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# Antibiotic resistance genes might serve as new indicators for wastewater contamination of coastal waters: Spatial distribution and source apportionment of antibiotic resistance genes in a coastal bay



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#### ABSTRACT

Rare information on using antibiotic resistance genes (ARGs) as new indicators for the wastewater contamination in coastal environment is available. This study investigated the distribution and sources of ARGs in a coastal bay influenced by anthropogenic activities including intensive wastewater discharge, which demonstrated that ARGs could be essential indicators for the wastewater contamination in coastal waters. Ten ARG subtypes encoding resistance to sulfonamides (sul1 and sul2), tetracyclines (tetB, tetG and tetX), quinolones (*anrA*, *anrB* and *anrS*) and macrolides (*ermF* and *ermT*) were detected with high frequencies. The absolute abundances of ARGs in different functional zones of Sishili Bay differed greatly in the range of  $10^{0}$ - $10^{8}$  copies/ mL. The total abundances of ARGs in the zones influenced by wastewater treatment plants (WWTPs) and sewage polluted rivers were relatively higher with the maximum up to  $6.1 \times 10^6$  copies/mL and  $6.2 \times 10^7$  copies/mL, respectively. The TN, nitrite, and silicate had extremely significantly positive correlations (p < 0.01) with the abundances of all target ARGs, as well as ammonium and TOC had significantly positive correlations with the target ARGs. A total of 402 pairs of strong and significant correlations between bacteria and ARGs were identified in samples from the zones influenced by wastewater pollution sources (rivers and WWTPs), while only 232 pairs of correlations were identified in other samples. It indicated the occurrence and prevalence of potential multi-antibiotic resistant bacteria in coastal waters contaminated by wastewater. These findings revealed that the occurrence and distribution of ARGs in the coastal bay were mainly influenced by the wastewater discharge. ARGs might serve as new indicators for the influence of wastewater discharge on coastal water qualities, since the wastewater discharge has critical influences on ARGs pollution in coastal waters.

### 1. Introduction

Antibiotics have been widely used for preventing or treating bacterial diseases as well as promoting animal growth in the world (Cai et al, 2019; Lu et al., 2018; Lu et al., 2020a; Qiao et al., 2018; Yang et al., 2016; Zhang et al., 2015). The global antibiotic consumption increased sharply in the 21st century (Van Boeckel et al., 2014). China is the largest producer and consumer of antibiotics in the world (Zhu et al., 2013). The overuse and abuse of antibiotics have promoted the spread of antibiotic resistance genes (ARGs) into various environments (Becattini et al., 2016; Lu et al., 2019a; Lu et al., 2019b; Lu et al., 2020a). Being one of the emerging environmental contaminants, ARGs originated from antibiotic resistant bacteria could make it difficult to control the bacterial diseases and pose potential risks to human health and eco-environment (Pruden et al., 2012; Zhang et al., 2020). Therefore, ARGs have attracted more attention from the public.

In recent years, wastewater treatment plants (WWTPs) and sewage from hospitals, ships, livestock, swine, chicken and aquaculture farms have been recognized as the hotspots of ARGs (Chen et al., 2016; Gao et al., 2018; Jia et al., 2017; Lu et al., 2019a; Lv et al., 2018; Mao et al., 2015; Wang et al., 2018; Zhu et al., 2017). Moreover, ARGs have been detected in various aquatic environments such as lakes, rivers, estuaries

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and marine which received the discharged sewage or effluents (Czekalski et al., 2015; Jia et al., 2017; Lu et al., 2020a; Luo et al., 2010). ARGs were even detected in the glaciers, reservoirs and groundwater (Huerta et al., 2013; Segawa et al., 2013). These studies indicated that ARGs were prevalent in various aquatic environments affected by human activities, and they might be transported to distant areas. Moreover, it has been reported that the resistance affected by anthropogenic activities exhibited regional characteristics.

