Antibiotic resistance gene levels in the highly urbanised coastal environment of Sydney Harbour

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**Abstract**

Antibiotic resistant bacteria are increasingly being found in aquatic environments, representing a potential threat to public health. To examine the dynamics and potential sources of antibiotic-resistant genes (ARGs) in urbanised waterways, we performed a six-month temporal study at six locations within the Sydney Harbour estuary. These locations spanned a salinity gradient from seawater at the mouth of the harbour to freshwater at the more urbanised western sites. We quantified the abundances of three ARGs (*sulI*, *tetA*, and *dfrA1*) and an anthropogenic pollution marker (*intI1*). To assess potential sources of environmental ARGs, we also quantified levels of the sewage marker (*Lachnospiraceae*), bird-associated faecal pollution markers (GFD), and a common wastewater pipe-dwelling genus of bacteria (Arcobacter). We assessed the impact of a major rainfall event on ARG levels during this period. The strong rainfall event led to increases in *intI1* and ARGs (*sulI* and *dfrA*) across sites, but the potential source for ARGs was different. Some sites experienced sewage intrusions, as defined using the human-faecal marker *Lachnospiraceae*, which were clearly correlated with ARG levels. However, at the two sites furthest from the ocean, links between ARG levels and sewage were less evident, with correlations to other contaminants, including heavy metals, apparent. These results highlight the potential complexities associated with identifying, and ultimately remediating, the causes and sources of antimicrobial resistance within natural aquatic ecosystems.

**Keywords**: Antibiotic resistance, Rainfall, Anthropogenic pollution, Aquatic environment, Heavy metals, Health risk.

**1. Introduction**

*1.1 Antibiotic resistance in natural environments*

The widespread use of antibiotics in medicine (Hofer, 2022), as well as in agricultural and veterinary practices (Mann et al., 2021), has led to an increased prevalence of antibiotic-resistant bacteria in natural environments (Bilal et al., 2020; Carney et al., 2019; Williams et al., 2022). Consequently, antibiotic resistance genes (ARGs) are now considered significant emerging environmental pollutants (Feng et al., 2021; Komijani et al., 2021). It was demonstrated that the incidence of antibiotic resistance in the gut microbiome of recreational beach users was three times higher than non-water users (Leonard et al., 2018). Elevated levels of antibiotic-resistant bacteria in natural environments have been increasingly recognised as a potential threat to human and ecosystem health (Leonard et al., 2022; Serwecińska, 2020), so understanding the sources and reservoirs of ARGs within ecosystems has become a key goal in most antibiotic resistance initiatives.

*1.2 Impact of wastewater on aquatic antibiotic resistance*

The introduction of antibiotic-resistant bacteria into aquatic environments via anthropogenic wastewater streams occurs largely due to the excretion of large quantities of antibiotics or active metabolites from human populations into sewage (Wang et al., 2023) . This leads to the selection of antibiotic resistance within bacteria inhabiting sewage waste-streams (Auguet et al., 2017; Medina et al., 2020), as well as within environments that are ultimately contaminated by sewage (Carney et al., 2019; Larsson and Flach, 2022; Williams et al., 2022). Sewage containing both antibiotics and antibiotic-resistant bacteria can contaminate aquatic environments via stormwater exposed to sewage infiltration, caused by aging and leaking infrastructure or during wet weather sewage over-flow events (Moors, 2015).

*1.3 Animal faeces as a source of antibiotic resistance*

In addition to sewage, contamination of aquatic environments by animal faeces can also be a source of antibiotic resistance (Xie et al., 2018; Yue et al., 2021). Antibiotics are frequently used in animal food to promote the well-being and growth of animals (Van et al., 2020). Livestock producers in the United States, use 70–80% of antibiotics sold across the country (Elliott et al., 2017), with most of the consumed antibiotics excreted into the environment (Xie et al., 2018). Detection of ARG’s in the faeces of domesticated (Bourne et al., 2019; Schmidt et al., 2018) and wild animals (Zhao et al., 2020), is also a potential source of ARGs in the environment (Larsson and Flach, 2022).

