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Antibiotic Resistance Gene Abundances Associated with Waste Discharges to the Almendares River near Havana, Cuba

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Considerable debate exists over the primary cause of increased antibiotic resistance (AR) worldwide. Evidence suggests increasing AR results from overuse of antibiotics in medicine and therapeutic and nontherapeutic applications in agriculture. However, pollution also can influence environmental AR, particularly associated with heavy metal, pharmaceutical, and other waste releases, although the relative scale of the "pollution" contribution is poorly defined, which restricts targeted mitigation efforts. The question is "where to study and quantify AR from pollution versus other causes to best understand the pollution effect". One useful site is Cuba because industrial pollution broadly exists; antibiotics are used sparingly in medicine and agriculture; and multiresistant bacterial infections are increasing in clinical settings without explanation. Within this context, we quantified 13 antibiotic resistance genes (ARG; indicators of AR potential), 6 heavy metals, 3 antibiotics, and 17 other organic pollutants at 8 locations along the Almendares River in western Havana at sites bracketing known waste discharge points, including a large solid waste landfill and various pharmaceutical factories. Significant correlations ($p < 0.05$) were found between sediment ARG levels, especially for tetracyclines and β-lactams (e.g., *tet*(M), *tet*(O), *tet*(Q), *tet*(W), bla_{OXA}), and sediment Cu and water column ampicillin levels in the river. Further, sediment ARG levels increased by up to 3 orders of magnitude downstream of the pharmaceutical factories and were highest where human population densities also were high. Although explicit links are not shown, results suggest that pollution has increased background AR levels in a setting where other causes of AR are less prevalent.

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Introduction

Antibiotics and antimicrobials have revolutionized the treatment of infectious disease and increased agricultural productivity worldwide; however, rapid and increasing development of antibiotic resistance (AR) has reached a point where untreatable pathogens are often found in hospital and community settings (*1, 2*). The dogma is that increasing AR is a consequence of indiscriminate use of antibiotics in medicine and agriculture (*3*). However, growing evidence suggests that industrial and other waste releases also might be changing background AR levels (*4*−*9*). As an example, recent work in India has found bacterial communities highly resistant to ciprofloxacin near an antibiotic production facility (*10*), and similar observations have been made elsewhere in the world (*11*−*13*).

Clearly, anthropogenic activities promote AR in different places in different ways, but the relative importance of different types of human activity on AR is largely unknown (or biased to discipline, such as medicine or agriculture), and quantitative data are needed from settings that have different historic antibiotic use behavior. One such location is Cuba, which only minimally uses antibiotics in agriculture and uses antibiotics very discriminately in medicine, whereas unregulated pollution is still quite common. Furthermore, the Almendares River near Havana is specifically useful for study because it has different pollutant inputs along its reach, it passes through areas with high population densities, and it has highly elevated heavy metal levels and other contaminants in its sediments (*14*). This is of local concern because the Almendares watershed provides over 47% of Havana's water supply (via groundwater) and contains one of the largest urban parks in Cuba (*15*).

This project evolved from previous water-quality studies on the river aimed at remediating the river prior to further development of the watershed for recreational purposes. However, early work showed heavy metals were very elevated in river sediments (*16, 17*), especially Cu (copper), Pb (lead), and Zn (zinc), and we speculated that this pollution might also be affecting AR because of known links between heavy metals and AR development (*18*). Further, the watershed contains pharmaceutical production and other facilities and untreated domestic wastewater discharges. Although the setting is complex, increased background AR in the River is especially pertinent to Havana because clinical studies have shown that multi-AR bacteria are increasing in local populations, but increases do not correlate with antibiotic use in medical applications (*19, 20*). Therefore, we sampled water and sediments at multiple sites along the river that bracket different waste inputs and quantified 13 AR genes (ARG) as biomarkers for AR potential in the river. These data then were statistically analyzed in association with water and sediment quality data to determine whether the ARG distribution in river sediments can be explained by environmental pollution, which has implications to public health in the city.

