Antibiotic-resistant bacteria in the Hudson River Estuary linked to wet weather sewage contamination

Suzanne Young, Andrew Juhl and Gregory D. O'Mullan

ABSTRACT

Heterotrophic bacteria resistant to tetracycline and ampicillin were assessed in waterways of the New York City metropolitan area using culture-dependent approaches and 16S rRNA gene sequence analysis of resultant isolates. Resistant microbes were detected at all 10 sampling sites in monthly research cruises on the lower Hudson River Estuary (HRE), with highest concentrations detected at nearshore sites. Higher frequency sampling was conducted in Flushing Bay, to enumerate resistant microbes under both dry and wet weather conditions. Concentrations of ampicillin- and tetracycline-resistant bacteria, in paired samples, were positively correlated with one another and increased following precipitation. Counts of the fecal indicator, *Enterococcus*, were positively correlated with levels of resistant bacteria, suggesting a shared sewage-associated source. Analysis of 16S rRNA from isolates identified a phylogenetically diverse group of resistant bacteria, including genera containing opportunistic pathogens. The occurrence of Enterobacteriaceae, a family of enteric bacteria, was found to be significantly higher in resistant isolates compared to total heterotrophic bacteria and increased following precipitation. This study is the first to document the widespread distribution of antibiotic-resistant bacteria in the HRE and to demonstrate clearly a link between the abundance of antibiotic-resistant bacteria and levels of sewage-associated bacteria in an estuary.

Key words | antibiotic resistance, *Enterococcus*, Hudson River, microbiology, sewage, water quality

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INTRODUCTION

The prevalence of antibiotic use has caused many bacteria, including pathogens and human-associated microbiota, to become resistant to commonly used forms (Williams & Heymann 1998; Huang *et al*. 2001; Levy & Marshall 2004), increasing the frequency of antibiotic-resistant infections. In the United States alone, approximately 2 million people a year now acquire an antibiotic-resistant infection, with approximately 90,000 lethal cases (Overbye & Barrett 2005). Antibiotic resistance has become one of the most pressing and urgent public health crises in the world (Wise *et al*. 1998). Thus, it is important to understand the sources and distribution of antibiotic-resistant bacteria in the environment.

In aquatic environments, the spread of antibiotics and resistant bacteria is a growing concern with implications for both ecosystem functions and public health (Costanzo *et al*. 2005; Baquero *et al*. 2008; Plano *et al*. 2011). Because antibiotics are not completely metabolized by the human or animal body, functional compounds can enter waterways through the waste products of humans or animals that have ingested antibiotics (Kummerer 2003). Even the most modern wastewater treatment plants (WWTPs) are not designed to remove antibiotics present in low concentrations (Batt *et al*. 2006). Therefore, treated effluent from WWTPs can contribute to antibiotic loading into waterways (Costanzo *et al*. 2005; Kim & Aga 2007; Spongberg & Witter 2008).

The presence of antibiotics in waterways can lead to bacterial antibiotic resistance through both selective pressure and horizontal gene transfer (Alonso *et al*. 2001). Genes related to antibiotic resistance are often located on plasmids prone to horizontal gene transfer, accounting for the main pathway by which antibiotic resistance genes are spread (Davies & Davies 2010). This mode of genetic

doi: 10.2166/wh.2013.131

transfer allows the abundance and diversity of resistant bacteria to increase rapidly in aquatic environments exposed to frequent contamination with either antibiotic-resistant bacteria or antibiotic compounds (Baquero *et al*. 2008). Such waterways may thus function as reservoirs or incubators for resistant bacteria (Biyela *et al*. 2004).

Many prior studies have reported that waterways influenced by sewage contain an increased concentration or occurrence of antibiotics (e.g. Hirsch *et al*. 1999; Alder *et al*. 2004; Watkinson *et al*. 2009) and that sewage-associated bacteria (e.g. *Escherichia coli*) isolated from WWTPs and urban waterways often exhibit a high frequency of resistance to common antibiotics (e.g. Goñi-Urriza *et al*. 2000; Reinthaler *et al*. 2003). Recent observations show that WWTP effluent can be a direct source of resistant microbes even following advanced treatment and disinfection. For example, Kim *et al*. (2010) measured tetracycline-resistant bacteria (TRB) within three WWTPs in New York and Connecticut through the treatment process and found high concentrations prior to secondary treatment. Treatment and disinfection did result in orders of magnitude reduction in counts, but TRB were still detected in treated secondary effluent. The relative importance of the direct supply of resistant bacteria to waterways from WWTP effluent, compared to the development of resistant populations *in situ* following inputs of antibiotics carried with the effluent, has not been fully assessed.

In many urbanized waterways, such as the lower Hudson River Estuary (HRE), which flows through the greater New York City (NYC) metropolitan area, raw or partly treated sewage enters the aquatic environment following rainfall through combined sewer overflows (CSOs) (NYCDEP 2010; Riverkeeper 2011a). Bypassing treatment, CSO effluent is likely to contain both high levels of antibiotics and antibiotic-resistant bacteria, creating a putative connection between rainfall and the occurrence of antibiotic resistance in urban aquatic environments. However, no direct association between the concentration of sewage or sewage-associated bacteria and the abundance of antibiotic-resistant heterotrophs in natural waterways has been previously reported (Garcia-Armisen *et al*. 2011).

In the heavily urbanized lower HRE, widespread sewage contamination has been reported based on measurements of the sewage indicator, *Enterococcus* (Riverkeeper 2011a; Suter *et al*. 2011), although contamination levels appear to be decreasing relative to past decades (NYCDEP 2010). However, no prior studies have been completed in NYC watersheds examining the distribution and diversity of antibiotic-resistant microbes or their association with water-quality standards, such as levels of fecal-indicator bacteria. The goals of this study were: (1) to examine the spatial distribution of microbes resistant to two commonly used antibiotics (ampicillin and tetracycline); (2) to test the correlation of resistant microbes with abundance of the sewage-indicating bacterium, *Enterococcus*; (3) to examine patterns of antibiotic-resistant microbes under both dry- and wet-weather conditions; and (4) to phylogenetically identify bacteria found to be resistant to ampicillin and tetracycline in the estuary. We hypothesized that antibiotic-resistant bacteria would be widely distributed in NYC waterways, that sewage would be a major source of resistant bacteria in the Hudson River, and that the abundance of antibiotic-resistant microbes would increase following wet weather.

