QUANTIFICATION AND IDENTIFICATION OF ANTIBIOTIC RESISTANT MICROBES IN THE HUDSON RIVER AND FLUSHING BAY

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ABSTRACT

Microorganisms resistant to tetracycline and ampicillin, two commonly used antibiotics, were detected in the Hudson River and other urban waterways of New York City. Culture dependent approaches were used to quantify the abundance of antibiotic resistant microbes and to examine their correlation to raw sewage inputs, while 16S rRNA gene sequences were used for taxonomic identification of microbes found to be resistant. Higher frequency sampling was conducted at Flushing Bay, NY in order to examine the patterns of antibiotic resistant microbes under both dry and wet weather conditions. Ten additional sites in the Hudson River Estuary were sampled during monthly research cruises to examine spatial variability in resistant microbes. Resistant microbes were detected at all sampling sites. Analysis of 16S rRNA genes amplified and sequenced from resistant colonies identified a phylogenetically diverse group of bacteria, including the genera *Aeromonas*, *Pseudomonas*, *Stenophomonas*, and *Escherichia.* All of these genera include opportunistic pathogens and have been associated with antibiotic resistant infections, especially in immuno-compromised individuals. The abundance of ampicillin and tetracycline resistant bacteria, in paired samples, were positively correlated with one another and both groups of microbes were found in greater abundance following precipitation events. The abundance of *Enterococcus,* a sewage indicating microbe, was also found to be positively correlated with levels of resistance, suggesting a shared sewage-associated source for the indicator microorganism and the phylogenetically diverse resistant bacteria.

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INTRODUCTION

Antibiotics are often referred to as miracle drugs and considered to be one of the greatest achievements in public health. Penicillin, among the first antibiotics to be investigated for use in medicine, was named and characterized by Alexander Fleming in 1928 (Overbye and Barrett 2005). Soon after, penicillin and other newly discovered antibiotics became widely used to treat and prevent infections. The prevalence of antibiotic use has caused many bacteria to become resistant to commonly used antibiotics. In the decades that followed the early use of penicillin, a greater number of resistant infections began to appear in hospitals throughout the developed world, as more and more antibiotics were used and sometimes over-prescribed. In the United States alone, approximately 2 million people a year now acquire an antibiotic-resistant infection, and approximately 90,000 of these cases are lethal (Overbye and Barrett 2005). Antibiotic resistance has become one of the most pressing and urgent public health crises in the world (Wise et al. 1998). The spread of antibiotic resistance in the environment is also a growing concern that has implications for ecosystem functions and public health.

Antibiotics and antibiotic resistant bacteria can enter surface water, groundwater systems, or drinking water sources in association with human and animal waste. Antibiotics are not completely metabolized by the human or animal body (Costanzo et al. 2005) and consequently, intact and functional compounds can enter waterways through the waste products of humans or animals who have ingested antibiotics. Waste can be efficiently disinfected at a treatment facility, but the antibiotic compounds are not efficiently removed from the effluent because even the most modern wastewater

treatment plants (WWTPs) are not designed to remove antibiotics present in small concentrations. Therefore, even treated effluent from WWTPs can contribute to antibiotic loading into waterways.

Other major sources which contribute to waterway contamination are concentrated animal farm operations (or CAFOs) where antibiotics are used as prophylactics to avoid disease and promote rapid growth. Concern over antibiotic use in CAFOs relates both to the release of antibiotic compounds associated with waste products from these facilities into the environment and to the evolution and stimulation of antibiotic resistant bacterial strains from their overuse. The European Union has banned prophylactic use in CAFOs, and a bill was introduced into the United States Congress in early 2010 to do the same. Measures such as these demonstrate the growing consensus that overuse of antibiotics is contributing to greater occurrence of resistant infections, affecting animal husbandry, human health and the environment (Conly 1998).

The presence of antibiotics in waterways leads to an increase in bacterial resistance to those antibiotics through selective pressure and horizontal gene transfer (Alonso et al. 2001). Selective pressure in the presence of antibiotics occurs due to the enhanced persistence or growth of bacterial strains carrying resistance genes that block a mechanism of cellular damage caused by antibiotic compounds. These resistance genes are often located on plasmids prone to horizontal gene transfer, accounting for the main pathway by which antibiotic resistant genes are spread. Plasmids are extrachromosomal gene structures that can be mobile, allowing for plasmid encoded genes to be transferred across cell membranes between bacterial cells. This mode of genetic transfer allows the abundance and diversity of resistant bacteria to increase rapidly in aquatic environments

exposed to frequent contamination with antibiotic compounds, and waterways may become reservoirs or incubators for resistant bacteria (Alonso et al. 2001).

