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Occurrences of Three Classes of Antibiotics in a Natural River Basin: Association with Antibiotic-Resistant *Escherichia coli*

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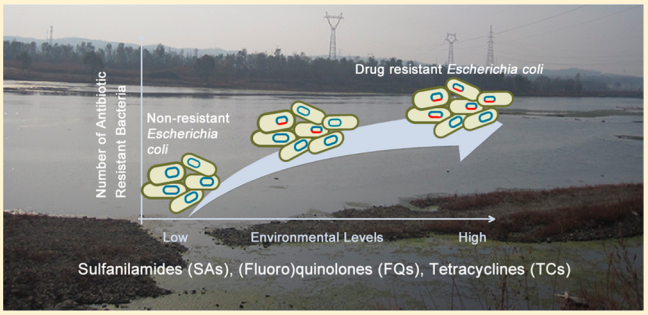
Supporting Information

ABSTRACT: To investigate the occurrence of antibiotics in urban rivers and their association with antibiotic-resistant *Escherichia coli*, 20 (fluoro)quinolone antibiotics (FQs), 16 tetracycline antibiotics (TCs) and their degradation products, and 25 sulfonamides (SAs) and some degradation products were determined in 45 river samples and 13 discharged wastewater samples collected from Wenyu River and its tributaries and 4 composite effluent samples from sewage treatment plants in Beijing, China. Fifteen FQs, eight TCs, including four degradation chemicals, and sixteen SAs, including four acetylated products, were detected in the river water. The SAs were the dominant antibiotic (total concentrations up to 3164.0 ng/L) in river water, followed by FQs (1430.3 ng/L) and TCs (296.6 ng/L). The sum concentrations for each class of detected antibiotic in the 13 discharge site samples were higher than those in river samples, up to 12326.7 ng/L for SAs, 6589.2 ng/L for FQs, and 730.1 ng/L for TCs, largely contributing to the high concentrations in the river basin. Log−linear regression analysis confirmed that the concentrations of FQs, TCs, and SAs in the Wenyu River basin were strongly correlated with the number of *E. coli* resistant to FQs ($p < 0.05$), TCs ($p < 0.05$), and SAs ($p < 0.05$), providing evidence for the environmental impacts of antibiotic usage.



INTRODUCTION

The presence of antibiotics in the environment has attracted increasing attention due to the induction and spread of antibiotic resistance genes.[1−6] The widespread occurrence of antibiotic-resistant *Escherichia coli* has been reported in river basins,[7] and 50−60% of infections in general in the United States have resulted from the spread of drug-resistance.[8] The potential physiological effects of antibiotics on nontarget organisms are also of special concern.[9−13] Some (fluoro)-quinolone antibiotics (FQs) such as ciprofloxacin (CIP) may interfere with the photosynthesis path of a plant, resulting in morphological abnormalities,[10] and almost all FQs, especially third- and fourth-generation FQs, elicit genotoxicity.[11] Tetracyclines (TCs) such as chlortetracycline (CTC) can inhibit plant growth,[14] and some sulfonamides (SAs) such as sulfadimethoxine (SDM) and sulfamerazine (SMR) have carcinogenic potential.[15−17]

Antibiotics are widely used throughout the world in human and veterinary medicine, as well as for agricultural purposes.[18] Annual worldwide antibiotic usage is estimated at 100 000−200 000 tons, and annual consumption in China of more than 25 000 tons.[19] A quantity of antibiotics are excreted in urine and then released into the aquatic environment via a sewage treatment plants (STP) due to limited removal efficiencies [e.g., moxifloxacin (MOXI, 40%), gatifloxacin (GATI, 43%), ofloxacin (OFL, 33−66%), lomefloxacin (LOME, 21−72%),[20] trimethoprim (TMP, 3%),[21] and oxytetracycline (OTC, 38%)[22]]. Some antibiotics, such as sulfamethoxazole (SMX), show increased concentrations in STP effluent.[23] Thus, various antibiotics discharged from STPs enter nearby rivers, creating potential ecological risks. Numerous antibiotics, including FQs, SAs, and TCs, have been detected downstream of STP discharge sites and in river basins and seawater in the United States, Japan, Korea, and China.[18,20,24−26] While many studies have demonstrated the ubiquity of antibiotics in the aquatic environment, most studies have only targeted one group or a narrow range of antibiotics and their metabolites: usually three FQs (OFL, CIP, and ENR), five SAs (sulfanilamide (SA), SMX, sulfadiazine (SDZ), sulfachloropyridazine (SCP), and TMP), and three TCs (TC, OTC, and CTC). To better assess their

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environmental risks, it is necessary to extensively investigate antibiotics and their metabolites in the aquatic environment.

Correlations between tetracycline residue and the level of tetracycline resistance genes, such as *tet*(O), *tet*(W), *tet*(Q), *tet*(M), *tet*(L), and *tet* (B), and the genes sum in soil surrounding swine feedlots have been reported previously.[27−29] Significant correlations have also been observed between the relative abundance of plasmid-mediated quinolone resistance (PMQR) genes and concentrations of 10 FQs in wastewater and soil adjacent to swine feedlots.[30] These correlations provide evidence to evaluate the effects of antibiotics on the levels of antibiotic resistance in the environment. To date, however, no studies have reported on the correlations between the resistance of bacteria that arise from various genes and the different resistance mechanisms. For example, 12 TC resistance genes have been reported in environmental samples, and the FQ resistance mechanism about mutations of chromosomal genes coding DNA gyrase or topoisomerase IV have been well accepted before the emergence of bacterial plasmid-mediated quinolone resistance (PMQR) genes. *E. coli* has been widely used as a fecal contamination indicator in natural river basins, and the correlation between its resistance (based on phenotypes) and the residue of medicinal antibiotics in natural river basins can help evaluate the effects of antibiotics on levels of resistance in aqueous environments.

