Marine Pollution Bulletin 84 (2014) 70–75

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Sulfonamide antibiotics in the Northern Yellow Sea are related to resistant bacteria: Implications for antibiotic resistance genes

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ARTICLE INFO

Article history:
Available online 11 June 2014

Keywords:
The Northern Yellow Sea
Sulfonamide
Antibiotic-resistance gene
Resistant bacteria
Correlation

ABSTRACT

Antibiotic resistance gene (ARG) residues and the mode of transmission in marine environments remain unclear. The sulfonamide (SAs) concentrations, different genes and total bacterial abundance in seawater and sediment of the Northern Yellow Sea were analyzed. Results showed the genes *sul I* and *sul II* were present at relatively high concentrations in all samples, whereas the gene *sul III* was detected fewer. The ARGs concentrations in the sediment were 10^3 times higher than those in water, which indicated sediment was essential ARG reservoir. Statistical analysis revealed the total antibiotic concentration was positively correlated with the relative abundance of the gene *sul I* and *sul II*. The relative abundances of the gene *sul I* and the gene *sul II* were also correlated positively with those of the gene *int1*. This correlation demonstrated that SAs exerted selective pressure on these ARGs, whereas the gene *int1* could be implicated in the propagation of the genes *sul I* and *sul II* in marine environments.

1. Introduction

Studies have focused on the release of antibiotics, antibiotic-resistant bacteria, and antibiotic resistance genes into natural environments because these organic materials may exacerbate environmental problems (Pruden et al., 2006; Knapp et al., 2010; Levy and Marshall, 2004). Furthermore, the use of antibiotics to prevent and cure infections in humans may result in increased resistance (Song et al., 2004; Hsu et al., 2010). For example, soil bacteria exhibit increased resistance to tetracycline after these microorganisms are exposed to this antibiotic; however, resistance decreases once exposure to this antibiotic is terminated (Rysz and Alvarez, 2004; Mei et al., 2013). After ARG-carrying bacteria die, their DNA can still be released into the environment and be transformed into other bacteria in the ecosystem (Zhu, 2006; Maurice and Philippe, 1972). Studies have described the occurrence of resistance gene pollution, but these studies have focused on the distribution of resistance genes in lagoons and rivers. For example, Mckinney (McKinney et al., 2010), Jiang et al. (2013), and Lou et al. (2010) detected approximately 10^1 and 10^{12} copies/mL ARGs in water and sediments, respectively. Nevertheless, ARG pollution in the ocean has not yet been reported.

Among different classes of antibiotics, sulfonamides (SAs) should be extensively investigated because of their widespread use, high excretion rate, high solubility, and persistence in the environment (Lamshöft et al., 2007; Sergio and Rossella, 2014). SAs are often detected at high concentrations in animal manure (Hu et al., 2006; Akin and Isil, 2009). Previous studies showed that SAs can be significantly drained into water bodies (Tolls, 2001; Hirsch et al., 1999). However, studies have yet to determine whether or not relatively high SAs concentrations contribute to the selection pressure of associated antibiotic-resistant bacteria and ARGs in marine environments.

The Yellow Sea is the second largest sea in China. Every year, large amounts of antibiotics flow into this water body as a consequence of human activities and industrial development. This area covers multiple inlets, coastal tourism areas, and city ports where economic development flourishes. Previous studies reported the presence of ARGs in inland waters in China (Hu et al., 2008). However, no comprehensive regional field studies have characterized the concentrations of antibiotic residues, the levels of the

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http://dx.doi.org/10.1016/j.marpolbul.2014.05.039
0025-326X/

associated antibiotic-resistant bacteria, and the amounts of ARGs in the Northern Yellow Sea. The persistence of antibiotic-resistant bacteria depends on the effects of resistance on fitness relative to antibiotic-sensitive genotypes in the absence of antibiotics (Hughes, 2010); resistance may be observed in the form of chromosomal mutations or horizontally acquired elements, such as plasmids, transposons, and integrons (Liezi and Hafizah, 2007). Transmissible plasmids are encoded, and integrons are a well-organized gene expression system that can capture and release some of these genes into natural environments; they can easily transfer from one bacterial species to another and become vulnerable to relocation by wind and water to agricultural soil, surface and groundwater, and human settlements (Pruden et al., 2006; Chee-Sanford et al., 2001; Pei et al., 2006; Novais et al., 2005; Zhang et al., 2009). In the absence of an evident selective pressure (Lili et al., 2007; Sandaa and Enger, 1994), ARGs can be transferred among prokaryotes as they exchange genomic information. Therefore, this study investigated the relationship between low SAs concentration in marine environments and SAs resistance genes (i.e., *sul I*, *sul II*, and *sul III*). This study also focused on the main source of ARGs in the Northern Yellow Sea combined with the *int1* gene that may facilitate ARGs propagation.

