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RESEARCH ARTICLE

Metagenome-Wide Analysis of Rural and Urban Surface Waters and Sediments in Bangladesh Identifies Human Waste as a Driver of Antibiotic Resistance

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ABSTRACT In many low- and middle-income countries, antibiotic-resistant bacteria spread in the environment due to inadequate treatment of wastewater and the poorly regulated use of antibiotics in agri- and aquaculture. Here, we characterized the abundance and diversity of antibiotic-resistant bacteria and antibiotic resistance genes in surface waters and sediments in Bangladesh through quantitative culture of extended-spectrum beta-lactamase (ESBL)-producing coliforms and shotgun metagenomics. Samples were collected from highly urbanized settings ($n = 7$), rural ponds with a history of aquaculture-related antibiotic use ($n = 11$), and rural ponds with no history of antibiotic use ($n = 6$). ESBL-producing coliforms were found to be more prevalent in urban samples than in rural samples. Shotgun sequencing showed that sediment samples were dominated by the phylum *Proteobacteria* (on average, 73.8% of assigned reads), while in the water samples, *Cyanobacteria* were the predominant phylum (on average, 60.9% of assigned reads). Antibiotic resistance genes were detected in all samples, but their abundance varied 1,525-fold between sites, with the highest levels of antibiotic resistance genes being present in urban surface water samples. The abundance of antibiotic resistance genes was significantly correlated ($R^2 = 0.73$; $P = 8.9 \times 10^{-15}$) with the abundance of bacteria originating from the human gut, which suggests that the release of untreated sewage is a driver for the spread of environmental antibiotic resistance genes in Bangladesh, particularly in highly urbanized settings.

IMPORTANCE Low- and middle-income countries (LMICs) have higher burdens of multidrug-resistant infections than high-income countries, and there is thus an urgent need to elucidate the drivers of the spread of antibiotic-resistant bacteria in LMICs. Here, we study the diversity and abundance of antibiotic resistance genes in surface water and sediments from rural and urban settings in Bangladesh. We found that urban surface waters are particularly rich in antibiotic resistance genes, with a higher number of them associated with plasmids, indicating that they are more likely to spread horizontally. The abundance of antibiotic resistance genes was strongly correlated with the abundance of bacteria that originate from the human gut, suggesting that uncontrolled release of human waste is a major driver for the spread of antibiotic resistance in the urban environment. Improvements in sanitation in LMICs may thus be a key intervention to reduce the dissemination of antibiotic-resistant bacteria.

KEYWORDS resistome, metagenome, antibiotic resistance, plasmids, aquaculture, Bangladesh, antimicrobial resistance, environmental microbiology, public health, sediment, surface water

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Metagenome analysis of surface water and sediments shows that the release of untreated sewage is a driver for the spread of environmental antibiotic resistance genes in Bangladesh, particularly in highly urbanised settings.

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The prevalence of antibiotic-resistant bacteria causing infections is increasing globally, but the clinical issues, including significant morbidity and mortality, posed by these bacteria are particularly alarming in low- and middle-income countries (LMICs) (1–4). Proposed drivers for the high burden of drug-resistant infections in LMICs include the unregulated sales of antibiotics and their misuse in clinical medicine, agriculture, and aquaculture; an inadequate sewerage infrastructure; poor governance; and low investments in health care (5, 6).

One of the challenges of studying antimicrobial resistance (AMR) is to disentangle the spread of resistant bacteria and antibiotic resistance genes between humans, animals, and the wider environment (7). For this reason, AMR is increasingly being studied from a collaborative and cross-disciplinary perspective that has been termed "One Health" (8). The One Health concept for studying the spread of AMR is particularly relevant for LMICs due to the crucially important role of agriculture and aquaculture in the livelihoods of billions of people in many of these countries, especially the poorest ones (9). Asia is home to an estimated 74% of the world's 570 million farms (10), and, in 2016, 89% of the global aquaculture production was estimated to originate from this continent (11). However, there are still major knowledge gaps on the spread of AMR in Asia from a One Health perspective.

Bangladesh is an LMIC in South Asia, where antibiotic-resistant infections are common among both hospitalized patients and the nonhospitalized population (12). The country has a number of unique characteristics that may contribute to the rapid spread of AMR. The capital city of Bangladesh, Dhaka, has a population of around 16 million people, with a population density that ranks among the highest of any megacity. Less than 20% of the households in Dhaka are directly connected to sewerage infrastructure (13). The prevalence of carriage of multidrug-resistant *Escherichia coli* among healthy humans is relatively high in Bangladesh, as it is in other LMICs (14–16).

Antibiotic-resistant bacteria that colonize the human gut can be passed into rivers, lakes, and coastal areas through the release of untreated wastewater, the overflow of pit latrines during monsoon season, or by practices such as open defecation (17, 18). These contaminated environments are often used for bathing and the washing of clothes and food preparation equipment, thus facilitating human gut colonization by antibiotic-resistant bacteria (19). The antibiotic resistance genes carried by human-associated bacteria are often found on mobile genetic elements, including plasmids, which are capable of transfer within and between bacterial species. Plasmid-mediated transfer of antibiotic resistance genes thus contributes to the rapid dissemination of antibiotic resistance genes in microbial ecosystems, including those in the human gut and the environment (20, 21).