Coastal zones were the important areas with frequent human disturbances, and more than 50% of global populations live in these areas (Liquete et al., 2013; Wang et al., 2020). Continuous economic growth might pose potential threats to coastal environments (He et al., 2014: Lu et al., 2018; Lu et al., 2019b; Lu et al., 2020a; Marques, 2019). especially to semi-enclosed bays with slowly hydrodynamic dispersion of pollutants. The total population and gross domestic product (GDP) were significantly correlated with the abundances of ARGs (Lu et al., 2019b; Zhu et al., 2017). Coastal areas were significantly influenced by anthropogenic activities and they have become hotspots of ARGs (Dash and Das, 2016; Fistarol et al., 2015; Lu et al., 2020a; Zhang et al., 2018; Zhu et al., 2017). The Sishili Bay is an important coastal bay in the north Yellow Sea of China with rapid economic development and intensive anthropogenic activities. In this research, the distribution of typical ARGs (tet, sul, qnr and erm) and the class 1 integron-integrase gene (intI1) was investigated in the Sishili Bay. The objective was to provide initial information for evaluating the impact of wastewater discharge on coastal ARGs pollution and searching for new indicators for coastal water pollutions.

# 2. Materials and methods

# 2.1. Sampling and physiochemical analysis

The sampling was performed in the Sishili Bay, northern Yellow Sea of China. Thirty-two sampling sites belonging to 6 functional zones were investigated. As shown in Fig. 1, the functional zones were classified based on different anthropogenic influences, including the sites impacted by WWTPs, the sites influenced by rivers receiving wastewater, the sites close to aquaculture farms, the sites close to ecological aquaculture farms, the sites close to bathing beaches, and the sites close to Yantai Port. Site S14 and S15 was the effluent of WWTP1 and WWTP2. Sites S30-S32 and Sites 27–29 were collected from the receiving sea of WWTP1 and WWTP2, respectively. S31/S28 was about 1 km away from S30/S27, and S32/S29 was 1 km away from S31/S28. WWTP1 was the biggest WWTP in Yantai City, combining the conventional anaerobic-anoxic-aerobic (AAO) system and the microfiltration membrane bioreactor (MBR) system. The wastewater treatment scale of WWTP1 was higher than 200,000  $m^3$ /day. WWTP2 was constructed with the AAO process, with the wastewater treatment scale up to 120,000  $m^3$ /day. Surface water samples (30 L, 3 replicates of 10 L for each sampling site) were collected for investigation. There were 4 rivers receiving wastewater in the investigated areas. River R1 passed through the countryside and received rural domestic sewage. River R2 which was close to a paper mill received the wastewater from the mill and large amounts of domestic sewage from surrounding urban residents. River R3 was close to the wastewater treatment plant 2 (WWTP2) which received domestic sewage from surrounding urban residents. River R4 was a short river that was surrounded by residential areas.

A conductivity-temperature-depth profiler (RBR Concerto, Canada) was used to measure salinity *in situ*. Environmental variables such as phosphate ( $PO_4^{3-}$ -P), total phosphorus (TP), ammonium ( $NH_4^+$ -N), nitrate ( $NO_3^-$ -N), nitrite ( $NO_2^-$ -N), total nitrogen (TN) and silicate ( $SiO_3^{2-}$ -Si) were measured by a continuous flow analyzer (Seal-Branlubble AA3, Germany). Total organic carbon (TOC) was quantified by a total organic carbon analyzer (Shimadu TOC VCPH, Japan). The water samples were filtered through 0.22 µm micropore membrane and kept at -80 °C for further analysis.

