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High-throughput profiling of seasonal variations of antibiotic resistance gene transport in a peri-urban river

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ABSTRACT

The rapid expansion of human activity in a region can exacerbate human health risks induced by antibiotic resistance genes (ARGs). Peri-urban ecosystems serve at the symbiotic interface between urban and rural ecosystems, and investigations into the dissemination of ARGs in peri-urban areas provide a basic framework for tracking the spread of ARGs and potential mitigations. In this study, through the use of high-throughput quantitative PCR and 16S rRNA gene high-throughput sequencing, seasonal and geographical distributions of ARGs and their host bacterial communities were characterized in a peri-urban river. The abundance of ARGs in downstream was 5.2–33.9 times higher than upstream, which indicated distinct antibiotic resistance pollution in the areas where human lives. With the comparison classified based on land use nearby, the abundance of ARGs in samples near farmland and villages was higher than in the background (3.47–5.58 times), pointing to the high load in the river caused by farming and other human activities in the peri-urban areas. With the co-occurrence pattern revealed by network analysis, *blaVEB* and *tetM* were proposed to be indicators of ARGs which get together in the same module. Furthermore, seasonal variations in ARGs and the transport of bacterial communities were observed. The effects of seasonal temperature on the dissemination of ARGs along the watershed was also evaluated. The highest absolute abundance of ARGs occurred in summer (2.81×10^9 copies/L on average), the trends of ARG abundances in four seasons were similar with local air temperature. The Linear discriminant analysis effect size (LEfSe) suggested that nine bacterial genera were implicated as biomarkers for the corresponding season. Mobile genetic elements (MGEs) showed significant positive correlation with ARGs ($P < 0.01$) and MGEs were also identified as the key-contributing factor driving ARG alteration. This study provides an overview of seasonal and geographical variations in ARGs distribution in a peri-urban river and draws attention to controlling pollutants in peri-urban ecosystems.

1. Introduction

Peri-urban ecosystems serve at the symbiotic interface between urban and rural ecosystems, which are designed and developed to produce food, as well as to assimilate domestic waste streams that might otherwise be transferred to water bodies and exported out of the urban zone (Zhu et al. 2017). However, human activities, including livestock and poultry breeding, agriculture and wastewater treatment, can lead to the presence of antibiotic resistance genes (ARGs) as pollutants in peri-urban ecosystems (He et al. 2016; Bondarczuk et al. 2016; Chen and Zhang 2013). Antibiotic resistance has been recognized as one of the biggest threats to public health by the World Health Organization. Research into ARGs in peri-urban ecosystems will provide a framework for tackling these global health challenges.

Rivers appear to be a significant reservoir of ARGs (Ouyang et al. 2015). Wastewater treatment plants (WWTPs) have limited influence on the removal of ARGs and the persistence of ARGs discharge into the receiving river directly, potentially affecting environmental resistomes (Amos et al. 2014; Rodriguez-Mozaz et al. 2015). Moreover, the vast majority of ARGs are discharged in WWTPs together with sewage sludge, but a land application for the sewage would result in the spread of ARGs into the soil and to underground (Threedeach et al. 2012; Xie et al. 2016). Livestock and poultry breeding is an important part of human activity in peri-urban areas. The abuse of antibiotics in animal farms leads to a high abundance of ARGs in the manure (Y. Xu et al., 2016; L. Xu et al., 2016; Cui et al. 2016); and higher levels of ARGs in manured soils have been detected in field sources (Fang et al. 2015). More importantly, bacteria have been proved to share genetic information by horizontal gene transfer (HGT) via mobile genetic elements (MGEs), the physical transport of bacteria between environment allowing the transfer of ARGs from soil to rivers (Pruden et al. 2006; Zhu et al. 2013). Previous research has reported that rivers appeared to

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play an important role in ARG transportation between various environments (Pruden et al. 2012; Y. Xu et al., 2016; L. Xu et al., 2016; Zheng et al. 2017). Furthermore, some studies focused on the influences of human activities on distribution and abundance of ARGs in rivers (Pruden et al. 2012; Graham et al. 2011; Luo et al. 2010), however specific changes of ARG profiles in river influenced by human is still unknown. Environment functional regionalization of peri-urban areas was clear, and peri-urban rivers were close to human beings and ARG pollutants. Studies on peri-urban rivers can directly show the relationship between ARG profiles and specific human activities, such as farming, human life and industry.

Bacterial communities have a significant effect on shifts in the ARG structure (Jia et al. 2015; Su et al. 2015). As well, the diversity and composition of a bacterial community are influenced by temperature, which varies across different seasons (Bertolini et al. 2013; Comte et al. 2017). However, to the best of our knowledge, there are few studies (Ahammad et al. 2014; Pehrsson et al. 2016) that have systematically reported the seasonal variations in ARGs in peri-urban rivers. In this study, we monitored ARGs and the bacterial community for four seasons in a peri-urban river, the Zhangxi River, located in eastern China. High-throughput quantitative PCR (HT-qPCR) was used to detect ARGs. By using HT-qPCR, 285 ARGs, 8 transposes and *intI-1* were efficiently detected in total (Chen et al. 2016). Additionally, the composition of the bacterial community was determined by performing 16S rRNA gene high-throughput sequencing. By combining the ARGs and bacterial community assessment, this study aims to (1) characterize the spatial distribution of ARGs and the core ARGs in this peri-urban river using network analysis; (2) explore the impact of seasons and human activities on the shift in the ARGs' structure; and (3) investigate the bacterial community and MGEs to address the links with ARGs and the potential factors affecting the diversity and abundance of ARGs.