In this study, three classes of antibiotics (20 FQs, 16 TCs and their degradation products, and 25 SAs) were determined by LC-MS-MS to investigate the occurrence and possible source of antibiotics in rivers of Beijing, China. The target antibiotics were selected due to their significant use, wide environmental occurrence and expected presence. In addition, they can be accurately measured in environmental samples using available technologies. Finally, the associations between the occurrences of SAs, FQs, and TCs and the corresponding incidence of resistant *E. coli* were assessed.

EXPERIMENTAL SECTION

Chemicals and Materials. Details of antibiotic standards and surrogate standards are shown in the Supporting Information. The methanol, dichloromethane, acetonitrile, and *n*-hexane from Fisher Chemicals (Fair Lawn, NJ) were all of HPLC grade. HPLC-grade formic acid was purchased from Dima Technology, Inc. (Richmond Hill, Ontario, Canada), ammonia was purchased from Alfa Aesar (Ward Hill, MA), and ethylenediamine tetraacetic acid disodium (Na_2EDTA) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). HPLC-grade water was prepared using a Milli-Q RC apparatus (Millipore, Bedford, MA). Solid phase extraction cartridges, Oasis HLB column (500 mg/6 cm^3) and Oasis MAX column (60 mg/3 cm^3, 150 mg/6 cm^3) were purchased from Waters Corporation (Milford, MA).

Sample Collection. The Wenyu River and its tributaries (Qing, Ba, and Tonghui rivers) cover an area of 2478 km^2 and are recipient rivers for most wastewater in urban areas of Beijing (Figure S1, Supporting Information). Four STPs (Gaobeidian, Qinghe, Jiuxianqiao, and Beixiaohe) are located upstream of the tributaries (Figure S1, Supporting Information). In August 2006, 62 environmental water samples were collected for the analysis of antibiotics and *E. coli* isolation to test antibiotic susceptibility. These samples included river water samples (n = 45), wastewater samples from discharge sites (n = 13), and STP effluent samples (n = 4; Table S1, Supporting Information). Brown glass bottles used for sample collection were previously washed with methanol, water, and deionized water. All water samples were immediately transported to the lab for *E. coli* isolation to test antibiotic susceptibility and were extracted on the same day after being filtered with a glass microfiber filter GF/C 1.2 μm (Whatman, Maidstone, UK). The cartridges were kept at −80 °C for 1 week prior to analysis.

Sample Preparation. After filtration, 800 mL of river water and 400 mL of wastewater were added with Na_2EDTA (0.5% w/v), acidified to pH 3.0 with hydrochloric acid (HCl), and then spiked with the surrogate standards before being passed through the HLB cartridges at a flow rate of approximately 5−10 mL/min. Na_2EDTA was used to reduce the formation chelate complexes of FQs and TCs with metal ions.[26,31] The HLB cartridges were preconditioned with 6 mL of methylene chloride, 6 mL of methanol, and 6 mL of ultrapure water containing 0.5 g/L of Na_2EDTA (adjusted to pH 3.0 with HCl). The cartridges were dried under a flow of nitrogen and finally eluted with 1 mL of methanol.

An aliquot of elute (0.5 mL) was dried by a weak stream of nitrogen and reconstituted with ethyl acetate (0.8 mL) and hexane (2.4 mL). The mixed solutions were then applied to silica cartridges (500 mg, 3 cc) preconditioned with 4 mL of hexane. The cartridges were rinsed with 3 mL of hexane, 3 mL of hexane/ethyl acetate (9:1, v/v) and 3 mL of hexane/ethyl acetate (3:2, v/v), and the analytes were finally eluted with 3 mL of methanol/acetone (1:1, v/v) followed by 3 mL of acetone. The solution was evaporated to dryness under a gentle stream of nitrogen and reconstituted with 0.5 mL of methanol/water (1:1) for LC-MS/MS analysis of trimethoprim, sulfonamides, and their metabolites.

The remaining elute (0.5 mL) in each HLB cartridges was diluted to 8 mL by adding ultrapure water (pH 7.0), and then applied to Oasis MAX cartridges conditioned with 3 mL of methanol and 3 mL of ultrapure water. After the cartridges were rinsed with 3 mL of ammonia (5%), the TCs, their metabolites, and FQs were eluted by 3 mL of methanol containing 1% formic acid. After the addition of 0.2 mL of 30% aqueous ammonia solution, the elutes were dried under a stream of nitrogen and reconstituted by 0.5 mL methanol/water (1:1). The standard samples were treated in the same way to counteract the effects of unexpected interference, as described in our previous papers.[20,32]

Instrumental Analysis. An Acquity Ultra Performance Liquid Chromatograph (Waters Corp., Milford, MA) and a Quattro Premier XE tandem quadrupole mass spectrometer (Waters Corp., Milford, MA) were used to analyze all target antibiotics. A Waters Acquity UPLC BEH C18 Column (100 × 2.1 mm, 1.7 μm) was used for separation of antibiotics, and the column temperature was 40 °C. Methanol (A) and ultrapure water containing 0.1% formic acid (B) were the mobile phases, and the injection volume was 2.0 μL. Analytical conditions are provided in Table S2 (Supporting Information). ESI-MS/MS detections were performed in the positive ion mode for all analytes. Data acquisition was performed in the selected reaction monitoring (SRM) mode (Table S3, Supporting Information). Common MS parameters were as follows: capillary voltage, 3.0 kV; source temperature, 110 °C; desolvation temperature, 400 °C; source gas flow, 50 L/h; and desolvation gas flow, 650 L/h.

