

INFLUENCE OF URBAN SEWAGE POLLUTION ON FISH MICROBIOMES

A Final Report of the Tibor T. Polgar Fellowship Program

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Abstract

The impact of urban pollution on fish microbiomes in a coastal megacity was evaluated. Fecal indicator bacteria (FIB) concentrations in water were paired to antibiotic resistant microorganism (ARM) concentrations in both fish guts and associated environmental water along a gradient of urbanization. A gradient of urban sewage impact across study sites was established based on historical data. This gradient was not significant based on sampling within the scope of this study; however, this was likely due to under-sampling. ARMs were detected in 95% of the individual fish tested. A positive correlation was found between FIB and ARMs at sites as well as between ARMs in water and in guts. Proteobacteria was the most abundant phyla detected in fish gut microbiomes; moreover, genus *Ralstonia* made up 63% of all ARM isolates. While environmental impacts on fish microbiomes are explored widely in the literature, this study is novel in its attempt to understand these impacts in the context of urban sewage pollution.

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INTRODUCTION

The majority of the world's metropolises have been built on coasts and estuaries because they have historically provided safe locations for ports with inland connectivity (Ross 1995). These environments are also typically rich in local biodiversity. As cities have grown, these environments have been subject to similarly rich and diverse contamination. Despite the substantial environmental degradation that has resulted from urban development, these environments still host diverse wildlife including species on high priority lists for conservation (Ives et al. 2015). While many resources are dedicated to the monitoring, management, and conservation of urban wildlife, a complete understanding of how urban pollution impacts resident species is still lacking.

Species that live in cities, particularly those that live in aquatic environments, are subject to both persistent historical and modern pollution sources. Urban waterways receive a variety of contaminants from stormwater runoff and wastewater, both treated and untreated. Of particular concern are the results of combined sewage systems, which confer untreated sewage into local waterways via combined sewage outfalls (CSOs) to avoid overwhelming sewage infrastructure during wet weather events. These sources discharge a variety of contaminants including heavy metals, PAHs, pesticides, pharmaceuticals, hormones, solvents, petroleum products, debris, and microorganisms (Rodenburg et al. 2010; Hendricks and Pool 2012; Eaton et al. 2013; Cantwell et al. 2018; Walter et al. 2019). Antibiotic resistant microorganisms (ARMs), in particular, have been found to enter waterways via CSOs, drastically increasing in concentration with wet weather events, closely correlated with fecal indicator bacteria (Young et al. 2013), suggesting that ARMs may be a helpful metric of urban sewage impact.

Furthermore, chemical contaminants can alter microbial environments through coselection (Nguyen et al. 2019). These conditions exhibit spatial heterogeneity, with highly localized conditions (Rouff et al. 2013). Furthermore, sediments can act as a reservoir for contaminants, including microorganisms both with and without antibiotic resistance (O'Mullan et al. 2019; Rodgers et al. 2018).

Recent developments recognizing the role that environment plays in shaping an organism's microbiome and the resultant impact this has on organismal health underscores a critical gap in the understanding of how urban pollution affects local aquatic species. Microbiomes made up of microbiota, the assemblage of microorganisms that reside within a given environment, their genes, and their habitat (Marchesi and Ravel 2015), are critical to organismal health. Gut microbiomes, in particular, impact fish development, immunity, nutrition, fecundity, survival, hormonal regulation, and energy homeostasis (Mehdinejad et al. 2019; Nayak 2010). Fish microbiomes are exposed to urban contamination through a variety of pathways. Early environment shapes the development of fish gut microbiomes (Giatsis et al. 2015); therefore, exposure to contaminants and microbiota originating from urban sewage sources can significantly impact fish in early life stages. Diet plays an integral role in shaping fish gut microbiomes (Tarnecki et al. 2017). Thus, benthivorous foraging behavior may expose fish microbiomes to contaminants and microbiota present in sediment reservoirs. Furthermore, while poorly documented, instances of fish feeding at outfalls of both treated and untreated wastewater have been recorded in both press (Tompkins 2015; Van Velzer 2017) and the literature (Moore 1932; Campbell 1939; Allen et al. 1976; Russo

1989). This suggests that fish microbiomes may reflect the highly localized spatial heterogeneity of urban pollution.

