Rainfall leads to elevated levels of antibiotic resistance genes within seawater at an Australian beach

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Keywords

Antimicrobial Resistance, Sewage Contamination, Water Quality and Water Safety.

Highlights

- Levels of human sewage markers were elevated during rainfall.
- Levels of ARGs correlated significantly with markers for human sewage.
- Not all ARGS displayed the same spatiotemporal distribution patterns.
- ARGs significantly correlated with stormwater drain indicator bacteria.
- Some ARGs were correlated with known human pathogens.

Abstract

Anthropogenic waste streams can be major sources of antibiotic resistant microbes within the environment, creating a potential risk to public health. We examined patterns in the occurrence of a suite of antibiotic resistance genes (ARGs) and their links to enteric bacteria at a popular swimming beach in Australia that experiences intermittent contamination by sewage, with potential points of input including stormwater drains and a coastal lagoon. Samples were collected throughout a significant rainfall event (40.8mm over 3 days) and analysed using both qPCR and 16S rRNA amplicon sequencing. Before the rainfall event, low levels of faecal indicator bacteria and a microbial source tracking human faeces (sewage) marker (Lachno3) were observed. These levels increased over 10x following rainfall. Within lagoon, drain and seawater samples, levels of the ARGs *sulI*, *dfrA1* and *qnrS* increased by between 1-2 orders of magnitude after 20.4mm of rain, while levels of *tetA* increased by an order of magnitude after a total of 40.8mm. After 40.8mm of rain *sulI*, *tetA* and *qnrS* could be detected 300m offshore with levels remaining high five days after the rain event. Highest levels of sewage markers and ARGs were observed adjacent to the lagoon (when opened) and in-front of the stormwater drains, pinpointing these as the points of ARG input. Significant positive correlations were observed between all ARGs, and a suite of Amplicon Sequence Variants that were identified as stormwater drain indicator taxa using 16S rRNA amplicon sequencing data. Of note, some stormwater drain indicator taxa, which exhibited correlations to ARG abundance, included the human pathogens *Arcobacter butzleri* and *Bacteroides fragilis*. Given that previous research has linked high levels of ARGs in recreationally used environments to antimicrobial resistant pathogen infections, the observed patterns indicate a potentially elevated human health risk at a popular swimming beach following significant rainfall events.

1.0 Introduction

The increasing occurrence of antibiotic resistance (AbR) is of large concern globally (Klein et al., 2021). While the majority of focus on the impacts of AbR has been placed on clinical settings (Paulus et al., 2019), the spread and environmental reservoirs of AbR within natural ecosystems are of increasing interest (Berendonk et al., 2015). While AbR occurs naturally within environmental microbiomes (Allen et al., 2010), high levels of antibiotic use and subsequent contamination related to the agricultural industry (Foley and Lynne, 2008; Sarmah et al., 2006), and inadequate management of human wastewater streams (Baquero et al., 2008) are contributing to concerningly high levels of AbR in aquatic environments (Auguet et al., 2017). This concern is in part due to the fact that environmental AbR presents a largely undefined health risk, particularly to community groups that are consistent users of aquatic environments, including swimmers (Leonard et al., 2015) and surfers (Leonard et al., 2018).

The spread of AbR into coastal environments via anthropogenic wastewater streams occurs because of excretion of large quantities of antibiotics from human populations into sewage (Steinbakk et al., 1992). Antibiotics are difficult to remove during wastewater treatment and therefore occur in both sewage influent and effluent waste streams, where they have the potential to select for AbR within resident bacterial communities (Baquero et al., 2008; Chow et al., 2015). Stormwater infrastructure is often contiguous to sewage infrastructure, where both pipe blockages during dry weather and wet weather sewer over-flow events during rainfall can lead to sewage leaks into the stormwater infrastructure, and as a result, can further lead to untreated sewage entering aquatic environments (Olds et al., 2018).

Recently, it has been shown that antibiotic resistance gene (ARG) levels linked to stormwater within the environment vary depending on the presence of sewage contamination within the stormwater (Carney et al., 2019; Karkman et al., 2019), however our knowledge on the spatial and temporal dynamics of ARGs at beaches where there are multiple potential input

points is limited. Moreover, sewage can also harbour microbial pathogens, however, while recent work has illustrated correlations between pathogens and ARGs in urbanised coastal settings (Carney et al., 2020), there is little research linking these ARGs and pathogens to a particular source i.e., sewage leaks within stormwater drains. Therefore, our aim was to determine the extent of ARG pollution within a coastal environment and if this pollution is linked to sewage contamination. Furthermore, we aimed to determine whether sewage contamination has the potential to transmit AbR pathogens into the environment.

Here we examined the potential links between sewage contamination and the occurrence of ARGs within a popular swimming beach Australia's East Coast. This site is characterised by a network of potential contamination sites, incorporating a coastal lagoon that is intermittently open to the ocean and the outputs from three stormwater drains. We performed sampling during a heavy rainfall event, with the central goal of defining the extent, spatiotemporal dynamics, and points of input of ARGs into a coastal environment used widely for human recreation.

2.0 Methods

2.1 Sampling Sites

Terrigal Beach is located on the east coast of Australia (3.4462° S, 151.4447° E), and is a popular site for recreational use by surfers and ocean swimmers. However, this beach regularly experiences poor water quality linked to faecal contamination, particularly after significant rainfall events (DPIE, 2020). However, the exact sources and impacts of this contamination are complex and unclear, with a broad network of potential input sources including three storm water drain outlets, as well as an intermittently closed and open saline coastal lagoon, Terrigal Lagoon, that is intermittently open to the ocean, particularly after intense rain events.

Water samples were collected from a suite of potential points of contamination (Figure 1), including the outlets of three stormwater drains (Drains 1, 2 and 4), and from five locations within Terrigal Lagoon. This lagoon receives water from 34 stormwater outlets, has a surface area of 30 ha, a mean depth of 50 cm and is mostly closed, but after intense rain events is manually or naturally opened resulting in drainage into the ocean. Surface seawater samples were also collected from 27 locations along Terrigal Beach, spanning 9 on-shore to off-shore transects. Each transect comprised of nearshore sampling points (1 metre from shore), sites where the water depth reached 5m (~150m offshore) and sites where the water depth reached 10m (~300m offshore). Three of these transects were located within Terrigal Haven, which is a 200m long beach, protected by 20m high Broken Head (Figure 1). These three transects originated immediately adjacent to three stormwater drains (Drain 1, 2 and 4, SW1.X, SW2.X and SW4.X). Drains 1 and 2 are separated by approximately 20m while Drain 4 is approximately 400m north of Drain 2 (Figure 1). A reference transect corresponding to the sampling point for the regional water monitoring program, Beachwatch (DPIE, 2020), was located approximately 200m north of Drain 4 and 350m south of the mouth of Terrigal Lagoon (SW5.X, Figure 1). To examine the spatial impacts of faecal contamination and related microbial hazards from Terrigal Lagoon, a transect was also established from the mouth of Terrigal Lagoon (SW6.X). Four additional reference transects were added in locations without nearby stormwater drains. The first was at Wamberal beach 1.5km north of Terrigal Beach, the second in front of Wamberal Lagoon 2km north of Terrigal beach, the third at Forresters Beach 4 km north of Terrigal beach (SW7.X, SW8.X and SW9.X) and the fourth at nearby Avoca Beach (SW0.X, Figure 1), which is approximately 3km south of Terrigal Haven. The regional water quality monitoring program, Beachwatch, (DPIE, 2020) indicates Forrester's beach as generally characterised by low Faecal Indicator Bacteria (FIB) levels, and samples collected

from this point were used as a reference for comparisons to more highly contaminated samples from Terrigal Haven.

