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Article

Developing Surrogate Markers for Predicting Antibiotic Resistance "Hot Spots" in Rivers Where Limited Data Are Available

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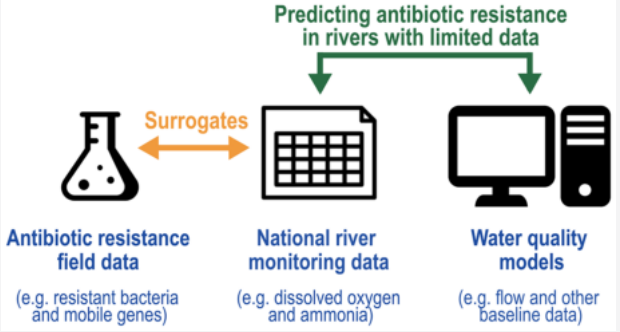
ABSTRACT: Pinpointing environmental antibiotic resistance (AR) hot spots in low-and middle-income countries (LMICs) is hindered by a lack of available and comparable AR monitoring data relevant to such settings. Addressing this problem, we performed a comprehensive spatial and seasonal assessment of water quality and AR conditions in a Malaysian river catchment to identify potential "simple" surrogates that mirror elevated AR. We screened for resistant coliforms, 22 antibiotics, 287 AR genes and integrons, and routine water quality parameters, covering absolute concentrations and mass loadings. To understand relationships, we introduced standardized "effect sizes" (Cohen's D) for AR monitoring to improve comparability of field studies. Overall, water quality generally declined and environmental AR levels increased as one moved down the catchment without major seasonal variations, except total antibiotic concentrations that were higher in the dry season (Cohen's D > 0.8, $P < 0.05$). Among simple surrogates, dissolved oxygen (DO) most strongly correlated (inversely) with total AR gene concentrations (Spearman's ρ 0.81, $P < 0.05$). We suspect this results from minimally treated sewage inputs, which also contain AR bacteria and genes, depleting DO in the most impacted reaches. Thus, although DO is not a measure of AR, lower DO levels reflect wastewater inputs, flagging possible AR hot spots. DO measurement is inexpensive, already monitored in many catchments, and exists in many numerical water quality models (e.g., oxygen sag curves). Therefore, we propose combining DO data and prospective modeling to guide local interventions, especially in LMIC rivers with limited data.



KEYWORDS: *antibiotic resistance, LMICs, SE Asian rivers, water quality, environmental monitoring, high-throughput qPCR, modeling*

INTRODUCTION

Increasing resistance in microorganisms to antibiotics and other drugs poses a global health threat.[1] When a pathogen becomes resistant to critical drugs, formerly easy-to-treat infections can be lethal.[2,3] Consequently, scientists and policy makers must better understand drivers of antibiotic resistance (AR) to reduce its global spread. The number of peer-reviewed AR papers has tripled in the last 10 years (title or abstract containing "antibiotic resistance" web of science from 2009 to 2019) with more than 10 000 papers published in 2019 alone. However, our understanding of environmental AR spread lags behind other contexts.[4] When insufficiently treated wastewater enters rivers, residues of antibiotics, antibiotic-resistant bacteria (ARBs), and antibiotic-resistant genes (ARGs) can radiate through the environment, potentially posing an exposure risk.[5,6] However, mitigating environmental AR spread is hindered by many factors, including: (1) inadequate data to make decisions about environmental AR exposures; (2) the complexity and diversity of environmental matrices; (3) conflicting definitions of AR and inconsistency in measuring methods; (4) reliance on overly expensive detection methods; (5) limited agreement on AR thresholds of possible concern; and (6) a limited understanding of how environmental AR levels translate to human health risk.[7]

Limited data and expensive AR detection methods are especially problematic in low- and middle-income countries (LMICs), particularly identifying sites of greatest concern.[8] This is partly because most studies are more academic rather than practical and also because researchers overly focus on testing statistical significance (P values) to report spatial or temporal differences. A lower P value is often interpreted as meaning a bigger difference between two settings, but

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statistical significance only means that it is unlikely for the null hypothesis to be true (such as H_0 = no difference in antibiotic concentration between up- and downstream river locations),[9] which often has limited value in quantifying the scale of differences.

In contrast to P values, we feel "standardized dimensionless effect sizes" better describe the size of differences and allow comparison of studies from different settings with different variables.[10] Effect sizes are easy to calculate and, unlike P values, provide a comparison independent of sample size.[10] Surprisingly, despite the popularity of effect size in meta-analysis and psychological studies, they have not been used in AR/water quality studies. We argue that to effectively interpret and compare AR levels, both statistical significance (P value) and substantive significance (standardized effect size with confidence intervals) should be reported.[10]

Increasing the informative value of monitoring data is especially critical in LMICs. While antibiotic use per person is increasing in LMICs compare with high-income countries (HIC), sewage treatment lags behind.[11−13] Southeast (SE) Asia with its rapid economic development has been proposed as an epicenter for emerging infectious diseases and AR.[14−16] In particular, extended-spectrum β-lactamase (ESBL)-producing and carbapenem-resistant pathogens pose major health threats in the region.[17,18] ESBL bacteria produce an extended-spectrum enzyme that breaks down the majority of β-lactam antibiotics such as penicillin.[17] Infections with ESBL bacteria are treated with the remaining β-lactam antibiotics, called carbapenems.[17] High rates of mortality occur when pathogens become resistant to these last-resort antibiotics.[17]

Malaysia, our study site here, has one of the fastest-growing economies in SE Asia.[19] Increased wealth has allowed more Malaysians to access healthcare, including antibiotics. A national study in 2014 found that antibiotics were prescribed in 21% of patient encounters, although 46% of these were for upper respiratory tract viral infections, where antibiotics are often not suitable.[20] In 2000, a National Surveillance of Antibiotic Resistance (NSAR) programme was initiated to monitor AR bacteria in hospitals.[21] Particular local concern are increasing ESBL-producing *Enterobacteriaceaea* and carbapenem-resistant pathogens, which underpins our focus herein on the environmental spread of ESBL strains and related ARGs.[22]

Despite LMICs carrying a higher burden of AR, including Malaysia, environmental AR surveillance is lacking.[12] As such, there is a shortage of data in most LMICs, especially the relative susceptibility of local populations to the effects of AR due to limited accurate health surveillance data.[8,23] AR transmission models have been proposed to estimate the risk of AR,[24] but environmental AR modeling, which might help fill in data gaps in LMICs, lags far behind.[25] While surface water quality models have existed for decades,[26] a few attempts have been made to model AR spread in watersheds,[27−29] often hindered by limited knowledge of AR fate processes in the environment, and missing AR and/or hydrological calibration data.

The aim of this study was to identify easy-to-measure water quality surrogates that would aid monitoring and modeling of AR in locations with limited data. For this, we examined the Skudai river catchment in Malaysia, using simple AR culturing methods and routine water quality markers in parallel to more sophisticated methods. Further, we show the value of effect sizes for environmental AR studies, which better account for spatial, seasonal, and dilution effects, as well as improve comparability of monitoring studies in LMICs and HICs.

