS*, *dfrA1* and *vanB* were all detected in a higher proportion (83% (n=25/30), 20% (n=6/30) and 6% (n=2/30) respectively) of seawater samples compared to the preceding sampling day. The highest levels of all antibiotic resistance genes

(except *tetA*) were observed within drain 3 and along the seawater sample transect adjacent to it, where, for example *vanB* was observed up to 500m offshore (Figure 3 C).

Three days after the rain event, levels of most of the ABR genes (except *tetA* and *dfrA1*) dropped significantly within the drains [$p<0.01$], but remained high in the nearshore seawater samples, as well as in samples collected 250m and 500m offshore, with levels statistically indistinguishable from those recorded during the rainfall event. In contrast to the other ABR genes*, tetA* levels displayed a similar spatial pattern to those observed during heavy rainfall, whereby levels of this gene within the drain and nearshore samples were significantly higher [$p<0.01$] than those within in the samples taken 250 m, 500 m and 1,000 m offshore.

3.5.0 16S Sequencing community data

3.5.1 Bacterial diversity

The total number of ASVs detected in the entire data set was 17, 158. There were 13, 574 ASVs detected within drain samples and 13, 565 ASVs detected within the seawater samples. Within Rose bay seawater samples, bacterial community diversity (Shannon's diversity [$F=44.4$, $p<0.01$]) and composition [$F=19.7$, $p<0.01$] differed significantly between dry (before the rain event) and wet weather conditions (43 mm on the 30/8/19) (Figure 4A). The most abundant ASVs in seawater samples were members of *SAR11 Clade I*, the *SAR86* clade and an *Actinomarinales.* The dissimilarity in seawater bacterial communities observed between dry (before the rain event) and wet weather conditions (3.8 mm rain on the 27/8/19 and 43 mm on the 30/8/19)was primarily driven by a significant [$F = 22.5$, $p < 0.01$] decrease

in the relative abundance of *SAR11 Clade I* following rain and a concomitant increase in an ASV classified as *Pseudarcobacter defluvii*.

Significant differences in the composition of bacterial communities inhabiting the stormwater drains relative to the seawater samples [$F=14.5$, $p < 0.01$] were also apparent (Figure 4B). Within stormwater drains, the most abundant ASVs included the same *Pseudarcobacter defluvii* ASV noted above, a member of the *Spirosomaceae* family and *Flavobacterium succinicans*. The dissimilarity in bacterial communities within the drains and seawater communities was largely driven by the same *Pseudarcobacter defluvii* ASV mentioned above and a member of the *Spirosomaceae* family, which were both over-represented within the drain samples, and a SAR 11 Clade I ASV that was over-represented in the seawater samples. Additionally, the bacterial community also differed significantly between each individual drain [$F = 5.5$, $p < 0.01$].

3.5.2 Indicator Species within the drains

A total of 6,651 ASVs were identified as 'bacterial indicators' of the stormwater drain microbial communities. After removing ASVs present in an average relative abundance of less than 0.1% within the drain samples, 6 ASVs remained, including the same *Pseudarcobacter* and *Spirosomaceae* ASVs that were most responsible for the significant differences between drain and seawater bacterial communities, as well as 4 *Comamonadaceae* ASVs.

During dry weather prior to the rainfall event, these drain indicator ASVs were detected in only 23% (n=7/30) of Rose Bay seawater samples, where their cumulative relative abundance was a mean of 0.05% ± 0.08 (n = 14). Most of these detections (85% n=6/7) were in the nearshore samples (Figure 5 A), with highest relative abundances observed in 5.1 (0.19%). Drain indicator ASVs were detected at one other site, 10.3, 500m offshore. No drain indicator ASVs were detected within samples 250m or 1000m offshore, nor were they detected at the control site.

Following 3.8 mm of rain, the occurrence of drain indicator ASVs in seawater samples increased significantly, with these ASVs now detectable in 60% (n=18/30) of Rose Bay seawater samples. Again, the prevalence of drain indicator ASVs was highest in the nearshore samples, where they were detected within all samples except 7.1 (mean cumulative relative abundance: 0.09% ± 0.02, n = 37). The highest levels of drain indicator ASVs were recorded at site 5.1, where the cumulative relative abundance of 1.2% was two orders of magnitude higher than the mean of all other nearshore samples (mean: 0.09% ± 0.14, n = 7). During this time, drain indicator ASVs were also detected at 11 of the sites located 250m and 500m offshore. No drain indicator ASVs were detected at the control site.

