

# Occurrence and distribution of antibiotic resistance genes in lakes and reservoirs from water-receiving area of Eastern Route of the South-to-North Water Diversion Project, Northern China

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## ABSTRACT

To characterize antibiotic resistance genes (ARGs) in surface waterbodies in Shandong Province, and to understand the occurrence, diversity and resistance mechanism of ARGs, nine aquatic ecosystems were selected and evaluated regarding occurrence of ARGs in the period from 2018 to 2019. Thirty-three types and 242 subtypes of ARGs were detected in the nine waterbodies with the most subtypes in Wohushan (242) and Jihongtan (235), and the fewest ARGs in Datun (213) and Mishan (221). Among the top nine ARG types, *MacB*, *BcrA*, *CarA* and *TetW* were the dominant ARGs in these samples. Four groups of the study sites were distinguished by cluster analysis, indicating different distribution characteristics of ARGs in these waterbodies, and regional differences in ARG contamination. Efflux pumps were the predominant resistance mechanism in all the waterbodies in terms of both subtype number (49.2%) and abundance (70.4%), followed by operon, antibiotic inactivation and others. Our findings provide comprehensive information to better understand the contamination level of ARGs on a local level as well as for global considerations, and may be practically helpful for the prevention and control of ARG pollution.

**Key words** | antibiotic resistance gene, cluster analysis, reservoir, resistance mechanism

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## HIGHLIGHTS

- Thirty-three ARG groups were detected, and tetracycline, vancomycin, multidrug were common.
- Four sampling groups indicated the regional differences in ARG contamination.
- Efflux pumps were the predominant resistance mechanism in these ecosystems.

## INTRODUCTION

In recent decades, hundreds of antibiotics have been widely used in many fields, e.g. medical treatment, aquaculture, and animal husbandry, and have made important contributions to the development of human society. However, widespread antibiotic resistance genes (ARGs) and emerging antibiotic-resistant bacteria have become a great public concern due to unmonitored use of antibiotics and release into the environment. In 2006, Pruden *et al.* (2006) formally proposed ARGs

as a new pollutant. At present, antibiotic-resistant bacteria (ARB) and ARGs are widely found in soil, waste water (Guo *et al.* 2017), surface water (Schwartz *et al.* 2003; Yang *et al.* 2017) and even deep ocean sediments, and in the remote Tibetan environment (Chen *et al.* 2016). Zhang *et al.* (2019) investigated 15 antibiotics and their responsible ARGs in water, sediments, and sewage treatment plants in the Yangtze River (Jiangsu section), indicating the relative

high risk of antibiotics and ARGs. The widespread occurrence and control of ARGs is a major public health issue and an emerging challenge to deal with worldwide (Pruden *et al.* 2013).

Although antibiotic resistance is recognized as a major threat to human public health worldwide, its diversity and distribution in urban water systems remains unclear (Guo *et al.* 2018; Tan *et al.* 2019). Liu *et al.* (2018) focused on the distribution characteristics of ARGs in 42 lakes and reservoirs in China for the first time and revealed the factors and mechanisms influencing their distribution. Most of these studies have used quantitative polymerase chain reaction (qPCR), which is an efficient tool to study the widespread occurrence and abundance of ARGs, however, PCR detection depends on available primers that are based on known resistance genes and is not suitable for the discovery of novel ARGs. With the fast development of next-generation sequencing techniques and the reduction in costs, metagenomics as a powerful technology overcomes the drawbacks of amplification-based methods, and could be used for broad-spectrum screening of ARGs (Chen *et al.* 2013; Fang *et al.* 2019). This culture-independent approach is a powerful tool for elucidating complex microbial diversities and the mechanisms of anti-microbial resistance in environmental samples. Recently, there have been a few studies based on metagenomic sequencing to detect ARGs in soil (Xiao *et al.* 2016), rivers (Jiang *et al.* 2018a), lakes (Didier *et al.* 2009; Vasileva *et al.* 2017) and sediments (Fang *et al.* 2019), including common tetracycline, macrolides and sulfonamides (Michael *et al.* 2013). Nevertheless, there are not many applications of metagenomics to study antibiotic resistance in surface water, and the knowledge of the diversity and abundance of ARGs in these aquatic ecosystems in the lower reaches of the Yellow River is poor, to date.