MATERIAL AND METHODS

Catchment Description. The Skudai river catchment in southern Malaysia (total drainage area 288 km^2;[30] see Supporting Information (SI) Figure S1) is composed of urban/developed, agricultural (80% oil palm, 20% rubber plantations), and forest land in roughly equal proportions.[31] The Skudai catchment lays within the Johor Bahru district (1865 km^2 with 1.4 million inhabitants[32]). Similar to many LMIC settings, sewage treatment in the Skudai catchment is inconsistent, sometimes with poorly defined discharge locations.[33,34] National data from 2017 show 79% of the Malaysian population connected to sewers with 20% serviced by septic tanks and <1% relying on latrines and other.[35] Improvement to secondary (biological) treatment has taken place in some areas,[36] but many suburban locations (such as within the Skudai catchment) still rely on septic tanks. To our knowledge, no major pharmaceutical production facilities are located in the catchment.[34]

The main Skudai river (42.8 km) passes rural and urban areas before it discharges into the sea. The Skudai has several tributaries, including the mostly rural Senai (11.8 km) and urbanized Melana (18.7 km).[30] Malaysia has a humid tropical climate and two monsoon seasons, the relatively dry Southwest Monsoon from May to September and the wetter Northeast Monsoon from November to March, but rainfall also occurs in the transitional periods.[37,38]

Sample Collection and Processing. River water samples were collected from eight sampling points in the Skudai catchment (SI Figure S1 and Table S1) across four seasonal sampling campaigns: two in March 2018 (trips I and II) in the "wet season" and two in July 2018 (trips III and IV) during the "dry season". The eight points were chosen based on land use and preliminary sampling data from 15 sites (results not shown) and included six locations on the Skudai itself (S1, S2, S5, S6, S7, and S8 during trips I−IV) and two sites on Senai and Melana tributaries (Se1 and M5; sampled during trips I, III, and IV), respectively. The campaign resulted in 30 samples from which technical triplicates were obtained.

Sampling events always were conducted over a single day in the morning, from up- to downstream, only at low tide, and on days when rainfall had not occurred within 24 h. Each sampling location was at a bridge, which allowed water collection from mid-river. Samples were collected in a prerinsed clean bucket (on a rope), waiting 2 min between taking each replicate. Sample water was stored in autoclaved glass bottles on ice in the dark (3 × 1 L, except 4 × 1 L for S1 to assure sufficient DNA yield for downstream processes). In the laboratory, technical replicates were processed separately, splitting 1 L of sample into 15 mL for chemical analysis, 2 mL for coliform plating, 500 mL for antibiotic analysis, and 80−250 mL for DNA extraction.

River water temperature, dissolved oxygen (DO), pH, and conductivity were measured on-site with an HQ40D portable multimeter (Hach). Conductivity was temperature-corrected (NaCl nonlinear with reference temperature 25 °C). River volumetric flowrates were estimated using the float method with an estimated accuracy of ± 20%.[39,40] River width and depth were used to calculate cross-sectional area at the time of sampling at each point, which was multiplied by the measured

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surface velocity to obtain the flowrate. A factor of 0.85 corrected for surface velocity data.[39,40]

Chemical Analysis. Water samples were filtered through 0.2 μm polyethersulfone (PES) syringe filters (VWR) and stored for a maximum of 24 h at 4 °C prior to chemical analysis. Ammonia (NH_3−N, salicylate method[41]), chemical oxygen demand (COD, USEPA reactor digestion method[42]), total phosphorus (TP, USEPA PhosVer 3 with acid persulfate digestion method[43]), and total nitrogen (TN, persulfate digestion method[44]) were measured using commercial colorimetric test kits with a UV−vis spectrophotometer DR5000 (all Hach). Where necessary, the samples were diluted with Milli-Q water prior to analysis.

The Malaysian Department of Environment (DOE) applies a Water Quality Index with three classifications ("clean", "slightly polluted" and "polluted") and the National Water Quality Standards for Malaysia (classes I−V) to evaluate river water quality based on selected parameters.[45] Combining both approaches, three water quality categories were created based on COD, NH_3−N, and DO concentrations in the catchment: clean (class I), slightly polluted (class II), and polluted (class III−V) (SI Table S2). We compiled chemical data from S1 and S8 with national DOE river water quality data collected for the same locations throughout 2018 (SI Table S3).

Coliform and Other Plating. Coliform ChromoSelect agar was used to quantify colony forming units (CFUs) of total coliform (TC), ESBL coliform (addition of ESBL supplement to agar), and carbapenem-resistant bacteria (CPB-0.5 and CPB-2; addition of meropenem in dimethyl sulfoxide (DMSO) to agar at final concentrations of 0.5 and 2 μg/mL) (all Sigma-Aldrich). Each ESBL plate contained following antibiotics in final concentrations: ceftazidime (3 μg/mL), cefotaxime (3 μg/mL), ceftriaxone (2 μg/mL), aztreonam (2 μg/mL), fluconazole (10 μg/mL).[46] Meropenem concentrations were selected based on preliminary screening experiments[47] and the intermediate meropenem CLSI minimum inhibitory concentration (MIC) breakpoint for *Enterobacteriaceae* of 2 μg/mL.[48] ChromoSelect agar allowed visual differentiation of presumptive *Escherichia coli* (subsequently referred to as *E. coli*[49]) versus other coliforms. Where necessary, water samples were diluted with sterile phosphate-buffered saline (VWR) to achieve 30−300 CFU per plate in three technical replicates.[50] Each plate was provided 100 or 200 μL of sample and incubated at 37 °C for 24 h. Negative controls and blanks were intermittently tested to verify that in-lab contamination was minimized. CPB-2 were only measured for trips II−IV.

Antibiotics Analysis. Solid-phase extraction (SPE) coupled with ultrahigh-performance liquid chromatography−tandem mass spectrometry (UHPLC-MS/MS) was used to quantify 22 antibiotics belonging to seven classes: β-lactams, lincosamides, macrolides, quinolones/fluoroquinolones, sulfonamides, tetracyclines, and others (see the SI for details and Tables S4 and S5). River antibiotic concentrations were compared to predicted no effect concentrations (PNECs).[51] PNECs are predictive indicators and only provide a relative sense of possible selection.

Antibiotic-Resistant Gene Quantification. River samples were analyzed using high-throughput quantitative polymerase chain reaction (HT-qPCR) for 283 ARGs (36 aminoglycosides, 52 β-lactams, nine fluoroquinolone (FCA, quinolone, florfenicol, chloramphenicol, and amphenicol ARGs), 46 MLSB (macrolide−lincosamide−streptogramin B ARGs), 51 nonspecific efflux pumps, seven sulfonamides, 39 tetracycline, 32 vancomycin, 11 others), 12 mobile genetic elements (MGEs; eight transposases, four integrases), and one 16S rRNA gene (SI Table S6).