*1.4 Non-faecal sources of ARG contamination*

As the faecal material of animals and humans is often enriched in bacteria harbouring ARGs (Larsson and Flach, 2022), close correlations between anthropogenic pollution and the presence ARGs are often observed (Gillings et al., 2015; Guan et al., 2022). However, there are also scenarios where ARG contamination can occur independently of faecal contamination. For example, wastewater infrastructure, such as stormwater pipes, contain bacterial biofilms that are regularly exposed to other forms of chemical pollution (i.e disinfectants and heavy metals), which can lead to the selection of ARGs in resident bacteria (Jia et al., 2023; Komijani et al., 2021). During rainfall events, flushing of these bacterial communities from stormwater pipes can introduce antibiotic-resistant bacteria into aquatic sites (Carney et al., 2019; Williams et al., 2022), meaning that allochthonous inputs of ARGs may occur independently of sewage contamination.

*1.5 Heavy metals and co-selection of ARGs*

In addition to allochthonous introduction of ARGs to aquatic environments, there is evidence that the presence of heavy metal and metalloids pollution can further increase antibiotic resistance through co-selection mechanisms (Komijani et al., 2021; Mahbub et al., 2020; Yonathan et al., 2021). Therefore, aquatic environments exposed to current or legacy contamination with heavy metals could also experience elevated levels of ARGs.

*1.6 ARGs dynamics in Sydney region*

Previously, we have provided evidence that elevated levels of ARGs occur at coastal sites in eastern Australia following rainfall events, with evidence for a key role of sewage as a vector for introducing ARGs to marine ecosystems (Carney et al., 2019; Williams et al., 2022). Here, we examined the dynamics of ARGs within Sydney Harbour, a large and highly urbanised estuary that runs through the centre of Australia’s most populated city (Sydney), spanning gradients of salinity and anthropogenic pressure. Here, we examined the abundance of ARGs and different anthropogenic pollution markers at six sites within the Sydney Harbour, spanning salinity and pollution gradients. We assume that the source for ARGs in different site across the harbour following rainfall events might be different. Defining, and potentially remediating, the sources of ARGs within aquatic ecosystems is important because there is a largely undefined, but potentially significant, exposure risk to humans using the ecosystems for recreation purposes.

**2. Methods**

*2.1 Description of study sites* *in Sydney Harbour Estuary*

Six study sites were chosen along the 250 km2 Sydney Harbour estuary system (Figure 1), spanning a salinity gradient from seawater at the mouth of the harbour to freshwater at the more urbanised western sites. The sampling locations included sites in the Parramatta River (33.811° S, 151.000° E), Homebush (33.825° S, 151.052° E), Putney Park (33.835° S, 151.109° E), Chiswick (33.846°S, 151.142° E), Barangaroo Reserve (33.857° S, 151.201° E) and Chowder Bay (33.839° S, 151.254° E) (Figure 1).

The Parramatta River site is located approximately 26 km from the mouth of the estuary and within a highly urbanised watershed and large industrial areas. High levels of heavy metals in sediments (Irvine and Birch, 1998) and herbicides and pharmaceutical compounds in water samples (Birch et al., 2015) have previously been detected in this area. The sampling point at this site is situated 50 m downstream from a large stormwater drain. The Homebush sampling site is located 10 m downstream from a stand of mangroves and has previously been reported to be impacted by legacy leachates including metal contamination from historical industrial pollution (Binet et al., 2003; Irvine and Birch, 1998). The Putney Park site is located in the middle harbour region of Sydney Harbour, is surrounded by a highly residential area, and is situated near to several stormwater drains and sewage overflow pipes. The Chiswick sampling site is located next to a wharf in a highly populated suburban area and is also situated near a sewage overflow pipe and a publicly accessible ocean pool. The Barangaroo Reserve site is located in the central business district (CBD) of Sydney. Finally, the Chowder Bay site is located 2.5 km from the mouth of the Sydney Harbour estuary and previously has been shown to be least affected by pollution within Sydney Harbour (Birch, 2017).

