Riverine antibacterial resistance gradient determined by environmental factors

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Abstract

Polluted waterbodies such as rivers provide a pathway or reservoir for bacterial resistance. We studied water quality and bacterial antibacterial resistance along the subtropical Qishan River in Taiwan as a case study of environmental resistance spread in a pristine to rural area. Human settlement densities increased generally from pristine mountain sites to the more polluted lowlands generally. Accordingly, as a working hypothesis, we expected antibacterial resistance level to increase towards downstream. We collected sediment samples from 8 stations along the Qishan river and where the Qishan river reaches the Kaoping river. The samples were processed in the lab for bacteriological and physicochemical analysis. Antibacterial resistance was tested with common antibacterials. A comparison was made among the sites where isolates began to occur at the upstream (site 1-6) with the downstream, including site 7 (Qishan town), site 8 (wastewater treatment plant) and site 9 (Kaoping river). The results of multivariate analysis for bacteriological and physicochemical parameters showed increasing water pollution levels downstream of the Qishan river. Bacterial isolates including *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Enterobacter* sp., *Acinetobacter* sp., *Staphylococcus* spp. and *Bacillus* spp. were analyzed and tested in the study. Their percentage of occurrence varied at each site. The resistance level was determined from the growth inhibition zone diameter (disk diffusion) and the minimum inhibitory concentration (micro-dilution). The results indicated that antibacterial resistance was related to certain environmental factors. Besides, the usage pattern of different classes of antibacterials in different places could alter trends of their resistance. Bacteria were found with increased resistance to antibacterials used in agriculture through the downstream sites. The WWTP emitting wastewater was demonstrated to be a hotspot of resistance in aquatic environments. In conclusion, bacterial resistance against antibacterials from the Qishan river has become a potential public health threat. This study could assist authorities by providing a reference for water quality risk assessment and management in Kaohsiung city and southern Taiwan.

Introduction

Human health risks associated with microbial pathogens provide an increasingly serious environmental and public health problem along rivers, in estuaries, and coastal zones which increased dramatically within a short time (Dahms 2018, Pereira et al. 2015). The most obvious ultimate sources of pathogens and fecal indicators in the aquatic environment are humans, animals and wildlife in general. Around 1.1 billion people worldwide lack clean water and 2.4 billion people had no access to sanitation in the late 2010s (Berendes et al. 2017). The more recent development and spread of antibacterial resistance is only one aspect of water pollution and unsafety worldwide (Wi et al. 2017). The present approach towards these issues contains a comparison of supposedly less contaminated places and highly contaminated places along a riverine gradient from highlands down to lowlands to understand real-time phenomena of antibacterial resistance on site.

The causal relationship between an ever increasing human population density along water ways and aquatic environmental changes are well known. Human health risks associated with resistant bacteria

are providing serious problems (Allen et al. 2010, Dahms 2018, O'Neill 2014). The misuse or overuse of antibacterials is a major cause of the development of resistant bacterial strains in normal as well as human pathogenic strains through evolutionary processes in time (Bengtsson-Palme & Larsson 2016, Holmes et al. 2016). The emissions of antibacterials, antibacterial resistant bacteria, and resistance genes into the aquatic environment cause antibacterial resistance among non-resistant bacterial communities for example through horizontal gene transfer by transduction, transfection and translation (von Wintersdorff et al. 2016). The source of these pollutants in the aquatic environments could be derived from domestic, clinical or industrial wastewater, or agricultural runoff. These wastewaters enter into water bodies and make surface waters, such as lakes and rivers, receiving sinks and reservoirs for antibacterial resistance (Cheng et al. 2020, Jia et al. 2018, Laquaz et al. 2020, Vaz-Moreira et al. 2014) The occurrence of antibacterial resistance is increasing in aquatic environments nowadays (Danner et al. 2019). Water from such reservoirs are commonly used as a drinking water source for humans but also for livestock in agriculture or fish in aquaculture. They themselves are sources of food for humans. The transfer of resistant bacteria from sewage sludge and manure to humans could ultimately occur via water or food (Danner et al. 2019, Ferri et al. 2017, Holmes et al. 2016). Transmission of resistance genes may occur within short periods of time so that antibacterial resistance would spread rapidly among bacterial communities.

The development and spread of antibacterial resistance are regarded as a universal threat of public health and environmental safety. It was predicted in a recent study that 10 million people will die per year by 2050 due to antibacterial resistance (Danner et al. 2019). Some studies demonstrated the significance of environmental settings such as water or soil as a pathway and reservoir for the spread of antibacterial resistance (Cheng et al. 2020, Rizzo et al. 2013, Sidrach-Cardona et al. 2014). Previous studies found antibacterial resistant bacteria in different water bodies including rivers, estuaries, lakes, and coastal waters. Water bodies are constantly exposed to environmental deterioration such as by wastewater pollution from domestic, agricultural, and industrial sources (Laquaz et al. 2020, Rizzo et al. 2013). As knowledge about the pressure and spread of resistant bacterial strains or genes in drinking/ recreational water of the coastal zone increases, new public health policies are providing awareness to academia and the public (W.H.O. 2015). Searching publicly available databases, only a few data could be found from the main water bodies of Taiwan (Miftahussurur &Yamaoka 2015, Rizzo et al. 2013).

The level of resistance to antibacterials by normal and pathogenic strains was increasing at an alarming rate for several decades (Danner et al. 2019). Since then, the uncontrolled use of pharmaceutical substances in industry, hospital, agriculture, and aquaculture has introduced several antibacterial to the aquatic environment (Bengtsson-Palme &Larsson 2016, Ryu et al. 2019, Van den Meersche et al. 2020). The abuse of antibacterials imposes new evolutionary pressure on non-pathogenic and pathogenic bacterial strains (Kummerer 2009b, a). In Taiwan, a large area of water bodies is used as a disposal and dumping place for medicinal, aqua-/ agricultural, industrial, and domestic wastes. However, there are very few reports about antibacterial resistance in aquatic ecosystems in Taiwan (Chang et al. 2007, Miftahussurur &Yamaoka 2015). The present study aims to identify and quantify bacterial antibacterial resistance from riverine waters of southern Taiwan.

The Qishan river, also called the Nanzihsiian river, originates at the foothills of Yushan Mountain in Namasia, in the northeast of Kaohsiung city. The terrain elevation varies greatly, with an average slope about 1/142 of the river bed. Due to uvial erosion, the undercutting of the river bank has formed many cliffs along the river. The river course is turbulent and meandering, flowing southwest for 65 km to the Jiasian district, where the channel begins to widen. It enters plain and merges with the Laonong river into the Kaoping river in Qishan district. The Qishan river has atotal length of 118 km and a drainage basin area of 842 km^2 (Yang 1997). Within the basin, agriculture dominates the socioeconomic structure with around 228.88 km^2 total agricultural area. The following administrative districts belong to the basin of the Qishan river: Namasia, Jiasian, Shanlin, Meinong, Qishan, Neimen, Taoyuan in Kaohsiung city, Alishan in Chiayi county, and Ligang in Pingtung county. Qishan river is the main tributary of the Kaoping river, of which the basin area covers the coastal region of Southern Taiwan. The upstream Qishan river fills the Nanhua Reservoir, which provides nearly 90% of the water supply for Tainan and Kaohsiung city. Its downstream supplies waters for domestic, agricultural, and industrial needs of various respects. The Kaoping river supplies water for domestic, agricultural, and industrial needs in Tainan city, Kaohsiung city, and Pingtung county (Yang 1997). Thus, the sanitation of the Qishan river could affects environmental and public health greatly. The Qishan river system provides a gradient of presumably pristine waters and adjacent environments to heavily populated and polluted areas where it discharges into the Kaoping river. From the river banks surface water is directly used for agricultural irrigation and drinking water supplies to husbandry. Since this provided health issues, we studied physicochemical properties and antibacterial resistance from the Qishan river. We collected eight sediment samples along the Qishan river and one at the confluence of the Qishan river and the Kaoping river.

The human population, settlement density, and husbandry area generally increase from pristine mountains to more polluted lowlands (Wang et al. 2019), so in the Qishan river catchment area. The upstream sampling sites (sites 1-6) belong to rural Namasia, Jiasian, and Shanlin districts with fewer human activities. We performed a physicochemical and bacteriological study to assess the water quality. From the results we could compare pollution extent among the sampling sites. Since the emitted water is from villages and towns with higher population densities in the Qishan district. The isolates from Qishan river are suspected to be more resistant (Hultman et al. 2018, Proia et al. 2018, Xu et al. 2015).

This study focusses on Qishan river water quality and bacterial antibacterial resistance from river samples. We obtained first-hand data about potential pathogenic resistant bacteria from the sampling sites. With the above aspects in view, detailed investigations of the research were pursuing the following objectives: to identify bacterial strains that represent communities with morphological and biochemical methods, to analyze physicochemical and bacteriological parameters, to perform a risk assessment and qualitative analysis, to compare antibacterial resistance levels among isolates from different sampling sites. We hypothesize an increase in the level of antibiotic resistance towards downstream.

Materials And Methods

Sample collection

We collected sediment samples at 9 sites along the Qishan river, from close to its source at the foot of Yushan Mountain (Xu et al. 2015) to its confluence with the Kaoping river (22°47'35.5"N 120°27'46.9"E (Google) in September 2020. The coordinates and GPS information were recorded from the Google map application ver. 10.39. 1. Sites 1-6 belong to the Qishan river upstream, sites 7-9 belong to the downstream (**Fig. 1**). Sampling was performed at about 10 km intervals on average along the course of the Qishan river, where it could be accessed at bridges or trails (**Table S1**). At each sampling site, we obtained water samples by using 1-liter sterile disposable bottles. Samples were stored at 4 ℃ and processed within 12 hours of collection (Moore et al. 2010, Vignesh et al. 2014, Vignesh et al. 2012). All samples were collected with precautions required for sterile microbiological sampling and personal protection.