2. Materials and methods

2.1. Sampling sites and sample collection

The Zhangxi River is located in Ningbo in the Zhejiang Province, China (29°48′ N, 121°18′ E) (Fig. 1). The local air temperature for the four seasons on average was: spring 2–5 °C; summer 21–26 °C; autumn 18–21 °C; and winter 6–13 °C. Based on the remote sensing image and drainage basin division, the whole watershed was divided into four areas. The extent of human activity in four areas was as following: Area4 > Area1 > Area2 > Area3 (Fig. S1).

Water samples were collected from 16 sampling sites (W1–W16) in March, June, September, and December 2016 (representing spring, summer, autumn, and winter, respectively). The numbers of sampling sites in four areas were 9, 2, 1 and 4 in Area1, Area2, Area3 and Area4, respectively. Water samples were collected from 10 cm below the middle of the river with sterile polyethylene bottles and stored at 4 °C before laboratory analysis. The DNA of each water sample was extracted using the FastDNA SPIN Kit (MP Bio, USA) according to the manufacturer's instructions. The concentration of purified DNA was quantified spectrophotometrically (NanoDrop ND-2000c, Thermo, USA) and stored at −85 °C until molecular analysis.

2.2. High-throughput quantitative PCR

High-throughput quantitative PCR (HT-qPCR) was performed with the SmartChip Real-time PCR system (Wafergen Inc. USA) (Wang et al. 2014). There were 295 primer sets targeting the 16S rRNA gene, 285 ARGs, 8 transposases, and 1 integron (Table S1). The 285 ARGs including the 8 groups divided by the antibiotics conferred resistance to aminoglycoside, beta-lactams, fluoroquinolone/quinolone/florfenicol/chloramphenicol/amphenicol (FCA), macrolide/lincosamide/streptogramin B (MLSB), sulfonamide, tetracycline, vancomycin and other/efflux. Amplification was conducted in a 100 nL reaction containing 1 × LightCycler 480 SYBR Green I Master Mix (Roche, USA), nuclease-free PCR-grade water, 1 ng/μL albumin from bovine serum, the DNA sample and each forward and reverse primer. After initial denaturation at 95 °C for 10 min, the thermal cycle included 40 cycles of denaturation at 95 °C for 10 min, and annealing at 60 °C for 30 s. A melting curve analysis was auto-generated by the program at the end. Amplification was conducted in triplicate and a non-template control sample was included for comparison. The results were analyzed using the SmartChip qPCR software (V2.7.0.1). For each primer set, a threshold cycle (Ct) had to be < 31 and amplification was confirmed with more than two positive replicates. The gene copy numbers were calculated by the following formula.

$$\text{Gene Copy Number} = 10^{((31-Ct)/(10/3))}$$

The relative copy number of each gene was calculated by normalizing each gene's copy number and bringing its ratio to the 16S rRNA's normalized copy number (Schmittgen and Livak 2008; Looft et al. 2012). The absolute abundance of 16S rRNA was determined by quantitative real time PCR (qPCR) described in previous research (Jiao et al. 2017). The absolute abundance of each gene was recorded as the product of the relative copy number and the 16S rRNA's absolute copy number.

2.3. 16S rRNA gene high-throughput sequencing

The 16S rRNA gene high-throughput sequencing was performed on an Ion Torrent PGM with the 314 Chip Kit using the Ion Sequencing 400-bp kit (Life Technologies) according to a standard protocol (Ion Xpress™ Plus gDNA and Amplicon Library Preparation, Life Technologies) (Wu et al. 2016). The V4-V5 hypervariable region of the 16S rRNA gene was selected for amplification with universal primer pairs 515F (5′-GTGCCAGCMGCCGCGG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) (Turner et al. 1999). The sequences were then checked further for chimeras on UCHIME and aligned to the reference data downloaded from Silva (http://www.arb-silva.de/). All sequences were trimmed off for primers, barcodes and adaptor sequences through the removal of low-quality reads. The sequencing results were clustered into operation taxonomy units (OTUs) at a similarity threshold of 97%. One representative sequence of each OTU was classified phylogenetically and assigned to one taxonomic identity using the Ribosomal Database Project with a bootstrap confidence of 60 (Huang et al. 2015). The differences in the bacterial community compositions of each sample were visualized by principal coordinate analysis (PCoA) in QIIME V1.3.0 (Caporaso et al. 2010).

2.4. Statistical analysis

The data was organized in Microsoft Excel 2010, and diagramming was performed with Origin 9.0 software. Correlation and statistical differences were analyzed using SPSS V22.0 (IBM, USA) at a $P < 0.05$ level of significance. In addition, to reduce the chances of obtaining false-positive re-sults, the *P*-values were adjusted with a multiple testing correction using the Benjamini-Hochberg (BH) method (Benjamini et al. 2001)To visualize the correlations in the network interface, a correlation matrix was constructed by calculating all the possible pair-wise Spearman's rank correlations between the ARG subtypes that occurred in at least 19 samples out of collected 64 samples (Steele et al. 2011). A correlation between two items was considered statistically robust if the Spearman's correlation coefficient (ρ) was > 0.75 and the $P < 0.01$, and the P-values were adjusted with a multiple testing correction using the Benjamini-Hochberg (BH) method (Benjamini et al. 2001; Li et al. 2015; Zhang et al. 2017). Network analysis was performed in an R environment using VEGAN packages and network visualization was conducted on the Gephi interactive platform and Cytoscape (version 3.5.1) (Li et al. 2015; Shannon et al. 2003). The Canoco V5.0 program was used to perform a Mantel test and