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# Diverse and abundant antibiotic resistance genes from mariculture sites of China's coastline

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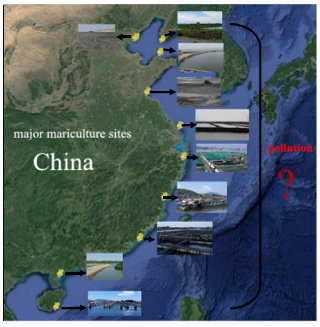
## HIGHLIGHTS

- Antibiotics and ARGs were widely distributed in most maricultural sediments.
- The abundance of *bac*A was the highest, followed by *mex*F and *mex*B.
- The resistance to antibiotic of bacteria isolated from almost all the maricultural sediments was high.
- The abundance of most ARGs correlated directly with antibiotics.

## GRAPHICAL ABSTRACT

The antibiotics and their corresponding ARGs were prevalent in major mariculture sites in China, and the number and proportion of antibiotic-resistant bacteria was large, which may pose a grave threat to marine environment and even human health. The relevant departments of Chinese government should strengthen supervision and reduce environmental damage.




The sampling locations along China's coastline

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## ABSTRACT

With the rapid development of mariculture in China, large amounts of antibiotics are being discharged into the aquatic environment. Little information is available regarding antibiotics and corresponding antibiotic resistance genes (ARGs) associated with maricultural environments in China. Sediments from eleven typical mariculture areas along the whole coastline of China were collected, and the sediment in Meijijiao in southern China was used as a non-mariculture control. The results revealed that antibiotics and their corresponding ARGs were widely distributed in most maricultural sediments, and present at low concentrations in samples from Meijijiao. The sulfonamide-resistance genes were prevalent, and the sul1 and sul2 in Penglai were the highest detected by using quantitative PCR. Moreover, remarkable differences in ARGs among different sites were observed. Due to the limited availability of primers to detect ARGs, illumina high-throughput sequencing was also used for profiling ARGs, and the results showed that the abundance of *bac*A in all samples was the highest compared to other ARGs, followed by *mex*F and *mex*B. This is the first study to comprehensively investigate the antibiotic resistance profile in typical mariculture areas along the whole coast of China. This study provides insights into the impacts of mariculture on the profiles of bacterial and ARG compositions in China.



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## 1. Introduction

Marine aquaculture (mariculture) plays an important role in the economic development within China. Many indigenous and introduced marine species have been successfully cultured along the 18,400 km coastline of China, which provide good, natural conditions for farming of marine animals. China has become the world's largest areas for marine farming production (Liu and Su, 2017). Cultured marine animals are frequently susceptible to bacterial infectious diseases. Therefore, antibiotics are widely and intensively used in mariculture for prevention and treatment of microbial pathogens (Bu et al., 2016). More than 8000 t are used as feed additives each year in China, which is the largest producer and consumer of antibiotics in the world (Ben et al., 2008). Although there is no government data, which accurately shows the exact quantity of antibiotics used in mariculture, official figures suggest that 210,000 t (46%) of antibiotics are used annually for livestock (Mo et al., 2017). It is conceivable that antibiotics are also heavily used in mariculture. The use of antibiotics is unmonitored in China, which often leads to high use, reflected by the high concentrations of antibiotic residues that are commonly detected in sediments around the coastline of China (Zhang et al., 2013).

The rise in use of antibiotics results in the emergence, spread, and dissemination of antibiotic resistance genes (ARGs), which ultimately challenges the efficacy of life-saving antibiotic therapies (Ji et al., 2012). The ARGs are becoming increasingly prevalent after introduction of antibiotics into aquaculture (Ozaktas et al., 2012). A significant correlation between the occurrence of ARGs and the concentration of antibiotics in aquaculture sites has been reported (Shah et al., 2014). To improve the production of fish and shellfish, there has been irresponsible overuse and misuse of antibiotics for treatment of a variety of bacterial pathogens. This has had a major effect on the propagation of ARGs, which is a major anthropogenic environmental threat to the natural environment. Once human pathogenic bacteria acquire the ARGs, this could consequently threaten human health. The intensive form of mariculture adopted by most Chinese fish farmers may greatly facilitate the dissemination of ARGs throughout the coastal aquatic environment (Dang et al., 2009; Gao et al., 2012). Therefore, antimicrobial resistance might have very serious implications for the maricultural environments of China, and it is thus necessary to investigate the antibiotic resistance profile in intensive farming areas along China's coastline.