METHODS

Estuarine sampling sites

The Hudson River watershed spans 34,000 sq. km, originating in the Adirondack Mountains and flowing south to NYC (Levinton & Waldman 2006). The water quality of the lower HRE is heavily impacted by NYC, which processes 1.4 billion US gallons of wastewater each day at 14 WWTPs, releasing treated effluent into the estuary (NYCDEP 2013). In addition, approximately 27 billion US gallons of raw sewage combined with rainwater are diverted into the lower estuary each year through CSOs (Riverkeeper 2011a). However, as a result of wastewater infrastructure investment, the water quality of the lower HRE has been improving in recent decades (NYCDEP 2010).

Fifty-six water samples were collected for this study from 10 sites throughout the lower HRE (Figure 1) in coordination with Riverkeeper's monthly water quality survey (www.riverkeeper.org/water-quality) aboard the survey vessel, R. Ian Fletcher, between May and September 2010. Sites were chosen to provide a cross-section of environments within the river based on location characteristics and historical data collected over the preceding 5 years by

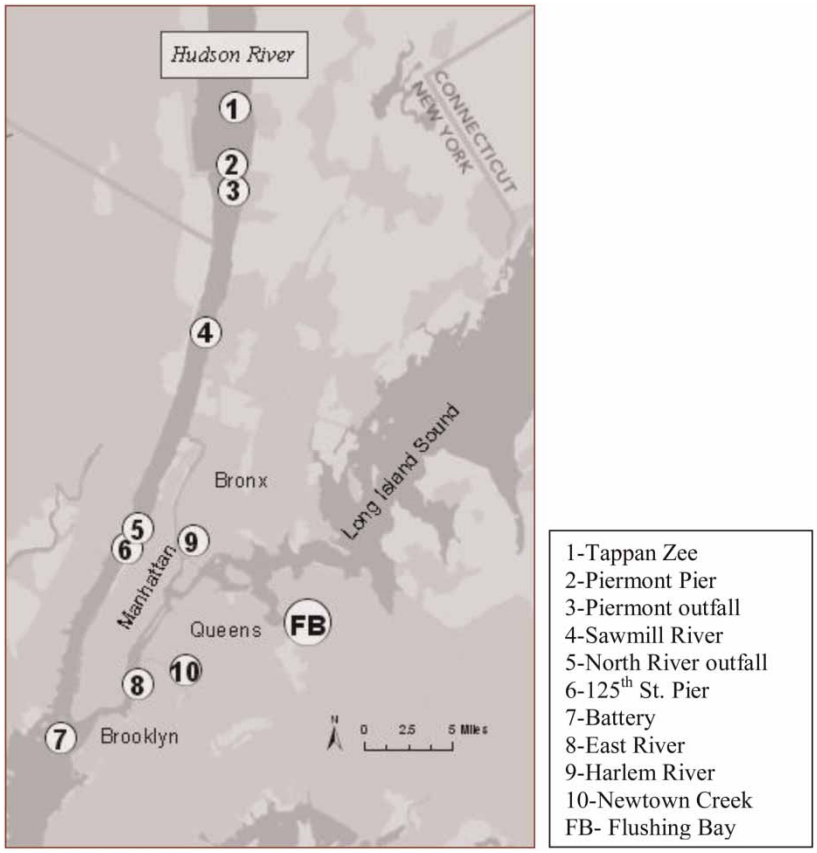

Figure 1 | Map of the Hudson River, flowing north to south, past the island and borough of Manhattan, into the mid-Atlantic Bight of the Atlantic Ocean. The 10 Riverkeeper sampling sites around Manhattan and throughout the lower HRE where spatial analyses were conducted are shown, along with the high frequency sampling site of Flushing Bay where temporal analyses were conducted. Sites 1–4 are north of New York City, while sites 5–10 and FB are in New York City waters.

the Riverkeeper survey. In addition to the boat-based sampling conducted during Riverkeeper surveys, a further 23 water samples were collected from a site in Flushing Bay (Figure 1), where frequent access was feasible from shore. The proximity of this site allowed for water sample collection during both dry weather and following rain events. Flushing Bay is located in northern Queens, NY, and is surrounded by various industrial and commercial establishments, as well as the World's Fair Marina and LaGuardia Airport. The sampling site for this part of the study (40°45′48.7″ N, 73°50′37.1″ W) is east of the World's Fair Marina, on a small dock adjacent to a frequently used public kayak and boat launch. Precipitation data for LaGuardia Airport and Central Park were taken from the Weather Underground historical data (www.wunderground.com). Wet days were classified by having >0.635 cm of rain within 3 days of sampling.

Microbial enumeration

Samples for microbiological analyses of antibiotic resistance were collected from surface waters in sterile 50-mL plastic tubes that were triple rinsed with sample water before collection. Samples were immediately stored on ice, and protected from sunlight in a cooler until processing. Samples for enumeration of antibiotic-resistant bacteria were processed within 12 h, while *Enterococcus* enumeration began within 6 h of collection, as specified in EPA method 1600 (US EPA 2006) using the Enterolert methodology (IDEXX 2011). All sampling stations were from brackish water sites and therefore a one in 10 dilution of sample water into sterile deionized water was performed prior to selective enrichment and enumeration, in accordance with the manufacturer's suggested protocol. Media were added to the diluted samples, which were then sealed in a Quanti-tray 2000 incubation

container, incubated at 41 °C for 24 h and then enumerated under UV light (IDEXX 2011).