After 43mm of rain, the occurrence of drain indicator ASVs increased further, with these indicator organisms now detected in 66% (n=20/30)of seawater samples. These indicator organisms were most prevalant in the nearshore samples 3.1 and 4.1, where their cumulative relative abundance was 0.1% and 0.7% respectively, with highest levels again observed in the near shore samples. The drain indicator ASVs were mainly restricted to transects 3, 4, 5, 8 and 9, extending from nearshore to 500m offshore (Supplementary Figure 3, C). Of note, the drain indicator taxa were detected in one site 1000m offshore and up to 500m offshore at the control site.

Three days after the rainfall event, the occurrence of drain indicator ASVs remained high within Rose Bay seawater samples, where they were detected in 87% (n=25/29) of samples. These ASVs were detected in all nearshore samples, and up to 500m offshore along transects 3, 5 and 10.

3.5.3 Impact of microbial signature from drain communities on Rose Bay

To estimate the influence of each stormwater drain on water quality at the Beachwatch reference site (BW.2) (Supplementary Figure 3), we used *SourceTracker* (Knights et al., 2011)

to examine the relative contribution of the microbial signature from each drain within at this reference sampling point used by local monitoring programs. The relative contribution of the microbial signature from each drain on BW.2 mostly consisted of the Rose Bay Entrance microbial community (99%), which in this test was our control. However, our main focus was the change in impact from the drains, which differed significantly over the time-course of this study [$F=37.69$, $p<0.01$].

Before the rain event (21/8/19), the microbial signature from Drain 4 was 3 times higher than the cumulative contribution of the other running drains (Supplementary Figure 3 A). After 3.8 mm of rain, the impact of all stormwater drains on the BW.2 bacterial community (mean: $0.001 \pm 0.0015\%$, $n = 12$) was significantly higher [$p<0.01$] relative to dry weather conditions before the rain event (mean: $0.005 \pm 0.003\%$, $n = 12$). The relative impact of the drains also shifted, with Drain 5 now contributing between 1-4 times more than any of the other drains (Supplementary Figure 3 B). After the more significant rainfall event, when 43 mm of rain fell, the microbial signature of Drains 3, 5 and 9 had the largest impact on the BW.2 bacterial assemblage (Supplementary Figure 3 C). The over-all level of impact from the drains on BW.2 did not change significantly 3 days after the rainfall event. The impact source however, shifted from Drain 3, Drain 5 and Drain 9, to Drains 4 and 6, which now had the largest level of impact on BW.2 (Supplementary Figure 3 D).

To investigate the spatial impact of specific stormwater drains on Rose Bay over the course of the rainfall event, we again, used *SourceTracker* (Knights et al., 2011) to investigate the contribution of the microbial assemblages within specific drains (used here as a source) on the microbial community within each sample within Rose Bay (sink). For each day of the study, the specific drain chosen was the drain that had the highest levels of the Lachno3 marker, which equated to Drain 5 before the rain event (21/8/19) and after 3.8mm of rain, Drain 3 after 43mm of rain and Drain 6 after the rainfall event.

Six days before the rain event (21/8/19), the microbial signature of Drain 5 impacted all nearshore samples and samples located 250m offshore, but only impacted 50% (n=4/8) of samples located 500m offshore (Figure 6 A). After 3.8mm of rain, the extent of impact from Drain 5 increased to 93% (n=28/30) of sites within Rose Bay, impacting all but one site 500m offshore and reaching two sites 1000m offshore. Whilst the impact of this drain increased spatially (mean: 0.002 ± 0.002, n = 80) the contribution was significantly lower [$p<0.01$] relative to dry weather conditions before the rain event (mean: 0.001 ± 0.0007, n = 92) (Figure 6 B). After 43mm of rain, the impact of the microbial signature from Drain 3 reached 1000m offshore (Figure 6, C) and was significantly greater [$p<0.01$] than the microbial impact from Drain 5 relative to conditions before the rain event (21/8/19) and after 3.8mm of rain (27/8/19). Indeed, using this approach, it was apparent that the impact of Drain 3 at this time was the greatest of any drain throughout the entire study period. Four days following the rainfall event, the spatial impact of Drain 6 on Rose Bay extended 500m offshore (Figure 6 D), but had a significantly lower [$p<0.01$] level of impact than Drain 3 during heavy rain.

