Science of the Total Environment 526 (2015) 366–373

Contents lists available at ScienceDirect

Science of the Total Environment

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

Fate and proliferation of typical antibiotic resistance genes in five full-scale pharmaceutical wastewater treatment plants

Jilu Wang [b], Daqing Mao [a,*], Quanhua Mu [b], Yi Luo [b,*]

[a] School of Environmental Science and Engineering, Tianjin University, Tianjin 300072, China
[b] College of Environmental Science and Engineering, Ministry of Education Key Laboratory of Pollution Processes and Environmental Criteria, Nankai University, Tianjin 300071, China

HIGHLIGHTS

- The ARGs in final discharges were 7–308 times higher than that in raw influents.
- The bio-treatment processes promote the proliferation of ARGs.
- Associated ARG subtypes were positively correlated with corresponding antibiotics.

ARTICLE INFO

Article history:
Received 21 January 2015
Received in revised form 11 May 2015
Accepted 13 May 2015
Available online 16 May 2015

Editor: Adrian Covaci

Keywords:
Antibiotic resistance gene
Pharmaceutical wastewater treatment plant
Bio-treatment process

ABSTRACT

This study investigated the characteristics of 10 subtypes of antibiotic resistance genes (ARGs) for sulfonamide, tetracycline, β-lactam and macrolide resistance and the class 1 integrase gene (*intI1*). In total, these genes were monitored in 24 samples across each stage of five full-scale pharmaceutical wastewater treatment plants (PWWTPs) using qualitative and real-time quantitative polymerase chain reactions (PCRs). The levels of typical ARG subtypes in the final effluents ranged from $(2.08 \pm 0.16) \times 10^3$ to $(3.68 \pm 0.27) \times 10^6$ copies/mL. The absolute abundance of ARGs in effluents accounted for only 0.6%–59.8% of influents of the five PWWTPs, while the majority of the ARGs were transported to the dewatered sludge with concentrations from $(9.38 \pm 0.73) \times 10^7$ to $(4.30 \pm 0.81) \times 10^{10}$ copies/g dry weight (dw). The total loads of ARGs discharged through dewatered sludge was 7–308 folds higher than that in the raw influents and 16–638 folds higher than that in the final effluents. The proliferation of ARGs mainly occurs in the biological treatment processes, such as conventional activated sludge, cyclic activated sludge system (CASS) and membrane bio-reactor (MBR), implying that significant replication of certain subtypes of ARGs may be attributable to microbial growth. High concentrations of antibiotic residues (ranging from 0.14 to 92.2 mg/L) were detected in the influents of selected wastewater treatment systems and they still remain high residues in the effluents. Partial correlation analysis showed significant correlations between the antibiotic concentrations and the associated relative abundance of ARG subtypes in the effluent. Although correlation does not prove causation, this study demonstrates that in addition to bacterial growth, the high antibiotic residues within the pharmaceutical WWTPs may influence the proliferation and fate of the associated ARG subtypes.

1. Introduction

Antibiotic resistance genes (ARGs) were emerging environmental contaminants and were extensively studied in various environments, including groundwater, surface water, soil, and sediments (Zhang et al., 2009a, 2009b, 2009c). The transformation of ARGs in environmental matrices increased the probability of antibiotic resistance dissemination into indigenous microorganisms and human pathogens. Antibiotic resistant microorganisms were able to spread their resistance into indigenous microbes, subsequently, aquatic systems became a reservoir of ARGs and played an important role in preserving and disseminating antibiotic resistance (Baquero et al., 2008; Taylor et al., 2011). Forsberg et al. (2012) have also documented that human pathogens shared their antibiotic resistome with soil bacteria, and ARGs carried by soil microorganisms could be integrated into the host cell genome in pathogens. Thus, antibiotic resistance potentially affects microbial structures in the environment and poses a risk to public health (Pruden et al., 2006).