The predominant source of antibiotics and resistant bacteria in the lower Hudson River Estuary (HRE) is thought to be human sewage (Kim et al. 2010), although this connection has yet to be carefully tested in the waterways of New York. New York City has as a combined sewer system, which means that sanitary sewers in homes and businesses are connected to storm drain sewers (City of New York 2011a). The benefit of such a system is that during dry weather, street runoff and other drainage can be treated before being released into local waterways, whereas separated systems would simply release storm drainage into the water untreated. Negative consequences of the combined sewer system become apparent during wet weather, when the excess volume, caused by rainwater combined with sanitary sewage, overwhelms the capacity of treatment plants and is diverted and released through combined sewer outfalls (CSOs) into the surrounding waterways (e.g. Hudson River, Harlem River, East River, Flushing Bay, and others) to avoid backups of drains and plumbing systems. This results in bacteria-laden human sewage, which may contain antibiotics or antibiotic resistant microbes, entering local waterways without treatment.

The presence of antibiotics and antibiotic resistant microorganisms in aquatic environments has been extensively documented and studied (Guardabassi et al. 1998; Goni-Urriza et al. 2000; Reinthaler et al. 2003), but no studies have been completed in urban NYC watersheds that may point to the possible environmental and public health risks presented by antibiotic contamination and the consequential promotion of antibiotic resistance. Due to the combined sewer system, sewage inputs associated with rainfall

events are predicted to contribute to the presence of antibiotics and antibiotic resistant microbes in the HRE.

The health of HRE ecosystem may also suffer when increased levels of antibiotics are introduced to aquatic communities. Bacteria that are essential to nutrient cycling are not immune to the effects of antibiotics, and studies have shown that microbial mediated nutrient cycling (e.g. denitrification rates) can be altered in the presence of antibiotics (Costanzo et al. 2005). In a system such as the HRE, which is known to experience excessive nutrient loading (Lampman et al. 1999), the disruption of microbial nutrient cycling could decrease the removal of gaseous nitrogen from the system, thereby increasing the intensity of eutrophication. Eutrophication can have deleterious effects on aquatic plant communities, invertebrates, and fish, and represents a large scale alteration to the aquatic ecosystem including increased potential for hypoxia or anoxia associated with the decomposition of microalgae blooms.

The goals of this study are to examine the spatial and temporal distribution of resistant microbes in correlation with the sewage indicating bacterium, *Enterococcus,* and to phylogenetically identify the species of bacteria in the HRE that were found to be resistant to two commonly used antibiotics: ampicillin and tetracycline. This research tested the hypothesis that the occurrence of antibiotic-resistant microbes is positively correlated with sewage loading in wet weather conditions indicated by the presence of *Enterococci*. This study is a preliminary step in understanding the patterns of antibiotic resistant bacteria in the lower HRE, and highlights the need for improvements in integrated approaches to stormwater management and public health risk assessment.

METHODS

Sampling

Samples for microbiological analysis were collected from surface waters into sterile 50 ml plastic tubes that were also triple rinsed with the sample water before collection. Sampling containers were immediately stored on ice, away from sunlight, in a cooler and transported to the lab within 12 hours for processing. Hach (www.hach.com) handheld sensor systems were used to measure levels of oxygen, temperature, salinity (HACH HQ40d) and turbidity (HACH 2100Q portable turbidimeter) at the time of sampling. Precipitation data were recorded over the prior 5 days from LaGuardia Airport and Central Park; wet days were classified by having >0.5 inches of rain within 2 days of sampling, based on the Weather Underground historical data (www.wunderground.com).

The majority of water samples were collected from Flushing Bay near College Point, Queens, a site in close proximity to Queens College campus where frequent access was feasible. The proximity of this site allowed water samples to be collected under both dry weather and following rain events. Water samples were also collected from various points in the HRE in coordination with the Riverkeeper monthly sampling cruises on the Hudson River, from June to October.

Study Site: Flushing Bay

Flushing Bay is located in Northern Queens, and surrounded by various industrial and commercial establishments, as well as the World's Fair Marina and LaGuardia Airport. The sampling site for this study is east of the marina, near a frequently-used kayak and boat launch. After some exploration of the area, this site was found to be the most conducive shore point on the Bay for the requirements of this project.   During the

sampling, police boats were seen using the bay as a training site for water rescue, fishermen were frequently on the dock (who also said they swim in the Bay in hot weather), and other recreational boats and kayaks were launched. The recreational use of this area was one desired characteristic in selecting a sampling site, along with access to the water and proximity to the lab at Queens College.

Study Site: Riverkeeper Sampling Sites

Sample collection from ten sampling sites throughout the lower HRE was performed in coordination with Riverkeeper's monthly water quality survey (www.riverkeeper.org/water-quality), The sites listed from North to South, and shown in Figure 1 are: Tappan Zee, Piermont Pier, Piermont Outfall, Sawmill River, Harlem River, 125th St. Outfall, Harlem Piers, East River, Newtown Creek, Battery.

Sites were chosen to provide a breadth of environments within the river based on location characteristics and historical data collected over the last five years. Samples were collected using the same procedure described above except that in some cases, *Enterococci* sewage indicator counts were processed while still aboard the boat because of time constraints.