Materials and Methods

Study Site. The Almendares River is 49.8 km in length and originates southeast of Havana City. The river initially flows westward south of the city itself and then loops northward on the western side of the city where it enters the Strait of Florida (see Figure 1). About 53% of the river watershed is within the city itself with the river passing through different land uses, including urban agricultural, parkland, and heavy and light industrial zones. For this study, the river was

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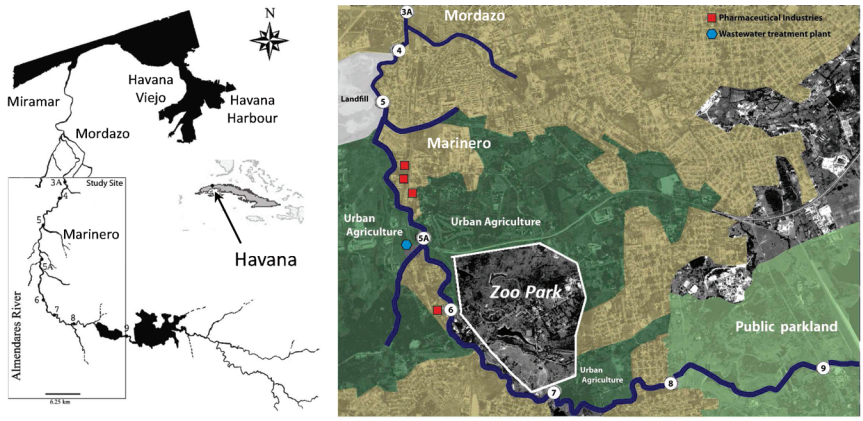

FIGURE 1. The Almendares River watershed near Havana, Cuba. Sampling stations are as indicated as well as general descriptions of land use within the watershed. The watershed includes a solid waste landfill, high-density urban development, urban agriculture, domestic waste discharges, and numerous pharmaceutical and other factories. The upper reaches are primarily urban agricultural, parkland, and low-density dwellings, whereas population densities and industrial intensity increase downstream, especially below station 6.

subdivided into eight reaches for sampling, which were carefully chosen based on multiple years of previous monitoring at 15 sites along the river and also locations of known waste inputs (*15*−*17*). Key elements of the river include reaches primarily influenced by urban agriculture, parkland (including a zoo), local industry, and a small community upstream of sta. 8 (sta. 9 to 6); untreated waste inputs from pharmaceutical factories and a malfunctioning domestic wastewater treatment with high effluent BOD and faecal coliform levels (stations 6 to 4) (*21*); and an unregulated solid waste landfill (stations 5 to 3A). Although the impacts of each waste input likely overlap, heavy metal and preliminary ARG data confirmed that the eight sites chosen for detailed evaluation described known waste inputs or were of possible public health significance.

Sample Collection and Initial Processing. Sampling was carried out between 2006 and 2008, although this report is based on data collected during the dry season between April-June 2007. Preliminary sampling of both metals and ARG indicated that water and sediment conditions was least variable at this time of year, which was important for assessing relationships between known waste inputs and ARG responses. In reality, ARG levels homogenize along the river during the rainy season (*22*); therefore, this first report is based on the one sample-season where comprehensive data were obtained. In that season, we collected five replicate samples per station per sampling event (there were three events in 2007) at sites chosen based on previous reconnaissance data. Although water column and sediment samples were collected, here we focus primarily on sediment data because sediment signals are much less variable over time in flowing aquatic systems (*23*−*26*).

All field sampling was performed as sterilely as possible with water and sediment samples being returned to the laboratory on ice in coolers for processing each sample-day. pH, dissolved oxygen (DO), and temperature were measured in the field using hand-held probes. Samples for water analysis were collected in 150-mL amber glass bottles, whereas 2-mL subsamples were transferred to microcentrifuge tubes and frozen at −20 °C in the laboratory for antibiotic analysis. Complementary sediment cores were obtained using ethanol-washed 1-L stainless steel cylinders. Immediately after coring to ∼10 cm depth, replicate ∼0.5-g aliquots were aseptically transferred into microcentrifuge tubes and frozen upon return to the lab for molecular biological analysis. Remaining sediments were transferred to clean plastic bottles and retained at 4 °C prior to metal analysis. Molecular biological samples were stored at −20 °C and analyzed *en masse* at the end of the sampling program.