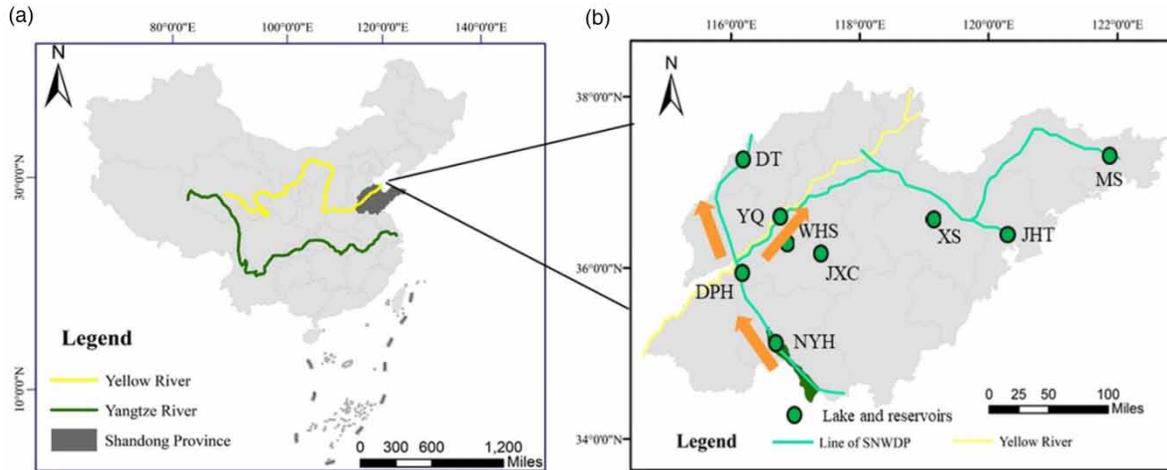
Shandong Province is the water-receiving area of the Eastern Route of the South-to-North Water Diversion Project (SNWDP). The SNWDP is a large-scale strategic project to relieve water shortages and sustain economic and social development in Northern China. The Eastern Route will span the four largest river basins in China (Yangtze River basin, Yellow River basin, Huai River basin and Hai River basin), and the diverted water will pass through and impound a chain of lakes and link up with

the northern section of the Beijing–Hangzhou Grand Canal (Feng *et al.* 2008). The Eastern Route will eventually transfer 14.8 billion m<sup>3</sup> of water annually from the Yangtze River to Northern China by a complex hydraulic system of interconnected lakes, rivers and canals. Most of these surface waterbodies are in a shallow, meso-eutrophic state (Hou *et al.* 2020). More important, after water diversion by SNWDP, there will be more multiple types of water source (e.g. mountainous reservoirs, Yellow River reservoirs, lakes), and the current characteristics of ARGs should be even more important for subsequent and comparable studies. As such, assessing the current status of water quality and contamination level of ARGs is critical for subsequent and comparable studies on a local level as well as for global considerations. A more comprehensive understanding of the occurrence and distribution in ARGs to adequately evaluate potential ecological impacts as the result of water transfer is essential. Nine aquatic ecosystems including lakes and reservoirs were selected in this work, and ARGs have been determined in 2018 and 2019. The aim of this study was to characterize ARGs in nine surface waterbodies in Shandong Province, and to understand the occurrence, diversity and resistance mechanism of ARGs by a comparison with samples from other environments.

## MATERIALS AND METHODS

### Sampling sites and sample collection

Nine representative surface water sources in the lower reaches of the Yellow River were selected in this study (Figure 1), including Wohushan Reservoir (WHS), Yuqing Lake Reservoir (YQ), Jinxiu Reservoir (JXC), Mishan Reservoir (MS), Datun Reservoir (DT), Dongping Lake (DPH), Jihongtan Reservoir (JHT), Nanyang Lake (NYH) and Xiaoshan Reservoir (XS), which spanned a large morphometric and biogeochemical gradient (Table 1). These waterbodies are distributed along the water-receiving area of the Eastern Route of the South-to-North Water Transfer Project (SNWTP) (Figure 1). Many of these reservoirs serve multiple purposes such as agricultural, drinking and industrial water supplies as well as fish-farming at various scales.



**Figure 1** | Map of (a) China and (b) Shandong Province showing the locations of the Yellow River, Yangtze River, as well as the nine lakes and reservoirs (green circles). The site codes are abbreviations for the waterbody names listed in Table 1. Please refer to the online version of this paper to see this figure in color: <http://dx.doi.org/10.2166/ws.2020.190>.

