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Trends in Antibiotic Resistance Genes Occurrence in the Haihe River, China

YI LUO,[†] DAQING MAO,[‡] MICHAL RYSZ,[§] QIXING ZHOU,[†] HONGJIE ZHANG,[‡] LIN XU,[†] AND PEDRO J. J. ALVAREZ*,[||]

Tianjin Key Laboratory of Environmental Remediation and Pollution Control, College of Environmental Sciences and Engineering, Nankai University, Tianjin 300071, China, School of Life Science and Bio-pharmaceutics, Shenyang Pharmaceutical University, Shenyang 110016, China, GSI Environmental Inc., 2211 Norfolk, Suite 1000, Houston, Texas 77098, and Department of Civil and Environmental Engineering, Rice University, MS 519, P.O. Box 1892, Houston, Texas 77251-1892

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The occurrence of antibiotics and antibiotic resistance genes (ARGs) was quantified in water and sediment samples collected from a 72 km stretch of the Haihe River, China. Tetracycline resistance genes (*tetW*, *tetQ*, *tetO*, *tetT*, *tetM*, *tetB*, and *tetS*) were not detected by quantitative PCR in many samples. In contrast, *sul1* and *sul2* (coding for sulfonamide resistance) were present at relatively high concentrations in all (38) samples. The highest ARG concentrations detected were $(7.8 \pm 1.0) \times 10^9$ copies/g for *sul1* and $(1.7 \pm 0.2) \times 10^{11}$ copies/g for *sul2,* in sediment samples collected during the summer. The corresponding total bacterial concentration (quantified with a universal 16S-rDNA probe) was $(3.3 \pm 0.4) \times 10^{12}$ cells/g. *Sul1* and *sul2* concentrations in sediments were 120−2000 times higher than that in water, indicating that sediments are an important ARG reservoir in the Haihe River. Statistical analysis indicated a positive correlation between the relative abundance of these ARGs (i.e., *sul1*/16S-rDNA and *sul2*/16S-rDNA) and the total concentration of sulfamethoxazole, sulfadiazine, plus sulfachlororyridazine, suggesting that sulfonamides exerted selective pressure for these ARGs. A class 1 integron was implicated in the propagation of *sul1*. Overall, the widespread distribution of sulfonamide ARGs underscores the need to better understand and mitigate their propagation in the environment and the associated risks to public health.

Introduction

Antibiotics are widely used and often abused in human medicine and stockbreeding operations, for both infectious disease therapy and growth promotion. Common veterinary and aquaculture antibiotics include tylosin, β-lactams, tetracycline, and sulfonamides. Globally an estimated 172 000 tons of antibiotics were produced in 2006, with approximately 68 000 tons utilized in animal husbandry applications (*1*). In China, which leads the world in antibiotic use, about 210 000 tons of antibiotics are produced every year, with 180 000 tons utilized in agriculture and medicine (*2*).

A significant fraction of the antibiotics fed to animals (25−75%) are excreted unaltered in feces and persist in soil after land application (*3*−*5*). These antibiotics may exert selective pressure for resistant bacteria (*6, 7*), which is a major public health concern due to the increased occurrence of associated clinical infections. Furthermore, antibiotics discarded to sewage are generally not efficiently removed in wastewater treatment plants (WWTPs) (*8, 9*), and residual antibiotics and resistant bacteria in WWTP effluents are released to the environment (*10, 11*). Feces from confined animal feeding operations (CAFOs), which are often used as fertilizer, can also be potential sources of environmental contamination by antibiotics and the associated antibiotic resistance genes (ARGs). Thus, there is a need to quantify the environmental occurrence of ARGs and understand the associated propagation and attenuation mechanisms.

Tetracycline resistance genes including *tetA*, *tetC*, *tetM*, *tetG*, *tetE*, *tetW*, *tetO*, *tetQ*, *tetB*/*P*, *tetS*, *tetT*, and *tetL*, *tetH*, *tetZ*, and four sulfonamides resistance gene types including *sul1*, *sul2*, *sul3*, and *sulA* have been detected in river and marine sediments, irrigation ditches, dairy lagoons, and WWTP effluents (*11*−*14*). The occurrence of these ARGs has been linked to the selective pressure exerted by residues of the corresponding antibiotics (*13, 15*). For example, tetracycline resistance in soil bacteria propagates following exposure to tetracycline, and attenuates with the subsequent interruption of exposure (*16*). Even after bacteria carrying ARGs die, the DNA can be released to the environment and transformed to other bacteria in the ecosystem (*17, 18*). Free DNA (including ARGs) generally persists longer in sediment than in water because soil and clay components adsorb DNases that would otherwise hydrolyze the free DNA. Thus, ARGs may persist in the environment even after the selective pressure has been removed (*19*). Recent historical ARG analysis has shown an overall increase in soil antibiotic resistance genes over the last 70 years (*20*).