Metal and Other Analysis. Flame Atomic Absorption Spectrometry (FAAS) was used to quantify sediment heavy metal levels, including Cd (cadmium), Pb, Cu, Zn, Co (cobalt), and Cr (chromium). These metals were chosen for this study based on results from previous samplings and past evidence of metals that most influence AR (*16, 17*). Each sample was digested using a $HF/HClO_4/HNO_3$ mixture (1:1:2) at 200 °C, and metal levels were quantified using a Buck Scientific 210VGP Atomic Absorption Spectrometer (East Norwick, CT, USA) equipped with an air-acetylene flame and deuterium background corrector. Quality control was provided by parallel analysis of certified reference SOIL 7 (IAEA). Detected levels in SOIL 7 were always ± 10% of defined standard values for all metals based on nine replicates, with represented coefficient of variations ranging from 0.035 to 0.089, depending upon the metal. Total dissolved solids (TDS) were analyzed according to Standard Methods (*27*).

Antibiotic and Organochlorine Pesticide Analysis. Water-column levels of three common antibiotics were quantified using the R-Biopharm Strip Reader (Darmstadt, Germany) and ELISA kits targeting tetracyclines (RIDASCREEN; R-Biopharm, Darmstadt, Germany), ampicillin (Gentaur; Kobe, Japan), and benzyl-penicillin (Gentaur; Kobe, Japan). The RIDASCREEN-Tetracycline assay is class-specific and detects various tetracycline derivatives, whereas ampicillin and benzyl-penicillin assays are more compound specific. Similar kits were not available for erythromycin. Although analyses were not comprehensive, these assays reflected classes of drugs of particular local importance and provided "example" antibiotic levels in the river. Fifty-μL sample volumes were employed in all assays (in duplicate) and detection limits were 50 ng/L. Analysis of sediment porewater levels of 17 organochlorine pesticides also was performed on parallel samples, which is summarized in the Supporting Information (SI).

Sample Processing and DNA Extractions. DNA was extracted within 24 h of sample collection using the Ultra-Clean Soil DNA Isolation kit (MoBio Laboratories Inc.) and the protocol for maximum yields. A combination of shaking and freeze/thaw cycles was used for cell disruption. Sediment samples (∼0.5 g) were transferred initially to microcentrifuge tubes preloaded with extraction buffer and glass beads. The samples were vortexed immediately and aggressively hand

TABLE 1. Selected Heavy-Metal and Antibiotic Levels in Sediments and the Water Column, Respectively, along the Almendares River[a]

station	9	8	7	6	5A	5	4	3A
Cd	0.24[b] (0.01)	0.44 (0.03)	0.64 (0.04)	0.73 (0.04)	0.68 (0.03)	0.49 (0.03)	1.59 (0.09)	0.28 (0.01)
Co	8.80 (0.54)	12.9 (1.02)	14.7 (1.16)	14.8 (0.78)	9.54 (0.59)	15.6 (0.82)	13.6 (0.60)	3.33 (0.20)
Cr	95.7 (5.88)	86.0 (3.02)	140 (7.33)	148 (10.4)	121 (5.29)	120 (5.26)	197 (10.4)	58.4 (4.60)
Cu	11.6 (0.41)	58.1 (3.57)	90.2 (5.54)	21.5 (0.95)	42.6 (1.49)	138 (8.44)	869 (30.5)	256 (20.2)
Pb	17.4 (1.07)	45.0 (3.55)	59.7 (4.19)	64.9 (2.27)	57.5 (3.52)	31.3 (2.19)	108 (4.04)	50.4 (2.21)
Zn	46.1 (1.61)	152 (5.22)	187 (9.81)	245 (15.1)	275 (7.63)	99.4 (7.84)	800 (28.3)	334 (11.7)
ampicillin	280[c] (167)	441 (69.2)	197 (116)	231 (164)	925 (349)	7570 (4820)	11400 (1990)	7940 (1940)
benzyl-penicillin	65.9 (12.4)	63.6 (10.5)	59.8 (7.49)	60.3 (16.4)	95.7 (21.4)	49.6 (19.3)	95.4 (41.8)	32.9 (10.9)
tetracycline	11.1 (19.8)	3.37 (4.64)	17.2 (23.9)	1.00 (0.01)	155.7 (91.8)	36.2 (46.0)	14.3 (16.5)	41.5 (26.4)