Sampling was conducted at each location on six occasions, which spanned the period before, during and after a significant rainfall event that resulted in 40.8mm of rain (Table 1). Due to large ocean swell conditions, it was not possible to safely sample SW9.2 on the 31/5/19, all sites 100m and 300m offshore on the 4/6/19, and SW0.2, SW8.2 and SW9.2 on the 6/6/19. Additionally, the stormwater drains were only sampled when running - Drain 1 was only running on the 4/6/19, Drain 2 on the 4/6/19 and 6/6/19, and Drain 4 on the 4/6/19, 6/6/19 and 11/6/19.

2.2 Water Sampling, Processing and Analysis

On each sampling occasion, physiochemical parameters including temperature, dissolved oxygen, salinity, and pH were measured in situ at each site using a WTW multiprobe meter (Multi3430, Germany). Samples were also taken for chlorophyll, nutrient, and *Enterococci* analysis (supplementary material section 1.1-1.3). Water samples were collected using 10L pre-washed plastic containers that were soaked in 10% bleach for 24 hours, thoroughly rinsed with miliQ, and washed three times with water from the collection point prior to sampling. Water samples were transported to a field laboratory (<2hrs) and filtered through 47mm diameter, 0.22µm pore-size membrane filters (Millipore, DURAPORE PVDF .22UM WH PL) using a peristaltic pump (100rpm). Between each sample, 500ml of 10% bleach was run through the pumping system, followed by 500ml of MilliQ water and then 2L of sample. The volume of sample water filtered was recorded for later normalisation and filters were immediately frozen and stored in liquid nitrogen prior to being stored at -80°C. Within a week of sample collection, DNA was extracted from filters using the PowerWater DNA

Isolation Kit (QIAGEN) according to the manufacturer's instructions, in batches of 45 samples, including 3 kit blanks per batch. DNA was stored at -80ºC prior to downstream analysis.

2.3 Quantitative PCR (qPCR) analysis

To determine the presence of human faeces, indicative of sewage contamination, we used quantitative PCR (qPCR) to measure levels of the Lachno3 assay (Feng et al., 2018), which targets human gut microbiome associated *Lachnospiraceae* and has been demonstrated to be an excellent marker of human sewage within the environment. We also quantified levels of *Arcobacter,* which is a genus of bacteria that is regularly associated with stormwater, sewage infrastructure (McLellan and Roguet, 2019) and sewage sludge (Collado et al., 2011). The *Arcobacter* genus includes species that are associated with human illness (Ferreira et al., 2016), and recently was shown to be correlated with some ARG in marine environments (Carney et al. 2019). *Arcobacter* were quantified using the ARCO assay, which targets the 23S rRNA gene (Bastyns et al., 1995). Furthermore, we characterised patterns in the class 1 integron-integrase gene (*intI1*), which facilitates the mobility of integron related ARG cassettes, is present within diverse pathogenic bacteria, and has been proposed as an excellent indicator of anthropogenic pollution within natural environments (Gillings et al., 2015). To quantify *intI1* gene abundances, we used the *intI1* primer set (Mazel et al., 2000) (Table 1, Supplementary Material).

Growing evidence suggests the birds are environmental reservoirs of ARB and ARGs (Bonnedahl and Järhult, 2014; Ahlstrom et al., 2018) and vectors for dissemination of ARGs in the environment (Jarma et al. 2021). In addition to sewage markers, we also used qPCR to determine levels of the GFD assay (Green et al., 2012) which is an indicator of bird feaces (Table 1, Supplementary Material).

Quantitive PCR was also used to quantify a suite of 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 *sulI, tetA, qnrS, dfrA1* and *vanB* genes. *sulI* encodes resistance to sulphonamide, an antibiotic used widely in clinical settings (Pei et al., 2006); *tetA* encodes resistance to tetracyclines, which are a group of antibiotics widely used to treat bacterial infections in both human and animals (Borjesson et al., 2009); *qnrS* confers resistance to quinolones, a group of antibiotics mostly used in clinical settings (Berglund et al., 2014); *dfrA1* confers resistance to trimethoprims (Grape et al., 2007), a group of antibiotics used widely to treat urinary tract infections; *vanB* encodes resistance to vancomycin (Berglund et al., 2014), which is a last line of defence antibiotic (Berglund et al., 2014). Details on all qPCR assays are provided in table 1, supplementary material and details on qPCR methods are provided in supplementary material section 1.4.

2.4 16S rRNA amplicon sequencing and analysis

To characterise bacterial community composition in all 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 a detailed description of 16S rRNA amplicon sequencing data processing details refer to supplementary material section 1.5.

2.5 Statistical Analysis

To test for differences in abiotic variables, and spatiotemporal differences in the levels of *Enterococci* and qPCR markers, data was first transformed using ln(x+1) and tested for normality. Normally distributed data was compared using the parametric ANOVA test, paired with Tukey pairwise comparisons, while non-normal data was compared using the nonparametric Kruskal-Wallis test, followed by Mann-Whitney pairwise comparisons with Bonferroni corrected p-values. To test for correlations between *Enterococci* plate counts (single replicate) and the qPCR samples (three biological replicates), average values from the qPCR data were used. Correlations between *Enterococci* counts, and data derived from qPCR assays were determined using Spearman's RS, with Bonferroni corrected p values.

To test for differences in microbial community composition (16S rRNA data) and alpha diversity between samples we used the Adonis function from Vegan (Dixon, 2003) and the pairwise.adonis function from the PairwiseAdonis (Arbizu, 2020) R package. Three separate tests were undertaken using the multipatt function within the indicspecies package (De Caceres and Jansen, 2018) to identify ASVs that represented 'indicator taxa' for: a) stormwater drain, b) lagoon and c) seawater communities. Three separate Mictools (Albanese et al., 2018) analysis were also run with the aim of determining the Spearman's RS correlations between the suite of ARGs detected via qPCR and a) drain indicator ASVs, b) lagoon indicator ASVs and c) seawater indicator ASVs. For complete R scripts used in statistical analysis of 16S data see (https://github.com/Nwilliams96/Terrigal_Wet_Weather_2019).

3.0 Results

3.1 Environmental Data and FIB (*Enterococci*) analysis

Substantial changes in abiotic parameters occurred over the course of the rainfall event. Following 20mm of rainfall (on 4/6/19), there was a significant decrease in both temperature [p=0.0006] and salinity [p=0.0026] from the preceding two sampling points taken before the

rain event. This initial rainfall event also led to significant increases in levels of total dissolved phosphate (TDP) [$p=1.36x10^{-5}$], total phosphate (TP) [$p=8.10x10^{-9}$], total dissolved nitrogen (TDN) [$p=1.19x10^{-8}$], filterable reactive phosphorus (FRP) [p=0.0178], and ammonia [p=0.0002], relative to the two sampling points that were taken before the rain event. After the opening of the entrance to Terrigal Lagoon (6/6/19), input of lagoon water into Terrigal beach resulted in significant increases [p=0.001] in levels of TP, FRP, TDN, ammonia and dissolved oxygen (DO%) in the Terrigal seawater samples, relative to samples taken on before the lagoon opening on the 6/6/19. Five days after the end of the rainfall event, levels of all measured abiotic parameters returned to levels that were statistically indistinguishable from what was observed before the rain event.