## 2.2. DNA extraction and PCR amplification

DNA was extracted by TIANamp Soil DNA Kit (TIANGEN Biotech, Beijing, China) according to the manufacturer's protocol. The concentration and quality of purified DNA was measured by NanoDrop Lite (Thermo, USA) and 1% agarose electrophoresis. The primers used in this study were listed in Table 1. The PCR assays for 16S rRNA gene, *intl*1 and 30 ARGs were conducted as previously (Wang et al., 2019). The target ARGs included 3 sulfonamide resistance genes (*sul*1, *sul*2, and *sul*3), 18 tetracycline resistance genes (*tet*A, *tet*B, *tet*C, *tet*D, *tet*E, *tet*H, *tet*G, *tet*J, *tet*K, *tet*L, *tet*M, *tet*O, *tet*Q, *tet*R, *tet*S, *tet*T, *tet*W, and *tet*X), 4 quinolone resistance genes (*qnr*A, *qnr*B, *qnr*D, and *qnr*S) and 5 macrolide resistance genes resistance genes (*erm*A, *erm*B, *erm*C, *erm*F, and *erm*T). The PCR products were checked on 2% agarose gel and purified by Qiagen Gel Extraction Kit (Qiagen, Germany) for further study.



Fig. 1. Sampling sites in the semi-enclosed bay (Sishili Bay of Yantai, northern Yellow Sea in China).

#### Table 1

Primers of target ARGs for PCR in this investigation.

Gene Types	Genes	Primer Sequence (F, R: 5'-3')
Sulfonamide (sul)	sul1	CGCACCGGAAACATCGCTGCAC, TGAAGTTCCGCCGCAAGGCTCG
	sul2	TCCGGTGGAGGCCGGTATCTGG,
		CGGGAATGCCATCTGCCTTGAG
	sul3	TCCGTTCAGCGAATTGGTGCAG,
		TTCGTTCACGCCTTACACCAGC
Tetracycline (tet)	tetA	GCTACATCCTGCTTGCCTTC,
		CATAGATCGCCGTGAAGAGG
	tetB	GGTTGAGACGCAATCGAATT,
	tetC	CTTGAGAGCCTTCAACCCAG
	1010	ATGGTCGTCATCTACCTGCC
	tetD	TGCCGCGTTTGATTACACA,
		CACCAGTGATCCCGGAGATAA
	tetE	TTGGCGCTGTATGCAATGAT,
	tetH	
	icuri	TTGCGCATTATCATCGACAGA
	tetG	CATCAGCGCCGGTCTTATG,
		CCCCATGTAGCCGAACCA
	tetJ	GGGTGCCGCATTAGATTACCT,
	t at V	TCGTCCAATGTAGAGCATCCATA
	letk	
	tetL	TCGTTAGCGTGCTGTCATTC,
		GTATCCCACCAATGTAGCCG
	tetM	ACAGAAAGCTTATTATATAAC,
		TGGCGTGTCTATGATGTTCAC
	tetO	GATGGCATACAGGCACAGACC,
	tetO	AGAATCTGCTGTTTGCCAGTG.
	μų	CGGAGTGTCAATGATATTGCA
	tetR	CGCGATAGACGCCTTCGA
		TCCTGACAACGAGCCTCCTT
	tetS	TTAAGGACAAACTTTCTGACGACATC
	totT	TGTCTCCCATTGTTCTGGTTCA
	1011	AGGTGTATCTATGATATTTAC
	tetW	GAGAGCCTGCTATATGCCAGC,
		GGGCGTATCCACAATGTTAAC
	tetX	CAATAATTGGTGGTGGACCC,
		TTCTTACCTTGGACATCCCG
Quinolone (qnr)	qnrA	TCAGCAAGAGGATTTCTCA,
		GGCAGCACTATGACTCCCA
	qnrB	TCGGCIGICAGIICIAIGAICG,
	anrD	AGTGAGTGTTTAGCTCAAGGAG.
	1	CAGTGCCATTCCAGCGATT
	qnrS	TGATCTCACCTTCACCGCTTG,
		GAATCAGTTCTTGCTGCCAGG
Macrolide (erm)	ermA	TTGAGAAGGGATTTGCGAAAAG,
		ATATCCATCTCCACCATTAATAGTAAACC
	<i>erm</i> B	CATGCGTCTGACATCTATCTGA,
	ama	CTGTGGTATGGCGGGTAAGTT
	GIIIG	TAGCAAACCCGTATTCCACG
	<i>erm</i> F	CAGCTTTGGTTGAACATTTACGAA,
		AAATTCCTAAAATCACAACCGACAA
	ermT	GTTCACTAGCACTATTTTTAATGACAGAAGT, GAAGGGTGTCTTTTTAATACAATTAACGA
IntI1	intI1	CCTCCCGCACGATGATC.
		TCCACGCATCGTCAGGC
16S rRNA gene	168	CGGTGAATACGTTCYCGG.
		GGWTACCTTGTTACGACTT