Laboratory Procedures

Sewage Indicators

The procedure for analysis of the sewage indicating bacteria, *Enterococci,* in water samples was performed using the IDEXX Enterolert methodology (www.Idexx.com). All sampling stations were from brackish water sites and therefore a one in ten dilution of sample water in sterile DI water was performed prior to selective enrichment and enumeration in accordance with the manufacturer suggested protocol for

1-Tappan Zee
2-Piermont Pier
3-Piermont Outfall
4-Sawmill River
5-North River WWTP
6-125th St. Pier
7-Battery
8-East River
9-Harlem River
10-Newtown Creek
FB- Flushing Bay

Figure 1. Map of the ten Riverkeeper sampling sites around Manhattan and throughout the lower HRE, and key for numbered sites. Additional sampling station of Flushing Bay marked as "FB". (Markers are not exact to GPS references, but represent approximate locations.)

Enterolert. Processing was always done within six hours of sample collection. The samples were added to liquid media, sealed in a Quanti-tray (IDEXX), and incubated at 41°C. After 24 hours incubation, the Quanti-tray was exposed to UV light and the number of wells displaying blue color in the trays was recorded used to calculate a most probably number (MPN) of *Enterococci* cells per volume of sample water.

Colony-based approaches for microbial counts

Quantification of heterotrophic bacteria and antibiotic resistant heterotrophic bacteria required the preparation of solid nutrient-rich R2A agar, and the addition of dry mass of antibiotics (Reasoner 2004). Media was autoclaved, then placed in a 55° C water bath to cool before the addition of antibiotics. Antibiotics were added to media for determination of antibiotic resistance in the following proportions: 50 mg/L ampicillin and 10 mg/L tetracycline. Plates were poured in a hepa filtered laminar flow hood and once liquid media solidified, plates were seasoned overnight at room temperature before being stored in a refrigerator until future use. For sample processing, two to four ten-fold dilutions of the sample water were created, using autoclaved and then 0.2 µm filter sterilized sample water as dilution water, and 100 µl of the dilutions was spread onto the plates using aseptic technique in a laminar flow hood. For each sample, plates were inoculated for growth on R2A agar with no antibiotic added, R2A agar with: 1) no antibiotic added (referred to as Heterotrophic or "Het" plates in the text); 2) with ampicillin added (referred to as "Amp" plates); and 3) with tetracycline added (referred to as "Tet" plates, in masses per volume as described previously). Control plates were created using sterile water spreads as a method blank. Inoculated plates were then incubated at 28˚C for three days.

Colonies were counted after three days of incubation, and then stored in the refrigerator for future molecular analysis. Counts of less than 300 colony forming units (CFUs)/100 µl were deemed "countable" numbers, and when multiple dilutions were processed, plates with less than 300 colonies were utilized in data analysis. Results were recorded in units of CFU/100 µl of initial sample water.

Molecular Techniques for Taxonomic Identification

Molecular analysis involved isolating colonies grown on R2A agar from the plate counts described above, including Het, Amp and Tet plates. Colonies were picked off of the media, in a sterile biosafety hood, using sterile pipet tips, and transferred into 40 µl of Hyclone molecular-grade sterile water in 0.2 ml stripe tubes. Strip tubes containing picked colonies were boiled at 95°C for 5 minutes using an Eppendorf thermocycler to lyse cells and then stored at -20°C into additional processing could be completed.

Polymerase Chain Reaction method (PCR) was used to amplify 16S ribosomal RNA genes from the DNA of lysed cells using the universal bacterial primers 8F and 1492R (Lane 1991) and cycling conditions as follows: 95° C for 10 minutes; 30 cycles of 95° C for 1 min, 55° C for 30 secs, 72°C for 1 min; 72° C for 5 mins; 4° soak. Amplification products were separated using gel electrophoresis in 1% (w/v) agarose gels, stained with SyberSafe dye (Invitrogen), and visualized using UV light and a Syngene gel documentation system. Amplification product sizes were estimated relative to a 1 kb ladder (Invitrogen) and products of approximately 1500 bp were selected for gene sequencing. Amplification product size was quantified using a Qubit fluorometer (Invitrogen), normalized in concentration and sent for DNA sequencing to SeqWright Inc. (Houston, TX). The sequence output files were edited using FinchTV (www.geospiza.com) and Geneious (www.geneious) software packages. Edited sequence files were exported in FASTA format and uploaded to the Ribosomal Database Project webserver (http://rdp.cme.msu.edu/) for alignment and classification, to the level of genus.

Statistical Analyses

Prism statistical analysis software (Version 4C, May 13, 2005) was used in order to perform non-parametric tests for statistically significant differences between wet and dry weather antibiotic resistance. Non-parametric tests were used because microbial counts were non-normally distributed. Spearman's coefficient was used to evaluate the relationship between sewage indicators and antibiotic resistant microbes. Values of zero were replaced with values of 0.1 when calculating geometric means for *Enterococci* measurements.