Urban contaminants can disrupt gut microbiome diversity, assemblages, and distribution. In fact, gradients of urbanization alone have been recently revealed to influence the microbiomes of resident species (Gaday et al. 2019; Murray et al. 2020). The Hudson River Estuary is home to New York City, its pollution, and a variety of fish species. Estuaries are unique in the critical roles they play in the life cycles of many fish species: providing nursery habitats for juvenile fish and passage for anadromous and catadromous species; therefore, New York City's waterways host both permanent and transient residents, some of which are at their most vulnerable life stages. Despite significant evidence that microbiomes influence fish health, and that urban contamination may alter these microbiomes, the influence of urban sewage pollution on the microbiomes of fish has not yet been directly studied.

In order to address this gap, the effect of urban pollution on fish microbiomes was explored along a gradient of sewage influence using antibiotic resistant bacteria as a metric of impact. The goals of this study were: (1) to establish a gradient of urban sewage impact using historical water quality data; (2) to confirm whether ARMs could be found in fish gut microbiomes; (3) to determine if there is a connection between ARM concentrations in the fish guts and contamination in their local environment; and (4) to characterize the taxonomic identity of the ARMs present in gut microbiomes. It was hypothesized that ARMs are present in fish gut microbiomes and vary along gradients of sewage impact in urban environments.

METHODS

Site Selection

Three sites with similar salinities and expected to have decreasing degrees of urban sewage pollution were chosen for sample collection: Flushing Creek; Little Neck Bay; and Hobart Beach (Figure 1; Table 1). Flushing Creek is located in a densely populated part of Queens, New York, and is adjacent to several high volume combined sewage outfalls which release upwards of 500 million gallons of untreated sewage into the waterway per year (Table 1; NYC DEP 2019). By comparison, Little Neck Bay, which lies on the boundary between Queens and Nassau County, receives wastewater/stormwater by separated stormwater pipes with only one major CSO approximately one mile from the study site, and has a lower population density (NYC DEP 2015). The Little Neck Bay study site itself is adjacent to a stormwater sewer designated solely for street water runoff (NYC DEP 2015). Finally, Hobart Beach, Eatons Neck, represents the least impacted of the study sites. This location exists on a recreational beach located along a spit, somewhat removed from direct street runoff, in an area of relatively low population density. In order to confirm a gradient of urban sewage pollution, historical datasets retrieved from the National Water Quality Monitoring Council (2020) for Hobart Beach (site ID 21NYBCH-NY477769-01) and the New York Water Trail Association (2020) for Little Neck Bay and Flushing Creek, were analyzed for overall fecal indicator bacteria trends.



Figure 1: Map of study sites, FC=Flushing Creek, LNB=Little Neck Bay, HB=Hobart Beach. Base map imagery retrieved from Esri (2021).

Table 1: Description of study sites. Population density data based on 2010 census data (United States Census Bureau 2010) of adjacent community districts Queens CD7, Queens CD11, and Eatons Neck representing Flushing Creek, Little Neck Bay, and Hobart Beach, respectively.

Site	Coordinates	Population Density	Site Description
Flushing Creek (FC)	40.761198, -73.835539	20,927 people/mile ²	This sample site is adjacent to Combined Sewer Overflow (CSO) outfalls TI-010 and TI-022, which release 463 million and 65 million gallons/year, respectively.
Little Neck Bay (LNB)	40.779155, -73.768005	12,386 people/mile ²	This sample site is adjacent to a storm sewer which is designated for street runoff only.
Hobart Beach (HB)	40.922675, -73.404104	1,396 people/mile ²	This sample site is adjacent to a beach designated for recreation which is located on a spit a distance from direct runoff from the street.