# 2.3. Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was used to determine the abundances of ARGs, *intl*<sup>1</sup> gene and 16S rRNA gene in Sishili Bay. Calibration standard curves for positive controls and quantification

were constructed by the plasmids DNA (cloned ARGs) extracted from *E. coli* DH5 $\alpha$ , with 10-fold dilution series from 10<sup>7</sup> to 10<sup>0</sup> copies/ $\mu$ L as described previously (Luo et al., 2010). The qPCR assays were performed on the Bio-Rad qPCR system (Bio-Rad CFX384 Touch, CA, USA) according to previous report (Wang et al., 2019). Each reaction was run in triplicate. The amplification efficiencies were between 90% and 110% and the R<sup>2</sup> values were higher than 0.99.

# 2.4. High-throughput sequencing

The extracted DNA were sent to Majorbio (Shanghai, China) for Illumina MiSeq sequencing. The V4-V5 hypervariable regions of the bacteria 16S rRNA gene were used for amplification with primers 515F (5'- GTGCCAGCMGCCGCGG-3') and 907R (5'-CCGTCAATTCMTTTRA-GTTT-3'). The sequences were generated on an Illumina MiSeq platform, and quality filtering on the raw tags was performed by FLASH. Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1) and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier (Version 2.2) algorithm against the Silva (SSU123) 16S rRNA gene database. The sequences derived from high-throughput sequencing were deposited in the National Center for Biotechnology Information (NCBI) database under accession number PRJNA517574.

#### 2.5. Statistical analysis

The statistical analysis and plotting were completed by Origin and Microsoft Office Excel. Pearson's and Spearman's correlation analysis were obtained by SPSS 19.0 (IBM, New York, USA). Contour map was conducted by Surfer. Co-occurrence patterns by network analysis between ARGs and the top 50 bacteria genera among samples from the zones influenced by wastewater pollution sources (rivers and WWTPs) (S11, S12, S14, S15, S16, S17, S18, S19, S20, S21, S22, S23, S27, S28, S29, S30, S31, S32) and other zones (S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S13, S24, S25, S26) were investigated. Network analysis was visualized by Cytoscape to investigate the relationship between bacteria and ARGs.

# 3. Results and discussion

# 3.1. Absolute abundances of ARGs in different functional zones of the bay

The absolute abundances of target ARGs and intl1 in seawaters from different functional zones were investigated (Fig. 2). The absolute abundances of ARGs in different functional zones of Sishili Bay differed greatly ranging from  $10^{0}$  to  $10^{8}$  copies/mL. For the ARGs encoding resistance to sulfonamides and tetracyclines, the average abundances of ARGs in the zones influenced by WWTPs and sewage polluted rivers were significantly higher than those in other functional zones (p < 0.05), demonstrating that the discharge of WWTPs and rivers had great influence on the ARGs abundances in coastal waters. The absolute abundances of quinolones resistance genes in bathing beaches was also relatively higher, as a result of the wide use of quinolones to cure human diseases. Besides, it was also probably because that the bathing beaches lied near the WWTP1 and river R4. Moreover, the average absolute abundances of macrolides resistance genes in the zones influenced by sewage polluted rivers were slightly higher than those in other functional zones. The average absolute abundances of erm genes in AF (aquaculture farms) and EA (ecological aquaculture farms) were higher than those in WT (WWTPs). On the one hand, the addition of macrolide antibiotics in the aquaculture farms contributed to the accumulation of erm genes, since the positive correlations between erm genes and macrolides have been confirmed (Yi et al., 2019). On the other hand, the abundances of erm genes in the discharge of WWTPs have been reduced to low levels after the wastewater treatment



Fig. 2. Box chart of the absolute abundance of ARGs and *intI*1 in different functional zones. RV was the zones influenced by rivers, AF was the zones close to aquaculture farms, EA was the zones close to ecological aquaculture farms, BB was the zones close to bathing beaches, YP was the zones close to Yantai Port and WT was the zones influenced by WWTPs.

processes (Lu et al., 2020b).

