Environmental Pollution xxx (2018) 1–9

Contents lists available at ScienceDirect

Environmental Pollution

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

Combined impact of fishmeal and tetracycline on resistomes in mariculture sediment☆

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ARTICLE INFO

Article history:
Received 2 April 2018
Received in revised form
27 June 2018
Accepted 22 July 2018
Available online xxx

Keywords:
Fishmeal
Antibiotic resistance genes (ARGs)
Bacterial communities
Mariculture microcosm
Human health

ABSTRACT

Mariculture sediment has been recognized as a major contributor of environmental antibiotic resistance genes (ARGs), which are challenging the treatment of infections worldwide. Both antibiotics and fishmeal are used in aquaculture, and each has the potential to facilitate ARG dissemination, however their combined impact on the sediment resistome and their relative contribution remain unclear. In this study, microcosms were exposed to varying concentrations of tetracycline with or without fishmeal (0.1% wt/wt) for 14 days. Sediment genomic DNA was analyzed using high throughput quantitative PCR and 16S rRNA gene amplicon sequencing to compare the contribution of fishmeal and tetracycline to antibiotic resistomes and bacterial communities in mariculture sediment. Sixty-seven ARGs were detected potentially correlating to resistance for several major antibiotics. Fishmeal, but not the dose of tetracycline, contributed to the significant increase of both ARG abundance and diversity in the sediment. Based on principle coordinate analysis and hierarchical clustering, ARGs were clustered into two groups depending on whether fishmeal was added. Aminoglycoside, macrolide–lincosamide–streptogramin b (MLSb) and tetracycline resistance genes were the most abundant when fishmeal was used, while a significant increase in mobile genetic element (MGE) abundance was also detected ($P < 0.05$). Meanwhile, bacterial community structures were detected with distinct patterns between the two groups (Adonis, $P < 0.05$). Using the Mantel test and partial least squares path modeling, we identified that sediment resistomes were significantly correlated with microbial community structures ($P < 0.05$) which were mainly driven by nutrients in fishmeal. Together our findings suggested that fishmeal plays a more important role than tetracycline in proliferation of ARGs in mariculture sediment. This study may provide new insights into the mitigation of ARG propagation in mariculture operations.

1. Introduction

Antibiotic resistance genes (ARGs) have been regarded as emerging environmental pollutants and global threats to public health (Garner et al., 2017; Pruden et al., 2006). Horizontal gene transfer (HGT) plays an important role in ARG dissemination and propagation via mobile genetic elements (MGEs). A set of MGEs, including plasmids, integrons and transposons, are the main vectors for the recruitment and spread of ARGs among bacteria (Reid et al., 2015). There is now evidence that most of ARGs acquired by human pathogens may originate from the environment (Baquero et al., 2008). Genetic elements and resistance determinants for quinolones, tetracyclines, and β-lactamases are shared between aquatic bacteria, fish pathogens, and human pathogens (Cabello et al., 2013). Yang et al. found that bacteria isolated from marine fish farm sediment harbor ARGs highly similar to those found in human pathogens (2013). Tomova et al. (2015) demonstrated the high similarity between quinolone resistance genes in *E. coli* from patients and intensive aquaculture areas. These findings suggest a linkage between bacteria present in aquaculture systems and human microbiota by potential uni- or bidirectional flow of ARGs and MGEs.

The use of antibiotics in aquaculture is widely recognized to facilitate the dissemination of antibiotic resistance, which may have unfavorable consequences for human health through food chains. The worldwide growth of aquaculture has been

☆ This paper has been recommended for acceptance by Dr. Harmon Sarah Michele.

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https://doi.org/10.1016/j.envpol.2018.07.101
0269-7491/

accompanied by a rapid increase in the use of antimicrobials to treat infectious diseases or promote production at sub-therapeutic level (Cabello et al., 2013; Chopra and Roberts, 2001). Considerable volumes of antibiotics are released into the marine environment or excreted by fish in their active form, and their residues may exert selective pressure for antibiotic-resistant bacteria (Berendonk et al., 2015). Tetracycline is commonly used in aquaculture worldwide and is critically important for human medicines (Chee-Sanford et al., 2009). In 2016, the estimated veterinary use of tetracycline in the United States was 5866.59 tons, which was the largest volume of its domestic use of veterinary antibiotics (USFDA, 2017). Tetracycline residues increased the rates and persistence of ARGs (Looft et al., 2012). Consequently, high levels of tetracycline and corresponding resistant genes have been reported in many aquaculture farms (Su et al., 2017; Xiong et al., 2015). For example, specific tetracycline resistant genes (*tetW*, *tetM*, *tetO*, *tetT* genes) were detected at six aquaculture farms in Tianjin, China (Gao et al., 2012).

The occurrence and dissemination of ARGs is far more complex than selection by antibiotics, and avoidance of antibiotics alone is therefore unlikely to solve the whole problem (Muziasari et al., 2014; Tamminen et al., 2011). Fishmeal is the fundamental feed ingredient in aquaculture production, providing amino acids and other nutrients, and accounting for 40–60% of the total dietary requirements for the fish (Fagbenro, 1999). Global aquaculture production has doubled over the past decade, now accounting for ~50% of fishery products for human consumption (FAO, 2014). Previous studies demonstrated that fishmeal for aquaculture foods harbor ARGs (Han et al., 2017), and is therefore an important contributor for ARG occurrence and proliferation in mariculture systems. Moreover, nutrients have the potential to promote the growth of the antibiotic resistant bacteria, and further contribute to the accumulation of ARGs (Udikovic-Kolic et al., 2014). Given that tetracycline and fishmeal are both routinely administered to fish in aquaculture systems via formulated food mixtures, their combined effect or potential synergistic effects on sediment resistomes are important to consider yet remain unexplored.