RESULTS

Spatial Variation

Ampicillin resistant colonies were detected at all of the eleven sampling sites in the HRE (Figure 2 and Table 1), including the ten sites sampled during Riverkeeper monthly water quality patrols and shore based sampling of Flushing Bay. Tetracycline resistant colonies were detected at all sites except the Battery. During monthly June to October Riverkeeper sampling cruises, 83% of samples collected at the ten sites were found to contain ampicillin resistant microbes and 36% of samples were found to contain tetracycline resistant microbes (Table 1). Ampicillin resistant heterotrophs were found more frequently (86% of the samples) than tetracycline resistant heterotrophs (28% of the samples) at all sites and were found to have a higher maximum abundance than tetracycline resistant heterotrophs at all sites except at the North River treatment plant (Figure 2 and Table 1). Newtown Creek and the Harlem Piers samples contained the highest maximum number of ampicillin resistant heterotrophs detected during monthly

Riverkeeper sampling patrols. In contrast, the highest maximum numbers of tetracycline resistant heterotrophs were detected at the two wastewater treatment outfalls.

Geometric means of *Enterococci* calculated over the entire sampling period did not exceed monthly geometric mean standards (35 CFU/100 ml) except at one site, Newtown Creek.   Maximum values of measured *Enterococci* exceeded single sample maximum standards (104 CFU/100 ml) at four sites: Newtown Creek, Sawmill River, Piermont Outfall and Piermont Pier. Across sites, the geometric mean of *Enterococci* at a site was found to be positively correlated with the percentage of samples found to contain tetracycline resistant microbes (Spearman r = 0.837; p = 0.003), but not with the percentage of samples with ampicillin resistant microbes (Spearman r = 0.563; p = 0.096).

Temporal Variation: Flushing Bay

Higher frequency sampling was conducted in Flushing Bay, NY (n=16, Table 1) to investigate patterns of temporal variation in antibiotic resistant microbes and correlations with environmental conditions, especially rainfall and sewage loading. Seven samples were collected following dry weather and nine samples were collected after rainfall.

The geometric mean for *Enterococci* at this site (250.87 CFU/100 ml, Table 1) exceeded the EPA recommended monthly geometric mean standard and the maximum recorded *Enterococci* also exceeded single sample maximum standard. All samples from Flushing Bay contained ampicillin resistant microbes and 88% contained tetracycline resistant microbes (Table 1).

A)

B)
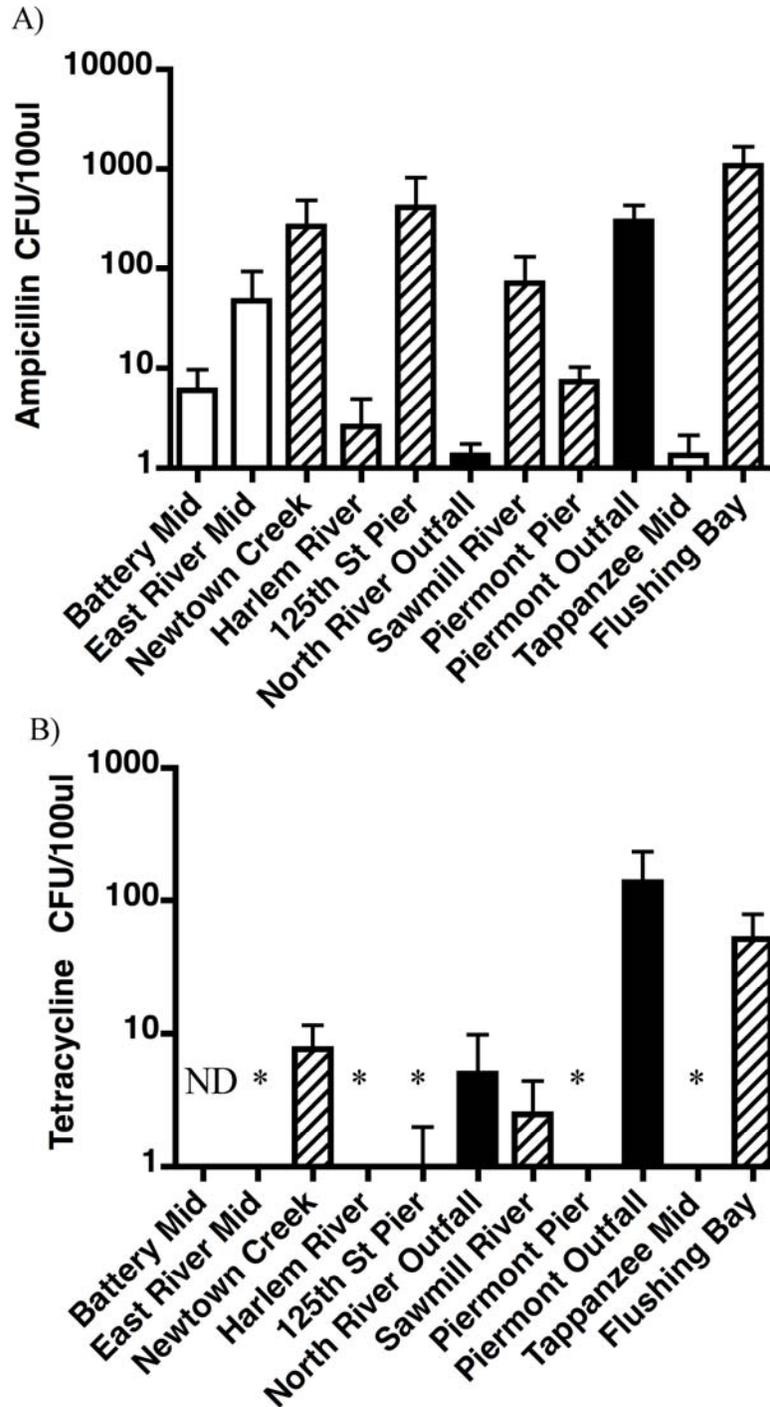
Figures 2A and 2B. Antibiotic resistant heterotrophs (A: ampicillin, B: tetracycline) in the HRE are variable based on location of sampling at eleven sites. White bars represent mid-channel stations, hatched bars are near-shore stations and black bars are WWTP outfall stations. In Figure 2B, ND indicates that no resistant organisms were detected on any of the sampling collection days and the asterisk (*) indicates a mean value of less than one.