*2.2 Sampling Methodology and Rainfall Data Collection*

Monthly sampling was conducted from September 2016 to February 2017. In addition, high resolution (every two days) sampling was conducted during a targeted, two-week long rainfall event February 2017 (8th February to 17th February), during which 57 - 116 mm of rain was recorded across the different sites. At each site (6 sites) and sampling time (11 time points), triplicate 2 L water samples were collected for downstream molecular analysis from each site using pre-washed 2 L plastic containers that were pre-rinsed with 10% bleach, washed three times with MiliQ water, and rinsed again three times with water from the collection point prior to sampling. Throughout the duration of the study, 198 water samples were collected for molecular analysis. Within three hours of collection, samples were filtered through 0.22 μm Durapore membrane filters (catalogue: GVWP04700) using a peristatic pump. Filters were then stored at -20 °C.

For nutrient analysis, approximately 50 mL of surface water was sampled in triplicate and immediately filtered through a 0.45 μm Sartorius Minisart filter (catalogue: 16533-k) before being transported back to the laboratory on ice and stored at -20 °C. For total metal and metalloid analysis, triplicate 15 mL of unfiltered surface water samples were collected, acidified with 7M nitric acid and stored at -4 °C.

Rainfall data were collected from the Australian Bureau of Meteorology from the weather station positioned closest to each sampling location (<http://www.bom.gov.au/climate/data/index.shtml>).

*2.3 DNA extraction and quantitative PCR (qPCR) analysis*

DNA was extracted from filters using the PowerWater DNA extraction kit (catalogue: 14900-100-NF) according to the manufacturer’s guidelines. A total of 198 water DNA samples were extracted. Microbial Source tracking (MST) assays designed to detect faecal pollution in water samples were applied to examine potential sources of contamination. To quantify the presence of human faeces, indicative of sewage contamination, we used the *Lachno12* assay (Feng et al., 2018). We also quantified levels of *Arcobacter*, a genus of bacteria which is regularly associated with stormwater, water-distribution pipe biofilms and sewage effluent (McLellan and Roguet, 2019) and has recently been shown to be correlated with some ARGs in near shore marine environments (Carney et al., 2019; Williams et al., 2022). The *Arcobacter* qPCR assay targets a variable regen within the 23S rRNA gene (Bastyns et al., 1995). Furthermore, we quantified the class 1 integron-integrase gene (*intI1*)*,* whichhas been demonstrated to be an excellent marker for antibiotic resistance, heavy metals and other anthropogenic pollution (Gillings et al., 2015). To quantify *intI1* gene abundances, we used the *intI1* primer set (Mazel et al., 2000) (Supplementary Table 1). Finally, in addition to sewage and anthropogenic pollution markers, we applied qPCR to determine levels of avian faeces contamination. To quantify levels of avian faeces contamination, we applied the *GFD* assay, which targets avian-specific *Helicobacter* spp. bacteria (Green et al., 2012) (Supplementary Table 1).

We quantified the abundance of three ARGs: *sul1*, *tetA* and *dfrA1*, which have all previously been detected in high abundances at multiple locations around Sydney including following rainfall events (Carney et al., 2019; Williams et al., 2022). Moreover, the genes *sul1* and *tetA* are the most common ARGs in wastewater treatment plants (Wang et al., 2020a). The gene *sul1* is part of the sulfonamide resistance gene family, which confers resistance to antibiotics used widely in clinical settings (Pei et al., 2006). The *tetA* gene encodes resistance to tetracyclines, which are a group of antibiotics widely used to treat bacterial infections and as a growth promoters in animal feedlots (Börjesson et al., 2009; Granados-Chinchilla and Rodríguez, 2017). Finally dfrA1 confers resistance to trimethoprim (Grape et al., 2007), a group of antibiotics used widely in human medicine, including the treatment of urinary tract infections (Supplementary Table 1).