The activated sludge and biofilms in wastewater treatment plants (WWTPs) are rich in nutrients and have a high density of bacteria, which played a vital role in controlling water pollution. The high load of organic matter and the bacterial density created an ideal environment in WWTPs for cell-to-cell contact and gene exchange (Sorensen et al., 2005). Besides, pollutants loaded in raw sewage (notably antibiotics

* Corresponding authors.
E-mail addresses: mao@tju.edu.cn (D. Mao), luoy@nankai.edu.cn (Y. Luo).

http://dx.doi.org/10.1016/j.scitotenv.2015.05.046
0048-9697/

and heavy metals) were also associated with increasing antibiotic resistance (Graham et al., 2011). Previous research has verified that antibiotic resistance determinants and antibiotic resistant bacteria (ARB) prevailed throughout the wastewater treatment process (Xu et al., 2015). Thus, the emission of effluent and sludge represented a key route of release of ARGs into the receiving environment. For example, West et al. (2011) detected a high abundance of ARGs in WWTP effluent receiving waters. Additionally, using dewatered sludge as land fertilizer also contributed to a high proportion of ARGs in soil, and the long half-life of ARGs in soil resulted in a higher persistence of antibiotic resistance (Burch et al., 2014).

Although studies have characterized ARGs in effluents of WWTPs, the fate and distribution of ARGs along the flow process of full-scale pharmaceutical wastewater treatment plants (PWWTPs) still remains unknown. Ideally, specific studies on the abundance of ARGs in various stages of PWWTPs will help to discern the proliferation of typical ARGs in different treatment stages. Additionally, PWWTPs should be further investigated due to the high load of antibiotics in influents and limited levels of other pollutants (e.g., heavy metals or detergents) (Fick et al., 2009). Concerning that the microbial community in the activated sludge has adapted to the high levels of antibiotics in pharmaceutical sewage (Li et al., 2011; Novo et al., 2013; Larsson et al., 2007), PWWTPs were an ideal model for studying the fate and removal of ARGs with respect to the influence of the associated antibiotics. Since antibiotics and typical ARGs discharged from PWWTPs might increase the prevalence of multi-antibiotic resistance among microbial communities in the receiving water (Kristiansson et al., 2011; Li et al., 2010; Zhang et al., 2009a, 2009b, 2009c; Guardabassi et al., 1998), the present study enabled the developed strategies and updated treatment technologies to control the dissemination of ARGs.

Twenty-two subtypes of ARGs conferring resistance to five classes of antibiotics were screened, including *sul1*, *sul2* and *sul3* resistant to sulfonamides; *tetO*, *tetQ*, *tetW*, *tetM* and *tetT* resistant to tetracyclines; *qnrA*, *qnrB*, *qnrD* and *oqxB* resistant to quinolones; $bla_{OXA\text{-}1}$, $bla_{OXA\text{-}2}$, $bla_{OXA\text{-}10}$, bla_{ampC}, bla_{mecA}, $bla_{CTX\text{-}M\text{-}1}$ and $bla_{TEM\text{-}1}$ resistant to β-lactams; and *ermB* and *ermC* resistant to macrolides. Concerning that mobile genetic elements (such as class 1 integrons) are conducive to forming multidrug resistance and accelerate the dissemination of ARGs in surface waters, soils (Wang et al., 2014; Xu et al., 2011) and WWTPs (Koczura et al., 2012), the class 1 integrase gene (*intI1*) as an indicator of the intercellular transfer potential of resistance was also assayed (Ling et al., 2013). The purpose of this study is to investigate the fate and proliferation of various ARGs in each processing stage of pharmaceutical wastewater treatment systems that employ various biotreatment operations. As the impacts of antibiotics on corresponding antibiotic resistance determinants, the selective pressures exerted by antibiotics were explored to seek a feasible solution to mitigate the discharge of ARGs into the receiving environment.