Sample Collection

During fall 2020, four sampling events occurred at FC and LNB and three sampling events occurred at HB. With each sampling event, three fish guts were sampled along with water from their environment. Mummichogs (*Fundulus heteroclitus*) were used as a model organism as they are found throughout the Harbor, including sites of extreme pollution, and have a small home range of <200 m (Skinner et al. 2012).

Mummichogs were collected using baited minnow traps, which were set to receive the incoming tide and left for no more than 2 hours. Once fish were collected, they were euthanized using a 0.4% solution of MS-222 followed by submersion in an ice slurry, consistent with methods outlined in the literature (e.g. Givens et al. 2015; Lloyd et al. 2016) in accordance with institutional guidelines (IACUC approved protocol #192). Euthanized fish were then wiped with 95% ethanol, dissected, and whole GI tracts were removed. Gut contents were extracted using sterile tweezers and placed in 1mL sterilized environmental water along with the whole gut. Samples were placed on ice and subsamples were processed for cultivation-based enumeration within six hours of extraction, and the remainder was frozen for future molecular analysis.

Microbial Enumeration

A spread plate technique was utilized to provide a relative enumeration of antibiotic resistant microorganisms (ARMs) across samples and to allow initial isolation of resistant colonies; therefore, both heterotrophic and antibiotic resistant microorganisms were quantified to enable a comparison. Four tenfold dilutions of water and gut samples were prepared, using sterilized (autoclaved) estuarine water. 0.1 mL sub samples of gut extractions and environmental water were added to plates containing solid R2A Agar

media with or without antibiotics to quantify heterotrophic and antibiotic resistant microorganisms, respectively. Following methodology outlined in Young et al. (2013), concentrations of 50mg/L and 10 mg/L of Ampicillin or Tetracycline, respectively, were used for antibiotic containing plates. Plates were subsequently incubated at 28°C for three days and colonies were visually enumerated heterotrophic (het), ampicillin resistant (ARB) and tetracycline resistant (TRB) bacteria.

Water samples were also processed for enterococci within six hours of collection, as specified in EPA method 1600 (US EPA 2006), using the IDEXX Enterolert methodology, consistent with prior literature (e.g., Eaton et al. 2013; Young et al. 2013; O'Mullan et al. 2019). Enterolert media was dissolved into 90mL of sterile deionized water, 10mL of sample water was added creating a ten-fold dilution, as recommended for brackish water samples (IDEXX 2019). The solution was sealed in a Quanti-Tray2000 container and incubated at 41°C for 24 hours in accordance with Enterolert procedures (IDEXX 2019). Post incubation enterococci concentration was quantified through visual enumeration of fluorescent quanti-tray wells under UV light and subsequently compared with the associated MPN table and multiplied by the dilution factor, consistent with IDEXX protocols (2019).

Molecular Techniques

Isolated bacterial colonies were picked from heterotrophic, ampicillin, and tetracycline plates using a micropipette tip and placed in 40 µL sterile hyclone water, and frozen until shipping. Single pass colony DNA sequences of the 16S rRNA gene were obtained through Sanger Sequencing performed by Eton Bioscience (Union, NJ). The DNA sequences were then aligned to a reference database from the Ribosomal Database

Project (Cole et al. 2014) and taxonomic identity determined at the genus level using the RDP Classifier tool (Wang et al. 2007).

Statistical Analysis

Non-parametric tests including Kruskal-Wallis and Dunn's adjusted multiple comparison were performed using GraphPad's Prism software (version 6.0; San Diego, CA) in order to compare enterococci concentrations and ARM concentrations between and across sites. These non-parametric tests were selected for comparison because of the non-normal distribution of microbial counts. Spearman's coefficient was used to conduct pairwise comparisons between concentrations of enterococci and ARMs in water as well as concentrations of ARMs in water and fish guts.