The water samples were filtered onto 0.22 μm cellulose-nitrate filters (Sartorius) to extract DNA with the FastDNA SPIN kit for soil (MP Biomedicals). Filtration volume varied depending on the sampling point (3 technical replicates of 80−250 mL each) with more water being filtered from upstream location S1 to collect sufficient DNA. The product DNA was cleaned with the QIAquick Nucleotide Removal Kit (Qiagen). DNA quality and quantity were measured with the NanoDrop and Qubit dsDNA HS assay (both Thermo Fisher Scientific), respectively. DNA absorbance ratios were 260/280 > 1.8 and 260/230 > 1.5. Replicate samples were pooled in equal DNA aliquots to reach 2 μg DNA and freeze-dried prior to further analysis. Between analysis steps, DNA was stored at −20 °C.

HT-qPCR was performed with SmartChip Real-Time PCR (Wafergen) as previously described.[52,53] Amplification efficiency always was between 90 and 110% and detection only was confirmed when all three technical replicates were positive. Relative copy number of ARGs and MGEs were calculated and transformed to absolute copy numbers by multiplying with 16S rRNA concentration for each sample. ARG and MGE cell concentrations were estimated by dividing the 16S rRNA concentration by 4.1, the estimated average 16S rRNA gene copy number per bacterium.[54]

Statistical Analysis and Data Visualization. Data can be accessed through the Center for Open Science, OSF (Ott, Amelie. 2021. "Monitoring and Modeling of Antibiotic Resistance in Southeast Asian Rivers". OSF. https://osf.io/gcpky/?view_only=90e614c2c6b64483aa503694af113789). Statistical analysis was performed in R.[55] Graphics were created using R package *ggplot2* version 3.3.3[56] and finalized in Inkscape.[57] The Skudai catchment map was composed in *ArcGIS* version 10.6.1.[58] The river catchment was extracted through digital elevation model (DEM) slope analysis.[59] Mass loading data was calculated by multiplying concentration data with the corresponding measured discharge (m^3/s) for each sampling site and trip.

The substitution method R2D was used to allow statistical analysis of left-censored data (e.g., antibiotic and coliform data).[60] For this, measurements under detection limit were substituted with $\sqrt{2}/2$ times the limit of detection, but only if less than 40% of all data points were under the detection limit.[60] Parameters with higher rates of "nondetects" were excluded from statistical analyses. Averages are reported as the mean with ± standard deviation (based on three or four biological replicates) throughout the paper.

Statistical significance testing employed P values and calculated Cohen's D effect sizes[61,62] to assess spatial and seasonal differences in water quality and AR parameters. Large statistically significant spatial or seasonal effects were defined for values of Cohen's D <−0.8 or >0.8 and $P < 0.05$.[61] Effect sizes can be negative or positive, depending on which mean is greater. Wet vs dry season data were compared with paired t-tests and corresponding Cohen's Ds (eq 1). Up- (S1) vs downstream (S8) data were compared with Welch's t-tests[63] and corresponding Cohen's Ds (eq 2).

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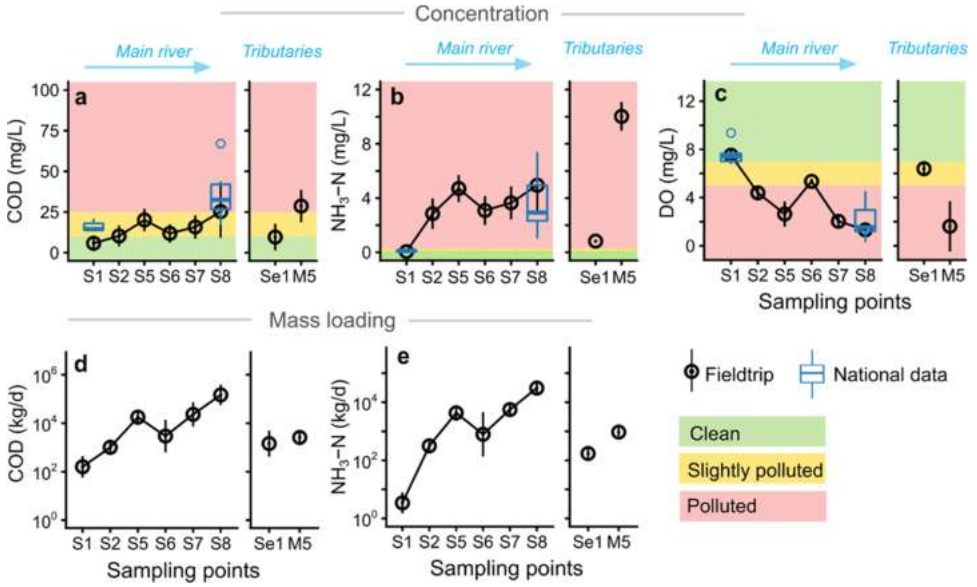

Figure 1. Chemical oxygen demand (COD; a, d), ammonia (NH_3–N; b, e), and dissolved oxygen (DO, c) concentrations (a–c) and mass loadings (d, e) in the river catchment. Data represented is based on four biological replicates for the main river (S1, S2, S5, S6, S7, and S8) and on three biological replicates for the tributaries (Se1, M5). Concentrations were compared to Malaysian water quality thresholds and Department of Environmental (DOE) monitoring data for S1 (DOE sampling point 3SI09) and S8 (DOE sampling point 3SI05). d: day.

$$\text{Cohen's D effect size for paired } t\text{ - test} = \frac{\text{mean}_A - \text{mean}_B}{\text{standard deviation}_A - \text{standard deviation}_B} \quad (1)$$

$$\text{Cohen's D effect size for Welch's } t\text{ - test} = \frac{\text{mean}_A - \text{mean}_B}{\sqrt{(\text{variance}_A + \text{variance}_B)/2}} \quad (2)$$

Benjamini–Hochberg P-adjustment was applied to correct for multiple testing.[64] Cohen's Ds were calculated with the "cohen.d" function in the R package *effsize* version 0.8.1.[65] Normality was assessed with the Shapiro–Wilk test. Where necessary, parameters were transformed using the Box–Cox transformation,[66] as implemented in the "boxcox" function in the R package *MASS* version 7.3–53.1[67] (SI Table S7). To visualize spatial and seasonal effects, Cohen's D effect sizes were plotted against *P* values for each parameter in volcano plots, using the R package *EnhancedVolcano* version 1.8.0.[68] To analyze water quality and AR parameter associations, Spearman's correlations were calculated with Benjamini–Hochberg multiple testing correction, using R packages *psych* version 2.1.3[69] and *corrplot* version 0.84.[70]

RESULTS

Water Quality and Microbiology. Water quality conditions in the catchment were characterized by generally low DO, high COD, and very high NH_3–N concentrations based on national Malaysian thresholds (Figure 1a–c, SI Tables S8–S10). Water quality declined in the Skudai from upstream being clean/slightly polluted (S1: 7.5 ± 0.5 DO mg/L, 0.05 ± 0.03 NH_3–N mg/L, 5.8 ± 4.8 COD mg/L) to downstream being slightly polluted/polluted (S8: 1.3 ± 0.3 mg DO/L, 4.9 ± 2 NH_3–N mg/L, 25.3 ± 16 COD mg/L). Measurements for DO, COD, and NH_3–N aligned well with the national 2018 DOE monitoring data (Figure 1a–c).