While a prescription is legally required to purchase antibiotics in Bangladesh, antibiotics can be readily acquired from many of the 200,000 drug stores across Bangladesh (22). In rural Bangladesh, aquaculture is widespread, with more than 2 million tons of freshwater fish produced in 2017 from inland freshwater fisheries (23). A survey performed from 2011 to 2012 revealed that antibiotics are widely used in Bangladeshi aquaculture for disease prevention and growth promotion. The most prominent classes of antibiotics employed are the tetracyclines, but other antibiotic classes, including β-lactams and sulfonamides, are also used (24). The use of antibiotics in Bangladesh is regulated in line with the European Union standards for antibiotic use in aquaculture, but Bangladesh has been found to be in breach of these regulations several times (25). The causes of antibiotics overuse in aquaculture are multifactorial: pharmaceutical companies provide food that is premixed with antibiotics without the farmers' knowledge, farmers administer antibiotics too often because they do not understand the instructions, and prophylactic use of antibiotics may be used to reduce the chance of damaging losses in production caused by disease (26). The combination of a densely populated country, intensive antibiotic usage in aquaculture, and the potential for the dissemination of antibiotic-resistant bacteria through surface water

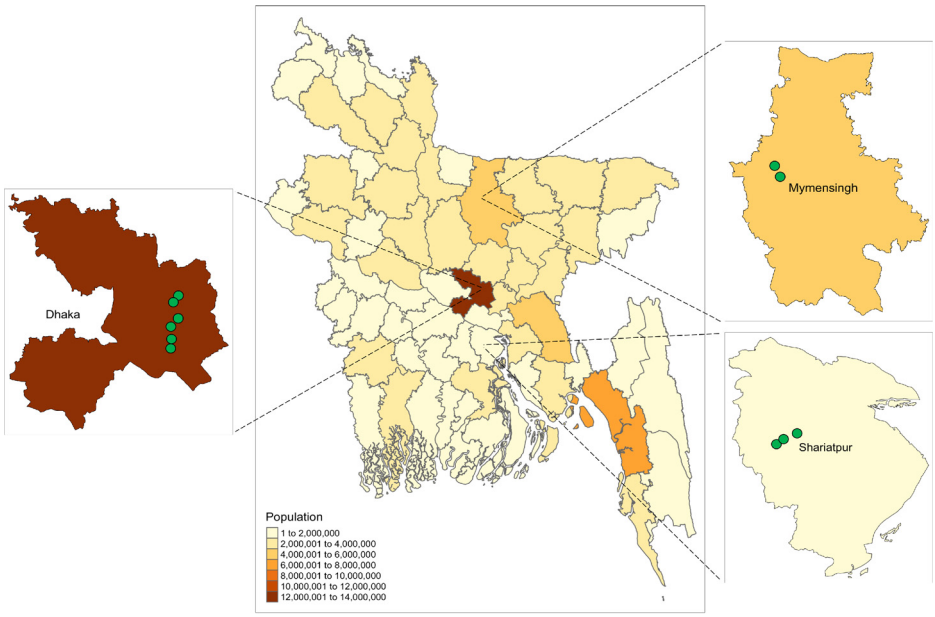

FIG 1 Map of Bangladesh showing the districts that the samples were collected from and the population of each district (obtained through https://data.humdata.org/dataset/bangladesh-administrative-level-0-3-population-statistics). Green circles represent sampling locations.

thus provides a unique opportunity to study the spread of AMR from a One Health perspective in Bangladesh.

In the manuscript, we use a combination of quantitative bacterial culture and metagenomic shotgun sequencing methods to disentangle pathways that contribute to the dissemination of antibiotic resistance. Specifically, we describe the abundance and diversity of microorganisms and antibiotic resistance genes in surface water in rural and urban settings in Bangladesh.

RESULTS

Sample collection across urban and rural sites in Bangladesh. Freshwater surface water and sediment samples were collected from 24 sites across 3 districts in Bangladesh (Mymensingh, Shariatpur, and Dhaka; Fig. 1). These sites spanned both rural and urban areas with different population densities. Among rural sites, ponds used for aquaculture with a history of antibiotic use ($n = 11$) and ponds with no history of antibiotic use ($n = 6$) were sampled. Further information on sampling locations and protocols is provided in Materials and Methods and Table S1 in the supplemental material. We used culture-dependent and culture-independent methods to study the abundance of antibiotic resistance genes and the diversity of microbiotas across the different sites.

ESBL-producing coliforms were more prevalent in urban samples than in rural samples. We quantitatively determined the burden of extended-spectrum beta-lactamase (ESBL)-producing coliforms in the water and sediment samples from the different sampling locations and found that ESBL-producing coliforms were detected in significantly more urban samples (12/14) than rural samples (15/34) (Fisher exact test; $P = 0.01$). However, there was no statistically significant difference in the viable counts of ESBL-producing coliforms in urban and rural samples (Fig. 2).

Microbiotas of surface water and sediments are distinct, with higher levels of human gut bacteria in urban samples. Shotgun metagenomic sequencing was used to study the diversity and composition of the microbial communities in the different samples. An important determinant shaping the communities was the sample type, with distinct (permutational multivariate analysis of variance [PERMANOVA], $P < 0.001$) clustering of sediment and water samples (Fig. 3A). The sediment samples were

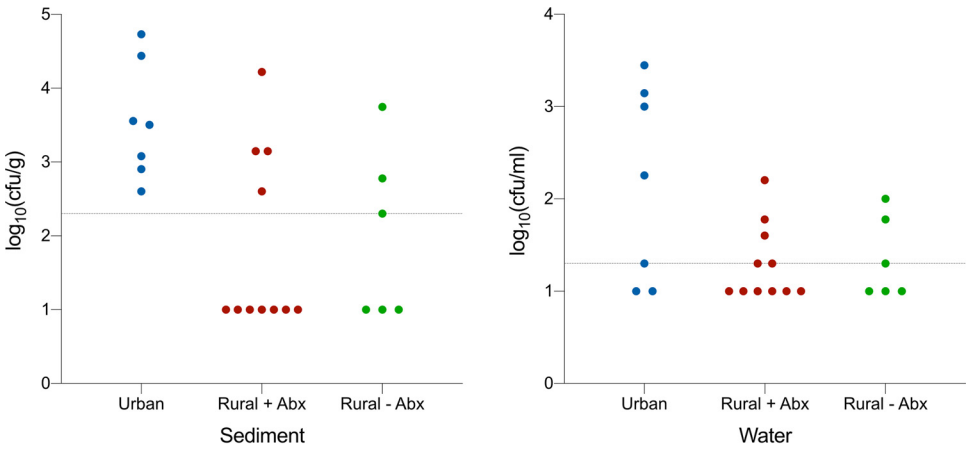

FIG 2 The abundance of ESB- producing coliforms isolated from sediment $\log_{10}$(CFU/g) and surface water $\log_{10}$(CFU/ml) in urban sites and rural settings with antibiotic use (+Abx) and without antibiotic use (−Abx) across Bangladesh. The horizontal dashed lines represent the detection limit of 200 CFU/g for sediment and 20 CFU/ml for water. Samples with ESBL-producing coliforms below the detection limit were plotted at $\log_{10}$(CFU/ml) of 1.

