



Organic compounds stimulate horizontal transfer of antibiotic resistance genes in mixed wastewater treatment systems



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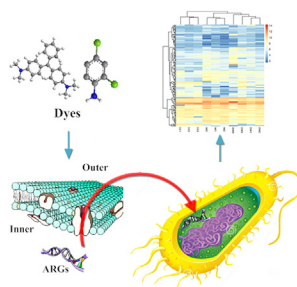
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HIGHLIGHTS

- Mixed wastewater contained high-abundant ARGs significantly differing from domestic sewage.
- Normalized copy number of MGEs in IDW3 was 8.5 fold higher than in domestic wastewater.
- Significant correlations were found between efflux pump genes and *Streptococcus*.
- Six representative organic compounds remarkably promoted conjugative transfer by plasmid RP4.

GRAPHICAL ABSTRACT



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ABSTRACT

Domestic wastewater treatment plants as a reservoir of antibiotic resistance genes (ARGs) have received much attention, but the effect of dyes on the propagation of ARGs has rarely been investigated. In this study, we investigated the differences in distributions of ARGs and microbial communities using high-throughput qPCR and 16S rRNA gene sequencing, respectively, between mixed (dyeing and domestic) wastewater and domestic sewage. The relative abundance of ARGs in inflows of mixed wastewater (IW2 and IW3) was higher than that of domestic wastewater (IW1). The relative abundance of mobile genetic elements in the inflow of textile dyeing wastewater (IDW3) was 3- to 13-fold higher than that in other samples. Moreover, in IDW3, some distinct high abundance ARGs, particularly operons encoding efflux pumps (such as *acrR-01*, *acrB-01* and *acrF*), were significantly correlated with *Streptococcus* of the Firmicutes. To explore why the abundance of ARGs was relatively high in mixed wastewater, six representative types of organic compounds in textile dyeing wastewater were used to test the effect on plasmid-based conjugative transfer from *E. coli* HB101 to *E. coli* NK5449. These six compounds all facilitated the transfer of resistance-carrying RP4 plasmid, and the highest transfer frequency (approximately 10^{-5} – 10^{-3}) was over 4- to 200-fold higher than that in the control group (approximately 10^{-6} – 10^{-5}). These results illustrated that the six common residual compounds, particularly low-dose substances in IDW3, could facilitate the dissemination of ARGs in aquatic environments. More importantly,

Abbreviations: ARGs, antibiotic resistance genes; ARB, antibiotic resistance bacteria; QACs, quaternary ammonium compounds; MACH, monoaromatic hydrocarbons; MGEs, mobile gene elements; HGT, horizontal gene transfer; HT-qPCR, high-throughput quantitative PCR; IW1, inflow of wastewater from W1; OW1, outflow of wastewater from W1; SS1, surplus sludge from W1; IDW3, inflow of dyeing wastewater from W3; FCA, fluoroquinolone, quinolone, florfenicol, chloramphenicol, and amphenicol; MLSB, macrolide-lincosamide-streptogramin B; RND, resistance nodulation division.

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this study revealed for the first time that dyeing contaminants influenced horizontal gene transfer (HGT) of ARGs.

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1. Introduction

The overuse of antimicrobials in medical, agricultural and veterinary applications during the past decades accelerated the emergence of microbial drug resistance (Allen et al., 2010). The increase of antibiotic resistance genes (ARGs) hampers the effectiveness of drugs, posing an overwhelming threat to the health of humans. The primary vehicles for the spread of ARGs are antibiotic resistant bacteria (ARB). Chromosomal mutations are an important pathway for bacteria to acquire antibiotic resistance (Dwyer et al., 2009). Moreover, for the spread of ARGs, the processes of transduction (via bacteriophages), conjugation (via plasmids and conjugative transposons), and transformation (via incorporation into the chromosomal DNA plasmids and other DNA from other organisms) (Levy and Marshall, 2004) are responsible. Equally worrying, ARGs can persist in the natural environment even when the antibiotic selecting pressure disappears (Salysers and Amabile-Cuevas, 1997).