Identification was accomplished by comparing the retention time (within 2%) and the signal ratio (within 20%) of two selected product ions in the environmental samples with standards. Quantitation was accomplished by choosing the

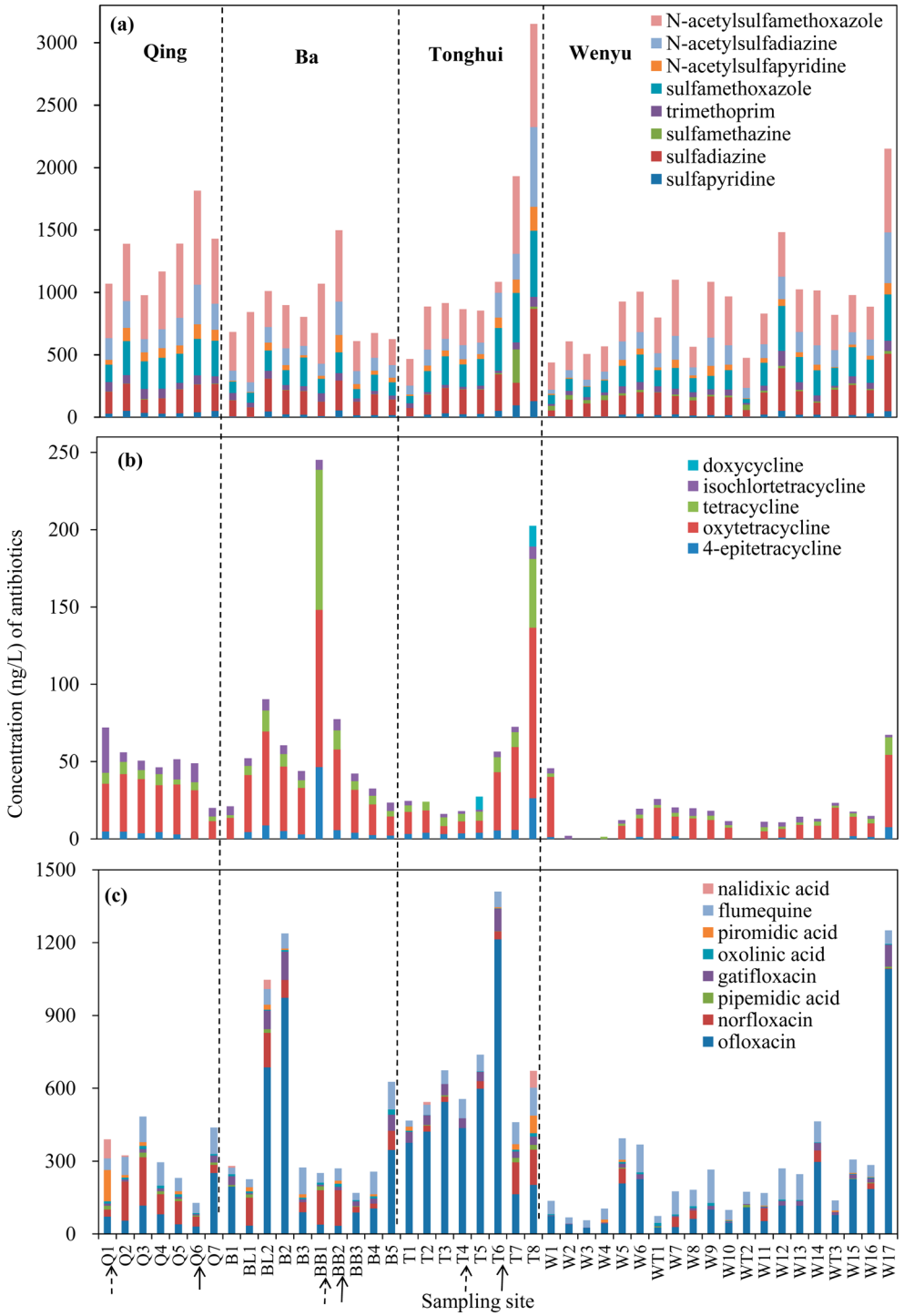

Figure 1. Distribution of the three classes of antibiotics in 45 river water sampling sites in Beijing. (a) SAs; (b) TCs; (c) FQs. Compounds with contribution percentages less than 1% are not shown. Sampling sites are shown in Figure S1 (Supporting Information). Solid arrows and dotted arrows indicate discharge sites and STP effluent, respectively.

select reaction monitoring conversion ion with the highest abundance and/or minimal interference or background. Internal standard (demeclocycline, DMC)[26] and surrogate standards (NOR-d_5, $^{13}C_6$–SMA, and NAcSMX-d_5) were used to compensate variations loss in the solid-phase extraction (SPE) process and instrument response.