Water quality testing

In this study, physicochemical and bacteriological parameters were included in the water quality analysis. First, we obtained the data of physicochemical parameters by instruments and bacteriological parameters by microbiological methods. And then we analyzed their variation along the river to see the pollution level change. Finally, we used these results for a multivariate analysis to detect possible interactions.

The physicochemical analysis of the water is assessed using several parameters amenable to water quality assessment. These are temperature, pH, conductivity, total dissolved solids, salinity, dissolved oxygen and biological oxygen demand. Temperature (Temp.) and potential of hydrogen (pH) were measured by immersing a portable sensor PH200 (CLEAN Instruments) into the field river water, conductivity (conduct.), total dissolved solids (TDS) and salinity (Sal.) by CON200 (CLEAN Instruments), dissolved oxygen (DO) by DO200 (CLEAN Instruments). After 3-5 minutes of immersion, each of these devices was removed for reading of the results. Biological oxygen demand (Lee &Nikraz 2015) was tested within 12 hours after sampling in the laboratory following a standard method (Baird 2017).

Bacterial strains were isolated and identified, and then used for antibacterial resistance testing. Bacterial numbers were estimated in each water sample by employing a plate count method. Samples were diluted to 10^{-2} with autoclaved river water and 100 µL of the sample solutions were spread on different agar media of Petri dishes. The isolation of bacteria was made using different growth media such as Nutrient agar for total colony counts and mostly present isolates, MacConkey agar for coliforms such as *E. coli* and *Enterobacteriaceae*, and Mannitol salt agar for *Staphylococcus* spp. Bacterial counts were used as indicators for water quality. The representation, which is the occurrence ratio of each isolate, was also calculated since previous studies have documented that polluted waters could impact the bacterial community composition (Lu et al. 2017, Tang et al. 2016).

The spread plates were incubated at 35 °C for 16-18 hours. Bacterial colonies were then developed and colony forming units (CFUs) were then counted from single bacterial cell counts. From each agar we told the difference among the colonies by their characteristics (color, size, texture, or borders etc.) (**Table 2**).

This way the number of viable counts per sample unit and their density within the original sample were calculated and expressed as the number of colony forming units (CFUs) per 1 mL of the sample. Our first steps were sub-culturing and isolating morphologically different CFUs until pure, uncontaminated cultures were obtained, followed by DNA extraction from bacterial cells. For 16S rRNA identification, genomic DNA extraction was performed using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, Waltham, MA, USA). After DNA extraction, we checked whether the isolation was successful and verified its presence by 0.8% agarose gel electrophoresis. Then Polymerase chain reaction (PCR) was performed in 50 µL volume which consisted of 1 × Taq buffer, 0.2 mM of dNTP mix each, 1 µM of each of the reverse and forward primers, 100 ng template DNA, 1.25 U Taq DNA polymerase (New England Biolabs) and sterile distilled water. The two universal primers (27F 5'-AGAGTTTGATCCTGGCTCAG3'; and 1492R: 5'-GGTTACCTTGTTACGACTT-3') were used to amplify the 16S rRNA gene sequence. The following cycling conditions were used: initial denaturation 95 °C for 5 mins, 35 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and 40 secs, and final elongation at 72 °C for 10 min. For purification the GeneJet PCR purification kit (Thermo Scientific, Waltham, MA, USA) was used. Finally, unknown sequences were compared with reference sequences from the NCBI database by BLAST-analysis to identify the bacterial strains. Gene sequences were aligned using the software Clustal W ver. 1.83. The gene sequences of strains were analyzed in the National Center for Biotechnological Information (NCBI) and an accession number is in the process of being obtained.

Multivariate statistics such as principal component analysis (PCA) have been used to demonstrate some of the variation in different samples. PCA has been adopted in a variety of scientific studies for simplifying a large volume of datasets containing several variables, e.g., the physicochemical characterization of surface water (Tanor et al. 2014), wastewater sludge (Tanor et al. 2016), different animal manures (Nnamdi et al. 2017) and rainwater (Wu et al. 2017a, Wu et al. 2017b). PCA identifies groups and sets of variables with similar properties and allows us to make our description of observations straightforward by discovering the trends or patterns in chaotic or confusing datasets. Our study concerned the characterization of river water from different sampling sites and the simultaneous analysis of physicochemical and bacteriological parameters.

Antibacterial resistance tests

To evaluate the antibacterial resistance of environmental bacteria in this study, we adopted two approaches, disk diffusion and broth micro-dilution as recommended by the CLSI (CLSI 2018). Isolated colonies of the same morphological type from an agar plate were suspended in 5 mL broth medium. The inoculated culture was incubated at 35 °C until it was harvested once its turbidity reached or exceeded an optical density (O.D.) at 600nm of 0.4-0.6 (being equivalent to $1–2*10^8$ colony forming unit mL^{-1}). Cell population growth was at the log phase when harvested. The following 10 common antibacterials were tested in this study: ampicillin, cefotaxime, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, tetracycline, trimethoprim, trimethoprim/sulfamethoxazole and vancomycin (all antibacterials were supplied by Sigma-Aldrich). They were dissolved in their respective solvents and diluted in their specific diluents (**Table S2**). And then they were sterilized by syringe filters (Sigma-Aldrich). We evaluated the

resistance by inhibition zone of disk diffusion and minimum inhibitory concentration (MIC) of micro-dilution (Jorgensen &Turnidge 2015). Breakpoints of susceptibility, intermediate resistance and complete resistance were based on CLSI criteria (CLSI 2018). For the results of downstream samples, which were found significantly different from upstream, and reaching the breakpoints of above intermediate resistance would be considered as increase of resistance.

For disk diffusion, we charged a sterile cotton swab with inoculum suspension and inoculated the surface of a Mueller-Hinton agar plate (Sigma-Aldrich) by streaking the swab in a back-and-forth motion. The plate was then rotated by 90° and the streak action was repeated for 4 times to ensure an even distribution of the inoculum. After the plate inoculation, we placed the antibacterial- impregnated disks onto the surface of the agar with forceps, which were tested at the indicated concentrations: ampicillin (10μg), cefotaxime (30μg), chloramphenicol (30μg), ciprofloxacin (5μg), erythromycin (15μg), gentamicin (10μg), tetracycline (30μg), trimethoprim (5μg), trimethoprim/sulfamethoxazole (1.25/23.75μg) and vancomycin (30μg). Negative controls were prepared by using blank disks with double-distilled water and ensuring that there was no inhibition zone around the control disk. Once all disks were inoculated, they were incubated at 35 °C for 16 to 18 hours. Following the incubation, the inhibition zone diameters were measured to the nearest millimeter by a ruler and recorded on sheets. The longer the diameter, the more effective the respective antibacterial was in the prevention of bacterial growth. All the tests were done in triplicate (Hudzicki 2009).

We performed the micro-dilution method on 96-well plates. There was a total of 200 μL volume in each well. An aliquot of 20 μL with an O.D. 600 of 0.4-0.6 inoculum suspension was added to all the wells except the negative control. Column 11 wells, the positive control, carried inoculated broth; and column 12 wells, as the negative control, carried broth only. Column 1-10 wells contained 180 μL of the 10 different antibacterials dissolved in Mueller-Hinton broth (Sigma-Aldrich). The range of antibacterial concentration in each well was 0.25–32 μg mL^{-1} (except for ampicillin, 0.125–16 μg mL^{-1}), totally 8 two-fold serial concentrations of an antibacterial were dispensed to a single row. The plates were incubated for 12 to 13 hours at 35 °C. Resazurin dye (Sigma-Aldrich) of an amount of 20 μL was added to each well and the plates were then incubated for 3-4 more hours. Living bacteria are maintaining a reducing microenvironment within their cells. This environment could cause the Resazurin color to change from blue (the oxidized-form) to red (the reduced form). Resazurin was prepared at 0.01%, sterilized with a syringe filter, and stored at 4 °C. The well of the dilution that showed no color changes (blue) at the antibacterial concentration was determined as the minimum inhibitory concentration (MIC) value. MIC is the lowest concentration of an antibacterial to inhibit visible bacterial growth. Bacterial cell viability could be measured by absorbance at 600 nm using a spectrophotometer. A higher absorbance value indicated more viable cells which coincided with the color change. All the tests were done in triplicates (Herbst et al. 2014).

Statistical analysis

Statistical tests were initially performed using Microsoft office Excel ver. 2016. The physicochemical and bacteriological parameters were analyzed by Principal Component Analysis (PCA) mean values of samples in PAST ver. 4.03. A one-way analysis of variance (ANOVA) in R Studio Desktop ver. 3.5.1. was used to analyze the results of inhibition zones and MICs. MICs were expressed as geometric mean. For statistical evaluations, MIC data were log-transformed (log_2MIC) and calculated. Results were considered as statistically significant at $p < 0.05$.

Results

The results of water quality tests indicated different pollution levels between the upstream and the downstream. It showed different variation patterns of physicochemical and bacteriological parameters along the watercourse. According to the PCA results, we found different degrees of pollution among the sampling sites.

Physicochemical parameters

Overall there was an increasing trend of temperature, conductivity, TDS, salinity and BOD values in the downstream. While pH and DO values went in an opposite trend. Temperature values gradually increased at downstream, it ranged from 20°C to 25°C in the upstream and from 25°C to 30°C in the downstream. For conductivity, TDS, salinity and BOD, the mean values slightly varied in the upstream from site 1 to site 6 (conduct. = 244.00 — 362.00 µS cm^{-1}, TDS = 120.20 — 174.00 mg L^{-1}, Sal. = 128.67 — 210.33 mg L^{-1}, BOD = 0.20 — 0.33 mg L^{-1}). For each of these values, there was a substantial increase in the downstream from site 7 to site 9 (conduct. = 557.33 — 758.33 µS cm^{-1}, TDS = 303.33 — 419.33 mg L^{-1}, Sal. = 381.67 — 519.00 mg L^{-1}, BOD = 0.97 — 1.27 mg L^{-1}). pH values were > 8.00 in the upstream and < 8.00 in the downstream. The DO were > 8.00 mg L^{-1} in the upstream and < 8.00 mg L^{-1} in the downstream (**Table 1.1**).