For all assays, quantitative PCR was performed using a Bio-Rad CFX384 Touch Real-Time PCR Detection System with a six-point standard curve and negative controls using the BIO-RAD CFX Maestro software. All standard curves were constructed from 10-fold dilutions of Qubit-quantified gene block fragments or cloned DNA product. For each sample, qPCR was performed with technical triplicates using a 5 μl reaction volume. Calibration curves and plate preparation were performed using a Liquid Handling Workstation epMotion 5075l (Eppendorf). Reactions volumes consisted of 2.5μl iTaq Universal SYBR green or Universal probes Supermixs with 0.2 µM of each forward and reverse primers (Supplementary Table 1), 0.1 µM probe (for the Lachno12 assay), 1 μl of diluted (1:10) DNA template and nuclease free water for a final volume of 5µL. The qPCR cycling conditions involved one cycle at 95°C for 3 min, 40 cycles at 95°C for 15 sec and the gene specific annealing/extension temperature (Supplementary Table 1) for 60 sec. A coefficient of variation (CV) was calculated for the Cq technical triplicates and for samples with CV > 2 % a technical triplicate was removed from the analysis to improve CV. A melting curve was added to the end of each SYBR qPCR assay to confirm the presence of a single PCR product. The resulting data were normalised to gene copies per litre of water.

*2.4 Nutrient analysis*

For samples from Homebush, Putney Park and Chowder Bay concentrations of ammonia, phosphate, nitrite, and total nitrogen were determined using flow injection analysis (FIA) on a Lachat Quikchem QC8500 Automated Ion Analyser (LACHAT Instruments, USA). A total of 45 samples were collected and processed for nutrient analysis. Prior to running samples, a working standard was made for each nutrient and calibration curves were prepared for Ammonia (NH3), Phosphate (PO4), NO2- and NO(x) (Total nitrogen: NO(x) - NO2- = NO3-). In addition to the water samples and method blanks, additional duplicates and spiked samples were included as controls.

*2.5 Heavy Metal Analysis at Homebush Site*

For samples from Homebush (21 samples in total), a site with a legacy of metal contamination from historical industrial pollution (Binet et al., 2003), concentrations of heavy metals and metalloids, namely vanadium (V), chromium (Cr), manganese (Mn), cobalt (Co), nickel (Ni), zinc (Zn), arsenic (As), selenium (Se), molybdenum (Mo), silver (Ag), cadmium (Cd), antimony (Sb), barium (Ba), and lead (Pb), were determined using an inductively coupled plasma mass spectrometry (ICP-MS, Agilent Technologies, Japan, Model 7900). Blanks, spikes, and continuing calibration verification standards (CCVs) were run during the analysis of the water samples. In addition, standard reference material (SRM), Trace elements in natural water (SRM 1640) from the National Institute of Standard and Technology (NIST) was also analysed to validate the results of trace elements in water (Supplementary Table 2).