Quantification of heterotrophic bacteria and antibiotic-resistant heterotrophic bacteria were conducted using solid R2A agar media (Reasoner 2004), with or without the addition of antibiotics, similar to the method of Garcia-Armisen *et al.* (2011) and Kim *et al.* (2010). Two classes of antibiotics were added to media based on previously published concentrations of 50 mg/L for ampicillin (De Souza *et al.* 2006) and 10 mg/L for tetracycline (de Cristóbal *et al.* 2006). For sample processing, two to four 10-fold dilutions of the sample water were created, using autoclaved and then 0.2 μm filter sterilized HRE water as dilution water, and 100 μL of the dilutions were spread onto the plates in a laminar flow hood. For each sample, plates were inoculated for growth on R2A agar with: (1) no antibiotic added (referred to as heterotrophic or 'Het' plates); (2) ampicillin added (referred to as ampicillin-resistant bacteria or 'ARB' plates); and (3) tetracycline added (referred to as tetracycline-resistant bacteria or 'TRB' plates). Control plates were created using sterile water spreads as a method blank and otherwise processed in parallel with samples. Inoculated plates were then incubated at 28 °C for 3 days before enumeration for colony forming units (CFU).

Molecular techniques for taxonomic identification

Isolated bacterial colonies were picked off the R2A media plates into 40 μL of sterile water for molecular analysis. Tubes containing picked colonies were heated to 95 °C for 5 minutes using an Eppendorf mastercycler to lyze cells and then stored at −20 °C until additional processing could be completed. The polymerase chain reaction (PCR) method was used to amplify the 16S ribosomal rRNA gene from the DNA of lyzed cells using universal bacterial primers 8F and 1492R (Teske *et al.* 2002) and cycling conditions as follows: 95 °C for 10 minutes; 30 cycles of 95 °C for 1 minute, 55 °C for 30 seconds, 72 °C for 1 minute; and final cycle of 72 °C for 5 minutes. DNA products were separated using gel electrophoresis to determine the length of amplified fragments and sent for sequencing to SeqWright Inc. (Houston, TX).

The resulting sequences were grouped into three libraries containing sequences from Het, ARB or TRB isolates. The sequence output files were edited using the Geneious software package (www.geneious.com), exported in FASTA format and uploaded to the Ribosomal Database Project (RDP) webserver (http://rdp.cme.msu.edu/) for alignment and classification, to the level of genus with 95% confidence unless otherwise noted. The RDP library comparison tool was used to assess significant differences, at the 0.05 level, between libraries with genera identified at the 80% confidence level for library comparisons. DNA sequences were deposited in the National Center for Biotechnology Information's GenBank database under accession numbers KC810066–KC810298.

Statistical analyses

Prism statistical analysis software (Version 4C, 13 May 2005) was used to perform non-parametric tests for differences between the abundance of wet and dry weather antibiotic-resistant bacteria. Non-parametric Mann–Whitney or Kruskal–Wallis 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 *Enterococcus*. For diversity analysis, rarefaction curves were created using the software Analytical Rarefaction (Hunt Mountain Software 2010) and Shannon–Weiner diversity and significance tests were run in the program Species Diversity and Richness (Pisces Conservation Inc. 2006).

RESULTS

Monthly sampling at estuarine monitoring stations

During monthly Riverkeeper sampling cruises from May to September, ARB were detected at all 10 sites sampled (Table 1). TRB were detected at all sites except Site 7, the NYC Battery, a mid-channel sampling site to the south of Manhattan. ARB were found more frequently (84% of samples) than TRB (38% of samples). ARB were also found to have a higher observed maximum abundance than TRB at every site except Site 5, the North River WWTP. The abundance of *Enterococcus*, ARB and TRB

were all significantly greater at nearshore sites as compared to mid-channel sites (Figure 2(a)–(c); Mann–Whitney, $p = 0.002$, $p = 0.021$, $p = 0.033$, respectively). The highest maximum numbers of ARB were measured at Newtown Creek, an urban waterway that has recently been listed as a Superfund site, and the 125th Street Pier, a site in close proximity to a CSO outfall on the upper west side of Manhattan, both nearshore sites. In contrast, the highest maximum numbers of TRB were measured at two WWTP outfalls occurring in the nearshore environment: Orangetown WWTP (Piermont outfall) and North River WWTP (North River outfall). Compared to previously published total cell count data (Suter *et al.* 2011) from five of these sampling sites, culturable heterotrophs represented approximately 10^{-5} to 10^{-2} of all bacteria cells, and resistant microbes represented a maximum of 10^{-3} of total cell counts.

Geometric means of the sewage indicator *Enterococcus* calculated over the entire May–September spatial sampling period did not exceed EPA guidelines for primary contact in recreational waters (geometric mean >35 CFU/100 mL; US EPA 2004) except for nearshore sites in two urban tributaries: Site 10, Newtown Creek, and Site 4, Sawmill River (Table 1). Considering samples from the survey cruises individually, 8.6% of all samples exceeded the EPA single sample maximum guideline for primary contact in recreational waters (104 CFU/100 mL; US EPA 2004).

Table 1 | Microbial data collected from surface water samples at 10 Riverkeeper patrol boat sampling sites show total percentage of samples at each site with antibiotic-resistant microbes detected. Bacteria were enumerated on R2A media in the presence of antibiotics (ampicillin-resistant-ARB; tetracycline-resistant-TRB) and without antibiotics added (Het). Mean and maximum (Max) values given are reported for ARB, TRB and Het. Geometric mean and maximum are reported for enterococci