4.0 Discussion

4.1 What is the principal cause of faecal contamination at Rose Bay?

Urban beaches are often characterised by poor water quality, which has implications for human and ecosystem health (McLellan et al., 2015). Routine FIB monitoring has indicated that water quality at Rose Bay has been regularly impacted by faecal contamination for at least the last 7 years (DPIE, 2020; OEH, 2013), yet the causes and point-sources of this contamination have not been identified. By applying a suite of molecular microbiological approaches, we have revealed significant levels of markers for human faeces, indicative of sewage contamination during both dry weather periods (before and after rainfall events), which increased further during rain (3.8 mm rain on the 27/8/19 and 43 mm on the 30/8/19), to levels

that are comparable to, and sometimes higher than, other aquatic environments known to be impacted by raw sewage (Liang et al. 2021). This was paired with intermittent impacts from dog faeces in dry weather (pre- and post-rain event) detected only in nearshore, and not drain, samples, indicating that it was sourced from dog faeces on the beach rather than the catchment serviced by the stormwater drains, and most likely from runoff in the catchment during wet weather (3.8 mm rain on the 27/8/19 and 43 mm on the 30/8/19) due to the large amount of DG3 present within the drains.

Before the rainfall event (21/8/19), *Enterococci* levels within the seawater samples were generally low, indicating good water quality, but these levels became substantially elevated following rainfall. The single exception to this pattern was the sample collected adjacent to Drain 5 (5.1), where *Enterococci* levels were 180 CFU/100ml. 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 was also elevated at site 5.1, but absent with the Drain 5 sample. These patterns imply one of two explanations for the moderate *Enterococci* levels observed in this nearshore site: (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 moderately high *Enterococci* levels measured at this location. Given that comparable levels of DG3 were recorded at other near-shore sites (3.1, 4.1, 5.1, 6.1, 9.1, 10.1) 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. This potential dry weather sewage overflow resulted in levels of HF183 within the seawater that were in some cases higher than those that have elsewhere been estimated to indicate a significant health risk from sewage-borne pathogens (Boehm and Soller, 2020). The *SourceTracker* analysis (Figure 6. A) showed that the microbial signature from Drain 5 was

the strongest around transects 3, 5, 6, 7, and offshore towards Neilson park. This aligns with locations that also had a higher level of HF183, therefore, a sewage leak within Drain 5 is a possible explanation for high background levels of HF183 before the rain event.

Following a 43mm rain on the 30/8/19, mean bacterial abundances within drains (as estimated by 16S rRNA qPCR) increased by an order of magnitude, and were over an order of magnitude higher than seawater bacterial abundances preceding the rainfall event. When this stormwater entered Rose Bay in a mixing ratio of 1:34, levels of bacteria increased by 95% and 23% within Rose Bay seawater. This pattern was confirmed by our SourceTracker analysis, which revealed that the bacterial assemblages within seawater samples surrounding drain 3 were comprised of between 10-50% bacteria from Drain 3. *Enterococci* levels also increased significantly within both stormwater drain samples and seawater 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, with the elevated seawater *Enterococci* levels spanning the Drain 3 transect into Rose Bay also 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, where levels of HF183 sometimes reached four orders of magnitude higher than those predicted to indicate a human health risk from sewage (Boehm and Soller, 2020). 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 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 (Ahmed et al., 2020). We posit that

this pattern of concentration in, and near to, the stormwater drains is likely indicative of dog faeces being washed into the stormwater system from the surrounding catchment, rather than significant levels of dog faeces being washed from the beach into the seawater at Rose Bay.