Domestic sewage and sludge constitute major reservoirs for ARB and ARGs (Chen and Zhang, 2013). Previous studies on ARGs in sewage primarily focused on urban (Rizzo et al., 2013), hospital (Yang et al., 2014) and pharmaceutical wastewater (Liu et al., 2014). The distribution of ARGs and ARB in industrial wastewater is often overlooked. In particular, wastewater from textile dyeing constitutes a huge proportion of the wastewater produced annually (Laera et al., 2012; Koprivanac et al., 1999). The gross production of dyes worldwide has reached over 600 thousand tons per year of which more than 50% are used for dyeing of textiles (Koprivanac et al., 1999). The wastewater from dyeing mills has put immense pressure on the aquatic environment and biodiversity. Preliminarily treated dyeing wastewater is transported via pipe to municipal wastewater treatment plants and further treated with domestic sewage. In most of the studies on dyeing wastewater, the focus is on the removal of organic contaminants. Therefore, little is known about the fate of ARGs in the water and sludge phases during mixed wastewater treatment processes.

Ubiquitous forms of organic matter occur in industrial processes that are collectively called quaternary ammonium compounds (QACs) (Jennings et al., 2015) and monoaromatic hydrocarbons (MACH). QACs serving as microbicides, surfactants, and dyes (Fadda and El-Mekawy, 2013) have decisive inhibitory effects on the growth of certain bacteria and fungi and membrane permeation (Bhattacharya and Samanta, 2011), and the microbial community structure is altered under high concentrations of these contaminants (Jennings et al., 2015). The *qac* genes encode a large family of bacterial efflux pumps, which are capable of expelling many QAC structures from bacterial cells (Aleksun and Levy, 2007) and leading to a decrease in susceptibility to QACs. Malachite green, belonging to QACs, is a common wool fabric dye, and although the compound is highly efficient in the elimination of fungal and bacterial infections, it is also highly toxic with carcinogenic, teratogenic, and mutagenic properties (Lin et al., 2016; Stamatati et al., 2005). Importantly, *qac* genes can be horizontally transferred via mobile gene elements (MGEs) (plasmids, integrons, and transposons) to other bacteria and are often transmitted in combination with other ARGs (Aleksun and Levy, 2007). Many reports show that plasmid-mediated conjugative transfer occurs both in the

environment and under laboratory conditions (Wang et al., 2015a; Bellanger et al., 2014; Qiu et al., 2012). Presumably, the input of dyeing wastewater could positively promote HGT in domestic sewage. To date, the influence of organic compounds from industrial dyeing wastewater on the spread of ARGs or MGEs has rarely been studied. Additionally, the effect of dyes on conjugative transfer has not yet been determined. The occurrence and abundance of ARGs can be affected by a shift in the microbial community; however, the effect of microbial community composition on ARG profiles in environmental samples remains largely unknown (Yang et al., 2014).

Therefore, we hypothesized that organic compounds from the dyeing industry (e.g., QACs) would induce an increase in the rate of gene transfer of ARGs, which could accelerate the spread of ARGs in mixed wastewater samples. The differences in composition of the microbial communities between dyeing wastewater and domestic sewage could explain the differences in the occurrence and distribution of distinct ARGs. Additionally, in this research, the differences in ARGs and microbial communities between dyeing wastewater and domestic sewage were analyzed using high-throughput quantitative PCR (HT-qPCR) and 16S rRNA gene sequencing. We intended to explore a) the diversity and abundance of ARGs in dyeing wastewater; b) the difference between domestic wastewater and mixed industrial wastewater on distribution of ARGs and microbial community structure in both the water and sludge phases; and c) the effect of six major organic compounds on HGT.

2. Materials and methods

2.1. Sampling sites and sample collection

All samples were collected from July to September 2015 in Huzhou, China. Inflow of wastewater (IW), outflow of wastewater (OW) and surplus sludge (SS) were collected from three wastewater treatment plants (WWTPs: W1, W2, and W3) in the same district. W1 is a domestic sewage treatment plant. W2 is a mixed wastewater treatment plant (approximately 70% domestic sewage, 27% dyeing wastewater and 3% steel mill wastewater), and W3 is also a mixed wastewater treatment plant (approximately 70% domestic sewage and 30% dyeing wastewater). The inflow of dyeing wastewater to W3 (IDW3) was also collected. The details of these WWTPs are shown in Table S1, and the geographical location of the sampling sites, all in the same district, is shown in Fig. S1. To avoid the effect of hydraulic loading fluctuations, all samples were collected twice a month, and water samples were blended in equal volumes per hour within 24 h. The samples collected from the same site every three months were treated as parallel samples. Water samples were kept in 5 L sterilized polypropylene containers, and sludge samples were kept in sterilized sealed plastic bags. All samples were placed in iceboxes during sampling and transported to the laboratory within 2 h after collection. All samples were pretreated within 12 h.

2.2. Sample pretreatment and DNA extraction

Water samples were concentrated using 0.22 μm vacuum

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