During dry weather, *Enterococci* levels were below limit of detection (10 MPN/100ml) within all seawater samples on the 20/5/19 and 29/31 (94 %) of samples on the 31/5/19 (Figure 2A). Following 20.4 mm of rain (4/6/19), *Enterococci* levels reached the maximum limit for detection (24,196 MPN/100 ml) within all three drains (Figure 2A). Within the nearshore water samples, *Enterococci* levels increased by over 450-times relative to samples taken before the rain event. On the 6/6/19, after a further 20.4mm of rain, average *Enterococci* levels within seawater samples decreased three orders of magnitude. *Enterococci* levels were notably high within Drain 2 and the adjacent seawater sample (SW2.1) and at one Terrigal Lagoon site (TL2) which exceeded 10,000 MPN/100ml. After the entrance to Terrigal Lagoon was mechanically opened to the ocean, *Enterococci* levels were elevated along transect 6 (immediately adjacent to the mouth of the lagoon Figure 2A). Five days after the rainfall event (11/6/19), *Enterococci* levels within all Terrigal Beach seawater samples had decreased to the lower limit of detection (10 MPN/100ml).

3.2 Sewage signals - qPCR markers Lachno3, *Arcobacter* and *intI1*

Throughout the study, there was both a statistically significant correlation between Lachno3, *intI1* and *Arcobacter* [Lachno3:r_s>0.59; p<0.01] as well as between each of these markers and *Enterococci* [*Arcobacter:* r_s=0.33; p<0.01; Lachno3:r_s=0.67,p<0.001; *intI1*:r_s=0.28;p<0.001]. Before the rain event concentrations of Lachno3 were detected within only 9/53 (17%) of samples taken from seawater on the 20/5/19 and 31/5/19, however *Arcobacter* and *intI* were detected in substantially more samples 50/53 (95%) and 52/53 (98%) of samples respectively, but concentrations of both were statistically indistinguishable between either Terrigal Beach or Lagoon samples with the 'pristine' reference site at Forrester's Beach.

Following 20.4mm of rain (4/6/19), within the nearshore seawater samples, Lachno3 was recorded at concentrations two orders of magnitude higher [mean=50979±130926 copies/100ml] than were observed before the rain event [mean=820±2693 copies/100ml], with highest levels within samples immediately adjacent to stormwater drains (Figure 2B). At these sites *Arcobacter* levels also increased substantially by between 16- to over 1000-fold and *intI1* levels increased over 100-fold. The maximum concentrations of Lachno3, *Arcobacter* and *intI1* all occurred in Drain 4. In accordance with this pattern, the highest levels of Lachno3 present in Terrigal Beach seawater samples occurred in the sample immediately adjacent to Drain 4 (Figures 2B, C and D). Unfortunately, due to rough ocean conditions, it was not possible to acquire offshore transect samples on this date, precluding an assessment of the seaward spatial extent of contamination from stormwater drains. During this period, concentrations of Lachno3 [mean=51860±433copies/100ml] and *intI1* [mean=7005±9147copies/100ml] also increased by three orders of magnitude and by 13-times respectively within all Terrigal Lagoon samples (Figure 2B and D) in comparison to what was observed during dry weather [Lachno3 mean=81±433copies/100ml, *intI1* mean=501±488copies/100ml].

On 6/6/19, after 40.8mm of rain in the preceding 48 hours, levels of Lachno3 decreased significantly [p=0.0377] within the drains and adjacent seawater samples, while *Arcobacter*

and *intI1* levels remained at statistically indistinguishable compared to levels detected two days prior. Lachno3 and *intI* levels remained elevated at other points along Terrigal Beach, in particular within samples adjacent to Drains 1 and 2. Although, significant concentrations of these markers did not extend beyond the shore-line samples in any of the off-shore transects (Figures 2B and D). Levels of Lachno3 [mean=$7.8x10^4$±$4.1x10^4$copies/100ml] and *Arcobacter* [mean=$1.47x10^7$±$6.03x10^6$copies/100ml] increased significantly [p=0.02, 0.0006 respectively] within Terrigal Lagoon compared to the 4/6/19 [Lachno3, mean=51860±433copies/100ml, *Arcobacter*, mean=$1.69x10^6$±$1.99x10^6$], (Figure 2C).

Following the opening of the lagoon entrance on the afternoon of 6/6/19, a significant increase in the concentrations of Lachno3 [p= $1.25x10^{-5}$], *intI1* [p=0.0019] and *Arcobacter* [p=0.003] compared to the morning, was observed at, and surrounding, the lagoon entrance (i.e., transects 4, 5 and 6). Of note, increased levels of Lachno3 were observed up to 300m offshore in transects 5 and 6.

Following 5 days without rain (11/6/19) concentrations of Lachno3 and *Arcobacter* decreased significantly [p=0.0239 and $3.46x10^{-6}$ respectively] from those observed during the peak rainfall events, but remained significantly [p=$2.69x10^{-4}$ and $2.81x10^{-9}$, $3.32x10^{-21}$ and $2.81x10^{-15}$ respectively] elevated relative to the samples taken during before any rain (i.e., 20/5/19 and 31/5/19). Most notably, high levels of Lachno3, *intI1* and *Arcobacter* occurred within Drain 4, where concentrations were higher than those observed during the rainfall event. Concomitantly high levels of Lachno3 and *Arcobacter* were observed in the adjacent seawater sample (SW4.1), indicating an impact of this drain on the surrounding environment.

3,3 Bird Faeces signal

Levels of the GFD Avian faecal marker were not significantly elevated in Terrigal seawater samples relative to the relatively pristine control site at Forrester's Beach, during

either dry (pre- or post-rain) or wet conditions (both days of rainfall). Within Terrigal Lagoon, rainfall led a significant [p=0.0016] increase in concentrations of this marker. Five days after rainfall, concentrations of the GFD marker were comparable to those observed during the rainfall event.

3.4 ARG dynamics

Throughout the study period, levels the *sulI* gene were significantly correlated with levels of Lachno3, *Arcobacter* and *intI1* [Lachno3 r_s>0.31, *Arcobacter* r_s>0.32, *intI1* r_s>0.41; p<0.05]. Levels of the *qnrS* gene were significantly positively correlated with *Arcobacter* [r_s=0.28; p<0.05], but not *Enterococci* or Lachno3, nor any other ARGs, while levels of the *dfrA1* gene were positively correlated with levels of *Enterococci* and *sulI* [*Enterococci* r_s=0.36, *sulI* r_s=0.23; p<0.05]. *vanB* was not detected in any samples.

Before the rain event (20/5/19 and the 31/5/19), *sulI* and *dfrA1* were present in 13/53 (24%) and 12/53 (22%) of samples respectively, with detected levels of both genes an order of magnitude higher in the nearshore samples than the samples taken approximately 100m and 300m offshore. The ARGs *tetA* and *qnrS* displayed substantially different patterns, whereby these genes were detected in 49/53 (92%) and 43/53 (81%)) of samples before the rainfall event (20/5/19 and 31/5/19) (Figure 3 C and D), however there was no statistically distinguishable differences between levels of either gene in the Terrigal Beach seawater samples compared to the samples taken at the relatively clean reference site, Forrester's Beach.