2. Materials and methods

2.1. Sampling sites

Surface water and sediment samples were collected from 16 sites, including typical regions containing ARGs. Sites 1 and 2 (S1 and S2) near the wastewater treatment plants (WWTP). Site 6 (S6) is found in the aquaculture area. Sites 7 and 8 (S7 and S8) are near the tourism area. Site 9 (S9) is situated in the medical wastewater area (Figs. 1 and 2). Water and sediment samples were collected from all of the sampling points. However, sediment was not collected from S6 to S7.

2.2. Sample collection

The upper 0.5 m of the surface water and upper 5 cm layer of the sediment samples were collected using water harvesting and dredging equipment along the coast of the Northern Yellow Sea. All of the samples were collected aseptically, placed in an ice bath, and transported to our laboratory. The water and sediment samples were stored at 4 and −20 °C, respectively, until processed for DNA extraction.

2.3. High-performance liquid chromatography–mass spectrometry (HPLC–MS/MS) analysis of antibiotics

Approximately 1 L of sea water and 5.0 g of the sediment samples were extracted with acetonitrile. Solid phase were performed using an Oasis®MCX cartridge (3 mL, 60 mg) to clean the SAs samples. The samples were then analyzed by TSQ Quantum HPLC–MS/MS (Thermo Fisher Scientific, USA). The antibiotics containing 14 SAs [*N*-((4-aminophenyl) sulfonyl) acetamide, sulfadiazine, sulfathiazole, sulfamerazine, sulfamethazine, sulfameter, sulfamethylthiazole, sulfamonomethoxine, sulfachloropyridazine, sulfamethoxazole, sulfamethoxypyridazine, sulfamethazine, sulfisoxazole, and sulfadoxine] were separated by C18 reverse phase columns. These antibiotics were subjected to mass spectrometer detections operated at selected reaction monitoring modes (Na and Fang, 2013). The quantification curves of antibiotics yielded good correlation coefficients ($R^2 > 0.990$). The limit of detection for each antibiotic was defined as the concentrations corresponding to the signal-to-noise of 3. The average water and sediment rates were 72.8% and 84.5%, respectively. All of the samples were analyzed in triplicates, and the relative standard deviation ($n = 3$) was less than 17%.

2.4. Isolation and identification of Escherichia coli

Tenfold serial dilution (i.e., 100, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) of each water sample and sediment sample were prepared in a sterile saline solution (0.85% NaCl) to obtain the recommended target range of *E. coli* (20–80 CFU per filter). Each diluted sample was filtered using a sterile membrane filter (pore diameter = 0.45 μm) with a vacuum filtration apparatus. The membrane filter was then removed using sterile forceps and rolled on the modified membrane-thermo tolerant *E. coli* agar (MI agar, BD, USA) The plates were then incubated at 36.5 ± 0.5 °C for 22 h.

An initial trial was conducted using *E. coli* strains with known antibiotic (SM_2) resistance profiles. *E. coli* ATCC 25922 was used as a control strain with no resistance to the selected antibiotics. According to the EPA protocols (U.S. EPA, 2002) in this method, *E. coli* are bacteria that produce blue colonies under ambient light after these bacteria were primary cultured on MI agar or broth. These colonies can be fluorescent under longwave ultraviolet light (366 nm).

2.5. Sample extraction optimization and DNA extraction

Water samples (1 L) were filtered using a 0.45 μm filter in a vacuum filtration apparatus. The filters were placed in extraction

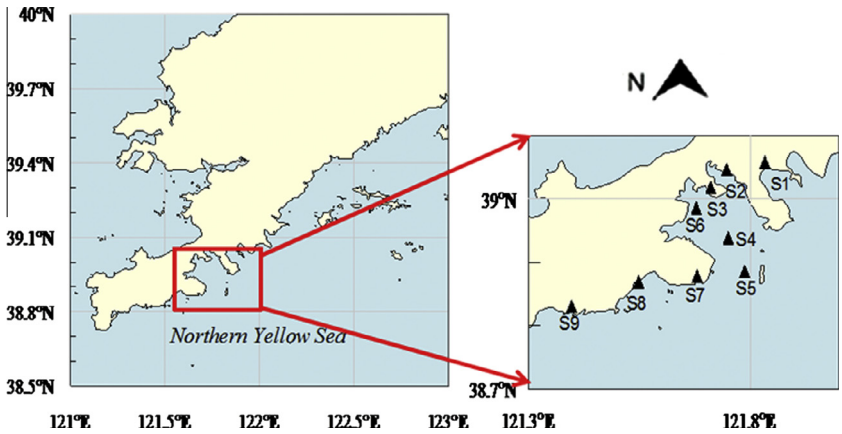

Fig. 1. Location of the sampling sites.