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Occurrence of intracellular and extracellular antibiotic resistance genes in coastal areas of Bohai Bay (China) and the factors affecting them☆

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ABSTRACT

Coastal areas are the transition zones between ocean and land where intracellular antibiotic resistance genes (iARGs) and extracellular antibiotic resistance genes (eARGs) could spread among marine organisms, and between humans and marine organisms. However, little attention has been paid to the combined research on iARGs and eARGs in marine environment. In this context, we collected water and sediment samples from the coastal areas of the Bohai Bay in China and performed molecular and chemical analyses. The results of quantitative real-time PCR (qPCR) showed that the relative abundance of eARGs was up to $4.3 \pm 1.3 \times 10^{-1}$ gene copies/16S rRNA copies in the water samples and $2.6 \pm 0.3 \times 10^{-3}$ gene copies/16S rRNA copies in the sediment samples. Also, the abundance of eARGs was significantly higher than that of iARGs. Furthermore, the average abundances of antibiotic resistance genes (ARGs, include iARGs and eARGs) were the highest in both the water and sediment samples from the estuaries. The results of liquid chromatography-mass spectrometry showed that the concentrations of antibiotics in estuaries and areas near the mariculture site were higher than that in the other sites. The class 1 integron gene (*int*1) and *sul*1 in the intracellular DNA were significantly correlated in the water samples. Moreover, significant correlation between *int*1 and *sul*2 in the extracellular DNA was also found in the sediment samples. The combination of sulfamerazine and tetracycline as well as the combination of sulfamethazine and dissolved oxygen can both explain the abundance of ARGs, implying the combined effects of multiple stresses on ARGs.

1. Introduction

In the first decade of the 21st century, global antibiotic consumption has increased by 35% (Van Boeckel et al., 2014). Coastal countries, such as India, China, and the United States, are the largest consumers of antibiotics in the world (Van Boeckel et al., 2014). Antibiotics used in medicine and agriculture could enter living organisms and set off selection for antibiotic resistance through induction of mutations; the antibiotics released into the environment could cause the enrichment of antibiotic resistant bacteria (Becattini et al., 2016; Kümmerer, 2009). Being one of the emerging environmental contaminants (Pruden et al., 2006), antibiotic resistance genes (ARGs) originating from antibiotic resistant bacteria have multiple ways of transmission in the natural environment. For instance, intracellular ARGs (iARGs), in addition to being passed on to the offspring of bacteria through self-replication, can also be transmitted to other species via conjugation or transduction. Extracellular ARGs (eARGs) can be taken up by bacteria *via* natural transformation (Zhu, 2006). On acquiring antibiotic resistance, human pathogenic bacteria could become resistant to antibiotics, and thus, could pose a potential risk to human health (Pruden et al., 2012; Rizzo et al., 2013). The antibiotic resistance has, therefore, gradually drawn the attention of people, and more and more studies focus on these new contaminants. A previous study showed that multiple factors could influence the spread of ARGs,

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for instance, mobile genetic elements (MGE) such as transposons and integrons play an important role (Ochman et al., 2000). In addition, environmental factors could contribute to the antibiotic resistance crisis (Fletcher, 2015). Sub-lethal concentrations of antibiotics can select and enrich the antibiotic resistant pathogens (Andersson and Hughes, 2012). Moreover, antibiotics, together with other environmental factors, can strengthen or weaken the antibiotic resistance in non-homogeneous environments (Chait et al., 2007, 2016). To devise methods to alleviate the widespread antibiotic resistance, it is important to comprehend the persistence and propagation of both iARGs and eARGs in the environment.

In recent studies, sewage treatment plants, hospitals, and swine farms have been recognized as the hotspots of ARGs (Bengtsson-Palme and Larsson, 2016; Picao et al., 2013; Zhu et al., 2013). The occurrence of iARGs has also been reported in rivers, lakes, and marshes, which were related to the discharge of sewage or effluents (Czekalski et al., 2015; Luo et al., 2010; Thi Thanh Thuy et al., 2012). Moreover, iARGs were also detected in glaciers, reservoirs, and groundwater, which are rarely influenced by human activities (Boeckelmann et al., 2009; Huerta et al., 2013; Segawa et al., 2013). These studies about ARGs focused on iARGs in aquatic environment, which are related to the occurrence of eARGs. eARGs could pose a serious threat to human health and ecosystems (Pietramellara et al., 2009; Pruden et al., 2006) because of the ability of transforming into bacteria (Chang et al., 2017). Although eARGs were detected in rivers, estuaries, and lakes (Guo et al., 2017; Mao et al., 2014; Wang et al., 2016), the studies about the effect of combined factors on total ARGs (including iARGs and eARGs) in aquatic environment are still few. Moreover, recent research reported that eARGs could be preserved in seawater (Bien et al., 2017), suggesting the occurrence and persistence of eARGs in the marine environment.