After 20.4mm of rain (4/6/19), *sulI* and *qnrS* levels increased significantly [$p=8x10^{-10}$], with levels of *sulI* increasing by almost three orders of magnitude within the nearshore samples. In contrast, *dfrA1* was only detected within a single seawater sample (SW4.1), but levels within this sample were two orders of magnitude higher than the nearshore levels observed before the rain event. Interestingly, highest levels of *sulI* were observed in sample SW4.1 (directly

adjacent to Drain 4), where levels of this gene were an order of magnitude higher than those observed in other nearshore samples. This was consistent with the patterns observed within Drain 4, which had significantly higher [$p=6.66x10^{-6}$] levels of *sulI* and *dfrA1* than any other drain. Levels of both *dfrA1* and *sulI* increased significantly [p=0.0002] in Terrigal Lagoon relative to conditions before the rain event. The ARG *tetA*, again, displayed substantially differing patterns to the other ARGs, whereby in the nearshore seawater sample levels were not statistically different to levels observed before the rain event. During this time, the highest levels of *tetA* were recorded within Drains 1 and 2 but decreased significantly [p=0.0001] within the lagoon samples (Figure 3C) compared to samples taken before the rain event.

On the morning of the 6/6/19 after a further 20.4mm of rain, levels of *sulI* and *qnrS* in nearshore samples were statistically indistinguishable from the preceding sampling point, and an order of magnitude lower within the drain samples relative to 4/6/19. However, in samples taken 100m and 300m offshore, concentrations of both genes were an order of magnitude higher than conditions before the rain event. At this time the *dfrA1* gene was not detected within any of the Terrigal Beach seawater samples, while within the stormwater drains concentrations of this gene dropped by two orders of magnitude relative to the 4/6/19. In contrast, levels of *tetA* within Terrigal Beach seawater samples were significantly higher [$p<0.01$] than those observed before the rain event. Within the lagoon samples, *sulI* and *dfrA1* levels remained high, but were statistically indistinguishable from those observed on the 4/6/19, while *tetA* levels increased significantly [p=0.0008]. At this time, *qnrS* was only detectable in 1/5 lagoon samples (Figure 3D). Consistent with this pattern, the opening of the mouth of Terrigal Lagoon on the afternoon of 6/6/19 did not lead to an increase in *qnrS* levels in Terrigal beach seawater samples. Conversely, the opening of the lagoon led to a 5 order of magnitude increase in levels of *sulI* at the site adjacent to the mouth of the lagoon (SW6.1) (Figure 3B, Supplementary Figure 1), while levels of *tetA* doubled in the seawater samples near to the mouth of the lagoon

(SW5.X, 6.X and 7.X) (Figure 3C) and *dfrA1* was detected in the nearshore sites SW5.1 and SW6.1, where, notably, it had been undetected on the same morning

Five days after the rain event (11/6/19), *dfrA1* was not detected, and *sulI* and *qnrS* levels dropped significantly [$p=3.57x10^{-12}$, $p=3.77x10^{-11}$] within both nearshore and offshore samples. Opposing this trend, levels of *tetA* were statistically indistinguishable from those recorded during the rainfall event (4/6/19 and the 6/6/19). At this time, levels of all ARGs remained high within Drain 4 and were statistically indistinguishable from those observed during the rainfall event. However, within Terrigal lagoon samples each ARG displayed a different pattern. Levels of *tetA* and *sulI* remained high and were statistically indistinguishable from those observed on the 4/6/19, while *qnrS* levels increased significantly [p=0.003]. The *dfrA1* gene was detected within a single lagoon sample (L5), but levels were 26-fold lower than those observed on the 6/6/19.

3.5 Bacterial Community Analysis

3.5.1 Patterns in bacterial community diversity and composition

Throughout the study period, bacterial diversity (Shannon's) was significantly lower within the Drain 1 [F=45.15, p<0.001], Drain 2 [F=127.3, p<0.001] and Drain 4 [F=309.9, p<0.001] samples relative to Terrigal Beach seawater samples. Bacterial community composition also differed significantly between Drain 1 [F=37.5, p<0.001], Drain 2 [F=78.4, p<0.001] and Drain 4 [F=101.5, p<0.001] samples and seawater samples. Stormwater drain bacterial communities were also significantly less diverse [F=123, p<0.001], and dissimilar in community composition to Terrigal Lagoon samples [F=46.2, p=0.001]. In contrast, bacterial communities within Terrigal lagoon were significantly [F=46.2, p=0.001] more diverse and significantly different in community composition [F=101.5, p=0.001] to the nearshore Terrigal

beach seawater samples. Bacterial composition was also significantly different between drains [F=5.04, p=0.001].

3.5.2 Associations between indicator taxa and AbR genes

To determine whether specific amplicon sequence variants (ASVs) resolved in the 16S rRNA amplicon sequencing data were indicative of stormwater, lagoons, or seawater samples, we identified 'indicator taxa' using the indicspecies package (De Caceres and Jansen, 2018). Network analysis was then used to identify putative links between these indicator taxa and the measured ARGs.

Within seawater samples, there were 507 indicator ASVs identified. Of these, none displayed a statistically significant correlation in their relative abundance to any of the measured ARGs. In contrast, 10,283 indicator ASVs were identified from stormwater drain samples, and from among these 5493 correlations occurred between the relative abundance of stormwater indicator ASVs and ARGs. The highest number of significant correlations with stormwater drain indicator ASVs occurred with *intI1* (Figure 4) (1,940/10283; 19%), followed by *sulI* (1,838/10283; 18%), *tetA* (1,167/10283; 11%), *dfrA1* (1,122/10283; 11%) and lastly *qnrS* (1,037/10283; 10%). Among stormwater sample indicator taxa, a number of positive correlations to ARGs occurred among members of the *Comamonadaceae* family, including 317 positive correlations with *intI1*. We also observed 319 *Comamonadaceae* ASVs displaying positive correlations with *sulI*, along with 150 with *dfrA1*, 149 with *tetA* and 71 with *qnrS*. Within the *Comamonadaceae* family, discrete ASVs identified as *C. testosteroni,* displayed four positive relationships with *intI1* [$p<0.05$, $r_s=0.77,0.44,0.40,0.33$], five with *sulI* [$p<0.05$, $r_s=0.78,0.66,0.36,0.09,0.07$], three with *dfrA1* [$p<0.05$, $r_s=0.56,0.50,0.36$] and three with *qnrS* [$p<0.05$, $r_s=0.25,0.18,0.05$]. Stormwater drain indicator taxa, identified as *Bacteroides* ASVs also displayed many statistically significant correlations with levels of the ARGs measured

here. *Bacteroides* ASVs in fact had the most (40) positive correlations with *intI1*, followed by 38 correlations with *sulI*, 8 with *dfrA1*, 8 with *tetA* and 6 with *qnrS*. Of note, *B. fragilis*, correlated with *intI1* and *sulI* [$p<0.05$, $r_s=0.76,0.08$]. *intI1* levels were also correlated with 12 *Arcobacter* ASVs, the strongest being with *A. butzleri* (B1000019013) [$p<0.05$, $r_s=0.82$]. Levels of the ARGs *sulI*, *dfrA1* and *qnrS* were also positively correlated with *A. butzleri* (B1000019013) [$p<0.05$, $r_s=0.46,0.36,0.10$].