Total coliform and β-lactam-resistant coliform concentrations all increased from upstream S1 ((1.1 ± 0.5) × 10^3 TC CFU/mL, (1.5 ± 1.3) × 10^2 ESBL coliform CFU/mL, (3.1 ± 4.1) × 10^1 CRB-2 CFU/mL) to downstream S8 ((4.1 ± 3.3) × 10^4 TC CFU/mL, (4 ± 4.1) × 10^3 ESBL coliform CFU/mL, (1.1 ± 0.2) × 10^2 CRB-2 CFU/mL) in the Skudai river (Figure S2 and Table S11). Across the catchment, we observed an approximately one $\log_{10}$ difference between TC > ESBL coliform > CRB-2 concentrations, meaning that ∼10% of total coliform produced ESBL and ∼1% of total coliform were resistant to 2 μg/mL meropenem. *E. coli* and ESBL *E. coli* concentrations increased from upstream S1 (3.5 ± 2) × 10^1 CFU/mL and (<0.5–2) × 10^1 CFU/mL, respectively, to downstream S8 (2.8 ± 2.1) × 10^3 CFU/mL and (<0.1–5) × 10^2 CFU/mL, respectively (Table S11).

Volumetric flowrate in the Skudai increased greatly from upstream (S1: 0.5 ± 0.3 m^3/s) to downstream (S8: 82.7 ± 30.7 m^3/s) with small variations observed between seasons (SI Table S8). Compared with concentration data, mass loadings showed much greater chemical and microbial pollutant transport down the Skudai river from rural to urban locations. NH_3–N concentrations increased almost 100-fold from S1 to S8, whereas NH_3-N mass loadings were >14 000-fold greater along the same reach (SI Tables S9 and S10). Similarly, TC, ESBL coliform, CRB-0.5, and CRB-2 concentrations increased 10^0- to 10^1-fold from S1 to S8 while mass loadings increased 10^2- to 10^3-fold (SI Tables S11 and S12).

Interestingly, water and microbial quality improved slightly mid-stream at S6 for most parameters in concentrations and mass loadings (Figure 1, SI Figure S2). However, water quality was much poorer in the heavily urbanized Melana tributary (M5), both relative to the Skudai itself and the predominantly rural Senai tributary (Se1) (SI Tables S8, S9, and S11). As indicator of conditions, elevated CRB-2 and CRB-0.5 *E. coli* levels only were found in the Melana tributary across the catchment (SI Table S11).

Antibiotics Levels. Out of 22 antibiotics tested (SI Table S4), eight antibiotics (meropenem, cefixime, ceftazidime, erythromycin, chlortetracycline, minocycline, oxytetracycline, tetracycline) were not detected in the Skudai catchment. Six

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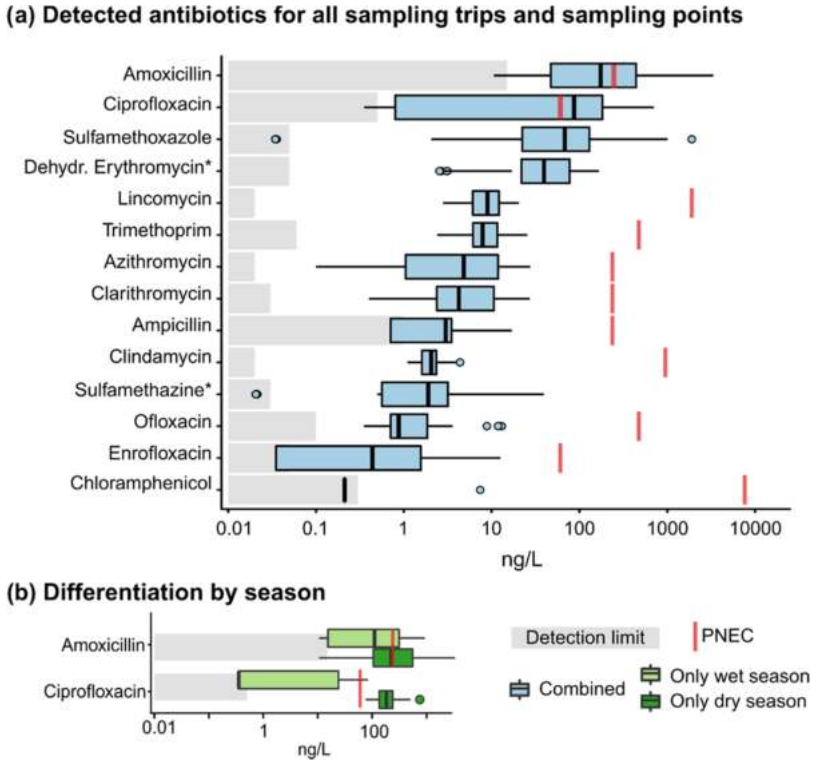

Figure 2. Antibiotic concentrations detected in the river catchment (n = 30) (a) with seasonal differentiation for amoxicillin and ciprofloxacin (b), compared to predicted no effect concentrations (PNEC).[51]

antibiotics/antibiotic derivates (clindamycin, lincomycin, azithromycin, clarithromycin, dehydrated erythromycin, trimethoprim) were detected in all river samples. Highest concentrations were observed for amoxicillin (all samples 510 ± 906 ng/L; max 3336 ng/L at S2), sulfamethoxazole (all samples 181 ± 383 ng/L; max 1933 ng/L at S8), and ciprofloxacin (all samples 131 ± 162 ng/L; max 705 ng/L at M5) with maximum values always detected in dry season samples (Figure 2). Only amoxicillin and ciprofloxacin were detected above PNEC values[51] with all ciprofloxacin and 50% of amoxicillin measurements in the dry season exceeding the PNEC thresholds.

In more than 40% of the samples, ampicillin and chloramphenicol concentrations were under the detection limit. Consequently, only 14/16 detected antibiotics were summarized into "total antibiotics" (SI Tables S9 and S10). Total antibiotics concentrations increased from up- (S1: 0.07 ± 0.05 mg/L) to downstream (S8: 1.27 ± 0.98 mg/L) and were higher in the dry than wet season.

Antibiotic-Resistant Gene Abundances. We detected 210 different ARGs (74% of assay) in the river catchment with 78 ARGs (28% of those assayed) shared between all river water samples (n = 30). All 12 MGEs assayed were detected in the catchment with nine MGEs (75% of assay) shared across all samples (n = 30). ARG and MGE levels increased from up- to downstream in the Skudai river (SI Tables S13−S17), except for lower levels found mid-stream at S6 (Figure 3), which parallels water quality conditions based on other measured parameters.

The number of detected ARGs increased from 119 ± 14 at S1 to 150 ± 8 at S8 (SI Table S13). Increases in ARG diversity were most apparent at the top of the river. The most upstream site, rural S1, and the next site, semiurban S2, shared a core resistome of 157 ARGs and MGEs (Figure 4). However, only five unique ARGs were detected at S1, whereas 41 unique ARGs (such as *bla*CTX-M and *vanA*) and 1 MGE were detected at S2.