Method Performance. All equipment rinses were conducted with methanol and ultrapure water to avoid sample contamination. While the HLB cartridges can simultaneously enrich all target antibiotics, a prevalent signal suppression (e.g., TCs 25−89% in STP effluent) occurred for the target analytes without the cleanup procedure. To optimize the simultaneous extraction of antibiotics, the silica cartridges were used for cleanup of SAs, and the Oasis MAX cartridges were used for cleanup of FQs and TCs. The efficiency of the extraction and purification procedure was assessed by spiking the river samples with standard solutions of target compounds and surrogate standards. To further evaluate the purifying effect on the

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response of target compounds, the matrix effects in three kinds of samples were calculated, with the results shown in Table S4 (Supporting Information). Recoveries were estimated by triplicate analysis of field water samples spiked with 200 ng/L of each target chemical in direct discharge wastewater, with 100 ng/L in STP effluent, and with 50 ng/L in river water, with low levels similar to the concentrations found in previously analyzed samples.[20,25,26,32] The recoveries of all target compounds, except for two TCs (EACTC, 54−57%; ACTC, 48−55%) and six FQs (PIPE, 53−63%; ENO, 58−61%; CIPRO, 59−75%; SARA, 58−66%; GATI, 46−59%; PIRO, 55−58%), in the discharge, STP effluent, and river water samples were 61−120, 60−116, and 61−120%, respectively, with a relative standard error (RSD) in the range of 0.1−20% (Table S4, Supporting Information). The limits of quantification (LOQs) of the method were based on a signal-to-noise ratio of 10:1 in field samples. For nondetected chemicals, samples were spiked using a mixture of standard solution. The LOQs for the target antibiotics were in the range of 0.4−19.1 ng/L in discharge site samples, 0.5−30.6 ng/L in STP effluent samples, and 0.5−32.6 ng/L in river water samples (Table S4, Supporting Information). The matrix effects after cleanup were in the range of 1.7−20.8%, except for ECTC, β-apo-OTC, EACTC, ACTC, SA, TMP, SIA, DANO, ENRO, OXO, and NALI (in the range of 21−32.7%) in the STP effluent samples (Table S4, Supporting Information), and using LC-ESI-MS/MS analysis these results showed no obvious signal suppression compared with the 24−49% signal suppression of six TCs in chlorinated drinking water and signal enhancement for TCs in surface waters reported previously.[33]

***E. coli* Isolation.** Of the 45 samples, numbers of *E. coli* resistant to antibiotics in 19 river water samples were reported in our previous papers (Table S5, Supporting Information),[6,7] and the incidence of antibiotic-resistant *E. coli* in 23 river water samples, 3 discharge samples, and 4 STP effluent samples were newly determined. Sample preparation and bacteriological tests for isolation of *E. coli* were performed by an established membrane filter method.[6] Briefly, water samples were filtered through nitrocellulose filters (0.45 μm pore-size) with the goal of obtaining colonies. The filters placed onto *E. coli* chromogenic agar (Chromagar Microbiology, France) and incubated at 44 °C for 24 h. The colonies that turned blue on *E. coli* chromogenic agar were chosen, recorded, purified, and collected for subsequent studies. The detailed isolation procedure of *E. coli* is provided in the Supporting Information.

Antibiotic Susceptibility Testing. Isolates were screened for susceptibility to three classes of antibiotics on Mueller-Hinton agar (Oxoid) by a disk diffusion method, as described by the CLSI 2005 guidelines[34] and our previous paper.[6,7] The following disks (Oxoid, UK) were used: tetracycline (TC, 30 μg), SXT (sulfamethoxazole/trimethoprim, 23.75 μg/1.25 μg), LEV (5 μg). *E. coli* ATCC 25922 was used as reference strain. The diameter of inhibition zones surrounding the antibiotic disks was interpreted according to the CLSI 2005 guidelines. The isolates that were shown to be resistant to antibiotics were recorded, purified, and collected for subsequent studies.

RESULTS AND DISCUSSION

Occurrence of Antibiotics in Wenyu Rivers. Figure 1 shows the concentrations of SAs, FQs, and TCs in the 45 water samples from Wenyu Rivers. Of the 61 antibiotics among three classes, 16 SAs, 15 FQs, and 8 TCs were detected, with total mean concentrations of 1046.7, 400.4, and 40.8 ng/L, respectively (Table S6, Supporting Information).