*2.6 Statistical analysis of ARGs abundance and correlations*

To compare ARG abundance between locations, a Kruskal-Wallis test was performed, followed by a Dunn’s post hoc test with Benjamini-Hochberg adjusted p-value (q-value) and a false-discovery rate (FDR) of 0.01. For comparing ARG abundance between the grouped baseline and the rainfall event, the same tests were used, but with an FDR of 0.05. To examine relationships between ARG abundance, nutrients (Homebush, Putney Park and Chowder Bay) and rainfall, Pearson’s correlations followed by Bonferroni correction were performed. To determine whether rainfall influences the concentrations of nutrients, heavy metals and metalloids at the Homebush site, a one-way ANOVA was performed. Homogeneity of variance was tested with Levene’s test and in instances where homogeneity of variance was not observed, a Kruskal-Wallis test was used. All aforementioned statistical tests except the correlations were performed in the R statistical environment (R Core Team, 2013) using the stats (R Core Team, 2013), FSA (Ogle et al., 2020) and car (Fox and Weisberg, 2019) packages, respectively. Correlations were performed in the PAST statistical environment (Hammer et al., 2001). To identify relationships between heavy metals/metalloids and ARGs within Homebush samples, network analysis was performed with a maximal information coefficient analysis (MICtools). Only significant correlations with Pearson correlation (r) above 0.3 or below -0.3 were included and visualised with Cytoscape (Shannon et al., 2003).

**3. Results**

*3.1 Spatial differences in ARGs abundance*

When comparing ARGs abundances across sites, spatial differences were observed for each of the ARGs - *sul1* (Kruskal-Wallis test; H = 77, p < 0.001), *dfrA1* (H = 68, p < 0.001) and *tetA* (H = 27, p < 0.001), as well as the anthropogenic pollution marker gene, *intI1* (H = 69, p < 0.001). Differences between locations were primarily driven by high abundances of *intI1*, *sul1* and *dfrA1* at Parramatta (Figure 2; Table 1), the location furthest from the mouth of Sydney harbour, and elevated levels of *tetA* and *intI1* at Homebush, the second-most distant location from the mouth of Sydney Harbour.

*3.2 Correlation of ARGs with rainfall and pollution markers*

Network analysis of qPCR assays and rainfall data across all sites identified positive Pearson correlations between *Arcobacter*, *Lachnospiraceae*, *sul1*, *intI1* and *dfrA* abundances and rainfall levels. In addition, positive correlations were detected between the human sewage marker *Lachnospiraceae* and *Arcobacter*, *sul1* and *dfrA* (Supplementary Table 3, Figure 5B).

In general, the rainfall event observed on the 8th of February 2017 led to increases in the abundance of each of the ARGs, as well as *intI1*, *Lachnospiraceae* and total *Arcobacter* (Figure 3). When grouping all baseline samples, each location separately (before 08/02/17), and comparing them to the rain event, abundances of *intI1* and *sul1* were consistently elevated at each location during the rainfall event (Table 2), while abundances of *dfrA1* were elevated at all locations during the rainfall event except the Putney site. No changes in *tetA* gene abundance were observed at any location.

Both *Lachnospiraceae* and total *Arcobacter* were elevated in response to rainfall at all sites except for Homebush and Parramatta (Table 2). In all sites except Parramatta, a significant decrease in the abundance of the GFD gene occurred during rainfall. In agreement with the above patterns, rainfall was positively correlated with the abundance of *Lachnospiraceae* (p < 0.001, r = 0.58), *Arcobacter* (p < 0.001, r = 0.44), *intI1* (p < 0.001, r = 0.35), *sul1* (p < 0.001, r = 0.35), and *dfrA1* (p < 0.001, r = 0.36) (Supplementary Table 3, Figure 5B). Interestingly, positive correlations were also found between *Lachnospiraceae* and *Arcobacter* (p < 0.001, r = 0.49) and *Lachnospiraceae* and *sul1* (p < 0.001, r = 0.46) (Supplementary Table 3, Figure 5B). Additional correlation analysis performed on a subset of the sites (Homebush, Putney Park and Chowder Bay) where nutrient data was available, identified positive correlations between NO3 and rainfall (p < 0.001, r = 0.72) and between NO3 to *Arcobacter* abundance (p < 0.001, r = 0.49).