2. Materials and methods

2.1. Pharmaceutical wastewater treatment plants

Five PWWTPs with different combinations of biological and physical/chemical technology processes in Northern China were investigated. PWWTPs A, B, C, and D, respectively treat oxytetracycline (tetracycline), oxytetracycline (tetracycline), trimethoprim (sulfonamide) and cephalosporin (β-lactam) contaminated wastewater, while PWWTP E receives non-antibiotic drugs (such as caffeine, ibuprofen) pharmaceutical wastewater. The flow chart layouts of the studied treatment plants are shown in Fig. 1, and the characteristics of the PWWTPs are listed in Table 1. Among the five different biological treatment processes, PWWTP A (Fig. 1a) used a cyclic activated sludge system (CASS), which was a sequencing batch reactor operated in a successively arrangement of aerobic and anaerobic stages. PWWTP B (Fig. 1b) combined an expanded granular sludge bed reactor (EGSB), which was a new type of anaerobic reactor, and a membrane bioreactor (MBR) system. PWWTP C (Fig. 1c) utilized an extended aeration activated sludge (EAAS) system, which involves retaining the activated sludge for 24–48 h in an aeration tank. The processes of conventional activated sludge (CAS) system were applied in PWWTP D (Fig. 1d), while anaerobic–anoxic–oxic (A/A/O) operation was applied in PWWTP E (Fig. 1e).

2.2. Sample collection and pretreatment

Two-liter grab samples of wastewater or sludge (including raw influents, activated sludge from biological treatment tanks, final effluent and dewatered sludge (Fig. 1)) were collected in triplicate using a GRASP refrigerated automatic sampler (GRASP Science & Technology Co., LTD, Beijing, China) in December 2013. To avoid confounding effects associated with hydraulic loading fluctuations, samples were collected every 2 h within a 24-hour period. Samples were kept on ice and transported to the laboratory for pretreatment within 24 h. Water samples were concentrated onto 0.22 μm sterile membranes and stored at −20 °C until DNA extraction. Activated sludge samples were centrifuged at 4000 ×*g* for 10 min at room temperature, and 0.5 g of the pellet was used for DNA extraction. Excess sludge samples were divided into two subsamples: one was subjected to DNA extraction and the other was used to determine the moisture content for further ARG quantification.

2.3. Antibiotic analysis

The targeted antibiotics across the five PWWTPs included sulfonamides (trimethoprim, TMP), tetracyclines (tetracycline, TC; oxytetracycline, OTC; chlortetracycline, CTC), and β-lactams (cephalosporin, CEP). Samples were analyzed for antibiotics using ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) in the positive-ion mode using a Waters Acquity UPLC equipped with a Waters 2777C auto-sampler system and a Waters Xevo TQ MS (Waters, USA). Details of the antibiotics analysis are presented in the Supplemental information.

2.4. DNA extraction

The pretreated pellets were placed in a 10 mL sterile centrifuge tube. In total, 1.5-mL DNA extraction buffer (100 mM Tris–HCl, 100 mM Na_2EDTA, 1.5 M NaCl, 1% 2-hydroxy-1-ethanethiol, 1 × PBS buffer, 1% PVP40000, and 2% CTAB) and 0.2-g glass beads (acid-washed) were added to the samples. The samples were then vortexed for 8 min. Lysozyme (20 μL) was then added, and the samples were then incubated at 37 °C for 30 min. Subsequently, a liquid nitrogen freeze–thaw process was performed on the mixture to further promote the release of DNA. To reduce the interference of proteins, 0.5 mL of SDS (20%) and 10 μL of proteinase K were added. After a 60 °C incubation, the mixture was separated by centrifugation at 6000 ×*g* for 15 min; the supernatant was transferred to a 2-mL sterile centrifuge tube. After the RNaseA treatment, the DNA was extracted using phenol/chloroform/isoamyl alcohol. The final products from the extraction were dissolved in TE buffer.

The quality of the DNA was determined by agarose gel electrophoresis, and the concentrations of DNA were normalized to the mass concentration of sludge (μg/g dry weight, dw) and volume concentration of sewage (μg/mL). The DNA extraction efficiency was also determined as in Mao et al. (2014). The purity of the extracted DNA was then verified by a spectrophotometer analysis to ensure that no contaminants were present that could inhibit the PCR.

2.5. PCR detection and qPCR quantification of ARGs

Polymerase chain reaction (PCR) assays were used to determine the presence and absence of targeted ARGs. All PCRs were conducted in