dominated by the phylum *Proteobacteria* (73.8%; standard deviation [SD], 27.1), while in the water samples, *Cyanobacteria* (60.9%; SD, 29.6) was the dominant phylum (Fig. 3B). However, among the different sample types (water and sediment), these phyla were not inevitably dominant, as in five of the nine sediment samples collected in Mymensingh, the abundance of *Euryarchaeota* was greater than 50%, while in five Dhaka water samples, *Proteobacteria* were present at levels greater than 45%. Water sample WAM6 had very high levels (>60%) of bacteriophage DNA. The sediment samples were dominated by typical soil bacteria such as *Pseudomonas*, *Azoarcus*, and *Anaeromyxobacter*, while the water samples were dominated by cyanobacteria such as *Cyanobium*, *Microcystis*, and other typical aquatic bacterial species from the phyla *Proteobacteria* and *Actinobacteria* (Fig. S1). Three bacteriophages (*Mycobacterium* phage Rizal, *Microcystis aeruginosa* phage Ma LMM01, and an Epsilon15-like virus) were also identified at different sampling sites. It was apparent that three of the Dhaka water samples contained bacteria which are typically found within the gastrointestinal tract, including *Escherichia coli*, *Streptococcus infantarius*, *Bifidobacterium adolescentis*, and *Prevotella copri*. Through microbial source-tracking analysis of our shotgun sequencing data using the FEAST algorithm (27), we found that the urban water samples had a significantly greater contribution from gut bacteria (Kruskal-Wallis, $P < 0.01$) than the rural samples without previous antibiotic use (Fig. 3C).

Based on species-level data (Fig. S1), the urban sediment samples were significantly more diverse than both the rural samples with and without previous antibiotic use (Browne-Forsythe and Welch, $P < 0.05$). There was no significant difference in diversity between either of the rural sediment sample types (Fig. 3D). On the other hand, the rural water samples without previous antibiotic use were significantly more diverse than the rural samples with previous antibiotic use (Browne-Forsythe and Welch, $P < 0.005$), but there was no significant difference between the urban water samples and either of the rural sample types.

Urban samples carry the highest antibiotic resistance gene loads. A total of 114 different antibiotic resistance genes (ARGs) that confer resistance to 16 antibiotic classes were identified in the 48 samples from sediment and surface water. The urban samples had the greatest number of ARGs ($n = 99$) followed by the rural samples with previous antibiotic use ($n = 49$), while the rural samples with no previous antibiotic use had the fewest resistance genes ($n = 36$) (Fig. 4). There was a large overlap between the ARGs present in the different sample types, with the urban and rural positive antibiotic samples sharing the greatest number of resistance genes ($n = 24$). There were 17