Using 16S rRNA gene amplicon sequencing data we revealed the occurrence of a set of "indicator bacteria", which were present at a high relative abundance (relative to seawater) within stormwater samples but became detectable within seawater samples after rainfall. Notably, these indicator bacteria, including *Pseudarcobacter* are known to be members of the bacterial communities inhabiting sewage (McLellan et al., 2015), providing further evidence for the impact of sewage within Rose Bay. Some of these taxa were intermittently detected within nearshore seawater samples before the rain event (21/8/19), but following the major rainfall event examined here were observed up to 1000m offshore, providing evidence for a spatially pronounced influence of sewage contamination across Rose Bay after rain.

While coastal environments can also be subject to faecal contamination from native animals, in particular water birds (Jarma et al., 2021), the marker for avian faeces 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 control site and (ii) bird faecal marker levels in the seawater were always within the range of those observed in other coastal habitats pre-rainfall (Ahmed et al., 2020), we conclude that bird faeces played a minimal role in driving the elevated total *Enterococci* levels observed during rainfall. This, however, does not negate the possible health risks associated with a high level of bird faeces. It has been demonstrated that the guts of birds can be colonised by antimicrobial resistant bacteria (ARB) when birds ingest food from polluted water sources containing antimicrobial bacteria (Franklin et al., 2020). This can then make them environmental reservoirs and vectors for ARB and ARGs (Bonnedahl and Järhult, 2014; Ahlstrom et al., 2018) and vectors for dissemination of

ARGs in the environment. Furhtermore it has been shown that bird faeces can contain human bacterial pathogens (Benskin et al., 2009).

Cumulatively, our results indicate that sewage input and input of dog faeces into Rose Bay contribute to high *Enterococci* counts 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, in some cases, dog faecal material from the catchment.

4.2 What are the primary points of contamination within Rose Bay?

Prior research has concluded that stormwater drains are responsible for input of sewage into recreationally used coastal environments during both dry and wet weather (Converse et al., 2011; Parker et al., 2010; Sauer et al., 2011; Sercu et al., 2011, 2009). Given the elevated levels of both FIB and the human faecal MST markers within stormwater drains, during both dry (before and after rainfall) and wet weather (3.8 mm rain on the 27/8/19 and 43 mm on the 30/8/19), 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 (before and after the rain event) or wet weather (3.8 mm rain on the 27/8/19 and 43 mm on the 30/8/19) periods.

Before the rain event on the 21/8/19, 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 5.1 seawater sample before the rain event. Indeed, at site SW5.1 levels of the Lachno3 marker were 1.1×10^5 copies/100ml

prior to the rainfall event and the Lachno3 and HF183 markers reached 1.98 x 10^7 copies/100ml and 2.45 x 10^6 copies/100ml respectively after 3.8mm of rain. While these levels are an order of magnitude lower observed in raw sewage (Sauer et al., 2011; D. Li et al., 2021), they are high relative to concentrations observed in other coastal environments (Liang et al., 2021; Rothenheber and Jones, 2018). It is not uncommon for dry weather sewage leaks to occur within stormwater drains (Sercu et al., 2009), and, notably, Drain 5 is adjacent to a sewage pumping station behind Rose Bay beach, which may contribute to high background levels of both sewage markers at Neilson park and is potentially worthy of further examination. These patterns are supported by our analysis of the 16S rRNA gene amplicon sequencing data using *SourceTracker*, which highlighted Drain 5 as the greatest point of impact on the Beachwatch reference sample after 3.8mm of rain. This impact was not only high at the Beachwatch site, but extended 1000m offshore both before the rain event and after 3.8mm of rain. Additionally, the 16S rRNA gene amplicon sequencing data also revealed the highest levels of sewage associated indicator species at site 5.1, both before the rain event and after 3.8mm of rain.

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 Rose Bay seawater 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 seawater samples, with Drains 3 and 10 clearly hotspots of sewage contamination, in Drain 3, HF183 reaching levels an order of magnitude lower than what has been previously detected in raw sewage HF183 (Sauer et al., 2011; D. Li et al., 2021). A similar pattern was observed upon inspection of the 16S rRNA gene amplicon sequencing data, whereby the *SourceTracker* data revealed drains 3 and 9 as the input points that had the highest impact on the Beachwatch reference sample on this day.