				ARB (CFU/100 μL)		TRB (CFU/100 μL)		Het (CFU/100 μL)		*Enterococcus* (CFU/100 mL)	
Site	***N* =**	**% samples w/ARB**	**% samples w/TRB**	**Mean**	**Max**	**Mean**	**Max**	**Mean**	**Max**	**Geomean**	**Max**
1-Tappan Zee											
Mid-channel	6	50%	33%	1	5	< 1	1	23	29	5	41
2-Piermont Pier											
Nearshore	6	100%	33%	7	21	< 1	3	327	1,110	26	740
3-Piermont outfall											
Nearshore WWTP	6	100%	83%	215	722	137	588	11,538	32,000	30	134
4-Sawmill River											
Nearshore	6	83%	33%	72	380	3	12	933	4,200	41	1,274
5-North River outfall											
Nearshore WWTP	6	83%	33%	1	3	5	29	64	120	8	20
6-125th St Pier											
Nearshore	5	100%	20%	414	2,060	1	5	312	1,120	9	86
7-Battery											
Mid-channel	6	83%	0%	6	24	0	0	45	107	2	20
8-East River											
Mid-channel	5	80%	20%	48	280	< 1	1	225	980	3	31
9-Harlem River											
Nearshore	4	50%	25%	3	12	< 1	1	31	51	5	63
10-Newtown Creek											
Nearshore	6	100%	67%	268	1,380	8	21	2,793	11,100	58	3,448
Estuary Sites Combined	**56**	**84%**	**38%**								
FB-Flushing Bay											
Nearshore	23	100%	91%	1,104	9,810	294	24,196	12,218	122,000	294	24,196

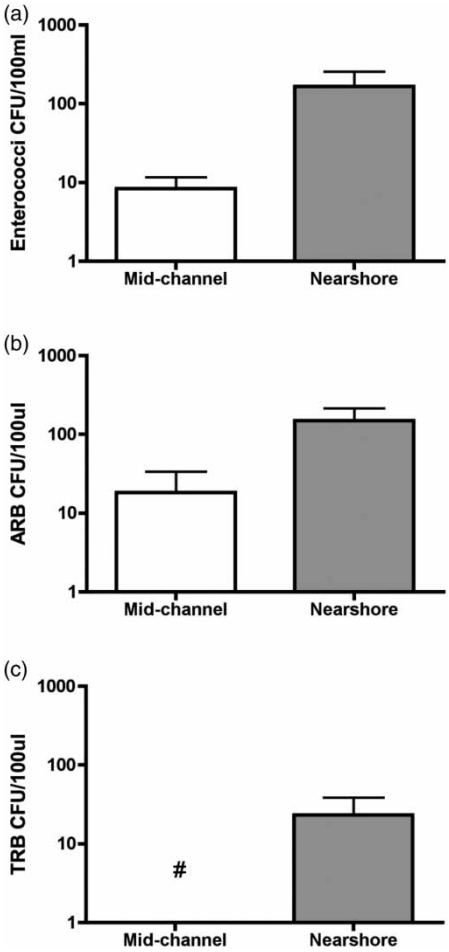

Figure 2 | Mean and standard error for *Enterococcus* (a), ampicillin-resistant bacteria, ARB (b), and tetracycline-resistant bacteria, TRB (c) in the HRE at 10 sampling sites used for spatial analyses. White bars represent data from mid-channel stations ($n = 17$), grey bars represent nearshore stations ($n = 39$). In (c), (#) indicates a mean value of less than one.

Maximum values of *Enterococcus* exceeded the single sample maximum guideline at four nearshore sites: Newtown Creek, Sawmill River, Piermont outfall and Piermont Pier. Pooling together observations from all sites, the abundance of *Enterococcus* in individual samples was positively correlated with abundance of both ARB (Spearman $r = 0.537$; $p < 0.001$) and TRB (Spearman $r = 0.394$; $p = 0.003$). When data were grouped based on the EPA single sample water quality guideline (i.e. greater or less than 104 CFU/100 mL), concentrations of resistant bacteria were significantly higher when *Enterococcus* concentrations exceeded the guideline (ARB: Mann–Whitney $p = 0.013$; TRB: Mann–Whitney $p = 0.005$).

Higher frequency, wet versus dry sampling

Higher frequency sampling was conducted at a nearshore site in Flushing Bay, NY ($n = 23$, Table 1) to investigate patterns of temporal variation in antibiotic-resistant microbes and correlations with environmental conditions, especially rainfall and sewage loading. The geometric mean for *Enterococcus* at the Flushing Bay site (294 CFU/100 mL) exceeded the EPA geometric mean guideline and 65% of samples exceeded the EPA single sample maximum guideline (EPA 2004). All samples from Flushing Bay contained ARB and 91% contained TRB. *Enterococcus* concentration showed a strong positive correlation with the abundance of resistant bacteria in the Flushing Bay dataset (Figure 3; Spearman, ARB $r = 0.888$ and $p < 0.001$, TRB $r = 0.849$ and $p < 0.001$).

Grouping the data based on rainfall, six samples were collected following dry weather and 17 samples were collected after rainfall. The frequency of sewage contamination based on the EPA single sample maximum guideline was higher (86%) after wet weather than dry weather (16%). Similarly, the abundance of antibiotic-resistant microbes increased significantly following rain events (Figure 4; Mann–Whitney, ARB: $p = 0.007$; TRB: $p = 0.005$). The proportion of culturable antibiotic-resistant bacteria (of total heterotrophic bacteria) increased in wet weather compared to periods of dry weather, but the difference was not statistically significant (Figure 5; unpaired one-tailed *t*-test, ampicillin $p = 0.10$, tetracycline $p = 0.20$). Less than 27% of culturable bacteria were resistant to ampicillin, and less than 2.5% were resistant to tetracycline. However, proportions of resistant microbes were positively correlated with *Enterococcus* concentrations (Spearman, ARB $r = 0.595$ and $p = 0.003$; TRB $r = 0.418$ and $p = 0.047$) and abundance of ARB and TRB were positively correlated with each other (Figure 6; Spearman $r = 0.918$, $p < 0.001$).