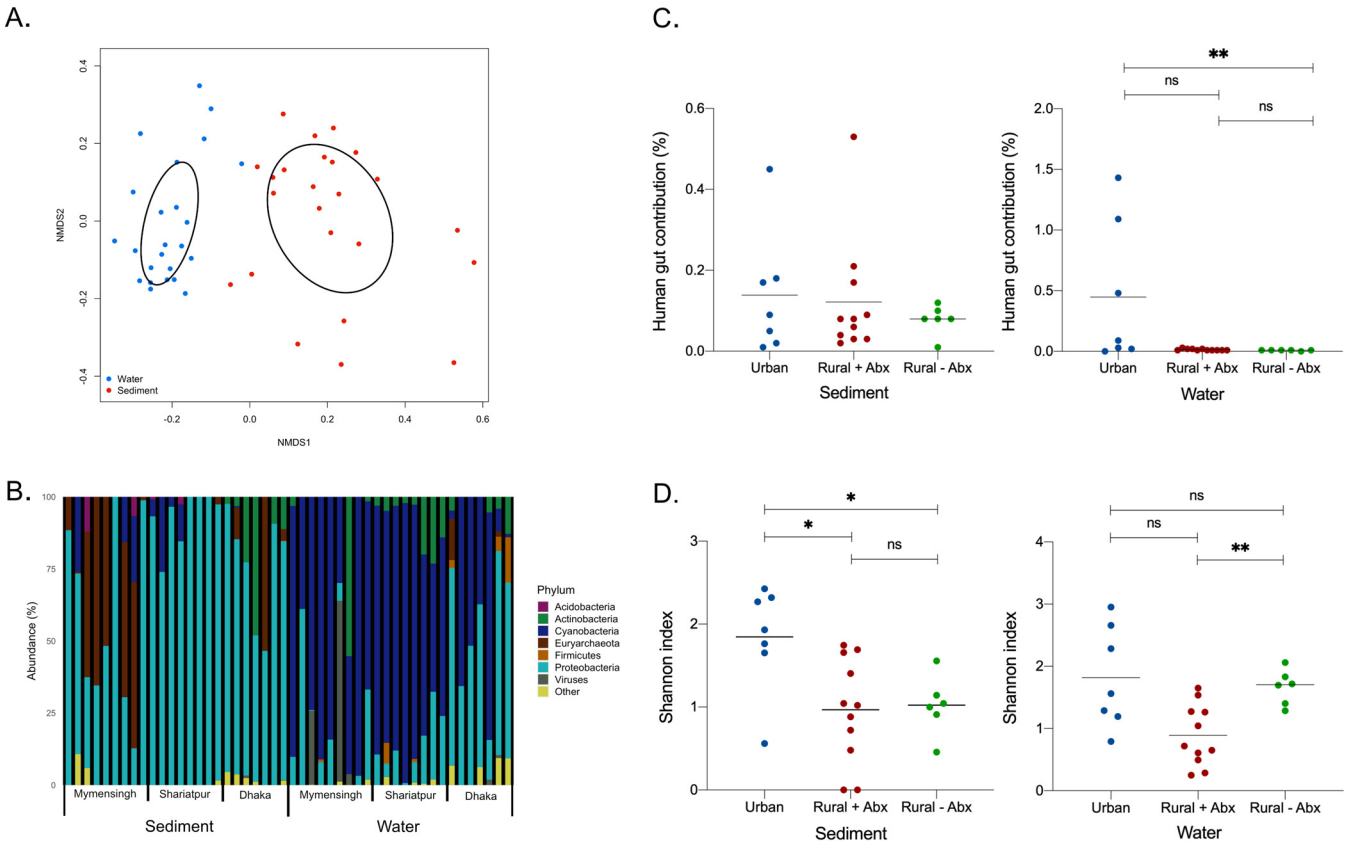

FIG 3 (A) Nonmetric multidimensional scaling (NMDS) analysis of a Bray-Curtis distance matrix of species abundance. Stress, 0.15. Ellipses represent standard deviation. (B) Relative abundance (%) of phyla across the 48 samples from sediment and surface water. (C) Source-sink analysis, percentage contribution of human gut bacteria to the bacterial composition of the water and sediments samples. Kruskal-Wallis test; **, $P < 0.01$. (D) Shannon diversity values of species present in sediment and water samples from across Bangladesh. Brown-Forsythe ANOVA. *, $P < 0.05$; **, $P < 0.005$; ns, not significant ($P > 0.05$).

ARGs shared between all three sample types, including five different beta-lactamase genes belonging to the bla_{OXA} and bla_{RSA} families.

The abundance of antibiotic resistance genes varied 1,525-fold between sites, with sample SAM6 (rural sediment sample with previous antibiotic exposure collected in Mymensingh) having the lowest abundance (0.078 reads per kilobase of reference sequence per million sample reads [RPKM]) and sample WD7 (surface water sample collected in Dhaka) having the highest ARG abundance (120.45 RPKM). Of the paired sediment and water samples, the ARG abundance was, on average, 3 times greater in the water samples than the sediment samples (Wilcoxon, $P < 0.0001$). The urban sediment samples collected from around the city of Dhaka were found to have a significantly greater (Kruskal-Wallis, $P < 0.05$) total ARG abundance (median RPKM, 4.01; interquartile range [IQR], 0.95 to 12.79) than the rural samples with prior antibiotic use (median RPKM, 0.60; IQR, 0.20 to 1.27) (Fig. 5A). However, the urban sediment samples were not significantly different from the rural samples without antibiotic use (median RPKM, 0.72; IQR, 0.64 to 1.36). There was also no statistically significant difference (Kruskal-Wallis, $P > 0.99$) between ARG abundance in rural sediment with prior antibiotic use versus sediment from rural sites in which antibiotics had not been used. ARG levels in the water samples reflected that of the sediment samples, with the total ARG abundance in urban samples (median RPKM, 37.08; IQR, 5.71 to 97.74) being significantly higher (Kruskal-Wallis, $P < 0.05$) than the rural samples with previous antibiotic use (median RPKM, 4.30; IQR, 2.39 to 7.60) but not significantly different from the rural samples with no previous antibiotic use (median RPKM, 5.09; IQR, 1.80 to 11.68). As with the sediment samples, there was no significant difference found between either of the rural sample types (Kruskal-Wallis, $P > 0.99$).

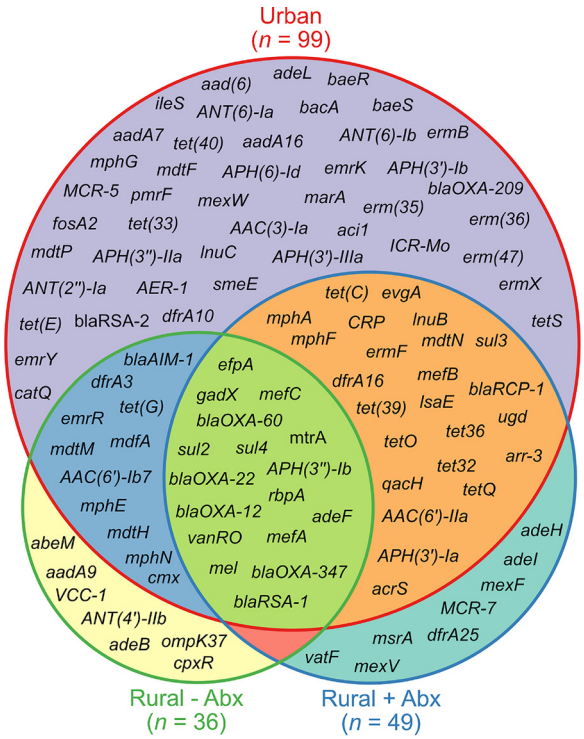

FIG 4 Distribution of antibiotic resistance genes across urban, rural without prior antibiotic use, and rural with prior antibiotic use sample types. Circles are proportional to the number of antibiotic resistance genes present within each sample type.

The individual antibiotic resistance genes were collated into 16 classes that cover resistance to specific antibiotics and a separate class for genes conferring antibiotic efflux mechanisms (Fig. 5B). Efflux genes were present in 47 of 48 samples, making it the most widespread ARG class. Other abundant antibiotic resistance classes were resistant to sulfonamides, macrolides, and aminoglycosides. Urban water samples WD2, WD6, WD7, and WD1 and an urban sediment sample SD7 clustered together, with high levels of resistance genes from these classes.

Abundance of human gut bacteria predicts levels of antibiotic resistance genes. There was a statistically significant correlation ($R^2 = 0.73$; $P = 8.9 \times 10^{-15}$) between the aggregated abundance of ARGs and the levels of human gut bacteria, as identified by FEAST, across our study (Fig. 6A). We also determined whether the levels of ESBL-producing coliforms are correlated with the total abundance of ARGs and observed a relatively weak but statistically significant correlation ($R^2 = 0.12$; $P = 0.009$) (Fig. 6B).