Bacteriological parameters

A total of 5 bacterial species were predominantly isolated and from samples inoculated on nutrient agar: *Bacillus subtilis* (*B. subtilis*), *Bacillus megaterium* (*B. megaterium*), *Bacillus cereus* (*B. cereus*), *Acinetobacter* sp. and *Serratia marcescens* (*S. marcescens)*; *Enterobacteriaceae* including *Escherichia coli* (*E. coli*), *Klebsiella pneumonia* (*K. pneumonia*), *Enterobacter* sp. and *S. marcescens* were isolated on MacConkey agar; *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermidis* (*S. epidermidis*) were isolated on Mannitol salt agar. *Bacillus* species, *Acinetobacter* sp. and *S. marcescens* began to occur at site 1, *E. coli* and *K. pneumonia* and *S. aureus* at site 3, *S. epidermidis* at site 4 and *Enterobacter* sp. at site 5. For abundance analysis, we calculated total viable counts (TVC), total *Enterobacteriaceae* (TE), total coliforms (TC), *E. coli* (EC), total *Staphylococcus* (TS), *Acinetobacter* sp. (AB) and total *Bacillus* (TB). As for representation analysis, we calculated the proportion of total *Enterobacteriaceae* (TEP), total coliforms (TCP), *E. coli* (ECP), total *Staphylococcus* (TSP), *Acinetobacter* sp. (ABP) and total *Bacillus* (TBP) (**Table 1.2**).

We present here the abundances of different bacteria or bacterial groups at different sampling sites. There was an increasing trend of CFU numbers for TVC, TE, TC and each of these bacteria in the downstream. Compared with the site where the bacteria or bacterial groups began to occur in the upstream (TVC = 4.35×10^4 CFU mL^{-1}, TE = 4.48×10^3 CFU mL^{-1}, TC = 3.46×10^3 CFU mL^{-1}, EC = 2.45×10^3 CFU mL^{-1}, TS = 2.45×10^3 CFU mL^{-1}), the mean values slightly increased at site 6 (TVC = 7.82×10^4 CFU mL^{-1}, TE = 1.37×10^4 CFU mL^{-1}, TC = 7.11×10^3 CFU mL^{-1}, EC = 3.11×10^3 CFU mL^{-1}, TS = 5.65×10^3 CFU mL^{-1}). For TVC, TE, TC, EC and TS, there were substantial increases of the values from site 6 to site 7 (TVC = 1.66×10^5 CFU mL^{-1}, TE = 3.06×10^4 CFU mL^{-1}, TC = 1.73×10^4 CFU mL^{-1}, EC = 8.76×10^3 CFU mL^{-1}, TS = 9.38×10^3 CFU mL^{-1}), and from site 7 to site 8 (TVC = 3.27×10^5 CFU mL^{-1}, TE = 6.46×10^4 CFU mL^{-1}, TC = 3.89×10^4 CFU mL^{-1}, EC = 1.75×10^4 CFU mL^{-1}, TS = 2.53×10^4 CFU mL^{-1}). At site 9, the values were close to site 8 (TVC = 3.27×10^5 CFU mL^{-1}, TE = 6.32×10^4 CFU mL^{-1}, TC = 3.87×10^4 CFU mL^{-1}, EC = 1.82×10^4 CFU mL^{-1}, TS = 2.65×10^4 CFU mL^{-1}). The AB and TB increase gradually. AB ranged from 8.08×10^3 CFU mL^{-1} to 1.12×10^4 CFU mL^{-1} in the upstream and from 1.67×10^4 CFU mL^{-1} to 2.63×10^4 CFU mL^{-1} in the downstream. TB ranged from 1.88×10^4 CFU mL^{-1} to 3.05×10^4 CFU mL^{-1} in the upstream and from 4.61×10^4 CFU mL^{-1} to 7.04×10^4 CFU mL^{-1} in the downstream. For representations of different bacteria or bacterial groups at different sampling sites, TBP maintained the highest value of the community along the river. Compared with site 1 (ABP = 18.59 %, TBP = 43.25 %), the mean values of ABP and TBP slightly decreased at site 6 (ABP = 14.25 %, TBP = 38.91 %). There was a substantial decrease of ABP and TBP from site 6 to site 7 (ABP = 10.06 %, TBP = 27.76 %) and from site 7 to site 8 (ABP = 7.28 %, TBP = 21.12 %). At site 9, the value was close to site 8 (ABP = 8.04 %, TBP = 21.54 %). Compared with the site where the bacteria or bacterial groups began to occur in the upstream (TEP = 10.31 % — 17.52 %, TCP = 5.68 % — 9.09 %, ECP = 3.97 % — 4.43 %, TSP = 4.43 % — 7.22 %), TEP, TCP, ECP, and TSP overall maintained the values in the downstream (TEP = 18.46 % — 19.73 %, TCP = 10.42 % — 11.88 %, ECP = 5.28 % — 5.55 %, TSP = 5.65 % — 8.11 %).

Principal components analysis

River water was characterized by 7 physicochemical parameters and 13 bacteriological parameters. The PCA analysis showed that of the 20 components, the first principal components accounted for 99.21%, while the second, third, fourth and fifth principal components accounted for 0.69%, 0.05%, and 3.83%, respectively (**Table S4**). Here we present a scatter plot consisting of PC1 and PC2 (**Fig. 2**). It demonstrates two clusters, one accommodates the samples which are from the upstream (Upstream cluster), while the other comprises those from the downstream (Downstream cluster). The two clusters differ in dispersion. The distribution of the 3 samples in the Downstream cluster are more dispersed than those in the Upstream cluster. This finding might imply that the pollution scenarios are similar among the sampling sites in the upstream but varied among the 3 sites downstream. Thus, for the following antibacterial resistance tests, we decided to make a comparison among 4 sites: a site where the isolate began to occur at the upstream of Qishan river, compared to the following downstream sites: Qishan town (site 7), WWTP (site 8) and Kaoping river (site 9).

Antibacterial resistance tests

Resistance level could be indicated by zone diameter in disk diffusion and MIC in micro-dilution. For each antibacterial activity on bacteria, resistance level that significantly increased and reached the breakpoints of above intermediate resistance were considered as increased resistance (**Table 3**). The results showed variable patterns of antibacterial resistance levels among sampling sites along the watercourse. Most increased resistance occurred at site 8 and site 9. Overall, the two methods showed that there were mainly 5-6 types of antibacterials showing increased resistance with bacteria in the downstream.

Disk diffusion

Fig. 3 shows the results of disk diffusion. All comparisons were made between a site where the isolate began to occur in upstream and sites in downstream. For *E. coli*, by comparison with site 3, increased resistances were found with ampicillin ($p < 0.0001$), chloramphenicol ($p < 0.0001$), ciprofloxacin ($p < 0.0001$) and tetracycline ($p < 0.0001$). Site 7 showed smaller inhibition zones with chloramphenicol. Both sites 8 and 9 showed smaller inhibition zones with ampicillin, chloramphenicol, ciprofloxacin and tetracycline (**Fig. 3A**). For *K. pneumonia*, by comparison with site 3, increased resistances were found with ampicillin ($p < 0.0001$), chloramphenicol ($p < 0.0001$), ciprofloxacin ($p < 0.0001$), and tetracycline ($p < 0.0001$). Site 7 showed smaller inhibition zones with chloramphenicol. Site 8 showed smaller inhibition zones with chloramphenicol, ciprofloxacin and tetracycline. Site 9 showed smaller inhibition zones with ampicillin, chloramphenicol, ciprofloxacin, and tetracycline (**Fig. 3B**). For *Enterobacter* sp., by comparison with site 5, increased resistances were found with ampicillin ($p < 0.0001$), chloramphenicol ($p < 0.0001$), ciprofloxacin ($p < 0.0001$) and tetracycline ($p < 0.0001$). Both sites 7 and 8 showed smaller inhibition zones with chloramphenicol and tetracycline. Site 9 showed smaller inhibition zones with ampicillin, chloramphenicol, ciprofloxacin and tetracycline (**Fig. 3C**). For *S. marcescens*, by comparison with site 1, increased resistances were found with ampicillin ($p < 0.0001$), ciprofloxacin ($p < 0.0001$), trimethoprim/ sulfamethoxazole ($p < 0.0001$), and tetracycline ($p = 0.0002$). Site 7 showed smaller inhibition zones with tetracycline. Site 8 showed smaller inhibition zones with tetracycline. Site 9 showed smaller inhibition zones with ampicillin, ciprofloxacin, trimethoprim/ sulfamethoxazole and tetracycline (**Fig. 3D**). The zone diameter breakpoints of *Enterobacteriaceae* were recommended by CLSI (CLSI 2018) (**Table S7.1**).

For *Acinetobacter* sp., by comparison with site 1, increased resistance was found with ciprofloxacin ($p < 0.0001$) and tetracycline ($p < 0.0001$). Both sites 7 and 8 showed smaller inhibition zones with tetracycline. Site 9 showed smaller inhibition zones with ciprofloxacin and tetracycline (**Fig. 3E**). The zone diameter breakpoints of *Acinetobacter* spp. were recommended by CLSI (CLSI 2018) (**Table S7.2**).