site	#	% samples w/amp resistance	% samples w/tet resistance	max amp-resistant (CFU/ml)	max tet-resistant (CFU/ml)	geomean ENT (CFU/100 ml)	max ENT (CFU/100 ml)
Battery	6	83%	0%	240	0	0	0
E.River	6	83%	17%	2800	10	0.82	31
Newtown	6	100%	67%	13800	210	58.34	3448
Harlem River	5	40%	20%	120	10	2.29	63
Harlem Piers	5	100%	20%	20600	50	3.49	86
North River WWTP	6	83%	33%	30	290	5.21	20
Sawmill	6	83%	50%	3800	120	27.71	1274
Piermont Outfall WWTP	6	100%	83%	7220	5880	20.74	134
Piermont Pier	6	100%	33%	210	30	25.92	740
Tappan Zee	6	50%	33%	50	10	1.52	41
Total	*58*	*83%*	*36%*				
Flushing Bay	16	100%	88%	9810	439	250.87	24196

Table 1. Microbial data collected from surface water samples at ten Riverkeeper patrol boat sampling sites show total antibiotic resistance (tetracycline or ampicillin), determined from heterotrophic plate counts on R2A media with and without antibiotics added. Percentages are based on the total number of samples with any resistant colonies grown divided by the total number of samples collected at the site over the study period.

The abundance of antibiotic resistant microbes in Flushing Bay increased significantly following rain events (Figure 3; Mann Whitney, ampicillin $p=0.005$, tetracycline $p=0.007$). The proportion of culturable heterotrophs that are resistant to antibiotics appears to increase in wet weather compared to periods of dry weather, but the difference was not statistically significant with the current sampling effort (Figure 4; Mann-Whitney, ampicillin $p=0.173$, tetracycline $p=0.211$).
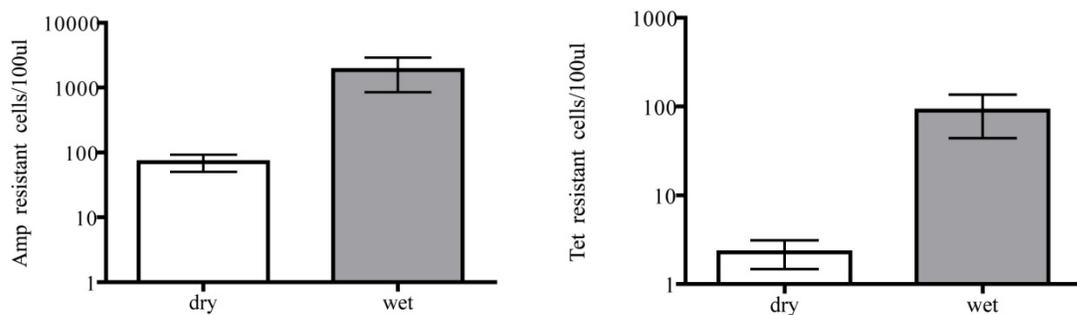
Figure 3. Abundance of cultured antibiotic resistant microbes from surface water samples at Flushing Bay following periods of dry weather (n=7) and wet weather (n=9).
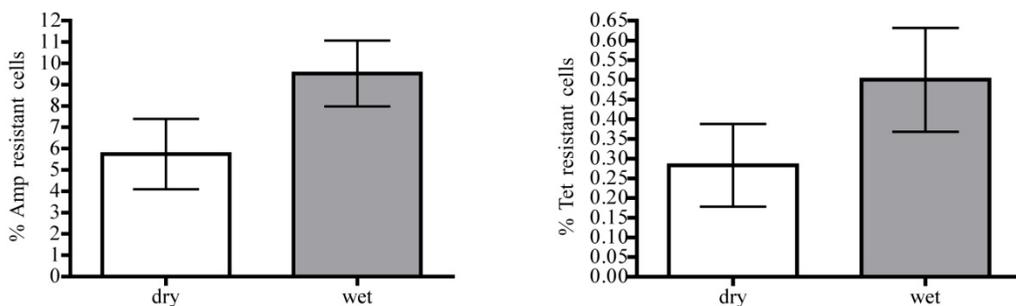

Figure 4. Proportions of culturable antibiotic resistant heterotrophs as a percentage of total culturable heterotrophs in Flushing Bay following periods of dry weather (n=7) and wet weather (n=9).