[a] Upstream to downstream: 9→3A. [b] Mean sediment concentrations based on five independent samples in μg/g-soil; 95% confidence intervals in brackets. [c] Mean water-column concentrations based on five independent samples in ng/L; 95% confidence intervals in brackets.

shaken for one minute to disrupt the soil matrix. Disrupted samples then were frozen at −20 °C and thawed at 70 °C four times in series to promote cell lysis. Subsequent extraction procedures followed manufacturer's protocols. Extracted DNA was stored at −20 °C and returned to the United Kingdom for qPCR analysis.

qPCR Detection of ARG. Extracted DNA was used to quantify levels of AR and 16S-rRNA genes, which allowed normalization of ARG abundances to baseline rRNA gene levels to account for differences between stations, differing DNA extraction efficiencies, and possible sample degradation. All genes were quantified in duplicate by qPCR (iCycler; BioRad, Hercules, CA USA), using carefully chosen probes and primers for specific ARG as "biomarkers" of AR (*5*). Table S2 in the SI provides details of the probes and primers used. Assays for tetracycline resistance genes (i.e., *tet*(L), *tet*(M), *tet*(O), *tet*(Q), *tet*(W)) and erythromycin-resistance-methylase determinants (i.e., *erm*(B), *erm*(C), *erm*(E) and *erm*(F)) were based on previously published methods (*5, 23, 28*). Other assays were adapted from published methods, including beta-lactamase genes: $bla_{\text{TEM-1}}$ (*29*), $bla_{\text{CTX-M}}$ (*30*), $bla_{\text{SHV-1}}$ (*31, 32*), and $bla_{\text{OXA-1}}$ (*33, 34*). Typically, DNA template (2 μL), appropriate primers (500 nM) and probes (200 nM, if used) were combined with iQ Supermix PCR reagent (BioRad). Reaction conditions included initial denaturation at 95 °C for one min, and 40−45 subsequent reaction cycles for annealing (55−60 °C for 30−60 s, depending on assay), elongation (20 s at 72 °C), and denaturation (20−30 s at 94 °C).

All reactions were run with parallel serially diluted DNA standards of known quantity and DNA-free negative controls (*23, 28*). The presence of inhibitory substances in the sample matrix was checked by spiking samples with known amounts of template and comparing differences in concentration threshold values (C_T) among the matrix and controls (one cycle difference between samples and controls was targeted). Based on pretesting, a 1:100 dilution of extracted DNA to molecular-grade water was used to minimize inhibitory effects of extraneous matter in the samples. PCR efficiencies (always ∼75−110%) were determined by comparing signals from serial dilutions of samples with high abundance of DNA with plasmid controls. Correlation coefficients were >0.99 for calibration curves, and log gene-abundance values were within the linear range.

Data Analysis. All data analyses were conducted using SPSS (Chicago, IL; v. 17.0) or Sigmaplot (Systat Software Inc.; v. 11.0). Bivariate correlation analysis was performed on antibiotic, heavy metal, and normalized ARG abundance data to identify general trends among parameters across all stations; all data were log-transformed to enhance sample normality prior to analysis. Principal components analysis (PCA) was undertaken to identify clusters of related parameters (as components) and assess how clusters varied along the river. The PCA used a correlation matrix with Varimax rotation and Kaiser normalization to maximize differences among loadings. Estimated local means were used to provide missing data to complete the matrix. Resulting factors were compared as bivariates to evaluate relationships with water quality conditions.