A further 1,160 ASVs were identified as indicators of lagoon samples. Of note, 1.5% of these taxa were identified as members of the *Arcobacteraceae* family, 3.6% were members of the *Comamonadaceae* family and 1% were members of the *Vibrionaceae* family. Interestingly, levels of the *qnrS* gene were positively correlated with 98% (n=1,141) of these lagoon indicator ASVs, which was more than those observed with drain indicator ASVs. Positive correlations were also observed between lagoon indicator taxa and *tetA* (1080) *intI1* (967), *sulI* (707), and *dfrA1* (471). Similar to the patterns observed with stormwater drain indicator ASVs, significant positive correlations were observed between all five measured ARGs and indicator ASVs identified as *Comamonadaceae* and *Arcobacter/Pseudarcobacter*.

4.0 Discussion

ARG contamination within coastal environments poses a potential health risk to people who use coastal environments recreationally (Leonard et al., 2018), but the extent of this issue and the underlying dynamics and mechanisms are not well resolved. The central goal of our study was to determine to what extent sewage contamination leads to an increased presence of ARGs in coastal environments. We focussed on a popular swimming beach in eastern Australia, which has regularly poor water quality ratings due to faecal contamination (DPIE, 2020). Terrigal Beach has multiple potential input points for faecal contamination, including a network of stormwater drains and an adjacent coastal lagoon, Terrigal Lagoon, which is

periodically opened to the ocean during heavy rain events. We hypothesised that these features may act as conduits for ARG contamination at Terrigal beach.

4.1 Sewage contamination of a beach leads to elevated ARGs

To determine if sewage contamination was linked to ARG abundance at Terrigal Beach, we first quantified two indicators of human faeces, *Enterococci* and the Lachno3 qPCR marker (Feng et al., 2018) as well as the *intI1* gene which has been identified as an excellent microbial measure of anthropogenic contamination in aquatic habitats (Gillings et al., 2015), and *Arcobacter*, a genus of bacteria linked to human sewage, urban stormwater, and sewage pipe infrastructure (McLellan and Roguet, 2019). All markers displayed a significant increase from low levels observed before the rain event, to high levels following 20.4mm of rain. Levels within all stormwater drains increased significantly relative to what was observed before rainfall, becoming highly elevated within nearshore seawater and Terrigal Lagoon samples. It is notable however, that in nearshore seawater samples that were not located immediately adjacent to stormwater drains, concentrations of Lachno3 remained low or often undetectable. This was particularly true for transects outside of the Terrigal Haven region (i.e., at North Avoca, SW0.1, Wamberal Beach SW8.1 and Forresters beach SW9.1 Figure 2B). The highest levels of all markers were detected adjacent to Drain 4, indicating it as a major source of sewage contamination. Notably, adjacent to Drain 4, levels of *Enterococci* were 49-times higher than the WHO safe swimming guidelines category D, which indicates a high level of illness transmission (WHO 2009) and levels of Lachno3 were only an order of magnitude lower than levels that have been detected within human faeces (Ahmed et al., 2019).

When Terrigal Lagoon was opened to the ocean, it had a pronounced impact on water quality at Terrigal Beach, particularly at sites immediately adjacent to the entrance of the lagoon. Near to the opening of Terrigal Lagoon, Lachno3, *intI1* and *Arcobacter* levels

increased significantly at the surrounding sites, demonstrating widespread sewage contamination following the opening of the lagoon entrance indicating that opening Terrigal Lagoon had an impact on the surrounding coastal environment that extended up to a few hundred metres to the east and south of the lagoon entrance. Taken together, these patterns confirm that both Terrigal Lagoon and the network of stormwater drains (particularly Drain 4) are sources of sewage contamination at Terrigal Beach, presenting a human health risk given that sewage has been previously linked to microbial pathogens (Collado 2011), and ARGs (Steinbakk et al., 1992).

Of note, concentrations of *intI1* and all ARGs investigated in this study (except *qnrS*), correlated positively with concentrations of either *Enterococci* or Lachno3. While the causes of elevated ARG levels within the environment are potentially multifaceted, the clear correlations between our MST markers for sewage and targeted ARGs indicate that sewage contamination following rainfall contributes to increased ARG levels at Terrigal Beach supporting our proposition that sewage contaminated water is the major conduit for ARG contamination at this site. While there was no statistically significant spatiotemporal links between *qnrS* and the human faecal marker, it should be noted that the samples with the highest levels of *qnrS* coincided with very high levels of Lachno3, particularly Drain 4 and the seawater sample directly adjacent to it, SW4.1. The strong evidence for sewage contamination through the stormwater drains and the lagoon being linked to a high load of ARG and *intI1* input into Terrigal Beach is in-line with other research in China, Europe, and the USA (Karkman et al., 2019).

After confirming the presence of sewage contamination at Terrigal Beach, and that levels of sewage markers correlated with 75% of detected ARGs, we next investigated the principal input points of these ARGs into the environment. Concentrations of all ARGs, as well as the *intI1* gene, displayed a dramatic increase during and after rainfall. After a total of

40.8mm of rainfall, levels of all four detected ARGs and *intI1* were highest within Drain 4, and among the seawater samples collected at Terrigal Beach, highest levels were observed in the samples adjacent to Drain 4 (SW4.1). Indeed, levels of *dfrA1* and *sulI* at this site were an order of magnitude higher and levels of *tetA* were two orders of magnitude higher, than levels of these genes previously observed in a highly contaminated urbanised beach in Sydney, which has previously been demonstrated to have high levels of sewage and ARG contamination (Carney et al., 2019).

While Drain 4 certainly appears to be a significant source of ARG contamination at Terrigal Beach, our analysis also revealed that Terrigal Lagoon also contained high levels of all four targeted ARGs following rainfall, and that the opening of the lagoon's entrance led to dramatic, and spatially extensive, increases in ARG levels at Terrigal Beach.

While the levels of 75% of the targeted ARGs were significantly correlated with either *Enterococci* or Lachno3 levels, not all the ARGs displayed the same spatial or temporal dynamics. Indeed, the only ARGs which correlated significantly with each other were *dfrA1* and *sulI*. In general, detectable and sometimes relatively high levels of *tetA* and *qnrS* occurred during dry weather both before and after the rain event, whereas *dfrA1* and *sulI* were largely undetectable at this time. This may be indicative of background levels of these genes in the environment, as both genes have been detected in remote areas that are subject to little or no anthropogenic pollution (Scott et al., 2021; Tan et al., 2018). Both tetracycline and quinolone resistance has been detected in avian faeces (Skarżyńska et al., 2021) which could explain them being widespread before the rain event, however no correlation was observed between either *tetA* or *qnrS* and the GFD qPCR marker (Green et al., 2012) for avian faeces (supplementary material 2.1).

Throughout the rain event on both the 4/6/19 and 6/6/19, both Drain 4 and Terrigal Lagoon displayed high levels of all 4 ARGs, however within Drains 1 and 2 the different ARGs

displayed different patterns. On the 4/6/19, high levels of both *sulI* and *tetA* were detected within Drain 1, but *qnrS* and *dfrA1* were undetectable, and from the 4/6/19–6/6/19 *sulI* and *dfrA1* levels decreased substantially within Drain 2, while *qnrS* and *tetA* levels increased substantially. Differing abundance patterns of ARGs has previously been linked to shifting bacterial communities (Zhu et al., 2017). Our analysis revealed different bacterial communities between the drains, presenting a possible explanation for these differing abundances of ARGs.