Among the different classes of antibiotics, sulfonamides (SAs) deserve special attention due to their widespread use, high excretion rate, high solubility, and persistence in the environment (*21*). SAs are often detected at high concentrations in animal manure (*22*), and previous studies show significant potential for SAs drainage to water bodies (*23, 24*). Whether the relatively high mobility of SAs contributes selective pressure for associated ARGs in different environmental compartments has not been addressed in the literature.

Previous studies in China reported the presence of ARGs in inland waters (*25*), coastal marine waters, and aquaculture ponds (*26*−*28*). However, to date no comprehensive regional field study has concurrently characterized the concentrations of antibiotic residues, the levels of the associated ARGs, the partitioning of these ARGs between environmental compartments, and the presence of other genetic elements such as integrons that may facilitate ARG propagation.

The Haihe River, the largest water system in Northern China, flows through an agricultural area before discharging into the Bohai Sea. It has a drainage area of 265 000 km^2, including 120 000 km^2 of farmland and CAFOs along its banks. Tetracyclines and sulfonamides were previously detected in the river in animal feces in this region (*22*). The flow in the Haihe River is limited during the winter by closing of sluice

* Corresponding author phone: (713) 348-5903; e-mail: alvarez@rice.edu.
[†] Nankai University.
[‡] Shenyang Pharmaceutical University.
[§] GSI Environmental Inc.
[||] Rice University.

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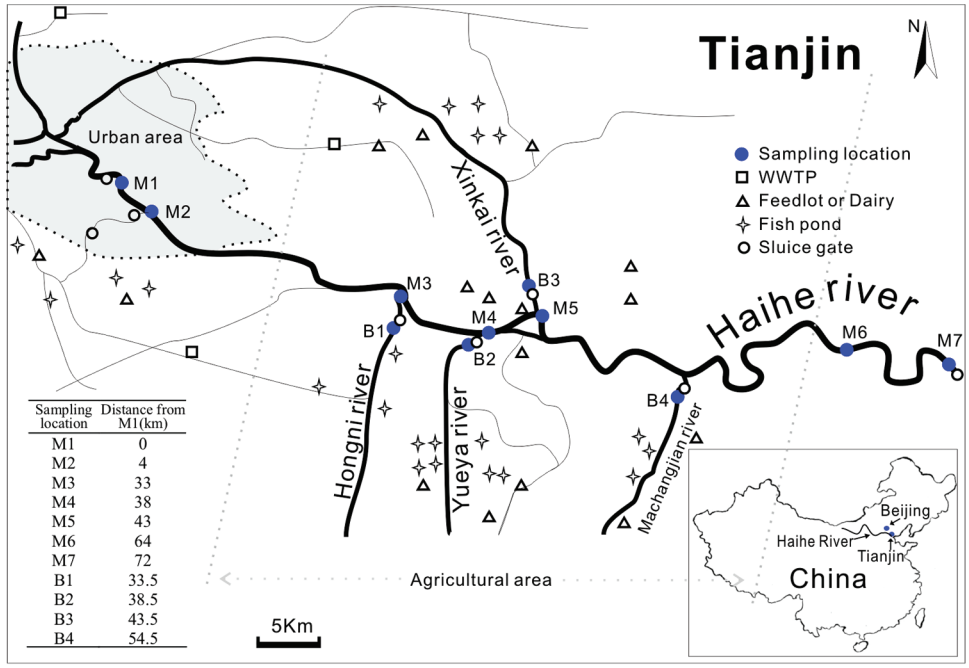

FIGURE 1. Sampling Locations.

gates which creates low-flow conditions. High-flow conditions are imparted in the summer months by the reopening of the gates. These alternating flow conditions allow for the investigation of seasonal and hydrologic effects on the establishment and maintenance dynamics of ARGs in aquatic environments.