Results and Discussion

Water and Sediment Quality Conditions in the Almendares River. The Almendares River provides varying conditions to compare the relative and net influence of different waste inputs on environmental AR signals. As background, the mean pH, and temperature along the river were 7.75 ± 0.3 and 28.4 ± 0.6 °C (±95% confidence intervals), respectively, and did not vary significantly among sites (*ANOVA*, $p > 0.05$). In contrast, DO and TDS levels in the water column differed significantly between upstream and downstream stations (e.g., *ANOVA*, $p < 0.01$) with DO being significantly higher and TDS significantly lower in the river upstream of sta. 5A, which is where the malfunctioning wastewater treatment plant and some pharmaceutical factory wastes enter the river. DO levels were consistently low further downstream of sta. 5A (0.81 ± 0.16 mg/L vs 4.2 ± 1.1 mg/L upstream), which suggests an oxygen sag due to waste input(s) at between sta. 6 and 5 and-or oxygen-demanding inputs further downstream. DO and TDS levels were inversely correlated ($r = -0.862$; $p = 0.006$), suggesting TDS included organic matter.

Table 1 summarizes mean heavy metal and antibiotic levels in river sediments and the water column, respectively, over the sampling program. Organochlorine pesticide data for sediment porewater are provided in the SI but are not included in subsequent analysis because levels were below detection limits for most compounds (>0.01 ng/L) or very low compared with known contaminated sites (*35*). In

contrast, heavy metal and antibiotic levels were generally high and followed spatial patterns similar to DO and TDS. With a few exceptions, metal levels upstream of sta. 5 were low but increased dramatically at sta. 5 and 4 near the landfill, especially Cu and Zn. Metals were lower at sta. 3A, presumably due to upstream metal precipitation. Antibiotic levels also were generally low upstream of sta. 5A, but levels increased substantially downstream of this point, probably due to inputs from the pharmaceutical factories. As background, the factories discharge their wastes directly into sewer lines that either enter the river or feed into the waste treatment plant (*21*), although no data on exact quantities exist (nothing publically available). Therefore, pharmaceutical and domestic wastes enter the river largely untreated, which explains the relatively high antibiotic levels downstream of sta. 5A and also possibly the low DO levels. It should be noted, however, even the highest antibiotic levels detected here are low compared with lagoons at feedlots using antibiotics non-therapeutically (*23*) and are more than 2 orders of magnitude lower than typical therapeutic doses. Regardless, antibiotic levels, especially ampicillin, profoundly increase at sta. 5A, which is particularly significant when one considers the river is warm and light-exposed; conditions that promote photochemical degradation of such compounds (*36*).

Ampicillin levels were the highest among the antibiotics tested (even above sta. 5A), approaching tetracycline levels observed at animal feedlots (*23*). Tetracycline levels also increased below sta. 5A but declined further downstream, presumably because tetracyclines are very photosensitive and likely degraded in the river (*32, 37*). It is noteworthy that even though antibiotic levels appear low, levels are concerning because actual exposures to environmental organisms are likely greater than the numbers imply because we are only detecting residuals after degradation. In fact, tetracyclines and β-lactams are rarely detected in surface waters due to dilution and chemical instability (*38*−*40*); therefore, detecting antibiotics at all, especially in the water column, suggests consequential inputs to the river.

Antibiotic Resistance Gene Abundances along the Almendares River. This study assessed relationships among environmental conditions, waste inputs, and ARG levels along the Almendares River, which are summarized in Figure 2. The figure presents ARG levels normalized to 16S-rRNA gene levels at each site. As background, normalized ratios of 10^{-6} to 10^{-8} *tet* to 16S-rRNA are typical of pristine areas, whereas highly contaminated sites often have $>10^{-4}$ *tet* to 16S-rRNA gene ratios (*23*−*26*), which are apparent for some *tet* ARG below sta. 5 in the river. No equivalent data are available for extended spectrum β-lactamase (*bla* genes) and *erm* gene levels.