**Table 1** | Information on waterbody morphology and hydrological data in the studied waterbodies

Waterbodies	Reservoir codes	Latitude and longitude	Year of construction	Capacity ( $10^6 \text{ m}^3$ )	CA ( $\text{km}^2$ )	WA ( $\text{km}^2$ )	Rainfall ( $\text{mm/y}$ )
Mishan	MS	121°56'E, 37°10'N	1960	107	440	22.3	628
Dongping Lake	DPH	116°27'E, 35°56'N	No data	4,000	627	124	766
Jihongtan	JHT	120°13'E, 36°21'N	1989	146	No data	14.4	583
Yuqing	YQ	116°59'E, 36°40'N	2000	48.5	No data	4.9	706
Nanyang Lake	NYH	118°26'E, 36°40'N	No data	16.1	31,700	1,266	493
Xiashan	XS	119°30'E, 36°28'N	1960	5.0	4210	144	632
Datun	DT	116°12'E, 37°15'N	2013	5.2	No data	No data	512
Wohushan	WHS	116°58'E, 36°29'N	1958	117	557	No data	694
Jinxiuchuan	JXC	117°09'E, 36°30'N	1970	30	166	No data	642

CA: catchment area; WA: water area.

All the samples were collected from June 2018 to April 2019. For each reservoir, water column samples were collected below 0.5 m of the surface from three pelagic sites in each lake and reservoir.

### DNA extraction

For ARG analysis, water samples (1 L) were filtered with a  $0.22 \mu\text{m}$  membrane (Millipore, USA) in a vacuum filtration apparatus, and then stored in the refrigerator at  $-80^\circ\text{C}$ . Then the filters were handled by Sangon Biotech® to purify DNA and to construct an Illumina standard shotgun library with an insert size of  $2 \times 150 \text{ bp}$ , and then genome sequences

were obtained by the Illumina HiSeq Xten platform. Three repetitions of analysis of YQ, WHS and JXC demonstrated good repeatability in the metagenomic profiling of ARGs, with a relative standard deviation (RSD) of less than 10% in relative abundance, ARG types and subtypes. DNA was extracted from the water samples using the E.Z.N.A.™ Mag-Bind Soil DNA Kit (OMEGA) according to the manufacturer's protocol. The DNA yields of the nine samples ranged from 1.0 to  $18.5 \mu\text{g}$ , as quantified using the Quant-iT Picogreen dsDNA HS assay kit (Invitrogen) according to the manufacturer's manual. Sequencing was performed at Sangon Biotech®, Inc., Shanghai, China, using an Illumina HiSeq 2000 sequencing system (Illumina), generating 6G with  $2 \times 150 \text{ bp}$  paired

end reads per sample. Raw reads containing three or more ambiguous nucleotides, or with an average quality score below 20, or with length less than 100 bp (101 bp in length) were removed to guarantee the quality of downstream analysis. Data are available at the NCBI Sequence Read Archive under project no. SRP039858. Although such a high similarity threshold most probably excluded some divergent ARGs from the analysis, a more conservative strategy was still used here and we only focus on those that are highly similar to the known ARGs.

### Statistical analysis

The proportions of different types or subtypes of ARG-like sequences in 'total ARG-like sequences' and 'total metagenome sequences' were defined as 'percentage' (%) (Yang et al. 2013). ARG diversity was defined at reference sequence level and represents the number of reference sequences in each ARG subtype identified in our dataset (Chen et al. 2013). The cluster analysis used to find the aquatic ecosystems with similar species and relative abundance of bacteria and ARGs was performed by R software using the 'hclust' function.

## RESULTS AND DISCUSSION

### Diversity and abundance of ARGs

In the present study, a total of 33 ARG groups were detected in the nine lakes and reservoirs, conferring resistance to most major classes of antibiotics, which were divided into nine categories of ARGs that included *tetracycline* (26–33 subtypes), *macrolides* (2–3 subtypes), *penicillin* (7–10 subtypes), *chloramphenicol* (14–17 subtypes), *fluoroquinolone* (9–11 subtypes), *vancomycin* (19–21 subtypes), *multidrug* (75–87 subtypes), *cephalosporin* (7–10 subtypes) and other genes (Figure 2). The results showed that *tetracycline*, *vancomycin*, *multidrug* and other genes were common in these aquatic ecosystems in the middle and lower reaches of the Yellow River.