In Zone RV influenced by rivers received wastewater, the total abundances of ARGs kept relatively higher in the range of  $3.0 \times 10^4$ – $2.7 \times 10^6$  copies/mL in estuaries and  $3.6 \times 10^5$ – $6.2 \times 10^7$ in rivers, respectively. The highest ARGs abundance in estuaries was detected in river R2 (S18). In Zone WP influenced by WWTPs, the total abundances of ARGs in the effluent of WWTPs were higher than 10<sup>6</sup> copies/mL, while the total abundances of ARGs detected in the seawater near the outlet of WWTPs were quantified in the range of  $2.7~\times~10^4\text{--}9.2~\times~10^5$  copies/mL. The ARGs were detected with the tendency of rivers > estuaries > seawater away from the estuaries, and the tendency of effluent of WWTPs > seawater near the outlet of WWTPs > seawater away from the WWTPs. Moreover, the total absolute abundances of ARGs in Zone BB (bathing beaches) were slightly lower than those in Zone RV and WP, but relatively higher than those in Zone AF (aquaculture farms), Zone EA (ecological aquaculture farms) and Zone YP (Yantai port). It could be speculated that the discharge from WWTPs and polluted rivers contributed significantly to ARGs pollutions in coastal bays. Studies on the distribution of ARGs along an urban river in Xi'an of China showed that the abundances of ARGs in areas near WWTPs and residential areas were higher than those in areas close to wetland parks (Guan et al., 2018). Zheng et al. (2018) found that the abundances of ARGs in the human-living areas were 5.2-33.9

times higher than those in the upstream of Zhangxi River in Ningbo, China. Investigations on the distribution of ARGs in estuaries showed that anthropogenic factors influenced the distribution of ARGs in river estuaries (Lu et al., 2020a; Zhu et al., 2017).

Furthermore, the average abundances of *int1* detected in Zone WP and Zone RV were significantly higher than those in other functional zones (p < 0.05). The highest absolute abundance of *int1* was up to  $4.9 \times 10^7$  copies/mL in Zone WP (S14) and  $3.4 \times 10^9$  copies/mL in Zone RV (S19). The abundances of *int1* in the areas near aquaculture farms, ecological aquaculture farms and Yantai Port were slightly lower. The lowest abundances of *int1* were detected in Zone BB with the abundances lower than 41.7 copies/mL, which might be attributed to the relatively weaker horizontal transfer of seawater in the bathing beaches with less discharge impacted by anthropogenic activities. The result indicated that the WWTPs and rivers might be the main sources of *int1* gene and ARGs.

# 3.2. Spatial distribution of ARGs in the bay

The spatial distribution of target genes (*sul, tet, qnr, erm*, total ARGs and *intI*1) in different functional zones in Sishili Bay were shown in Fig. 3. Different types of target ARGs exhibited different hotspots in the bay. The higher abundances of *sul* genes were detected in S19, S18 and



Fig. 3. The spatial distribution of ARGs and int/1 in the bay (Sishili Bay).