Figure 2 shows ARG levels vary among genes and from station-to-station, but general trends exist. Most detected *tet* genes and bla_{OXA} were locally elevated at sta. 8 but declined through sta. 6 and then increased again between sta. 5A and 3A. These data broadly suggest important inputs that affect ARG levels between sta. 9 and 8 and sta. 6 and 4. Similar patterns in ARG were seen for bla_{TEM} and *erm*(E), but variation among stations was less patterned. For example, bla_{TEM} levels increased at sta. 5A in parallel with increased ampicillin levels, but bla_{TEM} levels then declined further downstream. In contrast, *erm*(B) and bla_{SHV} were detected along the river, but no spatial patterns were apparent except *erm*(B) being locally elevated at sta. 8. Overall, three larger causes of increased ARG levels appear to exist on the river, although it is apparent that unknown causes also exist. In fact, some effort was made to identify unknown sources, but it proved impossible due to incomplete reporting information. However, this lack of background information is significant in understanding AR and pollution issues in an emerging country because increased industrialization does not necessarily equate with consistently strict waste management.

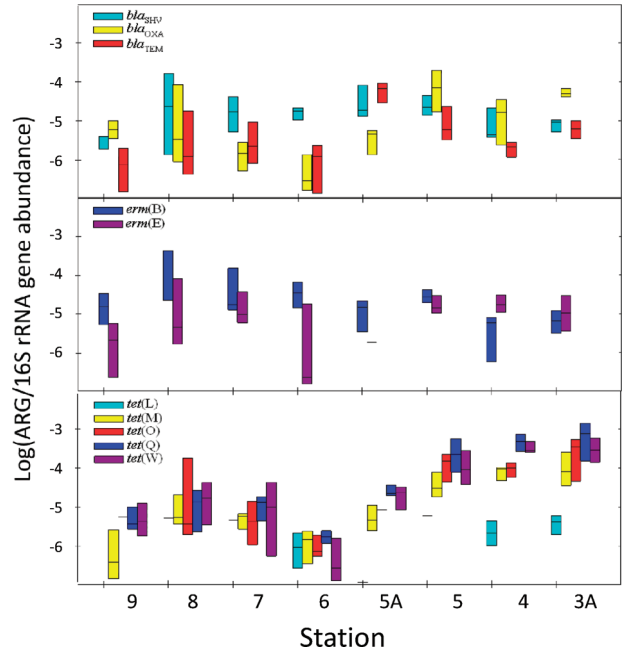

FIGURE 2. Relative abundances of ten antibiotic resistance genes (ARG) detected in sediments along the Almendares River. All values are normalized to 16S rRNA gene abundances to account for differences in background local gene abundances and differential extraction efficiencies. Local maxima of many ARG exist at station 8 and between stations 5 and 3A. Box plots represent median and range values ($n = 5$).

Relationships between Gene Abundances and Water Quality Parameters. To assess general relationships between ARG and water quality conditions, a PCA was performed on data from all stations, which included ten ARG (three ARG were below detection limits: *erm*(C), *erm*(F), and $bla_{CTX\text{-}M}$), six heavy metals, and three antibiotics. Table 2 shows four major clusters exist among data that account for ~70% of the total variance. The two most prominent clusters are component 1 (34.8% of variance), which includes many ARG, Cu, and ampicillin, and component 2 (19.2% of variance) that clusters the rest of the metals and benzyl-penicillin. Components 3 and 4, which include bla_{SHV} and bla_{TEM}, and *tet*(L), *erm*(B), Co, and tetracyclines, respectively, contribute to only 23.4% of variance combined.

Bivariate correlation analysis between each ARG and non-ARG parameters further elucidate ARG patterns along the river. Table 3 shows that elevated Cu and ampicillin levels most often correlate with detected ARG ($p < 0.05$; with seven different ARGs), although Pb, Co, Zn, and tetracycline levels also correlate with some ARG. Further, ampicillin significantly correlates with bla_{TEM} (data not shown). This is locally interesting because bla_{TEM} is associated with TEM-type extended spectrum β-lactamases (ESBL), the most common ESBL resistant phenotype found *Escherichia coli* in Havana hospital samples (*41*). Some unexpected (or lack of) relationships exist. For example, only *tet*(M) among the *tet* genes significantly correlated with tetracycline levels, whereas bla_{OXA} correlates with Cu. Such unexplained relationships result from differing attenuation rates between ARG and "their" antibiotic; unknown waste inputs along the river; differences between sediment and water signals in a flowing system; and secondary correlations between factors that affect ARG with other water quality factors that do not.