Figure 2 sums up the diversity of ARGs among the nine lakes and reservoirs; 213–242 ARGs were detected in the nine waterbodies, with the most ARGs in WHS (242) and JHT (235), and fewest ARGs in DT (213) and MS (221). The results suggested that this region under investigation has been exposed to detectable anthropogenic antibiotics,

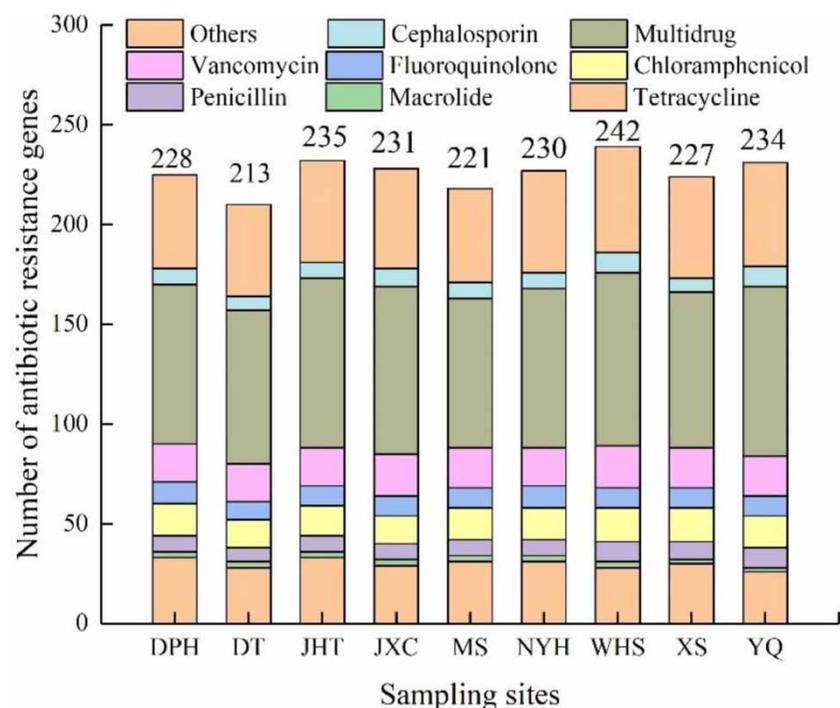
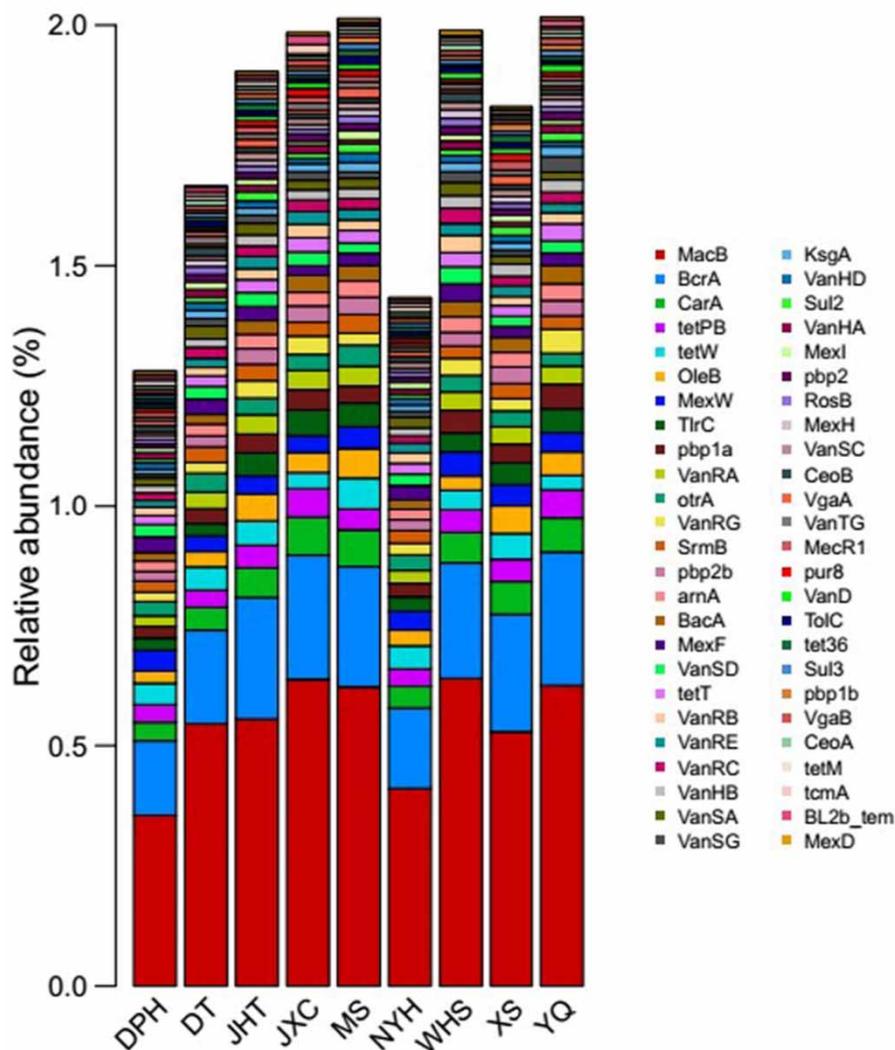