*3.3 Heavy metal and nutrient analysis at Homebush*

Since the Homebush site is known to be contaminated with trace elements (Suh et al., 2004), has a long record of industrial pollution (Binet et al., 2003) and had a significantly elevated level of ARGs during rainfall, it was chosen for further assessment of heavy metals/metalloid and nutrient concentrations. All tested nutrients (ammonia, phosphate, nitrite and nitrate) significantly increased in concentration during the rainfall event relative to their respective baselines (Figure 4; Supplementary Table 4). Concentrations of Mn, Zn, Sb and Ba also significantly increased in response to rainfall, whereas As, Mo and Cd all significantly decreased (Figure 4; Supplementary Table 4). Rainfall was positively correlated to ammonia (p < 0.001, r = 0.92), nitrite (p <0.001, r = 0.99) and nitrate (p = 0.001, r = 0.99). When considering correlations between rainfall and elements, rainfall was positively correlated to Zn (p < 0.001, r = 0.94) and Ni (p = 0.003, r = 0.49) and negatively correlated to Mo (p < 0.001, r = -0.93).

An additional network analysis was performed on the Homebush site to: (i) examine the co-occurrence of heavy metals, metalloids and ARGs (Figure 5, Supplementary Table 5) and (ii) to identify relationships between ARGs and pollution markers. This analysis revealed two discrete modules of correlations. The first module included heavy metals, metalloids and nutrients: Ba, Ni, Zn, Pb, Sb, Mn, NO3, NO2, NH3 and PO4 that generally involved positive correlations with rainfall levels, *sul1*, *intI1* and Lachno12. The second module included Ag, AS, Mo and Cd, which displayed mainly negative correlations to rainfall, *sul1* and *intI1* (Figure 5, Supplementary Table 5). The network analysis identified a positive relationship between rainfall and *sul1*, *dfrA*, *intI1*, Ba, Ni, Zn, Pb, Sb, Mn, PO4, NO3, NO2, NH3 and negatively correlated to As Mo and Cd.

**4. Discussion**

*4.1 The two sites farthest from the ocean had the highest level of ARGs*

The Sydney Harbour estuary is regularly impacted by anthropogenic contamination including stormwater runoff and raw sewage inputs (Birch et al., 2015; Birch and Rochford, 2010). In light of observations from other nearby aquatic environments that have revealed links between these types of contamination and high levels of ARGs (Carney et al., 2019; Williams et al., 2022), we hypothesised that at least one or more ARGs would be detected at all sites during the study period. It is important to note that we tested only a few ARGs, which do not represent the large distribution of ARGs in the environment (Zhuang et al., 2021). Therefore, our results do not necessarily extend to other ARGs. With this in mind, we found that the two sites farthest from the ocean (Parramatta and Homebush) contained highest levels of *intI1*, *sul1*, *tetA* and *dfrA1*. Moreover, these sites were also characterised by ARG spikes following a large rainfall event. These patterns are consistent with both sites having a history of pollution, including pharmaceuticals, heavy metals, metalloids and pesticides, that have been proposed to have arisen from leaky sewer pipes, polluted sediment or from polluted landfills leachates (Binet et al., 2003; Birch, 2017; Birch et al., 2015; Irvine and Birch, 1998).

*4.2 Rainfall increased the abundance of most AGRs*

The abundance of *intI1*, *sul1* and *dfrA1* significantly increased at all locations (except *dfrA1* at Putney) in conjunction with rainfall events, which is in line with similar observations at a nearby coastal site (Carney et al., 2019). Both *sul1* and *dfrA* are often found within the class 1 integron gene cassette (Domínguez et al., 2019) and may explain the co-occurrent spike of these genes. Interestingly, levels of *tetA* did not increase above baseline levels following rainfall and did not display a correlation with the anthropogenic pollution marker *intI1*. Similarly, other studies have failed to identify a correlation between tetracycline genes and *intI1* (Di Cesare et al., 2017; Wang et al., 2020b), and there is the potential that this pattern may be explained by the broad presence of these genes across environmental bacteria (Roberts, 2003; Zhang et al., 2009), which might indicate that tetracycline resistance genes are poor markers for anthropogenically sourced ARG pollution.