TABLE 2. Rotated Component Matrix[a] from Principal Components Analysis on Water-Column Antibiotic Levels and Sediment Heavy Metal and Normalized ARG Abundances in the Almendares River

pollutant		component 1	component 2	component 3	component 4
		34.8[b]	19.3[b]	12.0[b]	11.4[b]
ARGs	*tet*(L)	**.688**[c]	0.067	0.056	**−.614**
	tet(M)	**.931**	0.122	−0.034	0.143
	tet(O)	**.864**	−0.150	−0.075	0.230
	tet(Q)	**.903**	0.038	−0.097	0.284
	tet(W)	**.819**	−0.012	0.094	0.291
	erm(B)	−0.254	−0.209	0.366	**−.669**
	erm(E)	**.641**	0.041	0.203	−0.262
	bla_{OXA}	**.840**	−0.024	0.359	0.008
	bla_{TEM}	0.330	0.012	**.727**	0.392
	bla_{SHV}	0.126	0.187	**.854**	−0.111
heavy metals	Cd	0.141	**.977**	0.023	−0.032
	Cu	**.875**	0.401	−0.136	0.060
	Cr	−0.190	**.879**	−0.030	−0.145
	Pb	0.329	**.818**	−0.051	0.052
	Co	−0.305	**.576**	0.169	**−.550**
	Zn	0.483	**.696**	−0.081	0.245
antibiotics	tetracyclines	0.276	−0.027	0.285	**.677**
	ampicillin	**.728**	0.016	−0.116	0.288
	benzyl−penicillin	−0.406	**.578**	0.273	0.207

[a] Extraction method: principal component analysis; rotation method: Varimax with Kaiser normalization; rotation converged in 5 iterations. [b] % of variance. [c] Related factors are highlighted in bold.

TABLE 3. Antibiotic Resistance Genes (ARG) Correlated with Sediment Heavy−Metal and Water−Column Antibiotic Levels[a]

parameter	number of correlated ARG	specific ARG significantly correlated with indicated parameter
Cu	7	*tet*(M), *tet*(O), *tet*(Q), *tet*(W), *erm*(B), *erm*(E), bla_{OXA}
Cd	0	−
Cr	0	−
Pb	2	*tet*(M), bla_{OXA}
Co	2	*tet*(Q), *erm*(B)
Zn	3	*tet*(M), *tet*(Q), *tet*(W)
tetracyclines	2	*tet*(M), bla_{TEM}
ampicillin	5	*tet*(M), *tet*(O), *tet*(Q), *tet*(W), bla_{OXA}
benzyl−penicillin	0	−

[a] $\alpha = 0.05$ significance level.