RESULTS

Urban Sewage Impact at Sites

A gradient of urban sewage impact was confirmed using historical enterococci data retrieved from the National Water Quality Monitoring Council (2020) and the New York Water Trail Association (2020) (Figure 2). Enterococci concentrations were found to be significantly different across all three sites (Kruskal-Wallis, $p < 0.001$; Dunn's adjusted multiple comparison, $p < 0.001$ for all pairwise comparisons). Flushing Creek experienced the highest median enterococci concentration, followed by Little Neck Bay and finally Hobart Beach (Figure 2). Enterococci samples collected through the course of this study demonstrated a similar trend to the historical data among sites (Figure 2; Kruskal-Wallis, $p = 0.025$), but only the pairwise comparison of Flushing Creek and

Hobart Beach were significantly different (Dunn’s adjusted multiple comparison, $p=0.037$).

The abundance of enterococci had a positive correlation with both ARB (Figure 3; Spearman $r=0.813$, $p=0.004$) and TRB (Spearman $r = 0.7576$, $p = 0.009$, data not shown) in paired water samples.

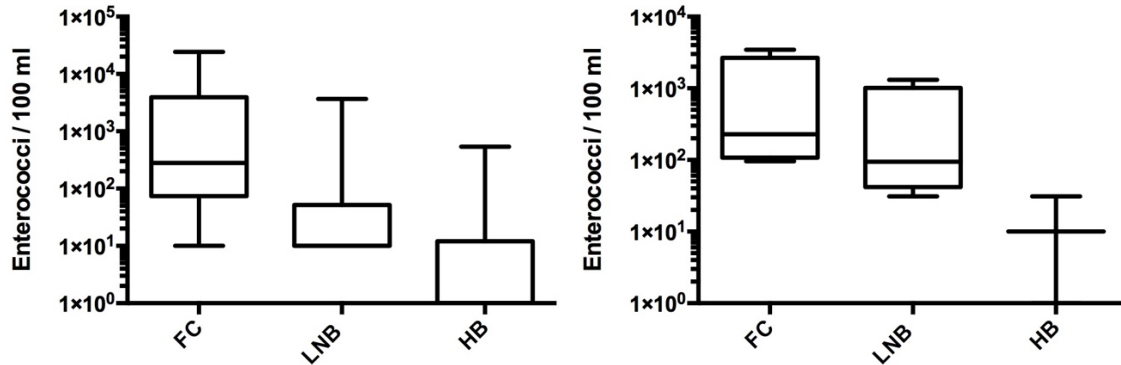


Figure 2: Box plots of enterococci concentrations based on historical data (left) and samples collected through the study (right) across sites Flushing Creek (FC), Little Neck Bay (LNB), and Hobart Beach (HB). Historical data: FC: $n=7$, LNB: $n=88$, HB: $n=132$. This study: FC: $n=4$, LNB: $n=4$, HB: $n=3$.

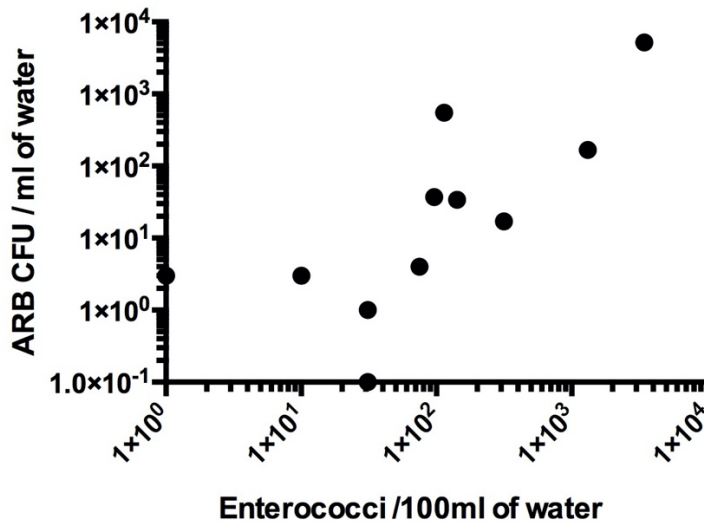


Figure 3: Pairwise comparison of Ampicillin Resistant Bacteria and enterococci in water samples.