*4.3 Avian faeces is not the source for ARGs pollution*

Based on observations elsewhere (Allen et al., 2010; Bourne et al., 2019; Hultman et al., 2018; Yue et al., 2021; Zhao et al., 2020), we assumed that ARG pollution would be associated with either animal faeces or sewage, and therefore applied microbial source tracking approaches to identify the potential sources of ARGs in the environment. Stormwater pipe and sewage related *Arcobacter* (McLellan and Roguet, 2019), avian specific *Helicobacter* spp. (Green et al., 2012), and sewage specific Lachnospiraceae (Feng et al., 2018) were quantified and related to levels of rainfall and ARG pollution. The avian specific assay displayed negative correlations with rainfall and ARGs, indicating that bird faecal material is not an abundant source of ARGs in the sampled sites during the weather event, despite wild bird faeces being identified as a source of ARGs in other locations (Zhao et al., 2020).

*4.4 During the rainfall event, sewage was a potential source for ARGs in most sites*

*Arcobacter* species have previously been identified as a possible host of ARGs at coastal beaches in Australia (Carney et al., 2019), while *Lachnospiraceae* species are widely used as sensitive indicators of sewage (Feng et al., 2018). The strong positive correlation between rainfall and *Lachnospiraceae* and *Arcobacter* abundances suggest that the strong rainfall event during this study was associated, in most sites, with raw human sewage through urban water infrastructures. While all three ARGs across the dataset were positively correlated with *Arcobacter* abundance, only *sul1* and *dfrA* were positively correlated with rainfall and *Lachnospiraceae* abundance. However, these patterns suggest that in most of the sites examined in this study, sewage was a potential source for two of the tested ARGs during the rainfall event. Strong weather events with heavy rainfall are a significant issue for Sydney Water and shown to cause sewer overflows in 36% of tested sewer and in 82% of the tested stormwater system (Besley and Cassidy, 2022).

*4.5 Rainfall increased heavy metals and ARGs in a site with legacy of contamination*

At the Homebush site, rainfall was linked to an increase of the level of heavy metals/metalloids and ARGs which is consistent with previous studies (Huo et al., 2023; Murray et al., 2024). A network analysis identified a significant cooccurrence between the correlations of rainfall, *intI1*, *sul1*, and to some extent, also *dfrA* to nutrients, metals and metalloids. Positive relationships between Zn and trimethoprim in sediment (Dickinson et al., 2019) and mixed correlations between heavy metals and sulfonamide resistance (Wu et al., 2016) have been reported previously. However, other studies have found that the association between metals or metalloids with ARGs in aquatic environments can be variable, potentially due to local differences in other environmental factors such as pH, which affects metal solubility (Nguyen et al., 2019; Ohore et al., 2020). Nevertheless, strong associations between ARGs and metals in aquatic sediments and terrestrial soils (Huo et al., 2023; Mahbub et al., 2020; Murray et al., 2024; Zhao et al., 2019) have been observed. While metals are likely to play a role in co-selection for pipe-dwelling bacteria due to constant exposure, the chemical complexity of stormwater and sewage likely makes it difficult to identify specific metal-ARG associations unless studies are conduct over longer periods.

*4.6 Co-occurrence of resistance genes in intI1 cassettes increased ARGs abundance*