Urban sites were enriched in plasmids carrying antibiotic resistance genes. As antibiotic resistance genes were particularly abundant in water samples, we performed a metagenomic assembly of the short-read data from the surface water samples to recover complete plasmid sequences and study their potential association with antibiotic resistance. The metagenomic assemblies were queried against the PlasmidFinder database (28) to identify contigs which contained plasmid replication (*rep*) genes. Eleven contigs in our data set contained *rep* genes (Table S2). Seven Gram-negative replicons were found, which were related to representatives of the P and Q incompatibility groups or to small theta- or rolling circle-replicating plasmids. A single Gram-positive replicon, repUS43, was identified in sample WD1. Two plasmid contigs, k141_206349 (2,113 bp) and k141_304072 (8,535 bp), could be circularized. The latter plasmid, which we named pWD1, contained the sulfonamide resistance gene *sul2*. pWD1 was found to have 99.97% identity over 81% of its sequence to the canonical broad-host-range mobilizable plasmid RSF1010 (29) (Fig. S2).

As metagenomic assemblies are often fragmented and plasmid replication genes may not be on the same contigs as ARGs that are carried on another region of the

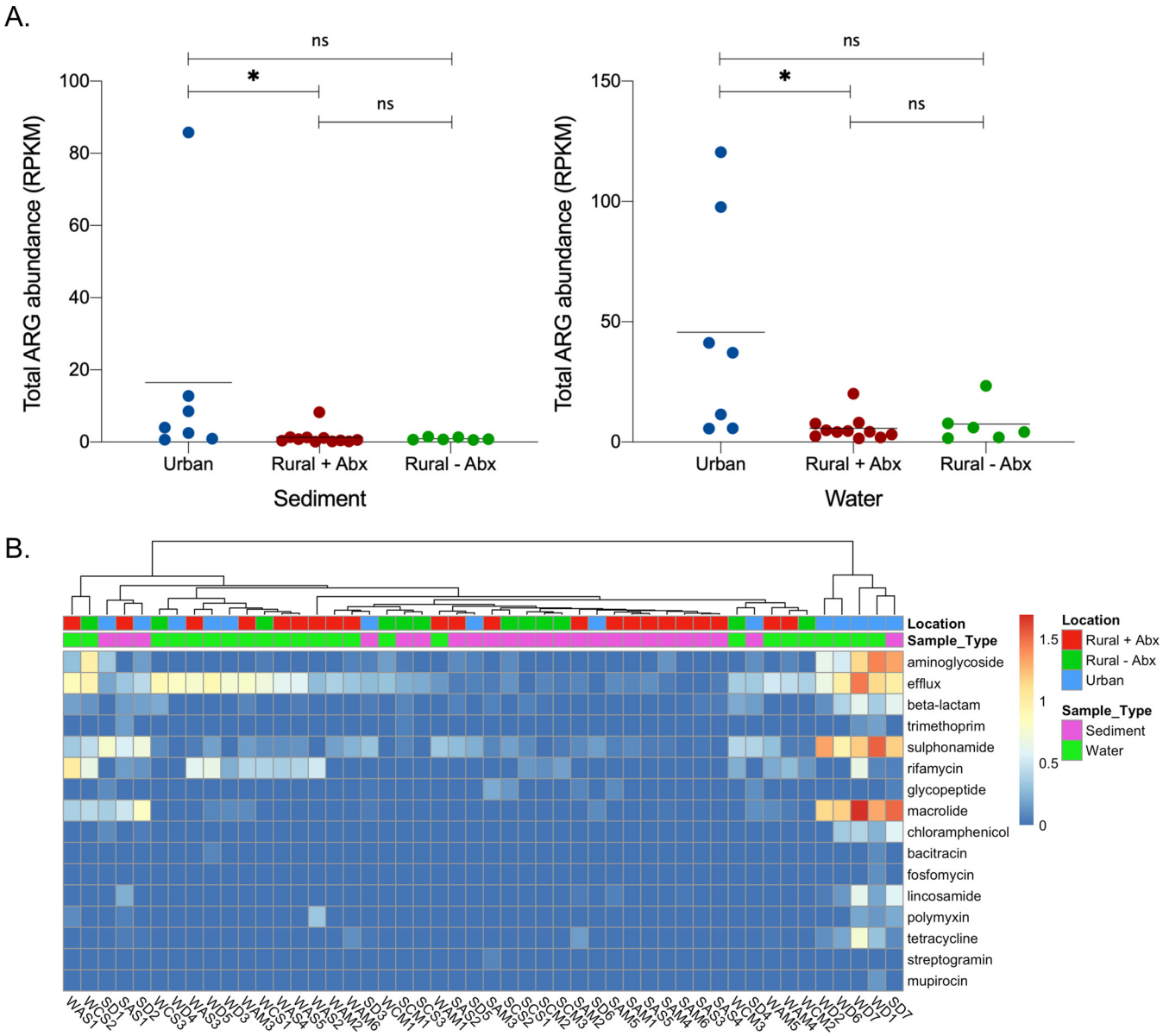

FIG 5 (A) Abundance in reads per kilobase of reference sequence per million sample reads (RPKM) of antibiotic resistance genes (ARGs) in each sample (sediments and surface water; urban, rural with antibiotic use, and rural without antibiotic use). Kruskal-Wallis test, *, $P < 0.05$. (B) Heatmap representing the summed abundance ($\log_{10}$-transformed RPKM) of antibiotic resistance gene classes present in water and sediment samples from surface water sites across Bangladesh.

plasmid, we employed PlasFlow (30) to classify contigs in our metagenomic assembly as either chromosomal or plasmid. We identified a total of 93 plasmid contigs containing ARGs. The urban sediment samples contained significantly more plasmid contigs with ARGs than either of the rural sample types (Kruskal-Wallis, $P < 0.001$), whereas the urban water samples had significantly more ARG-bearing plasmid contigs than the rural samples with no previous antibiotic use (Kruskal-Wallis, $P < 0.05$) (Fig. 7). There was no significant difference in the number of ARG-containing plasmid contigs between rural samples with and without prior antibiotic use. Of the 93 contigs identified which contained ARGs, 78 contigs contained only 1 resistance gene, with the remaining 15 contigs containing 2 or more ARGs (Table S3). All of the contigs that contained multiple resistance genes were found in urban samples and were closely related to known proteobacterial plasmids.