Figure 2 | Composition of ARGs in the nine selected waterbodies. They were based on the top nine ARGs at type and subtype level.

and that the bacterial inhabitants of this region have also suffered from the significant impacts of the wide use of antibiotics. Previous studies have reported the diversity of ARGs in reservoirs. Jiang et al. (2018b) pointed out that 118 ARGs were found in a reservoir located at the Yangtze River downstream during August, while 85–158 ARGs have been detected in 18 estuary sediments, at an average of 118. Diverse ARGs originating from natural environments were found in remote Tibetan soils, sheep waste, yak waste and sediments, and the sequence diversity was as high as ~200 (Chen et al. 2016). The top nine ARG types accounted for over 80% of the identified ARG sequences. Among them, *MacB*, *BcrA*, *CarA* and *TetW* were the dominant ARGs in

these samples (Figure 3), which have been detected in paddy soils from south China (Xiao et al. 2016) and Tibetan environments (Chen et al. 2016). The relative abundance ranged from 1.3% to 2.0% for the total of 50 ARGs. In general, the relative abundance of ARGs was significantly lower in lakes, including NYH and DPH, while higher in reservoirs (Figure 3), indicating the different distribution of ARGs between lakes and reservoirs.

Cluster analysis was conducted in the present paper to classify the samples according to microbial species similarity, where the paired group algorithm and Euclidean distance were used. In the dendrogram, where there is a smaller difference between samples, the samples will be in



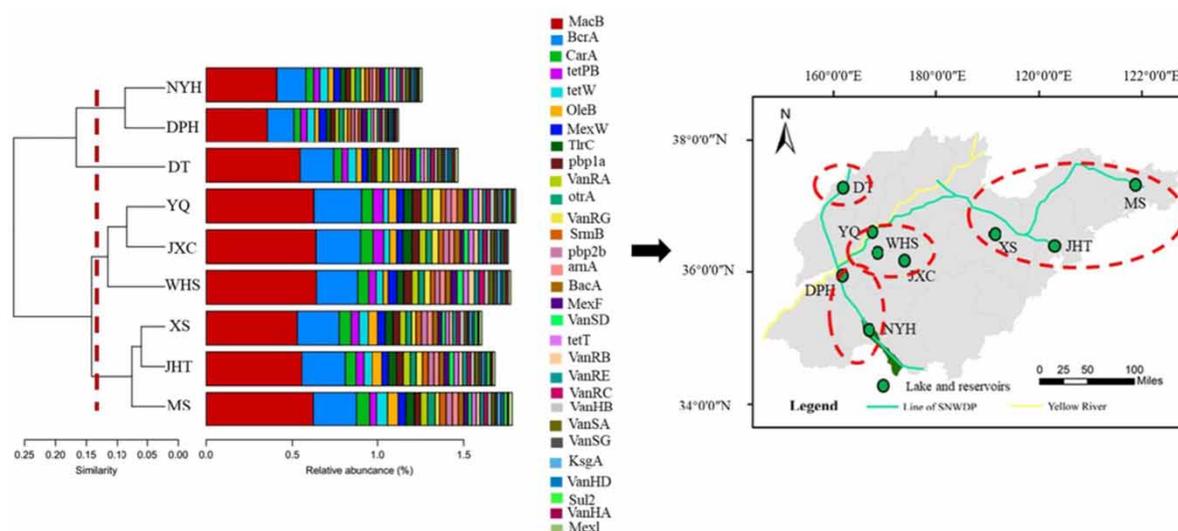
**Figure 3** | Composition and relative abundance of ARGs in the studied waterbodies based on percentages at ARG subtype level (%).