Of the tested 20 FQs, 15 FQs, including 4 first-generation quinolones (OXO, PIPE, PIRO, and NALI), 7 second-generation FQs (OFL, FLUM, NOR, LOME, CIP, FLER, and PEFL), 2 third-generation FQs (DIF and DANO), and 2 fourth-generation FQs (GATI and MOXI), were detected in all the samples from the rivers (Table S6, Supporting Information). OFL, GATI, and FLUM were detected in 100% of river samples, while OXO was detected in 84% (38/45). The mean concentration (702.0 ng/L) of FQs in Tonghui River was the highest, followed by Ba River (480.0 ng/L), Qing River (331.2 ng/L), and Wenyu River (264.7 ng/L). Of the detected FQs, OFL was the most abundant, with a concentration range of 25.1−1213.6 ng/L, followed by FLUM (24.2−137.0 ng/L), NOR (ND-199.4 ng/L), and GATI (1.6−116.4 ng/L). The total concentration of FQs in 45 water samples from the rivers ranged from 56.5 ng/L (site W3) to 1430.3 ng/L (site T6). The environmental occurrence of FQs has been well studied, but earlier research has focused on CIP, OFL, NOR, or enrofloxacin (ENR). The concentrations of CIP (maximum, 24.1 ng/L) and ENR (not detected, ND) in the present study were lower than those (80 ng/L for CIP and 15 ng/L for ENR) found downstream of STP discharge sites in New Jersey,[24] while the maximum concentration of OFL (1213.6 ng/L) was higher than that in New Jersey (920 ng/L). The lowest concentration of OFL (25.1 ng/L) in water samples from the rivers in the present study was higher than that in tributaries of the Seine in France (maximum, 18 ng/L),[35] and the median concentration of OFL (110 ng/L) in water samples from the rivers was comparable to that in the Pearl River (108 ng/L)[36] but higher than that in the Jiulong river basin (46 ng/L).[37] To our best knowledge, PIPE was detected for the first time at a median concentration of 7.2 ng/L and a frequency of 53.3%. It should be noted that GATI, a fourth-generation quinolone, was detected in all 45 samples from the rivers at a relatively high concentration (1.6−116.4 ng/L; median, 16.5 ng/L). This is the first report on its occurrence in natural rivers, and the concentration was higher than that found in STP effluent reported previously (16−42 ng/L).[32] Another fourth-generation quinolone, MOXI, was also detected for the first time with a detection frequency of 6.7%. Because fourth-generation FQs can cause hyperglycemia and hallucinations,[38,39] it has already been removed from clinical use in the North American market. The ubiquitous occurrence of fourth generation quinolones in natural river water is of concern.

Among the tested 16 TCs, 8 TCs (TC, OTC, DXC, and MINO, 4 degradation chemicals (ICTC, ETC, EACTC, and ATC)) were detected in all samples from the rivers (Table S6, Supporting Information). The total concentration of TCs ranged from ND to 296.6 ng/L, with OTC the most abundant (ND-110.2 ng/L), followed by TC (ND-90.7 ng/L), ICTC (ND-29.1 ng/L), and ETC (ND-46.5 ng/L), at detection frequencies of 91, 93, 91, and 64%, respectively (Table S6, Supporting Information). The mean concentration of TCs in Ba River (69.3 ng/L) was the highest, followed by Tonghui River (55.7 ng/L), Qing River (49.7 ng/L), and Wenyu River (17.5 ng/L). The concentrations of TCs in Wenyu River and its tributaries (ND−296.6 ng/L) were similar to those (20−180 ng/L) in the Cache la Poudre watershed in the USA,[40] and higher than those (ND ∼ 16 ng/L) in the Alzette and Mess rivers of Luxembourg.[42] However, the OTC concentration

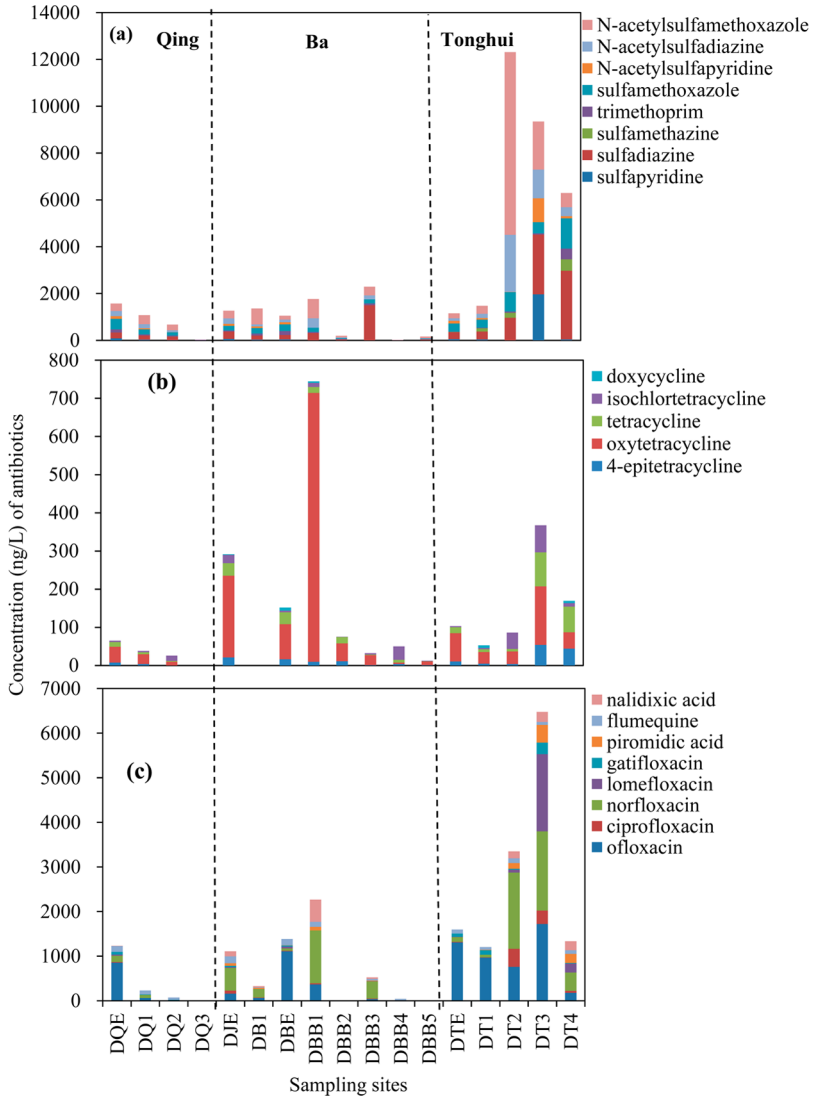

Figure 2. Concentrations of the three classes of antibiotics in 13 discharge site and 4 STP effluent samples in Beijing. (a) SAs; (b) TCs; (c) FQs. Compounds with contribution percentages less than 1% are not shown. Sampling sites are shown in Figure S1 (Supporting Information).