After determining links between human faecal markers and ARGs, we wanted to explore the possible vehicles for ARGs entering the environment. To do this, we employed the *indicspecies* package (De Caceres and Jansen, 2018), which enabled us to identify ASVs that are indicative of storm water infrastructure from the 16S rRNA sequencing dataset and coupled this with network analysis to determine correlations between these ASVs, and levels of ARGs identified via qPCR. We identified three groups of indicator taxa, that were representative of the stormwater drains, Terrigal Lagoon, and seawater at Terrigal Beach. All targeted ARGs displayed over 1000 correlations to bacterial indicators of the stormwater drains, which included a large proportion of *Comamonadaceae*, *Arcobacter* and *Bacteroides*. Notably, members of each of these groups are regularly associated with sewage (Fisher et al., 2014; Shanks et al., 2013). Similarly, the bacterial indicators for Terrigal Lagoon that showed the highest levels of correlations to ARGs included *Comamonadaceae* and *Arcobacter/Pseudarcobacter*. Of note, none of the bacterial indicators for seawater at Terrigal Beach displayed significant correlations to ARGs, further confirming that ARG levels at this swimming beach are linked to input of water from stormwater drains and Terrigal Lagoon, which is linked to sewage contamination of these samples. Differences in the putative vehicles for ARG input into Terrigal Beach may also account for the spatial differences in *tetA* compared to *sulI* and *dfrA1*, as discussed above.

Considering that many *Arcobacter* ASVs were correlated to levels of *tetA*, we examined levels of *Arcobacter* determined via qpCR finding strong correlations between *tetA* and the qPCR results. One example of this was in Drain 2 on the 6/6/19 after a total of 40.8mm of rain in the preceding 3 days, we found both *tetA* and *Arcobacter* to be at levels more than double than Drain 4 which had the highest levels of both markers for faecal contamination. An explanation for this is that *Arcobacter* may have been a vehicle for *tetA* entering the environment, indeed, of all the ARGs, *tetA* shared the strongest positive correlation (r_s=0.45) with *Arcobacter* detected via qPCR. This pattern is in line with previous research identifying *tetA* resistant *A. butzleri* (Jasim et al., 2020), however it should be noted that other bacteria may harbour tetA but weren't detected in this analysis.

In this study, we have shown a significant relationship between sewage markers and ARGs in the environment. We have also shown that both sewage and ARG contamination enter from two major sources, Drain 4 and Terrigal Lagoon, and we suggest these locations as the most relevant sites for pollution remediation strategies. Considering recent research has determined consistent surfing and swimming are activities that can lead to exposure to AbR bacteria (Leonard et al., 2018), high levels of ARGs in Terrigal represent a previously over-looked but potentially noteworthy health risk to recreational beach users.

Not only does sewage harbour ARGs, but it can also harbour numerous enteric pathogens (García-Aljaro et al., 2019) such as bacteria from the genus *Arcobacter* (Collado et al., 2011; Fisher et al., 2014; Millar and Raghavan, 2017). Amongst the *Arcobacter* ASVs detected via 16S sequencing we detected *A. butzleri*, which is a known human enteric pathogen (Levican et al., 2013). Relative abundances of this bacteria were correlated with the ARGs *sulI*, *dfrA1* and *qnrS*. ASVs identified as *Comamonadaceae testosteroni*, and *Bacteroides fragilis*, which are other known enteric pathogens (Tiwari and Nanda, 2019; Wexler, 2007) also displayed correlations to all targeted ARGs. While it should be noted that the resolution of 16S

rRNA sequencing is insufficient to provide unequivocal species or strain-level identification, and our correlative analysis does not prove that these bacteria host the measured ARGs, our results provide evidence that the potential human health impacts of coastal ARG contamination potentially require further attention. Further research could include culturing of pathogenic bacteria from untreated raw sewage at local sewage treatment plants, stormwater drains and the environment. These isolates could then be screened for ARGS, providing a more definitive conclusion.

5.0 Conclusions

The increase of AbR within natural environments is a point of rising global concern (Finley et al., 2013) and AbR within coastal environments represents a potential health threat to recreational users of these environments (Leonard et al., 2018). This study has shown a direct link between sewage contamination and an increase in ARGs at a popular coastal beach, and in doing so was also able to discriminate the point sources of ARGs, which were identified as stormwater drains and a nearby coastal lagoon that also receives sewage contaminated stormwater. Finally, we demonstrated that not all ARGs display the same spatial and temporal dynamics in coastal environments. This study serves as a warning of the potential health-risk associated with recreational use of sewage contaminated beaches and highlights the links between contaminated stormwater and ARGs in the environment, elevating the necessity for remediation of beaches impacted by faecal contamination.

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7.0 Author Contributions

NW, NS, SM, JP, PS, CJ, MJ, and JS planned sampling locations and designed experiments. NW, NS and CJ took and processed samples. NW performed qPCR, 16S analysis, MIC tools analysis and designed all figures. NW, NS and JS analysed data and wrote the manuscript.

8.0 References

Ahlstrom, C.A., Bonnedahl, J., Woksepp, H., Hernandez, J., Olsen, B., Ramey, A.M., 2018. Acquisition and dissemination of cephalosporin-resistant E. coli in migratory birds sampled at an Alaska landfill as inferred through genomic analysis. Sci. Rep. 8, 1–11. https://doi.org/10.1038/s41598-018-25474-w

Ahmed, W., Gyawali, P., Feng, S., McLellan, S.L., 2019. Host Specificity and Sensitivity of Established and Novel Sewage-Associated Marker Genes in Human and Nonhuman Fecal Samples 85.

Albanese, D., Riccadonna, S., Donati, C., Franceschi, P., 2018. A practical tool for maximal information coefficient analysis. Gigascience 7, 1–8. https://doi.org/10.1093/gigascience/giy032

Allard, J.D., Bertrand, K.P., 1992. Membrane Topology of the pBR322 Tetracycline Resistance Protein. https://doi.org/10.1016/S0021-9258(19)37116-9

Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J., Handelsman, J., 2010. Call of the wild: Antibiotic resistance genes in natural environments. Nat. Rev. Microbiol. 8, 251–259. https://doi.org/10.1038/nrmicro2312

Arbizu, M., 2020. Adonis., pairwiseAdonis: Pairwise multilevel comparison using. R Packag. version 0.4.