Identification and diversity of resistant microbes

Proteobacteria were the most abundant phyla detected from 16S rRNA gene sequences of isolates, accounting for 87% all sequences, and were the most abundant in each of the three sequence libraries, including 73% of Het sequences, 91% of ARB and 92% of TRB (Tables 2 and 3). Most Proteobacteria sequences were identified as

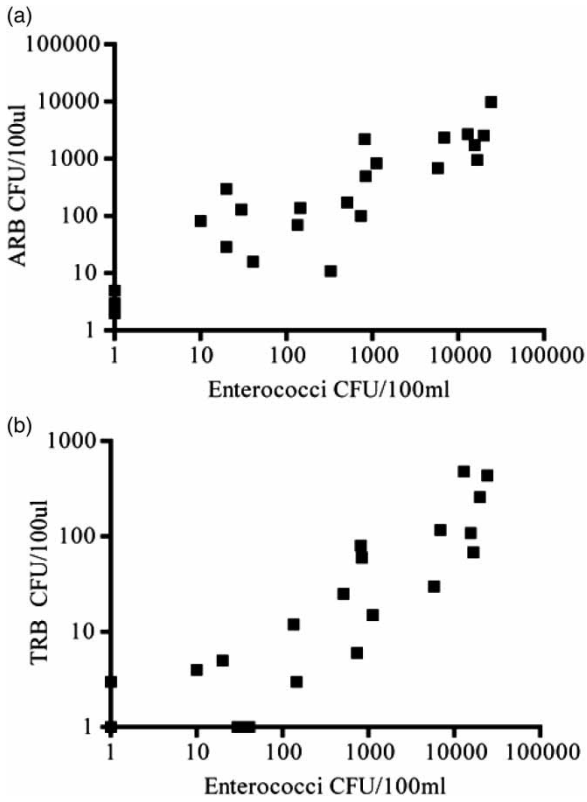

Figure 3 | Positive association of abundance of antibiotic-resistant heterotrophs ((a) ARB, $r = 0.888$, $p < 0.001$; (b) TRB, $r = 0.849$, $p < 0.001$) with the sewage indicator, *Enterococcus*, in Flushing Bay. Ampicillin-resistant bacteria were more abundant (note axis scales) than tetracycline-resistant bacteria.

Gammaproteobacteria (Table 3). *Pseudomonas* was the most abundant genus of all Proteobacteria sequenced from Het and ARB isolates, while *Escherichia/Shigella* was the most abundant genus from TRB. *Aeromonas* and *Pseudomonas* combined accounted for 54% of the ARB sequences identified.

Rarefaction curves showed significantly greater diversity, when normalized for sampling effort, in sequences from Het isolates compared to antibiotic-resistant (ARB or TRB) isolates (Figure 7). Similarly, the Shannon–Weiner diversity index (H′) for Het isolates had a value of 2.62, indicating greater diversity than ARB ($H' = 2.21$) or TRB ($H' = 2.18$), with no significant difference between ARB and TRB diversity.

The following library comparisons were performed using RDP's library comparison tool: resistant vs. non-resistant; ampicillin-resistant vs. tetracycline-resistant; resistant wet vs. resistant dry. In these comparisons, Enterobacteriaceae, a family of Gram-negative, fermenting facultative anaerobes often associated with the intestine, were found to be significantly more abundant (17% vs. 2%; $p = 0.005$) in the resistant isolate sequence libraries (ARB + TRB) than in the non-resistant heterotrophic (Het) isolate sequence library. Comparing the two resistant libraries, the TRB isolates contained significantly more Enterobacteriaceae than the ARB isolates (37% vs. 7%; $p = 0.035$). Similarly, sequences from resistant isolates obtained after wet weather, as compared to dry weather, also contained a significantly higher abundance of Enterobacteriaceae (21% vs. 5%; $p = 0.025$).

DISCUSSION

Monthly sampling at estuarine monitoring stations

Antibiotic-resistant bacteria were widespread and highly variable throughout the lower HRE. Overall, every site and 84% of samples contained some level of antibiotic-resistant bacteria, indicating that resistant microbes are commonly present in most of the lower estuary. This

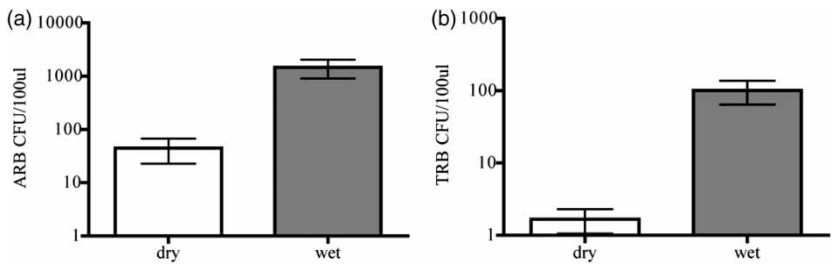

Figure 4 | (a) and (b) Mean abundance and standard error of cultured antibiotic-resistant bacteria (ARB and TRB) from surface water samples at Flushing Bay following periods of dry weather ($n = 6$) and wet weather ($n = 17$).

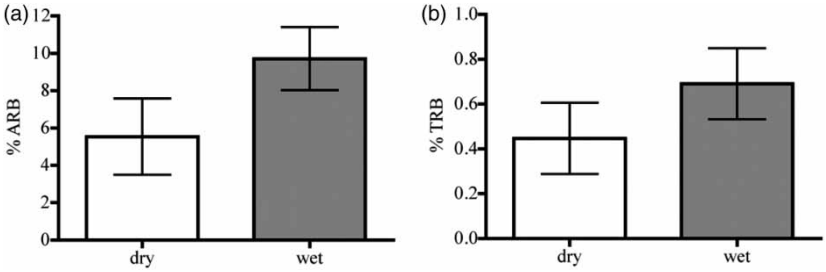

Figure 5 | (a) and (b) Mean and standard error for proportions of culturable antibiotic-resistant heterotrophs as a percentage of total culturable heterotrophs in Flushing Bay following periods of dry weather ($n = 6$) and wet weather ($n = 17$).