The objective of this study was to advance knowledge of the factors that contribute to ARG maintenance and propagation in the environment, by (1) characterizing the occurrence of tetracycline and sulfonamide residues and the associated ARGs in the Haihe River and its tributaries, (2) investigating ARG relative abundance in water versus sediment samples, and exploring correlations between ARG, integrons and antibiotic concentrations. To our knowledge this is the first regional study to report the presence of *sul1* and *sul2* genes as the predominant ARGs in regions where multiple antibiotic residues are present, and to provide direct field evidence of the selective pressure of antibiotic residues on the associated ARGs. The occurrence of class 1 integrons involved in *sul1* gene transfer was also addressed.

Materials and Methods

Sampling Sites. Surface water and sediment samples were collected at numerous sites along the Haihe River and its main tributaries (Figure 1) during the summer (July) and winter (December) of 2008. Both urban and agriculturally influenced regions were sampled within 10−20 km of potential ARG sources, such as WWTPs, agricultural feedlots, dairies, and fish ponds. For samples collected along the Haihe River, Sites M1 and M2 were located in urban areas near a WWTP receiving domestic sewage, pharmaceutical wastewater, and hospital sewage. Sites M3 to M5 were located in agriculturally influenced areas (Jinnan and Dongli District) in the vicinity of fish ponds, feedlots and dairy farms. Furthermore, CAFOs (72 in Dongli plus 105 in Jinnan) and aquaculture industries (40 000 km^2 in Dongli and 26 700 km^2 in Jinnan) are located between sites M3 and M5. Sites M6 and M7 are located at the mouth of the Haihe River, which discharges into the Bohai Sea. Because some animal feedlots and fish ponds drain into the four main tributaries of the Haihe River, four additional locations (B1 to B4) were also sampled. Each of these is located about 500 m from the confluence point.

Sample Collection. Surface water samples (2.5 L) from the top 0.5 m of the water surface were collected from a boat in the cross sectional midpoint of the river. Sediment samples (100 g) were collected from the top 5 cm layer using a core sampler. Three samples were collected from different locations along the river cross section, and were combined prior to analysis. All samples were collected into sterile containers, immediately placed on ice and maintained in the dark for up to 4 h until pretreatment.

HPLC-MS/MS Analysis of Antibiotics. Pretreatment of the surface water and sediment samples and solid phase extraction prior to HPLC-MS/MS analysis is detailed in Supporting Information (SI) sections SI-1 and SI-2. All HPLC-MS/MS analyses were conducted with an Alliance 2695 HPLC (Waters; Manchester, UK) equipped with a Waters Micromass Quattro MicroTM detector with electrospray ionization (ESI). Simultaneous chromatographic separation of 12 antibiotics (i.e., trimethoprim (TMP), sulfadiazine (SD), sulfamethazine (SM2), sulfamethoxazole (SMZ), sulfachlororyridazine (SCP), ciprofloxacin (CIP), enrofloxacin (ENR), ofloxacin (VFX), tetracycline (TC), oxyetracycline (OTC), eythromycin (ERY), and roxithromycin (ROX)), as well as an internal standard (simatone), and a surrogate (trimethyl-$^{13}C_3$ caffeine) was achieved with a 2.1 × 250 mm Intersil ODS-3 column (5 μm particle size, Scienes, JPN). MS conditions and compounds used as standards are described in the SI Table S1. Method validation and extraction recoveries and concentrations of other antibiotics are summarized in SI section SI-3 and Table S5. The limit of quantitation for the tested antibiotics ranged from 1.5 to 8 ng/L for water samples, and from 0.10 to 3.5 ng/g for sediment samples.

Sample Pretreatment, Solid Phase Extraction Optimization, and DNA Extraction. Water samples (0.2 L) were filtered through a 0.45 μm filter using a vacuum filtration apparatus, and the filters placed in extraction tubes provided in the Ultraclean Water DNA Kit (MoBio Laboratories, Inc.). DNA was extracted according to manufacturer's protocol. For sediments, DNA from 1 g of lyophilized samples was extracted with the Soil DNA Isolation Kit (MoBio Laboratories, Inc.) according to manufacturer's protocol. The extracted DNA was further purified using the DNA pure-spin kit (Vigorousbio, Beijing, China) to minimize PCR inhibition. An internal standard (*Escherichia coli* DH5a cloned with the

CESA9 gene, which codes for cellulose synthase A9 in *Arabidopsis thaliana*) was used to determine DNA extraction efficiency, as detailed in SI section SI-5. Extraction yield and quality of the DNA were verified by agarose gel electrophoresis and spectrophotometry (ND1000, Nanodrop). DNA extraction recoveries ranged from 48 to 83% for water samples and from 35 to 67% for sediment samples (SI Table S3), and were used to correct DNA concentrations.