For *S. aureus*, by comparison with site 3, increased resistances were found with chloramphenicol ($p < 0.0001$), erythromycin ($p < 0.0001$) and tetracycline ($p < 0.0001$). Site 7 showed smaller inhibition zones with chloramphenicol. Both sites 8 and 9 showed smaller inhibition zones with chloramphenicol, erythromycin and tetracycline (**Fig. 3F**). For *S. epidermidis*, by comparison with site

4, the increased resistances were found with chloramphenicol ($p < 0.0001$), erythromycin ($p < 0.0001$) and tetracycline ($p < 0.0001$). Site 7 showed smaller inhibition zones with chloramphenicol. Both sites 8 and 9 showed smaller inhibition zones with chloramphenicol, erythromycin and tetracycline (**Fig. 3G**). The zone diameter breakpoints of *Staphylococcus* spp. were recommended by CLSI (CLSI 2018) (**Table S7.3**).

For *B. megatium*, by comparison with site 1, the increased resistances were found with ciprofloxacin ($p = 0.0001$), erythromycin ($p = 0.0008$) and tetracycline ($p = 0.0056$). Both sites 7 and 8 showed smaller inhibition zones with erythromycin. Site 9 showed smaller inhibition zones with ciprofloxacin, erythromycin and tetracycline (**Fig. 3H**). For *B. cereus*, by comparison with site 1, the increased resistances were found with ciprofloxacin ($p = 0.0001$), erythromycin ($p = 0.0147$) and tetracycline ($p < 0.0001$). Site 8 showed smaller inhibition zones with erythromycin and tetracycline, site 9 showed smaller inhibition zones with ciprofloxacin, erythromycin and tetracycline (**Fig. 3I**). For *B. subtilis*, by comparison with site 1, the increased resistances were found with erythromycin ($p < 0.0001$) and tetracycline ($p < 0.0001$). Both sites 8 and 9 showed smaller inhibition zones with erythromycin and tetracycline (**Fig. 3J**). The zone diameter breakpoints of *Bacillus* spp. were recommended by CLSI (CLSI 2018) (**Table S7.4**).

Micro-dilution

Fig. 4 shows the results of micro-dilution. All comparisons were made between a site where the isolate began to occur in upstream and sites in downstream. For *E. coli*, by comparison with site 3, increased resistances were found with ampicillin ($p < 0.0001$), chloramphenicol ($p = 0.0001$) and tetracycline ($p < 0.0001$). Site 7 showed higher MICs with chloramphenicol and tetracycline. Both sites 8 and 9 showed higher MICs with ampicillin, chloramphenicol and tetracycline (**Fig. 4A**). For *K. pneumoniae*, by comparison with site 3, increased resistances were found with ampicillin ($p = 0.0001$), chloramphenicol ($p = 0.0012$), ciprofloxacin ($p = 0.0118$) and tetracycline ($p = 0.0001$). Site 8 showed higher MICs with chloramphenicol and tetracycline. Site 9 showed higher MICs with ampicillin, chloramphenicol, ciprofloxacin and tetracycline (**Fig. 4B**). For *Enterobacter* sp., by comparison with site 5, increased resistances were found with ampicillin ($p < 0.0001$), chloramphenicol ($p < 0.0001$), ciprofloxacin ($p < 0.0001$) and tetracycline ($p < 0.0001$). Site 8 showed higher MICs with ampicillin, chloramphenicol and tetracycline. Site 9 showed higher MICs with ampicillin, chloramphenicol, ciprofloxacin and tetracycline (**Fig. 4C**). For *S. marcescens*, by comparison with site 1, increased resistances were found with chloramphenicol, ciprofloxacin and tetracycline. Site 7 showed higher MICs with tetracycline. Site 8 showed higher MICs with chloramphenicol and tetracycline. Site 9 showed higher MICs with chloramphenicol ($p < 0.0001$), ciprofloxacin ($p < 0.0001$) and tetracycline ($p = 0.0054$) (**Fig. 4D**). The MIC breakpoints of *Enterobacteriaceae* were recommended by CLSI (CLSI 2018) (**Table S7.1**).

For *Acinetobacter* sp., by comparison with site 1, the increased resistance was found with tetracycline. Both sites 8 and 9 showed higher MICs with tetracycline ($p < 0.0001$) (**Fig. 4E**). The MIC breakpoints of *Acinetobacter* spp. were recommended by CLSI (CLSI 2018) (**Table S7.2**).

For *S. aureus*, by comparison with site 3, the increased resistances were found with chloramphenicol ($p < 0.0001$), erythromycin ($p < 0.0001$), tetracycline ($p < 0.0001$) and vancomycin ($p < 0.0001$). Site 7 showed higher MICs with chloramphenicol, erythromycin and tetracycline. Both sites 8 and 9 showed higher MICs with chloramphenicol, erythromycin, tetracycline and vancomycin (**Fig. 4F**). For *S. epidermidis*, by comparison with site 4, increased resistances were found with chloramphenicol ($p < 0.0001$), erythromycin ($p = 0.0006$), tetracycline ($p < 0.0001$) and vancomycin ($p < 0.0001$). Site 7 showed higher MICs with chloramphenicol and erythromycin. Both site 8 and site 9 showed higher MICs with chloramphenicol, erythromycin, tetracycline and vancomycin (**Fig. 4G**). The MIC breakpoints of *Staphylococcus* spp. were recommended by CLSI (CLSI 2018) (**Table S7.3**).

For *B. megatium*, by comparison with site 1, the increased resistances were found with erythromycin and tetracycline. Both site 8 and site 9 showed higher MICs with erythromycin ($p = 0.0012$) and tetracycline ($p < 0.0001$) (**Fig. 4H**). For *B. cereus*, by comparison with site 1, the increased resistances were found with erythromycin ($p = 0.0013$). Sites 7, 8 and 9 showed higher MICs with erythromycin (**Fig. 4I**). For *B. subtilis*, by comparison with site 1, the increased resistances were found with erythromycin ($p < 0.0001$). Both sites 8 and 9 showed higher MICs with erythromycin (**Fig. 4J**). The MIC breakpoints of *Bacillus* spp. were recommended by CLSI (CLSI 2018) (**Table S7.4**).

Discussion

Physicochemical analysis

Rivers act as important bodies of surface water, playing an essential role in the water cycle. They are polluted by the disposal of sewage and wastewater from human activities, which severely impact the physicochemical characteristics and bacterial communities. The results of physicochemical analysis showed differences in the degree of pollution between the upstream and downstream of Qishan river. Temperature could affect not only the physical and chemical properties of water but also the biological activities. Possibly due to the sea level, the temperature values in the downstream increased. The values of pH variation towards acidity in the downstream could be attributed to the anthropogenic activities or acidic precipitation (Singh et al. 2016, Wu et al. 2017b). Conductivity, TDS and salinity could correlate with each other (Rusydi 2018). They have been used to evaluate the purity of water. Their values substantially increased in the downstream, which might have implied the more dissolved salts and minerals within the river water. The presence of DO is essential to maintain the aquatic ecosystem and to keep the water bodies healthy from various pollutants. Decreased DO in the downstream might have been caused by the decreased solubility of oxygen at higher temperature. BOD could represent the amount of biodegradable organic matters (Lee &Nikraz 2015). The values increased in the downstream, indicating the higher pollution degree of organics in the river water.

Bacteriological analysis

Parameters such as TVC, TE, TC and EC and sometimes TS have been used as water quality indicators (Britz et al. 2013, Curtis et al. 2011, Rahmani et al. 2020). Their presences in water bodies were

associated with contamination. And they increased substantially from site 6 to site 7, which was the transition from upstream to downstream where agricultural activity and human settlement grow considerably. And these parameters increased substantially again from site 7 to site 8 where the river water received the WWTP effluent. The increase of TVC, TE, TC and EC and TS implied the impact of pollution on river water. Though AB and TB were not usual indicators, they are ubiquitous in natural environments (Adegoke et al. 2012, Baruzzi et al. 2011). In the bacterial enumeration, we commonly found and thus included them in bacteriological parameters. The AB and TB increase from upstream to downstream gradually. Different from those indicators, their counts were maintained in higher values from site 1 to site 6. It is somehow related to their ubiquity in the more pristine upstream. Though most bacteriological analyses take only bacterial enumeration as parameters, bacterial representation could provide information for water quality since contamination could alter the bacterial community (Lu et al. 2017, Tang et al. 2016). ABP and TBP decreased substantially from site 6 to site 7, which was the transition from upstream to downstream where agricultural activity and human settlement grow considerably. Both of them decreased substantially again from site 7 to site 8 where the river water received the WWTP effluent. As more polluting bacterial species brought into river water with the effluent, the values of ABP and TBP decreased. TEP, TCP, ECP and TSP maintained their values under the circumstance of the increase of TVC in the downstream. It is consistent with the fact that TE, TC and EC increased as TVC increased. In other words, the increase of TE, TC and EC could have somehow contributed to the increase of TVC.

Principal component analysis

Principal Component Analysis (PCA) is one of the most applied approaches in environmental sciences to study data. It is aimed at finding and interpreting hidden complex and casually determined relationships between dataset features. This is accomplished by studying the data structure in a reduced dimension while retaining the maximum of variability present in the data. To do this, it is necessary to estimate the number of significant components present in the data. More precisely, a matrix of pairwise correlations among parameters is decomposed into eigenvectors. These eigenvectors are sorted in descending order of their corresponding eigenvalues. The eigenvalue of PC1 overwhelms those of the other PCs (**Table 2**). All variables are well represented by the first components, PC1. Thus, we focused mainly on the first principal components that explain nearly 100.0% of the total variance of the dataset. Since most variables contributed to the first eigenvector, the first principal component can be interpreted as all of the parameters which are positively or negatively and highly correlated with each other. The downstream generally receives lots of polluted water from human settlement. The more it receives, the more pollutants (e.g. organics, heavy metals, nutrients, salt ions, coliforms, pathogens) be brought into river water. And for that the physicochemical parameters value change simultaneously as pollution happens. Though there is no cause-and-effect relationship between the parameters, they still strongly correlated with each other. Such case could lead to high percentages of variance which can be explained by PC1. And this might cause a considerable disparity in the eigenvalue for PC1 and the other PCs (Abdi &Williams 2010). The scatter plots (**Fig. 2)** indicated a different dispersion patterns within the Upstream and Downstream clusters. The 6 upstream sampling sites are relatively pristine with nearly no pollution effect, and thus

their status is close to each other, whereas the 3 downstream sampling sites are relatively separated. While the downstream sampling site 7 receives water from the town of Qishan, site 8 receives water from the Qimei wastewater treatment plant (WWTP) effluents, and site 9 locates at the Kaoping river receiving water from the Qishan river and the other tributaries. Thus these sampling sites are supposed to have a different pollution status. For the above results, we compared the antibacterial resistance between site 7 to 9 with only one site from upstream since sites 1 to 6 shared a similar status.