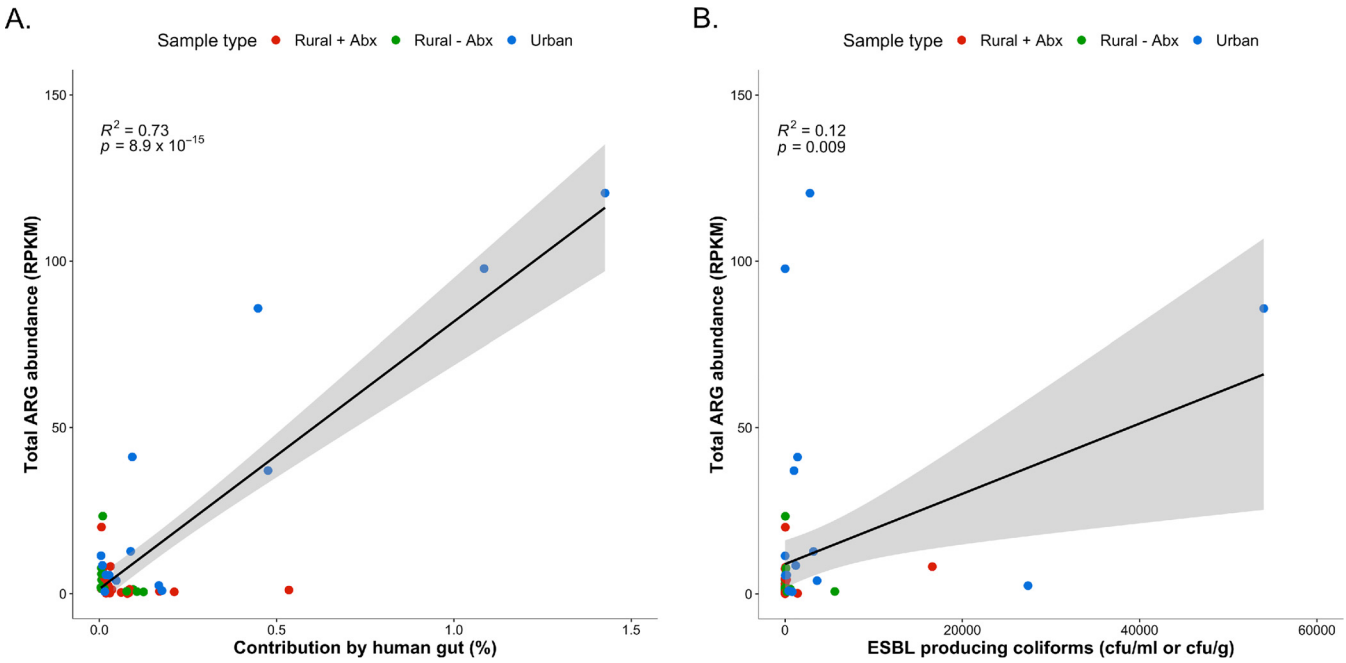

FIG 6 (A) Correlation between the total antibiotic resistance gene (ARG) abundance (reads per kilobase of reference sequence per million sample reads [RPKM]) and the percentage of bacteria contributed from the human gut within each sample. $R^2 = 0.73$; $P = 8.9 \times 10^{-15}$. (B) Correlation between the total ARG abundance (RPKM) and the number of ESBL-producing coliforms (CFU/ml) in each sample. $R^2 = 0.12$; $P = 0.009$. Gray area represents the 95% confidence interval.

DISCUSSION

In this study, we used quantitative culture and metagenomic techniques to understand the community composition and the level of antibiotic resistance genes in rural and urban surface water sites across Bangladesh. Selective plating showed that ESBL-producing coliforms were more prevalent in urban surface water than rural settings, consistent with reports of antibiotic-resistant fecal coliforms in rivers across Asia (31, 32). However, the predictive value of the abundance of ESBL-producing coliforms for the total abundance of antibiotic resistance genes was found to be limited, suggesting that ESBL-producing coliforms are not necessarily a valid proxy to determine the total load of antibiotic resistance genes in environmental ecosystems.

In addition to quantitative culture of ESBL-producing coliforms, a metagenomic shotgun sequencing approach was used to characterize the microbiota of each sample

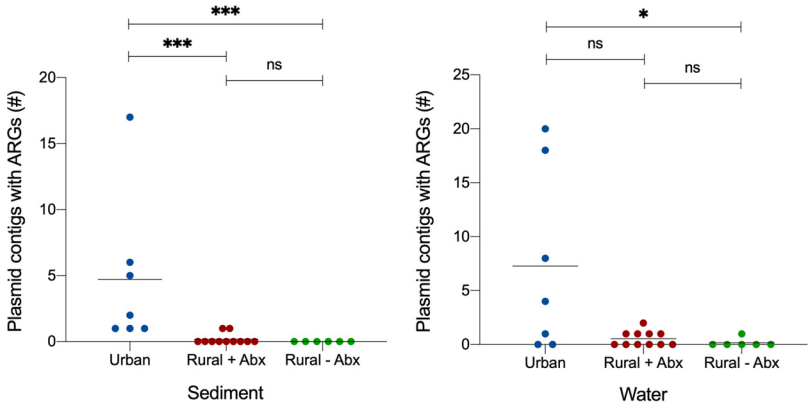

FIG 7 Number of plasmid contigs, as identified by Plasfow, carrying antibiotic resistance genes in urban and rural (with and without antibiotic use [Abx]) surface water and sediment samples. Kruskal-Wallis test; *, $P < 0.05$; ***, $P < 0.001$.

and quantify the abundance of antibiotic resistance genes in water and sediment samples. We found that the water and sediment samples grouped together by their type (water or sediment) rather than the location they were collected from. Sediment samples were dominated by bacteria belonging to the genera *Pseudomonas*, *Azoarcus*, and *Hydrogenophilacea*, which is in line with other studies which have shown that sediment is dominated by the phylum *Proteobacteria* (33). Water samples were dominated by the cyanobacteria *Cyanobium* and *Microcystis* that cause harmful blooms in aquaculture ponds (34). *Microcystis* produces potent toxins which can kill fish but are also harmful to humans (35). The two river water samples and a public pond water sample collected in Dhaka clustered away from the other water samples and were defined by an increased abundance of bacteria associated with the human intestinal tract (Fig. S1 in the supplemental material). The presence of increased amounts of the fecal indicator bacteria *E. coli* strongly suggests that human waste is contaminating the urban surface water (36).