Primer Design. New primers were developed to improve hybridization efficiency and enable the use of the same annealing temperature and concurrent analysis of the multiple genes targeted. Nucleotide sequences encoding sulfonamide resistance genes (*sul1, sul2, sul3, sulA*), tetracycline resistant genes (*tetB, M, O, Q, S, T, W*), integron 1 and integron 2 classes were downloaded from GenBank Database (http://www.ncbi.nlm.nih.gov/), and aligned with the multiple-sequence alignment program CLUSTALX 2.0.11 (*29*). Sequences within clusters were aligned separately and compared with each other to create consensus sequences for the primer design templates using primer premier 5.0 (*30*). PCR product sizes were specified in the range of 100−200 bp for q-PCR suitability. Specificity was verified using the BLAST alignment tool (http://www.ncbi.nlm.nih.gov/blast/). Purified PCR products from DNA extracts were cloned and sequenced to further confirm specificity. The four sets of *sul* primers and seven *tet* primers (from which verifiable target products were obtained) are shown in SI Table S2.

PCR Assays for Detection of Resistance Genes. Qualitative PCR assays were used to assess the presence of sulfonamide and tetracycline resistance genes in the surface water and sediment samples. The PCR mixtures (25 μL total volume) consisted of 2.5 μL of Taq reaction buffer, 0.2 mM dNTPs, 0.2 μM primers, 1.75 units of Taq DNA polymerase (Transtaq HiFi, Transgene, China), and 1 μL of template DNA. PCR conditions are detailed in the SI. Both positive controls (consisting of sequenced PCR amplicons obtained from Haihe River sediments that were cloned into *E. coli* DH5a) and negative controls (DNA extracted from bacteria which did not carry resistance genes) were included in PCR analysis (for details, see SI section S-5). The presence of the class 1 and the class 2 integrons was quantified by qPCR using the primers *int1* and *int2*, respectively (SI Table S2).

Real-Time Quantitative PCR (qPCR). All qPCR assays were performed using a Bio-Rad IQ5 instrument (Bio-Rad Company, U.S.). Calibration standard curves for positive controls were generated as described previously (*12*). Target genes in positive controls (including four *sul* genes, seven *tet* genes, class 1 and class 2 integrons and 16S-rDNA) were obtained from the sediment DNA extracts prior to PCR-amplification and cloning into *E. coli* DH5a (using the pEASY-T1 Simple Cloning Kit, Transgene, China), and were verified through sequencing results. The qPCR reactions were performed in 25 μL reaction mixtures (iQ SYBR Green Supermix, Bio-Rad), including 0.2 μM of each primer, and 1 μL of template DNA. Amplification details are provided in SI section SI-4. Limits of quantification ranged from 35 (for *sul1*) to 50 (for *sul2*) gene copies. The internal standard construction and the DNA extraction recoveries are detailed in SI section SI-5.

Statistical Analysis. The data were analyzed by partial correlation (SAS) to assess the potential selective pressure exerted by various variables on ARG proliferation. Correlations between the concentration of sulfonamides; that is, sulfamethoxazole (SMZ), sulfadiazine (SD) and sulfachloroyridazine (SCP), as well as the sum of SD, SCP, SMZ, versus ARG relative abundance (normalized *sul1*/16S-rDNA and *sul2*/16S-rDNA values) were evaluated. A regression analysis and paired-sample *t* test as well as ANOVA was used to infer about the influence of antibiotic concentrations and season (winter vs summer) on the absolute and relative ARG abundances.