Antibacterial resistance tests

Previous researches have documented intrinsic bacterial resistance to antibacterials (Cox &Wright 2013). Natural resistance recommended by CLSI (CLSI 2018) could be shown in the results of this study. All the *Enterobacteriaceae* had intrinsic resistance against erythromycin and vancomycin. As for *Acinetobacter* sp., there were ampicillin, chloramphenicol, erythromycin and vancomycin. Although antibacterial resistance is a natural phenomenon as a mechanism of bacteria for better competitiveness, the exposure of bacteria to selective pressure results in the emergence and spread of antibacterial resistance (Fonseca et al. 2015, Pereira et al. 2015).

Overall, chloramphenicol, erythromycin and tetracycline were found to show increased resistance on bacteria along the downstream including sites 7 to 9. In rural areas such as Qishan, agriculture and husbandry are important segments of the economy, and waste from these sectors represents an additional potential source of contamination with antibacterials used for farming. Due to the high oral bioavailability and ability to accumulate in many tissues and organs, chloramphenicol, erythromycin and tetracycline were widely used in veterinary practices to prevent and treat diseases in swine, poultry, cattle, and sheep. Resistance to these three antibacterials are often simultaneously found in environments such as ponds (Zhou et al. 2019), wastewater and its receiving river water (Jia et al. 2017), and even raw milk (Xie et al. 2017). Site 7 is located in Qishan Town and surrounded by agriculture area. The river water there received the agricultural effluents that brought these antibacterials or antibacterial-resistant bacteria. Though chloramphenicol, erythromycin and tetracycline resistances from site 7 could continue at site 8 as the river flows through., the resistance level could be higher in site 8 than site 7. According to the results of both the methods (**Table 3**), intensity of chloramphenicol and tetracycline activities reached intermediate resistance at site 7 while complete resistance at site 8. Site 8 is located near from the WWTP, receiving the effluent from not only agriculture but also human settlement in the whole Qishan and other neighbor districts. The effluent of WWTP provides an ideal environment for resistance gene transfer since environmental bacteria are kept in direct continuous contact with antibacterials and antibacterial resistant bacteria (Rizzo et al. 2013). The results of higher resistance level at site 8 than site 7 and even site 9 for few cases agreed with previous research that intensified pollution environments such as WWTPs could promote antibacterial resistance (Martinez 2009, Szekeres et al. 2018). Moreover, ampicillin, ciprofloxacin and vancomycin on bacteria began to show resistance at either site 8 or site 9. *Enterobacteriaceae* showed the increased resistance to ampicillin and ciprofloxacin. Though the results of micro-dilution showed that MICs of ampicillin for *S. marcescens* (site 9) and ciprofloxacin for *E. coli* (sites 8 and 9) did not reached the breakpoints, they are still significantly higher than the upper

stream sites. Pharmaceutical and personal care products (PCPPs) are household chemicals that are used in large amounts worldwide and regarded as potential environmental pollutants. Most of these products are discharged via domestic sewage system (Daughton 2003). Among the PCPPs, ampicillin and ciprofloxacin have been reported to be the most widely used antibacterials worldwide. The β-lactam antibacterials including ampicillin have been reported to account for over 65% of the world antibiotic market. Ciprofloxacin is one of the frequently used quinolones in hospitals, it is also available for limited use in veterinary medicine. Other report has also found the both antibacterials in significant quantities in wastewater (Githinji et al. 2010). Some bacteria belonged to *Enterobacteriaceae* are able to produce extended-spectrum β-lactamase (ESBL) enzymes, which confer resistance to most penicillin and its derivative antibacterials and most ESBL-producing bacteria could also be resistant to several other clinically-relevant antibacterial classes (Magwenzi et al. 2017). *Staphylococcus* spp. showed the increased resistance to vancomycin at both sites 8 and 9, according to the results of micro-dilution. Though in disk diffusion, the inhibition zone diameters of vancomycin for *S. aureus* and *S. epidermidis* are significantly smaller at both sites 8 and 9 than upper stream sites, there is no standard for intensity of vancomycin action on *Staphylococcus* spp. provided by the CLSI (CLSI 2018). Thus, we could not confirm if the resistance level reached the breakpoints. The previous research has reported that emergence of resistance to vancomycin is a threat to the already challenging therapy of MRSA (Methicillin-resistant *S. aureus*) or MRSE (Methicillin-resistant *S. epidermidis*). Our results indicated that *S. aureus* and *S. epidermidis* are not resistant to ampicillin, of which the structure is similar to methicillin. However, the other bacterial isolates' resistance to ampicillin implied that the stress caused by this type of antibacterial cannot be ignored. The spread of MRSA coupled with the emergence of VISA (Vancomycin-intermediate *S. aureus*) and VRSA (Vancomycin-resistant *S. aureus*) might become a major concern for public health further downstream or in the future (Tarai et al. 2013). Although there are fewer reports for the resistance of *S. epidermidis*, its antibacterial resistance to methicillin (MRSE/ Methicillin-resistant *S. epidermidis*) might extend an additional edge for VRSE pathogenesis that in turn complicates the management of these infections in healthcare settings (Sanober et al. 2017).

Conclusion

This study provides evidence that there is contamination with antibacterial resistant bacteria along the Qishan river. The water quality tests and analyses indicated different pollution status between the upstream and downstream of the Qishan river. The antibacterial-resistant bacteria from wastewater could be the potential source of the emergence and spread of antibacterial resistance in the Qishan river basin area. The results of our survey of the Qishan river indicates that levels of antibacterial resistance are related to environmental factors, such as agricultural area, population density and waste emission pathway. Resistance levels increased substantially at both the WWTP and Kaoping river with intensified pollution. The WWTP acts as a major reservoir and supplier of antibacterial resistance in riverine environments. The efficient and effective treatment of WWTP discharges should be considered with priority for counteracting the emergence of antibacterial resistance. Water quality assessment and management are critical public health issues. The protection of surface waters from pollutants, especially

antibacterials, resistant bacteria, and resistance genes, is fundamental to improve the water safety in Kaohsiung city and southern Taiwan. This study provides initial information of the distribution of antibacterial resistant bacteria. More detailed studies of the risks of water-borne diseases via the contamination with antibacterial resistance are warranted.

Declarations

Supplementary information

The online version contains supplementary material available at (DOI).

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

All authors agree to submit this work to above journal.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest. All authors read and approved the final manuscript. The authors declare no competing interests.

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tables

Table 1.1. Physicochemical parameters along the Qishan river. Temp. = temperature, Conduct. = conductivity, TDS = total dissolved solids, Sal. = salinity, DO = dissolved oxygen, BOD = biological oxygen demand.

Physicochemical parameters									
		Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	Site 9
Temperature	Mean	20.17	20.40	20.90	21.43	22.50	23.27	25.60	27.67	28.33
(°C)	SD	0.25	0.36	0.10	0.40	0.17	0.31	0.36	0.31	0.31
pH	Mean	8.45	8.41	8.35	8.26	8.26	8.24	7.70	7.41	7.20
	SD	0.03	0.03	0.02	0.04	0.03	0.04	0.04	0.02	0.05
Conductivity	Mean	244.00	293.33	338.00	333.00	348.67	362.00	557.33	758.33	694.33
($\mu S\ cm^{-1}$)	SD	3.61	4.51	5.57	3.61	6.11	7.00	4.73	5.13	1.15
TDS	Mean	120.20	134.43	154.67	169.33	173.00	174.00	303.33	419.33	386.00
($mg\ L^{-1}$)	SD	2.55	2.23	2.08	2.52	2.65	3.61	4.16	7.02	4.00
Salinity	Mean	128.67	155.33	198.67	203.67	217.67	210.33	381.67	519.00	470.67
($mg\ L^{-1}$)	SD	3.51	1.53	2.52	3.51	6.03	4.04	7.02	6.56	2.52
DO	Mean	8.38	8.27	8.25	8.23	8.25	8.18	7.58	7.32	7.21
($mg\ L^{-1}$)	SD	0.02	0.03	0.02	0.02	0.03	0.02	0.02	0.03	0.04
BOD	Mean	0.20	0.20	0.27	0.30	0.33	0.33	0.97	1.20	1.27
($mg\ L^{-1}$)	SD	0.00	0.00	0.06	0.00	0.06	0.06	0.12	0.10	0.15

Table 1.2. Bacteriological parameters along the Qishan river. TVC = total viable count, TE = total *Enterobacteriaceae*, TEP = total *Enterobacteriaceae* proportion, TC = total coliforms, TCP = total coliforms proportion, EC = total E. coli, ECP = total *E. coli* proportion, AB = total *Acinetobacter*, ABP = total *Acinetobacter* proportion, TS = total *Staphylococcus*, TSP = total *Staphylococcus* proportion, TB = total *Bacillus*, TBP = total *Bacillus* proportion.