On a wider scale, ARG and MGE concentrations increased more than 10^2-fold from up- to downstream (S8: 1.2 ± 0.9 × 10^8 ARG copies/mL and 1.1 ± 0.9 × 10^8 MGE copies/mL), while ARG and MGE mass loadings increased more than 10^5-fold from up- to downstream (S8: 8.6 ± 7.2 × 10^{20} ARG copies/d and 8.1 ± 7.3 × 10^{20} MGE copies/d; SI Table S13). The normalized copy number of ARGs and MGEs per cell increased from 0.1 ± 0.1 and 0.1 ± 0 upstream to 1.7 ± 0.6 and 1.6 ± 0.6 downstream, respectively. Detected numbers, concentrations, and normalized copy numbers for ARGs and MGEs were higher in both tributaries (M5 and Se1) than downstream in the Skudai river (S8) (SI Tables S13−S17).

Assessing Seasonal and Spatial Effects. Dimensionless Cohen's D effect sizes were calculated to inform the magnitude of spatial (up- vs downstream) and seasonal effects on water quality and AR levels. Reporting standardized effect sizes in concert with P values allows one to better compare findings within and across studies.[71] This is particularly important for LMIC settings where limited data availability hinders the identification of environmental AR "hot spots".

Seasonality only significantly influenced observed total antibiotic concentrations (paired t-test with $P < 0.05$ and large Cohen's D effect size >0.8, SI Table S18). For all other parameters, season did not have any significant effects on concentration or mass loading data (Figure 5a, SI Table S18). Conversely, spatial differences (up- vs downstream) were significant for all parameters, and more apparent in mass loading data (Cohen's D range −13.9 for S16 rRNA to −6.8 for ESBL coliform, SI Table S19) versus concentration data

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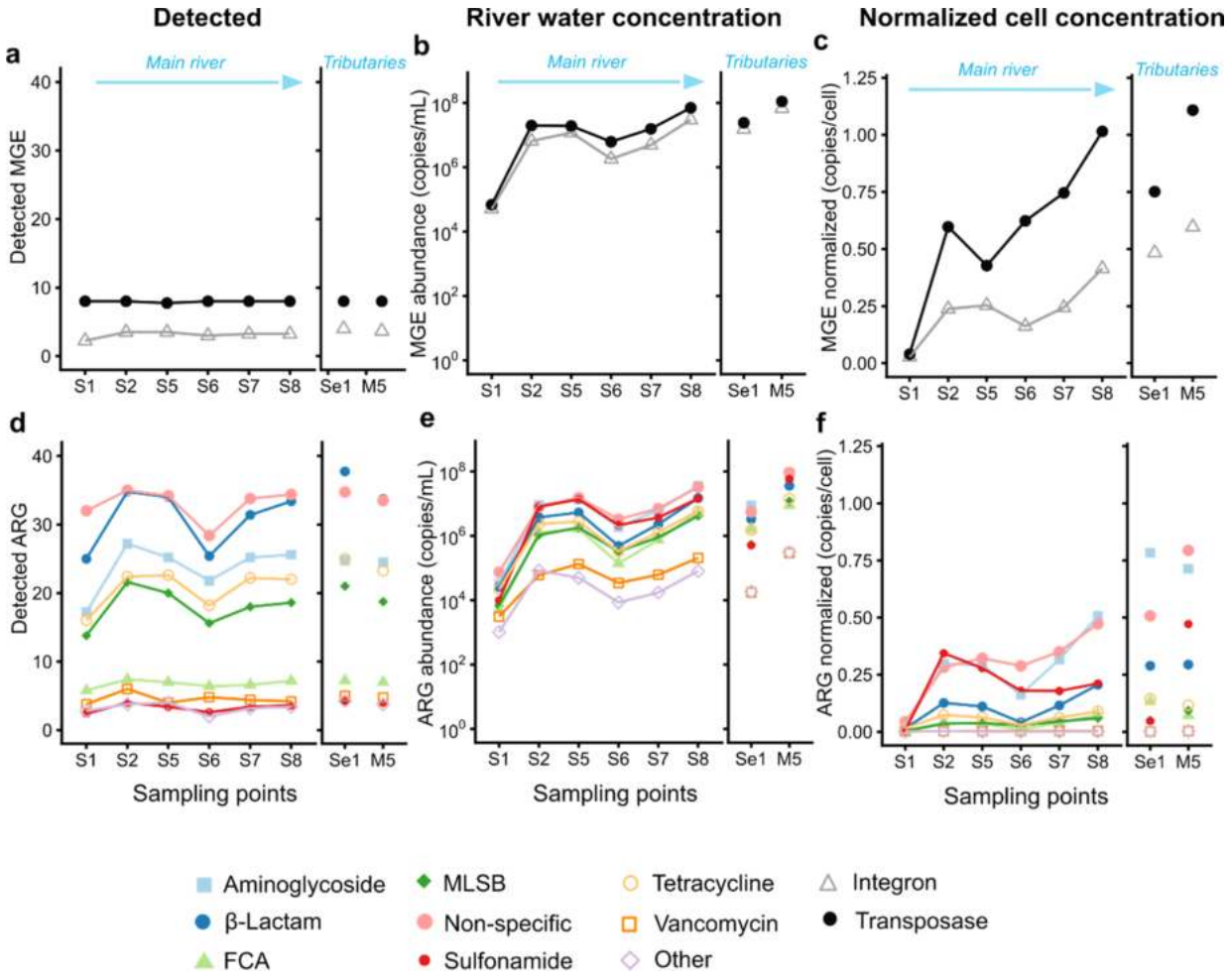

Figure 3. Antibiotic-resistant gene (ARG) and mobile genetic element (MGE) detected (a, d), river water concentrations (b, e) and normalized cell concentration (c, f) measured with HT-qPCR per sampling point in the Skudai catchment. Mean data represented is based on four biological replicates for the main river (S1, S2, S5, S6, S7, S8) and on three biological replicates for the tributaries (Se1, M5). For standard deviations, see SI Tables S14–S17. FCA: fluoroquinolone, quinolone, florfenicol, chloramphenicol, and amphenicol ARGs. MLSB: macrolide–lincosamide–streptogramin B ARGs.

(Cohen's D range MGE −6.85 to −1.6 for CRB-0.5, SI Table S19). For concentrations, the largest Cohen's D effect sizes were observed for DO (Cohen's D 15.6), MGE, and ARG river water concentrations (Cohen's D −6.5 for ARG and −6.85 for MGE) (Figure 5b).

Defining a Surrogate Marker for Antibiotic Resistance. Spearman correlation analysis was performed between all monitored parameters to identify possible "easy-to-measure" surrogates that associated with elevated AR in the catchment (Figure 6). For this, we focused on correlations between AR indicators (ESBL coliform, ESBL *E. coli*, total antibiotics, total ARGs, total MGEs, int1) and physicochemical water quality parameters (temperature, pH, DO, conductivity, NH_3–N, COD, TN, TP). These standard water quality parameters also are included in the Malaysian river water quality monitoring program.[72] Out of the physicochemical water quality parameters, DO and NH_3–N correlated strongest with total ARGs, the sum of all ARG copy number concentrations in river water (Spearman's ρ 0.81 and 0.83 with $P < 0.05$, respectively). Within the AR indicators, total ARGs correlated strongly with int1 (Spearman's ρ 0.98, $P < 0.05$) but less so with total antibiotics (Spearman's ρ 0.7, $P < 0.05$). When comparing correlations between total ARGs and each ARG class with total versus individual antibiotic concentrations, the strongest correlations always were between total ARGs and total antibiotics (SI Table S20). This was even true when comparing amoxicillin and ciprofloxacin with their ARG class, suggesting specific selection by individual antibiotics is not evident, even the detected antibiotics near their PNEC levels.