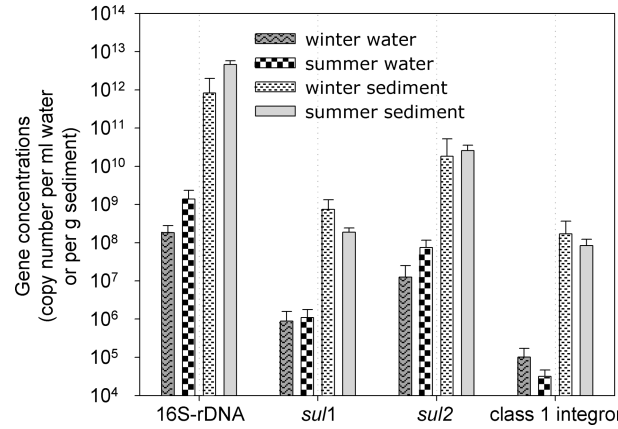

FIGURE 2. Seasonal and spatial variations in bacterial (16S-rDNA genes) and ARG (*sul1, sul2*) concentrations. Error bars represent one standard deviation from the average for all sampling sites ($n = 8$ for sediment, $n = 11$ for water).

Results and Discussion

Quantification of ARGs and 16S-rDNA Genes. Microbial concentrations were significantly higher ($p = 0.0002$) in sediments than in the water column (Figure 2), as indicated by 16S-rDNA analysis. Seasonal variations appear to have influenced 16S-rDNA gene levels in both surface water and sediment samples, with higher concentrations detected in the summer (26 °C average water temperature) than in winter (5 °C). Higher temperatures, nutrient availability and generally lower antibiotic concentrations in the water during the summer (SI Table S3) are conducive to faster microbial growth and consequently higher 16S-rDNA levels.

The sulfonamide ARGs s*ul1* and *sul2* were present in all 38 samples collected (including surface water and sediment), whereas *sul3* and *sulA* were not detected by conventional PCR analysis (Table 1). A recent field study similarly reported low frequency of detection of *sul3* (*31*). Why these genes were not detected despite the widespread presence of sulfonamides (SI Table S3) is unknown, which reflects our limited ecological understanding of ARG maintenance, amplification, and attenuation. Some tetracycline resistance genes (*tetW, tetQ, tetO, tetT, tetM, tetB,* and *tetS*) were detected, although less frequently than *sul1* and *sul2* (Table 1). This differs from recent surveys conducted in Colorado, India, and France (*13, 32, 33*), where *tetW, tetO,* and *sul1* were 100% detectable, whereas *sul2* was found only in a few samples. Such differences in prevalence of specific ARGs may be due to different patterns of antibiotic uses. This may reflect a higher usage and release of sulfonamides, resulting in higher residual concentrations (206 ± 10 μg/kg for sulfadiazine in sediment and 1.28 ± 0.11 μg/L for sulfamethoxazole in water) compared to tetracycline (17.71 ± 0.83 μg/kg for TC in sediment and 0.12 ± 0.01 μg/L for oxytetracycline in water), which may have contributed to the prevalence of *sul1* and *sul2* genes in the Haihe River. Accordingly, the subsequent qPCR analyses were focused on these two genes.

Both total sulfonamides concentrations (SI Table S3) and ARG (*sul1, sul2*) concentrations were approximately 120−2000 times higher in sediments than that in water (Figure 2), indicating that sediments are an important ARG reservoir in the Haihe River. Furthermore, under low-flow conditions (winter season, when the sluice gates are closed), a positive correlation was observed between the concentrations of *sul1* and *sul2* genes in sediments versus surface water (Figure 3). This suggests that a dynamic equilibrium exists between these environmental compartments, and implies that (even under

TABLE 1. PCR Analysis of Various ARGs in Water and Sediment Samples of Haihe River[a]