However, abundance of the sewage indicator *Enterococci* was positively correlated with resistant microbes (Figure 5; Spearman, ampicillin r=0.867 and p=0.005, tetracycline r=0.9 and p=0.002). Similarly, the abundance of ampicillin and tetracycline resistant cells were positively correlated with one another in Flushing Bay (Figure 6; Spearman r=0.918, p<0.001).
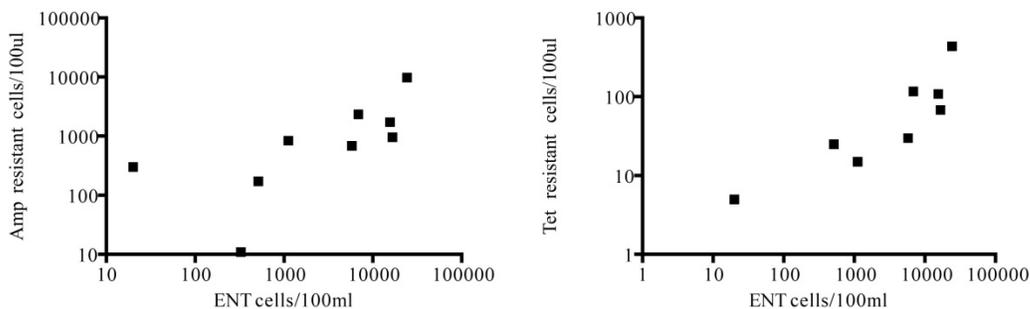
Figure 5. Positive association of antibiotic resistant heterotrophs (Amp or Tet) and sewage indicators *Enterococci (ENT)*. Ampicillin resistant microbes had a greater magnitude (note scale of axes) than tetracycline resistant microbes.
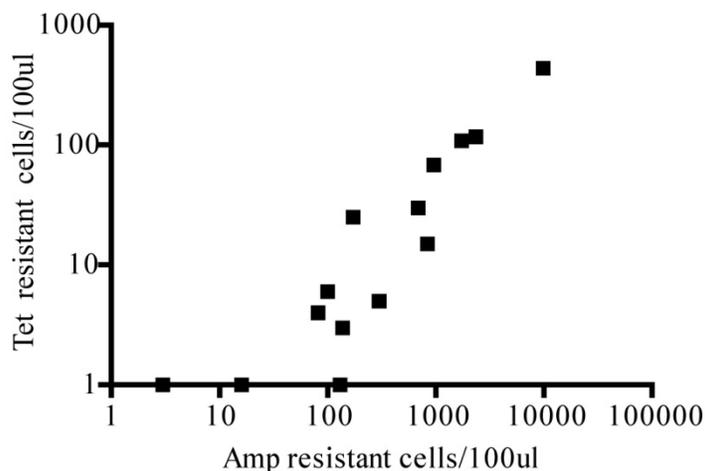

Figure 6. The abundance of both ampicillin and tetracycline resistant microbes at Flushing Bay were positively correlated.

Identification of Microbes

Proteobacteria were the most abundant phylum detected for all three types of culturable heterotrophs sequenced (Table 2). Proteobacteria accounted for 83% of total heterotroph sequences, 100% of ampicillin resistant heterotrophs, and 88% of tetracycline resistant heterotrophs. Of the total Proteobacteria sequenced from het plates, the most abundant genus was *Sphingobium*. Of the ampicillin resistant, the most abundant genus

was *Aeromonas* and of the tetracycline resistant, the most abundant genus was

Escherichia/Shigella.

Type	Phylum	#	Genus	conf.
All heterotrophs n=23	Proteobacteria (19)	6	*Sphingobium*	80%
		4	*Acinobacter*	
		2	*Shewanella*	
		1	*Aeromonas*	
		1	*Pseudomonas*	
		1	*Psychrobacter*	
		1	*Azospirillum*	
		1	*Erythrobacter*	
		1	*unclassified Gammaproteobacteria*	
	Actinobacteria (2)	1	*Arthrobacter*	
		1	*Brachybacterium*	
	Bacteroidetes (2)	2	*Flavobacterium*	
Amp-resistant heterotrophs n=60	Proteobacteria (60)	24	*Aeromonas*	95%
		19	*Pseudomonas*	
		7	*Stenotrophomonas*	
		2	*Brevundimonas*	
		2	*Comamonas*	
		1	*Variovorax*	
		1	*Acidovorax*	
		1	*Delftia*	
		1	*Escherichia/Shigella*	
		1	*unclassified Enterobacteriaceae*	
		1	*unidentified Xanthomonadaceae*	
Tet-resistant heterotrophs n=16	Proteobacteria (14)	2	*Actinobacter*	95%
		1	*Klebsiella*	
		1	*Citrobacter*	
		6	*Escherichia/Shigella*	
		3	*Stenotrophomonas*	
		1	*unidentified Xanthomonadaceae*	
	Bacteroidetes (2)	1	*Chryseobacterium*	
		1	*Flavobacterium*	

Table 2. Classifications of 16S rRNA sequences picked from Flushing Bay surface water samples based on Ribosomal Database Project (www.rdp.msu.edu). Results are reported at highest confidence interval at which the genus could be identified: 80% for het, 95% for amp and tet.