S14, indicating that river R2 and WWTP1 were hotspots of sul genes. The relatively higher abundances of tet genes occurred in S19 and S18, indicating that the river R2 was the hotspot of tetracycline resistance genes. However, the hotspots for qnr genes were widely distributed in the bay, and the absolute abundances of qnr genes were relatively higher in Zone WP (S14, S15 and S30), Zone BB (S24-26), Zone RV (S20-21, S18-19), indicating that the corresponding zones were the hotspots of qnr genes. As for erm genes, the hotspots were determined in river R1 (S16-17), river R2 (S18-19), and river R3 (S20-21). In terms of the total abundances of ARGs, river R2 (S18-19) and WWTPs (S14 and S15) were also identified as the hotspots. Occurrence of antibiotics in WWTPs and rivers might be one of the possible factors influencing the ARGs distribution. Furthermore, the higher abundances of intI1 were detected in river R2 (S18 and S19) and WWTPs (S14, S30-S32), revealing that the rivers and WWTPs might be the hotspots of *intI*1. It was speculated that the distribution of ARGs exhibited regional characteristics and affected by different anthropogenic activities. The genes of sul, tet, qnr, erm and intI1 in estuary of river R2 were abundant, the sul and qnr genes in zones near WWTP1 were more prevalent, and the erm genes in river R1 were widespread. The absolute abundances of ARGs in coastal areas under the influence of wastewater discharge were much higher than those in the offshore areas. These findings demonstrated that the distribution feature of ARGs in the bay was mainly dependent on the wastewater discharge. Therefore, a rational approach to strengthen the control of ARGs was wastewater reclamation due to the water resource shortage in the coastal zone.

# 3.3. ARGs in effluent of coastal WWTPs and inflow rivers receiving wastewater

The total absolute abundances of ARGs in the effluent of WWTP1  $(6.1 \times 10^6 \text{ copies/mL})$  and WWTP2  $(1.1 \times 10^6 \text{ copies/mL})$  were much higher than those in the bays, confirming that the WWTPs were essential pollution sources for coastal waters. The dominant ARG types in WWTP1 and WWTP2 were quite different (Fig. 4). According to the investigation on the sewage that the WWTPs received, WWTP2 mainly received the domestic wastewater from surrounding urban residents while WWTP1 not only received the domestic sewage from urban residents, but also received the wastewater from various enterprises in Yantai (including chemical, electroplating, smelting and medicine



Fig. 4. The absolute abundances of ARGs and *intl*<sup>1</sup> in samples from coastal WWTPs (WWTP1 & WWTP2) and inflow rivers (R1, R2, R3 and R4) receiving wastewater. *Sul: sul1* and *sul2; tet:* tetB, *tet*G and *tetX; qnr: qnrA, qnrB* and *qnrS; erm: ermF* and *ermT*.

industries). It has been reported that wastewater types could affect the distribution of bacterial communities and ARGs (Lu et al., 2020b; Fan et al., 2018).

Abundances of ARGs drop sharply from rivers to the bays along the inflow rivers, confirming that the rivers polluted by wastewater were the potential important pollution sources for ARGs in coastal waters. In addition, different types of ARGs in rivers differed significantly. River R2 was the hotspots of sul, tet, qnr, erm and intI1 while river R1 was the hotspots of qnr and erm. The absolute abundances of ARGs in samples collected from river R2 ( $2.7 \times 10^6$ – $6.2 \times 10^7$  copies/mL) were much higher than those in samples collected from other rivers (R1, R3 and R4). River R1 passed through the countryside and received rural domestic sewage while river R2 was close to a paper mill and received the wastewater from the mill and a large amount of domestic sewage from surrounding urban residents. River R3 was close to the wastewater treatment plant 2 (WWTP2), which might prevent large amount of domestic sewage from being discharged into the river. The influence of sewage on river R4 was limited since river R4 was much shorter that other rivers. Therefore, it was speculated that the distribution feature of ARGs was correlated to the wastewaters that discharged into the rivers. Previously, a total of 23 ARGs including 15 tet, 3 sul, 3 qnr, 2 erm were observed with high detection frequencies in all units of two municipal WWTPs in northern China (Mao et al., 2015). Total 13 ARGs (6 tet, 3



Fig. 5. Heatmap of the correlations between the target genes (16S rRNA gene, ARGs and *intl*1) and environmental factors. \*\*\* means the significant level at p < 0.001; \*\* means the significant level at 0.001 ; \*means the significant level at 0.01 <math>.