DISCUSSION

Comprehensive Environmental Antibiotic Resistance Monitoring. Discharge and mass loadings are rarely estimated in environmental AR monitoring studies. However, we show that both concentration and loading data provide valuable complementary information to understand processes occurring in a river catchment. In the Skudai, river health improved midstream at the semiurban sampling point S6 despite worse water quality conditions further up- and downstream. Considering the combination of lower pollutant concentrations and mass loadings, this was likely caused by a combination of reduced wastewater entering the river in this more agricultural reach (in comparison to more urbanized reaches up- and downstream) while simultaneously, rain and/or groundwater and/or cleaner tributaries (e.g., Senai) continued to dilute the river water with

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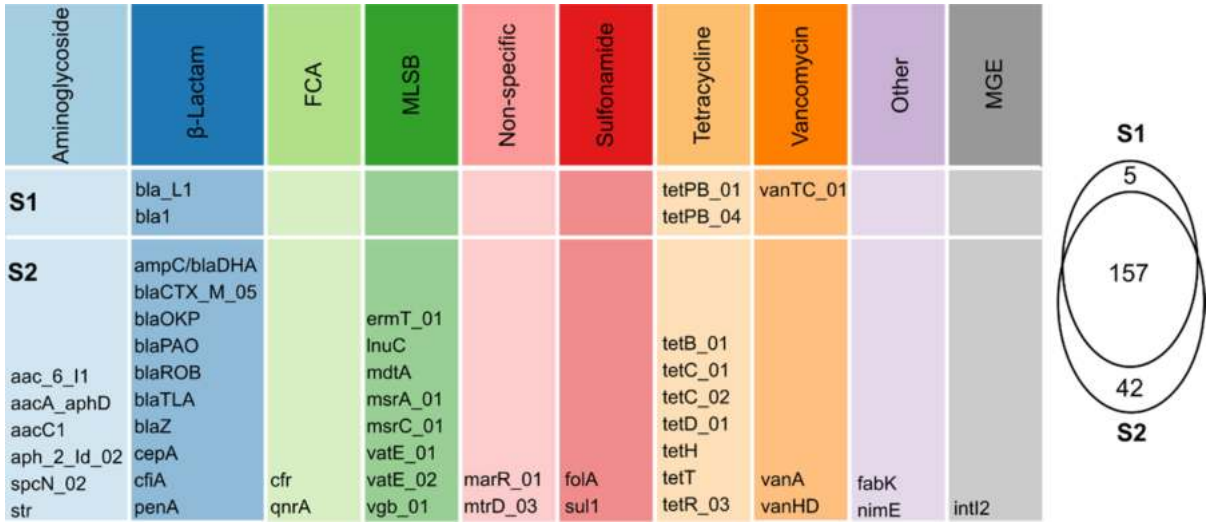

Figure 4. Differences in antibiotic-resistant gene (ARG) and mobile genetic element (MGE) detection between the most upstream rural sampling point S1 and the next, semiurban sampling point S2 on the Skudai. The Venn diagram indicates the number of ARGs and MGEs only detected at S1 (5), the number of shared ARGs and MGEs between S1 and S2 (157), and the number of ARGs and MGEs only detected at S2 (42). Data based on four biological replicates. FCA: fluoroquinolone, quinolone, florfenicol, chloramphenicol, and amphenicol ARGs. MLSB: macrolide−lincosamide−streptogramin B ARGs.

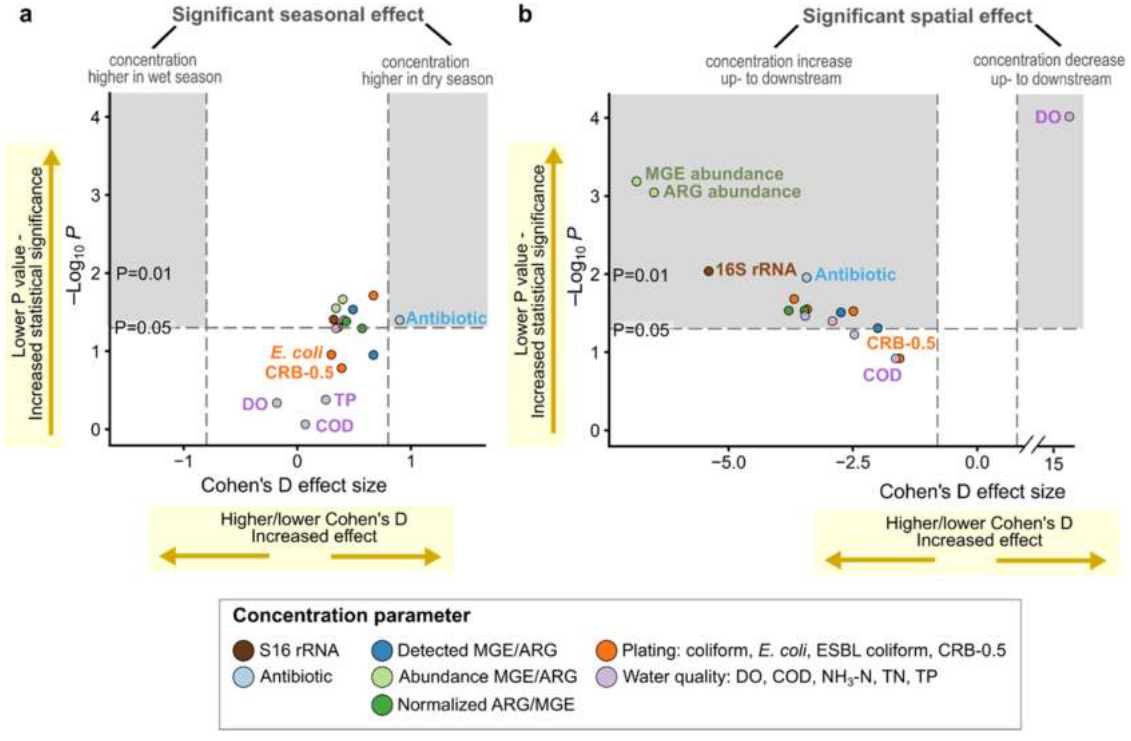

Figure 5. Comparing the effect of seasonality (a) and spatial variation between up- (S1) and downstream (S8) (b) for concentration parameters, based on statistical significance and Cohen's D effect size. Statistical comparisons performed with the paired *t*-test or Welch's *t*-test with Benjamini−Hochberg multiple testing correction. A high $-\mathrm{Log}_{10} P$ value indicates high statistical significance with $-\mathrm{Log}_{10} P(2) = P$ (0.01) and $-\mathrm{Log}_{10} P(3) = P(0.001)$. A Cohen's D effect size over 0.8 or under −0.8 indicates a large seasonal or spatial effect on the parameter. Only selected parameters are labeled; for more details, see SI Tables S18 and S19. ARG: antibiotic-resistant genes. COD: chemical oxygen demand. CRB-0.5: carbapenem-resistant bacteria selected for with 0.5 μg/mL meropenem. ESBL: extended-spectrum β-lactamase. DO: dissolved oxygen. MGE: mobile genetic elements. NH_3−N: ammonia. TC: total coliform. TN: total nitrogen. TP: total phosphorus.