(maximum, 110.2 ng/L) in Wenyu was lower than that (OTC, 340 ng/L) in the Suwannee River located in the United States.[41] The detection frequencies of OTC and TC in Wenyu were higher than those in 139 streams in the United States (1.2−2.4%).[43] It should be noted that while CTC (primary use in beef cattle) has been frequently reported in surface water,[40,44] it was not detected in Wenyu River or its tributaries, but its metabolite ICTC was detected (ND-29.1 ng/L). This phenomenon has been observed in previous research.[26] Apparently, ICTC is accumulated as CTC decomposes, suggesting ICTC to be moderately stable compared with CTC.[45]

Of the tested 25 SAs and metabolites, 12 sulfonamide antibiotics and four acetylated products were detected in all samples from the rivers (Table S6, Supporting Information). The detection frequencies of SMX, TMP, SDZ, SPD, SMA, SMM, and four N-acetylated metabolites (NAcSMX, NAcSPD, NAcSDZ, and NAcSMA) were 96−100%, and the detection frequencies of SGD, SA, SCP, SQX, STZ, and SME ranged from 51 to 87%, indicating their widespread occurrence in Beijing urban rivers. It has been reported that the removal efficiencies of SMX, SPD, and SDZ in sewage treatment plants are relatively low, or even negative,[23] which would lead to high detection frequencies and concentrations in the receiving rivers. Total concentrations of SAs ranged from 467.8 to 3164.1 ng/L in all samples from the rivers, with the highest total concentration observed downstream of Tonghui River (site T8). The mean concentration of SAs in Qing River (1328.0 ng/L) was the highest, followed by Tonghui (1276.9 ng/L), Wenyu (941.0 ng/L), and Ba (876.9 ng/L) rivers, and was significantly higher than that in the Liao River basin (192.1 ng/L) and adjacent Liaodong Bay (63.4 ng/L).[25] The occurrence of several SAs (SMX, TMP, SMA, SPD, and SDZ) has been reported in earlier studies.[18,46] The concentrations of SAs (1.3−129.3 ng/L for SPD, 33.2−528.1 ng/L for SMX, 267.3 ng/L for SMA, and 12.8−119.7 ng/L for TMP) in Wenyu River and its tributaries were about 1 order of magnitude higher than those in the Mekong River (21−132 ng/L for SPD, 3−33 ng/L for SMX, 7−54 ng/L for TMP, and <28 ng/L for SMA).[18] The mean concentrations of SDZ (185.0 ng/L) and SMX (186.4 ng/L) were comparable with those (140 ng/L for SDZ and 150 ng/L for SMX) in the Haihe River basin in China, but the mean levels of TMP (47.5 ng/L) and SCP (2.0 ng/L) were markedly lower (100 ng/L for TMP and 160 ng/L

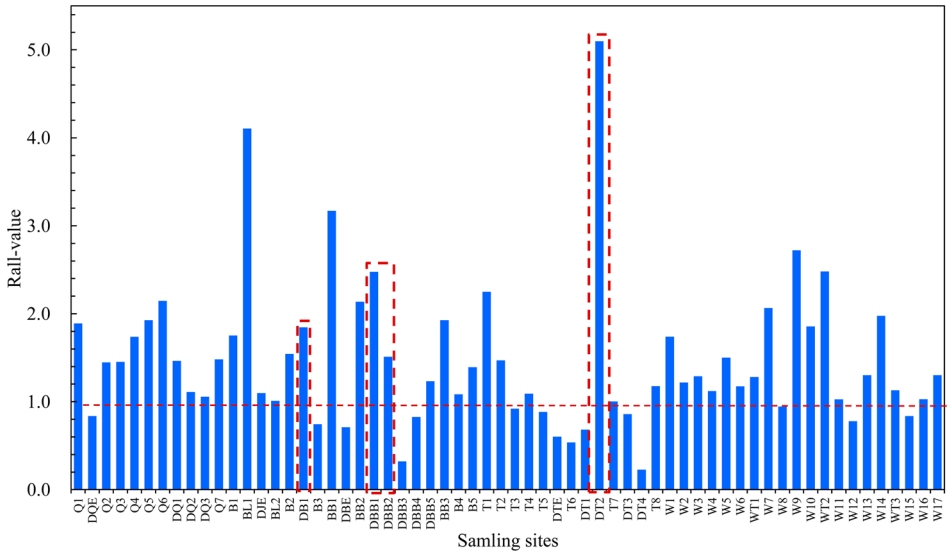

Figure 3. Ratio (R_{all}-value) of SAs and their metabolites in STP effluent, wastewater from discharge sites, and river water samples in Wenyu River Basin. Dotted boxes indicate discharge sites with R_{all}-values greater than 1.1.

for SCP in Haihe River basin).[46] Among the four detected N-acetylated sulfonamides, the concentration of NAcSMX (87.4−826.0 ng/L) was the highest, followed by NAcSDZ (33.2−638.9 ng/L), NAcSPD (4.5−192.6 ng/L), and NAcSMA (0.6−35.6 ng/L). While these N-acetylated sulfonamide metabolites have been detected in seawater samples from Liaodong Bay, these compounds were detected in the Wenyu urban rivers at higher concentrations than those reported in seawater samples.