The majority of previous research has considered only a few well-studied tetracycline resistance genes and integrons (Harnisz et al., 2015; Tamminen et al., 2011). Conventional quantitative PCR is the most commonly used molecular method to detect these broadly studied ARGs. Although high throughput qPCR method has once been used to study antibiotic resistome in mariculture sediment (Muziasari et al., 2016), the dominant factor to shape the resistome is still not clear. A comprehensive analysis of influencing factors on ARGs in mariculture sediments is therefore required. The present study aims to (1) characterize the resistomes in sediments following the application of tetracycline and fishmeal; (2) distinguish the respective contribution of fishmeal and tetracycline to the sediment resistomes and; (3) to address the factors influencing ARG variations. Our study will provide data to enable the development of effective ARG control strategies and human health risk assessment.

2. Materials and methods

2.1. Sample collection

Sediment and water were sampled in May 2016 from a mariculture farm in Dalian, China, where the shrimp and sea cucumber have were produced for 8 years. The farm owner claimed that they used blue clams as the food source and never used antibiotics or fishmeal in the eight years of production. Surficial sediment and seawater were randomly collected in the pond and mixed respectively to prevent the potential sampling bias. Samples were kept in sterile containers, stored in a portable icebox and transported back to the laboratory within 6 h where the microcosms were immediately established (Chen H. et al., 2011). Approximately 500 g fishmeal imported from Peru, was passed through a 1 mm sieve, spread and collected randomly. The fishmeal sample was stored in sterile amber bottles at 4 °C for subsequent uses. The detailed information about fishmeal could be found in the supplementary Table S1.

2.2. Microcosm setup

Microcosms were set up according to the methods outlined in "Aerobic and anaerobic transformation in aquatic sediment systems; Guideline for Testing of Chemicals No. 308" (OECD, 2002). Microcosms were established in 500 mL flasks by adding 200 g sediment and 300 mL seawater. All microcosms were kept in 20 °C and shaken at 100 rpm for 15 days' acclimation prior to treatments. The antibiotic group (A group) was set up by adding different concentrations of tetracycline (0, 0.1, 1 and 100 μg/kg) which were 1×, 10× and 100× environmentally relevant concentrations according to previous research (Berglund et al., 2014), while the fishmeal-antibiotic group (FA group) was set up in the same way, but with the addition of 0.2 g fishmeal into each microcosm (Han et al., 2017). There were three replicate microcosms per treatment. All microcosms were incubated under the same experimental conditions (20 °C and 100 rpm) and water loss was compensated by adding seawater to the microcosms every other day. To investigate the combined effect of tetracycline and fishmeal, a study period of 14 days was referred to some literature. Berglund et al. (2014) indicated that tetracycline concentration as high as 100 μg/kg was not detected in microcosm sediment after 14 days, and another previous experiment demonstrated that the abundance of total ARGs peaked after 14 days in microcosm sediment in which 0.2 g fishmeal had been added (Han et al., 2017). About 10 g of sediment sample were collected from each microcosm on day 14, and stored at −80 °C until subsequent analyses.

2.3. Detection of tetracycline in sediment by high performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS)

Sample pretreatment and extraction were performed, as previously described, with minor modifications (Gibs et al., 2013; Luo et al., 2011). Briefly, tetracycline was extracted from sediments using an accelerated solvent extraction system (ASE 350; Dionex, Sunnyvale, CA, USA) and loaded onto HLB columns (6 mL, 500 mg, 60 μm; Waters, Milford, MA, USA). The HPLC-MS/MS analysis was performed according to previously published methods (Han et al., 2017). The tetracycline concentration in 10 μL injection of sample extracts was measured by the LC/MS/MS coupled with multiple reaction monitoring system using an electron-spray ionization source (Agilent 1100 HPLC and tandem 6410B quadrupole mass spectrometer, Agilent, Lexington, MA, USA). The detailed pretreatment procedures, HPLC conditions and multiple reaction monitoring parameters are described in the supplemental materials.

2.4. Sediment characterization and DNA extraction

Total carbon (TC) and nitrogen (TN) in sediments were measured with an elemental analyzer (Vario EL, Elementar Co., Langenselbold, Germany; Wu et al., 2014). After sediments were air dried, pH was determined by adding 2 M KCl (volume ratio: 2.5:1) using a pH meter (Mettler Toledo Fiveplus FE20, Shanghai, China). We used the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) to extract total DNA from each sediment sample. DNA extracts were quantified with Qubit 2.0 (Life technologies) and qualified using a NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham,

Please cite this article in press as: Han, Y., et al., Combined impact of fishmeal and tetracycline on resistomes in mariculture sediment, Environmental Pollution (2018), https://doi.org/10.1016/j.envpol.2018.07.101