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Biomarkers of antibiotic resistance genes during seasonal changes in wastewater treatment systems☆

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ABSTRACT

To evaluate the seasonal distribution of antibiotic resistance genes (ARGs) and explore the reason for their patterns in different seasons and different systems, two wastewater treatment systems were selected and analyzed using high-throughput qPCR. Linear discriminant analysis (LDA) effect size (LEfSe) was used to discover the differential ARGs (biomarkers) and estimate the biomarkers' effect size. We found that the total absolute abundances of ARGs in inflows and excess sludge samples had no obvious seasonal fluctuations, while those in winter outflow samples decreased in comparison with the inflow samples. Eleven differentially abundant ARGs (biomarker genes, BmGs) (*aad*A5-02, *aac*-6-II, *cml*A1-01, *cml*A1-02, *bla*OXA10-02, *aad*A-02, *tet*X, *aad*A1, *ere*A, *qac*EΔ1-01, and *bla*TEM) in summer samples and 10 BmGs (*tet*-32, *tet*A-02, *aac*C2, *van*C-03, *aac*-6-I1, *tet*E, *erm*B, *mef*A, *tnp*A - 07, and *sul*2) in winter samples were validated. According to 16S rRNA gene sequencing, the relative abundance of bacteria at the phylum level exhibited significant seasonal changes in outflow water (OW), and biomarker bacteria (BmB) were discovered at the family (or genus) level. *Synechococcus* and vadinCA02 are BmB in summer, and *Trichococcus*, *Lactococcus*, *Pelosinus*, *Janthinobacterium*, *Nitrosomonadaceae* and *Sterolibacterium* are BmB in winter. In addition, BmB have good correlations with BmGs in the same season, which indicates that bacterial community changes drive different distributions of ARGs during seasonal changes and that LEfSe is an acute and effective method for finding significantly different ARGs and bacteria between two or more classes.

In conclusion, this study demonstrated the seasonal changes of BmGs and BmB at two wastewater treatment systems.

1. Introduction

In biological treatment, the main component of wastewater treatment systems, it has been reported that temperature influences performance in conventional activated sludge processes (Arevalo et al., 2014), which in turn could affect the bioconversion process, microbial community, and sludge morphology (Zhang et al., 2014). When the temperature drops to approximately 15 °C, methane-producing bacteria appear inactive, and at approximately 5 °C, autotrophic bacteria practically cease functioning (Gurung et al., 2017). In most areas of China, winter temperatures are generally below 15 °C, and studies have shown that seasonal variation could change feed water quality, water temperature, treatment efficacy and others. For example, the removal of ammoniacal nitrogen in winter is significantly lower than that in summer, and the number of ammonia-oxidizing bacteria in summer is 4.5 times than that in winter (Ducey et al., 2010). Acetylation enzyme gene is crucial for transcriptional regulation and plays an important role in a variety of biological functions. The expression level of acetylation enzyme gene can mark the activity of ammonia oxidizers. The Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway reveals that the acetylation enzyme gene was not detected in winter samples (Cui et al., 2012). These results indicate that low temperatures inhibited microbial growth and enzyme expression (Ducey et al., 2010; Cui et al., 2012).

Antibiotic resistance is an emerging biological pollutant that has become a hotspot issue for research, and wastewater treatment plants are a repository of antibiotic resistance genes (ARGs) and

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antibiotic resistant bacteria (ARB) (Munir et al., 2011). Li found that the abundance of ARGs in untreated domestic sewage was 2.28×10^{11}–3.41×10^{11} copies/mL (Li et al., 2016) and that ARG abundances decreased after the treatment process. In contrast to domestic sewage, studies on textile dye wastewater are a little-known area in ARGs research. According to the China Environment Statistical Yearbook in 2014, the discharge of dye wastewater was approximately 2.37 billion tons, which was the second largest volume of wastewater discharge according to the China Green National Accounting Study Report (2004). Various pollutants such as dyes, surfactants, degradable organics, and heavy metals exist in dye wastewater (Somasiri et al., 2008). Our previous study revealed that dye wastewater contains multiple ARGs and that their relative abundances were as high as those in domestic wastewater, although the diversity of ARGs was lower (Jiao et al., 2017). Another study suggested that the proportion of influent of industrial wastewater significantly influenced the β-lactamase C ($R^2 = 0.171$, $p < 0.05$) and multidrug toxic compound extrusion (MATE) enzyme families ($R^2 = 0.150$, $p < 0.05$), which were responsible for efflux of antibiotics (Sun et al., 2016). Several researchers have indicated that phylogeny, rather than horizontal gene transfer (HGT), is the main determinant of ARGs profiles (Forsberg et al., 2012, 2014). Jia et al. (2015) found microbial communities and their activity are the dominant factors for changing the efficiency of ARG removal during the wastewater treatment process. As far as we know, some studies have reported the integrated bacterial composition and characteristics in different seasons (Caucci et al., 2016; Karkman et al., 2016), while the influences of seasonal changes on ARGs and ARB in sewage treatment plants are rarely reported. A recent study reported that temperature was significantly correlated with ARGs ($r = 0.2938$, $p = 0.003$) (Sun et al., 2016), but temperature is not the only one factor affects the seasonal changes on ARGs and ARB, the complicated and main influences of seasonal changes on ARGs and ARB in sewage treatment plants are poorly understood. In this research, one sewage treatment plant was used to treat domestic sewage (70%) and dyeing wastewater (30%), one sewage treatment plant was only used to treat domestic sewage (100%). Few studies report the occurrence and distribution of ARGs and ARB in dyeing wastewater. Thus, ARGs and ARB over different seasons in dyeing wastewater deserve further investigation.