In addition to the river samples, we also analyzed 4 STP composite effluent samples collected over 1 week and 13 water samples collected from wastewater discharge sites along the tributaries of Wenyu River (Figure 2). Sites DQ2, DQ3, and DBB4 were close to fishing ponds. Sites DB1, DBB1, DBB3, DT3, and DT4 were located in residential areas; therefore, these sites were probably influenced by domestic sewage. The sources for other discharge sites were unclear. The levels of antibiotics at discharge sites DB1, DBB1, DBB2 (except for FQs), DBB3, DT1, DT3, and DT4, STP effluent sites DQE, DBE, DJE, and DTE, were much higher than those detected in the upstream and downstream river water samples, indicating that STP effluent and discharge sites were the main sources of antibiotics in the rivers. Total concentrations ranged from 1135.0 ng/L (site DJE) to 1621.6 ng/L (site DTE) for FQs, 65.1 ng/L (site DQE) to 297.2 ng/L (site DJE) for TCs, and 1051.1 ng/L (site DBE) to 1577.3 ng/L (site DQE) for SAs. The most abundant compounds in the STP effluent samples were OFL, OTC, and five SAs (SDZ, SMX, NAcSPD, NAcSDZ, NAcSMX). The highest concentrations of OFL, CIP, LOME, and OTC were 1.4-, 16.8-, 110.3-, and 6.4-fold higher than those in the river water samples. Four FQs (DANO, PEFL, DIF, and MOXI), four SAs (STZ, SME, SCP, and SQX), and three TCs (MINO, DXC, and ATC) were detected in a few discharge site and STP effluent samples and the detection frequencies were consistent with the river water samples. Very high concentrations of SAs at discharge sites (12 μg/L at site DT2 and 9.3 μg/L at site DT3) were detected in downstream Tonghui River into Wenyu River.

Profiles. Of the three major classes of antibiotics quantified in this study, SAs were the predominant antibiotic in all samples collected from rivers, discharge sites, and STP effluent, with an average contribution of 68%, followed by FQs (29%) and TCs (3%). Of the 16 detected sulfonamide antibiotics and N-acetylated metabolites, NAcSMX was the most abundant compound (35%), followed by SDZ (19%), SMX (18%), NAcSDZ (14%), TMP (5%), and NAcSPD (4%), with SMA, SMM, SGD, SME, SA, SCP, STZ, and SQX contributing to 5% of total SAs in Wenyu River and its tributaries. It was commonly thought that N-acetylated sulfonamides metabolites resulted from human and animal activities. It has been reported that during biological treatment, NAcSPD and NAcSMX can be transformed to SPD and SMX, respectively, and the ratio of an N-acetylated sulfonamide to the corresponding parent in STP effluent was lower than that in raw wastewater.[23] For example, previous research showed that the ratio (R = NAcSMX/SMX) in raw influent was 3.3, but was 0.03 (median) in effluent,[47] and the R_{all}-value (R_{all}-value = sum (four acetylated products)/sum (corresponding parents) in the effluent of four STPs ranged from 0.6 to 1.1 (Figure 3). It should be noted that the concentration ratios of N-acetylated sulfonamide to its parent compound (NAcSMX/SMX, NAcSPD/SPD) in all sampling sites, except for sites T6, W16, and B3, were greater than 1. The ratio for NAcSMA/SMA was in the range of 1.0−3.7 in 18 of all river samples, and the ratio of NAcSDZ/SDZ was 1.0−1.6 in 13 of all river samples. The higher than 1.1 R_{all}-value in 34 of 45 sites (R_{all}-value in discharge site samples was in the range of 0.2−1.1, except for DT2, DBB1, DB1, and DQ1 in the range of 1.2−5), and the significantly increased concentrations of SAs downstream of Wenyu River (R_{all}-value was 1.0 and 1.3 in W16 and W17, respectively) indicated the existence of freshly discharged treated sewage or naturally attenuated untreated sewage.

The main antibiotics in the river samples were ETC, OTC, TC, and ICTC, with mean percentages of 8.8, 62.5, 15.5, and 11.5%, respectively. These profiles were quite similar to the STP effluent and discharge site samples, indicating that TCs in the Wenyu River basin were affected by both municipal wastewater and discharge sites. For the profiles of FQs, OFL (45.7%) was dominant in the Wenyu rivers, followed by NOR (14.2%), FLUM (13.4%), and GATI (5.0%), which was different to the profiles in the discharge site samples in which

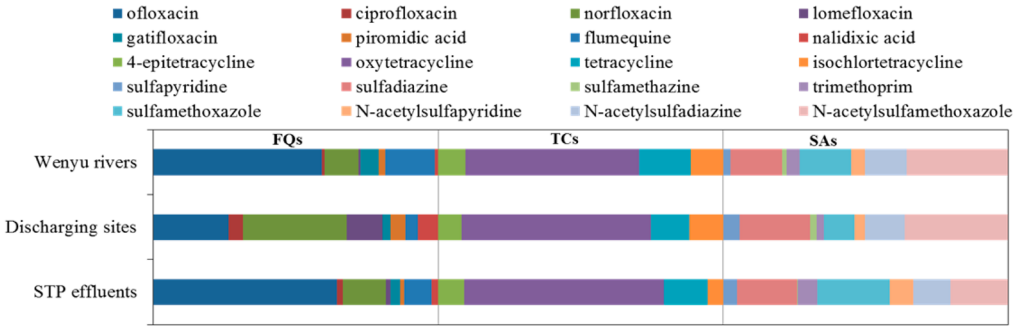

Figure 4. Comparison of chemical profiles detected in three kinds of samples (wastewater discharge sites, STP effluent, and Wenyu rivers). Compounds with contribution percentages less than 1% are not shown.