Bacteriological parameters										
		Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	Site 9
TVC	Mean	43.48	45.86	55.30	59.35	68.89	78.24	165.87	327.22	326.75
($\times 10^3$ CFU mL^{-1})	SD	4.26	9.51	11.86	6.49	8.20	8.57	12.76	38.89	37.48
TE	Mean	4.48	5.24	7.83	8.71	10.38	13.70	30.62	64.58	63.17
($\times 10^3$ CFU mL^{-1})	SD	1.53	1.10	1.82	2.65	2.69	3.11	1.72	7.93	11.35
TEP	Mean	10.31	11.43	14.16	14.67	15.07	17.52	18.46	19.73	19.33
(%)	SD	3.51	2.41	3.29	4.47	3.90	3.97	1.04	2.42	3.47
TC	Mean	0.00	0.00	3.46	4.47	3.91	7.11	17.29	38.89	38.66
($\times 10^3$ CFU mL^{-1})	SD	0.00	0.00	0.54	0.53	0.91	2.06	1.28	8.67	10.12
TCP	Mean	0.00	0.00	6.26	7.54	5.68	9.09	10.42	11.88	11.83
(%)	SD	0.00	0.00	0.97	0.89	1.32	2.63	0.77	2.65	3.10
EC	Mean	0.00	0.00	2.45	2.45	2.83	3.11	8.76	17.54	18.15
($\times 10^3$ CFU mL^{-1})	SD	0.00	0.00	0.55	0.55	1.17	1.41	2.26	6.14	4.99
ECP	Mean	0.00	0.00	4.43	4.13	4.11	3.97	5.28	5.36	5.55
(%)	SD	0.00	0.00	1.00	0.93	1.70	1.81	1.36	1.88	1.53
TS	Mean	0.00	0.00	2.45	4.16	4.16	5.65	9.38	25.33	26.50
($\times 10^3$ CFU mL^{-1})	SD	0.00	0.00	0.55	1.37	2.82	0.51	5.51	0.47	5.84
TSP	Mean	0.00	0.00	4.43	7.01	6.04	7.22	5.65	7.74	8.11
(%)	SD	0.00	0.00	1.00	2.31	4.10	0.65	3.32	0.14	1.79
AB	Mean	8.08	9.00	9.11	9.22	9.86	11.15	16.68	23.81	26.27
($\times 10^3$ CFU mL^{-1})	SD	2.27	2.74	2.24	3.23	3.40	2.21	3.62	3.23	1.90
ABP	Mean	18.59	19.62	16.47	15.54	14.32	14.25	10.06	7.28	8.04
(%)	SD	18.59	19.62	16.47	15.54	14.32	14.25	10.06	7.28	8.04
TB	Mean	18.80	19.83	20.57	23.27	26.96	30.45	46.05	69.12	70.38
($\times 10^3$ CFU mL^{-1})	SD	2.88	4.99	6.37	1.80	1.48	8.10	8.23	5.65	6.66
TBP	Mean	43.25	43.25	37.19	39.21	39.14	38.91	27.76	21.12	21.54
(%)	SD	6.62	10.89	11.52	3.03	2.15	10.36	4.96	1.73	2.04

Table 2. Culture media used for quantitative bacterial analysis.

Bacteria	Culture medium	Colonies	Reference
Total viable count	Nutrient agar	All	(Aditi et al. 2017)
Total *Enterobacteriaceae*	MacConkey agar	All	(Yao et al. 2017)
Total coliforms	MacConkey agar	Pink	(Aditi et al. 2017)
Escherichia coli	MacConkey agar	Flat, dry, pink	(Jung &Hoilat 2020)
Klebsiella pneumoniae	MacConkey agar	Mucoid, pink	(Jung &Hoilat 2020)
Enterobacter sp.	MacConkey agar	Mucoid, pink	(Jung &Hoilat 2020)
Serratia marcescens	MacConkey agar	Slow, small, pink	(Jung &Hoilat 2020)
Staphylococcus aureus	Mannitol salt agar	Yellow	(Aditi et al. 2017)
Staphylococcus epidermidis	Mannitol salt agar	Red	(Aditi et al. 2017)

Table 3. Intensity of antibacterial actions on bacteria along the Qishan river (S=Site)

Antibacterial	Disk diffusion/ Micro-dilution; *S* = susceptible, *I* = intermediate, *R* = resistant																		
	E. coli				*K. pneumoniae*				*Enterobacter* sp.					*S. marcescens*				*Acinetobacter* sp.		
	S3	S7	S8	S9	S3	S7	S8	S9	S5	S7	S8	S9	S1	S7	S8	S9	S1	S7	S8	S9
Ampicillin	*S/S*	*S/S*	*I/I*	*R/I*	*S/S*	*S/S*	*S/S*	*I/I*	*S/S*	*S/S*	*S/I*	*I/I*	*S/S*	*S/S*	*S/S*	*R/S*	*–/–*	*–/–*	*–/–*	*–/–*
Chloramphenicol	*S/S*	*I/I*	*R/R*	*R/I*	*S/S*	*I/S*	*R/I*	*R/I*	*S/S*	*I/S*	*R/R*	*R/I*	*S/S*	*S/S*	*S/I*	*S/I*	*–/–*	*–/–*	*–/–*	*–/–*
Ciprofloxacin	*S/S*	*S/S*	*I/S*	*R/S*	*S/S*	*S/S*	*I/S*	*I/I*	*S/S*	*S/S*	*S/S*	*I/I*	*S/S*	*S/S*	*S/S*	*I/I*	*S/S*	*S/S*	*S/S*	*I/S*
Cefotaxime	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*
Erythromycin	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*
Gentamicin	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*
Trimethoprin-sulfamethoxazole	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*I/S*	*I/S*	*S/S*	*S/S*	*S/S*	*S/S*
Tetracycline	*S/S*	*S/I*	*R/R*	*R/R*	*S/S*	*S/S*	*R/R*	*I/I*	*S/S*	*I/S*	*R/R*	*I/I*	*S/S*	*I/I*	*I/I*	*I/I*	*S/S*	*I/S*	*R/I*	*R/I*
Trimethoprin	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*
Vancomycin	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*

Table 3 cont. Intensity of antibacterial actions on bacteria along the Qishan river (S=Site)

Antibacterial	Disk diffusion/ Micro-dilution; *S* = susceptible, *I* = intermediate, *R* = resistant																			
	S. aureus				*S. epidemidis*				*B. megatium*				*B. cereus*				*B. subtilis*			
	S3	S7	S8	S9	S4	S7	S8	S9	S1	S7	S8	S9	S1	S7	S8	S9	S1	S7	S8	S9
Ampicillin	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*
Chloramphenicol	*S/S*	*I/I*	*R/I*	*R/I*	*S/S*	*I/I*	*R/R*	*R/I*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*
Ciprofloxacin	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*I/S*	*S/S*	*S/S*	*S/S*	*I/S*	*S/S*	*S/S*	*S/S*	*S/S*
Cefotaxime	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*	*–/–*
Erythromycin	*S/S*	*S/I*	*I/R*	*I/R*	*S/S*	*S/I*	*I/R*	*I/R*	*S/S*	*I/S*	*I/I*	*I/I*	*S/S*	*I/I*	*I/I*	*I/I*	*S/S*	*S/S*	*I/I*	*I/I*
Gentamicin	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*
Trimethoprin-sulfamethoxazole	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*
Tetracycline	*S/S*	*S/I*	*I/I*	*R/R*	*S/S*	*S/S*	*I/I*	*I/I*	*S/S*	*S/S*	*S/I*	*I/I*	*S/S*	*S/S*	*I/S*	*I/S*	*S/S*	*S/S*	*I/S*	*I/S*
Trimethoprin	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*	*S/S*
Vancomycin	*–/S*	*–/S*	*–/I*	*–/R*	*–/S*	*–/S*	*–/I*	*–I*	*–/S*	*–/S*	*–/S*	*–/S*	*–/S*	*–/S*	*–/S*	*–/S*	*–/S*	*–/S*	*–/S*	*–/S*