	M1		M2		M3		M4		M5		M6		M7		B1		B2		B3		B4		
ARG	w[b]	s[b]	w	s	w	s	w	s	w	s	w	s	w	s	w	s	w	s	w	s	w	s	control[c]
sul1	+	+	+	+	+	ns	+	ns	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sul2	+	+	+	+	+	ns	+	ns	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sul3	−	−	−	−	−	ns	−	ns	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
sulA	−	−	−	−	−	ns	−	ns	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
int1	+	+	+	+	+	ns	+	ns	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
int2	−	+	−	+	−	ns	−	ns	−	+	−	+	−	+	−	+	−	+	−	+	−	+	+
tetW	−	+	+	+	+	ns	−	ns	−	+	+	+	+	+	+	+	−	+	−	+	−	+	+
tetQ	−	−	−	−	−	ns	−	ns	−	+	−	−	+	+	−	−	−	+	−	−	−	+	+
tetO	−	−	−	−	−	ns	−	ns	−	+	−	−	−	−	−	+	−	−	−	+	−	+	+
tetT	−	−	+	+	+	ns	−	ns	+	+	−	−	−	−	+	−	−	+	+	+	−	+	+
tetM	−	−	+	−	−	ns	−	ns	−	−	−	+	−	+	−	+	−	−	−	+	−	+	+
tetB	−	−	−	+	−	ns	−	ns	−	+	+	+	+	+	−	−	−	+	−	+	−	−	+
tetS	−	+	−	−	−	ns	−	ns	−	−	−	−	−	−	−	+	−	−	−	−	−	+	+

[a] Samples were collected at the same locations along the Haihe River (M1-M7) and tributaries (B1−B4) in July and December 2008. [b] w-water, s-sediment, ns-not sampled due to lack of access. [c] The positive control was *E .coli* DH5a with cloning vectors and cloned target genes.

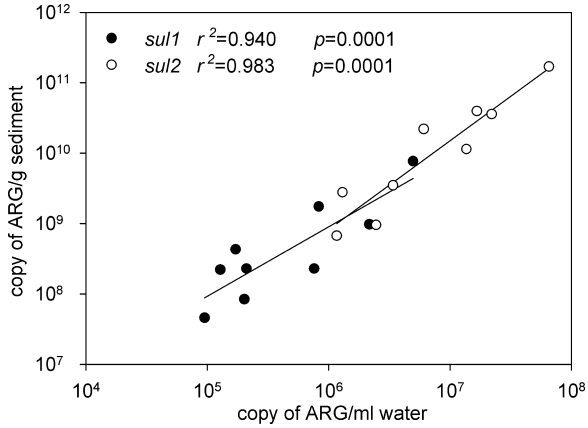

FIGURE 3. ARG concentrations (*sul1* and *sul2*) in surface water versus sediment during the winter.

relatively quiescent conditions) resistant bacteria and ARGs can migrate from sediments to water supplies (and vice versa, as previously reported (*34*)). *Sul1*, *sul2*, and total sulfonamides aqueous concentrations were significantly higher in the tributaries than in the main stream of the Haihe River (Table 2). Apparently, similar to the Poudre River study (*13*), the tributaries (which receive direct runoff from the agricultural operations and irrigation ditches) serve as important reservoirs for ARG maintenance and amplification, and are key sources for direct ARG loading into the main stream.

A Class 1 Integron May Contribute to *Sul1* Propagation. Integron gene sequences contribute to the spread of antimicrobial resistance by facilitating lateral ARG transfer and incorporation into bacterial chromosomes (*35*). Class 1 integrons, which are frequently detected in the environment, generally consist of two conserved segments, including the *int1* gene encoding type 1 integrase enzyme (*36*−*38*). The *sul1* gene is normally found in class 1 integrons, whereas *sul2* is usually located on small nonconjugative plasmids (*39*) or large transmissible multiresistance plasmids (*40*). However, *sul1* and *sul2* can also occur in class 2 integrons (*41*).

Class 1 and class 2 integrons were quantified by qPCR using the primers *int1* and *int2* (SI Table S2). Class 1 integrons were found in all tested samples, and their relative abundance (class 1 integron/16S-rDNA) correlated significantly to the total sulfonamides concentration (Figure 4a). Furthermore, the relative abundances of *sul1* and *int1* in sediments were significantly correlated (Figure 4b). Apparently, the propagation of *sul1* is facilitated by class 1 integrons, which may exhibit enhanced propagation characteristics compared to other mobile genetic elements (*42, 43*). Once sulfonamide resistance is established on such mobile genetic elements, it may be difficult to eliminate (*44*). The relative abundance of class 2 integrons was relatively low ($(3.61 \pm 1.27) \times 10^{-9}$), indicating that this is not likely the main transmissible vector in this system.