Antibiotic Resistant Microorganisms in Fish Microbiomes

ARMs were detected in 95% of fish gut samples (n=41) collected, with concentrations of ARMs varying by up to five orders of magnitude. Cross site comparisons of ARM concentrations yielded no significant difference among sites (Kruskal-Wallis $p=0.0628$ and $p=0.186$ for ARB and TRB, respectively). The median and range of values for both ARB and TRB, however, were several orders of magnitude greater at Flushing Creek compared to Little Neck Bay and Hobart Beach (Figure 4). Little Neck Bay ARB samples also varied by several orders of magnitude compared to Hobart Beach, but this was not reflected in TRB samples (Figure 4). Despite differences in the range of values, TRB samples reflected similar medians across sites (Figure 4b). Analysis of samples across sites indicated a positive correlation between ARMs in gut microbiomes and ARMs in water samples for both ARB (Figure 5; Spearman $r = 0.7527$, $p<0.001$) and TRB (Spearman $r = 0.6178$, $p=0.008$, data not shown).

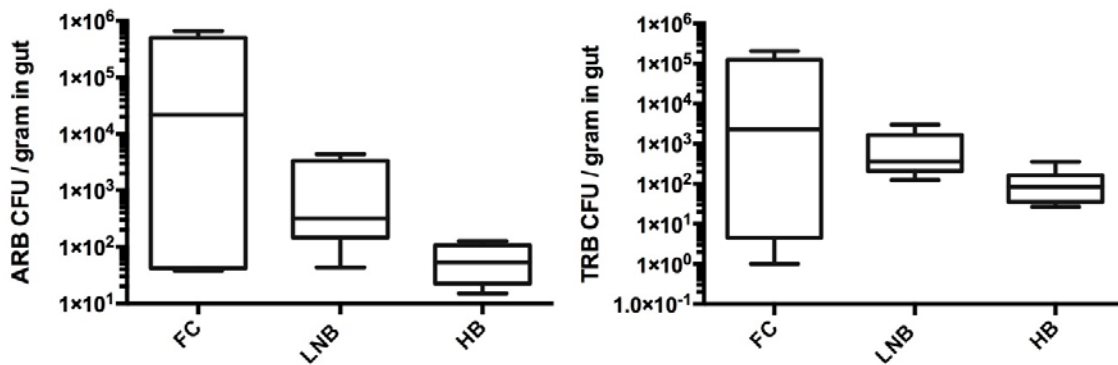


Figure 4: Box plots for ARM concentrations across sites. Both Ampicillin resistant bacteria (ARB) concentrations (left) and Tetracycline resistant bacteria (TRB) concentrations (right) are shown. Flushing Creek (FC; n=5), Little Neck Bay (LNB; n=6), and Hobart Beach (HB, n=6).