Figures

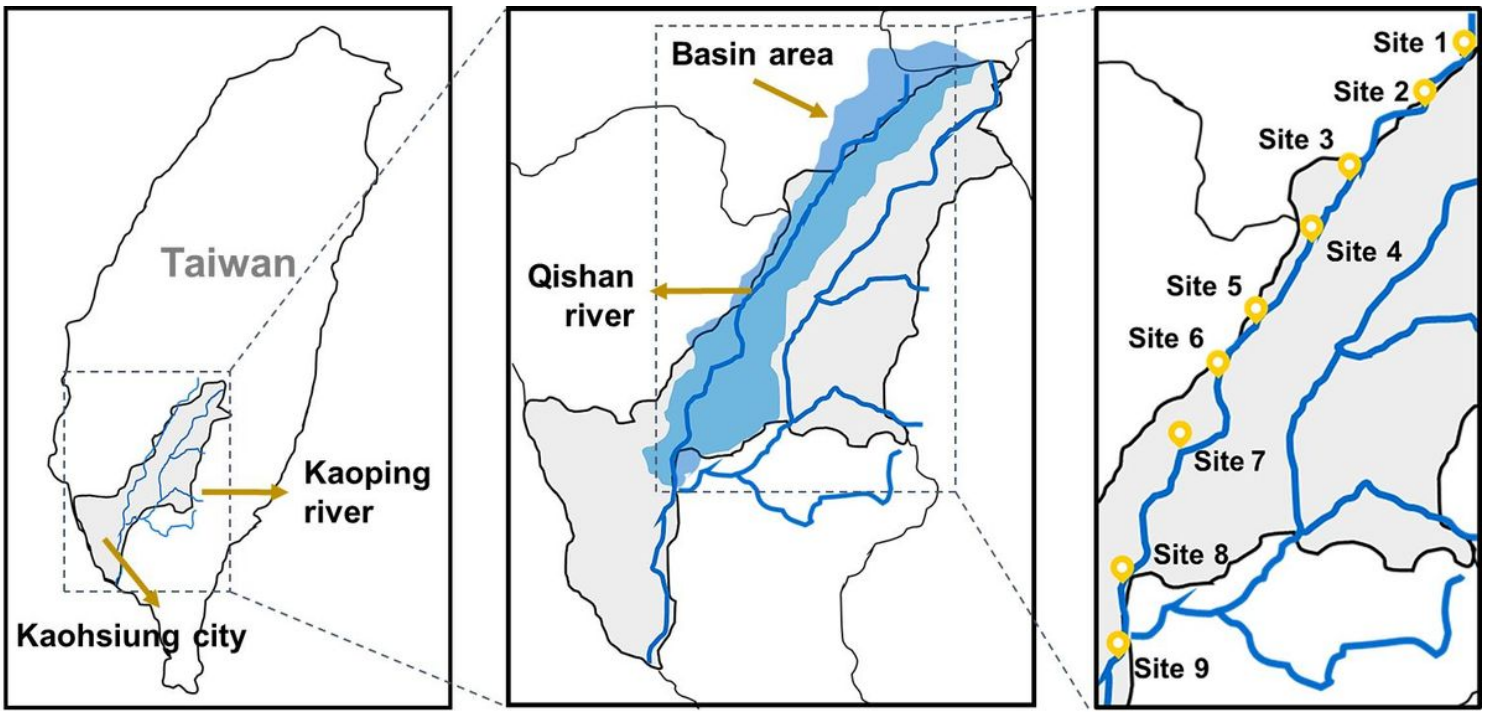

Figure 1

Locations of sampling sites along the Qishan river in Kaohsiung city.

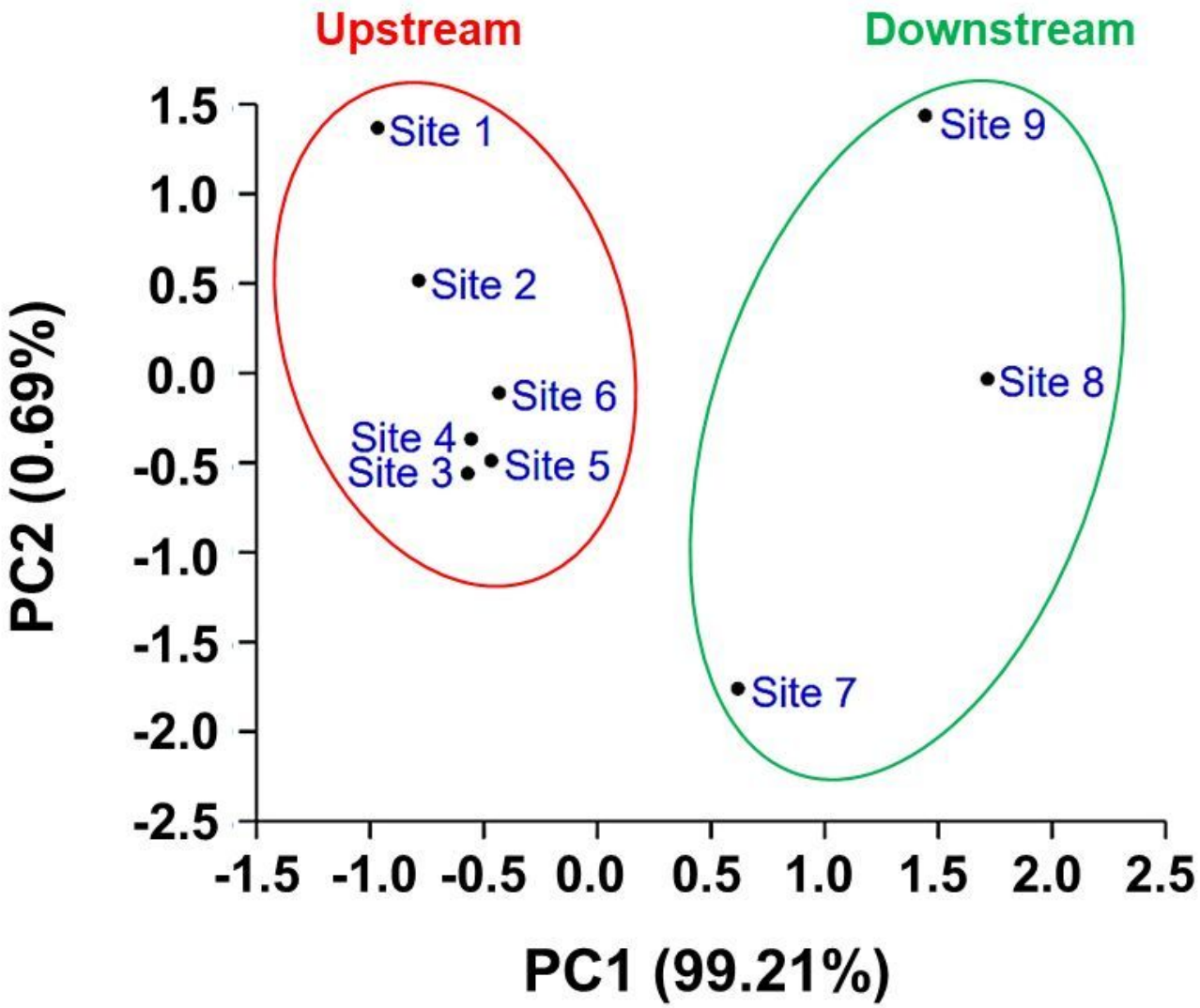

Figure 2

Principal component analysis (PCA) for physiochemical and bacteriological parameters in Qishan river water.

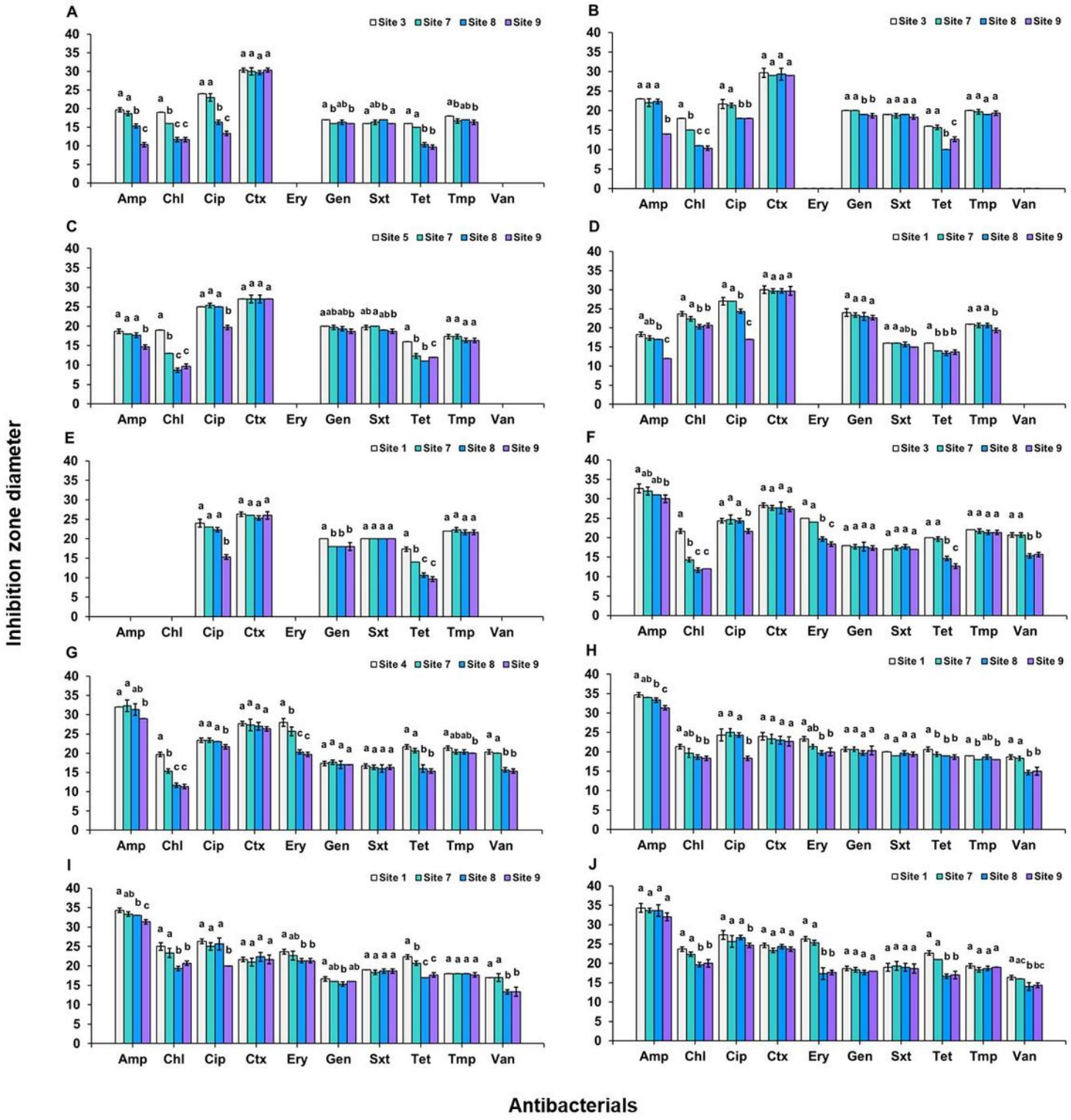

Figure 3

Inhibition zone diameter means of bacterial cultures with different antibacterials. **A =** *E. coli*, **B =** *K. pneumoniae*, **C** = *Enterobacter* sp., **D** = *Serratia marcescens*, **E** = *Acinetobacter* sp., **F** = *Staphylococcus aureus*, **G** = *Staphylococcus epidermidis*, **H** = *Bacillus megatium*, **I** = *Bacillus cereus*, **J** = *Bacillus subtilis*. Amp = Ampicillin, Chl = Chloramphenicol, Cip = Ciprofloxacin, Ctx = Cefotaxime, Ery = Erythromycin, Gen = Gentamicin, Stx = Trimethoprim-sulfamethoxazole, Tet = Tetracycline, Tmp = Trimethoprim, Van =

Vancomycin. The letter above each column indicates significant difference from other data with $p < 0.05$ based on Tukey's test and each error bar indicates standard deviation from three replications.