Influence of Antibiotic Concentration on *Sul1* and *Sul2* Enrichment. Consistent exposure to sulfonamides appears to have exerted selective pressure for *sul1* and *sul2* resistance genes in sediments, as inferred by a statistically significant increase in the relative abundance of these ARGs (i.e., normalized to 16S-rDNA genes) with increasing total sulfonamide concentrations (Figure 5). This was corroborated by ANOVA (SI Table S5). The relative abundance of these genes ranged from $(2.6 \pm 0.1) \times 10^{-5}$ to $(5.1 \pm 0.4) \times 10^{-2}$ copies per gram in sediment, and from $(3.2 \pm 0.2) \times 10^{-4}$ to

TABLE 2. Higher Aqueous Concentrations of Sulfonamides, *sul1* and *sul2* Genes Were Found in the Tributary (B1−B3) than in the Haihe River (M3-M5) Sampling Locations (Average of Three Replicates ± One Standard Deviation Shown)

	locations compared	*sul1* (copies/mL) in Haihe River/Tributary	*sul2* (copies/mL) in Haihe River/Tributary	total sulfonamide concentration (SD+SMZ+SCP) ug/L
winter	M3/B1	$(1.9 \pm 0.2) \times 10^5/(8.3 \pm 0.8) \times 10^5$	$(5.7 \pm 0.5) \times 10^6/(2.2 \pm 0.2) \times 10^7$	$(1.6 \pm 0.2) \times 10^{-1}/(1.9 \pm 0.2) \times 10^{-1}$
	M4/B2	$(9.3 \pm 0.9) \times 10^4/(2.0 \pm 0.1) \times 10^5$	$(3.9 \pm 0.4) \times 10^5/(2.5 \pm 0.2) \times 10^6$	$(5.3 \pm 0.6) \times 10^{-1}/(6.9 \pm 0.6) \times 10^{-1}$
	M5/B3	$(7.6 \pm 0.7) \times 10^5/(2.2 \pm 0.2) \times 10^6$	$(1.3 \pm 0.1) \times 10^7/(1.6 \pm 0.1) \times 10^7$	$(9.3 \pm 0.7) \times 10^{-1}/(9.2 \pm 0.8) \times 10^{-1}$
summer	M3/B1	$(8.3 \pm 0.7) \times 10^5/(1.8 \pm 0.2) \times 10^6$	$(3.0 \pm 0.3) \times 10^6/(2.8 \pm 0.2) \times 10^7$	$(9.0 \pm 0.8) \times 10^{-2}/(3.3 \pm 0.4) \times 10^{-1}$
	M4/B2	$(7.5 \pm 0.6) \times 10^5/(1.4 \pm 0.2) \times 10^6$	$(2.1 \pm 0.3) \times 10^6/(3.6 \pm 0.2) \times 10^7$	$(6.1 \pm 0.6) \times 10^{-2}/(2.9 \pm 0.3) \times 10^{-1}$
	M5/B3	$(8.5 \pm 0.7) \times 10^5/(1.2 \pm 0.1) \times 10^6$	$(1.4 \pm 0.2) \times 10^7/(2.5 \pm 0.2) \times 10^7$	$(3.5 \pm 0.3) \times 10^{-1}/(4.1 \pm 0.5) \times 10^{-1}$
	paired *t* test	$p = 0.015$	$p = 0.031$	$p = 0.042$

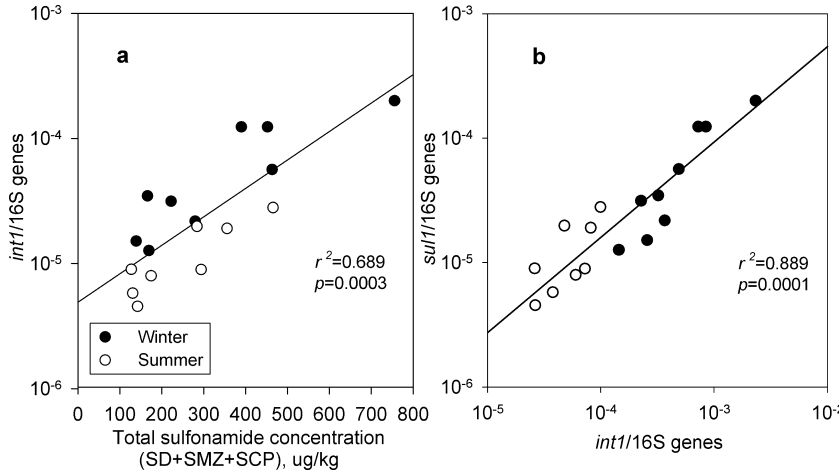

FIGURE 4. Correlation between relative abundance of class 1 integrons (*int1*/16S-rDNA gene levels) versus (a) total sulfonamides concentration; and (b) relative *sul1* abundance (*sul1*/16S-rDNA).