This data also revealed that Drain 3 had the highest impact on seawater samples across the entirety of Rose Bay.

Given that concentrations of the human faecal markers became significantly elevated within these drains following the major rainfall event, we suggest that drains 3 and 9 potentially represent sites most influenced by wet weather sewage overflows. This is supported by the *SourceTracker* data whereby we observed these two drains as having the highest impact on the Beachwatch site during the heavy rain event (Supplementary Figure 3 C).

4.3 Spatiotemporal dynamics of contamination

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 a rainfall event. This analysis revealed that FIB 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. While previous studies have shown increases of faecal contamination from either sewage or animal sources during rainfall events (Ahmed et al., 2020; Shrestha et al., 2020), only few studies (Newton et al., 2013) have assessed the spatial extent of impact.

For the most part, high levels of the human and dog faecal markers were restricted to near-shore samples. Drain 3 displayed highly elevated levels of the human faecal markers across several samples extending away from the shoreline, and when used as a "source" when 16S rRNA bacterial community data was employed as a tracer, contributed to up to 50% of the bacterial communities within "sink" samples up to 500m offshore along transects 3 and 4. This is indicative of a substantial influence of Drain 3 on water quality within Rose Bay following rainfall.

Four days after the major rainfall event, slightly elevated levels of both human faecal markers persisted within the environment. There are two potential explanations for this pattern: (i) The *Lachnospiraceae* and *Bacteriodes* targeted by their respective assays can persist for longer periods than FIB in the environment as shown by (W. Ahmed et al., 2020), or (ii) The DNA- based, rather than culture-dependent, approach used to quantify these markers detect unviable bacteria that would not grow via a culture-based approach.

4.4 Other microbiological hazards in Rose Bay?

Our results indicate that Rose Bay is extensively impacted by sewage contamination likely linked to sewage overflows into stormwater drains, which may consequently create several hazardous microbiological implications. Over the course of the experiment, we observed evidence for increased levels of ARGs in the environment following rainfall, which is consistent with recent studies in other urbanised beaches that are impacted by sewage contamination (Carney et al., 2019). The putative links between elevated ARG occurrence and sewage contamination (Akiyama and Savin, 2010; Auguet et al., 2017; Gaviria-Figueroa et al., 2019) was confirmed here by significant correlations between the HF183 human faecal marker and ARGs; *dfrA1, qnrS, sulI* and *vanB*. Notably, we observed levels of *qnrS* that were over two orders of magnitude higher than those previously reported within wastewater (Paulus et al., 2019) and levels of *vanB* that were over 3 times higher than those observed at other highly contaminated beaches in Sydney (Carney et al., 2019). This emerging occurrence of high levels of ARGs at Rose Bay represents a largely uncharacterised, but potentially significant (Leonard et al., 2018) health risk for swimmers.

4.5 Bacterial community analysis provides another powerful tool to analyse coastal water quality

We coupled 16S rRNA gene amplicon sequencing with the Bayesian *SourceTracker* package (Knights et al., 2011), which uses Bayesian statistics to predict the percentage of 'source' microbial communities within selected 'sink' samples. *SourceTracker* has previously been used to track the occurrence of sewage bacterial communities in the environment (Newton et al., 2013), and discern the relative contribution of faecal contamination from different sources, including sewage plants and animals (Ahmed et al., 2015; Brown et al., 2017). Similarly to our study, (Neave et al., 2014) used *SourceTracker* to analyse the microbial signature from different inputs, including a sewage outfall and a number of lakes (sources), at different beach sites (sinks). However, to the best of our knowledge, ours is the first study to use this approach to (i) track the spatiotemporal dynamics of specific bacterial signatures for individual stormwater drains and (ii) use this data to quantify the relative strength of the microbial signature from different stormwater drains.