the same branch. In this study, four groups of ARGs detected by conventional cluster analysis were conducted (Figure 4), implying different distribution characteristics of ARGs in the four groups of waterbodies. The first group included two lakes (NYH and DPH), with lower abundance of *MacB*, *BcrA*, *CarA* and total relative abundance of the main 30 types of ARGs. The second group (DT reservoir) showed the lowest abundance of the 30 types of ARGs. The third group included three reservoirs located in Jinan City, YQ, JXC and WHS, in which was the highest abundance of *MacB*, *BcrA*, *CarA* and total relative abundance of the main 30 types of ARGs. The last group had the intermediate relative abundance of the 30 types of ARGs, including XS, JHT and MS reservoirs. The results of cluster analysis indicated regional differences in ARG contamination. DPH and NYH were famous for regulated lakes for the Eastern Route of the SNWDP, and enclosure culture and pharmaceutical factories also had a great influence on pollution status (Lu et al. 2011; Wei & Sun 2016). The DT reservoir is located in the northwest of Shandong Province, where the land usage was dominated by agriculture (e.g. wheat, corn). Uyaguari-Díaz et al. (2018) found that the relative abundance and richness of ARGs were highest in agriculture-impacted watersheds compared with urban and protected watersheds, and environmental factors such as land-use and water quality parameters accounted for 45%

of the variability in ARGs observed in watershed locations. For the three reservoirs (WHS and YQ) located at the junction of urban and rural areas in Jinan City, domestic discharge and agricultural activities were the major threats to water quality (Hou et al. 2015). In addition, the reservoirs with larger surface area (e.g., JHT, XS, MS) are located in the east of Shandong Province, where there is drought and water is transferred from the Yangtze River by the SNWDP in the dry season. In a word, unique geomorphometric and land-use features may drive the differences in distribution of ARGs. Liu et al. (2018) also reported that the ARGs had a distinct biogeographical pattern in Chinese lakes and reservoirs, and spatial and physicochemical factors exhibited significant effects on the spatial distribution of ARG composition.

## Resistance mechanisms

Microbial resistance to antibiotics currently spans almost all known classes of natural and synthetic compounds (Pontes et al. 2009), while the underlying mechanisms vary, and sometimes two or three mechanisms coexist for one kind of antibiotic. The ARGs detected in these aquatic ecosystems encompassed the five major resistance mechanisms – extrusion by efflux pumps, antibiotic inactivation, operon, transposon and others or unknown. Extrusion by efflux



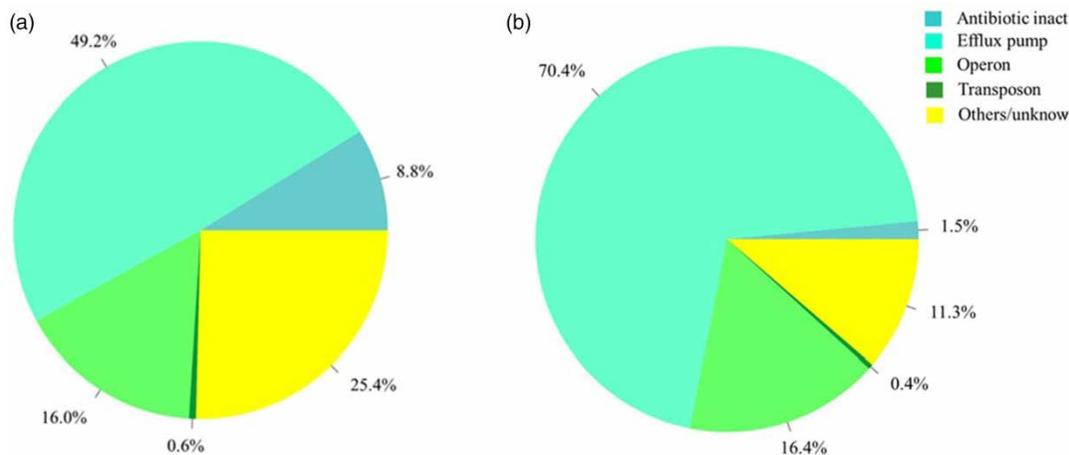
**Figure 4** | The classified groups on the left for qualitative analysis of ARGs in samples of the nine aquatic ecosystems. The histogram in the middle shows the distribution of ARGs in the samples, and different colors represent different species. On the right, the distribution of lake and reservoirs is shown in the map according to the classified groups. Abbreviations of waterbodies are the same as in Table 1. Please refer to the online version of this paper to see this figure in color: <http://dx.doi.org/10.2166/ws.2020.190>.