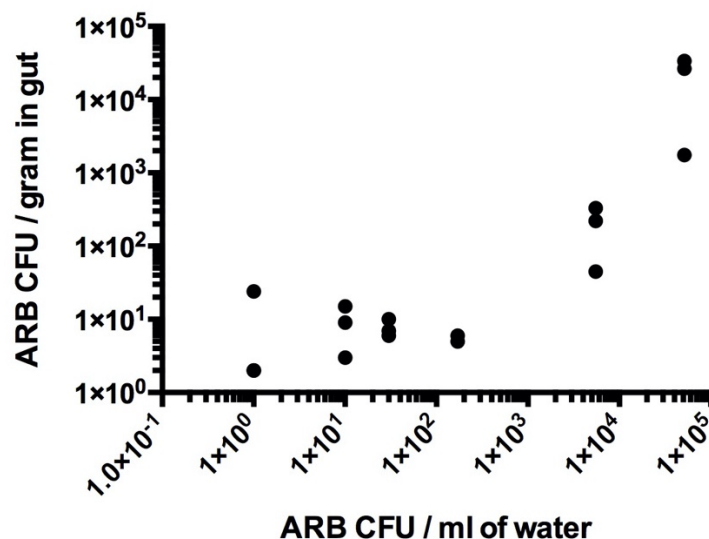


Figure 5: Pairwise comparison of Ampicillin Resistant Bacteria in fish guts and water samples.

Identity of Detected Microorganisms

Despite a relatively small number ($n=53$) of gut microbiome isolates being characterized, ARMs from fish microbiomes consist of a phylogenetically diverse assemblage, spanning the phyla Actinobacteria, Firmicutes, and Proteobacteria based on 16S rRNA gene sequences of isolates from the fish gut. Proteobacteria was the most abundant phyla detected in fish microbiomes (Table 2). The genus *Ralstonia* (phylum Proteobacteria) made up 63% of all ARM isolates and was dominant in both ARB and TRB isolates across all sites but was not detected among heterotrophic isolates. Genera *Aeromonas* and *Pseudomonas* were also common in both heterotrophic and ARB isolates, although not detected in TRB. Although very few isolates from water were sequenced ($n= 16$), two of the three most abundant genera in fish microbiomes (*Pseudomonas* and *Ralstonia*) were also detected in isolates from water (data not shown).

Table 2: Classification of 16S rRNA gene sequences from Het, ARB, and TRB isolates of fish gut microbiomes at Flushing Creek (Het: n=3; ARB: n=7; TRB n=10), Little Neck Bay (Het: n=11; ARB: n=9; TRB n=10), and Hobart Beach (Het: n=0; ARB: n=1; TRB n=2) based on the classifier tool of the Ribosomal Database Project with genera reported at 95% confidence unless otherwise indicated. ^a Indicates that all samples were between 57 and 85% confidence, ^b indicates 2 samples above 95%, remaining between 37 and 50% confidence in genus level assignment.

Microorganism Type	Phylum	Genus	Percent of identified colonies		
			FC (%)	LNB (%)	HB (%)
Het	Actinobacteria	<i>Microbacterium</i>		27	
	Firmicutes	<i>Lactococcus</i>		9	
		<i>Staphylococcus</i>		9	
		<i>Paenibacillus</i>	33 ^a		
	Proteobacteria	<i>Aeromonas</i>	33		
		<i>Citrobacter</i>		9	
		<i>Pseudomonas</i>		9	
		<i>Serratia</i>		36 ^b	
<i>Shewanella</i>		33			
ARB	Actinobacteria	<i>Streptomyces</i>		11	
	Proteobacteria	<i>Aeromonas</i>	43		
		<i>Pseudomonas</i>	14	22	
		<i>Ralstonia</i>	29	56	100
		<i>Raoultella</i>	14	11 ^a	
TRB	Firmicutes	<i>Enterococcus</i>	10		
		<i>Lactococcus</i>		10	
	Proteobacteria	<i>Klebsiella</i>	10		
		<i>Ralstonia</i>	50	70	100
		<i>Serratia</i>	30 ^b	20 ^a	

DISCUSSION

Establishing a Gradient of Impact

The gradient of impact established using sewage input is consistent with patterns of increasing urban characteristics such as population density (Table 1), impervious surface cover, and overall development supporting this study's hypothesis. Flushing Creek, the site with the greatest annual input of combined sewage overflow, containing both sanitary wastewater and stormwater runoff had the highest median and greatest range in enterococci concentrations (Figure 2). Little Neck Bay, being adjacent to a separated stormwater pipe is a site of intermediate sewage impact, which is reflected in the intermediate median enterococci concentrations. Finally, Hobart Beach, a recreational beach site, with no combined or separate stormwater pipes in the immediate vicinity, and relatively little direct runoff due to its location on a spit, had relatively low enterococci concentrations with a smaller range. The lack of significance of this gradient in the samples taken through the course of this study likely reflects the limited statistical power due to the small number of samples taken rather than a lack of gradient. An increase in sampling effort would likely result in a significant FIB gradient.