The *intI1* marker has been proposed to be an excellent indicator of anthropogenic pollution, as class 1 integrons are effective markers for gene mobility, facilitating the transfer of resistance genes, including those providing resistance to disinfectants, metals and antibiotics, found on plasmids and other mobile elements (Gillings et al., 2015). In most sites human sewage is a source of antibiotic resistance. We also know that the microbial community uniquely adapted to stormwater infrastructure such as *Arcobacter* can also be a potential source of ARGs (Fisher et al., 2014; McLellan and Roguet, 2019). However, the history of chemical pollution at the Homebush site, including metal contamination (Binet et al., 2003; Dafforn et al., 2012), together with only a small shift in abundance of human sewage associated markers (*Lachnospiraceae* and *Arcobacter*)at this site following the rain event, may suggest that the association of ARGs with *intI1* is link to resistance to metals and metalloids. It is likely that the level of polluted substance in the water column increase following strong rainfall event. In this case, the co-occurrence of metal resistance genes and ARGs within the *intI1* cassettes, potentially promotes the spread of ARGs among the bacterial community (Li et al., 2017) through co-selection mechanisms (Huo et al., 2023; Komijani et al., 2021; Mahbub et al., 2020; Murray et al., 2024; Yonathan et al., 2021).

*4.7 The potential source of ARGs is different between the sites*

This study has demonstrated that while there was a clear link between rainfall and environmental levels of ARGs, the source for these ARGs was potentially very different. In most of the sites, and consistent with the findings of other studies (Carney et al., 2019; Williams et al., 2022), sewage and urban water infrastructure appear to have been the main source of ARGs in Sydney Harbour. However, at Homebush and Parramatta, which are well known to be heavily polluted sites (Binet et al., 2003; Birch et al., 2015; Irvine and Birch, 1998), other factors appear to have played a role. Moreover, both sites locate at the upper estuary of Sydney Harbour, where higher concentration of pharmaceutical product and pesticides were reported (Birch et al., 2015). At both sites, rainfall did not lead to increases in the abundance of the sewage marker *Lachnospiraceae* or stormwater infrastructure marker *Arcobacter*. At Homebush, a site with a long record of metal and metalloid industrial pollution (Binet et al., 2003; Suh et al., 2004), we identified positive correlative links between rainfall, levels of metals and metalloids and ARGs that are potentially suggestive of co-selection in this site. In this instance, potential flushing of heavy metal enriched (and ARGs selected) contaminated water, or resuspension of sediments with legacy contamination, may have released heavy metals, metalloids, and antibiotic-resistant bacteria into the water column. These results highlight the potentially complex nature of ARGs contamination within aquatic ecosystems, whereby multiple sources of contamination, including sewage and stormwater inputs, as well as legacy industrial contamination may lead to elevated levels of ARGs within the environment.

**Figure legend**

**Figure 1**: Map of sampling locations across the Sydney Harbour estuary system.

**Figure 2:** Mean gene copies of different ARGs and pollution markers at individual locations. Each location is a different colour. Displayed data are mean gene copies L-1 and error bars are standard error of mean (n=3)

**Figure 3:** Time series of ARGs and anthropogenic pollution marker gene copies at individual locations. Grey bars represent rainfall with darker bars representing heavier rainfall. Each complete time-series at each location is a discrete colour. Solid black bars represent the baseline monthly sampling prior to the rainfall event. Vertical dashes represent the start of the rainfall event and the two-day sampling regimen following the rainfall event, except for the final sampling time-point which was five-days after the previous sampling time-point. Displayed data are averaged gene copies L-1 and error bars are standard errors of mean (n=3).

**Figure 4:** Heatmap of ARGs gene copies, heavy metals/metalloids and nutrients at Homebush. Data are scaled and the darker the blue the greater the number of gene copies or concentration. Asterisks represent significant comparisons between pooled baselines (before 08/02/17) and the rainfall event (08/02/17). Statistics and averaged values are provided in Supplementary Table 4. Green, orange, black and grey bars are nutrients, heavy metals/metalloids, quantified genes and rainfall, respectively.

**Figure 5:** Network analysis of: A) ARGs, nutrients, heavy metals and metalloids, rainfall and pollution markers at Homebush. Only significant relationships with absolute Pearson correlation above 0.3 are shown. B) ARGs, rainfall and pollution markers across all sites and time points. Coloured edges (lines) represent the correlation between nodes (variables). Red and blue edges are positive and negative correlations respectively and thicker and darker lines represent stronger correlations.

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