Auguet, O., Pijuan, M., Borrego, C.M., Rodriguez-Mozaz, S., Triadó-Margarit, X., Giustina, S.V. Della, Gutierrez, O., 2017. Sewers as potential reservoirs of antibiotic resistance. Sci. Total Environ. 605–606, 1047–1054. https://doi.org/10.1016/j.scitotenv.2017.06.153

Baquero, F., Martínez, J.L., Cantón, R., 2008. Antibiotics and antibiotic resistance in water environments. Curr. Opin. Biotechnol. 19, 260–265. https://doi.org/10.1016/j.copbio.2008.05.006

Bastyns, K., Cartuyvels, D., Chapelle, S., Vandamme, P., Goossens, H., De Wachter, R., 1995. A Variable 23S rDNA Region is a Useful Discriminating Target for Genus-Specific and Species-Specific PCR Amplification in Arcobacter Species. Syst. Appl. Microbiol. 18, 353–356. https://doi.org/10.1016/S0723-2020(11)80427-3

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum, H., Norström, M., Pons, M.N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., Martinez, J.L., 2015. Tackling antibiotic resistance: The environmental framework. Nat. Rev. Microbiol. 13, 310–317. https://doi.org/10.1038/nrmicro3439

Berglund, B., Khan, G.A., Weisner, S.E.B., Ehde, P.M., Fick, J., Lindgren, P.E., 2014. Efficient removal of antibiotics in surface-flow constructed wetlands, with no observed impact on antibiotic resistance genes. Sci. Total Environ. 476–477, 29–37. https://doi.org/10.1016/j.scitotenv.2013.12.128

Bonnedahl, J., Järhult, J.D., 2014. Antibiotic resistance in wild birds. Ups. J. Med. Sci. 119, 113–116. https://doi.org/10.3109/03009734.2014.905663

Borjesson, S., Dienues, O., Jarnheimer, P.Å., Olsen, B., Matussek, A., Lindgren, P.E., 2009. Quantification of genes encoding resistance to aminoglycosides, -lactams and tetracyclines in wastewater environments by real-time PCR. Int. J. Environ. Health Res. 19, 219–230. https://doi.org/10.1080/09603120802449593

Callahan, B.J., Mcmurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2 : High-resolution sample inference from Illumina amplicon data. Nat. Methods 13. https://doi.org/10.1038/nmeth.3869

Carney, R.L., Brown, M. V., Siboni, N., Raina, J.B., Kahlke, T., Mitrovic, S.M., Seymour, J.R., 2020. Highly heterogeneous temporal dynamics in the abundance and diversity of the emerging pathogens Arcobacter at an urban beach. Water Res. 171, 115405. https://doi.org/10.1016/j.watres.2019.115405

Carney, R.L., Labbate, M., Siboni, N., Tagg, K.A., Mitrovic, S.M., Seymour, J.R., 2019. Urban beaches are environmental hotspots for antibiotic resistance following rainfall. Water Res. 167, 115081. https://doi.org/10.1016/j.watres.2019.115081

Chow, L., Waldron, L., Gillings, M.R., 2015. Potential impacts of aquatic pollutants: Sub-clinical antibiotic concentrations induce genome changes and promote antibiotic resistance. Front. Microbiol. 6, 1–10. https://doi.org/10.3389/fmicb.2015.00803

Collado, L., Levican, A., Perez, J., Figueras, M.J., 2011. Arcobacter defluvii sp. nov., isolated from sewage samples. Int. J. Syst. Evol. Microbiol. 61, 2155–2161. https://doi.org/10.1099/ijs.0.025668-0

De Caceres, M., Jansen, F., 2018. indicspecies: Relationship between species and groups of sites. R package version 1.7.5.

Dixon, P., 2003. Computer program review VEGAN , a package of R functions for community ecology. J. Veg. Sci. 14, 927–930.

DPIE, 2020. State of the Beaches Report 2019-2020. Sydney.

Eaton, A.D., Franson, M.A.., 2005. Standard Methods for the Examination of Water and Wastewater, 23rd EditionTitle. American Public Health Association, American Water Works Association Water Environment Federation, Washington, Denver, Alexandria.

Feng, S., Bootsma, M., McLellan, S.L., 2018. Human-associated Lachnospiraceae genetic markers improve detection of fecal pollution sources in urban waters. Appl. Environ. Microbiol. 84, 1–14. https://doi.org/10.1128/AEM.00309-18

Ferreira, S., Queiroz, J.A., Oleastro, M., Domingues, F.C., 2016. Insights in the pathogenesis and resistance of Arcobacter: A review. Crit. Rev. Microbiol. 42, 364–383. https://doi.org/10.3109/1040841X.2014.954523

Finley, R.L., Collignon, P., Larsson, D.G.J., Mcewen, S.A., Li, X.Z., Gaze, W.H., Reid-Smith, R., Timinouni, M., Graham, D.W., Topp, E., 2013. The scourge of antibiotic resistance: The important role of the environment. Clin. Infect. Dis. 57, 704–710. https://doi.org/10.1093/cid/cit355

Fisher, J.C., Levican, A., Figueras, M.J., McLellan, S.L., 2014. Population dynamics and ecology of Arcobacter in sewage. Front. Microbiol. 5, 1–9. https://doi.org/10.3389/fmicb.2014.00525

Foley, S.L., Lynne, A.M., 2008. Food animal-associated Salmonella challenges: pathogenicity and antimicrobial resistance. J. Anim. Sci. 86. https://doi.org/10.2527/jas.2007-0447

García-Aljaro, C., Blanch, A.R., Campos, C., Jofre, J., Lucena, F., 2019. Pathogens, faecal indicators and human-specific microbial source-tracking markers in sewage. J. Appl. Microbiol. 126, 701–717. https://doi.org/10.1111/jam.14112

Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, K., Tiedje, J.M., Zhu, Y.G., 2015. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. ISME J. 9, 1269–1279. https://doi.org/10.1038/ismej.2014.226

Grape, M., Motakefi, A., Pavuluri, S., Kahlmeter, G., 2007. Standard and real-time multiplex PCR methods for detection of trimethoprim resistance dfr genes in large collections of bacteria. Clin. Microbiol. Infect. 13, 1112–1118. https://doi.org/10.1111/j.1469-0691.2007.01807.x

Green, H.C., Dick, L.K., Gilpin, B., Samadpour, M., Field, K.G., 2012. Genetic markers for rapid PCR-based identification of gull, Canada goose, duck, and chicken fecal contamination in water. Appl. Environ. Microbiol. 78, 503–510. https://doi.org/10.1128/AEM.05734-11

Huovinen, P., Sundstrom, L., Swedberg, G., Skold, O., 1995. Trimethoprim and sulfonamide resistance. Antimicrob. Agents Chemother. 39, 279–289. https://doi.org/10.1128/aac.39.2.279

Illumina, 2013. 16S Metagenomic Sequencing Library. Prep. 16S Ribosomal RNA Gene Amplicons Illumina MiSeq Syst. Introd. 1–28.

Jarma, D., Sánchez, M.I., Green, A.J., Peralta-Sánchez, J.M., Hortas, F., Sánchez-Melsió, A., Borrego, C.M., 2021. Faecal microbiota and antibiotic resistance genes in migratory waterbirds with contrasting habitat use. Sci. Total Environ. 783, 146872. https://doi.org/10.1016/j.scitotenv.2021.146872

Jasim, S.A., Al-abodi, H.R., Ali, W.S., 2020. Resistance rate and novel virulence factor determinants of Arcobacter spp., from cattle fresh meat products from Iraq. Microb. Pathog. 152, 104649. https://doi.org/10.1016/j.micpath.2020.104649

Karkman, A., Pärnänen, K., Larsson, D.G.J., 2019. Fecal pollution can explain antibiotic resistance gene abundances in anthropogenically impacted environments. Nat. Commun. 10, 1–8. https://doi.org/10.1038/s41467-018-07992-3

Klein, E.Y., Milkowska-Shibata, M., Tseng, K.K., Sharland, M., Gandra, S., Pulcini, C., Laxminarayan, R., 2021. Assessment of WHO antibiotic consumption and access targets in 76 countries, 2000–15: an analysis of pharmaceutical sales data. Lancet Infect. Dis. 21, 107–115. https://doi.org/10.1016/S1473-3099(20)30332-7