NOR (32.4%) was dominant, followed by OFL (18.0%), LOME (16.0%), and NALI (10.7%), but relatively similar to STP effluent samples (Figures 4). This suggested that STP effluent largely contributed to the occurrence of FQs in the river basin, which was consistent with the limited removal efficiencies of FQs (40−75%) in STPs.[20]

Relationship between Antibiotic Levels and Resistant *E. coli*. Antibiotics have been widely used in hospitals and aquaculture due to their good antibacterial, therapeutic, and preventive effects. This has led to the generation and distribution of resistant microorganisms in the natural environment. We determined the incidence of antibiotic-resistant *E. coli* isolated from samples in Wenyu River in Beijing, China, based on the phenotypic resistance testing of three classes of antibiotics, TCs (tetracycline (TC), SAs (sulfamethoxazole-trimethoprim (SXT)); and FQs (levofloxacin LEV; Table S5, Supporting Information). In addition to the numbers of *E. coli* resistant to antibiotics in 19 river samples reported in our previous research,[6,7] 23 river samples, 3 discharge site samples (DQ2, DQ3, and DBB4), and 4 STP effluent samples (DQE, DBE, DJE, and DTE) in the present study were used for regression analysis with antibiotic concentration (Table S5, Supporting Information). Log−linear regression analysis was performed between numbers of *E. coli* resistant to antibiotics and the antibiotic residue in the river basin to assess their associations. Results showed that the number of SA-resistant *E. coli* significantly increased with total concentrations of SAs in the river basin (r^2 = 0.18, p = 0.0016; Figure 5). Although the relationship was not as strong as that for SAs, a significant relationship between the number of FQ-resistant *E. coli* was also found (r^2 = 0.14, p = 0.0053). A significant correlation between the number of TC-resistant *E. coli* and TC residue in the river basin was also observed (r^2 = 0.28, p < 0.00005). As observed in the relationship between bacterial antibiotic resistance genes and TCs residue in soils adjacent to swine feedlots,[29] the logarithmic correlation suggested that the decay rate of antibiotic-resistance bacteria should be faster than the degradation of antibiotics in the natural environment, and the relatively weak correlations between the number of antibiotics-resistant *E. coli* and their concentrations may attribute to such phenomenon. To our best knowledge, only one prior study attempted to correlate the incidence of antibiotic-resistant *E. coli* with antibiotic residue in hospital wastewater, although no significant correlation was obtained.[2] The correlations observed in this study might be due to the same sources of SAs, FQs, and TCs and their resistance *E. coli*. An alternative explanation might be that the *E. coli* resistant to SAs, FQs, and TCs were positively selected following exposure to these classes of

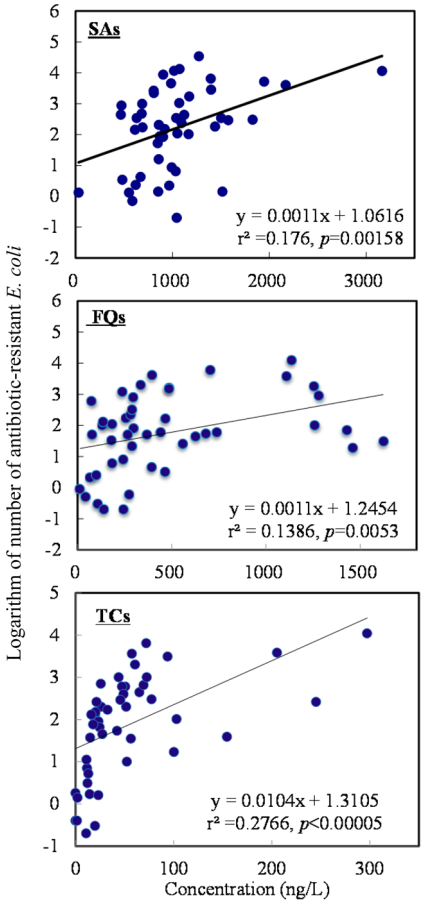

Figure 5. Correlation between resistant *E. coli* number to corresponding antibiotics and the antibiotic concentration residues in Wenyu River and its tributaries.

antibiotics during use or environmental contact. Horizontal gene transfer from other bacteria species at chronic low-level exposure to antibiotics[48] would also contribute to the increased antibiotic-resistance *E. coli* numbers as observed in our previous paper[6] where *tet*(M), a tetracycline-resistant gene originally detected in Gram-positive bacteria, was detected in *E. coli* isolated from the same natural river basin as in this study. This is the first report regarding the relationship between the number of *E. coli* and the levels of antibiotics found in the natural environment. Although determination of resistance genes can provide important information on the fate of resistant bacteria and antibiotics, as exemplified by the correlation between total TCs in soil adjacent to swine feedlots