*sul*, and 4 *qnr*) were found in a municipal WWTP and its effluent-receiving river (Xu et al., 2017). In six different full-scale municipal WWTPs, *bla*<sub>TEM</sub>, *erm*F, *mefA/E*, *qnr*S and *qnr*A were found as the dominant ARGs (Tong et al., 2019). All of these studies showed that the dominant ARGs varied in different wastewater pollution sources, indicating that the distribution feature of ARGs was also closely related to the characteristics of wastewater that discharged into the aquatic environments.

# 3.4. Correlations between water nutrients and ARGs in the bay

Relatively high nutrient levels were identified in the areas near the wastewater discharge sources such as the WWTPs and wastewater polluted rivers while the relatively high abundances of total ARGs were also identified in the same areas (Fig. S1). Previous investigations demonstrated that the wastewater discharge was the main pollution source of the nutrients and typical emerging contaminants such as endocrine-disrupting chemicals in coastal waters (Wu et al., 2019; Lu et al., 2020c). The increase of nutrient levels could enhance the accumulation of ARGs in the coastal bay, confirming that wastewater discharge might be the main pollution source for ARGs pollution in coastal waters. The correlations between target ARGs and water environment variables such as phosphate, TP, ammonium, nitrate, nitrite, TN, silicate and TOC were demonstrated in Fig. 5. The correlation analysis showed that the nutrients in water were positively correlated with the abundances of ARGs generally, but negatively correlated with the abundances of 16S rRNA genes. The TN, nitrite, and silicate had extremely significantly positive relation (p < 0.01) with the abundances of all target ARGs while ammonium and TOC had significantly positive relation with the abundances of all target ARGs. It has been reported that ammonium had significant effects on the distribution of ARGs in the sediments and it was positively correlated with the target ARGs (Li et al., 2018). Most of the detected ARGs (sul1, int/1, mphB and tetA) in the swine WWTPs were positively correlated with wastewater nutrients such as COD, NH4<sup>+</sup>-N, TP, and TN (Yuan et al., 2018). Nutrients, such as carbon, nitrogen and phosphate, could provide substrate for growth of bacteria including antibiotics resistance bacteria, and higher nutrients were more beneficial for the horizontal transfer of ARGs (Yuan et al., 2018).

#### 3.5. Correlations between bacterial communities and ARGs in the bay

Network analysis of the correlations between bacteria (top 50 genera) and ARGs was visualized in Fig. 6. A total of 402 pairs of strong and significant correlations were identified in samples from the zones influenced by rivers and WWTPs, while only 232 pairs of correlations were identified in other samples. The pairs of strong and significant correlations between bacteria and ARGs almost doubled in the areas under the influence of wastewater discharge. The result revealed the complicated and significant correlations between ARGs and bacteria in the functional zones near the rivers and WWTPs. Furthermore, total 7 pairs of correlations among target ARGs and int/1 were identified in samples collected from the zones near rivers and WWTPs (tetB & tetG, tetB & tetX, tetB & intI1, tetX & intI1, qnrA & qnrB, sul1 & tetX, and sul1 & intI1), while only 3 connections between tetB & intI1, sul1 & sul2 and ermF & intI1 were identified in other water samples. Positive correlations were determined between the same types of ARGs, reflecting the selective pressure exerted by the same antibiotics. The result demonstrated the co-occurrence of different ARGs within or across the antibiotic types, which could be supported by previous studies (Fan et al., 2018; Lu et al., 2020a; Sun et al., 2014; Wang et al., 2019). Moreover, more complicated correlations between different bacterial genera were confirmed in coastal water samples influenced by rivers and WWTPs.

For correlations between ARGs/*intl*1 and bacteria, there were 51 and 43 connections were determined in the zones near rivers and WWTPs and the remaining zones, respectively. More than three ARGs

were assigned to the bacterial genera of Acidovorax, Acinetobacter, Brachymonas and Pseudomonas with positive and significant correlations (Spearman's correlation coefficients > 0.6, p < 0.05), which were only identified in the zones influenced by rivers and WWTPs. The result indicated that the potential multi-antibiotic resistant bacteria tended to occur in coastal waters receiving the discharge of rivers and WWTPs. *Pseudomonas* was dominant in S14, S15 and S19, Acinetobacter was the dominant genus in S19, Acidovorax was predominant in S19 and S21, and Brachymonas was the predominant genus in S18 and S19. Besides, lots of potential host bacteria of ARGs related to one or two ARGs had been identified in these water samples, such as Candidatus\_Pelagibacter, Pseudoalteromonas, Candidatus\_Actinomarina, Psychrobacter and OM43 clade.