Leonard, A.F.C., Zhang, L., Balfour, A.J., Garside, R., Gaze, W.H., 2015. Human recreational exposure to antibiotic resistant bacteria in coastal bathing waters. Environ. Int. 82, 92–100. https://doi.org/10.1016/j.envint.2015.02.013

Leonard, A.F.C., Zhang, L., Balfour, A.J., Garside, R., Hawkey, P.M., Murray, A.K., Ukoumunne, O.C., Gaze, W.H., 2018. Exposure to and colonisation by antibiotic-resistant E. coli in UK coastal water users: Environmental surveillance, exposure assessment, and epidemiological study (Beach Bum Survey). Environ. Int. 114, 326–333. https://doi.org/10.1016/j.envint.2017.11.003

Levican, A., Alkeskas, A., Günter, C., Forsythe, S.J., Figueras, M.J., 2013. Adherence to and invasion of human intestinal cells by Arcobacter species and their virulence genotypes. Appl. Environ. Microbiol. 79, 4951–4957. https://doi.org/10.1128/AEM.01073-13

Lombardo, M.N., G-Dayanandan, N., Wright, D.L., Anderson, A.C., 2017. Crystal structures of trimethoprim-resistant DfrA1 rationalize potent inhibition by propargyl-linked antifolates. Physiol. Behav. 176, 139–148. https://doi.org/10.1021/acsinfecdis.5b00129.Crystal

Martin, M., 2011. Cutadapt Removes Adapter Sequences From High-Throughput Sequencing Reads. EMBnet,journal.

Mazel, D., Dychinco, B., Webb, V.A., Davies, J., 2000. Antibiotic resistance in the ECOR collection: Integrons and identification of a novel aad gene. Antimicrob. Agents Chemother. 44, 1568–1574. https://doi.org/10.1128/AAC.44.6.1568-1574.2000

McLellan, S.L., Roguet, A., 2019. The unexpected habitat in sewer pipes for the propagation of microbial communities and their imprint on urban waters. Curr. Opin. Biotechnol. 57, 34–41. https://doi.org/10.1016/j.copbio.2018.12.010

Millar, J.A., Raghavan, R., 2017. Accumulation and expression of multiple antibiotic resistance genes in Arcobacter cryaerophilus that thrives in sewage. PeerJ 2017. https://doi.org/10.7717/peerj.3269

Moré, E., Ayats, T., Ryan, P.G., Naicker, P.R., Keddy, K.H., Gaglio, D., Witteveen, M., Cerdà-Cuéllar, M., 2017. Seabirds (Laridae) as a source of Campylobacter spp., Salmonella spp. and

antimicrobial resistance in South Africa. Environ. Microbiol. 19, 4164–4176. https://doi.org/10.1111/1462-2920.13874

NHMRC Australia, 2008. Guidelines for managing risks in recreational water, Australian Government - National Health and Medical Research Council.

Paulus, G.K., Hornstra, L.M., Alygizakis, N., Slobodnik, J., Thomaidis, N., Medema, G., 2019. The impact of on-site hospital wastewater treatment on the downstream communal wastewater system in terms of antibiotics and antibiotic resistance genes. Int. J. Hyg. Environ. Health 222, 635–644. https://doi.org/10.1016/j.ijheh.2019.01.004

Olds, H.T., Corsi, S.R., Dila, D.K., Halmo, K.M., Bootsma, M.J., McLellan, S.L., 2018. High levels of sewage contamination released from urban areas after storm events: A quantitative survey with sewage specific bacterial indicators. PLoS Med. 15, 1–24. https://doi.org/10.1371/journal.pmed.1002614

Pei, R., Kim, S.C., Carlson, K.H., Pruden, A., 2006. Effect of River Landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). Water Res. 40, 2427–2435. https://doi.org/10.1016/j.watres.2006.04.017

Sarmah, A.K., Meyer, M.T., Boxall, A.B.A., 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere 65, 725–759. https://doi.org/10.1016/j.chemosphere.2006.03.026

Scott, L.C., Wilson, M.J., Esser, S.M., Lee, N.L., Wheeler, M.E., Aubee, A., Aw, T.G., 2021. Assessing visitor use impact on antibiotic resistant bacteria and antibiotic resistance genes in soil and water environments of Rocky Mountain National Park. Sci. Total Environ. 785, 147122. https://doi.org/10.1016/j.scitotenv.2021.147122

Shanks, O.C., Newton, R.J., Kelty, C.A., Huse, S.M., Sogin, M.L., McLellan, S.L., 2013. Comparison of the microbial community structures of untreated wastewaters from different

geographic locales. Appl. Environ. Microbiol. 79, 2906–2913. https://doi.org/10.1128/AEM.03448-12

Skarżyńska, M., Zając, M., Bomba, A., Bocian, Ł., Kozdruń, W., Polak, M., Wiącek, J., Wasyl, D., 2021. Antimicrobial Resistance Glides in the Sky—Free-Living Birds as a Reservoir of Resistant Escherichia coli With Zoonotic Potential. Front. Microbiol. 12, 1–12. https://doi.org/10.3389/fmicb.2021.656223

Steinbakk, M., Lingaas, E., Carlstedt-Duke, B., Høverstad, T., Midtvedt, A.C., Norin, K.E., Midtvedt, T., 1992. Faecal concentration of ten antibiotics and influence on some microflora-associated characteristics (MACs). Microb. Ecol. Health Dis. 5, 269–276. https://doi.org/10.3109/08910609209141594

Suzuki, M.T., Taylor, L.T., DeLong, E.F., 2000. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5'-nuclease assays. Appl. Environ. Microbiol. 66, 4605–4614. https://doi.org/10.1128/AEM.66.11.4605-4614.2000

Tan, L., Li, L., Ashbolt, N., Wang, X., Cui, Y., Zhu, X., Xu, Y., Yang, Y., Mao, D., Luo, Y., 2018. Arctic antibiotic resistance gene contamination, a result of anthropogenic activities and natural origin. Sci. Total Environ. 621, 1176–1184. https://doi.org/10.1016/j.scitotenv.2017.10.110

Tiwari, S., Nanda, M., 2019. Bacteremia caused by Comamonas testosteroni an unusual pathogen. J. Lab. Physicians 11, 087–090. https://doi.org/10.4103/jlp.jlp_116_18

Van Boeckel, T.P., Gandra, S., Ashok, A., Caudron, Q., Grenfell, B.T., Levin, S.A., Laxminarayan, R., 2014. Global antibiotic consumption 2000 to 2010: An analysis of national pharmaceutical sales data. Lancet Infect. Dis. 14, 742–750. https://doi.org/10.1016/S1473-3099(14)70780-7

Wexler, H.M., 2007. Bacteroides: The good, the bad, and the nitty-gritty. Clin. Microbiol. Rev. 20, 593–621. https://doi.org/10.1128/CMR.00008-07

WHO, 2009. Addendum To the Who Guidelines for Safe Recreational Water Environments , Volume 1 , Coastal and Fresh Waters. Water 1, 36.

Zhu, Y.G., Zhao, Y., Li, B., Huang, C.L., Zhang, S.Y., Yu, S., Chen, Y.S., Zhang, T., Gillings, M.R., Su, J.Q., 2017. Continental-scale pollution of estuaries with antibiotic resistance genes. Nat. Microbiol. 2. https://doi.org/10.1038/nmicrobiol.2016.270