Additionally, we coupled 16S rRNA gene amplicon sequencing with the *indicspecies* r package (de Caceres and Jansen, 2018), to identify specific microbial indicators of the water within stormwater drains and Rose Bay seawater samples. This analysis revealed that several of the bacteria that could be characterised as indicators for stormwater drains were often aligned with organisms previously identified as indicators of sewage (e.g., *Arcobacter* (McLellan et al., 2015)). One of the most prevalent indicator taxa was *Pseudarcobacter defluvii*, which was formerly known as *Arcobacter defluvii* (Pérez-Cataluña et al., 2018). *Pseudarcobacter defluvii* has been isolated from sewage (Collado et al., 2011), with strains of this organism isolated from sewage shown to be potential human pathogens (Levican et al., 2013). Furthermore, our microbial indicator analysis also revealed other, previously unrecognised, putative markers for contamination of urbanised coastal habitats. These included four *Comamonadaceae* ASVs and a *Spirosomaceae* ASV. *Comamonadaceae* are a family of bacteria which have also been reported as a core member of sewage sludge (Xu et al., 2017),

where they can make up to 10% of the cumulative relative abundance of effluent (Yasir, 2021), while *Spirosomaceae* has also been isolated from sewage sludge in Korea (Lu et al., 2007), implying that these ASVs are likely sewage markers.

We acknowledge that this was indeed a single rain event with only four time points and that while our results are reflective of what happened at this time, they may not be generalisable to Rose Bay at all times and in all rain events. However, in light of the outcomes of our analysis of 16S rRNA community profiles, we argue that DNA sequencing data provides a powerful and largely untapped means to trace the extent and impact of water contamination in aquatic ecosystems. This has the potential to augment other FIB and MST approaches.

5.0 Conclusions

Many urban beaches are characterised by poor water quality because of often undefined sources of faecal contamination. By employing molecular microbiological tools including MST assays and DNA sequencing we have delivered a precise assessment of the causes and sources of contamination at an intermittently contaminated beach within Sydney Harbour, Australia. Whilst traditional FIB methods indicated high levels of faecal contamination, we used MST approaches to precisely identify the likely source of this contamination (i.e., sewage vs animal). Our analysis demonstrated that Rose Bay is moderately impacted by dog and human faeces during dry weather (both before and after the rainfall event), but heavily impacted by human faeces (sewage) during wet weather (after 3.8mm and 43mm rain). Additionally, we identified the relative impact of individual stormwater drains on seawater quality by combining MST assays with DNA sequencing techniques to trace the spatial and temporal dynamics of contamination. This confirms that in cases where consistently high levels of FIB are recorded by regular monitoring practises, but the source of contamination remains ambiguous, MST tools like those used here provide a powerful means for informing subsequent

remediation and management efforts. Finally, we identified the spatial and temporal dynamics of microbiological hazards associated with contamination, including an increased occurrence of antibiotic resistance. These more nuanced insights into the contributing factors to poor water quality at this highly urbanised coastal environment will inform efforts to resolve the causes of contamination and subsequently help to safeguard public health.

Highlights

- Molecular MST methods identify faecal pollution cause (human and dog faeces).
- Molecular MST methods identify faecal pollution source location at urban beach.
- Microbial hazards including ARGs linked to faecal contamination were identified.
- 16S data paired with SourceTracker and Indicspecies augmented MST methods.

Conflicts of interest statement

There were no conflicts of interest to state.

Role of Funding Source

Acknowledgements

We acknowledge the NSW Department of Planning, Industry and Environment and Beachwatch for sampling, taking physiochemical measurements, and for organising *Enterococci* and nutrient analysis. We also acknowledge Martin Ostrowski for assistance in 16S rRNA data analysis and Amaranta Forcardi for assistance with interpolating *SourceTracker* data to map.

Author Contributions

Nathan L R. Williams[a], Nachshon Siboni[a], Meredith Campey[b], Colin Johnson[b], Jaimie Potts[b], Peter Scanes[b], Justin R. Seymour[a] contributed to experimental design, Nathan L R. Williams[a], Nachshon Siboni[a] and Colin Johnson[b] were responsible for sampling. Nathan L R. Williams[a] and Nachshon Siboni[a] were responsible for sample processing and qPCR analysis, while Nathan L R. Williams[a], Nachshon Siboni[a] and Anna Bramucci[a], were responsible for 16S analysis. Nathan L R. Williams[a], Nachshon Siboni[a] and Justin R. Seymour[a] contributed to writing the manuscript.

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