In short, a lot of bacteria detected in coastal water samples were confirmed to be correlated to target ARGs, especially the bacteria detected in water samples from the zones influenced by rivers and WWTPs, which were confirmed to have positive and significant correlations with more than three ARGs. The phenomena indicated that the study areas hold the potential risks due to the prevalence of potential host bacteria of ARGs, and the zones receiving the wastewater from WWTPs and rivers hold higher risks because of the occurrence and persistence of potential multi-antibiotic resistant bacteria. Although ARG-relevant bacteria were not in the first rank of risks for public health (Martínez et al., 2015; Ruppé et al., 2019), attention was still needed for them because ARGs could be transferred among different bacteria (Lu et al., 2020a; Wang et al., 2019; Zhang et al., 2020). Most of ARGs detected in a peri-urban river were found to be significantly correlated with Chloroflexi, Firmicutes, Gemmatimonadetes, Planctomycetes and Verrucomicrobia (Zheng et al., 2018). ARGs in the WWTPs were positively correlated with the functional genera and potential pathogens such as organic degrading bacteria, ammonia-oxidizing bacteria, nitrite-oxidizing bacteria, denitrifying bacteria and phosphorus accumulating organisms (Fan et al., 2018; Lu et al., 2020b). ARGs might have close connections with microbial ecology and biogeographic patterns in bacterial composition. Therefore, the differences of microbial communities in different sites might be important influential factors for the distribution of ARGs. More attentions should be attracted on the pollutions of ARGs and potential host bacteria of ARGs.

## 4. Conclusions

The occurrence, distribution and sources of ARGs in a coastal bay under the influences of intensive anthropogenic activities were investigated, which demonstrated that ARGs could be essential indicators for the wastewater contamination in coastal waters since wastewater discharge had great influences on the ARGs distribution in coastal waters. ARGs encoding resistance to sulfonamides, tetracyclines, quinolones and macrolides (sul1, sul2, tetB, tetG, tetX, qnrA, qnrB, qnrS, ermF and ermT) and intI1 were detected frequently. The higher abundances of ARGs were detected in the zones near the sewage outlet of WWTPs and the estuaries. The prevalence and abundances of ARGs depended closely on wastewater discharge. The increase in the nutrient levels caused by wastewater discharge could enhance the accumulation of ARGs in the coastal bay. The correlations between bacteria and ARGs in coastal waters influenced by wastewater discharge were much more complicated and significant than those without wastewater contamination. Potential multi-antibiotic resistant bacteria were prevalent in coastal waters collected from the zones influenced by rivers and WWTPs. The distribution of ARGs in the coastal bay was mainly influenced by the wastewater discharge, suggesting that the ARGs might serve as new indicators for wastewater contamination in coastal waters.

#### **CRediT** authorship contribution statement

Yuxuan Zhang: Investigation, Formal analysis, Data curation, Methodology, Writing - original draft. Jianhua Wang: Investigation,



**Fig. 6.** Co-occurrence patterns by network analysis between ARGs and the top 50 bacteria genera among samples from the zones (Left) influenced by wastewater pollution sources (rivers and WWTPs) and other zones (Right). The strong and significant correlations were visualized by purple connections (positive) and green connections (negative) (Spearman's correlation coefficients > 0.6, p < 0.05).

Formal analysis, Methodology, Writing - original draft. Jian Lu: Conceptualization, Funding acquisition, Project administration, Supervision, Resources, Writing - review & editing. Jun Wu: Methodology, Software, Writing - review & editing.

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# Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2020.106299.

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