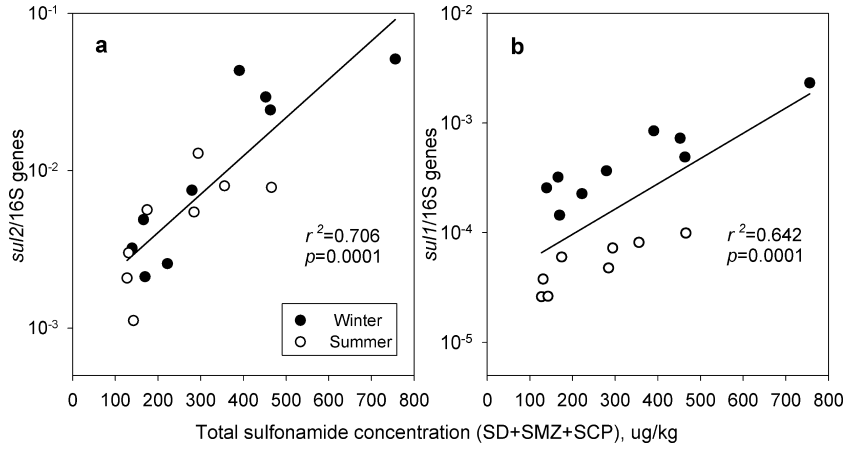

FIGURE 5. Correlations between relative abundance of *sul2* (a) and *sul1* (b) ARGs in sediments versus total sulfonamides concentration.

$(2.7 \pm 0.4) \times 10^{-1}$ copies per mL in water, which is comparable with levels reported for irrigation ditches and dairy lagoon water (*13*).

The effect of sampling season (summer versus winter) on ARG concentrations was less pronounced than that of antibiotic concentrations (SI Table S5). Seasonal effects could not be fully assessed due to data limitations, in addition to several confounding effects. These include differences in flow rate and antibiotic concentrations, which were generally lower in the summer due to faster photolysis and biodegradation as well as higher dilution from runoff. For example, total sulfonamides concentrations in water were 0.29 ± 0.22 μg/L in the summer versus 0.67 ± 0.39 μg/L in the winter, while the corresponding sediment concentrations were 246.8 $\pm$ 123.8 μg/kg in the summer versus 337.6 $\pm$ 200.2 μg/kg in the winter. This likely contributed to the lower ($p = 0.029$) relative abundance of the *sul1*gene (in sediment) in the summer than in the winter ($(5.6 \pm 2.6) \times 10^{-5}$ versus $(6.3 \pm 6.1) \times 10^{-4}$ *sul1*/16S-rDNA, respectively). On the other hand, there was no significant difference in the relative abundance of *sul2* genes between summer and winter samples ($p = 0.12$). Whether this reflects insensitivity of *sul2* carrier microorganism(s) to temperature variations, or to similar effect of season on *sul2* and 16S-rDNA reproduction and maintenance, or to different response of different genetic constructs (possibly class 1 integron for *sul1* and plasmid for *sul2*) was not determined. Albeit, the development, maintenance and propagation of ARGs in the environment will in part depend on the characteristics of the carrier bacteria and the stability of the genetic elements in which the ARGs are contained, which should be addressed in future studies.

Overall, this work shows a widespread distribution of *sul1* and *sul2* genes in the Haihe River and its tributaries, which is likely due to selective pressure exerted by prolonged exposure to sulfonamides. Given the importance to assess the risk posed by ARGs in the environment and to develop appropriate mitigation and control strategies, we conclude by underscoring the need for further research on the hydrogeochemical and biological factors that facilitate the transport, maintenance, amplification, and attenuation of ARG reservoirs in aqueous ecosystems.

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Note Added after ASAP Publication

The author names were reflected incorrectly, and the affiliation symbol for Hongjie Zhang was missing from the version of this paper published ASAP May 28, 2010; the correct version published on June 9, 2010.

Supporting Information Available

Detailed descriptions of extraction procedures, antibiotic and ARG analyses, PCR conditions, internal standards and primers, and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

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