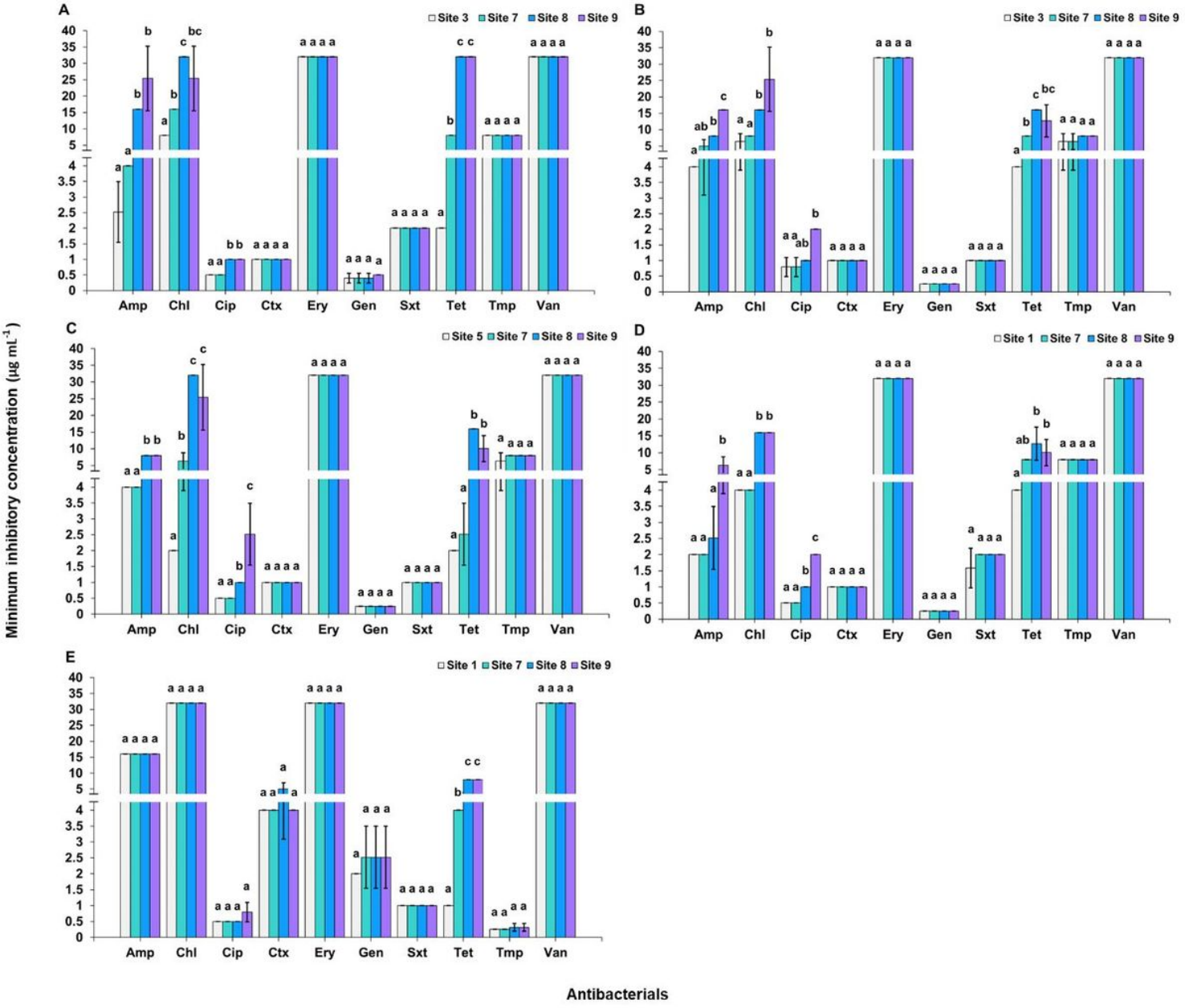

Figure 4

Minimum inhibitory concentration (MIC) geometric means of bacterial cultures with different antibacterials. **A =** *E. coli*, **B =** *K. pneumoniae*, **C** = *Enterobacter* sp., **D** = *Serratia marcescens*, **E** = *Acinetobacter* sp. Amp = Ampicillin, Chl = Chloramphenicol, Cip = Ciprofloxacin, Ctx = Cefotaxime, Ery = Erythromycin, Gen = Gentamicin, Stx = Trimethoprim-sulfamethoxazole, Tet = Tetracycline, Tmp = Trimethoprim, Van = Vancomycin. The letter above each column indicates significant difference from other data with $p < 0.05$ based on Tukey's test and each error bar indicates standard deviation from three replications.

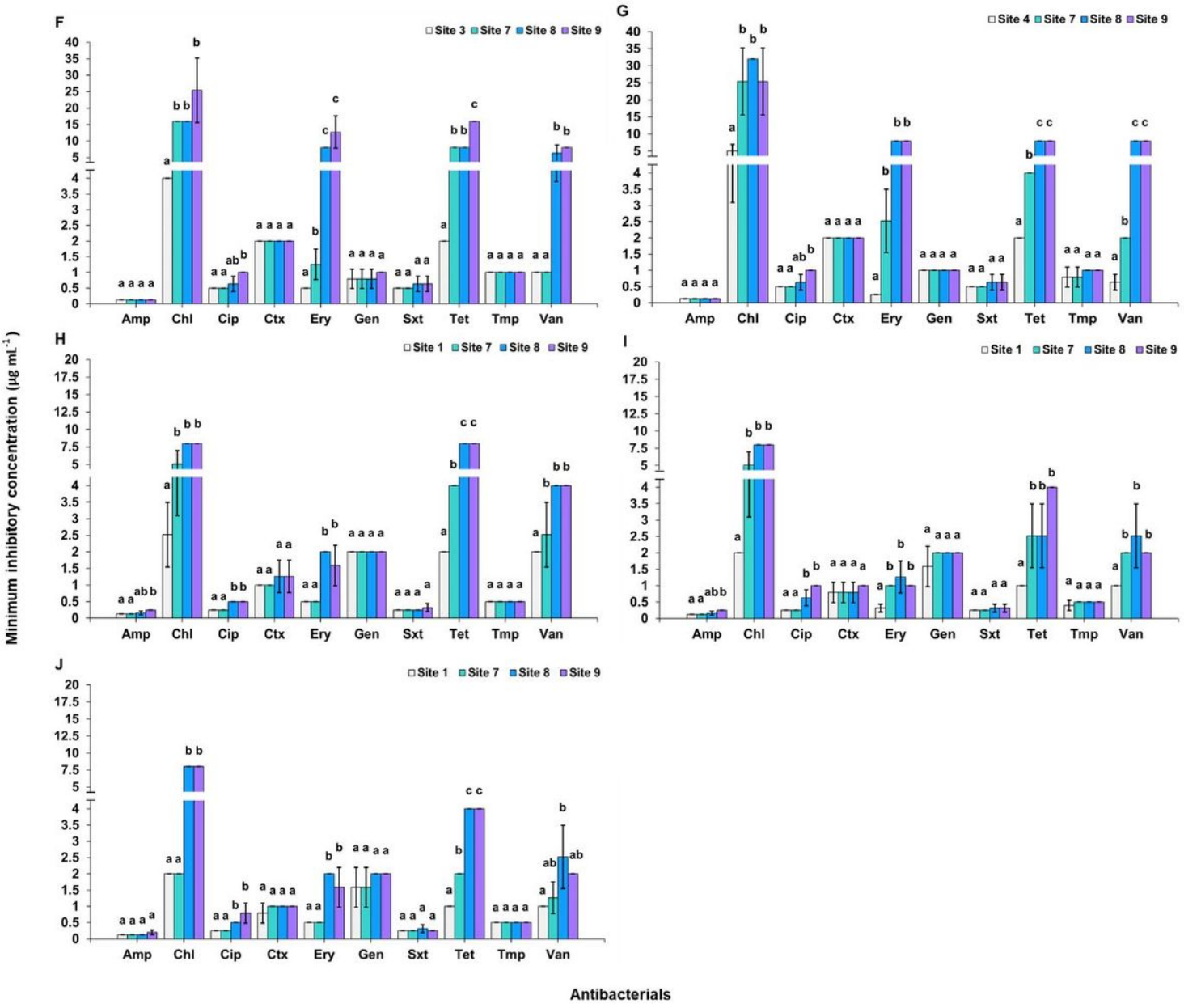

Figure 5

Minimum inhibitory concentration (MIC) geometric means of bacterial cultures with different antibacterials. **F** = *Staphylococcus aureus*, **G** = *Staphylococcus epidermidis*, **H** = *Bacillus megatium*, **I** = *Bacillus cereus*, **J** = *Bacillus subtilis*. Amp = Ampicillin, Chl = Chloramphenicol, Cip = Ciprofloxacin, Ctx = Cefotaxime, Ery = Erythromycin, Gen = Gentamicin, Stx = Trimethoprim-sulfamethoxazole, Tet = Tetracycline, Tmp = Trimethoprim, Van = Vancomycin. The letter above each column indicates significant difference from other data with $p < 0.05$ based on Tukey's test and each error bar indicates standard deviation from three replications.

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