Several different types of antibiotics were used in the rural aquaculture ponds which we surveyed (Table S1). The antibiotics were either mixed with feed or added directly to the ponds for the treatment of disease. Fluoroquinolone antibiotics such as ciprofloxacin and levofloxacin were the most widely used antibiotics in the rural aquaculture ponds; however, high levels of fluoroquinolone resistance were not observed in the rural sites with prior antibiotic use. Resistance to fluoroquinolone drugs is mainly mediated by chromosomal mutations in the *parC* and *gyrA* genes, so the absence of dedicated resistance genes in these ecosystems may be unsurprising (37). However, we note that the multidrug efflux pump genes *mexV*, *mexF*, *adeI*, and *adeH* were exclusively found in the rural sites with prior antibiotic use, and these efflux systems are capable of exporting fluoroquinolones from the cell (38–41). In addition, other multidrug efflux pump genes capable of exporting fluoroquinolones, such as *evgA* and *qacH*, were found in these sites and urban samples (42, 43). The macrolide drug erythromycin was another antibiotic which was widely used in aquaculture ponds that were sampled in this study. However, levels of macrolide resistance genes were low in the rural aquaculture ponds but extremely high in a subset of the urban samples. Notably, the erythromycin resistance gene *msrA* (44) was only present in the aquaculture ponds with prior antibiotic use. This gene was previously found in the intestinal contents of farmed rainbow trout and may thus be more commonly associated with aquaculture (45). We did not observe a difference in the total load of antibiotic resistance genes in rural ponds with and without a history of antibiotic use, suggesting that other factors than solely the historical use of antibiotics in fish farming are more important in shaping the resistome of the rural ponds. We postulate that heavy metals in the environment could be responsible for this observation. Previous work in Bangladesh has shown high levels of heavy metals in surface water, sediments, and fish (46–48), and heavy metals can coselect for antibiotic resistance in aquaculture ponds (49). Further research is needed to quantify the impact of different fish farming practices and environmental variables on the selection for antibiotic resistance in aquaculture.

Antibiotic resistance was the highest in urban areas, which suggests that human factors contribute to the accumulation of antibiotic-resistant bacteria in the environment. This was further corroborated by the correlation between the abundance of bacteria originating from the human gut and antibiotic resistance gene abundance observed in our study. The rivers and lakes of Dhaka are surrounded by slums with high population densities in which 13.7% of households report that human waste is directly released into lakes, ponds, or rivers (50). Our study thus extends on previous observations that link the introduction of human sewage into river and lake systems to high levels of antibiotic resistance genes (51).

By creating a metagenomic assembly of our short-read sequencing data, we were able to identify contigs which contained plasmid replication initiation genes and found that IncP, IncQ, and various small plasmid types were most common. Only the IncQ1

plasmid pWD1 contained an antibiotic resistance gene (*sul2*). Using PlasFlow on the metagenomic assemblies, we were able to assign antibiotic resistance genes to 93 plasmid contigs, revealing that urban samples had a higher number of plasmids carrying antibiotic resistance genes. This suggests that, particularly in urban water bodies, there exists an increased potential of horizontal gene transfer of mobile genetic elements carrying antibiotic resistance genes.

The microbiotas of surface water and sediment samples across Bangladesh are diverse, but antibiotic resistance genes are highly abundant in urban samples and are more commonly associated with plasmids in this setting. While the abundance of antibiotic resistance genes was considerably lower in rural than in urban settings, we nonetheless observed evidence for the selection for fluoroquinolone resistance mechanisms in ponds used for fish farming. Policies to minimize the use of antibiotics in aquaculture should thus remain a priority to reduce selection for antibiotic resistance. The presence of human gut bacteria was associated with high levels of antibiotic resistance genes, suggesting that contamination by human waste is an important driver for the presence of antibiotic resistance genes in surface water. Interventions aimed at improving access to clean water, sanitation, and sewerage infrastructure may thus be important to reduce the risk of AMR dissemination in Bangladesh and other low- and middle-income countries.

MATERIALS AND METHODS

Site selection. Paired surface water and sediment samples were collected in Bangladesh from 24 freshwater sites across three districts (Mymensingh, Shariatpur, and Dhaka; Fig. 1) in May and June of 2018. These sites spanned both rural and urban areas with different population densities. Samples were collected from 11 aquaculture ponds in the rural areas of 2 districts (Mymensingh and Shariatpur) with high commercial aquaculture activity. These ponds all had a history of antibiotic use within the past 3 months of collection. Six ponds with no history of antibiotic use were also sampled from these rural areas. In Mymensingh, 3 ponds used for domestic purposes were selected, while in Shariatpur, these were aquaculture ponds with no prior antibiotic use, which were used for culturing fingerlings. Antibiotic use information for the ponds was collected from local dealers who were responsible for supplying fish feed for these ponds. In addition to rural surface water sites, 7 water bodies (rivers, lakes, and public ponds) were sampled in Dhaka. The public ponds were heavily used for domestic purposes, and, while some had history of casual (noncommercial) fish cultivation, none of them had any prior antibiotic use.