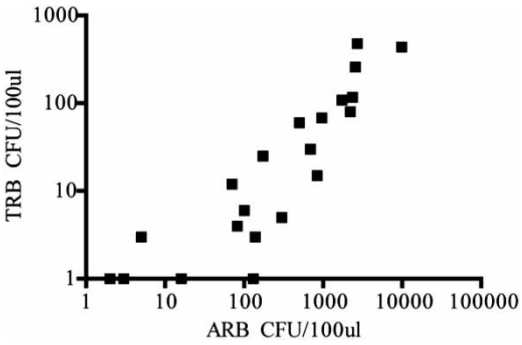

Figure 6 | The abundance of ARB and TRB at Flushing Bay were positively correlated ($r = 0.918$, $p < 0.001$).

is not surprising, given that in a 3-year study of 22 US rivers using similar methods, Ash *et al*. (2002) found measurable concentrations of ARB in all samples and that antibiotics and antibiotic-resistant microbes have been detected even in pristine aquatic sites (e.g. Boon & Cattanach 1999; De Souza *et al*. 2006). The Ash *et al*. study's sampling sites included rivers with a wide range of human population densities, therefore the common occurrence of ampicillin resistance described in this study is not restricted to urban waterways such as the lower HRE. The proportion of ARB as a fraction of total heterotrophs in the HRE overlapped with observations in the Ash *et al*. (2002) survey. Ash *et al*. found a range of 4–73% of total heterotrophs were ampicillin-resistant, while in our study the range was 0–27%.

Despite substantial overlap, the proportion of ARB as a fraction of total heterotrophs in the HRE was at the low end of results from the Ash *et al*. (2002) survey. The relatively low abundance of ARB measured in the HRE may partly be attributed to dry weather during and preceding our spatial sampling cruises. It is likely that this resulted in an underestimation of both sewage loading and the concentrations of antibiotic-resistant bacteria that would be found under more average conditions. When *Enterococcus* samples from a recent 5-year Riverkeeper study of the HRE (Riverkeeper 2011a) were grouped according to rainfall criteria, 32% of wet weather samples exceeded the US EPA single sample maximum guideline for primary contact in recreational waters (EPA 2004), while only 9% of dry weather samples exceeded the guideline. Thus, the *Enterococcus* results of this study (8.6% of samples from the spatial sampling exceeded the guideline) were more consistent with dry weather, and other results probably also represented such conditions. For comparison, Suter *et al*. (2011) detected *Enterococcus* concentrations in the lower HRE that exceeded the guideline in 29% of samples from many of the same Riverkeeper sites, likely because that study included a higher proportion of wet weather sampling days.

While the Ash *et al*. (2002) survey did not compare the prevalence of tetracycline resistance, in this study we observed that ARB were more abundant than TRB throughout the lower HRE. This result is consistent with data from Guardabassi *et al*. (2002) showing that more fecal coliforms and *Acinetobacter* from WWTPs were resistant to ampicillin than tetracycline or gentamicin. In addition, West *et al*. (2011) found 81–89% of cultured fecal coliforms were resistant to ampicillin in waterways upstream and downstream of WWTPs, the highest percentage of resistance in the study compared to four other antibiotics, including two tetracycline compounds.

In this study, the abundance of both antibiotic-resistant bacteria and *Enterococcus* were higher at nearshore, compared to mid-channel locations, similar to patterns previously reported for sewage indicating microbes in the

Table 2 | Classification of 16S rRNA gene sequences ($n = 234$) from Het, ARB and TRB isolates based on Ribosomal Database Project at 95% confidence unless otherwise noted

Plate type	#	Phylum	#	Genus
HET ($n = 52$)	38	Proteobacteria	13	*Pseudomonas*
			7	*Sphingobium*
			5	*Acinetobacter*
			3	*Psychrobacter*
			2	*Shewanella*
			1	*Aeromonas, Arcobacter, Azospirillum, Caulobacter, Erythrobacter, Escherichia/Shigella, Paraferimonus, Sphingomonas*
	10	Bacteriodetes	5	*Flavobacterium*
			1	*Chryseobacterium*
			4	Unclassified
	2	Actinobacteria	1	*Arthrobacter, Brachybacterium*
	2	Firmicutes	1	*Planomicrobium, Trichococcus*
ARB ($n = 123$)	11	Bacteriodetes	7	*Pedobacter*
			2	*Chryseobacterium*
			2	Unclassified
	112	Proteobacteria	39	*Pseudomonas*[a]
			27	*Aeromonas*
			10	*Stenotrophomonas*[a]
			4	*Comomonas, Ralstonia*
			3	*Acidovorax, Escherichia/Shigella, Raoultella*[a]
			2	*Brevundimonas*
			1	*Acinetobacter, Caulobacter, Delftia, Proteus, Variovorax*
			12	Unclassified
TRB ($n = 59$)	54	Proteobacteria	14	*Escherichia/Shigella*
			10	*Pseudomonas*
			8	*Acinetobacter*[a]
			6	*Stenotrophomonas*[a]
			3	*Citrobacter*[b]
			2	*Alcaligenes*
			1	*Achromobacter*[a], *Aeromonas, Enterobacter*[a], *Klebsiella, Sphingopyxis*
			6	Unclassified
	5	Bacteriodetes	4	*Chryseobacterium*
			1	*Flavobacterium*

[a]Indicates that one of the sequences was classified with 85–95% confidence; [b]indicates that two of the sequences were classified with 85–95% confidence; any sequence with a confidence level less than 85% for classification at the level of genus is listed as 'unclassified' in the table.

HRE (Riverkeeper 2011a; Suter *et al*. 2011) and supporting a link to pollution inputs that originate near the shoreline, such as CSOs. For example, the highest maximum level of ARB was measured at the 125th St Pier (Figure 1, Site 6), a site directly adjacent to a CSO, following a rain event. It should be noted that these nearshore environments are the areas where human contact with the water is most common. This highlights an important opportunity for improved management in the HRE, by demonstrating the need for shore-based sampling within regional monitoring programs or the use of monitoring boats capable of sampling near shore. Comprehensive monitoring programs in similar

Table 3 | Results from RDP library comparison of ARB, TRB and Het plates

Phylum	ARB (%)	TRB (%)	Het (%)
Actinobacteria	0	0	4
Bacteriodetes	9	8	19
Firmicutes	0	0	4
Proteobacteria[a]	91	92	73
Class			
Beta[a]	13	6	0
Epsilon	0	0	2
Alpha[a,b]	3	2	19
Gamma[a,b]	76	85	52

[a]Indicates significant difference in library comparison between ARB and Het.
[b]Indicates significant difference in library comparison between TRB and Het. There were no significant differences between ARB and TRB libraries.