Aeromonas and *Pseudomonas* accounted for 72% of the ampicillin resistant sequences identified. The most abundant genera of resistant bacteria identified, *Aeromonas, Pseudomonas, Stenophomonas*, and *Escherichia/Shigella*, all contain opportunistic pathogens that have been associated with antibiotic resistant infections (e.g. Varley et al. 2009).

DISCUSSION

Spatial Variation

Antibiotic resistance was found to be wide spread and highly variable throughout the lower HRE. Of the 56 total water samples collected, a majority (86%, Table 1) showed some level of antibiotic resistant bacteria, indicating that antibiotic microbes are commonly present across most of the lower estuary. The scope of this study does not specifically address where or how these resistant microbes develop resistance, but as discussed in the introduction, waterways may act as incubators for conferring resistance genes through horizontal gene transfer. The spread of resistance may be fostered in sewer systems, treatment plants and in near-shore environments where raw sewage is released.

The greatest mean values of ampicillin resistant microbes (Figure 2) and the highest maximum values of sewage indicators (Table 1) were recorded at three sites with historically high sewage contamination based on a four year sewage loading dataset collected by Riverkeeper (www.riverkeeper.org/water-quality). These sites are considered hot spots for contamination and include two tributaries (Newtown Creek and Sawmill River) and the Orangetown WWTP effluent at the Piermont Outfall. Maximum

levels of *Enterococci* were the highest in Newtown Creek and Sawmill River, both tributaries of the Hudson River. Microbial loads (het, Tet, Amp, and Entero counts) increased in tributaries following rainfall events and runoff. The high levels of *Enterococci* found at Newtown Creek and Sawmill River confirm that urban tributaries were a source of sewage loading into the major Hudson River during the course of this study.

Point sources near the shore also appear to contribute sewage to the Hudson River, evidenced by the high levels of sewage indicators measured from near-shore environments as compared to mid-river sampling sites (Figure 2). One such point source is a CSO, where wet weather release of combined sewage occurs. All monitoring cruises occurred during dry weather except for August sampling, which occurred after significant rainfall. Wet weather causes the delivery of sewage through CSOs, so the analysis of grouped data from the samples collected in this study are likely underestimated due to the predominance of dry weather during the Riverkeeper sampling days used for this study. The abundance of antibiotic resistant heterotrophs increased after wet weather events based on data collected from Riverkeeper sites. The highest maximum level of ampicillin resistance was recorded at the 125th Street Harlem Piers, following the wet weather in August. Raw data show either no resistance or low levels of resistance on dry sampling days at the Harlem Piers. The sampling point is directly adjacent to a CSO, and the extremely high value of resistant microbes recorded during wet weather is likely related to the release of sewage through the CSO during a rain event.

The highest measurements of tetracycline resistance and the third highest measurements of ampicillin resistance were recorded at the Piermont Outfall (maximum

values reported in Table 1). The Piermont Outfall site is located directly at the effluent

release from the Orangetown WWTP in Orange County, NY. This plant is known to

have significant problems with disinfection processes (State of New York 2010) and is

known to commonly have high fecal indicator bacteria counts associated with effluent

(Riverkeeper 2011a; Michaels 2008). The other wastewater treatment plant sampled,

North River (125th Street WWTP) had much lower levels of both antibiotic resistance and

sewage indicators. Although the North River WWTP serves most of northern Manhattan

and receives a much larger sewage load than the Orangetown plant, this result is not

surprising since the North River WWTP is among the newest wastewater treatment plants

in NYC and has been found to have low *Enterococcus* signals over the last four years

relative to the Piermont Outfall (Riverkeeper 2011b). The efficiency of disinfection can

vary widely across treatment plants and some have been suggested to be reservoirs

causing the spread of antibiotic resistance that can then be released into the environment.

Kim et al. (2010) found that tetracycline resistant bacteria were less efficiently removed

by NYC WWTPs using UV versus chlorination for disinfection. The primary clarifier

effluent in NYC WWTPs sampled by Kim et al. (2010) was found to contain tetracycline

resistant bacteria in concentrations ranging from 10^4 to 10^5 CFUs per milliliter. Based on

Kim's data, sewage in CSOs from the NYC area may contain tetracycline resistant

microbes at concentrations from 100 to 10,000 times the maximum surface water

concentrations detected at most sites in this study. Kim et al. (2010) also found that,

despite disinfection, the effluent from NYC WWTPs still contained tetracycline resistant

bacteria in concentrations from 10^1 to 10^3 CFUs per ml. Given the predominance of dry

weather during our sampling, it is not surprising that the highest tetracycline resistant

counts were found near the effluent of waste water treatment plants. It also suggests that the Orangetown WWTP's Piermont outfall, where a concentration of 5880 CFUs per ml was detected (Table 1), is inefficiently removing antibiotic resistant bacteria and is further evidence that disinfection procedures at this plant require additional attention.