pumps was the predominant resistance mechanism in all the waterbodies in terms of both subtype number (49.2%) (Figure 5(a)) and abundance (70.4%) (Figure 5(b)), partly owing to the high abundance/percentage of multidrug ARG types. Multidrug efflux pumps are ubiquitous in bacteria. The mechanism of an efflux pump is to excrete antibiotics out of the cell by specific or universal efflux pump, and reduce the antibiotic concentration in the cell, and show resistance in turn (Poole 2007; Debabov 2013). The antibiotic efflux pump systems usually make *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* multi-drug resistant to antibiotics, e.g. tetracycline, fluoroquinolone and macrolides (Liang 2013). Besides efflux pumps, a significant fraction of ARGs act by the mechanism of operon (16.0% by number and 16.4% by abundance) (Figure 5(a) and 5(b)), which is often considered to be mainly associated with resistance to common glycopeptide antibiotics, such as vancomycin (Ahmed & Baptiste 2017). High proportions of antibiotic inactivation were found in samples (1.5–8.8%), implying there is a selection effect of antibiotics on ARGs. The mechanism of antibiotic inactivation changes the structure of antibiotics by degrading antibiotics or replacing active genes, causing antibiotic inactivation. Transposon was another mechanism of resistance in this work, and accounted for the lowest percentage (0.4–0.6%). Other or unknown resistant mechanisms accounted for high percentages in this study, with values of 25.4% and 11.3% based on number and abundance of ARGs, respectively. It could be found in manifold

resistance, bleomycin resistance, and working independently or by facilitating resistance with other mechanisms.

## CONCLUSION

The Eastern Route of the SNWDP is a large-scale strategic project to relieve water shortages and sustain economic and social development in Northern China. Here for the first time, a metagenomic analysis was adopted to provide insights into the occurrence and distribution of ARGs in lakes and reservoirs from the water-receiving area of the project. A total of 33 types and 242 subtypes of ARGs were detected in the nine waterbodies with the most subtypes in WHS and JHT, and least ARGs in DT and MS. The top nine ARG types accounted for over 80% of the identified ARG sequences. Among them, *MacB*, *BcrA*, *CarA* and *TetW* were the dominant ARGs in these samples. Four groups of sampling sites detected by conventional cluster analysis were conducted, implying different distribution characteristics of ARGs in the four groups of waterbodies, and indicating regional differences in ARG contamination. Extrusion by efflux pumps was the predominant resistance mechanism in all the waterbodies in terms of both subtype number (49.2%) and abundance (70.4%), followed by operon, antibiotic inactivation and others. Overall, our results clearly demonstrated there is a great diversity of ARGs in sources of drinking water in Northern China, indicating that the occurrences, accumulation and spread of



**Figure 5** | Mechanism of antibiotic resistance. The ARGs detected in these samples were defined based on the mechanism of antibiotic resistance by (a) number and (b) abundance of ARG subtypes.

ARGs have threatened both public health and natural ecosystem health. In future, research will need to be directed towards the environmental factors (e.g. hydrological feature, eutrophication) influencing distribution and diversity of ARGs.

## ACKNOWLEDGEMENTS

We appreciate comments from two anonymous reviewers. This work was jointly supported by the Shandong Province National Science Foundation, China (ZR2017MC047), the Critical Patented Projects in the Control and Management of National Polluted Water Bodies (2017ZX07502003-06) and the Special Project of Taishan Scholar Construction Engineering (ts201712084).

## DATA AVAILABILITY STATEMENT

Data cannot be made publicly available; readers should contact the corresponding author for details.

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First received 13 January 2020; accepted in revised form 31 July 2020. Available online 13 August 2020