pollutants degrading and/or settling to the sediment.[73] More accurate methods exist to estimate flow than the applied float method. However, the easy and cost-effective application makes the float method particularly suitable for countries with limited resources.[74]

Accounting for volumetric flow is particularly important for countries with dry and wet seasons. Comparing total antibiotic concentrations and mass loadings, we demonstrate that while antibiotic releases into the catchment likely do not vary across seasons for this catchment, reduced rainfall during the dry season resulted in increased river antibiotic concentrations and

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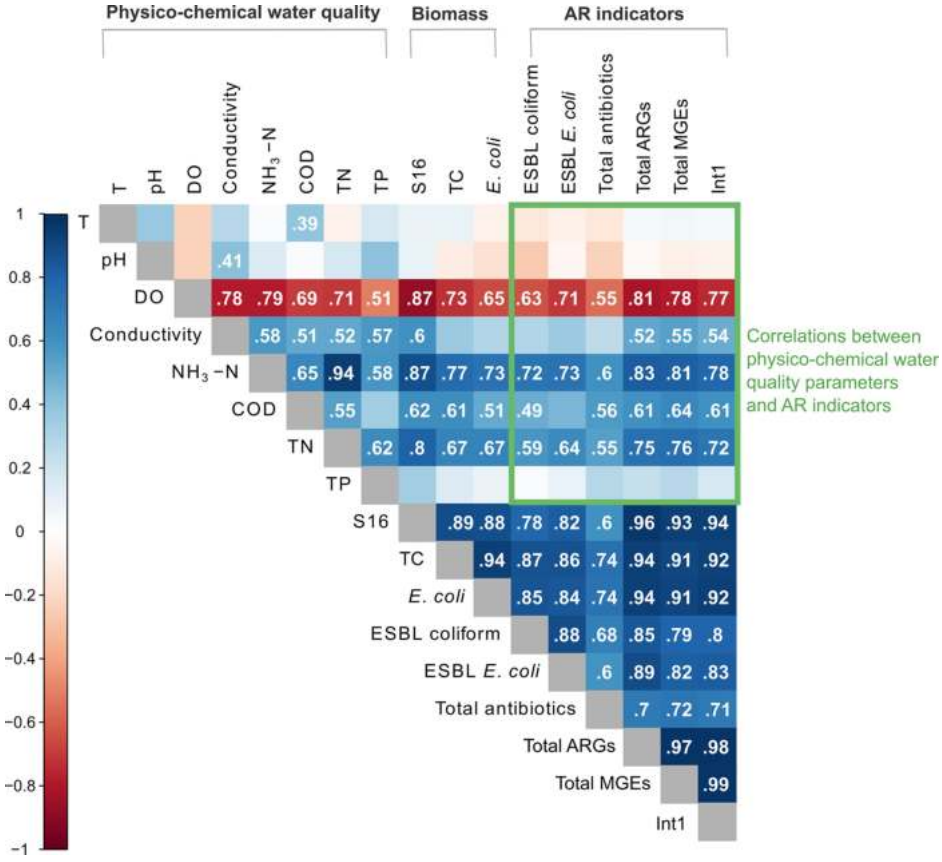

Figure 6. Spearman correlations between selected physicochemical water quality, biomass, and antibiotic resistance (AR) concentrations for the river catchment (n = 30). Correlation values only shown for $P < 0.05$ with P values corrected for multiple testing with the Benjamini−Hochberg approach. ARG: antibiotic-resistant genes. COD: chemical oxygen demand. DO: dissolved oxygen. MGE: mobile genetic elements. NH_3−N: ammonia. S16: S16 rRNA gene. T: temperature. TC: total coliform. TN: total nitrogen. TP: total phosphorus.

consequently, increased exposure. Seasonality is expected to have a much larger effect on water quality/AR parameters in other SE Asian regions with more pronounced dry/wet seasons than here in southern peninsular Malaysia.

The highest antibiotic concentrations in the catchment based on both, maximum and mean, were recorded for amoxicillin, ciprofloxacin, and sulfamethoxazole. A 2014 study found amoxicillin to be the most prescribed antibiotic in Malaysia.[75] Mean amoxicillin river concentrations were higher than previously recorded for European treated wastewater treatment plant effluents and surface waters.[76−78] Sulfamethoxazole and ciprofloxacin concentrations were higher than previously recorded for Malaysian surface waters,[79,80] but comparable to some other East and SE Asian surface water studies.[81] There is limited knowledge on which environmental antibiotic concentrations select for resistant bacteria.[51,82] In this study, only amoxicillin and ciprofloxacin exceeded the PNEC thresholds,[51] particularly during the dry season.

Comparing the Skudai ARG concentrations to other ARG HT-qPCR studies based on the same primer sets and analytical methods (SI Figure S3), we found the upstream ARG levels to be comparable to previous findings in upstream Chinese river reaches (10^5−10^6 ARG copies/mL[83,84]). Downstream Skudai ARG concentrations (~10^8 ARG copies/mL, this study and ref 83) were similar to wastewater treatment effluent ARG concentrations (10^7−10^9 ARG copies/mL) recorded in Spain and China but lower than influent ARG concentrations (10^9−10^{10} ARG copies/mL) from those same studies.[73,85] The detected number of ARGs upstream in the Skudai was higher than in any other of the reported upstream river water, upstream river sediment, lake, or soil samples. The number of detected ARGs downstream in this study also was the highest across all cited studies.

Movement from the rural (S1) to semiurban (S2) locale added over 40 additional genes, many associated with fecal matter and multidrug resistance, such as *bla*CTX-M and *vanA*. *Bla*CTX-M encodes for elevated resistance to β-lactam antibiotics.[86] *VanA* is a plasmid borne gene that confers high resistance to vancomycin and is most commonly associated with *Enterococcus faecium* and *Enterococcus faecalis*.[87] The S1 to S2 reach has limited wastewater treatment, which likely introduced these ARGs into the river, suggesting limited local wastewater treatment may be the dominant source of AR genes in this part of the river, which also was seen in an AR estuary study in southern Malaysian.[33]

Reporting Standardized Effect Sizes. Effect sizes are commonly applied in bioinformatics, medical drug trials, and meta-analysis.[88] However, to the best of our knowledge, this is the first study to apply the principle of standardized effect sizes to AR/river water quality monitoring. While unstandardized effect size statistics such as mean differences are important, additional reporting of standardized, dimensionless effect sizes such as Cohen's D effect size allows one to more easily compare seasonal and spatial effects on various parameters.[71] This is particularly crucial for understanding and comparing results from environmental AR monitoring studies where

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analysis costs are high, resulting in little available data, mostly existing for HICs.[12] Routine reporting of effect sizes will encourage researchers to view their results in the context of previous studies and facilitate the incorporation of results into future meta-analysis.[71] We support Nakawaga and Cuthill in their encouragement to report effect size statistics and their confidence intervals in all biological journals.[71] Using volcano plots, we provide an easy way to visualize seasonal and spatial effects together with *P* values to compare different water quality and AR parameters. For concentration data, we observed the largest statistically significant spatial effects (up vs downstream) for ARG, MGE, and DO concentrations. Spatial effects were even larger for all parameters based on their mass loadings than concentrations. This is not surprising when considering that the Skudai river increases in depth and particularly, width from 5 m at the most upstream sampling point to 75 m at the most downstream sampling point. For this study, we applied the Cohen's D threshold of over 0.8 or under −0.8 to define a large effect size as originally proposed by Cohen for behavioral studies.[89] However, depending on the study design, this threshold can be adapted.