Gaining insights into the structure, organization, and function of microbial communities has been highlighted as one of the major research challenges of the current decade, and experimental and computational meta-genomic analyses are used widely (Hamady and Knight, 2009). It is necessary to find tools that can ensure the reproducibility of the conclusions drawn from meta-genomic data. Linear discriminant analysis (LDA) effect size (LEfSe), which provides biological class explanations for establishing the statistical significance, biological consistency, and effect size estimation of predicted biomarkers, has been applied to detect differentially abundant features in the human microbiome and in a mouse model of colitis (Segata et al., 2011), and it can effectively aid in explaining the biology underlying the differences in microbial communities. LEfSe determines the features (organisms, flora, genes, or functions), which are called biomarkers, most likely to explain the differences between samples from different sources or different classes (Goecks et al., 2010; Segata et al., 2011). Ley et al. reported that microbial communities can be used as biomarkers for host factors such as lifestyle and diseases (Ley et al., 2006). Biomarker discovery is one of the most broadly successful and applicable ways to translate molecular and genomic data into real-world applications, especially for clinical practice (Golub et al., 1999; Segata et al., 2011). Biomarkers have also been used as a signal of environmental contamination in environmental risk assessments (Jin et al., 2015). Until now, high-throughput studies about ARGs have focused more on the holistic analysis of ARGs using antibiotic categories without identifying the differences in single genes. For instance, Su et al. found via high-throughput qPCR that aminoglycosides, tetracyclines, Macrolide-Lincosamide-Streptogramin B, and multidrug resistance genes were the most abundant during the composting process (Su et al., 2015), and Jia et al. (2015) used high-throughput sequencing and reported that multidrug, bacitracin and sulfonamide resistance genes were the dominant types of ARGs in drinking water.

We selected two representative municipal wastewater treatment plants and sought to investigate the seasonal characteristics of ARGs. Then LEfSe analysis was used to discover the specific genes (biomarkers) which can explain the different abundance in different seasons. Furthermore, microbial community was tested using 16S rRNA genes sequencing and analyzed from phylum to genus by LEfSe. This study explored the reason for ARGs distribution using the relationship between bacteria and ARG biomarkers.

2. Materials and methods

2.1. Sampling sites

The sampling sites are located in the same city. A total of 54 samples were collected from June 2015 to March 2016. Summer samples were collected in June, July and August 2015 (the average temperature was 35 ± 2 °C); autumn samples were collected in September, October and November 2015 (the average temperature was 20 ± 2 °C); winter samples were collected in December 2015 and January and February 2016 (the average temperature was 10 ± 2 °C).

Wastewater treatment plant one (W1) receives only domestic sewage, and inflow water, outflow water and excess sludge were named IW1, OW1 and SS1, respectively. Wastewater treatment plant two (W2), which is in the same area as W1, receives approximately 70% domestic sewage and 30% dye wastewater. The mixed inflow water, outflow water and excess sludge were named IW2, OW2 and SS2, respectively, and pure dye wastewater at W2 was collected before it was mixed with domestic sewage and labeled IDW2.

All water and sludge samples were collected in sterile containers using the sampling method referred to in our previous studies (Chen and Zhang, 2013a). The pretreatment of all samples was completed within 12 h.

2.2. Sample pretreatment and DNA extraction

The microbial biomass in water samples was collected using 0.22 μm vacuum suction filtration filters until the filter clogged and the volume after clogging was recorded. Then, the filters were covered with tinfoil and used for DNA extraction at −80 °C. The excess sludge samples were freeze-dried for 12 h at approximately −70 °C. DNA was extracted using the FastDNA SPIN Kit for Soil (MP, Biomedicals) using the protocol in the kit instructions. The concentration of the purified DNA was detected by micro-spectrophotometer analysis (NanoDrop ND-2000c, Thermo Fisher Scientific, USA). Then, the DNA samples were eluted with 75 μL DES solution and stored at −80 °C until use.

2.3. High-throughput quantitative PCR (HT-qPCR)

High-throughput qPCR (HT-qPCR) was used to detect the abundance of ARGs in the samples (SmartChip Real-time PCR, Warfergen Inc. USA). A total of 296 primer sets were designed using the findings of previous publications (Zhu et al., 2013; Su et al., 2015; Xu et al., 2016). These primer sets were designed to detect