Previous studies have demonstrated the close association of ARMs and FIB, which were positively correlated in water samples from New York Harbor increasing in abundance during wet weather events when combined sewage and stormwater runoff are actively flowing (Young et al. 2013). The findings of this study of a correlation between enterococci and ARMs (Figure 3) confirm this phenomenon, demonstrating that ARM concentration is a reasonable metric of sewage impact. Considering the historical pattern of enterococci differences when larger sample sizes are analyzed combined with the

correlated abundance of FIB and ARMs, these patterns suggest that with more sampling the site gradient would likely be reflected in significantly differing ARM concentrations as well, despite historical data of ARMs at sites not being available for verification and the inability to demonstrate this pattern with the current sampling effort. Expanded sampling could test this prediction.

ARMs in Fish Microbiomes

Mummichogs have a small home range, spending the majority of their lives in an environment of <200 m (Skinner et al. 2012). It is reasonable to predict that their microbiomes are likely to reflect this highly localized environment and the heterogeneous water quality conditions typical of New York Harbor; therefore, ARMs detected should be representative of the conditions associated with the immediate surroundings of the sample site. Furthermore, ampicillin and tetracycline attack microorganisms via two different pathways (by inhibiting cell wall growth and translation, respectively) commonly used by two major classes of antibiotics (NCBI 2021; Chopra and Roberts 2001) and, therefore, likely representing a range of organisms that utilize diverse pathways of resistance.

ARMs were detected in the gut microbiomes in the vast majority of fish sampled, including the lowest impact site, Hobart Beach. Hobart Beach fish did, however, exhibit a smaller range in microbiome ARMs compared to the highest impact site, Flushing Creek, which was consistent with trends in enterococci concentrations along the site gradient. Differences in salinity have been shown to impact fish microbiomes (Schmidt et al. 2015), but sites were intentionally chosen based on the similarity of their salinity profiles, to control for this factor. Overall, findings suggest that, similar to enterococci trends,

expanded sampling may result in a significant difference among sites that vary in sewage impact.

The combined positive correlations between ARMs in water and guts (Figure 5), and between ARMs and enterococci (Figure 3) suggest that sewage pollution may be impacting fish microbiomes, even if this current sampling effort did not resolve a significant difference along the site gradient. The implications of these correlations are twofold. First, that sewage pollution may impact fish microbiomes which, based on prior literature, would then be expected to impact fish health on several levels including development, nutrition, reproductive success, and survival (Mehdinejad et al. 2019; Nayak 2010). Second, the other contaminants present in discharge of both combined sewage and stormwater runoff, in particular heavy metals, PAHs, and pharmaceuticals, all which have been found to impact fish microbiomes in other contexts (Walter et al. 2019; Zhai et al. 2017; Navarrete et al. 2008) are likely impacting fish health through similar pathways in urban environments as well. ARMs have been previously detected in New York Harbor mummichog microbiomes, consistent with locations of high heavy metal contamination, which was attributed to co-selection of antibiotic and heavy metals resistance in microorganisms (Lloyd et al. 2016). CSO pipes are known contributors of heavy metals and ARMs to surrounding waterways (Eaton et al. 2013; Young et al. 2013), which affects the immediate environment around CSO discharge, but also may persist in sediments which may additionally influence benthivores. Co-selection in both environment and microbiomes may, therefore, play a role in the levels of ARMs detected in fish.