Surrogate Marker for Predicting Antibiotic Resistance. River water in more urban areas of the Skudai catchment are characterized by higher NH_3−N and lower DO levels, both indicators of wastewater pollution.[90] When sewage enters a river, the organic matter- and nitrogen-containing components are oxidized, decreasing DO levels.[91] This process has been known for many years and is mathematically described by DO sag curves.[92] Based on our data and local water quality thresholds, the Skudai catchment is classified in the slightly polluted to polluted range, which aligns with the Malaysian DOE classification.[93] Our DO and NH_3−N data aligns well with the long-term national Malaysian data set (Figure 1b,c), suggesting that our correlations between these parameters and AR markers might be used to extend existing Malaysian data sets to AR prediction, in theory suggesting places of elevated AR, using modeling, where no current AR data exists.

Modeling represents an efficient, cost-effective tool for LMICs to identify AR hot spots in rivers and propose engineering and/or social interventions. However, while many river water quality models exist,[94−96] no standardized, hydrological model yet includes an AR component. Conversely, many LMICs, including Malaysia,[93] operate long-standing national river monitoring programmes, but these do not capture AR either. Here, we investigated which easy-to-measure water quality parameter included in well-established river water quality models and captured in the Malaysian river water quality monitoring programme could act as a surrogate to predict AR levels in rivers with no/limited AR background data.

For the Skudai catchment, we found DO and NH_3−N exhibit the strongest correlations with high total ARG concentrations. This does not mean that low DO or high NH_3−N themselves directly causes higher ARG levels (or vice versa), although horizontal gene transfer (HGT) frequency can be higher under low-oxygen conditions,[97] suggesting that lower DO may increase local ARG HGT. In this catchment, lower DO and higher NH_3−N are likely associated with insufficiently treated sewage entering the river, which is probably also the major route for ARGs entering the river.

Given the above, DO is well suited as a surrogate for AR as it can easily be measured with a hand-held probe, relative differences often mirror sewage inputs, and DO potentially impacts in situ HGT frequency. DO is also one of the most commonly modeled indicators of stream, river, and lake health with a vast array of models available.[98] Consequently, we propose that for this catchment, DO concentrations are a useful surrogate to understand previous AR levels and model future AR levels. Future work should evaluate the applicability of this surrogate for other catchments in Malaysia and SE Asia. However, for such surrogates to have the greatest value, they should be coupled with other predictive AR approaches that do not heavily rely on directly monitored data, such as genomic and other modeling tools for AR bacteria.[99−101]

Interestingly, within the AR indicators, total antibiotic concentrations exhibited the lowest correlations with other AR parameters. The weaker correlation of total antibiotics with the other AR parameters might be due to the fact that many antibiotics quickly degrade in the environment while some ARGs and ARBs persist for longer.[102] However, even in the Skudai river that has relatively high antibiotic levels, any selective effect of antibiotics is probably minor (Figure 2) compared with the greater load of ARGs entering the river through less treated wastewater (Figure 3). This is best exemplified by the many "new" ARGs entering the river between S1 and S2 (Figure 4), which dwarfs any effect of antibiotics themselves. This does not mean low levels of antibiotics are incapable of influencing ARG selection in aquatic systems,[103] but data here suggest untreated sewage inputs have a much greater impact than in situ antibiotics on AR in catchments like the Skudai.

Taken together, this study shows that simple water quality markers, like DO and NH_3−N, can be valuable surrogates for local stakeholders to identify AR hot spots in rivers to target interventions. This does not mean that they are universally applicable, such as near major nonsewage organic waste inputs. However, DO and NH_3−N clearly mirror sewage, which often dominates ARG and AR bacteria inputs, especially in LMIC rivers. DO and NH_3−N also are inexpensive to measure and already exist in current monitoring programmes. Therefore, we propose DO and NH_3−N as the "first point of call" surrogates for AR in rivers. They clearly can be coupled with parameters such as ESBL *E. coli* for environmental AR monitoring, which the WHO is already using to monitor AR across environments (Tricycle project[104]). However, DO and NH_3−N are more amenable to water quality modeling, which might ultimately be the most affordable way of identifying AR "hot spots" in places with limited existing data.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c00939.

Methodological details on the antibiotics analysis; map of Skudai catchment with sampling points (Figure S1); total coliform, ESBL coliform, and carbapenem-resistant bacterial concentrations and mass loadings in the river catchment (Figure S2); comparison of HT-qPCR ARG detection and concentrations across different studies (Figure S3); sampling point coordinates (Table S1); modified water quality classes in Malaysia (Table S2); Malaysian Department of Environment water quality for the Skudai catchment (Table S3); monitored antibiotics from the Skudai river catchment across the four

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sampling campaigns (Table S4); relative SPE recovery, method detection limit (MDL), and method quantification limit (MQL) for antibiotics in surface water (Table S5); ARG and MGE primer list for the HT-qPCR (Table S6); Box−Cox transformations prior statistical analysis (Table S7); monitored physical parameters per sampling point across the four sampling campaigns (Table S8); monitored chemical parameters per sampling point across the four campaigns (Table S9); chemical mass loadings at each sampling point across the four campaigns (Table S10); coliform concentrations at each sampling point in CFUs in river water samples (Table S11); coliform mass loadings at each sampling point in CFUs per day (Table S12); summary of ARGs and MGEs in river water samples (Table S13); detected ARGs per antibiotic class in river water samples (Table S14); ARG concentration for each antibiotic class in river water samples (Table S15); normalized ARGs per cell in river water samples (Table S16); MGE concentrations in river water samples (Table S17); paired *t*-tests and Cohen's D effect sizes comparing seasonal mass loadings, concentrations, and detected numbers for water quality and antibiotic-resistant parameters (Table S18); Welch's *t*-test and Cohen's D effect sizes comparing spatial mass loadings, concentrations, and detected numbers for water quality and antibiotic-resistant parameters (Table S19); Spearman correlations between river water concentrations of total antibiotics, amoxicillin, ciprofloxacin, total ARGs, and ARGs reported by antibiotic class (Table S20) (PDF)

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Notes

The authors declare no competing financial interest.

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