Ampicillin resistance was found in much greater abundance than tetracycline resistance estuary-wide. Both antibiotics have been on the market for a similar length of time, so exposure time is not a likely explanation for the difference. The frequency of prescription or a difference in inherent resistance may explain greater abundance of organisms resistant to ampicillin than tetracycline.

Temporal Variation: Flushing Bay

Flushing Bay is an outlet for multiple CSOs and is surrounded by a highly urbanized industrial region, as well as LaGuardia Airport. In Flushing Bay, analyses showed significantly higher levels of resistant microbes and sewage indicators following wet weather as compared to dry weather (Figure 3). Over the course of the sampling period, *Enterococci* counts increased during wet weather, as would be predicted from earlier studies in the HRE (e.g. Young and Bower 2008). Diverted raw sewage from combined sewers can be detected at outfalls during rain events and antibiotic resistant microbes are in greater abundance during these overflow events. Total heterotrophs and resistant heterotrophs increased significantly during wet weather, demonstrating elevated microbial inputs from sewage loading.

The correlation of *Enterococcus* and antibiotic resistance (Figure 5) in combination with the increased levels of antibiotic resistance following wet weather (Figure 3) strongly suggests that sewage loading through CSOs introduces antibiotic

resistant microbes into Flushing Bay. The correlation of antibiotic resistance with sewage indicators, such as *Enterococcus*, indicates the value in fecal indicator monitoring programs. It also suggests the ability of fecal indicators to predict the abundance of other potentially harmful bacteria, such as resistant microbes, that provide a concern for public health.

The correlation of tetracycline resistant and ampicillin resistant microbes (Figure 6) also supports a shared source and may suggest that some microbes could be multi-drug resistant. Based on these data, testing for resistance to other antibiotics, such as vancomycin or streptomycin, would be predicted to produce similar patterns of distribution and environmental association to wet weather and sewage loading. Further molecular testing or characterization of cultures would be necessary to assess the extent of multi-drug resistance in the environment and in the colonies isolated in this study.

Identification of Microbes

These data provide a preliminary investigation of the diversity and identity of antibiotic resistant microbes in the HRE. The most abundant genera identified are known to contain opportunistic pathogens (Table 2) suggesting that these antibiotic resistant microbes are of potential concern to recreational users, especially immuno-compromised individuals. Potential human pathogens were isolated from both Amp and Tet plates. These include: *Aeromonas, Pseudomonas, Arthrobacter, Klebsiella* and *Escherichia/Shigella*. Many *Aeromonas* species, such as *Aeromonas hydrophilia*, are known to be associated with gastrointestinal disease in humans and infections in fish. The most common tetracycline resistant genus was *Escherichia/Shigella*, enteric bacterial groups that include known pathogens and are found in high density within human and

animal waste. Based on this limited sampling of sequences, the ampicillin resistant

colonies were found to be less diverse (all Proteobacteria) than the heterotrophs

(Proteobacteria, Actinobacteria and Bacteroidetes) and the tetracycline resistant bacteria

(Proteobacteria and Bacteroidetes). However, larger sequence libraries would be needed

to make additional quantitative statements about the relative diversity of the resistant

bacteria or the connection of diversity patterns to environmental conditions. This is an

area that the authors expect to pursue with future studies. These data also demonstrate

the importance of conducting future risk assessment studies of human exposure to these

resistant pathogens, especially in recreational waterways during warm weather where

contact is more likely to occur.

RECOMMENDATIONS

This work has confirmed that antibiotic resistant microbes are widespread in the

HRE system.   The study has clear management implications related to water quality and

public health, given the promotion of on-water recreational activities along the waterfront

in New York City. Decades of historical records exist for *Enterococci* and other fecal

indicator organisms in the New York Harbor Water Quality Surveys dating back to 1909

(City of New York 2011b). These measurements are common and the correlation of

antibiotic resistance with fecal indicators (*Enterococci*) strongly supports the value of

measuring such indicators as representative of other sources of sewage related concern in

the system.   The persistence and transport of resistant pathogens in the HRE is

currently unclear, and potential reservoirs within the system have yet to be confirmed.

The extent of the public health threat requires additional research and epidemiological study.

Urban stormwater management policy should integrate the linkage between wet weather discharges and potential spread of antibiotic resistant infections. Serious investigation should be pursued regarding the sources of antibiotics and antibiotic resistant microbes and possible solutions including: reduced CSO volumes through sustainable and green infrastructure; more discriminating prescription practices by the local healthcare community; potential upgrades to WWTPs to attempt to remove antibiotics and other emerging contaminants of great concern to society.

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