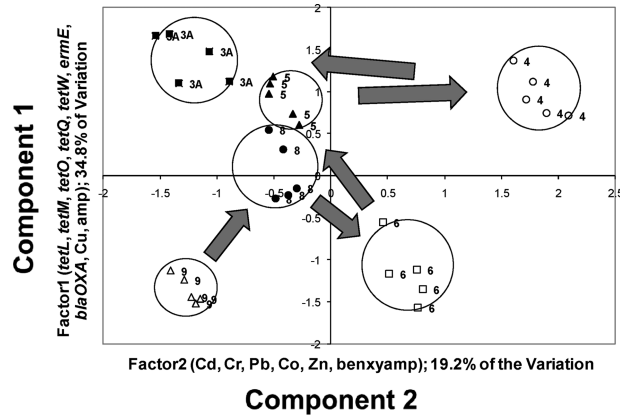

FIGURE 3. Spatial distribution of weighting factors for components 1 (many ARG, Cu and ampicillin) and 2 (primarily metals) from a Principles Components Analysis (PCA) of measured ARG, metals, and antibiotic data from the Almendares River. Shifts in the primary component between nonmetal (comp. 1) and metal-dominated (comp. 2) clusters are evident with metal-weighted factors being higher at sta. 4 and 6, and nonmetal factors being higher at sta. 5 and 3A. These patterns show the apparent influence of pharmaceutical factories and other wastewater discharges between sta. 6 and 5 and the landfill between sta. 5 and 4. Data for sta. 5A, 7, and 8 are not included for clarity, although trends are consistent with other stations.

To tease out relationships between water quality parameters and ARG levels, the spatial distribution of the weighting-factors of components 1 and 2 were plotted according to sampling station. Figure 3 shows that as one proceeds downstream from sta. 9 to 3A, the dominant component changes. For example, factors for both components were low at sta. 9, but both increased by sta. 8 in parallel with increasing ARG levels (see Figure 2). However, by sta. 6, component 1 (mainly ARG and Cu) becomes low again, whereas component 2 (non-Cu heavy metals) becomes large, which hints that an unknown metal-related factor may be responsible for elevated ARG levels at sta. 8. As one proceeds downstream to sta. 5, a shift toward component 1 occurs, implying that a nonmetal related factor is driving the large increase in ARG levels between sta. 6 and 5, which is likely related to the factories and waste treatment plant near sta. 5A. However by sta. 4, where highest ARG levels are detected, components 1 and 2 are both large, suggesting that metal and nonmetals factors affect ARG between sta. 4 and 5. This is consistent with heavy metal releases from the landfill and lingering effects of upstream inputs. Below sta. 4, the influence of metals becomes small again (i.e., component 2), probably due to metal precipitation above sta. 3A.

Segregation of ARG Causes-and-Effects along the Almendares River. Although Figures 2 and 3 and Tables 2 and 3 show significant relationships between the abundances of ARG, and water and sediment quality parameters, the relative influence of each factor on overall AR is not clear. Both water quality and metal/antibiotic suggest a major change in river conditions between sta. 6 and 5, which is downstream of the factories and waste treatment plant. However, Figure 2 also suggests important inputs that affect ARG upstream of sta. 8 and downstream of station 5. The landfill partially explains elevated ARG levels below sta. 5, but higher ARG levels at sta. 8 data cannot be fully explained, although they may relate to inputs from the small community upstream of the station. Regardless of exact causes, the river has increasing ARG levels

as one proceeds downstream with the highest levels detected in the general vicinity of one of the most heavily populated areas in Havana.

Unfortunately, little information exists on AR in resident human populations along the Almendares River, although some data exist on AR in Havana. As previously noted, Cuba is conservative in antibiotic use both in clinical and non-clinical settings (*42*), but there also is growing evidence of increasing multiple AR organisms in hospitals (*43*). Although it is speculation, the most common ESBL phenotype in resistant *E. coli* in Havana hospitals is associated with bla_{TEM} (*41*), which is a gene that is elevated in the river. This might be completely coincidental, but given the watershed is used for recreation and irrigation, ARG harbored in river organisms could have opportunity for human exposure and possibly transmission, although direct links have not been shown.

This work shows that unregulated "pollution" has the potential of affecting AR in exposed aquatic systems. In the Almendares River, possible sources include pharmaceutical wastes from factories, inadequate domestic treatment, and a large landfill, but these are not the only sources nor is this situation unique to Havana. Work in India suggests such issues are global in emerging and developing countries (*10*). Although this study was partially unsuccessful because it did not find any "smoking guns", it shows the power of unregulated pollutant releases on environmental AR, especially in an emerging country. However, such scenarios are a concern to all because they are costly to resolve, often beyond the resources of impacted countries, but also because once AR is gained in exposed species, it might translate across populations and borders.

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Supporting Information Available

Additional information on methods and results on porewater analysis of 17 organochlorine pesticide levels (including Table S1); probes/primers used in the study (Table S2); and relationships between DO and TDS (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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