Identity of Detected Microorganisms

The most dominant genus identified, *Ralstonia*, was not detected in heterotrophic isolates, indicating an ARM dominant genus. With larger sampling of heterotrophic isolates it is expected that *Ralstonia* would be detected, but they are of increasing importance when antibiotic selection decreases overall diversity of isolates. *Ralstonia* was also included within the ARB isolates detected in water by Young et al. (2013) in other New York Harbor locations. *Ralstonia* has also been previously identified in association with the guts of other fish species, making up one of the three dominant genera identified in the guts of marine fishes with diverse feeding habits and associated with several digestion pathways (Huang et al. 2020). *Ralstonia* may be common in a broader range of local fish species and may play a role in fish health through metabolism. Some species of *Ralstonia* are human pathogens (e.g., *R. picketti*); therefore, a species level identity may be useful in further understanding the implications of these microorganisms.

Additionally, ARM genera *Aeromonas* and *Pseudomonas*, which were common in fish gut samples, have been previously identified as among the most common ARMs detected in New York Harbor water samples (Young et al. 2013). This further supports a connection between microorganisms found in the aquatic environment and those found in fish guts. While *Aeromonas* was not detected in water samples from this study, this is likely due to the limited number of water isolates sequenced. It should also be noted that the cultivation-based methodologies used in this study do not provide a comprehensive insight into all microorganisms present in both water and fish guts, selecting only for those microorganisms that proliferate in the cultivation conditions used. Further

exploration using whole gut sequencing tools and known antibiotic resistant genes to better characterize these microbiomes would be beneficial.

Significance and Future Work

Sewage impacts such as FIB and ARMs are typically considered sanitary issues, of concern to the protection of human health but not necessarily ecological health. The relationship between urban sewage sources and fish health has wide implications for fisheries management and conservation. Current approaches focus on management planning and reporting, biodiversity and monitoring surveys, and creation of habitat structures. There is no current consideration of how urban sewage pollution impacts fish populations through their microbiomes. The local connection between sewage and organisms demonstrated in this study further illustrates the urgency with which urban centers like New York City should address sewage contamination. Solutions to FIB and ARM influx are often focused on limiting combined sewage through gray infrastructure which can increase conveyance and detention, but often disrupts urban landscapes and can be expensive (Hakimdavar et al. 2014). These impacts will be compounded by future increases in high intensity rainfall events and resulting increases in stormwater runoff and CSO discharge due to climate change (Horton et al. 2014; Fortier and Mailhot 2015). Green infrastructure captures stormwater before it enters sewage systems, providing a less invasive, lower footprint alternative with additional benefits, including the capture of direct runoff not fully addressed by grey infrastructure.

Management decisions related to upgrading urban infrastructure are often determined by cost-benefit considerations. This study highlights a negative consequence of sewage pollution on fish populations, an interaction generally overlooked when

considering possible benefits that would occur from sewage pollution reductions. This is similar to the mostly overlooked interaction of sewage pollution and greenhouse gas production (Brigham et al. 2019) or the connections between sewage pollution in water and microbial air quality (Dueker and O’Mullan 2014; Dueker et al. 2018). If the full range of impacts from sewage pollution would be considered, there would be added incentive to accept the cost of urban infrastructure upgrades.

The impact of urban sewage on fish microbiomes remains understudied, providing many pathways for future work. This study would benefit from an overall increase in sampling effort which may further support findings and produce significant insights. Future studies should consider exploring additional sites, fish species, and contaminants to provide a more comprehensive understanding of the relationship between urban sewage and fish microbiomes. Additionally, sampling that focused on comparing fish microbiomes in wet and dry weather and monitoring the shifts in microbiomes that occur after wet weather events would help to determine the timescale of microbiome responses that occur from CSO and stormwater influx and their role in shaping fish microbiomes.

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