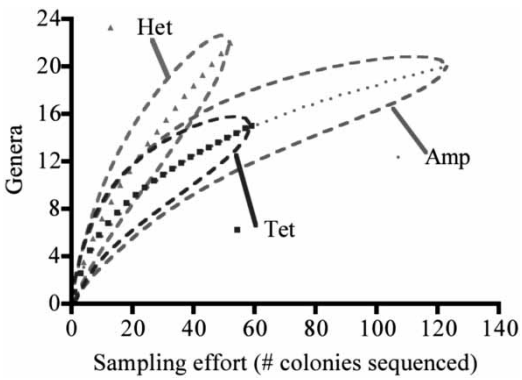

Figure 7 | Rarefaction curves showing greater diversity in Het samples than ARB and TRB samples, using Analytic Rarefaction. Dashed lines around the curves represent the 95% confidence intervals.

urban systems often emphasize sampling in nearshore environments to capture anthropogenic inputs (Rex 2011; Wicks *et al*. 2011). Rainfall-related impairment of water quality is frequently reported in urban areas, and this study supports the existing body of literature pointing to urban centers as having abundant sources of fecal contamination in association with stormwater released along the shoreline into local waterways (Noble *et al*. 2003; Petersen *et al*. 2005; Sauer *et al*. 2011).

WWTPs are well documented as sources of fecal indicator bacteria, allochthonous microbes and anthropogenic contaminants to urban waterways (Gannon *et al*. 1989; Petersen *et al*. 2005; Wakelin *et al*. 2008). Two WWTP outfalls were sampled during the spatial sampling portion of this study, and comparing the results from those two outfalls reveal important differences. The Piermont outfall releases effluent from the Orangetown WWTP in Rockland County, NY (Figure 1, Site 3). This plant has had significant problems with disinfection processes (State of New York 2010) and is known to have had commonly high *Enterococcus* counts associated with the effluent over the last 5 years (Riverkeeper 2011b). The other WWTP sampled, the North River WWTP (Figure 1, Site 5), is one of the newest plants in NYC. Over the last 5 years, its effluent has been found to have low *Enterococcus* counts, relative to the Piermont outfall, and relative to many other locations in the HRE (Riverkeeper 2011c).

The highest measurements of TRB and the third highest measurement of ARB from the spatial sampling were recorded at the Piermont outfall. In comparison, the North River WWTP had much lower levels of both antibiotic resistance and sewage indicators, although it still had the second highest maximum TRB concentration of the spatial survey sampling cruises. The TRB results were quantitatively consistent with extrapolations of data presented in Kim *et al*. (2010) on TRB concentrations at different stages of treatment in NYC WWTPs. TRB measurements during this study at the Piermont outfall (maximum 5.9×10^3 CFU/mL) were consistent with concentrations of 10^2–10^4 CFU/mL in plants where no disinfection processes were used (Kim *et al*. 2010). Meanwhile, the range of 0–290 CFU/mL at the North River WWTP outfall was consistent with measurements of 10^2 or fewer CFU/mL in plants where chlorination and ultraviolet disinfection processes were used (Kim *et al*. 2010). For comparison, Kim *et al*. (2010) found TRB concentrations of 10^4–10^5 CFU/mL in primary clarifier effluent. Based on those observations, active CSOs in the NYC area (i.e. during and after rainfall) could contain TRB at 1,000–10,000 times the maximum surface water concentrations detected at most sites in this study. These comparisons are a demonstration of the potential benefits from upgrading wastewater treatment infrastructure in the HRE and elsewhere (Koivunen *et al*. 2003; da Costa *et al*. 2006; Zhang & Farahbakhsh 2007).

Higher frequency, wet versus dry sampling

Flushing Bay is an outlet for multiple CSOs and is surrounded by a highly urbanized, partly industrial region, as well as

LaGuardia Airport. This nearshore site is prone to sewage contamination as demonstrated by the high percentage (86%) of single sample exceedances of the *Enterococcus* guidelines for primary contact. High-frequency sampling at this site allowed a number of rain events to be captured. The minimum concentration of *Enterococcus* measured at this site met EPA guidelines for acceptable primary contact, while the maximum (>24,196 CFU/100 mL) was more than 100-times higher than EPA guidelines, indicating a dynamic environment with a strong but episodic sewage connection. These characteristics made the location an ideal site to study the link between sewage contamination and antibiotic-resistant bacteria.

While significant correlations between *Enterococcus* counts and both ARB and TRB were found in the spatial sampling portion of this study, the relationships were much stronger within the higher frequency data from Flushing Bay. It is likely that the stronger relationships were related to the broader range of conditions sampled at Flushing Bay. This highlights the importance, and difficulty, of capturing the full range of environmental conditions within a water quality sampling program.

Prior studies have suggested a connection between sewage contamination and antibiotic resistance. First, waterways near sewage outfalls often show increased concentrations or occurrence of antibiotics (e.g. Hirsch *et al.* 1999), which sets the stage for resistance to develop. More directly, Goñi-Urriza *et al.* (2000) found increases in the proportion of resistant strains of *Aeromonas* and Enterobacteriaceae downstream from a WWTP, where fecal coliforms were also elevated. Similarly, Reinthaler *et al.* (2003) analyzed water 100-m downstream from three WWTPs and reported higher levels of tetracycline resistance near the plant where the highest levels of *E. coli* were detected. Garcia-Armisen *et al.* (2011) found high levels of antibiotic-resistant bacteria downstream from Paris and Brussels, in rivers where sewage indicators were also high, although in their overall data set, there was no significant correlation between sewage indicators and counts of antibiotic-resistant bacteria. Despite such prior indications, our study is the first to show positive correlations between the level of sewage indicators, such as *Enterococcus*, and the abundance of antibiotic-resistant bacterial heterotrophs. These correlations support the interpretation that the sewage-indicating and antibiotic-resistant bacteria share a common, sewage-related source, and thus, also support the value of fecal indicators to predict the abundance of other potentially harmful bacteria, such as other antibiotic-resistant microbes, that are a concern for public health.