Aquaculture environments have been considered as potential reservoirs for ARG pollution (Huang et al., 2017). Antibiotics and ARGs have been shown to be prevalent in the aquaculture environments (Su et al., 2017). ARGs in microorganisms could be easily transferred to indigenous environmental bacteria horizontally resulting in a poorer ecological environment, and a threat to humans living in certain environments (Boy-Roura et al., 2018; Freitas et al., 2018). Numerous reports have demonstrated that the direct application of antibiotics in mariculture increases the number of antibiotic resistant bacteria and ARGs in sediments at mariculture sites (Scarano et al., 2014). Although ARGs are recognized as a potentially serious threat to human health, they are only being viewed as ecological and environmental problems in China. Areas of mariculture in China may have developed a reservoir of antimicrobial resistant bacteria and ARGs due to the heavy use of antibiotics (Zhao and Dang, 2012). However, there are few studies focusing on the study of ARGs in maricultural environments.

The objective of the present study was to investigate the diversity of antibiotic resistant bacteria and to quantify the ARGs in typical mariculture environments of China. To our knowledge, this is the first study to analyze the diversity and abundance of antibiotic resistant bacteria and ARGs in the main mariculture areas along the coastline of China. These data provide an insight into the present situation of antibiotic pollution, antibiotic resistant bacteria, and ARGs in coastal waters of China.

## 2. Experimental procedures

### 2.1. Sample collection

A total of 100 samples were collected from September to October in 2015 from 10 typical maricultural areas in China, which included the following: DL (Dalian), TS (Tangshan), PL (Penglai), LYG (Lianyungang), QD (Qidong), XS (Xiangshan), ND (Ningde), DS (Dongshan), ZJ (Zhanjiang), and LS (Lingshui). These are the main mariculture areas in China, and the details of these are shown in Fig. 1. Data from 1S to 10S show the latitude and longitude positions of the 100 sampling sites in detail. MJJ (Meijijiao) is located in China's southernmost point where aquaculture activity is non-existent, and this site served as a non-aquaculture (control) site. The sediments from ten sampling points in the same maricultural area as above were chosen for collection and sampling, and then these were mixed as one field sample (Fig. 1S to 10S). All samples were kept in an icebox until they were brought to the lab, where they were mixed well according to the sampling sites and either processed immediately for plating the bacteria or stored at −80 °C for antibiotic quantification and DNA extraction.

### 2.2. Antibiotic quantification

With some minor modifications, the concentrations of two sulfonamides (sulfadiazine and sulfamethoxazole), two tetracyclines (tetracycline and oxytetracycline), and two quinolones (ciprofloxacin and enrofloxacin) were analyzed in this study according to previous studies (Chen et al., 2016; Luo et al., 2010). Briefly, solid phase extraction (SPE) was applied to extract sediment samples with HLB cartridges, and the target compounds in the final extracts were analyzed by high-performance liquid chromatography–tandem mass spectrometry (HPLC-MSIMS analysis).

### 2.3. Isolation and identification of antibiotic-resistant marine bacteria from sediment

According to the method described by Gao and colleagues, 1 g of wet sediment was thoroughly suspended in 9 ml of sterile physiological saline (0.85%) by vortexing, and a 10-fold serial dilution was created. The homogenized sample was diluted and serial 10-fold dilutions up to $10^3$ were plated onto nutrient broth plus 1.5% bacto agar containing tetracycline (10 μg/ml), oxytetracycline (20 μg/ml), ciprofloxacin (2 μg/ml), enrofloxacin (2 μg/ml), sulfadiazine (50 μg/ml), and sulfamethoxazole (100 μg/ml). The cultures were incubated at 30 °C for 48 h.

After isolation, the individual colonies were identified based on the analysis of the 16S rDNA gene sequence and using the primers, 27F and 1492R. Genomic DNA from pure strains was extracted using the Bacterial DNA Extraction Kit (Tiangen Biotech Co. Ltd., Beijing, China). The 16S rDNA was amplified by PCR and sent directly to Shanghai Sangon Biotechnology Co. Ltd. for sequencing. For identification of bacteria, the DNA sequences were analyzed by comparison with those available at the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov) using the Blast program (Tahrani et al., 2015).

### 2.4. Real-time qPCR of ARGs

Real-time qPCR was applied to quantify the presence of ARGs as previously described (Chen and Zhang, 2013). Total DNA was extracted from the sediment using the Soil DNA Isolation Kit (MiBio Laboratories, Inc., CA, USA) according to the manufacturer's instructions. The quality of the DNA was verified by agarose gel electrophoresis and spectrophotometry. Real-time DNA amplification was performed using SYBR Premix EXTaqTM (Takara Biotechnology, Dalian, China). Primers are listed in Table 1S. The 16S rDNA was quantified to minimize the variance in the abundance of ARGs caused by differences in the DNA