Similar to other water environments, bay and coastal areas largely influenced by human activities are also hot spot areas of ARGs (Dash and Das, 2016; Fistarol et al., 2015; Zhu et al., 2017). Moreover, ARGs have been detected in the marine sponge *Petromica citrina* along the Brazilian coast (Prichula et al., 2016). The identical sequences of *qnr*A1, *qnr*B1, and *qnr*S1 between *Escherichia coli* found in Chilean patients and Chilean marine bacteria indicates the occurrence of horizontal gene transfer between marine bacteria and human pathogens (Tomova et al., 2015). Moreover, the health risks caused by the human exposure to water bodies that contain antibiotic resistant bacteria, as a result of bathing in seawater (Leonard et al., 2015), should not be overlooked. These reports showed that ARGs could spread among marine organisms, as well as between marine organisms and humans, and the marine environment plays an important role in the spread of ARGs (Hatosy and Martiny, 2015). There are three possible sources of ARGs in the marine environment. The first is the terrestrial runoff flowing into ocean containing various ARGs (Na et al., 2014). The second source comprises of antibiotics and other pollutants from mariculture or coastal runoffs that select for antibiotic resistance (Muziasari et al., 2016). The third is the evolution of marine microorganisms resisting other populations that can produce antibiotics leading to the formation of ARGs (Cordero et al., 2012). Antibiotics and ARGs in the marine environment can not only be diluted by water exchange in the oceans but also be delivered to distant areas with ocean currents (De Souza et al., 2006; Zhang et al., 2013a). Continuous low concentrations of antibiotics might have a novel effect on ARGs owing to the unique physicochemical characteristics of the marine environment, such as high osmotic stress (Chait et al., 2016). Furthermore, eARGs in the marine environment could participate in the biogeochemical cycles or horizontal gene transfer (Corinaldesi et al., 2005), and thus enter different niches. However, until recently, there was a lack of knowledge about the distribution and levels of eARGs in the marine environment.

To better understand the persistence and fate of ARGs in the marine environment, iARGs, eARGs, and the environmental factors related to them in the coastal area of Bohai Bay in China were studied. The goals of this study were as follows: (1) to quantify the occurrence of eARGs and iARGs in the Bohai Bay, (2) to study the distribution of antibiotics in the Bohai Bay, and (3) to evaluate the relationship between ARGs and multiple factors.

2. Materials and methods

2.1. Sampling methods

Located in the west of Bohai Sea, Bohai Bay has a total area of 14,700 km^2 with a coastline of 1076.5 km. The bay is surrounded by the Bohai Economic Rim, which has a population over 100 million. The samples were collected from 10 sites in the coastal area of Bohai Bay (Fig. 1), including four sites from the estuary (EUY): Shahe estuary (H2), Haihe estuary (H4), Duliujianhe estuary (H5), and Tuhaihe estuary (H8); three sites near the mariculture area (MAC): Dashentang mariculture area with artificial reefs (M3), Lijiabao mariculture area by the bottom-sowing mode (M6), and Xinhu mariculture area with seawater ponds (M9); two sites that were rarely influenced by human activities (LIT): N7 and N10; and one site, i.e., I1 near the Caofeidian Industry Zone (IDY). The sampling was conducted in August 2016.

Surface seawater was collected with a water sampler and stored in a 1-L sterile brown polypropylene bottle. The sediment samples were collected with a sediment sampler and stored in a Ziploc bag. The water and sediment samples were placed in an ice bath, kept in darkness, and transported to the laboratory within 8 h of collection. The water and sediment samples were preserved at 4 °C and −20 °C, respectively, in the laboratory.

2.2. DNA extraction and PCR detection method

The extracellular and intracellular DNAs present in the sediments were extracted according to the method described by Corinaldesi et al. (2005), with some modifications. In brief, the sediment samples were divided into two identical subsamples. One subsample was lyophilized to calculate the percentage of moisture for further DNA quantification. The other one, which was equivalent to 1 g per unit dry weight, was used for extracting the DNA. Firstly, it was treated with an extraction buffer, and then the mixture was centrifuged at 10000 × *g* for 20 min at 4 °C. The supernatant containing the extracellular DNA was extracted using 1% cetyltrimethyl ammonium bromide (CTAB), and the cell pellet containing the intracellular DNA was extracted using a Soil DNA Extraction Kit (Omega, USA). All the extracted DNA samples were stored at 4 °C.

The water samples (0.5 L) were filtered through a 10-μm membrane to remove the impurities, as proposed by Yin et al. (2013); this was followed by filtration through a 0.22-μm membrane to separate the extracellular DNA from the intracellular DNA. The filtrates were centrifuged at 10000 × *g* for 20 min at 4 °C. The supernatant was diluted by adding an equal volume of deionized sterile water, as suggested by David and Megan (1989), and the extracellular DNA in the dilution was extracted by the CTAB method, as described above. The intracellular DNA retained on the 0.22-μm membrane was extracted using a Water DNA Extraction Kit (Omega, USA). All the extracted DNA samples were stored at 4 °C.

The quality of DNA was checked with 1% agarose gel electrophoresis. The yield and purity of DNA were examined using K5500 ultramicro spectrophotometer (Kaiao, Beijing, China). The $OD_{260/280}$