The magnitude of the increase in counts of antibiotic-resistant bacteria following rainfall, combined with the short time scale of the effect, strongly suggest that, for Flushing Bay, the majority of antibiotic-resistant bacteria were carried with wet-weather associated sewage inputs, as opposed to resistance developing *in situ*. The Flushing Bay site is known to be strongly influenced by CSOs and the episodic nature of the bacterial counts at this site also suggest that the populations of antibiotic-resistant bacteria at this site were highly dynamic, presumably experiencing rapid transport or mortality following input. Although CSO volumes released into many waterways are small in comparison to the total input of treated wastewater effluent, CSOs may represent a disproportionately large percentage of the total antibiotic and antibiotic-resistant bacterial load. Similar patterns have been described for hormones and other wastewater micropollutants and can result in complex patterns of contaminant concentration with increasing volume of rainfall (Phillips *et al.* 2012).

Identification and diversity of resistant microbes

This study is the first to investigate the phylogenetic identity and diversity of antibiotic-resistant microbes in the HRE. The ARB and TRB isolates were found to be less diverse (mostly Proteobacteria and some Bacteroidetes) than the Het isolates (Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes), suggesting that, although widely distributed, only a subset of the total estuarine microbes commonly carry resistance. It must be noted, however, that our study utilized cultivation-based methodologies and that only a small portion of the total estuarine microbial community can be assessed with these approaches due to biases associated with cultivation-based techniques.

The most abundant genera of ARB and TRB isolates included *Aeromonas*, *Pseudomonas*, *Stenotrophomonas*, *Klebsiella* and *Escherichia/Shigella*, and all of these genera contain strains that can act as opportunistic pathogens that have been associated with antibiotic-resistant

infections (e.g. Varley *et al.* 2009; Brussalaers *et al.* 2011), suggesting that these resistant microbes could be of potential concern to recreational users, especially immuno-compromised individuals. For example, some *Aeromonas* species have been associated with gastrointestinal disease in humans and infections in fish (Janda & Abbott 1998). These same genera have also been commonly found among the antibiotic-resistant isolates of other aquatic environments (Ash *et al.* 2002; De Souza *et al.* 2006; Garcia-Armisen, *et al.* 2011). *Pseudomonas* was the most abundant genera in the ampicillin-resistant isolates from Flushing Bay (32% of library) and also from Garcia-Armisen *et al.* (2011; 44% of library), suggesting that these bacteria are prone to resistance, widely distributed and easily cultured.

Despite the biases associated with cultivation-based approaches, a strong connection can be made between the types of resistant bacteria isolated and the gastrointestinal tract as a source of allochthonous bacteria in these waterways. The most common tetracycline-resistant genus identified was *Escherichia/Shigella*, a group of enteric bacteria found in high concentrations in human and animal waste. In addition, significantly more resistant isolates from the Enterobacteriaceae family were identified following wet weather compared to dry weather. This family of bacteria is commonly associated with the intestine, and increased detection following wet weather further supports a sewage source for the resistant bacteria. The other family of bacteria with significantly more wet weather sequences, Xanthomonadaceae, is not specific to the human gut but does contain pathogenic strains and again has been commonly identified in antibiotic-resistant isolates from prior studies (De Souza *et al.* 2006; Garcia-Armisen *et al.* 2011).

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

The results of this study showed widespread detection of antibiotic-resistant bacteria throughout the HRE, especially in the nearshore environment. Positive correlations between sewage indicators and antibiotic-resistant bacteria were demonstrated in both spatial and temporal sampling. These correlations point to sewage as a major source of bacteria resistant to antibiotics in urban environments. Phylogenetic identification of isolates from Flushing Bay confirms the presence of antibiotic-resistant bacteria from genera that are known to contain opportunistic pathogens and enteric bacteria. These findings further support the potential for a public health hazard to those exposed to the water during and after heavy rain events.

Recommendations

The widespread distribution of antibiotic-resistant microbes documented in this study has clear management implications related to water quality and public health in NYC and other urban areas prone to sewage contamination. The correlation of antibiotic resistance with *Enterococcus* supports the value of measuring such indicators as representative of other agents of concern in the source water. Significantly higher concentrations of sewage indicators and antibiotic-resistant bacteria in nearshore environments reinforce the need for shore-based monitoring or the use of boats capable of sampling in shallow waters near the shore.

The findings of this study also provide support for Sewage Right to Know initiatives, such as legislation recently passed in New York State, to alert the public when sewage overflows occur. Serious investigation should be pursued regarding the sources of antibiotics and antibiotic-resistant microbes in urban waterways and the potential link between wet weather stormwater discharges and antibiotic-resistant infections. Possible mitigation strategies could include: reduced CSO volumes through sustainable and green infrastructure; more discriminating prescription practices by the local healthcare community; and potential upgrades to WWTPs to remove antibiotics and other emerging contaminants of societal concern.

ACKNOWLEDGEMENTS

This study would not have been possible without the generous support provided by: The Hudson River Foundation, the Tibor T. Polgar committee, The Wallace Foundation, The Brinson Foundation and Riverkeeper. The authors gratefully acknowledge the assistance of Captain John Lipscomb, Carol Knudsen, Maren

Mellendorf, and Dr Timothy Eaton. This is Lamont Doherty Earth Observatory contribution number 7686.

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First received 27 July 2012; accepted in revised form 5 March 2013. Available online 19 April 2013