Sample collection. Samples were named using the following scheme: water (W) or sediment (S) followed by aquaculture (A) or control (C; ponds without antibiotic use). Sample sites were designated using Mymensingh (M), Shariatpur (S), or Dhaka (D), and a number was included to differentiate samples. Further metadata on the samples, including temperature, pH, and dissolved oxygen levels, are provided in Table S1 in the supplemental material. Water samples were collected by submerging a sterile 500-ml Nalgene plastic bottle approximately 15 cm below the water's surface. Bottles were capped before being removed from the water. The water samples were filtered through a 0.22-μm Sterivex-GP filter (Millipore) until the water would no longer be passed through the filter. The filter units were then capped and stored in a cool box and transported to the laboratory within 12 h of sampling. In addition to the water samples, approximately 10 g of sediment was taken from either the bed of the pond or from the bank 30 to 50 cm below the surface of the water. The sediment samples were stored in sterile 50-ml Falcon tubes and transported with the water samples.

Selective culturing for coliforms in surface water and sediment samples. Water and sediment samples were screened for the presence of ESBL-producing coliforms by quantitative plating on Brilliance ESBL agar (Oxoid). For sediment, 0.1 g of the sample was mixed with 0.9 ml of sterile saline solution, and 50 μl was spread onto the agar plates. For water samples, 50 μl of the undiluted sample was spread onto the media. Plates were incubated for 48 h at 37°C. In accordance with the manufacturer's instructions, blue, pink, and green colonies were designated coliforms and counted.

DNA extraction and Illumina sequencing. DNA was extracted from the Sterivex filters and sediment samples using the DNeasy PowerWater kit (Qiagen) and the DNeasy PowerSoil kit (Qiagen), respectively, in accordance with the manufacturer's instructions. DNA concentrations were quantified using the Qubit dsDNA HS assay kit (Thermo Fisher), with all samples yielding more than 0.2 ng/μl. Negative-control runs were performed for both kits by isolating DNA from sterile, distilled water: these yielded no detectable DNA. Metagenomic DNA libraries were prepared using the Nextera XT library prep kit (Illumina). The libraries were pooled and sequenced on the HiSeq 2500 sequencing platform (Illumina) using a 150-bp paired-end protocol. Paired reads were adapter trimmed, and both duplicates and reads less than 50 bp were removed using Trimmomatic 0.30 with Q15 as the sliding window quality cutoff (52).

Taxonomic profiling. To perform taxonomic profiling, the paired-end sequencing reads were mapped against clade-specific markers using the MetaPhlAn2 package v.2.7.7 (53). The MetaPhlAn2 package was run with default parameters. The utility script merge_metaphlan_tables.py was used to merge all of the output files into a single tab-delimited file.

Source-sink analysis. Raw sequence reads from BioProject accession nos. PRJNA254927, PRJEB7626, and PRJEB6092, which had previously been used as sources for source-sink analysis (54), were downloaded from the European Nucleotide Archive (ENA). These sequences represented freshwater, soil, and gut metagenomes, respectively. Adapters were removed from the sequence reads using fastp (55). Taxonomic counts were created for these metagenomic sequences and the 48 samples in this study by kraken2 v.2.0.9 (56) and Bracken v.2.6.0 (57) using a database containing bacterial, archaeal, viral, and fungal sequences. A metadata table was created which described the environment that the sample was from and designated it as either a source or a sink. The taxonomic count table and the metadata table were used as inputs to the R package FEAST v.0.1.0 (27), which determined the proportion that each source contributed to each sink.

Resistome profiling. Antibiotic resistance genes were identified using the ShortBRED package v.0.9.5 (58). The CARD database (59) (downloaded 1 July 2019) and the UniRef90 database (downloaded 4 July 2019) were used by ShortBRED-Identify to construct a marker database which the metagenomic reads could be mapped against. ShortBRED-Quantify.py was then used to map these paired-end reads against the database (cutoffs, ≥95% identity and ≥95% coverage). The relative abundance in reads per kilobase of reference sequence per million sample reads (RPKM) was generated for each resistance gene family in the database. The RPKMs were summed for antibiotic resistance genes belonging to the same class and visualized with the pheatmap package (https://cran.r-project.org/web/packages/pheatmap/pheatmap.pdf) in R (60).

Reconstruction of plasmids from metagenomic data sets. Metagenomic sequencing reads were assembled using the MEGAHIT v.1.1.3 assembler using default parameters (61). Contigs produced by MEGAHIT were then classified as plasmid or chromosomal by trained neural networks in the PlasFlow v1.1 program (30). Contigs designated to be of plasmid origin were queried against the CARD database by ABRicate v.0.9.8 (https://github.com/tseemann/abricate) to identify the presence of antibiotic resistance genes. Resistance genes were identified which had at least 95% identity and 50% coverage compared to the CARD database. Plasmid contigs were similarly queried against the PlasmidFinder database (28) to identify replication genes. Plasmids were circularized by comparing 300 bp from either end of putative plasmid-containing contigs using BLASTn (62). When ends were found to overlap, one copy of the overlapping sequence was removed to generate a complete, circularized plasmid sequence. To avoid spurious overlaps, overlapping regions were manually inspected to ensure that they were not part of translocatable elements.

Statistical analyses. The Shannon diversity index of the samples was calculated in R v.3.4.3 using the diversity function of the vegan package v.2.5-7 (63). Nonmetric multidimensional scaling (NMDS) was also performed in R using the metaNMDS function of the vegan package. Permutational multivariate analysis of variance (PERMANOVA) was performed on a Bray-Curtis distance matrix of species abundance in R using the adonis function of the vegan package. Correlation between total ARG abundance and human gut bacterial contribution was calculated using the lm function in base R. Additional tests for determining statistical significance were performed as described in the text and implemented in GraphPad Prism v.8.3.1.

Data availability. The short-read raw sequencing data have been submitted to the European Nucleotide Archive with accession number PRJEB39306.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, PDF file, 0.9 MB.

FIG S2, JPG file, 0.04 MB.

TABLE S1, PDF file, 0.3 MB.

TABLE S2, PDF file, 0.3 MB.

TABLE S3, PDF file, 0.1 MB.

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W.V.S. and M.S.I. conceived this study. R.S.M., M.H.Z., I.T.A., M.S.I., and W.V.S. collected and processed the environmental samples with support from J.D.C. R.S.M., S.F.S.H., and R.A.M. analyzed the data. R.S.M. and W.V.S. wrote the manuscript with input from all authors.

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